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PDE4 Inhibition as Potential Treatment of Epidermolysis Bullosa Acquisita

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Pemphigoid diseases such as epidermolysis bullosa acquisita (EBA) may be difficult to treat. In pemphigoid diseases, mucocutaneous blistering is caused by autoantibodies to hemidesmosomal antigens; in EBA the autoantigen is type VII collagen. Despite growing insights into pemphigoid disease pathogenesis, corticosteroids are still a mainstay of treatment. In experimental EBA, myeloid cell activation is a key event leading to blistering. Activation of these cells depends on phosphodiesterase (PDE) 4. We therefore evaluated the potential for PDE4 inhibition in EBA: PDE4 was highly expressed in inflammatory cells and in the epidermis of patients compared with healthy skin samples. PDE4 inhibitors rolipram, roflumilast, and roflumilast N-oxide prevented the release of immune complex-induced reactive oxygen species from polymorphonuclear leukocytes and separation of the dermal-epidermal junction of skin incubated with antibodies to collagen type VII and polymorphonuclear leukocytes. The PDE4 inhibitors also impaired CD62L shedding and decreased CD11b expression on immune complex-stimulated polymorphonuclear leukocytes. For in vivo validation, experimental EBA was induced in mice by transfer of anti-collagen type VII IgG or immunization with collagen type VII. Roflumilast dose-dependently reduced blistering in antibody transfer-induced EBA and also hindered disease progression in immunization-induced EBA. PDE4 inhibition emerges as a new treatment modality for EBA and possibly other neutrophil-driven pemphigoid diseases.

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INTRODUCTION

Pemphigoid diseases (PDs) are prototypical organ-specific autoimmune diseases caused by autoantibodies that are directed against proteins of basement membrane zone, such as type XVII collagen, type VII collagen (COL7), or laminin 332. Mucocutaneous blistering is the clinical hallmark of PD, but they are quite heterogeneous with respect to their overall clinical presentation, target antigens, and autoantibody isotype (Schmidt and Zillikens, 2013). The PD epidermolysis bullosa acquisita (EBA) is characterized and caused by autoantibodies against COL7 (Woodley et al., 1988). EBA may present as either classic, mechanobullous EBA with tense blisters and skin fragility preferentially localized to the extensor skin surfaces at trauma-prone areas or, more commonly, as an inflammatory skin disease mimicking other PDs such as bullous pemphigoid or mucous membrane pemphigoid (Buijsrogge et al., 2011; Gupta et al., 2012; Ludwig, 2013a; Zumelzu et al., 2011). Predominantly, the latter variant can be duplicated in mice by either antibody transfer or immunization.

Use of these animal models has greatly enhanced our understanding of the inflammatory type of EBA pathogenesis (Ludwig et al., 2013b): in genetically predisposed individuals (Gammon et al., 1988; Ludwig et al., 2012; Zumelzu et al., 2011), autoantibodies to COL7 are generated by a T-cell–dependent B-cell response (Iwata et al., 2013). Depletion of neutrophils impairs the formation of COL7-specific autoantibodies (Samavedam et al., 2014). Once formed, the half-life and biodistribution of the anti-COL7 IgG, which can be found in 54% of EBA patients (Buijsrogge et al., 2011), is controlled by the neonatal Fc receptor (Sesarman et al., 2008). Circulating anti-COL7 autoantibodies rapidly bind to their target antigen, mainly expressed in the skin (Ishii et al., 2011), which leads to the formation of skin-bound immune complexes (ICs). Through partially understood mechanisms involving complement activation and cytokine release (Ludwig et al., 2013b), an ICAM-1/CD18–dependent extravasation of myeloid cells into the skin is induced (Chiriac et al., 2007; Sadeghi et al., 2015b). After Fc gamma receptor IV-dependent binding to the IC (Kasperkiewicz et al., 2012) and an immunoreceptor

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Abbreviations: Akt, RAC-alpha serine/threonine-protein kinase; COL7, type VII collagen; EBA, epidermolysis bullosa acquisita; IC, immune complex; MAPK, mitogen-activated protein kinase; PD, pemphigoid disease; PDE4, phosphodiesterase-4; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocyte; ROL, rolipram; ROF, roflumilast; RNO, roflumilast N-oxide; ROS, reactive oxygen species

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tyrosine-based activation motif (ITAM)–dependent signaling cascade (Hellberg et al., 2013; Futosi et al., 2013; Kulkarni et al., 2011; Nimmerjahn et al., 2005), myeloid cells release reactive oxygen species (ROS) and proteases, which ultimately causes inflammation and blistering (Chiriac et al., 2007; Sitaru et al., 2002a).

Regarding intracellular signaling pathways in EBA, relatively little is known: IC-induced activation of myeloid cells and neutrophils (in the context of EBA) requires phosphoinositide 3-kinase-β (PI3Kβ), protein kinase B (Akt), extracellular signal-related kinase (ERK), p38, retinoic acid receptor-related orphan receptor–alpha (i.e., RORα) and Src family kinases (Hellberg et al., 2013; Kovacs et al., 2014; Kulkarni et al., 2011; Sadeghi et al., 2015a). In addition, increased phosphorylation of Akt and ERK 1/2 has been noted in bullous pemphigoid patient skin samples (Hellberg et al., 2013). More recently, inflammation-resolving mechanisms (i.e., modulators of wound healing), involving flightless I (Kopecki et al., 2013) have been identified. Therefore, myeloid cells (i.e., neutrophils) are key effector cells in EBA, acting by contributing to the generation of autoantibodies and by being essential for the induction of cutaneous inflammation and blistering. Despite these insights into EBA pathogenesis, corticosteroids are still a mainstay of treatment. In addition to a relatively poor response to treatment (Kim et al., 2011), adverse drug reactions contribute to the high morbidity of EBA patients. Hence, there is a significant unmet medical need for the development of new therapeutics for EBA and other PDs (Ludwig et al., 2013b).

In parallel to the growing understanding of EBA pathogenesis, small molecules modulating signal transduction have been developed to treat cancer and chronic inflammatory diseases (Ghoreschi and Gadina, 2014). The approval of the phosphodiesterase 4 (PDE4) inhibitor apremilast for the treatment of psoriasis (Papp et al., 2012; Papp et al., 2015) underscores the potential of targeting these pathways for the treatment of chronic inflammatory diseases. In general, PDE4 supports proinflammatory effects by degrading cAMP in inflammatory cells, smooth muscle cells, endothelial cells, and keratinocytes (Chujor et al., 1998; Torphy, 1998). Inhibition of PDE4 induces accumulation of the intracellular second messenger cAMP and activates protein kinase A, followed by phosphorylation of the transcription factor cAMP-response element binding protein, resulting in a decrease of proinflammatory mediators, including tumor necrosis factor-α, IL-17, and IFN-γ (Jimenez et al., 2001; Liu et al., 2000; Sheibanie et al., 2004). At the same time, inhibition of PDE4 increases the expression of anti-inflammatory mediators, such as IL-10 (Oger et al., 2005). Furthermore, the PDE4 inhibitors rolipram (ROL) and cilomilast inhibit promatrix metalloproteinase-9 activity (Oger et al., 2005), and roflumilast (ROF) hinders activation of NF-κB and phosphorylation of mitogen-activated protein kinase (MAPK), including c-Jun N-terminal kinase and p38MAPK, in murine macrophages (Kwak et al., 2005). In murine neutrophils, ROL has been reported to block activation of Akt and NF-κB (Sousa et al., 2010).

Hence, we hypothesized that PDE4 inhibition impairs IC-induced neutrophil activation, thus rendering it a potential target for the treatment of EBA and other PDs. Our assumption was based on the facts that (i) PDE4 is the key enzyme accounting for cAMP degradation in neutrophils (Schudt et al., 1991) and (ii) PDE4 inhibitors are highly effective to curb neutrophil functions (Sousa et al., 2010). In this study, we challenged this assumption and evaluated the impact of PDE4 inhibitors on key pathogenic steps in EBA in vitro. We also evaluated the effects of PDE4 inhibition on preclinical models of the disease.

RESULTS

Increased expression of PDE4 in EBA patient skin

We first compared the PDE4 expression of inflammatory-type EBA skin at lesional sites with normal human skin. We detected little PDE4 expression in normal human skin ($n = 4$). In contrast, a strong signal for PDE4 was noted in the epidermis and/or dermis in all four EBA-patient specimens (Figure 1a). Quantification of the PDE4 staining intensity confirmed the visual assessment: an increased florescence intensity in both epidermis and dermis in EBA patients compared with control skin (Figure 1b and c). We also determined the PDE4 expression in skin specimens from patients with the mechanobullous type of EBA. We observed no difference in PDE4 expression compared with healthy skin (data not shown).

PDE4 inhibition reduces reactive oxygen species (ROS) release from polymorphonuclear leukocytes (PMNs) activated by COL7/anti-COL7 IgG ICs

Having detected increased PDE4 expression at the site of inflammation, we next addressed the functional relevance of this finding. We focused on the dermal PDE4 expression (i.e., most likely corresponding to leukocytes), because myeloid cells (PMNs) are required to induce cutaneous inflammation and blistering in experimental models of EBA, whereas the contribution of keratinocytes to EBA pathogenesis remains to be elucidated. ROS release from activated PMN is a key effector in the pathogenesis of EBA (Ludwig, 2013a). We therefore evaluated the impact of PDE4 inhibitors ROF, ROL, and roflumilast N-oxide (RNO) on IC-induced ROS release from PMN. As previously reported (Recke et al., 2015), the addition of PMN to IC of COL7 protein and anti-COL7 IgG1 leads to their activation, as detected by ROS release. All tested PDE4 inhibitors reduced the IC-induced ROS release from PMN in a dose-dependent manner (Figure 2a). The observed changes in IC-induced ROS release were achieved at nontoxic concentrations of all compounds as judged by annexin V/propidium iodide staining (see Supplementary Figure S1 online).

PDE4 inhibition reduces autoantibody-induced, PMN-dependent separation of the dermal-epidermal junction in cryosections of human skin

Incubation of cryosections of normal human skin with anti-COL7 IgG induces PMN-dependent separation of the dermal-epidermal junction, reflecting their activation by the IC located at the dermal-epidermal junction (Ludwig, 2013a). Here, cryosections of human skin were incubated with rabbit anti-human COL7 serum or normal rabbit serum, followed by the addition of PMN obtained from healthy volunteers. As reported earlier (Sitaru et al., 2002a), cryosections incubated with anti-human COL7 serum showed separation of the

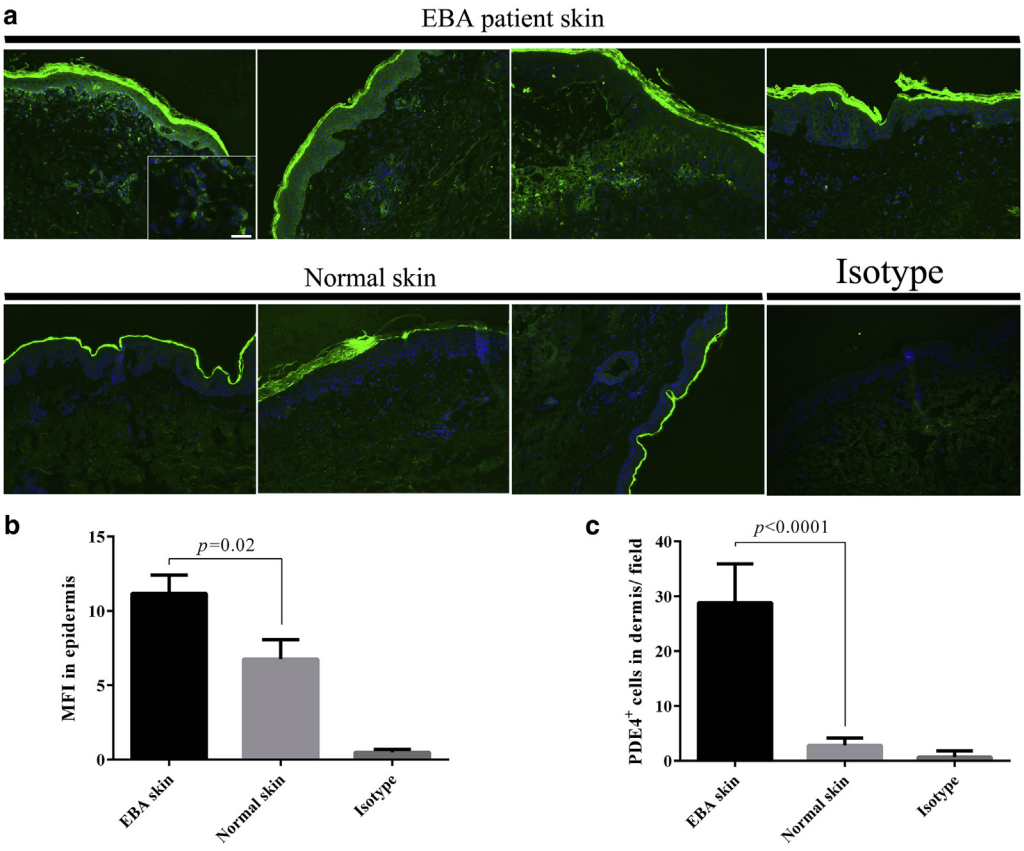

Figure 1. Increased expression of PDE4 in the skin of EBA patients. **(a)** PDE4 expression (green) was evaluated in EBA patient skin (n = 4) and healthy controls (n = 4). Nuclei were counterstained with DAPI (blue). Here, all stained EBA patient samples and three of four controls, as well as an isotype control staining, are shown. Scale bar = 20 μm. To quantify the expression of PDE4, **(b)** the MFI (arbitrary units) of the epidermis and the number of PDE4-positive cells in the dermis were analyzed. Both MFI in the epidermis and **(c)** $PDE4^+$ cells in the dermis were significantly increased relative to normal skin (two-tailed *t* test). MFIs and number of PDE4-positive cells were analyzed by Image J (National Institutes of Health, Bethesda, MD) and BZ-II analyzer (Keyence, Frankfurt, Germany). EBA, epidermolysis bullosa acquisita; MFI, mean fluorescence intensity; PDE4, phosphodiesterase 4.

dermal-epidermal junction, which was almost completely absent in sections incubated with normal rabbit serum. Addition of PDE4 inhibitors dose-dependently impaired autoantibody-induced, PMN-dependent separation of the dermal-epidermal junction: ROL prevented separation of the dermal-epidermal junction at 100 nmol/L, whereas ROF and RNO blocked separation of the dermal-epidermal junction at concentrations as low as 10 nmol/L (Figure 2b).

PDE4 inhibitors prevent specific, but not all, IC-induced neutrophil changes in surface molecule (CD11b, CD62L, and CD66b) expression

In addition to ROS release, decreased L-selectin (CD62L) expression and an increase in CD11b and CD66b are hallmarks of neutrophil activation (Faurschou and Borregaard, 2003). Therefore, we next analyzed the expression of these markers on PMN stimulated by COL7-IC. ROF and RNO slightly but significantly reduced CD11b expression and impaired shedding of CD62L. In contrast, ROL did not reduce either, although similar tendencies were observed (Figure 2c and d). CD66b expression did not change in the presence of any of the three PDE4 inhibitors (Figure 2e).

The PDE4 inhibitor roflumilast impairs disease induction in antibody transfer-induced EBA

The transfer of anti-COL7 IgG into mice induces experimental EBA, mostly resembling the inflammatory variant of the disease (Iwata et al., 2015a). Here, we confirmed these observations: mice were injected with anti-COL7 IgG and treated with vehicle (control treatment for ROF); they developed severe erosions, crusts, and alopecia on the skin. Preventive and continuous treatment with 5 mg/kg of ROF led to slightly but significantly milder symptoms, whereas 1 mg/kg had no effect (Figure 3a–c). The degree of dermal leukocyte infiltration at day 12 of the experiment was evaluated using hematoxylin and eosin-stained slides. Here, mice treated with 5 mg/kg of ROF presented with significantly milder inflammatory cell infiltration compared with either vehicle treatment alone or 1 mg/kg of ROF (Figure 3d). In the next set of experiments, we compared the effect of ROF with corticosteroid (methylprednisolone 20 mg/kg) treatment in this model, and treated mice simultaneously with both drugs to test for potential synergistic effects, which were reported in other models (Ogawa et al., 2002; Milara et al., 2014). In line with our previous results, treatment with ROF impaired disease induction. Compared with methylprednisolone, both treatments were equally effective. The combined treatment with corticosteroid and ROF did not, however, further decrease disease induction and thus did not point toward synergistic effects (see Supplementary Figure S2 online).

The PDE4 inhibitor ROF suppresses disease progression in mice with established immunization-induced EBA

To test if ROF treatment also affects disease severity in mice with already established immunization-induced EBA (Iwata et al., 2013), mice with 2% or more of their body surface area affected by skin lesions were randomly allocated to the vehicle or ROF (5 mg/kg/day, orally) treatment groups. In vehicle-treated mice, the magnitude of skin blistering further increased during the 6-week study period, reaching a maximum at week 2 after allocation to treatment. Thereafter,

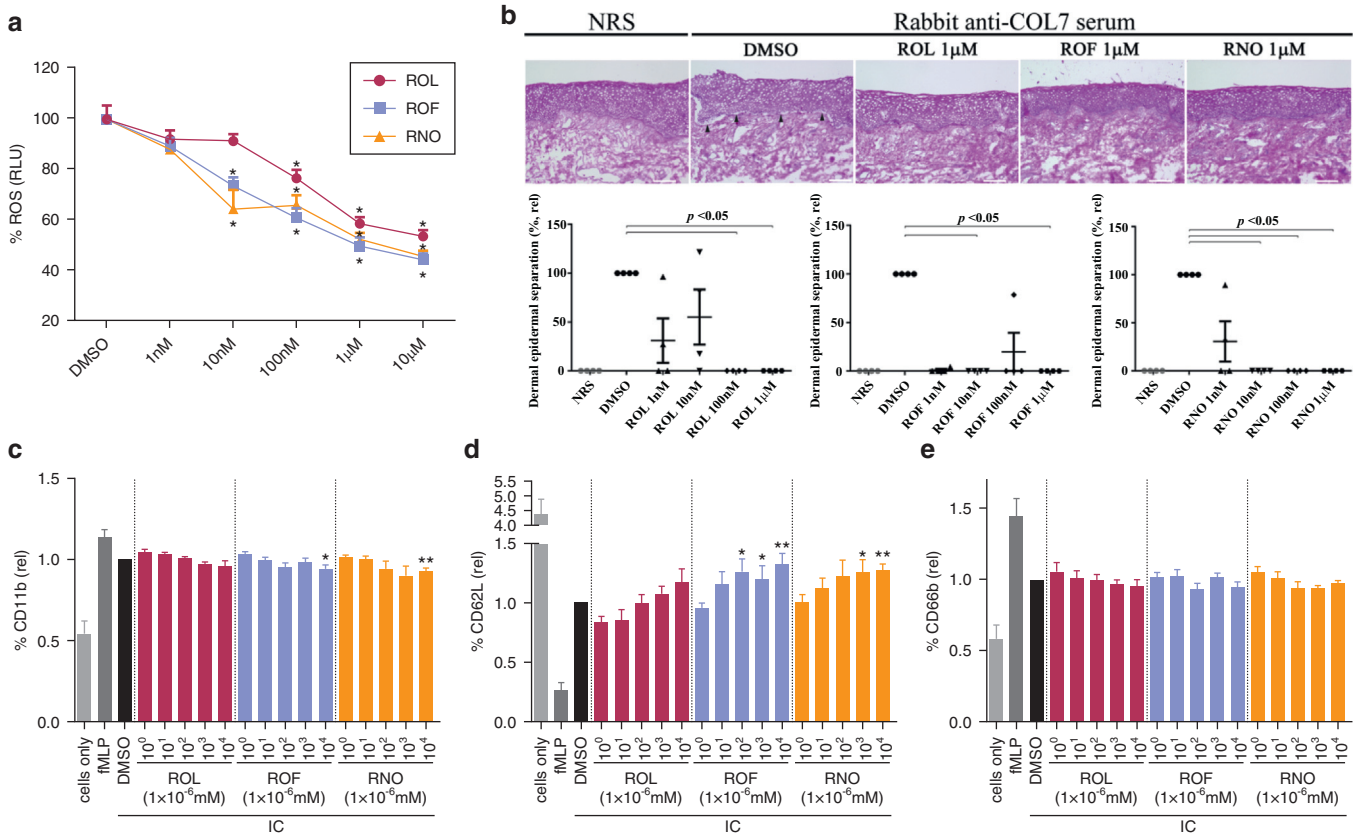

Figure 2. Suppression of in vitro and ex vivo PMN activation by PDE4 inhibitors. **(a)** Freshly isolated human PMN (n = 5) were activated by an immune complex (COL7/anti-COL7) in the presence of various concentrations of rolipram (ROL), roflumilast (ROF) or roflumilast-N-oxide (RNO). Their reactive oxygen species (ROS) release was measured over time. The cumulative ROS release, expressed as area under the curve of ROS production for 1 hour per sample, was related to the ROS release of vehicle-treated, immune complex-activated PMNs (DMSO). All PDE4 inhibitors dose-dependently reduced the immune complex-induced ROS release from PMN. **(b)** PMN activation can also be assessed by incubation on tissue-bound immune complexes (generated by exposing cryosections of human skin with anti-COL7 IgG), leading to separation of the dermal-epidermal junction (arrow). Consistent with the ROS release data, addition of PDE4 inhibitors dose-dependently impaired the induction of separation of the dermal-epidermal junction. The lower three graphs represent the separation of the dermal-epidermal junction in PDE4 inhibitor-treated samples compared with solvent control. Because of the high variability among different donors, data were normalized to the positive control (antibodies, PMN, and solvent). Scale bar = 100 μm. **(c)** PMN (n = 8) were incubated without (cells only) or with immune complexes (IC) and solvent (DMSO) in the absence or presence of PDE4 inhibitors. Stimulation with fMLP served as the control of PMN activation. Obtained data were normalized to the solvent group (DMSO). PDE4 inhibitors had only marginal, albeit statistically significant, effects on CD11b expression (expressed as relative changes of the percentage of cells expressing CD11b), whereas **(d)** a greater effect was noted regarding CD62L shedding, and **(e)** no effect was observed on the degranulation marker CD66b. Hence, PDE4 inhibition seems to preferentially inhibit ROS release and shedding of L-selectin on immune complex-activated PMN. $*P < 0.05$, $**P < 0.01$ (one-way analysis of variance). COL7, collagen type VII; IC, immune complex; NRS, normal rabbit serum; PDE4, phosphodiesterase 4; PMN, polymorphonuclear leukocyte; rel, relative; RLU, relative light unit; RNO, roflumilast N-oxide; ROF, roflumilast; ROL, rolipram; ROS, reactive oxygen species.

clinical disease activity gradually declined but was still almost 2-fold higher at the end of the study period than at time of allocation. Compared with vehicle-treated animals, the percentage of the affected body surface area in ROF-treated mice was significantly lower at 2 and 3 weeks after treatment initiation (Figure 4a and b). In each group, percentage of affected body area after 3 weeks gradually decreased. Overall disease activity, measured as area under the curve, was derived from the disease progression of individual mice in each treatment group and was also significantly lower in mice treated with ROF than in the vehicle-treated animals (Figure 4c). To investigate possible effects of ROF treatment on antigen-specific antibody production, we next analyzed the levels of circulating anti-vWFA2 IgG. However, we did not find significant differences between the two groups (data not shown). To test for the potential cytotoxicity of ROF in vivo, differential blood counts of the mice at the final day (6 weeks) were made. No significant differences in the peripheral blood counts were noted among the groups (see Supplementary Table S1 online).

PMN from mice with immunization-induced EBA treated with ROF show impaired neutrophil activation

So far, we have shown that PDE4 inhibitors reduced ROS production from PMNs in vitro (Figure 2a) and that the treatment of mice with the PDE4 inhibitor ROF impairs disease induction and progression in experimental EBA (Figures 3 and 4). To link these findings (and to possibly link the observed therapeutic effects of ROF to inhibition of neutrophil function in vivo), PMNs from vehicle-treated and ROF-treated (5 mg/kg) mice obtained at the end of the treatment period in the experiments with immunization-induced EBA (6 weeks) were purified and stimulated with

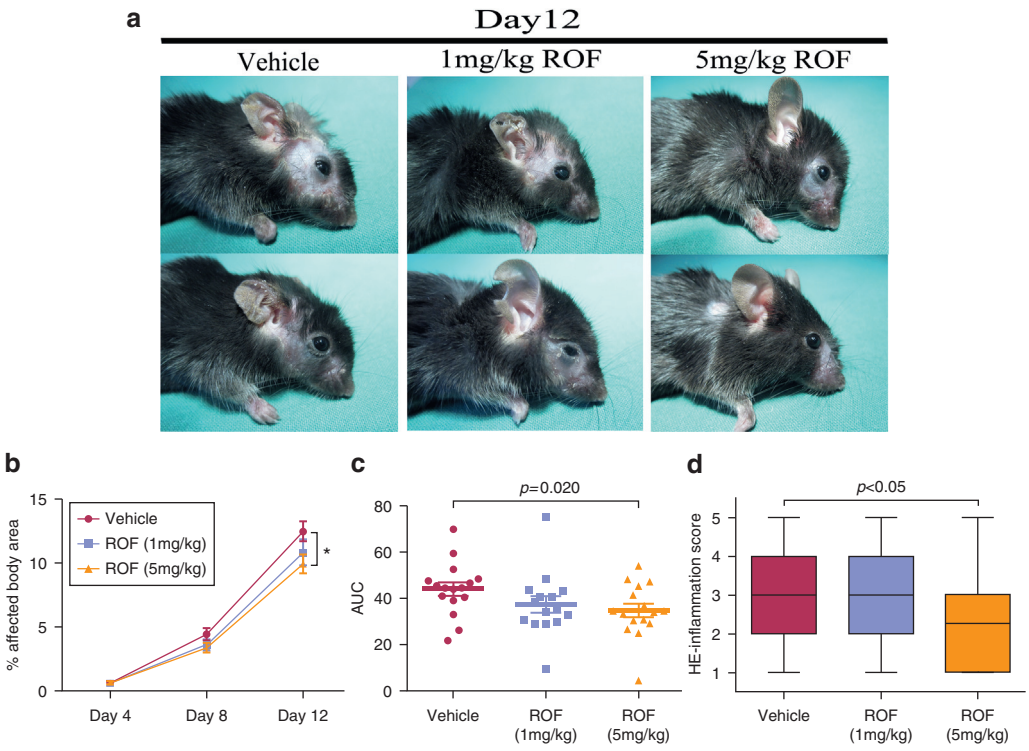

Figure 3. ROF prevents induction of subepidermal blistering in antibody transfer-induced EBA in mice. EBA was induced in C57BL/6J mice by anti-COL7 IgG transfer, and mice were treated with ROF at varying concentrations or vehicle (n = 15–16 mice/group). **(a)** Representative clinical pictures of the mice in the indicated treatment groups on day 12. **(b)** Indicates the percentage of affected body surface area in the treatment groups (*$P < 0.05$ for 5 mg/kg ROF vs. solvent, two-way analysis of variance, considering day and treatment type). **(c)** Cumulative clinical disease manifestation, expressed as area under the curve calculated from panel **b**. Comparison of the groups was performed using one-way analysis of variance with a Bonferroni *t* test as a posttest. **(d)** Hematoxylin and eosin-stained sections from ears (three fields, 15–16 mice/group) were semiquantitatively scored by three observers blinded to the treatment group. The scores ranged from 0 to 5, corresponding to degree of dermal leukocyte infiltration. The data are presented as the median (line), 25th/75th percentiles (boxes), and 5th/95th percentiles (error bars). Comparison of the amounts of the groups was performed using one-way analysis of variance with a Bonferroni *t* test as a posttest. AUC, area under the curve; COL7, collagen type VII; EBA, epidermolysis bullosa acquisita; HE, hematoxylin and eosin; ROF, roflumilast.

phorbol 12-myristate 13-acetate (PMA). The purified PMNs from both groups produced ROS when activated with PMA. After PMA stimulation, the PMNs from the ROF-treated mice produced less ROS than the vehicle-treated mice (Figure 5).

DISCUSSION

Collectively, we show the inhibitory properties of ROF on IC-activated PMN in vitro and ex vivo. Furthermore, we describe how PDE4 inhibition slows the onset of blistering in antibody transfer-induced murine EBA, an antibody-induced and myeloid cell/neutrophil-mediated disease. Even more strikingly, we found this drug to prevent further disease progression in mice with already established EBA. The reduced PMN response from ROF-treated mice to in vitro stimulation indicates that the observed clinical effects are at least partly due to inhibition of PMN activation. Other published treatments for experimental EBA, including methylprednisolone, high doses of intravenous IgG, and soluble CD32, like ROF, also all impaired disease progression (Hellberg et al., 2013; Hirose et al., 2015; Iwata et al., 2015b). HSP90 blockade, the IL-1 receptor antagonist anakinra and dimethyl fumarate were shown to improve even already established disease (Kasperkiewicz et al., 2011; Muller et al., 2016; Samavedam et al., 2013). Thus, the therapeutic efficacy of ROF in experimental EBA is comparable to those of established treatments in EBA patients, such as systemic corticosteroids or intravenous IgG (Kim and Kim, 2013).

In the context of EBA, IC-induced ROS release from neutrophils is triggered by the binding of specific activating Fc gamma receptors to the tissue-bound antibodies (Kasperkiewicz et al., 2012; Yu et al., 2010), ultimately leading to the NCF1-dependent release of ROS (Chiriac et al., 2007). So far, PI3Kβ, ERK2 (MAPK1), p38 (MAPK14), Akt, and the Src family kinases Hck, Fgr, and Lyn have been shown to mediate signaling downstream of Fc gamma receptor binding (Hellberg et al., 2013; Kovacs et al., 2014; Kulkarni et al., 2011; Sadeghi et al., 2015a). Independent of the EBA context, downstream meditators such as Ras, Rac, Rho, protein kinase C, and phospholipase C-gamma (PLCγ) have all been shown to be involved in downstream signaling that activates Fc gamma receptors (Nimmerjahn and Ravetch, 2008). To obtain insights into the interaction of these molecules, we used the STRING database, allowing the identification of functional interactions between proteins (Jensen et al., 2009). With the exception of *Rora*, all entered genes were linked into a complex network (see Supplementary Figure S3 online). *Fcgr4* is directly linked to *Hck*, which then evolves into a complex and interacting network, resulting in predominant activation of *Prkca*, *Rac1*, *Akt1*, and *Mapk14*, leading to ROS production by the *Ncf1* gene.

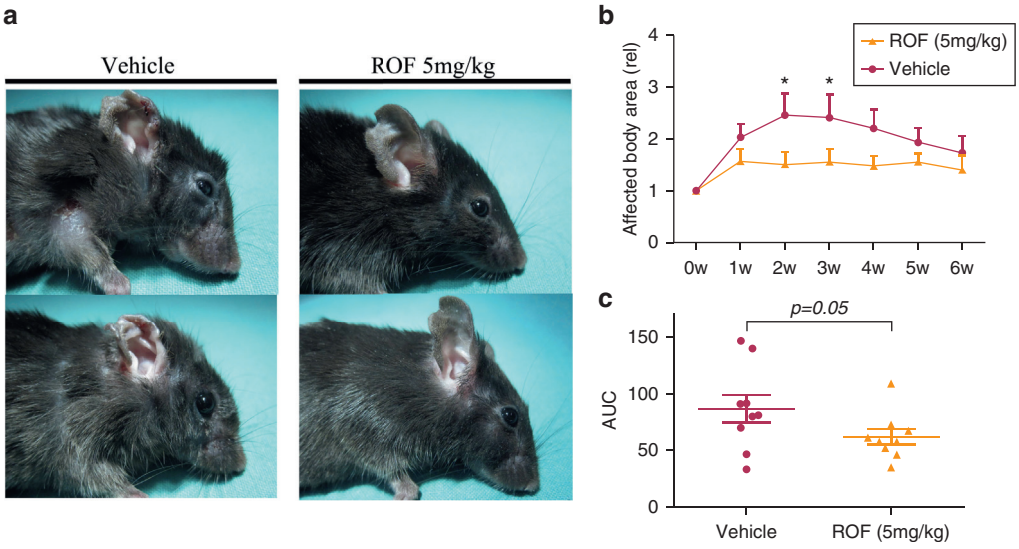

Figure 4. ROF hinders disease progression in mice already clinically manifesting immunization-induced EBA. EBA was induced in B6.SJL-H2s (B6.s) mice by single immunization. After immunization, mice were allocated to treatment groups when 2% or more of their body surface area was affected by skin lesions. **(a)** Representative clinical pictures of mice in the indicated treatment groups at 3 weeks of treatment. **(b)** Affected body surface area in ROF-treated (n = 9) or vehicle-treated (n = 9) animals during the 6-week treatment period (*$P <$ 0.05, two-way analysis of variance, taking week and treatment type into consideration). **(c)** AUC of percentage affected body area until 6 weeks in each group (Mann-Whitney Rank Sum Test). AUC, area under the curve; rel, relative; ROF, roflumilast; w, weeks.

Based on the incomplete inhibition of ROS release from IC-activated neutrophils using inhibitors of Akt, p38, or ERK (Hellberg et al., 2013), we assume that additional pathways can lead to the activation of NCF1. PDE4, especially PDE4B, is linked to MAPK1 and -14, which are linked to NCF1 (see Supplementary Figure S3). The interactions shown in Supplementary Figure S3 are based on the predictions of the STRING database and need to be experimentally validated in the context of EBA. We recently showed the crucial roles of both MAPKs in IC-induced ROS release from neutrophils (Hellberg et al., 2013), indicating that the interaction of these two MAPKs with PDE4 may be crucial for the release of ROS from IC-activated myeloid cells. This assumption is supported by the observation of a decreased signal intensity of MAPK in stimulated airway epithelial cells treated with PDE4 inhibitors (Victoni et al., 2014).

In addition to insights into downstream signaling that activates Fc gamma receptors and into the pathogenesis of blistering in EBA (Iwata et al., 2015c; Kasperkiewicz et al., 2016), our results are also of clinical relevance. First, the primary treatment of patients with PD, including EBA, is topical or systemic corticosteroid treatment (Joly et al., 2002; Kim et al., 2011). In accordance with this clinical practice, systemic corticosteroids are effective in mice with already established immunization-induced EBA (Hirose et al., 2015). In patients, the drawbacks of continued corticosteroid treatment include frequent relapses after withdrawal in bullous pemphigoid (Joly et al., 2009), insufficient efficacy in EBA and mucous membrane pemphigoid (Kim et al., 2011) and treatment-associated adverse drug reactions in both disorders (Buchman, 2001). The combination of corticosteroids with PDE4 inhibitors enhanced glucocorticoid sensitivity in human acute lymphoblastic leukemia and in human acute lymphoblastic leukemia cells (Ogawa et al., 2002), pointing to the synergistic effects of the two drugs. Similar observations were made in neutrophils from patients with chronic obstructive pulmonary disease. Here, the combination of RNO and dexamethasone showed additive/synergistic effects, which were consistent with the reversal of corticosteroid-resistant molecular markers by RNO (Milara et al., 2014). In contrast to these observations, the combination of both compounds did not show synergistic effects in our preclinical EBA mouse model. However, comparing the efficacy of ROF and high doses of systemic corticosteroids head to head in this model showed that both drugs are

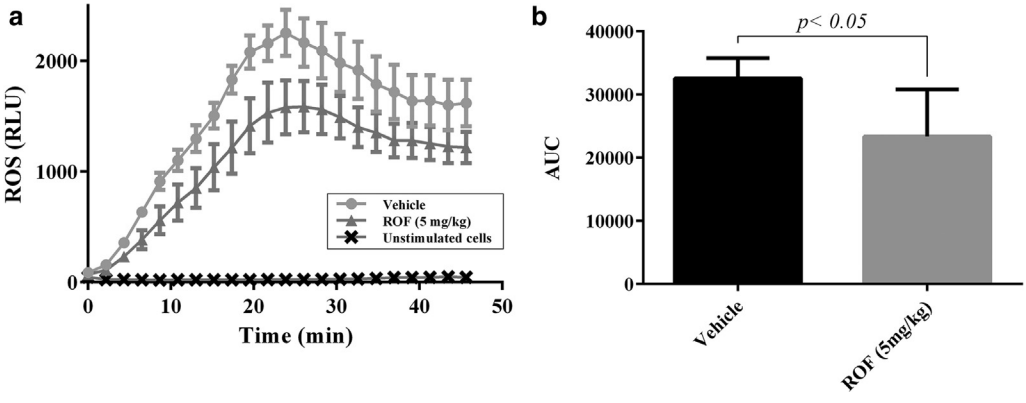

Figure 5. Reduced release of ROS in PMN isolated from ROF-treated mice. Murine PMN were isolated from the bone marrow of vehicle-treated (n=4) and ROF-treated mice (5mg/kg/day p.o.; n = 5) at 6 week. **(a)** PMNs were stimulated with PMA, and subsequent ROS production was monitored. **(b)** AUC of ROS production from vehicle treated- and ROF-treated mice ($P = 0.0465$, one-tailed *t* test). AUC, area under the curve; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocyte; RLU, relative light unit; ROF, roflumilast; ROS, reactive oxygen species.

equally effective in slowing down disease onset. This and the good tolerance of PDE4 inhibitors in both chronic obstructive pulmonary disease and psoriasis (Kumar et al., 2013; Lipworth, 2005) strongly suggests that patients with corticosteroid-treated EBA may benefit from adjuvant PDE4 inhibition. Recently, apremilast has been licensed for the treatment of psoriasis (Papp et al., 2012). However, other PDE4 inhibitors, that is, ROF, have a higher activity compared with apremilast (Man et al., 2009). We therefore focused on the effects of ROF, including its metabolites. ROF is marketed as a treatment for severe chronic obstructive pulmonary disease to reduce the frequency of exacerbations (Rabe, 2011; Tashkin, 2014). Neutrophils are a hallmark in exacerbations, and it is thought that the clinical efficacy of ROF is partly driven by its effects to inhibit neutrophils (Grootendorst et al., 2007; Hatzelmann et al., 2010). RNO is the active metabolite of ROF that largely accounts for overall PDE4 inhibition, although the clinical efficacy has not been tested (Bethke et al., 2007; Tenor et al., 2011).

Collectively, we showed that PDE4 inhibitors selectively affect PMN activation in vitro and ex vivo. We furthermore showed that PDE4 inhibition impairs both disease manifestation and progression in EBA mouse models and ultimately link this in vivo inhibitory effect to neutrophil activation. Hence, adjuvant PDE4 inhibition may be of potential use in patients with EBA and other forms of PD.

MATERIALS AND METHODS

Studies with human serum, leukocytes, and skin

Enrolled EBA patients (n = 6) fulfilled the following criteria: (i) presentation with skin lesions resembling EBA, (ii) linear IgG and/or IgA deposition in direct immunofluorescent microscopy, (iii) presence of an u-serrated pattern on direct immunofluorescent microscopy and/or detection of autoantibodies against COL7 by Western blot test in patient serum. Skin biopsy samples from EBA patients fulfilling these criteria were obtained from the lesional site of inflammatory type (n = 4) or mechanobullous type (n = 2) for analysis of PDE4 expression by immunofluorescent microscopy. Skin specimens from plastic surgery served as a reference. Leukocytes were collected from healthy blood donors (n = 21). All experiments using human samples were approved by the local ethics committee and were performed according to the Declaration of Helsinki. Blood donors and patients gave their written informed consent before study participation.

Mice

C57BL/6J (B6) and B6.SJL-H2s (B6.s) mice were obtained from colonies held at the animal facility at the University of Lübeck. Animals were fed acidified drinking water and standard chow ad libitum and were held on a 12-hour light-dark cycle at the animal facility of the University of Lübeck. Mice aged 6–10 weeks were used for the experiments. All clinical examinations, biopsies, and bleedings were performed under anesthesia with intraperitoneal administration of a mixture of ketamine (100 μg/g) and xylazine (15 μg/g). The experiments were approved by the Animal Care and Use Committee (Kiel, Germany) and performed by certified personnel.

Compounds and antibodies

ROL and ROF were purchased from Selleckchem (Munich, Germany). RNO was purchased from MyBioSource, Inc. (San Diego, CA). Rabbit anti-PDE4 polyclonal antibody, detecting all known PDE4 A and D variants with a lower affinity for PDE4B, was purchased from Abcam (Cambridge, MA), and FITC conjugated donkey anti-rabbit IgG was purchased from Jackson Immuno Research (West Grove, PA).

Generation of the vWFA2 recombinant protein and anti-murine vWFA2 IgG

Recombinant murine vWFA2 of the noncollagenous domain 1 (NC1) of COL7 (aa 1048–1238 with 5 additional amino acids [GRAMG] at the N-terminus) were produced as previously described using prokaryotic expression (Leineweber et al., 2011). Rabbit anti-murine vWFA2 IgG was generated as previously described (Iwata et al., 2015c). IgG from rabbit serum was isolated using Protein G Sepharose Fast Flow affinity column chromatography (Amersham Biosciences, Freiburg, Germany) as described (Sitaru et al., 2005). Reactivity of IgG fractions was analyzed by immunofluorescent microscopy on murine skin. Concentrations of the purified rabbit IgG were measured at 280 nm by spectrophotometer.

In vitro PMN activation (ROS release)

Immobilized IC-induced ROS release from PMN was performed as previously reported (Recke et al., 2014). White microtiter plates (Greiner BioOne, Frickenhausen, Germany) were coated for 3 hours with a recombinant protein located within the human noncollagenous domain of COL7 known as hCOL7EF (Recke et al., 2014) at 1 μg/well in carbonate buffer at pH 9.6. After washing with phosphate buffered saline with Tween 20, plates were blocked with 1% bovine serum albumin in phosphate buffered saline with Tween 20 overnight at 4 °C on a rocking platform. Subsequently, recombinant chimeric anti-hCOL7EF IgG1 (Recke et al., 2014) diluted in 1% bovine serum albumin in phosphate buffered saline with Tween 20 was added and incubated for 1 hour. In parallel, human PMNs were isolated using PolymorphPrep (Axis-Shield GmbH, Oslo, Norway) according to the manufacturer's instructions. After purification, PMN were resuspended in color-free RPMI 1640 chemiluminescence medium supplemented with 10 mg/ml luminol (5-amino-2,3-dihydro-1,4-phthalazindione; Roche Diagnostics, Mannheim, Germany). After preincubation with inhibitors for 15 minutes at room temperature, PMN were added to the plate-fixed IC. Immediately, chemiluminescence was measured for 60 minutes using a VICTOR 3 reader (PerkinElmer, Waltham, MA). The obtained data were expressed as relative light units. N-formylmethionyl-leucyl-phenylalanine (fMLP) at a final concentration of 500 nmol/L was used as positive control. Each measurement was repeated with PMN from five donors in duplicate. For a similar assay using murine PMN, the purification of PMN from femurs 1 hour after final administration of ROF or vehicle was performed as previously reported (Dorward et al., 2013). The purified PMN were incubated with 100 ng/ml of PMA and measured for 45 minutes, as described for the human PMN.

Determination of separation of the dermal-epidermal junction

PMN from healthy volunteers were isolated as previously described (Sitaru et al., 2002b). Separation of the dermal-epidermal junction of cryosections of human skin was evaluated using an ex vivo model as previously described (Sitaru et al., 2002a; Sitaru et al., 2002b). Finally, separation of the dermal-epidermal junction in the absence or presence of PDE4 inhibitors (preincubation with PMN for 15 minutes at room temperature) was calculated as length of separation divided by total length of the dermal-epidermal junction measured with BZ-9000 fluorescence microscope (Keyence, Frankfurt,

Germany). Evaluation was carried out by an investigator unaware of the section's treatments. Each compound was tested with PMN from four healthy donors.

Determination of CD62L, CD11b, and CD66b expression PMN by flow cytometry

Granulocytes from healthy adult volunteers (n = 8) were isolated as previously described (Aga et al., 2002; Laufs et al., 2002; Lotz et al., 2004). All procedures were conducted at room temperature. After preincubation with or without the presence of each PDE4 inhibitor at various concentrations for 15 minutes, PMN were applied to an IC-coated 96-well plate (prepared as described above) and incubated for 1 hour at 37 °C. Cells were stained using anti-human CD66b-FITC (BD, Heidelberg, Germany), anti-human CD62L-APC (BioLegend, München, Germany) and anti-human CD11b-R PE (Caltag, Hamburg, Germany). Samples were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and the data were evaluated using Cell Quest Pro (BD Biosciences).

Murine EBA models

For antibody transfer-induced EBA, C57BL/6J mice were subcutaneously injected with 2 mg of total rabbit IgG collected from rabbits immunized with murine vWFA2 (injections on experimental days 0, 2, 4, 6, 8, and 10). Administration of vehicle (0.5% carboxymethyl cellulose, 0,25% Tween 80), 1 mg/kg of ROF, or 5 mg/kg of ROF once daily by oral gavage was started on the day before the first IgG injection (day −1) and continued until day 11; 20 mg/kg of methylprednisolone (Urbason, Sanofi-Aventis, Frankfurt, Germany) in 0.9% NaCl was administrated intraperitoneally once daily. On day 12, serum and organs were obtained for analysis of secondary endpoints. For induction of immunization-induced EBA, B6.SJL-H2s mice were immunized via the hind footpad with 120 μg recombinant protein of murine vWFA2 domain emulsified in the nonionic block copolymer adjuvant TiterMax (ALEXIS Biochemicals, Norcross, GA). Thereafter, the mice were evaluated weekly for the presence of skin lesions. If an individual mouse had 2% or more of its body surface area affected, it was randomly allocated to treatment with vehicle (0.5% CMC, 0.25% Tween 80) or 5 mg/kg of ROF once daily by oral gavage. Disease severity was calculated as percentage of body surface area affected by skin lesions (i.e., erosions, crusts, and/or erythema), and total clinical disease severity during the observation period was expressed as area under the curve calculated from the disease manifestations over time for each individual mouse. After allocation to treatment, serum samples were collected every week in immunization-induced EBA. Ear skin samples were obtained at the final day and analyzed by histopathology. Peripheral blood cells taken from the immunization-induced mouse model (n = 5 from each group) on the final day were counted using a Hemavet 950FS (Drew Scientific, Oxford, CT). Serum samples from the immunization-induced mouse model (0−6 weeks of treatment, n = 6 from each group) were analyzed for the concentration of anti-vWFA2 IgG by ELISA as previously described (Iwata et al., 2013).

Statistical analysis

The data are presented as the mean ± standard error of the mean. Statistical calculations were performed using SigmaPlot version 12.0 (Systat Software, San Jose, CA). The tests used are indicated in the figure legends. A *P*-value of 0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.06.619.

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