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## **Eosinophils as putative therapeutic targets in bullous pemphigoid**

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### **Abstract**

Bullous pemphigoid (BP) is the most common autoimmune subepidermal blistering skin disease and is characterized by the presence of autoantibodies directed against the hemidesmosomal proteins BP180 and BP230 that can be detected in the skin and serum of BP patients. Histologically, the dermal infiltration of eosinophils is obvious. The objective of this review is to present evidence that eosinophils play a key role in the pathogenesis of BP. Eosinophils, together with cytokines and chemokines regulating their production, recruitment and activation, are abundantly present in lesional skin, in blisters and in peripheral blood of patients with BP.

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Recently, using a cryosection model, eosinophils were demonstrated to induce dermal-epidermal separation in the presence of BP antibodies. Thus, eosinophils and their products, as well as mediators regulating their function, present promising targets for the treatment of BP.

## **Introduction**

Bullous pemphigoid (BP) is the most common autoimmune, subepidermal blistering disease of the skin, mainly affecting elderly people. With the present aging demography, the incidence of BP has been increasing (1). In general, BP presents with a symmetrical, often generalized, severely itchy eruption of vesicles and blisters on eczematous and/or urticarial infiltrated lesions associated with severe pruritus (2). The diagnosis should be confirmed by histopathology, direct immunofluorescence analyses and immune serological tests (3). Characteristic for BP is the presence of autoantibodies directed against the hemidesmosomal proteins BP180 (BPAG2, collagen XVII) and BP230 (BPAG1epidermal isoform) that can be detected in the peripheral blood as well as skin of BP patients (3). Specifically, BP180 autoantibodies have been shown to play a crucial pathogenic role as they trigger an inflammatory cascade with subsequent tissue damage, and ultimately, blister formation (4, 5).

The management of BP should be stepwise and comprises topical and systemic corticosteroids, tetracycline with nicotinamide, dapsone, immunosuppressive drugs, e.g. azathioprine, methotrexate, mycophenolate and chlorambucil, as well as rituximab, omalizumab, intravenous immunoglobulins, and immunoadsorption for severe, refractory cases (3). These therapies have been proven to be effective in randomized trials or case series, however, they are often associated with serious adverse effects, in particular in elderly patients. Therefore, a better understanding of the cellular and molecular mechanisms of BP, as well as the development of targeted therapies, should be addressed for the unmet needs of BP patients. Most insights in the pathogenesis of BP have come from animal models, but until the present, little has been reported about the potential role of eosinophils that are prominent skin infiltrating cells in human BP.

## **Eosinophils: multifaceted immune cells**

Despite the fact that the skin is normally devoid of eosinophils, eosinophil infiltration can be found in a broad spectrum of infectious, allergic, autoimmune, and neoplastic skin diseases (6). So far, information about a direct pathogenic role of eosinophils in skin diseases is scarce; indeed most knowledge about eosinophil function has been generated in the context of parasitic infections, bronchial asthma and hypereosinophilic syndromes. Blood eosinophilia derives from either mutations or gene fusions in the genome of the eosinophils (intrinsic eosinophilia) or increased production and release of cytokines affecting eosinophils by inflammatory or tumor cells (extrinsic eosinophilia) (7). Eosinophils are generated in and differentiate in the bone marrow from where they are released into the circulation. In the peripheral blood, they represent 1% to 5% of the leukocytes under physiologic conditions (8). Interleukin (IL)-3, IL-5, and GM-CSF represent eosinophil hematopoietins regulating the production, differentiation, activation, trafficking, and survival of eosinophils (9, 10).

Eosinophils are innate immune cells. Their primary function has been related to host defense, in particular, the protection against helminth parasites (11). By generating extracellular DNA nets associated with toxic granule proteins, the so called eosinophil extracellular traps (EETs), eosinophils have been shown to bind and kill bacteria (12, 13). On the other hand, the toxic granule proteins normally released to defend against infections, also cause tissue damage (11, 14). Although eosinophils expressing MHC class II can serve as antigen presenting cells, they are relatively inefficient when compared to dendritic cells or other professional antigen-presenting cells (15). In addition, eosinophils play an important role in repair and remodeling processes as well as in immunomodulation (16, 17). For instance, eosinophils contribute to tissue fibrosis and remodeling since they express mediators such as fibroblast growth factor (FGF)-2, IL-4, IL-6, IL-11, IL-13, IL-17, IL-25, TGF- $\beta$ , nerve growth factor (NGF), matrix metalloproteinase (MMP)-9 and vascular endothelial growth factor (VEGF) (18, 19). By producing the proliferation-inducing ligand April, eosinophils support the survival of plasma cells that are the main producers of antibodies (20). Depending on the inflammatory milieu, eosinophils can modulate either T helper (Th) 1 or Th2 immune responses by releasing cytokines

such as IL-2, IL-12, IL-18, and IFN- $\gamma$  or IL-4, IL-5, IL-13, and IL-25 (16, 21-25). The characterization of the cytokine expression by eosinophils in lesional skin revealed typical patterns distinctive for infectious, allergic, autoimmune and tumor diseases (6). To note, eosinophils that respond to IL-31-mediated chemotaxis and activation may themselves release IL-31 and thus might be contributing to the pruritus associated with BP (26) (Figure 1).

Eosinophils are potent effector cells, equipped with weapons that are stored in cytoplasmic granules: primary and secondary granules, small granules and lipid bodies (27). The cytotoxic cationic proteins that are stored in the secondary granules are formed by a core containing major basic protein (MBP) and a matrix composed of eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN) (28). MBP is highly cytotoxic as it affects the charge of surface membranes resulting in disturbed permeability, disruption and injury to cell membranes (29, 30). Moreover, the toxicity of MBP has been shown to be regulated by aggregation (31). ECP forms pores or transmembrane channels in target cell membranes (32). ECP and EDN have been identified as ribonucleases (33) and possess antiviral activity (34). Eosinophils store abundant amounts of ECP and may release it upon repeated stimulation with the same agonist, implying that mature eosinophils do not require a significant ECP re-synthesis (35). Moreover, they are a source of MMP-9 that is released upon stimulation with IL-5, enabling eosinophil migration through basement membranes (36).

### **Eosinophil inflammation in BP**

Eosinophil infiltration is a prominent feature of BP (Figure 1). Eosinophils are located in the upper dermis, often lining the dermal-epidermal junction. They can also be found in blisters. In hematoxylin & eosin (H&E) stained skin specimens, eosinophils can easily be identified as round cells stuffed with coarse eosinophil granules. Immunofluorescence staining using antibodies directed against ECP or MBP allows a more sensitive detection of eosinophils and extracellular granular protein deposits than just H&E staining. In BP, extracellular granular proteins have been detected either as separate deposits, as a thin coating on collagen bundles, so called flame figures, or associated with EETs (37-40).

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That the presence of eosinophils is useful to distinguish BP from other blistering dermatoses such as dermatitis herpetiformis has long been recognized (41, 42). In skin lesions, mainly hypodense eosinophils were observed suggesting an activated state (43). Such activated eosinophils were located in the basement membrane zone and showed degranulation on keratinocytes (44). Recently, the expression of CD69, reflecting an activation of eosinophils, has been observed in BP (45). Eosinophil degranulation was most prominent in early erythematous and pre-bullous (urticarial) lesions and thus seemed to precede blister formation (34). The accumulation of eosinophils in the skin correlated with the serum levels of BP autoantibodies of the IgE type in the subgroup of patients with IgE to BP230 (45).

A Th2 milieu, and together with it, cytokines and chemokines affecting eosinophil recruitment, activation, and survival, have been found in lesional skin, blister fluids and peripheral blood of patients with BP. In the skin of BP and pemphigoid gestationis patients, a predominance of Th2 cytokines such as IL-4, IL-5 and IL-13 has been observed (46, 47), even though elevated levels of both Th1 and Th2 chemokines were also found in the blood (48, 49). The serum levels of chemokines such as interferon-gamma induced protein (IP)-10, monokine induced by gamma-interferon (MIG), monocyte chemoattractant protein (MCP)-1 and eotaxin significantly correlated with the severity and activity of BP (50). Activated eosinophils were shown to preferentially attach to BP-skin in the presence of IL-5 (46, 47). Moreover, eotaxins, in particular CC chemokine ligands (CCL)11 and CCL26, and CCL17 (TARC) as well as their receptors CCR3 and CCR4, respectively, have been demonstrated in BP lesions (50-52). In addition to epithelial cells, eosinophils themselves expressed Th2 chemokines such as eotaxin and MCP-4, in particular upon stimulation with IL-5 (53).

Blister fluids of BP have been reported to contain eosinophil chemotactic factors (54) and may stimulate eosinophil colony formation and activation (55). Eotaxin that was strongly expressed by the keratinocytes around blisters, was detected at high levels in blister fluids correlating with the number of tissue eosinophils (56). Moreover, blister fluids contain high amounts of Th2 cytokines including IL-5 (57) (Figure 1).

Peripheral blood eosinophilia is a common finding in BP and may range from 5 to 43% (58). In BP patients, the levels of ECP and MPO were increased compared to controls and decreased upon immunosuppressive therapy (59). Elevated levels of IL-5 in serum and blister fluid correlated with ECP levels and disease severity (60, 61). Anti-inflammatory therapies such as interferon-gamma or prednisolone plus intravenous immunoglobulins for patients with severe BP resulted in a decrease of IL-5 levels and blood eosinophil numbers in parallel with the clinical improvement (62, 63). Serum EDN levels strongly correlated with NC16A-specific IgG (64). It has been suggested that by releasing tissue factor (TF), eosinophils might activate the coagulation cascade in BP, subsequently resulting in inflammation, tissue damage, blister formation and possibly thrombotic risk (65).

### **Eosinophils and tissue damage in BP**

Granule proteins have been observed in intact eosinophils as well as in extracellular granule deposits in the tissue of herpes gestationis (66). In addition to granule deposits in the tissue and flame figures, EETs have been detected in BP lesions (40). Interestingly, some of the EETs formed by eosinophils lining the dermal-epidermal junction were directed toward the basement membrane zone (40) (Figure 1).

The 92 kD-gelatinase (MMP-9) was found to be actively expressed by eosinophils in BP lesions and to be present in blister fluids (67) (Figure 1). In vitro experiments showed that 92-kD gelatinase is able to cleave the extracellular, collagenous domain of recombinant 180-kD BP antigen (67). In addition to eosinophils, MMP-9, MMP-2 and MMP-13 were expressed by T cells (68). Gelatinase-deficient mice were resistant to the blistering effect of passively transfected anti-mBP180 antibodies (69). In an ex vivo BP model, the inhibition of leukocyte elastase and MMP-9 resulted in a suppression of blistering (70).

### **Eosinophils and experimental BP models**

Upon injection of blister fluid of BP patients in the skin of a guinea pig, a mixed dermal infiltration containing mainly neutrophils, but also eosinophils, has been reported (71). In SCID mice with engrafted human skin, the subcutaneous injection of an anti-basement membrane zone antibody led to an infiltration of eosinophils and

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histologically evident subepidermal blister formation (72). Similarly, a mixed skin infiltrate composed of eosinophils, neutrophils and mast cells was observed in human skin grafted to nu/nu mice upon receiving IgE antibodies isolated from BP sera (73). The importance of neutrophils and neutrophil elastase in inducing subepithelial blister formation has been shown in various models of BP (74-76). The recruitment of neutrophils to the tissue depended on mast cell activation and degranulation following stimulation by macrophages (77-79). The function of anti-mBP180 IgG was mediated by its Fc domain, whereas the F(ab')<sub>2</sub> of IgG had no substantial pathogenic activity (79, 80). Fc gamma receptors II, III and IV have been reported to be involved in BP (81, 82). Complement has been demonstrated to be necessary for blister formation in a mouse model, but was not essential in an ex vivo cryosection model of BP (79, 83). As shown for neutrophils, the generation of reactive oxygen species was a prerequisite for subepithelial blistering (76).

#### **The controversy about eosinophils and IgE in BP**

In a subgroup of BP patients, both tissue bound and circulating IgE autoantibodies directed to BP antigens can be detected and their presence correlated with disease severity (84-86). As mentioned above, when an IgE hybridoma to LABD97, a component of the shed ectodomain of bullous pemphigoid antigen 2, or IgE from BP patients were injected in animal BP models, erythema and intense scratching as well as eosinophil infiltration and degranulation of mast cells were observed in histology (72, 73). In humans, IgE anti-BP230 autoantibody levels showed a strong association with local eosinophil accumulation (45). However, it seems unlikely that eosinophils directly interact with IgE, since they lack functionally active high-affinity IgE receptors (FcεRI) (87, 88). In fact, BP antigen-specific degranulation of basophils and/or mast cells rather than eosinophils has been suggested as a mechanism by which IgE may contribute to lesion development (89). Recently, minimal expression of the FcεRI alpha-chain on eosinophils of BP patients has been shown, however, a functionality of the receptor has not been demonstrated (90). Moreover, the same authors reported that IgE to BP antigens did not mediate either eosinophil degranulation or eosinophil localization to the dermal-epidermal junction (65).

### **Eosinophil-induced DES in a cryosection model**

Eosinophilic cells derived from a human myeloid leukemia cell line were shown to localize at the dermal-epidermal junction in the presence of BP serum, but not upon incubation with control serum (64). The attachment to the DEJ was mediated by IgG and complement, whereas IgE had no effect (64). However, under these conditions, a subepithelial blistering could not be observed, presumably owing to a lack of eosinophil degranulation (64). In agreement with these findings, we have recently found that purified human eosinophils added to ex vivo skin sections in the presence of BP sera did not induce any DES while extensive DES was observed when the whole leukocyte fraction or purified neutrophils were used (90). However, eosinophils stimulated with IL-5 caused diffuse tissue damage including focal separation at the DEJ (91). Moreover, in the presence of BP sera, IL-5 activated eosinophils lined up at the DEJ and induced DES of approximately 20% of the DEJ (91). Under these conditions, eosinophil-induced DES depended on adhesion and Fc $\gamma$ R binding, suggesting that BP autoantibodies directly activate eosinophils and/or attract them to the DEJ (91). Next, de Graauw et al. investigated molecular mechanisms involved in DES induced by IL-5 activated eosinophils in the presence of BP sera. Upon incubation with diphenyleiodonium (DPI), a NADPH inhibitor, DES was decreased in a concentration-dependent manner (91). The observation that blocking of degranulation significantly reduced eosinophil-induced DES implied that toxic granule proteins directly contribute to blistering. Moreover, a role for EETs was assumed since adding DNase to eosinophils which had been activated by IL-5 and BP sera significantly decreased DES in the cryosection model (91) (Figure 1).

### **Therapeutic implications**

Recognizing eosinophils as major players in the pathogenesis of BP would open new perspectives for a targeted therapy. So far, topical and systemic steroids have been recommended as first line therapy of both localized and generalized BP (3). If steroids are not sufficient, immunosuppressives such as azathioprine, methotrexate, mycophenolate or chlorambucil might be added (3). Recently, rituximab and omalizumab, monoclonal antibodies targeting B cells (CD20) and IgE, respectively, have been shown to be effective in BP (92, 93). The mortality of patients with BP, in particular when treated with systemic steroids and immunosuppressive drugs, is

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significantly increased as compared to the general population (94, 95). Thus, there is an urgent need for effective, well-tolerated and safe therapeutic tools for BP patients.

The anti-IL-5 antibodies mepolizumab and reslizumab have demonstrated efficacy and safety in patients with allergic diseases and extrinsic hypereosinophilic syndromes (96). Moreover, blocking the IL-5 receptor led to a reduction of blood, bone marrow, and tissue eosinophilia in asthma patients (9). Based on the knowledge that IL-5 specifically regulates eosinophil production, activation and survival and on the observations that IL-5 is increased in the skin, blister fluids and peripheral blood in BP patients, there is a rationale for investigating the clinical effects of inhibiting IL-5 or its receptor in BP. A clinical trial studying the effect of mepolizumab on BP has been initiated ([www.clinicaltrials.gov](http://www.clinicaltrials.gov); NCT01705795).

Since considerable amounts of eotaxins and their receptor CCR3 have been found in lesional BP, the inhibition of eosinophil recruitment by blocking eotaxins or CCR3 would be a further approach to treating BP. A clinical trial with bertilimumab, an anti-eotaxin-1 antibody, in BP is ongoing ([www.clinicaltrials.gov](http://www.clinicaltrials.gov); NCT02226146).

Instead of inhibiting cytokines and chemokines regulating eosinophil function, T cells as their main source could be targeted. Current therapies directly (e.g. steroids, immunosuppressives) or indirectly (e.g. rituximab) suppress T cell function. A novel approach to inhibit Th2 cell activation is the application of a GATA-3 specific DNase (98). Prostaglandin D2 (PGD<sub>2</sub>) has been reported to mediate activation and recruitment of eosinophils, basophils, and Th2 cells, as well as to promote an enhanced Th2 cytokine production through its receptor chemoattractant receptor-homologous molecule on Th2 cells (CRTH2). Blocking CRTH2 has been shown to be effective in eosinophilic diseases such as asthma and eosinophilic esophagitis (99, 100). So far, studies with CRTH2 inhibitors in BP are lacking. In addition to inflammatory cells, epithelial cells may produce cytokines promoting Th2 reactions including eosinophil activation. In BP, keratinocytes were shown to express TSLP and eotaxin (6, 56). Thus, in addition to inhibiting eotaxin, suppressing TSLP would be worthwhile to study in BP.

## Perspective

Over the last decades, increasing evidence for a pathogenic role of eosinophils in BP in mediating tissue damage has been presented. The proof of an active contribution by eosinophils to subepithelial blister formation in BP patients, however, can only be indirectly adduced. Recently, eosinophils have been reported to be able to induce DES in a cryosection model of BP. So far, no animal model for BP showing that eosinophils contribute to blistering has yet been reported, but further evidence for the role of eosinophils in BP can be anticipated from clinical trials investigating targeted anti-eosinophil therapies in humans.

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### Figure legend

Figure 1 A proposal for the role of eosinophils in the pathogenesis of bullous pemphigoid (BP). Eosinophils are produced, recruited and activated by cytokines released by keratinocytes (TSLP, eotaxin) and inflammatory cells, e.g. T cells (IL-5). Via their Fc $\gamma$  receptor (Fc $\gamma$ R), eosinophils attach to the hemidesmosomes at the dermal-epidermal junction. Upon activation, eosinophils produce reactive oxygen species (ROS), form eosinophil extracellular traps (EETs) and degranulate, releasing toxic granule proteins (ECP, MBP), metalloproteinase (MMP)-9 and IL-31 and, in this way, cause blister formation and itching.

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### Authors' contribution

DS, LB and HUS designed and wrote this review article.

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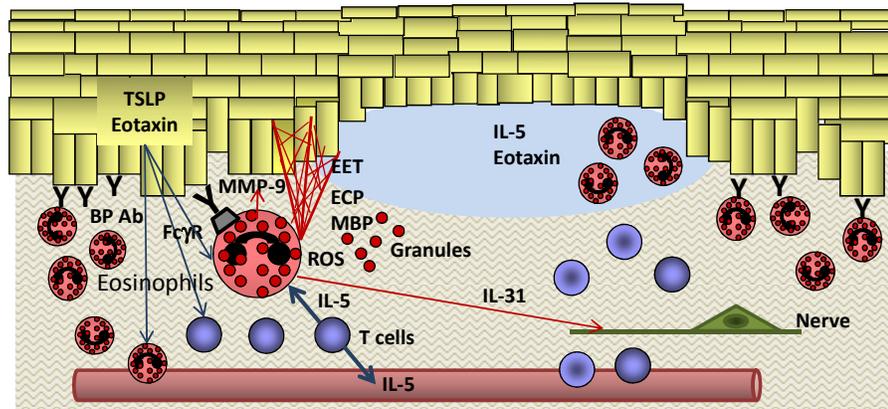


Figure 1