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Escherichia coli ghosts promote innate immune responses in human keratinocytes

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ABSTRACT

Bacterial ghosts (BGs) as non-living bacterial envelopes devoid of cytoplasmic content with preserved and intact inner and outer membrane structures of their living counterparts have been used to study the ability of their surface components for the induction of antimicrobial peptides and pro-inflammatory cytokines in human primary keratinocytes (KCs). Quantitative real-time PCR analysis revealed that incubation of KCs with BGs generated from wild-type *Escherichia coli* induced the mRNA expression of antimicrobial psoriasin (S100A7c) in a BGs particle concentration-dependent manner. Using immunoblot analysis we showed that BGs generated from the flagellin-deficient (ΔFliC) *E. coli* strain NK9375 were as effective as its isogenic wild-type (wt) *E. coli* strain NK9373 to induce psoriasin expression when normalized to BG particles being taken up by KCs. However, results obtained from endocytic activity of KCs reflect that internalization of BGs is greatly dependent on the presence of flagellin on the surface of BGs. Moreover, BGs derived from wt *E. coli* NK9373 strongly induced the release of the pro-inflammatory cytokines IL-6 and IL-8, compared to ΔFliC *E. coli* NK9375 BGs. Taken together, obtained data demonstrate that non-living BGs possessing all bacterial bio-adhesive surface properties in their original state while not posing any infectious threat have the capacity to induce the expression of innate immune modulators and that these responses are partially dependent on the presence of flagellin.

1. Introduction

The innate immune system serves to protect the host from invading microorganisms and upon activation triggers a series of host defense responses [1]. A central mechanism of these responses is the production of pro-inflammatory cytokines and antimicrobial defense molecules. Different components of microbial pathogens referred as pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors of the innate immune system. Among these receptors are the toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors and the peptidoglycan-like recognition protein, all contributing to early host defense against pathogens [2–4]. For example the bacterial PAMP flagellin, a monomeric subunit of the flagellum binds to TLR5 and activates the transcription factor NF-kappaB [5], ultimately leading to the expression of pro-inflammatory cytokines and antimicrobial peptides including psoriasin (S100A7c) and human β-defensin-2 in human keratinocytes (KCs) [6–8].

Bacterial ghosts (BGs) are empty cell envelopes devoid of cytoplasmic content which are produced by the controlled expression of plasmid-encoded lysis gene *E* of bacteriophage ΦX174 in Gram-negative bacteria. Inducible expression of gene *E* causes the fusion of the inner and outer membranes to form an intermembrane E-specific lysis tunnel through which the cytoplasmic content is expelled [9]. These non-living bacterial envelopes maintain the full cellular morphology of the native bacteria. All cell membrane structures, including the outer membrane proteins, adhesins, lipopolysaccharide (LPS) and peptidoglycan are preserved and remain intact [10].

BGs can be used for immunization either against their own envelope structures or as an efficient delivery system for foreign target antigens [11]. Furthermore, BGs can also be used as delivery vehicles for active substances such as doxorubicin [12], DNA [13,14], and enzyme [15]. Nevertheless, little is known about the capacity of BGs to be recognized by human skin cells and to induce the expression of in-

Abbreviations: BGs, bacterial ghosts; KCs, keratinocytes; NLRs, NOD-like receptors; PAMPs, pathogen-associated molecular patterns; qRT-PCR, quantitative real-time PCR; TLRs, toll-like receptors; wt, wild-type.

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nate immune modulators by skin epithelial cells especially epidermal KCs. In the present study the ability of *Escherichia coli* BGs to induce expression of antimicrobial psoriasin and pro-inflammatory cytokines in human primary KCs has been investigated. Moreover, we studied the capacity of BGs to be recognized and internalized by epidermal KCs. The results demonstrate that the presence of flagellin on the surface of BGs enhances the expression of psoriasin, and the release of IL-6 and IL-8 by human primary KCs, and plays an important role during internalization of BGs by KCs.

2. Materials and methods

2.1. Cell culture

Human primary KCs prepared from neonatal foreskin were obtained from Clonetics (San Diego, CA, USA) and cultured in serum-free keratinocyte growth medium (KGM, Clonetics) as described previously [16]. For stimulation, third passage KCs were cultured in 12-well tissue culture plates (Corning, NY, USA) and used at a confluence of 60–70%. Stimulations were performed in keratinocyte basal medium (KBM, Clonetics).

2.2. RNA isolation and qRT-PCR

After stimulation, cells were washed with phosphate-buffered saline (PBS) and RNA was isolated using TRIzol®Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For cDNA synthesis RNA was reverse-transcribed with MuLV-reverse transcriptase using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) and oligo dT primers (Roche Diagnostics, Basel, Switzerland). cDNA sequences of the genes under investigation were obtained from the GenBank. Primers were designed using the PRIMER3 software (http://frodo.wi.mit.edu/primer3) from the Whitehead Institute for Biomedical Research (Cambridge, MA, USA). The following forward (F) and reverse (R) intron-spanning primers were used for β-2-microglobulin (B2M): F, 5′-GATGAGTATGCCTGC CGTGTG-3′; R, 5′-CAATCCAAATGCGGCATCT-3′; for psoriasin: F, 5′-GGAGAACTTCCCCAACTTCCTT-3′; R, 5′-GGAGAAGACATTTTATTGT TCCT-3′. qRT-PCR was performed by the LightCycler technology using the Fast Start SYBR Green I kit for amplification and detection (Roche Diagnostics) as described earlier [17]. Briefly, in all assays, cDNA was amplified using a standardized program (10 min denaturing step and 55 cycles of 5 s at +95 °C; 15 s at +65 °C, and 15 s at +72 °C; melting point analysis in 0.1 °C steps; final cooling step). Each LightCycler capillary was loaded with 1.5 μl DNA master mix; 1.8 μl $MgCl_2$ (25 mM); 10.2 μl H_2O; and 0.5 μl of each primer (10 μM). Determinations of the relative quantification of target gene expression and amplification efficiencies were performed using a mathematical model by Pfaffl [18]. The expression of the target gene was normalized to the expression of the housekeeping gene B2M. All real-time PCRs were performed in triplicates. The specificity of PCR reactions was confirmed by sequencing of the PCR products.

2.3. BGs production and KCs stimulation

Escherichia coli NK9373 (wt *E. coli*) and *E. coli* NK9375 (ΔFliC *E. coli*), a flagellin-deficient strain having an in frame deletion within the *FliC* gene [19], were kindly provided by Dr. David Bates (Baylor College of Medicine, Houston, Texas). BGs were produced by the controlled expression of the phage-derived lysis gene *E*, as described previously [20]. For safety reasons, to avoid presence of potential non-lysed, non-culturable but viable cells, the BG's preparations were inactivated with gentamycin (50 mg/ml; Invitrogen) and streptomycin (100 mg/ml; Invitrogen). Subsequently the BGs were washed three times with PBS, resuspended in distilled water, lyophilized and stored at room temperature. Lyophilized BGs were resuspended in KBM medium before applying on KCs. For *in vitro* assays recombinant IL-1α (R&D Systems, Minneapolis, MN, USA) and purified flagellin from *S. typhimurium* (Invitrogen) were used. Culture supernatants of wt and ΔFliC *E. coli* were prepared as described previously [6]. Before KCs stimulation culture supernatants were diluted 1:100 in KBM.

2.4. Cytokine measurement

Culture supernatants of stimulated KCs were depleted of detached cells or cell fragments by centrifugation and stored at −20 °C until analysis. Concentrations of IL-6 and IL-8 were determined by enzyme-linked immunosorbant assay (ELISA; R&D Systems) according to the manufacturer's instructions.

2.5. Immunoblot analysis

Sample preparation, protein concentration measurement and immunoblot analysis were performed as described previously [21]. Equal loading of protein lysates was confirmed by Ponceau S staining of the membrane (data not shown). The following first step antibody was used: mouse monoclonal IgG_1 anti-psoriasin clone 47C1068 (dilution 1:500; Abcam, Cambridge, UK). The membranes were developed using the Chemiglow reagent (Alpha Innotech, San Leandro, CA, USA) according to the manufacturer's instructions. ImageJ software based analysis was applied to quantify the densities of bands obtained via immunoblot blot analysis (Rasband, W.S., ImageJ, U.S. NIH, Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij/, 1997–2009). Obtained values of psoriasin expression after stimulation of KCs with various particle concentrations of wt and ΔFliC *E. coli* BGs were normalized to the value of psoriasin expression obtained after stimulation of KCs with recombinant IL-1α.

2.6. Uptake of fluorescein-isothiocyanate (FITC)-labeled BGs

The efficiency of the endocytic activity of the human primary KCs was measured as described previously [13,14]. Briefly, human primary KCs cultured in 24-well plates (2×10^5 cells/well) were incubated with FITC-BGs (1×10^3 per cell) for 2 h at +37 °C. After the incubation cells were washed three times with PBS to remove the excess BGs. Finally the cells were detached using TrypLE™ Express (Invitrogen), washed twice with PBS, fixed in cold 1.5% paraformaldehyde (Sigma–Aldrich, St. Louis, MO) in PBS and analyzed on BD FACSCanto™ Flow Cytometer (BD Biosciences, Pharmingen, San Jose, USA). Dead cells were excluded according to their forward and side scatter properties. Obtained data were analyzed using FlowJo Software version 7.5 (Tree Star, Inc., Ashland, OR).

2.7. Statistical analysis

Obtained results were analyzed by GraphPad Prism version 5 (GraphPad Software, La Jolla, CA). The statistical significance of the difference between two groups was evaluated by Student's *t*-test and between more than two groups by the one-way ANOVA. Differences were considered to be significant with $p < 0.05$.

3. Results

3.1. Expression of antimicrobial psoriasin in human primary KCs after treatment with BGs

It has been reported that *E. coli* culture supernatants induce the expression of antimicrobial peptides in epidermal KCs [6,22]. To investigate the capacity of BGs to elicit innate immune responses

by epithelial cells, human primary KCs were stimulated for 24 h with different particle concentrations of BGs generated from wt *E. coli*. Stimulation of KCs by flagellin (10 ng/ml) and IL-1α (10 ng/ml) served as positive control for the up-regulation of the analyzed antimicrobial peptide, untreated cells served as negative control. The relative mRNA up-regulation of the antimicrobial psoriasin was determined by quantitative real-time PCR (qRT-PCR). As can be depicted from Fig. 1 the mRNA expression of psoriasin was up-regulated in KCs after incubation with BGs. The stimulated psoriasin mRNA production was dependent on the BG particles and the strongest up-regulation was observed at 2×10^8 BG particles/ml, BG concentrations below 2×10^8 particles/ml had no effect (Fig. 1).

We have previously reported that the induction of antimicrobial peptides including psoriasin in KCs is dependent on flagellin expression by living *E. coli* grown in standard conditions [6]. To investigate whether the flagellin of non-living BGs has a similar effect, we generated BGs from the wild-type flagellated and its isogenic flagellin-deficient (ΔFliC) *E. coli* strains. As positive controls KCs were stimulated with flagellin, IL-1α and culture supernatant from wt *E. coli*. KCs incubated in the presence of non-conditioned bacterial culture medium, culture supernatant from ΔFliC *E. coli*, and without stimulation served as negative controls. KCs were stimulated for 48 h and afterwards analyzed by immunoblot for psoriasin protein production. In contrast to the mRNA data a faint band of psoriasin was detected on the protein level at 2×10^7 particles/ml when stimulated with wt *E. coli* BGs (Fig. 2A). Strong induction of psoriasin was observed at 2×10^8 particles/ml which was even stronger at 2×10^9 particles/ml by wt *E. coli* BGs. For the isogenic ΔFliC strain a faint band of psoriasin was detected starting at 2×10^8 BG particles/ml with a prominent expression level at 2×10^9 BG particles/ml. These data indicate that KCs respond to BGs by the production of antimicrobial peptides in a particle-dependent manner (Fig. 2A and B).

3.2. Cytokine production by human primary KCs after treatment with BGs

The secretion of the pro-inflammatory cytokines IL-6 and IL-8 by KCs after incubation for 48 h with BGs were investigated by ELISA. The release of IL-6 and IL-8 by KCs after the incubation with wt and ΔFliC *E. coli* BGs was dependent on the BG source and particle numbers used (Fig. 3A and B). Obtained results showed that the effect of wt *E. coli* BGs on IL-6 and IL-8 release was detectable beginning at concentrations 2×10^7 particles/ml. While concentrations below 2×10^7 particles/ml were almost ineffective, 2×10^9 particles/ml strongly enhanced the secretion of IL-6 from 2 pg/ml (untreated) to 600 pg/ml and IL-8 from 60 pg/ml (untreated) to 1350 pg/ml, respectively (Fig. 3A and B). ΔFliC *E. coli* BGs increased the secretion of both cytokines when using 2×10^9 particles/ml only to 134 pg/ml and 750 pg/ml of IL-6 and IL-8, respectively, whereas BGs concentrations below that had no significant effect on the release of both cytokines (Fig. 3A and B). Furthermore, significant difference in secretion of both IL-6 and IL-8 related to the presence of flagellin was detected after incubation of KCs with culture supernatants from wt *E. coli* and ΔFliC *E. coli*, when cytokine production was nearly one order of magnitude lower after incubation of KCs in the presence of culture supernatant from ΔFliC *E. coli* compared to culture supernatant from wt *E. coli* (Fig. 3A and B). Altogether these results emphasize the importance of intact envelope surface structure of BGs and the role of flagellin in the stimulation of innate immune system.

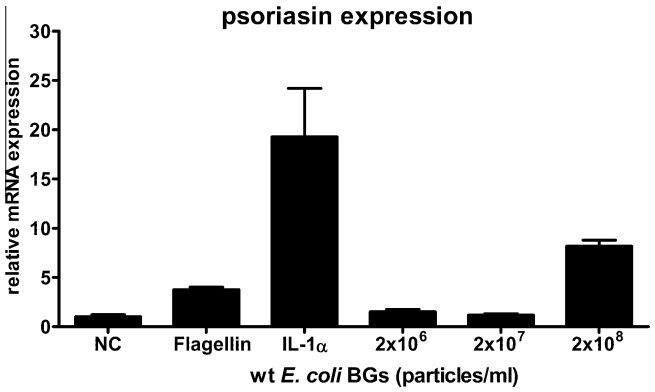

Fig. 1. BGs induce the expression of antimicrobial psoriasin in human primary KCs. KCs were incubated for 24 h with flagellin (10 ng/ml), IL-1α (10 ng/ml), wt *E. coli* BGs varying from 2×10^6 to 2×10^8 particles/ml or without stimulation (NC), thereafter total RNA was isolated and reverse-transcribed to cDNA. The relative expression of antimicrobial psoriasin was determined by qRT-PCR. The mean values are displayed in relation to untreated cells (NC). Relative gene expression levels were normalized to the expression of the housekeeping gene β-2-microglobulin. Data represent the mean ± SD of triplicate samples.

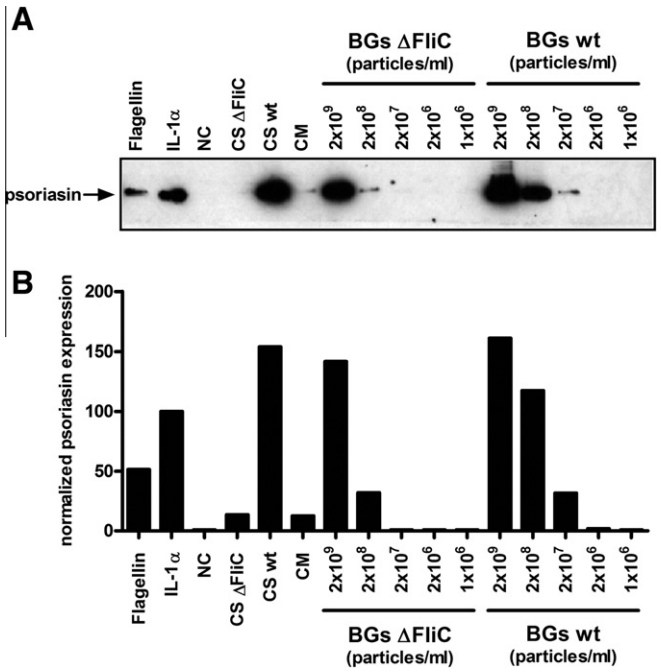

Fig. 2. Induction of psoriasin in KCs by wt and ΔFliC *E. coli* BGs. KCs were incubated for 48 h with wt and ΔFliC *E. coli* BGs from 1×10^6 to 2×10^9 particles/ml. Stimulation with flagellin (10 ng/ml), IL-1α (10 ng/ml), non-stimulated cells (NC), non-conditioned bacterial culture medium (CM), and culture supernatants of wt *E. coli* (CS wt) and ΔFliC *E. coli* (CS ΔFliC) were used as controls. Forty-eight hours after stimulation the cell lysates from KCs were subjected to immunoblot analysis for psoriasin production (A). Quantification of immunoblot of psoriasin protein expression (A) by densitometric analysis normalized to IL-1α stimulation of KCs (B). One representative experiment of three performed is depicted.

3.3. Endocytosis of wt and ΔFliC E. coli BGs by human primary KCs

As mentioned above, the up-regulation of antimicrobial psoriasin and enhanced release of pro-inflammatory cytokines by KCs is dependent on the presence of flagellin on the surface of BGs. To further investigate the role of flagellin in the recognition and uptake of BGs by KCs, the internalizations of wt *E. coli* and ΔFliC *E. coli* BGs were compared and analyzed. Missing flagellin on the surface of BGs caused a significant decrease of KCs capacity to bind and internalize BGs. FACs analysis clearly showed the difference between the internalization of FITC-labeled wt *E. coli* BGs (Fig. 4A) and ΔFliC *E. coli* BGs (Fig. 4B). As depicted in Fig. 4C

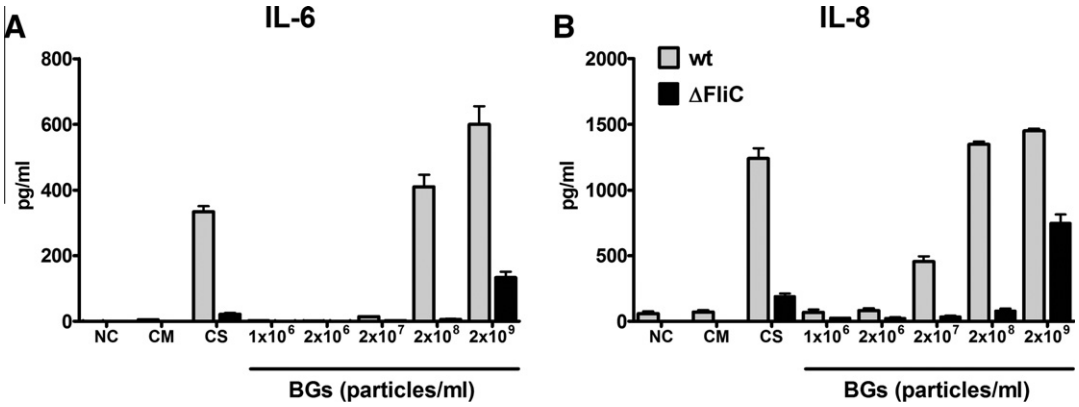

Fig. 3. BGs induce the secretion of pro-inflammatory cytokines. KCs were incubated for 48 h with wt *E. coli* and ΔFliC *E. coli* BGs from 1×10^6 to 2×10^9 particles/ml, and in the presence of culture supernatants (CS) of wt and ΔFliC *E. coli*. Non-stimulated cells (NC) and cells incubated in the presence of non-conditioned bacterial culture medium (CM) served as negative controls. After the incubation period KC's culture medium was collected and the concentrations of IL-6 (A) and IL-8 (B) were determined by ELISA. Data represent the mean ± SD of three independent experiments performed in triplicates.

approximately 6-fold increase of KC's capacity to bind and internalize wt *E. coli* BGs compared to ΔFliC *E. coli* BGs was observed due to the presence of the intact flagellin.

4. Discussion

BGs are non-living cell envelope preparations from Gram-negative bacteria, devoid of cytoplasmic contents, while their cellular morphology and native surface antigenic structures remain preserved [10]. In this investigation the effects of BGs on the regulation of innate immune modulators using human primary KCs were determined. Incubation of BGs generated from non-pathogenic *E. coli* with human KCs up-regulated the expression of the antimicrobial psoriasin. This was in agreement with earlier reports where culture supernatants or disrupted cells of *E. coli* strains enhanced the expression of antimicrobial peptides [6,22]. Therefore, this investigation confirmed that the envelope structures of BGs including flagellin are fully functional and intact, and are recognized by KCs promoting the innate immune responses similar to bacterial compounds used in the former studies [6,22].

As reported earlier, the responsiveness towards *E. coli* by KCs is mediated through TLR5 and its ligand flagellin [6]. The wt *E. coli* exhibited a BG concentration-dependent induction of psoriasin production (Fig. 1); whereas the ΔFliC *E. coli* showed the expression of psoriasin at BG concentrations of one order of magnitude higher than the wt strain (Fig. 2). Accordingly, the data obtained from the release of the pro-inflammatory cytokines IL-6 and IL-8 underlined the significance of the presence of flagellin in the BGs preparation when compared to the ΔFliC mutant strain (Fig. 3). From the previous studies with supernatants of *E. coli* as source of shad flagellin and/or purified flagellin, it is evident that flagellin is the major inducer of psoriasin in human KCs [6,22]. However, since ΔFliC BGs are capable of psoriasin induction there seems to be additional FliC-independent pathways.

Furthermore, investigation of BGs uptake by KCs derived from wt *E. coli* and ΔFliC *E. coli* showed that the BGs from the flagella bearing strain is taken up roughly one order of magnitude better than the ΔFliC BGs (Fig. 4). This observation suggests that the presence of flagellin on the BG surface considerably contributes to the binding and internalization of BGs by KCs. Moreover, the key role of flagellin in the induction of psoriasin production, and IL-6 and IL-8 secretion is reflected by decreased response of KCs in production of these proteins after incubation with ΔFliC *E. coli* BGs or incubation with culture supernatant from ΔFliC *E. coli*. The question remains whether other components on the surface e.g. LPS

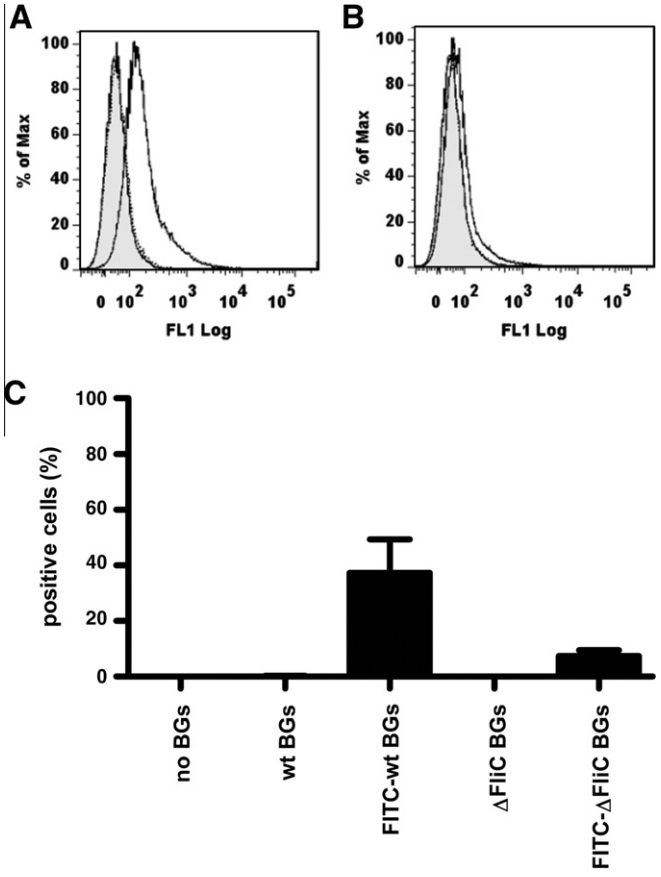

Fig. 4. Comparative flow cytometric analysis of the internalization of BGs by human primary KCs. KCs were incubated with FITC-labeled BGs (1×10^3 per cell) – wt *E. coli* (A) and ΔFliC *E. coli* (B) BGs (open histogram-full line) for 2 h at +37 °C. Cells incubated without BGs (shaded histogram) or with non-labeled BGs (open histogram-dotted line) served as the controls. Values were calculated as the percentage of cells with increased fluorescence incubated without or with non-labeled BGs subtracted from the percentage of positive cells incubated with FITC-labeled BGs. Each bar represents the mean of four independent experiments ± SD (C).

of the ΔFliC *E. coli* BGs have signaling activities for the production of antimicrobial peptides and/or pro-inflammatory cytokines.The release of IL-6 and IL-8 by KCs, however, were more sensitive to the presence of flagellin as no direct correlation with the BGs par-

ticle number could be detected (Fig. 3). Higher secretion of IL-8 compared to IL-6 by KCs after incubation with ΔFliC BGs (Fig. 3) and partial internalization of BGs from ΔFliC *E. coli* (Fig. 4) might be related to the presence of the LPS on the BG's shell. Presence of LPS from disrupted bacterial cells presented in the culture supernatant could explain detected low secretion of IL-8 by KCs after incubation with culture supernatant from ΔFliC *E. coli* (Fig. 3B). It was shown previously that activation of skin melanoma cells by LPS results in enhanced production of IL-8 and cell adhesion [23]. Although it has been reported that TLR4 is not involved in cellular LPS uptake by endothelial cells, the connection between TLR4-mediated epidermal KCs activation by LPS and their phagocytic activity remains to be defined [24]. Despite the fact that functional expression of TLR4 on the surface of KCs was observed by some investigators but not by other investigations [25–27], intact LPS on the BG's envelope might contribute to the complex process during recognition of bacterial components by human skin cells including KCs. Therefore, the exact role and the mechanisms involved during activation of epidermal KCs through LPS signaling have to be determined.

It is still possible that the acidic degradation of the flagella present in wt *E. coli* BGs in the lysosomal compartment of KCs leads to monomeric flagellin, which is able to bind to TLR5 and stimulate the expression of the cytokines IL-6 and IL-8 and the production of psoriasin. As flagellin binding to TLR5 [5] cannot occur with the ΔFliC BGs and consequently the signaling induced by this binding, other ways of internal signaling leading to NF-kappaB induced expression of psoriasin have to be induced by BGs in KCs. Possible intracellular receptors which might sense the presence of BGs or BGs constituents irrespective of flagellin expression are the NLRs such as NOD1 or NOD2. Recent investigations have reported the functional expression of NOD1 [28] and NOD2 [29] by peptidoglycan fragments in KCs. In particular, NOD1 mediates the sensing of peptidoglycan fragments containing the amino-acid *meso*-diaminopimelic acid and NOD2 mediates the sensing of muramyl dipeptide [1], which both of these fragments are degradation products of the still intact peptidoglycan of *E. coli* BGs [30]. The connection between activation of NOD2 by muramyl dipeptide (MDP) and increased antimicrobial peptides production in primary KCs has been reported [16], and in our study the use of BGs to induce psoriasin has been demonstrated. Therefore, obtained results demonstrate the ideal use of non-living and safe BGs in therapeutic approaches to enhance the innate immune defence system of the skin. Potential therapeutic effects of BGs can be combined by packaging drugs or other biological active substances into BGs which could also be delivered intracellular for the stimulation of additional beneficial health effects [12,31].

5. Conflict of interest

AA, MM, ET disclose no conflict of interest; PK, VJK, UBM, WL are employees of BIRD-C which has licensed the rights to the Bacterial Ghosts Technology.

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