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# Effects of thrombospondin-4 on pro-inflammatory phenotype differentiation and apoptosis in macrophages

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## Abstract

Thrombospondin-4 (TSP-4) attracted renewed attention recently as a result of assignment of new functions to this matricellular protein in cardiovascular, muscular, and nervous systems. We have previously reported that TSP-4 promotes local vascular inflammation in a mouse atherosclerosis model. A common variant of TSP-4, P387-TSP-4, was associated with increased cardiovascular disease risk in human population studies. In a mouse atherosclerosis model, TSP-4 had profound effect on accumulation of macrophages in lesions, which prompted us to examine its effects on macrophages in more detail. We examined the effects of A387-TSP-4 and P387-TSP-4 on mouse macrophages in cell culture and in vivo in the model of LPS-induced peritonitis. In tissues and in cell culture, TSP-4 expression was associated with inflammation: TSP-4 expression was upregulated in peritoneal tissues in LPS-induced peritonitis, and pro-inflammatory signals, INF $\gamma$ , GM-CSF, and LPS, induced TSP-4 expression in macrophages in vivo and in cell culture. Deficiency in TSP-4 in macrophages from *Thbs4*<sup>-/-</sup> mice reduced the expression of pro-inflammatory macrophage markers, suggesting that TSP-4 facilitates macrophage differentiation into a pro-inflammatory phenotype. Expression of TSP-4, especially more active P387-TSP-4, was associated with higher cellular apoptosis. Cultured macrophages displayed increased adhesion to TSP-4 and reduced migration in presence of TSP-4, and these responses were further increased with P387 variant. We concluded that TSP-4 expression in macrophages increases their accumulation in tissues during the acute inflammatory process and supports macrophage differentiation into a pro-inflammatory phenotype. In a model of acute inflammation, TSP-4 supports pro-inflammatory macrophage apoptosis, a response that is closely related to their pro-inflammatory activity and release of pro-inflammatory signals. P387-TSP-4 was found to be the more active form of TSP-4 in all examined functions.

## Introduction

Thrombospondin-4 (TSP-4) is a matricellular protein, one of the five members of thrombospondin family<sup>1,2</sup>. TSP-4 expression is low in adult tissues, but dramatically increases during tissue remodeling and regeneration. High levels of TSP-4 have been detected in remodeling and failing hearts<sup>3-7</sup>, several cancers<sup>8-13</sup>, and atherosclerotic lesions<sup>14</sup>. Recently, new functions have been

ascribed to TSP-4 in the cardiovascular system<sup>5-7,14-25</sup>, cancer<sup>8-12,24-29</sup>, skeletal muscle<sup>30,31</sup>, and nervous system<sup>32-36</sup>, but effects of TSP-4 on cellular responses and the regulation of TSP-4 expression remain poorly understood. For example, with an exception of TGF- $\beta$ <sup>25</sup>, stimuli inducing TSP-4 expression in tissues remain unknown. Although several cell surface receptors were shown to mediate TSP-4 effects<sup>14,24,32,37</sup>, signaling and regulation of intracellular processes by TSP-4 have received only limited attention.

While TSP-4 is best known as a secreted extracellular matrix (ECM) protein, it became clear that some of its

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effects depend on its intracellular interactions: its binding to the ER luminal domain of activating transcription factor 6 $\alpha$  (Atf6 $\alpha$ ) protects cardiomyocytes from injury caused by ER stress responses<sup>7</sup>. Thus, this protein exerts its effect in multiple complementary ways: by initiating intracellular signaling and transiting through secretory pathways<sup>38</sup>, as well as by binding to its extracellular ligands and receptors upon secretion<sup>5,6,14,24,30,33,37,39–48</sup>.

Tissue remodeling, inflammation, and angiogenesis often occur simultaneously and are regulated by the same stimuli. We recently reported that TSP-4 promotes angiogenesis<sup>24,25</sup> and vascular inflammation in a mouse atherosclerosis model<sup>14</sup>. These observations suggested that TSP-4 may play an important role in regulation of inflammation and inflammatory cell responses. In this study, we describe effects of TSP-4 and its common variant, P387-TSP-4, on macrophages. The P387-TSP-4 variant is associated with an increased risk of cardiovascular disease and myocardial infarction<sup>15–22</sup> and is more active than WT A387-TSP-4 in mediating cellular effects<sup>24,37,42,49</sup>. The region of TSP-4 molecule harboring P387 SNP appears to be important in regulation of many TSP-4 functions and was found to mediate the intracellular effects of TSP-4<sup>50</sup> and its effects in the nervous system<sup>32</sup>.

TSP-4 production by blood cells has not been reported to date. Here, we report several stimuli that induce or reduce TSP-4 production in macrophages. The effect of TSP-4 on macrophages has been examined in isolated macrophages and in LPS-induced peritonitis, an in vivo model of acute inflammation.

Macrophages play multiple roles in both the tissue homeostasis and inflammation, e.g., promote the initiation, progression, and healing of tissue injury; facilitate tissue remodeling associated with various pathologies; and participate in initiation and resolution of inflammation in tissues<sup>51,52</sup>. Macrophages are a heterogeneous cell types with high plasticity and display a variety of phenotypes regulated by external stimuli, including the extracellular matrix<sup>53</sup>. A continuum of phenotypes exists that can be pragmatically divided into pro-inflammatory and tissue-repair macrophages. Phenotypic polarization of macrophages is regulated by pro-inflammatory stimuli that include Toll-like receptor (TLR) ligands and IFN $\gamma$ , which induce pro-inflammatory phenotypes, and by anti-inflammatory stimuli such as IL-4/IL13, immune complexes, the anti-inflammatory cytokines IL-10, and transforming growth factor- $\beta$  that induce tissue-repair phenotypes<sup>54–57</sup>. Although the effects of microenvironment, including ECM proteins and matricellular proteins specifically, on phenotypic differentiation of macrophages are undeniable, they are still poorly understood. Here, we have uncovered previously unrecognized cellular mechanisms for regulation of phenotypes and functions of macrophages by TSP-4 and the P387-TSP-4 variant.

## Materials and methods

### Animals

Mice were of C57BL/6 background, 12–16 weeks of age, 30–35 g of body weight, and fed on regular chow diet. Both genders were used (there were no gender-specific differences). In total, 3–12 mice per group were used in in vivo experiments as indicated in the Figure legend, and the experiment was repeated three times. *Thbs4*<sup>-/-</sup> mice were described previously<sup>5,14,24,25,30</sup>, as were TSP-4-P387KI mice<sup>24</sup>. Briefly, these mice express P387-TSP-4 under the endogenous TSP-4 gene promoter (only a fragment of DNA where the SNP was found was substituted). P387-TSP-4 is expressed in the same pattern as the naturally occurring A387 variant in WT mice by immunocytochemistry (data not shown). The KI mouse colony has been maintained for more than 10 years in C57Bl/6 background, and are periodically crossed with WT mice to avoid genetic drift. Also, the TSP-4-P387KI mice are genotyped to insure integrity of the line. The TSP-4-P387KI mice develop and reproduce normally, and do not have an overt phenotype without challenge. Control wild-type (WT) mice were from the same mouse colony as *Thbs4*<sup>-/-</sup> mice or P387-TSP-4-KI mice.

For experiments exclusively using WT mice, WT C57BL/6 mice were purchased from the Jackson Laboratories. Animals were housed in the AAALAC-approved animal facilities of the Cleveland Clinic. Animal procedures were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic in agreement with the NIH Guide for Animal Use.

### LPS-induced peritonitis

Mice were treated with intraperitoneal (IP) injection of lipopolysaccharide (LPS, 0.5  $\mu$ g/g, ThermoFisher) to induce peritonitis 72 h prior to isolation of peritoneal cells by lavage and resection of visceral peritoneum. Mouse peritoneal macrophages (MPM) were used directly for cell adhesion and migration experiments. mRNA was isolated from MPM and peritoneal tissue. Immunofluorescence was also done on peritoneal tissues. IP injection with PBS was used for the control groups of animals.

### Isolation and culture of primary macrophages

Bone-marrow-derived macrophages (BMDM) were isolated from the hind leg tibia and femur of WT, *Thbs4*<sup>-/-</sup>, or P387-TSP-4 KI mice. In each experiment, the bone marrow cells were collected from three individual mice without pooling, and differentiated into BMDM using 30 ng/mL M-CSF for 7 days. Each experiment (Western blot, qRT-PCR, adhesion assay, migration assay, cell survival, and cellular apoptosis) was conducted using macrophages from independent mice of the noted genotypes. All experiments were repeated at least three times. These cells were treated with 50 ng/mL GM-CSF, 50 ng/mL M-CSF,

40 ng/mL IL-4, 2000 IU/mL IFN $\gamma$ , 0.5  $\mu$ g/mL LPS. Following stimulations, cells were analyzed by Western blot and quantitative RT-PCR for specific protein/gene expression patterns.

#### Cell culture and cell lines

Mouse macrophage cell line RAW 264.7 and WBC264-9C was purchased from ATCC (Virginia, USA). DMEM media (RAW 264.7) and grown in Eagle's minimum essential medium (WBC264-9C) supplemented with 10% fetal bovine serum (FBS), sodium bi-carbonate (1.5 gm/L), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin at 37 °C with 5% CO $_2$  atmosphere according to ATCC recommendations. Cells were treated with 20 ng/mL GM-CSF (#NBP2-35066, Novus Biologicals), 20 ng/mL M-CSF (#14-8983-62, ThermoFisher), 40 ng/mL IL-4 (#574306, BioLegend), 1000 IU/mL IFN $\gamma$  (#NBP2-35071, Novus Biologicals), 0.5  $\mu$ g/mL ILipopolysaccharide (#00-4976-93, ThermoFisher), 25  $\mu$ M cycloheximide (#0970, R&D Systems) dissolved in serum-free DMEM. All RAW 264.7 cell experiments were done with cells between passage 4 and passage 8. Cells were exposed to a brief period of serum starvation in serum-free DMEM for 3 h followed by stimulation with pro-inflammatory stimuli (LPS, GM-CSF, and IFN $\gamma$ ), anti-inflammatory stimuli (M-CSF and IL-4), and cycloheximide (CHX) at above-mentioned doses for stated durations.

#### Western blot analysis

Cells were harvested and lysed on ice using RIPA lysis and extraction buffer (# 89901, Thermo Scientific) supplemented with Halt™ protease and phosphatase inhibitor cocktail (#78442, Thermo Scientific). Cell lysates were boiled with 2-mercaptoethanol- (#21985-023, Life Technologies) activated Laemmli sample buffer at 100 °C for 5 min. In total, 50 microgram of total protein was loaded in each well of a 8% Bis-Tris gel, run and transferred to the PVDF blotting membrane using a semidry transfer module. After blocking with 5% skim milk in TBST for an hour at room temperature, membranes were probed overnight at 4 °C with primary antibodies (anti TSP-4 primary monoclonal Ab (#sc-390734, Santa Cruz Biotechnology) at 1:100 dilution; anti  $\beta$ -actin Ab (#A5316, Sigma-Aldrich) at 1:10000 dilution) followed by HRP anti-mouse secondary antibodies (#sc-516102, Santa Cruz Biotechnology and #1706516, Bio-Rad; respectively) incubation and developed with Pierce® ECL western blotting substrate on X-ray films for a time optimal to compare data points in each experiment.

#### Quantitative real-time RT-PCR analysis

The total RNA was extracted using TRIzol Reagent (#15596026, ThermoFisher) followed by cDNA synthesis using SuperScript™ First-Strand Synthesis System

(#11904018, ThermoFisher). The TaqMan® probe for *Thbs4* (Assay ID# Mm03003598\_s1, ThermoFisher), *CD68* (Assay ID# Mm03047343\_m1, ThermoFisher), *CD38* (Assay ID# Mm01220906\_m1, ThermoFisher), *Egr2* (Assay ID# Mm00456650\_m1, ThermoFisher), *Nos2* (Assay ID# Mm00440502\_m1, ThermoFisher), *Arg1* (Assay ID# Mm00475988\_m1, ThermoFisher) were used for detecting respective genes expressions with *Stx5a* (Assay ID# Mm00502335\_m1, ThermoFisher) and *ACTB* (Assay ID# Mm02619580\_g1, ThermoFisher) were used as housekeeping gene controls.

#### Macrophage differentiation and survival assays

Cells were seeded in 24-well plates at 300,000 cells/well in M1 and M2 differentiation media (#C-28055, #C-28056, Promo Cell). Initial attachment of cells was determined after 3 h using CyQUANT live-cell quantification kits. Cells were kept into M1 and M2 differentiation media for 5 days followed by total live-cell quantification using CyQUANT reagent. Each group of live-cell quantification after 5-day time points was normalized to their respective 3-h initial attachment quantification, and compared to assess cell survival of macrophages from WT or *Thbs4*<sup>-/-</sup> or P387-TSP-4-KI mice.

#### BMDM apoptosis assay

BMDM isolated from WT, *Thbs4*<sup>-/-</sup>, or P387-TSP-4-KI mice were differentiated with 50 ng/mL GM-CSF for 7 days. BMDM were treated with 0.5  $\mu$ g/mL LPS in serum-free media for 24 h followed by qRT-PCR for *Bax*, *Bcl2*, and *Caspase 3* (Casp3) expression.

For apoptosis assays, BMDM were seeded into 96-well plates (20,000 cells/well) and treated with 0.5  $\mu$ g/mL LPS for 48 h. Apoptosis was measured using Cell Meter™ No-Wash Live Cell Caspase 3/7 Activity Assay Kits (#20250, AAT Bioquest). Caspase 3/7 activity data were normalized to the total number of live cells (determined by CyQUANT kit) in each of the respective experimental groups.

#### In vitro adhesion assays

Adhesion assays were performed as previously described<sup>14,24,25,37,42</sup>. MPM were isolated from WT, *Thbs4*<sup>-/-</sup>, and P387-TSP-4-KI mice 72 h after treatment with 0.25  $\mu$ g/g LPS, and 5  $\times$  10<sup>3</sup> cells were added for 1 h at 37 °C to wells of 24-well plates (Corning) pre-coated with fibronectin (Sigma-Aldrich) or without coating.

#### In vitro migration assays

MPM were isolated from LPS treated WT, *Thbs4*<sup>-/-</sup>, and P387-TSP-4-KI mice, and 0.2  $\times$  10<sup>6</sup> MPM were resuspended in the serum-free DMEM and transferred into the trans-well chambers (Corning, Corning, NY, USA). FBS (20%) was used as a chemo-attractant in the

bottom chambers. The cells were incubated at 37 °C for 4 h, the medium was aspirated, and attached cells were removed from the surface of the upper chamber using Q-tips. The plates were frozen at −80 °C for 3 h, and DNA from remaining cells was quantified using the CyQUANT reagent (Invitrogen, Carlsbad, CA, USA).

#### Immunohistochemistry, immunofluorescence, confocal imaging, and quantification of macrophage markers

Sections of mice peritoneum (three mice of each genotype, with at least two sections of peritoneal tissue from each) were stained with primary rat anti-mouse CD68 antibody (MCA1957B, Bio-Rad), rabbit anti-mouse CD38 antibody (bs-0979R, Bioss Antibodies), rabbit anti-mouse Egr2 (ab90518, Abcam) antibody using the Vecta Stain ABC Kit. Visualization after staining with the antibodies was performed using a high-resolution slide scanner, Leica Aperio AT2 slide scanner (Leica Microsystems, GmbH, Wetzlar, Germany) was used to scan images of whole slides at ×20 magnification and quantified to determine the percentage of the stained area using Adobe photoshop CS6 (Media Cybernetics). The person performing quantification was blinded to the assignment of animals between groups. For immunofluorescence, rat anti-mouse CD68 (MCA1957B, Bio-Rad) and goat anti-human TSP-4 (AF2390, R&D Systems) were used with corresponding secondary antibodies (1:1000). Secondary antibodies were anti-goat NL557 conjugated donkey IgG (NL001 R&D Systems) and goat polyclonal antibody to rat IgG Alexa Fluor 488 (ab150161, Abcam). Images were taken at a high-resolution confocal microscope (Leica DM 2500) at ×63 magnification. All sections with primary antibodies were incubated for 2 h at 4 °C followed by incubating sections in secondary antibodies for 45 min at 4 °C.

#### Statistical analysis

Group size was calculated based on the previous data obtained in mouse models<sup>5,14,24,30</sup>. Analyses of the data were performed using Sigma Plot Software (Systat Software, San Jose, CA, USA): Student's *t* test and one-way ANOVA were used to determine the significance of parametric data, and Wilcoxon rank-sum test was used for nonparametric data. The significance level was set at  $p = 0.05$ . The data are presented as mean ± SEM; the number of biological repeats are listed in each figure legend. Dot plots for each figure are provided as Supplementary Figures.

## Results

### LPS and pro-inflammatory cytokines induce TSP-4 expression in macrophages

LPS induced TSP-4 in a time-dependent manner in RAW264.7 and BMDM (Fig. 1a). Most TSP-4 was

intracellular in both macrophage types (Fig. 1b). In both cell types, both the protein levels and mRNA levels were increased in response to LPS stimulation of cells (Fig. 1a, c). The decreases in protein levels in cells treated with cycloheximide were comparable with untreated cells, also consistent with the transcriptional mechanism or increased RNA stability.

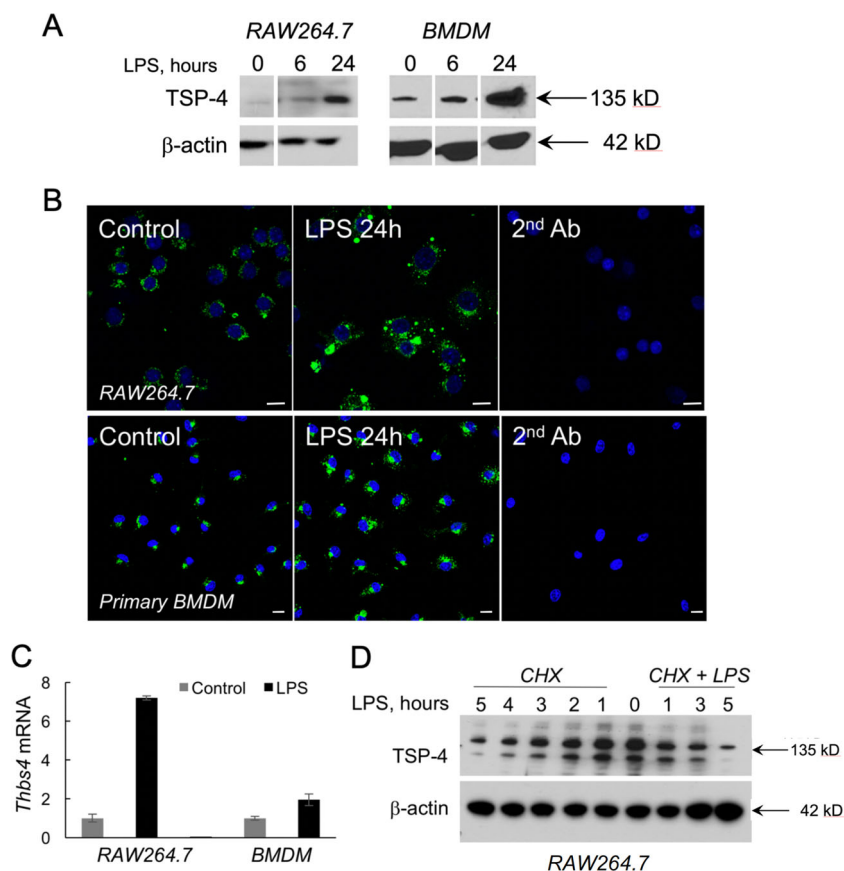
We examined the effects of pro-inflammatory (LPS, IFN $\gamma$ , and GM-CSF) and anti-inflammatory stimuli (M-CSF and IL-4) on the levels of TSP-4 protein and mRNA in RAW 264.7 cells (Fig. 2a–c) and in BMDM (Fig. 2d–f). BMDM were isolated from mice and cultured in macrophage differentiation media for 7 days. The purity was assessed by flow cytometry using anti-CD11b and F4/80 staining and was 93–98% (Supplementary Fig. 1). Western blot analysis showed a gradual increase in TSP-4 protein levels with pro-inflammatory stimuli LPS, IFN $\gamma$ , and GM-CSF. Conversely, TSP-4 protein levels reduced with anti-inflammatory cytokine IL-4 and M-CSF stimulation (Fig. 2 a, b, d, e; Supplementary Fig. 2). Average TSP-4 protein amount was quantified from three independent experiments, and normalized to  $\beta$ -actin expression as a loading control. Anti-inflammatory stimuli caused significant ( $*p < 0.05$ ) reduction in TSP-4 protein levels (Fig. 2b, d–f; Supplementary Fig. 2). In addition, equal loading and transfer of protein was confirmed by staining the membranes with Ponceau Red (Supplementary Fig. 3).

*THBS4* mRNA levels were measured by quantitative RT-PCR (Fig. 2c, f). There was a significant ( $*p < 0.05$ ) increase in *THBS4* expression upon stimulation with pro-inflammatory stimuli and a decrease in *THBS4* expression with anti-inflammatory stimuli.

Expression of markers of both pro-inflammatory (CD38 and *Nos2*, Fig. 3a–d; Supplementary Fig. 4A–D) and tissue-repair (Egr-2 and *Arg1*, Fig. 3e–h; Supplementary Fig. 4E–H) macrophages was measured in RAW 264.7 cells and BMDM in response to pro-inflammatory and anti-inflammatory stimuli, respectively. Following stimulation with IFN $\gamma$  (1000 IU/mL), GM-CSF (20 ng/mL), LPS (0.5  $\mu$ g/mL), M-CSF (20 ng/mL), or IL-4 (40 ng/mL), increased TSP-4 levels were detected (Fig. 2) and correlated with increased CD38 and *Nos2* expression. Decrease in TSP-4 levels upon stimulation with anti-inflammatory IL-4 and M-CSF (Fig. 2) was associated with increased Egr-2 and *Arg1* expression.

### TSP-4 promotes differentiation of macrophages into pro-inflammatory phenotype

To understand whether TSP-4 promotes the differentiation of macrophages into pro-inflammatory phenotype or whether the increased production of TSP-4 results from differentiation but does not affect the process, BMDM from WT and *Thbs4*<sup>−/−</sup> mice were stimulated



**Fig. 1** LPS induces TSP-4 in cultured macrophages. **a** Cultured macrophage-like RAW264.7 cells and mouse bone-marrow-derived macrophages (BMDM) were treated with LPS (0.5  $\mu$ g/ml for 1–24 h); western blotting with anti-TSP-4 Ab. **b** Cultured RAW264.7 (upper panels) and BMDM (lower panels) treated with LPS for 24 h were stained with anti-TSP-4 Ab; green = TSP-4, blue = nuclei, DAPI. Scale bar is 10  $\mu$ m. **c** RAW 264.7 and BMDM cells were treated with LPS (0.5  $\mu$ g/ml) for 24 h, and qRT-PCR was done (control = PBS treated); **d** RAW 264.7 cells were treated with CHX (25  $\mu$ M) for 1–5 h and CHX (25  $\mu$ M) + LPS (0.5  $\mu$ g/ml) for 1–3 h followed by western blot detection of TSP-4 ( $\beta$ -actin as loading controls). CHX was added at time 0, followed by addition of LPS.

with LPS (0.5  $\mu$ g/ml) and IFN $\gamma$  (1000 IU/ml), and the levels of mRNA of the markers of pro-inflammatory differentiation of macrophages CD38 and Nos2 were measured by real-time PCR (Fig. 4a; Supplementary Fig. 5A). The levels of both markers were significantly decreased in BMDM from *Thbs4*<sup>-/-</sup> mice, suggesting that TSP-4 promotes the differentiation into pro-inflammatory phenotype. The basal levels of CD38, a marker of pro-inflammatory macrophages, was unchanged in *Thbs4*<sup>-/-</sup> mice and increased in P387-TSP4-KI mice (Supplementary Fig. 6). The basal levels of Nos2, another marker of pro-inflammatory macrophages, was unchanged in *Thbs4*<sup>-/-</sup> mice (Supplementary Fig. 6B).

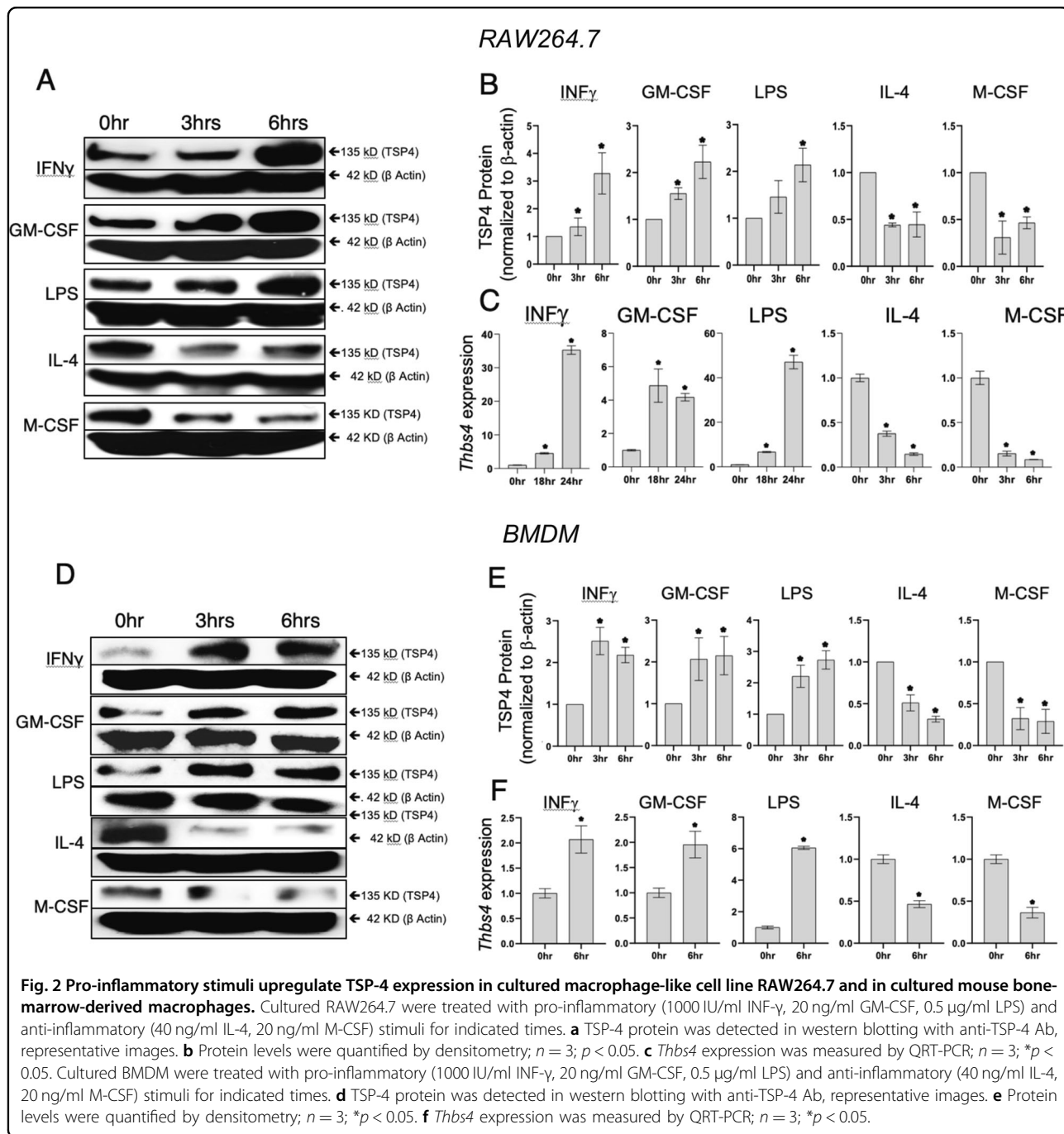
BMDM from WT and *Thbs4*<sup>-/-</sup> mice were incubated with recombinant TSP-4 (rTSP-4, 5  $\mu$ g/ml)<sup>14,24,37,49</sup> for 6 h, and the expression of markers of pro-inflammatory macrophages was measured by QRT-PCR (Fig. 4b; Supplementary Fig. 5B). rTSP-4 induced a ~100-fold increase in the level of CD38 expression in both WT and *Thbs4*<sup>-/-</sup>

cells and a 1000–4000 fold increase in NOS2 expression, suggesting that extracellular TSP-4 promotes pro-inflammatory polarization of macrophages.

When cultured BMDM from WT and *Thbs4*<sup>-/-</sup> mice were stimulated with IL-4 and M-CSF, the levels of both markers were higher in the knockout cells without stimulation and in knockout cells stimulated with IL-4 (Fig. 4c), suggesting that in the absence of TSP-4, polarization into a tissue-repair phenotype increases spontaneously.

#### TSP-4 regulates the survival of pro-inflammatory macrophages

Programmed death is a natural fate of pro-inflammatory macrophages and is associated with release of inflammatory signals<sup>58–65</sup>. Knowing that TSP-4 expression promotes pro-inflammatory phenotype of macrophages, we investigated the effect of TSP-4 on cultured macrophage apoptosis.



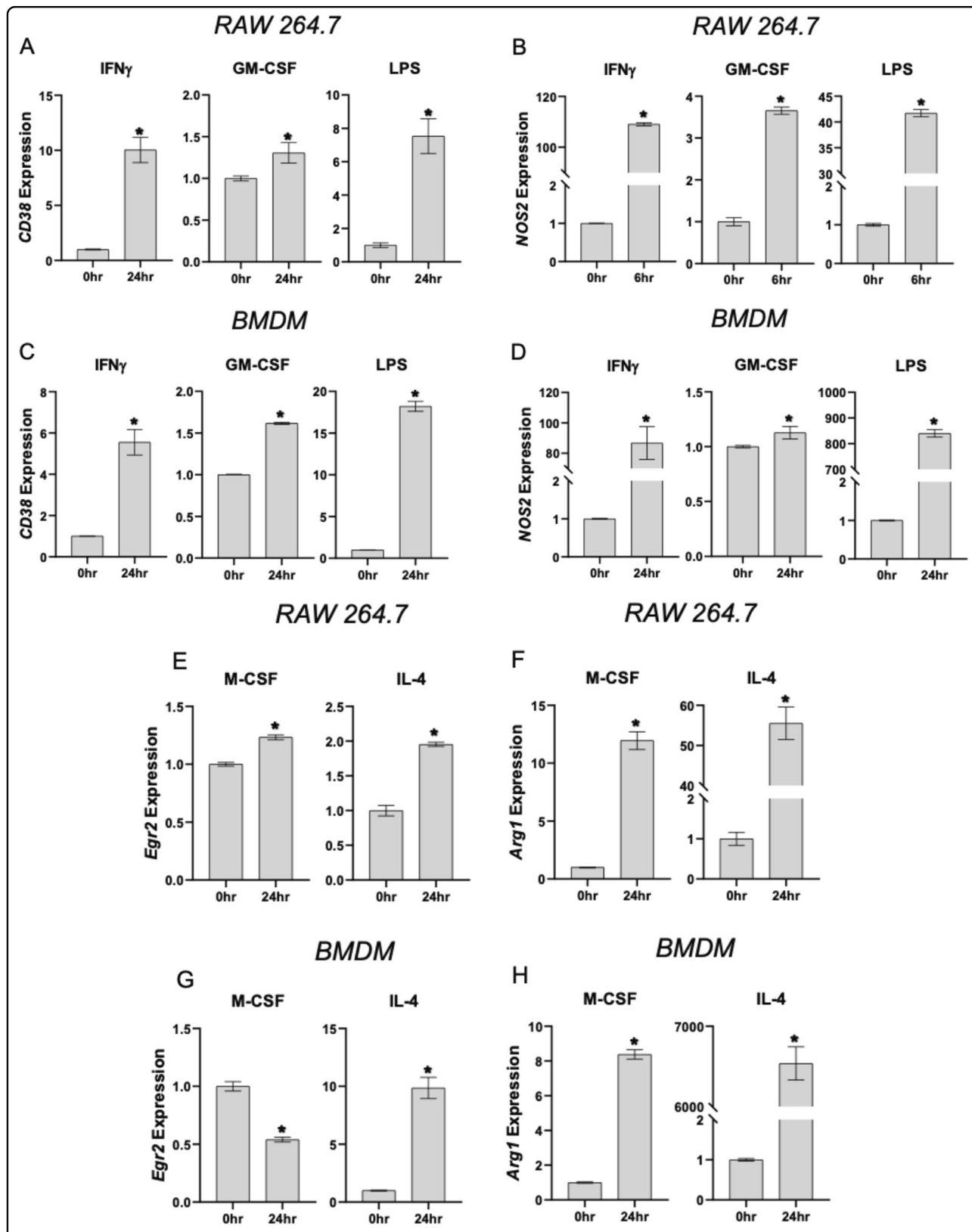
When BMDM from WT, *Thbs4*<sup>-/-</sup>, and P387-TSP-4-KI mice were differentiated in the pro-inflammatory (M1 medium) or tissue-repair differentiation (M2 medium), the survival of cultured pro-inflammatory BMDM but not tissue-repair BMDM was increased in the *Thbs4*<sup>-/-</sup> cells and decreased in P387-TSP-4-KI cells (Fig. 5a; Supplementary Fig. 7A).

Monocytes were isolated from mouse blood and differentiated in M1 or M2 media. In M1 or M2 media, lack

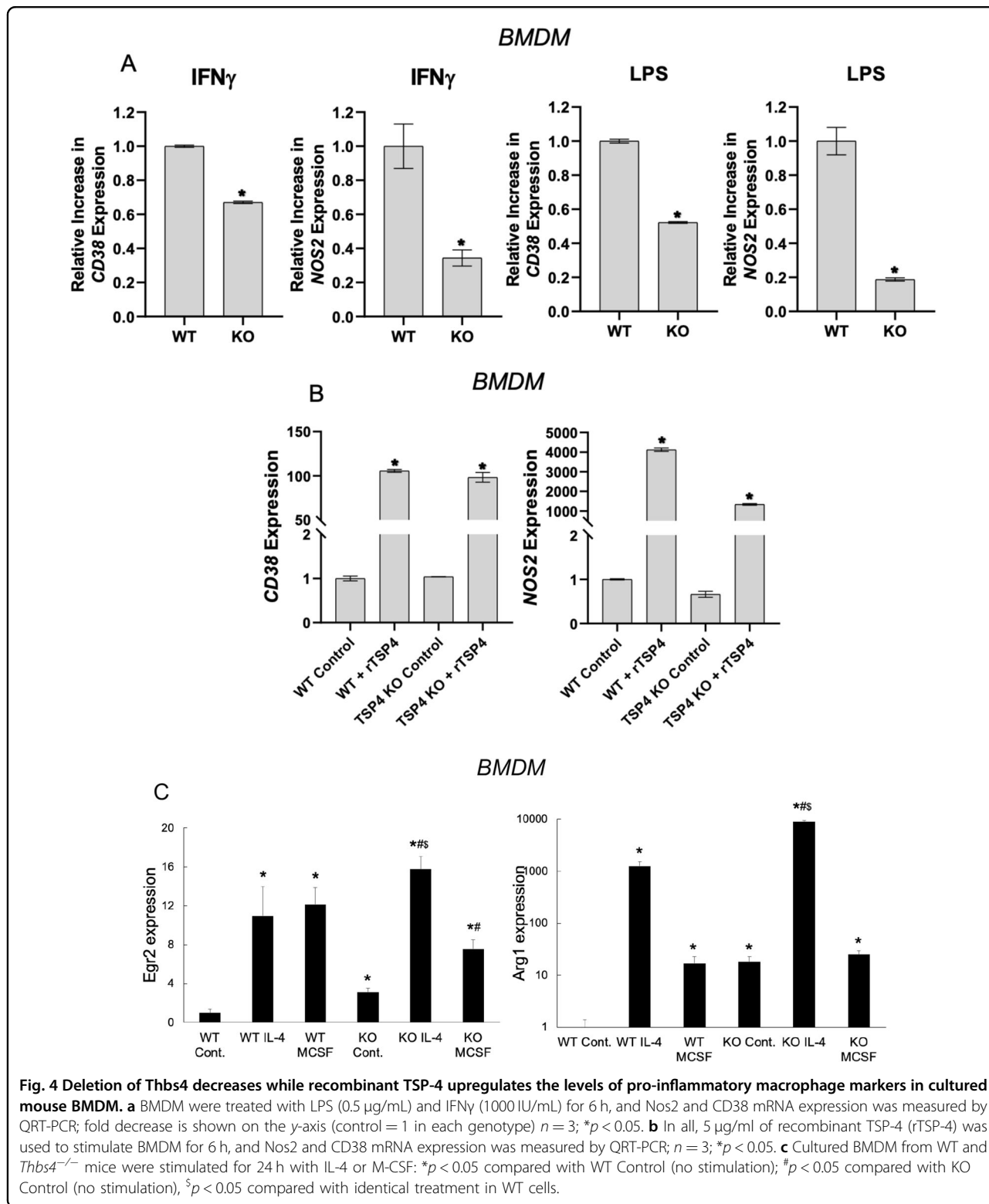
of TSP-4 resulted significantly higher survival of cells, but P387-TSP-4 did not affect cell number (Fig. 5b).

### TSP-4 promotes apoptosis in pro-inflammatory macrophages

Expression of *Bax*, *Bcl2*, and *Casp3* and the activity of Caspase 3/7 were measured in the BMDM differentiated into pro-inflammatory macrophages (Fig. 5c, d; Supplementary Fig. 7C). Lower apoptotic activity was



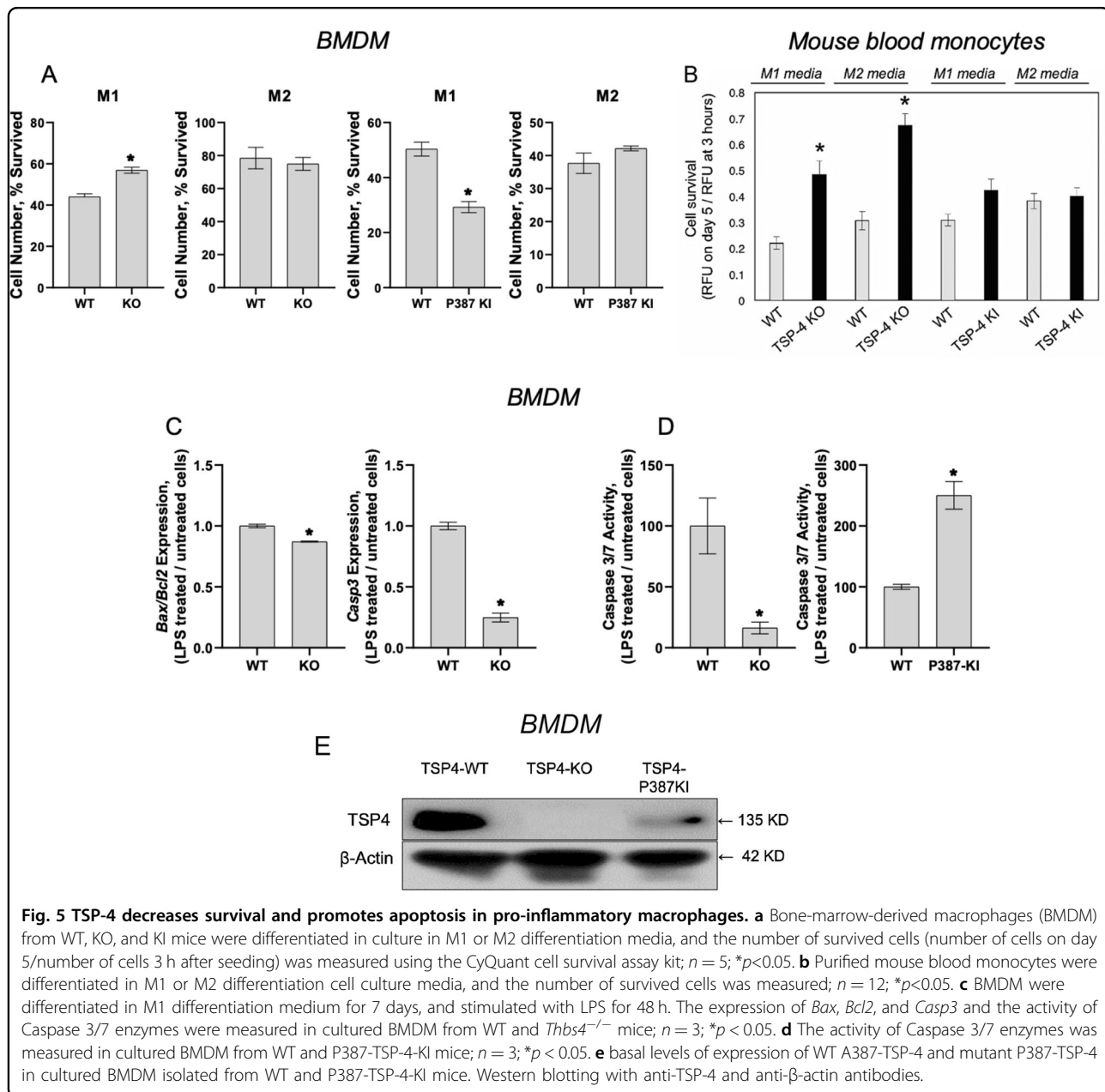
**Fig. 3 Expression of markers of pro-inflammatory and tissue-repair macrophages in response to stimulation with pro-inflammatory and anti-inflammatory stimuli.** Cultured RAW264.7 (a, b) and BMDM (c, d) were stimulated as described in the Materials and methods section, and mRNA of the markers CD38 and NOS2 was analyzed by QRT-PCR. Fold increase over unstimulated cells, C = control, no stimulation;  $n = 3$ ;  $*p < 0.05$ . Cultured RAW264.7 (e, f) and BMDM (g, h) were stimulated as described in the Materials and methods section, and mRNA of the markers Egr-2 and Arg1 was analyzed by QRT-PCR. Fold increase over unstimulated cells, C = control, no stimulation;  $n = 3$ ;  $*p < 0.05$ .



detected in *Thbs4*<sup>-/-</sup> cells as indicated by decreased *Bax/Bcl2* ratio, decreased expression of *Casp3*, and activity of Caspase 3/7 (Fig. 5c and 5d, left panel). In contrast, the activity of Caspase 3/7 was upregulated in

response to LPS in P387-TSP-4-KI BMDM (Fig. 5d, right panel). This differential effect is consistent with the higher activity of P387-TSP-4 in other cellular responses<sup>24,37,42,49</sup>.



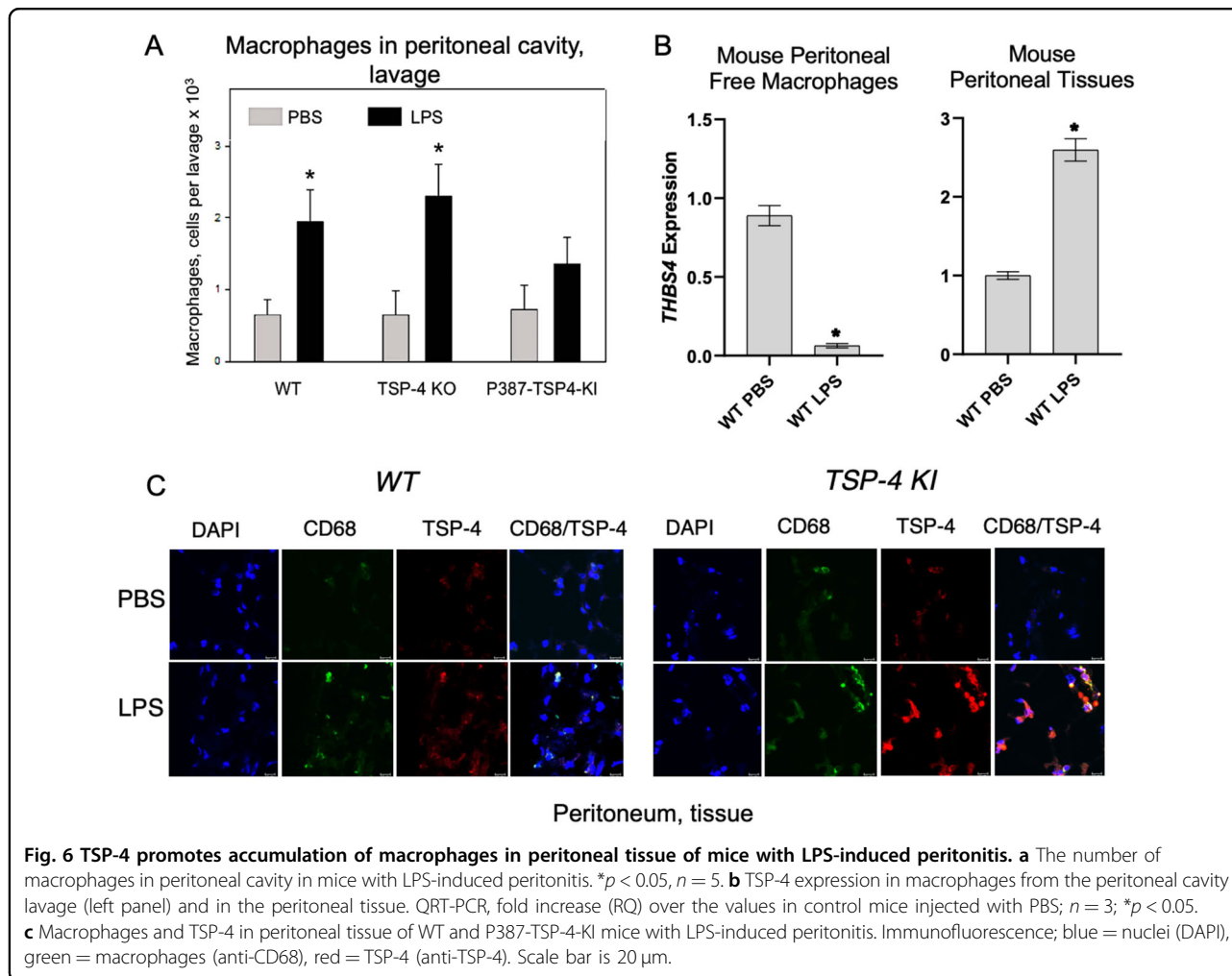


The basal production of TSP-4 protein was not detected in *Thbs4*<sup>-/-</sup> BMDM and was lower in P387-TSP-4-KI cells (Fig. 5e), and thus could not explain the differential effects of TSP-4 variants.

#### Macrophages in peritoneal cavity and tissue of mice with LPS-induced peritonitis

To evaluate the expression and the effect of TSP-4 on macrophages in a model of acute inflammation, we induced peritonitis in WT, *Thbs4*<sup>-/-</sup>, and P387-TSP-4-KI mice by IP injection of LPS. Mice were sacrificed 72 h later, and cells (> 90% macrophages) were collected by peritoneal lavage

and quantified (Fig. 6a; Supplementary Fig. 8). Surprisingly, TSP-4 deletion in the *Thbs4*<sup>-/-</sup> mice did not reduce the number of macrophages recovered in a saline lavage (free macrophages) compared with WT mice, but we collected fewer macrophages from the cavity of P387-TSP-4-KI mice than from WT mice after LPS-induced peritonitis (Fig. 6a). To find out whether the LPS-induced inflammation affects the expression of TSP-4 in peritoneal tissues and in free macrophages, we assessed the levels of *Thbs4* mRNA (Fig. 6b). TSP-4 expression was dramatically decreased in free peritoneal macrophages recovered by lavage and significantly increased in peritoneal tissue of WT mice,



suggesting that high levels of TSP-4 in tissue and in macrophages may promote their accumulation in the peritoneal tissue and/or prevent their egress into the cavity.

Immunofluorescence with anti-CD68 and anti-TSP-4 antibodies revealed that TSP-4 protein is associated with macrophages in peritoneal tissue in both WT and P387-TSP-4-KI mice (Fig. 6c).

There were no significant changes in monocyte counts in the blood from the two transgenic mice (Supplementary Fig. 9).

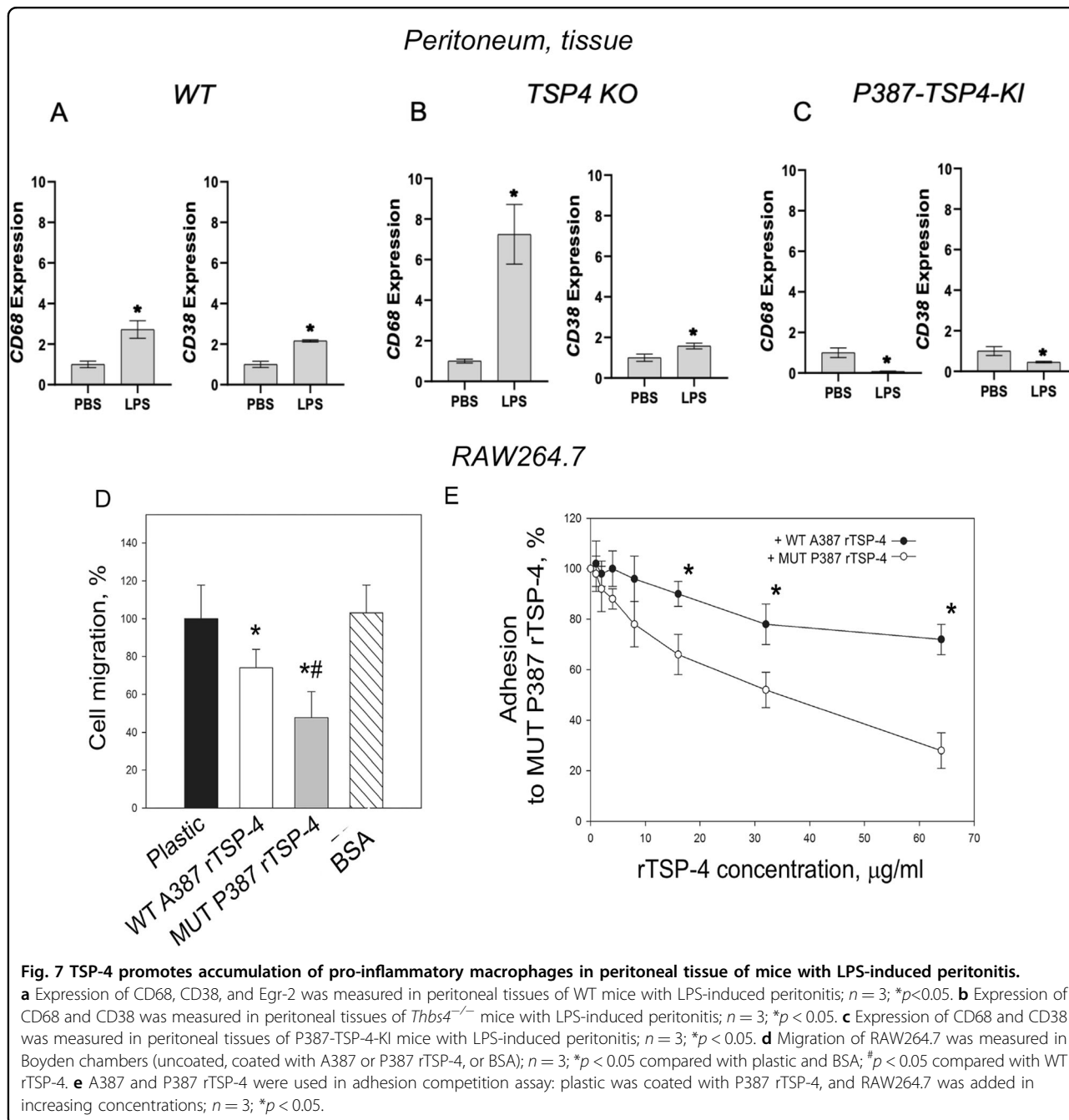
#### TSP-4 increases the number of pro-inflammatory macrophages in peritoneal tissue

Expression of CD68, a marker of macrophages, was upregulated in peritoneal tissues of WT (Fig. 7a; Supplementary Fig. 10A) and even more dramatically in *Thbs4*<sup>-/-</sup> (Fig. 7b) mice with LPS-induced peritonitis. TSP-4 deficiency resulted in higher accumulation of macrophages in peritoneal tissues. In a view of a lack of differences in numbers of macrophages in peritoneal cavity (Fig. 6a) and the equal numbers of macrophages in blood

(Supplementary Fig. 5), this suggested the effect of TSP-4 on macrophage survival in tissues, consistent with our observations in cultured macrophages. The expression of the marker of pro-inflammatory macrophages CD38 was upregulated in both WT and *Thbs4*<sup>-/-</sup> mice (Fig. 7a, b; Supplementary Fig. 10A, B), but the expression of *Egr-2*, a marker of tissue-repair macrophages, was decreased (Fig. 7a), suggesting that most macrophages retained in the peritoneal tissue are pro-inflammatory. Consistent with the difference between WT and *Thbs4*<sup>-/-</sup> mice and the P387-TSP-4, P387-TSP-4 reduced the accumulation of macrophages in peritoneal tissue (Fig. 7c; Supplementary Fig. 10C), suggesting more profound effect on their survival.

#### P387-TSP-4 is more active in supporting the adhesion of monocytes and macrophages

We used murine macrophage-like cell line RAW264.7 to assess the effect of A387-TSP-4 and P387-TSP-4 on macrophage adhesion and migration (Fig. 7). RAW264.7 cells migrated less on rTSP-4 as a substrate, and P387-



TSP-4 further decreased their migration (Fig. 7a). RAW264.7 adhered significantly better to P387-TSP-4 (Fig. 7b), although both A387 and P387-TSP-4 supported the adhesion of macrophage-like cells.

### Discussion

High expression of TSP-4 in remodeling tissues, particularly in heart disease and cancer<sup>5-12,14-29</sup>, suggested that this matricellular protein may regulate fibrosis and angiogenesis. The effects of TSP-4 on matrix remodeling

has been demonstrated in *Thbs4*<sup>-/-</sup> mice by us and others<sup>5-7,30</sup>, and we recently reported that TSP-4 is pro-angiogenic<sup>24,25</sup> in contrast to the prominent role of TSP-1 as an anti-angiogenic protein<sup>2,66,67</sup>. Tissue remodeling is associated with inflammation, and we reported that TSP-4 deficiency results in reduced inflammation in atherosclerotic lesions in *ApoE*<sup>-/-</sup> mice: the number of macrophages in the atherosclerotic lesion and the local vascular inflammation were reduced in *Thbs4*<sup>-/-</sup>/*ApoE*<sup>-/-</sup> mice<sup>14</sup>. To investigate the roles of TSP-4 and its variants

in inflammation, we examined the effects of TSP-4 on cultured macrophages and on macrophages in an LPS-induced mouse peritonitis model.

Several agents that are known to promote pro-inflammatory differentiation of macrophages (LPS, GM-CSF, and INF $\gamma$ <sup>68</sup>) or tissue-repair differentiation (IL-4<sup>68</sup> and M-CSF<sup>69</sup>) were used to stimulate cultured RAW264.7 and BMDM. LPS that was also used to induce peritonitis, increased TSP-4 production by macrophages in a time-dependent manner. mRNA of *Thbs4* was also upregulated, and production of the protein was efficiently blocked by cycloheximide, suggesting transcriptional regulation or reduced RNA stability. The newly synthesized protein accumulated in vesicle-like structures inside the cells, and was not efficiently secreted into the medium or matrix (not shown). Intracellular functions have been reported for TSPs<sup>7</sup>. Finding of increased amounts of intracellular TSP-4 suggested that such intracellular functions of TSP-4 may be activated by the treatment or that macrophages accumulate protein in vesicles that may be released in specific tissues where an inflammation response has been triggered. Alternatively, the intracellular protein could reflect uptake of the secreted TSP-4 by the cells. When exogenous recombinant TSP-4 was used in experiments with cultured BMDM, it efficiently induced expression of markers of pro-inflammatory macrophages, suggesting that this pro-inflammatory effect of TSP-4 on macrophages depends on TSP-4 interactions with cellular receptors upon TSP-4 secretion into ECM.

All pro-inflammatory signals that resulted in pro-inflammatory differentiation with increased expression of CD38 and *Nos2* (markers of pro-inflammatory macrophages) and decreased expression of *Egr-2* and *Arg1* (markers of tissue-repair macrophages) also increased TSP-4 expression and production. To the contrary, anti-inflammatory signals promoting the tissue-repair differentiation of macrophages (decreased CD38 and *Nos2* and increased *Egr-2* and *Arg1*) decreased the expression of *Thbs4* and the production of the TSP-4 protein. Thus, our results suggested a novel role for TSP-4 in inflammation: support of pro-inflammatory functions of macrophages. When BMDM from *Thbs4*<sup>-/-</sup> mice were stimulated with pro-inflammatory signals, the levels of markers of pro-inflammatory macrophages were decreased, demonstrating that TSP-4 actively promotes macrophage differentiation into pro-inflammatory phenotype.

We analyzed cultured primary BMDM and blood monocytes, and found that survival of both cell types was higher in *Thbs4*<sup>-/-</sup> macrophages cultured in the M1 medium. P387-TSP-4 in macrophages from P387-TSP4KI mice significantly decreased their survival. Knowing that TSP-4 expression is associated with pro-inflammatory macrophages, we hypothesized that the regulation of

macrophage number in the peritonitis model may be associated with their ability to differentiate into the pro-inflammatory phenotype and to commit to apoptosis, a process closely associated with pro-inflammatory differentiation. Pro-inflammatory macrophages produce and release inflammatory stimuli, and this release is associated with their transition to apoptosis<sup>58-65</sup>. Both our in vivo and in vitro results suggested that TSP-4 is needed for completion of pro-inflammatory differentiation of macrophages, their transition into apoptosis, and their release of pro-inflammatory signals. This sequence of events was observed using cultured RAW264.7 and BMDM. TSP-4 KO reduced caspase 3 gene expression and activity of caspase 3/caspase 7, as well as the ratio of expressed Bax/Bcl2 in BMDM in response to LPS stimulation, confirming that TSP-4 promotes apoptosis in pro-inflammatory macrophages. P387-TSP-4 BMDM from P387-TSP-4-KI mice had increased expression of apoptotic markers compared with BMDM isolated from WT mice, consistent with higher activity of this TSP-4 variant in many cellular effects and interactions that we previously reported.

TSP-4 increased accumulation of macrophages in peritoneal tissues, presumably by increasing their adhesive properties, reducing the macrophage migration as was demonstrated in cultured cells, and, as a result, decreasing the egress of macrophages from the peritoneal tissue into the cavity. LPS injection significantly increased the number of macrophages recovered in the lavage of the peritoneal cavity of WT mice and *Thbs4*<sup>-/-</sup> mice, but, in P387-TSP-4-KI mice, the number of recovered macrophages was not increased by LPS compared with the saline control. Free peritoneal macrophages recovered by lavage produced very little TSP-4, while the levels of TSP-4 mRNA and protein were increased in peritoneal tissue as was detected by QRT-PCR and immunofluorescence. TSP-4 production was associated with CD68-positive cells, macrophages, in the tissue. It remains an open question whether some blood monocytes are predisposed to produce larger amounts of TSP-4, restricting their egress into the peritoneal cavity, and whether a fraction of blood monocytes is unable to produce TSP-4 and thus, macrophages differentiated from these blood monocytes egress into the cavity more easily.

The production of TSP-4 by the blood cells has not been previously reported, and the ability of macrophages to produce TSP-4 protein has not been recognized. However, as we report here, we identified several pro-inflammatory agonists that stimulate macrophages to produce TSP-4 both in cell culture and in vivo.

P387-TSP-4 is a SNP variant of TSP-4 that is carried by > 30% of North American population<sup>18</sup>. In our previous studies of this TSP-4 variant in vitro in cell culture, and

in vivo in a mouse model, we found that P387-TSP-4 was more active in all cellular effects and interactions tested, and was less susceptible to proteolytic degradation<sup>5,14,24,25,30,37,42,49</sup>. Thus, the lack of increased macrophage egress into the peritoneal cavity of P387-TSP-4-KI mice was surprising and suggested that P387-TSP-4 prevented macrophage egress from tissues leading to increased accumulation in peritoneal tissues due to its higher adhesive properties than the WT, A387-TSP-4 form.

TSP-4 supports the adhesion of leukocytes<sup>14,37</sup> and macrophages specifically<sup>14</sup>. We have performed adhesion and migration assays with macrophage-like cells RAW264.7, and demonstrated that both A387- and P387-TSP-4 support the adhesion of macrophages and reduce their migratory activity, but, as was expected based on our previous publications<sup>24,37,42,49</sup>, P387-TSP-4 is more effective in these functions.

Although the accumulation of macrophages in peritoneal tissue was increased in response to LPS in all three mouse strains, WT expressing the A387-TSP-4 isoform, *Thbs4*<sup>-/-</sup>, and P387-TSP-4-KI, the relative increase in macrophage accumulation (in comparison with mice receiving PBS) was surprisingly higher in *Thbs4*<sup>-/-</sup> mice and significantly lower in P387-TSP-4-KI mice. Since the number of monocytes in blood was not significantly different between the three genotypes, and the number of macrophages in peritoneal cavity could not account for any differences in macrophage accumulation in tissues, these results suggested that TSP-4 levels might affect survival of macrophages in tissues. In addition to the effect of TSP-4 on CD68 levels, CD38 levels also followed the trend, suggesting that TSP-4 acts on pro-inflammatory macrophages rather than tissue-repair macrophages. Similar to other TSP-4 functions, the P387 variant of TSP-4 was more active in exerting these effects on macrophages, promoting inflammation, accumulation of macrophages in tissue, and their transition to apoptosis that allows macrophages to release pro-inflammatory stimuli and sustain a pro-inflammatory environment.

The association of TSP-4 with tissue inflammation and pro-inflammatory differentiation of macrophages was observed in vivo, in peritonitis model, as well as in cultured macrophages. TSP-4 carries out a dual role in inflammation by facilitating macrophage adhesion and promoting the pro-apoptotic response of macrophages at sites of tissue inflammation. This association suggests a new and unanticipated role for TSP-4 in inflammation: TSP-4 and its P387 variant are produced by macrophages in response to inflammatory stimuli and regulate the accumulation of macrophages in tissues and their pro-inflammatory functions.

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#### Conflict of interest

The authors declare that they have no conflict of interest.

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