ADVANCED HEALTHCARE MATERIALS

Supporting Information

for *Adv. Healthcare Mater.*, DOI 10.1002/adhm.202101534

Polysalicylic Acid Polymer Microparticle Decoys Therapeutically Treat Acute Respiratory Distress Syndrome

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Supplementary Materials:

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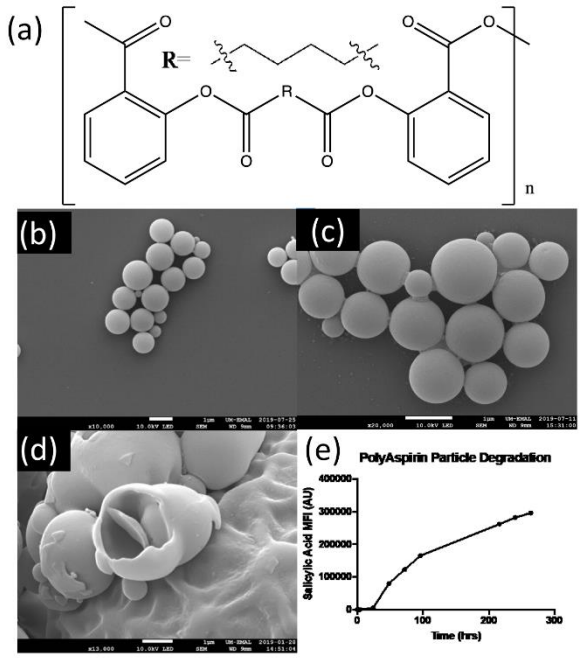
Supplemental Method 1: *ALI model determination of particle injection timeline.*

Given the transient nature of the influx of neutrophils into the lungs, we hypothesized that particle injection relative to LPS instillation time would play a role in the impact of intravenous (IV)-injected particles on the mitigation of acute inflammation in the lungs. Thus, we began by establishing the timeline of neutrophil influx into the pulmonary airways in LPS-induced ALI in C57BL/6J mice. Animals were euthanized at varying times after intratracheal LPS, which would impact the optimal injection time for our particles. To do this, we employed the bronchoalveolar lavage (BAL) procedure, where fluid flushed into the airway is collected and sampled for the cellular and protein contents. Here, we evaluated the BAL fluid (BALF) for the count and percentage of neutrophils (Fig. S2). As shown in Supplemental Figure 2D, we observed that the bulk of the neutrophil influx into the alveolar space occurs between 2 and 6 hours post-LPS instillation in C57BL/6J mice, with the neutrophil composition in the BALF peaking at 65% by 12 hours (Supplemental Fig 2E). Altogether, neutrophils and macrophages represented more significant than 98% of BALF cells in mice at all time points evaluated, in line with other published works (*27*, *28*).

Supplement Method 2: *P. aeruginosa model determination of particle injection timeline*

We quantified the timeline of neutrophil influx into the lungs in *P. aeruginosa* infection, as presented in Fig. S5A-C. After infection, mice were euthanized at various time points, and BALF

was collected. We observed a change in total BALF cells and neutrophils from 12 through 36 hours post-infection, with some plateauing seen at ~24-36 hours. Importantly, we saw the most significant influx of neutrophils into the lungs between 12 and 24 hours (Fig. S5C). Thus, we again chose two different injection times to explore the impacts of Poly-A particles on the resolution of inflammation and infection relative to PLGA and polystyrene of the same size and surface charge: 6 hours (Fig. S5D) and 18 hours (Fig 2A) post-infection, with a fixed harvest time of 24 hours post-infection.



Supplemental Figures

Fig S1—Fabrication and Physical Characteristics of Poly-A Microspheres. (A) Chemical structure of Poly-A polymer used to fabricate Poly-A microspheres**.** SEM images of freshly prepared **(B)** Poly-A and **(C)** Poly-A/Cy5.5 particles dried to a glass coverslip **(D)** SEM image of Poly-A particles after 13 days of degradation in water. **(E)** Hydrolytic degradation profile of Poly-A particles as measured by the release of salicylic acid.

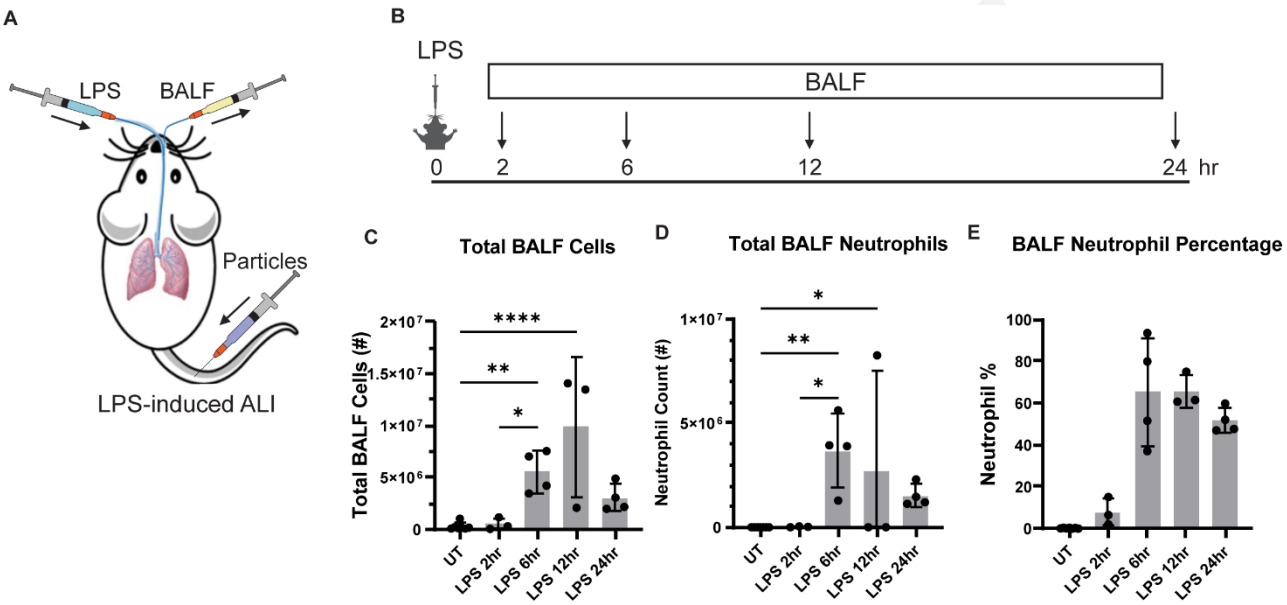


Fig. S2—Characterization of total BALF cells and neutrophils in the LPS ALI model in C57BL/6J mice. (A-B) Schematics of LPS-induced mouse lung injury, particle treatment, and BALF collection timeline. **(C)** Total cells in BALF 2, 6, 12, and 24 hours post-LPS instillation. **(D)** Neutrophils in BALF 2, 6, 12, and 24 hours post-LPS instillation. **(E)** The relative percentage of neutrophils and macrophages in the BALF 2, 6, 12, and 24 hours post-LPS instillation. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. $N \geq 5$ for all assays unless otherwise stated.

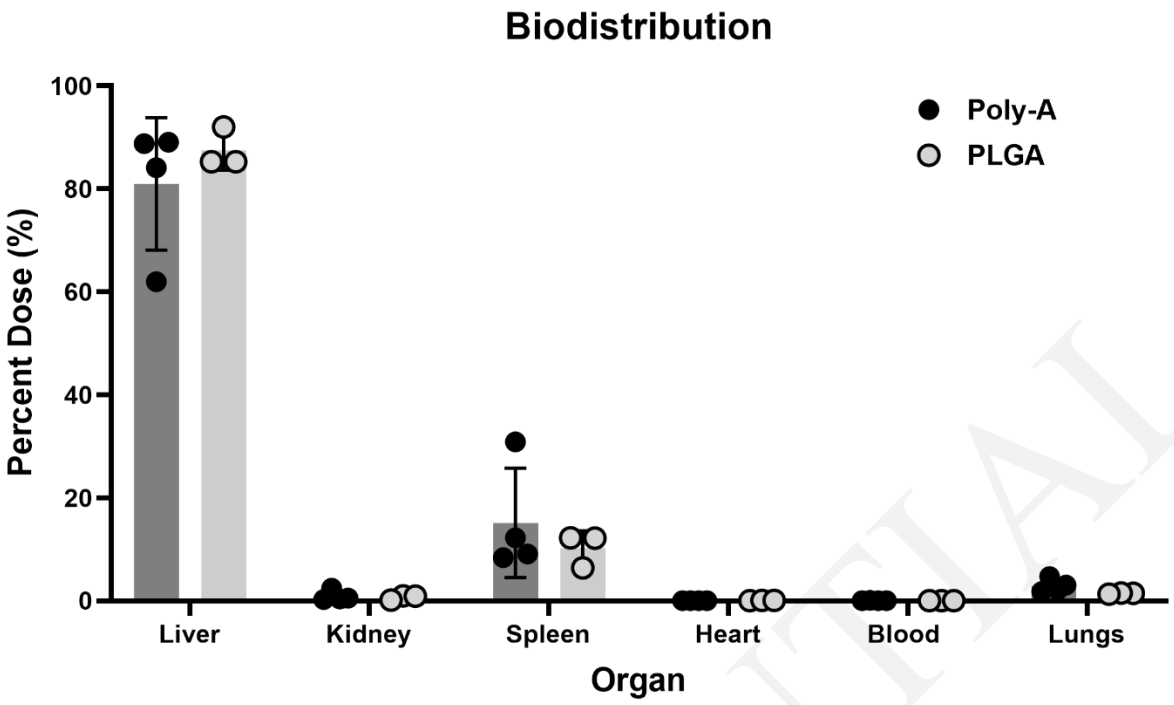


Fig. S3—Biodistribution of Poly-A and PLGA particles in mice 30-minutes post-injection. C57BL/6J Mice were injected with particles 2 hours after intratracheal LPS administration and sacrificed 30 minutes after particle injection. Particle distribution was quantified using a fluorescence signal as in our prior work (*27*). ***N* ≥ 3 for this experiment.**

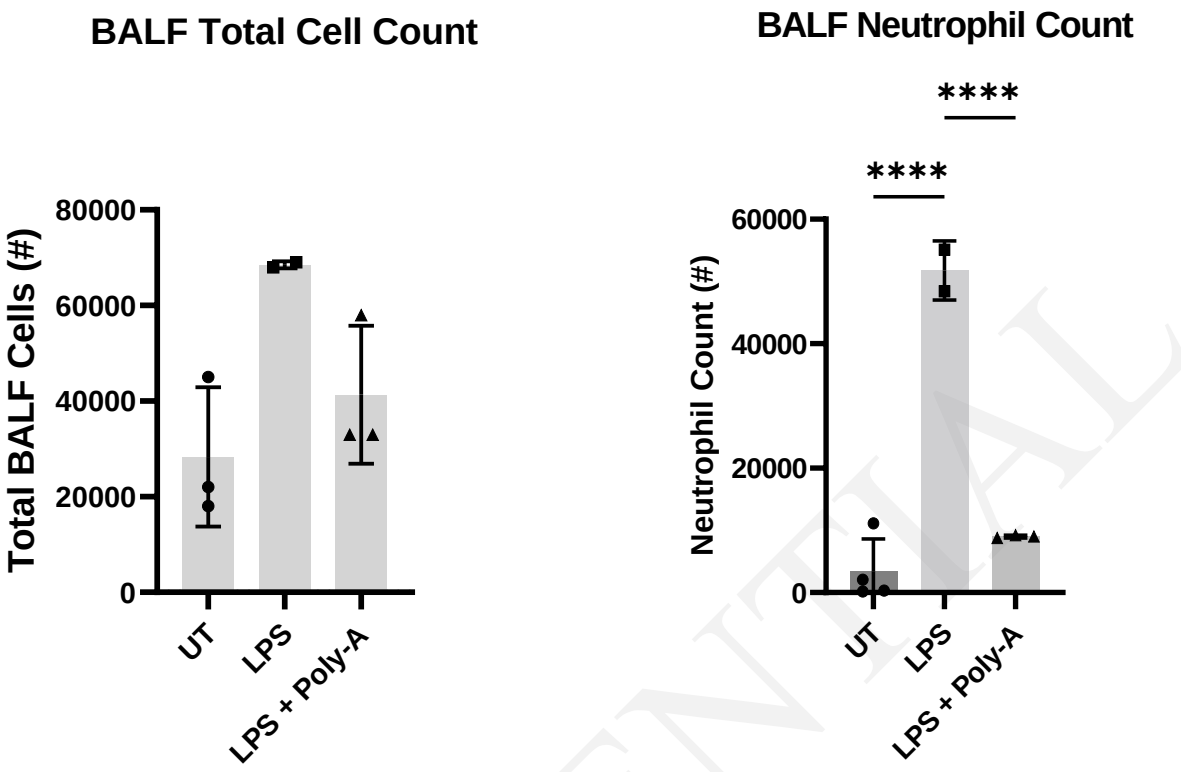


Fig. S4—BALF cell and neutrophils counts in the lungs of Balb/cJ mice in the LPS ALI model. **(A)** total cell in the BALF of Balb/cJ mice with no treatment, LPS instillation, and LPS instillation with Poly-A particle injection and **(B)** total neutrophils in the BALF of Balb/cJ mice with no treatment, LPS instillation, and LPS instillation with Poly-A particle injection. Particles were injected ($2x10^8$ particles/mouse) one hour after LPS instillation, and mice were euthanized 2 hours after particle injection. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. n.s. indicates "not significant." $N \geq 5$ for all assays unless otherwise stated.

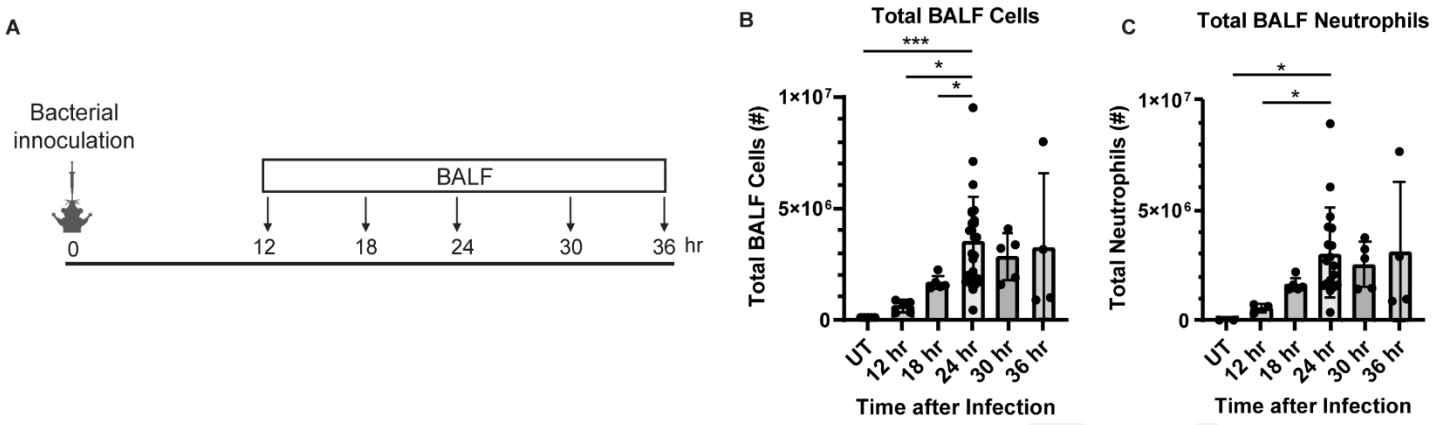


Fig. S5—Characterization of *P. aeruginosa* lung infection in C57BL/6J mice. (A) Representation of the timeline of bacterial infection and BALF sampling. **(B)** Total cells in BALF 12, 18, 24, 30, and 36 hrs post-infection. **(C)** Total neutrophils in BALF 12, 18, 24, 30, and 36 hrs post-infection. **(D)** Timeline for 6-hour particle injection and 24-hour BALF collection. **(E)** Total BALF cells and **(F)** neutrophils collected post 6-hour injection timeline. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. $\boldsymbol{N \geq 5}$ for a ss otherwise stated.

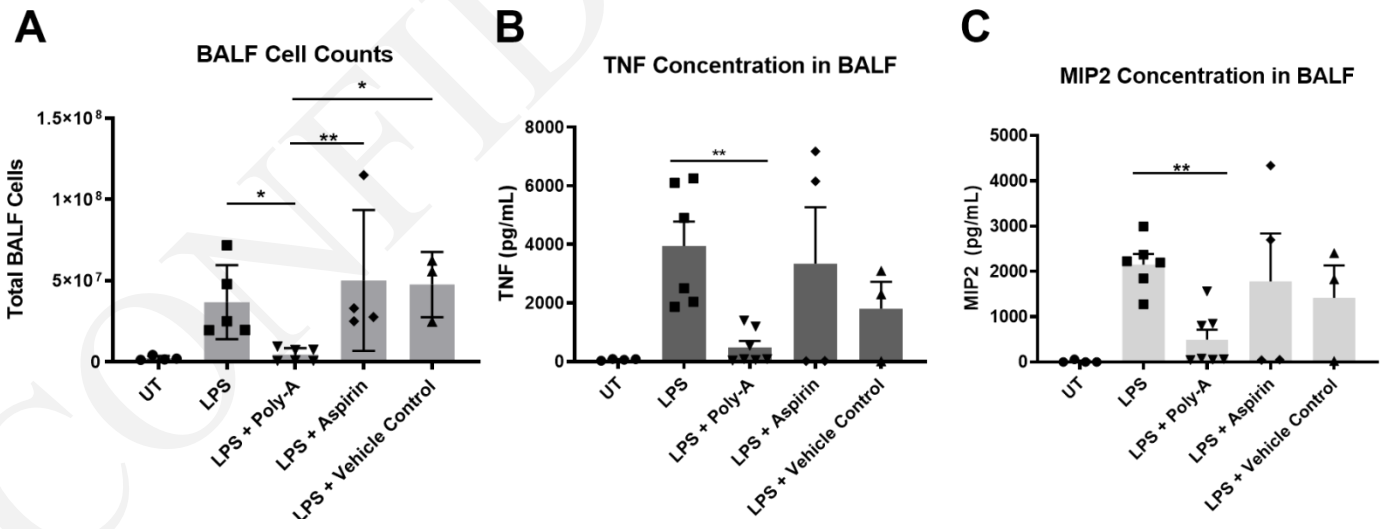


Fig. S6—Evaluating Poly-A particles as an anti-inflammatory agent in comparison with aspirin. Balb/cJ mice were used to evaluate lung damage 4 hrs post-ALI with either Poly-A particles or aspirin as a therapeutic. **(A)** Total BALF cells display a significant reduction for Poly-A treated mice compared to LPS + aspirin or LPS only. **(B)** TNF and **(C)** MIP2 concentrations in the BALF post-ALI are also only significantly reduced for Poly-A-treated mice. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test

with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. $\boldsymbol{N \geq 5}$ for all assays unless otherwise stated.

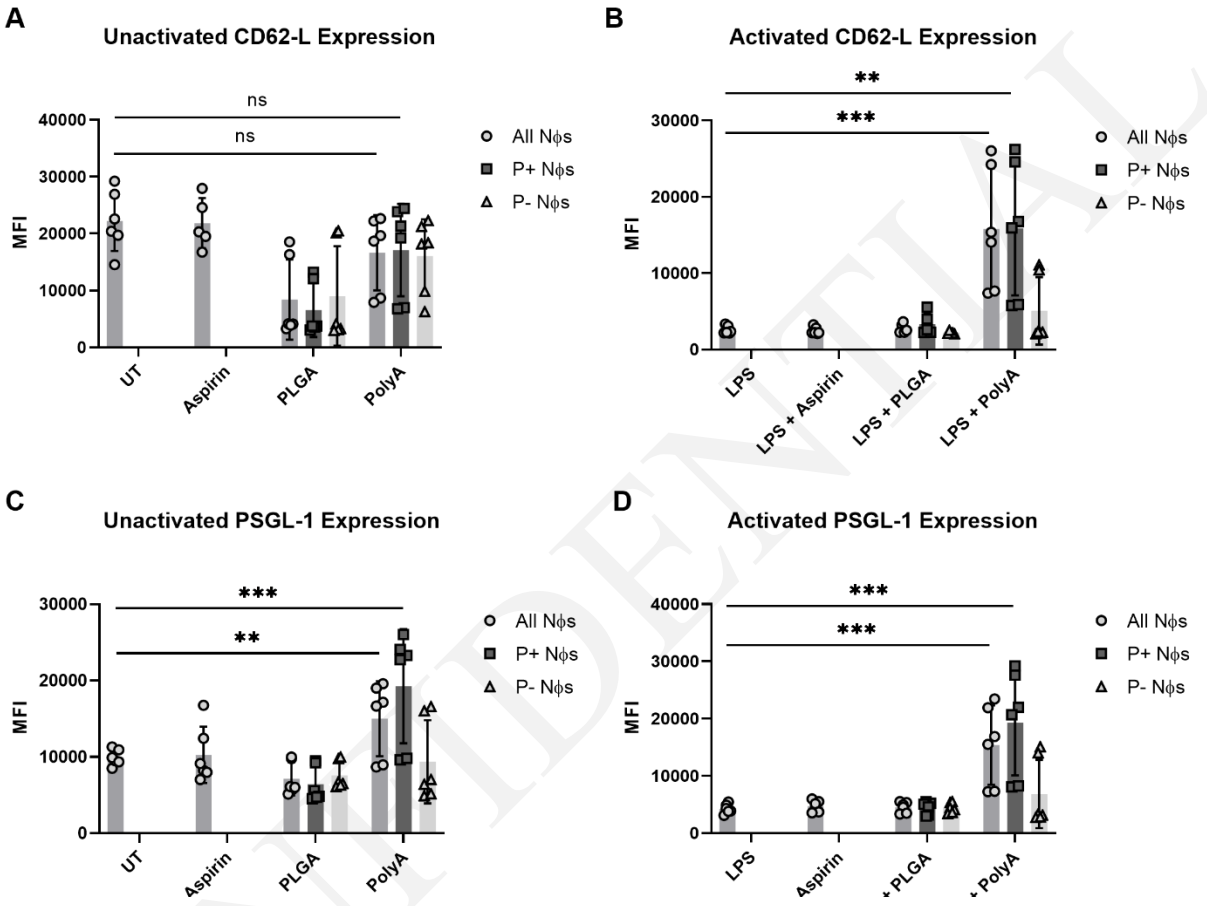


Fig. S7—Expression of inflammatory proteins on human neutrophils as measured by flow cytometry. Mean fluorescence intensity (MFI) of **(A)** unactivated and **(B)** LPS-activated human neutrophils treated with a fluorescent anti-L-selectin antibody after exposure to soluble aspirin or PLGA or Poly-A microspheres. Mean fluorescence intensity (MFI) of **(C)** unactivated and **(D)** LPS-activated human neutrophils treated with a fluorescent anti-PSGL-1 antibody after exposure to soluble aspirin or PLGA or Poly-A microspheres. UT = untreated, unactivated (naïve) neutrophils. P- Nøs = Particle negative neutrophils; P+ Nøs = Particle positive neutrophils. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. n.s. indicates "not significant." $N \geq 3$ for all assays unless stated.

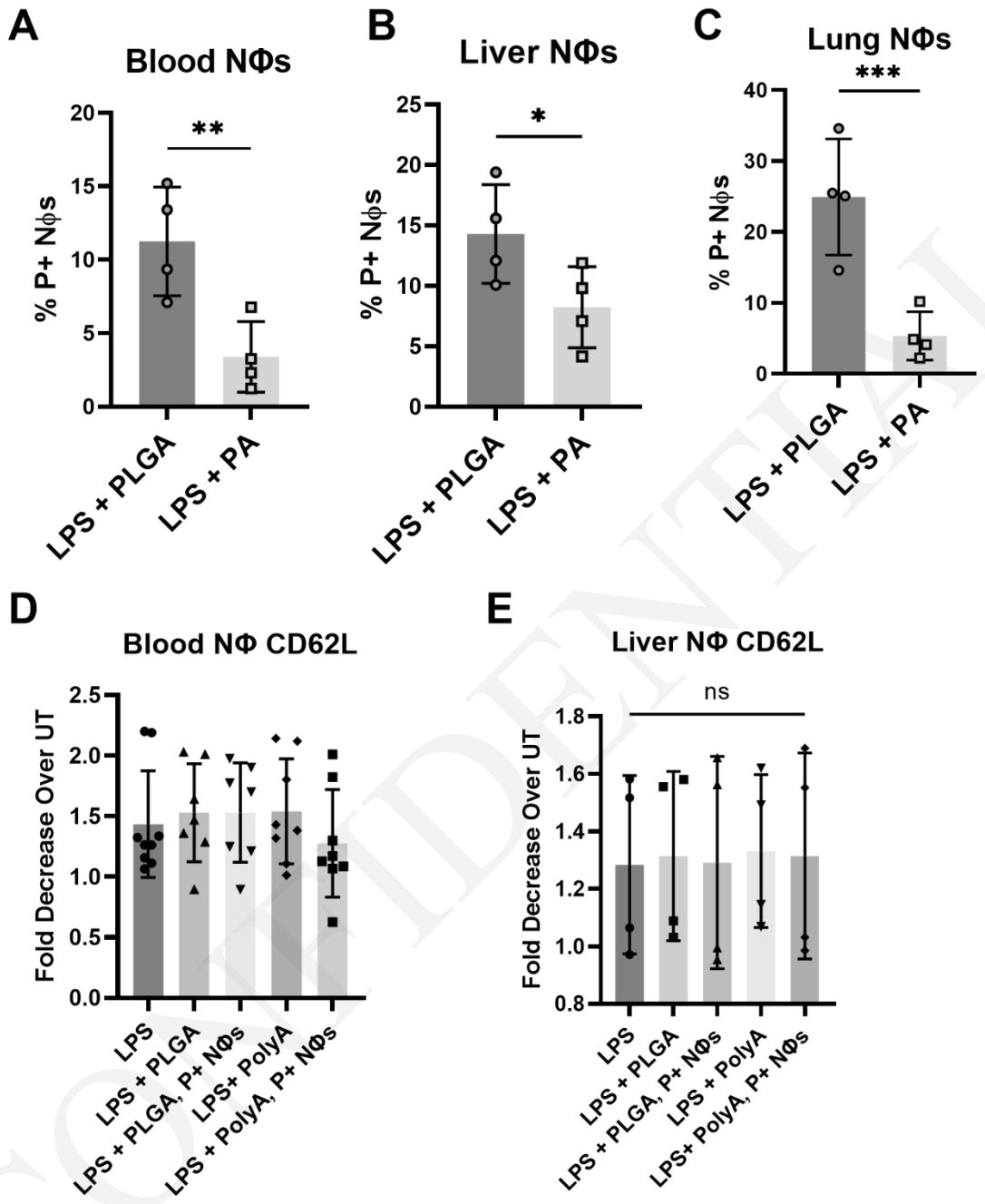


Fig. S8—Percent particle positive neutrophils in blood, liver, and lung. Particle positive neutrophils in **(A)** blood, **(B)** liver, and **(C)** lungs. Fold decrease of L-selectin on **(G)** blood neutrophils and **(H)** neutrophils isolated from the liver *P+ Nøs = Particle positive neutrophils* Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. n.s. indicates "not significant." $N \geq 3$ for all assays unless stated.

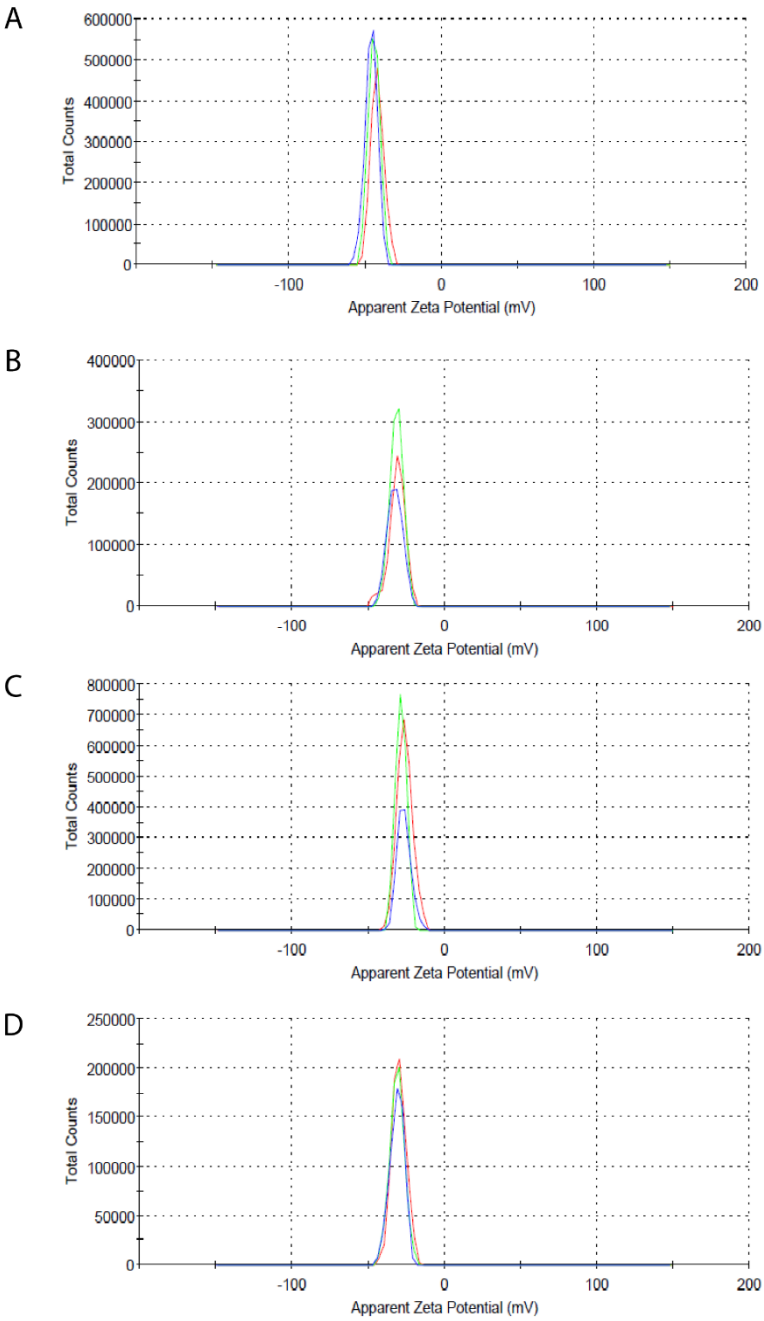


Fig. S9—Surface Charge of Particles. Histograms with three repeats of each particle type **(A)** PS **(B)** PLGA **(C)** Poly-A and **(D)** Poly-A/Cy5.5.

Table S1—Sizes and surface charges of Poly-A, PLGA, and polystyrene particles

Particle Type	Size	Zeta Potential (mV)	Surface Chemistry
PS	2.0 ± 0.16 µm	-44.2	-COOH
PLGA	0.8 ± 0.30 µm	-31.8	-COOH
Poly-A	0.9 ± 0.30 µm	-27.2	-COOH
Poly-A/Cy5.5	1.0 ± 0.24 µm	-30.0	-COOH

Table S2-Significance testing for figure 1.

Figure 1C, 2 hr injection: Total BALF Cells	**Uncorrected Fisher's LSD**	**Mean Diff.**	**95.00% CI of diff.**	**Below threshold?**	**Summary**	**Individual P-Value**
	UT vs. LPS	-2789524	-3813068 to -1765980	Yes	****	<0.0001
	UT vs. LPS + Poly-A	-351190	-1374735 to 672354	No	ns	0.4863
	UT vs. LPS + PLGA	-1228690	-2252235 to -205146	Yes	*	0.0206
	UT vs. LPS + PS	-1159857	-2237106 to -82608	Yes	*	0.0359
	LPS vs. LPS + Poly-A	2438333	1376151 to 3500515	Yes	****	<0.0001
	LPS vs. LPS + PLGA	1560833	498651 to 2623015	Yes	**	0.0057
	LPS vs. LPS + PS	1629667	515641 to 2743692	Yes	**	0.0059
	LPS + Poly-A vs. LPS + PLGA	-877500	-1939682 to 184682	No	ns	0.1013
	LPS + Poly-A vs. LPS + PS	-808667	-1922692 to 305359	No	ns	0.1474
	LPS + PLGA vs. LPS + PS	68833	-1045192 to 1182859	No	ns	0.8998
Figure 1D, 2 hr injection BALF Neutrophils	Uncorrected Fisher's LSD	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Individual P-Value
	UT vs. LPS	-1726767	-2310045 to -1143489	Yes	****	<0.0001
	UT vs. LPS + Poly-A	-197250	-762006 to 367507	No	ns	0.4816
	UT vs. LPS + PLGA	-659071	-1223828 to -94315	Yes	*	0.0236
	UT vs. LPS + PS	-586188	-1193283 to 20907	No	ns	0.0579
	LPS vs. LPS + Poly-A	1529517	964760 to 2094273	Yes	****	<0.0001
	LPS vs. LPS + PLGA	1067695	502939 to 1632452	Yes	***	0.0005
	LPS vs. LPS + PS	1140579	533484 to 1747674	Yes	***	0.0006
	LPS + Poly-A vs. LPS + PLGA	-461822	-1007428 to 83785	No	ns	0.0942
	LPS + Poly-A vs. LPS + PS	-388938	-978260 to 200384	No	ns	0.1881
	LPS + PLGA vs. LPS + PS	72884	-516439 to 662206	No	ns	0.8025
Figure 1E, 4 hr injection: Total BALF Cells	Uncorrected Fisher's LSD	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Individual P-Value
	UT vs. LPS	-5389217	-6907754 to -3870680	Yes	****	<0.0001
	UT vs. LPS + Poly-A	-1845050	-3468435 to -221665	Yes	*	0.0275
	UT vs. LPS + PLGA	-2612717	-4315337 to -910097	Yes	**	0.0041
	UT vs. LPS + PS	-3294717	-4997337 to -1592097	Yes	***	0.0005
	LPS vs. LPS + Poly-A	3544167	2025630 to 5062704	Yes	****	<0.0001
	LPS vs. LPS + PLGA	2776500	1173536 to 4379464	Yes	**	0.0015
	LPS vs. LPS + PS	2094500	491536 to 3697464	Yes	*	0.0125
	LPS + Poly-A vs. LPS + PLGA	-767667	-2470287 to 934953	No	ns	0.362

	LPS + Poly-A vs. LPS + PS	-1449667	-3152287 to 252953	No	ns	0.0918
	LPS + PLGA vs. LPS + PS	-682000	-2460329 to 1096329	No	ns	0.437
Figure 1F, 4 hr injection BALF Neutrophils	Uncorrected Fisher's LSD	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Individual P-Value
	UT vs. LPS	-3490961	-4939946 to -2041976	Yes	****	<0.0001
	UT vs. LPS + Poly-A	-615716	-2164747 to 933315	No	ns	0.4219
	UT vs. LPS + PLGA	-1090126	-2714763 to 534511	No	ns	0.1799
	UT vs. LPS + PS	-2524512	-4017195 to -1031828	Yes	**	0.0018
	LPS vs. LPS + Poly-A	2875245	1426260 to 4324230	Yes	***	0.0004
	LPS vs. LPS + PLGA	2400835	871290 to 3930380	Yes	**	0.0033
	LPS vs. LPS + PS	966449	-422135 to 2355034	No	ns	0.1647
	LPS + Poly-A vs. LPS + PLGA	-474410	-2099047 to 1150227	No	ns	0.5541
	LPS + Poly-A vs. LPS + PS	-1908796	-3401479 to -416112	Yes	*	0.0141
	LPS + PLGA vs. LPS + PS	-1434386	-3005389 to 136618	No	ns	0.0719