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The modulatory effects of prostaglandin-E on cytokine production by human peripheral blood mononuclear cells are independent of the prostaglandin subtype

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SUMMARY

The production of inflammatory mediators, relevant to (auto)immune diseases and chronic inflammatory conditions, can be modulated by dietary intake of *n*-3 and *n*-6 long chain polyunsaturated fatty acids (PUFAs). It was suggested that these effects are related to the formation of different series of eicosanoids, in particular prostaglandin-E (PGE). In this study we investigated whether prostaglandin subtypes metabolized from arachidonic acid (PGE_2), dihomo-γ-linolenic acid (PGE_1) or eicosapentaenoic acid (PGE_3) have different effects on T-cell proliferation and cytokine production *in vitro*. Freshly isolated human peripheral blood mononuclear cells (PBMC) were stimulated with concanavalin A (ConA) or lipopolysaccharide (LPS) in the presence or absence of exogenous PGE_1, PGE_2 or PGE_3. We found that tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and to a lesser extent interleukin (IL)-10 production was inhibited by all PGE-subtypes in ConA-stimulated PBMC concomitant with unaffected IL-2 levels. The modulated cytokine production of ConA stimulated cells was independent of T-cell proliferation. PGE_2 and PGE_1 moderately stimulated proliferation, while PGE_3 inhibited the proliferative response to some extent. In LPS-stimulated PBMC, TNF-α production was inhibited by all PGE-subtypes, whereas IL-6 remained unaffected and IL-10 production was increased. Time course experiments on the effects of PGE-subtypes on cytokine production after ConA or LPS stimulation showed these effects to be time dependent, but indifferent of the prostaglandin subtype added. Overall, the modulatory effects of PGE on cytokine production were irrespective of the subtype. This may implicate that the immunomodulatory effects of PUFAs, with respect to cytokine production, are not caused by a shift in the subtype of PGE.

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Abbreviations: AA, arachidonic acid; ConA, concanavalin-A; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FCS, fetal calf serum; GLA, γ-linolenic acid; HuS, human serum; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGE, prostaglandin-E; PUFAs, polyunsaturated fatty acids; RT, room temperature; Th, T-helper; TMB, tetramethylbenzidine; TNF-α, tumour necrosis factor-α.

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INTRODUCTION

Nutrition is a feasible tool for modulating immunity and its potential use was increasingly subjected to human studies during the last decade. Special attention has been paid to the modulatory effects of the nutritional components long chain poly unsaturated fatty acids (PUFAs) on immune function.[1,2] The inclusion of marine derived *n*-3 PUFA in the diet for instance was shown to decrease the production of proinflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by monocytes in healthy subjects[3–5] and in patients with rheumatoid arthritis.[6] From these studies on the modulatory effects on cytokine production a role was suggested for prostaglandins, a class of lipid mediators (eicosanoids), which are derived from 20 carbon atom PUFA.[7]

In non-supplemented individuals the prostaglandin-E (PGE) subtype usually synthesized in large quantities during an inflammatory response is PGE_2. The immediate precursor of PGE_2 is the *n*-6 fatty acid arachidonic acid

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(AA, 20:4*n*-6), which is one of the most abundant PUFA in mammalian cellular membrane phospholipids.[8] Consumption of fish oil rich in eicosapentaenoic acid (EPA, 20:5*n*-3) results in decreased AA levels and increased EPA levels. As EPA is the precursor fatty acid for the synthesis of the prostaglandin subtype PGE_3, this consequently results in increased production of PGE_3 at the expense of PGE_2.[7,9–12] Thus, the change in fatty acid composition consequently shifts the production of eicosanoids from AA, such as PGE_2, to eicosanoids derived from EPA, such as PGE_3. In addition, an alternative prostaglandin subtype PGE_1 is synthesized from the *n*-6 fatty acid dihomo-γ-linolenic acid (DGLA, 20:3*n*-6), which is a metabolite from γ-linolenic acid (GLA, 18:3*n*-6) that is naturally occurring for instance in borage oil.[13,14] Similar to fish oil, consumption of GLA rich oils was found to exert beneficial effects in inflammatory disorders[15–17] and to reduce production of pro-inflammatory cytokines.[18,19]

In vitro and *in vivo* studies have shown that prostaglandins can affect cytokine production directly during an inflammatory response. For instance, the production of T-helper cell-1 (Th1) cytokines like interferon-γ (IFN-γ)[20,21] and monocyte-derived cytokines like TNF-α are down-regulated by PGE_2 *in vitro*.[22–24] In general, PGE_1 and PGE_3 are thought to have a distinct biological potency than PGE_2 and are therefore usually associated with a decreased inflammatory response. Thus, it has been anticipated that as a result of changing the amounts and types of eicosanoids produced, fish oil and borage oil influence the magnitude of immune responses.[25]

In this study, we investigated whether the immunomodulatory effects of dietary PUFAs are mediated by a shift in production of the type of PGE by investigating the direct effect of exogenous PGE_1, PGE_2 and PGE_3 on cytokine production of human peripheral blood mononuclear cells (PBMC) induced by T-cell mitogen or endotoxin. From these experiments it becomes apparent that a shift in PGE_1 or PGE_3 over PGE_2 production by dietary fatty acid modulation can not solely explain the reduction in proinflammatory cytokine production observed in human dietary studies with GLA or EPA rich sources.

MATERIALS AND METHODS

Materials

Cells were cultured in RPMI-1640 containing 25 mM HEPES and 2 mM L-glutamine supplemented with 20% heat-inactivated human serum (HuS) (Bio-Whittaker, Verviers, Belgium), 12·5 mM D-glucose (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 100 U/ml penicillin/streptomycin and 1 mM sodium pyruvate (both obtained from Gibco BRL, Life Technologies, Breda, The Netherlands). PGE_1 and PGE_2 were obtained from Sigma-Aldrich Chemie BV, PGE_3 was obtained from Cayman Chemical Company (Ann Arbor, MI). Lipopolysaccharide (LPS, BO5 : 55), (+/–)-α-tocopherol and ascorbic acid were obtained from Sigma-Aldrich Chemie BV. Ficoll (Ficoll-Paque®) was obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Concanavalin A (ConA) and 5-bromo-2′-deoxyuridine (BrdU) assays were obtained from Boehringer Mannheim. WST-1 assay was obtained from Roche Diagnostics Nederland BV. (Almere, The Netherlands). Indomethacin was obtained from ICN Biochemicals (Zoetermeer, The Netherlands).

Cell isolation and culture conditions

PBMC were isolated from buffy coats by density-gradient centrifugation. In short, buffy coat cells were dispensed over five 50 ml falcon tubes, phosphate-buffered saline (PBS)/2% fetal calf serum (FCS) solution was added to reach a volume of 20 ml and 10 ml Ficoll-Paque® was gently added under the diluted buffy coat cells. Centrifugation was performed at 400 ***g*** for 20 min at room temperature (RT) and washing of PBMC was done three times with PBS/2% FCS. Culture of freshly isolated PBMC was performed in the presence of 20% HuS. Shortly before addition to cells, HuS was enriched with α-tocopherol and L-ascorbic acid to the final culture concentrations of 25 and 75 μM, respectively. PGE stock solution was prepared by dissolving PGE in ethanol and diluting the solution 1 : 10 in PBS to a concentration of 1 mg PGE per ml. Addition of PGE to culture medium was performed by adding the adequate amount of PGE stock solution to HuS resulting in final concentrations as indicated in the text. The ethanol concentration in the culture medium never exceeded 0·1%. Indomethacin (10^{-5} M) was added to the culture medium to inhibit endogenous production of prostaglandins. Cells were plated at $1{\cdot}5 \times 10^6$ cells/ml in flat-bottom 96-well culture plates in a volume of 200 μl per well and incubated at 37° in a humidified 5% CO_2 atmosphere. T-cell proliferation was induced by ConA stimulation (10 μg/ml) for 48 hr and monocyte stimulation was performed with LPS (100 ng/ml) for 20 hr.

PGE_1 and PGE_2 measurements in culture medium

Enzyme-linked immunosorbent assay (ELISA) kits from R & D systems were used (Wiesbaden-Nordenstadt, Germany) to measure PGE_1 in culture medium. PGE_2 was quantified by the Biotrak PGE_2 competitive enzyme immunoassay system (Amersham Pharmacia Biotech, Freiburg, Germany). The cross-reactivity of the PGE_1 ELISA was 21% with PGE_2 and 2% with PGE_3. The PGE_2 ELISA cross-reacted 4% with PGE_1 and 33% with PGE_3.

Proliferation assay

Cell proliferation was measured by a colorimetric immunoassay using incorporated nuclear BrdU according the manufacturers guidelines. In short, cells were stimulated with ConA for 48 hr and BrdU incorporation was allowed over the final 16 hr of culture. After removal of the culture medium, cells were fixed to the bottom of the plates and incorporated BrdU was quantified by anti-BrdU-peroxidase antibody and tetramethylbenzidine (TMB) substrate. Extinction was measured at 450 nm.

Measurement of cytokine production

The concentrations of cytokines in culture supernatants were determined by specific ELISA according to the manufacturer suggestions. TNF-α and IL-6 were measured with

ELISA kits from Biosource (Nivelle, Belgium). IFN-γ and IL-10 were measured with ELISA kits from the Central Laboratory for Blood Transfusion (Amsterdam, The Netherlands). IL-2 was determined using antibody pairs from Pharmingen (Hamburg, Germany). Results for all cytokines represent the mean of triplicate incubations.

Statistical analysis

Statistical evaluation of differences among PGE treated cells and control cells were calculated by two-way analysis of variance (ANOVA) with factors 'PGE concentration' and 'donor'. Parameters to be analyzed were viability, proliferation and cytokine production. Differences were considered significant at $P<0{\cdot}05$.

RESULTS

Kinetics of cytokine production during ConA or LPS stimulation of human PBMC

In order to characterize the *in vitro* model used, we measured the kinetics of cytokine production in supernatants at different time intervals after addition of ConA or LPS. TNF-α was the first cytokine detected after stimulation with ConA and increased in concentration during the incubation period of 48 hr. IFN-γ was detectable from 6 hr on and maximal amounts of IFN-γ were measured at 24 and 48 hr of ConA stimulation. IL-2 and IL-10 were not detected until 8 hr after addition of ConA, but increased during the remaining 40 hr stimulation period (Fig. 1).

LPS stimulation induced TNF and IL-6 production, which was detected within 1 hr of stimulation. TNF-α concentration rapidly increased during the first 5 hr of LPS stimulation and remained constant subsequently. In contrast, IL-6 concentrations greatly increased at time points 15 and 20 hr LPS stimulation compared to earlier time points. IL-10 was detectable 4 hr after LPS stimulation and slowly increased to plateau levels at 20 hr LPS stimulation (Fig. 1).

Effects of PGE-subtypes on production of T-cell cytokines and T-cell proliferation

To investigate the effects of PGE-subtypes on a T-cell dependent immune response, PBMC were cultured with different concentrations of PGE_1, PGE_2 or PGE_3 added to the culture medium and stimulated at the same time with ConA for 48 hr. The background of prostaglandins in the culture medium containing 20% human serum in absence of cells was 4·8 ng/ml ($1{\cdot}3\times10^{-11}$ M) PGE_1 and 0·5 ng/ml ($1{\cdot}4\times10^{-14}$ M) PGE_2.

PGE subtypes dose-dependently inhibited TNF-α, IFN-γ, and to a lesser extent IL-10 production, whereas IL-2 levels were unaffected (Fig. 2). TNF-α production was inhibited by approximately 80% (IC_{50} at $0{\cdot}5\times10^{-7}$ M PGE) and simultaneously IFN-γ production was inhibited by approximately 50% (IC_{50} at 1×10^{-6} M PGE). Production of IL-10 was decreased at concentrations of $1\times10^{-8}-1\times10^{-6}$ M by approximately 25%. Moreover, the effects seen on production of the four different cytokines were irrelevant of the subtype of PGE.

Subsequently, we investigated whether the modulatory effects of PGEs on cytokine production were reflected by a change in proliferation. PGE_2 moderately stimulated T-cell proliferation at concentrations of $1\times10^{-8}-1\times10^{-11}$ M, whereas PGE_1 induced similar increases in proliferation at $1\times10^{-9}-1\times10^{-11}$ M. In contrast, addition of PGE_3 induced a decrease in proliferation at concentrations of 1×10^{-7} and 1×10^{-6} M (Fig. 3). Therefore, it appears that the PGE-subtype independent effects on cytokine production are not closely mirrored by the proliferative response.

Effects of PGE-subtypes on LPS-induced cytokine production

We investigated the effects of PGE_1, PGE_2 and PGE_3 on cytokine production of LPS-stimulated PBMC. PGE_1, PGE_2 and PGE_3 induced a dose-dependent modulation of

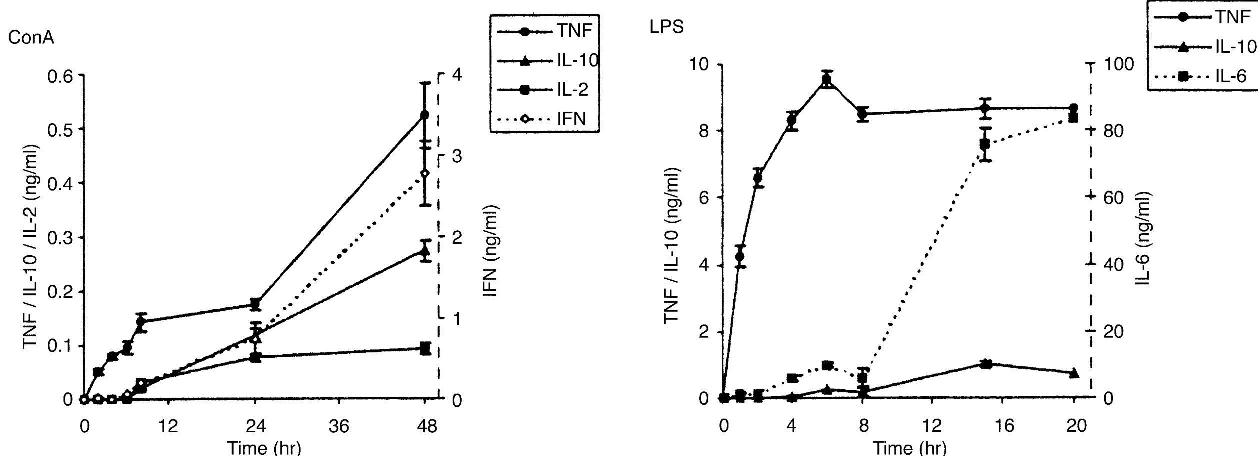

Figure 1. Kinetics of ConA- or LPS-induced cytokine production. Human PBMC were cultured with ConA (10 µg/ml) or LPS (100 ng/ml) and cytokine concentrations in culture supernatant were measured at the time points indicated by ELISA. Each data point represents the mean ± SD of five determinations from one representative donor ($n=3$).

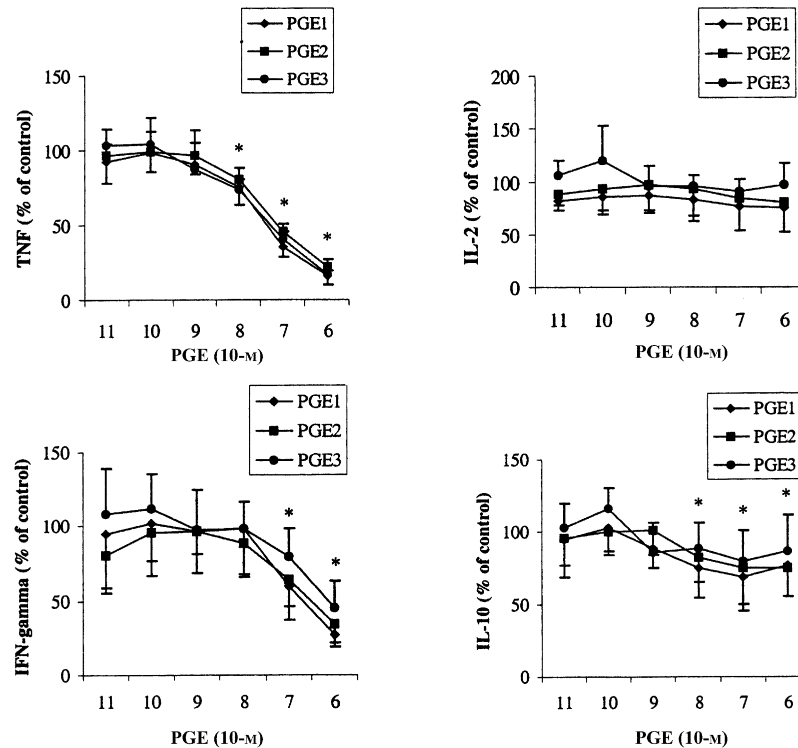

Figure 2. Effects of prostaglandins on ConA induced cytokine production. PBMC were stimulated with ConA for 48 h in presence or absence (control) of PGE_1, PGE_2 or PGE_3. Values are expressed as percentages of control and are mean $\pm$ SD for triplicate determinations using PBMC from six blood donors. The absolute values of the controls were 2471 ± 788 (TNF-α), 245 ± 137 (IL-2), 1678 ± 1432 (IFN-γ) and 68 ± 21 (IL-10). $^*P < 0{\cdot}05$ for values of both PGE_1, PGE_2 and PGE_3 compared to control values.

cytokine production (Fig. 4). A strong inhibition of TNF-α to almost undetectable levels was observed (IC_{50} at $0{\cdot}5 \times 10^{-7}$ M PGE), whereas IL-6 levels were unaffected. In contrast, IL-10 increased dose dependently to a maximum of approximately 200% compared to control incubations. Overall, we observed these dose–response effects to be independent of the subtype of prostaglandin added.

Time course of modulatory effects of PGE-subtypes on cytokine production

As we observed no differential effects between the PGE-subtypes on cytokine production when PGE was added just before cell stimulation, we investigated whether the subtypes differ in their effects at time points after stimulation. Therefore, we stimulated PBMC with ConA or LPS and added the PGE-subtypes just before or at different time intervals after addition of the stimulus. It was found that the ability of the PGE-subtypes to exert inhibitory effects on cytokine production declined during the culture period. The inhibition of TNF-α production remained constant during the first 6 hr of ConA stimulation and was abrogated when the PGE-subtypes were added at later time points (Fig. 5). The inhibitory effect on TNF-α production by PBMC after stimulation with LPS remained apparent for 1 hr after stimulation and then gradually declined to control levels within the following 5 hr (Fig. 5). Moreover, we observed no differential effects of the PGE-subtypes when added at different time intervals after ConA or LPS stimulation.

DISCUSSION

The modulation of immune function by dietary *n*-3 or *n*-6 PUFAs has been studied intensively.[1] It was suggested that the mechanism of these modulatory effects on the immune response by PUFAs might involve eicosanoid synthesis from DGLA, AA or EPA into PGE_1, PGE_2 or PGE_3, respectively.[7] PGEs are physiologically present in body fluids at low concentrations within the range of picomolar to nanomolar concentrations, but may locally rise to micromolar concentrations during inflammatory conditions.[26,27] By investigating relevant PGE concentrations, we found that PGE_1, PGE_2 and PGE_3 have similar effects on cytokine production by PBMC indifferent of the time point of PGE addition, that is, before or after induction of cell stimulation. This indicates that changes in cytokine production by specific dietary PUFAs may not be mediated by a shift from predominantly PGE_2 to other PGE subtypes. Therefore, *n*-3 and *n*-6 PUFA rich oils like fish oil (rich in EPA) and borage oil (rich in GLA) may exert their beneficial effects on cytokine production in inflammatory conditions via a PGE-subtype independent mechanism.

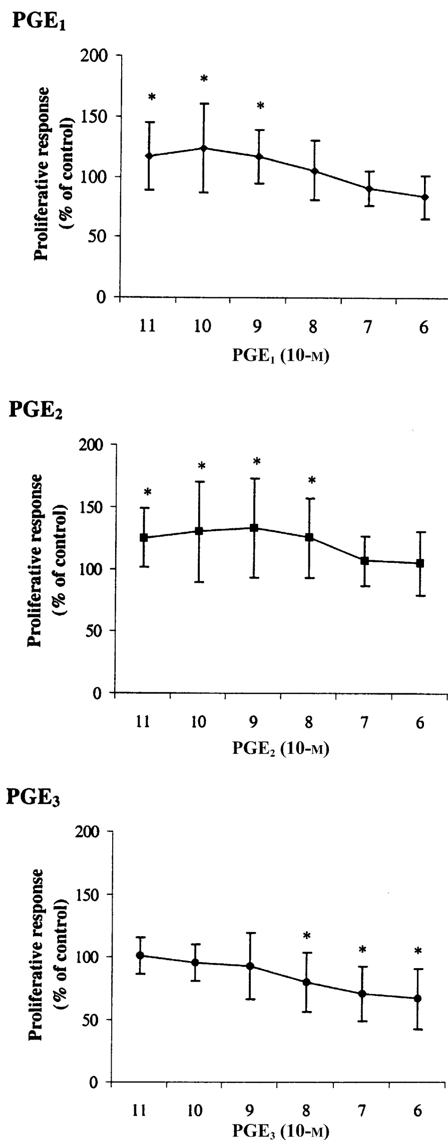

Figure 3. Effects of prostaglandins on ConA induced T-cell proliferation. PBMC were stimulated with ConA for 48 hr in presence or absence (control) of PGE_1, PGE_2 or PGE_3. Values are expressed as percentages of control and are mean ± SD for triplicate determinations using PBMC from eight blood donors. Mean stimulation index of controls was 2·51 ± 0·75. $^*P<0{\cdot}05$ from control value.

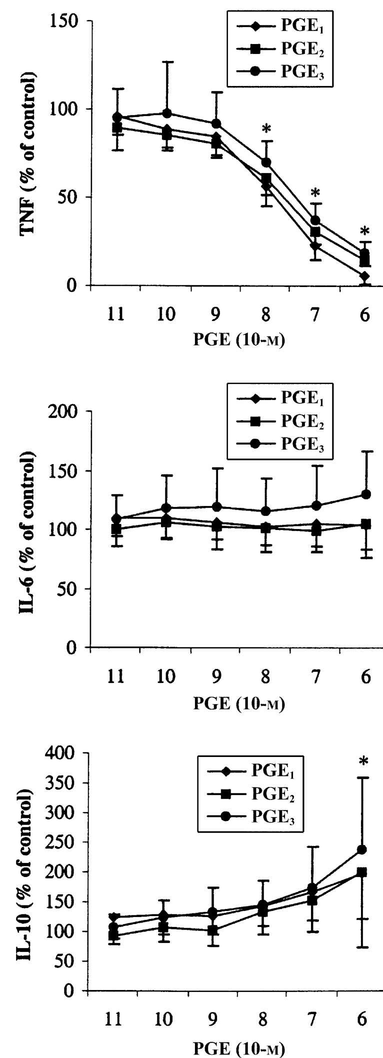

Figure 4. Effects of prostaglandins on LPS induced cytokine production. PBMC were stimulated with LPS for 20 hr in presence or absence (control) of PGE_1, PGE_2 or PGE_3. Values are expressed as percentages of control and are mean ± SD for triplicate determinations using PBMC from nine blood donors. The absolute values of the controls were 7028 ± 3314 (TNF-α), 32981 ± 22452 (IL-6) and 1059 ± 624 (IL-10) pg/ml $^*P<0{\cdot}05$ for values of both PGE_1, PGE_2 and PGE_3 compared to control values.

The effects of PGE_2 on cytokine production of T cells using an *in vitro* cell system have been described before. Down-regulation of Th1 cytokine production accompanied by largely unaffected Th2 cell function by PGE_2 was described for human T cells[28] and T-cell clones.[29,30] In line with this, we observed a bias towards down regulation of Th1 over Th2 cytokine production in ConA-stimulated PBMC dependent on the levels of PGE present. PGE (10^{-7} M) inhibited production of both Th1 cytokine IFN-γ and Th2 cytokine IL-10, whereas very high concentrations of PGE (10^{-6} M) inhibited IFN-γ production more than IL-10 production. The *in vivo* significance of this observation is unclear. PGE_2 determinations in joint fluid of rheumatoid arthritis patients have shown levels of approximately 10^{-10} M[31] and similar levels were found in plasma of mice after intraperitoneal injection of LPS.[32] However, it is anticipated that local PGE production may reach 10^{-6} M, because PGE levels within the micromolar range were found after *ex-vivo* restimulation of osteoarthritis affected human cartilage.[26,33] Moreover, the administration of cyclooxygenase inhibitors to BALB/c mice was found to promote

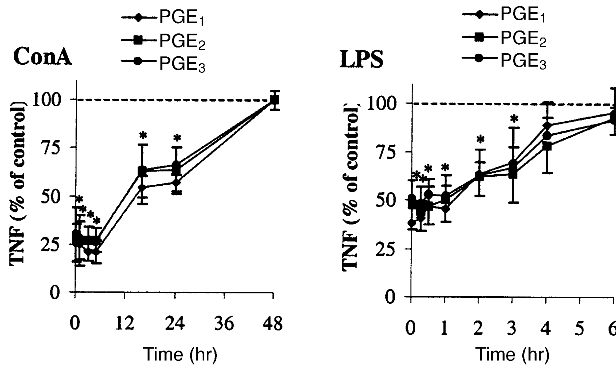

Figure 5. Time course of inhibitory effect of prostaglandins on TNF-α production. PBMC were stimulated with LPS at timepoint 0 and subsequently PGE_1, PGE_2 or PGE_3 (10^{-6} M for ConA and 10^{-7} M for LPS stimulation) was added at different time points. Supernatants were analysed for cytokine production 48 hr (ConA) or 20 hr (LPS) after stimulation. Values are expressed as percentages of control (no PGE added) and are mean ± SD for triplicate determinations using PBMC from four (ConA) or six (LPS) blood donors. $*P < 0{\cdot}05$ of values compared to control (no PGE added).

a Th1 response[34] and antigen-specific IFN-γ production[35] indicating involvement of PGE_2 in Th1 down-regulation *in vivo*.[29,36–38]

Despite the fact that we observed no differences in cytokine production between PGE-subtypes, we measured differential effects on T-cell proliferation. PGE_3 exerted inhibitory effects at high concentrations while PGE_1 and PGE_2 induced an increase in proliferation at low concentrations. This is in contrast with previous findings that have shown that both PGE_2 and PGE_3 induced a strong reduction of T-cell proliferation using rat[36,37] or human[29,38–40] lymphocytes. In the study using ConA-stimulated human PBMC[38] PGE_3 was described to be more potent than PGE_2,[36] which is in line with our data. However, currently described effects of PGE-subtypes on T-cell proliferation were marginal and insufficient to induce a PGE-subtype dependent cytokine production. Moreover, it has been known that the proliferative responses of T cells are strongly affected by IL-2 levels.[41] The IL-2 levels induced in the present study were easily detectable and within ranges to be expected after ConA stimulation of human PBMC *in vitro*.[42] Inhibitory effects on IL-2 production by PGE would therefore have been detected if present. Consequently, it is rational that the unaltered IL-2 production seen in our study is not accompanied by strong inhibitory effects on T-cell proliferation. Our data therefore suggest that during an inflammatory response cytokines like TNF-α and IFN-γ are more sensitive to PGE than other cytokines like IL-2 and the proliferative response. In conclusion, it is conceivable that the immunomodulatory effects of dietary fish or borage oil may not be induced by a PGE-subtype-dependent modulation of T-cell proliferation.

Stimulation of PBMC with LPS activates monocytes directly and induces production of a wide range of mediators including the cytokines TNF-α, IL-1β, IL-6, IL-10 and IL-12, and eicosanoids (prostaglandins, leukotrienes) by the activation of oxygenase enzyme expression.[43–45] Early after stimulation the dominating products in supernatants of PBMC are inflammatory cytokines (TNF-α, IL-1β and IL-6) followed by an increased production of anti-inflammatory mediators like IL-10[46] and prostaglandins at high concentrations,[47,48] which are associated with resolution of inflammation. In this model we observed that exogenous PGE induces a strong dose-dependent inhibition of TNF-α production accompanied by an increase in IL-10 production. This is in accordance with other studies describing down-regulation of endotoxin-induced TNF-α gene expression[22] and production by exogenous PGE_2 for human monocytes[22–24], rat macrophages[49] and murine macrophages.[50] In addition, up-regulation of the anti-inflammatory cytokine IL-10 by PGE_2 was observed in whole blood cultures.[51,52] Although IL-10 is sufficient to inhibit prostaglandin[53–57] and TNF-α[52] production, our data suggest that the inhibited TNF-α production by the PGE-subtypes is independent of IL-10. PGE inhibits TNF-α production until 6 hr after induction of LPS stimulation, whereas at that time point IL-10 is not yet produced. This is in accordance with the finding that anti-IL-10 treatment of human monocytes stimulated with LPS did not abolish the TNF-α down regulation induced by PGE.[52] Altogether, this suggests that the increased IL-10 levels seen after exogenous PGE treatment serves as an autoregulatory feedback to normalize PGE levels.

In conclusion, observations indicate that PGE_1, PGE_2 and PGE_3 similarly affect cytokine production in human PBMC. This may implicate that the immunomodulatory effects of PUFAs, with respect to cytokine production, are not caused by a shift in the subtype of PGE. Consequently, other mechanisms may be responsible for selective modulations by PUFAs, including synthesis of specific oxygenated metabolites from the different fatty acids. For example, it was found that DGLA is not converted to a leukotriene product but is converted to a 15-hydroxyl derivative that can block transformation of AA to (pro-inflammatory) leukotrienes.[58] Individual PUFAs may also differ in their ability to modulate intracellular signalling, such as binding to peroxisome proliferator-activated receptor-γ[59,60] or changing nuclear factor-κB[61] activity. Overall, further studies with focus on signaling pathways are necessary to clarify the mechanism of the immunomodulatory effects of PUFAs and the interactions of cytokines and prostaglandins during an inflammatory response.

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