Injury Is a Major Inducer of Epidermal Innate Immune Responses during Wound Healing

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We examined the importance of injury for the epidermal innate immune response in human skin wounds. We found that injury, independent of infiltrating inflammatory cells, generated prominent chemotactic activity toward neutrophils in injured skin because of IL-8 production. Furthermore, injury was a major inducer of the expression of antimicrobial (poly)peptides (AMPs) in skin wounds. In human skin, these injury-induced innate immune responses were mediated by activation of the epidermal growth factor receptor (EGFR). Consequently, inhibition of the EGFR blocked both the chemotactic activity generated in injured skin and the expression of the majority of the AMPs. The importance of injury was confirmed in mouse experiments *in vivo*, in which injury independent of infection was a potent inducer of AMPs in skin wounds. To our knowledge, these data thereby provide a previously unreported molecular link between injury and neutrophil accumulation and identify the molecular background for the vast expression of IL-8 and AMPs in wounded epidermis. Conceptually, these data show that the growth factor response elicited by injury is important for the recruitment of neutrophils in skin wounds.

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INTRODUCTION

The epidermis has an important role in the protection against microbes during skin infections and wound healing and is consequently an integral part of the innate immune system. In wound healing, the production and release of inflammatory cytokines ensure a rapid recruitment of neutrophils to the wound site. This recruitment of neutrophils is essential for keeping the wound free of infection (Martin and Leibovich, 2005). Epidermal keratinocytes are an important source of neutrophil chemotactic cytokines such as CXCL-1 (Gro-α) and IL-8 (CXCL-8), both found to be major chemotactic cytokines in human wound fluid *in vivo* (Rennekampff *et al.*, 2000). IL-8 is upregulated in keratinocytes by proinflammatory cytokines such as IL-1 and TNF-α (Larsen *et al.*, 1989). In addition, increased IL-8 production can be induced by various pathways and agonists including Toll-like receptors (TLRs) (Pivarcsi *et al.*, 2003), retinoic acid (Dai *et al.*, 2004), TNF-related apoptosis inducing ligand (TRAIL) (Leverkus *et al.*, 2003), LL-37 (Murakami *et al.*, 2004), and the ligands of the epidermal growth factor receptor (EGFR) (Miller *et al.*, 2005; Pastore *et al.*, 2005) *in vitro*. However, the pathway responsible for IL-8 production in wounded epidermis has not been identified.

Apart from production of chemotactic cytokines, the epidermis also produces antimicrobial (poly)peptides (AMPs). During wound healing, the infiltration of inflammatory cells is accompanied with expression of the major human epidermal AMPs, including hBD-2 (Schmid *et al.*, 2001), hBD-3 (Sørensen *et al.*, 2006), hCAP-18/LL-37 (Dorschner *et al.*, 2001), psoriasin (Lee and Eckert, 2007), calgranulins (Thorey *et al.*, 2001), and elafin (vanBergen *et al.*, 1996).

Microbes stimulate the AMP expression in human skin and keratinocytes mostly through TLR signaling (Gläser *et al.*, 2005; Büchau *et al.*, 2007; Abtin *et al.*, 2008; Gerstel *et al.*, 2009). Furthermore, IL-1 (Liu *et al.*, 2003; Sørensen *et al.*, 2005) and vitamin D (Weber *et al.*, 2005; Schauber *et al.*, 2007) induce AMP expression either alone or in combination with TLR signaling (Schauber *et al.*, 2007). Although induced expression has been found for many AMPs during wound healing, less is known about the pathways leading to their induced expression after wounding. For example, psoriasin, important for its prominent activity against *Escherichia coli* (Gläser *et al.*, 2005), is induced in skin wounds through unidentified pathways (Lee and Eckert, 2007).

We have previously shown that epidermal injury leads to the expression of three AMPs, SLPI, NGAL, and hBD-3, through activation of EGFR (Sørensen *et al.*, 2006). EGFR is known to be activated after injury by the so-called 'transactivation' (Tokumaru *et al.*, 2000). During this process, membrane-bound metalloproteases of the ADAM (A Disintegrin And Metalloprotease) family become activated in response to injury. Their subsequent shedding of the pool of

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Abbreviations: AMP, antimicrobial (poly)peptide; BD, β-defensin; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding EGF-like growth factor; PGN, peptidoglycan; SLPI, secretory leukocyte protease inhibitor; TAPI, TNF-α-converting enzyme protease inhibitor-1; TGF-α, transforming growth factor α; TLR, Toll-like receptor

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EGFR ligands on the keratinocyte cell surfaces lead to a local activation of the EGFR in an autocrine manner. Accordingly, injury-induced EGFR activation provides a molecular link between injury and the expression of these AMP during wound healing.

Inflammation is a potent inducer of AMP and cytokines, and in inflammatory skin diseases such as psoriasis, there is a potent induction of epidermal AMP expression (Harder and Schröder, 2005). Accordingly, it is not clear whether the major epidermal AMP and cytokine expression in wounds is caused by stimuli from the infiltrating inflammatory cells or represents an inherent epidermal reaction to injury. In this study, we show that epidermal reaction to injury is a major inducer of epidermal AMP and cytokine expression in skin wounds.

RESULTS

AMP protein expression increases over time in human skin wounds

To find the time point for maximal AMP expression, standardized human skin wounds were made by punch biopsy. New punch biopsy samples encompassing the wound edge were taken at variable time points after the initial biopsies from the same donor. The time course of the protein expression of SLPI, hBD-2, and elafin in these *in vivo* skin wounds were examined by immunohistochemistry. SLPI expression is induced by injury through activation of the EGFR, hBD-2 expression is known to be IL-1-dependent, and elafin is induced through an unknown signaling pathway. Despite being regulated by different pathways, we found that the AMP expression increased over time with significant presence of all three AMPs at days 3–4 after wounding (Figure 1).

The major part of AMP expression in human skin wounds is injury-induced and dependent on EGFR activation

Owing to the observed time course of AMP expression in the wounded human skin, we investigated the gene expression in the wounded skin/epidermis on day 4 after injury by cDNA microarray. To exclude that changes in gene expression levels were not simply due to variable amount of keratinocytes in our samples, we examined the expression of keratin 14 constitutively expressed in epidermal keratinocytes (Table 1). We also checked for infiltration of inflammatory cells in the different samples by monitoring markers for inflammatory cells. No major differences in expression between the samples were found (Supplementary Table 1). The microarray data confirmed the induced expression of SLPI, hBD-2, and elafin detected by immunohistochemistry (Figure 1). Furthermore, we found significantly increased expression of other AMPs reported to be induced in wounds: hBD-3 (Sørensen *et al.*, 2006), NGAL (Sørensen *et al.*, 2006), psoriasin (Lee and Eckert, 2007), and calgranulins A and B (Thorey *et al.*, 2001) (Table 1). In addition, we found increased expression of haptocorrin, lysozyme, S100A15, and lactoferrin (Table 1), which have not previously been described to be induced in skin wounds. Consequently, their

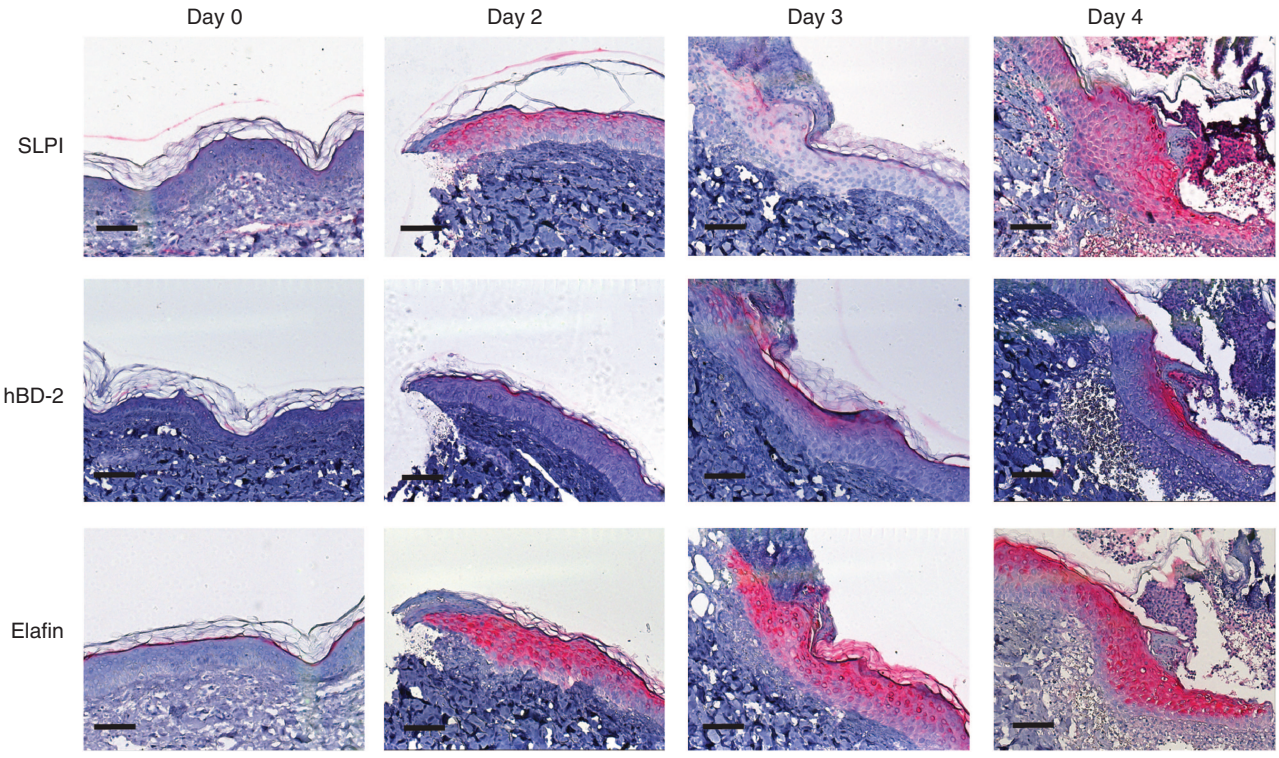

Figure 1. AMP expression increases over time in skin wounds *in vivo*. Samples of normal skin and of wounds 2, 3, and 4 days old were immunostained for SLPI, hBD-2, elafin, and lactoferrin (LF). Normal skin was obtained by punch biopsy. New biopsies of the wound samples were taken on days 2, 3, and 4 around the edges of the initial biopsy. Color was developed with Vulcan Fast Red Chromogen, and Harris Hematoxylin was used for counterstaining. Bars = 100 μm (black).

Table 1. Fold changes of AMPs and cytokines

		In vivo		*Ex vivo*			
		Day 4		Day 4		Day 4 + AG-1478	
Affymetrix ID	**Protein**	**Fold change[1]**	**Hybr. value[2]**	**Fold change[1]**	**Hybr. value[2]**	**Fold change[1]**	**Hybr. value[2]**
Antimicrobial peptides							
210397_at	hBD-1	0.9	(1.231)	1.8	(2.214)	0.8	(965)
207356_at	hBD-2	915.5	(2.364)*	25.4	(93)*	3.8	(12)
224239_at	hBD-3	2.3	(61)*	9.3	(196)*	1.9	(41)
205916_at	Psoriasin	25.1	(11.852)*	17.2	(5.597)*	1.5	(385)
203021_at	SLPI	2.6	(3.493)*	6.8	(9.010)*	2.1	(2.939)
203691_at	SKALP/elafin	176.7	(5.854)*	44.6	(3.861)*	4.1	(284)
234700_s_at	Rnase 7	0.9	(276)	43.3	(4.777)*	6.8	(866)
212531_at	NGAL	3.5	(207)*	5.5	(378)*	3.2	(175)
202018_s_at	Lactoferrin	73.1	(353)	16.6	(59)	10.0	(70)
1555745_a_at	Lysozyme	4.8	(319)*	0.3	(26)	0.7	(34)
210244_at	hCAP-18/LL37**	—	—	—	—	—	—
202917_s_at	Calgranulin A	19.9	(14.879)*	57.8	(13.324)*	29.2	(3.993)
203535_at	Calgranulin B	129.5	(11.348)*	161.1	(8.639)*	56.9	(2.607)
232170_at	S100A15	217.2	(1.426)*	1.4	(19)	1.0	(11)
205513_at	Haptocorrin	146.3	(346.1)*	2.9	(39.6)*	0.7	(8.4)
Interleukins							
210118_s_at	IL-1α	3.3	(117)*	7.8	(232)*	2.1	(54)
205067_at	IL-1ß	6.7	(178)	1.2	(44)	0.9	(32)
205207_at	IL-6	22.9	(252)*	119.4	(6.018)*	21.3	(3.226)
206693_at	IL-7	0.6	(39)	0.0	(6)*	0.1	(14)
202859_x_at	IL-8	68.3	(1.074)*	269.3	(7.169)*	81.4	(3.458)
231169_at	IL-14	1.2	(147)	0.3	(19)	0.3	(16)
209827_s_at	IL-16	1.2	(147)	0.2	(28)*	0.3	(37)
206295_at	IL-18	0.9	(840)	0.3	(273)*	0.6	(504)
224071_at	IL20	48.1	(73)*	111.7	(382)*	23.3	(165)
206569_at	IL24	64.3	(130)*	184.4	(1005)*	37.0	(166)
207113_s_at	TNF-α	2.3	(47)*	3.1	(43)	5.3	(77)
204470_at	CXCL1	7.1	(115)*	107.9	(2.493)*	18.1	(443)
Kerationcyte specific marker							
209351_at	KRT14	1.1	(13.004)	0.9	(9.455)	0.9	(9.734)

[1]Fold changes between non-wounded skin and wounded skin at day 4. Results are shown as the average of three donors in both the *in vivo* and *ex vivo* groups.
[2]The average expression intensity is depicted within the parentheses. The expression intensity was normalized to an average intensity of 100 for all genes. Only genes expressed above the threshold level are depicted with the exception for hCAP18/LL-37.
*Significantly differentially expressed between injured skin and non-injured skin according to Statistical Analysis of Microarray (SAM) with a false discovery rate of 1.56 %.
**Although the hCAP18/LL-37 probe set displayed absent calls in the arrays of all 15 skin samples a slight but not significant increase in hybridization values could be seen in wounded skin *in vivo* but not *ex vivo*.

induction was further investigated by quantitative real-time PCR (qRT-PCR) in two skin wound samples. This confirmed the induction of S100A15 and haptocorrin (data not shown). However, induction of lysozyme could not be detected by qRT-PCR. By qRT-PCR, lactoferrin expression was detected in wounded skin but not in normal skin. Lactoferrin expression

was therefore further investigated at the protein level by immunohistochemistry, where prominent expression was found indicating that lactoferrin was also induced in keratinocytes in skin wounds (Figure 1).

To examine the role of injury for the epidermal innate immune responses, we used an *ex vivo* skin injury model. In this model, there is limited stimulation from infiltrating inflammatory cells and changes in gene expression will therefore primarily be due to the epidermal response to injury (see Supplementary Table 1). We found good agreement between the AMP expression pattern in the *ex vivo* models and skin wounds *in vivo* (Table 1). Indeed, out of the 11 AMPs induced in skin wounds, S100A15 was the only AMP not to also be induced in the *ex vivo* injured skin. In addition, the expression levels of lactoferrin, hBD-2, and haptocorrin were much lower in the *ex vivo* skin injury model, suggesting that inherent epidermal reaction to injury was not a principal inducer of these AMPs in skin wounds. Apart from these exceptions, the microarray findings in the two models showed that out of the 11 AMPs significantly induced in skin wounds, only 4 were not induced primarily through the epidermal response to injury. Thus, expression levels of hBD3, psoriasin, SLPI, elafin, NGAL, and calgranulins A and B were all induced through an injury-induced mechanism.

To regard a gene as upregulated by injury-induced EGFR activation in keratinocytes in skin wounds *in vivo*, we established the following three criteria that should be met: (1) the gene should be upregulated both in skin wounds *in vivo* and in the *ex vivo* skin injury model; (2) AG-1478, a specific inhibitor of the EGFR-associated tyrosine kinase, should inhibit upregulation of the gene in the *ex vivo* skin injury model; and (3) the expression of the gene should be induced in primary keratinocytes after stimulation with transforming growth factor α (TGF-α), a potent and selective ligand of the EGFR. We have previously found that NGAL, SLPI, and hBD-3 meet these criteria (Sørensen *et al.*, 2003, 2006). Microarray and qRT-PCR analyses indicated that the induction of the other major injury-induced AMPs, that is psoriasin, elafin, and calgranulins A and B, in all cases was inhibited by AG-1478 in the *ex vivo* skin injury model, thereby meeting criterion 2 (Table 1). The inhibition with AG-1478 was confirmed by qRT-PCR data showing more than 90% inhibition in three independent experiments. Stimulation of primary keratinocytes with TGF-α further caused a robust induction of these AMPs both at the mRNA level determined by qRT-PCR and at the protein level, thereby fulfilling criterion 3 (Figures 2a and b). These data showed that psoriasin, elafin, and the calgranulins were induced by EGFR activation in skin wounds. To the best of our knowledge, these findings are previously unreported.

The major chemotactic activity in injured skin is generated by EGFR-dependent IL-8 expression

The gene expression for a set of chemokines and cytokines important in the epidermis was analyzed in skin wounds *in vivo* and in the *ex vivo* skin injury model. We found significant upregulation of IL-1, IL-6, and IL-8 mRNA levels,

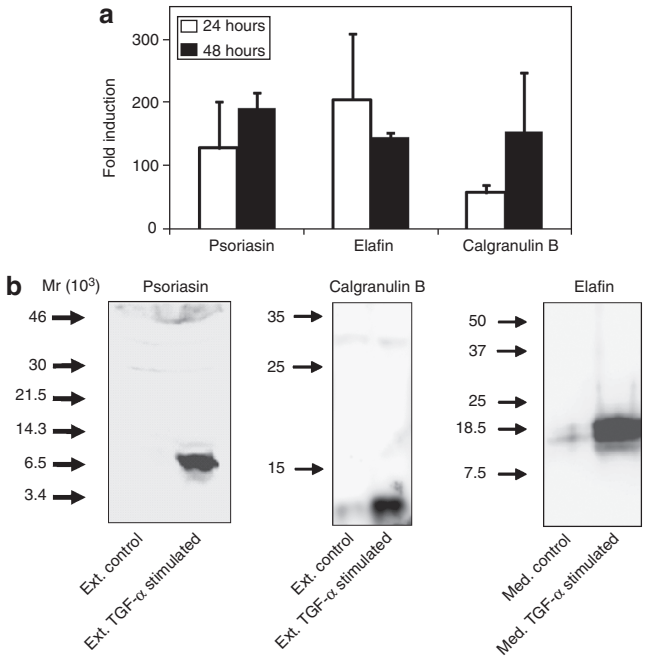

Figure 2. Expression of AMPs and cytokines in stimulated human keratinocytes. (**a**) Primary human keratinocytes were stimulated for 24 and 48 hours with TGF-α (50 ng ml^{-1}). RNA was purified and cDNA was synthesized. The gene expression for psoriasin, elafin, calgranulins, and S100A15 were analyzed by qRT-PCR and the expression was normalized to housekeeping gene *G3PDH* and depicted as fold-induction compared with non-stimulated controls. The average of three independent experiments is shown. (**b**) Medium (Med.) or extracts (Ext.) from stimulated human keratinocytes were subjected to SDS-PAGE, then blotted and probed with antibodies to psoriasin, calgranulin B, and elafin.

confirming previous reports. In addition, we found a significant increase in IL-20 and IL-24 mRNA levels. This induction was further confirmed by qRT-PCR (data not shown). The important neutrophil chemoattractant IL-8 was the IL most strongly induced both in skin wounds and in injured skin *ex vivo*. This was paralleled by the induction of CXCL-1 (GRO-α), another major chemoattractant for neutrophils (Table 1). There was again good agreement between the *in vivo* and *ex vivo* wound model.

The expression of all these injury-induced cytokines and chemokines was inhibited by AG-1478 in the *ex vivo* skin injury model (Table 1). For IL-8, this inhibition was confirmed at the protein level by western blot analysis of medium from *ex vivo* wounded skin (Figure 3a). In experiments with cultured keratinocytes, we observed a strong upregulation of IL-8 mRNA levels after stimulation with TGF-α (Figure 3b). We also found a 23-fold upregulation of CXCL-1, a 12-fold upregulation of IL-20, and a 47-fold upregulation of IL-24 by qRT-PCR (average of three independent experiments). The EGFR-dependent induction of IL-6 in keratinocytes is also in agreement with previous observations *in vitro* (Aragane *et al.*, 1996). According to our criteria, the five epidermal cytokines (IL-6, IL-8, IL-20, IL-24, and CXCL-1) were, thus, induced in skin wounds *in vivo* through activation of the EGFR pathway. To investigate a functional role of the injury-induced

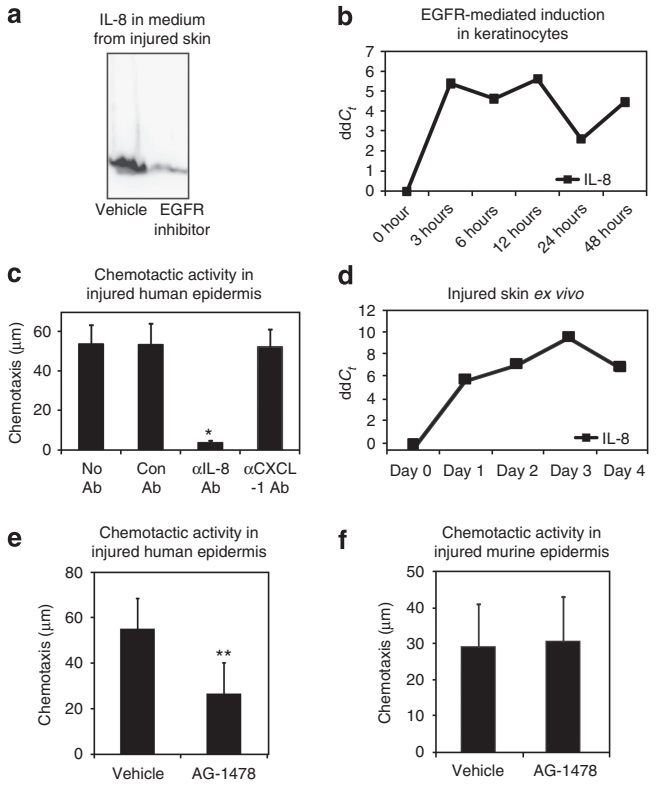

Figure 3. EGFR-dependent IL-8 is the major chemotactic cytokine released from injured skin. (**a**) IL-8 blot performed on medium from *ex vivo* wounded skin (WM.) incubated with or without AG1478. (**b**) The time course of induction of IL-8 was studied by monitoring the mRNA levels by qRT-PCR in TGF-α-stimulated keratinocytes and non-stimulated keratinocytes. The chart depicts the difference in mRNA levels (ddC_t) in normalized threshold cycles (dC_t) (calculated by subtracting the IL-8 threshold cycles (C_t) from the threshold cycles of the *G3PDH* housekeeping gene that was used as reference) between stimulated wells compared with non stimulated controls. One representative experiment out of three is shown. A considerable induction was always found already at day 0. (**c**) Chemotaxis of human neutrophils was measured with medium from the *ex vivo* injured skin/epidermis as neutrophil chemoattractant. IL-8-neutralizing antibodies inhibited the chemotactic activity of the medium from the *ex vivo* injured human skin ($P<0.02$, $n=3$), but pre-immune control antibodies or CXCL-1-neutralizing antibodies had no effect. (**d**) IL-8 expression in injured human skin *ex vivo*. The change in mRNA expression (ddC_t) is shown as the difference in normalized IL-8 threshold cycles (dC_t) from each day and the control at day 0. (*G3PDH* was used as reference gene.) One representative experiment out of three is shown. (**e**, **f**) The chemotactic activity in medium from *ex vivo* injured skin was measured both in humans and in mice. (**e**) Medium from *ex vivo* injured human skin treated with AG-1478 displayed significantly lower chemotactic activity toward neutrophils ($P<0.01$, $n=5$) than the medium from *ex vivo* injured skin treated with DMSO vehicle. (**f**) No significant difference in chemotactic activity could be seen between medium from *ex vivo* injured mouse skin treated with AG-1478 compared with medium from *ex vivo* injured mouse skin treated with DMSO vehicle ($P>0.75$, $n=4$). $^{**}P<0.01$.

cytokines, chemotaxis experiments were performed with the medium from the *ex vivo* injured epidermis. Prominent chemotactic activity toward neutrophils was found in the medium from *ex vivo* injured epidermis (Figure 3c). Neutralizing IL-8 antibodies completely abolished the chemotactic effect of the medium ($n=3$, $P<0.02$), whereas control antibodies or neutralizing antibodies against CXCL-1 had no significant effect.

To establish a link between the injury-induced EGFR activation and the attraction of neutrophils to the wound, chemotaxis experiments were performed with the medium from *ex vivo* injured skin treated with AG-1478. This treatment significantly reduced the chemotactic activity (>50%) generated in the *ex vivo* injured skin ($P<0.01$, $n=5$) (Figure 3e). Control experiments showed that AG-1478 by itself did not inhibit chemotaxis (data not shown).

Analysis of the time of IL-8 expression in the *ex vivo* injured skin showed induction already at day 1 (Figure 3d) (earliest time points tested). This is in contrast with the EGFR-dependent gradual increase in AMP expression (Figure 1). TGF-α stimulation of keratinocytes resulted in maximal IL-8 expression already after 3–6 hours (Figure 3c), whereas expression of AMPs such as SLPI, NGAL, and hBD-3 peaked after 48 hours (Sørensen *et al.*, 2003). Thus, EGFR activation leads to a much more rapid expression of IL-8 than that of AMPs both in cultured keratinocytes *in vitro* and in *ex vivo* injured skin. Stimulation of primary keratinocytes showed that the observed AMP expression by EGFR activation was not a direct effect due to IL-8 (data not shown).

Injury is independently of infection a major inducer of AMP expression in mouse skin wounds

To study the importance of injury for the epidermal innate immune responses *in vivo*, mouse skin experiments were performed. We have previously shown that injury of mouse skin induces the murine orthologs of SLPI and NGAL (named 24p3 in mouse) both *in vivo* and in the *ex vivo* skin injury model (Sørensen *et al.*, 2006). We further examined the mRNA expression of the murine β-defensins (mBD)-1 through 6 as well as the expression of mBD-14, the murine ortholog of hBD-3 (Hinrichsen *et al.*, 2008; Röhrl *et al.*, 2008), in murine skin wounds by qRT-PCR. Only mBD-3 was induced in the *ex vivo* mouse skin injury model (Figure 4a) and in skin wounds *in vivo* along with the previously shown SLPI and 24p3 (data not shown). To investigate the importance of injury compared with infection for AMP expression in skin wounds, we analyzed AMP expression during topical cutaneous infections in mouse skin. AMP expression was not induced in topically infected intact skin (data not shown). We subsequently perforated the skin using a syringe before the application of bacteria. No significant increase in expression levels of SLPI, 24p3, mBD-3, or any of the examined mBDs was found compared with non-infected skin controls perforated the same way (Figure 4b). To validate that this was not due to inadequate microbial stimulation, we incubated *ex vivo* injured mouse skin with peptidoglycan (PGN) and investigated the induction of AMP expression. This experimental setup gives very prominent AMP induction in human skin (Liu *et al.*, 2003; Sørensen *et al.*, 2005). However, the expression of SLPI, 24p3, and mBD-3 mRNA levels in the *ex vivo* injured mouse skin were not further induced by PGN stimulation (Figure 4c). Importantly, PGN

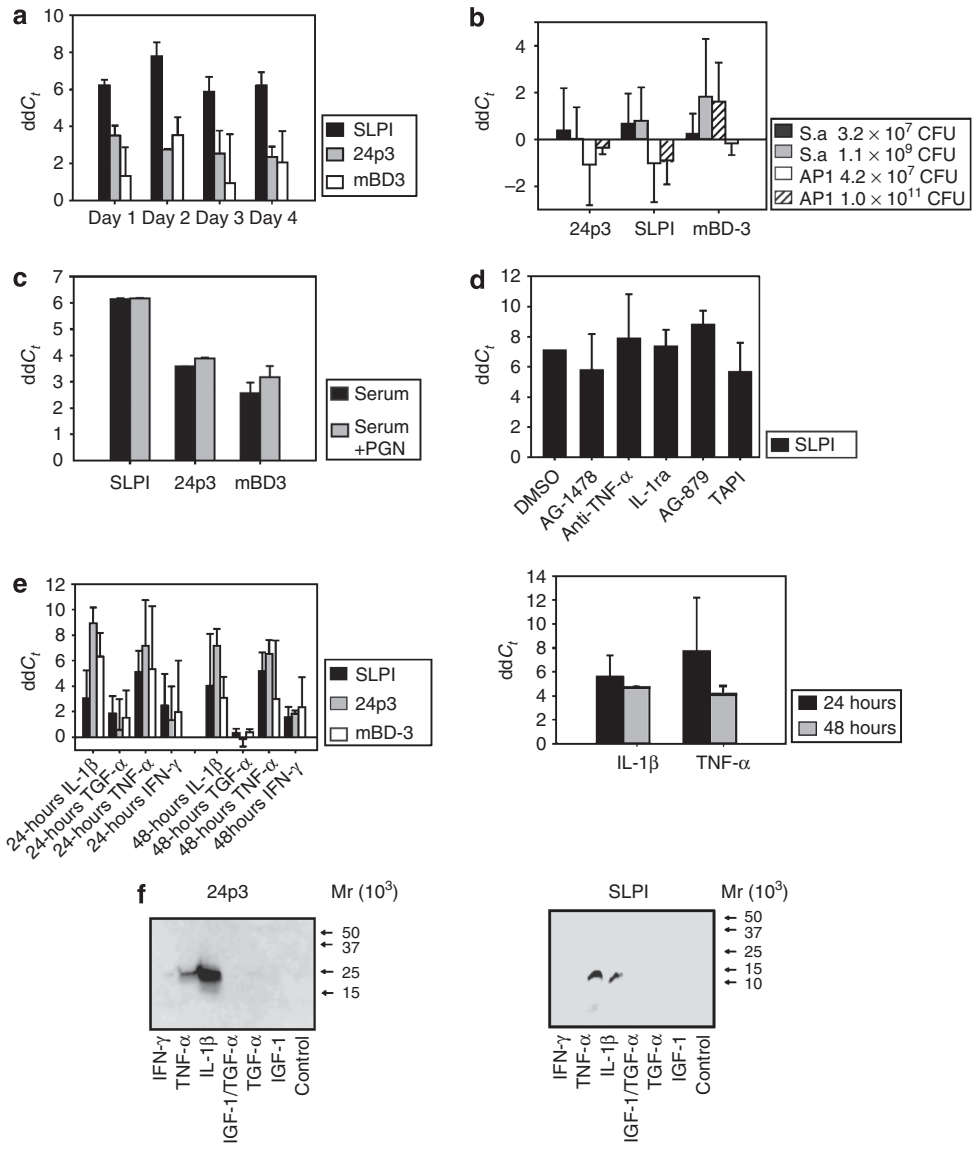

Figure 4. AMP expression in mouse skin wounds and keratinocytes. (**a**) The graph depicts injury-induced AMP expression over time in *ex vivo* wounded mice skin. Dorsal mouse skin was sliced in 1 × 10 mm pieces and cultured in keratinocyte medium before mRNA extraction at 24, 48, 76, and 96 hours. The change in mRNA expression (ddC_t) is shown as the difference in threshold cycles (C_t) of the gene of interest after normalizing each gene with G3PDH and control at day 0. A positive number of ddC_t denotes increased expression and negative ddC_t decreased expression. SLPI and mBD-3 reach maximum level at day 2 and 24p3 already at day 1. (**b**) Topical skin infection with *S. aureus* or *S. pyogenes* strain AP1 was performed in Balb/c mice (see Experimental procedures). The infected area of epidermis was excised and the gene expression was analyzed by qRT-PCR with non-infected epidermis from the same mouse as control. The gene expression was normalized to G3PDH and changes in threshold cycles (C_t) were compared with the non-infected control area (ddC_t). Each value represents an average of three mice. (**c**) *Ex vivo* wounded mouse skin was incubated with medium with 5% serum with or without peptidoglycan (PGN) and the gene expression analyzed by qRT-PCR. The mRNA expression is shown as the difference in threshold cycles (C_t) of the gene of interest (ddC_t) after normalizing each gene with G3PDH and control day 0. Results from two independent experiments are shown. (**d**) SLPI mRNA expression levels were monitored by qRT-PCR in *ex vivo* injured mouse skin after 48 hours. The skin slices were incubated with DMSO vehicle, AG-1478, AG-879, anti-TNFα, IL-1β, and TAPI-1. The mRNA expression is shown as the difference in threshold cycles (C_t) between the gene of interest (ddC_t) after normalizing each gene with G3PDH and day 0. Each value represents a minimum of four separate experiments. (Similar results were obtained for 24p3 and mBD-3.) (**e**) Primary murine keratinocytes were stimulated for 24 and 48 hours with cytokines and growth factors. RNA was purified and cDNA was synthesized. Gene expression was analyzed by qRT-PCR and the expression was normalized to housekeeping gene *G3PDH* and the changes in threshold cycles (ddC_t) were compared with non-stimulated controls. The average of three independent experiments is shown. Left panel: Gene expression of mBD-3, murine SLPI, and 24p3. Right panel: Gene expression of mBD-2 in response to IL-1β and TNFα. (**f**) Medium from stimulated murine keratinocyte was subjected to SDS-PAGE and then blotted and probed with antibodies to murine SLPI and 24p3.

also failed to induce the expression of any of the other mBDs (data not shown). Similar results were obtained when stimulating with lipopolysacharide (data not shown). These data showed that injury was a major inducer of epidermal AMP expression in mouse skin wounds independent of infection.

Injury induces chemotactic activity in mouse skin

The main chemokine induced in the experiments with injured human epidermis, IL-8, is not found in mice. By qRT-PCR, we failed to detect any expression of the murine neutrophil chemoattractants CXCL-1 (GRO-α) and CXCL-3 (KC) in the non-injured mouse skin. However, these cytokines were clearly expressed in the *ex vivo* skin injury model (data not shown). The mouse ortholog of IL-8, CXCL2 (MIP-2), could not be detected neither in non-injured nor in injured skin (data not shown). As with the human model, medium from the *ex vivo* injured mouse skin showed prominent chemotactic activity toward human neutrophils (Figure 3f).

Injury-induced epidermal innate immune responses are differently regulated in human and mouse skin

To investigate the molecular mechanism for the injury-induced AMP and cytokine expression in mouse skin, we first tested the ability of AG-1478 and AG-879 (a broader inhibitor of the homology to EGF receptor family) to inhibit the injury-induced expression of AMPs in the *ex vivo* injured mouse skin. However, these inhibitors did not block the injury-induced AMP expression (Figure 4d). Furthermore, neither the injury-induced chemotactic activity generated in the mouse epidermis nor the expression of CXCL-1 or CXCL-3 was inhibited by AG-1478 (Figure 3f and data not shown). We also tested TNF-α-converting enzyme protease inhibitor-1 (TAPI-1), a metalloprotease inhibitor that inhibits the shedding of membrane-bound EGFR ligands during transactivation. In contrast to in injured human skin (Sørensen *et al.*, 2006), TAPI-1 did not inhibit AMP expression in injured mouse skin (Figure 4d). Furthermore, stimulation of primary murine keratinocytes with TGF-α did not cause significant induction of SLPI, 24p3, and mBD-3 mRNA expression even in combination with insulin-like growth factor 1 (Figure 4e and f) that potentiates the AMP expression of TGF-α in human keratinocytes (Sørensen *et al.*, 2003). Accordingly, EGFR activation did not induce expression of AMPs in murine keratinocytes neither in *in vitro* nor in injured epidermis *ex vivo*.

Next, we investigated whether three pro-inflammatory cytokines known to induce AMPs in human keratinocytes, IL-1, TNF-α, and IFN-γ, could induce AMP expression in murine keratinocytes. We found induction of mBD-3, 24p3, and SLPI after stimulation with IL-1β and TNF-α after 24 and 48 hours as well as a minor effect of IFN-γ (Figure 4e and f). In addition, these cytokines induced the expression of mBD-2 (Figure 4e, right panel) but not of any of the other mBDs. We then tested whether the IL-1 receptor antagonist or TNF-α-neutralizing antibodies inhibited the injury-induced AMP expression in the *ex vivo* mouse skin injury model. Neither the IL-1 receptor antagonist nor the anti-TNF-α-neutralizing antibodies inhibited the injury-induced AMP expression in mouse skin (Figure 4d), indicating that injury-induced AMP expression in mouse skin was not mediated by IL-1 or TNF-α. This was substantiated by the fact that mBD-2, inducible by both IL-1 and TNF-α, was not expressed in the *ex vivo* skin injury model.

DISCUSSION

Epidermal injury represents a vulnerable condition where the normal physical barrier of intact skin protecting against microbes is disrupted. In accordance with the ''danger model of immunity'' (Matzinger, 2002), injury can therefore be seen as a danger signal that elicits innate immune responses in the wound, including production of AMPs and chemotactic cytokines attracting neutrophils. In this study, we attempted do delineate the importance of injury for the epidermal innate immune response during wound healing. We found that the major epidermal production of cytokines and AMPs in skin wounds was independent of stimulation from infiltrating inflammatory cells and that it represented an epidermal response to injury. The importance of injury for the epidermal innate immune responses found in the human models was corroborated *in vivo* by mouse experiments showing that injury has a major role in epidermal AMP expression in skin wounds independent of wound infections.

In human skin, the injury-induced EGFR activation was shown to be of major importance for the epidermal AMP expression. Interestingly, the same molecular pathway was clearly also involved in the production of chemotactic cytokines attracting neutrophils to the wound. Thus, we found that EGFR-dependent IL-8 production in response to injury was a major contributor to the potent chemotactic activity toward neutrophils generated in the injured epidermis. Interestingly, the dynamics of IL-8 expression found in the *ex vivo* injured epidermis, with a rapid induction of mRNA expression followed by a more gradual increase until days 3–4 after injury, fits well with the pattern of increasing concentrations of IL-8 in wound fluid from skin wounds *in vivo* peaking around day 5 (Rennekampff *et al.*, 2000). Thus, injury-induced EGFR-dependent IL-8 production provides a molecular link between tissue injury and neutrophil recruitment in human skin.

The injury-induced epidermal immune responses elicited by keratinocytes that encompass rapid production of IL-8 followed by a more gradual increase in the production of epidermal AMPs should be viewed in the context of other processes taking place after cutaneous injury (Werner and Grose, 2003). The keratinocyte response requires active protein synthesis and these cells are therefore not likely to be critically involved in the initial antimicrobial defense after epithelial injury. The first major event in a skin wound after injury is the exudation of plasma resulting both in activation of the complement system and in coagulation mediated through the intrinsic pathway (the contact system) (Kiritsy *et al.*, 1993). Recent studies have shown that activation of both these catalytic cascades leads to the generation of antimicrobial peptides (Nordahl *et al.*, 2004; Frick *et al.*, 2006). Additional AMPs are released from activated platelets (Tang *et al.*, 2002) at this stage. The complement activation also leads to attraction of leukocytes through the generation of chemotactic factors such as C3a and C5a. Simultaneously, epidermal injury leads to the activation of metalloproteases that results in shedding of growth factors, which subsequently activate the EGFR (Tokumaru *et al.*, 2000). Injury-induced EGFR activation causes IL-8 production in keratinocytes

which is likely to be important for the continued neutrophil recruitment to the wound. As inflammation starts to recede and neutrophil count diminish the epidermal EGFR-dependent AMP expression reaches its maximum.

Mice experiment confirmed the important role of injury independent of infection for AMP expression during wound healing. However, we found important differences in the regulation of AMP expression between murine and human skin wounds. Although the injury-induced production of cytokines and AMPs were dependent on EGFR-activation in human skin, this was not the case in murine skin. Indeed, EGFR activation failed to induce AMP expression in murine keratinocytes even *in vitro*. In addition, superficial skin infection did not induce AMP expression in murine skin or skin wounds *in vivo*. Lipopolysacharide and PGN, both potent microbial inducers of AMP expression in injured human skin *ex vivo* (Liu *et al.*, 2003; Sørensen *et al.*, 2005), failed to induce AMP expression in mouse skin when using the exact same experimental setup. Accordingly, both the molecular mechanism of injury-induced AMP and cytokine expression, as well as the response to cutaneous infection/microbial stimuli, differ between human and murine skin. These observed differences indicate that care must be taken when extrapolating mouse data regarding innate immunity in skin to the human situation.

In conclusion, we found that injury was a major inducer of the epidermal innate immune responses encompassing expression of AMPs and cytokines. Mouse experiments confirmed that injury was the major inducer of epidermal innate immune responses *in vivo* independent of the presence of wound infections. The epidermal injury responses generated chemotactic activity in the epidermis due to EGFR-dependent IL-8 production, thus, providing a, to our knowledge previously unreported, molecular link between tissue injury and neutrophil recruitment.

MATERIALS AND METHODS

Reagents

The anti-hBD-2 and anti hBD-3 antibodies have been described previously (Liu *et al.*, 2002; Sørensen *et al.*, 2005) and were generously provided by Dr. Tomas Ganz (University of California, Los Angeles). Antibodies to SLPI, IL-8, elafin, and murine IL-1 receptor antagonist were purchased from R&D Systems (Minneapolis, MN), whereas psoriasin antibodies were purchased from Biocarta (San Diego, CA) and haptocorrin antibodies from Abcam (Cambridge, UK). Normal rabbit immunoglobulins, anti-murine TNF-α, CXCl-1, and CXCL-3 antibodies, anti-human CXCL-1 antibodies, murine IFN-γ, murine insulin-like growth factor 1, murine IL-1β, murine IL-6, murine TNF-α, human TGF-α, and human IL-8 were all from Peprotech (Rocky Hill, NJ). *N*-formyl-methionyl-leucyl-phenylalanine, AG-1478, and AG-879 were purchased from Sigma (St. Louis, MO). TAPI-1 was from Peptides International (Louisville, KY). Lipopolysacharide and PGN were from Sigma.

Human skin wounds

Samples from human skin wounds were obtained under protocols approved by the Ethics Committee at Lund University (Lund, Sweden). Informed written consent was obtained from all subjects and all procedures in this study involving human samples were performed in accordance with the guidelines in the Declaration of Helsinki Principles. Non-wounded human skin was obtained by taking punch biopsies from three healthy donors and skin wound samples were retrieved by making new punch biopsies from the edges of the initial biopsies. These samples were fixed in formalin for immunohistochemistry. For analysis by cDNA microarray, as much dermal tissue as possible was removed by dissection from the biopsies. The remaining tissue was subsequently washed thoroughly in sterile NaCl to remove infiltrating inflammatory cells. This ensured that the samples mainly consisted of the epidermis (Figure 1 in the Supplementary material). RNA was isolated from these samples and used for cDNA microarray analysis.

Model of *ex vivo* injured human skin

Skin was obtained from three donors after informed written consent, following reduction surgery of the abdomen (normal skin) according to protocols approved by the Ethics Committee at Lund University and in accordance with the ethical principles in the Helsinki declaration. The skin biopsies were cut into slices of 1×10 mm and incubated in culture for 4 days (*ex vivo* injured skin). Furthermore, as much non-epidermal tissue as possible was removed by dissection and the samples were washed. The skin samples were cultured in serum-free keratinocyte medium (KGM2 Bullet kit) from Cambrex (Walkersville, MD) supplemented with transferrin, hEGF (0.15 ng ml^{-1}), 0.5 mg ml^{-1} hydrocortisone, gentamicin, amphotericin B, and epinephrine but without insulin (all supplied by Cambrex). We previously found that this medium does not induce the expression of AMPs in keratinocytes (Sørensen *et al.*, 2003). Indeed, even 100 ng ml^{-1} of epidermal growth factor gives a very limited AMP induction (Sørensen *et al.*, 2005). In the inhibition experiments, the skin slices were incubated with AG-1478 at a final concentration of 10 μmol l^{-1}. RNA was isolated from these samples and used for cDNA microarray analysis. The medium with injured skin was changed every 2 days, and medium from days 2 to 4 was used for chemotaxis experiments.

Experiments with *ex vivo* injured mouse skin

Mice were killed by CO_2 asphyxiation and cervical dislocation. A dorsal portion of the skin was shaved, surgically removed, and cut into slices of 1×10 mm. Approximately 25 skin pieces were incubated in six-well plates in 5 ml keratinocyte serum free medium (SFM) (Invitrogen, Carlsbad, CA) supplemented with 5 ng ml^{-1} bovine pituitary extract, 5 ng ml^{-1} recombinant EGF, and a antibiotic/antimycotic solution consisting of 1,000 units ml^{-1} penicillin G sodium, 1,000 units ml^{-1} streptomycin sulfate, and 0.25 μg ml^{-1} amphoteracin B (Invitrogen). For inhibition experiments, IL-1 receptor antagonist was used in a concentration of 250 ng ml^{-1}, AG1478 and AG879 at 10 μmol l^{-1}, and TAPI-1 at 50 μmol l^{-1} and anti-TNFα antibody at 7.5 μg ml^{-1}. For stimulation with microbe-derived molecules, PGN was used in a concentration 10 μg ml^{-1} and lipopolysacharide in a concentration of 100 ng ml^{-1}.

Isolation of polymorphonuclear neutrophils

Polymorphonuclear neutrophils were isolated at 4 °C from blood from healthy donors using gradient centrifugation (Böyum, 1968). EDTA-stabilized whole blood (5 ml) was placed on 3 ml Polymorfprep (Nycomed AS, Oslo, Norway) and centrifuged for 35 minutes at 400 *g*. The granulocyte layer was harvested and resuspended in Hanks' buffered salt solution (Gibco, Life Technologies, Rockville,

MD), washed three times, and centrifuged for 7 minutes at 900 *g*. Polymorphonuclear neutrophils were then resuspended (2.0×10^6 cells per ml) in Hanks' buffered salt solution with 0.2% human serum albumin (Behring, Marburg, Germany).

Chemotaxis

Neutrophil chemotaxis was assessed as previously described (Nybo *et al.*, 1998). Briefly, a 48-well microchemotaxis chamber (NeuroProbe, Gaithersburg, MD) with 3-μm Millipore filters, type SS (Millipore, Billerica, MA), was used. Media from *ex vivo* wounded skin were subsequently tested as chemoattractant. Survival of fibroblast and other non-keratinocyte cells are not supported by the growth media in the *ex vivo* skin injury model. Accordingly, we used media incubated with the *ex vivo* injured skin from days 2 to 4. Using this media made it possible to selectively investigate chemotactic factors secreted by the epidermal keratinocytes. In some experiments, neutralizing antibodies (5 μg/ml) toward the different chemokines were added to the incubation medium before the chemotaxis experiments. *N*-formyl-methionyl-leucyl-phenylalanine (10^{-8} mol l^{-1}) was used as a positive chemoattractant control. Polymorphonuclear neutrophils were placed in the microchemotaxis chamber for 30 minutes at 37 °C, and directed migration was subsequently measured by the leading-front principle (Zigmond and Hirsch, 1972) with three cells per high-power field. Three fields were read per well, and all samples were assayed in duplicate. All migration distances were subtracted, and the spontaneous migration was measured in non-stimulated polymorphonuclear neutrophils and expressed as micrometer per 30 minutes.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were carried out according to the instructions from the manufacturer (Bio-Rad, Hercules, CA). After transfer of proteins from the polyacrylamide gels, the polyvinylidene difluoride membrane was fixed for 30 minutes in Tris buffered saline with 0.05% glutaraldehyde (Sigma) and blocked with 3% skimmed milk. For visualization of the poly(peptides), the polyvinylidene difluoride membranes were incubated overnight with primary antibodies. The next day, the membranes were incubated for 2 hours with HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark) and visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Keratinocyte culture

Primary human keratinocytes were obtained from Cascade Biologics (Portland, OR) and cultured in serum-free medium (KGM2 Bullet kit) from Cambrex. Cells were stimulated, beginning 24 hours after complete confluence was reached and stimulated with TGF-α (50 ng ml^{-1}) for 3, 6, 12, 24, and 48 hours or left non-stimulated as control. Primary mouse keratinocytes (CellnTech, Bern, Switzerland) were cultured in six-well plates in CnT-02 medium (CellnTech). The medium was changed every 2 days until confluence was reached. Murine keratinocytes were stimulated the day after complete confluence was reached with the following mouse cytokines: IL-1β (25 ng ml^{-1}), IL-6 (100 ng ml^{-1}), TGF-α (50 ng ml^{-1}), TNF-α (25 ng ml^{-1}), insulin-like growth factor 1 (100 ng ml^{-1}), and INF-γ (50 ng ml^{-1}) for 24 and 48 hours before harvesting cells for mRNA extraction and medium for western blot analysis. Cells grown in medium were used as control.

Immunohistochemistry

The specimen were fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections of 5-μm thickness were placed on poly-lysine-coated glass slides, deparaffinized in xylene, and rehydrated in graded alcohols. The slides were then treated with Dako antigen retrieval solution (Dako) for 40 minutes at 97 °C. The slides were incubated for 24 hours at room temperature in a 1:1,000 dilution of polyclonal antibodies. The antibodies were diluted in TBS with 0.05% Tween 20, 1% BSA, and 5% serum from the same species as the secondary antibody. After three 20-minute washes in TBS with 0.05% Tween 20, the slides were incubated with alkaline phosphatase-conjugated secondary anti IgG (Dako) diluted 1:1,000 in the same buffer as the first antibody and incubated for another 24 hours followed by three 20-minute washes. Color was developed with Vulcan Fast Red chromogen (Biocare Medical, Concord, CA) and the slides were counterstained with Harris Hematoxylin (EM Science, Gibbstown, NJ).

Bacterial strains and growth conditions

The *Streptococcus pyogenes* strain AP1 of the M1 serotype was from the WHO Collaborating Centre for Reference and Research on Streptococci, Institute of Hygiene and Epidemiology (Prague, Czech Republic). *Staphylococcus aureus* was a clinical isolate from Department of Microbiology (Lund University Hospital). Bacteria from overnight cultured plates were grown in 10-ml Todd Hewitt broth (Becton, Dickinson and Company, Sparks, MD) with yeast extract (Becton, Dickinson and Company) at 37 °C and 5% CO_2. Bacteria were taken in stationary phase after a 16-hour culture. Before use, bacteria were washed twice in sterile physiological salt solution. Optical density was measured at 620 nm and bacteria were diluted to a calculated concentration estimated from previously established growth curves. All colony-forming unit (CFU) given values were based on triplicate plating assay results.

Topical skin infection *in vivo*

Wild-type C57BL/6 mice, 7- to 8-week old, were anesthetized with isoflorane and a small dorsal area of the skin was shaved and sterilized with ethanol. The shaved area was punctured four times at two places using a syringe needle (BD Microlance, Drogheda, Ireland, 0.3 × 19 mm) (MidMeds, Waltham Abbey, UK)). Two rubber rings with 10-mm inner diameter were subsequently attached using an ethylcyanoacrylate-based adhesive and the rings were covered with OpSite (Medisave, Weymouth, UK). CFU (2×10^7 or 1×10^{11}) of *S. pyogenes* and CFU (3.2×10^7 or 1.1×10^9) of *S. aureus* in a volume of 150 μl was thereafter injected through the OpSite into the cavity formed by one of the rubber rings. The other ring was injected with sterile buffer and used as control. Mice were killed after 2 days and the skin within the side of the rings was retrieved for RNA purification and qRT-PCR analysis. All animal experiments were approved by the Animal Welfare Committee at Lund University.

RNA isolation

Total RNA was isolated with Trizol (Invitrogen) according to the recommendations of the supplier and resuspended in 0.1 mmol l^{-1} EDTA. The concentration was determined by spectrophotometric measurement.

Real-time PCR

cDNA was synthesized from 200 ng purified RNA using iScript cDNA synthesis kit (Bio-Rad) according to the instructions given by the manufacturer. hBD-1, hBD-2, and hBD-3 together with G3PDH expression was analyzed using iQ SYBR Green Supermix (Bio-Rad). Primers for real-time PCR are described in Supplementary material. Amplification was performed at 55 °C for 40 cycles in iCycler Thermal Cycler (Bio-Rad) and data analyzed using iCycler iQ Optical System Software. The relative expression in each sample was calculated by a mathematical method based on the real-time PCR efficiencies (Pfaffl, 2001).

Microarray analysis

For gene expression analysis, total RNA was biotinylated and hybridized to Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) according to the instructions by the manufacturer. The microarray fluorescence signals were normalized using the GeneChip Operation Software (GCOS ver. 1.4, Affymetrix). All probe set lists were annotated with locus link identifications (IDs) provided by the NetAffx database (Affymetrix, www.affymetrix.com) and converged into gene/EST (expressed sequence tag) lists by exclusion of redundant probe sets with identical locus ID. Genes were defined as expressed in cell populations if all replicates were assigned a present call by the GCOS software (Affymetrix). Genes of potential interest for wound healing were therefore only genes with at least three present calls in either the *in vivo* control condition or in the *in vivo* wound condition. These genes were each assigned a *Z*-score, calculated on the basis of the log2 hybridization values of their probes on each microarray, in order to get a better comparison between arrays. The statistical technique Significance Analysis of Microarrays (SAM) was subsequently used in order to determine which of these genes that could be considered differentially expressed based on the *Z*-scores of the triplicate biological samples. The cutoff was set to include expression of three AMPs, hBD-3, NGAL, and SLPI, previously found to be induced in skin wounds. This gave a false discovery rate of 1.57 %.

Statistical analysis

For non-microarray data, two-tailed paired Student's *t*-test was used.

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

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