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Study programme: Immunology



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Evaluation of nanoconstructs for possible anti-cancer interventions and immunotherapy

Hodnocení nanokonstruktů pro možné protinádorové intervence a imunoterapii

Doctoral thesis

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Prague, 2024

Prohlášení

Tato disertační práce byla vypracována v Laboratoři imunoterapie Mikrobiologického ústavu AV ČR. K opravě gramatických aspektů v této práci byl použit AI program Grammarly.

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V Praze

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Declaration of honor

This work was developed in the Laboratory of Immunotherapy, Institute of Microbiology, Czech Academy of Sciences. The AI program Grammarly was used to correct grammatical aspects in this work.

I hereby declare that I have worked on the Thesis independently and that I have reported all the information, sources, and literature used. This work, or a substantial part of it, has not been submitted to obtain another or the same academic title.

In Prague

Mgr. Lenka Rajsiglová

Acknowledgements

I would like to express my most sincere gratitude to my supervisor, MUDr. Luca Vannucci, Ph.D., for his support of my Ph.D. study. His wide knowledge, patience, motivation, and guidance helped me at every step of the research and writing of this thesis. His most valuable insights and advice were crucial for creating this work.

In addition to my supervisor, I would like to express my deepest appreciation to my consultants doc. RNDr Daniel Smrž, Ph.D. and Dmitry Stakheev, M.D., Ph.D., for their continuous support, valuable insights, patience, and guidance during the study.

Many thanks also go to my colleagues from the Laboratory of Immunotherapy, particularly to RNDr. Pavol Lukáč, Mgr. Kateřina Krausová and our technician Ing. Jaroslava Vosmíková for their help, support, and great work environment.

Special thanks belong to all our local and international collaborators from the University Hospital in Motol, Institute of Macromolecular Chemistry, 1st Faculty of Medicine, Charles University, and CNR Rome, who collaborated on this project.

Lastly, I would like to thank my parents and partner for their continuous moral support and encouragement in accomplishing my studies.

This work was partially developed in the frame of the project Next Generation EU (NPO Exceles NCI, reg.n. LX22NPO5102)

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Abstract (EN)

Cancer represents the second leading cause of death in developed countries. Traditional cancer treatments, such as surgery, radiation, hormonal therapy, and chemotherapy, still demonstrate limits in their efficacy, especially in the late stages of the disease, despite the important progress already made. Immunotherapy and targeted therapies are becoming increasingly appealing for designing more selective and effective cancer treatments. Due to their unique properties, nanoparticles (NPs) have been investigated for inducing direct toxicity in the tumor, delivering anti-cancer drugs into the tumor microenvironment (TME), or improving tumor imaging. Some of them are already studied in preclinical and clinical settings. Consequently, we believe the NPs' potential to improve current anti-cancer treatment strategies deserves further investigation, including their possible impact on the patient's immune system.

In the first part of this study, we tested the biocompatibility, direct cytotoxicity, and biodistribution of upconversion/magnetic silica-coated NaGdF₄:Yb³⁺/Er³⁺ NPs. We found that the NPs were biocompatible at lower concentrations with direct dose-dependent cytotoxic effects on both tested murine melanoma and non-tumoral fibroblast cell line. Interestingly, they showed mainly perivascular distribution in the tumor, liver, and brain without any apparent signs of systemic toxicity.

Next, we examined the direct *in vitro* toxicity of TiO₂ prolate nanospheroids on human cancer cell lines, murine fibroblasts, and immune cells, in the dark and after ultraviolet (UVC) irradiation to test the NPs' potential as photosensitizers. TiO₂ NPs did not affect the viability of murine cells. A dose-independent cytotoxicity in two human cancer cell lines in the dark was observed. However, NPs did not potentiate the UVC-mediated toxic effects.

In the third part of the study, we investigated the potential of functionalized recombinant human ferritin-based NPs (THE-10 NPs) in boosting the response to anti-cancer treatments *in vitro* and *in vivo*. *In vitro*, the construct itself was not cytotoxic. However, it enhanced the dose-dependent anticancer effect of the antibody-drug conjugate (ADC) Trastuzumab-Deruxtecan (T-DXd) therapy, especially at lower concentrations, in a human melanoma cell line. *In vivo*, in syngeneic and xenograft mouse models of colorectal and pancreatic cancers, THE-10 NPs exerted synergistic effects with irinotecan and gemcitabine chemotherapy, sacituzumab govitecan ADC therapy, and even the anti-cancer effects of another ferritin-based NPs, THE-0504.

In the last part of the study, we decided to examine whether the NPs' cytotoxic effects, observed in previous experiments, could affect the immunogenicity of treated cancer cells and influence the consequent immune response. For this purpose, we used nickel-doped maghemite NPs (FN NPs), now considered promising nanocarriers, that showed direct dose-dependent cytotoxic effects on mouse colorectal carcinoma cell line *in vitro*. After incubating these cells with a cytotoxic dose of FN NPs, we evaluated if the treated cancer cells and/or their lysates could influence the expression of activation/maturation markers in murine macrophages, dendritic cells (DCs), and T cells. The NP-induced cytotoxicity on the cancer cells neither influenced the activation/maturation phenotype of the tested immune cells nor their responsivity. It also did not prevent DCs from generating cancer-cell reactive T lymphocytes in DC-T cell mixed cultures. To verify if these findings could be translated to humans, these experiments were repeated using human prostate cancer cell lines and *ex vivo*-produced immune cells obtained from hormone-refractory metastatic prostate cancer patients, obtaining similar results in the monocyte-derived DC and T lymphocyte mixed cultures. However, FN NPs were directly cytotoxic in only one of the two tested cancer cell lines, underlining the different susceptibility of cancer cells to treatments.

In summary, novel NP-based treatments appear viable tools for anti-cancer therapies, opening new possibilities for implementing modern chemo-or immunotherapeutic drugs' efficacy. However, further evaluation of their influence on immune cells' anti-cancer responses appears necessary.

Abstrakt (CZ)

Rakovina je druhou nejčastější příčinou úmrtí ve vyspělých zemích. Tradiční léčba rakoviny, jako je chirurgie, ozařování, hormonální terapie a chemoterapie, stále vykazuje limity ve své účinnosti, zejména v pozdních stádiích onemocnění, a to navzdory významnému pokroku, kterého již bylo dosaženo. Imunoterapie a cílené terapie jsou stále atraktivnější pro navrhování selektivnějších a účinnějších způsobů léčby rakoviny. Vzhledem ke svým jedinečným vlastnostem byly nanočástice (NP) zkoumány pro indukci přímé toxicity v nádoru, dodávání protirakovinných léků do mikroprostředí nádoru (TME) nebo zlepšení nádorového zobrazování. Některé z nich jsou již studovány v preklinických a klinických podmínkách. V důsledku toho se domníváme, že potenciál NP zlepšit současné strategie protinádorové léčby si zaslouží další zkoumání, včetně jejich možného dopadu na imunitní systém pacienta.

V první části této studie jsme testovali biokompatibilitu, přímou cytotoxicitu a biologickou distribuci upkonverzních/magnetických oxidem křemičitým potažených $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ NP. Zjistili jsme, že NP byly biokompatibilní v nižších koncentracích s přímými cytotoxickými účinky závislými na dávce jak na testovaný myší melanom, tak na nenádorovou fibroblastovou buněčnou linii. Je zajímavé, že vykazovaly hlavně perivaskulární distribuci v nádoru, játrech a mozku bez zjevných známek systémové toxicity.

Dále jsme zkoumali přímou *in vitro* toxicitu TiO_2 prolate nanosferoidů na lidské rakovinné buněčné linie, myší fibroblasty a imunitní buňky ve tmě a po ultrafialovém (UVC) ozáření, abychom otestovali potenciál NP jako fotosenzibilizátorů. TiO_2 NP neovlivnily životaschopnost myších buněk. Byla pozorována na dávce nezávislá cytotoxicita u dvou lidských rakovinných buněčných linií ve tmě. NP však nezesilovaly toxické účinky zprostředkované UVC.

Ve třetí části studie jsme zkoumali potenciál funkcionalizovaných rekombinantních NP na bázi lidského feritinu (THE-10 NP) při posilování odpovědi na protinádorovou léčbu *in vitro* a *in vivo*. *In vitro* nebyl konstrukt samotný cytotoxický. Zesílil však na dávce závislý protirakovinný účinek léčby konjugátem protilátka-léčivo (ADC) trastuzumabem-deruxtekanem (T-DXd), zejména v nižších koncentracích, v buněčné linii lidského melanomu. *In vivo* u syngenních a xenograftových myších modelů kolorektálního a pankreatického karcinomu vykazovaly THE-10 NP synergické účinky s chemoterapií irinotekanem a gemcitabinem, ADC terapií sacituzumabem-deruxtekanem a dokonce i s protirakovinnými účinky jiných NP na bázi feritinu, THE-0504.

V poslední části studie jsme se rozhodli prozkoumat, zda cytotoxické účinky NP, pozorované v předchozích experimentech, mohou ovlivnit imunogenicitu léčených rakovinných buněk a ovlivnit následnou imunitní odpověď. Pro tento účel jsme použili niklem dopované maghemitové NP (FN NP), nyní považované za slibné nanonosiče, které vykazovaly přímé cytotoxické účinky závislé na dávce na buněčnou linii myšího kolorektálního karcinomu *in vitro*. Po inkubaci těchto buněk s cytotoxickou dávkou FN NP jsme hodnotili, zda by ošetřené rakovinné buňky a/nebo jejich lyzáty mohly ovlivnit expresi aktivačních/maturačních markerů v myších makrofázích, dendritických buňkách (DC) a T buňkách. NP-indukovaná cytotoxicita na rakovinných buňkách neovlivnila aktivační/maturační fenotyp testovaných imunitních buněk ani jejich odezvu. Také nezabránila DC v generování T lymfocytů reaktivních na rakovinné buňky ve smíšených kulturách DC-T buněk. Aby se ověřilo, zda lze tyto nálezy přenést na lidi, byly tyto experimenty opakovány s použitím buněčných linií lidského karcinomu prostaty a *ex vivo* produkovaných imunitních buněk získaných od pacientů s hormon-refrakterním metastatickým karcinomem prostaty, přičemž se získaly podobné výsledky ve smíšených kulturách z monocytů odvozených DC buněk a T lymfocytů. Nicméně FN NP byly přímo cytotoxické pouze v jedné ze dvou testovaných rakovinných buněčných linií, což podtrhuje odlišnou náchylnost rakovinných buněk k léčbě.

Stručně shrnuto, nové léčby založené na NP se jeví jako životaschopné nástroje pro protirakovinné terapie, které otevírají nové možnosti pro implementaci účinnosti moderních chemo- nebo imunoterapeutických léků. Zdá se však nezbytné další hodnocení jejich vlivu na protinádorové reakce imunitních buněk.

List of abbreviations

ACT	adoptive cell transfer
ADC	antibody-drug conjugate
ADCC	antibody-dependent cell cytotoxicity
APC	antigen-presenting cell
BiTE	bispecific T-cell engager
BMDC	bone marrow-derived dendritic cell
CAR	chimeric antigen receptor
CD	cluster of differentiation
cGAS	cyclic guanosine monophosphate-adenosine monophosphate synthase
COVID	coronavirus disease
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
DAMP	danger-associated molecular pattern
DC	dendritic cell
Dox	doxorubicin
DR-5	death receptor 5
EBV	Epstein-Barr virus
EGFR	epidermal growth factor receptor
EMA	European Medicines Agency
EPR	enhanced permeability and retention effect
FDA	Food and Drug Administration
FN NP	nickel-doped ferric oxide-based nanoparticle
FtH	ferritin heavy chain
FtL	ferritin light chain
HBc	hepatitis B virus core antigen
HER2	human epidermal growth factor receptor 2
HFt	human ferritin
HIF-1	hypoxia-inducible factor 1
HPMA	N-(2-Hydroxypropyl)methacrylamide
HPV	human papilloma virus
HSV	herpes simplex virus
ICI	immune checkpoint inhibitor

IDO1	indoleamine 2.3-dioxygenase 1
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
KIR	killer immunoglobulin-like receptor
LAG-3	lymphocyte-activation gene 3
LA-ICP-MS	laser ablation inductively coupled plasma mass spectrometry
mAb	monoclonal antibody
MAGE-A4	melanoma-associated antigen 4
mCRPC	metastatic castrate-resistant prostate cancer
MHC I/II	major histocompatibility complex I/II
moDC	monocyte-derived dendritic cell
MPI	magnetic particle imaging
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRSA/VRSA	methicillin/vancomycin-resistant <i>Staphylococcus Aureus</i>
NAPOLI-1	NAnoliPOsomaLIrinotecan
NIR	near-infrared
NK	natural killer cell
NP	nanoparticle
NRP	neuropilin
NSCLC	non-small cell lung carcinoma
PACA	poly(alkylcyanoacrylate)
PAMP	Pathogen-associated molecular pattern
PD-1	programmed cell death 1
PD-L1/2	programmed cell death ligand 1/2
PEG	polyethylene glycol
PLGA	poly(lactide-co-glycolic) acid
PRR	pattern recognition receptor
RGD	arginine-glycine-aspartic acid peptide
ROS	reactive oxygen species
siRNA	small interfering RNA
SPION	superparamagnetic iron oxide nanoparticle

STING	stimulator of interferon genes
TAA	tumor-associated antigen
TCR	T cell receptor
TCR T	TCR-engineered T cells
T-DXd	Trastuzumab-Deruxtecan
TGFβ1	transforming growth factor β1
TIL	tumor-infiltrating lymphocyte
TIM-3	T-cell immunoglobulin and mucin-domain 3
TME	tumor microenvironment
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
UVC	ultraviolet C
VEGF	vascular endothelial growth factor
WHO	world health organization
α-MSH	alpha-melanocyte-stimulating hormone

1 Introduction

Even in 2024, cancer still remains a considerable global health challenge. As of now, it represents the second leading cause of death and morbidity in Europe, with more than 3,5 million new cases recorded every year and almost 2 million deaths predicted for 2024, according to World Health Organization (WHO) official statistics (WHO - Europe 2024). In addition to traditional cancer treatments such as surgery, radiation, chemotherapy, and recently immunotherapy, new methods have been developed to target tumors more directly and reduce the side effects of systemic treatments or enhance the overall effectiveness of the therapy. Thanks to the technical advancements over the past thirty years, it is now possible to prepare nanoconstructs of different origins and with different characteristics to employ as carriers of therapeutic molecules or as detectable materials for diagnostic purposes. These nanoparticles can target the tumor microenvironment directly or after coating with tumor-specific targeting molecules such as antibodies or ligands of tumor-expressed receptors. However, further research is needed to understand the possible effects that nanoconstructs can have not only on tumor cells but also on non-tumor cells, including immune cells. Studies on these aspects are essential for safe nanoparticle application in human patient treatments. The first part of this thesis provides an overview of the current knowledge on this topic, while the second part details our experimental experience.

1.1 Cancer treatment

Traditional cancer treatments, surgical resection, chemotherapy, and radiotherapy, or their combinations, proved to have only limited therapeutic efficacy, depending on the stage of the treated tumors, and are often associated with profound side effects and still account for significant rates of recurrence (Kaur, Bhardwaj and Gupta 2023). As yet, more novel and innovative approaches are still needed, and one such emerging approach seems to be selective tumor-targeting treatment and immunotherapy. Since the 1990s, immunotherapeutic approaches have rapidly developed and taken their spot next to traditional therapies as a new standard treatment for many patients (Zhang and Zhang 2020).

Immunotherapy, in general terms, represents approaches that exploit and/or modulate the body's own immune cells and boost immune mechanisms to specifically recognize cancer cells and fight them. However, even the most current immunotherapies are able to induce the desired complete response only in a limited number of patients and are often associated with severe side effects as well. Now, it is known that such a therapy failure may be due to an immunosuppressive microenvironment at the side of the tumor [more detailed at (Pitt, et al. 2016)].

Solid tumors are not exclusively composed of cancer cells; healthy stromal, endothelial, and nervous cells, together with tumor-infiltrating leucocytes, are also integral parts of the cellular tumor mass. The extracellular matrix, that comprises secreted proteins like collagen and other fibers, together with healthy and tumor cell products like cytokines, growth factors, and other molecules, form a complex structure called the tumor microenvironment (TME) (Seager, et al. 2017). All these components are in strict relationships and continuously networking. According to its conformation and function, the TME can be more or less permissive in being penetrated and aggressed by commonly used therapeutics (chemical, biological). The development of targeted therapies based on nanoconstructs has appeared as a promising advancement to overcome these limitations (Saha, et al. 2023, Vannucci 2015, Chenab, et al. 2024).

There are many ways to modulate immune responses against cancer cells. These interventions are based on our expanding understanding of the complicated relationship between cancer development and patients' immune responses and will be discussed in the next chapters.

1.2 The immune system vs. cancer

The immune system plays a critical role in protecting our body against infections and malignancies. It has two main branches: the innate and adaptive immune systems. Typically, the intrinsic part of the immune system, particularly macrophages and natural killer (NK) cells, represents a first line of defense because it identifies and destroys abnormal cells and pathogens. Macrophages act via pattern recognition receptors (PRRs), which recognize common patterns in pathogens by pathogen-associated molecular patterns (PAMPs) and danger signals from altered cells by damage-associated molecular patterns (DAMPs) (Chen, et al. 2024). NK cells can recognize and destroy transformed or virally infected cells that have altered major histocompatibility complex class I (MHC I) molecules, which are the molecules qualifying the selfness of an organism's cells (Karmakar, et al. 2024). In contrast, the adaptive immune system (T and B lymphocytes) creates a specific and long-term defense due to its capability to recognize and memorize unique antigens in pathogens or malignant cells (Gavil, Cheng and Masopust 2024). This happens by an antigen-specific response following the priming of T-lymphocytes by the antigen-presenting cells (APC), especially dendritic cells (DC). They phagocyte and elaborate the debris of innate immunity-killed cells to peptides that, complexed with the MHC class I molecules, are cross-presented and exhibited on their surface for priming and activating the specific T lymphocytes (CD8+, cytotoxic T lymphocytes - CTLs). These will specifically target and destroy the cells for which they were enabled to recognize as aberrant. The correct play of innate and adaptive immunity mediated by the DC cells is crucial for ablating the tumor cells (Ye, et al. 2024). Cellular regulation of the recognition and cytotoxic response by the CTL is an attribute of the immune checkpoint molecules [e.g. anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4), anti-programmed cell death 1 (PD-1), T-cell immunoglobulin and mucin-domain 3 (TIM-3)] through recognition of their ligands on regular cells [cluster of differentiation 86 (CD86), anti-programmed cell death-ligand 1/2 (PD-L1/2), Galectin-9, respectively]. This control mechanism is used by cancer cells that, over-expressing the ligands, can exhaust the CTL and block the cytotoxic anti-cancer response. The understanding of this immune escape mechanism by the tumor has led to the development of the currently used immunotherapies for rescuing the proper CTL cytotoxicity against the cancer cells (Sinicrope and Turk 2024).

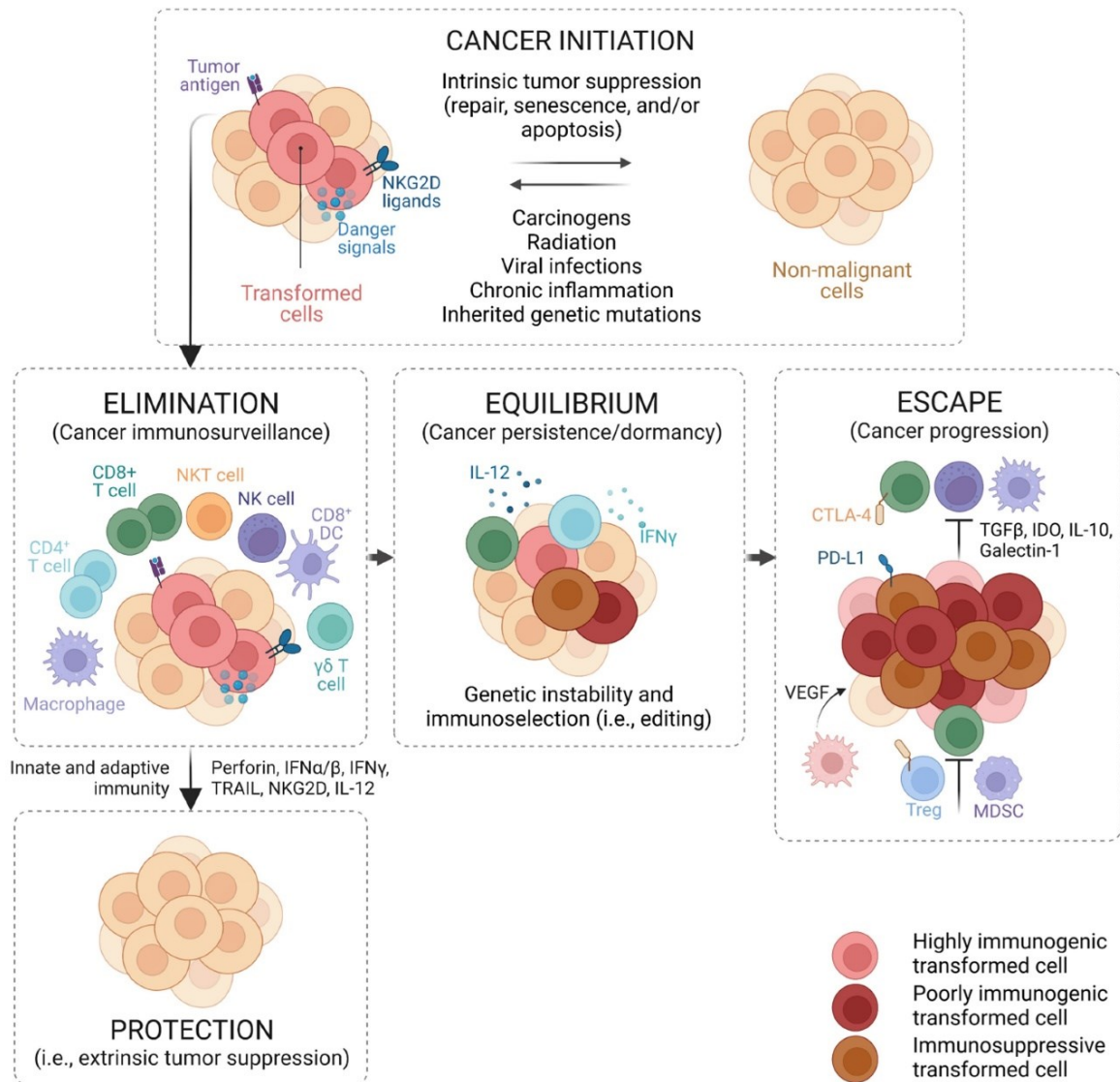
The importance of the immune system in fighting and preventing cancer was first brought about as early as the beginning of the 20th century. In 1909, Paul Ehrlich was the first to suggest that the immune system might be able to prevent cancer (Ehrlich 1909). F.M. Burnet and L. Thomas

revisited this hypothesis about 50 years later. They independently suggested the existence of tumor-associated antigens (TAA) that enable the recognition of aberrant cells by the immune cells with the subsequent elimination of the starting tumor (Burnet 1957, Thomas 1959). Even though this idea was initially rejected, it created a basis for the immune surveillance theory (Burnet 1970).

In the late 1990s and the beginning of the 21st century, the immune surveillance hypothesis was adapted and expanded to the now widely accepted theory of cancer immunoediting, or the “theory of 3E”. The 3E represents three distinctive phases of cancer immunoediting: Elimination, Equilibrium, and Escape (Fig. 1) (Dunn, Old and Schreiber 2004).

During the elimination phase, the cells of both innate and adaptive immune systems recognize transformed cells and destroy them. The main players during this phase are NK cells and T lymphocytes. The ideal outcome of this phase is the complete ablation of all transformed cells. This phase is often also referred to as immune surveillance. The equilibrium phase is set when the immune cells fail to completely clear all cancer cells. This phase is defined by a provisory balance between ablation activity by the immune cells and the replication capacity of the cancer cells. Some cancer cells can even enter the condition of “dormancy” by temporarily halting the reproductive activity but are ready to restart under appropriate stimulation (e.g., pro-angiogenic factors). During the last phase, the escape phase, the transformed cells overcome the immune eradication and start to divide uncontrollably, expanding the tumor mass. More and more dividing cancer cells give rise to progressive tumor genetic inhomogeneity, antigenic changes, and the development of less immunogenic clones that are less likely to be recognized by immune cells. Typical for this phase is the development of immunosuppressive mechanisms conditioning the immune response and the immune cell behavior in a way that, consequently, diminishes the anti-cancer response and helps the tumor establishment [(Dunn, Old and Schreiber 2004), more detailed at (Ribatti 2017), and (Lasek 2022)]. The most recent treatments try to target the tumor microenvironment and its immune compartment directly to rescue an effective anticancer immune response.

Fig.1 The phases of the 3E theory of cancer immunoediting (Suliman, et al. 2023).



1.3 Immunotherapy

As mentioned above, cancer immunotherapy emerged as a new approach to cancer treatment. It quickly gained interest and secured its spot next to traditional anti-cancer therapies. Cancer immunotherapy offers an alternative way of treatment based on harnessing/boosting/reactivating the patient's existing immune system to recognize and attack cancer cells selectively. Cancer immunotherapy can be generally classified as active or passive, depending on how it utilizes the immune system (Galluzzi, et al. 2014).

1.3.1 Active immunotherapy

Active immunotherapy induces the immune response by directly activating the patient's immune cells. From this point of view, oncolytic viruses, anti-cancer vaccines, DC-based vaccines, and checkpoint inhibitors (CTLA-4, PD-1, PD-L1/2)] may be classified as active immunotherapies (Galluzzi, et al. 2014).

1.3.1.1 Oncolytic viruses

Oncolytic viruses are non-pathogenic viral strains that infect and kill specifically cancer cells (Španielová and Brdička 2023). This type of immunotherapy can be further classified based on the anti-cancer activity of the viruses into two main categories.

a) Viruses with direct cytopathic effects on cancer cells. They overload the cellular metabolism upon infection or produce metabolites that are toxic selectively to cancer cells (Phan, et al. 2018).

b) Viral particles genetically engineered that are endowed with various desired properties. Genetically engineered viruses may:

- encode enzymes to convert pro-drug into its cytotoxic drug form (Lampe, et al. 2013)
- carry proteins to trigger cell death signaling selectively in transformed cells (Wyatt, Müller and Tavassoli 2019).
- deliver short-hairpin messenger RNA (mRNA), which inhibits factors necessary for the survival of cancer cells (Jiang, et al. 2015, Wang, et al. 2008).
- support the expression of immunostimulatory molecules or costimulatory receptors (Ylösmäki, et al. 2021).

By damaging and killing cancer cells, oncolytic viruses release many pro-inflammatory molecules and TAAs to induce or boost the anti-cancer response by a phenomenon known as the abscopal effect, where localized treatment with an oncolytic virus can lead to general immune system activation and systemic anti-tumor immune responses active also on tumor localizations far from the initially treated lesion (Kepp, et al. 2020, Kakiuchi, et al. 2021).

However, the effectiveness of oncolytic viruses in immunotherapy can be compromised by patients' innate and adaptive immune mechanisms. As with other viral particles, they may be neutralized by antibodies and complement system or sequestered by phagocytic cells, mainly tissue-resident macrophages [more detailed at (Raja, et al. 2018)].

There is currently one oncolytic virus therapy approved by the Food and Drug Administration (FDA) for the treatment of cancer: T-VEC (Imlygic®), a modified herpes simplex virus (HSV) approved for subsets of patients with melanoma (fda.gov).

1.3.1.2 Dendritic cell (DC)-based immunotherapy

DC-based immunotherapy, or DC-based vaccination, relies on patient-derived DCs loaded/pulsed with a source of TAA. Therapeutic DC vaccines are classified based on antigen loading or DC modification before infusion. The source of the TAA can vary from autologous (Liau, et al. 2023) or allogenic (Podrazil, et al. 2015) cancer lysates to cancer cell-derived mRNA (De Keersmaecker, et al. 2020), TAA-derived peptides (Ding, et al. 2021), or their coding mRNA (Zhu, et al. 2023). There is also evidence that DC functionality can be potentiated *in situ* (at the tumor site) by intra-tumoral administration of immunomodulators [(Asic, et al. 2024); more detailed at (Hammerich, et al. 2016)].

Nevertheless, it should be considered that DC-based vaccines may have a weak anti-tumor immune response, at least when used in the form of monotherapy, and they depend critically on the patient's function of the immune system (Lee, Yam and Mao 2023, Wculek, et al. 2020).

There is currently one DC-based anti-cancer immunotherapy approved by the FDA, sipuleucel-T (Provenge®) (fda.gov). It is a tumor-targeting DC-based therapy for patients with metastatic castrate-resistant prostate cancer (mCRPC) (Laureano, et al. 2022).

1.3.1.3 Anti-cancer vaccines

Anti-cancer vaccines are DNA/mRNA- or peptide-based vaccines designed to be employed as a prevention in high-risk patients or as a therapy. They rely on the activation/maturation of APCs followed by the subsequent activation of the adaptive immunity cells against the tumor. They can be delivered naked or carried by vectors like non-pathogenic viruses, bacteria, or yeast [more detailed in (Galluzzi, et al. 2014)].

a) DNA/mRNA-based anti-cancer vaccines

DNA or mRNA vaccines are based on the administration of TAA-coding DNA or mRNA. Promising results were obtained using oncolytic viruses as vectors, creating oncolytic vaccines, or with attenuated bacteria expressing TAA proteins (Rousseau, et al. 2001). This year, the FDA approved the application of WGC-043, an Epstein-Barr virus (EBV)- related mRNA cancer vaccine (fda.gov).

b) Peptide-based anti-cancer vaccines

Peptide-based anti-cancer vaccines are based on administering TAA peptides to the patient in combination with an immunostimulatory adjuvant, typically by intramuscular, intradermal, or subcutaneous route. These peptides are taken up by local APCs, mainly DCs, that mature in the presence of the adjuvant and prime an immune reaction against the introduced TAA (Liu, et al. 2021). One example of such therapy is the human papillomavirus 16 (HPV-16) targeting peptide vaccine for the E6 and E7 proteins in female patients with HPV-16-positive, high-grade vulvar intraepithelial neoplasia alone (Kenter, et al. 2009) or in combination with nivolumab (Sousa, et al. 2022)

1.3.1.4 Immunostimulatory cytokines

Another branch of anti-cancer immunotherapy is using immunostimulatory cytokines. Despite their potential, most immunostimulatory cytokines provide only a limited effect as a single therapy. As a result, they are primarily used in combination with other treatments as adjuvant to enhance the overall efficacy (Propper and Balkwill 2022).

There are currently two cytokines approved by the FDA as a standalone anti-cancer treatment: interleukin-2 (IL-2; known as Aldesleukin or Proleukin) is approved for the treatment of metastatic melanoma and renal cell carcinoma, and interferon α (IFN- α ; known as Intron-A or

Roferon A) is approved for patients with hairy cell leukemia, multiple myeloma, or melanoma (Rallis, et al. 2021).

The effectiveness of the cytokine treatment depends on the state of the patient's immune system and can potentially trigger even unwanted and dangerous cytokine cascades (Zhao, et al. 2021, Song 2024).

1.3.1.5 Checkpoint inhibitors (ICI)

Immune checkpoint inhibitor immunotherapy is based on inhibiting the immunosuppressive receptors expressed on activated immune cells, such as T cells or NK cells.

In healthy conditions, checkpoint receptors and their ligands represent a safety mechanism that regulates immune reactions and prevents the over-activation of immune cells and autoimmune responses. Tumor cells can exploit these protective mechanisms and inhibit the anti-cancer immune response (Yoo, et al. 2021).

The leading members of T-cell checkpoint receptors are CTLA-4 (Waitz, Fassò and Allison 2012), PD-1 (Munir, et al. 2013), TIM-3, and lymphocyte-activation gene 3 (LAG-3) (Farkona, Diamandis and Blasutig 2016). Checkpoint receptors are also present in NK cells and are often members of the killer cell immunoglobulin-like receptor (KIR) family (Long 2008).

Therapeutic interventions elicited mostly by monoclonal antibodies (mAb) targeting checkpoint receptors (PD-1, CTLA-4) or their ligands (PD-L1 or PD-L2 in the case of PD-1) aim to rescue the exhausted T lymphocytes. They attempt to reinstate prior anti-cancer responses or to induce a novel immune response by avoiding the checkpoint blockade caused by the tumor-expressed ligands (Naimi, et al. 2022).

Currently, 11 checkpoint inhibitors are approved by the FDA for cancer therapy, targeting CTLA-4, PD-1, or PD-L1 (Paul, et al. 2024).

1.3.1.6 Immunogenic cell death inducers

Some drugs with direct cytotoxic effects on tumor cells used in conventional anti-cancer therapy [i.e., doxorubicin (Dox), oxaliplatin, paclitaxel, cyclophosphamide, and others] can trigger an immune reaction against cancer cells by inducing an immunogenic type of cell death such as apoptosis, autophagy, necroptosis or pyroptosis resulting in a more potent anticancer immune response (Troitskaya, et al. 2022). Several immunogenic cell death inducers were

tested in clinical trials in patients with metastatic colorectal cancer (NCT03388190, NCT04262687), ovarian cancer (NCT01666444), breast cancer (NCT03409198) and other types of cancer [more detailed in (Choi, et al. 2023)].

1.3.1.7 Other active immunotherapies

Active anti-cancer immunotherapies can also employ other methods, such as:

- a) inhibition of immunosuppressive metabolism relies, i.e., on blocking the enzyme indolamine 2,3- dioxygenase (IDO-1) or inhibition of adenosine receptors (Vacchelli, Aranda, et al. 2014)
- b) neutralization of immunosuppressive factors in TME, such as transforming growth factor β 1 (TGF β 1) (Schnurr and Duewell 2013), or adenosine (Yamaguchi, et al. 2014)
- c) (re)-activation of costimulatory receptors: receptors of tumor necrosis factor (TNF) superfamily (TNFR) (Ye, Song and Powell 2013) (Chacon, et al. 2013)
- d) regulatory T cell inhibition by metronomic cyclophosphamide administration (van der Most, et al. 2009)
- e) immune suppressive M2 macrophages depletion by liposomes, including diphosphonates (Choi, et al. 2024)
- f) inhibition/depletion of myeloid-derived suppressive cells by commonly used anti-cancer chemotherapeutic agents 5-fluorouracil (Vincent, et al. 2010) and oxaliplatin (Kim and Kim 2019).

1.3.2 Passive immunotherapy

Passive immunotherapy utilizes pre-made immune components, such as monoclonal antibodies or adoptive cell transfer, to facilitate the anticancer response (Galluzzi, et al. 2014).

1.3.2.1 Tumor-targeting monoclonal antibodies

One of the clinic's most commonly and successfully used immunotherapies is tumor-targeting mAbs (Vacchelli, Eggermont, et al. 2013). Several mAbs have been tested in clinical trials and approved by the FDA and European Medicines Agency (EMA) for first-line treatment for patients with different types of hematological malignancies and solid tumors [more detailed at (Baldo 2016, Toledo-Stuardo, et al. 2024)]. mAbs specifically recognize either the surface

structures on tumor cells or signaling molecules produced in TME and affect the tumor in several different mechanisms, such as:

- a) Inhibition of signaling pathways that are crucial for the proliferation and survival of the tumor cells. For example, cetuximab, a human epidermal growth factor receptor (EGFR) inhibitor, was approved in patients with metastatic colorectal cancer and head and neck cancer (Zenda, et al. 2019, Park, et al. 2019). Bevacizumab is a vascular endothelial growth factor A (VEGFA)-neutralizing mAb that inhibits cancer-related neoangiogenesis and is approved in patients with various types of cancer (Marchetti, et al. 2019).
- b) Direct cell death induction by triggering surface receptors on the transformed cells. Tigatuzumab and drozitumab are mAbs that bind to death receptor 5 (DR-5), a member of the TNFR superfamily of receptors that, upon their activation, trigger an extrinsic way of apoptosis. They were tested in clinical trials in patients with pancreatic and colorectal cancer (NCT01220999, NCT00521404).
- c) mAbs with opsonizing properties can trigger antibody-dependent cell cytotoxicity (ADCC), antibody-dependent cellular phagocytosis, or complement-dependent cytotoxicity. Trastuzumab is a mAb that binds to human epidermal growth factor receptor 2 (HER2). The Fc fragment of the mAb triggers ADCC, leading to cell death of the cancer cells. It was approved and indicated as a treatment for patients with HER2-positive breast cancer and gastric cancer (NCT03613168, NCT04168931).
- d) The carrier or conjugated antibody with anti-cancer molecules or radionuclides. Gemtuzumab ozogamicin is an antibody-drug conjugate (ADC) of anti-CD33 mAb with an anti-cancer drug, calicheamicin, and was approved by the FDA and EMA for the treatment of myeloid leukemia (NCT03727750, NCT00882102, NCT00968071). Another example might be trastuzumab-deruxtecan (T-DXd), approved for patients with HER2-positive cancers. In this ADC mAb, trastuzumab is linked to deruxtecan, a topoisomerase I inhibitor that induces DNA damage to cancer cells (Keam 2020).
- e) “Bispecific T-cell engagers” (BiTE) are chimeric proteins made of two single-chain variable fragments. One fragment is a TAA, and the other is specific for T-cell surface antigen. One example of this mAb is CD19-CD3 BiTE Blinatumomab, approved for patients with B-cell acute lymphoblastic leukemia (Hoffman and Gore 2014)..

1.3.2.2 Adoptive cell transfer (ACT)

ACT immunotherapy is a cell-based treatment that employs collected lymphocytes from the patient's peripheral blood or tumor. The obtained lymphocytes are modified or activated, and after *ex vivo* expansion, the enriched tumor-reactive cells are re-administered to the patient (Humphries 2013, Met, et al. 2019). Genetic engineering often equips immune cells with improved therapeutic potential in different aspects of their function. It can alter or create unique antigen specificity in the cells [TAA-specific T cell receptors (TCR) or chimeric antigen receptors (CAR)], improve their proliferative potential or tumor-infiltrating capability, increase secretion of various molecules or enhance their direct cytotoxicity (Wang and Cao 2020).

a) Peripheral blood lymphocytes

There are currently two types of T cells with genetically altered receptors: TAA-specific TCR-engineered T cells (TCR T) (Baulu, et al. 2023) and CAR T cells (Dabas and Danda 2023). CAR is a transmembrane protein made of two parts. The TAA-targeting domain on the outer membrane is linked to the cytoplasmic domain of the CAR receptor. The cytosolic domain is bound to signal transducing molecules like the CD3 ζ chain of the TCR-CD3 complex, CD28, or 4-1BB costimulatory domains to activate the T cell upon antigen recognition. This conjugation allows the T cell to recognize TAA in a MHC I-independent fashion and directly activate killing mechanisms (Dabas and Danda 2023). In contrast, TCR T cells are able to recognize only the MHC-presented peptides (Shafer, Kelly and Hoyos 2022).

While this treatment is applied to hematologic cancers, this year, the FDA approved the use of Tecelra (Afamitresgene autoleucel) for adult patients with unresectable or metastatic synovial cell sarcoma. It is the first approved TCR T cell therapy for solid tumors, and it recognizes fragments of melanoma-associated antigen 4 (MAGE-A4) protein expressed in some synovial cell sarcomas (fda.gov).

b) Tumor-infiltrating lymphocytes (TIL)

Adoptive cell therapy with TILs utilizes tumor-specific T lymphocytes obtained from the patient's tumor. Harvested T cells are expanded *ex vivo* and re-administered to the patient after a lymphodepletion. Subsequent IL-2 administration supports the engraftment of the treatment (Met, et al. 2019).

Adoptive TIL treatment showed promising results in patients with metastatic melanoma, colorectal cancer, or ovarian cancer [(Mullard 2024), NCT04426669, NCT05902520, NCT03287674]. In 2024, the FDA approved Lifileucel (Amtagvi), the first tumor-infiltrating lymphocyte therapy to treat metastatic melanoma (Mullard 2024).

1.3.2.3 Nanoparticles in immunotherapy

As was mentioned above, current immunotherapies have limited response rates and, in some cases, severe side effects. To overcome these problems, new strategies or treatment modalities were elaborated to enhance the delivery and efficacy of immunotherapeutic agents. One such strategy is the combination of immunotherapies with nanoparticles, which can be engineered to interact with the immune system in specific ways and have numerous advantages compared to immunotherapy alone (Iscaro, Howard and Muthana 2019, Choi, et al. 2024).

1.4 Nanoparticles (NPs)

Nanoparticles are small constructs with a typical size below 100 nm. In the last decades, marked advancements have been made in this field, and various types of nanoparticles have been investigated in biomedical applications, including cancer therapy, cancer immunotherapy, and imaging (Siddique and Chow 2020).

1.4.1 Application of nanoparticles in anti-cancer therapies

Anti-cancer treatments can be administered either locally or systemically. Even though locally (intra-tumoral) administered treatment can be, in some circumstances, more effective, most anti-cancer therapies are administered systemically (intravenously, orally, subcutaneously) (Nelson, Fisher and Robinson 2014). Conventional anti-cancer drugs are known to have short circulation time and are relatively quickly cleared through renal filtration due to their small size. Furthermore, most of the approved chemotherapeutics cannot differentiate between healthy and transformed cells, which may lead to severe off-target side effects (Misra, Acharya and Sahoo 2010). Many anti-cancer drugs do not possess some necessary properties to be administered systemically, i.e., they can be unstable and quickly degradable in circulation, hydrophobic, or poorly soluble. These unfavorable characteristics can be overcome by encapsulating the drugs inside the nanoparticle structure or binding to the surface via a linker molecule. The size of the nanoparticles, bigger compared to the traditional molecular size of chemotherapeutic drugs,

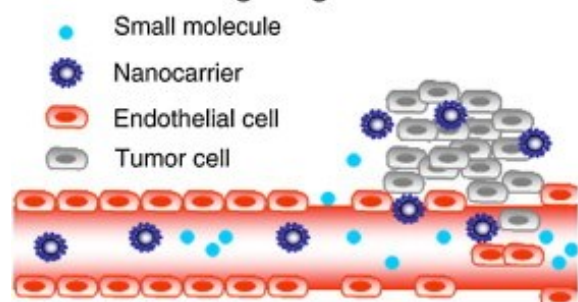
further allows them to exploit either passive or active tumor-targeting functions, taking advantage of the unique features of the tumor microenvironment structure (Rodríguez, et al. 2022). One of the most challenging aims of nanotherapeutics is to design a system for precise delivery and drug release to targeted tumor cells while decreasing the severe side effects on the healthy tissues and, notably, the immune system (Tyagi and Subramony 2018).

1.4.1.1 Active and passive tumor targeting

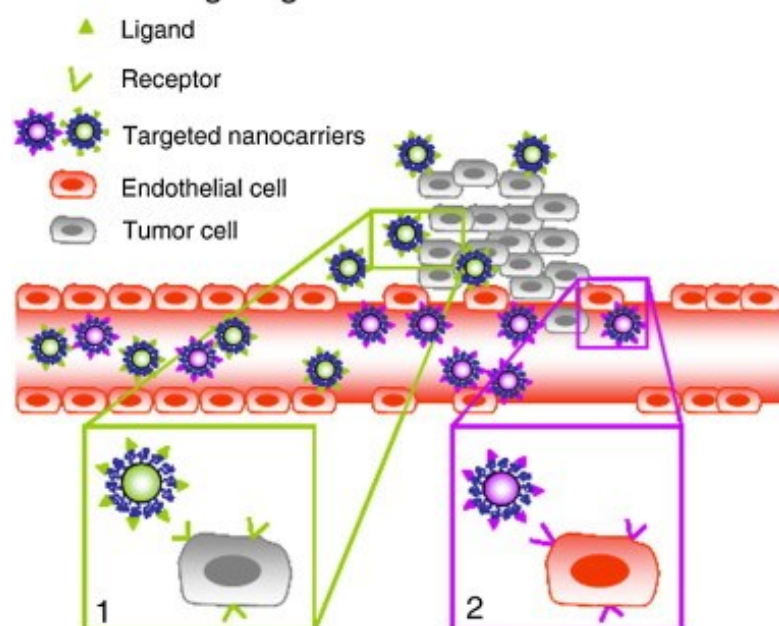
The targeting of nanoparticles in a tumor is primarily based on the fenestrated endothelium of the newly created vessels in the tumor. As the tumor grows, it quickly becomes starved for nutrients and oxygen due to its distance from capillary oxygen exchanges. As a result, it starts producing molecules, such as hypoxia-inducible factor 1 (HIF-1) and vascular endothelial growth factor (VEGF), that stimulate and support the growth of new blood vessels (neoangiogenesis) (Mantovani, et al. 2008). This neoangiogenesis in solid tumors generally happens too fast for the blood vessels to be appropriately formed. On the one hand, underdeveloped blood vessels with fenestrations between the endothelial cells of the veins and, on the other hand, subsequent insufficient lymphatic drainage of the tumor create the conditions for permitting the accumulation of nanoparticles in the TME. This is called the enhanced permeability and retention effect (EPR) (Fang, Islam and Maeda 2020, Matsumura and Maeda 1986). The first phase of this effect involves passive targeting of the tumor as the nanoparticles leak through the fenestrated vessels, followed by retention and accumulation in the extravascular spaces. Under this condition, nanoparticles can directly influence cells and tumor microenvironment. Furthermore, if the NPs carry tumor-specific targeting moieties with therapeutic molecules, they remain trapped inside the TME through active targeting, with even more selective release of the carried therapeutic agent (Shi, et al. 2020) (Fig.2). Similarly, the system can be used for imaging tumor localizations by NPs bringing molecules usable as a radiographic contrast (Dadfar, et al. 2019).

Fig.2: Active and passive targeting, EPR (Danhier, Feron and Pr  at 2010)

A. Passive targeting



B. Active targeting



The potential use of nanoparticles depends largely on their structure and the material(s) of which they are composed. To better understand this rapidly evolving field, nanoparticles are divided into main groups based on the materials they were made of.

1.4.2 *Synthetic nanopolymers*

Synthetic nanopolymers are a specific group of nanoparticles based on more or less complex macromolecular constructs that can be chemically synthesized as assembled repeats of one or more monomeric units. Polyethylenimine, polyamidoimine, PLGA [poly(lactide-co-glycolic acid)], or HPMA [N-(2-Hydroxypropyl)methacrylamide] are a few examples of synthetic

polymers used in creating these types of NPs. They can have different structures (dendrimers, polymersomes, micelles, or linear polymeric therapeutic conjugates) with a variety of possible surface modifications for ensuring the versatility of these constructs, allowing, for example, binding of additional molecules such as anti-cancer drugs [more detailed in (Mishra, Hubenak and Mathur 2013, Ding and Zhu 2018)].

It should be mentioned that these constructs were tested in the *in vitro* or preclinical animal models and have been applied as drug carriers in several clinical trials for cancer treatment (Avramović, et al. 2020, Rijcken, et al. 2022, Sambhi, et al. 2019). There are already synthetic constructs approved by the FDA and EMA as cancer treatments. Examples are TransdrugTM, doxorubicin-loaded PACA [poly(alkylcyanoacrylate)] nanoparticles, approved as a treatment in patients with hepatocellular carcinoma (NCT01655693) and Genexol-PM, paclitaxel loaded polymeric micelle nanoparticles, approved for patients with breast cancer (NCT01784120), also tested in clinical trials in patients with other cancers (NCT01426126, NCT00882973, NCT00877253) (ema.europa.eu, fda.gov).

1.4.3 Organic nanoparticles

As the name suggests, organic nanoparticles are made from organic materials or materials that mimic organic molecules. Thanks to the wide range of possibilities for modifying and functionalizing their basic structure, these types of NPs are mostly well-tolerated, biocompatible, biodegradable, and highly versatile (Mishra, Hubenak and Mathur 2013).

1.4.3.1 Lipid nanoparticles

Lipid nanoparticles are spherical constructs made from lipid molecules, mainly phospholipids, triglycerides, and cholesterol. Lipid NPs can reach bigger dimensions than other types of nanoparticles (up to 400nm) and are further subdivided based on their final structures into liposomes, micelles, and solid lipid nanoparticles (Mishra, Hubenak and Mathur 2013).

Liposomes are spherical nanoparticles with a lipid bilayer and are mainly used as carriers for hydrophobic molecules or drugs that can be encapsulated within their core. Liposomes showed promising results as effective nanocarriers for the targeted delivery of chemotherapeutic drugs for the treatment of brain cancer (Raju, et al. 2023) (Rajsiglova and Vannucci 2018), malignant melanoma (Mirzavi, et al. 2021) and colorectal cancer (Sang, et al. 2021). Clodronate-

encapsulating liposomes were also reported to selectively target and deplete M2 macrophages in a mouse model (Choi, et al. 2024).

Micelles are lipid monolayer nanoparticles organized to encapsulate lipophilic therapeutics within the structure. It was shown that micelles can bypass biological barriers and preferentially accumulate in tumors (Shen, et al. 2016). Such properties of micelles make them an attractive drug delivery system for cancer treatment (Fatfat, Fatfat and Gali-Muhtasib 2022, Rao, et al. 2019).

In contrast, solid lipid nanoparticles are more rigid because they are made of lipid molecules that remain solid even at body temperature. The high stability of solid lipid NPs makes them promising systems for the selective delivery of different types of cargo with controlled drug release (German-Cortés, et al. 2023).

In the last 30 years, several liposomal nanoparticles have been approved by the FDA or EMA as carriers with encapsulated anti-cancer drugs. For example, Doxil, Caelyx, and Myocet carry doxorubicin and are approved in patients with Kaposi's sarcoma, ovarian cancer, multiple myeloma, or metastatic cancer. Onivyde delivers irinotecan and is approved for patients with pancreatic and colorectal cancers, and Vyxeos carries daunorubicin with cytarabine in patients with acute myeloid leukemia (Rodríguez, et al. 2022).

1.4.3.2 Polysaccharide nanoparticles

Polysaccharide nanoparticles are made of monomeric carbohydrate molecules that are chained and linked together. The carbohydrates' origin varies from invertebrates (chitosan NPs) to algae (alginate NPs), bacteria (dextran or hyaluronic acid NPs), or plants (cellulose, starch or pectin, glycogen NPs) (Plucinski, Lyu and Schmidt 2021).

Polysaccharide-based nanoparticles are widely used in *in vitro* and pre-clinical studies as carriers for chemotherapeutic and photodynamic agents for the treatment of solid tumors [(Wang, et al. 2019), more detailed at (Zeng, et al. 2021)]. It also should be mentioned that polysaccharide NPs were tested in clinical trials as an immunomodulatory agent in patients with prostate (NCT00269555), esophageal (NCT03611712), and breast cancer (NCT03314805), and as a tumor-targeted drug carrier loaded with Cetuximab in patients with colorectal cancer (NCT03774680).

1.4.3.3 *Virus-like nanoparticles*

Virus-like nanoparticles are derived from virus capsid proteins. They exploit viruses' natural ability to enter targeted cells and release contents encapsulated inside the particle without the viral genome [nucleic acids, small interfering RNA (siRNA), therapeutic drugs, or pro-drugs]. Although virus-like NPs are used primarily in gene delivery applications and vaccines, they have been tested in drug delivery in cancer (Jeevanandam, Pal and Danquah 2019).

Bluetongue virus nanoparticles with fused HSV-1 thymidine kinase in combination with ganciclovir showed promising results in a human glioblastoma-derived cell line *in vitro* (Thuenemann, et al. 2021). Hepatitis B virus core antigen (HBc)-based virus-like nanoparticles superficially functionalized with an RGD (Arg-Gly-Asp) tumor-targeting moiety and encapsulated doxorubicin were tested in a murine melanoma model *in vivo*, and showed higher inhibition of tumor growth and reduced cardiotoxicity, compared to free Dox (Shan, et al. 2018).

1.4.3.4 *Protein nanoparticles*

Protein NPs are a promising group of nanoparticles made of proteins or polypeptides that might be of natural or synthetic origin. They could be directly conjugated with drugs, or the protein subunits could assemble into a protein cage nanoparticle, with the possibility of encapsulating drugs into their structure or attaching molecules via a linker to the surface. These nanoparticles can be based on various proteins, such as albumin, collagen, gelatin, heat shock proteins, silk, viral proteins, or ferritin (Tarhini, Greige-Gerges and Elaissari 2017).

Protein NPs possess unique properties and high versatility due to the natural characteristics of proteins, the possibilities of their surface modifications, functionalization, stimuli-sensitive controlled release, and biodegradability compared to other types of nanoparticles. They can be prepared through bioengineering strategies without using toxic chemicals or solvents. Protein-based NPs comprise numerous epitopes and domains that can be modified, especially by genetic engineering, to improve their functionality and targeting properties. Additionally, as proteins are naturally amphipathic, their hydrophobic domains enable interactions with hydrophobic anti-cancer drugs and their encapsulation. Lastly, they are naturally metabolized into amino acids, which makes them generally non-toxic and biodegradable in clinical settings (Miao, et al. 2022).

Protein nanoparticles were tested both in preclinical and clinical settings for anticancer therapies. Albumin nanoparticles represent interesting cancer-targeting carriers because many tumor cells overexpress albumin-binding proteins. In animal models, albumin NPs with encapsulated paclitaxel and fenretinide were used in targeted therapy of subcutaneous and intracranial glioma. They were reported to penetrate the blood-brain-barrier and accumulate in both models (Lin, et al. 2016). Silk-based NPs with surface RGD tumor-targeting functionalization showed selective targeting to human cancer cell lines that overexpressed integrins *in vitro* (Bari, et al. 2021). Apoferritin-based nanoconstructs loaded with paclitaxel were tested in a 4T1 mouse model as a photothermal-chemotherapy agent and showed promising results *in vitro* and *in vivo* (He, et al. 2020).

Protein-drug conjugate nanoparticles are also approved in clinical settings. The FDA and EMA approved paclitaxel-conjugated albumin-based nanoparticles (Abraxane/Pazenir) as a treatment for patients with breast cancer, pancreatic cancer, and non-small cell lung carcinoma (NSCLC) (ema.europa.eu, fda.gov).

Ferritin-based nanoparticles

Ferritin is a protein naturally present in most organisms and possesses excellent general biocompatibility and biodegradability as a material. Ferritin nanoparticles are assembled as carriers from 24 light-chain (FtL) and heavy-chain ferritin (FtH) subunits, creating a cage structure with a hollow core suitable for drug encapsulation and surface that can be functionalized with targeting moieties or drugs. The structure of ferritin NP is also pH sensitive as it self-assembles in neutral pH and disintegrates in a highly acidic environment, often found in the TME or lysosomes (Vannucci, Falvo and Failla, et al. 2015). Furthermore, the carrier's FtH subunit can bind to transferrin receptor 1 (TfR1) in humans, which is often overexpressed in rapidly proliferating cancer cells (Li, et al., 2010).

Ferritin nanoparticles were tested in animal models as drug carriers for targeted delivery, in photodynamic therapy, or imaging. Ferritin NPs with encapsulated photosensitizer and a surface targeting sequence for fibroblast activation protein were used in a 4T1 mouse tumor model as a photodynamic therapy sensitizer to target cancer-associated fibroblasts in the TME and to enhance the EPR effect in the tumor (Li, et al., 2018). Human ferritin (HFt)-based NPs superficially functionalized with melanocyte-stimulating hormone (α MSH) or melanoma targeting mAb were reported to selectively target melanoma mouse models both *in vitro* and *in vivo* (Vannucci, Falvo and Fornara, et al. 2012, Falvo, et al. 2013, Vannucci, Falvo and Failla,

et al. 2015). Epirubicin-loaded ferritin-based and mucin-functionalized NPs showed increased targeted drug delivery and anticancer effects *in vitro* and *in vivo* (Yazdian-Robati, et al., 2022). Doxorubicin-loaded ferritin NPs were tested in nude mice with human colorectal carcinoma, melanoma, and breast carcinoma and showed lower toxicity and prolonged survival compared to the free Dox (Liang, et al., 2014). They were also reported to target the HT29 carcinoma cell line and induce increased reactive oxygen species (ROS) production and ferroptosis (Yang, et al., 2019).

Even though many ferritin-based nanoparticles showed promising results in preclinical testing as cancer treatment, no ferritin NP-based anti-cancer therapies are currently tested in clinical trials. There are, however, several clinical trials with ferritin-based NPs as vaccines against EBV (NCT04645147, NCT05683834), COVID-19 (Coronavirus disease) (Ober Shepherd, et al., 2024; Weidenbacher, et al., 2022), influenza (Houser, et al., 2022) and treatment of HIV (NCT05903339).

1.4.4 Inorganic nanoparticles

The third big group of nanoparticles are inorganic NPs, which are made of non-organic materials such as carbon, silica, or metals (Au, Ag, Gd) or their oxides (Fe_2O_3 , Fe_3O_4 , TiO_2). They generally possess better stability when compared to organic NPs (Mauro, Utzeri, et al. 2021).

1.4.4.1 Carbon and silica nanoparticles

Carbon-based nanoparticles showed promising results in biosensing, bioimaging, drug delivery, and theranostic applications (Boakye-Yiadom, et al. 2019). They can be based on different carbon compounds, such as fullerenes, graphene, graphene oxide, or carbon nanotubes, and the material will determine the properties of the NP. For example, graphene NPs were tested in cancer phototherapy and imaging (Nurunnabi, et al. 2014, Chang, et al. 2023). Graphene oxide NPs were reported to selectively target cancer stem cells (Fiorillo, et al. 2015) and are often used as carriers in cancer therapy (Yang, et al. 2020). Fullerenes were tested in cancer treatments such as photodynamic therapy as photosensitizers. They were reported to suppress neoangiogenesis and used as carriers for chemotherapeutic drugs [more detailed in (Fernandes, et al. 2022)]. Carbon nanotubes were also investigated in anti-cancer treatments and imaging. They were applied in photothermal therapy, cancer imaging, or employed as carriers for targeted drug delivery [more detailed in (Tang, et al. 2021)].

Carbon nanoparticles were also tested in clinical trials for NP-guided lymph node resection in patients with advanced gastric cancer (NCT02123407) and as a contrast agent for lymph node tracing in colorectal cancer surgeries (NCT04759820). However, it must be mentioned that no carbon-based NPs are currently approved for cancer treatment by the FDA or EMA.

Silica-based nanoparticles are made of SiO_2 and have been used as carriers in tumor targeting therapy (Jia, et al. 2015), or as a system for controlled drug release, using various strategies such as pH-sensitive formulations, metalloproteinase-sensitive or temperature-responsive linkers for targeting the drug release to the specific milieu of TME (Avedian, et al. 2018, Cheng, et al. 2015, Feng, et al. 2018). Silica-based NPs were also tested in tumor imaging such as magnetic resonance imaging (MRI), photodynamic/photothermal therapy or ultrasound imaging, and in theranostic applications, which is an emerging field in cancer therapy that combines therapy and imaging/diagnostics into a single construct (Baeza and Vallet-Regí 2020).

Silica-based NPs are also being tested in clinical trials for real-time NPs-guided imaging of metastases in patients with head and neck melanoma (NCT02106598) and improved PET imaging in patients with melanoma and malignant brain tumors (NCT01266096).

1.4.4.2 Metal nanoparticles

Metals or their compounds (mainly oxides) within a 1-100 nm size are considered metal nanoparticles. They can be prepared from various metals, such as Gold, Silver, Zinc, Copper, Aluminum, Platinum, Titanium, Gadolinium, or Iron, and are used in varied applications, including cancer treatment, imaging, and theranostic (Mauro, Utzeri, et al. 2021).

Gold-based nanoparticles showed promising results for image-guided photothermal/photodynamic therapy (von Maltzahn, et al. 2009, Jang, et al. 2011, Li, et al. 2020) and as a contrast agent for x-ray imaging and computed tomography (Cole, et al. 2015). A spherical nucleic acid Au NPs composed of small interfering RNAs (siRNAs) targeting the Bcl-2-like protein 12 have been tested in clinical trials for the treatment of recurrent glioblastoma or gliosarcoma undergoing surgery (NCT03020017).

Silver-based nanoparticles have been primarily investigated for their antimicrobial properties but also as anti-cancer therapeutics (Takáč, et al. 2023). They were reported to induce apoptosis by modulating the expression of regulatory genes or signaling pathways, such as p53 or HIF

pathway (Yuan, et al. 2018, Jeong, et al. 2016). They were also suggested to prevent neoangiogenesis and inhibit metastatic migration (Kitimu, et al. 2022). Despite these promising results, no Ag-based nanoparticles are currently in clinical trials for anti-cancer treatment. However, Ag-based NPs have been tested in clinical trials for their antimicrobial properties against *Enterococcus faecalis*, *Candida albicans* (NCT06172023), and methicillin/vancomycin-resistant *Staphylococcus aureus* (MRSA/VRSA) (NCT04431440).

Gadolinium/Gold-based NPs as bimetallic radiosensitizers were reported to boost cancer radiotherapy in a murine breast cancer model (Nosrati, et al. 2023). Gadolinium-based compounds are approved for MRI as a contrast agent (Gadovist). Gd-based nanoparticles showed promising results in I-phase clinical trials in combination with chemoradiation in female patients with cervical cancer (Chargari, et al. 2024) and were also tested for MRI-guided radiation therapy in patients with centrally located lung tumors and pancreatic cancer (NCT04789486).

1.4.4.3 Metal nanoparticles in metalloimmunotherapy

Metal ions play an essential role in homeostasis and regulating immune reactions. Their employment in cancer therapy created a new branch of cancer immunotherapy called metalloimmunotherapy. The high stability, low self-immunogenicity, and toxicity of metal nanoconstructs make them attractive targets for cancer therapy and diagnostics. Various metal nanoparticles are being reported and tested for metalloimmunotherapeutic applications (Suliman, et al. 2023).

Manganese-based NPs, in combination with radiotherapy (Liu, et al. 2022) or doxorubicin (Hou, et al. 2020), can boost the anti-cancer immune response by potentiating the cGAS (cyclic guanosine monophosphate-adenosine monophosphate synthase)-STING (stimulator of interferon genes) pathway. This leads to increased ROS and IFN type I production, secretion of pro-inflammatory cytokines, improved immune cell tumor infiltration, and, as a result, a more robust anti-cancer immune response and prolonged lifespan of the tumor-bearing mice (Liu, et al. 2022, Hou, et al. 2020).

Zinc-based NPs can also stimulate the cGAS-STING pathway (Cen, et al. 2021), and they were also reported to enhance autophagy-dependent DC cross-presentation with subsequent antigen-specific T cell activation and tumor infiltration. It was achieved by the incorporation of an adjuvant (Alum) and targeting moiety (ovalbumin) into the structure, leading to increased ROS

production in the TME and eventually resulting in immunogenic cell death in B16F10 mouse melanoma model, with promising results both *in vitro* and *in vivo* (Zhao, et al. 2024). The combination of Zn-based NPs with doxorubicin can activate macrophages and induce M1 polarization in a 3D *in vitro* model while also inhibiting cancer cell adhesion and migration by downregulation of CD44 (Wang, et al. 2017).

Copper-based NPs were shown to induce M2-to-M1 macrophage polarization, enhance their phagocytic activity and ROS production, and decrease PD-L1 expression. When adoptively transferred into melanoma-bearing mice, they elicited a systemic anti-cancer reaction (Xu, et al. 2021). Other Copper-based nanoparticles were reported to induce DC maturation and activation and improve the efficacy of photothermal therapy *in vivo* (Ge, et al. 2023).

Calcium-based NPs were reported to promote DAMP secretion from cancer cells and induce pyroptosis through ROS induction, cytochrome c release, and activation of caspase-3, leading to immunogenic cell death, increased DC maturation, and T cell activation (Zheng, et al. 2022). They can also promote M2-to-M1 macrophage repolarization and block the expression of immunosuppressive molecules, such as PD-L1 (An, et al. 2022).

Iron-based nanoparticles

Iron is one of the most common earth elements. Iron-based nanoparticles are mainly iron oxides, such as magnetite (Fe_3O_4), maghemite ($\gamma\text{-Fe}_2\text{O}_3$), and mixed ferrites (MFe_2O_4 , where M= Co, Mn, Ni or Zn) (Dadfar, et al. 2019).

Iron-based nanoparticles were tested for targeted drug delivery and, thanks to their imaging properties, for cancer theranostic applications. Superparamagnetic iron oxide nanoparticles SPIONs are a part of this group of NPs, and they share unique magnetic properties that are employed in imaging, MRI and magnetic particle imaging (MPI), and also in applications such as local hyperthermia, magnetic-guided treatments, and magnetic biosensors. Local hyperthermia is created when accumulated SPIONs are exposed to an electromagnetic field, which leads to locally increased temperature and can be employed as an anti-cancer treatment (Dadfar, et al. 2019).

Fe-based NPs were further reported to promote M2-to-M1 macrophage repolarization and to catalyze ROS production through the Fenton reaction (Costa da Silva, et al. 2017). This reaction seemed also further supported by loading L-Arginine into the NPs, which can be converted to nitric oxide by inducible nitric oxide synthase iNOS (Wu, et al. 2021).

Several iron-based nanoparticles are clinically approved as contrast agents for imaging by MRI and MPI: Ferucarbotran (Resovist), Ferumoxide (Endorem), Ferumoxil (GastroMARK), and Ferumoxytol (Rienso), which is also approved as an iron replacement for patients with renal failure and anemia (Rodríguez, et al. 2022). In 2011, amino silane-functionalized superparamagnetic ferric oxide nanoparticles (NanoTherm®) were approved by EMA for the treatment and imaging of glioblastoma via MRI-guided hyperthermia. It was approved for direct intratumoral injection, which allowed for MRI imaging of the tumor mass and for the hyperthermia treatment by applying an alternating magnetic field. Later the EMA approved NanoTherm® for tumor ablation of prostate cancer (Rodríguez, et al. 2022, Foo, et al. 2022).

Furthermore, Ferumoxytol was also reported to enhance iron levels and increase ROS production via the Fenton reaction, which can lead to lipid peroxidation and DNA damage that result in activation of caspase-3 and terminally lead to cell death called ferroptosis (Zanganeh, et al. 2016). ROS are also known to be involved in redox regulation and cellular signaling. They regulate cell death, proliferation, motility, and phagocytic ability in macrophages and are linked to an M1-like polarization (Dadfar, et al. 2019).

Iron-based NPs and hafnium oxide crystalline NPs have recently shown clinical activity as radio-enhancer therapy in patients with metastatic hepatic cancer (NCT04682847), lung non-small cell carcinoma (NCT04505267), and soft tissue sarcoma (NCT02379845).

Despite numerous studies and clinical trials of cancer treatment by metal-based nanoparticles, the possibility of their clinical uses still remains somewhat elusive as only two metal-based nanoparticles were approved by EMA for anti-cancer therapy in patients. Hensify, Hafnium oxide crystalline NPs, were approved in radiotherapy in patients with soft tissue sarcoma, and NanoTherm, iron-based NPs, were approved for thermal ablation with magnetic field in patients with glioblastoma, prostate and pancreatic cancer. Currently, no metal-based nanoparticles are approved by the FDA for use in clinics for patients with solid tumors. It also should be considered that little is known about the immunomodulatory properties of metal-based NPs and their effect on the immune system's innate and adaptive routes (ema.europa.eu, fda.gov).

2 Aims of the study

The aim of this thesis was to attempt to find new strategies for approaching novel NP-based therapies as a new treatment modality. This includes not only their possible antitumor therapeutic performance *in vitro* and *in vivo* but also their safety, particularly towards the tumor microenvironment and the immune system.

The specific objectives of this thesis were:

- 1) To investigate the cytotoxic impact of upconversion/magnetic silica-coated NaGdF₄:Yb³⁺/Er³⁺ nanoparticles on B16F10 murine melanoma and 3T3 murine fibroblast cell lines *in vitro*
- 2) To evaluate NaGdF₄:Yb³⁺/Er³⁺ nanoparticles biodistribution *in vivo*, in a C57Bl/6 mouse model with subcutaneously developed B16F10 melanomas
- 3) To investigate the impact of TiO₂ NPs on viability of A375 human melanoma, PANC1 pancreatic, and SKBR3 breast cancer cell, 3T3 fibroblast and murine splenocytes *in vitro*.
- 4) To determine if the TiO₂ NPs could work as a potential photosensitizer in cancer therapy.
- 5) To evaluate the ability of THE-10 NPs to enhance the anti-cancer activity of Trastuzumab Deruxtecan (T-DXd) *in vitro*.
- 6) To find out whether the THE-10 nanoconstruct could potentiate cancer therapies *in vivo*:
 - To test combinations of THE-10 NPs and chemotherapeutic drug (irinotecan) in murine syngeneic CT26 colorectal carcinoma.
 - To explore if the THE-10 NP can increase the anti-cancer activity of the nanoferritin THE-0504 NP and the ADC sacituzumab govitecan in both syngenic and xenograft mouse models of pancreatic cancer.
- 7) To investigate the direct cytotoxic effect of nickel-doped ferric oxide-based NPs (FN NPs) on murine CT26 colorectal carcinoma and human PC-3 and LNCaP prostate cancer cell lines.
- 8) To test the potential changes in the immunogenicity of the cancer cells after incubation with a toxic dose of FN NPs:
 - To measure the changes in activation/maturation markers in macrophages, DCs, and T cells of human and murine origin upon their exposure to related live cancer cells and/or their lysates.

3 Results

3.1 Biodistribution of upconversion/magnetic silica-coated $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticles in mouse models

In this study, we investigated the direct *in vitro* toxicity of novel silica-coated Gadolinium-based nanoparticles doped with $\text{Yb}^{3+}/\text{Er}^{3+}$ on cancer cells, fibroblasts, and immune cells and their biodistribution *in vivo*. The upconversion property, i.e., the emission of high-energy photons following the absorption of low-energy photons by an immobilized photosensitizer, elicits red/green light emission under near-infrared light (NIR) irradiation. It is very promising in photodynamic tumor therapy and local immunomodulation due to the deep penetration in the tissues of the NIR irradiation and consequent photoactivation of the NPs in place.

For the purpose of the *in vitro* study, we investigated the viability of cancer and non-cancer cell lines. We cultivated B16F10 murine melanoma cell line and 3T3 murine fibroblast cell line with increasing concentrations of NPs. The viability of the treated cells was evaluated by MTT assay, and we observed dose-dependent direct toxic effects of the NPs on both tested cell lines.

To evaluate their biodistribution *in vivo*, we used a C57Bl/6 mouse model with subcutaneously developed B16F10 melanomas. 24h after the NPs treatment, biodistribution was evaluated on frozen sections by LA-ICP-MS (laser ablation inductively coupled plasma mass spectrometry) and compared with sequential sections stained with hematoxylin-eosin and, in immunohistochemistry for endothelia with anti-mouse CD31 antibody. We observed mainly perivascular biodistribution of the NPs in the tested exemplar tissues (tumor, liver, and brain). The treated mice exhibited no signs of systemic toxicity, as indicated by normal behavior and physiological functions. These findings are important in the perspective of possible future theranostic applications of upconversion/magnetic Gd-based NPs for MRI imaging and more efficient identification and treatment of pathological neoangiogenesis in cancer and other diseases.

Contribution: My work in this project focused on testing the *in vitro* toxicity of the provided NPs and the animal model. I was responsible for *in vitro* testing of the NPs' toxicity on tumor, non-tumor, and immune cells, including the final data analysis, and also for the *in vivo* model, which consisted of inoculating tumor cells to the animals, intracardial administration of the treatment, monitoring of the animals, and the final autopsy. I prepared the draft of the methods and results for which I was responsible.



Cite this: *RSC Adv.*, 2017, 7, 45997

Biodistribution of upconversion/magnetic silica-coated NaGdF₄:Yb³⁺/Er³⁺ nanoparticles in mouse models†

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Nanoparticles are constructs that can be used for cellular interventions and targeted drug delivery. They are useful for overcoming the dose-related toxic effects of drugs or diagnostic preparations by predominant or selective accumulation in the pathologic tissues. Gadolinium(III) compounds are largely used as contrast agents in magnetic resonance imaging (MRI) but may have toxic effects, especially in nephropathic patients, due to the dose required for use in MRI. Here, we describe the preparation of new multifunctional NaGdF₄:Yb³⁺/Er³⁺ nanoparticles, their characteristic properties, and some preliminary data about their effect on cell viability and tissue localization. Hexagonal-phase NaGdF₄ nanocrystals that were doped with optically active Yb³⁺ and Er³⁺ ions, were synthesized by coprecipitation of lanthanide chlorides in octadec-1-ene at high temperature, stabilized by oleic acid, and subsequently coated with a thin silica layer. The morphology, elemental composition, crystalline structure, and SiO₂ coating of the prepared NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles were characterized in detail by transmission electron microscopy (TEM) combined with energy-dispersive spectroscopy (TEM/EDX) and selected area electron diffraction (TEM/SAED) and attenuated total reflection Fourier transform infrared (ATR FTIR) spectroscopy. The upconversion and paramagnetic properties of the particles were measured using confocal microscopy and MRI, respectively. The biocompatibility of the NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles was tested *in vitro* using mouse 3T3 fibroblasts and B16F10 melanoma cells. Particle localization was evaluated *ex vivo* in tumor, liver, and brain tissues of B16F10 melanoma bearing mice after intravenous administration. The NaGdF₄:Yb³⁺/Er³⁺@SiO₂ particles proved to be non-toxic at moderate concentrations. Particle localization within the organs was demonstrated by analysis of the tissues using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and showed vascular localization.

Received 7th August 2017
Accepted 19th September 2017

DOI: 10.1039/c7ra08712h

rsc.li/rsc-advances

1. Introduction

Lanthanide-based nanocrystals have gained considerable attention for various biological applications due to their unique luminescent and magnetic properties, which can be easily

controlled by varying the lanthanide ion dopants.^{1–3} In particular, upconversion/magnetic NaGdF₄:Yb³⁺/Er³⁺ nanoparticles have great potential as promising multimodal probes for *in vitro* and *in vivo* bioimaging,⁴ targeted drug delivery,⁵ and image-guided surgery.⁶ Due to the presence of optically active Yb³⁺/Er³⁺ ion pair in the Gd³⁺-containing host lattice, the particles show not only a strong near-infrared (NIR) to visible upconversion luminescence but also a short longitudinal relaxation time *T*₁ that allows simultaneous optical and magnetic resonance (MR) images of healthy and diseased tissues.^{6–8} Moreover, the maximum light absorption of the NaGdF₄:Yb³⁺/Er³⁺ nanoparticles falls within the “optical window” of the biological tissues. The advantages of the particles include a large anti-Stokes shift of ~500 nm, absence of photobleaching and photoblinking, weak autofluorescence, deep light penetration, and reduced photodamage to living organisms.^{9,10} Integration of the magnetic and upconversion features in one particle provides more detailed and accurate non-invasive imaging information

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† Electronic supplementary information (ESI) available: *T*₁ relaxometry (Table S1), calibration curves (Fig. S1), and size distribution histograms (Fig. S2). See DOI: 10.1039/c7ra08712h

than using each method alone, facilitating diagnoses and follow-up therapeutic responses over long time periods.

Biocompatibility, dispersibility in water, and uniform particle size are prerequisite conditions to meet the demands of biomedical applications. Currently, $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticles are generally synthesized by high-temperature coprecipitation of lanthanide chlorides in an organic solvent, *e.g.*, octadec-1-ene, and stabilized by oleic acid.¹¹ However, these particles are hydrophobic and lack functional groups, which presents an obstacle for further surface conjugation of targeting ligands and therapeutic agents. Surface chemistry thus remains a key issue to minimize nonspecific uptake by the reticuloendothelial system, prolong blood circulation time, and improve diagnostic efficiency. Strategies to design water-dispersible particles include ligand exchange¹² or oxidation,¹³ and encapsulation with SiO_2 or polymers.^{14,15}

Gd is a paramagnetic lanthanide that was used more than 30 years ago in the formulation of contrast agents to enhance contrast in magnetic resonance imaging (MRI) due to its large magnetic moment and short relaxation time.¹⁶ Once in the circulation after intravenous administration, it demarks the vascularization of an organ and is widely used for magnetic resonance angiography. However, compromised kidney function is a contra-indication to its administration because Gd filtration is inefficient. Subsequent systemic toxicity is possible, even leading to fatal nephrogenic systemic fibrosis.¹⁷

Studies during the last twenty years have demonstrated the importance of the tumor microenvironment and of therapeutic interventions specifically directed toward cancer cells, infiltrating and surrounding immune cells, stroma cells, stromal structures, vessels, nerves and soluble products.¹⁸ A targeted therapy is not only critical to achieve highly efficient anticancer effects but also necessary to reduce unwanted side reactions in the rest of the organism. At present, the development of nanotherapeutics allows a selective targeting of the cancer cells and the tumor microenvironment. The availability of diagnostic tracers, *e.g.*, gold, iron oxide, gadolinium,^{19,20} or human ferritin loaded with maghemite^{21,22} as carriers of therapeutic agents has opened the field of theranostics, in which the dual functions of diagnosis and therapy are combined in a single nanoconstruct.²³ Moreover, a selective targeting of tumors can be obtained through the enhanced permeability and retention effect (EPR) due to endothelial leakages in the tumor neo-vascularization²⁴ and the reduced lymphatic circulation in the tumor microenvironment with possibility to reach concentrations 5–10 times higher than in normal tissue within 1–2 days.²⁵ Imaging of tumor vascularization and identification of the sites of EPR can help in deciding the type of tumor targeting, as well as the opportunity for anti-angiogenic therapy.

In our study, we synthesized $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@/\text{SiO}_2$ nanoparticles and evaluated them in the context of achieving a new system that, while reducing the general toxic effects by lowering the diagnostic dose, may maintain an efficient vascular localization. This might lead to targeted treatment of cancer or even other pathologies with aggressive neo-vascularization, such as neo-vascular eye diseases.²⁶ For a preliminary evaluation, three main tissues were analyzed (tumor, liver, and brain) as

examples of different vascular conditions: neo-angiogenesis, regular and abundant vascularization associated with reticular-endothelial cells, and vascularization with restricted permeability due to the brain–blood barrier. Biocompatibility, *i.e.*, cell viability, was also tested *in vitro* on tumor and non-tumor mouse cell lines and on immune cells.

2. Experimental

2.1 Chemicals

Lanthanide chlorides, such as anhydrous gadolinium(III), ytterbium(III), and erbium(III) chloride (99%), octadec-1-ene (90%), ammonium fluoride, tetramethoxysilane (TMOS), and Igepal CO-520 [polyoxyethylene (5) nonylphenylether], were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide was acquired from Lach-Ner (Neratovice, Czech Republic), and oleic acid, methanol, ethanol, and acetone were acquired from Lachema (Brno, Czech Republic). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). Ultrapure Q-water (pH 7; resistivity 18.2 M Ω cm) produced using ultrafiltration on a Milli-Q Gradient A10 system (Millipore; Molsheim, France) was used in all experiments.

2.2 Synthesis of $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticles

$\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticles were prepared according to previously described procedure with some modifications.^{27,28} In a typical synthesis, 1 mmol of lanthanide chloride (0.78 mmol GdCl_3 , 0.2 mmol YbCl_3 , and 0.02 mmol ErCl_3) was placed in a 100 ml three-neck flask to which octadec-1-ene (15 ml) and oleic acid stabilizer (6 ml) were added. The flask was heated to 160 °C for 30 min to obtain a homogenous solution and cooled to room temperature (RT), after which a methanolic solution (5 ml) containing NH_4F (4 mmol) and NaOH (4 mmol) was added dropwise. The resulting mixture was slowly heated to 70 °C until all the methanol evaporated, stirred under a gentle flow of argon, and heated to 300 °C for 90 min. After cooling to RT, the resulting $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticles were precipitated in acetone (30 ml), separated using a Sorvall Legend X1 centrifuge (ThermoFisher; Osterode, Germany), washed three times with ethanol, separated by centrifugation, and stored in hexane.

2.3 Synthesis of $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@/\text{SiO}_2$ nanoparticles

The surfaces of the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticles were coated with silica using a microemulsion technique.²⁹ $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticles (30 mg) were dispersed in hexane (10 ml) and Igepal CO-520 (0.5 ml) and 25% aqueous ammonia (80 μl) were added. After sonication for 20 min, a stable emulsion was formed; TMOS (20 μl) was added and the mixture was stirred (600 rpm) at RT for 2 days. The resulting $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@/\text{SiO}_2$ particles were precipitated with acetone (5 ml), washed three times with water/ethanol (1 : 1 v/v), and stored in water.



2.4 Viability test of cells incubated with NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles

Murine B16F10 melanoma and fetal 3T3 fibroblast cell lines (ATCC; Manassas, VA, USA) were used to evaluate the possible cytotoxic effects and biocompatibility of NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles using the MTT cell viability assay. The 3T3 cells were used not only as a common model of non-tumor cells, but also because fibroblasts are important component of the tumor microenvironment.³⁰ The cells were cultured at 37 °C under a 5% CO₂ atmosphere in Dulbecco's modified Eagle medium (DMEM; Institute of Molecular Genetics; Prague, Czech Republic) supplemented with 2 mM L-glutamine, 0.05 mg gentamycin, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol (Sigma-Aldrich) and 10% heat-inactivated fetal calf serum (FCS; Gibco; Grand Island, NY, USA).

Male Balb/c mice (Institute of Physiology, Prague, Czech Republic) were used. After adaptation to the conditions of local animal facility (Institute of Microbiology, Prague, Czech Republic), the spleen from a Balb/c mouse was harvested in sterile conditions into HMEM-d culture medium (Institute of Molecular Genetics). After mashing through a nylon net, half of the splenocytes was separated on the Ficoll-Paque™ 1085 (GE Healthcare; Stockholm, Sweden) using gradient density centrifugation (500 g/30 min at 18 °C) to isolate the mononuclear cells; the other half was used unseparated to include myeloid cells. Both types of splenocyte suspensions were then transferred to DMEM for culture.

The original colloid (2 mg of NaGdF₄:Yb³⁺/Er³⁺@SiO₂/ml) was diluted 50-fold in a phosphate-buffered solution (PBS) to avoid aggregation of the particles and sterilized using UV light. Further dilutions were obtained from this stock dispersion to evaluate the effect of the NaGdF₄:Yb³⁺/Er³⁺@SiO₂ particles at the different concentrations. Cells were seeded on 96-well plates in 150 µl of media at concentrations ranging from 20 000 to 30 000 cells per well. In the next step, the cells were incubated in the presence of 4.4, 22 or 44 ng of particles per well (equivalent to 0.03, 0.15 and 0.3 mg ml⁻¹), or 2.2, 22 or 45 µg of nanoparticles per well (corresponding to 0.015, 0.15 and 0.35 mg ml⁻¹). The cell viability was compared to control group in the absence of the nanoparticles. The tests were performed in triplicate, and 12 wells with medium alone were used as a blank. The cells were cultured in the incubator at 37 °C for 48 and/or 72 h under a 5% CO₂ atmosphere. The cell viability was evaluated by adding MTT (10 µl; 5 mg ml⁻¹) to each well containing the particles or control and to 6 wells containing only the medium. The plates were kept in the dark and incubated for 1 h longer. After formation of the formazan crystals, the color of which turned to violet, the medium (80 µl) was carefully removed using a multi-channel pipette, and dimethylsulfoxide (130 µl) was added to each well to dissolve the formazan crystals. The optical densities were obtained using a plate-reading spectrophotometer at 530 nm. The mean value of the blank (medium and MTT) was subtracted from each sample; the means and standard deviations for each group were determined using a Student's *t*-test; statistically significant results at *p* ≥ 0.05 are shown in the graphs.

2.5 Animal model and localization of NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles in mouse organs

B16F10 melanoma cells (1 × 10⁶) were subcutaneously inoculated into the lower back of syngeneic C57BL/6 mice (AnLab; Prague, Czech Republic), and the tumours were allowed to develop until they reached 10 mm in diameter. All experiments were approved by the Animal Care and Use Committee at the Institute of Microbiology (ID 64/2015). The animals were sacrificed 24 h after 3 intravenous injections (one per day every 24 h) of NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles (2 mg ml⁻¹ at 1 : 50 dilution in sterile PBS). The volume of each administered dose was 100 µl.

Both tumor (melanoma) and healthy tissues (liver and brain) were snap-frozen immediately after harvesting and preserved at -80 °C. The Gd localization and amount in the samples was determined by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). The deeply frozen tissues were allowed to warm up to -13 °C prior to the sectioning with a Leica cryomicrotome CM1950 (Leica Biosystems; Wetzlar, Germany). The tissues were cut to 30 µm-thick slices for the quantitative LA-ICP-MS study of Gd and Fe distributions in the tissues, which were conducted using an Analyte G2 laser ablation unit (Photon Machines; Bozeman, MT, USA) equipped with an ArF excimer nanosecond laser (193 nm) coupled to a 7700× ORS-ICP-MS spectrometer (Agilent Technologies; Tokyo, Japan). An octupole reaction system working in helium mode was used to eliminate spectral interferences observed mainly on ⁵⁶Fe. The raw data (csv files) were transformed to the elemental distribution images using ImageLab multisensor imaging software (v. 2.18, Epina; Pressbaum, Austria). The elemental quantitative data were achieved by the analysis of a set of dried droplet calibration standards with the increasing concentration of both elements: 0, 5, 50, 100, 250, 500, and 1000 µg g⁻¹ for Fe and 0, 0.1, 1, 10, 100, 500, and 1000 µg g⁻¹ for Gd, respectively. The linear relationships were established with the correlation coefficients >0.993 for all calibration curves (Fig. S1 in ESI†).

The resulting images showing the NaGdF₄:Yb³⁺/Er³⁺@SiO₂ particle localization and accumulation in the tissue were compared with classical histology after haematoxylin and eosin staining performed on sequential sections. The Gd accumulations were expressed as a colorimetric gradient scale corresponding to the µg of accumulated Gd/g.

The tissue samples used in this analysis were evaluated to demonstrate the vascular net distribution by immunohistochemistry detection of endothelia. The samples were fixed in 4% formalin solution, stored at RT for 24 h, placed in a Leica ASP6025 automated tissue processor (Wetzlar, Germany), and embedded in paraffin blocks using a Leica EG 1150H paraffin embedding station. Slices (2–3 µm thick) were cut from each sample using a Leica RM2255 microtome and mounted on special glass slides. The first slices were stained with haematoxylin-eosin (DiaPath; Martinengo, Italy). The second set, for detection of the endothelial cells by immunohistochemistry methods, was performed using the anti-mouse CD31 (PECAM-1) antibody (Zytomed Systems; Berlin, Germany). 3,3'-Diaminobenzidine chromogen (Dako/Agilent; Glostrup,



Denmark) was used for visualization of the detected vessels. The prepared slides were evaluated as light-microscopic images using a Carl Zeiss Axio Scope A1 (Oberkochen, Germany) and a Zeiss Axio Scan.Z1 slide scanner.

2.6 Characterization of the nanoparticles

Electron microscopy. The size, composition and crystal structure of the nanoparticles were analyzed using a Tecnai Spirit G² transmission electron microscope (TEM; FEI; Brno, Czech Republic). The microscope was equipped with an energy dispersive spectrometer (EDX; Mahwah, NJ, USA), which was used for the elemental analysis of the nanoparticles. Selected area electron diffraction (SAED) on the TEM microscope was used to verify the crystal structure of the nanoparticles. The electron diffraction patterns were processed using ProcessDiffraction software³¹ and compared with the diffraction patterns calculated using PowderCell software;³² the crystal structures for the diffraction pattern calculation were obtained from ICSD database.³³ Bright-field TEM micrographs were analyzed using Atlas software (Tescan; Brno, Czech Republic) to characterize the particle size distributions. Morphology of individual particles, including number-average diameter (D_n), weight-average diameter (D_w), uniformity (polydispersity index $PDI = D_w/D_n$), and ellipticity, were calculated using Atlas software (Tescan Digital Microscopy Imaging; Brno, Czech Republic) from evaluation of at least 500 individual particles from TEM micrographs by measuring long axis (morphological descriptor MaxFerret) and short axis (morphology descriptor MinFerret). The average diameters and ellipticity can be expressed as follows:

$$\text{Average diameter} = 1/2 \times (\text{MaxFerret} + \text{MinFerret}), \quad (1)$$

$$\text{Ellipticity} = \text{MaxFerret}/\text{MinFerret}, \quad (2)$$

$$D_n = \sum n_i D_i / \sum n_i, \quad (3)$$

$$D_w = \sum n_i D_i^4 / \sum n_i D_i^3, \quad (4)$$

where n_i and D_i are number and diameter of the i -th particle, respectively.

Dynamic light scattering. The hydrodynamic particle diameter (D_h), size distribution (polydispersity PI), and ζ -potential of the nanoparticles were determined using dynamic light scattering (DLS) on a ZEN 3600 Zetasizer Nano Instrument (Malvern Instruments; Malvern, UK). The particle suspension (0.1 mg mL^{-1}) was measured at 25°C , and the data were analyzed using the Malvern software.

ATR FTIR spectroscopy. Infrared spectra were recorded on a Nexus Nicolet 870 FTIR spectrometer (Madison, WI, USA) equipped with a liquid nitrogen-cooled mercury cadmium telluride detector. The spectra were measured using a Golden Gate single reflection ATR cell (Specac; Orpington, UK) equipped with a diamond internal reflection ATR crystal.

Upconversion luminescence. Upconversion luminescence was measured on a Leica TCS SP2 AOBS confocal inverted fluorescence microscope (Mannheim, Germany) using a PL APO

$100\times/1.40\text{--}0.70$ oil immersion objective (pinhole 1 Airy unit) and a Coherent Chameleon Ultra Ti:sapphire laser (Santa Clara, CA, USA) at 970 nm excitation. The $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticle (0.01 g) dispersions in hexane were placed on a cover glass.

Magnetic resonance. Magnetic resonance (MR) images were acquired using a 4.7 T Bruker Biospec spectrometer equipped with a commercially available resonator coil (both Bruker, Germany) at 25°C . A standard two-dimensional rapid acquisition with a relaxation enhancement multispin echo sequence was used with the following parameters: repetition time = 280 ms , echo time = 12 ms , turbo factor = 2 , spatial resolution = $137 \times 137 \mu\text{m}^2$, slice thickness = 0.7 mm , number of acquisitions = 16 , and acquisition time = $9 \text{ min } 36 \text{ s}$. The signal-to-noise ratio $\text{SNR} = 0.655 S/\sigma$, where S is signal intensity in a region of interest, σ is the standard deviation of the noise in background and constant 0.655 reflects Rician distribution of the background noise in a magnitude MR image.

The MR relaxometry was performed for different concentrations of the particles at magnetic field $B_0 = 0.5 \text{ T}$ and 20°C .

3. Results and discussion

3.1 Morphology, structure, phase, and composition of $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ and $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles

Upconversion/magnetic nanoparticles composed of NaGdF_4 host lattice doped with Yb^{3+} and Er^{3+} ions and stabilized with oleic acid were obtained by the high-temperature coprecipitation of lanthanide chlorides. The morphology and particle size were evaluated using the TEM microscopy (Fig. 1a). The particles exhibited an ellipsoidal shape with a mean size $D_n = 27 \text{ nm}$ and a reasonably narrow particle size distribution ($PDI = 1.20$). Size distribution histograms of all synthesized particles were presented in ESI (Fig. S2†). The hydrodynamic diameter of the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ nanoparticles in hexane was $D_h = 35 \text{ nm}$ with polydispersity $PI = 0.28$, which confirmed the relatively narrow size distribution. The difference between D_n and D_h of the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ particles can be explained by the presence of the adsorbed steric stabilizer (oleic acid) on the nanoparticle surface, which is not visible on the TEM micrographs of the particles in the dry state due to low contrast of oleic acid compared to the lanthanide particles.

Freshly synthesized $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ particles are hydrophobic in nature; hence they are dispersible only in organic

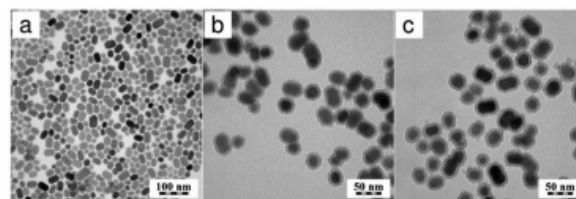


Fig. 1 TEM micrographs of (a) starting $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}$ and (b, c) $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles; (b) $20 \mu\text{l}$ and (c) $40 \mu\text{l}$ TMOS in the feed.



solvents (e.g., hexane), but not in water, which is required for all biological experiments. To render the particles dispersible in aqueous media, their surface was modified with a silica shell using the reverse microemulsion technique.²⁸ It is worth mentioning that the nanoparticle surface must be completely covered by a homogeneous silica shell without the formation of any additional pure silica particles without a core. In this report, the shell thickness of NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles was controlled by varying TMOS amount added to the mixture under otherwise constant reaction conditions, including the concentrations of the nanoparticles, NH₄OH, and Igepal CO-520. When 20 μ l of TMOS was added to the reaction mixture, the nanoparticles reached 36 nm in size (PDI = 1.18), i.e., they were covered by a 5 nm-thick silica shell (Fig. 1b). In contrast, the addition of a greater quantity of TMOS (40 μ l) increased the particle size and shell thickness to 41 and 7 nm, respectively. However, under the latter conditions, additional pure silica particles $\sim$ 10 nm in size were formed (Fig. 1c). Therefore, further experiments were performed using the optimal amount, 20 μ l of the TMOS precursor to achieve complete coverage of the nanoparticle surface with a homogeneous silica shell while avoiding the production of neat silica. The DLS measurements (*D_h*, PI, and ζ -potential) in water confirmed successful modification of the NaGdF₄:Yb³⁺/Er³⁺ particle surface with silica. The *D_h* of the NaGdF₄:Yb³⁺/Er³⁺@SiO₂ particles was 65 nm (PI = 0.3), and the particles exhibited a good colloidal stability due to a total negative surface charge (-30 mV) that originated from the silanol groups.³⁴

The crystal structure and elemental composition of the prepared nanoparticles were verified by TEM microscopy using

selected area electron diffraction (TEM/SAED) and energy-dispersive analysis of X-rays (TEM/EDX; Fig. 2). Regardless of the SiO₂ shell, the SAED diffractograms of the nanoparticles were identical; only one representative diffraction pattern of uncoated NaGdF₄:Yb³⁺/Er³⁺ nanocrystals is therefore shown on Fig. 2a. A comparison of the experimental SAED with theoretically calculated X-ray diffractograms (XRD) of known crystal structures from crystallographic databases³³ showed very good agreement between the experiment and the hexagonal phase of NaGdF₄ (Fig. 2b). Consequently, the most intense diffraction rings could be described (Fig. 2a) and the diffraction positions and intensities in the recalculated 1D-diffractogram were analyzed (Fig. 2b). The diffraction positions of SAED and XRD showed a perfect fit, which unambiguously confirmed that the prepared nanoparticles were the hexagonal phase of NaGdF₄. The diffraction intensities of the SAED and XRD exhibited certain differences that could be attributed to the quite complex preferred orientation (texture) of the nanocrystals lying on the flat, electron-transparent carbon film. The strongest SAED diffraction (*hkl*) was (101), and two additional SAED diffractions, (002) and (111), were remarkably stronger than the corresponding XRD diffractions (Fig. 2b). The first pair of strong SAED diffractions, (101) and (002), defined the first zone of strongly diffracting lattice planes that was described by the zone axis [*uvw*] = [0,1,0] and corresponded to the first preferred orientation of nanocrystals on the carbon film. The second pair of strong SAED diffractions, (101) and (111), defined the second zone of strongly diffracting lattice planes that was described by the zone axis [*uvw*] = [1,0,-1] and corresponded to the second preferred orientation of the nanocrystals on the carbon film (calculation of zone axes from the strong diffractions was described elsewhere³⁵). As a result, the strongest experimentally observed diffractions associated with the above two zone axes should obey the Weiss Zone Law (WZL): $hu + kv + lw = 0$, where (*h*, *k*, *l*) are the diffraction indices of the planes, and [*u*, *v*, *w*] are the indices of the zone axis.³⁵ For the first zone axis [*uvw*] =

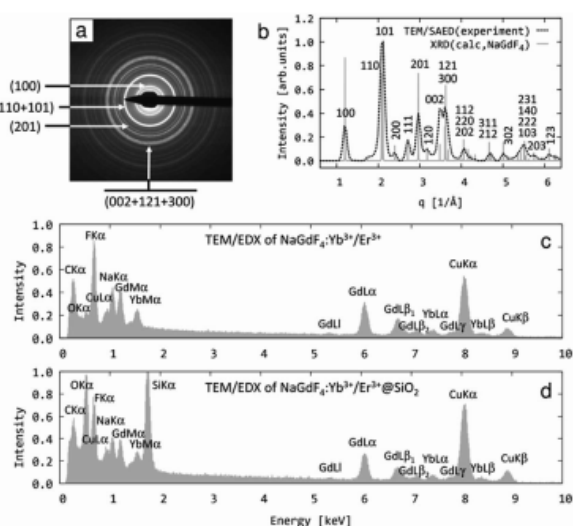


Fig. 2 (a, b) TEM analysis of the crystal structure and (c, d) the elemental composition of the prepared nanoparticles. (a) Selected area electron diffraction pattern (SAED) of NaGdF₄:Yb³⁺/Er³⁺, (b) comparison of the experimental SAED pattern with theoretically calculated X-ray diffraction pattern (XRD) corresponding to the hexagonal phase of NaGdF₄, and energy-dispersive spectrum (EDX) of (c) original NaGdF₄:Yb³⁺/Er³⁺, and (d) NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles.

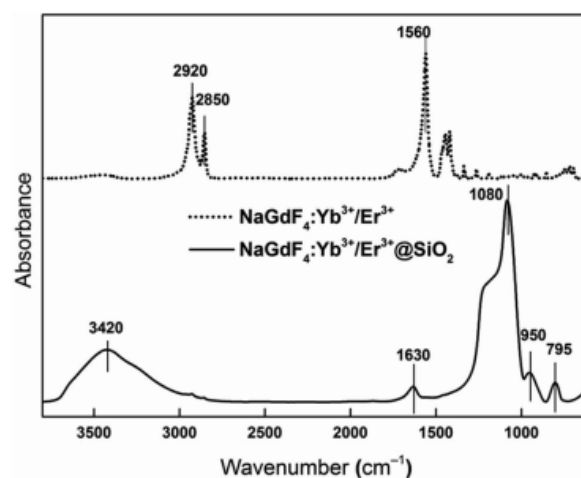


Fig. 3 ATR FTIR spectra of NaGdF₄:Yb³⁺/Er³⁺ (full line) and NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles (dashed line).

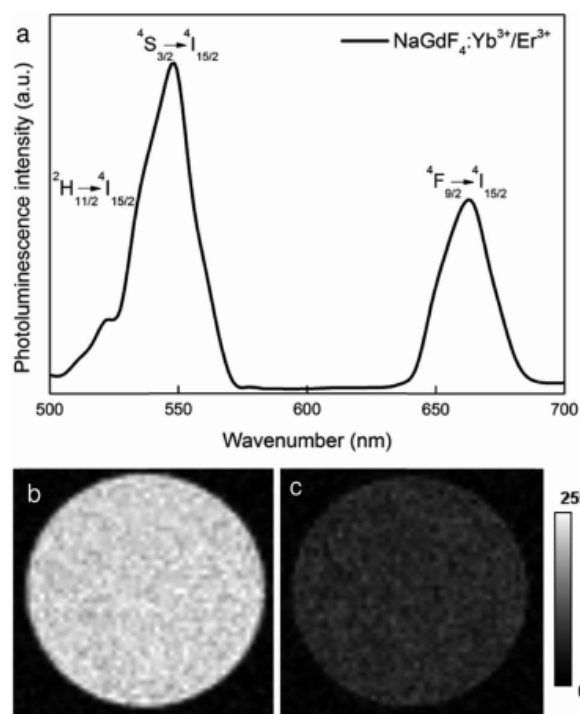


Fig. 4 (a) NIR-to-VIS emission spectrum of NaGdF₄:Yb³⁺/Er³⁺ nanoparticles at 970 nm excitation. T₁-weighted MR images of (b) a phantom containing NaGdF₄:Yb³⁺/Er³⁺ nanoparticles and (c) a control phantom filled with water.

[0,1,0], the WZL takes the form $k = 0$, and consequently, the strongest SAED diffractions should have indices of the type ($h0l$). For the second zone axis $[uvw] = [1,0,-1]$, the WZL takes the form $h - l = 0$, i.e., $h = l$, which means that the strongest SAED diffractions should have indices of the type (hkh), i.e., the first and the last diffraction indices are equal. Indeed, the four strongest SAED diffraction rings (Fig. 2a) were formed by

a combination of ($h0l$) and/or (hkh) diffractions (with just one exception of strong low-angle diffraction (110)), which proved that the above analysis of preferred orientation of nanocrystals deposited on the carbon film was correct. Moreover, the stronger higher-angle diffractions were usually ($h0l$) and/or (hkh) (Fig. 2b).

Embedding of the NaGdF₄:Yb³⁺/Er³⁺ nanoparticles in the SiO₂ shell was verified using TEM/EDX (Fig. 2c and d). The EDX spectrum of uncoated nanoparticles (Fig. 2c) showed only the peaks from the standard TEM support (carbon coated copper grid; peaks of C and Cu) and the peaks of the prepared particles (Na, Gd, and F, including the substituting Yb but lacking any of the substituting Er ions, whose concentration was below the detection limit). The very weak oxygen peak probably resulted from the slight oxidation of the carbon film. The EDX spectrum of the NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles exhibited the same peaks as described above plus strong peaks of the enveloping silica (Si and O; Fig. 2d).

Modification of NaGdF₄:Yb³⁺/Er³⁺ nanoparticles with the silica shell was also confirmed by ATR FTIR spectroscopy (Fig. 3). The peak at 1560 cm⁻¹ corresponding to the COO⁻ band and peaks at 2850 and 2920 cm⁻¹ belonging to the symmetric and asymmetric CH₂ stretching vibrations, respectively, confirmed the presence of oleic acid on the nanoparticle surface. After the addition of the silica shell to the NaGdF₄:Yb³⁺/Er³⁺ nanoparticles, the latter bands disappeared and characteristic SiO₂ peaks were recorded in the spectrum of the NaGdF₄:Yb³⁺/Er³⁺@SiO₂ particles. The symmetric and asymmetric vibrations of Si-O-Si and Si-OH appeared at 795 and 950 cm⁻¹, respectively. The peak at 1080 cm⁻¹ was attributed to the Si-O-Si stretching vibration, and the intense broad peaks at 1630 and ~3420 cm⁻¹ in the spectrum of NaGdF₄:Yb³⁺/Er³⁺@SiO₂ were induced by the bending and stretching vibrations of the OH groups of adsorbed water molecules, respectively.

NaGdF₄ nanoparticles that were doped with the optically active Yb³⁺/Er³⁺ ion pair exhibited a characteristic photoluminescence (Fig. 4a) that emitted high-energy visible photons

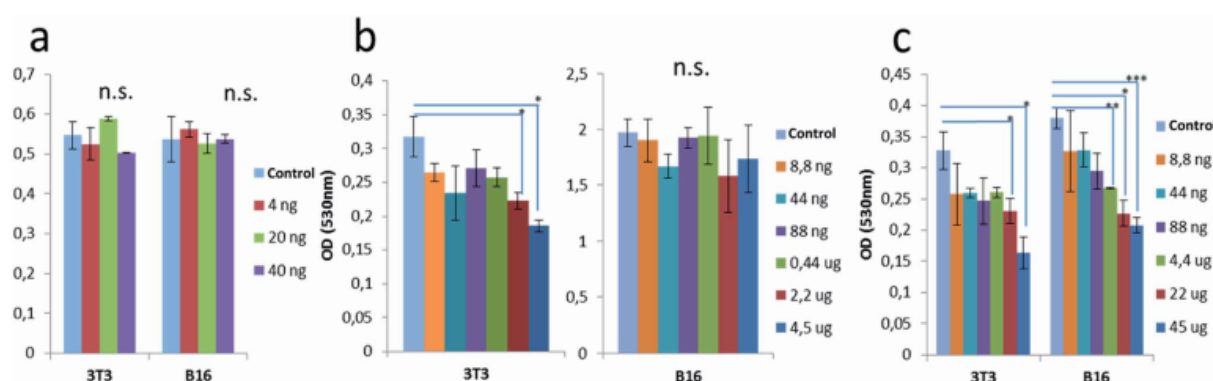


Fig. 5 The effect of increasing concentration of NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles (ng to µg per well containing 150 µl of cultivation medium) on the viability of non-tumor cells (3T3 mouse fibroblasts) and tumor cells (B16F10 mouse melanoma) after a 48 h incubation determined using the MTT assay. (a) 4–40 ng per well corresponding to 27–267 ng ml⁻¹ and (b) 8.8 ng per well to 4.5 µg per well, corresponding to 0.06–35 µg ml⁻¹, and (c) 8.8 ng per well to 45 µg per well corresponding to 60 ng ml⁻¹ to 0.35 mg ml⁻¹. OD: optical density. Student's *t*-test was considered significant for $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; n.s. = non-significant.



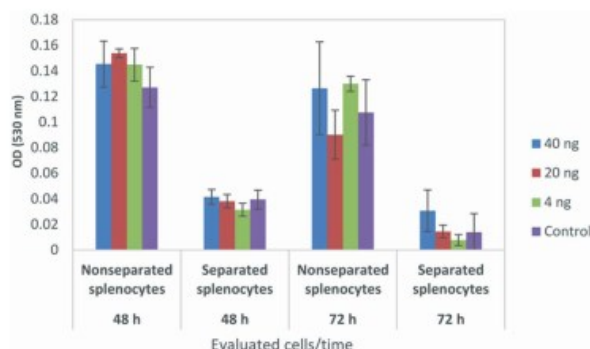


Fig. 6 Effect of increasing concentrations of NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles (ng per well) on the viability of immune cells obtained from the spleen homogenates of healthy mice after 48 and 72 h incubation. The viability was determined using the MTT assay. OD: optical density. No significance was found using the Student's t-test.

after being excited by two low-energy NIR photons *via* the energy transfer upconversion mechanism. Under NIR irradiation at 970 nm, the NaGdF₄:Yb³⁺/Er³⁺ nanoparticles emitted intense green (520 and 550 nm) and red light (660 nm), which can be ascribed to ²H_{11/2} → ⁴I_{15/2}, ⁴S_{3/2} → ⁴I_{15/2} and ⁴F_{9/2} → ⁴I_{15/2} radiative transitions of Er³⁺ ions, respectively, which originated from energy transfer between the Yb³⁺ and Er³⁺ ions. T₁-weighted MR images of NaGdF₄:Yb³⁺/Er³⁺ particles showed a noticeable higher MR signal (3.5×) compared to that obtained

from water phantoms (Fig. 4b). The SNR in the phantom containing NaGdF₄:Yb³⁺/Er³⁺ nanoparticles (0.5 mg ml⁻¹) was 22.5 (the control water phantom had an SNR = 6.4). This result suggested suitable paramagnetic properties that would allow use of the NaGdF₄:Yb³⁺/Er³⁺ nanoparticles as a contrast agent for magnetic resonance imaging. This was supported by the relaxometry measurements, where significant shortening of the relaxation time was observed even at low particle concentrations (Table S1†).

3.2 Viability of cells incubated with NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles assessed using the MTT assay

The viability of tumor and non-tumor mouse cell lines (B16F10 melanoma cells and 3T3 fibroblasts, respectively) was repeatedly investigated *in vitro* using MTT assay after 48 h of incubation with 4, 20, and 40 ng of the NaGdF₄:Yb³⁺/Er³⁺@SiO₂ particles per well containing 150 μl of cultivation medium (corresponding to 27, 133, and 267 ng ml⁻¹, respectively; Fig. 5a). None of these particle concentrations induced significant cytotoxicity. In further experiments, the 3T3 and B16F10 cells were incubated with progressively higher concentrations of the nanoparticles for 48 h (Fig. 5b). At concentrations ≤4.5 μg per well (corresponding to 30 μg ml⁻¹), the colloid did not cause any toxicity in the tumor cells, whereas in the range of 8.8 ng to 0.44 μg per well (corresponding to 0.06–3 μg ml⁻¹) the non-tumor cells (3T3) showed a slight reduction of viability and a progressive cytotoxic effect at 2.2 and 4.5 μg of particles per

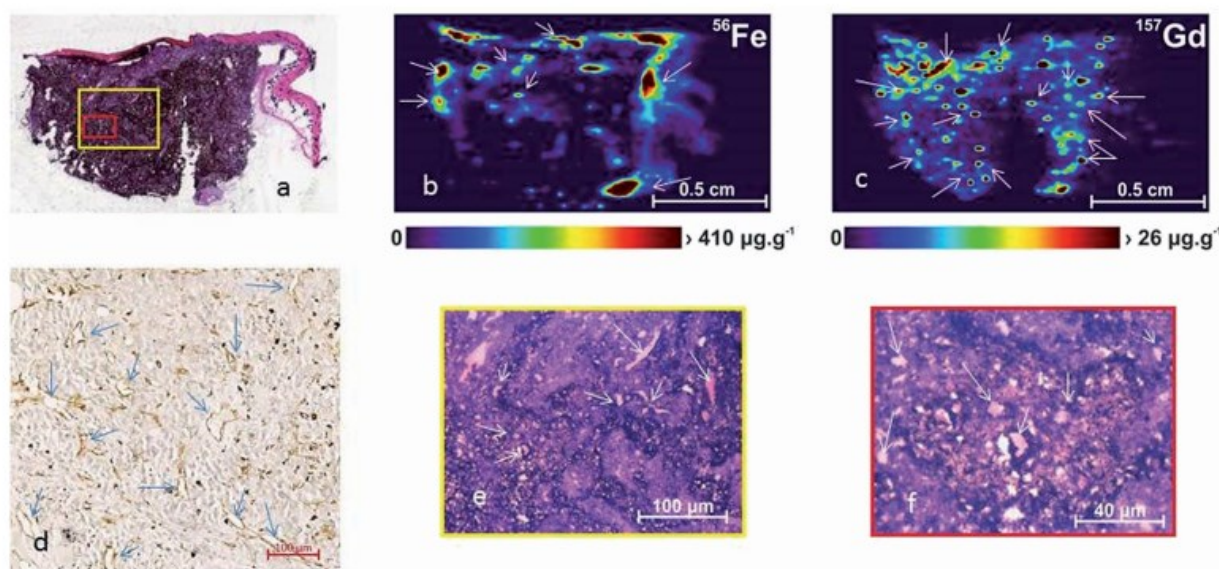


Fig. 7 The histology of melanomas in syngeneic C57BL/6 mice treated with intravenously injected NaGdF₄:Yb³⁺/Er³⁺@SiO₂ nanoparticles compared using LA-ICP-MS (first row). (a) Representative tumor slice stained with haematoxylin and eosin, elemental distribution map for (b) Fe, (c) Gd, (d) evidenced vascularization of melanoma tissue by endothelial staining in immunohistochemistry, (e, f) detailed histological images of LA-ICP-MS-analyzed melanoma tissue (haematoxylin-eosin staining), framed in yellow and red on the total tumor section, showing the tumor tissue with blood vessels, better evidenced by staining of vascular endothelia by immunohistochemistry methods (vessels are stained in brown). LA-ICP-MS for Fe demonstrated only the main vessels according to the Fe in the blood (hemoglobin), while the Gd analysis better revealed the details of the vascularization at an even lower concentration of the tracer than the Fe and with a very good correspondence with the vascular distribution revealed by immunohistochemistry. Arrows indicate the vessels.

well (corresponding to 15 and 30 $\mu\text{g ml}^{-1}$). The cytotoxic effect was evident and progressive with further increases in the particle concentrations in μg scale, up to 45 μg per well corresponding to 0.35 mg ml^{-1} ; (Fig. 5c). In this case, both kinds of cells presented cytotoxic effect when the particle concentration was $>4.4 \mu\text{g}$ per well (corresponding to 0.03 mg ml^{-1}). Interestingly, the toxic effect was more relevant on tumor cells.

Additional experiments were performed to evaluate the effects of various $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticle concentrations on the viability of immune cells after 48 and 72 h of cultivation. Immune cells were obtained from the spleens of healthy C57BL/6 mice after spleen homogenization. The complete suspension consisting of lymphocytes and myeloid cells was compared with a purified lymphocyte suspension obtained by density gradient separation with Ficoll-Paque™. This was performed because lymphocytes co-cultivated with myeloid cells are more resistant than those after separation. Therefore, the separated lymphocyte suspension was expected to be more sensitive to possible cytotoxic

agents. The incubation with $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles at concentrations 4–40 ng per well (27–267 ng ml^{-1}) did not produce a significant change in the cell viability in either preparations even after 72 h of culture. As expected, the viability of the isolated lymphocytes was lower than that of the non-separated cells, but in neither case did the doses of $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles induce cytotoxicity (Fig. 6).

3.3 Localization of $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles in mouse organs

The distribution of the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles in the tumor and body tissues was evaluated using LA-ICP-MS, which is a powerful analytical technique for highly sensitive direct examination of the content of elements, in the present case Gd, in biological tissues. The Fe present in the hemoglobin was evaluated for comparison. The experiments were performed in the tumor tissue (melanoma) and healthy tissue (brain and liver) in a mouse model after intravenous administration of

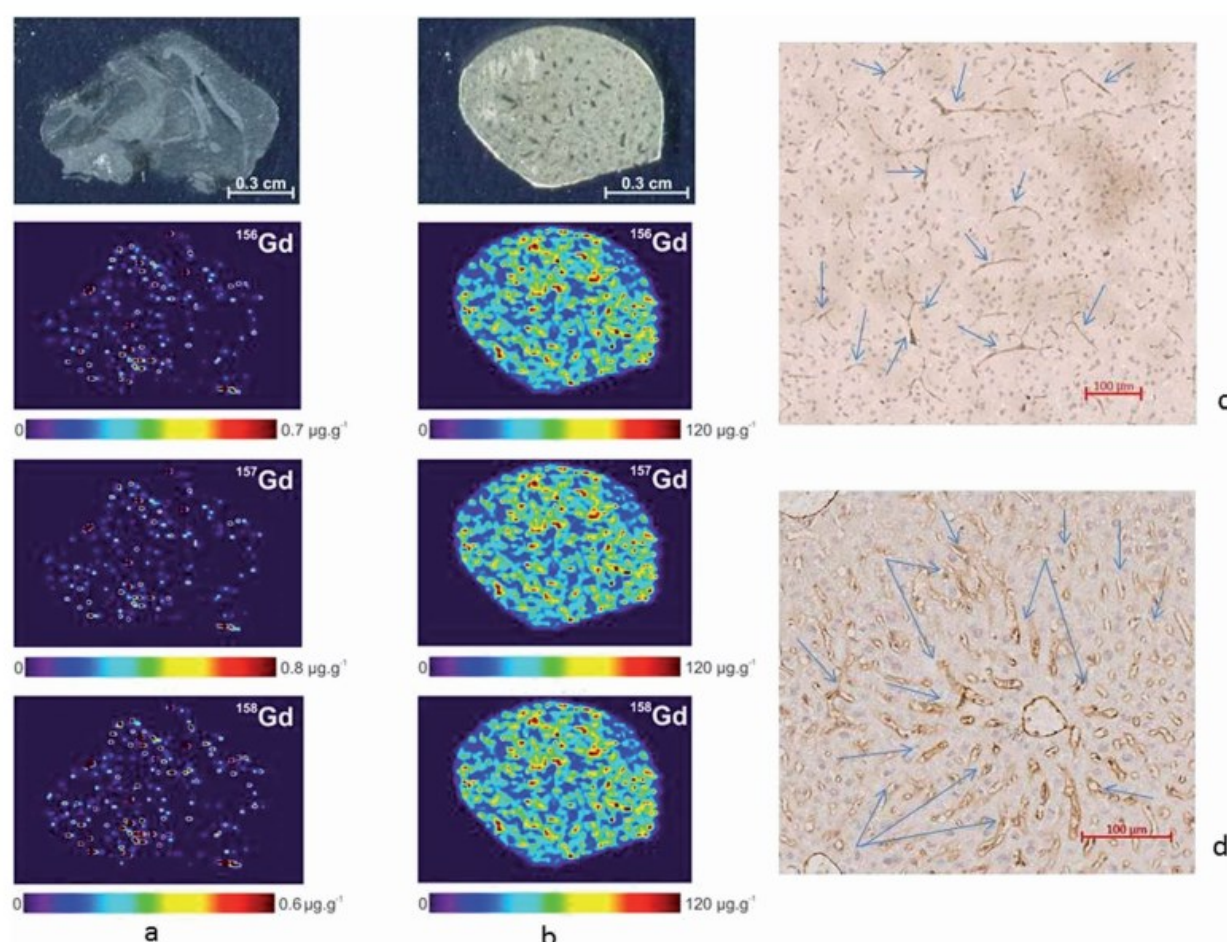


Fig. 8 Elemental distributions of gadolinium isotopes (^{156}Gd , ^{157}Gd , ^{158}Gd) in the brain (left) and liver vessels (right) after treatment of the tumor-bearing mice with $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles. LA-ICP-MS imaging data were collected with a 110 μm laser focus. The optical images of the analyzed tissue sections (top, grey) were obtained using a Scanner HP Scanjet 4890 with the resolution of 4800 dpi. LA-ICP-MS imaging shows accumulation of the particles at the vascular level according to the vascular distribution in the tissues, as confirmed by the immunohistochemistry (vessels stained in brown are indicated by arrows).



a standard concentration of the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles. First, the tumor localization was evaluated. The $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles (100 μl of a colloid; 2 mg of particles per ml diluted 1 : 50 in PBS) were injected into animals harboring murine B16F10 melanoma cells. As described in the Experimental section, sequential sections of frozen samples were prepared and analyzed to permit comparison of the histological picture (Fig. 7a, d and e) with the LA-ICP-MS analysis. The LA-ICP-MS images showed that the Fe was predominantly associated with large blood vessels due to high amounts of Fe present in hemoglobin (Fig. 7b) and a high background. In contrast, the Gd accumulation better defined the vascular component of the tumor tissue. The Gd signal was apparent at a much lower particle concentration (26 $\mu\text{g g}^{-1}$) than the Fe signal (410 $\mu\text{g g}^{-1}$; Fig. 7b and c). Many blood vessels with accumulated $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ particles can be seen in Fig. 7d. These nanoparticles have thus the substantial advantages of having a very low background and highly sensitive demonstration of blood vasculature as long as 48 h after the injection.

Second, the brain and liver tissues were analyzed, and the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ particles were distinctly detected in the vasculature net (Fig. 8). In the brain, the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ particles were present only in the blood vessels (Fig. 8a). In contrast, very high accumulation of the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles was observed in the vascular and perivascular spaces of the liver, as indicated by the presence of some background (Fig. 8b). This was expected because it is known that the Kupffer cells can easily take up circulating particles.³⁶ Chronic accumulation of the particles may represent a problem, especially within the kidneys, on the basis that Gd contrast agents, which are widely used in MRI studies, have been often associated with a risk of systemic toxicity and fibrosis in patients with reduced renal function.³⁷ However, the nanoparticle formulation may take advantage of the liver detoxification ability, implying that the particles are eventually removed and engulfed by macrophages.

4. Conclusions

Rare-earth upconversion nanocrystals with a thin silica coating layer, $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ particles, have been successfully prepared. Their morphology, composition, structure, and coating were verified by detailed TEM analysis, including energy dispersive spectroscopy (EDX) and electron diffraction (SAED). The comparison of experimental SAED diffraction patterns with theoretically calculated XRD patterns proved unambiguously that the prepared nanocrystals had a crystalline structure of hexagonal NaGdF_4 . The upconversion property in combination with an immobilized photosensitizer, namely, the emission of red/green light after irradiation with deep-tissue penetrating NIR light, is very important for future applications of the particles in photodynamic tumor therapy. Moreover, Gd renders the particles, when accumulated by efficient EPR effect, with contrast in MRI, which may permit non-invasive detection of $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ particle-engulfed cells of pathological sites in the organism.

Biological experiments confirmed that the cytotoxicity of $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles was dose-related with a threshold above 0.5 μg of particles per well (3 $\mu\text{g ml}^{-1}$) and clearly present at concentrations of 2.2 μg per well (15 $\mu\text{g ml}^{-1}$). Lower concentrations did not affect the viability of non-tumor, tumor, or immune cells. The LA-ICP-MS method proved to be very effective for the direct determination of the $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ particle distribution within the biological tissues. This technique enables visualization of even very small quantities of the particles (pico- to femtograms), thus facilitating highly sensitive (parts per billion) monitoring of the nanoparticle distribution inside a tissue. In this manner, the distribution of blood vessels containing $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles within the tumor was easily visualized. Even a very small quantity of accumulated particles was detectable, which is beneficial in terms of injection of very low particle doses. This might be applicable even in biopsies of investigated organs or tumor tissues, allowing extensive evaluation of the nanoparticle distribution in the organism to improve planning of highly precise theranostic interventions. A huge difference in the nanoparticle accumulation was found between brain and liver according to their vascular characteristics. The LA-ICP-MSI method may also be suitable for studies of the bio-distribution of the nanoparticles. In fact, the nanoparticles are actively investigated not only for therapeutic purposes³⁸ but also in terms of undesirable particle penetration into the organs, such as the lung and brain, due to the environmental pollution, as well as particle accumulation/elimination by the reticulo-endothelial system.^{39,40}

Our investigation provides the first useful information on the biocompatibility of the newly synthesized $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ nanoparticles and their vascular localization in the evaluated organs even 24 h after the administration. This is important for prospective applications of Gd compounds not only for MRI, but also for efficient targeting of pathological neoangiogenesis (cancer, diabetic retinopathy, *etc.*) and micro-environmental components of cancers. Moreover, $\text{NaGdF}_4\text{:Yb}^{3+}/\text{Er}^{3+}@\text{SiO}_2$ particles can be proposed for bi-modal upconversion luminescence/MR imaging and photodynamic therapy⁴¹ and, after functionalization or coupling with other molecules, as radiosensitizers for enhanced radiotherapy,⁴² or neutron capture therapy,⁴³ as well as therapeutic carriers.

Conflicts of interest

There are no conflicts to declare.

Ethical statement

All procedures were conducted in strict accordance with the European Convention for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee at the Institute of Microbiology, AS CR, approval ID: 64/2015.



Acknowledgements

The financial support of the Czech Science Foundation (15-01897S) is gratefully acknowledged. The biological experiments were supported by RVO 61388971 Institutional Grant and MŠMT COST CZ LD 15135, in the frame of TD1301 MiMed COST Action. Support for the electron microscopy experiments by the Technology Agency of the Czech Republic (TE01020118) is also acknowledged. The MR experiment was supported by the Ministry of Health of the Czech Republic (Institute for Clinical and Experimental Medicine, project IN 00023001).

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3.2 Biocompatibility of TiO₂ prolate nanospheroids as a potential photosensitizer in therapy of cancer

In this study, we examined the direct *in vitro* toxicity and intracellular localization of TiO₂ prolate nanospheroids in murine immune cells and human and murine cell lines. We also evaluated the potential of TiO₂ NPs to act as photosensitizers in human cancer cell lines.

To assess the potential direct *in vitro* toxic effect of the TiO₂ NPs on non-cancer cells, we cultivated mouse immune cells and murine 3T3 fibroblast cell line with increasing concentrations of TiO₂ NPs, and the viability of the cells was measured at set time points. We found a minimal toxic effect in the fibroblast cell line at the furthest time point. In contrast, a transient toxic effect was observed in immune cells directly upon exposure to NPs but was quickly recovered. TiO₂ NP's potential as a photosensitizer was tested in A375 human melanoma, PANC1 pancreatic, and SKBR3 breast cancer cell lines in the dark and with ultraviolet C (UVC) light irradiation. We observed a direct, dose-independent, cytotoxic effect in dark conditions in two of the tested human cancer cell lines (A375, SKBR3). However, no synergistic effects were observed after the UVC irradiation in either of the tested cancer cell lines. The internalization of TiO₂ NPs was evidenced in a human HeLa cancer cell line using confocal microscopy.

The results of this study showed that TiO₂ NPs are biocompatible at low and moderate concentrations, with a low and transitory cytotoxic effect on immune cell metabolic activity, and that cancer cells can be differently sensitive to TiO₂ NPs.

Contribution: My work in this project involved introducing part of the *in vitro* methods used to test for cell viability and toxicity of the NPs on the cell lines and splenocytes (including data analysis). I was also responsible for the autopsy of animals to obtain spleens and the preparation of splenocytes.



Biocompatibility of TiO₂ prolate nanospheroids as a potential photosensitizer in therapy of cancer

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Received: 17 October 2019 / Accepted: 2 June 2020
© Springer Nature B.V. 2020

Abstract TiO₂ prolatenanospheroids (PNSs) may be photosensitizers (PSs), which act by catalyzation of hydroxyl radical (OH) formation upon light illumination. OH might, in turn, contribute to killing of cancer cells. On the other hand, there is great concern about toxicity in the dark of TiO₂ nanoparticles in general. In this work, we have investigated the biocompatibility of TiO₂ PNSs of the anatase crystal form (length between 100 and 300 nm and width 50 nm) in the dark with immune cells and light-induced cytotoxicity on several cancer cell lines. The effects of the treatment of different cell lines with several concentrations of TiO₂ PNSs suspensions showed the specifics of cells' viability and the intracellular localization. The results of in vitro studies obtained by cytotoxicity assays adjusted to individual cell lines' metabolism point towards the biocompatibility of TiO₂ PNSs at low and

moderate concentrations in the dark, which neither kill the cells, nor induce activation of the immune system cells. Laser scanning confocal microscopy revealed that PNSs are taken up by cells, and insight into the intracellular distribution was obtained in this study.

Keywords TiO₂ prolate nanospheroids · Photodynamic therapy · Cancer therapy · Oxidative activity · Nanomedicine

Introduction

Photodynamic therapy (PDT) exploits reactive oxygen species (ROS) generated by a photosensitizer (PS) under the illumination of low-intensity light with appropriate

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wavelength to cause selective damage to the target tumor tissue. Based on the diagram proposed by Lucky and coauthors (Lucky et al. 2015), upon the light irradiation, PSs can be excited. Activation of PSs consequently triggers two types of photochemical reactions, one of them is electron transfer within cellular microenvironment that produces radicals which then react with O_2 . Consequently, various ROS are produced, such as superoxide anion radicals (O_2^-), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxides (H_2O_2 , type I). Alternatively, the direct energy transfer to nearby triplet oxygen ($^3O_2$) leads to the generation of singlet oxygen ($^1O_2$, type II). The literature suggests that type II is the dominant component of cytotoxic effect of PDT via apoptotic, necrotic, and autophagy-associated pathways (Hu et al. 2015; Zhang et al. 2018; Tang et al. 2015). On the other hand, with the aim to minimize the adverse impact on healthy tissue, PSs in the absence of light should not display toxicity to cells and tissues, even at high concentrations. This selectivity of the PSs is the most important advantage comparing to the widely used clinical cancer treatments—chemotherapy and radiotherapy. Furthermore, the possible side effects of the PDT itself can be eliminated, and the PDT efficiency can be improved by combining PSs with nanomaterials, i.e., carriers. The system PS/carrier delivers medications to the target tissues by preservation of a medication in the circulation and minimizing toxic side-effects. Thanks to the optical properties, this system enables the control of drug concentration in cancer tissues (Pandey 2017; Lee and Yeo 2015; Nešić et al. 2016). The final outcome of this synergistic effects of PSs and nanomaterials in PDT might be the overcoming drug resistance as the major cause of the observed treatments failure (Rizvi et al. 2018; Wu et al. 2010; Cho et al. 2008). Despite the positive aspects of merging PSs with nanomaterials, the clinical use is still questionable, as they usually have poor solubility in water. This property results in their aggregation under physiological conditions, which severely affects stability in human sera during drug administration and transportation. In addition, the agglomerates drastically decrease quantum yields of ROS generation (Hong et al. 2016).

PSs composed of the semiconducting nanocrystals are highly interesting for PDT. Nanocrystalline TiO_2 is the typical *n*-type semiconductor material that is widely used material in biomedical applications because of its large surface areas, tunable size, facile modification, and considerable biocompatibility (Savić et al. 2012; Hou

et al. 2015). Among those properties, biocompatibility is the greatest concern because contradictory data related to the toxicity of the nanocrystalline TiO_2 can be found in the literature (Adams et al. 2006). For instance, one study refers that the 5 g kg^{-1} of body weight of nanocrystalline TiO_2 is a safe dose for rats (Wang et al. 2007). Regarding humans, common exposure to TiO_2 nanoparticles (NPs) occurs through the food and cosmetic products, and it was reported that the overall toxicity depends on chemical and physical characteristics of TiO_2 NPs, including size, crystal structure, and the possibility of photo-activation (Wang et al. 2007).

We have previously demonstrated that colloidal NPs have potential to be used as the photosensitive carrier for metallo-drug delivery (Nešić et al. 2017). In the present study, we have synthesized TiO_2 PNSs with expected size of 300/50 nm of length/diameter ratio (Radoičić et al. 2012) and tested their potential to be used as photosensitive anti-cancer agent. In that sense, we have tested their biocompatibility in dark against immune and healthy cells, their potential for catalyzation of ROS production, localization after cellular uptake, as well as their potential for killing cancer cells in the dark and upon illumination.

Materials

TiO_2 powder (Fluka, Bucharest, Romania) was reagent-grade and used without further purification. Air and argon gases were of high purity (99.5%, Messer Tehnogas AD, Belgrade, Serbia). Spin trap DEPMPO for electroparamagnetic spectroscopy (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Phosphate-buffered saline salt solution (PBS) powder, Tris-hydroxymethylaminomethane (Tris), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Darmstadt, Germany). Trichloroacetic acid (TCA), ethanol, and acetic acid were obtained from Centrohem (Stara Pazova, Serbia).

Cell lines, mouse fetal fibroblasts, 3T3, human melanoma A375 cells, human breast cancer SKBR3 cells, and human pancreatic cancer PANC1 were acquired from American Type Culture Collection (ATCC; Manassas, VA, USA), whereas human cervical carcinoma cells (HeLa) were obtained from the cell bank of Shanghai (Shanghai, China). Medium for maintaining the 3T3

cells in the culture, Dulbecco's modified Eagle medium (DMEM) was from the Institute of Molecular Genetics, Czech Academy of Sciences (Prague, Czech Republic), L-glutamine, sodium pyruvate, and 2-mercaptoethanol were from Sigma Aldrich (Steinheim, Germany), and the heat-inactivated fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Hank's minimum essential medium (HMEM-d) was from the Institute of Molecular Genetics, Czech Academy of Sciences (Prague, Czech Republic) and cell culture medium, RPMI-1640, for splenocytes, was purchased from Sigma Aldrich (St. Louis, MO, USA). For A375, SKBR3 and PANC1 cells, DMEM medium, FBS and penicillin/streptomycin solutions were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), whereas for HeLa cells, DMEM medium supplemented with 10% (v/v) FBS was purchased from Sijiqing Inc. (Beijing, China).

Cell stain solution for HeLa cells, was Hoechst 33342 from Beyotime (Jiangsu, China). Stain for the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide was acquired from Serva Electrophoresis GmbH (Heidelberg, Germany), whereas for modified MTT assay, the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium, monosodium salt) was purchased from Beyotime (Jiangsu, China). Crystal violet stain (CVS), triaryl methane, and sulforhodamine B (SRB) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Methods

Synthesis of TiO₂ prolate nanospheroids

An acid digestion bomb, model 4746 (Parr Instruments), was used for the synthesis of titania nanotubes and finally prolate nanospheroids (PNSs). In order to prepare PNSs, nanotubes were synthesized by hydrothermal treatment of TiO₂ powder. Briefly, 250 mg of TiO₂ powder was dispersed in 10 mL in 10 M NaOH solution, treated hydrothermally at 150 °C for 20 h, and the powders was washed with 0.1 M HCl, until the solution became neutral. Solution was centrifuged (2000×g, 30 min) to spun down the TiO₂ nanotubes, and the pellet dried at 70 °C until it attained the constant mass. PNSs were then prepared from the nanotubes in an additional hydrothermal treatment, and both procedures have been

described in details in the previous work (Radoičić et al. 2012). Dry nanotubes (50 mg) were dispersed in 10 mL of distilled water (pH 7), transferred into a Teflon vessel and treated under saturated vapor pressure of water at 250 °C for 90 min. After autoclaving, the synthesized PNSs were dried at 70 °C to constant mass.

Characterization of TiO₂ prolate nanospheroids

The expected size, shape and appearance of the PNSs were confirmed by transmission electron microscopy (TEM), using a JEOL JEM-2100F field emission TEM operating at 200 kV. The samples for TEM observation were ultrasonically dispersed in deionized water and a drop of this solution was then placed onto a carbon film supported on a copper grid.

To measure size distribution of PNSs we used nanoparticle tracking analysis (NTA) measurements. NTA measurements were conducted using a NanoSight LM10 (NanoSight, Amesbury, UK), equipped with a 532-nm laser. Before acquisition, TiO₂ NPs were resuspended and further diluted in H₂O solution to a final concentration of about 10⁸–10⁹ particles ml⁻¹. In all measurements, the temperature was set to 25 °C. Each sample was measured at five different positions for 60 s in three independent experiments. The software NTA 3.0 was applied for capturing and analyzing the data.

Surface charge of TiO₂ PNSs suspension in deionized water (1 µg ml⁻¹) at 25 °C and pH = 7 was determined by zeta potential measurements performed on Zetasizer Nano ZS90 (Malvern 13 Instruments; Malvern, UK).

Oxidative activity of TiO₂ PNSs

For the detection of possible radical generation, we have used spin trap DEPMPO purified by a standard procedure given by Jackson et al. (2002). Suspension of TiO₂ PNSs (1 µg ml⁻¹) was prepared in deionized water and irradiated with an UV lamp (power of 600 µW, 2 min). Immediately after irradiation, DEPMPO solution was added and samples were transferred into the gas-permeable Teflon tube (Zeus Industries Inc., Orangenburg, USA) inserted into a special quartz tube in an Electron Paramagnetic Resonance (EPR) spectrometer (Bruker ELEXSYS-II E540, Bremen, Germany) operating at X-band, 9.51 GHz). EPR experimental settings were as follows: center field 3500 G, microwave power 10 mW, microwave frequency 9.85 GHz,

modulation frequency 100 kHz, and modulation amplitude 1 G. The spectra were recorded and analyzed using Xepr software (Bruker, Bremen, Germany).

Cell cultures and the treatment conditions

For viability tests and cell imaging, we have used various types of cell cultures prepared specifically for each test in order to investigate the response of the different cells to a similar treatment with TiO₂ PNSs. For all viability tests and cell imaging, the powder of PNSs was suspended in a PBS or deionized water to the concentration of 5 mg mL⁻¹, sterilized using UV light, following the agitation in an ultrasonic bath for 15 min.

The human melanoma cell line A375 and mouse melanoma cell line B16F10 were chosen given that this superficial type of skin cancer is the most accessible for the light treatment, which enables further in vivo tests. Human epithelial cervical cancer cells HeLa, human breast cancer cell line SKBR3, pancreatic ductal adenocarcinoma cell line PANC1, were also selected as some of the most common types of cancers. The mouse fetal 3T3 fibroblast cells represent noncancer cells type; fibroblasts have been reported to be an important component of the tumor microenvironment. Additionally, bearing in mind that we were testing the potential nanocarrier effects, we have assessed if the TiO₂ PNSs treatment may stimulate responses in immune cells obtained from mouse spleens.

Mouse fetal fibroblasts, 3T3 were cultured at 37 °C under a 5% CO₂ atmosphere in DMEM supplemented with 2 mM L-glutamine, 0.05 mg gentamycin, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 10% heat-inactivated fetal bovine serum.

The spleens from the 6 male Balb/c mice (Institute of Microbiology, Prague, Czech Academy of Sciences, Czech Republic) were harvested in sterile conditions into HMEM-d. After mashing through a nylon net, splenocyte suspensions were then transferred to RPMI-1640 medium for culture.

The 3T3 cells and splenocytes were seeded in 96-well plates. The treatment solutions in DMEM media were made from the stock of TiO₂ PNSs and added into the 96-well plates to culture cells to reach the final concentrations in medium per well for 3T3 (3, 6, 12, 24, 36, and 48 µg mL⁻¹) and for splenocytes (6, 24, and 48 µg mL⁻¹). After the treatment, one group of plates was immediately processed with assays, and the rest

were incubated at 37 °C for 24 and 120 h under a 5% CO₂ atmosphere in the dark.

HeLa cells were grown in 96-well plates or Petri dishes at 37 °C under a 5% CO₂ atmosphere in DMEM medium supplemented with 10% (v/v) FBS. After reaching 80% confluence, the culture medium was replaced by medium containing PNSs suspension of different concentrations. For viability test, the final concentrations in medium per well were 5, 50, and 150 µg mL⁻¹ and incubation time was 24 h in the dark. After the TiO₂ PNSs deposited and adhered to the cells, the medium was changed to the TiO₂-free DMEM solution supplemented with 10% fetal bovine serum for the viability test. For laser scanning confocal microscopy (LSCM) HeLa cells grown in Petri dishes were incubated with 5 and 25 µg mL⁻¹ TiO₂ in DMEM for 12 h before the LSCM observation (Olympus, FV-300, IX71; Olympus, Tokyo, Japan). Stain solution, Hoechst 33342, was used as a nucleus indicator by adding 0.5 µg mL⁻¹ into the growth medium for 5 min.

Human melanoma A375 cells, human breast cancer SKBR3 cells, and human pancreatic cancer PANC1 were cultured in DMEM medium supplemented with 10% FBS and penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C (Heraeus, Hanau, Germany).

Cell viability tests

Different cytotoxicity assays have been applied for each cell line tested, as each method is based on a specific parameter that could be observed in the cell culture, such as loss of membrane integrity, membrane metabolic activity, loss of monolayer adherence, and changes of cellular protein content (Patravale et al. 2012).

For all viability tests, measurements were made in quadruplicate during each experiment, while each experiment was repeated twice. The data were normalized to the particle-free control. The results were presented as the mean ± SD (standard deviation).

Influence of TiO₂ PNSs on 3T3 and splenocyte cells viability in dark conditions

Mouse fetal fibroblasts, 3T3, and mouse splenocytes suspension were used to assess the cytotoxic effects of TiO₂ PNSs using the MTT and CVS assays.

For both MTT and CVS tests, treatment procedures were done according to the manufacturer instructions, or

described previously in the literature, respectively (Feoktistova et al. 2016). The tests were performed in quadruplicate, whereas the 6 wells of cells in the absence of the treatment served as the control group and the 6 wells containing only the medium were used as a blank. In order to get the optimal wavelength for the tests, the spectrum was obtained from 400 to 700 nm, using a plate-reading spectrophotometer (Tecan infinite 200Pro, Männedorf Switzerland). After establishing the peak position in the spectrum, the optimal wavelength for each test was chosen for measurements of the optical densities of the samples.

For MTT cytotoxicity test, 3T3 cell line and splenocytes were mixed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The evaluation of the cell viability with MTT test (for 3T3 and splenocytes) commenced by adding MTT solution in deionized water ($10 \mu\text{L}$; 5 mg mL^{-1}) to each well, following the additional incubation for at least 1 h of plates in dark, to achieve the formation of the formazan crystals. After incubation, the generated formazan crystals were dissolved in DMSO. The optical densities were measured at 550 nm. The mean value of the blank was subtracted from each sample.

Since CVS test is only compatible with adherent cell types, it proceeded to the 3T3 cell line (Feoktistova et al. 2016). For the CVS assay, the cell culture medium was removed carefully from the wells and the cells washed gently with PBS. Then, cells were stained with 0.5% crystal violet in 30% ethanol for 10 min at room temperature, and washed off with water before absorbance measuring. The wavelength of 590 nm was chosen for measurements of the optical densities of the samples. The mean value of the blank was subtracted from each sample.

Modified MTT cytotoxicity test on treated HeLa cell line in dark conditions

The cell viability assays were conducted by a modified MTT method using WST-8. Each well containing $100 \mu\text{L}$ culture medium was supplemented with $10 \mu\text{L}$ of WST-8 reagent solution. The untreated cells were used as control groups. The cells were then incubated at 37°C with 5% CO_2 for 24 h, and the absorbance was measured at 450 nm using a microplate reader (Bio-Tek SynergyTM HT, Bio-Tek® 6 Instruments, Inc. Winooski, VT, USA).

Viability tests upon light irradiation

Exponentially growing cells, seeded into flat-bottom 96-well plates (Sarstedt, Newton, NC, USA) at a density of 2×10^3 cells per well, were incubated upon the treatment for 24 h and then exposed to the UVC light irradiation, i.e., the range 200–280 nm, with the maximum 254 nm ($600 \mu\text{W}$) for 5 min. The treatment used in this experiment slightly varied compared to the previous viability tests, as the final concentrations of TiO_2 PNSs in the medium were kept low (10, 20, and $40 \mu\text{g mL}^{-1}$).

The cytotoxicity was determined using a sulforhodamine B (SRB) assay, based on the measurement of cellular protein content. The assay was performed according to the method of Skehan and co-workers (Skehan et al. 1990) 24 h after treatment. In brief, cell monolayers were fixed with 10% TCA for 1 h at 4°C . After fixation plates were washed with water and stained with 0.4% SRB in 1% acetic acid for 15 min at room temperature. Subsequently, the excess stain was removed and the plates were washed with 1% acetic acid before air-drying. Protein-bound dye was dissolved in 10 mM Tris solution for 5 min. Absorbance was measured at 550 nm and a reference wavelength of 690 nm using a microplate reader (Wallac, VICTOR2 1420 Multilabel counter, PerkinElmer, Turku, Finland).

Statistical analysis

Statistical differences were assessed using one-way analysis of variance (ANOVA) with Tukey's multi-comparison post-test using GraphPad Prism 8.0.1. Tukey multi-comparison test was performed for the control groups of specific cells (nontreated cells) and cells treated with different TiO_2 PNSs concentration levels. The treated concentration levels have been compared to each other, too. In all cases, p value < 0.05 was considered as significant level. Data were averaged from four or six replicates ($n = 4$ or 6).

Interaction and localization of TiO_2 prolate nanospheroids within the cells

The cellular distribution of TiO_2 PNSs within HeLa cells was obtained by the means of a laser scanning confocal microscope (Olympus, FV300/IX 71; Japan) equipped with a 405 nm continuous laser excitation (Coherent, Palo Alto, USA) and a water immersion objective ($\times 60$, $\text{NA} = 1.2$).

The reflection images of the intracellular TiO₂ PNSs and the fluorescence images of nuclei were simultaneously obtained by the LSCM in the channels without filters for the reflecting light and a 430 to 460 nm bandpass filter for the fluorescence. The differential interference contrast micrographs were acquired simultaneously in a transmission channel to exhibit the cell morphology. The Z-scan mode of the microscope was used to acquire the three-dimensional (3D) distributions of TiO₂ PNSs and nuclei.

Results and discussion

NPs can be either anti-cancer agents alone or carriers for targeted drug delivery. They can exhibit cytotoxic effects after accumulation in cancer cells, or tumor tissues, or they can carry a drug and deliver it to the tumor tissue in a targeted way. In either case, their size and shape are critical for the cellular uptake and efficacy, because it has been postulated that particles smaller than 200 μm will be subjected to renal clearance quickly, before they reach the diseased tissue (Wang et al. 2007). Additionally, the uptake of particles by cells, where they exhibit their anti-cancer effect is more efficient in the case of elongated particles, compared to those with a smaller diameter and spherical shape (Lee and Yeo 2015; Xie et al. 2017). Therefore, for the future design of NPs acting as anti-cancer agents, a compromise in terms of the size and shape has to be found between the sufficient

size to avoid renal clearance, but to be efficiently taken up by target cells.

Morphology, structure, and phase of TiO₂ prolate nanospheroids

The morphology and size of the TiO₂ PNSs were evaluated using the TEM, and results given in Fig. 1, show that particles have a length in the range from 100 to 300 nm with a full width of 50 nm. This confirmed that the synthesized batch was of the same size as previously reported by Rabatić et al. (2006). A certain portion of agglomerates can be observed in Fig. 1, which is most likely the result of the air-drying of nanocrystalline suspension during the preparation step for TEM analysis, as already noticed by other authors (Vevers and Jha 2008).

The dynamic light scattering (DLS) and NTA are widely used for characterization of particle size distribution in aqueous solution. However, DLS and NTA provided different results concerning the hydrodynamic diameter. DLS measures an intensity weighted size distribution of the hydrodynamic diameter, where the intensity correlates with the particle diameter to the power of six. Hence, smaller particles are outshined by larger particles especially in samples containing polydisperse particles. On contrary, NTA delivers an undistorted number distribution of the hydrodynamic diameter. In this study we have used NTA since this method is more suitable for multimodal and polydisperse particles. The Fig. 2 reveals two-modal polydisperse particle

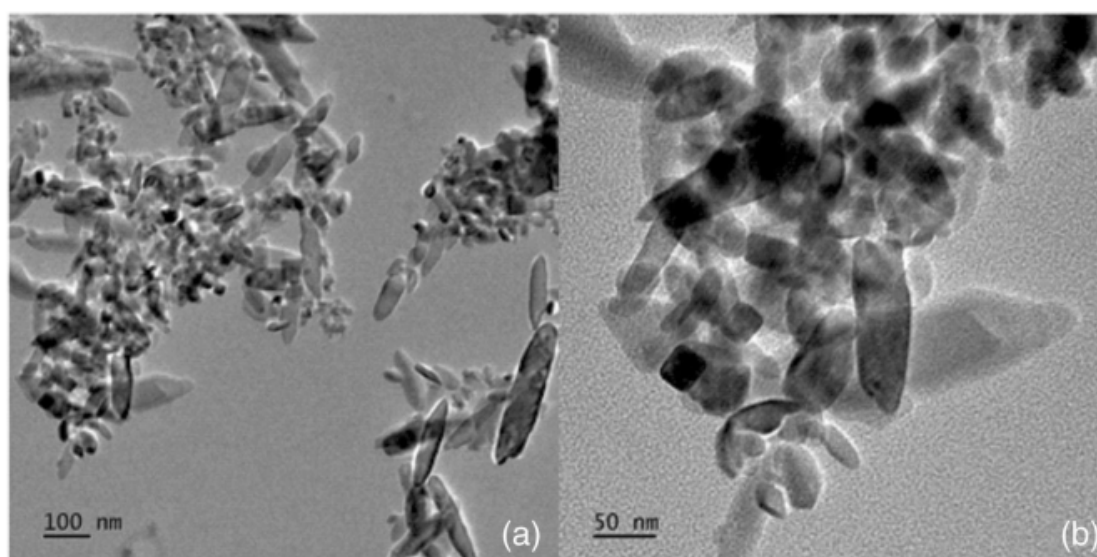


Fig. 1 TEM images of TiO₂ PNSs obtained at lower (a) and higher (b) magnification

distribution with the values of 95 nm and ca. 150 nm under assumption of spherical shape. Recalculation of equivalent spherical diameter of cylinder-shaped TiO₂ particles from TEM data reveal size ranged from 72 to 104 nm. Therefore, it may be concluded that particles aggregation in aqueous solution was very low. Only a small fraction of aggregates with the size of 380 nm have been observed.

For the uptake by the cells, the surface charge and stability in physiological conditions are of extreme importance. Determination of surface charge was done by measuring zeta potential, which revealed a good colloidal stability due to a total negative surface charge of the value of -22.4 mV on the TiO₂ PNSs. In terms of cellular uptake, if serum albumin were absorbed onto negatively charged NPs, it would retain its native structure, whereas with positively charged NPs it would undergo the conformational change. The conformational changes lead to the redirection of the protein–NP complex to scavenger receptors (Josefsen and Boyle 2008). This emphasizes the advantage of the use of negatively charged NPs, such as TiO₂ PNSs, for potential anti-cancer therapy for an intravenous administration. The potential workflow for anti-cancer therapy with TiO₂ PNSs could be the following: (I) to avoid potential aggregation of NPs immediately after the injection, PNSs should be incubated first with serum albumin; (II) intravenous administration of the suspension, which will be followed by accumulation of PNSs in tumor tissue; (III) finally, local application of UV

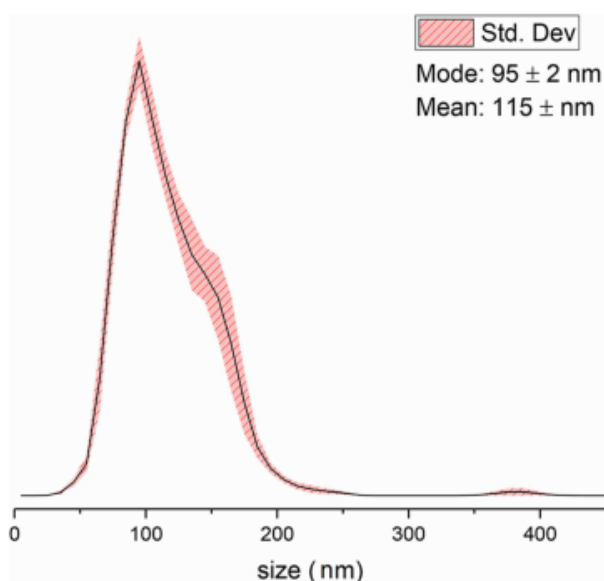


Fig. 2 NTA size distribution of TiO₂ PNSs

light (or infrared light, if therapy includes the up-conversion NPs) activates PNSs leading to the cell death. However, to achieve this step, a further study is required.

Oxidative activity of TiO₂ PNSs

The EPR technique is distinguished from other spectroscopic methods with a unique sensibility that allows detecting the paramagnetic species even at very low concentrations. It is known that UV irradiation of TiO₂ catalyst generates O_2^- , which plays an important role in biological pathways and in complex chemical reactions (Diaz-Urbe et al. 2010). Instead of O_2^- (or together with it), the presence of other radical species was confirmed by many studies (Dodd and Jha 2009; Coronado et al. 2001; Konaka et al. 1999). The generation of certain radical species depends on many factors, and to be useful as a PS agent, TiO₂ PNSs should be able to catalyze the production of ROSs upon light illumination. The production of ROS was monitored by EPR, and the TiO₂ PNSs solution was exposed to UV light for 2 min (600 μW), and immediately transferred to the EPR spectrometer.

Obtained EPR signals unambiguously showed the presence of DEPMPO– OH adduct both in irradiated and nonirradiated samples, Fig. 3, and no other radical species were detected. This finding that OH can be detected also in dark in the presence of investigated NPs implies a spontaneous oxidative activity of TiO₂ PNSs. The characterization of the small amount of ROS generated by TiO₂ NPs in the absence of UV light is rarely studied, as the main research in this area focuses

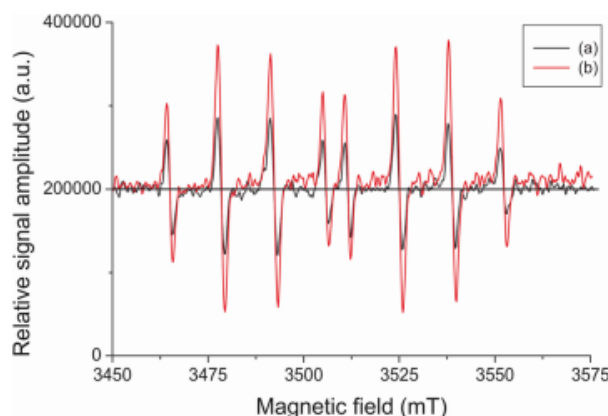


Fig. 3 EPR spectra of OH -spin adducts of DEPMPO in the presence of TiO₂ PNSs obtained (a) with measurements in dark and (b) after 2 min of irradiation with UV light

on the photocatalytic properties of TiO_2 . Studies of ROS generation in the dark have been limited to a few biological studies (Dalai et al. 2012; Gurr et al. 2005; Runa et al. 2016; Sayes et al. 2006) and more extensive one is done recently by Jayaram et al. (2017). Their study showed that both photocatalytic and food grade TiO_2 NPs, in the dark, generate low levels of ROS, and clearly demonstrated that source of ROS from TiO_2 NPs in the dark are surface defects, especially oxygen vacancies. In our hands, TiO_2 PNSs also catalyze the production of ROS in dark, but after illumination with UV light, the concentration of generated $\cdot\text{OH}$ increases 2 times (Fig. 3). It should be also mentioned that the measurements were done in air atmosphere, and under not completely dark conditions, so the presence of the molecular O_2 and maybe low background light with the UV component, might had also affected the formation of the certain amount of $\cdot\text{OH}$ even in nonirradiated samples.

However, the results imply that, if taken up in sufficient amounts, TiO_2 PNSs can act as PSs and, when stimulated, damage cellular biomolecules enough to trigger their death. The increased production of $\cdot\text{OH}$ leads to the peroxidation of lipid membranes and direct damage to proteins, so that could be the possible pathway of a biologically damaging effects of TiO_2 PNSs (Dodd and Jha 2009).

Viability of immune cells in the presence of TiO_2 PNSs

To check the biocompatibility of NPs, we have tested whether TiO_2 PNSs affect the viability of immune cells and fibroblasts in the dark. The cytotoxic effect of a TiO_2 PNSs was expected to be triggered only after cellular uptake and illumination, in which way the side effects could be minimized.

The viability of 3T3 fibroblasts and splenocytes was investigated in vitro using MTT assays after 24 and 120 h of incubation with TiO_2 PNS (Fig. 4). After incubation of 24 h, the cytotoxic effect was not observed, whereas the slight reduction of viability and a progressive cytotoxic effect was observed after 120 h for all concentrations except the concentration of $12 \mu\text{g ml}^{-1}$ at which the cytotoxicity was negligible. Still, the viability was more than 0.6 (60%) for each tested concentration of PNSs. Data assessed at all three time points (0, 24, and 120 h) have not shown any statistically significant differences ($p = 0.9700$, $F = 0.2034$, $df = 6, 14$). Splenocytes had different behavior,

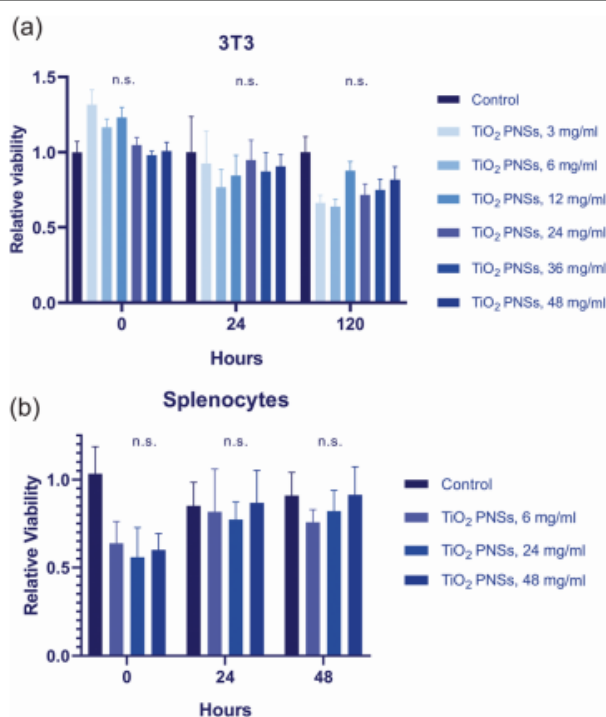


Fig. 4 The effect of increasing concentration of TiO_2 PNSs (presented the final concentrations in the cultivation medium) on the viability of nontumor cells after 24 and 120 h of incubation determined using the MTT assay: **a** 3T3 mouse fibroblasts and **b** splenocytes. Nontreated cells were used as a control (Control). Data were analyzed using a one-way ANOVA with the Tukey's test, and considered nonsignificant (n.s.) for $p \geq 0.05$

as immediately after treatment, viability was noticeably decreased for slightly less than double (0.63, 0.55, and 0.60 for final concentrations of 6, 24, and $48 \mu\text{g ml}^{-1}$, respectively). After incubation of 24 and 120 h the cells seemed to have been recovered, and the viability corresponded closely to the control group. The reason for immediate and "reversed" cytotoxic effect of this cell type is not known and requires further investigation. We can hypothesize that because these cells are freshly isolated, they are immediately more sensitive in their new environment with prompt adaptation and stabilization within 24 h. From the presented data, it could be assumed that the treatment with TiO_2 PNSs does not affect the immune cell viability, although a low proliferative activity could be observed in the dark. The later finding can imply a slight stimulation effect on the immune cells but, assuming a quick renal clearing from the system, NPs should leave the circulation before activation of an immune response. This discussion is, however, beyond the scope of the presented manuscript. The statistical analysis performed for the Splenocytes

have not shown statistically significant differences in all cases ($p = 0.241$, $F = 1.712$, $df = 3, 8$).

One-way ANOVA with Tukey multi-comparison test is performed for the control groups of 3T3 cells (nontreated cells) and 3T3 cells treated with different TiO₂ PNSs concentration levels. The treated concentration levels have been compared to each other, too. All data were assessed at three time points (0, 24, and 120 h) and have not shown any statistically significant differences ($p = 0.9700$, $F = 0.2034$, $df = 6, 14$). The same statistical analysis performed for the splenocytes also have not shown statistically significant differences in all cases ($p = 0.241$, $F = 1.712$, $df = 3, 8$).

As mentioned before, for chosen cell lines, different cytotoxicity assays have been applied, because each method is based on specific cellular processes. In further experiments, after the incubation under the same conditions with the same concentrations of TiO₂ PNSs treatment, the viability of 3T3 fibroblasts was assessed also with CVS assay, and in an analogous manner to previous one, the treatment did not cause any toxicity in the non-cancer cells (data not shown).

Viability of cancer cells in the presence of TiO₂ PNSs

To validate the impact of the TiO₂ PNSs treatment, in the dark, on the HeLa cancer cells, we have assessed the surviving fractions of HeLa cells in dependence on the treatment concentrations with modified MTT test, Fig. 5. One-way ANOVA using Tukey's test, was performed for the control group and between each concentration level of the treatments, and we did not observe any statistically significant differences ($p = 0.9700$, $F = 0.2034$, $df = 3, 12$). This result confirms a very weak cytotoxic effect of TiO₂ PNSs in the dark, with relative viability > 0.94 , even on cancer cells.

Effect of illumination on the cell viability in the presence of TiO₂ PNSs

As demonstrated with EPR (Fig. 2), TiO₂ PNSs produce an increased amount of ROS after illumination with light in the UV wavelength range. In order to get a better insight of the UV illumination effect, cytotoxicity was tested both in dark and after illumination with UV light. Three cancer cell lines (A375, SKBR3, and PANC1) were assessed by SRB assay, with additional UV light irradiation 24 h after incubation, and the results are presented in Fig. 6. In this experiment, A375 cell line

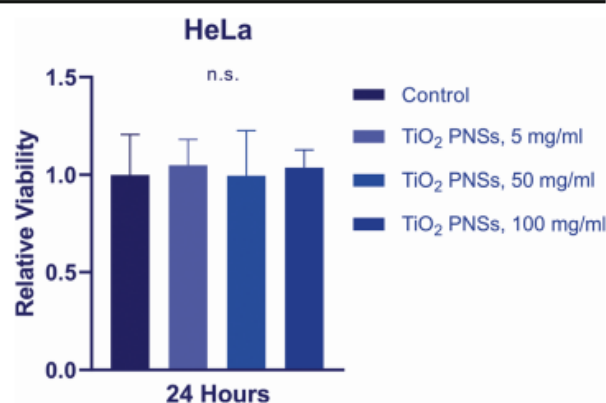


Fig. 5 The effect of increasing concentration of TiO₂ PNSs (presented the final concentrations in the cultivation medium) on the viability of HeLa cells after 24 h of incubation determined using the modified MTT assay. Data were analyzed using a one-way ANOVA with the Tukey's test and considered nonsignificant (n.s.) for $p \geq 0.05$

showed to be more sensitive to the treatment with TiO₂ PNSs after incubation, and the survival fraction decreased with increasing concentrations ($p < 0.05$ for $10 \mu\text{g ml}^{-1}$ TiO₂ PNS, $p < 0.01$ for $20 \mu\text{g ml}^{-1}$ TiO₂ PNS, $p < 0.001$ for $40 \mu\text{g ml}^{-1}$ TiO₂ PNS) (Fig. 6a). The measurements following the UV light irradiation showed a significant photo-killing effect, and the cell viability dropped to less than 0.29 after PNS treatment ($p < 0.001$ for all UV irradiated samples vs Control; $p > 0.05$ for all TiO₂ + UV-treated samples vs Control + UV). A similar effect was observed in the SKBR3 cell line (Fig. 6b). The cells were the most sensitive to the treatment of TiO₂ PNSs of $20 \mu\text{g ml}^{-1}$ ($p < 0.01$). Moreover, within A375 and SKBR3 cell lines, the UV light seemed to cause low survival fractions of control (less than 0.5; $p < 0.001$ for Control + UV vs Control), which makes the applied conditions inappropriate for the PDT. Unfortunately, under our experimental conditions, we were not able to change the power of light, and all results are compared to the control, i.e., the cell viability measured only upon UV light illumination. In contrast to A375 and SKBR3, PANC1 cell line exhibited quite different trend (Fig. 6c). All treatments have stimulated the cells grow progressively with the increased concentrations of TiO₂ PNSs and with more than doubled survival fraction for the highest concentration ($p > 0.05$ for $10 \mu\text{g ml}^{-1}$ TiO₂ PNS, $p < 0.05$ for $20 \mu\text{g ml}^{-1}$ TiO₂ PNS, $p < 0.001$ for $40 \mu\text{g ml}^{-1}$ TiO₂ PNS), and similar trend, only less intensive, continued after irradiation ($p > 0.05$ for all TiO₂ + UV-treated samples vs Control + UV). Pancreatic carcinoma is one of the deadliest

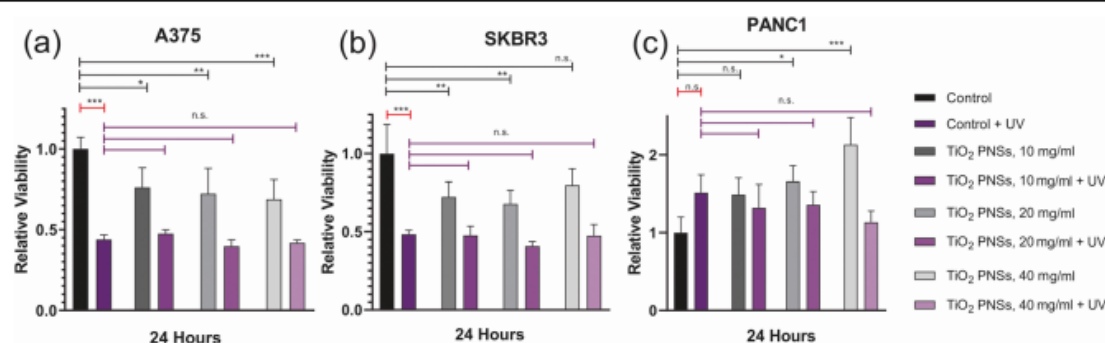


Fig. 6 The effect of the treatment with increasing concentrations of TiO₂ PNSs (presented the final concentrations in the cultivation medium) on the viability of (a) A375, (b) SKBR3, and (c) PANC1 after 24 h of incubation (left gray bars) and with additional irradiation with UV light (right violet bars) determined using the SRB

assay. Data were analyzed using a one-way ANOVA with the Tukey's test and considered non-significant for $p \geq 0.05$ (n.s.), whereas considered significant for $p < 0.05$ (*), $p < 0.01$ (**) and for $p < 0.001$ (***) compared to Control or Control + UV

cancers with few effective treatments, and in literature, the therapeutic effects of induced intracellular hyperthermia with radiofrequency radiation and targeted gold NPs were confirmed (Glazer et al. 2010). It is known that the impact of the same NPs on various cells is different and depends on cellular response to the numerous detoxification strategies that cell can utilize in response to NP treatment (Mahmoudi et al. 2012). It seems that PANC1 cell line had unexpected growth stimulation as a cellular response to the TiO₂ PNSs because they have not been recognized as intruders to the cells environment. However, these findings have to be further investigated in more details. Since PNSs have demonstrated oxidative activity in the solution, we have determined the content of MDA/HNE in the cells, i.e., the products of oxidative damage of lipids and proteins, but we failed to detect any significant changes in their content in all cells, treated with PNSs in dark and after illumination (data not shown). Therefore, the

mechanism of their action might be different, and this will be investigated further.

Cell imaging and localization of TiO₂ prolate nanospheroids

Having in mind that the photo-killing effect by induced TiO₂ PNSs redox reactions may only occur at the particles sites, the localization inside the cells and subcellular structures of TiO₂ is a crucial information for studying PSs (Li et al. 2011). In order to study the internalization of TiO₂ PNSs into cells and possible subcellular distribution, we have used a microscopic technique, LSCM.

The confocal micrographs of HeLa cells and the same cells treated with 25 $\mu\text{g ml}^{-1}$ TiO₂ PNSs are shown in Fig. 7a and b, respectively. Incubation time was 12 h, and we have presented the merged micrographs of the distribution of nuclei and TiO₂ PNSs. The most of seeded cells maintained the population 12 h

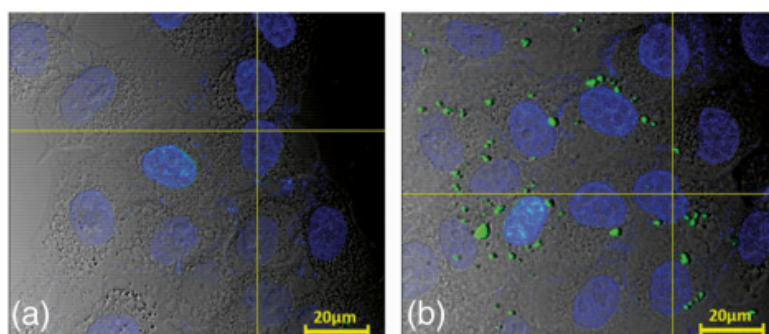


Fig. 7 Micrographs of distributions of nuclei and TiO₂ PNSs in HeLa cells. The distribution of nuclei (blue) is presented in (a); the distribution of TiO₂ PNSs (concentration of 25 $\mu\text{g ml}^{-1}$) is given with green color and presented in (b) in which the yellow crosshair denotes the co-localization of TiO₂ PNSs within nuclei. The

images displayed at the bottom and right side of the micrographs were the X-Z and Y-Z profiles measured along the lines marked in the main image, showing the 3D distributions of TiO₂ PNSs and nuclei

after treatment, and the agglomerates of TiO₂ PNSs are distributed in the outer- and inner-cellular space, as well as in the nuclei (presented at the Fig. 7b with yellow crosshair).

Conclusions

Results presented in this work demonstrate that along with colloidal TiO₂NPs, also nanorods made from the same material have potential to act as photosensitizing agents since they exhibit anti-cancer activity after cellular uptake and upon light illumination. Also, the biocompatibility of TiO₂ PNS in the dark is clearly demonstrated, in spite of the production of small amount of ROS. However, this study also opens new questions, which deserve further attention. For instance, the growth stimulating effect on the pancreatic cancer cell line and the elucidation of the mechanism of the anti-cancer cytotoxic activity.

Although TiO₂ PNSs stimulate production of ROS in water solution, this was most likely not the only mechanism through which TiO₂ PNSs exhibit their cytotoxic effect. Since they are localized in the cell cytoplasm, presumably around the cell nuclei, their cytotoxicity might be the consequence of triggering intracellular signaling pathways.

Author contributions M. Matijević performed most of experiments, and wrote the manuscript draft; M. Petković supervised integral part of the work, whereas M. Stepić, M. Vranješ, and Z. Šaponjić supervised and conducted the part of the work related to the light illumination experiments (both with cells and EPR) and synthesis and characterization of TiO₂ PNS, respectively; L. Mi, X. Liang, L. Korićanac, and M. Nešić performed most of cellular experiments with cancer cell lines, whereas L. Rajsiglova and L. Vannucci have done experiments with immune cells; M. Mojović and Đ. Nakarada were in charge for EPR spectra acquisition and data processing. I. Estrela-Lopis and J. Böttner performed and interpreted NTA measurements.

Funding information This study was funded by the Serbian Ministry of Education, Science and Technological Development (grant numbers: OI 172011, OI 172056, III 45010, III 41005, III 41027), Danube Region Strategies multilateral project – Danube meets Omics (grant number DS 052) and bilateral project with People's Republic of China (grant number 451-00-478/2018-09/16).

Compliance with ethical standards All procedures were conducted in strict accordance with the European Convention for the Care and Use of Laboratory Animals, and were approved by the

Animal Care and Use Committee at the Institute of Microbiology, AS CR, approval ID: 64/2015.

Conflict of interest The authors declare that they have no conflict of interest.

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3.3 Improving targeted therapy, chemotherapy and immunotherapy by a novel non-cytotoxic enhancer based on a recombinant human ferritin nanovector (in revision)

In this study, we investigated the potential of functionalized recombinant human ferritin-based NPs to boost the response to anti-cancer treatments *in vitro* and *in vivo*. For this purpose, human ferritin-based NPs (THE-10) were superficially functionalized with PD-L1 binding peptide, integrin $\alpha V\beta 3/5$, and neuropilin I (NRP-1) binding peptide.

To evaluate the ability of THE-10 NPs as an enhancer of therapy *in vitro*, we measured the viability of A375 human melanoma cells treated with Trastuzumab Deruxtecan (T-DXd), a clinically approved antibody-drug conjugate (ADC) alone and in combination with THE-10 NPs. We observed that the construct itself did not elicit any anticancer activity but could further potentiate the dose-dependent anti-cancer activity of T-DXd on the A375 cells, especially at lower concentrations.

To assess the construct's capacity to potentiate cancer therapies *in vivo*, we established a BALB/c mouse model with subcutaneously developed syngeneic CT26 colorectal carcinoma. In this model, animals were treated with chemotherapy (irinotecan) alone or in combination with the intravenous administration of THE-10 NPs on the following day for a total of six doses over three weeks. In our results, the anti-cancer activity of irinotecan was further potentiated when combined with THE-10 NPs. THE-10 NPs have also been shown to enhance the *in vivo* antitumor activity of the nanoferritin NPs, THE-0504, and the ADC sacituzumab govitecan in both syngeneic and xenograft models of pancreatic cancer.

These findings showed that co-administration of PD-L1 binding-peptide functionalized THE-10 NPs might expand therapeutic indications of currently approved anti-cancer drugs and that THE-10 NPs may also further improve the efficacy of these drugs in their approved indications, which may result in lowered dosages and side effects.

Contribution: My work in this project was focused mainly on the *in vivo* murine CT26 colorectal carcinoma model. I was responsible for writing the proposal for the animal welfare project (on our side of the project) and later became the person responsible for this project. My work involved all *in vivo* procedures, from inoculation of the cancer cells through intravenous/intraperitoneal administration of the treatments and follow-up of the animals to the final autopsy and harvesting of the organs. I wrote the draft for the methods and results of our part of the project.

International Journal of Biological Macromolecules
Boosting of targeted therapy, chemotherapy and immunotherapy by a novel non-cytotoxic nanovector THE-10 based on recombinant human ferritin
--Manuscript Draft--

Manuscript Number:	IJBIMAC-D-24-14855
Article Type:	Research Paper
Section/Category:	Proteins and Nucleic acids
Keywords:	human ferritin nanovector; peptide grafting; cancer therapy booster
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Abstract:	To address the challenge of drug accumulation and penetration at the tumor site(s), herein we describe a first-in-class nanovector containing 24 copies each of two bioactive peptides (BAPs) genetically fused in frame to the 24 N-termini of a human ferritin H-type construct, named THE-10. The two BAPs are specific for PD-L1, and integrin $\alpha V\beta 3/\alpha V\beta 5$ plus Neuropilin (iRGD) respectively, conferring immune checkpoint blockade and drug-internalization properties. In turn, the THE-10 backbone brings 48 BAPs contiguous for synergism, prolonged blood half-life, and release into the tumor microenvironment upon conditional cleavage of a metalloprotease-sensitive site. Predicted THE-10 multitasking activity was experimentally supported as follows. Size-exclusion chromatography and surface plasmon resonance demonstrated BAP cleavage/release and receptor binding (nanomolar KD). Live-cell/time-lapse imaging demonstrated 4-fold-increased internalization of naked therapeutic antibodies, mirrored by enhanced cytotoxicity of the corresponding Antibody-Drug Conjugate. Slight antitumor effects were observed in vivo by treating immune checkpoint-sensitive syngeneic mouse colorectal model with THE-10 alone. Drug boosting was instead considerable on colorectal and pancreatic tumor allografts when THE-10 was co-administered with both small and large chemotherapeutic agents, outperforming the original iRGD cyclic peptide. Thus, THE-10 may enhance target therapy, chemotherapy and immunotherapy altogether, e.g. it candidates as a multitasking, all-round, antineoplastic therapy booster

Boosting of targeted therapy, chemotherapy and immunotherapy by a novel non-cytotoxic nanovector THE-10 based on recombinant human ferritin

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Keywords: human ferritin nanovector; peptide grafting; cancer therapy booster

ABSTRACT. To address the challenge of drug accumulation and penetration at the tumor site(s), herein we describe a first-in-class nanovector containing 24 copies each of two bioactive peptides (BAPs) genetically fused in frame to the 24 N-termini of a human ferritin H-type construct, named THE-10. The two BAPs are specific for PD-L1, and integrin $\alpha V\beta 3/\alpha V\beta 5$ plus Neuropilin (iRGD) respectively, conferring immune checkpoint blockade and drug-internalization properties. In turn, the THE-10 backbone brings 48 BAPs contiguous for synergism, prolonged blood half-life, and release into the tumor microenvironment upon conditional cleavage of a metalloprotease-sensitive site. Predicted THE-10 multitasking activity was experimentally supported as follows. Size-exclusion chromatography and surface plasmon resonance demonstrated BAP cleavage/release and receptor binding (nanomolar K_D). Live-cell/time-lapse imaging demonstrated 4-fold-increased internalization of naked therapeutic antibodies, mirrored by enhanced cytotoxicity of the corresponding Antibody-Drug Conjugate. Slight antitumor effects were observed *in vivo* by treating immune checkpoint-sensitive syngeneic mouse colorectal model with THE-10 alone. Drug boosting was instead considerable on colorectal and pancreatic tumor allografts when THE-10 was co-administered with both small and large chemotherapeutic agents, outperforming the original iRGD cyclic peptide. Thus, THE-10 may enhance target therapy, chemotherapy and immunotherapy altogether, e.g. it candidates as a multitasking, all-round, antineoplastic therapy booster

1. Introduction

Cancer ranks as a leading cause of death and an important barrier to increasing life expectancy worldwide [1]. Despite fast-paced drug discovery, survival in advanced cancer remains low [2]. Major challenges of current anticancer regimens are poor drug penetration into the tumor mass, short blood half-life, prominent side effects, and induction of pharmacological resistance [3,4]. Enhancing drug accumulation at the tumor site(s) is possibly the single most important strategy to address the above limitations altogether, but to be effective therapeutic substances must penetrate well beyond the outer layers in a tumor lesion, e.g. deep into the tumor microenvironment (TME). Since TME acts as a penetration barrier and drug-inhibitory milieu, several approaches have been and/or are being developed by many groups to improve drug delivery and targeting [5]. Bioactive peptides (BAPs) are among the most promising tools [6,7]. For example, cerateptide (also known as

internalizing RGD, iRGD) is a TME-conduit agent that, administered in combination with several antitumor drugs, doubles their therapeutic activity [8–10]. iRGD is a 9-amino acid cyclic peptide (CRGDKGPDC) including an Arg-Gly-Asp (RGD) motif with a high binding affinity for $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins abundantly present in the tumor vasculature. Integrin binding is key to enhanced tumor penetration of iRGD-conjugated or co-administrated drugs. iRGD favorably compares with the RGD peptide (CRGDC) because when cleaved into CRGDK, iRGD exposes its C-terminal region, binds neuropilin-1 (NRP-1) receptors, and consequently triggers NRP-1-dependent endocytosis, further improving the tumor penetration of active drugs (C-end rule or CendR) [11]. Additional features of iRGD, that make it an attractive drug-delivery system, are its low toxicity for normal cells, easy and low-cost synthesis, and targeted release [11].

Along the same line, immune checkpoint inhibitors (ICI) are possibly the most successful therapeutic approach to relieve the negative impact of an immune-suppressive TME, but side effects require careful clinical management [11]. The classical approach, now routinely applied to most solid tumors in early as well as advanced clinical settings, is to unleash T-cells by administering therapeutic antibodies to ICI such as the Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) or programmed cell death protein 1 (PD-1) and its PD-L1 ligand [12]. Unfortunately, cost and *in vivo* immunogenicity of antibodies are two major drawbacks preventing a wider deployment of ICIs in the clinics. BAPs are seen as an effective tool to also mitigate these issues. Through the screening of phage-display peptide libraries, BAPs blocking critical checkpoint interaction sites have now been identified [13]. CLQKTPKQC and CVRARTR (PD-L1Pep-1 and PD-L1Pep-2 respectively) and GNWDYNSQRAQLYNQ, block PD-L1 and PD-1 respectively, and have shown promising activity both *in vitro* and *in vivo* [14,15]. Their major advantages over antibodies are improved tissue penetration, lower immunogenicity, and moderate manufacturing costs.

Unfortunately, short BAPs also have disadvantages, including weak binding affinity, low persistence in blood, and susceptibility to degradation [16]. Consequently, a single BAP (whether designed for enhanced drug delivery or checkpoint blockade) is not sufficient to elicit a strong and long-lasting biological effect, that instead requires the simultaneous engagement and delivery of multiple copies of distinct BAPs. An attractive way to achieve this, keeping the different BAPs in physical proximity, is to combine multiple BAPs into a single supramolecular structure, which is expected to result in exploiting BAP combinations and synergies. However, this approach requires careful selection of a recombinant protein with proven carrier ability, strong tropism for multiple tumors, resilient three-dimensional folding, tolerance to multiple simultaneous substitutions in

different regions, a controlled release safelock, and an effective, industrial-grade biomanufacturing pipeline.

Herein, we exploit the symmetrical cage-shaped structure of the human ferritin H-type (HfT) nanovector for the selective delivery of multiple BAPs to the tumor site. HfT is a highly symmetrical multimeric protein consisting of 24 subunits that assemble into a molecular structure with an essentially spherical shell. This shell encloses a hollow cavity that physiologically stores iron, but can be adapted to accommodate therapeutic drugs, as shown by several groups including ourselves [17–26][27–31]. In the present report, however, we do not exploit the cargo abilities of HfT, but rather its unique quaternary structure, whereby the N-terminus faces the external side of the spherical shell and can allow for the exposure of 24 copies of BAPs, once they are properly fused in frame by recombinant DNA technology. The novel HfT-based fusion protein described herein has now been engineered to include at the N terminus of each subunit two additional BAP amino acid sequences. The selected BAPs are two modified linear versions of the PD-L1 binding peptide (PD-L1Pep-1) and the integrin $\alpha V\beta 3/\alpha V\beta 5$ and NRP-1 binding peptide (iRGD).

In engineering these novel BAP-containing HfT nanovectors, we maintained two useful features already present in previous HfT constructs of ours. The first is PASylation, e.g. a masking polypeptide sequence rich in proline (P), alanine (A), serine (S) and Glutamic Acid (E) residues (PASE) that is genetically fused to the N-terminus of each HfT subunit. PASE was shown to improve HfT protein stability, *in vivo* half-life, and drug delivery, which provides a strong rationale for its inclusion in the present BAP-HfT constructs [32]. The second conserved feature is a tumor-selective sequence (MP) responsive to proteolytic cleavage by tumor metalloproteases (MMPs 2 and 9). Identical to previous constructs, the MP site was inserted between each HfT subunit and the outer PASE polypeptide [32].

Thus, the novel HfT construct described herein, named THE-10, contains 48 BAPs of two kinds with expected drug-delivery and checkpoint blockade activities. HfT-linked BAPs are incorporated into THE-10 in a way that they may be preferentially released in active form at the tumor site following proteolytic cleavage of two MP sites by TME metalloproteinases MMP2 and MMP9.

Herein, we provide evidence for conserved BAP activities and argue that the PASylated, cleavable HfT backbone is crucially involved in improving their anticancer properties, e.g. it acts as an efficacy booster for a large spectrum of co-administered drugs with different biochemical properties, sizes, and mechanisms of action.

2. Material and methods

2.1. Design of the THE-10 nanovector

The *THE-10* gene is a variant of the HfT-MP-PASE gene previously reported [32]. As shown in **Scheme 1**, THE-10 includes, C- to N-terminus: (a) a first domain comprising the amino acid sequence of the native human ferritin HfT; (b) a second domain comprising: (i) the amino acid sequence LQKTPKQ as PD-L1 binding peptide, which is conjugated to the N-terminal of said first domain through the amino acid sequence of a matrix metalloproteinase (MMP) cleavage site PLGLAG, and (ii) the amino acid sequence RGDKGPD as tumor penetrating peptide, which is conjugated to said amino acid sequence LQKTPKQ through the amino acid sequence of the MMP cleavage site; (c) a third N-terminal domain consisting of proline, serine, alanine and glutamate (PASE): ASPAAPAPASPAEPAPSAPAASPAAPAPASPAEPAPSAPA.

2.2. Cloning, overexpression and purification of HfT-MP-PASE construct

The *THE-10* sequence was codon-optimized for high level expression in *Escherichia coli*, generated by whole-gene synthesis at GENEART AG (Germany), cloned into the expression vectors pET-27b (Thermo Fisher Scientific Waltham, USA), and expressed in the BL21(DE3) bacterial host (New England BioLabs, Ipswich, USA). *E.coli* cells were grown to OD600 1.0 at 37°C in 1 L of terrific broth medium (Grisp, Porto, Portugal) containing kanamycin (Serva, Heidelberg, Germany) at a final concentration of 0.03 mg/mL. The cells were harvested by centrifugation at 5000 rpm for 20 minutes at 4°C. The recovered pellet was suspended in 90 mM Tris-HCl, 50 mM NaCl and EDTA 0.5 mM at pH 9 and incubated at 60°C for 15 minutes. After thermal treatment, the enzyme DENARASE (c-LEcta GmbH, Leipzig, Germany) was added at 15 U/mL to cleave DNA and RNA impurities, and then the cells were disrupted by sonication. The lysate was centrifuged at 14,000 rpm for 50 minutes at 4°C and the supernatant was further treated at 72°C for 8-10 minutes. The solution was centrifuged 14000 rpm for 50 minutes at 4°C. The supernatant was dialyzed overnight against phosphate-buffered saline (PBS) pH 7.5, concentrated by means of 100 kDa Amicon Ultra-15 centrifugal filter devices (Millipore, Billerica, USA) and loaded on a strong basic anion-exchange filter, Sartobind Q (Sartorius, Goettingen, Germany), previously equilibrated with the same buffer. THE-10 preparations were eluted from the column, whereas other *E. coli* impurities were retained

by the filter. Finally, the THE-10 samples were sterile-filtered and stored at 4 °C (short-term) or -20°C (long-term). Typical yields were 100 mg of pure proteins per 1 L of bacteria culture.

2.3. Protein characterization

The purity of all the preparations was assessed by SDS-PAGE, running the samples on 15% gels and staining with Coomassie brilliant blue. Protein concentrations were determined spectrophotometrically at 280 nm, using a molar extinction coefficient (on a 24-mer basis) of $4.54 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (ProtParam software, <http://www.expasy.org>). Size-Exclusion Chromatography (SEC) was performed using a Superose 6 gel-filtration column equilibrated with PBS at pH 7.4. All samples were prepared at 1 mg/mL in filtered H₂O_{dd}. All the traces of SEC experiments were analyzed with QtiPlot (IONDEV SRL, Bucuresti, Romania).

2.4. In vitro release of PASE and multiple peptides from THE-10 in the presence of MMP2/9 proteases

To assess the peptides release after enzymatic cleavage, THE-10 cleavage was studied in the presence of the specific proteases MMP-2/9. THE-10 solutions were mixed with collagenase IV (Collagenase Type 4 containing MMP2 and 9, Clostridium histolyticum, Pan-biotech, Leipzig, Germany) and incubated at 37 °C for 2 h. Samples were then analyzed by SEC using a Superose 6 gel-filtration column equilibrated with PBS at pH 7.4, and by SDS-PAGE gel chromatography. SDS PAGE was stained with Coomassie brilliant blue R-250.

2.5. Testing of the THE-10 fusion protein binding ability by Surface Plasmon Resonance experiments

Surface Plasmon Resonance (SPR) was carried out to assess the thermodynamic and kinetic parameters of the interaction between the construct THE-10 (analyte) and its specific receptors (ligands): $\alpha\text{V}\beta 3$, NRP-1 ($\alpha\text{V}\beta 3$ #IT3-H52E3, his-tagged human #NR1-H5228; ACROBiosystem, Germany), and PD-L1 (C-terminal his-tagged human PD-L1 #10084-H08H, Sino Biological Europe GmbH, Germany) in a Biacore X-100 apparatus. The ligands were immobilized on a Sensor Chip NTA. The NTA group was activated by nickel ions (Ni^{2+}) to bind selectively histidine-tagged ligands. Ligands ($\alpha\text{V}\beta 3$ or NRP-1: 25 $\mu\text{g/mL}$ and PD-L1: 50 $\mu\text{g/mL}$) were stripped by sequestering nickel ions with EDTA (regeneration phase). After ligand immobilization, THE-10 was injected at

different concentrations: α V β 3 and NRP-1: 96, 192, 384 (tested twice to ensure reproducibility of the results), 768 and 1536 nM; PD-L1: 6.25, 12.5, 25, 50, 100 and 200 nM.

2.6. Testing Flow cytometry analysis

To assess surface markers, A375 cells were stained with anti-HER2 Trastuzumab (provided from the hospital pharmacy of the Regina Elena Cancer Institute) or with the following fluorescently labeled monoclonal antibodies (mAbs): anti-human CD51/CD61 FITC (23C6) from eBioscience and anti-human CD304 PE (12C2) from Biolegend, respectively. Cells were stained with anti-CD51/CD61 or anti-CD304 mAbs (1:20) or with anti-Her-2 (10 μ g/ml) in cold PBS-2% FBS solution, 30 min on ice. Appropriate Goat anti-Human IgG (H+L) Secondary Antibody, FITC (1:100) was added to Trastuzumab stained cells. All acquisitions were performed with Attune NxT Acoustic Focusing Cytometer (Invitrogen, Thermo Fisher) and data analyzed with the FlowJo software v10.

2.7. Uptake and internalization experiments

Uptake and internalization were assessed using an Incucyte S3 live-cell analysis system (Sartorius, Goettingen, Germany). The test compound (recombinant antibody trastuzumab obtained from injectable preparations for human infusion, Roche Pharmaceuticals) was conjugated with the pH-sensitive dye pHrodo using the Deep Red Antibody Labeling kit (#P35355, Invitrogen, Thermo Fisher scientific), according to the manufacturer's instructions. Human melanoma cells A375 (obtained from the American Type Culture Collection, ATCC) were seeded at a density of 3×10^3 cells/well in 96-well plates one day before the addition of Trastuzumab-pHrodo (10 μ g/mL), alone or combined with THE-10 (1.0 mg/mL). Phase contrast and fluorescence images of cells cultured under standard conditions (37 °C, 5% CO₂, RPMI 1640 medium, 10% fetal bovine serum, 1% penicillin–streptomycin and 1% glutamine) were captured at 20X magnification every 60 min for 48 hours, and mounted in the time-lapse mode by on-board software. Red integrated fluorescence (from pHrodo-labeled antibodies internalized in acidic intracellular compartments) was elaborated by the integrated, automated Incucyte software, and quantitated after background subtraction. Data were plotted and analysed by onboard software. Internalization was expressed as per cent of baseline (time 0) fluorescence over incubation time.

2.8. Testing of the THE-10 fusion protein activity on cultured cells: anti-proliferative effect stimulation

To assess proliferation, human melanoma A375 cells were seeded at a density of 1×10^3 cells/well in 96-well plates (Costar #3595). On the following day, Trastuzumab Deruxtecan (T-DXd, MedChemExpress, USA), alone or combined with THE-10 (1.0 mg/mL), was added, and cells were grown under standard conditions. Cell viability was measured by the Cell Titer-Glo 2.0 kit (Promega, Madison, WI, USA). Each experimental condition was assessed in triplicate. After 96 hours of treatment, 100 μ l of Cell Titer-Glo reagent were added to each well. Plates were incubated for 10 minutes at room temperature in the dark, and luminescence was determined on a Synergy/LX multimode reader (Biotek), using the Gen5 3.10 Imager software. Results were plotted as mean $\pm$ SD, and analyzed by GraphPad Prism 9.

2.9. In vivo studies authorizations and response determination

Protocols for animal studies were approved by the Institutional Animal Care and Use Committees of the University of Udine (Italy), University of Chieti (Italy), and the Institute of Microbiology of the Czech Academy of Science (Prague, Czech Republic): Italian Ministry of Health IDs no. 414/2021-PR and 391/2022-PR; and ID 111/2019, respectively. Experiments were compliant with the principles laid down in the European Community Council Directives (86/609/EEC). In all experiments, tumor volume was measured twice a week with a digital caliper and mouse weight was monitored. Animals were sacrificed at the end of the study period or when the tumor had reached a volume ≥ 1000 mm³. For treated groups only, the percentage of tumor growth inhibition (% TGI) was defined as $100 \times (\text{MTV}_{\text{control}} - \text{MTV}_{\text{treated}}) / \text{MTV}_{\text{control}}$, where MTV is the median tumor volume.

2.10. In vivo anti proliferative enhancement effect of THE-10 using KPC pancreatic cancers

Seven-week-old female C57BL/6 mice (Charles River Laboratories, Lecco, Italy) were injected subcutaneously (i.e., right flank) with 0.5×10^6 pancreatic KPC cells resuspended in a 1:3 (v:v) solution of PBS:Matrigel in a final volume of 200 μ l. Three different experiments were carried-out using this cancer model.

In the first experiment, as test cytotoxic drug compound was evaluated the small molecule Gemcitabine (MedChemExpress, USA). When tumors had reached a volume of about 100-200 mm³, mice were randomized in groups of six animals and injected with 200 μ L of physiological saline

(intravenously, i.v.), 200 μ L Gemcitabine (100 mg/Kg; intraperitoneal, i.p.) or 200 μ L Gemcitabine (100 mg/Kg; i.p.) plus 200 μ L THE-10 (130 mg/Kg; i.v.). THE-10 was injected 24 hours before treatments. Mice were injected twice a week for two weeks. In the second experiment, as test cytotoxic drug compound the large macromolecule THE-0504 nanoferritin (Thena Biotech Srl) was evaluated [17,18,22]. When tumors had reached a volume of about 200-250 mm³, mice were randomized in groups of six animals and injected i.v. with 200 μ L of physiological saline (i.v.), 100 μ L THE-0504 (1.0 mg/kg), 100 μ L THE-0504 (1.0 mg/kg) plus 100 μ L iRGD peptide (3.0 mg/Kg; MedChemExpress, USA) or 100 μ L THE-0504 (1.0 mg/kg) plus 100 μ L THE-10 (130 mg/Kg, corresponding to 3.0 mg/kg in iRGD peptide). Mice were injected twice a week for three weeks. In the third experiment, as test cytotoxic drug compound the large macromolecule Sacituzumab govitecan (IMMU-132; MedChemExpress, USA) was evaluated. When tumors had reached a volume of about 100 mm³, mice were randomized in groups of six animals and i.v. injected with 200 μ L of physiological saline (i.v.), 100 μ L Immu-132 (45 mg/Kg) or 100 μ L Immu-132 (45 mg/Kg) plus 100 μ L THE-10 (130 mg/Kg). THE-10 was injected 5-10 minutes before the treatments with Immu-132. Mice were injected every 3-4 days for three times.

2.11. In vivo effects of THE-10 in CT26 colorectal cancers

Eight- to ten-week-old male Balb/c mice (AnLab, Prague, Czech Republic) were subcutaneously injected with 1×10^6 syngeneic colorectal adenocarcinoma cells CT26 (ATCC, USA). When tumors reached a volume about 50-100 mm³, mice were randomly subdivided into groups of 6 animal each. In the first experiment, animals were injected with 200 μ L of saline (i.v.) or THE-10 (120 mg/Kg) two times per week for a total of four injections. In the second experiment, animals were injected with 200 μ L of saline (i.v.), 300 μ L of irinotecan (100 mg/Kg) (i.p.) or 300 μ L of irinotecan (i.p.) plus THE-10 (120 mg/Kg) (i.v.), two times per week for a total of four injections. THE-10 was injected 24 hours before the treatment with irinotecan.

2.12. In vivo antiproliferative enhancement effect of THE-10 using MiaPaca2 pancreatic cancer

Four-week-old female athymic nude mice (Envigo, Italy) were injected subcutaneously (i.e., right flank) with 5×10^6 pancreatic MiaPaca2 cells resuspended in 200 μ L of DMEM medium plus 1% BSA. When tumors had reached a volume of about 80-100 mm³, mice were randomized in groups of six animals and injected i.v. with 200 μ L of physiological saline (i.v.), 100 μ L THE-0504

(1.0 mg/kg), or 100 μ L THE-0504 (1.0 mg/kg) plus 100 μ L THE-10 (130 mg/Kg). Mice were injected twice a week for three weeks.

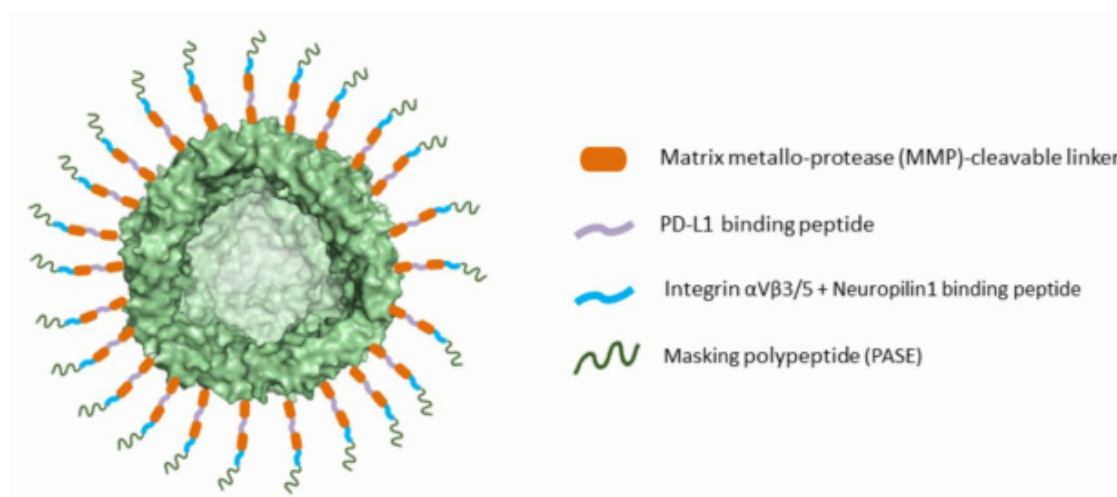
Statistical analysis. Values are presented as the means and standard deviation. Data analysis was performed by GraphPad Prism version 5. Statistical analysis of tumor growth curve and tumors weights at the sacrifice were done using one-way ANOVA analysis, adopting the Kruskal-Wallis test and using Dunn's Multiple Comparison test for coupled comparisons. Differences at $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Design and Production of THE-10

The goal of this study was to assess whether a novel BAP-fusion protein based on an engineered human ferritin enhances the antitumor activity of co-administered drugs ('booster' effect). We aimed at assessing whether the booster effect takes place with different classes of anticancer agents, in different cells, in various experimental conditions, and to which extent. Therapeutic boosting may be of interest to expand the indications of approved drug as well as to refine efficacy data of drugs under clinical developments.

The test compound, named THE-10, was derived by modification of our previously described PASylated protein HFt-MP-PASE [32]. The sequences selected for incorporation in the N-terminal domain of HFt-MP-PASE are linear versions of two previously reported cyclic BAPs: the PD-L1 binding peptide (PD-L1Pep-1), and another peptide binding integrin $\alpha V\beta 3/\alpha V\beta 5$ and NRP-1 (iRGD or Certepetide). THE-10 is shown in scheme 1.



Scheme 1. Schematic representation of the THE-10 construct, wherein the N terminus of each of the 24 monomers is genetically fused to a cleavable MMP peptide sequence, two bioactive peptides, and an outer PASE (masking) sequence.

3.2. THE-10: biochemical characterization and cleavable N-terminus

Purified (see methods) THE-10 was biochemically characterized and compared with THE-10 incubated at 37°C for two hours with a collagenase IV preparation containing matrix metalloproteases MMP-2 and 9. These are typically expressed in the tumor microenvironment. Size-exclusion chromatography (SEC, Fig. 1A) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 1B) revealed the expected differences in elution volumes and profiles before and after cleavage. The latter were slight but clearly detectable and resulted in a shift in electrophoretic band migration profiles compatible with the anticipated drop of about 5 kD in each HfT subunit. Therefore, highly purified THE-10 is cleavable, e.g. BAPs are expected to be released at the TME in the presence of MMPs.

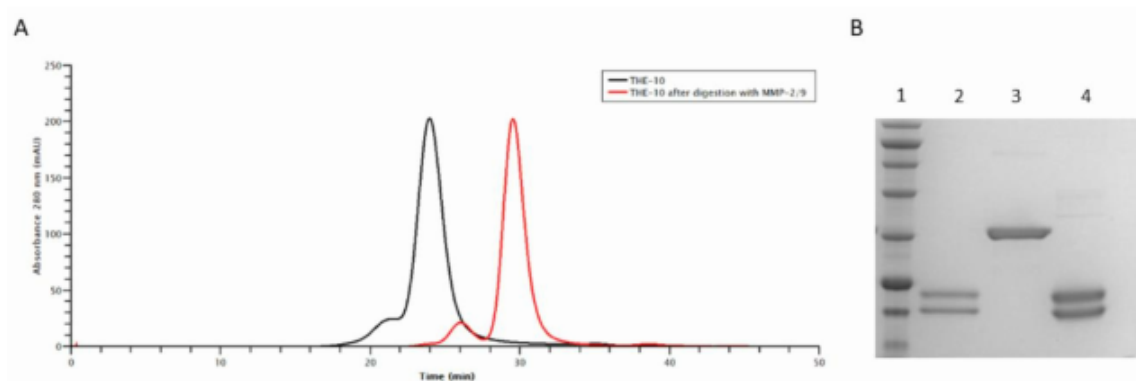


Fig. 1. Purity and polypeptides removal in vitro. (A) SEC profiles of THE-10 before (black line) and after (red line) PASE and BAP sequences removal by MMP-2/9 proteinase in vitro. (B) SDS-PAGE band migration profiles of THE-10 before and after MMP-2/9 digestion. *Lane 1*, protein marker; *Lane 2*, native Hft (5 µg); *Lane 3*, THE-10 (5 µg); *Lane 4*, THE-10 (5 µg) after in vitro digestion with 0.5 mg/mL MMP-2/9. SDS PAGE was stained with Coomassie brilliant blue R-250.

3.3. BAP-mediated receptor binding of THE-10

To determine whether BAPs confer receptor tropism, THE-10 binding was assessed by surface plasmon resonance (SPR) assay using recombinant $\alpha V\beta 3$, neuropilin-1 (NRP-1) and PD-L1 as ligands (Fig. 2A and B). All measured dissociation constants (K_D) were in the nanomolar range, indicating strong binding affinity between the THE-10 analyte and its ligands. Nevertheless, the two ligands show a different behaviour in the interaction with the functionalized construct. In fact, as shown in figure 2A, the interaction between THE-10 construct and $\alpha V\beta 3$ prompted out a strongly biphasic interaction, characterized by two association rate constants (k_{on}) and two dissociation rate constants (k_{off}). Consequently, the $\alpha V\beta 3$ SPR sensorgram required a heterogeneous ligand binding model compatible with the presence of one analyte (THE-10) and two possible binding sites, with high and low affinity respectively (Fig. 2A). Alternatively, a mixed population of the receptor may also be envisaged to interpret the kinetic data. However, the first interpretation is preferred, as the amplitude of the rapid dissociation phase is increasing with increasing concentration of the analyte, thus indicating that higher concentration of this species may populate more easily the alternative site at lower affinity. On the contrary, PD-L1 data fit a straightforward 1:1 interaction model (Fig. 2B),

with a very low K_D value consequent to the very slow k_{off} value. This indicates an extremely stable interaction between THE-10 and PD-L1.

As to NRP-1, no significant SPR response could be detected (data not shown). This result may be expected, because binding of THE-10 to NRP-1 requires cleavage of the iRGD peptide incorporated in THE-10 construct, and exposure of the internal RGDK binding motif (C-end rule). Unfortunately, this cannot be modelled by *in vitro* SPR experiments, since it is mediated by an unknown TME protease *in vivo*. Regardless, SPR experiments clearly show that at least 2 of the 3 BAPs grafted onto the THE-10 fusion HfT nanovector retain full biological *in vitro* activity.

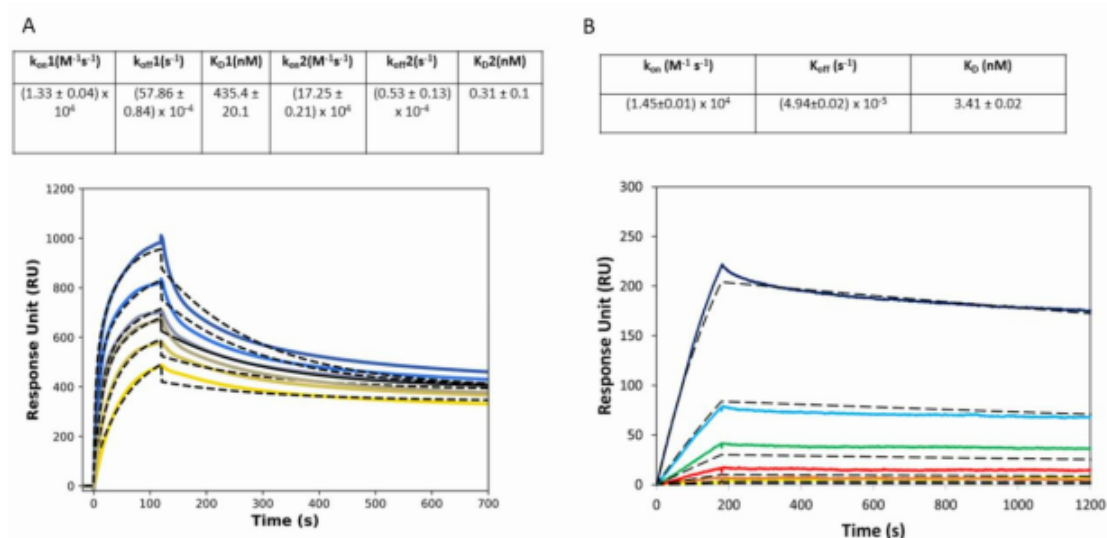


Fig. 2. SPR experiments. *Top:* Thermodynamic and kinetic parameters of the interaction of THE-10 (analyte) with **(A)** human $\alpha V\beta 3$ and **(B)** human PD-L1. *Bottom:* Sensorgrams showing the interaction of THE-10 with human his-tagged receptors immobilized onto a HisTag sensorchip. Sensorgrams show THE-10 injection, followed by buffer injections to monitor analyte dissociation. In **(A)**, curves were fitted using a heterogeneous ligand binding model. In **(B)**, curves were fitted as single 1:1 interactions; fittings are shown as black dashed curves.

3.4. THE-10 enhances the internalization of a therapeutic antibody

As a first step to determine whether biochemical activity translates into biological activity, THE-10 was assessed for its ability to enhance the uptake and internalization of a co-administered anti-cancer drug (booster effect). The humanized therapeutic antibody Trastuzumab (TTZ) was

selected as a test compound. TTZ binds the Human Epidermal Growth Factor Receptor 2 (HER2), that is typically overexpressed in breast cancer cells of the HER2-positive subtype, but is expressed at extremely low levels in most human tumors, including melanoma cells, which makes these tumors insensitive to anti-HER2 treatments with antibodies and small molecules. The melanoma cell line A375 recapitulates this typical HER2-negative phenotype (Fig. S1A), but it displays low and moderate expression of $\alpha V\beta 3$ and NRP-1, respectively (Fig. S1B and C), which makes it suitable to test booster THE-10 activity, if any.

On this basis, TTZ was conjugated to a pH-sensitive dye that turns red when the antibody is internalized into acidic intracellular vesicles. Time-lapse (every 60 min), live cell imaging of A375 melanoma cells growing for 48h under standard condition readily revealed that TTZ is poorly and slowly internalized, as expected, but addition of THE-10 to the culture medium accelerates uptake, and gradually enhances red fluorescence intensity, that reaches approximately four-fold the control values at endpoint (Fig. 3A, and compare panels B and C). In summary, THE-10 enhances cell uptake and internalization of a therapeutic antibody in a cell line with the right background of $\alpha V\beta 3$ and NRP-1 receptors. This conclusively demonstrates functionally successful BAP grafting onto the THE-10 nanovector backbone.

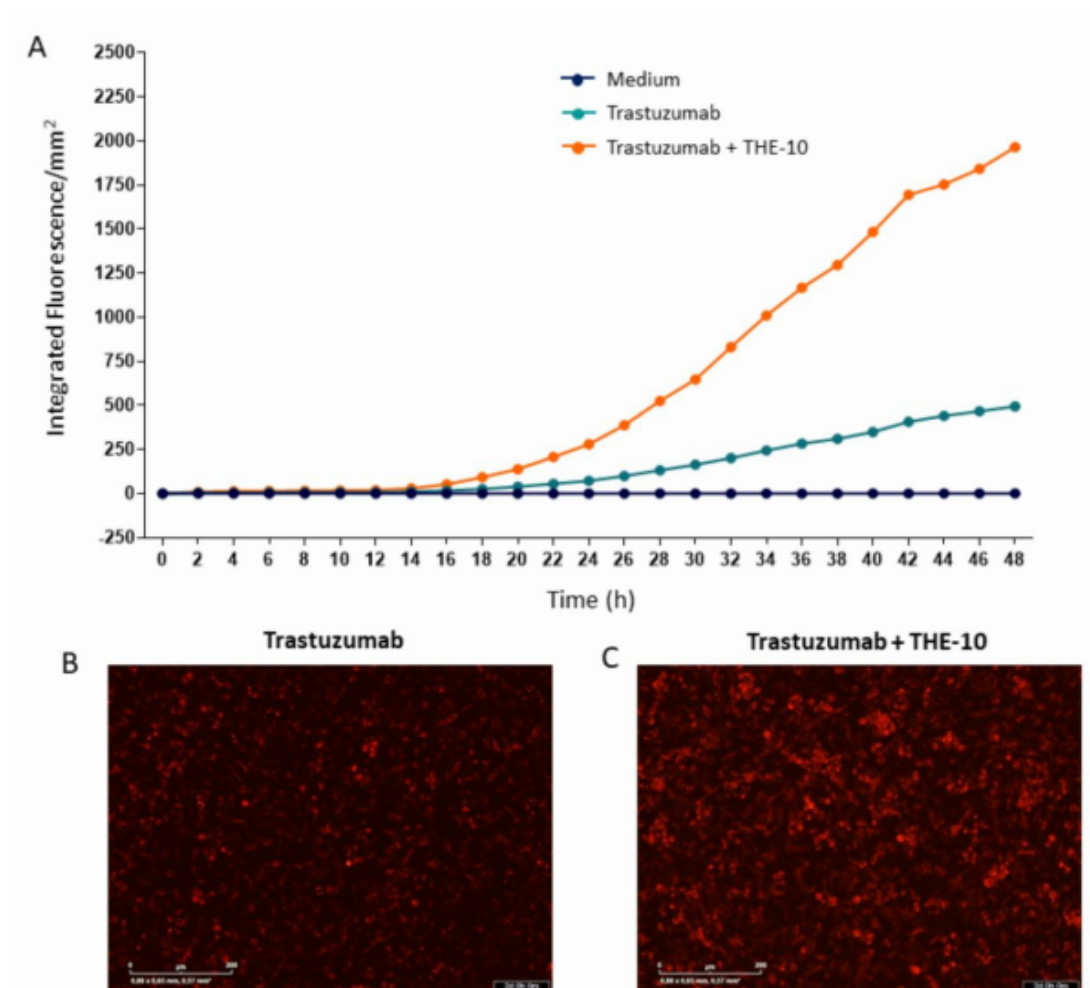


Fig. 3. Kinetics of antibody Internalization by A375 melanoma cells. A375 cells were treated with pHrodo Deep Red labeled Trastuzumab (10 μ g/mL), alone or combined with THE-10 (1 mg/mL), for 48 hours in an Incucyte Live-Cell system. **A.** A time course of red Integrated Fluorescence, corresponding to antibody internalized into acidic intracellular vesicles, is displayed (20X magnification) for the three conditions, with acquisition at 60 min intervals. Each point of the internalization kinetic is shown as the mean of 18 independent readings of 2 wells, which makes standard deviation negligible. **B, C.** Red images captured at time 48 h post treatment.

3.5. THE-10 enhances tumor cell killing by a therapeutic Antibody-Drug Conjugate (ADC)

To determine whether increased internalization translates into improved therapeutic efficacy, the same A375 human melanoma cells were exposed to the same antibody, albeit in a form

conjugated to a toxic topoisomerase I inhibitor, e.g. Trastuzumab deruxtecan (T-DXd). T-DXd was selected because, unlike other naked antibodies and ADCs, it has remarkable unprecedented activity on HER2-low breast cancers [33], although results of clinical trials with HER2-low cancers from other histotypes are not yet available.

T-DXd killing of A375 melanoma cells was dose-dependent, and at the lowest T-DXd dosage THE-10 enhanced killing approximately four times, whereas THE-10 alone displayed no significant effect (Fig. 4). Therefore, THE-10 mediates a booster effect on a cytotoxic antibody to HER2 in a cell line expected to be unresponsive to anti-HER2 treatments but capable to internalize the antibody in presence of THE-10.

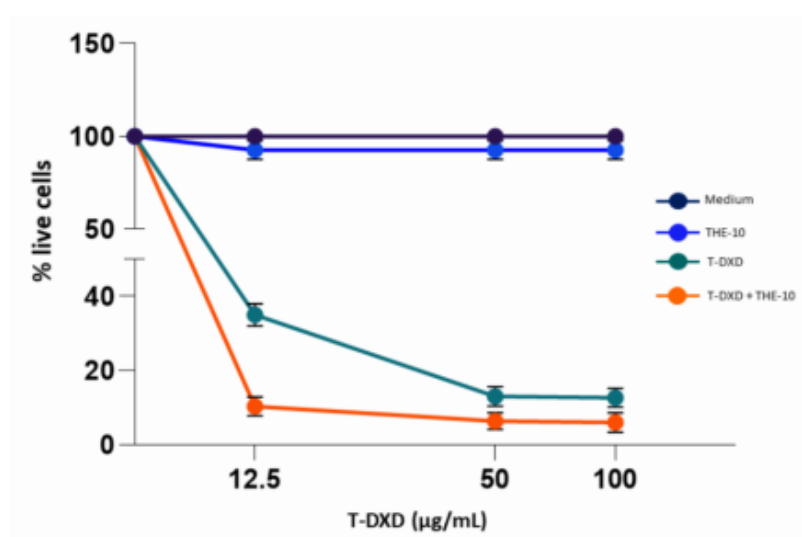


Fig. 4. Booster effect of THE-10 on A375 melanoma cells. Cell killing after 5 days of treatment with Trastuzumab deruxtecan (T-DXd) and THE-10, alone or combined. Each experimental condition was performed in triplicate. Bars correspond to mean $\pm$ SD from 3 replicates per condition.

3.6. THE-10 slows tumor growth in a colorectal cancer mouse model

A PD-L1 peptide (PD-L1pep1) fused to the N-terminal of the native human ferritin (HfT) was previously reported to block PD-1/PD-L1 interactions, inhibit the immune suppressive activity of PD-L1 and have some antitumor effects *in vivo* [34]. THE-10 contains a similar peptide, the only difference being the absence of the terminal N- and C-terminal cysteine residues. The antitumor activity of THE-10 was directly assessed in the same syngeneic mouse allograft model used by Jeon IS et al. Established CT26 mouse colon cancer grafts were treated by intravenous injection of THE-

10 (120 mg/kg) or saline two times per week for a total of four injections. Treatment with THE-10 mildly inhibited tumor growth compared with control mice treated with saline (Fig. 5). This mild antitumor efficacy was expected, and it is comparable to that reported for the HFt-PD-L1 fusion protein previously ascribed to the blocking PD-1/PD-L1 interaction and immunological restoring of T cell immune response against the tumor [34]. No significant differences in body weight were observed among experimental groups during treatment (not shown).

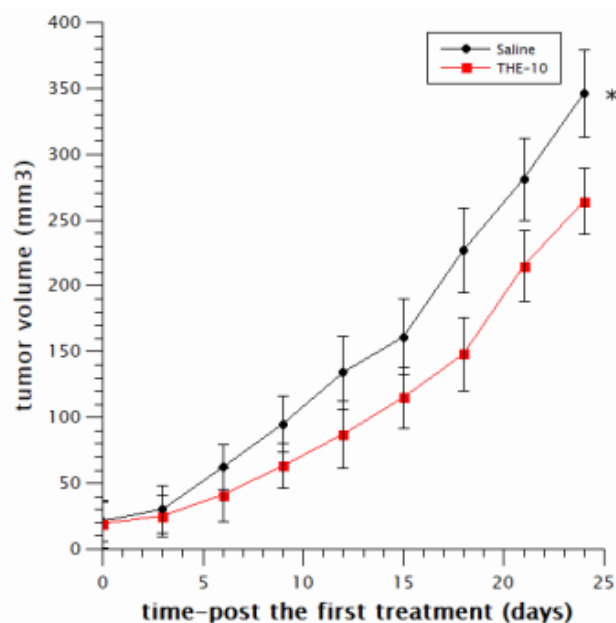


Fig. 5. THE-10 antitumor effect on CT26 syngeneic mouse colon tumors. Growth curves representing the media of the tumor volume for each of the treated groups $\pm$ SEM (* $p < 0.05$, t-test).

3.7. THE-10 enhances the *in vivo* antitumor activity of two small drugs: Irinotecan and Gemcitabine

Irinotecan and gemcitabine are two key drugs employed worldwide for the treatment of several solid tumors. However, therapeutic resistance, short half-life, and side effects owing to toxicity limit their success in clinical practice. For this reason, we examined the ability of THE-10 to boost drug accumulation and anticancer effects in two murine models. The anticancer booster effect of combination therapy with Irinotecan plus THE-10 was evaluated in the syngeneic mouse colon (CT26) cancer model (Fig. 6A). Four intravenous injections of THE-10 (120 mg/kg), followed by intra-peritoneal injections of irinotecan (100 mg/kg), two times per week for a total of four injections, significantly reduced tumor growth as compared to injection of the sole Irinotecan

($p < 0.05$; Fig. 6A). Tumor Growth Inhibition (TGI) was 29.0 % for the Irinotecan-treated group and 63.0 % for the group treated by irinotecan plus THE-10 (Table 1).

Similarly, co-administration of THE-10 (130 mg/Kg) with gemcitabine (100 mg/Kg) decreased growth of a syngeneic mouse pancreatic (KPC) tumor significantly more than gemcitabine monotherapy ($p < 0.001$, Fig. 6B), with TGI values of 82.2% vs 48.8% (table 1).

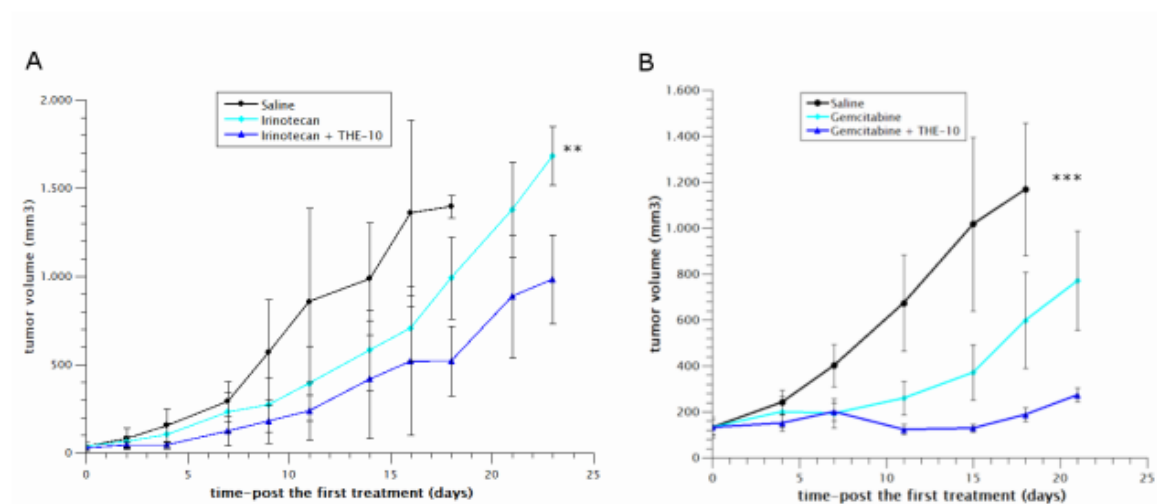


Fig. 6. Tumor treatment studies involving co-administration of THE-10 with irinotecan or gemcitabine. (A) Tumor growth curves of irinotecan and THE-10 combination therapy in CT26 colorectal cancer model. (B) Tumor growth curves of gemcitabine and THE-10 combination therapy in KPC pancreatic cancer model. Statistical differences (t-test) are as follows. * $p < 0.05$: Irinotecan vs control, Irinotecan plus THE-10 vs control, Gemcitabine vs control, Gemcitabine plus THE-10 vs control; ** $p < 0.001$: Irinotecan vs Irinotecan plus THE-10; *** $p < 0.0001$: Gemcitabine vs Gemcitabine plus THE-10.

3.8. THE-10 enhances the *in vivo* antitumor activity of the nanoferritin THE-0504 in two different models of pancreatic cancer

THE-0504 is a complex based on THE-05, a genetically modified human ferritin which acts as a drug carrier. THE-0504 encapsulates in its cavity, as payload, the cytotoxic drug Genz-644282, a non-camptothecin topoisomerase-I inhibitor. Recently, we evaluated THE-0504 in solid tumor animal models. Delayed tumor growth and durable complete remissions were observed in

combination with a manageable toxicity profile [17,18,35]. THE-0504 is currently at an advance development stage for first-in-human trials in solid tumors.

This prompted us to evaluate THE-10 as an antitumor booster of THE-0504. To this end, a murine model of pancreatic cancer was selected in which the activity of THE-0504 is very low, since THE-0504 binds the murine CD71 receptor much less than its human receptor orthologue [22]. Syngeneic mouse pancreatic (KPC) cancers, about 250 mm³ in volume, were treated by intravenous injection twice a week for three weeks. Mice received 200 µL of physiological saline, or THE-0504 (1.0 mg/kg), or THE-0504 (1.0 mg/kg) plus iRGD peptide (3.0 mg/Kg), or THE-0504 (1.0 mg/kg) plus THE-10 (130 mg/Kg, corresponding to about 3.0 mg/kg in iRGD peptide). Tumor growth curves show the expected low antitumor activity of THE-0504 alone, a limited booster effect of the original cyclic iRGD peptide (CRGDKGPDG) that is currently in phase 2 clinical trial for pancreatic cancer, and a much greater, and remarkable, boost by THE-10 (Fig. 7), as quantitatively estimated by TGI values (table 1). Since the plasma half-lives of iRGD and parental THE-05 are 15 min and about 40 hours respectively [35,36], these findings are consistent with a longer serum persistence and/or higher tumor accumulation/release of BAPs fused to HfT as compared to naked peptides.

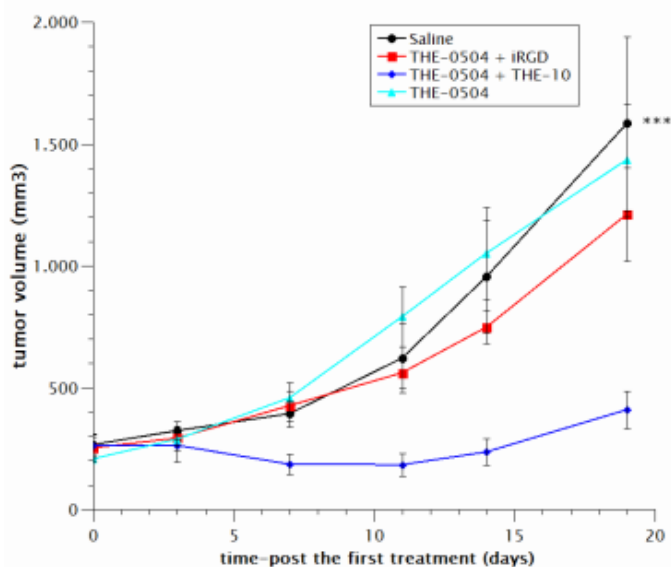


Fig. 7. Tumor treatment studies involving co-administration of THE-0504 drug with THE-10 or iRGD peptide. Tumor growth curves in KPC pancreatic cancer model. Statistical differences (t-test) are as follows. ** $p < 0.001$: THE-0504 vs control, THE-0504 plus THE-10 vs control, THE-0504 plus iRGD vs control; *** $p < 0.0001$: THE-0504 plus THE-10 vs THE-0504 plus iRGD.

The booster effect of THE-10 was assessed in a second pancreatic cancer model, that is instead susceptible to THE-0504. In this case, the tumor (MiaPaca2) was of human origin and was grown as a xenograft. Mice were intravenously injected twice a week for three weeks with 200 μ L of physiological saline, THE-0504 (1.0 mg/kg), or THE-0504 (1.0 mg/kg) plus THE-10 (130 mg/Kg). As shown in figure 8, THE-0504 alone greatly reduced tumor growth ($p < 0.001$), but despite this marked effect, addition of THE-10 resulted in a remarkable boost, revealed by an almost complete stall in tumor growth (Fig. 8A, table 1). This is best appreciated by considering the dramatic reduction (approximately four-fold) in the weight of residual tumor masses excised from the animals at the end of the experiment compared with THE-0504 alone (Fig. 8B).

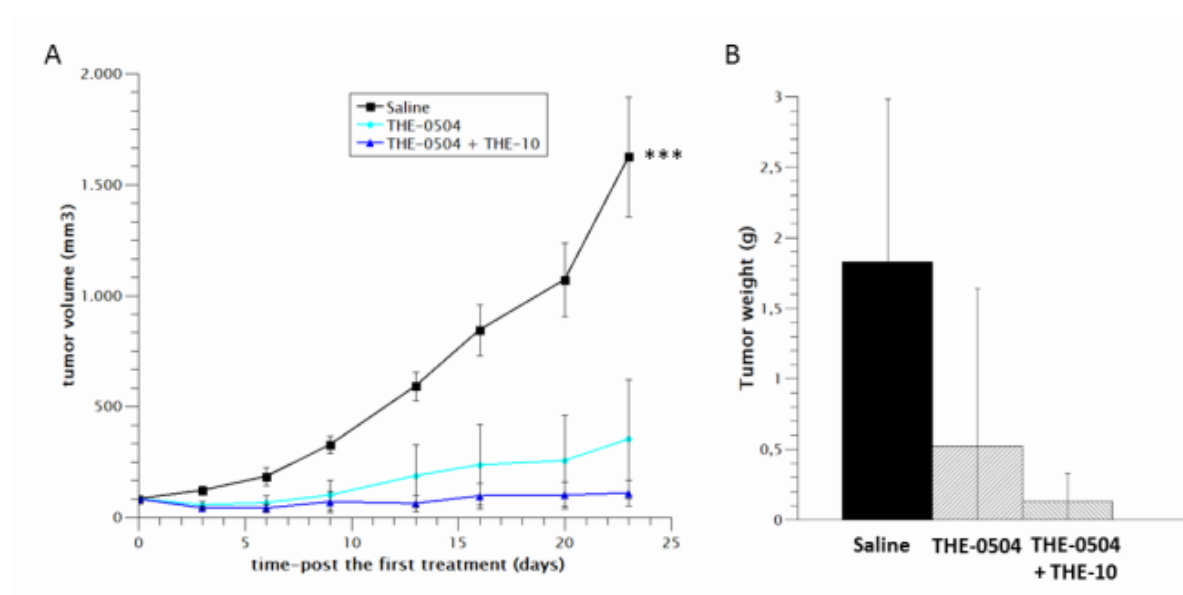


Fig. 8. Tumor treatment study involving co-administration of THE-0504 drug with THE-10 or iRGD peptide. (A) Tumor growth curves in MiaPaca2 pancreatic cancer model. (B) Tumor weights of animals sacrificed at the end of the experiment. Statistical differences (t-test) are as follows. ** $p < 0.001$: THE-0504 vs control; *** $p < 0.0001$: THE-0504 plus THE-10 vs control.

3.9. THE-10 enhances the *in vivo* antitumor activity of the Antibody-Drug Conjugate Sacituzumab govitecan in a murine model of pancreatic cancer

Sacituzumab govitecan (previously known as IMMU-132) is a first-in-class ADC that depends in its activity on the expression of the Trophoblast cell surface antigen-2 (Trop-2). IMMU-132 is

indicated for the treatment of metastatic triple-negative breast cancer and metastatic urothelial cancer [37].

To test THE-10 boosting on IMMU-132 antitumor activity, the same murine pancreatic cancer graft (KPC) was selected, used in previous experiments. In this model, the activity of IMMU-132 alone was expected to be absent or very low due to the lack of detectable Trop-2 expression in mice [38]. Then, mice were intravenously injected three times, one every 3-4 days, with 200 μ L of physiological saline, IMMU-132 (30.0 mg/kg), or IMMU-132 (30.0 mg/kg) plus THE-10 (130 mg/Kg). THE-10 was injected about 5-10 minutes before IMMU-132.

As expected, IMMU-132 did not exert any significant therapeutic effect, whereas in combination with THE-10 it resulted in an excellent anticancer activity, with a remarkable TGI of 93.0 %, supported by reduction in residual tumor masses (Fig. 9, table 1).

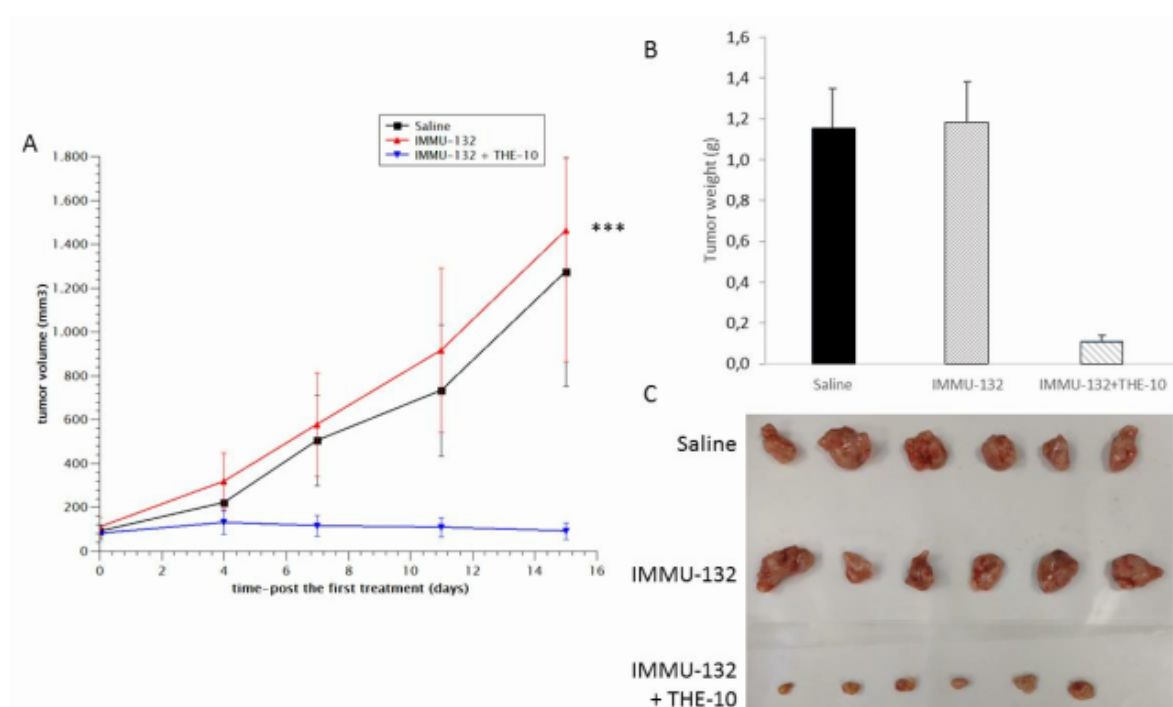


Fig. 9. Tumor treatment study involving co-administration of Sacituzumab govitecan (IMMU-132) with THE-10. (A) Tumor growth curves of IMMU-132 and THE-10 combination therapy in KPC pancreatic cancer model. (B) Tumor weights and images from animals sacrificed at the end of the experiment. Statistical differences (t-test) are as follows. ** $p < 0.001$: IMMU-132 vs control, IMMU-132 vs IMMU-132 plus THE-10; *** $p < 0.0001$: IMMU-132 plus THE-10 vs control.

In all in vivo experiments described above, no abnormal behavior or body-weight loss were observed in mice treated with THE-10. This is in agreement with the fact that THE-10 lacks any chemotherapeutic molecules and therefore should not negatively affect the toxicological profile of the co-administered drug. Nevertheless, further detailed studies are needed to obtain the complete toxicological and toxicokinetic profile of THE-10.

TABLE 1. Summary of the combination therapy of several drugs plus THE-10 in tumor-bearing mice models reported as Tumor Growth Inhibition percentage.

TUMOR MODEL	DRUG TYPE/COMBO	
	<i>Tumor Growth Inhibition (TGI)</i>	
Colorectal (murine, CT26)	Irinotecan 29.0 %	Irinotecan + THE-10 63.0 %
Pancreatic (murine, KPC)	Gemcitabine 48.8 %	Gemcitabine + THE-10 82.2 %
Pancreatic (murine, KPC)	THE-0504 9.4 %	THE-0504 + THE-10 74.2 %
Pancreatic (murine, KPC)	THE-0504 9.4 %	THE-0504 + iRGD peptide 23.5 %
Pancreatic (human, MiaPaca2)	THE-0504 82.5 %	THE-0504 + THE-10 98.3 %
Pancreatic (murine, KPC)	IMMU-132 0 %	IMMU-132 + THE-10 93.0 %

4. Conclusions

Current therapies for most solid tumor are suboptimal. The unique structure and physiology of the tumor microenvironment blocks intra-tumor penetration of the majority of chemotherapeutic and targeted agents, small molecules and large antibodies being both affected. In the present study, we developed a novel compound acting as a therapeutic booster for co-administered drugs in solid

tumors. This compound, named THE-10 incorporates two BAPs at the N-terminus region of the human H-type ferritin (HfT). Specifically, the selected BAPs are two modified linear versions of previously described peptides: (a) iRGD, that binds integrin $\alpha V\beta 3/\alpha V\beta 5$ and NRP-1, and (b) PD-L1Pep-1, that binds PD-L1. THE-10 can be expressed at high yields in *E. coli* and is produced through wild-type-like self-assembly as a highly homogeneous and water-soluble 24meric nanoparticle. The presence of the two BAPs confers to THE-10 outstanding booster properties providing an ICI-like effect and enhancing the anticancer effects (*in vitro* and/or animal models) of two ADCs (T-DXd and IMMU-132), one nanovector in the same therapeutic class (THE-0504), and two small molecules (gemcitabine and irinotecan), possibly with a preferential effect on large macromolecules. Evidence is provided that at least in some models THE-10 is superior to naked BAPs, possibly due to extended half-life in the bloodstream and conditional delivery into the TME (MMP cleavage).

In conclusion, THE-10 may expand therapeutic indications of co-administered drugs. Boosting with THE-10 may conceivably improve the efficacy of drugs in their approved indication and/or lower dosages and side effects. Alternatively, or in addition, it may help in drug repositioning, by expanding indications to resistant tumor histotypes and/or settings, e.g. low expression of the tumor target. Interestingly, THE-10 applies several principles of precision oncology and target therapy, but it may impact on target therapy, chemotherapy and immunotherapy altogether, e.g. it is an all-round booster. Additional preclinical studies are needed to evaluate the applicative spectrum of THE-10 and its possible limitations.

Conflict of Interest: Patrizio Giacomini and Pierpaolo Ceci are Members of the Scientific Board of Thena Biotech Srl. They received no compensation or payments from Thena Biotech.

Acknowledgements

This study was partially supported by grants from the project MUR-PRIN 2022WC7BL2 to E.F.; from the project PRIN MUR 2022HYF8KS to G.C.; from the European Union - Next Generation EU-NICR, Programme EXCELES, reg. n. LX22NPO5102 (CZ); from the Czech Ministry of Health, project NU23-08-00071; from the RVO 61388971 of the

Institute of Microbiology AS CR (institutional grant); from Fondazione-AIRC to G.S. (grant code: IG 2021 id 25696); from Italian Ministry of Health to V.D.L. (grant code: PNRR-MAD-2022-12376202). We also acknowledge CNCCS s.c.a.r.l. (National Collection of Chemical Compounds and Screening Center, www.cnccs.it); PNRR M4C2 - Dalla ricerca all'impresa - 3.1: Fondo per la realizzazione di un sistema integrato di infrastrutture di ricerca e innovazione "Potentiating the Italian Capacity for Structural Biology Services in Instruct-ERIC (ITACA.SB)" CUP: B53C22001790006; PNRR PE8 Age-IT., co-funding from Next Generation EU [DM 1557 11.10.2022], in the context of the National Recovery and Resilience Plan, Investment PE8 – Project Age-It: "Ageing Well in an Ageing Society"; Consiglio Nazionale delle Ricerche - CNR project DBA.AD005.225-NUTRAGE-FOE2021.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://>

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3.4 Immunogenic properties of nickel-doped maghemite nanoparticles and the implication for cancer immunotherapy

In this study, we investigated the direct cytotoxic effect of nickel-doped ferric oxide-based NPs (FN NPs) on murine and human cancer cell lines. For the second part of the study, we evaluated whether the FN NPs toxicity affected the immunogenicity of cancer cell lines.

For the purpose of this study, we first incubated a murine colorectal cancer cell line with increasing concentrations of FN NPs. We observed dose-dependent toxic effects on CT26 cells, and the most toxic concentration was used in all following experiments. To verify if these findings could be translated into human *in vitro* models, we decided to evaluate the toxicity of FN NPs in two human prostate cancer cell lines (PC-3 and LNCaP). We cultivated the cell lines with the toxic dose of NPs and observed toxicity only in the PC-3 cell line, while the LNCaP cell line seemed to be resistant.

To test the potential changes in the immunogenicity of the cancer cells after incubation with a toxic dose of FN NPs, we evaluated the changes in activation/maturation markers in murine macrophages, DCs, and T cells of both human and murine origin upon their exposure to related live cancer cells and/or their lysates.

We found that the NP-induced toxicity in the cancer cells did not affect the phenotype or responsivity of the tested immune cells. Additionally, it also did not prevent either murine or human DCs from stimulating cancer cell-reactive T lymphocytes in the mixed culture.

Our findings show that the tested NPs could deliver toxic effects to sensitive cancer cells without compromising the ongoing anti-cancer immune reactions, even though the human immune cells were obtained from prostate cancer patients. As iron-based NPs are considered a multifunctional therapeutic agent for combined imaging and anti-cancer therapy, it is vital to evaluate their effects on immune cells in cancer conditions.

Contribution: My work in this project involved participation in the experimental design and all experiments and analyses performed in the murine model. I was responsible for harvesting immune cells, preparing cancer cells of murine origin, and conducting experiments in the mouse *in vitro* model, including viability assays, flow cytometry, and final data analysis and interpretation. I wrote the paper under the supervision of doc. Smrz.

Immunogenic properties of nickel-doped maghemite nanoparticles and the implication for cancer immunotherapy

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ABSTRACT

Nanoparticles are commonly used in diagnostics and therapy. They are also increasingly being implemented in cancer immunotherapy because of their ability to deliver drugs and modulate the immune system. However, the effect of nanoparticles on immune cells involved in the anti-tumor immune response is not well understood. The study reported here showed that nickel-doped maghemite nanoparticles (FN NP) are differentially cytotoxic to cultured mouse and human cancer cell lines, causing their death without negatively impacting the subsequent anticancer immune response. It also found that FN NP induced cell death in the mouse colorectal cancer cell line CT26 and human prostate cancer cell line PC-3, but not in the human prostate cancer cell line LNCaP. The induced cancer cell death did not affect the phenotype and responsiveness of the isolated mouse peritoneal macrophages, or *ex vivo*-generated mouse bone marrow-derived, or human monocyte-derived dendritic cells. Additionally, the induced cancer cell death did not prevent the *ex vivo*-generated mouse or human dendritic cells from stimulating lymphocytes and enriching cell cultures with cancer cell-reactive T-cells. In conclusion, this study shows that FN NP could be a valuable platform for targeting cancer cells without causing immunosuppressive effects on the subsequent anticancer immune response.

ARTICLE HISTORY

Received 23 June 2024
Revised 29 September 2024
Accepted 10 October 2024

KEYWORDS

Nanoparticles; cancer cells; immunogenic cell death; macrophages; dendritic cells; T-cells

Introduction

Nanoparticles (NP) are widely used in medicine for diagnostic and therapeutic purposes. In diagnostics, NP can enhance imaging techniques such as magnetic resonance imaging (MRI), computed tomography (CT), and positron emission tomography (PET) (Pallares et al. 2022). They can also be utilized for early disease detection by identifying biomarkers (Combes et al. 2021). Many NP are also used for drug delivery (Yusuf et al. 2023), gene therapy to transform target cells (Mendes et al. 2022), and as therapeutic agents in cancer treatment using photothermal therapy (Duan et al. 2023). Additionally, they find their place in drug or antigen delivery and as immunomodulatory agents (Bezbaruah et al. 2022; Sau et al. 2024).

Iron-based NP have shown promise in cancer immunotherapy. Their magnetic properties make them useful for tumor-localized magnetic field-elicited hyperthermia, which can cause tumor cell death and make tumors more susceptible to oncologic treatment (Shestovskaya et al. 2023; Szwed and Marczak 2024). These NP can be further modified to carry immunomodulatory molecules to the tumor site, reshaping the tumor environment to enhance anti-tumor immune responses (Mulens-Arias

et al. 2021). Moreover, they can facilitate the delivery of tumor antigens to dendritic cells (DC), thus boosting adaptive anti-tumor immunity (Huang et al. 2023). The iron-based NP could also incorporate other metals (so-called 'doped nanoparticles'), such as nickel (Mokhosi et al. 2022). The incorporation of other metals into these NP then opens the possibilities not only to use these particles to be the effector components serving as scaffolds, molecular carriers, vectors, or even toxic agents to cancer cells but also to be markers whose localization could be detected and imaged using advanced imaging techniques, including the laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) (Arnaudguilhem et al. 2021; Clases and Gonzalez de Vega 2022) but also magnetic resonance imaging (Lu et al. 2022). Therefore, incorporating other metals into NP could turn them into traceable material by imaging techniques.

The effects of NP in cancer treatment may not necessarily work together to enhance therapeutic efficacy. One of the effects is the way through which cancer cells die. The death of tumor cells triggered by NP could be either immunogenic (Duan et al. 2019) or non-immunogenic (Garcia Garcia et al. 2023). In some studies, iron-based NP have also been found to induce ferroptosis (Bae et al. 2022; Fernández-Acosta et al. 2022), which was

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observed to have a negative impact on DC and hence the adaptive immune response (Wiernicki et al. 2022) or promote increased expression of the immune checkpoint inhibitor ligand PD-L1 on tumor cells (Chung et al. 2023; Desterke et al. 2023). However, whether ferroptosis is immunosuppressive or immunogenic remains to be further explored, as it is presumed to depend on the specific context (Catanzaro et al. 2024).

The study reported here examined how nickel-doped maghemite NP (FN NP) may affect mouse and human cancer cell lines and how this impact influences the immune response against these cancer cells. This study also evaluated the anti-tumor immune response using isolated mouse peritoneal macrophages, *ex vivo*-generated cancer cell-pulsed and matured mouse bone marrow-derived (BM). Further, to explore the possible clinical relevance of the FN NP impact on the immune system of cancer patients and cancer immunotherapy, human monocyte-derived DC (moDC) from patients with hormone-refractory metastatic prostate cancer were investigated. Finally, both the functionality of the *ex vivo*-generated DC through the DC-mediated lymphocyte stimulation and subsequent determination of the lymphocyte reactivity to cancer cells were analyzed in cells from mice and hormone-refractory metastatic prostate cancer patients.

Methods

Preparation of nickel-doped maghemite nanoparticles

The NP were prepared according to the previously published method for the preparation of maghemite nanoparticles with few modifications (Horak et al. 2007; Babič et al. 2008, 2009). The modification of the original method consisted of replacing half of the molar amount of FeCl_3 with NiCl_2 . Aqueous solutions of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (Sigma, St. Louis, MO) were prepared by dissolving the salts in water and then purified by MCE (mixed cellulose esters) membrane filtration prior to use. The solution of FeCl_3 (0.2 M, 100 ml) was treated with aqueous ammonia (0.5 M, 100 ml; Sigma) under sonication for 2 min. Then the mixture of FeCl_2 and NiCl_2 solutions (0.2 M, 25 + 25 ml) was added. The dispersion was poured into aqueous ammonia (0.5 M, 250 ml) and stirred. Black particles, presumably of a magnetite-like ferrite nature, were formed. The mixture was stirred for 1 h and then allowed to sediment. The supernatant was discarded, and the sedimented particles were purified by repeated addition of water followed by repeated (five times) magnetic separation and decantation. Trisodium citrate dihydrate solution (0.1 M, 12 ml; Sigma) and NaClO solution (5%, 19 ml; Sigma) were added to the colloid and sonicated for 10 min. Consequently, the colloids were repeatedly washed with ultrapure water (< 18.2 M Ω) via magnetic separation until achieving a spontaneous peptization (≈ 15 –20 cycles). Finally, the colloid was transferred to a laminar clean box, sonicated for 5 min, and filtered through a 0.45- μm sterile polyvinylidene fluoride (PVDF) syringe filter; its concentration was measured gravimetrically from two 0.5 ml samples of colloid.

Dynamic light scattering (DLS) and transmission electron microscopy (TEM) analyses

The colloidal stability, and hydrodynamic size of the particles in aqueous suspension were examined by dynamic light scattering (DLS), measuring the hydrodynamic diameter (Dh), polydispersity

index (PDI) and zeta potential (ζ) (Zetasizer Nano Series ZEN3500, Malvern, Worcestershire, UK). All values were measured for diluted aqueous particles dispersions in disposable folded DTS1070 capillary cells.

The shape, average diameter, and size distribution of the particles were evaluated directly from microphotographs obtained by a transmission electron microscope (FEI-TEM, Tecnai G2 Spirit, OR). Microphotographs were analyzed with ImageJ analysis software using at least 1000 particles. Particle sizes were measured manually from at least eight different images of one sample. Number-average diameter (D_n) (1) and dispersity (D) (2) were calculated according to the following formulas:

$$D_n = \frac{\sum n_i D_i}{\sum n_i} \quad (1)$$

$$D = \frac{D_w}{D_n}; [1, \infty) \quad (2)$$

where n_i is the number of the nanoparticles, D_i is the diameter of the nanoparticles. D_w (3), which is the weight-average diameter of the nanoparticles, was calculated as follows:

$$D_w = \frac{\sum n_i D_i^4}{\sum n_i D_i^3} \quad (3)$$

Mice and specimen

Male BALB/c mice (8–12-wk-of-age) were provided by the breeding colonies of the Institute of Microbiology of the Czech Academy of Sciences, Prague. Standard housing conditions (22°C, 55% relative humidity, 12-h light:dark cycle) were used throughout. All mice had *ad libitum* access to standard rodent chow and filtered tap water. All experimental procedures employed in the studies respected the rules of the European Convention for the Care and Use of Laboratory Animals (2010/63/EU) as approved by the Czech Animal Care and Use Committee and the Institutional Ethics Committee (Departmental Commission of the Academy of Sciences of the Czech Republic), according to the Act of the Czech National Council no. 246/1992 Coll., according to §5 of Decree #419/2012 Coll., the Act on the Protection of Animals Against Cruelty, under the project no. 111/2019.

Human peripheral blood mononuclear cells (PBMC) from six patients with hormone-refractory metastatic prostate cancer were from a previously described patient cohort (Taborska, Stakheev et al. 2021) and had been isolated from leukapheresis samples. The median age of the donor patients whose PBMC were used was 66.0 years (range 52–73 years), the median Gleason score was 7.5 (Gleason range 7–9), and the prostate-specific antigen (PSA) median level was 21.7 ng/ml (concentration range 6.21–78.68 ng/ml). Each patient provided signed written informed consent for using their blood-derived products for future research. The research with human specimens was approved according to the ethical standards of the institutional research committee - the Ethics Committee of the Motol University Hospital in Prague (approval date: Jun 19, 2019) and performed in compliance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Cancer cell line culturing with FN NP and evaluation of their cytotoxicity

The murine colon carcinoma cell line CT26 (Brattain et al. 1980) was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 100 U penicillin-streptomycin/ml (Sigma) and harvested with 0.02% EDTA in a phosphate-buffered saline (PBS). The human prostate cancer cell lines PC-3 (Kaighn et al. 1979) and LNCaP (Horoszewicz et al. 1983) were cultured in RPMI 1640 medium (Thermo Scientific, Waltham, MA) supplemented with 10% FBS, 100 U penicillin-streptomycin/ml, and 2 mM GlutaMax (Thermo Scientific). The cells were harvested with Trypsin-EDTA solution (500 mg/l-0.05%) (Thermo Scientific).

To preliminarily determine the cytotoxic impact of different concentrations of FN NP on CT26 cells and choose the working dose, the harvested cells were counted with a Countess II cell counter (Thermo Scientific) after staining with trypan blue at 0.2% (final dilution) for live cell identification and seeded in a flat-bottom 96-well plate. Once adherent after overnight incubation at 37°C and 5% CO₂, the cells were supplemented with vehicle alone, FN NP at progressive concentrations, or treated with Etoposide 100 nM (Sigma) used as a positive control. After 24 and 48 h, cells were fixed and stained with crystal violet solution (Thermo Scientific) as described (Tosetti et al. 2003). After removal of unbound dye, the cells were left to dry overnight, then dissolved with acidified ethanol [50% ethanol (VWR) and 1% acetic acid (VWR)], and the absorbance value in the wells was then measured at 595 nm using a Tecan Infinite 200 PRO spectrophotometer (Tecan Trading AG, Männedorf, Switzerland) (adapted from Tosetti et al. 2003). Absorbance at 650 nm was subtracted to correct for background.

Flow cytometry was used to determine the impact of FN NP on the viability of the cultured cells (CT26, PC-3, LNCaP) after they were supplemented with 30 µg/ml of FN NP and cultured for 2 or 7 days. The cells were then harvested, stained with Hoechst 33258 (1 µg/ml) or DAPI (100 ng/ml) (Thermo Scientific) and immediately analyzed. CT26 cells were evaluated using a ZE5 cytometer (Biorad, Hercules, CA). FACS Aria II flow cytometer (Becton Dickinson) was used to analyze the PC-3 and LNCaP cells. FlowJo software (Tree Star, Ashland, OR) was used to process all acquired data.

Isolation of murine peritoneal macrophages, their stimulation and analysis

To obtain murine peritoneal macrophages, mice were euthanized by cervical dislocation and their fur removed to expose their abdomen. Peritoneal cavity lavage was performed by injection of 3 ml HMEM medium (IMG) supplemented with 0.03% gentamycin (Sandoz, Princeton, NJ), 5 mM HEPES, and 0.025% NaHCO₃ (IMG). Harvested cells were kept on ice, washed with PBS, treated in 1X RBC lysis buffer (Biolegend, San Diego, CA) [5 min], and then rinsed with modified serum-containing RPMI-1640 medium including 10% FBS, 100 U/ml penicillin-streptomycin, 1 mM sodium pyruvate, 1X MEM/NEAA (Sigma), and 55 µM β-mercaptoethanol (Roth). The concentration of the isolated macrophages was then adjusted to 2 × 10⁶ live cells/ml.

CT26 cells were treated with vehicle or FN NP (30 µg/ml) for 48 h in DMEM medium. The cells were then harvested, rinsed with KM medium, adjusted to 0.5 × 10⁶ live cells/ml, and 100 µl (0.05 × 10⁶ live cells) aliquots of the suspension were transferred to wells of a flat-bottom 96-well plate (Thermo Scientific). After

2 h of incubation (37°C, 5% CO₂), the cells were co-cultured with 100 µl suspension (0.2 × 10⁶ live cells) of the previously isolated macrophages for 48 h. After co-culture, the cells were harvested, washed with PBS, and stained with a panel of primary antibodies to evaluate immune cell activation (e.g., FVD-eFluor780, F4/80-APC [eBioscience, San Diego, CA], CD80-PE, CD86-PE/Cy7, MHC-II-BV510, [Biolegend], CD206-FITC [Sony, San Jose]) for 30 min in dark and 4°C. The stained cells were washed with PBS and immediately analyzed with a ZE5 cytometer and processed with FlowJo software as above.

Preparation of murine BM-derived DC, their pulsing, maturation, and analysis

Bone marrow (BM) cells were isolated from BALB/c femurs and tibiae using a standard isolation technique (Lutz et al. 1999; Mikyskova et al. 2017). After pelleting and washing with PBS, isolated cells were re-suspended and cultured (37°C, 5% CO₂) at a density of 0.45 × 10⁶ live cells/ml in KM medium with 20 ng/ml of granulocyte-macrophage-colony-stimulating factor (GM-CSF) and 5 ng/ml of interleukin-4 (IL-4) (PeproTech, Cranbury, NJ), and repeated on Days 3 and 6.

On Day 7, the differentiated naïve bone marrow-derived dendritic cells (BMDC) were loaded with the lysate of murine CT26 cells to induce their maturation. To prepare the lysate, CT26 cells were treated with vehicle or FN NP (30 µg/ml) for 48 h, then rinsed with KM medium to remove residual FN NP, harvested, and re-suspended in KM medium at a final concentration of 1 × 10⁶ live cells/ml. The cells were then lysed by two cycles of freeze-thawing and two cycles of UV irradiation (Stakheev et al. 2022). After supplementation with 20 ng GM-CSF/ml and 5 ng IL-4/ml, 50 µl of lysate or KM medium (mDC; vehicle-pulsed DC) were combined with the BMDC suspension (200 µl, 2.5 × 10⁶ live cells/ml) in a flat-bottom 96-well plate, incubated (37°C, 5% CO₂) for 5 h, then supplemented with 5 µg/ml of R-848 (a TLR7/8 agonist; Enzo Life Sciences) and left to mature for 18–24 h. On Day 8, the cells were re-suspended, 180 µl of each sample was recovered, washed with PBS and stained for DC maturation markers using the primary antibodies: FVD-eFluor780, CD83-PE (eBioscience), CD11c-Alexa Fluor 700, CD80-FITC, CD86-PE/Cy7 and MHC-II-BV510 (Biolegend), CD14-PerCP/Cy5.5 (Exbio, Vestec, Czech Republic). After the staining and washing, the cells were immediately analyzed with the ZE5 cytometer, and processed with FlowJo software as above.

Preparation of human moDC, their pulsing, maturation, and analysis

The source material for the preparation of moDC was peripheral blood mononuclear cells (PBMC) isolated and cryopreserved as described in Taborska et al. (2018, 2020). Specifically, the PBMC used here were isolated from leukapheresis samples of the above-described patients with hormone-refractory metastatic prostate cancer.

The immature moDC were prepared from the cryopreserved PBMC as described in Stakheev et al. (2022). The vehicle- or FN NP-treated cancer cell line lysates either from PC3 (Kaighn et al. 1979) or LNCaP (Horoszewicz et al. 1983) cells were prepared as described above for the CT26 lysates, and added to the suspension of immature DC. After 5 h, the cells were supplemented with 10 µg/ml of the TLR7/8 agonist R-848 and matured overnight. The moDC were then stained as described in Stakheev et al. (2022) with the antibodies CD11c-APC (Exbio), CD86-PE

(Beckman Coulter, Brea, CA), and HLA-DR-PE-Cy7 (Becton Dickinson). The stained cells were then analyzed using the FACSaria II flow cytometer and all data processed using the FlowJo software.

Preparation and analyses of murine BM DC-expanded lymphocytes

BALB/c splenocytes were used to isolate lymphocytes employed for the DC-mediated expansion assays here. The spleen from each mouse was harvested under sterile conditions and dissociated through a 100-nm nylon mesh (Biologix, Jinan, Shandong, China). The liberated cells were washed with KM medium, treated with 1X RBC lysis buffer (Biolegend) for 10 min at 4°C, and then rinsed with KM medium. To obtain T-lymphocytes, the cells were re-suspended in PBS with 2 mM EDTA (PBSE) before undergoing negative magnetic separation using a Pan T-Cell Isolation Kit II (Miltenyi Biotec, Gladbach, Germany). The obtained T-cells were counted, re-suspended in KM medium (1×10^6 live cells/ml), and then 1 ml of the T-cell suspension was mixed with 50 μ l of matured BMDC (2×10^6 live cells/ml) in a flat-bottom 48-well plate. After a 24-h incubation (37°C, 5% CO₂), 20 IU murine IL-2/ml (PeproTech) was added, and the cells were incubated for another 3 days.

On Day 4 of co-culture, the DC-stimulated lymphocytes were divided into two portions. One portion was stimulated by co-culture with live CT26 cells at a ratio of 5:1 (lymphocytes: CT26 cells). The second part was co-cultured with KM medium only (vehicle). The cells were co-cultured (37°C, 5% CO₂) for 5 h in the presence of brefeldin A (Thermo Scientific) which was administered 1 h after starting the co-culture. After the stimulation, cells were washed with PBS and stained for surface markers and viability with a panel of primary antibodies, i.e., FVD-eFluor780, TIM-3 FITC, PD-1 PE (eBioscience), CD3-PE/Cy7 (Sony), CD4-PerCP, CD8-Alexa Fluor 700 (Biolegend). Cells were then fixed and permeabilized via an intracellular fixation/permeabilization buffer set (eBioscience), according to manufacturer protocols. Intracellular staining with anti-interferon (IFN)- γ -APC (Biolegend) and anti-tumor necrosis factor (TNF)- α -BV421 (eBioscience) was performed overnight (8–10 h). The next day, the stained cells were washed with PBS and analyzed with the ZE5 cytometer; acquired data was processed with FlowJo software as above. The percentages of CT26-reactive IFN γ - and/or TNF α -producing T-cell populations were calculated as the difference between the percentages of the IFN γ - and/or TNF α -producing T-cells in CT26- and vehicle-stimulated samples.

Preparation and analyses of human moDC-expanded lymphocytes

The DC-mediated stimulation of autologous lymphocytes was performed as described in Stakheev et al. (2022). After a 5-day co-culture, the DC-stimulated lymphocytes were processed, fixed, permeabilized, stained, and analyzed by flow cytometry as in Taborska, Lastovicka et al. (2021). The cells were stained with CD3-PerCP-Cy5.5 (eBiosciences), CD4-PE-Cy7, CD8-Alexa Fluor 700, Ki-67-APC (Exbio), and CD137-PE (Miltenyi Biotec). Percentages of the Ki-67⁺ or CD137⁺ T-cell populations were calculated as the difference in percentages of Ki-67⁺ or CD137⁺ T-cell populations in moDC- and vehicle-stimulated samples.

Statistical analysis

The values were calculated from the sample size (n) using Prism software (v.10.0, GraphPad Software, La Jolla, CA). Statistical significance ($*p < 0.05$) between or among variables was determined using the indicated tests.

Results

Characterization of the synthesized nickel-doped maghemite nanoparticles (FN NP)

The nickel-doped maghemite nanoparticles (FN NP) were synthesized as described in the method section. The prepared particles were visualized using TEM. As shown in Figure 1A, the particles were sphere-like shapes of comparable sizes. Picture analysis of the TEM micrograph showed the particles of 9.8 nm in diameter. The value of dispersity $D = 1.32$ confirms the narrow distribution of the particle sizes.

To further characterize the particles, their colloidal stability and hydrodynamic size of the particles in aqueous suspension were determined by dynamic light scattering (DLS), measuring the hydrodynamic diameter (Dh), polydispersity index (PDI), and zeta potential (ζ). All values were measured for diluted aqueous particle dispersions in disposable folded DTS1070 capillary cells. The acquired values of five independent measurements are presented in Figure 1B. As shown, the average hydrodynamic size (diameter) of the particles was 58.2 nm.

FN NP differentially induces cell death in mouse and human cancer cell lines

It is known that NP can induce cell death depending on their types (Mohammadinejad et al. 2019). This cell death is often observed in proliferating cells, including cancer cells. Cytotoxic agent-induced cell death is revealed by the detachment of adherent cells from the culture plate (Feoktistova et al. 2016). Exploring whether the FN NP generated here could induce cell death, it was found here that the FN NP-induced loosening of adherence by the murine CT26 cancer cell line was concentration-dependent after 24- and 48-h exposure, as determined by the crystal violet assay (Figure 2A). As an FN NP concentration of 30 μ g/ml was the most efficient at inducing cell death, it was selected as the reference dose for use hereafter. The impact of the chosen dose of NP on CT26 cell viability was also investigated by flow cytometry. Using the same FN NP concentration and 48-h or 7-day exposure periods (Figure 2B), the flow cytometry data confirmed the crystal violet assay results, even though the values were comparatively lower at the FACS analysis. However, the significance of the data remained, and the difference was explained by a more selective staining of dead cells in flow cytometry than in the crystal violet assay in which the detached cells may include both dead and dying cells.

The activity of the FN NP (respectively) on the PC-3 and LNCaP human prostate cancer cell lines was also evaluated. As shown in Figure 2C, D, both cell types were initially comparably affected by the FN NP after a 2-day treatment. However, the 7-day treatment showed that the viability of PC-3 cells was largely decreased, but no impact of the particles was observed in LNCaP cells. These data evidenced a differential susceptibility of the different cancer cell lines—including the human metastatic prostate cancer cell lines—to FN NP.

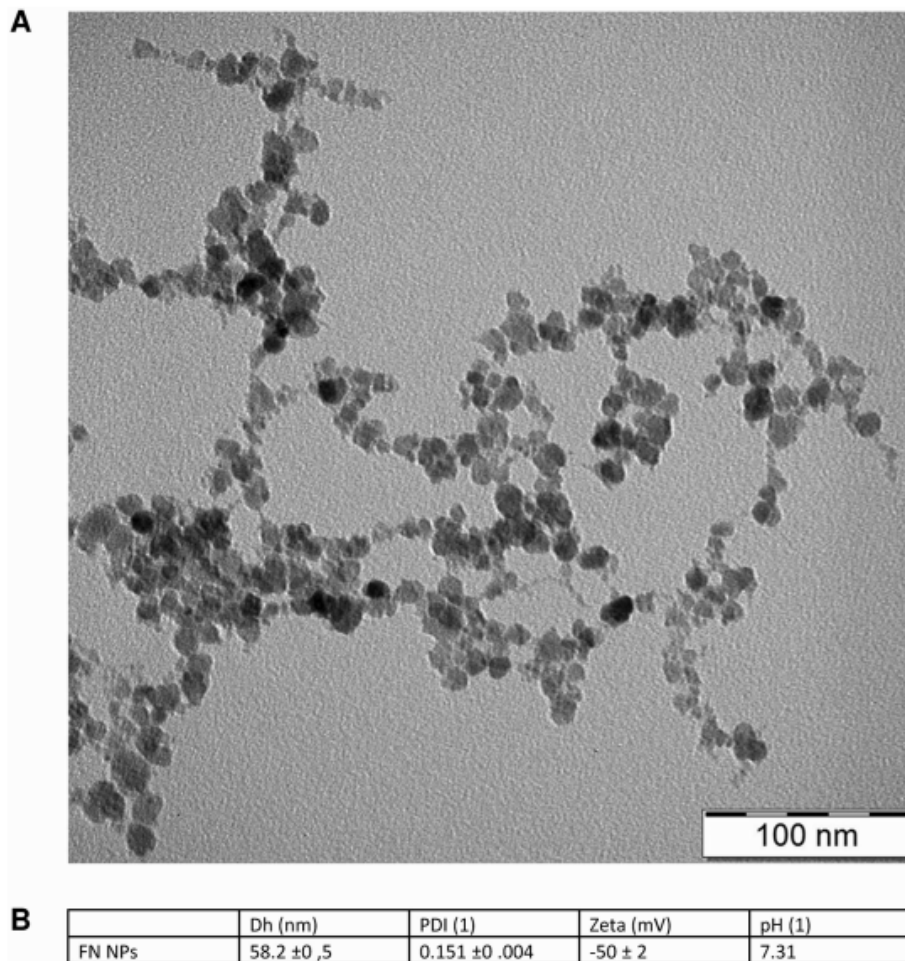


Figure 1. Transmission electron microscopy (TEM) of the synthesized FN NP and their characteristics. (A) Representative image of FN NP acquired by TEM. Bar = 100 nm. (B) The calculated means $\pm$ SD of diameter (Dn), hydrodynamic diameter (Dh), polydispersity index (PDI), zeta potential (ζ), and pH of synthesized FN NP ($n=5$).

FN NP-treated cancer cell line CT26 does not affect the phenotype and responsivity of mouse peritoneal macrophages

The manner of cell death determines whether dying or dead cells become immunogenic, non-immunogenic, or immunosuppressive (Hou et al. 2017; Kroemer et al. 2022; Garcia Garcia et al. 2023). To explore whether the FN NP-affected cancer cells could stimulate immune cells of the innate immunity, macrophages (more specifically, mouse peritoneal macrophages) were examined. These cells were isolated and then co-cultured with CT26 cells treated (or not) for 2 days with FN NP at a concentration previously found to deliver the cytotoxic effect (i.e., 30 μ g/ml). Thereafter, frequencies of M1 (F4/80, CD206^{low}) and M2 (F4/80, CD206^{high}) macrophage populations and their surface expression of MHC-II, CD80, and CD86 molecules, an increase in which is a marker of macrophage activation (Mosser and Zhang 2008) were analyzed. As shown in Figure 3A, B, the frequencies of M1 macrophages cocultured with CT26 cells slightly increased, and the FN NP-treatment of CT26 cells did not notably affect this increase. As

further shown in Figure 3A, B, M1 macrophages co-cultured with CT26 cells increased their surface expression of MHC-II, CD80, and CD86 molecules. Again, the FN NP treatment of CT26 cells did not affect this increase, showing that the manner of cell death induced by FN NP did not promote immunosuppressive features of the dying CT26 cells that could materialize during the M1 macrophage activation. Comparable results were obtained with M2 macrophages, except that a notable suppression of the MHC-II increase was observed with the FN NP-treated CT26 cells (Figure 3A, C), indicating a harmful impact of the treated CT26 cells on the activation of M2 macrophages.

Murine BMDC and human moDC are not affected by pulsing with FN NP-treated cancer cells

Since DC are professional antigen-presenting cells, they play a crucial role in activating and amplifying adaptive immune responses, thus making them central to adaptive immunity (Mellman and Steinman 2001). As maturation is key to their

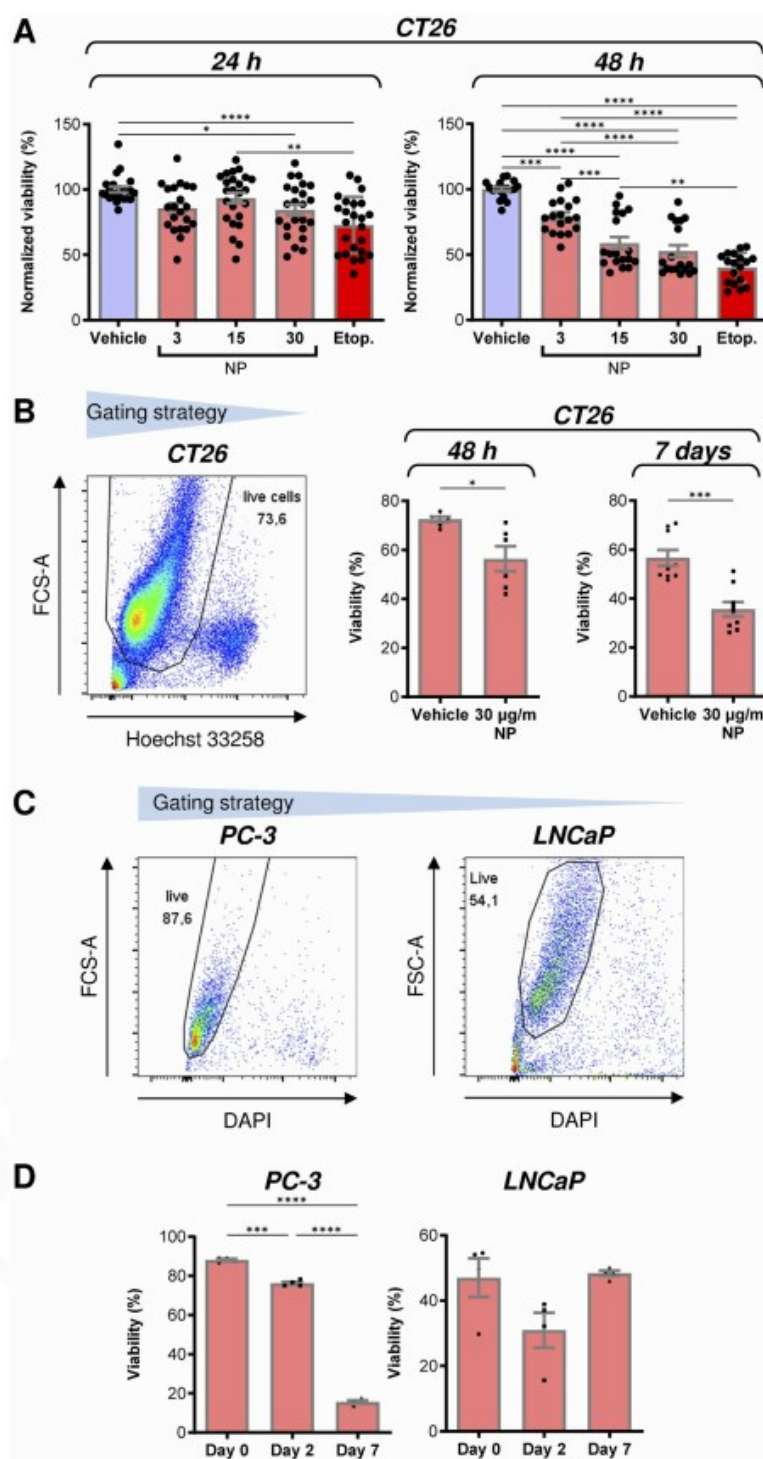


Figure 2. The FN NP differentially induce cell death in mouse and human cancer cell lines. (A) Crystal violet assay-determined viability of CT26 cells exposed (24 or 48 h) to vehicle, different concentrations (µg/ml) of FN NP (NP), and Etoposide (100 nM; positive control). (B) Flow cytometry-determined viability of CT26 cells exposed to vehicle or FN NP (NP, 30 µg/ml). Gating strategy of flow cytometry data (left panel) and evaluated cell viability using Hoechst 33258 staining (right panel). (C, D) flow cytometry-determined viability of PC-3 and LNCaP cells exposed to vehicle or FN NP (NP, 30 µg/ml). Gating strategy of flow cytometry data (C) and evaluated cell viability using DAPI staining (D). In B (left panel) and C, representative plots are shown. In A, B (right panels), and D, means ± SEM are shown and statistical significances of differences between the tested groups are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; A: $n = 24$ (24 h), $n = 18$ (48 h), B: $n = 6$ (48 h), $n = 7$ (7 days); D: $n = 3$; a and D: unpaired one-way ANOVA with a Tukey's post-test, B: unpaired two-tailed student's t-test).

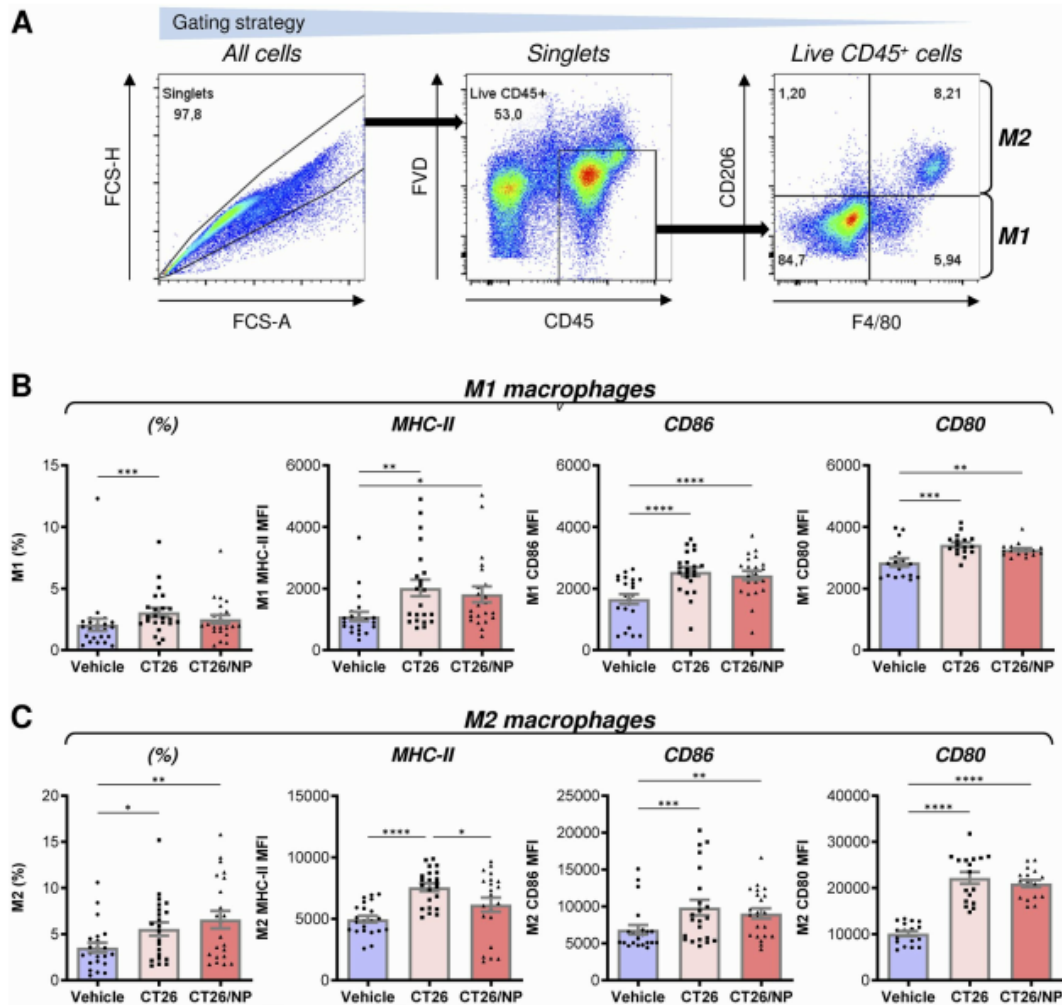


Figure 3. The FN NP-treated cancer cell line CT26 does not affect the phenotype and responsivity of murine peritoneal macrophages. (A) The gating strategy of flow cytometry data. (B, C) The isolated murine peritoneal macrophages were co-cultured for 48 h with vehicle alone (vehicle), CT26 cells (CT26), or CT26 cells that were 48 h-pre-treated with FN NP (30 µg/ml; CT26/NP). The co-cultured macrophages were then analyzed with flow cytometry, and the frequencies of the gated populations of M1 macrophages (M1) (B) and M2 macrophages (M2) (C) in a determined (left panels). The mean fluorescence intensities (MFI) of extracellular staining of MHC-II (second left panels), CD86 (second right panels), or CD80 (right panels) were determined in M1 (B) and M2 (C) macrophage populations. (A) Representative plots are shown. (B, C) Means $\pm$ SEM are shown, and statistical significances of differences between the tested groups are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; M1/2, MHC-II, CD86: $n = 22$ mice, CD80: $n = 17$ mice, paired one-way ANOVA with a Tukey's post-test).

function (Dalod et al. 2014), this study also investigated how treatment of CT26 cells with FN NP could affect their performance during the pulsing and maturation of murine BMDC. As shown in Figure 4A, B, the DC matured with the TLR7/8 ligand, R-848 (Napolitani et al. 2005), had high surface expression of DC maturation markers MHC-II, CD86, and CD80 (Taborska, Strizova et al. 2021; Stakheev et al. 2022). Once the cells were pulsed with antigen (i.e., lysate of untreated CT26 cells or lysate of FN NP-pre-treated CT26 cells), the surface expression of maturation markers was found to be only mildly modulated. These data thus showed that the FN NP-induced cell death in CT26 cancer cells had not notably affected the pulsing and maturation of DC, the key process necessary for effective induction of the adaptive immune response.

In the next experiments, the impact of the FN NP treatment of cancer cells on the pulsing and maturation of human moDC was explored. To better reflect the clinical relevance with respect to the tested PC-3 and LNCaP cells, which originated from tumors of metastatic prostate cancer patients, the moDC were prepared from PBMC isolated from hormone-refractory metastatic prostate cancer patients. Comparably to the murine BMDC, it was found that regardless of induction of cell death in human PC-3 cells and not in LNCaP cells, the treatment did not affect the pulsing and maturation process of the patients' DC (Figure 4C, D). These results showed that the DC, the central cells involved in adaptive anti-cancer immunity of mice and human patients, were not affected by FN NP-mediated death of cancer cells.

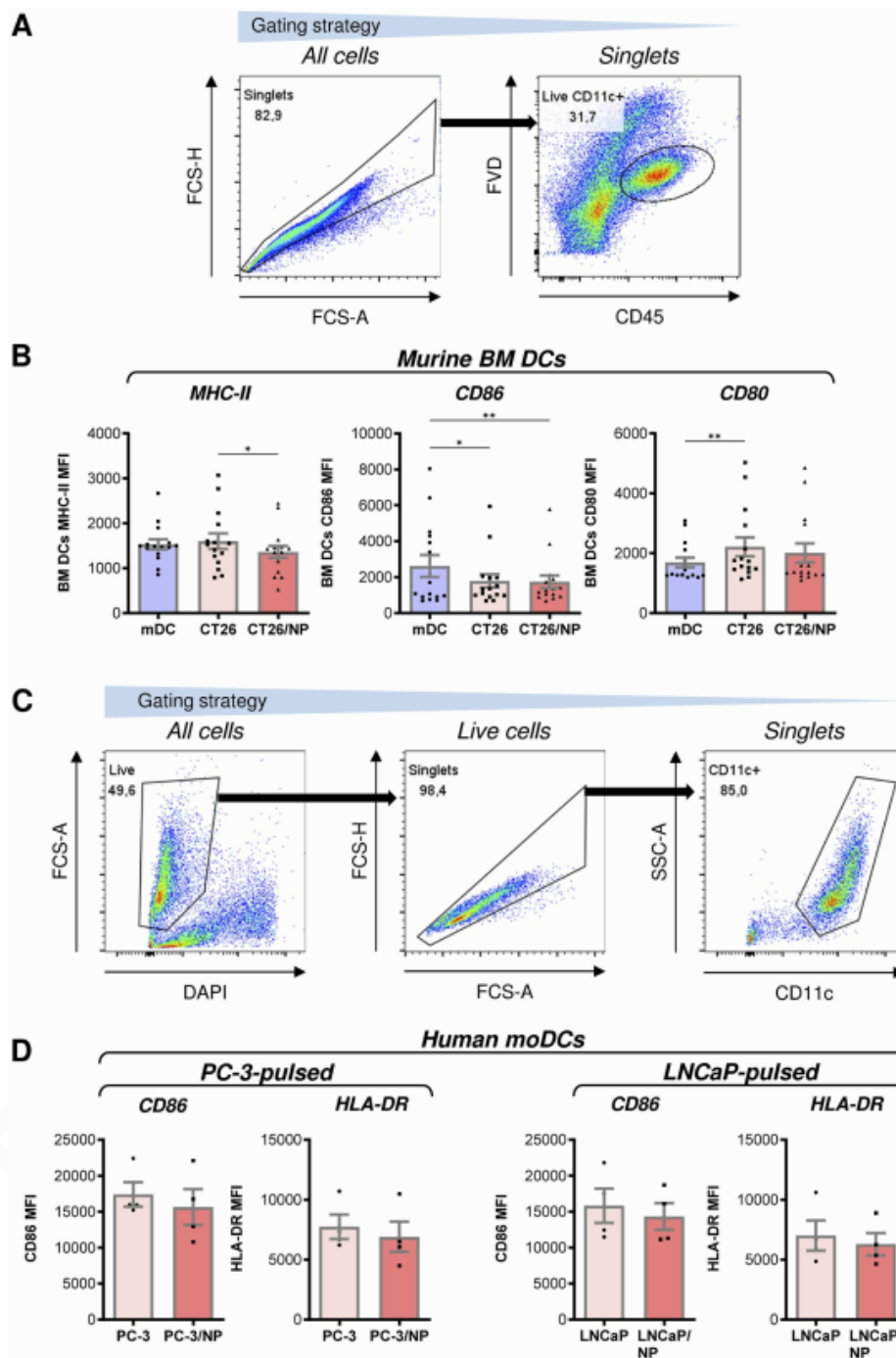


Figure 4. Murine BMDC and human moDC are not affected by pulsing with FN NP-treated cancer cells. (A) Gating strategy of flow cytometry data for ex vivo-produced murine BMDC. (B) Ex vivo-produced murine BMDC were pulsed for 5 h with vehicle alone (mDC), lysate of CT26 cells (CT26), or lysate of CT26 cells that were 48 h-pre-treated with FN NP (30 µg/ml; CT26/NP). Cells were then matured overnight (18–24 h) with R-848, and the mean fluorescence intensities (MFI) of extracellular staining of MHC-II (left panel), CD86 (Middle panel), or CD80 (right panel) determined by flow cytometry in the gated BMDC. (C) Gating strategy of flow cytometry data for ex vivo-produced murine BMDC. (D) Ex vivo-produced human moDC were pulsed for 4 h with lysate of PC-3 or LNCaP cells (PC-3, LNCaP), or lysate of PC-3 or LNCaP cells that were 48 h-pre-treated with FN NP (30 µg/ml; PC-3/NP, LNCaP/NP). The cells were then matured overnight (18–24 h) with R-848, and the MFI of extracellular staining of CD86 (left panels) or HLA-DR (right panels) determined by flow cytometry in the gated moDC. In A and C, representative plots are shown. In B and D, means ± SEM are shown, and statistical significances of differences between the tested groups are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; B: $n = 15$ mice, D: $n = 4$ patients; B: paired one-way ANOVA with a Tukey's post-test, D: paired two-tailed student's t -test).

Murine BMDC and human moDC pulsed with FN particle-treated cancer cells are not compromised in their ability to stimulate lymphocytes

The finding that DC maturation was not affected by FN NP treatment indicated that also the subsequent DC-mediated expansion of CT26-specific T-cells might not be compromised. To test this, CT26-pulsed and matured BMDC were co-cultured with isolated murine T-cells to induce expansion of CT26-specific T-cells in the co-culture. As seen, the frequencies of T-cells and their CD4⁺ and CD8⁺ T-cell populations were not affected by the CT26 treatment with FN NP (Figure 5A, B). The stimulation of the co-culture with CT26 cells showed comparable frequencies of the reactive T-cells, as determined by the intracellular production of inflammatory cytokines IFN γ and TNF α after the stimulation with CT26 (Figure 5A, C, D). Comparable results were also observed for co-cultures with human prostate cancer PC3-pulsed or LNCaP-pulsed and matured moDC with autologous lymphocytes (Figure 6). As shown in Figure 6A, B, the frequencies of T-cells and their CD4⁺ and CD8⁺ T-cell populations were not affected by the PC-3 or LNCaP treatment with FN NP. In addition, regardless of the FN NP treatment of cancer cells, moDC induced proliferation of autologous lymphocytes, as determined by Ki-67⁺ staining (Laštovička et al. 2016), and the moDC/lymphocyte co-cultures contained comparable frequencies of specific T-cells, as determined by staining with CD137—a surrogate marker of antigen-stimulated T-cells (Wolfl et al. 2007; Parkhurst et al. 2017). Collectively, these data demonstrated that although FN NP were cytotoxic to cancer cells, the manner of the induced cell death was not immunosuppressive toward the tested cells of both innate and adaptive immunity.

Discussion

The results of this study show that newly produced FN NP can *in vitro* differentially induce cell death in mouse and human cancer cells. However, this cell death was found *in vitro* not to compromise neither the innate nor the adaptive immune response against these cancer cells. Using isolated peritoneal macrophages, FN NP did not affect the stimulation of macrophages after coculture with cancer cells. The maturation of *ex vivo*-produced murine BMDC and human cancer patients' moDC was not affected by the cancer cell pretreatment with FN NP. No impact of this treatment was also determined on the subsequent expansion of CT26/PC3/LNCaP-reactive T-cells. These findings showed that the produced FN NP could deliver cytotoxic impact to cancer cells without modifying their immunotoxicity or promoting immune suppression and, consequently, without adverse effects on the following activation of the immune response against these cancer cells.

NP are increasingly implemented in oncologic treatments. Modification of NP can increase their homing to tumors and the treatment efficacy (Colombo et al. 2016; Abedin et al. 2021). The clinically approved iron-based NP have already been utilized in cancer diagnosis (Abedin et al. 2021) and cancer therapy (Trujillo-Alonso et al. 2019; Soetaert et al. 2020; Huang et al. 2022). Iron-based NP were also found to sensitize tumors to radiation therapy (Russell et al. 2021; Guerra et al. 2022). Many types of iron-based NP were reported to be cytotoxic to cancer cells (Namvar et al. 2014; Paunovic et al. 2020; Nizamov et al. 2022). In this study, it was found that the tested FN NP were also cytotoxic to CT26 and PC-3 cells but not to LNCaP cells, showing differential sensitivity of cancer cells to FN NP. This direct cancer cell-related cytotoxicity could favorably enhance

their therapeutic value if used as carriers of tumor-targeting molecules, antibodies and/or toxin conjugates (Juan et al. 2020; Xu et al. 2024), in the prospective of clinical applications in cancer diagnosis or therapy (theranostic approach). This consideration is even corroborated by the findings that the tested FN NP were not negatively affecting even the immune cells of the hormone-refractory metastatic prostate cancer patients whose immune system is already compromised by the advanced stage of the disease, previous treatments, and overall immunosuppression (Xu et al. 2022).

Cell death can be elicited *via* multiple mechanisms (Shen et al. 2023). The manner of the cancer cell death determines whether the cell death is immunogenic, non-immunogenic, or immunosuppressive (Hou et al. 2017; Duan et al. 2019; Arimoto et al. 2024). Many oncologic treatments, including immunotherapy, rely on the induction of immunogenic death of cancer cells, leading to the induction of specific immune responses (Fucikova et al. 2011, 2014; Podrazil et al. 2015; Mikyskova et al. 2017; Zhu et al. 2021; Huang et al. 2023). However, the opposite may occur when cancer cell death promotes immunosuppression, collaborating with the inhibition of anti-tumor immune responses in the tumor microenvironment (Hou et al. 2017), thus limiting also the efficacy of immunotherapeutic interventions. Therefore, growing the applications in which NP are used, the knowledge about their possible induction of immunosuppressive cell death should be considered to avoid unwanted effects. In this study, it was demonstrated that the synthesized FN NP did not elicit immunosuppressive cancer cell death-related events and did not prevent innate or adaptive immunity from activating an effective immune response.

Iron-based NP can induce ferroptosis (Bae et al. 2022; Fernández-Acosta et al. 2022; Nie et al. 2023). There is an ongoing debate on whether ferroptosis is immunosuppressive, immunologically neutral, or immunogenic. Many studies demonstrated opposite results, showing ferroptosis either as immunosuppressive (Dai et al. 2020; Wiernicki et al. 2022), tumor suppressive, or immunogenic (Kim et al. 2016; Efimova et al. 2020). Various studies indicate that the influence of ferroptosis toward the immune system is context-dependent (Catanzaro et al. 2024), and specific microenvironment conditions could determine the cutting edge between immunogenic and immunosuppressive ferroptosis (Kepp and Kroemer 2022). However, how the iron-based NP-induced ferroptosis can be differently directed is still largely unknown. As shown in the results, the FN NP investigated in this study were found to elicit cell death in two cultured cell lines, CT26 and PC-3, but not in LNCaP. Nevertheless, the treatment with NP did not influence the immune response by both innate and adaptive immunity cells, indicating that these NP could be safely used with immunotherapy. They may be useful for diagnostic imaging since the efficient signal is obtained with nickel as compared with gadolinium contrast, thus opening a perspective of possible theranostic applications (Lu et al. 2022).

Cancer cells could be differentially susceptible to NP-induced cell death (Lomphithak et al. 2023). The study here showed that two human metastatic prostate cancer cell lines, PC-3 and LNCaP, were differentially susceptible to FN NP-induced cell death. However, regardless of the susceptibility, no negative impact of the FN NP-induced cell death materialized in *ex vivo*-produced immune cells obtained from patients with hormone-refractory metastatic prostate cancer and the ability of these cells to elicit cancer cell-targeted immune response. These data thus suggested that the application of the particles in cancer patients could not compromise the patients' potential to elicit appropriate anti-tumor immunity even at advanced stages of the

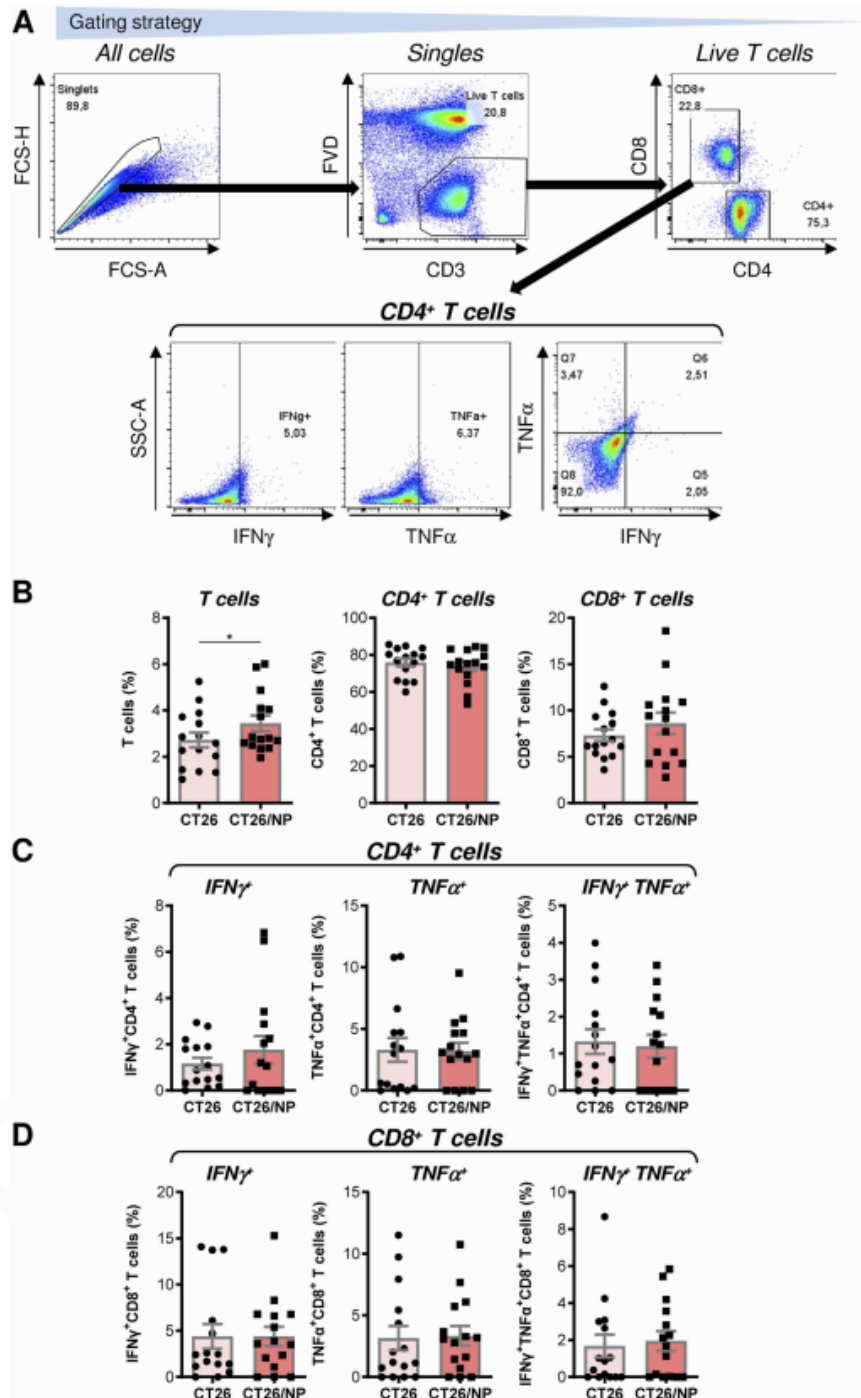


Figure 5. Murine BMDC pulsed with FN particle-treated CT26 cells are not compromised in their ability to stimulate lymphocytes. (A) Gating strategy of flow cytometry data for *ex vivo*-produced murine BMDC. (B–D) *Ex vivo*-produced murine matured BMDC pulsed with lysate of CT26 cells (CT26) or lysate of CT26 cells that were 48 h-pre-treated with FN NP (30 μ g/ml; CT26/NP) were used to stimulate isolated murine T-cells to enrich cell cultures with CT26-reactive T-cells. The 4-day-enriched cell cultures were stimulated with vehicle or live CT26 cells. The frequencies of T-cells, CD4⁺ and CD8⁺ T-cells were determined in the vehicle-stimulated samples (B). Frequencies of IFN γ - and/or TNF α -producing (C) CD4⁺ and (D) CD8⁺ T-cells were calculated as difference between frequencies of the IFN γ - and/or TNF α -producing T-cells in CT26- and vehicle-stimulated samples. (A) Representative plots are shown. (B–D) Means $\pm$ SEM are shown, and statistical significances of differences between the tested groups are indicated (* p < 0.05, n = 15 mice; paired two-tailed student's t -test).

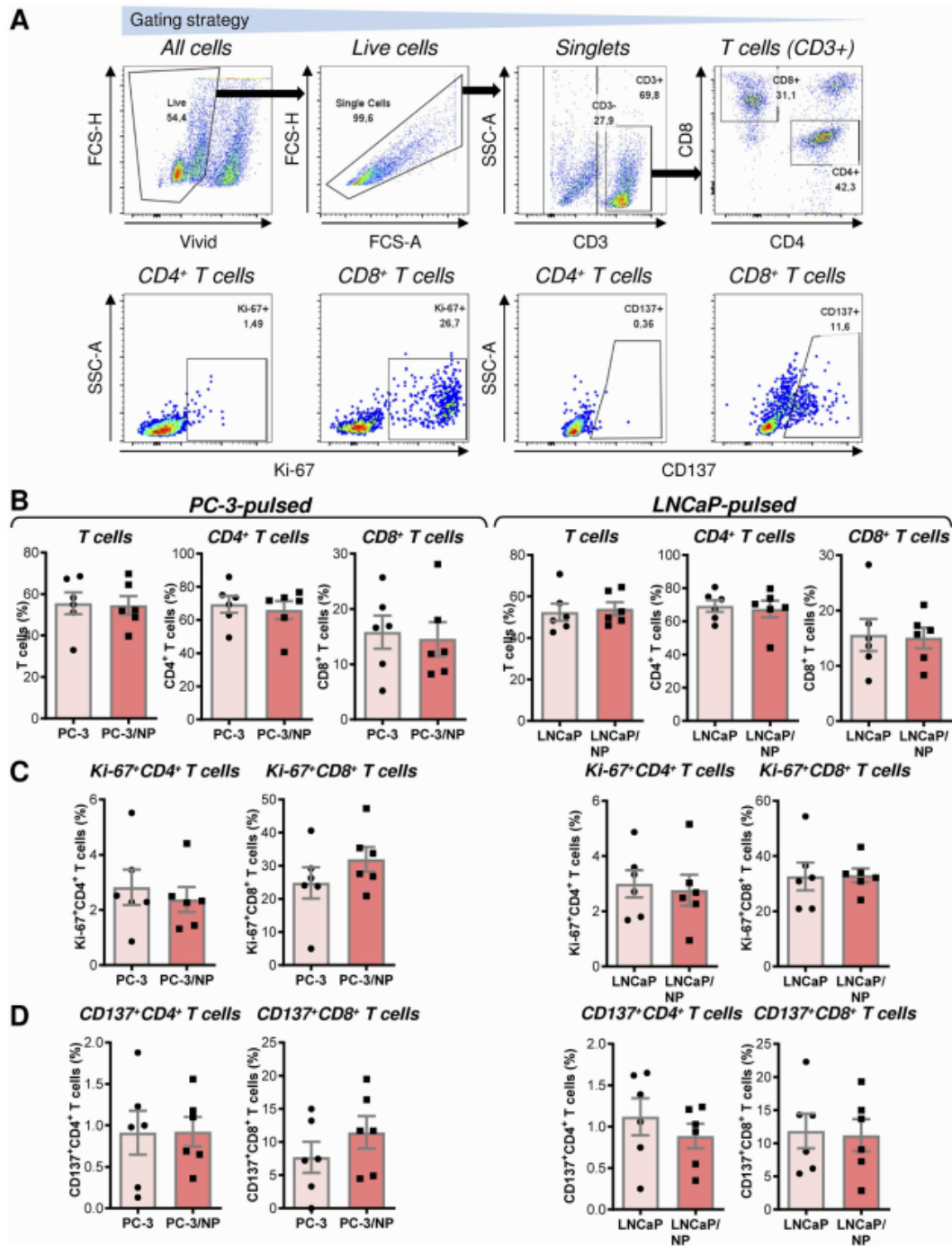


Figure 6. Human moDC pulsed with FN particle-treated PC-3 or LNCaP cancer cells are not compromised in their ability to stimulate lymphocytes. (A) Gating strategy of flow cytometry data for *ex vivo*-produced human moDC. (B-D) *Ex vivo*-produced human matured moDC pulsed with lysates of PC-3 or LNCaP cells (PC-3, LNCaP) or lysates of PC-3 or LNCaP cells that were 48 h-pre-treated with FN NP (30 µg/ml; PC-3/NP, LNCaP/NP) were used to stimulate autologous lymphocytes. After 5 days of co-culture, the frequencies of (B) T-cells, CD4⁺, and CD8⁺ T-cells, (C) Ki-67⁺ CD4⁺ (left panels) and CD8⁺ (right panels) T-cells, and (D) CD137⁺ CD4⁺ (left panels) and CD8⁺ (right panels) T-cells were determined by flow cytometry. (A) Representative plots are shown. (B-D) Means $\pm$ SEM are shown, and statistical significances of differences between the tested groups are indicated (* $p < 0.05$, $n = 6$ patients; paired two-tailed student's *t*-test).

disease, including hormone-refractory metastatic prostate cancer (Bernal et al. 2024). However, studies exploring the long-term impact of these particles on immune cells responsible for the induction and sustainability of anti-tumor immunity still warrant further attention.

Iron-based magnetic NP belong to one of the most used materials in nanomedicine (Dadfar et al. 2019; Montiel Schneider et al. 2022). Originally, they were mostly used as contrast agents for magnetic resonance (Rahman 2023). However, as they are more and more considered multifunctional therapeutic agents for drug delivery and anti-cancer thermal therapy (Shahbazi and Behbahani 2024), their impact not only on cancer cells but also on the subsequent anti-tumor immune response should be determined during their toxicological evaluations. This is particularly important when iron-based NP are increasingly considered also for implementation in cancer immunotherapy (Dias et al. 2022; Deng et al. 2024). Therefore, their identity toward anti-tumor immunity needs to be determined to effectively combine the benefits of nanomedicine and cancer immunotherapy. This study indicates possible avenues through which the search for this identity could be navigated.

Acknowledgments

The Authors wish to thank Jaroslava Vosmikova for assistance with the mouse cell isolation and cultivation.

Authors' contributions

M.B. synthesized and characterized FN NP. L.R., K.Kr., P.L., and D.St. performed the experiments and analyses with the murine cells. K.Ka. and P.T. performed the experiments and analyses with the human cells. L.R., D.St., and L.V. conceived and designed the research and data analysis. D.Sm. contributed to the study design. D.St., L.V., and D.Sm. supervised the experimental plan realization. L.S. and J.B. supervised the sampling and preservation of human blood. J.B. contributed to the supervision of the research and data interpretation. L.V. contributed to financial funding and research managing. L.R. and D.Sm. wrote the manuscript. All authors contributed to the writing and revisions of the manuscript and approved the submitted version.

Disclosure statement

L.S. and J.B. is a part-time employee, and J.B. is a minority shareholder of Sotio, a.s., a biotech company that is developing novel immunotherapies. Other authors declare no conflicts of interest.

Ethical statement

All experimental protocols were approved by the ethical standards of the institutional, national research committee – the Ethics Committee of the Motol University Hospital in Prague (approval date: Jun 19, 2019). All experiments were performed in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participant included in the study signed informed consent for the use of their blood-derived products for future research. The animal experimental procedures employed in the studies respected the rules of the European Convention for the Care and Use of Laboratory Animals (2010/63/EU) as approved by the Czech Animal Care and Use Committee and the Institutional Ethics Committee (Departmental Commission of the Academy of Sciences of the Czech Republic), according to the Act of the Czech National Council no. 246/1992 Coll., according to § 5 of Decree no. 419/2012 Coll., the Act on the Protection of Animals Against Cruelty, under the project no. 111/2019.

Funding

The research was supported by the Ministry of Health, Czech Republic - projects NU22-03-00300 and NU23-08-00071, by the Institutional grant RVO 61388971 of the Institute of Microbiology AS CR, v. v. i., by the project of the National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NP05102) - Funded by the European Union - Next Generation EU, by UniCredit Bank CZ&SK, and by Generali-CP Foundation.

Data availability

The raw data supporting the conclusions of this article will be made available from the corresponding author on reasonable request.

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4 Discussion

Despite the great success of immune checkpoint inhibitors (ICI) in cancer treatment, the low response rate and high incidence of severe side effects call for the implementation of novel therapeutic strategies. Nanoparticle-based therapy could be relevant for answering this call as it has the potential to effectively contribute to developing these novel strategies. However, their implementation also comes with new challenges that must be addressed. Among these challenges are safety concerns. The presented study attempted to unravel new possibilities of how novel nanoparticle-based therapies could be approached as to their evaluation, including not only their possible antitumor therapeutic performance *in vitro* and *in vivo* but also their safety, particularly towards the tumor microenvironment and the immune system.

The first part of the study focused on the “traditional” evaluation approaches, which concern nanoparticle characterization, biocompatibility, and biodistribution. The second part of the study describes the investigation of nanoparticles towards the remodeling of the tumor microenvironment, and the third part of the study attempts to elaborate on the indicia of the previous parts’ observations of the nanoparticle-elicited cytotoxic impact on cancer cells. This nanoparticle-elicited cytotoxic impact on cancer cells is investigated in the context of the immune system response, particularly considering the developing field of immunotherapy where the manner of cancer cell death could substantially influence the modulation of anticancer immunity.

Several strategies for NP-based immunotherapy have been utilized in the last decades and, in general, can be divided into targeted and non-targeted approaches (Yao, et al. 2020, Lu, et al. 2024, Riley 2019). Each of these approaches has some advantages and disadvantages but focuses on the same goals: direct targeting of tumor cells and the modulation of tumor microenvironment.

Biodistribution of nanoparticles is a basic parameter through which their use in nanomedicine is evaluated (Hoshyar, et al. 2016). Many technologies rely on the functional modification of nanoparticles to enhance their targeting to tissues of interest, including tumors (Finbloom, et al. 2020). However, *in vivo* milieu may challenge these modifications and cause its deterioration through its degradation or metabolism, decreasing the targeting capacity, and the “naked” (uncoated) particles become distributed based on the properties of their elemental composition and dimensions (Roy, et al. 2019). For this reason, the distribution of novel or newly modified nanoparticles needs to be evaluated. Our investigation on upconversion silica-coated

NaGdF₄:Yb³⁺/Er³⁺ nanoparticles in mouse models revealed these particles are well distributed at the vascular level, in the neo-generated vessels of the melanoma tumors, as well as in the brain vessels but not in the brain tissue. This was demonstrated by their accumulation detected and visualized with LA-ICP-MS imaging on the tumor and brain sections of the treated mice, using concentrations *in vitro* found to be well below the toxicity these particles could elicit *per se* toward either tumor or non-tumor cells (Kostiv, et al. 2017). These NPs, doped with Yb³⁺/Er³⁺ to generate an upconversion process, can produce high photothermal conversion coefficients and luminescence under NIR light, useful for focused deep tissue stimulation while lowering the peritumoral healthy tissue damage. The effect can be increased by adding photosensitizing molecules and obtaining a photodynamic reaction to induce cytotoxicity by eliciting the production of highly oxidative molecules in the tumor cells and microenvironment (Overchuk, et al. 2023). These findings showed that novel nanoparticles could be *ex vivo* very well traced with advanced tissue analysis and imaging techniques even at non-toxic concentrations, demonstrating their perspective use for imaging as well as safe carriers for therapeutic compounds.

The TiO₂ prolate nanospheroids were another type of these nanoparticles. They were investigated during the study for their potential as photosensitizers for cancer therapy. The results showed that these nanospheroids had minimal toxic effects on fibroblast and immune cells and exhibited dose-independent cytotoxicity in two of the tested human cancer lines. However, they did not seem to potentiate the UVC-mediated toxic effects (Matijević, et al. 2020).

A different approach can be followed using protein-based NPs. The protein NPs can be used as carriers or vectors of molecules with different purposes: therapeutic, diagnostic, or immunomodulation, depending on how they are functionalized. The possibility of their production as recombinant proteins conjugated with antibodies or specific proteins to target functional molecules expressed at the tumor cell's surface makes these NPs particularly promising. Moreover, they are fully metabolized and degraded (Hong, et al. 2020). A good example is the human ferritin-based (HFt) NPs (Khoshnejad, et al. 2018, Heger, et al. 2014). For instance, we previously reported that melanoma cells could be selectively targeted by HFt polyethylene glycol (PEG)-masked protein-based multifunctional nanoparticles *in vitro*, functionalized with the melanocyte-stimulating hormone (α -MSH), which receptor is overexpressed on the surface of the tumor cells (Vannucci, Falvo and Fornara, et al. 2012),.

Selective targeting and internalization of the HFt NPs were demonstrated both in the primary and metastatic cancer cells (Vannucci, Falvo and Failla, et al. 2015).

Generally, the HFt protein construct has several advantages in comparison with classical antibodies: first, the longer half-life in circulation; second, such a construct can be loaded with a chemotherapeutical or a contrast agent and, by this way, selectively deliver it to the tumor cells (Lei, et al. 2016, Li, et al. 2019). In fact, besides the selectivity of the construct, the HFt-based NPs can efficiently release their load of therapeutic molecules because of their sensitivity to low pH, like in the tumor microenvironment and in lysosomes. In fact, the low pH favors the opening of the HFt cage, and this can be an advantage to be considered in the choice of a targeting carrier for interventions to modulate the tumor microenvironment (Guo, et al. 2020).

There are several therapeutic approaches to facilitate the negative impact of an immune-suppressive TME, and the most successful of them is ICI; nevertheless, the problems mentioned above prevent its broader deployment in clinics. To solve this problem, we employed a novel HFt-based fusion protein, named THE-10, engineered to include two modified linear versions of the PD-L1 binding peptide (PD-L1Pep-1) and the integrin $\alpha V\beta 3/\alpha V\beta 5$ and NRP-1 binding peptide (iRGD). We demonstrated that THE-10 provides an ICI-like effect and significantly potentiates the anticancer effects of antibody-drug conjugates Trastuzumab (binds the EGFR2) conjugated to a toxic topoisomerase I inhibitor deruxtecan on A375 melanoma cells *in vitro* (Tisci and Rajsiglova, et al. 2024).

Irinotecan and gemcitabine are two widely used chemotherapeutic drugs employed for the treatment of different types of solid tumors. However, therapy-induced cancer cell resistance, short half-life, and high toxicity limit their clinical applications. Various approaches to solving such limitations have been made in the last decades, including the nano liposomal construct NAPOLI-1 (NAnoliPOsomaLIrinotecan) approved by the FDA for the treatment of pancreatic cancer (Wang-Gillam, et al. 2019) and recently in study for advanced biliary tract cancers (Malka and Colle 2024). We reported that THE-10 enhances the *in vivo* antitumor activity of both Irinotecan and Gemcitabine in the syngeneic colorectal carcinoma mouse model. The combination of immunotherapeutic molecules with the tumor targeting moieties exhibited by the nanoconstruct also determined the activation of a positive anti-tumor immune cell infiltration in the CT26 mouse adenocarcinoma *in vivo* (Tisci and Rajsiglova, et al. 2024). This observation provides a new strategy for combinatorial cancer treatment by small molecule inhibitors and nanoparticles.

Despite promising results in the clinic, antibody-drug conjugates (ADC) have several limitations. One of them is their limited internalization after conjugate-ligand interaction, which is critical to producing the needed therapeutic effect. To improve the efficacy of drug delivery into cancer cells, several strategies have been elaborated, including targeting antigens that are involved in the internalization pathways (Singh, et al. 2022), bispecific antibodies [(Sellmann, et al. 2016), NCT04695847, NCT05123482], conjugate linker designs (McCombs and Owen 2015), combinations with drugs that enhance endocytosis (Michigami, et al. 2021), or nanoparticle-based ADC delivery (Liu, et al. 2018, Tisci and Rajsiglova, et al. 2024). It is well known that ADC therapy has limitations due to the tumor microenvironment, which can significantly impact its efficacy (Lin and Sagert 2018). Therefore, therapy that affects both cancer cells and immunoinhibitory TME should be considered the most effective way to cure cancer. In this regard, we previously demonstrated that THE-10 mediated modulation of PD-L1 expression in the TME accompanied by selective endocytosis of ADC sacituzumab govitecan showed promising results in pancreatic cancer model *in vivo* (Tisci and Rajsiglova, et al. 2024).

In conclusion, THE-10 may expand the therapeutic indications of co-administered drugs. Boosting with THE-10 may conceivably improve the efficacy of drugs in their approved indication and/or lower dosages and side effects.

It must be mentioned that modern immunotherapy is mainly focused on direct targeting of tumor cells. Nevertheless, it is well known that the TME plays an important role in cancer development and progression. From this point of view, modulation of TME, even in an unspecific manner, could potentiate the effectiveness of anticancer therapy (Ruiz-de-Angulo, et al. 2020).

Inorganic NPs have been mainly used for diagnostic and theranostic properties (Mao and Yoo 2024, Narayana, et al. 2024). The studies about the therapeutic potential of inorganic NPs provided in this field were mainly focused on direct cytotoxic effects against cancer cells, and less is known about their immunomodulatory properties.

We showed that FN NPs can *in vitro* differentially mediate cell death in mouse and human cancer cells (Rajsiglova, Babic, et al. 2024). This direct cancer cell-related cytotoxicity demonstrates their therapeutic value if proposed as carriers for tumor-targeted therapy. It also should be mentioned that NP-induced cell death can be elicited via multiple mechanisms, which can have different impacts on the immune system, determining the cell death as immunogenic,

non-immunogenic, or immunosuppressive (Hou, Greten and Xia 2017, Duan, Chan and Lin 2019, Arimoto, et al. 2024). We demonstrated that our newly produced FN NPs do not affect the stimulation and phenotype of macrophages after co-culture with cancer cells, and we also did not observe changes in the maturation of *ex vivo*-produced murine bone marrow-derived DCs (BMDCs) pulsed with cancer cells pretreated with nanoparticles. These findings showed that FN NPs have a dual impact on cancer and immune cells, and their cytotoxic potential can be delivered without adverse effects on the following activation of the immune response against these cancer cells (Rajsiglova, Babic, et al. 2024).

It is well known that in the late stages of cancer, patients may be immunocompromised due to the progression of the disease or from ongoing treatments. Tumor mechanisms of immune escape limit the effectiveness of some therapies, such as ICI or DC-based vaccines, and decrease the therapeutic window for unspecific or less specific drugs, including chemotherapy or nanoparticles. Nevertheless, our newly produced FN NPs did not compromise the patients' anticancer immune response even at advanced stages of the disease, including hormone-refractory metastatic prostate cancer. We demonstrated that such a treatment had not affected the maturation of *ex vivo*-produced monocyte-derived DCs (moDCs) and subsequent expansion of prostate cancer cell line-reactive T-cells, supporting the possibility to develop more effective treatments (Rajsiglova, Babic, et al. 2024).

Iron-based magnetic NPs belong to one of the most used materials in nanomedicine (Dadfar, et al. 2019, Montiel Schneider, et al. 2022). Originally, they were mostly used as contrast agents for magnetic resonance (Rahman 2023). However, as they are more and more considered multifunctional therapeutic agents for drug delivery and anti-cancer thermal therapy (Shahbazi and Behbahani 2024), their impact not only on cancer cells but also on the subsequent antitumor immune response should be determined during their toxicological evaluations. This is particularly important when iron-based NPs are increasingly considered also for application in cancer immunotherapy (Dias, et al. 2022, Deng, et al. 2024). Therefore, their identity and role towards antitumor immunity needs to be determined to effectively combine the benefits of nanomedicine and cancer immunotherapy. Our study indicated possible avenues through which the search for this identity could be navigated (Rajsiglova, Babic, et al. 2024).

Altogether, the study of the characteristics and immune potential of different types of nanoparticles supports the importance of this specific approach in cancer management. It offers

the possibility of developing more effective anti-cancer interventions and even enhancing the newly applied immunotherapeutic approaches.

5 Conclusions

- 1) We observed dose-dependent direct toxic effects of the NPs on both B16F10 murine melanoma cell line and 3T3 murine fibroblast cell line *in vitro*.
- 2) We showed that the NaGdF₄:Yb³⁺/Er³⁺ has mainly perivascular biodistribution in the tumor, liver, and brain without any apparent signs of systemic toxicity in tested animals.
- 3) We found that TiO₂ NPs exhibited a direct cytotoxic effect on A375 and SKBR3 cancer cell lines but did not affect the viability of PANC1 pancreatic cells. We found a minimal toxic effect in the 3T3 fibroblast cell line at the furthest time point. In contrast, an immediate toxic effect was observed in immune cells directly upon exposure to NPs but quickly recovered.
- 4) We did not observe synergistic effects of the TiO₂ NPs with UVC irradiation in either of the tested cancer cell lines.
- 5) We showed that THE-10 nanoconstruct itself did not elicit any anticancer activity but was able to further potentiate the dose-dependent anticancer activity of T-DXd on the A375 cells, especially at lower concentrations *in vitro*.
- 6) We demonstrated that the PD-L1 binding-peptide functionalized THE-10 NPs potentiates the efficacy of anti-cancer therapy *in vivo*:
 - In the murine model of syngeneic CT26 colorectal carcinoma the therapeutic activity of irinotecan was significantly improved by co-administration with the THE-10 NPs.
 - THE-10 NPs have also been shown to enhance the *in vivo* antitumor activity of the nanoferritin THE-0504 and the antibody-drug conjugate sacituzumab govitecan in both syngeneic and xenograft models of pancreatic cancer.
- 7) We observed a direct *in vitro* toxic effect of FN NPs on murine CT26 colorectal carcinoma, and human PC-3 prostate cancer cell line, while the LNCaP cells resulted resistant.
- 8) We found that the NPs-induced toxicity in the cancer cells did not affect the phenotype or responsivity of the tested immune cells. Additionally, it also did not prevent either murine or human DCs from stimulating cancer cell-reactive T lymphocytes in the mixed culture.

6 Final remarks

In conclusion, our findings indicate that nanoparticles have the potential to play a significant role in anti-cancer therapy. They have demonstrated the ability to directly cause cell death in cancer cells and, importantly, to enhance the effects of combined treatments in in vitro and in vivo studies. Additionally, our study highlights not only the different sensitivity of different cancer cells to the effects of the same type of NP but also the importance of studying specific types of NPs in relation to the type of tumor being treated. Evaluating the induced changes in immunogenicity and cell death can help prevent unwanted adverse effects on the tumor-infiltrating immune cells. This is crucial for preventing any harmful effects that could compromise the overall effectiveness of anti-cancer treatment.

7 References

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8 List of publications

8.1 This thesis was prepared based on the following publications:

Kostiv, U., **Rajsiglová, L.**, Luptáková, D., Pluháček, T., Vannucci, L., Havlíček, V., . . . Horák, D. (2017). Biodistribution of upconversion/magnetic silica-coated NaGdF₄:Yb³⁺/Er³⁺ nanoparticles in mouse models. *RSC Advances*, 7(73), 45997-46006. <https://doi.org/10.1039/C7RA08712H>

IF: 2.936, original article

Contribution of the applicant: My work in this project focused on testing the *in vitro* toxicity of the provided NPs and the animal model. I was responsible for *in vitro* testing of the NPs' toxicity on tumor, non-tumor, and immune cells, including the final data analysis, and also for the *in vivo* model, which consisted of inoculating tumor cells to the animals, intracardial administration of the treatment, monitoring of the animals, and the final autopsy. I prepared the draft of the methods and results for which I was responsible.

Matijević, M., Nakarada, D., Liang, X., Korićanac, L., **Rajsiglova, L.**, Vannucci, L., . . . Stepić, M. (2020). Biocompatibility of TiO₂ prolate nanospheroids as a potential photosensitizer in therapy of cancer. *Journal of Nanoparticle Research*, 22(7). <https://doi.org/10.1007/s11051-020-04899-3>

IF: 2.253; original article

Contribution of the applicant: My work in this project involved introducing part of the *in vitro* methods used to test for cell viability and toxicity of the NPs on the cell lines and splenocytes (including data analysis). I was also responsible for the autopsy of animals to obtain spleens and the preparation of splenocytes.

Tisci, G.*, **Rajsiglova, L.***, Bibbod, S., Ziccheddu, G., Falvo, E., De Laurenzi, V., Sala, G., Colotti, G., Arcovito, A., Giacon, N., Vannucci, L., Giacomini, P. & Ceci, P. (2024). Boosting of targeted therapy, chemotherapy and immunotherapy by a novel non-cytotoxic nanovector THE-10 based on recombinant human ferritin. *International Journal of Biological Macromolecules*, in revision; * **These authors share first authorship**

IF: 7.7; original article

Contribution of the applicant: My work in this project was focused mainly on the *in vivo* murine CT26 colorectal carcinoma model. I was responsible for writing the proposal for the animal welfare project (on our side of the project) and later became the person responsible for this project. My work involved all *in vivo* procedures, from inoculation of the cancer cells through intravenous/intraperitoneal administration of the treatments and follow-up of the animals to the final autopsy and harvesting of the organs. I wrote the draft for the methods and results of our part of the project.

Rajsiglova, L., Babic, M., Krausova, K., Lukac, P., Kalkusova, K., Taborska, P., Sojka, L., Bartunkova, J., Stakheev, D.*, Vannucci, L.*, & Smrz, D.* (2024). Immunogenic properties of nickel-doped maghemite nanoparticles and the implication for cancer immunotherapy. *Journal of Immunotoxicology*. Accepted, provisory DOI: 10.1080/1547691X.2024.2416988

*these authors share senior authorship

IF: 2.4; original article

Contribution of the applicant: My work in this project involved participation in the experimental design and all experiments and analyses performed in the murine model. I was responsible for harvesting immune cells, preparing cancer cells of murine origin, and conducting experiments in the mouse *in vitro* model, including viability assays, flow cytometry, and final data analysis and interpretation. I wrote the paper under the supervision of doc. Smrz.

8.2 Other publications related to the theme of the thesis:

Rajsiglová, L., Vannucci, L., (2018). Local Treatment of Brain Tumors and the Blood-Brain Barrier. In: Crocco, L., Karanasiou, I., James, M., Conceição, R. (eds) Emerging Electromagnetic Technologies for Brain Diseases Diagnostics, Monitoring and Therapy. Springer, Cham. https://doi.org/10.1007/978-3-319-75007-1_7

Chapter in a book, review

Vannucci, L., Falvo, E., Failla, C. M., Carbo, M., Fornara, M., Canese, R., Cecchetti, S., **Rajsiglová, L.**, Stakheev, D., Krizan, J., Boffi, A., Carpinelli, G., Morea, V., & Ceci, P. (2015). In Vivo Targeting of Cutaneous Melanoma Using an Melanoma Stimulating Hormone-Engineered Human Protein Cage with Fluorophore and Magnetic Resonance Imaging Tracers. *Journal of biomedical nanotechnology*, 11(1), 81–92. <https://doi.org/10.1166/jbn.2015.1946>

IF: 5.068

8.3 Other publications:

Original articles:

Taborska, P., Lukac, P., Stakheev, D., **Rajsiglova, L.**, Kalkusova, K., Strnadova, K., Lacina, L., Dvorankova, B., Novotny, J., Kolar, M., Vrana, M., Cechova, H., Ransdorfova, S., Valerianova, M., Smetana, K., Jr, Vannucci, L., & Smrz, D. (2023). Novel PD-L1- and collagen-expressing patient-derived cell line of undifferentiated pleomorphic sarcoma (JBT19) as a model for cancer immunotherapy. *Scientific reports*, 13(1), 19079. <https://doi.org/10.1038/s41598-023-46305-7>

IF: 3.8

Sushytskyi, L., Lukáč, P., Synytsya, A., Bleha, R., **Rajsiglová, L.**, Capek, P., Pohl, R., Vannucci, L., Čopíková, J., & Kaštánek, P. (2020). Immunoactive polysaccharides produced by heterotrophic mutant of green microalga *Parachlorella kessleri* HY1 (Chlorellaceae). *Carbohydrate polymers*, 246, 116588. <https://doi.org/10.1016/j.carbpol.2020.116588>

IF: 9.381

Reviews:

Sushytskyi, L., Synytsya, A., Čopíková, J., Lukáč, P., **Rajsiglová, L.**, Tenti, P., & Vannucci, L. E. (2023). Perspectives in the Application of High, Medium, and Low Molecular Weight Oat β -d-Glucans in Dietary Nutrition and Food Technology-A Short Overview. *Foods (Basel, Switzerland)*, 12(6), 1121. <https://doi.org/10.3390/foods12061121>

IF: 4.7

Mishra, R., **Rajsiglová, L.**, Lukáč, P., Tenti, P., Šima, P., Čaja, F., & Vannucci, L. (2021). Spontaneous and Induced Tumors in Germ-Free Animals: A General Review. *Medicina (Kaunas, Lithuania)*, 57(3), 260. <https://doi.org/10.3390/medicina57030260>

IF: 2.948

Vannucci, L., Krizan, J., Sima, P., Stakheev, D., Caja, F., **Rajsiglova, L.**, Horak, V., & Saieh, M. (2013). Immunostimulatory properties and antitumor activities of glucans (Review). *International journal of oncology*, 43(2), 357–364. <https://doi.org/10.3892/ijo.2013.1974>

IF: 2.773