Cigarette Smoke Inhibits Engulfment of Apoptotic Cells by Macrophages through Inhibition of Actin Rearrangement.

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Online Supplementary Material

Supplementary Methods:

Cell isolation and culture. Human polymorphonuclear (hPMNs) and mononuclear leukocytes were isolated from peripheral blood of non-smoking subjects by ficoll gradient.[15,16] Briefly, heparinized blood was centrifuged at 300 g, for 20 min, at 22^{o}C, and plasma was replaced with phosphate buffered saline (PBS) containing dextran (MW: 400,000-500,000, final concentration 0.6 % w/v). Red blood cells (RBCs) were allowed to form a pellet by sedimentation, the leukocyte-rich supernatant was placed on Histopaque1077 (Sigma) gradient and centrifuged at 700 g, for 30 min, 22^{o}C. The mononuclear layer was washed 4 times in PBS containing 5 mM EDTA, and suspended in RPMI medium in 8 well chamber slides (Nulgen) culture dishes or culture plates. Adherent cells were cultured in RPMI supplemented with 20 % autologous serum and were maintained in culture for 8-10 days to allow differentiation into human monocyte-derived macrophages (hMDMs). hPMNs pellets from the ficoll gradient were resuspended in hypotonic lysis buffer to eliminate the residual RBCs, washed twice with PBS containing 0.25 % bovine serum albumin (BSA), and used to generate the apoptotic population.

Preparation of cigarette smoke extract (CSE). Research grade 1R5F cigarettes (Kentucky Tobacco Research Institute) were used to prepare the CSE. CSE was made fresh for each experiment by bubbling the smoke from one cigarette through 10 ml of medium in 15 ml conical tube and further passing through 0.22 μm filter to remove large particles and maintain sterility. This solution was designated as 100 % CSE and diluted in culture medium to yield concentrations specified for each experiment.

Assessment of cell viability. Cell viability was assessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma) according to manufacturer instruction. Briefly, hMDMs or Raw cells were grown in 24 well plates, MTT was added at indicated time points for 2 h, and the formation of formazan crystal was measured by absorbance at OD580 and OD690.

FACS Analysis. Cells obtained by BAL were collected by centrifugation (800g, 10 min at 4^{0}C), resuspended in PBS with 5 mM EDTA (10 min), pelleted and fixed with 4% paraformaldehyde (20 min). Fixed cells were washed twice with PBS, 5 % FBS, 5 mM EDTA (FACS buffer), nonspecific bindings was blocked by incubating cells in PBS supplemented with 10 % human serum and 2 μg of anti-mouse CD16/32 antibodies (BD, Fc blocker) (20 min, at 4^{0}C), cells were stained with anti-mouse CD11c antibodies conjugated to PE-Cy5.5 (Caltag) (30 min at 4^{0}C), washed (3×) and resuspended in FASC buffer for further analysis.

Human PMNs did not react with anti-mouse CD11 (data not shown) and present as a population of small negative cells, while macrophages represent

population of large positive cells (Sup Fig. 4B) Therefore, CD11-positive cells (FL3) were further analyzed for the presence of CTG-label (FL1), and the percent of phagocytosis was calculated as ratio of double positive cells to CD11-positive cells (Sup Fig.4C & D).

Each tube was run in FACSCaliber and analyzed with FlowJo software. Two-dimensional graph with FSC (cell size) and FL3 (CD11c) were used to gate macrophage population as $FSC^{high}FL3^{high}$ properties (R1 region in Fig. S3C, 10,000 macrophages were acquired for each sample). Note from Sup. Fig. 3C that free apoptotic hPMNs stained with CTG were relatively small and clearly negative for CD11c, therefore excluded from R1 region. Macrophages gated in R1 were further analyzed for CTG signals in two-dimensional graph with FSC (cell size) and FL1 (CTG) (Fig. S4C and D). FL1 positive macrophages indicated those containing CTG-stained apoptotic hPMNs. Ratio of CTG- containing macrophages to total macrophages was calculated for each tube.

Hema 3 staining. Cytospin slides were stained with Hema 3 (Fisher Scientific) according to manufacturer instructions. Briefly, slides were dipped sequentially in solution 1, 2 and 3 for 30 sec each, followed by washing out the excess of the dye under running water.

Annexin/propidium iodide stain and FACS analysis. 2×10^5 cells per sample were resuspended in 100 µl annexin binding buffer (Invitrogen) and incubated with FITC-labeled annexinV and propidium iodide (PI) solution (Invitrogen) at room temperature for 20 min. Samples were analyzed by FACS analysis to determine cell viability. AnnexinV binding, i.e. phosphatidylserine expression on outer leaflet if apoptotic cell, was expressed in X-axis (FL1) and PI staining properties were shown in Y-axis (FL-2) in two-dimensional graph. Cells population $FL1^{positive}FL2^{negative}$ was considered apoptotic.

Liposome Preparation. The lipids phosphatidylserine (PS) and phosphatidylchorine (PC) (Avanti polar lipids) were dried from chloroform stock solutions under a stream of nitrogen, hydrated in DMEM medium, freeze/thawed three times and sonicated for 15 min in a solid state Ultrasonic FS-14 as previously described.[19] The final concentration of liposomes for phagocytosis assays was 500 µM, and for Rac1 activation assay was 100 µM, where PC content was 100% mol for PC-liposomes and 70 % mol PC/ 30 % mol PS for PS-liposomes.

Figure S1. **Viability of cells after CS-exposure.** A. hMDMs were treated with various concentration of CSE for 24 h, and tested for viability by the MTT assay. B. Raw264.7 cells were treated with 10 % CSE for 8 or 24 h and their viability was tested using MTT assay *: $P<0.0001$ vs. no treatment. Of note, Raw cells were used after 8 hrs of CSE exposure in our studies to prevent cell death.

Figure S2. **Induction of apoptosis in hPMNs.** hPMNs were isolated from human peripheral blood of volunteers. Apoptosis of hPMNs was induced *in vitro* by UV irradiation. UV irradiation is a reliable and reproducible method and was chosen because, doses and duration are well known; UV-induced death is closer to CS-induced apoptotic

pathway, both agents cause damage to DNA from inside the cell; UV-induced death in culture is well synchronized.

Briefly, isolated hPMNs were irradiated at 305 nm UV for 5 and 10 min, followed by incubation at 37^0C in a humidified incubator for 1h, 2 h and 3 h. Apoptosis was assessed by (A) Hema3 staining and by (B) FACS using FITC conjugated AnnexinV and propidium iodide. Percentage of cells in each group is shown on the right of each graph. At all conditions tested more than 95 % of all cells were Trypan Blue negative, indicating that they were in the early stages of apoptotic process, before membrane permeability is affected and before cells become necrotic. (C) The percent of apoptotic cells between experiments was highly reproducible. For all experiments 10 min of UV-irradiation followed by 3 hours of recovery in RPMI, 0.25 % BSA was choosen.

Figure S3. Determination of optimal number of apoptotic cell for intratracheal instillation and timing of BAL after installation to assess the clearance of apoptotic cells by alveolar macrophages *in vivo*. Mouse bone marrow derived PMNs (BM-PMNs) were obtained from wild type C57BL/6 females. Femur and tibia bones were dissected and both ends of bones were cut off. Bone marrow was flushed out with RPMI medium into petri dish through 70 μm filter membrane. Bone marrow cells were incubated with anti mouse Gr-1 antibodies conjugated with biotin, followed by anti-biotin antibody conjugated microbeads (Miltenyi). Gr-1 positive cells were selected in MACS cell separation system and aged in culture for 24 h. Apoptosis was confirmed by MTT assay (data not shown). Apoptotic BM-PMNs were instilled intratracheally to mouse lungs. (A) Different amounts ($2\text{-}20\times10^6$) of apoptotic BM-PMNs were instilled intra-tracheally, 90 min after installation mice were lavaged and BAL fluids were analyzed for the presence of ingested apoptotic cells within alveolar macrophages by MPO staining. (B) 10×10^6 apoptotic BM-PMNS or apptotic neutrophils (left column) were installed intra-tracheally, BAL was performed 60, 90, and 120 min after instillation. Engulfment of apoptotic cells was assessed by MPO staining.

Figure S4. **Detection of alveolar macrophages containing apoptotic neutrophils by FACS.** (A) Histograms of CD11c staining of mouse alveolar macrophages collected from non-treated C57BL/6 mouse. Cells were blocked with anti mouse CD16/32 antibodies (Fc blocker) and stained for anti mouse CD11c antibodies conjugated with PE-Cy5.5. Representative plots for forward scatter and CD11c expression from smokers and controls. (B) $1X10^6$ CTG-labeled apoptotic hPMNs were instilled intratracheally to mice exposed to sham or to CS for 12 weeks. BAL was performed 2 h later, and BAL fluid was processed for FACS analysis. Cells were pre-incubated with Fc blocked and 10 % human serum to avoid non-specific binding of antibodies on Fc site and, stained with anti-mouse CD11c antibodies. Forward scatter (FSC) is shown in X axis and FL3 (CD11c) in Y-axis. R1 region contains alveolar macrophages, which are large cells (FSC) with high CD11c expression (FL3). Exogenously instilled CTG-labeled hPMNs are in the lower left quadrant. They are positive for CTG (data not shown) and negative for mouse CD11c. CD11c was expressed similarly in smokers and controls (C) Macrophages gated into R1 region (left) were analyzed for CTG. R2 region in the plot on the right represents macrophages containing CTG-labeled hPMNs, where FSC (cell size) shown in X-axis and CTG (FL1) in Y-axis. Phagocytosis was calculated as ratio of R2 to R1.

Of note we could not establish reliably a precise number of free apoptotic cells in BAL fluids obtained 2 h after initial installation, because after 2 h *in vivo* a lot of apoptotic cells were fragmented into small bodies known as 'apoptotic bodies'. Nevertheless, we can see a lot of CTG-positive small cells/pieces in FACS, which showed up in left lower corner in Sup Fig3C. (D) Morphology of cells in each gate is confirmed by cytospin and microscopy.

Figure S5. **CS-exposure does not affect the expression of surface markers on macrophages.** (A) Control and hMDMs were treated with 10 % CSE for 24 h, fixed and stained for indicated apoptotic cell recognition receptors. White-colored curves represent the expression of apoptotic cell recognition receptors analyzed by FACS, gray-colored curves represent isotype controls. Graphs are representative of 2 independent experiments.
(B) Mouse alveolar macrophages were collected from control mice by BAL, fixed and stained for indicated apoptotic cell recognition receptors. White-colored curves represent the expression of apoptotic cell recognition receptors analyzed by FACS, gray-colored curves represent isotype controls. Note that in mouse macrophages we were able to detect CD14, CD44, and integrin α_v only. (C) The mean fluorescence intensities of apoptotic cell recognition receptors expressed on mouse alveolar macrophages were compared between mice exposed or not to CS for 4 weeks. Data collected from 4 mice in each group.

Supplement Movie 1. **CSE inhibits actin rearrangements at the leading edge in polarized Raw264.7 cells**. 10^6 Raw264.7 cells were transfected by nucleoporation with 2 mg actin-GFP (peGFP-actin, BD Biosciences Clontech**)** using Amaxa nucleofector II system. 2×10^5 cells were plated on 35 mm glass-bottomed dishes (MatTek) and cultured for 40-48 h prior to imaging in DMEM containing 10% heat-inactivated FBS. To visualize the dynamics of actin at the leading edge, transfected Raw cells were rinsed with PBS and incubated in MEM medium (Sigma), supplemented with 10 mM MES, 10 mM HEPES, 2.4 mM Sodium BiCarbonate ($NaHCO_3$) pH 7.1 and 10 % FBS, at 37 ^{0}C on a temperature controlled stage. Images were acquired using an Olympus inverted microscope equipped with a 60×1.4 N.A. oil immersion objective. Pairs of DIC and fluorescence images were collected every 1 min using time-lapse spinning video microscopy (Perkin Elmer, Ultraview). Shutter and filter wheel timing and position were controlled by MetaMorph Software (Molecular Devices Corporation). Final images and movies were produced using MetaMorph Software and Adobe Photoshop.