Distinct Strains of *Propionibacterium acnes* Induce Selective Human β-Defensin-2 and Interleukin-8 Expression in Human Keratinocytes Through Toll-Like Receptors

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Acne is a chronic inflammatory disease of the pilosebaceous follicle. One of the main pathogenetic factors in acne is the increased proliferation of *Propionibacterium acnes* (*P. acnes*) in the pilosebaceous unit. We investigated whether direct interaction of *P. acnes* with keratinocytes might be involved in the inflammation and ductal hypercornification in acne. The capacities of different *P. acnes* strains to activate the innate immune response and the growth of cultured keratinocytes were investigated. We have found that two clinical isolates of *P. acnes* significantly induced human β-defensin-2 (hBD2) messenger RNA (mRNA) expression; in contrast a third clinical isolate and the reference strain (ATCC11828) had no effect on hBD2 mRNA expression. In contrast, all four strains significantly induced the interleukin-8 (IL-8) mRNA expression. The *P. acnes*-induced increase in hBD2 and IL-8 gene expression could be inhibited by anti-Toll-like receptor 2 (TLR2) and anti-TLR4 neutralizing antibodies, suggesting that *P. acnes*-induced secretion of soluble factors in keratinocytes are both TLR2 and TLR4 dependent. In addition, the clinical isolate *P. acnes* (889) was capable of inducing keratinocyte cell growth *in vitro*. Our findings suggest that *P. acnes* modulates the antimicrobial peptide and chemokine expression of keratinocytes and thereby contributes to the recruitment of inflammatory cells to the sites of infections.

Key words: human β-defensin-2/IL-8/keratinocyte/*Propionibacterium acnes*/Toll-like receptors
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Skin is the first barrier that invading microbes have to pass when entering the organism. The key cellular components of the pathophysiologic processes of the skin are the keratinocytes, cells that are in a unique position between the interface of the environment and the host organism. Through the secretion of soluble factors such as cytokines and antimicrobial peptides, keratinocytes are able to maintain the immune response of the skin. This regulation of the defense mechanisms is the key feature in the protection of the host as normal human skin is colonized with a wide variety of microorganisms such as *Propionibacterium acnes* (*P. acnes*), *Staphylococcus epidermidis*, and *Malessezia furfur*. In addition to normal flora, the skin is constantly challenged by pathogens, most of which do not cause clinical disease.

P. acnes is a Gram-positive anaerobe bacteria found in normal human cutaneous flora and is thought to play a major role in acne, a chronic inflammatory disease of the pilosebaceous unit (Koreck *et al*, 2003). It is proposed that hypercornification of the outer root sheath and the pilosebaceous duct, increased sebum production, abnormalities of the normal microbial flora, and inflammation are the major factors of the pathogenesis of acne (Toyoda and Morohashi, 2001). Although several lines of evidence suggest the direct role of *P. acnes* in acne (Leyden *et al*, 1998), little is known about the mechanism by which *P. acnes* contributes to the pathogenesis of acne. Kim *et al* (2002) have shown that in acne lesions, Toll-like receptor (TLR)2-expressing macrophages are surrounding the pilosebaceous follicles and that *P. acnes* induced cytokine production of monocytes via TLR2. The importance of *P. acnes* is further suggested by the observation that the presence of antibiotic-resistant strains of *P. acnes* may be associated with therapeutic failure (Zouboulis and Piquero-Martin, 2003; Oprica *et al*, 2004). Additionally, after complete sequencing of the *P. acnes* genome, Bruggemann *et al* (2004) identified surface-associated and immunogenic factors that might be involved in acne formation. As *P. acnes* is a resident of human cutaneous flora and more than 80% of the human population denotes acne in some stage of life; we hypothesized that the contribution of *P. acnes* in acne pathogenesis is isolate specific.

The human skin expresses a number of proteins that play a part in host defense. Antimicrobial peptides of the β-defensin family are small, cationic proteins produced in response to microbial infections and are expressed in all human epithelial tissues tested so far. To date, three human β-defensins (hBD) are shown to play a role in the defense of

Abbreviations: hBD2, human β-defensin-2; IL-8, interleukin-8; *P. acnes*, *Propionibacterium acnes*; PBS, phosphate-buffered saline; PGN, peptidoglycan; QRT-PCR, quantitative RT-PCR; RAPD, random amplification of polymorphic DNA; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α

the skin, namely human β-defensin-1, -2, and -3 (hBD1, hBD2, and hBD3). These defensins are expressed either constitutively or upregulated in response to microbial or pro-inflammatory stimuli (Raj and Dentino, 2002). Besides destroying various microorganisms, hBD2 contributes to the regulation of host-adaptive immunity as it is chemotactic for immature dendritic cells, induces the migration of CD45RO memory T cells (Yang *et al*, 1999), and also acts as a chemoattractant for neutrophils through CCR6 (Niyonsaba *et al*, 2004). Its murine homologue, mBD2, is an endogenous ligand of TLR4 (Biragyn *et al*, 2002).

TLRs play a crucial role in the induction of antimicrobial responses in different cells (O'Neill, 2004; Takeda and Akira, 2004). Seven of 11 human TLRs, TLR1–6, and TLR9 are known to have an important role in sensing microbial ligand-derived infections in keratinocytes (Mempel *et al*, 2003; Pivarcsi *et al*, 2003, 2004). In our previous study (Pivarcsi *et al*, 2003), we have shown that TLR2 and TLR4 expressed on keratinocytes are primarily responsible for sensing peptidoglycan (PGN) and lipopolysaccharide (LPS), respectively.

As diverse defense responses may influence the clinical course of infection, characterization of the host's antimicrobial peptide and cytokine production in response to *P. acnes* could lead to a better understanding of acne and development of more specific therapies. In this study, we analyzed the messenger RNA (mRNA) and protein expression of hBD2 and interleukin (IL)-8 in human keratinocytes exposed to different strains of *P. acnes*. The roles of TLR2 and TLR4 in sensing *P. acnes* as well as the effects of *P. acnes* strains on the viability of cultured keratinocytes were examined.

Results

Selective upregulation of hBD2 mRNA in response to *P. acnes* treatment in cultured keratinocytes To analyze the effect of different strains of *P. acnes* on hBD2 mRNA levels by quantitative RT-PCR (QRT-PCR), keratinocytes were treated with or without the bacterial strains for 3 and 24 h.

The hBD2 proved to be differentially inducible in a time-dependent manner when keratinocytes were treated with different strains of *P. acnes* (Fig 1*a*). Three hours after the treatment with two clinically isolated strains (889 and 2005), the increase in hBD2 mRNA level was 5-fold higher compared with 0 h ($p < 0.025$), whereas the other two strains (6609 and ATCC11828) did not modify hBD2 expression significantly. Twenty-four hours after the treatment, the hBD2 mRNA levels were back to the control level (Fig 1*a*).

hBD2 protein expression in cultured keratinocytes in response to *P. acnes* (889) treatment Based on QRT-PCR results, the clinically isolated *P. acnes* strain 889 was used in immunostaining experiments for the detection of hBD2 protein expression by keratinocytes in response to *P. acnes* treatment. The expression of hBD2 protein was not detectable at the beginning of the stimulation (0 h) and it was first detectable 6 h after the treatment with *P. acnes* (889) (data not shown). The treatment for prolonged time (12 and 24 h) elevated the number of cells that expressed hBD2 protein (Fig 2); however, not all of the cells expressed hBD2 protein.

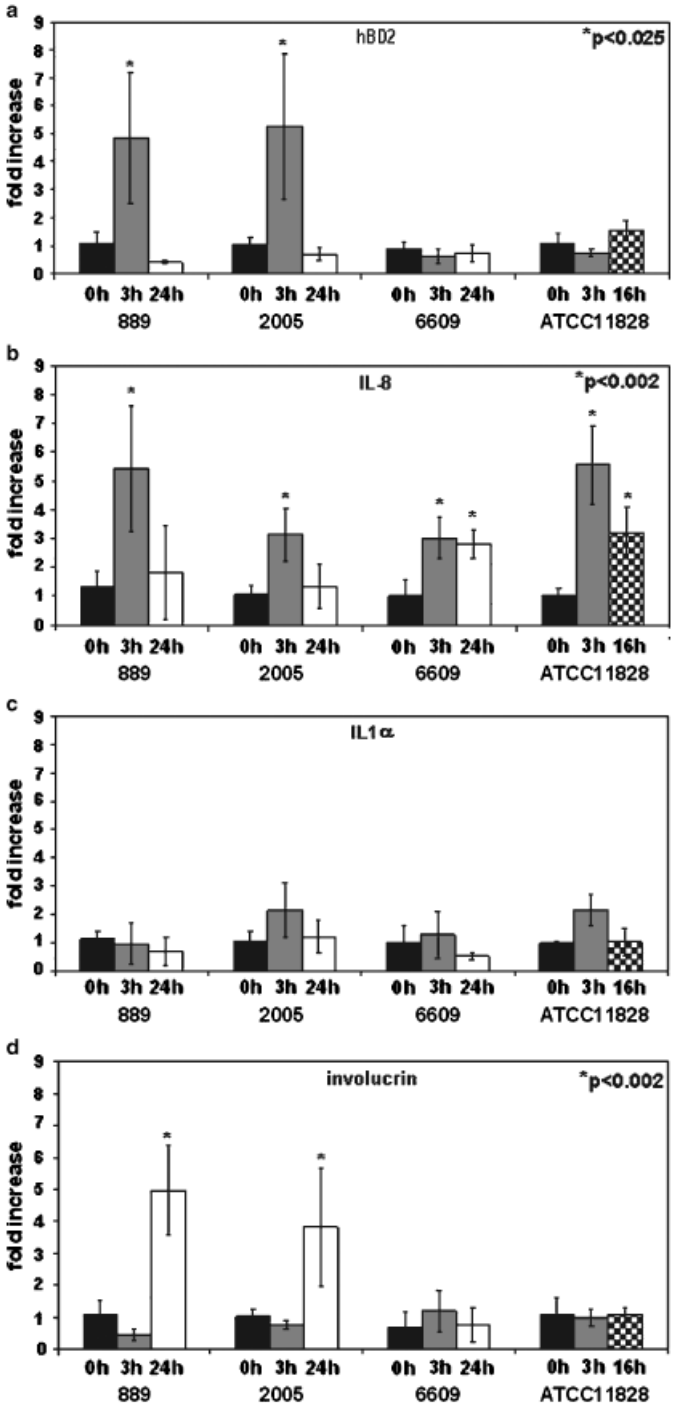

Figure 1
The induction of different genes in keratinocytes by *Propionibacterium acnes* is strain-specific. Temporal analysis of gene expression of human β-defensin-2 (*a*), interleukin (IL)-8 (*b*), IL-1α (*c*), and involucrin (*d*) in cultured keratinocytes infected with four different strains of *P. acnes* using quantitative RT-PCR. The levels of mRNA were normalized to the 18S ribosomal RNA (rRNA) level, and are presented as fold increases over the 0 h values.

Untreated keratinocytes used as controls did not show reactivity with the anti-hBD2 serum.

The effect of *P. acnes* treatment on cytokine production of cultured keratinocytes Changes in IL-8 and IL-1α mRNA levels were analyzed by QRT-PCR. All four strains

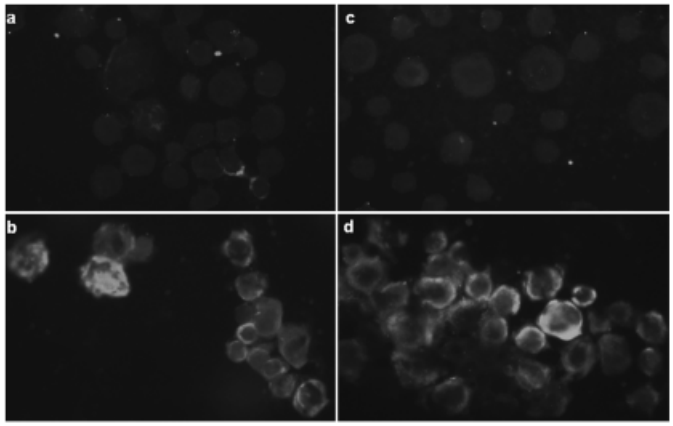

Figure 2
The expression of human β-defensin-2 (hBD2) protein in cultured keratinocytes is upregulated in response to *Propionibacterium acnes* (889) treatment as determined by indirect immunofluorescence staining. Keratinocytes were treated with *P. acnes* clinical isolate 889 and incubated with rabbit anti-hBD2 antibody; hBD2 staining was visualized with fluorescein isothiocyanate. Untreated keratinocytes lack the expression of hBD2 protein (*a* and *c*). Stimulation with *P. acnes* clinical isolate 889 induced the expression of the hBD2 protein 12 h post-treatment (*b*) and an intense staining was observed 24 h post-treatment (*d*), suggesting a high level of hBD2 expression. Magnifications × 40.

significantly ($p < 0.002$) increased IL-8 gene expression 3 h after the treatment (Fig 1*b*). At 24 and 16 h post-treatment, the increase in IL-8 gene expression in response to clinical isolate 6609 and reference strain ATCC11828 was still 3-fold ($p < 0.002$), respectively (Fig 1*b*). Expression of IL-8 gene was not changed significantly by treatment with clinical isolates 889 and 2005 for 24 h (Fig 1*b*).

No change in the IL-1α gene expression could be detected (Fig 1*c*).

Next, we investigated the effect of *P. acnes* on the IL-8 protein secretion of keratinocytes. As the QRT-PCR results for the expression of hBD2 mRNA revealed two patterns of keratinocyte response to *P. acnes* treatment, we show the effect of two strains of *P. acnes* (clinical isolate 889 that elevated hBD2 expression and reference strain ATCC11828 that did not change hBD2 mRNA expression) on the IL-8 protein secretion of cultured keratinocytes. Cells were treated with the two selected *P. acnes* strains and samples were taken at the same time points post-treatment as for the IL-8 mRNA expression analysis. The amount of secreted IL-8 protein in the cell culture supernatants was measured by ELISA. We found that both strains significantly ($p < 0.023$) increased the IL-8 protein secretion of keratinocytes at both time points (Fig 3).

Selective upregulation of involucrin mRNA in response to *P. acnes* treatment in cultured keratinocytes To determine the effect of distinct strains of *P. acnes* on involucrin gene expression, keratinocytes were treated with or without the bacterial strains for 3 and 24 h and the mRNA level of involucrin was determined by QRT-PCR. Involucrin proved to be differentially inducible in a time-dependent manner when keratinocytes were treated with different strains of *P. acnes* (Fig 1*d*). No effect was detected 3 h after the treatment; however, 24 h post-treatment, two clinically isolated

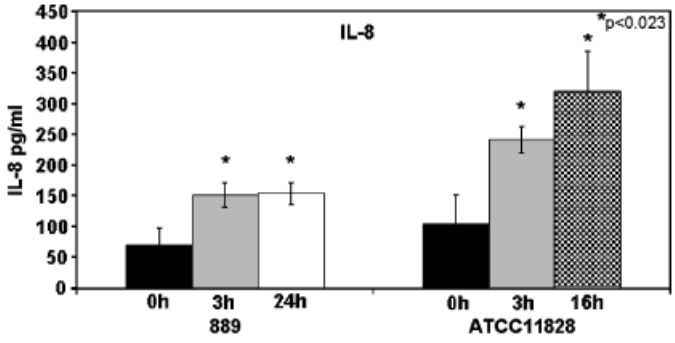

Figure 3
***Propionibacterium acnes* induces the secretion of interleukin (IL)-8 protein in keratinocytes.** Cultured keratinocytes were treated with two strains of *P. acnes* (clinical isolate 889 and reference strain ATCC11828) and the amount of IL-8 in the supernatant medium was measured by Quantikine human IL-8 ELISA.

strains (889 and 2005) significantly ($p < 0.002$) increased the involucrin mRNA levels (Fig 1*d*). The other two strains (6609 and ATCC11828) did not modify involucrin gene expression at all (Fig 1*d*).

***P. acnes* (889)-induced upregulation of hBD2 and IL-8 mRNA expression is both TLR2 and TLR4 dependent in cultured keratinocytes** We tested whether TLR2 or TLR4 is involved in the recognition of *P. acnes* (889) by keratinocytes. Similar to other Gram-positive bacteria, the major cell-wall component of *P. acnes* is PGN. The treatment with *P. acnes* (889) resulted in the upregulation of hBD2 and IL-8 mRNA expression in keratinocytes 3 h after the stimulation (Fig 1*a* and 1*b*). But, when cultured keratinocytes were pre-incubated either with anti-TLR2 monoclonal antibody (mAb) (10 μg per mL) or anti-TLR4 mAb (10 μg per mL) for 1 h before *P. acnes* (889) treatment, the upregulation of both hBD2 and IL-8 mRNA expression was abrogated (Fig 4). We have previously shown that keratinocytes recognize PGN

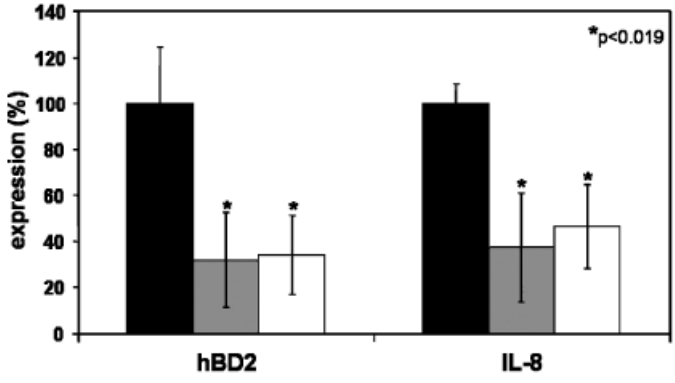

Figure 4
Blocking of Toll-like receptor (TLR)2 and TLR4 inhibits the *Propionibacterium acnes* (889)-induced expression of human β-defensin-2 (hBD2) and interleukin (IL)-8 in keratinocytes as determined with quantitative RT-PCR. Cultured keratinocytes were grown on six-well plates and pre-treated with anti-TLR2 (*gray bars*), anti-TLR4 (*white bars*), or isotype-matched control mouse immunoglobulin G (IgG) antibodies (*black bars*) for 1 h and then stimulated with *P. acnes* clinical isolate 889 for 3 h. The expression values are expressed relative to the values measured in the mIgG-treated controls. 18S ribosomal RNA was used as internal control.

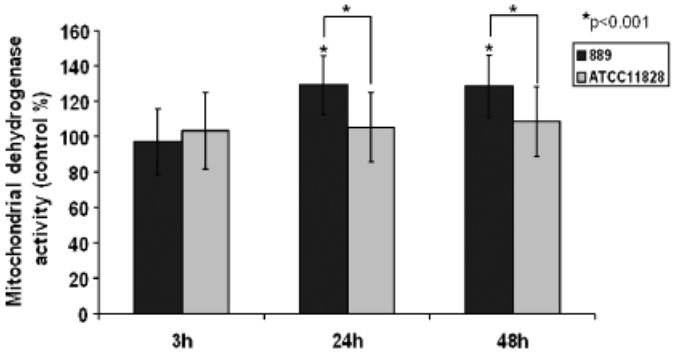

Figure 5
Selective effect of *Propionibacterium acnes* strains on the viability of keratinocytes. Cultured keratinocytes were treated with two strains of *P. acnes* (clinical isolate 889 and reference strain ATCC11828) for different times. The viability of cells was determined by 3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide assay.

through TLR2 (Pivarcsi *et al*, 2003); therefore, we used PGN as an internal control (data not shown).

We next investigated the effect of TLR2 and TLR4 blocking prior to *P. acnes* (889) treatment, and the levels of secreted IL-8 protein were determined 3 h post-treatment. As shown in Fig S1, the blocking of TLR2 significantly ($p < 0.004$) suppressed the IL-8 protein secretion of keratinocytes, whereas blocking of TLR4 had no effect on IL-8 protein secretion.

Different effects of *P. acnes* strains on the viability of cultured keratinocytes The effect of two previously selected *P. acnes* strains (clinical isolate 889 and reference strain ATCC11828) on the viability of cultured keratinocytes was examined by 3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assays. Neither of the *P. acnes* strains had any effect on the viability of keratinocytes 3 h post-treatment (Fig 5). In addition, the reference strain had no effect at any other time points (Fig 5). Still the clinical isolate significantly ($p < 0.001$) increased the number of viable cells compared with both control and *P. acnes* reference strain-treated keratinocytes 24 h post-treatment, and this effect was still detectable 48 h post-treatment (Fig 5).

Bactericidal activity of hBD2 on *P. acnes* strains As the expression of hBD2 is upregulated in response to some *P. acnes* strains we aimed to investigate the bactericidal activity of the protein against all four strains of the bacterium included in our study. Recombinant hBD2, in concentrations up to 2.5 μg per mL, had no effect on the viability of any of the tested four *P. acnes* strains (data not shown). These data are in good agreement with previous findings where hBD2 was active against Gram-negative bacteria (e.g., *Escherichia coli* and *Pseudomonas aeruginosa*) but it demonstrated only low, if any, microbicidal activity against Gram-positive bacteria (e.g. *Staphylococcus aureus*) (Harder *et al*, 1997; Schroder, 1999).

Random amplification of polymorphic DNA (RAPD) molecular typing of *P. acnes* strains As the treatment with the distinct strains of *P. acnes* gave different results, we have determined whether this is because of the variable genotypic characteristics of the strains. As shown in Fig S2, the RAPD profile is different for the four *P. acnes* strains, containing four to nine bands in the 100–1200 bp range. The RAPD profiles of the clinical isolates 889, 2005, and 6609 are comparable with the profile of a serotype I biotype 3 strain ATCC6919 (Johnson and Cummins, 1972; Perry *et al*, 2003). Moreover, the genotypic profiles of the clinical isolates 889 and 2005 are very similar to each other, both lacking the 350 bp band. The clinical isolate 6609 and the reference strain have six and nine bands, respectively. It is important to note that both of these strains contain the 350 bp band.

Discussion

P. acnes secretes various biologically active molecules like enzymes and chemotactic factors, which play a role in the initiation and perpetuation of the local inflammatory response (Vowls *et al*, 1995). Recently, Mouser *et al* (2003) have reported that *P. acnes* antigens triggers increased proliferative response in a subpopulation of T helper 1 cells generated from early inflamed acne lesions. Our results suggest that in addition to mononuclear cells, keratinocytes also recognize and respond to *P. acnes*.

Antimicrobial peptides produced in the skin play an important role in the elimination of the invading microorganisms (Gallo and Huttner, 1998; Bardan *et al*, 2004). β-defensins are antimicrobial peptides that protect the epithelia from microbial infection and colonization. hBD2 is one of the most important host defense molecules denoted by its inducibility with various pro-inflammatory agents such as tumor necrosis factor-α (TNF-α), LPS, bacteria, and yeast (Ali *et al*, 2001). This inducibility of hBD2 seems to be the key feature in the immune response of the host. Although our knowledge about the direct role of hBD2 in the innate immune response is still fragmentary, it is proposed that hBD2 has a dual role. Its direct effect is nonoxidative killing of primarily Gram-negative invading microorganisms (Schroder, 1999), but it also has an immune modulatory effect, recruiting macrophages and dendritic cells to the place of infection (Yang *et al*, 1999). Chronell *et al* (2001) found that hBD2 expression is upregulated in acne lesions. They suggested that this upregulation is most probably a secondary response to the marked perilesional infiltrate and that inflammatory cytokines may be responsible for this (Chronell *et al*, 2001). Our results demonstrate that keratinocytes might also be an important source of hBD2 in acne. In addition, we show that the inducibility of hBD2 in cultured keratinocytes by *P. acnes* is isolate specific. Still, only two of the tested four *P. acnes* strains upregulated the hBD2 mRNA expression of keratinocytes. There are two reasonable explanations. First, bacterial-derived proteins, such as lipoglycans of the Gram-positive cell envelope or the heat shock proteins are known TLR ligands. To date, several *P. acnes* cell envelope components such as GroEL and DnaK (Farrar *et al*, 2000) or lipoglycans (Whale *et al*, 2004) have been identified. Moreover, Graham *et al* (2004) have showed that *P. acnes* GroEL is able to upregulate the pro-inflammatory cytokine production of keratinocytes. Although there is no direct evidence for the interaction between the TLR receptors expressed by the keratinocytes and the *P. acnes*-

derived GroEL, DnaK, or lipoglycans one can expect that these components may act as ligands for the TLR receptors (Pivarcsi *et al*, 2005). Underhill and Ozinsky (2002) proposed a model in which host cells use multiple TLRs for the detection of numerous features on a microbe simultaneously. According to this model, keratinocytes recognize a number of *P. acnes* antigens and effective hBD2 secretion occurs only if several antigens are concurrently identified and all of the involved receptors are functional. Second, acne is a highly heritable disease with supposed genetic factors remaining to be determined (Bataille *et al*, 2002). Hollox *et al* (2003) showed that hBD2 gene (*DEFB4*) is polymorphic in copy number and there is a significant correlation between genomic copy number of *DEFB4* and levels of its mRNA transcript in different individuals. It is conceivable that the hBD2 mRNA level in individuals with low *DEFB4* copy number remains below detectable in response to *P. acnes* treatment. hBD2 transcripts were significantly induced as early as 3 h after treatment with *P. acnes* clinical isolates 889 and 2005 and cultured keratinocytes already expressed hBD2 protein 6 h post-treatment. Still, not all of the keratinocytes express hBD2 protein in response to *P. acnes* treatment. These data are in good agreement with the findings of Liu *et al* (2002), who showed that the expression of hBD2 peptide by human keratinocytes requires differentiation of the cells by growth and maturation as well as cytokine or bacterial stimulus. Moreover, Jeremy *et al* (2003) showed that the proliferative activity and the differentiation status of keratinocytes in the follicular epithelium of the pilosebaceous units are comparable with controls and are significantly lower than in early inflamed lesions. As determined by MTT assay, *P. acnes* clinical isolate 889 is able to stimulate the growth of keratinocytes. In addition, the same isolate induce differentiation of keratinocytes as demonstrated by increased expression of involucrin. In contrast, other stains have no effect on cell growth and differentiation. These data might explain the presence or absence of acne lesions in different individuals, depending on the *P. acnes* strain(s) that is/are present in the follicles. We also present data that hBD2 does not have any bacteriostatic or bactericidal effect against any of the four *P. acnes* strains included in our experiments. Our results together with the findings that hBD2 acts as a chemotactic agent for immature dendritic cells, memory T cells, and neutrophils (Yang *et al*, 1999; Niyonsaba *et al*, 2004) lead to the conclusion that the primary contribution of hBD2 to acne pathogenesis is the regulation of adaptive immunity by recruitment of T cells and neutrophils to the place of *P. acnes* infection.

Keratinocyte-derived soluble factors are fundamental in mobilizing leukocytes and neutrophils to the place of skin infection. Graham *et al* (2004) showed that *P. acnes* is able to stimulate keratinocytes to produce various pro-inflammatory cytokines. But only viable stationary-phase bacteria induced the secretion of IL-1α, whereas both exponential- and stationary-phase viable bacteria induced TNF-α and granulocyte/macrophage colony-stimulating factor secretion. None of the four exponential-phase *P. acnes* strains included in our experiments induced the expression of IL-1α. We provide evidence that cultured keratinocytes produce IL-8 in a time-dependent manner in response to *P. acnes* treatment. As IL-8 has mitogenic activity on keratinocytes and is a potent chemoattractant for neutrophils (Baggiolini and Loetscher, 2000), the major cell type in fully developed inflammatory lesions (Layton *et al*, 1998), we hypothesize that *P. acnes*-induced IL-8 secretion could also have a key role in the initiation of inflammatory events in acne. Moreover, the ductal hyperproliferation of the keratinocytes in acne could also be associated with elevated levels of IL-8 protein as it promotes proliferation of keratinocytes through their IL-8 receptor (Michel *et al*, 1992). We therefore suggest that the primary inflammatory event in acne pathogenesis is the hBD2 and IL-8 secretion accompanied by neutrophil infiltration and ductal hyperproliferation of keratinocytes.

The release of hBD2 and/or IL-8 is the response of keratinocytes to *P. acnes* exposure. The first step in the hosts defense against microbial infection, however, is the detection of pathogen(s). Kim *et al* (2002) have shown that *P. acnes* induces cytokine release of monocytes via TLR2. We investigated the molecular mechanism by which *P. acnes* elevated the hBD2 and IL-8 mRNA levels in keratinocytes and present evidence that *P. acnes*-induced upregulation in gene expression is both TLR2 and TLR4 dependent. We have previously shown that the induction of IL-8 gene expression of keratinocytes in response to treatment with *Mycobacterium tuberculosis* also depends on both TLR2 and TLR4 (Pivarcsi *et al*, 2003). Additionally, the induction of myeloid dendritic cells by *Mycobacterium bovis* bacillus Calmette-Guérin PGN can also be suppressed by simultaneous blocking of TLR2 and TLR4 (Uehori *et al*, 2003). Still only blocking of TLR2 prior to *P. acnes* treatment suppressed the IL-8 protein secretion of keratinocytes. As keratinocytes store IL-8 in vesicles, it is reasonable that only *de novo* synthesis is inhibited by blocking of TLR4 and the secretion of intracellularly stored IL-8 occurs independent of TLR4 signal transduction. According to the model of Underhill and Ozinsky (2002), we propose that some strains of *P. acnes* and also species of *Mycobacterium* have distinct ligands recognized by TLR2 and/or TLR4 and that both receptors are involved in the efficient response of keratinocytes to *P. acnes* and *Mycobacterium* infection, respectively. As the hBD2 and IL-8 induction is partially inhibited by TLR2 and TLR4 neutralizing antibodies, however, it is important to note that other TLRs such as TLR1 or TLR6 and in addition numerous signal transduction pathways might also play a role in sensing the bacteria. It is important to note that the TLR2-dependent bacterial sensing does not occur via PGN recognition (Travassos *et al*, 2004). Our results further suggest that the recognition of microbial pathogens is cell-type specific and also that distinct strains of bacteria exhibit different components that are potent TLR agonists. Recently, Bruggemann *et al* (2004) have identified numerous surface-associated and other immunogenic factors of *P. acnes*. Using the optimized RAPD method for typing *P. acnes* strains (Perry *et al*, 2003), we demonstrated that the strains included in our assays display a discrete RAPD profile. Still the clinical isolates 889 and 2005, strains that upregulate hBD2 and involucrin gene expression, display very similar profiles. Sequencing of the amplified products and further characterization of the clinical isolates of *P. acnes* may lead to the identification of specific immuno-

genic factors that might be involved in triggering acne inflammation.

This report first demonstrates that distinct strains of *P. acnes* upregulate hBD2 and IL-8 mRNA and protein expression in cultured keratinocytes. Here we show that distinct, genotypically different strains of *P. acnes* upregulate hBD2 and IL-8 mRNA and protein expression in keratinocytes via TLR2 and TLR4. In addition, the *P. acnes* clinical isolate 889 elevates the growth potential and differentiation of keratinocytes. This activity of *P. acnes* may be particularly important in the acute infection of the skin in which hBD2 participates in bacterial clearance and/or as a chemoattractant. The results also stress the importance of keratinocytes as possible sensors of abnormal *P. acnes* colonization. In conclusion, our results suggest that keratinocytes secrete hBD2 and IL-8 in response to *P. acnes* and that these molecules are potent chemotactic factors for leukocyte and neutrophil infiltration as well as for keratinocyte growth potential and differentiation at the place of *P. acnes* infections.

Materials and Methods

Culture and stimulation of cells Human epidermal keratinocytes were isolated and cultured as described previously (Pivarcsi *et al*, 2003). For detailed description, see supporting online information.

The tissue culture dishes and pipettes were free of endotoxin contamination, according to the manufacturers.

Clinical strains of *P. acnes* ($n = 3$; 889, 2005, and 6609) were obtained from the Department of Microbiology, University of Szeged. Routine phenotyping identification was carried out with the ATB ID 32A kit (BioMérieux, S.A., Marcy l'Etoile, France). *P. acnes* ATCC11828 was included as a commercially availably reference strain. The level of endotoxin contamination of *P. acnes* was quantified according to the European Pharmacopoeia IV and was found to be <2.6 IU per mL (<5 ng per mL). It has already been demonstrated that the concentration of LPS must be more than 100 ng per mL for the signaling via TLR4 expressed on keratinocytes (Pivarcsi *et al*, 2003). As the level of endotoxin contamination of *P. acnes* strains is at least 20-fold lower, this amount is not sufficient for signaling via TLR4. *P. acnes* strains were cultured on prereduced Columbia agar base (Oxoid, Basingstoke, UK) supplemented with 5% cattle blood, vitamin K_1, and hemin, incubated at 37°C under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, Cornelius, Oregon) for 48 h. Cells were then centrifuged at 2600 g for 15 min and the supernatant was discarded. After three washes in phosphate-buffered saline–ethylene diamine tetraacetic acid (PBS–EDTA), 15 min each, the cells were harvested in 5 mL PBS–EDTA and the cell number was calculated by measuring the optical density of the solution with a Cecil CE 2040 spectrophotometer (Cecil Instruments, Cambridge, UK) at 600 nm. After an additional centrifugation, the cells were harvested in a complete keratinocyte medium lacking antibiotic/antimicotic solution and stored at −80°C. The freezing had no effect on the viability of the bacteria, as 100% of the cells were viable after thawing (data not shown).

As *P. acnes* is very sensitive to different antimicrobial agents, the CKM was replaced with medium lacking antibiotic/antimicotic solution 24 h before stimulation of the cultured keratinocytes. *P. acnes* strains were thawed and keratinocytes were treated with bacteria (*P. acnes*:keratinocyte ratio 50:1). Keratinocytes and bacteria were co-cultured at 37°C in an atmosphere of 5% (vol/vol) CO_2 in air for 3 and 24 h for mRNA expression: 3, 6, 12, and 24 h for protein expression; and 3, 24, and 48 h for viability analysis before harvesting. For negative controls, keratinocytes were cultured with CKM without antibiotics. Following incubation, the supernatants were collected and the cells were harvested for mRNA or protein expression analysis.

For blocking experiments, cultured keratinocytes were grown on six-well plates and pre-treated with functional grade neutralizing anti-human TLR2 mAb TLR2.1 (10 μg per mL) (eBioscience, San Diego, California), anti-human TLR4 mAb HTA125 (10 μg per mL) (eBioscience), or isotype-matched control-purified mouse IgG2a antibodies (10 μg per mL) (Sigma, St. Louis, Missouri) for 1 h and then stimulated with *P. acnes* (889). After 3 h, keratinocytes were harvested for QRT-PCR analysis and supernatants were collected for ELISA.

QRT-PCR Total RNA was isolated with Trizol reagent (GibcoBRL, Eggstein, Germany) according to the manufacturer's instructions. RNA concentration was determined by the A_{260} value of the sample. Complementary DNA (cDNA) was generated from 1 μg total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California) in a final volume of 20 μL. After reverse transcription, amplification was carried out using iTaq DNA polymerase (Bio-Rad). QRT-PCR was used to quantify the relative abundance of each mRNA (iCycler IQ Real Time PCR; BioRad). For the specific primers used in the experiments see supporting online information (Table S1).

The amplification protocol contained one cycle of initial denaturation at 94°C for 5 min followed by varying number of cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and one cycle of terminal extension at 72°C for 10 min. Each primer set gave a unique product. As controls, we used the reaction mixtures without the cDNA.

Immunofluorescent staining The expression of hBD2 was determined by indirect immunohistochemistry. All the steps were carried out at room temperature (20°C). Cell suspensions of 1×10^5 cells in 50 μL PBS were loaded up in each cuvette of the cytospin (Wescor, Claremont, Ontario, Canada, Cytopro7620). The cells were spun at 600 rpm for 6 min and then fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min. The samples were then permeabilized with PBS containing 1% Triton-X (Sigma) for 30 min and blocked in PBS containing 1% bovine serum albumin (BSA) for 20 min. Samples were incubated with 1:500 dilution of polyclonal rabbit anti-hBD2 (a kind gift from Dr T. Ganz, UCLA, USA) in PBS containing 1% BSA and 0.1% Triton-X for 1 h and washed three times with PBS for 5 min each. The cells were then incubated with 1:100 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin (Sigma) in PBS containing 1% BSA and 0.1% Triton-X for 45 min. After three washes with PBS, 5 min each, the samples were mounted in mounting medium (100 mM TRIS pH 8.5; 25% glycerol) and analyzed using epifluorescent illumination of the Axioplan microscope (Opton, Carl Zeiss AG, Oberkochen, Germany). Images were recorded with an RT KE camera and SPOT RT v4.0 software (Diagnostic Instruments, Livingstone, Scotland). In all cases, negative controls consisted of preincubation with PBS or substitution of the primary antibody with pre-immune serum.

ELISA Human IL-8 protein was measured from the supernatants of third passage epidermal keratinocytes using the Quantikine human IL-8 immunoassay kit (R&D Systems, Minneapolis, Minnesota) following the manufacturer's instructions. We used serial dilutions of recombinant human IL-8 for standard curves. The optical density of the wells was determined using a microplate reader (Multiscan v3.0, Labsystems, Vantaa, Finland) set to 450 nm with a wavelength correction set to 540 nm.

MTT assay Keratinocytes were seeded in each well of the 96-well plate at a density of 5×10^3 cells per well. The treatment of keratinocytes was performed using the *P. acnes* clinical isolate 889 and the reference strain ATCC11828. Medium alone was used as control. Three, 24, and 48 h post-treatment, the supernatant was replaced with RPMI without phenol red and 50 μg of MTT (Sigma) was introduced into each well and incubated for 4 h at 37°C. Living cells degrade MTT by the mitochondrial succinate dehydrogenase resulting in MTT formazan. The converted dye was solubilized with

acidic isopropanol (0.04 M HCl in absolute isopropanol). The optical density of the wells was determined using a microplate reader (Multiscan v3.0, Labsystems) at 540 nm.

Antibacterial assay Assays measuring the antibacterial activity of hBD2 against all four strains of *P. acnes* included in our study were modified from the methods of Valore *et al* (1998) and Porter *et al* (1997). For detailed description, see supporting online information.

RAPD molecular typing of *P. acnes* strains RAPD analysis of the studied *P. acnes* strains was performed according to the method of Perry *et al* (2003). For detailed description, see supporting online information.

Data presentation PCR results were expressed as fold increases over the 0 h or control values.

For the evaluation of the viability assay, the average absorbance of the control wells was regarded as 100% and the percentage of cell growth in each well was calculated (% of control). Each plate was evaluated at one time point; at least 15 wells comprised one group. All the data were presented as mean $\pm$ standard deviation for at least three experiments. Data were compared using one-way ANOVA followed by Dunnett's *post hoc* test to determine statistical differences after multiple comparisons (SPSS, SPSS, Chicago, Illinois). A probability value of less than 0.05 was considered significant.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23705/JID23705.htm

Figure S1 Blocking of TLR2 but not TLR4 inhibits the *P. acnes* (889) induced secretion of IL-8 in kertinocytes 3 hours after treatment as determined by ELISA

Figure S2 RAPD profiles obtained from genomic DNA of *P. acnes* strains

Table S1 Sequences of primer pairs and probes used for QRT-PCR

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