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TATRasGap$_{317\text{-}326}$: a cancer cell sensitizer peptide with antimicrobial activity

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Présenté à la **F**aculté de Biologie et de Médecine de l'**U**niversité de Lausanne par

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Summary

The innate immune system plays an important role in host defenses and though in the viability of living being. An important capacity of this system is the recognition of pathogens through a panel of pattern-recognition receptors such as Toll-like receptors (TLRs). Triggering these receptors induces NF-κB, MAPK and IRF signaling pathways, gene activation, and production of pro-inflammatory molecules such as TNF or IL-6, required to fight infections. Unfortunately this defense system is sometimes overpassed and patients need external help such as antibiotherapy to clear infection.

Antibiotics exist since the discovery of penicillin by Fleming. Since then, numerous other antibiotics have been developed, but due to their great adaptation capacity bacteria became more and more resistant. Nowadays some bacteria are untreatable with the actual antibiotics panoply. The need of new antibiotics is a priority as stated by the WHO. A promising type of molecules with antimicrobial activity is the antimicrobial peptides (AMPs). AMPs directly act against pathogens and also, for some of them, modulate the immune system.

Previous studies have described the capacity of a peptide called TAT-RasGAP$_{317-326}$ to sensitize and kill tumor cells. During these studies, it was found that TAT-RasGAP$_{317-326}$ has a good bactericidal effect, suggesting it can act as an AMP. In this study, we explored the immunological modulation by TAT-RasGAP$_{317-326}$ *in vitro* and its antibiotics capacity in a mouse model of *E. coli*-induced peritonitis in mice.

We observed that TAT-RasGAP$_{317-326}$ modulates the immune response induced through TLR2, TLR4 and TLR9. TAT-RasGAP$_{317-326}$ decreased the production of IL-6 and TNF by mouse macrophages stimulated through TLR9, but not TLR2 and TLR4, while it increased cytokine production by human PBMCs stimulated through TLR2 and TLR9. The peptide had marginal effects on human whole blood. TAT-RasGAP$_{317-326}$ powerfully increased the survival of mice subjected to *E. coli* peritonitis when injected just after the onset if infection, an effect that was lost if the peptide was injected 3 hours after infection.

We conclude that TAT-RasGAP$_{317-326}$ is a promising molecule that fulfills some of the criteria to be useful in therapy against bacteria. However, more research is needed especially about the mode of action of TAT-RasGAP$_{317-326}$ and to improve its bio-distribution and stability to increase *in vivo* effectiveness.

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I want to thank greatly Thierry Roger and Didier Le Roy, for them great teaching, support and particularly for them patience and understanding, and also Christian Widmann without whom this work were not be possible. I am also particularly grateful to the great team of the laboratory, for they support and help in all little and important manipulations and for they friendly and easy integration feeling they give me.

1 Introduction

1.1 Innate Immunity

Plants and animals are confronted to external as well as internal aggressions. To combat these aggressions, living beings developed a more or less evolved defense system, called the immune system. In mammals, the immune system is traditionally divided into two branches: innate and adaptive immunity. The two branches are linked, as innate immune responses trigger adaptive immune responses.

Innate immunity has been conserved during evolution and is present in all living multicellular organisms including plants (Riera Romo, Perez-Martinez et al. 2016). This defense system is composed of three main actors: physical barriers, and humoral and cellular components (Riera Romo, Perez-Martinez et al. 2016). The skin and the mucosa (and their mucus and sebaceous liquid at specific pH), represent efficient physical barriers between the body and the external environment. Humoral components regroup molecules able to detect, kill or induce a response to eliminate the aggressor. Two main examples of humoral antimicrobial components are the anti-microbial peptides (AMPS), the central point of this study (see chapter 1.4), and molecules of the complement system. Cellular effectors are mainly myeloid professional phagocytes such as macrophages, dendritic cells and granulocytes (Riera Romo, Perez-Martinez et al. 2016). These cells express specialized receptors, called pattern-recognition receptors (PRRs) that sense microbial molecules, called microbial (or danger)-associated molecular patterns (MAMPs/DAMPs). PRRs are spatially located either in the cytosol or at the cellular membrane (Akira, Uematsu et al. 2006, Sellge and Kufer 2015). PRRs are constitutively expressed by innate immune cells and are highly conserved through evolution ((Akira, Uematsu et al. 2006). The main families of PRRs are the Toll-like receptors (TLRs), NOD-like receptor (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs) and cytosolic DNA sensors (CDSs). TLRs and CLRS are embedded in membranes, while NLRs, RLRs and CDSs are localized intracellularly (Riera Romo, Perez-Martinez et al. 2016).

A Toll molecule was first identified in *Drosophila* for its role in antifungal and antibacterial activities and for its structural similarities with the dorsal morphogen (Lemaitre, Nicolas et al. 1996). Later a study evidenced the existence of 5 homologous receptors in humans, named toll-like receptors (TLRs), suggesting that these receptors could play a role in innate immunity because of their conservation through evolution (Rock, Hardiman et al. 1998). Following this discovery, and until now, 10 functional TLRs were found in human (TLR1-10), while 12 TLRs are present in mice (TLR1-9, TLR11-13). TLRs have similar structure and are composed of a “leucine rich repeats” extracellular domain that detects the different MAMPs and of a cytosolic domain called Toll/IL-1 (TIR) with a tyrosine kinase activity that allows the activation of intracellular signaling pathways (Dowling and Mansell 2016). TLRs can be

classified according to their cellular localization, on the cytoplasmic membrane (TLR1, 2, 4, 5, 6) or in the endosomal compartment (TLR3, 7, 8, 9), or according to their dimerization either as homodimers (TLR3, 4, 5, 9) or heterodimers (TLR2/TLR1, TLR2/TLR6, TLR2/10) (Kawai and Akira 2006). TLR4, TLR2 and TLR9 are directly in relation with our experiments, therefore they will describe here. TLR4 is the most studied TLRs: its ligand is lipopolysaccharide (LPS, also called endotoxin) which is present in the outer membrane of Gram-negative bacteria. The triggering of TLR4 also depends on CD14, a GPI-anchored molecule that facilitates the binding between TLR4 and LPS and increases the intracellular signaling (Poltorak, He et al. 1998) through nuclear factor-κB (NF-κB), interferon response factor (IRF) and mitogen activated protein kinase (MAPK) pathways (Kawai and Akira 2006). TLR2 in association with either TLR1 or TLR6 reacts to diacetylated or triacetylated lipopeptides, respectively (Ku, Yang et al. 2005). Consequently, Pam_3CSK_4, a synthetic triacetylated lipopeptide that mimics lipopeptides from Gram-positive bacteria (Mintz, Mintz et al. 2013) leads to an augmentation of TNF production (Yu, Zhou et al. 2016) through binding with TLR1/TLR2 (Kawai and Akira 2006). TLR9 in endosomal compartment detects DNA fragments rich in unmethylated CG repeats called CpG motifs. Unmethylated CpG repeats are enriched in microbial genome (Krieg 2002). TLR9 detects DNA from viruses and bacteria captured in the endosome to activate NF-κB, MAPK and IRF pathways (Krieg 2002, Kawai and Akira 2006).

The NF-κB pathway is the main driver of inflammatory responses. In the cytoplasm, NF-κB transcription factor is constitutively inactivated by IκB. Following stimulation brought by for example TLRs, intermediate molecules like TIRAP-MYD88-IRAK are activated leading to IκB kinase activation. Then, IκB is phosphorylated, ubiquitinated and degraded to release free NF-κB. NF-κB translocates to the nucleus and binds to the promoter of immune related genes such as cytokine genes encoding for TNF, IL-1β and IL-6 (Beinke and Ley 2004).

1.2 Antibiotics and antibiotic resistance

Our immune system is very efficient at protection from microbial invasion. However, it happens that pathogens establish a local infection that can eventually disseminate into the blood inducing sepsis. In these cases, we need additional treatment such as antibiotics therapy.

Antibiotics kill bacteria or block (at least slow down) their growth (Smith, M'Ikanatha N et al. 2015). The first antibiotic to be identified, penicillin, was discovered by Alexander Fleming in 1928 (Tan and Tatsumura 2015). Following this pioneer discovery, more antibiotics have been developed and massively used for treating infections. At first antibiotic were efficient, but their widespread usage generated the emergence of resistant pathogens. Pathogens have several mechanisms to develop

resistance, which can be summarized in two classes. They act either on the quantity of active antibiotics by targeting destructive enzymes or efflux pumps, or on the target of the antibiotics by modifying the target, the transporter or by blocking the pre-antibiotics activation (Martinez and Baquero 2014). Resistance can be acquired in all types of pathogens (Smith, M'Ikanatha N et al. 2015). In bacteria there are two basic mechanisms: genomic mutation and horizontal transfer (Martinez and Baquero 2014, Smith, M'Ikanatha N et al. 2015). Horizontal transfer is particular to bacteria that use different method to share "antibiotic resistance genes" (ARGs). ARGs can be transferred from one bacterium to another in a plasmid, a transposon, an integron or a genomic island sequence (Brown-Jaque, Calero-Caceres et al. 2015). This transport needs a transporter that can be a phage or a "genes transfer agent" (GTI) or upon cell-to-cell contact (Brown-Jaque, Calero-Caceres et al. 2015). Transport by plasmids is a privileged transport because plasmids may carry genes able to create the cell-to-cell bridge and ARGs (Bennett 2008). ARGs origin is disputed, but a privileged hypothesis is the transfer from commensal or environmental bacteria used in the industrial production of antibiotics. Another hypothesis postulates that there are pre-resistance genes in bacteria. In opposition to these theories some obstacles are noted. For the first one, the physical communication between bacteria is necessary. For the second, if a resistance gene or pre-resistance gene exists, we should not be able to find functional antibiotics (Martinez and Baquero 2014).

If the acquisition of antibiotic resistance was sporadic, it would not be a problem. However, this is not the case. Antibiotic resistance is generalized and spread in the world, which is a main concern of sanitary authorities and WHO. The causes of acquiring and fast spreading of antibiotic resistance are mainly linked to behavioral and socio-political reasons (Larson 2007). Abused clinical prescription and self-prescription of antibiotics are a main problem, because misuse and/or overuse of antibiotics favor the development of resistances (Larson 2007). Another important problem is our close-built society in which resistant bacteria can easily move and spread, it is basically a hygienic problem. Particularly risky places are hospitals, which are often the source of spread resistant bacteria (Larson 2007, Xia, Gao et al. 2016). Last but not least, the use of antibiotics in food industry, notably cattle, sheep, poultry and fish, is of huge concern. However, it seems that interdiction of antibiotics for breeding have not significantly decreased usage (Larson 2007). In all cases, the need of new powerful antimicrobial molecules is a worldwide priority, because of the spread and the strength of resistance mechanism (Nikaido 2009). Paradoxically, the discovering of new molecules has steadily decreased over the years (Larson 2007). New hope emerged with the discovery of AMPs (antimicrobial peptides).

1.3 Anti-microbial peptide

AMPs, also called host defense-peptides, are important actors of humoral innate immunity (Riera Romo, Perez-Martinez et al. 2016). AMPs are a highly conserved defense mechanism highly present through evolution (Zasloff 2002, Riera Romo, Perez-Martinez et al. 2016). AMPs are produced by all living species, also by bacteria such as for example *Staphylococcus lugdunensis* (Zipperer, Konnerth et al. 2016). They play a central role of resistance against pathogens by plants and insects and also the "never-infected" cornea of animals (Zasloff 2002). All AMPs follow the same structure principle, which shows amphiphilic molecules with a hydrophobic and hydrophilic or cationic part (Zasloff 2002) (**Figure 1**). There is an important number and diversity of potential AMPs, with actually more than 6'500 sequences (Waghu, Gopi et al. 2014). AMPs are grouped in different subtypes, but this classification is complex (Brogden 2005). The diversity of AMPs is associated with differences in their structure that is frequently different between species except for conserved AMPs such as hepcidin, cathelicidins and defensins (Zasloff 2002, Riera Romo, Perez-Martinez et al. 2016). Some AMPs are active against all types of pathogens tested, while most of AMPS actually acts against bacteria (Waghu, Gopi et al. 2014) (**Figure 2**). AMPs are primarily secreted by epithelial tissues (skin or mucosa of digestive system) and expressed by phagocytes (Yeaman and Yount 2003, Riera Romo, Perez-Martinez et al. 2016). AMPs are rapidly mobilized to fight infection and constitutive expression protects more vulnerable mammalian tissues, such as epithelia and mucosa exposed to the external environment and inert tissues (keratinized skin for example) in which phagocytes poorly access (Yeaman and Yount 2003).

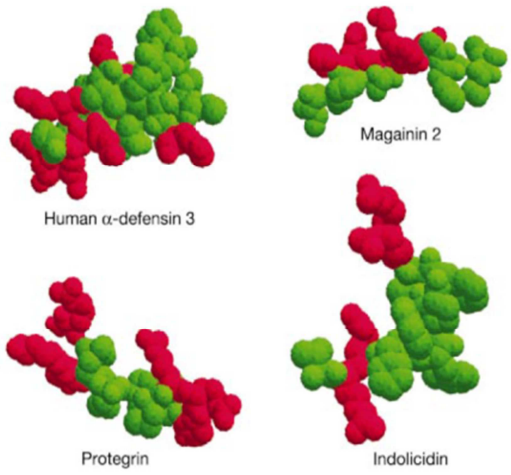

Figure 1. Example of different AMPs.
In red the cationic part; in green the hydrophobic part (from (Zasloff 2002).

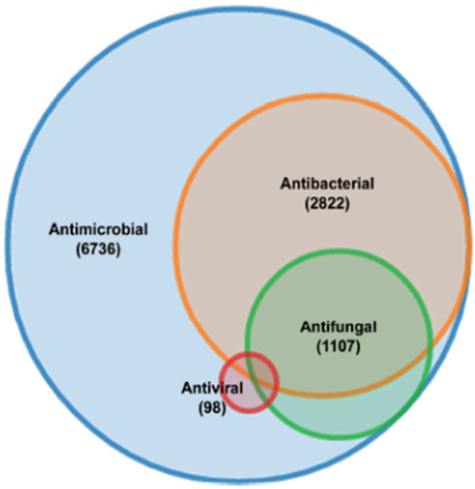

Figure 2. Venn diagram of classification of AMPs activity from (Waghu, Gopi et al. 2014).

AMPS are relatively well specified to target microbial cells. There are exceptions like α-defensin that can also attacks eukaryotic cells. This effect is probably linked with the fact that it is one of the most powerful AMPs (Yeaman and Yount 2003). AMPs target the cellular membrane; thereby the affinity specification could be easily understood by the important differences between mammalian and microbial cellular membranes. Mammalian membranes are quasi non-charged instead of microbial membranes that are negatively charged (Yeaman and Yount 2003). Moreover, the transmembrane potential varies between -90/-110 mV in mammalian cells and -130/-150 mV in microbial cells. Finally, microbial membranes offer binding sites for AMPs (Yeaman and Yount 2003). Mechanisms of binding between AMPs and microbial membranes follow these differences. The negatively charged membrane of bacteria attracts positively charged AMPs (cationic AMPs) and creates a first contact. Specific motifs exposed on microbial membranes also attract and bind some AMPs (Yeaman and Yount 2003, Brogden 2005). Following this contact, AMPs multimerize or aggregate to act on the microbial cells.

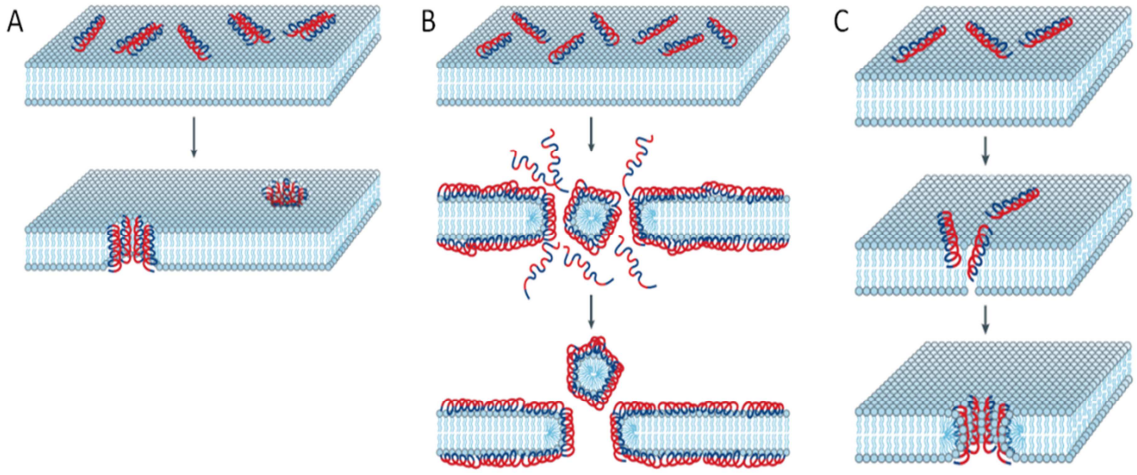

Figure 3. Model of pore formations by AMPs in microbial membranes (adapted of (Brogden 2005). In red and blue, the hydrophilic and hydrophobic domains are shown. **A**) Barrel-stave model: AMPs aggregate and insert into the membrane, hydrophobic part in contact with membrane phospholipids, hydrophilic in contact with hydrophilic domain of other AMPs. **B)** Carpet model: AMPs disrupt the membrane by forming an extensive carpet on the membrane, the extensity disrupt the carpet. **C**) Toroidal model: AMPs aggregate and induce the external lipid layer of the membrane to bend until they reach the internal lipid layer, hydrophilic domain of AMPs in addition with the phospholipid head composed the internal side of the pore.

AMPs are dynamic peptides that change of conformation according to the environment (Yeaman and Yount 2003). When linked to the membrane of microbial cells, two domains of the amphiphilic molecule rearrange to meet best energy. This arrangement and the following multimerization compose the first step of the “killing cell” mechanism. Basically, two types of action of AMPs exist: destabilization of

the membrane following pore formation and action on internal components of the cell (Brogden 2005). It is interesting to note that the effects of AMPs are concentration dependent, and that there is a threshold for acting on internal components because AMPs cannot reach the internal cavity before passing the membranes (Yeaman and Yount 2003). Three models of pore formation by AMPs have been proposed: the toroidal, barrel stave and carpet models (**Figure 3**). Intracellular AMPs interfere with cell membrane formation, nucleic acids, enzymes, proteins and their synthesis process (Brogden 2005).

Interestingly, some AMPs not only target microorganisms but also the host by modulating the inflammatory response, for example the production of TNF and IL-6 (Zasloff 2002). LL37, the only member of the cathelicidin family in humans, enhances immune response through activation of TLR/NF-κB pathway (Mookherjee, Brown et al. 2006). When released on inflammatory sites, AMPs are chemo-attractants of leukocytes such as neutrophils, monocytes and T lymphocytes (Yeaman and Yount 2003). This aspect is important for our investigation on the TAT-RasGAP$_{317-326}$ antimicrobial peptide.

AMPs, like all defensive mechanisms, generate adaptive countermeasures by the targeted microorganisms. The resistance to AMPs falls in two classes: inducible and passive resistance (Yeaman and Yount 2003). Inducible resistance is generated through the stress response of the pathogen exposed to AMPs. We find in this group: 1) the production of protease and peptidase degrading AMPs, 2) structural modifications of cytoplasmic membrane composition or extracellular membrane modification of lipid A and LPS impairing AMP binding, 3) trans-membrane potential variation with modification of Ca^{2+} and Mg^{2+}concentrations causing loss of the electro-attractive force, 4) activation of AMP efflux pumps, and 5) modification of intracellular targets (Yeaman and Yount 2003). Passive mechanisms of resistance are acquired and constitutively activated. Three principles support this type of resistant: 1) a normalization of the electric charge with a propensity to 0 charge of the membrane decreases the affinity of AMPs, 2) expression of a capsule/glycocalyx that impedes the binding of AMPs (expected by (Yeaman and Yount 2003) and proved by (Campos, Vargas et al. 2004), 3) the niche-specific resistance, that is the utilization by the pathogen of the anatomic or physiologic particular micro-environment of the host to overpass AMP action (Yeaman and Yount 2003). Pathogens acquire resistance capacities through mechanisms described for the acquisition of antibiotic resistances (see section 1.2), principally through plasmid exchange (Yeaman and Yount 2003). Despite resistance mechanisms against AMPs, AMPs are nevertheless interesting candidates to increase our arsenal to fight against multidrug resistant bacteria.

1.4 The TAT-RasGAP$_{317-326}$

TAT-RasGAP$_{317-326}$ (abbreviate TAT-P for the rest of the document) is a synthetic peptide of 10 amino-acids, born from the fusion of a p120 rasGAP derived peptide (RasGAP$_{317-326}$; *i.e.* WMWVTNLRTD) and a cell-permeable HIV-TAT derived peptide (TAT$_{48-57}$; GRKKRRQRRR) (Michod, Yang et al. 2004). TAT-P has been developed by the group of Prof Christian Widmann from the Department of Physiology of the University of Lausanne.

Ras is a proto-oncogene of the small GTPase family that, when permanently activated by mutation, increases cell growth, differentiation and survival. Ras changes from inactive to activate conformation in the presence of phosphate groups. This modulation by a phosphate group is led by the internal GDP/GTP action of Ras. This GDP/GTP action is regulated by GEFs, GDP to GTP and GAPs (GTPase-activating protein), GDP to GTP. If *Ras* is mutated, the RAS protein is blocked in active position. RasGAPs are the name of specific GAPs deactivating the RAS protein. There are several kinds of RasGAPs. For our study we will focus on the p120 RasGAP produced by the *Rasa1* gene.

TAT-P is an anticancer peptide. TAT-P acts on cells through two modes of action. First, TAT-P sensitizes tumor cells to genotoxins. Second, TAT-P increases adherence and inhibits migration and matrix invasion by cells (Barras, Lorusso et al. 2014). The underlying mechanisms of action of TAT-P are not well characterized. TAT-P binds the GAPs DLC 1, 2 and 3 (deleted in liver cancer 1, 2 and 3). Binding to DLC2 seems responsible of the sensitization of tumor cells and that to DLC1 for the increasing adherence effects (Barras, Chevalier et al. 2014), while binding to DLC3 has no effect. Interestingly, three amino-acids of TAT-P are sufficient for optimal tumor sensitization, while five amino acids (317-321) are necessary to increase adherence and the whole peptide (317-326) to obtain an optimal effect on adhesion (Barras, Chevalier et al. 2014). The tumor suppressor p53 is necessary to mediate apoptosis induced by TAT-P, but no modification in phosphorylation, acetylation or transcription of p53 was detected (Michod and Widmann 2007). The Ras effectors Akt (protein kinase B) and extracellular signal-regulated kinases (ERK) 1/2 MAPKs are not necessary to mediates TAT-P anticancer functions, suggesting that disruption of Ras signaling pathways by TAT-P is not implicate in the process. The p53 effector PUMA (p53 upregulated modulator of apoptosis), a pro-apoptotic protein, was indispensable to mediate TAT-P-induced apoptosis, while p21 a pro-apoptotic protein, was not (Michod and Widmann 2007). The expression of regulators of apoptosis of the BCL2 family, BAX (BCL2 associated X) and BAK (BCL2 antagonist/killer 1), is increased by TAT-P but this result is nuanced by a lack of effect in colon carcinoma cells (Annibaldi, Heulot et al. 2014).

Fortuitously, an anti-bacterial activity of TAT-P was uncovered during an episode of contamination of a mammalian cell culture. The growth of a contaminant, *Staphylococcus capitis*, was prevented when TAT-P was present in the culture medium. This observation stimulated the development of a research project to better characterize the antimicrobial activity of TAT-P.

2 Objectives

A bactericidal effect was demonstrated with utilization of the TAT-P, on different bacterial strains (Heulot, Jacquier et al. 2017). These results drive us to repeat the experiment and to test possible immune-modulatory effects of TAT-P. To investigate this aspect, we tested the impact of TAT-P on inflammatory and innate immune responses *in vitro* and *in vivo*.

3 Materials and Methods

3.1 Mice, cells & reagents

3.1.1 Mice

C57BL6/J and BALB/cByJ female mice (8-12 weeks old, Charles River Laboratories, L'Arbresle, France) were housed under specific pathogen-free conditions. All animal procedures were approved by the Office Vétérinaire du Canton de Vaud (Authorization numbers: 876-8 and 877-8) and performed in the respect of the institution and ARRIVE guidelines for animal experiments.

3.1.2 Mouse and human cells

Bone marrow cells from C57BL6/J mice were cultured 4-5 days (37°C, 5% CO_2) in IMDM medium (Life Technologies, Grand Island, NY, USA) containing 30% of L929 supernatant (containing M-CSF) and 10% FCS (Biochrome AG, Berlin, DE) to obtain bone marrow derived macrophages (BMDMs). Human blood was collected from different donors on heparin. Blood was either directly stimulated (50 µl per well, final dilution 1/5) or peripheral blood mononuclear cells (PBMCs) were purified by Ficoll Hypaque (GE Healthcare) gradient density centrifugation, washed with MACS buffer (Miltenyi Biotec, Bergisch Gladbach, DE), re-suspended in RPMI (Life Technologies) containing 10% FCS, seeded in 96 wells plate and incubated overnight at 37°C and 5% CO_2.

3.1.3 Reagents and bacteria

TAT-P and W_{317}a, (control peptide) are synthetic peptides provided by Christian Widmann (Barras, Chevalier et al. 2014). *Salmonella minnesota* ultra-pure LPS (List biological Laboratories, Campbell, CA), Palmitoyl-cys((RS)-2,3-di(palmitoyloxy)-Propyl)-Ser-Lys-Lys-Lys-Lys-OH trifluoroacetate salt (Pam_3CSK_4) (EMC microcollections, Tuebingen, Germany), cytidine-phosphate-guanosine oligonucleotides (CpG ODN) (Invivogen, San Diego, CA) were used in this study. *Escherichia coli* O18:K1:H8 is a pathogenic strain isolated from a septic patient.

3.2 Bactericidal assay

E.coli was cultivated in BHI (Eurogentec) for 3 hours at 37°C under agitation, washed 2 times in PBS, and OD_{600nm} was adjusted to 1.0 NTU (nephelometric turbidity unit) with a turbidimeter which corresponded to 3 x 10^8 bacteria/ml. This sample was diluted in PBS to reach 10^7 CFU/ml, 10^5 CFU/ml and 10^3 CFU/ml. Bactericidal activity of TAT-P was investigated using 0, 2 and 20 µg/ml TAT-P. Two minutes, 1, 3 and 20 hours post exposure, living bacteria were enumerated by plating serial dilutions of the samples on Columbia agar sheep blood Petri's dish (Becton Dickinson, Temse, Belgium). Plates were incubated overnight at 37°C and colonies were counted.

3.3 Stimulation

Fifty thousand cells (BMDMs or human PBMCs) or 50 µl of human blood were exposed to 0, 1 or 10 µg/ml of either TAT-P or W_{317}a. After 1 hour at 37°C and 5% CO_2, cells were exposed for 8 and 24 hours to 10-100 ng/ml LPS (Duffy, Rouilly et al. 2014), 10 ng/ml Pam_3CSK_4 or 1 µM CpG ODN. Each condition was performed in triplicate. Supernatants were collected (after centrifugation at 3000g for 3 min for whole blood stimulation) and stored at -20°C until cytokine measurement.

3.4 Cytokine measurement

Concentrations of IL-6 and TNF were measured by ELISA using human kits (BD Biosciences, San Diego, CA) or mouse kits (R&D Systems, Minneapolis, USA).

3.5 Measurement of cells viability by MTT test

The MTT method is a colorimetric method measuring the formation of formazan by active mitochondria, indicative of the relative activity and viability of cells. Briefly, 10 mg MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were dissolved in 5 ml of PBS. Fifty µl were added to cell cultures and incubated 2 hours at 37°C %CO2. Supernatants were discarded and then 150 µl of lysing solution (with a mixture of 6.7 ml isopropanol, 3.3 ml 20% sodium dodecylsulfate and 55 µl 5 N HCl) were added. This step dissolves the purple precipitate that had formed, and the optical density is measured at 570 nm. Cell viability was measured on PBMC and BMDM after 24 hours stimulation.

3.6 *In vivo* model

BALB/cByJ mice were infected intraperitoneally (i.p.) with the indicated inocula of *E. coli* O18. Two minutes or 3 hours after infection, mice were treated i.p. with 200 µl PBS or TAT-P at 100 µg/ml. After 24 hours, blood was collected under heparin from the tail vein and bacteria were numerated by plating serial dilutions of the blood. Mice survival and clinical signs of sickness (weight, ruffled fur, diarrhea...) were observed for 6 days.

3.7 Statistical analyses

Comparisons of cytokine and MTT values were performed by analysis of variance followed by two-tailed unpaired Student's t-test. The Kaplan-Meier method was used for building survival curves and differences were analysed by the log-rank sum test. Statistical analyses of bacterial counts were performed using the non-parametric Mann-Whitney test. All analyses were performed using PRISM (GraphPad Software). *P* values were two-sided, and $P < 0.05$ was considered to indicate statistical significance.

4 Results

4.1 TAT-P has a bactericidal effect on *Escherichia coli* O18

In previous collaborating experiments, it has been shown that TAT-P exerts bactericidal activity on gram-negative and gram-positive bacteria, including clinical strains of *Escherichia coli*, *Acinetobacter baumannii*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Heulot, Jacquier et al. 2017). Consequently, we first checked whether TAT-P has a bactericidal effect on a pathogenic strain of *E. coli* (*E. coli* O18) that the laboratory is regularly using in preclinical mouse models of peritonitis. We studied both the kinetic and the dose-dependent effects of the TAT-P. *E. coli* at 10^3, 10^5 and 10^7 CFU/ml exposed to 0, 2 and 20 µg/ml TAT-P. CFU were established 0, 1, 3 and 20 hours later.

As shown in **Figure 4 (Green line)**, *E. coli* did not grow in PBS and viable CFU counts decreased over time (50% to 70% after 20 hours of incubation). When TAT-P was added to the bacteria, a dose-dependent, inoculum-dependent and time-dependent bactericidal effect of TAT-P was observed: in panel A and B 100% of *E. coli* were killed using 20 µg/ml TAT-P (black line). Moreover, the killing activity of TAT-P was rapid, since 2 minutes after its addition more than 20% of *E. coli* were killed when using 10^3 or 10^5 CFU starting inocula (**Figure 4 A and B**). Using a higher inoculum of *E. coli* (**Figure 4 C**), the bactericidal activity of TAT-P seemed to require more time to develop.

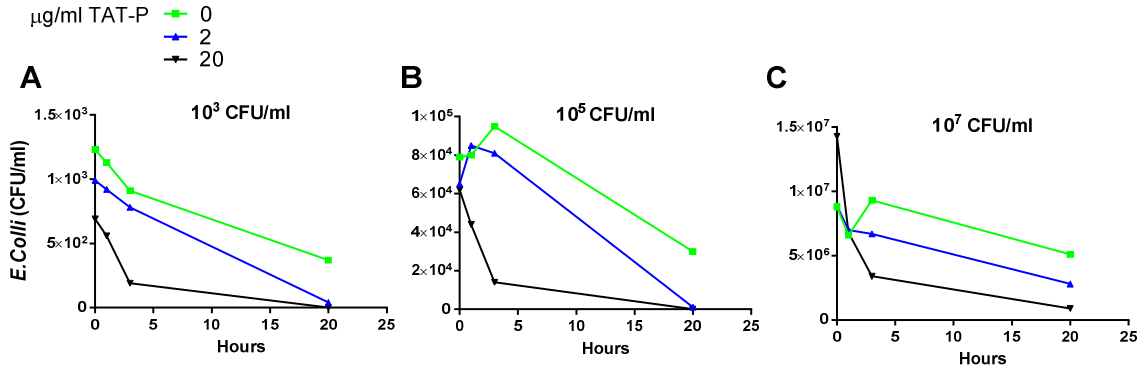

Figure 4. Bactericidal effect of TAT-P on Escherichia coli O18.
E. coli O18 (10^3, 10^5 and 10^7 CFU/ml) were exposed to 0 (green), 2 (blue) and 20 µg/ml (black) TAT-P and plated on agar plates after 2 minutes, 1, 3 and 20 hours. Results are expressed as the number of colonies obtained after one night of incubation. Data are from 1 experiment.

4.2 Effect of TAT-P on cytokine response by immune cells

4.2.1 TAT-P inhibits the production of TNF and IL-6 by BMDMs stimulated with CpG

To investigate whether TAT-P modulates cytokine production in a mouse system, C57BL6/J BMDMs were pre-incubated for 1 hour with 0, 2 and 20 µg/ml TAT-P and stimulated with 10 ng/ml LPS, µM CpG 1 and 10 ng/ml Pam_3CSK_4. IL-6 and TNF were measured in supernatants collected 8 and 24 hours post-stimulation. **Figure 5** shows LPS-stimulated IL-6 (**panel A**) and TNF (**panel B**) production by BMDMs. TAT-P had no impact on cytokine production. After 24 hours of stimulation, all stimuli induced the production of IL-6 (**panel C**) and TNF (**panel D**), albeit at different levels. TAT-P did not impact on LPS-induced IL-6 and TNF production, but reduced CpG and, to a minor extend, Pam_3CSK_4-induced IL-6 and TNF production.

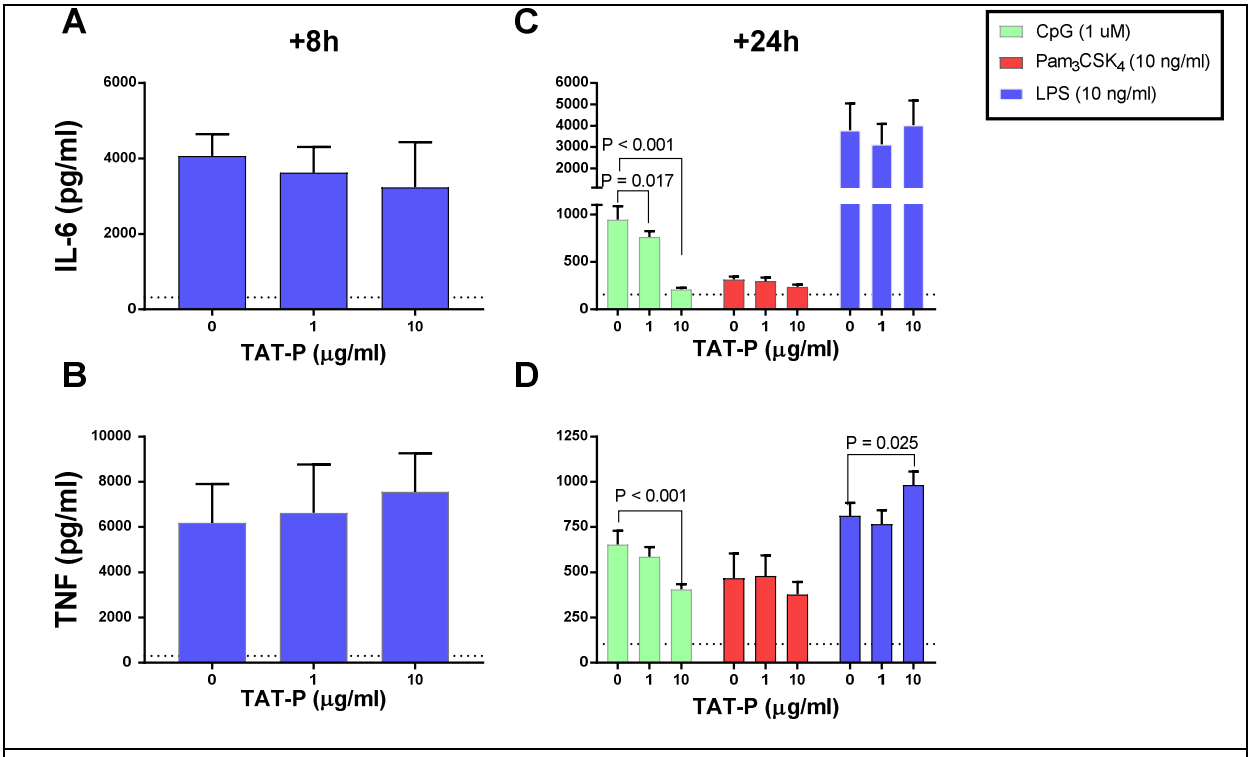

Figure 5 Effect of TAT-P on IL-6 and TNF production by BMDMs.
BMDMs were pre-incubated for 1 hour with 0, 1 and 10 µg/ml TAT-P and exposed for 8 and 24 hours to 1 µM CpG, 10 ng/ml Pam_3CSK_4 and 10 ng/ml LPS. IL-6 and TNF concentrations in supernatants were determined by ELISA. Data are means ± SD of triplicate samples and are representative of 2 experiments

To test whether the effects observed on cytokine production were related to cytotoxic effects of TAT-P, the mitochondrial activity of BMDMs was measured using the MTT assay performed 24 hours post-stimulation. Results showed that the mitochondrial activity of BMDMs was not affected by TAT-P, suggesting that TAT-P was not cytotoxic at the concentrations used in this experiment (**Figure 6**).

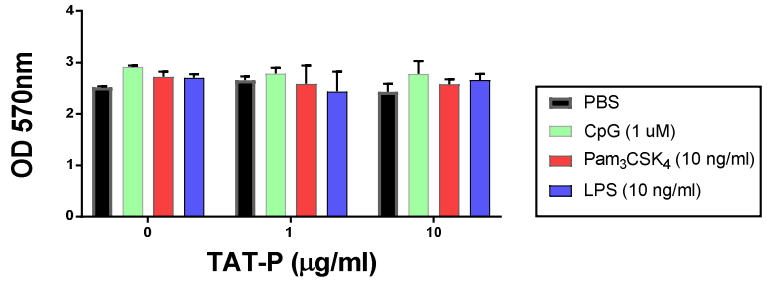

Figure 6. Mitochondrial activity of BMDMs incubated with TAT-P. BMDMs were pre-incubated for 1 hour with 0, 1 and 10 µg/ml TAT-P and exposed for 24 hours to 1 µM CpG, 10 ng/ml Pam_3CSK_4 and 10 ng/ml LPS before performing a MTT assay. Data are means ± SD of triplicate samples **from** 1 experiment.

4.2.2 TAT-P stimulates the early release of IL-6 and TNF by human PBMCs

To investigate whether TAT-P modulated cytokine production by human PBMCs, PBMCs were pre-incubated for 1 hour with 0, 1 and 10 µg/ml TAT-P and stimulated with 10 ng/ml LPS, 1 µM CpG and 10 ng/ml Pam_3CSK_4. IL-6 and TNF were measured in supernatants collected 8 and 24 hours post-stimulation. As shown in **Figure 7**, all ligands induced IL-6 (**panel A**) and TNF (**panel B**). Globally, after 8 hours, TAT-P dose-dependently increased IL-6 and TNF production (**panel A and B**). However, after 24 hours TAT-P either did not impact or inhibited IL-6 and TNF production (**panel C and D**).

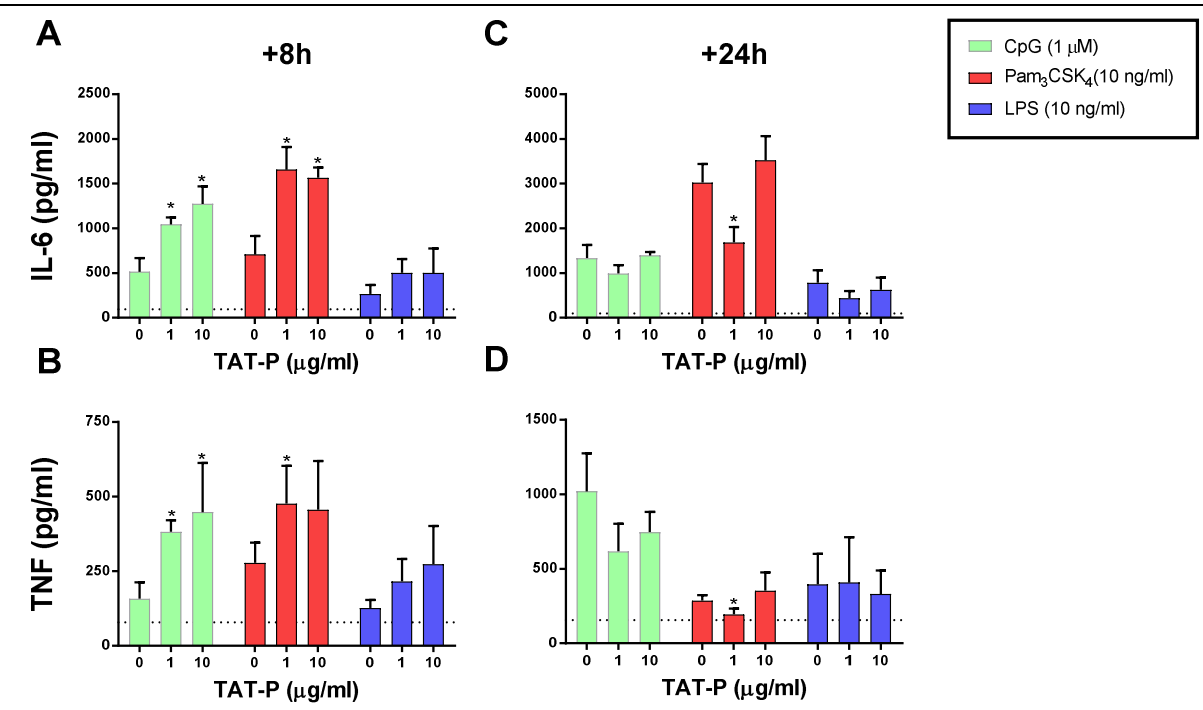

Figure 7. Effect of TAT-P on IL-6 and TNF production by PBMCs.
PBMCs were pre-incubated for 1 hour with 0, 1 and 10 µg/ml TAT-P and exposed for 8 and 24 hours to 1 µM CpG, 10 ng/ml Pam_3CSK_4 and 10 ng/ml LPS. IL-6 and TNF concentrations in supernatants were determined by ELISA. Data are means ± SD of triplicate measurements from 1 experiment representative of 3 experiments. *, $P < 0.05$ *vs* no peptide by unpaired t-test.

To test whether the effects observed on cytokine production were related to cytotoxic effects of TAT-P, a MTT assay was performed 24 hours post-stimulation of PBMCs. Results showed that the mitochondrial activity of PBMCs was not affected by TAT-P, suggesting that TAT-P was not cytotoxic at the concentrations used in this experiment (**Figure 8**).

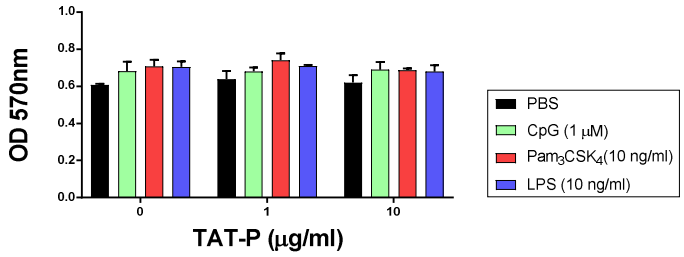

Figure 8. Mitochondrial activity of PBMCs incubated with TAT-P. PBMCs were pre-incubated for 1 hour with 0, 1 and 10 µg/ml TAT-P and exposed for 24 hours to 1 µM CpG, 10 ng/ml Pam_3CSK_4 and 10 ng/ml LPS before performing a MTT assay. Data are means ± SD of triplicate samples **from** 1 experiment.

4.2.3 TAT-P marginally affects IL-6 and TNF production by human whole blood

To investigate whether TAT-P modulated cytokine production by human whole blood, whole blood was collected from 3 healthy male volunteers, pre-incubated for 1 hour with 0, 1 and 10 µg/ml TAT-P and stimulated with 10 ng/ml LPS, 1 µM CpG and 10 ng/ml Pam_3CSK_4. IL6 and TNF were measured in supernatants collected after 24 hours.

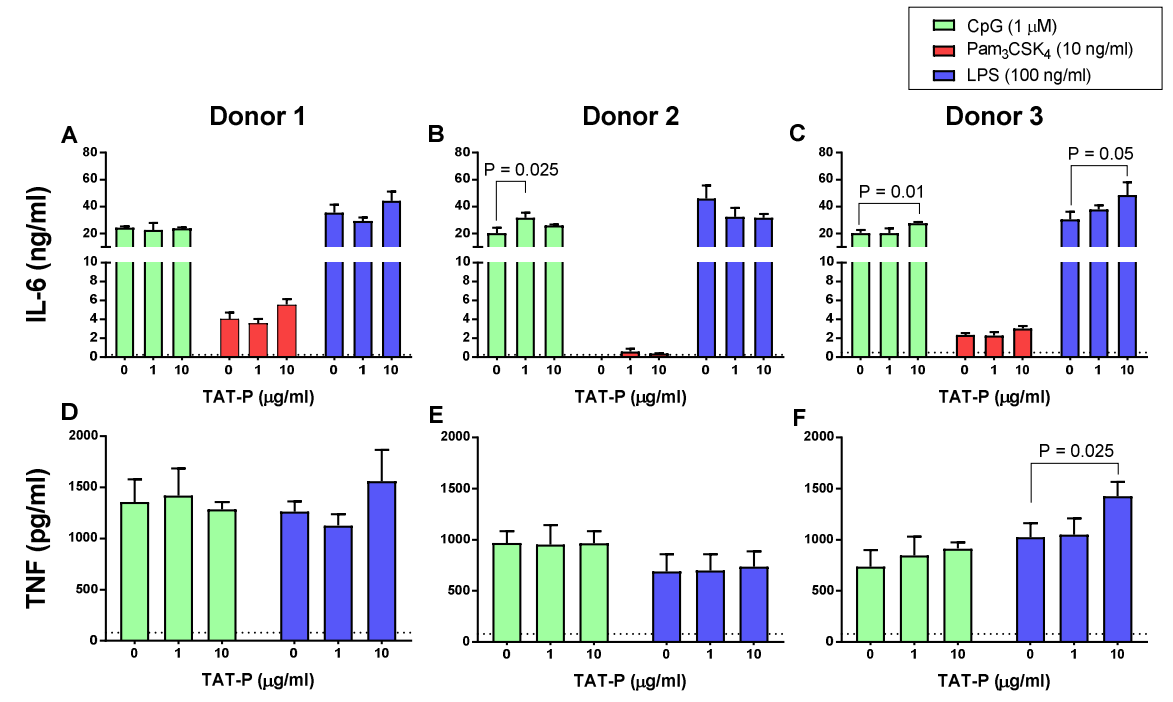

Figure 9. Effect of TAT-P on IL-6 and TNF production by whole blood. Whole blood from 3 healthy volunteers was pre-incubated for 1 hour with 0, 1 and 10 µg/ml TAT-P and exposed for 24 hours to 1 µM CpG, 10 ng/ml Pam_3CSK_4 and 10 ng/ml LPS. IL-6 and TNF concentrations in supernatants were determined by ELISA. Data are means ± SD of triplicate measurements from 1 experiment performed with 3 volunteers.

As shown in **Figure 9**, CpG, LPS and to a lower extend Pam_3CSK_4 induced IL-6 and TNF production by whole blood. Overall, TAT-P marginally affected IL-6 and TNF production, although sporadic significant increases were detected for IL-6 in donors 1 and 3 and TNF. Of note, this experiment also tested a control peptide of TAT-P (W_{317}) that did not modulate cytokine production (**data not shown**).

4.3 TAT-P injected locally at the onset of infection protects mice from *E. coli* peritonitis

To explore the antimicrobial effect of TAT-P *in vivo*, we used a mouse model of lethal peritonitis in which mice are injected intraperitoneally with 1.1 x 10^5 CFU *E.coli* O18. TAT-P (200 µg) or PBS were administrated i.p. 2 minutes later. TAT-P efficiently protected mice from death (80% *vs* 20% survival, P = 0.01, **Figure 6 A**). The severity scores and weight loss were also strongly decreased in TAT-P treated animals (**data not shown**). Accordingly, bacterial dissemination into the blood 24 hours post-infection was strongly reduced in TAT-P treated mice, with no detectable bacteria in 6/10 mice from the TAT-P group versus 1/10 mice from the PBS group (P = 0.01, **Figure 6 B**).

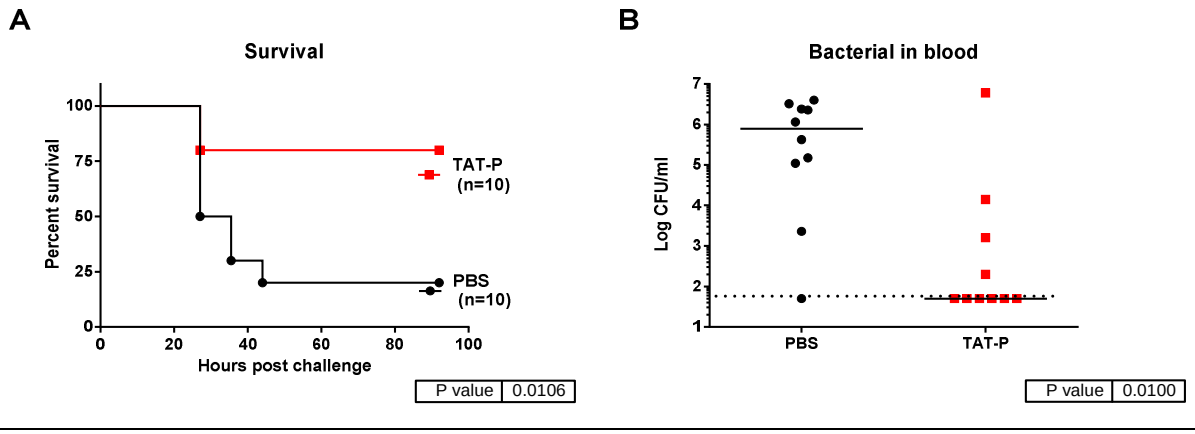

Figure 10. Effect of TAT-P injected into mice 2 min after infection with *E. coli* O18.
BALB/c mice (n = 10 per group) were injected i.p. with 1.1 x 10^5 CFU *E. coli* O18 and 2 minutes later with either PBS or 200 µg TAT-P. Survival was monitored. Blood was collected 24 hours post infection and CFU were determined.

4.4 TAT-P does not protect mice from lethal peritonitis if injected 3 hours post infection

To determine whether delayed application of TAT-P might protect from lethal peritonitis, mice were injected i.p. with 0.9 x 10^5 CFU *E.coli* O18 and 3 hours later with TAT-P (200 µg) or PBS. As shown in **Figure 7 A**, TAT-P did not improved survival (P = 0.146), although it should be noted that the overall mortality in the PBS group (20%) was much lower than the one in the previous experiment (80%). Bacterial dissemination into the blood 24 hours post-infection was not reduced by TAT-P treatment (P = 0.66, **Figure 7 B**).

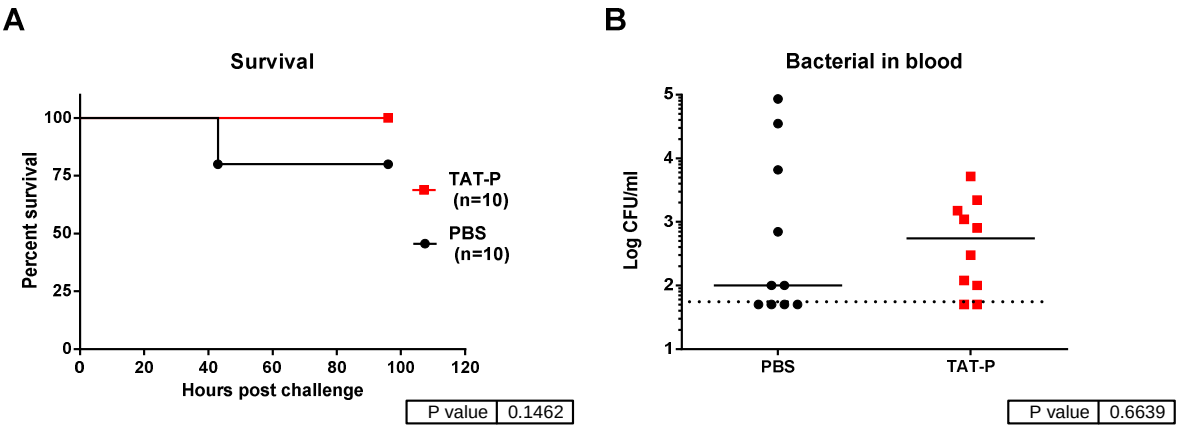

Figure 11. Effect of TAT-P injected into mice 3 hours after infection with *E. coli* O18.
BALB/c mice (n = 10 per group) were injected i.p. with 0.9 x 10^5 CFU *E. coli* O18 and 3 hours later with either PBS or 200 µg TAT-P. Survival was monitored. Blood was collected 24 hours post infection and CFU were determined.

5 Discussion

A major public health problem of the coming years would be the lack of treatment of infectious diseases, the discovery of new antibiotics being less important than the emergence of new resistant pathogens (Larson 2007). Actually, the number of patients with multi-drug-resistant infections increases and the WHO considers as a priority to find new efficient antimicrobial therapies.

One way to obtain new antimicrobial molecules could be the development of AMPs like TAT-P. Some studies identified efficient natural AMPs, such as *Staphylococcus lugdunensis*-derived lugdunin that suppressed the growth of *Staphylococcus aureus* in nares (Zipperer, Konnerth et al. 2016), and also proved that synthetics AMPs could have a significant effect in bacterial infections (Leon-Calvijo, Leal-Castro et al. 2015). The toxic effects of TAT-P against both cancer cells and bacteria have been demonstrated (Barras, Lorusso et al. 2014, Heulot, Jacquier et al. 2017). It's known that some AMPs target bacteria as well as eukaryotic cells. It could be explained by their mechanism of action, often by disrupting membrane integrity (Heulot, Jacquier et al. 2017), a mechanism suspected to underlie the action of TAT-P (Heulot, Jacquier et al. 2017).

Some AMPs have immunomodulation effects like LL37 (Mookherjee, Brown et al. 2006). Our results suggest that TAT-P modulates immune responses depending to the stimulus and the cells studied. Here we demonstrated using murine macrophages (BMDMs) that TAT-P significantly decreased the production of TNF and IL-6 when cells were stimulated by CpG and Pam_3CSK_4. This was not the case with LPS, which was unexpected since TAT-P was effective against Gram-negative bacteria *in vitro* and *in vivo* (Heulot, Jacquier et al. 2017)(**Figures 4 and 8**). Thus, the immunomodulation by TAT-P is probably not directly linked in the antibiotics effect of TAT-P against *E. coli* infection *in vivo*. Moreover the inflammatory process seems to be slowed down by the TAT-P in BMDMs.

TAT-P increased early (8 hours upon stimulation) secretion of TNF and IL-6 by human cells (PBMCs) in response to all stimuli tested. The effect was lost using late supernatant collected after 24 hours. A similar time-dependent impact on cytokine secretion was reported for LL37. LL37 is a human AMP produced by epithelial cells such as keratinocyte, cornea, ciliary lung epithelium (Mangoni, McDermott et al. 2016). LL37 increased in the first hour the level of expression of genes encoding pro-inflammatory cytokines such as TNF, but expression levels invariably decreases through time (Mookherjee, Brown et al. 2006), this effect is explained by the alteration of pro-inflammatory encoding genes due to the LL37 (Mookherjee, Brown et al. 2006). Interestingly, LL37 selectively modulated the inflammatory response, as LL37 decreased the levels of some pro-inflammatory cytokines while it increased the levels of others. In our study, TAT-P impacted on the production of IL-6 or TNF in a

similar fashion. Therefore, it could be interesting to test the impact of TAT-P onto the production of other pro-inflammatory cytokines. Moreover, it would be interesting to analyze cytokine expression at the mRNA level as it would tell us whether TAT-P impact on gene expression. The increased production of cytokine following stimulation using agonists of TLR4, TLR2 and TLR9 suggests that TAT-P acts through a common pathway downstream these receptors (i.e. MyD88 or below). Therefore, we could look at the activation of NF-κB, MAPK and IRF pathways. As an alternative, it can be hypothesized that TAT-P increases the recognition capacity of TLR4, TLR2 and TLR9.

We also tested the immunomodulation capacity of TAT-P on human whole blood, giving rise to results not as marked as in the previous experiments, showing sporadically tiny elevation of cytokine production. These slight effects might be explained by the theory of gene alteration, as the measures of cytokines were done 24 hours after stimulation. Another possible explanation is consumption or accelerated degradation of TAT-P in the blood. The results are correlated with previous experiment showing an increased cytokine production with all stimulation. During that experiment, a version of TAT-P was used in which tryptophan at position 317 was substituted by an alanine considered as inactive in cancer sensitization (Barras, Chevalier et al. 2014, Heulot, Chevalier et al. 2016). This modified peptide was totally inactive in immunomodulation capacity as expected (data not shown). It could be interesting in the future to repeat this experiment with a more important dose of TAT-P to overpass a hypothetical consumption in blood.

Overall, it appears that TAT-P has different effects on BMDMs, PBMCs and whole blood. It could be explained basically by differences of cells tested, which means that TAT-P could have a positive effect on TNF and IL-6 production by lymphocytes present in PBMCs, especially T cells, but a negative effect on macrophages. To test this hypothesis, experiments comparing the same populations of cells are needed.

TAT-P gave a significant survival advantage on *E. coli*-induced peritonitis in mice treated with the peptide immediately after infection at the site of infection (**Figure 7**). The protective effect was lost when TAT-P was injected 3 hours after infection. This difference may be explained by two arguments. First TAT-P has a short effect time as shown in (**Figure 4**). TAT-P acted maximally in the first minutes and its action decreased in a time-dependent manner (**Figure 4**). Second, previous data have shown that TAT-P was undetectable in blood 2 hours after injection (Michod, Annibaldi et al. 2009). The elimination of TAT-P was established through radiolabeled TAT-P measurements, showing a preferential accumulation of the peptide in liver, kidneys, stomach and pancreas and high concentrations in urine (Heulot, Jacquier et al. 2017). Overall, our hypothesis is that active TAT-P was unable to reach enough bacteria to avoid lethal sepsis due to its bio-distribution and short action time.

TAT-P is an antimicrobial peptide with some immunomodulation effects through action on TLR2, TLR4 and TLR9 pathways in mouse and human cells. The *in vivo* antimicrobial action is possible as shown in **figure 6** but only during a very narrow time window. The native form of TAT-P should be improved to reach a better antimicrobial activity and a possible clinical utilization. Considering that Heulot and al. showed an important accumulation of the peptide in urine, we could test the efficacy of TAT-P in a model of urinary tract infection, for example pyelonephritis in rats (Glauser and Bonard 1982). Finally the mechanisms underlying the antimicrobial as well as the immuno-modulation actions of TAT-P are unknown. Deciphering these mechanisms could help to improve the structure of TAT-P and its clinical applicability, and also to recognize and understand better new peptides with potential antimicrobial activity.

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