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Appendix E1

Extended Methods

^{64}Cu-DOTA-ECL1i in Vivo Stability Study

^{64}Cu-DOTA-ECL1i (3.7 MBq in 100 μL saline) was injected into the tail vein of mice. At 1 hour after injection, mice were euthanized and blood was collected in a glass tube containing acid citrate dextrose. The plasma was separated from cells by means of centrifugation and then analyzed with high-performance liquid chromatography (Ultimate 3000; Dionex, Sunnyvale, Calif). The high-performance liquid chromatography instrument that was equipped with a UV/VIS detector (Dionex), a radioactivity detector (B-FC-3200; BioScan, Poway, Calif), and a C-18 column (5 mm, 4.6 × 220 mm; Perkin Elmer, Waltham, Mass) (36). Similarly, mouse lung was harvested 1 hour after radiotracer injection and rinsed with PBS (5 mL, three times) before cell disruption by using a probe sonicator (Sonifier 185 cell disruptor; Branson, Danbury, Conn) for 30 seconds. The supernatant was isolated with centrifugation and analyzed by means of high-performance liquid chromatography. The eluate was separated by 1-mL fractions and counted in a well gamma counter (Wallac Wizard 1470; Perkin Elmer, Waltham, Mass).

Mouse Lung Injury Model

Wild-type mice in the C57B/6 J background and CCR2-deficient mice (CCR2$^{-/-}$, no. 004999) were obtained from Jackson Laboratories (Bar Harbor, Me). CCR2-deficient mice were backcrossed into the C57B/6 J background. Mice of both sexes, aged 8–12 weeks, and weighing approximately 25 g were used. Mice were anesthetized by administering a combination of ketamine HCl, 90 mg/kg, and xylazine HCl, 10 mg/kg, by means of intraperitoneal injection. To deliver PBS control vehicle or LPS into the lung, the trachea was surgically isolated and cannulated with a 22-gauge, 1-inch catheter (Exel Safelet; ThermoFisher Scientific, Waltham, Mass). Mice were administered PBS (1 μL/g) or LPS at a dose of 2.5 μg/g (LPS, endotoxin, *E coli* strain 055:B5; Sigma-Aldrich, St Louis, Mo). Other mice were administered a high dose (10 μg/g) or low dose (0.5 μg/g) of LPS by using stock solutions of LPS created to inject a volume of 1 L/g. Intratracheal injection was over approximately 2 seconds, after which mice were kept warm and allowed to recover.

Flow Cytometry

Mice that received intratracheal PBS or LPS were injected intravenously with ECL1i labeled with Dylight 550, as with the radiolabeled probe, to determine the type of inflammatory cell binding ECL1i. For each experiment, animals treated with PBS or LPS were paired with a control animal not injected with ECL1i-Dylight 550 to determine the background signal. Lungs were removed from mice without flushing blood from the vasculature, minced on ice, and digested in Roswell Park Memorial Institute 1640 medium containing Liberase TL 50 μg/mL (Roche, Indianapolis, Ind) and deoxyribonuclease (DNAse) I, Type II, 20 U/mL (Sigma-Aldrich) at 37°C for 60 minutes. The digested tissue was passed through a 70-μm sieve (Falcon 352350; Corning, Corning, NY) to create a single cell suspension and then incubated in ACK

Lysing Buffer (Lonza, Walkersville, Md) at room temperature to lyse red blood cells. After 5 minutes, the sample was neutralized with FACS buffer (PBS with 2% fetal bovine serum) and centrifuged at 500 *g* for 8 minutes at 4°C. Cells were suspended in FACS buffer, counted by means of a hemocytometer, and prepared for flow cytometry. Cells were immunostained with labeled antibodies (all from BioLegend, San Diego, Calif, unless indicated) specific for mouse, including CD31 PerCP/Cy5.5, CD326 Alexa Fluor 488, CD45.2 Alexa Fluor 488, Gr-1 APC (eBioscience), Ly-6C APC, CD11c PE/Cy7, CD11b APC, CD90.2 (Thy-1.2) APC, Ly-6G PE/Cy7 (BD Pharmingen, San Diego, Calif), and CD19 APC. Counts from 1 to 10×10^4 cells were collected for each sample on a FACSCalibur (Becton Dickinson, San Diego, Calif), dual laser flow cytometer by using CellQuest Pro software (BD Biosciences, San Diego, Calif), and analyzed by using FlowJo software (Ashland, Ore). Cell phenotype was determined by antibody binding shown in Table E2. The mean fluorescent intensity was determined by calculating the geometric mean of Dylight 550 fluorescence of the cell types identified in ECL1i-Dylight 550–injected mice and subtracting the fluorescent background of the same cell types from similarly treated mice not injected with ECL1i-Dylight 550.

Immunostaining and Microscopy

Tissue sections were fixed and processed for immunostaining as described (37). The monoclonal anti-CCR2 antibody (E68; Novus Biologicals, Littleton, Colo) was used in mouse and human tissues. In mouse lung, the CCR2 antibody was detected by using avidin-biotin amplification (Vectastain Elite ABC; Vector Laboratories, Burlingame, Calif) and horseradish peroxidase substrate 3, 3-diaminobenzidine, which produces a brown reaction product. Tissues were then counterstained blue with hematoxylin. In human tissues, the CCR2 antibody was detected with an Alexa Fluor 555–labeled secondary antibody (Molecular Probes, Carlsbad, Calif) and DNA was counterstained with DAPI. Photomicroscopy was performed by using a Leica DM5000 microscope and DFC7000T camera interfaced with LASX software (Leica Microscopy, Buffalo Grove, Ill). Images were adjusted globally by using Photoshop software (Adobe, San Jose, Calif). In human tissue, CCR2-staining cells were assayed from five representative photomicrographs from each tissue section. Images were acquired at ×200 magnification and counted by investigators with expertise in cell biology blinded to the hypothesis (Z.B.N., J.P., and S.P.G., with 2, 25, and 9 years of experience, respectively) that results are different between study groups and with experience in scoring these data. The CCR2 expression was calculated as a ratio of the area of CCR2 signal relative to DAPI determined by using ImageJ software v1.50 to establish a threshold setting for positive staining cells (38). Once determined, values from the five representative images from all samples were collected, analyzed by threshold, and then averaged, by one investigator (Z.B.N.). The percentage of CCR2-positive cells in the donor and COPD groups were compared by using the Mann-Whitney test. To further assess our findings, we also immunostained additional lung samples from different regions of the lung that were available for some individuals (two donors, three patients with COPD). Typically, there was little variation in the level of CCR2-positive cells within the same specimen. In these cases, the mean percentage CCR2-positive cell value was calculated and used for analysis.

References

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37. Pan J, You Y, Huang T, Brody SL. RhoA-mediated apical actin enrichment is required for ciliogenesis and promoted by Foxj1. J Cell Sci 2007;120(Pt 11):1868–1876.

38. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9(7):671–675.

Table E1. Donor and COPD Subjects Providing Lung Tissue for CCR2 Immunostaining

ID#	Disease	Age (y)	Sex	Race	Smoker	FEV_1/FVC	FEV_1% pred	Mean CCR2-positive Cells per Sample (%)
Donor (*n* = 11)								
D1		33	M	White	No	ND	ND	1.66
D2		19	M	White	No	ND	ND	0.53
D3		23	M	White	No	ND	ND	0.19
D4		15	F	Black	No	ND	ND	2.09
D5		17	M	White	No	ND	ND	23.97
D6		14	M	White	No	ND	ND	1.16
D7		17	M	White	No	ND	ND	0.57
D8		52	M	White	No	ND	ND	0.41
D9		54	M	White	No	ND	ND	4.52
D10		19	M	White	No	ND	ND	6.52
D11		62	M	White	No	ND	ND	9.30
Median		19						1.66
Range		14–62						0.19–23.97
COPD (*n* = 16)								
C1	COPD	58	M	White	Former	0.21	17	8.49
C2	COPD	51	M	White	Former	0.15	14	18.65
C3	COPD	58	F	White	Former	0.21	24	9.51
C4	COPD	54	M	White	Former	0.31	17	9.19
C5	COPD	43	M	White	Former	0.44	22	1.61
C6	COPD	61	F	White	Former	0.36	18	5.35
C7	COPD/A1E	35	F	White	Former	0.24	17	13.55
C8	COPD	54	F	White	Former	0.25	16	13.58
C9	COPD	62	M	White	Former	0.22	18	19.70
C10	COPD	57	F	White	Former	0.25	16	6.22
C11	COPD	60	F	White	Former	0.34	20	11.78
C12	COPD	57	F	White	Former	0.32	30	22.03
C13	COPD	54	M	White	Former	0.17	16	22.25
C14	COPD	63	F	White	Former	0.28	12	21.71
C15	COPD/A1E	52	M	White	Former	0.27	20	24.11
C16	COPD/A1E	58	F	White	Former	0.25	17	12.09
Median		57				0.25	17	12.82
Range		35–63				0.15–0.44	12–30	1.16–24.11

Note.—A1E = α-1 antitrypsin deficiency, FEV_1 = forced expiratory volume at 1 second, FVC = forced vital capacity, FEV_1% pred = predicted percent FEV_1, ND = not determined.

Table E2. Antibodies Used to Perform Phenotyping of Cells with Flow Cytometry

Cell Type	Fluorescent Label		
	AF 488	PerCP/Cy5.5 PE/Cy7	APC AF 647
Neutrophil	CD45.2+	$Ly6G^{Hi}$	$GR\text{-}1^{Hi}$
Monocyte	CD45.2+	$Ly6G^{Lo}$	$Ly6C^{Hi}$
Macrophage	CD45.2+	$CD11c^{Hi}$	$CD11b^{Lo}$
Dendritic cell	CD45.2+	$CD11c^{Hi}$	$CD11b^{Hi}$
T cell	CD45.2+	…	$CD90.2\ (Thy\ 1.2)^{Hi}$
B cell	CD45.2+	…	$CD19^{Hi}$