

T cell participation in autoreactivity to NC16a epitopes in bullous pemphigoid

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Summary

Bullous pemphigoid is a blistering skin disease characterized by autoantibodies against the NC16a domain of bullous pemphigoid 180. This study was performed to characterize and map the fine specificity of T cell responses to NC16a. Peripheral blood mononuclear cells (PBMC) from a total of 28 bullous pemphigoid patients and 14 matched controls were tested for proliferative and cytokine responses to recombinant NC16a and a complete panel of 21 overlapping peptides spanning this region of BP180. Proliferative responses to NC16A and the peptide panel in the patients with active disease were similar in frequency and magnitude to those in healthy donors, and included late responses typical of naive cells in approximately 60% of each group. Interleukin (IL)-4 responses were slightly stronger for six peptides, and significantly stronger for Nc16a, in patients than in controls. Factor analysis identified factors that separate responses to the peptide panel discretely into IL-4, T helper type 2 (Th2) pattern, interferon (IFN)- γ , Th1 pattern and IL-10 or transforming growth factor [TGF- β , regulatory T cell (T_{reg})] pattern. Factors segregating IL-10 *versus* IFN- γ were predicted by active blistering or remission, and TGF- β or IL-10 *versus* IFN- γ by age. Finally, we confirmed a significant up-regulation of IgE responses to BP180 in the patients with pemphigoid. This shows the complexity of T cell phenotype and fine autoreactive specificity in responses to NC16A, in patients and in normal controls. Important disease-associated factors determine the balance of cytokine responses. Of these, specific IL-4 and IgE responses show the strongest associations with pemphigoid, pointing to an important contribution by Th2 cytokines to pathogenesis.

Keywords: autoimmunity, cytokines, regulatory T cells, Th1/Th2

Introduction

Bullous pemphigoid (BP) is an autoimmune blistering skin disease [1], which is increasing in prevalence and has a high mortality [2,3]. It is characterized by the presence of immunoglobulin (Ig)G autoantibodies against the hemidesmosomal antigens BP230 (BPAG1) and BP180 (BPAG2), although IgE antibodies have also been reported to these targets [4]. BP230 is an intracellular component of the hemidesmosome [5]. BP180, a transmembrane glycoprotein located in basal keratinocytes, has a collagen type XVII extracellular NC16a domain [6–8] that contains immunodominant B cell epitopes [7,9,10]. There is mounting evidence that an autoimmune response to

BP180 is important in the initiation and evolution of BP [9,10], notably in animal models [11,12]. Recent reports also raise the possibility that CD4⁺ T helper (Th) cells play a role [13,14], but their phenotype, pathophysiology and specificity remain uncertain. Most investigators have relied on cloned T cells, which are highly selected and may not represent the phenotype or fine specificity of the polyclonal response *in vivo* [9,15,16]. Studies of polyclonal responses in BP have not clearly linked T cell responsiveness with disease. Thus, Budinger *et al.* [15] demonstrated proliferative ‘responses to BP180 in 10 of 12 BP patients, but also in 8/10 matched controls’, while Eming *et al.* [17] reported proliferation, interferon (IFN)- γ and interleukin (IL)-5 production in only one of seven patients *versus* four

of 11 control subjects. Th17 cells are increased in lesional skin [18], and circulating follicular Th cells which modulate B cells have also been shown to be increased in BP [19]. The pathogenicity of T cells in antibody-mediated autoimmune diseases can depend upon their precise specificity and phenotype [20,21], whether they are activated *in vivo* or remain ignorant [22], and whether effector responses are modulated by regulatory T cells (T_{reg}) [23]. T_{reg} include both the 'natural' subset that differentiates during thymic development and 'adaptive' types generated in the periphery, which can secrete transforming growth factor (TGF)- β or IL-10. Although one study failed to demonstrate deficiency of 'natural' T_{regs} in BP [24] and another no correlation with disease severity [18], recent studies suggest T_{regs} to be decreased in peripheral blood [25] and skin [26]. Adaptive T_{regs} secreting IL-10 [26], which increased in response to therapy, or TGF- β in response to particular epitopes, could be important in blunting BP180 autoaggression and have therapeutic potential. Gene gun transfection with NC16a encoding DNA on gold particles resulted in T_{reg} -mediated tolerance to BP180 [27].

While IgG antibody responses to BP180 are undoubtedly important in BP, evidence of disease-specific T cell responses is inconclusive, any roles of particular CD4⁺ T cell phenotypes such as Th2 and adaptive T_{reg} are unclear and the fine epitope specificity of the polyclonal response has not been mapped. We aimed to determine whether there are effector Th1, Th2 or regulatory cytokine responses to epitopes, using a complete panel of peptides spanning the NC16a region in patients with the disease and matched controls.

Materials and methods

Patients

We recruited patients with a diagnosis of BP (Table 1) prospectively, based on clinical history supported by histology and by immunofluorescence showing IgG deposited at the skin epidermal basement membrane. Diagnosis was confirmed in all cases by indirect immunofluorescence localizing circulating IgG autoantibodies to the roof of blisters in salt-split skin. Written consent was obtained from all

Table 1. Clinical and basic demographic features of patients included in the study.

Patient	Sex	Age (years)	Disease status	Medication	Proliferative response to NC16a	Repeat follow-up samples
1	Male	71	Active	Prednisolone Dermovate	Y	1
2	Male	66	Active	Dapsone	N	1
3	Male	80	Active	Dermovate	N	1
4	Male	70	Controlled	Dermovate	Y	3
5	Male	56	Controlled	Dermovate	Y	1
6	Female	75	Remission	No med	Y	1
7	Female	74	Active	Minocycline	Y	1
8	Female	73	Controlled	Dermovate	Y	0
9	Female	72	Remission	No	Y	1
10	Female	70	Remission	No	N	1
11	Male	65	Remission	No	Y	0
12	Male	84	Remission	No	N	0
13	Male	83	Controlled	Prednisolone	Y	1
14	Male	82	Active	Untreated	N	0
15	Female	46	Controlled	Prednisolone	N	0
16	Male	71	Active	Untreated	N	1
17	Male	84	Remission	No	Y	0
18	Male	67	Remission	No	Y	0
19	Female	74	Remission	No	Y	1
20	Male	70	Controlled	Dapsone	N	0
21	Female	71	Active	Prednisolone	Y	0
22	Female	92	Controlled	Prednisolone	Y	0
23	Male	71	Active	Untreated	Y	0
24	Male	71	Active	Untreated	Y	0
25	Male	72	Active	Untreated	Y	0
26	Male	83	Active	Untreated	N	0
27	Male	84	Active	Prednisolone dermovate	N	0
28	Male	86	Active	Untreated	N	0

patients and prior ethical permission for the study was granted by North Scotland Research Ethics Committees.

Blood samples were obtained from the patients and controls, and age- and sex-matched as closely as possible at a ratio of one control to two patients. Controls were healthy with no evidence of immune-mediated skin disease and receiving no immunosuppressive therapy. Patients with no blisters were classed as inactive, and with blisters present as active. To explore further the relationship of cytokine response to disease activity, samples were repeated where possible at 3–6-month intervals.

Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated from fresh blood samples by density gradient centrifugation (Lymphoprep; Nycomed, Roskilde, Denmark) and cultured in 1-ml wells at a concentration of 1.25×10^6 cells/ml in alpha modification of Eagle medium (Gibco, Glasgow, UK) supplemented with 5% autologous serum, 4 mmol/l L-glutamine (Sigma, Poole, UK), 100 U/ml sodium benzylpenicillin G (Sigma, Poole, UK), 100 µg/ml streptomycin sulphate (Sigma) and 20 mmol/l HEPES (pH 7.2; Sigma) at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air [22,28,29]. Viability of PBMCs by trypan blue exclusion was greater than 90% in all experiments.

Antigens and mitogens

The responsiveness of PBMC from patient and healthy controls was tested to recombinant NC16a (kindly donated by Dr Amo, Department of Dermatology, Kitasato University School of Medicine, Japan) [30], which had been dialysed and filtered before addition to cultures at 1 or 10 µg/ml. The responsiveness to a complete panel of 21 15-mer peptides spanning this region with 5-amino acid overlaps (Department of Biochemistry, University of Bristol, UK) (Table 2) was also assessed, using 20 µg/ml of each individual peptide to stimulate cultures.

The control recall antigen *Mycobacterium tuberculosis* purified-protein derivative (PPD; Statens Serum Institut, Copenhagen, Denmark) [22,31] or the control primary antigen keyhole limpet haemocyanin (KLH) (Calbiochem, London, UK) was added to cultures at 10 µg/ml.

Cell-proliferation assays

Cell proliferation was measured by tritiated thymidine incorporation in triplicate 100-µl volumes withdrawn from the PBMC culture wells on days 5 and 7 after stimulation of PBMC with antigen [28]. Previous kinetic studies [22,28,31] have demonstrated that human Th cell recall responses to antigen typically peak by day 5 of culture,

Table 2. Sequence of 15-mer peptides spanning the extracellular NC16a immunogenic region of bullous pemphigoid (BP)180; Numbering starts from the NH₃ 5' terminus.

BP peptide no.	Peptide sequence
1	EEVRKLKARVDELER
2	RKLKARVDELERIRR
3	KARVDELERIRRSIL
4	VDELERIRRSILPYG
5	LERIRRSILPYGDSM
6	IRRSILPYGDSMDRI
7	SILPYGDSMDRIEKD
8	PYGDSMDRIEKDRLQ
9	DSMDRIEKDRLQGMA
10	DRIEKDRLQGMAPAA
11	EKDRLQGMAPAAGAD
12	RLQGMAPAAGADLDK
13	GMAPAAGADLDKIGL
14	PAAGADLDKIGLHSD
15	GADLDKIGLHSDSQE
16	LDKIGLHSDSQEELW
17	IGLHSDSQEELWMFV
18	HSDSQEELWMFVRKK
19	SQEELWMFVRKKLMM
20	ELWMFVRKKLMMEQE
21	LWMFVRKKLMMEQEN

while measurements at later time-points from day 7 allow the slower primary responses of naive cells to become prominent. Results are expressed as the mean counts per minute (cpm) of each triplicate sample or as a stimulation index (SI) (ratio of mean cpm in stimulated *versus* unstimulated control cultures) [29], with an SI>3 being interpreted as representing a significant positive response [29].

Enzyme-linked immunosorbent assay (ELISA)

The production of the T cell cytokines IFN-γ, IL-4, TGF-β1 and IL-10 was assessed in duplicate 100-µl aliquots taken from the PBMC cultures 5 days after stimulation, using a sensitive cellular ELISA [29]. PBMC cultures were transferred into microtitre plates (Nunc, Roskilde, Denmark) coated with monoclonal anti-cytokine antibody (Pharmingen, Oxford, UK). After incubation of PBMC for 24 h at 37°C, the plates were developed with the appropriate biotinylated monoclonal detection antibody (Pharmingen), extravidin-alkaline phosphatase conjugate (Sigma) and p-nitrophenyl phosphate substrate (Sigma). Absorbance at 405 nm was measured using a multi-scan plate reader (Labsystems, Basingstoke, UK). Cytokine secretion was calculated by interpolation from a standard curve generated by incubating duplicate wells with doubling dilutions of recombinant human cytokines (Pharmingen). Results are presented as absolute concentration, or as SI, expressing the ratio of mean concentration in stimulated *versus*

unstimulated control cultures. An SI > 2.0 is interpreted as representing a significant positive response [29].

Flow cytometry

In some experiments flow cytometry was used to determine the phenotypes of cultured cells that proliferate or secrete cytokine in response to antigen. Aliquots of PBMC were taken from responding cultures and stained with anti-CD4 fluorescein isothiocyanate and with anti-CD25 phycoerythrin-cyanin 5.1 (Beckman Coulter, Brea, CA, USA) and with either anti-CD3 phycoerythrin-Texas Red^x-x or anti-forkhead box protein 3 (FoxP3), followed by rabbit anti-goat antibody-Texas Red (Abcam, Cambridge, UK). Activated cells in proliferating cultures were identified using anti-CD71-phycoerythrin (PE) or anti-CD69-PE (Beckman Coulter). Cells synthesizing IL-10 were labelled by incubating with anti-IL-10-PE (Pharmingen) after inhibition of protein secretion with brefeldin A (Sigma) or monensin protein transport inhibitor (GolgiStop) (BD Bioscience/Pharmingen, San Diego, CA, USA) and permeabilization with Intraprep[®] (Beckman Coulter). Stained cells were analysed using an EPICS XL cytometer (Beckman Coulter) and Expo version 2 analysis software (Applied Cytometry Systems, Sheffield, UK).

IgE ELISA

IgE antibodies against BP180 and BP 230 were detected in undiluted serum samples initially incubated twice in microplate wells coated with protein G (Sigma-Aldrich) to pre-absorb IgG and were subsequently tested with modified BP180/BP230 ELISA kits (MBL via Calbiochem, London, UK). Calibrators and pre-absorbed patient sera were added to microwells coated with BP180 antigen. After washing, horseradish peroxidase-conjugated anti-human IgE antibody was added followed by substrate, acid stabilization and photometric quantification by absorption at 450 nm (multi-scan plate reader).

Statistics

SIs all showed a skewed distribution. Therefore, non-parametric statistical tests were applied, including Mann-Whitney *U*-test, Wilcoxon's signed-rank test and Spearman's correlation tests. Exploratory factor analysis was performed on pooled data including the repeated samples using principal component analysis (PCA) on the matrix of peptide responses, with oblimin rotation applied as the optimal rotation to facilitate meaningful interpretation and constrained to the first two components with the highest eigenvalues. These components were then tested using multiple linear regression to explore the clinical variables which best predicted these components. Linear regression was performed using only the first sample for

each subject. Statistical tests were carried out using the PASW 18 version of SPSS statistical software. The potential for confounding by multiple comparisons was addressed by taking a *P*-value of 0.01 as statistically significant. Where *P*-values fell short of this more stringent threshold for statistical significance, results were explored instead for internal consistency, initially on first samples, and confirmed using the pooled data to support hypothesis generation.

Results

Demographics

Details of participating patients (*n* = 28) are listed in Table 1. As indicated, patients were sampled at different stages of disease, including newly presenting active disease, patients not controlled on therapy, patients controlled on therapy and patients in remission. Patients were 67% male, mean age 72 ± 11.46 [standard deviation (s.d.)]. Controls were 64% male, mean age 68 ± 7.4 (s.d.). To examine changes during the course of the disease, 14 repeat samples were taken from 10 of the patients, as indicated in Table 1, with repeat samples also taken from four matched controls.

Proliferative responses to recombinant NC16a

The initial PBMC sample from each donor was tested for the ability to proliferate in response to recombinant NC16a or control antigens (Fig. 1 summarizes the proliferative responses on days 5 and 7 after antigen stimulation, with day 7 included to reveal late responses mediated typically by previously unstimulated naive T cells [22,31]). There was no significant difference between BP patients and healthy controls at day 5 after stimulation with NC16a: 10 of 21 patients and seven of 13 controls tested demonstrated significant proliferative responses. Similarly, at day 7, PBMC from 14 of 23 patients and five of eight controls tested had a positive response to NC16a. However, the proliferative responses to NC16a by PBMC from both patients and controls at day 7 were significantly greater than those on day 5 (*P* = 0.007, Wilcoxon's signed-rank test), suggesting a contribution by naive cells [31]. PBMC from all subjects responded to the respective recall and primary control antigens PPD and KLH, as expected [22,28], with PPD responses falling, as expected, relative to KLH by day 7. Using flow cytometric analyses, in three patients we confirmed that there were increases in the numbers of CD71^{hi} (activated) and CD25⁺ (activated) cells with the CD4⁺ helper phenotype in proliferating PBMC cultures stimulated with NC16A (Fig. 2).

Cytokine responses to recombinant NC16a

PBMC were stimulated with recombinant NC16a and tested for cytokine responses representative of the major Th

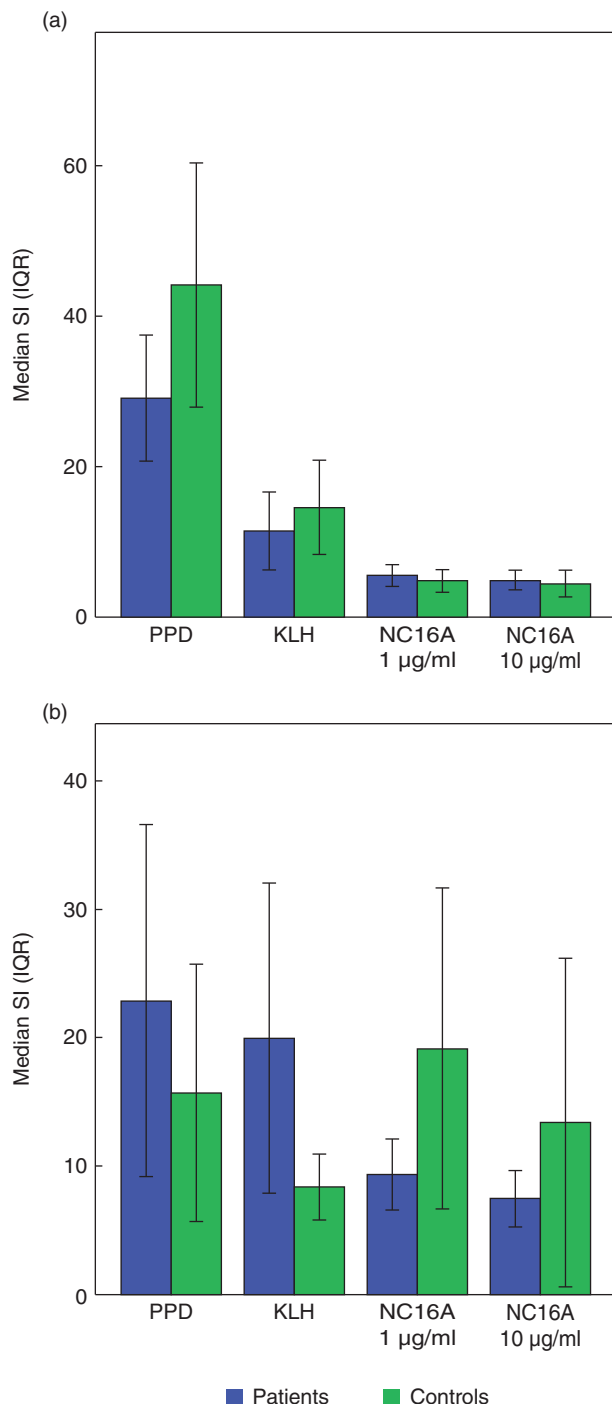


Fig. 1. Proliferative responses to recombinant NC16a or control antigens by peripheral blood mononuclear cells (PBMC) from patients and control subjects (mean SI $\pm$ standard error): (a) shows similar PBMC proliferative responses between the two groups 5 days after stimulation with the control antigens purified-protein derivative (PPD) or keyhole limpet haemocyanin (KLH), or with NC16a at two concentrations (1 and 10 µg/ml); (b) PBMC proliferative response to antigens after 7 days.

subsets Th1 (IFN- γ), Th2 (IL-4 and IL-10) and adaptive T_{reg} (IL-10 and TGF- β). The results are summarized in Fig. 3 and table S1 and demonstrate IFN- γ , IL-4, TGF- β 1 and IL-10 responses in all patients and control donors. Primary statistical analysis was performed on first samples from all subjects and controls. In 14 patients, samples were repeated 3–6 months apart and the results included to reflect different stages of disease ($n = 42$ patient samples, $n = 16$ control samples). Using either the first or pooled samples, patients showed a small but significant increase in the response to NC16a ($P = 0.01$) and approaching significance for IL-4 ($P = 0.026$) when compared to healthy controls (Mann–Whitney U -test), but not for IL-10, TGF- β or IFN- γ (Fig. 3).

Epitope mapping on NC16a

PBMC from BP patients and healthy controls were also screened for proliferative and cytokine responses to the panel of 21 peptides spanning the sequence of NC16a (Table 2). Mixed cytokine responses to multiple peptides were found in both groups, with considerable heterogeneity in the patterns of stimulatory sequences between different subjects illustrated across the range of peptides in examples in a patient and a control subject (Fig. 4a,b). In Fig. 4c the total number of significant positive responses (as opposed to SI) to NC16a and all the peptides in patients with BP are summarized for proliferation, IL-4, IL-10, IFN- γ and TGF- β . No particular peptides dominate in these mapping experiments. Analysis of the first sample data showed a significant increase in the IL-4 responses to peptide BP5 ($P = 0.007$) and marginal increases in BP3 ($P = 0.043$), BP6 ($P = 0.038$), BP18 (0.057) and BP20 (0.022) (Mann–Whitney U -test) compared to those in healthy controls (Fig. 3). Sensitivity analyses, which included repeat samples, were consistent, except for BP18 peptide ($P = 0.027$) and BP6 ($P = 0.133$).

Factor analysis

To look for correlated responses across the peptide array, a Spearman's correlation R matrix was analysed for individual cytokine responses to peptides BP1–21/NC16a, and revealed multiple correlations. Arbitrarily taking Spearman's rho 0.5–0.75 as a moderate correlation and >0.75 as a strong correlation, there were 131 moderate and 17 strong correlations between peptides for IL-4 responses, 119 moderate and three strong correlations between peptides for IL-10 responses, 38 strong and 128 moderate correlations between peptides for TGF- β responses and 0 strong and 59 moderate correlations between peptides for responses of IFN- γ . However, responses between different cytokines did not correlate. To study these complex data further, exploratory factor analysis was used as a method to reduce the complex inter-related variables to a smaller set of factors. We then plotted factor loading charts of the regression

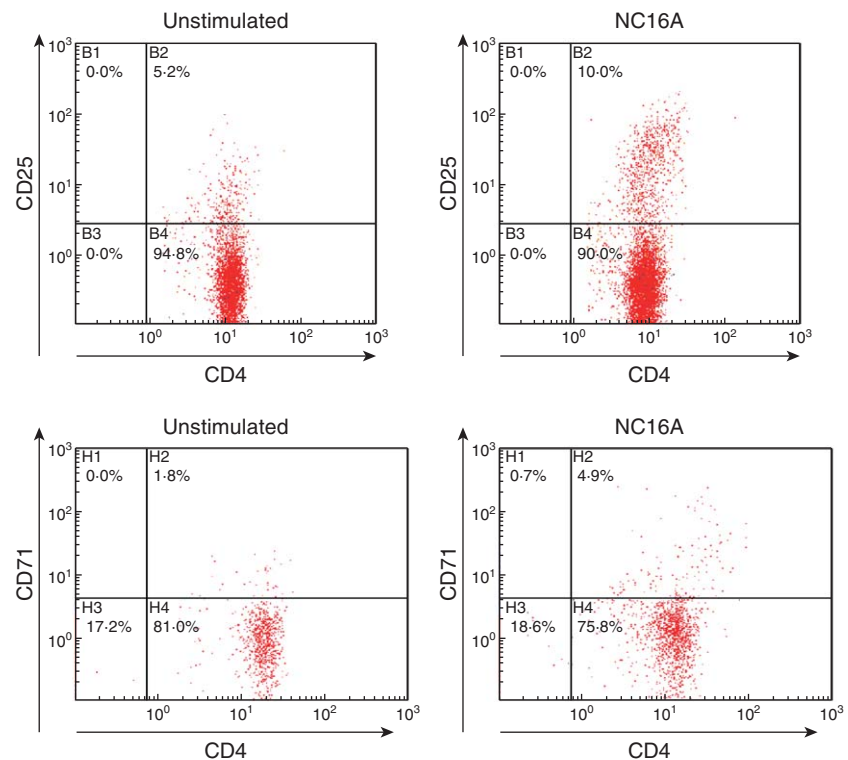


Fig. 2. Showing representative examples ($n = 3$ patients) of increased activated CD4 positive T cells after stimulation with NC16a in pemphigoid demonstrated by CD25 expression (top panels) and CD71 (transferrin receptor protein 1) as an activation marker (bottom panels).

coefficients which showed the difference between cytokine response and clustering of similar responses to the same cytokine (Fig. 5). Factor analysis showed a clear segregation of responses to each cytokine from each of the others. Linear regression analysis was performed to look for patient variables that predicted the factors separating the cytokine responses, with each of the factor scores as the dependent variable. The following independent variables were assessed in the model: whether or not the disease was active, and age (Table 3). Factor scores, segregating TGF- β *versus* IFN- γ and IL-10 *versus* IFN- γ , were predicted by the important determinant of susceptibility to BP, age [2]. Factor scores for IL-10 *versus* IFN- γ were predicted by whether or not disease was active (Table 3).

Anti BP180 and BP230 IgE serology

In view of the stronger IL-4 response to six peptides in patients compared to control donors and the segregation of IL-4 responses from IL-10 in patients in the factor analysis,

the Th2 cytokine pattern of responses to peptides in our BP cohort should have resulted in specific IgE synthesis. Stored sera were therefore analysed for specific IgE against BP180 or BP230. Figure 6 demonstrates a significant IgE response found to BP180 in patients compared to controls ($P = 0.004$, Mann–Whitney U -test), with IgE against BP180 showing reasonable specificity and sensitivity for BP in receiver operating characteristic (ROC) curves. Although three subjects showed strong IgE responses to BP230, this was not statistically significant.

Discussion

To our knowledge, this is the first study to characterize and to epitope map comprehensively the polyclonal cellular response to NC16a in patients with BP and in healthy controls. This work focuses on Th cells and the cytokine responses classically characteristic of T cell reactivity, although it is acknowledged that other cells such as CD8 $^{+}$, $\gamma\delta$ cells and B cells may have important pathogenetic roles.

Table 3. Results of multiple regression analysis for patient variables that predict the dominant cytokine response in the reduced dimensions (components) of the factor analysis, in which the coefficient β estimates the size of the effect.

Cytokine response	Compared to cytokine	Component	Independent variable	R^2	Standardized coefficient β	Model significance	Variable significance
IL-10	IFN- γ	2	Active disease	0.38	-0.49	0.03	0.04
IL-10	IFN- γ	2	Age	0.38	0.59	0.03	0.03
TGF- β	IFN- γ	2	Age	0.29	0.43	0.03	0.02

IFN, interferon; IL, interleukin; TGF- β , transforming growth factor.

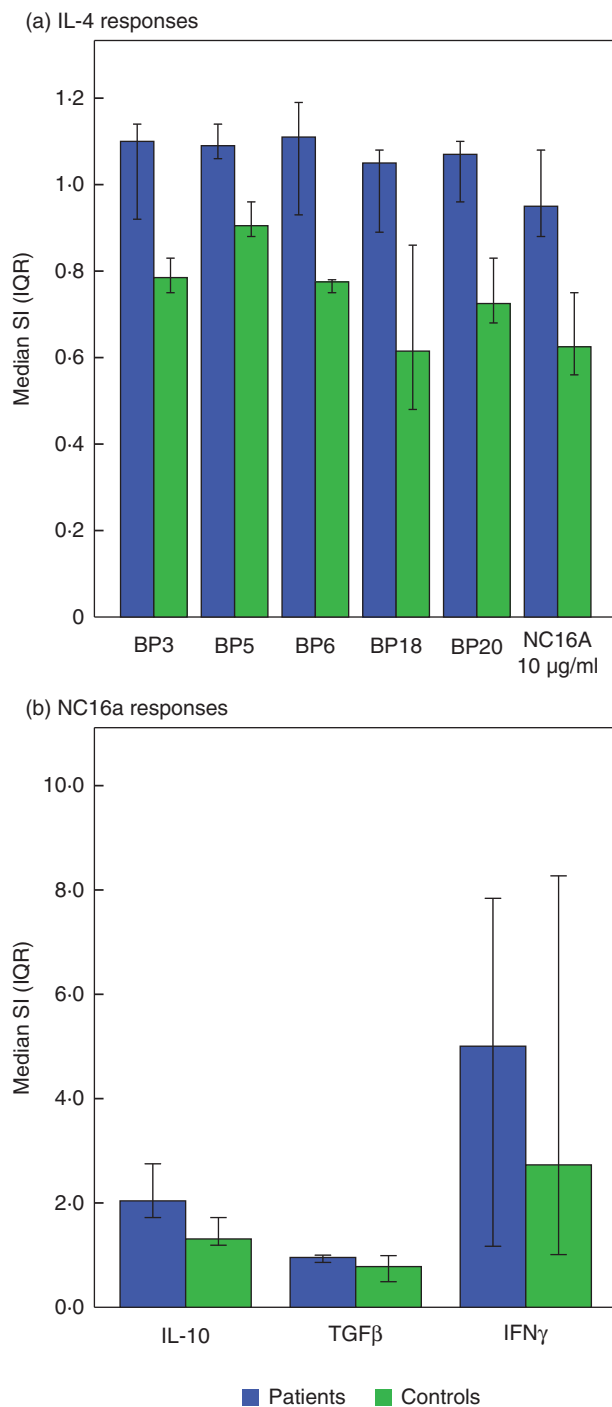


Fig. 3. (a) Shows interleukin (IL)-4 responses [median interquartile (IQR)] to NC16a showing six of the 22 peptides (Table 2 and Nc16a) with small but statistically significantly increased SI in patients compared to controls; others were not increased significantly. (a) Showing median SI (IQR) with no significance between patients and controls for IL-10, transforming growth factor (TGF)-β and interferon (IFN)-γ.

The results demonstrate a complex pattern of responsiveness, with heterogeneous cellular responses to NC16a and constituent peptides in both groups of individuals. Thus, there is no clear evidence of a simple causal relationship between T cell reactivity to NC16a and the development of BP. However, detailed analyses revealed a bias towards a Th2-type cytokine responsiveness *in vitro* which, together with confirmation of CD4⁺ T cell activation in selected cultures and the demonstration of specific IgE responses in BP, suggests that Th2 cells have a particular role in disease pathogenesis.

Although BP patients showed autoreactivity to NC16a and its epitopes, matched control populations also showed the same, as has been described in some other studies [32]. Thoma-Uszynski *et al.* [33] characterized autoantibody and Th cell responses to five overlapping peptides from NC16a in 35 patients. Unlike our study, these patients all had acute-onset disease and were not on therapy. The authors demonstrated T cell responses to NC16a in 82% of their patients. Eight of 12 (66%) patients responded to NC16a epitopes in another study [16] which tested five NC16a peptides. Our finding of 58% reacting to NC16a may be due to the higher SI threshold and to inclusion of patients with inactive disease, on treatment or in remission. Similar to our findings, Thoma-Uszynski *et al.* [33] demonstrated a correlation of T cell responses to peptides with overlapping sequences. Although they also observed IgG reactivity to NC16a epitopes, the association of this with Th cell proliferative responses was not significant, and they did not report T cell responses to NC16a in controls. Th1 responses to NC16a were seen in another study in healthy controls carrying the human leucocyte antigen (HLA) class 2 allele DQβ1*0301 [15], which is known to be associated with BP in North American Caucasians, approximately 35% of whom have this haplotype compared to 16% of controls [34]. However, this is not essential to the BP disease phenotype in other ethnic groups [35]. Recent computer modelling suggests affinity for this HLA binding in 13 peptide sequences in BP180. Only one of these sequences, IRRSILPYG (505–513), is predicted to bind HLA-DQ7 (DQβ1*0301) within the extracellular NC16A immunogenic region and is represented within our peptide panel in peptides 4, 5 and 6. These peptides did not appear to be biased predominantly to responses in any healthy or patient donor tested, with the exception of perhaps proliferative responses in matched control 8. As predicted, HLA binding is not necessarily a clear determinant of response type and is outside the remit of this study; it was considered that age- and sex-matched controls were more appropriate than HLA-matched controls. The responses we observed to the NC16a peptides cannot be explained by non-specific effects, as the response to each sequence varied between individuals, across both patient and healthy control groups, and was selective, as summarized in the factor analysis.

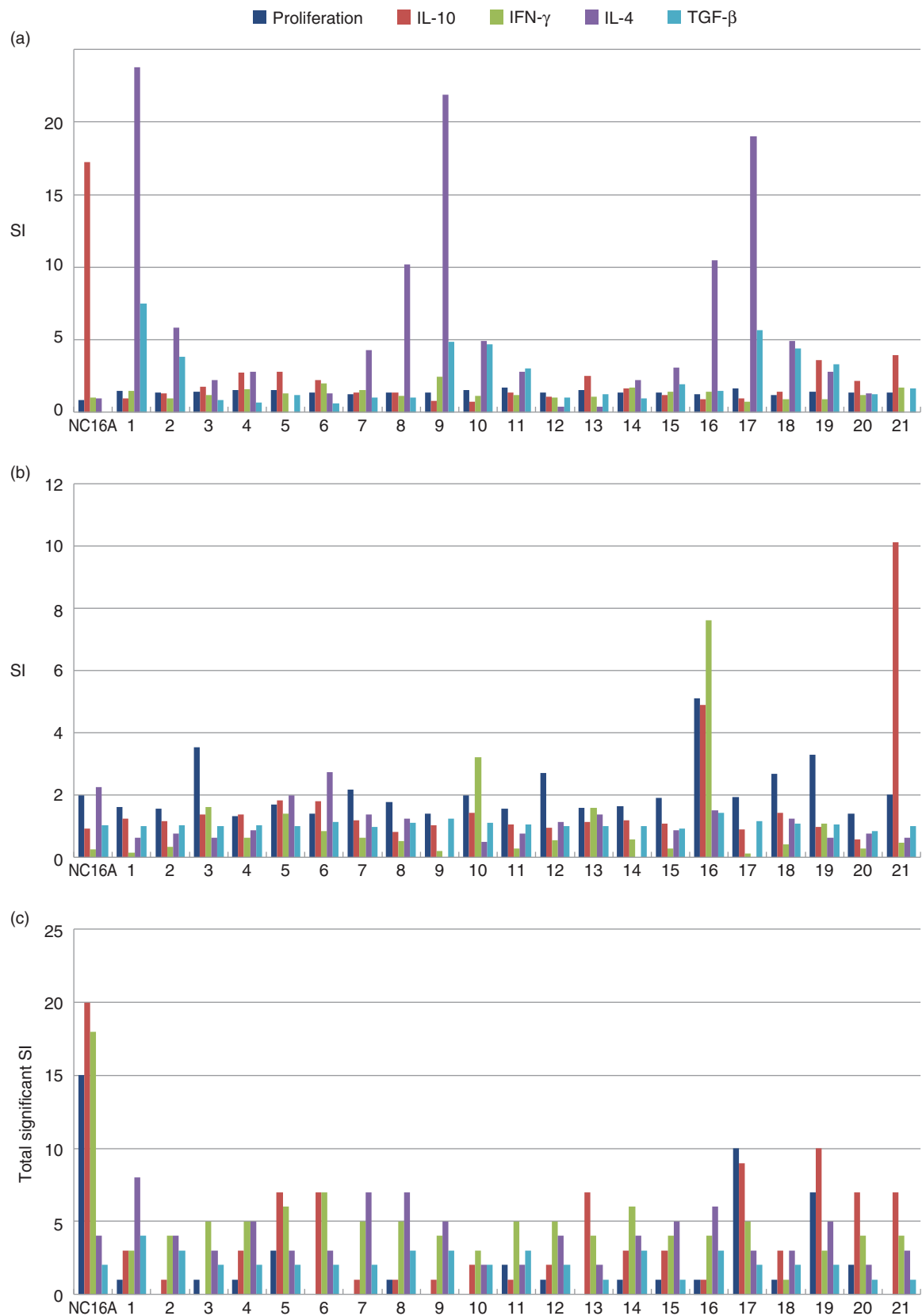


Fig. 4. Mapping of proliferative responses to recombinant NC16a and to the 21 peptides listed in Table 2 numbers on the x-axis represent the numbers of peptides, e.g. 1 = BP1: (a) responses (SI) in a typical patient ($n = 1$), with contiguous peptides eliciting interleukin (IL)-4; (b) responses of a control ($n = 1$); (c) the total count of significant SI reactions >2 from the cumulative data from all patients ($n = 28$).

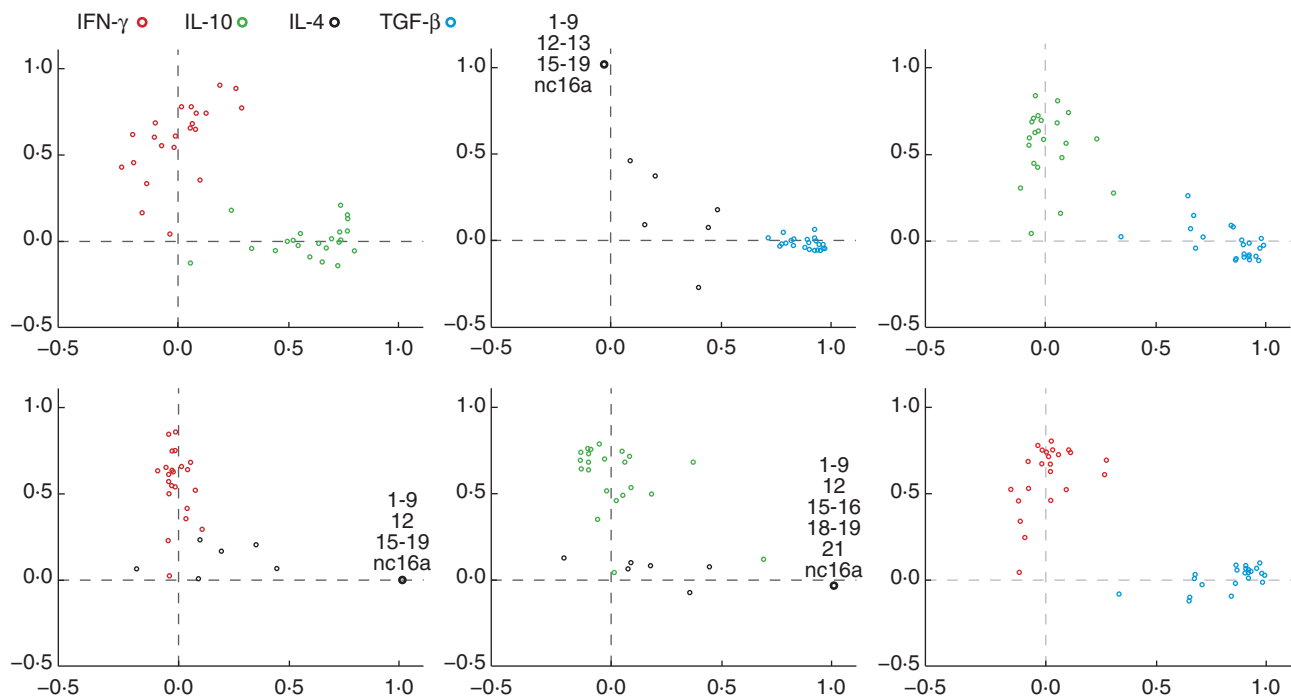


Fig. 5. Pairwise comparison of cytokines interleukin (IL)-10, interferon (IFN)- γ , IL-4 and transforming growth factor (TGF)- β in six factor loading plots. These represent the output of factor analysis of the two strongest components for each pair of cytokine responses. Each circle represents all subjects' cytokine responses to a particular peptide. These are plotted in rotated space (which assists meaningful interpretation). The x -axis represents component 1 (highest eigenvalue) and the y -axis component 2 for each of the factor analyses made. The plots show the extent to which similar cytokine responses to the 21 peptides cluster together and can be separated from other cytokine responses. These factors were then used to look for a meaningful clinical predictor (Table 3) that determines differential cytokine responses. Thus, in the top left panel, mathematically derived component 1 (x -axis) favours IL-10 and component 2 (y -axis) favours IFN- γ . Patients' age and the activity of disease were both predictors of the differential responses between these two cytokines.

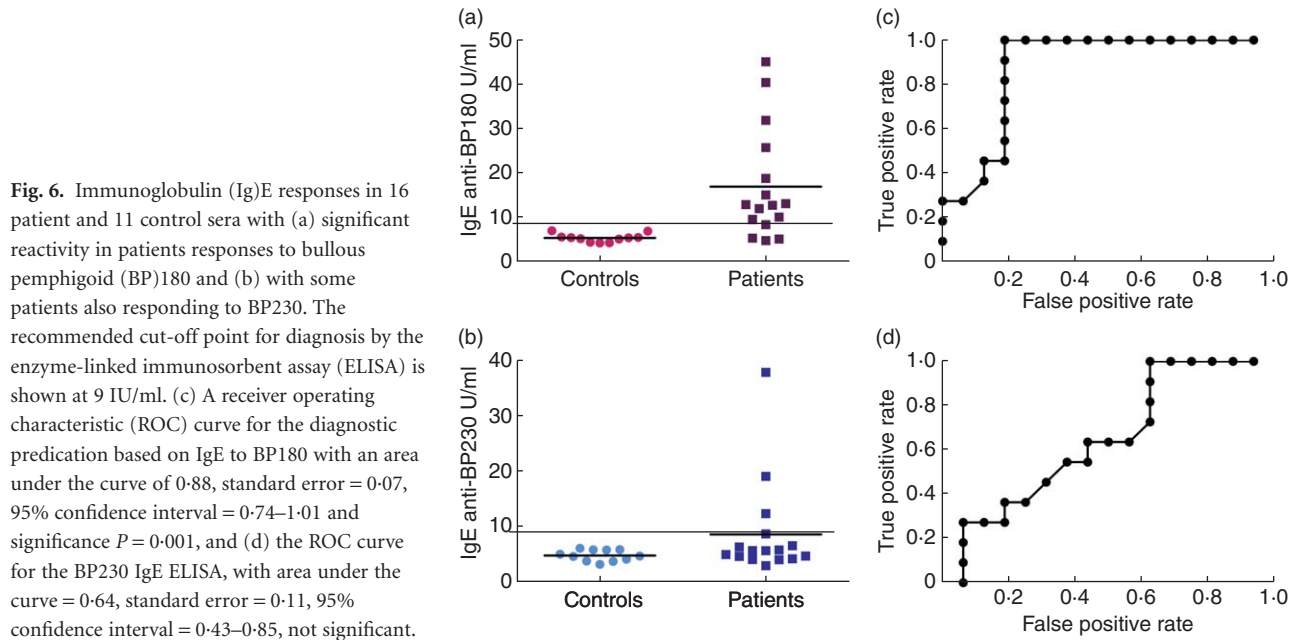


Fig. 6. Immunoglobulin (IgE) responses in 16 patient and 11 control sera with (a) significant reactivity in patients responses to bullous pemphigoid (BP)180 and (b) with some patients also responding to BP230. The recommended cut-off point for diagnosis by the enzyme-linked immunosorbent assay (ELISA) is shown at 9 IU/ml. (c) A receiver operating characteristic (ROC) curve for the diagnostic prediction based on IgE to BP180 with an area under the curve of 0.88, standard error = 0.07, 95% confidence interval = 0.74–1.01 and significance $P = 0.001$, and (d) the ROC curve for the BP230 IgE ELISA, with area under the curve = 0.64, standard error = 0.11, 95% confidence interval = 0.43–0.85, not significant.

Using factor analysis as a novel and powerful approach to represent the discrimination between cytokine responses served to illustrate that, despite the complex and apparently random different responses of four cytokines to the peptide array, there were patterns that could be revealed by this method and that these were meaningful. Thus, whether one or another cytokine was favoured was predicted by important differentials of age, itself a major determinant of the onset of pemphigoid [2], which determined relative responses to IFN- γ , IL-10 and TGF- β . Pemphigoid activity differentiated IL-10 from IFN- γ . This suggests that the relative contributions of cytokines associated with adaptive T_{reg}s and effector cells are important in BP activity. This is in keeping with suggestions that the effector and T_{reg} balance is particularly important in BP [18,25,26]. For example, Antiga showed that while circulating levels of IL-10 were not significantly different between BP patients and controls they were increased significantly after therapy for pemphigoid [26].

Dependent upon circumstance, IL-10 can be considered as either a Th2 or a regulatory cytokine [36]. The separation of IL-10 and IL-4 in the factor analysis is at least suggestive that the IL-10 responses we observed are more probably T_{reg}- than Th2-derived responses. Deficiency of FoxP3 in the peripheral blood in patients with BP [25], which is corrected by steroid therapy [26], and the significantly lower FoxP3/CD4 ratio in BP lesional skin compared to pemphigus [18], atopic dermatitis or psoriasis [26], all point to BP being a good example of an autoimmune disease in which the balance of T_{reg} and effector cells is particularly important.

This study, taken together with previous literature, suggests that cytokine responses to NC16a classically characteristic of T cell reactivity are not sufficient to lead to the clinical expression of disease. BP may be a heterogeneous disease in which NC16a is targeted by T cells in only some patients, or recruited into the response as a bystander effect due to epitope spreading [37]. Alternatively, the specific cytokine and proliferative responses detected *in vitro* here and elsewhere in patients or controls may be mediated in part by quiescent or naive populations that have not been activated *in vivo*. The readiness with which primary T cells can proliferate in culture is underestimated [38], and the slow kinetics of many responses to NC16a in the current work suggest that they contribute towards the reactivity observed. Why such cells remain unstimulated despite the presence of the antigen *in vivo*, and whether they can be recruited to a pathogenic response, remains to be established, but similar 'ignorant' autoreactive populations have been described for other autoantigens [22] and may be activated in disease [21]. Finally, the precise specificity or type of response, particularly the release of Th1, Th2 or regulatory cytokines, may influence its pathogenicity [21].

Previous mapping [15,16,33] of the fine specificity of T cell epitopes on BP has not been as extensive as in our

study, which revealed complex heterogeneous patterns of reactivity to peptides across the sequence of NC16a in both patients and controls. Factor analysis provided a useful tool to highlight factors that determine differential cytokine responses. These findings differ from Thoma-Uszynski *et al.* [33], who reported an association of more severe disease with responses to the NH₂ terminus, which also was immunodominant in antibody blotting [39]. However, epitopes recognized by autoreactive IgG are not confined to NC16a, but also encompass the intracellular domain of BP180 [39]. The IgG determinants also vary over time in individual patients, with epitope spreading and also loss of epitopes [37].

Cytokine responses to NC16a in BP and also other autoimmune bullous disease are heterogeneous. In BP, Budinger *et al.* [15] demonstrated that cloned T cells from controls produced IFN- γ only, while clones from patients produced Th1 and Th2 cytokines [15]. Similarly, mixed Th2 and Th1 cytokines were found in NC16a-reactive clones in BP [16]. Autoreactive T cell responses to NC16a have been demonstrated in pemphigoid gestationis as Th2 or mixed Th1/Th2 patterns [16,40], whereas in mucous membrane pemphigoid IFN- γ responses have been found [41]. Mixed Th1/Th2 responses have been reported in linear IgA bullous dermatosis [42]. When epitope mapping the entire sequence of NC16a we found that, although the signal was small, there was significantly more IL-4 secretion in patients compared to controls in response to NC16a and peptides BP3, BP5, BP6, BP18 and BP20.

Th1 cytokines preferentially induce IgG1 and/or IgG3, while Th2 cells preferentially induce IgG4 and IgE. Each of these isotypes has been documented in the antibody response to NC16a in BP [15–17,43,44], but their relative pathogenic importance is uncertain. In an animal model [45], IgG1 is proposed to be the main pathogenic subclass. If, as data from here and elsewhere suggest, a skew towards Th2 IL-4 responses is associated with BP, then this suggests a role for IgE autoantibodies. Fania *et al.* [46] show IgE responses in early pemphigoid [46], and Dresow *et al.* [4] found that eight of 10 sera contained IgE reactive with BP180, four of which were against extracellular NC16a, and also against the intracellular domain of BP180. Pomponi *et al.* [47] recently found 61% positivity to IgE against BP180 compared to 4% of controls [47]. Fairley *et al.* [43] described successful treatment of BP with an anti-IgE monoclonal antibody, with a similar report in infantile BP [48]. However, 7.7% of pregnant women and 2.2% of healthy controls had IgE reactive with NC16a without expressing blistering disease [49].

The results of the current study add to the complex picture of autoimmunity in BP. In contrast to some other antibody-mediated conditions [20,21], there is no clear association of particular types or specificities of T cell response with BP. Perhaps more than other inflammatory dermatoses the balance of T_{reg} and T_{effectors} is pivotal,

combined with increased drive from follicular T helper cells causing B cell activation and antibody production [19]. Further, the developments in gene therapy targeting epidermolysis bullosa have shown that transfection with DNA coding for NC16a is capable of inducing T_{reg}-mediated tolerance of this antigen in skin-grafted animals and point to enhancing specific immune tolerance as the future direction for therapy in BP [27]. Indications that Th2 and thus IgE responses may contribute to BP pathogenesis are further supported by our study, and agents specifically targeting IgE/Th2 are available or in development. Pursuing these developments could lead to better-targeted therapy for BP and help to reduce the high mortality [2,50] that is contributed to by current steroid therapy in elderly people [51].

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Disclosure

The authors have no conflicts of interest to declare.

Author contributions

W. J. P. planned the study, performed laboratory work, analysed data and was co-writer of the manuscript; V. G. planned the study, recruited patients, performed laboratory work and analysed data; A. M. H. supervised laboratory work and performed preliminary experiments; B. J. L. performed some of the experiments; R. H. supervised the IgE ELISA and diagnostic immunofluorescence and co-wrote the manuscript; R. N. B. designed, led and steered the project and co-wrote the manuscript; A. D. O. designed, led and steered the project, performed statistical analysis and co-wrote the manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Representative example (pemphigoid subject 17) showing typical ranges of CPM proliferation and cytokine concentrations.

Subject bp17	5day proliferation CPM	IL-4 pg ^{ml-1}	IFN γ pg ^{ml-1}	IL-10 pg ^{ml-1}	TGF β pg ^{ml-1}
Control	279.3333	1	734	169	71
PPD	5636.3333	4	39500	14.7	160
KLH	183.6667	6.1	576	89.9	88
BP1	408.3333	23.8	913	98.3	86
BP2	378.6667	5.8	570	137	74
BP3	392.3333	2.2	706	188	79
BP4	420.6667	2.8	976	287.5	85
BP5	431.3333	0	775	293.8	78
BP6	385.6667	1.3	1209	237.4	80
BP7	353.3333	4.3	921	140.87	73
BP8	381.3333	10.2	672	142.9	71
BP9	371.6667	21.9	1490	81.5	80
BP10	426	4.9	693	78.9	72
BP11	474.3333	2.8	714	142.9	70
BP12	373.3333	0.4	624	110.5	64
BP13	432	0.4	659	263.6	73
BP14	371.6667	2.2	1053	176	70
BP15	384	3.1	858	126.6	70
BP16	347	10.5	871	92.3	64
BP17	452.3333	19	431	100.4	68
BP18	329.6667	4.9	535	149.8	75
BP19	400.3333	2.8	563	382.8	99
BP20	377.6667	1.3	720	226	62
BP21	375.3333	0	1025	415.8	73