ONLINE SUPPLEMENT

MYELOID-DERIVED SUPPRESSOR CELLS AMELIORATE CYCLOSPORINE A-INDUCED HYPERTENSION IN MICE

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Short Title – MDSCs, CsA, and Hypertension

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MATERIALS AND METHODS

Animals and Treatments

Male C57Bl/6J mice (Jackson Laboratory) aged 10-18 weeks were utilized for the CsA treatment studies. Mice were maintained on a 12:12 light/dark cycle and had access to standard chow and water *ad libitum*. Systolic blood pressures were measured by tail-cuff (IITC, Inc.) daily, at the same time each day, following 3 days of training. Following blood pressure measures, mice were injected ip with CSA (50 mg/kg/day; Alomone, Israel) or diluent (saline and DMSO, 0.2% final concentration) as described previously.[1-4] Some mice were also given daily i.p. injections of IL-33 (0.5 ug/mouse/day; eBioscience).[5, 6]

Other control and CSA-treated mice were injected i.p. with primary MDSCs isolated from male control mice on days 1, 4, and 7. Male control mice were euthanized and MDSCs were isolated from the spleens and bone marrow according to the manufacturer's protocol (Miltenyi Biotech; #130-094-538).

Animals were anesthetized on day 8 with isoflurane and euthanized by cervical dislocation. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Serum Creatinine

Blood was obtained by cardiac puncture under anesthesia. Serum creatinine was measured according to the manufacturer's protocol (R&D Systems).

Vascular Reactivity

Vascular reactivity was measured in endothelium-intact aortas as described previously.[2, 3, 7, 8] Concentration-force curves were generated for the endothelium-dependent dilator acetylcholine (ACh) and the endothelium-independent dilator sodium nitroprusside following contraction with an EC_{70} concentration of phenylephrine (PE).

Immunoblotting

For protein analyses from whole aortic and kidney tissue, tissue lysates were processed and total protein concentration was determined as described previously.[4] Protein lysates (50 ug) were separated by 4-12% SDS PAGE (Invitrogen) and transferred onto a nitrocellulose membrane (BioRad). The membranes were blocked at room temperature with Blocking Buffer (LI-COR Biosciences) for 1 hour, incubated with appropriate antibodies (in LI-COR Blocking Buffer) overnight at 4°C, and washed three times with TBST. Secondary antibodies (1:10,000 in 50% Blocking Buffer/50% TBST) consisted of anti-rabbit and anti-mouse IgGs conjugated to Alexa-Fluor 680 and IR800Dye (LI-COR Biosciences). The blots were probed for Fibronectin (1:1,000; Abcam), E-selectin (1:1,000; Abcam), collagen (1:1,000; Millipore), calcineurin (1:1,000; Cell Signaling), and β-actin (1:5,000, Sigma) and were identified simultaneously (800 nm and 700 nm wavelengths, respectively) using near-infrared visualization (Odyssey System, LI-COR Biosciences). Densitometry was performed using the Odyssey software.

Histology and Morphometrical Analysis

Left kidney histological sections were prepared as described previously.[4] Three blinded investigators quantitated glomerular injury according to a scale described previously.[4] In brief, 15 glomeruli per tissue section were scored on a 0-4 scale, where 0=normal size, normal mesangium, and no congestion; 1=minimal hypertrophy, mesangial expansion, and/or congestion; 2=mild hypertrophy, mesangial expansion, and/or congestion; 3=moderate hypertrophy, mesangial expansion, and/or congestion; and 4=severe hypertrophy, mesangial expansion, and congestion. Mean glomerular injury index for each group was calculated by [(0 x N0)+(1 x N1)+(2 x N2)+(3 x N3)+(4 x N4)]/15, where N0-N4=number of glomeruli with scores of 0 to 4, respectively, as described previously.[4, 9]

Flow Cytometry

Spleens were harvested from the mice and single cell suspensions were generated as described previously.[4] One million cells from the spleen were stained with anti-mouse CD11b clone M1/70 (BD Pharmingen), anti-mouse Ly-6G and Ly6C clone RB6-8C5 (BD Pharmingen), or isotype controls for 1 hour at 4°C in the dark. The RB6-8C5 antibody reacts with a common epitope on Ly-6G and Ly-6C, previously known as the myeloid differentiation antigen Gr-1. Other splenocytes were stained with anti-mouse CD3e (BD Pharmingen), anti-mouse CD4 (BD Pharmingen), anti-mouse CD25 clone PC61.5 (eBioscience), or isotype controls. These cells were then fixed and permeabilized with the BD CytoFix/CytoPerm™ Fixation/Permeabilization Kit (BD Pharmingen) followed by intracellular staining using an anti-mouse FoxP3 antibody (eBioscience). Flow cytometry was performed on a BD FACS Canto II and analyzed using FlowJo. A negative control along with an isotype-matched control was used to determine the positive and negative cell populations for each sample. CD11b+/Gr1+ MDSCs and $CD3+/CD4^{+}/CD25+/FoxP3^{+}$ lymphocytes were quantified and averaged as described previously.[3, 4] Data are expressed as % of lymphocytes.

Isolated Aortas and Kidneys and Treatments

Aortas were excised from male control mice, cleaned of adipose tissue, and incubated with CsA (50 umol/L) for 24 hours in the absence and presence of 75,000 MDSCs isolated from male control mice. Segments 2 mm in length were cut from the thoracic portion, mounted in a myograph, and vascular reactivity studies were performed as above while the rest of the tissue was used for immunoblotting. Right kidneys isolated from the same control mice were treated with CsA (50 umol/L) for 24 hours in the absence and presence of 75,000 MDSCs and then immunoblotting for fibronectin and actin were performed as above.

Microvascular and Glomerular Endothelial Cells and Treatments

Murine microvascular and glomerular endothelial cells were obtained from Cell Biologics and cultured according the manufacturer's guidelines. Endothelial cells were grown in gelatin-coated tissue culture flasks containing Cell Biologics' Culture Complete Growth Medium. Endothelial cells were treated with CsA (50 umol/L) for 24 hours in the absence and presence of 75,000 MDSCs isolated from control mice. The cells were then processed for fibronectin immunoblotting as described above.

Isolated MDSCs and Treatments

MDSCs were isolated from male control mice spleens and bone marrow using the Miltenyi Biotech MDSC isolation kit. GM-CSF (10 ng/mL; R&D Systems) was added to the cells for survival. MDSCs were incubated for 24 hours with different concentrations of CsA (0, 10, 25, and 50 umol/L), the calcineurin autoinhibitory peptide (CAIP; 0, 10, 50 umol/L; Calbiochem), or the selective NFAT inhibitor VIVIT (0, 10, and 25 umol/L; Calbiochem) in the absence and presence of 75,000 MDSCs isolated from male control mice. The % of live MDSCs compared to 24-hour, DMSO vehicle-treated MDSCs were then determined by flow cytometry.

Statistical Analyses

Results are presented as mean + SEM. For multiple comparisons, either a 2-way ANOVA or a repeated measures 2-way ANOVA [main effects: group (Con or CsA) and treatment (Vehicle, IL-33, or MDSCs)] was used followed by the Student's-Newman-Keuls *post hoc* test. The significance level was set at 0.05. All analyses were performed using SigmaStat 3.5 software.

References

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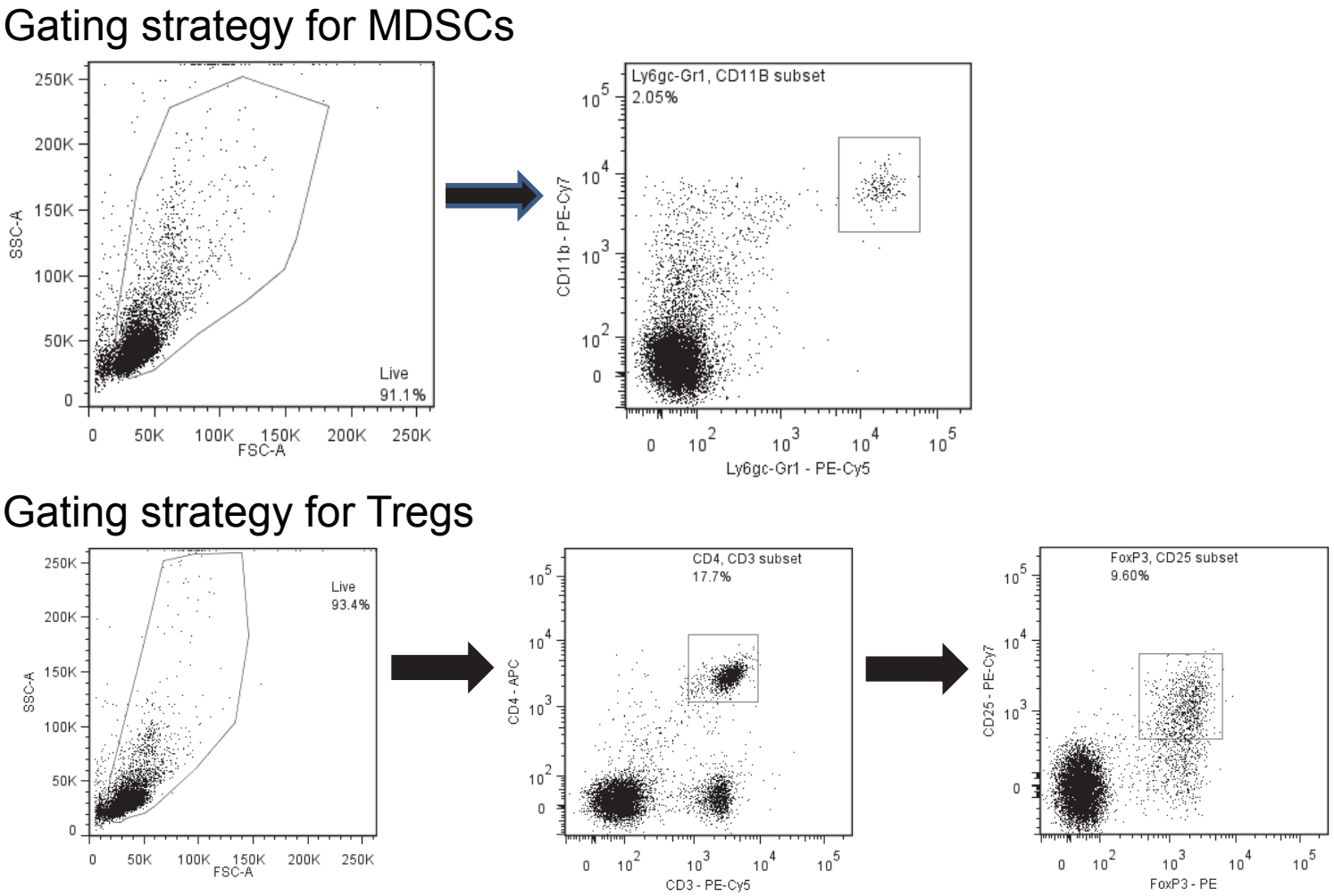

Figure S1. Gating strategies for flow cytometric analyses of splenic myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs). Detailed methods are provided in the Materials and Methods section.

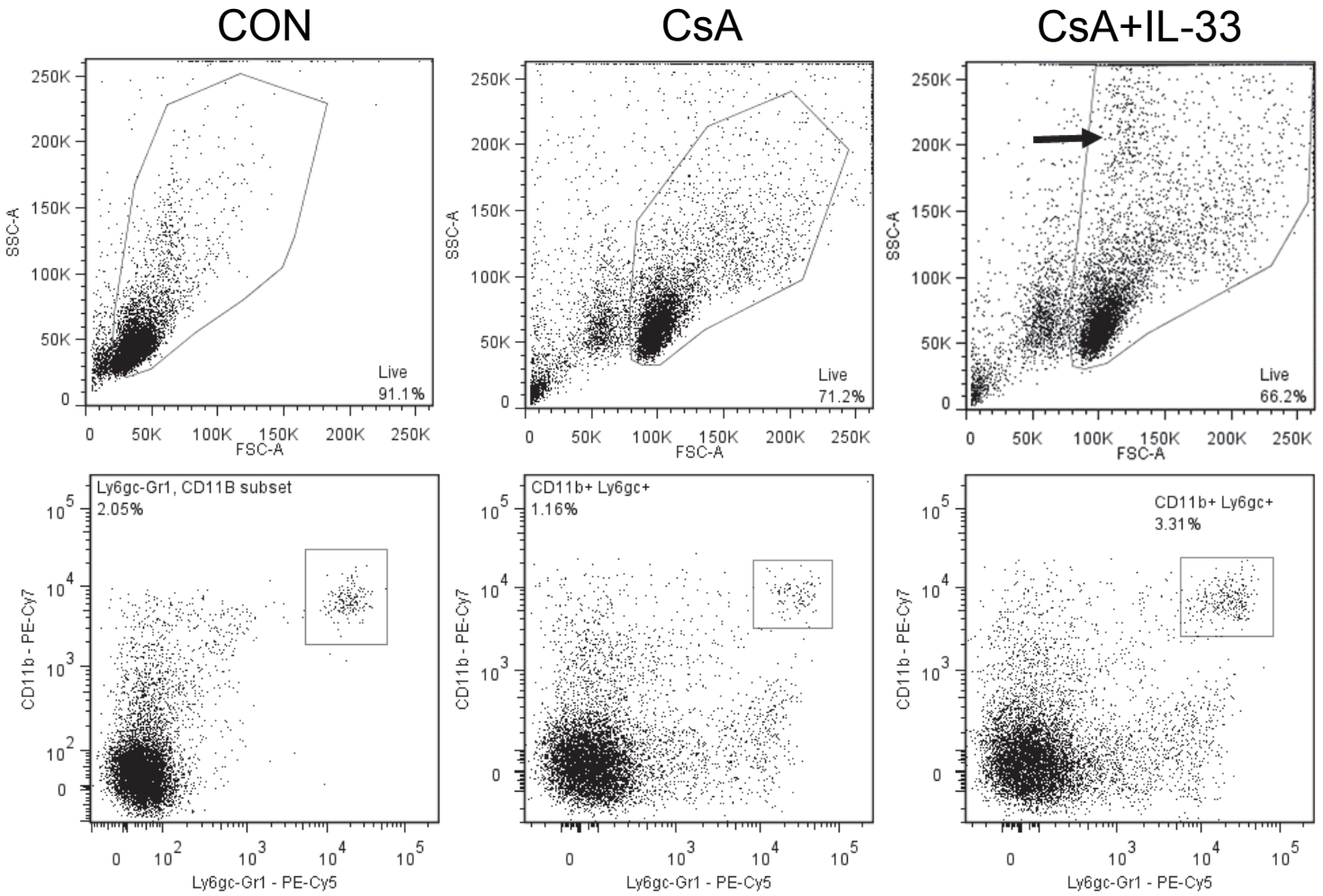

Figure S2. Representative dot plots for splenic myeloid-derived suppressor cells in control, CsA-treated, and CsA- and IL-33-treated mice. Detailed methods are provided in the Materials and Methods section. The arrow highlights the expanded MDSC population induced by IL-33.