Tumour-associated macrophages act as a slow-release reservoir of nano-therapeutic Pt(IV) pro-drug
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Therapeutic nanoparticles (TNPs) aim to deliver drugs more safely and effectively to cancers, yet clinical results have been unpredictable owing to limited in vivo understanding. Here we use single-cell imaging of intratumoral TNP pharmacokinetics and pharmacodynamics to better comprehend their heterogeneous behaviour. Model TNPs comprising a fluorescent platinum(IV) pro-drug and a clinically tested polymer platform (PLGA-b-PEG) promote long drug circulation and alter accumulation by directing cellular uptake toward tumour-associated macrophages (TAMs). Simultaneous imaging of TNP vehicle, its drug payload and single-cell DNA damage response reveals that TAMs serve as a local drug depot that accumulates significant vehicle from which DNA-damaging Pt payload gradually releases to neighbouring tumour cells. Correspondingly, TAM depletion reduces intratumoral TNP accumulation and efficacy. Thus, nanotherapeutics co-opt TAMs for drug delivery, which has implications for TNP design and for selecting patients into trials.
Approximately half of all cancer patients who receive chemotherapy are treated with one of three clinically approved platinum (Pt) drugs—cisplatin, carboplatin, and oxaliplatin. These Pt compounds are first-line agents in ovarian, lung, head and neck, testicular cancers, among others, and development of combination therapies using Pt compounds is an active area of research. Unfortunately, most cancers either exhibit intrinsic Pt resistance or ultimately develop resistance to treatment through mechanisms including reduced drug uptake, altered cellular metabolism, increased DNA repair, chemoprotective niche formation and/or activation of oncogenic and anti-apoptotic pathways. Dose escalation and combination drug regimens help overcome resistance. However, Pt-related toxic side-effects including neurotoxicity and nephrotoxicity limit some of these approaches, indicating the need for new strategies.

To circumvent these problems, specialized drug-delivery mechanisms such as nanoparticles (NPs) have been introduced to enhance local drug accumulation in tumours while simultaneously mitigating systemic toxicities. Non-encapsulated Pt compounds in particular are often plagued by poor pharmacokinetic (PK) properties. They generally must be intravenously administered over a prolonged period (sometimes over 24 h) because promiscuous Pt reactivity causes side effects and the covalent binding to plasma proteins such as albumin consequently neutralizes its activity. Recent work has demonstrated the possibility of using NPs to encapsulate Pt pro-drugs, thereby (i) extending the circulating half-life of the unreacted Pt compound and enabling controlled drug release; (ii) facilitating precisely engineered Pt-based combination therapies through co-encapsulation with additional drugs (or small interfering RNA treatments); (iii) enabling the administration of more lipophilic and newer-generation Pt compounds; and (iv) modifying tissue distribution and enhancing intratumoral accumulation. The last is thought to occur through enhanced permeability and retention (EPR) effects and has been termed ‘passive NP targeting’. In theory, enhanced permeability of the abnormal tumour microvasculature (TMV) should allow therapeutic nanoparticles (TNPs) to enter the tumour interstitial space, whereas suppressed lymphatic filtration and increased cellular uptake should allow them to stay there.

Although TNPs of Pt-based anticancer agents have advanced to clinical trials, results have been mixed, presumably due to heterogeneous EPR effects in different tumours combined with limited experimental data from patients on the effectiveness of this mechanism as related to enhanced drug accumulation. Furthermore, the inter-related in vivo PK and pharmacodynamic (PD) of TNPs are more difficult to understand than those of the unencapsulated drug. TNP drug delivery is inherently a multi-step process, defined by PK of the TNP vehicle, drug release dynamics that may change depending on the in vivo environmental context, and PK of the Pt payload itself. Little experimental evidence exists that describes how this multi-step drug delivery sequence performs in vivo and within tumours, despite its critical importance to overall therapeutic outcome. This lack of understanding clearly represents a bottleneck in the design and development of more efficacious therapies.

Here we use high-resolution microscopic imaging in live tumour-bearing mice to test the hypothesis that therapeutic nano-encapsulation not only affects traditional bulk PK properties, but also significantly influences heterogeneous drug uptake and response at the single-cell level within tumours. We engineer a platform that for the first time allows simultaneous imaging of both a ~100-nm TNP vehicle as well as its Pt payload, in addition to monitoring DNA damage at the single-cell level in real time. As expected, our results show that nano-encapsulated Pt exhibits a longer circulating half-life than traditional unencapsulated Pt compounds. However, we quite unexpectedly find that TNPs accumulate at high levels within tumour-associated macrophages (TAMs), and that TAMs serve as ‘cellular drug reservoirs’. Indeed, TAMs release the Pt payload into neighbouring tumour cells over time. Depletion of macrophages significantly decreases intratumoral Pt accumulation and correspondingly increases tumour growth. Overall, this work establishes a paradigm for NP drug delivery based on the principle that TNPs can sequester TNP payload and gradually release it into the surrounding tissue, thereby serving as ‘drug depots’.

Results

Dual imaging shows congruent vehicle and payload kinetics. For clinical applicability and generalizability, TNPs in this study were designed to incorporate desirable properties of previously described polymeric nano-formulations that have entered clinical trials, especially those of materials that have received Food and Drug Administration approval. TNPs were formulated by combining three compounds (compounds 1–3, Fig. 1a) via nano-precipitation, using spectrally complementary derivatives of BODIPYs as ideal fluorophores for efficient nano-encapsulation that convey lipophilicity and robust in vivo imaging arising from in vivo structural stability, environmentally robust fluorescence, high brightness and high photostability. Compound 3 (poly(D,L-lactic-co-glycolic acid)-b-poly(ethylene glycol); PLGA-b-PEG) self-assembles to form the hydrophobic PLGA core of the NP and hydrophilic PEG outer shell. For the cytotoxic payload, we used a novel Pt(IV) pro-drug (c.c.t-Pt(NH3)2Cl2-(hexadecylcarbamate)4-(2-(4,4-difluoro-1,3,5,7-tetramethyl-4-dora-3a,4a-diaza-s-Indacene)ethyl(amino)-4-oxobutanoate)) with a C16 aliphatic carbon chain and a BODIPY (λ_em = 498 nm, λ_ex = 543 nm; Supplementary Fig. 1a) as the two lipophilic ligands axially coordinated to the Pt(IV) centre. NPs were similar in size (135 ± 1 nm; standard error of the mean (s.e.m.), across n = 12; Supplementary Fig. 1b–c), polydispersity (PDI = 0.18 ± 0.01; s.e.m. across n = 12) and drug release rates (t1/2 release = 15 h; n = 2; Supplementary Fig. 1d) compared with previously published Pt(IV) nano-formulations and were stable in physiological saline (Supplementary Fig. 1e). Free carboxylic acid terminal groups on the PEG gave TNPs a slight negative charge (zeta-potential = −23 ± 1.4 mV; s.e.m. across n = 4), which has been shown in some cases to benefit tumour accumulation and mirrors TNPs in the clinic.

C16-Pt(IV) remains relatively stable in whole blood. However, upon reduction to the active Pt(II) compound cisplatin, dissociated BODIPY increases fluorescence by sevenfold owing to loss of quenching by Pt(IV) and mirrors TNPs in the clinic and in the late endosome 24 h post treatment (Supplementary Fig. 2c). Depletion of macrophages significantly decreases intratumoral Pt accumulation and correspondingly increases tumour growth. Overall, this work establishes a paradigm for NP drug delivery based on the principle that TNPs can sequester TNP payload and gradually release it into the surrounding tissue, thereby serving as ‘drug depots’.

We performed a series of in vitro experiments to characterize TNP behaviour in tumour cell culture (Supplementary Fig. 2a). Co-localization studies with fluorescent compartmentally localized proteins showed that the TNP vehicle moved from early to late endosomes over the course of 24 h (Supplementary Fig. 2b). Compared with the TNP vehicle, the TNP payload exhibited more diffuse intracellular localization, and co-localization peaked in the late endosome 24 h post treatment (Supplementary Fig. 2c). Payload fluorescence increased over time owing to Pt reduction and BODIPY de-quenching (Supplementary Fig. 2d). Dose–response TNP treatment indicated that intracellular fluorescence correlates well with overall Pt uptake (R² > 0.99) and resulting...
DNA damage (Supplementary Fig. 3), thereby validating the use of fluorescence as a surrogate marker of cellular payload content. Together, these results provide evidence that, over the course of 24 h, TNPs accumulate in endosomes and lysosomal compartments, where the Pt pro-drug is activated through reduction to form cisplatin.

TNPs exhibit long TMV half-life and perivascular cell uptake. We first used time-lapse intravital imaging to characterize PK within a xenograft tumour model often used for intravital studies (Fig. 1)\textsuperscript{16,26–28}. Tumours were generated from subcutaneously implanted HT1080 cells expressing a fluorescently tagged DNA damage response protein, 53BP1, which localizes to the nucleus\textsuperscript{29,30}. TNPs reached the TMV within 10–15 min of intravenous injection, and at early time points (t to 30 min) the vehicle and its payload exhibited strong co-localization within vessels (Fig. 2a; intravascular Pearson’s correlation r = 0.8 ± 0.02, s.e.m. across n = 4; Mander’s M1 and M2 > 0.95). Both the vehicle and its payload showed initial vascular PK similar to each
TNPs cause mitotic arrest and localized DNA damage. In addition to PK analysis, high-resolution intravital imaging allowed for simultaneous monitoring of the cellular response to TNPs at the molecular level. Fluorescently tagged 53BP1 is a key component of the non-homologous end-joining pathway that forms visible puncta at sites of DNA double-strand breaks. Based on the hypothesis that treatment with a cisplatin pro-drug would lead to increased DNA damage in tumour cells, we imaged 53BP1 puncta in response to TNP and quantitated the DNA damage response across populations of individual tumour cells at various time points (Fig. 3a). The results show a dose- and time-dependent increase in the fraction of tumour cells exhibiting high levels of DNA damage (Fig. 3b). We next tested whether there was an observable relationship between local payload accumulation and resulting single-cell DNA damage response. We used automated three-dimensional (3D) segmentation software to quantify TNP vehicle and payload accumulation within individual tumour nuclei, and correlated this information with DNA damage response in those cells. The results showed that cells exhibiting high levels of DNA damage also had a roughly 300% higher local payload concentration (Fig. 3c). Compared to TNP payload, local concentration of TNP vehicle also correlated with DNA damage, but to a lesser degree (Fig. 3c). DNA damage elicited by Pt treatment generally induces mitotic arrest in vitro, but it has been difficult to directly observe this response in vivo, where anti-mitotics often behave much differently. To determine TNP effects on cell growth, we used time-lapse intravital imaging to track individual tumour cells and mitotic events over the first 3 h following TNP injection in the HT1080 model (Supplementary Fig. 5) and found that TNP indeed caused a substantial reduction in tumour cell division (Fig. 3d). To test generalizability in another tumour model, we next examined local drug accumulation and DNA damage in an orthotopic model of disseminated ovarian cancer (OVCA). Human A2780CP OVCA cells were intraperitoneally injected in nu/nu mice, and 6 weeks later (once palpable tumours and/or ascites had developed), animals received TNP treatment. The following day, tumours were excised and imaged for TNP accumulation and DNA damage. Imaging shows similar TNP localization patterns as observed with the HT1080 model (Fig. 4a). We also found similar statistically significant correlation between local TNP payload levels and tumour cell DNA damage (Fig. 4b). Taken together, these results demonstrate that TNPs elicit substantial DNA damage response activity and corresponding growth arrest in vivo. In both the subcutaneous and orthotopic OVCA tumour models, the correlation between local drug accumulation and DNA damage response implies that NP-mediated drug delivery plays a significant role in governing such PD.

Identification of cell populations with drug in steady state. We next performed a combination of intravital imaging, flow cytometry and histology studies to determine in which immunologically defined cell populations the TNP vehicle and its payload accumulated within the bulk tumour mass at 24 h, after TNPs had largely cleared the circulation. With the same HT1080 xenograft model used in the previous imaging experiments, we labelled TAMs with a fluorescent dextran-coated NP and found significant TNP uptake in TAM-rich regions of the bulk tumour mass (Fig. 5a). For more detailed immunological examination, we next performed flow cytometry analysis of HT1080 tumours. Results show that tumour cells made up 61% of the bulk tumour; 31% of the cells within the bulk tumour were CD45+ host cells (erythrocytes, tumour associated fibroblasts, endothelium and others); and the remaining 8% were CD45+ leukocytes (Fig. 5b). TAMs made up the largest population of leukocytes within the bulk tumour and also accumulated the highest levels of both TNP vehicle and payload on a per-cell basis (Fig. 5c). Because tumour cells far outnumbered TAMs in the HT1080 xenograft model, the majority of TNP vehicle and payload within the bulk tumour still resided in tumour cells themselves (Fig. 5d). Nonetheless, even though TAMs comprised only 4% of cells within the total tumour mass, they accumulated more than 40% of the total injected TNPs relative to tumour cells, and 30% of the total TNP overall.

To directly visualize genetically defined tumour-associated host leukocytes, we imaged TNP vehicle uptake in tumour-bearing fractalkine Cx3cr1GFP+/- reporter mice, which are known to contain GFP+ macrophages. In this model, KRAS mutant p53-/-(KP) cells derived from autochthonous lung tumours were subcutaneously implanted into immunocompetent Cx3cr1GFP+/- reporter mice, and TNP vehicle (without the green fluorescent payload) was intravenously administered. The vast majority of TNP vehicle accumulated within these GFP+ host leukocytes, especially near TMV (Fig. 6a,b). Using the above described KP lung cancer and intraperitoneal OVCA xenografts, others; and the remaining 8% were CD45+ leukocytes (erythrocytes, tumour associated fibroblasts, endothelium and others); and the remaining 8% were CD45+ leukocytes (Fig. 5b). TAMs made up the largest population of leukocytes within the bulk tumour and also accumulated the highest levels of both TNP vehicle and payload on a per-cell basis (Fig. 5c). Because tumour cells far outnumbered TAMs in the HT1080 xenograft model, the majority of TNP vehicle and payload within the bulk tumour still resided in tumour cells themselves (Fig. 5d). Nonetheless, even though TAMs comprised only 4% of cells within the total tumour mass, they accumulated more than 40% of the total injected TNPs relative to tumour cells, and 30% of the total TNP overall.

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we confirmed by histology that high levels of TNP vehicle accumulate in F4/80⁺ host phagocytes in these disease models as well (Fig. 6c and Supplementary Fig. 6). These analyses ultimately corroborate results from the intravital imaging data showing uptake of TNPs in tumour cells but also host cells, which are confirmed here as TAMs.

**TNP payload spatially redistributes from TAMs to tumour cells.** Based on intravital imaging evidence showing that the Pt(IV)-payload was more diffusely distributed within tumour tissue compared with its TNP vehicle (Supplementary Fig. 4), we next quantified the extent of payload redistribution and its effect on drug response. Using flow-cytometry data, we confirmed that

**Figure 3 | TNP causes mitotic arrest and DNA damage in a spatially defined dose-dependent manner.** (a) Example images of 53BP1 puncta, 24 h post treatment with 1 mg kg⁻¹ TNP, scale bar, 10 μm. (b) TNP treatment increases the fraction of cells exhibiting many 53BP1 puncta, measured across n≥3 animals and n≥2,000 cells (*P < 0.01, two-tailed t-test). Positive control (Pos. ctrl); 24 h supra-therapeutic cisplatin, 9 mg kg⁻¹. (c) Heterogeneous local TNP vehicle and payload accumulation within 5 μm of single-cell nuclei correlate with corresponding DNA damage response in those cells (one-way analysis of variance; n>6,000 cells across n=5 animals; P<10⁻²¹ for both vehicle and payload); the local TNP payload correlates better with response compared with TNP vehicle (*P<0.05; two-tailed t-test). (d) Cell division events were quantified from time-lapse movies across n>1,000 cells (*P=0.001; two-tailed t-test; n=3 animals). All dosages in mg of Pt. Data are means ± s.e.m. Neg ctrl, negative control.

**Figure 4 | Local TNP payload accumulation correlates with DNA damage in OVCA.** (a) Single-cell DNA damage response correlates with local TNP payload levels in a model of disseminated metastatic OVCA. A2780CP OVCA cells were injected intraperitoneally; 6 weeks later, TNP was i.v. injected, and the following day intraperitoneal lesions were excised and imaged for DNA damage response (using 53BP1 puncta) and TNP uptake. Scale bar, 50 μm. (b) OVCA cells exhibiting detectable DNA damage by 53BP1-puncta have higher TNP payload levels, compared with those without (two-tailed t-test; s.e.m. across n=3 animals).
tumour cells exhibited more than twice the amount of payload accumulation as a ratio of vehicle accumulation, compared with TAMs (Fig. 7a), thereby suggesting that payload may transfer from TAMs to tumour cells. We hypothesized that local payload release from TAMs into neighbouring tumour cells would lead to elevated payload concentration in these tumour cells, which would in turn enhance DNA damage. By histology we also measured greater than threefold elevated payload concentrations within roughly one cell-length (15 μm) of TAMs (Fig. 7b,c), and analysis of intravital imaging data provided further evidence that relevant payload concentration gradients occur over length-scales of <25 μm (Supplementary Fig. 7a). One explanation for these observations is that TAMs induce neighbouring tumour cells to take up more TNP directly; however, the TNP vehicle itself was not similarly elevated in the same neighbouring region (Fig. 7c). Tumour cells within the same phagocyte-neighbouring region also exhibited higher DNA damage on average, as determined by histology (Fig. 7d) and supported by corroborating intravital imaging analysis (Supplementary Fig. 7b). These analyses cumulatively show local payload redistribution from TAMs to neighbouring tumour cells exhibiting elevated DNA damage.

We next performed a series of in vitro experiments to directly test whether macrophages could release functional, DNA-damaging TNP payload into their surrounding environment. Similar to in vivo observations, we found that in vitro macrophages accumulated TNP s more rapidly and to a greater degree than tumour cells (Supplementary Fig. 8a). Once taken up by macrophages, Pt-payload was released at high levels into the extracellular supernatant. In fact, over the course of 24 h, more Pt was found in the supernatant than in the macrophages themselves (Supplementary Fig. 8b,c). The Pt-containing macrophage supernatant was both DNA damaging (Supplementary Fig. 8d) and cytotoxic (Supplementary Fig. 8e). In contrast, supernatant from macrophages treated with unencapsulated cisplatin at concentrations ranging from 1 to 300 μM was not cytotoxic to tumour cells (Supplementary Fig. 8e). For supernatant from TNP-treated macrophages, filtration with molecular-weight cut-off filters (3 and 100 kDa) did not reduce its potency, which indicated that cytotoxic effects were independent of macrophage-derived cytokines or other signalling proteins (Supplementary Fig. 8f). In addition, Pt-containing macrophage supernatant exhibited 50% inhibitory effect on tumour cell viability/cytotoxicity at Pt concentrations of 1.5 ± 0.5 μM (s.e.m. across n = 3) within the macrophage supernatant (Supplementary Fig. 7e), which is comparable to the potency of the Pt-payload when TNP was directly applied to tumour cells (IC_{50} = 1.7 ± 0.02 μM; s.e.m. across n = 2; Supplementary Table 1). Taken together, these combined in vitro and in vivo data further confirmed that TAMs can serve as drug depots that accumulate high levels of TNPs and then release their DNA-damaging cytotoxic payload to the surrounding tumour tissue.

**TAM depletion reduces TNP accumulation and efficacy.**

Finally, we tested whether TAM depletion would reduce the therapeutic efficacy of TNP in cancers. Macrophages were systemically depleted by pretreatment with clodronate liposomes (clod-lip) before TNP injection. In this tumour model, macrophage depletion itself had no detectable impact on tumour growth (P = 0.2; two-tailed two-way analysis of variance; n = 38 mice). However, it substantially reduced the degree to which TNP treatment was able to slow tumour growth (Fig. 8a). Direct measurement of intratumoral TNP accumulation confirmed that macrophages play a key role in delivering drug payload: macrophage-depleted tumours exhibited both lower total intratumoral Pt (Fig. 8b) and a lower concentration of Pt (Fig. 8c) compared with macrophage-containing tumours, 24 h post administration of TNP, at which time no TNP remained circulating in the TMV of any tumour. The 24% decrease in Pt...
concentration (from 5 to 3.8 µM; Fig. 8c after converting to molarity) correlates well with the flow cytometry-based estimate that TAMs accumulate 30% of the total intratumoral TNP. It also correlates with the observation that unencapsulated Pt exhibits 37% lower intratumoral accumulation than nano-encapsulated Pt (Supplementary Table 1). This decrease disproportionately impacted TNP effects on tumour growth, probably because intratumoral Pt concentrations were near the threshold at which HT1080 tumour cells respond (in vitro IC_{50} = 3.7 ± 0.1 µM for unencapsulated Pt(IV) payload; s.e.m. across n = 2). TNP clearance kinetics, TMV leakiness and other EPR factors all may influence drug accumulation within the tumour; nonetheless, the experiments here show that all things being equal, macrophage uptake substantially contributes both to the delivery of TNP to the tumour and, consequently, to the therapeutic impact on tumour growth.

Liver TNP uptake is well tolerated and blocks metastasis. Given our evidence for TAMs in mediating TNP delivery, we next investigated TNP effects in the liver, which is a prominent site of the mononuclear phagocyte system and is known to accumulate NPs. Despite substantial liver accumulation (Supplementary Fig. 9a), TNPs did not significantly impact animal body weight (Supplementary Fig. 9b) and did not cause changes outside of the normal range in blood markers of renal or liver toxicity at the therapeutic dose (Supplementary Fig. 9c). Within the bone marrow, another important site of the mononuclear phagocyte system, TNP payload concentrations were much lower (Supplementary Fig. 9d,e). Although clinically relevant doses of un-encapsulated cisplatin caused a significant reduction in the number of nucleated cells in the bone marrow, TNP effects were not statistically significant (Supplementary Fig. 9f,g). Overall, these results demonstrate a positive safety profile despite liver accumulation.

We next tested whether TNP treatment could block the development of liver micro-metastases in a model of haematogenous breast cancer dissemination. 4T1 murine breast cancer cells were intravenously injected into immunocompetent balb/c mice, thus forming tumours in the lung, liver and other organs. To primarily deliver TNPs to host phagocytes rather than tumour cells, we pretreated naive mice with TNPs, waited 6 h for a majority of the drug to clear circulation, and then injected a single-cell suspension of 4T1 cells. Two weeks later, livers were excised and analysed by histology for tumour burden. Results show that pre-treatment with TNPs reduced the development of micro-metastases by over 65%, and repeated TNP doses caused a slightly greater reduction of 80% (Fig. 9). No treatment impacted tumour burden in the lung (two-tailed t-test; n ≥ 5). Taken together, these results demonstrate that TNPs can safely accumulate in host cells of the liver, thus allowing TNP payload to block the development of local metastases from haematogenously disseminated cancer cells.

Discussion

This work presents a new paradigm for employing TAMs as ‘drug depots’ for local delivery of nanotherapeutics to neighbouring...
tumour cells. Key to the development of this model was new technology for the in vivo visualization of multi-step drug delivery and the corresponding single-cell PD response at high spatio-temporal resolution. With this approach we demonstrated that nano-encapsulation allows for long circulating drug half-life and guides the delivery of drugs to TAMs, from which their functional cytotoxic payloads locally transfer to and damage neighbouring tumour cells. This paradigm most likely extends to other nano-encapsulated therapeutics and non-cancer disease indications in which macrophages accumulate near the target tissue. The clinically tested TNP platform used here\(^{14}\) can encapsulate other therapeutics including paclitaxel, doxorubicin and irinotecan, which exhibit release rates similar to the Pt(IV) payload \(^{5-7}\). Of relevance is that local macrophage accumulation occurs in multiple diseases including type 1 diabetes\(^{41}\), rheumatoid arthritis\(^{42}\), endometriosis\(^{43}\) and cardiovascular diseases such as atherosclerosis and myocardial infarction\(^{44,45}\). The imaging approach we describe may thus apply to these other pathologies, especially where intravital microscopy has already been successfully used\(^{46}\). Although macrophages themselves are often pharmacological targets\(^{47}\), concomitant drug delivery to surrounding tissue may be advantageous\(^{48}\). Overall, the drug delivery paradigm presented here, along with the complementary imaging technology, portend broad applicability to other TNP strategies and disease indications.

TAMs have been extensively studied and targeted for their roles in disease development, progression and therapeutic response\(^{48,49}\). Nonetheless, TAMs have received less consideration in the context of novel nanotherapeutics entering the clinic. Because of their secretion of growth factors, cytokines and proteases, TAMs have been implicated in supporting disease progression by enhancing angiogenesis, metastasis and cancer stem cell niche formation, all while suppressing anti-cancer immune responses. In multiple indications, high TAM accumulation at tumour sites correlates with poor prognosis\(^{50,51}\). Although TAM-mediated tumour neo-vascularization can increase vascular leakage and corresponding therapeutic delivery\(^{52}\), TAMs also provide chemoprotective cues to tumour cells, and TAM infiltration following chemotherapy correlates with poor treatment response\(^{52,53}\). For these reasons, therapeutic TAM killing or TAM re-polarization into an anti-cancer phenotype represents an active area of development, for

Figure 7 | DNA-damaging TNP payload redistributes from TAMs to neighbouring tumour cells. (a) Compared with TAMs, tumour cells exhibit elevated TNP payload as a fraction of TNP vehicle (measured by flow-cytometry), suggesting payload redistribution (*\(P = 0.005\); two-tailed t-test; \(n \geq 4\) per group). (b) Example immunofluorescence showing TNP vehicle uptake by F4/80\(^+\) macrophages and payload diffusion into surrounding tumour tissue. In lower left panel, red pseudo-colour indicates tumour cells with high levels of S3BP1 puncta. Of note, these cells are also adjacent to F4/80\(^+\) macrophages and contain elevated TNP payload. Scale bar, 50 \(\mu\)m. (c) By histological analysis, TNP payload but not vehicle is present at elevated levels within a 15-\(\mu\)m range of host phagocytes (*\(P = 0.047\), two-tailed t-test; \(n = 3\) animals). (d) By histological analysis, tumour cells immediately adjacent to F4/80\(^+\) macrophages exhibit elevated DNA damage response, compared with average DNA damage response seen in non-adjacent tumour cells for each of four animals shown by individual data points (*\(P = 0.007\); matched two-tailed t-test).
example, by targeting colony-stimulating factor 1 receptor (CSF1R)\(^\text{54–56}\). In several examples, blocking leukocyte infiltration via CSF1R inhibition or chemokine receptor type 2 ablation enhances response to chemotherapeutics including cisplatin\(^\text{52}\). However, very little experimental data describes how TAM depletion affects the response to nano-therapeutic formulations, and evidence presented here suggests that complete TAM elimination would be undesirable in such contexts.

In contrast to previous reports that focus chiefly on traditional unencapsulated cytotoxic therapies, we show here that TAMs are critical mediators of drug action for nano-encapsulated therapeutics. TAMs and other leukocytes are implicated in taking up a wide range of clinically relevant nano-therapeutics, including cisplatin\(^\text{52}\). By contrast, polymeric nano-formulations can be designed to release payloads at prescribed rates\(^\text{5} \) and even at programmed environmental (for example, pH) conditions\(^\text{62}\). Here, TNP were engineered to release their payload over a slightly longer time scale (50% drug release at 15 h; Supplementary Fig. 1d) relative to the observed PKs of cellular uptake (TAM uptake observable at 1 h and gradually increases by 24 h), and therefore were ideally suited for TAM-mediated drug delivery as described herein. Overall, this work builds on previous studies that implicate macrophages in TNP uptake; however, through engineered drug release and fluorescence imaging strategies, we demonstrate how the TNP payload can redistribute to neighbouring tumour cells at the site of local TAM accumulation in a therapeutically beneficial manner.

The results presented have implications for future development and clinical use of TNPs. Given that TNPs accumulate in TAMs at high levels, it follows that one criterion for selecting patients into TNP trials may be the degree of peritumoral TAM content, which can be assessed by magnetic resonance imaging using clinically approved NP contrast agents such as ferumoxytrol (Feraheme)\(^\text{63}\) or experimental theranostics including drug-conjugated ferumoxytrol derivatives\(^\text{64,65}\). TAM content is
especially important in the context of therapies that either increase or decrease infiltration of macrophages to tumours. For instance, doxorubicin52 and CD40 agonist antibodies56 enhance macrophage infiltration, whereas CSF1R inhibitors16 and the clinically approved trabectedin57 decrease TAM accumulation. The paradigm of TAM-mediated drug delivery targeting subsets targeting of both TAMs and their neighbouring TNP vehicles for more efficient (via improved kinetics and/or presentation) offers a compelling case for consideration of competing accumulation. The paradigm of TAM-mediated drug delivery especially important in the context of therapies that either increase or decrease infiltration of macrophages to tumours.

Animal models. All animal research was performed in accordance with guidelines from the Institutional Subcommittee on Research Animal Care (Massachusetts General Hospital). All experiments were performed using female mice that were 5- to 7-week old at the start of the experiment. For experiments with HT1080 tumours, 2 million cells in PBS were subcutaneously implanted into nu/nu mice; roughly 2-3 weeks later (once tumours reached ~8 mm diameter), imaging experiments were initiated. For the experimental model of haematogenous metastatic dissemination, 0.25 million 4T1 breast cancer cells suspended in PBS were intravenously injected into nu/nu mice. TNP injection and imaging were performed once ascites or tumour masses became evident (roughly 6 weeks later). For histology and imaging experiments using KP cells, 1 million cells were subcutaneously implanted into C57Bl6 background animals (all JAX), including C3.HK12/C3H.10T1/2 (mT) mice. For cellular differentiation, Cxcr3GFP/GFP animals were crossed with R26R−/−;mC/4T1−/− mice that ubiquitously express membrane-anchorized tdTomato66. The resulting Cxcr3GFP+/R26R−/−;mC/4T1−/− animals can be used to visualize GFP+;tdTomato+ macrophages, monocytes and dendritic cells, as well as tdTomato+ stroma and endothelium.

Cell lines. With some exceptions, all lines were obtained from American Type Culture Collection and routinely cultured according to the manufacturer’s guidelines. OVCA-429 were a kind gift from Dr Michael Birrer (MGH), and A2780CP were from Sigma. The murine lung adenocarcinoma cell line KP.19 was generated from lung tumour cells of KrasLSL;G12D+/+;p53Lo/Lo mice (C57/B6 background), which develop lung adenocarcinoma after infection with an adenovirus expressing Cre recombinase by intratracheal administration.52 KP cells were maintained in Iscove’s DMEM media supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin. All cell lines were routinely tested for contamination using mouse antibody production testing (VRL Laboratories) and assaying for mycoplasma (VRL Laboratories; Lonza MycoAlert). The fluorescent protein, mApple, was subcloned into the plVX-Apple expression vector (XhoI/BglII) and injected into the tail veins of 10-day-old C57Bl6 mice. Animals were crossed with C57Bl6 animals that ubiquitously express membrane-anchorized tdTomato66. The resulting Cxcr3GFP+/R26R−/−;mC/4T1−/− animals can be used to visualize GFP+;tdTomato+ macrophages, monocytes and dendritic cells, as well as tdTomato+ stroma and endothelium.

Intravital microscopic imaging. TNP injections were made at the indicated dose (1 mg kg−1 unless otherwise stated) via tail vein catheter immediately after mixing to a final 1 × PBS solution, at a final volume of 100 μl. Intravital microscopy was performed on an Olympus FV1000 multiphoton imaging system using a XPLUMPLFL×20 water immersion objective (numerical aperture = 0.8; Olympus America). Images were scanned sequentially using 405-, 473-, 559- and 633-nm diode lasers in combination with a DM405/488/559/635-nm dichroic beam splitter. Emitted light was then separated and captured using appropriate combinations of band splitters (SD473, SD556 and/or SD 640) and emission filters BA490–540, BA575–620, BA575–675 and/or BA655–755 (all Olympus America). Dextran Pacific blue (λex = 405 nm) was injected to initially image TMV previously described29. Briefly, 500-kDa amino-dextran (Thermo) was labelled with Pacific Blue succinimidyl ester (Thermo), purified using 30 kDa MWCO centrifugal filtration (Amicon), and 250 μg i.v. injected 10 min before imaging. Dorsal window chamber imaging was performed following previously described procedures30,60,61. Briefly, 2 million HT1080–3PBP1-mApple cells were suspended in 50 μl PBS and injected under the fascia of nu/nu mice (Cox7, MGH) 30 min following surgical chamber implantation, and imaged 2 weeks later.

Histology. Excised tumours were embedded in optimal cutting temperature (OCT) compound, flash-frozen, sectioned using a Leica CM1900 Rapid Sectioning Cryostat (Leica), stained using anti-mouse F4/80 antigen eFluor 450 (clone BM8;
In vitro NP characterization. For cytotoxicity assays (Supplementary Table 1), 5,000 cells per well were plated in 96-well plates, treated the next day with Pt-compound or the appropriate buffer control (DMF or drug-free PLGA-PEG 5,000) and assessed for viability 72 h later using PrestoBlue (Promega) or the appropriate buffer solution (DMF or drug-free PLGA-PEG 5,000). Corresponding measurements of intracellular Pt uptake (Supplementary Fig. 2d) were performed by treating 80% confluent cells in six-well plates with 24 h with TNP, rinsing with PBS, trypanosmitizing, centrifuging the cell pellet for 300 g for 5 min, re-suspending the pellet in 50 μl fresh PBS, and freezing it for subsequent AAS analysis. CellLight BacMam (Life Technologies) fluorescent protein-signal peptide fusions, delivered using baculovirus, were used for subcellular compartment co-localization experiments (Supplementary Fig. 3). Mice were treated with 20 μl CellLight reagent in 12-well plates, plated the next day on collagen-1-coated glass coverslips in six-well dishes, and the following day were treated with 0.5 μM TNP for either 3 or 24 h. Following treatment, cells were counterstained with Hoechst 33342 and immediately imaged. Collagen-coating was performed by treating coverslips with 100 μl/C0 1 rat-tail collagen-1 (BD Biosciences) overnight and then rinsing with PBS.

To analyse de-quenching of the C16-Pt(IV)-BODIPY compound (Supplementary Fig. 2d), TNP were added at 1 μM to full serum medium (DMEM + 10% fetal bovine serum + 1% penicillin/streptomycin) in the presence or absence of 90% confluent HT1080 in 96-well plates, monitored using a Tecan Safire 2 fluorescence plate reader. For monitoring TNP uptake kinetics (Supplementary Fig. 8a), equal numbers of RAW 264.7 macrophages or HT1080 cells were treated overnight with 20 μl CellLight BacMam in 96-well plates (Ibidi; Applied Biophysics), and incubated overnight. The following day cells were treated with 0.5 μM TNP for 24 h, washed, trypsinized, centrifuged, and the cell pellet for 300 g for 5 min, resuspended in 50 μl/C0 fresh media, incubated for another 24 h and then analysed for Pt concentration by AAS.

To analyse whether the Pt-containing macrophage supernatant could invoke a DNA damage response in tumour cells (Supplementary Fig. 8c), confluent 10 cm tissue-culture dishes of RAW macrophages were treated with 62 μM TNP for 24 h, rinsed in PBS, and incubated with fresh media, incubated for another 24 h and then analysed for Pt content by AAS. The supernatant was cleared by centrifugation (300 g for 5 min) before AAS analysis. Cells were harvested by trypsinization, centrifugation (300 g for 5 min), resuspension of the cell pellet in 50 μl PBS and freezing for subsequent AAS analysis.

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post injection with TNP, formalin-fixed overnight, decalciﬁed in EDTA and processed for FFPE sectioning. Immunohistochemistry was performed for apoptosis analysis by terminal deoxynucleotidyl transferase dUTP nick-end labelling, using ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore), counterstaining with Harris haematoxylin solution (Sigma-Aldrich), and scanning with a NanoZoomer 2.0RS (Hamatsu).

Metastasis burden in the lung and liver was quantiﬁed by morphological assessment in haematoxylin and eosin-stained liver FFPE sections that had been imaged using NanoZoomer 2.0RS, with organ excision at time of animal euthanasia according to the institutional guidelines (body condition score of 2) or 20 days post cancer injection, whichever came ﬁrst. No statistically signiﬁcant difference was observed in animal survival, body weight loss or lung tumour burden across the groups. One mouse from the group receiving repeated TNP dosing was excluded from analysis since no lung tumours (and very few liver tumours) were found by histology, presumably due to failed initial intravenous injection.

Clinical chemistry measurements in Swiss albino mice were performed using previously described TNP and methods2. Graphically re-plotted here for context (Supplementary Fig. 9a), bio-distribution of Pt in male rats was measured as described previously38.

References


