Supplementary Figure 1

Synthesis of polymer-TLR-7/8a conjugates (Poly-7/8a).

(a) Chemical structures of imidazoquinoline-based TLR-7/8a. Polymer reactive analogs of the commercially available TLR-7/8a, R848, were produced by replacing the isopropanol group with reactive linkers that are indicated by the shaded boxes overlaying the structures of SM 7/8a, SM 7/8a-alkane and SM 7/8a-PEG. Note that the alkane and PEG linkers are of comparable length but different composition (hydrophobic vs. hydrophilic). The terminal amine on each of the linkers permitted facile attachment to amine reactive polymer precursors. (b) Poly-7/8a were generated by reacting nucleophilic TLR-7/8a (e.g., SM 7/8a) with HPMA-based copolymers in a one step reaction, resulting in a stable amide bond between the TLR-7/8a and the polymer backbone. Note that the brackets represent
repeating units of each monomer, with the subscripts, x and y, representing the percentage composition (mol%) of each monomer. Poly = polymer; SM = small molecule; HPMA = N-(2-hydroxypropyl)methacrylamide; MA = methacrylamide; Ahx = aminohexanoic acid; PEG = Polyethylene glycol; TT = 2-Thiazolidine-2-thione.
Combinatorial synthesis of Poly-7/8a.

(a) Structures of Imidazoquinoline-based TLR-7/8a used in the generation of combinatorial libraries of Poly-7/8a. In addition to SM 7/8a...
described previously, a ~ 20-fold more potent TLR-7/8a with a xylene linker was prepared and is referred to as SM 20x7/8a. The potency of the two TLR-7/8a were determined in vitro using HEK293 hTLR7 reporter cells. Absorbance at 620 nm in this experiment is proportional to TLR-7 activity. Note that acetylated versions of the TLR-7/8a were used in these in vitro assays as this best represents the physicochemical characteristics of the compounds when they are attached to the polymers. (b) A combinatorial library of Poly-7/8a was generated by attaching 2 unique TLR-7/8a (SM 7/8a or SM 20x7/8a) to reactive HPMA-based copolymers at different densities (~ 2, 4, 8 mol %) using short, alkane or PEG linkers. By reacting 2 unique TLR-7/8a at 3 different densities with 3 different linkers, 18 unique products can be generated, as illustrated (c). Note that this cartoon representation is for illustrative purposes; not all Poly-7/8a represented in this schematic were evaluated in this study, nor does this schematic represent all the materials described herein.
Supplementary Figure 3

Screening a combinatorial library of Poly-7/8a in vivo.

Combinatorial library of Poly-7/8a with varying TLR-7/8a density and linker group composition. (b) Cartoon schematic of a combinatorial library of Poly-7/8a. (c) Poly-7/8a normalized for TLR-7/8a dose (12.5 nmol) were subcutaneously administered into both hind footpads of C57BL/6 mice. After 24 h, draining lymph nodes (n = 4) were harvested and processed to generate a cell suspension that was cultured for 8 h and then evaluated for IP-10 production by ELISA. Data are reported as mean; statistical significance is reported relative to naïve (ANOVA with Bonferroni correction); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01.
### Supplementary Figure 4

**Increasing agonist density is associated with particle formation and TLR-7 dependent lymph node cytokine production.**

(a) Properties of Poly-7/8a and controls. (b) Negative control polymers were generated using 2-aminopyridine (AP) to account for the contribution of the aromatic amine present on the Imidazoquinoline-based TLR-7/8a. AP was attached to polymers using a PEG or amphiphilic (AMPH) spacer to generate polymer coils and polymer particles, respectively. (c, d) Adjuvants were administered subcutaneously and after 24 h lymph nodes draining the site of immunization were harvested to create cell suspensions that were cultured for 8 h and then evaluated for (c) IFNα or (d) IFNγ by ELISA. (e, f) PP-7/8a (PEG, 10 mol% 7/8a) was administered subcutaneously to wild type (WT) or knockout mice and cytokines were evaluated from lymph node cell suspensions. All data are reported as mean ± SEM; except where indicated, statistical significance is relative to all other groups (ANOVA with Bonferroni correction, n = 4); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01

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SM = small molecule, PC = polymer coil, PP = polymer particle.

![Aminopyridine (AP) diagram](image)

![TLR-7 knockdown and Caspase 1/11 knockdown graphs](image)
Supplementary figure 5

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Note: Polymer-dye conjugates were prepared with either 0.5% mol% Alexa Fluor 488 (AF488) or 0.5% mol% IR-Dye800cw. All polymers were prepared from the same ~30 kDa reactive polymer precursor. The SM 7/8a is conjugated to a single dye molecule as described in Supplementary Materials and Methods.
Supplementary Figure 5

In vivo tracking of dye-labeled Poly-7/8a.

(a) Properties of fluorescent dye-labeled materials. (b) Example gating tree. (c) Gates designating adjuvant positive cells (AF488+) were set relative to naïve. (d) Percent adjuvant uptake by the major CD11c+ DC subsets. (e-g) Evaluation of polymer controls and polymer particles with different densities of TLR-7/8a (3 and 10 mol% 7/8a) reveals that pharmacokinetics and uptake by APCs is primarily dependent on the morphology of the carrier (i.e. submicron particle) and is independent of the attached agonist. All data are reported as mean ± SEM. DC = dendritic cell; pDC = plasmacytoid dendritic cell; Mac = Macrophage; Mon = monocyte.
Supplementary Figure 6

Characterization of DC populations in draining lymph nodes and spleen.

AF488-labeled materials normalized for dose of TLR-7/8a (62.5 nmol) were unilaterally administered subcutaneously into the hind footpad of C57BL/6 mice. (a-c) At serial timepoints thereafter, lymph nodes (n = 3) or spleen (n = 1) were isolated and enzyme-digested to create cell suspensions that were stained and evaluated by flow cytometry. (a) Magnitude of DCs and (b) expression of the costimulatory molecule CD80 were evaluated by flow cytometry. (c) DC populations in the spleen were evaluated for costimulatory molecule expression (CD80 MFI) at serial timepoints. Note that the small molecule TLR-7/8a (SM 7/8a) leads to transient activation of the major DC subsets and B cells in both spleen and lymph nodes, whereas PP-7/8a leads to persistent activation of CD8- B220- DC (monocyte-derived DCs, skin-derived DCs and CD8- resident DCs) and CD8+ DC. Serum (n = 3) was evaluated for the presence of (h) IL-12p40 at serial timepoints. All data are reported as mean ± SEM; except where indicated, significance is relative to all other groups (ANOVA with Bonferroni correction); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01.
Supplementary Figure 7

Orientation of the TLR-7/8a attached to the polymer carrier influences the timing of onset and magnitude of lymph node cytokine production.

Poly-7/8a were prepared with TLR-7/8a attached to the polymer carrier with either the C4-amine exposed (1) (PP-20x7/8a) or blocked (2) (PP-R20x7/8a). (b, c) Poly-7/8a with two different orientations of TLR-7/8a were administered subcutaneously into the hind footpads of C57BL/6 mice and lymph nodes (n = 4) were isolated at serial timepoints thereafter and cultured overnight. Supernatant from the ex vivo lymph node cell suspensions (n = 4) were evaluated for (b) IL-12 and (c) IP-10 by ELISA. Note that blocking the C4-amine delays onset and leads to lower magnitude of cytokine production. All data are reported as mean ± SEM; significance is relative to all other groups (ANOVA with Bonferroni correction); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01.

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Supplementary Figure 8

Anti-OVA antibody responses.

(a-d) Poly-7/8a, SM 7/8a or a control were formulated with 50 µg of OVA in PBS and given subcutaneously to C57BL/6 mice (n = 5) at days 0 and 14. Serum was collected from vaccinated mice at day 28 and evaluated for anti-OVA IgG1 and IgG2c antibodies. Doses of adjuvant and polymer are provided in the accompanying tables.
Particle-forming Poly-7/8a induces locally restricted Th1-polarizing cytokines.

(a, b) R848 (62.5 nmol), PP-7/8a (62.5 nmol) or CpG ODN 1826 (3.1 nmol) were administered subcutaneously into the footpad of mice. Cytokine bead array was used to quantify cytokines present (a) in the serum (n = 5) at 6 h (peak), or (b) from draining lymph nodes (n = 4) at 24 h. All data are reported as mean ± SEM; except where indicated, statistical significance is relative to both OVA alone and OVA + R848 (ANOVA with Bonferroni correction); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01.
Supplementary Figure 10

Polymer conjugates of TLR-2/6 and TLR-4 agonists.

Structures of polymer conjugates of TLR-2/6 (Pam2Cys) and TLR-4 (Pyrimidoindole) agonists are shown above. Both agonists were linked to the polymer backbones through hydrophilic PEG spacers at > 5 mol % agonist density to induce polymer particle (PP) formation in aqueous conditions. Synthesis and characterization of PP-Pam2Cys and PP-PI conjugates is provided in the Supplementary Materials and Methods.
Supplementary Figure 11

Local and systemic innate immune activation and morbidity by particulate and unconjugated TLRa.

(a-d) TLR-2/6 agonists (PP-Pam2Cys and unconjugated Pam2Cys, 20 nmol), heterocyclic TLR-4 agonists (PP-PI and PI, 20 nmol), lipid-based TLR-4 agonists (50 μg Alum / MPL 5 μg or MPL alone (5 μg, ~3 nmol)), TLR-7/8a (PP-7/8a and R848, 12.5 nmol), TLR-9a (CpG/polyplex and CpG alone, 3 nmol), and controls were delivered subcutaneously into both hind footpads of C57BL/6 mice. Draining lymph nodes were harvested at day 4 (early peak for local activity) and were evaluated for (a) total CD11c+ DCs per lymph node (n = 3) and (b) IL-12p40 production (n = 8). (c) Serum (n = 5) was collected at 4 h (peak for systemic activity) post-immunization and was evaluated for IL-12p40 by ELISA. (d) Percent body weight change (n = 5) at peak (24 h) following subcutaneous administration of...
different vaccine adjuvants. (e) Meta-analysis of 4 independent studies (n = 43 groups) showing the relationship between systemic IL-12 production and body weight change (relative to time = 0) for mice immunized with either particle carriers of TLRa, unconjugated (free) TLRa or controls. PP = polymer particle; PI = pyrimidoindole; MPL = Monophosphoryl Lipid A; Alum = Aluminum hydroxide; CpG/Polyplex = Poly(Lysine).HCl complexed to CpG ODN 1826 at 20:1 N:P. See Supplementary Materials and Methods for chemical synthesis and formulation of the different TLRa. All data are representative of two or more independent experiments that included multiple time points. Data on linear scale are reported as mean ± SEM and data on log scale are reported as geometric mean ± 95% CI; statistical significance is shown for specific comparisons and for adjuvant formulations relative to naive and particle controls (ANOVA with Bonferroni correction); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01.
Particle-forming Poly-7/8a elicit protective CD8 T cell responses.

(a-c) Poly-7/8a, SM 7/8a and polymer controls admixed with 50 μg of OVA in 50 μL of PBS were administered subcutaneously into the hind footpad of C57BL/6 mice (n = 6) at days 0 and 14. Three different small molecule TLR-7/8a were evaluated in this experiment: either commercially available R848, SM 7/8a, or SM 20x7/8a. Poly-7/8a were evaluated for either dose, comparing PP-7/8a at 12.5 and 62.5 nmol, or potency of the agonist attached, comparing PP-7/8a with PP-20x7/8a. (b) The proportion of tetramer+ positive CD8 T responses was evaluated from whole blood at day 24. (c) Mice (n = 6) were challenged intravenously at day 28 with *Listeria monocytogenes* expressing full-length OVA Albumin (LM-OVA) and bacterial burdens in spleen was evaluated at day 31. Note that protection (inverse of bacterial burden) is proportional to the tetramer+ response. All data are reported as mean ± SEM; except where indicated, statistical significance is relative to both OVA alone and OVA + R848 (ANOVA with Bonferroni correction); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01.
Supplementary figure 13

a) Antigen (MML, µg) 40 40 40 40 40 40 40 40 40 40

Adjuvant PP SM 7/8a SM 20x/7/8a PP 7/8a PP 20x/7/8a PP 7/8a CpG

Linker group PEG PEG PEG PEG PEG PEG — — —

Adjuvant dose (nmol) 62.5 12.5 12.5 12.5 62.5 3.1

b) 1 week post 3 immunizations

c) 4 weeks post 3 immunizations

d) Th1 CD4 T cell quality 4 weeks post 3 immunizations

(e) significant reduction in lesions between days 30-64
Supplementary Figure 13

Particle forming Poly-7/8a elicit Th1 cells that mediate protection against *Leishmania major*.

(a-f) C57BL/6 mice received subcutaneous immunizations of 20 µg of MML protein from *L major* either alone or admixed with an adjuvant on days 0, 21 and 42. (a) Splenocytes were isolated on either (b) day 56 (n = 4) or (c, d) day 70 (n = 5) and stimulated in vitro with a peptide pool derived from MML. Antigen-specific CD4 T cells were evaluated for their capacity to produce Th1 characteristic cytokines (IFNγ, IL-2 or TNFα); (b) and (c) report total cytokine producing CD4 T cells (magnitude), whereas (d) reports the frequency of CD4 T cells producing combinations of IFNγ, IL-2 and TNFα (quality). (e) Mice (n = 6) were challenged intradermally in both ears with *L major* at day 70. Ear lesion diameters were measured up to 12 weeks after challenge. All data are reported as mean ± SEM; except where indicated, statistical significance is relative to naïve, MML alone and SM 7/8a (ANOVA with Bonferroni correction); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01.
Supplementary figure 14

a

NIPAM-based (thermosensitive polymer)

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 \quad \text{CH}_2 \\
\text{NH} & \\
\text{CH}_3 & \quad \text{CH}_2
\end{align*}
\]

\[
X = (\text{CH}_2)_2\text{CO}(\text{NH})\text{(CH}_2\text{CH}_2\text{O})_8\text{CH}_2\text{CH}_2
\]

(16 atom PEG spacer)

\[
L = \text{TLR-7/8a (7/8a, 20x7/8a or AP)}
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b

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Percent tetramer+ (L-2k-SIINFEKL+) CD8 T cells

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Ratio IgG1 / IgG2c

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Nature Biotechnology: doi:10.1038/nbt.3371
Supplementary Figure 14

Temperature-responsive particle-forming Poly-7/8a (TRPP-7/8a) induce protective CD8 T cell responses.

(a) First-generation TRPP-7/8a are N-isopropylacrylamide (NIPAM)-based copolymers. Note that the TLR-7/8a (7/8a or 20x7/8a) or a control ligand (AP) were attached to the NIPAM-based copolymers using a similar reaction scheme as described in supplementary figure 1 (see materials and methods). (b) A series of TRPP-7/8a were produced with increasing densities of either SM 7/8a, SM 20x7/8a or the control, AP-AMPH. Note that increasing densities of the hydrophobic ligands attached to the polymers leads to decreasing transition temperatures, the temperature at which particle formation occurs in aqueous solution. (c, d) TRPP-7/8a and controls were evaluated in a vaccination and challenge model using OVA. C57BL/6 mice (n = 5) received 50 μg of OVA either alone or admixed with adjuvant that was administered subcutaneously in 50 μL of PBS at days 0 and 14. (c) At day 24, the proportion of tetramer+ CD8 T cells was evaluated from whole blood. (d) The capacity of the tetramer+ CD8 T cells to mediate protection was assessed by challenging the mice intravenously at day 28 with LM-OVA. Bacterial burdens were assessed in the spleen at day 31. (e, f, g) Serum was collected from vaccinated mice at day 28 and evaluated for (e) anti-OVA IgG1 and (f) IgG2c antibodies as well as (g) the ratio of the two isotype titers (geometric mean). Data are reported as mean ± SEM; statistical significance is relative to OVA alone (ANOVA with Bonferroni correction); ns, not significant (P > 0.05); *, P < 0.05; **, P < 0.01.
Supplementary Figure 15

(a) Schematic of a second-generation di-block copolymer-based TRPP-7/8a used for attachment of TLR-7/8a and a coil peptide. The
hydrophilic block consists of HPMA and propargyl(methacrylamide) (PgMA). The acetylene group on PgMA allowed for the attachment of ligands that are modified with azide groups. The hydrophobic block is comprised of poly(diethylene glycol(methacrylate)) homopolymer that allows for the transition temperature to be independent of the attached ligands and contains a biodegradable ester group. Ligands (TLR-7/8a, peptide or fluorophore) were attached to the diblock copolymer through copper-catalyzed 1,3-dipolar cycloaddition. (b) Summary of TRPP-7/8a and TRPP controls. (c) Amino acid sequence of the HIV Gag p41 coil fusion protein; note that the coil domain (KSK) on the protein is complementary to the ESE coil peptide attached to the polymers. (d) Schematic representation of the anti-parallel coil-coil interactions that occur between the ESE and KSK coil peptides.
Supplementary Materials and Methods

For

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


Correspondence should be addressed to R.A.S. (rseder@mail.nih.gov)
SUPPLEMENTARY MATERIALS AND METHODS

Chemicals
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) as reagent grade or higher purity, unless stated otherwise. Ethoxyacetic acid was obtained from Alfa Aesar (Ward Hill, MA). Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid (PEG4) was purchased from EMD Millipore (Darmstadt, Germany). N-Boc-1,4-diaminobutane\(^1\) and 2-Chloro-4,6-dimethoxy-1,3,5-triazine (CDMT)\(^2\) were prepared as previously described. Green fluorescent reactive dyes Alexa Fluor® 488 carboxylic acid tetrafluorophenyl ester, Alexa Fluar® 488 cadaverine and Carboxyrhodamine 110 PEG3 azide was purchased from Alfa Aesar. Amine reactive infrared fluorescent reactive dye IRDye® 800CW NHS Ester was purchased from LI-COR (Lincoln, NE). Nucleophilic infrared fluorescent reactive dye, CruzFluo\(sm^TM\) 8 amine, was purchased from Santa Cruz Biotechnology (Dallas, TX). Dibenzocyclooctyne (DBCO) modified PEG spacer (DBCO-PEG4-Amine) was purchased from Click Chemistry Tools (Scottsdale, AZ). Peptides were produced by solid phase peptide synthesis and were obtained from American Peptide Company (Vista, CA).

Instrumentation for synthesis, purification and chemical characterization
Microwave irradiation was carried out in a CEM Discover Synthesizer with 150 watts max power. Flash column chromatography was performed on a Biotage SP4 Flash Purification system (Uppsala, Sweden) using Biotage® SNAP Cartridges and SNAP Samplet Cartridges with KP-Silica 60 mm. Analytical HPLC analyses were performed on an Agilent 1200 Series instrument equipped with multi-wavelength detectors using a Zorbax Stable Bond C-18 column (4.6 x 50 mm, 3.5 mm) with a flow rate of 0.5 mL/min or 1.0 mL/min. Solvent A was 0.05% trifluoroacetic acid (TFA) in water (H\(_2\)O), solvent B was 0.05% TFA in acetonitrile (ACN), and a linear gradient of 5% B to 95% B over 10 minutes was used. ESI or APCI mass spectrometry (MS) were performed on an LC/MSD TrapXCl Agilent Technologies instrument or on a 6130 Quadrupole LC/MS Agilent Technologies instrument equipped with a diode array detector. ¹H NMR spectra were recorded on a Varian spectrometer operating at 400 MHz. Ultraviolet-Visible (UV-Vis) light spectroscopy was performed on a Lambda25 UV/Vis system from PerkinElmer (Waltham, MA) and fluorescence spectroscopy was carried out on a PerkinElmer brand Fluorescence Spectrometer, model LS 55.

Synthesis of polymer reactive small molecule TLR-7/8a
Synthesis of imidazoquinoline-based TLR-7/8a was based on previous reports\(^3\)\(^-\)\(^7\) and is described in more detail below.
The synthesis of tert-butyl (4-((2-chloro-3-nitroquinolin-4-yl)amino)butyl)carbamate was carried out as previously described.\(^5\) \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.11 (d, \(J = 7.6\) Hz, 1H), 7.91 (dd, \(J = 8.4, 1\) Hz, 1H), 7.74 (m, 1H), 7.52 (m, 1H), 6.40 (br s, 1H), 4.66 (br s, 1H), 3.48 (m, 2H), 3.20 (m, 2H), 1.80 (m, 2H), 1.65 (m, 2H), 1.47 (br s, 9H). MS (APCI) calculated for \(C_{20}H_{22}ClN_4O_4\), \(m/z\), 394.1, found 394.9 (M+H).\(^+\)

The synthesis of tert-butyl (4-(((2-chloro-3-nitroquinolin-4-yl)amino)methyl)benzyl)carbamate was carried out as previously described.\(^6\) \(^1\)H NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) 8.51 (d, \(J = 8.5\) Hz, 1H), 8.46 (t, \(J = 6.4\) Hz, 1H), 7.88 – 7.78 (m, 2H), 7.65 (dd, \(J = 8.4, 5.5\) Hz, 1H), 7.33 (t, \(J = 6.2\) Hz, 1H), 7.17 (q, \(J = 8.2\) Hz, 4H), 4.39 (d, \(J = 6.2\) Hz, 2H), 4.07 (d, \(J = 6.2\) Hz, 2H), 1.36 (s, 9H). MS (APCI) calculated for \(C_{22}H_{23}ClN_4O_4\), \(m/z\), 442.1, found 464.9 (M+Na).\(^+\)
(6) **tert-butyl (4-((3-amino-2-chloroquinolin-4-yl)amino)butyl)carbamate.** A 23 g solution of (5) and 230 mg of Na₂SO₄ in 200 mL of ethyl acetate was bubbled with Argon for 5 minutes to remove oxygen. To this solution, 230 mg of 10% Pt/c was added and the mixture was flushed with Argon for an additional 5 minutes and then pressurized with H₂(g) 55 mm Hg. The reaction mixture was agitated with a mechanical shaker. The reaction was considered complete (~3 hours) once the pressure remained constant at a constant volume of H₂(g). The reaction mixture was filtered through celite and evaporated to dryness to obtain yellow oil. Trituration with 1:1 hexanes / ether yielded white crystals that were collected by filtration. Drying overnight under vacuum yielded 20.12 g (94.7 % yield) of spectroscopically pure (>95% at 254 nm) white crystals. \(^1\)H NMR (400 MHz, DMSO-d₆) \(\delta 8.03 – 7.95\) (m, 1H), 7.70 – 7.61 (m, 1H), 7.44 – 7.34 (m, 2H), 6.73 (s, 1H), 5.14 (t, \(J = 6.7\) Hz, 1H), 5.00 (s, 2H), 3.19 (q, \(J = 7.0\) Hz, 2H), 2.87 (q, \(J = 6.5\) Hz, 2H), 1.55 – 1.34 (m, 4H), 1.33 (s, 9H). MS (APCI) calculated for \(C_{18}H_{25}ClN_2O_2\) m/z, 364.2, found 365.2 (M+H)⁺.

(7) **tert-butyl 4-((3-amino-2-chloroquinolin-4-yl)methyl)benzylcarbamate.** The synthetic protocol is the same as for (6), except 5 g of (5) was used as the starting material. Product was spectroscopically pure (>95% at 254 nm) following passage through celite. Solvent was removed under vacuum and yielded 4.57 g (93% yield) of white crystals. \(^1\)H NMR (400 MHz, DMSO-d₆) \(\delta 8.00 – 7.93\) (m, 1H), 7.63 (dd, \(J = 8.0, 1.7\) Hz, 1H), 7.35 (tt, \(J = 6.9, 5.2\) Hz, 2H), 7.31 – 7.25 (m, 3H), 7.11 (d, \(J = 7.9\) Hz, 2H), 5.79 (t, \(J = 7.1\) Hz, 1H), 5.04 (s, 2H), 4.40 (d, \(J = 7.2\) Hz, 2H), 4.04 (d, \(J = 6.2\) Hz, 2H), 1.36 (s, 9H). MS (APCI) calculated for \(C_{22}H_{29}ClN_4O_2\) m/z, 412.2, found 413.2 (M+H)⁺.

(8) **Tert-butyl (4-(4-chloro-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-1-yl)butyl)carbamate.** To 2.5 mL of 2-ethoxyacetic acid (0.026 mol, 1.2 eq) in 150 mL of ethyl acetate were added 4.6 g (0.026 mol, 1.2 eq) CDMT, followed by dropwise addition of 6.0 mL (0.055 mol, 2.5 eq) of N-methylmorpholine (NMM). After 5 minutes, 8 g (0.022 mol, 1.0 eq) of (6) was added and the reaction was refluxed using an oil bath. A white precipitate was formed after several minutes corresponding to the NMM.Cl salt. After 16 hours, the reaction mixture was filtered and washed 3x150 mL with 1M HCl. The organic phase was dried with Na₂SO₄, filtered and evaporated to dryness. The resulting crude product was added to 20 mL of methanol with 800 mg (10% w/w) CaO and then microwaved at 100°C for 3 hours. The CaO was removed by filtration and the resulting solution was evaporated to dryness to obtain an oily product that was purified by flash chromatography using a 0-6% methanol in DCM gradient, yielding 9.44 g of clear oil. Recrystallization from 5:1 hexane / ethyl acetate yielded 5.59 g (58.9 % yield) of spectroscopically pure (>95% at 254 nm) white crystals. \(^1\)H NMR (400 MHz, DMSO-d₆) \(\delta 8.37 – 8.28\) (m, 1H), 8.11 – 8.04 (m, 1H), 7.81 – 7.70 (m, 2H), 6.83 – 6.75 (m, 1H), 4.84 (s, 2H), 4.65 (t, \(J = 7.9\) Hz, 2H), 3.62 – 3.52 (m, 2H), 2.96 (q, \(J = 6.4\) Hz, 2H), 1.85 (t, \(J = 7.9\) Hz, 2H), 1.56 (t, \(J = 7.7\) Hz, 2H), 1.30 (s, 9H), 1.20 – 1.12 (m, 3H). MS (APCI) calculated for \(C_{22}H_{29}ClN_4O_3\) m/z 432.2, found 433.2 (M+H)⁺.

(9) **Tert-butyl 4-((2-butyl-4-chloro-1H-imidazo[4,5-c]quinolin-1-yl)methyl) benzylcarbamate.** The synthetic protocol is the same as for (8), except 2 g of (7) was used as the starting material and pentanoic acid was used in place of 2-ethoxyacetic acid. Flash purification was not required, but the product was recrystallized from methanol to obtain 1.4 g (58% yield) of spectroscopically pure (>95% at 254 nm) yellow crystals. NMR (400 MHz, DMSO-d₆) \(\delta 8.08\) (d, \(J = 8.3\) Hz, 1H), 8.02 (d, \(J = 8.4\) Hz, 1H), 7.63 (dd, \(J = 8.2, 6.8\) Hz, 1H), 7.50 (t, \(J = 7.7\) Hz, 1H), 7.30 (t, \(J = 8.0\) Hz, 1H), 7.15 (d, \(J = 7.9\) Hz, 2H), 7.01 – 6.94 (m, 2H), 5.94 (s, 2H), 4.04 (d, \(J = 6.2\) Hz, 2H), 2.96 (t, \(J = 7.7\) Hz, 2H).
Hz, 2H), 1.73 (q, J = 7.6 Hz, 2H), 1.38 (q, J = 7.4 Hz, 2H), 1.33 (s, 9H), 0.86 (t, J = 7.3 Hz, 3H). MS (APCI) calculated for C_{27}H_{31}ClN_{4}O_{2} m/z 478.2, found 479.2 (M+H)^{+}.

(10) Tert-butyl (4-(4-(benzylamino)-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-1-yl)butyl)carbamate. 6.5 g of (8) (0.015 mol, 1 eq) was added to 16 mL of benzylamine (0.15 mol, 10 eq) and reacted for 6 hours at 110°C in a microwave apparatus (CEM Discover Synthesizer). After completion, the reaction mixture was cooled to room temperature and then added to 100 mL of DCM and washed 4x 100 mL with 1 M HCl. The resulting yellow oil was recrystallized from 4:1 hexane / ethyl acetate to obtain 7.3g (97.1%) of spectroscopically pure (>95% at 254 nm) white crystals. \(^{1}\)H NMR (400 MHz, DMSO-d_{6}) \(\delta\) 7.99 (d, J = 8.0 Hz, 1H), 7.66 – 7.55 (m, 2H), 7.41 (d, J = 7.3 Hz, 3H), 7.25 (td, J = 7.5, 5.6 Hz, 3H), 7.20 – 7.12 (m, 1H), 6.80 (t, J = 5.7 Hz, 1H), 4.79 – 4.72 (m, 4H), 4.53 (t, J = 7.8 Hz, 2H), 3.54 (q, J= 7.0 Hz, 2H), 2.95 (q, J = 6.5 Hz, 2H), 1.85 (m, 2H), 1.54 (t, J = 7.7 Hz, 2H), 1.31 (s, 9H), 1.15 (t, J = 7.0 Hz, 3H). MS (APCI) calculated for C_{29}H_{37}N_{5}O_{3} m/z 503.3, found 504.3 (M+H)^{+}.

(11) Tert-butyl 4-((2-buty1-4-((2,4-dimethoxybenzyl)amino)-1H-imidazo[4,5-c]quinolin-1-yl)methyl)benzylcarbamate. The synthetic protocol was the same as for (10), except 300 mg of (9) was used as the starting material and 2,4-dimethoxy benzylamine was used in place of benzylamine. Product was recrystallized from 3:1 hexane / ethyl acetate to obtain 272 mg (78% yield) of a spectroscopically pure product (>95% at 254 nm). \(^{1}\)H NMR (400 MHz, DMSO-d_{6}) \(\delta\) 9.64 (s, 1H), 8.16 (s, 1H), 7.91 (s, 1H), 7.60 (t, J = 7.8 Hz, 1H), 7.34 (q, J = 7.1, 6.1 Hz, 2H), 7.18 (d, J = 8.0 Hz, 3H), 7.02 (d, J = 8.0 Hz, 2H), 6.60 (d, J = 2.3 Hz, 1H), 6.49 (dd, J = 8.3, 2.4 Hz, 1H), 5.91 (s, 2H), 4.89 (s, 2H), 4.05 (d, J = 6.2 Hz, 2H), 3.77 (s, 2H), 3.74 (s, 3H), 2.92 (t, J = 7.7 Hz, 2H), 1.75 – 1.66 (m, 2H), 1.37-1.19 (m, 11H), 0.84 (t, J = 7.3Hz, 3H). MS (APCI) calculated for C_{36}H_{43}N_{5}O_{4} m/z 609.3, found 610.3 (M+H)^{+}.

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Polymer reactive small molecule Toll-like receptor-7/8 agonists (TLR-7/8a) and aromatic heterocyclic base control ligands based on aminopyridine (AP).
(12) SM 7/8a, 1-(4-aminobutyl)-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-4-amine. Simultaneous debenzylation and Boc removal was achieved by adding 36 mL of 98% H$_2$SO$_4$ (36.8 N) to 7.2 g (0.014 mol) of (10). The solution turned from faint yellow to cloudy orange over several minutes. Reaction progress was monitored by HPLC. After 3 hours, the reaction mixture was slowly added to 200 mL of DI H$_2$O and stirred at room temperature for 30 minutes. This mixture was filtered through celite and the resulting clear aqueous solution was adjusted to pH 10 using 10 M NaOH. The aqueous layer was extracted with 6x100 mL DCM. The organic layer was dried with Na$_2$SO$_4$ and then evaporated to dryness, yielding 4.03 g (89.6% yield) of a spectroscopically pure (>95% at 254 nm) white solid.

The filtrate was made alkaline by the addition of 10 M NaOH, 50 mL of DCM. The organic phase was then dried with Na$_2$SO$_4$ and evaporated to dryness, yielding 4.03 g (89.6% yield) of C$_{17}$H$_{23}$N$_6$O m/z 313.2, found 314.2 (M+H)$^+$. After 16 hours at room temperature, the organic phase was dried with Na$_2$SO$_4$ and evaporated to dryness, yielding 0.74 g (50% yield) of C$_{17}$H$_{23}$N$_6$O m/z 314.2 (M+H)$^+$. 

(13) SM 7/8a-PEG, 1-amino-N-(4-(4-aminobutyl)-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-1-yl)butyl)-3,6,9,12-tetraoxapentadecan-15-amide. To 20 mL of ethyl acetate was added 500 mg (1.6 mmol, 1 eq) of (12), 281 mg (1.6 mmol, 1 eq) of CDMT and 643 mg (1.8 mmol, 1.1 eq) of Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid (PEG4), followed by the dropwise addition of 441 µL (4.0 mmol, 2.5 eq) of NMM, while stirring vigorously. After 16 hours at room temperature, the reaction mixture was filtered and then washed 3x50 mL with 1 M HCl. The organic phase was dried with Na$_2$SO$_4$ and then evaporated to dryness. The resulting solid was purified by flash chromatography using a 2%-15% methanol/dichloromethane gradient. The resulting clear oil was added to 5 mL of 30% TFA/DCM and reacted for 1 hour at room temperature. The TFA/DCM was removed by evaporation and the resulting residue was dissolved in 1M HCl and filtered. The filtrate was made alkaline by the addition of 10 M NaOH, followed by extraction with 3x50 mL of DCM. The organic phase was dried with Na$_2$SO$_4$ and evaporated to dryness to obtain 455 mg (51% yield) of spectroscopically pure (>95% at 254 nm) clear oil. $^1$H NMR (400 MHz, DMSO-d$_6$) δ 7.98 (d, J = 8 Hz, 1H), 7.63 – 7.56 (m, 1H), 7.47 – 7.38 (m, 1H), 7.30 – 7.21 (m, 1H), 6.55 (s, 2H), 4.76 (s, 2H), 4.54 (q, J = 6.3, 4.4 Hz, 2H), 3.54 (q, J = 7.0 Hz, 2H), 2.58 (t, J = 6.9Hz, 2H), 1.93-1.81 (m, 2H), 1.52 (m, 2H), 1.15 (t, J = 7.0Hz, 3H). MS (APCI) calculated for C$_{56}$H$_{46}$N$_6$O$_3$ m/z 560.3, found 561.3 (M+H)$^+$. 

(14) SM 7/8a-Alkane, 12-amino-N-(4-(4-aminobutyl)-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-1-yl)butyl)dodecanamide. To 20 mL of ethyl acetate was added 200 mg (0.64 mmol, 1 eq) of (12), 112 mg (0.64 mmol, 1 eq) of CDMT and 222 mg (0.70 mmol, 1.1 eq), of N-boc-aminodecanoic acid followed by the dropwise addition of 176 µL (1.6 mmol, 2.5 eq) of NMM while stirring vigorously. After 16 hours at room temperature, the reaction mixture was filtered and washed 3x50 mL with 1 M HCl. The organic phase was dried with Na$_2$SO$_4$ and then evaporated to dryness. The resulting solid was suspended in 5 mL of 30% TFA/DCM and reacted for 1 hour at room temperature. The TFA/DCM was removed by evaporation and the resulting residue was dissolved in 1M HCl and filtered. The filtrate was made alkaline by the addition of 10 M NaOH, followed by extraction with 3x50 mL of DCM. The organic phase was dried with Na$_2$SO$_4$ and evaporated to dryness to obtain 279 mg (85.4% yield) of spectroscopically pure (>95% at 254 nm) white solid. $^1$H NMR (400 MHz, DMSO-d$_6$) δ 7.98 (d, J = 8.1 Hz, 1H), 7.74 (t, J = 5.7 Hz, 1H), 7.60 (d, 8 Hz, 1H), 7.42 (t, J = 7.6 Hz, 1H), 7.24 (t, J = 7.5 Hz, 1H), 6.56 (s, 2H), 

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4.75 (s, 2H), 4.53 (t, J = 7.9 Hz, 2H), 3.54 (q, J = 7.0 Hz, 2H), 3.07 (q, J = 6.4 Hz, 2H), 2.60 (t, J = 7.1 Hz, 2H), 1.97 (t, J = 7.4 Hz, 2H), 1.87–1.78 (m, 2H), 1.55 (t, J = 7.6 Hz, 2H), 1.43–1.34 (m, 5H), 1.24–1.10 (m, 18H). MS (APCI) calculated for C_{29}H_{44}N_{6}O_{2} m/z 510.4, found 511.4 (M+H)^+.

(15) SM 20x7/8a, 1-(4-(aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine. Deprotection of (11) required milder conditions as compared with (12) so as to avoid removal of the xylene diamine linker. Simultaneous removal of the 2,4-dimethoxybenzyl and Boc groups was achieved by adding 300 mg of (11) to a 30 mL solution of 40% TFA/DCM that was stirred at room temperature for 30 hours. The reaction mixture turned from clear to deep red over several hours and the reaction was monitored by HPLC. After completion, the reaction mixture was evaporated to dryness and the resulting red oil was suspended in 200 mL of 1 M HCl. Insoluble pink material was removed by filtration and the resulting clear aqueous solution was adjusted to pH 10 using 10 M NaOH. The aqueous layer was extracted 6x100 mL using DCM as the organic phase. The organic layer was dried with Na2SO4 and evaporated to dryness, yielding 172 mg (89.6% yield) of spectroscopically pure (>95% at 254 nm) white powder. 1H NMR (400 MHz, DMSO-d6) δ 7.77 (dd, J = 8.4, 1.4 Hz, 1H), 7.55 (dd, J = 8.4, 1.2 Hz, 1H), 7.35–7.28 (m, 1H), 7.25 (d, J = 7.9 Hz, 2H), 7.06–6.98 (m, 1H), 6.94 (d, J = 7.9 Hz, 2H), 6.50 (s, 2H), 5.81 (s, 2H), 3.64 (s, 2H), 2.92–2.84 (m, 2H), 2.15 (s, 2H), 1.71 (q, J = 7.5 Hz, 2H), 1.36 (q, J = 7.4 Hz, 2H), 0.85 (t, J = 7.4 Hz, 3H). MS (APCI) calculated for C_{22}H_{25}N_{5} m/z 359.2, found 360.3 (M+H)^+.

(16) SM 20x7/8a-PEG, 1-(4-(aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine. The same reaction conditions and purification scheme were used as for the preparation of (13), except 100 mg of (15) was used in place of (12). 126.2 mg (96% yield) of spectroscopically pure (>95% at 254 nm) clear oil was obtained. 1H NMR (400 MHz, DMSO-d6) δ 8.31 (t, J = 6.0 Hz, 1H), 7.93 (d, J = 8.4 Hz, 1H), 7.78 (d, J = 8.3 Hz, 1H), 7.71 (s, 4H), 7.61 (t, J = 7.8 Hz, 1H), 7.35 (t, J = 7.8 Hz, 1H), 7.18 (d, J = 8.0 Hz, 2H), 7.00 (d, J = 8.0 Hz, 2H), 5.92 (s, 2H), 4.20 (d, J = 5.9 Hz, 2H), 3.62–3.44 (m, 16H), 3.00–2.90 (m, 4H), 2.33 (t, J = 6.4 Hz, 2H), 1.75–1.67 (m, 2H), 1.37 (q, J = 7.4 Hz, 2H), 0.85 (t, J = 7.3 Hz, 3H). MS (APCI) calculated for C_{33}H_{46}N_{6}O_{5} m/z 606.4, found 607.3 (M+H)^+.

(17) AP–PEG, 1-amino-N-((6-aminopyridin-3-yl)methyl)-3,6,9,12-tetraoxapentadecan-15-amide. The same reaction conditions and purification scheme were used as for the preparation of (13), except 50 mg of tert-Butyl 5-(aminomethyl)-2-pyridinylcarbamate was used in place of (12). 73 mg (88% yield) of spectroscopically pure (>95% at 254 nm) clear oil was obtained. 1H NMR (400 MHz, DMSO-d6) δ 8.32 (t, J = 5.9 Hz, 1H), 7.76 (d, J = 2.1 Hz, 1H), 7.66 (dd, J = 9.0, 2.2 Hz, 1H), 7.51 (s, 2H), 6.81 (d, J = 9.0 Hz, 1H), 4.10 (d, J = 5.8 Hz, 2H), 3.67–3.37 (m, 16H), 2.96 (s, 2H), 2.53 (p, J = 1.9 Hz, 1H), 2.43 (p, J = 1.9 Hz, 1H), 2.33 (t, J = 6.4 Hz, 2H). MS (APCI) calculated for C_{17}H_{36}N_{4}O_{5} m/z 370.2, found 371.2 (M+H)^+.

(18) AP–azide, N-((6-aminopyridin-3-yl)methyl)-5-azidopentanamide. The same reaction conditions and purification scheme were used as for the preparation of (13), except 50 mg of tert-Butyl 5-(aminomethyl)-2-pyridinylcarbamate was used in place of (12). 21.4 mg (39% yield) of spectroscopically pure (>95% at 254 nm) clear oil was obtained. 1H NMR (400 MHz, DMSO-d6) δ 8.25 (t, J = 5.7 Hz, 1H), 7.75 (d, J = 2.2 Hz, 1H), 7.65–7.57 (m, 1H), 7.22 (s, 2H), 6.75 (d, J = 8.9 Hz, 1H), 4.07 (d, J = 5.8 Hz, 2H), 2.43 (m,
2H), 2.11 (t, J = 7.0 Hz, 2H), 1.50 (m, 4H). MS (APCI) calculated for C₁₁H₁₆N₆O m/z 248.1, found 249.1 (M+H)⁺.

**Synthesis of SM TLR-7/8a dye conjugates**

(19) SM 7/8a-AF488.
The AF488 dye conjugate of the small molecule TLR-7/8a was synthesized by reacting 2 mg (2.3 µmoles, 1 eq) of Alexa Fluor® 488 carboxylic acid tetrafluorophenyl ester with 0.85 mg (2.7 µmoles, 1.2 eq) of (12) in 300 µL of anhydrous DMSO. The reaction was monitored by HPLC and the product, (19), was purified by semi-prep HPLC using a 25% to 35% ACN/H₂O gradient over 16 minutes. The reaction mixture was injected over 3 runs. Fractions containing (19) were consolidated, frozen and lyophilized to yield 1.6 mg (85.5% yield) of spectroscopically pure (>95% at 254 nm) product. MS (ESI) calculated for C₃₈H₃₃N₇O₁₁S₂ m/z 827.2, found 827.7 (M+H)⁺.

(20) SM 7/8a-IRDye800
For the IR Dye conjugate of the SM 7/8a, a PEG spacer was required to increase solubility. The same reaction conditions and purification scheme were used as for the preparation of (19), except 4 mg (3.4 µmoles, 1 eq) of IR Dye 800cw NHS ester was used as the dye and reacted with 2.3 mg (4.1 µmoles, 1 eq) of (13). 3.8 mg (71% yield) of spectroscopically pure (>95% at 254 nm) product was obtained. MS (ESI) calculated for C₇₄H₉₆N₈O₂₀S₄ m/z 1546, found 1547 (M+H)⁺.

**Synthesis of amine-reactive HPMA-based copolymers**
The N-(2-hydroxypropyl)methacrylamide (HPMA)-based statistical copolymer, p[(HPMA)-co-(Ma-ε-Ahx-TT)], was synthesized by free radical solution polymerization as previously described. Briefly, a mixture of HPMA (9.8 wt%), 2-Methyl-N-[6-oxo-6-(2-thioxo-thiazolidin-3-yl)-hexyl]-acrylamide (Ma-ε-Ahx-TT) (5.2 wt%) and azobisisobutyronitrile (AIBN) (1.5 wt%) were dissolved in DMSO (83.5 wt%) and polymerized at 60°C for 6 hours under argon atmosphere. The polymer was precipitated from a 1:1 mixture of acetone and diethyl ether and then dissolved into methanol and precipitated from a 3:1 mixture of acetone and diethyl ether. The content of TT reactive groups determined by UV-Vis spectrophotometry was 14.8 mol% (ε₃₀₅ =10,300 L/mol); the weight- and number-average molecular weights determined by size exclusion chromatography (SEC) were Mₘ = 31,850 g/mol and Mₙ = 20,330 g/mol, respectively.

**Synthesis of amine-reactive NIPAM-based (thermo-responsive) copolymers**
The N-isopropylacrylamide (NIPAM)-based statistical copolymer p[(NIPAM)-co-(Ma-Ahx-TT)] was prepared by free radical solution polymerization as described elsewhere. Briefly, a mixture of NIPAM (10.2 wt%), Ma-ε-Ahx-TT (4.8 wt%) and AIBN (1.5 wt%) was dissolved in DMSO (83.5 wt%) and polymerized at 60°C for 18 hours under argon atmosphere. The reaction mixture was diluted with an HCl aqueous solution (pH 2) and then extracted with dichloromethane (3x). The combined organic phases were dried and evaporated. The resulting residue was dissolved in methanol and precipitated into a 3:1 mixture of acetone and diethyl ether. The content of TT reactive groups determined by UV-Vis spectrophotometry was 10.2 mol% (ε₃₀₅ =10,300 L/mol); the weight- and number-average molecular weights determined by SEC were Mₘ = 26,830 g/mol and Mₙ = 17,650 g/mol, respectively.
Synthesis of polymer-TLR-7/8a (Poly-7/8a) conjugates

Example: To generate p[(HPMA)-co-(Ma-ε-Ahx-PEG4-7/8a)] with an agonist density of ~10 mol% TLR-7/8a, 10 mg (8.4 µmole TT, 1 eq) of p[(HPMA)-co-(Ma-ε-Ahx-TT)] with ~14 mol% TT was added to 1 mL of anhydrous methanol. To this solution, 470 µL (4.7 mg, 6.0 µmole, 0.7 eq) of a 10 mg/ml solution of 13 (SM 7/8-PEG) in anhydrous DMSO was slowly added while stirring vigorously. After 16 hours, 1.25 mg (16.8 µmole, 2 eq) of 1-aminopropanol was added to remove unreacted TT groups. After an additional 2 hours, the reaction mixture was dialyzed 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 dialysis tube was suspended in 1000 mL of methanol and the dialysis buffer was changed twice each day for 3 days. The methanol solution containing Poly-7/8a was evaporated to dryness and yielded 11.4 mg of p[(HPMA)-co-(Ma-ε-Ahx-PEG4-7/8a)]. The content of 7/8a-PEG determined by UV-Vis spectrophotometry was 7.9 mol% 7/8a (ε325 =5.012 L/mol); the weight- and number-average molecular weights determined by SEC were \( M_w = 55,680 \) g/mol and \( M_n = 33,850 \) g/mol, respectively.

Synthesis of second-generation TRPP-7/8a with ESE coil peptide

TRPP: p[(HPMA)-co-(PgMA)]-b-block-p(DEGMA)

Second generation TRPP-7/8a were produced as thermo-responsive A-B type di-block copolymers by RAFT polymerization in two synthetic steps. The hydrophilic block A was prepared by copolymerizing HPMA with N-propargyl methacrylamide (PgMA) using 4,4’-azobis(4-cyanovaleic acid) (ACVA) as an initiator and 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTP) as a chain transfer agent in molar ratios [M]:[CTP]:[ACVA] = 142:2:1 in 1,4-dioxane / H2O mixture. Briefly, a mixture of 7.6 mg CTP (27.3 µmol) and 3.8 mg ACVA (13.7 µmol) was dissolved in 647 µL of 1,4-dioxane and added to the solution of 250.0 mg HPMA (1.75 mmol) and 23.9 mg PgMA (0.19 mmol) in 1293 µL of DI H2O. The reaction mixture was thoroughly bubbled with Argon and polymerized in sealed glass ampoules at 70°C for 6 h. The resulting copolymer was isolated by precipitation into a 3:1 mixture of acetone and diethyl ether yielding 84.4 mg of the product. The content of dithiobenzoate (DTB) end groups determined by UV-Vis spectrophotometry was 31.1 µmol/g (ε302 =12,100 L/mol). The average molecular weights determined by SEC were \( M_w = 9,809 \) g/mol and \( M_n = 9,229 \) g/mol, respectively. The content of PgMA determined by 1H NMR was 9.8 mol%.

The hydrophilic polymer block A bearing DTB terminal groups was subjected to a chain-extension polymerization through the RAFT mechanism with di(ethylene glycol) methyl ether methacrylate (DEGMA) to introduce the thermo-responsive polymer block B. Briefly, a mixture of 50.0 mg p[(HPMA)-co-(PgMA)] (5.31 µmol ~DTB gr.), 53.0 mg DEGMA (0.28 mmol) and 0.30 mg ACVA (1.06 µmol) was dissolved in 477 µL of 1,4-dioxane / H2O (2:1) solution and thoroughly bubbled with argon gas before sealing the glass ampoule reaction vessel and carrying out the reaction at 70°C for 18 h. The di-block polymer was isolated by precipitation to diethyl ether followed by re-precipitation from methanol to 3:1 mixture of acetone and diethyl ether to yield 84.4 mg of the product. The content of dithiobenzoate (DTB) end groups determined by UV-Vis spectrophotometry was 31.1 µmol/g (ε302 =12,100 L/mol).
To remove the DTB end groups, the polymer and 12.9 mg of AIBN (0.79 µmol) were dissolved in 844 µL of DMF and the solution was heated to 80 °C for 2 h. The resulting polymer was isolated by precipitation in diethyl ether and purified by gel filtration using a Sephadex™ LH-20 cartridge with methanol as the eluent. The polymer solution was concentrated in vacuo and precipitated in diethyl ether yielding 72.4 mg of the product. The weight- and number-average molecular weights determined by SEC were $M_w = 22,020$ g/mol and $M_n = 16,790$ g/mol, respectively. The transition temperature (TT) of the polymer, determined by DLS, was 38°C at 1.0 mg/mL 15 M PBS (pH 7.4).

**Attachment of TLR-7/8a, ESE coil peptide and fluorophore to TRPP**

Different ligands (TLR-7/8a, ESE-coil peptide, scrambled peptide or fluorophore) functionalized with an azide group were attached to TRPP through the propargyl side chain moieties distributed along the hydrophilic block A of the copolymer by copper catalyzed 1,3 dipolar cycloaddition reaction. Reaction progress was monitored by HPLC.

**Example:** A mixture of 20.0 mg TRPP (7.1 µmol propargyl group), 1.0 mg TLR-7/8a-azide (2.1 µmol), 0.4 mg Carboxyrhodamine 110-azide (0.7 µmol), 4.6 mg ESE-coil peptide-azide (1.4 µmol) and 1.1 mg TBTA (2.1 µmol) was dissolved in 460 µL of DMSO and the solution was thoroughly bubbled with Argon. Then, 0.84 mg sodium ascorbate (4.2 µmol) in 168 µL of degassed water was added. Finally, a solution of 0.54 mg CuSO$_4$ in 108 µL of degassed water was pipetted to the reaction mixture to initiate the “click” reaction. The reaction was performed overnight at 45 °C until no unreacted ligands were detected by HPLC. The reaction mixture was diluted (1:1) with a saturated solution of EDTA in 0.15 M PBS (pH 7.4) and the conjugate was purified by gel filtration using a Sephadex™ PD-10 column with H$_2$O as the eluent. The resulting conjugate was isolated from an aqueous solution by lyophilisation yielding 18.6 mg of the product.
Attachment of HIV Gag-KSK to fluorescently labelled TRPP-ESE conjugate via the coiled coil interaction

Formation of TRPP-(coil-coil)-Gag complex was performed in PBS buffer by mixing TRPP-ESE with HIV Gag-KSK at 1.5/1.0 molar ratio (based on coil peptides). Formation of the coiled-coil complex was measured using SEC on MicroSuperose 12 column and by analytical ultracentrifugation (AUC) 1 hour after mixing.
Determination of TLR-7/8a and fluorophore content on polymers

The amount of ligand attached to the copolymers was determined by UV-Vis spectroscopy using the Beer-Lambert law relationship (\( A = \varepsilon c \); where \( A \) = absorption and \( c \) = mol/L). Samples were suspended in solutions of 1% triethylamine/methanol at known densities (mg/mL) and added to quartz cuvettes with a path length of 1 cm. Absorption was recorded over a spectrum from 250 – 775 nm using a Lambda25 UV-Vis spectrometer from Perkin Elmer. For example, a 0.1 mg/mL solution of Poly-7/8a in 1% triethylamine/methanol (\( \lambda_{\text{max}} = 325 \text{ nm} \), \( \varepsilon_{325} = 5012 \text{ L/mol} \)) has an optical density (OD, arbitrary units) of 0.25 at 325 nm. The concentration of TLR-7/8 can be calculated by solving for \( c \) in the Beer-Lambert law relationship and is \( 5 \times 10^{-5} \text{ mol/L} \), which can be expressed as the amount of TLR-7/8a per mass of copolymer (5e-4 mmol/mg).

The Beer-Lambert relationship was used to determine the amount of ligand molecules and dyes attached to the polymers based on known extinction coefficients.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Max absorption (( \lambda_{\text{max}}, \text{nm} ))</th>
<th>Extinction coefficient, (L/mol) 1% triethylamine / methanol</th>
<th>( A_{325} / A_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopyridine</td>
<td>305</td>
<td>3,511</td>
<td>---</td>
</tr>
<tr>
<td>TLR-7/8a (SM 7/8a)</td>
<td>325</td>
<td>5,012</td>
<td>1.00</td>
</tr>
<tr>
<td>AF488</td>
<td>495</td>
<td>167,415</td>
<td>0.12</td>
</tr>
<tr>
<td>Cruz Fluor 8</td>
<td>775</td>
<td>71,493</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Methods table 1: Absorption maxima and extinction coefficients were determined for different ligand and dye molecules in 1% triethylamine/methanol. Note that for copolymers with both TLR-7/8a and dye (AF488 or Cruz Fluor 8), the contribution of absorption at 325 nm by the dye was determined using the relationship described by \( A_{325} / A_{\text{max}} \).

Agonist density (mol% 7/8a) determination

UV-Vis can be used to estimate the agonist density (mol%) of co-monomers. Mol% of co-monomer \( y \), for a statistical copolymer comprised of monomers \( x \) and \( y \) is estimated using the following relationship:

\[
\text{mol}\%_y = \frac{1}{1 + \left( \frac{1}{\varepsilon \times A \times M_{w_x}} - \frac{M_{w_y}}{M_{w_y}} \right)} \times 100
\]

\( \text{mol}\%_y \) (agonist density) = percentage of copolymer that is \( y \) (e.g., TLR-7/8a containing monomer), for copolymer comprised of \( x \) and \( y \) monomers

\( \rho \) = volumetric mass density (mg/mL) of copolymer during UV-Vis measurement

\( \varepsilon \) = molar extinction coefficient for monomer \( y \) (e.g. for TLR-7/8a = 5,012)

\( A \) = Absorbance

\( M_{w_x} \) = molecular weight (g / mol) of majority monomer

\( M_{w_y} \) = molecular weight (g / mol) of minority monomer

Example calculation:

For poly-7/8a comprised of the majority monomer HPMA (\( M_{w_{\text{HPMA}}} = 143.2 \)) and minority monomer containing the TLR-7/8a (MA-Ahx-PEG4-7/8a; \( M_{w_{\text{MA-Peg47/8a}}} = 741.9 \)) that is suspended in methanol at 0.1 mg / mL and measures an average absorbance of 0.25 at 325 nm, the mol% of the minority unit, MA-PEG4-7/8a is:

\[
\text{mol}\%_{MA-Ahx-PEG4-7/8a} = \left( \frac{1}{1 + \left( \frac{0.1 \times 5012}{0.25 \times 143.2 - 741.9} \right)} \right) \times 100 = 10.2\%
\]
Synthesis of conjugatable TLR-4 agonists

(21) PI-NH₂, tert-butyl (4-(2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamido)cyclohexyl)carbamate. The pyrimidoindole carboxylic acid precursor (2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic acid) was prepared as recently described. 100 mg of this compound (0.28 mmol, 1 eq) and 67.1 mg (0.31 mmol, 1.1 eq) of N-Boc-trans-1,4-cyclohexanediamine were then added to 2 mL of DMF with triethylamine 80 µL Et₃N (0.56 mmol, 2 eq). A solution of 118 mg (0.31 mmol, 1.1 eq) of HATU in 400 µL of DMF was then added to the reaction mixture. The reaction was stirred at RT for 24 h. The solution was concentrated and recrystallized from methanol to provide the Boc-protected product as a white solid (108 mg, 70% yield).

1H NMR (500 MHz, DMSO-d6) δ 12.1 (s, 1H), 8.08 (d, J = 8, 1H), 7.63-7.61 (br m, 2H), 7.53 (t, J = 8, 1H), 6.72 (d, J = 8, 1H), 3.89 (s, 2H), 3.43 (br s, 1H), 3.17 (br s, 1H), 1.76 (br t, J = 13, 4H), 1.38 (s, 9H), 1.30-1.14 (br m, 4H). 13C NMR (500 MHz, DMSO-d6) δ 166.4, 155.4, 153.0, 139.4, 137.7, 136.6, 130.0, 129.7, 129.4, 128.5, 127.8, 120.8, 120.6, 119.7, 114.7, 113.3, 77.9, 48.1, 46.2, 37.2, 31.7, 31.6, 28.8. TLC: 100% Ethyl acetate, Rf 0.7. HRMS: m/z calcld for C29H33N5O4S [M+Na]+ 570.2, observed 570.2. 50 mg of the resulting Boc protected compound was then added to 5 mL of 30% TFA/DCM and reacted for 1 hour at room temperature. The TFA/DCM was removed by evaporation and the resulting residue was dissolved in 1M HCl and filtered. The filtrate was made alkaline by the addition of 10 M NaOH, followed by extraction with 3x50 mL of DCM. The organic phase was dried with Na₂SO₄ and evaporated to dryness to obtain 33 mg (80.8% yield) of a spectroscopically pure (>95% at 254 nm) white solid. MS (ESI) calculated for C24H25N5O2S m/z 447.17, found 448.2 (M+H)+.
**Example:** The polymer-particle forming TLR-4a conjugate (PP-PI) described in this study was prepared by reacting \((22)\) with amine reactive \(p((HPMA)-co-(Ma-β-Ala-TT))\). In short, 5 mg (3.7 µmol, TT, 1 eq) of \(p((HPMA)-co-(Ma-β-Ala-TT))\) with ~11.7 mol% TT was added to 500 µL of anhydrous methanol. To this solution was added 2.6 mg (3.7 µmol, 1 eq) of a 10 mg/ml solution of \((22)\) in anhydrous DMSO while stirring vigorously. After 16 hours, 2 eq of 1-amino-2-propanol was added to remove unreacted TT groups. After an additional 2 hours, the reaction mixture was dialyzed 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 dialysis tube was suspended in 1000 mL of methanol and the dialysis buffer was changed twice each day for 3 days. The methanol solution containing Poly-PEG-PI was evaporated to dryness and yielded 6.7 mg of \(p((HPMA)-co-(Ma-β-Ala-PEG-PI))\). The content of PI-PEG determined by UV-Vis spectrophotometry was 6.3 mol% (ε\(_{340}\) =7,272 L/mol). 2.744 ± 384.8 nm z-average diameter at 0.1 mg/mL PBS. 414.1 ± 135.4 nm z-average diameter at 0.1 mg/mL PBS.
Synthesis of conjugatable Pam2cys (TLR-2/6a)

(23) Pam2Cys-PEG-N3 14-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-1-azido-13-oxo-3,6,9-trioxa-16-thia-12-azanondecane-18,19-diyl dipalmitate, 1-amino-N-(4-(2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamido)cyclohexyl)-3,6,9,12-tetraoxapentaadecan-15-amide. To a 20 mL solution of 1:1 DCM/Methanol, was added 100 mg (0.11 mmol, 1 eq) of Fmoc-protected Pam2Cys-COOH (Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH) (Bachem, Bubendorf, Switzerland) 27 mg (0.12 mmol, 1 eq) of Amino-11-azido-3,6,9-trioxaundecane and 20 mg (0.11 mmol, 1 eq) of CDMT, followed by the dropwise addition of 25 µL (0.22 mmol, 2.0 eq) of NMM, while stirring vigorously. After 16 hours at room temperature, the reaction mixture was filtered and then washed 3x50 mL with 1 M HCl. The organic phase was dried with Na2SO4 and then evaporated to dryness to yield a white solid which was further purified by flash column chromatography using 0-10% methanol / DCM gradient. Fractions were combined and evaporated to dryness to obtain 75.6 mg (62% yield) of spectroscopically pure (>95% at 254 nm by TLC) white solid. MS (APCI) calculated for C61H99N2O10S m/z 1093.7, found 1113 (M+H3O)+ and 1208 (M+TFA)+.

Synthesis of Polymer-2/6 conjugates (PP-Pam2Cys)

Example: The polymer-particle forming TLR-2/6a conjugate described in this study was prepared by reacting (23) with amine reactive p[(HPMA)-co-(Ma-β-Ala-TT)] in a 3 step reaction. In the first step, 5 mg (3.7 µmol TT, 1 eq) of p[(HPMA)-co-(Ma-β-Ala-TT)] with ~11.7 mol% TT was added to 500 µL of anhydrous methanol. To this solution was added 98 µL (1.96 mg, 3.7 µmol, 1 eq) of a 10 mg/mL solution of the cross-linker, DBCO-PEG₆NH₂, in anhydrous DMCS while stirring vigorously. After 2 hours, 204 µL (2.04 mg, 3.7 µmol, 1 eq) of a 10 mg/mL solution of (23) was then added while stirring the reaction mixture vigorously. After 16 hours, 2 eq of 1-amino-2-propanol was added to remove unreacted TT groups. After an additional 2 hours, the reaction mixture was dialyzed 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 dialysis tube was suspended in 1000 mL of a 1:1 methanol/DCM solution and the dialysis buffer was changed twice over 1 day. The methanol solution containing Poly-PEG-Pam2Cys(Fmoc) was evaporated to dryness and then suspended in a 1 mL solution of 20% Piperidine/DMF for 1 hour to remove the Fmoc group. The reaction mixture was then dialyzed again against a solution of 1:1 methanol/DCM and the dialysis buffer was changed after 15 minutes, and then twice per day for 3 days. The methanol solution containing Poly-PEG-Pam2Cys was evaporated to dryness and yielded 8.1 mg of p([(HPMA)-co-(Ma-β-Ala-PEG-Pam2Cys)]). The content of Pam2Cys-PEG determined by UV-Vis spectrophotometry was 4.5 mol% Pam2Cys as determined using the TNBSA assay to measure primary amine content (ε₄₂₀ =11,500 L/mol). 2.744 ± 384.8 nm z-average diameter at 0.1 mg/mL PBS.
Formulation of MPL (TLR-4a) and CpG (TLR-9a) with particulate carriers
Both Monophosphoryl Lipid A (MPL) and CpG ODN 1826 were purchased from Invivogen as vaccine grade adjuvants. Alum/MPL for immunizations was comprised of a solution of PBS with 0.1 mg/mL MPL and 1 mg/mL Aluminum Hydroxide (Alhydrogel, Invivogen) that was allowed to incubate at room temperature for 2 hours prior to immunization. Polymer/CpG poly(plex) particles were prepared by formulating 16 kD Poly(L-lysine hydrochloride) (Alamanda Polymers, Huntsville, AL, USA) linear polymers with CpG ODN 1826 at 20:1 N:P in PBS.

References