

# ADVANCED HEALTHCARE MATERIALS

## Supporting Information

for *Adv. Healthcare Mater.*, DOI 10.1002/adhm.202200819

Temporal Control of Trained Immunity via Encapsulated Release of  $\beta$ -Glucan Improves  
Therapeutic Applications

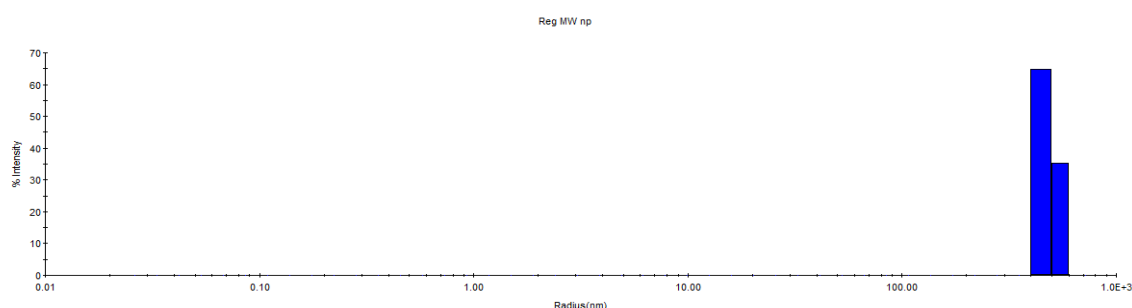
*Jainu Ajit, Britteny Cassaidy, Sophia Tang, Ani Solanki, Qing Chen, Jingjing Shen and Aaron P.  
Esser Kahn\**

## Supporting Information

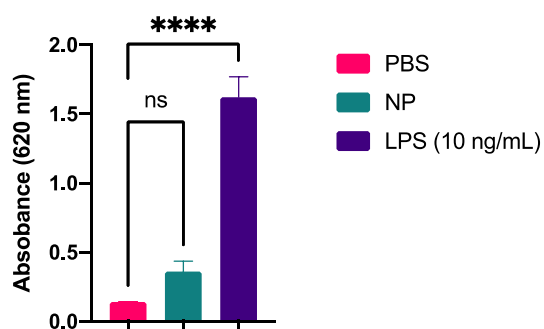
**Temporal control of trained immunity via encapsulated release of  $\beta$ -glucan improves therapeutic applications**

Jainu Ajit,<sup>‡</sup> Britteny Cassaidy,<sup>‡</sup> Sophia Tang, Ani Solanki, Qing Chen, Jingjing Shen, Aaron P. Esser Kahn\*

**Fig. S1.** Dynamic light scattering (DLS) for synthesized nanoparticles



**Fig. S2.** HEK<sub>m</sub>TLR4 assay for synthesized nanoparticles confirming no endotoxin. All values are expressed as mean  $\pm$  SEM. Samples were run in triplicate and statistics were conducted using a one-way ANOVA with Dunnett's multiple comparisons test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . n.s., not significant.



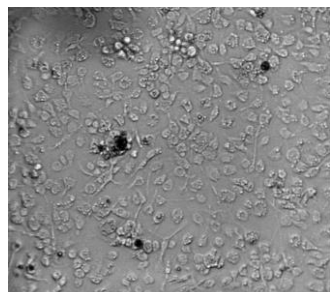
**Fig. S3.** *in vitro* TI assay

BMDMs were trained for 24 h with indicated materials (untrained or PBS (black bars), equivalent amount of free  $\beta$ -glucan (pink bars), empty or unencapsulated nanoparticles (green bars), mixture of empty nanoparticles and equivalent amount of free  $\beta$ -glucan (purple bars) or nanoparticles (lilac bars)). **Fig. S3a.** Image of BMDMs treated with indicated materials after PBS wash following 24 h training time. A separate batch of cells were rested for 5 days. On

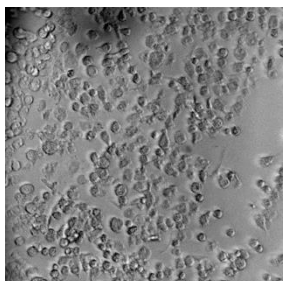
day 6, trained cells were challenged with 10 ng/mL of LPS. **Fig S3b.** IL-6 levels from the supernatant were quantified using ELISA. All values are expressed as mean  $\pm$  SEM. Samples were run in triplicate.

A)

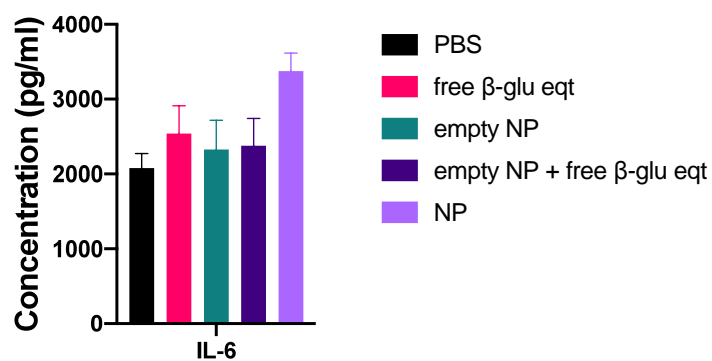
Nanoparticle treated BMDMs



free  $\beta$ -glucan treated BMDMs

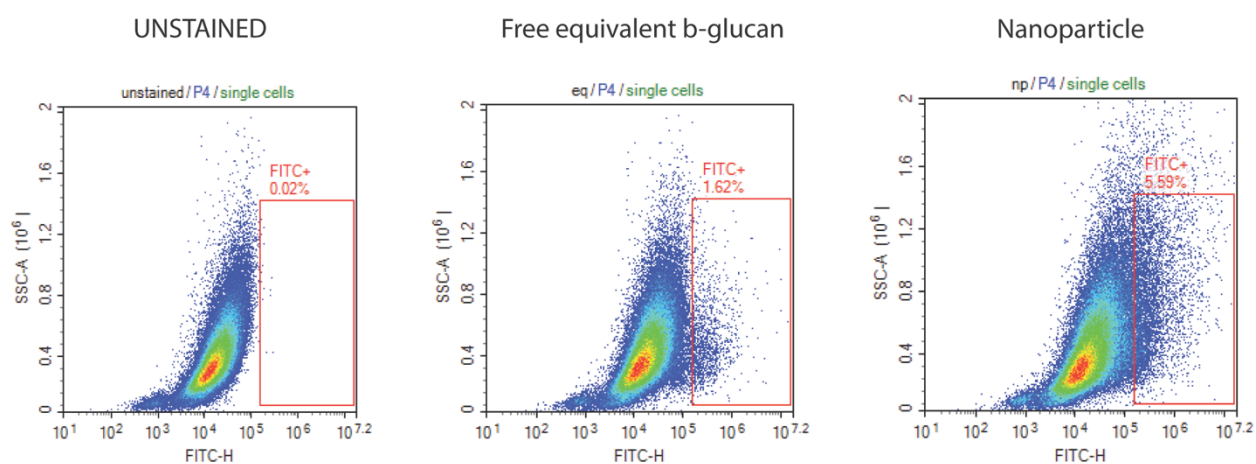


B)



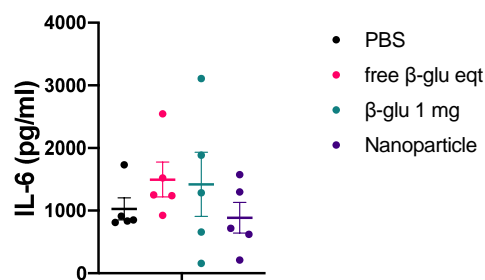
**Fig. S4. Gating strategy for measuring uptake of nanoparticles**

Uptake of free FITC-labelled  $\beta$ -glucan and FITC-labelled  $\beta$ -glucan encapsulated in nanoparticles by flow cytometry- representative plots shown. (n=3)

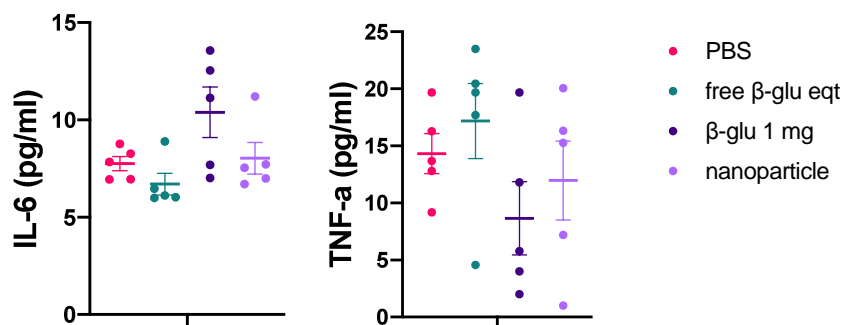


**Fig. S5. Day 35 serum cytokines after LPS challenge**

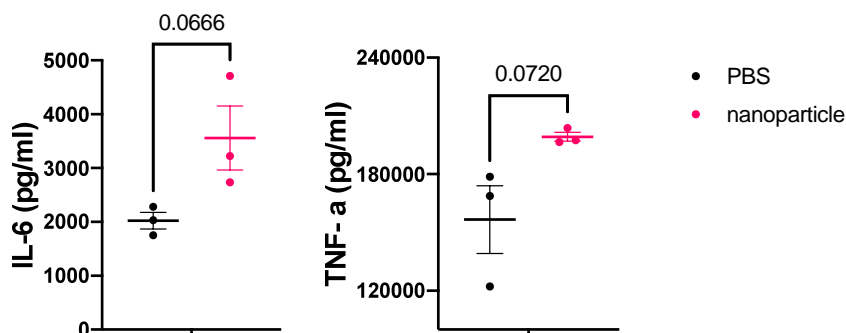
Day 35 serum cytokines for mice trained with indicated materials (PBS (black), equivalent amount of free  $\beta$ -glucan (pink), free 1 mg  $\beta$ -glucan (green) or nanoparticles (purple)) 1 h after challenge with LPS. No significant changes in cytokines were observed after LPS challenge 35 days post training. All values are expressed as mean  $\pm$  SEM. n=5, statistics were conducted using a one-way ANOVA with Dunnett's multiple comparisons test.

**Fig. S6. Ruling out innate immune priming for nanoparticles *in vivo***

Ruling out innate immune priming for nanoparticles *in-vivo*: Mice that were trained with indicated materials (PBS (pink), equivalent amount of free  $\beta$ -glucan (green), free 1 mg  $\beta$ -glucan (purple) or nanoparticles (lilac)) and serum cytokines were analyzed prior to LPS challenge on day 27. No significant changes in cytokines were observed in any of the groups before LPS challenge. All values are expressed as mean  $\pm$  SEM. n=5, statistics were conducted using a one-way ANOVA with Dunnett's multiple comparisons test.

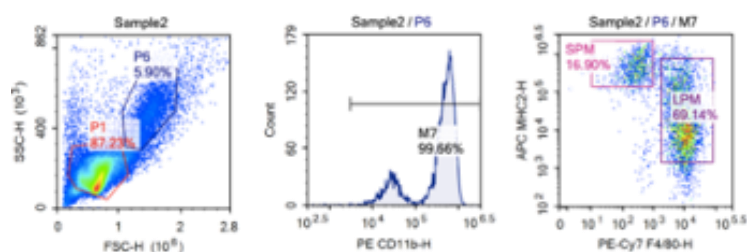
**Fig. S7. *in vivo* TI assay with NIR labelled PLGA nanoparticles**

Mice were trained with either PBS (black) or NIR labelled PLGA encapsulating  $\beta$ -glucan (pink). *in-vivo* degradation profile was recorded until 21 days later. The mice were then challenged with LPS and serum cytokines were quantified 3 h later. All values are expressed as mean  $\pm$  SEM. n=3, statistics were conducted using an unpaired T-test. p values are listed on the graphs.

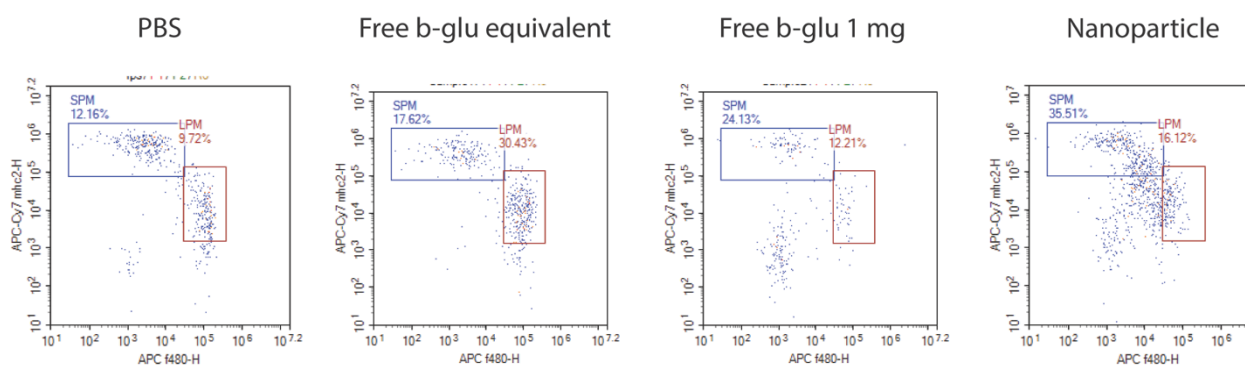


**Fig. S8. Gating strategy for small peritoneal macrophages (SPM) and representative flow plots**

Gating strategy for small peritoneal macrophages (SPM) characterized as CD11b<sup>+</sup>MHC2<sup>high</sup>F4/80<sup>-</sup> [42]

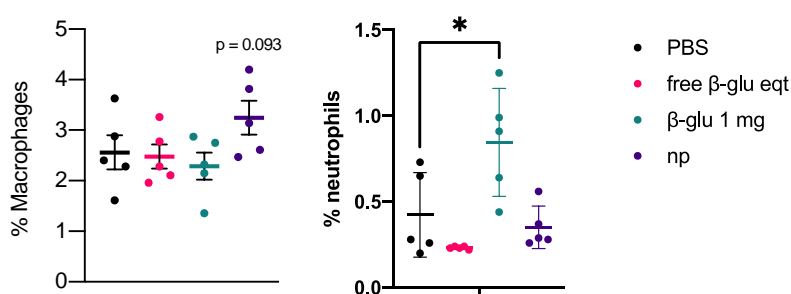


Representative flow cytometry plots for % SPMs in different groups tested (n=5)



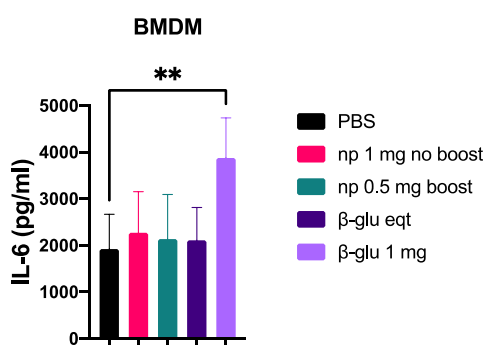
**Fig. S9. Percentage of splenic macrophages and neutrophils after training**

Mice were trained with indicated materials (PBS (black), equivalent amount of free β-glucan (pink), free 1 mg β-glucan (green) or nanoparticles (purple)) and challenged with LPS 7 days later. Spleens were harvested and analyzed. Splenic macrophages (CD45<sup>+</sup>B220<sup>-</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>) were higher in nanoparticle trained mice whereas neutrophils (CD45<sup>+</sup>B220<sup>-</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>) were higher in the conventional 1 mg β-glucan trained mice. All values are expressed as mean ± SEM. n=5, statistics were conducted using a one-way ANOVA, \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001. n.s., not significant.



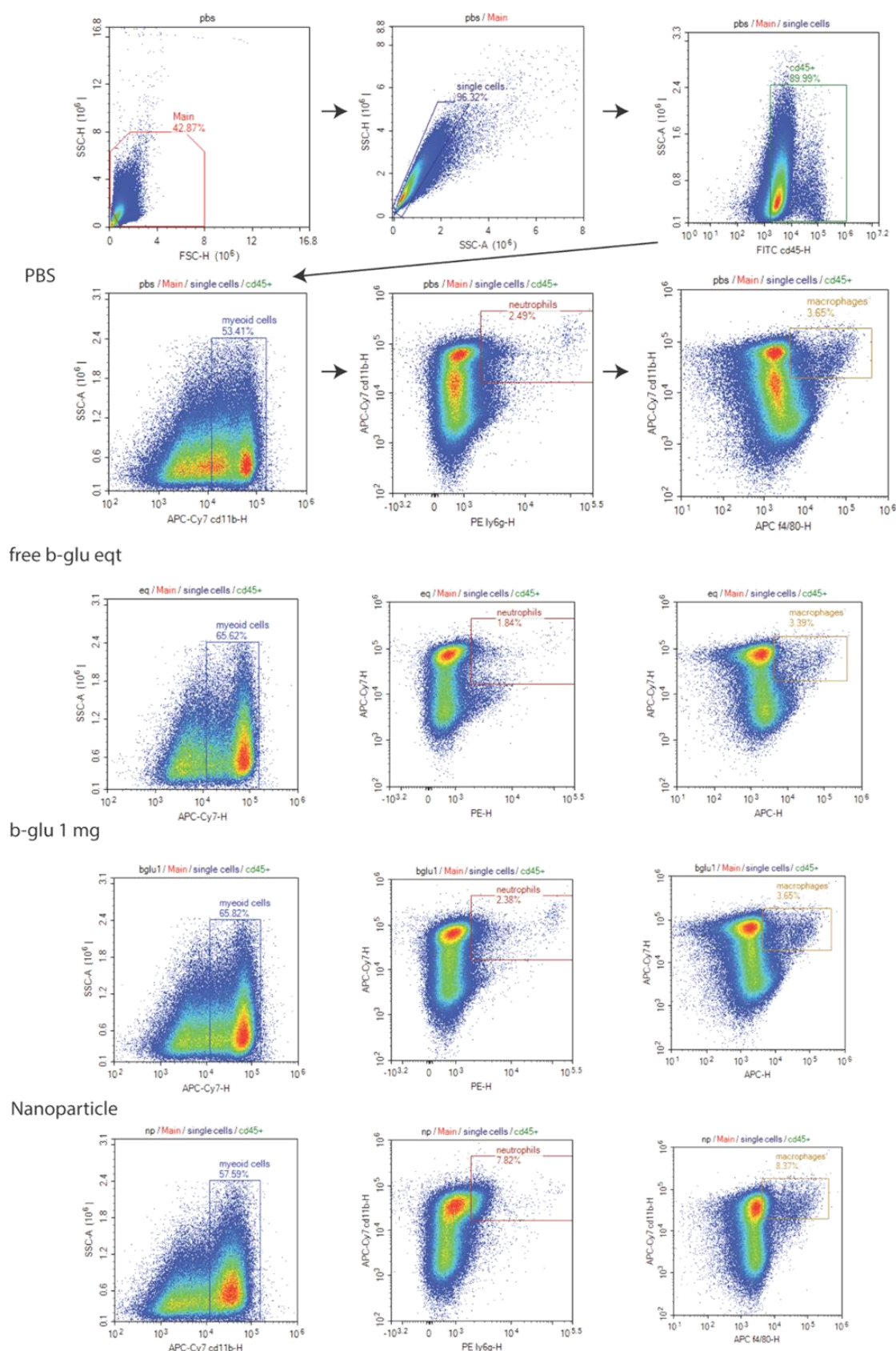
**Fig. S10. *ex vivo* challenge of BMDMs after training**

Mice were trained with indicated materials (PBS (black bars), 1 mg of nanoparticles on day 0 only (pink bars), 0.5 mg of nanoparticles on both day 0 and day 4 (green bars), equivalent amount of free  $\beta$ -glucan (purple), free 1 mg  $\beta$ -glucan (lilac). After 7 days, bone marrow was harvested and differentiated into BMDMs. 6 days after culture, cells were re plated at a density of 100,000 cells/ well and were challenged *ex-vivo* with LPS (10 ng/mL) and cytokines were quantified. Only mice trained with 1 mg of free b-glucan showed increased IL-6 levels confirming that nanoparticles did not act at the bone marrow. All values are expressed as mean  $\pm$  SEM. n=3, statistics were conducted using a one-way ANOVA, \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . n.s., not significant.



**Fig. S11. Tumor microenvironment analysis**

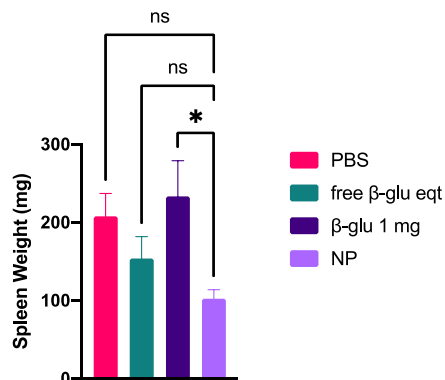
Tumor microenvironment analysis: At day 46, tumors were excised and single cell suspensions were prepared. Gating strategy for tumor associated macrophages ( $CD45^+CD11b^+F4/80^+$ ) and tumor associated neutrophils ( $CD45^+CD11b^+Ly6G^+$ ) and representative flow cytometry plots for % TAMs and % TANs in tumor micro-environment in the groups tested (n=5).



**Fig. S12. Spleen weight on day 46 after tumor challenge**

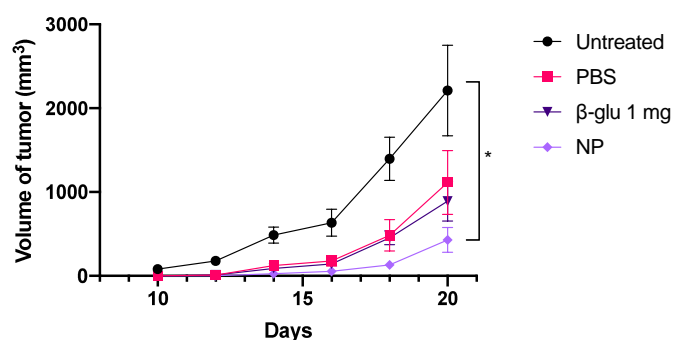
Mice were trained with indicated materials (PBS (pink), equivalent amount of free β-glucan (green), free 1 mg β-glucan (purple) or nanoparticles (lilac)) and were challenged with

B16.F10 tumors after 3 weeks. Mice were sacrificed at day 46 and spleen weights were measured. Splenomegaly was observed for all groups except nanoparticle trained mice. All values are expressed as mean  $\pm$  SEM.  $n=3$ , statistics were conducted using a one-way ANOVA,  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ . n.s., not significant.

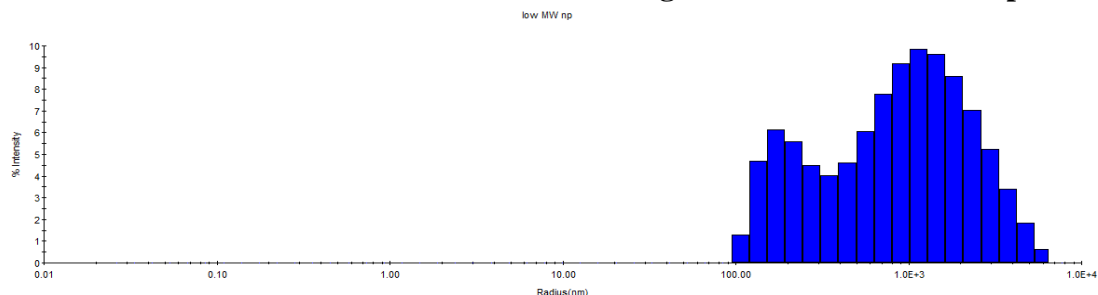


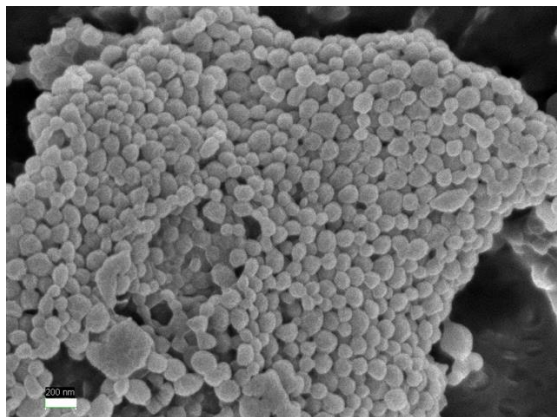
**Fig. S13. EG7.OVA tumor challenge.**

Mice were trained with indicated materials- PBS (black and pink), 1 mg of free  $\beta$ -glucan (purple) or 0.5 mg nanoparticles (lilac). One week after training, all groups except untreated (black) received CpG/OVA vaccination and were then challenged with EG7.OVA tumor after 2 weeks. Tumor growth was measured and quantified. All values are expressed as mean  $\pm$  SEM.  $n=3$ , statistics were conducted using two-way ANOVA with Dunnett's multiple comparison test,  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ . n.s., not significant.

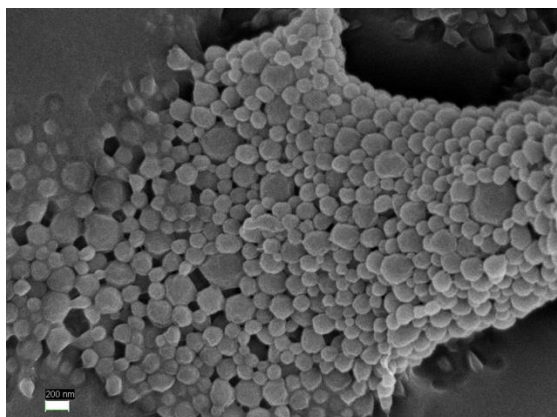
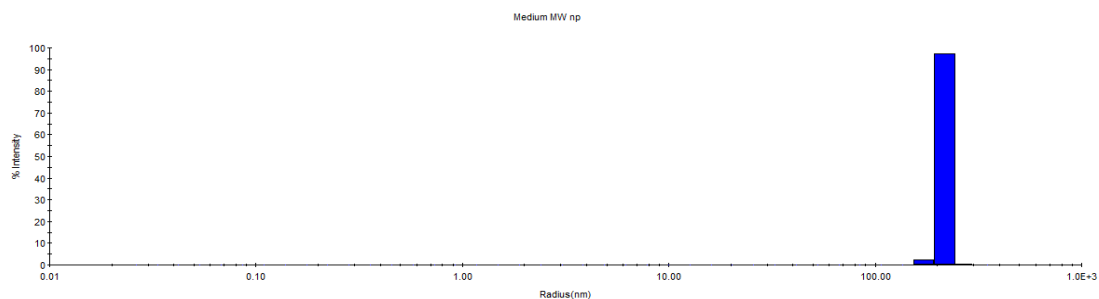


**Fig. S14a: DLS and SEM data for low molecular weight acid-terminated nanoparticles**

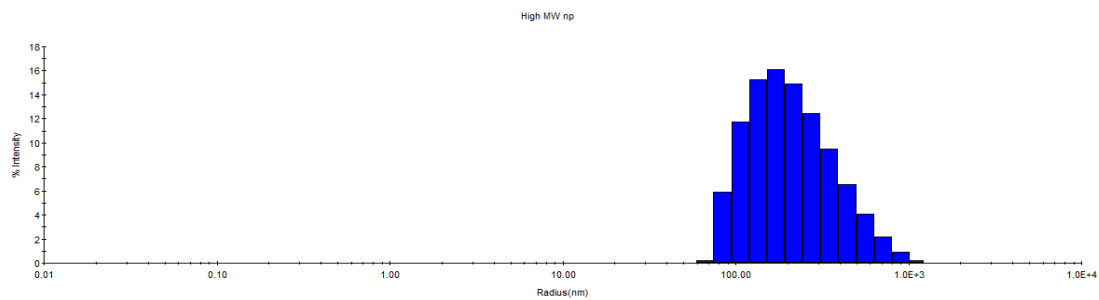


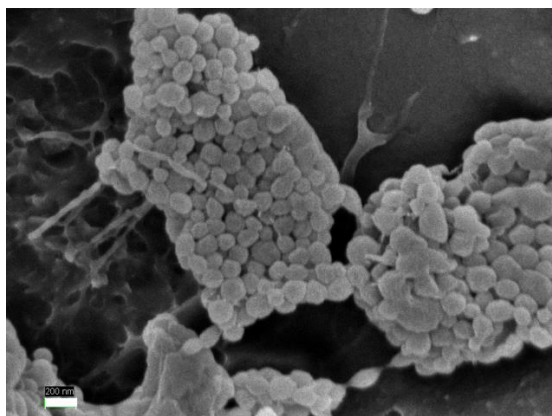


**Fig. S14b: DLS and SEM data for medium molecular weight ester-terminated nanoparticles (NIR-PLGA nanoparticles used for imaging)**

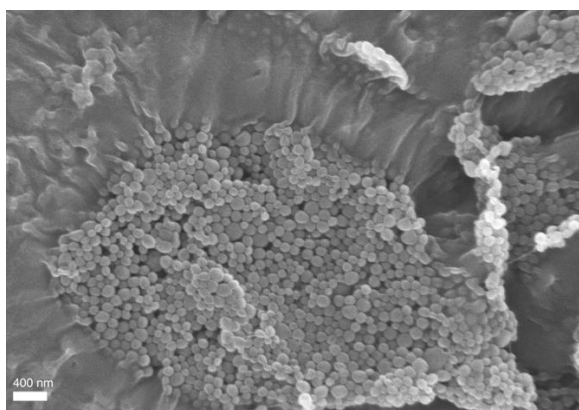
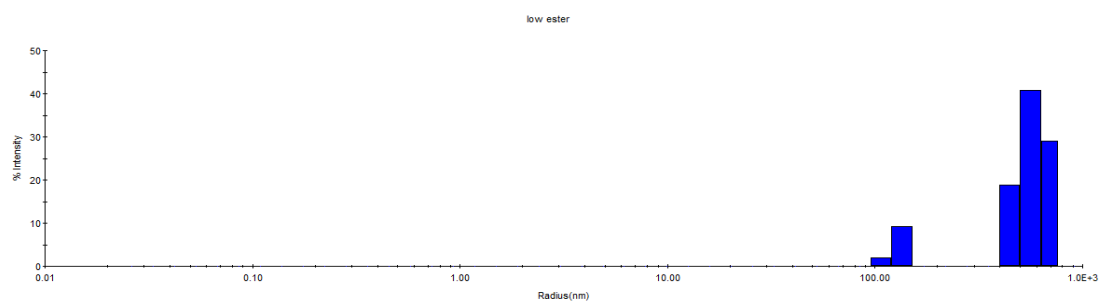


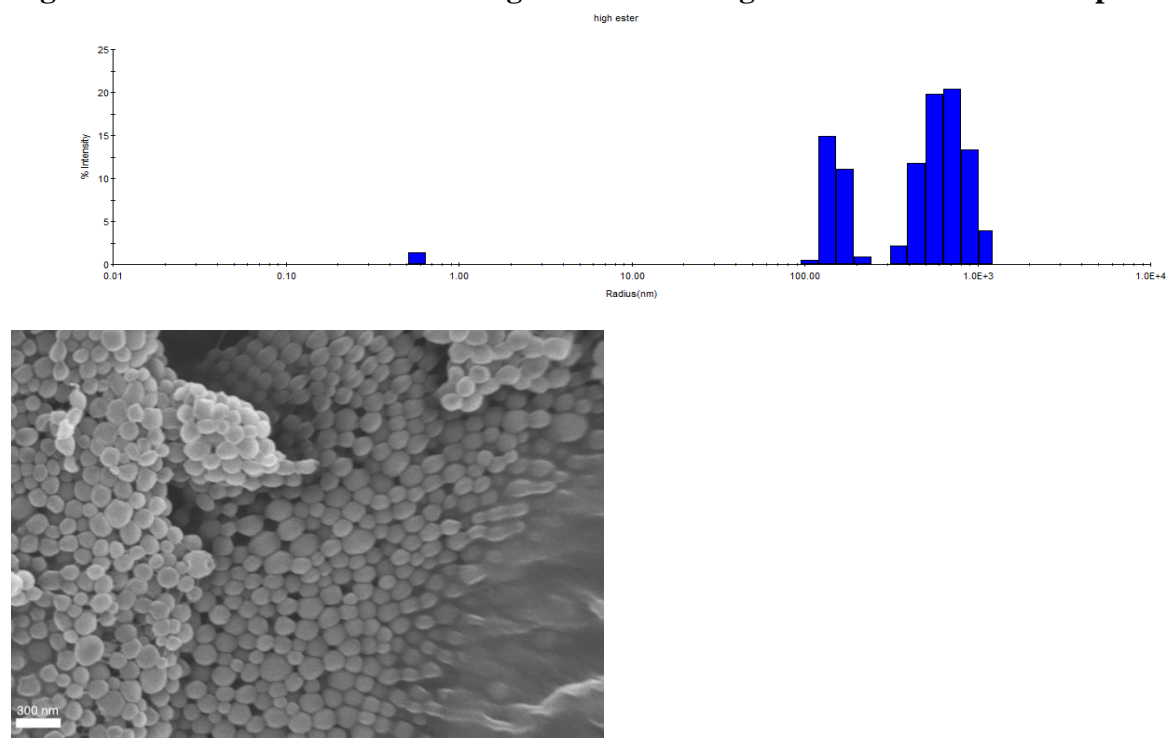
**Fig. S14c: DLS and SEM data for high molecular weight acid-terminated nanoparticles**





**Fig. S14d: DLS and SEM data for low molecular weight ester-terminated nanoparticle**



**Fig. S14e: DLS and SEM data for high molecular weight ester-terminated nanoparticles****Table 1: Characterization data for synthesized nanoparticles**

Nanoparticle	Scale	Recovered (mg)	Loading ( $\mu\text{g}$ $\beta$ -glucan/mg of particle)	SEM radius [nm]	DLS radius [nm]	encapsulation efficiency (%)
Regular ester	200	140	52.4	67.2	487.9	73.4
Low acid	100	44	41.0	66.8	687	72.4
Medium ester	100	57	56.4	100	217	63.8
High acid	100	43	24.1	104	245	41.3
Low ester	100	50	43.1	137	468	63.3
High ester	100	66	120	130	413	55.6

**Fig. S15: IL-6 levels from *in vivo* TI assay on day 30**

Mice were trained once on day 0 with the desired training materials – PBS (black), low molecular weight acid terminated nanoparticles (pink), low molecular weight ester-terminated nanoparticles (green), high molecular weight acid terminated nanoparticles (purple) or low molecular weight ester-terminated nanoparticles (lilac). Different set of mice were challenged with LPS at different time points- IL-6 levels from serum of mice challenged on day 30

showed no difference from untrained mice. All values are expressed as mean  $\pm$  SEM.  $n=3$ , statistics were conducted using one-way ANOVA with multiple comparison test,  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ . n.s., not significant.

