Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2011.04464.x

Up-regulation of CCL11 and CCL26 is associated with activated eosinophils in bullous pemphigoid

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Accepted for publication 25 July 2011
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Summary

Eosinophils contribute to the pathogenesis of bullous pemphigoid (BP) by secretion of proinflammatory cytokines and proteases. Trafficking of eosinophils into tissue in animal models and asthma depends on interleukin-5 and a family of chemokines named eotaxins, comprising CCL11, CCL24 and CCL26. Up-regulation of CCL11 has been described in BP, but the expression of the other two members of the eotaxin-family, CCL24 and CCL26, has not been investigated. In addition to these chemokines, expression of adhesion molecules associated with eosinophil migration to the skin should be analysed. We demonstrate that similar to CCL11, the concentration of CCL26 was up-regulated in serum and blister fluid of BP patients. In contrast, the concentration of CCL24 was not elevated in sera and blister fluid of the same BP patients. In lesional skin, CCL11 and CCL26 were detected in epidermis and dermis by immunohistochemistry. In contrast to CCL11, CCL26 was expressed strongly by endothelial cells. In line with these findings, eosinophils represented the dominating cell population in BP lesional skin outnumbering other leucocytes. The percentage of eosinophils expressing very late antigen (VLA): VLA-4 (CD49d) and CD11c correlated with their quantity in tissue. Macrophage antigen (MAC)-1 (CD11b/CD18) was expressed constitutively by tissue eosinophils. In conclusion, these data link the up-regulation of the eosinophil chemotactic factor CCL26 in BP to the lesional accumulation of activated eosinophils in the skin. Thereby they broaden the understanding of BP pathogenesis and might indicate new options for therapeutic intervention.

Keywords: bullous pemphigoid, CCL11, CCL26, chemokines, eosinophil

Introduction

Bullous pemphigoid (BP) is the most prevalent autoimmune blistering skin disease, presenting with pruritic erythema preceding urticarial infiltrates followed by tense blisters on erythematous skin, predominantly affecting elderly people [1]. The hallmark of BP is the deposition of immunoglobulin (Ig)G autoantibodies and/or complement component C3 at the basement membrane zone [2]. These autoantibodies target hemidesmosomal proteins, most notably a 180 kD transmembrane hemidesmosomal glycoprotein (BP180) whose non-collagenous stretch called NC16A harbours the major antigenic site [3]. Autoantibody titres to NC16A correlate with disease activity, while a correlation of disease activity to titres of autoantibodies to other BP antigens, such as BP230 or BP200, has not been demonstrated [4]. Autoantibody presence in the skin is a prerequisite, albeit not sufficient to induce a lesion, as autoantibody deposits can be demonstrated regularly in healthy skin of affected individuals.

In animal models, as well as in *in-vitro* models, blister formation upon autoantibody binding in BP in contrast to pemphigus vulgaris depends on the presence of granulocytes and their release of proteases [5]. In BP patients, lesional skin is characterized by the presence of a cellular, mainly granulocytic, infiltrate and complement deposition [5–7]. Eosinophils seem to play an essential role in the initiation and/or progression of human BP by secretion of eosinophil-derived proteases. These enzymes process basement membrane structural proteins [8] and activate neutrophilic elastase, a very potent cleaver of BP180 in human BP blister fluid [9,10] Their secretion and long persistence in the tissue may also explain the exquisite pruritus present in BP but not in pemphigus vulgaris [11].

The recruitment of eosinophils into tissues is a multi-step process. Tethering and rolling are followed by firm adhesion to endothelial cells and subsequent transmigration. Chemoattractants produced locally by endothelial or stromal cells can activate leucocytes and induce up-regulation of integrins. In BP and allergic conditions, interleukin (IL)-4 can induce production of CCL11 in fibroblasts [12]. This chemokine belongs to the eotaxin subfamily of CC-chemokines consisting of eotaxin/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26. CCL11 binds to CCR3 expressed on eosinophils and has been shown to induce eosinophil chemotaxis *in vitro* and *in vivo* [13]. CCR3 is the main chemokine receptor expressed on all eosinophils in blood, whereas other receptors such as CCR1, CCR2, CCR5, CCR8, CXCR1, CXCR2, CXCR3 and CXCR4 were found only in a minor subpopulation of the cells [14–16]. Beside eotaxins, the ligands CCL5, CCL7, CCL8, CCL13 and CCL15 can induce CCR3-mediated migration of eosinophils [17,18]. Of those, CCL13 has been found to be up-regulated in BP [19], whereas the weaker CCR3 ligands CCL5, CCL7 and CCL8 are not elevated significantly in sera of BP patients [20]. Activation of eosinophils through CCR3 results in enhanced avidity of $\alpha 4$ and $\beta 2$ integrins [21]. The $\beta 2$ integrins (CD11a/CD18 (LFA-1), CD11b/CD18 [macrophage antigen (MAC)-1, CD11c/CD18] are expressed by all granulocytes and mediate binding to intercellular adhesion molecule (ICAM)-1. Unlike neutrophils, eosinophils express the ligand of vascular cell adhesion molecule (VCAM)-1, $\alpha 4/\beta 1$ integrin [CD29/CD49d (very late antigen-4: VLA-4)], that is especially important for eosinophil arrest [22]. This selective expression of VLA-4 in eosinophils and the inducibility of the ligand VCAM-1 by IL-4 on endothelial cells might explain the predominance of eosinophils in diseases with high levels of IL-4-like allergic asthma or BP.

In order to investigate further the mechanism of eosinophil recruitment in BP, we analysed the serum of a large number of patients for expression of CCR3-specific chemotactic ligands, correlated the concentration of these ligands with blister fluid levels in selected patients and studied the expression of integrins on lesional eosinophils.

Materials and methods

Patients and samples

Blood serum samples were obtained for diagnostic purposes from caucasian individuals before systemic immunosuppressive treatment for autoimmune blistering diseases, including patients with BP (age 56–90 years, mean 77 years, 56% female), non-atopic healthy controls (age 16–87 years, mean 38 years, 58% female) and patients with pemphigus vulgaris (PV) (age 38–87 years, mean 62 years, 53% female). Included were only sera from BP patients with typical presentation with bullous disease and direct immunofluorescence of perilesional skin revealing linear deposits of IgG and/or C3 along the basement membrane zone, as well as indirect immunofluorescence (IIF) performed on monkey oesophagus and sodium chloride-split skin, revealing linear staining of the induced blister roof. Clinical severity was documented by the clinicians using a three-point clinical scoring system documenting the extension of patients lesions: mild = few lesions affecting maximally 10% of the body surface; moderate = fresh infiltrates and blisters covering up to 50% of the body; severe = heavy disease with infiltrates and blister formation on 50% of body surface or higher. In three patients with active blister formation, blister fluid and serum could be obtained. Diagnosis of PV was confirmed by staining intercellular IgG deposits in lesional epidermis and in indirect immunofluorescence on monkey oesophagus. Skin biopsies and blister fluid samples were obtained for diagnostic purposes. Patients did not take immunosuppressive drugs for other indications. Concomitant medication was taken in some patients for age-related diseases such as hypertension or diabetes.

Chemokine and cytokine measurement

CCL11, CCL24 and CCL26 levels in serum and blister fluid samples were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK), according to the manufacturer's instructions. The detection limits of the ELISAs were 5 pg/ml, 2·5 pg/ml and 2·3 pg/ml, respectively.

Immunofluorescence staining

Skin sections were stained with a panel of antibodies specific for cell markers and surface molecules (Table 1), as described previously [23]. Briefly, cryostat sections were stained with primary antibodies followed by secondary reagents labelled with fluorescence dye. Nuclei were stained by 4′,6-diamidine-2-phenylindole (DAPI). Analysis was performed using an Olympus IMT2 fluorescence microscope. Each antibody staining was controlled by staining with a respective unspecific antibody from the same species. For each eosinophil surface marker studied, five different subepidermal tissue areas of eight individual patients were photographed at 400-fold magnification. The cell density and percentage of double staining was calculated by manual counting of the cells in the photographed area (0·2 mm^2). In order to minimize observer-related errors, all slides were examined by three independent observers. The results show the means and standard deviation of those data.

Immunohistochemistry

For immunohistochemistry, cryostat sections of BP lesonal skin were incubated with antibodies against CCL11 (mouse anti-human IgG1, MAB320; R&D Systems) or CCL26 (goat anti-human IgG, AF693; R&D Systems). Detection of the

Table 1. Antibodies used for immunofluorescence staining.

Antibody against	Clone	Isotype/form	Source	Specificity relevant to present study
Lineage-specific markers				
Major basic protein (MBP)	Polyclonal	Mouse IgG1	Chemicon	Eosinophils
CD68	EBM11	Mouse IgG1	Dako	Monocyte lineage
CD3	UCHT1	Mouse IgG1–biotin	Pharmingen	T cells
Neutrophil elastase	AHN-10	Mouse IgG1	Pharmingen	Neutrophils
Secondary antibodies for lineage-specific markers				
Mouse IgG, IgM, IgA	Polyclonal	Goat-Texas red	Biozol	
Mouse Ig		Goat-rhodamin	Biosource	
Biotin		Streptavidin–FITC	Vector	
Rabbit IgG	Polyclonal	Alexa Fluor 488	Molecular Probes	
Mouse IgG1	Polyclonal	Alexa Fluor 546	Molecular Probes	
Antibodies against surface markers				
CD11c	EP1347Y	rabbit IgG	abcam	p150, integrin α
CD11c	3·9	Mouse IgG1-FITC	Biosource	p150, integrin α
CD49d	BU49	Mouse IgG1-FITC	Biozol	VLA-4
CD11b	LM2/1	Mouse IgG1-FITC	Biosource	MAC-1α, CR3
HLA-DR	EDU-1	Mouse IgG2b-FITC	Biosource	Human MHC class II
Factor VIII	F8/86	Mouse IgG1-FITC	Signet	Endothelial cells

Ig: immunoglobulin; FITC: fluorescence activated cell sorter; HLA: human leucocyte antigen; MAC: macrophage antigen; MHC: major histocompatibility complex; VLA: very late antigen-4.

primary antibodies was performed with donkey anti-mouse IgG biotin (Southern Biotec, Birmingham, AL, USA) or horse anti-goat IgG biotin (Vector Laboratories, Burlingham, USA) followed by streptavidin alkaline phosphatase (Biolegend, San Diego, CA, USA) and the substrate from LSAB-Kit-AP (Dako, Glostrup, Denmark). Unspecific mouse IgG1 and goat IgG were used as isotype controls.

Statistics

Data are presented as bars [indicating averages ± standard deviation (s.d.)] or box-plots (indicating average, median, 25–75% percentiles and s.d. (Origin 7·0 software; OriginLab Co., Northampton, MA, USA]. The Kolmogorov–Smirnov-test was used to test whether the samples were distributed normally. Statistical analysis was performed using the Mann–Whitney *U*-test for analysis of significant differences and Spearman's rank correlation test for testing correlation using WINstat software. In all cases, $P < 0{\cdot}05$ was considered to be significant.

Results

The eotaxins CCL11 and CCL26 but not CCL24 are up-regulated in serum and blister fluid of BP patients

In the search for chemotactic factors of the whole eotaxin family of chemokines, concentrations of CCL11, CCL24 and CCL26 were measured in serum and blister fluid of patients with BP compared to sera from patients with the autoimmune blistering disease pemphigus vulgaris (PV) and healthy controls. Values of CCL11 were higher in sera of BP patients than in healthy controls (mean values 133 pg/ml ± 108 *versus* 48 pg/ml ± 79, $P = 1{\cdot}5 \times$ E-6) or patients with PV (53 pg/ml ± 29, $P = 0{\cdot}002$) (Fig. 1a). The concentrations of CCL11 correlated with the extension of the disease indicated by the disease score. The highest levels of CCL11 were detected among patients with severe BP (Fig. 1b). CCL26 was up-regulated in 55% of the BP sera and mean values were 10-fold higher (34 pg/ml ± 69) compared to sera from patients with PV (3 pg/ml ± 6, $P = 0{\cdot}04$, Fig. 1a). The difference from healthy controls (9 pg/ml ± 17) was not statistically significant, because only patients with severe BP displayed elevated concentrations of CCL26 in serum (Fig. 1b). The mean of this subpopulation (105 pg/ml) was elevated significantly compared to healthy controls ($P = 4 \times$ E-5).

The levels of CCL24 were not significantly different in patients with BP (310 pg/ml ± 204) compared to patients with PV (362 pg/ml ± 317) or healthy controls (274 pg/ml ± 293; Fig. 1a). Concentrations of CCL24 did also not correlate with the disease score of BP (Fig. 1b).

Chemotaxis depends on a gradient of the chemotactic substance. To investigate the concentration-gradient from blood to tissue for the eotaxins we determined the concentration of the three eotaxins in blister fluid of three individuals with BP (Fig. 1c). In all blister fluid samples, the concentrations of CCL11 and CCL26 were increased, even in patients who presented with low serum concentrations of the respective chemokines. Blister fluid and serum of these two patients (patients 1 and 2, Fig. 1c) were collected during the first 48 h of blister formation. During this short disease

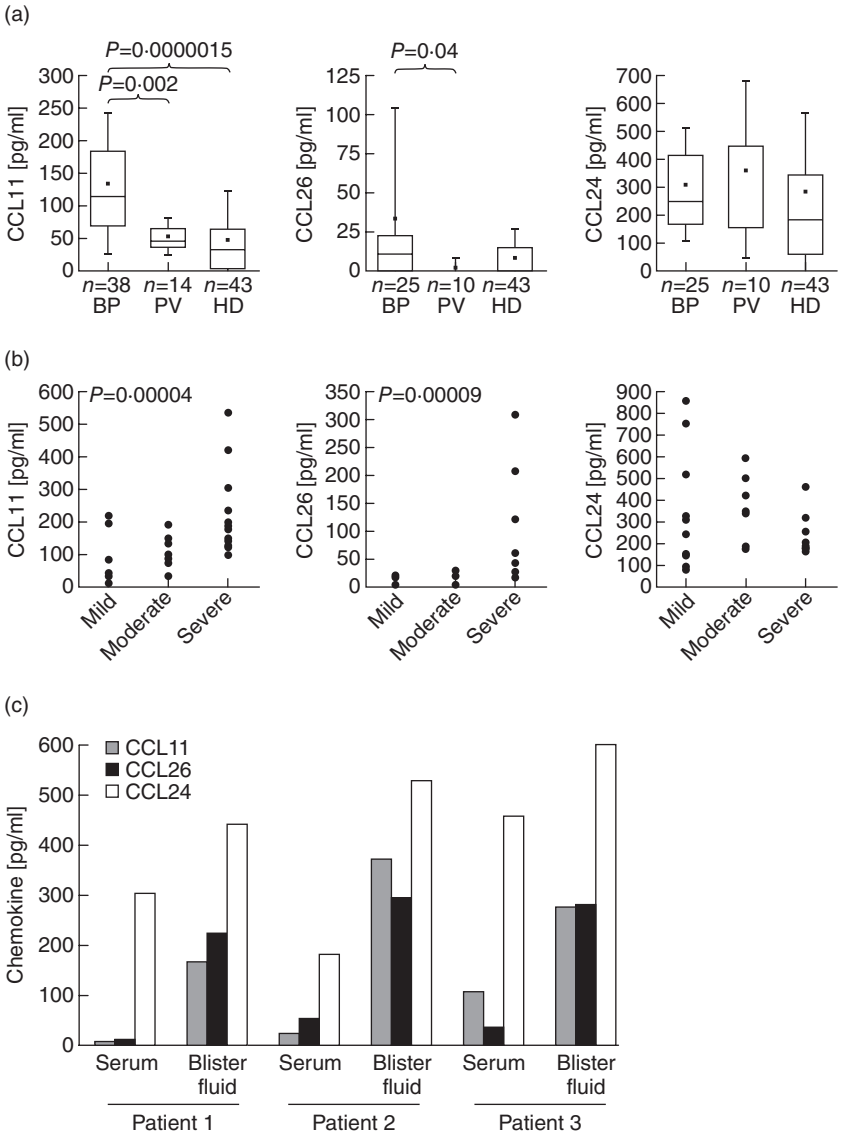

Fig. 1. CCL11 and CCL26 are up-regulated in bullous pemphigoid (BP). (a) CCL11 and CCL26 were determined in serum of patients with BP, patients with PV and healthy donors (HD) by enzyme-linked immunosorbent assay (ELISA). *P*-values determined by Mann–Whitney *U*-test indicate significant differences. (b) Concentrations of serum chemokines are grouped to the disease score of the patient. Mild = lesions covering maximally 10% of the body surface; moderate = BP lesions affecting up to 50% of the body; severe = heavy disease with infiltrates and blister formation on 50% of the body or higher. Significant Spearman's rank correlation test is indicated by the *P*-value (c) concentrations of CCL11, CCL24 and CCL26 were determined by ELISA in blister fluid of three individual BP patients compared to the respective serum concentration (*P*-values of the mean of three patients between serum and blister concentration: 0·04 for CCL11; 0·04 for CCL26 and 0·12 for CCL24).

period locally produced CCL11 might have not been distributed to the serum. We observed a ratio of blister fluid to serum for CCL11 of 2·5–33, with a mean of 45 pg/ml ± 55 in serum and 270 pg/ml ± 103 in blister fluid ($P = 0{\cdot}04$). The concentration of CCL26 detected in sera was 37 pg/ml ± 18 compared to 269 pg/ml ± 37 in the matched blister fluid ($P = 0{\cdot}04$). Thus, blister fluid concentration of CCL26 surpassed concentrations in matched sera at a ratio of 5·3–12·6. The blister fluid/serum ratios for CCL24 were only between 1·3 and 2·9 and the concentration was not increased significantly in blister fluid (525 pg/ml ± 80) compared to the sera (314 pg/ml ± 137, $P = 0{\cdot}12$).

The up-regulation of the chemokines in blister fluid prompted us to investigate their expression *in vivo*. Immunohistochemistry staining of CCL26 in lesional BP skin was performed and demonstrated a strong expression of the chemokine in lesional BP epidermis and dermis (Fig. 2a). CCL11 was also detected by immunohistochemistry in lesional BP epidermal and dermal skin (Fig. 2a). In contrast to CCL11, CCL26 was co-expressed by endothelial cells in the dermis, as indicated by double immunofluorescence staining with the endothelial cell marker factor VIII (Fig. 2b).

Eosinophils predominate in the inflammatory infiltrate in BP lesions

To evaluate whether the expression of eotaxins in BP is associated with increased numbers of eosinophils, eosinophils were quantified and compared to other inflammatory cells in the lesional tissue (Fig. 3). BP lesional skin exhibited large numbers of eosinophils (51/mm^2). Their amount was comparable to the total number of all cells of the CD68$^+$ monocyte/myeloid origin (59/mm^2) comprised of

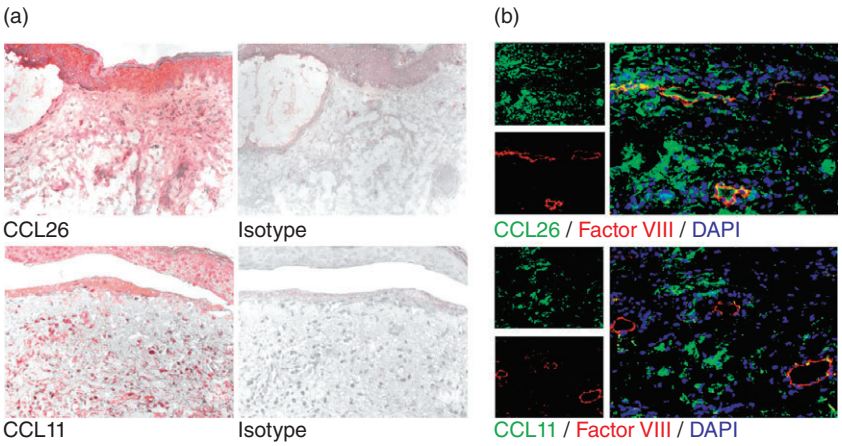

Fig. 2. CCL26 and CCL11 are detected in lesional bullous pemphigoid (BP) skin. (a) Immunohistochemical staining revealed deposition of CCL26 and CCL11 in BP skin. Isotype staining was negative. Representative stainings of five individual patients with BP are shown. Original magnification ×200 (b) Double immunofluorescence staining of Factor VIII marking endothelial cells (red) and CCL26 or CCL11 (green) in BP. Yellow fluorescence indicates coexpression of CCL26 and Factor VIII by endothelial cells. Nuclei are stained with 4′,6-diamidine-2-phenylindole (DAPI). Representative stainings of three individual patients with BP are shown. Original magnification ×200 .

macrophages and dendritic cells. $CD3^+$ T lymphocytes were less prominent, and eosinophils significantly outnumbered neutrophils (21/mm^2).

In lesional skin of patients with PV, we did not detect eosinophils. The density of the other inflammatory cell populations was comparable to BP lesions (Fig. 3).

Lesional eosinophils show signs of activation

Next we aimed at analysing the activation status of lesional eosinophils and the expression of adhesion molecules responsible for their immigration into the skin. Eosinophils were stained by antibodies to major basic protein (MBP), a major eosinophil derived-protease. Double immunofluorescence staining with antibodies against human leucocyte antigen D-related (HLA-DR) demonstrated that 38 ± 12% of tissue eosinophils in lesional skin expressed HLA-DR (Fig. 4a,b). This indicated the activation status of the infiltrated eosinophils, as eosinophils in blood do not express HLA-DR.

Further double immunofluorescence stainings of cryostat sections of eosinophils and adhesion molecules (CD11c, CD11b, CD49d) demonstrated that the percentage of eosinophils expressing CD49d (VLA-4) and CD11c correlated with the density of eosinophils in the lesions (Fig. 4a,b) The detection limit of surface molecules by immunofluorescence staining in lesional skin depends on their expression number on the cell surface. A higher percentage of positive cells can therefore indicate higher expression levels on the respective cells. The correlation of expression levels of CD49d and CD11c may suggest that these surface molecules can be recognized as activation markers for eosinophils in lesional skin. CD11b was constitutively highly expressed on eosinophils (Fig. 4b).

Discussion

In this study we demonstrate that in addition to CCL11, the chemokine CCL26 is expressed in lesional BP skin. The concentration of CCL26 is elevated in blister fluid and serum of BP patients, establishing a gradient from tissue to vessel. Both eotaxins are known to induce CCR3-mediated eosinophil migration [24]. Their up-regulation can therefore explain the high density of activated eosinophils in cutaneous BP lesions, which are required for blister formation, as has been proved in animal and *in-vitro* models [5].

The proof of significantly up-regulated levels of CCL11 in a large group of BP patients compared to PV and healthy

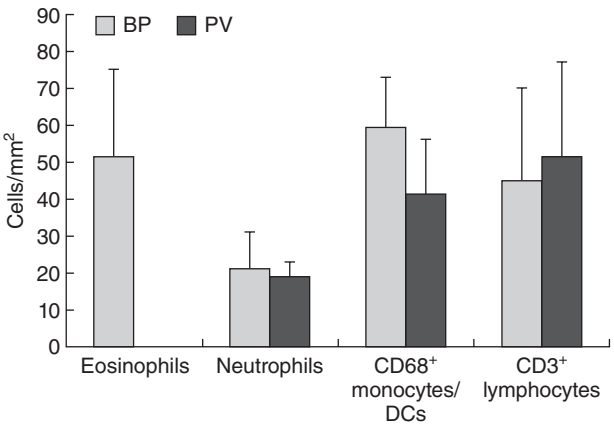

Fig. 3. Eosinophils predominate bullous pemphigoid (BP) lesions. Cells were stained by immunofluorescence and counted per visual field. Numbers were calculated per mm^2. The average densities and standard deviations of five different subepidermal tissue areas of nine individual patients with BP and four patients with pemphigus vulgaris (PV) for each cell type are shown.

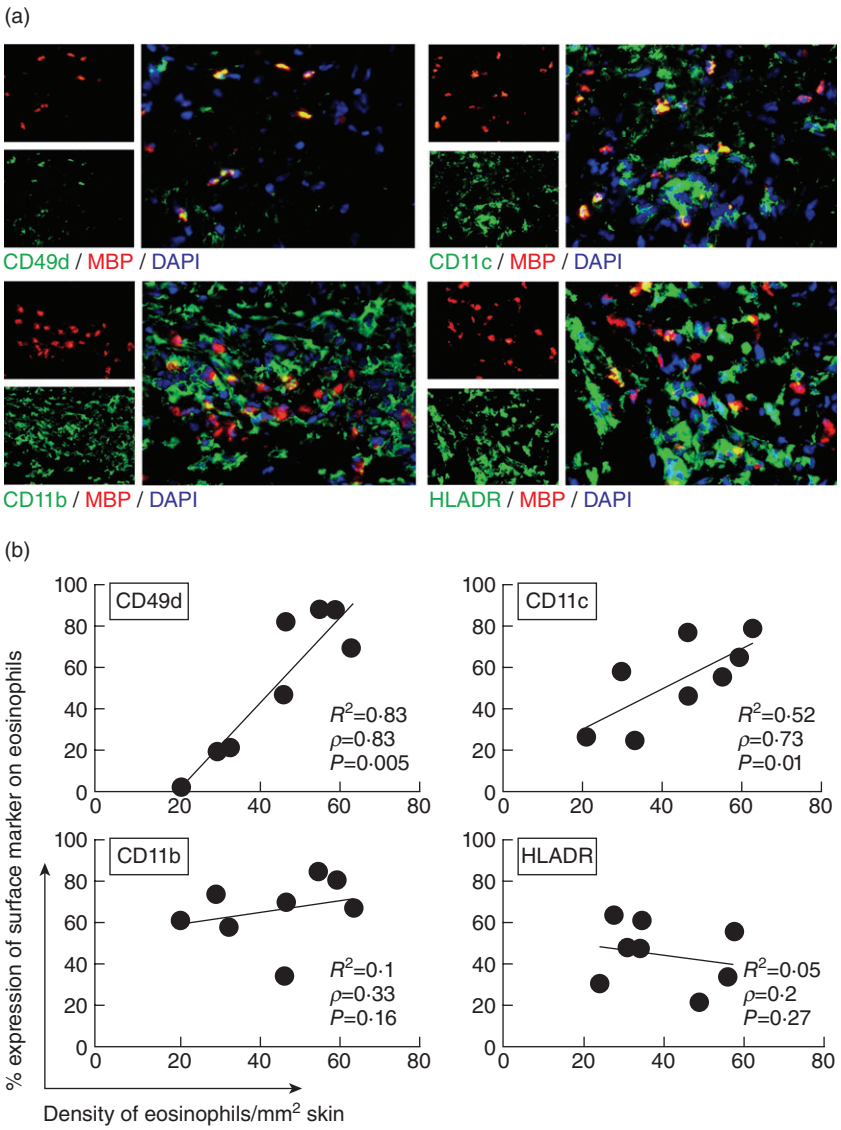

Fig. 4. Eosinophils up-regulate activation markers in bullous pemphigoid (BP). Two-colour immunofluorescence staining for eosinophils and surface markers was performed in eight lesional biopsies from BP patients. (a) Using yellow fluorescence, double immunofluorescence stainings demonstrate the co-expression of surface molecules CD49d, CD11c, CD11b or human leucocyte antigen D-related (HLA-DR) on eosinophils major basic protein (MBP). Nuclei are stained with 4′,6-diamidine-2-phenylindole (DAPI) (blue). Original magnification ×400. (b) Eosinophils were counted in lesional BP skin and the percentage of double-positive eosinophils for each surface marker was determined (e.g. 27% of 21 eosinophils/mm^2 express CD11c). Three sections for each marker were analysed by three independent investigators. The correlation of the density of eosinophils in tissue and the expression of adhesion molecules on the surface of eosinophils is given by Spearman's correlation coefficient ρ and the respective *P*-value, as well as the linear correlation coefficient R^2. Each point represents the mean values of an individual patient.

controls substantiates previous investigations on smaller numbers of patients [25,26]. In addition, up-regulation of CCL11 in association with dermal eosinophil accumulation was shown in pemphigoid gestationis, a variant of BP occurring in the peripartum period of pregnant women [23]. The serum values of CCL11 correlated with the disease severity [20].

CCL11 can be expressed by many cell types including eosinophils, keratinocytes, fibroblasts and macrophages [27]. Its expression is induced by IL-4 in human dermal fibroblasts [12] and by tumour necrosis factor (TNF)-α, IL-1α and interferon (IFN)-γ in human umbilical vein endothelial cells (HUVEC) [28]. Keeping in mind that IL-4 is up-regulated in lesional skin of BP patients, it is important that CCL26 but not CCL11 was inducible by this cytokine in HUVEC [24]. In line with these *in-vitro* data, immunohistochemical stainings in our study displayed the predominant expression of CCL26 by endothelial cells. It can therefore be proposed that eosinophils are activated by CCL26 expressed on endothelial cells in BP, and then transmigrate to the centre of the inflamed tissue through the action of CCL26 and CCL11. This sequential activation has been suggested previously to explain eosinophil migration into skin lesions in atopic dermatitis. Like BP, atopic dermatitis lesions are characterized by IL-4 expression during development of the disease [24]. CCL26 is also induced by IL-4 and IL-13 in keratinocytes [29,30], which supports our immunohistochemical staining of CCL26 in the epidermis.

The concentrations of CCL11, CCL24 and CCL26 in blister fluid reflect the intercellular concentration in tissue. Their several-fold higher levels in blister fluid compared to the serum of BP patients constitute a concentration gradient that enables the eosinophils to migrate further from the vessels to the basement membrane, where blister formation is induced. The up-regulation of CCL24 only in blister fluid but not in the patients' sera accounts for a limited impact of

CCL24 in eosinophil migration in BP. However, one possible function of up-regulated CCL24 in tissue can be its influence on the eosinophil adhesion molecule usage from VCAM-1 to ICAM-1 [31]. CCL24 was the only one of the three eotaxins not up-regulated in serum, but its total concentration in healthy controls as well as BP patients exceeded that of CCL11 and CCL26. This pattern has been described previously in atopic dermatitis, Churg–Strauss syndrome and leprosy [32–34]. Effects exerted by lower concentrations of CCL11 and CCL26 in the presence of higher concentrations of CCL24, which binds to the same receptor CCR3, may be a function of the receptor's affinity for the three chemokines. In Chinese hamster ovary (CHO) cells transfected with CCR3, CCL11 and CCL26 bound with higher affinity to CCR3 compared to CCL24 [35]. The base for this difference in affinity may be the three-dimensional structure of CCR3 after post-translational modification by sulphation, as Zhu *et al*. [36] described recently that double tyrosine sulphation of an N-terminal CCR3-derived peptide resulted in a 10-fold higher binding affinity of CCL11 and CCL26 to CCR3 compared to CCL24 [36]. Tyrosine sulphation of cell surface proteins occurs commonly in the trans-Golgi network, and has been detected in other chemokine receptors. Moreover, tyrosine residues have been modelled to be present in the binding site pocket of CCR3 [37,38]. The resulting higher affinity of CCR3 for CCL11 and CCL26 could be a reason for the effectiveness of CCL11 and CCL26 in the recruitment of eosinophils, despite the higher total concentration of CCL24.

Transmigration of eosinophils through the endothelium requires the expression of adhesion molecules and results in activation and up-regulation of specific eosinophil functions [39]. Consequently, eosinophils in BP expressed HLA-DR.

Selective expression of the adhesion molecule VLA-4 (CD49d/CD29) on eosinophils in contrast to neutrophils is one suggested mechanism by which predominant infiltration of eosinophils in asthma, atopic dermatitis and also BP is achieved [40]. VLA-4 interacts with VCAM-1 expressed by endothelial cells in skin [41] and lung [42], and mediates rolling, adhesion and transmigration of eosinophils.

In contrast to the suggested importance of the VLA-4–VCAM-1 interaction for emigration of eosinophils into tissue, therapeutic blockade of the α4-integrin chain by specific antibodies failed to prevent airway eosinophilia in humans [43]. In BP patients with low lesional eosinophil numbers, the percentage of CD49d was lower compared to highly infiltrated lesions. This low expression of CD49d might indicate that this integrin is not essential for transmigration, and could partially explain the lack of effectiveness of antagonizing drugs *in vivo*.

CD11b/CD18 is expressed on purified blood eosinophils [40]. Its interaction with ICAM-1 is influenced greatly by activation. CD11b/CD18 can also mediate a constitutive low-level adhesion of VCAM-1 and supports adhesion and transmigration together with $\alpha_4\beta_1$ [40]. Interaction of CD11b with its ligands on endothelial cells can also induce activation of eosinophils, as shown by CD11b-dependent CD69 up-regulation on eosinophils [44]. In its function as complement receptor 3, CD11b can support eosinophil degranulation [45]. The levels of CD11b expression on tissue eosinophils in BP were constantly high and did not correlate with the number of eosinophils. This might indicate a requirement of CD11b for transmigration [46] and function of eosinophils in BP [47].

All other integrins expressed on eosinophils, including CD11c/CD18, did not seem to play significant roles in eosinophil adhesion because antibodies to this ligand did not block adhesion to ICAM-1, albumin or fibrinogen *in vitro* [40]. In BP lesions we observed a weak correlation between the number of eosinophils infiltrated and CD11c. This could point to CD11c as a possible activation marker on these cells.

The findings of this study can be integrated into the current pathogenetic model of BP [48]: hemidesmosomal antigens are presented aberrantly by dendritic cells and induce a specific T and B cell response. Circulating antibodies bind to BP180 predominately in the skin of the great flexures, where most BP180 is expressed and the disease often starts [49]. A high density of these antibodies might induce complement binding and IL-5, IL-4, TNF-α and eotaxin production in mast cells, followed by additional chemokine secretion by fibroblasts and luminal presentation of chemotactic factors by endothelial cells. IL-5 can mediate release of eosinophils [50] from bone marrow and CCL26 supports adhesion of eosinophils to endothelial cells. Upon activation, eosinophils transmigrate mediated by VLA-4 (CD49d) and MAC-1 (CD11b) and follow a chemotactic gradient of CCL11, CCL26 and CCL13. Beside CCR3-induced chemotaxis, the chemokine receptor CCR1, highly expressed on eosinophils in about 20% of atopic and non-atopic donors [16], might contribute to eosinophil chemotaxis in some BP patients, in which the ligand CCL3 is up-regulated [20]. Other non-specific chemoattractants might also contribute to eosinophil migration: prostaglandin D2 by acting on the receptor CRTH2 on eosinophils, complement components C3a and C5a or platelet-activating factor through their respective receptors [51,52]. After transmigration eosinophils up-regulate HLA-DR and stimulate Th2 cells to further secrete IL-4 and IL-5 [53]. Concomitant immigration of further eosinophils leads to secretion of large amounts of eosinophil-derived proteases such as matrix metalloproteinases, eosinophil cationic protein and MBP [9,48]. These enzymes promote blister formation and induction of concomitant inflammation [8]. Because of their important function, eosinophil immigration is essential for the development of BP. Regulation of eosinophil influx might be achieved by sequestration of CCL11 by CXCR3-expressing T cells located in BP-skin [54,55] and the antagonistic function of CCL18 and CXCL10 on CCR3 [54,56], both of which have been shown to be up-regulated in BP lesional skin [20,57]. In addition, CCL26 could exert antagonistic functions on eosinophils expressing CCR1 [58].

Our data on the expression of CCL26, CCL11 and CCL24 in BP lesions and the up-regulation of adhesion molecules on eosinophils contribute to the understanding of the recruitment process for eosinophils in BP, and might open new possibilities for future therapeutic intervention in this autoimmune blistering skin disease. They further enhance the current knowledge of the fine interplay of chemokines and integrins in facilitating transmigration, tissue invasion and *in-situ* activation, linking biological knowledge and clinical observation to establish pathogenetic significance of immunological findings.

Acknowledgement

We thank Andreas Herrmann for collecting sera of healthy controls and highly acknowledge the technical support from Katharina Blümlein, Lysann Mehlig, Nick Zimmermann and Jana Phillip. This study was funded by a MeDDrive and a habilitation grant of the Medical Faculty of the Technical University of Dresden to C.P. and C.G., respectively.

Disclosure

None.

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