Simvastatin suppresses RANTES-mediated neutrophilia in poly I:C-induced pneumonia.

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Online data supplement

METHODS

Reagents and Materials

Simvastatin, donated by Prof. Yun-Song Lee, was prepared as 20 mM stock solution. This simvastatin stock solution was diluted with sterile phosphate buffer-saline (PBS) immediately before use. Polyinosinic-polycytidylic acid (poly I:C) was purchased from Sigma (St. Louis, MO, USA) and dissolved in PBS. The concentration of poly I:C employed was 25 μg/ml in vitro experiments. The kinase inhibitors were also purchased from Sigma (St. Louis, MO, USA). An antibody against alpha-tubulin was purchased from Neomarkers Inc. (Fremont, CA, USA). Phospho(Tyr701)-STAT3 and STAT3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz ,CA, USA) for western blotting, and were obtained from Cell Signaling (Danvers, MA, USA) for immunohistochemistry. An antibody against neutrophils marker protein for immunohistochemistry was purchased from Abcam (Cambridge, UK) and a neutralizing RANTES antibody was obtained from R&D systems (Minneapolis, MN, USA). S31-201, STAT3 inhibitor, was purchased from Merck (Darmstadt, Germany).

Cell Culture

Primary Normal human bronchial epithelial cells (NHBE) were grown in bronchial/tracheal epithelial-cell basal medium (Cambrex), to which bovine pituitary extract (13 μg/ml), hydrocortisone (0.5 μg/ml), human recombinant epidermal growth factor (0.5 μg/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), retinoic acid (0.1 μg/ml), triiodothyronine (6.5 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 μg/ml) were added. Before stimulation, NHBE was cultured in bronchial epithelial cell growth medium without hydrocortisone for at least 2 days because of the anti-inflammatory effect of corticosteroid. The human lung epithelial cell line A549 was maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Before stimulation, A549 cells were cultured in serum-free media for 12h.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cell lysates using easy-BLUE and cDNA was prepared using a Maxime RT-premix (iNtRON, Seoul, South Korea), according to the manufacturer's instructions. PCR was performed with 35 cycles of sequential reactions, specifically at, 94°C for 45 sec, 61°C for 45 sec, and 72°C for 1 min for RANTES and GAPDH; 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for STAT3. Oligonucleotide primers were purchased from Bioneer (Seoul, South Korea). The sequences of PCR primers were as follows: forward, 5′-ATGAAGGTCTCCGCGGCAGCCCT-3′ and reverse, 5′-CTAGCTCATCTCCAAAGAGTTG-3′ for RANTES, forward, 5′-TGCCTGGAGACAGTTGATGT-3′ and reverse, 5′- TGGAATTTGAATGCAGTGGC-3′ for STAT3, forward, 5′-CCATGGAGAAGGCTGGGG-3′ and reverse, 5′-CAAAGTTGTCATGGATGACC-3′ for GAPDH. PCR products were separated by electrophoresis in 1% agarose gels and detected under UV light.

Bronchoalveolar lavage fluid (BALF) collection

After the mice were sacrificed, their tracheas were cannulated with a 20-gauge catheter for bronchoalveolar lavage fluid (BALF) collection. The lungs were lavaged twice with 1 ml of a 0.1% BSA saline solution. BALF was centrifuged at 1,200 rpm for 5 min at 4 ℃. Then, supernatant is preserved for cytokine analysis, and cell pellet is resuspended in 200 μl of saline solution for differential cell analysis according to morphologic criteria using Diff-Quik staining.

Immunohistochemistry (IHC)

Tissue sections were then stained with haematoxylin and eosin (H&E) for general morphology. For immunohistochemistry, sections were deparaffinized with zylene and washed in ethanol. For antigen unmasking, slides were incubated in 10 mM sodium citrate buffer (pH 6.0) and maintained at a sub-boiling temperature for 10 min. Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide for 10 min. (consistency) Slides were then incubated with blocking solution for 1h at room temperature. After removing the blocking solution, slides were applied with primary antibody diluted in antibody diluent (Invitrogen, USA) and incubated at 4 ℃ for 24 h. After washing slides three times with PBS including Tween-20 (PBST), slides were incubated with biotylated secondary antibody diluted in antibody diluents for 30 min. Then, slides were incubated with ABC reagent (VECTOR, CA, USA) for 30 min at room temperature and treated with diaminobenzidine (DAB) as the chromogen (DakoCytomation, CA, USA) for 1-3 min. As soon as the sections develop, slides were immersed in distilled water and counterstained with hematoxylin. Finally, slides were mounted using Canada balsam reagent.

Western blotting

Cells were washed twice with cold PBS, and then lysed in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl) containing protease inhibitors (2 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 0.5 mM Na_3VO_4, 2 mM EDTA and 1 mM NaF). The lysate was centrifuged for 20 min at 13,000 rpm at 4°C, and the supernatant was collected. Proteins in lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with primary antibodies at 4 ℃ for 24 h, then washed with tris-buffered saline including 0.1% Tween-20 (TBST), peroxidase-conjugated secondary antibodies for 1 h at room temperature, washed and then visualized using an Enhanced Chemiluminescence (ECL) Plus kit (Amersham Biosciences Corp., Piscataway, NJ).

sFigure 1 Legend

sFig. 1 Simvastatin and neutralizing RANTES antibody suppress pro-inflammatory cytokine secretion in poly I:C-inhaled mice In lung tissues, for the detection of individual cytokine levels like a) TNFα, b) IL-6, c) GM-CSF, d) IL-12(p70), e) IL-4, f) IL-5, BALF of each mice group was collected as described in Materials and Methods. Cytokine secretion was determined by the Bio-Plex 200 system. Data are expressed as the mean ±S.E.M. (*n*=5). *, P<0.005 versus PBS group; **, P<0.001 versus PBS group.