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Amaranth peptides decreased the activity and expression of cellular tissue factor on LPS activated THP-1 human monocytes

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The effect of amaranth peptides on the activity and expression of tissue factor (TF) on THP-1 activated cells was evaluated *in vitro*. An active anticoagulant peptide fraction (AF) was found to inhibit TF expression (IC_{50} = 0.39 mg mL^{-1}) and activity. Immunocytochemical fluorescence confocal microscopy analysis showed that treated monocytes decreased TF membrane translocation by 49.0% and increased two-fold in nuclei compared to a positive control, indicating a decrease of active TF to initiate the coagulation cascade. Moreover, a cytokine array suggested that the AF mechanism of action implied the inhibition of the NF-κB pathway. Expression of MIP-3α, interleukin-1β, interleukin-1α, TARC, pentaxin 3, and PDGF-AA cytokines was highly suppressed by AF peptides, producing reductions of 78.8%, 61.8%, 54.1%, 42.6%, 37.9% and 37.8%, respectively, compared to a positive control. The results suggest a potential mechanism for the antithrombotic and anti-inflammatory effect of AF, by showing that amaranth peptides play a negative feedback regulatory role over the NF-κB pathway. In this research, we link for the first time the immunomodulatory activity of amaranth peptides with the inhibition of TF expression and therefore their antithrombotic potential.

1. Introduction

There is a global epidemic pattern of predominantly diet-related non-communicable diseases such as obesity, diabetes, cardiovascular disease (CVD) and cancer. According to the American Heart Association (AHA), CVD is listed as the underlying cause of death and accounts for about one of every three deaths in the USA.[1] An estimated 92.1 million US adults have at least one type of CVD, entailing health and economic burdens in the USA and globally. In 2010, the estimated global cost of CVD was $863 billion, and it is estimated to rise to $1044 billion by 2030.[1,2] Moreover, in Argentina, non-communicable diseases constitute more than 70% of deaths. Within this group, CVD represents the main cause of death (40.2%). Therefore, studies exploring innovative strategies for promoting healthful eating must be conducted to address these public health crises.

Among CVD, thrombosis implies the dysregulation of hemostatic balance leading to a pro-coagulation state and the pathologic formation of thrombi. Thrombosis is a common pathology underlying major cardiovascular disorders like ischemic heart disease, stroke, and venous thromboembolism.[3] There are many factors predisposing to thrombosis, including atherosclerosis, hypertension, diabetes mellitus, and obesity, among others. In addition to all these factors, chronic inflammation may also cause a tendency towards both arterial and venous thrombosis[4] as inflammation unleashes hemostatic mechanisms, which in turn can amplify inflammation.[5,6]

A major molecule involved in the thrombosis process is the cellular tissue factor (TF). TF is a plasma membrane-anchored glycoprotein involved in hemostasis regulation and is best known as the primary cellular initiator of blood coagulation under both physiological and pathological conditions.[7,8] It is expressed constitutively on cell surfaces of a variety of extravascular cell types not exposed to the bloodstream. However, TF expression could be induced in some blood cells, such as circulating monocytes, the primary source of intravascular TF,[9] as a consequence of injury or pathological inflammatory stimuli. Moreover, TF induction by inflammatory mediators in monocytes involves transcription factors from Jun/Fos, NF-κB and NFAT families.[10] They can trigger an uncontrolled

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TF-initiated upstream protease cascade, capable of supporting a self-perpetuating inflammatory response. Unregulated TF expression can exaggerate the pro-coagulant effects by altering cell membrane properties and by destroying the inhibitory control of the TF pathway.[11] Therefore, a proper regulation of TF expression and activity is critical for the maintenance of hemostatic balance and health, and crucial for thrombosis prevention.[12] Anticoagulant strategies that target both the pro-thrombotic and the pro-inflammatory signaling effects of the coagulation protease cascade may result in effective attenuation of morbidity and mortality caused by such disorders.

The bioactive components contained in functional foods include peptides with short sequences that are encrypted in food proteins.[13] Since about 1980, amaranth has been rediscovered as a promising food crop mainly due to its resistance to heat, drought, diseases, and pests, and the high nutritional value of its proteins.[14] Antithrombotic effects were observed in previous *in vitro*, *in vivo*, and *ex vivo* studies of amaranth peptides showing inhibition of coagulation in hemostasis tests and thrombin enzyme.[15–17] Moreover, anti-inflammatory activity of amaranth peptides has also been described.[18,19] However, there is a gap in knowledge about the effect of food peptides on TF expression in circulating monocytes. Thus, there is a need for new knowledge regarding the potential of food peptides to inhibit TF expression and activity as a possible strategy to target both the pro-thrombotic and the pro-inflammatory signaling effects of the coagulation cascade. Therefore, the objective of the present research was to determine the effect of amaranth peptides on pro-thrombotic and inflammatory crosstalk pathways, involving TF factor activity and expression, on THP-1 monocytic cells under inflammatory conditions.

2. Materials and methods

The THP-1 human acute monocytic leukemia cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Monocytic cells were maintained in Roswell Park Memorial Institute-1640 culture media (RPMI 1640, Gibco Thermo Fisher Scientific, Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Gibco, and streptomycin/penicillin and sodium pyruvate from Corning Inc. (Corning, NY, USA). Lipopolysaccharide from *Escherichia coli* O55:B5 (LPS) was purchased from Sigma-Aldrich (St Louis, MO, USA). CD142 mouse monoclonal TF antibody (IgG1, kappa Isotype; HTF-1 clone, catalog number 16-1429-85), functional grade primary antibody, was purchased from Invitrogen™ (Carlsbad, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-mouse IgG horseradish peroxidase conjugated secondary antibody from GE Healthcare (Bloomington, IL, USA). A tissue factor human chromogenic activity assay kit was purchased from Abcam (Cambridge, MA, USA) and a human XL cytokine array kit from R&D Systems (MN, USA).

2.1 Plant material and sample preparation

2.1.1 Isolation of amaranth protein. *Amaranthus mantegazzianus* seeds (Pass cv. Don Juan) were harvested at Río Cuarto, Córdoba, Argentina (2015). Seeds were ground using a cyclone mill (1 mm mesh), and the resulting flour was defatted with *n*-hexane (10 g flour per 100 mL *n*-hexane) for 24 h, dried at room temperature, and stored at 0 °C until use. Amaranth protein isolates were obtained according to Martínez and Añón (1996).[20]

2.1.2 Simulated gastrointestinal digestion of amaranth protein isolates. To simulate gastrointestinal digestion of amaranth protein isolates, an adaptation of published methods was used.[21,22] The protein isolate was suspended in a saline solution (pH 2.0, 0.1 mol L^{-1} HCl, 0.03 mol L^{-1} NaCl) and then treated with pepsin (0.1 g pepsin per g protein, 1 : 15 000 5× NF units, MP Biomedicals, Santa Ana, CA, USA) at 37 °C and stirred for 1 h to simulate passage through the stomach. Afterward, pH was adjusted to 6.0 and pancreatin was then added (0.1 g pancreatin per g protein, 4X-100 USP units per mg, MP Biomedicals, 0.1 mol L^{-1} NaHCO$_3$) to emulate intestinal fluid. After 1 h stirring at 37 °C, pancreatin activity was halted by heating at 85 °C for 10 min. Finally, suspensions were freeze-dried and stored at 0 °C until use of simulated gastrointestinal digestion peptides (SGD).

2.1.3 Production of amaranth bioactive peptides. The active anticoagulant peptide fraction (AF) was obtained as described in a previous publication.[15] Fractionation of the SGD was performed using size-exclusion chromatography utilizing a Pharmacia LKB, FPLC system, with a Superdex-75 10/300-GL molecular exclusion column (GE Healthcare, total volume 25 mL, range: 3–70 kDa). The AF, which represents 10.4% of the whole area of the chromatogram of SGD, was sequenced and many peptides were found. Potentially bioactive peptides, most of them located on the surface of 11S globulin and agglutinin amaranth protein structures, were then selected using the criteria described by Sabbione *et al.*,[15] where the sequences with potential anticoagulant capacity were considered. These selected peptides were synthesized by KareBay Biochem Inc. (Ningbo, Zhejiang, China).

2.2 Cell culture and treatments

2.2.1 Cell culture. The THP-1 human monocyte-derived cell line was maintained in culture media RPMI 1640 containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin–streptomycin, 1% (v/v) sodium pyruvate and 50 µmol L^{-1} β-mercaptoethanol (to prevent crosslinking of Fc receptors on the cell by the antibody in serum, which prevents damaging cell function). THP-1 cells were incubated at 37 °C in 5% CO$_2$/95% air.

2.2.2 LPS treatment. Human THP-1 monocytes were seeded at a density of 1×10^6 cells per 2 mL in a 6-well plate and treated with different concentrations of sterile-filtered LPS to induce TF expression.[23] In this research, LPS activation for 4 h in a concentration range of 1–100 µg mL^{-1} on THP-1 monocytes was used to determine the best concentration for future treatments.

2.2.3 Amaranth sample treatments. The different treatments used in this study consisted of SGD, AF and synthesized pure peptides. Sterile-filtered SGD and AF at a range of concentrations from 0.1 to 2 mg mL^{-1} and pure peptides at a concentration of 0.25 mg mL^{-1} were incubated for 24 h with 1×10^6 cells per 2 mL in a 6-well plate to study TF expression and activity. Two different types of treatments were performed: simultaneous and preventive effect of TF on LPS activated monocytes. To stimulate TF expression, THP-1 cells were treated with 10 μg mL^{-1} LPS dissolved in growth media. Cells treated with LPS served as the positive control while cells treated only with PBS served as the negative control. Simultaneous treatment consisted of a 24 h treatment with the different samples and LPS, whereas preventive treatment consisted of 24 h incubation with amaranth samples, removing old media and treating the monocytes with 10 μg mL^{-1} LPS for 4 h.

2.2.4 Cell viability. Cell viability of THP-1 human monocytes was performed using a Cell Titer 96 Aqueous One Solution Proliferation assay kit (Promega Corporation, Madison, WI, USA). The protocol required the use of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and an electron-coupling reagent, phenazine ethosulfate. All cell viability assays were performed at least in sixtuplicate.

2.3 Tissue factor studies

2.3.1 Obtaining of cell lysates. THP-1 cells were seeded at a density of 1×10^6 cells per well in 6-well plates, and the different treatments were performed at 37 °C under 5% CO$_2$/95% air incubation conditions. After the treatments ended, the cells were washed twice with PBS and harvested using a lysis buffer reagent. Afterwards, the cells were sonicated and centrifuged at 10 000g for 10 min at 4 °C. Supernatants were directly used for TF activity measurements or diluted using an equal volume of the lysates and Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 5% β-mercaptoethanol, and then boiled for 5 min for TF expression assay. Cell lysates were immediately frozen at −80 °C until used for analysis of TF activity or expression.

2.3.2 TF expression through western blot analysis. Protein concentration of the whole cell lysates was determined by a protein DC assay kit (Bio-Rad) in order to load equal amounts of protein (15 μg) from whole cell lysates in 4–20% gradient SDS-polyacrylamide gels (Bio-Rad). The separated proteins were transferred onto PVDF blotting membranes (GE Healthcare) and blocked with 5% nonfat dry milk in 0.1% Tris-buffered saline-Tween 20 (TBST) for 1 h at room temperature. The membranes were washed with 0.1% TBST and incubated with CD142 mouse monoclonal TF primary antibody overnight at 4 °C. Membranes were washed again with TBST (5 times, 5 min each) and incubated with anti-mouse IgG horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. Finally, membranes were washed with TBST (5 times, 5 min each) and prepared for detection using a 1 : 1 mixture of chemiluminescent reagents A (luminol solution) and B (peroxide solution) (GE Healthcare). The membrane pictures were obtained using a Gel Logic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY). The relative amount of each target protein was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All western blot procedures were run in at least two independent trials, performing each treatment in duplicate to confirm reproducibility.

2.3.3 TF human chromogenic activity assay. TF activity was measured by a tissue factor human chromogenic activity assay kit according to the manufacturer's instructions. Briefly, cell lysates were added to a 96-well plate. Human Factor VIIa, Factor X, and the chromogenic substrate Factor Xa were added as recommended and the optical density was monitored at 405 nm. The assay measured the ability of the TF/FVIIa complex to activate FX to FXa. The amidolytic activity of the TF/FVIIa complex was quantified by the amount of FXa produced using a highly specific FXa substrate releasing a *para*-nitroaniline (pNA) chromophore quantified by measuring changes in absorbance of the yellow pNA chromophore at 405 nm. A TF standard curve was used to quantify the TF concentration in every lysate. All activity measurements were determined in at least two independent trials, performing each treatment in duplicate to confirm reproducibility.

2.3.4 Immunocytochemical fluorescence confocal microscopy analysis. After AF (0.48 mg mL^{-1}) and LPS (10 μg mL^{-1}) treatment, monocytes were seeded in phenol red-free RPMI-1640® media (2×10^5 cells per mL) for 1 h onto flat-bottomed 12-well Ibidi chambers (Ibidi, Madison, WI, USA) precoated with poly-L-lysine solution (Sigma). The cells were centrifuged at 130g for 7 min at room temperature to promote cell attachment to the coating. The cells were fixed with 4% paraformaldehyde aqueous solution (Electron Microscopy Sciences, Hatfield, PA, USA) for 30 min at room temperature, washed twice, 5 min each, with PBS and permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 15 min at room temperature. The cells were washed once with PBS and incubated with ultra-cold HPLC-grade methanol for 15 min at −20 °C. Methanol was removed and replaced with PBS and incubated for 30 min at room temperature. Afterwards, the cells were blocked with an Image-IT FX Signal Enhancer (Invitrogen) for 30 min at room temperature and incubated with CD142 mouse monoclonal TF (Invitrogen, 1 : 200) overnight at 37 °C. After incubation, the cells were washed twice, 5 min each, with PBS and incubated with an Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen, 10 μg mL^{-1}) for 4 h avoiding light exposure. The cells were washed twice with PBS and covered with a ProLong Gold antifade reagent with DAPI (Invitrogen). The chambers were cured for 24 h at room temperature in the dark, and stored at 4 °C until further use. Cell image acquisition was obtained using a Carl Zeiss LSM 880 Laser Scanning Microscope (Carl Zeiss AG, Germany) with 63× oil immersion objective. The images were obtained using a 405 (10 mW) (415–470 nm emission) and a 488 (10 mW) (500–550 nm emission) laser line. The laser power, gain, and offset were kept constant across the samples, and the samples were scanned in

a high-resolution format of 1024 × 1024 pixels averaging four frames. Total intensities and area sums of regions of interest were quantified with ZEN 2 blue edition (Carl Zeiss) and were expressed as average relative intensity in arbitrary units (AU) per area of analysis (μm^2). All of the image panels were resized and consolidated, and the brightness of the final collage displayed was increased by 20%. Six independent fields of view from two independent cellular replicates were merged together per treatment.

2.3.5 Cytokine array analysis. The expression profile of thrombosis/inflammation-related proteins was assessed using a Human XL Cytokine Array (R&D systems, Minneapolis) following the manufacturer's instructions. Briefly, THP-1 cells were seeded at a density of 3×10^6 cells per well in 6-well plates and the treatment was performed at 37 °C under 5% CO_2/95% air incubation conditions. Cells treated with LPS alone were used as the positive control. After treatments were completed, the cells were washed twice with PBS and harvested using a lysis buffer reagent. The expression of proteins in the cell lysates was determined following the manufacturer's protocol. The cytokine arrays were performed in two independent trials, each treatment in duplicate to confirm reproducibility.

2.4 Statistical analysis

Data were analyzed using Statistical Analysis System software JMP version 7.0 (SAS Institute, Cary, NC, USA). The comparison analysis for all pairs was performed using one-way analysis of variance (ANOVA) with the *post-hoc* Tukey–Kramer HSD. Student's *t*-test was used to analyze comparisons against sample treatments and the positive control. Significant differences were reported at $p < 0.05$. All assays were performed at least in duplicate.

3. Results and discussion

3.1 Effects of LPS treatment on tissue factor expression and activity

Fig. 1 shows the expression and activity of TF after LPS treatment on THP-1 monocytes. Compared to the control without LPS, the TF expression increased significantly when 10 μg mL^{-1} or higher concentrations of LPS were used ($p < 0.05$) (Fig. 1A). TF activity showed covariation with western blot results. The LPS treatments at different concentrations for 4 h increased significantly TF activity when expressed as pM of TF concentration ($p < 0.05$) (Fig. 1B). According to various publications, the highest expression of TF occurs between 4–6 h using LPS concentrations between 1 and 10 μg mL^{-1}.[24,25] Brekke *et al.*[26] studied activated monocyte TF cell surface expression over time and observed a time-dependent increase of TF from 1 to 4 h treatment. The results observed in Fig. 1 agree with those reported by Kothari *et al.*[24] and Brekke
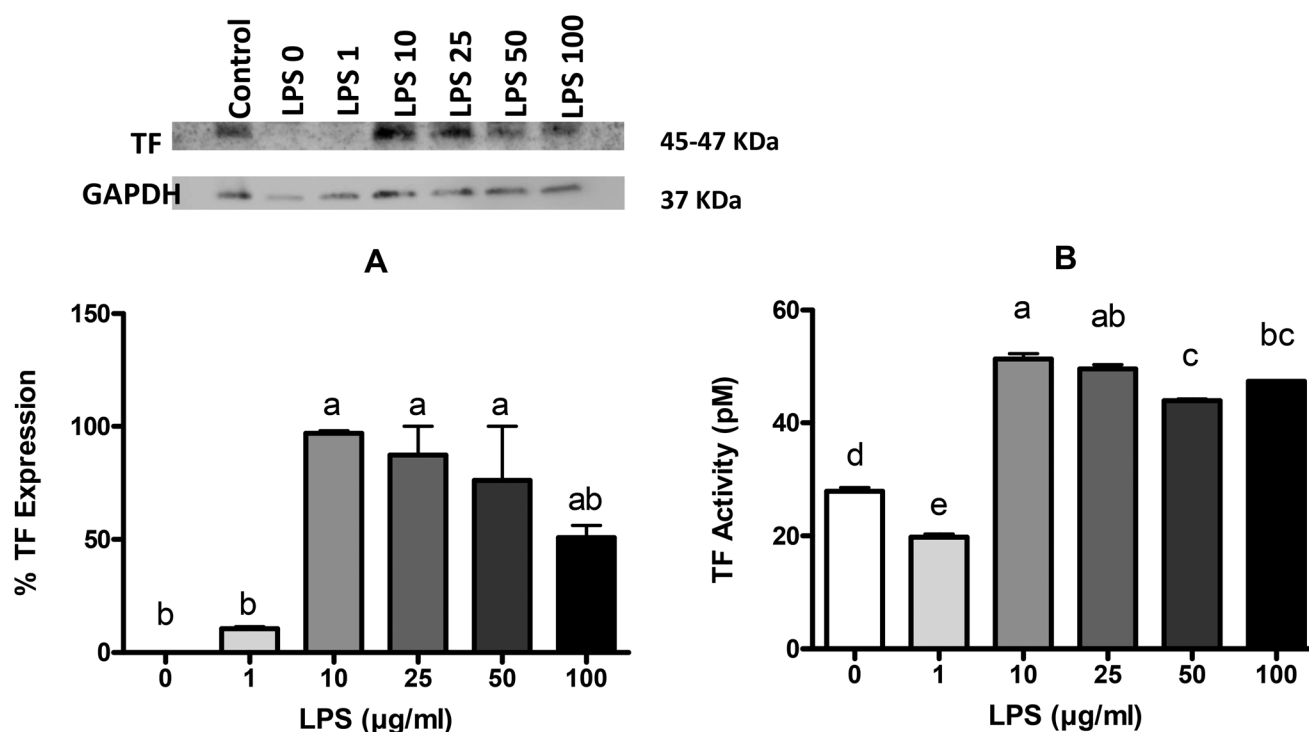

Fig. 1 LPS treatment. Effects of different concentrations of LPS on human THP-1 monocyte cell expression (A) and activity (B) of tissue factor (TF) measured using a TF standard curve after a 4 h treatment. All experiments were performed in at least two independent replicates. Bars with different letters mean that they are statistically different ($p < 0.05$) using comparisons for all pairs Tukey–Kramer HSD one-way analysis. Percentage of expression of TF was calculated considering LPS control as 100%. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping protein.

et al.,[26] and confirm the effectiveness of LPS to induce TF expression, which allowed us to select 10 μg mL^{-1} LPS treatment as the optimal condition for further experiments. Cell viability was not affected by the different LPS concentrations used (100% viability).

3.2 Effects of AF preventive treatment on tissue factor expression and activity

3.2.1 TF expression and activity. Fig. 2 shows the results obtained from TF expression and activity experiments after performing a preventive treatment with SGD and AF amaranth peptides at different concentrations. SGD did not produce significant reduction of TF expression at any concentrations when compared to LPS positive control ($p > 0.05$) (Fig. 2A). Similar results were observed for TF activity (Fig. 2B), confirming a good correlation between both methodologies. In contrast, AF at the highest concentration (0.48 mg mL^{-1}) significantly inhibited 61.8% TF expression ($p < 0.05$), while lower concentrations of these peptides did not reduce TF expression in comparison with the LPS positive control (Fig. 2A). AF exhibited a similar behavior according to the TF activity measures, where the highest concentration of AF produced a 32.9% decrease of the activity ($p < 0.05$, Fig. 2B). A dose–response inhibition of TF expression was observed with AF

treatments obtaining an IC$_{50}$ value of 0.39 ± 0.14 mg mL^{-1}. The viability of the cells was not affected by the different SGD and AF concentrations used in the present study (100% viability).

Rashid *et al.*[27] studied the inhibition of TF expression using different drugs. IC$_{50}$ values of approximately 10, 0.3, and 0.05 μg mL^{-1} were found for pentoxifylline, retinoic acid and cyclosporine A drugs, respectively. These values are at least 50 fold lower than those obtained for AF; however, it must be taken into account that AF contains amaranth seed peptides analyzed as possible functional ingredients and not as a targeting drug. Since AF preventive treatment at 0.48 mg mL^{-1} showed to be promising, further assays were performed under these conditions.

3.2.2 Immunocytochemical fluorescence confocal microscopy analysis. Fig. 3A and B exhibit images obtained using objective 63× oil immersion with the same treatments but different magnifications (10 and 5 μm scales, respectively). Cells without LPS treatment (Ctrl$^-$) showed that TF had mostly an intracellular location, whereas, in response to LPS stimulation (Ctrl$^+$), different patterns of intracellular accumulation and externalization of TF were observed. The Ctrl$^+$ cell image shows that most of the TF gets externalized (Fig. 3A and B). In accordance with these results, different studies analyzing TF
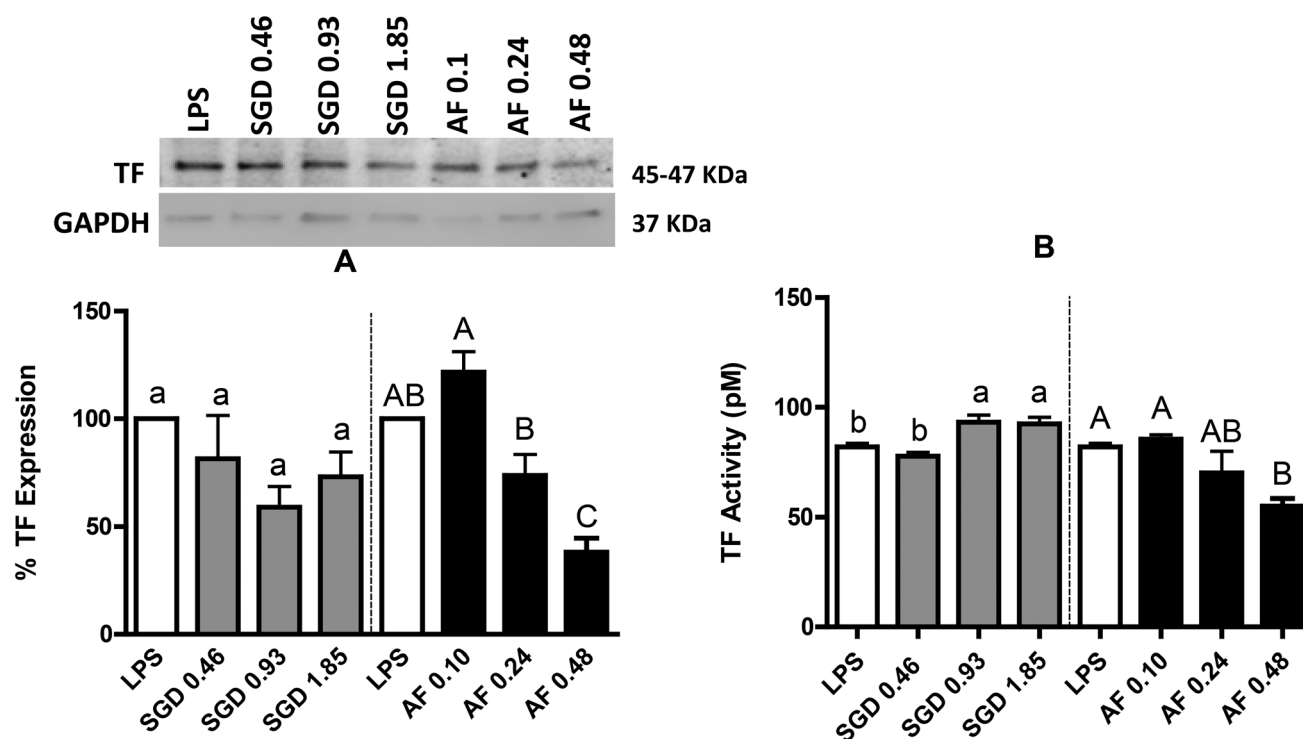

Fig. 2 Preventive treatment. Effects of different concentrations of simulated gastrointestinal digestion amaranth peptides (SGD) and the active anti-coagulant peptide fraction (AF) on (A) human THP-1 monocyte TF expression and (B) activity of TF measured using a TF standard curve. All amaranth peptide treatments were performed for 24 h and then treated with LPS (10 μg mL^{-1}) for 4 h. All experiments were done in at least two independent replicates. SGD and AF samples were compared with LPS control separately. Bars with different lowercase or uppercase letters (SGD and AF, respectively) mean that they are statistically different ($p < 0.05$) using comparisons for all pairs Tukey–Kramer HSD one-way analysis. Percentage of expression of TF was calculated considering LPS control as 100%. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping protein.
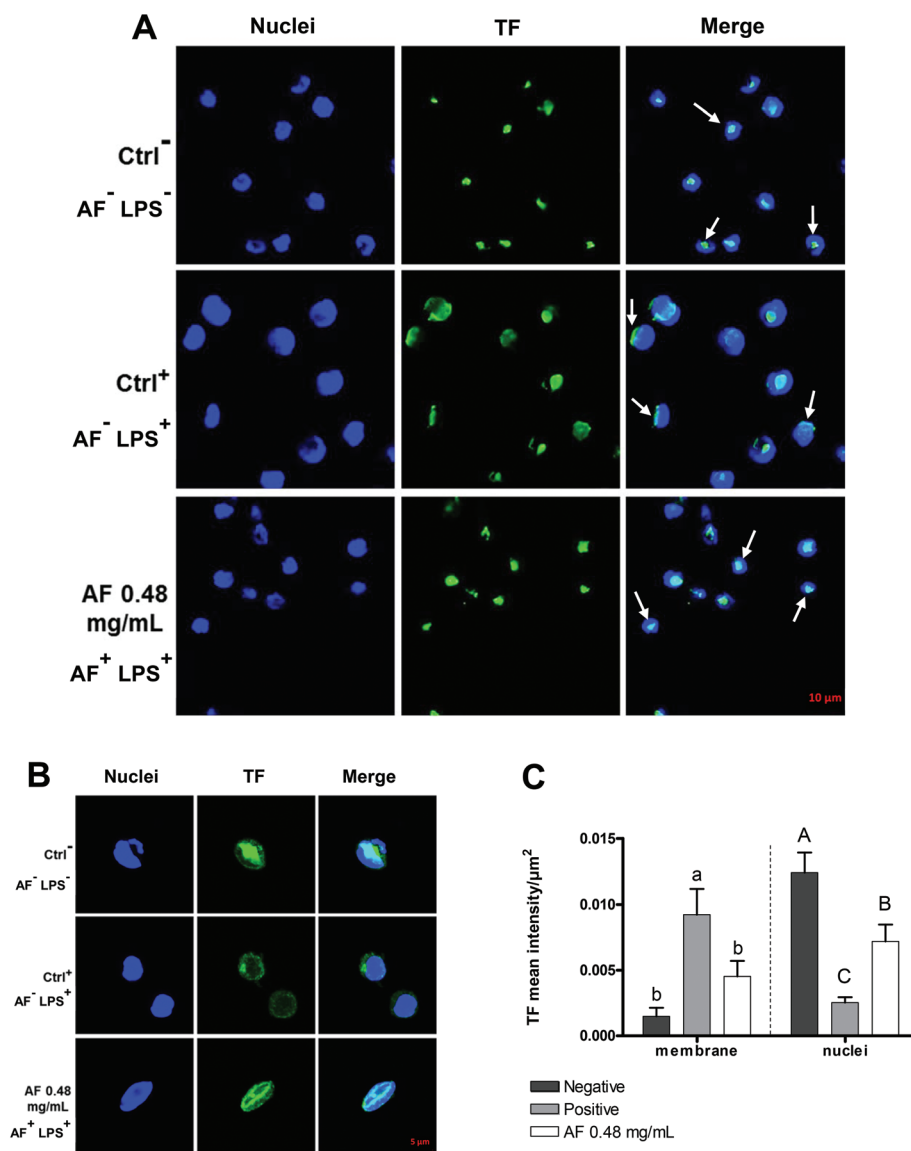
Fig. 3 Confocal laser scanning microscopy. Two-dimensional immunocytochemical localization of tissue factor (TF) in THP-1 monocytes. (A) and (B) Cells visualized using objective 63× oil immersion (10 μm scale) and digital zoom images of 63× oil immersion images (5 μm scale). (C) Quantification of TF in membrane and nuclei, expressed as intensity over their respective area sums, for controls and treatment. White arrows show TF nuclei or membrane localization. Means with different letters (membrane in lowercase letters, and nuclei in uppercase letters) are significantly different from controls ($p < 0.05$). Six independent fields of view from two independent cellular replicates were merged together per treatment. The panel to the left represents DAPI stained nuclei (blue), the panel in the middle shows the response to the primary antibody TF expression (green) and the third panel represents the merge of both.

localization in cells found the same distribution in activated and non-activated monocytes.[28,29] AF preventive treatment significantly decreased in 49.0 ± 12.8% TF membrane translocation compared to Ctrl+ ($p < 0.05$), and the level of TF in the membrane did not show significant differences compared to Ctrl− (Fig. 3C). The treatment with AF reduced the TF membrane translocation in LPS-stimulated monocytes since most of the glycoprotein was intracellularly located (Fig. 3A and B). These results indicate that AF preventive treatment reduces membrane TF in LPS stimulated monocytes implying a decrease of active TF to initiate the coagulation cascade,

related to a decrease in hypercoagulability and subsequent thrombosis.

3.2.3 Cytokine expression analysis. Variations in expression of thrombosis and inflammation related cytokines in response to AF preventive treatment were evaluated. The AF showed a reduction in expression of some protein markers and an increase of other markers when compared to LPS positive control (Ctrl+). Fig. 4A and B show the effect of 0.48 mg mL^{-1} AF on the expression of cytokines. Expression of MIP-3α, interleukin-1β, interleukin-1α, TARC, pentaxin 3, and PDGF-AA was highly suppressed by AF peptides, producing reductions of
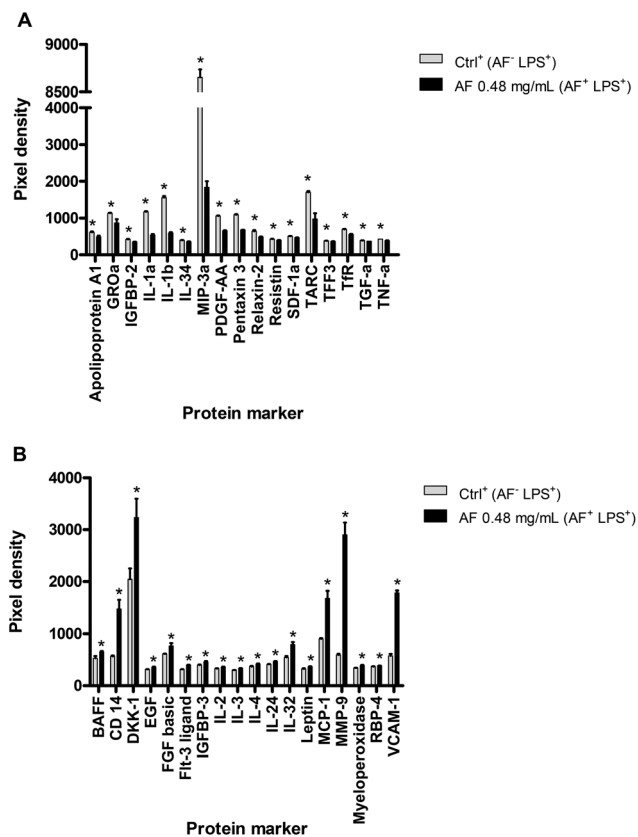
Fig. 4 Cytokine array analysis. Effects of AF treatment for 24 h and then 4 h of LPS (10 μg mL^{-1}) on human THP-1 monocyte cytokine expression. (A) and (B) Decrease and increase of cytokines, respectively. All the cytokines showed presented significant difference ($p < 0.05$) relative to the positive LPS control using Student's t test. All experiments were performed in at least two independent replicates.

matory processes or maintenance of tissue homeostasis.[18] Chronic inflammation is one of many factors that generates a predisposition to thrombosis.[4]

Moronta et al.[18] and Montoya-Rodríguez et al.[19] were able to identify amaranth peptides with immunomodulatory effects through the NF-κB signaling pathway. Moreover, Moronta et al.[18] studied the immunomodulatory effect of amaranth peptides on epithelial cells activated through the NF-κB signaling pathway. The results confirmed the inhibition of this inflammatory pathway through the amaranth peptides' capacity to suppress the expression of mRNA coding for the MIP-3α cytokine, also known as CCL20. In the present research, cytokine MIP-3α presented the highest inhibition after treatment with amaranth peptides. Even though there are different inflammatory pathways that involve the expression of MIP-3α, interleukin-1β, interleukin-1α, TARC, pentaxin 3, and PDGF-AA cytokines, the results mentioned above support the inhibition of the NF-κB signaling pathway by AF amaranth peptides.

In the present research, we confirm the presence of amaranth encrypted peptides with immunomodulatory activity and, for the first time, link this effect with the inhibition of TF expression and likely antithrombotic impacts. Moreover, the reduction of cytokines TARC, PDGF-AA and GROα related with coagulation platelet functions (Table 1) could be attributed to the TF decrease due to the fact that the presence of platelets influences TF expression by monocytes.[43]

The preventive treatment with AF also significantly reduced the expression of the cytokine tumor necrosis factor alpha (TNF-α) compared to Ctrl$^+$ ($p < 0.05$, Fig. 4A). This cytokine acts as pro-inflammatory stimuli and is expressed by the action of different stimuli, like LPS. However, low values of TNF-α expression were observed after the LPS treatment in Ctrl$^+$. This result may be related to the short times of LPS activation used in the preventive treatment.

Even though AF treatment reduced the expression of many cytokines, others were increased (Table 2). MMP-9 and MCP-1 cytokines were significantly increased after the AF treatment. Yang et al.[44] investigated the role of monocyte chemoattractant protein-1 (MCP-1) in human pathologies and concluded that this cytokine stimulates the secretion of MMP-9 directly through the MAPK/ERK pathway, which is related to cell proliferation, differentiation, migration, senescence, and apoptosis. However, further studies are needed to assess the effect of the increase of these cytokines and the way in which amaranth peptides differently affect MAPK/ERK and NF-κB signaling pathways.

Regarding the increase of cytokines observed (Table 2), Lepidi et al. (2001)[45] suggest that freshly isolated monocytes become predominately MMP-9 producing cells when cultured on plastic in the presence of serum; therefore, there might be some responses of the monocytes to the treatment that not necessarily mean an inflammatory response. Therefore, the increase of the MMP-9 cytokine in this research does not necessarily imply a pro-inflammatory effect of amaranth peptides.

78.8%, 61.8%, 54.1%, 42.6%, 37.9% and 37.8%, respectively (Fig. 4A). The proteins MMP-9, VCAM-1, CD 14, MCP-1, and DKK-1 showed the highest increase compared to Ctrl$^+$ (389.9%, 209.9%, 161.3%, 86.5%, and 58.2% higher than the control, respectively) (Fig. 4B). Tables 1 (ref. 30–37) and 2 (ref. 38–42) present the function of these proteins in the cell and the percent reduction or increase of expression, respectively. Only cytokines that had the highest modulation after treatment with AF, compared to Ctrl$^+$ ($p < 0.05$), are shown.

The cytokines with the highest inhibition of expression, compared to Ctrl$^+$, are involved in the induction of the NF-κB pathway and platelet adhesion, aggregation, and activation process (Table 1). The cytokine expression analysis showed that AF exerts potential anti-inflammatory and antithrombotic effects in LPS-induced THP-1 cells by reducing the expression of proteins associated with the NF-κB signaling pathway. The NF-κB comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory response, playing a key role in the cellular response to different stress situations. Extracellular or internal stress signals can activate this pathway and induce the expression of several genes that may be involved in pro-inflam-

Table 1 Cytokine expression significantly decreased after AF preventive treatment compared to LPS positive control ($p < 0.05$), their function and percent of reduction caused

Cytokine	Name of the marker	Function	% Reduction
MIP-3α (CCL20)	Macrophage inflammatory protein 3α	Pro-inflammatory chemoattractant that mediates downstream signaling through activation of the NF-κB pathway[30]	78.8 ± 2.9
IL-1β	Interleukin-1β	Important mediator of the inflammatory response, known inducer of the NF-κB pathway. Involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis[31]	61.8 ± 4.2
IL-1α	Interleukin-1α	Involved in hematopoiesis and various inflammatory processes, it can induce the activation of the NF-κB pathway[31]	54.1 ± 4.1
TARC	Chemokine (C–C motif) ligand 17	Potent and selective chemoattractant involved in activation of platelet aggregation and adhesion[32]	42.6 ± 11.3
PTX3	Pentaxin 3	Cytokine produced and released in response to primary inflammatory signals, associated with inflammatory cardiovascular disease[33]	37.9 ± 2.0
PDGF-AA	Platelet-derived growth factor-AA	Growth factor that plays a significant role in blood vessel formation linked to atherosclerosis and as a consequence of thrombosis[34]	37.8 ± 2.5
RLXH2	Relaxin-2	Peptide hormone that plays roles in the cardiovascular system, regulation of blood pressure and control of heart rate[35]	24.5 ± 6.8
GROα	Chemokine (C–X–C motif) ligand-1	α-Granule chemokines involved in platelet activation[32]	23.3 ± 10.6
Apo A1	Apolipoprotein A1	Component and a major structural protein of HDL, plays a vital role in reverse cholesterol transport[36]	20.2 ± 10.1
TfR	Transferrin receptor-1	Transmembrane glycoprotein involved in cellular uptake of iron. Is related with cell proliferation and differentiation[37]	20.2 ± 4.3

Table 2 Cytokine expression significantly increased after AF preventive treatment compared to LPS positive control ($p < 0.05$), their function and percent of increase caused

Cytokine	Name of the marker	Function	% Increase
MMP-9	Matrix metalloproteinase-9	Zinc endopeptidases that act as key effectors and regulators of tissue remodeling playing a role in the process of epithelial regeneration[38]	389.9 ± 9.0
VCAM-1	Vascular cell adhesion protein 1	Mediates adhesion of leukocytes to vascular endothelium and participates in cell signal transduction[39]	209.9 ± 4.4
CD 14	Cluster of differentiation 14	CD14 acts as a co-receptor for the LPS detection[40]	161.3 ± 13.2
MCP-1	Monocyte-chemoattractant protein-1	Key chemokine that regulates migration and infiltration of monocytes/macrophages[41]	86.5 ± 9.3
DKK-1	Dickkopf-related protein 1	Signaling pathway inhibitor of the WNT signaling pathway[42]	58.2 ± 17.8

In recent years, different studies have demonstrated that the inactivation of the NF-κB pathway may lead to the inhibition of TF expression on the monocyte surface, contributing to thrombosis prevention as a result of the inactivation of NF-κB intracellular signals.[46] Therefore, the NF-κB pathway is regarded as a promising target to inhibit inflammation and thrombosis related pathologies, and amaranth peptides could be used to produce a negative feedback regulator role over this pathway.

3.3 Effects of simultaneous LPS and AF treatment on tissue factor expression and activity

Incubation of THP-1 monocytes for 24 h with different amaranth samples and LPS did not produce significant difference of TF expression at any tested concentrations when compared to the LPS positive control, Ctrl⁺ ($p > 0.05$) (Fig. 5). The SGD sample showed no effect on TF activity while the highest concentration of AF significantly decreased TF compared to Ctrl⁺ ($p < 0.05$). Since THP-1 cells were incubated for 24 h with LPS, the TF activity of LPS positive control in this treatment was reduced (19.6%, Fig. 1B and 5B) compared to LPS 10 μg mL⁻¹ treatment for 4 h, in agreement with other studies, showing the highest expression of TF at short times.[24–26] Cell viability was not affected by the different SGD and AF concentrations used in the present study (100% viability).

3.4 Pure amaranth peptide preventive treatment

THP-1 monocytes were incubated for 24 h with different potential antithrombotic pure peptides and activated for 4 h with LPS (Fig. 6A); peptides were selected using the criteria described by Sabbione et al.[15] The pure peptide treatment (0.25 mg mL⁻¹) did not produce a significant difference on TF expression and activity when compared to Ctrl⁺ ($p > 0.05$) (Fig. 6B and C, respectively). Since multiple pathways and factors are involved in the blood coagulation, these peptides may interact with different coagulation cascade factors without affecting the TF expression and activity. Therefore, further sequencing analysis of amaranth peptides contained in AF must be conducted and other potential bioactive peptides need to be synthesized and studied.
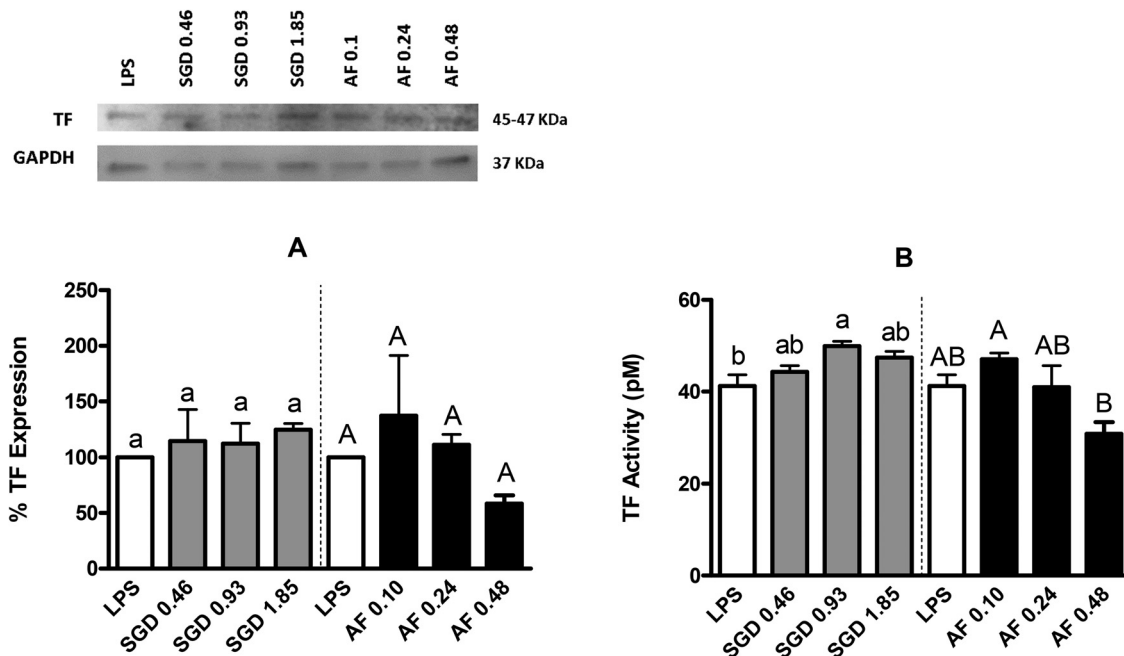
Fig. 5 Simultaneous treatment. Effects of different concentrations of simulated gastrointestinal digestion amaranth peptides (SGD) and the active anticoagulant peptide fraction (AF) on (A) human THP-1 monocyte TF expression and (B) activity of TF measured using a TF standard curve. All treatments were performed for 24 h with the different amaranth peptides from AF and LPS (10 µg mL^{-1}). All experiments were done in at least two independent replicates. SGD and AF samples were compared with LPS control separately. Bars with different lowercase or uppercase letters (SGD and AF, respectively) mean that they are statistically different ($p < 0.05$) using comparisons for all pairs Tukey–Kramer HSD one-way analysis. Percentage of expression of TF was calculated considering LPS control as 100%. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping protein.
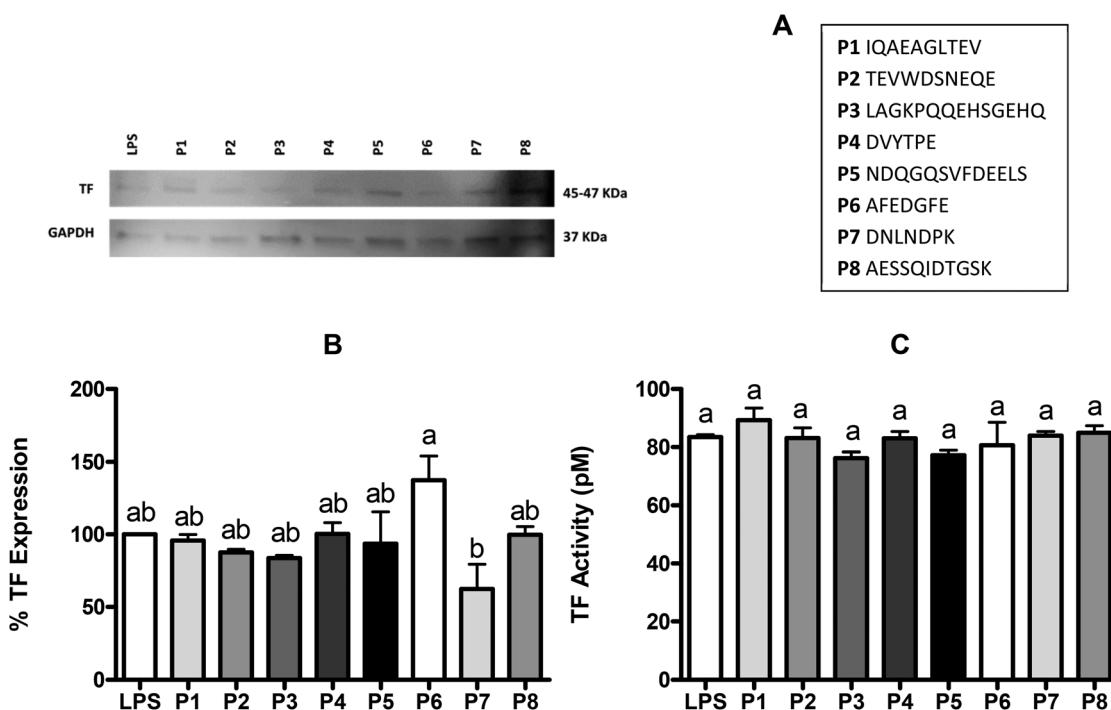

Fig. 6 Pure peptide preventive treatment. (A) Pure peptides sequences used. Effects of amaranth pure peptides (0.25 mg mL^{-1}) on human THP-1 monocyte cell expression (B) and activity (C) of TF measured using a TF standard curve. All pure peptide treatments were performed for 24 h and then treated with LPS (10 µg mL^{-1}) for 4 h. Bars with different letters mean that they are statistically different ($p < 0.05$) using comparisons for all pairs Tukey–Kramer HSD one-way analysis. Percentage of expression of TF was calculated considering LPS control as 100%. All experiments were performed in at least two independent replicates. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping protein.

4. Conclusions

A 24 h preventive treatment with amaranth peptides from AF produced a significant inhibition of TF expression and activity in THP-1 activated cells. Fluorescence confocal microscopy confirmed the effect of the peptides showing a decrease of TF migration to the membrane implying a reduction of active TF to initiate the coagulation cascade. Furthermore, the cytokine expression analysis suggested an AF mechanism of action related to an inhibition of expression of cytokines associated with the activation of the NF-κB signaling pathway and platelet adhesion, aggregation, and activation as part of the clotting process; the existence of other mechanisms involved cannot be discarded. Our findings suggest the presence of peptides encrypted in amaranth seed proteins with immunomodulatory activity and link for the first time this biological function to the antithrombotic potential, through the inhibition of TF expression. This effect could result in the prevention of hyper-coagulability states and subsequent thrombosis and inflammation pathologies, either of which presents cardiovascular risks. These findings support the promotion of amaranth massive consumption since it could be considered as a functional ingredient exerting potential health beneficial effects through the peptides released after digestion.

Abbreviations

AF   Active anticoagulant peptide fraction
CVD   Cardiovascular disease
LPS   Lipopolysaccharide from *Escherichia coli* O55:B5
SGD   Simulated gastrointestinal digestion amaranth peptides
TF   Tissue factor

Conflicts of interest

There are no conflicts of interest to declare.

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