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C-reactive protein is expressed and secreted by peripheral blood mononuclear cells

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Summary

C-reactive protein (CRP) protects against bacterial pathogens and is a predictor of cardiovascular events. CRP is produced by vascular and organ-specific cells but the generation of CRP from peripheral blood mononuclear cells (PBMC) is poorly established. In a randomized, double-blind, placebo-controlled, two-way cross-over trial six healthy volunteers received a bolus infusion of 20 IU/kg *Escherichia coli* endotoxin [lipopolysaccharide (LPS)] or placebo. Intracellular CRP protein and CRP secretion of peripheral blood mononuclear cells (PBMC) was measured at baseline and 6 h after LPS by flow cytometry and enzyme-linked immubosorbent assay (ELISA), respectively. CRP mRNA expression was determined by real-time polymerase chain reaction (PCR). Regulation of the expression pathway was assessed using specific inhibitors *in vitro*. Small amounts of CRP protein and mRNA were detectable in PBMC, which were up-regulated between two- and eightfold by endotoxaemia *in vivo*. Augmented expression and release of CRP by LPS was consistent in PBMC cell culture experiments. LPS, interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α increased and IL-10 reduced CRP expression in PBMC. Toll-like receptor (TLR)-4, nuclear factor (NF)-κB and protein kinase C (PKC) activation were identified as intracellular signal transduction pathways of LPS-induced CRP expression. Constitutive CRP expression and release in PBMC is enhanced by inflammatory stimuli *in vivo* and *in vitro*. LPS might induce CRP generation via activation of TLR-4, NF-κB and PKC.

Keywords: C-reactive protein, endotoxin, peripheral blood mononuclear cells, inflammation

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Introduction

The acute phase reactant C-reactive protein (CRP) is a predictor of future cardiovascular events in men and women [1–4] and participates in the pathogenesis of atherosclerosis directly [5–9].

CRP is synthesized and released by various cells in response to microbial infection, tissue injury and immunomodulatory stimuli. *In vitro* results have demonstrated that vascular and organ-specific cells can produce CRP in response to inflammatory stimuli [10–14]. Despite elaborate evidence on the generation of CRP by different cell types, the ability of peripheral blood mononuclear cells (PBMC) to express and release CRP has not yet been demonstrated. While some experiments have indicated that CRP is detectable in PBMC [15], this has been attributed to phagocytosis rather than to constitutive expression [12]. PBMC are cellular effectors of the innate immune response system and accumulate in compartments where CRP is highly concentrated, i.e. in arteriosclerotic plaques [15]. We therefore hypothesized that CRP may also be generated and released by PBMC.

In order to investigate the expression of CRP in PBMC we challenged healthy humans with low systemic doses of *Escherichia coli* endotoxin [lipopolysaccharide (LPS)]. This experimental model has been shown to reproduce many of the pathological alterations observed in severe inflammation, including haemodynamic changes [16,17]. As a corollary PBMC were exposed to LPS and cytokines *in vitro* and the mechanisms of signal transduction studied.

Materials and methods

Human endotoxin studies

The study protocol was approved by the Ethics Committee of the Medical University of Vienna and complies with the

Declaration of Helsinki, including current revisions and the Good Clinical Practice guidelines. After giving informed consent six healthy male subjects aged between 19 and 28 years were enrolled in this randomized, double-blind, two-way cross-over study. All subjects were given a complete health examination [including physical examination, electrocardiogram (ECG) and laboratory screening] within 14 days prior to the first study day. All subjects were non-smokers and had no history or signs of arterial hypertension, disorder in lipid metabolism or other cardiovascular risk factors. No additional medication, including 'over-the-counter' drugs, were allowed from 4 weeks prior to screening until the study was complete.

Twenty IU/kg body weight LPS (dose corresponding to 2 ng/kg; national reference endotoxin, *Escherichia coli*, USP United States Pharmacopeia Convention Inc., Rockville, MD, USA) or placebo was administered intravenously as a bolus infusion to induce acute inflammation. Venous blood was drawn before and 6 h after LPS administration and PBMC isolated using Ficoll columns. Laboratory monitoring was carried out according to standard procedures. Approximately 8–10 h after LPS subjects were discharged in good health and the alternate study day scheduled with a washout phase of 1 week.

Cell culture experiments

Reagents used

CRP goat anti-human/A80-125 (Bethyl Laboratories, Montgomery, TX, USA); Alexa 488 donkey anti-goat/A11055 (Molecular Probes, Invitrogen Detection Technologies, Karlsruhe, Germany); anti-human CD3, CD4, CD8 and CD14, all phycoerythrin (PE)-conjugated, were purchased from Becton Dickinson (Becton Dickinson Austria GesmbH, Schwechat, Austria); Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden); RPMI-1640 medium (Mediatech Cellgrow, Herndon, VA, USA); fetal calf serum (FCS; Gibco, Carlsbad, CA, USA); Saponin (Sigma Chemical Co., St Louis, MO, USA); Amp RNA easy kit (Qiagen, Valencia, CA, USA); RT-reagent kit, MicroAmp optical plates, ABI Prism 7700 sequence detection instrument (Applied Biosystems, Foster City, CA, USA); *Porphyromonas gingivalis* LPS antagonist TLRL–MKLPS and LPS (*E. coli* 0111:B4) from Invivogen (San Diego, CA, USA); MAPK inhibitors SB203580, PD98059, UO126, protein kinase B (AKT) inhibitor and the nuclear factor (NF)-κB inhibitor SN50 from Calbiochem (La Jolla, CA, USA); Ro 31–8220 and wortmannin (Sigma, St Louis, CA, USA); hs-CRP enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems, Vienna, Austria), tumour necrosis factor (TNF)-α and interleukin (IL)-1α, IL-6 and IL-10 were obtained from Strathmann Biotec AG (Hamburg, Germany).

Intracellular CRP expression

Flow cytometry (FACSCalibur, Becton Dickinson, NJ, USA) was used to detect CRP protein expression by 10 000 PBMC. Cells were washed in saponin 0·1% buffer directly after isolation and incubated with the primary antibody against CRP. Isotype-matched non-specific antibody was used as control. Cells were spun and washed again in saponin 0·1% and incubated with the secondary antibody for another 30 min. Cells were respun and washed in saponin 0·1% and incubated further using monoclonal antibodies (mAb) against the leucocyte differentiation antigens CD3, CD4, CD8 and CD14. Stained cells were suspended in 3% bovine serum albumin (BSA) for immunodetection.

CRP protein secretion

PBMC isolated before and 6 h after systemic LPS administration were cultured in RPMI-1640 medium and 10% FCS for 24 h. The supernatant was analysed after centrifugation and 100-fold concentration using Sartorius Vivaspin2-tubes with a commercial hs-CRP ELISA. Basal CRP production was below the limit of quantification in unstimulated PBMC *in vitro*.

Quantitative CRP mRNA analyses

Total mRNA was prepared using the Amp RNA-easy kit, transcribed directly into cDNA using the RT-reagent kit and stored at −80°C until analysis. Real-time polymerase chain reaction (PCR) was performed in 96-well MicroAmp optical plates and optical adhesive covers with reaction volumes consisting of 2·5 μl of the quantitative PCR core reagent Kit. The 25 μl reaction in each well contained 1 μl of total cDNA, 300 nm of sequence-specific primers and 200 nm of dual-labelled fluorogenic probe in 1 × *Taq*man® Universal PCR master mix. Controls with and without templates of known amplification were included in each assay. The reaction was performed in an ABI PRISM 7700 Sequence Detection System. For analysis of CRP, the cDNA-specific pre-developed assay reagents kit was purchased from Applied Biosystems.

LPS and cytokine signal transduction pathways

PBMC were incubated with LPS (100 ng/ml) [18] alone and with specific antagonist of LPS at the Toll-like receptor 4 (TLR-4; *Porphyromonas gingivalis* LPS antagonist, 1 μg/ml) [19]; nuclear factor κB (NF-κB; SN50, 100 μg/ml) [20]; protein kinase C (PKC; Ro 31–8220, 1 μM) [21]; AKT inhibitor (AKT/PKB; 10 μM), phosphatidylinositol 3-kinase (PI3; wortmannin, 100 nM), p38 (SB203580, 10 μM), MAP kinase (MEK) (UO126, 10 μM) and extracellular-regulated kinase (ERK) (PD98059, 10 μM). Inhibitors used had no effect on constitutive CRP expression or on cell viability.

Table 1. Systemic response to lipopolysaccharide (LPS) administration.

	Baseline	4 h after LPS
Heart rate, bpm	64 ± 13	95 ± 15*
Systolic blood pressure, mmHg	118 ± 13	116 ± 7
Diastolic blood pressure, mmHg	63 ± 7	55 ± 7
Body temperature, °C	35·7 ± 0·2	37·7 ± 0·7*
White blood count, G/l	6·6 ± 1·1	11·1 ± 3·0*
CRP, mg/l	0·52 ± 0·21	1·27 ± 0·68*

Data are means ± s.d., $n = 6$. *$P < 0·05$, Wilcoxon matched pairs test. bpm: beats per minute; CRP: C-reactive protein.

Table 2. Differential haemogram before and after lipopolysaccharide (LPS) administration.

	Baseline	4 h after LPS
Neutrophils (G/l)	4·0 ± 1·7	8·1 ± 2·9*
Monocytes (G/l)	0·6 ± 0·3	0·2 ± 0·2*
Lymphocytes (G/l)	1·8 ± 0·3	0·4 ± 0·2*
Thrombocytes (G/l)	215 ± 61	167 ± 56*

*$P < 0·05$ *versus* baseline.

mRNA and protein expression was detected by real-time PCR and flow cytometry as above. Control experiments were conducted using different cytokines (IL-1α and TNF-α at 10 ng/ml, IL-6 and IL-10 at 50 ng/ml).

Statistical analyses

Due to the skewed distribution of the data, non-parametric tests were carried out using the Statistica® software package (release 6·1, Statsoft Inc., Tulsa, OK, USA). The Wilcoxon matched pairs test and the Mann–Whitney *U*-test were used for comparisons within and between groups, respectively. A $P = 0·05$ was considered significant. Values are expressed as means ± s.d. unless indicated otherwise.

Results

CRP expression *in vivo*

Following LPS, the expected mild and transient flu-like symptoms with elevated body temperature ($P < 0·05$), increased pulse rate ($P < 0·05$) and total CRP ($P < 0·01$) after 4 h were observed (Table 1). LPS altered the composition of the PBMC population with an increase in neutrophils, and a decrease in monocytes and lymphocytes (Table 2).

CRP protein expression was detectable in PBMC from all subjects under resting conditions. Expression was similar between cells positive for CD14, CD3, CD4 and CD8, and there was no difference between study days or within the observation period during placebo. In contrast, a significant increase in CRP protein expression by two- to eightfold was detectable in all cell types 6 h after LPS ($P < 0·05$ *versus* baseline, Wilcoxon matched pairs test, $P < 0·01$ *versus* placebo, Mann–Whitney *U*-test; Fig. 1). This induction by LPS was also consistent with augmentation on a translational level, as observed by real-time polymerase chain reaction (RT–PCR) and with enhanced protein secretion as measured by ELISA ($P < 0·05$ *versus* baseline, Wilcoxon matched pairs test, $P < 0·05$ *versus* placebo, Mann–Whitney *U*-test; Fig. 2). A representative immunofluorescence blot is shown in Fig. 3.

Regulation of LPS-induced CRP expression *in vitro*

The regulation of CRP expression by LPS was studied further in isolated PBMC (Figs 4 and 5). Co-incubation with

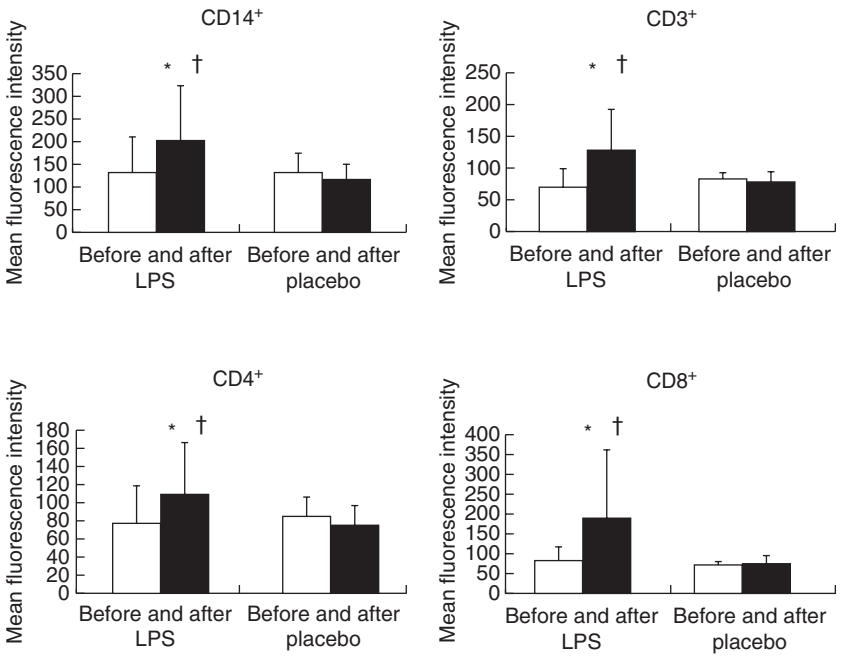

Fig. 1. C-reactive protein (CRP) expression of different peripheral blood mononuclear cell (PBMC) subsets at baseline and 6 h after lipopolysaccharide (LPS) or placebo administration as assessed by fluorescence activated cell sorter (FACS) analyses. LPS increases CRP protein expression *versus* baseline (*$P < 0·05$) and compared with placebo after 6 h (†$P < 0·01$). Data are means ± s.d., $n = 6$ placebo per group.

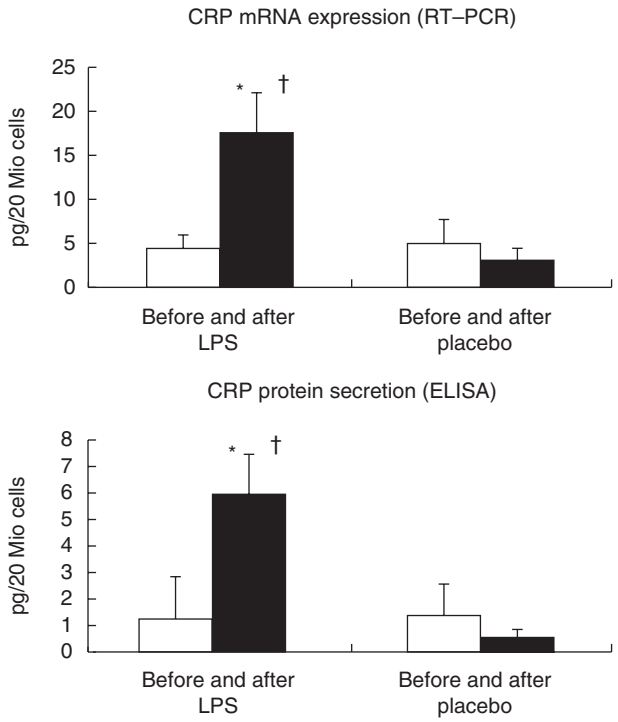

Fig. 2. Lipopolysaccharide (LPS) increases C-reactive protein (CRP) mRNA expression and protein secretion (*$P < 0{\cdot}05$ *versus* baseline, †$P < 0{\cdot}05$ *versus* placebo after 6 h) as measured by real-time polymerase chain reaction (RT–PCR) and enzyme-linked immunosorbent assay (ELISA). Data are means ± s.d., $n = 6$.

100 ng/ml and 100 pg/ml of LPS revealed comparable results in augmenting intracellular CRP protein expression in all cell types by approximately 50% using fluorescence activated cell sorter (FACS) analysis ($P < 0{\cdot}05$, $n = 3$, not shown). When incubated with LPS, TNF-α, IL-1α or IL-6 CRP protein expression increased. In contrast, incubation with IL-10 reduced basal CRP expression. Co-incubation of LPS with specific inhibitors of intracellular inflammatory pathways indicates that MAPK, PI3 or AKT are not involved in LPS-induced signal transduction. Inhibitors of TLR-4, NF-κB and PKC prevented the LPS-induced up-regulation of CRP mRNA and protein expression. This effect was most pronounced in $CD14^+$ and $CD3^+$ cells but consistent in all subsets of PBMC. Basal CRP production was below the limit of quantification of the ELISA in unstimulated PBMC *in vitro*.

Discussion

This study demonstrates that PBMC constitutively express and release CRP, which is potentiated by LPS *in vivo* and *in vitro*. This was detectable in different subsets of PBMC. Further, these experiments provide evidence that stimulation of CRP generation by LPS involves activation of TLR-4, NF-κB and PKC. Accumulation of PBMC at sites of inflammation may therefore play a significant role in the compartmentalized formation and secretion of CRP but may also influence systemic protection mechanisms against pathogens. This observation is of particular interest considering potential proatherogenic and prothrombotic effects of CRP in smooth muscle cells, alveolar macrophages and endothelial cells [22].

Induction of CRP expression in PBMC was not specific for LPS, but also observed after incubation with inflammatory cytokines. This is in good agreement with findings of increased cellular CRP expression to LPS, TNF-α or other cytokines [10–14] and also compatible with results obtained in alveolar macrophages [22]. However, it is important to note that alveolar macrophages may respond differentially to CRP [23] and divergent effects of cytokine stimulation, depending on the target cells, may therefore occur. The potential involvement of TLR-4, NF-κB and PKC is also consistent with other *in vitro* data [24,25]. CRP production in PBMC might therefore represent a feedback-loop during systemic or local inflammation, as CRP can protect LPS signal transduction through interactions with the Fcγ-receptor [26].

Interestingly, CRP protein and RNA expression is suppressed by the anti-inflammatory cytokine IL-10. It has been demonstrated previously that IL-10, which limits and down-regulates inflammation [26], inhibits CRP-induced tissue factor expression of monocytes [27]. This observation strengthens the close link between IL-10 and CRP in the modulation of the inflammatory disease progress.

The human endotoxin model was employed as a standard setting to study inflammatory responses *in vivo*. Endotoxin doses used in this model are substantially lower than in animal experiments and changes in circulating cytokine concentrations in the subjects under study are smaller accordingly. We therefore cannot exclude that stimulation with substantially higher LPS doses or different pathogens as seen in sepsis have different effects. However, the notion that various cytokines as mediators of the immune response system result in similar regulatory actions as LPS alone argue for a potential extrapolation of our findings to clinical conditions. Further, changes in plasma CRP concentrations 4 h after LPS were detectable only using a highly sensitive CRP assay. Comparable plasma CRP levels as caused by transient experimental endotoxaemia are seen in patients with subclinical chronic inflammatory diseases. This also suggests that the CRP release from PBMC may be even more relevant when the inflammatory stimulus is more potent or maintained over a longer time period.

The quantitative importance of localized CRP release, e.g. in atherosclerotic plaques, and production by PBMC is unclear. Our study expands these discussions, having observed that PBMC also participate in CRP production. Several *in vitro* studies demonstrated that CRP might be a source of local inflammation in the vascular interstitium [28,29]. However, there is no direct evidence of whether PBMC, or the amount of CRP released, play an important

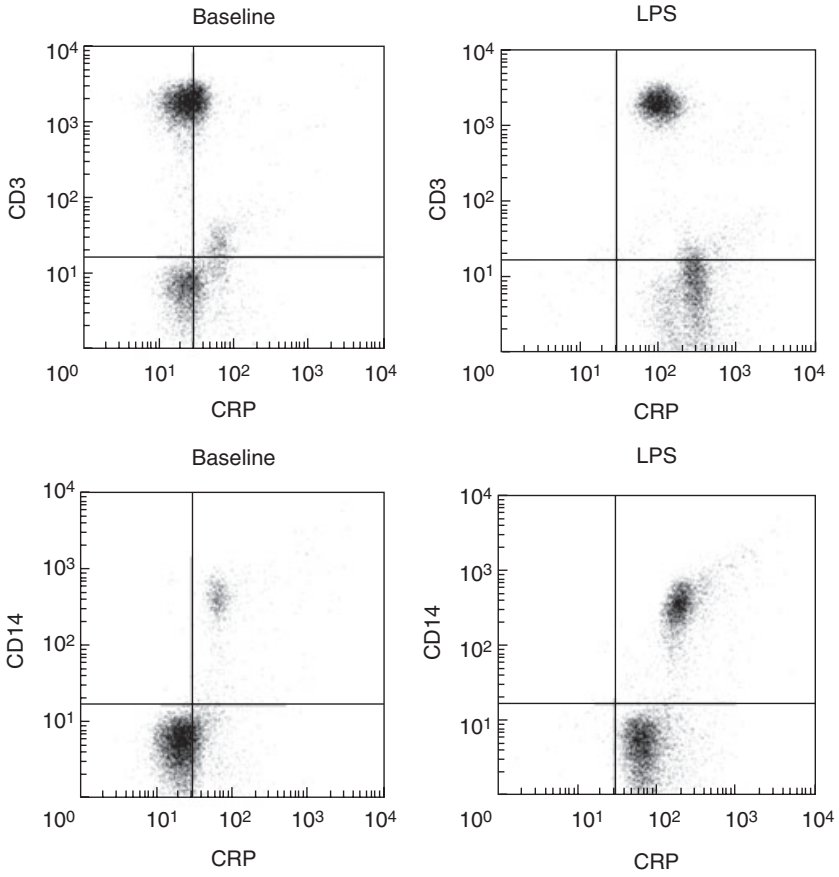

Fig. 3. Representative immunofluorescence blots of C-reactive protein (CRP) expression in $CD3^+$ and $CD14^+$ peripheral blood mononuclear cell (PBMC) at baseline and 6 h after lipopolysaccharide (LPS) or placebo.

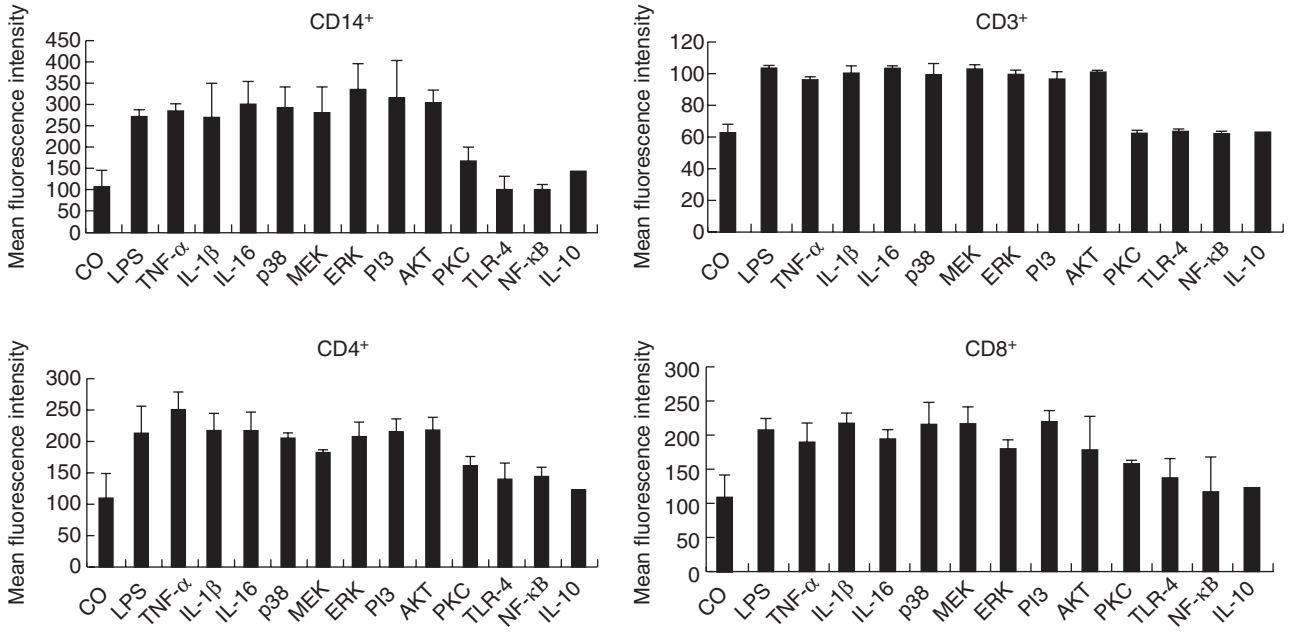

Fig. 4. C-reactive protein (CRP) expression of different peripheral blood mononuclear cell (PBMC) subsets by fluorescence activated cell sorter (FACS) analysis. CRP expression was increased by incubation with lipopolysaccharide (LPS), tumour necrosis factor (TNF)-α, interleukin (IL)-1α or IL-6, and decreased by IL-10. Co-incubation of LPS with inhibitors of Toll-like receptor (TLR)-4, nuclear factor (NF)-κB and protein kinase C (PKC) signalling, but not of protein kinase B (AKT), PI3, p38, MAP kinase (MEK) and extracellular-regulated kinase (ERK) suppressed CRP expression. Data are means ± s.d., $n = 3$.

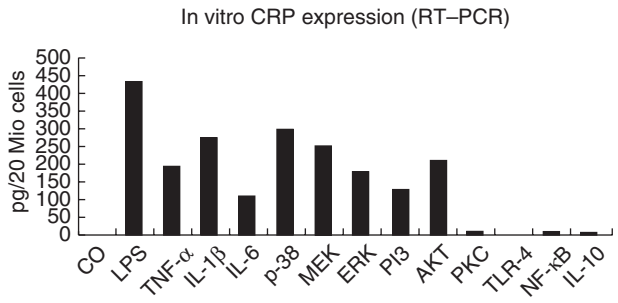

Fig. 5. C-reactive protein (CRP) expression in peripheral blood mononuclear cells (PBMC) by real-time polymerase chain reaction (RT–PCR) analysis. CRP expression was increased by incubation with lipopolysaccharide (LPS), tumour necrosis factor (TNF)-α, interleukin (IL)-1β or IL-6, and decreased by IL-10. Co-incubation of LPS with inhibitors to Toll-like receptor (TLR)-4, nuclear factor (NF)-κB and protein kinase C (PKC) signalling, but not against protein kinase (AKT), PI3, p38, MAP kinase (MEK) and extracellular-regulated kinase B (ERK) suppressed CRP expression.

role for this finding and thus for atherogenesis and the development of cardiovascular complications.

In summary, CRP mRNA and protein is expressed by PBMC under resting and stimulated conditions *in vitro* and during endotoxaemia. TLR-4, NF-κB and PKC may represent important intracellular signalling pathways. CRP activation in PBMC can also be induced by other cytokines such as IL-1α, IL-6 and TNF-α, which are released abundantly during inflammatory processes.

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