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## Toll-like receptor stimulation differentially regulates vasoactive intestinal peptide type 2 receptor in macrophages

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### Abstract

Vasoactive intestinal peptide (VIP) was originally isolated as a vasodilator intestinal peptide, then as a neuropeptide. In the immune system, VIP is described as an endogenous macrophage-deactivating factor. VIP exerts its immunological actions in a paracrine and/or autocrine manner, through specific receptors. However, very little is known about the molecular regulation of VIP type 2 receptor (VPAC<sub>2</sub>) in the immune system. We now report that different toll-like receptor (TLR) ligands selectively regulate the VPAC<sub>2</sub> receptor gene and show a gene repression system controlled by key protein kinase signalling cascades in macrophages. VPAC<sub>2</sub> gene expression is regulated by gram-positive (TLR2 ligands) and gram-negative bacteria wall constituents (TLR4 ligands). Moreover, VPAC<sub>2</sub> is tightly regulated: TLR2- or TLR2/6- but not TLR2/1-mediated mechanisms are responsible for the induction of VPAC<sub>2</sub>. TLR stimulation by viral or bacterial nucleic acids did not modify the VPAC<sub>2</sub> mRNA levels. Remarkably, imiquimod – a synthetic TLR7 ligand – led to a potent up-regulation of VPAC<sub>2</sub> gene expression. TLR5 stimulation by flagellin present in gram-positive and gram-negative bacteria did not affect VPAC<sub>2</sub> mRNA. The p38 mitogen-activated protein kinase (MAPK) activity accounted for the TLR4-mediated induction of VPAC<sub>2</sub> gene expression. Surprisingly, our data strongly suggest for the first time a tightly repressed control of VPAC<sub>2</sub> mRNA induction by elements downstream of MAPK kinase 1/2, PI3K/Akt, and particularly Jun-NH<sub>2</sub>-terminal kinase signalling pathways.

**Keywords:** VIP • neuroimmunology • neuropeptides • inflammation • toll-like receptors

### Introduction

Research on endogenous negative feedback or compensatory mechanisms that serve to limit deleterious immune responses is critical in the attempts to identify effective therapeutic targets [1, 2]. In recent years, vasoactive intestinal peptide (VIP) and VIP receptors have shown their relevance as endogenous factors that regulate inflammatory immune responses and immune tolerance [3, 4]. The mechanisms involved include the deviation towards

Th2-driven inflammatory pathways, the specific recruitment and development of Th2 cells and the peripheral expansion of regulatory T cells [3]. Endogenously, VIP is produced and secreted by antigen-stimulated Th2 cells [5–7], resembling a cytokine-like acting molecule [3, 4]. Interestingly, VIP knockout mice expressed an asthmatic phenotype that is partially corrected after VIP treatment [8]. This is agreement with current views of bronchial asthma as a non-exclusive Th2 polarized immune response [9, 10]. VIP exerts its immunological actions in a paracrine and/or autocrine manner, through three different specific receptors: VIP type 1 receptor (VPAC<sub>1</sub>), VPAC<sub>2</sub> and PAC<sub>1</sub> [11, 12]. Although seminal works in the immune system described VIP as an anti-inflammatory agent, acting as an endogenous macrophage-deactivating factor (see reviews [3, 11, 13]), very little is known about the molecular regulation of VPAC receptors in macrophages. Delgado *et al.* [14] have reported that in contrast to VPAC<sub>1</sub> and PAC<sub>1</sub> receptors – which

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seem to be constitutively expressed in mice macrophages – VPAC<sub>2</sub> is selectively expressed only after lipopolysaccharide (LPS) stimulation. In lymphocytes, VPAC<sub>2</sub> mRNA is an activation-induced gene in several experimental conditions [15–18]. Despite the inducible character of the VPAC<sub>2</sub> gene, detailed data about the promoter and regulatory elements scarcely exist and are limited to the human genome [19, 20]. Very recently, Lutz and collaborators have identified the core promoter region of the mouse VPAC<sub>2</sub> receptor gene [21].

The present work represents the first study of the regulation of VPAC<sub>2</sub> mRNA by toll-like receptors (TLRs) in macrophages. TLRs are the best-characterized signal generating receptors among the pathogen-associated molecular patterns; they initiate key inflammatory responses and also shape the adaptive immunity [22, 23]. TLRs can be divided into two groups according to their cellular localization: TLRs 1, 2, 4, 5, 6 are located mainly on the cell surface and primarily recognize bacterial components, while TLRs 3, 7, 8 and 9 are mostly found in the endocytic compartments and mainly recognize viral products [22, 23]. TLR-mediated innate immune activation also induces several molecules shown to negatively regulate TLR signalling [24]. Although studies on VIP immunoregulatory activities are of notable therapeutic interest [3], the effects of TLRs on the induction of VPAC<sub>2</sub> have not yet been examined. Thus, the VPAC<sub>2</sub> regulation by TLRs may play an important role in positive and negative regulation of normal and aberrant immune responses.

Our findings indicate that the VPAC<sub>2</sub> gene is differentially regulated by subsets of TLRs. Furthermore, VPAC<sub>2</sub> mRNA is tightly repressed by key protein kinase pathways: c-Jun-NH<sub>2</sub>-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) kinase 1/2 (MEK1/2), p38 MAPK and phosphatidylinositol-3 kinase (PI3K/Akt).

## Materials and methods

### Animals

Female C57BL6/Jlco mice (4–8 weeks old) were maintained in the Animal Breeding Center of the University of Seville. Experiments were carried out under the supervision and guidelines of the Animal Welfare Committee.

### Cell culture and reagents

Raw 264.7 cells (ECACC, Porton Down, Wiltshire, UK) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (BioWhittaker, Verviers, Belgium), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA). Peritoneal macrophages were isolated and pooled after intraperitoneal injection of 1 ml of 3% thioglycolate at day 5 (Fluka, Milwaukee, WI, USA). TLR ligands were as follows: LPS (*E. coli* serotype 0127: B8, Sigma), oligodeoxynu-

cleotide containing unmethylated CpG motifs (CpG<sup>+</sup>: CpG-DNA 1668: 5'-TCCATGACGTTCCCTGATGCT-3', TIB MolBioL, Berlin, Germany), polyinosinic: polycytidylic acid (poly I: C), flagellin, lipoteichoic acid (LTA), synthetic bacterial lipopeptide Pam<sub>3</sub> CSK4, synthetic lipoprotein derived from Mycoplasma (FSL-1), peptidoglycan (PGN), imiquimod and ssRNA40 were obtained from InvivoGen (San Diego, CA, USA). SB203580 (a p38 MAPK inhibitor), SP600125 (a JNK inhibitor), PD98059 (a MEK1/2 inhibitor), LY294002 (a PI3K/Akt inhibitor) were all from Sigma.

### Cytokine assays

Supernatants from Raw 264.7 cells and thioglycolate-elicited peritoneal macrophages were harvested 24 hrs after TLR stimulation. Interleukin 6 (IL-6) ELISA determinations were carried out according to the manufacturer's instructions (OptEIA Mouse IL-6 set, BD Pharmingen, San Diego, CA, USA).

### Quantitative real-time PCR (qPCR)

Raw 264.7 cells were seeded into 12-well plates in a final volume of 2 ml. At 70–80% confluence, cells were stimulated with LPS (1 µg/ml), CpG<sup>+</sup> (1 µg/ml, CpG-DNA 1668), Poly (I: C) acid (50 µg/ml), LTA (10 µg/ml), Pam<sub>3</sub> CSK4 (300 ng/ml), FSL-1 (1 µg/ml), PGN (10 µg/ml), imiquimod (10 µg/ml) or ssRNA40 (0.25 µg/ml). Peritoneal macrophages were adherent-isolated by overnight culture into 12-well plates in 1.5 ml RPMI 1640. Then, non-adherent cells were removed and peritoneal macrophages were treated as indicated above. Twenty-four hours later, RNA from cell cultures was extracted using Tripure™ isolation reagent (Roche, Basel, Switzerland) and qPCRs of VPAC<sub>2</sub>, IL-6 or the housekeeping gene HPRT were carried out. For qPCR determinations (MiniOpticon detector, BioRad, Hercules, CA, USA), 1 µg of RNA was reversely transcribed and genomic DNA removed using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). One hundred nanograms of cDNA were amplified using 12.5 µl of 2× FastStart SYBR Green Master Mix (Roche), 200 nM of each primer and H<sub>2</sub>O up to 25 µl. The PCR amplification scheme was: 10 min. at 95°C; followed by 50 cycles at 95°C 15 sec., 65°C 30 sec. and 30 sec. at 72°C. HPRT (ID genebank accession number 15452) was used as housekeeping gene (forward 5'-GTAATGATCAGTCAACGGGGGAC-3', reverse 5'-CCAGCAAGCTTGCAACCTTAACCA-3' (174 bp in length spanning from nucleotide 464 to 638). The VPAC<sub>2</sub> sequence (ID genebank accession number 22355) was used to design the VPAC<sub>2</sub> primers. The oligonucleotides were as follow: forward 5'-GGACAGCAACTCGCCTCTCT-3' (nt 825–844), reverse 5'-CCCTGGAAGGAACCAACACATAAC-3' (nt 1129–1152) (328 bp in length). IL-6 primers (ID genebank accession number 16193) were: forward 5'-TTCCATCCAGTTGCCTTCTT-3', reverse 5'-ATTTCACGATTTCCAGAG-3' (170 bp in length spanning from nucleotide 55 to 225). SYBR-Green I detection was followed by generation of melting curves and visualization of the products to confirm specificity. Quantitative PCR results were obtained using the  $\Delta\Delta C_t$  method [25]. The induction of mRNA was calculated as  $2^{-\Delta\Delta C_t}$ .

### Statistical significance

For statistical evaluation, Mann-Whitney rank sum tests were performed to differentiate between experimental groups.

## Results and discussion

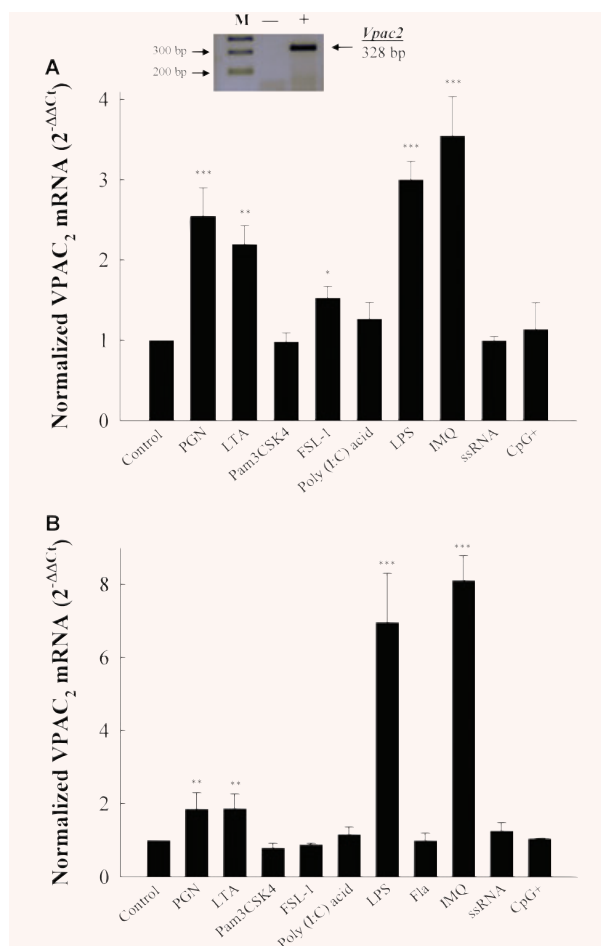
The VPAC<sub>2</sub> gene is constitutively expressed in both Raw 264.7 cells and peritoneal macrophages (Fig. 1). VPAC<sub>2</sub> mRNA levels were up-regulated upon LPS treatment, as previously reported by Delgado and collaborators [14]. In our experimental setting, we were able to detect a constitutive expression of VPAC<sub>2</sub> mRNA in resting macrophages while Delgado and collaborators [14] did not detect it by conventional RT-PCR in resting Raw 264.7 cells. This discrepancy could be due to differences in the sensitivity of the systems used (conventional *versus* real time PCR) or the efficiency of the primers used in each study. Besides, what is constitutive or not relies on the experimental technique or reagents used in each case. In this sense, we reproduced the responsive character of the VPAC<sub>2</sub> gene pioneered reported by Delgado and collaborators [14] and this information about the inducible nature of the VPAC<sub>2</sub> gene is the relevant message in terms of biological interpretation.

LPS treatment caused about three-fold induction of VPAC<sub>2</sub> mRNA levels in Raw 264.7 cells. Interestingly, PGN, a ligand for TLR2, or ligands for TLR2/6 heterodimers such as LTA or the lipopeptide FSL-1 – a synthetic analogue of mycoplasmal lipoprotein – were able to up-regulate VPAC<sub>2</sub> gene expression in Raw 264.7 cells (Fig. 1A). In contrast, Raw 264.7 cells treated with Pam<sub>3</sub> CSK4 – a TLR2/1 ligand – did not modify the mRNA levels of VPAC<sub>2</sub> receptors (Fig. 1A). Therefore, VPAC<sub>2</sub> gene expression is regulated by components of gram-positive (TLR2 ligands) and gram-negative bacteria (TLR4 ligands) cell walls. These data also imply that different TLR-mediated signalling pathways may result in the selective regulation of the VPAC<sub>2</sub> gene.

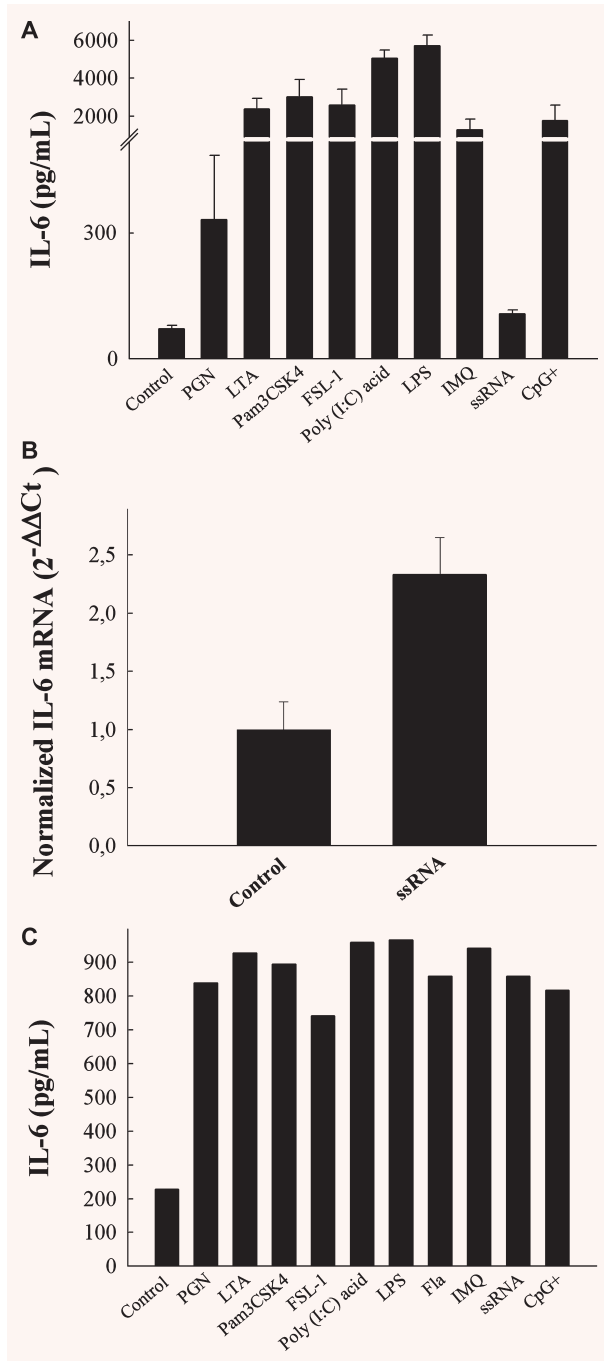
VPAC<sub>2</sub> is tightly regulated by TLR2- or TLR2/6-mediated mechanisms. TLR2/1 heterodimers recognize triacyl lipopeptides such as Pam<sub>3</sub> CSK4, and TLR2/6 heterodimers recognize diacyl lipopeptides. Findings of differential responses mediated by TLR1 are scarcely known. Recently, defects in TLR1 recognition have been related to ineffective responses to lipoprotein antigens in mice and human beings, providing a link between TLR1 and acquired antibody responses [26]. The reason why VPAC<sub>2</sub> up-regulation is unresponsive to TLR2/1 stimulation is uncertain. Synthetic bacterial lipopeptide Pam3CSK4, LTA and PGN have been shown to exert a beneficial decrease in the Th2 phenotype in allergic contexts [27–30], although there is no such a comprehensive comparative analysis of the Th1 responses elicited by these ligands. Although future studies should clarify the differences observed with Pam3CSK4 regarding VPAC<sub>2</sub> expression, they might be related with specific features of the Th1-mediated response by Pam3CSK4.

This is the first report of VPAC<sub>2</sub> regulation by TLRs sensing gram-positive bacteria. Recent studies support the concept that chronic or recurrent microbial infections may contribute to atherosclerotic disease acting through TLR2 [31, 32]. The putative role of VPAC<sub>2</sub> in the modulation of innate responses affecting atherosclerosis is presently unknown and deserves further attention.

Optimal stimulation with CpG DNA motifs for TLR9, single stranded RNA for TLR7, or poly (I: C) acid as a dsRNA synthetic



**Fig. 1** Differential TLRs regulation of VPAC<sub>2</sub> mRNA in macrophages. **(A)** Raw 264.7 cells were seeded into 12-well plates in a final volume of 2 ml. At 70–80% confluence (750,000 cells/well), cells were stimulated with LPS (1 μg/ml), CpG<sup>+</sup> (1 μg/ml), CpG-DNA 1668: 5'-TCCATGACGTTCCCTGATGCT-3'), Poly (I: C) acid (50 μg/ml), LTA (10 μg/ml), Pam<sub>3</sub> CSK4 (300 ng/ml), FSL-1 (1 μg/ml), PGN (10 μg/ml), imiquimod (10 μg/ml) or ssRNA40 (0.25 μg/ml). Twenty-four hours, RNA was extracted and qPCR of VPAC<sub>2</sub> was carried out. Results are the mean ± S.D. of five independent experiments performed in triplicate. (Inset) Samples from qPCR samples on 1.7% agarose gel electrophoresis. Lanes showed the DNA molecular size markers (M), reaction performed in the absence of RT (-) and presence (+) of RT from resting Raw 264.7 RNA. **(B)** Female C57BL/6Jlco (*n* = 20) peritoneal macrophages were isolated and pooled after i.p. injection of thioglycolate medium at day 5 as described in the 'Materials and methods' section. For qPCR determinations of VPAC<sub>2</sub> mRNA levels, cDNA (100 ng) was amplified as described in the 'Materials and methods' section. Quantitative PCR results were obtained using the ΔΔCt method [25]. The induction of mRNA was calculated as 2<sup>-ΔΔCt</sup> (normalized for HPRT as housekeeping gene). For statistical evaluation, Mann-Whitney rank sum tests were performed. Asterisks indicate statistical significance (\*, *P* < 0.05 versus control; \*\*, *P* < 0.01 versus control; \*\*\*, *P* < 0.001 versus control).



**Fig. 2** IL-6 production after TLRs stimulation in macrophages. **(A)** Raw 264.7 cells were seeded into 12-well plates in a final volume of 2 ml. At 70–80% confluence, cells (750,000 cells/well) were stimulated with LPS (1  $\mu\text{g/ml}$ ), CpG<sup>+</sup> (1  $\mu\text{g/ml}$ , CpG-DNA 1668: 5'-TCCATGACGTTCTGATGCT-3'), Poly (I: C) acid (50  $\mu\text{g/ml}$ ), LTA (10  $\mu\text{g/ml}$ ), Pam<sub>3</sub> CSK4 (300 ng/ml), FSL-1 (1  $\mu\text{g/ml}$ ), PGN (10  $\mu\text{g/ml}$ ), imiquimod (10  $\mu\text{g/ml}$ ) or ssRNA40 (0.25  $\mu\text{g/ml}$ ). Twenty-four hours later, supernatants were harvested for IL-6 ELISA determination. **(B)** mRNA IL-6 stimulation after ssRNA treatment in Raw 264.7 cells. RNA was extracted for IL-6 gene expression study after ssRNA treatment as described in the 'Materials and methods' section. For qPCR determinations of IL-6 mRNA levels, cDNA (100 ng) was amplified as described in the 'Materials and methods' section. Quantitative PCR results were obtained using the  $\Delta\Delta\text{Ct}$  method [25]. The induction of mRNA was calculated as  $2^{-\Delta\Delta\text{Ct}}$ . Results are the mean  $\pm$  S.D. of five independent experiments performed in triplicate. **(C)** Female C57BL/6Jlco ( $n = 20$ ) peritoneal macrophages were isolated and pooled after intraperitoneal injection of 1 ml of 3% thioglycolate at day 5 as described above. Afterwards, non-adherent cells were removed and peritoneal macrophages were treated as indicated above and 24 hrs later, supernatants were harvested for IL-6 ELISA determination.

There is still a long way to go regarding the imiquimod molecular mechanism of action and many questions remain unanswered. This is the reason why ssRNA and imiquimod does not always show similar effects. It has been shown that imiquimod has mechanisms of action that are TLR7-independent, suggesting a role for adenosine receptors [33]. Topical imiquimod cream is an FDA-approved treatment for superficial basal cell carcinomas [34]. Predominant among its actions is the induction, in transcutaneous immunization protocols, of efficient Th1-antitumoral cellular immunity [35, 36]. Fabricius and collaborators have reported an inhibitory role for VIP in the production of interferon (IFN)- $\alpha$  stimulated by TLR9 ligands in human plasmacytoid cells [37]. Recently, a strong inhibitory effect after TLR7 stimulation on the IFN- $\alpha$  production induced by TLR9 ligands has been shown [38]. Even though the regulation of specific TLR7-mediated responses by VIP has not been addressed, our results might provide grounds for a possible counter regulatory mechanism mediated by VPAC<sub>2</sub> receptors.

We extended our observations to peritoneal macrophages, a more physiological context. Moreover, peritoneal macrophages, contrary to Raw 264.7 cells, express TLR5 [39, 40]. TLR5 stimulation is mediated through the interaction with gram-positive or gram-negative bacterial flagellin [22, 24]. VPAC<sub>2</sub> mRNA levels were not affected by treatment with this bacterial constituent (Fig. 1B). Using peritoneal macrophages, we confirmed the pattern of VPAC<sub>2</sub> gene regulation by TLR ligands (Fig. 1B). VPAC<sub>2</sub> is differentially up-regulated by ligands of TLR2, TLR2/6 and TLR4 receptors. The synthetic ligand for TLR7, imiquimod, also increased the mRNA levels of the VPAC<sub>2</sub> gene. Interestingly, the increase in VPAC<sub>2</sub> gene expression by LPS or imiquimod was two-fold compared with that observed in Raw 264.7 cells, while the induction mediated by TLR2 and TLR2/6 remained at the same levels (Fig. 1B). To be certain that Raw 264.7 cells or peritoneal

analogue for TLR3 did not modify VPAC<sub>2</sub> mRNA levels (Fig. 1). Therefore, VPAC<sub>2</sub> gene is not affected by TLR-stimulation detecting nucleic acids derived from viruses and bacteria. Imiquimod – a specific TLR7 ligand – is an imidazoquinoline-like molecule. Interestingly, imiquimod treatment of Raw 264.7 cells led to a potent up-regulation of VPAC<sub>2</sub> mRNA levels, causing  $3.5 \pm 0.5$  fold induction of VPAC<sub>2</sub> mRNA levels in Raw 264.7 cells (Fig. 1A).



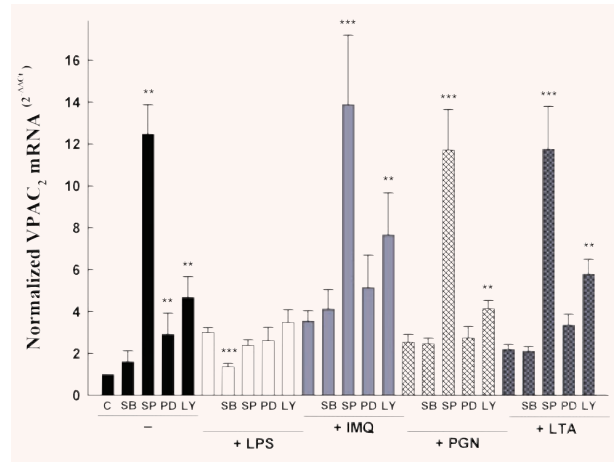
macrophages have responded appropriately to the different TLR ligands used, we determined IL-6 secretion or expression in both Raw 264.7 cells and peritoneal macrophages as a well-recognized marker of TLR-mediated activation [24] (Fig. 2).

TLRs have been shown to activate four major intracellular kinase signalling pathways; p38 MAPK, JNK, MEK1/2 and PI3K/Akt [24, 41, 42]. We studied the signalling pathways involved in the induction of VPAC<sub>2</sub> mRNA by TLR4, TLR2, TLR2/6 and TLR7 in Raw 264.7 macrophages. Previously, we checked whether the specific inhibitors of the different kinase signalling pathways or the TLR ligands used in the present study affected cell viability at the concentration used herein (Fig. S1). No statistically significant differences were observed in terms of cell viability compared to the control experimental situation. The proteasome inhibitor MG132 was used as a positive control for cytotoxicity.

The induction of VPAC<sub>2</sub> expression triggered by LPS was completely abrogated by the blockade of the p38 MAPK-dependent signalling pathway with the specific inhibitor SB203580 (Fig. 3) [43, 44]. However, the up-regulation of VPAC<sub>2</sub> mRNA levels observed after treatment with PGN (TLR2), lipoteichoic acid (TLR2/6) or imiquimod (TLR7) were not dependent on p38 MAPK activity (Fig. 3). This is the first report that ascribes a signal transduction cascade to the LPS induction of VPAC<sub>2</sub> gene expression, although we cannot exclude a partial indirect effect mediated by pro-inflammatory cytokines triggered by LPS treatment as it has been reported in the epidermal keratinocyte cell line DJM-1 for VPAC<sub>1</sub> [45]. Strikingly, SP600125 – a highly selective inhibitor of JNK activity [46] – increased the mRNA levels of VPAC<sub>2</sub> up to  $12.5 \pm 1.4$  fold compared to control values (Fig. 3). The co-treatment of the different TLR ligands with the specific JNK inhibitor always led to a dramatic increase in VPAC<sub>2</sub> gene expression despite TLR2-, TLR2/6- or TLR7-mediated stimulation without a synergistic effect (Fig. 3). Whether LPS triggers additional counter mechanisms is presently unknown, but only the LPS co-treatment with SP600125 abolished the effect that the JNK inhibitor produced by itself in Raw 264.7 cells (Fig. 3). Addition of the MEK1/2 signalling blocker [44, 47] PD98059 led to an increase in VPAC<sub>2</sub> mRNA levels which did not synergize with the TLR stimulation (Fig. 3). However, LPS-induced VPAC<sub>2</sub> expression was not reversed by blocking MEK1/2 activity, as was the case when p38 MAPK activity was inhibited (Fig. 3), nor did LPS affect the up-regulation of VPAC<sub>2</sub> mRNA levels by MEK1/2 inhibition as in the case of the inhibition of JNK (Fig. 3).

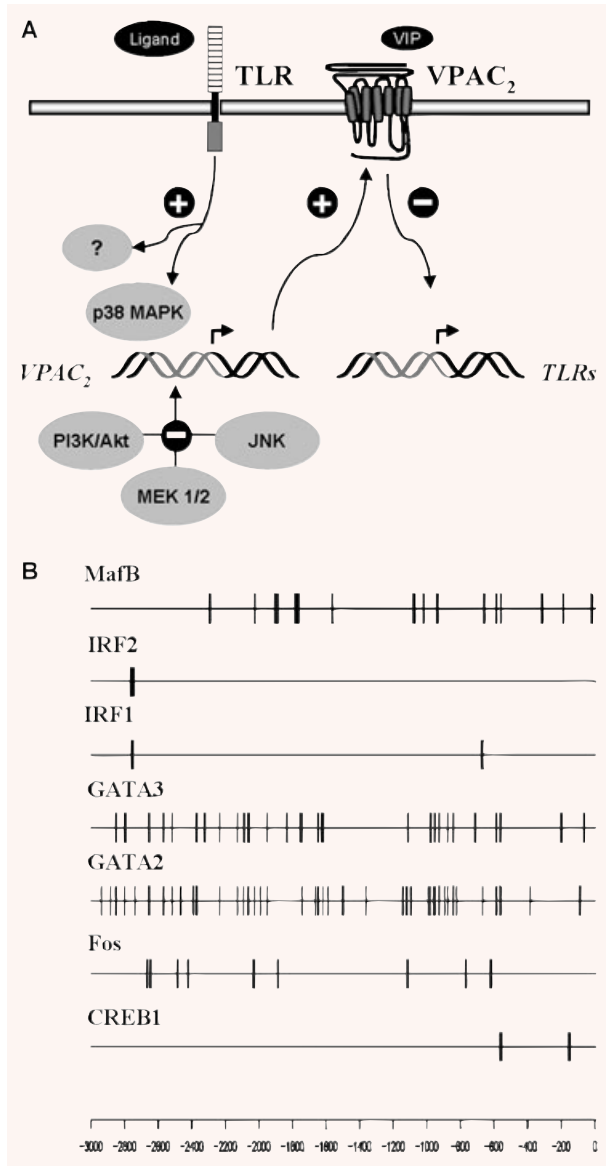
We blocked PI3K/Akt activity with LY294002 [48, 49]. PI3K/Akt inhibition produced a  $4.7 \pm 1.0$  fold induction of VPAC<sub>2</sub> mRNA in Raw 264.7 macrophages (Fig. 3). PI3K/Akt inhibition did not reverse the TLR-mediated up-regulation of VPAC<sub>2</sub> mRNA. Moreover, PI3K/Akt inhibition synergized with TLR ligands for TLR7, but not for TLR4, TLR2 or TLR2/6 (Fig. 3). These data strongly suggest a tightly repressed control of VPAC<sub>2</sub> mRNA induction by elements downstream of MEK1/2, PI3K/Akt and especially JNK signalling pathways.

Recent studies have been highlighting that VIP decreases the up-regulation of TLR2 and TLR4 in human monocytes [50], as



**Fig. 3** Effects of protein kinase inhibitors on TLR-mediated VPAC<sub>2</sub> regulation in Raw 264.7 macrophages. Raw 264.7 cells were grown and treated as above. Briefly, at 70–80% confluence, cells (750,000 cells/well) were stimulated for 24 hrs with LPS (1 µg/ml), LTA (10 µg/ml), PGN (10 µg/ml) or imiquimod (10 µg/ml) after 30 min. pre-treatment in the presence or absence of 10 µM SB203580 (a p38 MAPK inhibitor), 25 µM SP600125 (a JNK inhibitor), 50 µM PD98059 (a MEK1/2 inhibitor) and 10 µM LY294002 (a PI3K/Akt inhibitor). Twenty-four hours later, RNA was extracted and qPCR of VPAC<sub>2</sub> was carried out according to the 'Materials and methods' section. Results are the mean ± S.D. of three independent experiments performed in triplicate. For statistical evaluation, Mann-Whitney rank sum tests were performed. Asterisks indicate statistical significance (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.0001$  versus the experimental condition in the absence of inhibitor).

well as in lymphocytes, macrophages and dendritic cells after the trinitrobenzene sulfonic acid induced colitis in mice [13, 51, 52]. Interestingly, VIP has also been reported to inhibit the LPS-induced RNA expression of downstream adapters molecules involved in TLR4 signalling pathways in fibroblast-like synoviocytes from patients with rheumatoid arthritis or osteoarthritis [53]. We should notice the importance of understanding how the TLR, associated kinases pathways and VIP receptors serve to maintain the homeostasis and the integrity of the immune response. In this sense, it has been shown very recently that VIP suppresses TLR4 expression in macrophages *via* PI3K/Akt [54]. This is in agreement with increasing experimental evidence showing that PI3K/Akt signalling pathway has a negative regulatory role in inflammatory processes [55–57]. The VIP-mediated down-regulation of the pro-inflammatory response is partly explained by activation of PKA and subsequent inhibition of p38 MAPK activity and JNK activity in macrophages [1, 3]. Thus, it is conceivable to propose that up-regulation of VPAC<sub>2</sub> expression might be part of a counter balance mechanism that allows a VIP-mediated anti-inflammatory effect in situations that produce extensive inhibition of the PI3K/Akt signalling pathway. In addition, the up-regulation of VPAC<sub>2</sub> expression could be part of the inflammatory deactivating cascade produced by VIP, partly due to its inhibitory effects on p38 MAPK and JNK activities. Further experiments should address



**Fig. 4** TLR-mediated control of VPAC<sub>2</sub>. **(A)** VPAC<sub>2</sub> up-regulation by TLRs represents an additional mechanism to ensure the modulator capabilities of VIP while at the same time a down-regulation of TLRs occurs. **(B)** Distribution of putative binding sites for transcription factors involved in TLR signalling across the 3000 long upstream region of mouse VPAC<sub>2</sub> gene. Thicker lines correspond to more densely packed putative binding sites.

protein expression levels of VPAC<sub>2</sub>. Unfortunately, although there are no reliable/specific antibodies against VPAC<sub>2</sub> receptors available so far, our data point out to new fine-tuned mechanisms of control of VPAC<sub>2</sub> receptors. Very recently, two findings highlight the importance of the expression levels of VIP receptors in the onset and evolution of autoimmune/inflammatory human

diseases. Delgado and collaborators have shown a genetic association involving VPAC<sub>1</sub> receptor genetic polymorphisms and susceptibility of human rheumatoid arthritis, suggesting that an impaired expression of VPAC<sub>1</sub> could be related to the pathogenesis of rheumatoid arthritis [58]. Juarranz and collaborators have shown that the inhibitory effects of VIP on fibroblast-like synoviocytes production of pro-inflammatory mediators were mimicked by VPAC<sub>1</sub> and VPAC<sub>2</sub> specific agonists in these cells from patients affected by osteoarthritis and rheumatoid arthritis, respectively, with a predominant role for VPAC<sub>2</sub> receptor in the latter [59]. Taken together, despite difficulties of homogenizing cell cultures from synovial tissues from patients with different degrees of rheumatoid arthritis and osteoarthritis, it is evident that VPAC receptors regulate the threshold of autoreactivity in these diseases where TLRs and their signalling pathways might have great potential as additional targets for new treatments. Last, we should not rule out the possibility that the observed changes in the VPAC<sub>2</sub> gene expression were based on post-transcriptional regulation of its mRNA stability due to the inhibition of p38 MAPK, JNK or PI3K/Akt signalling pathways as growing experimental evidence has been showing [60–62].

Figure 4A summarizes our findings. Based on these results, we postulate that up-regulation of VPAC<sub>2</sub> by TLRs represents an additional mechanism to ensure the modulator capabilities of VIP, while at the same time a down-regulation of TLRs occurs. In this sense, we used the Jaspar database (<http://jaspar.genereg.net>) to search for transcription factors possibly involved in TLR-mediated signalling (Fig. 4B). The 3000 nucleotide long upstream region of the *Mus musculus* VPAC<sub>2</sub> gene (ENSMUSG00000011171) was obtained from the Ensembl database (<http://www.ensembl.org>). We found putative binding sites with a relative score threshold of 0.8 in the positive strand for CREB1, Fos, GATA2, GATA3 and IRF1/2 transcription factors. It must be noted that most of the predictions will correspond to sites bound *in vitro*, and therefore their functional significance must be further analysed.

The present data shed light on how TLRs differentially decode innate stimuli, not only to achieve protection but also to avoid damage by uncontrolled inflammatory responses, in this case, by means of VPAC<sub>2</sub> receptor.

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## Supporting Information

The following supplementary material is available for this article:

**Fig. S1** Effects of TLR ligands and protein kinase inhibitors on cytotoxicity. Raw 264.7 cells were previously seeded into 96-well plates to a final volume of 100  $\mu$ l. After 24-hrs treatment with protein kinase inhibitors, LDH release was calculated according to the following equation: LDH release = [LDH supernatant/(LDH supernatant + LDH cells)]\*100. The proteasome inhibitor MG-132 (6  $\mu$ M) was used as a positive control for cytotoxicity. Results are the mean  $\pm$  S.D. of two independent experiments performed in duplicate.

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## **Appendix S1**

### **Lactate dehydrogenase (LDH) release**

LDH release from cells was measured to quantify the cytotoxicity by using the Cytotoxicity Detection kit (Roche). Raw 264.7 cells were previously seeded into 96-well plates in a final volume of 100  $\mu$ L. After treatment, 100  $\mu$ L of the supernatants and 100  $\mu$ L of 1% Triton X100-lysed cells were assayed for LDH content under each experi-

mental condition. LDH determinations were carried out according to the manufacturer's guidelines. LDH release was calculated according to the following formula: LDH release = [LDH supernatant/(LDH supernatant + LDH cells)]\*100. The proteasome inhibitor MG-132 (Sigma) was used as a positive control for cytotoxicity.