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## Activation of innate immunity by 14-3-3 $\epsilon$ , a new potential alarmin in osteoarthritis

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1                   **ACTIVATION OF INNATE IMMUNITY BY 14-3-3  $\epsilon$ , A NEW POTENTIAL**  
2                   **ALARMIN IN OSTEOARTHRITIS**

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## Abstract

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Objective: The innate immune system plays a central role in osteoarthritis (OA). We identified 14-3-3 $\epsilon$  as a novel mediator that guides chondrocytes toward an inflammatory phenotype. 14-3-3 $\epsilon$  shares common characteristics with alarmins. These endogenous molecules, released into extracellular media, are increasingly incriminated in sustaining OA inflammation. Alarmins bind mainly to TLR2 and TLR4 receptors and polarize macrophages in the synovium. We investigated the effects of 14-3-3 $\epsilon$  in joint cells and tissues and its interactions with TLRs to define it as a new alarmin involved in OA.

Design: Chondrocyte, synoviocyte and macrophage cultures from murine or OA human samples were treated with 14-3-3 $\epsilon$ . To inhibit TLR2/4 in chondrocytes, blocking antibodies were used. Moreover, chondrocytes and bone marrow macrophage (BMM) cultures from KO TLRs mice were stimulated with 14-3-3 $\epsilon$ . Gene expression and release of inflammatory mediators (IL-6, MCP-1, TNF $\alpha$ ) were evaluated via RT-qPCR and ELISA.

Results: *In vitro*, 14-3-3 $\epsilon$  induced gene expression and release of IL6 and MCP1 in the treated cells. The inflammatory effects of 14-3-3 $\epsilon$  were significantly reduced following TLRs inhibition or in TLRs KO chondrocytes and BMM.

Conclusions: 14-3-3 $\epsilon$  is able to induce an inflammatory phenotype in synoviocytes, macrophages and chondrocytes in addition to polarizing macrophages. These effects seem to involve TLR2 or TLR4 to trigger innate immunity. Our results designate 14-3-3 $\epsilon$  as a novel alarmin in OA and as a new target either for therapeutic and/or prognostic purposes.

1 **Keywords :** 14-3-3  $\epsilon$ , alarmin, osteoarthritis, Innate immunity, TLR, synovitis

2 **Running headline :** 14-3-3  $\epsilon$  : a new alarmin in osteoarthritis

3

#### 4 **Abbreviations**

5 BMM: bone marrow derived macrophages

6 BSA: bovine serum albumin

7 cDNA: complementary deoxyribonucleic acid

8 DAMP: damage-associated molecular pattern

9 DMEM: Dulbecco's modified Eagle's medium

10 EDTA: Ethylenediaminetetraacetic acid

11 ELISA: enzyme linked immunosorbent assay

12 FBS: fetal bovine serum

13 FLS: Fibroblast-like synoviocyte

14 Glu: glutamine

15 H&E: Hematoxylin and eosin

16 HPRT: Hypoxanthine-guanine phosphoribosyltransferase

17 IL : interleukin

18 KO: Knockout

19 LPS: lipopolysaccharides

20 mAB: monoclonal antibody

- 1 MCP1 : monocyte chemoattractant protein-1
- 2 MMP: matrix metalloproteinases
- 3 mRNA: messenger ribonucleic acid
- 4 OA: osteoarthritis
- 5 OxPAPC: Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
- 6 PBS: phosphate buffered saline
- 7 PRRs: pattern-recognition receptors
- 8 PS: penicillin/streptomycin
- 9 RA: rheumatoid arthritis
- 10 RNA: ribonucleic acid
- 11 RT-PCR: real-time polymerase chain reaction
- 12 TLR: toll like receptor
- 13 TNF $\alpha$ : Tumor necrosis factor alpha
- 14 WT: wild-type
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## Introduction

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Osteoarthritis (OA) is a highly complex and the most prevalent joint disorder with a total of 242 million people affected worldwide<sup>1</sup>. Clinical symptoms include severe pain, joint stiffness and reduced function, which seriously decrease quality of life<sup>1</sup>. Unfortunately, only a few drugs are weakly effective for treating symptoms, and no disease-modifying osteoarthritis drugs (DMOADs) are available to date. Initially considered cartilage driven, OA is a much more complex disease with inflammatory mediators released by cartilage, bone and synovium<sup>2</sup>. Increasing evidence suggests that inflammation is present in OA and has raised the possibility that inflammation and the innate immune system could be active players in the development and progression of OA<sup>3,4</sup>. Innate immune responses involve the activation of resident leukocytes such as macrophages, production of inflammatory mediators (cytokines, chemokines, and lipid metabolites), recruitment of neutrophils and monocytes/macrophages, and aims to eliminate invading microorganisms and injured tissues<sup>5</sup>. Histopathological studies have confirmed that immune cell infiltration is extremely common in OA histological specimens<sup>6</sup>. The innate immune system participates in inflammation triggered by host molecules or fragments collectively called DAMPS (danger-associated molecular patterns) or alarmins<sup>5,7,8</sup>.

The term “alarmin” was proposed by Oppenheim and co-workers in 2005 to classify proteins that are rapidly released during infection or tissue damage, activating immune cells after interaction with their specific receptors<sup>7</sup>. Alarmins are now considered to be markers of destructive processes in joints<sup>9</sup>. Because of their fast release as a result of cell stressor nonprogrammed cell death, alarmins are among the first factors to be secreted and, as such, act as first responders to stimuli.

1 In addition to their role in disease initiation, alarmins also amplify and sustain  
2 inflammatory processes and, thus, play a notable role in the pathogenesis of  
3 inflammatory conditions<sup>10,11</sup>. A number of alarmins have been detected at high levels  
4 in OA tissues and synovial fluid, including HMGB1, UA, ATP, thymosin  $\beta$ 4 and  
5 various S100 proteins, enhancing catabolic processes and inflammatory responses  
6 that contribute to disease progression<sup>8,12-14</sup>.

7  
8         Synovitis, the inflammation of the synovium, can occur in early stages of OA<sup>15</sup>.  
9 OA synovitis directly contributes to several clinical signs and symptoms including joint  
10 swelling and effusion, and reflects the structural progression of the disease<sup>15,16</sup>.  
11 Moreover, synovitis is significantly associated with OA severity<sup>17</sup>. Macrophages are  
12 the main immune cell type in the healthy synovium and are likely the front-line cells  
13 that sense joint damage. These cells also contribute to OA progression in response  
14 to alarmins by producing MMPs and cytokines<sup>18</sup>. The main morphological  
15 characteristic of synovitis is macrophage accumulation in the intimal lining<sup>19</sup>.  
16 Macrophages are characterized by heterogeneity and plasticity in response to stimuli  
17 from their microenvironment, leading to a continuum of phenotypes where  
18 M1/M2 are the 2 extremes<sup>20</sup>. M1 macrophages are activated by interferon- $\gamma$  and  
19 lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF- $\alpha$ ), resulting in the  
20 secretion of large amounts of proinflammatory cytokines and mediators such as TNF-  
21  $\alpha$ , interleukin (IL)-1 and IL-6<sup>21</sup>. M2 macrophages have been further divided into  
22 specific subtypes and possess anti-inflammatory activity<sup>22</sup>. Studies have shown that  
23 macrophages accumulate and become polarized (M1 or M2) in the synovium during  
24 OA development<sup>23</sup>. The classification of macrophages into M1/M2 subtype is  
25 reductive. Recently, the heterogeneity of macrophage phenotypes in OA patients has

1 been studied and revealed a more complex classification <sup>24</sup>. However, the role of  
2 macrophages, their polarization in OA development and the underlying mechanisms  
3 are still unknown.

4 Activated synovial cells (synoviocytes or macrophages) secrete several  
5 degenerative enzymes and inflammatory mediators, as well as alarmins <sup>14,25</sup>. These  
6 alarmins activate pattern-recognition receptors (PRRs) including TLRs (toll-like  
7 receptor) in the OA-affected cartilage and synovium <sup>26,27</sup>, which in turn amplifies  
8 inflammation and degeneration of cartilage. TLRs are transmembrane receptors that  
9 display binding affinity for a variety of DAMPs. According to their ligands, location and  
10 signaling pathways, ten different genes can be distinguished in humans <sup>28</sup>. TLRs  
11 activate signaling pathways that result in the production of cytokines, chemokines,  
12 and various inducible molecules associated with the immune response. TLR2 and  
13 TLR4 are overexpressed in OA cartilage, and their presence correlates with  
14 histopathological damage <sup>29,30</sup>.

15 Our team identified 14-3-3 $\epsilon$  as a novel soluble mediator that is critical in the  
16 communication between subchondral bone and cartilage in OA <sup>31</sup>. The 14-3-3  
17 proteins, a family of seven isoforms ( $\beta$ ,  $\epsilon$ ,  $\gamma$ ,  $\eta$ ,  $\theta$ ,  $\sigma$ ,  $\xi$ ), are involved in a wide range  
18 of vital regulatory processes by binding to more than 200 intracellular proteins <sup>32</sup>.  
19 Under normal conditions, these proteins reside intracellularly; however, some reports  
20 indicate its presence extracellularly. Externalization of 14-3-3 appears to be mediated  
21 by a non-classical pathway, similar to IL1- $\beta$ , due to the absence of a signal peptide  
22 sequence <sup>33</sup>. 14-3-3 proteins are released into the extracellular space through an  
23 exosomal pathway <sup>34</sup>. Extracellular 14-3-3s are now thought to play an important role  
24 in the pathogenesis of certain inflammatory conditions. In OA, our team showed that  
25 14-3-3 $\epsilon$ , released by osteoblasts in response to mechanical stress, skews



1 chondrocytes toward a pro-catabolic phenotype by strongly inducing the expression  
2 of MMPs in an CD13/APN-dependent manner<sup>31,35</sup>. 14-3-3ε seems to share common  
3 characteristics with other alarmins (such as ATP and thymosin β4) in inducing  
4 degradation of the cartilage matrix<sup>8</sup>. Extracellular 14-3-3ε proteins could, therefore,  
5 be classified as alarmins that are derived from activated or damaged osteoblasts.

6 Our aim is to investigate the effects of 14-3-3ε on the different cell types of the  
7 joint and its role in the activation of innate immunity by studying interactions with its  
8 potential receptors TLR2 and TLR4.

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## Methods

### **Materials**

All reagents were purchased from Sigma-Aldrich (Lyon, France), unless stated otherwise. Fetal bovine serum (FBS) was obtained from Invitrogen (Cergy-Pontoise, France). Liberase TM and complete protease inhibitor mixture were from Roche Diagnostics (Meylan, France). Recombinant human 14-3-3 $\epsilon$  was from Enzo Life Sciences. Anti-TLR2 antibody and OXPAPc (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine) were from InvivoGen (Toulouse, France). Anti-TLR4 antibody was from Santa Cruz Technology (Heidelberg, Germany).

### **Collection of human OA synovium**

Human OA knee explants were obtained from patients undergoing total knee arthroplasty due to OA at Saint-Antoine Hospital (Paris) or at the Maussins clinic (Paris) (BioJOINT, a biobank of OA human knee , legal authorization: CPP Paris Ile de France V, CNIL reference: MMS/ HGT/AR177404). Informed consent for the use of tissue and clinical data was obtained from each patient before surgery. Experiments with human samples were approved by a French Institutional Review Board (Comité de Protection des Personnes, Paris Ile de France V and Commission Nationale de Informatique et des Libertés).

1

## 2 **Mice**

3 Mice on a C57BL/6 J background, 8–12 weeks old, were used in all experiments.  
4 Wild-type (WT) mice were purchased from Janvier Laboratories. The animal housing  
5 facility was granted approval (C 75-12-01) by the French Administration. All  
6 experiments were conducted according to the European Communities Council  
7 Directive (2010/63/UE) and approved by the Regional Animal Care and Use  
8 Committee (Ile-de-France, Paris, n°5; agreement number 00917.02 and 4625).

9 *Tlr2*<sup>-/-</sup>, and *Tlr4*<sup>-/-</sup> mice were kind gifts from Professor Shizuo Akira (Osaka  
10 University, Japan) and with the collaboration of Professor F. Pene (Institut Cochin,  
11 France). All knockout (KO) were maintained in the specific pathogen-free (SPF)  
12 animal facility of the Cochin Institute.

13

## 14 **Cell cultures**

15 Detailed protocols are described in supplemental data and (36).

16 Synovial explants : For explants, synovium from OA patients was cut into small  
17 pieces aseptically and incubate in 24-well plate before treatment.

18 Primary culture of synovial fibroblasts: Primary cultures of fibroblast like synoviocytes  
19 are obtained after enzymatic digestion (collagenase and DNase) of human synovial  
20 membrane samples (Biojoint biobank). The digestion solution was then placed in  
21 culture flasks during an overnight incubation. The digestion solution was removed,  
22 and adherent cells were washed 3 times with phosphate buffered saline (PBS) and  
23 cultured in FLS growth medium at 37°C in a humidified atmosphere (5% CO<sub>2</sub>). After

1 reaching their confluence, FLS were counted and cultured in 12-well plates for  
2 stimulations <sup>37</sup>.

3 Primary culture of murine articular chondrocytes: Mouse primary chondrocytes were  
4 isolated from the articular cartilage of 5 to 6-day-old C57Bl6 mice from Janvier (St.  
5 Berthevin, France). All experiments were performed according to protocols approved  
6 by the French and European ethics committees (Comité Régional d'Ethique en  
7 Expérimentation Animale N°3 de la région Ile de France). Each littermate among the  
8 mice was used for one experiment. After 1 week, the cells were incubated in fasting  
9 medium for 24h before treatment.

10 In addition, experiments were also performed using *TLR2*<sup>-/-</sup> and *TLR4*<sup>-/-</sup> mice.

11 Primary culture of murine bone marrow mononuclear (BMM) cells: Bone marrow  
12 mononuclear (BMM) phagocytic precursor cells were isolated from femurs and tibiae  
13 of *WT* and *TLR2*<sup>-/-</sup> and *TLR4*<sup>-/-</sup> mice. These precursors were differentiated into  
14 adherent mature macrophages (BMM) for 7 days in complete medium containing 10  
15 ng/mL of macrophage colony stimulating factor (PeproTech, Neuilly-sur-Seine,  
16 France).

17 Culture of the THP-1 cell line: Human monocytic THP1 cells (American Type Culture  
18 Collection, Rockville, MD, USA) were kind gifts from Professor Rouis (Sorbonne  
19 University, France). Cells were routinely grown to a primary macrophage culture  
20 following differentiation from monocytes with a 24h treatment with Phorbol 12-  
21 Myristate 13-Acetate (50 nM) PMA, Sigma, Saint-Louis, USA). After 72h, the  
22 macrophages were starved before treatment.

1 Treatment by recombinant 14-3-3ε: All cell cultures were stimulated with  
2 recombinant 14-3-3ε at 1 µg/ml for 24h. Supernatants and total mRNA collected after  
3 cell lysis were harvested and stored at -80°C.

4 For blocking antibody and pharmacological experiments, murine articular  
5 chondrocytes were pretreated for 20 minutes with increasing concentrations (1 and 5  
6 µg/ml) of a mouse TLR2 or TLR4 antibody or oxidized 1-palmitoyl-2-arachidonoyl-sn-  
7 glycerol-3-phosphocholine (OxPAPC) at 0.3, 3 and 30 µg/ml before the treatment by  
8 recombinant 14-3-3ε.

9

#### 10 ***RNA extraction and quantitative RT-PCR***

11 Total RNA was extracted from murine chondrocytes using the ReliaPrep RNA Cell  
12 Miniprep System kit (Promega, Madison, WI, USA) from human synovial fibroblasts,  
13 BMM and THP1 by Trizol chloroform. Concentrations were determined by  
14 spectrophotometry (Eppendorf, Le Pecq, France). Reverse transcription was  
15 performed with 500 ng of total RNA with the Omniscript RT kit (Qiagen). mRNA levels  
16 were quantified with the Light Cycler LC480 (Roche Diagnostics, Indianapolis, IN,  
17 USA). PCR amplification conditions are described in supplemental data. Product  
18 formation was detected at 72°C in the fluorescein isothiocyanate channel. The mRNA  
19 levels were normalized to those of murine HPRT or Human 18S. Specific primer  
20 sequences are presented in Table S1.

21

#### 22 ***Protein secretion quantification by ELISA***

23 Total mouse and human IL6, MCP1, TNFα, MMP-3 and 14-3-3ε secretion were  
24 assayed in cell-free supernatants using an enzyme-linked immunosorbent assay

1 (ELISA) kit (R&D Systems and Abbeva) according to the manufacturer's instructions.  
2 Concentrations were analyzed in duplicate at serial dilutions and determined by  
3 comparison against a standard curve.

4

#### 5 ***Endotoxin tests:***

6 Protocol of the experiments is described in supplemental data

7

#### 8 ***Statistical Analysis***

9 The choice of the number of experiments was established by power analysis tests  
10 and previous and published work from our laboratory. The small number of  
11 experiments used for each part of the work can be considered as a limitation. All data  
12 were showed as mean values +/- s.e.m. In Fig. 5a and b, the stimulated condition  
13 (14-3-3ε) was normalized to 1 in order to study the inhibition rates and data were  
14 showed as mean values with 95% confidence intervals (CI). Statistical analyses  
15 were performed with the Mann Whitney test to compare mean values between 2  
16 groups or by the Wilcoxon test when analyses were based on patient paired-matched  
17 samples (Fig.1 and 2). One-way analysis of variance (ANOVA) and two-way ANOVA  
18 with the Bonferroni multiple comparisons post-test were used to compare mean  
19 values between more than 2 groups using GraphPad Prism software (GraphPad  
20 Software, San Diego, CA). P < 0.05 was considered statistically significant. \*P<0.05;  
21 \*\*P<0.01; \*\*\*P<0.001; ns: not significant.

## Results

### **Stimulation of synovium explants from OA patients by 14-3-3 $\epsilon$ elicits the release of pro-inflammatory factors.**

To verify whether 14-3-3 $\epsilon$  is able to induce synovium inflammation in humans, we used synovium explants from OA patients to mimic the pathophysiological environment as closely as possible. Stimulation of these human synovium explants with recombinant 14-3-3 $\epsilon$  (1  $\mu$ g/ml) induced the secretion of MCP1 and IL6 protein (Fig. 1A, B). Indeed, mean difference of IL6 protein release showed a 5.7-fold increase between control and 14-3-3 $\epsilon$  stimulation (0.6, 95%CI [0.3;0.9] vs 3.4, 95%CI [2.7;4.1] respectively) (Fig. 1A) and mean difference of MCP1 protein release showed a 9.5-fold increase between control and 14-3-3 $\epsilon$  stimulation (4.8, 95%CI [0.9;8.6] vs 45.6, 95%CI [9.9;81.3] respectively) (Fig. 1B).

### **14-3-3 $\epsilon$ elicits a pro-inflammatory phenotype in fibroblast-like synoviocytes (FLS).**

To more precisely study the impact of 14-3-3 $\epsilon$  on the two main cell types residing in the synovium, FLS and macrophages, we stimulated primary cultures of FLS from OA patients with 14-3-3 $\epsilon$  recombinant protein (1  $\mu$ g/ml) for 24h. The levels of IL-6 and MCP1 in controls samples are below the detection threshold. We found that stimulated synoviocytes had increased mRNA expression and secretion of both IL6 and MCP1 (Fig. 2 A-D). Mean difference of IL6 mRNA expression showed an increase of 7.5 fold between control and 14-3-3 $\epsilon$  stimulation (0.5, 95%CI [0;1.0] vs 3.7, 95%CI [2.1;5.3] respectively) and MCP1 mRNA expression showed an increase of 4.4 fold mean difference between control and 14-3-3 $\epsilon$  (0.6, 95%CI [0.1;1.1] vs 2.6, 95%CI [0.9;4.3] respectively) (Fig. 2A, B). Mean difference of protein secretion of IL6

1 and MCP1 showed a fold increase of 3.7 (5.5, 95%CI [-0.6;11.6] vs 20.54 95%CI  
2 [-0.7;41.7]) and 31.8 (1.7, 95%CI [1.0;2.4] vs 54.2, 95%CI [32.7;75.7]) between  
3 control and 14-3-3 $\epsilon$  stimulation respectively (Fig. 2C, D).

4  
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6 **14-3-3 $\epsilon$  skews macrophages toward a pro-inflammatory phenotype involving**  
7 **Toll-like receptors signaling.**

8 Stimulation of human macrophages derived from the THP1 cell line with 14-3-3 $\epsilon$   
9 induced mRNA expression and secretion of both IL6 and MCP1 (Fig. 3A, B, D, E).  
10 Mean difference of mRNA expression levels of IL6 and MCP1 showed a fold increase  
11 of 7.2 (0.1, 95%CI [0;0.2] vs 0.7, 95%CI [0.5;0.8]) and 3.1 (0.2, 95% CI [0;0.4] vs 0.7,  
12 95%CI [0.5;0.8]) between control and 14-3-3 $\epsilon$  stimulation respectively. Similarly, the  
13 release of IL6, MCP1 and TNF $\alpha$  protein was increased in the supernatants of  
14 macrophages stimulated with 14-3-3 $\epsilon$ , showing a fold mean difference of 17.8 (0.03,  
15 95%CI [0;0.05] vs 6.2, 95%CI [1.9;10.4]), 18.9 (23.9, 95%CI [23;24.8] vs 453, 95%CI  
16 [379.8;525.6]) and 15.1 (0.04, 95%CI [0;0.07] vs 0.6, 95%CI [0.2;1.1]) between  
17 control and 14-3-3 $\epsilon$  stimulation respectively (Fig. 3D, E, F). Moreover, macrophages  
18 subjected to 14-3-3 $\epsilon$  stimulation displayed increased mRNA expression of CD38,  
19 another marker associated with the M1 pro-inflammatory phenotype, with a 6.6-fold  
20 mean difference between control and 14-3-3 $\epsilon$  stimulation (0.1, 95%CI [0;0.2] vs 0.9,  
21 95%CI [0.7;1.1]) (Fig. 3C),

22 To assess the implications of TLR2 and TLR4 in the cellular response to 14-3-3 $\epsilon$ , we  
23 used primary cultures of bone marrow macrophages (BMM) from TLR2 or TLR4 KO  
24 mice and stimulated them with recombinant 14-3-3 $\epsilon$ . Untreated WT, TLR2 KO and  
25 TLR4 KO BMM showed no protein release of IL6 and MCP1, whereas IL-6 level and



1 MCP1 release were increased by WT BMM cells stimulated with 14-3-3ε. IL-6 protein  
2 release was significantly decreased in the supernatants of TLR2 and TLR4 KO BMM  
3 stimulated with 14-3-3ε compared to the WT BMM stimulated with 14-3-3ε (64%  
4 inhibition for TLR2, WT 2.5, 95%CI [0.9;4.1] vs TLR2 KO 0.9, 95%CI [0.4;1.3] and  
5 84% inhibition for TLR4; TLR4 KO 0.4, 95%CI [0.2;0.6]) (Fig. 4A). MCP1 showed the  
6 same tendency (22% inhibition for TLR2 WT 2.8, 95%CI [-2.0;7.7] vs TLR2 KO 2.2,  
7 95%CI [-1.5;6.0] and 68% inhibition for TLR4; TLR4 KO 0.9, 95%CI [-2.1;3.8]) (Fig.  
8 4B).

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## 12 **14-3-3ε elicits a catabolic and inflammatory phenotype in murine articular** 13 **chondrocytes involving TLR2 and TLR4.**

14 Murine chondrocytes were sensitive to stimulation with 14-3-3ε recombinant protein  
15 and showed increased mRNA expression and secretion of pro-catabolic (MMP3) and  
16 pro-inflammatory (IL6) factors.

17 To confirm the involvement of TLR2 and TLR4 in 14-3-3ε signaling, murine articular  
18 chondrocytes were pre-treated with specific TLR2, TLR4 blocking antibody or the  
19 pharmacologic inhibitor (OxPAPC) inhibiting both receptors followed by 14-3-3ε  
20 stimulation. MMP3 mRNA expression and protein secretion induced by 14-3-3ε were  
21 significantly and dose-dependently reduced by TLR4 blocking antibody treatment  
22 (MMP3 mRNA expression inhibition fold: 78%, (95%CI [-0.1;0.6]) (1 µg/ml) and 77%  
23 (95%CI [-0.02;0.5]) (5 µg/ml) (Fig. 5A); secretion: 69%, (95%CI [-0.18;0.81]) (1 µg/ml)  
24 and 84%, (95%CI [-0.09;0.42]) (5 µg/ml) (Fig. 5D)). Anti-TLR2 blocking antibody  
25 reproduced the same pattern (mRNA expression: 79%, (95%CI [-0.01;0.43]) (1

1  $\mu\text{g/ml}$ ) and 83%, (95%CI [0.04;0.30]) (5  $\mu\text{g/ml}$ ) (Fig. 5B); secretion 73%, (95%CI [-  
2 0.07;0.61]) (1  $\mu\text{g/ml}$ ) and 74%, (95%CI [0.04;0.48]) (5  $\mu\text{g/ml}$ ) (Fig. 5E)). Furthermore,  
3 the pharmacologic inhibitor OxPAPC, inhibiting both receptors, markedly decreased  
4 MMP3 mRNA expression and protein release in a dose-dependent manner (mRNA  
5 expression: from 71% (95%CI [0.04;0.54]) (0.3  $\mu\text{g/ml}$ ) to 98% (95%CI [-0.01;0.04])  
6 (30  $\mu\text{g/ml}$ ) (Fig. 5C); secretion: from 64% (95%CI [0.14;0.58]) (0.3  $\mu\text{g/ml}$ ) to 96%  
7 (95%CI [-0.01;0.08]) (30  $\mu\text{g/ml}$ ) (Fig. 5F)).

8 Inhibition of TLR2 and/or TLR4 also impacted the mRNA and protein expression of  
9 the pro-inflammatory cytokine IL6 by murine chondrocytes. (IL6 mRNA expression:  
10 anti-TLR4 treatment: 73% (95%CI [-0.15;0.68]) (1  $\mu\text{g/ml}$ ) and 71% (95%CI [-  
11 0.10;0.69]) (5  $\mu\text{g/ml}$ ) (Fig. 5G); secretion: 79% (95%CI [-0.12;0.55]) (1  $\mu\text{g/ml}$ ) and  
12 81% (95%CI [-0.10;0.48]) (5  $\mu\text{g/ml}$ ) (Fig. 5J); anti-TLR2 treatment: IL6 mRNA  
13 expression 53% (95%CI [-0.02;0.97]) (1  $\mu\text{g/ml}$ ) and 51% (95%CI [0.04;0.94]) (5  
14  $\mu\text{g/ml}$ ) (Fig. 5H); secretion 69% (95%CI [-0.03;0.64]) (1  $\mu\text{g/ml}$ ) and 78% (95%CI  
15 [0.01;0.43]) (5  $\mu\text{g/ml}$ ) (Fig. 5K)). Inhibiting both receptors simultaneously with  
16 OxPAPC resulted in decreases IL6 expression and secretion (IL6 mRNA expression:  
17 from 69% (95%CI [0.01;0.60]) (0.3  $\mu\text{g/ml}$ ) to 92% (95%CI [0;0.15]) (30  $\mu\text{g/ml}$ ) (Fig.  
18 5I); secretion: from 69% (95%CI [0.05;0.58]) (0.3  $\mu\text{g/ml}$ ) to 96% (95%CI [-0.02;0.09])  
19 (30  $\mu\text{g/ml}$ ) (Fig. 5L). Stimulations of chondrocytes with the different treatments  
20 showed high variability between cultures.

21 To confirm the results obtained in KO BMM, we also stimulated TLR2 or TLR4 KO  
22 murine articular chondrocytes with 14-3-3 $\epsilon$ . TLR2 and TLR4 KO murine chondrocytes  
23 exhibited decreased mRNA expression and protein release of IL6 but also mRNA  
24 expression of MMP3 and MMP13 compared to WT chondrocytes (Fig. 6). IL6 mRNA  
25 expression was significantly decreased in TLR2 and TLR4 KO chondrocytes treated

1 with 14-3-3 $\epsilon$  compared to the WT chondrocytes (75% for TLR2; WT 65, 95%CI  
2 [-53.7;183.8] vs TLR2 KO 16.5, 95%CI [3.6;29.4] and 97% for TLR4; TLR4 KO 1.8,  
3 95%CI [1.0;2.5]) (Fig. 6B), and its release was greatly attenuated in TLR2 (77% for  
4 TLR2 (WT 21.8, 95%CI [-1.5;45.1] vs TLR2 KO 5.0, 95%CI [2.0;8.0]) and TLR4 (by  
5 97% for TLR4, TLR4 KO 0.7, 95%CI [0.5;0.9]) KO chondrocytes in response to 14-3-  
6 3 $\epsilon$  stimulation respectively (Fig. 6A). Similarly, MMP3 and MMP13 mRNA expression  
7 levels were also decreased in TLR2 and TLR4 KO chondrocytes (For MMP3: 60% for  
8 TLR2, WT 988, 95% CI [-588;2566] vs TLR2 KO 394.1, 95%CI [242.3;545.8] and  
9 94% for TLR4; TLR4 KO 61.4, 95%CI [46.9;75.9]; For MMP13: 22% for TLR2, WT  
10 0.5, 95% CI [0;1] vs TLR2 KO 66.4, 95%CI [48.6;84.2] and 77% for TLR4; TLR4 KO  
11 20.2, 95% CI [10.4;30.1]) (Fig. 6C and 6D). Taken together, these results  
12 demonstrate the involvement of both TLR2 and TLR4 receptors in 14-3-3 $\epsilon$  signaling.

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## Discussion

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17 We have recently identified 14-3-3 $\epsilon$  as a new soluble mediator involved in  
18 deleterious biochemical interactions between bone and cartilage<sup>30</sup>. In the  
19 present study, we showed that 14-3-3 $\epsilon$  is a new alarmin and can act particularly in  
20 the synovium during OA pathogenesis. Its role in the activation of innate immunity  
21 leading to synovitis could be due to interactions with its potential receptors, TLR2 and  
22 TLR4.

23 It is now established that up to 50% of OA patients have synovitis, as  
24 demonstrated by magnetic resonance imaging, ultrasonography and arthroscopy<sup>38</sup>.  
25 Based on these results and to study the involvement of 14-3-3 $\epsilon$  in the synovium of

1 the OA joint, we analyzed the effects of 14-3-3 $\epsilon$  on whole synovial tissue explants  
2 from OA patients by measuring the release of pro-inflammatory factors. We found  
3 that IL6 and MCP1 release in culture media was significantly increased after 14-3-3 $\epsilon$   
4 stimulation compared with the control explants. In these complete synovial explants  
5 from OA patients, the pathophysiological environment was conserved. There was  
6 obvious heterogeneity in the degree of inflammation of the synovium between patient  
7 samples, which could be due to the different pathological mechanisms leading to  
8 OA among patients<sup>39,40</sup>. Although fewer synovial macrophages are present in OA  
9 compared with RA, they are crucial for the production of proinflammatory cytokines  
10 such as IL6<sup>18</sup>. Previous studies have shown that selective depletion of synovial  
11 macrophages during experimental OA largely reduces cartilage damage and  
12 osteophyte formation, which are 2 major hallmarks of OA<sup>41</sup>. Thus, we would like to  
13 separately analyze the role in synovitis of 2 main cell types residing in the synovium:  
14 fibroblast-like synoviocytes (FLS) and macrophages. We found that 14-3-3 $\epsilon$  was able  
15 to increase the mRNA expression and protein secretion of IL6 (7.5 and 4.3-fold  
16 increase respectively) and MCP1 (4.4 and 32-fold increase) in FLS. These cells were  
17 able to respond to 14-3-3 $\epsilon$  resulting in an inflammatory phenotype. Moreover, Thp1  
18 cells, a human monocyte cell line, were used to address whether 14-3-3 $\epsilon$  could  
19 polarize these cells toward a pro-inflammatory macrophage phenotype. In our current  
20 study, we showed that 14-3-3 $\epsilon$  increased the mRNA expression and protein secretion  
21 of IL6 (7.2 and 17.8-fold increase respectively) and MCP1 (3.1 and 19-fold increase).  
22 In addition to the experiments on Thp1, we also performed stimulations of primary  
23 cultures of murine macrophages (Bone marrow macrophages: BMM) by 14-3-3 $\epsilon$  and  
24 studied the expression of different markers. mRNA expressions of pro-inflammatory  
25 mediators such as iNOS, IL-1 $\beta$  and TNF $\alpha$  were increased whereas mRNA

1 expression of anti-inflammatory marker (EGR2) was decreased in BMM (Fig. S1  
2 Supplemental data). This protein subsequently appears to polarize macrophage  
3 toward the M1 phenotype. In the case of knee OA, a study analyzing M1  
4 macrophages and M2 macrophages in synovial fluid in normal versus OA knees  
5 found a higher ratio of M1/M2 in OA versus normal knees, and the ratio was  
6 significantly correlated to the Kellgren-Lawrence grade<sup>42</sup>. These results suggest that  
7 macrophage polarization may indeed play a role in the control and even progression  
8 of OA disease. However, it should be noted that the classification of macrophages  
9 into M1/M2 subtype is reductive. This ability of macrophages to modify their  
10 phenotype in response to external signals gives rise to a broad spectrum of  
11 possibilities depending on their interactions<sup>24</sup>. Our study indicates that 14-3-3ε can  
12 induce a proinflammatory environment in the OA synovium through stimulation of  
13 macrophages which possibly contributes to the joint destruction that occurs during  
14 OA. Similar results have been obtained previously for the alarmin S100A9 in the  
15 synovium<sup>43</sup>.

16 Interestingly, high levels of many alarmins have been described in the synovial  
17 fluid of OA patients<sup>12</sup>. Numerous studies have shown that these DAMPs stimulate  
18 synovial cell proliferation, influence hypertrophic chondrocyte differentiation and  
19 induce inflammatory and pro-catabolic events *in vitro*, and they promote synovitis and  
20 cartilage degradation *in vivo* in murine OA models<sup>44</sup>. We hypothesized that 14-3-3ε is  
21 a new alarmin involved in OA. Our results showed that this protein was able to lead  
22 to an inflammatory and catabolic response in joint similarly to other alarmins.  
23 Moreover, OA is mainly linked to activation of innate immunity by the binding of  
24 damage-associated molecular patterns (DAMPs) to so-called pattern recognition  
25 receptors (PRRs)<sup>45</sup>. Of central importance in the PRR family are the Toll-like

1 receptors (TLRs)<sup>45</sup>. TLR2 and TLR4 are overexpressed in OA cartilage, and their  
2 presence correlates with histopathological damage<sup>29</sup>. Blockade of TLR signaling -as  
3 shown in TLR2/TLR4 and in MyD88 knockout mice- downregulates cartilage  
4 catabolic response *in vitro*, and it can protect animals from experimental OA<sup>46</sup>.  
5 Moreover, synovial fluid proteins from patients with OA activate macrophages via  
6 TLR2/TLR4 receptors, in turn translocating NF-κB to the nucleus<sup>9</sup>. Synovial  
7 fibroblasts are sensitive to both mechanical alterations and DAMPs due to the  
8 expression of different TLRs on the cell membrane, resulting in increased synthesis  
9 of pro-inflammatory mediators<sup>27</sup>. In particular, TLR-2 and TLR-4 are used by many  
10 alarmins<sup>47</sup>.

11 To examine whether 14-3-3ε response was driven by TLR, we used TLR2 and TLR4  
12 KO mice to study their potential involvement in macrophage and chondrocyte  
13 responses. Our results showed that these two receptors were involved in the  
14 inflammatory and catabolic phenotype after stimulation with 14-3-3ε, with TLR4  
15 showing predominant involvement. To further investigate the involvement of TLR2  
16 and TLR4, we used blocking antibodies against them and a pharmacological inhibitor  
17 (OXPAPC) that is able to inhibit both TLR2 and TLR4. Our results validated the  
18 results obtained in the KO mice and confirmed that 14-3-3ε could elicit a catabolic  
19 and inflammatory phenotype in murine articular chondrocytes and a pro-inflammatory  
20 phenotype in BMM macrophages. Interestingly, a TLR4 monoclonal antibody has  
21 recently been demonstrated to have an adequate safety profile, and a phase II  
22 clinical trial in patients with RA has been launched<sup>48</sup>.

23 In the present study, we used recombinant 14-3-3ε protein produced in  
24 *Escherichia coli*, similarly to many commercially available recombinant proteins.  
25 Although this expression system has many advantages, including rapid expression,

1 high yields, ease of culture and low cost<sup>49</sup>, the proteins recovered may be  
2 contaminated with endotoxin, a highly complex lipopolysaccharide (LPS) constitutive  
3 of the outer membrane of most gram-negative bacteria<sup>50</sup>. LPS is recognized by a  
4 receptor complex composed of TLR4, CD14 and MD-2<sup>51,52</sup>. Consequently, using  
5 recombinant 14-3-3 $\epsilon$  in this study, we wanted to be sure that the effects of 14-3-3 $\epsilon$  on  
6 joint tissues were due to the protein itself and not to endotoxin contamination. Low  
7 levels of endotoxins measured by a LAL kit (14-3-3 $\epsilon$  contained less than 0.15 ng/ml  
8 of LPS, data not shown) and no significant inhibition by the PMB on 14-3-3 $\epsilon$   
9 chondrocyte stimulation (Fig. S2 supplemental data) and 14-3-3 $\epsilon$  macrophages  
10 stimulation (data not shown) confirmed the proper effect of 14-3-3 $\epsilon$  recombinant  
11 protein. Moreover, in our previous study<sup>31</sup>, we demonstrated that immunodepletion  
12 and blocking of 14-3-3 $\epsilon$  in conditioned media of compressed osteoblasts inhibited its  
13 catabolic effect on chondrocytes confirming that 14-3-3 $\epsilon$  itself is involved in the  
14 establishment of a pro-catabolic phenotype in chondrocytes.  
15 Thus, although we cannot rule out that a small component of the effects observed  
16 herein were due to endotoxin contamination, we are confident that the cellular  
17 responses resulted from the activity of 14-3-3 $\epsilon$  protein itself.

18 Taken together, our results designate 14-3-3 $\epsilon$  as a novel alarmin for further  
19 exploration in OA for either therapeutic or prognostic purposes.

20  
21

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23

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6

### **Contributions**

7

8 - Conception and design: MM, FB, CJ, FP

9 - Collection and assembly of data: MM, LS, AR, AP, MN, CR

10 - Analysis and interpretation of the data: MM, CJ, FB

11 - Drafting of the article: MM, CJ, FB,

12 - Critical revision of the article for important intellectual content: MM, CJ, FB,

13 FP, GA-L,

14 - Final approval of the article: MM, LS, MN, FP, CR, AR, AP, GA-L, FB, CJ

15

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21

### **Conflict of interest**

22

23 No potential conflicts of interest relevant to this article were reported. All authors disclose  
24 any financial and personal relationships with other people or organizations that could  
25 potentially and inappropriately influence their work and conclusions.



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## Figure legends

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4 **Fig. 1: Stimulation of MCP1 and IL6 protein release by synovium explants from**  
5 **OA patients with 14-3-3 $\epsilon$ .**

6 Synovium explants from OA patients were incubated with medium in the presence or  
7 absence of 14-3-3 $\epsilon$  (1  $\mu$ g/ml) for 24 hours. A, B: Protein levels of IL6 (A) (n=5) and  
8 MCP1 (B) (n=5) released by human synovium explants treated with the control or 14-  
9 3-3 $\epsilon$  were measured using the enzyme-linked immunosorbent assay (ELISA). Bars  
10 show the mean  $\pm$  s.e.m. (Wilcoxon test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001, ns, not  
11 significant.

12

13 **Fig. 2: Stimulation of IL6 and MCP1 expression and protein release in response**  
14 **to 14-3-3 $\epsilon$  in primary cultures of human fibroblast-like synoviocytes.**

15 Synovium explants from OA patients were digested and seeded in 6-well culture  
16 plates until confluence. The primary cultures were then stimulated with the control  
17 medium in the presence or absence of recombinant 14-3-3 $\epsilon$  (10  $\mu$ g/ml) for 24 hours.  
18 A,B: Total RNA was extracted, and mRNA levels of IL6 (A) (n=6) and MCP1 (B) (n=6)  
19 were determined by qRT-qPCR. Protein levels of IL6 (C) (n=6) and MCP1 (D) (n=6)  
20 in cell supernatants were measured by ELISA. Bars show the mean  $\pm$  s.e.m.  
21 (Wilcoxon test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns, not significant.

22

23 **Fig. 3: Stimulation of IL6, MCP1 and CD38 mRNA expression and IL6, MCP1,**  
24 **and TNF $\alpha$  protein release in human macrophages (derived from the THP1 cell**  
25 **line).**

1 Stimulation of macrophages (THP1 cells) was performed using control medium or 14-  
2 3-3ε (1 μg/ml) treatment. A,B,C: Total RNA was extracted, and mRNA levels of IL6  
3 (A) (n=5), MCP1 (B) (n=5), and CD38 (C) (n=5) were determined by qRT-PCR. D, E,  
4 F: Protein levels of IL6 (D) (n=5), MCP1 (E) (n=5) and TNFα (F) (n=5) in cell  
5 supernatants were measured by ELISA. Bars show the mean ± s.e.m. (Mann  
6 Whitney test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns, not significant.

7

8 **Fig. 4: Stimulation of IL6 and MCP1 protein release by bone marrow**  
9 **macrophages from WT, TLR2 or TLR4 KO mice in response to 14-3-3ε treatment.**

10 Stimulation of bone marrow macrophages (BMM) were performed using control  
11 medium or 14-3-3ε (1 μg/ml) treatment. Protein levels of IL6 (A) (n=4) and MCP1 (B)  
12 (n=4) in cell supernatants were measured by ELISA. Bars show the mean ± s.e.m.  
13 (Two way ANOVA with a Bonferroni post test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns, not  
14 significant.

15

16

17 **Fig. 5a: Involvement of TLR2 and TLR4 in 14-3-3ε-induced MMP-3 (degradative**  
18 **enzyme) release by murine articular chondrocytes.**

19 Murine articular chondrocytes were treated with TLR2, TLR4 blocking antibodies or  
20 the pharmacologic inhibitor OxPAPC for 15 minutes and then stimulated with  
21 recombinant 14-3-3ε (1 μg/ml) for 24 h. (A, B, C) Total RNA was extracted, and  
22 mRNA levels of MMP-3 were determined by qRT-PCR to examine the inhibitory  
23 effects of anti-TLR4 (A) (n=5), anti-TLR2 (B) (n=5), and OxPAPC (C) (n=5). (D, E, F)  
24 Protein levels of MMP-3 in cell supernatants were measured by ELISA to examine  
25 the inhibitor effects of anti-TLR4 (D) (n=5), anti-TLR2 (E) (n=5), and OxPAPC (F)

1 (n=5). 14-3-3 $\epsilon$ -stimulated cells released an average of 350 ng/ml of MMP-3. Bars  
2 show the mean values with 95% confidence intervals (One-way ANOVA with a  
3 Bonferroni post test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns, not significant.

4

5 **Fig 5b: Involvement of TLR2 and TLR4 in 14-3-3 $\epsilon$ -induced IL-6 (pro-**  
6 **inflammatory cytokine) release by murine articular chondrocytes.**

7 Murine articular chondrocytes were treated with TLR2, TLR4 blocking antibodies or  
8 the pharmacologic inhibitor OxPAPC for 15 minutes and stimulated with recombinant  
9 14-3-3 $\epsilon$  (1  $\mu$ g/ml) for 24 h. (G, H, I) Total RNA was extracted, and mRNA levels of IL6  
10 were determined by qRT-PCR to examine the inhibitory effects of anti-TLR4 (G)  
11 (n=5), anti-TLR2 (H) (n=5), and OxPAPC (I) (n=5). (J, K, L) Protein levels of IL6 in  
12 cell supernatants were measured by ELISA to examine the inhibitory effects of anti-  
13 TLR4 (J) (n=5), anti-TLR2 (K) (n=5), and OxPAPC (L) (n=5). 14-3-3 $\epsilon$ -stimulated cells  
14 released an average of 2 ng/ml of MMP-3. Bars show the mean values with 95%  
15 confidence intervals (One-way ANOVA with a Bonferroni post test) \*P<0.05;  
16 \*\*P<0.01; \*\*\*P<0.001; ns, not significant.

17

18 **Fig. 6: Stimulation of IL6 mRNA expression and IL6, MMP3 and MMP13 protein**  
19 **release by murine articular chondrocytes from WT, TLR2 or TL4 KO mice in**  
20 **response to 14-3-3 $\epsilon$  treatment.**

21 Stimulation of murine chondrocytes was performed using control medium or 14-3-3 $\epsilon$   
22 (1  $\mu$ g/ml) treatment. Total RNA was extracted, and mRNA levels of IL6 (B) (n=4),  
23 MMP3 (C) (n=4) and MMP13 (D) (n=4) were determined by qRT-PCR. Protein levels  
24 of IL6 (A) (n=4) in cell supernatants was measured by ELISA. Bars show the mean  $\pm$   
25 s.e.m. (Two way ANOVA post test)\*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns, not significant.



1

2 **Supplemental data:**

3 **Fig. S1:** Stimulation of M1 markers (iNOS, CD38, IL-1 $\beta$ , TNF $\alpha$ ) and decrease of M2  
4 marker (EGR2) in response to 14-3-3 $\epsilon$  treatment murine bone marrow macrophages.

5 Murine BMM were stimulated with 14-3-3 $\epsilon$  (1  $\mu$ g/ml). Total RNA was extracted and  
6 mRNA levels of CD38 (A) (n=5), iNOS (B) (n=5), IL-1 $\beta$  (C) (n=5), TNF $\alpha$  (D) (n=5) and  
7 EGR2 (E) (n=5) were determined by qRT-PCR. Bars show the mean  $\pm$  s.e.m. (Mann  
8 Whitney test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns: not significant. mRNA expression  
9 levels of CD38, iNOS, IL-1 $\beta$  and TNF $\alpha$  showed significant fold increase of 28; 216;  
10 13 and 7.3 respectively compared with the controls which show the property of 14-3-  
11 3 $\epsilon$  to induce the expression of BMM type M1 genes markers. Moreover, mRNA  
12 expression levels of EGR2, a M2 phenotype marker, was significantly reduced (5.3-  
13 fold decrease) after a 14-3-3 $\epsilon$  stimulation compared to the BMM controls.

14

15 **Fig. S2:** Assessment of 14-3-3 $\epsilon$  recombinant protein proper effect by inhibiting  
16 endotoxins with polymyxin B on murine articular chondrocytes.

17 Murine articular chondrocytes cultures were stimulated with 14-3-3 $\epsilon$  (1  $\mu$ g/ml) or LPS  
18 (10 ng/ml) with or without PMB (30  $\mu$ g/ml). Total RNA was extracted and mRNA  
19 levels of MMP13 was determined by qRT-PCR (A) (n=3). Protein level of MCP1 in  
20 cell supernatants was measured by ELISA (B) (n=3). Bars show the mean  $\pm$  s.e.m.  
21 (One-way ANOVA with a Bonferroni post test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns: not  
22 significant. Treatment with PMB inhibited MMP13 mRNA expression (15% compared  
23 to 14-3-3 $\epsilon$  stimulation), MCP1 release (1% compared to 14-3-3 $\epsilon$  stimulation) whereas  
24 MMP13 mRNA expression and MCP1 protein release in response to LPS stimulation

- 1 were totally inhibited by PMB. This result show that recombinant 14-3-3 $\epsilon$  has its self-
- 2 effect independent from endotoxins.

Figure 1

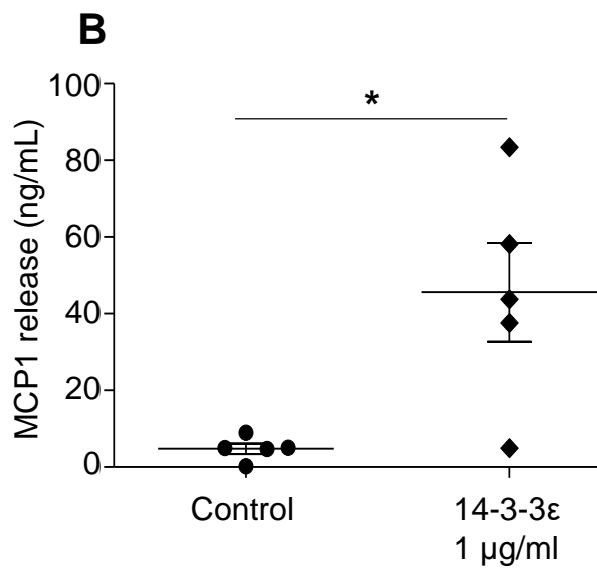
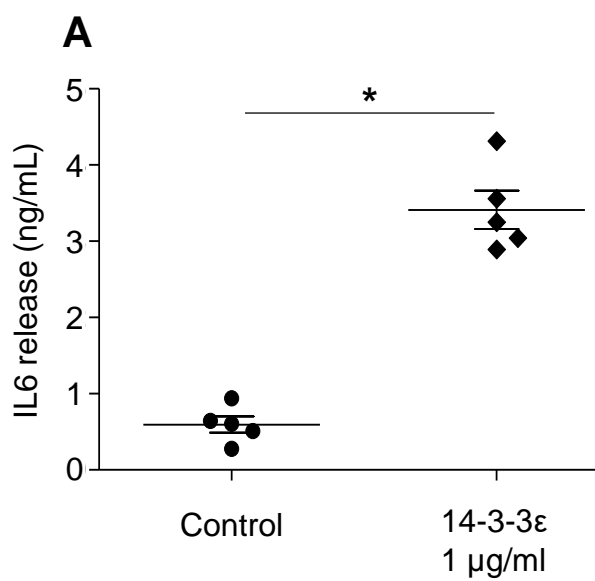


Figure 2

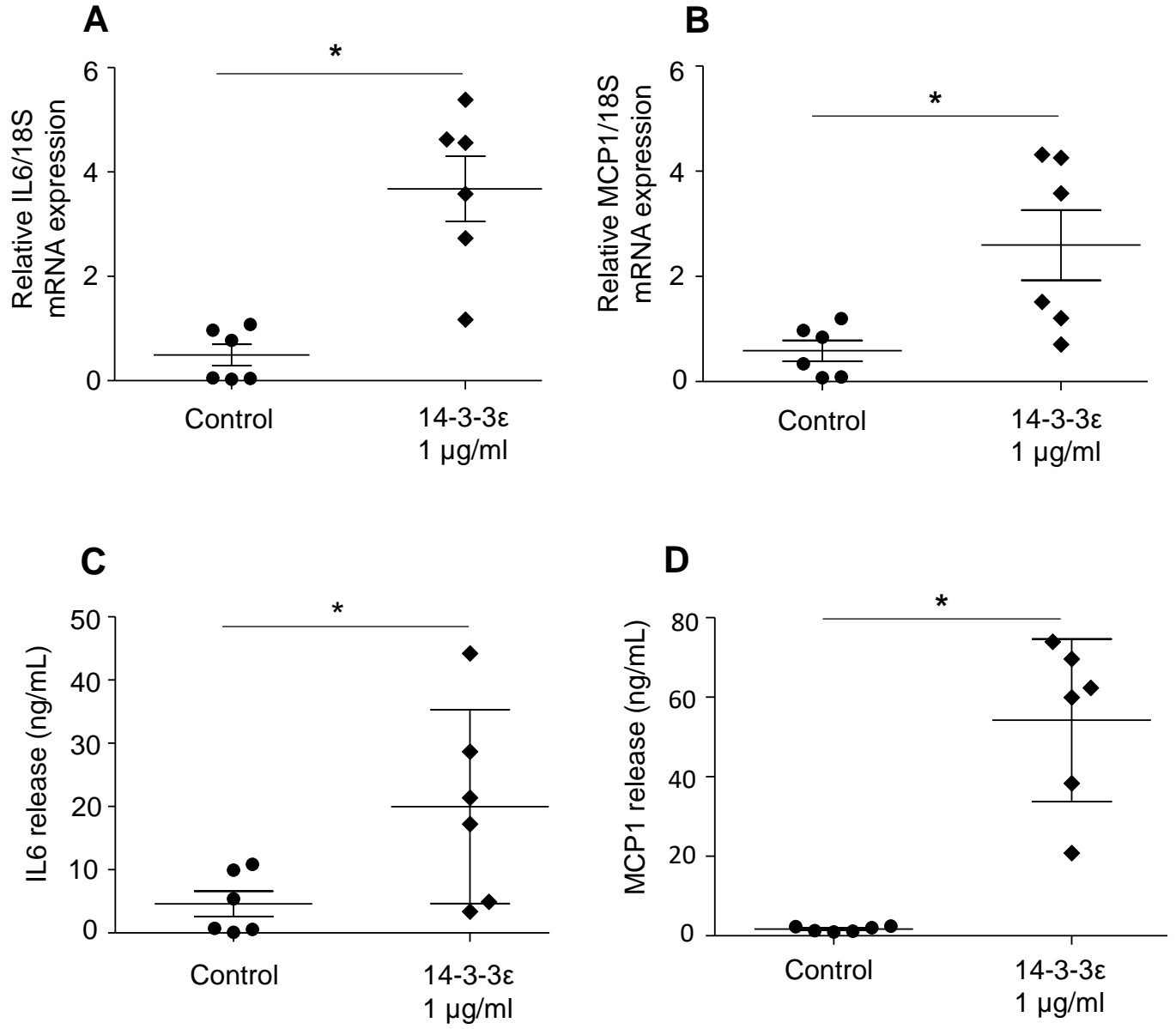
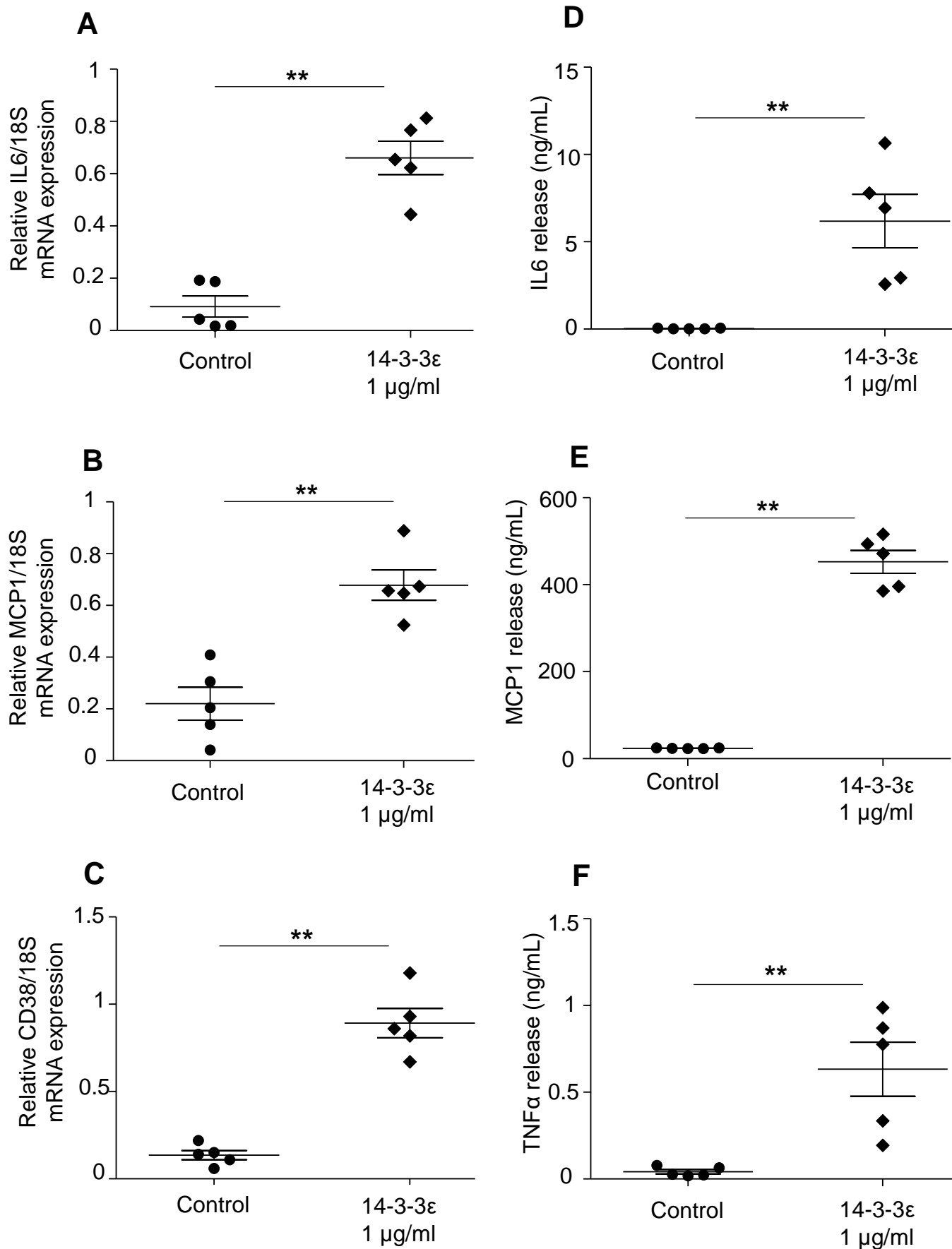


Figure 3



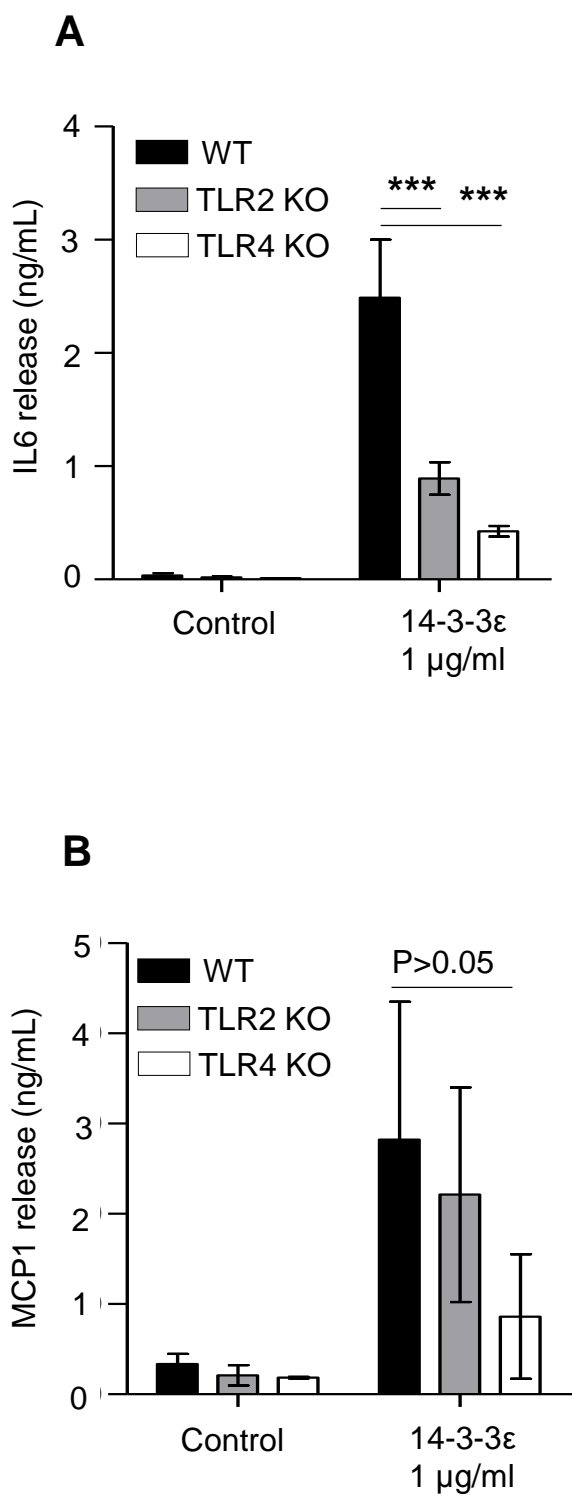
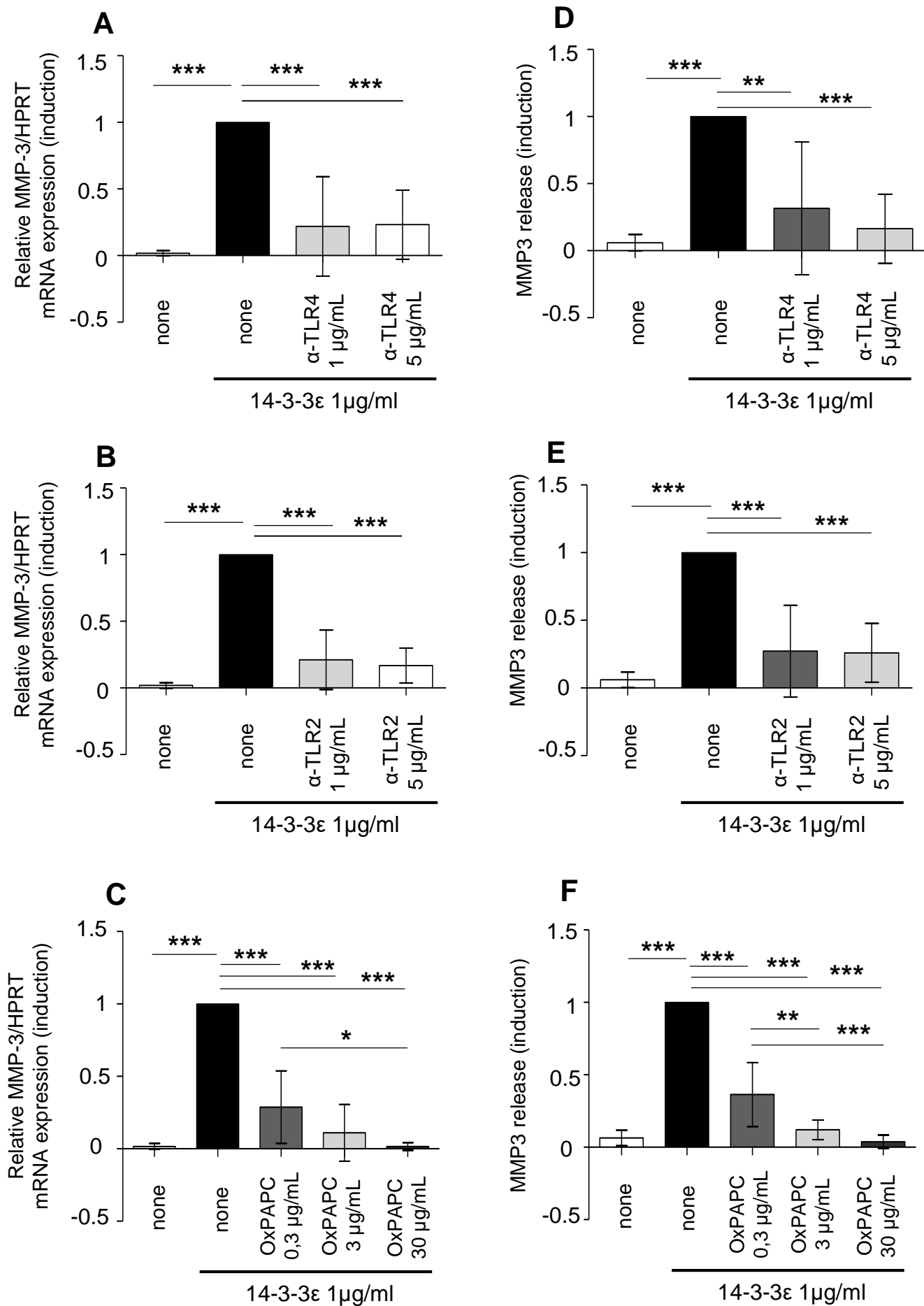


Figure 5a



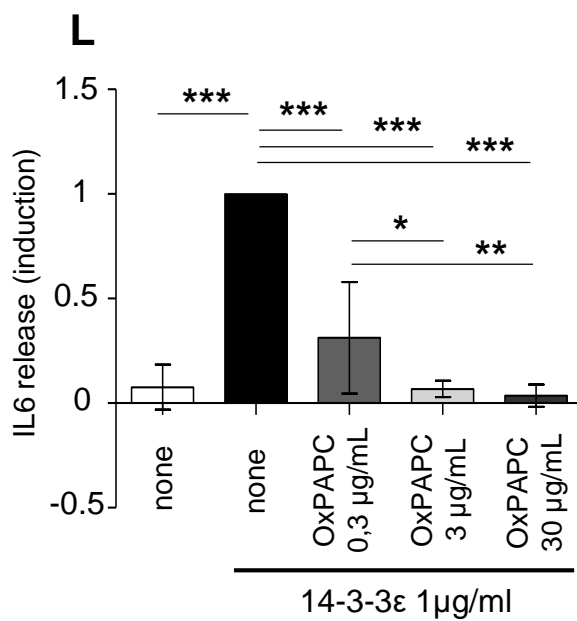
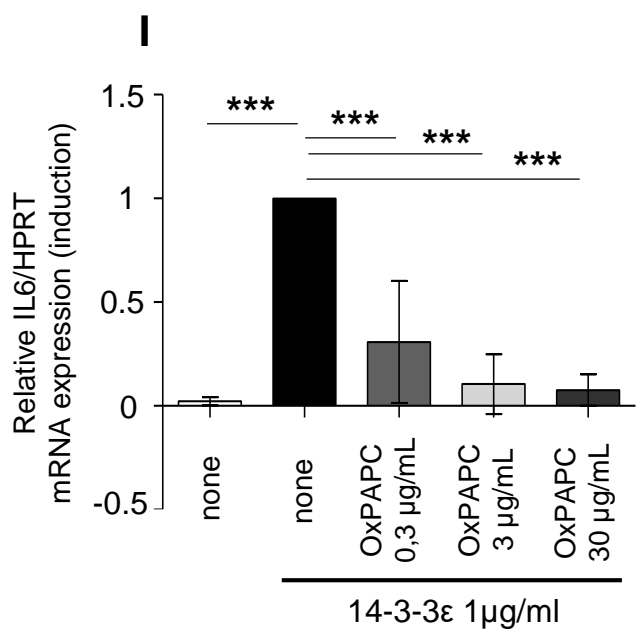
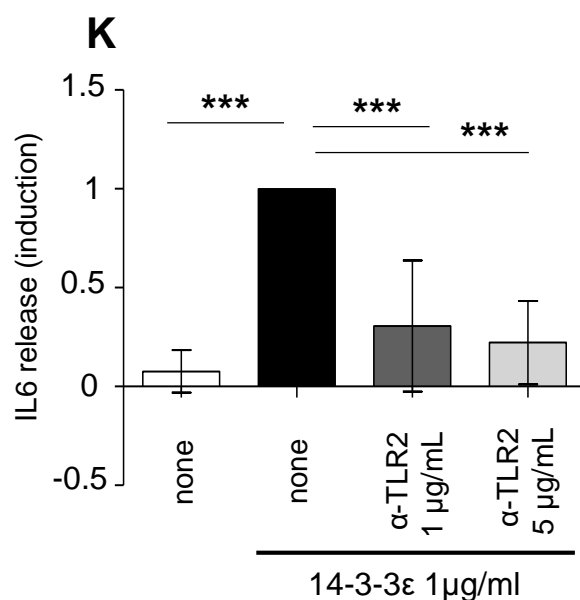
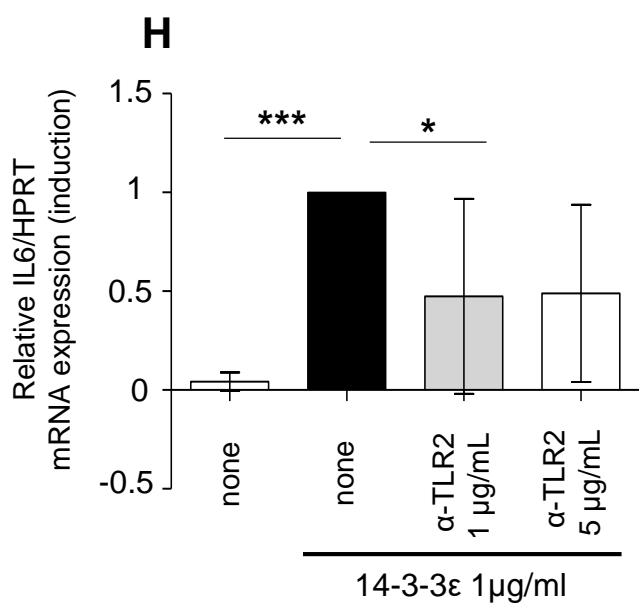
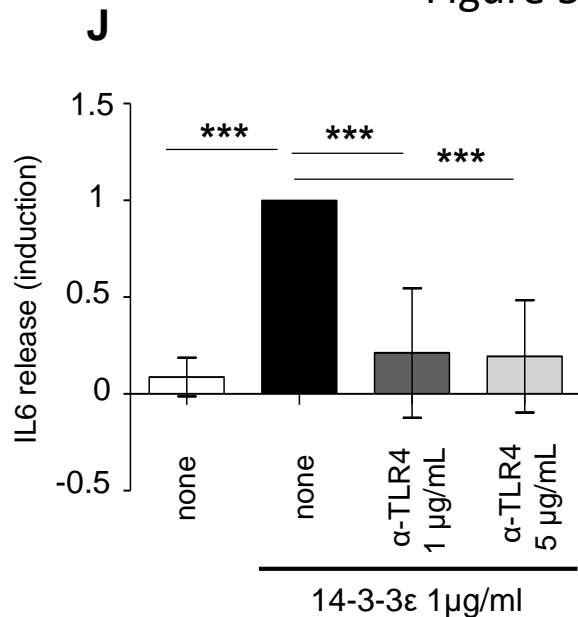
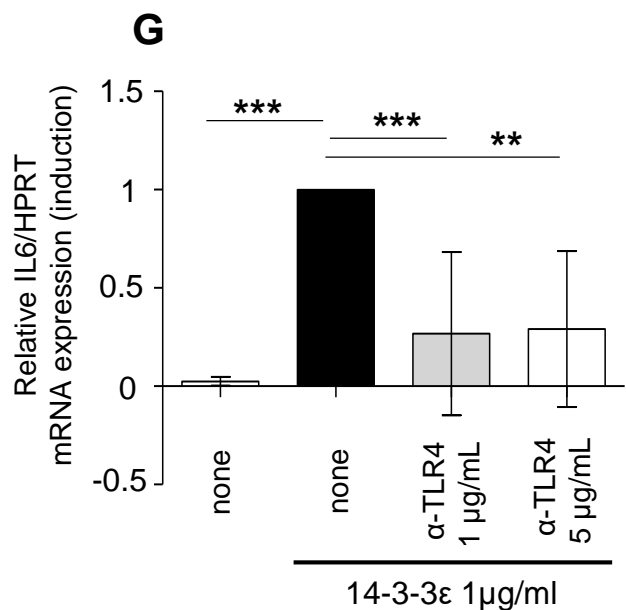
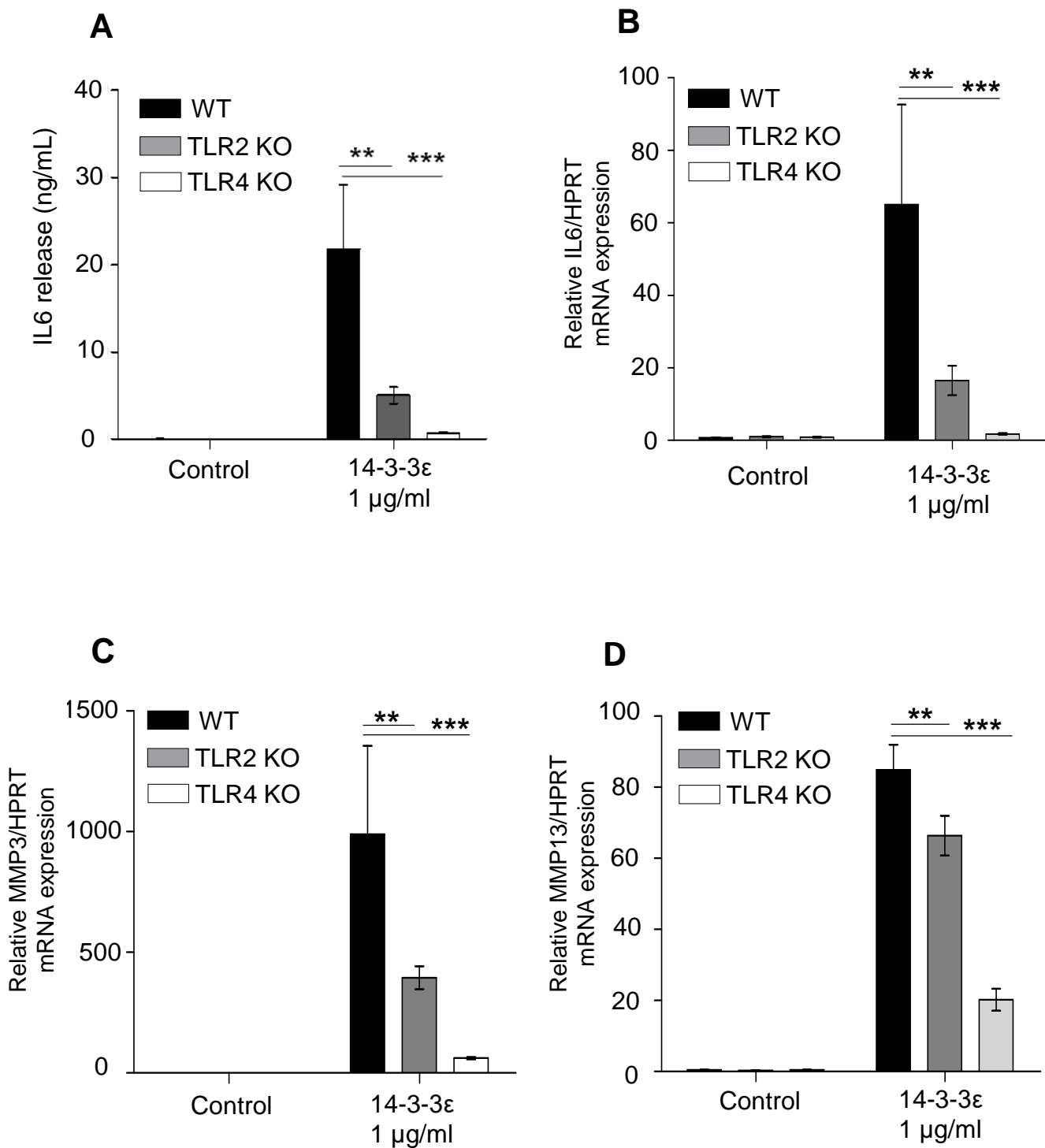
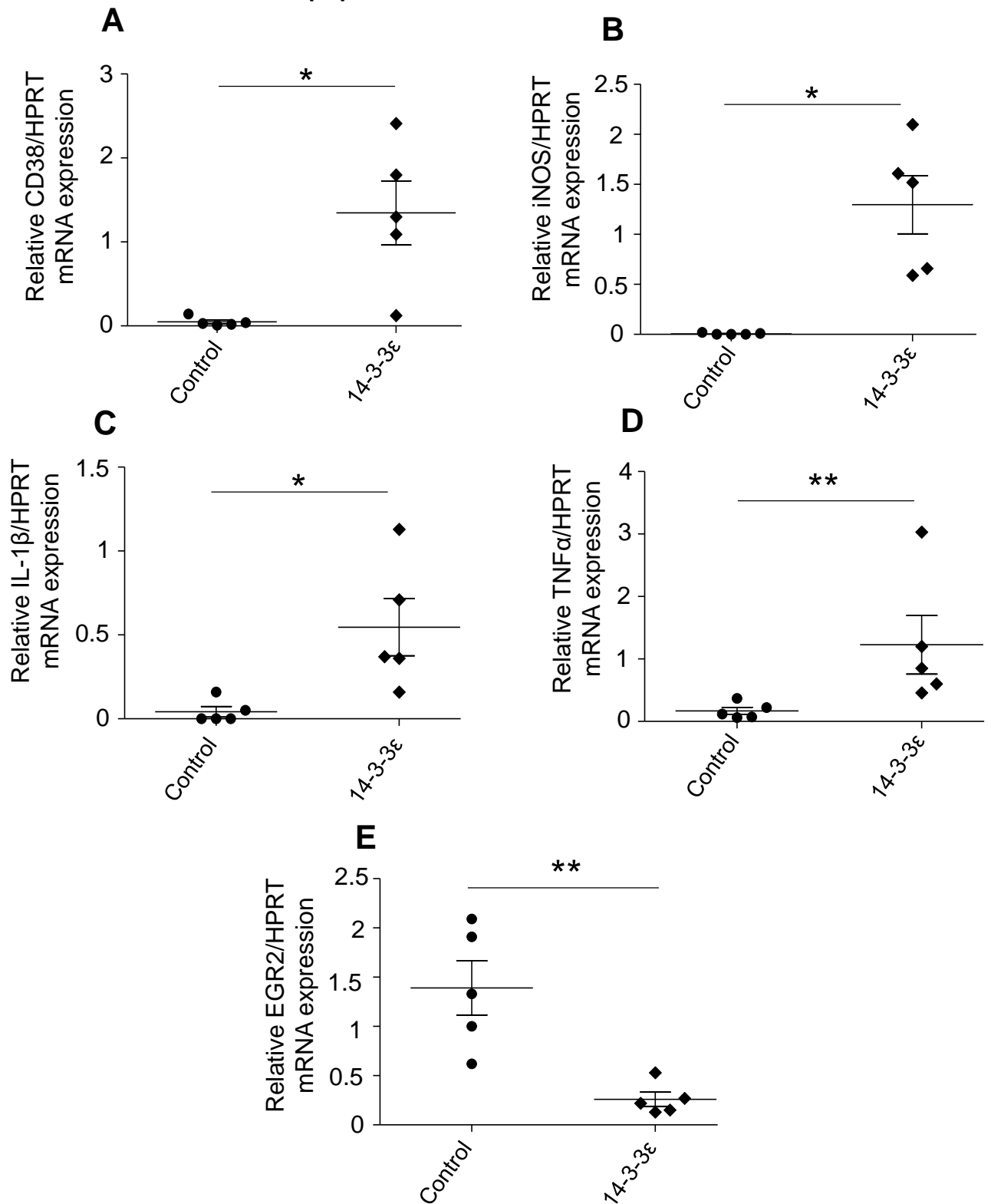




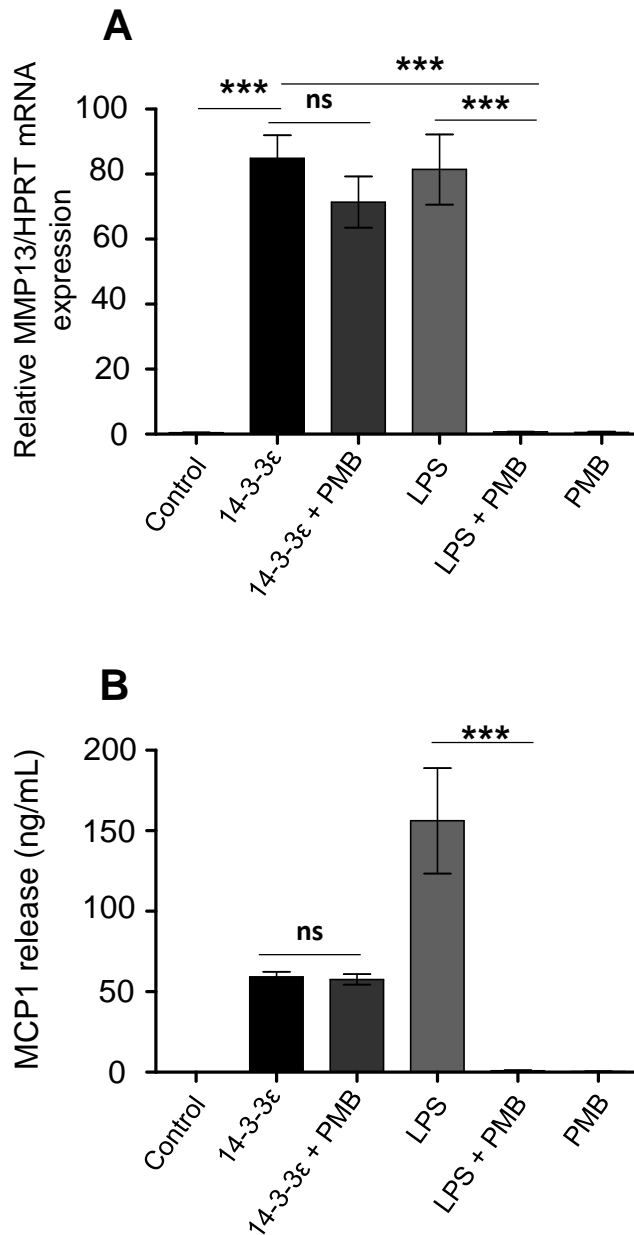
Figure 6





**Fig. S1: Stimulation of M1 markers (iNOS, CD38, IL-1β, TNFα) and decrease of M2 marker (EGR2) in response to 14-3-3ε treatment murine bone marrow macrophages.**

Murine BMM were stimulated with 14-3-3ε (1 μg/ml). Total RNA was extracted and mRNA levels of CD38 (A) n=5, iNOS (B) n=5, IL-1β (C) n=5, TNFα (D) n=5 and EGR2 (E) n=5 were determined by qRT-PCR. Bars show the mean ± s.e.m. (Mann Whitney test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns : not significant.



**Fig. S2: Assessment of 14-3-3ε recombinant protein proper effect by inhibiting endotoxins with polymyxin B on murine articular chondrocytes.**

Murine articular chondrocytes cultures were stimulated with 14-3-3ε (1 µg/ml) or LPS (10 ng/ml) with or without PMB (30 µg/ml). Total RNA was extracted and mRNA levels of MMP13 was determined by qRT-PCR (A) n=3. Protein level of MCP1 in cell supernatants was measured by ELISA (B) n=3. 1% for MCP1 release and 15% for MMP13 mRNA expression, as compared to 100% inhibition between LPS and LPS+PMB in the cellular response, but the major effect of 14-3-3ε was retained even in the presence of polymyxin. (One-way ANOVA with a Bonferroni post test) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; ns : not significant.

Species	Name	Forward primer (5'→3')	Reverse primer (5'→3')
Murine	MMP-3	TGAAAATGAAGGGTCTTCCGG	GCAGAAGCTCCATACCAGCA
	MMP-13	GATGGCACTGCTGACATCAT	TGTAGCCTTTGGAAGTCTT
	IL6	GTCACAGAAGGAGTGGCTA	AGAGAACAACATAAGTCAGATACC
	MCP-1	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT
	HPRT	AGGACCTCTCGAAGTGT	ATTCAAATCCCTGAAGTACTCAT
	CD68	AAGATGTTCAACCCTGGAGGA	ACTCCAATGTGGGCAAGAGA
	iNOS	AAGGTGAGCTGAACGAGGAG	GGAGCCTTTAGACCTCAACAGA
	IL-1 $\beta$	GGGCCTCAAAGGAAAGAATC	TCCACTTTGCTCTTGACTTCTATC
	TNF $\alpha$	CAAGGACTCAAATGGGCTTTCCG	TCTGTGAGGAAGGCTGTGCATTG
Human	EGR2	CTACCCGGTGGAAAGACCTC	AATGTTGATCATGCCATCTCC
	18S	GCAATTATTCCCATGAACG	GGGACTTAATCAACGCAAGC
	IL6	CAATGAGGAGACTTGCCTGG	GCACAGCTCTGGCTTGTTCC
	MCP-1	TCAAACCTGAAGCTCGCACTCT	GTGACTGGGGCATTGATTG
	CD38	CCTCACATGGTGTGGTGAAT	TTGCTGCAGTCCTTTCTCC

**Table S1 : PCR Primers**

95°C: 5min	Denaturation (1 cycle)
Denaturation: 10sec at 95°C	Amplification
Hybridation of primers: 15sec at 60°C	
Elongation: 10sec at 72°C	
95°C during 10sec then 60°C during 30sec	Fusion (1 cycle)
40°C: 30sec	Cooling (1 cycle)

**Table S2: RT-qPCR amplification conditions**