TLR7/8-agonist-loaded nanoparticles promote the polarization of tumour-associated macrophages to enhance cancer immunotherapy

Christopher B. Rodell1, Sean P. Arlauckas2, Michael F. Cuccarese1, Christopher S. Garris2,1, Ran Li1, Maaz S. Ahmed1, Rainer H. Kohler3,1, Mikael J. Pittet1 and Ralph Weissleder1,3*

Tumour-associated macrophages (TAMs) have recently attracted much attention, as they play key roles in tumour metastasis and therapeutic resistance. TAMs often accelerate the progression of untreated tumours1–5, influence the efficacy of anticancer drugs6–10, including checkpoint blockade immunotherapies8–10, and increased counts of TAMs are associated with worse outcomes11–12. TAMs are considerably plastic and assume opposing phenotypes and functions that can be either tumour-supportive (M2-like cells) or tumoricidal (M1-like cells)13–15. In most tumours, the tumour-suppressive M2 phenotype prevails, and efforts have been underway to either deplete M2-like cells or convert (re-educate) the phenotype into tumoricidal M1-like cells. There has been intense interest in the latter strategy, exploiting the use of small molecules to inhibit receptors16,17, tyrosine kinases18 or other transcription pathways in TAMs. Despite early preclinical studies19 and ongoing clinical trials, hurdles remain for developing new therapeutic agents that can overcome some of the limitations of current immunotherapies. Specific limitations include (i) a deficient understanding of which small molecules most efficiently impact M2 → M1 phenotypic conversion, (ii) an inadequate ability to preferentially deliver small molecules to TAMs in vivo and (iii) resulting sub-optimal therapeutic efficacy.

To address these challenges, we set out to develop a high-throughput and low-cost phenotypic screening assay to directly compare the efficiency with which different small molecules re-educate M2-like macrophages into M1-like cells. We furthermore hypothesized that the drugs emerging from this screen could be delivered to TAMs more efficiently in vivo when encapsulated in nanotherapeutics, as nanoparticles are often rapidly internalized by macrophages16,20. Systemic therapy using such an approach would be particularly useful in treating surgically inaccessible tumours, where intratumoural injections are not feasible. The kinetics and TAM-associated distribution of many nanoformulations, including modified dextran21, copolymers22 and liposomes23 have been demonstrated using intravital microscopy; however, we found many of these materials to have only modest capacity for incorporation of small molecule TAM modifying agents. Thus, we sought to capitalize on the use of β-cyclodextrin (CD) as a supramolecular drug reservoir. CD has an extensive history in industrial and pharmaceutical trials24–28. We therefore hypothesized that covalent crosslinking of CD would enable formation of cyclodextrin nanoparticles (CDNPs) with macrophage affinity and high drug-loading capacity. Using different mouse and tumour models that allowed direct visualization of TAM subsets in vivo, we demonstrate the ability to leverage macrophage affinity for drug-loaded nanoparticles to achieve efficient TAM delivery, preferentially alter myeloid phenotype and subsequently improve immunotherapy response including through cooperation with checkpoint therapy, which resulted in remarkable anti-cancer efficacy.

Results
Development of a morphometric polarization screen. The tumour microenvironment (TME, Fig. 1a) is home to diverse host cell types. Tumour-associated macrophages (TAMs) often represent a dominant proportion of the immune cell infiltrate21, and a continuum of macrophage phenotypes exist that can be challenging to fully recapitulate in vitro. TAMs predominantly assume a tumour-supportive M2-like signature that includes the expression of mannose receptor-1 (MRC1) and the metabolic checkpoint enzyme

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1Center for Systems Biology, Massachusetts General Hospital Research Institute, Boston, MA, USA. 2Graduate Program in Immunology, Harvard Medical School, Boston, MA, USA. 3Department of Systems Biology, Harvard Medical School, Boston, MA, USA. *e-mail: ralph.weissleder@hms.harvard.edu
arginase-1 (ARG1), which are induced by IL4 treatment (Fig. 1b). In contrast, classically activated (M1-like, LPS and IFN-γ treated) cells are characterized by the expression of nitric oxide synthase (NOS2) and interleukin 12 (IL12) (Fig. 1b)\textsuperscript{29,30}. This dichotomy provides a simplified, tractable model for in vitro examination of macrophage polarization phenotypes\textsuperscript{36}, where computationally assigned shape-feature weights were determined from supervised training data sets (Supplementary Fig. 1) and reflected the relative changes in cell-shape features between M1- and M2-like population states (Fig. 1c). The proportional increase in M1-like cells within the examined population is expressed simply as M1 enrichment.

**Morphometric HCS identifies re-polarizing drugs.** Having established a HCS for examination of cell state, we next sought to identify drugs capable of macrophage re-education. A panel of 38 drugs was curated from the literature, representing specific drugs or drug classes which have been implicated in macrophage polarization (Fig. 2a, Supplementary Table 2). Freshly isolated murine monocytes were differentiated into an M2-like phenotype, followed by drug treatment spanning six orders of magnitude in drug concentration.

Morphometric analysis readily stratified drug activity (Fig. 2b). Macrophage colony-stimulating factor 1 receptor (CSF1R) activation is a driving signal in M2-like polarization and experimental CSF1R inhibition is known to bias macrophage polarization in addition to altering TAM recruitment and distribution in vivo\textsuperscript{35,36}. With the exception of GW2580, inhibitors of CSF1R demonstrated enrichment of the M1 population in a dose-dependent manner (Fig. 2b). We observed similar enrichment in the M1 population
with a number of tyrosine kinase inhibitors, often to a lesser degree, including for imatinib (Bcr-Abl), dabrafenib (B-raf), gefitinib (EGFR), XL228 (IGF1R) and UNC2025 (MerTK). However, the largest polarization effects were observed for agonists of the pattern recognition receptors toll-like receptor 7 and 8 (TLR7/8). Agonists including motolimod (TLR7 agonist), GS9620 (TLR8 agonist) and R848 (resiquimod, dual TLR7/8 agonist) yielded M1 enrichments that were as pronounced as standard M1 induction by LPS and IFN-γ. In a direct comparison of the latter three TLR agonists, R848 emerged as the most potent driver of macrophage re-education, with a half-maximal effective concentration (EC50) and TLR8-specific agonists examined (Fig. 2c). In vitro, murine macrophages re-educated by R848 closely resembled M1 controls of 14.1 nM, an order of magnitude improvement relative to TLR7-specific agonists (Fig. 2d). Activity in the nanomolar range was likewise observed for macrophages re-educated by R848 by qPCR analysis of representative M1-like (nos2, il12b and cd80) and M2-like (mrc1, arg1) transcripts (Supplementary Fig. 3). Strong correlation (R² > 0.92) between transcriptional and morphological phenotypes was observed, validating the ability of M1 enrichment to predict expression of an inflammatory transcriptome. Although differences between human and murine TLR7/8 may exist, including a reduced expression and activity of TLR8, we observed similar results in human cells (Supplementary Fig. 4).

**Design of nanoparticles with drug-binding affinity and TAM avidity.** In prior work, we have shown that certain dextran nanoparticles have native macrophage avidity, which results in rapid, preferential distribution to TAMs relative to other cells present in the TME. β-Cyclodextrin (CD) shares a similar chemical composition with linear dextran, suggesting potential for macrophage avidity. Moreover, host–guest inclusion by macrocycles, such as CD, is an established mechanism for drug solubilization and nanoparticle-mediated drug delivery that forgoes chemical modification of established drug compounds. Therefore, we sought to leverage the interaction of CD with R848 to enable formation of drug-loaded nanoparticles.

CDNPs were formed through amide bond formation between succinyl-β-cyclodextrin and l-lysine under aqueous conditions. Concentration-dependent studies showed that nanoparticles with a diameter of approximately 30 nm, preferable for phagocytic uptake, were reliably synthesized overnight starting with a 3.3%w/v solution of succinyl-β-cyclodextrin. A molar...
To further interrogate the intratumoural kinetics and cellular distribution of the CDNP, we employed a dorsal window chamber setup for intravital imaging\(^\text{43,44}\). Tumours were generated by inoculation with \(1 \times 10^6\) MC38-H2B-mApple cells, allowing identification of tumour cells. To enable identification of TAMs, we utilized a recently described reporter mouse wherein TAMs are readily detectable through MerTK\(^{\text{GFP+}}\) expression\(^\text{45}\). In following intravenous administration of CDNP-VT680 by time lapse microscopy, we observed its rapid vascular distribution adjacent to and throughout the tumour (Fig. 5a) and accumulation within GFP\(^+\) perivascular macrophages within 60 min (Fig. 5b, Supplementary Video 1), demonstrating nanoparticle accumulation in TAMs to be the primary mode of uptake as opposed to intratumoural migration of CDNP-loaded monocytes from the vasculature. At 24 h following administration, CDNPs were cleared from the vasculature and had accumulated within TAMs throughout the tumour (Fig. 5c,d). When macrophage uptake was compared across tissues, it was highest in tumours on a per cell basis (Supplementary Fig. 8). Moreover, CDNP accumulation was dominated by macrophages in the TME, relative to other immune cell types, and accumulation in tumour cells was not observed (Supplementary Fig. 9).

We also examined R848 delivery to TAMs in an orthotopic lung adenocarcinoma model (eGFP-expressing Kras\(^{\text{G12D}}\) p53\(^{-/-}\)-mutant (KP) lung adenocarcinoma\(^\text{17,46}\)) by concurrent imaging of CDNP-VT680 and a newly developed fluorescent drug conjugate, R848-BODIPY TMR (Supplementary Fig. 10). R848 and the CDNP distribution of the CDNP, we employed a dorsal window chamber examination in vivo by fluorescence microscopy. Systemic circulation and biodistribution were examined in an immunocompetent mouse model of colorectal cancer (MC38) in C57BL/6 mice. First, we employed time-lapse confocal fluorescence microscopy of vessels within the ear for assessment of systemic circulation, demonstrating a vascular half-life \((t_{1/2})\) of 62.5 ± 4.75 min (Fig. 4a). Subsequently, organ biodistribution was examined by fluorescence reflectance imaging at 24 h post-injection (Fig. 4b). Interestingly, CDNP accumulation was highest in tumours \((94.9 \pm 1.9%\text{ID per g tissue, } 4.1 \pm 1.15\%\text{ID})\) followed by draining lymph node \((93.0 \pm 6.6%\text{ID per g tissue})\). Retention in other reticuloendothelial system organs was lower than within the tumour, including in the liver \((78.4 \pm 3.3%\text{ID per g tissue})\) and spleen \((35.6 \pm 3.5%\text{ID per g tissue};\) Fig. 4c). Temporal analysis (Supplementary Fig. 7) demonstrated nanoparticle accumulation in macrophage-rich tissues over time.

To further interrogate the intratumoural kinetics and cellular distribution of the CDNP, we employed a dorsal window chamber setup for intravital imaging\(^\text{43,44}\). Tumours were generated by inoculation with \(1 \times 10^6\) MC38-H2B-mApple cells, allowing identification of tumour cells. To enable identification of TAMs, we utilized a recently described reporter mouse wherein TAMs are readily detectable through MerTK\(^{\text{GFP+}}\) expression\(^\text{45}\). In following intravenous administration of CDNP-VT680 by time lapse microscopy, we observed its rapid vascular distribution adjacent to and throughout the tumour (Fig. 5a) and accumulation within GFP\(^+\) perivascular macrophages within 60 min (Fig. 5b, Supplementary Video 1), demonstrating nanoparticle accumulation in TAMs to be the primary mode of uptake as opposed to intratumoural migration of CDNP-loaded monocytes from the vasculature. At 24 h following administration, CDNPs were cleared from the vasculature and had accumulated within TAMs throughout the tumour (Fig. 5c,d). When macrophage uptake was compared across tissues, it was highest in tumours on a per cell basis (Supplementary Fig. 8). Moreover, CDNP accumulation was dominated by macrophages in the TME, relative to other immune cell types, and accumulation in tumour cells was not observed (Supplementary Fig. 9).

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Having demonstrated the TAM uptake of CDNP and improved distribution of R848 to TAMs through nanoformulation, we sought to explore the ability of CDNP-R848 to enhance TAM re-education. In vitro, qPCR analysis demonstrated enhanced M1-like transcription

**Fig. 3 | Development and characterization of CDNPs.** **a.** Schematic of CDNP preparation by lysine crosslinking of succinyl-β-cyclodextrin (orange) and subsequent drug loading by guest–host complexation of R848 (blue). **b.** Dynamic light scattering measurement of hydrodynamic diameter, dependent on the concentration of CD in solution during crosslinking. Data represent the mean ± s.d. of a single sample (N>1,000 reads); polydispersity index (PDI) is indicated in parentheses. **c.** Representative scanning electron microscopy image (N=3 independent samples) of CDNPs. Average diameter: 29.3 ± 1.70 nm. Scale bar, 200 nm. **d.** Drug loading (%wt/wt R848 relative to CDNP-R848) as a function of the molar ratio of guest-to-host. Results represent analytical estimation of the mean loading; calculated at reaction equilibrium.
in murine and human M2 macrophages re-educated by R848, which was further enhanced by CDNP-R848, while the CDNP alone elicited no response (Supplementary Fig. 11). In vitro models of murine or human macrophage polarization do not fully represent the immunosuppressive TAM phenotype, which may only be accurately achieved within the in vivo tumour environment. We therefore explored the pharmacodynamics of M1 induction in vivo by employing an IL12-eYFP reporter mouse in which TAMs co-express YFP with the prototypical M1 marker IL12-p40 (Fig. 6a). CDNP alone (without R848) accumulated in TAMs but did not elicit an IL12 response, and R848 itself also failed to elicit a robust IL12 response in vivo. In contrast, CDNP-R848, which showed potent accumulation in TAMs, induced a robust IL12 response (Fig. 6b,c). Flow cytometry of tumour tissue independently confirmed IL12 induction by CDNP-R848, and this response was correlated with nanoparticle uptake by TAMs (Supplementary Fig. 12). R848 and similar TLR agonists have demonstrated some degree of efficacy as vaccine adjuvants and cancer therapeutics⁴⁷,⁴⁸; though, as of recent review, these approaches have been clinically hindered by poor bioavailability⁴⁹. The unique distribution of CDNP-R848 to TAMs is a promising methodology to overcome the challenge to clinical translation, as we have directly observed that TAM-targeted CDNPs increase the delivery of R848 to TAMs and this is correlated with improved re-education of macrophages within the tumour microenvironment.

**Therapeutic efficacy.** Having shown that CDNP-R848 elicited TAM re-education in vivo, we set out to determine the agent’s therapeutic efficacy. CDNP by itself did not have an effect on MC38 tumour growth relative to control animals (Fig. 7a), which was independently confirmed by proliferation assays in cell culture (Supplementary Fig. 13). When given repeatedly in the free drug form, the small molecule R848 provided marginal benefits in terms of tumour control (Fig. 7a), not attributable to direct effects on tumour cell proliferation (Supplementary Fig. 13). CDNP-R848 treated mice showed noticeably smaller tumours than in any other repeated treatment group, reduced tumour growth rates and improved survival (Fig. 7a–c). As a single dosage of CDNP-R848 was observed to re-educate TAM phenotype in vivo (Fig. 6), resulting in observation of tumour regression for CDNP-R848 treatment (Supplementary Fig. 14), we repeated the tumour growth experiments using single dosage of free or nano-encapsulated R848 and found CDNP-assisted delivery of R848 to significantly improve therapeutic response (Fig. 7d, Supplementary Fig. 15). However, CD8+ T cell depletion (Supplementary Fig. 16) mitigated the effects of CDNP-R848 on tumour growth. Given the productive diversion of TAMs from immune-suppressive to immune-supportive phenotypes and the demonstrated involvement of adaptive immunity through T cell involvement, we reasoned that CDNP-R848 monotherpay could potentiate checkpoint blockade. Combination of CDNP-R848 with anti-PD-1 was synergistic and resulted in tumour shrinkage, stabilization and homogenization of response (Fig. 7e, Supplementary Figs. 17, 18). Complete tumour regression was observed in 2/7 tumors, and animals cured in the course of treatment resisted secondary tumour challenge, further indicating that the treatment had triggered anti-tumour memory.

**Fig. 4 | In vivo biodistribution and pharmacokinetics of CDNP.** a, Nanoparticle blood half-life in MC38-tumour-bearing C57BL/6 mice, quantified by time-lapse confocal fluorescence microscopy of CDNP-VT680. Data represent mean ± s.d. (shaded), N = 3 animals. b, Fluorescence reflectance imaging of CDNP-VT680 accumulation in the tumour and representative organs at 24 h following administration (λ_{ex} = 620–650 nm, λ_{em} = 680–710 nm; N = 3 animals from a single experiment). Tissues are outlined (cyan) for clarity. Scale bars, 5.0 mm. c, Corresponding quantified biodistribution of CDNP-VT680. Data are presented as mean ± s.e.m., N = 3 animals.
to anti-PD-1 treatment, indicating the nanotherapeutic treatment can sensitize the tumour environment toward effective combination therapy, which is a major focus of ongoing clinical trials. These results support the combination of immunotherapies that rationally target both innate and adaptive immune components to improve therapeutic efficacy.

**Discussion**

Immune cells play a critical role in regulating tumour growth and can potentially be harnessed for therapy. For example, immunotherapies targeting T cell immune functions are beginning to show impressive survival benefits. Furthermore, considering that the TME is home to diverse host cell types and that current immunotherapies only benefit a minority of patients, it is critical to consider other cell types as additional therapeutic targets. TAMs have recently attracted much attention as they frequently emerge as abundant immune cells in the tumour stroma in a broad range of cancers, and an abundance of these cells is often associated with poor clinical outcome.

TAM-targeted therapies have primarily sought to deplete TAM populations. For example, several small molecule and antibody drugs have been developed to antagonize CC chemokine receptor-type 2 (CCR2) or colony-stimulating factor-1 receptor (CSF1R, also called MCSF-R) signalling, as these pathways are involved in local recruitment and maintenance of the TAM population. These and other strategies have shown promise in that they can delay tumour progression in animal studies, and are currently being evaluated in the clinic.

However, experimental studies have also shown that TAM depletion may not suffice to trigger durable anti-cancer response. An alternative therapeutic approach consists of re-educating these cells. Re-education strategies offer the possibility to not only ablibish phagocytes’ tumour-supportive functions but also to actively promote their anti-tumour immune actions and could thus be more efficacious when combined with immune checkpoint blocking therapies. Several approaches have been proposed to re-educate TAM populations. For example, it was found in a mouse model of glioblastoma that a CSF1R small molecule inhibitor did not ablate TAMs but instead altered their polarization and substantially controlled tumour progression. Similar observations have recently...
be made for the class IIa HDAC inhibitor TMP195, among others\textsuperscript{55,56}. Despite these observations, significant challenges remain in the development of TAM re-education therapies; these challenges are twofold and include the ability to preferentially deliver therapeutics to TAMs as well as the identification of therapeutics that drive re-education with high potency.

Here, we leveraged sugar polymers for systemic TAM targeting given the extensive research into these materials, which has demonstrated their general safety\textsuperscript{19,57}, biocompatibility and biodegradation\textsuperscript{58}. We initially considered dextrans for TAM targeting based on our own work\textsuperscript{19,21,23} and that of others\textsuperscript{59} but low loading efficiencies led us to explore alternatives. Here, we demonstrated that engineered CDNPs similarly display high TAM affinity in vivo while perpetuating a considerable loading efficiency of small molecule payloads. These features are due to the unique composition of the nanoparticles developed herein, where base components (for example, l-lysine and cyclodextrin) are recognized by the US Food and Drug Administration as safe for medical use. Moreover, the facile nanoparticle formulation yielded optimal properties for systemic delivery (for example, hydrodynamic radius and zeta potential) resulting in 4.1 ± 1.2% of the injected dose being delivered to a solitary tumour, as compared to a modest 0.7% median for conventional nanoparticle preparations\textsuperscript{60}. We also attained a higher cyclodextrin content (78%wt/wt) than conventional approaches to cyclodextrin-mediated drug delivery, such as post-modification of existing nanoparticles\textsuperscript{39}. These properties, in conjunction with the demonstrated ability to alter biodistribution of encapsulated therapeutics through preferential delivery in tumour tissue, make the

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\caption{Therapeutic efficacy. a–c, Efficacy of repeated dosing regimen. a, Tumour area at day eight following the start of treatment. Data are expressed as mean ± s.e.m.; N = 12 tumours; **P = 0.0017, ****P < 0.0001 (Dunn’s multiple comparison) relative to vehicle control. b, Survival following start of treatment. **P = 0.005 (log-rank test, two-sided) relative to vehicle controls; N = 6 animals. c, Macroscopic images of tumours at day eight following initiation of treatment, representative of N = 6 mice per group. d, Individual tumour growth curves for mice treated with a single dose of R848 or CDNP-R848. e, Change in individual tumour area at day eight following treatment with a single dose of CDNP, CDNP-R848, aPD-1 or the combination therapy. All studies were executed in C57BL/6 mice, and treatment was initiated when tumours reached an area of 25 mm\textsuperscript{2} (approximately 100 mm\textsuperscript{3}).}
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described nanoparticles a useful system for the in vivo delivery of TAM-targeted therapeutics.

We found that the drugs most efficient at re-educating TAM in vitro were TLR7/8 agonists. We examined GS9620 (TLR7 agonist), motolimod (TLR8 agonist) and R848 (dual TLR7/8 agonist) based on their approval or prior use in a clinical setting. These drugs and related analogues have been used for topical treatment of cutaneous cancers (for example, resiquimod, Alumder) or for intratumoral injections and can trigger tumour infiltration by T cells. These studies indicate the effectiveness of TLR7/8 agonists in humans. Accordingly, we observed anti-tumour effects in mice that are consistent with involvement of adaptive immunity, such as (i) a change in myeloid phenotype, including IL12 production that can drive Th1 responses, (ii) an anti-tumour response that was blunted by CD8+ T cell depletion and (iii) long-term anti-tumour memory that resisted re-challenge. These data indicate profound anti-tumour immune effects following TAM targeting by CDNP-R848. It is also possible that the drug affects myeloid cells other than TAM, including dendritic cells. Indeed, other myeloid-targeted therapies have demonstrated modulation of dendritic cell homeostasis.

Replicative systemic administration of small-molecule TLR agonists has not yet been pursued, owing to dose-limiting adverse events (fever, headache), which limit clinical efficacy. Instead, CDNP-R848 favours TLR agonist delivery to TAMs and alters the tumour immune microenvironment in ways that were not observed with systemic administration of the free drug. We expect that increasing target payload with nanoparticle delivery may further decrease systemic side effects, as has been seen with encapsulation of chemotherapeutic agents. Furthermore, systemic TAM targeting, rather than image-guided intratumoral injection, would allow applications in the metastatic setting.

In summary, we believe that the described approach of targeting the myeloid-cell compartment with small molecules will be an exciting addition to cancer immunotherapy. Beyond TLR agonists, we envision the encapsulation of a variety of small molecules with effects on myeloid effector function, especially as a strategy that would be synergistic with checkpoint inhibitors.

**Methods**

Materials. Unless otherwise indicated, solvents and reagents were purchased from Sigma-Aldrich and used without further purification. Water used for all experiments was purified using a MilliQ filtration system (Waters). All pharmacological drugs were purchased from Selleckchem, MedChemExpress, InvivoGen or LC Laboratories. The Rat IgG2a kappa anti-mouse PD1 29F.1A12 clone was kindly provided by G. Freeman (Dana Farber Cancer Institute). Ferumoxytol (AMAG Pharmaceuticals) and amino-dextran (500 kDa, Thermo Fisher Scientific) used for intravital imaging were fluorescently labelled by Pacific Blue (label concentration: 40.1 ± 2.6 nmol mg−1 dextran, 0.6 mg injected) as previously described.

Cell models. Cells were maintained in the indicated medium at 37 °C and 5% CO2 and screened regularly for mycoplasma. RAW 264.7 cells used in nanoparticle uptake assays were sourced from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Atlanta Biologicals), 100 IU penicillin (Invitrogen), and 100 μg ml−1 streptomycin (Invitrogen), and 200 mM l-glutamine (Invitrogen). The MC38 mouse colon adenocarcinoma cell lines were kindly provided by M. Smyth (QIMR Berghofer Medical Research Institute) with stable transfection of the H2B-Apple reporter as previously described. M0, M1-like or M2-like phenotypes. The fast gentle algorithm was trained on the selected cells for unsupervised determination of weights and thresholds for cell shape features. The resulting set of parameters was used to score all other images. The enrichment score for M1 cells was output back into the database and imported into KNIME to generate per-well and per-treatment averages.

For transcriptional analysis, derived murine macrophages were treated with 10 ng ml−1 recombinant mouse IL4 (PeproTech 214-14) for 24 h to induce an M2-like polarization state and subsequently dosed with fresh media supplemented by pharmacologic drugs at the prescribed concentrations. Murine macrophages treated only with IL4 (10 ng ml−1) or LPS (100 ng ml−1) were similarly treated and processed prior to analysis for expression of β-actin (Mm00711660_m1), cell membrane (5.0 μg ml−1 DyLight 554 Phalloidin, Cell Signaling Technology), cell membrane (5.0 μg ml−1 Alexa Fluor 647 wheat germ agglutinin, Thermo Fisher) and nuclei (DAPI, Invitrogen) for 25 min at 4 °C in PBS. Plates were washed by PBS prior to use with 10 μM CFP (Invitrogen) and 3 μM Cy5-labeled (3-dimethylaminopropyl)-'N'-ethylcarbodiimide (Sigma, 5.0 eq. to carboxylate) were combined and dissolved in MES buffer (10 mM, pH 6.0) at the desired glucose concentration (1.25 to 20.0 mM/t). The reaction was stirred for 30 min at room temperature prior to the addition of t-lysine (0.5 eq. to carbohydrate, unless otherwise noted) and overnight crosslinking. The product was recovered by addition of brine (0.05 volumetric equivalents) and precipitation from a tenfold excess of iced ethanol. Upon re-dissolution in water, the product was concentrated by centrifugal filtration (10kDa MWCO, Amicon), washed repeatedly by water, passed through a 0.22 μm filter (Costar, Costar) and lyophilized. The final products were re-dissolved at a concentration of 20 mg ml−1 prior to use. Particle size was calculated by dynamic light scattering (Malvern, Zetasizer NanoS) with a standard protocol (ZS300) at a typical concentration of 5 mg ml−1 in 10 mM PBS. Zeta potential was determined at 100 μg ml−1 in 10 mM PBS (Malvern, Zetasizer NanoS) following calibration measurements on manufacturer standards. For scanning electron microscopy, samples were prepared at 1.0 μg ml−1 in water, spotted on silica wafers, freeze-dried and sputter coated prior to imaging. Analysis of R848 affinity for CD was performed by a standard colourimetric competitive binding assay. Briefly, phenolphthalein (200 μM) was freshly prepared in
Intravitral examination CDNP-VT680 distribution into macrophages and tumour cells was examined using dorsal skinfold window chambers installed on recently developed NOD SCID MetTERTm mice inoculated with MC38-H2A-mApple tumours similar to those previously described. Mice received CDNP-VT680 i.v. (0.5 mg, 50 μl saline) 24h prior to imaging. Intravitral examination of IL12 expression was similarly performed using p40-IREs-eYFP IL12 reporter mice (#015864, Jackson) described previously. Prior to imaging, mice received intravenous administration of R848 (2.0 mg kg−1). CDNP-VT680 (16.5 mg kg−1 CDNP) or CDNP-VT680 + R848 (16.5 mg kg−1 R848: 1:1 R848/CDNP molar ratio) each prepared in 50 μl sterile saline. IL12 expression was examined at 24h following treatment. In both cases, macrophages and vasculature were labelled by Pacific Blue–ferumoxyl and Pacific Blue–dextran, respectively.

Intravitral examination CDNP-VT680 and R848-BODIPY TMR-X distribution to macrophages was further carried out in C57BL/6 mice inoculated by tail vein injection of 2.5 × 106 eYFP expressing KRAS/p53 mutant lung adenocarcinoma (KP1.9) cells in 100 μl PBS, similar to previous reports. At 30 days following tumour inoculation, mice received intravenous administration of R488–BODIPY TMR X (5.0 mg kg−1) or CDNP-VT680 + R848–BODIPY TMR X (41.25 mg kg−1 CDNP + 5.0 mg kg−1 R848) in 50 μl sterile saline. The fluorescence intensity was determined for YFP within each ROI, and data are presented following normalization to the average intensity for CDNP control treatment. For quantification of R488–BODIPY distribution, the macrophages were identified and masked by automated thresholding of the Gaussian filtered Pacific Blue channel using the RenRandomGT method. The fluorescence intensity was determined for BODIPY within each ROI, and data are presented following background normalization.

Flow cytometry. For examination of CDNP-VT680 biodistribution in MerTERTm mice, MC38 tumours were harvested 9 days after intradermal implantation into IL12-eYFP mice, 24h after intravenous injection of CDNP-VT680 (0.5 mg). For examination of IL12 expression, MC38 tumours were harvested nine days after intradermal implantation into IL12-eYFP mice, 24h after intravenous injection of R848 (2.0 mg kg−1). CDNP-VT680 (16.5 mg kg−1 CDNP) or CDNP-VT680 + R848 (16.5 mg kg−1 + 2.0 mg kg−1 R848; 1:1 R848/CDNP molar ratio) each prepared in 50 μl saline. Tissues were minced, incubated in RPMI containing 0.2 mg ml−1 collagenase I (Worthington Biochemical) for 30 min at 37°C and then passed through a 40 μm filter. Red blood cells were lysed using ACK lysis buffer (Thermo Fisher Scientific) prior to pre-treatment with low-affinity Fc receptor blocking reagent (TruStain FcX anti-CD16/32 clone 93, BioLegend) and staining for macrophages (CD45+Ly6C−Ly6G−), neutrophils (CD45+Ly6C+Ly6G+), and other immune cells (CD45+) in 4% paraformaldehyde. Cells were washed and resuspended in 50 μl of PBS. Tumours were allowed to grow to an established size of 50 mm2 (100 mm3) at which time treatment cohorts were assigned such that tumour size and body weight were normalized across groups at baseline. For repeated dosing experiments, animals were treated three times weekly by i.v. administration of R488 (2.0 mg kg−1). CDNP-VT680 (16.5 mg kg−1 CDNP) or CDNP-VT680 + R848 (16.5 mg kg−1 + 2.0 mg kg−1 R848; 1:1 R848/CDNP molar ratio) each prepared in 50 μl saline. For single dosing experiments, animals were treated by i.v. administration of R488 (3.0 mg kg−1) in saline. For aPD-1 treatment, the 29 F.1.A12 aPD-1 clone was administered at a dose of 200 μg by intraperitoneal injection. In the case of CD8+ T cell depletion, anti-mouse CD8 antibody (BioXcell, clone 53-6.72) was administered every three days. At set time points, tumour growth was assessed by caliper measurement (A = length x width) and values are reported following normalization to area at the time treatment was initiated.

Articles

125 mM carbonate buffer (pH 10.5). Decrease in absorbance at 550 nm due to nanoparticle–phospholipid complex formation and absorbance recovery due to R848 competitive binding were measured (Tecan, Spark), and results are presented as absorbance relative to nanoparticle–free controls. The dissociation constant, Kd, was determined by treatment of β-cyclodextrin by increasing concentrations of R848 and fit to a one-site competitive inhibition model in GraphPad Prism 6. Drug loading in CDNP-R848 was analytically determined as a function of the molar ratio of R848 to CD in the nanoparticle, assuming the appropriate reaction equilibrium for one-to-one association: [CDNP/R848]1 or ([CDNP]×[R848])/(CDNP + R848), where (R848) is the concentration of unbound R848, (CD) is the concentration of unbound cyclodextrin in the nanoparticle, and (CD-R848) is the concentration of R848 bound by the nanoparticle. A molar ratio of guest-to-host ranging from 0.01 to 100 was examined, and drug loading (%w/w) was defined as 100×([CDNP]/([CDNP] + [R848])).

Fluorescence derivatization. For intravitral imaging and assessment of biodistribution, cyclodextrin nanoparticles were fluorescently labelled. The CDNP nanoparticle was dissolved at 20 mg ml−1 in carbonate buffer (0.1 M, pH 8.5) prior to addition of VivoTag 680 XL. (PerkinElmer, 1.0 mg ml−1 in anhydrous DMSO) at a final concentration of 50 μM. The reaction was allowed to proceed for 3 h at room temperature prior to product recovery by centrifugal filtration (10kDa MWCO, Amicon), repeated washing by water to remove unbound dye and lyophilization. Resultant CDNP-VT680 was re-dissolved at a concentration of 10 mg ml−1. Absorption spectra (668 nm) were used to determine the labelled concentration (1.72 ± 0.03 mg ml−1) by the Beer–Lambert equation, (A = b×c, where A is the absorbance, b is the molar absorptivity 210,000 M−1 cm−1, and c is the concentration).

For assessment of drug biodistribution, R848 was fluorescently labelled. R848 (7.4 mg, 25 μmol), EDC (4.7 mg, 25 μmol), N,N-diisopropylethylamine (3.2 mg, 25 μmol) and NHS (3.8 mg, 50 μmol) were dissolved in DMSO (3.8 mg, 50 μmol) and added to BODIPY TMR-X SE (5.0 mg, 8.2 μmol, Fisher Scientific) in a 5 ml amber-tinted vial with a magnetic stir bar. The reaction was allowed to proceed for 16h at 50°C with protection from light. After which, the reaction was concentrated under reduced pressure to produced a dark purple solid. The solid was suspended in the minimal amount of methanol and purified via high-performance liquid chromatography equipped with an Atlantis Prep T3 OBD 5 m, 19 mm × 50 mm using a gradient of water (0.5% formic acid) and acetonitrile (0.5% formic acid) and collected via Waters Fraction Collector III. The fractions of similar purity were combined and concentrated to produce a dark purple solid, R848-BODIPY TMR X (1.3 mg, 20%), which was protected from light and stored at 4°C. The purity of the product was determined with high-performance liquid chromatography with a gradient of water (0.5% formic acid) and acetonitrile (0.5% formic acid) and separated with an Atlantis T3 5 m, 19 mm × 50 mm and mass ions were detected on a Waters 3100 Mass Detector in positive mode. The product eluted at approximately 1.12 mins (m/z = 808.59 Da, expected 810.50 Da Supplementary Fig. 20). The purified product was dissolved in DMSO-d6 and 1H and 13C NMR spectra were recorded on a Bruker AC-400 MHz spectrometer, which were consistent with reported spectra for the parent compounds, R848 and BODIPY TMR-X.

Pharmacokinetic and biodistribution analysis. The blood half-life of CDNP-VT680 was determined by intravenous injection of 257BL/6 mice into the ear during and immediately following tail vein injection of Pacific Blue–dextran and CDNP-VT680 (0.5 mg, 50 μl saline). Time-lapse images were acquired continually over the first 3h after CDNP-VT680 injection, after which the mice were allowed to recover before subsequent imaging at 24h. Across three separate animals, multiple fields of view were analysed by identification of regions of interest within the labelled vasculature. Mean fluorescence intensity was determined as a function of time, background subtracted and normalized to the peak fluorescence intensity. Resulting data were fit to a mono-exponential decay in GraphPad Prism 6.

At 1, 4 and 24h following injection, examination of CDNP biodistribution was performed in C57BL/6 mice. Surgically resected tissues of involved in DFM (2 ml) were thoroughly washed in PBS, weighed and placed in an OV110 ( Olympus) for brightfield imaging to identify regions of interest and fluorescence reflectance imaging (1,000 ms exposure time; λex = 620–650 nm, λem = 680–710 nm). Integrated fluorescence density was determined for regions of interest (ROIs) representing each tissue (Image, NIH). Values were background-subtracted for tissue autofluorescence by imaging of corresponding tissues from a vehicle-treated control. Percentage of injected dose was determined relative to standards of CDNP-VT680 prepared in 1% intralipid (McKesson, 988248) to account for optical scattering of tissue and, values are presented following normalizations to tissue mass.

Intravitral microscopy. Images were acquired on an EV1000MP confocal imaging system (Olympus). Pacific Blue, GFP/YFP, mApple/BODIPY TMR X, and VT680 were excited sequentially using 405, 473, 559 and 635 nm diode lasers and BA430-455, BA490-540, BA575-620 and BA655-755 emission filters with SDM473, SDM560 and SDM640 beam splitters.
Statistical analysis. Data are presented as mean ± standard error unless otherwise indicated. Statistical analyses were performed using GraphPad Prism 6. Statistical significance was determined by analysis of variance, using repeated measures where appropriate, in conjunction with post-hoc Tukey’s honest significant difference test. For in vivo studies of tumour growth, temporal comparisons were made by Friedman’s test and comparisons at set time points were performed by Kruskal–Wallis, each using post-hoc Dunn’s test for multiple comparisons. Survival analysis was performed by log-rank test. Significance was determined at P < 0.05.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data supporting the findings of this study are available within the paper and its Supplementary Information. Data acquired in the course of this study are available from the corresponding author upon reasonable request.

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**Author contributions**

R.W. and C.B.R. conceived and designed the CDNP–drug conjugate. C.B.R., S.P.A., M.F.C., C.S.G., R.L., M.S.A. and R.H.K. performed the experiments and data analysis. C.B.R., S.P.A., M.J.P. and R.W. wrote the manuscript. All authors contributed feedback on the final manuscript.

**Competing interests**

C.B.R. and R.W. are listed on a patent filed by Partners Healthcare. The remaining authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41551-018-0236-8.

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Correspondence and requests for materials should be addressed to R.W.

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### Experimental design

1. **Sample size**
   Describe how sample size was determined.

   Sample size was based on prior reports by our group using these and similar animal models (e.g., Arlauckas, Science Translational Medicine, 2017). Power analysis used in the development of these models indicates that sample sizes of 6-7 animals are sufficient, given SD = 25% with a 99% difference between treatment and control groups; $a = 0.05$, $b = 0.2$.

2. **Data exclusions**
   Describe any data exclusions.

   No data were excluded in analysis.

3. **Replication**
   Describe whether the experimental findings were reliably reproduced.

   All attempts for replication were successful.

4. **Randomization**
   Describe how samples/organisms/participants were allocated into experimental groups.

   Mice were randomly assigned to the groups.

5. **Blinding**
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   Investigator were not blinded to group allocation in tumour measurement; however, efficacy of the treatments were apparent both in quantification and in representative images of outcomes. Survival endpoints were determined by blinded veterinary staff.

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Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   | n/a | Confirmed |
   |-----|-----------|
   |     | The **exact sample size** (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
   |     | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
   |     | A statement indicating how many times each experiment was replicated |
   |     | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
   |     | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
   |     | The test results (e.g. $P$ values) given as exact values whenever possible and with confidence intervals noted |
   |     | A clear description of statistics including **central tendency** (e.g. median, mean) and **variation** (e.g. standard deviation, interquartile range) |
   |     | Clearly defined error bars |

*See the web collection on [statistics for biologists](#) for further resources and guidance.*
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

All analysis was performed in GraphPad Prism v6. Image processing was performed in FIJI (ImageJ, NIH) as detailed in the Methods. Analysis of cell shape was performed in CellProfiler (Broad Institute) and the pipeline used in analysis is defined in the Supplementary Information.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

The rat IgG2a kappa anti-mouse PD1 29F.1A12 clone was kindly provided by Gordon Freeman (DFCI). All other reagents are commercially available.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Antibodies used include those for 1) flow cytometry: anti-CD45 (30-F11, eBioscience), anti-CD11c (N418, BioLegend), anti-Ly6G (1A8, Biolegend), and anti-F4/80 (BM8, BioLegend) which were all used according to manufacturers instructions; 2) T-cell depletion: 200 ug anti-CD8 (BioXcell, clone 53-6.72) administered every 3 days via i.p. injection per the manufacturers instructions; and 3) tumor treatment: 200 ug of the rat IgG2a kappa anti-mouse PD1 (clone 29F.1A12) was administered via i.p. injection as per our previous reports (Arlauckas et al, Science Translational Medicine, 2017).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Cell sources are defined in the Methods (cell models): RAW 264.7 and B16.F10 cells were sourced from ATCC. MC38 cell lines were provided by Mark Smyth (QIMR Berghofer Medical Research Institute). KP1.9 cells were provided by Dr. A. Zippelius (University Hospital Basel, Switzerland). Murine bone marrow-derived macrophages (BMDMs) were isolated and derived from C57BL/6 mice, and human macrophages were derived from peripheral blood mononuclear cells.

b. Describe the method of cell line authentication used.

Cell lines were used from the source without authentication.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cells were tested monthly and found to be negative for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Studies were conducted in compliance with the Institutional Animal Care and Use Committees at Massachusetts General Hospital (MGH). Unless otherwise stated, experiments were performed using female C57BL/6 (#000664, Jackson) that were 6- to 8-weeks old at the start of the experiment. Examination of CDNP-VT680 distribution into macrophages was examined in recently developed NOD MerTKGFP/+ mice (Mohan, Proc Natl Acad Sci U S A, 2017), crossed into NOD SCID mice (#001303, Jackson). IL12 expression was examined in p40-ires-eYFP IL12 reporter mice (#015864, Jackson) described previously (Reinhardt, J Immunol, 2006).
12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

▪ Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

▪ Methodological details

5. Describe the sample preparation. Sample preparation is described in detail in Methods.

6. Identify the instrument used for data collection. LSR2

7. Describe the software used to collect and analyze the flow cytometry data. FACS Diva and FlowJo vX.0.7

8. Describe the abundance of the relevant cell populations within post-sort fractions. No cell sorting was performed.

9. Describe the gating strategy used. Briefly, single cells were selected using forward and side scatter linearity. Live cells were selected as defined by 7AAD- negativity. Immune cell populations were gated from CD45+ cells, as defined in detail in Methods. The expression gating of TAM IL12-eYFP and CDNP-VT680 positive cells is indicated in Supplementary Fig. 12.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒