Aqueous cigarette smoke extract promotes the adhesion of monocytic cells to human coronary arterial endothelial cells in direct and indirect-dependent ways

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INTRODUCTION

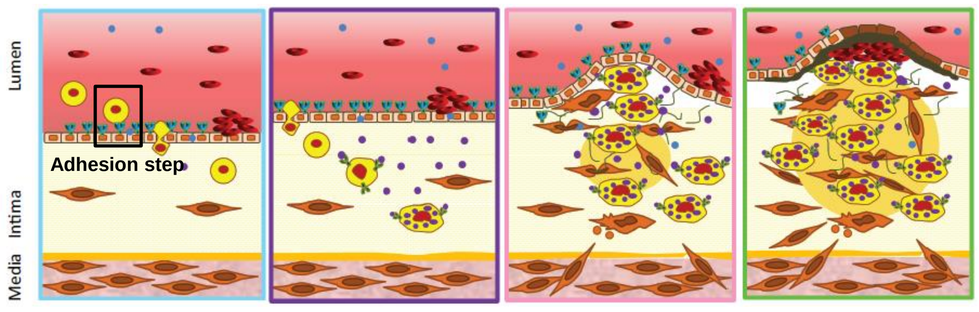
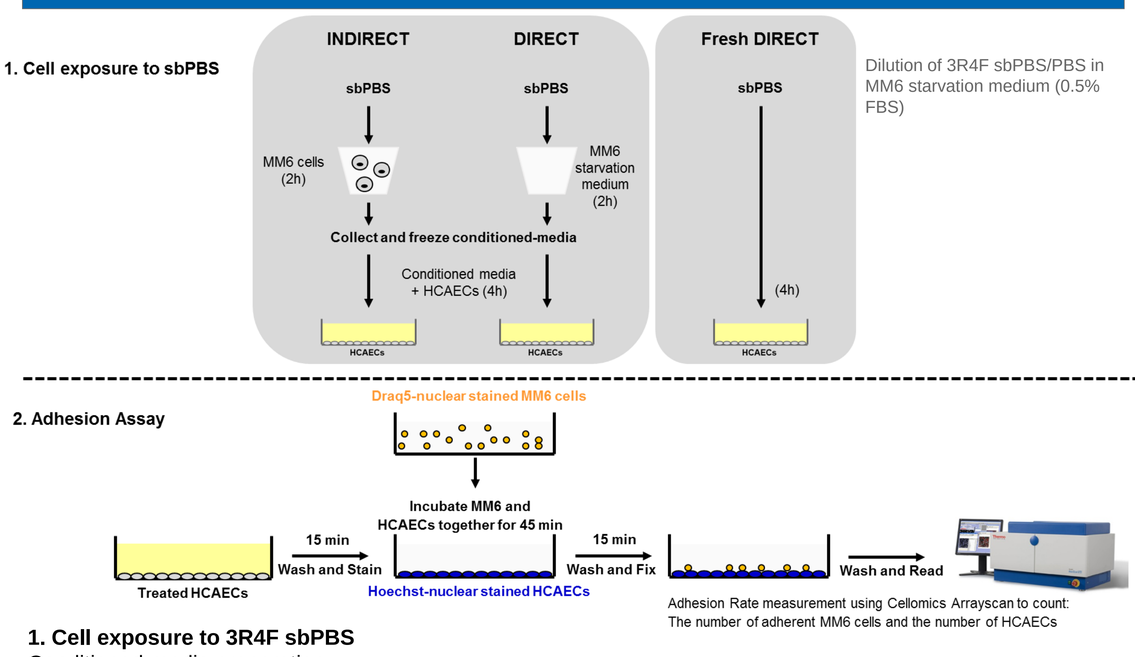


Figure 1: Atherosclerotic plaque development steps

Atherosclerosis is a progressive silent process leading to arterial narrowing. It is the most common cause of myocardial infarction, stroke and peripheral vascular disease. Adhesion of monocytic cells to a "dysfunctional" endothelium (Figure 1) constitutes a critical step in the initiation of atherosclerotic plaque formation (1). Cigarette smoke (CS) has been shown to affect the adhesion of monocytes to endothelial cells (2,3). However, the molecular mechanisms underlying the pathophysiology of monocytic cell adhesion in the presence of CS is poorly understood.

MATERIALS & METHODS



1. Cell exposure to 3R4F sbPBS

Conditioned-media preparation

MM6 cells (2 million/mL) were starved in MM6 starvation medium (0.5% FBS) for 2h and, then exposed for 2h to 3R4F sbPBS (or PBS, 15%v/v). Medium without MM6 cells underwent identical incubation conditions. Both conditioned-media were collected and frozen down.

Treatment of HCAECs

- Indirect (I) and Direct (D) treatments: 24h-starved (0.1% FBS instead of 2%) HCAECs were treated with conditioned-media: thawed PBS/sbPBS exposed-MM6 supernatants or PBS/sbPBS-MM6 starvation medium for 4h.
- Fresh Direct (FD) treatment: 24h-starved HCAECs were exposed to freshly generated 3R4F sbPBS (or PBS, 15%v/v) for 4h.

HCAECs and MM6 lysates were collected and stored at -80°C for RNA extraction.

2. Adhesion Assay

Untreated MM6 cells and 4h-treated HCAECs were nuclear-stained for 15min with Draq5 and Hoechst fluorescent dyes, respectively and then incubated together for 45min. After cell fixing (formaldehyde 4%; 15min) and washing, remaining adherent MM6 cells and HCAECs were counted using a Cellomics ArrayScan instrument. The adhesion rate was calculated as follows: AR=(MM6 cell count/HCAECs cell count) X 100.

3. Other endpoints

Cell viability: MM6 and HCAECs viability was determined using a resazurin assay (Sigma-Aldrich).

Inflammatory mediators: A panel of 45-biomarkers was measured in MM6 supernatants by Myriad-RBM (Austin, TX, USA) using their Human InflammationMAP® v. 1.0 kit.

Transcriptomics: mRNA extracted from MM6 and HCAEC cell lysates was analysed in our transcriptomics laboratory using Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array.

4. Computational analysis

Transcriptomics data were processed using GCRMA R package. Pairwise differential gene expression (contrast) analysis comparing sbPBS vs PBS for each exposure-condition type (I, D, FD) was computed with limma R package. Relative biological impact factor (RBIF) analysis was conducted for each contrast using two-layer causal network models representative of different biological processes (4,5). The approach enables to identify significantly perturbed biological networks, and to quantify their respective contribution to the overall biological impact of the treatment on cells. Additionally, principal component analysis was performed using the fold change matrix.

SUMMARY / CONCLUSION

MM6 cells exposed to 3R4F sbPBS:

- released inflammatory mediators at low concentration, however, with an inhibitory effect of sbPBS at higher concentrations.
- showed regulation of genes involved in (1) inflammatory and oxidative stress response at low concentration, (2) cell cycle arrest / senescence and apoptosis at both concentrations, (3) MAPK signaling (increased and decreased at low and high concentrations, respectively).

The adhesion of MM6 cells to HCAECs was increased with Indirect (low concentration) and Fresh Direct (high concentration), but not in Direct sbPBS treatments.

Additionally, the analysis of HCAECs gene expression changes for Indirect, Direct and Fresh Direct exposure-condition groups revealed the following observations:

- Indirect: MM6-soluble mediators (e.g., TNFα) present in supernatants increased the expression of adhesion molecules (e.g. ICAM1, SELE) probably *via* NFkB-mediated inflammatory response, while CS components alone inhibited their expression as observed in microarray data.
- Fresh Direct: unstable CS compounds present only in freshly generated sbPBS were accountable for the adhesion of MM6 cells to HCAECs, maybe *via* non-classical adhesion molecules (e.g., CERCAM, ICAM3), and completely impaired the expression induction of xenobiotic metabolism genes observed in all other conditions in HCAECs.

Aqueous CS extract promoted the adhesion of monocytic cells to human coronary endothelial cells via distinct mechanisms in indirect and fresh direct exposure conditions.

RESULTS

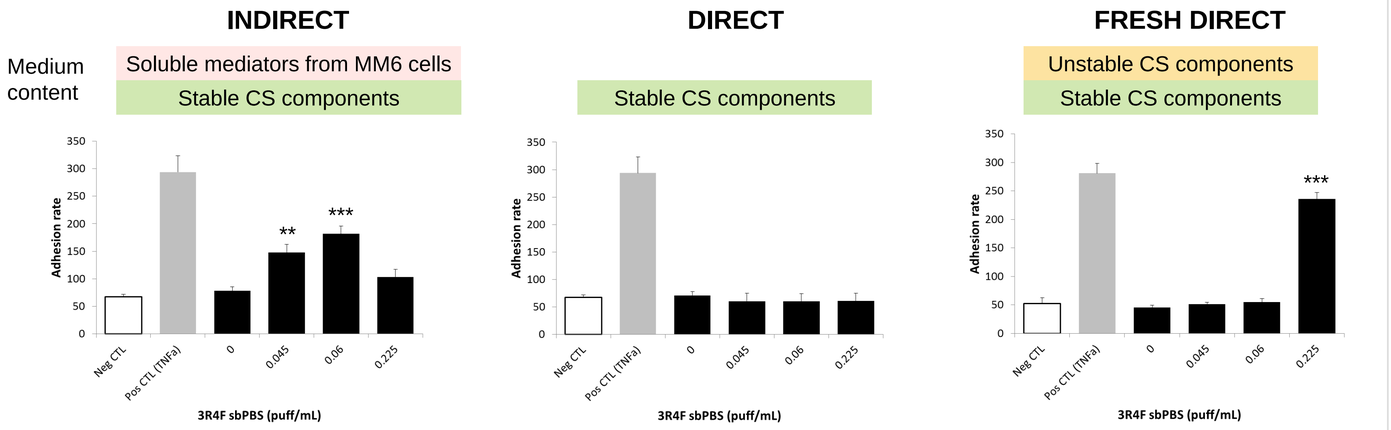


Figure 1: Effect of 3R4F sbPBS on the adhesion rate of MM6 cells to HCAECs after Indirect, Direct and "Fresh" Direct exposure of HCAECs (Mean ± SD; N=1 representative experiment out of 2-3; (n=4). Adhesion rate: number of adherent MM6 cells relative to 100 HCAECs. Neg CTL: starvation medium, negative control; Pos CTL: 10 ng/mL TNFα, positive control. Concentration 0: starvation medium + 15% PBS. *p≤0.05; **p≤0.01; ***p≤0.001 (Bonferroni adjusted p-value). At the highest concentration of 3R4F sbPBS, HCAEC viability exceeded 80%.

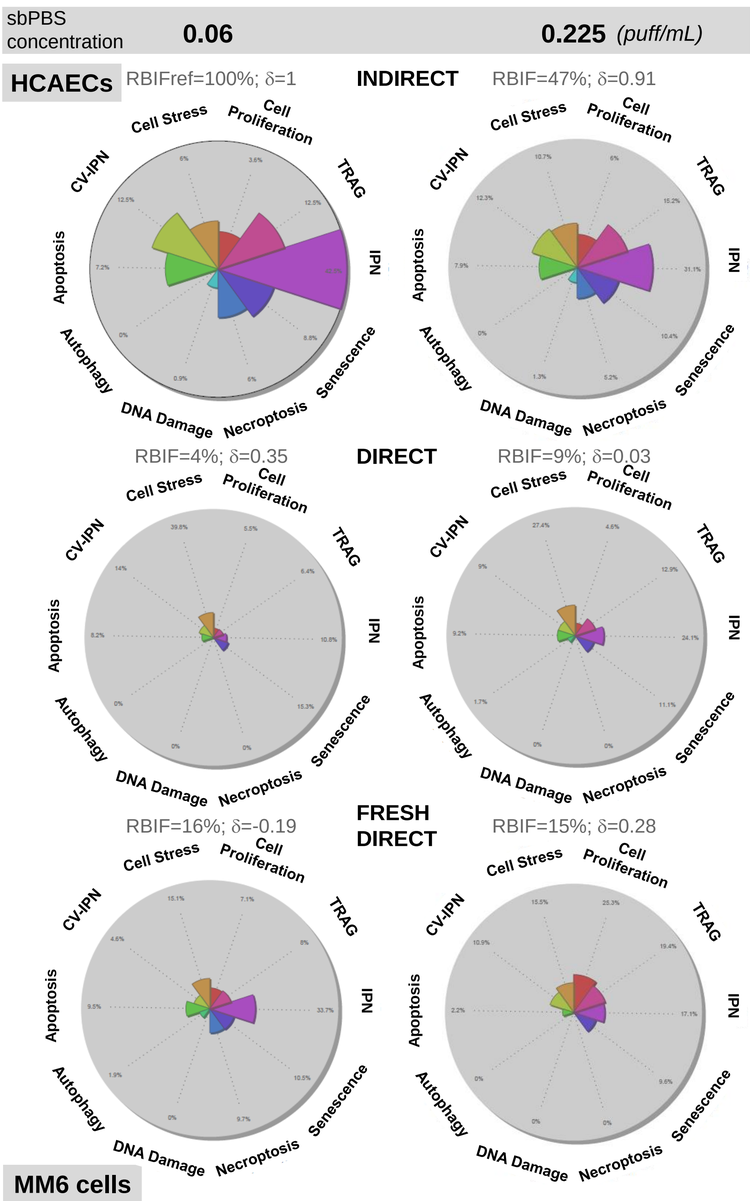


Figure 2: Concentration-dependent biological perturbation induced by 3R4F sbPBS relative to PBS control in HCAECs and MM6 cells. Relative biological impact factor (RBIF) is computed using HCAECs and MM6 gene expression data (comparison PBS vs 3R4F sbPBS) and two-layer causal network models representative of different biological processes (4,5). RBIF of the gene expression comparison that induces maximum network perturbations is automatically set to 100% as a reference (RBIFref). RBIF of other comparisons is expressed as percent of the RBIFref. Colored surfaces covering circular plots are comparable between exposure-conditions within each cell type. For each comparison, the contribution (indicated in percent inside grey circle) of significantly perturbed biological networks to the overall BIF is shown on starplots. The delta (δ, [-1,1]) value reflects how much the underlying perturbed biology modeled in networks is similar to the reference comparison.

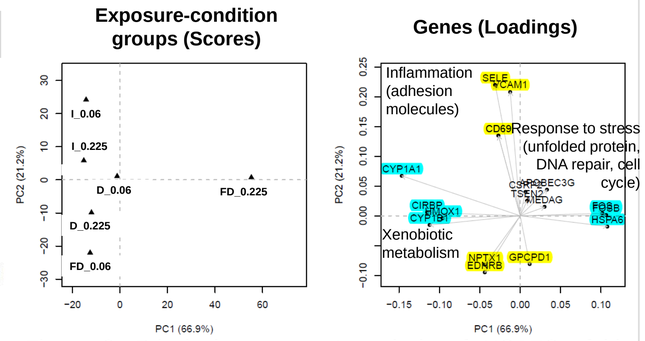


Figure 3: Principal component analysis of HCAECs fold change matrix (data are centered). Left plot: different exposure-condition groups (I: indirect, D: direct, FD: fresh direct) are plotted in PC1-PC2 sub-space accountable for ~88% of the total variance. Right plot: genes that contribute the most to the separation of exposure-condition groups in the first 3 PCs are plotted in the PC1 (cyan)-PC2 (yellow) sub-space (non-colored genes from PC3, 7.7%).

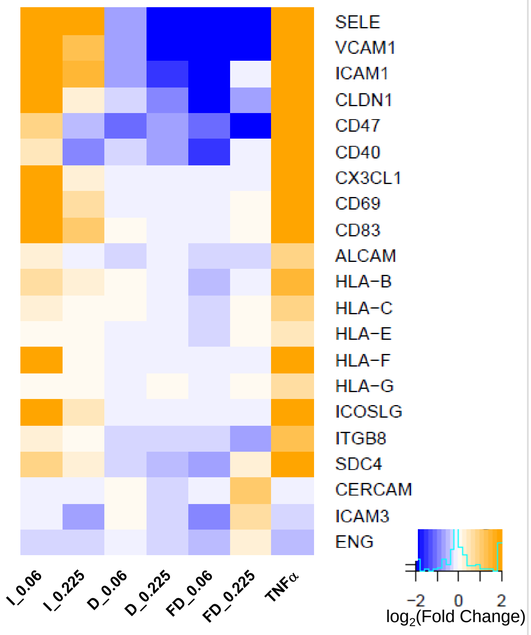


Figure 4: Heatmap of adhesion molecule encoded-gene expression fold changes (as $\log_2$ scale) comparing the effect of 3R4F sbPBS relative to PBS in different exposure-conditions (I: indirect, D: direct, FD: fresh direct).

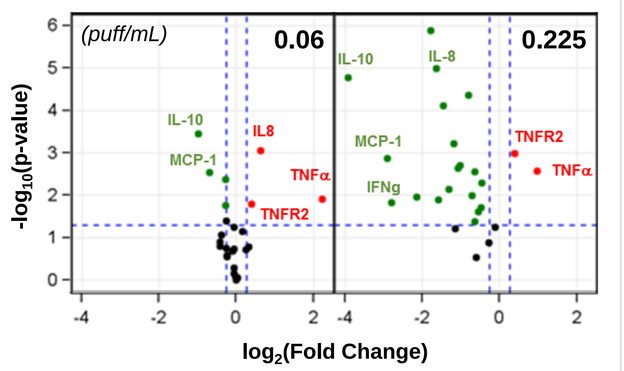


Figure 5: Measure of 45 inflammatory markers released by MM6 cells exposed to 3R4F sbPBS. Fold changes (FC) between PBS-control and 3R4F sbPBS treatment (x-axis) and associated p-values (y-axis) are visualized on volcano plots. Vertical and horizontal dotted lines correspond to FC and p-value thresholds of 1.2 and 0.05, respectively. Up- and down-regulated inflammatory mediators are colored in red and green, respectively. FC correspond to the mean of 3 supernatant replicates.

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Abbreviations. **CS**: cigarette smoke; **sbPBS**: smoke-bubbled phosphate buffered saline; **ICAM-1**: intercellular adhesion molecule 1; **VCAM-1**: vascular cell adhesion protein 1; **MM6**: mono mac 6 cells; **HCAECs**: human coronary arterial endothelial cells; **SD**: standard deviation; **RBIF**: relative biological impact factor. **CV-IPN**: cardiovascular-inflammatory processes network ; **IPN**: inflammatory processes network ; **TRAG**: tissue repair and angiogenesis network.

Eurotox conference – Edinburgh (7th – 10th of September 2014)

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