Basic Res Cardiol (2013) 108:319
DOI 10.1007/s00395-012-0319-8

ORIGINAL CONTRIBUTION

Cytochrome P4502S1: a novel monocyte/macrophage fatty acid epoxygenase in human atherosclerotic plaques

Timo Frömel · Karin Kohlstedt · Rüdiger Popp · Xiaoke Yin · Khader Awwad · Eduardo Barbosa-Sicard · Anita C. Thomas · Ralf Lieberz · Manuel Mayr · Ingrid Fleming

Received: 30 April 2012 / Revised: 2 November 2012 / Accepted: 26 November 2012

Abstract Cytochrome P450 (CYP) epoxygenases metabolize endogenous polyunsaturated fatty acids to their corresponding epoxides, generating bioactive lipid mediators. The latter play an important role in vascular homeostasis, angiogenesis, and inflammation. As little is known about the functional importance of extra-vascular sources of lipid epoxides, we focused on determining whether lipid epoxide-generating CYP isoforms are expressed in human monocytes/macrophages. Epoxides were generated by freshly isolated human monocytes and production increased markedly during differentiation to macrophages. Mass spectrometric analysis identified CYP2S1 as a novel macrophage CYP and CYP2S1-containing microsomes generated epoxides of arachidonic, linoleic and eicosapentaenoic acid. Macrophage CYP2S1 expression was increased by LPS and IFN-γ (classically activated), and oxidized LDL but not IL-4 and IL-13 (alternatively activated), and was colocalised with CD68 in inflamed human tonsils but not in breast cancer metastases. Prostaglandin (PG) E_2 is an immune modulator factor that promotes phagocytosis and CYP2S1 can metabolize its immediate precursors PGG_2 and PGH_2 to 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid (12-HHT). We found that CYP inhibition and siRNA-mediated downregulation of CYP2S1 increased macrophage phagocytosis and that the latter effect correlated with decreased 12-HHT formation. Although no Cyp2s1 protein was detected in aortae from wild-type mice it was expressed in aortae and macrophage foam cells from $ApoE^{-/-}$ mice. Consistent with these observations CYP2S1 was colocalised with the monocyte marker CD68 in human atherosclerotic lesions. Thus, CYP2S1 generates 12-HHT and is a novel regulator of macrophage function that is expressed in classical inflammatory macrophages, and can be found in murine and human atherosclerotic plaques.

Keywords Epoxygenase · Linoleic acid · Macrophage polarization · Omega 3 fatty acids · Phagocytosis

Electronic supplementary material The online version of this article (doi:10.1007/s00395-012-0319-8) contains supplementary material, which is available to authorized users.

T. Frömel · K. Kohlstedt · R. Popp · K. Awwad · E. Barbosa-Sicard · I. Fleming (✉)
Institute for Vascular Signalling, Centre for Molecular Medicine, Johann Wolfgang Goethe University and DZHK (German Centre for Cardiovascular Research) partner site Rhine-Main, Theodor Stern Kai 7, 60596 Frankfurt am Main, Germany
e-mail: fleming@em.uni-frankfurt.de

X. Yin · M. Mayr
King's College London, King's British Heart Foundation Centre, London, UK

A. C. Thomas
Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol BS2 8HW, UK

R. Lieberz
Institute of Pathology, Johann Wolfgang Goethe University, Frankfurt am Main, Germany

Introduction

In addition to being important xenobiotic-metabolizing enzymes, extra hepatic cytochrome P450 (CYP) epoxygenases can utilize endogenous substrates such as arachidonic acid, linoleic acid, eicosapentaenoic acid and docosahexenoic acid to generate bioactive lipid mediators. The epoxides thus generated can acutely affect vascular tone and stimulate a spectrum of signaling pathways that affect growth-promoting kinase and transcription factor activity in different cell types [16]. More recently, it has

Published online: 07 December 2012

become clear that CYP-derived fatty acid epoxides, such as the arachidonic acid epoxides or epoxyeicosatrienoic acids (EETs), can attenuate inflammation by interfering with the activation of nuclear factor κB (NFκB) [33]. The latter effects are not restricted to endothelial cells as inhibitors of the soluble epoxide hydrolase, which can be used to prevent EET metabolism and thus increase tissue and circulating EET levels, can attenuate inflammatory cell infiltration into the different tissues [12, 24, 36, 43, 47].

Despite the fact monocytes are an integral part of the inflammatory response and the cyclooxygenase and lipoxygenase pathways have been extensively studied in these cells, little is known about the ability of monocyte-derived macrophages to generate fatty acid epoxides. To date, CYP2J2 and CYP2C8 are the only EET-generating CYP enzymes reported to be expressed in monocytes/macrophages [5, 28], but whether these are the most relevant enzymes is unclear, as are the biological consequences of CYP activation. Certainly, the characteristics of the two enzymes are such that different biological reactions would be expected following activation. For example, while CYP2J2 activation clearly decreases NFκB activity in cardiac myocytes [12], other CYP epoxygenases, such as CYP2C8 can simultaneously generate physiologically relevant levels of superoxide anions which tend to activate NF-κB and thus functionally antagonize the inhibitory effects of EETs [15]. The aim of this study was therefore to better characterize CYP epoxygenase expression in human monocyte-derived macrophages and the consequences of CYP expression on the macrophage phenotype.

Materials and methods

For detailed methods please refer to the online supplement.

Cell culture

Human monocytes were isolated from blood obtained from healthy donors (DRK-Blutspendedienst Baden-Württemberg-Hessen, Germany) and differentiated to macrophages in the presence of human serum as described [22]. In some experiments, cells were stimulated with human LDL and oxidized LDL (ox-LDL), isolated and prepared as described [29]. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki and the isolation of human cells was approved by the ethics committee at the Goethe University. For proteomic studies, cells were further purified via immunomagnetic positive selection using an Easysep CD14+ cell separation kit (Stem Cell Technologies, Grenoble, France) according to the manufacturer's instructions.

Proteomics

Samples were denatured with 2× SDS sample buffer (Invitrogen) at 97 °C for 5 min. Proteins were separated on 5–20 % Tris–glycine polyacrylamide gels. The gel was silver stained and gel bands within a mass range of 37–75 kD were excised, digested with trypsin using a robotic digestor (ProGest Genomic Solutions). Peptides were separated by HPLC on a reverse-phase column (C18 PepMap 100, Dionex) and identified by a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Spectra were acquired with full scan (m/z 450–2,000) followed by six dependent MS2 scans using dynamic exclusion. The results were searched against human protein database (UniProt Release 12.5, 17658 entries) using SEQUEST v2.7 using the following parameters: fragment tolerance = 1.0 Da, parent tolerance = 1.5 Da, carbamidomethylation on cysteine as fixed modification, oxidation on methionine as variable modification, and 2 mixed cleavage was allowed. The search results were loaded into Scaffold software (Proteome Software). Proteins were filtered according to the following criteria: peptide probability >95 %, protein probability >99.9 % with at least 2 peptides [30].

Immunohistochemistry

Monocytes were grown on IBIDI chambers (Martinsried, Germany) coated with fibronectin, fixed in paraformaldehyde (PFA, 4 % in PBS, 15 min), permeabilized, blocked and stained with polyclonal goat anti-human CYP2S1 antibody (Santa Cruz) and rabbit anti-CD68 antibody (Thermo Scientific, Fremont, CA, USA) and the respective secondary antibodies (Alexa546 conjugated anti-goat, Alexa488 conjugated anti-rabbit, Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 20 min at 37 °C before mounting.

For tissue samples, informed consent was obtained in accordance with the Declaration of Helsinki and approval of the ethics committee of the University Hospital of Frankfurt. Paraffin embedded tissue sections were processed by the Institute of Pathology (Goethe University, Frankfurt) and fixed with 10 % buffered formalin. After the deparaffinization and rehydration in a series of xylol, ethanol (100, 90, 70, and 50 %) and distilled water, epitopes were retrieved using a sodium acetate solution (10 mmol/L, pH 6), in a steamer for 20 min before staining of CYP2S1 and CD68.

Phagocytosis assay

Human macrophages were incubated with pHrodo™ *E. coli* BioParticle® conjugates (Invitrogen) in RPMI

medium (37 °C, 5 % CO_2) in an Axiovert time lapse microscope (Zeiss, Jena, Germany). The uptake of bioparticles was monitored by recording the pH-dependent bioparticle emission spectra.

Murine brachiocephalic arteries, macrophage foam cells and non-foam cells

The housing and care of the animals and all the procedures used in these studies were performed in accordance with the guidelines and regulations of the University of Bristol and the United Kingdom Home Office.

Foam cell macrophages (FCM) were isolated from sponges implanted into ApoE$^{-/-}$ mice given a high fat diet, while non-foamy macrophages were obtained from sponges from C57Bl6 control mice fed a normal diet. The macrophage preparations were isolated from sponges 4 weeks after surgery. Cells were purified on the basis of their buoyant density and/or differential adherence as described [44]. RNA from purified foam cells (from the ApoE$^{-/-}$ mice) was compared with non-foam cell (NFC) RNA using quantitative PCR and the results normalized to 18S. Partially purified samples were cytospun or smeared onto slides for immunocytochemistry.

An additional group of ApoE$^{-/-}$ mice commenced a high-fat diet at 5 weeks of age and were killed 12 weeks later and perfusion fixed at normal pressure with PBS and formalin. Paraffin sections from their brachiocephalic arteries were prepared and used for immunohistochemistry.

RT-qPCR

The analysis of macrophage polarization was performed as described [22]. CYP2S1 mRNA was determined using primers for mouse Cyp2S1 (forward: 5′-CCAGCCGA CCTGCAGAGATGGAGGC-3′ and reverse: 5′-ACGCA GCTGCAGGAGGTTCCCCAG-3′; Sigma) or human CYP2S1 (QuantiTect Primer Assay; QIAGEN, Hilden, Germany) and normalized against 18S mRNA expression (ΔΔCT method).

Statistics

Data are expressed as mean ± SEM. Statistical evaluation was performed with Student's *t* test for unpaired data, one-way ANOVA followed by a Bonferroni *t* test or ANOVA for repeated measures where appropriate. Values of $P < 0.05$ were considered statistically significant.

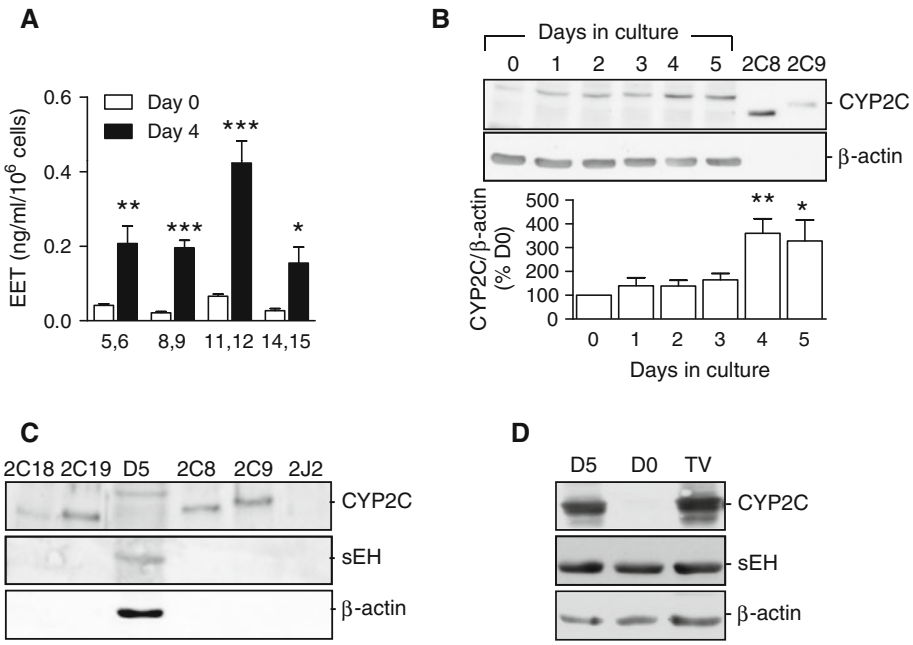

Fig. 1 EET production and CYP expression in peripheral blood-derived monocytes. **a** EET production in freshly isolated monocytes (Day 0) and in monocytes maintained in culture for 4 days (Day 4). **b** Expression of CYP2C in monocytes maintained in culture for up to 5 days in the presence of human serum. **c** Comparison of the CYP2C protein at day 5 (D5) with supersomes overexpressing CYP2C8, 2C9, 2C18, 2C19 or 2J2. **d** Comparison of the effects of 5 days treatment with human serum (D5) the combination of TGF-β (2 ng/nL) and vitamin D3 (TV; 50 nmol/L) on the expression of CYP2C and sEH. The *graph* represents data obtained in 5 independent experiments; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus D0

Results

Production of epoxyeicosatrienoic acids in human blood-derived monocytes

The generation of 5,6-, 8,9-, 11,12- and 14,15-EET was detectable in freshly isolated human monocytes but production increased markedly when cells were maintained in culture over 4 days (Fig. 1a). Western blot analysis using a non-specific CYP2C antibody revealed a time-dependent increase in the expression of a protein with a molecular mass distinct to that of CYP2C8 or 2C9 (Fig. 1b). A direct comparison with the human CYP2C epoxygenases revealed that the protein was distinct from CYP2S8, 2C9, 2C18 and 2C19 (Fig. 1c). Moreover, the antibody did not recognize CYP2J2, which was previously reported to be present at low levels in monocytes [28]. Induction of the same CYP2C-like protein was observed following cell incubation with TGF-β and vitamin D3 for 4 days while expression of the soluble epoxide hydrolase (sEH) was not affected (Fig. 1d).

Identification of CYP2S1 in human monocytes

Next we isolated human CD14+ monocytes (CD14 is a co-receptor for LPS that is expressed mainly by macrophages and markedly less so by neutrophil granulocytes) and as the arachidonic acid epoxygenases are normally endoplasmic CYP enzymes, we generated samples enriched in this fraction and analyzed the microsomal proteins after gel separation by mass spectrometry. Using this approach, we detected 8 unique peptides in human monocyte-derived macrophages which covered 15 % of the amino acids of CYP2S1 (EC 1.14.14.1; Fig. 2). The CYP2S1 gene has been recently identified from homology searches with the known sequences of CYP enzymes against the entire human genome sequence [18, 37, 38]. The human and mouse isoforms are 78 % identical at the amino acid level [37] and in both species 2S1 seems to be the only member of the new 2S family [39].

Using a combination of RT-qPCR (Fig. 3a), specific antibodies and microsomes from CYP2S1 overexpressing Sf-9 cells it was possible to confirm the expression of CYP2S1 in human monocytes and that protein levels increase during differentiation towards macrophages (Fig. 3b). We next tested the ability of a series of different siRNAs to target CYP2S1 in the monocytes studied and identified two siRNAs that effectively decreased CYP2S1 RNA and protein levels (Fig. 3c, d). Immunohistochemistry also revealed an increase in the perinuclear staining of CYP2S1 in monocyte-derived macrophages over 5 days

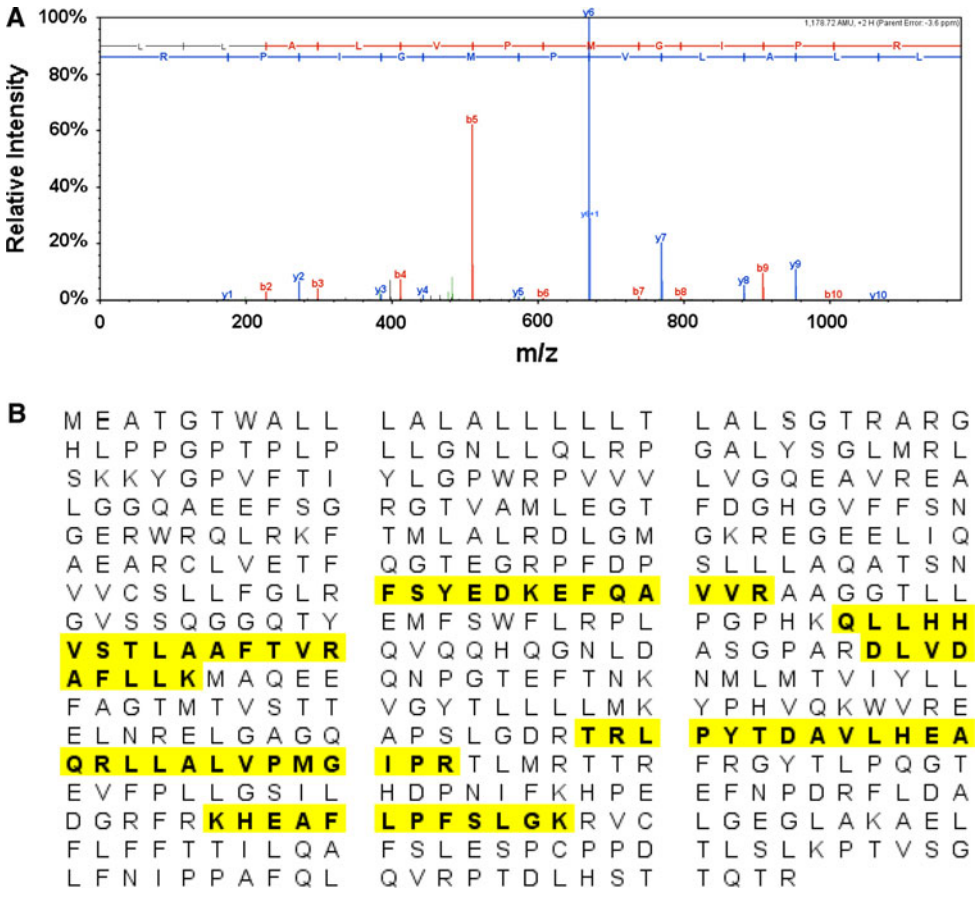

Fig. 2 Identification of cytochrome P450 2S1 (CYP2S1_HUMAN) in human monocytes. **a** Representative MS2 spectrum of peptide LLALVPMGIPR. **b** Amino acid sequence of CYP2S1. Identified peptides are highlighted (total sequence coverage 15 %)

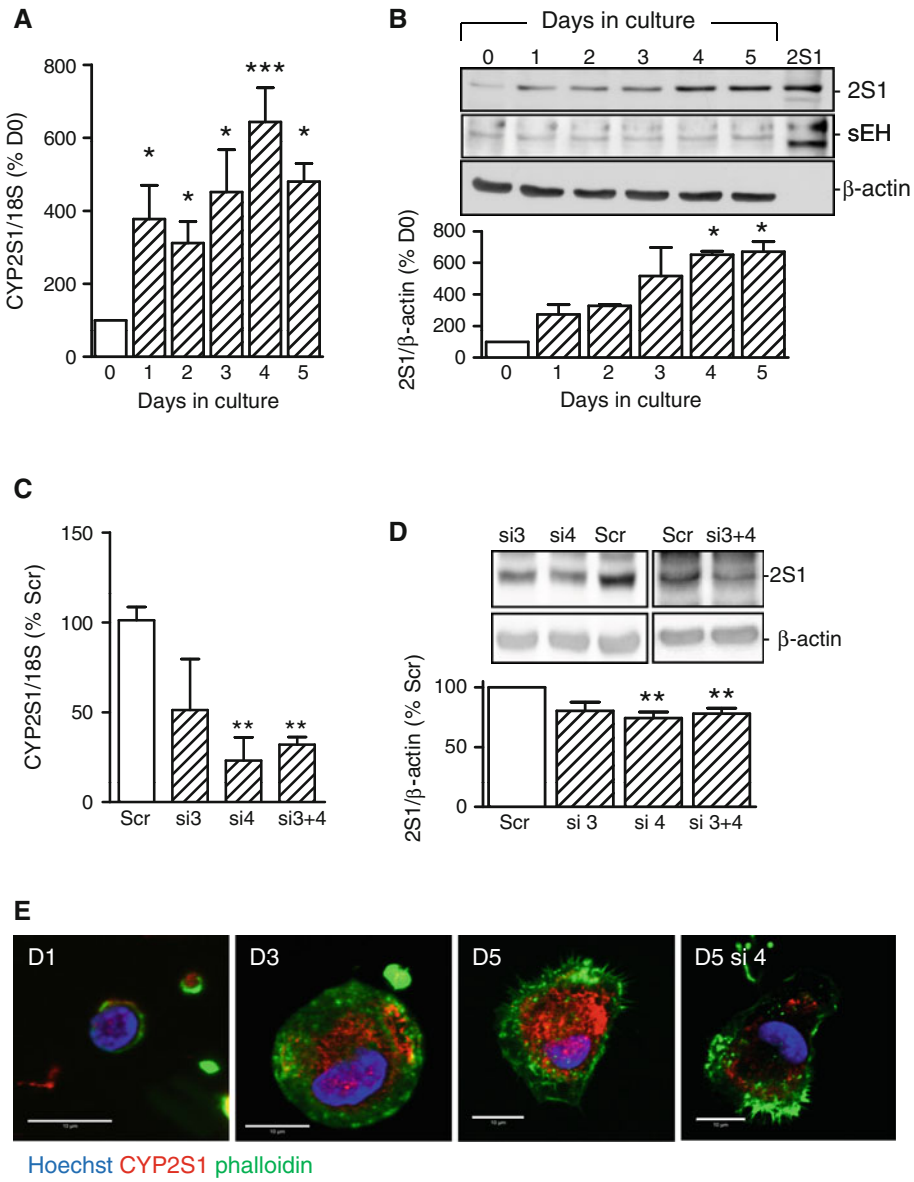

Fig. 3 Expression of CYP2S1 in human monocyte-derived macrophages. Effect of time in culture on the expression of **a** CYP2S1 mRNA and **b** CYP2S1 and sEH protein. **c**, **d** Effect of different CYP2S1 siRNA sequences (*si3*, *si4* alone and in combination) on **c** CYP2S1 mRNA and **d** protein expression in human macrophages. The *graphs* represent data obtained in 4–5 independent experiments; $*P < 0.05$, $***P < 0.001$ versus freshly isolated monocytes i.e., D0. **e** Intracellular localization of CYP2S1 during differentiation of human monocytes to macrophages day (D) 1 to 5. To ensure specificity of CYP2S1 staining, cells were treated with a specific siRNA (*si4*) for 48 h. The images represent data obtained in 3 independent experiments, *red* = CYP2S1, *green* = phalloidin, *blue* = nucleus, *bar* = 10 μm

culture and downregulation of signal in response to CYP2S1 siRNA (Fig. 3e).

CYP2S1 is a fatty acid epoxygenase

The major substrates for human CYP2S1 have not been established. Preliminary reports suggested that the recombinant CYP2S1 is involved in the metabolism of retinoic acids [26, 39, 42] and naphthalene [21], although the latter reports were poorly quantified and apparently controversial [45]. The ability of CYP2S1 to generate EETs from arachidonic acid was assessed after overexpression of the enzyme together with the cytochrome NAPDH reductase in Sf-9 cells. CYP2S1-containing microsomes prepared from the cells were able to generate EETs from arachidonic acid in the absence of additional NADPH with a slight (non significant) preference for the 11,12- and 14,15-EET regioisomeres (Fig. 4a). However, the production of all the regioisomers was increased following NADPH addition. Other polyunsaturated fatty acids e.g., linoleic acid (Fig. 4b) and eicosapentaenoic acid (Fig. 4c) were also accepted as substrates to generate 14,15- and 17,18-epoxyeicosatetraenoic acid (EpETE) as well as 9,10- and 12,13-epoxyoctadecenoic acid (EpOME). We were unable to detect any significant epoxygenation of docosahexaenoic acid (data not shown).

The latter results were not entirely expected as it was previously reported that CYP2S1-catalyzed epoxygenation proceeds by a free radical mechanism and that the enzyme is unable to accept electrons from NADPH via the P450 reductase [4]. To address this point in more detail, we determined EET production by CYP2S1 in the presence of

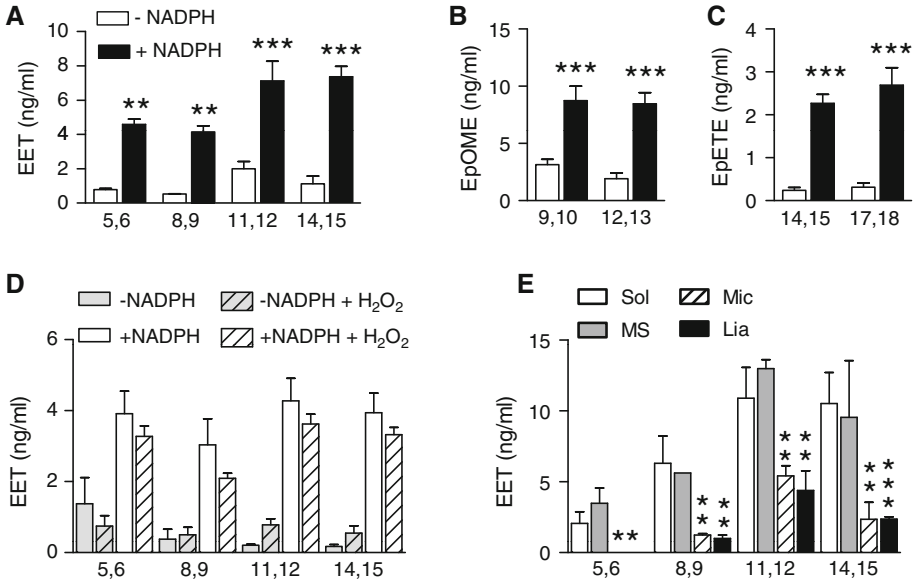

Fig. 4 Epoxide generation by CYP2S1. Epoxides of **a** arachidonic acid **b** linoleic acid and **c** eicosapentaenoic acid generated by CYP2S1-containing microsomes prepared from overexpressing Sf-9 cells in the absence and presence of NADPH (10 µmol/L). **d** Lack of effect of H_2O_2 (10 mmol/L) on the activity of CYP2S1. **e** Sensitivity of CYP2S1-overexpressing microsomes to solvent (*Sol*; 0.1 % DMSO), MS-PPOH (MS; 10 µmol/L), miconazole (*Mic*, 3 µmol/L) and liarozole (*Lia*, 10 µmol/L). The *graphs* represent data obtained in 3 independent experiments, each performed in duplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus NADPH or Sol

NADPH (10 µmol/L) or H_2O_2 (10 mmol/L), but found that the epoxygenation of arachidonic acid was unaffected by the addition of H_2O_2 (Fig. 4d). The epoxygenase activity of CYP2S1 could be inhibited in vitro using either miconazole or liarozole, an inhibitor of CYP26 and CYP2S1, but not by the presumably global epoxygenase inhibitor MS-PPOH (Fig. 4e).

CYP2S1 tissue expression

To determine whether macrophage polarization into the classical inflammatory (M1) or alternatively activated phenotype (M2) could affect CYP2S1 expression, monocyte-derived macrophages were incubated with LPS and IFN-γ (M1 polarized) or IL-4 and IL-13 (M2 polarized). While LPS and IFN-γ increased the expression of CYP2S1 mRNA, IL-4 and IL-13 had no effect on enzyme expression (Fig. 5a). In agreement with these findings, inflamed human tonsils which should contain predominantly M1 polarized macrophages, showed a high level of CYP2S1 and CD68 colocalization (Fig. 5b). To analyze the effect of CYP expression on macrophage function, differentiated macrophages were pretreated with compounds targeting the CYP/sEH axis. While the inhibition of CYP enzymes with the CYP inhibitor miconazole and liarozole led to an increased phagocytic uptake of bioparticles, inhibition of the sEH tended to decrease phagocytosis (Fig. 5c). The increase in phagocytosis induced by the CYP inhibitors could be reproduced by down regulating CYP2S1 expression using two different siRNAs (Fig. 5d). As our data indicated that alternatively activated macrophages, such as those observed in tumors, should express less CYP2S1 than classically activated macrophages we assessed CYP2S1 expression in breast cancer metastases in the lung. In line with previous observations, we detected CYP2S1 in lung epithelial cells [40] and found that macrophages adhering to the lung epithelium expressed high levels of CYP2S1 (Fig. 5e). However, the CD64 positive cells within the tumor (usually alternatively activated or M2–type) were negative for CYP2S1 (Fig. 5e). Tissue environment is a critical factor for macrophage polarization and the downregulation of CYP2S1 in the metastases is likely the results of a combination of factors, as hypoxia alone (1 % O_2, 24 h) had no effect on macrophage CYP2S1 levels (Supplementary Figure 1a).

The anti-inflammatory effects associated with CYP2S1 expression and activity may be due to the ability of the enzyme to generate an anti-inflammatory factor (such as EET's) or the metabolism/downregulation of an immune modulator factor. As one such factor is prostaglandin (PG) E_2 and CYP2S1 has recently been reported to metabolize its immediate precursors PGG_2 and PGH_2 to 12(S)-hydroxy-heptadeca-5Z, 8E, 10E-trienoic acid (12-HHT) [3, 23], we determined the consequences of CYP2S1 siRNA on monocyte 12-HHT. We found that down regulating macrophage CYP2S1 markedly decreased the generation of 12-HHT (Fig. 5f).

As activation of the aromatic hydrocarbon receptor (AhR) can increase dihydroxyeicosatrienoic acid

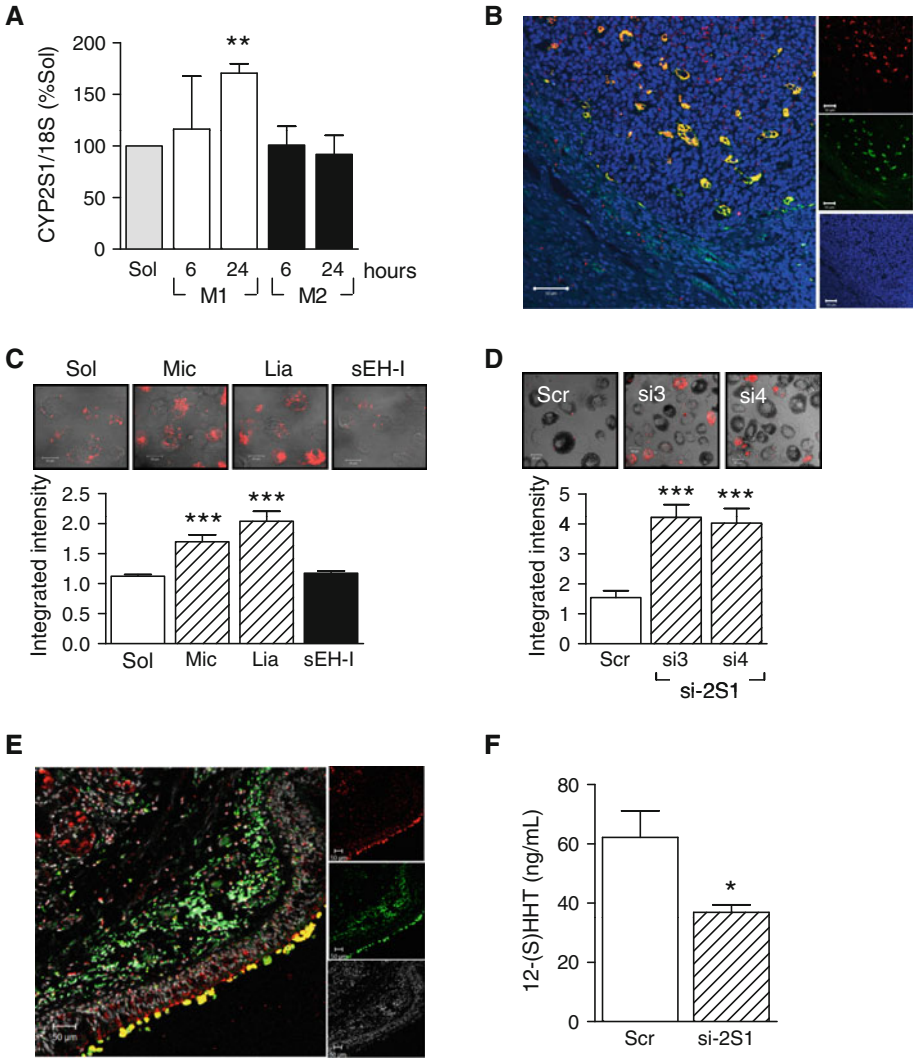

Fig. 5 CYP2S1 and macrophage polarization. **a** Effect of 6 and 24-h treatment with IFNγ (100 U/L) and LPS (1 μg/mL; M1) or IL-4 and IL-13 (each 10 μg/mL; M2) on CYP2S1 mRNA expression. **b** Immunofluorescence images showing colocalisation of CYP2S1 and CD68 in tonsillitis. **c** Phagocytosis of pHrodo bioparticles after 2-h pretreatment with solvent (0.1 % DMSO), miconazole (*Mic*, 3 μmol/L), liarozole (*Lia*, 10 μmol/L) or the sEH inhibitor *t*-AUCB (sEH-I, 3 μmol/L). (D) Phagocytosis of pHrodo bioparticles after treatment with either a scrambled siRNA (Scr) or 2 separate siRNAs directed against CYP2S1. **e** Immunofluorescence images demonstrating CYP2S1 localization in breast cancer metastases in the human lung. *Red* = CYP2S1, *Green* = CD68, *white* = DAPI; *size bar* = 50 μm. **f** 12-HHT levels in the supernatant (collected over 24 h) of macrophages pre-treated with a scrambled siRNA probe (*Scr*) or different CYP2S1 siRNAs (si-CYP2S1, si3 + 4). The *graphs* represent data obtained in 5–7 independent experiments; $*P < 0.05$, $***P < 0.001$ versus solvent (*Sol*) or scrambled siRNA (*Scr*)

production in wild-type but not AhR-deficient mice [3], and has been reported to increase CYP2S1 expression in mouse Hepa-1 cells [37], we determined whether the β-naphthoflavone regulated CYP2S1 expression. In monocyte-derived macrophages, stimulation with β-naphthoflavone only slightly increased CYP2S1 protein expression in human macrophages (Supplementary Figure 1b). Although the role of the AhR in physiology and toxin-independent pathophysiology is largely unclear, we found that resveratrol, a non-flavonoid polyphenolic phytoalexin that acts as an AhR antagonist [6, 9] and inhibits the JAK/STAT-1 pathway that controls inflammatory responses in IFN-γ-activated macrophages [8], resulted in a concentration-dependent decrease in CYP2S1 protein (Supplementary Figure 1c). The role of AhR in the regulation of CYP2S1 expression, however, requires further investigation as the AhR agonist L-kynurenine and the AhR antagonist StemRegenin-1 failed to alter macrophage CYP2S1 levels (data not shown).

CYP2S1 and atherosclerosis

Macrophages play a key role in the initiation and progression of atherosclerotic plaques [27]. As classically activated macrophages expressed more CYP2S1, we determined whether LDL and oxidized LDL modulated expression of the enzyme. In monocyte-derived macrophages ox-LDL significantly increased CYP2S1 levels in vitro (Fig. 6a). Analysis of RNA from macrophage foam cells recovered from ApoE$^{-/-}$ mice fed a high fat diet versus non-foam cells recovered from wild-type mice on a normal diet, revealed that the former expressed significantly more Cyp2s1 (the mouse ortholog of the human CYP2S1 is Cyp2s1, Fig. 6b). While no Cyp2s1 could be detected in aortae from non-atherosclerotic mice it was clearly expressed in aortae from ApoE$^{-/-}$ mice fed a high fat diet (Fig. 6c). Partially purified macrophage preparations isolated from sponges from fat-fed ApoE$^{-/-}$ mice

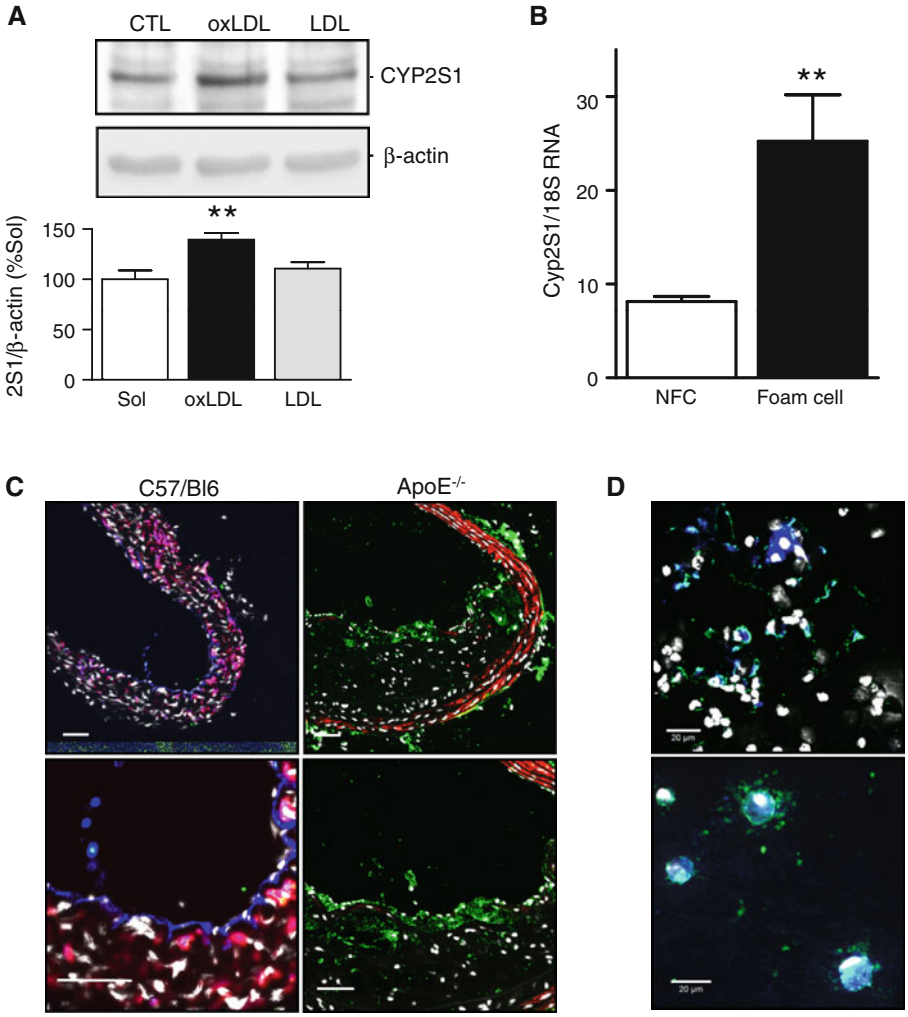

Fig. 6 CYP2S1 and atherosclerosis. **a** Effect of LDL and ox-LDL (both 50 μg/mL, 24 h) on CYP2S1 expression in differentiated human monocyte-derived macrophages. **b** CYP2S1 RNA expression in macrophage foam cells and non-foam cells (NFC) obtained from subcutaneous sponges. The *graphs* represent data obtained in 4–8 independent experiments; $*P < 0.05$, $**P < 0.01$, versus solvent (*Sol*) or NFC. **c** Immunofluorescent image showing lack of Cyp2s1 positive cells in an aorta from a control (C57/Bl6) mouse and Cyp2s1 positive cells in an atherosclerotic plaque from a fat-fed ApoE$^{-/-}$ mouse. *Green* = Cyp2s1, *red* = smooth muscle actin, *white* = DAPI, *bar* = 50 μm. **d** CYP2S1 in partially purified macrophage preparations isolated from sponges from fat-fed ApoE$^{-/-}$ mice. *Green* = Cyp2S1, *blue* = F4/80, *white* = DAPI, *bar* = 20 μm

also stained positively for Cyp2s1 and colocalised with F4/80 (Fig. 6d).

In human atherosclerotic plaques CYP2S1 was colocalised with the monocyte marker CD68 and could be detected in intimal xanthoma, fibrous cap atheroma as well as in fibrous cap atheroma with hemorrhage (Fig. 7).

Discussion

The results of the present study demonstrate that CYP2S1 is expressed in human monocyte-derived macrophages. Enzyme expression increases during the differentiation to macrophages and can be detected in classically activated or M1 macrophages and macrophages present in atherosclerotic plaques and inflamed tonsils. CYP2S1 was not present in macrophages polarized towards the M2 or alternatively activated phenotype and was not detected in tumor macrophages. The enzyme is able to metabolize arachidonic acid, linoleic acid and eicosapentaenoic acid as well as PGG_2/PGH_2 and its inhibition increased macrophage phagocytic activity.

Although quite a lot is known about the role played by fatty acid epoxides in vascular signaling and they are thought to prevent the development of cardiovascular disease in some animal models [14], surprisingly little is known about their role in monocytes and macrophages. Indeed, research in monocytes has tended to focus on CYP hydroxylases and cholesterol-metabolizing enzymes. The only macrophage arachidonic acid epoxygenases reported to date are CYP2J2 [5, 28] and CYP2C8 [5], but the protein levels reported either tended to be low or (in the case of CYP2C8) were not demonstrated, and expression in situ was not addressed.

In looking for a CYP epoxygenase in monocyte-derived macrophages, the first step was to assess epoxide production and whether this altered during differentiation. Indeed, while very low levels of EET were generated just after monocyte isolation all four regioisomers were detectable after 4 days in culture with human serum. The increase in

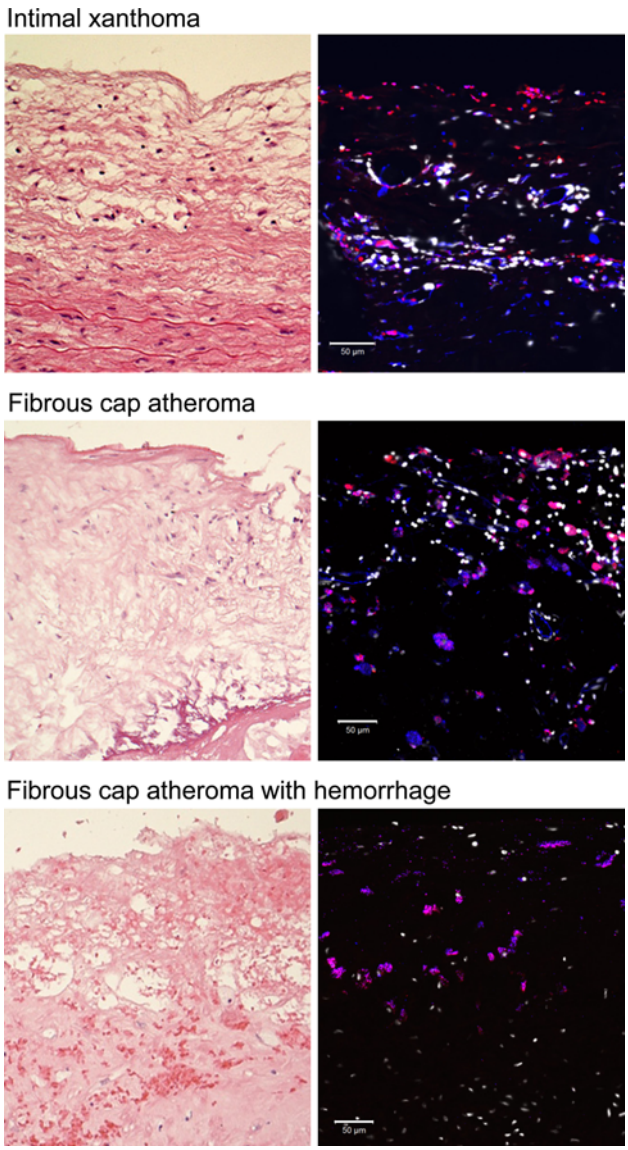

Fig. 7 CYP2S1 and human atherosclerotic plaques. Histological (*left*) and immunofluorescent (*right*) images demonstrating CYP2S1 localization in sequential sections from the same preparations of different stage human atherosclerotic plaques. *Red* = CYP2S1, *blue* = CD68, *white* = nuclei, *bar* = 50 μm

EET generation correlated with the appearance of a band recognized by a non-specific CYP2C antibody in Western blots, but this band did not display the same mass as any of the human 2C epoxygenases, i.e., CYP2C8, 2C9, 2C18 or 2C19. Our findings contrast with a recent report that CYP2C8 is expressed in human monocyte-derived macrophages [5]. However, the latter conclusion was based on mRNA and was not backed up by Western blotting. While we found a positive signal in monocyte-derived macrophages using a commercially available 2C8 antibody, this detected all four 2C enzymes. Careful comparison of the molecular mass of the antibody-labeled band clearly indicated that the macrophage enzyme could not be any of the four human 2C isoforms. Using a proteomic approach, it was possible to identify CYP2S1; an enzyme previously referred to as an orphan P450 with no clear physiological function [11], as a novel macrophage CYP epoxygenase.

CYP2S1-catalyzed epoxygenation was previously reported to proceed by a free radical mechanism and the enzyme was thought to be unable to accept electrons from NADPH via the P450 reductase [4]. This was apparently substantiated by the fact that CYP2S1 lacks amino acids known to be important for the interaction of other P450s with the P450 reductase [10, 31, 41]. However, our data indicate that CYP2S1 can oxidize several substrates in the presence of NADPH, a fact that has recently been confirmed by others [32, 46]. We also found that the generation of EETs by CYP2S1 was sensitive to miconazole or liarozole (an inhibitor of CYP26) but not by the reportedly global epoxygenase inhibitor, MS-PPOH.

Unlike the common CYP2 genes, which are predominantly expressed in the liver, hepatic levels of CYP2S1 are low but the enzyme is expressed abundantly in the skin, intestine, lung and spleen [11]. CYP2S1 expression is reported to be high in epithelial cells and relatively strong in lymphocytes [38] and its expression is elevated during the late stages (24–48 h) of adenovirus type 12 infection in HeLa cells [13]. All of these observations indicated that there may be a role for 2S1 in the immune response/inflammation. Certainly, our finding that CYP2S1 is present in macrophages fits well with this proposal. Interestingly, CYP2S1 positive macrophages were previously been detected (in situ hybridization) in the lung [40], even though the authors of the latter study apparently dismissed this result as an experimental artifact.

As the expression increased during monocyte differentiation we determined whether CYP2S1 was preferentially expressed when macrophages were activated with LPS and IFN-γ (classically activated or M1 polarized) or alternatively activated (M2 polarized) with IL-4 and IL-13. While M2 polarization had no effect on CYP2S1 mRNA levels, stimulation with LPS and IFN-γ did increase enzyme expression. As the human CYP2S1 mRNA transcript has a long 3′-untranslated region and is thus a potential target for microRNAs [20], it was important to confirm these observations and show altered CYP2S1 protein expression in different tissues. We used inflamed tonsils and breast cancer metastases in the human lung as examples of tissues that should contain M1 and M2 polarized macrophages, respectively, and found that CYP2S1 was detectable in CD68 positive cells in the tonsil and in the lung but not within the tumor samples. Thus, CYP2S1 is preferentially expressed in classically activated macrophages, but is there any link to macrophage function? To address this point, we focused on LPS and IFN-γ-stimulated macrophages and

their ability to phagocytose *E. coli* bioparticles and found that phagocytotic activity was increased in cells treated with the CYP inhibitors, miconazole or liarozole as well as following the siRNA-mediated downregulation of CYP2S1. This finding suggests that CYP2S1 activation attenuates macrophage activation. It will be interesting to determine how CYP2S1 expression is affected by signaling molecules such as the class A scavenger receptor, which has been suggested to suppress polarization toward a skewed M1 phenotype [19].

How is it that the expression of CYP2S1 can have an anti-inflammatory effect? One possibility is that the lipid epoxides generated attenuate NFκB activation and thus the expression of pro-inflammatory gene products [33]. The other possibility is that CYP2S1 actively detoxifies a pro-inflammatory substance that plays a key role in macrophage polarization. We focused on the latter possibility as CYP2S1 was recently reported to metabolize PGG_2 and PGH_2 to TxA_2, 12-HHT and malondialdehyde and thus decrease cellular production of the pro-inflammatory eicosanoid, PGE_2 [3, 23]. We were able to confirm these results in human monocyte-derived macrophages and demonstrate that the siRNA-mediated downregulation of CYP2S1 decreased the generation of 12-HHT. Since PGE_2 inhibits phagocytosis [7], a decrease in its production (associated with the upregulation of CYP2S1) would be expected to increase phagocytotic activity and is exactly what was observed in monocytes treated with CYP inhibitors or siRNA directed against CYP2S1. Whether the effect on phagocytosis can be completely attributed to altered PGE_2 or can be partly attributed to the generation of 12-HHT is currently unclear.

How is the expression of CYP2S1 regulated? Again relatively little is known about the mechanisms involved, but we found no effect of hypoxia on the expression of CYP2S1 in macrophages (or in endothelial cells; unpublished observation) and only weak effects following interference with the AhR pathway using β-naphthoflavone and resveratrol. Resveratrol is a potent activator of the anti-inflammatory transcription factor nuclear factor-erythroid 2–related factor 2 (NRF2) [34]. Given that CYP2S1 can be regulated by AhR which in turn can cross-talk with other transcription factors, including NRF2 [1] it is tempting to speculate that NRF2 underlies the effects observed. Additional regulatory mechanisms almost certainly exist as glucocorticoid receptor activation represses CYP2S1 expression in A549 cells via a mechanism that is sensitive to trichostatin A and may therefore require histone deacetylase activity [2]. We did, however, find that ox-LDL increased the expression of CYP2S1 in human monocyte-derived macrophages in vitro, and that Cyp2s1 was detectable in foam cells isolated from $ApoE^{-/-}$ mice as well as in atherosclerotic plaques from the same animals. The F4/80 staining in $ApoE^{-/-}$ samples was not successful and colocalization of Cyp2s1 with a macrophage marker will need to be performed in future studies. However, the finding that Cyp2s1 was expressed in murine atherosclerotic plaques could be confirmed in sections from human atherosclerotic plaques and CYP2S1 was found colocalised with CD68 in all the lesion stages assessed. Given that ox-LDL has been reported to increase levels of hypoxia-inducible factor 1α in primary human macrophages [35] it will be necessary to readdress the effects of ox-LDL alone and in combination with hypoxia on Cyp2s1 expression.

A difficult question to answer is whether the CD68-expressing cells really reflect "true" M1 (classically activated) macrophages. Certainly macrophage polarization has become increasingly complicated [25] and numerous different subgroups have been described—including an intermediate phenotype induced by ox-LDL [17]. However, we clearly observed that CYP2S1 was expressed in macrophages in inflamed and atherosclerotic tissue while it was absent from tumor-associated macrophages. Although the enzyme was able to accept several substrates and to generate bioactive epoxides from arachidonic acid, linoleic acid and eicosapentaenoic acid in an NADPH-dependent manner, perhaps from the macrophage polarization point of view the most relevant substrates seem to be PGG_2 and PGH_2. The resulting decrease in the immunomodulator PGE_2 would certainly be expected to result in a macrophage subtype with attenuated angiogenic potential, but whether the CYP2S1 product 12-HHT actively contributes to inflammation, remains to de determined. The latter can however, only be addressed once appropriate selective inhibitors have been developed.

Acknowledgments The authors are indebted to Marie von Reutern for expert technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft (SFB-TR 23/A6 and Exzellenz-cluster 147 "Cardio-Pulmonary Systems") and by the European Vascular Genomic Network, a Network of Excellence supported by the European Community's sixth Framework Program (Contract N° LSHM-CT-2003-503254). M.M. is a Senior Fellow of the British Heart Foundation.

Conflict of interest The authors have no disclosures to declare.

References

1. Anttila S, Raunio H, Hakkola J (2011) Cytochrome P450-mediated pulmonary metabolism of carcinogens: regulation and cross-talk in lung carcinogenesis. Am J Respir Cell Mol Biol 44:583–590. doi:10.1165/rcmb.2010-0189RT
2. Bebenek IG, Solaimani P, Bui P, Hankinson O (2012) CYP2S1 is negatively regulated by corticosteroids in human cell lines. Toxicol Lett 209:30–34. doi:org/10.1016/j.toxlet.2011.11.020
3. Bui P, Solaimani P, Wu X, Hankinson O (2012) 2,3,7,8-Tetrachlorodibenzo-p-dioxin treatment alters eicosanoid levels in

several organs of the mouse in an aryl hydrocarbon receptor-dependent fashion. Toxicol Appl Pharmacol 259:143–151. doi:10.1016/j.taap.2011.12.009
4. Bui PH, Hankinson O (2009) Functional characterization of human cytochrome P450 2S1 using a synthetic gene-expressed protein in *E. coli*. Mol Pharmacol 76:1044–1052. doi:10.1124/mol.109.057752
5. Bystrom J, Wray JA, Sugden MC, Holness MJ, Swales KE, Warner TD, Edin ML, Zeldin DC, Gilroy DW, Bishop-Bailey D (2011) Endogenous epoxygenases are modulators of monocyte/macrophage activity. PLoS ONE 6:e26591. doi:10.1371/journal.pone.0026591
6. Casper RF, Quesne M, Rogers IM, Shirota T, Jolivet A, Milgrom E, Savouret JF (1999) Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. Mol Pharmacol 56:784–790
7. Chuang PC, Lin YJ, Wu MH, Wing LY, Shoji Y, Tsai SJ (2010) Inhibition of CD36-dependent phagocytosis by prostaglandin E_2 contributes to the development of endometriosis. Am J Pathol 176:850–860. doi:10.2353/ajpath.2010.090551
8. Chung EY, Kim BH, Hong JT, Lee CK, Ahn B, Nam SY, Han SB, Kim Y (2011) Resveratrol down-regulates interferon-γ-inducible inflammatory genes in macrophages: molecular mechanism via decreased STAT-1 activation. J Nutr Biochem 22:902–909. doi:10.1016/j.jnutbio.2010.07.012
9. Ciolino HP, Daschner PJ, Yeh GC (1998) Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. Cancer Res 58:5707–5712
10. Crespi CL, Miller VP (1997) The R144C change in the CYP2C9*2 allele alters interaction of the cytochrome P450 with NADPH: cytochrome P450 oxidoreductase. Pharmacogenetics 7:203–210. doi:10.1097/00008571-199706000-00005
11. Deb S, Bandiera SM (2009) Characterization and expression of extrahepatic CYP2S1. Expert Opin Drug Metab Toxicol 5:367–380. doi:10.1517/17425250902865586
12. Deng Y, Edin ML, Theken KN, Schuck RN, Flake GP, Kannon MA, Degraff LM, Lih FB, Foley J, Bradbury JA, Graves JP, Tomer KB, Falck JR, Zeldin DC, Lee CR (2011) Endothelial CYP epoxygenase overexpression and soluble epoxide hydrolase disruption attenuate acute vascular inflammatory responses in mice. FASEB J 25:703–713. doi:10.1096/fj.10-171488
13. Dorn A, Zhao H, Granberg F, Hosel M, Webb D, Svensson C, Pettersson U, Doerfler W (2005) Identification of specific cellular genes up-regulated late in adenovirus type 12 infection. J Virol 79:2404–2412. doi:10.1128/JVI.79.4.2404-2412.2005
14. Fleming I (2011) Cytochrome P450-dependent eicosanoid production and cross-talk. Curr Opin Lipidol 22:403–409. doi:10.1097/MOL.0b013e32834a9790
15. Fleming I, Michaelis UR, Bredenkötter D, Fisslthaler B, Dehghani F, Brandes RP, Busse R (2001) Endothelium-derived hyperpolarizing factor synthase (cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. Circ Res 88:44–51. doi:10.1161/01.RES.88.1.44
16. Fleming I (2011) The cytochrome P450 pathway in angiogenesis and endothelial cell biology. Cancer Metastasis Rev 30:541–555. doi:10.1007/s10555-011-9302-3
17. Hirose K, Iwabuchi K, Shimada K, Kiyanagi T, Iwahara C, Nakayama H, Daida H (2011) Different responses to oxidized low-density lipoproteins in human polarized macrophages. Lipids Health Dis 10:1. doi:10.1186/1476-511X-10-1
18. Hoffman SM, Nelson DR, Keeney DS (2001) Organization, structure and evolution of the CYP2 gene cluster on human chromosome 19. Pharmacogenetics 11:687–698. doi:10.1097/00008571-200111000-00007
19. Hu Y, Zhang H, Lu Y, Bai H, Xu Y, Zhu X, Zhou R, Ben J, Xu Y, Chen Q (2011) Class A scavenger receptor attenuates myocardial infarction-induced cardiomyocyte necrosis through suppressing M1 macrophage subset polarization. Basic Res Cardiol 106:1311–1328. doi:10.1007/s00395-011-0204-x
20. Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C (2007) Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. Pharmacol Therapeut 116:496–526. doi:10.1016/j.pharmthera.2007.09.004
21. Karlgren M, Si Miura, Ingelman-Sundberg M (2005) Novel extrahepatic cytochrome P450s. Toxicol Appl Pharmacol 207:57–61. doi:10.1016/j.taap.2004.12.022
22. Kohlstedt K, Trouvain C, Namgaladze D, Fleming I (2011) Adipocyte-derived lipids increase angiotensin-converting enzyme (ACE) expression and modulate macrophage phenotype. Basic Res Cardiol 106:205–215. doi:10.1007/s00395-010-0137-9
23. Madanayake TW, Fidler TP, Fresquez TW, Bajaj N, Rowland AM (2012) Cytochrome P450 2S1 depletion enhances cell proliferation and migration in bronchial epithelial cells, in part, through modulation of prostaglandin E_2 synthesis. Drug Metab Dispos 40:2119–2125. doi:10.1124/dmd.112.046466
24. Manhiani M, Quigley JE, Knight SF, Tasoobshirazi S, Moore T, Brands MW, Hammock BD, Imig JD (2009) Soluble epoxide hydrolase gene deletion attenuates renal injury and inflammation with DOCA-salt hypertension. Am J Physiol Renal Physiol 297:F740–F748. doi:10.1152/ajprenal.00098.2009
25. Mantovani A, Garlanda C, Locati M (2009) Macrophage diversity and polarization in atherosclerosis. Arterioscler Thromb Vasc Biol 29:1419–1423. doi:10.1161/ATVBAHA.108.180497
26. Marill J, Idres N, Capron CC, Nguyen E, Chabot GG (2003) Retinoic acid metabolism and mechanism of action: a review. Curr Drug Metab 4:1–10. doi:10.2174/1389200033336900
27. Martinet W, Schrijvers D, Meyer G (2012) Molecular and cellular mechanisms of macrophage survival in atherosclerosis. Basic Res Cardiol 107:297. doi:10.1007/s00395-012-0297-x
28. Nakayama K, Nitto T, Inoue T, Node K (2008) Expression of the cytochrome P450 epoxygenase CYP2J2 in human monocytic leukocytes. Life Sci 83:339–345. doi:10.1016/j.lfs.2008.06.026
29. Namgaladze D, Kollas A, Brune B (2008) Oxidized LDL attenuates apoptosis in monocytic cells by activating ERK signaling. J Lipid Res 49:58–65. doi:10.1194/jlr.M700100-JLR200
30. Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 75:4646–4658. doi:10.1021/ac0341261
31. Nikfarjam L, Izumi S, Yamazaki T, Kominami S (2006) The interaction of cytochrome P450 17α with NADPH-cytochrome P450 reductase, investigated using chemical modification and MALDI-TOF mass spectrometry. Biochim Biophys Acta 1764:1126–1131. doi:10.1016/j.bbapap.2006.04.003
32. Nishida CR, Lee M, de Montellano PRO (2010) Efficient hypoxic activation of the anticancer agent AQ4N by CYP2S1 and CYP2W1. Mol Pharmacol 78:497–502. doi:10.1124/mol.110.065045
33. Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK (1999) Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 285:1276–1279. doi:10.1126/science.285.5431.1276
34. Palsamy P, Subramanian S (2011) Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling. BBA-Mol Basis Dis 1812:719–731. doi:10.1016/j.bbadis.2011.03.008
35. Poitz D, Augstein A, Weinert S, Braun-Dullaeus R, Strasser R, Schmeisser A (2011) OxLDL and macrophage survival: essential and oxygen-independent involvement of the Hif-pathway. Basic Res Cardiol 106:761–772. doi:10.1007/s00395-011-0186-8
36. Revermann M, Schloss M, Barbosa-Sicard E, Mieth A, Liebner S, Morisseau C, Geisslinger G, Schermuly RT, Fleming I,

Hammock BD, Brandes RP (2010) Soluble epoxide hydrolase deficiency attenuates neointima formation in the femoral cuff model of hyperlipidemic mice. Arterioscler Thromb Vasc Biol 30:909–914. doi:10.1161/ATVBAHA.110.204099

37. Rivera SP, Saarikoski ST, Hankinson O (2002) Identification of a novel dioxin-inducible cytochrome P450. Mol Pharmacol 61:255–259. doi:10.1124/mol.61.2.255
38. Rylander T, Neve EPA, Ingelman-Sundberg M, Oscarson M (2001) Identification and tissue distribution of the novel human cytochrome P450 2S1 (CYP2S1). Biochem Biophys Res Commun 281:529–535. doi:10.1006/bbrc.2001.4390
39. Saarikoski ST, Rivera SP, Hankinson O, Husgafvel-Pursiainen K (2005) CYP2S1: a short review. Toxicol Appl Pharmacol 207:62–69. doi:10.1016/j.taap.2004.12.027
40. Saarikoski ST, Wikman HAL, Smith G, Wolff CH, Husgafvel-Pursiainen K (2005) Localization of cytochrome P450 CYP2S1 expression in human tissues by in situ hybridization and immunohistochemistry. J Histochem Cytochem 53:549–556. doi:10.1369/jhc.4C6576.2005
41. Schulze J, Tschop K, Lehnerer M, Hlavica P (2000) Residue 285 in cytochrome P450 2B4 lacking the NH_2-terminal hydrophobic sequence has a role in the functional association of NADPH-cytochrome P450 reductase. Biochem Biophys Res Commun 270:777–781. doi:10.1006/bbrc.2000.2495
42. Smith G, Wolf CR, Deeni YY, Dawe RS, Evans AT, Comrie MM, Ferguson J, Ibbotson SH (2003) Cutaneous expression of cytochrome P450 CYP2S1: individuality in regulation by therapeutic agents for psoriasis and other skin diseases. Lancet 361:1336–1343. doi:10.1016/S0140-6736(03)13081-4
43. Smith KR, Pinkerton KE, Watanabe T, Pedersen TL, Ma SJ, Hammock BD (2005) Attenuation of tobacco smoke-induced lung inflammation by treatment with a soluble epoxide hydrolase inhibitor. Proc Natl Acad Sci USA 102:2186–2191. doi:10.1073/pnas.0409591102
44. Thomas AC, Sala-Newby GB, Ismail Y, Johnson JL, Pasterkamp G, Newby AC (2007) Genomics of foam cells and nonfoamy macrophages from rabbits identifies arginase-I as a differential regulator of nitric oxide production. Arterioscler Thromb Vasc Biol 27:571–577. doi:10.1161/01.ATV.0000256470.23842.94
45. Wu ZL, Sohl CD, Shimada T, Guengerich FP (2006) Recombinant enzymes overexpressed in bacteria show broad catalytic specificity of human cytochrome P450 2W1 and limited activity of human cytochrome P450 2S1. Mol Pharmacol 69:2007–2014. doi:10.1124/mol.106.023648
46. Xiao Y, Shinkyo R, Guengerich FP (2011) Cytochrome P450 2S1 is reduced by NADPH-cytochrome P450 reductase. Drug Metab Dispos 39:944–946. doi:10.1124/dmd.111.039321
47. Zhang LN, Vincelette J, Cheng Y, Mehra U, Chen D, Anandan SK, Gless R, Webb HK, Wang YX (2009) Inhibition of soluble epoxide hydrolase attenuated atherosclerosis, abdominal aortic aneurysm formation, and dyslipidemia. Arterioscler Thromb Vasc Biol 29:1265–1270. doi:10.1161/ATVBAHA.109.186064