Laura Andrea Barrero Guevara
Directed by: Helena Groot de Restrepo
Co-directed by: Carolina Muñoz-Camargo

New peptides with immunomodulatory activity in macrophages and antibacterial activity against multiresistant *Staphylococcus aureus*

Abstract:

Staphylococcus aureus infections are a common concern worldwide due to the increasing number of bacterial strains with multiresistant properties to existing antibiotics, incrementing the need for novel molecules and therapy approaches for their treatment. This study evaluated the antibacterial and immunomodulatory activity of eight new peptides as the basis for the search of new antibacterial and therapeutic agents for topic prevention and treatment against *S. aureus* infections. The methodology consisted of the *in silico* characterization of the eight peptides in order to identify their antimicrobial peptide characteristics, stability features, sequence similarities with already described proteins and immunogenicity. Following, antibacterial activity microdilution assays were performed with the eight new peptides individually and in combination. Furthermore, the cytotoxic activity of the peptides was evaluated against VERO fibroblasts, THP-1 monocytes and THP-1 macrophages. Finally, immunomodulatory activity assays were performed with three of the peptides (GF, AT and AA) in macrophages and under three scenarios: non-stimulation, *Escherichia coli* LPS stimulation and *S. aureus* lysate stimulation. Results showed that the GF, AT and AA peptides individually showed the best antibacterial activity against the *S. aureus* strains evaluated. On the other hand, the combination of the eight peptides showed antibacterial activity against multiple *S. aureus* strains and was able to decrease the bacterial growth of the clinically isolated strains more than the individual antibiotic controls. In addition, the majority of the peptides, except for LK, AT and the peptide combination, did not present cytotoxic activity against the cell lines tested. Finally, AA, GF and RN peptides presented immunomodulatory activity in THP-1 macrophages by displaying different profiles of enhancement and diminution of four cytokines (IL-1 β , TNF- α , IL8 and MCP-1). This activity depended on the peptide concentration and the stimulation which the macrophages were exposed to. Taken together, these results demonstrate the potential of these peptides to be used in further studies as novel antimicrobial molecules for the prevention and treatment of *S. aureus* infections.

Introduction

Antimicrobial peptides are commonly defined as small, cationic molecules that consist of 10 to 50 amino acid residues found in a broad range of organisms (Zhang, 2016). The presence of antimicrobial peptides in the skin secretions of frogs has been confirmed since some decades ago. Such peptides are now considered part of the immune response of these and other animals (Conlon, 2011). Still, their precise role in the biology and survival of the organisms remains largely unknown, although their antimicrobial activity against bacteria (Groot et al. 2012), fungi (Rollins-Smith et al., 2002) and viruses (Muñoz et al., 2016) have long been studied. Recently, it has been described that some of these peptides have further functions (Yeung, Gellatly & Hancock, 2011) such as immunomodulatory (Conlon et al., 2014 y Pantic et al., 2014), chemoattractant (Guaní-Guerra et al., 2010) and cytotoxic (Guaní-Guerra et al., 2010) activity, which has lead to rename and group them as host defense peptides.

Assuming a worldwide panorama of increasing numbers of infections caused by multiresistant bacteria, it is important to keep searching for new therapeutic molecules (WHO, 2014). Taking into account the potential which has been found in the peptides secreted from glands on the frog's skin, not only because of their antibacterial activity but also as immunomodulatory molecules, these peptides are considered a promising alternative in clinical applications (Conlon et al. 2014). This simultaneous action

should be considered for presenting an integrated therapeutic solution against infections. The purpose of immunomodulatory therapy for example is the prevention and healing of infectious and non-infectious diseases. In this, coadjuvant molecules, such as pattern recognition receptors, bacterial ligands and antimicrobial peptides, regulate the immune response (Hancock et al, 2012).

Staphylococcus aureus is one of the microorganisms which has caught the greatest attention worldwide, not only because of its role in nosocomial infections, but also in community-acquired infections. This Gram-positive bacteria is responsible for many diseases ranging from simple skin infections to more serious conditions such as sepsis (Fournier & Philpott, 2005). *S. aureus* can colonize the host in an asymptomatic manner as a commensal microorganism, however, it can also arise as a pathogen. Nearly the 20% of the human population is consistently colonized by *S. aureus* and around 60% are intermittent bearers, which is considered an important risk factor. Generally, the main entry sites for infections of this pathogen are skin wounds, where resulting minor infections may appear. Although *S. aureus* can infect many other tissues, the skin is where the majority of the infections start out (Kluytmans, Van Belkum & Verbrugh, 1997).

Studies have indicated, that the pathogenicity of *S. aureus* is caused by a great repertoire of toxins, exo-enzymes, adhesins, and proteins capable of immunomodulation (Fournier & Philpott, 2005). Additionally, during the innate immune response against *S. aureus* it has been found, that the severity of the infection varies according to the host's differential secretion of cytokines (Brown et al., 2014). Specifically, in skin infections, it is known that the local inflammation, which is addressed by proinflammatory cytokines secreted by monocytes and macrophages, exacerbate the severity of the bacterial infection (Montgomery et al., 2013 y Myles et al., 2013). Therefore, it has been proposed recently, that the *S. aureus* infection prevention and treatment should include immunomodulatory therapy (Wardenburg, Williams & Missiakas, 2006 y Thangamani, Younis & Seleem, 2015).

Currently, the treatment of infections with *S. aureus* is selected depending on three factors: the tissue infected, the severity of the infection and the pathogen resistance profile. *S. aureus* can infect soft tissues and the skin, blood (bacteremia), and endocardium, lungs, bones, articulations and/or the central nervous system. The infections of the skin and of soft tissues can occur as single or as recurrent events (Liu et al., 2011). In these cases, the severity of the infection is classified according to the presence of impetigo (minor infection), eczema, ulcers and lacerations (secondary infection), which can be treated with topical solutions. Additionally, the infection can proceed to a purulent or non-purulent cellulitis state, which is usually treated in a systematic manner. For the prevention and treatment of minor and secondary infections and in recurrent infections in soft tissues, mupirocin and bacitracin are the recommended topic agents (Bamberger & Boyd, 2005). Nevertheless, recent studies have shown, that the resistances to these antibiotics are increasing worldwide (Patel, Gorwitz & Jernigan, 2009). A study in New York showed, that nearly 15% of the *S. aureus* strains studied in 2013 presented resistance to the aforementioned antibiotics (Antonov et al., 2015), although, there is still insufficient information on the prevalence of the resistance in the population. In conclusion, taking into account the worldwide *S. aureus* panorama, the need to increase effort in the search of new treatments and preventing measures, including topical options, becomes evident.

The Human Genetics Laboratory of the Los Andes University in Bogotá, Colombia, has a research group working on antimicrobial peptides secreted by frog skin (Groot et al. 2012 & Muñoz et al. 2016). Here, previous studies have identified peptides with antimicrobial activity (individually and in combination) against bacteria of clinical importance, including multiresistant strains of *S. aureus*. Additionally it was found, that these peptides do not show cytotoxic activity, which makes them interesting candidate drugs for their use in human. In this study, taking into account the new clinical applications that have been proposed for these molecules, we aimed to identify if a group of eight new peptides presented immunomodulatory and antibacterial activity, with the purpose of searching for new

agents which could be used for the prevention and therapeutic topic treatment of early *S. aureus* skin infections.

Hence, we divided the following study into two parts. The first part aimed to evaluate the antibacterial activity of eight new peptides as the basis for the search of new antibacterial agents for the topic prevention and treatment against *S. aureus* infections. With this objective, firstly, the general peptide characteristics, stability related features, sequence similarities with previously described proteins and antimicrobial peptides, as well as the immunogenicity were analyzed *in silico* with a broad repertoire of bioinformatic tools. Secondly, the resistance profile of five *S. aureus* strains (one reference strain and four clinical isolations) against four antibiotics was determined. Finally, the antibacterial effect of the peptides against the five aforementioned *S. aureus* strains was identified and compared with that of the antibiotics. The second part aimed to identify the potential immunomodulatory activity of these eight new peptides as a basis for the search of new therapy agents for topic prevention and treatment against *S. aureus* infections. Firstly, the differentiation of human macrophages from the human THP-1 monocytic cell line was established. Then, the cytotoxic effect of the peptides was evaluated in VERO fibroblasts, human monocytes (THP-1 cell line) and differentiated human macrophages (from THP-1 cell line). Finally, the immunomodulatory effect of the peptides in differentiated human macrophages, stimulated with and without *E. coli* lipopolysaccharides (LPS) or *S. aureus* lysate, was identified by evaluating their capacity to enhance and/or inhibit the production of 13 different cytokines.

Part 1: *In silico* description of eight new peptides and evaluation of their antibacterial activity against *S. aureus*

Methodology

Peptide Synthesis

Eight new antimicrobial peptides previously described in the Human Genetics Laboratory of the Los Andes University in Bogotá, Colombia, named after their first and last amino acid of their sequence (AA, KS, NS, RN, AT, GF, KV and LK) were used in this study. Their synthesis was performed by GL Biochem Ltd. (Shanghai, China) via the solid phase method. Afterwards, the analysis and purification of the peptides were performed by reversed-phase HPLC using a Venusil XBP-C18 RP-HPLC column (4.6mmX250mm) at a flow rate of 1mL/min and a linear gradient of 20:80% of acetonitrile:water with 0.1% trifluoroacetic acid with a detection of 250nm. Finally, electrospray mass spectrometry was used to corroborate the purity (>98%) and for the identification of the synthetic peptides.

***In silico* characterization of eight new peptides individually**

General peptide characteristics, stability related features, sequence similarities with previously described proteins and antimicrobial peptides, as well as the immunogenicity of the eight new peptides were analyzed with a broad repertoire of databases and bioinformatic tools. Firstly, the “APD3: Antimicrobial Peptide Calculator and Predictor” tool (Wang & Wang, 2016) of the Antimicrobial Peptide Database (APD) was used to identify the following general characteristics of antimicrobial peptides in the eight new peptides: Net charge, hydrophobic and hydropathy (GRAVY) ratio, potential capacity to attach to lipid bilayers and other proteins (W-W hydrophobicity and Boman Index) and special features like potential for alpha helices formation or sequence richness in some specific amino-acids. The ProtParam Tool (Gasteiger et al., 2005) was used to identify the stability features of the eight new peptides, allowing recognition of their suitability to remain stable in specific environments. Based on the N-terminal amino acid of each peptide, the half-life *in vitro* and *in vivo* was predicted along with the instability and aliphatic index and finally classification of the stability of the peptide. Likewise, the “HLP: Web server for predicting half-life of peptides in intestine-like environment” (Sharma et al., 2014) permitted the prediction of the half-life and stability of the new peptides in an intestine-like, proteolytic, environment.

Alignments of the eight new peptides with the Protein Information Resource (PIR) and the APD (Chen et al., 2013 & Wang, 2016) were developed to predict the possible precursor sequences of the new peptides and to identify the characteristics as well as the antimicrobial activity of similar described peptides. Finally, the immunogenicity of the new peptides was analysed *in silico*. The “Predicted Antigenic Peptide Tool” (Reche, 2002) was used to determine the potential binding capacity to antibodies. Furthermore, the possible antigenic presentation of the peptides, by the MHC class I and II pathways, was identified using the SYFPEITHI Tool (Rammensee, 1999). Additionally, the immunogenicity (Calis et al., 2013) and affinity to class I and II MHC were calculated using the Immune Epitope Database and Analysis Resource (IEDB). The MHC I binding predictions were made on 4/17/2017 using the IEDB Analysis Resource Consensus Tool for MHC I (Kim et al., 2012), which combines predictions from AMM (Nielsen et al., 2003 and Lundegaard et al., 2008), SMM (Peters & Sette, 2005) and comblib (Sidney et al., 2008). The MHC II binding predictions were made on the same date and using the IECB Analysis Resource Consensus Tool for MHC II (Wang et al., 2008, Wang et al., 2010 and Sturniolo et al., 1999).

Determination of the antibacterial activity of eight new peptides individually and in combination against a reference strain, a clinically isolated non-resistant strain and three clinically isolated resistant strains of *S. aureus*

A reference strain (ATCC□ 25923), a non-resistant clinically isolated strain (39413) and three resistant clinically isolated strains (34026, 36055, 39791) were used in this study. To ascertain the integrity of the bacteria strains resistance profile, antibiotic sensitivity assays were developed with a qualitative method based on diffusion (antibiotic-discs with 200μM concentration of each antibiotic: Ampicillin, Gentamicin, Methicillin and Vancomycin) and a quantitative method based on microdilution (seven concentrations: 0, 3.12, 6.25, 12.5, 25, 50 and 100μM of each antibiotic). Determination of the resistance (P-value < 0.05) was performed by a linear model comparing the growth of the clinically isolated strains against the reference strain.

Then, according to previous studies (Groot et al., 2012), microdilution assays were conducted to evaluate the antibacterial activity of the peptides following the guidelines suggested by the Clinical and Laboratory Standards Institute (CLSI, 2012). All the *S. aureus* strains mentioned above were exposed to the eight peptides individually and in combination in the assay. Briefly, a standardized bacterial inoculum (OD_{595nm}=0.001) of each strain was exposed to seven different concentrations (0, 3.12, 6.25, 12.5, 25, 50 and 100μM) of the peptides individually and in combination in Luria Bertani (LB) Media (LB-Media, Miller, Sigma-Aldrich) in 96-well microplates for 6, 8 and 24 hours at 37°C. The antibiotics Ampicillin, Gentamicin, Methicillin and Vancomycin were used as control and were tested in the same concentrations of the peptides. After incubation, the optic density was determined reading the microplates at 595nm in a spectrophotometer. Each peptide, the eight peptide combination and the control experiments were done with two replicates with three repetitions per concentration.

Statistical Analysis

All statistical analysis was performed using the computing environment R (R Development Core Team, 2008). In all cases, a residual analysis was performed to evaluate the normality and homoscedasticity of the data. All plots were created with the package ggplot2 (Wickham, 2009). In addition, for the creation of the dose-response sigmoidal models and calculations of the IC₅₀ and EC₅₀, when evaluating the bacterial resistance profile and the peptides' antibacterial activity, the package GRmetrics (Clark et al., 2016) from the Bioconductor project was used (Gentleman et al., 2004).

Results

General potential antimicrobial features of the eight new peptides: The eight new peptides studied show important features which predict a promising antimicrobial potential

The positive net charge and the amphipathic composition are considered predictors of the peptides' potential for presenting antimicrobial activity, as they might indicate their capacity to interact with living cell membranes. Although the eight peptides used in this study had these characteristics, NS and AT peptides showed the most promising values (Table 1). Furthermore, according to the hydrophobic ratio, the peptides were classified as high hydrophobic peptides (AT, GF, KV and LK) and low hydrophobic peptides (AA, KS, NS and RN). Meanwhile, other features ranked the potential of the peptides differently, for example, the W-W Hydrophobicity indicated that the GF peptide might have a higher possibility to bind to a membrane, while NS might not stay attached (Table 1). Also, the Boman Index showed, that all the peptides evaluated in this study have a low potential to bind to other proteins. Finally, the APD predicted that some of these peptides might form alpha helices or have sequences rich in rare amino acids, special features which some described antimicrobial peptides present (Table 1). In conclusion, although the eight peptides described in this study do not share the same characteristics, all predictions indicated their potential to be antimicrobial peptides.

Table 1. General potential antimicrobial features of the eight new peptides. *In silico* analysis using the APD were conducted to identify general characteristics of antimicrobial peptides of the eight new peptides described in this study. All the peptides have consistently shown general characteristics of typical antimicrobial peptides, such as positive net charge and amphipathic composition. Nevertheless, the peptides were classified into two groups: high hydrophobicity peptides (AA, KS, NS and RN) and low hydrophobicity peptides (AT, GF, KV and LK). Furthermore, GF was identified as the peptide with the highest possibility to bind to membranes, while none of the peptides were considered to have the potential for binding to other proteins.

ID	Isoelectric Point ¹	Net Charge ²	Hydrophobic ratio ²	GRAVY ^{1,2}	W-W Hydrophobicity (kcal/mol) ²	Boman index (kcal/mol) ²	Predicted Antimicrobial Features ²
AA	10	+2	45%	0,36	2,54	0,07	Formation of alpha helices
KS	10,3	+3	25%	-1,30	2,49	2,06	
NS	10,18	+4	35%	-0,84	7,27	2,14	Formation of alpha helices
RN	11	+2	47%	0,73	0,92	-0,26	Formation of alpha helices
AT	10,3	+3	53%	0,37	1,87	0,28	Formation of alpha helices
GF	11	+2	54%	0,89	-0,94	-0,43	Rich in Prolines
KV	8,75	+1	50%	0,92	1,45	-0,91	Formation of alpha helices
LK	8,75	+1	52%	1,06	0,78	-1,22	Formation of alpha helices

ID = Code given to each peptide according to the first and last amino acid of its sequence. Hydrophobic ratio= Total Hydrophobic Ratio as defined by APD. GRAVY= Grand Average Hydropathy Value of the peptide. W-W Hydrophobicity= Wimley-White Whole-Residue Hydrophobicity of the peptide, calculated as the sum of whole-residue free energy of transfer of the peptide from water to lipid bilayer interface. Boman Index= Protein Binding Potential to other proteins. Predicted Antimicrobial Feature= APD Predictions of Antimicrobial Features. 1. ExPASy, Bioinformatics Resource Portal, ProtParam Tool (Gasteiger et al., 2005). 2. APD, APD3: Antimicrobial Peptide Calculator and Predictor (Wang & Wang, 2016)

Predicted stability features of the new peptides *in vivo*, *in vitro*, in test tubes, in an intestine-like environment and in presence of proteolytic enzymes and chemicals. Predictions indicate the peptides are stable in proteolytic environments, but susceptible to peptidases originating from bacteria of clinical importance

The half-life predictions obtained according to the N-terminal amino acid indicated, that the AA, AT, GF and NS peptides might maintain their structural integrality *in vivo* inside of yeast and bacteria cells, probably also keeping their functionality (Supplementary Material Table 1). However, KV, KS, RN and LK might disintegrate within minutes, but their antimicrobial activity could be interacting with the membranes outside the cell. Inside mammalian cell lines (*in vitro*), all peptides would not disappear for hours (Supplementary Material Table 1). Furthermore, the instability index allowed to classified the

AA, NS, RN and AT peptides as “stable” in test tube conditions. Although the GF, KV and LK were classified as “unstable”, their instability index (all values under 53) were not too high above the threshold (40) (Supplementary Material Table 1). In regards of the intestine-like environment stability predictions, none of the peptides were classified with “low” stability. Instead, AA and KS were classified with “high” stability in this proteolytic environment (Supplementary Material Table 1).

The predictions performed with the Peptide Cutter Tool established, that lowly specific enzymes involved in the digestive system functions of vertebrates (Trypsin) or isolated from a fungus (LysN and Proteinase K) or bacteria (LysC and Thermolysin) can degrade all the peptides evaluated in this study (Supplementary Material Table 1). Also, chymotrypsin, a protein isolated from the porcine pancreas gland, and pepsin, a protein isolated from gastric juice of dogs, were predicted to degrade all the peptides except for AA. Furthermore, the inorganic molecule CNBr might possibly fragment KS, NS, AT and GF (Supplementary Material Table 1). In addition, some of these eight new peptides are susceptible to be degraded by proteins isolated from bacteria of clinical importance. For example, RN and GF peptides might be susceptible to Clostripain originating from *Clostridium sp.* and the NS peptide may be fragmented by the Asp-N endopeptidase originating from *Pseudomonas sp.* as well as the Asp-N and Glutamyl endopeptidase and Staphylococcal peptidase I originating from *S. aureus* (Supplementary Material Table 1).

Proteins and antimicrobial peptides already described have sequence similarities to the eight new peptides: KS, NS, AT, KV and LK might have a possible precursor sequence from proteins involved in gene regulation and all the peptides of this study presented similarities with peptides already described displaying antibacterial activity

Protein alignments with the peptides' sequences performed with the PIR showed, that the new peptides might have a possible precursor sequence from proteins already described in several organisms and taxonomic groups (Table 2). Three of these proteins have been found to be involved directly or indirectly in regulating gene expression. In addition, the KV and LK peptides' sequences, as other described peptides like for example Buforin (Park et al., 1996), matched with those of histones. Analysis of the taxonomic groups from the proteins matching with the sequence of each peptide showed, that all peptides had a possible precursor sequence from proteins of amphibian origin. On the other hand, the Peptide Match Tool of the APD indicated, that the new peptides of this study had similarities of between 36 to 50% to already described peptides (Table 2). Surprisingly, the KS peptide had its highest similarity with a synthetic peptide. All the peptides already described which matched with the eight new peptides of this study showed antibacterial, antifungal, antitumorigenic, cytotoxic, antiparasitic and/or antiviral activity (Table 2).

Table 2. Proteins and peptides with similar sequences to the eight new peptides. The PIR and the APD were used to identify proteins and peptides already described, having sequence similarities with the new antimicrobial peptides described in this study. All the peptides, except for GF, were found to have a total match with the sequence of proteins according to PIR. The majority of the protein and peptide matches were identified in the amphibian taxonomic group.

ID	Protein ¹	Protein matches (Taxonomic groups) ¹				Peptide ²	Peptide Source ²	Peptide Similarity ²	Peptide Activity ²
		Birds	Reptiles	Amphibian	Mamalian				
AA	Decaprenyl-diphosphate synthase	72%	13%	10%	5%	Dominulin B ³	<i>Polistes dominulus</i> (Invertebrates)	44%	Anti-Gram + & -
KS	Myoneurin	0%	0%	100%	0%	BP100 and L5K5W ⁴	Library approach	36%	Anti-Gram + & -
NS	Nuclear receptor corepressor of (SMART)	0%	0%	100%	0%	Latarcin 3b ⁵	<i>Lachesana tarabaevi</i> (Invertebrates)	43%	Anti-Gram + & - and Antifungal

RN	Malate dehydrogenase	0%	67%	33%	0%	Alyteserin-2c and 2b and Maximin H1 and H4 ⁶	<i>Alytes obstetricans</i> and <i>Bombina maxima</i> (Amphibians)	42%	Anti-Gram + & -, Antiviral, Antifungal, Antitumorigenic
AT	MGC81605 protein	0%	0%	100%	0%	Lasioglossin LL-1 ⁷	<i>Lasioglossum laticeps</i> (Invertebrates)	39%	Anti-Gram + & - and Antitumorigenic
GF	-	-	-	-	-	Temporin-SN3 and SN2 ⁸	<i>Hylarana spinulosa</i> (Invertebrates and Amphibians)	50%	Anti-Gram+, Antifungal, Cytotoxic
KV	Histone H2A	6%	68%	3%	23%	Maculatin 1.4 ⁹	<i>Litoria eucnemis</i> (Amphibians)	42%	Anti-Gram + & - and Antitumorigenic
LK	Histone H2A	6%	68%	3%	23%	XPF-St7 ¹⁰	<i>Silurana tropicalis</i> (Amphibians)	45%	Anti-Gram + & -, Antifungal, Antiparasitic and Cytotoxic

ID = Code given to each peptide according to the first and last amino acid of its sequence. Protein = Protein that exactly matches the sequence of the peptide according to the PIR. Protein Matches Taxonomic Groups = Percentage of Matches from the total classified according to the taxonomic group. Peptide = Most similar peptide found to our new peptide after alignment in the APD. Peptide Source = Source (organism) of the most similar peptide found to our new peptide. Peptide Sim. = Percentage of peptide similarity between our new peptide and the best match found. Peptide activity = Antimicrobial activity reported for the most similar peptide found. 1. PIR, Peptide Match Tool (Chen et al., 2013). 2. APD, APD3: Antimicrobial Peptide Calculator and Predictor (Wang & Wang, 2016). 3. APD ID= AP01639 (Turillazzi et al., 2006). 4. APD ID= AP02671 and AP00143 (Badosa et al., 2007). 5. APD ID= AP01013 (Kozlov et al., 2006). 6. APD ID= AP01463, AP01462, AP00832 and AP00527 (Conlon et al., 2009 and Lai et al., 2002). 7. APD ID= AP01465 (Čeřovský et al., 2009). 8. APD ID= AP02274 and AP02273 (Yang et al., 2013). 9. APD ID= AP00770 (Apponyi et al., 2004). 10. APD ID= AP02292 (Roelants et al., 2013).

Immunogenicity features of eight new peptides: KV and LK might interact with antibodies and class I and II HLA

The affinity of the peptides to antibodies was considered according to their antigenic propensity and antigenic determinants. Results showed, that the peptides are potentially antigenic and that GF, KV, and LK have antigenic determinants which might activate B cells (Supplementary Material Table 2). Consequently, the immunogenicity, taken into account as the peptide - HLA I complex score to trigger an immune response, also predicted that these last three peptides (GF, KV, and LK) have the highest probability to be immunogenic (Supplementary Material Table 2). On the other hand, the probability of the antigenic presentation of the peptides was calculated with the SYFPEITHI Tool. The results showed that RN, AT, KV and LK of the peptides might be presented by >10 HLA I alleles, but only KV and LK by HLA II alleles. Furthermore, the predictions showed, that all peptides except KS might bind to the HLA I. Regarding the HLA II, the RN, AT, KV and LK peptides might bind to the molecule with high affinity (Supplementary Material Table 2). Taken together, these results indicate that although all peptides had important predictions about their immunogenicity features, the KV and LK peptides were ranked in all variables analyzed as the ones that have the greatest probability to interact with the immune system pathways analyzed.

Determination of the clinically isolated bacteria resistance profiles: Three clinically isolated *S. aureus* strains are resistant to Ampicillin, Gentamicin and/or Methicillin

In accordance with the expected results, the reference *S. aureus* strain (ATCC® 25923) was classified as susceptible to all of the antibiotics tested (Figure 1 and Supplementary Material Table 3 and 4). Similarly, the clinically isolated strain (39413) showed also not to be resistant to any of the antibiotics in the diffusion assay (Supplementary Material Table 3). However, the quantitative methodology demonstrated, that the IC50 and EC50 of the ampicillin were higher in this strain, although, the concentrations were still too low to indicate that the strain was ampicillin resistant (Figure 1 and Supplementary Material Table 4). Regarding the three clinically isolated resistant strains, all showed different resistance profiles, but none of them was resistant to Vancomycin (Figure 1 and Supplementary

Material Table 3 and 4). Specifically, the analysis showed that the 34026 strain only displayed resistance to Ampicillin, while the 36055 and 39791 strains showed resistance to Ampicillin, Gentamicin and Methicillin (Supplementary Material Table 3 and 4 and Figure 1). Taken together, these results classified the C. I. strain (39413) as non-resistant to the antibiotics tested, the C. I. strain (34026) as Ampicillin resistant and the C. I. strains (36055) and (39791) as Methicillin-resistant *Staphylococcus aureus* (MRSA), multiresistant to Ampicillin and Gentamicin.

Figure 1. Clinically Isolated (C. I.) *S. aureus* strain resistance profile to four antibiotics. Five *S. aureus* strains, one of reference (ATCC 25923) and four clinically isolated, were exposed to four antibiotics (Ampicillin, Gentamicin, Methicillin and Vancomycin) with the microdilution methodology described above. Their viability was measured after 6, 8 and 24 hours (only viability after 8 hours exposure results are shown). The black line at 100% was calculated as the total growth of the strains without exposure to antibiotics. Comparison with the reference strains was realized with a linear model to determine the C. I. strains resistances to the antibiotics. The C. I. (39413) strain was found to be susceptible to all the antibiotics, while the other three strains (34026, 36055 and 39791) were classified as ampicillin resistant. Furthermore, the 36055 and 39791 C. I. strains showed to be Methicillin resistant *Staphylococcus aureus* (MRSA) and resistant to Gentamicin. None of the strains were identified as Vancomycin resistant *Staphylococcus aureus* (VRSA).

Determination of antimicrobial activity of eight new peptides individually: The peptides individually showed their best activity against the *S. aureus* strains after 24 hours of exposure

As mentioned above, AA, KS, NS and RN peptides had low values in their hydrophobic features, this allowed us to dilute them in LB media for the antimicrobial assays. Meanwhile, the high hydrophobic peptides (AT, GF, KV and LK) were diluted in 5% DMSO in LB media solution for the antimicrobial assays. Microdilution assays were performed with the peptides individually, the bacterial growth was measured after 6, 8 and 24 hours of exposure and the inhibitory concentration of the 50% of the bacterial growth (IC₅₀) of the peptides was calculated. Firstly, after 6 hours of exposure, only AT, GF and LK inhibited the 50% of the bacterial growth of a multiresistant MRSA C. I. strain (Supplementary Material Table 5). After 8 hours of exposure, AT and GF continued inhibiting the multiresistant MRSA C. I. strain, but showed a higher IC₅₀ (Figure 2 and Supplementary Material Table 5). Furthermore, the peptide KV showed to be capable to inhibit the bacterial growth of the multiresistant MRSA C. I. strain (39791) (Figure 2 and Supplementary Material Table 5).

Most of the peptides reached a peak in their antibacterial activity against the *S. aureus* strains after 24 hours of exposure (Supplementary Material Table 5). For example, AA, KS and RN inhibited the *S. aureus* strains after 24 hours of exposure, a capacity that was not displayed in the shorter exposure. Furthermore, GF inhibited the reference strain growth at the lowest IC₅₀ found between the individual peptides results (22.8µM) (Supplementary Material Table 5). Although overall, none of these peptides individually inhibited the bacteria growth of all the strains tested, AA, AT and KS peptides had the

capacity to inhibit the bacteria growth of at least two strains of *S. aureus* (Supplementary Material Table 5). On the other hand, in general, the most susceptible bacteria to the peptides were the reference strain (ATCC® 25923) and, surprisingly, the clinically isolated strain 36055 (Supplementary Material Table 5). Also, the only peptide which did not show antibacterial activity against the *S. aureus* strains tested at any time was the NS peptide.

In addition, statistical analysis of the antibacterial activity of the peptides showed that after 24 hours of exposure, the capacity of the peptides to inhibit the bacterial growth was significantly reduced, depending on the C. I. strain it was exposed to when comparing with the reference strain (Supplementary Material Table 6). Furthermore, between the hydrophobic peptides, this antibacterial activity diminution was displayed by more C. I. *S. aureus* strains. For example, the GF peptide's antibacterial activity decreased when it was exposed to all the C. I. strains tested. However, the NS and RN peptides' antibacterial activity did not decrease against any of the C. I. strains they were exposed to (Supplementary Material Table 6). Interestingly, taken together, these results showed that the C. I. strain 39413 growth was significantly less inhibited by the peptides when comparing with the reference strain (Supplementary Material Table 5). Still, these decreases in the antibacterial activity are not comparable to those of the antibiotics when they are exposed to resistant bacteria (calculated values lie above a 20% decrease in the antibacterial activity of the antibiotics). Finally, the antibacterial activity of the individual peptides were compared with the antibiotic controls. Results showed that overall, the antibiotics decreased the growth of all the *S. aureus* strains significantly more than the peptides individually (Supplementary Material Table 7).

Determination of antimicrobial activity of eight new peptides in combination: The peptides in combination showed their best activity against the MRSA *S. aureus* strains after 8 hours of exposure

In consequence of the low antibacterial activity that the individual peptides exhibited, antibacterial assays were realized with all the peptides in combination. Results showed that the combination had a significantly more potent activity against the bacteria strains than the peptides individually. Consequently, the combination exhibited the capacity to inhibit four of the *S. aureus* strains and in three of them, this capacity was shown until after 24 hours of exposure. Specifically, after 6 hours of exposure, the eight peptide combination inhibited the bacterial growth of the reference strain and the two C. I. multiresistant MRSA strains (Supplementary Material Table 8). Then, after 8 hours of exposure, the combination showed its broader antibacterial activity inhibiting the bacterial growth of the reference strain and three C. I. strains with a very low IC₅₀ (Figure 2 and Supplementary Material Table 8). Finally, after 24 hours of exposure, the eight peptide combination had the capacity to keep inhibiting the bacterial growth of the reference strain (ATCC® 25923) and the two C. I. multiresistant MRSA strains (Supplementary Material Table 8). In addition, this antibacterial activity did not decrease in a significant way when the combination was exposed to C.I. reference when comparing with the reference strain. Nevertheless, the combination was not able to inhibit the bacterial growth of the C. I. non-resistant strain 39413.

In addition, comparisons of the antibacterial activity of the peptides (combined) with the antibiotic controls were performed. Interestingly, all the antibiotics inhibit both the bacterial growth of the reference strain and the non-resistant C. I. strain significantly more than the eight peptide combination. Similarly, Gentamicin, Methicillin and Vancomycin inhibited the bacterial growth of the C. I. Ampicillin-resistant strain significantly more than the peptide combination and Vancomycin had the capacity to inhibit the bacterial growth of the multiresistant MRSA C. I. strains more significantly than the peptide combination (Supplementary Material Table 8). However, the eight peptide combination was able to inhibit the bacterial growth of these last strains more effectively than Ampicillin, Gentamicin, and Methicillin (Supplementary Material Table 8).

Figure 2. Antibacterial activity of the peptides (individually and in combination) against *S. aureus* strains. Microdilution assays with the peptides individually and in combination were performed in order to identify the antibacterial activity of the peptides against a reference *S. aureus* strain (ATCC 25923) and four C. I. *S. aureus* strains with different antibiotic resistance profiles (39413, 34026, 36055 and 39791). The bacteria viability was measured after 6, 8 and 24 hours of exposure (Only the 8 hour exposure viability results are shown). The individual results for each peptide were separated according to their hydrophobicity. High hydrophobicity individual peptides were more potent inhibiting bacterial growth than low hydrophobicity individual peptides. The eight peptide combination was more potent than the peptides individually and it was able to inhibit bacterial growth of four of the *S. aureus* strains evaluated. Furthermore, this antibacterial activity was stronger in the multiresistant clinically isolated strains (36055 and 39791).

Discussion

The worldwide emergence of multi-antibiotic resistant infectious diseases, such as the ones caused by *S. aureus*, enhance the need to search for new antibacterial agents. As mentioned previously, this study aimed to identify the potential of eight new peptides as new antibacterial agents as a basis for the search for topic prevention and treatment against *S. aureus*. With this intention, *in silico* analysis of the antimicrobial activity, stability, sequence similarities and immunogenicity of the eight new peptides features were performed. Secondly, the resistance profile of the *S. aureus* strains used in this study were described, allowing to identify two multiresistant MRSA strains among them. Then, the antibacterial activity of the eight peptides individually and in combination were evaluated after 6, 8 and 24 hours of exposure. Finally, the described antibacterial activity was compared between strains and with that of the antibiotics.

In silico results determined that, although the eight peptides described in this study do not share the same characteristics, all predictions regarding their general characteristics indicated their potential to be antimicrobial peptides. The predictions about their stability features suggested the possible environments where they would be active as well as the ones where they could easily degrade. Positive net charge, short length, amphipathic composition and formation of an α -helix are common characteristics presented by host defense peptides with antimicrobial activity, especially because these features are associated with the capacity of the peptides to interact with living cell membranes (Zhang & Gallo, 2016). This interaction is important, because most of the mechanisms of action of the antimicrobial peptides described focus on the capacity of the peptide to disrupt or translocate living membranes (Nguyen, Haney & Vogel, 2011). Hence, these characteristics are usually correlated with the biological activity of the peptides (Soltani, Keymanesh, & Sardari, 2007). Although the aforementioned peptide characteristics are the ones most taken into account in the search for new antimicrobial peptides, we also evaluated the peptides stability in certain environments *in silico* in this study. A major limitation of the peptides biological activity is their susceptibility to proteases (Meng & Kumar, 2007). However, according to the *in silico* analysis of the peptides in this study, three would be considered highly stable in proteolytic environments. Nevertheless, all the peptides were determined to be susceptible to a wide range of proteases, indicating an important limitation of the peptides. Studies have proposed alternative modifications to increase the stability of antimicrobial peptides in proteolytic environments, such as the amidation and acetylation of the terminals or by introducing tryptophan substitutions at cleavage sites (Strömstedt et al., 2009). These alternatives should be taken into consideration for further studies with these peptides.

Alignments *in silico* of the peptides' sequences found proteins and antimicrobial peptides already described with sequence similarities to the eight new peptides of this study. Previous studies have found, that many antimicrobial peptides are derived of the cleavage of bigger proteins (Stewart et al., 2008). For example Buforin I, a potent antimicrobial peptide, is the product of the cleavage of the α Histone H2A by pepsins (Kim et al., 2000). Similarly, LK and KV peptides were found to have sequence similarities with histones. These results might predict the possible protein precursor of seven of the eight new frog peptides described in this study, an assumption supported by the fact that the majority of these possible protein precursors were identified in the taxonomic group of amphibians. On the other hand, all the eight new peptides were identified to have sequence similarities with antimicrobial peptides already described. Some studies have considered, that many peptides are derived from other larger peptides. In some cases, the shorter new peptides present more potent biological activity (Zhao et al., 2016). In addition, these sequence similarities allow classifying the peptides into the different families of antimicrobial peptides already described (Khamis et al., 2014), however further studies would be needed to ascertain the pertinence of each peptide to the families that are suggested here.

Immunogenicity is one of the biggest concerns in the search for new antimicrobial agents (Yeaman, & Yount, 2003). As peptides are amino acid sequences of short length, they are susceptible to be recognized via class I and class II HLA or simply by antibodies. Hence, in this study, we performed analysis with bioinformatic tools, which are commonly used to identify antigens that might be recognized by the immune system, along with specific tools that directly predict the immunogenicity of the peptides. The *in silico* analysis determined, that LK and KV peptides were predicted to have a major probability to trigger an immune response. Nonetheless, AA, KS and GF peptides were considered too short to be recognized by some of the aforementioned recognition molecules, which is considered a major advantage for their subsequent use as therapeutic agents (Fox, 2013).

S. aureus infections are one of the major concerns worldwide due to their increased resistance to antibiotics, especially because they can cause several severe health issues when they are acquired in healthcare environments (Fournier & Philpott, 2005). Here, we evaluated the antibacterial activity of the peptides against a reference strain and four clinically isolated strains, including two methicillin-resistant *Staphylococcus aureus* (MRSA) strains. The results with the MRSA strains indicated, that the use of the peptides instead of antibiotics significantly decreased the bacterial growth more, indicating the importance of evaluating the new antibacterial agent candidates against multiresistant bacteria. Although the peptides individually decreased the bacterial growth, the IC₅₀ of their antibacterial activity ranges between 20-100µM. These results contrast with other synthetic peptides, which with a calculated IC₅₀ range of in-between 4µM and 8µM, have shown a much more potent antibacterial activity against *S. aureus* (Mohamed, Abdelkhalek & Seleem, 2016).

Interestingly, among the antibacterial assays performed with the peptides individually, the GF peptide showed the best results against one of the MRSA strains, AT the most prolonged activity and AA the best antibacterial activity against several *S. aureus* strains. Meanwhile, the *in silico* results determined, that the eight new peptides have sequence similarities with described peptides reported to have antibacterial activity. Impressingly, the peptides that matched the sequence of AT, GF and KV were described in other studies as highly active against reference strains of *S. aureus* (Čeřovský et al., 2009, Yang, et al., 2013 and Apponyi et al., 2004). Although highly homologous peptides can greatly vary in their biological activity, peptide families usually show an overall antimicrobial activity addressed against a specific group of bacteria. This is the case of the Temporin SN3 and SN2 peptides, which showed a 50% of sequence similarity with the GF peptide, and belong to the Gram + antibacterial peptide family of Temporins (Conlon, 2011). On the other hand, the NS peptide was the only peptide that did not present antibacterial activity against any evaluated *S. aureus* strain. We consider that, as shown in the *in silico* results, its susceptibility to the *S. aureus* staphylococcal peptidase I degraded it during the bacterial exposure in the assays. These results highlight the importance of bioinformatic tools available for the analysis of these molecules and should be used in the search for new host defense peptides.

Finally, in consequence of the variation in the antibacterial activity shown by the individual peptides, antibacterial assays were performed with all the eight peptides in combination. In accordance with previous results in the Human Genetics Laboratory, the combination of peptides showed the most potent and broad antimicrobial activity. It has been proposed, that this increased activity might be consequence of the synergic action of the peptides, a suggested common phenomenon in antimicrobial peptide interactions (Yu, 2016). Nonetheless, the combination of molecules with biological activity can result in synergistic, additive or antagonistic interactions (Yu, 2016). Therefore, further studies should be conducted with the eight peptides in combination, in order to understand their action mechanisms and to sort out irrelevant combinations of peptides in order to find combinations enhancing the desired antibacterial activity.

In conclusion, this study describes the potential antimicrobial activity, stability, sequence similarities and immunogenicity features of eight new peptides (*in silico*), and the *in vivo* antibacterial

activity against one reference and four multiresistant strains *S. aureus*. The analysis identified that GF, AT and AA individually showed the best antibacterial activity against the *S. aureus* strains evaluated. However, this effect depended on the *S. aureus* strain and was not significantly better than the use of antibiotics to decrease the bacteria growth. Instead, the combination of the eight peptides showed antibacterial activity against multiple *S. aureus* strains and was the better treatment to decrease the bacterial growth, especially of the clinically isolated strains, when compared with the individual antibiotics.

Part 2: Cytotoxic and immunomodulatory activity of eight new peptides

Methodology

Cell culture of fibroblasts (VERO) and human monocytes (THP-1) and differentiation to macrophages

The African green monkey fibroblast-like kidney cells (VERO) were maintained at 37°C and 5% CO₂ in DMEM media with 10% FBS and 1% Penicillin/Streptomycin. The human monocytic cell line (THP-1) was maintained at 2×10^5 cells/mL in RPMI 1640 media with 10% FBS and 1% Penicillin/Streptomycin at 37°C and 5%CO₂. Macrophages were obtained using the protocol proposed by (Daigneault et al. 2010), exposing THP-1 monocytes to 200nM phorbol 12-myristate 13-acetate (PMA, Sigma, P8139) for three days, followed by five days of incubation in RPMI media with supplements. After treatment, the confirmation of the monocytes-to-macrophages differentiation was performed by analysis of the expression of recognized macrophage markers (CD11b, CD14 and CD36). Briefly, cells were detached by exposing them to 10mM EDTA for 40 minutes at 4°C. Supplemented media was added to the flasks and the cell suspension put in FACS tubes. Afterwards, two washes were performed by centrifuging for five minutes at 2500 rpm and 21°C and the pellet resuspended in 3mL of PBS. The final suspension was incubated with 5µL of APC Mouse Anti-Human CD11b (Clone D12, #333143, BD Biosciences), PE Mouse Anti-Human CD14 (Clone M5E2, #555398, BD Biosciences) and Anti-Human CD36 (Clone CD38, #55545, BD Biosciences) for 30 minutes. After incubation, two more washes were performed and ultimately, the pellet with the marked cell was resuspended in 100µL of PBS. Finally, cells were analyzed via flow cytometry (FACSCanto™, BD Biosciences).

Determination of the cytotoxic activity of eight new peptides individually and in combination in fibroblasts (VERO), monocytes (THP-1) and macrophages (THP-1 differentiated) cell cultures

MTT cytotoxic assays were realized with the three cell lines described above, including the differentiated macrophages. Firstly, 1×10^5 cells/well were seeded in 96-well microplates for 24 hours. Following, they were exposed to seven concentrations (0, 3.12, 6.25, 12.5, 25, 50 and 100µM) of the peptides, individually and in combination, the antibiotics (Ampicillin, Gentamicin and Vancomycin) and the positive control (Doxorubicin). After incubation for 24 hours, MTT reagent (Sigma-Aldrich, St. Louis, USA) was added and the cells were incubated for two more hours. Then, the media was removed and 100µL of DMSO were aggregated to each well and the plates were read at 595nm with a spectrophotometer (Bio-Rad, Philadelphia, PA, USA). Ultimately, cytotoxic concentration 50 (CC50) was calculated as the concentration at which the peptides produced the 50% of the death of the cells in two replicates with three repetitions each.

Determination of the immunomodulatory activity of three new peptides individually in human macrophage (THP-1 differentiated) cell culture in three scenarios: without stimulation, with *E. coli* LPS stimulation and with *S. aureus* lysate stimulation

After differentiating the THP-1 monocytes to macrophages as mentioned previously, 1×10^5 cells were seeded in 96-well plates. The macrophages were exposed to three concentrations (0, 6.25, 25µM) of three peptides selected for their strong antibacterial and weak cytotoxic activity. Experiments were performed with and without 0.5µL/mL of *Escherichia coli* LPS (Sigma-Aldrich, St. Louis, USA) or *S.*

aureus lysate with two repetitions in two replicates. The *S. aureus* lysate was obtained previously by heat-killing (98°C for 30 minutes) the ON growth of the *S. aureus* reference strain (ATCC 25923), followed by the lyophilization of the pellet obtained after the centrifugation (2500 rpm for five minutes) of the bacterial culture and washing with ddH₂O. Afterwards, the exposed macrophage culture plates were incubated for 48 hours at 37°C with 5%CO₂ and the cells and supernatant were collected for further analysis. Firstly, in the supernatants the concentration of 13 cytokines (IL-1 β , IFN- α , IFN- γ , TNF- α , MCP-1 (CCL2), IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IL-18, IL-23 e IL-33) was determined with the LEGENDplex™ Human Inflammation Panel (13-plex) kit (Biolegend) following the recommendations of the kit. The results were obtained using flow cytometry (FACSCanto™, BD Biosciences). Secondly, the expression of the cell markers CD11b, CD14 and CD36 was evaluated in the macrophages following the methodology described above.

Statistical Analysis

As in part 1, all statistical analysis were performed using the computing environment R (R Development Core Team, 2008). In all cases, a residual analysis was performed to evaluate the normality and homoscedasticity of the data and all plots were created with the package ggplot2 (Wickham, 2009). Also, for the evaluation of the cytotoxic activity, the GRmetric package (Gentleman et al., 2004) was used for the creation of the dose-response sigmoidal models and calculations of the CC₅₀. In addition, the model selection analysis, Bartlett's test and DFA analysis were performed using the packages MuMin (Gelman, 2008), psych (Revelle, 2017) and candisc (Friendly & Fox, 2017) respectively.

Results:

The cytotoxic activity of the peptides was evaluated in three cell lines (VERO-Fibroblasts, THP-1-Monocytes and THP-1-Macrophages): The eight peptide combination and the AT and LK peptides individually displayed cytotoxic activity only against THP-1 monocytes

The overall results indicate that the peptides individually and in combination presented significant differences between the cell culture types (Figure 4 and Supplementary Material Table 9). Firstly, assays performed in the VERO cell line showed, that the peptides did not have cytotoxic activity against these fibroblasts, neither individually nor in combination. In contrast, the antibiotics evaluated (Ampicillin, Gentamicin and Vancomycin) negatively affected the cells viability, even though none decreased the cells viability more than 50% (Figure 4 and Supplementary Material Table 9). The positive control, Doxorubicin, inhibited the cells growth significantly and presented an IC₅₀=56.71 μ M as expected (Figure 4 and Supplementary Material Table 9).

Figure 3. THP-1 monocytes differentiation into macrophages after 200nM PMA exposure. THP-1 monocytes were exposed to 200nM PMA for three days, followed by five days of incubation in RPMI media with supplements free of PMA. Photos taken at day 0 of the THP-1 monocytes before PMA treatment show their suspension phenotype and small size. At day two cells started to change their morphology and to adhere. After four days, macrophages elongated and remained in their adherent phenotype. After eight days, macrophages were fully differentiated.

Contrary to the above results, cytotoxic activity assays performed in the THP-1 monocytes cell culture showed, that all high hydrophobicity peptides and the eight peptide combination inhibited the cells viability in a significant way. However, only AT and LK individually and the eight peptide combination inhibited more than the 50% of the viability of the cells between the concentrations tested (Figure 4 and Supplementary Material Table 9). In addition, the antibiotics evaluated did not inhibit the monocytes viability (Figure 4 and Supplementary Material Table 9). However, the positive control, Doxorubicin, significantly inhibited the THP-1 cells growth and presented a lower IC₅₀ (Figure 4 and Supplementary Material Table 9) than the one calculated for the VERO cell line.

Figure 4. Cytotoxic activity of eight new peptides (individually and in combination) in three cell lines. The peptides' effect (individually and in combination) on the viability of the cells was evaluated in three cell types (VERO-fibroblasts, THP-1-monocytes and THP-1-macrophages) via MTT assays. Cytotoxicity assays presented significant differences between cell lines. The individual peptides and the eight peptide combination did not show cytotoxic activity in the VERO – fibroblasts nor in THP-1 macrophages. In contrast, AT, LK peptides and the eight peptide combination showed cytotoxic activity against THP-1 monocytes.

Ultimately, THP-1 monocytes were differentiated into macrophages using PMA. Cells rapidly changed their suspension phenotype typical for monocytes to the adherent expected phenotype of macrophages, further morphological changes and changes in size were observed (Figure 3). In addition, in order to confirm the differentiation, analysis of the expression of recognized macrophages markers (CD11b, CD14 and CD36) were performed. As expected, the results showed that macrophages expressed

the CD11b marker significantly more than monocytes, while the CD14 and CD36 markers were expressed with no significant difference in both macrophages and monocytes. Following, we performed cytotoxic activity assays with the THP-1 macrophages as described previously. Results showed that all the peptides individually and in combination inhibited the cell viability significantly, however, none of them was able to inhibit the 50% of the viability of the macrophages between the different concentration treatments (Figure 4 and Supplementary Material Table 9). In contrast with the previous results in the monocyte cell line, antibiotics inhibited the cells viability, but still, none were able to decrease the 50% of the cells viability (Figure 4 and Supplementary Material Table 9). Finally, consistent with the monocyte assays results, Doxorubicin decreased the macrophages viability significantly and presented a similar IC50 (Figure 4 and Supplementary Material Table 9) to the one calculated for the THP-1 monocytes.

Immunomodulatory activity of the peptides: Each evaluated peptide (AA, GF and RN) displayed dose-dependent immunomodulatory activity, which was significantly different depending on the molecules which the macrophages were stimulated with

After differentiating the THP-1 monocytes to macrophages as mentioned previously, the macrophages were exposed to three concentrations (0, 6.25, 25 μ M) of three peptides selected for their high antibacterial and low cytotoxic activity (AA, GF and RN). Furthermore, experiments were performed in three different stimulation scenarios: without stimulation, with *E. coli* LPS stimulation or with *S. aureus* lysate stimulation. In the supernatants of the exposed cells, the concentration of 13 cytokines (IL-1 β , IFN- α , IFN- γ , TNF- α , MCP-1 (CCL2), IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IL-18, IL-23 e IL-33) was determined with the LEGENDplex™ Human Inflammation Panel (13-plex) kit (Biolegend). The results obtained indicated that the peptides could modulate the secretion of four (IL-1 β , MCP-1, TNF- α and IL-8) of the 13 cytokines analyzed.

A Bartlett's test was performed to identify if the cytokines secretion variables were correlated. Results indicated a strong correlation between the variation of the variables, hence a DFA was performed to determinate whether each peptide modulated the cytokine secretions in a significantly different manner (Figure 5). A DFA was performed for each different stimulation scenario, results demonstrated that each peptide modulated the cytokine secretion differently. Specifically in the non-stimulation scenario (Figure 5), the discriminant function Can 1 explained the 95% of the variation between the variables related to cytokine secretion, where the AA peptide increased the secretion of IL-8 and decreased that of the IL-1 β , MCP-1 and TNF- α cytokines. On the contrary, while both the GF and RN peptides decreased the secretion of IL-8, only GF increased the secretion of TNF- α and IL-1 β and only RN increased the secretion of MCP-1. Regarding the *E. coli* LPS stimulation scenario (Figure 5) the discriminant function Can 1 explained 72.7% of the variation, while the Can 2 explained 27.3%. In this analysis, the RN peptide increased all the cytokines secretions, while the AA and GF peptides decreased the cytokine secretions, especially IL-1 β /TNF- α and MCP-1/IL-8 respectively. Finally, the *S. aureus* lysate stimulation scenario (Figure 5) showed, that the peptides modulated the cytokine secretions in an again different manner. The discriminant function Can 1 explained 97% of the variation, where the AA peptide increased the IL-8 and MCP-1 cytokine secretion, while RN and GF decreased these cytokine secretions. In addition, in this scenario, the RN peptide also decreased the IL-1 β and TNF- α secretion, while the GF peptide increased their secretion.

To determine the existence of significant differences in the effect the peptides had on each of the cytokines secretions between the two peptide concentrations tested, a model selection with the Akaike information criterion was performed. In all the cases, the best linear model was the one that considered the peptide, the peptide concentration, and the stimulation scenario. Then, a linear model analysis was performed with each cytokine as the response variable. The results showed, that IL-1 β secretion, without the presence of the peptides, significantly increased when the macrophages were stimulated with the *E. coli* LPS, while there were no differences in IL-1 β secretion when the macrophages were stimulated with

the *S. aureus* lysate. When the macrophages were exposed to the AA and GF peptide in the non-stimulation scenario, the cytokine secretion increased at the lowest peptide concentration. In addition we observed an increment in this effect, especially when the GF peptide concentration was higher. In contrast, at the low concentration, RN peptide increased the IL-1 β secretion significantly more, while there was no observable modulation of the cytokine secretion at the high concentration (Figure 6 and Supplementary Material Table 10). In the *E. coli* LPS stimulation scenario, the three peptides decreased the IL-1 β secretion at their lowest concentration, but then at the highest concentration, they significantly increased the cytokine secretion, especially the RN peptide (Figure 6 and Supplementary Material Table 10). Finally, when the macrophages were stimulated by the *S. aureus* lysate, the three peptides increased the IL-1 β secretion at low concentrations. Furthermore, when the GF and RN concentration increased, the concentration of the IL-1 β further increased, while it remained similar when the AA concentration increased (Figure 6 and Supplementary Material Table 10).

The MCP-1 cytokine concentrations in the non-stimulation and the *E. coli* LPS stimulation scenarios were not significantly different, while they decreased significantly in the *S. aureus* lysate stimulation scenario. When the macrophages were exposed to the AA and GF peptides in the non-stimulation scenario, the MCP-1 secretion decreased, and this effect did not change when the peptides concentration increased. In contrast, when the cells were exposed to the low concentration of the RN peptide, the cytokine secretion increased, but when they were exposed to its high concentration, the cytokine secretion decreased (Figure 6 and Supplementary Material Table 10). In the *E. coli* LPS scenario, the AA and GF peptides kept decreasing the MCP-1 secretion, and in this case, this effect augmented when the peptides concentration increased. On the contrary, exposing macrophages to the RN peptide at a low concentration did not have any effect on the cytokine secretion, while the peptide at a high concentration increased its secretion (Figure 6 and Supplementary Material Table 10). In the *S. aureus* lysate stimulation scenario, all the peptides increased the MCP-1 secretion in their low concentration, but this effect decreased when the AA peptide was presented at a higher concentration, while it remained stable when the GF and RN concentration increased (Figure 6 and Supplementary Material Table 10).

Regarding the IL8 cytokine concentration, our results show that after the macrophages were not stimulated and when they were stimulated with *E. coli* LPS and *S. aureus* lysate, there were no significant differences in the cytokine secretion and with *S. aureus* lysate. However, when the macrophages were not stimulated but exposed to the peptides, the low concentration of the GF and RN peptides increased the IL8 secretion and remained unaffected when the RN concentration increased, but it decreased when the GF concentration increased (Figure 6 and Supplementary Material Table 10). In the *E. coli* stimulation scenario, the three peptides slightly decreased the IL-8 at their low concentration and increased the cytokine secretion in their high concentration. Similarly, in the *S. aureus* stimulation scenario, the three peptides marginally decreased the cytokine concentration and increased it at their high concentration, especially the GF peptide (Figure 6 and Supplementary Material Table 10).

Finally, when the macrophages were stimulated with the *E. coli* LPS, the TNF- α concentration significantly increased compared to the non-stimulation scenario, while in the *S. aureus* lysate stimulation scenario the cytokine concentration decreased. When the macrophages were not stimulated, the exposure to a low concentration of the peptides increased the TNF- α concentration, but exposed to a high peptide concentration, both the RN and the AA peptides decreased the cytokine secretion, while it was further increased by the GF peptide (Figure 6 and Supplementary Material Table 10). On the other hand, in the *E. coli* LPS stimulation scenario at a low concentration exposure, all the peptides decreased the TNF- α concentration and increased it at their high concentration, especially the RN peptide. Finally, when the macrophages were stimulated with the *S. aureus* lysate, the peptides at a low concentration increased the TNF- α concentration, and this effect further increased when the peptides were presented at a higher concentration (Figure 6 and Supplementary Material Table 10).

Figure 5. DFA analysis of the immunomodulatory activity of three peptides in three stimulation scenarios. The immunomodulatory activity of the peptides individually was evaluated with the LEGENDplex™ Human Inflammation Panel (13-plex) kit (Biolegend). THP-1 macrophages were exposed to the peptides in three stimulation scenarios (non-stimulation, stimulation with *E. coli* LPS and stimulation with *S. aureus* lysate) and the concentration of the cytokines secreted to the culture media was calculated with flow cytometry. Results of four cytokines (IL-1 β , MCP-1, TNF- α and IL8) were analyzed via DFA. Depending on each stimulation scenario, the peptides modulate the cytokine secretion to the media. Furthermore, this cytokine secretion modulation showed significant differences between the peptides.

Figure 6. Analysis of the effect of peptide concentration on the secretion of four cytokines. To determine if the concentration of the peptides had an effect on the secretion of each of the four cytokines (IL-1 β , MCP-1, IL-8 and TNF- α), a model selection was performed using the Akaike information criterion, followed by a linear model analysis. The concentration of the peptides significantly affects the cytokine secretions, and this effect further depends on the stimulation scenario. The grey line and its corresponding standard deviation represent the reference cytokine concentration for each stimulation scenario. Each treatment consists of two peptide concentrations (6.25 μ M and 25 μ M), the lightly coloured dots represent the raw data, the bigger dots represent the mean and the error bars its corresponding standard deviation.

Discussion

The need to search for new alternatives against multiresistant infectious diseases, comprehend not only the exploration for new antibiotic agents, but also for new therapeutic agents for alternative therapies such as the immunomodulatory therapy. With the objective of healing *S. aureus* infections, it

has been proposed that treatment should include immunomodulatory therapy (Wardenburg, Williams & Missiakas, 2006 and Thangamani, Younis & Seleem, 2015). Therefore, as mentioned previously, this study aimed to identify the potential of eight new peptides as immunomodulatory agents as a basis for the search of new therapeutic agents for the topic prevention and treatment against *S. aureus*. With this intention, cytotoxicity assays in three cell lines, including two of immune-related cells were performed. Those results, together with the antibacterial activity of the peptides evaluated in part 1, allowed to select AA, GF and RN for the immunomodulatory activity assays. Finally, the evaluation of the capacity of the peptides to stimulate and/or inhibit the production of cytokines were performed in three different simulation scenarios.

After PMA exposure, the THP-1 monocyte cell line was successfully differentiated to macrophages using the protocol of Daigneault et al. (2010). This protocol was used, because it has been reported to differentiate the THP-1 cell into macrophages which resemble the phenotype of primary human monocyte-derived macrophages more. Specifically, the study showed that this treatment enhances cytoplasmic growth and increased the number of mitochondria and lysosomes as well as a change in differentiation-dependent cell surface markers such as CD11b. Concomitantly, our results allowed to visualize these differences. After eight days of treatment, according with the flow cytometry assays, the THP-1 macrophages expressed the CD11b marker more and were significantly bigger and more granular than the THP-1 monocytes. In consequence we expect that, as found by Daigneault et al., the expressed cytokine profile in response to stimuli resembled that of the human monocyte-derived macrophages.

Although the expected results of the cytotoxic activity anticipated that the peptides might not have effect on the exposed cell lines for their possible subsequent use as a topic therapeutic agent, the results showed that the peptides had differential cytotoxic activity on the cells depending on the exposed cell line. This variation was also demonstrated between cells, which were and were not differentiated. Previous studies performed not only with antimicrobial peptides but also with other molecules, such as antibiotics and antitumorigenic agents (Florento et al., 2012), showed that the effect of the molecules depends heavily on the cell type which they were exposed to. Taken together, these results illustrate the need of testing the peptides not only with one cell line, but instead with a multitude of different cell types.

In this study the THP-1 monocytes were more susceptible to the AT and LK and the eight peptide combination than the THP-1 macrophages. Accordingly, it has been reported that the monocytes are especially susceptible to apoptosis and are easily stimulated to apoptosis, while macrophages are considered to be resistant to many apoptotic stimuli (Liu et al., 2001). On the other hand, the *in silico* analysis performed in part 1 identified that AT have a sequence similar to Lasioglossin LL-I, a peptide reported to have antitumorigenic activity in a wide range of cancer cell lines, including the leukemia THP-1 cell line used in this study (Čeřovský et al., 2009). Meanwhile, antitumorigenic activity assays have not been performed with the peptide that has a similar sequence to the LK peptide, XPF-St7, but it was reported to have cytotoxic activity (Roelants et al., 2013). Hence, we propose that as two peptides (AT and LK) and the eight peptide combination showed a potent cytotoxic activity against the THP-1 cell line, further studies could be of interest in a subsequence analysis as antileukemic agents. Nevertheless, because of their cytotoxic activity and despite the antibacterial activity of these peptides and the peptide combination found in part 1, AT, LK and the eight peptide combination were discarded for the immunomodulatory activity assays.

Regarding the immunomodulatory activity, this study evaluated the capacity of three host defense peptides to modulate the macrophages secretion of a wide cytokine panel for the first time. Similar studies evaluating a smaller number of cytokines have found, that some peptides can modulate the macrophages' cytokines secretion, and that this activity is usually described as complex effect in different cytokines (Pantic et al., 2014). For example, Tigerinin 1R, Tigerinin 1V and Tigerinin 1M

demonstrated to have the capacity to increase the production of the IL-10 anti-inflammatory cytokines and to decrease the IFN- γ secretion in a mouse macrophage cell line (Pantic et al., 2014). Other studies have shown, that peptides can increase the production of pro-inflammatory cytokines, such as Frenatin 2D (TNF- α , IL-1 β and IL-12) (Conlon et al., 2013) and Escuelin-2CHA (TNF- α) (Attoub et al., 2013). Concomitant to our results, each peptide tested (AA, GF and RN) showed a different profile of increasing or decreasing the cytokine secretions by THP-1 macrophages. Interestingly, these profiles varied according to the stimulation scenario which the macrophages were exposed to. These results might suggest the existence of some specificity of the peptides to modulate the immune response depending on the microorganism to which they are exposed to, or to which the macrophages respond. Studies have proposed that this immunomodulatory activity of the antimicrobial peptides found in frogs might indicate, that the peptides also regulate the immune response in terms of pro- and anti-inflammatory cytokine secretions (Pantic et al., 2014, Conlon et al., 2011 y Conlon et al., 2014).

In regards to the objective of this study, interestingly all the peptides evaluated, but especially the GF peptide, increased the cytokine secretion (IL-1 β , MCP-1, TNF- α and IL8) when the macrophages were stimulated with *S. aureus* lysate. Studies have determined, that the IL-1 β secretion by bone-marrow derived cells, such as macrophages, is required for neutrophil recruitment and bacterial clearance in *S. aureus* infections (Miller et al., 2007). Furthermore, the increase in the IL-8 interleukin concentration allows neutrophils to significantly reduce the bacterial load. In addition, an increase in this cytokine decrease the neutrophils apoptosis that is usually enhanced by *S. aureus* (Zurek, Pallister & Voyich, 2015). However, as *S. aureus* induces inflammatory cytokines and causes inflammatory diseases, during infection the MCP-1 and TNF- α cytokine concentrations are commonly increased during infection, a further increment in these cytokines' secretion would exacerbate the infection. The aforementioned and several other studies have demonstrated, that cytokines play an important role in *S. aureus* clearance and infections healing. This has been confirmed in recombinant cytokine treatments *in vivo* in mammals studies, where the enhancement of IL-1 β concentrations successfully cured the infections in 75% of the cases (Daley et al., 1993). Therefore, new agents capable to modulate the cytokine secretion in a *S. aureus* infection scenario are of medical interest for developing new therapies.

In conclusion, this part describes the cytotoxic and immunomodulatory activity of eight new peptides. The assays demonstrated that, the majority of the peptides (except LK and AT), did not present cytotoxic activity, a desirable feature for their subsequent use as drugs. Nevertheless, the LK, AT peptides and the eight peptide combination are proposed as molecules of interest for new anti-leukemia agents studies. Finally, AA, GF and RN peptides presented immunomodulatory activity in THP-1 macrophages. This feature depended on the peptide and the stimulation which the macrophages were exposed to. Results indicate that AA, GF and RN might be of interest for immunomodulatory therapy against *S. aureus* infections. Nevertheless, further studies should be performed to better understand the peptide interactions with the immune system.

General Conclusions

The worldwide emergence of multi-antibiotic resistant infectious diseases, as the ones caused by *S. aureus*, enhance the need to search for new alternatives for therapeutic agents. As mentioned previously, this study aimed to identify the potential of eight new peptides as immunomodulatory or antibacterial agents, or both, as a basis for the search of new agents for the topic prevention and treatment against *S. aureus*. With this intention, *in silico* analysis, antibacterial and cytotoxic assays and the evaluation of the capacity of the peptides to stimulate and/or inhibit the production of cytokines were performed. The analysis identified AA, GF and AT as the peptides with best antibacterial performance individually against the *S. aureus* strains evaluated. However, the combination of the eight peptides was the better treatment to decrease the bacterial growth, especially of the clinically isolated strains. Furthermore, the majority of the peptides, except for LK, AT and the peptide combination, did not

present cytotoxic activity, which is a desirable feature for their subsequent use as drugs. Nevertheless, LK and AT peptides are proposed as molecules of interest for the study of new anti-leukemia agents. Finally, AA, GF and RN peptides presented immunomodulatory activity in THP-1 macrophages. This feature depended on the peptide and the stimulation which the macrophages were exposed to. Results indicate that AA, GF and RN might be of interest for immunomodulatory therapy against *S. aureus* infections. Nevertheless, further studies should be performed to better understand the peptide interactions with the immune system.

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Appendix

Supplementary Material Table 1. Predicted stability features of the eight new peptides. The ProtParam Tool and PeptideCutter Tool of ExPASy and the HLP: “Web server for predicting half-life of the peptide in an intestine-like environment” were used to predict stability features of the eight new peptides. All the peptides were predicted to stay stable for hours *in vitro* in mammalian reticulocytes, while only AA, AT and GF might conserve their structure *in vivo* in yeast and bacteria. The instability index classified KS, GF, KV and LK as unstable in test tube conditions, while the aliphatic index predicted that RN might be the peptide with the highest potential to be thermostable. Interestingly, the predictions in an intestine-like environment showed, that all the peptides might be stable in a proteolytic environment. The PeptideCutter Tool predicted that NS would be susceptible to the Staphylococcal peptidase I.

	Half-life ¹						Intestin-like environment ²		
	<i>in vivo</i>								
ID	<i>in vitro</i>	Yeast	Bacteria	Instability index ¹	Class ¹	Aliphatic index ¹	Half-life	Stability	Cutters ³
AA	4.4h	>20h	>10h	-21,7	Stable	89,09	1,03	High	LysC, LysN, Proteinase K, Thermolysin, Trypsin
KS	1.3h	3min	3min	119	Unstable	48,75	2,41	High	CNBr, Chymotrypsin-low specificity, LysC, LysN, Pepsin (pH1.3 and >2), Proteinase K, Thermolysin, Trypsin
NS	1.4h	3min	>10h	23,0	Stable	68,82	0,36	Normal	Asp-N endopeptidase + N-terminal Glu, CNBr, Chymotrypsin-low specificity, Glutamyl endopeptidase, LysC, LysN, Pepsin (pH1.3 and >2), Proteinase K, Staphylococcal peptidase I, Thermolysin, Trypsin
RN	1h	2min	2min	12,4	Stable	148,5	0,74	Normal	Arg-C proteinase, Chymotrypsin-low specificity, Clostripain, LysC, LysN, Pepsin (pH1.3 and >2), Proteinase K, Thermolysin, Trypsin
AT	4.4h	>20h	>10h	-6,27	Stable	110,7	0,83	Normal	CNBr, Chymotrypsin-high specificity, Chymotrypsin-low specificity, LysC, LysN, Pepsin (pH1.3 and >2), Proteinase K, Thermolysin, Trypsin
GF	30h	>20h	>10h	42,2	Unstable	106,3	0,52	Normal	Arg-C proteinase, CNBr, Chymotrypsin-high specificity, Chymotrypsin-low specificity, Clostripain, LysC, LysN, Pepsin (pH1.3 and >2), Proteinase K, Thermolysin, Trypsin
KV	1.3h	3min	3min	44,9	Unstable	151	0,90	Normal	Chymotrypsin-low specificity, LysC, Pepsin (pH1.3 and >2), Proteinase K, Thermolysin, Trypsin
LK	5.5h	3min	2min	52,4	Unstable	165,2	1,00	Normal	Chymotrypsin-low specificity, LysC, LysN, Pepsin (pH1.3 and >2), Proteinase K, Thermolysin, Trypsin

ID = Code given to each peptide according to the first and last amino acid of its sequence. N-terminal = Amino acid, which initiates the peptide sequence and that is responsible for the free amine group of the molecule. Half life - *in vitro* = Predicted time required for half of the molecules in mammalian reticulocytes to disappear after synthesis. Half life - *in vivo* - Yeast = Predicted time required for half of the molecules in yeast cells to disappear after synthesis. Half life - *in vivo* - Bacteria = Predicted time required for half of the molecules in *Escherichia coli* cells to disappear after synthesis. Instability index = Estimate of the stability of the peptide in a test tube. Class = Peptide stability classification according to the instability index, values above 40 are classified as unstable. Aliphatic index = Prediction of the relative volume occupied by aliphatic amino acids, positively correlated with thermostability. Intestine-like - Half-life = Predicted time required to half of the molecules in an intestine-like, proteolytic environment. Intestine-like - Stability = Peptide stability classification according with the intestine-like - half-life, <0.1 = low, 0.1-1.0 = normal and >1.0 = high. Cutters = Proteases or chemicals with potential cleavage sites in the peptide. 1. ExPASy, Bioinformatics Resource Portal, ProtParam Tool (Gasteiger et al., 2005). 2. Institute of Microbial Technology, HLP: Web server for predicting half-life of the peptide in intestine-like environment (Sharma et al., 2014). 3. ExPASy, Bioinformatics Resource Portal, PeptideCutter Tool (Gasteiger et al., 2005).

Supplementary Material Table 2. Immunogenicity features of eight new peptides. The Predicted Antigenic Peptide Tool, the Immune Epitope Database and Analyze Resource and the SYFPEITHI, Database of MHC Ligands and Peptide Motives were used to identify immunogenicity features of the peptides considered as their potential to bind or interact with antibodies and the HLA (I and II). Seven of the peptides were classified as potentially antigenic, while only GF, KV, and LK were predicted to have antigenic determinants which might activate B cells. Results about the immunogenicity ranked these last three peptides also as the peptides with a higher potential to trigger an immune response via HLA I. In addition, RN, AT, KV and LK might be presented by >10 HLA I alleles, while KV and LK would be presented by >4 HLA II alleles. In regards to the peptide affinity to HLA molecules, NS, AT, KV and LK peptides might bind with high affinity to HLA type I and RN, AT, KV and LK to the HLA type II. Results of potential presentation and affinity of the KS peptides to HLA (I and II) molecules were not developed because the peptide sequence was too short. This was also the case for the AA and GF analysis prediction for their affinity to HLA (I and II) molecules.

ID	Antigenic Determinants ¹	Antigenic Propensity ¹	Immunogenicity ²		Presentation ⁵		Affinity				
			HLA I Score	HLA I	HLA II	Perc. Rank	HLA I ³		HLA II ⁴		
							SMM Align IC50 (nM)	Sturniolo Score	Perc. Rank	SMM Align IC50 (nM)	
AA	0	1,0233	-0,199670	2	0	4,15	105,99	-	-	-	
KS	0	1	-0,389600	-	-	-	-	-	-	-	
NS	0	1	-0,136308	9	3	0,2	3,48	[-1,5-5,1]	0,87		727
RN	0	1,0849	-0,270060	11	3	0,9	171,91	[-2,9-3,2]	5,92		17
AT	1	1,0572	-0,129580	15	3	0,2	38,18	[0,7-4,4]	0,38		36
GF	0	0,9631	0,886000	8	0	0,3	107,84	-	-	-	
KV	1	1,0607	0,325080	12	5	0,5	29,28	[-2,4-2.7]	0,09		26
LK	1	1,0986	0,325120	20	5	0,4	50,85	[-2,4-2.7]	0,85		79

ID = Code given to each peptide according to the first and last amino acid of its sequence. Antigenic determinants = Predicted number of antibody binding sites in each peptide. Antigenic propensity = Average antigenic propensity of the peptide, values above 1,0 are potentially antigenic. Immunogenicity = Immunogenicity of the peptide-MHC (pMHC) complex, the higher score indicates a greater probability to trigger an immune response. Presentation = Predicted HLA presentation of the peptide as a number of HLA alleles that present >18 scores with the peptide according to the SYFPEITHI database. Affinity - Percentile rank = Peptide representative percentile rank, a small numbered percentile rank indicates high affinity. Affinity - SMM Align IC50 = Peptide predicted IC50 using the scoring matrix-based predictor method (SMM), peptides with IC50 values <50 nM are considered to have high affinity. Binding - HLA II - Sturniolo Score = Peptide predicted range of affinity score according to the Sturniolo Database, values above -1 are considered to have high affinity. 1. Universidad Complutense Madrid, Immuno-Medicine Group, Predicted Antigenic Peptide Tool (Reche, 2002). 2. Immune Epitope Database and Analyze Resource, T Cell Epitope Prediction, MHC I Immunogenicity Tool (Calis et al., 2013). 3. Immune Epitope Database and Analyze Resource, T Cell Epitope Prediction, MHC I Binding Tool (Kim et al., 2012, Nielsen et al., 2003, Lundegaard et al., 2008, Peters & Sette, 2005 and Sidney et al., 2008). 4. Immune Epitope Database and Analyze Resource, T Cell Epitope Prediction, MHC II Binding Tool (Wang et al., 2008, Wang et al., 2010 and Sturniolo et al., 1999). 5. SYFPEITHI, Database of MHC Ligands and Peptide Motives, SYFPEITHI Tool (Rammensee, 1999)

Supplementary Material Table 3. Qualitative classification of the resistance profile of each of the *S. aureus* strains used in this study to the four antibiotics tested. Antibiotic sensitivity assays were performed using a qualitative method based on diffusion, antibiotic-discs with 200µM concentration of each antibiotic were seeded in LB agar plates where the bacteria was grown. The concentration used is shown in µM and µg/mL units for each antibiotic. Results resembled what was expected and the quantitative results are shown below.

			<i>S. aureus</i> strain				
	Concentration		Reference	Clinically Isolated			
Antibiotic	[μM]	[μg/mL]	ATCC 25923	39413	34026	36055	39791
Ampicillin	200	69.88	Susceptible	Susceptible	Resistant	Resistant	Resistant
Gentamicin	200	95.00	Susceptible	Susceptible	Susceptible	Resistant	Resistant
Vancomycin	200	289.9	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Methicillin	200	76.08	Susceptible	Susceptible	Susceptible	Resistant	Resistant

Supplementary Material Table 4. Quantitative results of the resistance profile of each of the *S. aureus* strains used in this study to the four antibiotics tested.

Microdilution assays were performed exposing the strains to the antibiotics at concentrations between 3.12 and 100µM, results were obtained after 6, 8 and 24 hours of exposure. The half maximal inhibitory concentration (IC50), and the half maximal effective concentration (EC50) are shown. In all cases the significance was <0.05 and the coefficient of determination of the model (r^2) > 0.77.

<i>S. aureus</i> Strain	Hours of exposure	Antibiotic							
		Ampicillin		Gentamicin		Methicillin		Vancomycin	
		IC50 (uM)	EC50 (uM)	IC50 (uM)	EC50 (uM)	IC50 (uM)	EC50 (uM)	IC50 (uM)	EC50 (uM)
ATCC 25923	6	2,24	2,06	1,27	1,23	0.566	0.551	0,98	0,96
	8	1,68	1,6	1,12	1,09	0,299	0,295	1,05	1,04
	24	1,22	1,17	1,27	1,23	0.521	0.515	0,9	0,89
Clinically isolated 39413	6	3,88	3,74	1,59	1,51	0.322	0.318	1,56	1,56
	8	2,98	2,98	1,06	1,04	0,640	0,635	0,95	0,94
	24	8,73	8,74	4,58	4,58	0.419	0.416	0,62	0,61
Clinically isolated 34026	6	17,47	17,5	1,57	1,5	2.15	2.13	1,13	1,11
	8	26,51	26,5	1,06	1,01	1,52	1,50	0,85	0,84
	24	32,67	32,7	12,3	12,3	9.66	8.49	1,08	1,08
Clinically isolated 36055	6	125	125	-	66,1	-	2.09	0,92	0,92
	8	42,1	24,2	-	-	-	-	0,7	0,69
	24	-	27,9	-	-	-	0.26	0,97	0,96
Clinically isolated 39791	6	58,5	32,1	-	18,5	-	21.62	1,07	1,07
	8	32,94	9,56	-	-	-	-	0,82	0,81
	24	-	12,76	-	15,3	-	0.247	0,88	0,88

Supplementary Material Table 5. Antimicrobial activity of eight new peptides individually. Microdilution assays were performed exposing the *S. aureus* strains to the peptides individually at concentrations between 3.12 and 100µM, results were obtained after 6, 8 and 24 hours of exposure. In all cases the significance was <0.05 and the coefficient of determination of the model (r2) > 0.77.

<i>S. aureus</i> Strain	Hours of Expos.	Low Hydrophobic Peptides								High Hydrophobic Peptides							
		AA		KS		NS		RN		AT		GF		KV		LK	
		IC50	EC	IC50	EC	IC50	EC	IC50	EC	IC50	EC	IC50	EC	IC50	EC	IC50	EC
ATCC□ 25923	6	-	-	-	16,6	-	4,8	-	5,9	-	11,1	-	-	-	4,8	-	-
	8	-	-	-	12,3	-	5,6	-	12,2	-	20,9	-	11,6	-	5,6	-	92,1
	24	52,1	41,1	87,4	26,4	-	-	-	-	86,1	23,5	22,8	7,0	-	-	-	75,5
Clinically isolated 39413	6	-	-	-	18,4	-	28,1	-	9,5	-	-	-	35,3	-	28,1	-	7,1
	8	-	-	-	-	-	6,0	-	9,0	-	-	-	36,2	-	6,0	-	9,7
	24	89,0	12,5	-	23,3	-	19,1	-	25,8	100	78,6	-	20,5	-	19,1	-	26,9
Clinically isolated 34026	6	-	-	-	-	-	10,9	-	5,6	-	-	-	33,1	-	10,9	-	-
	8	-	-	-	-	-	6,8	-	5,0	-	-	-	38,4	-	6,8	-	45,5
	24	97,4	97,4	82,4	10,1	-	16,5	-	43,3	-	11,8	-	17,4	-	16,5	-	11,2
Clinically isolated 36055	6	-	21,1	-	75,6	-	5,2	-	-	69,3	10,6	37,7	13,0	-	5,2	-	10,4
	8	-	24,7	-	22,7	-	-	-	-	88,0	24,7	67,5	67,5	-	-	-	-
	24	-	7,2	-	29,6	-	37,2	98,8	49,4	-	88,0	-	45,1	-	37,2	98,8	25,0
Clinically isolated 39791	6	-	14,4	-	-	-	10,4	-	17,2	-	65,8	-	-	-	35,3	39,9	25,9
	8	-	23,9	-	-	-	63,0	-	-	-	52,0	-	-	-	80,6	80,6	-
	24	76,7	38,2	-	-	-	35,8	-	14,3	-	10,3	-	-	-	8,1	-	20,8

Supplementary Material Table 6. Comparison of the antimicrobial activity of eight new peptides individually between the clinically isolated *S. aureus* strains. The antibacterial activity of the peptides individually against the four clinically isolated strains were compared with the corresponding antibacterial activity identified for the reference *S. aureus* strains. Only results after 24 hours of exposure are shown. The estimate (decrease (%)) in the antibacterial activity) and the significance (P-value = ***<0.001, **<0.01, *<0.05) are shown. In all cases, the coefficient of determination of the model (r2) was >0.57.

<i>C. I. S.</i> <i>aureus</i> Strain	Peptide															
	Low Hydrophobic								High Hydrophobic							
	AA		KS		NS		RN		AT		GF		KV		LK	
	Est. (%)	Sig.	Est. (%)	Sig.	Est. (%)	Sig.	Est. (%)	Sig.	Est. (%)	Sig.	Est. (%)	Sig.	Est. (%)	Sig.	Est. (%)	Sig.
39413	-		3.80	*	-		-		8.81	***	9.92	***	5.72	***	3.52	*
34026	-		-		-		-		6.55	***	10.9	***	-		4.58	**
36055	4.21	*	10.9	***	-		-		3.13	*	16.5	***	-		-	
39791	-		-		-		-		-		8.67	***	-		5.55	***

Supplementary Material Table 7. Comparison of the antimicrobial activity of eight new peptides individually with antibiotics. Microdilution assays were performed exposing the *S. aureus* strains to the peptides individually at concentrations between 3.12 and 100µM, results were obtained after 6, 8 and 24 hours of exposure. The half maximal inhibitory concentration (IC50), and the half maximal effective concentration (EC50) are shown. In all cases the significance was <0.05 and the coefficient of determination of the model (r2) > 0.77.

<i>S. aureus</i> Strain	Antibiotic	Peptide															
		Low Hydrophobic										High Hydrophobic					
		AA		KS		NS		RN		AT		GF		KV		LK	
		Est.	Sig.	Est.	Sig.	Est.	Sig.	Est.	Sig.	Est.	Sig.	Est.	Sig.	Est.	Sig.	Est.	Sig.
ATCC 25923	Ampicillin	-46.4	***	-49.0	***	-52.6	***	-53.8	***	-45.6	***	-36.6	***	-50.5	***	-48.9	***
	Gentamicin	-45.5	***	-48.2	***	-51.8	***	-52.9	***	-44.8	***	-35.8	***	-49.7	***	-48.1	***
	Methicillin	-49.7	***	-52.3	***	-55.9	***	-57.1	***	-48.9	***	-39.9	***	-53.8	***	-52.2	***
	Vancomycin	-50.4	***	-53.1	***	-56.7	***	-57.8	***	-49.7	***	-40.6	***	-54.6	***	-52.9	***
C. I. 39413	Ampicillin	-26.5	***	-32.5	***	-27.2	***	-28.2	***	-34.2	***	-25.1	***	-35.9	***	-32.1	***
	Gentamicin	-38.2	***	-44.2	***	-38.8	***	-39.8	***	-45.8	***	-36.8	***	-47.6	***	-43.8	***
	Methicillin	-51.0	***	-57.0	***	-51.7	***	-52.6	***	-58.6	***	-49.6	***	-60.4	***	-56.6	***
	Vancomycin	-51.3	***	-57.3	***	-51.9	***	-52.9	***	-58.9	***	-49.9	***	-60.7	***	-56.9	***
C. I. 34026	Ampicillin	-		-	***	-12.0	***	-	***	-11.9	*	-		-12.9	*	-13.2	*
	Gentamicin	-24.9	***	-18.7	***	-27.8	***	-24.9	***	-27.8	***	-23.1	***	-28.8	***	-29.1	***
	Methicillin	-24.7	***	-18.5	***	-27.6	***	-24.7	***	-27.5	***	-22.8	***	-28.5	***	-28.8	***
	Vancomycin	-53.4	***	-47.2	***	-56.3	***	-53.3	***	-56.2	***	-51.5	***	-57.2	***	-57.5	***
C. I. 36055	Ampicillin	-		-		-14.7	***	-		-		-		-		-	
	Gentamicin	12.1	**	-		-9.90	***	13.3	**	13.5	**	9.66	*	10.9	*	12.7	**
	Methicillin	-10.7	*	-20.1	***	-13.0	***	-9.60	***	-9.35	**	-13.2	**	-11.9	**	-10.1	*
	Vancomycin	-54.2	***	-63.6	***	-56.4	***	-53.0	***	-52.7	***	-56.6	***	-55.4	***	-53.5	***
C. I. 39791	Ampicillin	-		-		-		-		-		-		-		-	
	Gentamicin	-		-		-		-		-		-		-		-	
	Methicillin	-		-		-9.78	*	-8.57	*	-		-		-		-11.6	**
	Vancomycin	-50.2	***	-55.1	***	-56.8	***	-55.5	***	-52.8	***	-49.3	***	-53.9	***	-58.5	***

Supplementary Material Table 8. Antibacterial activity of eight new peptides in combination. Microdilution assays were performed exposing the *S. aureus* strains to the peptides in combination at concentrations between 3.12 and 100µM, results were obtained after 6, 8 and 24 hours of exposure. The antibacterial activity of the peptide combination was compared with the antibiotic controls. The half maximal inhibitory concentration (IC50), the half maximal effective concentration (EC50), the estimate (Estim.) (difference shown compared to the antibiotic (%)) and the significance (Sign.) (P-value = ***<0.001, **<0.01, *<0.05) are shown.

<i>S. aureus</i> Strain	Hours of exposure	Eight Peptide Combination		Comparison results with antibiotics							
				Ampicillin		Gentamycin		Methicillin		Vancomycin	
		IC50	EC50	Estim.	Sign.	Estim.	Sign.	Estim.	Sign.	Estim.	Sign.
ATCC□ 25923	6	58,6	8,4	-33.4	***	-41.0	***	-42.6	***	-43.7	***
	8	52,4	52,4	-41.9	***	-46.6	***	-48.8	***	-48.3	***
	24	33,3	33,3	-40.8	***	-39.9	***	-44.1	***	-44.8	***
Clinically isolated 39413	6	-	12,3	-34.4	***	-43.8	***	-50.3	***	-47.2	***
	8	-	5,6	-33.9	***	-42.7	***	-45.8	***	-44.7	***
	24	-	11,1	-24.3	***	-35.9	***	-48.7	***	-49.0	***
Clinically isolated 34026	6	-	17,1	-13.1	*	-41.6	***	-45.6	***	-45.8	***
	8	58,2	6,8	-		-40.9	***	-41.0	***	-43.1	***
	24	-	3,7	-		-19.4	***	-19.2	***	-47.9	***
Clinically isolated 36055	6	15,8	2,1	23.6	***	21.1	***	8.80	*	-37.0	***
	8	7,7	2,0	11.9	**	32.4	***	20.4	***	-32.6	***
	24	90,8	5,5	17.2	***	22.4	***	-		-43.9	***
Clinically isolated 39791	6	14,0	3,8	11.3	**	18.1	***	11.2	**	-34.8	***
	8	9,3	2,4	-		29.1	***	25.9	***	-32.7	***
	24	56,7	3,2	10.2	*	14.9	***	-		-42.6	***

Supplementary Material Table 9. Cytotoxic activity of eight new peptides individually and in combination. MTT assays were performed, exposing three cell lines to the peptides individually and in combination at concentrations between 3.12 and 100µM. Results were obtained after 24 hours of exposure. The half maximal cytotoxic concentration (CC50) is shown. In all cases the significance was <0.05 and the coefficient of determination of the model (r2) > 0.50.

[illegible]

Supplementary Material Table 10. Immunomodulatory activity of three new peptides. After exposing the peptides to the macrophages in three different stimulation scenarios and at different concentrations (6.25 and 25µM), the cytokine concentration in the cell culture media was calculated. Results were obtained after 48 hours of exposure. The cytokine concentration, as well as the standard deviation are shown. Furthermore, the significance of the difference between the peptide concentrations are shown (P-value = ***<0.001, **<0.01, *<0.05).

Peptide	Stimulation Scenario	Cytokine											
		IL-1β			MCP-1			TNF-α			IL-8		
		Without stimulation	<i>E. coli</i> LPS	<i>S. aureus</i> lysate	Without stimulation	<i>E. coli</i> LPS	<i>S. aureus</i> lysate	Without stimulation	<i>E. coli</i> LPS	<i>S. aureus</i> lysate	Without stimulation	<i>E. coli</i> LPS	<i>S. aureus</i> lysate
Without Peptide	Cytokine [pg/mL]	1195.9	1853.4	1128.1	9109.6	8058.4	9052.5	640.8	1188.2	453.2	8058.3	8057.7	8078.9
	<i>S. D.</i> (pg/mL)	100.4	127.5	4.9	0.985	5.67	5.18	24.5	18.9	22.0	9.99	2.96	5.24
AA [6.25µM]	Cytokine [pg/mL]	2031.4	1442.0	1811.9	9048.7	9075.5	9098.8	922.7	541.9	903.0	8063.3	8036.0	8043.0
	<i>S. D.</i> (pg/mL)	46.0	7.29	46.5	3.75	3.87	7.67	51.0	10.4	21.5	2.86	1.09	6.26
AA [25µM]	Cytokine [pg/mL]	2170.2	1986.5	1770	9046.4	9047.5	9080.4	813.5	661.9	1028.9	8069.3	8066.4	8105.8
	<i>S. D.</i> (pg/mL)	7.34	22.4	28.6	2.60	1.06	7.88	51.6	2.64	26.8	3.32	5.35	8.85
	Significance	**	***	*				***	***	***			**
GF [6.25µM]	Cytokine [pg/mL]	2098.8	1540.9	1799.8	9085.6	9074.9	9073.3	1028.4	678.5	725.9	8110.3	8061.5	8069.7
	<i>S. D.</i> (pg/mL)	55.8	25.9	16.5	3.57	5.67	9.69	72.5	1.59	13.9	3.20	3.34	3.83
GF [25µM]	Cytokine [pg/mL]	3146.9	2329.2	3201.3	9087.1	9053.0	9081.8	1507.7	784.6	1390.9	8120.5	8107.2	8303.6
	<i>S. D.</i> (pg/mL)	122.9	132.5	120.1	6.59	4.38	1.19	167.5	33.6	86.1	10.3	6.74	44.6
	Significance	***	***	***		*	*	***	***	***			***
RN [6.25µM]	Cytokine [pg/mL]	2876.4	1605.1	2113.0	9158.4	9116.5	9069.2	1729.7	683.8	739.2	8116.9	8038.3	8069.8
	<i>S. D.</i> (pg/mL)	10.3	4.47	149.3	6.15	4.30	5.77	14.9	5.01	5.95	13.3	4.54	4.94
RN [25µM]	Cytokine [pg/mL]	1447.0	3255.0	2658.1	9055.1	9165.5	9080.9	633.8	1935.5	1099.7	8038.4	8188.5	8071.9
	<i>S. D.</i> (pg/mL)	72.1	110.3	23.4	8.12	14.2	3.29	16.3	235.6	13.5	1.27	16.1	726
	Significance	***	***	***	***	***	*	***	***	***	***	***	