Polyunsaturated Fatty Acids Block Dendritic Cell Activation and Function Independently of NF-κB Activation*

Maximilian Zeyda‡, Marcus D. Säemann§, Karl M. Stuhlmeier‡, Daniel G. Mascher‖, Peter N. Nowotny‡, Gerhard J. Zlabinger**, Werner Waldhäusl‡ ‡‡, and Thomas M. Stulnig† ‡‡§§

From the Clinical Divisions of &Endocrinology and Metabolism and &Nephrology and Dialysis, Department of Internal Medicine III, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria, the Ludwig Boltzmann Institute for Rheumatology and Bioimaging, Kärntnerstrasse 10, POB 78, A-1107 Vienna, Austria, and the JPharm-analyt Laboratory GmbH, Ferdinand-Pichler-Gasse 2, A-2500 Baden, Austria, the Institute of Immunology, Medical University of Vienna, Borschkegasse 8A, A-1090 Vienna, Austria, and the CeMM Center of Molecular Medicine of the Austrian Academy of Sciences, Schönhauerstrasse 40, A-7780 Vienna, Austria

Polyunsaturated fatty acids (PUFAs) modulate immune responses leading to clinically significant beneficial effects in a variety of inflammatory disorders. PUFAs affect T cells have been extensively studied, but their influence on human dendritic cells (DCs), which are the most potent antigen-presenting cells and play a key role in initiating immune responses, has not been elucidated so far. Here we show that PUFAs of the n-3 and n-6 series (arachidonic and eicosapentaenoic acid) affect human monocyte-derived DC differentiation and inhibit their activation by LPS, resulting in altered DC surface molecule expression and diminished cytokine secretion. Furthermore, the potency to stimulate T cells was markedly inhibited in PUFA-treated DCs. The PUFAs-mediated block in LPS-induced DC activation is reflected by diminished TNF-α, IL-12p40, CD40, and COX-2 mRNA levels. Strikingly, typical LPS-induced signaling events such as degradation of IκBα and activation of NF-κB were not affected by PUFAs, even though DC membrane lipid composition was markedly altered. Arachidonic and eicosapentaenoic acid both altered DC prostaglandin production, but inhibitors of cyclooxygenases and lipoxygenases did not abolish PUFA effects, indicating that the observed PUFA actions on DCs were independent of autoregulation via eicosanoids. These data demonstrate a unique interference with DC activation and function that could significantly contribute to the well known anti-inflammatory effects of PUFAs.

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Polyunsaturated fatty acids (PUFAs)† provoke clinical benefits in a variety of inflammatory diseases, particularly rheumatoid arthritis, inflammatory bowel disease, and IgA nephropathy (1, 2). Most studies elucidating PUFA effects on the adaptive immune response focused on lymphocyte function and cytokine production (3–5). While mechanisms underlying the distinct effects of PUFAs on T cell activation have been elucidated in detail (6), little is known about PUFA effects on antigen-presenting cells (APCs) in general and dendritic cells (DCs) as the primary and most potent APCs in particular.

DCs are the only APCs capable of initiating immune responses, i.e. activation of naïve T cells (7). In vivo, DCs constitutively patrol through peripheral tissues and upon stimulation by pathogenic microbial compounds such as LPS DCs are activated to mature into efficient APCs. Mature DCs migrate into lymph nodes to promote T cell-mediated immune responses (8). In vitro, DCs are generated by differentiation from monocytes e.g. to be used in immunotherapy (9, 10). The resulting cells are CD14 negative immature monocyte-derived DCs expressing class II MHC molecules, stimulatory ligands for T cell surface receptors such as B7.1 (CD80), B7.2 (CD86), and CD40, and endocytic receptors such as the mannose receptor (MR).

Activating signals such as pathogenic compounds derived from microorganisms induce maturation of DCs via different toll-like receptors (TLR, Ref. 11). Upon LPS stimulation, TLR4 associates with the serine/threonine kinase IL-1 receptor-associated kinase (IRAK)-1 that is subsequently autophosphorylated, ubiquitinated, and thus primed for degradation (12, 13). Phosphorylated IRAK-1 dissociates from the receptor complex to induce phosphorylation of MAP kinases such as p38 that mediate activation of NF-κB and subsequent DC maturation characterized by strongly up-regulated surface markers CD40, CD80, CD83, and CD86, cyclooxygenase (COX)-2, and secretion of inflammatory mediators such as IL-12 and TNF-α (8). LPS stimulation of DCs also results in activation of the PI3K/Akt pathway that is crucial for DC survival (14, 15).

Here we show that PUFAs affect differentiation of human monocytes-derived DCs and severely interfere with DC functions such as cytokine production and T cell stimulation by blocking their responsiveness to LPS even at low micromolar concentrations. This block is associated with diminished levels of relevant mRNAs. However, degradation of IκBα and NF-κB activation remained unaffected by PUFA treatment.
Cells and Pufa Treatment—Peripheral blood mononuclear cells were isolated from buffy coats obtained from healthy donors by density gradient centrifugation over Ficoll-Paque (Amersham Biosciences) and depleted from T cells by sheep erythrocyte rosetting. For DC differentiation, monocytes were incubated in vitro for 5–7 days in human albumin-containing X-Vivo 10 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 50 ng/ml (555 international units/ml) GM-CSF (Leucomax, Aescia, Triaskirchen, Austria) and 10 ng/ml IL-4 (Specific activity > 4 × 105 international units/mg, Strathmann Biotec, Hannover, Germany). Serum-free conditions were generally chosen to avoid significant amounts of variable serum fatty acids, and such DCs have been shown to possess functions that are dramatically similar to those differentiated in serum-supplemented media (16–19). For DC activation, 100 ng/ml LPS (Escherichia coli O111B4, Sigma) was added to the cultures for indicated durations. Experiments were additionally performed in presence of 1 and 10% FCS (Hyclone, Logan, UT) during the activation period, which did not influence the inhibitory effects of PUFAs (data not shown). Palmitic acid (PA, C16:0), oleic acid (OA, C18:1(n-9)), arachidonic acid (AA, C20:4(n-6)), and eicosapentaenoic acid (EA, C20:5(n-3), all Sigma) of highest available quality were added from 10 mM stocks in ethanol to prewarmed X-Vivo 10. The medium was mixed for 1 h to allow binding of fatty acids to albumin prior to addition to cells at indicated concentrations. Solubilization of added fatty acids was confirmed by GC-MS analysis of medium after high speed centrifugation revealing a recovery of >90% of added fatty acids. The fatty acid concentration of the medium without fatty acid supplementation was 15 ± 2 μM. Neither treatment affected harvested cell numbers and viability, as detected with trypan blue exclusion and propidium iodide staining.

Cytokine Production and Cell Surface Marker Expression—Secreted cytokines were measured in cell supernatants by sandwich ELISA using matched-pair antibodies and human recombinant standards (R&D Systems, Minneapolis, MN). Cell surface proteins were stained with FITC-labeled anti-CD40 (Immunotech, Marseille, France), FITC-conjugated anti-CD83, and PE-labeled anti-CD80, anti-CD86, and anti-DR (all BD Biosciences, San Jose, CA) and analyzed on a FACS Calibur flow cytometer (BD).

Stimulation of T Cells—For assessment of the stimulatory capacity of fatty acid-treated DCs, irradiated (3000 rad, 137Cs source) DCs as stimulator cells were added at increasing cell numbers to 1 × 10^6 allogeneic T cells in 96-well culture plates in RPMI 1640 medium (Invitrogen). The cultures were supplemented with 10% fetal calf serum (total volume, 200 μl/well). After 5 days, cells were pulsed with 1 μCi of [3H]thymidine (ICN Pharmaceuticals, Irvine, CA) for 18 h. Cells were harvested on Topcount glass fiber filters (Packard, Meriden, CT), and radioactivity was determined using a microplate scintillation counter (Packard). T cell proliferation was expressed as mean cpm of triplicate cultures. Supernatants from cocultures were obtained 48 h after culture initiation and analyzed for T cell cytokines (IL-2, IFN-γ) by sandwich ELISA (Abs from R&D Systems).

Analysis of Membrane Fatty Acid Composition—Membrane purification and gas chromatography-mass spectrometry (GC-MS) were performed essentially as described in Ref. 21. Briefly, cells were washed, swollen in hypotonic buffer (42 mM KCl, 5 mM MgCl2, 10 mM Heps, pH 7.4) in the presence of protease inhibitors, and mechanically broken. Membranes were pelleted from postnuclear supernatants by centrifugation at 50,000 × g for 30 min and resuspended in phosphate-buffered saline. Samples were freeze-dried overnight before addition of 2 ml of methanol (Merck/benzene (Riedel-de Haen, Seelze, Germany), 4:1 (v/v), including heptadecanoic acid as internal standard. 20 μl of acetyl chloride (Fluka, Buchs, Switzerland) were added and methanolation performed by incubation for 60 min at 100 °C under continuous stirring. Fatty acid methyl esters were separated by gas chromatography on a DB-23 (J & W Scientific, Folsom, CA) and electron-impact ionization mass spectrometry. Fatty acid methyl esters of highest available quality (Alltech, Deerfield, IL) were used as standards.

Early Signal Transduction—For biochemical analyses, cells were harvested and resuspended at 2 × 10^6 cells/ml. After stimulation with 100 ng/ml LPS for 15–45 min reactions were stopped by addition of ice-cold washing buffer. Cells were pelleted by short centrifugation (12,000 × g for 20 s) and lysed on ice for 30 min in TBS, pH 7.4, containing 1% Nonidet P-40 (Pierce), phosphatase (1 mM sodium orthovanadate, 10 mM NaN3, 5 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 5 mM EDTA), and protease inhibitors. Nuclei were removed by short centrifugation. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences). The following Abs were used for Western blotting: anti-phospho-Akt (BioVision, Mountain View, CA), anti-phospho-p38 (Cell Signaling, Beverly, MA), anti-IkBα (Calbiochem), anti-IRAK-1, and anti-p28 (both Santa Cruz Biotechnology, Santa Cruz, CA). Antigens were detected by horseradish peroxidase-labeled secondary antibodies. Chemiluminescence was generated by BM chemiluminescence substrate (Roche Applied Science) and quantified on a Lumi-Imager (Roche Applied Science).

For intracellular analyses of protein phosphorylation, cells were fixed, permeabilized, and stained using a kit (Fix and Perm, Ancell,compared to the manufacturer’s instructions, followed by flow cytometric analysis. Anti-phospho-p38 and anti-phospho-p42/44 MAPK (Erk-1, -2) rabbit polyclonal Abs, and anti-phospho-JNK and anti-phospho-c-Jun mouse monoclonal Abs were obtained from Cell Signaling. Secondary Alexa Fluor® 488- and PE-labeled Abs were obtained from Molecular Probes (Eugene, OR).

NF-κB Activation—NF-κB nuclear translocation and DNA binding were visualized by biotinylated mobility shift assays (EMSA). DCs were activated with 100 ng/ml LPS for 70 min or left unstimulated in presence of 1% fetal calf serum. Nuclear extracts were prepared as described (22). Oligonucleotides resembling the consensuses binding site for NF-κB (5‘-AGTTTGGGGACTCTTCCGACC-3‘) and CRE (5‘-AGAGATTGCTGACGTCAGAGAGCTAG-3‘) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The double-stranded oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-32P]ATP. Nuclear extracts (2 μg of protein) were incubated with 120,000 cpm labeled probe in the presence of 3 μg of poly(dI-dC) at room temperature for 20 min. This mixture was separated on a 6% polyacrylamide gel in Tris/glycine/EDTA buffer at pH 8.5. Control experiments to verify the obtained shifted band to be specific for NF-κB, were performed as described (23, 24). For specific or nonspecific competition, 5 μM unlabeled NF-κB or double-stranded CRE oligonucleotides were used, respectively.

For assessing NF-κB transcriptional activity, cells were transiently transfected with a NF-κB firefly luciferase reporter construct (Stratagene, La Jolla, CA) plus a constitutive Renilla luciferase expression vector (Promega, Madison, WI) applying the Nucleofection® technology for hard-to-transfect cells using the appropriate kit (both Amnax BioSystems, Cologne, Germany, according to the manufacturer’s instructions. Subsequently, cells were stimulated for 6 h with 100 ng/ml LPS. Luminescence was detected applying dual luciferase reporter assay system (Promega) as described (25). To quantify NF-κB activation firefly luciferase light units were divided by Renilla luciferase light units and related to the ratio obtained from samples transfected with Renilla luciferase only. Luminescence from Renilla luciferase was similar in all investigated samples indicating comparable transfection efficiency (not shown).

Quantification of Specific mRNA—Total RNA of fatty acid-treated DCs, stimulated for 4 to 8 h with 100 ng/ml LPS, was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. One microgram of RNA was reverse-transcribed by Superscript II (Invitrogen) using random hexamer priming. Expression of TNF-α, IL-12, COX-2, and CD40 mRNAs was quantitated and normalized to 18 S RNA using Taqman Assays-on-Demand (Applied Biosystems, Foster City, CA) for quantitative real-time RT-PCR.

Analyses of Prostanoid Synthesis—DCs were differentiated, treated with fatty acids, and stimulated with LPS for 48 h as described above. For some cultures no IL-4 was added to obtain macrophage-like cells that are not suppressed in phospholipase A2 activity (26). In addition, 50 μM AA was included in indicated cultures for the last 40 h of LPS stimulation to bypass phospholipase A2 activity. Following stimulation, cell-free supernatants were harvested and analyzed by LC-MS/MS. After addition of deuterated prostaglandin (PGIE2 (Cayman Chemical, Ann Arbor, MI) as internal standard and BHT the samples were extracted in ethyl acetate and the organic phase was evaporated to dryness at room temperature after addition of Me3SO. Samples were separated by HPLC on a Luna phenylhexyl column (2 × 150 mm, Phenomenex, Torrance, CA) and detected in MRM-mode using negative electrospray ionization (Sciex API 4000). Calibration was linear in the range of interest (50 pg/ml to 20 ng/ml for PGE2, and 150 pg/ml to 20 ng/ml for PGE3). Quality control samples were prepared in medium with or without supplemented AA. Precision was better than 15%.
Statistics—Data are presented in means ± S.E. Comparisons were performed by two-tailed unpaired Student’s t test and a *p* < 0.05 was considered statistically significant.

RESULTS

PUFAs Affect DC Differentiation and Function—DC surface molecule expression and cytokine production are hallmarks of DC differentiation and activation. To elucidate PUFA effects on these events, isolated human monocytes were differentiated to DCs in the presence of 20 μM of a variety of fatty acids. DCs treated with saturated PA and monounsaturated OA did not differ in any investigated surface molecule expression from cells incubated in medium alone of six independent experiments. 

PUFAs Affect DC Activation and Function—PUFAs affect DC phenotype. Human DCs were differentiated in the presence of 20 μM of saturated PA, monounsaturated OA, and polyunsaturated AA and EA, or EtOH as solvent control. Surface marker expression after 5 days of differentiation was determined by flow cytometry. A, representative histograms for indicated markers. Dotted line, isotype control; open histogram, PA; filled histogram, EA. B, diagrams show mean fluorescence intensities related to that of cells incubated in medium alone of six independent experiments. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001 versus untreated cells.

Statistics—Data are presented in means ± S.E. Comparisons were performed by two-tailed unpaired Student’s t test and a *p* < 0.05 was considered statistically significant.

Following activation with LPS for 2 days, PUFA-treated DCs completely failed to up-regulate the investigated surface molecules CD40, CD80, CD83, and CD86 (Fig. 2, A and B). Expression of CD86 tended to be increased in PUFA-treated DCs, but this increase failed to reach statistical significance.

Thus, PUFA treatment leads to moderately altered molecule expression in unstimulated DCs, whereas expression of T cell costimulatory molecules was severely impaired in LPS-activated DCs.

In parallel with impaired surface molecule expression, TNF-α and IL-12p40 production of LPS-stimulated DCs treated with either PUFA (AA or EA) was drastically reduced compared with cells treated with PA or OA (Fig. 3A). PUFAs inhibited cytokine secretion in a concentration-dependent man-
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PUFA Treatment Leads to Altered Membrane Lipid Composition of Human Monocyte-derived DCs—Previous studies showed that PUFAs treatment alters T cell membrane lipid composition in vitro and in vivo (21, 27, 28) causing functional defects in T cell signaling (29, 30). Hence, we investigated whether also DCs incorporate exogenous PUFAs into cell membranes leading to altered membrane lipid composition. Membranes of DCs differentiated in presence of 20 μM saturated PA (C16:0), monounsaturated OA (C18:1(n-9)), and polyunsaturated fatty acids (AA, EA) and EtOH. A, T cell proliferation induced by fatty acid-treated DCs. Cytokine levels related to that from T cells stimulated with PA-treated LPS-activated DCs of six independent experiments are shown. ***, $p < 0.001$ versus PA.

The Inhibitory Effect of PUFAs on DCs Is Reflected at mRNA Level but Does Not Involve Impaired NF-κB Activation—To investigate possible mechanisms underlying the PUFAs-mediated block in DC activation we analyzed PUFAs effects on LPS-induced gene expression and crucial membrane-proximal signaling events. Quantitation of mRNA revealed that the reduced levels of secreted TNF-α and IL-12, as well as decreased CD40 surface expression, were related to diminished mRNA expression (Fig. 5). The reduction was particularly pronounced for IL-12p40 mRNA amount, which was inhibited by 90 and 98% after 4 h and 8 h of stimulation, respectively, in PUFA (AA, EA)-treated cells compared with PA-treated cells. The level of TNF-α mRNA was decreased by about 70% at both time points (Fig. 5). CD40 mRNA levels were least affected by
PUFAs with a reduction of about 50% detectable after 4 h of stimulation (Fig. 5). Also expression of mRNA for COX-2, the key enzyme of induced eicosanoid synthesis, was inhibited by more than 80% at both time points.

Surprisingly, early LPS-induced signaling events such as IRAK degradation and phosphorylation of Akt and p38 were not altered in DCs treated with polyunsaturated AA and EA (Fig. 6A). Degradation of 1x3, crucial for nuclear translocation of NF-kB, was also not affected by PUFAs (Fig. 6A). Also phosphorylation of MAP kinases ERK and JNK, as well as the JNK substrate c-Jun was not significantly affected by PUFA-treatment (Fig. 6B). Furthermore, we determined DNA binding of NF-kB to its consensus response element, a correlative of NF-kB activation, by EMSA. In DCs differentiated in the absence of AA or EA, LPS-stimulated DNA binding of NF-kB was comparable to that of PA-treated cells (Fig. 6C). To test the activity of NF-kB, we applied a luciferase reporter system. A high basal NF-kB activity was found in reporter-transfected DCs that was somewhat higher in PA-treated compared with EA-treated DCs (Fig. 6D). However, independently of fatty acid treatment, LPS stimulation increased NF-kB activity 2-fold independently of fatty acid treatment (Fig. 6D). Thus, PUFAs affect LPS-induced DC cytokine mRNA expression levels without interfering with well known early signaling events and NF-kB activation.

**PUPFA Treatment of DCs Post-differentiation Affects DC Function and Responsiveness to LPS Independently of Early LPS Signaling.—**To investigate whether PUFAs affect also fully differentiated DCs, or if the effect of PUFAs is restricted to the differentiation period, DCs were differentiated without addition of fatty acids to the culture for 5–7 days. Subsequently, 50 μM of fatty acids were added for 24 h before cells were stimulated with LPS for another 48 h. Unlike DCs differentiated in presence of PUFAs, short-term PUFAs-treated DCs were markedly inhibited in LPS-induced expression of CD40, while basal CD86 expression was increased (Fig. 7A). Production of TNF-α and IL-12p40 was significantly reduced in AA- and EA-treated cells compared with PA (Fig. 7B). Also stimulation of allogeneic T cells was reduced when DCs were treated with either PUFAs (Fig. 7C). Furthermore, PUFAs treatment post differentiation reduced LPS-induced TNF-α, IL-12p40, CD40, and COX-2 mRNA levels (Fig. 8A), while leaving early LPS signaling unaffected (Fig. 8B). These data demonstrate that PUFAs treatment of differentiated DCs also potently affects their activation by LPS.

**PUPFA Treatment Increases DC PG Production, but Inhibitory PUPFA Effects Are Independent of COX and LOX Activity—**Prostanoids are synthesized from C20 PUFAs and modulate inflammatory responses. Therefore, we analyzed concentration of pro-inflammatory, n-6-derived, PGE2, and n-3-derived PGE3, from supernatants of DCs differentiated and LPS-activated in presence of various fatty acids. Interestingly, both PUFAs increased PGE2 concentrations compared with PA-treated DCs (Fig. 9A) even though changes in the AA group failed to reach statistical significance due to high interindividual variability. PGE2 production in AA-treated cells was not increased by LPS (Fig. 9A). The presence of IL-4 during DC differentiation was reported to block phospholipase A2 activity (26). When bypassing phospholipase A2 by addition of exogenous AA after LPS stimulation, PGE2 production was increased by more than 10-fold in DCs treated with PA but only 2-fold in PUFAs-treated DCs (Fig. 9A), which is well in line with reduced COX-2 expression of these cells (Fig. 5). In the absence of IL-4 during the differentiation period the resulting macrophage-like cells produced markedly higher amounts of PGE2. PGE2 was detectable only following EA treatment and in similar concentrations as PGE2 (Fig. 9A). While, however, IL-12p40 production was decreased by COX inhibitor indomethacin, presence of COX and LOX inhibitors during differentiation and activation did not influence the inhibitory effects of PUFAs on CD80 and IL-12p40 expression (Fig. 9B). In summary, PUFAs treatment enhanced production of prostaglandins, but PGE2 production of monocyte-derived DCs was low compared with macrophage-like cells unless exogenous AA was provided, and blockade of crucial enzymes for eicosanoid synthesis did not attenuate PUFAs action on DC.

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**Table I**

Modification of DC membrane fatty acid composition by PUFAs

| Fatty acid | EtOH | PA | OA | AA | EA |
|-----------|------|----|----|----|----|
| C14:0     | 1.6±0.5<sup>a</sup> | 1.0±0.3 | 1.2±0.1 | 1.2±0.1 | 1.3±0.1 |
| C16:0     | 37.3±1.9 | 38.8±0.6 | 30.0±4.8<sup>c</sup> | 36.2±5.8<sup>c</sup> | 37.4±3.8<sup>c</sup> |
| C16:1(n-7) | 6.2±0.4 | 8.3±0.8 | 2.9±1.0<sup>c</sup> | 0.5±0.2<sup>c</sup> | 0.8±0.1<sup>c</sup> |
| C18:0     | 15.8±0.8 | 13.7±1.1 | 12.9±1.2<sup>c</sup> | 19.3±1.7<sup>c</sup> | 123±5.3<sup>c</sup> |
| C18:1(n-9) | 19.7±0.6<sup>c</sup> | 11.4±1.4 | 41.7±5.8<sup>c</sup> | 8.8±0.4<sup>c</sup> | 9.2±0.9<sup>c</sup> |
| C18:1(n-7) | 8.9±1.2<sup>c</sup> | 18.5±2.7 | 3.7±1.2<sup>c</sup> | 1.2±0.2<sup>c</sup> | 3.6±2.4<sup>c</sup> |
| C18:2(n-6) | 2.0±0.0 | 1.2±0.2 | 0.9±0.1 | 2.3±0.2<sup>c</sup> | 2.8±0.5<sup>c</sup> |
| C20:3(n-6) | 0.8±0.4 | 0.2±0.2 | 0.1±0.1 | 3.5±0.8<sup>c</sup> | 0.7±0.4<sup>c</sup> |
| C20:4(n-6) | 4.0±1.6 | 4.0±0.4 | 1.8±0.8<sup>c</sup> | 16.5±3.3<sup>c</sup> | 3.7±0.2<sup>c</sup> |
| C20:5(n-3) | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| C22:1(n-9) | 0.1±0.1 | 0.1±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| C22:4(n-6) | 1.4±0.4 | 1.0±0.4 | 1.5±0.2 | 8.6±1.6<sup>d</sup> | 0.6±0.1 |
| C22:5(n-3) | 0.2±0.2 | 0.0±0.0 | 0.2±0.2 | 0.1±0.1 | 7.6±2.1<sup>d</sup> |
| C24:0     | 0.8±0.2 | 0.8±0.2 | 0.3±0.1 | 0.5±0.2<sup>c</sup> | 5.7±2.7<sup>c</sup> |
| C24:1(n-9) | 0.7±0.1 | 0.6±0.1 | 2.0±0.4<sup>c</sup> | 0.5±0.1<sup>c</sup> | 0.5±0.0<sup>c</sup> |
| Saturated | 55.7±1.3 | 54.4±0.8 | 44.5±3.8 | 57.3±4.7 | 66.6±3.3<sup>c</sup> |
| Monounsaturated | 35.7±1.8 | 38.9±0.8 | 50.6±3.8<sup>c</sup> | 11.3±0.5<sup>c</sup> | 14.1±2.2<sup>c</sup> |
| Polyunsaturated | 8.7±1.3 | 6.8±0.3 | 5.0±0.3 | 31.5±4.9<sup>c</sup> | 19.4±2.9<sup>c</sup> |

**a** Fatty acids are expressed in mol%; means and S.E. of three independent experiments are given; fatty acid species occurring less than 1 mol% in all groups were omitted.

**b** Asterisks indicate significant differences to PA, which served as control in all experiment.

**c** p < 0.05.

**d** p < 0.001.

**e** p < 0.01.

**f** Average number of double bonds per fatty acyl residue.

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DISCUSSION

DCs are the most powerful APCs for stimulating T cell responses and serve as an essential link between innate and adaptive immunity (31). Therefore, interference with DC differentiation, maturation, and function is of interest for a broad field of immunology including prevention and treatment of allograft rejection and autoimmune diseases (9, 18). We show here that PUFAs moderately inhibit differentiation of DCs and severely block their responsiveness to LPS. Monocyte-derived DCs are a well accepted model for myeloid DCs, although the detailed mechanisms and requirements for in vivo differentiation of DCs are unclear (32). Therefore, we were interested in effects of PUFAs on DC differentiation as well as on activation of differentiated DCs, thus covering the full DC life cycle. As demonstrated in this study, DCs are affected in a similar manner when treated with PUFAs during or post-differentiation differing only quantitatively. Of note, PUFAs concentrations for effective inhibition of DC cytokine production were very low with EC50 of about 2 μM (Fig. 3B) and the extent of inhibition, which exceeded 90%, was much more pronounced compared with PUFAs effects on other cell types of the immune system, e.g. T cell activation (EC50 5–25 μM; maximal inhibitory effect ~50%; Refs. 25, 28, 33). Together, these in vitro findings indicate that the immunomodulatory activity of PUFAs in vivo may involve inhibition of DC function.

PUFA treatment of DCs effectively inhibited production of cytokines such as TNF-α and IL-12p40, the tightly regulated
differentiated DCs inhibits T cell activation. The diagram shows significant differences stimulated PA-treated cells of six independent experiments. Statistical diagrams show mean fluorescence intensities related to that of un-

PUFAs. Surface marker expression was determined by flow cytometry. Diagrams show mean fluorescence intensities related to that of unstimulated PA-treated cells of six independent experiments. Statistical significant differences versus activated or unstimulated PA-treated samples, respectively. **, p < 0.01 versus saturated fatty acid (PA). A, PUFA-treatment of differentiated DCs inhibits cytokine production. The diagram shows cytokine concentrations in supernatants of four independent experiments. *, p < 0.05; **, p < 0.01 versus saturated fatty acid (PA). B, PUFA treatment of differentiated DCs inhibits T cell activation. The diagram shows [3H]thymidine uptake of T cells stimulated with LPS-activated DCs treated with indicated fatty acids expressed in detected cpm of four independent experiments.

FIG. 7. PUFA treatment post-differentiation affects DC activation. DCs were differentiated for 5–7 days without addition of fatty acid subunit of functional IL-12p70 (34). In vivo studies investigating effects of n-3 PUFAs on peripheral blood mononuclear cells showed no reduction of TNF-α and other cytokine production by oral PUFAs. In human DCs, as shown here, a n-3 PUFAs enriched lipid infusion (36). However, IL-12 is exclusively produced by professional APCs and is essential for differentiation of Th1 cells and promotes activation of NK cells (37). The lack in IL-12 production of DCs shown here correlates to PUFA feeding studies showing a selective inhibition of Th1 responses (4, 38). IL-12 plays a critical role in rheumatoid arthritis and inflammatory bowel disease (39) and IL-12 neutralizing agents are considered for clinical use in treatment of inflammatory disorders (40). Thus, an exquisite sensitivity of DCs to PUFA-mediated inhibition could be particularly important for the beneficial effects of PUFAs in these diseases (41, 42). Moreover, elucidations of mechanisms that block DC IL-12 production as mediated by PUFAs are of general interest for the development of novel anti-inflammatory drugs.

Interestingly, unaltered NF-κB activation in PUFA treated human DCs as shown here is in contrast to results obtained from murine monocyte/macrophage-like cell line models. In these studies, addition of lauric acid alone was shown to activate TLR2 and 4 pathways, while polyunsaturated as well as monounsaturated fatty acids inhibited NF-κB activating pathways induced by agonists to different TLRs (43–46). Unaltered NF-κB activation after PUFA treatment as well as the lack of impact of a monounsaturated fatty acid on any investigated event could thus be specific for DCs or non-immortalized cells. Furthermore, cells were treated with fatty acids directly before activation in these studies (43–46), in contrast to our study that investigated PUFAs treatment for at least 1 day. The difference in the duration of fatty acid treatment could add to the differences in the observed effects.

NF-κB is supposed to play a predominant role in DC activation including IL-12p40 production (47–49). However, despite unaltered NF-κB nuclear transmigration and DNA binding, as well as transactivation activity, PUFAs-treated DCs did not adequately respond to LPS by production of relevant mRNAs. Similarly, it was shown recently that IL-10 primed macrophages are severely blocked in IL-12p40 gene transcription despite unaltered DNA binding of NF-κB (50). Of note in this regard, IL-10 was shown to suppress DC activation (51, 52), but
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IL-10 concentration was not increased in supernatants of PUFAtreated DCs (not shown). Though PUFAs apparently do not act via IL-10, they could utilize similar molecular mechanisms to block gene transcription. The nature of these mechanisms remain to be elucidated. PUFAtreatment could hence be a useful tool to discover novel molecular mechanisms that provoke immunomodulation by potent DC inhibition.

Inhibitory effects of PUFAs could be due to altered gene expression as a consequence, e.g., of activation of nuclear receptors or altering levels of fatty-acid derived inflammatory mediators (53). Of known nuclear receptors that can mediate anti-inflammatory responses, DCs predominantly express peroxisome proliferator-activated receptor (PPARy) (54). The presence of PPARy antagonists (bisphenol A diglycidyl ether (55), PD068235 (56)) did not antagonize PUFAtion on DCs (data not shown), making PUFAtion via PPARy rather unlikely to be critical for the described effects.

PUFAs, particularly AA, are substrates of COX and LOX for synthesis of eicosanoids (prostaglandins, leukotrienes) that influence DC function (19, 57, 58). In general, EA interferes with synthesis and effects of AA-derived eicosanoids that are probably biologically more active (59). PGE2 that is derived from AA is generally regarded as pro-inflammatory, but down-regulates IL-12 production of DC (52, 58). Hence, a PGE2-dependent autocrine negative regulation of DCs may exist (60). However, a significant impairment of rat DC antigen presentation could only be detected in cells isolated from animals fed a diet enriched with fish oil (n-3 PUFAs) but not from those fed a linoleic acid-enriched (n-6 PUFAs) diet (61). n-3 as well as n-6 PUFAs somewhat increased production of PGE2 and PGE3 in case of EA in our analyses despite down-regulation of COX-2 gene expression in LPS-stimulated DCs. Together with the lack of increased PGE2 production by LPS these data suggest that PG production in DCs is mainly driven via COX-1 rather than COX-2. Of note, the amounts of PGs produced by monocyte-derived DCs were generally low presumably because DC lack active phospholipase A2 (26).

Moreover, though the detected concentrations of PGE2 were within a range which could affect DCs (52, 58) our data of unaltered PUFAtion effects in presence of COX an LOX inhibitors argue against a PUFAtduced autocrine eicosanoid-dependent inhibitory mechanism thereby confirming results obtained with murine bone marrow-derived DCs (62). However, a large variety of eicosanoids with the potential to influence DC function remains that has to be analyzed in detail before a possible implication of eicosanoids in mediating PUFAtion effects on DCs can definitely be ruled out.

Lipid raft modification has been shown to underlie inhibitory effects of PUFAs on T cell signaling (21, 30). PUFAs were incorporated into DC membrane lipids in our studies and probably also in raft lipids since raft lipid alterations by exogenous fatty acids generally reflect those in cellular membranes (21). We did not detect any functional differences in early LPS-induced signaling events in PUFAtreated DCs though rafts could be involved in DC activation and LPS signal transduction as suggested by other studies (63, 64). However, lipid raft-dependent LPS signaling in DCs has not yet been characterized in enough detail in order to completely understand a possible interference of PUFAs with DC lipid raft signaling and its contribution to the inhibitory effects of PUFAs on DC activation.

In conclusion, PUFAs of the n-3 and n-6 series affect human DC differentiation, and inhibit their function by blocking their response to LPS. This block in DC activation appears to be independent of altered eicosanoid synthesis, well-known steps of early LPS signaling and critical downstream events like NF-xB activation. The central position of DCs to link innate and adaptive immune responses and the high efficiency of PUFAtion inhibition of DC function indicate that this cell type could be critically implicated in the anti-inflammatory action of PUFAs in vivo.

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