ONLINE DATA SUPPLEMENT

Low sphingosine-1-phosphate impairs lung dendritic cells in cystic fibrosis

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Supplemental Materials and Methods

Cells

$CD11c^+$ cells were isolated from lung single cell suspensions from WT and CF mice using anti-CD11c magnetic beads (Miltenyi, Auburn, CA) on a LS column (Miltenyi). The conventional DC (cDC; $CD11b^+CD11c^+CD103^-$) and plasmocytoid DC (pDC; $CD11c^+CD11b^-B220^+$) and $CD103^+$ DC ($CD11c^+CD11b^-CD103^+$) were defined with the rat anti mouse antibodies CD11c, CD11b, CD103 and B220 (BD Biosciences, San Jose, CA) and analyzed by FACSCalibur flow cytometer (BD Biosciences) using the Flowjo software (Tree Star Inc., Ashland, OR). For extraction of RNA, cDC and pDC were isolated by fluorescence-activated cell sorting with BD FACSAria Cell Sorter (BD Biosciences). Maturation and activation of cDC, pDC and $CD103^+$ DC were assessed by quantification of the surface expression of CD40, CD86, MHCI and MHCII using rat anti-mouse antibodies (BD Biosciences).

CFTR expression in DC

CFTR protein expression was determined in $CD11c^+$ cells or homogenized lungs from WT mice by Western analysis with a rabbit anti-mouse CFTR antibody (R&D System, Minneapolis, MN). GAPDH protein, the loading control, was detected using a rabbit anti-mouse GAPDH antibody (Sigma-Aldrich, St Louis, MO). Messenger RNA levels of CFTR in purified cDC, pDC, and lung tissue from WT mice were measured by real-time RT-PCR using a CFTR specific probe (Mm00445197_m1, Applied Biosystems, Carlsbad, CA). CFTR mRNA levels were quantified using the ΔΔCt method (Applied Biosystems) and normalized to expression of murine GAPDH (Applied Biosystems).

Allostimulatory capacity of DC

The allostimulatory capacity of DC was assessed by mixed leukocyte reaction (MLR). Allogeneic T cells were isolated from spleens of BALB/c mice using the Dynal Mouse T Cell Negative Isolation Kit (Invitrogen). T cells were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen). The CFSE-labeled T cells ($1x10^5$/well) were then stimulated with DC isolated from CF and WT mice at DC to T cell ratios of 1:2, 1:4, 1:8 and 1:16 for 5 d. Proliferation of CFSE-labeled splenic T cells was assessed by flow cytometry and the proliferation index was determined as mean fluorescence intensity of proliferated cells to nonproliferated cells.

Infection with RSV

To assess the effect of infection of the respiratory tract with a viral pathogen relevant to CF, CF or WT mice were infected with RSV strain A2. Details in Supplemental Methods.RSV strain A2 (ATCC, Manassas, VA) was propagated in HEp-2 cells (ATCC) maintained in Eagle's minimal essential medium (Invitrogen) supplemented with 50 mg/ml of Penicillin/Streptomycin (Invitrogen) and 10% FBS (Invitrogen). RSV was administered intranasally (10^6 pfu/mouse). Three and 6 d following infection the mice were sacrificed, lungs were harvested and DC were isolated and analyzed as described above.

Co-culture of DC with BALF

BALF was collected by intratracheal instillation of 0.5 ml PBS. BALF was centrifuged at 1200 rpm for 10 min to remove cells and the supernatant was added to the DC. Expression levels of CD40, CD86, and MHCII in DC were determined at the beginning of the culture and after 24 h by flow cytometry.

To assess the effect supplementing S1P to the CF BALF on lung DC, DC were cultured in WT BALF, CF BALF, or CF BALF supplemented with 0.1, 1 and 10 μM S1P (Enzo Life Sciences International, Plymouth Meeting, PA). S1P in the nM to uM range affects DC chemotaxis (1). Expression of CD40, CD86, and MHCII in DC were determined by flow cytometry as described above after 24 h. Human peripheral blood derived DC (PBDC) were also analyzed to evaluate the effect of CF BALF. Monocytes obtained from Buffy coats (New York Blood Bank, New York, NY) were differentiated to mature DC under stimulation of 100 μg/ml IL-4 (R&D System) for 6 days. PBDC cultured in WT BALF, CF BALF, or CF BALF supplemented with 0.1 μM S1P for 24 h were evaluated for expression levels of HLA-DR, CD40 and CD86 by flow cytometry.

To evaluate the effect of S1P uptake WT lung DC were cultured in WT BALF or CF BALF supplemented with the S1P analogue FTY720 (20 nM; Cayman Chemical Company, Ann Arbor, MI), a dose previously reported to have effects on endothelial cells (2), or the S1PR2 receptor blocker JTE013 or the S1PR1 and 3 receptor blocker VPC23019 (Tocris Bioscience, Bristol, United Kindom) for 24 h.

To evaluate other potential factors in BALF affecting DC maturation neutrophil elastase (NE) and type 1 interferon (IFN-α/β) were measured in the BALF by ELISA (NE; antibodies-online Inc, Atlanta, GA; and IFN-α/β, Enzo Life Sciences International, Plymouth Meeting, PA, respectively). To assess if NE or IFN-α/β could impair activation and maturation of DC, WT lung DC were cultured in WT BALF supplemented with 50nM NE (R&D System, Minneapolis, MN) or 100nM interferon (R&D System).

S1P analysis

The level of S1P in BALF was analyzed from single–phase extracts and separated by reverse phase liquid chromatography. BALF was fortified with internal standards, lyophilized to reduce total volume, and then the extract was resuspended in methanol and an aliquot of the sample was used for the high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) analysis (3). Protein concentration were measured by BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) and used for normalization.

Supplemental Figure Legends

Supplemental Figure 1. Activation and maturation of splenic CF DC. Surface expression of **A.** MHCII, **B.** CD40 , and **C.** CD86 on cDC, pDC and $CD103^+$ DC isolated from spleens of CF or WT mice. Shown are the means ± SEM from a representative of six independent experiments, each with n=4-5 mice/group. * denotes $p<0.05$.

Supplemental Figure 2. Early effect of pulmonary infection with RSV on DC in CF mice. CF and WT mice were infected with RSV strain A2 (10^6 pfu/mouse) via intranasal installation. The percentages of cDC (**A**) and pDC (**B**) from CF and WT mice were quantified by flow cytometry after three days. Data is expressed as mean ± SEM of the percentage of stained cells of all lung cells. (**C**) Allostimulatory capacity of DC from WT and CF mice following infection with RSV were assessed by co-cultured with allogeneic T cells purified from spleens of BALB/c mice labeled with CFSE at ratio of 1:2 for five days. Proliferation index (mean fluorescence intensity

of proliferated cells to nonproliferated cells) was determined by flow cytometry. * denotes $p<0.05$.

Supplemental Figure 3. Cytokine expression of lung T cells from CF and WT mice following pulmonary infection with RSV. CD4 and CD8 T cells isolated from lung suspensions after six days were analyzed by intracellular staining of IL-4 (**A, C**) and INF-γ (**B, D**) by flow cytometry. Shown are the means ± SEM from a representative of six independent experiments, each with n=4-5 mice/group. * denotes $p<0.05$.

Supplemental Figure 4. S1P increases maturation and activation of human blood DC cultured with CF BALF. Human peripheral blood derived DC were cultured in WT BALF, CF BALF, and CF BALF with 0.1 μM S1P for 24 h. Surface expression of MHCII (**A**), CD40 (**B**), and CD86 (**C**) in human PBDC were assessed by flow cytometry. Data represents the mean ± SEM of mean fluorescence intensity. *,*** denote $p<0.05$, 0.001.

Supplemental Figure 5. Elevated levels of neutrophil elastase and type 1 interferon do not affect maturation of lung DC. Neutrophil elastase (NE, **A**) and interferon-α/β (IFN -α/β□ □**B**) were analyzed in BALF of WT and CF mice by ELISA. Lung DC from WT mice were cultured in WT BALF supplemented with 50 nM NE or 100 nM IFN -α/β for 24 h. Surface expression of MHCII (**C**), CD40 (**D**), and CD86 (**E**) in DC were assessed by flow cytometry. * denotes $p<0.05$.

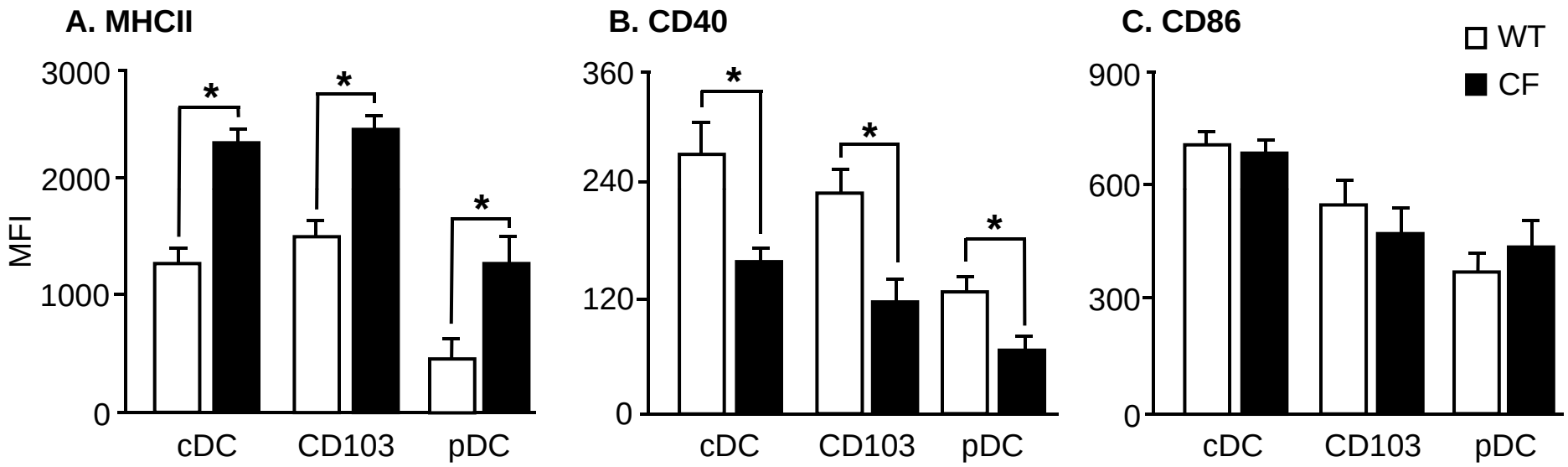
References

E1. Idzko M, Panther E, Corinti S, Morelli A, Ferrari D, Herouy Y, Dichmann S, Mockenhaupt M, Gebicke-Haerter P, Di Virgilio F, et al. Sphingosine 1-Phosphate Induces Chemotaxis of Immature Dendritic Cells and Modulates Cytokine-Release in Mature Human Dendritic Cells for Emergence of Th2 Immune Responses. *FASEB J*. 2002, 16:625-7.

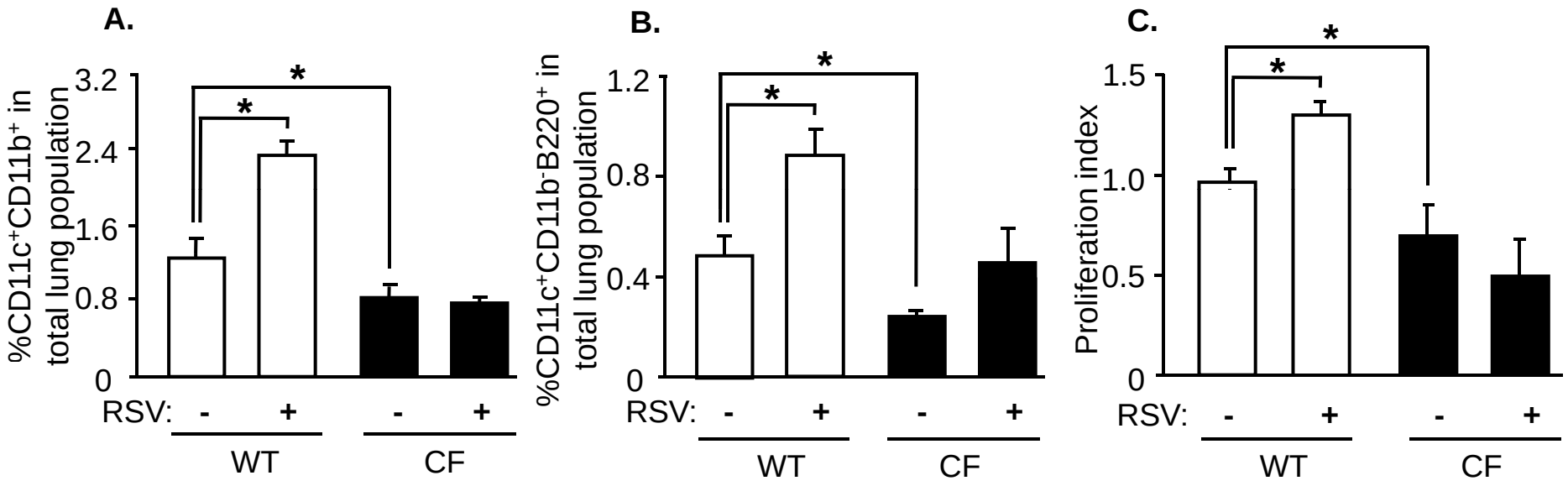
E2. Muller HC, Hocke AC, Hellwig K, Gutbier B, Peters H, Schonrock SM, Tschernig T, Schmiedl A, Hippenstiel S, N'Guessan PD, et al. The Sphingosine-1 Phosphate Receptor Agonist FTY720 Dose Dependently Affected Endothelial Integrity in Vitro and Aggravated Ventilator-Induced Lung Injury in Mice. *Pulm Pharmacol Ther* 2011;24:377-385.

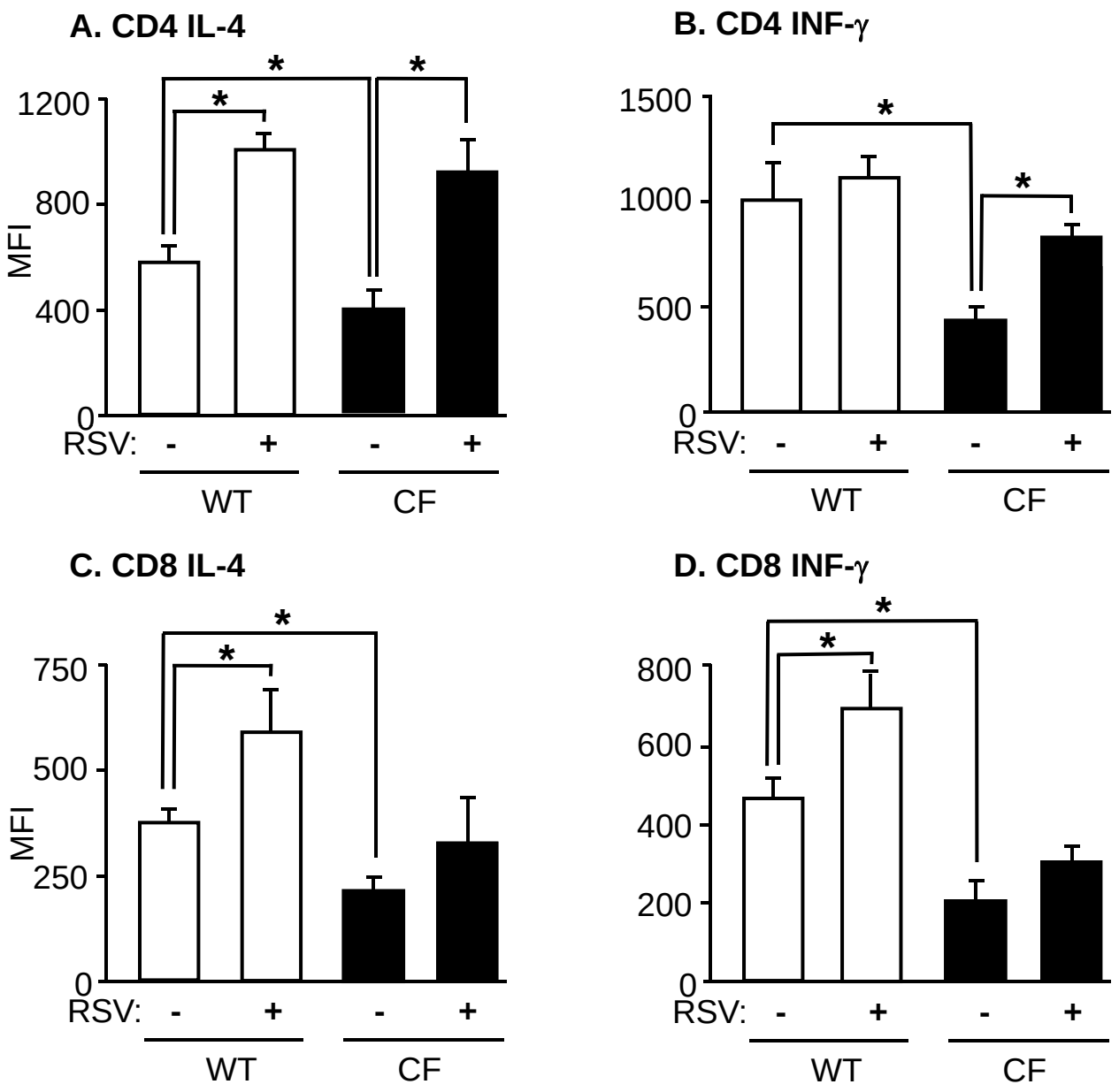
E3. Shaner RL, Allegood JC, Park H, Wang E, Kelly S, Haynes CA, Sullards MC, Merrill AH. Quantitative Analysis of Sphingolipids for Lipidomics Using Triple Quadrupole and Quadrupole Linear Ion Trap Mass Spectrometers. *J Lipid Res* 2009;50:1692-1707.

Supplemental Figure 1



Supplemental Figure 2

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MFI
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800
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RSV: - + - +
WT
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WT
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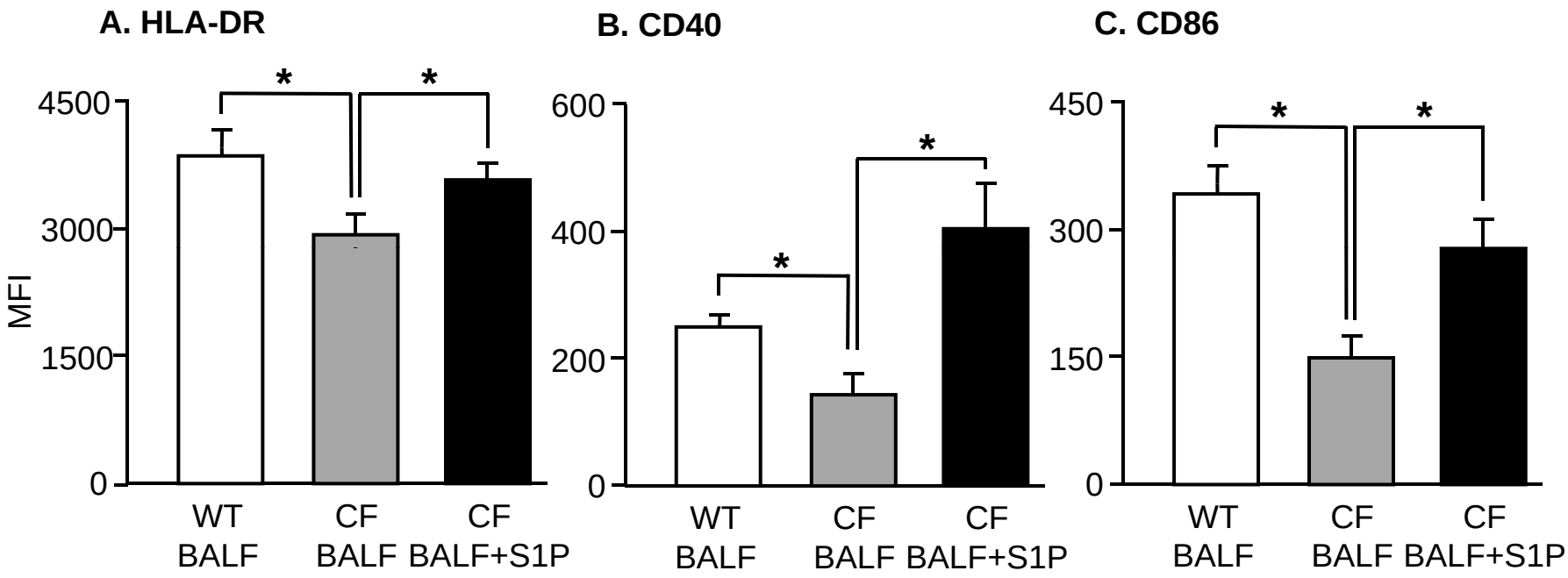
Supplemental Figure 4



Supplemental Figure 5