Supplemental material

Materials
5-methoxy-7’-azaindirubin (E847) was kindly provided by Prof. G. Eisenbrand (Kaiserslautern, Germany).

Methods

BrdU incorporation assay
Vascular smooth muscle cells (VSMCs) were seeded at the density of 15 000 cells/well in a 96-well plate (Microtest™, Optilux™, black/clear bottom, TC Surface, sterile with lid, BD Biosciences, San Diego, CA, USA) to grow for the 24 h and starved over the next 24 h. The starvation medium was freshly exchanged and cells were stimulated with either ethanol (0.33%), increasing concentrations of LTB₄ and cysLT-mixture (1-1000 nM), or 20 ng/ml PDGF-BB as a positive control for 24 h. Cells were loaded with bromodeoxyuridine (BrdU, 10 µM) 4 h after stimulation. BrdU incorporation was measured according to the manufacturer’s instructions using the BrdU Cell Proliferation ELISA kit (chemiluminescent) from Roche Diagnostics GmbH (Vienna, Austria). Chemiluminescence was measured in Tecan Genios™ Pro (Tecan, Mannersdorf, Switzerland) plate reader.

Information on buffy coats
Monocytes were isolated from buffy coats obtained from the Austrian Red Cross with the following identification codes: A004012108935Q (*1969, male), A0040121175443 (*1949, male) and A004012137409R (*1947, male).

Isolation of neutrophils and monocytes for lipoxygenase activity studies
Human peripheral blood was taken from fasted (12 h) healthy donors that had not taken any anti-inflammatory drugs during the last 10 days, with venipuncture in heparinized tubes (16 IE heparin/ml blood). The blood was centrifuged at 4000xg for 20 min at 20 °C for preparation of leukocyte concentrates (University Hospital Jena, Germany). Leukocyte concentrates (buffy coats) were subjected to dextran sedimentation and centrifugation on Lymphocyte separation medium LSM 1077 (PAA Laboratories, Coelbe, Germany). Contaminating erythrocytes of pelleted neutrophils were lysed by hypotonic lysis. Neutrophils were washed twice in ice-cold PBS and finally resuspended in PBS pH 7.4 containing 1 mg/ml glucose and 1 mM CaCl₂ (PGC buffer) (purity > 96-97%). Monocytes were isolated from the peripheral blood mononuclear cell (PBMC) fraction by adherence for 1.5 h at 37 °C to culture flasks (Greiner, Nuertingen, Germany), cell density was 2x10⁷ cells/ml RPMI 1640 medium containing 2 mM L-glutamine and 100 U/ml penicillin, 100 µg/ml streptomycin(all from PAA Laboratories, Coelbe, Germany), which gave a purity of > 85%, defined by forward- and side-light
scatter properties and detection of the CD14 surface molecule by flow cytometry (BD FACS Calibur, Heidelberg, Germany). Monocytes were finally resuspended in ice-cold PGC buffer.

**Purity of isolated monocytes**

To confirm the purity of isolated monocytes, samples containing 1 x 10⁶ cells were either left untreated or stained with anti-human CD14 FITC-labeled antibody as instructed by the manufacturer (eBioscience, Vienna, Austria), for 20 min at RT in staining buffer (0.2 g KCl, 0.2 g KH₂PO₄, 8 g NaCl, 1.92 g Na₂HPO₄, 0.9 g NaN₃, 3% fetal bovine serum, ad 1000 mL Aqua dest.). Cells were then centrifuged, followed by one washing step and resuspension in 1 ml staining buffer. The populations of 10000 cells were analyzed for CD14 positive cells with flow cytometer BD FACSCalibur™ at the Exc/Em wavelength 488/530 nm (FL1 channel).

**Determination of lipoxygenase products in intact cells**

Determination of LO products in intact neutrophils or monocytes (5x10⁶ cells, each) was performed as described.¹ The cells (5x10⁶) were resuspended in 1 ml PGC buffer, preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO), and incubated for 10 min at 37 °C with the indicated stimuli. Alternatively, monocytes were first primed at 37 °C with lipopolysaccharide (LPS, 1 μg/ml) for 5 min and addition of compounds (or 0.1% DMSO) for 15 min at 37 °C. Formation of 5-LO products was then started by addition of 1 μM formyl-methionyl-leucyl-phenylalanine (fMLP) and after 10 min the reaction was stopped by methanol. 30 μl 1 N HCL and 500 μl PBS, and 200 ng prostaglandin B₁ were added and the samples were subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). 5-LO products (LTB₄ and its trans-isomers, and 5-H(P)ETE) and the 12/15-LO products 12(S)- H(P)ETE and 15(S)- H(P)ETE, respectively, were analyzed by HPLC and quantities calculated on the basis of the internal standard PGB₁. Cysteinyl-LTsC₄, D₄ and E₄ were not detected (amounts were below detection limit), and oxidation products of LTB₄ were not determined. The HPLC system consists of a Hitachi L-2130 pump and MeOH/H₂O/TFA (76/24/0.007) as mobile phase with a flow rate of 1.2 ml/min, equipped with a C₁₈ column (Nova-Pak cartridge, 5 × 100 mm, 4 μm particle size, Waters, Eschborn, Germany). Detection was performed with a Hitachi L-2400 UV/Vis detector. PGB₁, LTB₄ and its trans-isomers were detected at 280 nm and H(P)ETEs at 235 nm, respectively. The sensitivity of the analysis of 5-LO products in the HPLC assay is as follows: LTB₄ and its trans-isomers: 3 ± 1 ng, 5-H(P)ETE: 5 ± 2 ng. The recovery of PGB₁ is 72 +/- 7%.

**Boyden Chamber Assay**

Serum- starved cells (0 % serum, 24 hrs) were seeded at a density of 200 000 cells/ well into pre-collagen I coated (5 μg/cm²) rat collagen I; BD Biosciences, San Diego, CA, USA) cell culture inserts (BD Biosciences, San Diego, CA, USA). Chemotaxis was initiated by addition of 10 ng/ml PDGF or 1 μM LTB4 to the lower chamber. Cells were then stained with 8 μM Calcein- AM (Merck Millipore, Billerica, MA, USA) for 20 minutes. Cells on the upper side of the inserts (non- migrated cells) were completely scraped off with a cotton bud and washed away with PBS. Cells on the lower side of the
inserts (migrated cells) were visualized under the microscope (Olympus Corporation, Tokyo, Japan) and photographed.

Figure legends

Supplemental figure 1. Representative HPLC chromatogram of stimulated monocytes (2.5 µM A23187, 10 min, 37 °C). The vehicle control (DMSO, red) and a sample preincubated with 10 µM I3MO is shown (grey). Characteristic peaks: a = compound I3MO; b = PGB2; c = LTB4; d = 12-HETE; e = 5-H(P)ETE. Enlarged peaks for LTB4 and 5-H(P)ETE are shown in the lower panels.

Supplemental figure 2. LTB4 and cysLT-mixture do not induce VSMC proliferation. VSMCs were treated with either vehicle (0.33% ethanol; veh), increasing concentrations of LTB4 or cysLT-mixture as indicated, or with PDGF-BB (10 ng/ml) as a positive control, and labeled with BrdU (10 µM) for 24 h. A graph shows relative mean values ± S.E. of BrdU incorporation out of 3 biological replicates with each measured in technical triplicates; *** p<0.001.

Supplemental figure 3. LTB4 acts as chemotactic stimulus for VSMC, but is weaker than PDGF. Quiescent VSMC were subjected to a Boyden Chamber chemotaxis assay as described in the Methods section, using 10 ng/mL PDGF or 1 µM LTB4 as chemotactic stimuli. Migrated cells were visualized by calcein-AM staining and photographed. Representative pictures of two independent experiments with consistent results are shown. For comparison, the bar graph depicts compiled data of three independently performed biological replicates (wound healing) assays (mean ± S.E., * p<0.05, ***p < 0.001).

Supplemental figure 4. Isolation technique using CD14 Microbeads resulted in >95% pure monocyte population. Flow cytometry analysis of CD14 unstained vs. stained cells. Results of one representative experiment (upper panel) and a graph with percentage of cells ± SEM in lower right (LR) quadrant (results of quadrant statistics) out of 3 biological replicates (lower panel) are shown. UL = upper left quadrant; UR = upper right quadrant; LL = lower left quadrant; LR = lower right quadrant; Ab = antibody.

Supplemental figure 5. Media conditioned with AA and A23187 in the absence of monocytes does not induce VSMC migration. VSMCs were stimulated for 21 h in the course of a wound healing assay either with vehicle (0.33% ethanol; veh), LTB4 (1 µM), or medium conditioned in the absence of monocytes with vehicle (0.33% ethanol + 0.1% DMSO), or AA (20 µM) + A23187 (2.5 µM). A graph indicates the change in area occupied by cells as fold induction of VSMC migration from 3 independent biological replicates; mean ± S.E.; * p<0.05.

Supplemental figure 6. The indirubin derivatives E847 fails to induce HO-1 and to inhibit migration in VSMC. (A) VSMC were treated with I3MO or E847 for 18 hrs before their cell lysates were subjected to immunoblot analysis for HO-1 and actin. Representative blots of 2 independent experiments are depicted. (B) I3MO and E847 were tested for inhibition of PDGF-induced migration in VSMC in a scratch assay. Compiled data of two independent biological replicates are depicted.
References

1. Pergola C, Rogge A, Dodt G, Northoff H, Weinigel C, Barz D, et al. Testosterone suppresses phospholipase D, causing sex differences in leukotriene biosynthesis in human monocytes. *FASEB J* 2011; **25**:3377-87
Suppl. fig. 1
Suppl. fig. 2

A

BrdU incorporation (x-fold basal)

|        | LTB4 (nM) | cysLT (nM) |
|--------|-----------|------------|
| veh    |           |            |
| 1      |           |            |
| 10     |           |            |
| 100    |           |            |
| 1000   |           |            |
| 1      |           |            |
| 10     |           |            |
| 100    |           |            |
| 1000   |           |            |
| PDGF   |           |            |

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Suppl. fig. 3

**TRANSWELL ASSAY**

| Ctrl | Exp #1 | Exp #2 |
|------|--------|--------|
|      | ![Image](image1.png) | ![Image](image2.png) |
| PDGF | ![Image](image3.png) | ![Image](image4.png) |
| LTB4 | ![Image](image5.png) | ![Image](image6.png) |

**SCRATCH (WOUND HEALING) ASSAY**

![Bar chart showing relative migration](chart.png)
Suppl. fig. 4

CD14-dependent fluorescence

- Without CD14 Ab
- With CD14 Ab

Cell size/granularity

% CD14 positive cells

CD14 Ab

- 
  +
Suppl. fig. 5

On VSMC: cell-free incubation with:
veh LTB4 conditioned medium
veh AA + A23187

change in area occupied (x-fold vehicle control)
Suppl. fig. 6

A

- - 5 - 5 μM I3MO
- - - - μM E847

[Image of HO-1 and actin Western blots]

B

[Bar chart showing relative migration with different treatments]

- - 3 5 - - μM I3MO
- - - - 3 5 μM E847

PDGF