Decreased indoleamine 2, 3-dioxygenase activity and IL-10/IL-17A ratio in COPD patients: *Online Supplement*

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SUPPLEMENTAL METHODS

Sputum induction and processing

Sputum induction was performed as previously described.[E1] The supernatants were kept frozen at –70° C until further analysis. For immunocytochemistry, cytospins were fixed with 4% paraformaldehyde (BDH Ltd., Poole, UK) and stored at –20°C. Total cell counts were recorded with on a hemocytometer, using Kimura staining. Cell viability was determined by Trypan blue exclusion before cytospins were undertaken. The slides were stained with May-Grunwald-Giemsa stain and differential cell counts were made by a blinded observer. Four hundred inflammatory cells were counted on two slides for each sample in a blinded manner. Differential cell counts are expressed as the percentages of total inflammatory cells. Samples with cell viability of greater than 70% and less than 30% squamous cell contamination were considered adequate for analysis.

Alkaline phosphatase immunostaining

Indirect staining of sputum cells was performed using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method using a commercial kit (Vectastain Laboratories, Burlingame, CA), as previously described.[E2] Cells were permeabilised for 10 minutes with 0.5% Nonidet P-40 (NP-40, Sigma Chemicals, St Louis, MO) and blocked in 20% normal swine nonimmune serum (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Polyclonal anti-rabbit antibodies were used to detect IDO (1:100; Genescript International Inc, Temecula, CA). After incubating with the secondary biotinylated goat anti-rabbit antibody, the immunoreaction was detected using the APAAP system to produce red staining. Slides were counterstained with hematoxylin for cellular identification, and examined under light microscopy. Anti-IDO positive cells, which were identified by red immunoreactive signals on sputum cytospins, were counted by an experienced observer blind to the clinical characteristics of the subjects. Immunoreactive macrophages were counted among 300 cells and expressed as a percentage of total cells.

ELISA

For detection of human IL-6, CXCL8, IL-10 and IL-17A sandwich ELISAs were performed according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). The sensitivity was 0.7 pg/mLfor IL-6, 3.5 pg/mL for CXCL8, 3.9 pg/mL for IL-10 and 15 pg/mL for IL-17A. Cytokine output was normalised to the concentration of protein.

Determination of IDO enzymatic activity

Enzymatic activity of IDO was determined by measuring kynurenine (Kyn) and tryptophan (Trp) concentrations in sputum supernatants, as previously described.[E2]

Preparations of cigarette smoke extract

Cigarette smoke extract (CSE) was prepared using two full-strength Marlboro cigarettes with filters removed (Phillip Morris, VA), which were combusted through a modified 60-ml syringe apparatus into 20 ml RPMI 1640 as described.[E3] Macrophages were treated with CSE for 24 h. Supernatants were collected and stored at -80^{o}C for determination of IDO activity.

Knockdown of IDO expression

RNAi was used to suppress IDO in macrophages. Two ds 21-mer RNA oligonucleotides directed against IDO were synthesized by Sigma-Proligo (St. Louis, MO). The sequences are AAGAAGTGGGCTTTGCTCTG and CAGAGCAAAGCCCACTTCTT. Cells were transfected with siRNA using DOTAP Liposomal Transfection Reagent (Roche Applied Science, USA), as described by the manufacturer. IDO protein expression was monitored by flow cytometry after 48 h.

Fluorescence-activated cell sorting (FACS) analysis

Flow cytometry was performed as described.[E4] Macrophages were incubated with the corresponding primary monoclonal antibodies for 60 min at 4^{o}C, washed twice, and resuspended in PBS, 1% BSA. Analysis on 20,000 cells was performed using a FACSort (Becton Dickinson, CA). The employed primary monoclonal antibodies were Alexa Fluor 488-conjugated anti-IDO (R&D Systems, MN), phycoerythrin-conjugated anti-IL-10 (eBioscience, CA) and Allophycocyanin-conjugated anti-IL-17A mAbs (R&D Systems, MN). The respective negative controls were matched isotype controls. Data were analyzed using WinMDI version 2.9.

RNA isolation and real-time RT-PCR analysis

Total RNA was extracted from monocyte-derived macrophages by using an RNeasy Mini kit (Qiagen, Valencia, Calif), according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA by using Improm-II Reverse Transcription system (Promega,

Madison, Wis). Real-time RT-PCR was performed with FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) with an ABI PRISM 7900 thermal cycler (Applied Biosystems, Warrington, United Kingdom), according to the manufacturer's instructions. IDO primers were purchased from First BASE Laboratory (Singapore): sense: AGT CCG TGA GTT TGT CCT TTC AA, antisense: TTT CAC ACA GGC GTC ATA AGC T, and glyceraldehyde-3-phosphate dehydrogenase (GADPH) sense: GAA ATC CCATCA CCATCT TCC, antisense: AAA TGA GCC CCA GCC TTC TC. The 7300 System SDS software version 1.4.0.25 was used to analyse the relative quantity of the target cDNA, according to the ΔΔCt method.

Whole protein extraction and Western blot analysis

Whole cell protein extraction and western blot analysis were performed as previously described.[E5] Whole cell lysates were prepared in NP-40 lysis buffer (0.5% Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl) in the presence of complete protease cocktail inhibitor. Lysates were centrifuged at 4^{o}C for 10 min at 12,000 rpm in an Eppendorf microcentrifuge to remove cellular debris. Western blot analysis was performed using polyclonal anti-rabbit antibody against IDO (Genescript International, Inc, Temecula, Calif).

REFERENCES FOR SUPPLEMENT

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