Peptides ToAP3 and ToAP4 decrease release of inflammatory cytokines through TLR-4 blocking

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ABSTRACT

Antimicrobial peptides (AMPs) are small molecules with microbicidal and immunoregulatory activities. In this study we evaluated the anti-inflammatory and antimicrobial activities of peptides ToAP3 and ToAP4, AMPs from the venom of the Brazilian scorpion *Tityus obscurus*. To test the peptides' activity, murine bone marrow-derived macrophages (BMDMs) or dendritic cells (BMDCs) were stimulated with peptides plus LPS to analyze their ability to modulate cytokine release as well as phenotypic markers. For antimicrobial analysis, we evaluated the indirect activity against macrophage-internalized *Cryptococcus neoformans* and direct activity against *Mycobacterium massiliense*. Our data demonstrate that they were able to reduce TNF-α and IL-1β transcript levels and protein levels for BMDM and BMDC. Furthermore, the reduction of TNF-α secretion, before LPS- inflammatory stimuli, is associated with peptide interaction with TLR-4. ToAP4 increased MHC-II expression in BMDC, while ToAP3 decreased co-stimulatory molecules such as CD80 and CD86. Although these peptides were able to modulate the production of cytokines and molecules associated with antigen presentation, they did not increase the ability of clearance of *C. neoformans* by macrophages. In antimicrobial analysis, only ToAP3 showed potent action against bacteria. Altogether, these results demonstrate a promising target for the development of new immunomodulatory and anti-bacterial therapies.

1. Introduction

Antimicrobial peptides (AMPs) are a diverse class of small molecules that are widely distributed as a conserved and primitive defense of innate immunity [1–4]. Most are small cationic and amphipathic molecules, which are able to interact with cell membranes, cellular receptors and ionic channels [5,6]. Among the abilities ascribed to AMPs, one is microbicidal activity against a variety of pathogens, and another is limiting sepsis by interacting with endotoxins and cytotoxic effects against some tumors [7]. Another possibility for AMPs, as defensins, is their capacity to regulate the immune system, thereby exerting indirect

influence on pathogen clearance or even acting in inflammatory contexts [3,8].

AMPs can be found in venom glands of many animals such as scorpions [9,10]. The scorpion venom peptides can be classified in two groups: disulfide bridge peptides (DBPs) and non-disulfide bridge peptides (NDBPs). The NDBPs are a minor part of scorpion venom, but interest in their activity as microbicidal molecules and mammal cell metabolism regulators has been increasing in recent years, besides their mechanisms of action,. Nevertheless, little is known about their mechanism of action [11,12].

Many peptides from invertebrates have recently been studied as a

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therapeutic alternative to conventional drugs. They are usually less toxic to host cells and effective against the multiresistant microorganisms which are currently a major issue in public health [13]. Some conventional drugs have shown decreased efficiency in controlling infections caused by many microorganisms, including *Mycobacterium sp.* and *Cryptococcus neoformans.*

Our group recently carried out *in silico* prediction of 11 NDBPs of *Tityus obscurus* cDNA library, and seven of them showed microbicidal activity *in vitro* against *Candida* genus and *C. neoformans,* with minimum inhibitory concentration values ranging from 3.12 to 200 μM [14]. Three peptides, named ToAP1, ToAP3 and ToAP4, showed similarity to previously described AMPs belonging to NDBP subfamily 4. ToAP1 showed high hemolytic activity in all concentrations tested, so its structure should be modified to carry out more studies. ToAP3 showed anti-*C. albicans* and anti-*C. neoformans* activity, while peptide ToAP4 did not show any microbicidal activity. ToAP4 showed similarity with Stigmurin, a peptide from *Tityus stigmurus,* which showed antibacterial and antiproliferative activity on tumor cells as well as a reduction in leukocyte migration and TNF-α levels [15,16]. Several peptides have been described as modulators of the immune system, besides showing antimicrobial activities, e.g. LL37 and clavanin, which promote the reduction of proinflammatory cytokine secretion [17,18]. Therefore, we aimed to evaluate if ToAP3 and ToAP4 presented immunomodulatory functions in the activation of macrophage and dendritic cells. Furthermore, we also analyzed the indirect or direct activity of these peptides against *C. neoformans* and *Mycobacterium abscessus* subsp. *massiliense,* respectively.

2. Material and methods

2.1. Microorganisms' growth conditions

C. neoformans (var. grubii serotype A, H99) was inoculated in Sabouraud broth medium (Acumidia®) and kept at 37 °C for 24 h at 120 rpm. After this time, yeasts were centrifuged at 3000 x g for 5 min and washed in phosphate-buffered saline (PBS) three times. Cells were counted using a Neubauer chamber and concentration adjusted to 10^6 cells/ml. *M. abscessus* subsp. *massiliense* was cultivated in Mueller Hinton (MH) medium for three days using the same conditions as described for *C. neoformans*. After adjusting the OD to 0.5 at 600 nm, the bacterial suspension was plated in MH medium (Himedia) for 5 days to evaluate the colony forming units (CFU), which corresponded to 1.5×10^8 CFU/ml.

2.2. Animals

C57BL/6 (*Mus musculus*) wild type and TLR4 KO mice were used in all experiments, aged 8–12 weeks old. The animals were maintained with food and water *ad libitum* in the animal facility of the Institute of Biological Sciences (IB), University of Brasilia. All treatments and experimental procedures were performed after approval by the Ethics Committee on Animal Use (CEUA) of the University of Brasília - UNB (UnBDoC no. 66704/2016) and according to the guidelines presented by the National Council for the Control of Animal Experimentation (CONCEA).

2.3. Effects of AMPs on LPS-stimulated cells

FastBio Ltda (Ribeirão Preto, SP, Brazil) synthesized the peptides ToAP3 and ToAP4 using the Fmoc (N-9-fluorophenylmethoxy-carbonyl) solid phase strategy as previously described [14]. Murine macrophage cell line J774 cells were cultivated in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS - Gibco) at 37 °C and 5% CO_2. Then, 1×10^5 cells/well were plated in two 96-well plate cultures, one for each peptide. Cells were treated for 24 h simultaneously with *Escherichia coli* lipopolysaccharides (LPS - 500 ng/mL cod.

L3012, (*Escherichia coli* O111:B4), Sigma Aldrich) and different concentrations of ToAP3 or ToAP4 peptides (0.78–100 μM) to verify TNF-α release. Cells treated with LPS without peptide treatment were considered the positive control. The levels of TNF-α were analyzed by ELISA (Enzyme-Linked Immunosorbent Assay) (eBioscience; BD Bioscience) following the manufacturer's recommendations.

2.4. Cell culture conditions to analyze the inflammatory activity of ToAP3 and ToAP4 in innate immune cells in vitro

Primary cultures of macrophages and dendritic cells derived from bone marrow (BMDMs and BMDCs, respectively) of C57BL/6 mice were used throughout the study. The generation of the BMDMs and BMDCs was performed from the differentiation of murine hematopoietic stem cells [19]. BMDMs or BMDCs were plated at 1×10^6 cell/well in a 24-well plate in a final volume of 0.5 mL of RPMI-1640 (Sigma-Aldrich) and 10% FBS (Gibco). Then, cells were treated for 24 h with different stimuli and/or the peptide (25 μM) simultaneously, previously or afterwards (indicated in the caption to the figure), to verify cytokine production. As the positive control, LPS (500 ng/mL – Sigma-Aldrich) was used for TNF-α and IL-10 release and nigericin (20 - μM invivogen) was added (40 min prior to supernatant collection) for IL-1β production. Levels of TNF-α, IL-10 and IL-1β were analyzed by ELISA (Enzyme-Linked Immunosorbent Assay) following the manufacturer's recommendations - (TNF-α and IL-1β from eBioscience; IL-10 from BD Bioscience).

The production of nitric oxide (NO) was estimated based on the quantification of nitrite (NO_2^-) present in the culture supernatants from BMDMs and BMDCs after interaction with ToAP3 or ToAP4. To assess NO production, we used a co-stimulation of LPS (20 ng/mL) and IFN-γ (100U - ImmunoTools). Briefly, samples were incubated in a 1:1 vol/vol mix with Griess reagent (1% sulfanilamide diluted in orthophosphoric acid and 0.1% naphthylethylenediamine hydrochloride, Sigma-Aldrich), and the resulting absorbance was measured at 540 nm in a "Multiskan ™ FC Microplate Photometer" (Thermo Scientific) spectrophotometer. The molar concentration of nitrite was determined in comparison to a standard curve (concentration range of 100-1.56 μM) obtained from the serial dilution of $NaNO_2$ (Vetec). The results are expressed as [nitrite] (μmol) [20].

Peritoneal macrophages from C57Bl/6 wild type (WT) and Toll-like receptor 4 (TLR4) knockout (KO) mice were aseptically harvested by washing the peritoneal cavity with cold RPMI medium at 72 h after thyoglicolate injection (3%) (Laborclin). Cells were plated at 1×10^6 cell/well in a 24-well plate in a final volume of 0.5 mL of RPMI-1640 and 10% FBS. Then cells were incubated at 37 °C and 5% CO_2 for 24 h. Afterwards, macrophages were treated for 24 h with different stimuli and/or the peptide (25 μM) simultaneously, to verify TNF-α production. As the positive control, LPS (500 ng/mL), LPS ultrapure (500 ng/mL (tlrl-pb5lps, (E. coli 055:B5), InvivoGen)) and Zymosan (20 μg/mL invivoGen) were used.

2.5. Quantitative real-time PCR (RT-qPCR)

The evaluation of the expression of some important inflammatory genes was carried out through RT-qPCR. BMDCs were plated at 1×10^6/well on a 24-well plate with RPMI-1640 (Sigma-Aldrich) and 10% FBS (Gibco), and were then stimulated with LPS (500 ng/mL – Sigma-Aldrich) and ToAP3 or ToAP4 (25 μM) for 6 h at 37 °C and 5% CO_2. Then, cell total RNA was extracted using the TRIzol reagent (Invitrogen), and reverse transcriptase reactions were performed using the high capacity RNA-to-cDNA kit (Applied Biosystems), according to the manufacturer's instructions. RT-qPCR was performed using SyBr Green Master Mix and StepOne real-time PCR system (Applied Biosystems). The genes evaluated were Tnfa, Il1b, Nosi, Il-10, Arginase-1 and Nfkb. Results were calculated by the $2^{-\Delta\Delta CT}$ method [21] normalized to the expression of the housekeeping gene Rps9 and expressed

Table 1
Sequence of oligonucleotides.

GENES	FORWARD	REVERSE
Rps9	CGCCAGAAGCTGGGTTTGT	CGAGACGCGACTTCTCGAA
Arginase 1	GTTCCCAGATGTACCAGGATTC	CGATGTCTTTGGCAGATATGC
iNOS	CGAAACGCTTCACTTCCAA	TGAGCCTATATTGCTGTGGCT
Nfkb	AGCCAGCTTCCGTGTTTGTT	AGGGTTTCGGTTCACTAGTTTCC
Tnfa	GTACCTTGTCTACTCCCAGGTTCTCT	GTGGGTGAGGAGCACGTAGTC
Il1b	GTGTGTGACGTTCCCATTAGACA	CAGCACGAGGCTTTTTTGTTG
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG

as "Fold change". A FC $\geq$ 5.0 was used as the cut-off of mRNA differential expression.

The total RNA used for miRNA analysis was extracted using the mirVana™ miR Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's specifications. The miRNA-155 and miRNA-125b levels were determined using TaqMan® MicroRNA Assay probes (Thermo Fisher Scientific). For cDNA synthesis, total RNA was reverse-transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The qPCR reactions were performed using TaqMan® Universal PCR Master Mix (Applied Biosystems), and carried out in the 7500 Fast Real-Time PCR System (Applied Biosystems). The housekeeping control used was U6 snRNA TaqMan® MicroRNA Control Assay (Thermo Fisher Scientific,). The fold-change in stimulated samples was compared to the non-stimulated control group, using the $2^{-\Delta\Delta CT}$ method [21]. A FC $\geq$ 2.0 was used as the cut-off of miRNA differential expression. The primers used were previously validated [21], and the sequences of oligonucleotides are described in Table 1.

2.6. Flow cytometry analysis

Immunophenotypic characterization of ToAP3- or ToAP4-stimulated BMDCs in the presence or absence of LPS was performed by flow cytometry. First, 2×10^6 cells were plated in a Petri dish (49 x 12 mm) with RPMI-1640 medium (Sigma-Aldrich) and 10% FBS (Gibco). Then, cells were stimulated with LPS (Sigma-Aldrich) at 1 μg/mL in the presence of ToAP3 or ToAP4 (25μM). After 24 h of interaction, the cells were harvested, re-suspended in labeling buffer (PBS supplemented with 2% FBS) and incubated with anti-MHC-II (FITC fluorophore conjugate; eBioscience) anti-CD80 (FITC; eBioscience) or anti-CD86 (FITC; eBioscience). Simultaneously, the suspension of BMDCs was added to anti-CD11c antibodies (conjugated to the fluorophore APC; eBioscience). Isotypic FITC and APC-conjugated controls were used. All antibodies were used following the manufacturer's recommendations. Cells were acquired in the FACSVerse flow cytometer (BD Biosciences), and the data analyzed in the FlowJo software (version X).

2.7. Indirect microbicidal activity

Peritoneal macrophages were plated in a 48-well plate at concentration of 5×10^5 cells/ml in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Gibco) and kept in a humid chamber at 37 °C and 5% CO_2. *C. neoformans* inoculum (MOI 1:2) opsonized with 18B7 antibody (5 μg/mL) (kindly granted by Dr. Arturo Casadevall, AECOM/USA) was added to the culture and, after 1 h at 37 °C and 5% CO_2 in humid chamber, the non-internalized yeasts were removed with PBS. Then, 200 μl of RPMI 1640 supplemented with 10% FBS, fluconazol (20 μg/ml – Sigma-Aldrich) and 12.5 μM, 25 μM or 50 μM of peptides was added to each well and kept in a humid chamber at 37 °C and 5% CO_2 for an additional 20 h. The plate was washed three times with PBS to remove the remaining non-internalized fungi, and then macrophages were lysed with 0.05% of SDS in H_2O; released yeasts were plated in Sabouraud dextrose agar (SDA - Acumidia) and kept at 30 °C. Fungal viability was assessed by counting the colony forming units (CFU), counted after 48 h of incubation.

2.8. Minimum bacterial concentration (MBC)

The culture in *M. abscessus* subsp. *massiliense* broth for the susceptibility assessment was adjusted to a turbidity standard equivalent to the McFarland 0.5 scale (1.5×10^8 CFU/ml). This suspension was used to make serial dilutions. Bacterial suspensions (50 μl, 1.5×10^3 CFU/ml) were distributed on the plate, which had previously had the solution containing the peptides added. ToAP3 and ToAP4 synthesized peptides were used at the concentrations of 100 μM, 12.5 μM and 1.56 μM, incubated with 1.5×10^3 CFU/ml of *M. abscessus* subsp. *massiliense*, under agitation (120 rpm) at 37 °C for 24 h. After this time, the three-well bacterial suspensions were collected and seeded in MH agar medium (Himedia). After incubation of these plates for 5 days at 37 °C, the colony forming units were counted. The count of the colony-forming units of the control cultures that did not receive treatment was considered as 100% growth. The percent inhibition of bacterial growth was estimated by comparing the growth control (100%) in relation to the count of the colony forming units obtained from each of the treatments at the three dilutions. Growth controls incubated on the same plate contained only the inoculum and PBS, while controls for the mycobacteria treatment were incubated with different concentrations of Clarithromycin (8-0.06 μg/ml – Sigma-Aldrich).

2.9. Scanning Electron Microscopy (SEM)

Colonies of *M. abscessus* subsp. *massiliense* grown on MH agar medium for 5 days were cut and then exposed to the peptide at the concentration corresponding to 50% IC for 24 h. Colonies were then fixed with Karnovsky's solution (2% paraformaldehyde – Sigma-Aldrich, 2% glutaraldehyde – Sigma-Aldrich in 0.07 M cacodylate – Sigma-Aldrich buffer pH 7.2) for 30 min at 4 °C. They were then dehydrated with increasing concentrations of ethyl alcohol (Vetec) (30%, 50%, 70%, 90% and 100%) for 10 min, after which a solution of acetone (J.T. Backer) and hexamethyldisilazane (HMDS – Sigma-Aldrich) was added volume by volume for 5 min and HMDS plus 5 min. The samples were left in a dry chamber containing silica for 24 h until they were covered with a thin layer of gold by the Denton Vacuum Desk V. The images were made using a Jeol microscope, JSM-6610, equipped with EDS (Thermo scientific NSS Spectral Imaging). Metallization and analyses were performed at the High Resolution Microscopy Multiuser Laboratory of the Institute of Physics of the Federal University of Goiás.

2.10. Statistical analyses

Assays were analyzed by one-way ANOVA followed by the Tukey post-test, and results were expressed as the mean ± SEM of representative data of three experiments conducted in triplicates. Statistical analyses were performed using GraphPad Prism version 6.0. Data were considered significant when p < 0.05.
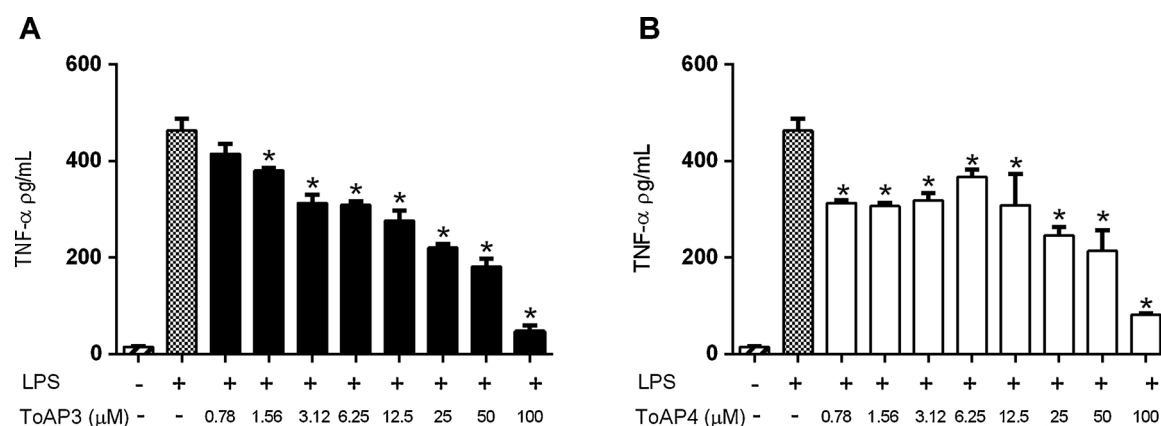
Fig. 1. Effects of ToAP3 and ToAP4 on TNF-α levels in LPS-stimulated J774 cell supernatant. Levels of TNF-α secretion by J774 cells were measured at 24 h post interaction with LPS (500 ng/mL) and/or different concentrations of ToAP3 (a) or ToAP4 (b) peptides (0.78–100 μM). Data are expressed as mean ± SEM from three independent experiments performed in triplicate. *p < 0.05 compared to LPS group, one-way ANOVA and Tukey's test.

3. Results

3.1. ToAP3 and ToAP4 show activity in TNF-α production and are not cytotoxic to cells

To corroborate the sequence of both peptides used in this work, we conducted mass spectrum analysis of the synthetized peptides (Supplementary Fig. 1). The interpretation of the spectra allowed us to identify ions corresponding to the series of type b and y related to the following amino-acid sequences: FIGMIPGLIGGLISAIK-NH2 (ToAP3) (Supplementary Fig. 1B) and FFSLIPSLIGGLVSAIK-NH2 (ToAP4) (Supplementary Fig. 1D). Low-mass ions (ions) corroborate the presence of most amino acids present in the peptide structure (Supplementary Fig. 1 A, C). After confirming peptides' primary structure, their biological activities were evaluated.

Besides the antimicrobial activities previously described for these AMPs [14], we assessed their immunomodulatory effects using different peptide concentrations. The peptides alone did not increase TNF-α secretion by J774 cells (data not shown); however, both peptides were able to reduce TNF-α secretion after LPS stimulation. For ToAP3, a dose dependent effect was noted (Fig. 1A), and for ToAP4 a decrease in all tested concentrations was observed, but the dose dependent effect was seen with doses of 25 μM and higher (Fig. 1B).

The peptide concentrations were determined after their capacity to cause toxic effects in human erythrocyte and monocyte cells. It was verified that both AMPs have low activity in inducing erythrocyte lysis (Supplementary Fig. 2A, B) or monocyte cytotoxicity (Supplementary Fig. 2C, D). After 24 h of stimulation, peptides showed high cytotoxicity only in elevated concentrations of ToAP3 (EC50 = 52.35 μM) and ToAP4 (EC50 = 55.71 μM) (Supplementary Fig. 2C, D). Therefore, we considered 25 μM to be suitable for subsequent assays. Another important function of AMPs in the host innate immune response is their activity in the modulation of immune cell migration [18]. As a result, considering the low cytotoxicity (Supplementary Fig. 2) and the modulation of TNF-α in LPS-stimulated cell cultures (Fig. 1), assays to evaluate the effect of ToAP3 and ToAP4 in the cell migration to mouse peritoneum were performed. Therefore, peptides ToAP3 and ToAP4 were injected in mouse peritoneal cavity, and the cell population was measured at 4, 24 and 72 h post inoculation. There were no statistically significant differences in the migration of neutrophils, monocytes and lymphocytes compared with non-treated mice (data not shown).

3.2. ToAP3 and ToAP4 modulate the release of inflammatory mediators

To get a more detailed view of the immunomodulatory activities of these peptides, the production of other immunological mediators by LPS-stimulated BMDM and BMD was analyzed. The concomitant treatment of BMDMs and BMDCs with ToAP3 or ToAP4 with LPS or LPS plus Nigericin resulted in reduced levels of pro-inflammatory cytokines TNF-α (Fig. 2A, B) and IL-1β (Fig. 2C, D) in cell supernatant. On the other hand, ToAP4 increased the levels of anti-inflammatory cytokine IL-10, especially for BMDM (Fig. 2E, F). No increase in cytokine levels was observed when both cells were treated with ToAP3 or ToAP4 peptides alone, when compared with non-treated cells. Furthermore, no changes were observed in the production of NO when the cells were co-stimulated or not with LPS and IFN-γ and treated with the peptides (Fig. 2G, H).

To better explore the influence of the peptides on the cytokine release profile, we also investigated the transcript level of some cytokines. Considering the similarity of these peptides' effects on the secretion of immune mediators in both BMDM and BMDC, as described above, a RT-qPCR was performed to evaluate the level of some of these transcripts only in BMDCs. The cell stimulation with peptide alone did not increase the transcript levels for Tnfa, Nosi, Arginase-1 and Nfkb (Fig. 3A, C–E). As shown in panels 3B and 3E (Fig. 3), only ToAP3 increased the transcript levels of Il10 and Il1b; however, these transcripts increased were not correlated with protein secretion after 24 h of peptide stimulation, as presented in Fig. 2. When BMDCs were treated with ToAP3 or ToAP4 combined with LPS stimulation, there were reductions in the transcript level of TNF-α, IL-1β and Nfkb (Fig. 3A, B, F), suggesting that the peptides modulate cytokine secretion at the transcriptional level. With respect to nitric oxide synthase (Nosi), only ToAP4 induced a small reduction in its transcript levels (Fig. 3C). However, no change in the Arginase and Il-10 transcript abundance was observed in the presence of both peptides plus LPS (Fig. 3D, E). As described above, the treatment of LPS-stimulated cells with both AMPs resulted in a decrease of TNF-α and IL-1β secretion and also in the levels of TNF-α, IL-1β and NFkB transcripts. However, it is important to note that although the reduction of both proteins and their respective transcripts occurs, an increase in the levels of both proteins and mRNAs is still observed in comparison to control cells (untreated).

MicroRNAs (miRNAs or miR), which are small regulatory RNA molecules of about 22 nucleotides, act in a plethora of biological processes, including regulation of host immune response at both innate and adaptive levels. MicroRNAs generally promote gene expression silencing through mRNA degradation or translational repression [22]. They have emerged as important regulators of immune response, modulating the intensity of inflammatory responses, while they themselves are modulated in host immune cells submitted to different stimuli. For example, it was described that LPS stimulation of macrophages induces the up-regulation of miR-155 and down-regulation of miR-125b [23,24]. Due to their role in the modulation of immune response
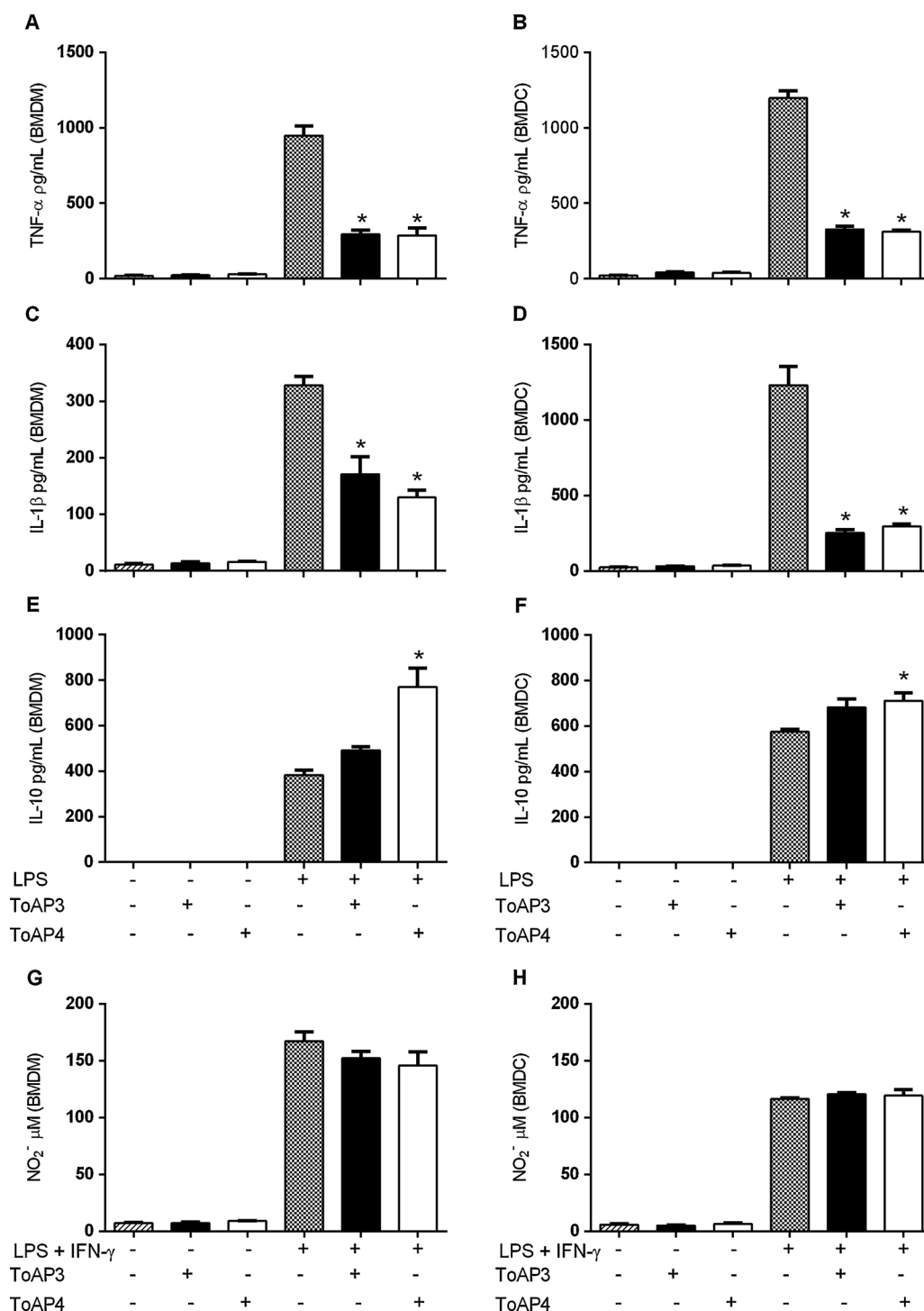
Fig. 2. Effect of ToAP3 and ToAP4 on the release of LPS-induced inflammatory mediators in macrophages (BMDMs) and dendritic cells (BMDCs) derived from murine bone marrow. Levels of TNF-α, IL-1β and IL-10 secretion of BMDM (a, c and e, respectively), and BMDC (b, d and f, respectively) were measured at 24 h post interaction with LPS (500 ng/mL) and/or ToAP3 or ToAP4 (25 μM). For IL-1β dosage, a co-stimulus of LPS (500 ng/mL) and nigericin (20 μM) was used. For the quantification of nitric oxide production, NO_2 was measured at 24 h post-interaction with LPS (500 ng/mL) and IFN-γ (20 ng/mL) by Griess reagent in BMDMs and BMDCs (A and B, respectively). Data are expressed as mean ± SEM from three independent experiments performed in triplicate. $^{*}p < 0.05$ compared to LPS or LPS and nigericin or LPS and IFN-γ group, one-way ANOVA and Tukey's test.

intensity, here we showed that the BMDCs treated with ToAP3 or ToAP4 plus LPS presented a reduced miR-155 transcript level (Fig. 3G), and no changes in the transcript levels of miR-125b (Fig. 3H). Collectively, these data suggest an anti-inflammatory role of ToAP3 and ToAP4, which probably act in preventing the exacerbation of the inflammatory response.

To better understand if the TNF-α modulation by these AMPs occurs before or after LPS cell activation, we treated BMDMs or BMDCs with ToAP3 or ToAP4 for 6 h before or after the addition of LPS. The previous treatment with ToAP3 or ToAP4 before LPS stimulation reduced TNF-α secretion more efficiently (Fig. 4A, B). However, after LPS priming, the ability of these peptides to reduce TNF-α levels was only

observed in BMDCs (Fig. 4B). The explanation for these results may be the interaction between peptides and Toll-like receptor 4 (TLR4). To investigate this question, we tested the production of TNF-α macrophages from TLR4KO mice previously treated with peptides, and we observed that they did not produce TNF-α after stimulation with ultrapure LPS; however, they released low levels of this cytokine when stimulated with LPS, which can interact with both TLR4 and TLR2 (Fig. 4D), when compared with wild type mice under the same conditions (Fig. 4C). These results corroborate the hypothesis that these peptides can interact with the LPS receptor, inducing low levels of transcript and small protein release of TNF-α.
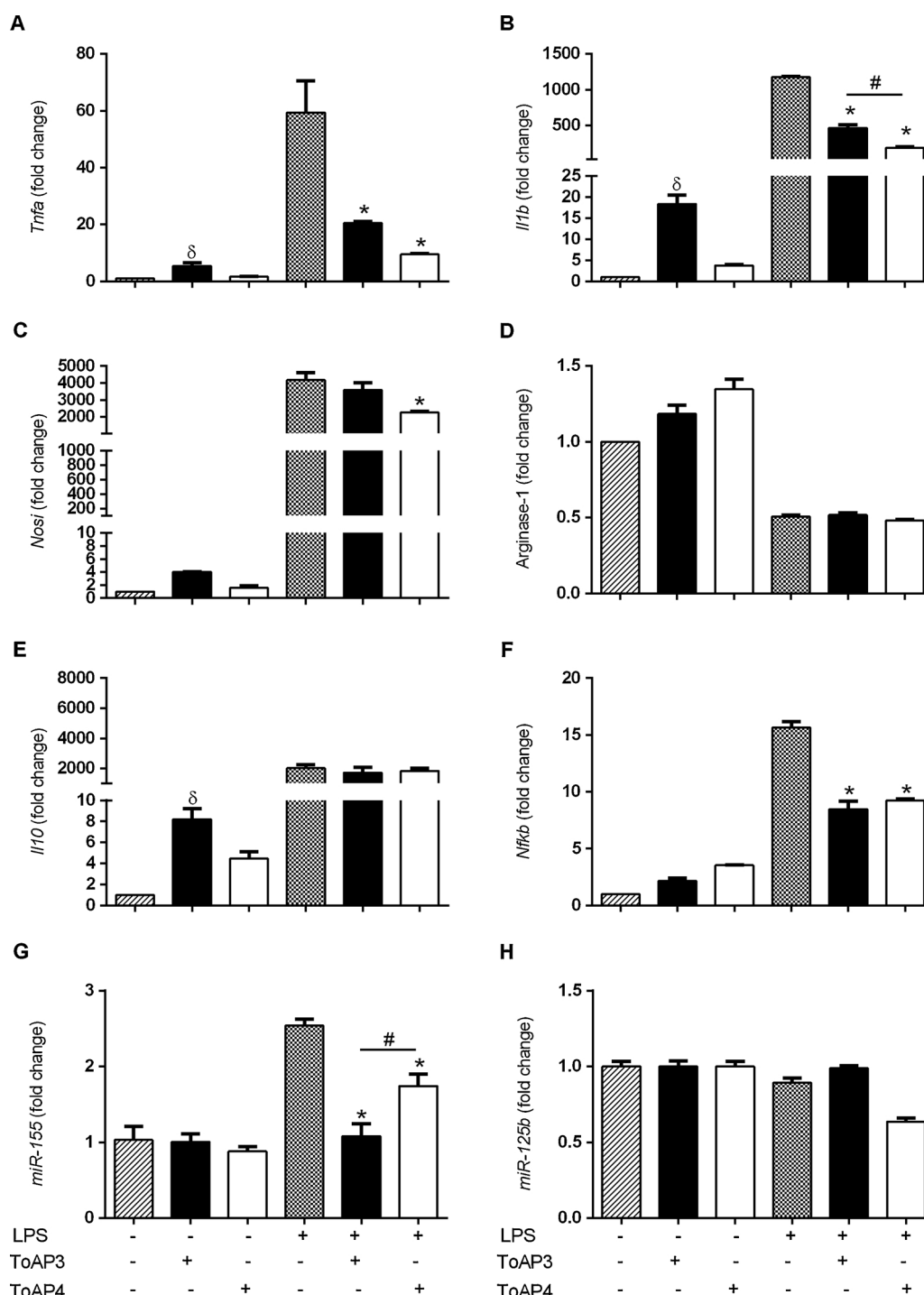
Fig. 3. **Effect of ToAP3 and ToAP4 peptides on LPS-induced transcript level in dendritic cells (BMDCs) derived from murine bone marrow.** Levels of *Tnfa* (a), *Il1b* (b), *Nosi* (c) *Arginase-1* (d), *Il10* (e), *Nfkb* (f), miR-155 (g) and miR-125b (h) transcript in BMDCs were measured at 6 h post interaction with LPS (500 ng/mL) and ToAP3 or ToAP4 (25 μM). Data are expressed as mean ± SEM from three independent experiments performed in triplicate. $^{\delta}p < 0.05$ compared to untreated group, $^{*}p < 0.05$ compared to LPS group, $^{\#}p < 0.05$ compared to AMPs group one-way ANOVA and Tukey's test.

3.3. ToAP3 and ToAP4 can differentially modulate BMDC maturation and differentiation after LPS stimulation

The main aspect of BMDC maturation is the expression of membrane proteins associated with antigen presentation, providing the complete activation signaling for T lymphocytes. The first signal consists of the antigen presentation of the complex peptide-MHC, the second signal is the expression of co-stimulatory molecules such as CD80 and CD86, and the third signal is the cytokine secreted by DCs that will modulate the T cell response. Here, we investigated if ToAP3 and ToAP4 could intervene in the BMDC maturation process and consequently in antigen presentation. For this assay, BMDCs were stimulated with LPS concomitantly with ToAP3 or ToAP4 and, after 24 h of interaction, we analyzed the expression of dendritic cell markers for maturation (MHC-II, CD80) and differentiation (CD86). The ToAP3 or ToAP4 peptides alone did not induce an increase in MHC-II and co-stimulatory molecules' expression. However, ToAP4 enhanced the increase of MHC-II expression induced by LPS (Fig. 5A). In contrast, the expression of the
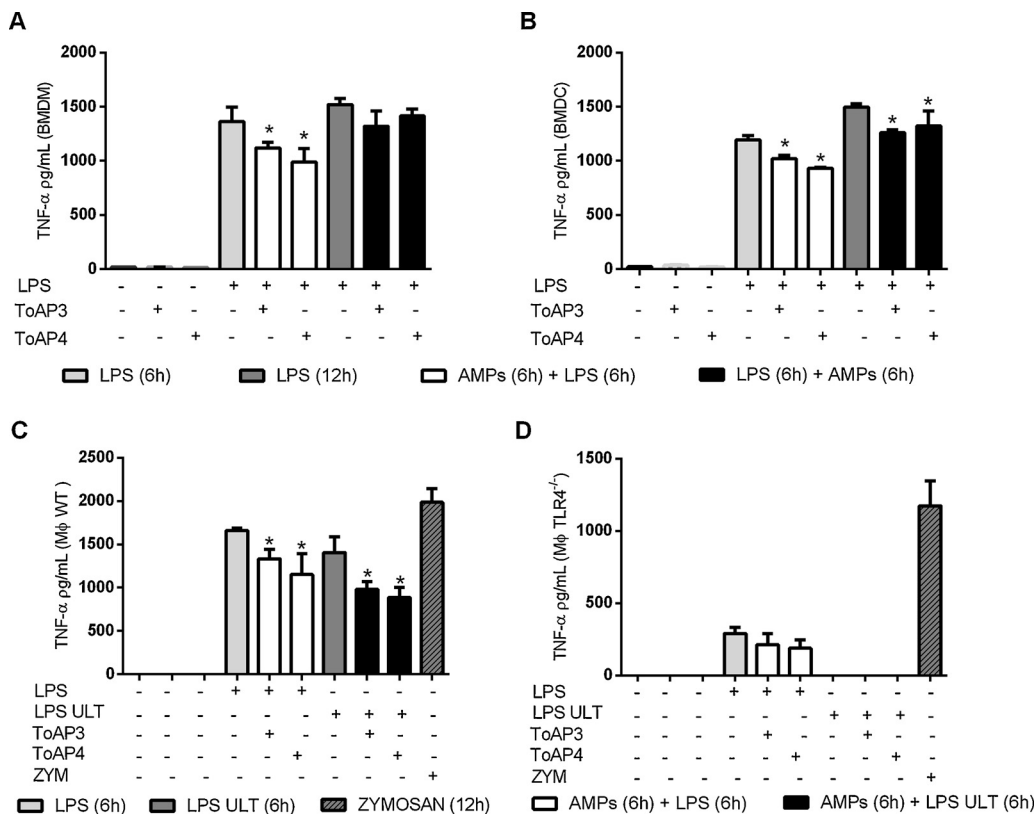
Fig. 4. Effect of ToAP3 and ToAP4 peptides addition at different times on LPS-induced TNF-α production. Levels of TNF-α secretion in BMDMs (a) and BMDCs (b) in the presence or absence of peptides ToAP3 or ToAP4 peptides (25 μM) before or after 6 h of LPS interaction (500 ng/mL). Levels of TNF-α secretion in peritoneal macrophages obtained from WT (c) or TLR4$^{-/-}$ (d) mice in the presence or absence of ToAP3 or ToAP4 peptides (25 μM) 6 h previously addition of LPS or an ultrapure LPS TLR4-specific (LPS ULT) (both at 500 ng/mL). In addition, Zymosan (20 μg/mL) was used as a TLR4-independent stimulus. Data were also obtained from cells cultured in the presence of ToAP3 or ToAP4, without LPS stimulation. Data are expressed as mean ± SEM from three independent experiments performed in triplicate. $^*p < 0.05$ compared to LPS or LPS ULT groups, one-way ANOVA and Tukey's test.

co-stimulatory molecules CD80 and CD86, which are induced in response to LPS stimuli, showed a small decrease when BMDCs are co-stimulated with ToAP3 (Fig. 5B, C).

3.4. ToAP3 and ToAP4 indirect microbicidal activity

Our previous results showed that ToAP3 and ToAP4 were not able to induce direct microbicidal activity in C. albicans or C. neoformans in vitro [14]. However, considering their ability to modulate the inflammatory cytokines, we speculated that these peptides could increase the clearance by macrophage. Therefore, peritoneal macrophages were harvested from C57BL/6 mice and stimulated for 4 h with the peptides. After this time, they were challenged with C. neoformans, and after 20 h the viability of the phagocyted yeast cells was assessed. We did not notice any significant decrease in the viability of the fungus in this assay (Fig. 6). These results corroborate the low levels of nitric oxide production induced by ToAP3 and ToAP4.

3.5. ToAP3 and ToAP4 activity against Mycobacterium abscessus subsp. Massiliense

Finally, we wondered whether ToAP3 and ToAP4 could exert activity against a different class of microorganism, such as *Mycobacterium sp.* Therefore, we incubated *M. abscessus subsp. massiliense* in presence of the peptides for subsequent CFU analysis. Although ToAP4 showed no interference with *M. abscessus* subsp. *massiliense* growth (data not shown), ToAP3 was able to kill *M. abscessus* subsp. *massiliense* in a dose-dependent manner (Fig. 7A). Moreover, the mycobactericidal effect of ToAP3 altered the shape of the cell surface, as evidenced by SEM analysis (Fig. 7B–D).

4. Discussion and conclusion

AMPs are a diverse group of molecules, with huge differences in their structure, and some of them show positive-charged amphipathic small sequences. Considering their biological properties, some of them show ability to kill or inhibit microorganism growth and/or modulate host immunity [7,25–27]. A considerable number of studies regarding AMPs have confirmed their broad spectrum of action. A number of these studies focused on identifying peptides with potential microbicidal activity, elucidating their probable mechanisms of action and evaluating their cytotoxic effects on eukaryotic cells and erythrocytes [28]. Recently, the ability of AMPs to act as modulators of host immunity has been extensively studied [2,29]. Our group has been studying the AMP activities shown by scorpion venom glands.

Venoms are a mixture of many peptides, among other diverse molecules with biological activities. As described before, scorpion venom peptides can be separated into two groups, DBPs and NDBPs. The focus on NDBPs is very recent, and they have been identified and functionally characterized only during the last decade. This interest in NDBPs is due to their diverse biological activities, which include antimicrobial, anticancer, hemolytic, anti-inflammatory and immune-modulatory action [12,30]. *T. obscurus* is an endemic scorpion species from Northern Brazil, which has 5% of its crude venom composed of NDBPs. Among all the NDBPs detected, only a few peptides have been structurally and functionally characterized [31].

In this study, we report new insights into the functions of ToAP3 and ToAP4, including their anti-inflammatory activity, which was demonstrated by decreasing the released transcript levels and the protein release of proinflammatory cytokines (TNF-α and IL-1β). These lower transcript levels can be an outcome of decreased levels of NFκb transcript, which directly influence the transcript levels of several cytokines [32] (Liu et al., 2017). Another possible explanation is that the levels of mRNA can be modulated through post-transcriptional mechanisms, such as mRNA stability. This stability can be regulated by proteasome, exosome, microRNAs and RNA processing-body (P-body) [33]. Among the microRNAs, some of them act downstream of TLR4 signaling, such as miR-146, miR-155, miR-125b and miR-181 [34,35], modulating the immune response intensity, as mentioned above. Usually, miRNAs act in the cytoplasmic control of gene expression, generally by repressing
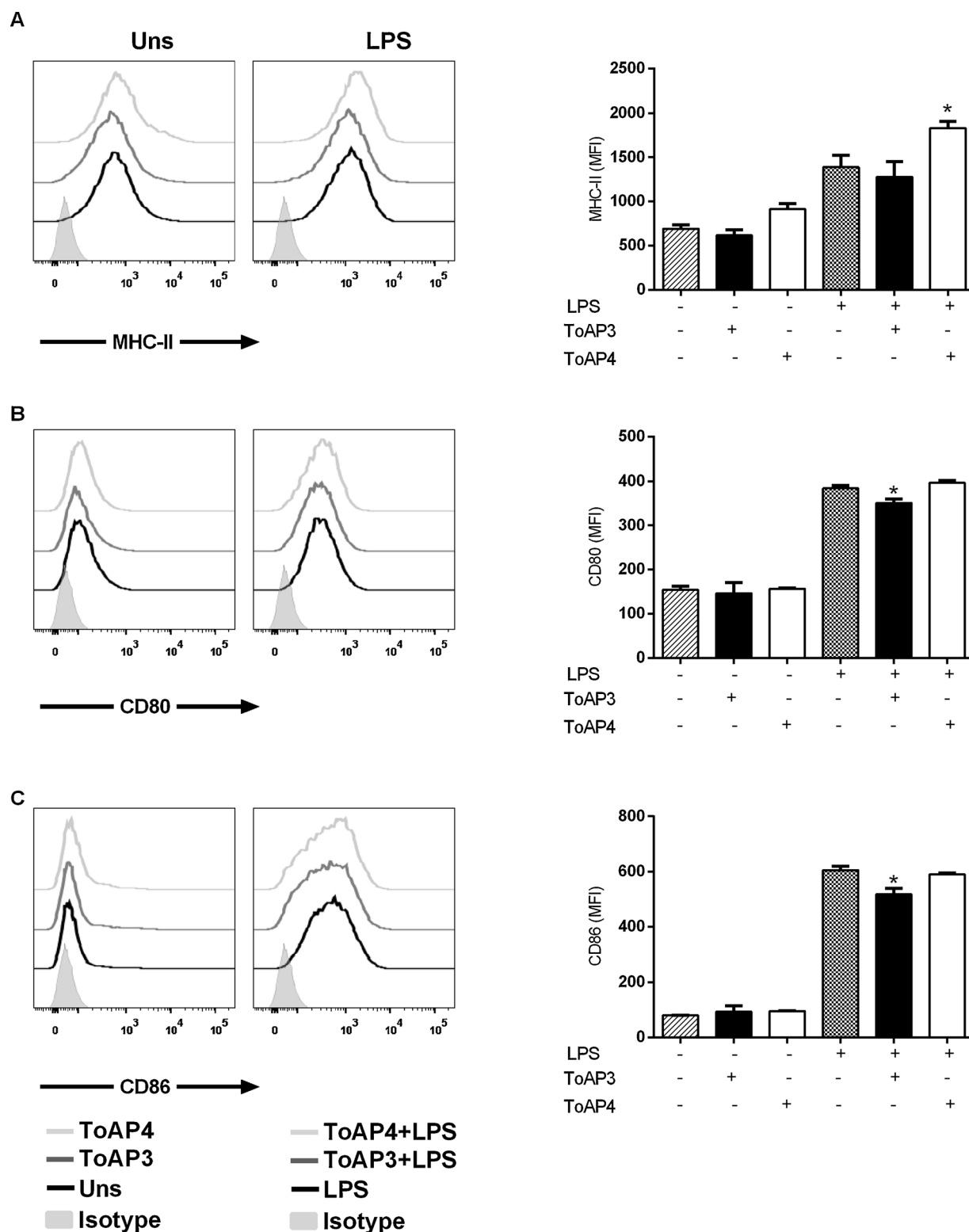
Fig. 5. Phenotyping for activation markers of bone marrow-derived dendritic cells (BMDCs) stimulated with ToAP3 or ToAP4 peptides and/or LPS. BMDCs were stimulated for 24 h with ToAP3 or ToAP4 (25 μM) and/or LPS (1 μg/ml) and subsequently labeled with antibodies to evaluate MHC II, CD80 and CD86 markers. The expression of MHC II (a), CD80 (b) and CD86 (c) is represented as a histogram (panels) and as median fluorescence intensity (MFI) (bar graph). Data are expressed as mean ± SEM of one experiment representative of three independent experiments performed in triplicate. *$p < 0.05$ compared to LPS group (one-way ANOVA followed by Tukey's test).

the translation or promoting mRNA degradation as, for example, mRNAs encoding inflammatory mediators (e.g. TNF-α). The miR-155 expression increases in inflammatory situations and decreases in anti-inflammatory situations, respectively. This lower expression is an important aspect of the negative feedback in the regulation of immune response [36]. The levels of miR-155 are modulated by several TLR ligands within myeloid differentiation factor 88 (MyD88)-, TRIF- and JNK -dependent pathways. Macrophages stimulated with LPS induced
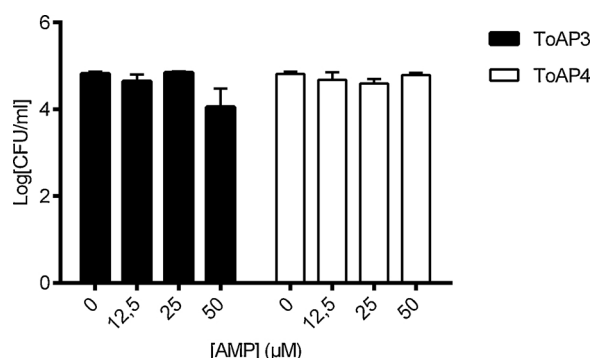
Fig. 6. Peptides do not increase the killing ability of macrophages. Peritoneal macrophages harvested from the C57BL/6 mice stimulated with thioglycolate were exposed to 25 μM of ToAP3 or ToAP4 for 24 h. After this, they were challenged with *C. neoformans* to verify their ability to kill this fungus. The results are representative of three independent experiments in triplicate.

hornet venom, is able to bind with LPS and lipid A with high affinity, neutralizing this endotoxin and reducing the expression of TLR-4 and TNF-α release [37]. Moreover, some AMPs (including NDBPs) can interact with membrane proteins, making LPS unable to bind to its receptor and thus suppressing subsequent cytokine production [30,38]. The incubation of peptides and BMDM or BMDC before LPS stimulation showed a higher reduction of TNF-α secretion, while incubation of peptides with previously LPS-stimulated BMDM did not interfere in cytokine level. So, this result showed that these peptides preclude the interaction between receptors and LPS. This hypothesis was confirmed with TLR4 KO macrophages. Although our results showed the possibility of interaction with the TLR4, the addition of ToAP3 and ToAP4 in BMDC culture after LPS stimulation reduced TNF-α levels, albeit at different levels, suggesting that these peptides can have more than one way of interacting with BMDCs.

Inflammatory cytokines, such as TNF-α and IL-1β, are important for DC activation, differentiation and maturation [39]. DCs are an important type of antigen presentation cell (APC) because they present the processed antigen to naive antigen-specific T cells. According to our results, ToAP3 did not intervene in LPS-induced MHC class II molecules' expression in murine BMDCs. Instead, we observed that the peptide ToAP4 increased the expression of these molecules and did not alter the expression levels of CD80 and CD86, which suggests that ToAP4 can induce DCs maturation. Moreover, ToAP4 increased IL-10 secretion, and in this context, DCs' function can shift from immunogenic to tolerogenic phenotype [40]. The tolerogenic DCs can act by inducing apoptosis or anergy in the absence of co-stimulatory signals and induce T regulatory cell differentiation [41], among other functions. On the other hand, only ToAP3 inhibited the expression of the T-cell co-stimulatory molecules CD80 and CD86 under LPS-induced dendritic cell maturation without changes in IL-10 production. A successful antigenic
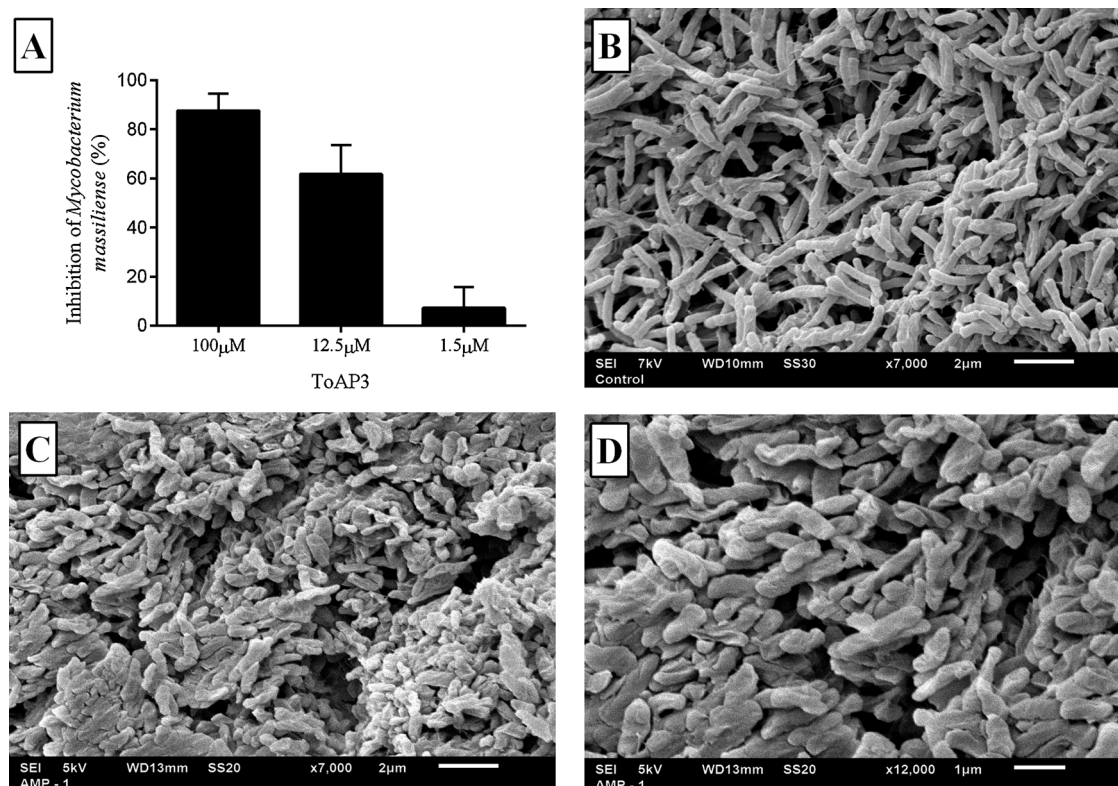
up-regulation of miR-155 and down-regulation of miR-125b [24]. In the assays described here, the levels of miR-125b were not modified thorough peptide incubation, either in the presence or absence of LPS. However, BMDC stimulated with LPS has shown lower levels of miR-155 when treated with both peptides. The decrease in miR-155 levels can be correlated with lower TLR-4 downstream pathway activation.

Considering the possibility that lower levels of IL-1β and TNF-α mRNA are associated with a weak interaction between LPS and CD14/TLR-4, we investigated this pathway. Some studies have already shown that AMPs can interact directly with LPS, interfering in its attachment to receptors. Mastoparan-1, a tetradecapeptide toxin isolated from

Fig. 7. Inhibition of *Mycobacterium abscessus* subsp. *massiliense* growth and morphology affected by the ToAP3 scorpion peptide. (a) Percentage of growth inhibition and Minimal bactericidal concentration (MBC) definition of ToAP3 against *M. a. s. massiliense*. The percentage of growth inhibition was calculated by plating serial dilutions of each culture and determining the number of colony-forming units (CFUs). (b) Scanning Electron Microscopy of untreated *M. a. s. massiliense* cells. (c) *M. a. s. massiliense* exposed to 12.5 μM of ToAP3 peptide for 24 h, presenting surface alterations. (D) *M. massiliense* with cell wall disruptions after treatment with ToAP3 for 24 h. (B) and (C) 7000 × magnification and (D) 12,000 × magnification.

presentation requires the expression of both B7-family members, CD80 and CD86, the major ligands to T-cell receptor CD28, together with MHC-II, stabilizing the immune synapse and facilitating antigen presentation. In the absence of those co-stimulatory molecules, antigen presentation is frustrated, and may lead to T cell anergy and possibly immune tolerance. In this context, regulation of the CD80/86-CD28 axis has been extensively explored in therapies for the treatment of chronic inflammatory (auto) immune diseases [42]. Considering these results together, we can conclude that ToAP4 is able to modulate the maturation and antigen-presenting function of dendritic cells, probably increasing the T regulatory cells, whilst ToAP3 is able to modulate the DCs, increasing the anergic T cells. A myriad of peptides have been successfully screened for the treatment of diseases resulting from autoimmune dysregulation such as psoriasis, rheumatoid arthritis and systemic lupus erythematosus [42,43].

As mentioned before, ToAP4 is similar to Stigmurin from *T. stigmuruns*, which has been characterized as an antibacterial and antifungal agent [15]. The analogs of this peptide, StigA25 and StigA31, have shown stronger microbicidal activity when compared with the native peptide [44]. Since these peptides did not present the capacity to inhibit *C. neoformans* growth [14], and considering the similarity between them with StigA25 and StigA3, we questioned if these peptides could have an effect on indirect antifungal activity, through macrophage activation. The results obtained showed that these peptides had no influence on fungalclearance by macrophages. This indicates that these peptides cannot be used as antifungal therapy, but there is still the possibility of using them as coadjutant in treatments when associated with antifungal drugs.

To verify the microbicidal activity of these peptides, we tested ToAP3 and ToAP4 against *M. abscessus* subsp. *massiliense*. We could only see microbicidal activity for ToAP3, which also altered the shape of this microorganism's cell surface. So, ToAP3 showed immunomodulatory and antibacterial activities. The AMP clavanin-MO also showed that it had immunomodulatory properties, increased leukocyte recruitment to the site of infection, and affected the production of GM-CSF, IFN-γ and MCP- 1 [18]. It suppressed an excessive and potentially harmful inflammatory response, increasing the synthesis of anti-inflammatory cytokines such as IL-10 and suppressing the levels of proinflammatory cytokines IL-12 and TNF-α. In addition, treatment with the peptide protected mice against lethal infections caused by Gram-negative and drug-resistant positive strains [18].

In summary, the present study showed that ToAP3 and ToAP4 could inhibit inflammatory responses, decreasing the production of various inflammatory mediators and modulating dendritic cells' activation and maturation, avoiding exacerbated inflammatory reactions. Besides, ToAP3 showed antibacterial activity. These observations raise the possibility that ToAP3 and ToAP4 could be a promising target for the development of new immunomodulatory and antimicrobial therapies.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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.2019.109152.

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