



**HAL**  
open science

## **IL-1 $\beta$ potently stabilizes IL-6 mRNA in human astrocytes**

Anneleen Spooren, Pieter Mestdagh, Pieter Rondou, Krzysztof Kolmus, Guy Haegeman, Sarah Gerlo

► **To cite this version:**

Anneleen Spooren, Pieter Mestdagh, Pieter Rondou, Krzysztof Kolmus, Guy Haegeman, et al.. IL-1 $\beta$  potently stabilizes IL-6 mRNA in human astrocytes. *Biochemical Pharmacology*, 2011, 10.1016/j.bcp.2011.01.019 . hal-00681627

**HAL Id: hal-00681627**

**<https://hal.science/hal-00681627>**

Submitted on 22 Mar 2012

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

Title: IL-1 $\beta$  potently stabilizes IL-6 mRNA in human astrocytes

Authors: Anneleen Spooren, Pieter Mestdagh, Pieter Rondou, Krzysztof Kolmus, Guy Haegeman, Sarah Gerlo



PII: S0006-2952(11)00076-1  
DOI: doi:10.1016/j.bcp.2011.01.019  
Reference: BCP 10815

To appear in: *BCP*

Received date: 6-12-2010  
Revised date: 25-1-2011  
Accepted date: 27-1-2011

Please cite this article as: Spooren A, Mestdagh P, Rondou P, Kolmus K, Haegeman G, Gerlo S, IL-1 $\beta$  potently stabilizes IL-6 mRNA in human astrocytes, *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2011.01.019

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14 IL-1 $\beta$  potently stabilizes IL-6 mRNA in human astrocytes.  
15  
16  
17  
18  
19  
20  
21

22 Anneleen Spooren<sup>a</sup>, Pieter Mestdagh<sup>b, #</sup>, Pieter Rondou<sup>b, #</sup>, Krzysztof Kolmus<sup>a</sup>, Guy  
23 Haegeman<sup>a</sup> and Sarah Gerlo<sup>a, \*</sup>  
24  
25  
26  
27  
28  
29  
30  
31

32 <sup>a</sup>Laboratory of Eukaryotic Gene Expression and Signal Transduction (LEGEST), University of Ghent,  
33 K.L. Ledeganckstraat 35, 9000 Ghent, Belgium; [Anneleen.Spooren@UGent.be](mailto:Anneleen.Spooren@UGent.be),  
34 [Krzysztof.Kolmus@UGent.be](mailto:Krzysztof.Kolmus@UGent.be), [Guy.Haegeman@UGent.be](mailto:Guy.Haegeman@UGent.be), [Sarah.Gerlo@UGent.be](mailto:Sarah.Gerlo@UGent.be)  
35  
36

37  
38 <sup>b</sup>Center for Medical Genetics, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium;  
39 [Pieter.Rondou@UGent.be](mailto:Pieter.Rondou@UGent.be), [Pieter.Mestdagh@UGent.be](mailto:Pieter.Mestdagh@UGent.be)  
40  
41

42 <sup>#</sup>The following authors contributed equally to this paper.  
43  
44

45 <sup>\*</sup>To whom correspondence should be addressed: Dr. Sarah Gerlo, K.L. Ledeganckstraat 35, 9000  
46 Ghent, Belgium, [Sarah.Gerlo@ugent.be](mailto:Sarah.Gerlo@ugent.be), phone +32 9 264 51 35, fax +32 9 264 53 04  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

**Abstract**

1  
2  
3  
4  
5  
6 Uncontrolled expression of IL-6 in the central nervous system is associated with  
7  
8 neurodegenerative pathology and glioma development. Astrocytes are the predominant source  
9  
10 of IL-6 in the central nervous system, and they are characteristically susceptible to synergistic  
11  
12 IL-6 expression. Combined  $\beta$ -adrenergic and TNF-receptor triggering induces synergistic IL-  
13  
14 6 expression in 1321N1 cells via a transcriptional enhancer mechanism. Here, we have  
15  
16 investigated the molecular basis of the very potent “super”-synergistic IL-6 expression that is  
17  
18 apparent after combined treatment of astrocytes with a  $\beta$ -adrenergic agonist, isoproterenol,  
19  
20 and the inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ . We found that IL-1 $\beta$  treatment strengthens  
21  
22 the IL-6 synergy by inducing a distinct stabilization of IL-6 mRNA. Surprisingly, the mRNA-  
23  
24 stabilizing effect seems to be dependent on protein kinase C (PKC), but not on the  
25  
26 prototypical mRNA-stabilizing kinase p38. Moreover, although the mRNA-binding protein  
27  
28 HuR basally stabilizes IL-6 mRNA, the mRNA-stabilizing effect of IL-1 $\beta$  is independent of  
29  
30 HuR. Our data using pharmacological inhibitors suggest PKC is an important modulator of  
31  
32 IL-6 expression in the central nervous system and this might have therapeutic implications.  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42

43 Keywords: mRNA stability, IL-6, astrocytes, IL-1 $\beta$ , PKC, p38  
44  
45  
46

47 Abbreviations: ARE, AU-rich element; CNS, central nervous system; COX-2, cyclo-  
48  
49 oxygenase 2; ELAV, embryonic lethal abnormal vision; HuR, human antigen R; IL-6,  
50  
51 interleukin-6; IL-1 $\beta$ , interleukin-1 $\beta$ ; iso, isoproterenol; MAPK, mitogen-activated protein  
52  
53 kinase; miRNA, microRNA; PKC, protein kinase C; TNF, tumor necrosis factor; 3'UTR, 3'  
54  
55 untranslated region; veh, vehicle  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## 1. INTRODUCTION

1  
2  
3  
4  
5 Control of the expression levels of cytokines is essential for retaining cellular homeostasis and  
6  
7 the orchestration of inflammation. In the central nervous system (CNS), excessive expression  
8  
9 of cytokines, such as interleukin-6 (IL-6), has been associated with the pathogenesis and  
10  
11 progression of different CNS affections [1, 2].

12  
13  
14 For instance, IL-6 has been demonstrated to play an important role in the development and  
15  
16 malignant progression of brain glioma by promoting angiogenesis, cell proliferation and  
17  
18 resistance to apoptosis and radiation [3-5]. Moreover, a positive correlation between IL-6  
19  
20 gene expression and shortened survival in glioblastoma patients has been shown [6]. During  
21  
22 the last decade, more insight has been gained into the physiological consequences of IL-6  
23  
24 upregulation in gliomas; however, the molecular mechanisms leading to excessive IL-6  
25  
26 expression in astrocytes remain largely unclear.

27  
28  
29  
30  
31 Apart from being involved in tumor development, IL-6 dysregulation in the CNS has been  
32  
33 associated with various neurodegenerative diseases, such as Alzheimer's disease or  
34  
35 Parkinson's disease. The accompanying neuro-inflammatory reaction is believed to become  
36  
37 an enhancer of neurodegeneration at the moment it escapes the normal control mechanisms to  
38  
39 restrict the expression of inflammatory mediators, such as cytokines [7].  
40  
41  
42  
43  
44  
45

46 To guarantee their transient expression, many cytokines contain AU-rich elements (AREs) in  
47  
48 their 3' untranslated regions (3'UTR). The AREs allow rapid mRNA degradation by  
49  
50 promoting the recruitment of mRNA-destabilizing proteins to the 3'UTR. The IL-6 3'UTR  
51  
52 contains six AREs [8]. In several systems, IL-6 has been described to have a short half life,  
53  
54 varying from 30 minutes [8-10] to 50 minutes [11]. Several extracellular stimuli have been  
55  
56 described to have a stabilizing effect on IL-6 mRNA, such as IL-1 $\beta$  [9, 12]; IL-17 [13, 14];  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

TNF- $\alpha$  [11] and IL-6 itself [10]. Relatively little is known about the signaling pathways responsible for IL-6 mRNA stabilization. It has been found by multiple groups that the mitogen-activated protein kinase (MAPK) p38 stabilizes IL-6 mRNA [12, 15-18]. Another kinase often associated with mRNA stabilization is protein kinase C (PKC). PKC activation has been implicated in mRNA stabilization of, among others, GAP-43 [19, 20], COX-2 [21], IL-1 $\beta$  [22] and p21 [23]. Moreover, PKC and p38 target several RNA-binding proteins that bind 3'UTRs and stabilize or destabilize the mRNA. More specifically for IL-6, direct binding of both AUF-1 [8] and HuR (also known as ELAV-1 (embryonic lethal, abnormal vision, *Drosophila*-like 1)) [24, 25] has already been described, with the former protein both stabilizing and destabilizing IL-6 mRNA, depending on its expression level, and the latter stabilizing IL-6 mRNA.

Astrocytes are the main source of IL-6 in the CNS [26]. In light of the pathological consequences of uncontrolled IL-6 expression in the CNS, we aimed to unravel the pathways leading to IL-6 production in a human astrocytoma cell line. The 1321N1 cells used in this study were originally isolated from primary cultures of a cerebral glioma multiforme [27], and are often used as an astrocyte model [28]. We have shown before that combined  $\beta$ -adrenergic and TNF-receptor triggering induces synergistic IL-6 expression in 1321N1 cells via a transcriptional enhancer mechanism, involving the cooperative recruitment of CREB, NF- $\kappa$ B and CBP to the IL-6 promoter [29]. Here, we describe that IL-1 $\beta$  can further enhance IL-6 production via a post-transcriptional mechanism involving mRNA stabilization. Surprisingly, this effect does not involve a prototypical p38- or HuR-dependent mechanism and, based on pharmacological inhibitor experiments, seems to be PKC-dependent.

## 2. MATERIALS AND METHODS

### 2.1 Reagents

Isoproterenol, GF109203X, Brefeldin A (BFA) and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO). IL-1 $\beta$  was from Invitrogen (Carlsbad, CA). Anti-P-p65-Ser536, anti-P-ERK, anti-P-p38, anti-P-JNK and anti-P-CREB-Ser133 were from Cell Signaling (Danvers, MA); anti-p65 and anti-PARP were from Santa Cruz (Santa Cruz, CA) and anti-tubulin was from Sigma-Aldrich (St. Louis, MO). siHuR SMART pool (M-003773-04) and siControl (D-001210-05) were from Dharmacon (Lafayette, CO). SB203580 was from Alexis Biochemicals (Buttler Pike, PA), Ro31-8220 was from Calbiochem (Gibbstown, NJ). Recombinant human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was obtained from the Department of Molecular Biology of Ghent University (DMBR, Ghent, Belgium) (with specific activity of  $3.37 \cdot 10^5$  U/ $\mu$ g).

### 2.2 Cell culture

The human astrocytoma cell line 1321N1 was a kind gift from Prof. Dr. Müller (University of Bonn). 1321N1 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FCS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all from Invitrogen, Carlsbad, CA). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. All cells were passaged using 0.05% (w/v) of trypsin in 0.4% (w/v) EDTA. Cells were starved overnight prior to inductions in DMEM supplemented with 1% FCS.

### 2.3 Human IL-6 ELISA

1  
2 Human IL-6 levels were determined using a specific ELISA kit (Biosource, Camarillo, CA)  
3  
4 with detection limits of 5 pg/ml, according to manufacturer's instructions.  
5  
6  
7  
8  
9

#### 10 11 **2.4 RNA isolation and Quantitative real-time PCR** 12 13

14  
15  
16 Total RNA was extracted with the acid-guanidinium-thiocyanate-phenol chloroform method  
17  
18 using Trizol Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed on 0.5  
19  
20  $\mu$ g of total mRNA using MMLV (Promega, Madison, WI). For real time cDNA amplification  
21  
22 we used the Biorad SYBR Green Mastermix (Biorad, Hercules, CA) and the following  
23  
24 primers: h-IL-6 fw: GAC AGC CAC TCA CCT CTT CA, h-IL-6 rv AGT GCC TCT TTG  
25  
26 CTG CTT TC, h-HPRT fw TGA CAC TGG CAA AAC AAT GCA, h-HPRT rv GGT CCT  
27  
28 TTT CAC CAG CAA GCT, h-COX-2 fw GCC CTT CCT CCT GTG CC, h-COX-2 rv AAT  
29  
30 CAG GAA GCT GCT TTT TAC CTT T, h-IL-8 fw CTC TCT TGG CAG CCT TCC TGA,  
31  
32 h-IL-8 rv CAA TAA TTT CTG TGT TGG CGC. Fluorescence was monitored using the  
33  
34 BioRad iCycler (BioRad, Hercules, CA). A serial dilution of a cDNA mix standard was used  
35  
36 to determine the efficiency of the PCR reaction and to calculate relative mRNA inputs.  
37  
38  
39  
40  
41  
42  
43 Absolute values were normalized to the HPRT reference gene.  
44  
45  
46  
47  
48  
49

#### 50 51 **2.5 Reporter gene assays** 52 53

54  
55 The IL-6 promoter luciferase construct containing 1168 bp of the human IL-6 promoter  
56  
57 (1168-IL-6-luc) has been described before [30]. The 3'UTR IL-6 luciferase vector (IL-6  
58  
59 3'UTR-luc, with the IL-6 3'UTR (1-403) cloned in the pGL3 promoter vector, under control  
60  
61  
62  
63  
64  
65

1 of an SV40 promoter) was a kind gift of Prof. Kirkwood (University of Michigan) and has  
2 been described before [15]. FLAG-HuR was a kind gift of Dr. Doller (J.W.Goethe-  
3 Universität, Frankfurt am Main) and has been described before [31]. Cells were seeded in 24-  
4 well plates (50 000 cells/well) and transfected using the calcium phosphate method with 0.8  
5  $\mu\text{g}$  IL-6 construct and 0.2  $\mu\text{g}$  neogal constructs per well or with 0.5  $\mu\text{g}$  of IL-6 constructs, 0.5  
6  $\mu\text{g}$  expression vector plasmid DNA and 0.2  $\mu\text{g}$  neogal-construct. 48 hours following  
7 transfection, cells were starved overnight and induced for the indicated time period. Total  
8 lysates were subsequently incubated with luciferase or galactosidase reagent and  
9 luminescence was measured on the Viktor3 system (Perkin Elmer Life Sciences, Boston,  
10 MA). Luciferase output was normalized to  $\beta$ -galactosidase values transcribed from the  
11 cotransfected galactosidase vector.  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30

## 31 **2.6 Western immunoblotting**

32  
33  
34  
35  
36 Total cellular extracts were made in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS,  
37 10% glycerol, 0.5%  $\beta$ -mercapto-ethanol). Equal amounts of total lysates from each condition  
38 were resolved by 10% SDS-PAGE, transferred onto nitrocellulose membranes and analysed  
39 by Western blotting. Chemiluminescent detection was performed using fluorophore-coupled  
40 secondary antibodies (Rockland, Gilbertsville, PA) in combination with the Odyssey Imaging  
41 System (Licor, Lincoln, NA). Quantification was performed using ImageJ software and based  
42 on 3 independent experiments. Tubulin was used as a loading control.  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53

54 Nuclear and cytosolic extracts were obtained as described earlier [32]. Briefly, cells were  
55 lysed in B1 buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1 mM  $\text{MgCl}_2$ , 5 % glycerol, 0,5 mM  
56 EDTA pH 7.5, 0.1 mM EGTA pH 7.5, 0.5 mM DTT, protease inhibitors and 0.65 % (v/v)  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 NP40). Nuclei were pulled down by centrifugation at 800 rpm for 15 min and nuclear pellets  
2 were lysed in B2 buffer by shaking for 15 min at 4°C (20 mM HEPES pH 7.5, 1 % NP40, 1  
3 mM MgCl<sub>2</sub>, 400 mM NaCl, 10 mM KCl, 20 % glycerol, 0.5 mM EDTA pH 7.5, 0.1 mM  
4 EGTA pH 7.5, 0.5 mM DTT and protease inhibitors). Lysates were subsequently analyzed by  
5 Western blotting. Fractionation was verified using tubulin as a cytoplasmic and PARP as a  
6 nuclear control.  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17

## 18 **2.7 siRNA silencing of HuR**

19  
20  
21  
22  
23 For siRNA experiments, cells were seeded in 6-well plates (200 000 cells/well) and  
24 transfected using the calcium phosphate method with a final concentration of 40 nM  
25 siGENOME SMART pools. siControl transfected samples were used to assess for aspecific  
26 effects. 48 hours after transfection and after overnight starvation, cells were induced for the  
27 indicated time periods. Subsequently, cells were lysed in SDS sample buffer or RNA was  
28 isolated, as described in section 2.4 and 2.6.  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41

## 42 **2.8 Immunofluorescent PKC translocation assay**

43  
44  
45  
46  
47 Immunofluorescence assay of PKC translocation was adjusted from [33]. Briefly, cells were  
48 starved overnight, followed by induction for the indicated time periods. Cells were fixed and  
49 permeabilized in ice cold methanol for 5 min at -20°C. Next, samples were blocked for 1  
50 hour at room temperature with PBS 3% BSA and then washed 3 times with PBST (PBS with  
51 0.1 % Tween). Nuclei were coloured with DAPI and the coverslips were mounted using  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 Mowiol (Calbiochem, Gibbstown, NJ). Samples were analyzed using an Axiovert 200M  
2 Zeiss Microscope (Thornwood, NY).  
3  
4

## 5 **2.9 Actinomycin D assays**

6  
7  
8  
9

10 For actinomycin D assays, cells were seeded in 6-well plates (250 000 cells/well). After  
11 overnight starvation, cells were preinduced with the indicated stimuli and/or inhibitors.  
12  
13 Subsequently media were aspirated and replaced by starvation medium containing 5 µg/ml of  
14  
15 actinomycin D to block transcription. After incubation for the indicated time periods with  
16  
17 actinomycin D, RNA was isolated, reverse transcription was performed and cDNA was  
18  
19 amplified using qPCR with SYBR Green and specific primers for IL-6. Absolute mRNA  
20  
21 values were normalized to a housekeeping gene (HPRT) and subsequently recalculated as  
22  
23 percentages of mRNA values after preinduction with the respective inductantia without  
24  
25 actinomycin D treatment (0 min, 100%).  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35

## 36 **2.10 Analysis of let-7a expression levels**

37  
38  
39  
40

41 Let-7a expression levels were determined using a specific Taqman microRNA assay (Applied  
42 Biosystems, Carlsbad, CA) according to manufacturer's instructions. Briefly, 1321N1 cells  
43  
44 were seeded in 6-well plates (250 000 cells/well), starved overnight and induced for 6 hours.  
45  
46 RNA was isolated as described under 2.4, and 100 ng of non-denatured RNA was used for  
47  
48 RT-PCR using specific stem-loop primers. cDNA was subjected to qPCR using specific  
49  
50 Taqman probes according to manufacturer's instructions. For normalization, the following  
51  
52 small RNA controls were used: RNU6B, RNU44, RNU58B. Stability of these small RNA  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

controls was assessed using the geNorm algorithm implemented in the qbasePLUS Software package (Biogazelle, Ghent, Belgium) [34].

## 2.11 Statistical Analysis

Results are represented as mean values  $\pm$  standard error of mean (SEM). Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's multiple comparison test, except in figure 2i, 4d and 6b where a Student's *t*-test was used. Both tests were performed using Graphpad Prism 4 software (Graphpad Software Inc., San Diego, CA). Results were considered as significant when  $P < 0.05$ .

### 3 RESULTS

#### 3.1 IL-1 $\beta$ potently enhances the isoproterenol/TNF- $\alpha$ -induced IL-6 synergy

We have shown before that combined  $\beta$ -adrenergic and TNF receptor triggering elicits synergistic IL-6 expression in human astrocytoma 1321N1 cells at protein, mRNA and promoter level [29]. Here we show that addition of the pro-inflammatory cytokine IL-1 $\beta$  to the isoproterenol and TNF- $\alpha$  treatment protocol substantially strengthened this synergistic IL-6 production, inducing a “super”-synergy. This synergy is prominently present at the IL-6 protein level, as measured by ELISA (Fig. 1a), as well as at the mRNA level, as measured by RT-qPCR (Fig. 1b). Analysis of the ratio of IL-6 mRNA after triple (iso+TNF+IL) versus double (iso+TNF) induction indicates a  $10.9 \pm 1.32$  fold enhancement.

#### 3.2 IL-1 $\beta$ does not enhance isoproterenol/TNF- $\alpha$ -induced IL-6 promoter activation

The prototypical signaling cascades activated by isoproterenol, TNF- $\alpha$  and IL-1 $\beta$  were screened to investigate if a) there was synergistic activation of one of the downstream kinases or transcription factors and b) if there were factors that were exclusively activated by IL-1 $\beta$ , thereby explaining the synergy. The MAP kinases p38, JNK and ERK1/2, previously shown to be potently activated by TNF- $\alpha$  in 1321N1 cells [29], were also activated by IL-1 $\beta$  (Fig. 2a, b, c and f). However, triple induction did not lead to synergistic activation of any of the MAP kinases compared to double induction. Additionally, combined isoproterenol and TNF- $\alpha$  treatment activated CREB [29], and IL-1 $\beta$  also induced minor CREB phosphorylation, but

1 again triple induction did not induce synergistic CREB activation as compared to double  
2 induction (Fig. 2d and f). Lastly, NF- $\kappa$ B activation, measured by translocation (Fig. 2g) and  
3  
4 Serine 536 phosphorylation of p65 (Fig. 2e and f), was induced by both iso-TNF and IL-1 $\beta$ .  
5  
6 Again triple induction did not lead to synergistic NF- $\kappa$ B activation compared to double  
7  
8 induction at 30 minutes. Investigation of the main signaling cascades activated by  
9  
10 isoproterenol, TNF- $\alpha$  and IL-1 $\beta$ , thus showed that IL-1 $\beta$  did not activate any 'extra' signaling  
11  
12 cascades, i.e. that were not already activated by isoproterenol or TNF- $\alpha$ , nor did IL-1 $\beta$  induce  
13  
14 synergistic activation of any of the investigated factors. In line with this, we found that, at the  
15  
16 promoter level, as measured by a reporter gene assay, triple induction did not significantly  
17  
18 strengthen promoter activation, as compared to double induction (Fig. 2h). The ratio of the  
19  
20 triple versus double induction was reduced to  $1.3 \pm 0.28$  at the promoter level (Fig. 2i). This  
21  
22 ratio does not differ significantly from 1 ( $P = 0.0959$ ), which indicates that the synergy has  
23  
24 disappeared from mRNA to promoter level.  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35

### 36 **3.3 IL-1 $\beta$ stabilizes IL-6 mRNA.**

37  
38  
39  
40  
41  
42

43 Because the strengthening effect of IL-1 $\beta$  was not conserved at the promoter level, a potential  
44  
45 explanation for the synergy could be the stabilization of IL-6 mRNA by IL-1 $\beta$ . It has been  
46  
47 shown that IL-1 $\beta$  stabilizes multiple mRNA species, including IL-6, in a variety of cell types  
48  
49 [9, 12, 14, 16, 35, 36]. A paradigm commonly used to study effects on mRNA stabilization is  
50  
51 the blockade of new transcription by a transcriptional inhibitor, such as actinomycin D,  
52  
53 followed by quantification of the mRNA levels at several time points after inhibition of  
54  
55 transcription [37]. This allows measuring the degradation rate of a certain mRNA species.  
56  
57  
58  
59 When we investigated the degradation rate of IL-6 mRNA after double versus triple  
60  
61  
62  
63  
64  
65

1 induction, we observed a very clear stabilizing effect of IL-1 $\beta$ . Double induction with  
2 isoproterenol and TNF- $\alpha$  yielded IL-6 mRNA with a half-life of approximately 60 minutes.  
3  
4 Adding IL-1 $\beta$  to the isoproterenol/TNF- $\alpha$  induction clearly stabilized the IL-6 mRNA by  
5  
6 blocking virtually all IL-6 mRNA degradation (Fig. 3a). This effect was sustained for up to 6  
7  
8 hours after addition of actinomycin D (Suppl Fig. 1). We also investigated whether IL-1 $\beta$   
9  
10 induces mRNA stabilization on its own, and, although the IL-1 $\beta$ -mediated mRNA induction is  
11  
12 relatively small, complicating establishment of the degradation rate, it was clear from our  
13  
14 experiments that IL-1 $\beta$  by itself also stabilizes IL-6 mRNA (Suppl Fig. 2). Furthermore, we  
15  
16 analyzed the degradation rates of two other genes that are induced by isoproterenol, TNF- $\alpha$   
17  
18 and IL-1 $\beta$  in 1321N1 cells, namely COX-2 and IL-8 [29, 38]. COX-2 showed a synergistic  
19  
20 mRNA induction and an mRNA degradation pattern that was very comparable to the one  
21  
22 observed for IL-6 (Fig. 3c and d). Triple induction stabilized COX-2 mRNA completely  
23  
24 compared to double induction. IL-8, on the other hand, showed a slower, but progressive  
25  
26 mRNA degradation pattern, with no significant stabilization by IL-1 $\beta$  at the earlier time points  
27  
28 (60 and 120 min, Suppl Fig. 3).  
29  
30  
31  
32  
33  
34  
35  
36

37 As the 3'UTR is the main target for mRNA-(de)stabilizing mechanisms, we also studied IL-6  
38  
39 mRNA stability using a reporter gene assay. In this system, the IL-6 3'UTR is cloned behind  
40  
41 the luciferase coding sequence, thus reporting 3'UTR-mediated effects on mRNA stability via  
42  
43 changes in the luciferase signal. Both the double induction with isoproterenol and TNF- $\alpha$  and  
44  
45 the individual induction with IL-1 $\beta$  induced a small, but insignificant increase in the RLU  
46  
47 values after 6, 16 and 24 hours induction (Fig. 3b). Only the triple induction with  
48  
49 isoproterenol, TNF- $\alpha$  and IL-1 $\beta$  elicited a significant increase in RLU values after 6, 16 and  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

### 3.4 The effect of IL-1 $\beta$ seems to be mediated by PKC and not by p38.

Two kinases that have been particularly implicated in mRNA-stabilizing effects are p38 and PKC [39, 40]. Importantly, both can be activated by IL-1 $\beta$  and have been convincingly implicated in COX-2 mRNA stabilization [21, 31, 41-46]. Moreover, in multiple studies, IL-1 $\beta$ -induced mRNA stabilization of IL-6 was shown to be crucially dependent on p38 [12, 16], whereas dependence on PKC has not yet been described. Therefore, we used pharmacological inhibitors of p38 and PKC to investigate if they are involved in the mRNA-stabilizing effect of IL-1 $\beta$  in our experimental system. When we blocked p38 with SB203580, IL-1 $\beta$  was still able to stabilize IL-6 mRNA in the actinomycin D assay (Fig. 4a), indicating that p38 activation is not necessary for IL-6 mRNA stabilization. The effectiveness of SB203580 was demonstrated by the complete inhibition of p38-mediated MSK phosphorylation after treatment with the inhibitor (Fig. 4b). On the other hand, when we blocked PKC with GF109203X, we observed complete inhibition of the stabilizing effect of IL-1 $\beta$  (Fig. 4a). This suggests that IL-1 $\beta$  stabilizes IL-6 mRNA via PKC. To further substantiate the finding that PKC was involved, we used a second PKC inhibitor, namely Ro31-8220. Again we observed complete inhibition of the IL-1 $\beta$ -mediated IL-6 mRNA stabilization (Fig. 4c), whereas parallel inhibition by SB203580 had no effect. Additional evidence for the involvement of PKC came from the ability of PMA, a PKC activator, to mimic IL-1 $\beta$ -induced stabilization of the IL-6 3'UTR reporter gene (Fig. 4d). Moreover, like IL-1 $\beta$ , PMA also induced 'super'-synergistic IL-6 mRNA production, when added to isoproterenol and TNF- $\alpha$ , imitating the super-synergy induced by IL-1 $\beta$  (Fig. 4e).

### 3.5 Identification of the PKC isoform involved

1  
2  
3 PKC is a family of kinases composed of 10 isoforms [47]. The family is subdivided into 3  
4  
5 classes, based on their  $\text{Ca}^{2+}$ , diacylglycerol and phospholipid dependency, namely, the  
6  
7 classical ( $\alpha$ ,  $\beta\text{I}$ ,  $\beta\text{II}$ ,  $\gamma$ ), novel ( $\eta$ ,  $\theta$ ,  $\delta$ ,  $\epsilon$ ) and atypical PKCs ( $\iota$ ,  $\zeta$ ). In order to identify the PKC  
8  
9 isoform responsible for the IL-1 $\beta$ -induced IL-6 mRNA stabilization, we set up a large  
10  
11 screening experiment using different previously validated PKC-GFP constructs [31, 48-52].  
12  
13 Our aim was to assess which PKC isoform was activated after triple induction, as manifested  
14  
15 by PKC translocation to the membrane, and sometimes also to the nucleus. However, at  
16  
17 various time points ranging from 1 minute to 60 minutes, we did not observe translocation of  
18  
19 any of the 10 isoforms after triple induction, even though PMA, used as a positive control,  
20  
21 induced clear membrane translocation of the classical and novel isoforms. An exemplary  
22  
23 image of PKC distribution after triple induction or PMA treatment is shown in supplementary  
24  
25 figure 4 for the various PKC isoforms at the 30 minute time point. Similar results were  
26  
27 obtained at earlier and later time points (1', 5', 10, 15', 30', 60' 6h; data not shown).  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38

### 39 **3.6 The IL-1 $\beta$ -induced IL-6 mRNA stabilization is a late, indirect effect, independent of** 40 41 **protein secretion**

42  
43  
44  
45  
46  
47  
48 Since we could block the IL-1 $\beta$ -induced IL-6 mRNA stabilization by PKC inhibitors on the  
49  
50 one hand, but did not observe rapid PKC activation after triple induction on the other hand,  
51  
52 we hypothesized that the mRNA-stabilizing effect of IL-1 $\beta$  was possibly indirect and delayed.  
53  
54 In line with this, the full-blown IL-1 $\beta$ -mediated mRNA-stabilizing effect was not apparent  
55  
56 when we reduced the preinduction time to 1 or 3 hours in our actinomycin D experiments  
57  
58 (Fig. 5a). Similarly, IL-1 $\beta$  did not induce COX2 mRNA stabilization after 1 or 3 hours  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

preincubation with isoproterenol, TNF- $\alpha$  and IL-1 $\beta$  combined (Fig. 5b). These data suggest that although IL-1 $\beta$  stabilizes IL-6 mRNA, it does so via triggering a late-onset, possibly indirect, mechanism. To investigate if a secreted factor was responsible for the effect of IL-1 $\beta$ , we blocked secretion of proteins using brefeldin A (BFA). Pretreatment with 5  $\mu$ g/ml BFA did not inhibit IL-1 $\beta$ -induced IL-6 mRNA stabilization (Fig. 5c). The effectiveness of BFA was demonstrated by visualizing the Golgi network, using the Golgi-marker GM130 (Fig. 5d). Treatment of 1321N1 cells with BFA resulted in the transformation of the discrete perinuclear, vesicular structure to a largely dispersed, dotted structure, reflecting the desintegration of the Golgi network. In addition, we could not mimic the IL-6 mRNA-stabilizing effect by using conditioned medium fractions, suggesting the mRNA-stabilizing effect is not dependent on an IL-1 $\beta$ -induced secreted factor (data not shown). We also investigated the role of novel protein synthesis in the stabilizing effect of IL-1 $\beta$ , using the translational elongation inhibitor cycloheximide. However, as described before by several other authors, translational inhibition led to “superinduction” of IL-6 mRNA [35, 37] (data not shown). This effect has been attributed to the freezing of the translation machinery on the IL-6 mRNA, thereby protecting it from degradation, and thus rules out the possibility of using translational inhibitors to study whether protein synthesis is required for IL-1 $\beta$  induced IL-6 mRNA stabilization [35]. However, when 1321N1 cells are treated with IL-1 $\beta$  in the presence of actinomycin D (instead of the here employed treatment with IL-1 $\beta$  before actinomycin D treatment), IL-6 mRNA is no longer stabilized, indicating the importance of transcription for the mRNA stabilizing effect of IL-1 $\beta$  (data not shown).

### 3.7 IL-1 $\beta$ does not stabilize IL-6 mRNA via a prototypical HuR-dependent pathway

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

One of the main mRNA-stabilizing proteins, is the ELAV protein HuR, which upon stimulation with mRNA-stabilizing stimuli translocates from the nucleus to the cytoplasm where it binds and stabilizes the 3'UTRs of several mRNAs [53]. HuR has previously been shown to stabilize IL-6 [24, 25] and COX-2 mRNA [21, 31, 54]. Moreover, HuR is a major effector of PKC-mediated mRNA stabilization [21, 31, 54] and can be induced by IL-1 $\beta$  [55, 56]. Therefore, we sought to determine if HuR was involved in the IL-6 mRNA-stabilizing effect of IL-1 $\beta$ .

First, we analyzed the effect of overexpressing HuR in the IL-6 3'UTR luciferase assay.

Although HuR overexpression did clearly enhance the basal luciferase signal (Fig. 6a), there was no effect on the magnitude of the triple induction (Fig. 6b). This suggests that HuR is able to stabilize IL-6 mRNA via its 3'UTR, which corresponds to findings in other studies, but nevertheless the effect of IL-1 $\beta$  does not seem to be mediated via HuR. In line with this, we found that the nuclear/cytoplasmic distribution of endogenous HuR after triple induction at different time points (0-6 hours) did not change (Fig. 6c). In 1321N1 cells, HuR is already present in the cytoplasm in small amounts, possibly due to the fact that they are tumor-derived cells [57]. Since HuR has also been shown to stabilize mRNAs in response to IL-1 $\beta$  in the absence of cytoplasmic translocation, but via direct binding to the 3'UTR [56], we next employed an siRNA approach to finally exclude the involvement of HuR. Knockdown of HuR using siRNA did not inhibit IL-1 $\beta$  mediated IL-6 mRNA stabilization (Fig. 6d), even though there was substantial knockdown of HuR protein as assessed by Western blot (Fig. 6e), indicating HuR was not involved in IL-1 $\beta$ -mediated IL-6 mRNA stabilization.

### 3.8 Let-7a microRNA is not involved in the IL-1 $\beta$ -induced IL-6 mRNA stabilization

1  
2  
3 Because a prototypical p38- or HuR-dependent mechanism did not seem to be involved in IL-  
4  
5 6 mRNA stabilization in our experimental system, we additionally investigated whether  
6  
7 microRNAs (miRNAs) played a role in IL-6 mRNA stabilization [58]. miRNAs are  
8  
9 particularly abundant in the brain and are suggested to have a pivotal role in several aspects of  
10  
11 CNS functioning [59]. Recently, the let-7a miRNA was shown to bind the IL-6 3'UTR [60].  
12  
13 The let-7 miRNA family is comprised of 12 members. Importantly, it is was shown that NF-  
14  
15  $\kappa$ B activation led to downregulation of let-7a and this subsequently leads to upregulation of  
16  
17 IL-6, resulting in a positive feedback loop [60]. Since IL-1 $\beta$  has been shown to cause long-  
18  
19 term, persistent NF- $\kappa$ B activation in 1321N1 cells [28], we hypothesized that IL-1 $\beta$  might  
20  
21 downregulate let-7a in 1321N1 cells. However, when we analyzed let-7a levels after  
22  
23 treatment with isoproterenol, TNF- $\alpha$  and IL-1 $\beta$ , alone or in combinations, we did not observe  
24  
25 significant differences in the expression level of this miRNA, indicating that let-7a is not  
26  
27 involved in the IL-6 mRNA-stabilizing effect of IL-1 $\beta$  in 1321N1 cells (Fig. 6f).  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

#### 4 DISCUSSION

1  
2  
3  
4  
5  
6 Here, we have reported that IL-1 $\beta$  potently enhances TNF- $\alpha$ /isoproterenol-induced IL-6  
7  
8 expression in astrocytes. Susceptibility to synergy is a hallmark of IL-6 expression by  
9  
10 astrocytes [26]. IL-6 has paradoxical effects in the CNS, combining pro-inflammatory and  
11  
12 neurotrophic traits [61], and dose and timing of IL-6 expression are regarded as crucial  
13  
14 determinants in shifting the balance between beneficial and detrimental effects. Therefore, it  
15  
16 is important to understand the molecular mechanisms underlying synergistic IL-6 expression  
17  
18  
19  
20  
21 in astrocytes.

22  
23  
24  
25  
26  
27 In 1321N1 cells, IL-1 $\beta$  did not enhance isoproterenol- and TNF-induced IL-6 expression via a  
28  
29 transcriptional mechanism, but instead very potently enhanced IL-6 mRNA stability. IL-1 $\beta$   
30  
31 has been previously demonstrated to stabilize multiple mRNA species, such as COX-2 [42,  
32  
33 43, 62-64], IL-8 [56] and NGF [65, 66]. Importantly, it has already been reported that IL-1 $\beta$   
34  
35 stabilizes IL-6 mRNA in osteoblasts [16, 35], fibroblasts [9], myofibroblasts [14], astrocytes  
36  
37 [36] and fibroblast-like synoviocytes [12], although relatively little was known about the  
38  
39 molecular mechanisms underlying this stabilization. Moreover, other studies found no  
40  
41 stabilization of IL-6 mRNA after IL-1 $\beta$  induction, but instead found that TNF- $\alpha$  [11] or  
42  
43 cAMP [67] were the stabilizing agents in this instance, indicating the cell type specificity of  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65 IL-1 $\beta$ -mediated mRNA stabilization.

66  
67  
68  
69  
70  
71  
72  
73  
74  
75  
76  
77  
78  
79  
80  
81  
82  
83  
84  
85  
86  
87  
88  
89  
90  
91  
92  
93  
94  
95  
96  
97  
98  
99  
100  
101  
102  
103  
104  
105  
106  
107  
108  
109  
110  
111  
112  
113  
114  
115  
116  
117  
118  
119  
120  
121  
122  
123  
124  
125  
126  
127  
128  
129  
130  
131  
132  
133  
134  
135  
136  
137  
138  
139  
140  
141  
142  
143  
144  
145  
146  
147  
148  
149  
150  
151  
152  
153  
154  
155  
156  
157  
158  
159  
160  
161  
162  
163  
164  
165  
166  
167  
168  
169  
170  
171  
172  
173  
174  
175  
176  
177  
178  
179  
180  
181  
182  
183  
184  
185  
186  
187  
188  
189  
190  
191  
192  
193  
194  
195  
196  
197  
198  
199  
200  
201  
202  
203  
204  
205  
206  
207  
208  
209  
210  
211  
212  
213  
214  
215  
216  
217  
218  
219  
220  
221  
222  
223  
224  
225  
226  
227  
228  
229  
230  
231  
232  
233  
234  
235  
236  
237  
238  
239  
240  
241  
242  
243  
244  
245  
246  
247  
248  
249  
250  
251  
252  
253  
254  
255  
256  
257  
258  
259  
260  
261  
262  
263  
264  
265  
266  
267  
268  
269  
270  
271  
272  
273  
274  
275  
276  
277  
278  
279  
280  
281  
282  
283  
284  
285  
286  
287  
288  
289  
290  
291  
292  
293  
294  
295  
296  
297  
298  
299  
300  
301  
302  
303  
304  
305  
306  
307  
308  
309  
310  
311  
312  
313  
314  
315  
316  
317  
318  
319  
320  
321  
322  
323  
324  
325  
326  
327  
328  
329  
330  
331  
332  
333  
334  
335  
336  
337  
338  
339  
340  
341  
342  
343  
344  
345  
346  
347  
348  
349  
350  
351  
352  
353  
354  
355  
356  
357  
358  
359  
360  
361  
362  
363  
364  
365  
366  
367  
368  
369  
370  
371  
372  
373  
374  
375  
376  
377  
378  
379  
380  
381  
382  
383  
384  
385  
386  
387  
388  
389  
390  
391  
392  
393  
394  
395  
396  
397  
398  
399  
400  
401  
402  
403  
404  
405  
406  
407  
408  
409  
410  
411  
412  
413  
414  
415  
416  
417  
418  
419  
420  
421  
422  
423  
424  
425  
426  
427  
428  
429  
430  
431  
432  
433  
434  
435  
436  
437  
438  
439  
440  
441  
442  
443  
444  
445  
446  
447  
448  
449  
450  
451  
452  
453  
454  
455  
456  
457  
458  
459  
460  
461  
462  
463  
464  
465  
466  
467  
468  
469  
470  
471  
472  
473  
474  
475  
476  
477  
478  
479  
480  
481  
482  
483  
484  
485  
486  
487  
488  
489  
490  
491  
492  
493  
494  
495  
496  
497  
498  
499  
500  
501  
502  
503  
504  
505  
506  
507  
508  
509  
510  
511  
512  
513  
514  
515  
516  
517  
518  
519  
520  
521  
522  
523  
524  
525  
526  
527  
528  
529  
530  
531  
532  
533  
534  
535  
536  
537  
538  
539  
540  
541  
542  
543  
544  
545  
546  
547  
548  
549  
550  
551  
552  
553  
554  
555  
556  
557  
558  
559  
560  
561  
562  
563  
564  
565  
566  
567  
568  
569  
570  
571  
572  
573  
574  
575  
576  
577  
578  
579  
580  
581  
582  
583  
584  
585  
586  
587  
588  
589  
590  
591  
592  
593  
594  
595  
596  
597  
598  
599  
600  
601  
602  
603  
604  
605  
606  
607  
608  
609  
610  
611  
612  
613  
614  
615  
616  
617  
618  
619  
620  
621  
622  
623  
624  
625  
626  
627  
628  
629  
630  
631  
632  
633  
634  
635  
636  
637  
638  
639  
640  
641  
642  
643  
644  
645  
646  
647  
648  
649  
650  
651  
652  
653  
654  
655  
656  
657  
658  
659  
660  
661  
662  
663  
664  
665  
666  
667  
668  
669  
670  
671  
672  
673  
674  
675  
676  
677  
678  
679  
680  
681  
682  
683  
684  
685  
686  
687  
688  
689  
690  
691  
692  
693  
694  
695  
696  
697  
698  
699  
700  
701  
702  
703  
704  
705  
706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718  
719  
720  
721  
722  
723  
724  
725  
726  
727  
728  
729  
730  
731  
732  
733  
734  
735  
736  
737  
738  
739  
740  
741  
742  
743  
744  
745  
746  
747  
748  
749  
750  
751  
752  
753  
754  
755  
756  
757  
758  
759  
760  
761  
762  
763  
764  
765  
766  
767  
768  
769  
770  
771  
772  
773  
774  
775  
776  
777  
778  
779  
780  
781  
782  
783  
784  
785  
786  
787  
788  
789  
790  
791  
792  
793  
794  
795  
796  
797  
798  
799  
800  
801  
802  
803  
804  
805  
806  
807  
808  
809  
810  
811  
812  
813  
814  
815  
816  
817  
818  
819  
820  
821  
822  
823  
824  
825  
826  
827  
828  
829  
830  
831  
832  
833  
834  
835  
836  
837  
838  
839  
840  
841  
842  
843  
844  
845  
846  
847  
848  
849  
850  
851  
852  
853  
854  
855  
856  
857  
858  
859  
860  
861  
862  
863  
864  
865  
866  
867  
868  
869  
870  
871  
872  
873  
874  
875  
876  
877  
878  
879  
880  
881  
882  
883  
884  
885  
886  
887  
888  
889  
890  
891  
892  
893  
894  
895  
896  
897  
898  
899  
900  
901  
902  
903  
904  
905  
906  
907  
908  
909  
910  
911  
912  
913  
914  
915  
916  
917  
918  
919  
920  
921  
922  
923  
924  
925  
926  
927  
928  
929  
930  
931  
932  
933  
934  
935  
936  
937  
938  
939  
940  
941  
942  
943  
944  
945  
946  
947  
948  
949  
950  
951  
952  
953  
954  
955  
956  
957  
958  
959  
960  
961  
962  
963  
964  
965  
966  
967  
968  
969  
970  
971  
972  
973  
974  
975  
976  
977  
978  
979  
980  
981  
982  
983  
984  
985  
986  
987  
988  
989  
990  
991  
992  
993  
994  
995  
996  
997  
998  
999  
1000

Two kinases in particular emerge as possible candidates for mediating IL-1 $\beta$ -induced IL-6 mRNA stability, namely p38 and PKC. p38 has been identified as an essential kinase in

1 regulating IL-1 $\beta$ -mediated mRNA stabilization in multiple studies [12, 15-17, 41-44, 56], of  
2 which several have specifically investigated IL-1 $\beta$ -mediated COX-2 and IL-6 mRNA  
3 stabilization. On the other hand, PKC has been implicated in the stabilization of a variety of  
4 other mRNAs such as, p21, GAP-43 and IL-1 $\beta$  itself [19, 20, 22, 23]. Importantly, PKC has  
5 been convincingly implicated in COX-2 mRNA stabilization in several recent studies [21, 31,  
6 44, 54, 68] and, indirectly, in IL-6 mRNA stabilization in one older study [69]. In astrocytes,  
7 PKC was previously shown to be important for IL-6 induction after IL-1 $\beta$  [70]. Moreover, IL-  
8 1 $\beta$  has been shown to activate several PKC isoforms, such as PKC- $\zeta$  [71, 72], PKC- $\gamma$  [45],  
9 PKC- $\delta$  [46] and PKC- $\beta$  [73], in some of these studies in the context of mRNA stabilization.  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25

26 To the best of our knowledge we are the first to show that the mRNA-stabilizing effect of IL-  
27 1 $\beta$  on IL-6 mRNA can also be p38-independent and might instead be mediated via PKC.  
28

29 However, the conclusion that the mRNA-stabilizing effect of IL-1 $\beta$  is late, and thus probably  
30 indirect, substantially complicated our attempts to identify the involved PKC isoform. Since  
31 PKC activation can be a very fast process, sometimes disappearing already after a few  
32 minutes [74], and since the time frame in which PKC could be activated in our experimental  
33 system ranged from approximately 4 to 6 hours, it unfortunately became virtually impossible  
34 to identify the responsible PKC isoform. Moreover, the existence of 10 different PKC  
35 isoforms complicates a traditional siRNA-based approach. Although the fact that PMA  
36 mimicked the effect of IL-1 $\beta$  indicates that the involved isoform belongs to the novel or  
37 classical subfamily, 8 isoforms are still left. The pharmacological PKC inhibitors that we used  
38 are not very suitable for distinguishing between the different PKC isoforms, since they both  
39 interact at the homologous ATP-binding site, and therefore they do not allow clear distinction  
40 between the different isoforms [75]. Lastly, although the involvement of PKC in mRNA  
41 stabilization is well established [76], and the pharmacological inhibitors we used have been  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 intensively employed previously to investigate PKC involvement in a variety of processes, we  
2 cannot exclude that aspecific inhibition of another kinase might occur, causing inhibition of  
3  
4 IL-1 $\beta$ -mediated IL-6 mRNA stabilization. For example, both Rsk-2 and p70 S6 kinases can  
5  
6 be inhibited by both GF103209X and Ro31-8220 [77]. The involvement of these kinases in  
7  
8 mRNA stabilization processes has however, to our knowledge, not yet been documented. A  
9  
10 well characterized downstream target of PKC in mRNA stabilization processes is the ARE-  
11  
12 binding protein HuR [76]. Moreover, the importance of the PKC-HuR axis in COX-2 mRNA  
13  
14 stabilization is well established [21, 31]. Nevertheless, the mRNA-stabilizing protein HuR,  
15  
16 which has formerly been shown to bind the 3'UTR of IL-6 [24], did not mediate IL-1 $\beta$ -  
17  
18 induced mRNA stabilization of IL-6 in 1321N1 cells, although a constitutive stabilization of  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Whereas the actinomycin D experiments pointed to a very potent mRNA-stabilizing effect,  
IL-1 $\beta$  had only a modest, although highly reproducible and significant effect in the IL-6  
3'UTR assay. This could mean that other elements in the IL-6 mRNA, such as introns or the  
5'UTR, are involved in IL-6 mRNA stabilization after IL-1 $\beta$  treatment in astrocytes. On the  
other hand, in the IL-6 3'UTR assay, luciferase mRNA levels were assessed indirectly, via  
measurement of enzymatic activity of the translated luciferase protein. It could be that the  
mRNA-stabilizing effect in this assay is blurred by additional (post)translational mechanisms  
targeting the luciferase protein and we also cannot rule out that IL-1 $\beta$  affects the half-life of  
the luciferase protein.

Interestingly, in a recent paper, treatment of 1321N1 cells with  $\beta$ -adrenergic agonists inhibited  
their proliferation [78]. In view of the known proliferative effects of IL-6 on brain glioma [4,  
5], this seems contradictory to the results we present here. This contradiction can however be

1 explained by, on the one hand, the differences in timing of the treatment with  $\beta$ -adrenergic  
2 receptor agonists (20 hours by Toll et al. and 6 hours in this paper); and on the other hand, the  
3 fact that IL-1 $\beta$  or TNF- $\alpha$  might possibly overrule the effects isoproterenol has on 1321N1  
4 proliferation.  
5  
6  
7  
8  
9

10  
11  
12  
13 The exact molecular cascade leading to IL-6 mRNA stabilization in our model system  
14 remains unclear. Nevertheless, we can hypothesize on several possible mechanisms, although  
15 we did not explore all of them. First of all, it was recently demonstrated that APOBEC-1  
16 complementation factor (ACF) controls IL-6 mRNA stability in liver [79]. ACF is present in  
17 brain tissue [80], so could be a potentially interesting target. Another recently identified novel  
18 mechanism involving IL-6 mRNA stabilization is the TLR-inducible RNase Zc3h12a [81].  
19 Zc3h12a was shown to destabilize IL-6 mRNA after TLR-triggering, thus providing an  
20 essential mechanism for controlling immune reactions *in vivo*. Interestingly, Zc3h12a is also  
21 expressed in brain and was recently shown to be involved in glial differentiation of NT2 cells  
22 [82]. Thirdly, alternative polyadenylation of IL-6 mRNA, i.e. the use of an upstream  
23 polyadenylation signal, resulting in a shorter mRNA, could possibly contribute to changes in  
24 mRNA stability. This mechanism has been convincingly described for COX-2 [83] and HuR  
25 [84]. The human IL-6 gene contains an alternative polyadenylation signal less than 100 bp  
26 upstream from the canonical polyadenylation site [85], and alternative polyadenylation would  
27 lead to the exclusion of several AU-rich regions in the alternative mRNA species. The  
28 possibility that alternative polyadenylation of the human IL-6 cDNA influences mRNA  
29 metabolism is intriguing, but remains uninvestigated up to now. Finally, AUF-1, another  
30 RNA binding protein, has been shown to bind IL-6 mRNA and to influence its stability [8].  
31  
32 We have investigated if AUF-1 is involved in IL-1 $\beta$ -induced IL-6 mRNA stabilization by  
33 overexpressing the different AUF-1 isoforms (p37, p40, p42 and p45) in the IL-6 3'UTR  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 reporter gene assay, but our preliminary data did not suggest a role for AUF-1 in this system  
2 (data not shown).  
3  
4  
5  
6  
7

8  
9 In conclusion, IL-1 $\beta$  stabilizes IL-6 and COX-2 mRNA via a novel, not yet completely  
10 characterized mechanism. This mRNA stabilization translates into a very potent synergistic  
11 IL-6 protein expression. Based on pharmacological inhibitor data, the stabilization process  
12  
13 seems to depend on PKC, but not on p38, contrary to the earlier described modes of IL-6  
14  
15 mRNA stabilization [12, 15-17]. Moreover, the prototypical mRNA-stabilizing protein HuR is  
16  
17 not involved. Finally, the effect is late and probably indirect, although it seems to be  
18  
19 independent of protein secretion.  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29

30 Further elucidation of the molecular mechanism underlying this novel mode of IL-1 $\beta$  induced  
31 mRNA stabilization is required, but in any case the very potent enhancement of IL-6 levels by  
32  
33 combining two classical pro-inflammatory stimuli, TNF- $\alpha$  and IL-1 $\beta$  with a  $\beta$ -adrenergic  
34  
35 agonist, isoproterenol, is bound to have profound physiological effects *in vivo*, specifically in  
36  
37 the context of gliomas and neurodegenerative diseases. Moreover, the finding that PKC  
38  
39 inhibitors are powerful tools to reduce astrocytic IL-6 expression might have interesting  
40  
41 therapeutic implications.  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Acknowledgements

1  
2  
3  
4  
5  
6 The authors would like to thank Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO)  
7  
8 for financial support. A.S. and K.K. are predoctoral FWO fellows and S.G. is a postdoctoral  
9  
10 FWO fellow. P.M. is supported by the Ghent University Research Fund (BOF 01D31406).  
11  
12 P.R. is a postdoctoral BOF fellow (Bijzonder Onderzoeksfonds). The authors are very grateful  
13  
14 to Dr. Doller (Goethe-universität, Frankfurt am Main) for the generous gift of the FLAG-HuR  
15  
16 plasmid, to Prof. Gorospe (NIH, Baltimore) for the generous gift of the AUF-1 expression  
17  
18 plasmids, to Prof. Saito (University of Kobe), Prof. Ferguson (University of Western Ontario),  
19  
20 Prof. Larrison (Lund University) and Dr. Doller (Goethe Universität) for the very generous  
21  
22 gifts of the PKC-GFP expression plasmids, to Prof. Kirkwood (University of Michigan) for  
23  
24 the generous gift of the IL-6 3'UTR-luciferase vector and to Prof. Muller (University of  
25  
26 Bonn) for the generous gift of the 1321N1 cells. The authors would also like to thank R. Dahl.  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## References

- 1  
2  
3  
4  
5  
6 [1] Trikha M, Corringham R, Klein B, Rossi JF. Targeted anti-interleukin-6 monoclonal antibody  
7  
8 therapy for cancer: a review of the rationale and clinical evidence. *Clin Cancer Res*  
9  
10 2003;9:4653-65.  
11  
12 [2] Munoz-Fernandez MA, Fresno M. The role of tumour necrosis factor, interleukin 6,  
13  
14 interferon-gamma and inducible nitric oxide synthase in the development and pathology of the  
15  
16 nervous system. *Prog Neurobiol* 1998;56:307-40.  
17  
18 [3] Weissenberger J, Loeffler S, Kappeler A, Kopf M, Lukes A, Afanasieva TA, et al. IL-6 is  
19  
20 required for glioma development in a mouse model. *Oncogene* 2004;23:3308-16.  
21  
22 [4] Saidi A, Hagedorn M, Allain N, Verpelli C, Sala C, Bello L, et al. Combined targeting of  
23  
24 interleukin-6 and vascular endothelial growth factor potently inhibits glioma growth and  
25  
26 invasiveness. *Int J Cancer* 2009;125:1054-64.  
27  
28 [5] Goswami S, Gupta A, Sharma SK. Interleukin-6-mediated autocrine growth promotion in  
29  
30 human glioblastoma multiforme cell line U87MG. *J Neurochem* 1998;71:1837-45.  
31  
32 [6] Tchirkov A, Rolhion C, Bertrand S, Dore JF, Dubost JJ, Verrelle P. IL-6 gene amplification  
33  
34 and expression in human glioblastomas. *Br J Cancer* 2001;85:518-22.  
35  
36 [7] Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease--a double-edged sword.  
37  
38 *Neuron* 2002;35:419-32.  
39  
40 [8] Paschoud S, Dogar AM, Kuntz C, Grisoni-Neupert B, Richman L, Kuhn LC. Destabilization  
41  
42 of interleukin-6 mRNA requires a putative RNA stem-loop structure, an AU-rich element, and  
43  
44 the RNA-binding protein AUF1. *Mol Cell Biol* 2006;26:8228-41.  
45  
46 [9] Ng SB, Tan YH, Guy GR. Differential induction of the interleukin-6 gene by tumor necrosis  
47  
48 factor and interleukin-1. *J Biol Chem* 1994;269:19021-7.  
49  
50 [10] Weigert C, Dufer M, Simon P, Debre E, Runge H, Brodbeck K, et al. Upregulation of IL-6  
51  
52 mRNA by IL-6 in skeletal muscle cells: role of IL-6 mRNA stabilization and Ca<sup>2+</sup>-dependent  
53  
54 mechanisms. *Am J Physiol Cell Physiol* 2007;293:C1139-47.  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [11] Elias JA, Lentz V. IL-1 and tumor necrosis factor synergistically stimulate fibroblast IL-6 production and stabilize IL-6 messenger RNA. *J Immunol* 1990;145:161-6.
- [12] Miyazawa K, Mori A, Miyata H, Akahane M, Ajisawa Y, Okudaira H. Regulation of interleukin-1beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. *J Biol Chem* 1998;273:24832-8.
- [13] Andoh A, Shimada M, Bamba S, Okuno T, Araki Y, Fujiyama Y, et al. Extracellular signal-regulated kinases 1 and 2 participate in interleukin-17 plus tumor necrosis factor-alpha-induced stabilization of interleukin-6 mRNA in human pancreatic myofibroblasts. *Biochim Biophys Acta* 2002;1591:69-74.
- [14] Shimada M, Andoh A, Hata K, Tasaki K, Araki Y, Fujiyama Y, et al. IL-6 secretion by human pancreatic periacinar myofibroblasts in response to inflammatory mediators. *J Immunol* 2002;168:861-8.
- [15] Zhao W, Liu M, Kirkwood KL. p38alpha stabilizes interleukin-6 mRNA via multiple AU-rich elements. *J Biol Chem* 2008;283:1778-85.
- [16] Patil C, Zhu X, Rossa C, Jr., Kim YJ, Kirkwood KL. p38 MAPK regulates IL-1beta induced IL-6 expression through mRNA stability in osteoblasts. *Immunol Invest* 2004;33:213-33.
- [17] Winzen R, Kracht M, Ritter B, Wilhelm A, Chen CY, Shyu AB, et al. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J* 1999;18:4969-80.
- [18] Neininger A, Kontoyiannis D, Kotlyarov A, Winzen R, Eckert R, Volk HD, et al. MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. *J Biol Chem* 2002;277:3065-8.
- [19] Pascale A, Amadio M, Scapagnini G, Lanni C, Racchi M, Provenzani A, et al. Neuronal ELAV proteins enhance mRNA stability by a PKCalpha-dependent pathway. *Proc Natl Acad Sci U S A* 2005;102:12065-70.
- [20] Perrone-Bizzozero NI, Cansino VV, Kohn DT. Posttranscriptional regulation of GAP-43 gene expression in PC12 cells through protein kinase C-dependent stabilization of the mRNA. *J Cell Biol* 1993;120:1263-70.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [21] Doller A, Huwiler A, Muller R, Radeke HH, Pfeilschifter J, Eberhardt W. Protein kinase C alpha-dependent phosphorylation of the mRNA-stabilizing factor HuR: implications for posttranscriptional regulation of cyclooxygenase-2. *Mol Biol Cell* 2007;18:2137-48.
- [22] Gorospe M, Kumar S, Baglioni C. Tumor necrosis factor increases stability of interleukin-1 mRNA by activating protein kinase C. *J Biol Chem* 1993;268:6214-20.
- [23] Park JW, Jang MA, Lee YH, Passaniti A, Kwon TK. p53-independent elevation of p21 expression by PMA results from PKC-mediated mRNA stabilization. *Biochem Biophys Res Commun* 2001;280:244-8.
- [24] Nabors LB, Gillespie GY, Harkins L, King PH. HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3' untranslated regions of cytokine and angiogenic factor mRNAs. *Cancer Res* 2001;61:2154-61.
- [25] Zhou H, Jarujaron S, Gurley EC, Chen L, Ding H, Studer E, et al. HIV protease inhibitors increase TNF-alpha and IL-6 expression in macrophages: involvement of the RNA-binding protein HuR. *Atherosclerosis* 2007;195:e134-43.
- [26] Van Wagoner NJ, Benveniste EN. Interleukin-6 expression and regulation in astrocytes. *J Neuroimmunol* 1999;100:124-39.
- [27] Foster SJ, Perkins JP. Glucocorticoids increase the responsiveness of cells in culture to prostaglandin E1. *Proc Natl Acad Sci U S A* 1977;74:4816-20.
- [28] Griffin BD, Moynagh PN. Persistent interleukin-1beta signaling causes long term activation of NFkappaB in a promoter-specific manner in human glial cells. *J Biol Chem* 2006;281:10316-26.
- [29] Spooren A, Kooijman R, Lintermans B, Van Craenenbroeck K, Vermeulen L, Haegeman G, et al. Cooperation of NFkappaB and CREB to induce synergistic IL-6 expression in astrocytes. *Cell Signal* 2010;22:871-81.
- [30] Vanden Berghe W, Plaisance S, Boone E, De Bosscher K, Schmitz ML, Fiers W, et al. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J Biol Chem* 1998;273:3285-90.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [31] Doller A, Akool el S, Huwiler A, Muller R, Radeke HH, Pfeilschifter J, et al. Posttranslational modification of the AU-rich element binding protein HuR by protein kinase Cdelta elicits angiotensin II-induced stabilization and nuclear export of cyclooxygenase 2 mRNA. *Mol Cell Biol* 2008;28:2608-25.
- [32] Plaisance S, Vanden Berghe W, Boone E, Fiers W, Haegeman G. Recombination signal sequence binding protein Jkappa is constitutively bound to the NF-kappaB site of the interleukin-6 promoter and acts as a negative regulatory factor. *Mol Cell Biol* 1997;17:3733-43.
- [33] Saha RN, Jana M, Pahan K. MAPK p38 regulates transcriptional activity of NF-kappaB in primary human astrocytes via acetylation of p65. *J Immunol* 2007;179:7101-9.
- [34] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034.
- [35] Kirkwood K, Martin T, Andreadis ST, Kim YJ. Chemically modified tetracyclines selectively inhibit IL-6 expression in osteoblasts by decreasing mRNA stability. *Biochem Pharmacol* 2003;66:1809-19.
- [36] Van Wagoner NJ, Oh JW, Repovic P, Benveniste EN. Interleukin-6 (IL-6) production by astrocytes: autocrine regulation by IL-6 and the soluble IL-6 receptor. *J Neurosci* 1999;19:5236-44.
- [37] Ross J. mRNA stability in mammalian cells. *Microbiol Rev* 1995;59:423-50.
- [38] Aloisi F, Care A, Borsellino G, Gallo P, Rosa S, Bassani A, et al. Production of hemolymphopoietic cytokines (IL-6, IL-8, colony-stimulating factors) by normal human astrocytes in response to IL-1 beta and tumor necrosis factor-alpha. *J Immunol* 1992;149:2358-66.
- [39] Dean JL, Sully G, Clark AR, Saklatvala J. The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation. *Cell Signal* 2004;16:1113-21.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [40] Shim J, Karin M. The control of mRNA stability in response to extracellular stimuli. *Mol Cells* 2002;14:323-31.
- [41] Faour WH, He Y, He QW, de Ladurantaye M, Quintero M, Mancini A, et al. Prostaglandin E(2) regulates the level and stability of cyclooxygenase-2 mRNA through activation of p38 mitogen-activated protein kinase in interleukin-1 beta-treated human synovial fibroblasts. *J Biol Chem* 2001;276:31720-31.
- [42] Ridley SH, Dean JL, Sarsfield SJ, Brook M, Clark AR, Saklatvala J. A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA. *FEBS Lett* 1998;439:75-80.
- [43] Mifflin RC, Saada JI, Di Mari JF, Adegboyega PA, Valentich JD, Powell DW. Regulation of COX-2 expression in human intestinal myofibroblasts: mechanisms of IL-1-mediated induction. *Am J Physiol Cell Physiol* 2002;282:C824-34.
- [44] Subbaramaiah K, Marmo TP, Dixon DA, Dannenberg AJ. Regulation of cyclooxygenase-2 mRNA stability by taxanes: evidence for involvement of p38, MAPKAPK-2, and HuR. *J Biol Chem* 2003;278:37637-47.
- [45] Lin CH, Sheu SY, Lee HM, Ho YS, Lee WS, Ko WC, et al. Involvement of protein kinase C-gamma in IL-1beta-induced cyclooxygenase-2 expression in human pulmonary epithelial cells. *Mol Pharmacol* 2000;57:36-43.
- [46] Carpenter L, Cordery D, Biden TJ. Protein kinase Cdelta activation by interleukin-1beta stabilizes inducible nitric-oxide synthase mRNA in pancreatic beta-cells. *J Biol Chem* 2001;276:5368-74.
- [47] Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem J* 1998;332 ( Pt 2):281-92.
- [48] Kajimoto T, Ohmori S, Shirai Y, Sakai N, Saito N. Subtype-specific translocation of the delta subtype of protein kinase C and its activation by tyrosine phosphorylation induced by ceramide in HeLa cells. *Mol Cell Biol* 2001;21:1769-83.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [49] Villalba M, Bi K, Hu J, Altman Y, Bushway P, Reits E, et al. Translocation of PKC[theta] in T cells is mediated by a nonconventional, PI3-K- and Vav-dependent pathway, but does not absolutely require phospholipase C. *J Cell Biol* 2002;157:253-63.
- [50] Raghunath A, Ling M, Larsson C. The catalytic domain limits the translocation of protein kinase C alpha in response to increases in Ca<sup>2+</sup> and diacylglycerol. *Biochem J* 2003;370:901-12.
- [51] Babwah AV, Dale LB, Ferguson SS. Protein kinase C isoform-specific differences in the spatial-temporal regulation and decoding of metabotropic glutamate receptor 1a-stimulated second messenger responses. *J Biol Chem* 2003;278:5419-26.
- [52] Faisal A, Saurin A, Gregory B, Foxwell B, Parker PJ. The scaffold MyD88 acts to couple protein kinase Cepsilon to Toll-like receptors. *J Biol Chem* 2008;283:18591-600.
- [53] Brennan CM, Steitz JA. HuR and mRNA stability. *Cell Mol Life Sci* 2001;58:266-77.
- [54] Doller A, Gauer S, Sobkowiak E, Geiger H, Pfeilschifter J, Eberhardt W. Angiotensin II induces renal plasminogen activator inhibitor-1 and cyclooxygenase-2 expression post-transcriptionally via activation of the mRNA-stabilizing factor human-antigen R. *Am J Pathol* 2009;174:1252-63.
- [55] Yeh CH, Hung LY, Hsu C, Le SY, Lee PT, Liao WL, et al. RNA-binding protein HuR interacts with thrombomodulin 5'untranslated region and represses internal ribosome entry site-mediated translation under IL-1 beta treatment. *Mol Biol Cell* 2008;19:3812-22.
- [56] Suswam EA, Nabors LB, Huang Y, Yang X, King PH. IL-1beta induces stabilization of IL-8 mRNA in malignant breast cancer cells via the 3' untranslated region: Involvement of divergent RNA-binding factors HuR, KSRP and TIAR. *Int J Cancer* 2005;113:911-9.
- [57] Khabar KS. Post-transcriptional control during chronic inflammation and cancer: a focus on AU-rich elements. *Cell Mol Life Sci* 67:2937-55.
- [58] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008;9:102-14.
- [59] Kosik KS. The neuronal microRNA system. *Nat Rev Neurosci* 2006;7:911-20.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [60] Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell* 2009;139:693-706.
- [61] Gadiant RA, Otten UH. Interleukin-6 (IL-6)--a molecule with both beneficial and destructive potentials. *Prog Neurobiol* 1997;52:379-90.
- [62] Repovic P, Mi K, Benveniste EN. Oncostatin M enhances the expression of prostaglandin E2 and cyclooxygenase-2 in astrocytes: synergy with interleukin-1beta, tumor necrosis factor-alpha, and bacterial lipopolysaccharide. *Glia* 2003;42:433-46.
- [63] Srivastava SK, Tetsuka T, Daphna-Iken D, Morrison AR. IL-1 beta stabilizes COX II mRNA in renal mesangial cells: role of 3'-untranslated region. *Am J Physiol* 1994;267:F504-8.
- [64] Tamura M, Sebastian S, Yang S, Gurates B, Fang Z, Bulun SE. Interleukin-1beta elevates cyclooxygenase-2 protein level and enzyme activity via increasing its mRNA stability in human endometrial stromal cells: an effect mediated by extracellularly regulated kinases 1 and 2. *J Clin Endocrinol Metab* 2002;87:3263-73.
- [65] Vige X, Costa E, Wise BC. Mechanism of nerve growth factor mRNA regulation by interleukin-1 and basic fibroblast growth factor in primary cultures of rat astrocytes. *Mol Pharmacol* 1991;40:186-92.
- [66] Pshenichkin SP, Szekely AM, Wise BC. Transcriptional and posttranscriptional mechanisms involved in the interleukin-1, steroid, and protein kinase C regulation of nerve growth factor in cortical astrocytes. *J Neurochem* 1994;63:419-28.
- [67] Szabo-Fresnais N, Blondeau JP, Pomerance M. Activation of the cAMP pathway synergistically increases IL-1-induced IL-6 gene expression in FRTL-5 thyroid cells: involvement of AP-1 transcription factors. *Mol Cell Endocrinol* 2008;284:28-37.
- [68] Doller A, Schlepckow K, Schwalbe H, Pfeilschifter J, Eberhardt W. Tandem phosphorylation of serines 221 and 318 by protein kinase Cdelta coordinates mRNA binding and nucleocytoplasmic shuttling of HuR. *Mol Cell Biol* 30:1397-410.
- [69] Akashi M, Loussararian AH, Adelman DC, Saito M, Koeffler HP. Role of lymphotoxin in expression of interleukin 6 in human fibroblasts. Stimulation and regulation. *J Clin Invest* 1990;85:121-9.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [70] Norris JG, Tang LP, Sparacio SM, Benveniste EN. Signal transduction pathways mediating astrocyte IL-6 induction by IL-1 beta and tumor necrosis factor-alpha. *J Immunol* 1994;152:841-50.
- [71] Limatola C, Barabino B, Nista A, Santoni A. Interleukin 1-beta-induced protein kinase C-zeta activation is mimicked by exogenous phospholipase D. *Biochem J* 1997;321 ( Pt 2):497-501.
- [72] Ganz MB, Saksa B, Saxena R, Hawkins K, Sedor JR. PDGF and IL-1 induce and activate specific protein kinase C isoforms in mesangial cells. *Am J Physiol* 1996;271:F108-13.
- [73] Kracht M, Heiner A, Resch K, Szamel M. Interleukin-1-induced signaling in T-cells. Evidence for the involvement of phosphatases PP1 and PP2A in regulating protein kinase C-mediated protein phosphorylation and interleukin-2 synthesis. *J Biol Chem* 1993;268:21066-72.
- [74] Ohmori S, Shirai Y, Sakai N, Fujii M, Konishi H, Kikkawa U, et al. Three distinct mechanisms for translocation and activation of the delta subspecies of protein kinase C. *Mol Cell Biol* 1998;18:5263-71.
- [75] Way KJ, Chou E, King GL. Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* 2000;21:181-7.
- [76] Doller A, Pfeilschifter J, Eberhardt W. Signalling pathways regulating nucleo-cytoplasmic shuttling of the mRNA-binding protein HuR. *Cell Signal* 2008;20:2165-73.
- [77] Alessi DR. The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1beta (Rsk-2) and p70 S6 kinase. *FEBS Lett* 1997;402:121-3.
- [78] Toll L, Jimenez L, Waleh N, Jozwiak K, Woo AY, Xiao RP, et al. {beta}2-Adrenergic receptor agonists inhibit the proliferation of 1321N1 astrocytoma cells. *J Pharmacol Exp Ther*.
- [79] Blanc V, Sessa KJ, Kennedy S, Luo J, Davidson NO. Apobec-1 complementation factor modulates liver regeneration by post-transcriptional regulation of interleukin-6 mRNA stability. *J Biol Chem* 285:19184-92.
- [80] Mehta A, Kinter MT, Sherman NE, Driscoll DM. Molecular cloning of apobec-1 complementation factor, a novel RNA-binding protein involved in the editing of apolipoprotein B mRNA. *Mol Cell Biol* 2000;20:1846-54.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [81] Matsushita K, Takeuchi O, Standley DM, Kumagai Y, Kawagoe T, Miyake T, et al. Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature* 2009;458:1185-90.
- [82] Vrotsos EG, Kolattukudy PE, Sugaya K. MCP-1 involvement in glial differentiation of neuroprogenitor cells through APP signaling. *Brain Res Bull* 2009;79:97-103.
- [83] Hall-Pogar T, Zhang H, Tian B, Lutz CS. Alternative polyadenylation of cyclooxygenase-2. *Nucleic Acids Res* 2005;33:2565-79.
- [84] Al-Ahmadi W, Al-Ghamdi M, Al-Haj L, Al-Saif M, Khabar KS. Alternative polyadenylation variants of the RNA binding protein, HuR: abundance, role of AU-rich elements and auto-Regulation. *Nucleic Acids Res* 2009;37:3612-24.
- [85] Tanabe O, Akira S, Kamiya T, Wong GG, Hirano T, Kishimoto T. Genomic structure of the murine IL-6 gene. High degree conservation of potential regulatory sequences between mouse and human. *J Immunol* 1988;141:3875-81.

**Figure captions****Fig. 1**

Synergistic IL-6 induction at the protein and mRNA level, after combined induction with isoproterenol, TNF- $\alpha$  and IL-1 $\beta$  in 1321N1 cells.

- a) IL-6 secretion is synergistically induced in 1321N1 cells by various combinations of isoproterenol (iso, 10  $\mu$ M), TNF- $\alpha$  (TNF, 2000 IU/ml) and IL-1 $\beta$  (IL, 2 ng/ml). Cells were induced for 6 h, after which medium was collected and IL-6 concentrations in medium were determined by ELISA. Data shown are means of 3 independent experiments, each performed in triplicate. <sup>a</sup> $P < 0.05$  versus all other treatments (veh, iso, TNF, IL, TNF+IL, iso+TNF and iso+IL).
- b) IL-6 mRNA expression is synergistically induced in 1321N1 cells by combinations of isoproterenol (iso, 10  $\mu$ M), TNF- $\alpha$  (TNF, 2000 IU/ml) and IL-1 $\beta$  (IL, 2 ng/ml). Cells were induced for 6 h, and RT-qPCR analysis was performed. Data shown are the means of 4 independent experiments, with <sup>a</sup> $P < 0.05$  versus all individual inductions (veh, iso, TNF, IL); <sup>b</sup> $P < 0.05$  versus TNF+IL; <sup>c</sup> $P < 0.05$  versus iso+TNF and <sup>d</sup> $P < 0.05$  versus iso+IL.

**Fig. 2**

Effect of combined induction with isoproterenol, TNF- $\alpha$  and IL-1 $\beta$  on downstream signaling cascades and IL-6 promoter activation.

1 a-e) After induction of 1321N1 cells for 30 min with vehicle (veh), isoproterenol (iso, 10  
2  $\mu\text{M}$ ), TNF- $\alpha$  (TNF, 2000 IU/ml) and IL-1 $\beta$  (IL, 2 ng/ml) individually or combined, cells were  
3  
4 lysed in SDS sample buffer and subjected to Western analysis. Activation of p38 (a), JNK (b),  
5  
6 ERK (c), CREB (d) or p65 (e) pathways was assessed using phospho-specific antibodies and  
7  
8 tubulin as a loading control. Three independent experiments were used for quantification of  
9  
10 Western blots. <sup>ns</sup> $P > 0.05$  versus iso+TNF.  
11  
12  
13  
14

15 f) A representative blot after treatment of 1321N1 cells for 30 min as described under a-e).  
16  
17

18 g) Effect on nuclear translocation of p65. 1321N1 cells were induced with isoproterenol (10  
19  $\mu\text{M}$ ), TNF- $\alpha$  (2000 IU/ml), IL-1 $\beta$  (2 ng/ml) or combinations thereof for 30 minutes.  
20  
21

22 Subsequently nuclear extracts were subjected to Western blot analysis to determine p65  
23  
24 levels. Data shown are representative for 3 independent experiments.  
25  
26

27 h) IL-6 promoter activation after treatment with isoproterenol, TNF- $\alpha$  and IL-1 $\beta$ . 1321N1  
28  
29 cells were transiently transfected with an IL-6 promoter construct (1168-IL-6-luc).  
30  
31 Reporter gene activation was measured with a luciferase reporter gene assay after  
32  
33 induction for 6 hours with isoproterenol (iso, 10  $\mu\text{M}$ ), TNF- $\alpha$  (TNF, 2000 IU/ml) and IL-  
34  
35 1 $\beta$  (IL, 2 ng/ml), individually or combined. Data shown are means of 5 independent  
36  
37 experiments. <sup>a</sup> $P < 0.05$  versus all individual inductions (veh, iso, TNF, IL); <sup>ns</sup> $P > 0.05$  for  
38  
39 iso+TNF versus iso+TNF+IL.  
40  
41  
42  
43  
44

45 i) Comparison of the ratio of triple (iso+TNF+IL) versus double (iso+TNF) induction at  
46  
47 mRNA and promoter level. Mean ratios were calculated on the basis of 3-4 independent  
48  
49 experiments, performed as described under Fig. 1b (mRNA level) and Fig. 2h (promoter  
50  
51 level). <sup>a</sup> $P < 0.05$  versus promoter; <sup>ns</sup> $P > 0.05$  in one sample  $t$  test with the theoretical mean  
52  
53  
54  
55  
56  
57  
58  
59  
60 of 1.  
61  
62  
63  
64  
65

**Fig. 3**

1  
2 Effect of isoproterenol, TNF- $\alpha$  and IL-1 $\beta$  treatment on IL-6 and COX-2 mRNA stability.  
3

- 4  
5 a) mRNA degradation rates were determined using actinomycin D. 1321N1 cells were  
6  
7 pretreated for 6 hours with isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml) (iso+TNF) or  
8  
9 isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml)+IL-1 $\beta$  (2 ng/ml) (iso+TNF+IL).  
10  
11 Subsequently, iso, TNF- $\alpha$  and IL-1 $\beta$  were removed and actinomycin D (5  $\mu$ g/ml) was  
12  
13 added to block new transcription. mRNA was isolated at different time points after  
14  
15 addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR analysis.  
16  
17 Data shown are means of 8 independent experiments. <sup>a</sup>*P* < 0.05 versus iso+TNF at that  
18  
19 time point.  
20  
21  
22  
23  
24 b) Kinetics of mRNA stability as measured by a reporter gene assay. 1321N1 cells were  
25  
26 transiently transfected with an IL-6 3'UTR construct (IL-6 3'UTR-luc). Reporter gene  
27  
28 expression was measured with a luciferase reporter gene assay after induction for 6, 16  
29  
30 and 24 hours with isoproterenol (iso, 10  $\mu$ M), TNF- $\alpha$  (TNF, 2000 IU/ml) and IL-1 $\beta$  (IL, 2  
31  
32 ng/ml), individually or combined. Data shown are mean values calculated from 4  
33  
34 independent experiments. <sup>a</sup>*P* < 0.05 versus iso+TNF at that time point; <sup>ns</sup>*P* > 0.05 between  
35  
36 the inductions with iso+TNF, IL and veh at the corresponding time points.  
37  
38  
39  
40  
41 c) mRNA degradation rates were determined using actinomycin D. 1321N1 cells were  
42  
43 pretreated for 6h with isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml) (iso+TNF) or  
44  
45 isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml)+IL-1 $\beta$  (2 ng/ml) (iso+TNF+IL).  
46  
47 Subsequently iso, TNF- $\alpha$  and IL-1 $\beta$  were removed and actinomycin D (5  $\mu$ g/ml) was  
48  
49 added to block new transcription. mRNA was isolated at different time points after  
50  
51 addition of actinomycin D (0, 30, 60, 120 and 180 min) and subjected to RT-qPCR  
52  
53 analysis. Data shown are mean values calculated from 3 independent experiments. <sup>a</sup>*P* <  
54  
55  
56  
57  
58 0.05 versus iso+TNF at that time point.  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

d) COX-2 mRNA expression is synergistically induced in 1321N1 cells by combinations of isoproterenol (iso, 10  $\mu$ M), TNF- $\alpha$  (TNF, 2000 IU/ml) and IL-1 $\beta$  (IL, 2 ng/ml). Triple (iso+TNF+IL) versus double (iso+TNF) induction clearly induces a “super” synergy. Cells were induced for 6 h, after which RT-qPCR analysis was performed. Data shown are mean values of 3 independent experiments. <sup>a</sup>*P* < 0.05 versus veh; <sup>b</sup>*P* < 0.05 versus iso+TNF.

**Fig. 4**

Effect of pharmacological inhibitors of p38 and PKC on IL-1 $\beta$ -induced mRNA stability of IL-6 mRNA.

a) Effect of a p38 inhibitor (SB) and a PKC inhibitor (GF) on mRNA degradation rate of IL-6, determined using actinomycin D. 1321N1 cells were pretreated for 6h with isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml) (iso+TNF) or isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml)+IL-1 $\beta$  (2 ng/ml) (iso+TNF+IL) with or without SB203580 (SB, 10  $\mu$ M) or GF109203X (GF, 10  $\mu$ M). Subsequently iso, TNF- $\alpha$ , IL-1 $\beta$ , SB and GF were removed and actinomycin D (5  $\mu$ g/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 30, 60 and 120 min) and subjected to RT-qPCR analysis. Data shown are mean values calculated from 3 independent experiments. <sup>a</sup>*P* < 0.05 between iso+TNF+IL+GF versus iso+TNF+IL at that time point; <sup>ns</sup>*P* > 0.05 between iso+TNF+IL versus iso+TNF+IL+SB at the corresponding time points.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- b) Effectiveness of SB203580. 1321N1 cells were induced for 30 min using isoproterenol (10  $\mu$ M), TNF- $\alpha$  (2000 IU/ml) and IL-1 $\beta$  (2 ng/ml) (iso+TNF+IL) with or without SB230580 (10  $\mu$ M). Lysates were subjected to Western analysis. Activation of MSK was assessed using a phosphospecific antibody (Anti-P-MSK (Thr 581)).
- c) Effect of a p38 inhibitor (SB) and a PKC inhibitor (Ro) on the mRNA degradation rate of IL-6, determined using actinomycin D. 1321N1 cells were pretreated for 6h with isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml) (iso+TNF) or isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml)+IL-1 $\beta$  (2 ng/ml) (iso+TNF+IL) in combination with SB203580 (SB, 10  $\mu$ M) or Ro31-8220 (Ro, 5  $\mu$ M)). Subsequently, iso, TNF- $\alpha$ , IL-1 $\beta$ , SB and Ro were removed and actinomycin D (5  $\mu$ g/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR. Data shown are mean values calculated from 3 independent experiments. <sup>a</sup>*P* < 0.05 iso+TNF+IL+Ro versus iso+TNF+IL at that time point; <sup>ns</sup>*P* > 0.05 between iso+TNF+IL versus iso+TNF+IL+SB at the corresponding time points.
- d) PMA mimics IL-1 $\beta$ -induced stabilization of the IL-6 3'UTR reporter gene. 1321N1 cells were transiently transfected with an IL-6 3' UTR construct (luc-IL-6 3'UTR). Reporter gene expression was measured with a luciferase reporter gene assay after induction for 6 h with vehicle (veh) or PMA (1  $\mu$ g/ml). Data shown are mean values calculated from at least 5 independent experiments. <sup>a</sup>*P* = 0.0021 in a two-tailed *t* test versus veh.
- e) PMA mimics IL-1 $\beta$ -induced 'super'synergistic IL-6 mRNA expression. IL-6 mRNA expression is synergistically induced in 1321N1 cells by combinations of isoproterenol (iso, 10  $\mu$ M), TNF- $\alpha$  (TNF, 2000 IU/ml), IL-1 $\beta$  (IL, 2 ng/ml) and PMA (1  $\mu$ g/ml). Triple induction with iso+TNF+PMA mimics the triple induction with iso+TNF+IL-1 $\beta$ . Cells were induced for 6 h, and RT-qPCR analysis was performed. Data shown are mean values

of 3 independent experiments. <sup>a</sup>*P* < 0.05 versus iso+TNF; <sup>ns</sup>*P* > 0.05 for iso+TNF+PMA versus iso+TNF+IL.

### Fig. 5

Effect of kinetic preinduction on IL-6 mRNA degradation rate.

a-b) 1321N1 cells were preinduced with isoproterenol (10 μM) and TNF-α (2000 IU/ml) (iso+TNF) or isoproterenol (10 μM), TNF-α (2000 IU/ml) and IL-1β (2 ng/ml) (iso+TNF+IL) for 1, 3 or 6 hours. Subsequently iso, TNF-α and IL-1β were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR. Data are means of 3-4 independent experiments. <sup>ns</sup>*P* > 0.05 between iso+TNF versus iso+TNF+IL at that time point; <sup>a</sup>*P* < 0.05 versus iso+TNF at that time point.

c) Effect of blocking protein secretion using brefeldin A (BFA) on IL-6 mRNA degradation rate. 1321N1 cells were preinduced for 30 min with or without BFA (5 μg/ml) and subsequently induced with isoproterenol (10 μM) and TNF-α (2000 IU/ml) (iso+TNF) or isoproterenol (10 μM), TNF-α (2000 IU/ml) and IL-1β (2 ng/ml) (iso+TNF+IL) for 6 hours. Iso, TNF-α, IL-1β and BFA were removed and actinomycin D (5 μg/ml) was added to block new transcription. mRNA was isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min) and subjected to RT-qPCR. Data shown are means of 2 independent experiments. <sup>ns</sup>*P* > 0.05 between iso+TNF+IL versus iso+TNF+IL+BFA for the corresponding time points; <sup>a</sup>*P* < 0.05 versus iso+TNF+IL at that time point.

d) Effectiveness of brefeldin A. Cells were treated with BFA (5 μg/ml) for 6,5 hours and fixed with formaldehyde. Immunofluorescence staining was performed using an antibody for a Golgi marker (anti-GM130) to visualize the Golgi compartment.

**Fig. 6**

1  
2  
3  
4  
5 Involvement of the mRNA stabilizing protein HuR and let-7a in IL-1 $\beta$ -induced IL-6 mRNA  
6  
7 stabilization.

- 8  
9  
10 a) Effect of overexpression of HuR on an IL-6 3'UTR reporter gene assay. 1321N1 cells  
11 were transiently transfected with an IL-6 3' UTR construct (luc-IL-6 3'UTR) combined  
12 with an empty vector (pcDNA3) or a HuR expression vector (HuR). Reporter gene  
13 expression was measured with a luciferase reporter gene assay after induction for 6 hours  
14 with isoproterenol (iso, 10  $\mu$ M), TNF- $\alpha$  (TNF, 2000 IU/ml) and IL-1 $\beta$  (IL, 2 ng/ml),  
15 individually or combined. Data are means of 3 independent experiments. <sup>a</sup>*P* < 0.05 versus  
16 the corresponding pcDNA3 condition; <sup>b</sup>*P* < 0.05 versus the corresponding veh condition.  
17  
18  
19 b) The fold enhancement after triple induction with isoproterenol, TNF- $\alpha$  and IL-1 $\beta$  is not  
20 affected by HuR overexpression. 1321N1 cells were transfected, induced and subjected to  
21 reporter gene analysis as described under a). Values were obtained by normalizing to non-  
22 induced condition (vehicle). <sup>ns</sup>*P* > 0.05 versus pcDNA3.  
23  
24  
25 c) Nuclear-cytoplasmic shuttling of HuR after induction for increasing time points. 1321N1  
26 cells were treated with isoproterenol (10  $\mu$ M), TNF- $\alpha$  (2000 IU/ml) and IL-1 $\beta$  (2 ng/ml)  
27 for 0 to 6 hours with 30 min intervals. Nuclear and cytoplasmic fractions were isolated  
28 and subjected to Western analysis. Data shown are representative for 3 independent  
29 experiments.  
30  
31  
32 d) Effect of knockdown of HuR using siRNA on IL-6 mRNA degradation rate, determined  
33 using actinomycin D. 1321N1 cells were transiently transfected with siControl (siCtr, 40  
34 nM) or siHuR (40 nM). 48 hours after transfection, cells were pretreated for 6 hours with  
35 isoproterenol (10  $\mu$ M)+TNF- $\alpha$  (2000 IU/ml) (iso+TNF) or isoproterenol (10  $\mu$ M)+TNF- $\alpha$   
36 (2000 IU/ml)+IL-1 $\beta$  (2 ng/ml) (iso+TNF+IL). Subsequently iso, TNF- $\alpha$  and IL-1 $\beta$  were  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 removed and actinomycin D (5  $\mu$ g/ml) was added to block new transcription. mRNA was  
2 isolated at different time points after addition of actinomycin D (0, 60, 120 and 180 min)  
3  
4 and subjected to RT-qPCR. Data are means of 3 independent experiments. <sup>a</sup> $P < 0.05$   
5  
6 between iso+TNF siCtr versus iso+TNF+IL siCtr for that time point; <sup>ns</sup> $P > 0.05$  between  
7  
8 iso+TNF+IL siCtr versus iso+TNF+IL siHuR for the corresponding time points.  
9

10  
11  
12 e) Control of knockdown of HuR. 1321N1 cells were transiently transfected with siControl  
13  
14 (40 nM) or siHuR (40 nM). 48 hours after transfection, cells were lysed in SDS sample  
15  
16 buffer and subjected to Western analysis.  
17  
18

19 f) Analysis of let-7a expression levels. 1321N1 cells were induced for 6 hours with  
20  
21 combinations of isoproterenol (iso, 10  $\mu$ M), TNF- $\alpha$  (TNF, 2000 IU/ml) and IL-1 $\beta$  (IL, 2  
22  
23 ng/ml). RNA was isolated and let-7a expression levels were determined using a specific  
24  
25 Taqman microRNA assay. Data shown are the mean of 4 independent experiments. <sup>ns</sup> $P >$   
26  
27 0.05 between all treatments.  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

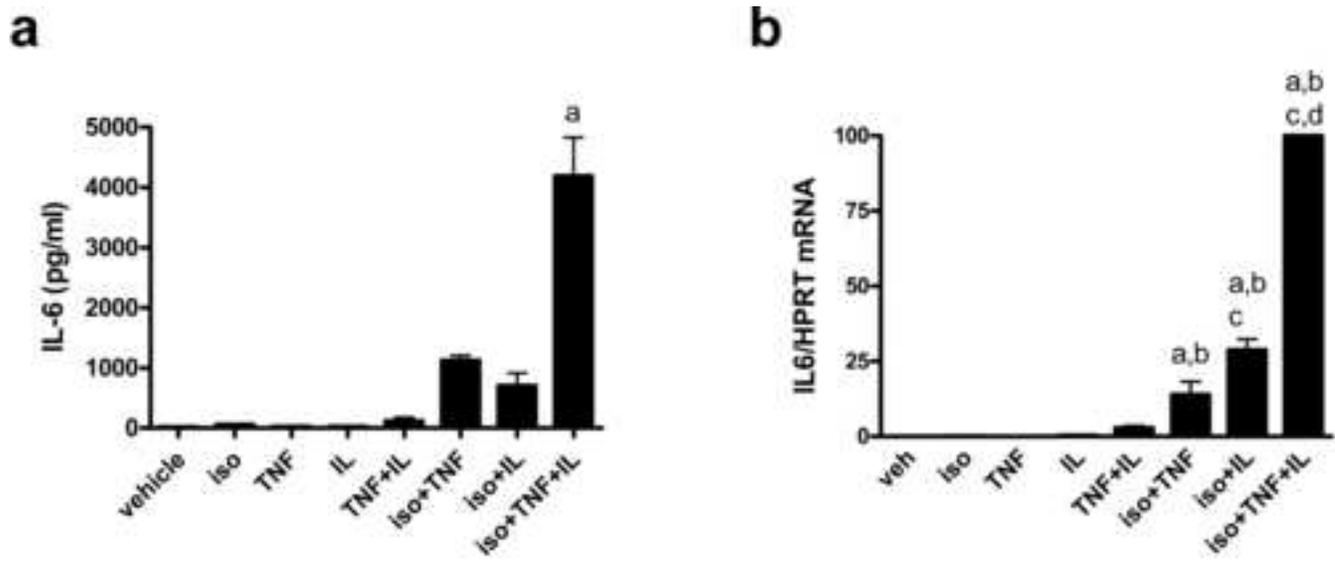


Figure 1

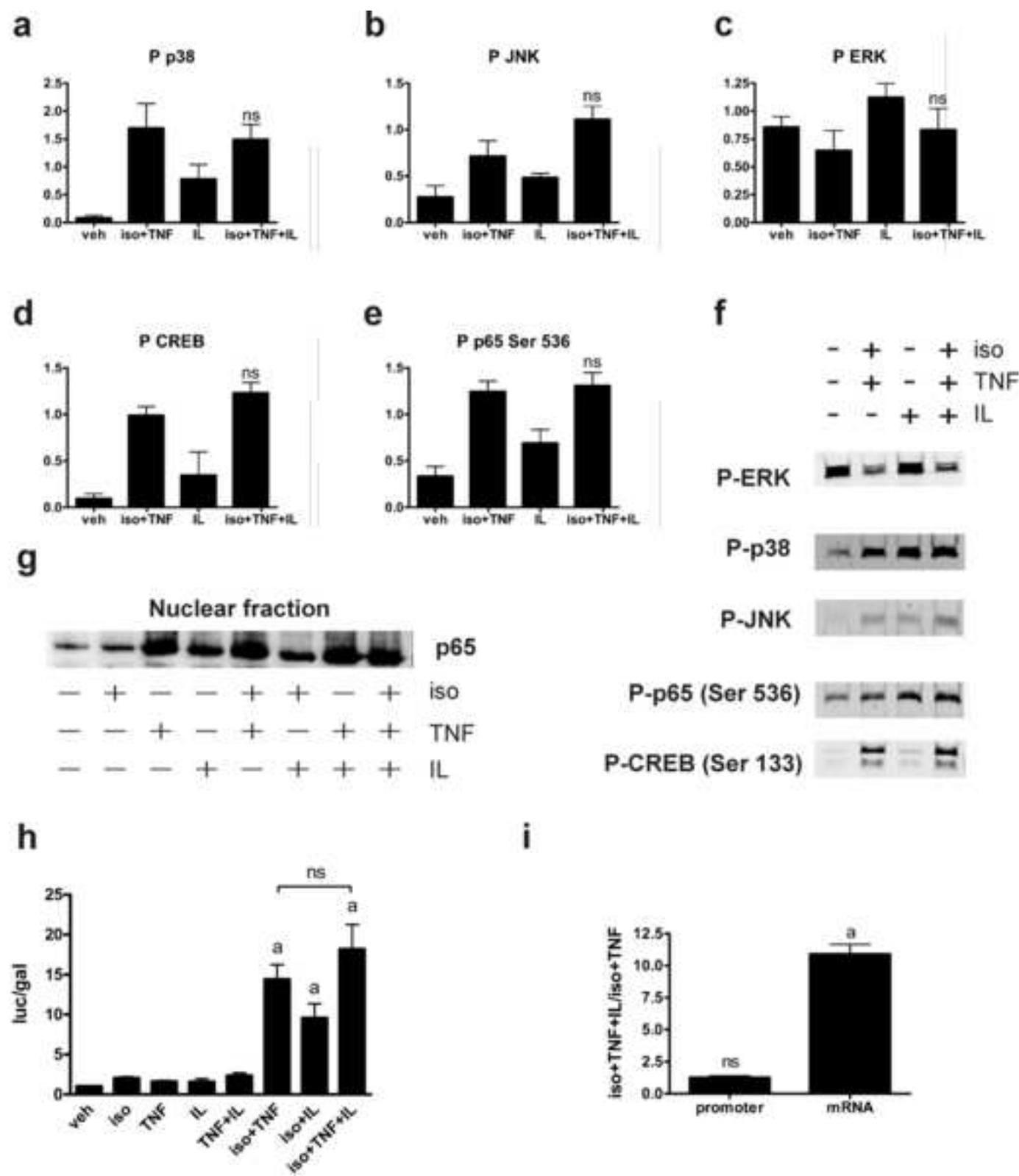


Figure 2

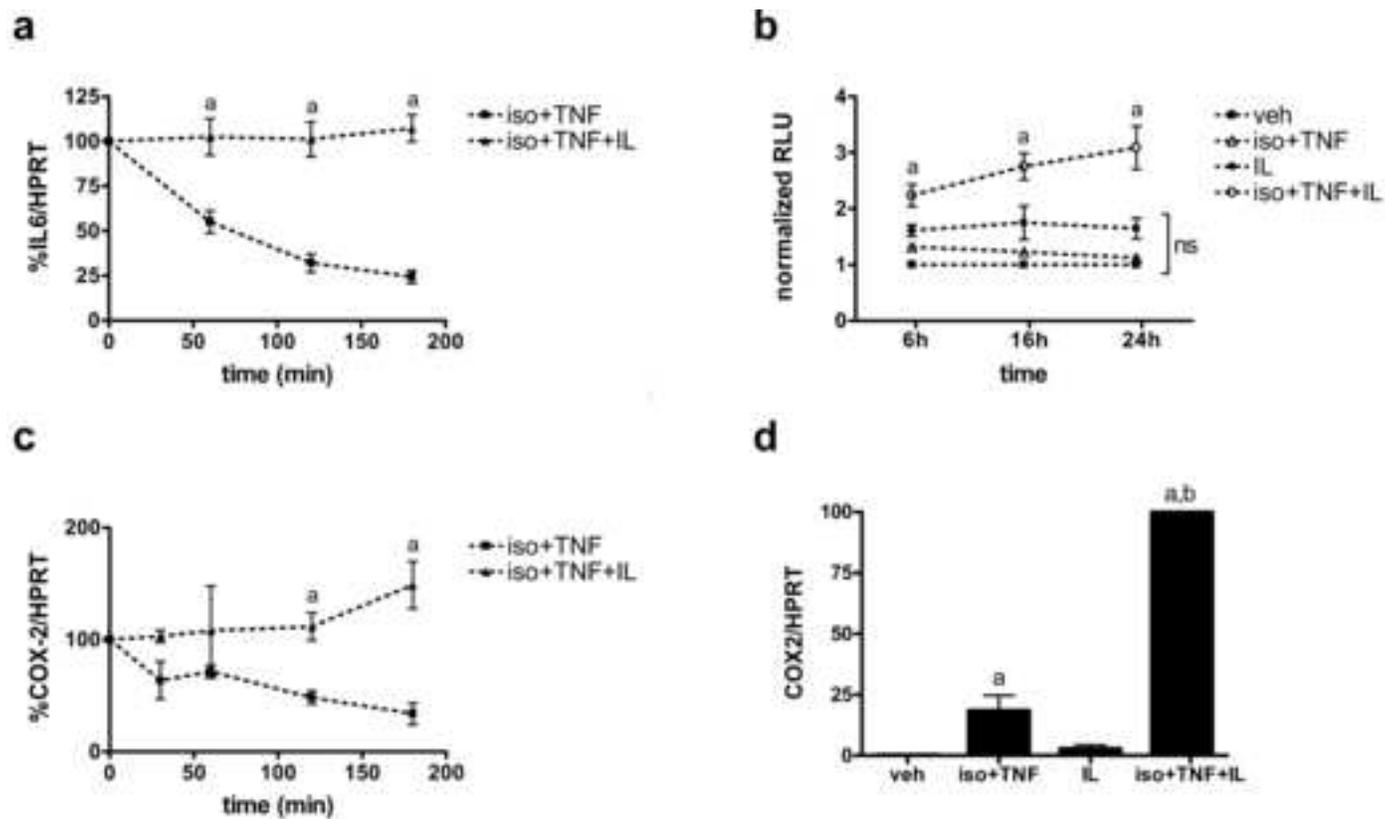


Figure 3

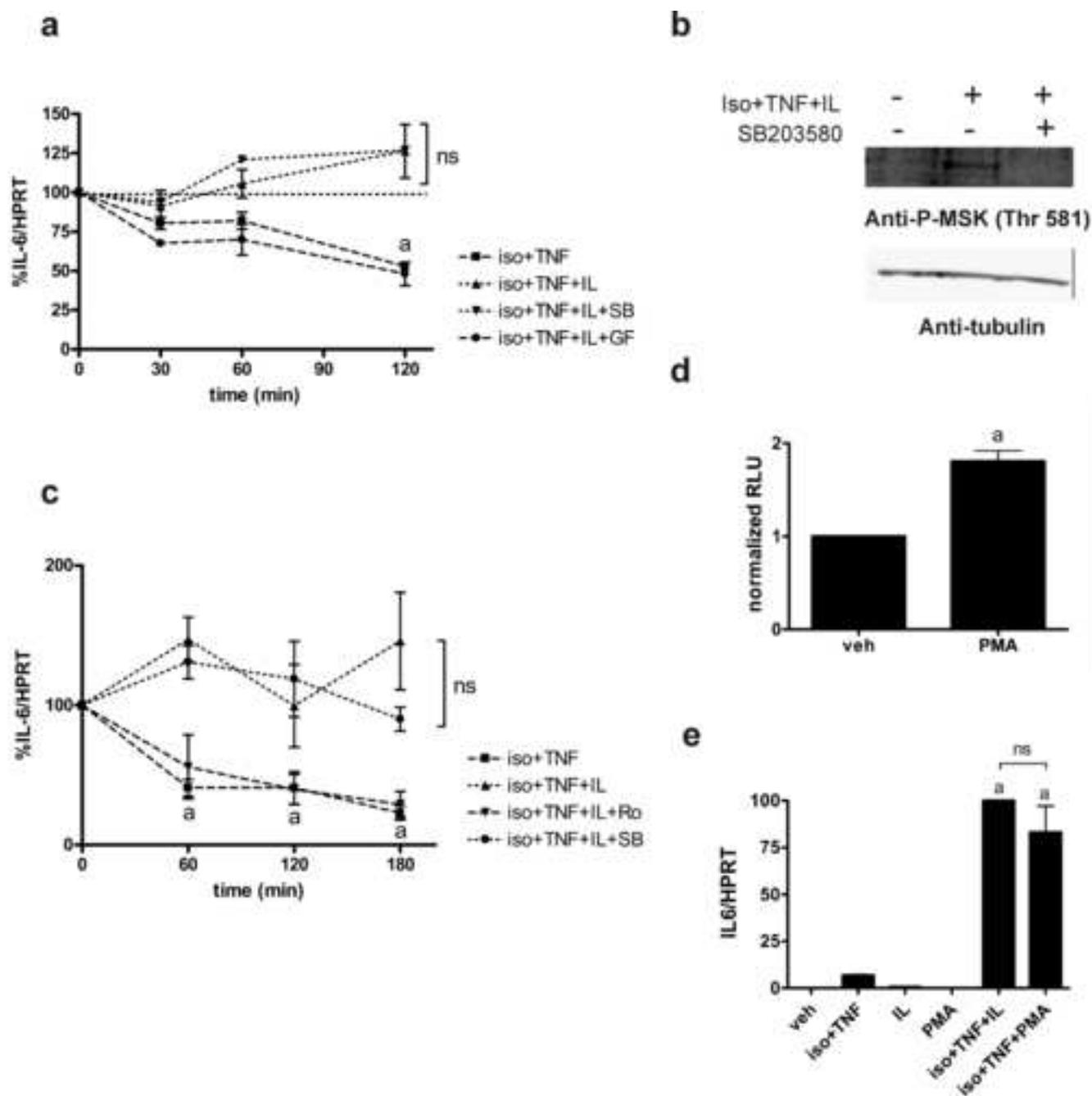


Figure 4

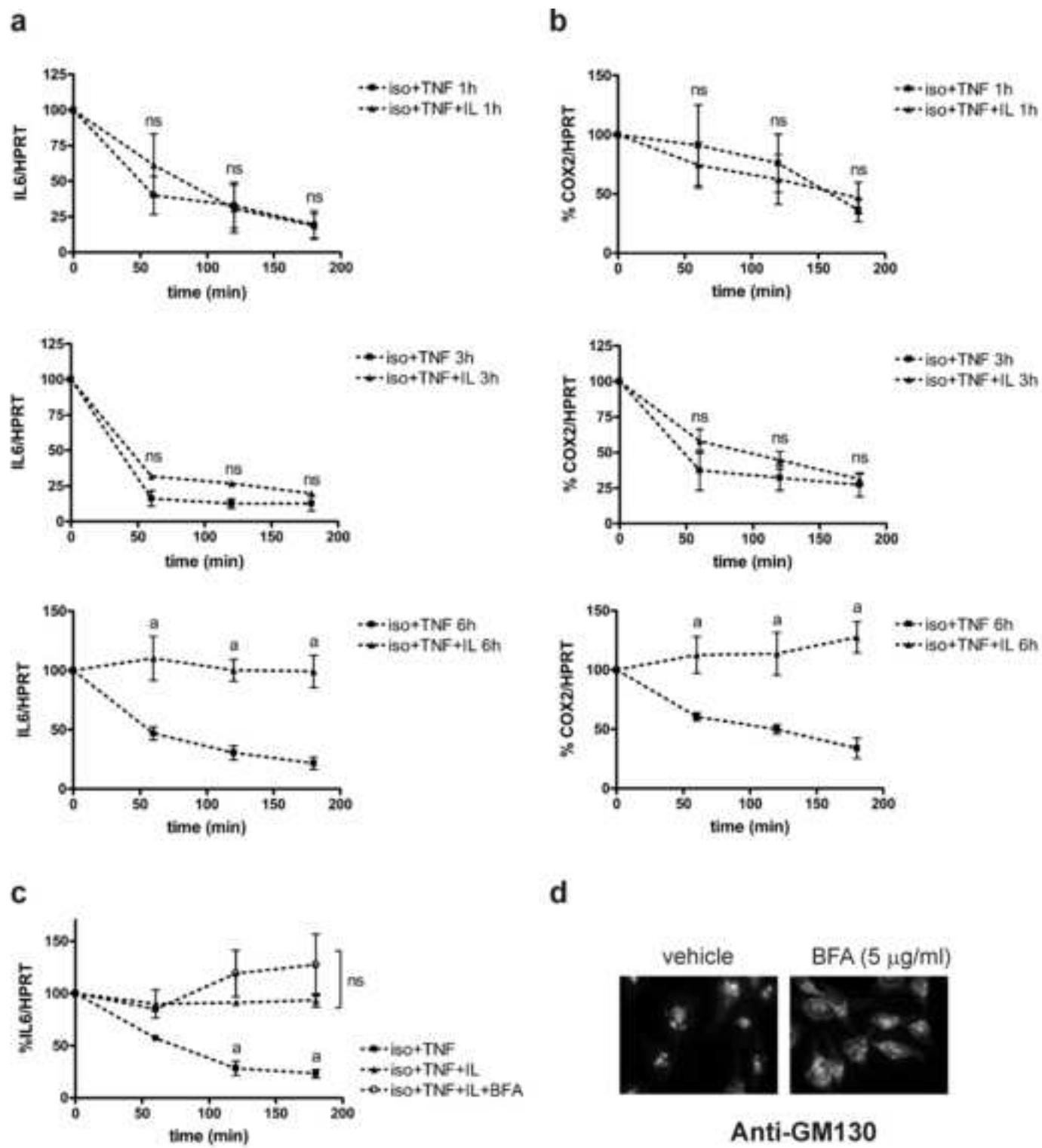


Figure 5

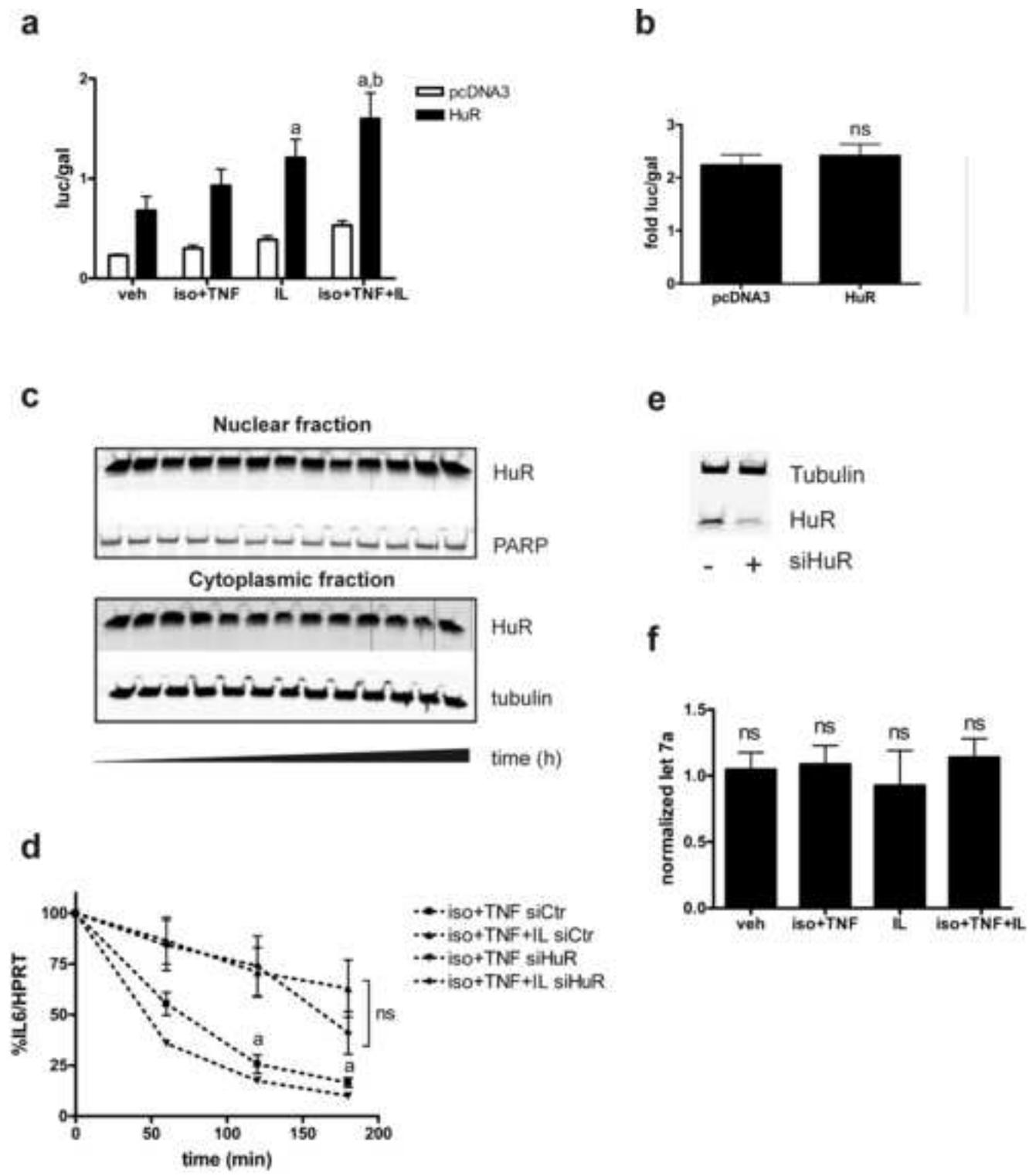


Figure 6  
 Page 47 of 48

IScrip

