Dendritic active T cells and mediate inflammation in smoke inhalation injury mouse models

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Abstract

Acute pulmonary and systemic inflammation induced by smoke inhalation is crucial to the outcome and complications of burn patient, and immune dysfunction is implicated in the development of organ injury. To reveal the immune cytokines change and immune cell interactions is helpful to know the immune response following smoke inhalation injury. We were analyzing hematoxylin-eosin (HE) staining of the lung tissues of Smoke inhalation mouse model, and detect the immune cytokine expression after 2,6,24,48,72 hours by The Real-Time qPCR. Flow cytometry was used to identify the expression of DC cells, T cells in the spleen, bone marrow derived-dendritic cells (BMDCs) and respective subtypes at 48h post inhalation. CCK-8 detected at the level of BMDCs on T cell proliferation in a mixed lymphocyte response. Smoke inhalation induced inflammation as evidenced by the significantly altered inflammatory constituent, including inflammatory cytokines and factors such as TNF-α, IFN-γ, IL-2, IL-4, RAGE, TLR4 and HMGB1, as well as inflammatory cells such as dendritic cells, regulatory T cells and BMDCs. Further research on BMDCs revealed that after smoke exposure, there was an increased expression of co-stimulatory molecules, such as CD80 and MHC-II, which aided T cell proliferation. We found that cytokines and immune cells get activation after smoke inhalation in the mouse model, and the activated BMDCs prompt the proliferation of normal T cells.

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Running title: Dendritic and T cells in inhalation injury

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List of abbreviations

BMDCs: bone marrow derived-dendritic cells

SI: Smoke inhalation

ARDS: acute respiratory distress syndrome

MODS: Multiple Organ Dysfunction Syndrome

ALI: acute lung injury

PAMPS: pathogen-associated molecular patterns

DAMPS: damage associated molecular patterns

TLRs: Toll-like receptors

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Abstract

Acute pulmonary and systemic inflammation induced by smoke inhalation is crucial to the outcome and complications of burn patient, and immune dysfunction is implicated in the development of organ injury. To reveal the immune cytokines change and immune cell interactions is helpful to know the immune response following smoke inhalation injury. We were analyzing hematoxylin-eosin (HE) staining of the lung tissues of Smoke inhalation mouse model, and detect the immune cytokine expression after 2,6,24,48,72 hours by The Real-Time qPCR. Flow cytometry was used to identify the expression of DC cells, T cells in the spleen, bone marrow derived-dendritic cells (BMDCs) and respective subtypes at 48h post inhalation. CCK-8 detected at the level of BMDCs on T cell proliferation in a mixed lymphocyte response. Smoke inhalation induced inflammation as evidenced by the significantly altered inflammatory constituent, including inflammatory cytokines and factors such as TNF-α, IFN-γ, IL-2, IL-4, RAGE, TLR4 and HMGB1, as well as inflammatory cells such as dendritic cells, regulatory T cells and BMDCs. Further research on BMDCs revealed that after smoke exposure, there was an increased expression of co-stimulatory molecules, such as CD80 and MHC-II, which aided T cell proliferation. We found that cytokines and immune cells get activation after smoke inhalation in the mouse model, and the activated BMDCs prompt the proliferation of normal T cells.

Keywords: smoke inhalation injury, dendritic cell, T cell, inflammation.

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Conflict of interest statement

No conflict of interest was declared.

1. Introduction

Smoke inhalation (SI) injury is induced by smoke, toxins as well as heat. It is a complex multifaceted injury that initially affects the airway, and then rapidly manifests into a life-threatening systemic disease [1]. The main manifestation of smoke inhalation injury is acute lung injury [2]. Toxic particles and substances inhaled damage the alveolar epithelial and lung vascular endothelial cells, and both stimulation and inhibition of immune systems occur within minutes or hours of injury [3, 4], which resulting in "cytokine storm", pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IFN-γ, secreted by immune cells, especially macrophages, cause a sustained and intense inflammatory response, leading to apoptosis of immune cells and cellular dysfunction [5], may leads to acute respiratory distress syndrome(ARDS) and Multiple Organ Dysfunction Syndrome(MODS) [6]. In general, pro- and anti-inflammatory effects are regulated through chemotaxis, cytokine release, and reactive oxygen species [7-9]. These inflammatory cytokines spread with the blood circulation and cause systemic inflammation and secondary tissue and organ damage,may lead systemic inflammatory response syndrome(SIRS),and multiple organ dysfunction syndrome(MODS)[10]. Certain plasma inflammatory biomarkers were substantially increased in patients who succumbed to their injuries compared to those who survived, abnormal lung function, including inflammation, coagulation and fibrinolysis could predict patient mortality in patients with acute lung injury (ALI) [11].

Elucidating the mechanism of initiation and regulation of early inflammatory response in acute lung injury (ALI) patients would aid in the management of the inflammatory process [12, 13]. In this study, we built a mouse model of smoke inhalation injury by a self-made smoke generator, and we detected pathological changes in the lungs of mice at different time points after smoke inhalation injury, as well as the expression levels of various anti-inflammatory and pro-inflammatory cytokines, and also the expression levels of spleen DC cells and T cells and their subtypes. Additionally, the mlr study supported the hypothesis that mice with smoke inhalation damage had DC cells in their bone marrow that stimulated T cell proliferation. It is helpful to further understanding of the evolution of lung inflammation and the expression and interaction pattern of spleen immune cells following smoke inhalation injury.

2. Materials and methods

2.1. Ethics statement

This study was approved by The Institutional Animal Care and Use Committee of The First Affiliated Hospital of Nanchang University Nanchang, China (CDYFY-IACUC-202304QR021), all methods were carried out in accordance with relevant guidelines and regulations. This study was carried out in compliance with the ARRIVE guidelines. Animal care was performed according to the Guidelines for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Sciences, and followed the guidelines of the Animal Welfare Act.

2.2. Animals

Mice (BALB/c and C57BL/6) (4-6 weeks old, male, body weight 25±5g) were purchased from Hunan slake jingda laboratory animal co. LTD. In summary, we used thirty-six BALB/c mice(n=6) for smoke inhalation to hartvest lung, eighteen BALB/c mice(n=3 for three times) for BMDCs isolation, and extra nine C57BL/6 mice(n=3 for three times) to harvest spleen for MLR studys. Mice were housed in the Experimental Animal Science Center of The First Affiliated Hospital of Nanchang University and under pathogen-free conditions with controlled temperature (20-25), relative humidity (50-70%) on a 12/12 light-dark schedule. Water and food are available *ad libitum* .

2.3. Smoke Inhalation-Acute Lung Injury (SI-ALI) modeling

Six BALB/c mice were randomly selected as the control group, while a total of thirty-six BALB/c mice were used in the SI-ALI model group, based on the handmade machine and methods published before[14]. Briefly, sawdust (150 g/kg, sawdust/body weight) was used to generate smoke in our handmade smoke generator, mice were exposed to smoke in the animal chamber three times for two-and-a-half minutes each, with a two-minute rest between two exposures to avoid carbon monoxide poisoning. Mice in the SI-ALI group were

abdominally injected with pentobarbital sodium (50mg/kg mouse weight) at2, 6, 24, 48, and 72 hrs after smoke inhalation. Lung tissues and spleens were then harvested from both the SI-ALI and control group for following experiments.

2.4. Isolation and in vitro culture of dendritic cells from the bone marrow (BMDCs)

BMDCs were isolated from the control and SI-ALI group of BALB/C mice (n=3), and cultured *in vitro* . Briefly, femur bones were aseptically harvested after removing the muscle and tissues attached at 48 hours post-smoke inhalation. The bones were rinsed with PBS and both ends of the bones were cut to expose the marrow cavity. The cavity was washed using 3-4mls of PBS, and the marrow contents were extracted using a 5ml injection syringe until the marrow cavity turned white. The marrow was then collected and filtrated 3 times using a mesh (#200). Bone marrow cells were then centrifuged at 1500 rpm/min for 5 minutes and re-suspended in serum-free Roswell Park Memorial Institute RMPI-1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). The cell suspensions were supplemented with 10ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Thermo Fisher, USA) and 10ng/ml IL-4 (Peprotech, Co, Ltd, US). On day 2, 4 and 6, half the media was changed and replenished with GM-CSF and IL-4 to ensure a concentration of 10ng/ml for each cytokine. After 7 days of culture, cells in suspension that were the BMDCs were collected and analyzed by flow cytometry (BD, USA).

2.5. Isolation of spleen leukocytes from control mice

Three C57BL/6 mouses were euthanized by cervical dislocation. Then sterilized through immersed below the chest in 75% alcohol for 10 minutes. Spleens were then harvested in a sterile manner and then cut/chopped finely in 2 mls of sterile PBS. Tissue and cell suspensions were filtered twice using a mesh (# 200) and then centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and 3 mls of erythrocyte lysing solution (Sigma-Aldrich, USA) was added and incubated at room temperature for 5 minutes. Afterwards, 10 mls of sterile PBS were added and centrifuged at 1500 rpm for 5 minutes. Supernatant was removed and the cell pellet was re-suspended in RPMI-1640 media containing 10% FBS. Cell concentration was adjusted to $1x10^6$/ml.

2.6. Histological assessment

The inferior lobe of the right lung was harvested from the control and SI-ALI group at 2, 6, 24, 48, and 72 hrs after the control (air) or smoke inhalation procedure. Tissues were fixed in 4% paraformaldehyde solution for 48 hours, and then tissues were sliced into 4 μm sections. After H&E staining, histological examinations were performed using a light microscope and evaluated by two pathologists who were blinded to the study.

2.7. Quantitative real-time PCR (qRT-PCR)

Freshly dissected lung tissues were harvested from the control and SI-ALI group at 2, 6, 24, 48, and 72 hr post control (air) or smoke inhalation. RNA was extracted using TRIZOL (Invitrogen, USA). Total RNA(1μg) was reverse transcribed (Omniscript, QIAGEN) into cDNA and then qRT-PCR (TB Green Premix Ex Taq, Takara, Japan) was performed to measure the following mRNA expression. TNF-α (F: GCCAGGAGGGAGAACAGAAACT, R: AAGAGGCTGAGACATAGGCACC), IFN-γ (F: ATGAACGCTACACACTGCATC, R: CCATCCTTTTGCCAGTTCCTC), IL-4 (F: GGTCTCAACCCCCAGCTAGT, R: GCCGATGATCTCTCTCAAGTGAT), IL-2 (F: GTGCTCCTTGTCAACAGCGC, R: GGGGAGTTTCAGGTTCCTGTA), TLR-4 (F: ATGGCATGGCTTACACCACC, R: GAGGCCAATTTTGTCTCCACA), FoxP3 (F: CCCATCCCCAGGAGTCTTG, R: ACCATGACTAGGGGCACTGTA), HMGB1 (F: GGCGAGCATCCTGGCTTATC, R: GGCTGCTTGTCATCTGCTG) and RAGE mRNA GAPDH (F: TGTGTCCGTCGTGGATCTGA, R: TTGCTGTTGAAGTCGCAGGA) was used as a control to normalize gene expression (WUHAN GENECREATE BIOLOGICAL ENGINEERING CO, ltd.).

2.8. Flow cytometry

Spleens were harvested from the control and SI-ALI group at 48 hrs after control (air) or smoke inhalation, and then immersed into 1ml of PBS. Spleens were ground and then filtrated using a screen mesh (#200).

Cells were then transferred to a 15 ml centrifuge tube and centrifuged. The supernatant was discarded and 5mls of erythrocyte lysing reagent was used to lyse red blood cells and purify the leukocytes by incubating for 5 minutes at room temperature. Afterwards, 10mls of PBS were added, and the tube centrifuged. The supernatant was discarded and the leukocytes were re-suspended in PBS.

Labeling was performed using fluorochrome-conjugated antibodies against mouse CD11C, CD80, CD86, MHC II, CD4, CD25 and FoxP3 (eBioscience, Co, Ltd, US) at a concentration of 0.5ul/10^5 cells. Stained leukocytes were analyzed by flow cytometry (BD FACSCanto II, USA).

After 7 days of *in vitro* culture, BMDCs were harvested and the cell concentration was adjusted to 1x10^6/ml. Characterization was performed using flow cytometry with antibodies against mouse CD11c, CD86 and MHC II (eBioscience, Co, Ltd, US).

2.9. Mixed lymphocyte reaction

After phenotypic characterization, BMDCs (1×10^6/ml, 20μl) were co-cultured with normal spleen leukocytes (1×10^6/ml, 100μl) in round-bottomed 96-well plates (Nune, Roskilde, Denmark), at a ratio of 1:5 (BMDCs: leukocytes). Cells were cultured in DMEM supplemented with 10% fetal bovine serum. Viable cells were counted using the CCK-8 assay kit (Solarbio, USA) after 3-days of co-culture for 1 hour and then Optical density (OD) for each well was measured at 450 nm using the Thermo Scientific Varioskan Flash (Molecular Devices, USA). At a ratio of 1:10 (BMDCs: leukocytes), cells were co-cultured in 24-well plate round bottom medium, collected at day 5, stained and the T cell phenotypes were detected using flow cytometry.

2.10. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software 9.0. Numerical data were presented as mean ± standard error of the mean (SEM). ANOVA with multiple comparison tests were used to analyze intergroup differences. Student's unpaired t-test was used to determine differences between the two groups. Data was considered statistically significant if $p<0.05$.

3. Results

3.1. Lung appearance change after smoke inhalation

Mice smoke inhalation injury model was set by our handmade smoke generator, the macroscopic images shows 2 hours after smoke inhalation, the SI-ALI appears more swollen than the control group, and diffused hemorrhage are observed (figure 1A). As shown in figure 1B, different histological changes were observed at different time points after smoke inhalation. Thickened alveolar wall and inflammatory cell infiltration were observed at 2 and 6 hours post smoke inhalation. After 24 hours, alveolar fusion and expansion was observed, while inflammatory cell infiltration was notably reduced. 48 or 72 hours post-smoke inhalation, large alveoli fusions, expansion, widening of the septum and fibrous tissue was observed.

3.2. Inflammatory response to smoke inhalation

Acute pulmonary inflammatory response was observed after smoke inhalation tissue injury. The representative inflammatory mediators that were measured were TNF-α, IFN-γand IL-2. The mRNA levels of TNF-α reduced at 2 and 6 hours post inhalation, and then subsequently increased at 24, 48 and 72 hours (figure 2A). The changes in IFN-γ mRNA expression levels were similar, however the increase in mRNA expression was only observed after 48 hours (figure 2 B). Interestingly, IL-2 mRNA expression was strongly induced 24 hours post smoke inhalation, and then gradually increased in expression during the following 72 hours (figure 2D). Both inflammatory and anti-inflammatory factors have been demonstrated to be induced after smoke inhalation, hence we measured the levels of IL4 (inflammatory cytokine) and the expression of Foxp3 (a marker of regulatory T cells). IL-4 mRNA expression levels decreased at 2 or 6 hours post-inhalation and then dramatically increased after 24 hours, peaking at 48 hours. In contrast, Foxp3 expression levels continuously decreased after smoke inhalation.

The mechanism of acute smoke injury is similar to the pathogen-associated molecular patterns (PAMPS)

and damage associated molecular patterns (DAMPS) [15]. Toll-like receptors (TLRs) are the congenital immune recognition receptors and belong to type I transmembrane protein receptors. The airway epithelium is the first defense against infections and contain numerous TLRs that are able to detect pathogenic antigens. TLR signaling pathway activates the innate immune system by identifying PAMPs. HMGB1 secreted by dendritic cells and macrophages binds to the TLR4 and RAGE which subsequently leads to NF-kB mediated production of pro-inflammatory cytokines. In this study, we measured the expression levels of HGMB1, RAGE, and TLR4. We observed that the expression levels of HMGB1, RAGE and TLR4 were increased after smoke inhalation (figure 2E-G).

3.3. Treg expression and immune function of DCs in the spleen after smoke inhalation

Our results demonstrated that smoke inhalation induced global changes in the entire immune system to suppress immune function during the initial phases of smoke injury. We observed that the number of Foxp3$^+$Treg cells was notably increased after injury (figure 3D). Treg cells function to attenuate DCs maturation and stimulatory function. We measured the co-stimulatory molecules CD80 and MHC-II on the surface of DCs in the spleen using flow cytometry. As shown in figure 3B, DCs from the spleens of mice after smoke injury had increased surface expression of CD80 and MHC-II. We then investigated the function of DCs in the bone marrow (BMDCs) of mice after smoke inhalation. Similar to DCs obtained from the spleen, CD80 and MHC-II expression levels on the surface of BMDCs were increased. These BMDCs were able to reduce T-cell proliferation when *in vitro*co-cultured for three days with xenogeneic splenocytes.

BMDCs were isolated from the control group or SI-ALI group, and *in vitro* cultured for seven days. To identify the cells isolated from bone marrow, we used a light microscope to observe the characteristics of the cells (Fig.4A). Presence of synapse indicated that those cells are BMDCs. To determine whether smoke inhalation injury would affect BMDCs activation and maturation, we used flow cytometry to detect the surface markers on BMDCs (Fig.4B and C). The result showed a notable increase in the expression of CD86 and MHC-2 after smoke inhalation. Since mature DCs generally have immunostimulatory properties of T cells, we used the mixed lymphoctyte reaction to determine the T-cell stimulatory effect of BMDCs. BMDCs were co-cultured with xenogeneic splenocytes (after removal of erythrocytes), Then CCK8 assays were used and showed that BMDCs from the inhalation group has a more potent effect to activate T cell proliferation compared to the control group (Fig.5).

4. Discussion

Smoke inhalation injury is the main is the leading cause of morality in burn patients, which commonly leads to pulmonary injury. In this study, we measured the levels of inflammatory factors in the lung and immune cells in the spleen to evaluate the role of smoke inhalation injury on innate and systemic inflammation. In a mouse model, we discovered that smoking activates cytokines and immune cells, and that activated BMDCs promote the growth of normal T cells.

After inhaling smoke, pathogen-recognizing receptors such as TLR-4 and RAGE might identify the danger signals and instantly activate the innate immune response, the first line of defense. Activated immune cells and cytokines cause pulmonary tissue damage and increase blood vessel permeability[16]. Resulting in the blood enters the bronchoalveolar space and the lung interstitial [17, 18]. Later, pathophysiological adaptations are induced to regulate the over-activated inflammation. Furthermore, carbon monoxide (CO), the major component of smoking, plays a crucial role in immune modulation. CO content is low at the beginning of smoke inhalation, and it has cytoprotective properties and decreases the inflammatory response by suppressing pro-inflammatory cytokine release and promoting anti-inflammatory cytokine secretion. However, as the CO concentration gradually rises, it may lead cell apoptosis and impede respiratory enzyme function[19]. We can see from our results that smoke inhalation immediately activates pulmonary inflammation, but later, damage to the lung tissue by inflammatory mediators leads to the fusion of pulmonary alveoli, which, when paired with high CO concentration, contributes to a decrease in inflammatory cell infiltration. We conduct experiments to monitor the expression level of a range of inflammatory mediators to learn more about the pathomechanism and immune modulators involved in the inflammatory process.

Our findings suggest that the inflammatory mediators TNF-α, IFN-γ, IL-4 and HMGB1 have a similar trend following smoke inhalation, their secretion is lower than the control group initially, but subsequently increases significantly by the second day. However, once damaged, the expression of pathogen recognition receptors TLR-4 and RAGE is stimulated. The cause for the variability of inflammatory mediator expression is still unclear, hence further research in this area is needed.

The spleen, human body's largest peripheral immune organ, was chosen as the experimental target to observe the systemic inflammatory response. Dendritic cells (DC) are one of the most prominent antigen-presenting cells, and they are in charge of antigen capture, processing, and presentation[20, 21]. DCs move to T-cell regions in immune organs such as the spleen and lymph nodes after receiving antigenic stimuli from damaged tissue to increase the proliferation of T cells, especially CD8+ T cells [22-26]. MHC-2 and CD86 are markers of DC maturation, which may offer co-stimulatory signals required for T cell activation, expansion, and differentiation. And immature DCs would decrease T-cell responses and induce immune tolerance [27]. Regulatory T cells (Treg) have the potential to modulate the immune response by inhibiting T cell proliferation and activation. Analyzing our findings, we discovered that both pro-inflammatory DCs and anti-inflammatory Tregs are more activated than the control group. The underlying reason for overexpressed Tregs might be to regulate overactive inflammation. Previous researches also found that keep the balance between pro- and anti-inflammatory signaling could improve the prognosis of smoke inhalation injury. We assume that the organism is endeavoring to maintain immune system homeostasis.

TNF-α is a pro-inflammatory cytokine that is produced by macrophages, T cells and keratinocytes[28, 29].IFN-γ is mainly secreted from NK cells and T cells in the lung to boost macrophage phagocytosis. Aside from being the main cytokine that identifies Th1 cells, it also stimulates the development of Th0 cells into Th1 cells, resulting in a positive feedback loop[30]. The primary cytokine released by Th2 cells is IL-4. IL-2 is an inflammatory mediator that can stimulate T cell proliferation and differentiation[31]. HMGB1 is a vital regulator engaged in both innate and adaptive immune responses. It is released upon cell death or actively from immune cells and acts as a DAMP to trigger the production of inflammatory chemokines and cytokines by binding to RAGE and TLR receptors[32]. The development of lung injury brought on by various etiologies is significantly influenced by the activation of the toll-like receptor, a transmembrane protein whose activation leads to intracellular NF-ϰB signal transduction and the generation of stimulating cytokines, such as TNF-α and IL-6 [33]. The strong endocytosis activity allows immature DCs to locate and collect antigens. Subsequently, these DCs gradually lose phagocytic activity, MHC-2 is transported to the cell surface, and the expression of co-stimulatory molecules, like CD80, CD86, is increased. At last, the mature DC presents antigens to T cells[34]. Therefore, the maturation of DC is crucial for the initiation of systemic immunity.

In this study, flow cytometry findings revealed that SII may be able to increase BMDC surface markers, while MLR findings suggested that SII may be able to enhance DC's ability to trigger T cell proliferation. Since the ability of DC to activate T cells necessitates a combination of the peptide-MHC complex's action on the T cell receptor, production of co-stimulatory molecules, as well as cytokines produced by T cells that have been activated[35]. Thus, the enhanced expression of MHC-2 and CD86 molecules might be the reason for the increased T cells' ability for activation.

5. Conclusions

We found that cytokines and immune cells get activation after smoke inhalation in mouse model, and the activated BMDCs prompote the proliferation of normal T cells.

Ethics Statement

This study was approved by The Institutional Animal Care and Use Committee of The First Affiliated Hospital of Nanchang University, Nanchang, China (CDYFY-IACUC-202304QR021). Animal care was performed according to the Guidelines for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Sciences, and followed the guidelines of the Animal Welfare Act. There are no human subjects performed in this article.

Author Contributions

All authors contributed to the study conception and design. Material preparation by XC Liao, data collection and analysis were performed by JQ Li. The first draft of the manuscript was written by CX Gan, reviewed and edited by ZH Fu, Funding acquisition by MZ Liu, Supervision by GH Guo and HM Wang. All authors read and approved the final manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1 Pathological features in lung tissues after smoke inhalation

BALB/c mice were divided into two groups: the control group (N=6) was exposed to normal air and the inhalation - acute lung injury (SI-ALI) group (N=6 for each timepoints) was exposed to smoke inhalation. The inferior lobe of the right lung from mice in the SI-ALI group at 2, 6, 24, 48, or 72 hrs after smoke inhalation, and from mice in the control group. (A) Representative macroscopic images of the lungs from control BALB/c mice (left) and from mice in the smoke-inhalation group (right). (B) Histology of lung tissues from control BALB/c mice and mice in the smoke inhalation group at 2, 6, 24, 48 hours and 72 hours. Representative images for each group are shown.

Figure 2 Quantitative real-time PCR (qRT-PCR) of inflammatory factors in lung tissues

The mRNA expression levels of TNF-α (A), IFN-γ (B), IL-4 (C), IL-2 (D), FoxP3 (E), HMGB1 (F), RAGE (G) and TLR-4 (H) in lung tissues were measured using qRT-PCR. qPT-PCR was performed from lung tissues obtained from three mice in each group and performed in triplicate. Results were expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P<0.001 and ****P<0.0001 compared to the control group.

Figure 3 The expression of T cells and DCs in spleens

(A) Percentage of $CD11^+CD80^+$DCs in mice exposed to either air (control) or smoke. (B) Percentage of $CD11^+$MHC-II^+ DCs in mice exposed to either air (control) or smoke. (C) Percentage of $CD4^+CD25^+Foxp3^+$ T cells in mice exposed to either air (control) or smoke. Lymphocytes were isolated from the spleens of six mice from each group and measured by flow cytometry in triplicate. Results were expressed as mean ± SEM. * significant difference ($p < 0.05$) between the air and smoke group.

Figure 4 The expression of BMDCs in bone marrow

(A) Images of dendritic cells were taken after 1 week of *in vitro*culture. The black arrow denotes the synapse of mature DCs. (B) Percentage of $CD11^+CD86^+$BMDCs in mice exposed to either control or smoke. (C) Percentage of $CD11^+$MHC-II^+ BMDCs in mice exposed to either control or smoke. Results were obtained from three mice from each group and analyzed in triplicate. Results were expressed as mean +- SEM. *significant difference ($p < 0.05$) between the control and smoke group. ** significant difference ($p < 0.01$) between the control and smoke group.

Figure 5 CCK8 assays measure T-cell proliferation after co-culture with BMDCs

Samples were obtained from five mice from each group and analyzed in triplicate. Results were expressed as mean +- SEM. ** significant difference ($p < 0.01$) between the control and smoke group.