Fetal Human Keratinocytes Produce Large Amounts of Antimicrobial Peptides: Involvement of Histone-Methylation Processes

Maria Gschwandtner[1,4], Shaomin Zhong[1,2,4], Antonia Tschachler[1], Veronika Mlitz[1], Susanne Karner[1], Adelheid Elbe-Bürger[3] and Michael Mildner[1]

Antimicrobial peptides (AMPs), an important part of the innate immune system, are crucial for defense against invading microorganisms. Whereas AMPs have been extensively studied in adult skin, little is known about the impact of AMPs in the developing human skin. We therefore compared the expression and regulation of AMPs in fetal, neonatal, and adult keratinocytes (KCs) *in vitro*. The constitutive expression of human β-defensin-2 (HBD-2), HBD-3, S100 protein family members, and cathelicidin was significantly higher in KCs from fetal skin than in KCs from postnatal skin. The capacity to further increase AMP production was comparable between prenatal and postnatal KCs. Analysis of skin equivalents (SEs) revealed a strong constitutive expression of S100 proteins in fetal but not in neonatal and adult SEs. The elevated AMP levels correlated with reduced H3K27me3 (tri-methyl-lysine 27 on histone H3) levels and increased expression of the histone demethylase JMJD3. Knockdown of JMJD3 in fetal KCs elevated H3K27me3 levels and significantly downregulated the expression of HBD-3, S100A7, S100A8, S100A9, and cathelicidin. Our data indicate a crucial contribution of histone modifications in the regulation of AMP expression in the skin during ontogeny. The elevated AMP expression in prenatal skin might represent an essential defense strategy of the unborn.

Journal of Investigative Dermatology (2014) **134,** 2192–2201; doi:10.1038/jid.2014.165; published online 8 May 2014

INTRODUCTION

Antimicrobial peptides (AMPs) are a diverse group of mostly cationic polypeptides with antimicrobial cytotoxic activities against bacteria, fungi, and viruses. The mode of action of AMPs against bacteria include the formation of transmembrane pores, as well as metabolic interference (Brogden, 2005). The main groups of AMPs identified so far in adult human skin include defensins, cathelicidins, and members of the RNase and the S100 families (Harder and Schröder, 2005; Schröder and Harder, 2006). Whereas some AMPs, such as RNase 7 and S100 proteins, are constitutively expressed in the upper layers of the epidermis, others such as human β-defensin-2 (HBD-2) and HBD-3 are inducible in response to pathogen invasion of the skin or by inflammatory mediators (Harder and Schröder, 2002, 2005; Schröder and Harder, 2006; Suter *et al.*, 2009; Afshar and Gallo, 2013). In addition to their direct antimicrobial effect, AMPs also have important immunomodulatory properties (Yang *et al.*, 2001b, 2002, 2004). Although β-defensins are chemotactic for dendritic cells and memory T cells by binding to CCR6 (Yang *et al.*, 2002), cathelicidins are chemotactic for neutrophils, monocytes, and T cells but not for dendritic cells (Yang *et al.*, 2001a). In addition, AMPs not only attract immune cells but are also able to activate them directly. S100A7, for example, has been shown to induce secretion of cytokines and chemokines from neutrophils, which in turn contribute to innate immunity through enhancing neutrophil host defense functions (Zheng *et al.*, 2008).

Recent studies showed that histone modifications, especially lysine methylation, have an important function during cell differentiation and organ development. In particular, the methylation status of histone H3K27 has been associated with the activation and repression of a variety of developmental genes (Nottke *et al.*, 2009). In addition, Sen *et al.* (2008) identified an important role of H3K27 trimethylation in epidermal cells during epidermal differentiation. KCs lacking

[1]*Research Division of Biology and Pathobiology of the Skin, Department of Dermatology, Medical University of Vienna, Vienna, Austria;* [2]*Department of Dermatology, Peking University First Hospital, Beijing, China and* [3]*Laboratory of Cellular and Molecular Immunobiology of the Skin, Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna, Vienna, Austria*

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

Correspondence: Michael Mildner, Department of Dermatology, Research Division of Biology and Pathobiology of the Skin, Medical University of Vienna, Anna-Spiegel Research Building, Lazarettgasse 14, 1090 Vienna, Austria. E-mail: michael.mildner@meduniwien.ac.at

Abbreviations: AMP, antimicrobial peptide; H3K27me3, tri-methyl-lysine 27 on histone H3; HBD, human β-defensin; KC, keratinocyte; SE, skin equivalent; siRNA, small interfering RNA; TLRL, Toll-like receptor ligand

Received 30 September 2013; revised 6 March 2014; accepted 18 March 2014; accepted article preview online 2 April 2014; published online 8 May 2014

JMJD3 expression, the enzyme responsible for demethylation of H3K27, showed an impaired KC differentiation process, resulting in reduced levels of KC differentiation–associated proteins such as certain keratins, involucrin, and S100A8. Accordingly, depletion of EZH2, a histone methyltransferase trimethylating H3K27, resulted in premature epidermal differentiation (Sen *et al.*, 2008). The importance of H3K27, JMJD3, and EZH2 has also been demonstrated in tissue regeneration and wound healing (Shaw and Martin, 2009; Ezhkova *et al.*, 2011).

In contrast to adult human skin, only few data have been published on AMP expression and regulation in developing prenatal human skin. Cathelicidin, human neutrophil defensins, and S100A7 have been detected in vernix caseosa and full-term amniotic fluid (Marchini *et al.*, 2002; Yoshio *et al.*, 2003; Porre *et al.*, 2005), and RNAse 7 and S100A7 have been found to be present in fetal human epidermis (Schuster *et al.*, 2013), indicating that AMPs have a role in prenatal antimicrobial defense. Furthermore, systematic studies on the human skin innate immune system during prenatal development have recently demonstrated that prenatal skin expresses the same spectrum of pattern recognition receptors as adult skin and that Toll-like receptors (TLRs) 1–5, NODs 1/2, NALPs 1/3, and DECTIN-1 are even significantly higher expressed in prenatal than in postnatal skin. Functional experiments revealed that fetal human KCs strongly react to the activation of TLRs, suggesting an enhanced innate immune response of prenatal skin (Iram *et al.*, 2012). Taken together, these data demonstrated that fetal KCs are equipped with specific immune functions to combat invading pathogens.

Once born, the neonate is exposed to an abundance of microbial agents. As the neonatal immune system is not yet fully responsive (Chorro and Geissmann, 2010; Elbe-Bürger and Schuster, 2010; Ginhoux and Merad, 2010; PrabhuDas *et al.*, 2011; Palin *et al.*, 2013), innate immunity presumably has a crucial role for protection of the newborn against invading pathogens. To further explore the role of AMPs as a rapid first line of defense in the developing human skin, we investigated the expression profile of several groups of AMPs in prenatal and postnatal KCs.

RESULTS

Fetal KCs express high levels of mRNA from antimicrobial peptides

To assess comparatively the expression pattern of several AMPs in proliferating and differentiated prenatal and postnatal KCs, we performed real-time PCR for HBD-1, HBD-2, HBD-3, S100A7, S100A8, S100A9, RNase5, RNase7, and cathelicidin. As shown in Figure 1, mRNA levels of HBD-2, HBD-3, S100A7, S100A8, S100A9, and cathelicidin were significantly higher in KCs from several fetal donors as compared with KCs derived from skin of neonatal and adult donors in proliferating (Figure 1a) and differentiated KCs (Figure 1b). In contrast to postnatal KC expression, the level of HBD-1 was only weakly inducible by differentiation of fetal KCs. S100A8 and RNase5 were not inducible at all in neonatal KCs (Table 1). Differences between the AMP expression levels of proliferating neonatal and adult KCs were not detected (Figure 1a), whereas in differentiated KCs the expression of HBD-2, S100A7, and S100A8 was constantly higher than in adult KCs (Figure 1b). Regulation of the KC differentiation–dependent proteins keratin 14, keratin 10, filaggrin, and loricrin was comparable in all three KC groups before and after induction of KC differentiation (Supplementary Figure S1 online), suggesting a comparable differentiation process in prenatal and postnatal KCs. These data demonstrate that despite comparable capacities of KCs derived from different age groups to proliferate and differentiate, fetal KCs constitutively express significantly higher levels of HBD-2, HBD-3, S100A7, S100A8, S100A9 (only in differentiated KCs), and cathelicidin than adult and neonatal KCs.

Fetal KCs constitutively produce high levels of HBD-2 and S100 proteins

We next analyzed the production of AMP proteins by ELISA and western blot analysis. As HBD-2 belongs to a group of inducible AMPs, we also included IL-1α and the TLR5L flagellin, two known inducers of HBD-2 expression in postnatal KCs (Abtin *et al.*, 2008), as stimulants. In line with our real-time PCR data, significantly higher levels of HBD-2 protein were detected in cell lysates of nonstimulated fetal KCs ($2{,}204 \pm 490\,pg\,ml^{-1}$) than in neonatal ($754 \pm 35\,pg\,ml^{-1}$) and adult ($232 \pm 8\,pg\,ml^{-1}$) KCs (Figure 2a). The capacity of IL-1α and flagellin to upregulate HBD-2 was largely comparable in lysates of prenatal and postnatal cells (Figure 2a). Surprisingly, considerable amounts ($224 \pm 92\,pg\,ml^{-1}$) of HBD-2 were already released by unstimulated fetal KCs but were not detectable in neonatal KCs and only barely detectable in adult KCs (Figure 2b). Both IL-1α and flagellin induced the strong release of HBD-2 in fetal KCs ($1{,}237 \pm 349$and $850 \pm 256\,pg\,ml^{-1}$) (Figure 2b), but induction was much less pronounced in neonatal (193 ± 21 and $409 \pm 76\,pg\,ml^{-1}$) and adult (76 ± 57 and $243 \pm 73\,pg\,ml^{-1}$) KCs (Figure 2b).

Our mRNA results on S100A7-9 were corroborated by studying the production of S100 proteins. As shown in Figure 3, western blot analysis revealed strong baseline expression of S100A7, S100A8, and S100A9 in fetal proliferating KCs, whereas their expression was marginal in neonatal and adult KCs (Figure 3a). As mRNA expression of the S100 proteins was induced in differentiated KCs (Table 1), we also established SE cultures from KCs of different age groups and analyzed their protein production by western blot analysis (Figure 3b) and immunofluorescence staining (Figure 3c). As in monolayer cultures, the expression of all three S100 proteins was significantly higher in SEs established with fetal than with neonatal and adult KCs (Figure 3b). This observation was confirmed by immunostaining of SEs. All three S100 proteins were strongly present throughout all epidermal layers in SE cultures of fetal KCs. Whereas in SE cultures of neonatal and adult KCs only the expression pattern of S100A8 was comparable with the one in fetal SEs, S100A7 and S100A9 were only focally expressed (Figure 3c). Although cathelicidin mRNA was present in all KCs tested, we could not detect the protein expression of cathelicidin in monolayer cultures and SEs using ELISA (data not shown).

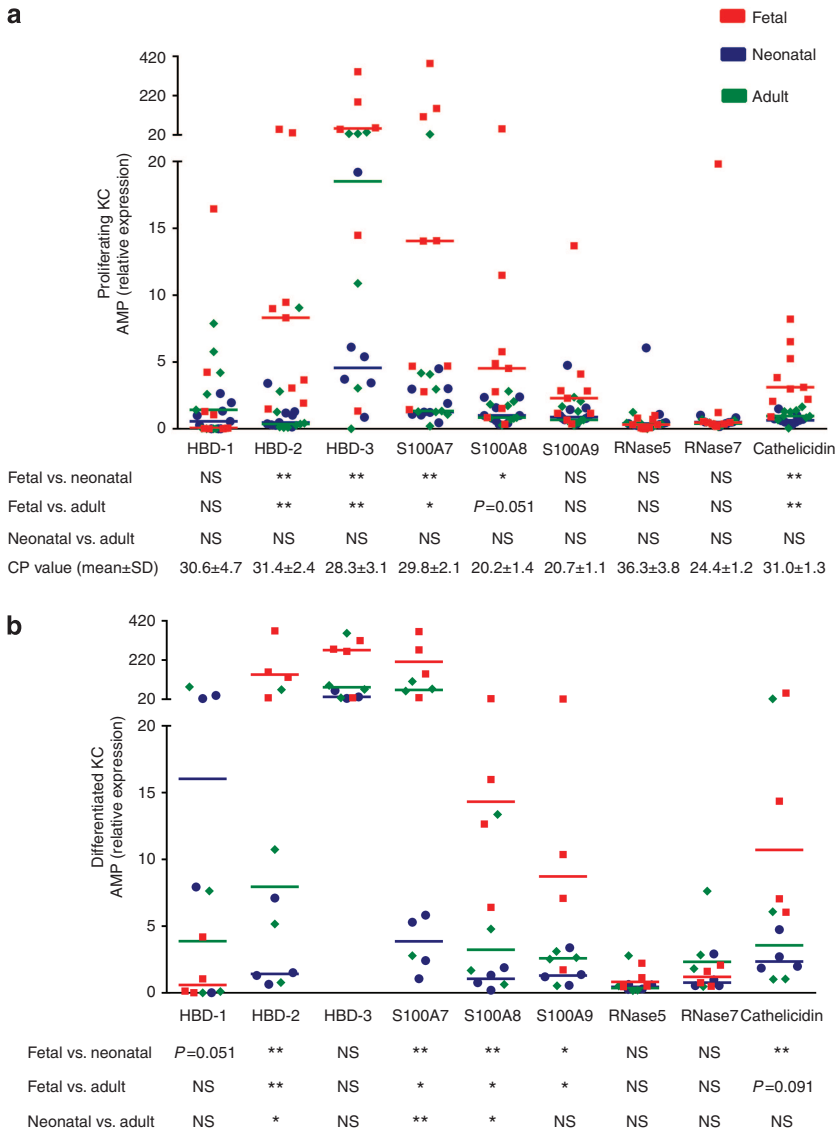

Figure 1. Fetal keratinocytes (KCs) have a different antimicrobial peptide (AMP) mRNA expression pattern when compared with neonatal and adult KCs. Fetal, neonatal, and adult KCs cultured under proliferating and differentiated conditions were investigated for AMP expression levels using quantitative real-time PCR. (**a**) Proliferating fetal KCs expressed significantly higher levels of human β-defensin-2 (HBD-2), HBD-3, S100A7, S100A8, and cathelicidin as compared with neonatal and adult KCs. (**b**) Differentiated fetal KCs expressed significantly higher levels of HBD-2, S100A7, S100A8, S100A9, and cathelicidin as compared with neonatal and adult KCs. The median and individual data points of (**a**) six to nine different donors from each age group and (**b**) four donors from each age group are shown. Each data point represents the mean of a measurement with three replicates; Mann–Whitney test, $*P<0.05$, $**P<0.005$; CP value: mean PCR crossing point of all proliferating KC samples (for comparison: the CP of the housekeeping gene β2-microglobulin (*β2M*) was 19.3 ± 1.1).

TLR activation differently regulates the expression of HBD-2, S100A7, and S100A8 mRNA in prenatal and postnatal KCs

To investigate the regulation of AMPs after TLR signaling in prenatal and postnatal KCs, proliferating cells were stimulated with the TLR ligands (TLRLs) 1–9 and analyzed by real-time PCR. A regulation was only observed for HBD-2, HBD-3, and the S100 proteins (Table 2, gray) but not for HBD-1, RNase5 and 7, as well as cathelicidin independent of KC source and TLRL (Table 2). S100A8 showed the strongest response to cytokine and TLR activation in fetal KCs, whereas HBD-2 and S100A7 were most potently induced in postnatal KCs. Regulation of S100A9 was comparable between prenatal and postnatal KCs. HBD-3 was only weakly induced in fetal and neonatal KCs after TLR-3 activation. IL-1α served as a positive control and upregulated HBD-2 and the S100 proteins in fetal and adult KCs, and slightly upregulated HBD-3 in fetal KCs. The TLRL poly(I:C) (TLR3L), flagellin (TLR5L), and FSL1 (TLR6L) most potently upregulated AMP expression (Table 2, highlighted in gray). However, the total amount of HBD-2 mRNA was still slightly higher and the total amount of S100A7 and S100A8 mRNA was more than 100-fold higher in fetal KCs compared with neonatal and adult KCs (Supplementary Figure S2 online).

Table 1. Induction of AMPs in differentiated KCs

Gene	Fet.	Neo.	Ad.
HBD-1	2	14*	7*
HBD-2	8*	5*	7*
HBD-3	2	5*	8*
S100A7	3*	2*	7*
S100A8	6*	1	6*
S100A9	5*	2*	4*
RNase5	2	1	3*
RNase7	2*	18*	7*
Cathelicidin	7*	5*	8*
JMJD3	5*	1	1

Abbreviations: Ad., adult; AMP, antimicrobial peptide; Fet., fetal; Neo., neonatal; HBD, human β-defensin; KC, keratinocyte.
Fold induction of AMPs in differentiated Fet., Neo., and Ad. KCs compared with proliferating KCs is shown. Statistically significant values are highlighted in gray. The mean fold induction was calculated from four independent donors of each age group, each performed in triplicate, and statistical significance was calculated with Wilcoxon's signed-rank test: $*P<0.05$.

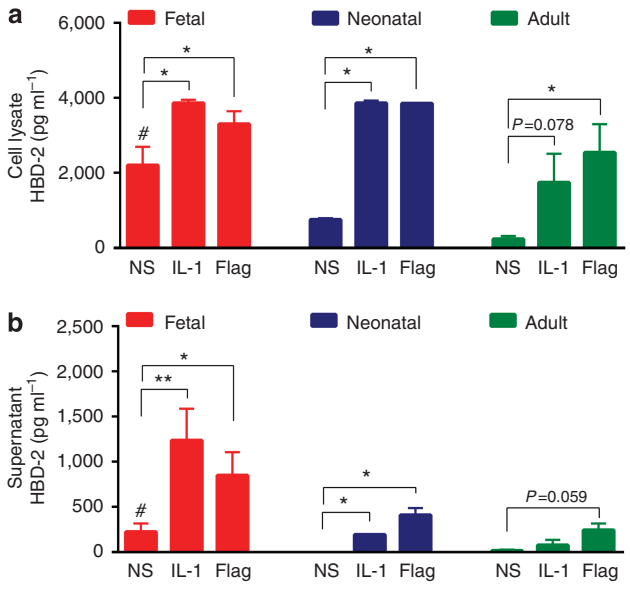

Figure 2. Human β-defensin-2 (HBD-2) expression and secretion is increased in fetal keratinocytes (KCs). Fetal, neonatal, and adult KCs were cultured for 24 hours in the presence or absence of IL-1α or the TLR5L flagellin, and the concentration of HBD-2 in (**a**) cell lysates and (**b**) supernatants was determined by ELISA. Basal HBD-2 levels (NS) were significantly higher in supernatants and cell lysates from fetal KCs as compared with neonatal and adult KCs ($^{\#}P<0.05$). Stimulation with IL-1α or TLR5L induced HBD-2 expression in all KC groups ($*P<0.05$, $**P<0.005$). The mean and SEM of two to six independent experiments for each group are depicted. Flag, flaggelin; NS, nonstimulated control; TLR, Toll-like receptor.

Expression of the histone demethylase JMJD3 influences the expression of HBD-3, S100A7, S100A8, S100A9, and cathelicidin

The histone demethylase JMJD3 and histone methyltransferase EZH2 are involved in gene regulation during KC differentiation by erasing and adding trimethyl groups to H3K27 present at diverse promoters including the S100A8 promoter (Sen *et al.*, 2008). We therefore investigated the expression of JMJD3 and EZH2 mRNA in fetal, neonatal, and adult KCs. We found that JMJD3 expression was significantly higher in proliferating fetal KCs as compared with both neonatal and adult KCs (Figure 4a). Moreover, KC differentiation in monolayer cultures additionally increased JMJD3 expression in fetal cells, whereas no significant increase was observed with neonatal and adult KCs (Figure 4b). In contrast, EZH2 expression was comparable in prenatal and postnatal KCs and was not regulated during KC differentiation in monolayer cultures (Figure 4c and d). Western blot analysis revealed that, in contrast to postnatal KCs, H3K27me3 (tri-methyl-lysine 27 on histone H3) was barely detectable in fetal KCs (Figure 4e). Spearman's correlation between JMJD3 and the AMPs tested in our study showed a high correlation for HBD-3 ($R=0.861$), S100A7 ($R=0.758$), S100A8 ($R=0.585$), and cathelicidin ($R=0.698$) (Supplementary Figure S3 online). To further investigate the contribution of JMJD3 to AMP expression, we next performed JMJD3 knockdown experiments using small interfering RNA (siRNA) technology. Knockdown of JMJD3 in fetal KCs led to significantly reduced expression levels of HBD-3, all S100 proteins tested, and cathelicidin (Figure 4f). In addition, H3K27me3 was increased in JMJD3 knockout cells (Figure 4g).

DISCUSSION

The innate component of the skin immune system is the first line of defense, protecting the body from invasion of microbial pathogens. This first line consists of the physical barrier of the stratum corneum and the presence or induction of AMPs produced by epithelial cells (Wiesner and Vilcinskas, 2010; Afshar and Gallo, 2013). Under physiological conditions, these mechanisms prevent microbial invasion on exposed body sites such as the skin but also colonization of sterile body sites such as the cerebrospinal fluid and the amniotic cavity by pathogenic microbes. Because of the fact that the adaptive immune system is not yet fully functional *in utero* (Chorro and Geissmann, 2010; Elbe-Bürger and Schuster, 2010; Ginhoux and Merad, 2010; PrabhuDas *et al.*, 2011; Palin *et al.*, 2013; Schuster *et al.*, 2012), the innate immune system, especially AMPs and pattern recognition receptors, has been suggested to have central roles for the survival of the developing fetus and the newborns (Dorschner *et al.*, 2003; Iram *et al.*, 2012). In the present study, we have investigated the expression and regulation of a variety of AMPs in KCs derived from fetal, neonatal, and adult skin.

The sterility of the amniotic cavity is thought to be established and maintained by the action of multiple physical and chemical barriers such as the vaginal (Mildner *et al.*, 2010b) and cervical epithelium (Svinarich *et al.*, 1997), the cervical mucus plug (Hein *et al.*, 2002), and the chorioamniotic membranes (Kjaergaard *et al.*, 2001). Although these epithelia and membranes represent a powerful defense against invading pathogens, some are still able to cross these barriers (Evaldson *et al.*, 1983; Galask *et al.*, 1984). Although

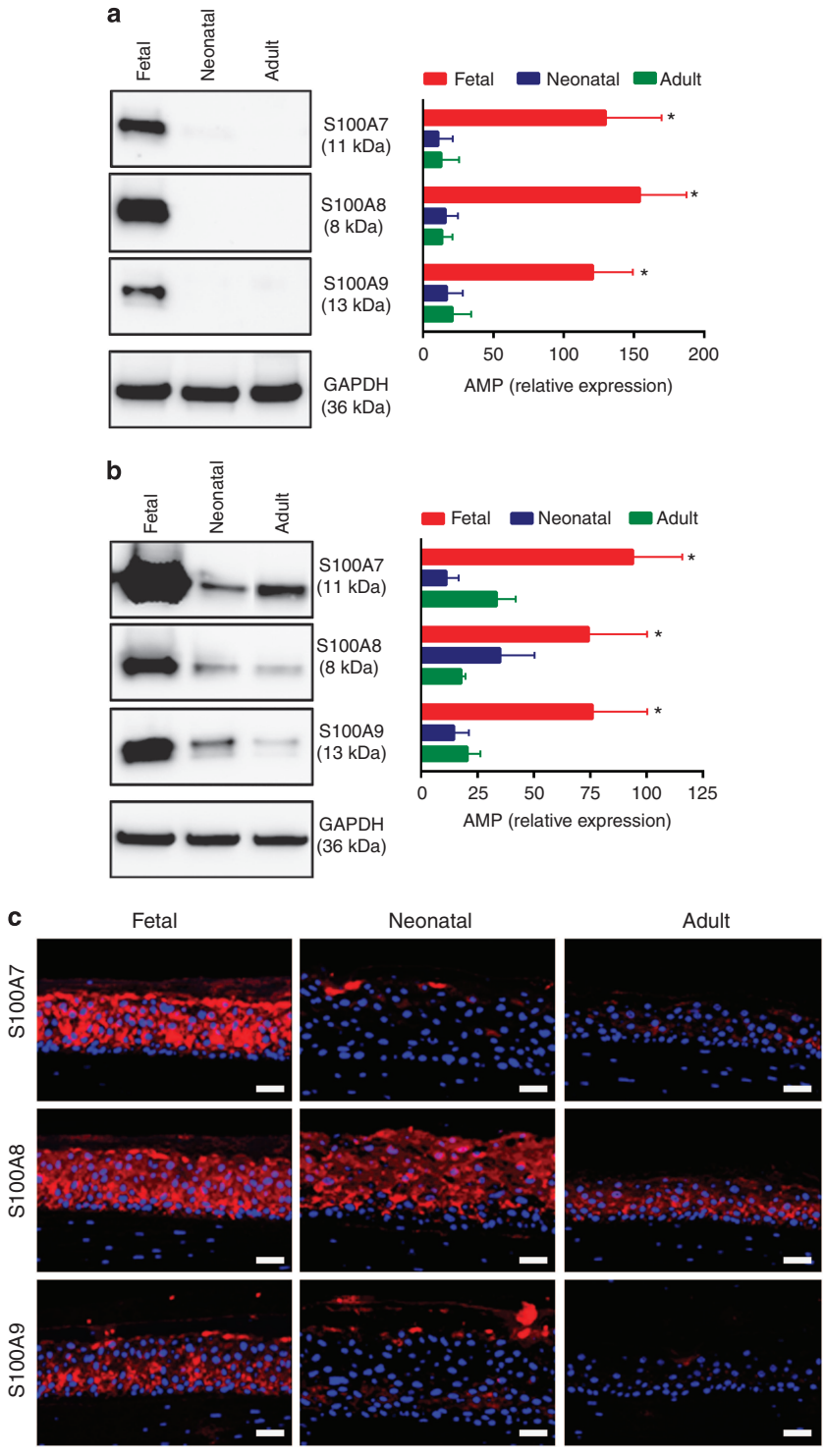

Figure 3. Fetal keratinocytes (KCs) express high levels of S100A7, S100A8, and S100A9 protein in monolayer and skin equivalent (SE) cultures. Fetal, neonatal, and adult KCs were cultured under proliferating conditions in monolayer cultures and analyzed for the expression of S100 proteins (**a**). Western blot analysis of KCs showed higher expression of S100 proteins in fetal KCs, as compared with neonatal and adult KCs. Western blots of representative samples and densitometric analysis of four to six independent experiments in each group are shown. *$P<0.05$ for fetal KCs compared with neonatal and adult KCs; SEs from fetal, neonatal, and adult KCs were established and analyzed by (**b**) western blot analysis and (**c**) immunfluorescence staining. Western blot analysis showed higher expression of S100 proteins in SEs from fetal KCs, as compared with neonatal and adult KCs. Western blots of representative samples and densitometric analysis of five to seven independent experiments in each group are shown. *$P<0.05$ for SEs from fetal KCs compared with neonatal and adult KCs. (**c**) Immunofluorescence staining of SE cultures showed higher expression of S100 proteins in SEs cultured from fetal KCs as compared with neonatal and adult KCs; one representative experiment out of three is shown. Bars = 100 μm. AMP, antimicrobial peptide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 2. Induction of AMPs in response to IL-1α and TLRL

Gene	IL-1			TLR1L (Pam3CSK4)			TLR2L (HKLM)			TLR3L (poly-I:C)			TLR4L (LPS)		
	Fet.	Neo.	Ad.	Fet.	Neo.	Ad.	Fet.	Neo.	Ad.	Fet.	Neo.	Ad.	Fet.	Neo.	Ad.
HBD-1	2	1	2	1	2	2	2	2	2	4	4	3	2	2	2
HBD-2	78*	25*	160*	4	3	14	3	3	3	38*	154*	227*	2	2	2
HBD-3	4*	2	1	1	2	1	4	1	1	4*	5*	1	1	1	1
S100A7	8*	8*	54*	3	3	3	4	3	3	4	12*	14*	2	2	2
S100A8	118*	4	8*	128*	3	3	40*	3	3	3	6*	7*	31*	2	2
S100A9	7*	3	6*	3	2	2	3	2	2	5*	7*	8*	2	2	2
RNase5	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2
RNase7	1	2	2	1	2	2	1	1	2	1	3	2	1	2	2
Cathelicidin	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2
JMJD3	1	1	1	1	1	1	2	1	1	3*	4*	2	1	1	1

Gene	TLR5L (flaggelin)			TLR6L (FSL1)			TLR7L (imiquimod)			TLR8L (ssRNA40)			TLR9L (ODN 2006)		
	Fet.	Neo.	Ad.	Fet.	Neo.	Ad.	Fet.	Neo.	Ad.	Fet.	Neo.	Ad.	Fet.	Neo.	Ad.
HBD-1	2	2	2	2	2	2	2	2	2	1	2	2	2	5	3
HBD-2	17*	24*	195*	19*	5*	42*	2	2	2	3	3	52*	2	2	2
HBD-3	1	1	1	3	2	1	1	1	1	1	1	1	ND	ND	ND
S100A7	4	9*	14*	6*	3	6*	2	2	2	2	2	3	1	2	3
S100A8	83*	4	6*	79*	3	3	52*	3	3	23*	3	3	7	5	5
S100A9	6*	4	6*	6*	3	6*	4	3	3	3	2	3	3	2	4
RNase5	2	2	2	6	1	2	6*	1	3	3	1	2	4	2	2
RNase7	1	2	2	1	2	2	1	2	3	1	2	3	1	2	2
Cathelicidin	2	2	2	2	2	2	3	2	2	2	2	2	1	2	3
JMJD3	1	1	1	2	1	1	2	1	1	2	1	1	1	1	1

Abbreviations: Ad., adult; AMP, antimicrobial peptide; Fet., fetal; HBD, human β-defensin; HKLM, heat-killed *Listeria monocytogenes*; KC, keratinocyte; LPS, lipopolysaccharide; Neo., neonatal; ND, not done; ODN, oligodeoxynucleotides; ssRNA, single-stranded RNA; TLRL, Toll-like receptor ligand.

Fold induction of AMPs in Fet., Neo., and Ad. KCs in response to stimulation with IL-1α or TLRLs 1–9 compared with nonstimulated controls is shown. Statistically significant values are highlighted in gray. The mean fold induction given in the table was calculated from five independent donors of each age group, each performed in triplicate, and statistical significance was calculated with Wilcoxon's signed-rank test: $*P<0.05$.

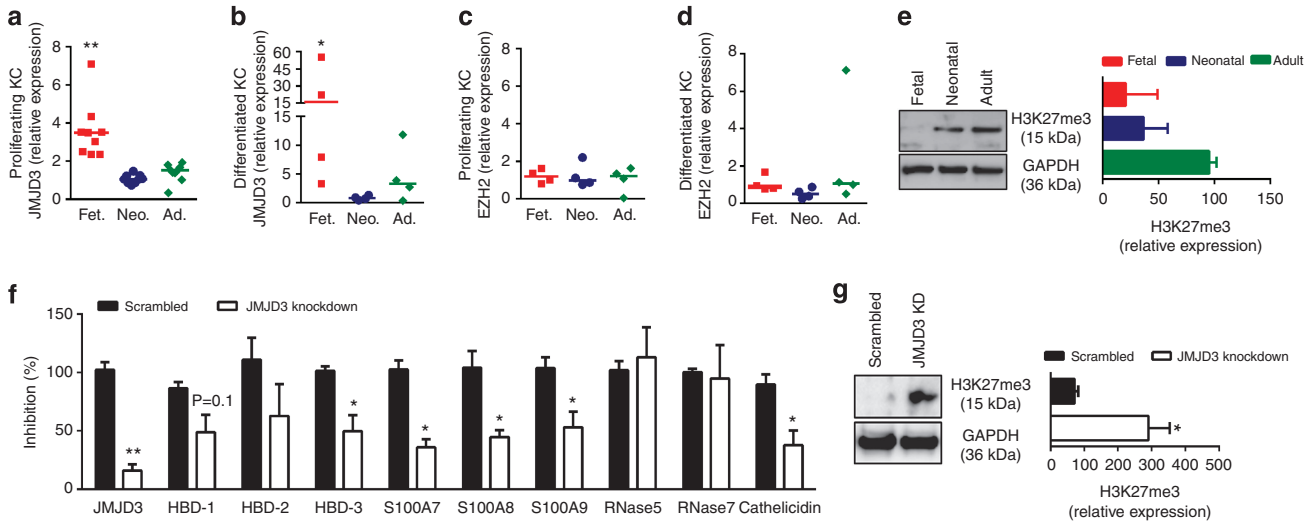

Figure 4. JMJD3 knockdown (KD) influences the expression of human β-defensin-3 (HBD-3), S100A7, S100A8 S100A9, and cathelicidin. Fetal, neonatal, and adult keratinocytes (KCs) cultured under (**a**, **c**) proliferating and (**b**, **d**) differentiated conditions were investigated for (**a**, **b**) JMJD3 and (**c**, **d**) EZH2 expression levels using quantitative real-time PCR. Fetal KCs were found to express significantly higher levels of JMJD3 as compared with neonatal and adult KCs (**a**, **b**; Mann–Whitney test, $*P<0.05$, $**P<0.005$). The median and individual data points of four to nine different donors per group are shown; each data point represents the mean of a measurement with three replicates. (**e**) Western blot analysis showed lower expression of H3K27me3 (tri-methyl-lysine 27 on histone H3) in fetal KCs, as compared with neonatal and adult KCs. Western blot of one representative experiment and densitometric analysis of two independent experiments are shown. (**f**) Small interfering RNA (siRNA)-mediated KD of JMJD3 in fetal KCs resulted in significantly lower expression of JMJD3, HBD-3, S100A7, S100A8, S100A9, and cathelicidin. The mean and SEM of two independent experiments with three different siRNAs each performed in triplicate are depicted; paired *t*-test, $*P<0.05$, $**P<0.005$. (**g**) Western blot analysis showed higher expression of H3K27me3 in JMJD3 KD cells. Western blot of one representative experiment and densitometric analysis of three independent experiments are shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

it is well established that amniotic fluids have strong antimicrobial activity and contain several AMPs including human neutrophil peptides 1–3, bactericidal/permeability-increasing protein, S100A7 (Porre *et al.*, 2005), and S100A8/A9 (Sampson *et al.*, 1997; Espinoza *et al.*, 2003), our knowledge of AMP expression in fetal skin is scarce. The facts that (i) KCs are a rich source of AMPs, (ii) S100A7 is primarily of epithelial origin, (iii) AMPs of the cathelicidin and β-defensin families are present in human newborn foreskin specimens (Dorschner *et al.*, 2003), and (iv) RNase7 is expressed in the periderm and prenatal epidermis (Schuster *et al.*, 2013) prompted us to investigate the contribution of fetal KCs to the innate immune defense of the fetus. We found that already under normal culture conditions fetal KCs produce significantly more HBD-2, HBD-3, S100A7-9, and cathelicidin than either neonatal or adult KCs. Our finding was surprising, as Schuster *et al.* (2013) did not find increased HBD-2 and S100A7 expression levels in fetal skin. Possible explanations for this discrepancy could be a different experimental design of the two groups. In contrast to our work, Schuster *et al.* (2013) investigated supernatants from cell suspensions derived from full-thickness skin samples (dermis and epidermis), thus probably having a dilution effect. In addition, they cultured their cells in a medium that was not favorable for the survival and growth of KCs. As to the differences seen in their *in vivo* staining, it is tempting to speculate that the aqueous environment in which the fetus is embedded together with the not yet fully established cornified layer of the epidermis favors a more rapid release of soluble factors, resulting in a weaker staining intensity. Also striking was the fact that fetal KCs constitutively express much higher levels of AMPs than neonatal KCs, suggesting that the elevated levels described in newborn skin specimens (Dorschner *et al.*, 2003) may be due to the exposure to the new environment. The fact that the high constitutive production of AMPs was maintained even after several passages under sterile conditions and in the presence of antibiotics excludes the possibility that bacterial contamination accounts for activation of fetal KCs in our cultures. It rather indicates that AMP production is due to cell-autonomous mechanisms that differ in the cells of different age. Indeed, when we transferred cell culture supernatants from fetal and adult KCs onto fresh cultures of adult KCs, we could not detect any changes in AMP expression levels (Supplementary Figure S4 online). This finding is in line with our previously published secretome analysis of prenatal and postnatal KCs (Iram *et al.*, 2012), where we could not detect any differences in the baseline expression levels of a variety of cytokines and chemokines, suggesting that AMPs are not indirectly enhanced in fetal KCs via the production and release of other soluble factors. Interestingly, the capacity to further increase AMP production after stimulation with inflammatory triggers was largely comparable between prenatal and postnatal KCs. Our *in vitro* data of postnatal KCs are in line with previously published *in vivo data*, showing that baseline expression of most AMPs is quite low or even absent in the epidermis, but can be strongly

upregulated after pathogen contact or inflammation (Kim *et al.*, 2006; Abtin *et al.*, 2008; Gläser *et al.*, 2009). The high baseline production and upregulation of AMPs in response to inflammatory stimuli in fetal KCs suggest an important defense strategy of the skin before birth.

We further present evidence for a methylation-dependent regulation of at least some AMPs during skin development. The histone demethylase JMJD3 has been shown to be involved in epidermal development and antimicrobial defense, as its expression is required for the transcription of the KC differentiation locus and the *S100A8* gene (Sen *et al.*, 2008). Transient histone methylation changes that are strongly tissue- and developmental stage–specific are constantly ongoing in the fetus, leading to the regulation of multiple genes (Yuen *et al.*, 2011). Here we could demonstrate that proliferating and differentiated fetal KCs express significantly higher JMJD3 mRNA levels than neonatal and adult KCs. We further identified significant correlations between the expression of JMJD3 and the AMPs HBD-3, S100A7, S100A8, and cathelicidin. Moreover, we found that the trimethylated form of H3K27 was indeed less abundant in fetal KCs as compared with neonatal and adult KCs. siRNA-mediated knockdown of JMJD3 confirmed our assumption that histone methylation is involved in the regulation of AMPs in fetal KCs. These results show that in addition to S100A8 also other AMPs are regulated by JMJD3. Whether this is a direct regulation by demethylation of H3K27m3 in their promoter regions, as previously shown for S100A8 (Sen *et al.*, 2008), or indirect via other mechanisms is currently not known. Our observation of high JMJD3 and low H3K27me3 levels in fetal KCs are in line with a study describing a similar phenomenon in healing epidermis (Shaw and Martin, 2009).

In summary, we have shown a quantitative comparison of several AMPs in prenatal and postnatal KCs. The data presented expand our understanding of the complex mechanisms involved in maintaining the sterility of the amniotic cavity, and show that fetal KCs possess a high capacity to produce AMPs, thereby contributing to innate immune responses of the developing organism.

MATERIALS AND METHODS

Cell culture

KCs prepared from fetal skin (20–23 weeks of estimated gestational age) were purchased from CellSystems (Troisdorf, Germany) and Tebu-bio (Offenbach, Germany). KCs prepared from neonatal foreskin and adult skin were purchased from Lonza (Basel, Switzerland). All KCs were cultured in serum-free KC growth medium (KGM; Lonza), as described previously (Mildner *et al.*, 2006). For stimulation, third-passage KCs were cultured in 12-well tissue culture plates (Corning, Corning, NY) and used at a confluence of 60–70%. Stimulation was performed in KC basal medium (Lonza). For the analysis of differentiated KCs, cells were kept in culture for 4 days after reaching 100% confluence.

Reagents used for the treatment of KCs

For *in vitro* assays, 10 ng ml^{-1} recombinant IL-1α (R&D Systems, Minneapolis, MN) and the following TLRLs (InvivoGen, San Diego, CA) were used: 1 μg ml^{-1} synthetic tripalmitoylated lipopeptide (Pam3CSK4; TLR1/2L), 10^8 cells per ml heat-killed *Listeria monocytogenes* (TLR2L), 25 μg ml^{-1} poly(I:C), a synthetic analog of double-stranded RNA (TLR3L), 100 ng ml^{-1} ultrapure lipopolysaccharide from *Escherichia coli* K12 (TLR4L), 500 ng ml^{-1} purified flagellin from *Salmonella typhimurium* (TLR5L), 500 ng ml^{-1} synthetic lipoprotein of *Mycoplasma salivarium* (FSL1; TLR6/2L), 10 μg ml^{-1} imiquimod (R837; TLR7L), 1 μg ml^{-1} single-stranded RNA40 (TLR8L), and 2.5 μM ml^{-1} ODN2006 (CpG oligonucleotide type B; (TLR9L)). For certain experiments, supernatants from fetal and adult KCs were collected after 24 hours of culture, mixed with the same volume of fresh medium, and transferred onto confluent adult KCs. After 24 hours, mRNA was prepared and RT-PCR was performed as described below.

siRNA-mediated JMJD3 knockdown

Third-passage fetal KCs (50–60% confluence) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to a published protocol (Mildner *et al.*, 2010a). A measure of 1 ml of OPTI-MEM medium (Invitrogen) was mixed with 10 μl of Lipofectamine 2000 and 20 μl of a 20 μM siRNA (Invitrogen) solution or the scrambled control RNA (Invitrogen) solution. Three different JMJD3 siRNAs were used (siRNA ID: HSS146206, HSS146208, and HSS177200). After incubation at room temperature for 30 minutes, the solution was added to 20 ml of KGM (Lonza) and transferred to the KCs. KCs were then incubated for 48 hours before mRNA was isolated.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

After stimulation for 16 hours, KCs were washed with phosphate-buffered saline (PBS), and RNA was isolated using the RNeasy 96 Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cDNA synthesis, RNA was reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), and real-time PCR was carried out with LightCycler480 SYBR Green I Master (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. The primers used are listed in Supplementary Table S1 online. The relative expression of the target genes was calculated by comparing with the housekeeping gene *β2-microglobulin* or *keratin 14* (for stimulation experiments in Table 2, as the expression of *β2-microglobulin* was modulated by TLR3 stimulation) using a formula described previously (Pfaffl, 2001). All real-time PCR experiments were performed at least in triplicate, and the specificity of the reactions was confirmed by sequencing of the PCR products.

Preparation of 3D SE cultures

In vitro 3D SEs were generated as described previously (Mildner *et al.*, 2010a). Briefly, a suspension of collagen type I (PureCol; Advanced Biomatrix, San Diego, CA) containing 1×10^5 fibroblasts per ml was poured into cell-culture inserts (3 μm pore size; BD Bioscience, Bedford, MA) and allowed to gel for 2 hours at 37 °C. After equilibration with KGM for 2 hours, 1.5×10^6 KCs, in a total volume of 2 ml of KGM, were placed on the collagen gel. After overnight incubation, the medium was removed from both the inserts and external wells and replaced in the external wells only by serum-free KC-defined medium consisting of KGM but without bovine pituitary extract and supplemented with 1.3 mM calcium, 10 μg ml^{-1} transferrin, 50 μg ml^{-1} ascorbic acid, and 0.1% bovine serum albumin (all Supplements from Sigma-Aldrich, Vienna, Austria). Serum-free KC-defined medium was changed every second day for 7 days.

Western blot analysis

For the analysis of protein expression, monolayer cultured KCs or epidermis taken from SE cultures were lysed in a buffer containing 50 mM Tris (pH 7.4) and 2% SDS. After sonication, insoluble cell debris was removed by centrifugation, and the protein concentration was measured by BCA assay (Pierce, Rockford, IL). After denaturing with 0.1 M DL-dithiothreitol (Sigma-Aldrich), SDS-PAGE was conducted on 8–18% gradient gels (GE Amersham Pharmacia Biotech, Uppsala, Switzerland). The proteins were then electrotransferred onto nitrocellulose membranes (Bio-Rad) and immunodetected with the following primary antibodies: mouse monoclonal anti-S100A7 (5 $\mu g\,ml^{-1}$; clone 47C1068; Abcam, Cambridge, UK), mouse monoclonal anti-S100A8 (2 $\mu g\,ml^{-1}$; clone S13.67; Acris, Hiddenhausen, Germany), mouse monoclonal anti-S100A9 (1 $\mu g\,ml^{-1}$; clone 47-8D3; Abcam), anti-H3K27me3 (4 $\mu g\,ml^{-1}$; clone mAbcam 6002m; Abcam), and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (dilution 1:2,000; Biogenesis, Poole, UK). Secondary antibody was sheep anti-mouse horseradish peroxidase (1:10,000; Amersham, Buckinghamshire, UK) and the reaction products were detected by chemiluminescence with the ImmunStar Western C Substrate kit (Bio-Rad) according to the manufacturer's instructions. The band intensity of four to seven independent experiments was quantified by densitometric analysis using the ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence labeling

Five-µm-thin sections of formalin-fixed, paraffin-embedded SEs were stained by immunofluorescence labeling, as described previously (Mildner *et al.*, 2010a). Briefly, fixed sections were deparaffinized, rehydrated, and washed in PBS (2×5 minutes), incubated with 10% goat serum at room temperature for 30 minutes, and then with primary antibodies overnight at 4 °C (the same antibodies as described for western blotting were used). Sections were then washed in PBS (2×5 minutes) and incubated with goat anti-rabbit Alexa Fluor 488 (Alexa, Eugene, OR). Slides were washed in PBS (2×5 minutes) and counterstained with Hoechst to visualize nuclei. Images were recorded using the AX70 microscope with the imaging software MetaMorph from Olympus (Hamburg, Germany).

ELISA

After 24 hours of stimulation of KCs, cell culture supernatants were harvested and KCs were washed with PBS and lysed with 0.1% IGEPAL lysis buffer at 4 °C for 15 minutes. Supernatants and cell lysates were stored at −20 °C until further analyses. Concentrations of HBD-2 in the supernatant and in the cell lysate were determined by ELISA Development Kit (Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's instructions. Optical density at 450 nm was measured using a microplate reader (Fluostar Optima; BMG Labtech, Offenburg, Germany).

Statistical analysis

Statistical analysis was performed using the program GraphPad Prism version 5 (GraphPad Software, San Diego, CA), and calculations are based on the means of independent experiments (each individual experiment with three to four replicates; Figures 1, 2, and 4a, and Tables 1 and 2) or on the individual values of all experiments (Figures 3a, b and 4). Data are represented as individual values (mean of three to four replicates per experiment) and the median (Figure 1 and Figure 4a–d) or as mean and SEM (Figures 2–4f and Supplementary Figures S1 and S2 online). Statistical significance was calculated using the Mann–Whitney test (unpaired, nonparametric data; Figures 1 and 4a, b), Wilcoxon's signed-rank test (paired, nonparametric data, Tables 1 and 2), unpaired *t*-test (Figure 2 (comparison of nonstimulated controls) and Figure 3), or paired *t*-test (Figure 2 (comparison of stimulations) and Figure 4f). The correlation coefficient *R* was calculated by Spearman's correlation test (nonparametric data; Supplementary Figure S3 online). A *P*-value below 0.05 was regarded as significant and is depicted with *, and a *P*-value below 0.005 is depicted with **.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are very grateful to Heidemarie Rossiter and Erwin Tschachler for critical reading of the manuscript and helpful discussions. This project was funded in part by Austrian Science Fund grants (P19474-B13 to A E-B and T545-B19 to MG).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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