Dimethylfumarate Impairs Neutrophil Functions

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Susen Müller[1,4], Martina Behnen[2,4], Katja Bieber[1], Sonja Möller[2], Lars Hellberg[2,3], Mareike Witte[1], Martin Hänsel[1], Detlef Zillikens[1], Werner Solbach[2], Tamás Laskay[2,4] and Ralf J. Ludwig[1,4]

Host defense against pathogens relies on neutrophil activation. Inadequate neutrophil activation is often associated with chronic inflammatory diseases. Neutrophils also constitute a significant portion of infiltrating cells in chronic inflammatory diseases, for example, psoriasis and multiple sclerosis. Fumarates improve the latter diseases, which so far has been attributed to the effects on lymphocytes and dendritic cells. Here, we focused on the effects of dimethylfumarate (DMF) on neutrophils. In vitro, DMF inhibited neutrophil activation, including changes in surface marker expression, reactive oxygen species production, formation of neutrophil extracellular traps, and migration. Phagocytic ability and autoantibody-induced, neutrophil-dependent tissue injury ex vivo was also impaired by DMF. Regarding the mode of action, DMF modulates—in a stimulus-dependent manner-neutrophil activation using the phosphoinositide 3-kinase/Akt-p38 mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 pathways. For in vivo validation, mouse models of epidermolysis bullosa acquisita, an organ-specific autoimmune disease caused by autoantibodies to type VII collagen, were employed. In the presence of DMF, blistering induced by injection of anti-type VII collagen antibodies into mice was significantly impaired. DMF treatment of mice with clinically already-manifested epidermolysis bullosa acquisita led to disease improvement. Collectively, we demonstrate a profound inhibitory activity of DMF on neutrophil functions. These findings encourage wider use of DMF in patients with neutrophil-mediated diseases.

INTRODUCTION

Neutrophils are the vanguard of the cellular innate immune system to invading pathogens (Amulic et al., 2012). In addition, they have been recently shown to orchestrate important functions of the adaptive immune system (Puga et al., 2012). The overall importance of neutrophils for host defense is underscored by the detrimental effects of congenital or acquired defects in neutrophil function, that is, recurrent infections in patients with chronic granulomatous disease (Leiding and Holland, 1993). Furthermore, the dysregulation of neutrophil function also leads to chronic inflammation (Leiding and Holland, 1993). Uncontrolled neutrophil activation has also been demonstrated to contribute to the pathogenesis of several chronic inflammatory diseases. For example, in the absence of neutrophils, mice are completely resistant to the arthritogenic and inflammatory effects of the K/BxN serum. Furthermore, the depletion of neutrophils in mice with already-established arthritis reverses the inflammatory reaction (Wipke and Allen, 2001). Similar observations were made in murine experimental autoimmune encephalomyelitis, a model for multiple sclerosis (McColl et al., 1998). The key role of neutrophils has also been documented in several models of autoimmune skin blistering diseases (Ludwig et al., 2013). In autoimmune skin blistering diseases, autoantibodies to structural proteins directly or indirectly induce blister formation in skin and/or mucous membranes (Schmidt and Zillikens, 2013; Stanley and Amagai, 2006). In two model systems of autoimmune skin blistering diseases, bullous pemphigoid (BP) with autoimmunity to type XVII collagen (COL17) and epidermolysis bullosa acquisita (EBA) with autoimmunity to type VII collagen (COL7), the depletion of neutrophils completely protected mice from blistering induced by transfer of auto-antibodies (Chiriac et al., 2007; Liu et al., 1997, 2008).

In addition to the diseases described above, where the pathogenic relevance of neutrophils has been demonstrated in experimental models, the presence of neutrophils has been documented in several chronic inflammatory diseases, such as psoriasis (Schön and Boehncke, 2005), severe asthma (Fahy et al., 1995; Nakagome et al., 2012), and systemic lupus erythematosus (Hakkim et al., 2010; Kaplan, 2011; Villanueva et al., 2011). Over the past decades, the incidence of chronic inflammatory diseases has increased dramatically (Bach, 2002). Despite improved treatments, morbidity and mortality of these patients are still high (Bernatsky et al., 2006; Joly et al., 2002). The associated cardiovascular comorbidity further adds to the high medical burden (Asanuma et al.,

[1]*Department of Dermatology, University of Lübeck, Lübeck, Germany;* [2]*Institute for Medical Microbiology and Hygiene, University of Lübeck, Lübeck, Germany; and* [3]*Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany*

[4] *These authors contributed equally to this work.*

Correspondence: Ralf J. Ludwig, Department of Dermatology, University of Lübeck, RatzeburgerAllee 160, D-23538 Lübeck, Germany. E-mail: ralf.ludwig@uksh.de

Abbreviations: BP, bullous pemphigoid; COL7, type VII collagen; COL17, type XVII collagen; DMF, dimethylfumarate; EBA, epidermolysis bullosa acquisita; fMLP, formyl-methionylleucyl-phenylalanine; iIC, immobilized immune complex; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NET, neutrophil extracellular trap; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α

2003; Gelfand et al., 2006; Ludwig et al., 2007). Hence, there is a great medical need for the development of novel, safe, and effective treatment modalities for these patients.

Intriguingly, *Fumaria officinalis,* a plant rich in fumaric acid, has been used as a remedy for inflammatory skin complaints since the 17th century. The clinical use of fumaric acid derivatives started in the late 1950s when the German chemist Schweckendiek, suffering from psoriasis, successfully treated himself with fumaric acid derivatives (Schweckendiek, 1959). Several decades later, a more standardized preparation of fumaric acid derivatives was approved for the treatment of psoriasis in Germany (Mrowietz and Asadullah, 2005). On an anecdotal basis, fumarates have been noted to be therapeutically effective in other diseases associated with neutrophil infiltration (Meissner et al., 2012).

Different modes of action of fumaric acid derivatives have been described, including modulation of cytokine production, lymphocyte apoptosis, lymphocyte as well as endothelial cell adhesion molecule expression, NF-κB activity, and cellular redox systems (Ghoreschi and Rocken, 2004; Ghoreschi et al., 2011; Milenkovic et al., 2008; Mrowietz and Asadullah, 2005; Rubant et al., 2008; Treumer et al., 2003; Vandermeeren et al., 1997; Wallbrecht et al., 2011). However, only a few studies have addressed the role of fumaric acid derivatives, namely dimethylfumarate (DMF) and its main metabolites methylhydrogenfumarate and monomethylfumarate, in human monocytes and neutrophils after activation. One study demonstrated that monomethylfumarate activated neutrophils evidenced by increased elastase release and intracellular killing of bacteria; in this study, monomethylfumarate also inhibited formyl-methionyl-leucyl-phenylalanine (fMLP)-induced reactive oxygen species (ROS) burst (Nibbering et al., 1993). In another study, DMF and methylhydrogenfumarate dose-dependently enhanced superoxide anion generation from monocytes, but not from neutrophils (Zhu and Mrowietz, 2005). Despite the latter observations, based on the effectiveness of fumaric acid derivatives in psoriasis and multiple sclerosis as well as the predominance of neutrophils in these diseases (Braun-Falco and Burg, 1970; McColl et al., 1998), we hypothesized that fumaric acid derivatives, namely DMF, impair neutrophil functions.

To address this assumption, we first evaluated the effect of DMF on neutrophil function in vitro and then went on to characterize the prophylactic and therapeutic effect of DMF in animal models of an organ-specific autoimmune disease, where tissue injury depends on neutrophils.

RESULTS

In vitro effects of DMF on neutrophil effector function and activation

The luminol-amplified chemiluminescence assay was used to measure the sum of intra- and extracellular ROS (Briheim et al., 1984; Hasegawa et al., 1997; Stevens and Hong, 1984). We here demonstrate that DMF significantly and dose-dependently (doses ranging from 5 to 15 μg/ml) reduced the lipopolysaccharide (LPS)/fMLP, phorbol 12-myristate 13-acetate (PMA), tumor necrosis factor-α (TNF-α), and immobilized immune complex (iIC)-induced ROS production of human neutrophils (Figure 1a and b). Furthermore, PMA- and iIC-induced neutrophil extracellular trap (NET) formation

was sensitive to DMF (Figure 1c–e). All used DMF concentrations (5, 10, and 15 μg/ml) significantly impaired the PMA-induced NET formation (Figure 1d), whereas only 15 μg/ml DMF had a significant inhibitory effect on iIC-induced NET formation (Figure 1d). The effects on PMA-induced NET production were verified by fluorescence microscopy of SYTOXgreen-stained neutrophils, which confirmed a dose-dependent inhibitory effect of DMF on PMA-induced NET release (Figure 1e). TNF-α and IL-8 did not induce NETs in our experimental settings (data not shown). We also assessed the impact of DMF on phagocytosis. The LPS+IFN-γ-induced phagocytosis was inhibited by the pretreatment of neutrophils with DMF (Supplementary Figure S1 online). Whereas DMF doses below 15 μg/ml had no significant effect on phagocytosis, 15 μg/ml of DMF significantly reduced the number of neutrophils with phagocytosed beads (Supplementary Figure S1a and c). Similarly, the number of beads per neutrophil was also reduced (Supplementary Figure S1b and c). The shedding of CD62L and degranulation (increased expression of CD11c and CD66b) are also key features of neutrophil activation. The exposure of resting neutrophils to DMF had no effect on CD62L expression (data not shown). In iIC-activated neutrophils, 15 μg/ml DMF led to increased CD62L shedding, whereas DMF treatment (10 and 15 μg/ml) of LPS+IFN-γ-activated neutrophils significantly inhibited CD62L shedding (Supplementary Figure S2a and b online). DMF had no significant effect on IL-8- or TNF-α-induced CD62L shedding. In resting neutrophils, exposure to DMF had no effect on the expression of the granule markers CD11b and CD66b (not shown). DMF treatment also had no effect on the IL-8-, TNF-α-, or iIC-induced enhanced cell surface expression of CD11b (Supplementary Figure S2c and d) and CD66b (Supplementary Figure S2e and f). Preincubation with DMF, however, strongly diminished the LPS+IFN-γ-induced enhanced CD11b (Supplementary Figure S2c and d, 15 μg/ml DMF has an effect) and CD66b (Supplementary Figure S2e and f) expression in a dose-dependent manner. Lastly, the effect of DMF on neutrophil migration was evaluated in vitro. DMF had no effect on neutrophil chemotaxis in the transwell system when IL-8 was added to the lower chamber (Figure 2a). By using TNF-α as chemoattractant, DMF significantly blocked migration in a dose-dependent manner (Figure 2b). Whereas 5 μg/ml DMF had no significant effect, 10 or 15 μg/ml of DMF significantly reduced migration (Figure 2b). Similar observations, that is, no effect on IL-8-induced migration (Figure 2c), but the impairment of TNF-α-directed migration (Figure 2d), were obtained when the effect of DMF on neutrophil transendothelial migration was evaluated (Figure 2c and d). These results indicate that inhibitory effects by DMF on neutrophils functions are dependent on the stimulus and may involve specific intracellular signaling cascades. All of the above-mentioned changes were achieved at nontoxic doses of DMF (Supplementary Figure S3 online).

DMF treatment results in reduced autoantibody-induced neutrophil-dependent tissue injury ex vivo

Autoantibodies directed against antigens located at the dermal-epidermal junction (e.g., type VII or type XVII collagen) induce subepidermal split formation in the presence of neutrophils on cyrosections of human skin (Gammon
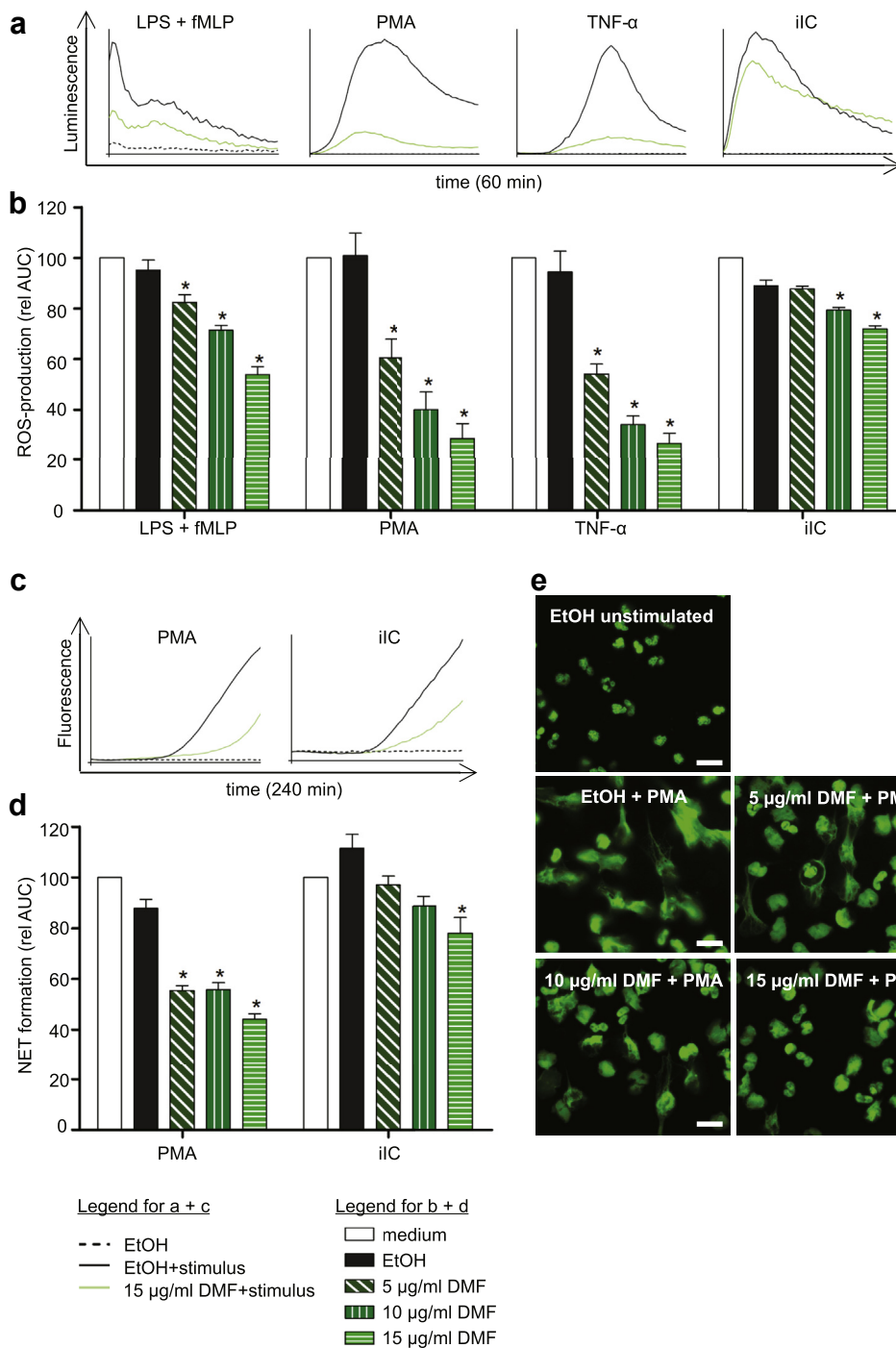
et al., 1982; Sitaru et al., 2002a, 2002b). Confirming previous work, we demonstrate that in contrast to normal human serum, serum obtained from patients with BP induced split formation in cryosections of human skin (Figure 3). DMF (5, 10, and 15 μg/ml) reduced split formation in this assay (Figure 3). Similar results were obtained when serum from patients with EBA was used (not shown).

DMF affects Akt, p38 MAPK, and ERK phosphorylation in neutrophils in a stimulus-dependent manner

To assess which activating pathways are targets of DMF, we evaluated the effect of DMF on p38 mitogen-activated protein kinase (MAPK), extracellular signal−regulated kinase (ERK) 1/2, and Akt phosphorylation in human neutrophils on stimulation with TNF-α, IL-8, and iIC. Moreover, we used fMLP or LPS as stimuli. fMLP is known to activate p38 MAPK and preferentially the ERK 1/2 pathway (Zu et al., 1998) and stimulates Akt phosphorylation in neutrophils (Tilton et al., 1997), whereas LPS is mainly involved in p38 MAPK signaling (Detmers et al., 1998). A marked inhibition of Akt phosphorylation was observed in IL-8-, TNF-α-, and fMLP-activated neutrophils when treated with DMF, but not in iIC- and LPS-stimulated neutrophils (Figure 4a). The effects of DMF on p38 MAPK phosphorylation were diverse. Although

Figure 2. DMF specifically inhibits TNF-α, but not IL-8-induced neutrophil chemotaxis and transendothelial migration.
Neutrophils were pretreated with various concentrations of DMF or with EtOH as a solvent control. (a, b) Chemotaxis and (c, d) transendothelial migration in response to IL-8 (a, c) or TNF-α (b, d) values (n = 3) were normalized to migrated cells without exposure to DMF or EtOH (medium). Data show mean + standard error. *P < 0.05; differences were calculated with analysis of variance followed by the Bonferroni t-test for multiple comparisons versus control (EtOH). DMF, dimethylfumarate; TNF-α, tumor necrosis factor-α.
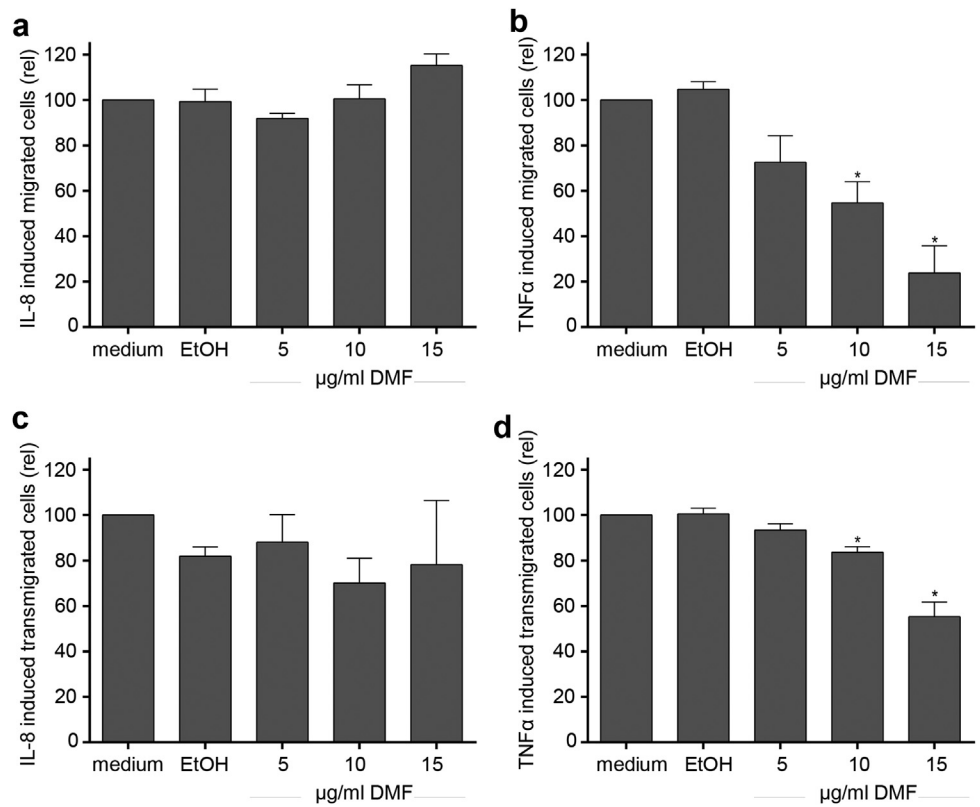
Figure 3. DMF reduces autoantibody-induced and neutrophil-dependent dermal-epidermal separation ex vivo.
Neutrophil-dependent ex vivo dermal-epidermal separation was induced by autoantibodies against type XVII collagen; NHS served as negative control. (a) Mean ± SEM values are shown (n = 10). *P < 0.05; differences were calculated with repeated measures analysis of variance followed by Dunnett's method for multiple comparisons versus control (EtOH). (b) Representative sections from the indicated treatment groups from the above experiments. The arrows indicate dermal-epidermal separation. DMF, dimethylfumarate; EtOH, positive controls incubated with solvent (ethanol); NHS, normal human serum; PC, positive control (BP sera).
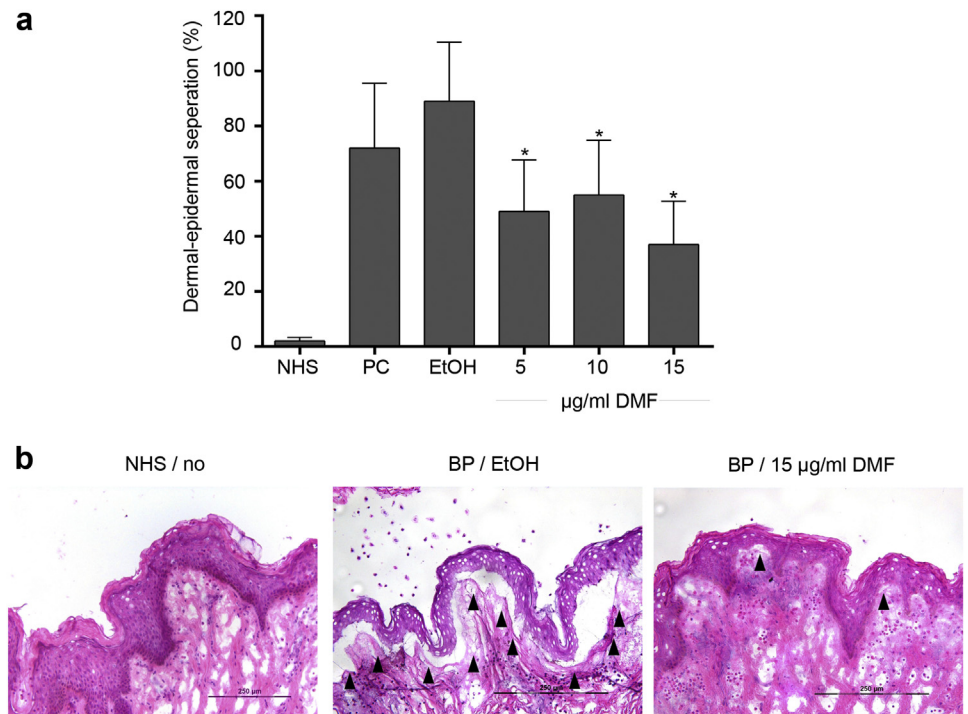
DMF reduced p38 phosphorylation in LPS-stimulated neutrophils, it induced and/or enhanced p38 phosphorylation in unstimulated and IL-8-stimulated neutrophils (Figure 4b). DMF had no effect on p38 phosphorylation of TNF-α-, iIC-, or fMLP-activated neutrophils. ERK 1/2 phosphorylation was inhibited by DMF in IL-8-, TNF-α-, and LPS-activated cells, whereas no effect of DMF was observed on ERK 1/2 phosphorylation in iIC- and fMLP-treated cells (Figure 4c). The inhibitory effects of DMF on phosphorylation of Akt and ERK were also obvious when different concentrations of the
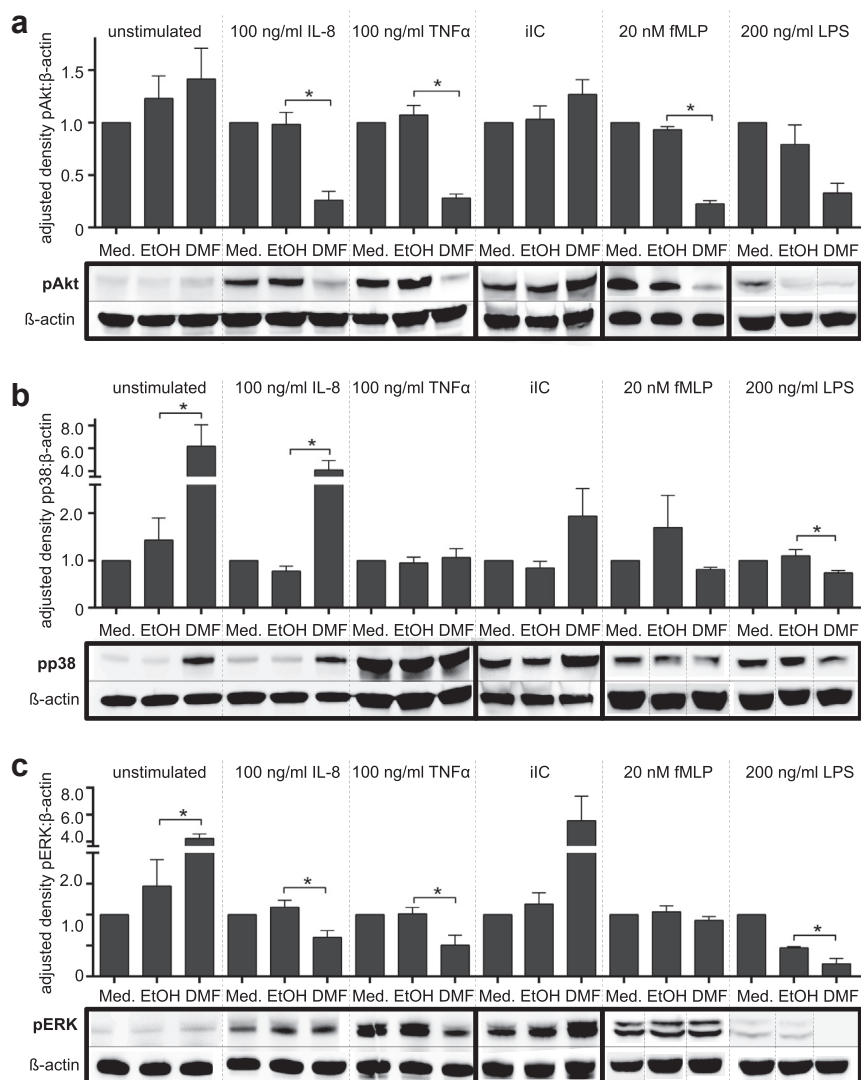
Figure 4. DMF affects Akt, p38 MAPK, and ERK phosphorylation in neutrophils. Neutrophils were pretreated with DMF, solvent control or left untreated before activation by IL-8, TNF-α, iIC, fMLP, or LPS. Phosphorylation of Akt (**a**), p38 MAPK (**b**), and ERK (**c**) was detected and quantified by the Western blot analysis of whole cell lysates. Representative blots of one from a total of three experiments and/or conditions and quantitative analyses (adjusted densities) are shown. For the quantitative analysis, the pAkt, pp38 MAPK, and pERK signals from three independent Western blots were adjusted to the β-actin signal detected on the same blot or from the same sample and normalized to the respective medium control. Quantitative data are shown as mean ± standard error. *$P < 0.05$ correspond to a statistical significant difference of DMF-treated samples compared with the ethanol control as analyzed by analysis of variance with the Bonferroni post-test. DMF, dimethylfumarate; ERK, extracellular signal-regulated kinase; fMLP, formyl-methionyl-leucyl-phenylalanine; iIC, immobilized immune complex; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor-α.

activation agents (1−10 μg/ml IL-8 or TNF-α) were used (data not shown). These results suggest that DMF modulates neutrophil functions stimulus dependent by specifically affecting the phosphoinositide 3-kinase/Akt, p38 MAPK, and ERK 1/2 pathways.

DMF has prophylactic and therapeutic effects in mouse models of organ-specific neutrophil-dependent autoimmune disease

We next evaluated the impact of prophylactic DMF application in the antibody transfer model of EBA. This model was chosen because disease manifestation strongly depends on activated neutrophils (Ludwig, 2012). The affected body surface area in mice treated with DMF was lower compared with mice receiving methylcellulose, during the observation period (Figure 5a). The overall disease severity showed a great variation, which was mainly attributed to the gender of the mice, that is, female mice presented with a significantly higher disease activity than male mice ($P < 0.001$, two-way analysis of variance [ANOVA]). Despite this variability, disease severity of DMF-treated mice was significantly lower

compared with those treated with methylcellulose (Figure 5b and d). These differences were independent of changes of the IgG and C3 deposition at the dermal-epidermal junction (not shown). Moreover, we could observe that DMF treatment results in a lower number of activated bone marrow neutrophils (CD62L negative/GR-1 positive) in EBA diseased mice (Figure 5c), but not in healthy mice (data not shown).

To test for a possible therapeutic activity of DMF in experimental EBA, mice with already-clinically established EBA were allocated to DMF treatment or methylcellulose. At the time of randomization to treatment, clinical EBA manifestation was identical in all groups (Supplementary Table S1 online). Furthermore, mice were allocated to treatment at a similar time point after immunization (data not shown). Treatment with DMF at 2 × 50 mg/kg, but not at lower doses, significantly improved blistering (Figure 6). A more detailed analysis of changes in disease severity showed an increase of skin blistering in methylcellulose treated mice for 3 weeks, which then remained at a plateau until the end of the observation period (Figure 6). In contrast, DMF led to a decrease in skin blistering, which was evident as early as 1

Figure 5. DMF hinders neutrophil activation and the induction of autoantibody-induced, neutrophil-dependent tissue injury in vivo.
(**a**) Mean (standard error) percentage of the body surface area affected by experimental EBA skin lesions in C57BL/6 mice treated with solvent (methylcellulose, gray circles) or DMF (green circles) during the 12-day observation period. Data are based on 16 solvent and 17 DMF-treated C57BL/6 mice. (**b**) Mean ± SEM values are shown for overall disease activity in the same mice. *$P < 0.05$; differences were calculated with two-way analysis of variance followed by the Student-Newman-Keuls method for multiple comparisons taking treatment and gender of the mice as independent variables. (**c**) Mean ± SEM values are shown for percentage of activated (CD62L-negative/Gr-1-positive) bone marrow neutrophils from EBA diseased C57BL/6 mice treated with solvent (n = 6) or 50 mg/kg DMF (n = 6). *$P < 0.05$; differences were calculated with the *t*-test. (**d**) Representative clinical presentations of four individual mice per treatment at the end of the experiment (day 12). DMF, dimethylfumarate; EBA, epidermolysis bullosa acquisita.
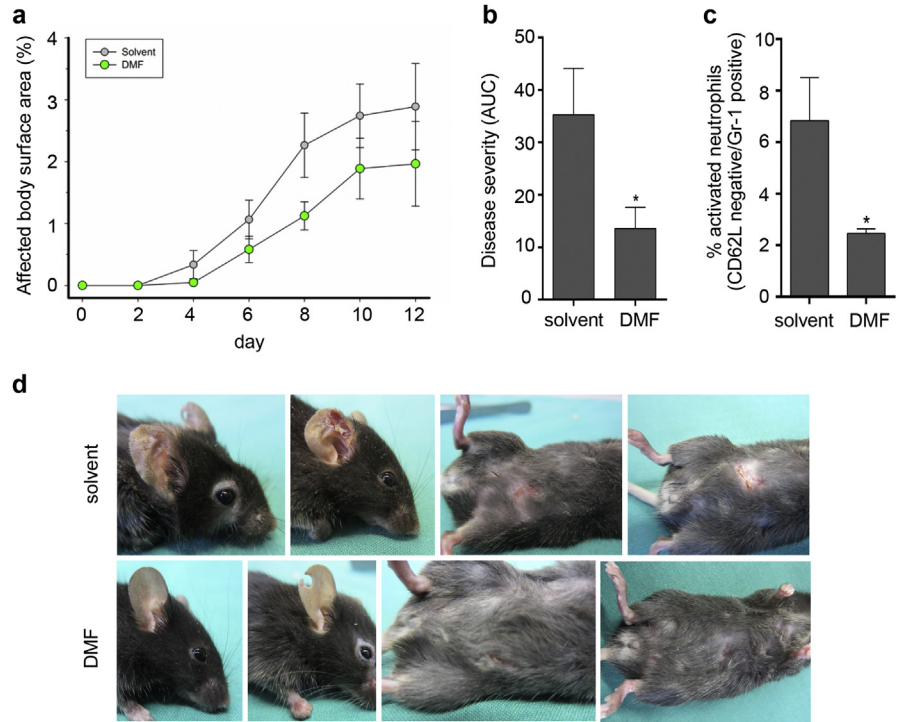

Figure 6. DMF treatment improves skin blistering in mice with already-established immunization-induced EBA. (**a**) Mean (standard error) percentage of the body surface area affected by experimental EBA skin lesion in SJL/J mice treated with solvent (methylcellulose, gray circles) or DMF (2 × 50 mg, green circles) during the 4-week treatment period. Scoring (and treatments) was initiated after individual mice had 2% or more of their body surface area affected by EBA skin lesions. (**b**) Mean (boxes) SEM values (error bars) are shown for the overall disease activity in SJL/J mice with already-established skin blistering in experimental EBA treated with solvent (methylcellulose, gray circles) or DMF (green circles) during the 4-week treatment period. Data are based on 12 solvent, six 2 × 25 mg, and ten 2 × 50 mg DMF-treated mice. *$P < 0.05$; differences were calculated with two-way analysis of variance followed by the Student-Newman-Keuls method for multiple comparisons taking treatment and gender of the mice as independent variables. (**c**) Representative clinical presentations of three individual mice per treatment at the end of the experiment (week 4). DMF, dimethylfumarate; EBA, epidermolysis bullosa acquisita.
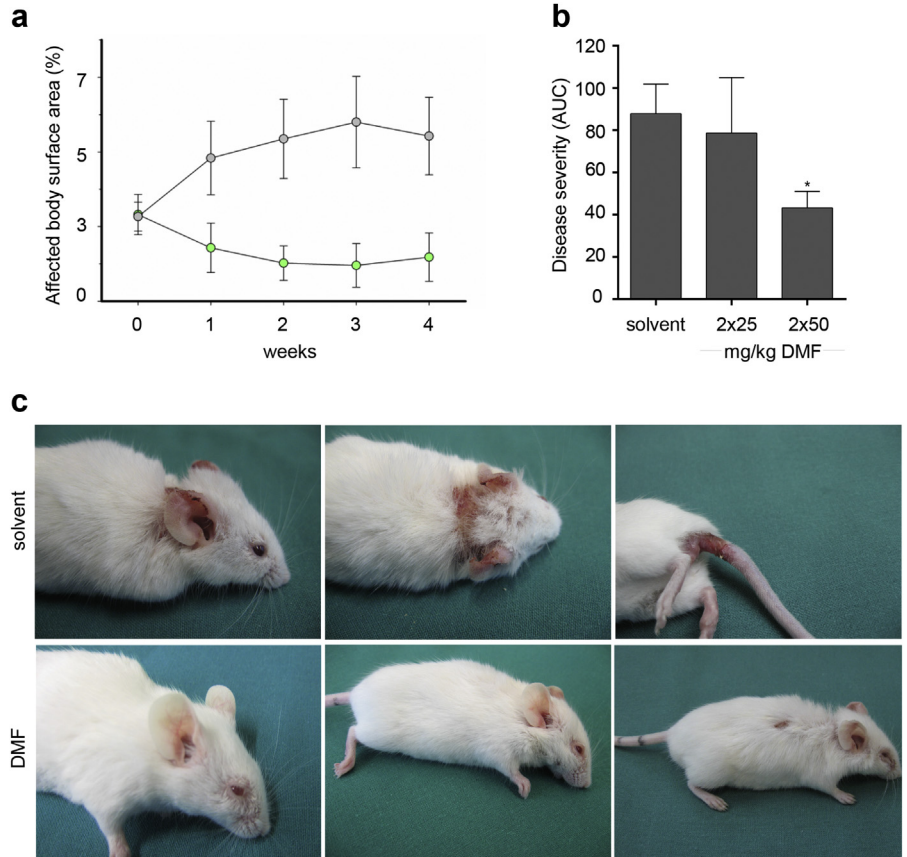
week after the initiation of treatment. This therapeutic effect was maintained throughout the observation period. Again, these differences in clinical disease severity were independent of changes of the IgG and C3 deposition at the dermal-epidermal junction, as well as the concentration of circulating anti-COL7 antibodies (not shown).

DISCUSSION

Recruitment and activation of neutrophils are crucial in the pathogenesis of many chronic inflammatory diseases. We here demonstrate a stimulus-dependent inhibitory activity of DMF on activation-induced functions of neutrophils. These effects of DMF are likely associated with the inhibitory effect of DMF on the phosphoinositide 3-kinase/Akt and p38 MAPK signaling pathways. We further demonstrate that both prophylactic and therapeutic application of DMF significantly impairs and/or improves neutrophil-dependent tissue injury and improves clinical disease manifestation in mice with experimental EBA.

Because of the rising incidence of pemphigoid diseases, predominantly BP, and the increased, partially treatment-associated mortality (Joly et al., 2002, 2012; Langan et al., 2008), there is a so far unmet high medical need for safe and effective treatment strategies for patients with BP and other pemphigoid diseases. Having demonstrated a therapeutic effect of DMF application in mice with already-established EBA, clinical trials using either Fumaderm or BG-12 (Kappos et al., 2008; Mrowietz et al., 2011) in patients with EBA or BP are warranted. Despite the recognition of different target antigens, namely COL7 in EBA (Woodley et al., 1988) or COL17 (Diaz et al., 1990) and BP230 (Stanley et al., 1988) in BP, both diseases share a similar pathogenesis. In detail, after binding of autoantibodies to their respective target antigen in the skin, a proinflammatory milieu is generated. In experimental models of BP and EBA, this process is predominantly mediated by complement activation (Karsten et al., 2012; Liu et al., 1995; Mihai et al., 2007; Nelson et al., 2006). Furthermore, the binding of anti-COL17 antibodies to keratinocytes has been demonstrated to induce IL-6 and IL-8 secretion in vitro (Schmidt et al., 2000). The synthesis of these cytokines is controlled by NF-κB, and DMF has potent inhibitory functions on NF-κB through inhibition nuclear translocation of p65 (Loewe et al., 2001) and indirectly inhibiting the phosphorylation and ubiquitination of IκB through increasing glutathione levels (Mrowietz and Asadullah, 2005). The short time frame of the in vitro experiments suggests that the observed inhibitory effects of DMF were not due to a modulation of NF-κB function. In our in vivo experiments, however, NF-κB modulation by DMF cannot be completely excluded. Overall, this proinflammatory milieu leads to a CD18-dependent neutrophil extravasation into the skin of mice with experimental BP or EBA (Chiriac et al., 2007; Liu et al., 2006). Expression (decrease) of the endothelial ligands for leukocyte integrins is mediated by DMF (Vandermeeren et al., 1997; Wallbrecht et al., 2011). Furthermore, DMF affects the expression of different additional leukocyte and endothelial adhesion molecules (Rubant et al., 2008; Vandermeeren et al., 1997; Wallbrecht et al., 2011). Although a contribution of adhesion molecules other than CD18 has not been demonstrated

experimentally in EBA and BP, the multistep nature of leukocyte extravasation into inflamed skin (Schön and Ludwig, 2005) suggests that the downregulation of different adhesion molecules, including leukocyte integrins, is another possible mode of action of DMF in BP and EBA.

In experimental BP and EBA, neutrophils are activated by iIC located in the skin. Interestingly, this process is completely dependent on Fcγ receptor III in experimental BP (Zhao et al., 2006), whereas the same process is solely dependent on Fcγ receptor IV in experimental EBA (Kasperkiewicz et al., 2012). In human models of BP, Fcγ receptors IIA and IIIB were responsible for binding of neutrophils to immune complexes (Yu et al., 2010). Intracellular signaling pathways, including phosphoinositide 3-kinase beta, Akt, p38 MAPK, and ERK phosphorylation, lead to neutrophil activation in experimental EBA (Hellberg et al., 2013; Kulkarni et al., 2011). In human keratinocytes, DMF was shown to specifically inhibit mitogen and stress-activated kinases 1 and 2 within the p38 MAPK pathway and transcription factors such as NF-κB and ATF1 that are downstream targets of mitogen and stress-activated kinase signaling (Gesser et al., 2007). Recent work also identified that DMF inhibits ERK phosphorylation in microglia (Wilms et al., 2010). Although the effects of fumaric acids on certain innate immune functions have been investigated in previous studies (Nibbering et al., 1993; Zhu and Mrowietz, 2005), no information regarding the mode of action of DMF on neutrophils has been available. Results from our present study show that DMF regulates neutrophil functions by the inhibition of phosphoinositide 3-kinase/Akt, p38 MAPK, and ERK signaling pathways. Of note, DMF did predominantly affect cytokine-induced phosphorylation of these molecules. Lately, the contribution of cytokines to blister formation in experimental EBA has been demonstrated (Ludwig et al., 2013). Moreover, because DMF increases glutathione levels (Spencer et al., 1990), DMF may counteract the proinflammatory effects induced by ROS.

In addition to the effects on pathways involved in autoantibody-induced and neutrophil-mediated tissue injury, DMF may also influence the production of pathogenic autoantibodies. In immunization-induced EBA, the induction of complement-fixing IgG2 anti-COL7 autoantibodies leads to skin blistering (Sitaru et al., 2006). This production of IgG2 autoantibodies occurs in the context of a Th1 polarization in peripheral lymph nodes of immunized mice (Hammers et al., 2011). As DMF can shift Th1-polarized immune responses toward a Th2 polarization (Ghoreschi et al., 2011; Ockenfels et al., 1998), this may be yet another possible therapeutic target for DMF in BP and EBA. However, we here did not observe any difference in autoantibody levels or changes of autoantibody isoforms, which may be due to the relatively long half-life of circulating and tissue-bound autoantibodies in experimental EBA (Kasperkiewicz et al., 2010).

In summary, we here identified neutrophils as a cellular target of DMF. Given the profound therapeutic effect of DMF in patients with psoriasis and multiple sclerosis, as well as our observation of the beneficial effect of DMF in already-established experimental EBA, clinical trials in patients with EBA, related autoimmune skin blistering diseases, and other neutrophil-dependent diseases are warranted.

MATERIALS AND METHODS

A more detailed description of the experiments can be found in the Supplementary Materials and Methods online.

Experiments with human cells and sera

Sera from patients with BP, patients with EBA, and from healthy volunteers used for the ex vivo studies were obtained after written informed consent, approved by the ethical committee of the Medical Faculty of the University of Lübeck, and performed in accordance with the Declaration of Helsinki.

Mice

C57BL/6J and SJL/J mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Animal experiments were approved by local authorities of the Animal Care and Use Committee (Kiel, Germany) and performed by certified personnel.

Isolation of primary human and mouse neutrophils

For isolation of human neutrophils, peripheral blood was collected by venipuncture from healthy adult volunteers using lithium heparin. Neutrophils were isolated as described previously (Aga et al., 2002). Cell purity and viability was greater than 99.9%. Mouse neutrophils were isolated from femur and tibia.

DMF treatment and culture of human neutrophils

For in vitro studies, DMF was solubilized at 15 mg/ml in ethanol and diluted with an appropriate medium as used in the assays to final concentrations of 5, 10, and 15 µg/ml (0.1% v/v EtOH). Neutrophils were preincubated with DMF for 1−4 hours at 37 °C. An LPS-free medium and EtOH (0.1% v/v EtOH) were used as controls. None of the applied DMF concentrations exerted a toxic or apoptosis-inducing effect within 4 hours, as determined by Annexin V and propidiumiodide counterstaining (Supplementary Figure S3 online). As stimuli for neutrophil activation, IL-8 (R&D Systems, Minneapolis, MN), TNF-α (PeproTech, Hamburg, Germany), PMA (Sigma Aldrich), LPS (Sigma Aldrich, Hamburg, Germany), fMLP, (Sigma Aldrich, Hamburg, Germany), IFN-γ (R&D Systems), or iIC were used. iIC were formed by using human serum albumin (Baxter) antigen and rabbit polyclonal anti−human serum albumin IgG (Sigma Aldrich) as described previously (Behnen et al., 2014).

Flow cytometry analysis of cell surface molecules

Following a preincubation with the medium EtOH or DMF, human neutrophils were left unstimulated or activated by 100 ng/ml IL-8, 100 ng/ml TNF-α, Iic, or 100 ng/ml LPS + 200 U/ml IFN-γ. Cells were then stained with FITC-conjugated mouse anti-human CD62L or FITC-conjugated mouse anti-human CD66b or PE-conjugated mouse anti-human CD11b mAb or appropriate isotype controls. For staining of mouse neutrophils from bone marrow, a PE-conjugated rat anti-mouse GR-1 and an FITC-conjugated rat anti-mouse CD62L antibodies were used. Fixed cells were analyzed with a FACSCalibur flow cytometer using CellQuest pro software (BD Biosciences, San Diego, CA).

Analysis of ROS production

The luminol (5-amino-2,3-dihydro-1,4-phthalazindione)-amplified chemiluminescence assay was used to measure the total of intra- and extracellular ROS as described previously (Behnen et al., 2014).

Assessment of NET formation

NET release was induced for 4 hours by 20 nM PMA or iIC and measured using a previously described kinetic assay (Brinkmann et al., 2004; Kirchner et al., 2012)

Phagocytosis assay

Neutrophils were preincubated for 3 hours in the presence or absence of DMF and ethanol. On stimulation of cells with LPS and IFN-γ, FluoSpheres carboxylate-modified microspheres (Invitrogen) were added. Subsequently, phagocytosis of the yellow-green fluorescent FluoSpheres by neutrophils was assessed by flow cytometry (FACS Calibur, BD) and by fluorescence microscopy (Axioskop 40, Zeiss).

Chemotaxis assay

To analyze the effect of DMF on neutrophil chemotaxis toward 100 ng/ml IL-8 or 100 ng/ml TNF-α, a modified chemotaxis assay was performed using a 24-well transwell system with 3 µm pore filters (Costar, Bodenheim, Germany) as previously described (Wilde et al., 2007).

Transendothelial migration assay

Similar to the chemotaxis assay, a 24-well transwell system (Costar) was used to analyze the effect of DMF on neutrophil transendothelial migration. The number of cells that migrated through a human umbilical vein endothelial cell-endothelial layer toward IL-8 or TNF-α into the lower chamber was calculated by using the glucuronidase assay, as described in the chemotaxis assay part.

Western blot analysis

Neutrophils were preincubated for 3 hours with 15 µg/ml DMF or solvent control after 15 minutes of stimulation with 100 ng/ml IL-8, 100 ng/ml TNF-α, iIC, 200 ng/ml LPS, or 5 minutes of stimulation with 20 nM fMLP. To analyze the effect of DMF on ERK 1/2, Akt, and p38 MAPK phosphorylation, Western blot analysis with whole cell lysates was carried out as described previously (Behnen et al., 2014).

Assessment of ex vivo dermal-epidermal separation induced by autoantibodies

Dermal-epidermal separation of cryosections of human skin was evaluated using an ex vivo model as previously described (Sitaru et al., 2002b).

Induction of experimental EBA in mice and treatment protocols

Passive transfer studies for EBA followed published protocols with minor modifications (Sitaru et al., 2005). The loss of tolerance toward COL7 and subsequent clinical EBA manifestation was induced by immunization with an immunodominant fragment located within murine COL7 as described elsewhere (Kasperkiewicz et al., 2011; Ludwig et al., 2011). Please see the Supplementary Materials and Methods for a detailed description.

Statistical analysis

Data were analyzed using SigmaPlot (Systat Software, Chicago, IL). Applied tests and confidence intervals are indicated at the respective text and figure legends. A P-value < 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1038/JID.2015.361.

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