SUPPLEMENTAL MATERIAL

Cell Culture

Human umbilical arterial endothelial cells (HUAEC) were isolated by collagenase treatment [1] and maintained in human endothelial cell specific medium (EBM-2) supplemented with endothelial growth media (EGM-2) and 10% FCS. Cells up to passage 1 were grown to confluence to preserve endothelial features. Prior to every experiment, cells were incubated 16 h in medium containing 1% FCS. Previous studies carried out by our group have shown that human umbilical vein endothelial cells (HUVEC) do not behave like HUAEC in response to relevant cardiovascular stimuli such as angiotensin II [2,3]. In this context, HUAEC and HUVEC showed dissimilar mononucleal cell and neutrophil adhesion when the same stimulus was applied.

Cigarette smoke extract (CSE) preparation

The composition of 3R4 research grade cigarettes was as follows: total particulate matter, 10.9 mg/cigarette; tar, 9.5 mg/cigarette; and nicotine, 0.726 mg/cigarette. 10% CSE was prepared by bubbling smoke from one cigarette 3R4F into 10 ml of EGM-2 culture media without FBS at a rate of 1 cigarette / 2 min. The pH of the CSE was adjusted to 7.4 and sterile filtered through a 0.22 μm filter. CSE preparation was standardized by measuring the absorbance (optical density= 0.86 ± 0.05) at a wavelength of 320 nm. The pattern of absorbance (spectrogram) observed at λ320 showed very little variation between different preparations of CSE. CSE was freshly prepared for each experiment and diluted with culture media supplemented with 0.1% FBS immediately before use. Control medium was prepared by bubbling air through 10 ml of culture media without FBS, the pH was adjusted to 7.4, and the

medium was sterile filtered as described above. In initial experiments, a range of concentrations of CSE were tested (0.1-3%). Based on these preliminary studies a final concentration of 1% was used in all subsequent experiments.

RT-PCR

The reverse transcription was performed in 300 ng of total RNA with TaqMan reverse transcription reagents kit. cDNA was amplified with specific primers for fractalkine (CX_3CL1), TNFα , Nox2, Nox4, Nox5 and GAPDH (all pre-designed by Applied Biosystems, Carlsbad, CA) in a 7900HT Fast Real-Time PCR System (Applied Biosystem) using Universal Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using GAPDH as endogenous control and normalized to control group.

Flow cytometry

The cells were washed and incubated at 2 x 10^6 cells/ml with a PE-conjugated mAb against human CX_3CL1 (1.25 µg/ml) in PBS with 0.2% BSA and 0.05% NaN_3 for 1 h on ice. After 2 washes, cells were suspended in PBS containing 2% paraformaldehyde. The fluorescence signal of the labeled cells was then analyzed by flow cytometry (FACSCanto Flow cytometer, BD Biosciences, Frankiln Lakes, NJ). The expression of CX_3CL1 (PE-fluorescence) was expressed as the mean of fluorescence intensity (MFI).

Leukocyte-endothelial cell interactions under flow conditions

Human mononuclear cells were obtained from buffy coats of healthy donors by Ficoll Hypaque density gradient centrifugation [1]. The Glycotech flow chamber was assembled and placed onto an inverted microscope stage, and then freshly isolated mononuclear cells (1×10^6/ml) were perfused across the endothelial monolayers. In all experiments, leukocyte interactions were determined after 5 min at 0.5 dyn/cm^2. Cells

interacting on the surface of the endothelium were visualized and recorded (×20 objective, ×10 eyepiece) using phase-contrast microscopy (Axio Observer A1 Carl Zeiss microscope, Thornwood, NY).

Immunofluorescence

Confluent endothelial cells were grown on glass coverslips and stimulated with 1% CSE or vehicle for 24 h. The cells were fixed with 4% paraformaldehyde, and blocked in a PBS solution containing 1% BSA. Then, they were incubated at 4°C overnight with a primary mouse mAb against human CX_3CL1 (1:200 dilution) in a 0.1% BSA/PBS solution, followed by incubation with a secondary antibody Alexa Fluor 488-conjugated goat anti-mouse mAb (1/1000 dilution) at room temperature for 45 min. Cell nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). Images were captured with a confocal microscope (Leica TCS/SP2, Solms, Germany).

Western Blot.

After treatment, cells were washed, detached, collected, and centrifuged at 15,000 g at 4ºC for 30 min to yield the whole extract. Protein content was determined by the Bradford method. Samples were denatured, subjected to SDS-PAGE using a 10% running gel, and transferred to nitrocellulose membrane. Nonspecific binding sites were blocked with 3% BSA in TBS solution, and were then incubated overnight with rabbit polyclonal antibody against human CX_3CL1 (0.2 µg/ml), a mouse polyclonal antibody against human Nox2 (0.2 µg/ml), a rabbit polyclonal antibody against human Nox4 (2 µg/ml), a rabbit polyclonal antibody against human Nox5 (2 µg/ml) or a goat polyclonal anti-human TNFα (0.1 µg/ml). Then they were washed and further incubated for 1 h with the corresponding secondary HRP-linked antibody: anti-rabbit IgG (1:2000 dilution), anti-goat IgG or anti-mouse IgG (1:2000 dilution) and developed using the ECL procedure. Signals were recorded using a luminiscent

analyser (FujiFilm image Reader LAS1000, Fuji, Tokyo, Japan) and analyzed using the software ImageJ (Windows free version).

Transfection of TNFα, Nox2, Nox4 or Nox5 siRNA

The transfection reagent used was Lipofectamine RNAiMAX, following the manufacturer's instructions. The mRNA expression for transcripts was determined by real time RT-PCR after 48 h post-silencing and compared with siRNA control at the respective time to determine silencing efficiency. Cells were also tested for TNFα, Nox2, Nox4 or Nox5 expression by western blot of cells lysates. In addition, cell viability after control or siRNA transfection was assessed by MTT assay. Cells were 94-97% viable.

Experimental protocols

In a first set of experiments, HUAEC were grown to confluence and stimulated with 0.1-3% CSE or TNFα (20 ng/ml) for 1, 4 or 24 and CX_3CL1 mRNA expression was determined by RT-PCR and protein expression by flow cytometry, immunoflorescence analysis and western blot.

In another group, HUAEC were stimulated with with 1% CSE for 24 h. Freshly isolated human mononuclear cells were perfused across the endothelial cell monolayers and leukocyte-endothelial interactions were determined under flow conditions. To determined the effect of endothelial CX_3CL1 expression on mononuclear recruitment, endothelial cells were incubated with a monoclonal neutralizing antibody against human CX_3CL1 (5 μg/ml) or with an isotype matched control antibody (MOPC-21, 5 μg/ml) 10 min prior to mononuclear cell superfusion.

To evaluate the potential involvement of NADPH and xanthine oxidase (XO) on CSE-induced responses, cells were incubated for 1 h with a NADPH oxidase inhibitor

(apocynin, 30 μM) or with a XO inhibitor (allopurinol, 100 μM) and then stimulated with 1% CSE for 24 h. The doses of these compounds were used as previously described.[4] and, no direct toxicity was found by MTT assay (viability 95-98%). Since, the NADPH oxidase isoforms Nox2, Nox4, and Nox5 are all expressed in endothelial cells,[5,6] in subsequent experiments, HUAEC were transfected with either control or Nox2, Nox4 or Nox5–specific siRNA. Forty eight h post-transfection they were stimulated with 1 % CSE and CX_3CL1 expression and mononuclear cell arrest evaluated.

To investigate the possible contribution of TNFα to CSE-induced CX_3CL1 expression and mononuclear cells recruitment, we first incubated the cells with 1% CSE for 24 h and TNFα expression was determined by western blot. Next, HUAEC were transfected for 48 h with control or TNF-α-specific siRNA before CSE stimulation and CSE-induced responses were measured 24 h later.

To extend these findings, in additional experiments, the phosporylation/ activation of p38MAPK and NFκB were determined by flow cytometry as previously described.[3] HUAEC were stimulated or not with 1% CSE for 30 – 60 min. The endothelial cells were then fixed and permeabilized with BD Cytofix/Cytoperm solution and sequentially stained with a Alexa Fluor-conjugated mouse anti-human p38MAPK (pT80/pY182) and with a PE-conjugated mouse anti-human p65 subunit (pS529) mAbs.

To further elucidate the signalling pathways involved in CSE-induced responses, endothelial cells were pretreated with the inhibitors of p38MAPK (SB202190, 20 μM) or NFkB (MOL294, 2.5 μM) 1 h before CSE stimulation. These concentrations have previously been employed to inhibit p38MAPK and NFkB.[7,8] and, cell viability by MTT assay was higher than 95%. After 24 h stimulation with 1% CSE, both CX_3CL1 expression and mononuclear cell arrest were determined.

Studies in COPD patients and age-matched controls

A total of 52 subjects (29 COPD patients and 23 control age-matched subjects without COPD) were included in this study. COPD patients and control subjects were recruited by the Pneumonology Unit of University Clinic Hospital of Valencia, Valencia, Spain. All patients had COPD confirmed by medical history, clinical and functional examinations according to criteria established by the American Thoracic Society (Standards of diagnosis and care of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1995;152 (Suppl):77-120): smoking history of ≥ 10 pack-year , the post-bronchodilator ratio of low forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC), FEV_1/FVC ratio was < 0.70 and the post-bronchodilator FEV_1 was <80%. One pack-year was defined as smoking 20 cigarettes per day for one year. The control group was volunteers seen at the respiratory function laboratory for routine preoperative assessment. They had no history of pulmonary disease or respiratory symptoms, and had a normal spirometry. In order to study homogeneous samples of both COPD patients and controls, only subjects older than 60 years of age were included. Written informed consent was obtained from all volunteers. Spirometry was performed on a Master Scope (Jaeger, Germany), after inhalation of 0.4 mg of salbutamol. A minimum of three airflow and volume tracings were obtained and the highest value for FEV1 and FVC as percent predicted normal were used for calculations. Most of the patients used in this study presented moderate COPD according with the criteria of GOLD classification (Global Initiative for Chronic Obstructive Lung Disease. Global strategy for the diagnosis, management, and prevention of COPD. [December, 2011]. Available from: http://www.goldcopd.org). In this regard, 8% were GOLD1 (mild), 60% were GOLD2 (moderate), 28% were GOLD3 (severe) and 4% were GOLD4 (very severe). Clinical features of patients and age-matched controls are shown in Table 1.

Table 1: Patient demographics of the subjects studied (data expressed as mean ± SEM)

	Control non smoker volunteers	COPD subjects
Numbers per group (n)	23	29
Smoking (pack-years)	none	49.1 ± 7.07
Age	69.48±1.79	68.52±1.88
FEV_1 (% Predicted)	94.23±3.95	59.32±3.54**
FEV_1/FVC (%)	74.85±1.40	57.84±2.03**
Gender (M)	100%	100%

Pack-year (nº cigarettes per day per smoking years / 20). FEV_1 % Predicted, forced expiratory volume in 1 s (%); FVC, forced vital capacity. **p<0.01 relative to values in the control group.

To determine the expression of CX_3CL1 receptor (CX_3CR1) on circulating monocytes and lymphocytes from COPD patients and control-matched individuals, a flow cytometry analysis was employed. Duplicate samples (100 µl) of heparinized whole blood were incubated on ice in the dark for 20 min with saturated amounts (10 µl) of the carboxyfluorescein (CFS)-conjugated mAb against human CX_3CR1. RBCs were lysed and leukocytes were fixed using an automated EPICS Q-PREP system (Coulter Electronics, Hialeah, Florida). Samples were run in a Flow cytometer (FACSCanto Flow cytometer, BD Biosciences, Frankiln Lakes, NJ). The expression of CX_3CR1 (CFS fluorescence) was measured on monocytes and lymphocytes by their specific features of size (forward scatter) and granularity (side scatter) and expressed as the mean of fluorescence intensity (MFI) as it is illustrated in Figure II (online supplemental data 2).

In another set experiments, a dynamic flow chamber assay was performed using heparinized whole blood from both groups under investigation. Diluted whole blood (1/10 in HBSS) of COPD patients and control-matched subjects was perfused across unstimulated or 1% CSE-stimulated endothelial monolayers as previously described and leukocyte-endothelial cell interactions were determined. Some plates were incubated with a monoclonal neutralizing antibody against human CX_3CL1 (5 μg/ml) or with an isotype matched control antibody (MOPC-21, 5 μg/ml) 10 min before blood perfusion.

Finally, heparinized human whole blood (10 U heparin/ml) from COPD patients and healthy control-matched volunteers was collected. Before centrifugation to obtain plasma, further heparin was added to the blood sample (to 100 U/ml). This procedure was used to help to dissociate chemokines from blood cells. Plasma samples were stored at −80°C. Human CX_3CL1 was measured in plasma by ELISA, as previously described.[9] Results are expressed as pM chemokine in the supernatant.

Animals

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the ethics review board of the Faculty of Medicine, University of Valencia.

Male mice of C57BL/6 background carrying targeted knock in of GFP to disrupt the CX_3CR1 gene have been used in numerous studies[10] in which male $CX_3CR1^{gfp/+}$ mice were used as heterozygote controls ($CX_3CR1^{-/+}$) and homozygote $CX_3CR1^{gfp/gfp}$ animals that do not express CX_3CR1 receptor as CX_3CR1 deficient mice ($CX_3CR1^{-/-}$). Animal colonies were bred and maintained under specific pathogen-free conditions. For all the experimental period the mice were fed with autoclaved balanced diet and water. The animals used were 22–30 g weight.

Cigarette smoke exposure

Mice were placed in a plexiglass chamber (volume of 20 l) covered by a disposable filter. The smoke produced by cigarette burning was introduced at a rate of 25 ml/min into the chamber with the continuous airflow generated by a mechanical ventilator, with no influence on the chamber temperature (<0.1°C variation). The animals received smoke from 5 research grade cigarettes (3R4F) per exposure, 2 exposures a day during 3 days. Experiments were carried out 16 h after the last exposure.

Intravital microscopy

The mouse cremaster preparation used in this study was similar to that described previously.[11] Mice were anesthetized by i.p. injection with a mixture of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (200 mg/kg). Additional anesthetic (30 µl, i.v.) was administered as required to maintain profound anesthesia. A polyethylene catheter was placed in the jugular vein to permit the intravenous administration of additional anesthetic. The cremaster muscle was dissected free of tissues and exteriorized onto an optical clear viewing pedestal. The muscle was cut longitudinally with a cautery and held flat against the pedestal by attaching silk sutures to the corners of the tissue. The muscle was then perfused continuously at a rate of 1 ml/min with warmed bicarbonate-buffered saline (pH 7.4).

The cremasteric microcirculation was then observed using an intravital microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, Netherlands) equipped with a 50x objective lens (Nikon SLDW, Badhoevedorp, The Netherlands) and a 10x eyepiece. A video camera (Sony SSC-C350P, Koeln, Germany) mounted on the microscope projected the image onto a color monitor and the images were CCD recorded for playback analysis. Cremasteric arterioles (20-40 µm in diameter) were selected for study. Vessel diameter

was measured on-line by using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas).

The number of adherent leukocytes was determined off-line during playback of the recorded images. A leukocyte was defined as adherent to arteriolar endothelium, if it was stationary for at least 30 s. Leukocyte adhesion was expressed as the number per 100 μm length of vessel per 5 min. In each animal, leukocyte responses were averaged in three to five randomly selected arterioles.

RT-PCR

Real time RT-PCR was performed using standard protocols employing the following primers: mouse CX_3CL1 forward, 5′-GGACAGGACCTCAGTCCAGA- 3′, reverse 5′-TCGGGGACAGGAGTGATAAG -3′, (256 bp product). β-actin forward, 5´-GTGGGCCGCTCTAGGCACCAA-3´, reverse 5´-CTCTTTGATGTCACGCACGATTTC-3´(539 bp product). The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. CX_3CL1 mRNA abundance was determined by comparison with β-actin.

Histology and Immunofluorescence

Immunofluorescence studies were performed following a similar protocol to that previously described. [12] Once intravital microscopy determinations were performed, mice were sacrificed and the cremaster muscle was isolated and fixed in 4 % paraformaldehyde for 10 minutes. Muscles were incubated in 0.2% Triton X-100, 1% BSA and 0.5% horse serum in phosphate-buffered saline (PBS) for 2 h. Then muscles were incubated overnight at 4°C with a primary Ab rabbit anti-mouse CX_3CL1 (1/100 dilution) or eFluor 450-conjugated anti-mouse CD31 (PECAM-1) (1/100 dilution). Samples were then washed with PBS and incubated for 1.5 h at room temperature with Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1/500 dilution). All

antibodies were diluted in 0.1% PBS/BSA. Muscles were then mounted with Slowfade Gold Reagent (Invitrogen, Eugene, Oregon, USA). Images were acquired by using a fluorescence microscope (Axio Observer A1, Carl Zeiss, NY) equipped with a 40x objective lens and a 10x eyepiece.

In some animals, lungs were removed, fixed with 4% paraformaldehyde and embedded in paraffin. Sections (4 μm-thick) were obtained and then stained with hematoxylin/eosin. Cells were counted in 10 different fields and averaged.

Materials

Endothelial basal medium-2 (EBM-2) supplemented with endothelial growth medium-2 (EGM-2) were acquired from Lonza Iberica (Barcelona, Spain). Ketamine and xylazine hydrochloride were from ORION Pharma (Espoo, Finland). Apocynine, allopurinol, SB202190, Thiazolyl Blue Tetrazolium Bromide, the mouse anti-human β-actin mAb (clone AC-15), the mAb IgG1 (MOCPC21) and the rabbit polyclonal anti-human Nox 5 Ab were purchased from Sigma-Aldrich (Madrid, Spain). The rabbit polyclonal anti-mouse CX_3CL1 and the PE-conjugated conjugated rat monoclonal anti-mouse CD31 (clone 390) were from eBioscience (Hatfield, UK). Recombinant human TNFα and the rabbit polyclonal anti-human CX_3CL1 employed for western blotting were acquired from Peprotech (London, UK). The PE-conjugated mouse monoclonal anti-human CX_3CL1 (clone 51637), the CFS-conjugated mouse monoclonal anti-human CX_3CR1 (clone 528728), the mouse monoclonal anti-human CX_3CL1 (clone 81506), the biotinylated mouse monoclonal anti-human CX_3CL1 (clone 51637) and the goat polyclonal anti-human TNFα were purchased from R&D Systems (Abingdon, UK). The rabbit polyclonal anti-human Nox 4 was from Abcam (Cambridge, UK), and the mouse monoclonal anti-human Nox 2 (clone NL7) Ab was purchased to Serotec (Oxford, UK). The sodium heparin (5000 U/ml or 50 mg/ml)

was from Pharmaceutical Laboratories Rovi SA (Madrid, Spain). Neutravidin-HRP was supplied by Perbio Science (Northumberland,UK) and the K-Blue substrate by Neogen (Ayr, Scotland, UK). The cytotoxicity detection Kit plus LDH were obtained from Roche Applied science (Mannheim, Germany), Ficoll-Paque TM plus and ECL developer were purchased from GE Healthcare (Chalfont St Giles, UK). DAPI, TRIzol isolation reagent and Alexa Fluor 488-conjugated secondary antibodies were from Molecular Probes-Invitrogen (Carlsbad, CA). The secondary HRP-linked anti-rabbit IgG Ab was supplied by Cell Signalling Technology (Grand Island, NY). The secondary Abs, HRP-linked anti-goat IgG and HRP-linked anti-mouse IgG were purchased from Dako (Glostrup, Denmark). BD Cytofix/Cytoperm solution, PE-conjugated mouse anti-p65 (pS529) (clone K10-895.12.50) and the Alexa Fluor-conjugated mouse anti-p38MAPK (pT80/pY182) (clone 36/p38; pT180/pY182) were from BD Biosciences (San Jose, CA). TNFα, Nox2, Nox4 or Nox5–specific siRNA were purchased to Dharmacon (Lafayette, CO). TaqMan reverse transcription reagents kit were from Applied Biosystems, (Perkin-Elmer Corporation, Carlsbad,CA). MOL-294 was kindly donated by Dr. Kahn (Department of Pathobiology, University Washington, Seattle, WA.

Statistical Analysis

Values were expressed as mean ± SEM. Differences between two groups were determined by paired or unpaired Student's t test, as appropriate. Data within multiple groups were compared using an analysis of variance (one-way ANOVA) including a Newman–Keuls post hoc test for multiple comparisons. Data were considered statistically significant when $p<0.05$.

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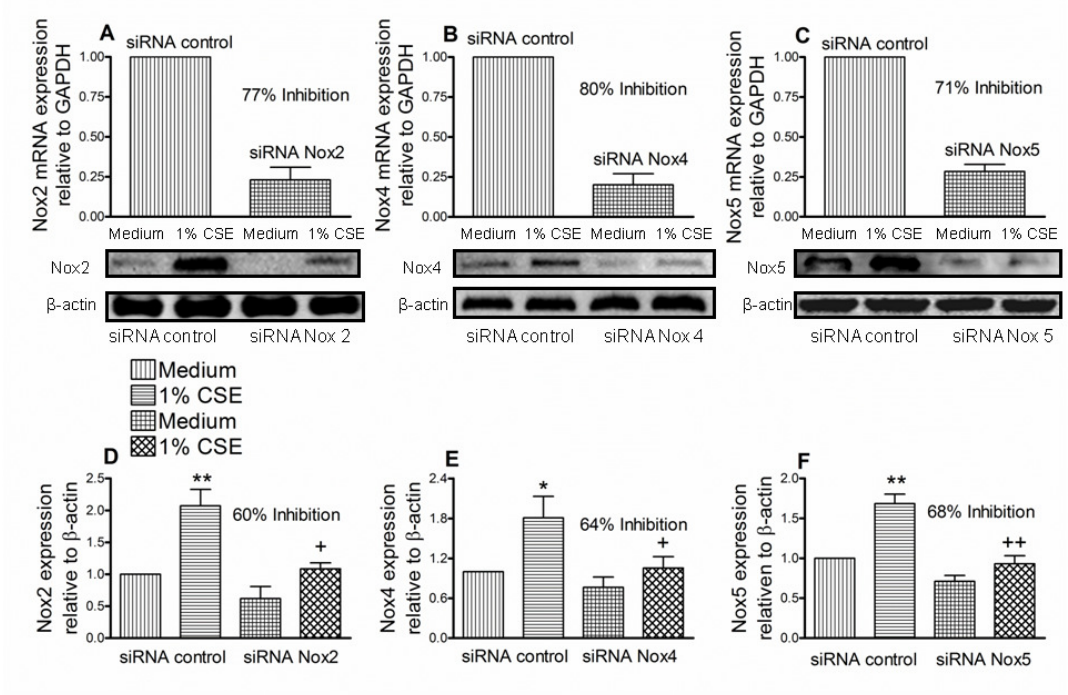

Figure I: 1% CSE increases Nox2, Nox4 or Nox5 expression in HUAEC which is abolished in HUAEC transfected with siRNAs targeting Nox2, Nox4 or Nox5. Endothelial cells were transfected with control siRNA or Nox2siRNA, Nox4siRNA or Nox5siRNA. 48h post-transfection cells were stimulated or not with 1% CSE for 24 h. Relative quantification of the mRNA levels of the different Nox isoforms and GADPH was determined by real time quantitative RT-PCR by the comparative Ct method. Columns show the fold increase in the expression of Nox mRNA, relative to control values, calculated as the mean ± SEM of the $2^{-\Delta\Delta Ct}$ values of n= 4-5 independent experiments. Protein expression of the different Nox isoforms was determined by western blot. Results (mean ± SEM of at least 4 independent experiments) are expressed as fold increase of the Nox isoform relative to β-actin. Representative gels are also shown. *p<0.05 or **p<0.01 relative to values in the control group; +p < 0.05 relative to the 1% CSE group.

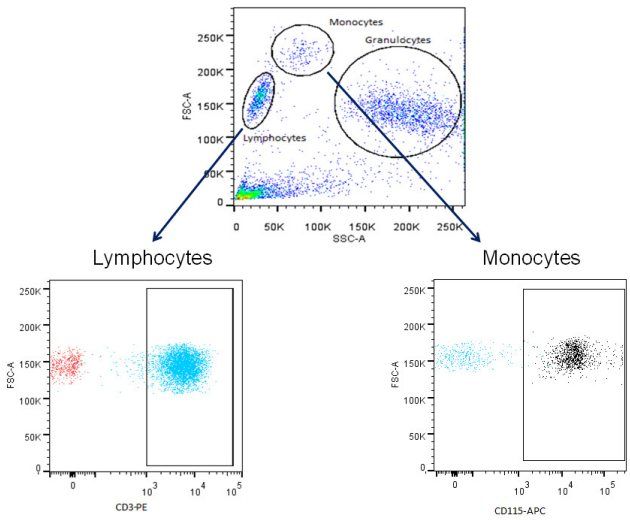

Figure II: Flow-cytometry detection and morphologic gating of human monocytes and lymphocytes in whole blood. In initial experiments lymphocytes were stained with a PE-labeled anti-CD3 mAb and monocytes with an APC-labeled anti-CD115 mAb. Once detected, they were gated based on their specific features of size (forward scatter) and granularity (side scatter).