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Temporin L-derived peptide as a regulator of the acute inflammatory response in zymosan-induced peritonitis

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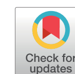

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ABSTRACT

Antimicrobial peptides (AMPs) are an ancient group of defense molecules distributed in nature being found in mammals, birds, amphibians, insects, plants, and microorganisms. They display antimicrobial as well as immunomodulatory properties. The aim of this study was to investigate, for the first time, the anti-inflammatory activities of two synthetic temporin-L analogues (here named peptide **1** and **2**) by an *in vivo* model of inflammation caused by intraperitoneal sub-lethal dose of zymosan. Our results show that peptide **1** and **2** exert anti-inflammatory activity *in vivo* in response to zymosan-induce peritonitis. Simultaneous administration of 10 mg/kg of both temporins, with a sub-lethal dose of zymosan (500 mg/kg), significantly rescued mice from the classical hallmarks of inflammation, including leukocyte infiltration and synthesis of inflammatory mediators including IL-6, TNF-α and MCP-1. More importantly, flow cytometry analysis highlighted a selective modulation of infiltrating inflammatory monocytes (defined as $B220^{-}/GR1^{hi}$-$F480^{hi}/CD115^{+}$) after peptide **2** treatment. Our results and presented models offer the possibility to test, in a preclinical setting, the potential of temporin analogues as anti-inflammatory agents.

1. Introduction

The high interest in antimicrobial peptides (AMPs), as new therapeutic agents, is stimulated by their possible use for the treatment of several infectious diseases, due not only to their antimicrobial activity but also to their immunomodulatory properties [1].

AMPs represent an abundant group of molecules that are an intrinsic component of innate immunity in multicellular organisms and they show a potent activity against Gram-positive and -negative bacteria, fungi and viruses [2]. According to their mode of action, AMPs can be divided in two main categories, that are membrane disruptive and no-membrane disruptive AMPs [3]. Membrane disruptive AMPs present a net positive charge, ranging from +2 to +9, crucial to create electrostatic interactions with negatively charged cellular membrane of bacteria, hence disturbing the membrane permeability and causing a rapid cell death. In this scenario, temporins, first isolated from the skin secretions of the red frog *Rana temporaria*, are the largest family of membrane disruptive AMPs [4]. Structurally, they are among the smallest sized peptides (10–14 residues) endowed with antimicrobial activity found in nature to date. The amphibian peptide temporins have generally, an amidated C-terminus, a low positive charge due to the presence of 2 or more basic residues in their whole sequence and adopt an amphipathic α-helical-like preferential conformation in a hydrophobic environment [5].

In previous work, we reported a novel library of antimicrobial peptides correlated to [Pro^3, $DLeu^9$]TL (temporin-L derivate), herein named peptide **1** (Fig. 1), with improved antimicrobial activity [6,7]. In this context, we uncovered an interesting analogue, herein referred to as peptide **2** (Fig. 1), which showed a slight increase in activity against Gram-positive (*B. megaterium Bm 11*, *S. aureus Cowan I*, *S. epidermidis ATCC12228*), and Gram-negative bacteria (*A. baumannii ATCC* 19606 and *P. aeruginosa ATCC 2785*), compared to the reference peptide **1**. Furthermore, peptide **2** was devoid of hemolytic activity and showed a weak toxicity against human keratinocytes at its antimicrobial concentration (12.5 μM) in comparison to **1**.

In addition to their antimicrobial activity, the potential therapeutic

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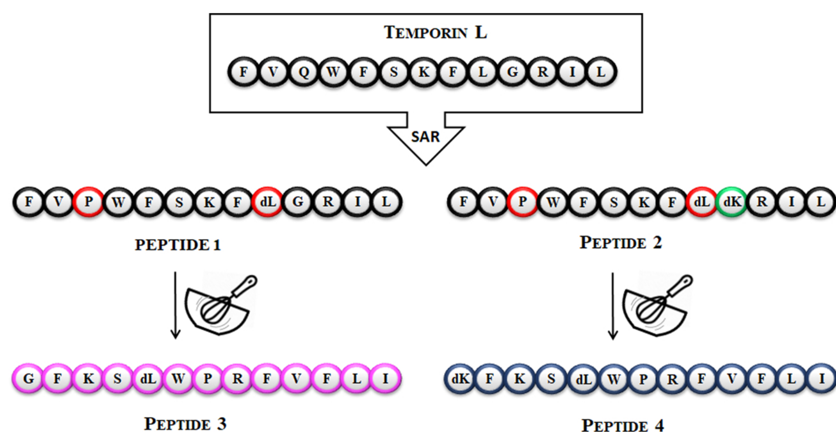


Fig. 1. Representation of peptide **1** and peptide **2** obtained through a structure-activity relationship (SAR) study of temporin-L. Peptide **3** and **4** represent the scrambled sequence of peptide **1** and **2**, respectively.

application of AMPs includes an anti-inflammatory and anti-proliferative activity [8–10]. Inflammation is an essential component of the immune system and represents a defense mechanism against endo- and exogenous- stimuli, such as pathogens, toxic agents and damaged cells [11,12]. More recently, different studies have reported that AMPs have several functions in inflammatory diseases [13] related to their ability to reduce the levels of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in lipopolysaccharide (LPS)-activated macrophages [14].

With this in mind, the aim of our current study was to extend, by means of both *in vitro* and *in vivo* assays, the evaluation of the potential anti-inflammatory activity of these temporin-L derived peptides in order to shed some light on the cellular and molecular profiles responsible for their mode of action.

2. Materials and methods

2.1. Materials

N^{α}-Fmoc-protected amino acids, Fmoc-Phe, Fmoc-Val, Fmoc-Pro, Fmoc-Trp(Boc), Fmoc-Ser(*t*Bu), Fmoc-Lys(Boc) and Fmoc-DLys(Boc), Fmoc-Leu and Fmoc-DLeu, Fmoc-Gly, Fmoc-Ile, Fmoc-Arg(Pbf) were purchased from GL Biochem Ltd (Shanghai, China). Coupling reagents such as *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uranium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt), as well as the Rink amide resin used were commercially obtained by GL Biochem Ltd (Shanghai, China). *N,N*-diisopropylethylamine (DIEA), piperidine, and trifluoroacetic acid (TFA) were purchased from Iris-Biotech GMBH. Moreover, peptide synthesis solvents and reagents, such as *N,N*-dimethylformamide (DMF), dichloromethane (DCM), diethyl ether (Et_2O), water and acetonitrile (MeCN) for HPLC, were reagent grade acquired from commercial sources (Sigma-Aldrich and VWR) and used without further purification. Unless otherwise stated, all the other reagents were from BioCell (Milan, Italy).

2.2. Peptide synthesis

The *syntheses* of peptides (peptide 1: Phe-Val-Pro-Trp-Phe-Ser-Lys-Phe-DLeu-Gly-Arg-Ile-Leu NH_2, peptide 2: Phe-Val-Pro-Trp- Phe-Ser-Lys-Phe-DLeu-DLys-Arg-Ile-Leu-NH_2, peptide 3: Gly-Phe-Lys-Ser-DLeu-Trp-Pro-Arg-Phe-Val-Phe-Leu-Ile-NH_2, peptide 4: DLys-Phe-Lys-Ser-DLeu-Trp-Pro-Arg-Phe-Val-Phe-Leu-Ile-NH_2) were performed by applying the innovative ultrasound-assisted solid-phase peptide strategy (US-SPPS), as elsewhere reported [15]. In brief, each peptide was constructed on a Rink amide resin (0.1 mmol from 0.64 mmol/g of loading substitution) as solid support, using the orthogonal strategy Fmoc/tBu [Fmoc-deprotections: 20 % Piperidine/DMF, 0.5 + 1 min; couplings: Fmoc-aa (2 equiv), HBTU/HOBt (2 equiv) and DIEA (4 equiv), 5 min]. Finally, peptides were purified and characterized by RP-HPLC and HR−MS spectroscopy, as previously reported [6,15].

2.3. Cell culture

Murine macrophage cell line, J774, was cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (FBS, Cambrex, Verviers, Belgium), L-glutamine (2 mM, Sigma, Milan, Italy), penicillin (100 units/ml, Sigma) and streptomycin (100 μg/ml, Sigma), and cultured in a humidified 5 % carbon dioxide atmosphere at 37 °C. Cell monolayers were regularly collected by gentle scraping with a cell scraper, diluted 1:10 in fresh medium and cultured to confluency at 37 °C. To determine cell responses to peptide 1 and 2, macrophages were seeded into 96-microwell culture plates at a density of 10^4 cells/well and allowed to grow for 24 h. The medium was then replaced with fresh medium and cells were treated for 4 and 24 h, with a range of concentrations (1 → 25 μM) of peptide 1 and 2. Positive control incubations were carried out by equivalent concentrations of Diclofenac and Betamethasone, commonly used preclinically as anti-inflammatory drugs. In another set of experiments cell were pre-treated with zymosan (100 ng/ml) followed by peptide 1 and 2 for 4 and 24 h, with the same range of concentrations. Biological activity of peptides was investigated by the estimation of a "cell survival index", arising from the combination of cell viability evaluation with cell counting. Cell viability was evaluated using MTT assay, which measures the level of mitochondrial dehydrogenase activity using the yellow 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) as substrate. Briefly, after treatments, medium was removed, and cells were incubated with 20 μl/well of MTT solution (5 mg/mL) for 1 h in a humidified 5 % CO_2 incubator at 37 °C. The incubation was stopped by removing the MTT solution and by adding 100 μl/well of dimethyl sulfoxide (DMSO) to solubilize the obtained formazan. Finally, the absorbance was monitored at 550 nm using a microplate reader (iMark microplate reader, Bio-Rad, Milan, Italy). Cell number was determined by TC20 automated cell counter (Bio-Rad, Milan, Italy), providing an accurate and reproducible total count of cells and a live/dead ratio in one step by a specific dye (trypan blue) exclusion assay. Bio-Rad's TC20 automated cell counter uses disposable slides, TC20 trypan blue dye (0.4 % trypan blue dye w/v in 0.81 % sodium chloride and 0.06 % potassium phosphate dibasic solution) and a CCD camera to count cells based on the analyses of captured images. [16].

2.4. Animals

CD-1 male mice (10–14 weeks of age, 25–30 g of weight) were obtained from Charles River (Margate, UK) and kept in an animal care

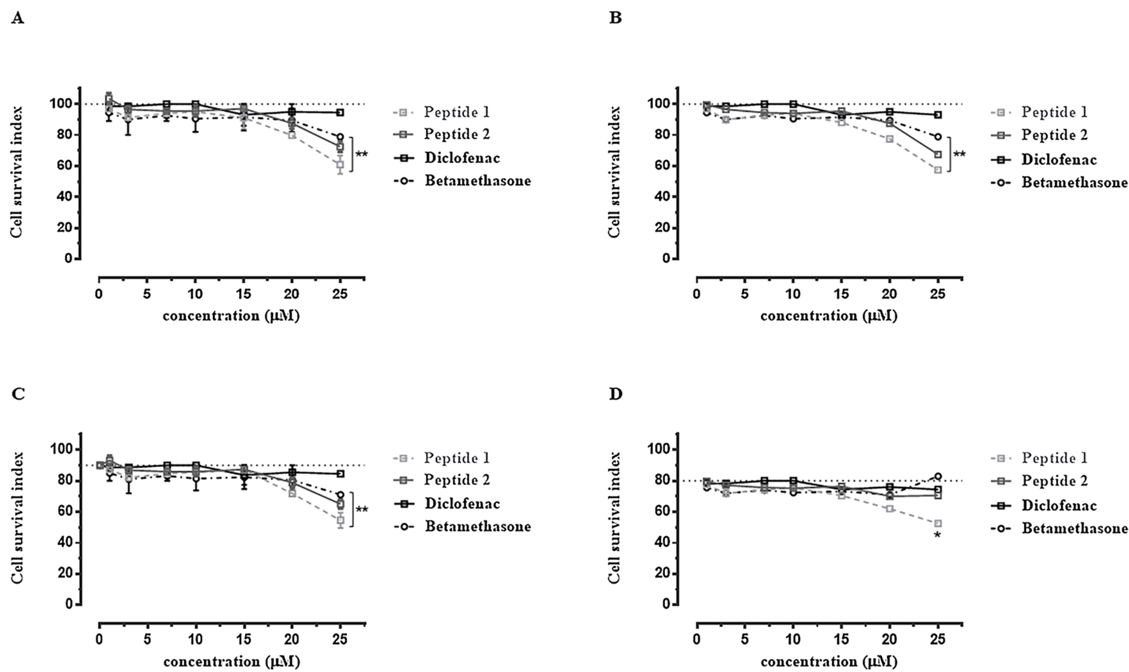


Fig. 2. Bioactivity profile of peptides in J774 macrophages. Cell survival index, evaluated by MTT assay, on murine macrophage cell line, following 4 (**A**) and 24 h (**B**) of treatment with selected concentrations (1→25 μM) of peptide **1** and **2**. In another set of experiments cells were pre-treate with zymosan (100 ng/ml) followed by peptide **1** and **2** for 4 (Fig. 2C) and 24 h (Fig. 2D), with the same range of concentrations. Anti-inflammatory positive control incubations were carried out by equivalent concentrations of Diclofenac and Betamethasone. Data are expressed as percentage of untreated control cells and are reported as mean of five independent experiments ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, *vs.* control cells.

facility under controlled temperature, humidity and on a 12 h:12 h light:dark cycle, with *ad libitum* access to water and standard laboratory chow diet. All experimental procedures were carried out in compliance with the international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept) [17,18] and approved by Biomedical Services Unit at the University of Birmingham (UK). All procedures were carried out to minimize the number of animals (n = 7 per group) and their suffering.

2.5. Induction of peritonitis in mice

To examine the anti-inflammatory action of peptide 1 and 2, mice were randomly divided into different groups: control group (Ctrl), model group (Zymosan), zymosan + peptides (1, 3 and 10 mg/kg) groups (peptide 1 and 2), and zymosan + dexamethasone (3 mg/kg) group (Dex). Animals received the peptides or Dex intraperitoneally (i.p.) 30 min after i.p. injection of zymosan (500 mg/kg) [19]. Ctrl and model group received an equal volume of vehicle (PBS) according to the same schedule. Peritonitis was induced in mice as previously described [20]. In brief, 500 mg/kg of zymosan were dissolved in PBS and then boiled before the i.p. injection (0.5 mL) at selected time points (4 and 24 h). Peritoneal exudates were collected by washing the cavity with 2 mL of PBS. Then cell number of lavage fluids was determined by TC10 automated cell counter (Bio-Rad, Milan, Italy) using disposable slides, TC10 trypan blue dye (0.4 % trypan blue dye w/v in 0.81 % sodium chloride and 0.06 % potassium phosphate dibasic solution) and a CCD camera to count cells based on the analyses of captured images. The remaining lavage fluids were centrifuged at 3000 rpm for 20 min at 4 °C and supernatants frozen at −80 °C for further ELISA analysis [21].

2.6. Flow cytometry

Cells collected from the peritoneal cavities were first washed with PBS and then re-suspended in FACS buffer (PBS containing 1 % FCS and 0.02 % NaN_2) containing CD16/CD32 FcγIIR blocking antibody (clone 93; eBioscience, Wembley, UK) for 30 min at 4 °C. Thereafter, cells were labelled for 60 min at 4 °C with the following conjugated antibodies (all from BioLegend, London, UK): GR1 (1:300; clone RB6-8C5), F480 (1:300; clone BM8), B220 (1:200; clone RA3-6B2), CD115 (1:200; clone AFS98). Neutrophils, macrophages and resident/inflammatory monocytes were defined according to the flow cytometry procedure previously described [22]. At least 1×10^4 cells were analysed per sample, and determination of positive and negative populations was performed based on the staining obtained with related IgG isotypes. Flow cytometry was performed on BriCyte E6 flow cytometer (Mindray Bio-Medical Electronics, Nanshan, China) using MRFlow and FlowJo software operation.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-6, IL-10 and MCP-1 in the peritoneal exudates at 24 h were measured using commercially available enzyme-linked immunosorbent assay kit (ELISA kits, eBioscience Co., San Diego, CA, USA) according to the manufacturer instructions. Briefly, 100 μl of peritoneal exudates, diluted standards, quality controls, and dilution buffer (blank) were applied on a pre-coated plate with the monoclonal antibody for 2 h. After washing, 100 μl of biotin-labeled antibody was added and incubation continued for 1 h. The plate was washed and 100 μl of the streptavidin–HRP conjugate was added and the plate was incubated for a further 30 min period in the dark. The addition of 100 μl of the substrate and stop solution represented the last steps before the reading of absorbance (measured at 450 nm) on a microplate reader [23,24].

2.8. Statistical analysis

The results obtained were expressed as the mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test when comparing more than two groups. In some cases, one sample

t-test was used to evaluate significance against the hypothetical zero value. Statistical analysis was performed by using GraphPad Prism 7.0 software (San Diego, CA, USA). Data were considered statistically significant when a value of $P \leq 0.05$ was achieved. The data and statistical analysis comply with the recommendations on experimental design and analysis [25].

3. Results

3.1. Macrophages biological response to treatments in vitro

The biological activity of peptide **1** and **2** was tested with murine macrophages J774 cell line. As reported in Fig. 2, the concentration-effect curves show no interference with cell growth and proliferation up to the concentration of 20 μM after 4 (**A**) and 24 h (**B**) of treatment with both peptides and anti-inflammatory reference drugs, *i.e.* Diclofenac and Betamethasone. However, at 25 μM a slight interference with cell viability was found for peptide **1** and peptide **2**. Indeed, the calculated IC_{50} values of both peptides were higher than 80 μM after 4 h and 24 h of treatment. These values were similar to those measured for betamethasone and diclofenac (~85 and ~200 μM respectively), herein used as reference drugs. In another set of experiments cells were pretreate with zymosan (100 ng/ml) followed by peptide **1** and **2** for 4 (Fig. 2C) and 24 h (Fig. 2D), with the same range of concentrations. Although we observed a small reduction in cell vitality after zymosan treatment, the effect of both peptides was similar to that observed in “non-stimulated” conditions.

3.2. Anti-inflammatory effect of peptides 1 and 2 in zymosan-induced peritonitis in mice

In light of the safety of peptide **1** and **2** evidenced through *in vitro* evaluations on J774 macrophages, we investigated the role of both peptides in an *in vivo* model of inflammation that allowed for the characterization of leukocyte recruitment and local inflammatory mediator production. Mice were subjected to i.p. injection of 500 mg/kg zymosan, in the presence or absence of peptide **1** and **2** (1 − 10 mg/kg dissolved in PBS). As internal control, i.p injection of PBS alone without zymosan and i.p. injection of Dex (3 mg/kg) post zymosan administration were also assessed. As shown in Fig. 3A, zymosan injection elicited a strong leucocyte recruitment at 4 h (~ 4.5×10^6) which was significantly reduced after peptide **2** administration at a dose of 10 mg/kg ($P \leq 0.05$). At this time-point the effect of peptide **1** (1 − 10 mg/kg) was not significant compared to mice injected with peptide **2** and Dex ($P \leq 0.005$). Conversely, after 24 h both peptides at 10 mg/kg significantly reduced ($P \leq 0.05$ and $P \leq 0.01$ respectively for peptide **1** and **2**) leukocyte infiltration (~11×10^6 after zymosan injection) with a profile, for peptide **2**, comparable to Dex ($P \leq 0.01$). Interestingly, peptide **2** also displayed significant effects at a lower dose of 3 mg/kg ($P \leq 0.05$) (Fig. 3B). Other experimental groups also included mice that received zymosan with peptide **1** and **2** at the highest dose (10 mg/kg) inactivated by denaturation or peptides with scrambled aminoacidic assembly. As shown in Fig. 4, both peptides at 24 h did not display any significant inhibitory effects on leukocyte recruitment.

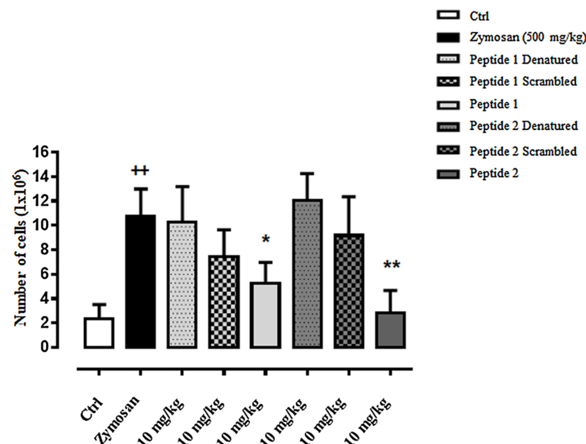
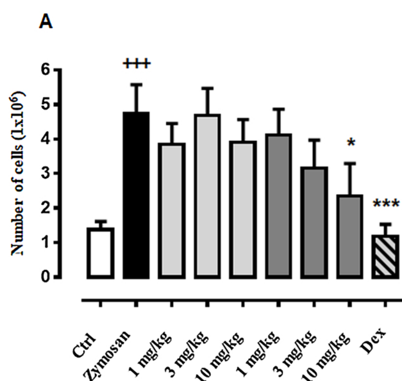


Fig. 4. Effect of denatured and scrambled temporin-derived peptides in zymosan-induced peritonitis in mice. Mice (n = 7) were injected intraperitoneally with 500 mg/kg zymosan, with 500 mg/kg zymosan and 10 mg/kg of peptides **1** and **2** or 500 mg/kg zymosan and peptides (**1** and **2**) denatured and scrambled (10 mg/kg). At 24 h post injection, peritoneal essudate from each mouse was recovered and total cell number (expressed as 1×10^6 and normalized to exudate levels) was evaluated. Results are expressed as mean ± S.E.M. $^{++}P \leq 0.01$ compared *vs.* ctrl group, $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ *vs.* zymosan-treated mice.

3.3. Modulation of cellular inflammatory infiltrates by peptides 1 and 2

Previous studies have shown that a single administration of zymosan into the peritoneal cavity causes a transient infiltration of leukocytes that becomes evident between 4–24 h, which declines by 48 h. To test the effects of an acute systemic administration of peptide **1** and **2**, we administered these peptides with the same dose (10 mg/kg) that evoked the optimal inhibition of leukocyte recruitment to the peritoneum. All mice were sacrificed 24 h after zymosan administration. Consistent with our previous findings, 24 h after a single injection of zymosan (500 mg/kg) i.p there was a significant increase in the levels of TNF-α, IL-6, IL-10 and MCP-1 compared to the control group (Fig. 5A,



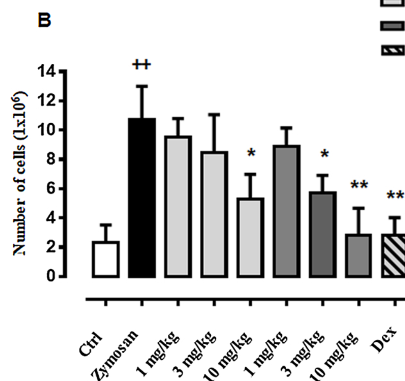


Fig. 3. Anti-inflammatory effect of temporin-derived peptides in zymosan-induced peritonitis in mice. Mice (n = 7) were injected intraperitoneally with 500 mg/kg zymosan, with 500 mg/kg zymosan and 1 − 10 mg/kg of peptides **1** and **2** or 500 mg/kg zymosan and 3 mg/kg of dexamethasone (Dex). At 4 (**A**) and 24 h (**B**) after zymosan injection, peritoneal essudate from each mouse was recovered and total cell number (expressed as 1×10^6 and normalized to exudate levels) was evaluated. Results are expressed as mean ± S.E.M. $^{++}P \leq 0.01$, $^{+++}P \leq 0.005$ *vs.* ctrl group, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$ compared to zymosan-treated mice.

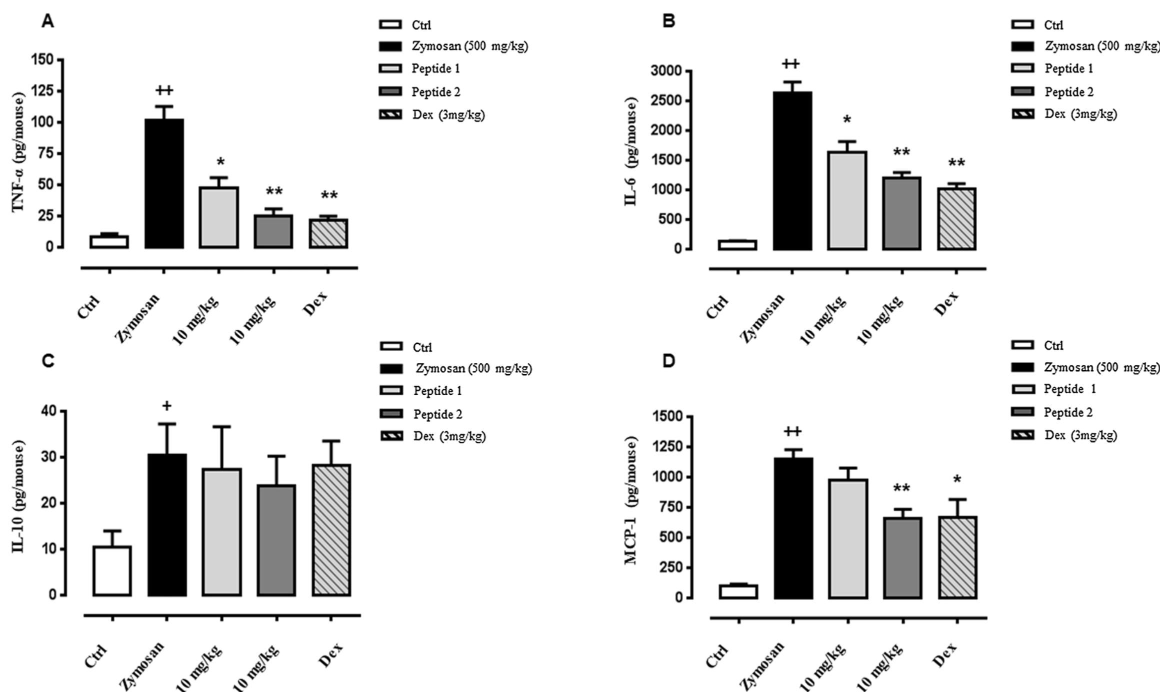


Fig. 5. Cyto-chemokines analysis of collected peritoneal exudates. Analysis of collected peritoneal exudates identified heightened levels of the classical pro-inflammatory cyto-chemokines TNF-α (**A**), IL-6 (**B**), IL-10 (**C**), and MCP-1 (**D**) in the peritoneal cavity of mice from zymosan groups (n = 7). Significant differences were found in relative levels after temporin **1** and **2** administration (n = 7). Results (normalized to exudate levels) are expressed as mean ± S.E.M. $^{+}P \leq 0.05$ and $^{++}P \leq 0.01$ *vs.* ctrl group, $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ *vs.* zymosan-treated mice.

B, C and D respectively). Interestingly, peptide **1** at 10 mg/kg significantly ($p \leq 0.05$) reduced the levels of TNF-α (Fig. 5A) and IL-6 (Fig. 5B). The levels of IL-10 (Fig. 5C) and MCP-1 (Fig. 5D) remained unaffected. Similarly, peptide **2**, at the same dose, also reduced ($p \leq 0.01$) the levels of TNF-α (Fig. 5A) and IL-6 (Fig. 5B), but a reduction was also observed in ($p \leq 0.05$) MCP-1 (Fig. 5D). Injection of the positice control Dex (3 mg/kg) decreased the values of mentioned cyto-chemokines (Fig. 5A, B, C and D) with a profile comparable to peptide **2**.

Based on these results, we selected the most active temporin L-derived peptide (peptide **2**) to investigate the phenotype of inflammatory leukocytes recruited to the peritoneal cavity. Leucocytes collected at 24 h time-point post zymosan injection were stained with an anti-B220, anti-F480, anti-GR1, anti-CD115 antibodies and then analyzed by flow cytometry. We did not observe any significant difference in terms of neutrophils ($GR1^{+}$ cells) and macrophages ($F4/80^{+}$ cells) reduction after peptide **2** treatment (data not shown). However, to identify potential differences in monocyte subpopulations, total cells were gated on their totality (Fig. 6A, gate R1) and singlet (Fig. 6B, gate R2) for the identification of $B220^{-}$ population (Fig. 6C, gate R3) followed by GR1 and F480 expression (Fig. 6D–F). Double high positive population for these markers (Gate R4, 9.05 ± 0.32, 41.20 ± 2.78 and 31.70 ± 2.55 of double high positive population respectively for Ctrl, zymosan and zymosan + peptide **2**; Fig. 6J) were then further interrogated for CD115 (Fig. 6G–I) as its expression level is commonly correlated with the degree of maturation of inflammatory monocytes. Our results show that in zymosan-injected mice, most cells recovered were $B220^{-}/GR1^{hi}$-$F480^{hi}/CD115^{+}$ (10.70 ± 1.14 compared to 2.70 ± 0.24 of Ctrl) with a significant lower expression in peptide **2**-treated group (3.24 ± 1.41) (Fig. 6K). These values were strengthened by a low percentage of positive cells found in the staining for the isotype control antibodies (Supplementary Fig. 1).

4. Discussion

The most important result of this work is that peptide **2**, a synthetic analog of temporin-L, displayed potent anti-inflammatory activity in a well characterised *in vivo* model of acute inflammation. This was exemplified by its ability to inhibit the recruitment of inflammatory monocytes and dampen the production of pro-inflammatory mediators IL-6, MCP-1 and TNF-α. This activity of peptide **2** was also shown not to detrimentally impact the ability of macrophages to grow or proliferate *in vitro*.

In fact peptide **2** showed a remarkable biocompatibility profile in our experimental model especially at the lower micromolar range allowing for a safe animal experimentation. The concentration-effect curves on J774 cell line showed no interference with cell growth and proliferation up to the concentration of 20 μM after 4 and 24 h of treatment with both peptides and anti-inflammatory reference drugs, *i.e.* Diclofenac and Betamethasone. Interestingly, this profile was similar to that observed after co-stimulation of murine macrophages with zymosan and both peptide **1** and **2** [27,28].

Zymosan, a polysaccharide cell wall component derived from *Saccharomyces cerevisiae*, has been reported to elicit a multiple organ failure and a massive recruitment of innate immunity cells in the peritoneal cavity, mainly characterized by neutrophils and monocytes [26]. The organ dysfunction in zymosan treated animals may be, in part, dependent on bacterial translocation [29]. The role of the production of pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α [30,31] and of prostaglandin metabolites [29] with consequent cellular infiltration and exudate formation is well established in the pathophysiology of zymosan-induced shock. TNF-α plays a pivotal role characterized by the release of IL-1 and IL-6 that orchestrate neutrophil, macrophage, and fibroblast accumulation to the site of inflammation [32,33]. This scenario is supported by the CC chemokine MCP-1, as one of the most potent chemotactic factors for monocyte migration to the site of tissue injury [34]. On the other hand, IL-10 is an anti-inflammatory cytokine that mainly suppresses inflammatory response by

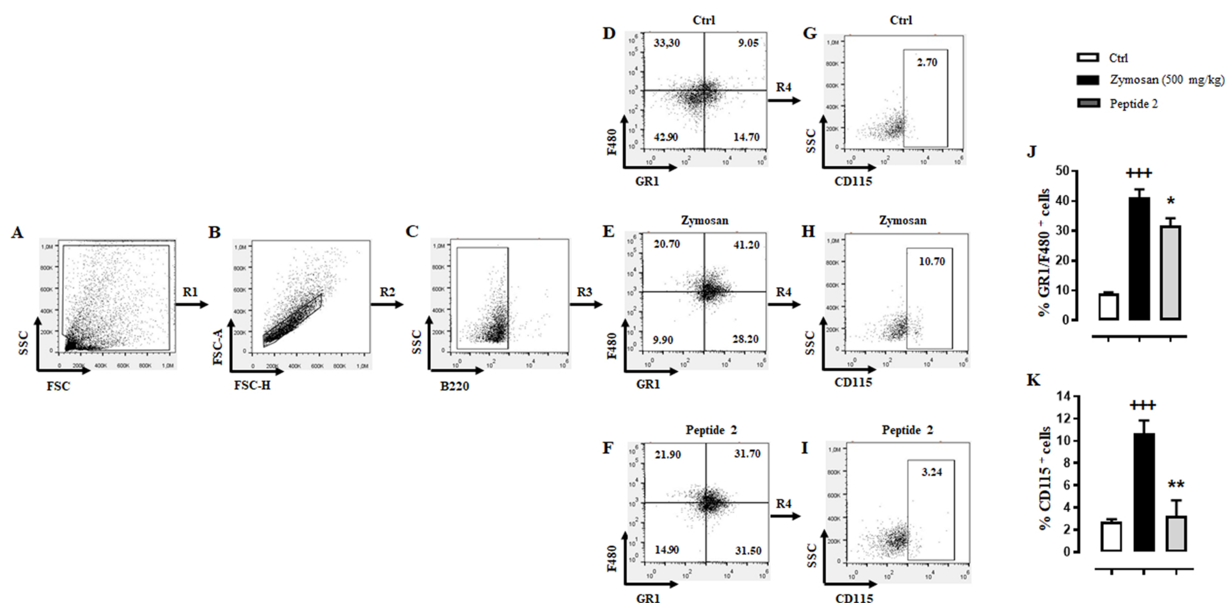


Fig. 6. Flow cytometry strategy applied to identify the modulation of inflammatory monocytes in zymosan and zymosan + peptide **2**-treated group. Cells obtained at 24 h time-point post zymosan injection in all experimental conditions were washed and stained with the following panel of antibodies: anti-B220, anti-F480, anti-GR1 and anti-CD115. Specifically, to identify potential differences in monocyte subpopulations, total cells were gated for their totality (**A**, gate R1) and singlet (**B**, gate R2) to identify $B220^-$ population (**C**, gate R3) followed by GR1 and F480 expression (**D-F**). Double high positive population for these markers (gate R4) was then further interrogated for CD115 (**G-I**) The numbers in the dot plots indicated the percentage of positively stained cells after gating strategy. FACS plots are representative of seven samples with similar results. Results (normalized to exudate levels) are presented as mean ± S.E.M (J and **K**) of n = 7 mice per group. $^{+++}P \leq 0.001$ *vs.* ctrl group, $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ *vs.* zymosan-treated mice.

increasing anti-inflammatory factors and inhibiting the activation and function of T cells and monocytes [35]. Accordingly, it is well known that the use of zymosan as experimental model of inflammation results in a range of benefits. Following the injection with zymosan, it is possible to collect a reasonable amount of exudate for the analysis of several inflammatory mediators, furthemore, injection into a serosal cavity instead of an artificiall formed cavity, such as a sterile air pouch, means that leukocytes exit from the site of inflammation *via* their natural conduits to the draining lymph nodes [36,37].

Our results demonstrate that the zymosan-induced leukocytes recruitment was attenuated by treatment with peptide **2**, at the highest dose examined, starting from 4 h post zymosan administration. A similar trend was observed for peptide **1**, however, the degree of attenuation was not similarly significant. Interestingly, at 24 h both peptides significantly attenuated inflammatory cell migration to the peritoneal cavity, but even at this time point peptide **2** appeared to be more potent when compared to littermate compound. Moreover, denaturized or scrambled sequence of both peptides administrated at 10 mg/kg did not display any biological effects highlighting the hypothesis that only “selected peptides sequence” elicited observed anti-inflammatory effects. Lending support to these findings, a screening of the main pro-inflammatory cytokines showed a significant reduction in the levels of IL-6, MCP-1 and TNF-α after peptide **2**, and to a lesser extent with peptide **1**. However, we did not reveal any modulation of IL-10 after both peptides administration. This difference in leukocytes accumulation and cytokines level may be due to the difference in potency of these two analogues and to assess this possibility, further animal studies will be carried out using other *in vivo* models as well other structural analogues.

To investigate and compare the phenotype of the inflammatory leukocytes recruited by zymosan injection into peritoneal cavity, we first gated on cells isolated from peritoneal exudates for $B220^-$ population, followed by $GR1^{hi}/F480^{hi}$ expression to finally identify the level of $CD115^+$, commonly correlated with the degree of maturation of inflammatory monocytes [22,23]. We did not observe any significant difference in terms of neutrophil and macrophage levels after peptide **2** treatment. However, our FACS analysis showed that in zymosan-injected mice, most cells recovered were inflammatory monocytes with a significant lower expression observed in peptide **2**-treated mice.

5. Conclusion

In conclusion, we have demonstrated, for the first time, the anti-inflammatory activity of peptide **2** reinforcing the idea that selected AMP could be effectively used as antisepsis agents *in vivo* due to its ability to modulate the recruitment of inflammatory monocytes. In future studies, it would be interesting to explore the antiendotoxin properties of other temporins-derivatives and synthetic analogues even in combination with conventional antibiotics, in way to acquire key information needed to assist the design of improved endotoxin-neutralizing temporin-based peptides for therapeutic applications.

Author contributions

RB, FR, FM, MP, MGF performed the experiments. CI, RS, AJI, EN, NM, PG and FM designed the study, drafted and wrote the manuscript. PG and FM edited and revised the manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109788.

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