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Temporal Control of Trained Immunity via Encapsulated Release of β-Glucan Improves Therapeutic Applications

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Emerging diseases require generating new vaccines, which can often be time consuming. An alternate method to boost host defense is by inducing nonspecific innate immune memory, called trained immunity, to develop novel prophylactics. Many molecules, most notably β-glucan, induce trained immunity, but their effects are often short-lived and uncontrolled. This lack of temporal control limits both the therapeutic ability of training and provides fundamental questions about its nature. To achieve temporal control of trained immunity, controlled release nanoparticles encapsulating only 3.5% of the standard dose of β-glucan to attain sustained release over a month are engineered. Nanoparticle-trained mice exhibit prolonged training effects and improve resistance to a B16F10 tumor challenge compared to mice that receive an equivalent amount of free β-glucan. The duration of trained immunity is further fine tuned by synthesizing nanoparticles composed of different molecular weights to modulate the release kinetics. These results demonstrate that dosing and temporal control can substantially alter the trained response to unanticipated levels. As such, this approach using sustained release platforms might lead to a novel prophylactic strategy for improved disease resistance against a wide variety of diseases.

1. Introduction

Innate immune cells form the first line of defense against pathogens.[1] Recent work has revealed that innate immune cells experience nonspecific "memory," which allows modulation of responses against repeated pathogenic insult, which was previously not considered an innate function. This enhanced responsiveness conferred by innate cells, called trained immunity, can improve the first line of defense and subsequent adaptive immune responses by increasing antigen presentation and T-cell activation.[2] Certain pathogenic stimuli, such as whole-cell inactivated mycobacteria, induce epigenetic and metabolic changes that rewire innate immune cells.[3] This rewiring improves pathogen detection and immune reactivity of the "trained" cells to better respond to a second attack by both the same or an unrelated pathogen.

Many whole-pathogen vaccines, such as *Bacillus* Calmette-Guérin (BCG),[4] polio,[5] and measles,[6] have been reported to induce trained immunity and protect against several unrelated viral and bacterial infections.[4,7,8] Less active trained immunity inducers, such as the yeast-derived β-glucan, are functional substitutes and protect mice against a diverse range of pathogens.[9–12] However, their protective effects are transient—lasting for only a few days—and the high dosages have raised concerns about inducing adverse responses.[10,13] Therefore, methods to control and extend trained immunity could help provide new tools in generating durable disease resistance. This paper demonstrates using conventional materials methods to control trained immunity responses over time and its application to a disease model.

Since macrophages and monocytes have a relatively short lifespan, methods to prolong trained immunity have thus far focused on targeting the bone marrow to generate trained myeloid precursors.[14,15] Priem et al. developed high-density lipoprotein (HDL) based nano-biologics encapsulating trained immunity-inducing muramyl tripeptide (MTP) that suppressed tumor growth in mice.[16] However, bone marrow-targeted nanomaterials do not provide precise temporal control over trained immunity and can potentially lead to unwanted side effects due to accumulation in the liver.[17] We sought to employ an efficient method of controlling the kinetics of trained immunity through a more generalizable approach using the sustained release of trained immunity-inducing molecules from biodegradable nanoparticles. Poly (lactic-co-glycolic acid) (PLGA) is a widely used FDA-approved polymer that exhibits slow degradation via hydrolysis of ester bonds with broad applications.[18] PLGA nanoparticles can be easily formulated to deliver the encapsulated cargo to their

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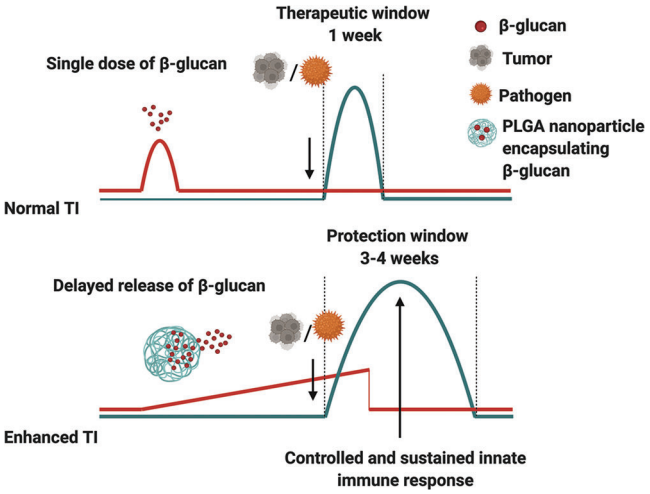

Figure 1. Schematic representation of the proposed mechanism of action of PLGA nanoparticles encapsulating β-glucan. Trained immunity mediated by free β-glucan confers protective effects that last only one week. Sustained release of β-glucan from engineered PLGA nanoparticles enable prolonged trained immunity effects that can last up to four weeks depending on the release profile (created with BioRender.com).

intended target cells. Moreover, the release kinetics can be controlled by modulating the properties of PLGA.[19,20] Therefore, to test the hypothesis of if sustained release would alter the temporal control of trained immunity—PLGA nanoparticles were an excellent test bed.

We investigated if the sustained release of β-glucan from PLGA nanoparticle formulation would prolong trained immunity effects by controlling release kinetics (**Figure 1**). We synthesized and characterized PLGA nanoparticles varying the rate of release of β-glucan over 30 d. We observed enhanced proinflammatory cytokine production in nanoparticle-trained bone-marrow-derived macrophages (BMDMs) challenged with lipopolysaccharide (LPS) in a 7 d in vitro assay. To better capture slow release and its effect on inducing trained immunity, we tested the synthesized nanoparticles in an in vivo model. While the standard dose of 1 mg of free β-glucan conferred trained immunity for more than a week, nanoparticle-trained mice demonstrated peak systemic effects extending over a month of training. This prolonged response enabled the nanoparticle-trained mice to resist tumor growth better than conventionally trained animals when challenged with B16.F10 melanoma three weeks after training. We demonstrated that different molecular weight PLGA could fine-tune the duration of training for various applications by controlling release kinetics. We report for the first time that sustained release from biodegradable polymer nanoparticles can be used for temporal control of trained immunity over a predictable window of a week to a month. This work can potentially lead to the development of a new class of prophylactics for safe, effective, and improved disease resistance against a wide range of pathogens.

2. Results

To test the hypothesis that sustained release can prolong trained immunity, we first synthesized nanoparticles composed of PLGA encapsulating β-glucan. Nanoparticles were synthesized using a modified version of a previously reported double emulsion technique,[21] owing to its ease and consistency in creating homogenous nanospheres that degrade in a controlled release fashion. In brief, nanoparticles were synthesized by ultrasonication of an aqueous solution of β-glucan and PLGA dissolved in dichloromethane. The solution was ultrasonicated again after adding 5% PVA and left stirring for 6 h in a 0.5% PVA stabilizing solution. The resulting precipitated nanoparticles were washed and lyophilized for characterization.

In examining the consistency of nanoparticles, scanning electron microscopy (SEM) images revealed nanosphere morphology of particles that were around 67 ± 20 nm in diameter (**Figure 2**a). However, we detected larger radiuses under DLS measurements suggesting that the particles significantly aggregate in aqueous solutions (Figure S1, Supporting Information). The amount of β-glucan encapsulated within the nanoparticles was quantified using a total carbohydrate assay.[22] Encapsulation efficiency was determined by the percentage of β-glucan as measured by the carbohydrate assay divided by the amount of β-glucan initially loaded in each formulation. Synthesized nanoparticles had a high encapsulation efficiency of 73%. We also evaluated the in vitro release profile at pH 7.4, which showed a sustained release profile of β-glucan over the 4 week period of testing (Figure 2b). We confirmed that the synthesized nanoparticles were devoid of endotoxin using a HEK-mTLR4 reporter assay (Figure S2, Supporting Information). Concluding that the particles were loaded with β-glucan and devoid of any other immunostimulatory materials, we next tested them for their ability to train innate cells.

As previous work had shown that PLGA nanoparticles exhibit slow-release, we first tested if the synthesized nanoparticles encapsulating β-glucan improved trained immunity using a standard BMDM training model.[23] In this model, macrophages are trained with added material (e.g., β-glucan) and later challenged with a conventional secondary stimulant (e.g., LPS). If training occurs, the macrophages increase transcription of proinflammatory cytokines like IL-6 and TNF-α. Following a standard 7 d training assay, murine BMDMs at a density of 100 000 cells per well were incubated for 24 h with a set of "training material." Each group of cells was trained for 24 h, rested for 4 d, and later challenged with 10 ng mL^{-1} of LPS in a total volume of 200 µL. We compared how the release of β-glucan affected training by using nanoparticles containing either β-glucan (100 µg mL^{-1}) or administering the free form of an equivalent amount of β-glucan (3.7 µg mL^{-1}). We included an untrained (or PBS trained) group as our negative control. Some nanoparticles were found to stick to the well plate even after washing with PBS after 24 h (Figure S3a, Supporting Information). We observed significantly higher IL-6 and TNF-α levels confirming that nanoparticles enhanced trained immunity in vitro (Figure 2c). In fact, encapsulating the β-glucan in nanoparticles resulted in an approximately 1.5-fold increase in both IL-6 and TNF-α produced by BMDMs in response to the same stimulus compared to the unencapsulated equivalent. In addition, we observed that nanoparticles with no β-glucan did not induce training (Figure S3b, Supporting Information). Together, these results suggested that encapsulation of the training material, β-glucan, could strongly enhance the training effect in preliminary in vitro assays.

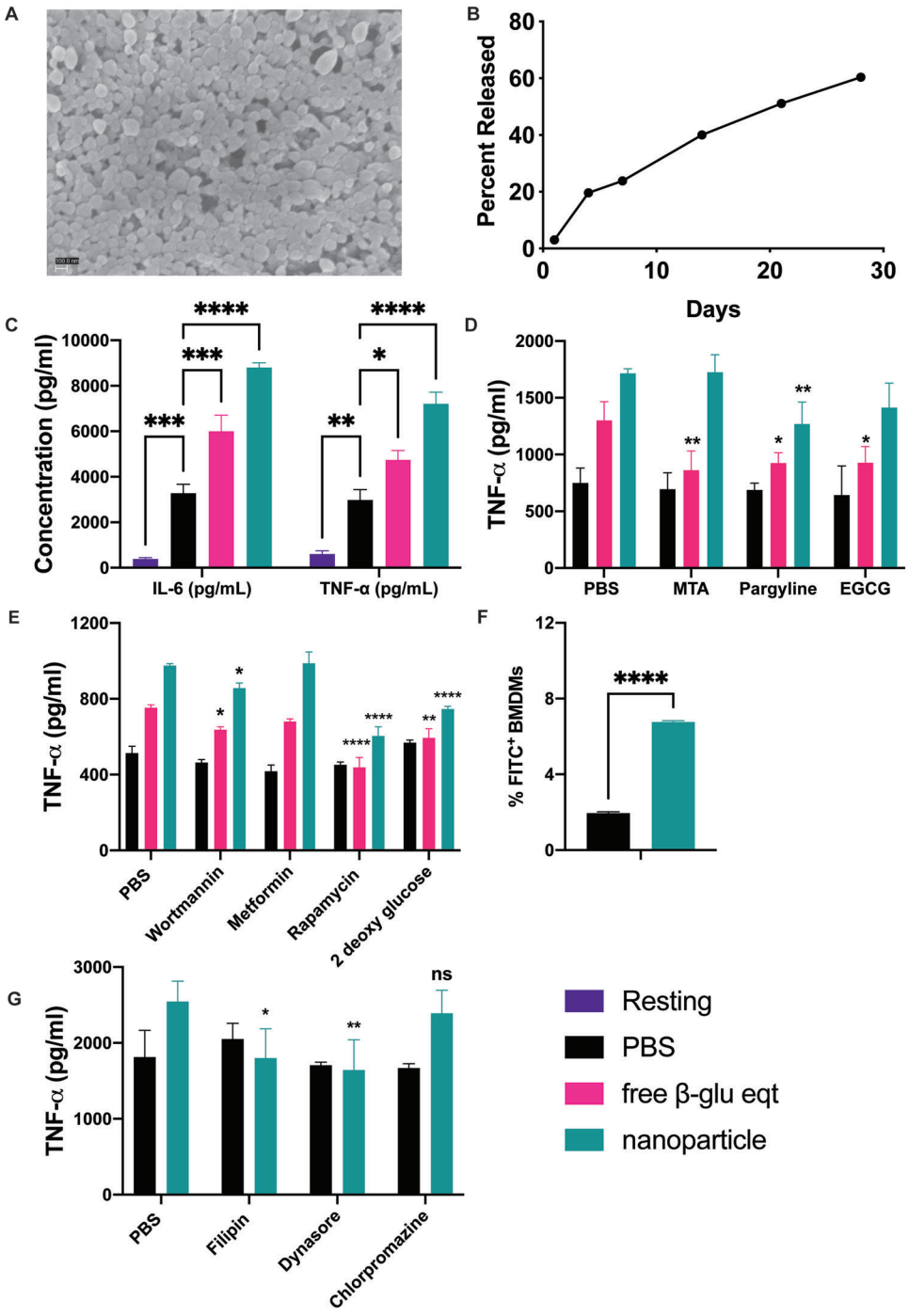

Figure 2. Nanoparticle characterization and in vitro TI assays. a) Scanning electron microscopy image of PLGA nanoparticles synthesized by double emulsion technique. (scale bar = 100 nm). b) 7 d in vitro release kinetics of encapsulated β-glucan from the synthesized PLGA nanoparticles at pH 7.4. c) In vitro trained immunity assay comparing cytokine levels in resting cells (purple bar) or following an LPS challenge in BMDMs trained with either PBS (black bar), the free equivalent amount of β-glucan (pink bar) or PLGA nanoparticles encapsulating β-glucan (green bar), $n = 3$, significance compared with PBS. d) Assay determining changes in epigenetic training response. BMDMs were pretreated for 30 min with inhibitors targeting epigenetic modifications, namely MTA (histone methyltransferase inhibitor), EGCG (histone demethylase inhibitor), or pargyline hydrochloride (histone acetyltransferase inhibitor). Cytokine levels were measured after an LPS challenge on day 5 with BMDMs trained with PBS (black bar), the free equivalent amount of β-glucan (pink bar), or PLGA nanoparticles (green bar) $n = 3$, significance compared with PBS (no inhibitor) group. e) Assay determining changes in metabolic training response. BMDMs were pretreated for 30 min with inhibitors targeting metabolic pathways, namely wortmannin (Akt inhibitor), metformin (AMPK activator), rapamycin or 2-deoxy D-glucose (glycolysis inhibitor). Cytokine levels were measured after an LPS challenge on day 5 with BMDMs trained with– PBS (black bar), the free equivalent amount of β-glucan (pink bar), or PLGA nanoparticles (green bar), $n = 3$, significance compared with PBS (no inhibitor) group. f) BMDMs were treated with either free FITC-labeled β-glucan (pink bars) or nanoparticles encapsulating FITC-β-glucan (green bars) for 24 h. BMDMs were analyzed by flow cytometry to measure the percentage of cells that were FITC+. Statistics was

Additionally, the remaining nanoparticles after the 24 h wash period, might also contribute to slow release of β-glucan and enhanced training.

While the initial in vitro data was promising, one important consideration was if encapsulation affected the mechanism of action of trained immunity mediated by β-glucan. In previous experiments, others established that β-glucan induces epigenetic and metabolic changes via histone methylation and acetylation to control transcription of pro-inflammatory cytokines.[24] We tested if the nanoparticles activated similar pathways in trained immunity using our in vitro assay. To do this, BMDMs were pretreated with small-molecule epigenetic and metabolic inhibitors for 30 min before exposing them to each training material in the standard 7 d BMDM training assay used in the previous experiment.[23,25] All the epigenetic inhibitors reduced trained immunity induced by free β-glucan, as reported previously—validating the assays. Histone demethylase inhibitor–pargyline hydrochloride significantly reduced training by nanoparticles, whereas histone methyltransferase inhibitor (methylthioadenosine or MTA) did not affect training. Histone acetyltransferase inhibitor (Epigallocatechin-3-gallate or EGCG) reduced nanoparticle-induced training in vitro; however, the difference was not significant ($p = 0.073$) (Figure 2d). The results confirm that the nanoparticles induce training through an epigenetic mechanism. However, the results also suggest that nanoparticle-based training operates through a slightly different overall temporal process, perhaps owing to the timing and delivery of the β-glucan. This may bear further examination in fundamental studies in the future.

Apart from epigenetics, metabolic changes also contribute to β-glucan-induced training. Dectin-1 activation by β-glucan results in phosphorylation of Akt, thereby activating the mammalian target of rapamycin (mTOR). Akt inhibitor- wortmannin reduced trained immunity mediated by free β-glucan but not by nanoparticles. Inhibiting mTOR by pretreating cells with rapamycin significantly reduced training effects in both the free β-glucan equivalent and nanoparticle groups. Trained innate cells switch from oxidative phosphorylation to glycolysis to meet the energy demands to induce epigenetic changes for higher effector function, such as secretion of proinflammatory cytokines. This process also generates metabolites that further modulate their epigenetic profile.[26,27] To test the metabolic effects of training with nanoparticles, we depleted glucose by pre-treating cells with 2-deoxy D-glucose and observed reduced training effects by all training materials tested, confirming that glycolysis is a key to trained immunity effects by nanoparticles (Figure 2e). Taken together, these results demonstrate that nanoparticles mediate trained immunity through much the same pathways targeted by free β-glucan encompassing both metabolic and epigenetic reprogramming of BMDMs. However, they show distinctive differences in how the particles may alter the pathways, perhaps in the kinetics of the overall dose of β-glucan, which may explain the resulting improvements in training observed in the preliminary in vitro results.

While β-glucan stimulates Dectin-1 and complement receptors, previous reports have shown that trained immunity mediated by β-glucan is not fully dependent on this receptor–ligand interaction.[25,43] One potential explanation for these subtle mechanistic differences observed with our nanoparticle system would be the kinetics and delivery of β-glucan to the cells. We reasoned that better uptake of nanoparticles by BMDMs might be one reason for better training in vitro compared to an equivalent amount of free β-glucan. To test this hypothesis, we labeled β-glucan with fluorescein isothiocyanate (FITC) and monitored their uptake by BMDMs at the end of 24 h, mimicking the in vitro training assay using flow cytometry. After 24 h of training, we observed higher levels of FITC+ cells in the nanoparticle trained groups than in the free equivalent group (Figure 2f and Figure S4, Supporting Information). We performed the 7 d in vitro assay by pretreating BMDMs with known inhibitors targeting caveolae and clathrin-mediated endocytosis pathways to test if endocytosis is involved.[28] We observed a significant reduction in nanoparticle-mediated training with inhibitors of both caveolae-specific endocytosis (filipin) and both caveolae and clathrin-dependent endocytosis (dynasore) (Figure 2g). Clathrin inhibitor (chlorpromazine) did not cause any change in nanoparticle-mediated training. These results strongly support that nanoparticle-induced trained immunity in vitro proceeded through increased uptake by BMDMs revealing an alternate method for inducing trained immunity.

To accurately test how controlled release might affect trained immunity in a prophylactic model, we tested the particles using an in vivo model (**Figure 3**a). Mice were trained twice with either nanoparticles or an equivalent amount of free β-glucan. To account for the potential effects of age and injection, we included an "untrained" (PBS) group. For comparison, we also included a conventional standard training regimen—1 mg of free β-glucan. To determine the increase in innate response from training, mice were then challenged intraperitoneally with a secondary stimulus—LPS, at defined time points after the training regimen. In a standard training assay, LPS challenge results in systemic pro-inflammatory cytokines (IL-6 and TNF-α). Higher cytokine response indicates better trained mice. Since the synthesized nanoparticles exhibit slow release of the encapsulated β-glucan over 30 d, we hypothesized that the training response in the nanoparticle-trained group would coincide with its release profile, peaking after 30 d of training. To test this hypothesis, we analyzed systemic cytokines following an LPS challenge at two different time points—7 and 28 d after the first training material was administered. In previous work, mice trained with standard 1 mg free β-glucan elicited peak systemic cytokines after 7 d of training.[13] At 7 d, for the β-glucan (1 mg) group, systemic cytokines following an LPS challenge were highest, matching previous work. The group trained with

conducted using an unpaired student's T-test. g) BMDMs were pretreated for 30 min with inhibitors targeting endocytosis pathways, namely dynasore (caveolae and clathrin inhibitor), filipin (caveolae inhibitor), or chlorpromazine (clathrin inhibitor). IL-6 levels were measured after an LPS challenge on day 5 with BMDMs trained with—PBS (black bar), the free equivalent amount of β-glucan (pink bar), or PLGA nanoparticles (green bar), $n = 3$, significance compared with PBS (no inhibitor) group. All values are expressed as mean ± SEM. $n = 3$ and statistics were conducted using a one or two-way ANOVA with Dunnett's multiple comparisons test (significance compared with PBS group) or student's T test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s., not significant.

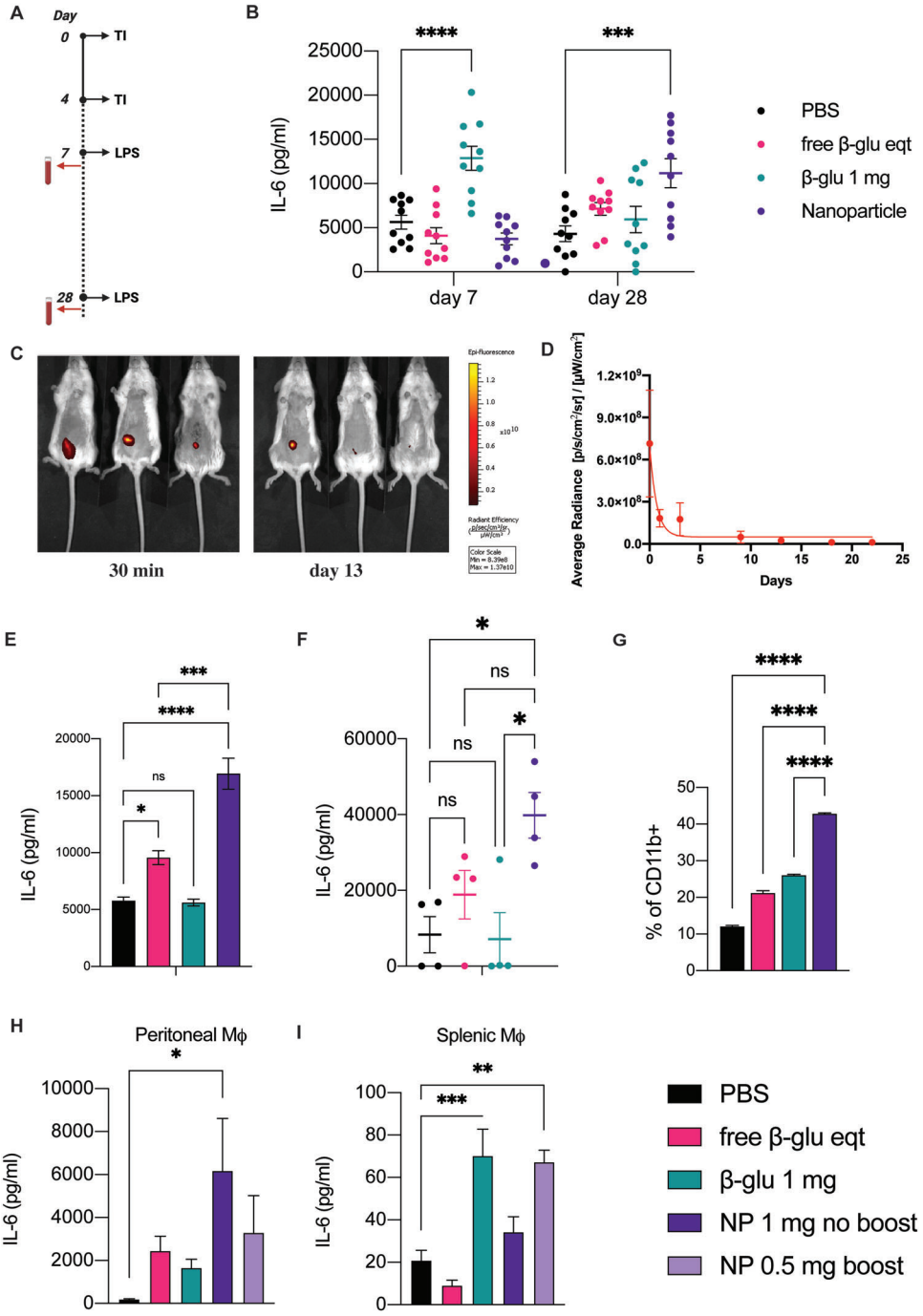

Figure 3. Altering the temporal response of trained immunity in vivo results in changes in trained macrophage populations. a) In vivo trained immunity schematic: Training materials were administered on day 0 and day four, and separate sets of mice were challenged 7 or 28 d later. Systemic cytokines were measured 1 h after the LPS challenge. b) Systemic IL-6 levels 1 h after LPS challenge on day 7 and day 28 with mice trained with PBS (black), the free equivalent amount of β-glucan (pink), 1 mg of β-glucan (green) or nanoparticles (purple), $n = 10$, significance compared with PBS (untrained) group. c) In vivo biodistribution assay of NIR-labeled PLGA nanoparticles encapsulating β-glucan at 30 min and 13 d measured by IVIS, $n = 3$. d) PLGA degradation profile in vivo measured by fluorescence intensity over time. Data were fitted to one-phase decay characteristic of reported PLGA degradation kinetics. e) Analysis of trained immunity phenotype in peritoneal macrophages isolated from mice 28 d after training. Training was administered on day 0 and day 4 with PBS (black), the equivalent amount of β-glucan (pink), 1 mg of β-glucan (green), or nanoparticles (purple). After 28 d, peritoneal macrophages were harvested and challenged ex vivo with LPS (10 ng mL^{-1}), and IL-6 levels were quantified, $n = 5$. f) Analysis of the effect of adoptive transfer of peritoneal macrophages from trained mice in conferring trained immunity phenotype in naïve mice. Training was administered on day 0 and day 4 with PBS (black), the equivalent amount of β-glucan (pink), 1 mg of β-glucan (green), or nanoparticles (purple). After 28 d, peritoneal macrophages were harvested and cultured for 1 d. 1 million peritoneal macrophages per group were adoptively transferred (i.p) to naïve mice. After 24 h, these mice were challenged with LPS, and serum cytokines were analyzed 3 h post-challenge; $n = 4$. g) Analysis of percentage of SPMs in the peritoneal cavity after LPS challenge after 7 d of training. Training was administered on day 0 and day 4 with PBS (black), the equivalent amount of β-glucan (pink), 1 mg of β−glucan (green), or

the free equivalent amount of β-glucan (35 µg per mouse) did not show any systemic responses. This result may be due to the very low dosage—only 3.5% of a responsive amount. Also, mice trained with nanoparticles at 7 d somewhat surprisingly showed no systemic response (Figure 3b). However, when challenged 28 d after the first training, nanoparticle-trained mice showed higher peak systemic responses than mice trained with 1 mg of β-glucan or the nanoparticle-free equivalent dosage. Of note, the level was nearly as high as that of the standard training at day 7 with only 3.5% as much β-glucan added. This result provided strong support for our initial hypothesis. Additionally, we observed no increase in cytokines in any category when mice were challenged with LPS 35 d after training (Figure S5, Supporting Information)—indicating the transient nature of training. To rule out innate immune priming effects, we analyzed serum cytokines before the LPS challenge on day 28. We observed no significant differences between untrained and nanoparticle-trained mice (Figure S6, Supporting Information), indicating that the system was not actively responding to previous stimulation. Taken together, these results strongly support that the controlled release from nanoparticles prolonged training, but only peak around 28 d. They did not extend training beyond a predictable time window-preventing potential uncontrolled inflammation.

A critical question remained: how long do the particles persist upon injection, and how does that timing impact the controlled training regimen? To assess the biodistribution of the synthesized nanoparticles, mice were injected intraperitoneally with NIR-labeled PLGA nanoparticles with the same formulation of encapsulated β-glucan (Table S1, Supporting Information). The injected nanoparticles localized to the peritoneal cavity 30 min after injection (Figure 3c). To confirm that nanoparticles exhibited sustained release, we monitored the fluorescence intensity emitted from the nanoparticles. Fluorescence intensity measurement was reduced to zero by three weeks post-training and followed first-order kinetics. (Figure 3c,d). After confirming the absence of residual nanoparticles, we challenged the mice with LPS and observed higher systemic cytokines from the nanoparticle-trained mice (Figure S7, Supporting Information). These results add further support to our hypothesis that it is the sustained release of β-glucan from the nanoparticles which increases the effect of training. We also confirm that peak training effects induced in the nanoparticle-trained group occurred after the nanoparticles appeared fully dispersed.

After confirming that the nanoparticles localized at the peritoneal cavity, we examined if peritoneal macrophages contributed to the response of nanoparticle-induced training when peak systemic responses were induced at day 28. To test this hypothesis, we isolated peritoneal macrophages from mice that received training material four weeks earlier. The isolated trained peritoneal macrophages were challenged ex vivo with LPS, and proinflammatory cytokines were quantified. We observed 77% higher IL-6 levels from peritoneal macrophages isolated from nanoparticle-trained mice than those isolated from unencapsulated free equivalent β-glucan trained mice (Figure 3e). We performed an adoptive transfer experiment to determine if peritoneal macrophages could act as a sole mediator of training. After 28 d of training in mice administered standard nanoparticles, the free-equivalents of β-glucan, or 1 mg of β-glucan, peritoneal macrophages were adoptively transferred to naïve mice. The recipient mice were challenged with LPS 1 d later, and systemic cytokines were quantified. Analogous to the ex vivo challenge experiment, we observed that peritoneal macrophages isolated from nanoparticle-trained mice conferred naïve mice with improved training effects against LPS challenge compared to all other groups tested (Figure 3f). Mice that received peritoneal macrophages from nanoparticle-trained mice produced a twofold increase in IL-6 compared to the group with an unencapsulated amount of free β-glucan. This result provided evidence that peritoneal macrophages are one of the primary cellular mediators of training for nanoparticle-induced training and that sustained-release platforms can target macrophages to improve training effects.

We then explored if nanoparticle-trained mice exhibit localized training effects, particularly at the administration site, i.e., the peritoneal cavity, at earlier times (one week after training). In previous work, inflammation in the peritoneal cavity was observed to induce the recruitment of monocyte-derived small peritoneal macrophages (SPMs).[9,29] Mice were trained with the same regiment of desired training materials, challenged with LPS after 7 d and their peritoneal cavity cells were harvested. We observed that mice trained with nanoparticles had much higher populations of SPMs (40%) than the free β-glucan groups (Figure 3g and Figure S8, Supporting Information), confirming that the particles induced localized effects. However, training may also modulate immune cell populations at the spleen. Analysis of splenic cell populations between nanoparticles and other free formulations revealed a slight but nonsignificant increase in the percent of macrophages in mice trained with nanoparticles. We observed no changes in other cell types like neutrophils which were upregulated in mice trained with 1 mg free β-glucan (Figure S9, Supporting Information). These results were inconclusive but indicated that the nanoparticles might exert some localized effects at the injection site, and

nanoparticles (purple). After one week, mice were challenged with LPS, and peritoneal cells were harvested 2 h later. The percentage of small peritoneal macrophages (SPMs) (CD11b+ F4/80-) was analyzed by flow cytometry, $n = 5$. h) Analysis of trained immunity phenotype in peritoneal macrophages after 7 d. Training was administered on day 0 and day 4 with PBS (black), the equivalent amount of β-glucan (pink), 1 mg of β-glucan (green), 1 mg only on day 0 (purple), or 0.5 mg nanoparticles on both day 0 and day 4 (lilac). Peritoneal macrophages were harvested on day 7 and challenged ex-vivo with LPS. Cell supernatants were analyzed for inflammatory cytokines-IL-6, $n = 5$, significance compared with PBS (untrained) group. i) Analysis of trained immunity phenotype in splenic macrophages after 7 d. Training was administered on day 0 and day 4 with PBS (black), the equivalent amount of β-glucan (pink), 1 mg of β-glucan (green), 1 mg only on day 0 (purple), or 0.5 mg nanoparticles on both day 0 and day 4 (lilac). Splenic macrophages were harvested and challenged ex-vivo with LPS. Cell supernatants were analyzed for inflammatory cytokines- IL-6. $n = 5$, significance compared with PBS (untrained) group. All values are expressed as mean ± SEM. $n = 3$–5, Statistics was conducted using one or two-way ANOVA with Dunnett's multiple comparison testing to compare groups to PBS group or Tukey's multiple comparison testing to compare all groups as indicated. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s., not significant.

further study of the localized effects of controlled release is warranted.

To explore if the dosing regimen would alter the activity and cellular makeup at the site of action in nanoparticle-induced training at earlier time points (day 7), mice were trained with either two doses of 0.5 mg spaced 4 d apart or a single dose of 1 mg of nanoparticles. In this case, both groups would ultimately release an equivalent amount of β-glucan. Yet, the distributions would differ, resulting, theoretically, in different cellular compositions in the injection site and altered training responses. After a standard training injection of either 0.5 mg of nanoparticles at intervals of 4 d or 1 mg of nanoparticles, we collected cells from the peritoneal cavity, spleen, and bone marrow. Cells were challenged with LPS ex vivo, and cytokines released into the supernatant were analyzed.[13,30,31] We observed a single dose of 1 mg nanoparticles resulted in better training in peritoneal macrophages. (Figure 3h) However, two doses of 0.5 mg of nanoparticles resulted in better-trained immunity effects in splenic macrophages (Figure 3i). No significant differences were observed in bone marrow-derived macrophages (BMDMs) harvested from nanoparticle trained mice, confirming that the site of action was restricted to the peritoneal cavity and spleen at earlier time points (Figure S10, Supporting Information). These results indicate that the dosing regimen of nanoparticles could provide more spatial control over the site of training at earlier time points. Taken together, these results indicate that the training with nanoparticles occurs mainly within the local tissue but can be altered via dosing and distribution. Future therapeutic approaches might take that timing and dose into consideration. However, further experiments are needed to evaluate the extent of local versus systemic training thoroughly.

Trained immunity has been proposed and demonstrated to be useful in both infectious diseases and immune therapy. In initial experiments, we explored how altering the temporal persistence of trained immunity could be applied to cancer immunotherapy owing to the potential to generate a long-lived response. Generating durable responses against tumor growth is an oftcited goal to sustain therapeutic effects. We tested to see if nanoparticle-trained mice would provide greater resistance to a tumor challenge owing to its sustained release. Using a similar setup to previous experiments, mice were injected intraperitoneally, either with nanoparticles containing β-glucan, an equivalent amount of unencapsulated free β-glucan, or a standard training dose of β-glucan at 1 mg. In this experiment, injections were given twice, 4 d apart, indicated as day 0 and day 4. Mice were challenged with B16.F10 tumors three weeks (day 21) after the training. Tumors take about a week to form a visible mass (day 28) which we had previously measured to be the time by which nanoparticles elicited complete release of encapsulated β-glucan (**Figure 4**a). Measuring tumor volume, we observed that the nanoparticle-trained mice significantly resisted tumor growth compared to the free β-glucan trained groups (Figure 4b). Looking for a mechanism, an analysis of the tumor-infiltrating innate immune cells at the end of the experiment revealed increased neutrophils and macrophages in the tumor microenvironment, confirming that the antitumor effects in nanoparticle-trained mice were due to changes in the tumor microenvironment[32] (Figure 4c–e and Figure S11, Supporting Information). In previous work, β-glucan was shown to skew TAMs into an inflammatory M1 phenotype and train neutrophils to improve tumor resistance.[33–35] From this preliminary examination, we conjecture that the nanoparticles may sustain this same result for a more extended period, resulting in the observed phenotype. Additionally, spleen weights corresponded with tumor size as well—splenomegaly was observed for both the PBS and 1 mg free β-glucan groups (Figure S12, Supporting Information). Beyond this model, we also tested the anti-tumor effects of the nanoparticles in an EG7.OVA tumor model. We observed similar effects with nanoparticle-trained mice exhibiting the highest tumor resistance (Figure S13, Supporting Information). These results suggest that sustained-release training platforms could potentially be combined with existing anticancer therapies, including checkpoint blockade, to prolong protection as they provide an alternate method to influence the tumor microenvironment.[36]

One of the well-established properties of PLGA is its control over the release kinetics of encapsulated cargo.[19] Increasing the molecular weight of PLGA decreases diffusion of the encapsulated cargo, thereby prolonging the duration of release and vice versa.[20,37] We hypothesized that this property might be uniquely suited to improve temporal control of trained immunity. Additionally, PLGA end groups can also affect release kinetics—carboxylic acid end groups degrade faster than those with ester end groups.[38] To test this hypothesis, we encapsulated β-glucan using two different molecular weight PLGA polymers of molecular weights ranging from 7 to 17 kDa and 35 – 55 kDa. We also used PLGA with two different end groups—acid terminated and ester terminated. For simplicity, the formulations are hereby categorized as low acid/ester and high acid/ester, respectively. SEM characterization revealed a spherical morphology for all the PLGA nanoparticles. Encapsulation efficiency was in the range of 41–72% for all the synthesized nanoparticles (Figure S14 and Table S1, Supporting Information). In vitro release data confirmed that nanoparticles composed of smaller molecular weight PLGA degraded faster than higher molecular weight PLGA nanoparticles (**Figure 5**a). Because our experiment was solely focused on the temporal control of the training and not on inducing a specific magnitude of training, we proceeded with this level of control over loading and release.

To study the kinetics of training, we tested the different molecular weight nanoparticles in an in vivo model with a time-course analysis of serum cytokines following an LPS challenge (Figure 5b). Mice were trained as previously described with the various molecular weight nanoparticles and challenged with LPS at increasing time points—7, 21, 25, or 30 d after the first training. We observed peak systemic IL-6 responses at day 7 for both the acid and ester terminated low molecular weight nanoparticles. By day 21, the high molecular weight acid terminated group demonstrated significantly higher systemic IL-6 levels after LPS challenge. However, the high molecular weight ester terminated group peaked at day 25 (Figure 5c) suggesting that controlled release could be achieved by modulating both molecular weight as well as polymer end groups used. Continued analysis through day 30 confirmed the absence of sustained inflammatory responses (Figure S15, Supporting Information). These results further supported our hypothesis that fine-tuning the properties of sustained-release platforms can be used for precise temporal control over the duration of trained immunity. These results suggest

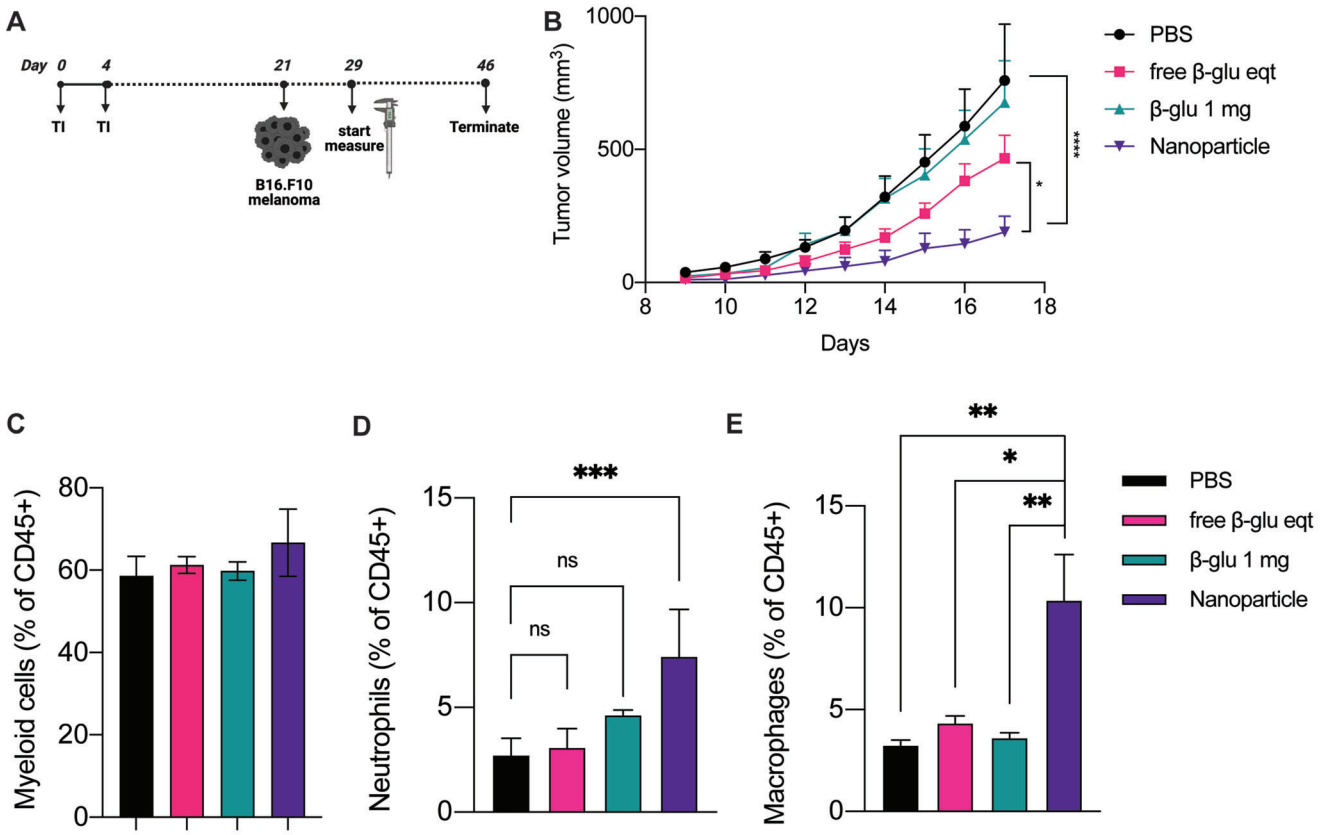

Figure 4. Tumor challenge. a) Experimental scheme for B16.F10 tumor challenge (created with BioRender.com). b) Mice were trained on day 0 and day 4 with the indicated training materials- PBS (black bars), the free equivalent amount of β-glucan (pink bars), 1 mg β-glucan (green), or PLGA nanoparticles encapsulating β-glucan (purple bars). Mice were then challenged subcutaneously with B16.F10 tumor cells. Tumor volume was recorded after the tumors were palpable—day 29 onwards. c) Tumors were excised at the end of the experiment on day 46, and the percentage of myeloid cells (Cd45+Cd11b+) was analyzed using flow cytometry. d) Tumors were excised at the end of the experiment on day 46, and the percentage of neutrophils (Cd45+Cd11b+Ly6g+) was analyzed using flow cytometry, significance compared to the PBS group. e) Tumors were excised at the end of the experiment on day 46, and the percentage of macrophages (Cd45+Cd11b+Ly6g-F4/80+) was analyzed using flow cytometry, significance compared to the PBS group. Statistics was conducted using one-way ANOVA with Dunnett's multiple comparison testing to compare groups as indicated. All values are expressed as mean ± SEM. $n = 5$,*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, n.s., not significant.

that with different formulations, it is possible to partially control the timing of training and lengthen or shorten the window over which higher innate responses are induced via training. Controlled training is an important step to preventing potential over-inflammatory or chronic-inflammatory responses which might cause adverse reactions.

3. Conclusion

Trained immunity improves the innate immune response to pathogenic challenge and tumor burden. However, current methods only provide transient approaches to induce training due to the short lifespan of the trained cells. While methods to direct training at the bone-marrow level ensure long-term effects, this approach suffers from the potential disadvantage of inducing long-term adverse inflammatory responses. Precise control of timing and dosage of innate immune cell activation may provide a method to improve training duration while managing unwanted adverse reactions due to prolonged activation.

We report on a nanoparticle-based sustained delivery platform for temporal control over training. Controlled release of training material, β-glucan, resulted in both more sustained and higher training than a comparable injection of standard-dose β-glucan or an equivalent amount of β-glucan to that in the particle. Of note, only 3.5% of the standard dose of β-glucan was incorporated into the nanoparticles. We observed that particle training proceeded through similar pathways and mechanisms as free β-glucan training with the potential difference that some kinetic elements are altered. These results indicate that the enhanced training effect resulted mainly from the altered timing of the release. We further studied the pharmacokinetics by examining the release in animals using IVIS imaging, concluding that sustained release occurred for at least the first 14 d. This altered and improved training resulted in differences in the location of training and the degree of trained cells in the peritoneal cavity and spleen, demonstrating that control of cell training can be achieved through material programming.

To demonstrate the potential of temporal control for trained immunity, we also demonstrated that training induced by

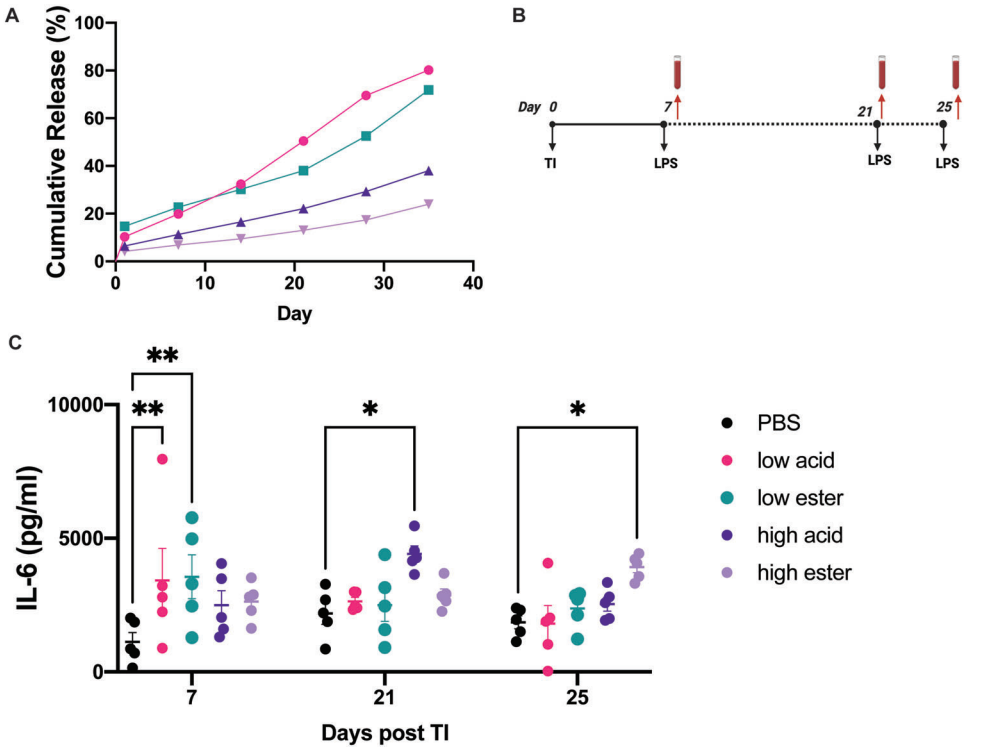

Figure 5. Modulating PLGA properties for temporal control of trained immunity. a) 7 d in vitro release profile of low molecular weight ester-terminated PLGA nanoparticles (green), high molecular weight acid-terminated nanoparticles (purple), or high molecular weight ester-terminated nanoparticles (lilac) at pH 7.4, $n = 3$. b) In vivo experimental protocol (created with BioRender.com). c) Mice were trained with either PBS (black) low molecular weight acid-terminated PLGA nanoparticles (pink), low molecular weight ester-terminated PLGA nanoparticles (green), high molecular weight acid-terminated nanoparticles (purple), or high molecular weight ester-terminated nanoparticles (lilac) on day 0. Different sets of mice ($n = 5$) were challenged with LPS on the indicated days (7, 21, or 25), and systemic cytokines were quantified. Significance compared with PBS group. All values are expressed as mean ± SEM. $n = 3$–5 and statistics were conducted using a two-way ANOVA with Dunnett's multiple comparisons test,*$P < 0.05$ and **$P < 0.01$.

nanoparticles improved tumor resistance in mice than those trained with free β-glucan. This approach can be potentially used in conjunction with current immune therapies such as checkpoint blockade therapies to prolong antitumoral effects and prevent recurrence. Finally, to show the potential of this technology for future application, we showed that different training windows could be achieved with different molecular weights and end groups of nanoparticle formulation PLGA. This novel nanoparticle-based delivery platform reduces bulk inflammation and provides effective temporal control of trained immunity.

In this study, we focused on an intraperitoneal route of training that has been extensively explored in the field. The use of PLGA-based delivery platforms makes it easy to translate to other clinically relevant routes of training to target specific tissues that are key to disease resistance.[39] For example, detailed aerosolized techniques for PLGA nanoparticle delivery have been documented and explored to target pulmonary macrophages [40] that are important in resisting respiratory diseases. Trained immunity also enhances antitumor effects, and surface modification of PLGA with suitable ligands can help target specific tumors such as those affecting the pancreas.[44,45] Similarly, local release platforms can be leveraged to decrease trained immunity to decrease allograft rejection for prolonged periods of time. For example, myeloid cell targeting high-density lipoprotein nanobiologics delivered intravenously were recently shown to reduce training to ensure prolonged allograft survival in a heart transplant model in mice.[14] It is also important to eliminate training after achieving the desired outcome to reduce unwanted inflammation.[17] Taken together, our nanoparticle-based delivery system offers two advantages. First, our platform enables easy surface modifications to achieve targeted delivery. Secondly, our sustained release formulations utilize significantly less β-glucan that releases slowly over time thereby eliminating any adverse immunostimulation. We present this work as its potential for biomaterials and formulations methods employed in drug delivery to modulate and improve trained immune responses. This work can potentially lead to the development of novel, safe, and effective prophylactics for improved resistance against a variety of diseases.

4. Experimental Section

HEK mTLR4, B16F10, and EG7.OVA cells were obtained from InvivoGen. All cell culture reagents were obtained from Thermo Fisher Scientific. mTLR4 cells were cultured in DMEM supplemented with 10% FBS and selective antibiotics. Cells were maintained at 37 °C and 5% CO2. C57Bl/6J mice were obtained from Jackson Laboratories and acclimatized for 1 week prior to experimentation. All animal experiments were conducted with approval from the University of Chicago Institutional Animal Care and Use Committee (approval number 72517). All statistical analyses were performed using GraphPad Prism.

Formulation of Nanoparticles: β-glucan was purchased from Sigma (G5011). The payload was added in 200 µL to 2 mL of 100 mg mL^{-1} of PLGA in methylene chloride. The mixture was ultrasonicated at 40% amplitude for 3 minutes (30 seconds stirring following 30 seconds of rest). After ultrasonification, a determined amount of 5% (w/v) PVA solution was added to the primary w/o emulsion and ultrasonicated for 60 s to produce the w/o/w double emulsion. The w/o/w emulsion was poured into 200 mL of chilled 1% (w/v) PVA and stirred at room temperature for 6 h. The microspheres were centrifuged at 4000 rpm for 40 min and the pellet was washed three times with ultrapure deionized water and then lyophilized.

Characterization of Nanoparticles: For SEM imaging, nanoparticles were first sputter coated with 12 nm of Pt/Pd using Sputter Coater—Cressington 208HR. SEM images were taken with Carl Zeiss Merlin SEM at 3.0 kV. Particles were immersed in PBS at 1 mg mL^{-1} concentration for DLS measurements. Measurements were taken with 10 acquisitions with 5 s for each acquisition.

In Vitro Release: Graham vials containing 20 mg of β-glucan nanoparticles were treated with 0.5 mL of pH 7.45 PBS buffer. The vials were gently stirred at 90 rpm at 37 °C. The particle solution was collected by centrifuging the vials at 4000 rpm for 5 min and stored at -20 °C. The β-glucan concentration was determined by using a total carbohydrate assay kit (Millipore Sigma).

BMDM Harvest and Culture: Bone marrow-derived macrophages (BMDMs) were harvested from the femurs of 6 week old C57BL/6 mice (Jackson Laboratory). BMDMs were cultured in primary medium: RPMI 1640 (Life Technologies), 10% heat inactivated fetal bovine serum (HIFBS) 2×10^{-3} M L-glutamine (Life Technologies), antibiotic antimycotic (1×) (Life Technologies), and 10% MCSF (mycoplasma free L929 supernatant) for 5 d at 37 °C and 5% CO_2. The cells were then released with 5×10^{-3} M EDTA in PBS, counted and plated at desired densities.

In Vitro Training Assays: BMDMs were plated at a density of 100 000 cells per well in flat bottom 96-well plates (Corning) at a final volume of 200 µL) and rested for a few hours to adhere at 37 °C and 5% CO_2. After the cells were adherent, training material was added at the desired concentration and incubated for 24 h (Nanoparticle concentration used was 100 µg mL^{-1} unless otherwise stated). Then cells were washed and rested for 3 d. On day 4, BMDMs were washed again and primed with 25 ng mL^{-1} IFN-γ (BD Biosciences) for 24 h. On day 5, a final wash was performed, and cells were stimulated with 10 ng mL^{-1} standard *Escherichia coli* LPS (serotype O55:B5; Invivogen). Cell supernatant was collected after 24 h to measure IL-6 and TNF-α levels using ELISA (BioLegend) according to manufacturer's instructions.

For inhibition studies, BMDMs were preincubated for 30 min with inhibitors before stimulating with training materials and were left in the media during the 24 h training period.[23] For epigenetic pathway analysis, cells were pretreated with 500×10^{-6} M 5′-deoxy-5′-(methylthio)adenosine (MTA), 6×10^{-6} M pargyline and 50×10^{-6} M (-)-epigallocatechin-3-gallate (EGCG) (all from Sigma Aldrich). For metabolic pathway analysis, cells were pretreated with 100×10^{-9} M rapamycin (Invivogen), 10×10^{-3} M Wortmannin, 30×10^{-3} M Metformin and 1×10^{-3} M 2' deoxyglucose (all from Sigma). For testing different pathways contributing to endocytosis, cells were pretreated with 5×10^{-3} M Filipin (Sigma), 100×10^{-3} M Dynasore (Abcam) and 3×10^{-3} M chlorpromazine hydrochloride (Sigma).

To quantify uptake, β-glucan was labeled with fluorescein isothiocyanate (FITC) using a previously described protocol.[40,41] BMDMs were treated with an equivalent amount of free FITC-labeled β-glucan or 100 µg mL^{-1} nanoparticles encapsulating FITC-labeled β-glucan for 24 h. Then, media was removed and cells were released by gentle scraping and the cells were analyzed by flow cytometry (NovoCyte Benchtop Flow Cytometer).

After in vitro training assay, the cells were incubated in 100 mL PBS and 10 mL of cell counting kit-8 (CCK-8) solution was added to each well and incubated at 37 °C. Absorbance was measured at 540×10^{-9} M using Multiskan FC plate reader (Thermo Scientific).

HEK mTLR4 cells were passaged and plated in a 96-well plate at 100 000 cells per well in 180 µL DMEM containing 10% HI-FBS and selective antibiotics. The cells were stimulated with the synthesized nanoparticles for 24 h at 37 °C and 5% CO_2. TLR4 binding was measured by a QUANTI-Blue (Invivogen) assay and the absorbance was measured at 620 nm using a Multiskan FC plate reader (Thermo Scientific).

In Vivo Training Assay: Mice were trained intraperitoneally either once or twice on days 7 and/or day 4 with the indicated amount of nanoparticles, an equivalent amount of free β-glucan, or 1 mg free β−glucan. Sterile PBS was always used as a control. After training, different set of mice were challenged intraperitoneally with 5 µg LPS (serotype O55:B5; Invivogen) at desired time points and serum was collected after 1 or 3 h. None of the mice received repeated LPS challenges. Serum cytokines were analyzed using Legendplex Mouse Inflammation Panel (BioLegend) according to the manufacturer's protocol. 2 h after LPS challenge, mice were euthanized and peritoneal cells were harvested by lavage. Cells were flow cytometry analysis was performed to determine the percentage of small peritoneal macrophages. All antibodies were purchased from BioLegend or BD Biosciences. Anti-mouse phycoerythrin (PE) CD11b, APC- MHC2, Pe/Cy7-F4/80, Brilliant violet 570- Ly6G, APC/fire 750-CD11c, Alexa fluor 700- Ly6C, APC- NK1, PE-cd115, PerCP/Cy5.5- cd19, Alexa flour 488- CD3, Brilliant violet 788- MHC2, Brilliant violet 711 CD8a, Brilliant violet 650-F4/80, BUV 395 CD45R/B220. In other experiments, spleens and peritoneal exudate cells were isolated prior to LPS challenge. Spleens were homogenized, and cells were filtered through a 70 µm strainer. Red blood cells were lysed by incubating with ACK Lysing Buffer for 5 min at 25 °C. Cells were plated in Petri dishes for 2 h and washed to remove nonadherent cells. Peritoneal cells were harvested by lavage according to the previously described protocol.[40] Briefly, the skin was peeled off and clamped, followed by injection of 5 mL PBS containing 3% heat-inactivated fetal bovine serum (HIFBS) into the peritoneal cavity. The peritoneal content was then collected and centrifuged (400 g for 10 min). Peritoneal cells were plated in Petri dishes for 2 h and washed to remove non-adherent cells. Peritoneal and splenic macrophages (0.1 million cells) were plated in a 96-well plate and allowed to adhere for 2 h before being challenged with LPS.

Training was administered on day 0 and day 4 with the desired material. After 28 d, mice were euthanized and cells were harvested by peritoneal lavage. The peritoneal macrophages were split for two sets of experiments. In one set, 10 0000 cells were plated in 96 well flat bottom plates in a final volume of 200 µL. These cells were allowed to adhere for 3 h and then challenged with LPS (10 ng mL^{-1}). Supernatant was collected after 24 h and IL-6 levels were quantified. In a second set of experiments, 1 million peritoneal macrophages per group were adoptively transferred (ip) to naïve mice. After 24 h, these mice were challenged with LPS (5 µg per mouse) and serum cytokines were analyzed 3 h postchallenge using Legendplex Mouse Inflammation Panel (BioLegend).

To determine the biodistribution, nanoparticles were synthesized using NIR-labeled PLGA (Poly(lactide-co-glycolide)-Flamma Fluor near-IR, Millipore Sigma). Fluorescence intensity was measured using Xenogen IVIS 200 Imaging System at F stop = 2, 1 s Exposure, Ex = 745 nm, and Em = 800 nm.

Tumor Challenge: Mice were anesthetized and shaved of hair from their right-side using clippers. Following grooming, the mice were injected with 200000 B16F10 melanoma cells or 1 million EG7OVA cells in 50 mL of PBS subcutaneously. For B16.F10 tumor model, mice were first trained with indicated materials and rested for three weeks. After three weeks, all the animals were challenged with B16.F10 tumor. For EG7.OVA tumor model, mice were trained first and were vaccinated with CpG (50 µg) and OVA (100 µg) a week later. Tumor cells were injected two weeks after vaccination. Tumor progression was monitored and measured with a caliper, recording the width, length, and height of the tumor every other day throughout the experiment. Mice with tumor sizes exceeding 20 mm along any direction were sacrificed.

Statistical Analysis: Preprocessing was not performed for any of the data. All values are expressed as mean ± SEM. Sample size is as indicated in figure captions in all in vivo and in vitro experiments. Student's T-test was applied for comparing two groups and one- or two-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's multiple comparisons for comparison of multiple groups (indicated in the figure captions) using the GraphPad Prism 9 software. *P* values less than 0.05 were considered statistically significant. Significance $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, n.s., not significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

innate immunity, nanoparticles, poly(lactic-co-glycolic acid), prophylactics, sustained release

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