Neutrophil extracellular traps induced by cigarette smoke activate plasmacytoid dendritic cells

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Materials and methods

Animals and Cigarette Smoke Exposure

Male BALB/c mice between the ages of 6 and 8 weeks (20–25 g body weight) were purchased from the Guangxi Medical University Laboratory Animal Center (Nanning, China). All mice were housed in sterilized cages in a room maintaining a constant temperature and on a 12-hour light/12-hour dark cycle at Guangxi Medical University Laboratory Animal Center, and all mice received sterilized diet and water. All animal studies were approved by the Laboratory Animal Ethics Committee of Guangxi Medical University.

Mice were exposed to cigarette smoke as we previously described.[1] Briefly, mice were exposed to five commercial cigarettes (Nanning zhenlong unfiltered cigarettes: 12 mg of tar and 0.9 mg of nicotine) per time, four times each day and were given rest for 30 minutes between each cycle of cigarette smoke exposure. In total, mice were exposed to cigarette smoke 5 days a week up to 24 weeks. Mice tolerated cigarette smoke exposure without evidence of toxicity (carboxyhemoglobin levels ~10 % and no weight loss). The control mice were exposed to air for 24 weeks.

Evaluation of experimental mouse model of emphysema

The mice were anesthetized with 10% chloralhydrate solution and sacrificed 24 hours after the last cigarette smoke exposure. The left lungs were fixed with instillation of 4％neutral paraformaldehyde through a tracheal cannula at a constant pressure of 25 cm H_2O, followed by paraffin embedding, then cut sagittally into 5-μm sections and stained with hematoxylin and eosin (H&E) for histopathological studies. Emphysema is the hallmark of COPD characterized by enlargement of alveolar spaces and destruction of the alveolar architecture. We therefore evaluated the enlargement of alveolar spaces by determining the mean linear intercept (Lm) as described previously.

Mouse immune cell preparation and isolation

Mouse blood samples were collected in EDTA-treated tubes via retro-orbital bleeding. For isolation of mouse peripheral blood mononuclear cells (PBMCs) and polymorphonuclear neutrophils (PMNs), fresh mouse blood samples were carefully placed on the surface of mouse peripheral blood neutrophil separation medium (TBD;CHINA) and were centrifuged at 500×g for 25min at 28℃. PBMCs were recovered from the upper layer of separation medium and washed two times with cold PBS at 300×g for 10min, and then the cells were resuspended in complete RPMI-1640 medium containing 10% FCS. The remaining interphases were collected and washed with PBS by centrifugation for 10 min at 300×g. Contaminating erythrocytes were removed by hypotonic lysis, and PMNs were harvested and resuspended in serum-free RPMI supplemented

with 1% bovine serum albumin (BSA). The purity of isolated PMNs was>85%.

To obtain spleen single-cell suspensions, spleens were minced and cut into small pieces, subsequently were ground gently into single-cell with a plunger from a 5-ml syringe and filtered through nylon mesh to eliminate debris. The spleen cell suspensions were centrifuged at 300×g for 10 min at 4 ◦C. After that, the erythrocytes of cell suspensions were removed as described previously and the cell pellets of spleen were washed twice with cold PBS.

pDCs in suspensions of mouse spleen cells were isolated by Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec, Aubum, CA) according to the manufacturer. Briefly, non-pDCs were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies and an anti-biotin monoclonal antibody conjugated to Microbeads. Isolation of highly pure pDCs was achieved by passing cell suspensions through a LS column for depletion of magnetically labeled non-target cells. The purity of mPDCA-1^+ pDCs was > 85%, as measured by flow cytometry.

Naive $CD4^+$T cells from suspensions of spleen were isolated by Naive $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Aubum, CA) according to the manufacturer. Briefly, non-naive $CD4^+$T cells including cytotoxic T cells, regulatory T cells, activated T cells, B cells, NK cells, macrophages, granulocytes, endothelial cells, and erythroid cells were indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies and Anti-Biotin Microbeads. Memory T cells were directly magnetically labeled with CD44 Microbeads. Isolation of highly pure naive $CD4^+$ T cells was achieved by depletion of magnetically labeled non-target cells. The purity of $CD44^{low}$ $CD62L^+$ naive $CD4^+$ T cells was > 90%, as measured by flow cytometry.

Mice lung single-cell suspensions were isolated by modifying established protocols, using a combination of mechanical fragmentation, enzyme digestion, and centrifugation procedures as described previously.[1 2] Firstly, pulmonary circulation was flushed via the right ventricle with 10 ml PBS to remove the intravascular pool of cells. The lungs were then thoroughly minced into 0.1cm pieces and followed by digestion in RPMI 1640 medium supplemented with 1mg/ml collagenase type IV (Sigma-Aldrich) for 30 to 45 min at 37℃. To improve tissue disintegration, the lung pieces were incubated in a shaker and vigorous pipetted with a Pasteur pipette every 15 minutes. Subsequently, the digested lung tissues were triturated through a metal screen with a plunger from a 5-ml syringe, and the resulting cell suspension was filtered through a 70-μm cell strainer, washed, followed by red blood cells lysis.

Flow cytometry

The maturation status of pDCs in spleen of cigarette smoke exposed mice was determined by using following anti-mouse antibodies: FITC-MHC-Ⅱ, PE-CD40, APC-CD86. All antibodies and isotype control were purchased from BD Pharmingen. Cell surface staining was performed according to standard procedures.

For intracellular cytokine analysis, fresh isolated PBMCs, spleen and lung single-cells were stimulated for 4 h with 25ng/ml phorbol-myristate-acetate (PMA; Sigma-Aldrich) and 1ug/ml

ionomycin (Sigma-Aldrich) in the presence of GolgiPlug™ (BD Pharmingen) at 37 ℃ in 5% CO_2. The cells were then washed and stained for surface marker Percp- CD4. After surface staining, cells were fixed/permeabilized with fixation/permeabilization solution (Cytofix/Cytoperm™; BD Pharmingen), and stained with APC- IFN-γ and PE-IL-17 mAbs for 30 min at 4℃. Cells were then washed with 1×Perm/Wash Buffer (BD Pharmingen) and resuspended in PBS + 2% FBS for flow cytometric analysis. Flow cytometry was performed on a BD FACS Canto II (BD Biosciences) and analyzed using FCS Express 4 software (De Novo Software, Los Angeles, CA).

Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) was prepared by using a modification of the method as we previously described.[1] Briefly, The smoke of 3 cigarettes (Nanning Zhen long unfiltered cigarettes: 12 mg of tar and 0.9 mg of nicotine) was slowly drew into 3ml of RPMI 1640 medium. The concentration of CSE was determined by using a dual-wave violet spectrophotometer (Lambda Bio 20, Perkin Elmer) at 320 nm absorbance, then the solution was filtered through 0.2-μm filters and 0.1%~0.3% CSE was used immediately.

Induction of NETs formation and immunofluorescence

NETs were induced as previously described.[3 4] Briefly, freshly isolated PMNs were seeded on poly-D-lysine coated coverslips in 24-well round bottom culture plates (1.0×10^6 cells per well) in 500μl serum-free RPMI 1640, and allowed to settle down for 1h in CO_2 incubator at 37°C. PMNs were stimulated with PMA (100nmol/L final concentration) or 0.3%CSE for 4h in CO_2 incubator at 37°C. In some experiment, NETs was incubated with 10% serum from air-control or cigarette smoke exposed mice or DNase-Ⅰ(10U/ml) for additional 30min at 37°C after induction. Cells were fixed in 4% paraformaldehyde(end concentration) for 20min at room temperature. The cells were then permeabilized with 0.5%Triton X-100 for 1 min, and were washed three times with PBS. To visualize NETs, the DNA was stained with 5μmol/l propidium iodide (PI) (Sigma Aldrich) for 30min at 37°C. Slides were washed and mounted with 20μl glycerol and immediately examined with fluorescence microscope. To quantify NETs formation at the microscopical level, 200 neutrophils were analyzed in each sample by two independent investigators. In fluorescence microscopy, only cells that featuring the morphological characteristics of NETosis (nuclear became flat and associated with chromatin decondensation) were identified as NETosis cells.

Preparation of CSE induced-NETs and stimulation of pDCs

After induction of NETs with 0.3%CSE, medium was removed and cells were carefully washed 3 times with 1ml PBS and then treated with 20 U/ml restriction enzyme AluI or MNase (20 U/ml; TAKALA) in HBSS for 20 min at 37°C to recover soluble NETs. The wells were collected by vigorous aspiration and supernatants were harvested and centrifuged at 300×g for 5 min at 4°C to remove contaminating cells and debris. dsDNA in NETs was quantified by Picogreen® dsDNA Assay kit (Invitrogen; P7589) according to the manufacturer's instructions.

To determine the effect of CSE-induced NETs on pDCs maturation, primary pDCs isolated

from spleens of air-control mice or pDCs isolated from cigarette smoke exposed mice were plated in 24-well plates (Costar) at a density of 1×10^6 cells/ml in serum-free RPMI-1640 medium in the presence or absence of CSE-induced NETs for 18 h at 37℃ under a humidified atmosphere with 5%CO_2. Subsequently, cells were harvested, washed and surface molecule expression was determined by flow cytometry as described above. The supernatant of culture mediums was stored at -80°C immediately for later cytokine measurement. In some experiments, pDCs were collected for assessing the capacity to promote the differentiation of naïve $CD4^+$ T lymphocytes.

Coculture of naïve $CD4^+$ T cells with pDCs *in vitro*

pDCs were stimulated with CSE-induced NETs as described above, then were harvested and cocultured with naive $CD4^+$ T lymphocytes isolated from spleens of air-control mice (1:1 ratio) in 24-well plates. In some experiments, naïve $CD4^+$T cells were cultured with primary pDCs from air-control mice or pDCs from cigarette smoke exposed mice.

Control naive $CD4^+$ T lymphocytes were cultured in 24-well plates precoated with anti-mouse CD3 mAbs (5ug/ml; ebioscience) and soluble anti-CD28 (2mg/ml; 37.51; ebioscience) for 4 days. Mediums that naive $CD4^+$T lymphocytes cultured alone were supplemented with IL-2 (10ng/ml; ebioscience) on days 2. Four days later, cells were stimulated with 25ng/ml PMA and 1ug/ml ionomycin in the presence of GolgiStop™ for 4 h. Cells were then collected and stained for surface markers PerCP-CD4 followed by intracellular labeling of PE-IL-17 and APC-IFN-γ. Alternatively, some cells were permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and intracellular stained with PE-anti-mouse T-bet (eBio4B10; eBioscience) and APC-anti-mouse ROR-γt (B2D; eBioscience).

Statistical analysis

Data were expressed as median. Differences between groups were compared using nonparametric test (Mann-Whitney test between two groups or Kruskal-Wallis one-way ANOVA on ranks between different groups). Analysis was completed with SPSS version 16.0 Statistical Software, and $p < 0.05$ was considered statistically significant.

References

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