**Online data supplement**

# Epithelial Cell-derived Secreted and Transmembrane 1a (Sectm1a) Signals to Activated Neutrophils During Pneumococcal Pneumonia

Hirofumi Kamata[1], Kazuko Yamamoto[1,2], Gregory A. Wasserman[1,3], Mary C. Zabinski[1], Constance K. Yuen[4], Wing Yi. Lung[4], Adam C. Gower[5], Anna C. Belkina[3], Maria I. Ramirez[1,6,7], Jane C. Deng[4], Lee J. Quinton[1,6,7], Matthew R. Jones[1,6], and Joseph P. Mizgerd[1,3,6,8]

## Supplemental Materials and Methods

### Experimental reagent

Recombinant mouse Sectm1a-Fc chimera protein (R&D systems, Mineapolis, MN), composed of the extracellular portion of mouse Sectm1a (Gln28-Thr165, Swiss-Prot Accession number Q921W8) and an Fc portion of mouse IgG2a (Glu98-Lys330), derived from the NS0 mouse myeloma cell line and with a molecular mass is 42.6 kDa, was reconstituted with PBS.

Recombinant mouse Fc (R&D systems), with the same amino acid sequence (Glu98-Lys330) as those of rm Sectm1a-Fc chimera protein described above and also derived from the NS0 mouse myeloma cell line with a molecular mass of 26.6 kDa, was also reconstituted with PBS.

### Antibodies

PE-conjugated anti-mouse IgG2a was obtained from R&D systems (Monoclonal Rat IgG1 Clone # 344701). Pacific Blue rat anti-mouse CD45 (clone 30-F11), FITC rat anti-mouse CD45 (clone 30-F11), Brilliant Violet rat anti-mouse CD45 (clone 30-F11), FITC rat anti-mouse Ly6G (clone 1A8), PE/Cy7 rat anti-mouse/human CD11b (clone M1/70), Brilliant Violet 421 rat anti-mouse CD62L (clone MEL-14), PE rat anti-mouse CD18 (cloneM18/2), APC/Cy7 hamster anti-mouse CD3 (clone 145-2C11), APC rat anti-mouse CD19 (clone 6D5), PE/Cy7 rat anti-mouse NK1.1 (clone PK136), PE hamster anti-mouse Podoplanin (clone 8.1.1) antibody were from Biolegend (San Diego, CA). APC rat anti-CD326 (EpCAM) (clone G8.8) antibody was from ebioscience (San Diego, CA).

### Lung epithelial cell sorting

Mouse left lungs were digested into single-cell suspensions using elastase (Roche, Basel, Switzerland) as previously described (1, 2). Lung epithelial cells identified as $CD45^{-}EpCAM^{+}$ or non-epithelial cells were sorted using a FACS Aria III (BD Biosciences, San Jose, CA) (Fig E1). For

microarray, cells were sorted into PBS containing 1% BSA and immediately centrifuged at 500xg for 5 min at 4°C and resuspended in RNAProtect Cell Reagent (Qiagen, Valencia, CA). For Quantitative real-time PCR, cells were sorted into PBS containing 1% BSA and then resuspended in TRIzol reagent (Life Technologies, Grand Island, NY). FITC-conjugated rat anti-mouse CD45 and APC-conjugated rat anti-mouse CD326 (EpCAM) were used. Dead cells were excluded using 7-aminoactinomycin (7AAD).

**Microarray analysis**

The left lungs of C57BL/6 mice that received intratracheal pneumococcus were harvested 15 hours after instillation to sort all epithelial cells and all non-epithelial cells, and epithelial cells from uninfected mice were also sorted (Fig E1). Total RNA was purified from sorted cells using RNeasy Micro Kit (Qiagen). RNA quality was validated using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA), and microarrays were performed for each sample using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix, Santa Clara,CA) (3). All data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (accession number GSE71623; http://www.ncbi.nlm.nih.gov/geo/). CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average (RMA) in the affy Bioconductor package (version 1.28.1) and an Entrez Gene-specific probeset mapping (version 14.0.0) from the Molecular and Behavioral Neuroscience Institute (Brainarray) at the University of Michigan (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF) (4). Differential expression was assessed using Student's two-sample *t* test as implemented in the multtest Bioconductor package (version 2.6.0). Correction for multiple hypothesis testing was accomplished using the Benjamini-Hochberg false discovery rate (FDR). Human homologs of mouse genes were identified using HomoloGene (version 65) (5). All microarray analyses were performed using the R environment for statistical computing (version 2.15.1).

**Gene Set Enrichment Analysis (GSEA)**

GSEA (6) was used to identify biological terms, pathways and processes that were coordinately up- or down-regulated with respect to pneumonia in epithelial cells. The Entrez Gene identifiers of the human homologs of the genes interrogated by the array were ranked according to the *t* statistic computed for the epithelial infected versus uninfected comparison. Mouse genes without a human homolog were removed, and the *t* statistics for multiple mouse genes with the same human homolog were averaged prior to ranking. This ranked list was then used to perform pre-ranked GSEA analyses (default parameters with random seed 1234) using the Entrez Gene versions of the Biocarta, KEGG, Reactome, and Gene Ontology (GO) gene sets obtained from the Molecular Signatures Database (MSigDB), version 3.1 (7).

**Quantitative real-time PCR**

Total RNA was purified from lung homogenate and cell lysates using RNeasy Mini kit (Qiagen) or TRIzol reagent (Life Technologies) and an RNase-free DNase set (Qiagen). Quantitative RT-PCR was performed using the StepOnePlus real-time PCR systems and the TaqMan RNA-to-CT 1-step kit (both from Applied Biosystems, Foster City, CA). The primers and TaqMan probes for mouse CXCL1, CXCL2, TNF-α, IL-1β were previously published (1). The primers and TaqMan probe set were designed for mouse Sectm1a, Sectm1b, CXCL10 and IFN-β using the CLC DNA Workbench software (CLC bio, Boston, MA) with the following sequences: Sectm1a, forward, 5'-CCTCAGCCTAA GAGTCAAGAAGA-3', reverse, 5'-ACAAGTCCACTGGGACACAAA-3', probe, 5'-ACACAGCCCAAGGAGGAAATGATCACAGC-3'; Sectm1b, forward, 5'-TTCCCCTGGTCACTGTCAGC-3', reverse, 5'-CACAGCTTGTAGGTGGCACAC-3', probe, 5'-ACCATCCCTGCCTCTGCCCCGAAG-3';CXCL10, forward, 5'-TCCTGTGTGCTCATCCTTCTT-3', reverse, 5'-TCCAGAGCAACGCAAAATCCAT-3', probe, 5'-TTCCTGCA

ACACAACTACCTGCCCCTT-3'; IFN-β, forward, 5'-TCCTGTGTGCTCATCCTTCTT-3', reverse, 5'-TCCAGAGCAACGCAAAATCCAT-3', probe, 5'-TTCCTGCAACACAACTACCTGCCCCTT-3'. TaqMan gene expression assays were obtained from Applied Biosystems and used for, Caveolin-1 (Mm00483057_ m1), Sftpc (Mm00488144_m1), Foxj1 (Mm01267279_m1), CC10 (Mm00442046_m1). Expression values were normalized to 18s rRNA content (8) and expressed as fold induction.

**Enzyme-linked immunosorbent assay**

The levels of CXCL2 in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using a matched antibody pair from R&D systems.

**Cell culture**

The murine alveolar epithelial cell-like E10 cells (obtained from Dr. Alvin Malkinson, University of Colorado, Aurora, CO) were cultured in CMRL-1066 medium containing 10% FBS and, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. E10 cells were stimulated with recombinant IFN-β (1000U/m) for 6 hours to measure Sectm1a expression by qRT-PCR.

**STAT1 knockdown by siRNA**

E10 cells were seeded in 6 well plates and cultured for 18 hours, followed by transfection with either non-targeting or STAT1 siRNA (Dharmacon, Piscatway, NJ; L-058881-00 or D-001810-01, respectively) using the DharmaFECT1 transfection reagent (Dharmacon) for 48 hours. After 48 hours, cells were stimulated with IFN-β (1000U/ml) for 6 hours.

**Immunoblot**

Immunoblots were performed to determine siRNA-mediated knockdown of STAT1 in E10 cells. Briefly, E10 cells were lysed in buffer containing 25mM Tris pH 7.4, 50mM sodium chloride, 0.5% sodium

deoxycholate, 2% NP-40, 0.2% SDS, 1x protease inhibitor, cleared by centrifugation and protein concentrations determined by BCA assay (Sigma, St. Louis, MO). Protein lysates were resolved on a 4-12% Bis-Tris gel and transferred to PVDF membrane using the NuPAGE gel system and XCell Blot II system (Novex by Life Technologies, Carlsbad, CA) (9, 10). Membranes were probed using anti-STAT1 and pan-actin antibodies (#9172 and #4968, respectively; Cell Signaling Technologies, Danvers, MA). Primary antibodies were detected using an anti-rabbit-HRP conjugated secondary antibody (Cell Signaling; #7074) and visualized using the ECLPlus Western Blotting Detection System (GE Healthcare, Little Chalfont, UK) (9).

**Sectm1a binding assay for blood leukocytes**

Blood was collected from inferior vena cava from uninfected or infected C57BL/6 mice that received intratracheal instillation of pneumococcus 20 hours prior to blood collection. After lysing red blood cells using BD Pharm Lyse lysing solution (BD Biosciences), blood cells were suspended in PBS and Sectm1a binding assay was performed as described in *Materials and Methods*.

***Ex vivo* neutrophil stimulation**

Bronchoalveolar lavage (BAL) was performed 20hrs after intra-bronchial instillation of pneumococcus to mice. Neutrophils were isolated using anti-Ly6G MicroBead Kit (Miltenyi Biotec). Purity of neutrophils was evaluated by flow cytometry, and was determined to be greater than 98%. Isolated neutrophils were cultured in 96 well plates ($2.5x10^5$ cells per well, suspended in media composed of RPMI plus 5% FBS) and stimulated with vehicle, recombinant mouse IgG2a Fc, or recombinant mouse Sectm1a-Fc in the presence of rat anti-mouse CD16/CD32 antibody, for 2, or 8 hours at 37 °C. Cellular mRNA and supernatants were collected for quantitation of cytokine transcripts and protein concentrations, respectively. Further, isolated neutrophils were stimulated for 4 hours in the same way and were collected to determine expression of CD11b, CD18, and CD62L using flow cytometry.

After washing, cells were stained with Brilliant Violet 521-conjugated anti-mouse CD45, FITC-conjugated anti-mouse Ly6G, PE/Cy7 conjugated anti-mouse/human CD11b, PE-conjugated anti-mouse CD18, and Brilliant Violet-conjugated anti-CD62L (Biolegend). Dead cells were excluded using 7-aminoactinomycin D (7AAD) (BD Pharmingen).

**Supplemental References**


E1. Yamamoto K, Ferrari JD, Cao Y, Ramirez MI, Jones MR, Quinton LJ, Mizgerd JP. Type I alveolar epithelial cells mount innate immune responses during pneumococcal pneumonia. *Journal of immunology* 2012; 189: 2450-2459.

E2. Yamamoto K, Ahyi AN, Pepper-Cunningham ZA, Ferrari JD, Wilson AA, Jones MR, Quinton LJ, Mizgerd JP. Roles of lung epithelium in neutrophil recruitment during pneumococcal pneumonia. *American journal of respiratory cell and molecular biology* 2014; 50: 253-262.

E3. Quinton LJ, Mizgerd JP, Hilliard KL, Jones MR, Kwon CY, Allen E. Leukemia inhibitory factor signaling is required for lung protection during pneumonia. *Journal of immunology* 2012; 188: 6300-6308.

E4. Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, Watson SJ, Meng F. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic acids research* 2005; 33: e175.

E5. Coordinators NR. Database resources of the National Center for Biotechnology Information. *Nucleic acids research* 2013; 41: D8-D20.

E6. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* 2005; 102: 15545-15550.

E7. Subramanian A, Kuehn H, Gould J, Tamayo P, Mesirov JP. GSEA-P: a desktop application for Gene Set Enrichment Analysis. *Bioinformatics* 2007; 23: 3251-3253.

E8. Jones MR, Quinton LJ, Simms BT, Lupa MM, Kogan MS, Mizgerd JP. Roles of interleukin-6 in activation of STAT proteins and recruitment of neutrophils during Escherichia coli pneumonia. *The Journal of infectious diseases* 2006; 193: 360-369.

E9. Hilliard KL, Allen E, Traber KE, Yamamoto K, Stauffer NM, Wasserman GA, Jones MR, Mizgerd JP, Quinton LJ. The Lung-Liver Axis Facilitates Pulmonary Innate Immunity and Hepatoprotection during Pneumonia. *American journal of respiratory cell and molecular biology* 2015.

E10. Traber KE, Hilliard KL, Allen E, Wasserman GA, Yamamoto K, Jones MR, Mizgerd JP, Quinton LJ. Oncostatin M Induces STAT3-dependent CXCL5 Expression and Neutrophil Recruitment During Pneumonia. *American journal of respiratory cell and molecular biology* 2015.

**Supplemental Figure Legends**

**E1.** Separation of cell populations consisting of all epithelial cells, identified as $CD45^{-}EpCAM^{+}$ and all non-epithelial cells, from mouse lungs.

**E2.** Sectm1a binds to NK cells and T cells, but only to small fractions. (A-D) Mice received intratracheal instillation of pneumococcus and left lungs were harvested 24 hours after instillation. Sectm1a binding assay was performed as described in *materials and methods* section using the same concentrations of Fc (25μg/ml) and Sectm1a-Fc (40μg/ml). Pacific Blue-conjugated anti-mouse CD45, APC/Cy7-conjugated anti-mouse CD3, PE/Cy7-conjugated anti-mouse NK1.1 and APC-conjugated anti-mouse CD19 antibodies were used to differentiate lymphocytes. Gates were determined based on vehicle group (data not shown). Results are representative of three independent experiments.

Epithelial cells

Non-Epithelial cells

P5

P6

EpCAM

CD45

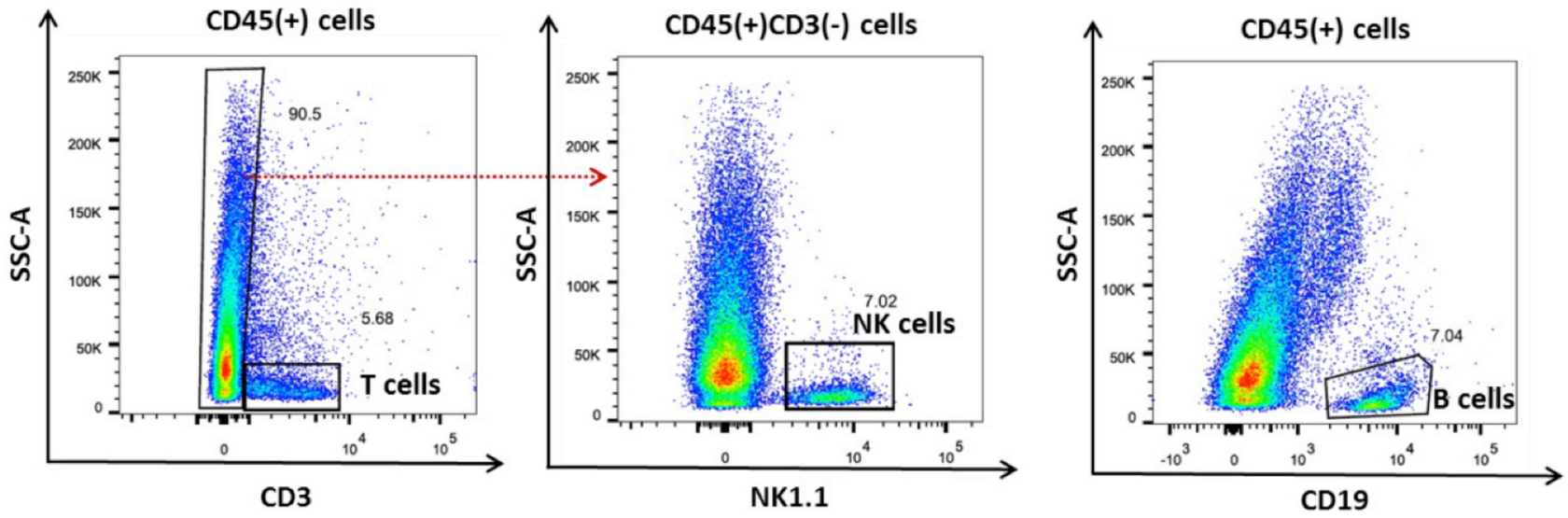
$10^2$

$10^3$

$10^4$

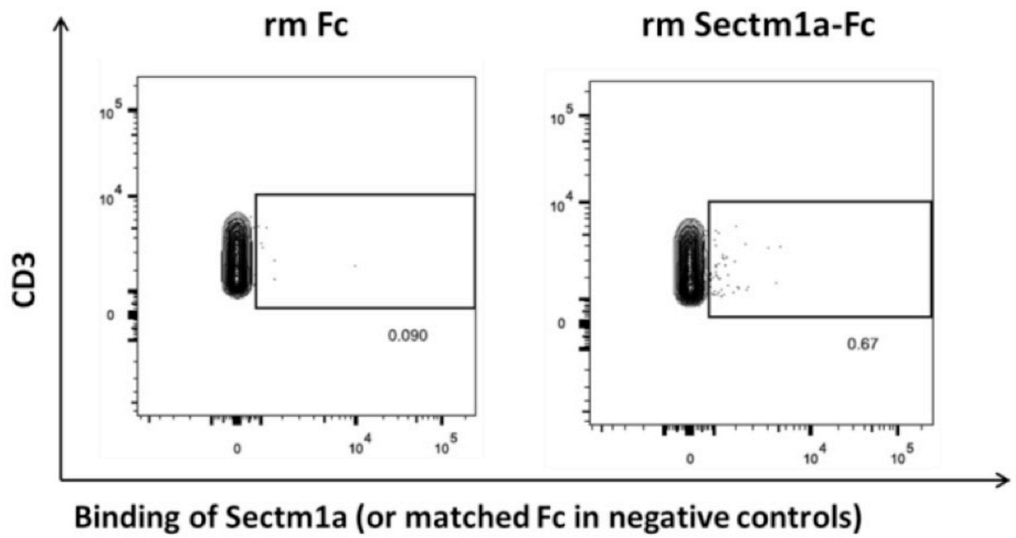
$10^5$

**A.**



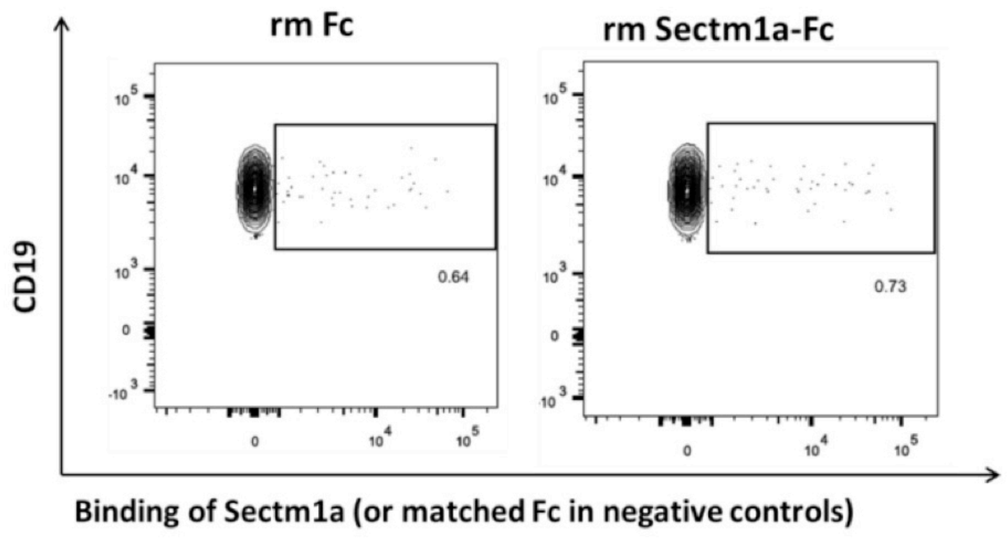

**B.**

T cells; CD45(+)CD3(+)




**D.**

B cells; CD45(+)CD19(+)




**C.**

NK cells; CD45(+)CD3(-)NK1.1(+)