SUPPLEMENTAL DATA

CCR5 as a Treatment Target in Pulmonary Arterial Hypertension

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SUPPLEMENTAL METHODS

Animals. Mice (C57Bl/6j) were used according to institutional guidelines that complied with national and international regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee of the French National Institute of Health and Medical Research (INSERM)-Unit 955, Créteil, France. Transgenic CCR5-deficient mice were produced and bred at the INSERM-Unit 945 as previously described [1]. In addition, h-CCR5 ki mice were generated and provided by Pfizer. Briefly, the sequence encoding human CCR5 was cloned in a target vector and transfected to embryonic stem (ES) cells in order to obtain an ES clone containing the homologous recombination. Chimeric mice were then produced through aggregation to obtain heterozygous mice. First-generation of h-CCR5 ki mice were obtained by breeding two heterozygous mice. The h-CCR5 ki mice thus obtained were backcrossed to obtain a C57/BL6 background. To generate bone-marrow (BM) chimeras, we irradiated wild-type (WT) and CCR5 knock-out mice with a total dose of 10 Gy. Then, on the same day, each mouse received an intravenous injection of $5 \cdot 10^6$ total BM cells from WT and CCR5 donors. Animal experiments were approved by the institutional animal care and use committee of the Pitié-Salpêtrière School of Medicine, Paris, France.

Antibodies, recombinant proteins and reagents. Mouse monoclonal Ab (mAb) Zy12 against human CCR5 was obtained from TEBU-Bio, Santa Cruz Biotechnology, Santa Cruz, CA), rat mAb against F4/80 from Serotec (Düsseldorf, Germany), and mouse mAb against CD68 from Dako (Glostrup, Denmark). Polyclonal rabbit Abs against α-SMA, vWF, and Ki67 were purchased from Abcam (Abcam, Cambridge, MA). TRPC4 (#ACC-018) was obtained from Alomone Laboratories (Jerusalem, Israel) and mouse mAb against β-actin from Sigma (Saint Louis, MO). As negative controls, we used corresponding IgG antibodies (R&D Systems, Lille, France) in appropriate concentrations with the same protocol as for the anti-CCR5, anti-vWF, anti-α-SMA, anti-CD68 and anti-F4/80 antibodies. All Alexa secondary Abs were from Molecular Probes (Life Technologies, Carlsbad, CA). The mAbs used for flow cytometry were anti-CD11b (clone M1/70), anti-Ly-6G (clone 1A8), anti-NK1.1 (clone PK136), and anti-CD11c-PE (Clone HL3) from Becton Dickinson (Franklin Lakes, NJ); and anti-

neutrophil (clone 7/4) and anti-F4/80 (clone BM8) from Serotec. All cytokines, PDGF-BB, mouse recombinant CCL5, and human recombinant CCL5 were obtained from R&D Systems. Gp120 R5 tropic (97CN001 strain) was obtained from Interchim (Montluçon, France); heat-inactivated gp120 was obtained by heating at 90°C for 30min. SU5416 was obtained from SIGMA (Lyon, France).

Tissue preparation and immunostaining. The left lung from each animal was inflated with a 1:1 mixture of phosphate-buffered saline (PBS) and OCT embedding medium or 4% formalin (m/v). Formalin-inflated lungs were embedded in paraffin and cut into 5 µm-thick sections for hematoxylin and eosin staining and immunochemistry staining. In addition, OCT-inflated lungs were embedded in OCT then frozen at -80°C and cut into 10 µm-thick slices using a Leica Cryostat (Leica Microsystems, Wetzlar, Germany). The cryosections were used for immunofluorescence staining.

Paraffin-embedded sections were deparaffinized using xylene and a graded series of ethanol dilutions then incubated in citrate buffer (0.01 M, pH 6) at 90°C for 20 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 and 10% methanol in PBS for 10 minutes. Ki67 staining was preceded by a permeabilization step with 0.1% Triton X-100 in PBS for 10 min. Slides were incubated for 60 minutes in 1% bovine serum albumin and 5% goat serum in PBS then incubated overnight with the primary antibody. We used the ABC Vectastain kit (Vector Labs, Burlingame, CA) to mark the primary antibodies according to the user's guide. The staining substrate was DAB (FastDAB, Sigma) and the sections were counterstained with hematoxylin.

Cryosections were defrosted, fixed with acetone at 4°C for 10 min, and dried for 10 min. After hydration in PBS, endogenous peroxidase activity was blocked with 3% H_2O_2 and 10% methanol in PBS for 10 minutes. For CCR5 staining, sections were saturated using the MOM kit according to the manufacturer's instructions (Vector Labs) and incubated overnight at 4°C with the primary antibody (CCR5/α-SMA or CCR5/vWF or CCR5/Mac-3 or CCR5/CD68). We used α-SMA for SMC detection, vWF for endothelial cell (EC) detection, Mac-3 for mouse macrophage detection, and CD68 for human macrophage detection. On the next day, the sections were washed with PBS. Primary CCR5 was revealed using an amplification step and vWF/α-SMA or Mac-3 using a secondary antibody directly conjugated with Alexa (see Antibodies section). For CCR5 revelation, which required an amplification

step, we used the ABC Vectastain kit (Vector Labs) to mark the primary antibodies according to the user's guide. Substrate staining was detected using fluorescent peroxidase according to the manufacturer's instructions (PerkinElmer, Waltham, MA). All nuclei were visualized using DAPI (Sigma). Adobe Photoshop was used according to guidelines to construct multi-channel images, select specific regions of interest, and apply minor alterations to contrast and brightness uniformly across the entire figure panel.

Flow cytometry. The lung cells were prepared as follows. The lungs were isolated, minced, and digested with 400 units of collagenase D (Roche Diagnostics, Mannheim, Germany) for 30 minutes. Cell suspensions were filtered through a 70-µm cell strainer (BD Biosciences, Bedford, MA) and the leukocytes were collected using Ficoll-Paque (GE Healthcare, Cleveland, OH) and washed in phosphate buffer saline (PBS) prior to FACS staining. BAL fluid cells were collected as previously described [2]. For FACS staining, lung or BAL fluid cells were labeled for flow cytometry using the appropriate Ab (see online supplemental methods section). Samples were acquired on a FACS Calibur cytometer (Becton Dickinson, Franklin LAKES, NJ) using Cell Quest Pro then analyzed using FlowJo (Tree Star, Ashland, OR,) software [3]. Absolute cell counts were computed using a fixed number of nonfluorescent 10-µm Polybead® Carboxylate Microspheres (Polysciences, Niles, IL) as a reference.

Alveolar macrophage isolation, culture, and conditioned media preparation. Animals aged 3 months were anesthetized with ketamine/xylazine (60 mg/Kg and 10 mg/Kg, respectively). Macrophages from BAL fluid were obtained as previously described[2]. For cell-culture experiments, BAL fluids were obtained as previously described [2]. The fluorescence-activated cell-sorting (FACS) results showed that over 90% of the cells were macrophages (see flow cytometry section). To assess cell proliferation, macrophage-conditioned media were obtained as follows. Macrophages from BAL fluid were seeded onto 48-well plates in DMEM supplemented with 10% fetal calf serum (FCS), 10 mM L-glutamine, 100 IU penicillin, and 100 µg/mL streptomycin then left to grow for 4 hours. Cell viability as assessed by Trypan Blue exclusion was greater than 85% in all groups. To produce conditioned-medium cells, the medium was replaced by serum-free DMEM with or without murine

recombinant CCL5 (2 ng/mL) and with or without CCR5 antagonist (maraviroc, 10 µM). For the PA-SMC proliferation assay, macrophage-conditioned media were diluted 2-fold with fresh serum-free medium and applied on PA-SMCs for 72 hours.

Exposure to hypoxia. For in vitro experiments, cells were placed in a hypoxic chamber containing 1% O_2 and 5% CO_2 at the indicated times**.**

Cell migration assay. The cell migration assay was performed as previously described [4]. Briefly, PA-SMCs were subjected to growth arrest in FCS-free medium for 24 or 48 hours then resuspended at $40 \cdot 10^6$ cells/mL in culture medium containing 15% FCS and 0.3% agarose. These cells were plated by forming an agarose spot in the center of each well of a 24-well tissue culture plate. The preparation was left at 4°C for 20 min to allow the agarose to gel. Then, 900 µL of medium was added to cover the drops. The preparation was incubated at 37°C in 5% CO_2 for 24 or 48 hours. Samples were fixed and stained using the Diff-Quick kit (Siemens Healthcare Diagnostics, Saint Denis, France). Images were imported into Image J analysis software (http://rsbweb.nih.gov/ij/) for calculation of cell migration under each condition.

Measurement of intracellular free calcium concentrations ($[Ca^{2+}]i$). Human PA-SMCs were loaded with 2 µM Fura-2-AM for 45 min at 37°C then kept in serum-free medium for 30 min before the experiment. HEPES buffer (in 116 mM/L NaCl, 5.6 mM/L KCl, 1.2 mM/L $MgCl_2$, 5 mM/L $NaHCO_3$, 1 mM/L NaH_2PO_4, and 20 mM/L HEPES, pH 7.4) was used for the experiments. Single images of fluorescent emission at 510 nm under excitation at 340 and 380 nm were taken every 5 s. Changes in $[Ca^{2+}]i$ in response to the indicated agonist were calculated using the Fura-2 340/380 fluorescence ratio according to the equation of Grynkeiwicz or using the 380em/380em(basal) ratio [5, 6].

Cell immunostaining. PA-SMCs were cultured on Labteck (10 000 cells/well), fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and immunostained with the indicated

primary antibodies. Visualization was achieved using the appropriate Alexa Fluor-conjugated Ab (see Antibodies section).

Fluorescence microscopy analysis. Tissue sections were examined with an AxioCam microscope (Zeiss, Oberkochen, Germany). Immunocytochemistry slides were examined under a Zeiss LSM-510 confocal scanning laser microscope equipped with a 25 mW argon laser and a 1 mW helium-neon laser, using a Plan Apochromat 63X objective (NA 1.40, oil immersion). Green fluorescence was observed with a 505-550 nm band-pass emission filter under 488 nm laser illumination. Red fluorescence was observed with a 560 nm long-pass emission filter under 543 nm laser illumination. Pinholes were set at 1.0 Airy units. Stacks of images were collected every 0.7 µm along the z-axis. All settings were kept constant to allow comparisons. For double immunofluorescence, dual excitation using the multitrack mode (images taken sequentially) was achieved using the argon and He/Ne lasers.
ImageJ software was used according to the guidelines to construct multi-channel images and Z-projection of serial slides.

Protein extraction and ELISA on homogenized lung tissue. Lung-tissue specimens each weighing 30 mg and stored at -80°C were defrosted and homogenized in a Tissue Lyser® homogenizer (Qiagen, Courtaboeuf, France) using 300 µL of T-PER® tissue-protein extraction reagent (Thermo Scientific Pierce, Illkirch, France). The homogenate was centrifuged at 10 000 *g* for 5 min to remove tissue debris, and the supernatant containing the total lung lysate was used for ELISA. The total protein level in the lung lysate was quantified using the Biorad DC protein assay (Biorad, Marnes-la-Coquette, France). Before each ELISA, various dilutions of protein lysates were tested to ensure that the detected level fit within the standard linear range. All ELISA kits were obtained from R&D Systems.

Western-Blot. Total proteins were extracted as described above. Immunoblots were carried out using the above-listed antibodies and detected using an enhanced chemiluminescence detection system (Millipore, Molsheim, France). Densitometric quantification was normalized for the β-actin level using Gene Tools software (Ozyme, Montigny le Bretonneux, France).

Real-time quantitative PCR (RT-qPCR). Total mRNA was extracted from pulmonary artery ECs using RNeasy Protect Mini Kit (Qiagen, ZA Courtaboeuf, France). First-strand cDNA was synthesized in reversed transcribed samples, as follows: 1 µg total RNA isolated from cells, 100 ng Random Primers, 0.4 mM mixed dNTP, 40 U RNaseOUT, and 200 U Superscript II. All reagents were obtained from Life Technology. Quantitative PCR was performed using an ABIPRISM 7000 detection system (Applied Biosystems, ZA Courtaboeuf, France), with SYBR Green from Life Technology. To normalize for cDNA input load, mouse 18s and human SF3A1 were used as endogenous standards. The analysis was performed using the standard ΔΔCt method. Specific primers were as follows:

Mouse CCR5

Fwd 5'-CGGTGTTCAATTTTCCAGCAA-3'

Rev 5'- TCTCCTGTGGATCGGGTATAGAC- 3';

Mouse CCL5

Fwd 5'- GCCCACGTCAAGGAGTATTTCT-3'

Rev 5'- CAAACACGACTGCAAGATTGGA-3'

Mouse CCL3

Fwd 5'-GCCCTTGCTGTTCTTCTCTGT-3'

Rev 5'- GGCATTCAGTTCCAGGTCAGT -3'

Mouse CCL4

Fwd5'- GCTCGTGGCTGCCTTCTG-3'

Rev 5'- GAGGTGTAAGAGAAACAGCAGGAAGT-3'

Human CCR5

Fwd5'-GCTCTAACAGGTTGGACCAAGCT-3'

Rev 5'-TGATGCAGCAGTGCGTCAT-3'

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: CCR5 expression in SIV-infected macaques. Representative micrographs of lung tissue from an SIV-infected macaque with remodeled pulmonary vessels and uninfected, normal control macaque. CCR5 (Red), α-SMA for SMC staining or CD68 for macrophage staining (Green). No positive immunoreactivity was detected in sections incubated with the appropriate control IgG followed by secondary anti-rabbit and anti-mouse antibodies. Bar= 40 μm**.**

Supplemental Figure 2: Comparison of inflammatory and resident monocyte counts in lungs of wild-type (WT) and $CCR5^{-/-}$ mice under normoxia or hypoxia. Graph of the number of $7/4^{hi}$ inflammatory monocytes ($CCR5^{hi}$ monocytes) and $7/4^{low}$ resident monocytes ($CX3CR1^{hi}$ monocytes) in 1 mg of lung tissue, analyzed using FACS. Both individual and median values are shown.*P<0.025 and **P<0.005. Micrographs show representative FACS profiles of cells from hypoxiaWT and $CCR5^{-/-}$ mouse lung tissue.

Supplemental Figure 3: Effect of the pharmacological CCR5 inhibitor maraviroc (MVC) on PH induced by chronic hypoxia combined with SU5416. (A)- Graphs of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RV/[left ventricle plus septum (LV+S)] weight), pulmonary vessel muscularization, and percentages of dividing Ki67-positive cells in h-CCR5ki mice subjected to 18 days of chronic hypoxia combined with SU5416, with daily MVC (CCR5ki SU+MVC) or vehicle (CCR5ki SU+Ve). Representative micrographs of pulmonary vessels stained for Ki67. Red arrows show Ki67-positive nuclei. No immunoreactivity was detected in sections incubated with rabbit IgG control and secondary anti-rabbit antibody. Bar=50 μm. (B)- Medial wall thickness as a percentage of fully muscularized intraacinar arteries. Representative micrographs of pulmonary vessels stained with hematoxylin and eosin. Bar=50 μm. For all graphs, both individual and median values are shown. *P<0.025, **P<0.005.

Supplemental Figure 4: Reversal of pulmonary hypertension by maraviroc (MVC) treatment in h-CCR5ki mice. RVSP, RV/[LV+S] weight ratio, and pulmonary vessel muscularization in normoxic and chronically hypoxic mice studied on days 15 and 30 after hypoxia exposure. MVC 200 mg/Kg/day was given from day 15 to day 30. Both individual and median values are shown. *P<0.025. Representative micrographs of pulmonary vessels stained with hematoxylin eosin. Bar=50 µm

Supplemental Figure 5: Effect of hypoxia on CCR5 expression in human PA-SMCs treated with maraviroc (MVC) or vehicle. CCR5 mRNA levels were quantified by RT-qPCR after 4 h exposure to hypoxia. Data are mean±SEM of 9 values of three different experiments. *P<0.05 (Wilcoxon Rank test).

Supplemental Figure 6: Mobilization of intracellular calcium in hu-PA-SMC by native or heat-inactivated gp120. Intracellular calcium imaging in FURA-2 loaded cells. The curve represents the mean of three cells responding to native gp120 (100 pM) recorded in the same experiment. The capacity of gp120 to trigger the mobilization of Ca^{2+} from intracellular store though receptor activation was assessed by addition of heat-inactivated (90°C, 30 min) gp120 (100 pM) followed by addition of native gp120 (100 pM). Heat-inactivated gp120 had no effect on intracellular Ca2+, whereas the addition of native gp120 triggered the calcium mobilization in three out of four cells.

Supplemental Figure 7: Effect of CCL5 (250 pM) on growth of PA-SMCs from h-CCR5ki, WT, and $CCR5^{-/-}$ mice in the presence of maraviroc (MVC, 10 µM) or vehicle. Values are expressed as percent of control values in DMEM starvation medium. Data are mean±SEM of 8-12 values of three different experiments. *P<0.05.

Supplemental Figure 8: Effect of CCL5 (10 nM) on migration of PA-SMCs from h-CCR5ki, WT, and $CCR5^{-/-}$ mice. Values are expressed as percent of control values in DMEM starvation medium. Data are mean ±SEM of 8 values. *P<0.05. Representative micrographs of agarose spots after migration for 48 hours.

Supplemental Figure 9: CCL5 levels measured by ELISA in media conditioned by BAL fluid macrophages from normoxic and hypoxic mice. Both individual and median values are shown. *P<0.05

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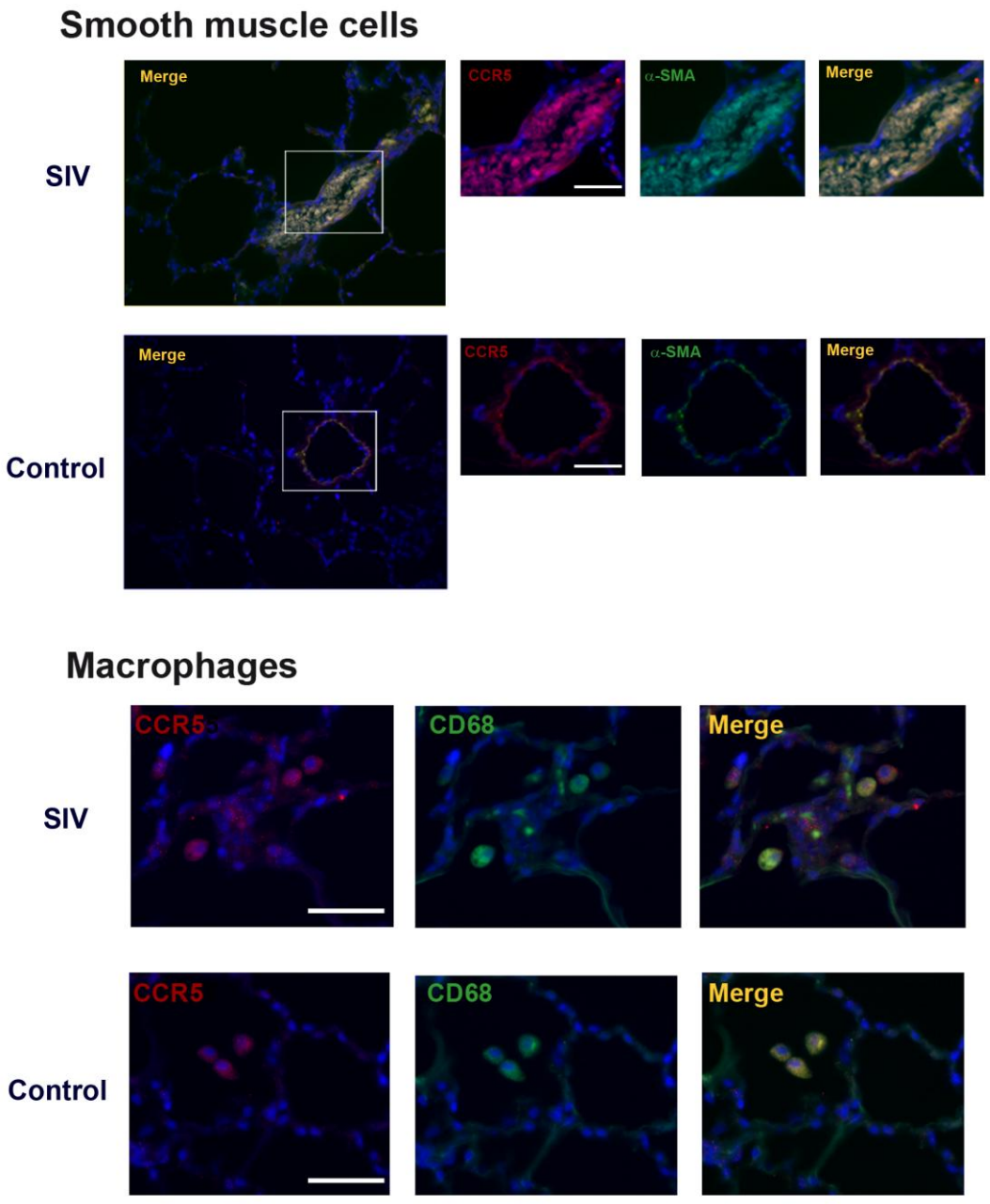
Supplemental Figure 1



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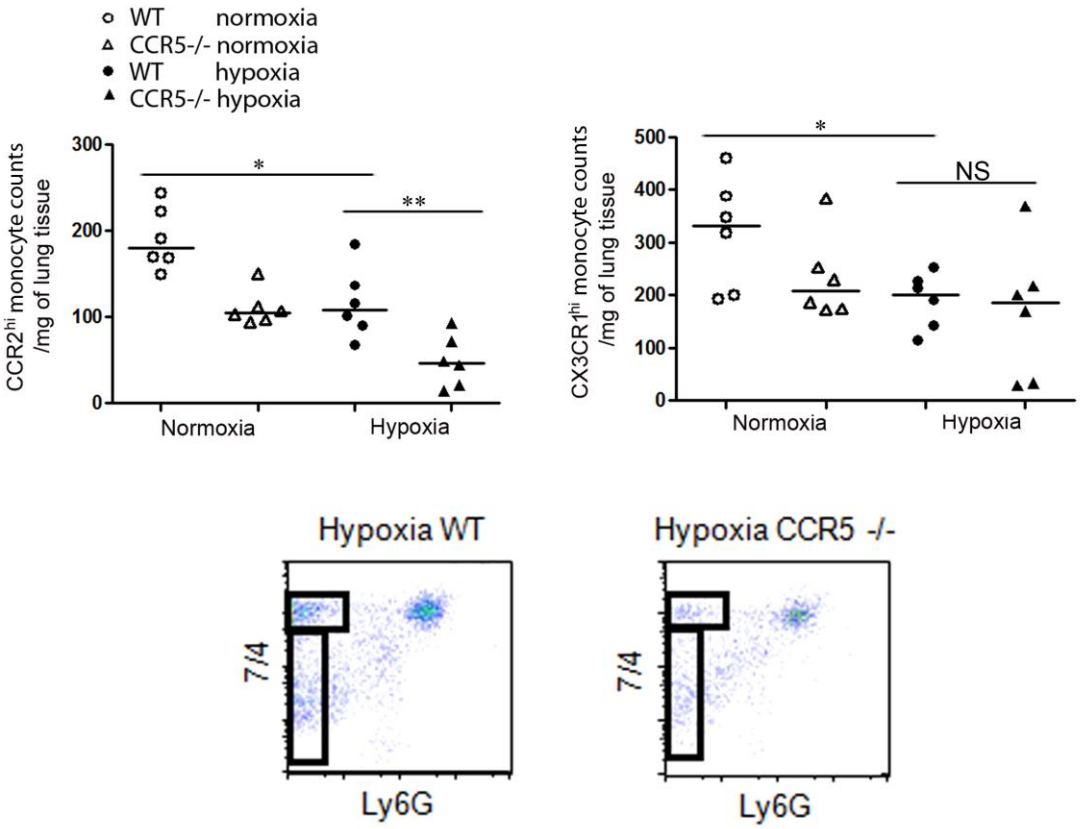

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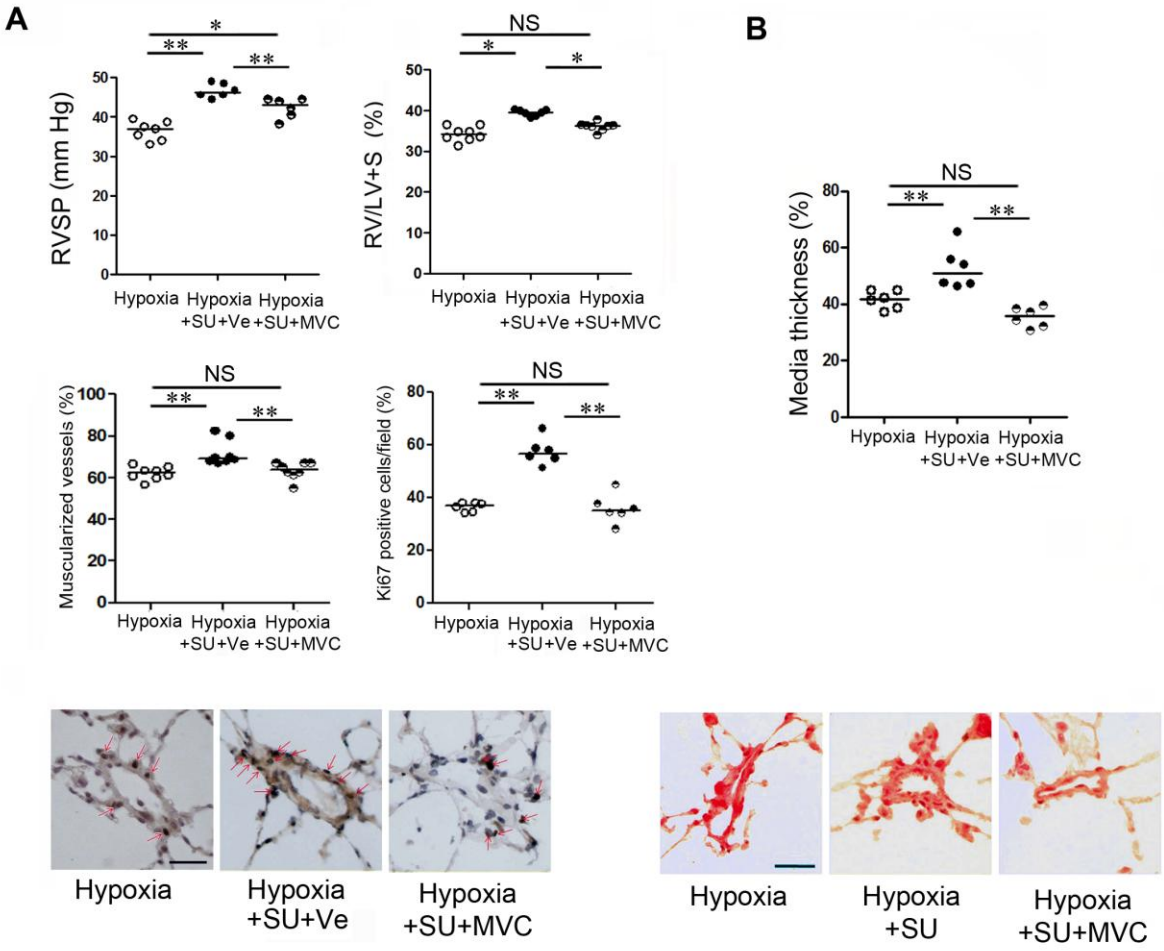

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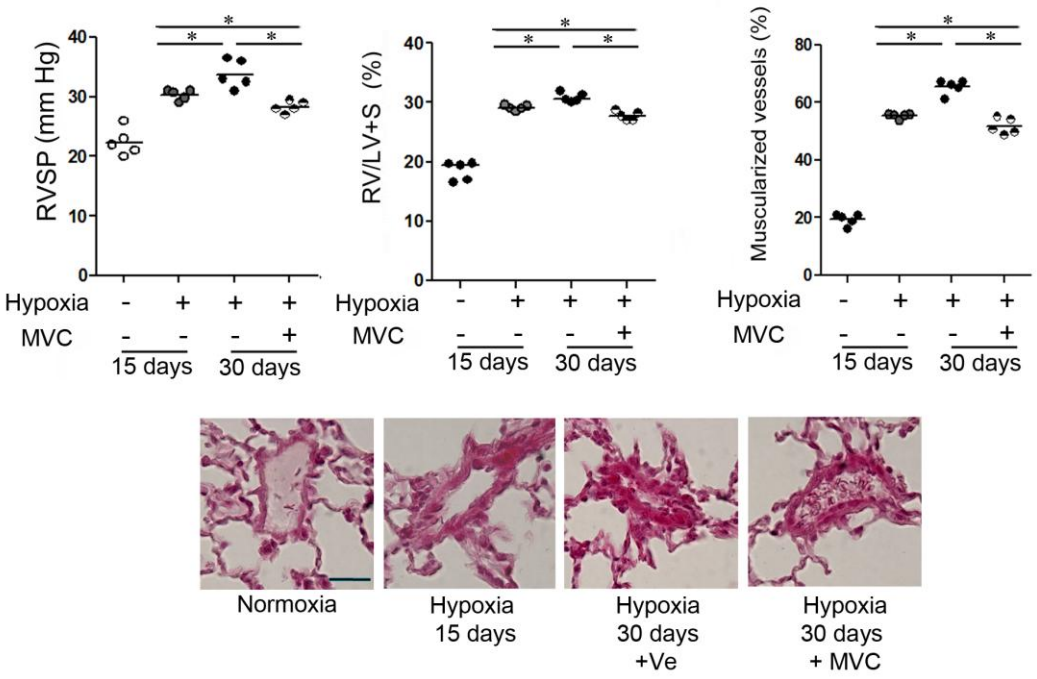

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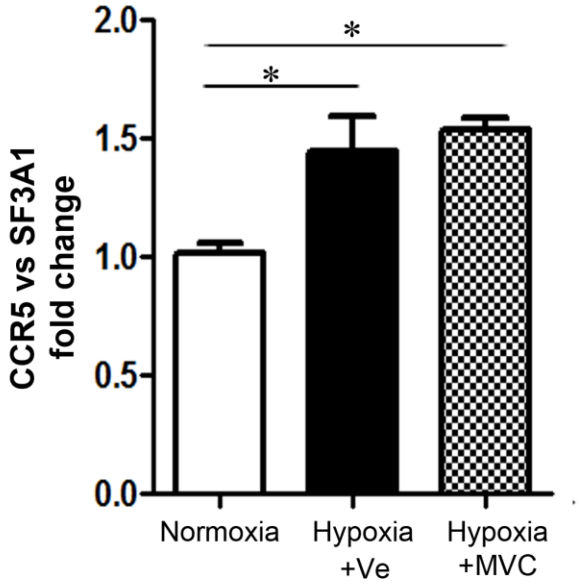

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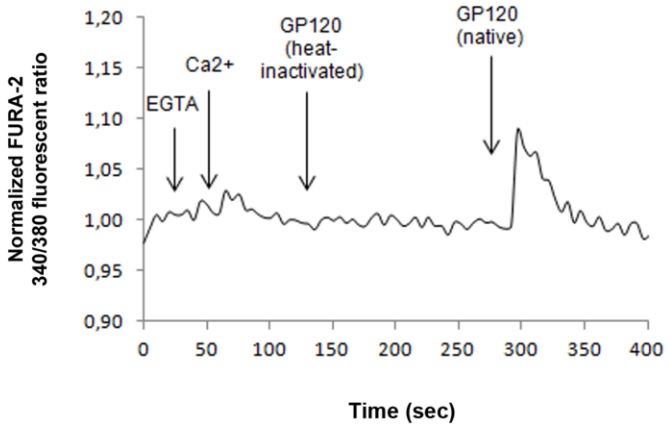

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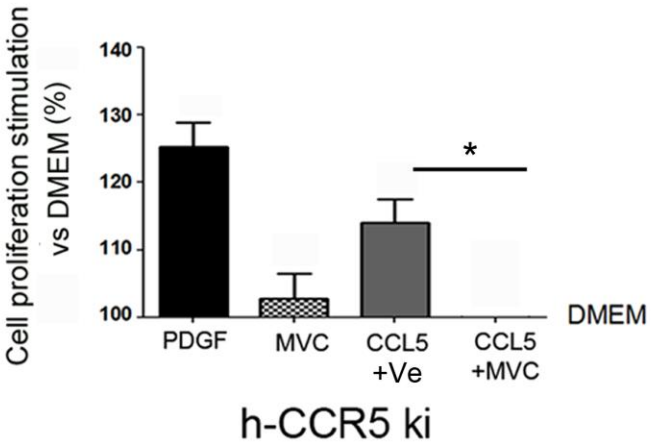

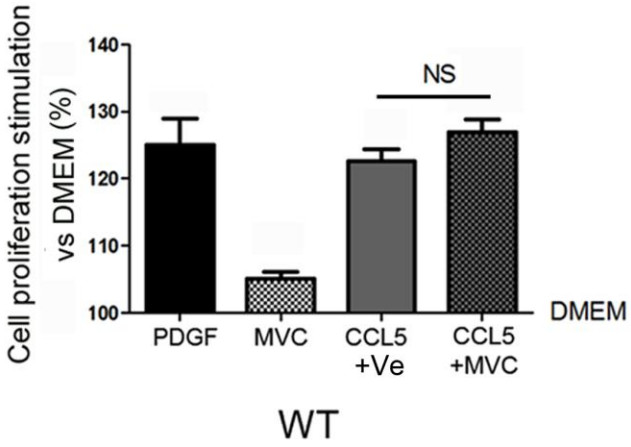

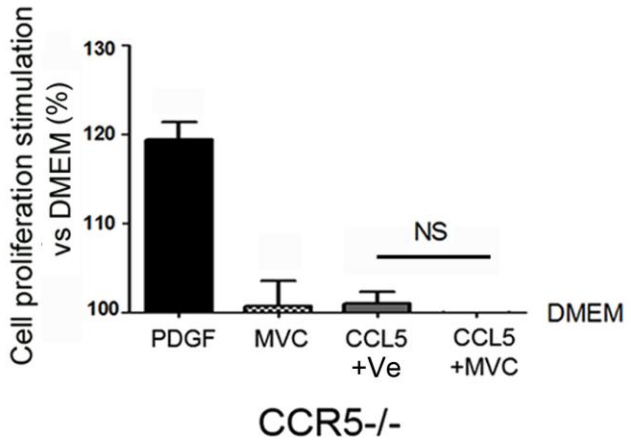

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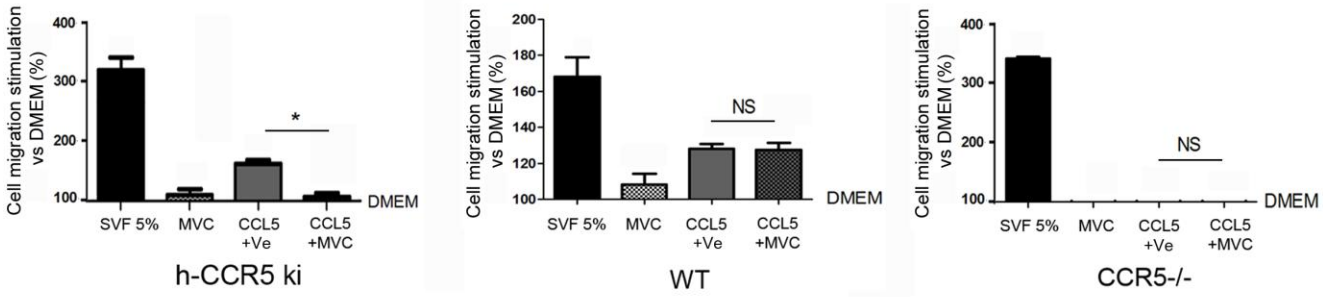

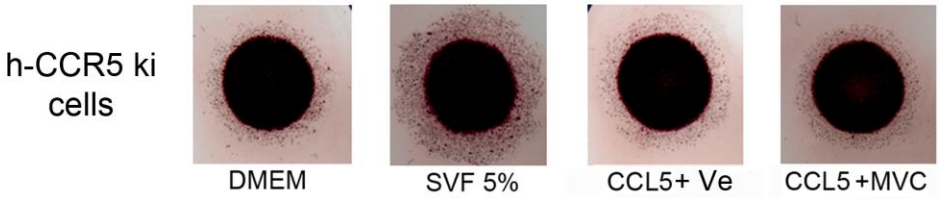

Supplemental Figure 9