Mechanical and Metabolic Injury to the Skin Barrier Leads to Increased Expression of Murine β-Defensin-1, -3, and -14

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Protection of the skin against microbiological infection is provided by the permeability barrier and by antimicrobial proteins. We asked whether the expression of murine β-defensins (mBDs)-1, -3, and -14—orthologs of human β-defensins hBD-1, -2, and -3, respectively—is stimulated by mechanically/physicochemically (tape stripping or acetone treatment) or metabolically (essential fatty acid–deficient (EFAD) diet) induced skin barrier dysfunction. Both methods led to a moderate induction of mBD-1 and mBD-14 and a pronounced induction of mBD-3 mRNA. Protein expression of the mBDs was increased as shown by immunohistology and by western blotting. Artificial barrier repair by occlusion significantly reduced the increased expression of mBD-14 after mechanical injury and of all three mBDs in EFAD mice, supporting an interrelationship between permeability and the antimicrobial barrier. mBD-3 expression was stimulated *in vitro* by tumor necrosis factor-α (TNF-α), and a neutralizing anti-TNF-α antibody significantly reduced increased mBD-3 expression after barrier injury in mouse skin, indicating that induction of mBD-3 expression is mediated by cytokines. The expression of mBD-14 was stimulated by transforming growth factor-α and not by TNF-α. In summary, we demonstrated upregulation of mBD1, -3, and -14 after mechanically and metabolically induced skin barrier disruption, which may be an attempt to increase defense in the case of permeability barrier dysfunction.

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INTRODUCTION

The skin is constantly exposed to a variety of microbial challenges. The permeability barrier, localized in the stratum corneum, is a mechanical protective barrier against bacterial infection. Impairment of the physical barrier by injury is a prerequisite for experimental skin infection by application of a bacterial suspension (Singh *et al.*, 1971). In eczema or psoriasis—skin diseases accompanied by disturbed permeability barrier function—the skin is colonized with potentially pathogenic microorganisms (Aly *et al.*, 1976, 1977). It is well known in clinical dermatology that occlusive conditions in the skin body folds and the use of occlusive latex gloves and shoes can cause hyperhydration and skin irritation (Denda *et al.*, 1998). Irritation results in impaired barrier function, which in turn predisposes to skin infections such as intertrigo, impetigo, and tinea (Warner *et al.*, 2003; Fluhr *et al.*, 2005).

Despite an impaired permeability barrier and significant bacterial colonization, psoriatic skin is usually free of infection. In atopic dermatitis, clinical signs of bacterial infection are predominantly found in severe cases or after extensive scratching (Lübbe, 2003). Therefore, additional defense mechanisms besides the physical barrier have been suggested, leading to the discovery of antimicrobial peptides produced by epithelial cells (reviewed by Ganz and Lehrer, 1994; Gallo and Huttner, 1998; Schröder, 1999; Schröder and Harder, 1999; Harder and Schröder, 2005a, b; Izadpanah and Gallo, 2005). Several types of antimicrobial peptides, including β-defensins, cathelicidin (Gallo *et al.*, 1997), catestatin (Radek *et al.*, 2008), RNase-7 (Harder and Schröder, 2002), and psoriasin (Gläser *et al.*, 2005), have been identified in the skin. In addition, three human β-defensins (hBD-1, -2, and -3) with a specific spectrum of antimicrobial activity have been characterized in human skin and are expressed in keratinocytes (Harder *et al.*, 1997, 2001). Constitutive expression of hBD-1 has been detected in human skin (Fulton *et al.*, 1997; Ali *et al.*, 2001), but its role in cutaneous defense is still emerging because of a lack of the respective studies. In contrast, hBD-2 and hBD-3 were originally isolated in native form from psoriatic scale extracts (Harder *et al.*, 1997, 2001), and both peptides are inducibly expressed in keratinocytes. The expression of hBD-2 and hBD-3 can be upregulated by bacteria as well as endogenous mediators such as cytokines (e.g., IL-1β and IL-17 for hBD-2) and growth factors (e.g., transforming growth factor-α (TGF-α)

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Abbreviations: EFAD, essential fatty acid–deficient; hBD, human β-defensin; mBD, murine β-defensin

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for hBD-3) (Schröder and Harder, 1999; Sørensen *et al.*, 2003; Pazgier *et al.*, 2006; Yin *et al.*, 2006). In addition, it has been reported that hBD-3 is induced upon epidermal injury, suggesting a role for hBD-3 in wound healing (Sørensen *et al.*, 2006). Moreover, it has been documented that hBD-3 as well as hBD-2 stimulates keratinocyte migration, proliferation, and production of proinflammatory cytokines and chemokines (Niyonsaba *et al.*, 2007).

To further elucidate the role of β-defensins after epidermal barrier disruption, we aimed to study the role of β-defensins in a mouse model using mechanical/physicochemical (via tape stripping or acetone treatment) as well as a metabolic (via essential fatty acid–deficient (EFAD) diet) disruption of the skin barrier. We focused on the expression of mouse orthologs of the human β-defensins hBD-1, -2, and -3. Mouse β-defensin-1 (mBD-1) is the ortholog of hBD-1 (Huttner *et al.*, 1997; Morrison *et al.*, 1998), but its expression in mouse keratinocytes has not yet been investigated. Mouse β-defensin-3 (mBD-3) is considered an ortholog of hBD-2 (Morrison *et al.*, 2003). mBD-3 is expressed in epithelia, especially in the lung and gastrointestinal tract, and its expression is induced by *Pseudomonas aeruginosa* (Bals *et al.*, 1999). Expression and induction of mBD-3 in mouse skin after barrier disruption and by UVB exposure have recently been described (Aberg *et al.*, 2007, 2008; Hong *et al.*, 2008). Synthetic mBD-3 inhibited the growth of *Escherichia coli, P. aeruginosa, Staphylococcus aureus,* and *Candida albicans* at concentrations from 25 to 50 $\mu g\,ml^{-1}$ (Burd *et al.*, 2002). Recently, mouse β-defensin-14 (mBD-14) has been identified as the ortholog of hBD-3 (Hinrichsen *et al.*, 2008; Röhrl *et al.*, 2008; Taylor *et al.*, 2008). mBD-14 exhibited a broad spectrum of potent antimicrobial activity against various microorganisms, including Gram-positive and Gram-negative bacteria and the yeast *C. albicans* (Hinrichsen *et al.*, 2008).

Both the permeability barrier and the antimicrobial barrier are regulated by specific signaling mechanisms, including cytokines and growth factors (Sørensen *et al.*, 2003, 2005). Oren *et al.* (2003) showed that hBD-2 is localized in epidermal lamellar bodies and that IL-1α stimulates movement from the endoplasmatic reticulum to lamellar bodies (post-transcriptional). We and others previously described an important role for proinflammatory cytokines in the regeneration of the physical barrier (Wood *et al.*, 1992; Jensen *et al.*, 1999). Also, a role for tumor necrosis factor-α (TNF-α) in the induction of hBD-2 has been described (Harder *et al.*, 1997; Varoga *et al.*, 2004). Cytokines and growth factors, which are necessary for the potential induction of β-defensins during barrier repair in mouse skin, are only partly known. In the present study, we observed that mBD-1, -3, and -14 are upregulated by mechanical/physicochemical as well as metabolic barrier injury in mouse skin *in vivo,* and we present evidence that cytokines and growth factors are involved in this process.

RESULTS

Mechanical/physicochemical barrier injury induces expression of mBD-1, -3, and -14

The major goal of our study was to assess a potential interaction between the permeability barrier and the antimicrobial barrier and whether acute and chronic disruption of the physical skin barrier induces changes in the antimicrobial barrier. We performed mechanical injury to the skin barrier through repeated tape stripping of the skin of shaved BALB/c mice, which potentially leads to an invasion of environmental germs into the living epidermal layers. Subsequently we examined mRNA and protein expression of mBD-1, -3, and -14 at different time points after barrier disruption. mRNA levels revealed a significant, slight increase in mBD-1 and mBD-14 expression 6 hours after barrier disruption as well as a significant, pronounced increase of mBD-3 expression. At 16 hours after barrier disruption, a very slight but significant increase in the mRNA expression could be detected only for mBD-14 (Figure 1a).

mBD-1 protein immunostaining in untreated skin was visible in granular and spinous layers (Figure 1b). A moderate increase in staining density occurred at 4–48 hours after barrier injury, especially in granular layers, and staining also involved spinous and basal layers. For mBD-3, only faint staining was detected in untreated skin, which was concentrated in basal and spinous layers (Figure 1b). Staining strongly increased at 4–48 hours after skin barrier disruption. Immunoreactivity for mBD-3 was visible in the entire nucleated epidermis with slightly higher staining density in granular layers. Staining for mBD-14 was already moderate in untreated skin, concentrated in granular layers, and also visible in spinous and basal layers (Figure 1b). At 4–48 hours after injury of the skin barrier, the staining of mBD-14 protein increased as seen for mBD-1 and -3.

Occlusion partially reduced expression of mBD-1, -3, and -14 after barrier injury

In previous studies, artificial skin barrier repair reduced the expression of several, but not all, metabolic events in the skin induced by skin barrier disruption (Proksch *et al.*, 1990; Wood *et al.*, 1994). For technical reasons, we used hairless SKH-1 mice—a tight fit of the occlusive foil (with the thumb of a latex glove used as a tube) was more easily obtained than with shaved hairy mice. We used acetone treatment as well as tape stripping for barrier disruption. However, we show occlusion data only for acetone-induced barrier disruption because tape stripping yielded a high variance in mRNA expression (data not shown). After acetone-induced barrier injury, we found a significant but moderate increase in mBD-1 and -14 expression and a significant, pronounced increase in mBD-3 mRNA expression (Figure 2a). However, the increase in mRNA expression, especially for mBD-1 and mBD-14, was more pronounced after acetone treatment compared with tape stripping. Occlusion for 24 hours after acetone treatment reduced mRNA expression of the investigated β-defensins significantly for mBD-14—however, not to normal levels (Figure 2a).

Immunohistology revealed increased staining. A reduced increase in staining intensity, in particular for mBD-14, occurred with occlusion (Figure 2b). Western blotting revealed an increased protein expression for mBD-1, -3, and -14 after barrier injury in SKH-1 mice (Figure 2c).

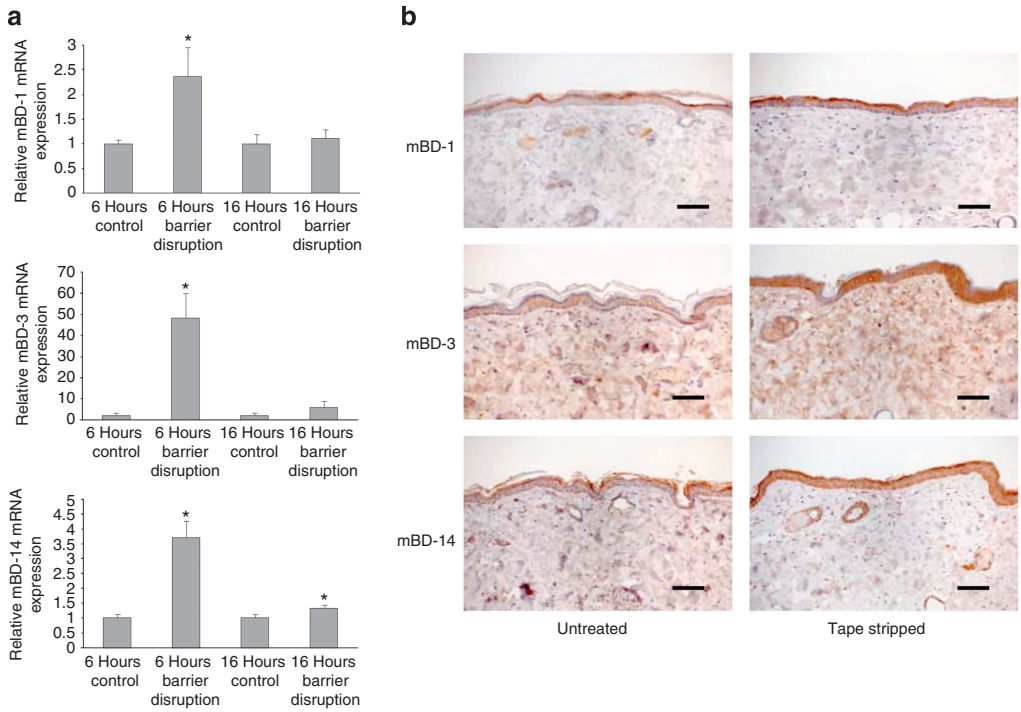

Figure 1. Expression of mouse β-defensins (mBDs) is enhanced in skin after barrier injury caused by tape stripping. (**a**) Stratum corneum barrier injury in BALB/c mice caused an increase in mBD-1, -3, and -14 mRNA expression 6 hours after barrier injury. Relative mRNA expression was quantified by real-time PCR (columns represent means ± SE; *$P<0.05$; $n=8$–11). (**b**) Immunohistochemical staining in mouse skin showed expression of mBD-1, -3, and -14 in the suprabasal epidermis. At 6 hours after barrier injury an increase in staining density for mBD-1, -3, and -14 occurred. Staining of all three β-defensins extended to the basal layer and was most pronounced in the granular layer. Bars = 50 μm.

Metabolically induced barrier dysfunction–induced expression of mBD-1, -3, and -14

Mice fed an EFAD diet develop a red and scaly skin, impaired epidermal differentiation, a disturbed skin barrier function, and increased bacterial colonization (Bibel *et al.*, 1989; Proksch *et al.*, 1992). In the present study, transepidermal water loss as a marker of skin barrier function increased from 9.0 ± 0.35 to $26.9 \pm 1.15\,g\,m^{-2}$ per hour ($n=10$, $P<0.005$). An EFAD diet resulted in a significantly increased mRNA expression of all analyzed β-defensins (Figure 3a).

Immunostaining for mBD-1 was increased in the skin of EFAD mice compared with untreated mice; it was clearly visible in the entire nucleated epidermis and concentrated in the granular layer. Immunostaining for mBD-3 also increased. Highest staining density was found in the granular layers and less pronounced in the granular layer. An increased staining intensity occurred for mBD-14. Staining was most pronounced in the granular layer (Figure 3b). Western blotting revealed an increased protein expression for all mBDs (Figure 3c).

Occlusion reduced expression of mBD-1, -3, and -14 in metabolically induced skin barrier dysfunction

Occlusion by latex foil for 48 hours significantly reduced the EFAD-induced increased mRNA expression for mBD-1, -3, and -14 (Figure 3a). Similarly, mBD staining intensity in immunohistology was reduced (Figure 3b).

mBD-3 expression was stimulated by TNF-α and mBD-14 expression was stimulated by TGF-α in mouse keratinocyte culture *in vitro*

After mechanical/physicochemical barrier injury by tape stripping or acetone treatment, inflammatory processes take place with expression of different cytokines and growth factors (Wood *et al.*, 1992; Jensen *et al.*, 1999; Werner *et al.*, 2007). To evaluate whether inflammatory reactions may affect β-defensin expression, we stimulated primary mouse keratinocytes with the proinflammatory cytokine TNF-α and with TGF-α. Concentrations of $10\,ng\,ml^{-1}$ for TNF-α and $50\,ng\,ml^{-1}$ for TGF-α proved to be most effective. TNF-α induced the expression of mBD-3 mRNA 16 hours after treatment (Figure 4a). mBD-14 mRNA expression was induced by TGF-α 24 hours after treatment (Hinrichsen *et al.*, 2008). This correlated with an induction of mBD-14 protein secretion after 24 hours as measured by ELISA (Figure 4b).

Injection of a neutralizing anti-TNF-α antibody led to reduced expression of mBD-3 after barrier injury

To examine whether the stimulation of mBD-3 expression by TNF-α is relevant for an *in vivo* situation, we intraperitoneally injected a neutralizing anti-TNF-α antibody in mice 24 hours before physicochemical barrier injury (Plessner *et al.*, 2007). A significant reduction in mBD-3 mRNA expression occurred 6 hours after barrier disruption.

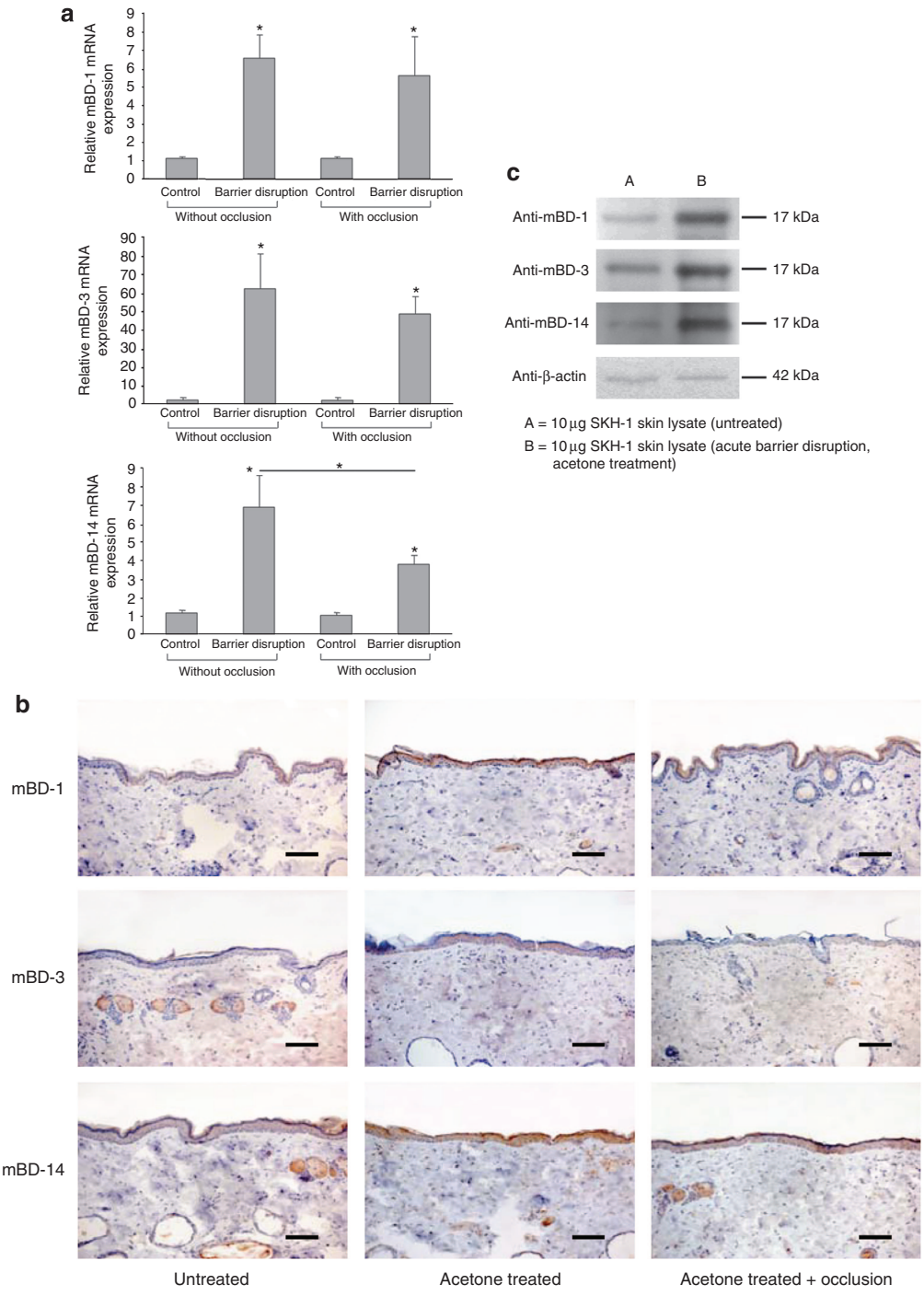

Figure 2. Expression of mouse β-defensins (mBDs) is induced after barrier injury by acetone treatment and can be reduced by occlusion. (**a**) Stratum corneum barrier injury by acetone treatment led to an increased expression of mBD-1, -3, and -14 in SKH-1 mice 6 hours after barrier injury. The increase was reduced by application of an occlusive foil immediately after barrier disruption, though no basal levels were obtained (significant for mBD-14 only). Relative mRNA expression was quantified by real-time PCR (columns represent means ± S.E. $*P<0.05$; $n=10$–16). (**b**) Staining for mBD-1, -3, and -14 in immunohistochemistry increased 6 hours after barrier injury by acetone treatment in SKH-1 mice. Staining density was reduced after artificial barrier repair by latex occlusion. Bars = 50 µm. (**c**) Six hours after barrier disruption by acetone treatment, SKH-1 mice showed an increased protein staining for mBD-1, -3, and -14 in western blot compared to untreated controls. β-Actin served as loading control.

A similar trend was noted for mBD-1 and mBD-14 mRNA expression (Figure 5a).

Similar results were observed at the protein level investigated by immunohistochemistry (Figure 5b).

DISCUSSION

The skin is permanently exposed to a variety of potentially harmful microorganisms but usually remains free of infection. An intact permeability barrier prevents infection.

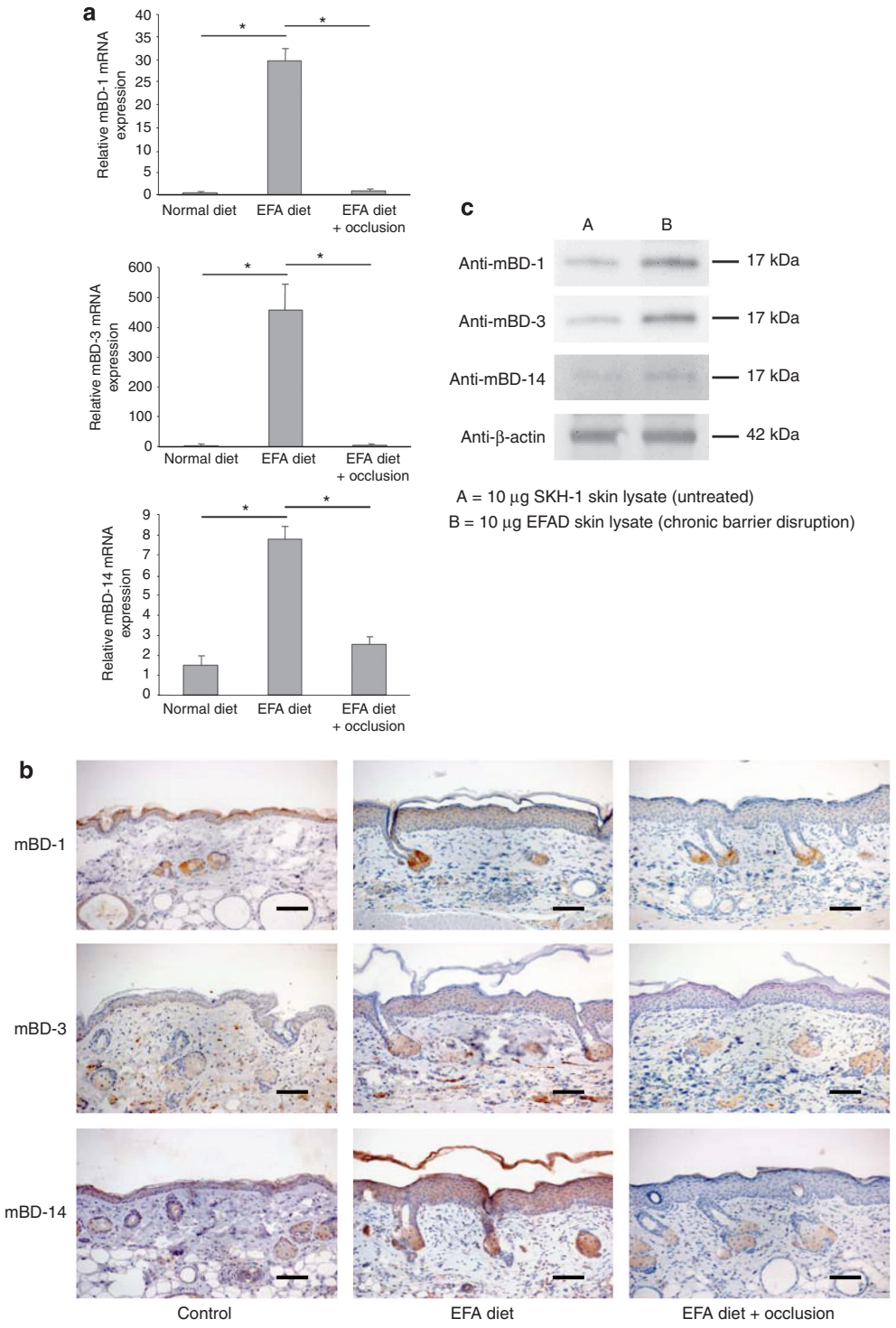

Figure 3. Expression of murine β-defensins (mBDs) is enhanced in metabolically barrier-disrupted skin and can be reduced by occlusion. (**a**) In metabolically barrier-disrupted skin of mice fed an essential fatty acid–deficient (EFAD) diet, an increased mRNA expression was noted for mBD-1, -3, and -14 compared with control mice fed a normal diet. Occlusion of EFAD mouse skin with a latex foil for 48 hours did significantly reduce the mRNA expression for all three mBDs (columns represent means ± S.E. $*P<0.05$; $n=11$–12). (**b**) In EFAD mice, immunohistochemical staining density markedly increased for all three defensins. Occlusion by a latex foil for 48 hours in EFAD mice caused reduction in staining intensity. Bars = 50 μm. (**c**) In EFAD mice all three investigated mBDs showed an increase in protein staining in western blot. β-Actin served as loading control.

However, impairment of the permeability barrier by scratches and other minor injuries is common and does not necessarily result in skin infection. This indicates that the skin has additional defense mechanisms. Several studies have suggested that a major cutaneous defense mechanism may be the inducible release of antimicrobial

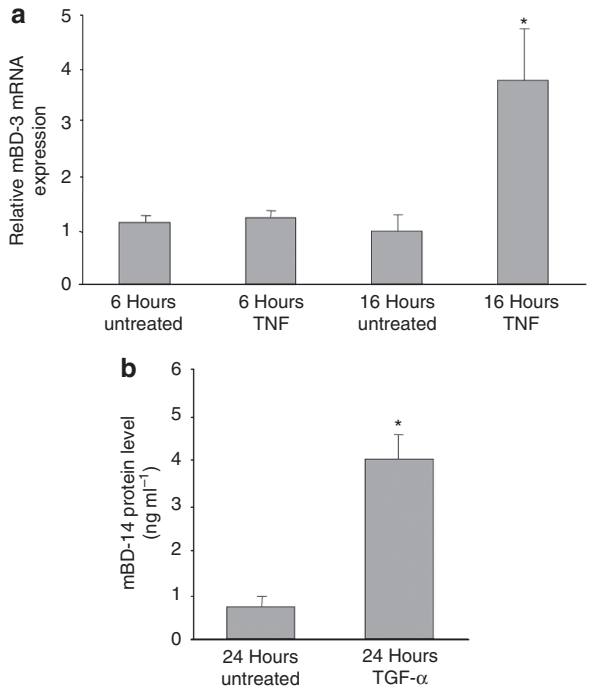

Figure 4. TNF-α and TGF-α influenced the expression of murine β-defensins (mBDs) in primary mouse keratinocytes *in vitro*. (**a**) Primary mouse keratinocytes were stimulated with 10 ng ml^{-1} TNF-α for 6 and 16 hours, and mBD-3 mRNA expression was analyzed by real-time PCR. The mRNA expression of mBD-3 was increased. Bars represent the relative mBD-3 transcript levels normalized to GAPDH transcript levels. Results are presented as means ± standard errors (*$P<0.05$; $n=9$). (**b**) Expression of mBD-14 protein in supernatants of primary mouse keratinocytes stimulated for 24 hours with TGF-α (50 ng ml^{-1}) was measured by ELISA. The protein release of mBD-14 was significantly increased after stimulation with TGF-α compared with medium controls (*$P<0.05$; $n=9$).

peptides (Schröder and Harder, 2006; Schauber and Gallo, 2008).

To gain more insight into the role of antimicrobial peptides upon barrier disruption, we used mouse models to investigate the expression of mouse β-defensins mBD-1, -3, and -14—orthologs of human defensins hBD-1, hBD-2, and hBD-3, respectively—following skin barrier disruption. Acute stratum corneum barrier injury can easily be caused by tape stripping or acetone treatment, and the EFAD mouse is a model for metabolically induced permeability barrier disruption (Proksch *et al.*, 1992). We found that barrier injury and metabolically induced barrier dysfunction led to a slight to moderate induction of mBD-1 and mBD-14 and a pronounced increase in mBD-3 mRNA expression. An induction of protein expression was confirmed by immunohistological staining and western blotting, with highest induction for mBD-3. Immunoreactivity for mBD-1, -3, and -14 in unstimulated mouse epidermis was localized in the suprabasal layers with increased staining intensity toward the outer layers of the epidermis. In injury and metabolically induced barrier-disrupted skin, staining was extended to the entire epidermis, although staining intensity was still most pronounced in the outer layers. This is in agreement with the proposed function of β-defensins as defense molecules against invading microorganisms and is in concordance with findings for human orthologs (Liu *et al.*, 1998, 2002; Sørensen *et al.*, 2006; Jensen *et al.*, 2007; Harder *et al.*, 2010). These results are also in line with a recent study showing upregulation of mBD-3 and CRAMP (cathelin-related antimicrobial peptide) expression after acute skin barrier injury (Aberg *et al.*, 2008).

Although there is a global increase in β-defensin expression after barrier injury, there are differences in the level of induction. In contrast to the slight to moderate induction of mBD-1 and mBD-14, we observed a pronounced induction of mBD-3 after barrier injury. The upregulation of mBD-1 upon barrier injury was unexpected, because mBD-1 and the human ortholog hBD-1 are generally considered constitutively expressed β-defensins that are not induced by infection or inflammation (Zhao *et al.*, 1996; Morrison *et al.*, 1998; Mathews *et al.*, 1999). The mechanism of mBD-1 (and hBD-1) induction is unknown. In primary mouse keratinocytes stimulated with various cytokines and growth factors upregulation of mBD-1 could not be observed (data not shown).

Previous publications showed that the expression of mBD-3 is induced by inflammation and infection (Bals *et al.*, 1999; Burd *et al.*, 2002). Thus, one can speculate that the strong induction of mBD-3 after skin barrier disruption may be mediated by an increased expression of proinflammatory cytokines stimulated by skin injury. The hypothesis that the induction of β-defensins in barrier dysfunction may be mediated via proinflammatory cytokines and growth factors is supported by several studies reporting that proinflammatory cytokines and growth factors are of crucial importance for the repair of the permeability barrier in mouse skin *in vivo* (Wood *et al.*, 1992; Liou *et al.*, 1997; Jensen *et al.*, 1999). To further investigate this hypothesis, we intraperitonally injected mice with a neutralizing anti-TNF-α antibody before performing barrier disruption. This treatment significantly reduced the increase in mBD-3 expression. This indicates that TNF-α contributes to the induction of mBD-3 upon barrier injury.

It has been reported that sterile wounding induced expression of hBD-3, the human ortholog of mBD-14, a process that is mediated via activation of the EGFR (Sørensen *et al.*, 2006). The observation that expression of mBD-14 mRNA (Hinrichsen *et al.*, 2008) and protein can be induced by the EGFR ligand TGF-α in mouse keratinocytes *in vitro* prompts the interesting hypothesis that increased levels of TGF-α and other growth factors may mediate the induction of mBD-14 after barrier disruption.

Artificial barrier restoration by occlusion with a latex foil reduced the increase in β-defensin mRNA expression although significantly only for mBD-14. It has been shown that several biochemical mechanisms involved in barrier repair can be reduced by artificial barrier repair (Proksch *et al.*, 1990). However, not all biochemical effects are reduced; in particular, cytokine expression is reduced only under certain circumstances. Wood *et al.* (1994) reported that

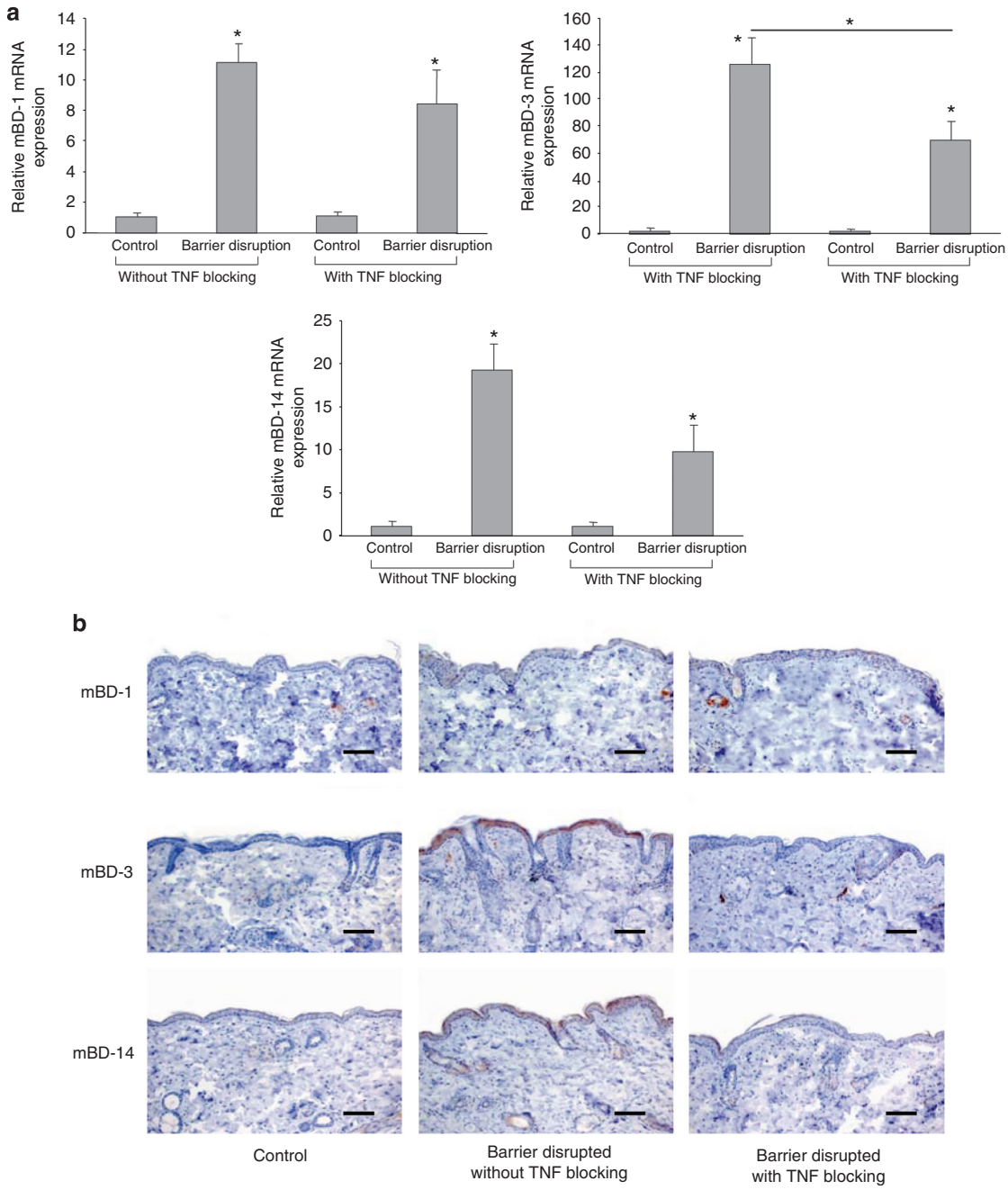

Figure 5. Treatment of mice with a tumor necrosis factor-α (TNF-α) neutralizing antibody before barrier disruption reduced induction of murine β-defensin-3 (mBD-3) expression. (**a**) To block the activity of TNF-α, a neutralizing anti-TNF-α antibody was intraperitoneally injected into SKH-1 mice 24 hours before barrier disruption by acetone treatment. Six hours later, the mRNA expression of mBD-3 was significantly reduced in mice treated with anti-TNF-α antibody compared with mice treated with phosphate-buffered saline. No significant difference was recognized in the expression of mBD-1 and mBD-14 mRNA. Relative mRNA expression was quantified by real-time PCR (columns represent means ± SE. $*P<0.05$; $n=6$). (**b**) Immunohistochemical analyses of skin samples from mice treated with anti-TNF-α antibody showed a reduction of β-defensin protein staining, most pronounced for mBD-3. Bars = 50 μm.

occlusion does not lower cytokine mRNA levels after acute barrier disruption. Similarly, we found a reduction only in mBD-14 expression, and not for mBD-3, because the expression of mBD-3 is induced by proinflammatory stimuli such as TNF-α, whereas the expression of mBD-14 is induced by TGF-α. Growth factor expression is partially blocked in occluded skin barrier disruption (Liou *et al.*, 1997). Wood *et al.* showed that cytokine expression was reduced in EFAD mice after occlusion. Consistent with this, we found reduction of all three mBDs after occlusion in these mice.

In summary, we found increased expression of mBD-1, -3, and -14 after mechanical and metabolic skin barrier disruption in mouse skin. The increased expression may reflect a defense response to protect the skin against harmful

microorganisms that can invade skin with a dysfunction of the permeability barrier.

MATERIALS AND METHODS

Mice

SKH-1 mice (Crl:(hr/hr) BR) were supplied by Charles River (Sulzfeld, Germany). BALB/c mice were bred in the central animal facility at the University of Kiel. For purposes of investigating barrier disruption, male 6- to 8-week-old mice were used. EFAD mice were obtained by feeding 3-week-old SKH-1 mice an EFAD diet (Bavandi *et al.*, 1992), modified by adding $MgSO_4 \cdot 7H_2O$. Mice were individually and conventionally maintained in plastic cages under standardized conditions (room temperature 25 °C, relative humidity 45–55%, circadian rhythm 12 hours, and standard laboratory animal chow (V1534, SSNIFF Spezialdiäten, Soest, Germany) and water supplied *ad libitum*. The University of Kiel Committee for Animal Care approved the study.

Barrier injury of mouse skin and artificial barrier repair

The barrier of one flank of mouse skin was disrupted by repeated tape stripping (cellophane tape) or by application of acetone with a small cotton stick until a six- to eightfold increase in transepidermal water loss (Tewameter TM 210, Courage & Khazaka, Cologne, Germany) occurred. In one set of animals, the disrupted skin barrier was immediately occluded with a latex foil after treatment. Hairy mice were shaved with a razor blade 1 day before treatment. EFAD mice were compared with a normally fed control group and occluded for 48 hours.

Injection of neutralizing anti-TNF-α antibody in mice

To evaluate a possible role for TNF-α in induction of β-defensins, a neutralizing anti-TNF-α antibody (0.5 mg in phosphate-buffered saline (PBS); eBioscience, Frankfurt, Germany, MP6-XT3) was intraperitoneally injected into SKH-1 mice 24 hours before acetone treatment. Control mice were injected with PBS.

Cell culture and stimulation

Primary keratinocytes were isolated from the skin of 1- to 2-day-old BALB/c mice and cultivated in EpiLife-Medium (Sigma, Taufkirchen, Germany) in collagenized six-well culture plates (9.6 cm^2 per well; Sarstedt, Nürnbrecht, Germany) under standardized conditions (37 °C, 0.5% CO_2). Cells were stimulated at a confluence of 90–100%.

mRNA isolation, reverse transcription, and real-time PCR

mRNA isolation from cultured primary keratinocytes was performed using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). Skin samples were minced in liquid nitrogen, and RNA was isolated using the TRIzol reagent (Invitrogen, Karlsruhe, Germany). Reverse-transcription reagents were obtained from Applied Biosystems (HighCap cDNA RT Kit; Darmstadt, Germany). cDNA was analyzed by real-time PCR (LightCycler 2.0; Roche Diagnostics GmbH, Mannheim, Germany) using the SYBR Advantage qPCR Premix (TaKaRa Bio Europe, Saint-Germain-en-Laye, France). cDNA corresponding to 10 ng RNA was used as a template. Samples were incubated for an initial denaturation at 95 °C for 10 minutes followed by 45 cycles, each cycle consisting of 95 °C for 10 seconds, 60 °C (touchdown of −1 °C per cycle from 66 °C to 60 °C) for 5 seconds, and 72 °C for 15 seconds. To confirm amplification of specific transcripts, melting curve profiles were produced. (See Supplementary Table 1 online for a list of real-time PCR primers (Eurofins MWG Operon, Ebersberg, Germany).) Quantifications were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Relative expression is given as the ratio between target gene expression and *GAPDH* expression.

Generation of mBD-14-specific antibodies

Expression of recombinant mBD-14 protein was described previously (Hinrichsen *et al.*, 2008). In the present study, a fusion protein (2.2 mg) was used for immunization. This (1.5 mg) was conjugated to keyhole limpet hemocyanine (Sigma) using glutaraldehyde. Keyhole limpet hemocyanine (1 mg) in 1 ml PBS was mixed with 1 µl 25% glutaraldehyde (Serva, Heidelberg, Germany) and incubated for 1 hour at room temperature with gentle shaking. The reaction mixture was diafiltrated and concentrated in 400 µl PBS using a Vivaspin 0.5 ml concentrator column (30 kDa cutoff, Vivascience, Hannover, Germany). The concentrate was incubated with 600 µl (1.5 mg) mBD-14 fusion protein in PBS for 1 hour at room temperature with gentle rotation. The reaction was stopped by the addition of 5 µl 1 M Tris (pH 8.0). mBD-14 fusion protein (0.7 mg) in 500 µl of PBS was added. The preparation was divided into one 450 µl aliquot for initial immunization and three 350 µl aliquots for booster immunization of a goat. Immunization was carried out by ZIKA-Kaninchenbetrieb (Gottin, Germany). We generated an mBD-14 affinity column (1 ml HiTrap NHS-activated columns, Amersham Pharmacia Biotech, Vienna, Austria) using 500 µg recombinant mBD-14 to selectively isolate mBD-14-specific antibodies from the serum. Also, a pET-32 affinity column using 650 µg pET-32 was generated to remove pET-32-specific antibodies. The mBD-14 affinity column was loaded with 750 µl goat anti-mBD-14 serum and washed with 10 mM sodium phosphate buffer, pH 7.4. Low-affinity antibodies were eluted using 1 M NaCl (pH 7), and high-affinity mBD-14 antibodies were eluted using 200 mM glycine (pH 3), immediately neutralized with 1 M Tris (pH 7.5) and dialyzed against PBS. Antibodies with affinity to pET-32 were removed by pET-32 affinity column. Specificity of mBD-14 antibodies was verified by immunohistochemistry and western blotting.

Western blot analysis

Peptides were extracted from mouse skin using a lysis buffer containing 62.5 M Tris, 5% SDS, and 10 mM DTT. Proteins were separated by 16.5% SDS-tricine polyacrylamide gel containing 6 M urea (Schägger and von Jagow, 1987). Proteins were transferred to a nitrocellulose membrane (0.2 µm, Amersham Hybond-ECL, GE Healthcare, Freiburg, Germany), blocked for 1 hour in blocking buffer (5% (wt/vol) BSA in PBS + 0.05% Tween), then incubated overnight at 4 °C in 3% (wt/vol) BSA in PBS + 0.05% Tween containing 1:250 anti-mBD-1 antibodies, 1:250 anti-mBD-3 antibodies (both Santa Cruz Biotechnology, Santa Cruz, CA), or 1:10,000 mBD-14 affinity-purified antibodies (1.85 mg/ml). The membrane was washed with PBS + 0.05% Tween six times for 5 minutes each and incubated for 1 hour in 3% (wt/vol) BSA in PBS + 0.05% Tween containing 1:20,000 dilution of goat anti-rabbit or rabbit anti-goat IgG HRP conjugate (Dianova, Hamburg, Germany). After another six washes, the membrane was incubated

for 5 minutes with chemiluminescent peroxidase substrate (Lumi-Light PLUS Western Blotting Substrate, Roche Diagnostics, Mannheim, Germany) and visualized using a Diana III cooled CCD-camera imaging system (Raytest, Straubenhardt, Germany). β-Actin served as loading control in all western blots.

Immunohistochemistry

For immunohistochemistry, 5 μm vertical paraffin sections were deparaffinized and rehydrated followed by heat-induced antigen retrieval in citrate acid buffer (10 mM, pH 6.0). Slides were incubated with 3% aqueous H_2O_2 for 5 minutes at room temperature to block endogenous peroxidase activity. After blocking nonspecific antibody binding by incubation with normal rabbit or swine serum (1:5 in Tris-buffered saline, Dako Diagnostics, Hamburg, Germany), sections were incubated with antibodies for mBD-1, mBD-3 (1:100, both Santa Cruz Biotechnology), and mBD-14 (1.85 mg ml^{-1}, 1:100) diluted in Tris-buffered saline (0.15 M NaCl, 0.05 M Tris, pH 7.6) overnight at 4 °C. Sections were incubated for 30 minutes with secondary biotinylated antibodies (Dianova, Hamburg, Germany) followed by incubation for 30 minutes with StreptABComplex/HRP (Dako Diagnostics), developed with Liquid DAB (Biogenex, San Ramon, CA), and counterstained with Mayer's hemalaun (Merck, Darmstadt, Germany).

ELISA

Ninety-six-well immunoplates (MaxiSorp, Nunc, Roskilde, Denmark) were coated at 4 °C overnight with 50 μl anti-mBD-14 antibody diluted 1:2,000 to 1 μg ml^{-1} in 0.05 M carbonate buffer, pH 9.6. Wells were blocked with 200 μl 1% BSA in PBS for 10 minutes at 37 °C. After being washed three times with 200 μl PBS + 0.1% Tween 20, 50 μl cell culture supernatants and serial dilutions of recombinant mBD-14 protein were incubated for 30 minutes at 37 °C. Plates were washed three times and incubated for 30 minutes at 37 °C with 50 μl biotinylated anti-mBD-14 antibody diluted 1:4,000 to 0.23 μg ml^{-1} in PBS + 0.1% Tween 20. Plates were washed again three times, filled with 50 μl Streptavidin-POD (Roche Diagnostics; 1:10,000 in PBS + 0.1% Tween 20) and incubated for 30 minutes at 37 °C, washed six times, and incubated with 2,29-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Roche Diagnostics) for 30–45 minutes at 37 °C. Absorbance was measured at 405 nm with a multichannel photometer (Sunrise; Tecan, Crailsheim, Germany).

Statistical analysis

All calculations were performed using the unpaired Student's *t*-test.

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 http://www.nature.com/jid

REFERENCES

Aberg KM, Man MQ, Gallo RL *et al.* (2008) Co-regulation and interdependence of the mammalian epidermal permeability and antimicrobial barriers. *J Invest Dermatol* 128:917–25

Aberg KM, Radek KA, Choi EH *et al.* (2007) Psychological stress downregulates epidermal antimicrobial peptide expression and increases severity of cutaneous infections in mice. *J Clin Invest* 117:3339–49

Ali RS, Falconer A, Ikram M *et al.* (2001) Expression of the peptide antibiotics human beta defensin-1 and human beta defensin-2 in normal human skin. *J Invest Dermatol* 117:106–11

Aly R, Maibach HE, Mandel A (1976) Bacterial flora in psoriasis. *Br J Dermatol* 95:603–6

Aly R, Maibach HI, Shinefield HR (1977) Microbial flora of atopic dermatitis. *Arch Dermatol* 113:780–2

Bals R, Wang X, Meegalla RL *et al.* (1999) Mouse beta-defensin 3 is an inducible antimicrobial peptide expressed in the epithelia of multiple organs. *Infect Immun* 67:3542–7

Bavandi A, Meingassner JG, Becker S (1992) Diet-induced dermatitis response of hairless rats to systemic treatment with cyclosporin A (Sandimmun), cyclosporin H and FK506. *Exp Dermatol* 1:199–205

Bibel DJ, Miller SJ, Brown BE *et al.* (1989) Antimicrobial activity of stratum-corneum lipids from normal and essential fatty acid-deficient mice. *J Invest Dermatol* 92:632–8

Burd RS, Furrer JL, Sullivan J *et al.* (2002) Murine beta-defensin-3 is an inducible peptide with limited tissue expression and broad-spectrum antimicrobial activity. *Shock* 18:461–4

Denda M, Sato J, Masuda Y *et al.* (1998) Exposure to a dry environment enhances epidermal permeability barrier function. *J Invest Dermatol* 111:858–63

Fluhr JW, Akengin A, Bornkessel A *et al.* (2005) Additive impairment of the barrier function by mechanical irritation, occlusion and sodium lauryl sulphate *in vivo*. *Br J Dermatol* 153:125–31

Fulton C, Anderson GM, Zasloff M *et al.* (1997) Expression of natural peptide antibiotics in human skin. *Lancet* 350:1750–1

Gallo RL, Huttner KM (1998) Antimicrobial peptides: an emerging concept in cutaneous biology. *J Invest Dermatol* 111:739–43

Gallo RL, Kim KJ, Bernfield M *et al.* (1997) Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *J Biol Chem* 272:13088–93

Ganz T, Lehrer RI (1994) Defensins. *Curr Opin Immunol* 6:584–9

Gläser R, Harder J, Lange H *et al.* (2005) Antimicrobial psoriasin (S100A7) protects human skin from *Escherichia coli* infection. *Nat Immunol* 6:57–64

Harder J, Bartels J, Christophers E *et al.* (1997) A peptide antibiotic from human skin. *Nature* 387:861

Harder J, Bartels J, Christophers E *et al.* (2001) Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* 276:5707–13

Harder J, Dressel S, Wittersheim M *et al.* (2010) Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury. *J Invest Dermatol* 130:1355–64

Harder J, Schröder JM (2002) RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. *J Biol Chem* 277: 46779–84

Harder J, Schröder JM (2005a) Antimicrobial peptides in human skin. *Chem Immunol Allergy* 86:22–41

Harder J, Schröder JM (2005b) Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins. *J Leukoc Biol* 77:476–86

Hinrichsen K, Podschun R, Schubert S *et al.* (2008) Mouse beta-defensin-14, an antimicrobial ortholog of human beta-defensin-3. *Antimicrob Agents Chemother* 52:1876–9

Hong SP, Kim MJ, Jung MY *et al.* (2008) Biopositive effects of low-dose UVB on epidermis: coordinate upregulation of antimicrobial peptides and permeability barrier reinforcement. *J Invest Dermatol* 128:2880–7

Huttner KM, Kozak CA, Bevins CL (1997) The mouse genome encodes a single homolog of the antimicrobial peptide human beta-defensin 1. *FEBS Lett* 413:45–9

Izadpanah A, Gallo RL (2005) Antimicrobial peptides. *J Am Acad Dermatol* 52:381–90

Jensen JM, Pfeiffer S, Akaki T *et al.* (2007) Barrier function, epidermal differentiation, and human beta-defensin 2 expression in tinea corporis. *J Invest Dermatol* 127:1720–7

Jensen JM, Schutze S, Forl M *et al.* (1999) Roles for tumor necrosis factor receptor p55 and sphingomyelinase in repairing the cutaneous permeability barrier. *J Clin Invest* 104:1761–70

Liou A, Elias PM, Grunfeld C *et al.* (1997) Amphiregulin and nerve growth factor expression are regulated by barrier status in murine epidermis. *J Invest Dermatol* 108:73–7

Liu AY, Destoumieux D, Wong AV *et al.* (2002) Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J Invest Dermatol* 118:275–81

Liu L, Wang L, Jia HP *et al.* (1998) Structure and mapping of the human beta-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* 222:237–44

Lübbe J (2003) Secondary infections in patients with atopic dermatitis. *Am J Clin Dermatol* 4:641–54

Mathews M, Jia HP, Guthmiller JM *et al.* (1999) Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infect Immun* 67:2740–5

Morrison GM, Davidson DJ, Kilanowski FM *et al.* (1998) Mouse beta defensin-1 is a functional homolog of human beta defensin-1. *Mamm Genome* 9:453–7

Morrison GM, Semple CA, Kilanowski FM *et al.* (2003) Signal sequence conservation and mature peptide divergence within subgroups of the murine beta-defensin gene family. *Mol Biol Evol* 20:460–70

Niyonsaba F, Ushio H, Nakano N *et al.* (2007) Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J Invest Dermatol* 127:594–604

Oren A, Ganz T, Liu L *et al.* (2003) In human epidermis, beta-defensin 2 is packaged in lamellar bodies. *Exp Mol Pathol* 74:180–2

Pazgier M, Hoover DM, Yang D *et al.* (2006) Human beta-defensins. *Cell Mol Life Sci* 63:1294–313

Plessner HL, Lin PL, Kohno T *et al.* (2007) Neutralization of tumor necrosis factor (TNF) by antibody but not TNF receptor fusion molecule exacerbates chronic murine tuberculosis. *J Infect Dis* 195:1643–50

Proksch E, Elias PM, Feingold KR (1990) Regulation of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase-activity in murine epidermis—modulation of enzyme content and activation state by barrier requirements. *J Clin Invest* 85:874–82

Proksch E, Feingold KR, Elias PM (1992) Epidermal Hmg CoA reductase-activity in essential fatty-acid deficiency—barrier requirements rather than eicosanoid generation regulate cholesterol-synthesis. *J Invest Dermatol* 99:216–20

Radek KA, Lopez-Garcia B, Hupe M *et al.* (2008) The neuroendocrine peptide catestatin is a cutaneous antimicrobial and induced in the skin after injury. *J Invest Dermatol* 128:1525–34

Röhrl J, Yang D, Oppenheim JJ *et al.* (2008) Identification and biological characterization of mouse beta-defensin 14, the orthologue of human beta-defensin 3. *J Biol Chem* 283:5414–9

Schägger H, von Jagow G (1987) Tricine sodium dodecyl-sulfate polyacrylamide-gel electrophoresis for the separation of proteins in the range from 1- to 100-Kda. *Anal Biochem* 166:368–79

Schauber J, Gallo RL (2008) Antimicrobial peptides and the skin immune defense system. *J Allergy Clin Immunol* 122:261–6

Schröder JM (1999) Epithelial antimicrobial peptides: innate local host response elements. *Cell Mol Life Sci* 56:32–46

Schröder JM, Harder J (1999) Human beta-defensin-2. *Int J Biochem Cell Biol* 31:645–51

Schröder JM, Harder J (2006) Antimicrobial skin peptides and proteins. *Cell Mol Life Sci* 63:469–86

Singh G, Marples RR, Kligman AM (1971) Experimental staphylococcus aureus infections in humans. *J Invest Dermatol* 57:149–62

Sørensen OE, Cowland JB, Theilgaard-Monch K *et al.* (2003) Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol* 170:5583–9

Sørensen OE, Thapa DR, Rosenthal A *et al.* (2005) Differential regulation of beta-defensin expression in human skin by microbial stimuli. *J Immunol* 174:4870–9

Sørensen OE, Thapa DR, Roupe KM *et al.* (2006) Injury-induced innate immune response in human skin mediated by transactivation of the epidermal growth factor receptor. *J Clin Invest* 116: 1878–85

Taylor K, Clarke DJ, McCullough B *et al.* (2008) Analysis and separation of residues important for the chemoattractant and antimicrobial activities of beta-defensin 3. *J Biol Chem* 283:6631–9

Varoga D, Pufe T, Harder J *et al.* (2004) Production of endogenous antibiotics in articular cartilage. *Arthritis Rheum* 50:3526–34

Warner RR, Stone KJ, Boissy YL (2003) Hydration disrupts human stratum corneum ultrastructure. *J Invest Dermatol* 120:275–84

Werner S, Krieg T, Smola H (2007) Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol* 127:998–1008

Wood LC, Elias PM, Sequeiramartin SM *et al.* (1994) Occlusion lowers cytokine messenger-RNA levels in essential fatty acid-deficient and normal mouse epidermis, but not after acute barrier disruption. *J Invest Dermatol* 103:834–8

Wood LC, Jackson SM, Elias PM *et al.* (1992) Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J Clin Invest* 90:482–7

Yin C, Dang HN, Zhang HB *et al.* (2006) Capacity of human beta-defensin expression in gene-transduced and cytokine-induced cells. *Biochem Biophys Res Commun* 339:344–54

Zhao C, Wang I, Lehrer RI (1996) Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett* 396:319–22