In vivo characterization of the physicochemical properties of polymer-linked TLR agonists that enhance vaccine immunogenicity

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The efficacy of vaccine adjuvants such as Toll-like receptor agonists (TLRa) can be improved through formulation and delivery approaches. Here, we attached small molecule TLR-7/8a to polymer scaffolds (polymer–TLR-7/8a) and evaluated how different physicochemical properties of the TLR-7/8a and polymer carrier influenced the location, magnitude and duration of innate immune activation in vivo. Particle formation by polymer–TLR-7/8a was the most important factor for restricting adjuvant distribution and prolonging activity in draining lymph nodes. The improved pharmacokinetic profile by particulate polymer–TLR-7/8a was also associated with reduced morbidity and enhanced vaccine immunogenicity for inducing antibodies and T cell immunity. We extended these findings to the development of a modular approach in which protein antigens are site-specifically linked to temperature-responsive polymer–TLR-7/8a adjuvants that self-assemble into immunogenic particles at physiologic temperatures in vivo. Our findings provide a chemical and structural basis for optimizing adjuvant design to elicit broad-based antibody and T cell responses with protein antigens.

Although vaccines that mediate protection through antibodies are in routine clinical use1, vaccines that generate robust and durable T cell immunity are still needed for protection against certain infections (such as malaria2 and tuberculosis3) and as therapies for cancer4. One means of improving T cell immunity to vaccines is through the rational choice of adjuvants formulated with defined protein or peptide antigens5. Some of the most effective adjuvants for promoting T cell immunity are Toll-like receptor agonists (TLRa), which stimulate distinct populations of antigen-presenting cells (APCs), particularly dendritic cells (DCs), to present antigen, express co-stimulatory molecules and produce selective cytokines that drive T cell responses6.

The formulation and delivery of TLRa play a critical role in influencing their in vivo activity when used with protein-based vaccines7–9. Accordingly, linking TLRa directly to protein antigen10–12 and co-administering protein with particle-based carriers9,13–17 of TLRa have each been shown to markedly improve both T cell and antibody responses. Recent reports show that the ability of formulations to spatially restrict TLRa activity to the sites of vaccine administration and draining lymph nodes are critical for enhancing antibody and T cell-mediated protection18,19. The capacity of particulate delivery platforms to target distinct APC populations in lymph nodes on the basis of size has been studied20–23, but it is not clear how these and other physical and chemical properties of TLRa delivery influence the location, magnitude and duration of innate immune activation to enhance T cell immunity to protein vaccines in vivo.

Here, we investigated how delivery of TLRa on biocompatible polymer scaffolds can modulate innate immune activation in vivo to enhance T cell immunity to protein antigens. Small molecule agonists of endosomally localized TLR-7 and TLR-8 (TLR-7/8a) were selected as model TLRa for these studies because they have been shown in both humans24 and mice6–25 to activate all major DC subsets and macrophages and induce the production of specific cytokines (IL-12 and type I interferons (IFNs)) that promote T cell immunity26. In terms of other TLRa to consider as adjuvants, while the TLR-9a CpG has been used as a model adjuvant for vaccine formulations in a number of pre-clinical mouse studies16,18, the more restricted expression of TLR-9 to pDCs and B cells in humans6 may limit its effectiveness for inducing T cell immunity when used in protein subunit vaccines in patients. In contrast, formulations with combined TLR-7/8a may be potentially more clinically translatable for enhancing T cell immunity due to the

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broader expression of TLR-7 and TLR-8 across all major human APC subsets. Although the US Food and Drug Administration’s approval of two imidazquinoline-based TLR-7/8a (imiquimod and resiquimod (R848)) for treating skin pathologies provides evidence to support their safety and efficacy as immunotherapies in humans, the unfavorable pharmacokinetic properties of these agonists for use in vaccines reduces their effectiveness for inducing adaptive immunity and gives rise to systemic toxicity. To improve their pharmacokinetics and evaluate the ways that various properties of delivery influence their activity for use in vaccines, we linked TLR-7/8a to polymers (creating molecules referred to as Poly-7/8a) in a combinatorial process and evaluated how different physiochemical properties, such as the density of TLRa arrayed on polymers and inducible particle formation, influenced innate immune activation, adjuvant distribution, kinetics and uptake by different APCs in vivo. The data show that particle formation by Poly-7/8a is critical for enhancing the magnitude and duration of innate immune activation in draining lymph nodes while reducing systemic distribution and toxicity. The improved activity of particle-forming Poly-7/8a was associated with TH1 CD4+ and CD8+ T cell-mediated protection in two infectious challenge models and enhanced TH1-skewed antibody responses. To extend these findings and develop an approach that combines the benefits of soluble antigens and adjuvants—high chemical definition and stability—with the superior activity of particulate vaccines, we developed temperature-responsive polymers co-delivering TLR-7/8a and protein antigen that exist as water-soluble molecules in vitro at room temperature but undergo temperature-dependent particle formation in vivo. This report highlights the flexibility and chemical tunability of using polymers to modulate delivery of antigen and TLRa so as to enhance innate and adaptive immunity in vivo.

RESULTS

In vivo screening of polymer–TLR-7/8a conjugates

The first studies focused on optimizing the delivery of small molecule TLR-7/8a (Supplementary Fig. 1a) on polymer carriers. We chose HPMA-based polymers (Supplementary Fig. 1b) as scaffolds on the basis of their demonstrated safety in humans and because they provide a modular platform for delivering bioactive molecules. To evaluate how properties such as the density of TLR-7/8a arrayed on the polymer or the chemical composition (hydrophobic versus hydrophilic, longer or shorter) of the linkers anchoring the agonist to the polymer backbone (Supplementary Fig. 1a) influence biological activity, we generated a library of different Poly-7/8a (Fig. 1a,b and Supplementary Fig. 1b) through combinatorial synthesis (Supplementary Fig. 2) and then screened them in vivo. As the hydrocarbon backbone of HPMA polymers is nonbiodegradable, Poly-7/8a conjugates were kept at or below the previously reported glomerular filtration limit (~45 kDa) to facilitate renal excretion.

Increasing the density of TLR-7/8a arrayed on polymers resulted in substantially higher lymph node production of cytokines critical for priming T(H1) CD4+ and CD8+ T cell immunity (IL-12, IP-10 and IFNs), even though the same total dose of TLR-7/8a (12.5 nmol) was administered (Fig. 1c,d and Supplementary Figs. 3 and 4a–d). Notably, the enhanced in vivo activity observed for increasing densities of TLR-7/8a was associated with Poly-7/8a assemblage into particles in aqueous conditions (Fig. 1d,e). Whereas Poly-7/8a with low to intermediate agonist densities (1–4 mol% TLR-7/8a) exist predominantly as unimolecular polymer coils (PC, ~10–20 nm diameter) and induce no measurable cytokine production in vivo, Poly-7/8a with high agonist density (8–10 mol% TLR-7/8a) assemble into submicron polymer particles (PP, ~700-nm diameter) and induce substantially higher lymph node cytokine production than the unformulated small molecule TLR-7/8a (SM 7/8a) and Poly-7/8a with low agonist density (1–2 mol% 7/8a) that exist as unimolecular polymer coils (Fig. 1d,f and Supplementary Fig. 4a–d). These data show that particle formation, increasing densities of TLR-7/8a on the polymers, or both are critical for in vivo cytokine responses by Poly-7/8a. Such innate cytokine production by particle-forming Poly-7/8a is dependent on TLR-7 and independent of caspase 1/11 signaling, a major inflammasome pathway that is activated by certain particulate delivery systems (Supplementary Fig. 4e,f).

Particulate Poly-7/8a promotes local retention and APC uptake

Cytokine production by lymph node APCs depends on the capacity of TLR-7/8a to access endosomally localized receptors within these cells. However, the relative efficiency of TLR-7/8a delivery for targeting cells by different carrier morphologies is presently unknown. Thus, we assessed how the morphology of the TLR-7/8a carrier, either small molecule (SM 7/8a), polymer coil (PC-7/8a) or particle-forming polymer (PP-7/8a), influences uptake by immune cells in lymph nodes draining the site of immunization following their subcutaneous administration in the hind footpads of mice. All TLR-7/8a constructs were fluorescently labeled (Supplementary Fig. 5a) to facilitate in vivo tracking. PC-7/8a and PP-7/8a were prepared with the same density of TLR-7/8a, while an inert hydrophobic ligand was attached to PP-7/8a to induce particle formation (Supplementary Figs. 4a,b and 5a). Whereas the unformulated SM 7/8a exhibited systemic distribution (Fig. 2a) and rapid clearance (Fig. 2b), both PC-7/8a and PP-7/8a were primarily focused at the injection site (Fig. 2a) and persisted in draining lymph nodes for up to 20 d (Fig. 2b). Moreover, PP-7/8a led to ~400- and 4-fold higher levels of TLR-7/8a (area under the concentration-time curve, AUC) in draining lymph nodes as compared with SM 7/8a and PC-7/8a, respectively (Fig. 2b).

Analysis of lymph node sections by confocal microscopy (Fig. 2c) provided additional spatiotemporal resolution by revealing that PP-7/8a localized primarily within APCs situated in or near the medullary and cortical lymphatic sinuses of draining lymph nodes on day 1, but was also associated with a large influx of migratory APCs in the T cell zone at later time points (days 4 and 8). In contrast, only a limited amount of PC-7/8a was detectable and localized with APCs in the lymph node periphery. The SM 7/8a was undetectable in all lymph node sections (Fig. 2c), confirming that the low-molecular-weight agonist was rapidly eliminated (Fig. 2b).

Further assessment of specific lymph node APC populations by flow cytometry (Supplementary Fig. 5b,c) showed that PP-7/8a leads to a substantially greater (>10-fold) influx of CD11c+ DCs and macrophages/monocytes (CD11c+CD11b+Ly6F4/80+) as compared with SM 7/8a and PC-7/8a (Fig. 2d). Furthermore, although PP-7/8a and PC-7/8a localized in draining lymph nodes with similar proportions of total DCs (~40–60%) and macrophages/monocytes (~60–80%) (Fig. 2e), the relative amount of material taken up by APCs on a per-cell basis was markedly higher (~10–20-fold) for PP-7/8a as compared with PC-7/8a (Fig. 2f).

Further stratification of DC subsets revealed uptake of PP-7/8a by monocyte-derived DCs (CD11c+CD11b+Ly6F4/80+CD8−CD202+) as well as the mixed population of migratory and lymph node–resident CD8− DCs (CD11c+CD11b+Ly6F4/CD8−B220+) and lymph node–resident CD8+ DC (CD11c+CD11b+CD8+B220+) populations at day 1, whereas the CD11c+CD8−B220+ population enriched for migratory APCs accounted for the majority of uptake at later time points (Supplementary Fig. 5d). These findings are consistent with...
Figure 1  Increasing densities of TLR-7/8a arrayed on polymer carriers is associated with particle formation and enhanced lymph node cytokine production. (a) Poly-7/8a were generated by reacting linear biocompatible polymers with nucleophilic TLR-7/8a. (b) Combinatorial synthesis was used to generate Poly-7/8a with varying linker-group composition and TLR-7/8a density. The density of agonist arrayed on the polymers (mol% 7/8a) is reported as the percentage of monomers that are linked to TLR-7/8a (for example, 10 mol% 7/8a indicates 10 out of 100 monomers are linked to TLR-7/8a). (c) Different Poly-7/8a (12.5 nmol) or controls (HPMA polymer alone or small molecule TLR-7/8a (SM 7/8a)) were subcutaneously administered into hind footpads of mice. After 24 h, lymph nodes draining the site of immunization were harvested and processed to generate a cell suspension that was cultured for 8 h and then evaluated for the presence of IL-12p40 (n = 4 per group). (d) Polymer controls (see Supplementary Fig. 4a,b), SM 7/8a or Poly-7/8a with increasing densities of TLR-7/8a (normalized to 12.5 nmol TLR-7/8a) were evaluated for particle formation by dynamic light scattering (n = 3) and the capacity to induce IP-10 (n = 6 per group) and IL-12p40 (n = 6 per group) cytokine production in draining lymph nodes at 24 h and 96 h, respectively, after administration. (e) Size-distribution plots from dynamic light scattering are shown for selected samples; a confocal microscopy image is shown for a Poly-7/8a with 10 mol% 7/8a that forms particles in aqueous conditions. (f) Data from d for which Poly-7/8a with 1–2 mol% 7/8a that exist as unimolecular polymer coils (PC-7/8a) or Poly-7/8a with 8–10 mol% 7/8a that exist as submicron polymer particles (PP-7/8a) are grouped together to correlate the effect of Poly-7/8a morphology with lymph node IP-10 (n = 12 per group) and IL-12p40 (n = 12 per group). In vivo screens are representative of two independent experiments. Data on linear axes are reported as mean ± s.e.m. Data on log scale are reported as geometric mean with 95% confidence interval (CI). Comparison of multiple groups for statistical significance was determined using Kruskal-Wallis ANOVA with Dunn’s post hoc test; Student’s t-test was used for comparison of two groups; ns, not significant (P > 0.05); **P < 0.01. SM, small molecule; PC, polymer coil; PP, polymer particle.
PC-7/8a across a range of molecular weights (5–300 kDa) were still inactive (data not shown). Altogether, these results show that particle formation by Poly-7/8a is critical for mediating local DC activation and cytokine production.

Increasing agonist density (3–10 mol% 7/8a) on PP-7/8a administered at the same dose of TLR-7/8a (62.5 nmol) led to higher expression of co-stimulatory molecules by macrophages/monocytes (Fig. 3b) and certain DC subsets (Supplementary Fig. 6b), as well as more persistent cytokine production (Fig. 3c). Furthermore, using PP-7/8a either at different doses of TLR-7/8a (1–62.5 nmol) or with a ~20-fold more potent agonist (PP-20x7/8a, Supplementary Fig. 2a,b) led to higher-magnitude and more persistent (>8 d) cytokine responses (Fig. 3d). Consistent with recent in vitro findings that the C4 amine can be blocked to delay onset of immune activity36, we also found that reversing the orientation of TLR-7/8a on the polymers delayed the onset and reduced the magnitude of lymph node cytokine responses (Supplementary Fig. 7a–c).
Based on the striking increase in innate immune activation by PP-7/8, CD8+ T cell and antibody responses were assessed following co-administration of the different adjuvant formulations with a model protein antigen, ovalbumin (OVA). After two immunizations, the magnitude of CD8+ T cell responses (Fig. 3e,f) was concordant with that of lymph node cytokine responses (Fig. 3c,d). PP-7/8a with high TLR-7/8a density (10 mol% TLR-7/8a) elicited substantially higher CD8+ T cell responses than were seen in all other groups (Fig. 3e). Moreover, increasing either the dose of TLR-7/8a delivered with PP-7/8a or the potency of the agonists attached to PP-7/8a resulted in significantly increased magnitude of CD8+ T cell responses (Fig. 3f). In assessing humoral immunity, although the magnitude of anti-OVA total IgG antibody responses was comparable between the different Poly-7/8a formulations (Fig. 3g), increasing density, potency and dose of TLR-7/8a delivered on polymer particles was associated with strikingly more biased antibody class switching to IgG2c (Fig. 3h,i and Supplementary Fig. 8a–d), consistent with the persistence of local IL-12 and IFN production driving the skewing of antibody responses to isotypes associated with T111 immunity.

Particulate formulation of other TLRa limits systemic toxicity

The local and systemic innate immune responses induced by PP-7/8a were then benchmarked against two commercially available TLRa, a small molecule TLR-7/8a, R848 (Resiquimod), and a TLR-9 agonist, CpG (ODN 1826). Whereas the low-molecular-weight agonist, R848, induced only systemic (serum) cytokines (Fig. 4a and Supplementary Fig. 9a), the PP-7/8a induced predominantly local (lymph node) cytokine production (Fig. 4b and Supplementary Fig. 9b). In contrast, CpG induced high levels of both local and systemic cytokine production (Fig. 4a,b and Supplementary Fig. 9a,b). The systemic cytokine production...
induced by R848 and CpG was associated with transient decreases in body weight (Fig. 4c), which were not observed for locally retained PP-7/8a (Fig. 4c,d).

To extend these findings to other TLRa, particulate carriers of TLR-2/6a, TLR-4a and TLR-9a (Supplementary Fig. 10 and Supplementary Methods) were compared with their respective

Figure 4 Persistent, local innate immune activation is necessary and sufficient for eliciting protective CD8+ and T\textsubscript{H}1 CD4+ T cell responses. (a–c) CpG ODN 1826 (3.1 nmol, 20 µg), R848 (62.5 nmol, 20 µg) or PP-7/8a (62.5 nmol, 120 µg) were delivered subcutaneously into both hind footpads of C57BL/6 mice. (a) Supernatant of ex vivo–cultured lymph node cell suspensions (n = 4 per group per time point) and (b) serum (n = 5 per group per time point) were assessed for IL-12p40 by ELISA at serial time points. (c) Percent body weight change (n = 3 per group) following subcutaneous administration of different vaccine adjuvants (significance is shown for comparison with naive; two-way ANOVA with Bonferroni correction). (d) Relationship between biodistribution and local and systemic innate immune activation. DLN, draining lymph nodes; i.m., intramuscular; s.c., subcutaneous. (e,f) C57BL/6 mice received subcutaneous administration of protein antigen (either 50 µg of OVA or 20 µg of SIV Gag p41) formulated with adjuvant at days 0 and 14. At day 24, tetramer+ CD8+ T cell responses were assessed from whole blood by flow cytometry (n = 6 per group). (g,h) Mice were challenged intravenously at day 28 with either (g) LM-OVA or (h) LM-Gag, and bacterial burden in spleens (n = 6 per group) was evaluated on day 31 and 30, respectively. CFU, colony-forming units. (i,j) C57BL/6 mice received subcutaneous immunizations of 20 µg of MML (a polyprotein subunit antigen derived from L. major) with or without adjuvant on days 0, 21 and 42. (i) Splenocytes were isolated on day 70 and stimulated in vitro with an MML peptide pool. CD4+ T cells in the mixed splenocyte cultures were evaluated for T\textsubscript{H}1-characteristic cytokine (IFN\textsubscript{y}, IL-2 and TNF\textsubscript{a}) production (n = 4 per group). (j) Mice were challenged intradermally in both ears with L. major at day 70. Ear lesion diameters (n = 6 per group) were measured for 12 weeks (significance is shown for comparison with protein alone). All data are representative of two or more independent experiments, except that the L. major ear lesion kinetic is from a single study. Data on log scale are reported as geometric mean with 95% CI. Unless stated otherwise, comparison of multiple groups for statistical significance was determined using Kruskal-Wallis ANOVA with Dunn’s post hoc test; ns, not significant (P > 0.05); *P < 0.05; **P < 0.01.
unconjugated TLRas for local and systemic innate immune activation and morbidity (Supplementary Fig. 11a–d). Delivery of the various TLRas on particulate carriers largely resulted in enhanced DC activation and lymph node cytokine production while reducing systemic cytokine production and morbidity relative to the unconjugated TLRas. Grouping data from several studies in a meta-analysis showed that the systemic cytokine production induced by unconjugated TLRas is associated with acute TLRa toxicity and morbidity (Supplementary Fig. 11e).

Particulate TLRa enhances protective T cell immunity

The adjuvant capacity of PP-7/8a, R848 and CpG to elicit protective CD8+ T cell responses was determined using OVA and SIV Gag protein as immunogens and Listeria monocytogenes (LM) expressing either OVA (LM-OVA) or SIV Gag (LM-Gag) for challenge to assess vaccine efficacy (Fig. 4e–h and Supplementary Fig. 12a–c). After two immunizations with OVA protein, PP-7/8a and CpG (Fig. 4b) were associated with increased OVA-specific CD8+ T cell responses (12% and 8%, respectively) (Fig. 4e) and significant protection (5- and 4-log reductions in bacterial burden, respectively) against LM challenge (Fig. 4g). Of note, the magnitude of CD8+ T cell responses observed with PP-7/8a coadministrated with OVA was ∼10-fold higher than with SIV Gag (Fig. 5), and this was associated with far less protection against LM-Gag challenge (Fig. 4h). Last, these studies were extended to show that PP-7/8a conferred T11 CD4+ T cell mediated protection in the mouse model of Leishmania major when used as an adjuvant (Fig. 4i) and Supplementary Fig. 13).

In vivo particle formation with temperature-responsive Poly-7/8a

To substantiate the observation that particle scaffolds are critical for optimizing TLR-7/8a activity as an adjuvant and provide a potentially more flexible commercial platform for manufacturing and storage, we developed temperature-responsive polymer particle (TRPP)-7/8a conjugates (Supplementary Fig. 14a) that exist as water-soluble macromolecules during manufacturing and storage (T < 30 °C) but undergo temperature-driven (T > 36 °C) particle assembly in vivo (Fig. 5a). Although temperature-responsive polymers have been used as adjuvants alone37 or to physically entrap adjoined (that is, nonlinked) immunomodulators38,39, herein we evaluated how varying densities of TLR-7/8a that are covalently linked directly to the polymer backbone influenced immune activity in vivo.

The transition temperature of TRPP-7/8a is tunable through modulation of the density and the hydrophilic or hydrophobic character of ligands attached to the polymer backbones (Supplementary Fig. 14b), allowing for production of TRPP-7/8a that form particles at precisely defined temperatures either in vitro or at body temperature in vivo (Fig. 5b,c). Consistent with our earlier findings with PP-7/8a, only TRPP-7/8a capable of forming particles in vivo led to persistent and high-magnitude local cytokine production that was associated with protective CD8+ T cell responses and Th1-skewed antibodies (Fig. 5d–f and Supplementary Fig. 14c–g).

Steps were taken to further refine the structure of TRPP-7/8a to promote biodegradability and improve generalizability of the approach. First, di-block copolymers were used with ester side chains to promote degradation of the particles to individual polymer chains. Second, as prior studies by others and us have shown that synchronous delivery of protein antigen with innate immune stimulation is a highly efficient approach for optimizing T cell priming10,40,41, we prepared a TRPP-7/8a with coil peptides to provide a generalizable strategy for site-specifically linking antigen-coil fusion proteins to polymer carriers through coiled-coil interactions as described in previous reports42,43. To demonstrate the utility of this approach for ensuring
Figure 6 Co-delivery of TLR-7/8a and protein antigen on a self-assembling temperature-responsive vaccine particle. (a) Cartoon schematic of a temperature-responsive Poly-7/8a (TRPP-7/8a) modified with a coil peptide that forms heterodimers with a recombinant HIV Gag-coil fusion protein to form TRPP-7/8a-(CC)-Gag. Heterodimerization occurs at room temperature and particle formation results at temperatures greater than 33 °C. (b) Temperature-dependent particle formation illustrated by dynamic light scattering. (c) Aqueous solutions of TRPP-7/8a-(CC)-Gag at 25 °C and 37 °C. (d,e) Colocalization of HIV Gag (labeled with anti-Gag PE) with TRPP-7/8a (labeled with carboxyrodamine 110) was confirmed by flow cytometry (d) and confocal microscopy (e). (f–i) BALB/c mice received subcutaneous administration of 50 μg of HIV-Gag coil formulated with either a control or TRPP-7/8a normalized for TLR-7/8a dose (1× dose = 2.5 nmol or 3× dose = 7.5 nmol) at days 0 and 14. At day 28, draining lymph nodes (DLN), spleen and serum from vaccinated mice were collected for analysis. Splenocytes were stimulated in vitro with an HIV Gag peptide pool. Antigen-specific IFNγ-producing CD4+ T cells (n = 5 per group) (f) and CD8+ T cells (n = 5 per group) (g) in the mixed splenocyte cultures, as well as Th cell responses (n = 5 per group) in draining lymph nodes (h), were quantified by flow cytometry. (i) Serum was evaluated for anti-HIV Gag total IgG antibody titers (n = 5 per group). In vivo studies are representative of two independent experiments. Data on linear axes are reported as mean ± s.e.m., and data on log scale are reported as geometric mean with 95% CI. Comparison of multiple groups for statistical significance was determined using Kruskal-Wallis test; ns, not significant (P > 0.05); *P < 0.05; **P < 0.01. CC, coil-coil heterodimer.

Co-delivery of antigen and adjuvant, we prepared a recombinant HIV Gag-coil fusion protein site-specifically linked to a TRPP-7/8a through self-assembly using peptide-based coiled-coil interactions (Fig. 6a and Supplementary Fig. 15a–d). Mixing aqueous solutions of the HIV Gag-coil protein with a TRPP-7/8a modified with a complementary coil peptide resulted in self-assembly of a TRPP-7/8a-(coil-coil)-Gag heterodimer that undergoes particle formation (Fig. 6b,c) at temperatures greater than 34 °C and ensured co-delivery of Gag with TRPP-7/8a (Fig. 6d,e). Co-delivery of Gag with TRPP-7/8a (TRPP-7/8a-(coil-coil)-Gag) resulted in enhanced T cell and antibody responses as compared with Gag-coil admixed with either the free TLR-7/8a or TRPP-7/8a (Fig. 6f–i).

Discussion

Herein, we systematically refined properties of polymer–TLR-7/8a to elicit robust T cell and antibody responses to protein antigen. These studies established that particulate carriers (PP-7/8a) displaying high densities and potencies of TLR-7/8a are critical properties for promoting high-magnitude and persistent (>8 d) innate immune activation restricted to draining lymph nodes that is necessary for eliciting protective T_{H1} CD4+ and CD8+ T cell responses and high antibody titers, while mitigating systemic innate immune activation that is associated with adjuvant toxicity and morbidity.

The data presented here extend earlier findings on particulate delivery of TLRα to additional TLRs (TLR-2/6a, TLR-4a, TLR-7/8a and TLR-9a) and show that, in the context of TLR-7/8a delivery, improved retention is necessary but not sufficient for enhancing T cell immunity. We find that despite improved retention at the injection site and draining lymph nodes by all polymer carriers of TLR-7/8a, only the particles (PP-7/8a) are taken up efficiently by APCs and induce persistent innate immune activation in draining lymph nodes. These findings provide additional mechanistic insights from studies by others and us that TLR-7/8a attached to protein antigens are markedly more immunogenic upon aggregation or when adsorbed to alum microparticles.

The patterns of DC uptake observed for the particulate Poly-7/8a (~700 nm) used in this study are consistent with earlier findings that 100- to 1,000-nm particles are primarily trafficked to lymph nodes.
through uptake by migratory and monocyte-derived DCs; however, it will be important to determine whether particulate carriers of TLR-7/8a that passively traffic to lymph nodes, or larger particles that require uptake by DCs in the periphery, are preferred for generating T cell immunity. Notably, most existing vaccines use alum microparticles that form depots to promote DC activation and antigen uptake in the periphery. Moreover, direct intranodal injection of microparticle carriers of TLR-3a have been shown to be more immunogenic than intranodal injection of nanoparticles. Thus, additional studies are needed to carefully characterize which sizes of particulate TLRa carriers are optimal for enhancing vaccine immunogenicity.

Although this study used linear polymers based on HPMA and NIPAM as scaffolds for TLR-7/8a delivery, other particle-based delivery platforms have been used in formulations with TLRa (for example, poly(lactic-co-glycolic acid) (PLGA) and liposomes and polymerosomes) and can likely be adapted to satisfy the key physicochemical requirements for TLR-7/8a adjuvant activity defined herein. However, particulate delivery systems used in vaccines can be unstable during long-term storage and often require specialized manufacturing and storage conditions that contribute significantly to cost. The temperature-responsive Poly-7/8a (TRPP-7/8a) reported herein could potentially overcome the manufacturing and storage limitations of pre-formed particles and may allow for the use of single-vial water-soluble preparations of both antigen and adjuvant.

Finally, it should be noted that other delivery approaches, such as albumin hitchhiking or linear polymers that do not form particles but can promote local retention and efficient uptake by APCs in draining lymph nodes, may also provide the persistence of lymph node restricted innate immune activity that is needed to drive antibody and T cell responses. Indeed, as shown here, phosphorothioate-modified CpG oligodeoxynucleotides can induce protective T cell responses to protein antigen despite being relatively low-molecular-weight oligomers. This may be accounted for by local retention of CpG through extracellular matrix protein binding durability through endocytosis and uptake by APCs through lectin receptors and polymerosomes and can likely be adapted to satisfy the key physicochemical requirements for TLR-7/8a adjuvant activity defined herein. However, particulate delivery systems used in vaccines can be unstable during long-term storage and often require specialized manufacturing and storage conditions that contribute significantly to cost. The temperature-responsive Poly-7/8a (TRPP-7/8a) reported herein could potentially overcome the manufacturing and storage limitations of pre-formed particles and may allow for the use of single-vial water-soluble preparations of both antigen and adjuvant.

In conclusion, polymer carriers of TLRa represent a diverse and versatile class of adjuvant that can be systematically tuned to achieve the optimal magnitude, quality and spatiotemporal characteristics of innate immune activity required for eliciting antibody and T cell immunity for applications in preventive and therapeutic vaccines for infections and tumors.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

G.M.L., R.L., K.D.F., L.W.S. and R.A.S. were involved in experimental planning, interpreting data and writing the manuscript. G.M.L., R.L., A.E.D., O. Vaněk, J.T., K.A.R. and A.P.E.-K. planned and carried out the synthesis, purification and characterization of small molecules. R.L., R.P., M.F., T.E., O. Vaněk and G.M.L. planned and completed the synthesis, purification and characterization of the polymer precursors and polymer conjugates. P.A.D., A.S.I., A.J.B., A.Y., K.M.Q., C.R.B., K.K. and J.R.F. planned and conducted many of the biological studies. M.Y.G. and M.G.S. carried out the confocal microscopy studies on lymph node sections and polymer particles, respectively. T.H. and R.C. developed the plasmids to express the HIV Gag-coil fusion protein. R.L., M.P., R.P. and T.E. devised the coil-coil strategy. A.P.E.-K., T.E., K.D.F., L.W.S. and R.A.S. are principal investigators who advised the studies.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details accompany the online version of the paper.

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ONLINE METHODS

Synthesis of small molecule TLR-7/8a (SM 7/8a). A detailed description of the synthesis, purification and chemical characterization of the small molecules used in this study is provided in the Supplementary Methods. The conjugatable imidazoquinoline-based small molecule TLR-7/8a (SM 7/8a, SM-7/8a-Alkane, SM-7/8a-PEG, SM 20x7/8a and SM R20x7/8a) and TLR-7/8a dye conjugates were prepared according to previous reports27,35,51. The conjugatable imidazoquinoline-based small molecule TLR-7/8a (SM 7/8a, SM-7/8a-Alkane, SM-7/8a-PEG, SM 20x7/8a and SM R20x7/8a) and TLR-7/8a dye conjugates were prepared according to previous reports27,35,51. 

Synthesis of polymer–TLR-7/8a (Poly-7/8a) conjugates. A detailed description of the synthesis, purification and characterization of the Poly-7/8a is provided in the Supplementary Methods. Briefly, polymer–TLR-7/8a (Poly-7/8a) conjugates were synthesized in a combinatorial approach by reacting varying reactive copolymers based on N-(2-hydroxypropyl) methacrylamide (HPMA) and N-isopropylacrylamide (NIPAM) that have been previously described27,51. For dye-labeled polymers, Alexa Fluor 488 (AF488) Cadaverine (Life Technologies, Carlsbad, CA) or Cruz Fluor 8 amine (Santa Cruz Biotechnology, Dallas, TX) were reacted with the amine reactive copolymers for 2 h before the addition of the nucleophilic TLR-7/8a. Poly-7/8a were purified by dialysis against methanol using Spectra/Por7 Standard Regenerated Cellulose dialysis tubing with a molecular weight cut-off (MWCO) of 25 kDa (Spectrum Labs, Rancho Dominguez, CA). The solution of methanol was changed twice each day for 3 days to ensure efficient removal of the unreacted small molecules. Poly-7/8a were collected following evaporation of the solvent and characterized according to the methods described below. 

Determination of polymer molecular weights. Molecular weights and polydispersities of polymers and copolymers were measured by gel permeation chromatography using an HPLC system (Shimadzu, Japan) equipped with refractive index (RI), UV and multangle light scattering (MALS) DAWN 8 EOS detectors (Wyatt Technology, USA). Either a Superose 6 column (GE Healthcare, USA) was used with a 0.3 M acetate buffer (pH 6.5) eluent or a MicroSuperose 6 column was used with PBS (pH 7.4) as the eluent at 0.1 mL min⁻¹. Weight-average molecular weights (Mw) were calculated from the light-scattering detector based on the known injected mass while assuming 100% mass recovery. Number-average molecular weights (Mn) were determined by refractive index measurements and were calculated assuming a dn/dc value of 0.167 mL/g. Polydispersity is defined as (Mw/Mn). 

Determination of TLR-7/8a and fluorophore content on polymers. The amount of TLR-7/8a or fluorophore attached to the copolymers was determined by UV-Vis spectroscopy. Briefly, samples were suspended in 1% triethylamine/methanol at known concentrations and added to quartz cuvettes with a path length of 1 cm. Absorption was recorded over a spectrum from 250 to 775 nm using a Lambda25 UV-Vis spectrophotometer from PerkinElmer (Waltham, MA). The amount of TLR-7/8a or fluorophore in solution was calculated from the Beer-Lambert law relationship. Example calculations are provided in the Supplementary Methods. 

Particle size determination by dynamic light scattering. Z-average diameter (nm) of copolymers in PBS (pH 7.4) was determined by dynamic light scattering using a Zetasizer Nano ZS purchased from Malvern (Malvern Instruments, Malvern, UK). 

Transition temperature determination for temperature-responsive polymers. Temperature-responsive polymers were suspended at 0.1 mg/mL PBS (pH 7.4) in 200 µL volumes in Costar polystyrene 96 well flat bottom culture plates (Corning, NY). Absorption at 490 nm was evaluated as a function of temperature (1 °C/5 min steps) on a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). The Data was analyzed using Prism (GraphPad, La Jolla, CA) and nonlinear regression fit was used to calculate transition temperature (approximate temperature at half-maximum absorbance). 

Cloning and expression of the HIV Gag-coil fusion protein. The HIV Gag-KSK coil fusion protein was produced using an expression construct encoding the p41 region of the HIV Gag (HX2) gene fused to the KSK coil peptide sequence IAALKSKIAALKSKIAALKSKIAALKSKK. Briefly, a 1,131 bp fragment from HIV Gag p41 was prepared with the following additions: an N-terminal His6 tag followed by the Tobacco Etch Virus endopeptidase (TEV) cleavage site ENLYFQS, and a C-terminal Glu6 spacer followed by the KSK coil. This sequence was codon optimized for expression in Escherichia coli and synthesized commercially (DNA2.0, USA) before being subcloned into a T7-driven expression plasmid (OG37) (Oxford Genetics, UK) for expression and purification from E. coli. The His-tagged Gag-coil fusion protein was purified from cell lysates and Sigma Protease Inhibitors (P8849, Sigma) were added to prevent proteolysis. Cell lysate was loaded onto a 10 mL IMAC column (Thermo Scientific) and eluted with imidazole. Fractions containing the Gag-coil fusion protein were extracted with Triton X-114 (Sigma) at 1% to remove endotoxin and then buffer exchanged into 20 mM HEPES, pH 7.3, 300 mM NaCl and 2 mM β-mercaptoethanol to a final concentration of 1.7 mg/mL HIV Gag-coil (<2.9 EU/mg protein). 

Animal protocols. All animal experiments were conducted at the Vaccine Research Center (VRC) at the National Institutes of Health (Bethesda, MD) and were in compliance with the guidelines set by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and the Institutional Animal Care and Use Committee (ACUC). All experimental animal protocols underwent review and were approved by the ACUC before the start of experiments. 

Animals. BALB/c mice, C57BL/6 (B6) mice, and B6N.129S2-Casp1tm1Flv/J (Caspase 1/11, Inflammasome KO) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained at the Vaccine Research Center’s (VRC) Animal Care Facility (Bethesda, MD) under pathogen-free conditions. TLR7 KO mice were kindly provided by R. Kedl at the University of Colorado (Denver, CO, USA) and were bred and maintained at the VRC. BALB/c and B6 mice used in this study were female and were between 8 and 12 weeks old at the start of experiments. Both female and male Caspase 1/11 and TLR7 KO mice were used in this study and were between 6 and 20 weeks old at the start of experiments. Animals were randomly assigned to either control or experimental groups. 

Immunizations. Vaccines were prepared in sterile, endotoxin-free (<0.05 EU/mL) PBS (Gibco, Life Technologies) and administered subcutaneously in a total volume of 50 µL. All immunogens were certified endotoxin free (<1 EU/mg) by the manufacturer or were prepared in-house with <5 EU/mg as determined by LAL assay (Genscript, Piscataway, NJ). EndoFIT Ovalbumin was obtained from Invivogen (San Diego, CA). SIV Gag p35 was obtained from Protein Sciences Corporation (Meriden, CT) and MML (also known as Leish-111f) was produced as previously described36 by the Protein Expression Laboratory at the National Cancer Institute (Frederick, MD). Adjuvants had <1 EU/mg endotoxin and were either prepared in-house as described elsewhere or were acquired from commercial sources. CpG ODN 1826 and R848 were obtained from Invivogen (San Diego, CA). 

Ex vivo lymph node cultures for cytokine determination. Proximal draining lymph nodes were harvested at various time points following subcutaneous administration of different adjuvants or controls and placed in 300 µL of RPMI supplemented with 10% (v/v) FCS, 50 µM penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine in 1.5 mL DNAse, RNase, Pyrogen free Kontes Pellet Pestle Grinders (Kimble-Chase, Vineland, NJ) sitting on ice. Lymph nodes were gently mechanically disrupted using sterile pestles and the resulting suspensions were vortexed for 5 s and added to a 96 well round bottom culture plate that was incubated at 37 °C/5% CO₂ for 8 h. Supernatant was collected and stored at -80 °C until analyzed by ELISA or cytokine bead array. 

Cytokine measurements. Cytokines in lymph node culture supernatants and sera were determined using ELISA and cytokine bead array kits according to the manufacturer’s recommended guidelines. ELISA kits for murine IL-12p40, IP-10 and IFNγ were obtained from Peprotech (Rocky Hill, NJ), and an ELISA kit for the detection of murine IFNα (Verikine Mouse IFNA Alpha ELISA Kit) was purchased from PBL Assay Science (Piscataway, NJ). Customized mouse cytokine/chemokine magnetic bead panels were purchased from EMD...
Millipore (Billerica, MA) and were run on a FLEX MAP3D multiplex system from Luminex (Austin, TX) according to the manufacturer’s guidelines. Concentrations of individual cytokines in the supernatants were determined from standard curves.

**Whole animal imaging.** Biodistribution and kinetics of IR Dye-labeled materials was evaluated using a Bruker In Vivo Xtreme (Bruker, Billerica, MA) combined optical and X-ray small animal imaging system. Following subcutaneous administration of IR dye-labeled materials, mice were imaged at serial time points using a two-step imaging protocol: (1) epifluorescence (excitation = 760 nm; emission = 830 nm; 0.915 s exposure); followed by (2) X-ray (45 kVp; 1.0 s exposure). Amount of TLR-7/8a (nmol) within a region of interest (ROI) was calculated using a standard curve of log(photons/mm² × seconds) versus TLR-7/8a amount (nmol).

**Confocal microscopy of lymph node sections.** 20 µm lymph node sections were prepared and imaged as previously described, with minor modifications. In brief, lymph node sections were fixed with 0.05 M phosphate buffer containing 0.1 M L-lysine (pH 7.4), 2 mg/ml NaCl, and 10 mg/ml paraformaldehyde overnight at 4 °C, equilibrated in 30% sucrose solution for 24 h, fixed and sectioned using a Leica cryostat. Tissue sections were then blocked for 1–2 h at 4 °C with 1% normal mouse serum and bovine serum albumin solution containing 0.3% Triton X-100 and stained with directly conjugated antibodies for 6–10 h at 4 °C. Sections were then imaged using a Leica SP8 confocal microscope equipped with a 1.3NA 40x objective and a motorized ziling stage. Potential fluorescence spillover was removed using Channel Dye Separation analysis module in the Leica LAS AF software. Final image analysis was conducted using Imaris software (Bitplane, South Windsor, CT). For quantification, 3D isosurfaces of whole imaged lymph node sections were first generated using the Imaris surface creation module and statistics for the total surface volume and sum of AF488 fluorescence signal were exported into Excel (Microsoft, Redmond, WA) for further calculations.

**Flow cytometry analysis of innate immune cells from lymph nodes and spleen.** The magnitude, activation status and adjuvant uptake of innate immune cells in draining lymph nodes and spleen were evaluated as previously described, with slight modifications. Briefly, lymph node sections were prepared and imaged as previously described, with minor modifications. In brief, lymph nodes were fixed with 0.05 M phosphate buffer containing 0.1 M L-lysine (pH 7.4), 2 mg/ml NaCl, and 10 mg/ml paraformaldehyde overnight at 4 °C, equilibrated in 30% sucrose solution for 24 h, fixed and sectioned using a Leica cryostat. Tissue sections were then blocked for 1–2 h at 4 °C with 1% normal mouse serum and bovine serum albumin solution containing 0.3% Triton X-100 and stained with directly conjugated antibodies for 6–10 h at 4 °C. Sections were then imaged using a Leica SP8 confocal microscope equipped with a 1.3NA 40x objective and a motorized ziling stage. Potential fluorescence spillover was removed using Channel Dye Separation analysis module in the Leica LAS AF software. Final image analysis was conducted using Imaris software (Bitplane, South Windsor, CT). For quantification, 3D isosurfaces of whole imaged lymph node sections were first generated using the Imaris surface creation module and statistics for the total surface volume and sum of AF488 fluorescence signal were exported into Excel (Microsoft, Redmond, WA) for further calculations.

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**Analysis of polyclonal CD4+ T cell responses by flow cytometry.** Evaluation of polyclonal CD4+ T cell responses was determined as previously described. Briefly, splenocytes were isolated from vaccinated mice at several time points and 1.5 × 10⁶ splenocytes were cultured in 96 well plates with 2 µg/ml anti-CD28 (37.51, eBioscience) alone (background response), or in combination with 20 µg/ml MML protein (Leish-111f, derived from *Leishmania spp.*) or a 2 µg/ml solution of MML peptides (Mimotopes Pty Ltd; 15-mers overlapping by 11). Cells were incubated for 2 h before brefeldin A (BFA, Sigma-Aldrich) was added to a final concentration of 10 µg/ml and cells were incubated for an additional 4 h. After washing with PBS, cells were stained with Violet Dead Cell Stain (VIVID, Life Technologies), then washed and blocked with anti-CD16/CD32 (clone 2.4G2, BD) for 10 min at room temperature. After blocking, the following surface antibodies were added for 30 min at room temperature: APC-Cy7-anti-CD8 (clone 53-6.7, BioLegend) and Alexa700-anti-CD4 (RM4-5). Cells were then fixed and permeabilized using Fix/Perm solution (BD) and incubated at 4 °C for 30 min. Cells were washed and then suspended in Perm/Wash buffer containing PerCP-Cy5.5-anti-CD3 (145-2C11, BD) and incubated at 4 °C for 30 min. Cells were washed and suspended in Perm/Wash buffer and then evaluated by flow cytometry.

**Flow cytometry.** Samples were acquired on a modified LSR II flow cytometer (BD). Results were analyzed using Flowjo version 9.3, Pestle version 1.6.2 and SPICE version 5.22 software (M. Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

**Listeria monocytogenes.** Recombinant *L. monocytogenes* expressing-OVA (LM-OVA) was a gift from H. Shen (University of Pennsylvania, Philadelphia, PA) and attenuated (ΔactA, ΔinfB) *L. monocytogenes* expressing-SIV Gag (LM-Gag) from SIV strain mac239 was provided by ANZA Therapeutics (Concord, CA). Both strains were maintained at ~80 °C as a stock in brain-heart infusion/50% glycerol (BHI/50% glycerol).

**Listeria monocytogenes infectious challenge.** Evaluation of CD8+ T cell mediated protection using *L. monocytogenes* was carried out as previously described. For infection with recombinant LM-OVA and LM-Gag, mice were infected with 3 × 10⁴ and 2 × 10⁶ CFU, respectively, in 0.3 mL PBS that was delivered intravenously into the tail vein. Splenocytes were harvested 48 or 72 h after infection and mechanically homogenized in 1 mL PBS using a Tissue Ruptor (Qiagen, Valencia, CA). Splenocyte homogenate was plated in duplicate as a tenfold dilution series on brain heart infusion agar (Difco, Detroit, MI). Plates were incubated at 37 °C for 24 h and colonies were counted by personnel blinded to group assignment to determine total CFU per spleen (bacterial burden).

**Leishmania major challenge.** *L. major* clone V1 (MHOM/IL/80/Friedlin) promastigotes were grown as previously described. Briefly, infectious-stage metacyclic promastigotes of *L. major* were isolated from stationary cultures (4–5 days old) by negative selection using peanut agglutinin (Vector Laboratories).
Mice were infected 4 weeks after boost by injecting 750 metacyclic promastigotes intradermally within both ears. Personnel blinded to the vaccination assignments of the infected mice recorded weekly ear measurements using a metric caliper.

**Serum antibody measurements.** Immulon 4HBX plates (Thermo Scientific) were coated overnight at 4 °C with protein antigen (OV A or HIV Gag) diluted in 0.1 M carbonate buffer (pH 9.8). Plates were then blocked with 1% FBS/PBS, and plasma from immunized mice was applied in serial tenfold dilutions in ELISA diluent (0.1% FBS/PBS) and incubated for 1 h at 37 °C. For determining total IgG, biotin conjugated goat anti-mouse total IgG (Jackson ImmunoResearch, Westgrove, PA) was added and incubated for 1 h at 37 °C. Alternatively, for antibody isotyping, either biotin conjugated goat anti-mouse IgG1 (Jackson ImmunoResearch) or biotin conjugated goat anti-mouse IgG2c (Jackson ImmunoResearch) were added in diluent incubated for 1 h at 37 °C. For detection, Avidin-Horse Radish Peroxidase (BD) was added and incubated for 30 min at room temperature, followed by the addition of TMB substrate-chromogen (Dako, Glostrup, Denmark) and a 2 N sulfuric acid stop solution. Washing was performed between steps with PBS + 0.05% Tween 20. Plates were read on a SpectraMax Plus spectrophotometer (Molecular Devices) at 450 nm. Data was analyzed in Prism to generate a nonlinear regression fit to estimate EC50 (midpoint titer) or EC 05 (endpoint titers).

**Evaluation of TLR-7 activity in vitro using HEK293 reporter cells.** HEK293 expressing human TLR-7 (HEK-TLR7) were purchased from Invivogen (San Diego, CA) and used according to the manufacturer’s guidelines.

**Statistics and graphs.** Sample sizes for biological studies were chosen based on calculations using JMP statistical analysis software (Cary, NC); standard deviations and pre-specified differences in groups (“differences to detect”) were based on historical data, and type I and type II error rates were set at 0.05 and 0.2, respectively. Data on linear axes are reported as mean ± s.e.m. Data on log scale are reported as geometric mean with 95% CI. Statistical analyses were carried out using Prism software (GraphPad). Unless stated otherwise within the figure legends, Kruskal-Wallis one-way ANOVA with Dunn’s post hoc test was used to calculate P values for comparisons between >2 groups. Differences were found to be significant when P was less than 0.05 or 0.01, as indicated by single (*) or double asterisks (**) within the figures. Most graphs were produced using Prism. Flow cytometry data was processed using FlowJo (Tree Star). To evaluate the multifunctional cytokine response for antigen-specific T cells, Boolean gating was performed in Flow Jo and the resulting data was processed in Pestle (M. Roederer, YRC, NIAID, NIH, USA) and graphed using SPICE (http://exon.niaid.nih.gov/spice/) provided by M. Roederer.

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