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NEUTROPHIL-TARGETED, PROTEASE-ACTIVATED PULMONARY DRUG DELIVERY BLOCKS AIRWAY AND SYSTEMIC INFLAMMATION

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SUPPLEMENTARY MATERIAL

Supplementary Methods:	2 pages
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Supplementary methods

Nanoparticle fabrication. Nanoparticles were fabricated using a single emulsion solvent evaporation method. 0.8-1 mg of Nexinhib20 (Cayman Chemicals), 0.2 mg of DiR (ThermoFisher), and 130 mg of Poly (D,L-lactide-co-glycolide) (PLGA RG502H, Sigma) was dissolved in 2 mL of ethyl acetate. The PLGA mixture was then added to 4 mL of 2.5 % w/v poly(vinyl) alcohol (PVA; Sigma) and sonicated at 65% for 10 min on ice using a Sonics Vibra-Cell VCX130 with CV18. The emulsion was then added to 50 mL of 0.3 % w/v PVA solution and magnetically stirred for 5 hours. The nanoparticles were centrifuged at 8,000 g for 10 minutes, pellet discarded, and nanoparticles in the supernatant were then washed in DI water by centrifuging at 100,000 g for 30 minutes. After the second DI rinse, the nanoparticles were resuspended in 5 mL 1% w/v trehalose solution, snap frozen in LN, lyophilized for 48h, and stored at -20 C. Nanoparticles were resuspended at 1 μg/mL in water and run on a NanoSight to determine the mean particle size from 5 replicate runs, table S1. Nanoparticles (100 mg/mL) were degraded in DMSO to measure Nexinhib20 concentration by absorbance ($\lambda = 274$); Nexinhib20 was loaded at 1.48 ug/mg of PLGA formulation (21% encapsulation efficiency).

N-in-M fabrication. Microgels were fabricated as reported elsewhere with slight modifications to the polymer mixture. A 20 % w/v solution of equimolar amounts of di-sulfhydryl elastase peptide, CGAAPVRGGGGC (Chi Scientific), and 4-arm PEG maleimide (10kDA, Laysan Bio) were dissolved in PBS. 1 μL of 10 mg/mL DyLight 488-maleimide or DyLight 650 4x PEG-maleimide and nanoparticles were added to the PEG macromer solution was added to mineral oil (1% v/v surfactant: Span 80/Tween 80, HLB = 5) and homogenized on a PRO Scientific D Series homogenizer for three minutes at 4,000 rpm in a 35-40C water bath. After several centrifuge washes microgels were then strained through a 40 μm filter and run on an Accuri to determine the % of microgels positive for PLGA nanoparticles and microgel/mL concentration and sterilized by UV. Nanoparticles incorporated were either 10 mg of the PLGA formulations or fluorescent carboxylated polystyrene beads; 60 nm Dragon Green (Bangs Laboratories) or 100 nm blue fluorospheres (ThermoFisher).

Flow cytometry. Cells were pre-stained with Fc block and Zombie live/dead dye (BioLegend) for 10 minutes on ice, followed by staining for 15 minutes on ice with antibodies against the following markers: CD45, Ly6G,

Ly6C, MHCII, CD11b, F4/80, CD206, CD11c, Siglec F, CD63 (BioLegend). Cells were washed with PBS-EDTA (2.5 mM), fixed with Lyse/Fix PhosFlow reagent (BD Biosciences) and acquired on a FACS LSRII (BD Biosciences) and on a Cytoflex S (Beckman Coulter). Robust acquisition of samples over time was ensured by the utilization of stringent calibration methods employing rainbow bead-based laser and channel standardization. Data analysis and fluorescence compensation were performed with FlowJo V9.9.5 and V10.4.2 (Treestar). PMNs were gated as CD45+Ly6G+CD11b+, and macrophages as CD45+F4/80+MHCII+Ly6G- (**Fig. S5)**.

Immunofluorescent staining. Left lung lobes were fixed in 4 % paraformaldehyde at 4 ºC overnight, allowed to settle in 30 % w/v sucrose for 24-48 h at 37 ºC and then frozen in OCT by a dry ice isopentane mixture. 10 µm sections were sliced and stored at –80 ºC until stained. Slides were washed in 1x TBS for 5 minutes, blocked for 2 h at room temperature in 5 % goat serum with 0.5 % Triton X, incubated at 4 ºC overnight with the primary antibody, 1:100 dilution MPO (ThermoFisher) in 5% goat serum. Slides were then washed and stained with secondary, 1:250 dilution goat anti-rabbit Alexa Fluor 555 (ThermoFisher) with 5 µg/mL DAPI for 1 h at room temperature. Then samples were mounted using Prolong Gold. Average background of each channel from the secondary stain was used to linearly adjust the contrast equally across all images.

Table S1. PLGA nanoparticle size

	Size (nm)	D90 (nm)
DiR PLGA	177.2 ± 4.3	265 ± 9.5
Nex20 DiR PLGA	145.6 ± 3.3	196.5 ± 8.9

N = 5

Table S2. Antibodies used in the study

Antibody	Clone	Species	Company
CD45	30-F11	anti-mouse	BioLegend
Ly6G	1A8	anti-mouse	BioLegend
Ly6C	HK1.4	anti-mouse	BioLegend
MHCII	M5/114.15.2	anti-mouse	BioLegend
CD11b	M1/70	anti-mouse	BioLegend
F4/80	BM8	anti-mouse	BioLegend
CD206	C068C2	anti-mouse	BioLegend
SiglecF	S17007L	anti-mouse	BioLegend
CD11c	N418	anti-mouse	BioLegend
CD63	NVG-2	anti-mouse	BioLegend
NE	887105	anti-mouse	R&D Systems
MPO	RRID: AB_11006367	anti-mouse	ThermoFisher
AF555	Polyclonal	Goat anti-rabbit	ThermoFisher
CD66b	G10F5	anti-human	BioLegend

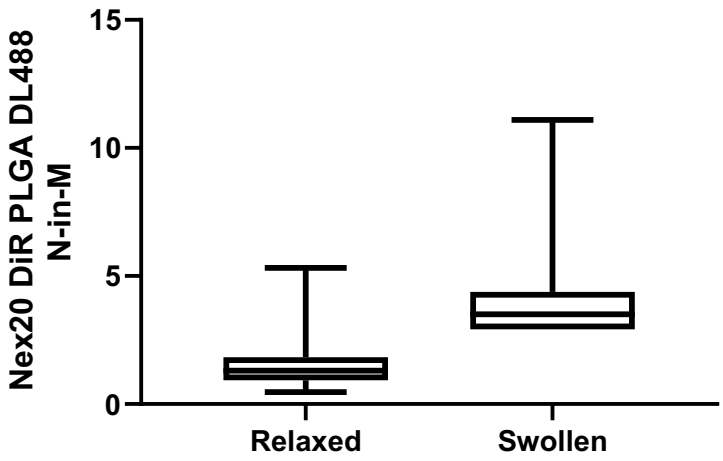

Figure S1. N-in-M size in relaxed and swollen states. PLGA nanoparticles were encapsulated in DyLight 488 labeled elastase-responsive microgels and size was measured by microscopy in the relaxed (oil; N = 1,047) and swollen states of the microgels (water; N = 161). Boxplot whiskers from min to max.

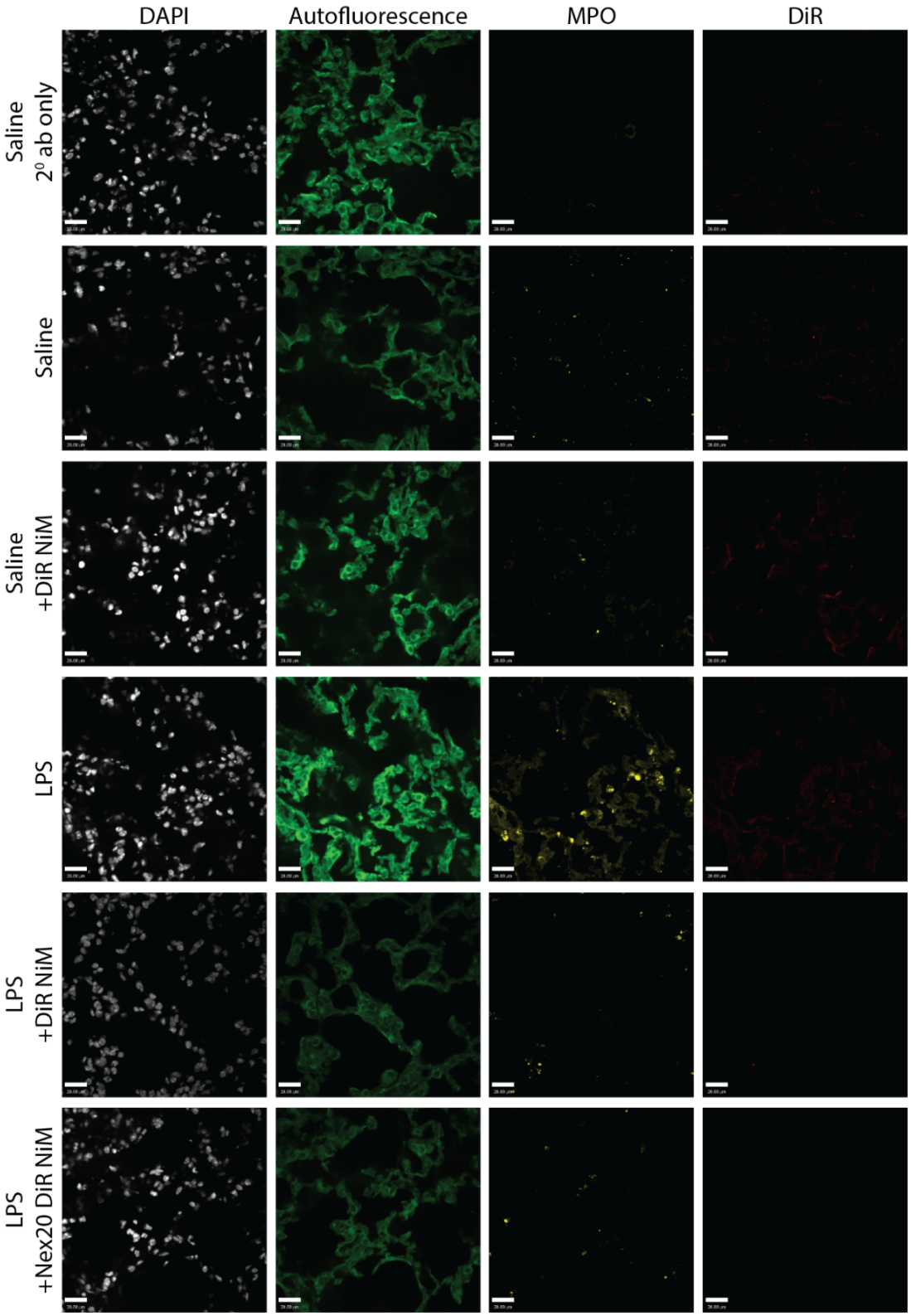

Figure S2. **Microscopic analysis of PMN-driven inflammation in the lung**. Treatment with Nex20 N-in-M show decreased presence of myeloperoxidase (MPO) presence in tissue sections relative to the LPS control, scale bar = 20 µm, linearly contrasted for clarity.

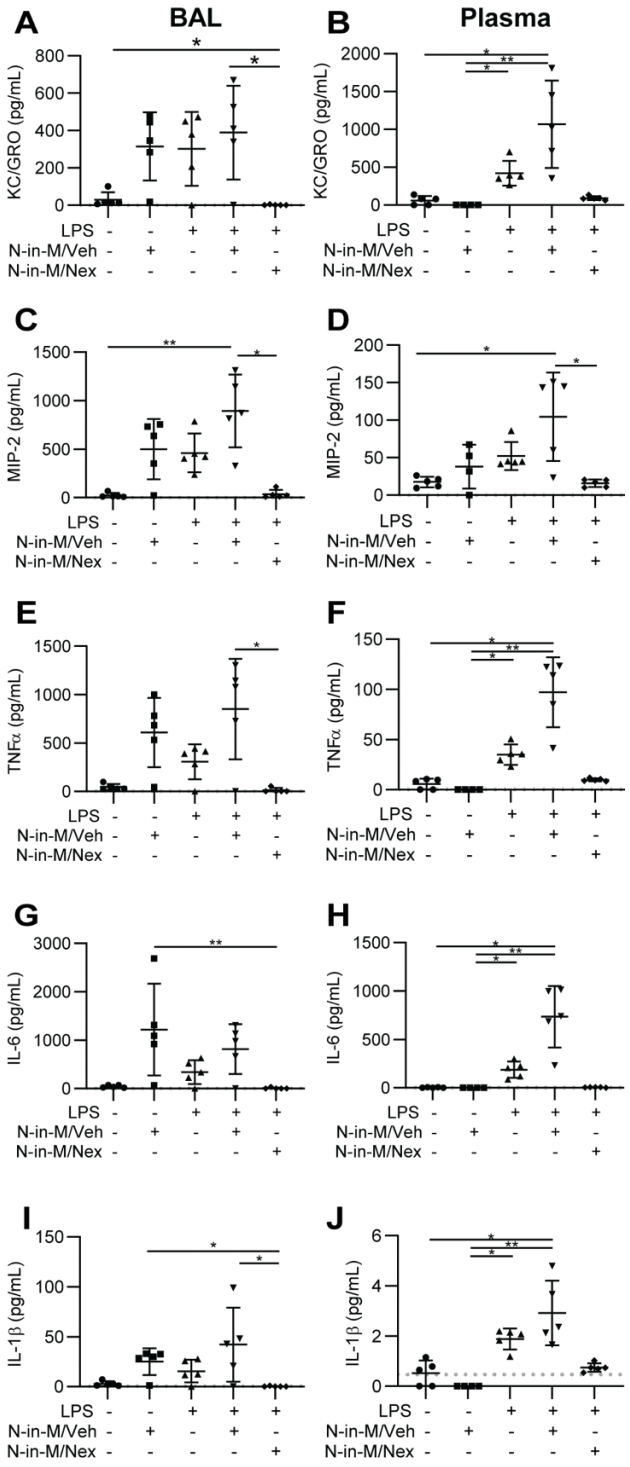

Figure S3. Delivery of Nexinhib20 reduces critical inflammatory mediators. Pro-inflammatory cytokines in the BAL and plasma were measured using a multiplex chemiluminescent multiplex ELISA platform. Mice treated with the N-in-M loaded with Nexinihib-20 showed decrease of pro-inflammatory cytokines in both compartments **(A-J)**. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ between groups indicated. Shown are means +/- IQR; N=4-5.

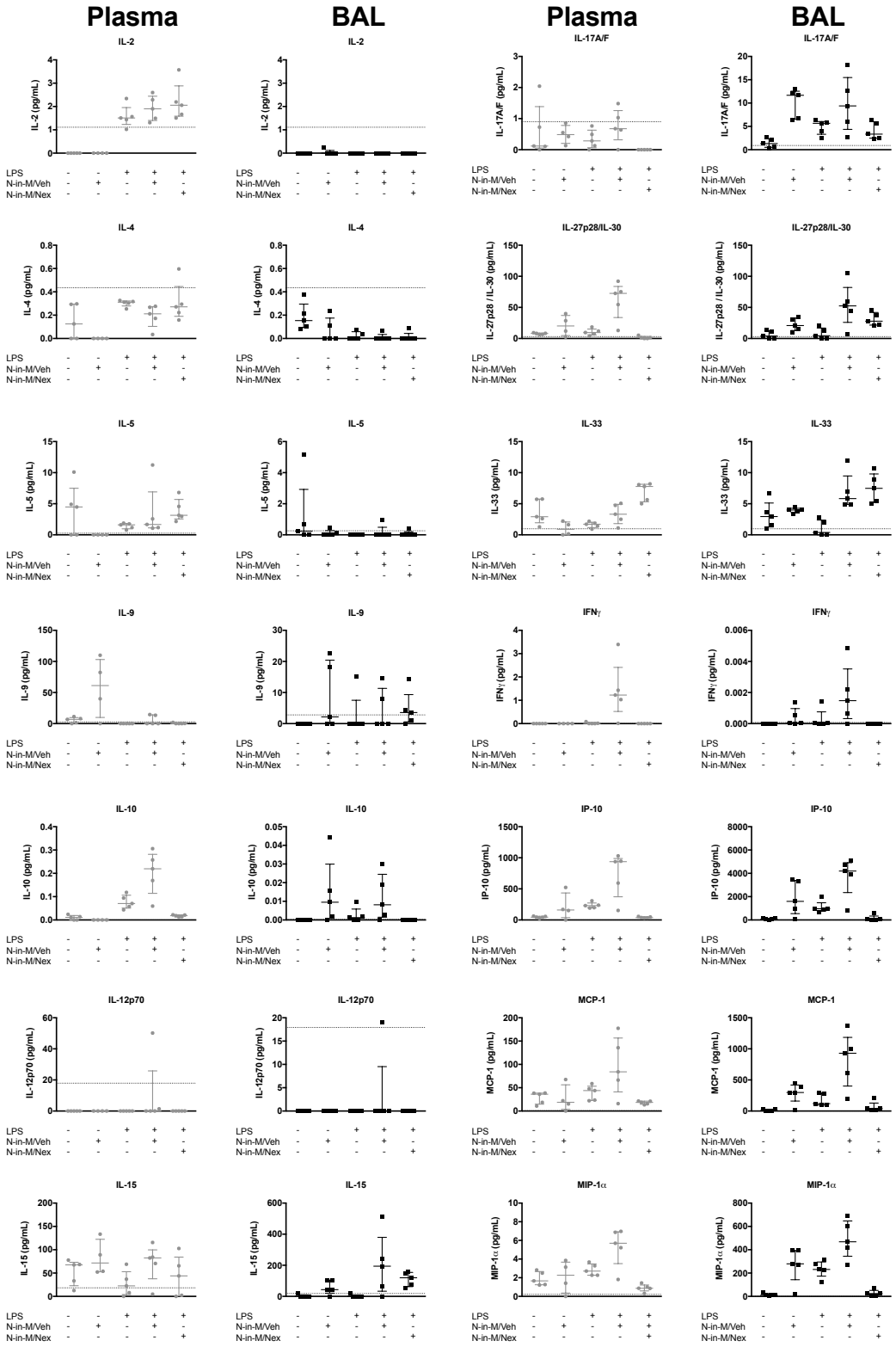

Figure S4. Extended plasma and BAL mediator profiling. Mediator levels in plasma (grey) and BAL (black) were measured using the multiplexed Mesoscale platform. Dashed lines on graphs represent detection limits. Shown are means +/- IQR; N = 4-5.

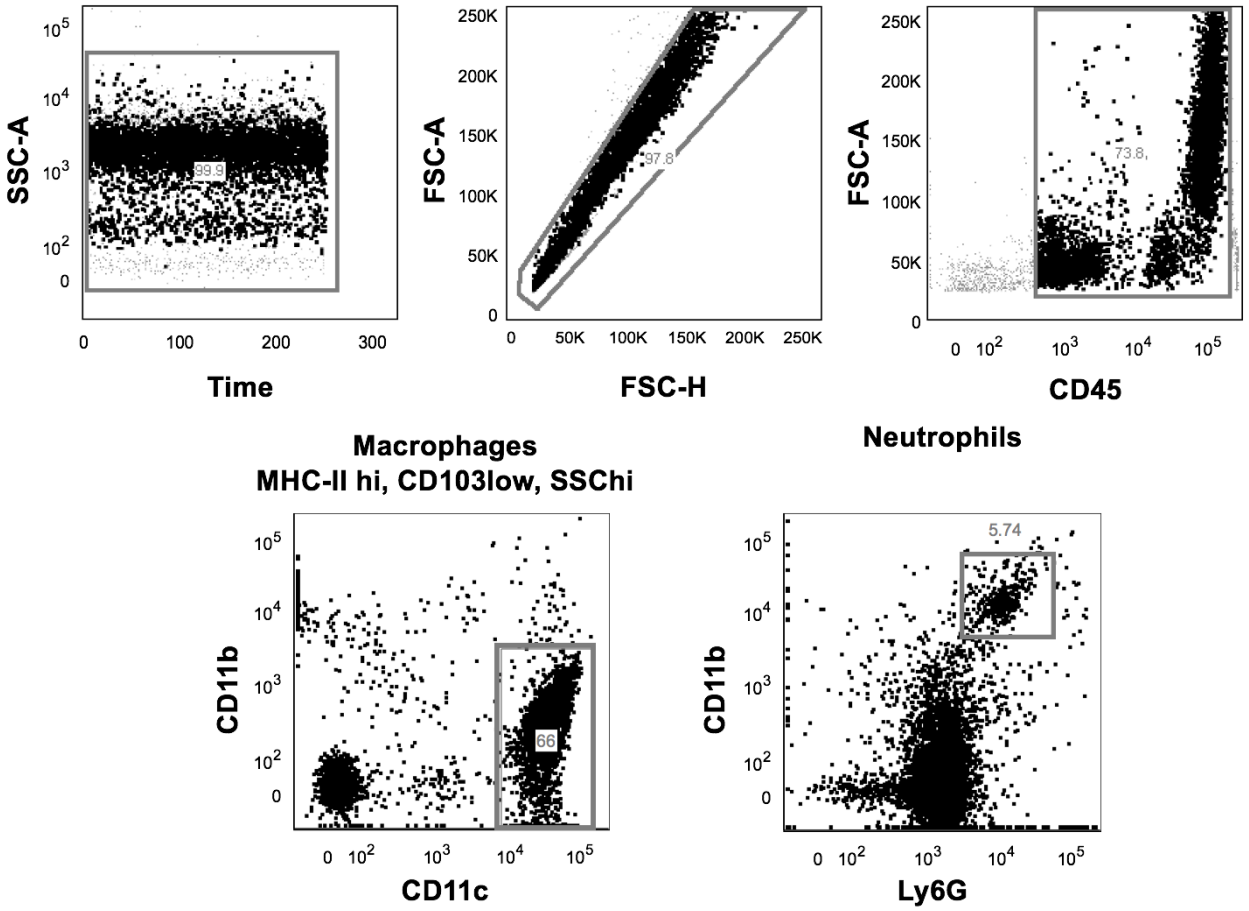

Figure S5. **Flow cytometry gating scheme**. After singlet gating, PMNs were gated as CD45+Ly6G+CD11b+ and macrophages as CD45+F4/80+MHCII+Ly6G-.