Peptides 33 (2012) 1–8

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Antimicrobial decapeptide KSL-W enhances neutrophil chemotaxis and function

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ARTICLE INFO

Article history:
Received 16 November 2011
Received in revised form
30 November 2011
Accepted 30 November 2011
Available online 16 December 2011

Keywords:
Antimicrobial peptide
Multifunctional
Neutrophil
Chemotaxis
Actin polymerization

ABSTRACT

Mammalian cationic antimicrobial peptides have received increased attention over the last decade, due to their prokaryotic selectivity and decreased risk of microbial resistance. In addition, antimicrobial peptides display differential biological effects on mammalian immune cell function, such as migration, adhesion, and modulation of respiratory burst, which make them even more attractive as therapeutic agents. Synthetic combinatorial libraries provide a time-efficient and cost-effective source for these diverse molecules. The novel synthetic antimicrobial peptide, KSLW (KKVVFWVKFK-NH_2), has been shown to display a broad spectrum of antimicrobial activity against Gram (+) and Gram (−) bacteria, fungi and viruses. In this study, we evaluated the alternative biological activity of the decapeptide on neutrophil migration and function. KSLW was demonstrated to be chemotactic for neutrophils in micromolar amounts, and neutrophil treatment with KSLW, after 1 min, resulted in significant increases in F-actin polymerization. KSLW was shown to inhibit oxygen radical production in PMA- and LPS-stimulated neutrophils. Future studies, to determine if KSLW regulates neutrophil phagocytosis, adhesion, and apoptosis, or examining the effect of KSLW on other mammalian cell types, such as cell populations of healing-impaired wounds, would provide significant insight for the potential therapeutic strategies offered by antimicrobial peptides.

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1. Introduction

During the last decade, attention has focused on antimicrobial peptides due to their prokaryotic selectivity and minimal risk of microbial resistance. Mammalian endogenous cationic antimicrobial peptides such as defensins, cathelicidins, and histatins have been shown to display a broad range of activity against Gram-positive and Gram-negative bacteria, as well as against fungi and enveloped viruses [1]. In addition to their direct antimicrobial activity, endogenous antimicrobial peptides have been characterized as multifunctional molecules, with functions in inflammation, wound repair, and regulation of the innate [23] and adaptive [1,23] immune responses. It has become increasingly apparent that cationic antimicrobial peptides function via alternative mechanisms in response to pathogens, with biological activities contributing to both the recruitment of immune cells and direct inactivation of pathogens [10]. The α- and β-defensins, which are members of the best-studied class of human antimicrobial peptides [10,21], display chemotactic properties for monocytes, T-cells, dendritic cells and mast cells [10,21,38]. The neutrophil-derived proline-arginine (PR)-rich antibacterial peptide PR-39 possesses several distinct functional properties, and has been shown to function as a chemoattractant to polymorphonuclear leukocytes [17] and alter oxidative burst responses by inhibiting NADPH oxidase [35,48].

The development of synthetic combinatorial libraries as a source for antimicrobial peptides has gained interest as an alternative approach to overcoming time constraints and limitations of designing novel antibacterial and antifungal agents, with desired structure in the lipid membranes [14]. The decapeptide KKVVFKVKFK (KSL), was originally identified from combinatorial libraries with activity against *Candida albicans*, and later demonstrated to display a broad range of activity against bacteria [14]. The much smaller KSL when compared with the *Xenopus*-derived peptide antibiotic magainin II, was shown to have lower minimum inhibitory concentrations (MICs) for *C. albicans* (almost seven times lower), as well as nine tested bacterial species [5]. Stability studies of KSL and its analogs revealed the critical peptide bonds susceptible to enzymatic cleavage, Lys^6-Val^7 and Phe^5-Lys^6, in human saliva and simulated gastric fluids, respectively [25]. The analog KSLW, whereby the sixth residue (lysine) is substituted

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0196-9781/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.peptides.2011.11.022

Report Documentation Page

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JAN 2012	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Antimicrobial decapeptide KSL-W enhances neutrophil chemotaxis and function	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Williams R. L., Sroussi H. Y., Leung K., Marucha P. T.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a REPORT	b ABSTRACT	c THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**8**	

with tryptophan proved to be most stable in saliva, while still maintaining its antimicrobial activity [25]. Because of the known multifunctional activities associated with many antimicrobial peptides, we became interested in examining the effect of KSLW on host cell functions, which result in pathogen inactivation. Particular interest developed in the regulatory activity of this novel synthetic antimicrobial decapeptide, via its potential effect on the inflammatory response, specifically leukocyte migration and oxidative metabolism. The purpose of this study was to examine the effect of KSLW on neutrophil chemotaxis and respiratory burst.

2. Materials and methods

2.1. Synthesis and Preparation of KSLW decapeptide

The antimicrobial decapeptide KSLW (KKVVFWVKFK-NH2) (lot 05US11311-B, molecular weight [MW] ~ 1307) was synthesized, using standard solid-phase procedures, using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on a model 90 automatic peptide synthesizer [18] (Advanced ChemTech), by SynPep (Dublin, CA). Peptide purity was confirmed by mass spectrometry with the molecular mass found at 1308.3. The final product was stored in lyophilized form at −20 °C until use. For various experiments, stock solutions of KSLW were prepared at either 200 mg/ml, 20 mg/ml, or 2 mg/ml, in molecular grade water (Mediatech, Inc., Herndon, VA), RPMI 1640 (Cellgro®, Mediatech, Inc., Herndon, VA), or Hank's Balanced Salt Solution (HBSS) (SAFC Biosciences™, Lenexa, KS) and filter sterilized though 0.22-μm mixed cellulose esters membrane filters (Fisher Scientific, Fairlawn, NJ).

2.2. Isolation of neutrophils

Human peripheral blood was collected in 6.0 ml lithium heparin tubes (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ) and diluted with Dulbecco's phosphate buffered saline (DPBS) (BioWhittaker®, Lonza, Walkersville, MD). An equal volume of diluted blood was layered over a double gradient with specific gravities 1.077 and 1.119 (Histopaque®, Sigma–Aldrich™, St, Louis, MO), and centrifuged at 1730 rpm for 30 min. The neutrophil-rich fraction was removed and washed twice in 1× DPBS. Residual erythrocytes were lyzed via hypotonic-hypertonic rescue, yielding >97% neutrophil purity. All experiments were done within 2.5-h in vitro age of cell [15]. Cells were resuspended in either RPMI 1640 for migration studies or HBSS for FITC-phalloidin staining. Neutrophils used for intracellular oxidant studies were resuspended in 1× DPBS, and were not subjected to hypotonic lysis, during isolation.

2.3. Neutrophil chemotaxis

Chemotaxis assays were conducted with human neutrophils isolated from whole blood 3.3×10^6 cells/ml. Purified neutrophils were washed and resuspended in 1× RPMI 1640 containing L-glutamine, and quantified via counting on hemacytometer (Hausser Scientific, Horsham, PA). Then, 8.5×10^4 cells in 75 μl RPMI 1640 were transferred to the upper compartment (insert) of a Corning® HTS Transwell® 96-well plate, with a 3 μm-pore polycarbonate membrane (Corning, Inc., Corning, NY). Serial 1:2 dilutions of the synthetic antimicrobial peptide (KSLW) in 235 μl RPMI 1640, at an initial concentration of 2.0 mg/ml, were loaded in the bottom chambers of the wells. Plates were incubated at 37 °C and 5% CO_2 for 1.5 h in a cell culture CO_2 incubator (Thermo Fisher Scientific, Milford, MA). Relative comparisons were made with 10^{-9} M, 10^{-10} M and 10^{-11} M *N*-formyl Methionyl-Leucyl-Phenylanine (FMLP) (Sigma–Aldrich™), a known neutrophil chemotactic factor, and fresh RPMI 1640 media, as the negative control. Neutrophils migrating across the membrane were quantified by counting vital cells in suspension via hemacytometer, following staining with 0.4% Trypan Blue solution (Mediatech, Inc., Herndon, VA). Checkerboard analyses were conducted with KSLW concentrations ranging from 10^{-7} M to 10^{-4} M, to differentiate between chemotaxis and chemokinesis. Checkerboard migration levels were determined as ratios relative to the control 10^{-10} M FMLP. All migration studies correspond to at least three individual experiments conducted in quadruplicate.

2.4. Pertussis toxin treatment of neutrophils

Reconstituted *Bordetella pertussis* toxin containing 0.01 M sodium phosphate, pH 7.0, with 0.05 M sodium chloride, was added, at a concentration of 1.0 μl/ml, to purified neutrophils suspended in either RPMI 1640 for chemotaxis studies or HBSS for FITC-phalloidin staining. For the migration assays, the chemotactic responses of toxin-treated neutrophils were analyzed using the same parameters and volumes previously stated.

2.5. Neutrophil treatment and measuring actin polymerization

Purified human neutrophils were treated with HBSS, FMLP (10^{-7} M and 10^{-10} M), or various concentrations of the KSLW decapeptide. Neutrophils were exposed to the following concentrations of KSLW: 1.25×10^{-4} M (K4), 6.25×10^{-4} M (K5), 3.125×10^{-4} M (K6), 1.5625×10^{-4} M (K7), and 1.25×10^{-5} M (K 1:10) in 300 μl HBSS.

2.6. FITC-phalloidin staining of neutrophils

For the flow cytometry and fluorescence experiments, we prepared FACS buffer containing 1 mg/ml fluorescein isothiocyante-phalloidin with calcium and magnesium (Sigma–Aldrich™, St, Louis, MO), and 0.5% BSA (Sigma–Aldrich™) in 1× DPBS. Neutrophils were isolated, as previously described, and resuspended in HBSS. Then, 300-μl aliquots containing 9×10^5 cells were transferred into 1.5 ml Eppendorf centrifuge tubes and treated with different concentrations of KSLW containing 1.25×10^{-4} M, 6.25×10^{-4} M, 3.125×10^{-4} M, 1.56×10^{-4} M, and 1.25×10^{-5} M. Positive and negative control groups were treated with 10^{-7} M/10^{-10} M FMLP and HBSS, respectively. After 60 s of exposure, unstimulated and treated cells were fixed with 125 μl of neutral buffered formalin, at a final concentration of 3.7%, vortexed, and allowed to incubate at room temperature for 30 min. Fixed cells were then centrifuged for 5 min at 300 g and resuspended in 1 ml permeabilization buffer containing 0.1% Triton®-X-100 [$C_8H_{17}(C_6H_4)(OCH_2CH_2)_xOH$, Avg. MW 628 (Fisher Scientific)] in 1× DPBS. Following brief centrifugation, the supernatants were aspirated, and the cells were resuspended in 125 μl of permeabilization buffer and allowed to incubate for 5 min. Permeabilized cells were then transferred to 5-ml polypropylene round-bottom tubes (BD #2063, Becton Dickinson). One milliliter of FACS buffer was added to each tube and incubated on ice for 30 min, before flow cytometry or spectrofluorometric analysis. Unstained control neutrophils were resuspended in 1 ml of 1× DPBS.

2.7. Actin polymerization

F-actin content in unstimulated and FMLP-/KSLW-treated neutrophils was quantified by FITC-phalloidin fluorescence. For spectrometric analysis of actin polymerization, 150 μl of 1× DPBS containing 4.5×10^4 unstained neutrophils and 150 μl of FACS buffer solution containing 4.5×10^4 of FITC-phalloidin-stained neutrophils, were transferred to a black, 96-well, clear-bottom

plate (Corning, Inc.) and fluorescence was measured at excitation 488 nm/emission 538 nm. Fluorescence readings were normalized to unstained control cells. Data were collected and analyzed using SOFTmax® PRO software (Molecular Devices Corp., Sunnyvale, CA).

2.8. Flow cytometry

Neutrophils were fixed and stained as described above and analyzed using a BD LSR Benchtop Flow Cytometer (Becton Dickinson). Fluorescence was exited at 488 nm with a 20-mW laser power output and detected using a 530/28 bandpass emission filter. Voltage and gain settings were adjusted to produce a normalized fluorescence of unstained control cells. The data were analyzed to yield distribution histograms of cell number versus log fluorescence intensity and forward angle light scatter for a minimum of 10,000 cells per sample. Polystyrene beads, 10-μm diameter (Beckman-Coulter, Miami, FL) were used for calibration and normalization of the flow cytometer from experiment to experiment. All experiments were performed at least three times.

2.9. Inhibition of oxidative burst

The use of intracellular fluorochromes to monitor the production of oxidants by neutrophils is a popular and valuable research tool [32]. To determine the effect of KSLW peptide treatment on neutrophil oxidative burst, we performed spectrometric analysis using a 2,7-dichlorofluorescein diacetate (DCFH-DA) assay. Briefly, 50-μl aliquots of DPBS containing 0.5 μg of DCFH-DA were added to the wells of black, clear bottom 96-well plates. In the experimental wells, either *Eschericia coli* O55:B55 lipopolysaccharide (LPS) (Sigma–Aldrich) at 1 mg/ml, or phorbol myristate acetate (PMA) (Sigma–Aldrich) at 62.5 μg/ml were added to the mixture. KSLW peptide at final concentration of 2 mg/ml (after addition of cells) was then added to wells containing LPS and PMA, followed by serial 1:2 dilutions. Neutrophils were isolated, as described above, and resuspended in 1× DPBS. Then 50-μl aliquots containing 1.5×10^5 cells were added to wells containing LPS, PMA, and DPBS. Plates were incubated, for various time points following stimulation, at 37 °C and 5% CO_2. Spectrophotometric analyses were performed at baseline, 30 min, 1, 2, and 3 h following stimulation of neutrophils. Fluorescence was excited at 488 nm and emission detected at 530 nm. For the DCFH-DA assays, four individual experiments were performed in quadruplicate.

2.10. Statistical analyses

Data are expressed as the mean ± S.E.M. Differences between means were calculated by means of the Student's *t*-test for paired samples. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Neutrophil chemotaxis

To assess the chemotactic effect of KSLW antimicrobial peptide on human neutrophils, the migratory response was tested in transwell plates, as indicated above. Neutrophils demonstrated a capacity to migrate toward KSLW in a concentration-dependent manner, after 90 min of exposure. Typical responses [41] of neutrophils to the positive control, FMLP, occurred at nanomolar to micromolar levels, with the greatest response at 10^{-11} M, with marked reductions beyond the picomolar range (Fig. 1). We observed neutrophil chemotactic responses to KSLW concentrations ranging from 10^{-7} M to 10^{-3} M, with the highest level of migration (>90%) at 10^{-5} M (Fig. 2). Between 20% and 92% of the neutrophils added to the upper chambers of the transwell plates migrated into the bottom chamber, in response to 10^{-7}–10^{-3} M KSLW. At doses outside this range the chemotactic effect was absent, resulting in a bell-shaped dose–response relationship. The effective concentrations of KSLW generating neutrophil chemotactic responses, were similar to effective concentrations of other antimicrobial peptides, such as PR-39 [9,17], which displayed maximal effects at the micromolar level.

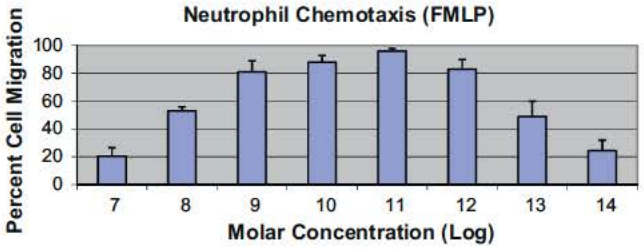

Fig. 1. Neutrophil chemotactic response to FMLP. The neutrophil response to the positive control, FMLP, occurred at nanomolar to picomolar concentrations, with the greatest response at 10^{-11} M, tapering beyond the picomolar range.

3.2. Pertussis toxin-sensitivity inhibits neutrophil migration toward KSLW

G-alpha$_i$ family G proteins are central to neutrophil migration and activation [43]. Pertussis toxin inhibits chemotaxis of neutrophils by preventing chemoattractant receptors from activating heterotrimeric G proteins in the Gi subfamily [26]. Pertussis toxin (PTX) inhibits Gα_i signaling by NAD^+-mediated ADP-ribosylation of a specific cysteine residue near the carboxyl terminus, as well as ADP-ribosylation-independent structural changes of Gα proteins [31]. To test whether the neutrophil migration toward KSLW is mediated through chemotactic receptor signaling mechanisms, we conducted migration assays with neutrophils treated with PTX. Incubation of neutrophils with PTX yielded negative responses to both 10^{-10} M FMLP and the various concentrations of KSLW tested (Table 1). In fact, less than one percent of neutrophils migrated toward either chemotactic factor, which suggests the involvement of chemotactic receptors in responses to KSLW.

3.3. Checkerboard analysis

To discriminate whether the observed neutrophil migration associated with KSLW peptide exposure was chemotactic or chemokinetic, as a result of unspecific responses, a standard checkerboard analysis was performed [3,29,44,45]. The checkerboard analysis revealed that neutrophil responses to KSLW possess

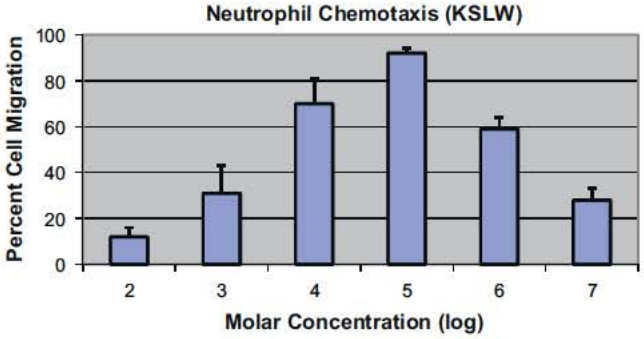

Fig. 2. Neutrophil chemotactic response to KSLW. Neutrophil migratory responses to KSLW occurred at concentrations ranging from 10^{-7} M to 10^{-3} M, with the highest level of migration at 10^{-5} M. Between 20% and 92% of the neutrophils added to the upper chambers of the transwell plates migrated into the bottom chamber in a dose-dependent manner.

Table 1
Pertussis toxin treatment blocks neutrophil migration.

Mean percent migration (%)		
Media/chemotactic factor (lower chamber)	Migrating neutrophils in RPMI (upper chamber)	Migrating PTX-treated neutrophils in RPMI (upper chamber)
RPMI	14	<1
K11 (KSLW 10^{-7} M)	28	<1
K10 (KSLW 10^{-6} M)	58.6	<1
K4 (KSLW 10^{-5} M)	92	<1
FMLP (Control 10^{-10} M)	96	<1

To test whether the neutrophil migration toward KSLW was mediated through chemotactic receptor signaling mechanisms, we conducted migration assays with neutrophils treated with PTX. Incubation of neutrophils with PTX yielded negative responses to both 10^{-10} M FMLP and the various concentrations of KSLW tested.

Table 2
Checkerboard analysis confirms neutrophil chemotaxis to KSLW.

Checkerboard analysis (mean percent migration, %)				
	RPMI	K11 + RPMI (KSLW 10^{-7} M)	K10 + RPMI (KSLW 10^{-6} M)	K4 + RPMI (KSLW 10^{-5} M)
RPMI	14.1	9	8	8
K11 (KSLW 10^{-7} M)	45.12	43	46	32
K10 (KSLW 10^{-6} M)	57.34	54	47	48
K4 (KSLW 10^{-5} M)	86.48	87	77	63
FMLP (Control 10^{-10} M)	94			

The checkerboard analysis revealed that neutrophil responses to KSLW possess both chemotactic and chemokinetic components. However, migratory responses to uniform concentrations in the upper and lower chambers of the transwell plates were reflective of responses to a positive gradient. Thus, incubation with KSLW resulted in a chemotactic response. These differences were significant ($P < 0.05$) for KSLW at 10^{-5} M concentration.

both chemotactic and chemokinetic components. As shown in Table 2, the values along the diagonal, demonstrate migratory responses to an increasing concentration gradient [29]. At lower concentrations, 10^{-11} M, KSLW demonstrated migratory responses to a negative gradient, reflecting chemokinetic activity. However, the migratory responses to uniform concentrations in the upper and lower chambers of the transwell plates were lower than the values below the diagonal, reflective of responses to a positive gradient [29]. Thus, incubation with KSLW resulted in a chemotactic response. These differences were significant ($P < 0.05$) for KSLW at 10^{-5} M concentration.

3.4. F-Actin polymerization in response to KSLW antimicrobial peptide

Chemotactic molecules, such as FMLP [15] and IL-8 [42] induce actin assembly and changes in actin distribution in neutrophils. Increases in F-actin content correlates with increased locomotion at FMLP concentrations $\leq 10^{-8}$ M [15,16]. In this study, we evaluated the effect of KSLW to induce actin assembly in isolated neutrophils. Spectrofluorometric assays on 96-well plates demonstrated a significant ($P < 0.05$) increase in F-actin content following neutrophil stimulation with KSLW or FMLP, relative to FITC-labeled control (Fig. 3). These results were confirmed using flow cytometric analysis. Fluorescence emission of single cells after FITC-phalloidin staining was recorded as a measure of the F-actin content of individual neutrophils [41]. As shown in Fig. 4, stimulation with different concentrations of KSLW decapeptide, resulted in a 1–2-log increase in fluorescence intensity in a dose-dependent manner. Stimulation with the positive control 10^{-10} M FMLP resulted in a 2-log increase in the fluorescence intensity, which reflects actin polymerization [15,19]. Pre-treatment of neutrophils with PTX, before stimulation with FMLP or KSLW, resulted in a reduction in F-actin content, as demonstrated by the decrease in FITC fluorescence (Fig. 5). These results suggest that the changes in F-actin content are influenced by KSLW within a specific range of concentrations, and the neutrophil actin polymerization response to KSLW, is regulated by G-α_i signaling mechanisms [39,43], which are associated with neutrophil chemotactic (e.g. formyl peptide [4]) receptors.

3.5. Neutrophil oxidative burst inhibition

Neutrophils inactivate and destroy virulent pathogens through the release of superoxide and enzymes and by phagocytosis [6,11,20,22]. We evaluated the effect of KSLW on neutrophil respiratory burst. After 3 h of incubation, neutrophils stimulated with PMA displayed a significant reduction ($P < 0.05$) in superoxide production when treated with KSLW, at concentrations ranging from 10^{-8} M to 10^{-3} M, with the greatest reduction observed at 2.5×10^{-7} M (Fig. 6). Similarly, after 3 h of incubation, we observed a dose-dependent effect with KSLW treatment of LPS-stimulated neutrophils. However, the range of concentrations displaying, a significant reduction ($P < 0.05$) in superoxide production was more limited, 10^{-7}–10^{-4} M. Interestingly, the peak concentration for KSLW-inhibition of superoxide production in LPS-stimulated neutrophils was at 2.5×10^{-4} M, and fluorescence values were not different from control (Fig. 7). We must note that the overall level of superoxide production was higher in PMA-stimulated neutrophils, relative to LPS-stimulated neutrophils, establishing different baselines from which to evaluate inhibitory effects.

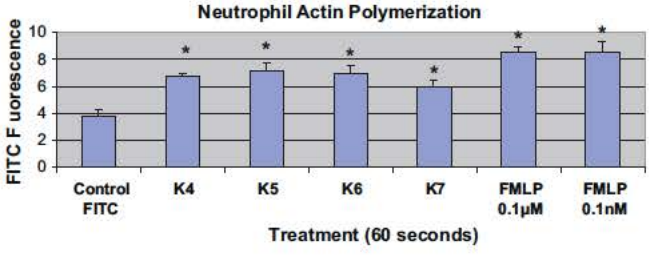

Fig. 3. FITC-phalloidin fluorescence correlates with actin polymerization. Spectrofluorometric assays on 96-well plates demonstrated a significant ($P < 0.05$) increase in F-actin content following neutrophil stimulation with KSLW on FMLP, relative to FITC-labeled control. (*, statistically different from control).

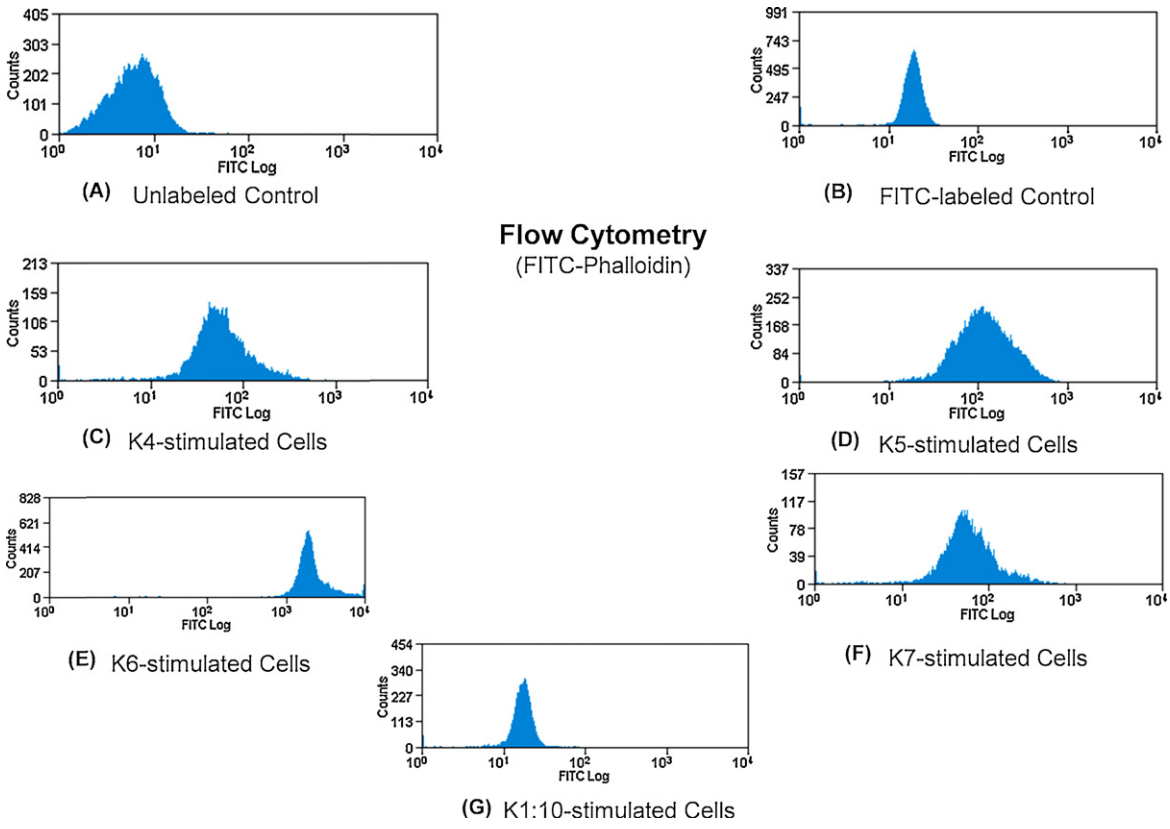

Fig. 4. Effect of KSLW on F-actin content. KSLW-treatment demonstrated a positive effect on actin polymerization. The distribution of the F-actin content of unstimulated control neutrophils (unlabeled and FITC-labeled in A and B, respectively), and cells stimulated with varying concentrations of KSLW, in C–G, was measured as the log fluorescence emission of 10,000 cells. The distribution of FITC-phalloidin-stained cells in this representative experiment after 60 s of treatment with KSLW concentrations: (C) 1.25×10^{-4} M (K4), (D) 6.25×10^{-5} M (K5), (E) 3.125×10^{-5} M (K6), (F) 1.5625×10^{-5} M (K7), and (G) 1.25×10^{-5} M (K 1:10) demonstrated a dose-dependent effect on actin polymerization.

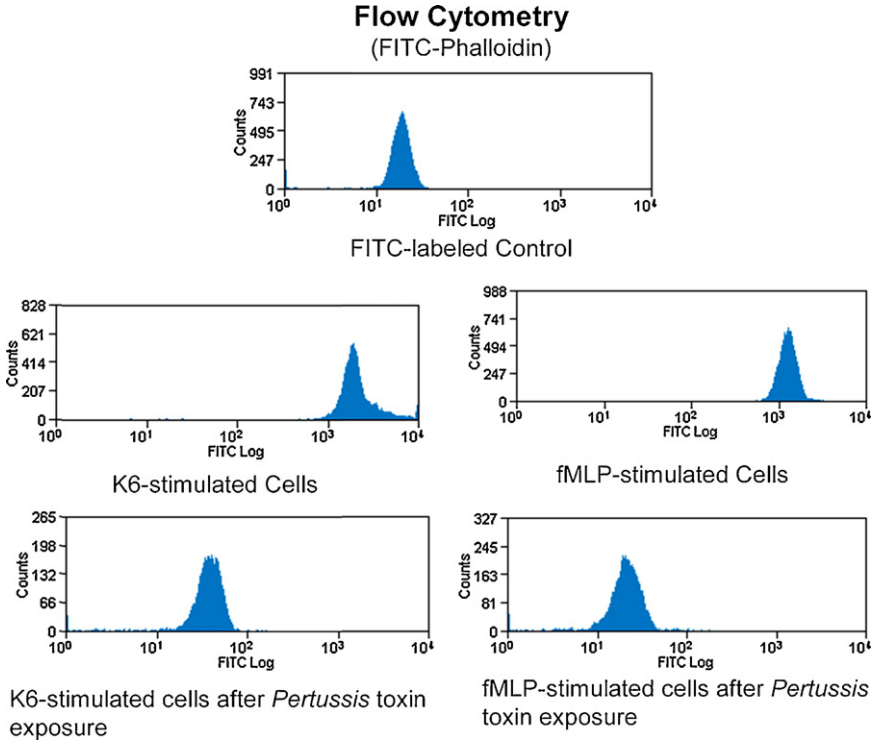

Fig. 5. Pertussis toxin-treatment results in decreased F-actin content. Incubation with pertussis toxin, prior to 10^{-10} M FMLP- and KSLW-stimulation [3.125×10^{-5} M (K6)], reduced actin polymerization. FITC fluorescence decreased by more than 1 log and to near the levels of FITC-labeled control.

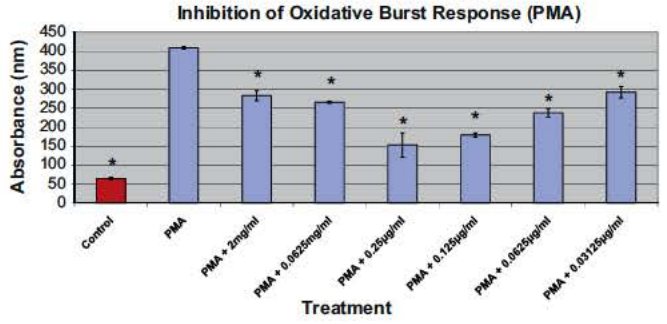

Fig. 6. KSLW inhibits oxidative burst in PMA-stimulated neutrophils. After 3 h of incubation, neutrophils stimulated with PMA displayed a significant reduction ($P < 0.05$) in superoxide production when treated with KSLW, at concentrations ranging from 10^{-8} M to 10^{-3} M, with the greatest reduction observed at 2.5×10^{-7} M. The asterisks (*) represent values statistically different from PMA positive control.

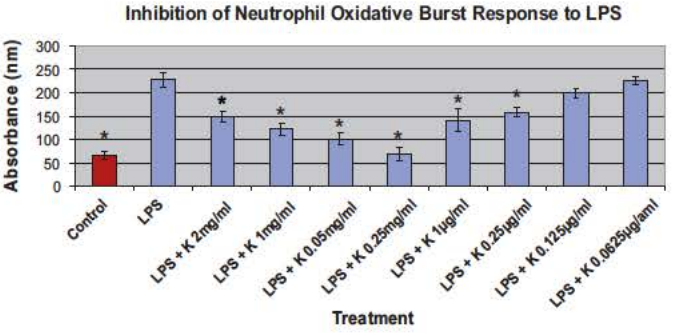

Fig. 7. KSLW inhibits oxidative burst in LPS-stimulated neutrophils. Following 3 h of incubation, KSLW inhibited oxidative burst in LPS-stimulated neutrophils, in a dose-dependent manner. The effective range of inhibitory concentrations, displaying significant reduction ($P < 0.05$) in superoxide production was more limited ($10–10^{-7}$ M) than PMA-stimulated neutrophils. The peak concentration for KSLW-inhibition of superoxide production in LPS-stimulated neutrophils was at 2.5×10^{-4} M, and fluorescence values were not different from control. The asterisks (*) represent values statistically different from LPS positive control.

4. Discussion

Cationic antimicrobial peptides were initially recognized for their antimicrobial properties, which alone make them highly attractive for clinical applications [1]. Their multifunctional properties and abilities to manipulate mammalian cell function have led to increased interest in their utilization as therapeutic molecules. Cationic host-defense peptides have been shown to have a broad range of immunomodulatory and biological properties. The diverse effects, on cellular behavior, include regulation of gene expression in various cell types (e.g. monocytes and epithelial cells) [13,28], enhanced chemokine (IL-8) release from human airway smooth muscle cells [49] and stimulation of epithelial cell proliferation and wound closure [34]. Additionally, human LL-37 promotes angiogenesis [13] and demonstrates chemotactic properties for neutrophils, monocytes, mast cells, and T cells [10,37,48]. The neutrophil-derived proline-arginine (PR)-rich cathelicidin PR-39 possesses several distinct functional properties, and was shown to function as a chemoattractant to porcine leukocytes [17]. In addition to its antibacterial and chemotactic activities, PR-39 was demonstrated to induce syndecan expression in mesenchymal cells of wounds [12,17], promote angiogenesis [48], and inhibit phagocyte NADPH oxidase [35,48] indicating a role in wound repair.

In reciprocal fashion, a number of known chemoattractant molecules, particularly chemokines, have been shown to display antimicrobial activity against Gram-negative and Gram-positive bacteria and fungi [46], with varying potency. Chemokines displaying antimicrobial activity include: CCL20/MIP-3α, CXC12/SDF-1α, CCL11/eotaxin, CCL13/MCP-4 [46], CXCL4, CCL5 (RANTES), CTAP-3 [10,37], as well as CXCL9, and the INF-γ-inducible chemokines CXCL10/IP-10 and CXC11/ITAC [10,46]. The fundamental structural principle common to all or many antimicrobial peptides, is the topological amphipathic design, where clusters of hydrophobic and cationic amino acids organize in discrete surface areas [46]. Thus, central to the antimicrobial activity of chemokines, is the topological formation of a large, positively charged electrostatic patch on their surfaces [46]. Moreover, other endogenous peptides and proteins with roles in inflammation display multifunctional activities (at varying concentrations), which influence leukocyte function and migration. Small peptide molecules, such as the tripeptide FEG, derived from the submandibular gland peptide T, reduces platelet activating factor-induced neutrophil movement and adhesion (at picomolar concentrations), by binding CD11b and CD16b [22]. The anti-inflammatory, calcium-binding, myeloid related proteins S100A8 and S100A9 were demonstrated to cause neutrophil chemotaxis at picomolar to nanomolar concentrations [33]. In contrast, S100A8 was demonstrated to possess fugetactic activity [40] or repulsive effects down a gradient, on neutrophils, with a peak concentration of 10^{-9} M, in an oxidation-dependent manner [36].

Synthetic combinatorial libraries represent a major approach in the development of antimicrobial agents as a response to the increasing rate of bacterial resistance to existing drugs. Novel antimicrobial compounds having activities equal to or greater than those of existing antimicrobial agents, can be rapidly identified by using synthetic combinatorial library technology [2]. KSLW is an analog of the novel decapeptide, Lys-Lys-Val-Val-Phe-Lys-Val-Lys-Phe-Lys-NH_2 (KSL), originally identified by combinatorial chemistry, in which the sixth residue (lysine) is substituted with tryptophan. Na and coworkers confirmed that this analog improved the stability of the peptide in salivary fluids [25], while still maintaining its broad spectrum of antimicrobial activity, relative to other analogs, which also demonstrated improved stability. KSLW displays potent antimicrobial activities against Gram-positive and Gram-negative bacteria, and fungi. Here, we endeavored to explore the possibility of multifunctional roles for KSLW in inflammation.

Neutrophils are the key cellular elements of the innate immune system, providing protection from bacterial invasion [4]. Migration to sites of infection and tissue damage occur through the process of chemotaxis, in which chemoattractants bind cell-surface receptors and trigger downstream signaling events, which result in activation of the cytoskeletal machinery. Neutrophil chemoattractants include a variety of molecules, such as: bacterial and mitochondrial formylated peptides [4], C5a (a complement cascade product) [4,47], fibrinopeptide b [7], leukotriene B_4 (product of phospholipid metabolism), and chemokines, such as IL-8 [4,47]. Our data show that KSLW displays chemoattractant properties for neutrophils, in micromolar amounts, and is capable of inducing actin polymerization in neutrophils, which is consistent with other chemotactic antimicrobial molecules [17,35]. Pertussis toxin (PTX) inhibits $G\alpha_i$ signaling by NAD^+-mediated ADP-ribosylation, as well as ADP-ribosylation-independent structural changes of Gα proteins [31]. Inactivation of G_i family G proteins by PTX strongly impairs leukocyte migration in vitro [43]. In this study, we demonstrated that pretreatment of neutrophils with PTX inhibited migration toward KSLW, as well as FMLP. Taken together, these data support the hypothesis that KSLW is chemotactic for neutrophils and likely mediates its effect via chemotactic receptor signaling.

Various substances differentially affect oxidative burst phenomena. Earlier studies showed that alteration of the surface of human neutrophils with p-diazobenzene-sulfonic acid (DASA) prevented activation of the respiratory burst by some stimuli, but

not others [24]. Production of superoxide anion (O_2^-) stimulated by concanavalin A or the chemotactic peptide formyl-methionyl-leucyl-phenylalanine, FMLP, was inhibited by DASA-pretreatment, whereas O_2^- production stimulated by phorbol myristate acetate (PMA), sodium fluoride, or the ionophore A23187 was not inhibited by DASA [24]. McPhail and coworkers concluded that more than one mechanism exists for activation of the respiratory burst oxidase in human neutrophils and that the neutrophil possesses at least one oxidase that is not an ectoenzyme [24]. Bacterial components, such as lipopolysaccharides, are known to stimulate oxidative burst in phagocytes [20,30]. Lipopolysaccharides (LPS) were shown to have a direct effect on oxygen radical production via LPS-binding protein and CD14 [20]. Minimization of the host-mediated tissue destruction associated with inflammatory reactions [6,11], may be a possible role of antimicrobial peptide-inhibition of leukocyte respiratory burst. Although the molecular mechanisms have yet to be delineated, our experiments demonstrated that KSLW has a concentration-dependent inhibitory effect on neutrophil oxidative burst, in response to stimulation by LPS and PMA. In addition to their antimicrobial activity, defensins and cathelicidins can bind to LPS and inactivate its biological functions [1]. Indeed, KSLW, has been shown to display direct anti-endotoxic effects against *E. coli* O55:B5 LPS and lipopolysaccharides of several oral pathogens [8]. The observed reduction in O_2^- production, in LPS-stimulated cells, is perhaps, due to a combination of LPS-binding and direct inhibition of oxidative burst. A synergistic effect would explain the low levels of O_2^- production (not significantly different from control values) in KSLW-treated neutrophils stimulated with LPS, compared with reduced levels observed following PMA-stimulation.

5. Conclusion

Bacterial pathogens, such as group A *Streptococcus* and *Staphylococcus aureus*, have evolved mechanisms to evade the innate immune response. These include: resistance to endogenous cationic antimicrobial peptides, by altering membrane chemistry (incorporating positively charged amino acid residues into their cell wall), cytoplasmic escape from the phagosome, interference with antibody-mediated opsonization, and impairment of leukocyte recruitment (via cleavage and inactivation of chemokines, such as IL-8) [27]. Recognition of such evasive strategies necessitates the development of therapeutic agents to improve host cell function in eliminating pathogenic microbes. The multifunctionalities displayed by antimicrobial peptides, particularly their chemotactic properties, indicate important overlapping usage in immune modulation, and support expanding our views of the biological roles of these diverse molecules. Future investigations, to determine if KSLW regulates neutrophil phagocytosis and adhesion, or if KSLW exacerbates the proinflammatory activities of infiltrating neutrophils, by delaying neutrophil apoptosis are plausible. Also, extended studies to discern the mechanisms involved in the peptide's effect on respiratory burst are in order. Moreover, determining the responses of other mammalian cell types, such as cell populations of healing-impaired wounds, would provide significant insight for potential therapeutic strategies with antimicrobial peptides. The continued exploration of alternative biological properties of synthetic antimicrobial peptides, such as KSLW, will offer advantages in the treatment of resistant organisms, and yield time-efficient and cost-effective approaches to managing host-mediated responses to infectious agents, and thus improve clinical outcomes.

Acknowledgment

This study was supported by a grant from the United States Army.

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