# Regulatory Tcells Reduce Acute Lung Injury Fibroproliferation By Decreasing Fibrocyte Recruitment

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## Online Data Supplement

## Supplemental Methods

*Isolation of Regulatory Tcells*

Tregs were isolated by bead separation as previously described.[1] Spleens were removed from GFP-labeled, Foxp3+ mice at time of sacrifice. Single cell suspensions were prepared by mashing the spleens through a 70μm filter in RPMI media. Total splenocytes were depleted of CD8 (Ly-2), CD11b (Mac-1), CD45R (B220), CD49b (DX5), Ter-119 cells using biotin-labeled specific mAbs, anti-biotin magnetic beads, and an LD magnetic bead column (Miltenyi Biotec). To isolate CD4+CD25+T cells, purified CD4+ T cell populations were incubated with PE-labeled anti-CD25 Ab and anti-PE magnetic beads and then passed through a MACS separation column. CD4+CD25– T cells were isolated from CD4+CD25+ cells by negative selection using anti-CD25 micro-beads. The purity of CD4+, CD4+CD25+, and CD4+CD25– T cell fractions was confirmed by flow cytometry and averaged 85-90% across all experiments (Supplemental Figure E1A). Success of adoptive transfer was confirmed by flow cytometry at time of animal sacrifice to identify CD4+Foxp3-GFP+ cells (Supplemental Figure E1B).

*Adoptive Transfer of Regulatory Tcells*

After isolation of Tregs and non-Treg CD4+ cells as described above, cells were resuspended at a concentration of $10^6$ cells/100μl in sterile PBS. Cell suspensions were then injected retro-orbitally into *Rag-1*$^{-/-}$ mice within 1 hr of their i.t. LPS injection.

*Sircol Assay*

Left lungs were excised and flash frozen in liquid nitrogen. Lungs were homogenized in 1ml of Complete Lysis Buffer (Roche) using an Ultra-Turrax tissue

homogenizer (Janke and Kunkel). Homogenates were centrifuged at 10,000 RCF for 10 minutes and the supernatant filtered through a 0.8μm filter (Nalgene). 20 μl of homogenate was incubated with 1 ml of Sircol reagent for 30 minutes. The resulting dye-homogenate complex was centrifuged at 16,000 RCF for 10 minutes and all supernatant removed by inverting the microcentrifuge tube. The dye was released from the remaining pellet using an Alkali reagent and the absorbance at 540nm measured using a microplate reader. Collagen content was derived by comparing absorbance to a standard curve generated using type 1 Collagen from rat tail extract (BD Biosciences). Collagen content in BAL fluid cell-free supernatants was determined by the Sircol assay using 100μl samples.

*CXCL12 ELISA*

CXCL12 ELISA assays were performed using a CXCL12 ELISA Duoset (R&D Systems) per the manufacturer's protocol. 100μl of lung homogenate was incubated in a 96-well plate with capture antibody for 2 hours. After washing, 100μl of detection antibody was added and incubated for 2 hours. 100μl of streptavidin-HRP was then added for 20 minutes. Substrate solution was added for 20 minutes followed by stop solution. The optical density of each sample was determined at a wavelength of 450nm (with a correction at 540nm) and final CXCL12 concentration determined by comparing to a standard curve created from CXCL12 standards provided by the manufacturer.

*Immunohistochemistry*

Lungs were prepared for histologic examination as previously described.[1] Left lungs were inflated to 25 cm H2O with 1% low-melting agarose (Invitrogen). The left mainstem bronchus was tied and the lung was excised and placed in 10% formalin for

24hrs. The fixed lungs were embedded in paraffin and 5 μm sections were mounted on slides before SDF-1 staining was performed using anti-SDF-1 Ab (R&D Systems, clone 79018, catalog number MAB350). Sections were de-paraffinized, re-hydrated, and treated for antigen retrieval. Blocking steps were: peroxidase (Dual Enzyme Block, Dako, Carpinteria, CA, for 30 min), mouse-on-mouse immunodetection technique (M.O.M. kit; Vector Laboratories as per kit), and serum block (ImmPress Reagent Kit, Vector, for 60 min), all at room temperature. Slides were incubated in SDF-1 primary antibody (diluted 1:50 in PBS containing 1% BSA and 0.1% Triton x-100, 20h, 4ºC), rinsed, then incubated with a peroxidase-conjugated secondary Ab (ImmPress Reagent Kit, Vector, 60 min, RT). The slides were then developed with 3,3'-diaminobenzidine and counterstained with hematoxylin. Appropriate IgG control stains were also performed (R&D Systems).

*Flow cytometry*

Cells were prepared for flow cytometry as previously described.[1] Right lungs were excised, minced and incubated at 37°C in an enzyme cocktail of RPMI containing 2.4 mg/ml collagenase I and 20 μg/ml DNase (Invitrogen), then mashed through a 70-μm nylon cell strainer (BD Falcon). Red blood cells were lysed using ACK lysis buffer (Invitrogen) and the cell pellet resuspended in FACS buffer. Lung cells or BAL cells were incubated with Fc Block-2.4G2 Ab (BD Biosciences) to prevent non-specific staining. The following surface stains were used: Alexa-700 conjugated CD4 (Ebiosciences), CD25 (BD Biosciences), allophycocyanin-conjugated (APC) anti-mouse CXCR4 (BD Biosciences). GFP-labeled, Foxp3 expression was examined using the FITC channel. For intracellular staining of Col-I, cells were fixed and permeabilized

using BD Cytofix/Cytoperm (BDPharmingen) and then stained with a biotinylated rabbit anti-Col-I Ab (Rockland Chemicals), followed by staining with PE-Texas Red conjugated streptavidin (BD Biosciences).

For epithelial cell isolation, lungs were instilled with 1.2 ml Dispase (BDBiosciences) with 3U/ml Elastase (Worthington) at the time of excision. Lungs were then minced as described above and single cell suspensions prepared. Surface stains included APC-Cy7- conjugated anti-CD326, and biotinylated anti-CD45 and CD31 with V450-conjugated streptavidin secondary (Biolegend). Cells were prepared for intracellular staining using Fixation/Permeabilization solution (Ebiosciences). Intracellular stains included biotinylated rabbit anti-Col-I Ab (Rockland Chemicals) with a PE-Texas Red conjugated streptavidin secondary (BD Biosciences) and APC-conjugated anti-CXCL12 (R&D Systems).

**Supplemental References**

E1. D'Alessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF, Pipeling MR, Brower RG, Tuder RM, McDyer JF, and King LS. CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. J Clin Invest 119[10], 2898-2913. 2009.

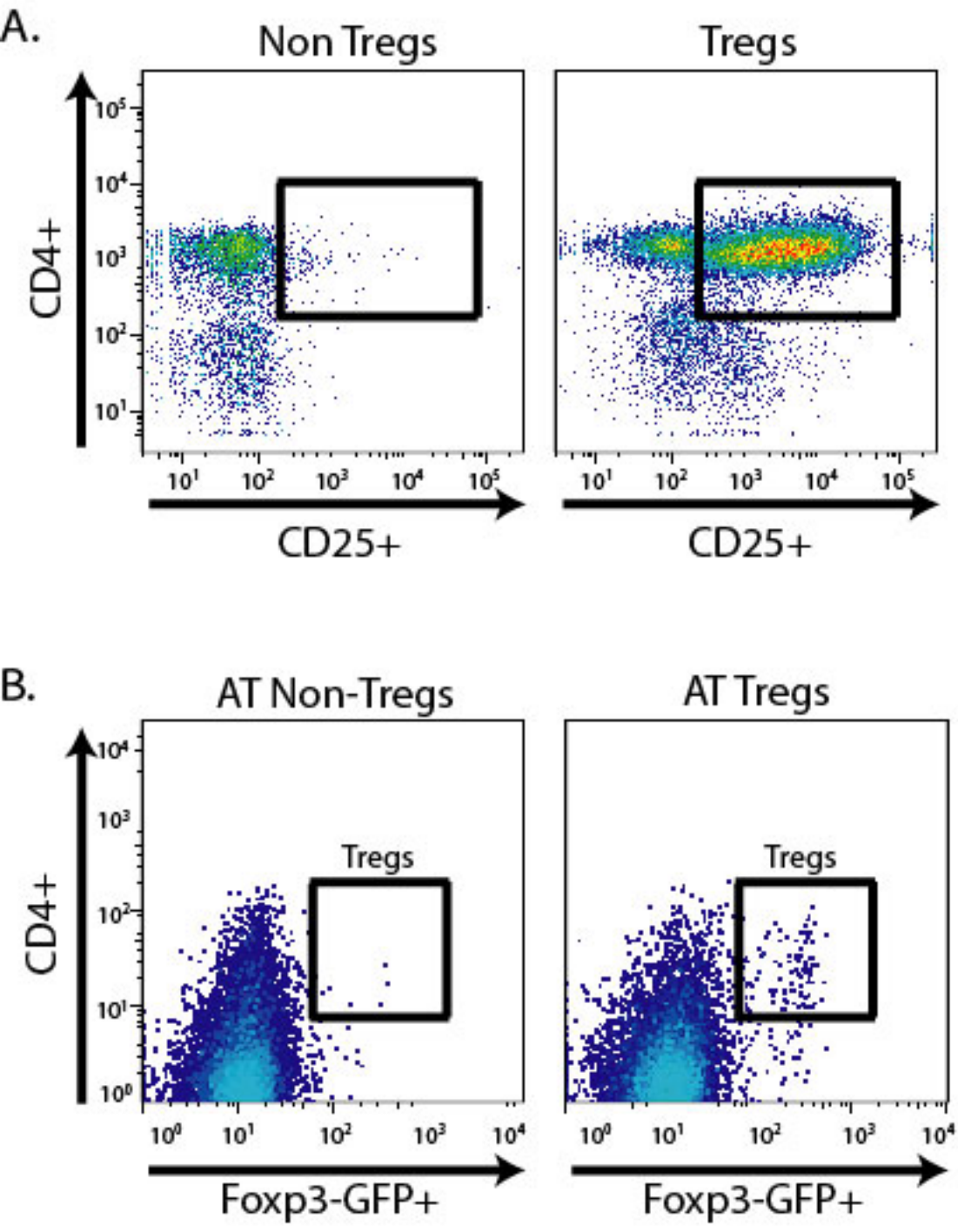
**Supplemental Figure Legends**

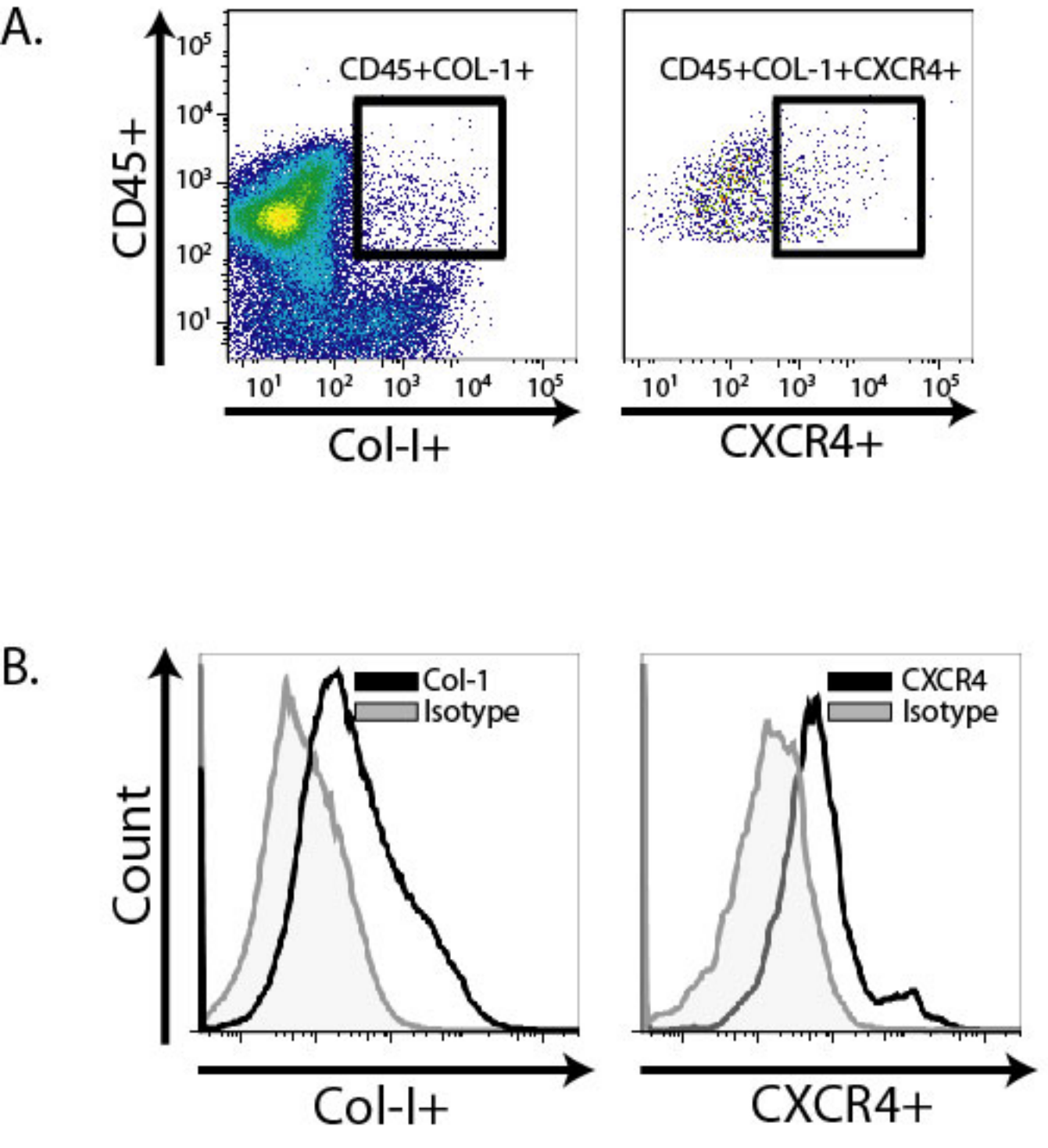
**Supplemental Figure E1. Adoptive transfer of Tregs into *Rag-1*$^{-/-}$ mice was successful using Miltenyi beads to sort CD4+CD25+ cells.** A.Flow cytometry plot shows successful bead separation of CD4+CD25+ cells from spleens of Foxp3$^{GFP}$ mice. B. CD4+Foxp3-GFP+ cells are present in the lungs of *Rag-1*$^{-/-}$ mice 7 days after adoptive transfer of Tregs but not non-Treg cells.

**Supplemental Figure E2. Fibrocytes were identified in BAL and Lung using flow cytometry.** A. Typical flow gate used to identify fibrocytes. Cells that stained positive for both CD45 and Col-I were identified. CXCR4+ cells were then further selected to obtain a population of CD45+Col-I+CXCR4+ cells. B**.** Isotype antibodies for Col-1 and CXCR-4 were used to gate on Col-I+ and CXCR4+ cells by flow cytometry.

**Supplemental Figure E3. CXCL12 can be detected in murine lungs by immunohistochemistry after LPS injury.** Lung sections were stained for CXCL12 by immunohistochemistry. A. CXCL12 expression was identified using a monoclonal mouse IgG1 antibody against CXCL12 (R&D Systems, MAB350, Clone 79018). B. Mouse IgG1 isotype Ab (R&D systems, MAB002) was used as a control to exclude non-specific antibody staining (characteristic images shown at 200x).

**Supplemental Figure E4.** Blockade of CXCR4 with the chemical AMD3100 did not reduce markers of lung injury at day 7 after LPS. A. BAL protein was not significantly decreased in *Rag-1*$^{-/-}$ mice that received AMD3100. B. BAL cell count was not significantly decreased in *Rag-1*$^{-/-}$ mice that received AMD3100.


A.
Non Tregs
Tregs
CD4+
CD25+
CD25+
B.
AT Non-Tregs
AT Tregs
Tregs
Tregs
CD4+
Foxp3-GFP+
Foxp3-GFP+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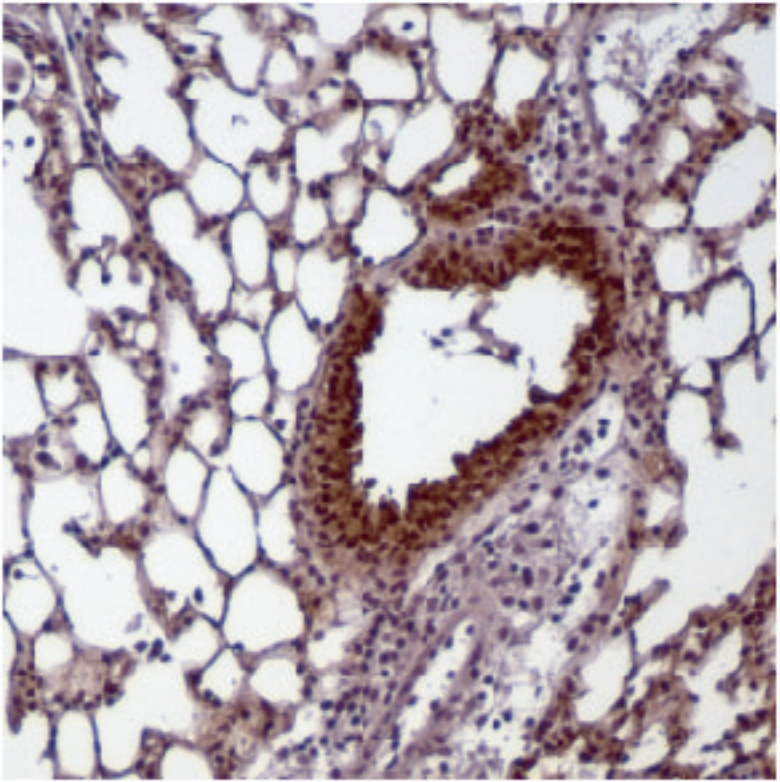
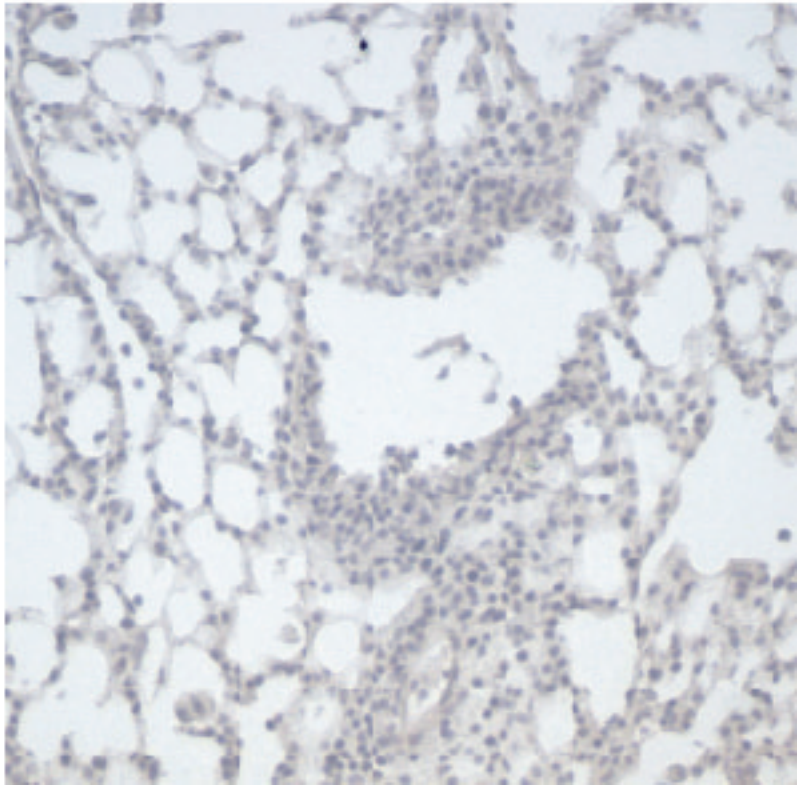
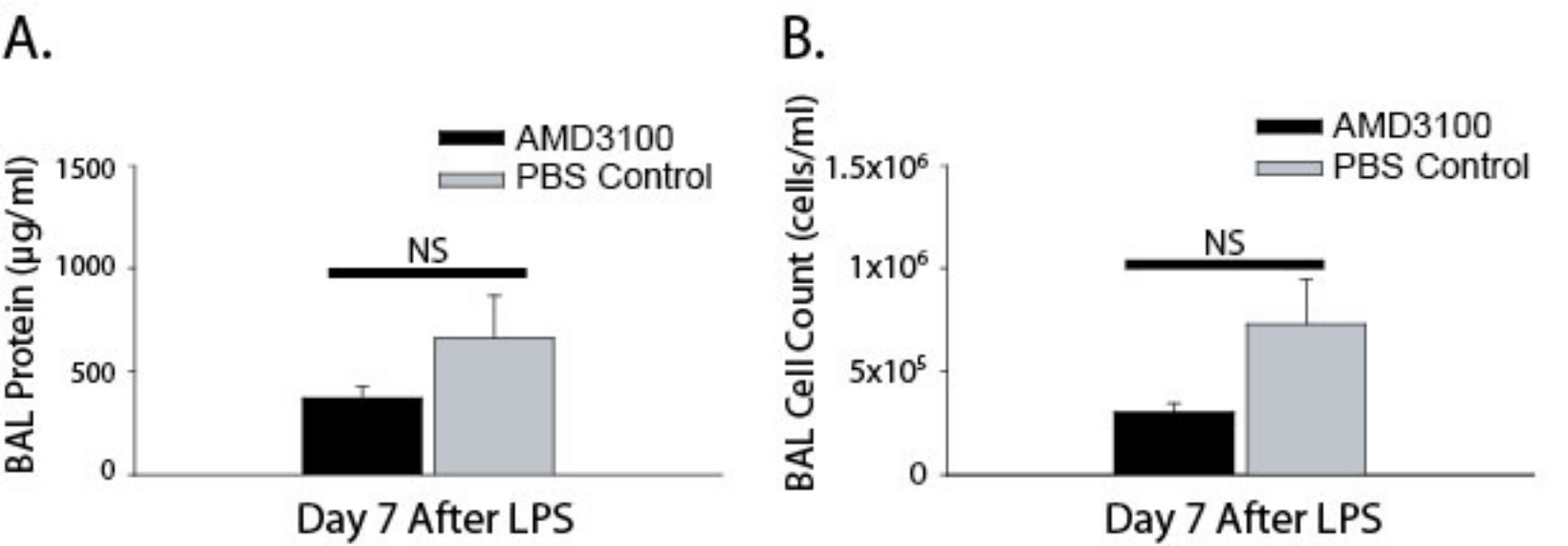
CD45+COL-1+
CD45+COL-1+CXCR4+
CD45+
Col-I+
CXCR4+
B.
Col-1
Isotype
CXCR4
Isotype
Count
Col-I+
CXCR4+

A.



B.




A.
AMD3100
PBS Control
BAL Protein (µg/ml)
1500
1000
500
0
NS
Day 7 After LPS
B.
AMD3100
PBS Control
BAL Cell Count (cells/ml)
1.5x10⁶
1x10⁶
5x10⁵
0
NS
Day 7 After LPS