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Crosstalk Between Co-cultured A549 Cells and THP1 Cells Exposed to Cigarette Smoke

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Abstract

Cigarette smoke (CS) is considered as a major etiological factor in the pathogenesis of chronic obstructive pulmonary disease. In this study we used A549 cells and THP-1 cells grown for 24 h in monoculture or in co-culture in CS-conditioned media and changes in their proliferation, viability, acetylated histone H3 levels and expression of extracellular antigens CD14, HLA-DR, CD11a, and CD11b were assessed. CS was highly toxic to A549 cells but not to THP1 cells. In A549 cells, oxidative stress reached the highest values after 1 h of CS exposure and then decreased. In THP1 cells oxidative stress was lower and increased progressively with time. CS decreased proliferation of A549 and THP1 cells by about 80 % and 21 %, respectively. CS did not alter acetylated histone H3 levels in A549 cells, while in THP1 cells the levels were reduced by about 35 %. CS significantly increased expression of CD14, HLA-DR, CD11a, and CD11b in THP1 cells. In co-culture, naïve or CS-pretreated THP1 cells significantly protected A549 cells against CS toxicity but had higher death rates. These results show that epithelial cells are more fragile to CS than monocytes and that CS-activated monocytes may protect epithelial cells against CS-induced cytotoxicity.

Keywords

A549 cells • Cell culture • COPD • Inflammation • THP1 cells

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1 Introduction

Cigarette smoke (CS) is considered a major etiological factor in the pathogenesis of chronic obstructive pulmonary disease (COPD), which is characterized by a progressive development of airflow limitation. In COPD lowered lung function is associated with local and systemic

inflammation, resistant to anti-inflammatory drugs including steroids. Activated leukocytes, especially macrophages but also T-cells, B-cells, and neutrophils release cytokines, chemokines and proteases, affect physiology of non-immune cells of respiratory tract and produce increased proliferation of lining epithelial cells, airway remodeling and peribronchial fibrosis leading to emphysema (GOLD 2013). Moreover, a number of compounds found in CS can directly damage lung tissue and induce oxidative imbalance, adding noxious exogenous chemical stimuli to complex endogenous inflammation. The molecular mechanism responsible for COPD is unknown and several animal and cellular models were described to mimic human disease and to study different aspects of the disease (Adamson et al. 2011). Cell lines are commonly used in *in vitro* studies to model COPD. It was shown that human bronchial epithelial cell lines NCI-H292, 16HBE14σ, and BEAS-2B are affected when grown in CS-conditioned media and several biochemical and functional alterations have been described (Heijink et al. 2010). In the present study we used human alveolar epithelial cell line A549, which is the most exploited alveolar epithelial cell line. In our experimental model, naïve or CS-treated A549 cells were grown in monoculture or in co-culture with intact or CS-exposed THP-1 cells, a human monocyte cell line which is able to produce inflammatory mediators, to examine how both cell types respond to CS and how naïve and CS pretreated cells, which share common culture medium, interact in a co-culture system that does not allow physical contact between both cell types.

2 Methods

2.1 Cell Culture

Two types of cells were used: A549 (ATCC® CCL185™) cells growing in ATCC-formulated F12K medium supplemented with 10 % fetal bovine serum (FBS) and THP1 (ATCC® TIB202™) cells growing in ATCC-formulated RPMI 1640 medium, supplemented with 2-mercaptoethanol to a final concentration of 0.05 mM and with FBS to a final concentration of 10 %. Cells were maintained in 37 °C in an incubator in a humidified atmosphere containing 5 % CO_2. For particular experiments, cells were plated out onto 6, 24, or 96 well plates and grown in control or smoke conditioned media for 24 h.

2.1.1 Cell Co-cultures

Naïve A549 cells or A549 cells grown for 24 h in smoke-conditioned media were co-cultured in 6-well cell culture plates with naïve or smoke-pretreated (24 h) THP1 cells. THP1 cells were added to the co-culture inserts (Translucent PET membrane, RoTrac; Greiner Bio-one, Courtaboeuf, France) which were placed in upper parts of culture dishes. The pores of membranes in co-culture inserts were 0.4 μm large and did not allow THP1 cells to pass and to contact physically with A549 cells. Cells were kept in co-culture in CS-free medium for 24 h.

2.2 Preparations of CS-Conditioned Media and Cells Treatment

The smoke of four full-strength Red Marlboro cigarettes (Phillip Morris, Cracow, Poland) containing 8.0 mg of tar, 0.6 mg of nicotine and 9.0 mg of carbon monoxide per cigarette was passed through 100 ml of culture media using low pressure vacuum pump. Cigarette filters were removed before the procedure. The pump pressure was set to give a combustion time for each cigarette of about 1 min. To ensure similar level of smoke saturation between different batches of smoke-conditioned media, the nitrate/nitrite levels were measured in media using Griess reagent. Freshly prepared stem cell were diluted with standard media to obtain 30 μM nitrate/nitrite content in each batch. Stem cell media were subsequently sterilized using 0.22-μm filters and were used immediately to cell culture. A549 or THP1 cells were plated at low density in 6 or 24-well plates, and 24 h after seeding cultures were switched to smoke conditioned media and were further incubated

maximally for 24 h. In some experiments cell treatment was shorter to acquire time-effect data. Samples of culture media were collected during incubation to estimate the activity of lactate dehydrogenase (LDH). In co-culture experiments naïve or CS-pretreated (24 h) A549 cells were grown without physical contact with naïve or CS-pretreated (24 h) THP1 cells which were added to co-culture inserts allowing to diffuse soluble molecules to common culture medium (smoke free 1:1 mixture of F12K and RPMI 1640). Cells were kept in co-culture for 24 h.

2.3 Cell Growth, Proliferation, and Viability

Cell growth and proliferation was quantified in flow cytometry (Epics XL flow cytometer, Coulter Electronics, High Wycombe, UK) using propidium iodide DNA staining and cell cycle analysis (Brown et al. 1996). Histograms of propidium iodide fluorescence distributions were quantified using MultiCycle software and cells were quantified by their relative distribution in the damaged-subdiploid ('early' G0/G1 cells), diploid (G0/G1 zone)-pre-DNA synthesis/resting, S-phase-DNA synthesis, and G2/M-post-DNA-synthesis/mitosis phases (Fig. 1). Each histogram was derived from analysis of 5,000 cells and six samples were analyzed in each group. Cytotoxicity was expressed as a fraction of damaged – 'early' G0/G1 cells while proliferating cells were quantified as S + G2/M cells.

Cell viability was quantified after 24 h of cell growth in CS-conditioned media using mitochondrial-dependent MTT (3-[4,5-dimethylthiazolyl-2] 2,5-diphenyltetrazolium-bromide) reduction to purple formazan, with colorimetric detection (Niks and Otto 1990). Changes in absorbance in viable cells were measured at 570 nm, with 630 nm as a reference wavelength. Cell viability was estimated as a percentage of the control. Time-dependent toxicity was assessed using lactate dehydrogenase (LDH) release assay (LDH cytotoxicity kit, ScienCell,

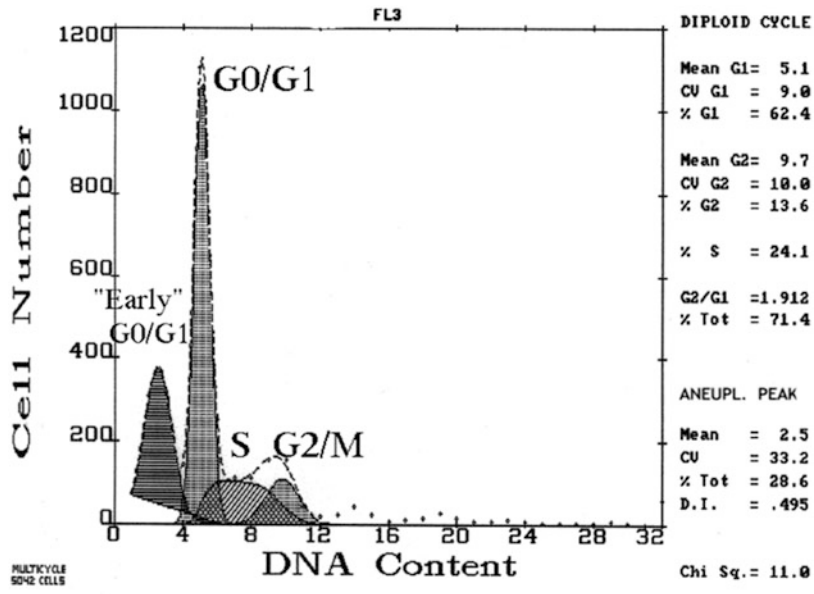

Fig. 1 MultiCycle software-transformed histogram of propidium iodide-DNA fluorescence of THP1 cells grown for 24 h in co-culture with A549 cells. Original histogram was obtained using epics XL flow cytometer. Gating was set for the control probe (THP1 cells) and applied to all experimental samples. Cell distribution was quantified using MultiCycle software as subdiploid ('early' G0/G1 cells), diploid (G0/G1 peak)-pre-DNA synthesis/resting, S-phase-DNA synthesis, and G2/M-post-DNA-synthesis/mitosis phases

Carlsbad, CA). LDH release to the culture medium was compared to total enzyme activity in sonicated cells.

2.4 Oxidative Stress

Reactive oxygen intermediates were quantified using dichlorodihydrofluorescein diacetate (H2DCFDA; Sigma-Aldrich, St. Louis, MO) (Ubezio and Civoli 1994). Cells were loaded with 5 μM H2DCFDA for 30 min, washed, resuspended in phosphate-buffered saline, and assayed by flow cytometry. Green dichlorofluorescein (DCF) fluorescence was captured on F11 channel of flow cytometer (Epics XL, Coulter Electronics, High Wycombe, UK) and registered as histograms of fluorescence distribution.

2.5 Expression of Acetylated Histone 3 and Extracellular Activation Markers

Acetylated histone H3 levels were measured in formaldehyde (1 %)-fixed and ethanol (70 %)-refixed cells with acetylated histone H3 (AcH3)-specific monoclonal fluorescent antibodies (Acetyl-Histone H3 (Lys9) Antibody; Rabbit mAb Alexa Fluor 488 Conjugate; Cell Signalling Technology Inc., Danvers, MA), corresponding isotype control antibody, and flow cytometry (Coulter Electronics, High Wycombe, UK) detection (Ronzoni et al. 2005).

The percentages of $CD14^+$, $HLA\text{-}DR^+$, $CD11a^+$, and $CD11b^+$ THP1 cells were determined using specific monoclonal fluorescent antibodies (Beckman-Coulter, Warsaw, Poland), corresponding isotype controls, and flow cytometry detection. Cells were diluted to 10^5 cells per ample and 10 μl of a commercial antibody solution was added to cell suspension and allowed to bind for 30 min at room temperature in darkness. The cells were washed with phosphate buffered saline, fixed with CellFIX™ (Becton Dickinson, Oxford, UK) and run on an Epics XL flow cytometer (Coulter Electronics, High Wycombe, UK). Three thousand total events were collected per sample.

2.6 Statistical Analysis

Results were expressed as means ± SD of 6–10 assays. Statistical analysis was performed with a statistics package-Statistica 6.0 software (Statsoft, Cracow, Poland) using one-way or two-way ANOVA followed by Bonferroni *post-hoc* tests for selected pairs of data. A p value of less than 0.05 was considered statistically significant.

3 Results

Cytotoxicity of CS applied for 24 h to the cells was tested initially by the MTT test (Table 1). In A549 cells grown for 24 h in CS-conditioned medium very significant toxicity was detected. MTT values in this group were lower by about 78 % ($p < 0.01$), while no toxicity was noticed in THP1 cells. Since cytotoxicity data in the MTT test reflect not only cell damage but also alterations in cell proliferation, CS cytotoxicity to A549 cells was further characterized in time-dependent experiments. Increased LDH levels were found in the culture medium of A549 cells exposed to CS already after 1 h of cell treatment ($p < 0.05$). Then, LDH levels increased with incubation time to reach 36 % ($p < 0.01$) of total enzyme activity (sonicated cells) after 24 h of cell treatment.

We also examined oxidative stress in cells grown in CS-conditioned media. In A549 cells, the stress was the highest after 1 h. At that time registered values of DCF fluorescence were more than 8 times higher ($p < 0.01$) than baseline reference values. In the 6th hour, oxidative stress was still very high (increased by more than 5 times; $p < 0.01$) and then lower values were observed, but after 12 h of cell growth in CS-conditioned media the stress was still

Table 1 Cigarette smoke (CS) cytotoxicity (MTT test), proliferation (PI-DNA assay), oxidative stress (DCF fluorescence), expression of acetylated histone H3 (flow cytometry) and expression of extracellular markers of immune activation (flow cytometry) in control alveolar epithelial cells (A549) and in human monocyte cell line (THP1) as well as in cells grown in smoke-conditioned medium for 24 h

	A549 cells		THP1 cells	
	Control	CS	Control	CS
MTT (% of control)	100 ± 16	22 ± 6**	100 ± 17	118 ± 24
LDH (% of total activity)				
1 h	–	11 ± 5*	–	–
6 h	–	18 ± 7**	–	–
12 h	–	27 ± 9**	–	–
24 h	–	36 ± 11**	–	–
Oxidative stress (relative units)				
1 h	100 ± 11	844 ± 55**	100 ± 16	153 ± 66**
6 h	100 ± 13	527 ± 66**	100 ± 17	187 ± 34**
12 h	100 ± 17	161 ± 41*	100 ± 21	221 ± 42**
24 h	100 ± 19	62 ± 33*	100 ± 18	266 ± 48**
Cytotoxicity (% of 'early' G0/G1 cells)	7 ± 3	47 ± 11**	6 ± 3	11 ± 6*
Proliferation (% of S-G2/M cells)	43 ± 7	9 ± 3**	27 ± 5	19 ± 4*
Expression of acetylated histone H3 (relative units)	100 ± 14	85 ± 17	100 ± 19	65 ± 17**
$CD14^+$ (% of cells)	–	–	5	94 ± 11**
$HLA\text{-}DR^+$ (% of cells)	–	–	5	47 ± 9**
$CD11a^+$ (% of cells)	–	–	5	69 ± 10**
$CD11b^+$ (% of cells)	–	–	5	46 ± 8**

To visualize time-dependent cell membrane damage and alterations in oxidative stress during 24 h of cell growth in CS-conditioned medium Lactate dehydrogenase (LDH) liberation to the culture medium and oxidative stress were assessed in the 1st, 6th, 12th, and 24th hour of experiment. All other parameters were quantified after 24 h of cell growth in CS-conditioned medium

*$p < 0.05$; **$p < 0.01$ for comparisons with the corresponding control cells

significant (increased by 61 %; $p < 0.05$). After 24 h, the DCF fluorescence was lower than control values but at that time DCF fluorescence histograms became broad and bimodal (results not shown) due to significant toxicity. In THP1 cells, oxidative stress increased progressively with time attaining the highest values (about 2.5 times higher than baseline) after 24 h of cell treatment with CS.

Table 1 shows flow cytometry data of PI-DNA fluorescence reflecting both cytotoxicity of CS and changes in proliferation rates of A549 and THP1 cells grown for 24 h in CS-conditioned media. Damaged cell numbers were assessed as 'early' G0/G1 cells, while cell proliferation was quantified as fractions of S + G2/M cells (Fig. 1). About 47 % of A549 cells were damaged ($p < 0.01$) by CS, while in THP1 cells the fraction of damaged cells was about 11 % ($p < 0.05$). CS decreased cell proliferation particularly in A549 cells, where an almost 81 % ($p < 0.01$) decrease in cell growth dynamics was observed comparing to control cells. In THP1 cells, proliferation was also reduced, by about 21 % ($p < 0.05$).

Next we determined the expression of acetylated histone H3 (AcH3) in both cell types. CS did not alter AcH3 levels in A549 cells, while in THP1 cells AcH3 level was reduced by about 35 % ($p < 0.01$). It should be stressed however, that expression of AcH3 was assessed only in viable cells. Incubation of THP1 cells in a smoke-conditioned medium resulted in increased expression of antigens typical for monocyte activation. At baseline, 5 % of cells in each sample were set as antigen positive. CS treatment significantly increased CD14-positive cell numbers (to 95 % of cells; $p < 0.01$), HLA-DR

Table 2 Cytotoxicity and alterations in cell proliferation in naïve or CS-treated A549 or THP1 cells grown in co-cultures

	A549 cells		THP1 cells	
	Cytotoxicity (% of 'early' G0/G1 cells)	Proliferation (% of S + G2/M cells)	Cytotoxicity (% of 'early' G0/G1 cells)	Proliferation (% of S + G2/M cells
Control	5 ± 2	42 ± 6	7 ± 3	22 ± 5
CS	48 ± 7**	2 ± 1**	14 ± 3**	29 ± 6*
A549 + THP1	8 ± 3	30 ± 6*	22 ± 4**	34 ± 7**
$A549^{CS}$ + THP1	18 ± 4**$^{\#\#}$	10 ± 3**$^{\#\#}$	27 ± 6**$^{\#\#}$	27 ± 8
A549 + $THP1^{CS}$	5 ± 2	26 ± 5**	26 ± 6$^{\#\#}$	49 ± 8^{++}**
$A549^{CS}$ + $THP1^{CS}$	24 ± 6$^{\#\#}$	9 ± 3$^{\#\#}$	53 ± 9$^{\#\#\wedge\wedge}$	19 ± 4$^{\#\#\wedge\wedge}$

Naïve or CS-pretreated (24 h) A549 cells were grown in co-cultures without physical contact with naïve or CS-pretreated (24 h) THP1 cells which were grown in co-culture inserts allowing to diffuse soluble molecules to common culture medium (1:1 mixture of F12K and RPMI 1640 media). Cells were kept in co-culture in cigarette smoke-free medium for 24 h

*$p < 0.05$; **$p < 0.01$ for comparisons with the corresponding control cells

$^{\#\#}p < 0.01$ for comparisons with the CS-treated cells

$^{++}p < 0.01$ for comparisons with A549 + THP1 group

^^$p < 0.01$ for comparisons with $A549^{CS}$ + THP1 group

was expressed in 47 % ($p < 0.01$) of cells, while corresponding values in CD11a and CD11b were 69 % ($p < 0.01$) and 46 % ($p < 0.01$), respectively.

In co-culture experiments we estimated CS cytotoxicity using propidium iodide DNA staining and alterations in cell proliferation (Table 2). Co-incubation of CS-pretreated A549 cells with naïve THP1 cells or THP1 cells pretreated with CS resulted in significantly lower ($p < 0.01$) toxicity and increased ($p < 0.01$) but not normalized cell proliferation. Naïve THP1 cells and CS-pretreated THP1 cells exerted similar cytoprotection to CS-pretreated A549 cells. CS was toxic ($p < 0.01$) to THP1 cells grown in co-culture medium and increased ($p < 0.05$) THP1 cell proliferation. In THP1 cells, CS toxicity was about 2 times higher when CS–pretreated cells were incubated in co-culture with A549 cells ($p < 0.01$) and almost 4 times higher ($p < 0.01$) when CS–pretreated THP1 cells were grown in co-culture with CS-pretreated A549 cells. Both naïve A549 cells and CS-pretreated A549 cells exerted similar cytotoxic effect, but only to naïve THP1 and not to CS-pretreated THP1 cells coincubated with naïve or smoke-pretreated A549 cells, where striking ($p < 0.01$) differences in THP1 cells growth and cytotoxicity were observed.

4 Discussion

This study demonstrated that the alveolar epithelial cell line and monocyte cell line significantly differed in their response to CS. When naïve or CS-pretreated cells were grown in a co-culture system physically separating monocytes from epithelial cells, changes in cell viability and growth rates were observed. A substantial fraction of epithelial cells died in the first few hours of culture in CS-conditioned medium, while monocytes not only survived but also became activated and to some extent protected epithelial cells against CS-induced cytotoxicity.

Alveolar epithelial cell line A549 is considered as relatively resistant to chemical-induced cytotoxicity, but it has been shown that CS induces apoptosis and alters immunity of A549 cells (Sohn et al. 2009). It should be stressed that CS extract contain several toxic compounds as nicotine, acrolein, formaldehyde, hydrogen cyanide, polycyclic aromatic hydrocarbons, and nitrosamines (Moylan et al. 2013) and A549 cells have low activity of cytochrome P450 enzymes, which are responsible for metabolism of several xenobiotics (Yatzeck et al. 2008). Published CS toxicity data in A549 cells are highly variable

depending on experimental systems and smoke exposure, but it has been reported that CS extract dose-dependently decreases glutathione concentration, increases 4-hydroxy-2-nonenal levels, and induces necrosis in A549 cells (Kode et al. 2006). In our experimental model, cell exposure to CS was rather extensive and nitrate/nitrite levels in smoke-conditioned media were relatively high comparing to other models (Naik et al. 2014). Nonetheless, our smoke-conditioned medium was not toxic to the monocyte cell line, which apparently is more resistant to noxious compounds of CS. It is possible that in A549 cells CS cytotoxicity starts with chemically induced oxidative stress and is followed by cell membrane damage as evidenced by changes in DCF fluorescence assay and LDH release. A major role in CS cytotoxicity in A549 cells may be played by acrolein and hydrogen peroxide (Aoshiba and Nagai 2003). Monocytes are more resistant to oxidative stress, but activation of THP1 cells by CS may be mediated by nicotine, which is able to activate immune cells (Zhou et al. 2013). It has been shown that antioxidants prevent CS toxicity in A549 cells (Banerjee et al. 2008) and decrease protein damage, inflammation, apoptosis, and lung injury in smoke-exposed animals (Rahman 2012), supporting the major role of oxidative stress in CS-induced cytotoxicity.

Another parameter, which may be related to altered inflammatory signaling and steroid resistance in COPD is acetylated histone H3 (Sundar et al. 2013). Histones are responsible for transcriptional regulation of inflammatory signaling and it has been shown that acetylation of core histones may increase expression of inflammatory genes in inflammatory lung diseases (Marwick and Chung 2010). In our model, decreased levels of acetylated histone H3 were detected in monocytes exposed to CS but not in epithelial cells, where only slight but not significant decrease was observed. Our data show that histone acetylation may depend on the cell type. It should be stressed that we quantified acetylated histone H3 in viable cells, without strenuous cell homogenization and histone extraction, which may affect labile histone acetylation status. The quantity of acetylated histones may be relevant to inflammation in COPD and experimental data indicate that in COPD histones are hypercetylated due mostly to decreased activity of histone deacetylases (Yao and Rahman 2012). Recently published data seem to support our observations, since it has been shown that a potential major carcinogen of CS-acrolein inhibits acetylations of N-terminal tails of cytosolic histones H3 and H4 (Chen et al. 2013). It seems that the role of epigenetic signaling in COPD may be different in immune and non-immune cells and should be further evaluated.

CS has been shown to either activate or inhibit activation of cells in culture (Adamson et al. 2011). We have shown that smoke increases expression of CD14, HLA-DR, CD11a, and CD11b antigens on THP1 cells. In COPD, there is increased expression of adhesion molecules and increased differentiation of inflammatory cells (GOLD 2013). Clinical studies also evidenced specific distribution of adhesion molecules in the airways and parenchyma that was consistent with the inflammatory response (González et al. 1996). It has been shown that CS activates human monocytes and macrophages to release chemokines and increases proinflammatory potential of cytokines and tumor necrosis factor-α (Walters et al. 2005). Another study has shown that after 24 h of CS exposure more than 300 genes in THP1 cells are activated, while a similar number of genes is repressed including inducible antioxidants, chaperone proteins, and the ubiquitin/proteosome proteins (Wright et al. 2012). It seems that increased adherence of monocytes may help to protect injured or damaged cells but also to induce an allergic response in the airways.

There are only few studies on airway cell co-cultures exposed to CS. In a co-culture model using A549 cells and fetal lung fibroblasts, low CS concentrations have induced epithelial-mesenchymal transition, observed but in co-cultured A549 cells and not in cell monoculture (Wan et al. 2009). When human alveolar epithelial type II (AT-II) cells were co-cultured with human pulmonary microvascular endothelial

cells, AT-II cells differentiated into AT-I like cells (Hermanns et al. 2009). In another study, lung microvascular endothelial cells (MVECL) grown in co-culture with AT-I cells have been treated with CS extract. Endothelial cells have demonstrated about 50 % reduction in hydrogen peroxide production comparing to monocultures (Downs et al. 2011). Also in our model, co-incubation of CS-pretreated A549 cells with naïve THP1 or CS-pretreated THP1 cells decreased CS cytotoxicity to A549 cells and partly restored A549 cell proliferation. Considering CS cytotoxicity in THP1 cells exposed to CS in monoculture or in co-culture, there was no toxicity when cells were exposed to CS in a dedicated medium and only small toxicity when THP1 cells were grown in co-culture medium mixture (1:1). It should be stressed that CS cytotoxicity becomes more relevant in co-culture with naïve A549 cells and remarkably high when smoke pretreated THP1 cells were co-cultured with smoke pretreated A549 cells.

In conclusion, we demonstrated that during co-culture of A549 cells and THP1 cells there is a bi-directional crosstalk between both cell types cells *via* medium-soluble mediators and that THP1 cells may to some extent protect A549 cells against CS toxicity.

Conflicts of Interest The authors had no conflicts of interest to declare in relation to this article.

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