Modulation of cytokine responses by adrenomedullin and adrenomedullin binding protein-1 in macrophages: a novel pathway in sepsis

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KEY MESSAGES

1. Adrenomedullin augments the production of interleukin-10, a cytokine that limits inflammation.
2. Interferon-γ, a cytokine that is increased in inflammation, downregulates the receptor and binding protein of adrenomedullin.
3. Cyclic AMP mediates the increased expression of adrenomedullin, interleukin-6, and interleukin-10 in response to endotoxin.
4. The production of interleukin-6 is mediated by p38-mitogen-activated protein kinase (MAPK), p42/44-MAPK, protein kinase C, and protein kinase K, whereas that of interleukin-10 is mediated by p38-MAPK and protein kinase K.
5. These downstream pathways could be targets for therapeutic intervention.

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Introduction

Polymicrobial sepsis is a life-threatening disorder. Patients die of septic shock and multiple organ failure caused by lipopolysaccharide (LPS) and other bacterial products. A hyperdynamic phase is followed by shock and circulatory collapse. Myocardial dysfunction frequently accompanies severe sepsis and septic shock secondary to circulating depressant factors, including tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Macrophages produce proinflammatory cytokines including TNF-α, IL-1β, and IL-6 that lead to tissue injury.

Adrenomedullin (AM) is a vasorelaxant peptide originally isolated from the adrenal medulla. It relaxes vascular smooth muscle cells through the elevation of intracellular cyclic adenosine 3'-5'-monophosphate (cAMP).[1] It also acts on endothelial cells by activating adenylyl cyclase and nitric oxide synthase, resulting in dilation of blood vessels. The macrophage produces AM in inflammation and sepsis. Transgenic mice over-expressing AM are resistant to septicaemic shock. AM markedly increased IL-6 production in both resting and LPS-stimulated macrophages, but significantly suppressed LPS-induced TNF-α secretion.[2] IL-6 and IL-10 can inhibit the production of pro-inflammatory cytokines such as TNF-α and IL-1. These results suggest that AM may play an important role as an anti-inflammatory regulator of the inflammatory response in the macrophages, at least partly, via its effect on production of inflammatory cytokines.

Elevated levels of AM play a major role in initiating the hyperdynamic response during the early stage of sepsis, yet transition to the late, hypodynamic phase occurs, despite continued high circulating levels of AM.[3] A specific AM binding protein-1 (AMBP-1) carries AM in human plasma and is complement factor H.[4] The decreased vascular response to AM in advanced sepsis is related to a decrease in AMBP-1. Administration of AM/AMBP-1 to septic animals prevents the drop in blood pressure and reduces mortality. AM and AMBP-1 in combination down-regulate pro-inflammatory cytokines in septic animals, and suppress LPS-induced TNF-α expression and release from the macrophages.[5]

The interaction between AM and AMBP-1 has opened a new avenue for research into the transition from the hyperdynamic to the hypodynamic phase of sepsis. AM has been reported to induce its effect through the cAMP-, Ca^{2+}, or mitogen-activating protein kinase (MAPK) mediated pathway. Expression of AMBP-1 is detectable in both monocytes and macrophages, and AMBP-1 given in conjunction with AM further raises AM-induced cAMP production. Nonetheless, little is known about the regulation of AMBP-1, the cause of reduced AMBP-1 production in sepsis, or the regulatory

pathways involved to elicit the physiological actions of AM and AMBP-1.

The study aimed to (1) investigate the expression of AM, AMBP-1 and AM receptor proteins in the LPS-induced inflammatory response in a rat alveolar macrophage cell line; (2) examine the effect of AM and AMBP-1 on LPS-induced inflammatory cytokine production in macrophages and determine whether feedback loops or desensitisation of AM receptors affect the production of AM and AMBP-1; and (3) identify a potential role of second messenger-dependent kinases in the regulation of AM and AMBP-1 expression and AM-induced cytokine responses in LPS-stimulated macrophages.

Methods

This study was conducted from October 2006 to March 2008. NR8383 rat macrophages were stimulated by IFN-γ, TNF-α, IL-6, and IL-10 with or without LPS (at 1 to 1000 ng/mL) for 6 and 24 hours.[2,6] Levels of AM, AMBP-1, and AM receptor proteins (calcitonin receptor-like receptor [CRLR]) and receptor activity-modifying protein (RAMP2 and RAMP3) mRNA were quantified using real-time PCR (ABI PRISM Sequence Detection System 7000, Applied Biosystems Group) or semi-quantitative RT-PCR after reverse transcription. Immunoreactive AM was measured by a radioimmunoassay (Phoenix Pharmaceutics, Belmont, CA, USA).

NR8383 rat macrophages and human monocytic cell line THP-1 were studied. THP-1 cells were incubated with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma, USA) for 72 hours to induce differentiation into macrophage-like cells. The cells were stimulated by LPS (10 ng/mL) and AMBP-1 (500 nM) in the presence or absence of AM or AM receptor antagonists (AM_{22-52} or $CGRP_{8-37}$) for 6 and 24 hours. Concentrations of TNF-α, IL-6, and IL-10 in the culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA, R&D Systems, USA). To study desensitisation of the AM receptor, cells were pre-incubated with AM (1 to 1000 nM) for 2 hours followed by a washout period and a re-stimulation by LPS with or without AM/AMBP-1. The concentration of TNF-α, IL-6, and IL-10 in the supernatant was measured.

NR8383 macrophages were stimulated by LPS (10 ng/mL) with or without addition of AM, forskolin (an adenylyl cyclase activator), SQ22536 (Qbiogene, an adenylyl cyclase inhibitor) or dibutyril-cAMP (a membrane permeable cAMP analogue) or a signalling pathway inhibitor for 6 and 24 hours. Inhibitors of protein tyrosine kinase (PTK) (genistein, Merck, USA), p38 and p42/44 MAPK, protein kinase A (PKA), and protein kinase C (PKC) were used. TNF-α, IL-6, and IL-10 in the supernatant were measured.

Changes in cytokine levels were analysed using analysis of variance or t-test as appropriate. A P value of <0.05 was considered significant.

Results

Expression of AM, AMBP-1, and AM receptor proteins in the LPS-induced inflammatory response in a rat alveolar macrophage cell line

In unstimulated NR8383 cells, AMBP-1 was constitutively expressed, with a RT-PCR cycle threshold of 26.62±0.08 compared with 18.35±0.07 for β-actin. Basal mRNA expression of CRLR and RAMP-2 were low, with a mean RT-PCR cycle threshold of 30.77±0.03 and 35.52±0.07, respectively, compared with 18.35±0.07 for β-actin.

LPS increased expression of CRLR by 5- to 26-fold at 6 hours (P<0.01), which declined to 5- to 11-fold above basal at 12 hours and to 2- to 4-fold above basal at 24 hours. IFN-γ, TNF-α, IL-6, or IL-10 had no effect on CRLR mRNA level in unstimulated NR8383 cells. In 10 ng/mL LPS-stimulated cells, IL-10 and IFN-γ reduced CRLR mRNA level at 6 hours from 12.2-fold basal level to 8.0-fold and 5.6-fold, respectively (P<0.05), whereas IL-6 increased the CRLR mRNA level to 16.9-fold (P<0.05). LPS reduced RAMP-2 expression to 0.35-fold basal level at 6 and 12 hours.

AMBP-1 expression was not altered by LPS, but IFN-γ significantly reduced AMBP-1 mRNA level in unstimulated and LPS-stimulated cells to 0.66-fold and 0.47-fold of basal level, respectively, at 6 hours (Fig 1).

IFN-γ at 10 ng/mL increased AM concentration in the culture medium from 12.9±2.3 fmol/mL to 215.9±35.7 fmol/mL (P<0.05), whereas addition of IFN-γ to LPS-stimulated cells increased AM concentration from 120.6±23.8 fmol/mL to 329.7±58.4 fmol/mL (P<0.05).

Effect of AM and AMBP-1 on inflammatory cytokine production in NR8383 and PMA-activated THP-1 cells

AMBP-1 at 500 nM did not alter the concentration of TNF-α, IL-6, or IL-10 in LP-stimulated macrophages. IL-10 secretion from unstimulated NR8383 cells was undetectable and not increased by AM. LPS stimulated IL-10 production that was augmented by 100 nM AM (Fig 2a).

PCR products of IL-10 and TBP are shown (Fig 2b). IL-10 mRNA expression was significantly up-regulated at 6 hours after LPS, paralleled the IL-10 peptide levels and further increased by 140%, 120%, and 65%, with addition of exogenous AM for LPS at 1, 5, and 100 ng/mL, respectively (Fig 2c). LPS-induced IL-10 production was also increased by TNF-α and IL-6 by 71% and 70%, respectively, but was markedly reduced by IFN-γ by 82% (Fig 2d).

In PMA-activated THP-1 cells, LPS markedly increased IL-10 production from as low as 1 ng/mL. 100 nM AM increased LPS-induced IL-10 production by 69% to 112% while IFN-γ reduced it by 77%.

The AM receptor antagonists (AM_{22-52} and $CGRP_{8-37}$) did not significantly alter the concentration of TNF-α, IL-6, or IL-10. Pre-incubation with AM followed by washout had no effect on TNF, IL-6, or IL-10 production, compared with LPS stimulation alone.

Role of second messenger-dependent kinases in the regulation of AM expression and AM-induced cytokine responses in LPS-stimulated macrophages

Dibutyril-cAMP (10 μM) and forskolin (1 μM) reduced TNF-α production by 50% and increased the production of IL-6 and IL-10 by 105% to 128%. Dibutyril-cAMP also increased AM concentration from 120.6±23.8 fmol/mL to 514.5±102.6 fmol/mL (P<0.05). SQ22536 had no effect on IL-6, IL-10, or AM concentration.

SB203580 inhibited LPS-induced IL-10 production by 56%; the production was partially reversed by exogenous AM and IL-6 but not TNF-α (Fig 3a). In contrast, PD98059 did not significantly affect IL-10 production that could be increased by adding AM or IL-6 (Fig 3b). SB203580 reduced IL-6 production by 13% to 29%; the produciton was completely reversed by adding AM or TNF-α (Fig 3c). PD98059 reduced IL-6 production by 22% to 26%; the production was reversed by AM but not TNF-α (Fig 3d).

In PMA-activated human THP-1 cells, PD98059 had no effect on LPS-induced IL-10 production but SB203580 inhibited it by 66%. This blockade was not reversible by AM, TNF-α, or IL-6.

PKA-inhibitor did not significantly affect IL-6 and IL-10 production. PKC-inhibitor reduced IL-6 production by 32% (P<0.05), but did not alter IL-10 production. Genistein reduced both IL-6 and IL-10 production by 87%. IL-6 production was partially restored by exogenous AM, but IL-10 production was not rescued by AM, TNF-α, or IL-6.

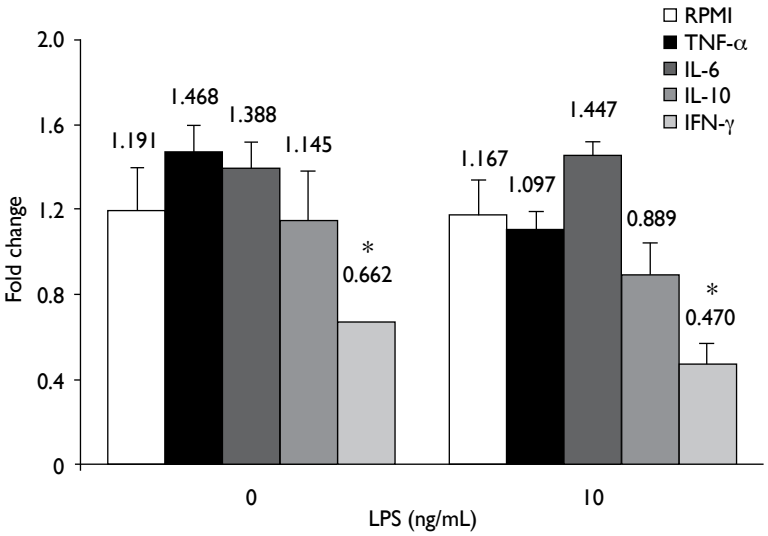

FIG 1. Relative expression of adrenomedullin-binding protein-1 (AMBP-1) in NR8383 cells in response to lipopolysaccharide (LPS) and inflammatory mediators at 6 hours

NR8383 cells were stimulated by different inflammatory mediators in the presence or absence of LPS. Expressions of AMBP-1 mRNA were measured by real-time PCR at 6 hours of incubation

* P<0.05, vs no inflammatory mediator

Discussion

In this study, LPS increased the initial expression of CRLR but reduced the expression of RAMP-2. Expression of CRLR in LPS-stimulated cells was attenuated by IL-10. This might suggest a negative feedback or dampening effect of IL-10 on CRLR expression. IFN-γ increased AM production in LPS-stimulated cells, while it reduced both CRLR and AMBP-1 expression.

In this study, AM significantly augmented LPS-induced IL-10 production in both NR8383 alveolar macrophages and PMA-activated THP-1 cells. This indicates a role of AM production in macrophages and 'macrophage-like' cells. On the contrary, AM receptor antagonists had no significant effect on the production of these inflammatory cytokines on LPS-stimulated NR8383 cells. These indicate that other mediators or alternative pathways independent of AM may be sufficient to stimulate the IL-10 production in these cells. Furthermore, IFN-γ reduced LPS-induced IL-10 production in both NR8383 alveolar macrophages and PMA-activated THP-1 cells that might be related to its inhibitory effect on the AM receptor and AMBP-1 expression. Thus, AM and IFN-γ might have opposite roles in the regulation of the anti-inflammatory cytokine IL-10 in both rat and human macrophages.

In this study, cAMP mediated the increased expression of AM, IL-6 and IL-10, and the decreased expression of TNF-α following LPS stimulation. Moreover, the production of IL-6 was mediated by p38-MAPK, p42/44-MAPK, PKC, and PTK, whereas that of IL-10 was mediated by p38-MAPK and PTK. Activation of the p42/44- and p38- MAPK pathways is common early signals necessary for LPS-induced cytokine production in monocytes and macrophages.[7]

In this study, blockade of the p38-MAPK pathway by SB203580 could be reversed by exogenous AM and IL-6, suggesting that other

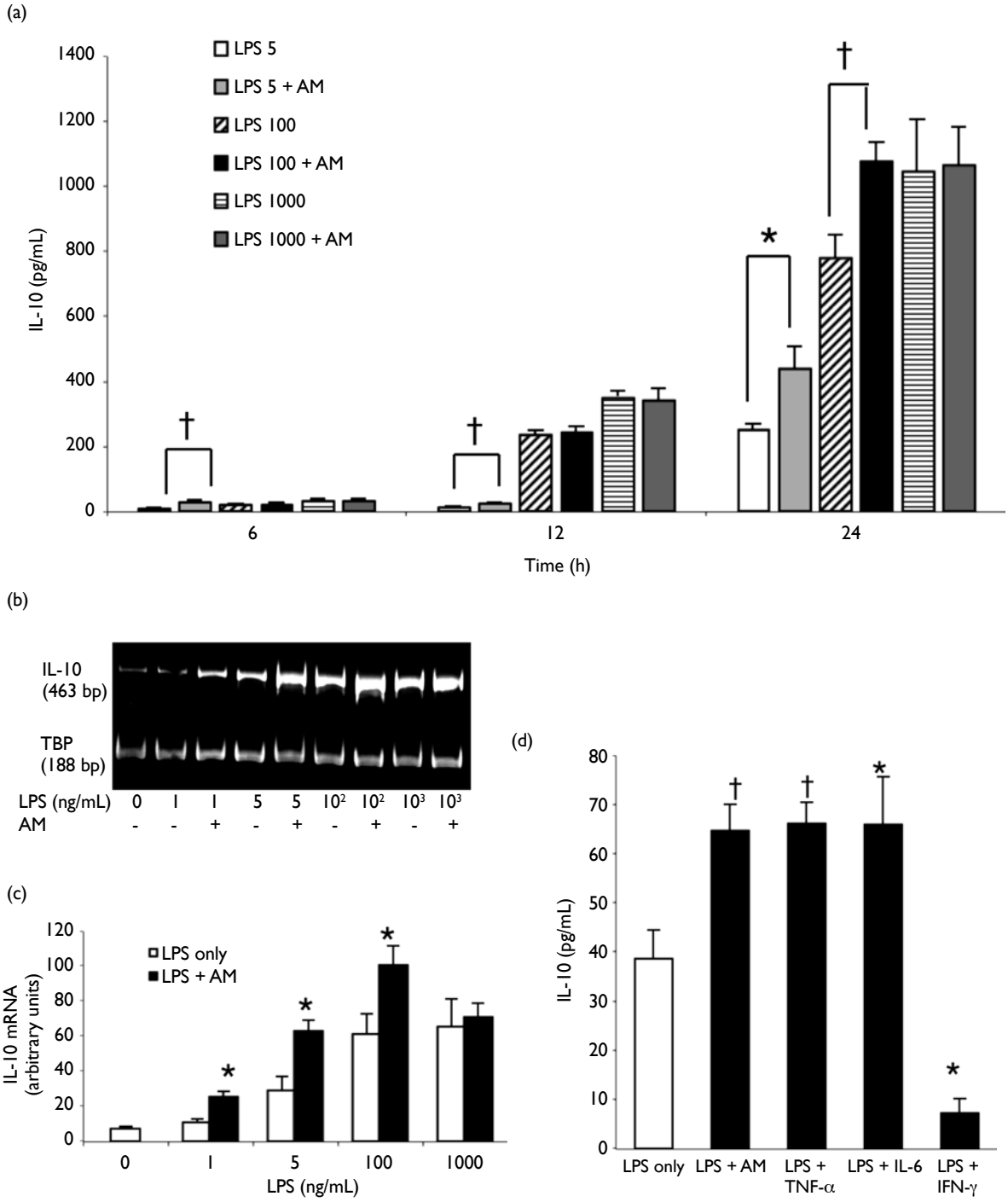

FIG 2. Effect of adrenomedullin (AM) and inflammatory mediators on lipopolysaccharide (LPS)-induced IL-10 production in NR8383 cells

(a) NR8383 cells were stimulated with LPS in the presence or absence of exogenous AM at 100 nM. IL-10 concentrations in the culture media were measured at 6, 12, and 24 hours by ELISA. (b) Representative results of gene-specific PCR products of IL-10 and TBP mRNA at 6 hours of incubation analysed by gel electrophoresis. (c) Semi-quantitative RT-PCR analysis was performed to determined IL-10 mRNA expression after normalising with TBP mRNA level at 6 hours after LPS-stimulation. (d) NR8383 cells were stimulated with 1 ng/mL of LPS in the presence or absence of AM (at 100 nM) or TNF-α, IL-6, and IFN-γ (each at 30 ng/mL). IL-10 concentrations were measured at 24 hours of incubation

* P<0.05, vs LPS alone

† P<0.01, vs LPS alone

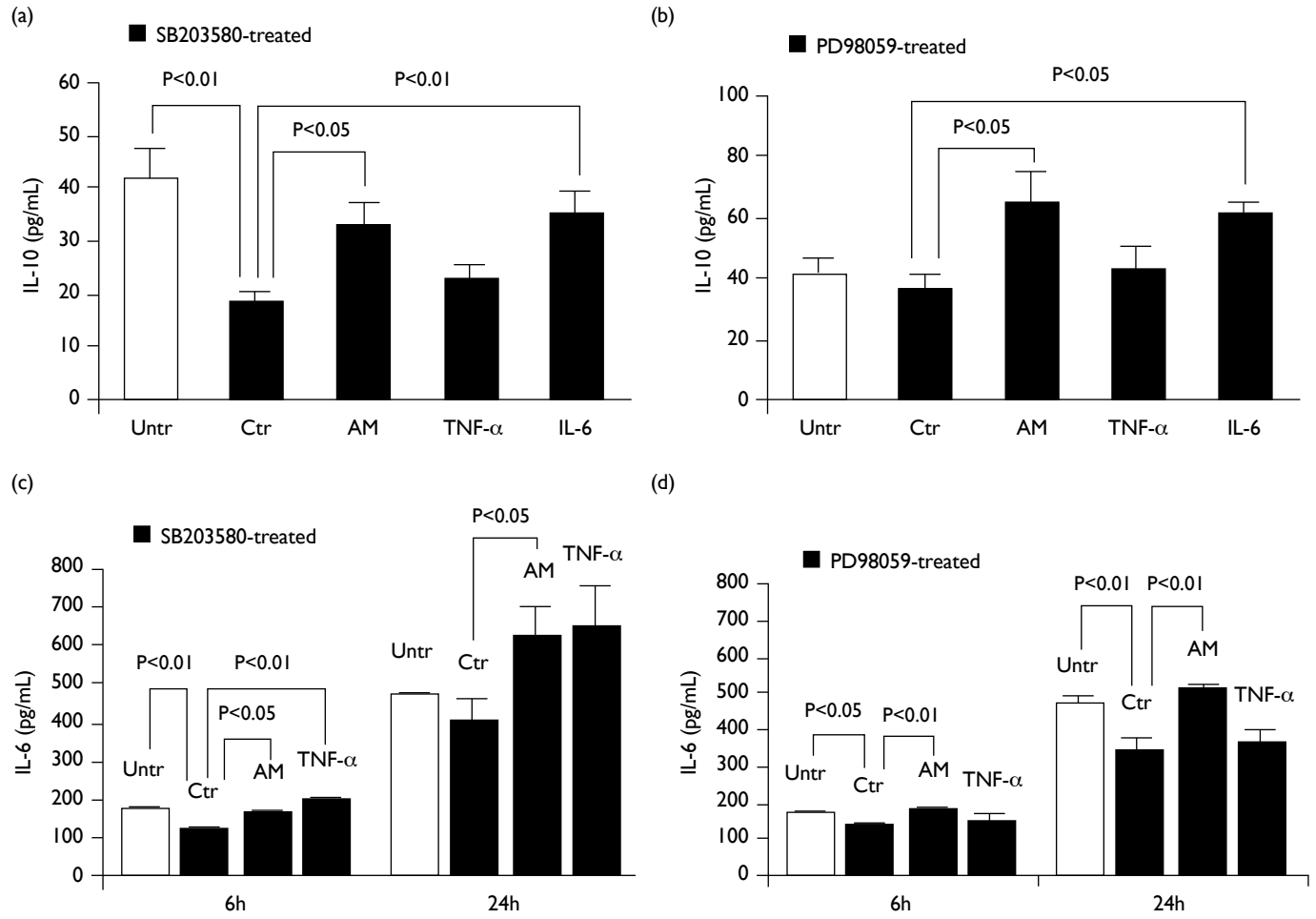

FIG 3. Effect of SB203580 and PD98059 inhibitors of p38- and p42/44-MAPKs on lipopolysaccharide (LPS)-induced production of IL-10 and IL-6 in NR8383 cells*

NR8383 cells were pretreated with SB203580 (2.5 μM) or PD98059 (25 μM) for 1 hour before stimulation with LPS (1 ng/mL) in the presence or absence of exogenous AM (100 nM), TNF-α or IL-6 (each at 30 ng/mL). (a) and (b) IL-10 concentrations in culture media were measured by ELISA at 24 hours of incubation. (c) and (d) IL-6 concentrations at 6 and 24 hours after stimulation were measured by ELISA

* Untr denotes untreated sample with 0.05% DMSO but no inhibitor added for pretreatment prior to LPS stimulation; Ctr denotes control pretreated with inhibitor and stimulated with LPS alone; AM, TNF-α, and IL-6 denote samples pretreated with inhibitor and co-stimulated with LPS and AM, TNF-α, or IL-6, respectively.

pathways may exist to induce IL-10 production upon LPS-stimulation. LPS-induced IL-10 and IL-6 production were markedly reduced by genistein, indicating that PTK-mediated pathways play an important role. The fact that IL-10 synthesis could not be rescued by any of the mediators that increase IL-10 production indicates a decisive role of PTKs in IL-10 production in this macrophage cell line. In contrast, IL-6 production could be partially rescued by AM in the presence of genistein, suggesting that AM activates more than one pathway to induce IL-6 production.

Conclusions

IFN-γ increases the expression of AM but downregulates CRLR and AMBP-1. AM augments LPS-induced IL-10, independently of increases in TNF-α and IL-6, and acts in opposition to IFN-γ. cAMP mediates the increased expression of AM, IL-6, and IL-10, and the decreased expression of TNF-α following LPS stimulation. The production of IL-6 is mediated by p38-MAPK, p42/44-MAPK, PKC and PTK, whereas that of IL-10 is mediated by p38-MAPK and PTK. AM plays a role in the regulation of inflammation. It augments the production of IL-10, a cytokine that limits inflammation. Nonetheless, the effectiveness of AM, although elevated in inflammation, may be diminished by IFN-γ that down-regulates AM's receptor and its binding protein. The downstream pathways that

mediate IL-10 and IL-6 production are identified. These findings suggest new targets for therapeutic intervention.

Acknowledgements

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