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Letter to the Editor

Immunomodulatory effects of cigarette smoke condensate in mouse macrophage cell line

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Abstract

Increasing evidence has demonstrated that the secretion of cytokines may be associated with cigarette smoke–induced immunomodulatory effects, but a comprehensive analysis of the cytokine profile for cigarette smoke condensate (CSC) exposure is lacking. The aims of this study were to (1) examine the release of 20 cytokines induced by CSC from 12 brands of cigarettes in macrophages cells (Ana-1) and (2) to investigate the general characteristics of the immunomodulatory effects of CSC. Luminex technology was used to simultaneously determine the levels of 20 cytokines (interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), keratinocyte-derived Chemokine (KC), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), induced protein 10 (IP-10), tumor necrosis factor α (TNF-α), vascular endothelial growth factor (VEGF), monkine inducible by γ interferon (MIG), and fibroblast growth factor (FGF)-basic) in the supernatants from Ana-1 cells treated with the CSC. The results showed that the release of eight cytokines was altered (IL-5, IL-6, IL-12, TNF-α, VEGF, IP-10, MCP-1, and MIP-1α) compared with the control. These cytokines fall into two major subtypes: proinflammatory cytokines, including IL-5, IL-6, IL-12, TNF-α, and VEGF, and chemokines, including IP-10, MCP-1, and MIP-1α. Compared with control, the remaining 12 cytokines were not significantly affected by CSC from the 12 brands of cigarettes. As a general characteristic, CSC exerts potently suppressive immunomodulatory effects on cytokine production of Ana-1 cells. Proinflammatory cytokines and chemokines may account for or contribute to the immunosuppressive properties of CSC.

Keywords

cigarette smoke condensate, cytokines, immunomodulatory, macrophage cells

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Introduction

Tobacco smoke is a complex mixture of thousands of different chemicals, many of which have immunotoxicity.[1] Many constituents of cigarette smoke not only modulate the function of immune cells in vitro and/or after in vivo administration[2] but also impact the immunomodulatory response in a variety of experimental animal models and humans.[3] Increasing evidence has demonstrated that the effects of cigarette smoke on the immune system

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Table 1. Characteristics of study samples.

Brand	Type	Specificity			Origin
		Tar[a]	Nicotine[a]	CO[a]	
C1	Blended-type cigarette	9	0.7	11	Anhui Province, China
C2	Flue-cured cigarette	12	1.1	12	Yunnan Province, China
C3	Flue-cured cigarette	10	1.0	13	Hunan Province, China
C4	Flue-cured cigarette	11	1.1	13	Hubei Province, China
C5	Flue-cured cigarette	10	1.0	11	Henan Province, China
C6	Blended-type cigarette	7	1.0	9	Fujian Province, China
C7	Flue-cured cigarette	11	1.0	11	Zhejiang Province, China
C8	Flue-cured cigarette	6	0.6	8	Fujian Province, China
C9	Blended-type cigarette	11	1.0	12	Guangdong Province, China
C10	Flue-cured cigarette	8	0.8	9	Yunnan Province, China
C11	Flue-cured cigarette	8	0.8	10	Jilin Province, China
C12	Blended-type cigarette	8	0.7	10	Beijing, China

[a]Label of cigarette case.

may reflect the cumulative effects of both immunosuppressive and immunostimulatory components in cigarette smoke. Cytokines are small molecules with large roles in modulating immune reactions. Macrophages may be activated by cigarette smoke condensate (CSC) to release inflammatory mediators. Several studies have shown that cigarette smoking is associated with mitogenic responses in lung lymphocytes and the production of interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α) by peripheral blood mononuclear cells (PBMCs) and macrophages.[4] Macrophages play an important role in all organisms and are essential mediators of the immune system that contribute to inflammation and regulate tissue homeostasis.[5] As one type of mouse macrophages, the Ana-1 cell line has been used to assess toxicological effects due to cigarette smoke.[6] Macrophages play a major role in releasing cytokines, which regulate innate, adaptive immunity, cell growth, and tissue destruction.

The aim of this study was to investigate the general characteristics of the immunomodulatory effects of CSC. We selected the Ana-1 cell line and used Luminex technology to assess the release of 20 cytokines (IL-1a, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), keratinocyte-derived chemokine (KC), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), induced protein 10 (IP-10), TNF-α, vascular endothelial growth factor (VEGF), monkine inducible by γ interferon (MIG), and fibroblast growth factor (FGF)-basic) from macrophages stimulated with CSC from 12 brands of cigarettes.

Materials and methods

Reagents and equipment

Ana-1 is a macrophage cell line, isolated from mouse thymus, obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (China). RPMI 1640 culture medium and fetal bovine serum (FBS) were purchased from Gibco Co. Ltd (USA). The Cell Counting Kit-8 (CCK-8) was purchased from the Tokyo Institute of Japan. The Mouse Cytokine 20-Plex Panel was purchased from Life Technologies (USA). A high-speed centrifuge was purchased from Thermo Fisher Scientific (USA). A Bio-Plex Liqui Chip was purchased from Bio-Bad (USA), and 96-well plates were purchased from Corning Costar (USA). RM-20H smoking machine was purchased from Borgwaldt KC (Germany). Fully humidified incubator was purchased from Thermo Fisher Scientific (Germany). The 12 brands of cigarettes used are shown in Table 1.

Preparation of CSC

Cigarettes of 12 brands were conditioned unpacked in open containers following ISO standards[7] (at least 48 h at target conditions of 22°C ± 1°C and a relative humidity of 60% ± 3%). Borgwaldt RM-20H smoking machine was used to collect cigarette smoke. Total particulate matter was collected on glass fiber filters extracted with dimethyl sulfoxide (DMSO) followed by sterile filtration and then stored at −70°C.

Cell culture

Ana-1 cells were cultured in RPMI 1640 medium, which was supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C in a fully humidified incubator containing 5% CO_2.

Cell inhibition assay

The exposure experiment of Ana-1 cells to CSC with the concentrations of 0, 12.5, 25, 50, 100, and 200 μg/mL was conducted. Cell viability was measured by CCK-8 assay. Each sample was assayed in sixtuplicate, and the values were expressed as mean of six experiments. Next, 2×10^5 cells/well were seeded into a 96-well culture plate at a final volume of 200 μL. After 24 h of treatment, 10 μL of CCK-8 was added into each well and incubated for 90 min at 37°C in a 5% CO_2 incubator. The absorbance at 490 nm was measured using a microplate reader. The inhibition rate was calculated using the following equation: Inhibition rate = [(experimental group − blank group)/(control group − blank group)] × 100%. An inhibition curve was plotted based on the CSC concentration and the inhibition rate. The concentrations of CSC selected (cell viability is more than 80%) were used for testing cytokines in this study.[8]

Detection of cytokines after treatment with CSC

Detection of cytokines of CSC treated with the concentrations of 0, 2.5, 5, 10, and 20 μg/mL was assessed. The assay was performed in five replicates for each group. Next, 2×10^5 cells/well were seeded into a 96-well culture plate to a final volume of 200 μL. After 24 h of treatment, cells were centrifuged at 1000 *g* for 10 min and then cell-free supernatants were recovered and stored at −80°C for subsequent analysis. All assays were performed according to the Mouse Cytokine 20-Plex Panel kit instructions. Median fluorescence intensities were collected on a Luminex-100 instrument using Bio-Plex Manager software version 6. Standard curves for each cytokine were generated using the premixed lyophilized standards provided in the kits. Cytokine concentrations in samples were determined from the appropriate standard curve. Each sample was run the five replicates, and the average of these replicates was used as the present data.

Statistical analyses

The results are shown as the mean of the observed concentration of cytokines for CSC. A statistical analysis of the results was performed, and the statistical significance of the differences was tested using Fisher's test. Differences between groups were considered significant at $P < 0.05$, and the actual P values are indicated for each series of experiments. A complete statistical analysis of the data was conducted with the statistical software package, SPSS Statistics 19.0 (IBM, USA).

Results

Effect of CSC exposure on viability

CSC differentially induced cytotoxicity and reduced cell viability in a concentration-dependent manner in the macrophage cell line Ana-1, as measured by CCK-8 assay. For 12 brands of CSC, CCK-8 assay indicated that the number of Ana-1 cells in the CSC-treated group was lower than that of the control group ($P < 0.05$). CSC significantly inhibited the proliferation of Ana-1 cells after 24 h of treatment. The data are shown in Figure 1.

Levels of cytokines after exposure to CSC from 12 brands of cigarette

Compared with control (Table 2), the release of IL-5, IL-6, IL-12, TNF-α, IP-10, MCP-1, MIP-1α, and VEGF (expect C10) (Table 3) was significantly different for all 12 brands of cigarettes ($P < 0.05$). However, there was no significant difference between some cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-10, IL-13, IL-17, GM-CSF, IFN-γ, KC, MIG, and FGF-basic) and control ($P > 0.05$) (Table 2).

Cytokine profiling of lower limits of detection with CSC treatment

Lower limits of detection for 12 brands of CSC were IL-6 and IP-10, which were significantly reduced compared with the control group (⩽10 μg/mL). For CSC from all 12 brands, relatively low amounts (5–20 μg/mL) of IL-5, IL-12, and MCP-1 were detected. In contrast, the lower limits of detection for VEGF, MIP-1α, and TNF-α were not very sensitive (⩾20 μg/mL). The results are shown in Table 3.

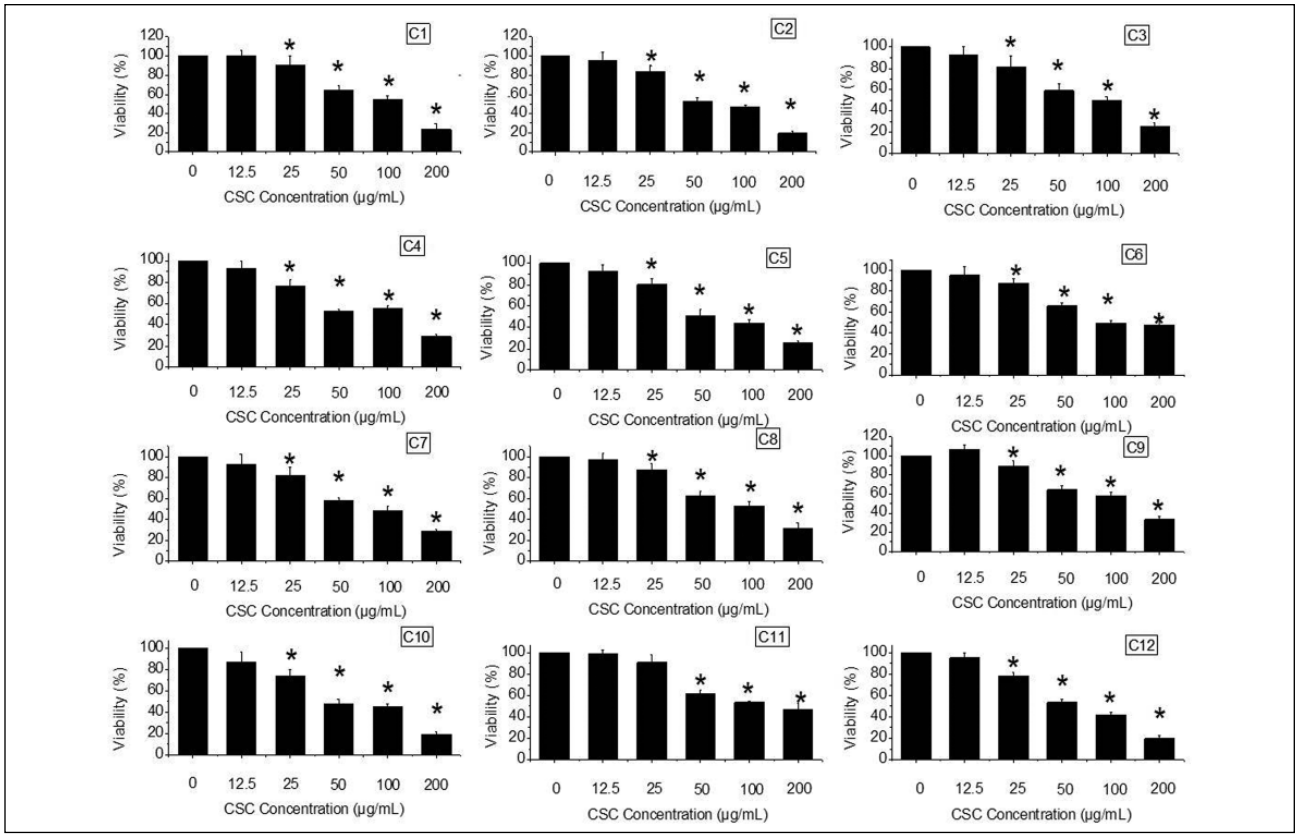

Figure 1. The results show cell viability after exposure for 24 h to CSC from 12 brands (n = 6) as the mean ± standard deviation.

Table 2. Release of 20 cytokines for the control (non-stimulated macrophages).

Cytokine	Release level (mean ± SD) (pg/mL)
FGF-basic	192.47 ± 8.20
GM-CSF	23.98 ± 1.40
IFN-γ	25.18 ± 2.14
IL-1α	36.49 ± 1.96
IL-1β	91.78 ± 6.98
IL-2	33.75 ± 2.37
IL-4	240.59 ± 18.23
IL-5	1092.68 ± 106.58
IL-6	5137.66 ± 257.06
IL-10	1297.45 ± 84.43
IL-12	173.01 ± 16.31
IL-13	108.56 ± 9.36
IL-17	7.56 ± 0.64
IP-10	3332.97 ± 678.80
KC	1004.74 ± 41.33
MCP-1	3498.78 ± 466.58
MIG	361.47 ± 21.89
MIP-1α	54,242.08 ± 8325.12
TNF-α	2028.48 ± 464.00
VEGF	1995.03 ± 252.24

SD: standard deviation; FGF: fibroblast growth factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin; IP-10: induced protein 10; VEGF: vascular endothelial growth factor; MCP-1: monocyte chemoattractant protein 1; MIP-1α) macrophage inflammatory protein 1α; TNF-α: tumor necrosis factor α; IFN-γ: interferon-γ.

Discussion

Several studies have demonstrated that macrophages from smokers are functionally impaired and secrete significantly lower levels of cytokines.[9] However, the impact of CSC exposure on macrophage cytokine secretion in vitro is not known. To investigate the general characteristics of the immunomodulatory effects of cigarette smoke, we used Luminex technology to assess the secretion of 20 cytokines from Ana-1 cells exposed to CSC from 12 brands of cigarettes. The results demonstrated that 8 of the 20 cytokines measured were secreted at significantly lower levels after exposure to CSC from 12 brands of cigarettes.

Our data showed that CSC differentially induced cytotoxicity. It has been demonstrated that cigarette smoke extract not only decreased cell viability but also induced apoptosis of Ana-1 cells.[6] Previous studies have also shown that exposure of human umbilical vein endothelial cells (HUVECs) to cigarette smoke extract led to phase cell cycle arrest.[10]

Our study shows that CSC induced reduction in release of several cytokines, such as IL-5, IL-6,

Table 3. Cytokine profiling of lower limits of detection with CSC exposure.

Cigarette samples	Biologic function	Cytokine	Lower limit of detection (μg/mL CSC)	Release level (mean ± SD) (pg/mL)	*P* value
C1	Interleukins	IL-5	10	1049.07 ± 8.62	0.045
		IL-6	2.5	4958.46 ± 243.25	0.013
		IL-12	5	178.87 ± 3.14	0.006
	Chemokines	IP-10	2.5	4003.55 ± 450.70	<0.001
		MCP-1	2.5	3091.60 ± 281.95	0.004
		MIP-1α	20	5346.08 ± 1496.87	<0.001
	Growth factors	VEGF	5	2114.88 ± 88.70	0.005
	Tumor necrosis factor	TNF-α	5	2076.27 ± 59.73	<0.001
C2	Interleukins	IL-5	10	999.75 ± 54.01	<0.001
		IL-6	2.5	3910.23 ± 311.79	0.001
		IL-12	10	144.21 ± 9.30	0.003
	Chemokines	IP-10	2.5	3896.13 ± 575.93	<0.001
		MCP-1	2.5	3020.66 ± 118.96	0.006
		MIP-1α	20	8186.74 ± 1945.78	<0.001
	Growth factors	VEGF	20	1402.73 ± 47.66	<0.001
	Tumor necrosis factor	TNF-α	2.5	1969.39 ± 79.45	0.048
C3	Interleukins	IL-5	20	543.54 ± 21.24	0.019
		IL-6	5	2461.313 ± 1262.00	<0.001
		IL-12	20	80.01 ± 6.81	0.030
	Chemokines	IP-10	10	1278.39 ± 231.75	0.043
		MCP-1	20	1030.51 ± 68.08	<0.001
		MIP-1α	20	2114.36 ± 370.80	0.001
	Growth factors	VEGF	20	1012.15 ± 326.24	0.041
	Tumor necrosis factor	TNF-α	20	248.90 ± 72.43	<0.001
C4	Interleukins	IL-5	10	884.12 ± 101.93	0.004
		IL-6	5	3202.08 ± 979.88	0.001
		IL-12	10	117.45 ± 31.74	0.001
	Chemokines	IP-10	10	1492.63 ± 83.89	<0.001
		MCP-1	20	1137.29 ± 185.70	<0.001
		MIP-1α	20	2037.31 ± 187.56	<0.001
	Growth factors	VEGF	20	854.11 ± 156.70	0.005
	Tumor necrosis factor	TNF-α	20	167.52 ± 66.78	<0.001
C5	Interleukins	IL-5	5	1114.46 ± 29.83	0.021
		IL-6	2.5	3648.16 ± 511.83	<0.001
		IL-12	10	147.88 ± 10.40	0.005
	Chemokines	IP-10	2.5	2265.18 ± 102.72	<0.001
		MCP-1	10	2517.43 ± 93.67	<0.001
		MIP-1α	20	8232.52 ± 898.63	<0.001
	Growth factors	VEGF	20	1285.77 ± 86.27	<0.001
	Tumor necrosis factor	TNF-α	10	1775.41 ± 63.58	0.002
C6	Interleukins	IL-5	20	549.72 ± 36.72	0.039
		IL-6	2.5	5123.25 ± 349.12	0.001
		IL-12	5	177.77 ± 7.27	0.001
	Chemokines	IP-10	2.5	40,003.55 ± 450.70	0.001
		MCP-1	10	2550.83 ± 224.64	<0.001
		MIP-1α	20	1977.30 ± 512.06	<0.001
	Growth factors	VEGF	10	1945.85 ± 145.88	0.001
	Tumor necrosis factor	TNF-α	10	2019.75 ± 116.60	0.001
C7	Interleukins	IL-5	20	648.54 ± 145.99	<0.001
		IL-6	5	5528.61 ± 1373.02	0.025
		IL-12	5	184.23 ± 18.68	0.006
	Chemokines	IP-10	2.5	4866.26 ± 1112.07	0.042

(Continued)

Table 3. (Continued)

Cigarette samples	Biologic function	Cytokine	Lower limit of detection (μg/mL CSC)	Release level (mean ± SD) (pg/mL)	*P* value
		MCP-1	5	2950.79 ± 216.11	0.013
		MIP-1α	20	46,429.79 ± 57,254.80	0.003
	Growth factors	VEGF	20	3237.31 ± 533.89	0.028
	Tumor necrosis factor	TNF-α	20	498.53 ± 49.62	<0.001
C8	Interleukins	IL-5	10	1145.75 ± 44.02	0.003
		IL-6	10	2768.47 ± 251.34	0.010
		IL-12	20	91.44 ± 4.78	0.047
	Chemokines	IP-10	5	3542.57 ± 23.92	0.042
		MCP-1	5	4698.25 ± 496.07	0.038
		MIP-1α	20	3388.37 ± 747.96	0.036
	Growth factors	VEGF	20	1279.67 ± 96.17	0.024
	Tumor necrosis factor	TNF-α	20	214.95 ± 17.30	<0.001
C9	Interleukins	IL-5	10	1053.83 ± 52.70	<0.001
		IL-6	2.5	5078.52 ± 188.87	<0.001
		IL-12	5	178.15 ± 6.58	0.047
	Chemokines	IP-10	10	2153.12 ± 276.80	0.044
		MCP-1	5	3403.97 ± 357.54	0.008
		MIP-1α	20	2841.48 ± 547.03	<0.001
	Growth factors	VEGF	20	1582.33 ± 56.27	<0.001
	Tumor necrosis factor	TNF-α	2.5	2357.16 ± 88.24	<0.001
C10	Interleukins	IL-5	20	638.38 ± 31.08	<0.001
		IL-6	5	5528.61 ± 1373.01	0.038
		IL-12	20	104.41 ± 6.70	<0.001
	Chemokines	IP-10	5	7768.87 ± 2750.43	<0.001
		MCP-1	10	2614.74 ± 177.56	0.001
		MIP-1α	20	27,647.41 ± 5080.00	0.000
	Tumor necrosis factor	TNF-α	20	634.19 ± 22.88	<0.001
C11	Interleukins	IL-5	10	1037.19 ± 195.45	0.022
		IL-6	5	4666.14 ± 272.45	0.002
		IL-12	20	100.31 ± 3.70	0.007
	Chemokines	IP-10	5	1798.974 ± 549.03	<0.001
		MCP-1	5	4975.74 ± 426.89	0.043
		MIP-1α	20	2790.32 ± 804.59	<0.001
	Growth factors	VEGF	20	1612.03 ± 168.47	0.002
	Tumor necrosis factor	TNF-α	20	286.30 ± 48.72	<0.001
C12	Interleukins	IL-5	20	475.36 ± 25.89	<0.001
		IL-6	10	2659.19 ± 134.47	0.004
		IL-12	20	74.18 ± 6.83	0.001
	Chemokines	IP-10	5	1867.55 ± 101.76	0.002
		MCP-1	10	2148.69 ± 139.00	0.042
		MIP-1α	20	2176.66 ± 383.20	<0.001
	Growth factors	VEGF	20	1306.18 ± 67.25	0.026
	Tumor necrosis factor	TNF-α	10	1614.70 ± 66.95	0.037

CSC: cigarette smoke condensate; SD: standard deviation; IL: interleukin; IP-10: induced protein 10; MCP-1: monocyte chemoattractant protein 1; MIP-1α: macrophage inflammatory protein 1α; TNF-α: tumor necrosis factor α; VEGF: vascular endothelial growth factor.

IL-12, TNF-α, IP-10, MCP-1, MIP-1α, and VEGF. The affected cytokines constitute two major subtypes: proinflammatory cytokines, including IL-5, IL-6, IL-12, TNF-α, and VEGF, and chemotactic cytokines, including IP-10, MCP-1, and MIP-1α. These subtypes are both known to play an important role in the immune response to infections. In light of our results, changes in the expression of these cytokines might explain, at least in part, the delayed wound repair process, increased susceptibility to infections, and relative resistance to some inflammatory diseases observed in smokers.[11] The effects

on cytokines observed in this study can be supported by other studies.[12,13]

In this report, lower limits of detection with CSC exposure showed that IL-6 and IP-10 were reduced significantly compared with the control group in a dose-dependent manner in Ana-1 cells treated with the majority of CSC. Specifically, 5 μg/mL CSC did not affect cell viability but significantly induced the secretion of these cytokines. In contrast, VEGF, MIP-1α, and TNF-α were not sensitive to CSC. Indeed, 20 μg/mL CSC significantly reduced the secretion of some cytokines. For example, IL-5, IL-12, and MCP-1 had distinct lower limits of detection. These phenomena indicate that the diverse components of cigarette smoke may impact its immunomodulatory effects in Ana-1 cells.

Limitation

We do only tested one mouse macrophage for viability and cytokine production. This is the limitation of our study. We learned that the Ana-1 cell has been used to assess toxicological effects due to cigarette smoke. So, Ana-1 was selected in this study. In the further work, we will test CSC's effects in primary macrophage for viability and cytokine production.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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