Preclinical Evaluation of Drug Delivery Systems for Immunotherapy

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Publication date: 2019

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Preclinical Evaluation of Drug Delivery Systems for Immunotherapy

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PhD Dissertation
Submitted December 1st 2019

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Preface

This dissertation has been submitted to the Technical University of Denmark. The research was carried out from 2016 to 2019 under supervision of Thomas L. Andresen and Ladan Parhamifar at the Technical University of Denmark. The work was carried out in the Colloids & Biological Interfaces group in the Department of Health Technology, Technical University of Denmark and in the Cluster for Molecular Imaging group at the Department of Biomedical Sciences, University of Copenhagen.

Enclosed is the following manuscripts that presents a significant portion of the research conducted during the PhD. The reference list for all chapters, including manuscripts, is combined in the end of the dissertation.


*Contributed equally to the work

The data produced during the PhD has been part of one patent application;

Acknowledgements

I am very grateful to all the people making this PhD project possible. A project so exciting it has been hard to take time off from what officially was just work. It has by no means been possible alone and all the people involved has greatly improved the quality of the work while making it fun and inspiring journey.

First, I would like to thank my supervisor Professor Thomas L. Andresen for his leadership and providing me the opportunity and facilities to do incredibly exciting and inspiring science. Thomas’ opinions on specific matters and aspiration to do impactful research that always aims to affect the life of patients has been truly inspiring and enlightening.

Second, I owe my thanks to Ladan Pharmifar and Anders E. Hansen for providing vital sparring, co-supervision, and help with several aspects of the work. The research would certainly not have been anywhere near as good without.

Third, I owe my thanks to all the great colleagues at both the Colloids & Biological Interfaces group and the Cluster for Molecular Imaging group, where Professor Andreas Kjær has been so kind to provide a great working atmosphere and excellent facilities. A special thanks to Camilla Stavnsbjerg and Lars Ringgaard, whom I worked with on most of my research and has been invaluable for the projects. Additionally, Rasmus Münter deserves a special thanks for being instrument for the biophysics and chemistry related to our research. Although too many to list, so many additional colleagues have been vital for the research performed and has also led to several great friendships and social activities. To name a few: Anja Brus, Hólmfrídur Halldórsdóttir, Matilda Weywadt, Kasper Johnsen, Ditte Jæhger, Mie Hübbe, Trine Engel, Jennifer Jørgensen, Jonas Henriksen, Gael Veiga, and Martin Bak. Thank you!

Fourth, I owe a thanks to the people at MonTa Biosciences for collaborating on the project related to Manuscript III.

Last, thanks to all my friends and family encouragements throughout the PhD and putting up me being ever so slightly absentminded.
English Summary

Cancer is among the leading causes of global deaths. This is despite significant advances in cancer treatment and understanding where especially immunotherapy has shown promising results in recent years. Overall response rates are, however, still modest and have been correlated to composition and polarization of the infiltrating immune cells in the tumor microenvironment (TME). Here, infiltration of myeloid immune cells into the TME is typically associated with worse prognosis and treatment response while CD8+ T cells are generally positive for prognosis and treatment outcome. Despite primarily having a harmful role in cancer, myeloid cells can be repolarized using immune stimulatory compounds and thus become beneficial.

The first manuscript in this dissertation provides comparative data of the TME across 11 murine subcutaneous cancer models with a focus on myeloid cells. Many of the investigated models are commonly used in preclinical evaluation and development of immunotherapies but prior to this work, comparative data has been lacking. The results demonstrated major differences in tumor-infiltrating myeloid cells across the evaluated cancer models. By providing comparative data across cancer models, the manuscript is valuable for explaining differences in treatment responses across cancer models and thereby presents a valuable tool for selecting relevant preclinical cancer models and helps to bridge the gap between preclinical research and clinical results.

The second manuscript investigates the obstacles associated with delivery of innate immune stimulatory molecule in nanoparticles. The manuscript demonstrates that nanoparticles containing immune stimulatory molecules may be recognized by the immune system and subsequently be rapidly eliminated from blood circulation. This was accompanied by acute hypersensitivity symptoms and associated with IgG specific for the nanoparticles.

The third enclosed manuscript describes preclinical evaluation for a nanoparticle containing an innate immune stimulatory compound developed within the group. The nanoparticle could be safely administered in preclinical animals and demonstrated potent anti-cancer effect and synergistic effect with other anti-cancer therapies in murine cancer models. Upon intravenous injection, cell viability within murine tumors was found drastically reduced while spleens were unaffected.
Danske Resumé

Kræft er blandt de ledende globale dødsårsager. Dette er på trods af betydelige fremskridt i kræftbehandling, hvor især immunterapi har vist lovende resultater i de seneste år. Dog er den overordnede responsrate stadig beskeden og er blevet korreleret til sammensætningen og polariseringen af de infiltrerende immunceller i tumormikromiljøet (TMM). Her er infiltrationen af myeloide immunceller i TMMet forbundet med forværret prognose og behandlingsrespons, mens CD8⁺ T celler er generelt positive for prognose og behandlingsresultat. På trods af at myeloide celler primært har en skadelig rolle i kræft, kan myeloide celler blive repolariseret ved brug af immunstimulatoriske stoffer og derved blive fordelagtige.

Det første manuskript i denne afhandling leverer komparativt data for TMMet på tværs af 11 murine subkutane kræftmodeller med fokus på myeloide celler. Mange af de undersøgte modeller er almindeligt anvendt i præklinisk evaluering og udvikling af immunterapier. Forinden dette arbejde har komparativt data dog manglet. Resultaterne heri demonstrerer store forskelle i tumorinfiltrerende myeloide celler på tværs af de evaluerede kræftmodeller. Ved at levere komparativt data på tværs af kræftmodeller er manuskriptet værdifuldt til at forklare forskelle i behandlings respons på tværs af kræftmodeller og udgør dermed et værdifuldt værktøj til at vælge relevante prækliniske kræftmodeller samt hjælper med at indsnævre kløften mellem præklinisk forskning og kliniske resultater.

Det andet manuskript undersøger de forhindringer, der er forbundet med levering af innate immunstimulatoriske molekyler i nanopartikler. Manuskriptet demonstrerer, at nanopartikler, der indeholder immunstimulatoriske molekyler, kan blive genkendt af immunsystemet og efterfølgende blive hurtigt elimineret fra blodcirkulationen. Dette var ledsaget af akut overfølsomhedssymptomer og associeret med IgG specifikke for nanopartiklerne.

Det tredje vedlagte manuskript beskriver præklinisk evaluering af en nanopartikel udviklet i gruppen, der indeholder et innat immunstimulatorisk molekyle. Nanopartiklen kunne administreres sikkert i prækliniske dyr og demonstrerer potent effekt imod kræft samt synergestisk effekt med andre kræftbehandlinger i murine kræftmodeller. Efter intravenøs administration var celle overlevelsen i murine tumorer kraftigt reduceret, mens milte var upåvirket.
Abbreviations

ABC         Accelerated blood clearance
APC         Antigen-presenting cell
CARPA       Complement activation-related pseudoallergy
cDC         Classical dendritic cell
cT          Cytotoxic T cell
DC          Dendritic cell
eNOS        Endothelial nitric oxide synthase
EPR         Enhanced permeability and retention
iNOS        Inducible NO synthase
LAMP-1      Lysosomal-associated membrane glycoprotein-1
moDC        Monocyte-derived dendritic cell
Mo-MDSC     Monocytic myeloid-derived suppressor cell
ODN         Oligonucleotide
pDC         Plasmacytoid dendritic cell
PDI         Polydispersity index
pDNA        Plasmid DNA
PMN-MDSC    Polymorphnuclear myeloid-derived suppressor cell
PMo         Patrolling monocyte
TAA         Tumor-associated antigen
TAM         Tumor-associated macrophage
TAN         Tumor associated neutrophil
TCR         T cell receptor
TLR         Toll-like receptor
TME         Tumor microenvironment
t-SNE       t-distributed Stochastic Neighbor Embedding
RT          Radiotherapy
MHC         Major histocompatibility complex
MPS         Mononuclear phagocytic system
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Chapter 1 – Scope and Manuscripts

The scope of this PhD dissertation has been to improve evaluation of anti-cancer immunotherapy and apply this knowledge to anti-cancer immunotherapies developed within the group. The immunotherapies evaluated in this dissertation were formulated in two types of nanoparticles; liposomes and micelles. The dissertation contains one background chapter, essential to understanding the concepts used in the research, three research manuscripts, a perspectivation of the included manuscripts, and concluding remarks. As a starting point a brief background and summary of main findings from the enclosed manuscripts are provided;

Manuscript I: Myeloid characterization of 11 syngeneic cancer models for optimized treatment evaluation

The association between poor prognosis and reduced treatment effect due to suppressive phenotypes in the myeloid compartment of the tumor microenvironment (TME) is of great importance to cancer therapy. Due to the plasticity and repolarization potential within the myeloid compartment it is becoming an increasingly intriguing target as potent activation can lead to immune recognition and thus cancer regression. Evaluation using syngeneic cancer models are key for successful development of novel anti-cancer treatments but to date, comparative studies showing the differences between cancer models are lacking.

This manuscript characterizes the myeloid compartment in the TME of 11 syngeneic murine tumor models including markers of activation and cytokine milieu. Additionally, the manuscript investigates how radiotherapy affects tumor-associated antigen (TAA)-uptake in myeloid cells within the TME. A side-by-side comparison of the cancer models revealed TMEs ranging from immune-deserted, dominated by a single myeloid subset to general high myeloid infiltration. Ultimately providing a tool for selecting the relevant tumor models for anti-cancer treatment evaluation that also strives to improve the translational value of treatments.
Manuscript II: *Accelerated blood clearance in mice following repeated systemic dosing of high lipid dose PEGylated liposomes containing TLR agonists*

Innate immune stimulators, like toll-like receptor (TLR) 7/8 agonists, have shown impressive preclinical anti-tumor effects following systemic administration. However, clinical use has been limited due to toxicity associated with systemic activation. To this end, liposomes are commonly investigated for their potential to alter cellular uptake, prolong circulation, and alter biodistribution, whereby systemic toxicity may be reduced. However, studies have shown that liposomes may be recognized by the immune system which subsequently leads to accelerated blood clearance (ABC) of nanoparticles upon repeated dosing. However, most studies investigating this phenomenon use liposomes at nontherapeutic levels and without cargo, whereby the translational value is limited.

In this manuscript, we demonstrate that formulating toll-like receptor (TLR) 7/8 agonists in commonly used liposomal formulations lead to immune recognition of nanoparticles which ultimately can cause ABC and acute hypersensitivity symptoms upon repeated dosing due to the humoral anti-nanoparticle response.

Manuscript III: *Nanoformulation of TLR7/8 agonist provides safe and potent anti-cancer immunity*

This manuscript describes preclinical evaluation of micelles developed within the group containing a TLR7/8 agonist. The treatment resulted in impressive efficacy in syngeneic murine tumor models and synergistic effects with radiotherapy and immune checkpoint inhibitor treatment. The formulation could be safely administrated intravenously in mice and non-human primates. Upon intravenous administration in tumor-bearing mice, the TME was found to have drastically reduced cellular viability and was accompanied by both an innate and adaptive immune response.
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2.1 Cancer

Cancer develops from cells due to cumulative mutational changes at a genetic and epigenetic level leading to cancerous cells (1). The TME is comprised of cancer, immune, and stromal cells (1) that all influence prognosis and treatment outcome. However, not only total infiltration matters, the topography of the infiltrating cells also have significant importance (2,3). Certain tumor characteristics have been defined as critical for tumor-formation and progression, commonly known as the hallmarks of cancer, which includes proliferative signaling, angiogenesis, replicative immortality, resisting cell death, invasive characteristics, and immune evasion. The latter being critical for disease progression and dissemination, which is mediated by both cancer cells and the immune system (1).

Despite significant advancements in cancer treatment it remains among the most prevalent causes of death globally and is responsible for an estimated 9.6 million deaths in 2018 (4). Advancements in anti-cancer treatment have greatly improved the prognosis for many cancer patients (4) but warrants further research into better understanding and ultimately more effective and more tolerable treatments. Manipulation of the immune system for anti-cancer therapy is highly desirable based on its potential to break immune evasion to cause immune mediated killing of cancer cells (5,6). However, the potency of immunotherapy may cause severe toxicity due to systemic activation of the immune system and therefore must be restrained. To this end, drug delivery platforms may be advantageous to alter the biodistribution and pharmacokinetics, whereby systemic toxicity may be reduced (7).

2.2 Cancer and the Immune system – a Game of Cat and Mice

The dynamic process between cancer cells and the immune system is known as cancer immunoediting which is divided into three phases: Elimination, equilibrium, and escape. During the elimination phase, the immune system readily recognizes and destroys cancer cells that do not present as ‘self’. During the equilibrium phase, cancers are controlled by the immune system that despite cytolytic activity is not capable of fully eradicating the heterogeneous population of cancer cells that may have acquired immune-evading
characteristics. Finally, during the escape phase, the immune system can no longer eliminate cancer cells at the rate that cancer cells are proliferating (8).

To escape immune recognition, cancer cells dysregulate proteins that would otherwise lead to recognition followed by cytolysis by immune effector cells. Effector immune cells recognizes different molecules including peptides presented on major histocompatibility complex (MHC) I, that is present on all nucleated cells and displays peptide fragments of proteins produced by the cell (9). Normally, failing to display MHC I leads to cytolysis by NK cells. In contrast, displaying non-self peptides on MHC I may lead to recognition and cytolysis by antigen-specific T cells (5,10). However, while cancer cells may display altered levels and non-self peptides on MHC class I, other suppressive mechanisms causes immune evasion, including 1) paracrine molecules, e.g, transforming growth factor-β (1), 2) suppressive ILs (e.g IL-10) (11), and immune checkpoint inhibitors (e.g. PD-L1) (11). Interestingly, stromal and immune cells are generally the predominant source of immunosuppressive cytokines in the TME (11).

Successful elimination of cancers requires a series of critical steps by the immune system – known as the cancer-immunity cycle (Figure 1). Here cancer antigens are released from dying cancer cells and are subsequently taken up by antigen-presenting cells (APCs). APCs presents the processed cancer antigen and delivers priming and activating signals to effector cells. Activated T cells traffics to tumors where they kill cancer cells upon recognition of MHC molecules that ultimately leads to release of cancer cell antigens. In progressing tumors, the cycle is halted by excessive inhibitory signals. Immunotherapy strives to break suppression by repolarizing the TME into a milieu dominated by stimulatory factors that supports a functional cancer-immunity cycle (5).
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Figure 1. The cancer immunity cycle. Released cancer antigens are processed by antigen presenting cells that primes and activates T cells that traffics to tumors and facilitates antigen-specific cytolysis. Each step has stimulatory and inhibitory factors (marked as green or red, respectively). Adapted from (5).

2.3 Immune cells in the TME

The composition of tumor infiltrating immune cells vary greatly across tumors due to differences in recruitment signals (mainly chemokines). Tumor infiltrating immune cells have functions based on cell type and polarization. Crucial cell types for anti-cancer immunity includes CD8\(^+\) T cells, dendritic cells (DCs), B cells, macrophages, monocytes, and neutrophils. Other immune cells with significance in cancer that have not been touched upon in this dissertation includes T helper cells, regulatory T cells, γδ T cells, NK cells, NKT cells, mast cells, eosinophils, and basophils.

Stratifying patients into the most relevant therapy may be assisted by understanding the composition of the TME. For instance, in contrast to conventional tumor node metastasis
staging, quantity of infiltrating CD8+ T cells have been shown superior in predicting outcome and important for stratification into treatment with immunotherapy (2,6,12–15). Contrary to CD8+ T cell infiltration, myeloid cells are generally associated with poorer prognosis and may negatively impact treatment outcome (15–21) and constitutes a major portion of tumor-infiltrating immune cells (22–26). Although immune subset infiltration correlates with survival, immune cell polarization is likely just as important as partially evident by how immune subsets differ in being positive or negative prognostic factors across cancer types (15). Furthermore, it is important to understand that looking at a single cell subset is less relevant as the TME presents a complex interplay between many cell types that have high plasticity and all affect each other. Therefore, obtaining broad information on the immune subsets within the TME also provides better prognostic value (27) and is important for evaluating anti-cancer treatments.

2.3.1 Myeloid Polarization
The high plasticity of immune cells allows phenotypes of either anti- or pro-tumorigenic function. Indeed, immune cell types may be associated with good prognosis in some cancers and associated with poor prognosis in other cancers (15,28). Due to the conditions in tumors that is characterized by low oxygen, acidic environment, and poor vascularization, the TME is reminiscent of tissue damage. The resulting signals causes immune cells to attempt to ‘repair’ the tissue by aiding in de novo vascularization, tissue remodeling, and immunosuppression (29). Additionally, TMEs are often characterized by chronic inflammation, causing immunosuppressive phenotypes. Interestingly, many of the same signals would in acute inflammation be associated with anti-tumorigenic phenotypes (30). The low grade systemic inflammation resulting from cancers are linked to exhaustion of bone marrows whereby released immune cells (mainly monocytes and neutrophils) display immature and pro-tumorigenic phenotypes (31,32). Despite the consensus on the importance of anti- and pro-tumorigenic myeloid phenotypes, these phenotypes are reported with large indiscrepancies in literature. This is further discussed in manuscript I.

Anti-tumorigenic myeloid phenotypes are characterized by increased antigen processing and presentation (mainly evaluated by MHC II expression or MHC I molecules carrying specific epitopes), increased levels of co-stimulatory molecules (e.g. CD86), and
production of cytokines that are central for anti-tumor immunity (e.g. IL-12, IFNγ, and IFNα) (32–34).

In contrast, pro-tumorigenic myeloid phenotypes are characterized by absence or low levels of molecules involved in antigen presentation and co-stimulation, production of proangiogenic factors (e.g. VEGF), production of enzymes starving the microenvironment for arginine (e.g. arginase 1), and cytokines with immunosuppressive activity (e.g. IL-10 and TGF-β) (31,32,34).

2.3.2 CD8+ T cells

CD8+ T cells are central effector cells in the adaptive immune system and performs antigen specific cytolysis. Antigen-specific recognition occurs through the T cell receptor (TCR), a highly polymorphic molecule allowing a pool of CD8+ T cells to react towards a large pool of antigens, that together with CD8 recognizes antigens presented on MHC class I (35). Recognition and signal strength is based on affinity towards the presented antigen for which affinity may differ by magnitudes between T cell clones (36). Each T cell have a single TCR arrangement and as TCRs may recognize any presented antigen, T cells are subject to thymic selection to avoid strong recognition of self-antigens during their development (37).

Naïve T cells are not able to lyse target cells and need licensing and co-stimulatory factors, known as signal 1, 2, and 3, to become fully functional (38). All the necessary signals may be provided by professional APCs which are constituted by macrophages, monocytes, B cells, and DCs (39). Signal 1 is provided through TCR on the T cell when it recognizes its target peptide on MHC class I (MHC class II for CD4+ T cells) (40). The signal increases the avidity for signal 2 to occur (41). Signal 2 is most notably provided through CD28 on the T cell that recognizes co-stimulatory molecules CD80 and CD86 on APCs and promotes survival, differentiation, and production of cytokines. Lack of signal 2 induces T cell anergy (41). Signal 3 is cytokines provided by APCs and is essential to develop optimal effector and memory function and also affects the expression of genes important for survival, proliferation, and trafficking. Notable cytokines able to deliver signal 3 include IL-12 and type 1 IFNs (IFNα and IFNβ) (38). Additionally, CD8+ T cells must traffic and extravasate into the TME to exert effector function. This is mediated by chemotactic signals produced in the TME and adhesion molecules on tumor endothelium. Centrally to
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this is CXCR3 on CD8+ T cells which respond to the CXCL9, 10, and 11 chemokines, which have been correlated to intratumoral T cell infiltration (42).

T cells may also receive inhibitory signals provided by APCs, cancer cells, and stromal cells through co-inhibitory molecules or inhibitory cytokines (11,43,44). Inhibitory signals may be provided by co-inhibitory molecules like PD-L1 signaling through PD-1 on T cells (43) and CTLA-4 on T cells competing with CD28 (co-stimulatory molecule) to bind CD80 and CD86 on APCs (45). Inhibitory signals cause effector and proliferative functions to diminish and may be regarded as a continuum of cell states that may culminate in terminally differentiated exhausted states. Exhausted T cells are further characterized by the expression of inhibitory receptors (notably PD-1, TIM3, LAG3, CTLA-4, and TIGIT) and have epigenetic changes responsible for the diminished effector and proliferative functions. However, it is important to understand that highly functional effector cells also express inhibitory receptors. Thus defining exhausted T cells should be based on expression of both functional markers and inhibitory receptors or epigenetic program. Despite their diminished function, exhausted T cells can still exert effector functions (46). A complete understanding of T cell exhaustion is still being pursued with important questions including how terminally differentiated exhausted T cells may be reinvigorated.

Tumor infiltrating T cells may also be in an anergic or senescent state. Anergic T cells are induced due to a lack of adequate co-stimulatory factors, lack effector function, and have low proliferative capacity. Like exhaustion, this is a continuum of states that may be functionally rescued in the early states (45,46). Senescent T cells have effector function but low proliferative capacity due to repetitive stimulation (44,45).

Tumor infiltrating T cells may also be divided into memory and effector states which differ in proliferative capacity and effector function. However, differentiation into memory and effector states are not within the scope of this dissertation. These have been extensively reviewed previously and comprise T memory stem cells, central memory T cells, effector memory, tissue resident memory T cells, and effector T cells (47–49) and determining factors for which subset is generated depends on the strength of signal 1, 2, and 3 (49).

Elimination of cancer cells by CD8+ T cells is most notably mediated by the combination of perforins and granzymes although other cytolytic mechanisms also exists (e.g. Fas ligand). Upon recognition through TCRs, an immunologic synapse is formed where
pore forming proteins (perforins) and proteases (granzymes) are released that ultimately
cause cell death (mostly apoptotic) (50). Evaluation of cytotoxicity is important and desirable
in evaluating anti-cancer therapies. Previous studies have shown that surface staining of
lysosomal-associated membrane glycoprotein-1 (LAMP-1, also known as CD107a) on
effector cells correlates well with degranulation events and with cytotoxicity. Furthermore,
determining T cell antigen specificity may be performed using fluorescent MHC I multimers
loaded with known peptide sequences (e.g. dextramers) (51).

2.3.3 B cells
B cells are a central component of the adaptive immune system. Their role as tumor-
infiltrating immune cell is however still not well understood with contributing factors being
their relatively low infiltration (22–24,52), high plasticity (53), and inconsistencies in
phenotyping of B cell subsets. Accompanied by the increasing understanding and
appreciation of tumor infiltrating immune cells there is also an increasing appreciation of B
cells function in cancer (54). Main functions of B cells include the ability to differentiate into
other specialized subsets (e.g. plasma cells), present antigen, activate T cells, produce
antibodies of different immunoglobulin classes, and secrete cytokines (54,55).

Depending on cancer type and other factors within the TME, B cells may be
either anti- or pro-tumorigenic (15,28,54). Their antitumorigenic factors include activation
and polarization of T cells and producing antibodies that mediate killing of cancer cells
through antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity
(54). Their pro-tumorigenic function are mainly contributed to their polarization state as
regulatory B cells, that induce tolerance by suppressing immune cells with IL-10 and T cell
function through PD-L1 (54).

2.3.4 Dendritic cells
DCs are constituted by four distinct main subsets; classical DC 1 (cDC1), cDC2, monocyte-
derived DCs (moDCs), and plasmacytoid DCs (pDCs). cDCs can be further divided into
resident and migratory subsets as well as subpopulations based on anatomical position.
Each subset has distinct specialization and are conserved from mice to human (24,56). Due
to the large inconsistencies in phenotyping of moDCs in literature, these are not investigated
in this dissertation.
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cDC1s excel in presentation of extracellular antigens on MHC I to CD8+ T cells (57). Evidence supporting their role as key activators of CD8+ T cells includes correlation with good prognosis in various clinical cancers (58) and cDC1 infiltration correlating positively with CD8+ T cell infiltration in various studies (57). Indeed, studies have shown that in some clinical tumors cDC1 transcriptomic signatures may be just as powerful a predictor for survival as that of CD8+ T cells (58). Furthermore, murine knock-out models (e.g. Batf3−/−) supports their central role in anti-tumor immunity (58) and importance in immunotherapy (59–61). cDC1s have antigen processing pathways specialized for presentation on MHC class I for CD8+ T cells. Although specialized in cross-presentation, cDC1s can also present antigens to CD4+ T cells on MHC class II, although less potently than cDC2s. Furthermore, cDC1s are key producers of IL-12 production that polarizes CD4+ T cells towards a Th1 response and licenses NK cells that subsequently maintains CD8+ T cell cytotoxicity and cDC1 maturation (57).

cDC2s are the most potent APC for activation and expansion of CD4+ T cells (57). Compared to cDC1s they are inferior at cross-presentation. However, it is important to appreciate that most murine studies exploring the importance of cDC1 also affected cDC2s by their approach; e.g. Batf3 and Zbtb46 is expressed in both cDC1s and cDC2s and thus some value given to cDC1s may be from cDC2s (58).

pDCs are key producers of type 1 IFNs with the capability to produce >100 times more type 1 IFNs compared to monocyte-derived cells (62). Although pDCs are APCs, their positive anti-cancer effect is mostly contributed to modulation of other key immune populations with type 1 IFNs. These effects include enhancing the function of other APCs, potentiation of NK cells and CD8+ T cells, and suppression of regulatory T cells (62). Infiltration of pDCs in tumors have varying (albeit mostly negative) effect on prognosis in human patients (57,63,64) due to immunosuppressive functions which include expression of PD-L1 and secretion of IL-10 (64). Indeed, depletion of pDCs in highly pDC-infiltrated murine tumors may delay tumor growth. Conversely, pDCs and IFNα can be essential for anti-tumor responses in the same tumor model when using TLR7 agonists as immunotherapy (63).
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2.3.5 Monocytes
Monocytes are blood-circulating precursors to macrophages. However, it has become apparent that monocytes are able to extravasate into tissues without differentiating into macrophages and mediate functions including antigen trafficking, cytokine production, phagocytosis, and antigen presentation (65). Two main subsets exist: Patrolling monocytes (PMos) and inflammatory monocytes (in cancer best known as monocytic myeloid derived suppressor cells; Mo-MDSCs). Each with their own specialties and implications in cancer and are further described in manuscript I.

2.3.6 Macrophages
Macrophages may differentiate from monocytes or from derive from tissue-resident macrophages that developed during embryogenesis. Their physiological role includes phagocytosis, antigen presentation, and secretion of immune-modulating factors (66). Although tumor-associated macrophages (TAMs) may perform anti-tumorigenic functions (e.g. stimulation of immune cells, antigen presentation, and phagocytosis of cancer cells), infiltration of TAMs is mostly associated with poor prognosis, metastasis, and poor response to therapy (29). Despite their association with poor prognosis, studies have reported that anti-tumorigenic phenotypes may be obtained using immunotherapy (e.g. TLR7 agonists) (67). More specific functions of TAMs are further described in manuscript I.

2.3.7 Neutrophils
Tumor associated neutrophils (TANs) are a heterogeneous population. While functional neutrophils are regarded as anti-tumorigenic, the low-grade inflammation caused by tumors exhausts the bone marrow, causing pro-tumorigenic neutrophils with an immature phenotype (known as polymorphnuclear-MDSCs; PMN-MDSCs) to be released (31,68). Subsequently, TANs are generally associated with negative prognosis (24,69). Current research indicates different methods for determining whether TANs are pro-or anti-tumorigenic including based on density (70), surface Siglec-F expression (24,71), or arginase-1 (72). However, a consensus on differentiating pro-tumorigenic from anti-tumorigenic has yet to be made. Anti-tumorigenic functions include cytolysis of tumor cells through ROS secretion, neutrophil-derived extracellular DNA secretion, antigen presenting capabilities, and mediating antibody-dependent cell cytotoxicity through its Fc-receptors
(69,73). Pro-tumorigenic functions are mainly contributed to potent T cell inhibition by production of arginase-1 and ROS that causes reduced effector function, halts proliferation, inhibits interactions with MHC molecules, and induces apoptosis (69,73,74).

2.4 Antigen Uptake and Processing

All nucleated cells, including tumor cells, can present antigen on MHC molecules. However, only professional APCs can provide all the necessary signals to fully activate naïve T cells. Professional APC are highly efficient in processing antigen and presenting it to T cells and is constituted by DCs, monocytes, macrophages and B cells. APCs reside throughout the body and may be either migratory or tissue-resident. In cancers, the most critical places for presentation of TAA occurs in tumor-draining lymph nodes, in the TME, and in tertiary lymphoid structures in tumors.

The most relevant pathway of antigen uptake to this dissertation is phagocytosis. Here, large insoluble particulates such as apoptotic cells or nanoparticles are endocytosed through a variety of receptors including opsonic (Fc and complement) receptors (75) and receptors recognizing ‘eat me’ signals on apoptotic cells (76). Although phagocytosis is a corner stone in antigen presentation, phagocytosis of apoptotic cells is linked to obtaining immunosuppressive phenotypes due to its role in wound healing, homeostasis, and immune tolerances (76).

Following uptake, particulates are processed through phagosomal and endosomal compartments that are subject to progressive maturation (39,77). Maturation occurs by fusion with vesicles leading to progressively more acidic environments containing progressively more harsh proteases. Early compartments have mildly reducing environments (77) in which retention is inducible and associated with enhanced MHC I antigen presentation (78). Maturation of early compartments occurs by fusion with later compartments that may culminate in endo-lysosomal compartments containing a highly reducing environments (77). Processing in late compartments is associated with MHC II antigen presentation (79). Notably, APCs differs in antigen processing by regulating surface receptors responsible for antigen uptake and by regulating internal proteins responsible for antigen processing (e.g. protease inhibitors and proton pumps) differently. Thereby APCs subset can determine whether proteins are preferentially presented on MHC I or II.
Additionally, the receptor taking up antigen and ligation within endosomal compartments (e.g. TLR ligation) may also steer antigen processing and presentation (78).

MHC I loading occurs in the vacuolar and cytosolic pathway. In the vacuolar pathway, endocytosed antigen are processed by lysosomal proteases in endolysosomes and loaded directly onto MHC I molecules (80). In the cytosolic pathway, endocytosed antigens are partly degraded in endosomes or phagosomes, exported from the phagosome, processed to small peptides by immunoproteasomes in the cytosol, and transported into the endoplasmic reticulum by Transporter associated with Antigen Processing. Here peptides are trimmed to the 8-10 residues preferred for MHC I binding and high-affinity peptides loaded onto MHC I molecules and transported to the cell surface where it may be presented to CD8\(^+\) T cells (9,80). The MHC I molecules recognized by CD8\(^+\) T cells on potential target cells are conversely derived from endogenous proteins. These proteins are also generated by proteasomes in the cytosol and follow the subsequent steps described previously for MHC I loading and presentation (9,80). During steady state, proteasomes do not generate MHC I epitopes efficiently but upon IFN\(\gamma\) stimulation the proteasome exchanges subunits to become an immunoproteasome whereby processing is greatly enhanced (81,82).

MHC II loading occurs directly in late compartments. MHC II molecules are assembled in the ER and transported through the Golgi apparatus to late compartments. Here high affinity peptides, resulting from proteasomal degradation in late endosomal or lysosomal compartments, are loaded onto MHC II molecules followed by transportation to the cell surface where it may be presented to CD4\(^+\) T cells (9).

### 2.5 Immunotherapy

Today, immunotherapy constitutes a broad range of immunomodulating therapies that continues to expand with novel treatments. Treatments aim at affecting different steps within the cancer immunity cycle to ultimately cause anti-cancer immunity by shifting the TME-infiltrating immune cells from pro-tumorigenic towards anti-tumorigenic phenotypes. Notable trends within immunotherapy includes the use of immune checkpoint blockade and TLR agonists amongst others (83). Desirable key outcomes in the TME includes higher infiltration of CD8\(^+\) T cells and cDC1s, repolarization of immune cells towards anti-tumorigenic phenotypes, infiltration into the tumor (beyond the tumor margin) (11), and systemic anti-cancer immunity (84).
2.5.1 Immune Checkpoint Blockade

Immune checkpoints are a corner stone in the immune system. As described previously, immune checkpoint molecules are overexpressed in the TME and are associated with exhausted T cells with low cytotoxicity. By administering antibodies that bind checkpoint molecules it is possible to provide steric hindrance which prevents inhibitory signaling for T cells. The antibodies differ in immunoglobulin isotype and Fc-regional mutations whereby the mechanism of action also may occur through antibody-dependent cellular cytotoxicity for some immune checkpoint inhibitors (85). FDA approved immune checkpoint inhibitors have shown remarkable results and includes antibodies directed against PD-1, PD-L1, and CTLA-4 in various cancer types (86). As expression of immune checkpoints differ greatly between tumors, stratification of patients based on immune checkpoint expression greatly improves response rates (87). However, current clinical methods for stratification is far from perfect. Recent studies have demonstrated that a subpopulation of PD-1+ T cells is predictive for response to αPD-1 treatment (specifically PD-1+ TCF1+ CD8+ T cells) (46,88). Additionally, studies have demonstrated that the myeloid compartment can cause resistance to αPD-1 treatment (19,20).

Due to the protective role of immune checkpoints, significant adverse events can be observed due to treatments with immune checkpoint inhibitors. Furthermore, only a minority of patients exhibit long-term durable responses and patients may develop resistance (89). Consequently, additional targets (89) and combinational approaches with other anti-cancer therapies are actively being explored, as impressive synergy with other anti-cancer treatments is common (90).

2.5.2 Toll-like Receptor Agonists

TLR agonists are widely investigated for their anti-cancer potential based on promising preclinical results (91). TLRs recognizes pathogen-associated molecular patterns and damage-associated molecular patterns with subsequent potent immune stimulatory signals following activation. Furthermore, TLRs are comprised of several families that are localized in different cellular compartments and are expressed to varying degrees depending on cell type. Of these, much research aims for delivery to TLR7 remains which has shown promising results for anti-cancer immunotherapy (91).
Chapter 2 – Background

TLR7 is mainly located in intracellular endosomal compartments and recognizes purine-rich ssRNA which may be found in ssRNA viruses like hepatitis B and HIV. Expression of TLR7 is reported to be highest in pDCs, cDCs, and B cells but is also expressed in other immune and non-immune cells and is inducible by inflammatory signals (92,93). Ligation of TLR7s is generally associated with type 1 IFN responses and its anti-tumor effects, when systemically administered, have been associated to be dependent on DCs, CD8+ T cells, and NK cells (91,94–97). Furthermore, repolarization of myeloid cells in the TME have been shown following administration of TLR7 agonists (63,67,96–99). However, the contribution to treatment effect for each cell type has been reported with conflicting results and no exhaustive studies have been concluded that investigates which cells are mediating the anti-cancer immunity of TLR7 agonists. Additionally, studies have shown that upon systemic TLR7 administration endothelial cells respond by up-regulation of adhesion molecules leading to a transiently reduced availability of peripheral blood leukocytes due to extravasation into tissues (100).

Translational value of studies in mice are limited as many TLR7 agonists are also recognized by TLR8 and recognition of ligands by TLR8 differs between mice and humans (91,101–103). In contrast to TLR7, TLR8 is primarily located in myeloid cells and have signaling pathways similar to that of TLR7 (101). In addition to promising results as monotherapy, TLR7 agonists also synergizes well with other treatments including radiotherapy and immune checkpoint blockade (91,96,104). FDA approved TLR7 agonists is limited to topical administration of Imiquimod that is approved for basal cell carcinoma (91). Administration routes that target systemic activation in patients have shown narrow therapeutic windows. Oral administration of the TLR7/8 agonist resiquimod in humans caused severe adverse events at 0.2 mg/kg (105). The TLR7 agonist 852A has been investigated in a phase I study for subcutaneous and intravenous administration where it could be safely administered up to 1.2 mg/m² (~0.04 mg/kg) (106). However, additional studies demonstrated that only modest anti-cancer activity could be obtained at 0.9 mg/m² (107). In summary, despite promising preclinical results the clinical results warrant research into methods of improving the therapeutic window of TLR7 agonists to obtain the same impressive outcomes in the clinical setting as those observed pre-clinically.
2.6 Improving Drug Delivery using Nanocarriers

Delivering sufficient therapeutic compounds to the desired tissue without causing adverse reaction in other organs is of paramount importance for obtaining a clinically feasible treatment. Nanocarriers are widely investigated for their potential of increasing target-to-off-target ratios by either targeted or untargeted approaches and thereby reduce adverse reactions. Additionally, nanoparticles can protect drugs from undesirable enzymatic degradation, renal filtration, and ultimately greatly improve circulation time. Nanocarriers are nano-sized particles (typically 10-200 nm) that can be made in a variety of forms including liposomes and micelles and consist of various materials (108).

To the immune system, nanoparticles typically appear as a foreign object which results in clearance from circulation. Elimination of nanoparticles in circulation occurs by the mononuclear phagocytic system (MPS) and renal clearance (for < 5 nm nanoparticles and destroyed nanoparticles) (109,110). The MPS is constituted of phagocytes (mainly monocytes, macrophages and DCs) which are highly abundant in the spleen and liver and recognizes complement factors and antibodies binding to nanoparticles. To avoid binding of complement factors, nanoparticles may be shielded using for instance polyethylene glycol (PEG) (109). However, PEG may be recognized by B cells due to its repetitive structure across the nanoparticle surface which enables crosslinking of B cell receptors to an extent that overrules the need for co-stimulation from T cells, ultimately leading to production of αPEG IgM. Additionally, αPEG IgG has been reported but have been much less investigated (111). The large quantity of antibodies against nanoparticles means that subsequent infusions of nanoparticles will be opsonized and cleared by the MPS resulting in ABC. Several factors influence the degree of ABC including nanoparticle size, charge, and composition, time between infusions, animal species, lipid dose, and encapsulated drug. Additionally, complement binding of nanoparticles may cause complement activation-related pseudoallergy (CARPA) which, in contrast to ABC, typically occur on the initial infusion. Importantly, both ABC and CARPA has been shown to occur clinically and greatly affect biodistribution and thus treatment effect (109).

Accumulation of nanoparticles in tumors may be based on either active or passive accumulation, each with advantages and disadvantages. For active accumulation, nanoparticles are decorated with ligands or antibodies that binds overexpressed proteins on cancer cells, e.g. folate decoration to target tumors with high folate receptor expression.
However, the heterogeneity in solid tumors, opsonization, and off-target protein expression may impact tumor accumulation negatively. Passive accumulation mainly relies on the enhanced permeability and retention (EPR) effect present in tumors due to the leaky vasculature. Here macromolecules (>40 kDa) extravasate into the tissue at a much higher rate than in healthy vasculature (112,113). However, relying on the EPR effect may be risky as it varies greatly between tumors and may be affected by prior treatments (e.g. surgery, radiotherapy, and chemotherapy) (113,114). Interestingly, a metareview from 2016 categorized data from publications with biodistribution in mice of nanoparticles from 2005 to 2015 and found only minor differences in tumor accumulation based on investigated parameters like size, charge, nanoparticle type, and tumor model. Reported data revealed that small nanoparticles (<100 nm) had a tendency to have slightly better reported tumor accumulation. However, a median of just 0.7% injected dose per gram tumor was observed across all nanoparticles studies included and concluding that most preclinically evaluated nanoparticles may be inadequately accumulated for clinical use (110). Although low accumulation of nanoparticles are typically observed, free drugs tend to accumulate to an even lower degree in tumors (113,115,116).

In this dissertation, two types of nanoparticles (liposomes and micelles) were investigated for their potential to mediate anti-cancer immunity by delivery of TLR7 agonists; Liposomes are typically developed as unilamellar vesicles around 100 nm but may also be multilamellar and significantly smaller or larger than 100 nm (7). Several liposomal formulations for anti-cancer treatments has been approved, most of which focuses on delivery of chemotherapeutics to tumors (117). In contrast, micelles are self-assembled monolayered vesicles that are typically smaller than liposomes (10-100 nm) (7,108).
2.7 Preclinical Evaluation of Anti-Cancer Treatments in Mice

Although *in vitro* assays are useful for screening drug potency and cytotoxicity, the complexity of cancer and influences on the immune system by anti-cancer treatments (in particular nanoparticles) makes *in vitro* testing inadequate for most preclinical evaluation. In contrast, preclinical *in vivo* cancer models allow evaluation in a more relevant setting where biodistribution, immune interaction throughout the body, and several other parameters may be evaluated and influence results and is consequently central to oncology research. Several *in vivo* models exist that each has advantageous (118). In the current dissertation, subcutaneous implantation of cancer cells in immunocompetent mice were used to model tumor biology and anti-tumor effects of immunotherapies. While this method is widely used for preclinical oncology, it is also criticized for skipping the phases of cancer clonal selection and progression and for treatments being started at very small tumor volumes (118,119).

There is a growing amount of murine syngeneic cancer cell lines available. These vary in several relevant aspects including tissue of origin, mutations, growth rate, and the immune infiltration in the resulting tumor of which the latter is most relevant to this dissertation. However, currently only one comprehensive peer-reviewed study has investigated the TME immune infiltration across models. Here, Mosely et al. characterized the general immune infiltration with focus on the T cell compartment, mutational status of the cancer cell line, and response to αPD-L1 and αCTLA-4. Striking differences in the immune composition within the TME could be observed and the differences could explain the differences seen in response to immunotherapy (120). These findings, together with the previously described importance of the immune system in cancer development and treatment outcome, demonstrates a need for further understanding the differences between murine tumor models to improve the translational value of treatment evaluation.
Chapter 3 – Manuscript I

Myeloid characterization of 11 syngeneic cancer models for optimized treatment evaluation

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Manuscript in preparation.

Abstract

Syngeneic murine cancer models are instrumental for the development of anti-cancer treatments including immunotherapies. The composition and polarization of infiltrating immune cells in the tumor microenvironment (TME) has been demonstrated to be predictive for immunotherapy outcome. Myeloid cells constitute a major part of immune cells in the TME and are central to prognosis and treatment efficacy but functional evaluation in preclinical models remains inadequate. Myeloid cells have potent anti-cancer potential due to their tumor antigen presentation abilities and production of anti-tumor signaling proteins. Thus, myeloid cells present attractive immunotherapeutic targets. However, the vast differences in TMEs observed clinically and preclinically lack better comparative characterizations to improve translation of treatment outcome.

Here, we provide a side-by-side characterization of the myeloid compartment across several syngeneic murine cancer models. This work offers a tool for model selection based on intervention strategy. The evaluated subcutaneous syngeneic models presented with TMEs ranging from immune-excluded, dominated by a single myeloid subset to TMEs characterized by general myeloid infiltration and high infiltration of cytotoxic T cells.
Furthermore, we observed correlation tendencies across cancer models between immune populations and cytokines levels in the TME. This work highlights the importance of considering differences in the TME and provides researchers with a comparison of relevant cancer models to facilitate translation of preclinical anti-cancer treatment.
Introduction

The clinical success of cancer immunotherapy has expanded the treatment paradigm to further improve patient outcomes. Some of the major challenges with anti-cancer strategies include stratified responses to immunotherapy with only subpopulations of patients achieving durable remissions (6). The composition and functional status of immune cells in the tumor microenvironment (TME) have emerged as major contributors to both immune evasion and tumor elimination (6). The myeloid compartment constitutes a major part of the immune-infiltrating populations in the TME and contains subpopulations that are major contributors to immune evasion of cancer cells (22–24,52). Consequently, infiltration of myeloid cells are generally correlated with worse prognosis (16,17) but with potential for potent anti-cancer effects through repolarization. Central immunosuppressive myeloid populations include polymorphnuclear myeloid-derived suppressor cells (PMN-MDSCs) and monocytic MDSCs (Mo-MDSCs). These are released from the bone marrow in high numbers following continuous low-grade inflammatory signals produced by tumors. This ultimately exhausts the bone marrow and leads to immature phenotypes of what would otherwise be effector cells (31). Furthermore, the MDSCs are implicated in producing pre-metastatic niches, which makes them important for cancers ability to metastasize (121).

The immune modulating properties of intratumoral myeloid cells directly influence the infiltration, activation, and maintenance of anti-cancer T cell populations. The infiltration of cytotoxic T cells (cTs) are used to identify tumors as T cell inflamed (“hot”) or non-inflamed (“cold”) based on high or low cT infiltration, respectively. Indeed, hot tumors correlate with favorable response to immunotherapy and positive patient outcomes (6). The complexity of myeloid cells, TME differences, and the potential of myeloid cells as targets for immunotherapy shows the necessity for correct characterization to enable optimal evaluation of therapy.

For translational purposes, it was recently shown by single cell transcriptomics that major myeloid populations are conserved between mice and humans (24). The functional plasticity of myeloid cells is generally divided into type 1 and type 2 based on whether they contribute towards immune recognition or evasion, respectively. Polarization of cells towards either type 1 or 2 is determined by factors in the TME. Immunostimulatory factors support type 1 phenotypes that in turn supports the cancer immunity cycle leading to effector responses against cancer cells (5). Here, tumor associated antigens (TAA)
released from cancer cells during immunogenic cell death are processed and presented by e.g. type 1 classical dendritic cells (cDCs) leading to infiltration, priming, and activation of cTs. Key factors include CXCL10 that recruits cTs (122) and IFNγ that polarizes antigen presenting cells, increases antigen presentation, and promotes Th1 differentiation (123). However, progressing tumors are dominated by type 2 phenotypes (24), thus restricting the cancer immunity cycle (5). Type 2 cytokines include CXCL1 causing immunosuppression by recruitment of PMN-MDSCs to the TME (124).

Dividing myeloid cells into type 1 and 2 has generally been based on surface markers that differ between type 1- and type 2-like induced cells in vitro under inconsistent experimental settings (125–128) and probably has little functional meaning for tumor growth in vivo. This is supported by recent transcriptomic comparison analysis of patient and murine tumors that further supports the theory that conventional type 1/2 division are not distinctive states (24). Nonetheless, macrophage subsets were found strongest associated with type 2-like gene signatures and with gene signatures correlating to neutral and unfavorable patient outcome (24). These data suggest anti-cancer treatments should not only focus on polarization of tumor-associated macrophages (TAMs) but should always result in improved cT infiltration for positive patient outcome.

Conventional type 2 markers for macrophages includes the scavenger receptors CD163 and CD206 that are correlated with type 2 signatures in vitro and negative prognosis in melanoma patients (128–130). Although depletion of CD163+ TAMs alleviates αPD-1 resistance (131), it has yet to be shown whether polarizing treatment, like toll-like receptor 7 agonists, can diverge CD163+ and CD206+ myeloid cells into anti-tumorigenic populations. Conversely, functional markers like the type 2 marker arginase-1 directly affect anti-tumor immunity and consequently is more relevant to evaluate. Other relevant functional markers include the co-stimulatory molecule CD86, which is type 1 associated, and directly involved in T cell licensing and activation (129,132). Functionally type 1 TAMs are commonly described as MHC IIhigh (133). Consequently, we divided TAMs according to MHC II expression.

In the present study, we characterized the following myeloid TME populations across 11 syngeneic cancer models: PMN-MDSCs, Mo-MDSCs, patrolling monocytes (PMos), TAMs, and cDCs. These myeloid subsets are described in more detail in supplementary.
Materials and Methods

Cancer Models
All culture media were supplemented 2 mM Glutamax. 10% fetal bovine serum was supplemented for all cell lines except J558, which was supplemented with 10% horse serum. No additional independent validation of tumor cell lines was performed. Cancer cell lines were inoculated within 5 passages from thaw.

All experimental procedures were approved by the institutional ethical board and the Danish national Animal Experiment Inspectorate (license no. 2016-15-0201-00920). Female BALB/cJRj, C57BL/6JRj (Janvier Labs), and DBA/2JRccHsd (Envigo) mice age 7-12 weeks were anesthetized with 3-5% sevoflurane and inoculated subcutaneously in the right flank with 100 µl cancer cells in unsupplemented ice-cold media. Tumor volume was measured with electronic calibers as length x width² / 2. Additional details on cancer cell lines maintenance and inoculation can be seen in Supplementary Table S1.

Radiotherapy
Mice were irradiated under lead-shielding exposing the tumor-bearing flank once tumors reached a mean volume of 300-600 mm³ using a small animal irradiator (X-RAD 320, PXi, US) with a dose rate of 1.0 Gy/min (320 kV, 12.5 mA, 1.5 mm Al, 1.25 mm Cu, 0.75 mm Sn filter).

Flow cytometry
Tumors (100-1,000 mg, n=3-19) were excised, weighted, minced with scalpels, and digested in tumor dissociation enzyme mix for murine tumors (Miltenyi Biotec) for 40 minutes at 37°C in a shaking water bath and mashed through 70-µm cell strainers. Total cell yield was determined by flow cytometry (Muse®, Merck Millipore). Tumor-draining lymph nodes (tdLN)s were excised and mashed through 70-µm cell strainers.

Processed cells from tumors (>1x10⁶) or total tdLN cells were resuspended and blocked in 50 µg/ml Fc-block (clone 2.4G2, BD Biosciences) in FACS buffer (PBS + 0.5% BSA + 0.1% NaN₃) for 5 minutes on ice. Fc-blocked samples were mixed with a mastermix of FACS buffer, brilliant stain buffer, and antibodies listed in Supplementary Table S2 (5 µg/ml final concentration of Fc-block) for 30 minutes on ice, washed, filtered through 70-µm strainers, and acquired on a BD LSRFortessa X-20 (equipped with a 405, 488, 561,
and 640 nm laser; BD Biosciences) using BD FACSDiva v.8.0.1 software (BD Biosciences). All surface staining was performed in a single staining step. Intracellular staining of CD68 was performed by fixation in 1% freshly diluted formaldehyde in PBS for 20 minutes at room temperature and staining under similar conditions to surface stain but in permeabilization buffer (PBS + 0.5% BSA + 0.1% saponin).

**Cytokine Analysis**

Tumor tissue (20-110 mg) was snap frozen in liquid nitrogen and stored at -80°C until use. Tumors were pulverized using CP02 cryoPREP (Covaris), resuspended in 5 µL lysis buffer/mg tumor (50 mM TRIS HCl, 150 mM NaCl, 10% UltraPure Glycerol, 1% NP-40 Surfact-Amps, pH 7.5 supplemented with 10 µL/mL Halt Protease and Phosphatase Inhibitor Cocktail lysis buffer (Life Technologies) (61)), shaken at 300 rpm for 1 hour at 4°C followed by centrifugation at 15,000 g for 15 min at 4°C. Supernatant was collected, added 25 U (0.1 µL/mL supernatant) Pierce Universal Nuclease for Cell lysis (ThermoFisher Scientific), and incubated shaking at 300 rpm for 1h at 20°C. Samples were analyzed for murine GM-CSF, IL-1β, IFN-γ, CXCL10 (IP-10), CXCL1 (KC/GRO), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), TNF-α, and VEGF using a U-PLEX assay (MSD Mesoscale) according to the manufacturer’s instructions. Plates were read using MESO QuickPlex SQ 120 (MSD Mesoscale). Data analysis was performed in the Prism 8 Software (GraphPad).

**Immunohistochemistry**

Paraffin-embedded formalin-fixed tumors were sectioned at 4 µm, mounted on SuperFrost ULTRA PLUS slides (Thermo Fisher Scientific), and deparaffinized. Following rehydration in a series of alcohols, sections were microwaved in citrate buffer pH=6 for heat-induced epitope retrieval. Sections were blocked with Peroxidase Blocking Solution (Dako) for 10 min and 2% bovine serum albumin (BSA) in PBS for 20 min. Sections were stained with 0.125 µg/ml primary rabbit anti-mouse arginase-1 antibody (#PA5-29645, Thermo Fisher Scientific) in 2% BSA for 1 hour. Primary antibody was detected using the EnVision+ System-HRP Labeled Polymer and Liquid DAB+ Substrate Chromogen System (Agilent Technologies) for 40 min and sections were counterstained with Mayer’s acidic Hematoxylin (Region H Apotek, Denmark). All procedures were performed at room temperature and all
tumors were stained in the same analysis. Sections were digitalized and quantified in viable tumor areas using Visiopharm Software.

**Data Analysis**

Data analysis was performed in FlowJo (v10.6.0 and v10.6.1; BD Biosciences) and Prism (v8.2.1; GraphPad Software) unless stated otherwise. T-distributed Stochastic Neighbor Embedding (t-SNE) analysis was performed in FlowJo on downsampled concatenated viable CD45+ cells. t-SNE was performed using all parameters except for forward scatter, side scatter, viability dye, CD45, CD68, and mCherry. To compare samples, number of cells per 100 mg tumors was determined as [%gated as cells] x [total cell count] / [tumor weight in mg] x 100.

R (v.3.5.1) was used for the following figures: Heatmaps of individual sample marker expression levels, cytokine levels, correlation plots, and t-SNE plots for demonstrating sample clustering and visualizing differences following radiotherapy in CT26, MC38, 4T1, and B16-F10. Heatmaps of marker expression and cytokine levels were obtained using the ComplexHeatmap package using flow cytometry data obtained using FlowJo. Correlation plots of cell population sizes and cytokine levels were obtained utilizing the corrplot package. For visualizing differences in untreated and radiotherapy-treated CT26, MC38, B16-F10, and 4T1 tumors, gating and t-SNE clustering was performed in R using flowCore and Rtsne packages. t-SNE was performed on 4 randomly chosen downsampled samples (5000 viable CD45+ events) for each of the cancer models. All parameters were included in the analysis except for viability dye and CD45, then inverse sine transformed and scaled with a cofactor of 5.

**Results**

**Syngeneic cancer models display large heterogeneity in immune cell infiltration and cytokine profiles**

The myeloid populations with central importance for tumorigenesis and anti-cancer therapies were analyzed across 11 syngeneic cancer models. The comprehensive analysis was performed in commonly used syngeneic cancer models, including four carcinomas (ASB-XIV, MC38, LL/2, and 4T1), two adenocarcinomas (CT26 and Renca), two lymphomas
(A20 and E.G7-OVA), one mastocytoma (P815), one melanoma (B16-F10), and one myeloma (J558).

Cancer models were analyzed by flow cytometry. t-SNE of analyzed parameters demonstrated clustering of samples according to models and mouse strains (Supplementary Fig. 1A). The evaluated cancer models displayed large variations in both viability and immune cell (CD45+ infiltration, ranging from immune excluded (J558 and B16-F10 with 4±0.4% and 7±5%, respectively) to highly immune-infiltrated (ASB-XIV with 58±14%) (Figure 1A). Overall cell density ranged from 2 (ASB-XIV and Renca) to 14±8 (CT26) million cells/100 mg tumor (Supplementary Fig. S1B).

We observed a tendency of increased cT infiltration in the tumors with overall high myeloid infiltration (Figure 1B). Several models were dominated by a single myeloid population including TAMs in MC38, Mo-MDSCs in LL/2, and PMN-MDSCs in 4T1. Furthermore, ASB-XIV was dominated by B and T cell infiltration. Interestingly, the A20 and the P815 models have quite high infiltration of cTs compared to the rest of the myeloid cells in the TME. Separation of TAMs into MHC II high and low was used to show differences in polarization across cancer models. Here, several of the established immunogenic models (CT26, MC38, E.G7-OVA, and Renca) (120,134) had a two to three fold higher infiltration ratio between MHC II^{high} over MHC II^{low} TAMs.

Cytokines with central importance for immune cell recruitment and polarization were analyzed to correlate immune infiltrating cells and the cytokine milieu. Cytokine milieus were found to be highly variable across cancer models with highly cT-infiltrated models having highest cytokine levels (Figure 1C). The immune infiltrating cells were correlated with cytokine milieu across all models due to clinical observations of correlation between patient outcome and infiltration in several different cancer types (Figure 1D). Intriguingly cT infiltration was found to correlate with both IFN\(\gamma\) (R = 0.75) and cDC1s (R = 0.53) despite the many confounding parameters of comparing across cancer models. IFN\(\gamma\) further correlated with CCL3/4 (R = 0.78 and 0.71, respectively) that are under IFN\(\gamma\) control. The monocyte chemoattractant CCL2 correlated with MHC II^{high} TAMs (R = 0.56). The neutrophil-attracting chemokine CXCL1 had negative correlation tendency with cTs (R = -0.42). Notably, cytokine expression was generally higher in cancer models with high leukocyte infiltration.
Figure 1. Immune subset infiltration display large variations across common syngeneic cancer models. (A) Viability and immune infiltration as percentage of total cells and of viable cells, respectively. Shown is mean ± SD, n = 3-9. (B) Heatmap of investigated cell types per 100 mg tumor (mean, n = 3-9 per model). cTs, TAMs, Mo-MDSCs, PMos, PMN-MDSCs, and B cells is x10^4 cells per 100 mg tumor, cDC1s is x10^2 per 100 mg tumor. White color denotes the median and red the highest infiltration of each immune population across investigated models. B16-F10 infiltration is pooled from two independent experiments with similar results between each experiment. n.d. = not determined, as it is based on a B cell lymphoma. (C) Heatmap of log2 transformed
cytokine expression levels across all cancer models. Expression levels were averaged across samples (n = 3 per model) and scaled by the median within each cytokine. For gating strategy used in (A, B), see Supplementary Fig. S2. (D) Kendall correlation between average of cell populations and log2 transformed, median scaled average cytokine expression levels across all cancer models. Kendall correlation score is shown in correlation heatmap. Individual correlation plots are shown in Supplementary Fig. S3.

CD86 associates with type 1 signatures in the tumor microenvironment

The differences in myeloid infiltration across the evaluated cancer models demonstrate their value as attractive tools for guidance of immune modulating therapies with respect to the infiltration observed across patient populations. To expand the characterization we profiled activation markers, including; three co-stimulatory type 1 markers (CD80, CD86, and MHC II), two type 2 scavenger receptors (CD163 and CD206), and two checkpoint inhibitor molecules (PD-1 and PD-L1) (Figure 2A–B). We observed clustering of samples, with respect to overall marker expression, within cancer models. However, clustering of markers across populations showed less consistency (Figure 2A).

MHC II was expressed highest on TAMs, and to lesser extent on PMos and Mo-MDSC. The use of MHC II as a type 1/2 marker was supported by the tendency of CD86 expression being higher in MHC II\textsuperscript{high} compared to MHC II\textsuperscript{low} TAMs in all models. The only exception was low expression of CD86 in the J558 model, which might be explained by low myeloid infiltration and sparse cytokine milieu. CD80 expression was not specific to MHC II and CD86 and was broadly expressed in relation to myeloid cell type and tumor model (Figure 2B).

CD163 was highly expressed in PMos and TAMs in ASB-XIV and 4T1 and to a lesser extent in other investigated cancer models. PMos displayed the highest expression of CD206, which is consistent with previous reports on expression in healthy tissues (135). CD206 expression displayed scattered expression in TAMs. Importantly, we did not observe any tendency of CD163 and CD206 associating with co-stimulatory molecule expression in syngeneic subcutaneous cancer models (Figure 2B).

Expression of PD-L1 was homogenously expressed on PMos and TAMs across cancer models with few exceptions. Highest expression was observed in TAMs with PMos as the only other investigated subsets with high expression. Notably, E.G7-OVA, LL/2, and 4T1 were characterized by low myeloid PD-L1 expression and are reported not to respond to αPD-L1 therapy (136–138). PD-1 expression on T cells have been studied extensively,
but recently the expression on myeloid cells in the TME have gained interest. Here, PD-1 has been linked to type 2 phenotypes and lower phagocytic capacity (139). Our findings show high PD-1 expression on Mo-MDSCs, PMos and TAMs in 7/11 models that was irrespective to MHC II levels (Figure 2B).

Arginase-1 expression was investigated by immunohistochemistry and did not appear to associate with the extent of MDSC infiltration, indicating that overall arginase-1 expression level is not only dependent on myeloid cell infiltration in the TME (Figure 2C).
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Figure 2. Expression of scavenger receptors, checkpoint inhibitor molecules, and co-stimulatory molecules have distinct expression patterns in the myeloid tumor microenvironment. (A) Heatmap of marker expression levels across cell populations and all cancer models. Shown is data that was mean normalized and inverse sine transformed. Average linkage hierarchical clustering was performed using Manhattan distance. (B) Heatmaps of each investigated surface marker across myeloid populations colored based on the mean MFI (arbitrary unit) within each myeloid population in each tumor model. For (B, C) shown is mean, n = 3-9 and is based on the dataset and gating used in Figure 1A-B. (C) Relative arginase-1 expression in the TME determined by immunohistochemistry. Shown is mean + SEM, n = 2-3.

CD11c expression separates monocytic subsets, macrophages, and cDC1s in the TME
Phenotyping myeloid cells in the TME is challenging as commonly used markers for discrimination of monocytic cells, TAMs, and cDCs do not provide a clear subset identification. Studies often rely on CD64, CD68, and F4/80 but due to the continuum of expression of these markers in tumors there is a risk of misidentification. Therefore, we performed a comprehensive analysis of myeloid compartments in CT26 and MC38 tumors, which are characterized by high but very different myeloid infiltrations, with a panel that included commonly used markers for myeloid delineation. The panel focused on CD68, CD64, F4/80, and CD11c due to their widespread use in the literature – with the addition of key markers for delineation of the myeloid subsets.

CD68 is associated with antigen processing and presentation (140) and highest expression was observed in PMos (2.5-fold higher than in TAMs in CT26 and 1.8-fold higher in MC38). Notably, myeloid cells in MC38 tumors expressed more CD68 compared to those in the CT26 model, where CD68 was expressed at comparable levels on TAMS, cDC1s, and cDC2s (Figure 3A-B).

CD64 (FcyR1) is a high-affinity IgG receptor important for effector responses and is besides on macrophages also expressed on monocytes, cDC1s, cDC2s (24,132,141). We found CD64 equally expressed by TAMs, PMos, and Mo-MDSCs and to a lesser degree cDC2s (Figure 3A-B) in the TME, thus confirming its usefulness for delineation of cDCs (132). Of interest, our data indicate that CD64 may be used instead of CX3CR1 to define PMos in the CD11b+ CD11c– Ly6g– Ly6c– population (Supplementary Fig. 4A).

F4/80 is a macrophage subset marker in healthy tissues where it is also expressed by eosinophils and cDCs (132,142). At a transcripational level, TANs and
monocytes express more Adgre1 (F4/80) than TAMs in the TME (24). Although we did not observe significant F4/80 expression in PMN-MDSCs, we found it highly expressed in TAMs, PMos, and Mo-MDSCs (Figure 3A-B).

CD11c has commonly been used as a pan-DC marker, which is potentially problematic due to tissue and subset dependent expression levels of CD11c in macrophages (132). Consequently, additional markers are necessary to delineate these populations. We found that CD11c may be used to discriminate between TAMs and other monocytic subsets in the TME but additional markers are necessary to separate TAMs and cDC2s.

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Figure 3. CD11c, CD64, CD68, and F4/80 is expressed across myeloid populations in the TME. (A) Expression of surface CD11c, CD64, F4/80, and intracellular CD68 in myeloid subsets and B cells. Populations were gated on t-SNE using data from four separately processed CT26 (no fill pattern) and MC38 (with fill pattern) tumors. t-SNE was run on concatenated samples after downsampling to 25,000 viable CD45+ cells (see Supplementary Fig. 4B for gating prior to t-SNE). Box displays 25th to 75th percentiles, whiskers 10th to 90th percentile, line the median, and plus symbol the mean. Dotted line marks 0. (B) t-SNE projection of data used in A. Colored circles indicate areas with the respective cell type. Top left panel: t-SNE projection overlaid with gated populations. CT26 and MC38 plots are density plots of the respective tumors with black indicating highest cell density. Additional panels are overlaid with signal for each marker. Blue denotes minimal, green low, yellow intermediate, red high expression of each marker. A.U., arbitrary unit.

Radiotherapy reshapes the myeloid TME and induces uptake of tumor-associated antigens

It is increasingly evident that durable anti-cancer effect is dependent on modulation of the immune infiltration in the TME. However, the mechanisms in responsive and non-responsive tumors are not fully understood. Here we investigated how high dose radiotherapy affects the myeloid TME in the early phase after irradiation using two radiation sensitive (CT26 and MC38) and two radiation resistant (4T1 and B16-F10) cancer models. Radiation doses were based on previous studies that have demonstrated potent tumor control of CT26 and MC38 tumors but only temporary tumor control of 4T1 and B16-F10 tumors. The early response was found to have model-specific effects on population sizes with only Mo-MDSCs being similarly affected across all models (Figure 4A-B). In the radiation sensitive models, CD86 was increased significantly more on MHC II\textsuperscript{high} TAMs (2.3-2.5 fold, p < 0.05) than in resistant models (1.2-1.9 fold, p < 0.2). Similar changes were observed for PD-L1 on MHC II\textsuperscript{high} TAMs while CD206 was found to be downregulated on PMos in radiation sensitive models (p < 0.05) but not in radiation resistant models (p > 0.05) (Figure 4C).
Figure 4. Radiotherapy reshapes and repolarizes the TME in radiation sensitive models. Tumors were irradiated with 10 Gy (CT26, MC38, & 4T1) or 20 Gy (B16-F10) and processed for flow cytometry four days later. (A) t-SNE projection of the TME in untreated and radiotherapy treated CT26, MC38, 4T1, and B16-F10 (n = 4). t-SNE was run on concatenated sampled after downsampling to 7,000 viable CD45+ cells. Colored circles indicate areas with the respective cell type. Black indicates highest cell density. (B) Barplots showing populations out of total CD45+ in tumors. (C) Expression of CD86 and PD-L1 on MHC II\textsuperscript{high} TAMs and CD206
on PMos. Data for untreated samples are based on the dataset used in Figure 1A-B and gating strategy is also similar to the one used for Figure 1A-B. For (B-C), data is plotted as mean ± SEM, n = 4-5. Statistical significance was determined using Mann-Whitney U test (B-C). ns: nonsignificant, p ≥ 0.05; *, p < 0.05; ***, p < 0.001. MFI, median fluorescent intensity. A.U., arbitrary unit.

The increase in co-stimulatory markers led us to investigate how myeloid cells take up TAA in response to radiotherapy. Radiation therapy induces immunogenic cell death whereby TAAs are released and presented directly within the TME or in tumor-draining lymph nodes. To investigate the in vivo kinetics of TAA-uptake following local radiotherapy we used B16-F10 with stable mCherry expression as a surrogate marker for TAA (143) (Figure 5A).

In untreated tumors, TAMs and PMos were the main myeloid populations responsible for TAA-uptake (>3 fold to Mo-MDSCs, PMN-MDSCs, cDC1s, and cDC2s; Figure 5B-C). Radiotherapy induced increased TAA-uptake in TAMs, PMos, Mo-MDSCs, and cDC1s with subset-specific uptake kinetics. No significant increased TAA-uptake was observed in PMN-MDSCs and cDC2s, indicating that these cells are not significant contributors to TAA-trafficking following anti-cancer therapy. Notably, TAA-uptake kinetics differed between the cell types responsible for taking up TAA: TAMs peaking in uptake 1 day post radiotherapy while PMos and cDC1s peaked 7 days post treatment (Figure 5C). Consistent with CD86 and MHC II being type-1 markers, TAA-uptake was found highly associated with CD86+ and MHC II+ phenotypes of investigated cell types (Figure 6D and Supplementary Fig. S5A). Furthermore, we observed a peak in cDC1 activation in the tdLN the day after radiotherapy (Figure 5E).
Figure 5. TAA-uptake in the TME of B16-F10 following 15 Gy radiotherapy show cell type specific uptake kinetics and is associated with CD86. (A) Cancer cells expressing mCherry was used a model for TAA-uptake where mCherry fluorescence in immune cells indicates the amount of TAA taken up. (B) t-SNE projection of the TME in B16-F10-mCherry tumors. t-SNE was run on concatenated samples after downsampling to 11,000
viable CD45+ cells. Colored circles indicate areas with the respective cell type. Left panel: t-SNE projection overlaid with manually gated populations. Additional panels are overlaid with signal for each marker. (C) TAA (mCherry)-uptake in myeloid populations in the TME using manually gated populations. (D) TAA-uptake in CD86+ phenotypes of relevant myeloid cell types in the TME. n = 3-4 (n = 1 for mCherry FMO) for (C, D). (E) Expression of CD86 (top) and I-A/I-E (bottom) in cDC1s in tdLNs following radiotherapy. Data is plotted as mean ± SEM. Statistical significance was determined using one-way ANOVA with Dunnett’s multiple comparison test (C, E) or unpaired two-tailed student t test with Bonferroni correction for multiple comparisons (D). ns: nonsignificant, p ≥ 0.05; *, p < 0.05; ***, p < 0.001. For gating strategy used in (A, B), see Supplementary Fig. S5B. For gating strategy used in (E), see Supplementary Fig. S5C. MFI, median fluorescent intensity. A.U., arbitrary unit.

Discussion

Evaluation of immunotherapies and other anti-cancer treatments in murine syngeneic cancer models is key to understanding how to develop better treatment with good translational value across patients. Importantly, preclinical and clinical tumors both display vast differences in immune infiltration and phenotypes depending on a multitude of factors (24,122). Correct characterization of the myeloid subsets allows researchers to optimize their model choice and evaluation. Single-cell analysis methods like flow cytometry have facilitated in-depth understanding of the TME, but the plasticity of the myeloid cells in particular have caused a lot of discrepancies in the way data have been evaluated. Recent literature on single cell transcriptomic profiles of patient biopsies revealed conserved myeloid populations with clinical relevance (24). These data should be used to improve data obtained by flow cytometry and immunohistochemistry and thereby improve translational value.

The discrepancies of classifying myeloid population in the TME of murine subcutaneous tumors are illustrated by our findings. CD11c, CD64, CD68 and F4/80 was investigated across myeloid populations in two cancer models. None were found suitable as pan markers in the myeloid compartment due to the continuum of expression. While CD64, CD68, and F4/80 improved delineation of cDCs from monocytes and TAMs, only CD11c could delineate TAMs from monocytic subsets. Notably, variation in CD68 expression was observed between TMEs and better associated with an antigen presenting cell/phagocyte phenotype than as a TAM specific marker in murine tumors.

In the present study, the myeloid compartment of 11 syngeneic models were characterized. Several findings were comparable with the only previous comprehensive
study that compared several cancer models (120). Here, characterization of six cancer models was performed with focus on the T cell compartment, mutational differences, and response to αCTLA-4 and αPD-L1 treatment. Similarities include the dominant infiltration of Mo-MDSCs in LL/2 and PMN-MDSCs in 4T1. B16-F10 was found to have overall low infiltration and Mo-MDSCs as the largest population. Furthermore, the cytokine levels of IFNγ and TNFα were found to be comparable. Significant differences include MC38 having high Mo-MDSC but low TAM infiltration. We found MC38 to be dominated by TAMs, in line with previous studies using MC38 as a model for investigating TAMs (144). Furthermore, in contrast to our findings where MC38 was found to have low CXCL1 (neutrophil attractant) and low PMN-MDSC infiltration, Mosely et al. reported MC38 to have high levels of CXCL1 but low PMN-MDSC infiltration.

In addition to MC38 being dominated by TAMs, we identified vast differences between models in terms of both chemokine levels and myeloid immune infiltration. Several other models were also dominated by a single myeloid subset. Thus, indicating that few chemokines drive the immune infiltration in many cancer models. This is exemplified by the PMN-MDSC infiltrated 4T1 model also containing high levels of CXCL1 and MC38 being highly infiltrated by Mo-MDSCs and TAMs and containing high levels of monocyte-attractants (CCL2, 3, & 4). Furthermore, levels of favorable cytokines (e.g. CXCL10 and IFNγ) were observed in the cancer models with high cT infiltration, but as expected, the cytokine landscape was very heterogeneous across models.

PMos remains the least characterized myeloid subset within the TME. However, like other myeloid cells they have both pro- and anti-tumorigenic functions and phagocytic capacity (145–149). Due to their expression of CD206, F4/80, and CD68 they may have been misidentified as TAMs in previous studies. Phenotypical difference between TAMs and PMos included lower expression of CD11c, MHC II, CD86, and PD-L1 and higher expression of CD64, CD68, F4/80, and PD-L1. Furthermore, we found that although PMos and TAMs have similar capacity to take up TAAs during tumor progression, their TAA-uptake kinetics differ significantly following radiotherapy. Interestingly, uptake of TAA in Mo-MDSCs were found negligible in progressing tumors and following radiotherapy. The phagocytic capacity of PMos and their proximity to vasculature (149) indicates a significant role of PMos in drug delivery of systemic treatments, including intravenously administered nanoparticles.
Additionally, we explored markers commonly used to describe myeloid cells as type 1 or type 2 and found interesting differences and similarities across cancer models. The checkpoint inhibitor PD-L1 was found mainly expressed by TAMs and PMos, which is in line with previous studies (24,150). Interestingly, the expression of PD-L1 was found to be extremely low in J558, Renca, and LL/2. CD86 was mainly associated with MHC II^{high} TAMs and PMos but also showed distinct patterns based on tumor model. Furthermore, CD86^{+} myeloid cells were associated with TAA-uptake. Following radiotherapy, we observed a higher increase in CD86 on TAMs in radiosensitive models (~2-fold) compared to non-responsive models. In contrast to CD86, CD80 was more broadly expressed across myeloid subsets and less specifically with MHC II^{high} expression. Likewise, we investigated the traditional type 2-associated markers, CD163 and CD206, across myeloid subsets in the TME. Studies continue to use CD163 and CD206 interchangeably to define type 2 TAMs but the markers have been criticized for not being specific to type 2 phenotypes. Furthermore, CD163^{+} TAMs and CD206^{+} TAMs are separate subsets of TAMs and correlate separately with patient survival (23,133). Notably, we found CD206 predominantly expressed by PMos rather than TAMs. CD163 expression was low in most models, irrespectively of cT and TAM infiltration. Thereby, our results support the notion that functional markers, e.g. CD86 and arginase-1 expression, are more relevant for evaluation of cancer therapy.

In summary, we have performed a comprehensive characterization of the myeloid TME in a broad selection of murine syngeneic cancer models commonly used to evaluate anti-cancer therapies. This work provides comparative data that could assist in selecting relevant models as well as providing foundation for relevant analysis methods in anti-cancer therapy development and evaluation.
Supplementary Information

Supplementary text

Classical Dendritic Cells
Tumor-associated cDCs control T cell activation and licensing by providing co-stimulatory or suppressive signals and presenting tumor-associated antigen (TAA). The level of T-cell interacting molecules expressed by the cDCs (e.g. CD86, PD-L1 and IL-12) dictate whether DCs facilitate or suppress T cell responses (151). Previous clinical studies have focused on tumor-infiltrating monocyte-derived DC signatures in patient TMEs for cDC infiltration. Hindering correct interpretation of the cDC anti-cancer potential (58). Two main subsets, cDC1 and cDC2, have been defined, of which cDC1s are the most significant in anti-tumor immunity and is associated with response to immunotherapy (57,58). cDC1s were defined as CD45+ CD11c^{high} MHC II^{high} CD11b^{low}, in accordance with literature (152,153).

Polymorphnuclear Myeloid-Derived Suppressor Cells
PMN-MDSCs are a subpopulation of tumor-associated neutrophils (TANs) with an immature and pro-tumorigenic phenotype. However, consensus on classification to differentiate anti-tumorigenic from pro-tumorigenic TANs has not been established (31). Suppression of T cell function is mediated through production of arginase-1 and endothelial nitric oxide synthase (eNOS). eNOS produces peroxynitrites that together with arginase-1 leads to arginine starvation and inhibition of protein function. Ultimately, these factors cause apoptosis, inhibition of proliferation, and impairing T cell interaction with MHC molecules (74,154). In mice, PMN-MDSCs are characterized as CD11b^{+} Ly6c^{int} Ly6g^{+} (31).

Monocytes
Monocytes have a diverse functions, including phagocytosis, cytokine production, and de novo differentiation into macrophages and DCs upon entering tissues (145). Two primary subsets exist in mice and humans: Classical/inflammatory and non-classical/patrolling monocytes (145). Recent literature demonstrates a more complex function of monocytes beside the classical paradigm of monocytes as sources of tissue macrophages following excavation into tissue. Non-differentiating monocytes excavate, take up antigen, and transport it to lymph nodes or even prime T cells without differentiating into macrophages.
Upon uptake of antigen, monocytes upregulate the co-stimulatory markers CD86 and MHC II but does not upregulate maturation markers like CD11c (65).

In cancers, the inflammatory subset is the best described and referred to as Mo-MDSCs based on their potent immunosuppressive activity (31). Inhibition of T cells are mediated by both arginase-1 and inducible NO synthase (iNOS). iNOS produces NO that together with arginase-1 metabolizes arginine from the TME and inhibits T cells by mechanisms including non-specific downregulation of IL-2R on T cells (74,155).

PMos role in cancer have only recently been explored. In circulation they patrol vasculature and respond to injury or tumor endothelium with upregulated CX3CL, ligand of CX3CR1. Studies have shown their protective role by preventing tumor formation in the lungs from intravenously injected B16-F10 cancer cells due to their high phagocytic ability and potent ability to recruit NK cells (146–148). However, PMos may also exert suppressive effects in the TME by secretion of IL-10 and CXCL5, inducing regulatory T cells and recruits PMN-MDSCs, respectively (145,149). Both monocytic subsets express CD11b, are Ly6g−, and are further defined as Ly6c<sup>high</sup> and Ly6c<sup>c</sup>−CX3CR1<sup>high</sup> for Mo-MDSCs and PMos, respectively (145).

**Macrophages**

TAMs are involved in phagocytosis of tumor cells, antigen-presentation of TAA, and production of soluble factors (e.g. iNOS, TGF-β, IFN-γ, and IL-12) (18,143,156). Furthermore, expression of surface markers such as CD86 and PD-L1 influences T cell function towards cancer recognition or evasion, respectively (5). Clinically, infiltration of TAMs (based on CD68) is a positive prognostic marker in colorectal cancer but is generally a negative prognostic marker in several other cancer types (17). The regulating effects of TAMs on anti-cancer treatments is well-studied. For instance, high TAM infiltration have been shown to reduce the effect of αPD-1 checkpoint inhibitor therapy clinically and preclinically (18–21). Synergistic effect can be observed when combining αPD-1 that promotes cytotoxicity of T cells with αCSF1R that blocks proliferation, differentiation, and survival of macrophages (19).

TAMs are described in literature with huge discrepancies contributed to phenotypic differences in macrophage subsets that overlaps phenotypically to monocyte and cDC2 markers. For instance, macrophages and cDC2s shares expression of CD11b,
CD11c, MHC II, CD68, and F4/80 (127,132,142,146). Based on transcriptional phenotyping and FLT3−/− mice TAMs may be defined as CD11b+ CD11c+ (156). Likewise, we defined TAMs as CD11b+ CD11c+ as in (18,126,156).

In summary, myeloid cells in the TME are of high interest regarding both tumor progression and treatment efficacy based on the previously described properties. Further characterization of preclinical cancer models bridges the gap between preclinical and clinical treatment responses and further develops the understanding of cancer models.

**Supplementary Figure S1**

**A** t-SNE projection of tumor samples

**B** Cell density in investigated cancer models as determined by flow cytometric counting. Shown is mean ± SD, n = 3-9.

**Supplementary Figure 1.** (A) t-SNE analysis of data used to generate Figure 2B and Figure 3A. (B) Cell density in investigated cancer models as determined by flow cytometric counting. Shown is mean ± SD, n = 3-9.
Supplementary Figure S2

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Supplementary Figure S2. Gating strategy used in Figure 1, 2, and 4. All populations were gated on time, singlets, scatter, viable, CD45+. B cells were defined as CD19-. PMN-MDSCs were defined as CD19-CD11b-CD11c-Ly6g+. Mo-MDSCs were defined as CD19-CD11b+CD11c-Ly6g Ly6c<sup>high</sup> CX3CR1+. PMos were defined as CD19-CD11b+CD11c-Ly6g Ly6c CX3CR1<sup>+</sup>. TAMs were defined as CD19-CD11b+CD11c-Ly6g I-A/I-E<sup>+</sup>. cDC1s were defined as CD11c<sup>high</sup>CD11b<sup>-</sup>.
Supplementary Figure S3. Kendall correlations between average of cell populations and log2 transformed, median scaled average cytokine expression levels across all cancer models. Corresponds to Kendall ranking correlation scores shown in correlation heatmap in Figure 1D.
Supplementary Figure S4

Supplementary Figure S4. (A) CD64 expression overlaid on a dot plot where PMos and Mo-MDSCs could be gated, demonstrating that PMos may also be gated based on CD64 expression instead of CX3CR1. (B) Representative dot plot overlays showing myeloid populations from Figure 5D overlaid with mCherry expression on I-A/I-E and CD86 displaying dot plots. For (A-B), blue denotes minimal, green low, yellow intermediate, red high fluorescent signal.
Supplementary Figure S5. Gating strategy for identification of mCherry+ myeloid cells. (A) Representative dot plot overlays showing myeloid populations from Figure 5D overlaid with mCherry expression on I-A/I-E and CD86 displaying dot plots. Blue denotes minimal, green low, yellow intermediate, red high fluorescent signal. (B) Gating strategy for Figure 5B-D. All populations were gated on time, singlets, scatter, viable, CD45+. PMN-
MDSCs were defined as CD11b⁺ CD11c⁻ Ly6c⁺ Ly6g⁻. Mo-MDSCs were defined as CD11b⁺ CD11c⁺ Ly6c⁻ Ly6g⁻ Ly6c<sup>high</sup>. PMos were defined as CD11b⁺ CD11c⁺ Ly6g⁻ Ly6c⁻ CX3CR1<sup>+</sup>. TAMs were defined as CD11b⁺ CD11c⁻ CD64<sup>high</sup>. cDC1s were defined as CD11c⁺ CD64<sup>low</sup> CD11b⁻ XCR1<sup>+</sup>. cDC2s were defined as CD11c⁻ CD64<sup>low</sup> XCR1⁻ CD11b⁻. (C) Gating strategy for Figure 5E. cDC1s were identified as viable CD45<sup>+</sup> Siglec-H⁻ CD64<sup>low</sup> CD11c⁺ CD11b⁻ XCR1<sup>+</sup>.

### Supplementary Table S1

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**Supplementary Table S1.** Information on cell lines and cancer models used in the study.
## Supplementary Table S2

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<td>Brilliant Violet 786</td>
<td>2 µg/ml</td>
<td>BD Biosciences</td>
<td>3, 4</td>
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<tr>
<td>CD86</td>
<td>GL-1</td>
<td>Alexa Fluor 488</td>
<td>5 µg/ml</td>
<td>BD Biosciences</td>
<td>3, 5</td>
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<tr>
<td>CX3CR1</td>
<td>SA011F11</td>
<td>FITC</td>
<td>6.67 µg/ml</td>
<td>BioLegend</td>
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<tr>
<td>CD3</td>
<td>17A2</td>
<td>FITC</td>
<td>2.5 µg/ml</td>
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<tr>
<td>I-A/I-E (MHC II)</td>
<td>M5/114.15.2</td>
<td>FITC</td>
<td>1.25 µg/ml</td>
<td>BD Biosciences</td>
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<tr>
<td>Ly6g</td>
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<tr>
<td>CD19</td>
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<td>Brilliant Blue 700</td>
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<td>BD Biosciences</td>
<td>4, 3</td>
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</tr>
<tr>
<td>CD163</td>
<td>TNKOPJ</td>
<td>PE</td>
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<td>Thermo Fisher</td>
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<tr>
<td>CD66 (FcyRI)</td>
<td>X54-5/7.1</td>
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<td>BD Biosciences</td>
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<tr>
<td>CD19</td>
<td>1D3</td>
<td>PE-Cy5.5</td>
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<td>Thermo Fisher</td>
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<td></td>
</tr>
<tr>
<td>CD64 (FcyRI)</td>
<td>X54-5/7.1</td>
<td>PE-Cy7</td>
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<td>BioLegend</td>
<td>3, 5</td>
<td></td>
</tr>
<tr>
<td>PD-1 (CD279)</td>
<td>J43</td>
<td>PE-Cy7</td>
<td>4 µg/ml</td>
<td>Thermo Fisher</td>
<td>1, 2</td>
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<td>PD-L1 (CD274)</td>
<td>10F.9G2</td>
<td>PE-Cy7</td>
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<tr>
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<td>H194-112</td>
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<tr>
<td>CX3CR1</td>
<td>SA011F11</td>
<td>APC</td>
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<td>BioLegend</td>
<td>3, 5</td>
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</tr>
<tr>
<td>Ly6c</td>
<td>AL-21</td>
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<tr>
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<td>M1/70</td>
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<td>CD45</td>
<td>30-F11</td>
<td>Alexa Fluor 700</td>
<td>1 µg/ml</td>
<td>BD Biosciences</td>
<td>1, 2, 3, 4, 5</td>
<td></td>
</tr>
<tr>
<td>Amines (dead cells)</td>
<td>-</td>
<td>eFluor 780 Viability Dye</td>
<td>1:400</td>
<td>Thermo Fisher</td>
<td>1, 2, 3, 4, 5</td>
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</table>

**Supplementary Table S2.** Flow cytometry panels used in the present study. For panel 1 and 2, FMO controls was performed for all parameters in 3 tumor lines. For panel 3, FMO controls for mCherry was performed using wildtype B16-F10 tumors. For panel 4, FMO controls was performed for all parameters in CT26, a CD68-FMO was performed in both CT26 and MC38, and an CD68-FMO-isotype was performed in CT26.
Chapter 4 – Manuscript II

Accelerated blood clearance in mice following repeated systemic dosing of high lipid dose PEGylated liposomes containing TLR agonists

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Abstract

Systemic administration of toll-like receptor (TLR) agonists has demonstrated impressive preclinical results as anti-cancer therapy due to their potent innate immune stimulatory properties. Their clinical advancement has, however, been hindered by severe adverse effects due to systemic activation of the immune system. Liposomal drug delivery systems may modify biodistribution, cellular uptake, and extend blood circulation. Thereby potentially make systemically administered TLR agonists tolerated at therapeutic doses.

In this study, we investigated potential barriers for administration of TLR agonists in PEGylated liposomes in regards to liposome formulation, TLR agonist, administration route, administration schedule, biodistribution, blood clearance, and anti-PEG antibodies. We found that TLR agonists formulated in PEGylated liposomes are likely recognized as a type 1 T cell-independent antigen by B cell. This led to high anti-PEG antibody titers which upon multiple intravenous administrations led to accelerated blood clearance and acute hypersensitivity reactions. The latter was found to be associated with anti-PEG IgG and not anti-PEG IgM opsonization. This study highlights the need to carefully design and evaluate
nanoparticular immunotherapy delivery systems as their activation of the immune system may challenge their therapeutic application.

**Introduction**

Cancer immunotherapy has brought new hope for cancer therapy after the potential of harnessing the body’s own immune response to combat cancer has been demonstrated. Innate activating cancer immunotherapy aims at stimulating the immune system to mount an inflammatory recognition of the cancerous tissue. One way to achieve this is to activate the pattern recognition receptors (PRR) of the innate immune system. Upon activation PRRs induce production of inflammatory cytokines such as type I interferons and tumor necrosis factor α that have anti-viral and anti-tumor effects (157). The most studied PPRs are toll-like receptors (TLRs). Currently, three TLR agonists are approved by the U.S. Food and Drug Administration as anti-cancer drugs: Bacillus Calmette Guérin, Monophosphoryl lipid A, and Imiquimod. All are administered locally at the tumor-site or as adjuvants in anti-cancer vaccines (158). However, local administration can be challenging in the clinic as the tumor-site is not always accessible for injection or disease may be disseminated and require a very large number of injections. Systemic administration of TLR agonists for anti-cancer therapy has been performed in clinical studies. Unfortunately, the maximally systemically tolerated doses induced none or limited therapeutic effect (107,159).

Toxicity associated with systemic administration can potentially be reduced by applying drug delivery systems that alter the biodistribution and pharmacokinetics of the drug. Polyethylene glycol (PEG) decorated liposomes have shown tumor accumulating properties in both clinical and translational studies (113). PEGylation of the liposome increases the circulation time of the drug (160) and allows the PEGylated liposomes to accumulate in tumors due to the enhanced permeability and retention effect (161). However, repeated dosing of PEGylated liposomes leads to accelerated blood clearance, which has been shown in mice, rats, rabbits, dogs, minipigs, and monkeys (162–167). Here, marginal zone (MZ) B cells in the spleen recognizes the PEG moiety on liposomes as a type 2 T cell-independent (TI-2) antigen, leading to production of anti-PEG IgM and subsequent opsonization and clearance of liposomes from circulation (168–170). Previous studies have shown that increasing the lipid dose of the initial liposomal injection reduces accelerated blood clearance (171,172). Thus, indicating that high lipid doses induces tolerance or anergy.
in MZ B cells. However, most research regarding accelerated blood clearance of liposomes have been performed using lipid doses with limited translational value or without liposomal cargo (172,173).

To potentially overcome systemic side-effects and thereby improve the therapeutic window for TLR agonists, we formulated TLR agonists in different PEGylated liposomal carriers. For translational purposes, we administered liposomes in high lipid doses to reach relevant therapeutic TLR agonist doses. However, we observed acute hypersensitivity following repeated administration that did not occur in response to the initial administration. Subsequently, we investigated whether this was caused by accelerated blood clearance of liposomes upon repeated dosing and whether the benefit of administering high lipid doses also applies to therapeutic liposomes that contains TLR agonists.

**Materials and Methods**

**Materials for liposome preparation**

Unless, otherwise stated, all chemicals were acquired from Sigma Aldrich. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG2000) and cholesterol were acquired from Lipoid (Germany). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]) (DOPE-PEG2000), dioleoyl-3-trimethylammonium-propane (DOTAP) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG) were acquired from Avanti Polar Lipids (AL, US). 1V270 (also known as TMX-201) was synthesized by Corden Pharma (Germany) and Chimete (Italy). Gardiquimod was acquired from Invivogen (CA, US). FITC-5’-CpG-3’-Cholesterol oligodeoxynucleotides (ODN) were purchased from Eurofins Genomics (Germany). Formulation and characterization of liposomes is further described in supplementary material and methods and summarized in supplementary table 1 and 2.

**Mice**

BALB/cJRj mice (Janvier) mice ages 8-13 weeks old were used. Mice were subjected to at least 1 week of acclimation upon arrival and were kept under controlled environmental conditions (constant temperature, humidity and 12:12h light-dark cycle). All experimental
procedures were approved by the institutional ethical board and the Danish National Animal Experiment Inspectorate.

**Liposome treatments**
Liposomes were administered intravenously or subcutaneously and administered either q1d or q4d. Mice were monitored for up to an hour after administration to assess signs of acute hypersensitivity. Additional information on administration of liposomes can be found in Table 1 and 2 and extended information can be found in Supplementary Table 1 and 2.

**Efficacy studies**
The colorectal cancer cell line CT26 was obtained from ATCC and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM Glutamax, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO₂ in a humidified atmosphere.

For inoculation, mice were anesthetized with 3-5% sevoflurane and inoculated with 3x10⁵ CT26 cells in unsupplemented media in the right flank. Tumor volume was measured with calibers as length x width² / 2 and randomized based on tumor volume. Tumors were allowed to grow to a mean size of 95 mm³. Liposome treatments were given intravenously. Mice receiving radiotherapy (RT), were irradiated under lead-shielding exposing the right flank at a dose rate of 1.0 Gy/min in a X-RAD 320 (PXi).

**Liposome treatments and blood sampling for anti-PEG IgM and IgG determination**
Mice were randomized according to weight. All liposome formulations were administered in a q4d schedule for a total of three injections (day 0, 4 and 8). Liposomes were administered intravenously in tail vein or subcutaneously in the neck of the mouse. Blood samples were collected in hirudin blood tubes (Roche) 1 h prior the third administration and 5 min after. Samples were centrifuged at 2000 g for 15 min a 4°C and resulting plasma fractions were collected and stored at -80°C until ELISA analysis.

**ELISA procedure**
The ELISA procedure were adapted from (174). Briefly, 96-well plates were coated with 20 nmol DOPE-PEG in 99.9% ethanol (100 µL) overnight. Plates were washed three times with washing buffer (50 mM Tris, 0.14 M NaCl, 0.05% CHAPS). Subsequently, plates were
blocked in blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA) for 1 h at room temperature (RT) and washed washing. For the anti-PEG IgM assay: Standards were prepared using pooled plasma samples from mice injected with DOPE-PEGylated stealth-like 1V270 liposomes on day 0 and 4. Plasma was collected on day 8 and diluted 100 fold with blocking buffer followed by 2-fold serial dilution series to create a standard curve (256, 128, 64, 32, 16, 8, 4, 2, 1 arbitrary units; A.U./ml). For the anti-PEG IgG assay: A standard was prepared from purified mouse IgG1k anti-PEG (clone 5D-3; Abcam) diluted in a 2-fold serial dilution series from 17,920 to 70 pg/ml. Plasma samples (diluted 1:400 in blocking buffer) and standards (100 µl volume) were incubated for 1 h at RT. Plates were subsequently washed 5 times and added 100 µl detection antibodies and incubated for 1 h at RT. Bound IgM was detected using 100 ng/ml HRP-conjugated goat anti-murine IgM (Bethyl Laboratories, US) in blocking buffer. Bound IgG was detected using 100 ng/ml HRP-conjugated goat anti-murine IgG (Jackson Immunoresearch Europe) blocking buffer. Plates were washed 5 times and developed using 100 µl SigmaFast OPD (Sigma Aldrich) substrate for 9 min (IgM) or 25 min (IgG). The reaction was stopped by the addition of 100 µl 2 M sulfuric acid. Absorbance was measured at 490 nm (target) and 800 nm (reference) on a FluoStar Omega instrument (BMG Labtech). A non-linear 4 parameter regression was done in Prism software (Graphpad).

**Biodistribution study**

Radiolabelling of liposomes was conducted as previously described (175). Briefly, 1.4 mL of each set of liposomes (empty DOPE-PEGylated stealth-like, 1V270 DOPE-PEGylated stealth-like, 36mM) was added to 180 MBq dry $^{64}$CuCl$_2$, and the samples were heated and stirred at 55°C for 75 min. The samples were equilibrated at room temperature, and 2 µl was spotted for Radio-TLC and 50 µl was pipetted onto pre-equilibrated PD10 columns. Radio-TLC were performed on silica gel 60 F254 plates (Merck) with 5% (w/v) NH$_4$OAc in H$_2$O-MeOH (1:1) as eluent, and the PD10 columns were eluted and equilibrated by ISO-HEPES (150 mM NaCl, 10 mM HEPES, pH 7.4). Both the empty PEGylated stealth-like and 1V270 PEGylated stealth-like formulation displayed >95% loading on PD10. Radio-TLC further showed that the empty DOPE-PEGylated stealth-like ($^{64}$Cu-lip) and 1V270 DOPE-PEGylated stealth-like ($^{64}$Cu-lip-1V270) liposome formulation contained 0.6% and 3.1%
unloaded $^{64}$Cu$^{2+}$ respectively. $^{64}$Cu-lip had an activity concentration of 94.4 MBq/ml and $^{64}$Cu-lip-1V270 an activity concentration of 98.4 MBq/ml.

To investigate biodistribution and blood clearance on the first and third administration with or without liposomal 1V270, mice were randomized based on weight into four groups. Liposomes were administered q4d. Groups: 1) 1st injection with $^{64}$Cu-lip-1V270, 2) 1st injection $^{64}$Cu-lip, 3) 1st and 2nd injection with non-labelled 1V270 PEGylated stealth-like liposomes and 3rd injection with $^{64}$Cu-lip-1V270 4) 1st and 2nd injection with non-labelled empty PEGylated stealth-like liposomes and 3rd injection with $^{64}$Cu-lip. PET/CT was performed on a small animal PET/CT system (Inveon®, Siemens Medical Systems, USA). Mice were anesthetized using 3-5% sevoflurane. PET images were acquired over 5 min (10 min, 2 h, 24 post administration) or 10 min (48 h post administration) followed by a CT scan (tube setting: 70 kV, 500 µA, 350 ms exposure time, 0.21x0.21x0.21 mm pixel size). PET data were arranged into sinograms and reconstructed using a maximum posteriori (MAP) reconstruction algorithm (0.388x0.388x0.796 mm pixel size) with attenuation corrected based on the corresponding CT scan. Image analysis was performed in Inveon Software (Siemens Medical System). Region of interests (ROIs) were manually constructed on co-registered PET/CT images. ROIs were drawn on heart, spleen, kidney, liver, muscle, and entire mouse. Blood activity was estimated from a ROI covering the heart and subsequently segmented to only include the voxels above 80% of the maximum activity in the original ROI. $^{64}$Cu liposome activity was as decay-corrected, subtracted with muscle activity, and reported as % injected dose per gram (%ID/g).

**Antibody transfer**

Mice were intravenously administered DOPE-PEGylated stealth-like 0.75% 1V270 liposomes (200 nmol 1V270) or empty DOPE-PEGylated stealth-like liposomes on day 0 and 4. On day 8, blood was collected by cardiac puncture in hirudin blood tubes. Plasma was obtained by centrifugation at 2000 g for 15 min at 4°C. Total IgG was purified from plasma with the Nab Protein A/G spin kit (Thermo Fisher) according to the manufacturer’s recommendations. The unbound fraction was processed with the NAb protein L spin kit (Thermo Fisher Scientific) to purify remaining antibodies with kappa chains including IgM’s. The antibody fractions was analyzed by size exclusion-high performance liquid chromatograph (SEC-HPLC) on a Yarra™ 3 µm SEC-3000 Column 300 x 7.8 mm
(Phenomenex) with 0.1 M phosphate buffer pH 7 as mobile phase on an isocratic method with a flow of 1 ml/min. The sample was analyzed at 280 nm (Supplementary Fig. S1). Zeba spin desalting columns (Thermo Fisher Scientific) were used to exchange the buffer to sterile PBS. Antibody solutions were upconcentrated using Amicon Ultra-4 Centrifugal Devise to obtain a final volume of 50 µL. IgG or IgM from mice receiving stealth-like 1V270 liposomes or empty stealth-like liposomes were given to naïve mice intravenously, followed by intravenous injections of PEGylated stealth-like 1V270 liposomes (200 nmol 1V270) 10 min later. Mice were subsequently monitored for hypersensitivity symptoms.

Inhibition of platelet-activating factor
Mice were administered intravenously twice with DOPE-PEGylated stealth-like 0.75% 1V270 liposomes in a q4d schedule. 5 min prior to the third administration, mice were given 50 µg CV6209 (platelet-activating factor, PAF, antagonist) by intraperitoneal injection.

Statistical analyses
Statistical analyses were performed using Prism 8. The appropriately used statistical test is stated in figure legends. A p-value ≤ 0.05 was considered statistically significant.

Results
PEGylated liposomes containing TLR agonists induce a hypersensitivity reaction upon repeated administration
To investigate if PEGylated liposomes containing a TLR7/8 agonist could be intravenously administered safely, we evaluated repeated administrations of PEGylated anionic, PEGylated stealth-like, and PEGylated cationic liposome formulation containing 1V270 in mice. Liposomes were injected intravenously on day 0, 4, and 8 with 1V270 liposomes (2 µmol/kg) in lipid doses of 230-270 µmol/kg. 5-15 min after the third administration hypersensitivity symptoms were consistently observed in mice treated with the stealth-like and cationic 1V270 liposomes. Symptoms included inactivity, piloerection, eye squinting and hunched back. In contrast, the third injection of the anionic formulation lead to hypersensitivity symptoms in the majority of studies (in 8/11 studies). Notably, we observed loss of anti-cancer effect in studies where hypersensitivity occurred upon multiple administrations of DOPE-PEGylated anionic 1V270 liposomes (Supplementary Fig. S2A-B).
To prevent potential hypersensitivity reaction, liposome formulations were modified on multiple parameters, including 1V270 content (0.25, 0.75, 1.5 and 5 mol%), PEGylation, different lipid anchors for PEG (DOPE, DSPE, DSG and Cholesterol), different TLR agonists (1V270, gardiquimod, and CpG), schedule (q1q and q4d) and delivery route (subcutaneous and intravenous). See Table 1 for liposomes administered intravenously and Table 2 for liposomes administered locally. Varying 1V270 content did not prevent the hypersensitivity, nor did changing the lipid anchor to DSPE or DSG, but the cholesterol anchor did not induce hypersensitivity. Empty variations of PEGylated liposomes did not induce hypersensitivity. Additionally, no hypersensitivity was observed for daily administered DOPE-PEGylated anionic 1V270 liposomes and q4d-administered non-PEGylated anionic 1V270 liposomes. However, no anti-cancer effect was observed for these treatments (Supplementary Fig. S1C). Hypersensitivity was also observed when liposomes were formulated with CpG (TLR9 agonist) or gardiquimod (TLR7 agonist). Based on these results, the remainder of the studies were conducted using DOPE-PEGylation and 0.75% 1V270, unless stated otherwise.

Formulations that gave rise to hypersensitivity did so with different severities, for instance PEGylated stealth-like 1V270 liposomes induced more severe symptoms than PEGylated anionic 1v270 liposomes. These results show that PEGylated liposomes containing TLR agonists can induce hypersensitivity when administered intravenously every fourth day and that the majority of these formulations did induce hypersensitivity. This also indicates that the anti-cancer effect is attenuated when PEGylated TLR agonist liposomes induce hypersensitivity.

<table>
<thead>
<tr>
<th>Intravenously administered liposomes</th>
<th>Dosing schedule</th>
<th>Hypersensitivity</th>
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</thead>
<tbody>
<tr>
<td><strong>Anionic formulations</strong></td>
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<td></td>
</tr>
<tr>
<td>Anionic DOPE-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>q1d × 10</td>
<td>No</td>
</tr>
<tr>
<td>Anionic DOPE-PEG 5% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Anionic DSPE-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>q1d × 5</td>
<td>No</td>
</tr>
<tr>
<td>Anionic DSPE-PEG 5% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Anionic DSPE-PEG 0.75% 1V270 (10% POPG)</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Anionic DSPE-PEG 0.75% 1V270 (30% POPG)</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Anionic DSPE-PEG 1.5% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Anionic DSPE-PEG 0.25% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
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</table>
### Table 1. Overview of liposomal formulations administered intravenously and investigated for acute hypersensitivity reaction. Liposomes were administered in indicated dosing schedule. Subsequently, mice were monitored for symptoms of acute hypersensitivity. Additional information on liposomal formulations and administered doses can be found in Supplementary Table 1.

<table>
<thead>
<tr>
<th>Anionic formulations</th>
<th>Dosing schedule</th>
<th>Hypersensitivity</th>
</tr>
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<tbody>
<tr>
<td>Anionic non-PEG 0.75% 1V270</td>
<td>q4d × 5</td>
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</tr>
<tr>
<td>Anionic DSG-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Anionic Chol-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>No</td>
</tr>
<tr>
<td>Anionic DOPE-PEG empty</td>
<td>q4d × 3</td>
<td>No</td>
</tr>
<tr>
<td>Anionic DOPE-PEG Gardiquimod</td>
<td>q4d × 3</td>
<td>No</td>
</tr>
<tr>
<td>Anionic DSPE-PEG Gardiquimod</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Anionic DSG-PEG Gardiquimod</td>
<td>q4d × 3</td>
<td>Yes</td>
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<table>
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<tr>
<th>Stealth-like formulations</th>
<th>Dosing schedule</th>
<th>Hypersensitivity</th>
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<tbody>
<tr>
<td>Stealth-like DOPE-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Stealth-like DSG-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Stealth-like DOPE-PEG 5% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Stealth-like non-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>No</td>
</tr>
<tr>
<td>Stealth-like PEG empty</td>
<td>q4d × 3</td>
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<th>Stealth formulations</th>
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<tr>
<td>Stealth DSPE-PEG Gardiquimod</td>
<td>q4d × 3</td>
<td>Yes</td>
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<tr>
<td>Stealth DSPE-PEG CpG ODN</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Stealth DSPE-PEG non-CpG ODN</td>
<td>q4d × 3</td>
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<th>Cationic formulations</th>
<th>Dosing schedule</th>
<th>Hypersensitivity</th>
</tr>
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<tbody>
<tr>
<td>Cationic DOPE-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
</tr>
<tr>
<td>Cationic non-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>Yes</td>
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</table>

### Table 2. Overview of liposomal formulations administered subcutaneously in the neck of mice and investigated for acute hypersensitivity reaction. Liposomes were administered in indicated dosing schedule. Subsequently, mice were monitored for symptoms of acute hypersensitivity. Additional information on liposomal formulations and administered doses can be found in Supplementary Table 2.

<table>
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<th>Subcutaneously administered liposomes</th>
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<tr>
<td>Anionic DOPE-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>No</td>
</tr>
<tr>
<td>Anionic non-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>No</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Cationic formulations</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic DOPE-PEG 0.75% 1V270</td>
<td>q4d × 3</td>
<td>No</td>
</tr>
</tbody>
</table>
Rapid clearance of anti-PEG antibodies from circulation following administration of liposomes is associated with hypersensitivity

The aforementioned findings regarding different PEGylated liposomal formulations result in varying hypersensitivity severity and that previous studies have observed anti-PEG antibodies prior to hypersensitivity reaction (176) led us to investigate whether differences in liposomal formulations that contain 1V270 affects the induced anti-PEG antibody levels. To this end, we investigated intravenously administered formulations including PEGylated stealth-like, cationic, and anionic liposomes containing 1V270. For anionic liposomes the PEG anchor and 1V270 content was varied to investigate how it affected anti-PEG antibody induction. Additionally, subcutaneous injections were investigated as a control for slower systemic availability and subsequently slower liposomal opsonization. All formulations were administered in a q4d schedule. Anti-PEG antibodies were determined from blood samples collected 1-2 h prior to the third liposomal administration and 5 minutes after administration.

Anti-PEG antibodies were detected for all liposomal formulations containing 1V270 regardless of investigated composition. Following intravenous administration of liposomes, anti-PEG antibodies were rapidly cleared from circulation, which also corresponded to observed hypersensitivity reaction. In contrast, minimal antibody production could be detected for empty liposomes (Figure 1A). Although subcutaneously administered liposomes containing 1V270 did induce anti-PEG, clearance of antibodies upon third administration occurred at a reduced rate and was not associated with hypersensitivity reaction (Figure 1B). These results demonstrate that addition of a TLR7/8 agonist to PEGylated liposomes induces generation of anti-PEG IgM and IgG and rapid clearance of these antibodies from circulation is associated with hypersensitivity.
Figure 1. Liposomes containing 1V270 induces anti-PEG IgM and anti-PEG IgG. Mice were administered intravenously (A) or subcutaneously (B) with different liposome formulations on day 0 and 4. Formulations further varied in PEGylation and 1V270 content. Formulations containing 1V270 was administered as 2.0 µmol/kg per administration. Plasma was collected on day 8 and anti-PEG IgM and anti-PEG IgG determined by ELISA. Formulations that induced acute hypersensitivity reaction after the third administration on day 8 are indicated on the graph by “+” and formulations that did not is indicated by “−”. Shown is mean ± SD. A.U. = arbitrary units.
Next we sought to determine whether anti-PEG IgM or anti-PEG IgG is linked to the observed hypersensitivity reaction following repeated administration of PEGylated stealth-like liposomes with and without 1V270. The former formulation having previously resulted in acute hypersensitivity upon the third administration in a q4d schedule. Mice were administered liposomes on day 0 and 4. Cardiac blood was collected on day 8 from which total IgG and IgM fractions were isolated using purification columns. Subsequently, the respective immunoglobulin fractions were administered into naïve mice followed by administration of PEGylated liposomes with or without 1V270 10 minutes later. Naïve mice receiving IgG fractions from mice pretreated with PEGylated liposomes containing 1V270 developed hypersensitivity 10-15 minutes after liposome administration. In contrast, naïve mice receiving the IgM fraction did not develop any signs of hypersensitivity. Likewise, no symptoms developed in naïve mice receiving the IgG or IgM fraction from mice pretreated with empty PEGylated liposomes.

Previous studies have shown that hypersensitivity occurring upon multiple administrations of PEGylated liposomes containing TLR9 agonists can be avoided by prophylactic treatment with platelet-activating factor (PAF) antagonists. This indicates the reaction to be IgG-mediated (176,177). Therefore, we investigated whether a PAF antagonist could prevent hypersensitivity following the third administration of PEGylated stealth-like 1V270 liposomes administered in a q4d schedule and found that pretreatment with a PAF antagonist did prevent hypersensitivity. Altogether, these results indicate that anti-PEG IgG mediates the acute hypersensitivity reaction in response to repeated administration of PEGylated liposomes containing TLR agonists.

**PEGylated liposomes containing 1V270 are subject to accelerated blood clearance**

Accelerated blood clearance of PEGylated liposomes has been demonstrated for empty PEGylated liposomes and PEGylated liposomes containing an immunogenic compound (e.g. TLR9 agonist) and that this is associated with the presence of anti-PEG antibodies (172,173,176). Therefore, we sought to investigate whether accelerated blood clearance occurs for repeated administrations of PEGylated stealth-like liposomes containing a TLR7/8 agonist. To this end, circulation and biodistribution were investigated on the first and third administration in a q4d schedule using PET/CT. Mice imaged by PET/CT on the third administration were pretreated twice with unlabeled PEGylated stealth-like liposomes. For
PET/CT, mice were intravenously administered $^{64}$Cu radiolabeled PEGylated stealth-like liposomes with 1V270 ($^{64}$Cu-lip-1V270) or without 1V270 ($^{64}$Cu-lip) on the PET/CT was performed 10 min, 2 h, 24 h and 48 h after injection.

Groups receiving $^{64}$Cu-lip displayed comparable circulation profiles on the first and third administration, thereby demonstrating that high lipid doses effectively abolished accelerated blood clearance for empty PEGylated liposomes. $^{64}$Cu-lip-1V270 displayed similar circulation profile on the initial administration compared to administrations of $^{64}$Cu-lip. In contrast, the third administration of $^{64}$Cu-lip-1V270 was subject to rapid elimination from circulation which was evident as early as 10 minutes post injection. Similarly, blood activity levels were significantly lower at the 2 hour and 24 hour PET/CT scans ($p \leq 0.05$, Figure 2A-B). In addition, increased uptake in the liver and spleen was evident after the third administration of $^{64}$Cu-lip-1V270 at the 2 h time point. Activity in the liver and spleen was lower at the 24 h and 48 h scans for the third administration of $^{64}$Cu-lip-1V270. Interestingly, an accumulation of $^{64}$Cu was observed at all imaged time points in the mouth and front limbs of mice following the third administration of $^{64}$Cu-lip-1V270 (Figure 2B). Altogether, these data demonstrate that administration of high lipid doses of liposomes circumvents the accelerated blood clearance effect, but encapsulation of immune agonists in liposomes (here a TLR7/8 agonist) may lead to accelerated blood clearance regardless of lipid dose.
Figure 2. Biodistribution and blood clearance of PEGylated stealth-like liposomes with and without 1V270 is subject to accelerated blood clearance. Mice were intravenously administered $^{64}\text{Cu}$ labelled DOPE-PEGylated stealth-like liposomes with or without 0.75% 1V270 on the first injection or on the third injection. The latter having received two unlabeled pretreatments that were administered q4d. The lipid dose administered was 270 µmol/kg for all formulations and the 1V270 dose was 2.0 µmol/kg for liposomes containing 1V270. PET/CT scans were acquired 10 min, 2 h, 24 h and 48 h after administration. (A) Show is mean %ID/g ± SD (n=4) for blood, spleen, and liver ROIs. (B) Representative PET/CT images, “1V270” indicates liposomes with 1V270, “empty” indicates liposomes without 1V270. Arrow points to the spleen and L indicates the position of the liver. Statistical significance was determined for blood using a two-way ANOVA with Tukey’s multiple comparisons test. Displayed comparisons are for 1V270 stealth-like liposomes on the third administration compared to all other administrations. *, p ≤ 0.05; ****, p ≤ 0.0001. p.i. = post injection.
Discussion

In the present study, we have shown that repeated administration of PEGylated liposomes containing a TLR agonist leads to accelerated blood clearance at high lipid doses (>40 µmol/kg). Accelerated blood clearance was associated with an acute hypersensitivity reaction and generation of both anti-PEG IgM and IgG. In accordance with previous studies in mice and rats, we did not see observe significant anti-PEG antibodies and accelerated blood clearance when administering empty PEGylated liposomes at high lipid doses (178,179). A previous study by Judge et al. also investigated the use of PEGylated liposomes containing a TLR agonist. Here plasmid DNA (pDNA; recognized by TLR9) was formulated in PEGylated liposomes for gene therapy, which was subject to accelerated blood clearance upon the second administration and was found to induce acute hypersensitivity within 5-10 minutes following administration. This manifested as lethargy, facial puffing, labored respiration 5-10 minutes after administration and was fatal in high doses (176). Although not yet demonstrated in mice as a timeseries experiment, studies in rats have shown that anti-PEG IgG titers following administration of empty PEGylated liposomes are barely produced at detectable levels and returns to baseline levels shortly after administration (170). In contrast, administration in mice of PEGylated liposomes containing pDNA induced anti-PEG IgG titers that remained at high levels beyond 28 days after administration and can cause acute hypersensitivity reaction at a dosing interval of 28 days (176). Additional other studies have also reported accelerated blood clearance after repeated administration of PEGylated liposomes containing pDNA (180,181). Semple et al. observed accelerated blood clearance after repeated dosing (4-6 days between) of PEGylated liposomes containing ODNs, pDNA, and ribozymes. They also reported morbidity after second dosing including difficulty bleeding and in extreme cases seizures and fatality (182). Likewise, we also experienced difficulty taking sublingual blood samples 15 min after third administration in mice receiving PEGylated stealth-like 1V270 liposomes.

Including the findings in this study, accelerated blood clearance after repeated dosing of PEGylated liposomes has now been shown for liposomes containing 1v270 (TLR7/8 agonist), gardiquimod (TLR7 agonist), CpG ODNs (TLR9 agonist), non-CpG ODNs, pDNA (TLR9 agonist), ribozymes, and siRNA (TLR3 agonist) at high lipid doses (176,180–182). Although non-CpG ODNs does not act as a TLR agonist, non-CpG ODNs can exert adjuvant activity (183). These observations clearly demonstrate that encapsulating an
immune agonist in PEGylated liposomes induces anti-PEG antibody generation, leading to accelerated blood clearance and hypersensitivity reactions when dosing repeatedly, ultimately resulting in lack of disease targeting. Additionally, this may also occur for alternatives to PEGylation and other nanoparticles. As several TLR agonists, including TLR7 agonists, can act as direct B cell mitogens affect antibody production and class switching (184–187), PEGylated liposomes containing such agonists are likely recognized as a TI-1 antigen. Here, the PEG chains on the liposomes binds to the BCR on specific B cells and is subsequently internalized and processed. During this process TLRs may recognize immune agonists in liposomes, leading to high production of anti-PEG IgM and IgG. However, immunogenicity studies in mice deficient in T cells (e.g. nude) and with defective B cells (e.g. xid) or with cell-specific deficiencies in TLR7-signalling would be necessary to confirm this (188–190). In contrast, empty PEGylated liposomes act as a TI-2 antigen (170,173,191) where the repetitive structures of PEG chains on the liposome surface crosslink multiple B cell receptors on the surface of specific B cells, causing activation and production of mainly anti-PEG IgM (173,191,192). At high antigen concentrations, i.e. as present when high lipid dose of empty PEGylates antibodies are administered, B cells become anergic due to continuous BCR stimulation without co-stimulatory signals, resulting in no anti-PEG IgM production (193,194).

The acute hypersensitivity reaction observed upon repeated administration of PEGylated liposomes with TLR agonist further demonstrates the problematic nature of encapsulating immune agonists in PEGylated liposomes (176,180,182). Judge et al. demonstrated that PAF antagonists could alleviate the acute hypersensitivity observed upon repeated administration with PEGylated liposomes containing a TLR agonist and hypothesized that the hypersensitivity was mediated by IgG opsonization (176). Particles opsonized by IgG can activate FcγRIII expressing macrophages and platelets that in response release PAF and may cause an anaphylactic reaction (177). In support of this, we found that a PAF antagonist could alleviate hypersensitivity in this study. Additionally, we found that transfer of IgG but not IgM from pretreated mice indicated a key role of anti-PEG IgG in mediating acute hypersensitivity upon repeated dosing of PEGylated liposomes with a TLR agonist. It may be speculated that the $^{64}$Cu signal we observed in the mouth and front legs of mice administered a third dose of 1v270 PEGylated stealth-like liposomes are depositions of IgG immune complexes. IgG mediated hypersensitivity to PEGylated
liposomes has not yet been demonstrated in humans but a clinical study with PEGylated RNA aptamers indicates that this may also occur in humans (195). Here, pre-existing anti-PEG IgG in patients caused severe allergic responses which terminated the study prematurely. The study also found correlation between anti-PEG IgG levels and severity of allergic responses. Importantly, anti-PEG antibodies have been reported in healthy individuals and are associated with non-responders (196,197), which further complicates the usage of PEGylated liposomes.

In summary, the present study demonstrated by PET/CT that PEGylated liposomes containing a TLR7/8 agonist is subject to accelerated blood clearance. Additionally, the liposomes induced high anti-PEG antibody titers and acute hypersensitivity upon repeated administrations. The latter of which was found to be associated to anti-PEG IgG. Ultimately, this study highlights the need to carefully consider the immunogenicity of nanoparticle compounds and comprehensively evaluate antibody responses generated against the nanoparticle.

**Acknowledgements**

The authors would like to thank Professor Tatsuhiro Ishida, Ph.D. for kindly sharing the full PEG-IgM and PEG-IgG ELISA procedure used in his publications.
Supplementary Information

Supplementary Materials and Methods

1V270 Liposomes formulation
Lipids were dissolved in tert-butanol:milliQ water (9:1), mixed to the desired lipid compositions in glass vials, snap-frozen in liquid nitrogen and freeze-dried overnight using a Scanvac Coolsafe lyophilizer (Labogene). Dry lipids were re-hydrated in PBS buffer (pH 7.4) to a concentration of 50 mM total lipid and placed stirring 65 °C for 1 hour. Size of liposomes was controlled by extruding one time through three stacked Whatman filters (GE Healthcare) with a pore size of 400 nm, 200 nm, and 100 nm, followed by seven extrusions through two stacked 100 nm Whatman filters. Extrusion was done at 20 bar nitrogen pressure using a 10 mL LIPEX thermobarrel pressure extruder on a heating block at 65 °C.

Gardiquimod liposome formulation
Liposomes with encapsulated gardiquimod were formulated following the same procedure as described above, with the exception that the dry lipids were rehydrated in an Ammonium Sulphate (150mM) buffer (pH 5.75) instead of PBS. After extrusion, the liposomes were left at 4°C overnight before proceeding with remote loading protocol. The liposomes were transferred to Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific) and dialyzed for 86 hours against a dialysis buffer (10% Sucrose, 25 mM HEPES, pH 7.4), with buffer exchanges after 18 hours and 26 hours. After dialysis, the phosphorus concentration was measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as described below. The liposomes were then transferred to new glass vials with gardiquimod in powder form, weighed out to achieve a final drug/lipid ratio of 1:10. The solution was left stirring at 55°C for 4 hours. Due to the high loading efficiency (>95%) the liposomes were used with no further purification.

Determination of gardiquimod loading efficiency
For measuring the encapsulation efficiency of gardiquimod, liposome-encapsulated gardiquimod was separated from free gardiquimod using PD10 Desalting Columns containing Sephadex G-25 resin (GE Healthcare), equilibrated in dialysis buffer. The loading percentage was calculated by comparing the gardiquimod concentration in the void volume from the column to the total gardiquimod concentration in a liposome before separation.
CpG liposome formulation
Liposomes were prepared using a stealth lipid mix (HSPC/DSPE-mPEG2k/Cholesterol: 3:1:1 w/w/w) as scaffold. Dry lipid mix was hydrated with a mixture of FITC-CpG-Chol and 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. Hydration was carried out at 70°C for 1 h under vigorous mixing of the sample. Liposomes were obtained by extruding the lipids 21 times through a 100 nm nucleopore membrane (Whatman) at 70°C using a mini extruder set (Avanti Polar Lipids). 500 µL of the crude Liposomes were purified using a self-packed Sephadex G100 superfine (GE Healthcare) column (250 mg, h 45 mm x d 10 mm) using 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl as eluent.

Determination of phospholipid concentration
The total lipid concentration of the gardiquimod liposome stocks was determined by measuring the phosphorus concentration using ICP-MS. Samples were first diluted 10,000 times in an ICP-MS diluent (2% HCl, 10 ppb Ga) to fall within a standard range of 25-100 ppb phosphorus. The phosphorus content was then measured on an ICAP-Q from Thermo Fisher Scientific. The total lipid concentration was calculated based on the assumption that 70% of the lipids in our formulations contain a phosphorus atom (50% for DOTAP liposomes). For 1V270 formulations, the total lipid concentration was estimated based on the TMX201 concentration measured by HPLC, which was in agreement with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) measurements.

Determination of 1V270, gardiquimod and CpG concentration
Concentration of 1V270 and gardiquimod was measured using analytical reversed-phase (RP)-HPLC on a LC-20AD liquid chromatograph (Shimadzu Corporation) equipped with a DGU-20A SR degassing unit and a SIL-20AC HT autosampler. The HPLC Eluent A consisted of a 5% CH₃CN aqueous solution with 0.1% trifluoroacetic acid; HPLC Eluent B consisted of 0.1% TFA in CH₃CN. 1V270 liposomes were diluted 5 times in PBS, and compared to a 1V270 standard (50-200µM diluted in DMSO). A XBridge C8 (5 µm, 4.6 x 150 mm) column (Waters) was used and quantification of 1V270 was done using UV detection at 280 nm with an SPD-M20A Photodiode Array Detector fitted with a Deuterium Tungsten lamp (Shimadzu Corporation). A gradient from 40% to 100% B over 15 minutes
was applied (flow rate 1 mL/min). Gardiquimod liposomes were diluted 4 times in dialysis buffer and compared to a gardiquimod standard (25-100 µg/mL diluted in MQ). A Waters XTerra C18 (5 µm, 4.6 x 150 mm) column (Waters, MA, US) was used for measuring Gardiquimod and quantification was done using the same detector as for 1V270 at 320 nm. Quantification was done with a gradient from 0% of 100% B over 15 minutes (flow rate 1 mL/min). The ODN content of CpG liposomes was determined by measuring the fluorescence (ex: λ = 490 nm; em: λ = 525 nm) in comparison to free FITC-CpG-Chol (+/-) in triplets using a Spark® (Tecan) plate reader. A 20-fold dilution of purified ODN-bearing liposomes with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl was used.

**Sterile filtering**

Before usage, the liposomes were diluted to a final drug concentration, so the desired dose could be reached by injection 150 µl. 1V270 liposomes were diluted in PBS buffer, gardiquimod liposomes were diluted in the dialysis buffer. Finally, the liposomes were sterile filtered through 0.45 µm syringe filters (Frisenette) and transferred to sealed sterile vials.

**Liposome characterization**

Total lipid concentration of the liposome stocks was determined by measuring the phosphorus concentration using ICP-MS. Samples were diluted 10,000 times in an ICP-MS diluent (2% HCl, 10 ppb Ga) to fall within a standard range of 25-100 ppb phosphorus, and the phosphorus content was measured on an ICAP-Q (Thermo Fisher Scientific). Lipid concentration was calculated based on the assumption that 70% of the lipids in our formulations contain a phosphorus atom (50% of the lipids for the DOTAP liposomes). The hydrodynamic diameter and polydispersity index (PDI) of the liposomes were measured by dynamic light scattering using a ZetaSizer Nano ZS (Malvern Instruments), equipped with a 633 nm laser. The liposomes were diluted to about 120 µM total lipid in the buffer the liposomes were formulated in (PBS buffer for 1V270 liposomes, dialysis buffer for the Gardiquimod liposomes), and the size measured as the average from 3 runs of 15 cycles each. The zeta potential of the liposomes was measured using the same instrument by Mixed Measurement Mode Phase Analysis Light Scattering in glucose buffer (300 mM glucose, 10 mM HEPES, 1 mM CaCl₂ at pH 7.4) at 120 µM total lipid. Each measurement consisted of 3 individual runs in automatic mode (10-100 cycles)
# Supplementary Table 1

<table>
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<tr>
<th>Name</th>
<th>Formulation</th>
<th>Batches</th>
<th>Size (nm)</th>
<th>Charge (mV)</th>
<th>Drug dose (µmol/kg)</th>
<th>Lipid dose (µmol/kg)</th>
<th>Dosing schedule</th>
<th>Hypersensitivity</th>
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<td><strong>Anionic formulations</strong></td>
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<td>q4d × 3</td>
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<td>q4d × 3</td>
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<td>86</td>
<td>q4d × 3</td>
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<td>125.1</td>
<td>-13.3</td>
<td>0.06</td>
<td>ND</td>
<td>q4d × 3</td>
<td>No</td>
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</table>
### Cationic formulations

| Cationic DOPE-PEG 0.75% 1V270 | POPC:Chol:DOTAP:1V270:DOPE-mPEG2k (44.25:30:20:0.75:5) | 2 | 135.0 ±25.1 | +16.3 ±3.0 | 2.0 | 270 | q4d × 3 | Yes |
| Cationic non-PEG 0.75% 1V270 | POPC:Chol:DOTAP:1V270 (61:30:8.25:0.75:5) | 1 | 100.0 | 38.3 | 2.0 | 270 | q4d × 3 | Yes |

**Supplementary Table 1.** Overview of liposomal formulation given intravenously throughout the study. ND = not determined.

## Supplementary Table 2

<table>
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<th>Name</th>
<th>Formulation</th>
<th>Batches</th>
<th>Size (nm)</th>
<th>Charge (mV)</th>
<th>Drug dose (µmol/kg)</th>
<th>Lipid dose (µmol/kg)</th>
<th>Dosing schedule</th>
<th>Toxicity</th>
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<td>Anionic 0.75% 1V270 DOPE-PEG</td>
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<td>-8.17</td>
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<td>q4d × 3</td>
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<td><strong>Cationic formulations</strong></td>
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<tr>
<td>Cationic 0.75% 1V270 DOPE-PEG</td>
<td>POPC:Chol:DOTAP:1V270:DOPE-mPEG2k (44.25:30:20:0.75:5)</td>
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<td>117.2</td>
<td>+14.2</td>
<td>0.7</td>
<td>90</td>
<td>q4d × 3</td>
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**Supplementary Table 2.** Overview of liposomal formulation given subcutaneously throughout the study.
Supplementary Figure S1. HPLC chromatograms of IgM (A) or IgG (B) antibody fractions purified using protein A/G and protein L spin columns from plasma from mice the fourth day after two intravenously administrations of DOPE-PEGylated stealth-like 0.75% 1V270 liposomes (2.0 µmol/kg 1V270 per administration) given q4d.
Supplementary Figure S2. CT26 bearing mice were treated with 1V270 liposomes. All groups received 2 Gy RT on 5 consecutive days from the day of the first liposome treatment under lead-shielding exposing the tumor-bearing flank. (A) Mice were randomized on day 11 post inoculation (mean tumor volume: 90 mm$^3$) and treated with RT in combination with DOPE-PEGylated anionic 0.75% 1V270 liposomes q4d for a total of 5 treatments. Mice were administered 2.0 µmol/kg 1V270 and 266 µmol/kg lipid per liposome administration. No acute hypersensitivity reaction was observed for administrations. (B) Mice were randomized on day 12 post inoculation (mean tumor volume: 100 mm$^3$) and treated with RT in combination with DOPE-PEGylated anionic 0.75% 1V270 liposomes q4d for a total of 3 treatments as acute hypersensitivity reaction was observed following the third administration. Mice were administered 2.0 µmol/kg 1V270 and 263 µmol/kg lipid per liposome administration. (C) Mice were randomized on day 11 post inoculation (mean tumor volume: 95 mm$^3$) and treated with RT in combination with non-PEGylated anionic 0.75% 1V270 liposomes given q4d for a total of 5 treatments (2.0 µmol/kg 1V270 and 270 µmol/kg lipid per administration) or DOPE-PEGylated anionic 0.75% 1V270 liposomes given q1d for 10 treatments (0.7 µmol/kg 1V270 and 74 µmol/kg lipid per administration).
Chapter 5 – Manuscript III

Nanoformulation of TLR7/8 agonist provides safe and potent anti-cancer immunity

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Data from an ongoing study.

Abstract

Toll-like receptor (TLR) 7/8 agonists are potent innate immune stimulators that can bridge the innate and adaptive immune system. TLR7/8 agonists have shown very promising results as anti-cancer therapy against various tumors. However, clinical success has been restricted to topical administration due to limited systemic tolerability.

Here, we demonstrate potent anti-cancer effect in murine cancer models and safe systemic administration in mice and non-human primates of a micellar nanoparticle formulation containing a TLR7/8 agonist. Following intravenous administration, micelles induced massive cell death in the CT26 cancer model accompanied by substantial influx of neutrophils and an antigen-specific CD8⁺ T cell response. Additionally, the TLR7/8-micelles demonstrated good synergy with immune checkpoint inhibitor blockade and radiotherapy in the CT26 cancer model. Further testing in other cancer models revealed good anti-cancer effect in combination with radiotherapy in the EL4 and MC38 cancer model but no significant anti-cancer effect in the B16-F10 cancer model. Consequently, the TLR7/8-micelles provide a promising candidate for clinical testing based on potent anti-cancer effects and the ability to be safely administered in mice and non-human primates.
Introduction

Toll-like receptor (TLR) 7/8 agonists are potent immune stimulators that have shown promising preclinical results as anti-cancer immunotherapy in multiple murine cancer models (91). Following TLR7 agonist therapy, the otherwise suppressive tumor microenvironment (TME) may be repolarized whereby pro-tumorigenic phenotypes become anti-tumorigenic (67,97,99). Anti-cancer immunity induced by TLR7 agonists has previously been shown to be mediated through a type 1 interferon response with effects on both innate and adaptive immune cells (91,94–97). Additionally, previous studies have shown impressive synergy with other therapies, including radiotherapy (RT) and immune checkpoint blockade (94,96,198,199). The TLR7 agonist, Imiquimod (Aldara), is approved by FDA for topical treatment of basal cell carcinoma and is also used for treating melanoma and lentigo maligna with effective responses (91,200,201). However, compounds like Imiquimod can only be applied on superficial cancers. Despite promising results in mice of systemically administered TLR7/8 agonists, translation into the clinic has been limited by adverse reactions owed to systemic activation (91,105–107). Thus, efforts to reduce systemic activation following administration of TLR7 agonists present a promising approach to obtain safe and potent immunotherapy for anti-cancer therapy. Drug delivery systems have the potential to improve efficacy and safety of drugs by altering the circulatory properties and biodistribution (110). Liposomes are widely used as drug delivery systems to reduce adverse effects. For instance, the first FDA-approved liposomal formulation of doxorubicin, Doxil, lowers cardiac toxicity of the drug and thereby in some cases improves the therapeutic window (117,202). However, our group has recently demonstrated that PEGylated liposomes containing TLR agonists are highly problematic for repeated dosing due to induction of antibodies against liposomes (unpublished data).

To this end, we sought to produce smaller nanoparticles containing a TLR7/8 agonist to improve the systemic tolerability and minimize anti-nanoparticle immune responses. We developed a micellar formulation that is easily produced in GMP-grade and contains a lipid-anchored TLR7/8 agonist (1V270). This formulation could be administered safely in both mice and non-human primates and demonstrated potent anti-cancer effect in murine syngeneic cancer models both as monotherapy and combined with RT or immune checkpoint inhibitor blockade.
Materials and Methods

Materials
1-Palmitoyl-2- oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-mPEG2000) and Cholesterol (Chol) were acquired from Lipoid HmbH. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]) (DOPE-mPEG2000), dioleoyl-3-trimethylammonium-propane (DOTAP) were acquired from Avanti Polar Lipids. (2-(4-((6-amino-2-(2-methoxyethoxy)-8-oxo-7H-purin-9(8H)-yl)methyl)benzamido)ethyl 2,3-bis(oleoyloxy)propyl phosphate (1V270, also known as TMX-201; molecular weight = 1085.4 Da) was obtained from Chimete or Corden Pharma. Resiquimod (R848) was obtained from Invivogen.

Synthesis and characterization of nanoparticles
Micelles were prepared from DSPE-mPEG2k and 1V270. Lipids were dissolved in tert-butanol:water (9:1; v/v) at 50-60°C. Lipid dispersions were mixed in a molar ratio of 90:10 (DPSE-PEG2k:1V270). Solvent was removed by lyophilization (Scanvac Coolsafe lyophilizer, Labogene). Micelles were re-hydrated in a buffered solution (150 mM NaCl and 10 mM phosphate, pH 7.1-7.4) with gentle vortexing followed by ultrasonication. The micelle dispersions were sterile filtered through 0.22 µm syringe filters (Frisenette). Micelles were stored at 2-8°C until use.

Liposomes were prepared by mixing the indicated components in a glass vial in tert-butanol:water (9:1; v/v). Cationic liposomes were prepared in a molar ratio of 44.25:30:20:5:0.75 (POPC:Chol:DOTAP:DOPE-mPEG2k:1V270. Stealth-like liposomes were prepared in a molar ratio of 64.25:30:5:0.75 (POPC:Chol:DOPE-mPEG2k:1V270). Lipid mixtures were lyophilized (Scanvac Coolsafe lyophilizer). Dried lipids were then re-hydrated in a buffered solution (150 mM NaCl and 10 mM phosphate, pH 7.1-7.4) and heated to 65°C for 1 h under stirring. The solution was extruded once through three stacked Whatman filters (GE Healthcare) with a pore size of 400, 200, and 100 nm followed by seven extrusions through two stacked 100 nm Whatman filters. Extrusion was performed at 10-30 bar nitrogen pressure using a 10 ml LIPEX thermobarrel pressure extruder on a heating block at 65°C. Liposomes were stored at 2-8°C until use.
Hydrodynamic diameter of nanoparticles was measured by dynamic light scattering using a Zetasizer (Malvern Instruments), in the same buffer as prepared in, and reported as distribution according to particle number. Vehicle micelles had a mean size of 10-12 nm while 1V270-micelles had a mean size of 11-15 nm. PDI for micelles ranged 0.1-0.5. Stealth-like liposomes had a mean size of 111 nm (<0.1 PDI) and cationic liposomes had a mean size of 70 nm (<0.1 PDI).

Zeta potential was measured in 5% (w/w) glucose, 10 mM HEPES, 1 mM CaCl₂ in milliQ water, pH 7.4 using a Dip cell Kit (Malvern Instruments) and Zetasizer. Vehicle micelles were -4 mV while 1V270-micelles were -5 to -10 mV.

**TLR7 stimulation assay**

HEK-Blue™ mTLR7 cells were obtained from Invivogen. In accordance with the manufacturer’s instructions, 40,000 cells in a 96-well plate were incubated for 17 h with PBS, R848, 1V270, 1V270-micelles, or vehicle micelles in duplicates. All compound stocks, except free 1V270, were dissolved in PBS while the 1V270 stock was dissolved in DMSO. Serial dilution was performed in PBS. Activity of the secreted embryonic alkaline phosphatase (SEAP) reporter gene was monitored in HEK-Blue detection media (Invivogen) and was measured as absorbance at 655 nm using a FLUOstar Omega Microplate Reader (Ramcon). Response ratio was calculated as absorbance in target wells divided by absorbance in control wells.

**Cancer cell lines**

The colorectal cancer cell lines CT26 and MC38, melanoma cancer cell line B16-F10, and lymphoma cell line EL4 were obtained from ATCC. CT26, MC38, and B16-F10 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM Glutamax, 100 units/ml penicillin, and 100 µg/ml streptomycin. EL4 was maintained in DMEM with 4.5g/l glucose supplemented with 10% fetal bovine serum, 2 mM Glutamax, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37°C and 5% CO₂ in a humidified atmosphere.
Mice
When not stated otherwise, female mice aged 8-12 weeks (BALB/cJRj and C57BL/6JRj) were obtained from Janvier Labs. All experimental procedures were approved by the institutional ethical board and the Danish National Animal Experiment Inspectorate. For inoculation, mice were anesthetized using 3-5% sevoflurane and inoculated subcutaneously in the right flank with 100 µl cancer cells in unsupplemented media containing 3x10^5 CT26, MC38, EL4, or B16-F10 cells. Tumor volume was measured with calibers as length x width^2 / 2 and randomized based on tumor volume. Mice were euthanized when tumors reached 1200 mm^3, weight loss >15%, or displaying sign of failure to thrive. Tumor volume curves are reported with last observation carried forward for euthanized mice.

Studies evaluating plasma cytokines and hematology were conducted at EPO-Berlin (Germany). Efficacy studies addressing combination treatment with αPD-1 antibodies were conducted at Crown Biosciences (China). All protocols were approved by local ethical committees.

Treatment of mice
Micelles or liposomes were given intravenously in doses of 50, 100, or 200 nmol. For efficacy studies, 50, 100, or 200 nmol 1V270-micelles or lipid-matched vehicle controls were given intravenously in a q4d schedule. Mice given αPD-1 were given 50 µg (10 mg/kg) αPD-1 (RMP1-14, BioXcell) intraperitoneally in a q4d schedule. Mice given RT were irradiated with a dose rate of 1.0 Gy/min under lead-shielding exposing the tumor-bearing flank using an X-RAD 320 (PXi).

Mouse cytokine analysis and hematology
For cytokine analysis, mice were injected intravenously with 1V270 micelles (50, 100 or 200 nmol) or 1V270 PEGylated cationic liposomes. Blood samples were taken 2 h (retro-orbital) and 6 h (cardiac) post injection. Plasma was prepared using K_3EDTA vials (Greiner) according to the manufacturer’s protocol and stored at -80°C until analysis. Plasma samples were diluted 15-fold and analyzed for CXCL1 (KC/GRO), IL-6, IL-12p70, IFNγ, and TNF-α using a V-plex assay (MSD Mesoscale) according to the manufacturer’s instructions. IFNα was measured in 25-fold diluted plasma by ELISA (PBL Assay Science). Data analysis was
performed using Prism 8 Software (GraphPad). Hematology was analyzed 1 h prior to 1V270-micelle treatment and 3, 8, 24, 72, 120, and 192 h post treatment.

Mouse anti-PEG antibody determination
Mice were injected intravenously with 1V270-micelles (200 nmol), vehicle micelles or stealth-like 1V270 PEGylated liposomes (200 nmol). Blood samples were collected on day 5, 10, 14, 21, and 28 into tubes containing 10 µl 50 mM EDTA. Plasma was prepared by centrifugation at 2000 g, 4°C for 15 min. αPEG IgM and αPEG IgG was measured in 400-fold diluted plasma by ELISA as done in Stavnsbjerg et al. 2019 (unpublished manuscript).

Flow cytometry
Tumors (80-800 mg) were excised, weighed, cut into pieces, and digested in tumor dissociation enzyme mix for murine tumors (Miltenyi Biotec) for 40 minutes at 37°C in a shaking water bath. Resulting solutions were mashed through 70-µm cell strainers. Spleens were excised and mashed through 70-µm cell strainers, erythrolyzed in VersaLyse (Beckman Coulter) for 10 minutes, and passed through another 70-µm cell strainer. Cell yield was determined using a Muse® cell analyzer (Merck Millipore).

Processed cells (1-10x10⁶) were Fc-blocked (clone 2.4G2, BD Biosciences) in FACS buffer (PBS + 0.5% BSA + 0.1% NaN₃). Subsequently, samples were stained with an amine-reactive dye and antibodies (listed in Supplementary Table 1) and FACS buffer and brilliant stain buffer (BD Biosciences) for 30 minutes on ice. Stained tumor samples were filtered through a 70 µm cell strainer before acquisition on a BD LSRFortessa X-20 (BD Biosciences) using FACSDiva v8.0.1 (BD Biosciences). Data analysis including T-distributed Stochastic Neighbor Embedding (t-SNE) was performed in FlowJo v.10.6.1 (BD Biosciences). t-SNE was performed on a concatenated sample consisting of an equal number of down-sampled samples. t-SNE was run using the Barnes-Hut algorithm with 1000 iterations and all fluorescent parameters except viability signal and including forward and side scatter signals. Number of cells per 100 mg tumors was determined as [%gated as cells] x [total cell count] / [tumor weight in mg] x 100.
Dose escalation study in cynomolgus monkeys
Three naïve male cynomolgus monkeys (*Macaca fascicularis*) were treated with 1V270-micelles at escalating doses of 0.01, 0.03, 0.1, 0.3, 0.9 and 2.7 mg/kg administered once every 14 days by intravenous infusion (5 ml / 20 min). Clinical observations were conducted twice a day. Food consumption and rectal temperature were monitored daily. Body weight was checked once a week. Blood pressure was monitored at 2 h, 4 h and 8 h after each administration and then once a week between dosing. Hematology and CRP were analyzed at day -13 and day -6 during acclimation period, immediately prior to each drug administration and 8 h, 24 h and 72 h after infusion. Blood biochemistry was analyzed during the acclimation period and 14 days after each administration. The study was conducted by Cynbiose, France. The study protocol was approved by the local ethical committee.

Statistical analysis
Statistical analyses were performed using Prism 8. The appropriately used statistical test is stated in figure legends. A p-value ≤ 0.05 was considered statistically significant.
Results

Characterization and potency of 1V270-micelles

Micelles were formulating using DSPE-mPEG2k and 1V270 resulting in micelles with a size of approximately 15 nm (Figure 1A-B). The ability of 1V270 and 1V270-micelles to stimulate the TLR7 was determined using murine TLR7 transfected HEK-293 cells with NF-κB reporter activity (Figure 1C). Free 1V270 was suspended in DMSO due to poor water solubility, thereby explaining the low signal observed at the highest assayed concentrations (31.6 and 100 µM). Micellar 1V270 provided much lower reporter signal compared to free 1V270 but due to the higher solubility micellar 1V270 could be assayed at higher concentrations. We speculate that the lower signal of 1V270-micelles compared to free 1V270 is due to a slower uptake. Additional preliminary studies also indicate human TLR 7 and human TLR8 activity by 1V270 (data not shown).

The therapeutic effect of 1V270-micelles was evaluated in the syngeneic murine cancer model CT26. 1V270-micelles were injected either intravenously (200 nmol/mouse) or intratumorally (100 nmol/mouse due to volume constraints) in animals bearing established tumors (~90 mm³). Treatment with 1V270-micelles in mice resulted in transient weight losses and no additional clinical symptoms. Encouragingly, we observed significantly increased survival for both intravenously and intratumorally administered 1V270-micelles compared to vehicle-treated mice (p < 0.001) and 3/8 complete responders. Immunological memory was established as evident by all complete responders rejecting cancer cell rechallenge (Figure 1C-F). As systemic delivery was well tolerated, provided good efficacy, may access all cancerous lesions including metastases, and can be used to treat tumors that are difficult to inject intratumorally, we focused on systemic delivery.
Figure 1. Characterization and potency of 1V270 micelles. (A) Graphical illustration of 1V270-micelles. (B) Dynamic light scattering profile of 1V270-micelles and vehicle micelles. (C) HEK-Blue mTLR7 cells were incubated with 1V270 in DMSO, R848, 1V270-micelles, and vehicle micelles in PBS in duplicate wells. Response ratio was calculated in relation to PBS-treated cells and displayed as mean ± SEM of measurements. (D) Mice were inoculated with CT26 subcutaneously, \( n = 8 \). On day 12 post inoculation (~90 mm\(^3\) tumors) mice were treated intravenously (i.v.) or intratumorally (i.t.) with 1V270-micelles or vehicle every fourth day for 5 consecutive treatments. Shown is mean tumor volume ± SEM. Individual growth curves can be found in Supplementary Fig. S1. Weight change was compared to the day of randomization. Surviving mice
were challenged with CT26 on the opposite flank on day 103 after primary tumor challenge. Shown is the percentage of mice rejecting rechallenge. Survival analysis was performed using log-rank (Mantel-Cox) tests. ***, p < 0.001. CR = complete responders.

Administration of 1V270-micelles induces favorable systemic cytokine release
To further evaluate the safety and mechanism of action of 1V270-micelles we investigated the systemic cytokine response, hematology, and antibody responses against the 1V270-micelles and compared these to a liposomal formulation containing 1V270 as a large nanoparticle comparator.

To evaluate the systemic cytokine profile, we investigated dosing 50, 100, and 200 nmol 1V270-micelles and compared to 100 nmol 1V270-cationic liposomes. Micellar 1V270 could be administered at twice the dose of 1V270-liposomes before resulting in similar plasma concentrations of IL-6 and TNFα. Similar results were observed for IL-12p70 and IFNγ. However, systemic IFNα was induced to a much lower extent by micelles compared to liposomal 1V270 while CXCL1 was induced to a similar extent by micelles compared to liposomal 1V270 (Figure 2A). As previous studies has demonstrated that systemically administered TLR7 agonists can lead to transiently reduced availability of peripheral blood leukocytes (100), we performed hematology and found indications of this also occurring following 1V270-micelle administration (Supplementary Fig. S2).

As nanoparticles may be subject to accelerated blood clearance due to antibodies produced against the PEG layer or other immunogenic compounds (109,164,173), we compared the αPEG antibody response generated against the 1V270-micelles with that generated against stealth-like liposomes. Injection with the micelle formulation produced markedly lower αPEG IgG and IgM (2.3-fold and 18-fold, respectively, on day 5 post injection; Figure 2B-C).
Figure 2. Evaluation of systemic cytokine profile and antibodies directed against 1V270-micelles. To determine cytokine levels in plasma, BALB/c mice were treated with a single intravenous dose of 1V270-micelles, vehicle micelles, PBS, or cationic liposomes containing 1V270. (A) Plasma samples were collected 2 and 6 h post injection and measured by cytokine multiplex and ELISA, n = 5. IgG (B) and IgM (C) antibody production against PEG was determined by treating BALB/c mice with a single intravenous dose of 1V270-micelles, vehicle micelles, PBS, or stealth-like liposomes; n = 2 - 7. Plasma was collected on indicated days post injection. Shown is mean + SEM. Statistical significance in (A) was determined between 100 nmol 1V270-micelles and 100 nmol 1V270-liposomes. In (B-C) statistical significance was determined on day 5 post injection between 200 nmol 1V270-micelles and 1V270-liposomes. Statistical significance was determined by multiple t tests with Holm-Sidak correction for multiple comparisons. ns: nonsignificant, p > 0.05; *, p ≤ 0.05; **, p < 0.01; ***, p < 0.001.
1V270-micelle treatment leads to massive cell death in the tumor microenvironment

To decipher the anti-cancer effect of 1V270-micelles, we treated CT26-bearing mice with 1V270 micelles and analyzed tumors by flow cytometry (Figure 3A) with the hypothesis that changes in myeloid and lymphoid subsets may explain the anti-cancer effect. The TME was analyzed for infiltration of cancer cells, classical dendritic cells (cDCs), plasmacytoid DCs (pDCs), tumor-associated macrophages (TAMs), monocytic myeloid-derived suppressor cells (Mo-MDSCs), patrolling monocytes (PMos), T cells, and neutrophils. In this experiment, 1V270-micelles were produced in a molar ratio of 80:20 (DSPE-PEG2k:1V270) which is slightly different than the formulation used in the other experiments. Preliminary studies indicate that the molar ratio of 80:20 in 1V270-micelles leads to slightly lower anti-cancer effect in the CT26 cancer model (data not shown).

Two days after the second treatment with 1V270-micelles, cell viability in the TME was reduced from 22% in untreated tumors to 4% in treated tumors (p < 0.01; Figure 3B). Immune infiltration was 2.4-fold increased two days after the second treatment (p < 0.01; Figure 3C) and two days post the fourth treatment was accompanied by a 64% increase in active tumor specific CD8+ T cells out of the total CD8+ T cell population (p = 0.052; Figure 3D). The investigated immune populations were found to be greatly affected by 1V270-micelle treatment with all investigated cell types being depleted from the TME except for neutrophils, which were found to be heavily recruited (quantified as total count per 100 g tumor in Figure 3E, as percentage of CD45+ cells in Supplementary Fig. S3A, and displayed on t-SNE projection in Figure 3F). Interestingly, viability and infiltration of neutrophils in spleens was unaffected by 1V270-micelle treatment (Supplementary Fig. S3B-C).
Figure 3. 1V270-micelles induces massive cell death in tumors. (A) Mice, n = 4 - 5, were inoculated with CT26 tumors, grown to ~190 mm³ tumor volume (day 15 post inoculation), and treated with 200 nmol 1V270-micelles every fourth day. Tumors and spleens were processed for flow cytometry on day 21 (two days post 2nd treatment) and day 29 (two days post 4th treatment), indicated as D21 and D29, respectively. (B) Viability of cells in tumors. (C) CD45 infiltration in tumors. (D) CD8⁺ T cells positive for surface CD107a and AH-1
dextramer (H2-Ld SPSYVYHQF). (E) Cells per 100 mg tumor of respective populations. Shown is mean + SEM. (F) t-SNE projection of viable cells from 1V270-micelle treated and untreated. Top left panel: t-SNE projection overlaid with manually gated populations. Remaining top panels: t-SNE projection divided into the respective groups and displayed as density plots with black indicating highest cell density. Additional panels are overlaid with signal from the respective parameter. Blue indicates minimal, green low, yellow intermediate, and red high expression of each parameter. Tumor weight at take-down can be found in Supplementary Fig. S3D. For gating strategy used in (B–F), see Supplementary Fig. S4A–B. Statistical significance was determined using Kruskal Wallis with Dunn’s multiple comparison tests. ns: nonsignificant; **, p < 0.01

Treatment with micelles containing 1V270 synergizes with radiotherapy and immune checkpoint blockade

To investigate the potential for synergy with other treatments, we combined treatment of 1V270-micelles with fractionated RT and αPD-1 immunotherapy. Combining micelles with RT indicated a threshold for anti-cancer effect as demonstrated by minimal synergistic effect of 50 nmol micelles but potent efficacy when RT was combined with 100 and 200 nmol 1V270-micelles, resulting in 2/10, 4/9, and 8/8 complete responders, respectively. As demonstrated by rejection of subsequent cancer cell rechallenge, all surviving mice had established a memory response (Figure 4A). Investigation of synergistic anti-cancer effect of 1V270-micelles and αPD-1 was performed using CT26 tumors with treatment started on day 9 while treatment in other CT26 studies in this article started treatment on day 12-15. Here, monotherapy with 1V270-micelles provided 8/10 long term survivors and combination with αPD-1 provided 10/10 long term survivors (Figure 4B). One mouse treated with 1V270-micelles in combination with αPD-1 was found dead of unknown reasons on day 76 post inoculation.
Figure 4. Synergy of 1V270-micelles with RT and aPD-1. Mice were inoculated with CT26 subcutaneously, n = 8-10. On day 9 or 12 post inoculation (~90-100 mm³ tumors) mice were treated intravenously with 1V270-micelles every fourth day in combination with 5x2Gy RT given daily (A) or in combination with aPD-1 given as 50 µg intraperitoneally every fourth day for a total of 6 treatments (B). Surviving mice in (A) were challenged with CT26 on the opposite flank on day 101 after primary tumor inoculation. Shown is the percentage of mice rejecting rechallenge. Survival analysis was performed using log-rank (Mantel-Cox) tests. ns: nonsignificant, p > 0.05; *, p ≤ 0.05. CR = complete responders.
Treatment efficacy is dependent on cancer model

Based on the impressive results in CT26 as monotherapy and in combination with RT and αPD-1, we sought to investigate the effect in less immunogenic models. Like with CT26, subcutaneous tumors were grown to a volume of ≈75-100 mm³ and treated with 1V270-micelles in combination with fractionated RT (Figure 5A-C). In the EL4 model, 1V270-micelles had no significant anti-cancer effect as monotherapy. Despite not reaching statistical significance, 1V270-micelles appear to synergize with RT in the EL4 model, resulting in 4/9 complete responders while RT combined with vehicle micelles resulted in 2/9 complete responders. Immunological memory was induced as evident by 3/4 complete responders receiving 1V270-micelles in combination with RT rejecting rechallenge (Figure 5A). In the MC38 model, 1V270-micelles showed potent effect in combination with RT, resulting in 3/9 complete responders and induced immunological memory (Figure 5B). In the B16-F10 model, 1V270-micelles in combination with RT did not improve median survival time compared to control (p = 0.27; Figure 5C). These data indicate that immunogenicity in tumors or other model-dependent differences are relevant for stratification into responders of 1V270-micelle treatment.
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**Figure 5.** Efficacy studies of 1V270-micelles combined with RT in EL4, MC38, and B16-F10 cancer models. Mice were inoculated subcutaneously with EL4, MC38, and B16-F10 cancer cells. (A) EL4 tumors were grown to a mean volume of 170 mm$^3$ (day 7 post inoculation). Mice were treated intravenously with 1V270-micelles every fourth day for a total of 5 treatments in combination with 3x2 Gy RT given daily. (B) MC38 tumors were grown to a mean volume of 75 mm$^3$ (day 10 post inoculation). Mice were treated intravenously with 1V270-micelles every fourth day for a total of 5 treatments in combination with 5x2 Gy RT given daily. (C) B16-F10 tumors were grown to a mean volume of 95 mm$^3$ (day 8 post inoculation). Mice were treated intravenously with 1V270-micelles every fourth day for a total of 5 treatments in combination with 3x6 Gy RT given daily. Surviving mice were rechallenged with the respective cancer cell line on the opposite flank on day 100 with EL4 (A) and on day 80 with MC38 (B) after primary tumor inoculation. Shown is the percentage of mice rejecting rechallenge. Survival analysis was performed using log-rank (Mantel-Cox) tests. ns: nonsignificant, $p > 0.05$; **, $p < 0.01$. CR = complete responders.

1V270-micelles can be safely administered in non-human primates

To obtain additional translation information regarding 1V270-micelles, cynomolgus monkeys were administered 1V270-micelles in escalating doses of 0.01 to 2.7 mg/kg. Doses were increased by factor three every 14$^{th}$ day. Body temperature remained within normal range throughout the dose-escalating study (Figure 6A). Likewise, clinical symptoms were not observed except for mild flu-like symptoms following 2.7 mg/kg 1V270-micelles. CRP increased in a dose-dependent manner (Figure 6B), indicating a dose-dependent response to treatment. In contrast to the findings in mice, hematology revealed marked increase in white blood cell count 8 h following administration and returning to baseline values within 24 h (Figure 6C). Similar findings were observed across the analyzed leukocyte populations at low 1V270-micelle doses. At higher doses we observed a transient increase in neutrophils but decrease in monocytes and lymphocytes in peripheral blood in response to treatment (Supplementary Fig. S5). These results demonstrate that 1V270-micelles can be safely administered at the evaluated dose levels in non-human primates and display a dose-dependent response.
Figure legend on next page
Figure 6. 1V270-micelles can be safely administered to non-human primates from 0.01 mg/kg to 2.7 mg/kg. 1V270-micelles were intravenously administered in cynomolgus monkeys (n = 3) in a dose escalating scheme from 0.01 mg/kg to 2.7 mg/kg with 14 days between administrations. (A) Body temperature measurements performed daily, mean ± SEM. CRP (B) and leukocytes (C) determination in individual cynomolgus monkeys. Measurements were performed on day -13 and -6 to the initial administration, immediately prior to administration, 8 h, 24 h, and 72 h after administration. Grey to black solid lines indicate individual monkeys (B-C). Dotted horizontal lines indicate the range measured during the acclimatization period. Vertical dotted line or arrows indicate administration of the indicated 1V270-micelle dose.

Discussion

Potent anti-cancer effect of 1V270-micelles was observed in the murine CT26 syngeneic cancer model with the establishment of lasting immunological memory against the cancer. Evaluation of the TME by flow cytometry after systemic administration of 1V270-micelles revealed substantial cell death in tumors occurring after the second treatment. Tumor cells, T cells, PMos, Mo-MDSCs, TAMs, pDCs, cDC1s, and cDC2s were depleted from treated tumors compared to untreated tumors. The mechanism of cell death induction was not fully elucidated in the current study but the cell death was associated with a large influx of neutrophils. Previous studies have also linked influx of neutrophils following administration of TLR2 and TLR4 agonist to be essential for anti-cancer effect and to link the innate and adaptive immune response (203). In the current study, adaptive immunity following administration of 1V270-micelles was observed by enrichment of cytotoxic antigen-specific CD8+ T cells following the fourth micelle treatment and by rejection of rechallenge with cancer cells.

1V270-micelles could be safely administered in mice despite induction of systemic CXCL1, IL-6, IL-12p70, IFNγ, and TNFα. Administration was only accompanied by transient weight loss and no clinical observable symptoms. Systemic IL-6, IFNγ, and TNFα have been associated with cytokine release syndrome which may limit the therapeutic window (204). To this end, we investigated the safety in non-human primates and found it could be safely administered with mild flu-like symptoms observed only at the highest investigated dose (2.7 mg/kg). Peripheral blood cells were affected by 1V270-micelles in both mice and cynomolgus monkey, however the investigated cells types generally returned to normal levels within 8-72 h in both species. Interestingly, we found the peripheral blood response kinetics to differ between mice and non-human primates. Mice exhibited a
decrease in peripheral blood leukocytes (including neutrophils) in response to 1V270-micelles while cynomolgus monkeys exhibited a marked increase in peripheral blood leukocytes. In cynomolgus monkeys, we observed that low doses of 1V270-micelles resulted in transiently increased levels of peripheral blood monocytes, neutrophils, and lymphocytes while high doses lead to transiently increased levels of peripheral blood neutrophils but decreased peripheral blood monocytes and lymphocytes. Similarly, Kwissa et al. has reported a strong transient increase in peripheral blood neutrophils and decrease in peripheral blood monocytes in response to intradermal injection of the TLR7/8 agonist R848 (205).

Synergy with αPD-1 was demonstrated in the CT26 cancer model, resulting in 10/10 long term survivors. Synergy with RT was investigated in CT26, EL4, MC38, and B16-F10. Complete responders in combinational treatments were observed in all except for the B16-F10 cancer model. All mice were cured in the CT26 model, whereas 3/9 and 4/9 were cured in the MC38 and EL4 model, respectively. CT26 tumor have a high infiltration of CD8$^+$ T cells and granzyme B$^+$ NK cells. MC38 tumors have an intermediate infiltration and B16-F10 a poor infiltration of CD8$^+$ T cells (120). EL4 has still to be comprehensively compared to other models in regards to immunogenicity and response to immunotherapy but previous studies have demonstrated good anti-cancer effect of TLR7/8 agonists in combination with RT in EL4 (206). These results indicate that successful response to 1V270-micelles is dependent on prior immune infiltration.

In summary, 1V270-micelles can be safely administered in mice and cynomolgus monkeys and demonstrates potent anti-cancer effect in murine cancer cell models as monotherapy and in combination with αPD-1 and RT. Thus, 1V270-micelles presents an attractive candidate for further exploration in a clinical setting.
Supplementary Information

Supplementary Figure S1

Supplementary Figure S1. CT26-bearing mice (n = 8) were treated intravenously (i.v.) or intratumorally (i.t.) with 1V270-micelles or vehicle every fourth day for 5 consecutive treatments starting from day 12 post inoculation.
Supplementary Figure S2. Hematology of mice treated with a single dose of 200 nmol 1V270-micelles or vehicle micelles. Shown is mean (black line) and individual values, n=7-8. Dotted lines indicate the range measured prior to treatment. X axis is hours post 1st injection in all graphs.
Supplementary Figure S3. Changes in the TME and spleens following treatment with 1V270-micelles. CT26-bearing mice, n=4-5, were treated with 200 nmol 1V270-micelles every fourth day starting from day 15 post inoculation. Take-down was conducted two days post second treatment (D21) and two days post fourth treatment (D29). (A) Changes in the immune compartment in CT26 tumors treated with 1V270-micelles. (B) Cellular viability in spleens. (C) Infiltration of neutrophils in spleens. (D) Tumor weight at take-down. Gating strategy used in (A) can be found in Supplementary Figure S4A. Gating strategy used in (B-C) can be found in Supplementary Figure S4C. Statistical significance was determined using Kruskal Wallis with Dunn’s multiple comparison tests (B) or Mann-Whitney U test (C-D). ns: nonsignificant; *, p < 0.05.
**Supplementary Figure S4.** Gating strategies used in Figure 3 and Supplementary Figure S3. All populations were gated on time, singlets, scatter, and viability. (A) Gating strategy for myeloid populations in the TME and for downsampling of viable events prior to t-SNE projection. Cancer cells were identified as CD45− FSC<sup>mid</sup> SSC<sup>mid</sup>. Neutrophils were identified as CD45<sup>+</sup> CD11b<sup>+</sup> Ly6g<sup>+</sup>. TAMs were identified as CD45<sup>+</sup> Ly6g− CD11c<sup>+</sup> CD11b<sup>+</sup> CD64<sup>high</sup>. PMos were identified as CD45<sup>+</sup> Ly6g<sup>−</sup> CD11b<sup>−</sup> CD11c<sup>−</sup> Ly6c<sup>−</sup> CD64<sup>+</sup>. Mo-MDSCs were
identified as CD45$^{+}$ Ly6g$^{-}$ CD11b$^{+}$ CD11c$^{-}$ Ly6c$^{\text{high}}$. cDC1s were identified as CD45$^{+}$ Ly6g$^{+}$ CD11c$^{+}$ CD64$^{\text{low}}$ CD11b$^{\text{low}}$ XCR1$^{+}$. cDC2s were identified as CD45$^{+}$ Ly6g$^{+}$ CD11c$^{+}$ CD64$^{\text{low}}$ XCR1$^{+}$ CD11b$^{\text{high}}$. pDCs were identified as CD45$^{+}$ Ly6g$^{+}$ CD11c$^{+}$ CD64$^{\text{low}}$ XCR1$^{+}$ CD11b$^{\text{high}}$. T cells were identified as CD45$^{+}$ SSC$^{\text{low}}$ CD3$^{+}$. CD8$^{+}$ T cells were identified as CD45$^{+}$ SSC$^{\text{low}}$ CD3$^{+}$ CD4$^{+}$ CD8$^{+}$. sCD107a$^{+}$ and AH-1 dextramer$^{+}$ were based on FMOs. (B) Gating strategy for T cells in the TME. T cells were identified as CD45$^{+}$ SSC$^{\text{low}}$ CD3$^{+}$. (C) Gating strategy for neutrophils and viability of spleens. Neutrophils were identified as CD11b$^{+}$ CD11c$^{+}$ Ly6c$^{\text{int}}$ Ly6g$^{+}$.
Supplementary Figure S5

Figure legend on next page
Supplementary Figure S5. Hematology in cynomolgus monkeys following intravenous administration of 1V270-micelles. 1V270-micelles were administered to cynomolgus monkeys (n = 3) in a dose escalating scheme from 0.01 mg/kg to 2.7 mg/kg with 14 days between administrations. Hematology was performed on day -13 and -6 to the initial administration, immediately prior to administration, 8h, 24h, and 72h after administration. Shown is mean. Vertical dotted line indicates infusion of the indicated 1V270-micelle dose.

Supplementary Table 1

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Supplementary Table 1. Overview of fluorescent antibodies and amine reactive dye used for flow cytometry.

Panel 1 was used to investigate myeloid cells in tumors. Panel 2 was used to investigate T cells in tumors. Panel 3 was used to investigate populations in spleens.
Chapter 6 – Perspectivation

In the following sections, a brief perspectivation is given for the manuscripts enclosed in this dissertation, focusing on relevant additions to the enclosed manuscripts and their clinical relevance.

6.1 The Importance of Characterizing Tumor Microenvironments

Manuscript I provided a side-by-side comparison of the myeloid compartment in the investigated tumor models to help guide rational model selection. Additionally, the manuscript highlighted the plasticity of the myeloid compartment with striking differences in several parameters across models and myeloid subsets. While the plasticity in tumor infiltrating immune cells currently limits the accuracy of phenotyping, recent technological improvements are likely to improve the shortcomings. More specifically, the addition of DNA-barcoded antibodies that enables single cell simultaneous analyses of both mRNA and protein level are likely to revolutionize how cells are phenotyped.

Manuscript I also highlighted characteristics of the underappreciated PMo subset and the distribution of commonly used surface markers across myeloid subsets. Although providing valuable information for designing and evaluating preclinical studies, it was beyond the scope of the manuscript to address other important parameters like mutational status of cancer cells, response to therapy, stromal cells in the TME, spatial information within the TME, and the lymphoid compartment within the TME. Although the focus of the current work was myeloid infiltration, it was highly evident that preclinical tumor models, like their clinical counterparts, are highly heterogeneous. The heterogeneous nature of tumors is a major hurdle for the translational value of preclinical studies and for stratifying patients to the treatment that would benefit them the most. Compared to murine tumors, defining the TME in the clinical setting is further complicated by the relatively large tumor volumes, larger topological differences within the tumors, sampling errors, and individual differences (e.g. genomic). Despite these hurdles, stratifying patients for treatments based on TMEs remains clinically relevant (6).

While much effort is put into individualizing treatments (e.g. treatments against specific mutation or adoptive transfer of T cells trained against individualized peptide pools), this approach presents a much more costly approach compared to stratification based on
TME (e.g. highly infiltrated by CD8+ T cells). Additionally, individualized treatments typically depend on epitope spreading to ensure the heterogeneous tumor does not escape during the selection pressure. Thus, obtaining a better understanding of the TME presents an important research area of which the importance of the myeloid compartment is gaining increasing recognition.

6.2 Overcoming Accelerated Blood Clearance for Liposomes

Manuscript II pursued to overcome the ABC effect that may be induced against liposomes containing TLR agonists as antibodies recognizing nanoparticles poses a significant safety issue for clinical use of nanoparticles (202). To investigate this, we investigated repeated intravenous administration in mice of PEGylated liposomal formulations of different charge, with TLR agonists bound to the phospholipid layer or encapsulated, and different lipid anchors for PEG. Although not exhausting possibilities, the manuscript demonstrates that repeated systemic administration of PEGylated liposomes with immune stimulatory molecules is problematic due to the potential antibody response. Although it was beyond the scope of the manuscript to investigate compounds besides PEG for liposomal shielding, these would likely result in similar outcomes unless their immunogenicity is lower.

A popular method of inhibiting antibody production and subsequent ABC for liposomes includes delivery of cytotoxic drugs, whereby B cells are killed if they take up the drug (111). However, we determined, in preliminary studies that are not shown in this dissertation, that combining cytotoxic drugs and TLR7/8 agonists in PEGylated liposomes also causes severe safety problems. Several studies have investigated alternatives to PEG that are less immunogenic. However, no well-characterized solution exists to the best of the author’s knowledge. Solutions put forward in literature includes pre-infusion of free PEG polymer, modifications to PEG, alternatives to PEGylation, waiting so long between infusions that circulatory antibodies are at negligible levels, using more immunosuppressive nanoparticle components, including cytotoxic drugs, or very high lipid doses (109,117,172,176,207).

Additionally, the manuscript investigated whether the acute hypersensitivity reaction associated with repeated dosing of liposomes containing TLR7/8 agonists could be attributed to IgM or IgG and found strong indications of IgG being the most significant
contributor. Although beyond the scope of the manuscript, it would be interesting to
determine whether IgG is also the most significant contributor to ABC by transfer of
antibodies from immunized mice to naïve mice.

6.3 Advancing 1V270-micelles to the Clinic

As evident by the data presented in Manuscript III, 1V270-micelles presents a promising
anti-cancer immunotherapy. The micellar formulation demonstrated a good safety profile
while maintaining potent efficacy in preclinical studies.

In summary, safety was evaluated in mice and cynomolgus monkeys with the
investigated parameters indicating a safe treatment. The dose escalating study in
cynomolgus monkeys determined that the range of investigated doses (0.01 to 2.7 mg/kg)
could be safely administered with the highest dose resulting in flu-like symptoms. In
comparison, the TLR7 agonist 852A was only shown tolerable to 1.2 mg/m² (~0.04 mg/kg)
in a phase I study (106). In contrast to what have been reported for other systemically
administered TLR7 agonists, we found very low systemic IFNα levels after administration of
micelles containing 1V270 in mice (97,205). This was found only for the micellar formulation
and not for the liposomal formulation, indicating that the biodistribution and/or cellular uptake
is markedly altered by formulating TLR7 agonists in micelles. We also observed that
treatment efficacy appeared to depend on existing immune infiltration as evident by being
highly efficacious in the highly immunogenic CT26 cancer model, having intermediate effect
in the MC38 and EL4 models, and having no effect in the poorly immunogenic B16-F10
tumor model. Furthermore, the micelles demonstrated great synergy with radiotherapy and
αPD-1 treatment. Following infusion of 1V270-micelles, we observed a high degree of cell
death within the TME accompanied by a massive infiltration of neutrophils and an antigen-
specific CD8+ T cell response. The finding could not be observed in spleens of treated mice.
Additional preliminary studies, that are not included in this dissertation, indicated that 1V270-
micelles exhibit very low cytotoxicity in vitro. As this does not fully elucidate the mechanism
of action future studies will investigate the overall changes at mRNA level followed by
determination of the mechanism of action at a cellular level by flow cytometry and cytokine
determination within the TME. Additionally, knockout and depletion studies will demonstrate
the importance of relevant cell types. Based on the findings in manuscript I, CT26 is also
the only of the investigated models that is heavily infiltrated by PMN-MDSCs. Therefore, it
would be interesting to investigate the potential of 1V270-micelles in another heavily PMN-MDSC infiltrated cancer model, like 4T1.

Although not included in this dissertation, unpublished data from the group indicates that the micelles are unstable in plasma. Thus, 1V270 are most likely transported in circulation by other serum components. As demonstrated in Manuscript II, PEGylated liposomes containing 1V270 elicit an αPEG response leading to ABC upon repeated infusion. Here, not-included unpublished data from the group, indicates that although antibodies are raised against nanoparticles upon 1V270-micelle infusion, these cannot recognize the micelle formation but can recognize a liposomal formulation containing 1V270. Additionally, we observed that injecting 100 nmol 1V270-micelles intratumorally provided efficacy comparable to injecting 200 nmol 1V270-micelles intravenously. Altogether, these data indicate that 1V270-micelles are not subject to the ABC effect and that antibodies against the treatment will not pose translational complication. Furthermore, the data indicates that either a high degree of systemic activation is favorable or that approximately half of the injected intravenous dose reaches the tumor. To further elucidate whether the formulation will be subject to the ABC effect, biodistribution, cellular uptake, and circulatory properties will be investigated using \( ^{14} \text{C} \)-labeled 1V270.

Based on these results and following additional toxicology and dose range finding studies, we hope to start phase I clinical trials in 2020 or 2021 with 1V270-micelles as monotherapy and combined with αPD-1 in patients with recurrent/relapsing solid tumors.
In conclusion, the manuscripts enclosed in this dissertation have provided additional insight into preclinical evaluation of anti-cancer therapies and demonstrate the preclinical evaluation of a novel immunotherapy developed within the group.

The presented side-by-side characterization of several murine syngeneic tumor models provides a valuable tool for evaluating anti-cancer therapies and demonstrates the heterogeneous nature and functionality within the myeloid compartment of the TME. The observed differences may be useful in explaining why immunotherapies are efficacious in some tumor models but not in others. Additionally, the study highlighted the plasticity of myeloid subsets across tumor models as evident by differences in expression of immune cell-interacting molecules (e.g. PD-L1).

The presented evaluation of PEGylated liposomes containing TLR agonists indicates that unless immunostimulatory PEGylated liposomes can avoid recognition by B cells, repeated infusions will be subject to the ABC effect and possibly lead to acute hypersensitivity reactions. Previous studies have indicated that IgM is the most significant in causing ABC of nanoparticles but our results indicate that hypersensitivity related to ABC following administration of PEGylated liposomes containing TLR agonists are mediated by IgG opsonization of nanoparticles and not IgM.

The presented micelle formulation containing a TLR7/8 agonist could be safely administered to mice and non-human primates. Furthermore, it provided excellent efficacy in murine syngeneic tumor models as monotherapy and exhibited synergistic effects in combination with radiotherapy and immune checkpoint blockade. Following infusion, we observed massive cell death within the tumors accompanied by substantial influx of neutrophils and a cancer antigen-specific CD8+ T cell response. This was not reflected in spleens. All together, these results demonstrate that 1V270-micelles present a promising new anti-cancer treatment and highlights the usefulness of nanotechnology to increase the therapeutic window.


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