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Anti-inflammatory activity of small-molecule antagonists of Toll-like receptor 2 (TLR2) in mice

G. Wietzorrek[a], M. Drexel[b], M. Trieb[c], S. Santos-Sierra[c,*]

[a] *Section of Molecular and Cellular Pharmacology, Medical University of Innsbruck, A-6020, Innsbruck, Austria*
[b] *Department of Pharmacology, Medical University of Innsbruck, A-6020, Innsbruck, Austria*
[c] *Section of Biochemical Pharmacology, Medical University of Innsbruck, A-6020, Innsbruck, Austria*

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ABSTRACT

Toll-like receptor 2 (TLR2) is currently investigated as a potential therapeutic target in diseases with underlying inflammation like sepsis and arthritis. We reported the discovery, by virtual screening and biological testing, of eight TLR2 antagonists (AT1-AT8) which showed TLR2-inhibitory activity in human cells (Murgueitio et al., 2014). In this study, we have deepened in the mechanism of action and selectivity (TLR2/1 or TLR2/6) of those compounds in mouse primary cells and *in vivo*. The antagonists reduced, in a dose-dependent way the TNFα production (*e.g.* AT5 IC_{50} 7.4 μM) and also reduced the nitric oxide (NO) formation in mouse bone marrow-derived macrophages (BMDM). Treatment of BMDM with the antagonists showed that downstream of TLR2, MAPKs phosphorylation and IkBα degradation was reduced. Notably, in a mouse model of tri-acylated lipopeptide (Pam3CSK4)-induced inflammation, AT5 attenuated the TNFα and IL-6 inflammatory response. Further, the effect of AT5 in the stimulation of BMDM by the endogenous alarmin HMGB1 was investigated. Our results indicate that AT4-AT7 and, particularly AT5 appear as good starting points for the development of inhibitors targeting TLR2 in inflammatory disorders.

1. Introduction

Toll-like receptors (TLR) are key components of the innate immune response. They recognize conserved molecular structures (pathogen associated molecular patterns –PAMPS-) in pathogens that come into contact with the host (Janeway and Medzhitov, 2002). The signaling cascade initiated by TLR ligation in cells of the innate immune system like monocytes and dendritic cells leads to activation of several transcription factors (*e.g.* NF-kB, AP-1) (Jones et al., 2001). In turn, the released cytokines and chemokines contribute to the activation of the adaptive immune response (Akira and Takeda, 2004). Recent studies demonstrate that the inflammation underlying several autoimmune diseases may be mediated by TLRs due to their capacity to recognize host derived molecules or alarmins (also called DAMPS -danger associated molecular patterns-) which are produced after trauma and stress conditions (Mills, 2011; Takeuchi and Akira, 2010).

From the ten known human TLRs, TLR2 is distinctive as it recognizes, by hetero-dimerization with TLR6 or TLR1, various PAMPs derived from Gram-positive bacteria, like tri- and di-acylated lipopeptides (Bessler, 1992; Henneke et al., 2008), yeast lipoproteins (Akira et al., 2006) and DAMPs like HMGB1 or hyaluronan (Park et al., 2006). It has been demonstrated that TLR2/TLR1 and TLR2/TLR6 signaling mediate non-sterile inflammatory diseases like sepsis (Henneke et al., 2008). In this case, the initial inflammatory response to microbes is deregulated and causes an uncontrolled release of cytokines (*e.g.* TNF-α) and nitric oxide (Cauwels, 2007), what can lead to tissue damage and multiorgan failure (Sriskandan and Altmann, 2008). In a polymicrobial sepsis model, TLR2 deficient mice have increased survival rates compared to wild type mice (Bergt et al., 2013). Moreover, blockade of TLR2 (and also TLR4) signaling by antagonistic antibodies successfully decreases disease severity in sepsis models of Gram-positive and Gram-negative bacteria (Daubeuf et al., 2007; Meng et al., 2004).

Other studies suggest that the over-activation of TLRs contributes to the development and progression of diseases like atherosclerosis (Michelsen et al., 2004), diabetes (Zipris et al., 2005) and Alzheimer's disease (Landreth and Reed-Geaghan, 2009). TLR activation is fast and happens in the early stages of inflammation. Then inflamed tissue produces alarmins which further activate TLRs strengthening the cytokine production feedback. For this reason, it has been suggested that it would be of advantage to pharmacologically block TLR-activity before the stage of chronic inflammation arises (Hennessy et al., 2010). Promising results have been obtained with a TLR2-blocking antibody

* Corresponding author.
E-mail address: Sandra.santos@i-med.ac.at (S. Santos-Sierra).

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(OPN-305) in several murine models of autoimmune diseases (Arslan et al., 2008), and the discovery of small-molecule antagonists of TLR2 represents an encouraging therapeutic approach in the above mentioned disorders.

Previously, we employed virtual screening and molecular modeling to discover potential TLR2 antagonists (Murgueitio et al., 2014). This approach allowed us to pre-select a reduced number of molecules to be *in vitro* tested. The antagonistic activity of eight selected hits was confirmed by assessing the inhibition of TNFα production by human monocytes which were stimulated with di- and tri-acylated lipopeptides (TLR2 ligands). However the effect of the antagonists in mouse cells following TLR2 activation was not tested, neither whether the compounds would be effective in a mouse-model of inflammation. In the present study, the eight TLR2 antagonists (1–8, renamed AT1-AT8) are characterized for their inhibitory activity in mouse cells and *in vivo*.

2. Materials and methods

2.1. Cell culture

The mouse macrophage cell line RAW264.7 (ATCC collection) was cultured in DMEM (Sigma Aldrich) with 10% FBS (Sigma Aldrich) and 0.5% (v/v) Ciprofloxacin (Sigma Aldrich) at 37 °C and 5% CO_2 atmosphere. The cell line HEK293 was cultured as the macrophages. For the selection of BMDM we followed the protocol described by Manzanero, S. (Manzanero, 2012). The bone-marrow from tibias and the femurs of wild-type 9–12 week old mice (C57BL/6; Charles River Laboratories) were isolated and differentiated during 5 days in RPMI 1640 media with 2 mM L-glutamine (Sigma Aldrich), 10% heat-inactivated FBS (Sigma Aldrich), 0.5% Ciprofloxacin and 100 ng/ml M-CSF (Biolegend) to induce monocytic lineage proliferation and differentiation. Afterwards the cells were washed and detached with Accutase (Sigma Aldrich), suspended in the media indicated above and plated as described in each experiment.

2.2. Luciferase and ELISA assays

Luciferase and ELISA (mTNF, mIL-6, mIL-10 –RandDSystems-) assays were basically as described previously (Murgueitio et al., 2017, 2014). A modification for the luciferase assay with RAW264.7 macrophages was the increased amount of DNA used (plasmids *Elam.luc* 200 ng and *Renilla* 100 ng per 1×10^5 cells).

2.3. Antibodies and reagents

Antibodies IkBα, p-p38, p-JNK and Actin-ß (Cell Signalling Technology), HRP-conjugated anti-rabbit (Sigma Aldrich), HRP conjugated anti-mouse (Cell Signalling Technology); mouse recombinant HMGB1 (Biolegend). Pam2CSK4 –P2-, Pam3CSK4 –P3-, Flagellin, IL-1ß (Invivogen). Plasmid preparations were done using PureYield plasmid endotoxin-free kit (Promega). Compounds: Stock solutions of the compounds were prepared in DMSO (Sigma Aldrich) at a concentration of 10 mM and filtered through a 0.2 µm PTFE sterile-filter, then aliquoted and kept at −20 °C for immediate use or −80 °C for long term storage. All tested small molecules (AT1-AT8 and NCI35676) were ordered from the National Cancer Institute (NCI) database, USA, and the available ones were ordered from the next providers: Enamine, Maybridge, SpecsOmega (Murgueitio et al., 2014).

2.4. Immunoblot

BMDM differentiated as specified above were grown in 24 well-plates (1×10^5 cells per well) in RPMI media. Next day the cells were treated as indicated. After stimulation, the cells were washed with PBS (room temperature) and then lysed with ice-cold lysis buffer (20 mM Tris-HCl, pH7.6, 150 mM NaCl and 0.1% Tween20 and protease and phosphatase inhibitor mix -Roche Applied Science-). The lysates were frozen at −20 °C and after thawing they were cleared by centrifugation at 10^4 x g for 10 min (4 °C) and equal amounts of the supernatant were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane (CarlRoth). The membrane was blocked for 1 h in 5% milk in TBST (20 mM Tris-HCl, pH7.6, 150 mM NaCl and 0.1% Tween20), then it was incubated overnight at 4 °C with primary antibody (in 5% bovine serum albumin –BSA-) and subsequently with horseradish peroxidase (HRP)-conjugated secondary antibody in 5% BSA for two hours. Immunoreactive proteins were detected with Immobilon detection reagents (Millipore) and the Fusion analyzer (Peqlab). Quantification was done with the FusionCapt software.

2.5. Nitric oxide (NO)

NO measurement was performed as described previously (Nussler et al., 2006). In short, 1×10^5 macrophages were grown in RPMI plus 10% FBS overnight and next day they were incubated with compounds or DMSO and P3. After overnight incubation, 130 µl of the supernatant were added to a 96-well black plate (Nunc, Denmark). Then, 70 µl of 2,3-diaminonaphthalene (0.05 mg/ml in 0.62 M HCl) was added to each well and incubated 15 min at 30 °C. The reaction was quenched by addition of 50 µl of 3 M NaOH and the fluorescence was measured with excitation at 360 nm and emission at 430 nm in a Victor plate reader (Perkin Elmer).

2.6. Mice experiments

8-week old, male, pathogen free C57BL/6 mice (Charles River Laboratories) were maintained at the animal facility of the Medical University Innsbruck (12 h light/dark cycle; standard rodent chow and water available *ad libitum*). For lipopeptide induced inflammation 5 µg/g of compounds or vehicle were administered intraperitoneally. After one hour Pam3CSK4 (1 µg/g) was injected and 25–50 µl of vein tail blood were collected in Lithium-heparin Microvette tubes (Sarstedt) at time point 0 h, and 2 h after. The blood was centrifuged at 5×10^3 x g and the supernatant frozen at −20 °C until further cytokine measurement by ELISA. Animal experiments were conducted according to national guidelines and European Community laws and were approved by the Committee for Animal Protection of the Austrian Ministry of Science.

2.7. Statistical analysis

GraphPad Prism (San Diego, CA, USA) was used to perform statistical analysis. One-way analysis of variance (ANOVA) was used for multiple group comparison with Dunnette´s *post hoc* test and unpaired t-Student test for comparison between two groups.

3. Results

3.1. Compounds AT1-AT8 inhibit TLR2-induced NF-kB transcriptional activity in mouse macrophages and subsequently, pro-inflammatory cytokine and nitric oxide production

Previously we described the screening and selection of small-molecules with antagonistic properties on TLR2 activity (Murgueitio et al., 2014). For easier identification the antagonists 1–8 have been renamed AT1-AT8 (Fig. 1A). We demonstrated that AT1-AT8 reduced NF-kB activity following TLR2 stimulation in a human cell line overexpressing TLR2 (HEK293-TLR2) and the TNFα production in primary human monocytes. In order to address the activity of the compounds in *in vivo* experiments, we now have investigated their activity in mouse cells. Because primary macrophages (*e.g.* bone-marrow derived macrophages –BMDM-) are difficult to transfect, we initially used RAW264.7

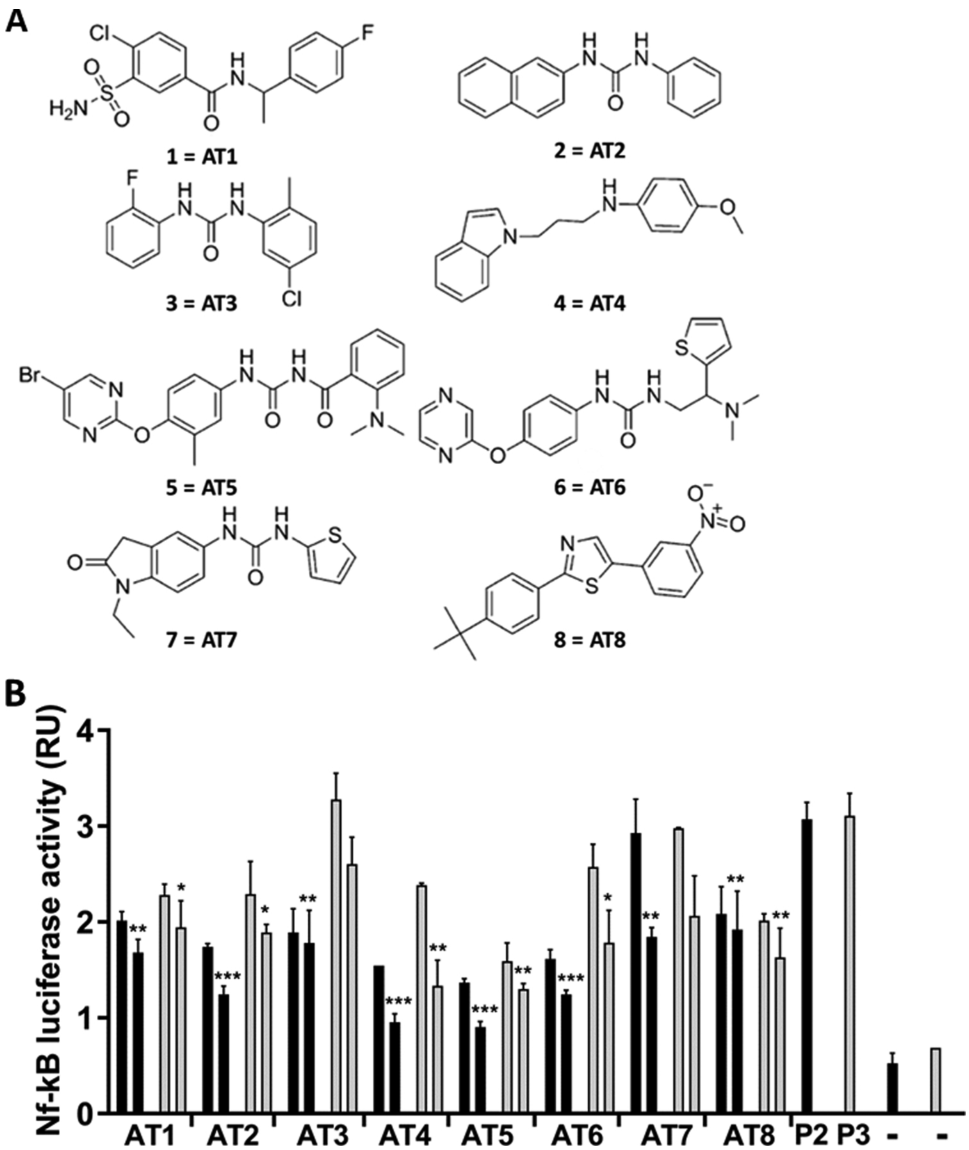

Fig. 1. A) Chemical structure of the TLR2 antagonists (AT), here renamed for clarity. Numbers indicate the nomenclature used in Murgueitio et al., 2014. **B)** RAW264.7 macrophages were transfected with an NF-kB dependent luciferase-reporter plasmid and an NF-kB independent Renilla producing plasmid. After incubation with AT1-AT8 (black: P2, grey: P3. Same colour adjacent bars are 25 and 50 μM) or vehicle (DMSO), the cells were stimulated with P2 (25 ng/ml; blue bars), P3 (80 ng/ml; grey bars), or media (-). Then the luciferase activity was measured after five hours and the ratio to the Renilla activity calculated. Bars represent mean and SEM of triplicates. RU: relative units. One experiment representative of three is shown. The values obtained with P2 or P3 plus antagonist (50 μM) were compared with the P2 or P3 values plus vehicle by one way ANOVA with Dunnett´s *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

macrophages to study the compounds effect in the NF-kB transcriptional activation. The cells were transiently transfected with the NF-kB-dependent reporter plasmid *Elam.luc* and a plasmid that constitutively expresses *Renilla*. Then, the cells were treated with two concentrations of AT1-AT8 for 1 h and afterwards stimulated with Pam3CSK4 (P3) or Pam2CSK4 (P2) for additional five hours. As shown in Fig. 1B, the antagonists decreased the P2 and P3 induced activity in a dose-dependent way in RAW264.7 macrophages. AT2-AT6 exhibited a preference to inhibit P2 induced NF-kB transcriptional activity while AT1, AT7 and AT8 inhibited equally TLR2/TLR6 (P2) and TLR2/TLR1 (P3) activity.

To confirm the previous result and to assess whether inhibition of transcriptional activation paralleled inhibition of cytokine production after TLR2 stimulation, we measured the TNFα secretion in the supernatant of primary mouse macrophages. BMDM were treated with increasing concentrations of AT1-AT8 and stimulated either with P2 or P3. As shown in Fig. 2, AT1-AT8 inhibited TNFα production in a dose-dependent way. In these cells, we did not observe a significant preference of any of the antagonists for inhibition of P2 or P3 activation of TLR2. The most potent compounds were AT5 ($IC_{50} \approx$ 7 μM), AT7 ($IC_{50} \approx$ 9 μM) and AT4 ($IC_{50} \approx 10$ μM) (Fig. 2). This confirmed our previous results in HEK293-TLR2 cells where AT5 was the most potent inhibitor (Murgueitio et al., 2014). Further, we tested the effect of the antagonists in the production of IL-6 (Fig. 3A). Pre-incubation of BMDM with the antagonists lead to a reduction of cytokine production induced by P3 (1095.65 ± 139.36 pg/ml), most markedly by AT5 (decreased to 47.58 ± 4.09%). AT8, at the same concentration, did not have an effect in the IL-6 production. TLR activation leads to pro-inflammatory cytokine production but in some instances also to activation of anti-inflammatory mechanisms that initiate the resolution of inflammation (*e.g.* IL-10, IL-4, IL-5 and IL-13) (Netea et al., 2004; Re and Strominger, 2004). Thus, we tested the effect of AT1-AT8 in the P3-induced IL-10 production. Pre-treatment of BMDM with the antagonists lead to a significant reduction of P3 activity (501.54 ± 103.59 pg/ml) only in the case of AT8 (73.97 ± 7.258%, Fig. 3B).

Other groups have described small-molecule antagonists of TLR2 which were discovered by high throughput screening (*e.g.* (Cheng et al., 2012)). These antagonists were selected in a screening assay testing for the inhibitory effect in the production of nitric oxide by RAW264.7 mouse macrophages stimulated with P3. Thus, we wanted to assess the activity of our compounds in an equivalent assay. BMDM were incubated with AT1-AT8 (20 μM) or the initial lead compound from Cheng et *al.* NCI35676 (purpurogallin). Then they were stimulated with P3 and the nitric oxide production was assessed after overnight incubation (Fig. 3C). The nitric oxide levels obtained with P3 (2.74 ± 0.51 μM) were decreased by incubation with the antagonists.

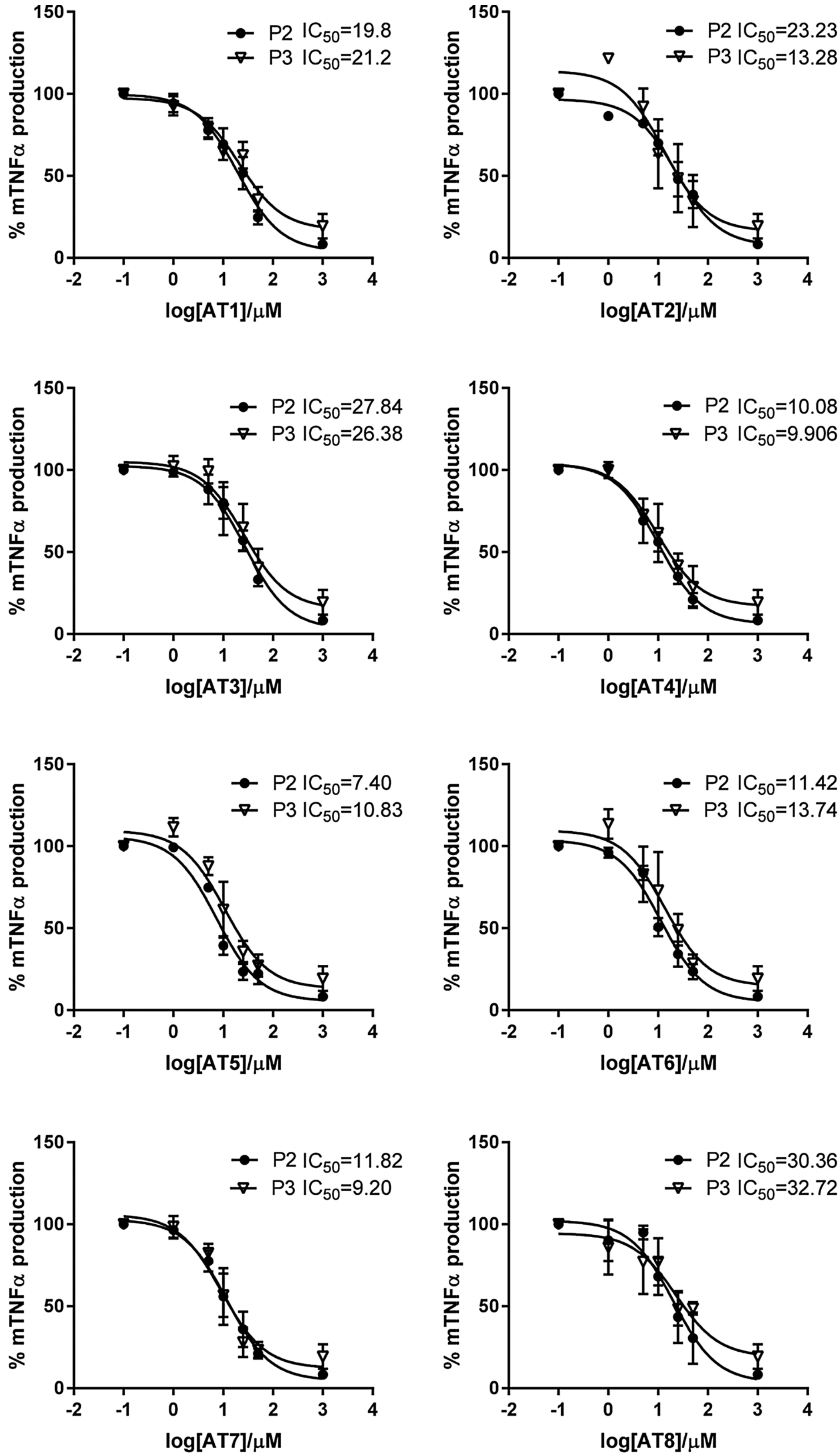

Fig. 2. BMDM were treated with increasing concentrations of AT1-AT8 (1, 5, 10, 25, 50 μM) or vehicle and then stimulated overnight with P2 (15 ng/ml) or P3 (45 ng/ml). Then, the TNFα concentration was measured by ELISA. Circles represent the mean and SEM of three independent experiments in duplicates. The IC_{50} constants were obtained by non-linear regression fit (three parameters curve).

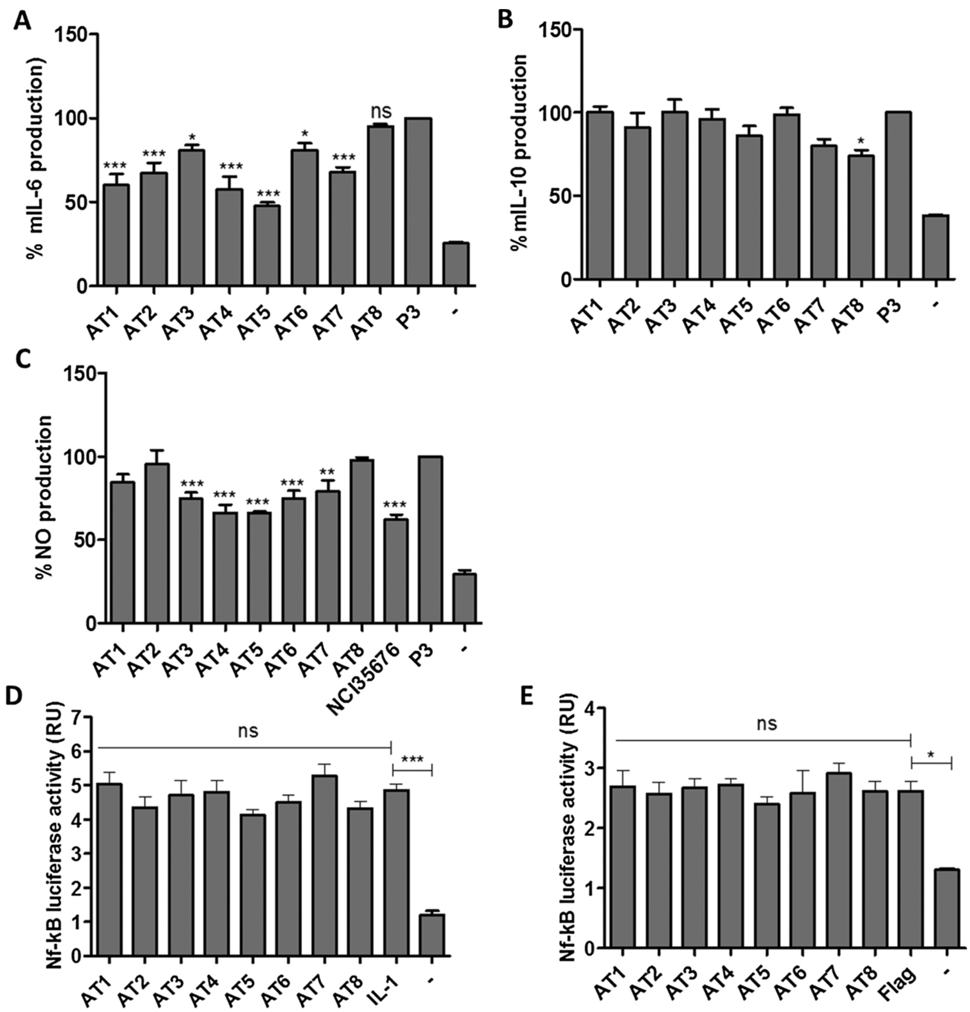

Fig. 3. A) BMDM were treated with AT1-AT8 (25 μM) or vehicle (DMSO) and then stimulated with P3 (45 ng/ml). After overnight incubation, the IL-6 released into the supernatant (and IL-10, **B**) was quantified by ELISA**. C)** BMDM macrophages were treated with AT1-AT8 (20 μM) or vehicle (DMSO) and then stimulated with P3 (5 μg/ml). After 24 h the NO in the supernatant was measured by the 2,3-diaminonaphthalene method. The NO produced in the P3 samples plus vehicle was normalized as 100%. Bars represent media and SEM of three independent experiments. **D)** HEK293 cells were transfected with an NF-kB reporter plasmid and an NF-kB independent Renilla plasmid and next day the cells were incubated with AT1-AT8 (25 μM) or vehicle (DMSO) and then stimulated with IL-1 (50 ng/ml) or Flagellin (**E**; 100 ng/ml). After five hours the luciferase activity was measured and the fold to the Renilla values were calculated. Bars represent media and SEM of three independent experiments. RU: relative units. The statistical significance was calculated by one way ANOVA with Dunnett´s *post-hoc* test (A–E). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In our experimental conditions, the compound NCI35676 reduced the nitric oxide activity to 62.33 ± 2.96% and performed similarly to AT4 (66.33 ± 4.97%) and AT5 (66.67 ± 1.20%). The values reported by Cheng at *al.* were in the order of 30% remaining nitric oxide activity at 3 μM of NCI35676. This discrepancy might be due to the use of different cells in both cases.

3.2. Compounds AT1-AT8 do not decrease TLR5 or IL-1R activity

Some small-molecules are cell-permeable thus, we wondered if any of the selected TLR2 antagonists would also interfere with the activity of other inflammatory receptors which share components of the intracellular signaling with TLR2, like TLR5 or IL-1R. In Murgueitio et al. (2014), we showed that AT1-AT8 do not interfere with TLR3 or TLR4 activity. Yet, this test does not discriminate if the compounds may be cell-permeable and inhibit TLR2 at the level of downstream adaptors since TLR3 and TLR4 function through MyD88-independent pathways (i.e TRIF for TLR3, TRIF/TRAM for TLR4) (Bin et al., 2003; Murgueitio et al., 2014; Oganesyan et al., 2006).

To discriminate whether the antagonism occurred at the TLR2 receptor level or downstream proteins, we performed luciferase assays in HEK293 cells which express both TLR5 and IL-1R (Jiang et al., 2003; Semnani et al., 2008). The cells were incubated with AT1-AT8 (25 μM) or vehicle (DMSO) and then they were activated either with IL-1β, flagellin or media. After five hours the luciferase activity was measured (Fig. 3D, E). We did not observe a significant inhibition of either of the pathways by AT1-AT8, which indicates that the inhibitory effect in the TLR2 pathway should happen at the level of the receptor. These results are in accordance with our previous report where we did not observe inhibition of TLR3 and TLR4 activity.

3.3. MAPK activity and IkBα degradation downstream of TLR2 is reduced by the TLR2 antagonists

TLR2 stimulation leads to the activation of downstream kinases, IkBα degradation and finally to NF-kB activation. To unravel the effect of AT1-AT8 on the proteins downstream of TLR2, we treated BMDM with the compounds and then stimulated them with P3. We assessed the effect of the compounds on the phosphorylation of the kinases JNK and p38, and the degradation of IkBα by immunoblot. As shown in Fig.4A, compounds AT5 and AT6 decreased significantly the phosphorylation of p38 induced by P3 stimulation. Moreover AT1 and AT3-AT8 inhibited significantly the phosphorylation of JNK and although not significant, an inhibitory effect on the degradation of IkBα could be detected for all the compounds. AT2 and AT8 showed the lowest inhibitory effect in MAPK phosphorylation and IkBα degradation. We had previously tested the effect of the compounds in RAW264.7 macrophages without observing any toxic effect at the concentrations used in the assays (not shown). However, at very high concentration, AT7 produced a decreased in cell viability of RAW264.7 macrophages (Murgueitio et al., 2014). In our own experience, primary cells are less robust than stable cell lines. Thus, to exclude that the inhibitory effects obtained in the immunoblot experiments were due to toxic effects of the compounds, BMDM were incubated with AT1-AT8 (same concentration, 50 μM) during the same time frame used in the previous experiment (125 min). Furthermore, longer incubation times were also tested

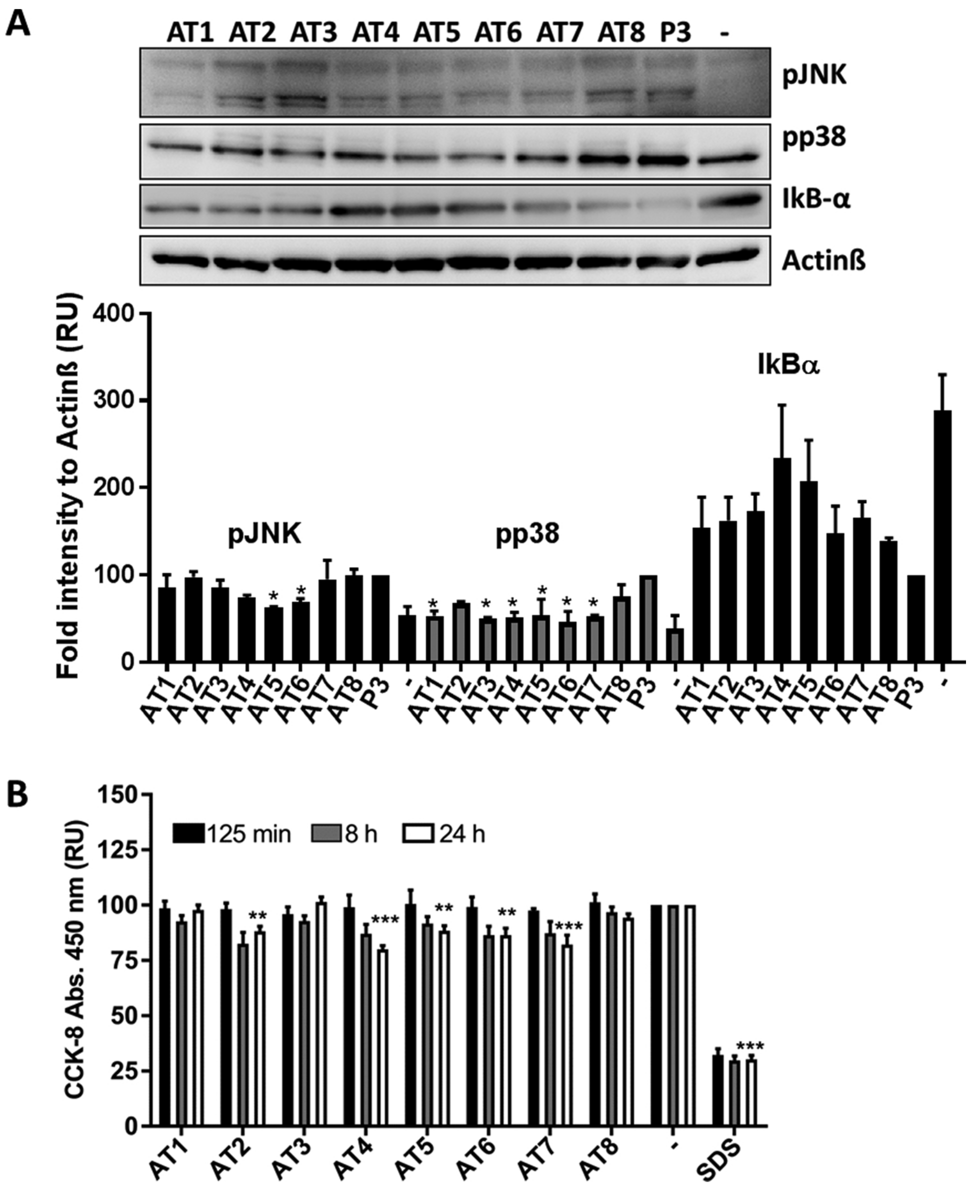

Fig. 4. A) BMDM were incubated with compounds AT1-AT8 (50 µM; 125 min) or vehicle. Then, P3 (100 ng/ml) was applied to the media (35 min). The phosphorylation degree of JNK, p38 and the degradation of IkBα were detected with the corresponding antibodies and densitometry analysis was assessed as fold to the loading control (Actinß). RU: relative units. The values represent the mean and SEM of two independent immunoblots. The values for AT1-AT8 were compared to P3 by one way ANOVA with Dunnett´s *post hoc* test. *$p < 0.05$. **B)** Viability of BMDM incubated with AT1-AT8 (50 µM). The dehydrogenase activity of the cells produces a colored formazan-derivative. The absorbance at 450 nm is indicative of cell viability. The experiment was repeated two times in triplicates. The values were compared to control by one way ANOVA with Dunnett´s *post-hoc* test. ** $p < 0.01$, *** $p < 0.001$.

(8 h and 24 h). Then the cell viability was assessed with the CCK-8 assay. The treatment of the macrophages with AT1-AT8 during 125 min did not result in toxicity (Fig. 4B). However, we observed a decrease in cell viability after 24 h of incubation with AT2 (88.24%), AT4 (81.00%) AT5 (88.46%) and AT7 (82.25%). Yet, the concentration used (50 µM) is above the IC_{50} of these compounds (Fig. 2).

3.4. AT5 decreases inflammatory cytokine production in a mouse model of P3 induced inflammation

Sepsis is a severe clinical condition with a high incidence that develops as a response to an infection and if not timely diagnosed it may lead to shock, multiple organ failure and death (Minasyan, 2017). Unfortunately, the clinical trials with anti-inflammatory agents in sepsis and anti-cytokine therapies have not rendered positive results until now (Marshall, 2014). In comparison to healthy individuals, monocytic expression of TLR2 and TLR4 in septic patients is significantly up-regulated, and it has been suggested that modulation of TLR signalling pathways might show therapeutic potential in the treatment of organ injury developed in this pathology (Tsujimoto et al., 2008).

Two of the compounds that performed better in *in vitro* assays, AT4 and AT5, were chosen to study the effect of antagonizing TLR2 *in vivo*. We used a lipopeptide-induced sepsis mouse model as it has been already described for LPS and P3 (Copeland et al., 2005; Mersmann et al., 2010). These studies have indicated that intraperitoneal injection of LPS induces a maximum of TNFα production after 2 h (Copeland et al., 2005) and P3 1 h after injection, in the case of IL-1β the maximum secretion appears 6 h post-injection (Mersmann et al., 2010). Thus, we wanted to establish the time point of full TNFα production after P3 injection in our conditions.

C57BL/6 J mice were intraperitoneally injected with saline or P3 (1 µg/g). Then blood was obtained from the vein tail at time points 0, 2, 6 and 24 h (Fig. 5A). At 2 h there was a maximum of TNFα production (898.1 pg/ml). Therefore we chose this time point to test the effect of AT5. The compound was i.p. injected (5 µg/g), followed by i.p. P3 (1 µg/g) injection one hour after. The TNFα and IL-6 levels in blood serum were quantified by ELISA. Mice that were treated with AT5 had a decreased level of TNFα in comparison with the control group (from 1118 ± 95.31 pg/ml to 775.4 ± 97.53 pg/ml); Fig. 5B). Additionally, the levels of IL-6 after AT5 treatment decreased from 2895 ± 442.8 pg/ml to 1495 ± 305.4 pg/ml. On the contrary, mice treated with AT4 did not show a reduction in TLR2 mediated TNFα

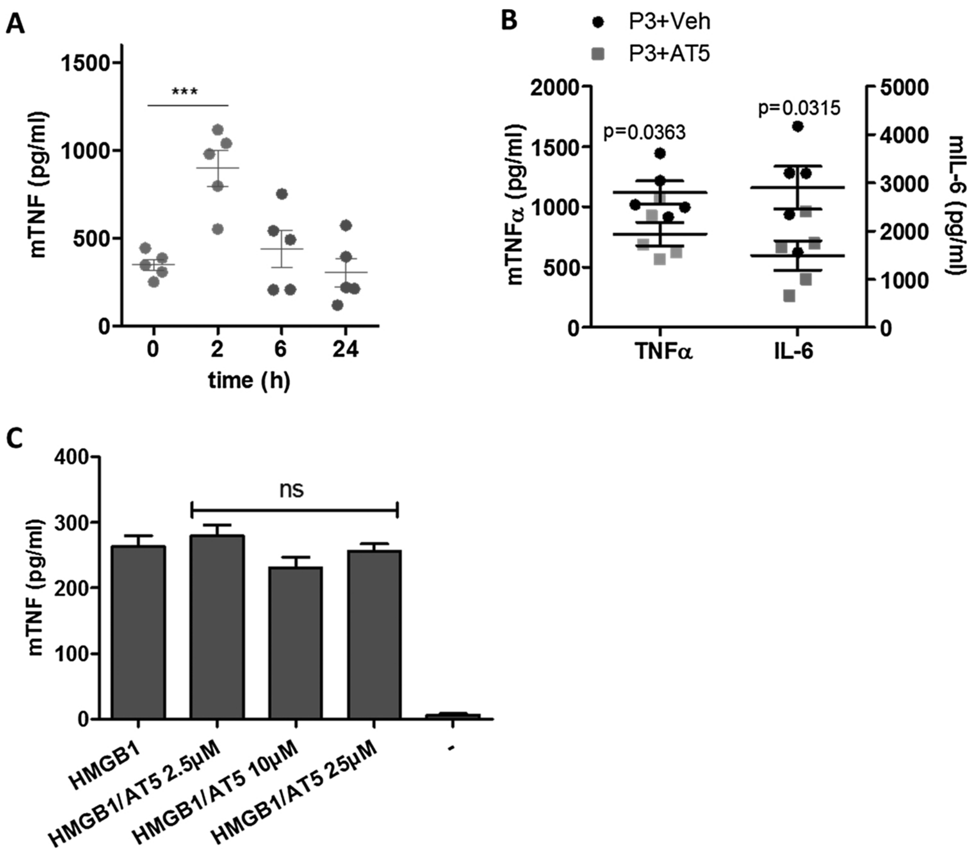

Fig. 5. A) C57BL/6 J mice were injected with P3 (1 μg/g) and blood was taken at different time points. The TNFα level was assessed by ELISA. **C)** Mice were pre-treated with AT5 (5 μg/g) i.p. or with vehicle (DMSO/NaCl) for 1 h. Then they were challenged with P3 (1 μg/g) and vein-blood was taken 2 h after AT5 injection. The TNFα and IL-6 level was quantified by ELISA. The statistical significance was assessed with unpaired t-student test. ***$p < 0.001$. **D)** BMDM macrophages were incubated with AT5 or vehicle and then stimulated with HMGB1 (15 μg/ml). After overnight incubation the TNFα released in the supernatant was quantified by ELISA. The bars represent mean and SEM of four independent experiments in duplicates. The difference between incubation with AT5 plus HMGB1, or HMGB1 plus vehicle was not significant by one way ANOVA with Dunnett´s *post hoc* test.

production at the same concentration (not shown). During the course of these experiments we did not observed other effects in the animals treated with the compound (*e.g.* weight loss, abnormal movements, dyspnoea). Thus, our results indicate that AT5 reduces the TNFα and IL-6 production *in vivo* after P3 injection.

3.5. AT5 does not inhibit the cellular response to HMGB1

The fast release and accumulation of pro-inflammatory cytokines (*e.g.* TNFα, IL-1) in the very early stages of sepsis make these cytokines a difficult target to be treated with neutralizing antibodies (Abraham, 1999). On the contrary, in septic mice and patients, HMGB1 which is released by damaged cells, has a delayed kinetic making it a potential target in sepsis (Musumeci et al., 2014). It is thought that the inflammatory responses caused by released HMGB1 might be mediated by TLR2 and TLR4 (Andersson and Tracey, 2011; Park et al., 2004; Yu et al., 2006) and it has been shown that HMGB1 is elevated in the serum of septic patients (Nogueira-Machado and de Oliveira Volpe, 2012). Therefore, either neutralization of HMGB1 with antibodies, or disruption of the interaction with TLRs has been suggested as a possible strategy in the therapy of sepsis (Yu et al., 2006). We questioned whether the antagonist which showed higher activity, AT5, could decrease the inflammatory response induced by HMGB1. To this end, we pre-incubated BMDMs with increasing concentrations of AT5 and then stimulated the cells with a high concentration of HMGB1. As shown in Fig. 5C, quantification of the TNFα released in the supernatant demonstrated that AT5 did not affect the inflammatory response produced by HMGB1 stimulation of BMDM macrophages (262.8 ± 48.35 pg/ml). This value is in line with the value obtained by Yu et al. (Yu et al., 2006) after stimulation of wild type macrophages with 10 μg/ml of HMGB1 (250–450 pg/ml). It is plausible that either the recognition epitope in TLR2 for HMGB1 is different than the one for AT5 or that the interaction surface between both proteins is extensive and can not be competed by a small-molecule like AT5.

4. Discussion

The search for antagonists of TLRs has flourished in the last years in an attempt to find novel immunomodulators to control the progress of inflammatory diseases like sepsis or rheumatoid arthritis (Elshabrawy et al., 2017; Gao et al., 2017). Several groups, including ours, have reported the discovery of antagonists and also agonists of TLR2 (Guan et al., 2010; Murgueitio et al., 2017, 2014; Zhou et al., 2010) and some other TLRs (Balak et al., 2017; Kokatla et al., 2014). In our previous study (Murgueitio et al., 2014), we identified 8 TLR2 antagonists (AT1-AT8) which decreased NF-kB activation in HEK293-TLR2 cells after di-acylated (P2) or tri-acylated (P3) lipopeptide challenge. They were also effective inhibitors in primary human cells as demonstrated by the reduction in the TNFα production by human monocytes. Now, in order to test the efficacy of the compounds *in vivo* we have further characterized their activity. In a luciferase assay in mouse macrophages we showed that AT1-AT8 decreased the NF-kB transcriptional activity after TLR2 stimulation, therefore, they are active in mouse cells additionally to the already reported activity in human cells. These results were confirmed by assessing the decrease in TNFα production by primary mouse macrophages. In these experiments AT4 –AT7 were the most potent inhibitors. In general we could not observe a marked preference for TLR2/TLR6 or TLR2/TLR1 inhibition. AT1-AT8 did not inhibit significantly the activity of other TLRs, namely TLR3 and TLR4 (Murgueitio et al., 2014) and neither did we observe inhibition of IL-1R or TLR5 signaling in the current study. Thus, we conclude that the antagonists are selective for TLR2 activity and primarily AT4- AT7 inhibit equally TLR2/TLR1 and TLR2/TLR6 unlike other reported antagonists which inhibit only the heterodimer TLR2/TLR1 (Chang et al., 2009; Cheng et al., 2012).

AT7 is a derivative of AT2 and although AT2 had a lower IC_{50} in human cells, AT7 seems to be more potent than AT2 in mouse cells. AT4 and AT5 are derivatives of compound E567 which was shown by Zhou et al. to have anti-viral activity mediated by TLR2 (Zhou et al., 2010).

However, we did not test AT4 or AT5 in Lymphocytic choriomeningitis virus infection, model used by the authors. Pathogenic gram positive bacteria (*e.g. Staphylococcus aureus, Streptococcus pneumoniae*) produce both kind of ligands, di- and tri-acylated lipopeptides (Nguyen and Gotz, 2016). Thus, we hypothesize that AT4-AT7 may be superior inhibitors of TLR2-mediated inflammation caused by gram positive bacteria than other small-molecule antagonists which inhibit only TLR2 when it hetero-dimerizes with TLR1.

Downstream of TLR2, particularly AT5 reduced the phosphorylation of the MAPKs p38 and JNK and the degradation of IkBα (Fig. 4A). Thus, it might be feasible that the compounds would be cell permeable and thus inhibit TLR2 activity intracellularly by targeting TLR2-downstream proteins. As mentioned, in previous studies we demonstrated that the compounds were selective for TLR2 as they did not inhibit TLR3 or TLR4 activity (Murgueitio et al., 2014). Yet, this experiment did not discard intracellular effects of AT1-AT8 since those two receptors activate different pathways than TLR2: LPS (TLR4 receptor ligand) can activate MyD88 signalling (similar to TLR2) but also a TRIF/TRAM dependent pathway; PolyI:C (TLR3 receptor ligand) functions through TRIF and TRAF3. In the current study two pathways that are intracellularly common to TLR2, (*i.e.* IL-1R, TLR5) were challenged with AT1-AT8 and the response obtained with IL-1β or flagellin was not inhibited (Fig. 3D**,** **E**).

We further studied the capacity of AT1-AT8 to reduce nitric oxide production, another important mediator of inflammation, and we compared this effect to purpurogallin inhibition. Purpurogallin was the primary compound used in the screening for TLR2 antagonists by Cheng et al. Purpurogallin performed similarly to AT4 and AT5. However, there was a disparity in the inhibitory values obtained by us and Cheng et al., most likely due to the use of different model cells.

The most active molecules AT5 and AT4 were subsequently challenged in a mouse model of P3-induced inflammation. After treatment of the mice with AT5 the TNFα and IL-6 production induced by P3 was decreased in comparison to the vehicle-treated group. In contrast AT4, although with good inhibitory properties *in vitro*, did not have a significant inhibitory effect at the tested concentration *in vivo.* In future studies, compounds AT6 and AT7 will be also tested.

The alarmin HMGB1 has been described to be involved in the development of bacterial sepsis, and it has been reported to signal through TLR4 in primary cells and through TLR2 in stable cell lines (Akin et al., 2011). Therefore, a small-molecule able to antagonise TLR2 over-stimulation by bacterial lipopeptides and simultaneously being able to partially inhibit the stimulatory activity of HMGB1 would be an ideal candidate for further development with therapeutic potential in sepsis. In BMDM stimulated with high concentrations of HMGB1 we obtained a relative low amount of TNFα (260 pg/ml) in comparison to classical lipopeptide ligands (*e.g.* P2, P3, FSL-1), however in the range of what was previously described for BMDM (250–450 pg/ml (Yu et al., 2006)). Nonetheless, we did not observe a reduction in the TNFα production by increasing the concentration of AT5. A plausible explanation for this result might be that HMGB1 and AT5 have a different binding epitope in TLR2 and therefore, AT5 can not compete with HMGB1 recognition by TLR2 as it does with P3.

In future studies, further screening rounds to discover compound derivatives of AT4-AT7 will be done in order to develop selective TLR2 inhibitors with higher potency.

Conflict of interest statement

The authors declare no conflict of interest according to the ICMJE recommendations.

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