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further adds to the increasingly recognized redundancy of innate immunity. The multifunctional HDP GKY25 (GKYGFYTHVFRL-KWQIKVQIDQFFE), derived from the C terminus of human throbmin, exerts direct antimicrobial activities as well as anti-endotoxic effects in vitro and in vivo (17, 29, 30). In animal models of LPS-induced shock and Pseudomonas aeruginosa sepsis, GKY25 significantly improves the outcome by modulation of tissue factor--induced coagulation, but also by inhibition of proinflammatory responses in combination with antimicrobial effects (17, 29). Despite its observed direct interaction with LPS, lipid A, and bacterial surfaces (29), the detailed mode of action for GKY25 underlying the observed inhibition of cytokine responses still remained unknown. We therefore set out to further explore the mechanisms of action of this HDP. The results demonstrate that GKY25 functions by binding LPS, thereby preventing TLR4 dimerization at the surface of macrophages and monocytes, leading to inhibition of activation of MAPK and NF-κB activity and, finally, reduced proinflammatory cytokine production.

Materials and Methods
Ethics statement
The use of human blood was approved by the Ethics Committee at Lund University, Lund, Sweden (permit no. 657-2008). Animal experiments were conducted according to national guidelines (Swedish Animal Welfare Act SFS 1988:534) and were approved by the Laboratory Animal Ethics Committee of Malmö/Lund, Sweden (permit no. M252-11). Animals were housed under standard conditions of light and temperature and had free access to standard laboratory chow and water.

Peptides
The thrombin-derived peptide GKY25 (GKYGFYTHVFRLKKWIQKVIDQFFE), the control peptides WFF25 (WFFFYLYIEGVVTYHQ-RKKAQED) and IVE25 (IVEGSDAEIGMSPQWMLFRSQE), the murine GKY24m (GKYGFYTHVFRLKWQKVIDQFFEM), and their T-forms were synthesized by Biopeptide (San Diego, CA). The purity (>95%) of these peptides was confirmed by mass spectral analysis (MALDI-TOF Voyager).

Cells
RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM (PAA Laboratories, Pasching, Austria) supplemented with 10% (v/v) heat-inactivated FBS (FBS; Invitrogen, Carlsbad, CA) and 1% (v/v) antibiotic-antimycotic solution (Invitrogen). The mouse macrophage cell line RAW-Blue (InvivoGen, San Diego, CA), a reporter cell line derived from RAW 264.7 cells and the human monocyte cell line THP1-Xblue-CD14 (InvivoGen), was cultured according to the manufacturer’s instruction. Human PBMCs separated from healthy donors were isolated from fresh heparinized blood by Lymphoprep (p = 1.077 g/ml; Axis-Shield, Oslo, Norway). CD14+ human monocytes were isolated from buffy coats by Lymphoprep density centrifugation at 700 × g for 20 min followed by a purification step using anti-CD14-coated microbeads (Milenyi Biotec, Bergisch Gladbach, Germany). Monocyte purity was >96%. Human monocytes were collected in RPMI 1640 (PAA Laboratories) with 10% (v/v) FBS.

RNA isolation
RAW 264.7 cells (1.8 × 10⁶/cell) in DMEM supplemented with 10% (v/v) FBS were seeded in six-well tissue culture plates and incubated overnight. The cells were thereafter stimulated for 3 h with 100 ng/ml Escherichia coli (0111:B4) LPS (Sigma-Aldrich, St. Louis, MO; ∼500,000 endotoxin units/ml) in the presence or absence of 1, 5, or 10 μM GKY25. Total RNA was isolated with TRIzol (Invitrogen) according to the recommendation of the supplier and resuspended in RNase-free water. For reverse transcription–quantitative real-time PCR (RT-qPCR), RNA concentrations were determined by spectrophotometric measurements.

Nitrite assay
RAW 264.7 cells (3.5 × 10⁶/ml) in phenol red-free DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution were seeded in 96-well tissue culture plates (Nunc, Roskilde, Denmark). Following 20 h of incubation to allow adherence, cells were washed with cell medium and first incubated for 1 h with GKY25 or WFF25 at the indicated concentrations, washed twice, and stimulated for another 2 h with 20 ng/ml E. coli (0111:B4) LPS followed by another wash step. The level of NO in culture supernatants was determined after incubation for 20 h. In aqueous solutions, such as cell medium, NO rapidly oxidizes to nitrite (31), which was measured using the Griess reaction (Sigma-Aldrich) as described previously (28).

Cytokine assay
The levels of IL-6, IL-10, TNF-α, and IL-12p40 were measured either in supernatants of primary monocytes or human blood (anticoagulated with lepurdin, 50 mg/ml) stimulated with 100 ng/ml E. coli LPS with or without GKY25, using CytoSets (Invitrogen), according to the manufacturer’s instructions. Cytokines in cell supernatants of RAW 264.7 cells were determined using a mouse inflammation kit (BD Biosciences, San Jose, CA) according to the manufacturer’s protocol.

QUANTI-Blue assay
RAW-Blue cells (1 × 10⁶/ml) were allowed to adhere overnight before they were stimulated with 10 ng/ml E. coli (0111:B4) LPS with or without peptides in various fashions (see specific for Results). THP1-Xblue-CD14 cells (1 × 10⁶/ml) were stimulated with 100 ng/ml E. coli LPS, 10 μg/ml zymosan, 1 μg/ml purified lipoteichoic acid (LTA), or 1 μg/ml E. coli–derived peptidoglycan (PGN-EB; all from InvivoGen), with or without the immediate addition of GKY25, GKY24m, WFF25, or IVE25. NF-κB activation was determined 20 h later by using the QUANTI-Blue assay (InvivoGen). In brief, through activation of the cells by various antagonists, NF-κB/IB-1 activation is induced, leading to secreted embryonic alkaline phosphatase (SEAP) into the cell supernatant. For detection, the cell supernatant and a SEAP detection reagent (QUANTI-Blue) were mixed and the absorbance was measured at 600 nm.

RT-qPCR analysis
cDNA was synthesized from 1 μg RNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instruction. IFN-β, CXCL10, and GAPDH expression was analyzed by using the iQ SYBR Green Supermix kit (Bio-Rad Laboratories) on a CFX96 (Bio-Rad Laboratories). Samples were run in triplicates in 15 μl reaction volumes containing 1× iQ SYBR Green Supermix, 0.3 μM of each primer, and 1:10 diluted cDNA template. The following primers were used: mouse IFN-β, 5′-CAGCTTCAAGAAGGACCAGAC-3′ and 5′-GGCGATGTGAATCTTCTTCATC-3′; mouse CXCL10, 5′-CAAGTGTCGTCGTCATTCTTT-3′ and 5′-GGCCCTCGAGGAGTTGATTTCA-3′; mouse GAPDH, 5′-TGGACACCACTGCTTGAAG-3′ and 5′-GGATGGCAGGATGAGTGC-3′. Amplification was performed at 55°C for 40 cycles in an iCycler iQ5 optical system software (Bio-Rad Laboratories). Expression of genes of interest was quantified by applying the formula ΔΔCt = ΔCt (target, untreated) − ΔCt (target, treated) − ΔCt (GAPDH, untreated) (32).

Dimerization assay
RAW 264.7 cells (1 × 10⁶/ml) were seeded into 24-well plates (Nunc). After adherence, cells were stimulated with 1 μg/ml E. coli (0111:B4) LPS in presence of 10 μg/ml GKY25 or IVE25 for 30 min at 37°C. Next, cells were washed twice with cold PBS; 1% (v/v) BSA (Sigma-Aldrich) and stained at 4°C for 30 min using a monoclonal rat anti-mouse TLR4/MD2-PE Ab (clone MTS510, BD Biosciences) or the corresponding isotype control IgG2a-PE (BD Biosciences). Cells were analyzed using the FACSCalibur (BD Biosciences) in combination with FlowJo v.9.3.1 software.

Phosphorylation assay
THP1-Xblue-CD14 (1 × 10⁶/ml), incubated in six-well plates in RPMI 1640 medium with 10% FBS, were stimulated with 1 μg/ml E. coli (0111:B4) LPS in the presence of 10 μg/ml GKY25 or IVE25 for 1 h at 37°C. After stimulation, cells were washed with PBS and lysed for 15 min at 4°C using lysis buffer 6 from the Proteome Profiler array kit (R&D Systems, Minneapolis, MN). The cell lysate was centrifuged at 14,000 × g for 5 min and the supernatant was analyzed by using the Proteome Profiler human kinase array kit (R&D Systems) according to the manufacturer’s instructions.

Binding of peptide to cell lines and primary cells
RAW 264.7 cells (1 × 10⁶/ml) were seeded into 24-well plates and incubated overnight to allow adherence. Cells were then incubated with the indicated concentrations of TAMRA-labeled (T-)GKY25, T-GKY24m, or T-WFF25 for 15 or 30 min at 37°C or 4°C. In other experiments, RAW 264.7 cells were incubated with T-peptides in the presence of 1 μg/ml E. coli (0111:B4) LPS or 10 μg/ml cytochalasin B (Sigma-Aldrich). Upon incubation, cells were
washed twice, suspended in PBS, and analyzed by flow cytometry. To evaluate peptide binding ex vivo, human lepentin-treated blood, diluted with HEPES buffer (1:3), was incubated with T-GKY25 for 30 min with or without 1 μg/ml E. coli (0111:B4) LPS. After lysis of erythrocytes (using Ulti-Lyse erythrocyte lysing reagent, Dako, Carpinteria, CA), the binding of peptide to monocytes was determined using a FACScalibur system.

Confocal microscopy

RAW 264.7 cells and CD14⁺ human monocytes (1.5 × 10⁵ cells/well) were seeded in eight-well chamber slides (Nunc) and allowed to adhere. E. coli T-IVE25 in cell medium for 30 min at 37°C or 4°C in presence of 1 × 10⁶ cells were incubated with 1 μM T-GKY25, T-WFF25, or T-IVE25 in cell medium for 30 min at 37°C or 4°C in presence of 1 μg/ml E. coli (0111:B4) LPS or 10 μM cytochalasin B as indicated in Results. CD14⁺ human monocytes were incubated with 1 μM T-GKY25, T-WFF25, or T-IVE25 in cell medium for 30 min at 37°C or 4°C in presence of 1 μg/ml E. coli (0111:B4) LPS. After washing with PBS, cells were fixed with 2% formaldehyde for 30 min at 4°C. Intraperitoneal cells from mice were treated as described above, except that the monocyte/macrophage population was stained after fixation with a F4/80 Alexa Ab in PBS with 0.5% BSA (1:100; Affymetrix/eBioscience, San Diego, CA). Fixed cells were washed with PBS and mounted with ProLong Gold with DAPI (Invitrogen) to stain the nuclei blue. Mounted samples were examined using an LSM 510 META inverted confocal laser-scanning microscope (Zeiss, Jena, Germany) with a ×63-1.25 numerical aperture oil objective. Images were obtained using a diode pump solid-state laser at 405 and 561 nm to detect DAPI (350 nm) with a (420–480 nm) bandpass excitation filter and TAMRA (544 nm) with a (>561 nm) longpass excitation filter. Images were collected with Zen 2009 software and analyzed by using ImageJ software (version 1.46).

Electron microscopy

For transmission electron microscopy and visualization of the interaction of GKY25 with LPS and cells, RAW 264.7 cells were incubated for 30–60 min with 20 μM GKY25 and LPS (1 μg/ml) using different experimental settings at 37°C. The cells were harvested, centrifuged at 250 × g, and resuspended in fixation solution. Sections mounted on gold grids were blocked with 50 mM glycine, incubated for 15 min with 5% goat serum in 0.2% BSA-c in PBS (pH 7.6), and then incubated overnight at 4°C with polyclonal Abs against a C-terminal region of thrombin (VFR17, VFRKKWQKVIDQP-FGE; 1 mg/ml, LifeSpan BioSciences, Seattle, WA), or TLR4 (10 μg/ml; Affymetrix/eBioscience). Next, grids were washed, incubated for 2 h at 4°C with 1 μg/ml various species specific gold-labeled IgGs (BBI Solutions, Cardift, U.K.), washed, and postfixed in 2% glutaraldehyde. Finally, sections were washed with water and poststained with 2% uranyl acetate and lead citrate. Samples were examined with an FEI/Philips CM 100 electron microscope (operated at 8 kV accelerating voltage) connected to an Olympus Veleta camera.

RESULTS

GKY25 reduces LPS-induced cytokine production

Previous studies have shown that the peptide GKY25 inhibits proinflammatory responses, leading to reduced lung damage and increased survival in mouse models of endotoxin shock and P. aeruginosa sepsis.
Of relevance is that monocytes and macrophages are important producers of cytokines in blood in response to bacterial stimuli (1). To assess the anti-endotoxin effects of GKY25 on primary human monocytes, purified monocytes were stimulated with 100 ng/ml _E. coli_ LPS together with GKY25 and the levels of cytokines were determined after 20 h in the cell supernatants. GKY25 reduced the levels of IL-6, TNF-α, IL12p40, and IL-10 in a dose-dependent manner (Fig. 1A). When added to human blood, GKY25 also reduced the release of the proinflammatory cytokines IL-6, TNF-α, IL12p40, and IL-10 (Fig. 1B). Thus, the anti-inflammatory effects of GKY25 observed in human blood are in accordance with the data on isolated monocytes and show that GKY25 is able to inhibit LPS also in a physiologically relevant environment. Further studies demonstrated that mRNA expression of the proinflammatory cytokine IFN-β and chemokine CXCL10 was reduced when LPS-stimulated RAW 264.7 cells were subjected to GKY25 (Fig. 1C).

**GKY25 reduces LPS-induced NF-κB activation**

As outlined above, LPS-induced cytokine production is dependent on the activation of NF-κB (6). Therefore, the effect of GKY25 on LPS-induced NF-κB and AP-1 activation was investigated in more detail by using specific reporter cell lines. The data demonstrate that NF-κB/AP-1 activation in LPS-stimulated mouse macrophages (RAW-Blue cells) was significantly inhibited by increasing concentrations of GKY25 (Fig. 2A). In contrast, the peptide WFF25, which binds LPS with higher affinity than GKY25 (17, 33, 34), and IVE25, which is derived from the N terminus of thrombin and does not bind LPS, did not block NF-κB/AP-1 activation (Fig. 2A). Furthermore, addition of GKY25 2 h after LPS stimulation was sufficient to reduce NF-κB/AP-1 activation, whereas WFF25 and IVE25 did not show any inhibitory effects (Fig. 2B).

Next, we tested whether pretreatment of macrophages with peptide affected LPS-induced cell responses. RAW-Blue cells

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**FIGURE 2.** Inhibition of NF-κB/AP-1 activation by GKY25. Cells were stimulated as indicated below and the NF-κB/AP-1 activity was determined 20 h after addition of stimuli. NF-κB/AP-1 activation induces production of SEAP. SEAP in cell supernatants was mixed with QUANTI-Blue reagent and absorbance was measured at 600 nm. (A) RAW-Blue cells were stimulated with 10 ng/ml _E. coli_ LPS in the presence of the indicated concentrations of GKY25, WFF25, and IVE25. Mean ± SEM. _n_ = 4. *_p_ < 0.05 one-way ANOVA. (B) RAW-Blue cells were stimulated with 10 ng/ml _E. coli_ LPS. GKY25, WFF25, or IVE25 (all at 10 μM) was added to cells in LPS-containing medium at the indicated time points. Mean ± SEM. _n_ = 4. *_p_ < 0.05 one-way ANOVA. (C) RAW-Blue cells were incubated for 1 h with different concentrations of GKY25 or WFF25 followed by removal of peptides and wash prior to addition of 10 ng/ml _E. coli_ LPS for 20 h and determination of NF-κB/AP-1 activity. Mean ± SEM. _n_ = 4. *_p_ < 0.05 one-way ANOVA. (D and E) RAW 264.7 cells were incubated with GKY25 or WFF25 for 1 h, washed, and stimulated for 2 h with 20 ng/ml _E. coli_ LPS. After another 20 h incubation, NO (D) and IL-6 and IL-10 (E) were determined in cell supernatants. Mean ± SEM. _n_ = 3. **_p_ < 0.01, ***_p_ < 0.001, one-way ANOVA.
were incubated with GKY25 or WFF25 for 1 h, allowing peptide binding to the cells. Subsequently, the medium was removed and cells were washed before *E. coli* LPS (10 ng/ml) was added and cell activation measured. Preincubation of macrophages with GKY25 (40 μM) significantly reduced NF-κB/AP-1 activation (27 ± 4.2% reduction; *p* < 0.029, Mann–Whitney *U* test), whereas 40 μM WFF25 did not show any inhibitory effects (1.8 ± 3.7% reduction) (Fig. 2C). Pretreatment experiments with peptides present during LPS stimulation yielded NF-κB/AP-1 inhibition (Supplemental Fig. 1) similar to experiments in which LPS and peptide were added simultaneously (Fig. 2A). To evaluate effects of pretreatment with GKY25 and WFF25 using a short exposure time to LPS, thus avoiding a continuous LPS stimulation during incubation, RAW 264.7 cells were pretreated for 1 h with GKY25 or WFF25, washed, and incubated with *E. coli* LPS (20 ng/ml) for 2 h. Twenty hours later, NO production and cytokines were analyzed in the cell supernatants. Compatible with the initial pretreatment experiments, GKY25, but not WFF25, significantly inhibited LPS-induced NO release (Fig. 2D) and reduced IL-6 and IL-10 levels (Fig. 2E). MTT assays using the above peptide doses showed no reduction of cell viability, thus excluding that the above observed inhibitory effects were due to peptide-mediated toxic effects on these cells (data not shown). These data demonstrate that GKY25 exerts anti-inflammatory effects while being associated with cells.

**Effects of GKY25 on phosphorylation of MAPKs**

LPS stimulation of monocytes and macrophages induces the activation of the NF-κB and AP-1 pathway via the phosphorylation of MAPKs. To investigate whether GKY25 has an influence on LPS-induced MAPK phosphorylation, human monocytes were stimulated with LPS with or without GKY25 or with the control peptide IVE25. The activation of p38α and JNK1/2/3 MAPKs was significantly decreased in the presence of GKY25 when compared with monocytes stimulated with LPS alone (Fig. 3). Additionally, GKY25 significantly reduced the phosphorylation of HSP27 and the activation of the transcription factor c-Jun (Fig. 3). The peptide alone did not significantly influence the phosphorylation of ERK1/2 during LPS stimulation. Furthermore, the control peptide IVE25 did not reduce phosphorylation during LPS stimulation (data not shown).

**Effects of GKY25 on LPS-induced TLR4/MD2 dimerization**

Upon binding of LPS to the TLR4/MD2 complex, dimerization of the receptors occurs (5). To test whether GKY25 prevents dimerization of TLR4 during LPS stimulation, experiments employing FACS and utilizing a specific anti-TLR4/MD2 Ab were performed. This Ab recognizes the receptor conformation and cannot bind properly upon LPS-induced dimerization (35). The histograms in Fig. 4A show the unstained isotype control (black) and the PE-labeled anti-TLR4/MD2 control (blue). LPS-induced dimerization was demonstrated by a reduction in fluorescence intensity (green). Incubation of RAW 264.7 cells with GKY25 for 30 min allowing the peptide to interact with the cells, before the addition of LPS for 30 min (red), prevented TLR4 dimerization. This indicates that LPS binding to the receptor was disturbed when GKY25 was present at the cells prior to the addition of LPS (histogram; GKY25 > LPS). The control peptide IVE25 did not affect the TLR4 dimerization (histogram; IVE25 > LPS). In a similar experiment, peptides were preincubated together with LPS before this mix was added to the cells. Also in this case, GKY25 was found to prevent TLR4 dimerization by binding to LPS (histogram; GKY25 + LPS) whereas IVE25 (histogram; IVE25 + LPS) did not influence the TLR4 dimerization.

Electron microscopy studies were employed to further gain mechanistic insights into how GKY25 prevents the binding of LPS to TLR4. For this purpose, RAW 264.7 cells were incubated with GKY25 and/or LPS for 30 min, washed, fixed, and finally stained with gold-labeled Abs against GKY25, LPS, and TLR4 (Fig. 4B). TLR4 receptors (medium-sized particles) were found to be distributed as a monomer on the cell surface (control), whereas the addition of *E. coli* LPS (large particles) induced dimerization of TLR4. GKY25 (small particles) was distributed on the cell surface and also found in the cytoplasm, and it did not affect dimerization of TLR4. In subsequent experiments, GKY25 was preincubated with the cells, washed, and LPS was added after 30 min (GKY25 > LPS). The results showed that GKY25 was bound to the cells, and concomitantly it was observed that TLR4 dimerization was blocked. Furthermore, it was noted that the peptide was inter-

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**FIGURE 3.** GKY25 reduces the phosphorylation of MAPKs. THP-1 XBlue CD14 cells were treated for 1 h with 1 μg/ml LPS in the presence of 10 μM GKY25 or IVE25. p38α, JNK1/2/3, HSP27, and c-Jun were measured by using a phospho-kinase assay. Mean ± SEM. *n* = 2–4. **p < 0.01, ***p < 0.001; test with Welch’s correction.
nalized in early endosomes, together with LPS and TLR4. In a different setting, GKY25 was added simultaneously with LPS to RAW 264.7 cells (GKY25 + LPS). Similarly to the data above, GKY25 was found localized with LPS on the cell surface, preventing TLR4 dimerization. Additionally, early endosomes contained GKY25 together with LPS and TLR4.

**FIGURE 4.** Effects of GKY25 on TLR4 dimerization. (A) Flow cytometry analysis of TLR4 dimerization using a specific TLR4/MD2-PE (MTS51) Ab recognizing only monomeric TLR4/MD2 complexes. RAW 264.7 cells were incubated with 10 μM GKY25 or IVE25 and after 30 min E. coli LPS (1 μg/ml) was added (GKY25 > LPS, IVE25 > LPS). In another experimental setting GKY25 or IVE25 was preincubated with LPS (GKY25 + LPS, IVE25 + LPS). Histogram shows the isotype control (black) and the PE-labeled anti-TLR4/MD2 expression (blue), LPS (green), and LPS with GKY25 (red) or IVE25 (orange). One representative experiment out of three is shown (n = 3). (B) For transmission electron microscopy RAW 264.7 cells were incubated with E. coli LPS (1 μg/ml) and GKY25 (20 μM) in two different settings. Left panel, Images show gold-labeled Abs against mouse TLR4 (medium) (control), gold-labeled Abs against E. coli LPS (large) together with TLR4 (LPS), and gold-labeled VFR17 Abs against GKY25 (small) with TLR4 (GKY25). Right panel, RAW 264.7 cells were preincubated with GKY25 and LPS was added after 30 min to the cells for and additional 30 min (GKY25 > LPS). GKY25 and LPS were added to the cells simultaneously for 30 min (GKY25 + LPS). Scale bar, 200 nm.
Interactions of GKY25 with macrophages

Next, we further studied cell binding of GKY25. For this purpose, RAW 264.7 cells were incubated for 30 min at 37°C with increasing concentrations of T-GKY25 or T-WFF25. Cell binding was analyzed by flow cytometry. Mean ± SEM. n = 3–4. (B) Binding of 1 μM T-GKY25 and T-WFF25 to RAW 264.7 cells at 37°C after indicated incubation times was visualized by confocal microscopy. The nucleus DNA was stained in blue with DAPI and the TAMRA peptide by red fluorescence. One representative image out of three experiments is shown. Scale bar, 5 μm. RAW 264.7 cells were incubated with of 10 μM T-GKY25 and T-WFF25 at 37°C (C) or 4°C (D) and binding was determined at indicated time points. Mean ± SEM. n = 3. (E) Binding of 1 μM T-GKY25 and T-WFF25 to RAW 264.7 cells after 15 min of incubation at indicated temperatures was analyzed by confocal microscopy. One representative image out of three to four experiments is shown. Scale bar, 10 μm.

Interactions of GKY25 with macrophages

Next, we further studied cell binding of GKY25. For this purpose, RAW 264.7 cells were incubated for 30 min at 37°C with increasing concentrations of T-GKY25 and T-WFF25. Using FACS, a significantly higher dose-dependent increase in median fluorescence intensity (MFI) was observed for T-GKY25 as compared with T-WFF25 (Fig. 5A), which corresponds to the pretreatment studies above. The presence of FBSi (10%) did not significantly influence the peptide binding at 37°C (data not shown). Confocal microscopy analyses confirmed the FACS results. As shown in Fig. 5B, T-GKY25 (1 μM) was detected inside macrophages after incubation for 15–30 min, showing a homogeneous distribution. T-WFF25 (1 μM) was significantly less bound, whereas T-IVE25 did not show any binding to the RAW 264.7 cells at all (Supplemental Fig. 2). FACS analyses demonstrated the time dependency of the GKY25 interaction. After 60 min at 37°C, the maximum binding was reached for T-GKY25, but not for T-WFF25 (Fig. 5C), with the results corresponding to those obtained using confocal microscopy (Fig. 5B). Additionally, the temperature dependence of peptide–cell interactions was investigated, showing that the internalization of T-GKY25 was significantly reduced at 4°C (Fig. 5D, 5E). This suggests that factors such as membrane fluidity, temperature-induced phase separation and lateral ordering transitions, or temperature effects on raft size and composition could be important for peptide uptake. Next, experiments using cytochalasin B were performed to investigate whether peptide uptake may depend on endocytosis. Confocal microscopy as well as FACS analysis showed that cytochalasin B affected the uptake of T-GKY25, as well as T-WFF25, only to a minor extent. (Supplemental Fig. 3, Table I).

GKY25 binds to monocytic cells ex vivo and in vivo

Having shown a direct interaction of GKY25 with macrophages in vitro, the binding of T-GKY25 to monocytes during ex vivo conditions was investigated. Therefore, human blood was incubated with T-GKY25 for 30 min and the MFI of monocytes was determined by flow cytometry. In agreement with the in vitro data, GKY25 was found to bind to human monocytes ex vivo (Fig. 6A).

Table I. Effect of cytochalasin B on peptide interactions with RAW 264.7 macrophages

<table>
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<tr>
<th>Peptides</th>
<th>MFI Signal Reduction with Cytochalasin B (%)</th>
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<tr>
<td>T-GKY25</td>
<td>124.9 ± 16.3 90.8 ± 7.3 26.3 ± 4.1</td>
</tr>
<tr>
<td>T-WFF25</td>
<td>21.8 ± 5.9 15.4 ± 3.0 25.2 ± 9.4</td>
</tr>
</tbody>
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Ten micromolar cytochalasin B and peptides were used. Data represent mean ± SEM of three independent experiments.
The presence of LPS (1 μg/ml) did not significantly alter peptide binding. Confocal microscopy analysis of primary monocytes showed that the binding and uptake of GKY25 was similar to the results obtained with the RAW 264.7 cells (Fig. 6B). Furthermore, addition of LPS did not change the distribution of the peptide within the cell. To investigate a possible cell binding of GKY25 also in vivo during endotoxin shock, C57BL/6 mice were injected with LPS, followed by injection of T-GKY25 after 30 min. FACS analysis of cells from peritoneal washes indeed showed a significant binding of T-GKY25 to monocyte/macrophage populations from peritoneal washes were analyzed in the flow cytometer. Mean ± SEM. n = 4. *p < 0.05 one-way ANOVA (C) or by confocal microscopy (D). For confocal microscopy the monocyte population was stained with the Ab F4/80 Alexa 488 (green), nuclear DNA was stained with DAPI (blue), and T-GKY25 was used for visualization of GKY25 (red) (19 mononuclear cells were inspected in five separate view fields, p = 0.015, paired t test). Scale bar, 5 μm.

GKY25 reduces peptidoglycan, LTA, and zymosan-induced NF-κB activation

Next we tested whether GKY25 reduces the NF-κB activation induced by other microbe-derived agonists. Similarly to mouse macrophages (Fig. 2B), GKY25 significantly reduced the NF-κB/AP-1 activation in THP-1 XBlue-CD14 cells in response to LPS, PGN-EB, LTA, and zymosan (Fig. 7). Moreover, we noted that WFF25, despite its high LPS-binding capacity (33), reduced the response to LPS to a minor extent, and a similar effect was noted with IVE25. Thus, these data demonstrate that GKY25, besides exerting broad antimicrobial properties (29), also prevents cell activation induced by various stimuli from Gram-negative and Gram-positive bacteria as well as fungi by reducing the TLR4- and TLR2-induced NF-κB/AP-1 activation.

Discussion

The peptide GKY25 exerts multiple biological effects involving direct bacterial killing, inhibition of coagulation, and blocking of LPS-induced cytokine responses in vitro and in vivo (17, 29). The mode of action of GKY25 on LPS-mediated signaling thus depends on multiple and interdependent molecular mechanisms. As demonstrated previously, the peptide binds to LPS, for example, via the lipid A epitope of LPS (33), compatible with its previously reported anti-endotoxic effect (17, 30). The observation that the LPS-binding peptide WFF25 showed considerably less binding to mononuclear cells, with significantly lower LPS inhibition, provided further evidence that combined endotoxin and cell interactions are involved in the anti-inflammatory activity of the endogenous GKY25 peptide. Furthermore, the results that the GKY25 was able to block not only effects of LPS, but also peptidoglycan, LTA, and zymosan, suggest a possible role for the peptide as an inhibitor of other microbial-derived and anionic agonists. In combination with previous findings showing that GKY25 inhibits activation of mouse macrophages by TNF-α, as well as ODN1826 (a TLR9 agonist) (17), the data further indicate that the inhibitory activity is not restricted to a specific TLR.

FIGURE 6. Effects of GKY25 ex vivo and in vivo. (A) Binding of 20 μM T-GKY25 to monocytes in lepirudin-treated blood with or without simultaneous addition of E. coli LPS (1 μg/ml) was determined after 30 min of incubation at room temperature by flow cytometry (MFI). Mean ± SEM. n = 4. *p < 0.05 one-way ANOVA. (B) Confocal microscopy images of representative primary human monocytes incubated for 30 min with buffer (control) or 10 μM T-GKY25 alone or in the presence of 1 μg/ml E. coli LPS. For T-GKY25 only, 28 cells were T-GKY25+ of 29 cells inspected in five separate view fields (paired t test, p = 0.0008). For GKY25 and LPS, 146 cells were T-GKY25+ of 151 cells inspected in three separate view fields (p = 0.0042, paired t test). T-IVE25 (10 μM) was used as negative control. Scale bar, 5 μm. (C) C57BL/6 mice were injected i.p. with 1 mg/kg LPS. Thirty minutes after LPS injection mice were treated i.p. with 0.5 mg T-GKY25 or buffer. After 1 h mice were sacrificed and the monocyte/macrophage populations from peritoneal washes were analyzed in the flow cytometer. Mean ± SEM. n = 4. *p < 0.05 one-way ANOVA (C) or by confocal microscopy (D). For confocal microscopy analysis the monocyte population was stained with the Ab F4/80 Alexa 488 (green), nuclear DNA was stained with DAPI (blue), and T-GKY25 was used for visualization of GKY25 (red) (19 mononuclear cells were inspected in five separate view fields, p = 0.015, paired t test). Scale bar, 5 μm.

FIGURE 7. GKY25 inhibits NF-κB activation by pathogen-associated molecular patterns. THP-1 XBlue-CD14 cells were stimulated for 20 h with 100 ng/ml E. coli LPS, 1 μg/ml of PGN-EB, LTA, or 10 μg/ml zymosan in the presence of 10 μM GKY25, WFF25, or IVE25 before the NF-κB activity was measured. Dashed line indicates values for untreated cells. Mean ± SEM. n = 3–4. *p < 0.05 one-way ANOVA. **p < 0.01, ***p < 0.001.
Additionally, new evidence presented in this study shows that GKY25 may also be bound to macrophages and monocytes, in turn causing LPS binding/scavenging and interfering with downstream signaling by MAPKs p38α and JNK1/2/3. Thus, considering the latter, results from experiments seeking to explore the possibility of such cell-associated effects showed that pretreatment of monocytes cells with GKY25 indeed reduced the following LPS-induced NF-kB/AP-1 activation. Furthermore, FACS, confocal microscopy, and electron microscopy analyses, addressing GKY25 binding to macrophages, demonstrated a direct interaction of the peptide with the cells. Interestingly, the finding that GKY25 was present together with LPS in early endosomes pointed to a role in TLR4-TRAM/TRIF-dependent signaling, and indeed it was found that GKY25 also reduced LPS-induced production of IFN-β and CXCL10. Taken together, the results show that GKY25 affects both MyD88-dependent and -independent signaling pathways.

The finding that GKY25 binding was significantly reduced at low temperature suggests that membrane fluidity is a possible factor enabling the peptide’s cell binding. However, because temperature also affects receptor-mediated endocytosis, the presence of other uptake mechanisms cannot be ruled out. Likewise, the fact that cytochalasin B did not significantly affect binding, as assessed by FACS and confocal microscopy, does not exclude a specific internalization route, perhaps preceded by initial membrane interactions. For example, it is possible that GKY25 may bind, as a ligand, to a carrier protein in the membrane or in plasma and get shuttled into the monocytes and macrophages, as shown in Fig. 5. Nevertheless, the observation that the peptide also binds to neutrophils (36) suggests an affinity to a common structure on monocytes/macrophages and neutrophils. Clearly, whether the peptide also interacts specifically with other receptors or mediators at cell surfaces or in the cytoplasm, or whether it interferes with other pathways such as the autophagic clearance pathway, is beyond the scope of the present study and needs to be further investigated in follow-up studies.

It is notable that apart from direct receptor interactions, possibly followed by endocytosis, some cationic peptides have been reported to be taken up by eukaryotic cells through other mechanisms, including membrane internalization such as that described for cell-penetrating peptides (37). The question of how these latter peptides reach the cytoplasm of cells is still widely debated, with both translocation and endocytosis reported as internalization pathways (38). Regarding the former, the amphiphatic profile and charge distribution of amphiphilic peptides influences peptide-mediated perturbation mechanism on the membrane bilayer (39), as well as translocation (37). Through adopting a random coil conformation, GKY25 has the ability to adopt an amphipathic helical conformation in specific environments, such as in the presence of LPS or negatively charged liposomes (29, 33). It is also notable that the peptide contains a helix-stabilizing N-cap motif as well as interspersed hydrophobic residues enabling helix formation (40). Thus, GKY25 shares many of the characteristics with other membrane-active peptides of diverse functions and structures (41).

From a physiological perspective, it is notable that the peptide dose required to achieve anti-inflammatory effects in vitro was in the range of 1–5 μM. Given that the physiological concentration of human prothrombin in plasma is ~1.5 μM, proteolysis of the molecule could lead to the generation of C-terminal thrombin fragments at effective concentrations also in vivo. Furthermore, the fact that thrombin binds with high affinity to fibrin, and that proteolysis of this matrix by neutrophil elastase leads to release of C-terminal thrombin fragments (29), further implies a role of such thrombin peptides in vivo. Indeed, in ongoing studies, C-terminal fragments of thrombin similar in size to GKY25 have been identified in acute wound fluids derived from patients after surgery. Current investigations address the exact structures of these thrombin fragments and their physiological concentrations and roles during wounding and infection (data not published).

Besides their physiological roles, such thrombin-derived peptides may have potential as therapeutics, and exploration of their mode of action is therefore clearly motivated from a clinical and developmental perspective. Previous results show that the prototypic GKY25 inhibits the proinflammatory response, reduces tissue factor–mediated coagulation, as well as mortality in experimental models of endotoxin shock and P. aeruginosa sepsis (17). Furthermore, in a nonbacterial but TLR4-dependent murine pancreatitis model, GKY25 demonstrates potent anti-inflammatory effects (36). In the present study, the insights on the peptide’s interference with TLR signaling, along with the demonstration that the peptide binds to monocytes cells also in vivo, thus indicate that GKY25 mediates its activity during infection and inflammation via multiple interactions involving bacteria, endotoxins, and inflammatory cells.

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Disclosures
A.S. is a founder of in2cure AB, a company developing anti-inflammatory peptides of thrombin for human therapy. The other authors have no financial conflicts of interest.

References


