**Online Supplementary Materials**

# miR-511-3p Protects Against Cockroach Allergen-Induced Lung Inflammation by Antagonizing CCL2

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## METHODS

### Flow cytometry analysis

Single-cell suspensions from whole lung tissues were prepared from minced lung tissues following by collagenase D (2 mg/mL) and DNase I (40 U/mL) digestion in DMEM supplemented with 10% v/v FBS for 60 min at 37$^{o}$C with shaking. The digested lung solution was passed through a sterile 40 μm strainer and centrifuged for 10 min at 400 x g, 4$^{o}$C. Red blood cells were lysed with 5 mL of ACK buffer (ThermoFisher) and resuspended in cold PBS for antibody staining. To prepare stained cells for flow cytometric analysis, Fc-receptors were blocked with anti-CD16/32 and stained for the macrophage surface antigens F4/80 and CD11c, In a separate sample, the T-cell surface antigens CD4 and CD25 were also stained. Cells were fixed and permeabilized using the Transcription Factor Fixation/Permeabilization Buffer Set (BioLegend) as instructed by the manufacturer and stained for either the intracellular marker iNOS, arginase-1, or FoxP3. In each test, a minimum of 100,000 cells was analyzed using a FACSCalibur (BD) flow cytometer. M1 and M2 macrophages (1), and Tregs are defined as $F4/80^{+}CD11c^{+}iNOS^{+}$, $F4/80^{+}CD11c^{+}Arg\text{-}1^{+}$, or $CD4^{+}CD25^{+}FoxP3^{+}$, respectively. Cells collected from the BAL fluids were also stained and analyzed using this method.

### miR-511-3p overexpression in macrophages with Lentivirus transduction

Murine bone marrow (BM) cells were obtained from the femurs and tibias of 6-8 week-old mice. The BM cells were cultured at a starting density of $5x10^{6}$ cells/mL at 37°C in a humidified atmosphere containing 5% CO2 and differentiated into macrophages (BMDMs) in DMEM containing 10% FBS, 1% penicillin /streptomycin, and 20 ng/mL of recombinant murine M-CSF (BioLegend) for 7 days. BMDMs were seeded onto a 6-well tissue culture plate (CellTreat,

Pepperell MA) at a density of $1x10^5$ cells/well 24 h prior to treatment. Over-expression of miR-511-3p in BMDMs were accomplished by LV transduction at a calculated MOI=3 48 h prior to treatment. Classically (M1) or alternatively (M2) activated macrophages were induced with 100 ng/mL of LPS (ThermoFisher) or 20 ng/mL of murine recombinant IL-4 (ThermoFisher). Total RNA was isolated from the cells using the Ribospin$^{TM}$ II kit after 6 or 18 h prior to LPS or IL-4 stimulation, respectively. In some experiments, BMDMs were treated with recombinant murine Ccl2 (BioLenged) at 5, 20, or 50 ng/mL in DMEM containing 10% FBS and 1% penicillin /streptomycin. Total RNA was isolated from the cells ~2 h post-treatment.

**Isolation of murine lung macrophages**

The isolation of murine lung macrophages from the BAL was accomplished using the method described by Zhang *et al* (2) with minor modifications. In brief, 6-8 weeks old $Mrc1^{-/-}$ mice were terminally anesthetized using a ketamine/xylazine cocktail. Circulating red blood cells were flushed from the lungs with 10mL of phosphate-buffered saline (PBS) through the right ventricle. The lungs were infused with 1 mL of prewarmed (37°C) calcium and magnesium-free PBS supplemented with 0.5 mM EDTA via a tracheal catheter and gently withdrawing the fluid into the syringe. This was repeated 9 more times, the collected volumes were pooled together, and centrifuged for 10 min at 400 x g, 4°C. The cells were resuspended in DMEM/F12 medium supplemented with 10% (v/v) fetal bovine serum, 10 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Macrophage purity was assessed by flow cytometry and defined as $CD45^+F4/80^+CD11c^+$ cells.

**RT-PCR**

Total RNA was isolated using Ribospin$^{TM}$ II (GeneAll, Seoul Korea) with on-column DNase I digestion as instructed by the manufacture. RNA quality and concentration was assessed by Nanodrop-2000 spectrometer (Agilent Technologies, Santa Clara CA). cDNA templates were synthesized with SuperScript II (Invitrogen, Carlsbad CA) as instructed. qRT-PCR was performed in duplicates using Power SYBR Green Universal 2X qPCR Master Mix (Applied Biosystems, Foster City CA) on an ABI Prism 7300 detection system. mRNA levels were normalized to the internal control gene (β-actin) and data were analyzed using the $2^{-\Delta\Delta CT}$ method (3). Primers sequences are annotated by PrimerBank (4) and provided in the online depository.

**Co-immunoprecipitation**

Co-immunoprecipitation (IP) was performed as previously described (5). THP-1 cells were treated with 50 ng/mL of recombinant human CCL2 (BioLegend) for 2 h and rinsed with ice-cold 1X PBS. Cells were lysed with Cell Lysing Buffer (Cell Signaling) containing Protease/Phosphatase Inhibitor Cocktail (Cell Signaling). Cell lysate was pre-cleared with Protein A magnetic beads (Cell Signaling) and incubated with anti-CCR2 (clone D14H7, Cell Signaling) or isotype control (clone DA1E, Cell Signaling) overnight at 4$^{o}$C with rotation. Antibody-protein complexes were enriched using Protein A magnetic beads and washed 5 times with 1X Cell Lysing Buffer. The beads were resuspended in SDS lysing buffer and heated to 95-100°C for 5 min. The pellet beads were separated using magnetic rack and the the supernatant was analyzed by Western Blot.

**REFERENCE**


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5. Li C, Zhen G, Chai Y, Xie L, Crane JL, Farber E, Farber CR, Luo X, Gao P, Cao X, Wan M. RhoA determines lineage fate of mesenchymal stem cells by modulating CTGF-VEGF complex in extracellular matrix. *Nature communications* 2016; 7: 11455.

**FIGURE LEGENDS**

**Figure E1. Confirmation of AAV-miR-511-3p delivery into the mouse lung and infected major cell populations.** (**A**) Flow cytometry analysis of single cell suspensions from mouse lung on day 35 after AAV-miR-511-3p delivery and AAV-Mock control. Gating was made on GFP with F4/80 and CD11C (macrophages), CD11C, and MHCII (DCs), and MHCII and EpCaM (epithelial cells). (**B**) Representative images of co-immunostaining for eGFP with F4/80 (macrophages) and EpCaM (epithelial cells) from paraffin embedded lung section of AAV-treated mice. Original magnification x20. (**C**) Quantitative RT-PCR analyses for miR-511-3p expression in the single cell suspensions from mouse lung on day 35 after AAV-miR-511-3p delivery or AAV-Mock control. n=3/group. Data represent means ± SEMs, comparisons were made using 2-tailed Student's t test between groups. $^{**}P < 0.01$.

**Figure E2. Gating strategies to measure bronchoalveolar lavage fluid cells by flow cytometry.** Eosinophils were gated $SSC^{high}$ $SiglecF^{+}Mac\text{-}3^{-}$cells, macrophages on $SSC^{high}$ $SiglecF^{+}$ $Mac\text{-}3^{+}$ cells, granulocytes on $SSC^{high}$ $Gr\text{-}1^{+}$ cells, and lymphocytes on $FSC^{low}/SSC^{low}$ $CD3^{+}$ cells. Flow cytometry was performed on a FACSCalibur cytometer.

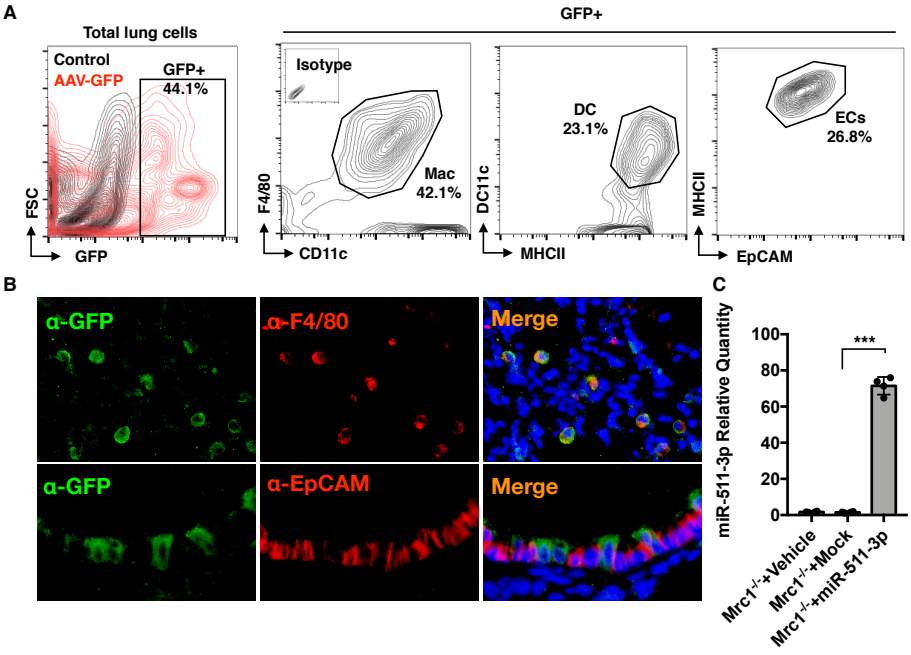
**Figure E3. Confirmation of purity of macrophages enriched from bronchoalveolar lavage fluids by flow cytometry analysis.** Macrophages: $CD45^{+}F4/80^{+}/CD11C^{+}$.

**Figure E4. Expression of miR-511-3p in different cells.** The figure was generated based on data provided online by Geome.ucsc.edu.

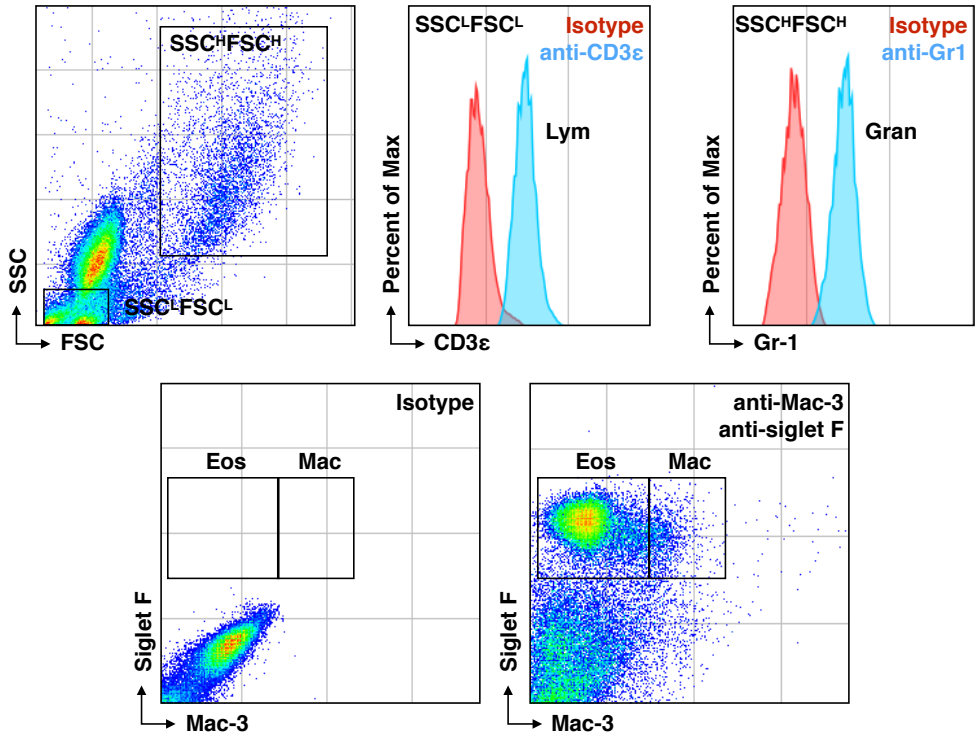
**Figure E5**. **Flow cytometry analyses of T regulatory cells (Tregs) in the lung tissues and bronchoalveolar lavage fluids of mice treated with or without AAV-miR-511-3p. (A)** Representative flow cytometric gating of Tregs from the single cell suspension of whole lung tissue and BALs of mice treated with AAV-miR-511-3p (top panel) and AAV-Mock control

(bottom panel). (**B-C**) Quantitation of Tregs as indicated by the percentage of $CD4^+CD25^+FoxP3^+$ cells among total lung single cells from lung tissues (**B**) and BALs (**C**). n=5/group. Data represent means ± SEMs, comparisons were made using 2-tailed Student's t test between groups. $^{**}P < 0.05$, $^{**}P < 0.01$, and $^{**}P < 0.001$.

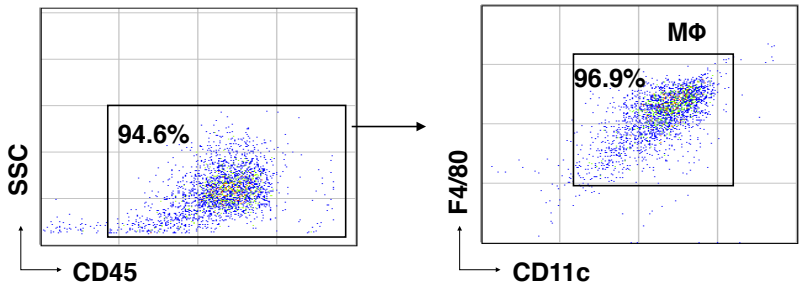
**Supl Figure 1**



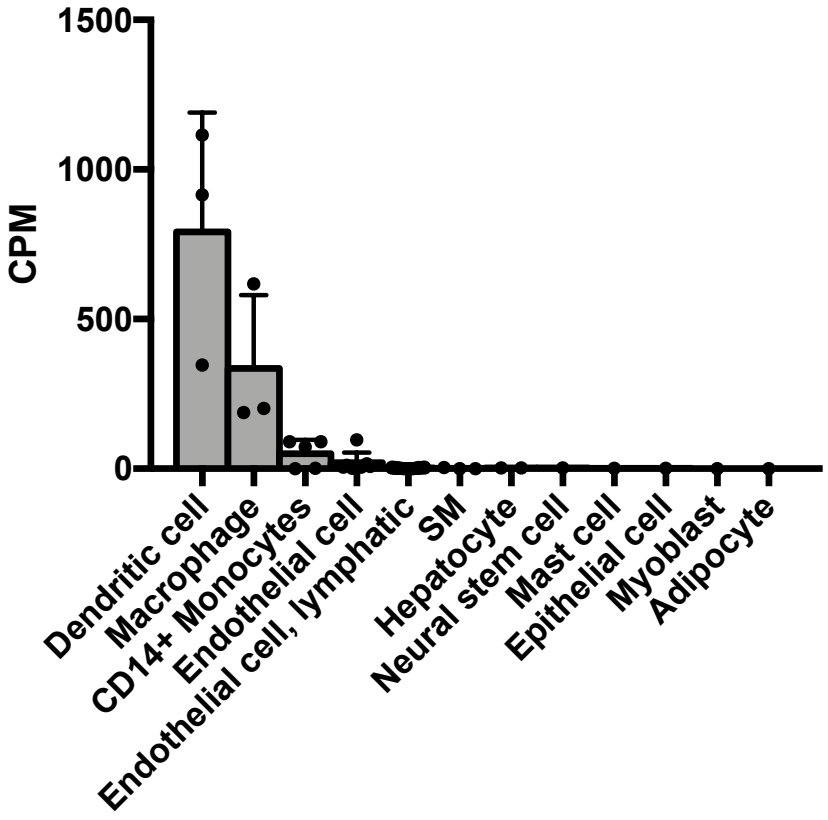
**Supl Figure 2**



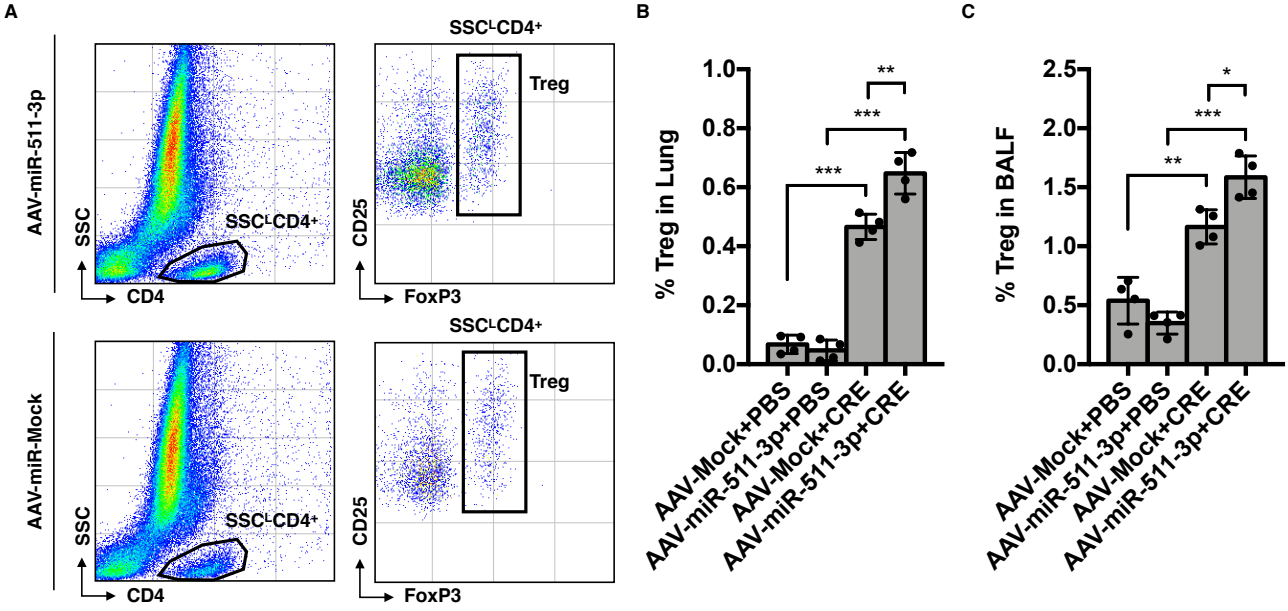
**Supl Figure 3**



**Supl Figure 4**



**Supl Figure 5**



**Supl Table 1**

**Table E1: mRNA-miR-511-3p Target Region**

| Target region (top)<br>miRNA (bottom) | Location | Predicted consequential pairing |
|---|---|---|
| Position 872-894 of Ak2<br>mmu-miR-511-3p | 3'UTR | 5' CCCUGCUCUUAGGUGCUGGGCAG<br>3' UAGGAC AGAAA ACGAUGUGUAA |
| Position 5067-5097 of Clcn3<br>mmu-miR-511-3p | 3'UTR | 5' UAUCUGAUGUGUCUUACUGCUGAAACACAUG<br>3' UAGG ACAGAAA ACGA UGUGUAA |
| Position 3735-3761 of Plek<br>mmu-miR-511-3p | 3'UTR | 5' UGUUCUGUUACUGUGAAAAUGCACGUA<br>3' UAGGACAGAAA ACG AUGUGUAA |
| Position 693-722 of Srgn<br>mmu-miR-511-3p | 3'UTR | 5' UAUCUUGUUGUUCUGGAAAAUGCCUGCAUU<br>3' UAGGACAGAAA AC GAUGUGUAA |

**Supl Table 2**

**Table E2: Antibody Characteristics**

| Antibody | Clone | Species | Application | Source |
|---|---|---|---|---|
| Arg-1 | D4E3M<br>IC5868P | Rabbit<br>Sheep | IF (1:100)<br>FC (1:200) | Cell Signaling<br>R&D |
| β-Actin | 2F1-1 | Mouse | WB (1:1000) | BioLegend |
| CCR2 | D14H7<br>3G7 | Rabbit<br>Mouse | IP (1:100)<br>WB (1:1000) | Cell Signaling<br>Novus |
| CD11c | HL3 | Hamster | FC (1:100) | BD |
| CD25 | PC61 | Rat | FC (1:200) | BioLegend |
| CD3ε | 145-2C11 | Hamster | FC (1:100) | BioLegend |
| CD4 | RM4-5 | Rat | FC (1:200) | BioLegend |
| CD45 | 30-F11 | Rat | FC (1:50) | BioLegend |
| EpCAM | G8.8 | Rat | FC (1:200) | BioLegend |
| F4/80 | BM8 | Rat | IF (1:100<br>FC (1:50) | BioLegend |
| FoxP3 | FJK-16s | Rat | FC (1:200) | ThermoFisher |
| Gr-1 | RB6-8C5 | Rat | FC (1:100) | BioLegend |
| iNOS | NB300-605<br>CXNFT | Rabbit<br>Rat | IF (1:50)<br>FC (1:200) | Novus<br>ThermoFisher |
| Mac-3 | M3/84 | Rat | FC (1:100) | BioLegend |
| RhoA | 1A11-4G10 | Mouse | WB (1:500) | Novus |
| RhoA-GTP | 26904 | Mouse | IF (1:100) | NewEast |
| SiglecF | 1RNM44N | Rat | FC (1:100) | ThermoFisher |

**Supl Table 3**

**Table E3: Primer Sequences**

| Gene | NCBI GeneID | Primer Sequence | Amplicon size | PrimerBank ID |
|---|---|---|---|---|
| Ak2 | 11637 | Fwd: 5'GGCTTCCGAACCGGAGATTC<br>Rev: 5'CAGACACAAAAGTTTTCAGCCAG | 108 | 34328230a1 |
| Arg-1 | 11846 | Fwd: 5'CATTGGCTTGCGAGACGTAGAC<br>Rev: 5'GCTGAAGGTCTCTTCCATCACC | 124 | NA |
| Actb | 11461 | Fwd: 5'GGCTGTATTCCCCTCCATCG<br>Rev: 5'CCAGTTGGTAACAATGCCATGT | 154 | 6671509a1 |
| Ccl2 | 20296 | Fwd: 5'TTAAAAACCTGGATCGGAACCAA<br>Rev: 5'GCATTAGCTTCAGATTTACGGGT | 121 | 6755430a1 |
| Ccr2 | 12772 | Fwd: 5'ATCCACGGCATACTATCAACATC<br>Rev: 5'CAAGGCTCACCATCATCGTAG | 104 | 6753466a1 |
| Chi3l3 | 12655 | Fwd: 5'TACTCACTTCCACAGGAGCAGG<br>Rev: 5'CTCCAGTGTAGCCATCCTTAGG | 135 | NA |
| Clcn3 | 12725 | Fwd: 5'AGCTACAACAGCATAACCAGC<br>Rev: 5'GTCCCCGTCTAACAAATTGTCAT | 108 | 2599550a1 |
| Fizz1 | 57262 | Fwd: 5'CAAGGAACTTCTTGCCAATCCAG<br>Rev: 5'CCAAGATCCACAGGCAAAGCCA | 151 | NA |
| IL-1β | 16176 | Fwd: 5'TGGACCTTCCAGGATGAGGACA<br>Rev: 5'GTTCATCTCGGAGCCTGTAGTG | 148 | NA |
| IL-6 | 16193 | Fwd: 5'TAGTCCTTCCTACCCCAATTTCC<br>Rev: 5'TTGGTCCTTAGCCACTCCTTC | 76 | 13624311a1 |
| iNOS | 18126 | Fwd: 5'GAGACAGGGAAGTCTGAAGCAC<br>Rev: 5'CCAGCAGTAGTTGCTCCTCTTC | 179 | NA |
| Plek | 56193 | Fwd: 5'CAAAGCGGATCAGGGAGGG<br>Rev: 5'TTGACAAGGGCTAGTGAGAGT | 173 | 11464971a1 |
| Rhoa | 11848 | Fwd: 5'AGCTTGTGGTAAGACATGCTTG<br>Rev: 5'GTGTCCCATAAAGCCAACTCTAC | 138 | 31542143a1 |
| Srgn | 19073 | Fwd: 5'CTCGCCTTCGTCCTGGTTT<br>Rev: 5'CCTCGATGCAGTTCGCAAAAA | 112 | 6997243a1 |