PPARγ antagonist attenuates mouse immune-mediated bone marrow failure by inhibition of T cell function

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Received: 2/12/2014.

Accepted: 17/11/2015.

Pre-published: 20/11/2015.

doi:10.3324/haematol.2014.121632

Methods

BM failure mouse models. Immune-mediated AA failure mice were generated as previously described[1, 2]. Briefly, recipient CB10 mice were exposed to 5 Gy of total body irradiation (TBI). On the same day, inguinal, brachial, and axillary lymph node (LN) cells were obtained from B6 donors, homogenized, and washed in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA), and filtered through 90 μM nylon to obtain single cell suspension. Then, cells were counted and infused into recipients by tail vein injection at 5.0 x 10^6 cells per mouse. FVB BM failure model was created as the CB10 AA model except that recipient FVB mice were irradiated at 6.8 Gy and injected with LN cells from MHC H2-matched donor DBA mice at 10 x 10^6 per mouse.

Treatment of mice. PPARγ antagonists, BADGE or GW9662 (Sigma, St. Louis, MO), were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. To assess improvement of hematopoiesis and inhibition of BM adipogenesis in different mouse models, the aliquots were diluted with PBS to a final concentration of 10% DMSO and administrated by daily intraperitoneal injection at 30 mg/kg for BADGE or at 1 mg/kg for GW9662 from one day before experiment and continued for up to 2 weeks. In FVB BM failure model, some mice were injected cyclosporine A (CsA, 50 mg/kg/day, Sigma) starting 1 hour after LN injection, and continued for 5 days as immunosuppression. At the end of experiments, mice were euthanized by CO_2 inhalation.

Peripheral blood (PB) and BM cell counting. PB was obtained by retro-orbital sinus bleeding and anti-coagulated with ethylenediaminetetraacetic acid (EDTA). Complete blood counts (CBCs) were performed using a Hemavet 1700 analyzer (Drew Scientific, Oxford, CT) and plasma was separated for cytokine measurement. BM cells were flushed from 1 femur and 2 tibias of each mouse for BM cell counting. Red blood cells were lysed with Gey's solution for 10 minutes on ice. Then, cells were filtered, and counted by a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Fullerton, CA), and processed for various analyses. Another femur from each mouse was used for histology or extraction of protein or RNA.

Flow cytometry. Antibodies specific to mouse CD3 (clone 145-2C11), CD4 (clone L3-T4 and H129. 19), CD8 (clone 53-6.7), CD11b (clone M1/70), CD34 (clone RAM34), CD45R (B220; clone RA3-6B2), CD25 (IL-2Ra; clone p55), CD117 (c-kit; clone 2B8), erythroid cells (clone Ter119), granulocyte (Gr-1/Ly6-G; clone RB6-8C5), stem cell antigen 1 (Sca1; clone E13-161), CD150 (clone Q38-480), and 7-AAD were purchased from BD Biosciences (San Diego, CA). Cells were stained with antibodies for 20 minutes at room temperature in dark and analyzed by a BD LSR II Flow cytometer (BD Biosciences). Data were analyzed with FlowJo software Version 7.6.5 (TreeStar, Ashland, OR).

Histology and confocal microscopy. Femurs fixed in 10% neutral buffered formalin were paraffin-embodied, and sectioned followed by staining with H&E. Immunofluorescence labeling and confocal imaging were performed using sternums whole mounts preparations as previously described[3]. Specimens were

fixed with 4% paraformaldehyde at room temperature (RT) for 1 hr. After washing with PBS three times for 15 minutes, specimens were immediately stained with boron-dipyrromethene dye (BODIPY493/503, 1:500 dilution; Invitrogen) for lipid droplets in cytoplasm and 4',6-diamidino-2-phenylindole (DAPI, 1:1000 dilution; Invitrogen) for nuclei at RT for 40 minutes. In some samples, rat anti-mouse CD117 antibody (1:50, BD Biosciences) was used for staining of hematopoietic stem cells (HSC), followed by Texas-Red conjugated donkey anti-rat IgG (1:200, Jackson Immunoresearch, West Grove, PA); In different samples, BM megakaryocytes were stained with anti-mouse CD41- phycoerythrin (PE) (1:50, BD Biosciences). All images were acquired by CLSM with a Zeiss LSM 510 confocal system (Confocal Zeiss MicroImaging). 3D tiled-images comprising the entire sternum fossae volume were collected for assessment of adipocyte and hematopoietic cells number and distributions and comparisons between different experimental conditions.

Cytokine measurement. Cytokines in plasma were measured using MILLIPLEX MAP Mouse Magnetic Bead Panel (EMD Millipore, Billerica, MA), according to the manufacturer's instructions.

RNA isolation and gene expression analysis by PCR array. Whole femurs were crushed and sonicated using Sonicator XL2020 (Misonix, Farmingdale, NY) at 4°C for RNA isolation. Total RNA was isolated with the RNeasy Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Then RNA was digested with RNase free DNase I (Qiagen) and assessed using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). First strand

cDNA was synthesized with 500 ng of total RNA using RT^2 First Strand Kit (Qiagen). Quantitative analysis of mRNA expression of inflammasome- and adipogenesis-related genes was performed using mouse PCR arrays (PAMM-097Z and PAMM-049Z, Qiagen), according to the manufacturer's instructions.

Protein extraction and immunoblot. Proteins from whole bone cavities were extracted with extraction reagents (Thermo Scientific, Rockford, IL) with a complete cocktail of protease inhibitors (Roche, Madison, WI) and quantitated using the BCA Protein Assay Kit (Thermo Scientific). Proteins (20 μg) were separated in Novex 4-12% Tris-Glycine Mini Gel and electro-transferred onto 0.2 μm pore size PVDF membranes (Life Technologies, Grand Island, NY). Non-specific sites on membranes were blocked with 3% non-fat dry milk in 0.1% Tween 20/PBS. PPARγ, AGT, and β-action were detected with anti-PPARγ antibody (H-100, 1:200 dilution), anti-AGT antibody (1:500 dilution), and anti-β-actin antibody (I-19, 1:200 dilution) (Santa Cruz Biotechnology), respectively. Immunoblotting was performed using enhanced chemiluminescence reagents to visualize immunoreactive proteins.

Cell culture. Mouse LN cells (1.0×10^6) were cultured in 1.0 ml of RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 ug/ml streptomycin (Invitrogen) in a 24-well tissue culture plate. To stimulate naïve T cells, phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma) were used at concentrations of 5 ng/ml and 500 μM, respectively. BADGE was added at different concentrations 2 hours post PMA stimulation. T cell activation was

evaluated by flow cytometry after 16-hr incubation. Mouse LN cells were labeled with the carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), and stimulated with PMA (5 ng/ml) and ionomycin (500 μM), BADGE was added at different concentrations 2 hours post PMA stimulation and cultured for 3 days. T cell proliferation based on CFSE dye dilution was evaluated by flow cytometry.

Calcium flux assay: Mouse LN cells were cultured with or without BADGE (50 or 100 μM) for 4 hours, and were then loaded with Fluo-4 (Invitrogen) according to the manufacturer's instruction. The cells were added with biotinylated anti-CD3 antibodies (clone 145-2C11, Biolegend), and crosslinked with streptavidin (Thermo Scientific) at 20 sec of the analysis to initiate calcium flux. The changes of Fluo-4 intensity along the time were recorded by BD LSR Fortessa Flow cytometer (BD Biosciences), and the kinetics was analyzed by FlowJo software (TreeStar).

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