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

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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 they 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 erythematosides (SLE) or anti-cylcic 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 anti-inflammatory activity [16,17]. This compound is a second-generation derivative of 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...
samples from corresponding anatomical sites were obtained from (25 ng/ml). This IL-8 concentration was then used to test the impact of results, the optimal IL-8 dose for neutrophil activation was determined by chemiluminescence as described [8]. Based on these experiments, 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 ^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 SJL/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 (Aquaa.ad injectabilia, 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
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 (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 leisional 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].
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 ± 0.12 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.
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 indispensable 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 CXC1 and CXC2 genes, one polymorphism at 5:9321410 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 CXC1R/2 inhibitor DF2156A, clinical trials with this compound in patients with EBA and other AIIBD with similar pathogenesis should be performed.

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