Published in final edited form as:

ACS Nano. 2018 December 26; 12(12): 12015–12029. doi:10.1021/acsnano.8b04338.

Quantitative Imaging of Tumor Associated Macrophages and Their Response to Therapy Using ^{64}Cu -Labeled Macrin

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Abstract

Tumor associated macrophages (TAMs) are widely implicated in cancer progression, and TAM levels can influence drug responses, particularly to immunotherapy and nanomedicines. However, it has been difficult to quantify total TAM numbers and their dynamic spatiotemporal distribution in a non-invasive and translationally relevant manner. Here, we address this need by developing a pharmacokinetically optimized, ^{64}Cu -labeled polyglucose nanoparticle (Macrin) for quantitative positron emission tomography (PET) imaging of macrophages in tumors. By combining PET with high resolution *in vivo* confocal microscopy and *ex vivo* imaging of optically cleared tissue, we found that Macrin was taken up by macrophages with >90% selectivity. Uptake correlated with the content of macrophages in both healthy tissue and tumors ($R^2 > 0.9$), and showed striking heterogeneity in the TAM content of an orthotopic and immunocompetent mouse model of lung carcinoma. In a proof-of-principle application, we imaged Macrin to monitor the macrophage response to neo-adjuvant therapy, using a panel of chemotherapeutic and γ -irradiation regimens. Multiple treatments elicited 180–650% increase in TAMs. Imaging identified especially TAM-rich tumors thought to exhibit enhanced permeability and retention of nanotherapeutics. Indeed, these TAM-rich tumors accumulated >700% higher amounts of a model poly(D,L-lactic-co-glycolic acid)-*b*-polyethylene glycol (PLGA-PEG) therapeutic nanoparticle compared to TAM-deficient tumors, suggesting that imaging may guide patient selection into nanomedicine trials. In an

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Author contributions: M.A.M., M.P. and R.W. developed the concept; H.Y.K., R.L., M.A.M., and R.W. designed the experiments; all authors contributed to data generation; all authors analyzed the results; M.A.M., H.Y.K., R.L., and R.W. wrote the paper; all authors edited the manuscript.

Competing interests: The authors declare no competing interests.

Data and materials availability: All cell lines were obtained through material transfer agreements. Requests for collaboration involving materials used in this research will be fulfilled provided that a written agreement is executed in advance between Massachusetts General Hospital and the requesting parties.

Supporting Information for Publication. Supplementary figures (S1–S10) can be found in a single PDF document of supporting information available online.

orthotopic breast cancer model, chemoradiation enhanced TAM and Macrin accumulation in tumors, which corresponded to the improved delivery and efficacy of two model nanotherapies, PEGylated liposomal doxorubicin and a TAM-targeted nanoformulation of the toll-like receptor 7/8 agonist resiquimod (R848). Thus, Macrin imaging offers a selective and translational means to quantify TAMs and inform therapeutic decisions.

Graphical Abstract

Keywords

personalized medicine; prognostic biomarker; EPR effect; immunogenic chemotherapy; tumor microenvironment; myeloid infiltration

For many solid cancers, tumor associated macrophages (TAMs) are the most abundant type of immune cell found within the tumor mass.¹ TAMs exist across a spectrum of molecular phenotypes, but the most dominant type are generally immunosuppressive macrophages.^{1, 2} Consequently, TAMs are frequently associated with increased risk of metastasis, poor response to cytotoxic, targeted, and immune checkpoint blockade therapies, and negative overall clinical outcomes.^{1–4} Furthermore, TAMs potentially have prognostic roles in the context of TAM-targeted drugs, including many that have reached clinical trials.⁵ In oncology applications, TAM-targeted therapies have been designed to act through several mechanisms, including to block their infiltration into tumors;⁶ to stimulate TAMs to shift from an M2-like tumor-promoting phenotype to a M1-like pro-inflammatory one;^{7, 8} to kill and deplete TAMs;⁹ and to block interaction of TAMs with other immune and tumor cell populations.^{10, 11} Thus, an accumulation of clinical and preclinical data collectively indicate that quantitative measurement of TAM density is a potentially useful tool for interpreting the tumor-immune milieu and predicting therapeutic outcomes. For these reasons, measurements of TAMs alongside other immune and stromal populations are becoming increasingly appreciated in the molecular classification, patient stratification, and prognosis of solid cancers.

Companion and complementary diagnostic approaches have begun to take TAMs into consideration. TAMs can be the primary source of the immune checkpoint programmed death ligand 1 (PD-L1) in tumors² and can modulate antibody action through Fc-receptor binding,¹² thus making them highly relevant for monoclonal antibodies blocking the PD1/PDL1 axis. For their role in angiogenic signaling and phagocytosis, recent work has

highlighted how TAMs can promote the delivery of drug-loaded nanomedicines to solid tumors.^{13–16} This suggests that TAM imaging is a potential complementary diagnostic for nanotherapeutics, and hints at the need for the advantages of PET imaging.

Molecularly targeted agents have been developed to image TAMs in patients, but most face obstacles with respect to specificity. Ongoing trials, some with positive initial results,^{14, 17} are examining whether magnetic resonance imaging of the iron-oxide nanoparticle ferumoxytol (which has been shown to largely accumulate in TAMs^{13, 18}) can predict delivery of liposomal irinotecan (Onivyde®; NCT01770353). In disseminated, osseous or pulmonary disease, PET imaging may be preferable. The translocator protein TSPO is highly expressed in resident macrophages of the brain (microglia) and clinical trials are using the PET TSPO tracers ¹¹C-PBR28 and ¹¹C-(R)-PK11195 to image neuroinflammation; however, in other clinical contexts, TSPO is expressed in many cell types including cancer cells and thus its imaging can be less selectively informative.¹⁹ A range of nanomaterials designed for tumor imaging,²⁰ for example the integrin-targeted silica nanoparticles ¹²⁴I-cRGDY-PEG-dot²¹ and lipid/lipoprotein-based nanoparticles,²² can indeed accumulate in macrophages but also are taken up by tumor cells and thus face similar specificity issues. Macrophage markers such as CD206 and CD163 have been imaged, but their expression may be limited to certain subpopulations and may fail to encompass macrophages across diverse phenotypes. In contrast, polyglucose nanoparticles consisting of cross linked dextrans and their derivatives (DNP, dextran nanoparticles) have remarkable affinity for tissue macrophages.²³ The biological behavior of these materials is largely determined by their building blocks, chain lengths, cross linking strategies, overall particle size, zeta potential, imaging labels (chelators, fluorochromes) and protein coronas. Different materials with variable pharmacokinetics (PK) have been described including i) a 5 nm ¹⁸F-DNP (¹⁸F-Macroflor) with rapid renal clearance (blood $t_{1/2}$ < 5 min in mice) for imaging in cardiovascular applications,²⁴ ii) a 13 nm ⁸⁹Zr labeled DNP;²⁵ and iii) similar DNPs optimized for imaging atherosclerosis and cardiac allograft rejection.^{26, 27} However, none of these preparations have ideal PK and sufficiently high tissue accumulation for tumoral TAM imaging.

In an effort to develop TAM imaging capabilities for clinical oncologic applications, we screened different compounds and settled on a ~20 nm diameter, ⁶⁴Cu-labeled DNP (⁶⁴Cu-Macrin), which is currently slated for human clinical development. Macrin differs from previous cross linked dextran nanoparticles in that all components are biodegradable and safe, the crosslinker is *L*-lysine, and biological properties are optimized to avoid rapid renal clearance and maximize accumulation in macrophages within solid tissues and tumors. The radioactive half-life of ⁶⁴Cu ($t_{1/2}$ = 12.7 h) also enables imaging at 24 h post-injection of Macrin, thus providing high signal-to-noise imaging of macrophage uptake within tumors. To fully characterize Macrin cellular accumulation, we complemented PET studies with fluorescence confocal microscopy and flow cytometry of a co-administered near-infrared labeled analog. These studies revealed that quantitative PET signals correlate with efficient and selective uptake in well-defined TAM populations, including in an orthotopic model of lung adenocarcinoma. As a proof-of-principle, Macrin imaging monitored heterogeneous TAM response to neo-adjuvant “tumor priming.” This approach identified positively-responding tumors that accumulated up to 730% higher levels of a subsequently

administered nanomedicine compared to Macrin-low tumors. Thus, here we demonstrate the potential of using Macrin to efficiently and selectively image TAMs for monitoring the innate immune response to therapy and to predict drug action.

Results and Discussion

Synthesis of Macrin for PET and optical imaging.

A streamlined Macrin synthetic scheme was designed starting with EDC/NHS activated 4 kDa carboxymethylated polyglucose, crosslinked by *L*-lysine (Fig. S1a). Macrin hydrodynamic diameter was optimized to be above the renal excretion thresholds (~8 nm) and to maximize tissue penetration and uptake in TAM rather than in the liver and spleen (< 100 nm). Time and temperature of cross-linking were tuned to yield nanoparticles with a mean hydrodynamic diameter of ~20 nm in aqueous media, as measured by dynamic light scattering (Fig. S1b–f). Macrin formation was completed after ~5 h of cross-linking at room temperature, with minimal additional change observed with extended reaction time (Fig. S1d–g). The particles showed a comparable size and spherical morphology by scanning electron microscopy (Fig. 1a) and contained roughly 200 primary amines per particle (Fig. 1a schematic) as determined by colorimetry using trinitrobenzene sulfonic acid (Fig. S1g). Free amines were then functionalized with either a radionuclide chelator (NODA-GA) or a near infrared (NIR) fluorophore, VT680XL (see Fig. S2 for characterization and purity). All remaining amines were then succinylated to generate particles with a consistent negative zeta-potential regardless of imaging functionalization (–12 mV with NODA-GA compared to –9.5 mV with VT680XL). Macrin size or morphology were not substantially impacted by functionalization (Fig. S2f–g; Mw = ~554 kDa; Mp = ~520 kDa). Chelator conjugated Macrin can be stored in lyophilized form at 4 °C for over 9 months without degradation (Fig. S2h) and is stable at room temperature in a physiological buffer for over a month. The nanoparticle yielded ⁶⁴Cu labeling efficiencies of > 99% and specific activities of ~10 GBq nmol^{–1}, ~500 mCi mg^{–1} (Fig. S2). The hydrophilic ⁶⁴Cu-Macrin (LogP = –2.78 ± 0.15) was stable in serum at 37°C over prolonged period of times (Fig. S2b–c) and exhibited a plasma half-life (t_{1/2}) of 45 min in mice, which is appropriately matched to the kinetics of ⁶⁴Cu radioactive decay (Fig. S3a–b).

Macrin enables specific imaging of tumor associated macrophages in vivo.

To first confirm the ability of Macrin to efficiently accumulate in TAMs, we performed positron emission tomography/X-ray computed tomography (PET/CT) using a murine model of colon adenocarcinoma, based on the intradermal implantation of MC38 cells in C57BL/6 immunocompetent mice. This TAM-rich model¹² displayed clearly elevated nanoparticle accumulation (10 ± 1 percent injected dose per gram tissue, %ID g^{–1}) with 16-fold higher uptake compared to adjacent muscle at 24 h post-injection (Fig. 1b and Fig. S3c–d). In principle, high tumoral uptake could be due to a host of physicochemical properties known to promote nanoparticle accumulation in solid malignancies. Neo-vascularization, high vessel permeability, and other features collectively contribute to passive nanoparticle accumulation in tumors *via* the enhanced permeability and retention (EPR) effect.^{28–31} Thus to parse the cellular-level mechanisms of Macrin uptake in tumors, we performed *in vivo* confocal fluorescence (intravital) microscopy in the same MC38 xenograft model.

Surgically implanted dorsal window chambers were used to image the distribution of near-infrared labeled Macrin in live MC38 xenografts at single cell resolution. Tumor cells were transgenically engineered to express a fluorescent histone 2B fusion protein for labeling nuclei (MC38 H2B-mApple). The *Mertk*^{GFP/+} genetically engineered reporter mouse, based on CRISPR knock-in at the *Mertk* locus on a NOD SCID background, was used to selectively image GFP⁺ cells (confirmed as specific to macrophages based on extensive validation).^{15, 32} As a *caveat* in analysis, side-by-side comparison in organ biodistribution showed an average 30% decrease in Macrin accumulation across all organs in the *Mertk*^{GFP/+} mice compared to age-matched C57BL/6 (Fig. S3e), consistent with past reports of strain-specific nanoparticle uptake and clearance;³³ nonetheless, no significant differences were found on an organ-by-organ level after correcting for this decrease (Fig. S3c). Confocal images clearly indicate selective and efficient Macrin uptake in GFP⁺ macrophages but not in other cell populations or compartments, including blood vessels and tumor cells (Fig. 1c and Fig. S4a–b). Although uptake was heterogeneous across GFP⁺ cells, >90% of them accumulated measurable amounts of the probe (Fig. 1d). *In vitro* treatment of bone marrow derived macrophages with Macrin confirmed its selective uptake, compared to tumor cells (Fig. 1e).

To complement imaging, we also performed flow cytometry to measure Macrin accumulation across immunologically defined leukocyte populations in MC38 tumors. Tumors from immunocompetent C57BL/6 mice were excised 24 h post-injection. Upon disaggregation of the bulk tumor mass, the single-cell suspension was stained for various CD45⁺ immune cell populations including lymphocytes, Ly6C^{high} inflammatory monocytes, Ly6C^{low} monocytes, neutrophils, and macrophages (Fig. 2a). Gated cell populations were analyzed for single-cell nanoparticle uptake, showing highest levels in TAMs on a per-cell basis (Fig. 2b). Compared to the second highest accumulating leukocytes in the tumor (lymphocytes), TAMs took up 6-fold higher levels of Macrin on average, and cells with highest Macrin uptake (>5000 mean fluorescence intensity, MFI) were almost exclusively TAMs (Fig. 2b). In this study, TAMs were the most abundant leukocytes in the tumor (Fig. 2c), as seen in many patients.¹ Although substantial heterogeneity was found in the composition of tumor cell types within the bulk tumor mass (Fig. 2c), >90% of Macrin signal in the tumor could be reliably attributed TAM uptake, due to both high TAM prevalence and consistently high Macrin uptake on a cell-by-cell basis (Fig. 2d). Saturated macrophage uptake of Macrin could in principle impact the specificity of its imaging; however, the imaging dose (10 nmol of VT680-Macrin, 740 µg) used here was well below *in vivo* saturation limits estimated from controlled *in vitro* experiments (Fig. S4c–d). Furthermore, the Macrin imaging dose is roughly an order of magnitude less than the dose of material used in past reports to partially saturate nanoparticle clearance by the mononuclear phagocyte system.^{34–36}

Macrin accumulation correlates with macrophage density across the body.

We next examined whether similar macrophage-specific uptake of Macrin occurred in tissues outside of the tumor. By flow cytometry we found that CD11b⁺ F4/80⁺ macrophage populations accumulated the nanoparticle on average >100-times higher than other immune cells across a panel of tissues (Fig. 3a; Fig. S5). On a per-cell basis, macrophages took up

Macrin at particularly high level in the heart, which may be attributable to its ample perfusion and vascularization. Nonetheless, the differences in Macrin uptake per macrophage were not substantial enough to correlate with overall uptake in the bulk tissue (Fig. 3a, right). In contrast, overall Macrin uptake in tissues (determined by scintigraphy) correlated exceptionally well with corresponding macrophage content (determined by flow cytometry), as examined across organs from MC38 tumor-bearing mice (Fig. 3b). Liver was excluded in this correlation due to properties that are characteristic of most clinical nanomaterials, namely residual nanoparticle accumulation in liver vasculature and the observation that hepatic phagocytes internalize a disproportionately high amount of nanoparticles, in part attributed to slow hepatic blood flow.³⁷ With this *caveat*, the results suggest that macrophage concentration in tissues is the prevailing factor in determining bulk Macrin accumulation, outweighing the heterogeneity in phagocytic activity that is seen on a per-cell basis.

To further test whether uptake across tissues correlated with bulk macrophage content, we performed confocal microscopy of excised tissue to co-image the *in situ* localization of tissue macrophages and Macrin. Guided by biodistribution data (Fig. 3b), we imaged fat and spleen as representative tissues with very low and high Macrin uptake, respectively. The *Mertk*^{GFP/+} mouse model was again used to image GFP⁺ macrophages. Although fewer GFP⁺ macrophages were present in fat compared to the spleen, Macrin labeled these cells with comparable efficiency and selectivity in both tissues (Fig. 3c–d). Confocal microscopy likewise confirmed selective nanoparticle accumulation in GFP⁺ macrophages in the liver and lymph nodes (Fig. S6), although issues with perfusion (liver) and imprecision in cell density due to small tissue size (lymph node) precluded their inclusion in the above correlation analysis. Taken altogether, these results suggest that Macrin accumulation in bulk tissue can serve as a correlative readout of the underlying macrophage density and their phagocytic activity.

Macrin quantitatively visualizes heterogeneous TAM density in orthotopic lung tumor model.

We next tested whether Macrin imaging could usefully extend to an orthotopic model of lung adenocarcinoma. As a proof of principle, we used a *Kras*^{G12D/+} *p53*^{-/-} mouse model that is particularly known for its heterogeneity in TAM levels and responsiveness to TAM-targeted therapies.¹⁶ KP tumor cells derived from this model (KP1.9 cells) were intravenously injected into a syngeneic host to form lung tumors. Four weeks after inoculation, Macrin PET/CT was performed to quantify TAM levels across individual lung tumor nodules (Fig. 4a). Macroscopic tumor lesions were clearly identifiable by CT, allowing for anatomic segmentation and corresponding Macrin standardized uptake value (SUV) measurements within the tumor masses. Inspection of one representative subject revealed varying TAM levels across tumor nodules, clearly visualized by 3D volume rendering and corresponding transverse sections (Fig. 4a): although some tumors exhibited substantial Macrin accumulation (some of which are highlighted in cyan in Fig. 4a, labeled #1–5), other segmented tumors (shown as blue in the rendering) displayed less dramatic nanoparticle signal. Analyses of Macrin uptake in whole lungs (measured by tissue scintigraphy, Fig. 4b) and segmented tumors (measured by quantitative PET, Fig. 4c) both

showed similarly heterogeneous, yet overall enhanced accumulation compared to tumor-free lung tissue.

To better understand the cellular-level mechanisms of uptake in the lung, we co-registered Macrin PET with *ex vivo* confocal microscopy. Excised lungs were imaged by *ex vivo* PET and autoradiography (Fig. 4d–e). Subsequently, tissue was then optically cleared using an optimized CUBIC solution, scanned by confocal fluorescence microscopy, and co-registered with Macrin PET (Fig. 4e). Despite differences in image resolution, good spatial concordance was observed between radio- and fluorescent-labeled Macrin signals (Fig. 4f; see S7a for sampling method), therefore suggesting that confocal microscopy faithfully reflects observations by PET.

Analysis of Macrin accumulation by confocal microscopy enabled clear identification of even microscopic tumor nodules and unambiguous delineation of neighboring masses. Consistent with PET findings, Macrin varied substantially across tumors (coefficient of variation CV = 52%), which correlated only mildly with lesion size (Fig. 5a, Spearman's correlation $R^2 = 0.2$). To better understand the underlying mechanism of this variability, tumors associated with the highest or lowest Macrin PET signal were inspected by high-resolution microscopy. In high PET signal tumors, the greater observed Macrin accumulation correlated with a significant increase in the density of phagocytic cells (Fig. 5b). The Macrin signal was assessed on a per-cell basis to account for any variations in the amount of nanoparticle uptake for each phagocytic cell between the high and low PET signal tumors. No significant differences in such uptake were found between the high and low PET signal tumors, thus indicating that any observed changes in nanoparticle accumulation were due to heterogeneity in the phagocytic cell densities (Fig. 5c). Likewise, tumor size did not correlate with Macrin uptake on a per-cell basis (Fig. S7b). Finally, we imaged the same lung cancer model using the *Cx3Cr1^{GFP/+}* reporter mouse, which expresses GFP⁺ macrophages, and found >95% of Macrin signal in GFP⁺ cells (Fig. 5d). No tumor regions were found containing disproportionate densities of Macrin-negative GFP⁺ cells (Fig. S7c). These data suggest that highly variable tumor environments do not significantly restrict the ability of macrophages to take up Macrin, at least in the KP model, and indicate that Macrin-PET accurately reports TAM density in orthotopic lung tumors.

Detection of enhanced TAMs and nanomedicine delivery following neo-adjuvant treatment.

Recent work has highlighted how TAMs can become enriched in tumors following certain chemotherapeutic treatments. This behavior has been implicated in affecting drug response and is also seen as a potential opportunity for enhancing the delivery of drug-loaded nanomedicines that rely on TAMs to accumulate in the bulk tumor mass. However, mixed response to such neo-adjuvant “tumor priming” strategies — including in recent clinical trials³⁸ — has impeded their reliable clinical implementation. We hypothesized that Macrin could serve as an effective readout of neo-adjuvant response for identifying tumors that are likely to accumulate high levels of nanotherapeutics. To test this hypothesis, we treated mice bearing orthotopic KP lung tumors with a panel of “tumor priming” regimens known to be promising for enhancing TAMs: single-dose lung radiation therapy (RT),¹⁵ the combination of oxaliplatin and cyclophosphamide (OC), and the combination of paclitaxel and

carboplatin (PTX/carbo).³⁹ Following these treatments (and using optimized dose and timing regimens indicated by prior studies),^{15, 39} we subsequently co-administered Macrin and a polymeric nanoformulation that was modeled after drug-encapsulated (e.g., docetaxel-loaded) nanomedicines undergoing clinical trials⁴⁰ or clinically approved, for instance in Korea.⁴¹

RT and OC treatments enriched TAMs by an average of 180–650% compared to the untreated cohort (Fig. 6a–b). In contrast, PTX-carbo failed to enhance accumulation of TAMs or the model nanomedicine, in agreement with prior reports that indicated PTX-carbo is poorly immunogenic in the KP model³⁹ (Fig. 6a–c). TAM enrichment by RT was dose-dependent, with the 20 Gy high-dose causing more Macrin and nanomedicine uptake compared to the 5 Gy low-dose. However, high-dose RT also enhanced accumulation of both nanoparticles in non-tumor lung tissue, suggesting high-dose RT may cause off-target effects in surrounding tissue (Fig. 6d). The degree of TAM enrichment was highly variable: while some treated tumors exhibited TAM levels that were no higher than the average seen in the untreated cohort, the top responders showed extraordinary TAM enrichment of over 10-fold (Fig. 6b). Analysis of the tumor nodule size *versus* Macrin uptake revealed that TAM enrichment was not associated with substantial tumor regression in the short term, therefore suggesting the need for additional therapy (Fig. S8a–b). With continued rounds of OC treatment, past studies have shown that these enriched TAMs have anti-tumor activity (perhaps *via* TLR4 signaling) associated with eventual tumor regression.³⁹ Interestingly, tumor irradiation especially enriched TAMs in the tumor interior, suggesting a potential for not just higher tumor drug delivery but also greater drug penetration (Fig. S8c–d). Along these lines, both OC and RT treatments enhanced the uptake of the model nanomedicine in the lung tumor nodules (Fig. 6c). Importantly, Macrin uptake in tumors correlated strongly with delivery of the model nanomedicine (Fig. 6e). Across all tumors, high Macrin uptake denoted on average 730% higher nanomedicine accumulation compared to tumors with low signal (Fig. 6f). This correlation between uptake of Macrin and the model nanomedicine was independent of tumor size (Fig. S9). Past work has shown that intratumoral nanoformulation levels correlate with longitudinal drug response, including in patients.^{13, 14, 17}

We next tested whether tumor-priming — as monitored by Macrin — could successfully correlate with improved nanotherapeutic efficacy. Using the 4T1 orthotopic mouse model of breast cancer, we pre-treated tumors with a previously optimized neo-adjuvant regimen¹⁵ consisting of a combination of 5 Gy RT and 170 mg kg⁻¹ of cyclophosphamide (RT/C). RT/C treatment induced a time-dependent enrichment in TAMs (Fig. 7a) and a corresponding increase in Macrin uptake (Fig. 7b), with highest accumulation observed 3 days post-RT/C. Based on this data, we then administered PEGylated liposomal doxorubicin (dox-lip) as a model nanotherapeutic similar to a clinically-approved formulation (DOXIL®, Janssen), 3 days following RT/C. Confocal imaging of excised tumors revealed that RT/C substantially improved dox-lip delivery (Fig. 7c), which correlated with an enhanced blockage of tumor growth (Fig. 7d) and an increase in the percentage of dead cells found in excised tumors (Fig. 7e). Overall, these results indicate that tumors with enriched TAM and increased Macrin accumulation (those treated with RT/C) respond better to nanotherapeutic treatment.

Past reports have suggested that tumor-priming can influence not just the numbers of TAMs but also their polarization - in particular, that low-dose RT can elicit pro-inflammatory macrophage phenotypes.⁴² Indeed, we found by flow cytometry that RT/C and dox-lip increased the TAM surface expression of the co-stimulatory molecule CD80 (B7-1) but not the mannose receptor CD206, suggestive of pro-inflammatory polarization (Fig. 7f).² As discussed above, macrophage density is the principal factor in determining Macrin accumulation in tissues (Fig. 2), and *in vitro* tests confirm that macrophage polarization only exerts a modest secondary effect on per-cell Macrin uptake (Fig. S10). Nonetheless, we hypothesized that nanotherapeutics designed to influence TAM inflammatory signaling would be more effective in tumors with high TAM accumulation. To test this, we treated RT/C-primed tumors with a recently described and extensively validated TAM-targeted nanoformulation, CDNP.⁸ CDNP is a cyclodextrin nanoparticle carrying the immunostimulatory toll-like-receptor 7/8 agonist resiquimod (also known as R848).⁸ Using confocal microscopy of excised tumors, we found that TAM-rich RT/C-treated tumors accumulated higher levels of CDNP (Fig. 7g) and demonstrated correspondingly decreased tumor growth following treatment (Fig. 7h). These data thus indicate that Macrin accumulation correlates with efficacy of not just traditional anti-mitotic nanoformulations, but also with nanotherapies designed to stimulate TAM inflammatory signaling.

Discussion of Macrin design and translational potential.

Although nanoparticle-based PET imaging agents are developing rapidly,⁴³ economic, regulatory, and safety issues remain substantial barriers to clinical translation. Here, we designed Macrin with feasible clinical development in mind: ⁶⁴Cu is a widely used isotope in the clinic for investigational PET studies; the toxicology of similar polysaccharide materials has been extensively studied and deemed acceptably safe; and the facile synthetic route aims to mitigate economic and regulatory costs. Broad macrophage uptake of ⁶⁴Cu may potentially result in increased radiation exposure to macrophage-rich healthy tissues. Encouragingly, imaging doses of ⁶⁴Cu have already been demonstrated as well-tolerated in patients, even for liposome agents that strongly accumulate in the liver.³⁸ Furthermore, the long half-life of ⁶⁴Cu, compared to ¹⁸F, enables imaging at 24 h post-injection, thus allowing Macrin to strike a balance between long-circulation for efficient tissue distribution, and sufficient blood pool clearance to allow the imaging of selective retention in macrophages.

Broad clinical applicability typically motivates translation of an imaging tool, and Macrin exhibits several advantages in this regard. The advantages of Macrin over previously described macrophage-avid nanoparticles such as liposomes are especially highlighted when considering the ratio of TAM to tumor cell nanoparticle uptake. For Macrin, this ratio was 20-fold (Fig. 1d-e), compared to ~6-fold seen in previous lipid²² or polymeric⁴⁴ nanoformulations. Nanoparticle-based TAM imaging may in principle be problematic in tumors with inadequate or dysfunctional vasculature; nonetheless, we found no evidence that these effects prevented Macrin from labeling TAM, at least in the orthotopic lung adenocarcinoma model used here (Fig. S7). Moreover, since we found high Macrin accumulation in nearly every macrophage population we examined across multiple tissues and tumor models, it appears that Macrin imaging can quantify tissue macrophages in a manner that is relatively robust to potential differences in their molecular phenotypes.

Macrin offers a complementary approach to methods that focus on measuring particular proteins or enzymatic activities. Surface receptors for cell adhesion molecules, sugars (e.g., mannose), folate, immune checkpoint ligands, and many others have all been proposed, along with readouts of enzymatic activities, for example of metalloproteinases, cathepsins, and myeloperoxidase. While these tools may offer insight into the molecular phenotype and function of macrophages, this is often at the cost of only examining a subset of all macrophages. More importantly, those strategies often lack the exquisite macrophage specificity seen by Macrin. In some instances, it may be preferable to only image a subset of macrophages based on their molecular phenotype. However, molecular markers that are expressed by TAMs and used as imaging targets may also exist in other cell types. For example, CD68 has also been reported expressed in some fibroblasts, tumor cells, endothelial cells, and neutrophil subsets;^{45–48} translocator protein TSPO can be moderately expressed in multiple cell types and tissues;^{45, 49} and CD163 has been reported in non-myeloid cell types including neurons⁵⁰ and carcinoma cells.^{51, 52} Some polarization-selective TAM-targeted nanoparticles exhibit only a ~2-fold preference for “M2” over “M1” macrophages.⁵³ Ideally, selectivity for M2 over M1 macrophages should exceed variability in overall TAM levels to be able to unambiguously ascertain whether nanoparticle accumulation is due to M2-like macrophages, or simply high overall TAM levels. Broad uptake into a wide range of macrophage populations thus offers a complementary perspective. We anticipate that the generalizability of Macrin for macrophage imaging will be even more important as applications extend beyond cancer, for instance to image chronic and acute inflammation in a myriad of contexts.

Diversity in applicability also applies to drug delivery applications. Radiolabeled nanopharmaceuticals have been developed, including ^{99m}Tc-labeled Doxil⁵⁴ as an early example, to monitor intratumoral drug delivery and evaluate the EPR effect in patients. In contrast to imaging or theranostic agents based on particular drug formulations, Macrin enables measurement of a specific biological feature (TAM accumulation) that has been found to be predictive of drug delivery across a variety of tumor models and clinically-relevant nanomedicines, including polymeric micelles and liposomes.¹⁴ Furthermore, the regulatory costs and toxicity risks are inherently lower for Macrin as it lacks a therapeutic payload. Thus, we anticipate it will become a useful complement to existing nanoparticles for both imaging macrophages and predicting macromolecular delivery in tumors.

More than a tool for the detection of solid tumors, we anticipate Macrin will be especially useful as a complementary diagnostic for predicting response, especially to TAM-targeted therapies. These drugs frequently block signaling pathways that mediate the infiltration and behavior of TAMs, most prominently using antibodies (e.g., LY3022855; PD0360324; Ph 2) and small molecule kinase inhibitors (e.g., Pexidartinib; Ph 3) that block the colony stimulating factor 1 (CSF1/CSF1R) axis. Other targets include the C-C chemokine receptors CCR2⁵⁵ and CCR5, which regulate recruitment of monocytes to the tumor and are for instance antagonized by BMS-813160 (Ph1/2). As other examples, drugs have been developed to target macrophage migration inhibitory factor (MIF1) and its oxidized form (oxMIF1; imalumab Ph 2), macrophage stimulating 1 receptor (RON; narnatumab Ph1), CD47 (TTI-621; Ph 1), and the macrophage activator EF-022 (Ph 1), all of which can impact TAMs directly. Small molecule inhibitors that target kinases such as PI3K γ and MERTK, or

class IIa histone deacetylase, have also shown promise and act at least in part through TAMs. The mere presence or absence of TAMs is likely to have a significant impact on the efficacy for at least some of these therapies, and we anticipate that the ability of Macrin to image TAM distribution in response to treatment will be highly informative for interpreting and optimizing drug action. Our results with CDNP — the TAM-targeted nanoformulation of the immunostimulant resiquimod, which has been shown to elicit pro-inflammatory TAM signaling in tumors⁸ — serves as one example. Macrin imaging was predictive of enhanced CDNP delivery and efficacy in response to neo-adjuvant (RT/C) tumor priming.

Conclusions

Macrophages play critical roles in immune defense and surveillance throughout the body, and their behaviors are coordinated through a network of regulatory pathways across distant tissues and organs. For this reason, TAM targeting through systemic macrophage depletion carries substantial risk of negative side effects. Even therapies that more selectively target TAM signaling pathways can be susceptible to unanticipated compensatory reactions, for instance through off-target mechanisms or through action in off-target tissues. The ability to identify TAM concentrations in lesions at a whole-body level, and to longitudinally evaluate TAM changes, will thus be important as new TAM-targeted therapies are used in the clinic. The high sensitivity and macrophage-targeting specificity of Macrin PET are uniquely suited to address this need.

Methods and Experimental

Solvents, reagents, and enzymes were purchased from Sigma–Aldrich (St. Louis, MO) and used without further purification, unless otherwise stated. Carboxymethylated polyglucose (4 kDa MW, 5% COOH) was purchased from TdB consultancy (Uppsala, Sweden). Aqueous solution was prepared using MilliQ water (Millipore). VT680XL-NHS and NODA-GA-NHS were obtained from PerkinElmer (Norwalk, CT) and Macrocyclics, Inc. (Plano, TX), respectively. ⁶⁴CuCl₂ was purchased from the Department of Medical Physics in the University of Wisconsin (Madison, WI). Particle size and zeta-potential were measured in PBS and 10 mM NaCl, respectively, by Zetaziser ZS and APS dynamic light scattering devices (Malvern Instruments). Size-exclusion chromatography (SEC) was performed using Superdex 200 Increase (10 × 300 mm, GE Healthcare) and Yarra SEC-X300 (1.8 μm, 4.6 mm × 150 mm) columns, on an Agilent 1200 Series high performance liquid chromatography (HPLC) machine, with a multichannel-wavelength UV/Vis detector (G1365D), fluorescence detector (G1321A), and a flow-through γ-detector (35900E). PBS was used as a mobile phase at a flow rate of 0.7 mL min⁻¹ and 0.2 mL min⁻¹, for semi-preparative and analytical SEC columns, respectively. Radio thin-layered chromatography (Radio-TLC) was performed on ITLC-SG paper, using 50 mM EDTA as a mobile-phase, on a Bioscan AR-2000 radio-TLC scanner (Bioscan Inc., Washington, DC), operated by WinScan V3 software. Radioactivity from excised organ tissues was counted using a Perkin-Elmer WIZARD gamma counter (Waltham, MA). Fluorescence reflectance imaging of tissues was performed with OV110 (Olympus). Distribution of radioactivity in excised lungs was visualized by autoradiography using a Typhoon 9410 scanner (Amersham Biosciences).

Macrin-NP.

Carboxymethylated polyglucose (550 mg, 2.3 mmol COOH) was activated with EDC (2.4 g, 12.5 mmol) and NHS (457.2 mg, 4.0 mmol) in 6.2 mL MES buffer (50 mM, pH 6.0–6.5) for 10 min at room temperature. *L*-Lysine (401.8 mg, 3.5 mmol) in 0.7 mL MES buffer (50 mM, pH 6.0–6.5) was added into the glass vial and stirred for 5 h at room temperature. The clear reaction mixture was drop-wise added into ethanol (30 mL), and a white pellet was obtained from centrifugation ($2.5k \times g$, 3 min). The pellet was dissolved in H₂O (MilliQ) and passed through a 0.22 μ m nylon syringe filter (Thermo). This crude material was dialyzed (MWCO 8–10 kDa, Spectrum Labs, Rancho Dominguez, CA) against H₂O (MilliQ) for 3 days at room temperature. Macrin nanoparticle (Macrin-NP) was filtered (0.22 μ m) and lyophilized to give off-white solid particles, resulting in 825.3 ± 54.3 mg.

NODA-GA-Macrin.

Macrin-NP (0.25 g, 94.0 μ mol amines) was dissolved in 3.8 mL H₂O (MilliQ) and mixed with 5.9 mL of NaHCO₃ (100 mM). NODA-GA-NHS (175 mg, 239.0 μ mol) was dissolved in 6.8 mL DMSO and added into the Macrin solution. The reaction mixture was stirred for 5 h at room temperature. The light beige solution was precipitated in ethanol and pelleted by centrifugation ($2.5k \times g$, 5 min). This pellet was dissolved in H₂O (2.5 mL, MilliQ) followed by NaHCO₃ (5 mL, 100 mM). Succinic anhydride (0.6 g, 6 mmol) in 4 mL DMSO was added, and the reaction mixture was stirred overnight at room temperature. The crude mixture was purified by dialysis (MWCO 8–10 kDa) against H₂O (MilliQ) for 3 days at room temperature. NODA-GA-Macrin in water was filtered (0.22 μ m) and lyophilized to give 168.6 mg of NODA-GA-Macrin as a white solid. The contents of dextran and primary amine per Macrin were measured by colorimetry using trinitrobenzene sulfonic acid and following manufacturer guidelines.^{56, 57}

The molecular weight of NODA-GA-Macrin was determined from calibration curves using SEC and DLS. Briefly, standard proteins with known molecular weights (M_w 13.7 – 669 kDa, Gel Filtration Calibration Kits, GE Healthcare) were injected to SEC, and retention times (t_R at 280 nm) were empirically measured to plot log M.W. *versus* t_R ($R^2=0.97$).⁵⁸ To confirm this measurement, serial dilutions of the polystyrene standard (M_p 300 kDa, Agilent) with known concentrations were prepared and used to obtain a Debye plot, which resulted in a calculation plot of M.W. and A₂.

VT680XL-Macrin.

Macrin-NP (20 mg, 7.6 μ mol amines) was dissolved in MES buffer (200 μ L, 50 mM, pH 6.0–6.5) followed by triethylamine (2 μ L, 14.4 μ mol) and VT680XL-NHS (0.5 mg in 215.5 μ L DMF, 0.27 μ mol). The reaction mixture was shaken for 3 h at room temperature on a thermomixer (900 rpm) and purified by a PD-10 column (GE Healthcare) using water as an eluent. Fractions containing fluorophore-conjugated Macrin were combined and concentrated down to ~150 μ L using centrifugal filters (MWCO 10-kDa, Amicon Ultra). This was further diluted with MES buffer (200 μ L, 50 mM, pH 6.0) and treated with Et₃N (2 μ L) and succinic anhydride (100 μ L, 750 mM in DMSO, 75.0 μ mol). This mixture was shaken at 900 rpm for 18 h at room temperature. Succinylated VT680XL-Macrin was purified by PD-10 and concentrated using centrifugal filters (MWCO 10-kDa, Amicon

Ultra). The purity was confirmed by TLC, SEC, and DLS. Fluorescently labeled Macrin (13.5 nmol mg⁻¹ Macrin) was analyzed by a SEC, a Varian Cary 100 UV/VIS spectrophotometer, and a Varian Cary Eclipse fluorescence spectrometer.

Nanoformulation and characterization.

Poly(lactic-co-glycolic acid)-b-polyethyleneglycol (PLGA-PEG) polymeric micelles were used as a model for drug-encapsulated nanomedicines being testing in the clinic, synthesized as previously described and labeled with co-encapsulation of the polymer-fluorophore conjugate, PLGA-BODIPY-TMR ($\lambda_{\text{ex}}/\lambda_{\text{em}}$ 545 nm/570 nm).¹⁶ Briefly, nano-precipitation was used, first combining 5 mg PLGA(75:25 lactide:glycolide)_{8.3kDa}-PEG_{5.5kDa} (Advanced Polymer Materials, Inc.; 70% functionality by ¹H NMR, PI 1.38 according to GPC according to manufacturer), and 1 mg BODIPY-TMR labeled PLGA(50:50 lactide:glycolide)_{30–60kDa} (Sigma) in a 200 μ L mixture of 1:1 dimethylformamide (DMF) : acetonitrile (MeCN), then added drop-wise to 10 mL H₂O under room temperature stirring for at least 6 h, then filtered through a cellulose acetate 0.45 μ m filter (Cole-Parmer), and concentrated using Amicon 100 kDa molecular-weight-cutoff centrifugal filters (Millipore). PLGA-BODIPY-TMR was synthesized and characterized as previously described.¹³ Size and zeta potential measurements were performed using DLS (Malvern Zetasizer) and determined to be ~75 nm (PDI=0.137) and -16.5 mV, consistent with previous results using similar nanoformulation routes.

Scanning electron microscopy (SEM).

Macrin was dissolved with H₂O (MilliQ), and residual salts were removed by centrifugal filtrations (10 kDa MWCO). Diluted samples were applied on a Si substrate (University wafers, 452), frozen at -80 °C, and lyophilized. The samples were then coated with a 2-nm thick platinum/palladium layer using a sputter coater (208HR, Cressington Scientific Instruments) to reduce charging effects on the non-conducting materials. The samples were imaged with the Field Emission Scanning Electron Microscope (FESEM, Ultra Plus, Carl Zeiss) in the Center for Nanoscale Systems at Harvard University.

⁶⁴Cu labeling of NODA-GA-Macrin.

Metal free buffer solutions were prepared using Chelex® 100 Chelating Resin (100–200 mesh, BioRad). NODA-GA-Macrin (10 μ g) was dissolved with acetate buffer (100 μ L, 0.1 M, pH 6.0). ~5 mCi (~185 MBq) of ⁶⁴CuCl₂ in 0.1 N HCl was mixed with acetate buffer (0.1 M, pH 8.0) to form ⁶⁴Cu(OAc)₂ and pH was adjusted to ~6. The Macrin was labeled with ⁶⁴Cu at 90 °C for 30 min on a thermomixer (900 rpm). The labeling efficiency was monitored by iTLC showing >99% labeling with a specific activity of 20.5 MBq (554 μ Ci) ⁶⁴Cu μ g⁻¹ Macrin. Trace amounts of unchelated ⁶⁴Cu were removed by EDTA chelation (final concentration ~5 mM) followed by centrifugation (MWCO 10 kDa) at 12k $\times$ g for 5 min. The buffer was exchanged to saline, and ⁶⁴Cu-Macrin was sterilized using a 0.22 μ m HT Tuffryn Membrane string filter (PALL) in ~167 MBq (~4.5 mCi) ⁶⁴Cu-Macrin with ~95% average decay-corrected radiochemical yield (RCY). Radio HPLC and iTLC demonstrated >99% radiochemical purity of ⁶⁴Cu-Macrin.

LogP measurement.

^{64}Cu -Macrin (100 μCi in 50 μL) was added into the 1:1 mixture of 1-octanol and 0.1 M citric acid (500 μL for each). The mixture was vigorously vortexed for 15 min at room temperature followed by centrifugation ($11\text{k} \times g$) for 10 min. 2 μL was taken from both layers and spotted on a piece of iTLC paper for radioactivity measurement using a gamma counter. The logP value is the mean from three replicate tubes.

Cell lines and animal models:

All animal research was performed in accordance with guidelines from the Institutional Subcommittee on Research Animal Care. Previous imaging guided experimental sample sizes in this study.^{13, 15, 24} MC38 H2B-mApple¹² tumors were generated by 2 million cells intradermally injected in 50 μL PBS, in the flanks of 7–12 week old female C57BL/6 mice (Jackson Laboratory) or *Mertk*^{GFP/+} NOD.CB17-Prkdcscid/J mice (bred at MGH) as indicated. 4T1 orthotropic breast tumor models was established in 6–8 week old female BALB/c mice by mammary fat pad injection of 500,000 4T1 cells suspended in 50 μL PBS. Imaging was performed approximately 2 weeks post-implantation once tumors reached a palpable size of roughly 5 mm. For the KP lung tumor model, 2.5×10^5 KP1.9 tumor cells (provided by Dr. Annette Zippelius, University Hospital Basel), derived from the *Kras*^{G12D} *p53*^{-/-} autochthonous tumor model as described previously,^{16, 59} were injected by tail-vein catheter in 100 μL PBS using either 6–12 week old wild-type or *CX3CR1*^{GFP/+} mice on a C57BL/6 background, as indicated. Macrin imaging was performed roughly 5 weeks later. When indicated, eGFP-expressing version of the KP1.9 tumor cells¹⁶ were used. For all procedures, mice were anesthetized with an isoflurane vaporizer on a heated stage; euthanasia was performed by CO₂ chamber when necessary, and all treatment groups underwent procedures and monitoring consecutively on the same day when possible, but in a randomized order. MC38 mouse colon adenocarcinoma cells were provided by Mark Smyth (QIMR Berghofer Medical Research Institute) and transduced using the pLVX-H2B-mApple lentiviral vector (Clontech) as previously described.⁶⁰ Bone marrow-derived macrophages (BMDM) were isolated from fresh femurs of 6–8 week old female C57BL/6 mice, cultured in Iscove's modified growth medium (IMDM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, Invitrogen), 10 ng mL⁻¹ M-CSF (Peprotech), 100 IU penicillin, and 100 μg mL⁻¹ streptomycin (Invitrogen) for 7 days before experiments. When indicated, BMDM were treated with either 10 ng mL⁻¹ IL4 (Peprotech) or 100 ng mL⁻¹ LPS (Sigma) and 50 ng mL⁻¹ IFN γ (Peprotech). MC38, 4T1, or Raw 264.7 cells were maintained in IMDM, RPMI1640, or Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% heat inactivated fetal calf serum (Atlanta Biologicals), 100 IU penicillin, and 100 μg mL⁻¹ streptomycin (Invitrogen) at 37°C and 5% CO₂.

Blood half-life ($t_{1/2}$) measurement and biodistribution studies.

Mice (C57BL/6J) received tail-vein injections of $\sim 100 \mu\text{Ci}$ ^{64}Cu -Macrin under anesthesia (2% isoflurane with 2 L min⁻¹ O₂), and blood was collected by serial retro-orbital bleeds at the indicated time-points. Whole-body biodistribution studies were performed 24 h after ^{64}Cu -Macrin administration. Mice were euthanized and perfused with PBS (20 mL) through a left ventricle prior to organ harvesting. Excised organs were weighed and subjected to

radioactivity measurement using a gamma counter (1480 Wizard 3-inch, PerkinElmer, Waltham, MA). Macrin tumor accumulation (%ID g⁻¹) was represented as an average of the two tumor values on left and right flanks. Biodistribution data were obtained after corrections of radioisotope decay and residual activity at the injection site.

Biodistribution of VT680XL-Macrin was performed in age matched C57BL/6J (n=3) and NOD-SCID mice. 10 nmol of VT680 labeled Macrin (740 µg) was injected intravenously *via* tail vein. The procedure for organ preparation for biodistribution study was the same as performed for the ⁶⁴Cu-Macrin. Tissues were placed on an OV110 (Olympus) for fluorescence reflectance imaging (exposure time: 122 ms, λ_{ex} = 620–650 nm, λ_{em} = 680–710 nm). Fluorescence intensity was obtained from background subtraction of the saline treated control (Image J, NIH).

Serum stability of ⁶⁴Cu-Macrin.

Mouse blood was collected from WT mice and left at room temperature for 30 min. Clots were removed by centrifugation (2k × g, 10 min), and mouse serum (60 µL) was incubated with ⁶⁴Cu-Macrin (~90 µCi in 20 µL) at 37 °C. The aliquots at different time points were analyzed by iTLC and size-exclusion chromatography (SEC). Duplicated chromatograms were normalized for relative intensity.

PET/CT and ex vivo autoradiography.

In vivo and *ex vivo* PET-CT was performed in a similar manner as in prior studies²⁴ using a small animal hybrid PET/CT system (Inveon, Siemens, Munich, Germany). *In vivo* imaging was performed 24 h after tail-vein injection of Macrin (8.5 ± 2.4 MBq/230 ± 64.3 µCi in 150 ± 10 µL PBS). For correlation studies with optical imaging of tumor bearing lungs, near-infrared fluorescent Macrin (10 nmol of VT680-Macrin (10 nmol of VT680 in 740 µg Macrin)) was also given i.v. to the metastatic lung tumor model and WT mice at 4 h after Macrin-PET tracer injection and ~18 h before organ extraction. High-resolution CT (Inveon, Siemens, Munich, Germany) was conducted prior to the PET scan with the following parameters: beam energy of 80 kVp, current 500 µA, and exposure time of 425 ms over 360 projections. CT images were reconstructed using a modified Feldkamp cone beam reconstruction algorithm (Cobra, Exxim Inc.). *In vivo* PET images were acquired using 30 min static acquisitions, while *ex vivo* PET images of excised lungs were acquired for 60 min duration. PET images were reconstructed using a 3D ordered-subset expectation maximization/maximum a posteriori (3D-OSEM/MAP) algorithm with 2 iterations of OSEM and 18 MAP iterations. The image data were normalized to correct for non-uniformity of response of PET, dead-time count losses, positron-branching ratio and decay correction. Energy and coincidence windows of 350–650 keV and 3.432 nsec were used. The count rates in the reconstructed images were converted to a standardized uptake value (SUV) using the animals' weight in grams and by use of a system calibration factor derived from Ge-68 cylindrical phantom to determine Bq per mL. Lungs were immediately harvested after the 24 h imaging time point, measured by scintillation counting, and then placed in custom-built glass chambers for *ex vivo* PET and autoradiography. This was performed to enable spatial co-registration between modalities, as well as facilitating optical-clearing of the lung tissue. For autoradiography, lungs were exposed to a storage

phosphor screen in a cassette (GE Healthcare) for ~12 h and read with a Typhoon 9410 laser scanner (Amersham Biosciences). PET regions of interest (ROI) analyses were guided by anatomic CT data. Further confocal microscopy of selected tumor nodules was performed based on comparisons between the PET/CT, autoradiographs and whole volume optical microscopy of intact lungs.

Intravital and confocal microscopy.

All surgeries were performed under sterile conditions. NOD-SCID *Mertk*^{GFP/+} fluorescent reporter mice^{15, 32} were anesthetized while titanium dorsal skin-fold window chambers (DSCs: APJ Trading Co, Inc., Ventura CA) were implanted as described previously.¹⁵ To develop tumor xenograft in DSC, the top skin layer was removed under anesthesia, and MC38 cells (2×10^6 in 20 μ L PBS) stably expressing H2B-mApple by viral transduction were inoculated in the fascia layer. After injection, a small amount of sterile saline was applied, and the DSC was closed with a sterile cover slip. The mice were kept on analgesic for 72 hours afterwards. Tumors were allowed to grow for 10–14 days. Macrin (10 nmol of VT680 in 740 μ g Macrin) and/or BODIPY-TMR labeled PLGA nanoparticles in saline were administered *via* tail vein injections 24 h prior to imaging. During imaging, vital signs of mice were carefully monitored under anesthesia (2% isoflurane with 2 L min⁻¹ O₂) and on a heating pad (37 °C). Images were acquired using a FluoView FV1000MPE confocal imaging system (Olympus America). Tumor cell uptake was quantified by selecting perinuclear ROIs (proximal to H2B-mApple signal). After imaging, mice were euthanized and perfused with PBS (10 mL). Excised tissues were placed on glass slides and covered with cover slips for imaging. For the orthotopic lung cancer model, whole lungs were harvested after clearance of ⁶⁴Cu-Macrin and further processed according to published procedure:¹⁶ lung tissues were imaged after fixation with 4% paraformaldehyde followed by tissue optical clearance using a modified CUBIC solution. Lung tissues containing ⁶⁴Cu-Macrin were allowed to radioactively decay for at least 5 days prior to confocal imaging. Nuclei were labeled with 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Life technologies). Prior to confocal imaging, the lungs were positioned in a custom glass chamber to facilitate tissue immobilization. The microscopy setup has been optimized and described previously:¹² 405, 473, 559 and 635 nm lasers were used with dichroic beam filters (DM405/473/559/635 nm), beam splitters (SDM473, SDM560, and SDM 640) and emission filters (BA430–455, BA490–540, BA575–620, and BA575–675), all from Olympus America.

Confocal images were analyzed using FIJI (NIH) and with Matlab-based scripts.^{13, 61} When appropriate, the images were pseudo-colored, and the z-projections of 3D images were created. To define the boundary of the cells and tumors for fluorescence quantification, ROIs were generated by automated thresholding segmentation followed by water-shedding. To quantify the co-localization of CX3CR1-GFP and VT680-Macrin signals at the single cell level, average fluorescence intensity in each cell was measured, with fluorescent signal 10% higher than that of the background intensity considered GFP⁺ or VT680⁺. For quantifying VT680-Macrin and BODIPY-TMR-PLGA nanoparticle uptakes in eGFP expressing KP1.9 lung tumor, ROIs were generated by segmenting the images in the GFP channel, and the average fluorescent intensities (normalized to background and the intensity in the non-tumor

tissues) in the BODIPY-TMR and VT680 channels were calculated for each tumor. Finally, to assess the co-localization of nuclear and optical Macrin signals, PET, autoradiographic, and optical images were co-registered using the boundary of the lung tissues.

Flow cytometry.

MC38 tumor bearing C57BL/6 mice (n=3) or 4T1 tumor bearing BALB/c mice (n=22) were euthanized 24 h after i.v. injection of VivoTag (VT) 680-Macrin (10 nmol VT680XL in 740 μ g Macrin). PBS was injected in age-matched control mice. All animals were perfused with PBS (20 mL) from the left ventricle, and organs were excised and weighed prior to tissue digestions for single cell suspension. Tissues were minced in a 37 °C solution containing collagenase I (450 U mL⁻¹), collagenase XI (125 U mL⁻¹), DNase I (50 U mL⁻¹), and hyaluronidase (60 U mL⁻¹) and incubated for 1 h with an agitation (750 rpm). The digested tissues were then passed through 40 μ m cell strainers using PBS containing 0.5% bovine serum albumin (FACS buffer) and centrifuged (4°C for 7 min at 340 $\times$ g). The cell pellets were re-suspended with FACS buffer (300 μ L) and incubated with a fluorophore-labeled antibody cocktail containing the following: anti-CD45 (30-F11), anti-CD11b (M1/70), anti-F4/80 (BM8), anti-Ly6C (HK1.4), and anti-Ly6G (1A8) antibodies, all purchased from Biolegend. Dead cells were stained with BD Horizon™ fixable viability stain 700 (BD Bioscience) or Zombie Aqua fixable viability stain (Biolegend) and were excluded from analysis, with the exception of Fig. 7e which quantified % dead cells. After washing and resuspension, samples were run on LSR II flow cytometer (BD Biosciences), and data were analyzed by FlowJo software (Treestar). Macrophages were defined as CD45⁺/CD11b⁺/F4/80^{high}, lymphocytes as CD45⁺/CD11b⁻/F4/80⁻, neutrophils as CD45⁺/CD11b⁺/F4/80⁻/Ly6G^{high}, and monocytes as CD45⁺/CD11b⁺/Ly6C^{low/high}. Interstitial macrophages in lung were identified as CD45⁺/CD64^{high}/F4/80^{high}/MHCII^{high}/CD11b⁺. In some experiments, the samples were further stained with anti-mannose receptor (C068C2) and anti-CD80 (16-10A1), purchased from Biolegend. In Fig. 2, relative tumor cell abundance, and Macrin accumulation in tumor cells compared to TAMs and other immune cells, were deduced from a combined flow-cytometry measurement of CD45⁻ cell abundance (which includes tumor and other cells) and Macrin uptake in CD45⁻ cells within the bulk tumor mass; intravital microscopy of MC38, TAM, and Macrin, showing proportionate levels of each cell population and corresponding amounts of Macrin uptake on a per-cell basis; and supporting *in vitro* Macrin uptake experiments which quantitatively agreed with intravital and flow cytometry measurements (see Fig. 1). In all cases, the ratio of TAM to tumor cell uptake of Macrin was 20:1 or greater.

Chemotherapy, radiotherapy, and tumor growth model.

For radiotherapy, dual source ¹³⁷Cs Gammacell 40 Exactor (Best Theratronics) with a custom lead shield was used for irradiation of the whole lung, using a setup described previously.¹⁵ Irradiation was performed in mice anesthetized *via* 87.5 mg kg⁻¹ ketamine and 12.5 mg kg⁻¹ xylazine i.p., immobilized in the lead shielding chamber, and irradiated at 0.6 Gy min⁻¹ three days prior to administering Macrin. A single dose of 2.5 mg kg⁻¹ oxaliplatin and 50 mg kg⁻¹ cyclophosphamide, or 10 mg kg⁻¹ paclitaxel and 10 mg kg⁻¹ carboplatin, was given to mice i.p. four days prior to administering Macrin.³⁹ For the 4T1 tumor model, a combination of 5 Gy γ -irradiation and 170 mg kg⁻¹ cyclophosphamide (given to mice i.p.)

was used,¹⁵ followed by a single dose of 10 mg kg⁻¹ PEGylated liposomes loaded with doxorubicin (FormuMax) or previously formulated cyclodextrin nanoparticle loaded with 10 mg kg⁻¹ R848 (Selleckchem) delivered intravenously.⁸

4T1 orthotropic tumors on BALB/c mice were allowed to grow to 20 mm³ before the initiation of the treatment. Prior to treatment, the mice were assigned to each cohort so that the average mice weight and tumor volume were similar across different cohorts. Tumor volume was monitored every day for 10 days post neo-adjuvant treatment using a caliper, and calculated according to the following formula: volume = (1/2)*(width²)*length.

In vitro VT680-Macrin uptake experiments.

10,000 BMDM were seeded in 96-well plates, and subsequently treated with VT680-Macrin. For the saturation experiment (Fig. S4c–d), 125,000 Raw macrophages were treated with 1 mL of culture media containing varying doses of Macrin (ranging from 1 to 100 nmol of VT680-Macrin). Given that total number of macrophages in mice (and even in the mouse liver alone) is estimated to be > 10⁷,⁶² our *in vitro* data (8×10⁻⁴ nmol VT680-Macrin (60 ng of Macrin) uptake per macrophage, without apparent saturation) predict that > 10× dose would be required for substantial saturation effects compared to the dose used for imaging. 24 hours after the treatment, the cells were carefully washed with PBS and fixed with 4% PFA. The cells were then stained with DAPI to label nuclei. Fluorescent images of VT680-Macrin inside the cells were then acquired using DeltaVision (Applied Precision) modified Olympus BX63 microscopy system with a Neo sCMOS monochrome camera (Andor) and an environmental chamber. Fluorescent intensities were quantified using Image J (NIH).

Statistics.

Unless otherwise indicated, results are expressed as mean ± standard error of the mean throughout. Statistical analyses were performed using Prism (GraphPad), MATLAB (Mathworks), and Excel (Microsoft). Two-tailed t-tests, ANOVA tests, Spearman correlation tests, and Pearson correlation tests were used with false-positive thresholds of α = 0.05. When appropriate, the Tukey test for multiple comparison was used following ANOVA tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments.

The author would like to acknowledge the help of Dr. Lisa Honold, Dr. Juhyun Ou, and Dr. Hyungsoon Im (MGH-CSB). We acknowledge Benoit Tricot, Stephen Schmidt, and Greg Wojtkiewicz for assistance with imaging and biodistribution through the MGH-CSB MIP program. Part of this work was supported by NIH/NCI grants R00CA207744, R01CA206890, U01CA206997, R01HL131495, and T32CA079443.

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Figure 1. Macrin enables selective *in vivo* imaging of TAMs.

a At top, Macrin schematic shows polyglucose (black) crosslinked and functionalized with *L*-lysine (red); primary lysine amines are used to attach chelators for ⁶⁴Cu or fluorophore labels. Distribution of Macrin particle diameters as measured by SEM, along with representative images (right), are shown with scale bar = 20 nm. **b** PET/CT reconstruction of Macrin in bilateral hindleg MC38 tumors, imaged 24 h post-injection in C57BL/6 mice. **c** Representative *in vivo* confocal fluorescence imaging of MC38 tumors at low (left) and high (right) magnification in *Mertk*^{GFP/+} reporter mice. Scale bars are 100 μm and 10 μm respectively. **d** Quantification of *in vivo* Macrin uptake using microscopy data from *c*. **e** *In vitro* VT680-Macrin uptake after 24 h incubation with MC38 or bone marrow derived macrophages. Data are means ± s.e.m. across n > 25 cells. ****P < 10⁻⁴, two-tailed student's t-test.

Figure 2. Flow cytometry confirms predominant TAM uptake of Macrin.

10 nmol of VT680-Macrin, corresponding to 740 μg of total Macrin material, was injected into MC38 tumor-bearing C57BL/6 mice. Macrin distribution within the tumor tissue was assessed. **a)** From single-cell suspensions of dissociated MC38 tumors, host cell populations were gated as shown, with gate color corresponding to cell-type in the legend. **b)** Histogram shows single-cell distribution of the boxed leukocyte populations above (see *a*, left), determined by near-infrared fluorescence (TAMs, blue; lymphocytes, red; Mo/neutrophils, black; macrophages from PBS vehicle control treated mice, shaded gray). **c)** Gated cell populations (see *a*) were tabulated according to relative prevalence, with colors corresponding to cell-type legend in *a*. **d)** From data in *b-c*, distribution of Macrin was calculated as a function of cell-type in the tumor, again with colors corresponding to cell-type legend. Relative tumor cell abundance and their Macrin accumulation were calculated from a composite analysis of both flow cytometry and imaging (see Fig. 1; *methods*). Data are means $\pm$ s.d. ($n = 3$).

Figure 3. Macrin accumulation correlates with macrophage density across the body.

a) Tissues from MC38 tumor-bearing C57BL/6 mice were excised and analyzed by flow-cytometry for accumulation of VT680-labeled Macrin ($n = 3$; see Fig. S5 for gating scheme). Interstitial macrophages and Kupffer cells are shown for lung and liver, respectively. **b)** In the MC38 xenograft model, tissue macrophage density was calculated from flow cytometry (x-axis) and correlated with ⁶⁴Cu-labeled Macrin biodistribution ($n=6$). Data are means $\pm$ s.e.m. (t-test for Pearson's correlation). **c-d)** Fat and spleen were confocally imaged from *Mertk*^{GFP/+} reporter mice (containing GFP⁺ macrophages), 24 h post-injection with VT680-labeled Macrin, shown as representative images (c; scale bar, 10 μ m) and corresponding quantification (data are means $\pm$ s.e.m. across $n > 20$ cells, **** $P < 10^{-4}$, two-tailed student's t-test).

Figure 4. Multiscale analysis of Macrin accumulation in an orthotopic model of lung adenocarcinoma.

a-f) ^{64}Cu and VT680 labeled Macrin were administered to image TAMs at 24 h post-injection, using the KP lung adenocarcinoma model in C57BL/6 mice. **a)** Reconstructed PET/CT showing segmented lung tumors (blue and cyan) and Macrin (orange). Representative tumors with high Macrin uptake (cyan tumors with arrows) were further highlighted in corresponding transverse sections. **b)** Whole tissue biodistribution scintigraphy was performed using tumor-bearing and healthy (control) whole lungs ($n > 5$, means $\pm$ s.e.m.; two-tailed t-test). **c)** SUV analysis of PET data was performed in tumor-bearing lungs ($n > 5$, means $\pm$ s.e.m.; two-tailed t-test). **d-e)** *Ex vivo* imaging corresponding to *in vivo* imaging data, especially for the highlighted tumors #1–5 (a), based on *ex vivo* PET (d) and confocal microscopy of optically cleared lung tissue (e). Autoradiography (ARG) is overlaid on the optical images for reference (d and e, scale bar=2 mm). **f)** Correlation between optical (VT680-) and nuclear (^{64}Cu -) Macrin imaging using data in e, calculated from tumor-sized regions of interest (Spearman's correlation reported, $n = 25$ tumors). Numbered points correspond to highlighted tumors in a (see Fig. S7 for correlation method).

Figure 5. Macrin imaging captures heterogeneity in TAM accumulation across metastatic lung tumor nodules.

a) 24 h post-injection, Macrin concentration was measured through whole tumors in optically cleared KP-tumor bearing lungs in C57BL/6 mice (see Fig. 4e). Data are shown as individual tumor measurements across 264 tumors from 5 lungs, correlated with each tumor's size (scatterplot, red) and shown as a corresponding histogram describing tumoral Macrin uptake (blue). CV, coefficient of variation. **b)** Tumors with exceptionally high or low Macrin uptake by PET were analyzed for their corresponding abundance of Macrin⁺ TAMs using *ex vivo* confocal microscopy (n = 4 tumors, student's two-tailed t-test; means $\pm$ s.e.m). **c)** Macrin⁺ TAMs in **b** were quantified for accumulation of Macrin on a per-cell basis (n>50 single-cells shown per group). **d)** KP-tumor bearing lungs from *CX3CR1^{GFP/+}* C57BL/6 reporter mice were imaged by confocal microscopy to quantify overlap between GFP⁺ macrophages and VT680-labeled Macrin. Representative low and high magnification images are shown (top, scale bars=1 mm; bottom, scale bar= 50 μ m, insert scale bar=20 μ m).

Figure 6. Monitoring TAM response to neo-adjuvant treatment enables prediction of nanomedicine delivery.

KP-tumor bearing lungs from C57BL/6 mice were pre-treated with one of four neo-adjuvant treatments. 24 h prior to excising the lungs, VT680-labeled Macrin and a model BODIPY-TMR-labeled PLGA-PEG nanomedicine were co-administered. The lungs were optically cleared and imaged. **a**) Representative confocal images showing accumulation of Macrin, PLGA-PEG nanoparticles, and GFP⁺ KP tumors in whole lung lobes (outlined blue). Representative tumors are outlined yellow for reference (Scale bar= 2 mm). **b-c**) Macrin (b) and PLGA-PEG NP (c) uptake in tumors following neo-adjuvant treatment ($n > 700$ tumors in 21 lungs across 5 conditions; box and whiskers show median $\pm$ I.Q.R. and min/max, respectively; *** $P < 10^{-3}$, ANOVA). **d**) NP accumulation in non-tumor tissues ($n \sim 500$ regions across >14 lungs; means $\pm$ s.e.m; *** $P < 10^{-3}$, ANOVA). **e**) Correlation of tumor uptake of co-administered NPs, shown as average tumor intensity for each lung (Spearman

correlation R and P values reported, n=21 lungs). **f)** Average PLGA-PEG NP uptake in KP tumors stratified according to their Macrin uptake (n > 50 tumors per group; means $\pm$ s.e.m).

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Fig. 7. Macrin correlates with nanomedicine delivery and response.

BALB/c mice bearing 4T1 mammary fat pad tumors were pre-treated with a combination of 5 Gy of radiation and 170 mg kg⁻¹ cyclophosphamide (RT/C), 72 h prior to subsequent treatment unless otherwise stated. **a-b**) Flow cytometry of tumors analyzed 24 h following Macrin administration, which was injected at the indicated times following RT/C (n=20 total tumors). **c**) Representative confocal microscopy of intrinsic doxorubicin fluorescence (left, tumor outlined in yellow, scale bar=100 μ m) and quantification (right), 48 h after dox-lip treatment (n~300 regions across 8 tumors). **d**) Tumor size was monitored over time (left); corresponding single-tumor data are shown at right (n=24 total tumors). **e-f**) Flow cytometry of excised tumors, 48 h post-treatment with dox-lip or vehicle (n=16 total tumors). **g**) Representative confocal microscopy of tumors, 48 h after treatment with AlexaFluor555-labeled CDNP (left, tumor outlined in yellow, scale bar=100 μ m), quantified at right (n~250 regions across 8 total tumors). **h**) Tumor size was monitored over time (left); corresponding

single-tumor data are shown at right (n=24 total tumors). For all, data are means $\pm$ s.e.m, *** $P < 10^{-3}$, * $P < 0.05$, ANOVA.

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