Journal of Genetic Syndromes & Gene Therapy

Hirose et al., J Genet Syndr Gene Ther 2013, S3
DOI: 10.4172/2157-7412.S3-005

Research Article Open Access

The Allosteric CXCR1/2 Inhibitor DF2156A Improves Experimental Epidermolysis Bullosa Acquisita

Hirose M[1], Brandolini L[2], Zimmer D[3], Götz J[1], Westermann J[3], Allegretti M[2], Moriconi A[2], Recke A[1], Zillikens D[1], Kalies K[3] and Ludwig RJ[1]*

[1]*Department of Dermatology, University of Lübeck, Germany*
[2]*Dompé s.p.a., L'Aquila, Italy*
[3]*Institute of Anatomy, University of Lübeck, Germany*

Abstract

Cytokines are an integral part of host response. However, aberrant cytokine responses are linked to chronic inflammatory diseases, including autoimmune bullous dermatoses (AIBD). AIBD are characterized by autoantibodies against cutaneous structural proteins, mucocutaneous tissue injury, and increased mortality. From the variety of differently expressed cytokines in AIBD, CXCR2 ligands, such as IL-8, seem to be promising therapeutic targets, as these have profound activating effects on neutrophils, which are indispensable for induction of skin lesions in different experimental models of AIBD. Therefore, we analyzed the contribution of CXCR2 ligands in experimental epidermolysis bullosa acquisita (EBA), an AIBD with autoimmunity to type VII collagen (COL7). We first evaluated the impact of the allosteric CXCR1/2 inhibitor DF2156A on neutrophil activation in vitro. The compound significantly reduced IL-8- and combined IL-8/immune complex-induced reactive oxygen species (ROS) release from neutrophils. Next, we noticed an increased expression of CXCL1 and CXCL2 in the skin of mice with experimental EBA. Interestingly, genetic (CXCR2-deficient mice) or pharmacologic inhibition (DF2156A) of CXCR2 function had only moderate and strain-dependent inhibitory effects in an antibody transfer model of EBA. In contrast, in a chronic, immunization-induced EBA mouse model, DF2156A showed profound therapeutic effects, which were comparable to high doses of systemic corticosteroids. Improvement of skin disease by blocking CXCR2 ligand function was independent of changes in circulating and tissue-bound anti-COL7 antibodies, excluding effects on adaptive immune cells. In summary, we here provide evidence that modulation of CXCR1/2 has therapeutic effects in EBA, a prototypical organ-specific autoimmune disease.

Keywords: Skin; CXCR2; Skin blistering; Autoimmunity; Animal model; Modulation; Allosteric inhibitor

Introduction

Autoimmune diseases have become a significant health burden, as the prevalence has continuously increased over the last decades [1], patients suffer from a high morbidity and mortality [2], and therapeutic options are mainly still limited to general immunosuppressive therapies. In several autoimmune diseases, autoantibodies are associated with certain diseases and their detection is used for diagnosis. Examples are antinuclear antibodies (ANA) in patients with systemic lupus erythematodes (SLE) or anti-cyclic citrullinated protein (CCP) antibodies in rheumatoid arthritis [3]. In other autoimmune diseases, the presence of autoantibodies has been clearly linked to disease pathogenesis, e.g. in autoimmune bullous dermatoses (AIBD). In AIBD, autoantibodies to desmosomal or hemidesmosomal adhesion proteins directly or indirectly induce tissue injury, manifesting as blisters on skin and/or mucous membranes [4].

Epidermolysis bullosa acquisita (EBA) is a prototypic AIBD, in which the autoimmune response is directed to type VII collagen (COL7), an important structural protein of the skin [5]. In animal models of the disease, Gr-1 positive leukocytes are a prerequisite for blister formation [6,7]. Furthermore, dermal-epidermal separation *ex vivo* depends on the Fc fragment of autoantibodies [6] and expression of specific activating Fc gamma receptors [8]. Correspondingly, F(ab')2 fragments of anti-COL7 IgG or anti-COL7 IgY failed to induce clinical disease in mice, while injection of rabbit, human or murine anti-COL7 IgG was shown to induce skin blisters [9]. This blister formation requires binding of Fc gamma RIV to autoantibodies deposited along the dermal-epidermal junction [10]. Interestingly, in this model, histological examination of skin biopsies from common gamma chain or Fc gamma RIV-deficient mice are completely devoid of dermal leukocyte infiltration [10]. This finding indicates that immune complex-induced activation of Gr-1 positive cells may contribute to a sustained influx of these cells into the skin. Activation induces manifold biological responses in neutrophils, including release and production of pro- and anti-inflammatory cytokines. In highly purified neutrophil preparations, IL-1ra, IL-8, M-CSF, GRO-α, MIP-1 and MIP-1β have been described to be released upon activation [11-13].

Based on these observations, we assumed that neutrophil-derived CXCR2 ligands such as CXCL2 may modulate disease manifestation in experimental EBA. Different small molecule CXCR1 and CXCR2 antagonists have been developed [14,15]. Among these, DF2156A is a novel, potent and selective dual non-competitive allosteric CXCR1 and CXCR2 inhibitor, which impairs leukocyte migration and shows therapeutic activity in acute and chronic experimental models of inflammation [16,17]. This compound is a second-generation derivative from reparixin (formerly repertaxin), a well-known CXCL8 inhibitor, and emerged for its optimal pharmacokinetic profile, including long half life and oral bioavailability [16]. DF2156A is currently under clinical development (Phase 2) with the aim to assess the efficacy of the

***Corresponding author:** Ralf J. Ludwig, Department of Dermatology, University of Lübeck, RatzeburgerAllee 160, D-23538 Lübeck, Germany, Tel: +49 451 500-2541; Fax: +49 451 500-5162; E-mail: ralf.ludwig@uk-sh.de

Received March 11, 2013; **Accepted** April 15, 2013; **Published** April 18, 2013

Citation: Hirose M, Brandolini L, Zimmer D, Götz J, Westermann J, et al. (2013) The Allosteric CXCR1/2 Inhibitor DF2156A Improves Experimental Epidermolysis Bullosa Acquisita. J Genet Syndr Gene Ther S3: 005. doi:10.4172/2157-7412.S3-005

Copyright: © 2013 Hirose M, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

molecule in preventing graft dysfunction after islet transplantation in type 1 diabetes patients.

To address the role of CXCR1/2 ligands and to evaluate their therapeutic potential in AIBD, we first analyzed the impact of allosteric CXCR2 inhibition on neutrophil activation *in vitro*. Subsequently, we studied CXCL1 and CXCL2 expression in the skin of mice with experimental EBA and evaluated the functional impact of CXCR2 inhibition.

Materials and Methods

Experiments with human cells

Peripheral blood leukocytes from healthy volunteers were isolated as previously described [6]. Cryosections from skin for *ex vivo* experiments were obtained from neonatal human foreskin. Serum samples from EBA patients (n=3) fulfilling the following criteria were included: (i) presentation with skin lesions resembling EBA, (ii) linear IgG deposition in direct immunofluorescent (IF) microscopy, (iii) an u-serrated pattern in direct IF microscopy and/or detection of autoantibodies against COL7 by western blotting. Approval for studies using biological material from humans was obtained from the Institutional Review board at the University of Lübeck (Lübeck, Germany), and informed consent was provided by each blood donor according to the Declaration of Helsinki.

Mice

All mice, aged 6-8 weeks, were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were maintained on a 12 h light–dark cycle at the animal facility of the University of Lübeck and were fed acidified drinking water and standard chow ad libitum. Animal keeping protocols were approved by the responsible governmental administration.

Evaluation of neutrophil ROS release

First, IL-8 at the indicated dose-range was added to freshly isolated human neutrophils in the presence and absence of immune complexes (COL7 and monoclonal anti-COL7 antibodies [18]), and ROS release was determined by chemiluminescence as described [8]. Based on these results, the optimal IL-8 dose for neutrophil activation was determined (25 ng/ml). This IL-8 concentration was then used to test the impact of DF2156A (10^{-8} to 10^{-4} M) on IL-8-induced ROS release in the presence and absence of immune complexes.

RT-PCR

For RT-PCR analysis, lesional (n=4) and non-lesional (n=3) skin samples from corresponding anatomical sites were obtained from SJL/J mice 3 weeks after immunization. RT-PCR from mouse skin was performed as described [10,19,20] using primer sets detailed in table 1.

Induction of EBA and phenotype assessment

EBA was induced in C57BL/6, BALB/c and CXCR2-deficient (BALB/c background) by repetitive injections of rabbit anti-mouse COL7 IgG as described [21]. Extent of skin disease was the primary endpoint of these experiments and was expressed as percent of body surface area affected by skin lesions and determined at 3 time points (day 4, 8, 12). From these time points, the area under the curve (AUC) was calculated, taking both disease onset and maximal disease activity into account. For secondary endpoints, (i) semiquantitative determination of the dermal leukocyte infiltration in skin specimen [22], (ii) IgG and (iii) C3 deposition at the dermal-epidermal junction specimen were obtained at the end of the experiment (day 12). Analysis of these endpoints was performed as described elsewhere [23].

Immunization-induced EBA was provoked by immunization of SLJ/J mice with an immunodominant fragment of the murine COL7 as described [24-26]. After immunization, mice were weekly monitored and treatment was started in individual mice when at least 2% of the body surface area was affected by lesions. DF2156A (15 mg/kg or 30 mg/kg) was emulsified in aqua injectabilia (Auqua.ad iniectabilia, Diaco, Naila Germany) and administered once per day orally by gauge for the period of four weeks. Methylprednisolone (Sanofi, Frankfurt/Main, Germany, 20 mg/kg) was injected i.p. once per day. Treatments were carried out over a 4-week period. The percentage of affected body surface area was determined weekly during this time, and from these data the AUC was calculated, reflecting the overall disease activity. Secondary endpoint analysis was performed as described above; skin and serum samples were obtained at the end of the treatment period.

Statistics

All data are presented as mean ± SEM, if not otherwise indicated. SigmaPlot 12.0 (Systat Software Inc, www.systat.de) was used to determine statistical differences. For comparison of differences among two groups, *t*-test or Rank Sum test were used. For comparison of differences among treatments, ANOVA or ANOVA on ranks for non-parametric distributed data was applied. A p-value of <0.05 was considered statistically significant.

Results

Allosteric CXCR1/2 inhibition hinders activation-induced reactive oxygen species (ROS) release from neutrophils

First, the effect of IL-8 on ROS release from neutrophils was evaluated. IL-8 dose-dependently increased ROS release from both resting neutrophils and cells, activated by immune complexes of COL7 and anti-COL7 antibodies. In detail, at doses below 25 ng/ml, no statistically significant effect of IL-8 on neutrophil ROS release

Gene	Sequence	Size (bp)	Accession number
CXCL1 probe	CCTCGCGACCATTCTTGAGTGTGGCTATGAC	142	NM_008176.2
CXCL1 for	CAGACCATGGCTGGGATTC		
CXCL1 rev	GAACCAAGGGAGCTTCAG		
CXCL2 probe	CCCTGCCAAGGGTTGACTTCAAGAACATCC	155	X53798
CXCL2 for	AGTGAACTGCGCTGTCAATG		
CXCL2 rev	GCTTCAGGGTCAAGGCAAAC		
MLN 51 probe	CACGGGAATCTCGAGGTGTGCCTAAC	134	NM_138660.2
MLN 51 for	CCAAGCCAGCCTTCATTCTTG		
MLN 51 rev	TAACGCTTAGCTCGACCACTCTG		

Information obtained from the National Resource for Molecular Biology Information (www.ncbi.nlm.nih.gov). Abbreviations: *for*, forward; *rev*, reverse

Table 1: Primer sequences, amplicon sizes, and gene accession numbers of the analyzed genes.

Citation: Hirose M, Brandolini L, Zimmer D, Götz J, Westermann J, et al. (2013) The Allosteric CXCR1/2 Inhibitor DF2156A Improves Experimental Epidermolysis Bullosa Acquisita. J Genet Syndr Gene Ther S3: 005. doi:10.4172/2157-7412.S3-005

was observed (ANOVA with (Bonferroni t-test for comparisons of treatments versus control). For both resting and immune complex-activated neutrophils, the maximal ROS release was achieved with an IL-8 concentration of 50 ng/ml, which declined if concentrations above 50 ng/ml were used in figure 1A and 1B). Addition of the allosteric CXCR1/2 inhibitor DF2156A to either IL-8 or IL-8 immune complex-activated neutrophils significantly impaired ROS release within a dose range of 10^{-8} to 10^{-4} M (Figure 1C and 1D). At the same dose-range, the compound did not modify spontaneous migration properties of PMN in the absence of IL-8 stimulation (reduction to 88 ± 13% compared to solvent control, p=ns, *t*-test).

Increased CXCL1 and CXCL2 expression in experimental mouse models of EBA

We had recently reported an increased CXCL1/KC expression in mice with autoantibody transfer-induced EBA [20]. Following up on this observation, we also evaluated the expression of CXCL1 and CXCL2 mRNA in immunization-induced EBA. For this purpose, CXCL1 and CXCL2 expression were determined in lesional and non-lesional skin from mice with immunization-induced EBA. We observed little expression of both chemokines in non-lesional skin; compared to the housekeeping gene MLN51, only 0.009 ± 0.002 CXCL1 and 0.015 ± 0.004 CXCL2 copies were detected (Figure 2). In contrast, in lesional skin of EBA mice, CXCL1 expression was 50-fold increased to 0.0458 ± 0.187 copies per MLN51 (Figure 2A). This was even more pronounced for CXCL2, where we noted an over 1,500-fold increase in CXCL2 expression in lesional compared to non-lesional skin samples from corresponding anatomical sites (Figure 2B).

Strain-dependent, moderate inhibitory effects of allosteric CXCR1/2 inhibition in autoantibody transfer-induced EBA

Interestingly and despite increased serum CXCL1 expression [20]

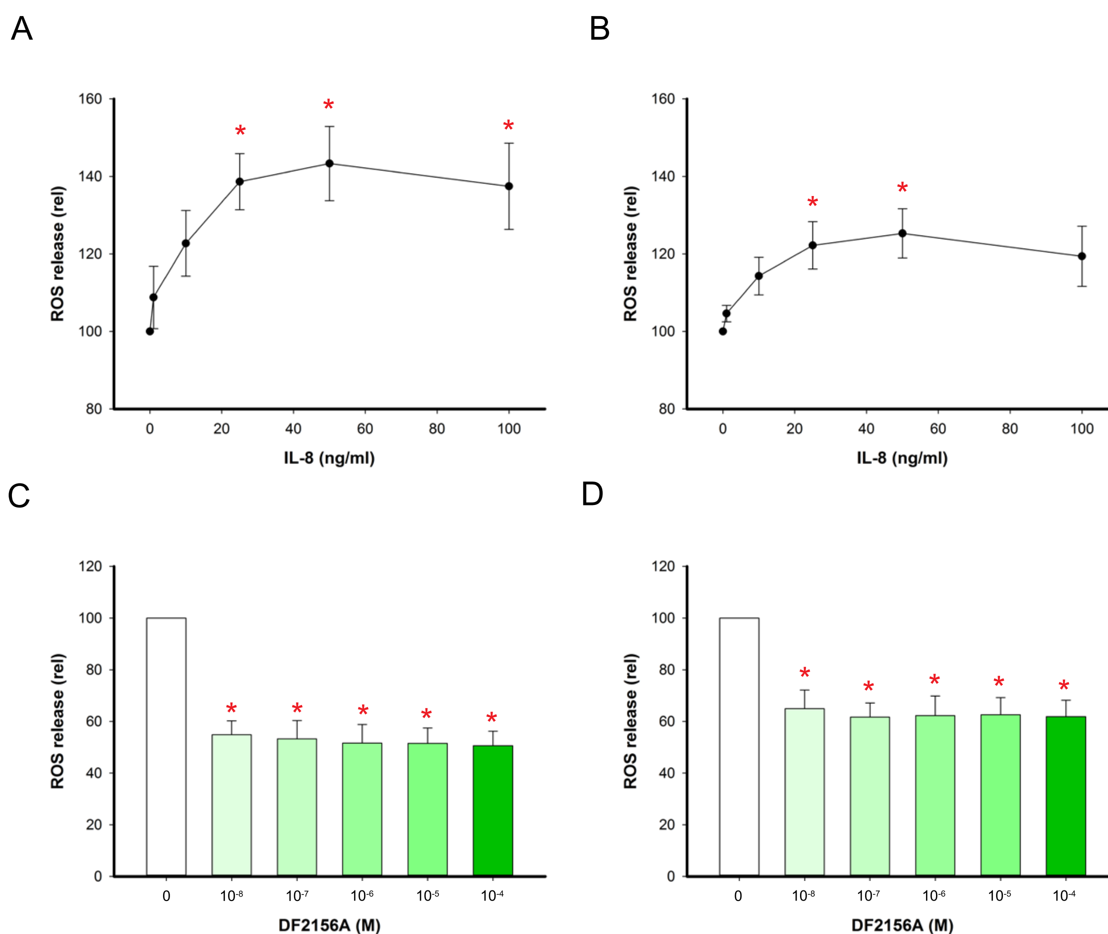


Figure 1: Allosteric CXCR1/2 inhibition impairs the IL-8-induced ROS release from neutrophils

A. Isolated human neutrophils were incubated with increasing doses of IL-8 and ROS release (in relation to unstimulated cells) was assessed by chemiluminescence. While IL-8 at concentrations of 1 ng/ml and 10 ng/ml had no significant impact on ROS release, addition of 25 ng/ml, 50 ng/ml or 100 ng/ml significantly increased the ROS release, which reached its maximum at 50 ng/ml. Data is based on 6 samples/group.

B. Similar findings were obtained when neutrophils were concurrently stimulated with immune complexes of COL7 and monoclonal COL7 antibodies. Data is based on 6 samples/group.

C. In the presence of DF2156A within the indicated dose-range, IL-8 (25 ng/ml)-induced ROS release was reduced to approximately 50% of ROS release from neutrophils treated with vehicle. Data is based on 5 samples/group.

D. Compared to control, DF2156A treatment reduces ROS release to 60% in neutrophils stimulated with IL-8 (25 ng/ml) and immune complexes. Data is based on 5 samples/group. *indicates statistic significance (ANOVA followed by Bonferroni t-test for multiple comparisons to control).

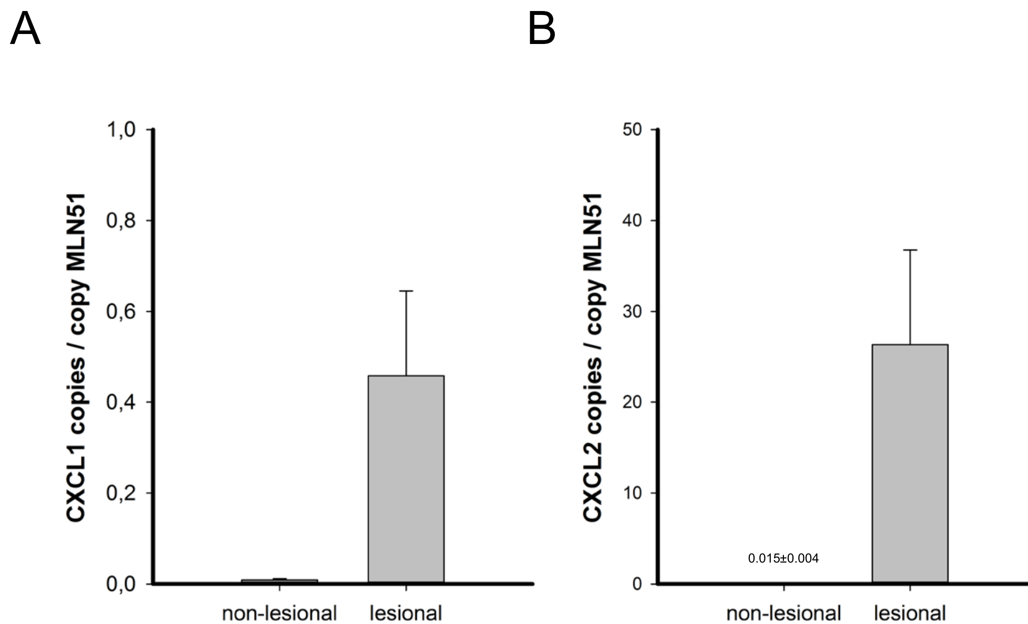


Figure 2: Increased cutaneous CXCL1 and CXCL2 expression in mice with experimental EBA

A. CXCL1, and

B. CXCL2 mRNA expression in non-lesional (healthy appearing) und lesional skin of SJL/J mice with immunization-induced EBA. Graphs represent mean ± SEM CXCL1/2 expression in relation to copies per MLN51. *indicates statistical difference (t-test). Data is based on 3 samples form non-lesional and 4 samples from lesional skin (from corresponding anatomical sites).

and increased CXCL1 expression in the skin (Figure 2), treatment of BALB/c mice with DF2156A within doses as high as 30 mg/kg had no effect on skin blistering induced by transfer of anti-COL7 IgG. In detail, BALB/c mice treated prophylactically with DF2156A at doses ranging from 3 to 30 mg/kg had a cumulative disease score expressed as area under the curve (AUC) of 18 ± 1 (3 mg/kg), 17 ± 3 (15 mg/kg) and 14 ± 1 (30 mg/kg). BALB/c mice treated with vehicle (saline) had scores of 14 ± 2, while mice treated with methylprednisolone (MP) had an AUC of 12 ± 2 (p=ns, ANOVA). Corresponding results were obtained when EBA was induced in CXCR2-deficient and wild type mice on the BALB/c genetic background (AUC of 40.3 ± 5.8 in BALB/c and 56.7 ± 5.2 in CXCR2-deficient mice, p=ns; *t*-test).

As pharmacological interventions have been noted to depend on the genetic background of the mice [27], we also evaluated the effect of DF2156A in C57BL/6 mice. In contrast to BALB/c mice, DF2156A application at 15 mg/kg or 30 mg/kg reduced extent of skin disease, while injection of MP had no effect (Figure 3A). These changes induced by DF2156A treatment were independent of changes in dermal infiltration as well as IgG and C3 deposition at the dermal-epidermal junction, all assessed at the ears of the mice (Figure 3B).

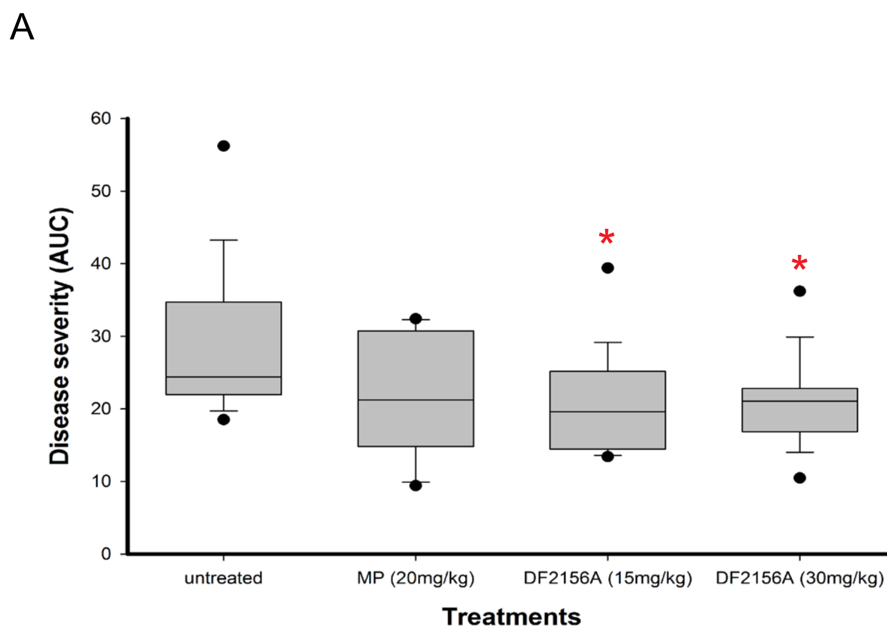
Therapeutic efficacy of allosteric CXCR1/2 inhibition in immunization-induced EBA

Based on these observations, we next investigated if inhibition of CXCL1/2 function has therapeutic effects in already established immunization-induced EBA. Upon allocation to treatment, extent of skin disease was identical in all groups. In detail, 2.9 ± 0.2%, 3.0 ± 0.4%, 2.9 ± 0.3% and 2.9 ± 0.3% of the body surface area were affected in mice allocated to control, MP, DF2156A (15 mg/kg) or DF2156A (30 mg/kg) treatment, respectively. After completion of the 4-week treatment period, cumulative skin disease was significantly reduced in all treatment groups compared to the control group (Figure 4A and 4B). Detailed analysis of the disease development during this period in individual mice showed that skin disease progressed in all mice allocated to the control group. In contrast, in all treatment groups, a significant proportion of mice showed a decline of disease activity during the 4-week treatment period (Figure 4C). At the end of the treatment, dermal inflammatory infiltrate as assessed by semiquantitative scoring reached 1.60.2 in control mice, 0.8 ± 0.2 in MP-, 1.2 ± 0.2 in 15mg/kg DF2156A- and 1.9 ± 0.2 in 30mg/kg DF2156A-treated animals ($p<0.05$ for MP versus control treatment; no significant differences for the different DF2156A doses, ANOVA with Bonferroni t-test for multiple comparisons). Changes in clinical disease were independent of IgG and C3 deposition at the dermal-epidermal junction (Figure 4B).

We recently reported that mice affected by immunization-induced EBA gain less weight compared to age-matched control mice [23]. Evaluation of the weight gain during our 4-week observation period revealed that EBA mice in the treatment control group gained 1.4 ± 0.5 g. In contrast, methylprednisolone-treated animals lost 1.5 ± 0.5 g weight during the same observation period. Treatment of EBA mice with DF2156A 15 mg/kg or 30 mg/kg lead to marginal weight changes (-0.2 ± 0.4 g and -0.1 ± 0.3 g, respectively). Collectively, compared to the treatment control group, after our 4-week treatment period, weight differences were significantly lower in mice treated with methylprednisolone, while the weight of DF2156A-treated mice did not change (Figure 5).

Discussion

Altered expression patterns of several cytokines, including CXCR1 and CXCR2 ligands, have been reported in patients with AIBD [28-



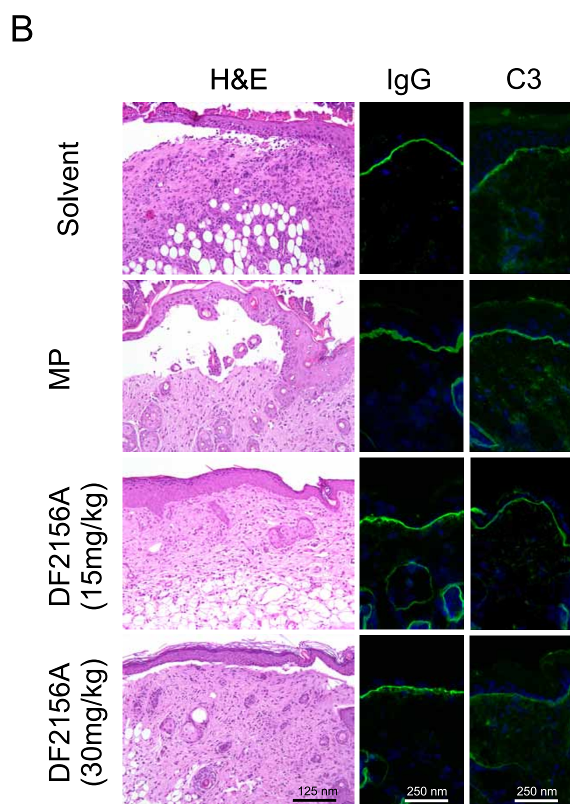


Figure 3: Inhibitory effects of prophylactic DF2156A application in antibody transfer-induced EBA in C57Bl/6 mice

A. Overall disease severity, expressed as the area under the curve (AUC), in the indicated treatment groups: untreated (saline), n=17; DF2156A (15 mg/kg), n=18; DF2156A (30 mg/kg), n=16; methylprednisolone (MP), n=10. Due to the non-parametric distribution of the numbers, data is presented as median (black line), 75-percentile (box) and 95-percentile (error bars). Numbers beyond the 95-percentile are indicated as dots. *indicates statistic significance (ANOVA on Ranks followed by Dunn's Method for multiple comparisons versus untreated).

B. Representative H&E-stained sections as well as IgG and C3 deposition at the dermal-epidermal junction by direct immunofluorescence microscopy (counterstained with DAPI) 12 days after first IgG injection. Scale bars at the bottom indicate the original magnification.

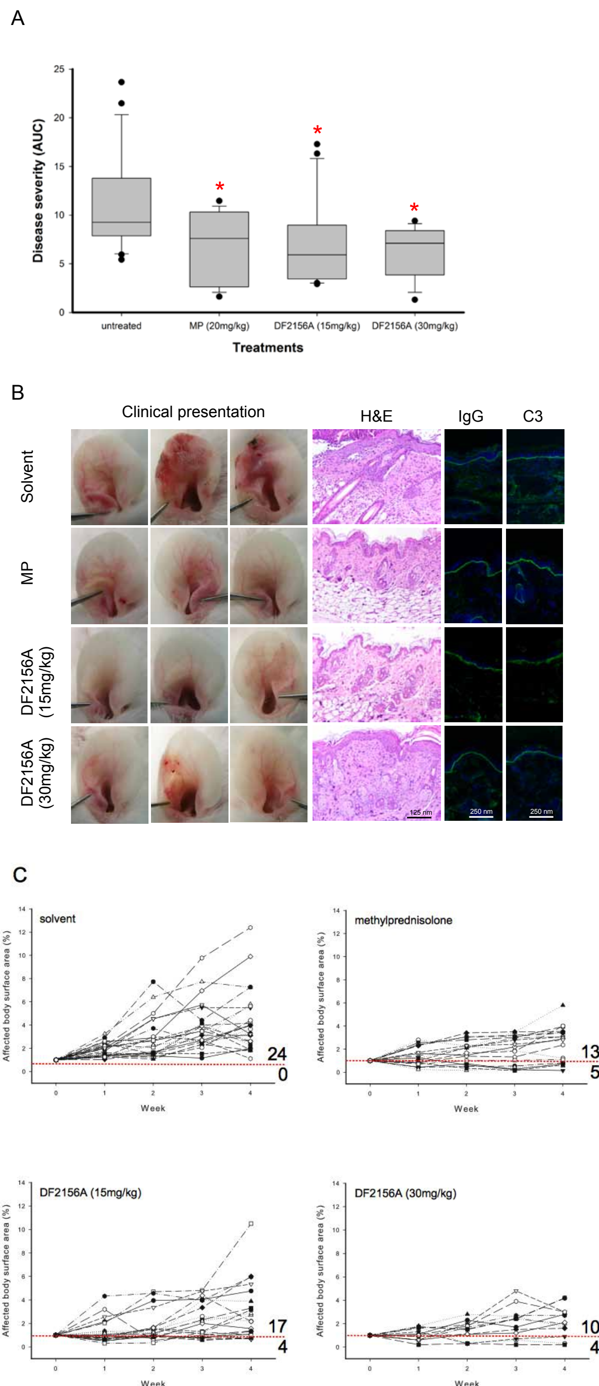


Figure 4: DF2156A improves established disease in immunization-induced EBA

A. Cumulative severity of skin disease in the indicated groups during the 4-week observation period (untreated (saline), n=24; DF2156A (15 mg/kg), n=21; DF2156A (30 mg/kg), n=14; methylprednisolone (MP), n=18). Due to the non-parametric distribution of the numbers, data is presented as median (black line), 75-percentile (box) and 95-percentile (error bars). Numbers outside the 95-percentile are indicated as dots. *indicates statistic significance (ANOVA on Ranks followed by Dunn's Method for multiple comparisons versus untreated).

B. Representative clinical presentations, H&E-stained skin specimen (ear), and IgG and C3 deposits at the dermal-epidermal junction by direct immunofluorescence microscopy (counterstained with DAPI); skin specimens were obtained at the end of the 4-week treatment period. While MP-treated animals showed a significant reduction in the dermal inflammatory infiltrate, a not significant trend towards a lower infiltration was observed in DF2156A (15 mg/kg)-treated mice. Scale bars at the bottom indicate the original magnification.

C. Relative disease activity (in relation to the affected body surface area at the time of inclusion) of individual mice during the 4-week treatment period. Scores below 1 indicate improvement of experimental EBA, which is indicated by the red dotted line. Compared to untreated mice, scores in all treatment groups were lower at the end of the observation period. While skin disease increased in all 24 control mice, 5/18 (28%) of MP-treated, 4/21 (19%) of DF2156A (15mg/kg) - and 4/14 (29%) of DF2156A (30mg/kg)- treated mice showed a decreased disease severity at the end of the observation period.

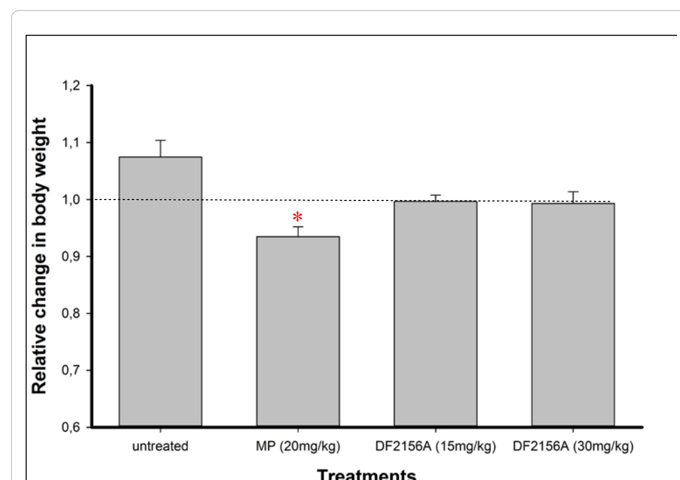


Figure 5: Allosteric CXCR1/2 inhibition has no significant effect on body weight, while methylprednisolone (MP) treatment reduces the weight in immunization-induced EBA during the 4-week observation period.

Body weight of mice with immunization-induced EBA was recorded at the beginning and at the end of the 4-week treatment period. While untreated mice with immunization-induced EBA gained weight (n=7), immunized mice treated with MP (20 mg/kg/day, n=6) showed a significant weight loss and DF2156A-treated mice showed no significant difference in weight compared to untreated controls (n=7, 15 mg/kg and n=6, 30 mg/kg)). Data is presented as mean ± SEM. *indicates statistical difference in comparison to control mice (ANOVA followed by Bonferroni t-test for multiple comparisons versus mice with untreated EBA).

33]. However, little information on the functional relevance of this altered expression has been available. One report showed that IL-1 and TNF-α were required to mediate blister formation in a neonatal mouse model of pemphigus [34]. Recent work from our laboratory identified a complex role of IL-6 in experimental EBA. Surprisingly, IL-6, which showed significantly elevated serum levels in both human and experimental EBA, had a profound anti-inflammatory activity in this model, by regulating IL-1ra concentrations [20]. This latter work indicates that despite an increased cytokine expression, modulation of this cytokine function does not necessarily lead to an improvement of the disease phenotype. In line with this notion, use of anti-TNF therapy in rheumatoid arthritis patients was reported to occasionally induce AIBD [35].

From a medicinal chemistry program on the identification of novel small molecular weight inhibitors targeting CXCR1 and CXCR2, a series of allosteric inhibitors had been identified and subsequently extensively characterized. Among these, a second-generation dual CXCR1/CXCR2 inhibitor (DF2156A) with optimized pharmacokinetic properties and suitable for chronic administration emerged [16,17]. This drug is currently evaluated for safety in patients with BP (NCT01571895). Data on cell transfectants and primary leukocyte populations show that DF 2156A effectively and selectively inhibits CXCR1/CXCR2-mediated chemotaxis in the low nanomolar range. *In vitro*, DF 2156A prevented proliferation, migration and capillary-like organization of HUVEC cells in response to human IL-8. *In vivo*, in a murine model of sponge-induced angiogenesis, DF2156A reduced leukocyte influx, TNF-α production and neovessel formation [16]. In a rat model of liver and cerebral ischaemia and reperfusion (I/R) injury, DF2156A reduced PMN infiltration and associated tissue damage [16,17]. DF2156A showed no relevant toxicity in rodent and non-rodent animal species after single or repeated dose administrations. In particular, a repeated dose administration study conducted by oral route in rats (for 28 days up to the dose of 200 mg/kg) did not cause any effect on the immune system (unpublished observation).

We therefore here systematically analyzed the contribution of CXCL1 and CXCL2 in a mouse model of EBA to evaluate the potential therapeutic application of DF2156A. We first demonstrate that DF2156A blocks IL-8-induced neutrophil activation in vitro and document increased CXCL1 and dramatically increased CXCL2 expression in experimental EBA. Interestingly, the compound only had moderate and strain-dependent effects when applied prophylactically in a previously developed passive transfer model of murine EBA [21]. In contrast, DF2156A significantly improved blistering in already established disease using an active, immunization-induced mouse model of EBA [25,36]. The effects of DF2156A were comparable with those of high doses of corticosteroids, and (based on differences in body weight) apparently associated with less adverse events.

In experimental EBA, blister formation is initiated by the binding of anti-COL7 antibodies to COL7 located at the dermal-epidermal junction [21,37,38]. Subsequently, a pro-inflammatory milieu is generated within the skin. This process is mainly, but not exclusively, mediated by alternative and classical activation of the complement cascade [26,39]. We recently demonstrated, that cytokines are also critically involved in this process. Interestingly, some of the cytokines (such as IL-6), which correlate with the severity of skin disease in experimental EBA, have strong anti-inflammatory, rather than pro-inflammatory, effects. The anti-inflammatory effects of IL-6 were at least partially mediated by regulation of IL-1ra by IL-6 [20]. In addition, there was circumstantial evidence for pro-inflammatory activities of IL-1, as administration of the IL-1 receptor antagonist anakinra improved the skin disease impaired induction of blister formation in experimental EBA [20]. In the present study, we show an increased expression of two additional cytokines, namely CXCL1 and CXCL2 (Figure 2). Potential cellular sources for both cytokines are macrophages as well as neutrophils and epithelial cells for CXCL1 [40-42]. Both CXCL1 and CXCL2 have a strong chemoattractant activity for neutrophils [40,43]. Therefore, CXCL1 and CXCL2 could potentially contribute to neutrophil extravasation into the skin after antibody binding. In experimental EBA, neutrophil migration into the skin has been demonstrated to depend on CD18 expression [7]. In the skin, neutrophils bind to immune complexes at the dermal-epidermal junction in an Fc gamma receptor-dependent fashion [10], which leads to PI3K beta- and LTB4-dependent [44] release of proteolytic enzymes and ROS, which are indispensible for blister formation [7,45]. The crucial role of neutrophils in the pathogenesis of experimental EBA in mice, induced by antibody transfer, is further supported by the observation, that administration of the neutrophil depleting Gr-1 antibody completely protects mice from induction of skin lesions [7]. As shown in figure 1 and elsewhere [46], inhibition of CXCR1/2 not only prevents recruitment of neutrophils, but also their activation. However, our observation that administration of DF2156A lead to improvement of skin lesions in EBA mice in the presence of an unaltered neutrophil infiltration into the skin (Figure 4) indicates, that DF2156A predominantly affected neutrophil activation rather than neutrophil recruitment in our model.

We can only speculate on reasons for the different therapeutic responses to DF2156A in antibody transfer- and immunization-induced EBA. The differences in CXCR2 dependency in anti-COL7 IgG transfer-induced EBA in BALB/c and C57BL/6 mice may be due to differences of the genetic background. Indeed, while no polymorphism

between BALB/c and C57BL/6 mice have been reported in CXCR1 and CXCR2 genes, one polymorphism at 5:91321410 has been described in the CXCL1 gene and three polymorphisms between the strains within the CXCL2 locus have been identified (http://phenome.jax.org, accessed September 4th, 2012). Similarly, a different response to IVIG treatment has been noted in BALB/c and C57BL/6 mice [27].

Overall, the modulation of cytokines such as CXCL1 and CXCL2 are promising new avenues for the treatment of autoimmune skin blistering. Based on the therapeutic activity of the allosteric CXCR1/2 inhibitor DF2156A, clinical trials with this compound in patients with EBA and other AIBD with similar pathogenesis should be performed.

Acknowledgments

Grant support: DFG Excellence Cluster “Inflammation at Interfaces” (DFG EXC 306/1 and 2), DFG LU877/5-1, The B. Braun-Foundation, Focus Program “Autoimmunity” of the University of Lübeck, and a research grant from Dompe pha.r.ma.

References

1. Bach JF (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 347: 911-920.
2. Langan SM, Smeeth L, Hubbard R, Fleming KM, Smith CJ, et al. (2008) Bullous pemphigoid and pemphigus vulgaris--incidence and mortality in the UK: population based cohort study. BMJ 337: a180.
3. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ (1998) Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest 101: 273-281.
4. Sitaru C, Zillikens D (2005) Mechanisms of blister induction by autoantibodies. Exp Dermatol 14: 861-875.
5. Chen M, Kim GH, Prakash L, Woodley DT (2012) Epidermolysis bullosa acquisita: autoimmunity to anchoring fibril collagen. Autoimmunity 45: 91-101.
6. Sitaru C, Kromminga A, Hashimoto T, Bröcker EB, Zillikens D (2002) Autoantibodies to type VII collagen mediate Fcgamma-dependent neutrophil activation and induce dermal-epidermal separation in cryosections of human skin. Am J Pathol 161: 301-311.
7. Chiriac MT, Roesler J, Sindrilaru A, Scharffetter-Kochanek K, Zillikens D, et al. (2007) NADPH oxidase is required for neutrophil-dependent autoantibody-induced tissue damage. J Pathol 212: 56-65.
8. Yu X, Holdorf K, Kasper B, Zillikens D, Ludwig RJ, et al. (2010) FcγRIIA and FcγRIIIB are required for autoantibody-induced tissue damage in experimental human models of bullous pemphigoid. J Invest Dermatol 130: 2841-2844.
9. Ludwig RJ (2012) Model systems duplicating epidermolysis bullosa acquisita: a methodological review. Autoimmunity 45: 102-110.
10. Kasperkiewicz M, Nimmerjahn F, Wende S, Hirose M, Iwata H, et al. (2012) Genetic identification and functional validation of FcγRIV as key molecule in autoantibody-induced tissue injury. J Pathol 228: 8-19.
11. Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, et al. (2000) The neutrophil as a cellular source of chemokines. Immunol Rev 177: 195-203.
12. Kasama T, Miwa Y, Isozaki T, Odai T, Adachi M, et al. (2005) Neutrophil-derived cytokines: potential therapeutic targets in inflammation. Curr Drug Targets Inflamm Allergy 4: 273-279.
13. Schröder AK, von der Ohe M, Kolling U, Altstaedt J, Uciechowski P, et al. (2006) Polymorphonuclear leucocytes selectively produce anti-inflammatory interleukin-1 receptor antagonist and chemokines, but fail to produce pro-inflammatory mediators. Immunology 119: 317-327.
14. Bertini R, Allegretti M, Bizzarri C, Moriconi A, Locati M, et al. (2004) Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury. Proc Natl Acad Sci U S A 101: 11791-11796.
15. Barsante MM, Cunha TM, Allegretti M, Cattani F, Policani F, et al. (2008) Blockade of the chemokine receptor CXCR2 ameliorates adjuvant-induced arthritis in rats. Br J Pharmacol 153: 992-1002.
16. Bertini R, Barcelos LS, Beccari AR, Cavalieri B, Moriconi A, et al. (2012) Receptor binding mode and pharmacological characterization of a potent and selective dual CXCR1/CXCR2 non-competitive allosteric inhibitor. Br J Pharmacol 165: 436-454.
17. Garau A, Bertini R, Mosca M, Bizzarri C, Anacardio R, et al. (2006) Development of a systemically-active dual CXCR1/CXCR2 allosteric inhibitor and its efficacy in a model of transient cerebral ischemia in the rat. Eur Cytokine Netw 17: 35-41.
18. Recke A, Sitaru C, Vidarsson G, Evensen M, Chiriac MT, et al. (2010) Pathogenicity of IgG subclass autoantibodies to type VII collagen: induction of dermal-epidermal separation. J Autoimmun 34: 435-444.
19. Hammers CM, Bieber K, Kalies K, Banczyk D, Ellebrecht CT, et al. (2011) Complement-fixing anti-type VII collagen antibodies are induced in Th1-polarized lymph nodes of epidermolysis bullosa acquisita-susceptible mice. J Immunol 187: 5043-5050.
20. Samavedam UK, Kalies K, Scheller J, Sadeghi H, Gupta Y, et al. (2013) Recombinant IL-6 treatment protects mice from organ specific autoimmune disease by IL-6 classical signalling-dependent IL-1ra induction. J Autoimmun 40: 74-85.
21. Sitaru C, Mihai S, Otto C, Chiriac MT, Hausser I, et al. (2005) Induction of dermal-epidermal separation in mice by passive transfer of antibodies specific to type VII collagen. J Clin Invest 115: 870-878.
22. Ludwig RJ, Zollner TM, Santoso S, Hardt K, Gille J, et al. (2005) Junctional adhesion molecules (JAM)-B and -C contribute to leukocyte extravasation to the skin and mediate cutaneous inflammation. J Invest Dermatol 125: 969-976.
23. Ishii N, Recke A, Mihai S, Hirose M, Hashimoto T, et al. (2011) Autoantibody-induced intestinal inflammation and weight loss in experimental epidermolysis bullosa acquisita. J Pathol 224: 234-244.
24. Kasperkiewicz M, Hirose M, Recke A, Schmidt E, Zillikens D, et al. (2010) Clearance rates of circulating and tissue-bound autoantibodies to type VII collagen in experimental epidermolysis bullosa acquisita. Br J Dermatol 162: 1064-1070.
25. Ludwig RJ, Recke A, Bieber K, Müller S, Marques Ade C, et al. (2011) Generation of antibodies of distinct subclasses and specificity is linked to H2s in an active mouse model of epidermolysis bullosa acquisita. J Invest Dermatol 131: 167-176.
26. Ludwig RJ, Müller S, Marques Ad, Recke A, Schmidt E, et al. (2012) Identification of quantitative trait loci in experimental epidermolysis bullosa acquisita. J Invest Dermatol 132: 1409-1415.
27. Leontyev D, Katsman Y, Branch DR (2012) Mouse background and IVIG dosage are critical in establishing the role of inhibitory Fcγ receptor for the amelioration of experimental ITP. Blood 119: 5261-5264.
28. Center DM, Austen KF, Wintroub BU (1978) Identification of chemoattractant activity for lymphocytes in bullous pemphigoid bullous fluid. Trans Assoc Am Physicians 91: 242-252.
29. Grando SA, Glukhenky BT, Drannik GN, Epshtein EV, Kostromin AP, et al. (1989) Mediators of inflammation in blister fluids from patients with pemphigus vulgaris and bullous pemphigoid. Arch Dermatol 125: 925-930.
30. Schmidt E, Bastian B, Dummer R, Tony HP, Bröcker EB, et al. (1996) Detection of elevated levels of IL-4, IL-6, and IL-10 in blister fluid of bullous pemphigoid. Arch Dermatol Res 288: 353-357.
31. Ameglio F, D’Auria L, Bonifati C, Ferraro C, Mastroianni A, et al. (1998) Cytokine pattern in blister fluid and serum of patients with bullous pemphigoid: relationships with disease intensity. Br J Dermatol 138: 611-614.
32. Nakashima H, Fujimoto M, Asashima N, Watanabe R, Kuwano Y, et al. (2007) Serum chemokine profile in patients with bullous pemphigoid. Br J Dermatol 156: 454-459.
33. Ludwig RJ, Schmidt E (2009) Cytokines in autoimmune bullous skin diseases. Epiphenomena or contribution to pathogenesis? G Ital Dermatol Venereol 144: 339-349.
34. Feliciani C, Toto P, Amerio P, Pour SM, Coscione G, et al. (2000) In vitro and in vivo expression of interleukin-1alpha and tumor necrosis factor-alpha mRNA in pemphigus vulgaris: interleukin-1alpha and tumor necrosis factor-alpha are involved in acantholysis. J Invest Dermatol 114: 71-77.
35. Boussemart L, Jacobelli S, Batteux F, Goulvestre C, Grange P, et al. (2010) Autoimmune bullous skin diseases occurring under anti-tumor necrosis factor therapy: two case reports. Dermatology 221: 201-205.

36. Sitaru C, Chiriac MT, Mihai S, Büning J, Gebert A, et al. (2006) Induction of complement-fixing autoantibodies against type VII collagen results in subepidermal blistering in mice. J Immunol 177: 3461-3468.

37. Woodley DT, Chang C, Saadat P, Ram R, Liu Z, et al. (2005) Evidence that anti-type VII collagen antibodies are pathogenic and responsible for the clinical, histological, and immunological features of epidermolysis bullosa acquisita. J Invest Dermatol 124: 958-964.

38. Woodley DT, Ram R, Doostan A, Bandyopadhyay P, Huang Y, et al. (2006) Induction of epidermolysis bullosa acquisita in mice by passive transfer of autoantibodies from patients. J Invest Dermatol 126: 1323-1330.

39. Mihai S, Chiriac MT, Takahashi K, Thurman JM, Holers VM, et al. (2007) The alternative pathway of complement activation is critical for blister induction in experimental epidermolysis bullosa acquisita. J Immunol 178: 6514-6521.

40. Wolpe SD, Sherry B, Juers D, Davatelis G, Yurt RW, et al. (1989) Identification and characterization of macrophage inflammatory protein 2. Proc Natl Acad Sci U S A 86: 612-616.

41. Iida N, Grotendorst GR (1990) Cloning and sequencing of a new gro transcript from activated human monocytes: expression in leukocytes and wound tissue. Mol Cell Biol 10: 5596-5599.

42. Becker S, Quay J, Koren HS, Haskill JS (1994) Constitutive and stimulated MCP-1, GRO alpha, beta, and gamma expression in human airway epithelium and bronchoalveolar macrophages. Am J Physiol 266: L278-286.

43. Moser B, Clark-Lewis I, Zwahlen R, Baggiolini M (1990) Neutrophil-activating properties of the melanoma growth-stimulatory activity. J Exp Med 171: 1797-1802.

44. Kulkarni S, Sitaru C, Jakus Z, Anderson KE, Damoulakis G, et al. (2011) PI3Kß plays a critical role in neutrophil activation by immune complexes. Sci Signal 4: ra23.

45. Shimanovich I, Mihai S, Oostingh GJ, Ilenchuk TT, Bröcker EB, et al. (2004) Granulocyte-derived elastase and gelatinase B are required for dermal-epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid. J Pathol 204: 519-527.

46. Lazaar AL, Sweeney LE, MacDonald AJ, Alexis NE, Chen C, et al. (2011) SB-656933, a novel CXCR2 selective antagonist, inhibits ex vivo neutrophil activation and ozone-induced airway inflammation in humans. Br J Clin Pharmacol 72: 282-293.

This article was originally published in a special issue, **Autoimmune Diseases** handled by Editor(s). Dr. Kenneth Blum, University of Florida, USA