In vivo imaging of pro- and anti-tumor cellular components of the tumor microenvironment

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ABSTRACT

Tumor development and growth as well as metastatic spread are strongly influenced by various, mostly innate, immune cells, which are recruited to the tumor site and driven to establish a specific tumor-supportive microenvironment. The contents of this microenvironment such as myeloid cells are a major factor for the overall prognosis of malignant disease, addressed by a constantly growing armament of therapeutic interventions, targeting tumor-supportive immune cells. Current clinical imaging has long ignored the growing need for diagnostic approaches addressing these microenvironmental contents, enabling a sensitive and specific classification of tumor immune crosstalk and resulting tumor-associated immune cell activity.

In this focus article, we review the present status and promising developments of in vivo molecular imaging approaches of tumor immune components, designed to allow for inference on the cross-talk between tumor cells and the immune system.

Based on the infiltrating cell types current imaging modalities are briefly discussed.

Key Words: Tumor microenvironment, tumor-induced inflammation, tumor immunology, in vivo imaging

INTRODUCTION

The prognosis of cancer is most notably dependent on tumor cell invasion and metastasis, both being promoted by cellular and molecular mechanisms in the primary tumor and at distant pre-metastatic sites. Already at early stages of their development, tumors are infiltrated by mainly innate immune cells and build a characteristic tumor microenvironment (TME). The active recruitment of various immune cells results in tumor-associated inflammation, which is an important condition of tumor growth and expansion, and has been described by Hanahan and Weinberg as a new hallmark of cancer (*1*).

These observations have led to an increasing amount of targeted therapeutic approaches in oncology. However, specific in vivo biomarkers for tumor-mediated immune cell activity and monitoring under therapy are still missing.

Therefore, there is a growing need for visualization and measurement of molecular and cellular components of the TME in vivo, to aid development of new therapeutic options and enable their theranostic monitoring as well as to gain better understanding of tumor biology.

Anti-tumoral cellular components of the tumor microenvironment

During cancer development tumors enforce a shift in the TME from normal tissue homeostasis to promoting malignant progression. The

TME is composed of pro-tumoral immune cells, which enable tumor cell invasion and metastasis and, in part, modify certain inflammatory cell types to render them tumor-promoting rather than suppressive (*2*). However, especially in early tumor development, anti-tumoral characteristics exist. Immunosurveillance of cancer is mainly driven by natural killer cells (NK) and CD8-positive T cells.

Both are known as effector cells of the immune system and are cytotoxic to cancer cells through perforin- and granzyme mediated apoptosis. CD8-positive T cells are activated by antigen presentation of dendritic cells via the major histocompatibility complex I and induce apoptosis in antigen presenting cells (*3*). Additionally to their endogenous protective effects, NK cells are also mediators of anti-tumoral cytokines like IL-2 and IL-12 (*4*). Indirect evidence for the effect of immunosurveillance is provided by reduced risk of cancer in patients with high levels of NK cells (*5*). Furthermore, NK cells induce a reinforced T cell response via interferon γ mediated activation of dendritic cells (*6*), which increases anti-tumoral immune response in a feedback-loop.

Pro-tumoral cellular components of the tumor microenvironment

Various interactions between cellular components of the TME result in the retention of a pro-tumoral setting that enhances tumor progression.

Tumor-associated macrophages (TAM) constitute the biggest population of pro-tumoral components of the TME (*6*). TAM are among the first non-neoplastic cells, infiltrating the tumor. They are attracted by chemokines secreted by both, malignant and stromal cells, especially via chemokine (C-C motif) ligand 2 (*7,8*). TAM share characteristics with M2 macrophages: they promote the degradation of the extracellular matrix by releasing proteolytic enzymes, whereas in hypoxic tumor areas, they induce neoangiogenesis through hypoxia induced factor dependent release of vascular endothelial growth factor (*9*).

TAM aid the expansion of the proinflammatory microenvironment by inflammatory cytokines like tumor necrosis factor α, resulting in a self-augmenting process (*10*).

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of premature granulocytes, macrophages, dendritic cells and myeloid precursors, expanding during tumor development (*11*). They are associated with tumor progression and neoangiogenesis (*8*). Through the production of arginase 1 and iNOS, MSDC are potent suppressors of CD4- and CD8-positive T cells, but also NK cells (*12*). MDSC may also differentiate into TAM under hypoxic

conditions (*11*), which underlines the close connection between TAM and MSDC.

Levels of neutrophils are increased in several types of cancer like colon, gastric or lung cancer (*8*) and are associated with poor prognosis (*2*) as they are associated with increased invasion and metastasis (*2*)

While T helper type 1 cells act as tumor opponents, CD4+ T helper type 2 cells can steer polarization of tumor-associated immune cells away from anti-tumoral activity (*13*).

Regulatory T cells (T_{reg}) have a central role in tumor development by directly suppressing effector T cells and establishing an immunosuppressive environment, inter alia via secretion of various cytokines like IL-10 or transforming growth factor β (*14*).

Detailed characteristiscs of anti- and pro-tumoral cell types are provided in table 1.

Molecular imaging of the tumor microenvironment

Molecular imaging approaches offer the opportunity to examine tumor immune interaction in vivo non-invasively. Single cells or molecules as TME components can be visualized sensitively and specifically by either cell tracking strategies or targeting of effector molecules. Labeling strategies for cell tracking can include direct labeling of isolated cells and transfection of cells for stable expression of traceable molecules or, less invasively, the administration of a specific tracer for cell-bound target structures in vivo (*15*).

In principle, all conventional imaging technologies, including optical imaging, radionuclide driven approaches as well as magnetic resonance imaging (MRI) enable visualization of TME components following either of theses strategies.

In this article we review exemplary approaches of imaging tumor immune interaction based on the most important tumor-infiltrating immune cells (Figure 1).

Tumor associated macrophages

Macrophages are the most abundant cells in the TME, their crucial role in tumor development has been recognized early.

Their ability for effective phagocytosis renders TAM ideal cells for direct labeling using particles of different sort: mannosylated liposomes loaded with ^{64}Cu and taken up by TAM, have been used for PET imaging in a mouse model of pulmonary adenocarcinoma. Integration of a fluorescent dye in the lipid bilayer of liposomes

allowed for correlative fluorescence microscopy (*16*). While phagocytosis of mannosylated liposomes as well as macrophage mannose receptor expression are not exclusive to TAM but have been reported for resident macrophages and other phagocytes as well, the uptake of high-density lipoprotein (HDL) is supposed to be more specific for macrophages (*17*). ^{89}Zr modified reconstituted HDL served as a label for PET imaging of TAM in an orthotopic murine breast cancer model. Due to the biological function of HDL concerning the cholesterol efflux from macrophages (*18*), the authors concluded to selectively target TAM rather than witnessing passive HDL accumulation. PET imaging allowed noninvasive visualization of labeled HDL at tumor sites and TAM could be identified as main HDL-container in ex vivo analyses (*17*).

For MRI cell tracking, Shih et al. injected superparamagnetic iron oxide nanoparticles (SPIO) systemically for longitudinal assessment of TAM accumulation during tumor development. Macrophages are known to be the major cell type, internalizing SPIOs. MR signal alteration, reflective of local SPIO accumulation, was found in close vicinity of tumor vessels and interpreted as anchor points for tumor expansion. Correlative post-mortem analyses provided proof of SPIO-labeled TAM in tumors (*19*). Other MRI techniques focus on nanoparticle based imaging of macrophages and have been reviewed in detail elsewhere (*20*). Referring to tumor associated macrophages, a magneto-fluorescent nanoparticle (AMTA680) has been presented that addresses a subset of myeloid cells with M2 like phenotype and showed specific labeling of CD11b+ myeloid cells.

The particle was equipped with two reporter tags, a fluorescent dye and a superparamagnetic core, and could, upon intravenous injection, be detected in the TME by microscopy, MRI and fluorescence mediated tomography (*21*).

Ultra small SPIO have a longer circulation time as compared to SPIOs as they are not as quickly recognized and eliminated by the reticulo-endothelial system. The result is a comparatively stronger accumulation in the TME.

USPIO-driven MRI allowed for non-invasive imaging of F4/80 positive phagocytes. In vitro studies showed particle internalization by F4/80+ TAM, but not by tumor cells. Accordingly, TAM depletion led to a significant inhibition of tumor nanoparticle enhancement (*22*). With the USPIO ferumoxytol being clinically available, a high translational potential for this application is evident.

All these direct labeling approaches suffer from only limited specificity as not only TAM but also resident macrophages and other phagocytes can in principle accumulate the label/particle and contribute to the image signal. An additional constraint always is the potential influence of the label on cell homeostasis and function.

For more selctive in vivo imaging of M2 oriented macrophages, including TAM, ^{99m}Tc labeled nanobodies, binding the macrophage mannose receptor specifically, were introduced in a preclinical proof of concept (*23*).This receptor is strongly expressed on pro-angiogenic TAM, that reside in hypoxic tumor areas. After intravenous injection of ^{99m}Tc-labled anti-macrophage mannose receptor nanobodies, TAM could be detected successfully by Single

Photon Emission Computed Tomography (SPECT) in breast and lung cancer.

Other SPECT and fluorescence imaging driven approaches targeting macrophage mannose receptor in murine breast cancer showed high and specific expression in M2 macrophages after sorafenib-induced polarization in tumors. The specific probe IRD-aCD206 could also suppress tumor growth in sorafenib-resistant tumors by light irradiation and consecutive reduction of M2 activity (*24*).

Intravenous injection of luciferase-expressing murine macrophages enabled in vivo cell tracking in a murine colon cancer model with mCherry-labeld cancer cells visualized separately (*25*). The injected cells did have an influence on tumor growth, responsive to dexamethasone. However, the cells were not endogenous and changes of biological behavior and cell cell interaction due to the expression of luciferase remain unclear.

As a marker of monocyte activity in inflammation and chemokine for TAM and MDSC attraction, the locally released protein heterodimer S100A8/A9 gained attention in immunology and oncology (*26*). We have introduced an approach for targeted imaging of S100A9 secreted actively by monocytes as well as MDSC within the TME (*27,28*). We could visualize monocyte activation beyond sheer abundance in the primary tumor and target tissue of metastasis by in vivo optical and radionuclide imaging (*29*), Figure 2.

Myeloid-derived suppressor cells

TAM and MSDC share cathepsin secretion as a means for environmental remodeling. Commercially available optical probes address this protein upon secretion, enabling for measurement of TAM and MDSC activity. ProSense680 has been used for visualization of cathepsin B activity in high vascularized polyps. Genetic ablation of of cathepsin B reduced polyposis (*30*), reflecting the strong influence on tumor development and progression. Co-localization of CD11b and Gr1 staining with ProSense680 signal identified TAM and MDSC as main sources of cathepsin

MDSC could also be visualized using a ^{111}In-labeled anti-S100A9 antibody in murine breast cancer models in SPECT/CT (*29*). Imaging 6 h post injection identified already small tumors with 3 mm diameter but revealed also high tracer accumulation in bone marrow and spleen, which are preferred localizations of MSDC (*31*).

Due to the phenotypical overlap between TAM and MDSC and the heterogeneity within the MDSC population (*32*), the currently available imaging approaches offer little opportunity to differentiate between the two cell populations in vivo and further research needs to resolve how best to define and distinguish the individual actors within the TME.

Neutrophils

For imaging of neutrophil activity, the secreted protease neutrophil elastase has been targeted. The enzyme neutrophil elastase contributes to tumor growth, invasion and metastasis (*33*). In vivo optical Imaging revealed a highly specific enzyme signal using the

neutrophil elastase 680 fluorescent activatable fluorescent imaging agent in a xenograft model of colon cancer. Accordingly, the neutrophil elastase inhibitor sivelestat could reduce tumor growth and tracer uptake (*34*).

T cells

In recent years, T cells became the target of several therapeutic agents that aim to revert the immune-modulation, exerted by e.g. T_{reg} (Figure 3). The in part amazing success of these immune check point therapies is hampered by a lack of means for identification and screening of patients, who would likely benefit from e.g. an expensive anti cytotoxic T lymphocyte-associated antigen 4 (anti-CTLA-4) driven therapy.. Moreover, adoptive T cell transfer is a means of therapy in itself and in vivo monitoring of the course of treatment using cell tracking techniques became a popular option (*35*). The numerous approaches for T cell tracking and to image T cell-driven anti-tumor immunity have been reviewed elsewhere (*36,37*).

Numerous imaging approaches have been employed in the context of adoptive T cell transfer for monitoring of cell distribution and fate. Aside from those, basicall resembling labeling methodology, described earlier, such as iron-loaded particles for MRI or ^{111}In-oxine for SPECT (*38*), both mostly carried out on isolated and purified cell populations ex vivo before transfer, other approaches make use of the specific characteristics of T cells. T cells exert cytolysis through interaction between T cell receptor and multi-histocompatibility complexes (MHC), usually representing pathogens. These MHC can be modeled and multiplied and, equipped with an imaging label, used for specific visualization of T cells in vivo (*39*).

Similarly using the CD3 T cell membrane receptor, bivalent antibodies, e.g. binding carcinoembyonic antigen and CD3 can serve as theranostic agents enabling in vivo (fluorescence) imaging while

triggering strong cytotoxic T cell activation, resulting in killing of tumor cells (*40*).

CTLA-4 is expressed on T cells and – similar to PD-1 – exerts inhibitory effects on anti-tumor immunity via multiple signaling pathways. Specific tracers for CTLA-4 have been presented, e.g. ^{64}Cu-DOTA-anti-CTLA-4 for PET imaging in murine syngeneic colon carcinoma (*41*).

The PD1 - PD-L1 signaling axis was one of the first to be addressed successfully for immune check point therapy. Binding T cell PD1 by tumor cell PD-L1 inhibits cytotoxic anti-tumor activity and aides tumor immune evasion. Inhibition of PD1 binding on T cells can revert this tumor-mediated immune remodeling and unleash an effective anti-tumor immune response. Albeit frequently successful, the therapy fails in a significant number of patients and so far, means for safe identification of those patients, likely to benefit, are missing. A first attempt was to visualize PD-L1 directly, using the therapeutic agents as tracers for identification of PD-L1 positve tumors (*42*).

In vivo imaging of the effector cells, endogenous helper $CD4^+$ and cytotoxic $CD8^+$ cells has been realized by immuno-PET detecting ^{89}Zr-labeled anti-CD4 and anti-CD8 antibody fragments (*43,44*).

As T cells exert many of their effects via membrane-bound receptor interaction, membrane labels can inhibit cell function and viability at relatively low doses already, as has exemplary been shown with anti-CD4 diabodies for ^{89}Zr-driven PET (*45*).

Natural Killer Cells

Several techniques for imaging NK cells in vitro and in vivo, mainly focussing on direct labeling methods, have been introduced and reviewed elsewhere (*46*).

Imaging NK cell marker CD56 using a ^{99m}Tc labeled anti-CD56 allowed for estimation of tumor infiltrating NK cell abundance; in vivo imaging signals correlated with good prognosis (*47*).

In accordance with T cell tracking techniques, trafficking of murine NK cells has been described by radiolabeling of isolated NK cells with ^{111}In-oxine. Although migration was not impaired in exemplary studies cell viability and function was allegedly unchanged, the translational potential of NK cell tracking is limited due to reportedly high background activity using ^{111}In-oxine driven approaches (*47*).

CONCLUSION

In recent years, the cellular and molecular composition of the TME as well as interactions between tumor and TME components became increasingly relevant for cancer research and tumor therapy.

It is well known that various infiltrating immune cells either promote or hamper tumor growth and therefore have significant influence on cancer prognosis.

Novel treatment strategies – among them highly expensive regimens – require biomarkers for estimation and continuous measurement of the malignant potential or treatment response. Thus, non-invasive in vivo imaging approaches that allow for visualization of specific components of the tumor microenvironment are essential to gain further understanding of tumor pathogenesis and tumor-immune

interaction. An ideal imaging technology in this context addresses endogenous imaging targets specifically and non-invasively and permits longitudinal measurements after single-dose injection of a tracer. Rapid tracer elimination reduces unspecific accumulation in liver or kidneys and prevents accumulation errors in target tissue in case of repeated examinations. Several imaging approaches targeting either immune cells directly or soluble factors, reflecting the activity of TME components, have been developed and already showed promising results. Although so far, the ideal imaging agent for the TME has yet to be developed, current research results underline the great potential for clinical translation.

A frequent limitation of current cancer imaging research is the use of xenograft models for cell tracking studies for imaging of immune cell infiltration. Animals in this context lack a completely developed immune system and consecutively exhibit an altered composition of the TME.

Nevertheless, in vivo imaging represents an excellent tool for real-time visualization of tumor immune interaction, contributing to a better understanding of tumor biology and – potentially – for estimation and monitoring of therapy effects.

However, further research is needed and can help to raise cancer diagnostics in terms of personalized medicine to the next level.

TABLES

Cell type	Cell surface markers	Functions in the TME
TAM	$CD11b^+$ $CD14^+$ $CD31^+$ $CD34^+$ $CD45^+$ $CD68^+$ $CD117^-$ $CD133^-$ $CD146^-$ $CD204^+$ $CD206^+$ $CCR2^+$ $CSF1R^+$ $MHCII^+$ $VEGFR1^+$ $VEGFR2^-$ (human/mouse) F4/80 (mouse) $CD23^+$ $CD16^+$ $CXCR4^+$ (human)	Enhancement of angiogenesis and remodelling, tumor promoting, association with poor prognosis
MDSC	$CD11b^+$ $CD14^+$ $MHCI^+$ $MHCII^{low}$ (human/mouse) $GR1^+$ $CD11b^+$ (mouse) $CD11b^{+/-}$ $CD33^+$ $CD34^+$ $CD68^-$ (human)	Increased in almost all patients/animals with cancer, ability to suppress T cells as a defining trait
Neutrophil	$CD11b^+$ $CD14^{low}$ $CD31^+$ $CD66B^+$ $CXCR2^+$ (human/mouse) $GR1^+$ $VEGFR1^+$ $CXCR1^-$ (mouse) $CD15^+$ $CXCR1^+$ (human)	Enhancing angiogenesis and metastasis in animal models, increased levels in patients with colon, gastric and lung cancer, associated with poor prognosis in bronchoalveolar carcinoma
$CD4^+$ T cell	$CD3^+$ $CD4^+$ $CD45^+$ (human/mouse)	T helper 1 cells aid $CD8^+$ cells in tumor rejection, T helper 2 cells polarize immunity away from an anti-tumor response
$CD8^+$ T cell	$CD3^+$ $CD8^+$ $CD45^+$ (human/mouse)	Effector cells of the adaptive immune system, specific recognition and destroying of cancer cells through perforin- and granzyme-mediated apoptosis
T_{reg}	$CD4^+$ $CD25^+$ $FOXP3^+$(human/mouse)	Central role in tumor maintenance via suppression of anti-tumor immune response, blocking of $CD8^+$ cell activation and NK cell killing, infiltration is associated with poor prognosis (*14*)
NK cell	$CD11b^+$ $CD27^+$ $CD3^-$ $CD16^{+/-}$ $CD56^+$ $CD3^-$ $CD335^+$ $NKp46^+$ (human/mouse)	Effector lymphocytes, cytotoxicity to cancer cells through perforin-granzyme mediated apoptosis, contribution to immunosurveillance of cancer, low NK-like cytotoxicity in peripheral blood is associated with increased risk of cancer

Table 1: Characteristics of immune cells in the tumor micro-environment. CCR – C-C chemokine receptor, CSF – colony-stimulating factor, CXCR – C-X-C chemokine receptor, FOXP –

forkhead box protein, MDSC – myeloid derived suppressor cell, MHC – major histocompatibility complex, NK cell – natural killer cell, TAM – tumor associated macrophage, T_{reg} – regulatory T cell, VEGFR – vascular endothelial growth factor, adapted with permission from (*2*)**.**

FIGURES

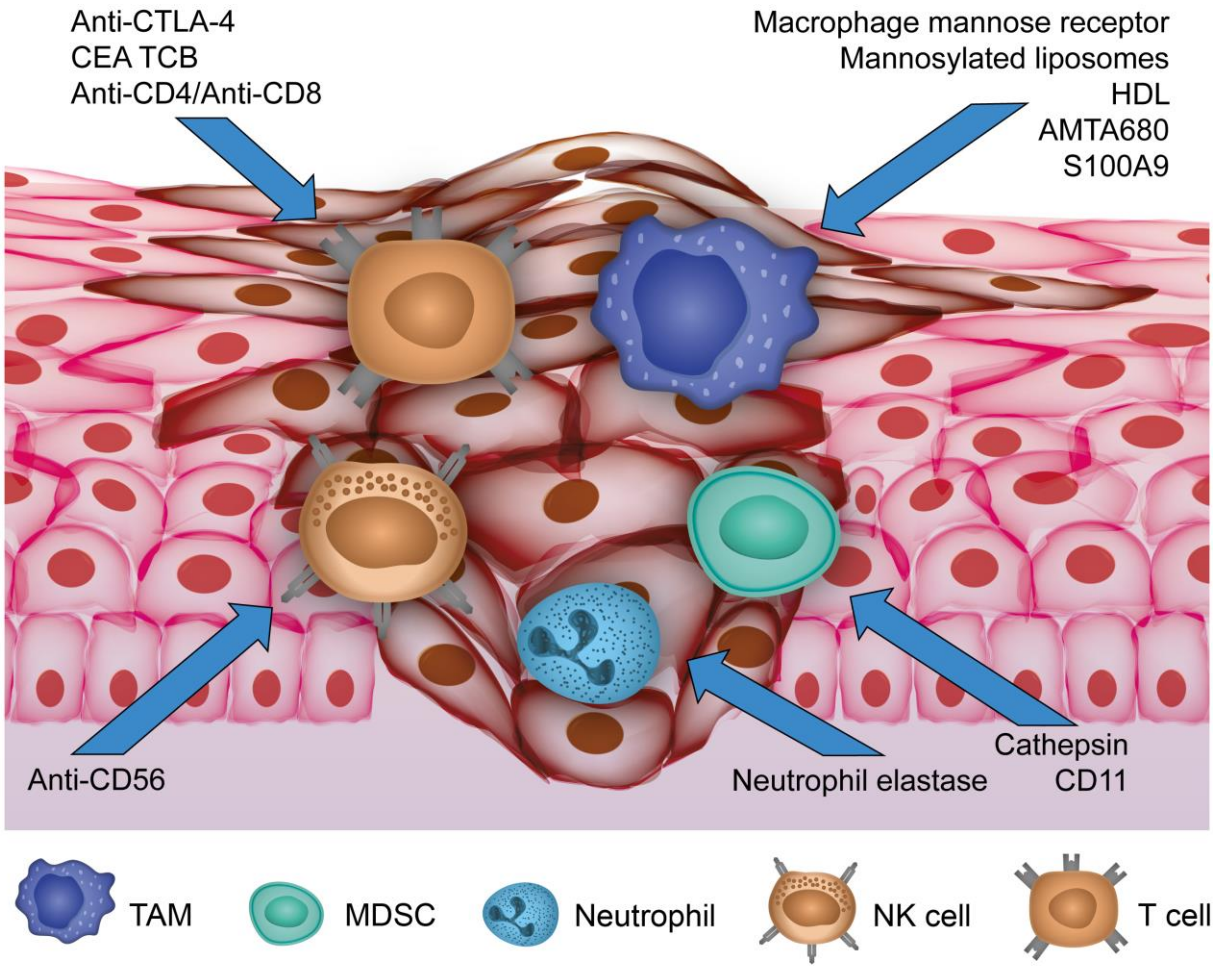

Figure 1

Overview of current imaging approaches targeting cellular compounds of the tumor microenvironment. The activity of tumor associated macrophages (TAM), myeloid derived suppressor cells (MDSC) and neutrophils as pro-tumoral immune cells infiltrating the primary tumor is reflected by visualizing specific targets for current molecular imaging approaches. Anti-tumoral natural killer cells have been addressed by anti-CD56, whereas approaches targeting anti-

CTLA-4, anti-CD4/CD8 and carcinoembyonic antigen T cell specific antibody for T cell imaging have been reported.

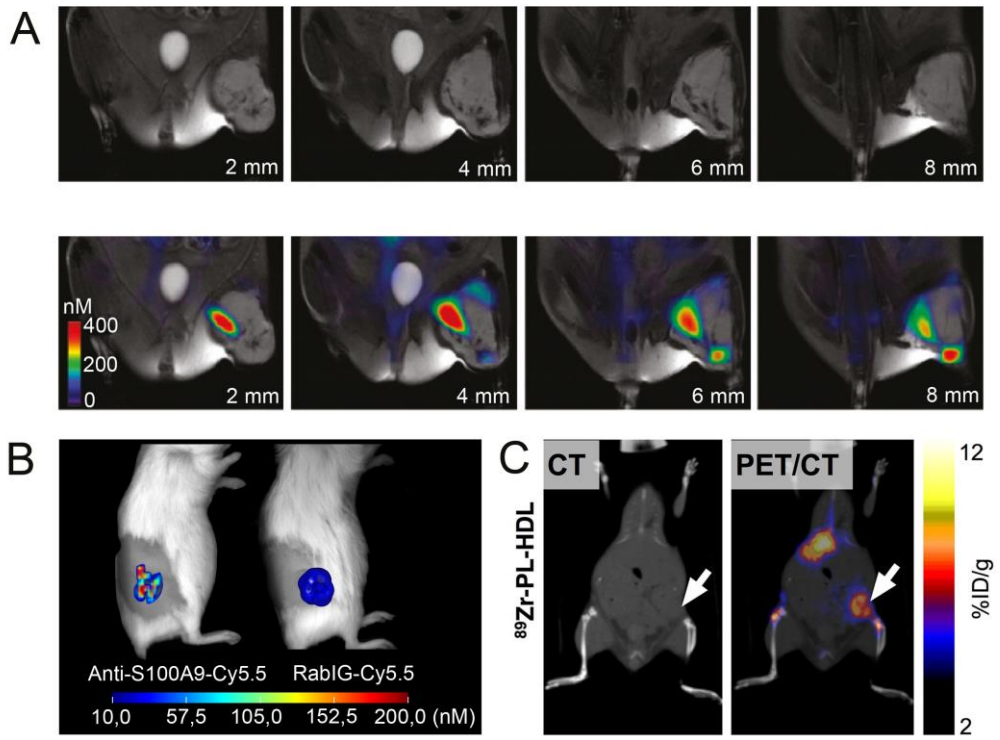

Figure 2

In vivo imaging of tumor associated macrophages.

A Imaging of TAM distribution in a mouse with soft tissue sarcoma (naïve MRI images shown in upper row) 24 h after i. v. injection of AMTA680 (fusion of fluorescence mediated tomography and MRI in second row), adapted with permission from (*21*). **B** Fluorescence imaging of TAM activity in murine 4T1 breast cancer. The specific tracer anti-S100A9-Cy5.5 shows high accumulation within the tumor lesion, whereas the homogenous signal of unspecific binding rabIgG-Cy5.5 reflects tumor perfusion. **C** ^{89}Zr-HDL-driven in vivo PET for imaging TAM in murine 4T1 breast cancer 24 h after tracer injection. CT (left) and PET/CT fusion images (right), adapted with permission from (*17*).

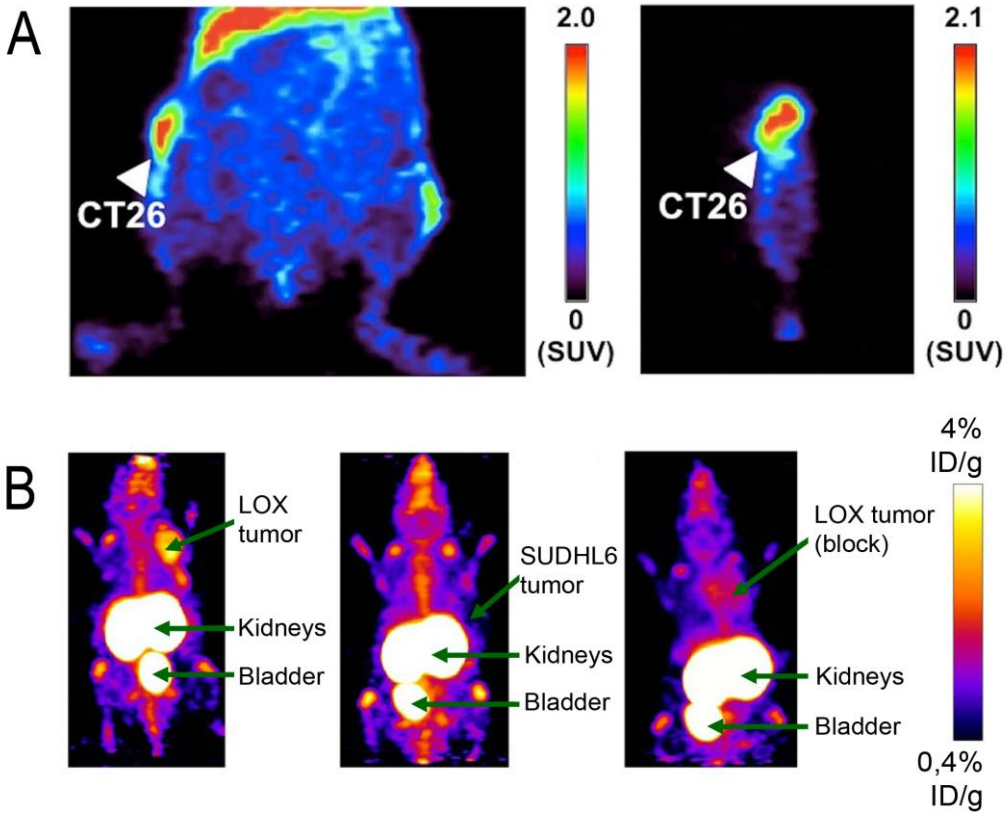

Figure 3

T cell in vivo imaging within the tumor microenvironment.

A PET imaging using ^{64}Cu-DOTA-labeled anti-CTLA-4 showed specific tracer accumulation in murine CT26 colon carcinoma in representative coronal (left) and sagittal (right) slices. Results suggest a promising tool for evaluating targeted therapy by anti-CTLA-4 monoclonal antibodies, adapted with permission from (*41*). **B** Representative PET images after injection of 18F labeled anti programmed cell death protein (PD) L1 affibody molecule. The tracer allows for imaging of PD-L1 expressing LOX malignant melanoma (left) in comparison with the negative controls of the non-expressing lymphoma SUDHL6 (middle) and ,a blocked LOX tumor (right), adapted with permission from (*48*).

DISCLOSURE

The authors have no conflicts of interest to disclose.

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REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144:646-674.

2. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer.* 2009;9:239-252.

3. Barnes TA, Amir E. HYPE or HOPE: the prognostic value of infiltrating immune cells in cancer. *Br J Cancer.* 2017;117:451-460.

4. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol.* 2008;9:503-510.

5. Malmberg KJ, Carlsten M, Bjorklund A, Sohlberg E, Bryceson YT, Ljunggren HG. Natural killer cell-mediated immunosurveillance of human cancer. *Semin Immunol.* 2017;31:20-29.

6. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene.* 2010;29:1093-1102.

7. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer.* 2004;4:71-78.

8. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer.* 2008;8:618-631.

9. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol.* 2002;196:254-265.

10. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature.* 2008;454:436-444.

11. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* 2009;9:162-174.

12. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. *Immunol Rev.* 2008;222:162-179.

13. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer.* 2006;6:24-37.

14. Silverio KA, Patel SA. Harnessing antitumor immunity: Employment of tumor recall antigens to optimize the inflammatory response to cancer. *Oncol Lett.* 2017;13:2015-2020.

15. Kim MH, Lee YJ, Kang JH. Stem Cell Monitoring with a Direct or Indirect Labeling Method. *Nucl Med Mol Imaging.* 2016;50:275-283.

16. Locke LW, Mayo MW, Yoo AD, Williams MB, Berr SS. PET imaging of tumor associated macrophages using mannose coated 64Cu liposomes. *Biomaterials.* 2012;33:7785-7793.

17. Perez-Medina C, Tang J, Abdel-Atti D, et al. PET Imaging of Tumor-Associated Macrophages with 89Zr-Labeled High-Density Lipoprotein Nanoparticles. *Journal of Nuclear Medicine.* 2015;56:1272-1277.

18. Marcel YL, Ouimet M, Wang MD. Regulation of cholesterol efflux from macrophages. *Curr Opin Lipidol.* 2008;19:455-461.

19. Shih Y-YI, Hsu Y-H, Duong TQ, Lin S-S, Chow K-PN, Chang C. Longitudinal study of tumor-associated macrophages during tumor expansion using MRI. *NMR in Biomedicine.* 2011;24:1353-1360.

20. Weissleder R, Nahrendorf M, Pittet MJ. Imaging macrophages with nanoparticles. *Nat Mater.* 2014;13:125-138.

21. Leimgruber A, Berger C, Cortez-Retamozo V, et al. Behavior of Endogenous Tumor-Associated Macrophages Assessed In Vivo Using a Functionalized Nanoparticle. *Neoplasia.* 2009;11:459-IN454.

22. Daldrup-Link HE, Golovko D, Ruffell B, et al. MRI of Tumor-Associated Macrophages with Clinically Applicable Iron Oxide Nanoparticles. *Clinical Cancer Research.* 2011;17:5695-5704.

23. Movahedi K, Schoonooghe S, Laoui D, et al. Nanobody-Based Targeting of the Macrophage Mannose Receptor for Effective In Vivo Imaging of Tumor-Associated Macrophages. *Cancer Research.* 2012;72:4165-4177.

24. Zhang C, Gao L, Cai Y, et al. Inhibition of tumor growth and metastasis by photoimmunotherapy targeting tumor-associated macrophage in a sorafenib-resistant tumor model. *Biomaterials.* 2016;84:1-12.

25. Choi YJ, Oh S-G, Singh TD, et al. Visualization of the Biological Behavior of Tumor-Associated Macrophages in Living Mice with Colon Cancer Using Multimodal Optical Reporter Gene Imaging. *Neoplasia.* 2016;18:133-141.

26. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J Leukoc Biol.* 2009;86:557-566.

27. Becker A, Grosse Hokamp N, Zenker S, et al. Optical in vivo imaging of the alarmin S100A9 in tumor lesions allows for estimation of the individual malignant potential by evaluation of tumor-host cell interaction. *J Nucl Med.* 2015;56:450-456.

28. Vogl T, Eisenblatter M, Voller T, et al. Alarmin S100A8/S100A9 as a biomarker for molecular imaging of local inflammatory activity. *Nat Commun.* 2014;5:4593.

29. Eisenblaetter M. Visualization of tumor-immune interaction - target-specific imaging of S100A8/A9 reveals premetastatic niche establishment. *Theranostics.* 2017.

30. Gounaris E, Tung CH, Restaino C, et al. Live imaging of cysteine-cathepsin activity reveals dynamics of focal inflammation, angiogenesis, and polyp growth. *PLoS One.* 2008;3:e2916.

31. Cheng D, Zou W, Li X, et al. Preparation and Evaluation of99mTc-labeled anti-CD11b Antibody Targeting Inflammatory Microenvironment for Colon Cancer Imaging. *Chemical Biology & Drug Design.* 2015;85:696-701.

32. Bronte V, Brandau S, Chen SH, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun.* 2016;7:12150.

33. Hurt B, Schulick R, Edil B, El Kasmi KC, Barnett C, Jr. Cancer-promoting mechanisms of tumor-associated neutrophils. *Am J Surg.* 2017.

34. Ho AS, Chen CH, Cheng CC, et al. Neutrophil elastase as a diagnostic marker and therapeutic target in colorectal cancers. *Oncotarget.* 2014;5:473-480.

35. Liu Z, Li Z. Molecular imaging in tracking tumor-specific cytotoxic T lymphocytes (CTLs). *Theranostics.* 2014;4:990-1001.

36. Hammoud DA. Molecular Imaging of Inflammation: Current Status. *J Nucl Med.* 2016;57:1161-1165.

37. Ehlerding EB, England CG, McNeel DG, Cai W. Molecular Imaging of Immunotherapy Targets in Cancer. *J Nucl Med.* 2016;57:1487-1492.

38. Srinivas M, Aarntzen EH, Bulte JW, et al. Imaging of cellular therapies. *Adv Drug Deliv Rev.* 2010;62:1080-1093.

39. Dolton G, Tungatt K, Lloyd A, et al. More tricks with tetramers: a practical guide to staining T cells with peptide-MHC multimers. *Immunology.* 2015;146:11-22.

40. Lehmann S, Perera R, Grimm HP, et al. In Vivo Fluorescence Imaging of the Activity of CEA TCB, a Novel T-Cell Bispecific Antibody, Reveals Highly Specific Tumor Targeting and Fast Induction of T-Cell-Mediated Tumor Killing. *Clin Cancer Res.* 2016;22:4417-4427.

41. Higashikawa K, Yagi K, Watanabe K, et al. 64Cu-DOTA-anti-CTLA-4 mAb enabled PET visualization of CTLA-4 on the T-cell infiltrating tumor tissues. *PLoS One.* 2014;9:e109866.

42. Maute RL, Gordon SR, Mayer AT, et al. Engineering high-affinity PD-1 variants for optimized immunotherapy and immuno-PET imaging. *Proc Natl Acad Sci U S A.* 2015;112:E6506-6514.

43. Tavare R, McCracken MN, Zettlitz KA, et al. Immuno-PET of Murine T Cell Reconstitution Postadoptive Stem Cell Transplantation Using Anti-CD4 and Anti-CD8 Cys-Diabodies. *J Nucl Med.* 2015;56:1258-1264.

44. Tavare R, Escuin-Ordinas H, Mok S, et al. An Effective Immuno-PET Imaging Method to Monitor CD8-Dependent Responses to Immunotherapy. *Cancer Res.* 2016;76:73-82.

45. Freise AC, Zettlitz KA, Salazar FB, Lu X, Tavare R, Wu AM. ImmunoPET Imaging of Murine CD4+ T Cells Using Anti-CD4 Cys-Diabody: Effects of Protein Dose on T Cell Function and Imaging. *Mol Imaging Biol.* 2017;19:599-609.

46. Galli F, Histed S, Aras O. NK cell imaging by in vitro and in vivo labelling approaches. *Q J Nucl Med Mol Imaging.* 2014;58:276-283.

47. Galli F, Rapisarda AS, Stabile H, et al. In Vivo Imaging of Natural Killer Cell Trafficking in Tumors. *J Nucl Med.* 2015;56:1575-1580.

48. Gonzalez Trotter DE, Meng X, McQuade P, et al. In vivo Imaging of the Programmed Death Ligand 1 by 18F Positron Emission Tomography. *J Nucl Med.* 2017.