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CANCERTO - NANOSCIENCE IN CANCER IMMUNOTHERAPY

Virtual Event - March 9–11, 2021

frontiers Event Abstracts

CANCERTO - NANOSCIENCE IN CANCER IMMUNOTHERAPY

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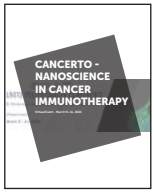

March 9-11, 2021, Virtual Event

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Welcome to the CancerTO - Nanoscience in Cancer Immunotherapy

Cancer is a complex systemic disorder arising from the altered function of multiple cellular and molecular mechanisms. One of the systems malfunctioning in cancer is the immune system. Restoring and improving the ability of the immune system to effectively recognize and eradicate cancer is the main focus of immunotherapy, a topic which has garnered recent and significant interest. The initial excitement about immunotherapy, however, has been challenged by its limited efficacy in certain patient populations and the development of adverse effects such as therapeutic resistance and autoimmunity. At the same time, a number of advances in the field of nanotechnology could potentially address the challenges faced by modern immunotherapeutics and allow immunotherapy to reach full therapeutic success.

This e-book collects the abstracts presented to the international workshop "CancerTO: Nanoscience in Cancer Immunotherapy" virtually held on March 9-11th, 2021 and based in Turin, Italy.

The online meeting allowed to share the most recent scientific advances made in the nanotechnology field that could help to overcome some of the limitations that cancer immunotherapy is currently facing in terms of inadequate efficacy and side effects.

Unito-Polito Conference Series in Cancer Nanoscience in Cancer Immunotherapy

March, 9 th – 11 th 2021

Virtual Event

AIMS OF THE WORKSHOP – CALL FOR ABSTRACTS

The Nanoscience in Cancer Immunotherapy Workshop (NCIW), to be held in Turin from March 9-11 2021, is an international meeting jointly organized by the Università and Politecnico of Turin with the aim to provide a platform for discussion on the most recent advances in tumor immunology with a focus on nano bio-technology as a strategy to foster the impact of immunotherapy on cancer treatment.

The main goal of the NCIW is to provide a challenging transdisciplinary environment where life and nano scientists can exchange their knowledge and latest findings to accelerate and improve the coordinated progress of oncology, drug development and delivery. The program covers topics from basic research in tumor immunology to the latest advancements in the design of complex tissue models and of nanotechnology based delivery systems in cancer immunotherapy.

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March, 9th 2021

14.30 – 14.45 Official Greetings

14.45 – 14.55 – Introduction and Program Presentation (Federico Bussolino – Eugenio Brusa)

Opening Lecture

14.55 – 15.20 Haifa Shen - Methodist Hospital Research Institute, Houston (TX, USA)

Therapeutic Cancer NanoVaccine

A Role for Nanoscience in Cancer Immunotherapy Chairs: Gloria Berlier – Valentina Cauda

15.20 – 15.45 Jen Morton - Beatson Institute, CRUK Glasgow Center, Glasgow (UK)

Exploring Opportunities for Immunotherapy in Mouse Models of Pancreatic Cancer

15.45 – 16:10 Cornelis Melief - Department of Immunohematology and Blood Transfusion Leiden University Medical Center, Leiden, (NL)

Combination Immunotherapy of Cancer Involving Therapeutics Vaccination

16.15 – 16.45 Workshop – Merck Life Science Srl Ludivine Grosjean

Immunology & Oncology - A Review of Milliplex Multiplex and SMC™ Assays for Immunology and Oncology Research

16.50 – 17.20 **Short Talks Selected Among the Submitted Abstracts**

16.50 – 17.05 **Veronica Vighetto**

Zinc Oxide Nanocrystals Combined with Ultrasound for the Controlled Generation of Reactive Oxygen Species

17.05 – 17.20 **Lorenzo Pallante**

Understanding the Molecular Binding Mechanism of Colchicine Derivatives Targeting βIII Human Tubulin Isotype

17.20 – 17.35 **Gloria Delfanti**

Dual Targeting of Cancer and Suppressive Myeloid Cells by Tumor-redirected iNKT Cells and Antigen-carrying Microparticles

17.35 – 17.50 **Annarita D'urso**

In Vitro and In Vivo Magnetic Drug Targeting by Using Biomimetic Magnetite Nanoparticles

17.50 – 18.15 **Luisa De Cola – Istituto di Ricerche Farmacologiche Mario Negri, Milan (I)**

Nanocarriers for Biomolecules Delivery

18.20 – 18.40 **Virtual room for poster presentation**

"Just The Woman I am" Keynote Lecture

18.45 – 19.10 **Fiona Watt – Centre for Stem Cells & Regenerative Medicine King's College London (UK)**

Exploring the Ecosystem of Oral Cancer

March, 10th 2021

Tumor Microenvironment and Models Chairs: Valeria Chiono – Clara Mattu

14.55 – 15.20 **David Ting - Massachusetts General Hospital - Cancer Center, Boston (MA, USA)**

Single Cell Technologies to Define Intratumoral Heterogeneity

15.20 – 15.45 — **Giulia Adriani - Singapore Immunology Network (SIgN) Agency for Science, Technology and Research. Singapore**

3D Microfluidic Model of Tumor Microenvironment for Pre-clinical Screening of Immunotherapies

15.45 – 16.15 — **– Short Talks Selected Among the Submitted Abstracts**

15.45 – 16.15 — **Chiara Ambrogio**

Novel Approaches to Target Oncogenic KRAS Complex Assembly at the Cell Membrane

16.00 – 16.15 — **Gianluca Mucciolo**

Interleukin 17A Depletion: A Promising Adjuvant Strategy to Increase DNA-Vaccination Efficacy in Pancreatic Ductal Adenocarcinoma Treatment

16.15 – 16.45 — **Kairbaan Hodivala-Dilke - Barts Cancer Institute London, London (UK)**

New Routes for Vascular Modulation

16.45 – 17.10 — **Joao Mano – Department of Chemistry - University of Aveiro, Aveiro (Portugal)**

Soft Biomaterials for Engineering 3D Cancer Models

17.15 – 17.55 — **Short Talks Selected Among the Submitted Abstracts**

17.15 – 17.30 — **Martina Godel**

P-glycoprotein Inhibitors as Inducers of Immunogenic Cell Death

17.30 – 17.45 — **Antonino DI Lorenzo**

Toll-Like Receptor 2 Targeting Impairs Breast Cancer Progression and Overcomes Resistance to Chemotherapy

17.45 – 18.00 — **Claudia Voena**

The Perivascular Niche Protects ALK+ Lymphoma Cells from ALK Inhibition through the CCL19/21-CCR7 Axis

18.00 – 18.15 **Marco Campisi**

In Vitro Model of ALK+ Anaplastic Large Cells Lymphoma and Vascular Interactions and Prediction of Drug Efficacy in a 3D Microfluidic Chip

18.20 – 18.40 **Virtual Room for Poster Presentation**

Cell-Based Immunotherapy
Chairs: Paola Cappello – Marco Agostino Deriu

18.45 – 19.10 **Mariolina Salio - MRC Human Immunology Unit, WIMM, University of Oxford (UK)**

Harnessing Innate-like T cells in Cancer Immunotherapy

19.10 – 19.35 **Samuel MOK - University of Texas - MD Anderson Cancer Center, Houston (TX, USA)**

Anticancer Immunotherapy by MFAP5 Blockade Suppresses Ovarian Cancer Progression

March, 11th 2021

Myeloid Cells Orchestrate Tumor Response
Chair: Federico Bussolino

14.30 – 14.55 **Paola Allavena - Humanitas University, Rozzano (I)**

Macrophage Reprogramming Using TLR-Agonist-loaded Polymeric Nanocapsules for the Treatment of Cancer

14.55 – 15.20 **Dmitry Gabrilovich - AstraZeneca I Oncology R&D (USA)**

Regulation of Tumor Microenvironment by Myeloid-derived Suppressor Cells

15.20 – 15.30 **Awards Assignment**

15.35 – 16.05 **Workshop - Fluidigm**

Roberto Spada

Advancing Breakthroughs in Immuno-Oncology Research from Cell Types to Tissue: Analyse >40 Functional and Phenotypic Markers Simultaneously on the Helios and Hyperion™ Imaging System, Powered by CyTOF® Technology

Keynote Lecture

16.10 – 16.35 **Melody Swartz - Pritzker School of Molecular Engineering - University of Chicago (MI, USA)**

Emerging Technologies In Cancer Immunology
Chairs: Luca Primo – Alberto Puliafito

16.35 – 17.10 **Massimiliano Pagani – Ifom (The FIRC Institute for Molecular Oncology) and UNIMI, Milan (I)**

Organoid Models to Decipher the Interplay between Tumor and the Immune System

17.10 – 17.35 **Giovanni Ciriello - Université de Lausanne, Lausanne, (CH)**

Cancer Evolutionary Dependencies

17.35 – 18.00 **Jérome Galon - Laboratory of Integrative Cancer Immunology, Sorbonne Université, Sorbonne Paris (F)**

The Immune Contexture in the Era of Cancer Immunotherapy

18.00 – 18.25 **Henner Farin - Georg-Speyer-Haus - Institute Tumor Biology and Experimental Therapy, Frankfurt (D)**

Patient-derived Tumor Organoids to Model Stromal Interactions and Cell-based Immunotherapies

18.25 – 18.40 **Concluding Remarks - (Federico Bussolino – Eugenio Brusa)**

List of abstracts

Editorial by chairmen

Federico Bussolino[1]*, Eugenio Brusa[2]*

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Interdisciplinary research is a way to solve problems whose solutions cannot be found in a single area of research but need the integration of tools, concepts, theories and results from more disciplines. This strategy comes at a cost for researchers because interdisciplinarity has its own set of unique challenges, ranging from communication issues to the requirement of specific grants and allocation of credits among a team.

However, some of the most relevant leaps forward in biomedicine have been arisen from interdisciplinary researchers: from radioactivity and nuclear Chemistry, to magnetic nuclear resonance, optical imaging, bio-inspired materials and so on and so forth.

Since 2016 the Università of Torino (UniTo) and the Politecnico of Torino (PoliTo) have been organizing in Torino city (Italy) the UniTO-PoliTO Conference Series in Cancer. These workshops aim at discussing challenging and groundbreaking topics in Oncology, which require interdisciplinary approaches encompassing Medicine, Biology, Chemistry, Physics, Technology, Engineering and Computer Science. These workshops are sponsored by the running race Just the Woman I am, held every March 8th in Torino and organized by "Centro Universitario Sportivo -Torino"(www.custorino.it) to celebrate the Women's Day.

The first ever workshop (Enabling technologies in 3D cancer organoids) took place March 8th -9th, 2016 focused on the interdisciplinary effort to improve 3D cell culture by the use of innovative materials, favoring organoid assembly and the development of new imaging tools to navigate inside tumor organoids. The second workshop (Imaging of Cancer Dynamics) took place March 7th -9th, 2018 and discussed the newest imaging technologies at different spatial scales to improve in vitro and in vivo knowledge in cellular and tissue oncology. Due to the Sars-2-Covid outbreak, the III edition (Nanoscience in Cancer Immunotherapy) was postponed from March 2020 to March 9th-11th 2021 and organized in a virtual way.

The workshop has provided a platform for discussion on the most recent advances in tumor immunology with a focus on nano-biotechnology as a strategy to foster the impact of immunotherapy on cancer treatment from the drug development and delivery to the design of more suitable preclinical models.

The exploitation of immune system to fight cancer has characterized the whole history of oncology with up and down promises and enthusiasms [1,2]. The first clinical attempts to treat cancer date back to 1883 when Fehleisen and Busch independently observed a remarkable tumor shrinking after erysipelas infection. At the end of eighteenth century, William Cooley continued and improved these studies by showing an evident regression of sarcoma lesions in patients inoculated with bacterial culture derived from erysipelas lesions. However, the vague scientific bases of this treatment and the risk of infections suggested physicians to abandon this strategy for many decades and to pursue surgery and radiotherapy for the treatment of tumors.

The second half of nineteenth century has been characterized by the identification of many cellular players (T cells, NK, dendritic cells, macrophages) of the immune system as well as of many soluble mediators (cytokines, chemokines) involved in the immune response to cancer. These findings have been represented the background for the current renaissance of cancer immunotherapy, but already they allowed to pioneer bone marrow transplant as a treatment for hematological cancers, a method that is still used today.

It is possible to date the restart of immunotherapy as a powerful tool to contrast cancer at the beginning on this century with the discovery that the immune checkpoint pathways, which normally maintain self-tolerance, can be co-opted by cancer cell to evade immune attacks. Monoclonal antibodies halting immune checkpoints can unleash anti-tumor immunity and control the progression of many tumors. Furthermore, new positive impulses are characterizing the progress in vaccine-mediated strategies (autologous and allogenic cancer vaccine) [3] and a new frontier is emerging: the adoptive cell therapy. It exploits the tools of gene and cell therapy and involves the isolation of patient's T cells, which are tumor-specific, genetic modification and multiplication of those cells in the laboratory and then re-injection back to the patient circulation to kill cancer cells [4].

Nevertheless, immune check point blockade, adoptive cell therapy and vaccination have limitations, which are reflected by the restricted patient

populations that benefit from these therapeutic regimens. Therefore, increasing research attention has shifted to understanding the biological basis of these variable responses, to identifying biomarkers that can predict which patients are likely to respond or not respond to these therapies, to delaying the occurrence of resistance, to improving the drug delivery.

The main themes discussed and developed by the workshop aimed at focusing on the most significant problems in cancer immunotherapy and how technology can contribute to their solutions. This unique gathering of international experts presented current research and discussed key issues to apply nanoscience to improve immunotherapy and offered a good opportunity for young scientists to present their data.

There were 20 faculty speakers, 10 short-oral presentations dedicated to young scientists, 2 poster sessions including 42 different posters, 2 independent industry expert sessions and more than 300 attendees. Additionally, abstracts were approved for publication, poster presentation and/or oral presentation, as reported in the following.

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CancerTO: Nanoscience in cancer immunotherapy

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Cancer is a complex systemic disorder arising from the altered function of multiple cellular and molecular mechanisms. One of the systems malfunctioning in cancer is the immune system. Restoring and improving the ability of the immune system to effectively recognize and eradicate cancer is the main focus of immunotherapy, a topic which has garnered recent and significant interest. The initial excitement about immunotherapy, however, has been challenged by its limited efficacy in certain patient populations and the development of adverse effects such as therapeutic resistance and autoimmunity. At the same time, a number of advances in the field of nanotechnology could potentially address the challenges faced by modern immunotherapeutics and allow immunotherapy to reach full therapeutic success.

This e-book collects the abstracts presented to the international workshop "**CancerTO: Nanoscience in Cancer Immunotherapy**" virtually held on March 9-11th, 2021 and based in Turin, Italy.

The online meeting allowed to share the most recent scientific advances made in the nanotechnology field that could help to overcome some of the limitations that cancer immunotherapy is currently facing in terms of inadequate efficacy and side effects.

Study infiltrating lymphocytes in a 3D microfluidic pancreatic tumor model

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OBJECTIVES

Immunotherapy applications have achieved tremendous advances in cancer treatment and *in vitro* assays can be beneficial to study interactions of immune cells with tumor. Two dimensional (2D) *in vitro* systems have demonstrated a great value in the biomedical research but they fail to reproduce the complexity of *in vivo* systems. Therefore, we developed a three dimensional (3D) pancreatic tumor model within a microfluidic device with the aim of overcoming some limitations of 2D systems and provide more physiologically relevant results. Specifically, we investigated the mechanism of T cell infiltration across the vasculature in pancreatic ductal adenocarcinoma.

METHODS

The microfluidic chip is composed by three channels, the central gel region and two lateral media channels. T cells were isolated from peripheral blood mononuclear cells from blood of healthy donors. T cell were activated with CD3/CD28 magnetic beads. Human pancreatic adenocarcinoma cells (Panc-1) were cultured in a collagen type I matrix in the central chamber of the device. Human umbilical vein endothelial cells (HUVEC) expressing green fluorescent protein (GFP+) and pancreatic stellate cells (PSC) were cultured in the lateral fluidic channels. After HUVEC formed an endothelial monolayer, T cells were added into the vascular channel and allowed to transmigrate across the endothelial barrier to reach the tumor region.

RESULTS

The 3D PDAC model was used to assess the ability of T cell to infiltrate within the tumor region. T cell population was divided in T cell activated and T cell not activated as verified by flow cytometry. T cell infiltration was quantitatively evaluated as a function of the T cell activation, presence of vasculature and tumor microenvironment. The presence of the endothelial barrier reduced T cell infiltration, confirming the key role played by the blood vessels in controlling T cell trafficking. Activated T cells migrated more than not-activated T cells towards the tumor and, indeed, without tumor cells in the matrix both T cells presented a similar migration toward the PSC.

CONCLUSIONS

Our results support the use of 3D microfluidic systems to reproduce a physiological 3D tumor model to study trans-endothelial migration of T cell.

ACKNOWLEDGMENTS

This work was supported by a core grant to Singapore Immunology Network (SIgN) from Agency for Science, Technology and Research (A*STAR) to GA and by a National Medical Research Council (NMRC) Open Fund Young Individual Research Grant (OFYIRG18nov-0002) to AP and GA.

In pancreatic cancer patients chemotherapy, but not immune checkpoint blockade, rescues effector T cell response to tumor associated antigens

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OBJECTIVES

Pancreatic Ductal Adenocarcinoma (PDA) is an almost incurable tumor mostly resistant to chemotherapy (CT). Clinical trials with immune checkpoint blockade (ICB) failed to increase PDA patient survival. We recently demonstrated that CT enhances the adaptive immune response of PDA patients to four tumor associated antigens (TAA), namely ENO1, FUBP1, K2C8 and G3P [1]. Understanding how to better induce an effector response following TAA stimulation is critical to set up efficient precision immunotherapy in PDA.

METHODS

Peripheral blood mononuclear cells (PBMC) from 18 PDA patients, before and after one round of gemcitabine-based CT, were stimulated *in vitro* with recombinant protein of TAA (ENO1, FUBP1, K2C8 or G3P) in the presence or absence of ICB (anti-CTLA4 and anti-PD1) to evaluate proliferative T cell response. Cytokine production of IFN-g and IL-10 was assessed by ELISA. Clonal T cell receptor beta (TRB) gene rearrangements were identified and tracked using Next-Generation Sequencing.

RESULTS

ICB abolished high proliferative responses to TAA before and after CT, while the same were maintained after CT with an increased number of TAA recognized per patients that positively correlates with their survival. Both before and after CT, ICB decreased the number of high IFN-g responses, whereas these were significantly increased after CT. Clustering analysis of the effector versus regulatory tone based on IFN-g/IL-10 ratio, identified three distinct groups of patients: in "CT responder" group the effector tone was increased or maintained after CT; in "exhausted" one the effector tone was lost after CT and in "non-responder" one the regulatory tone was predominant before and after CT. The expansion of TAA-specific T cell clones was observed by TRB repertoire sequencing and, of note, the percentage of these TAA-specific V-J rearrangements was to a larger extent after CT.

CONCLUSIONS

Better than ICB, CT alone shifted the immunological tone toward an effector phenotype, with a significant gain of TAA-induced effector T cell response. The expansion of V-J rearrangements due to TAA stimulation was enhanced by CT, suggesting a stronger reactivity of precise TAA-specific T cell clones after CT. These data suggest a strategy based on CT in combination with precision immunotherapy might be considered in selected responder PDA patients.

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Effectiveness of DNA vaccination and PI3Kgamma inhibition in pancreatic cancer

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BACKGROUND

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer mortality in developed countries, with one of the poorest prognoses among all cancers. Although 10-15% of patients are candidates for gross total surgical resection, recurrence is frequent, and the overall 5-year survival rate is around 10%. Using a proteomic approach, we have identified alpha-Enolase 1 (ENO1) as PDA-associated antigen. We have shown that ENO1 DNA vaccination efficiently prolongs survival of engineered mice that spontaneously develop PDA (both KC and KPC mice). Targeting of Phosphoinositide-3- kinase (PI3K) gamma and delta isoforms is an effective way to inhibit the suppressive activity of immune-regulatory cells, thus strengthening the anticancer immune response. Recently, we also have demonstrated that PI3K gamma play a critical role in PDA by driving the recruitment of myeloid derived suppressor cells into tumor tissues and it's genetic or pharmacologic inhibition effectively inhibits PDA progression and metastasis. In this study we assessed the hypothesis that the targeting of myeloid derived suppressor cells, via pharmacological PI3Kgamma inhibition (iPI3Kg), synergizes with ENO1 DNA vaccine by inducing a strong and sustained immune response.

METHODS

KPC mice were vaccinated four times with ENO1 starting at 4 weeks of age; 2 weeks later mice were treated with the iPI3Kg, for further 2 weeks. At sacrifice humoral and cellular responses were analyzed by enzyme-linked

immunosorbent assays, flow cytometry, and enzyme-linked immunosorbent spot, cytotoxicity assays, histology and immunohistochemistry (IHC).

RESULTS

Mice that received ENO1+ iPI3Kg therapy showed a significant decrease in tumor size in comparison to both ENO1 alone and PBS treated mice. This correlated with a decrease of CD11bGr1 positive cells in the blood and an increase of circulating anti-ENO1 specific antibodies in ENO1+ iPI3Kg group in comparison to control mice.

Moreover, the analysis of pancreatic tissues by IHC demonstrated that combined therapy induced an increased number of CD8 and F4/80. In addition, the analysis of the mRNA extracted from formalin fixed paraffin embedded pancreas tissues displayed an increase of Granzyme B in both ENO1 and ENO1+ iPI3Kg and a down modulation of genes involved in fibroblast and stellate cell activation suggesting a modulation of microenvironment in the combined therapy group. Magnetic Resonance Imaging and in particular diffusion-weighted imaging (DWI) was used to monitor tumor progression. DWI showed that mice vaccinated with ENO1 alone displayed an increase of mean apparent diffusion coefficient that significantly decrease after iPI3Kg treatment, correlating with an increase in tumor infiltrating cells.

CONCLUSION

The treatment with ENO1 plus iPI3Kg is able to reduce tumor size in pancreas, increases immune cell infiltration and modulates stroma cell compartment, making the therapy a suitable approach for PDA treatment.

Interleukin 17A depletion: A promising adjuvant strategy to increase DNA-vaccination efficacy in Pancreatic Ductal Adenocarcinoma treatment

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OBJECTIVES

Pancreatic Ductal Adenocarcinoma (PDA) remain one of the most lethal tumors because of its highly immunosuppressive microenvironment and the absence of specific therapeutic target. Our lab has demonstrated that, the glycolytic enzyme alpha-enolase (ENO1) is a promising PDA-associated antigen exploitable to boost the cancer immune response. In fact, an ENO1 DNA-based vaccination strategy significantly prolongs survival in genetically engineered mice (GEM) by increasing the T Helper-1 and T Helper- 17 detectable in the spleen. Because of the controversial role of the interleukin-17A (IL17A) and T helper-17 in PDA, we deeply investigated its role in combination with the ENO1 DNA-vaccination.

METHODS

GEM and IL17A-/- mice were crossed and vaccinated at 8 weeks of age every 2 weeks for a total of four vaccination rounds. Mice were constantly monitored and some mice were sacrificed 2 weeks after the last vaccination to collect serum and spleen for in vitro experiments: ELISA, ELISPOT and Flow Cytometry analysis. Moreover, PDA cells were orthotopically injected into the pancreas of syngeneic mice, which were treated or not with anti-IL17A in combination or not with ENO1-DNA vaccine. After one month, mice were euthanized; tumor masses were collected and analyzed by immunohistochemistry and quantitative PCR.

RESULTS

DNA vaccination prolonged survival of KPC/IL17A-/- mice compared to the non-vaccinated or IL17A proficient mice. Moreover, we observed an enhanced production of anti-ENO1 antibodies and increased number of IFNg-secreting T cells in KPC/IL17A-/- vaccinated mice. The analysis of the tumor infiltrating immune-cells revealed an increase of antigen-presenting cells and more effector/memory CD4+ and CD8+ T cells. PDA orthotopically injected mice treated with the vaccine in association with anti-IL17A antibody displayed a significantly reduced tumor growth that paralleled the induction of cytotoxic T cells not observed in the other groups.

CONCLUSIONS

Overall these results candidate the antibody-mediated depletion of IL17A as a potential adjuvant to increase the DNA-vaccination efficacy in PDA treatment.

Reprogramming Tumor Associated Macrophages using Polymeric Nanocapsules loaded with TLR ligands for the treatment of lung cancer

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Tumor-associated macrophages (TAMs) play a key role in tumor progression, metastasis, and recurrence after treatment. In solid tumors, such as lung cancer, they are a major source of immunosuppression in the tumor microenvironment, hampering and even blocking completely the immune response to fight against cancer cells. Toll like receptors (**TLRs**) ligands are well-known activators of immunostimulation, however their ability to reprogram TAMs from their M2-tumor-promoting phenotype towards an M1-antitumor mode has still not been effectively achieved in vivo.

Thus, we aimed to develop Polymeric Nanocapsules (NCs) to improve the pharmacokinetics of selected TLR ligands and their ability to reach their receptors (intracellular TLRs) inside TAMs in the tumor microenvironment. **We developed a series of NCs loaded with TLR ligands single or combined into an unique nanostructure.** Classical drugs were loaded into the NCs to reach and activate their respective receptors, TLR-3 and TLR-7/8. These TLR-loaded-NCs were characterized by their shape, size, surface charge and drug encapsulation efficiency. In vitro 2D cell cultures, using primary human monocyte derived macrophages alone or combined with cancer cells, have been used to study the toxicity of the TLR-loaded-NCs and their ability to **program macrophages into an M1-anti-tumor phenotype**. These experiments demonstrated their favorable biocompatibility profile and their ability to enhance the cytotoxic activity of NCs-treated-macrophages towards cancer cells. In an *in vivo* murine subcutaneous lung cancer model, **TLR-loaded-NCs were able to reduce tumor growth**, the most effective formulation being the NCs loaded with TRL ligands combination. Future experiments will be carried out to test these NCs in orthotopic murine lung cancer models.

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The extracellular matrix produced by tumor cells harboring TKRs substitution R1032Q modifies adhesive and migratory characteristics of endothelial cells

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OBJECTIVES

Tumor progression is profoundly influenced by interactions of cancer cells with their environment, consisting of resident and infiltrating host cells, secreted factors, and extracellular matrix proteins that ultimately determine whether the primary tumor can be eradicated, metastasize or establish dormant micrometastases. The tumor microenvironment also modulates tumor metabolism. A tumor may obtain ATP for its anabolism from extracellular microenvironmental macromolecules as well as the therapeutic response. In this highly dynamic environment, tumor-associated endothelial cells (tECs) are the interface between circulating blood cells, tumor cells, and the extracellular matrix (ECM), thereby playing a central role in controlling tumor cell behavior and metastasis formation. In a recent study, we characterized the effects of oncogenic point mutations of VEGFR2 on the growth and metastatization of SK-MEL-31 cells, a model of human melanoma. Among other effects, these mutations lead to an alteration of the ECM. Here we address the role of melanoma-derived ECM on tEC behavior as a mechanical support and metabolic regulator. Understand the interplay between tECs-ECM during the occurrence of R1032Q mutation might therefore to help to clear tumor aggressiveness and propensity to metastasize.

METHODS

Here we use these melanoma models, which differ only for the expression of VEGFR2 point mutation, to deepen the ECs-ECM interaction in vitro and in vivo using high resolution and FLIM/FRET fluorescent technologies. Endothelial cells behavior was analyzed on decellularized ECM produced by SK-MEL-31 harboring $VEGFR2^{wt}$ or mutated in terms of haptotaxis, forces of

adhesion, migration, and cytoskeleton organization. The ECM remodeling was also analyzed in vivo.

RESULTS

In vivo experiments confirmed the oncogenic activities of R1032Q substitution in VEGFR2. SK-MEL-31 VEGFR2^{R1032Q} growth faster with higher metalloproteases activities and metastatization ability compared to VEGFR2^{wt} expressing tumor. qPCR and SHG highlighted that although there is no huge variation in the amount of matrix deposited, in tumors harboring VEGFR2 mutated ECM has a higher organization with the formation of directed matrix bundles and is highly vascularized. In *in vitro* experiments, decellularized ECM of SK-MEL-31 VEGFR2^{R1032Q} supports ECs migration and a cellular monolayer's wound repair. ECs seeded on SK-MEL-31 VEGFR2^{R1032Q} decellularized ECM show disorganization of actin filaments with fewer focal adhesions that also present lower degrees of tyrosine-phosphorylated residues. Despite these results, the adhesion force to ECM, measured with the mechanical biosensor, is similar in ECs plated on ECM produced by SK-MEL-31 expressing wild-type or mutated receptor.

CONCLUSION

Our data suggest that ECs are not only recruited by ECM produced by SK-MEL-31 VEGFR2^{R1032Q} but also are able to modify their phenotype in order to assume adhesive and migratory characteristics that might promote aggressiveness and metastatization of tumors harboring the mutation R1032Q in TKRs.

Generation of ALK CAR-T Cells for Neuroblastoma therapy

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Neuroblastoma (NB) is the most deadly cancer in children with dismal survival in high-risk patients. The majority of NB express the full length anaplastic lymphoma kinase (ALK) receptor, that typically acts as driver oncogene together with MYCN. In contrast to ALK-driven lung cancer or lymphoma, targeted therapies with ALK tyrosine kinase inhibitors (TKIs), despite encouraging, induce only partial responses in NB. Therefore, additional tools to improve NB treatment are strongly needed. To specifically target NB cells, we developed a series of ALK chimeric antigen receptor (CAR) T constructs from antibodies that recognize both human and mouse ALK. Murine ALK CAR-T cells are able to control the growth of ALK+ leukemia and NB in syngeneic tumor models without detectable toxicity. From the leading candidate, we generated fully humanized ALK CAR-T cells that showed potent in vitro killing activity against a large panel of human NB lines, with activity comparable to GD2 CAR-T cells that are currently in clinical trials for NB patients. Remarkably, ALK CAR-T treatment synergized in vivo with the ALK inhibitor lorlatinib. Mechanistically, lorlatinib not only reduced tumor growth, but enhanced ALK expression on the surface of tumor cells, thereby facilitating ALK CAR-T targeting. Combination of ALK CAR-T cells with lorlatinib resulted in enhanced killing of NB cells and cure or markedly increased survival in mouse models of human metastatic NB even with low ALK expression. These findings support the clinical development of ALK CAR-T cells for NB therapy.

Dual targeting of cancer and suppressive myeloid cells by tumor-redirected iNKT cells and antigen-carrying microparticles

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BACKGROUND

Adoptive immunotherapy with T cells engineered with tumor-specific TCRs or CARs hold promise for the treatment of hematological and solid malignancies. However, suppressive cues generated by the tumor microenvironment (TME) can dampen the efficacy of engineered T cells. Hence, reprograming the TME is considered critical to optimize the current cell therapy approaches. CD1d-restricted invariant natural killer T (iNKT) cells are active component of the TME and participate in the tumor immunosurveillace by restraining cancer-supporting myeloid populations. Retargeting iNKT cells against cancer cells, by transducing tumor-specific TCR genes, may produce enhanced effectors able to concurrently kill malignant cells and modulate detrimental myeloid cells in TME.

METHODS

Mouse iNKT cells were expanded *in vitro*, engineered with TCRs specific for MHC-restricted tumor-associate peptide antigens and assessed either *in vitro* or upon transfer *in vivo* against tumors expressing the nominal tumor associate antigens. Moreover, the adoptive iNKT cell transfer was combined with their local restimulation with the strong agonist αGalactosylCeramide (αGalCer) delivered using porous silicon microparticle-based nanotherapeutics, which sequentially overcome biological barriers and accumulate at the tumor site.

RESULTS

iNKT cells engineered with MHC-restricted TCRs specific for tumor-associate peptide antigens are indeed bi-specific for CD1d- and MHC-restricted antigens *in vitro*. Upon adoptive transfer *in vivo*, TCR-engineered iNKT cells effectively delay the progression of tumors expressing the cognate antigens and remodel the local myeloid components. These dual anti-tumor functions are further sustained by delivering *in vivo* αGalCer using porous silicon microparticles resulting in enhanced tumor control.

CONCLUSIONS

Collectively, these results support the use of tumor-retargeted iNKT cells plus local restimulation to enhance adoptive cell transfer efficacy, suggesting a rational for future therapeutic strategies in cancer patients.

A chimeric Human/Dog CSPG4 DNA vaccine reveals potential therapeutic effects for the treatment of melanoma

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OBJECTIVES

Among the most interesting targets for immunotherapeutic approaches, the Chondroitin Sulfate Proteoglycan (CSPG)4 stands out, with low expression in healthy tissues, high expression in several solid tumors and with a key role in cancer progression [1]. Thanks to the translational power of spontaneous-tumor bearing dogs as pre-clinical models for human malignancies and the CSPG4 over-expression by both human and canine malignant melanoma (MM), we demonstrated the safety and the clinical effectiveness of a xeno-geneic human (Hu)-CSPG4 DNA vaccine in prolonging the overall survival of client-owned canine patients with stage II-III surgically resected CSPG4+ MM [2]. However, Hu-CSPG4 vaccine was barely effective in activating canine and human T cells in vitro. Based on these results, we aimed to increase the effectiveness and the translational power of our approach and to extend it for the treatment of CSPG4+ tumors other than MM.

METHODS

We generated a hybrid plasmid, derived in part from the Hu- and in part from the dog (Do)-CSPG4 sequences (HuDo-CSPG4). We tested the safety, immuno-genicity and anti-tumor potential of HuDo-CSPG4 DNA vaccine in mice, in dogs with stage II-IV surgically resected CSPG4+ MM and in a human setting in vitro.

RESULTS

Chimeric HuDo-CSPG4 vaccination is immunogenic and endowed with an anti-tumor potential in mice. In canine patients, the procedure is safe and clinically effective. Indeed, HuDo-CSPG4 significantly increased the overall survival of adjuvantly vaccinated canine MM patients as compared to controls treated with conventional therapies alone. These clinical results were related to the induction of antibodies against both the Hu- and Do-CSPG4, with a higher affinity as compared to those induced by the Hu-CSPG4 DNA vaccine. Moreover, a cytotoxic response against canine MM CSPG4+ cells was detected. Preliminary results obtained in vitro with T cells from human subjects suggested HuDo-CSPG4 could be more immunogenic than Hu-CSPG4 in a human setting.

CONCLUSIONS

These results provide the rationale to propose HuDo-CSPG4 vaccination for the treatment of canine CSPG4+ tumors. Thanks to the power of naturally occurring cancers in dogs as valuable predictive models for cancer immunotherapy response, these data represent a solid basis to stimulate the translation of this approach in a human clinical setting.

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AXL-based chimeric aptamers for targeted cancer therapy

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OBJECTIVES

miRNAs are small non-coding RNAs acting as negative regulators of gene expression and playing a central role in tumor progression. We previously demonstrated that miR-214 is upregulated in malignant melanomas and triple negative breast tumors and promotes metastatic dissemination by affecting a complex pathway including transcription factors and adhesion molecules as well as the anti-metastatic miR-148b [1, 2]. We recently showed that inhibition of the pro-metastatic miR-214 or increased expression of the anti-metastatic miR-148b could strongly reduce melanoma and breast cancer metastasis formation, thus suggesting the possibility to exploit the miR-214/miR-148b axis for miRNA-based therapeutic approaches [3]. To explore the therapeutic potential of miR-148b and miR-214, we generated conjugated molecules aimed to target miR-148b or miR-214 expression, selectively in tumor cells.

METHODS

To modulate miR-148b and miR-214 in cancer cells, we linked miR-148b or miR-214sponge to GL21.T, an aptamer able to specifically bind to AXL, an oncogenic tyrosine kinase receptor highly expressed on several cancer cells [4] and obtained axl-miR-148b (axl-148b) or axl-miR-214sponge chimeric aptamers. Then, we used these conjugates to evaluate metastatic traits *in vitro* and *in vivo* by treating breast cancer and melanoma cells in cultures or mice bearing xenotransplants.

RESULTS

The efficacy of the chimeric aptamers was appreciated when migration of tumor cells through a porous membrane or an endothelial cell monolayer or invasion of a matrigel layer resulted impaired compared to controls. Moreover, the expression of ALCAM and ITGA5, two known miR-148b direct targets, and of ITGA3 and TFAP2G, both shown to be miR-214 targets, was modulated. More importantly, the chimeric aptamers acted on mammosphere formation *in vitro*, promoted apoptosis and necrosis in primary tumors and blocked tumor cell dissemination and metastatization in mice when injected intratumorally. Relevantly, the GL21.T aptamer *per se* also participated to cell dissemination inhibition. Moreover, with the goal to make a systemic delivery possible, we increased the molecular weight of our conjugate by adding Polyethylene Glycol (PEG) to reduce its renal clearance, and obtained promising results.

CONCLUSIONS

From our data, we can conclude that axl-miR-148b and axl-miR-214sponge can be specifically delivered to AXL-expressing cancer cells to block tumor cell spreading. We believe that axl-based chimeric aptamers are promising tools against tumor progression that could be soon transferred to the clinics.

ACKONOWLEDGMENTS

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A new chemo-immune-sensitizing strategy based on self-assembling nanoparticles encapsulating zoledronic acid

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OBJECTIVES

Zoledronic acid is a clinically used aminobisphosphonate indicated for treatment of patients with bone metastases from solid tumors. Moreover, by up-regulating the efflux of the phosphoantigen isopentenyl pyrophosphate (IPP) via ABCA1, it is a strong activator of the antitumor Vγ9δ2 T-lymphocytes [1]. The aim of this work is to validate the use of self-assembling lipid nanoparticles (NanoZol, NZ) as an immune-therapeutic treatment against osteosarcoma refractory to standard chemotherapy, in order to propose new chemo-immune-therapy adjuvant protocols.

METHODS

In ex-vivo experiments, we compared doxorubicin-sensitive human U-2OS and Saos-2 osteosarcoma cells, their doxorubicin resistant (DX) sublines and the 3D cultures derived from parental doxorubicin-sensitive cells. Vγ9δ2 T-lymphocytes were obtained from healthy donors. We examined the effects of NZ on the molecular circuitries up-regulating ABCA1 and on the Vγ9δ2 T-lymphocyte-mediated killing. The immune-activating and the anti-tumor effects of NZ were validated in NSG mice engrafted with human hematopoietic CD34+ cells (Hu-CD34+ mice) bearing doxorubicin-resistant osteosarcoma.

RESULTS

Differently from 2D sensitive cells, 2D DX sublines and 3D cultures have low levels of ABCA1, a feature that makes them resistant to the Vγ9δ2 T-lymphocyte-mediated killing. By targeting the farnesyl pyrophosphate synthase step in the isoprenoid synthetic pathway, NZ inhibits Ras/Akt/mTOR axis and re-activates LXRα. In consequence of the LXRα-driven up-regulation of ABCA1, NZ restores the immune-killing by Vγ9δ2 T-lymphocytes. At the same time, NZ inhibits Ras/ERK1-2/HIF-1α, down-regulates ABCB1 (i.e. the main transporter effluxing of doxorubicin) and restores chemosensitivity. This phenotype is recapitulated in Hu-CD34+ bearing chemo-immuno-resistant osteosarcomas derived from 3D cultures, where NZ reduces tumor growth, increases the ABCA1/ABCB1 ratio, the intratumor Vγ9δ2 T-lymphocytes and the sensitivity to doxorubicin.

CONCLUSIONS

We propose nanoformulations of aminobisphosphonates as multi-target chemo-immune-sensitizing tools against high-grade osteosarcomas, thanks to their high intratumor delivery, enhanced Vγ9δ2 T-lymphocyte tumor cell killing and simultaneous rescue of chemosensitivity. killing and simultaneous rescue of chemosensitivity.

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Novel approaches to target oncogenic KRAS complex assembly at the cell membrane

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OBJECTIVES

Mutations in *KRAS* are among the most frequent alterations in human cancers and the prevalent driver event in lung adenocarcinoma (LUAD). There are still no effective targeted therapies for KRAS-driven LUAD, although specific KRAS G12C inhibitors are showing very promising results in clinical trials. We aim at finding innovative therapeutic strategies against KRAS based on molecular dynamics simulations of KRAS complex formation at the cell membrane.

METHODS

By unbiased molecular dynamics (MD) simulations, we built an atomistic structural model of KRAS nanoclusters starting by a GTP-mediated asymmetric KRAS dimer which results in an open-ended KRAS helical assembly. In parallel, we generated inducible cellular systems of both KRAS LOH (loss of heterozygosity) and KRAS clustering at the cell membrane to force either wild-type/mutant or mutant/mutant KRAS dimerization *in vitro* and *in vivo*.

RESULTS

The KRAS complex model obtained by molecular dynamics simulations is anchored to the plasma membrane, with the RAS-binding domains (RBDs) and cysteine-rich domains (CRDs) of CRAF along with Galectin-3 (Gal-3) interacting directly with the KRAS helical assembly. This complex produces a millimolar local concentration of CRAF at the plasma membrane, sufficient to ensure dimerization-dependent activation of CRAF.

In the cellular systems, we observed that loss of wild-type KRAS enhances tumor fitness in *KRAS* mutant cancer cells while concomitantly increasing sensitivity to MEK inhibition. We also showed that forced dimerization between wild-type/mutant KRAS resulted in impaired cell growth as compared to forced mutant/mutant KRAS dimerization and that dimerization/oligomerization between KRAS proteins is a key regulator for lung adenocarcinoma biology and a determinant of treatment response. Given the challenges of reanalyzing prior clinical trials, future clinical studies of targeted inhibitors should evaluate and/or stratify patients based on the relative expression of wild-type and mutant KRAS alleles to determine their correlation with treatment outcome.

CONCLUSIONS

Loss of wild-type KRAS enhances tumor fitness in *KRAS* mutant cancer cells while concomitantly increasing sensitivity to MEK inhibition. Dimerization of wild-type KRAS with mutant KRAS results in growth inhibition and changes the therapeutic index for MEK inhibitors. Mutant-mutant KRAS dimerization is critical for the full oncogenic properties of mutant KRAS. Collectively these observations suggest that strategies designed to interfere with KRAS dimerization should be evaluated as a therapeutic approach in *KRAS* mutant cancers.

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Maternal immunization against ALK: A new weapon against Neuroblastoma

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OBJECTIVES

Neuroblastoma (NB) is the most common extracranial solid tumor in infancy. Due to the nature of NB, occurring in the early postnatal age or even during fetal life and given our previous findings attesting the efficacy of maternal immunization against a breast cancer-associated antigen in delaying cancer development in genetically predestined offspring [1, 2], a pre-birth immunotherapy approach against one of the major oncogene involved in familial NB development, the anaplastic lymphoma kinase (ALK), has been evaluated. To this aim, I am exploiting a NB mouse model (ALK/MYCN) which spontaneously develops early-onset multifocal lesions due to MYCN amplification coupled with the expression of F1174L-mutated ALK in neural crest-derived cells.

METHODS

MYCN transgenic females underwent immunization against ALK by DNA electrovaccination by using a plasmid coding for the extracellular and transmembrane domains of human ALK (ALK-ECTM) followed by electroporation, and then they were mated with an ALK^{F1174L} transgenic male. In ALK^{F1174L}/MYCN offspring, the presence of abdominal, cervical and paraspinal tumors has been evaluated and quantified by Magnetic Resonance Imaging. The humoral immune response induced against ALK in the mothers and their offspring, as well as the presence of immune complexes containing ALK, have been evaluated by ELISA. ALK expression in tumor tissue was assessed by Western blot.

RESULTS

Pre-birth immunization against ALK leads to an extended survival time and to a lower tumor growth kinetic in ALK^{F1174L}/MYCN offspring born from ALK-ECTM-vaccinated mothers (ALK-ECTM offspring) as compared to controls born from control empty vector vaccinated mothers. Similar results have been obtained with a NB transplantable tumor model. As expected, maternally derived anti-ALK antibodies were successfully transferred from mothers to newborns; moreover, anti-ALK IgM were found in the sera of five- and six-week old ALK-ECTM offspring, suggesting the induction of the pups' own immune response against ALK. This effect could be due to the breast milk-mediated transfer of immune-complexes containing ALK, found in the milk of vaccinated mothers and in their offspring sera. Finally, MI against ALK induces a decrease in ALK expression in ALK-ECTM offspring tumor tissue.

CONCLUSIONS

Overall, these results indicate that maternal immunization against ALK is able to induce an active immunization against this oncoantigen in the offspring, impairing tumor development and enhancing survival time in a preclinical model of high-risk NB.

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Water soluble NIR absorbing chromophores based on perylene and Si-Rhodamine scaffolds for fluorescence guided surgery

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OBJECTIVES

Low molecular weight organic dyes emitting in the near infrared (NIR) (650-1000 nm) represent optimal contrast agents in fluorescence image guided surgery of tumors as the low energy photons are not absorbed neither scattered by endogenous chromophores [1]. To date, the main limitations that the existing chromophore possess, are low water solubility, photobleaching and tendency to aggregation due to their extended conjugated systems [2]. We are working on the modification of the perylene monoimide scaffold to develop water soluble, highly photostable NIR "always ON probes" for *in vivo* NIR Angiography and sentinel lymph node mapping. Additionally, we are investigating pH activatable ON-OFF Si-Rhodamine probes that can be conjugated with antibodies as targeting moieties toward overexpressed receptors in cancer cells to detect diseased tissues and their boundaries with high signal to noise ratio.

METHODS

Modern methods of characterization of organic compounds and *in vivo* angiography.

RESULTS

We designed APMI dyes by installing alkyl amine substituents on the peri positions of the perylene monoimide core to improve their water solubility and extended the conjugated system through the imide group achieving,

for the first time, red shifted emission maximum of 780 nm in aqueous solution. Furthermore, we could improve their optical properties by protecting the donor nitrogen atoms by introducing aminal and urea bonds, which ultimately led to higher extinction coefficients, 15 k and 21k respectively. We observed an increased Stoke shift up to 131 nm that makes them ideal candidates for applications *in vivo*. We investigated the dye biodistribution profile in rats by using angiography of the main abdominal organs (small bowel, gastric, spleen, ureteral and bladder vessels) following the hepatic excretion, a slow process that allow the dyes to easily stain the liver and the bile. We are currently working on mapping the lymphatic system in cancer and control tissues in pigs. Additionally, we designed pH activatable dyes based on Si-Rhodamine scaffold through the installation of a pH responsive group on the 9th position of the xanthene core. The chromophore showed ON-OFF activation of the fluorescence in the physiological pH range of 7.4.

CONCLUSIONS

We are developing water soluble perylene dyes as photostable contrast agents for *in vivo* angiography and lymph node mapping in animal models and pH responsive Si-Rhodamine dyes for site-specific label of targeting peptides or antibodies against overexpressed receptors in cancer tissues.

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In vitro model of ALK+ Anaplastic Large cells lymphoma and vascular interactions and prediction of drug efficacy in a 3D microfluidic chip

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OBJECTIVES

Microfluidic technology has the potential to revolutionize cancer diagnosis and therapy. Indeed, several *ex vivo* models are being developed specifically to evaluate the preclinical efficacy of novel and personalized cancer therapeutics.

Those complex *in vitro* models are needed to overcome the limitations of 2D culture to uncover the continuous interactions and chemokine signaling that exist between tumor cells and nontumor cell elements of the tumor microenvironment [1,2]. Among others, patients with Anaplastic Large cell lymphoma (ALCL) typically respond to Anaplastic Lymphoma Kinase (ALK) inhibitor therapies (e.g. crizotinib)[3,4]; however, resistance appears once the treatment is concluded. Histologically, ALCL tumors are identified in and around blood and lymphatic vessel in the lymph node and further evidences have shown that CCL19/21-CCR7 chemokine-receptor signaling axis regulating T cell trafficking might be involved in the resistance of ALCL cells. To prove this mechanism, we developed a microphysiological model of ALCL interacting with a 3D vasculature using a microfluidic chip.

METHODS

A commercial microfluidic chip with a central gel channel, flanked by two fluidic media channels was used to develop the model. A cell line of ALCL

(COST), GFP-transfected, with CCR7 receptor knockout (KO), and human umbilical vein endothelial cells (HUVECs) were cultured in RPMI and EGM-2MV. A collagen hydrogel (2.5 mg/ml) was injected into the central gel region of the 3D chamber and incubated (30 mins at 37 °C) in sterile humidity chambers while channels were hydrated with RPMI. After coating with 150 µg/ml collagen, 50 µL of 3x106 cells/ml HUVECs suspension were injected twice on the fluidic channel to create a 3D macrovessel. To allow the cells to attach to the media-gel interface and to upper part of the 3D channel, the chip was rotated twice and placed in incubator to form a confluent 3D macrovessel in 1 day. Then, 2x105 cells/ml ALCL were added inside of the 3D vascular channel. Medium was refreshed daily, supplemented with +/- 300 nM crizotinib. Image capture and analysis were performed using a confocal microscope.

RESULTS

The 3D vascular model consisted of a well-formed and perfusable macrovessel (Fig.1a,b) with ALCL flowing inside and interacting with the endothelial cells. Cell viability was evaluated with a luminescent readout reagent. COST macrovessel co-culture showed increased viability of ALCL cells after 3 days of crizotinib treatment compared to COST cultivated in the absence of macrovessels. In addition, CCR7 KO cells showed decreased viability compared to wild type (WT) control cells (Fig.1c).

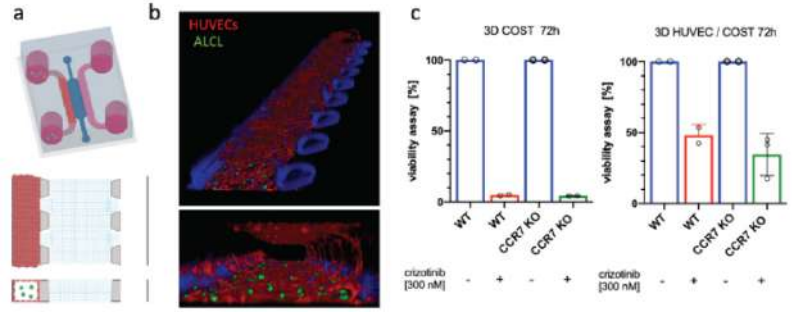

FIGURE 1: (**a**) Schematics of the microfluididc chip and LCLvascular model. (**b**) Confocal images of the macrovessels with ALCL cells. (**c**) ALCL viability assay from the model.

CONCLUSIONS

These preliminary results suggest that the 3D ALCL-vascular model might contribute to unveil the mechanisms of interaction and resistance in a complex microenvironment with a more reliable prediction of drug efficacy in humans. However, further studies are necessary to confirm that CCL19/21-CCR7 signaling driven by ALCL-vascular interactions might contribute to ALCL resistance to ALK inhibitor. This robust and physiologically relevant LCL vascular model offers an innovative platform for drug testing and development of novel nanocarriers to predict therapeutic efficacy in pre-clinical applications and to further recapitulate patient-specific cell to cell interactions.

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Squaraine dyes as fluorescent turn-on probes for the detection of mucin

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OBJECTIVES

Mucins are a family of long polymeric glycoconjugates and represent the principal components of mucus which cover all the mucosal surfaces of the human body. In the last years great attention was addressed to expression of mucins in various cancer diseases such as pancreatic adenocarcinomas, colon, rectal, breast and ovarian cancer, as well gastric carcinoma. Mucins have been identified as an important biomarker of adverse prognosis and attractive therapeutic target [1-2]. To date, several techniques have been developed to detect mucins; in particular, fluorometric assays mediated by fluorescent probes gained increasing attention thanks to their convenience, simplicity, non-invasive monitoring capability and usability in biological samples. Among the most promising fluorescent probes, squaraines are characterized by sharp and intense absorption and emission in the visible up to the NIR region, but in aqueous environments, they tend to form aggregates that lead to fluorescence quenching. Despite this drawback, squaraine dyes are proved to turn-on their fluorescence in response to a biological target, finding promising application for living processes, medical diagnosis and biological imaging at the molecular, cellular and organism level [3-4]. The aim of this work is to understand if different squaraine dyes can be used to detect mucin, but also to identify a structure-activity relationship in order to design more effective and selective fluorescent dyes to be use as fluorescent turn-on probes.

METHODS

The interaction in aqueous media between Porcine Gastric Mucin (PGM) and several squaraines with different substitutions have been investigated by using UV–Vis, circular dichroism and fluorescence spectroscopies. Binding parameters as association (KA) and dissociation (KD) constants of each squaraine-protein

complex were calculated, and the fluorescence quantum yield of the complexes was measured.

RESULTS

Squaraine dyes showed a structure-relationship influence upon the kinetic interaction with mucin. In addition, squaraine-mucin complexes displayed interesting emission characteristics since a fluorescence "turn-on" behavior was observed upon increasing additions of mucin in aqueous medium with a good increase of fluorescence quantum yield.

CONCLUSIONS

The squaraine molecular structure play an important role in the kinetics' of reaction with mucin particularly, the bulkier the squaraine the slower the interaction. Moreover, the hydrophobic interactions seem to play an important role, and based on the fluorescence turn-on observed in presence of other proteins, we hypothesize that the turn-on mechanism can be related to proteins' surface hydrophobicity. These results make the evaluated squaraines as potential biosensors for different biological applications.

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Development of multifunctional iron-doped ZnO nanoparticles addressing pancreatic cancer cells

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OBJECTIVES

Pancreatic ductal adenocarcinoma (PDAC) is currently one of the most devastating diseases for which current therapeutics are of limited benefit. PDAC has an incidence of 5 per 100'000 women worldwide in developed country and an enormous mortality rate of about 90 % [1]. The overall patient survival is improved by "only" 4 months in the last decade. Therefore, novel therapeutic and early diagnostic techniques are required to address this tumor.

Zinc oxide nanoparticles (ZnO NPs) are gaining attention in nanomedicine, due to their high versatility and properties, which can be easily tailored by means of various strategies. One of the most promising ones is doping: introducing ions of different elements may induce new functionalities to ZnO NPs [2] which can be exploited to design a theranostic nanoparticle. Here iron-doped ZnO NPs (Fe:ZnO NPs) functionalized with oleic acid and amino groups are developed to achieve a multipurpose theranostic nanomaterial: on the one hand, Fe doping elicits magnetic responsiveness with potential uses as contrast agent in magnetic resonance imaging (MRI); on the other hand the amino-functionalized ZnO NP is able to develop reactive oxygen species (ROS) generation under ultrasound (US) activation, aiming to kill pancreatic cancer cells.

METHODS

Fe:ZnO NPs were synthesized with different iron concentrations (0, 6, 12 at.%) by a wet chemical method with an oleic acid shielding, while the

amino group functionalization was performed with a post-synthetic grafting approach [3] and exploited for dye labelling. The resulting nanoparticles were characterized from the crystallographic and morphological standpoints by X-Ray diffractometry (XRD) and scanning electron microscopy respectively. Energy Dispersive X-ray Spectrometry (EDS), Fourier transform infrared spectroscopy (FTIR) and X-ray photoemission spectroscopy were used to establish the composition of the resulting particle. Furthermore, their magnetic response was evaluated through a DC magnetometer [3], while the ROS generation under US stimulation was established through electron paramagnetic resonance spectroscopy (EPR) coupled with the spin-trapping technique [4].

To assess the biological behavior of the Fe:ZnO NPs, viability and nanoparticles uptake tests were carried out on pancreatic cancers cells (e.g. BxPC-3) by means of the WST-1 assay and cytofluorimetry analysis.

RESULTS

Fe:ZnO NPs developed in this work revealed to be spherical 8 nm ZnO particles presenting a wurtzitic crystalline structure (Figure 1a) and an actual doping level of 0, 4.8 and 7.8 at. %. EDS and FTIR analyses confirmed the functionalization with oleic acid and amino groups. Iron doping was proven to be effective, with concentration dependence, in increasing the magnitude of the paramagnetic signals obtained in DC magnetization measurements, as well as the reactive oxygen species generation under US stimulation (Figure 1b and c).In addition, Fe:ZnO NPs showed to be safe up to 20 μg/mL concentration and no significant cell viability reduction was found at this concentration, considering the different levels of doping. Nanoparticles uptake experiments were used to assess the number of NPs that could contribute to intracellular reactive oxygen species generation upon US stimulation, establishing that a high percentage of cells have internalized the NPs with a fast internalization rate.

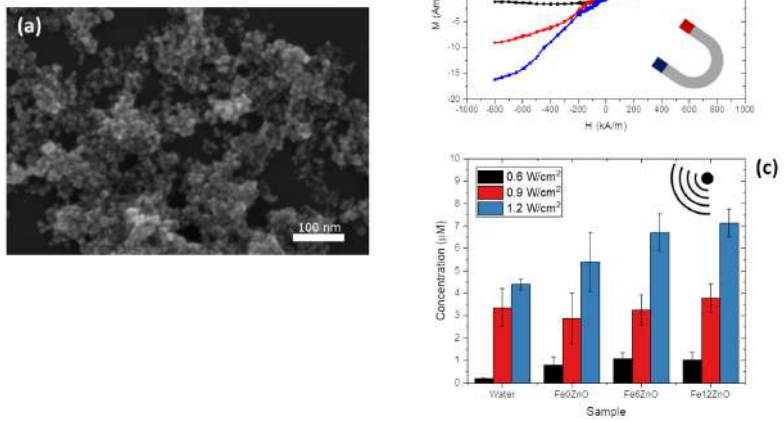

FIGURE 1: (**a**) FESEM, (**b**) magnetic properties and (**c**) ROS generation under US stimulation characterization of iron-doped ZnO nanoparticles.

CONCLUSIONS

Iron-doped ZnO NPs were successfully synthetized and characterized. The physical and chemical characterizations reveal the potential use of these nanoparticles as a powerful theranostic system for cancer cells, where the imaging is accomplished by MRI and the therapy is performed through NP-assisted US stimulation. Viability tests suggest the safety of the developed device up to 20 μg/mL, allowing to go further with biological tests in which cells are physically stimulated with NP-assisted US.

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Teneurin 4 as novel triple negative breast cancer-associated antigen and potential biomarker

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OBJECTIVES

Triple negative breast cancer (TNBC) is one of the most aggressive type of human breast cancer. Being negative for the expression of estrogen and progesterone receptors and Her2, chemotherapy and radiotherapy remain the two main options for treatment. However, these therapies display a reduced efficacy in the elimination of the small population of cancer stem cells (CSC) able to give rise to recurrence and metastases. For this reason, the objective of this work is to identify novel TNBC stem cells targets suitable for immunotherapy.

METHODS

To enrich the CSC population, we established tumorsphere cultures from mouse 4T1 and human MDA-MB231 mammary cancer cell lines. RNA-Seq was used to identify differences in gene expression between tumorspheres and their monolayer counterpart. TNBC cells were silenced through RNAi and 4T1 knock-out (KO) cells were obtained exploiting the CRISPR/Cas9 technology. To evaluate the role of TENM4 in self-renewal and in migratory ability, silenced and KO cells were tested in vitro with tumorsphere-forming ability assay and transwell invasion assay. To evaluate TENM4 as TNBC biomarker TENM4 expression in exosomes from supernatant of 4T1 and MDA-MB231 cells were tested with western blot. Finally, TENM4 presence in the sera and in the exosomes of 4T1 and MDA-MB231 tumor bearing mice and breast cancer patients was tested by using ELISA assay.

RESULTS

TENM4 was identified as one of the transmembrane proteins overexpressed in mouse and human TNBC stem cells-enriched tumorspheres as compared to epithelial ones. TENM4 silencing significantly impairs the tumorsphere-forming potential of 4T1 and MDA-MB231 cells and their ability to migrate, suggesting a possible role of TENM4 in cancer stem-like features, as well as in invasion capacity. A lower tumorsphere-forming ability was also observed in 4T1 TENM4 KO cells. Moreover, while injection of 4T1 KO or wild-type cells in BALB/C mice didn't affect tumor growth, a lower number of lung metastasis was observed in mice injected with 4T1 KO cells, further supporting an involvement of TENM4 in cell invasion. Interestingly, publicly available datasets revealed a trend of correlation between TENM4 higher expression in TNBC and shorter patients' relapse-free survival. Furthermore, preliminary data demonstrate the presence of TENM4 in the sera of 4T1 and MDA-MB-231 tumor bearing mice, in the plasma of some breast cancer patients and in exosomes of 4T1 and MDA-MB231 cells supernatants.

CONCLUSIONS

Overall, our results demonstrate that TENM4 expression is up-regulated in TNBC stem cells. Moreover, the role of TENM4 in tumor-initiating potential and cell migration points out the possibility to use TENM4 as valuable immune-target for TNBC treatment. Furthermore, the presence of TENM4 in the supernatant of TNBC cells and in the sera of breast cancer patients, suggest its potential use as biomarker.

ACKNOWLEDGMENTS

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Bypassing cancer drug resistance mechanisms by tuning ERCC1-XPF and p53 activity

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OBJECTIVES

The ERCC1-XPF 5'-3' DNA endonuclease complex is involved in the nucleotide excision repair (NER) pathway, which is one of the key mechanisms responsible for resistance development to chemotherapeutic agents [1] [2]. A strategy to improve effects of traditional crosslinking drugs and reduce development of resistance is to target both the endonuclease and p53. These proteins play a key role in the efficacy of DNA damage induced apoptosis. Indeed, a loss in function of p53 allows cells to survive despite great genomic impairment [3]. The overall objectives of the present study are: i) to evaluate, by in vitro tests, the efficacy of in silico predicted ERCC1-XPF inhibitors [1] on both p53 WT cells and mut-p53 expressing cells [4]. ii) trying to reactivate p53 function in mut-p53 cells and restore their susceptibility towards ERCC-XPF inhibitors and cisplatin.

METHODS

Using cytotoxicity studies, synergy between inhibitors and DNA damaging drugs has been assessed. MTT assays were performed and the dose-response curves were fitted with the aid of software CompuSyn, to quantitatively assess the level of synergy for specific drug combinations. Doing so, the key parameter CI (combination index) was obtained, which value indicates if synergy is present or not. PLA was performed on p53 WT cells for further investigation on the nature of the drug synergy, targeting the complex ERCC1-XPF. This allowed to observe the abundance and the location of the complex in the cell trough a fluorescence microscope. Moreover, Annexin-PI studies were

preformed to investigate whether p53 reactivators could restore synergy in mutp53 cells.

RESULTS

MTT data provided evidence for a possible synergistic effect between compounds, that correlates with affinity values from the in silico predictions. In particular, on two p53 WT cell lines, the CI value of new compound combinations seemed to reflect a synergistic effect. Moreover, the synergy itself, was evident in the p53 WT cells, but not in the mut-p53 cells. From the PLA, shown in figure, the new compounds (A4 and B9) were macroscopically observed to reduce the ERCC1-XPF interaction, even in presence of cisplatin, which upregulates this complex. Therefore, experimental results demonstrated that inhibition of the ERCC1-XPF complex is stronger upon usage of the new compounds (A4 and B9), compared to the reference compound (F06). Annexin-PI FACS experiments showed improvement of apoptosis in association with p53 reactivator PRIMA-1.

CONCLUSIONS

Results [4] evidence the efficacy of the ERCC1-XPF inhibitors in disrupting this DNA repair complex, thus preventing the development of drug resistance mechanisms and improving the effects of DNA damaging drugs. The efficacy improvement seems to be conserved in p53 WT cells only, for this reason we have further tested p53-activating compounds in combination with the inhibitors of ERCC1-XPF. Preliminary data evidence that toxicity of cisplatin and B9 is conserved in the reactivated mut-p53 cells, paving the way for possible clinical applications of our inhibitors.

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Investigating the role of CSPG4 as a target for immunotherapy in osteosarcoma

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OBJECTIVES

Osteosarcoma (OSA) is a fatal tumor mainly due to the high frequency of recurrence, metastasis and resistance to standard therapies, representing the leading cause of cancer death among children and adolescents. The chondroitin sulfate proteoglycan (CSPG)4 is overexpressed by several tumor types that fail to respond to conventional therapeutic regimens, including OSA. Therefore, it could be an ideal target for innovative therapeutic approaches such as immunotherapy, especially for those patients that do not benefit from currently available treatments.

Since OSA is a rare disease and the young age of patients diagnosed hampers the understanding of its pathogenesis, the possibility of identifying and testing new therapies is generally limited. Thus, the finding of adequate pre-clinical models for testing anti-OSA therapies is required. Canine OSA is now widely recognized as a reliable avatar of the human disease, making OSA-bearing dogs a valuable translational pre-clinical model for testing new therapies. We have previously investigated the effect of CSPG4 targeting by means of monoclonal antibodies, alone or combined with chemotherapy, in both human and canine OSA cells *in vitro* [1]. As a step forward, the importance of understanding whether CSPG4 is able to confer a survival advantage and aggressive behavior in OSA tumorigenesis, and the pre-clinical efficacy of an anti-CSPG4 immunotherapy in dogs is under investigation.

METHODS

We downregulated CSPG4 expression through siRNAs in a CSPG4+ OSA cell line and overexpressed it in a CSPG4- cell line. Functional assays were performed to evaluate the effects of CSPG4 downmodulation in terms of proliferation, migration, tumorspheres formation and chemoresistance. The activation of the ERK signaling pathway following CSPG4 overexpression was evaluated by western blot. Naturally-occurring OSA-bearing dogs are being enrolled in a clinical veterinary trial and adjuvantly treated with an anti-CSPG4 DNA vaccine. Flow cytometry analysis was performed to evaluate the vaccine-induced antibody response.

RESULTS

Our previous comparative study established the role of CSPG4 immune-targeting in both human and canine OSA *in vitro* [1]. CSPG4 down-modulation in human U2-OS cells, naturally over-expressing CSPG4, demonstrated to be significantly effective in reducing CSPG4 dependent proliferation, migration, tumorspheres generation and viability *in vitro*. CSPG4 overexpression in canine D22 cells activated the downstream Erk signaling pathway and conferred resistance to cytotoxic drug treatment. Adjuvant anti-CSPG4 vaccination in spontaneously occurring OSA-bearing dogs is demonstrating to be safe and able to induce a humoral immune response.

CONCLUSIONS

Overall, these results suggest the possible role of CSPG4 in sustaining OSA progression. Further investigations are required to provide more insights toward the understanding of the immune response triggered by vaccination and its possible effect on prolonging the survival of canine patients. In the future, these findings could be eventually translated in a human clinical setting, hopefully improving life expectancy of OSA patients that cannot benefit from current available therapies.

ACKONOWLEDGMENTS

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BRAF and VEGF targeting in melanoma stimulates tumor immunity that increases efficacy of immune checkpoint inhibitor

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OBJECTIVES

VEGFA is a key promoter of both tumor angiogenesis and tumor immunosuppressive functions and for this reason is an attractive target for combinatorial cancer therapy. VEGF pathway inhibition may endorse and facilitate the development of antitumor immunity and enhance the efficacy of anti-cancer therapies.

METHODS

Here, we used 2 different BRAFV600E melanoma syngeneic models to study in vivo the simultaneous targeting of VEGF and BRAF pathways within immunocompetent melanoma models. It has previously demonstrated that targeting VEGFA enhanced the antitumor effect of BRAFi by normalizing the tumor vasculature, remodeling the extracellular matrix and recruiting M1 macrophages in $BRAF^{V600E}$-driven melanoma and colorectal xenograft models [1].

RESULTS

We showed that the concurrent BRAF and VEGFA inhibition promotes antitumor immunity and sensitizes mouse melanoma tumors to PD-1 checkpoint blockade better than single BRAF and VEGFA inhibition. In particular, we demonstrated that contrasting the VEGF effect along with simultaneous BRAF inhibition can turn into a promotion of both innate and adaptive immunity. Immune phenotype analysis revealed that the combinatorial regimen activated the host immune system inducing the tumor infiltration of several

key effectors of antitumor immunity: macrophages with tumor suppressive features, NKs, CD4+ and cytotoxic CD8+ lymphocytes.

Interestingly, we observed that blocking PD-L1/PD-1 signaling could help to prevent the early exhaustion od CD8+ T cells and the addition of anti-PD-1 antibody to VEGFA and BRAF co-targeting improved tumor growth control. Moreover, we demonstrated that triple combination therapy (BRAFi + anti-VEGFA + anti-PD-1) promoted complete and durable regression in 50% of $BRAF^{V600E}$-driven mouse melanomas. In vivo neutralization of $CD8^{+}$ T cells and M1 macrophages revealed that these 2 immune cell populations were required for the efficacy of triple combination therapy (BRAFi + anti-VEGFA + anti-PD-1).

CONCLUSIONS

Our preclinical findings revealed that dual BRAF and VEGF blockade shifted the tumor immune environment toward an antitumor phenotype and this study demonstrated the potential of tumor-conditioning strategy that may unleash or increase the efficacy of anti-PD-1/PD-L1 antibodies for cancer immunotherapy.

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In vivo imaging of macro-environment before tumor appearance: A novel approach to identify early tumor biomarkers in pancreatic cancer

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OBJECTIVES

In the entire body the organ fates are determined by cellular and molecular interplay between tissues, micro-environment, and macro-environment. We have recently developed a mouse model engineered to express the luciferase reporter gene in cells undergoing active proliferation [1,2]. We noticed that in the hematopoietic system of these mice with pancreatic cancers invariably, light increases several weeks before tumor appearance. Based on this consideration we hypothesize that systemic proliferation events occur in the animal macro-environment at early stages, before tumor appearance. Emerging evidences highlight the temporal and spatial activation of hematopoiesis and immune response during cancer progression. We aims to characterize the cellular components of the macro-environment during early steps of transformation, identify critical biomarkers specific of early steps of cancer cell transformation and validate them for clinical application.

METHODS

Using noninvasive imaging tools to measure in longitudinal studies cell proliferation in the entire living animal of mouse model for pancreatic ductal adenocarcinoma (PDAC), the LSL-KrasG12D/+; Pdx-1 Cre (KC), we spatio-temporally identified early steps of pre-neoplastic transformation, involving waves of BM and spleen cell proliferation before tumor take place when molecular events pro- or anti-tumor growth could take place [3]. To clarify whether the proliferating cells during early steps of pre-neoplastic transformation are multipotent, pluri-potent or committed cells we will perform Cytof analysis on isolated BM and spleen cells before, during and after increase

of photon emission from MKC mice. Once the proliferating cell type has been identified, expression of chemokine and cytokine receptors will be investigated. Levels of cytokines and chemokines in sera will be analysed using mouse multiplex arrays.To translate these results in clinic we plane to analyzed the novel identified early tumor biomarkers in sera of patients with PANin, PDAC, and in healthy volunteers.

RESULTS

We crossed MITO-Luc and KC mice [3] and we used these mice (MKC) to measure proliferation in longitudinal studies by in vivo BLI. Although PDAC arise in a variable time span (22-56 weeks of life), PANins are already present in 100% of mice at 12 weeks of life [3]. In MKC animals (n=14), but not of the control mice (n=10), we noticed that photon emission increases at 9/10 weeks of life. These data indicate that systemic proliferation occurs at early stages of pancreatic tumor development. To characterize the cellular component of the macro-environment during early steps of transformation we injected EdU in MKC mice at 9 weeks of life and after 2 hours spleen cells were purified and analyzed by FACS analysis with an anti-EdU and an anti-CD3 antibody. In the spleen of MKC mice at 9 weeks of life there is an increase of EdU-incorporating cells (0,37% in MK versus 1,47% in MKC) thus demonstrating that in this model the increase in photon emission is due to cell proliferation. Interestingly, only a small fraction of proliferating cells from MKC mice are CD3+ (6,45%), versus 15,2% in proliferating cells from MK mice [4].

CONCLUSIONS

The use of the MITO-Luc mice to study how incipient tumors potentially affect the proliferation of cells at distant sites, prior to the appearance of detectable tumors is an innovative application of the imaging technology.

ACKONOWLEDGMENTS

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Toll-Like Receptor 2 targeting impairs breast cancer progression and overcomes resistance to chemotherapy

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OBJECTIVES

Toll-Like Receptor 2 targeting impairs breast cancer progression and overcomes resistance to chemotherapy Objectives Toll-Like Receptor 2 (TLR2) acts as a double-edge sword in cancer. Indeed, it is a fundamental player of innate immunity that recognizes molecules associated to pathogen invasion and tissue damages, promoting inflammation and immune responses. However, TLR2 is also expressed on tumor cells of different origin, including breast cancer, where its expression is associated with poor prognosis. Indeed, we have previously demonstrated that TLR2 is overexpressed in breast cancer stem cells (CSCs), responsible for cancer progression, therapy resistance and invasion, and promotes their self-renewal through an autocrine pathway initiated by high mobility group box 1 (HMGB1). To dissect the dual role of TLR2 in breast cancer and develop new anti-cancer therapies, here we further investigated the effects exerted by TLR2 in breast cancer progression and resistance to chemotherapy in preclinical models.

METHODS

We crossed transgenic mice expressing the rat HER2-neu oncogene, which spontaneously develop mammary tumors, with TLR2 wild type or knockout mice, to generate TLR2WT-neuT and TLR2KO-neuT mice, respectively. We compared tumor progression and performed FACS analysis of CSCs and immune cells in tumors and other compartments. We derived cancer cell lines from primary tumors of TLR2WT and TLR2KO mice in order to characterize in vitro the TLR2- mediated cancer cell intrinsic pro-tumor effects. Moreover, we analyzed the effect of TLR2 inhibitors in combination with chemotherapy on mouse and human breast cancer cell lines.

RESULTS

TLR2KO-neuT mice showed a delayed tumor onset and increased survival as compared to TLR2WT-neuT mice, which was accompanied by a reduction in CSCs and T regulatory cells. Transplantation experiments demonstrated that TLR2 acts mainly through cancer cell intrinsic mechanisms. The in vitro characterization of tumor-derived TLR2WT and TLR2KO cell lines demonstrated that TLR2 affects cancer cell proliferation and CSC self-renewal. Furthermore, TLR2 conferred resistance to chemotherapy in mouse and human breast cancer cell lines. This effect was mediated by chemotherapy-induced release of TLR2 ligands, such as the (HMGB1), which through TLR2 signaling promote breast CSC self-renewal and proliferation. Treatment with a TLR2 inhibitor impaired viability and induced apoptosis of breast cancer cell lines, and a synergistic effect was observed when combined with chemotherapy.

CONCLUSIONS

Taken together, our results enlightened the pro-tumorigenic role of TLR2 in breast cancer progression and chemoresistance in preclinical models of breast cancer. Moreover, we demonstrated that TLR2 silencing or inhibition impair breast tumor progression and restore sensitivity to therapy, opening new perspectives in the treatment of breast cancer.

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Zinc oxide nanocrystals shielded by extracellular vesicles as hybrid nanoconstructs against cancer cells

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OBJECTIVES

Thanks to their cellular origin and biological role of intercellular messengers, extracellular vesicles (EVs) have been recently evaluated as interesting candidates for the formulation of specific, biomimetic and stable delivery vehicles for therapeutic payloads. However, the efficient encapsulation of cargos and the maintenance of the EVs integrity and functionalities after the loading process are still major challenges [1]. In this context, we optimize the encapsulation of zinc oxide nanocrystals (ZnO NCs) in cell-derived EVs for the creation of a nanoconstruct for the treatment of cancer cells. The hybrid product will combine the intrinsic and selective cytotoxicity of ZnO nanostructures [2] with the natural delivery features of EVs.

METHODS

Chemically synthesized ZnO NCs were combined with EVs, isolated from cell culture supernatants, by different procedures designed to maximized the loading efficiency. These were based on the optimization of thermodynamic and electrostatic interactions between the two components [3] or on the application of freeze-thaw cycles as active stimulus to permeabilize the EVs membrane. The final nanoconstructs were fully characterized in terms of loading efficiency and morphology, paying particular attention also to their colloidal and biological behaviors, analyzed by nanoparticle tracking analysis (NTA) and *in vitro* tests.

RESULTS

The encapsulation of ZnO NCs in EVs was successfully achieved, obtaining hybrid nanoconstructs characterized by promising loading efficiency and well-preserved morphology, even after the application of an active loading method. Stability tests performed on the obtained nanoconstructs demonstrated also that the EVs-shielding efficiently prevents the aggregation of ZnO NCs, ensuring a great colloidal stability in physiological media. *In vitro* tests [3] demonstrated that the presence of EVs envelope guaranteed also a more efficient delivery inside cancer cells while preserving the intrinsic toxicity of ZnO NCs.

CONCLUSIONS

In this study, we show the successful encapsulation of ZnO NCs in cell-derived EVs. The obtained hybrid nanoconstructs combine the cytotoxic potential of ZnO with the biostability and capability of interaction with target cells of EVs. The biological origin of EVs would also improve the biomimetic and biocompatible features of our hybrid nanoconstruct, making it a promising tool for therapeutic applications against cancer cells.

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In vitro and in vivo magnetic drug targeting by using biomimetic magnetite nanoparticles

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BACKGROUND

Nanotechnology and nanoparticles (NPs) have become very attractive for their applications in different fields, comprising biology, medicine and oncology in particular, since cancers are still the second cause of morbidity and mortality in western countries, and thus require new therapeutic strategies. In this context, magnetite nanoparticles are even more interesting as they can be manipulated by an external magnetic field, besides being multifunctional platforms. We have recently shown that MamC-mediated biomimetic magnetic nanoparticles (BMNPs) can be considered effective multifunctional doxorubicin (DOXO) nanocarriers that allow a targeted chemotherapy driven by a gradient magnetic field (GMF) [1], a pH-sensitive release of DOXO, which is even enhanced by the exposure of the nanoparticles to an alternating magnetic field (AMF) [2]. The latter can also develop hyperthermia. Objectives We have now investigated the in vitro magnetic behavior of these BMNPs on cells, their in vivo biocompatibility, magnetic drug targeting, and ability to mediate thermoablation of tumors.

METHODS

Cytocompatibility/cytotoxicity of DOXO-free and DOXO-BMNPs in presence/absence of a gradient magnetic field (GMF) in 4T1 cell (MTT assay, ROS production, apoptotic/proliferation pathways in western blot), as well as their cellular uptake (iron quantification and Pearl's blue staining) were evaluated. The biocompatibility and in vivo distribution of DOXO-free BMNPs, as well

as the effect of DOXO-BMNPs injected in BALB/c mice bearing 4T1 induced mammary carcinomas after applying a GMP and an AMF were analyzed.

RESULTS

In vitro in the presence of GMF, DOXO-free and DOXO-BMNPs were uptaken by cells more efficiently than in its absence; in GMF+ conditions DOXO-BMNPs were more cytotoxic discharging the drug in the cell nuclei and activating caspases more efficiently, while DOXO-free BMNPs were not toxic. In vivo DOXO-free BMNPs, administered systemically, were biocompatible and did not induce morphological alterations in the major organs. Furthermore, when DOXO-BMNPs were intravenously injected twice a week for six time in BALB/c mice bearing 4T1 induced carcinomas and a GMF was applied over the tumor for 1 hour, higher amounts of BMNPs were accumulated in the tumor site and tumor growth was inhibited compared to mice injected with DOXO-BMNPs but not treated with the GMF. In another set of in vivo experiments with the same animal model, the injection of DOXO-BMNPs in situ together with the application of an AMF, resulted in a significant tumor weight decrease of ~40% compared to mice injected only with soluble DOXO (~25%), relative to untreated control animals.

CONCLUSIONS

These promising results show the suitability of the BMNPs as magnetic nanocarriers for local targeted chemotherapy, which can be combined with hyperthermia.

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IL-33 activates eosinophils to influence tumor cell phenotype and the microenvironment

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OBJECTIVES

Eosinophils (EO) are innate effectors of Th2-related pathologies that infiltrate most tumors, playing diverse roles [1]. Upon activation, these cells can exert direct cytotoxic functions against tumor cells. We recently showed that IL-33 is a potent activation stimulus for EO, inducing contact-dependent degranulation and tumor cell killing [2]. Here, we evaluated whether IL-33 activated EO could affect the phenotype of "surviving" tumor cells, by investigating several biological processes related to melanoma progression: epithelial to mesenchymal transition, cell cycle and immunovisibility/immunogenicity. Finally, we evaluated the mechanisms underlying such effects through analysis of EO extracellular vesicles (EO-EVs).

METHODS

We co-cultured murine EO exposed to IL-33 (EO33) or to IL-5 (EO5) with tumor cells (B16.F10 melanoma, MC38 colon carcinoma or TC1 lung adenocarcinoma) and investigated the expression of genes involved in tumor progression and immunovisibility in tumor cells by qRT-PCR. We assessed the capacity of conditioned medium derived from EO-tumor cell

co-cultures to attract dendritic cells (DCs) both in transwell migration assay and in microfluidic devices. We evaluated the EO33-EVs engagement in order to understand their role in several biological activities in a 0.4 µm transwell separated co-culture and following EO-EVs purification. In human and mouse model, we analysed the inhibition of tumor 3D spheroid formation due to EO33-EVs' secretome and EO33-tumor cells co-culture medium.

RESULTS

Our data demonstrated that tumor cell phenotype endures alterations following co-culture with EO33, but not EO5, in contact-dependent and independent manner. Upon co-culture with EO33, tumor cells expressed increased levels of p21, involved in cell cycle G1/S transition blockade. We also observed upregulation of epithelial marker CDH1 and a downregulation of mesenchymal marker CDH2, indicating a lower metastatic phenotype. Moreover, tumor cells co-cultured with EO33 expressed higher levels of MHC-II and CD74 and released soluble factors capable of attracting DCs, meaning an increased tumor cell immunogenicity. In human and mouse models, our experiments demonstrated that conditioned medium derived from EO33-tumor cell co-cultures inhibits tumor spheroid formation determined by tumor cell aggregation loss of capability (Figure 1).

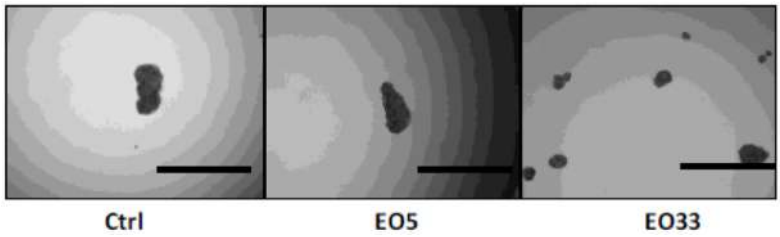

FIGURE 1: Inhibition of cell 3D spheroid formation due to EO33-tumor cells co-culture medium, in human melanoma model. Scalebar 1000 µm.

CONCLUSIONS

Our studies suggest that activation of eosinophils with IL-33 not only promotes tumor cell killing, but can also modify tumor cell features, preventing metastatic progression and increasing immunovisibility and immunogenicity. These results further extend the knowledge on the potential of these granulocytes within the tumor microenvironment, which may be exploited for novel therapeutic options in cancer patients. CtrlEO33EO5

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P-glycoprotein inhibitors as inducers of immunogenic cell death

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OBJECTIVES

Dying cancer cells produce different damage-associated molecular patterns (DAMPs) molecules, recognized by the immune cells which induce the so-called immunogenic cell death (ICD). ICD starts with the exposure of calreticulin (CRT) on the cell surface and the release signal molecules, such as ATP, allowing the interaction of cancer cells with dendritic cells (DCs), that phagocytize tumor cells and activate anti-tumor CD8+ T-lymphocytes [1]. Doxorubicin is a strong ICD inducer, but doxorubicin-resistant cells are also ICD-resistant [2]. The main mechanism is the high presence of ATP Binding Cassette transporter B1 or P-glycoprotein (ABCB1/Pgp), which effluxes doxorubicin and interacts with CRT impairing its immunesensitizing function [2]. This work investigated if Pgp inhibitors, initially designed as chemosensitizers, may restore ICD by inhibiting the immune-suppressive functions of Pgp.

METHODS

We screened three different cell lines, human doxorubicin-sensitive colon cancer HT29 cells, non-small cell lung cancer A549 cells and triple-negative breast cancer MDA-MB-231, and the counterparts with acquired resistance to doxorubicin (HT29/DX, A549/DX and MDA-MB-231 DX). Cells were treated with the first-in-class Pgp-inhibitor Tariquidar and compound R-3, a N,N-bis(alkanol)amine aryl ester derivative with the same potency, calculated as EC50, of Tariquidar. DCs were generated from monocytes, isolated from peripheral blood of healthy donors, and phagocytosis assays were performed as performed in [3]. DCs were co-cultured for 10 days with autologous T-cells, and the presence of active antitumor CD8+ lymphocytes was determined by flow cytometry. Pgp KO clones were produced by CRISPR-Cas technology.

RESULTS

Our results showed that both Tariquidar and R-3 decreased Pgp activity and increased doxorubicin retention and toxicity. Both compounds induced the exposure of CRT on the cell surface of cells when with doxorubicin, as well as the release of DAMP molecules such as ATP, thus increasing the signals triggering ICD. However, Tariquidar-treated cells were not phagocytized by DCs and were not able to activate CD8+ lymphocytes, while R-3-treated cells went through a complete ICD. While Tariquidar did not alter the presence of Pgp on the cell surface, whereas R-3 promoted Pgp internalization, ubiquitination and disruption of its interaction with CRT, restoring a proper cancer cells-DCs interaction. The need of disrupting the interaction between Pgp and CRT to restore ICD was confirmed in Pgp-KO cells that underwent a complete ICD, as R-3-treated cells.

CONCLUSIONS

We demonstrated that highly-expressed Pgp impairs the complete ICD by preventing the immune-sensitizing functions of CRT on cell surface. We suggest that Pgp inhibitors, able to destabilize and ubiquitinate Pgp, may be re-purposed as immunesensitizer agents against chemoresistant tumors.

ACKONOWLEDGMENTS

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Predictive factors gender-related of response to Pembrolizumab in non-small cell lung cancer

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OBJECTIVES

The recent introduction of immune-checkpoint inhibitors (ICPI) – mainly the anti-programmed death 1 (PD1)/PD-ligand 1 (PD-L1) Pembrolizumab – has improved the prognosis of non-small cell lung cancer (NSCLC) patients in 20-35% cases. Recent network meta-analyses reported a differential response to immunotherapy (IOT) between the two genders but it is not known if they are responsible for the gender-differential benefit of IOT. Our aims are to identify gender-related biomarkers, predictive of ICPI response in NSCLC and build a gender-tailored IOT for NSCLC patients. Clarifying specific gender-dependent and/or hormonal-dependent molecular circuitries will help to identify new druggable targets in order to improve the efficacy of IOT treatment in NSCLC patients.

METHODS

We analyzed a panel of 80 genes with NanoString technology to identify the most significantly associated with lower metastasis rate and better outcome of 42 patients treated with Pembrolizumab in monotherapy. ERα expression and 17-β-estradiol production were analyzed with qRT-PCR and ELISA. Key signalling pathways activating Erα was evaluated by immunoblot, the ERα binding to CD274/PD-L1 promoter was evaluated by ChIP. We analyzed the combined effects of Pembrolizumab and letrozole in humanized animals injected with male and female NSCLC lines, in terms of tumor growth, overall survival (OS), intra-tumor PD-L1 expression, proliferating and active CD8+ and NK cells among the tumor-infiltrating lymphocytes (TILs).

RESULTS

According to NanoString analysis in patients, ESR1 gene, encoding for ERα, is the only one significantly associated with lower metastasis rate, better

progression free survival (PFS) and OS in patients treated with Pembrolizumab in monotherapy, particularly in females. To investigate if there was a molecular link between ERα and Pembrolizumab efficacy we analyzed a panel of 30 human NSCLC cell lines of female and male origin. The amount of ERα and 17-β-estradiol, produced by endogenous aromatase, was directly related to the expression of PD-L1. ERα transcriptionally up-regulated CD274/PD-L1 gene, with higher effects in females. In cells with high EGFR activity, EGFR-downstream effectors Akt and ERK1/2 increased the amount of transcriptionally active phospho(Ser118)ERα, which in turns up-regulates PD-L1. The efficacy of Pembrolizumab in humanized mice bearing NSCLC xenografts was significantly enhanced by the aromatase inhibitor letrozole that reduced PD-L1 and increased the percentage of anti-tumor CD8+ and NK TILs. The benefit was maximal in 17-β-estradiol/ERα highfemale xenografts, minimal in 17-β-estradiol/ERα lowmale xenografts.

CONCLUSIONS

Our data demonstrate that 17-β-estradiol/ERα status predicts the response to Pembrolizumab in NSCLC, potentially explaining the gender-related differential benefit of the IOT. Aromatase inhibitors may be explored in pivotal studies as adjuvant agents in gender-tailored IOT studies for NSCLC.

Doxorubicin-decorated attenuated *Listeria monocytogenes* (Dox-Lmat): A new chemo-immunotherapeutic "Smart Pill"

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OBJECTIVES

Attenuated *Listeria monocytogenes* (Lmat) is a bacterium used as anticancer vaccine, because it shows (i) selective tropism for cancer tissues and (ii) the intrinsic capability to stimulate the immune system against primary tumors and metastases[1]. Our aim is to generate a chemo-immunotherapeutic tool, using Lmat as carrier for chemotherapeutics.

METHODS

We firstly functionalized Lmat with Alkyne groups, upon metabolic incorporation of Alkyne-bearing probes. Then, we performed a bio-compatible Copper-catalyzed-Alkyne-Azide-Cycloaddition (CuAAC) to obtain Lmat decorated with Azide-bearing Doxorubicin (Dox-Lmat).

RESULTS

We successfully decorated Lmat surface with the fluorescent drug Dox, generating a "Lmat-PILL", as confirmed by FACS analysis, fluorescent microscopy,

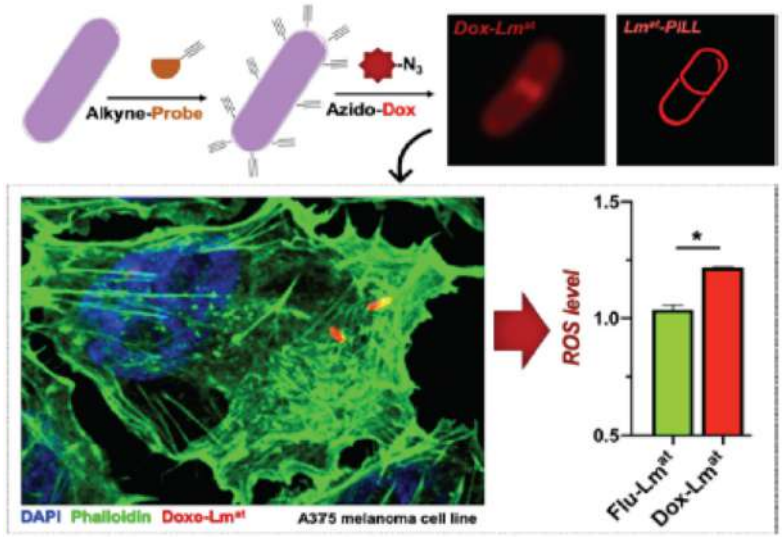

FIGURE 1: (***upper***) Lmat is fed with a probe bearing the Alkyne chemical handle needed for the second conjugation step, which consists in the attachment of the Azide-bearing doxorubicin (Dox), by means of Copper-catalyzed-Alkyne-Azide-Cycloaddition (CuAAC). (***lower***) After cell wall decoration with the drug, Dox-Lmat was investigated *in vitro* to assess the retention of its physiological features, such as the ability to infect melanoma cells (***left***). Furthermore, the increased cytotoxicity of Dox-Lmat compared to control Flu-Lmat (Lmat decorated with a green fluorophore) was verified by measuring intracellular ROS level (***right***).

and Fluorescence Lifetime Imaging (FLIM) (Figure 1). Dox-Lmat not only retains the physiological features of control Lmat, but our preliminary results show its increased cytotoxicity on infected cells, as demonstrated by measuring intracellular ROS, which are known to be triggered by both Lmat and Dox.

CONCLUSIONS

We successfully generated a Dox-decorated Lmat and our preliminary results *in vitro* indicates its increased cytotoxicity on melanoma cells, compared to control Lmat. Therefore, we propose our Lmat-PILL as a new chemoimmunotherapeutic tool.

ACKNOWLEDGMENTS

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Immobilization of LDH enzyme on mesoporous silica with FDH as a cofactor regeneration system for the evaluation of novel cancer drugs

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OBJECTIVES

Tumor cells are characterized by the uncontrolled growth and the production of high amount of lactic acid via the Cori cycle (anaerobic conditions). The production of lactic acid is catalyzed by the A form of Lactate dehydrogenase (LDH A) [1]. Thus, the immobilization of LDH has shown to serve as a simple method to study the inhibition by means of anticancer inhibitors [2]. In our previous work, we successfully immobilized the Formate dehydrogenase (FDH) on synthesized mesoporous silica, Mesoporous Cellular Foams-type (MCF). In this scenario, the similarities between the protein structure of FDH and LDH allows predicting a good performance of MCF as support for LDH enzyme.

METHODS

The glyoxyl functionalized MCF support was prepared as reported in literature [3]. The enzyme activity was evaluated by the variation of the concentration of NADH in the presence of sodium formate (for the FDH) or sodium pyruvate (for the LDH) [2]. Immobilization time, enzyme loading and the presence of protecting glycerol as protecting agent can be studied to optimized the immobilization process.

RESULTS

The immobilization of FDH on MCF support was successful achieved, where an immobilization yield (IY) of 60 % and a specific activity (SA) of 1 UI/g was obtained. By labelling the enzyme with fluoresceine isothiocyanate (FITC),

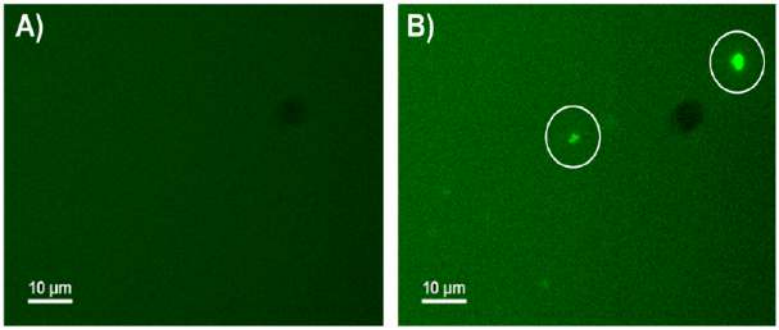

FIGURE 1: Optical fluorescence microscopy images of the FITC-labelled (**A**) MCF and (**B**) FDH/MCF.

the presence of immobilized FDH was evidenced by optical fluorescence microscopy (Figure 1B) in contrast with the control (Figure 1A). Since the immobilization protocol was well consolidated and simple, it could be modified and be used for the LDH immobilization.

CONCLUSIONS

Thanks to our previous study, we were able to immobilize FDH on mesoporous silica support (MCF). This method presents many advantages, as it is modulable to be used with different supports and enzymes and mass transfer limitations are avoided, at certain pore size. The aim is to study the immobilization of LDH on the same mesoporous silica supports and target LDH with anticancer inhibitors.

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Understanding the molecular binding mechanism of colchicine derivatives targeting βIII human tubulin isotype

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OBJECTIVES

Microtubules (MTs), made up by the self-association of α and β tubulin, have been widely studied as targets for anti-cancer treatment due to their cardinal role during mitosis. In recent years, colchicine derivatives have been extensively tested on different cell lines as promising antimitotic agents able to reduce well-known colchicine drawbacks [1]. In this study, we performed a virtual screening of recently synthesized colchicine derivatives to pinpoint the molecular mechanisms driving the binding to the human βIII tubulin isotype, which is considered an excellent drug-target for anti-tumoral therapies.

METHODS

Human 3D models of βIII tubulin isotype were generated by homology modelling and optimized by molecular dynamics (MD) simulations. Colchicine derivatives were docked to the tubulin structure and each obtained complex was further investigated by short MD simulation (1ns). The binding energy of the complex was estimated by MM-GBSA and the ligand pose was compared to colchicine. The best colchicine derivative was chosen for further long (100 ns) MD investigations.

RESULTS

Among all ligands, short MD trajectories have remarked a peculiar 4-chlorinethiocolchicine derivative, named as C19. This ligand was characterized

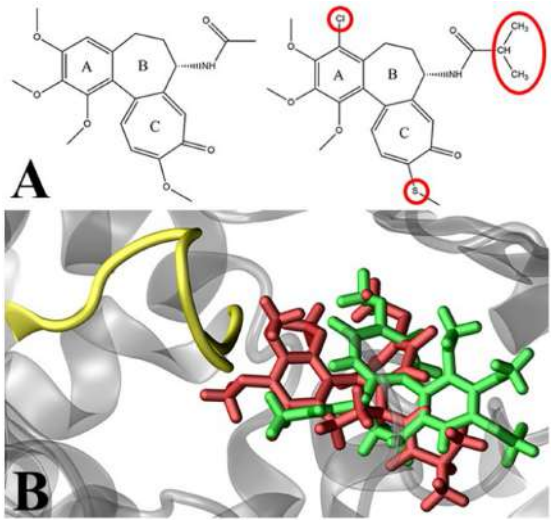

FIGURE 1: (**A**) colchicine and C19, (**B**) final MD snapshot of colchicine (green) and C19 (red) interacting with the αT5 loop (yellow).

by binding energy similar to colchicine and a unique docking pose in the tubulin-binding cleft. All other derivatives, instead, showed binding poses very similar to colchicine. To further investigate the peculiar features of C19, 100ns-long MD simulations have been performed on C19-βIII tubulin and colchicine-βIII tubulin complexes to characterize the conformational dynamics associated with the ligand binding. Interestingly, C19 was able to create stronger interactions with an unstructured region of the binding cleft, namely the αT5 loop, thanks to an increase of the ligand-receptor interacting surface (Figure 1).

CONCLUSIONS

Among all considered compounds, the C19 has demonstrated a profound different conformational behaviour in the colchicine binding site and higher affinity for the αT5 loop. It is worth mentioning that this loop has been already indicated as a key structure to bind antiproliferative compounds [2]. The uniqueness of the C19 binding mechanism is a promising feature to

rationally drive future studies aimed at refining the action of the colchicine derivatives and overcoming colchicine drawbacks. Therefore, this compound deserves further in-depth investigation to effectively prove its potency and affinity for the βIII tubulin isotype, which is mostly expressed in cancer cells and related to drug resistance.

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Collective aggregation dynamics of cancer cells

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OBJECTIVES

The ability of cancer cells (CC) to spread by invading neighboring tissue is a hallmark of cancer. Invasion can occur both by isolated cells and by aggregates that migrate collectively. This work arises from previous evidence of chemotaxis-driven aggregation (CDA) of CC: they are able to migrate toward each other in a directional manner forming larger clusters when seeded in a 3D matrix, by following a gradient of a diffusible factor [1]. Prompted by this evidence, we aim at understanding the universality of such phenomenon and at investigating the dynamics of such process.

METHODS

30 CC lines selected on the basis of their aggressiveness, were seeded in a 3D gel and observed by means of time-lapse microscopy for about three weeks. To gain insights into the dynamics of CDA, we performed time-lapse experiments at high spatiotemporal resolution in a subset of cell lines stable transduced with fluorescent reporters. Chemotaxis assays were performed to see whether cells were attracted by their own and by other cell lines conditioned media.

RESULTS

We showed that 6 CC lines derived from different tissues of origin are able to perform CDA. This behaviour is not common to all CC lines and is independent of the tissue of origin. Indeed, we identified cell lines which just grow as spheroids when seeded in 3D gel, without moving directionally toward other clusters. We showed that CDA is mediated by the emission of

actin-rich protrusions. The perturbation of actin or myosin polymerization leads to an impairment of CDA, by reducing the number of protrusions or changing the structure of the clusters respectively. Measurements of cluster coalescence rate demonstrate that aggregation time is independent of initial seeding density, suggesting that aggregation is not due to the random motion of cells. Aggregating cell lines are able to migrate toward their own conditioned media and toward conditioned media collected from other cell lines that are able to aggregate, supporting the hypothesis that CDA is mediated by autocrine loops of ligand-receptor couples. Furthermore, we showed that different cell lines can form heterotypic aggregates by means of CDA.

CONCLUSIONS

Our results point at CDA as a universal mechanism present in multiple cancer types. Results about the dynamics of the aggregation are not consistent with a random motion of cells, but point to a long-distance interaction between clusters. This could provide new insights into the mechanisms of collective aggregation of CC, which is emerging as a mechanism by which CC detach from the primary tumor and spread into the body, in opposition to the detachment of single cells from a multicellular structure.

Future elucidation of the molecular mechanisms underlying CDA might unfold the impact of such a migration strategy on cancer progression.

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HLA-independent killer lymphocytes and chemo-targeted therapy: An integrated approach against uterine leiomyosarcoma and other soft tissue sarcomas

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OBJECTIVES

Uterine leiomyosarcomas (UL) are aggressive soft tissue sarcomas (STS) expressing smooth muscle antigens. Advanced STS have severe prognosis and no curative options. We showed that chemotherapy with trabectedin (TR) and targeted-therapy with the PARP-1 inhibitor olaparib (OL) is feasible in STS patients. Nonetheless, different degrees of response from initial complete remission to partial response and finally relapses were observed. Drug resistance and relapses are likely attributed to a subpopulation of quiescent cancer cells with stem cell features (CSCs). In this work, we explored adoptive cell immunotherapy with Natural Killer (NK) or Cytokine–induced killer cells (CIK) against UL and STS cell lines surviving to TR+OL treatment. Moreover, we investigated the effect of this integrated approach on the putative CSC compartment.

METHODS

Two UL cell lines (DMR, MES-SA) and two STS cell lines (HT-1080 and S018) were treated with TR+OL at IC75 for 72 h to obtain the 25% surviving fraction. The expression of the NK/CIK activating ligands (MICA/B, ULBPs, CD112, CD155) were analyzed by flow cytometry on UL and STS cell lines. UL and

STS cells were challenged with adoptive NK/CIK immunotherapy with different scalar effector/target ratios (1:1; 1.3; 1:12 and 10:1; 3:1; 1:1, respectively) and tumor cell viability were tested after 72h with ATP assay and after 7 days with colony growth assays. The CSCs compartment was visualized by OCT-4 marker on the surviving UL and STS fractions both after trabectedin+ olaparib treatment and NK co-culture.

RESULTS

NK/CIK activating ligands were expressed on UL and STS cells at variable levels, and these molecules were maintained (in S018) or even increased after TR+OL treatment (1.7-9-fold increase in DMR, MES-SA, HT-1080, $p<0.05$). Tumor cells surviving TR+OL treatment were killed by NK/CIK equally (S018) or even more efficiently than untreated cells (0.6-10; 1.4-3.3; 1.2-2.9 fold increase of killing at 1:12; 1:3; 1:1 NK/T ratio with DMR, MES-SA, HT-1080, $p<0.05$; 1.6-1.9 fold increase of tumor killing of DMR by CIK, $p< 0.001$). The putative CSCs fraction were visualized in MES-SA, DMR, HT-1080 based on OCT-4 expression showing that TR both as single agent or in combination with OL, caused a relative increase in the proportion of this subpopulation if compared to untreated controls (1.2-2.8 fold, $p<0.05$). The subsequent co-culture with NK cells did not significantly change this proportion showing the CSCs are relative resistant to chemotherapy but sensitive to immunotherapy.

CONCLUSIONS

In conclusion, we showed that NK/CIK based adoptive immunotherapy was active against UL and STS cells surviving TR+OL therapy. Moreover, NK acted both on bulk population and on supposed CSCs compartment. Our results support the potentiation of TR+OL treatment by the subsequent HLA-independent adoptive cell immunotherapy with NK/CIK. This integrated approach warrants further investigations both in in vivo models and in clinical settings.

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Synthesis and characterization of organic and hybrid photosensitizers for photodynamic therapy

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OBJECTIVES

Over the recent years, extensive efforts have been devoted to the development of near-infrared (NIR) dyes for biological applications, especially for photodynamic therapy (PDT). Polymethine dyes must be considered as innovative photosensitizers (PS) due to the easy and low-cost synthesis along with remarkable absorption property in the far-red NIR region, perfectly matching the biological tissues' transparency window (600-900 nm) [1]. In particular, NIR polymethine cyanines (CY) and squaraines (SQ) are well suited for this purpose and have been extensively studied for these applications, thanks to their high molar absorption coefficients, remarkable brightness, fluorescence and photostability, especially in organic media [2]. However, despite their excellent photodynamic activity, their chemical instability and self-aggregation properties when in contact with biological media still limit their effective clinical application. To overcome these drawbacks, the incorporation of these dyes in nanoparticles (NPs) is extremely important to prevent the formation of dye aggregates in aqueous environment. The present contribution deals with the design and synthesis of SQ and CY polymethine dyes with different substitution groups. These dyes were then encapsulated in solid lipid nanoparticles (SLN), in order to improve their bioavailability and pharmacokinetic profile in view of possible applications in the clinical field.

METHODS

New series of SQ and CY dyes, based on indolenine and benzo-indolenine rings, were synthetized by microwave irradiation and photophysically characterized (UV-Vis and fluorescence spectroscopy) before being in vitro tested for the evaluation of their cytotoxicity and photo-toxicity. In addition, with the aim to increase solubility and stability in aqueous solutions, some of the dyes were entrapped into solid lipid nanoparticles.

RESULTS

A cyanine and a squaraine dye based on bromo benzoindolenine ring were efficiently encapsulated into SLN in order to overcome their solubility issues in aqueous solutions. Dye-loaded SLN displayed a homogeneous size of <200 nm and high entrapment efficiency, preserving dyes' excellent spectroscopic properties. In particular, CY-SLN led to a photoactivity on MCF-7 cells showing a good uptake and a partial mitochondrial localization, suggesting the potential application as PS for photodynamic anticancer treatment.

CONCLUSIONS

SLN are a valuable delivery strategy for polymethine dyes in biomedical applications, although further investigation on in vivo models is needed in order to assess the real applicability of these nanosystems in both diagnostic and therapeutic fields.

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Zinc oxide nanocrystals and ultrasound: A new strategy to fight cervical cancer

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OBJECTIVES

Cervical cancer is the fourth most common cancer in women, and is in the top three cancers affecting women younger than 45 years old. Traditional therapeutic protocols include surgery, radiation therapy and chemotherapy [1]. Nanomedicine, however, could represent a new strategy to decrease the mortality of this pathology, reducing the negative outcomes related to traditional anticancer approaches [2]. An innovative proposal in this field is the administration of nanotools remotely activated by an external physical stimulation. In this study thus the possibility to exploit the synergism between ZnO NCs (ZnO NCs) and ultrasound (US) to affect the viability of cervical cancer cells was investigated.

METHODS

The cytotoxicity and internalization of aminopropyl-functionalized ZnO NCs was evaluated on cervical adenocarcinoma KB cells. Furthermore, the presence of a synergistic effect between ZnO NCs and US was evaluated performing single and multiple US treatments per day on KB cells pre-incubated with ZnO NCs. Pilot studies on the mechanism of the observed synergism have been performed, evaluating cell proliferation, after the incubation of ZnO NCs and US treatments, whit the addition of reactive oxygen species (ROS) scavengers, and the kinetics of cell death.

RESULTS

ZnO NCs resulted to be non-toxic for KB cells and they were efficiently internalized in cancer cells. Regarding the evaluation of the synergism, a significant decrease of cell viability was recorded when cells incubated with

ZnO NCs were treated multiple times with US. The addition of two different ROS scavengers revealed ROS marginal role in the pathways involved, whereas the kinetic evaluation of cell death highlighted the progressive increase of apoptosis and secondary necrosis caused by the combination of ZnO NCs and US.

CONCLUSIONS

Herein, for the first time, the synergistic action of ZnO NCs and US to achieve cervical cancer cell death was demonstrated. Further studies are going to focus on the evaluation of the US-assisted therapy with ZnO NCs shielded into a lipid envelop, to improve their biocompatibility and let them a biomimetic property, decorated with monoclonal antibodies for targeting.

ACKNOWLEDGMENTS

This work has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 678151 – Project Acronym "TROJANANOHORSE" – ERC starting Grant). We wish to gratefully thank the ELvation Medical company for the supply free of charge of the PW2 instrument (R. Wolf).

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Investigating the cystine/glutamate antiporter xCT in the interaction between mammary cancer and the immune system

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OBJECTIVES

The transmembrane protein xCT imports extracellular cystine in exchange for intracellular glutamate and is fundamental for intracellular redox balance. Enhanced xCT expression in cancer cells provides protection against harmful reactive oxygen species (ROS) generated by altered metabolic states and by chemotherapeutic drugs. Therefore, xCT represents a relevant target for cancer treatment. xCT expression is also needed for the activation and function of different immune cell populations, and cystine and glutamate are important players in the biology of both transformed cells and immune cells. However, whether the overall outcome of xCT depletion in the tumor and in the immune system results in promotion or suppression of tumor growth is unknown. The aims of this project are to investigate how xCT depletion in tumor cells affects tumor progression in a fully immunocompetent mouse model and if xCT depletion in the immune system negatively or positively affects tumor growth.

METHODS

We generated xCT-KO mouse mammary cancer cells (4T1) and tested their malignant properties including proliferation, motility, and resistance to oxidative stress. Flow cytometry was used to characterize the immune cell populations. We generated xCTnull BALB/c mice to investigate the role of xCT in the immune system of tumor-bearing mice, and assessed both humoral and cellular immune response through ELISA and in vivo cytotoxicity assay, respectively. Finally, we generated xCTnull / ErbB2-transgenic BALB-neuT mice to study the contribution of xCT to tumor initiation and progression in a mammary cancer-prone model.

RESULTS

xCT depletion in 4T1 cells impaired clonogenic potential, sensitized cells to oxidative stress and affected VEGF and GM-CSF secretion. Furthermore, xCT-KO 4T1 cell showed reduced migration in vitro and lung metastasization in vivo. On the other side, depletion of xCT in the immune system partially altered the composition of the tumor immune infiltrate and prevented activation/polarization of immune cells cultured ex vivo. Nevertheless, lack of xCT in the immune system did not impair the proper mounting of both humoral and cellular immune response in vivo. Finally, xCT depletion in BALB-neuT mice did not impair autochthonous tumor initiation.

CONCLUSIONS

While xCT is required for both cancer cell malignancy and proper immune system functionality in vitro, it is partially dispensable in vivo. The mechanisms at the basis of this discrepancy need to be identified. Moreover, further in vivo analysis will reveal if the immune system plays a role in the reduced metastatic ability of xCT-KO cancer cells. Finally, while xCT depletion does not interfere with tumor initiation, xCT depletion could sensitize otherwise normally growing tumors to oxidative stress-inducing therapies in vivo, opening up possibilities for a better design of combinatorial approaches involving xCT targeting. Further experiments are required to confirm or reject this hypothesis.

Characterization of PIRB role in modulating pancreatic cancer immune infiltrate

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OBJECTIVES

Murine paired immunoglobulin-like receptor B (PIRB) and its human ortholog leukocyte immunoglobulin-like receptor B (LILRB) are widely expressed inhibitory receptors with multiple tissue-dependent functions. PIRB is a type I transmembrane protein expressed on the surface of macrophages, granulocytes, mast cells, dendritic cells and B lymphocytes. PIRB presents an intracellular tail with 3 Immunoreceptor Tyrosine-based Inhibitory Motives (ITIM), acting as an immune-checkpoint by contacting its principal ligand, class I major histocompatibility complex (MHC) molecules. Previous studies have underlined how PIRB loss is associated with the presence of hypersensitive B cells which display a higher production of antibodies, of highly cytotoxic T cells and of myeloid cells polarized toward an M1-like anti-tumoral phenotype [1]. The potential involvement of PIRB in the adaptive immune response prompted us to better characterize its role in modulating the anti-tumoral immune response in models of pancreatic ductal adenocarcinoma (PDA). PIRB human homologs were also demonstrated to directly support leukemia and solid cancer development [1]. Therefore, we investigated its intrinsic roles in pancreatic tumoral cells by generating a Pirb-/- PDA cell line (K-PIRB).

METHODS

Mice lacking PIRB gene (Pirb-/-) were crossed with pancreatic cancer–prone KrasG12D/Cre (KC) and KrasG12D/Trp53R172H /Cre (KPC) mice. Pancreata from Pirb-/- and wild-type (WT) genetically engineered mice (GEM) were collected at the indicated times, fixed in formalin and paraffin embedded.

Tissues were used for Hematoxylin and Eosin and Immunohistochemical stainings. K-PIRB cells were obtained from a tumor mass arose in a KPC Pirb-/- and purified from stromal cells in consecutive steps.

RESULTS

We found that PIRB ablation significantly improved survival of Pirb-/- GEM compared to WT counterpart and this correlated with a reduced percentage of transformed ducts in a late-stage disease. Preliminary histological and Immunohistochemical analyses of pancreatic tissues demonstrated an increased in tumor infiltrating CD8+ and CD4+ T lymphocytes (TIL), as well as an increased count of intra-tumoral B cells in Pirb-/- GEM mice compared to WT GEM. In parallel, Pirb-/- GEM mice showed a significant increase in blood circulating CD8+ T lymphocytes together with a significant reduction in CD11b+Gr1+ myeloid derived suppressor cells (MDSCs) population. Concerning intrinsic roles, preliminary results showed how K-PIRB, in comparison to a Pirb proficient PDA cell line (K8484), had a lower viability on a two-dimension surface and were able to significantly increase their MHC class I expression after 24, 48 and 72 hours of stimulation with IFNg.

CONCLUSIONS

Overall, our results support the need to further characterize PIRB molecules as potential candidate to develop novel immunotherapy strategies for the treatment of PDA.

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Cell penetrating peptides folding mechanism and cellular translocation

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OBJECTIVES

Cell-penetrating peptides (CPPs) are short peptides able to penetrate the plasmatic membrane at low micro-molar concentrations, without binding any chiral receptor [1]. In the past, CPPs have demonstrated a great ability to covalently or non-covalently link several cargoes. In this context, CPPs could increase the uptake of drugs in tumor cells and thereby increase the efficacy of the treatment, allowing to administer lower doses of the drug and reducing side effects [2]. A better understanding of CPPs conformational dynamics is of paramount interest and computational techniques represent the right tool to explore at atomistic level the CPPs folding in different biological environment. The outcome of this work could help the design of new chimeric CPPs with an improved cell penetration ability.

METHODS

We have performed a systematic investigation of CPPs conformational rearrangements in two environments: water and POPC lipid bilayer. Classical Molecular Dynamics (MD) and Metadynamics (MTD) enhanced sampling technique have been employed to address this issue. Several CPPs were considered: Cady, MAP, MPG, TAT, PEP, VEC, TP10, pAntp, Trasportant and EP1. The AMBER99SB-ILDN force field was used for defining system topology.

RESULTS

Considering the simulations in pure water environment, most of the CPPs showed mostly a random coil conformation with the only exception of PEP peptide, which exhibited a prevalent alpha helix configuration. MTD simulations have highlighted fruitful information about the conformation

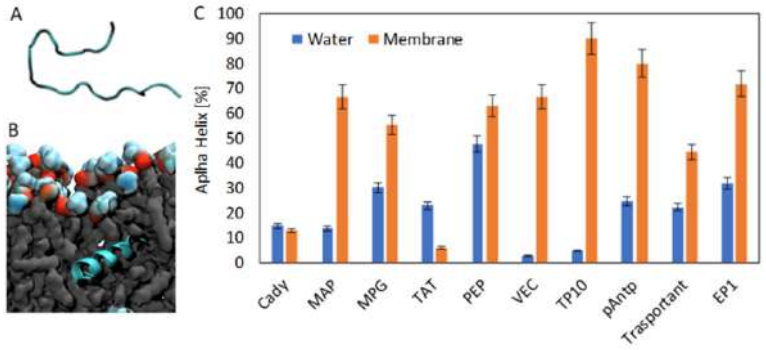

FIGURE 1: Representative conformation of the MAP CPP in (**A**) water and (**B**) membrane. (**C**) The percentage of alpha helix for the two environments are reported.

of the CPPs in presence of the lipid bilayer, showing a high level of alpha helix structures. Instead, results from MD have confirmed the tendency of the most of CPPs considered to re-arrange their structure in a random coil conformation (Figure 1). It is worth remark that the environment may result a key feature to predict the CPPs conformation, which may play a crucial role in the penetration mechanism [3].

CONCLUSIONS

This work shed light how the surrounding environment may influence the shape and folding of CPPs. Our study may be considered as a first step for designing of engineered CPPs able to acquire specific conformations as dependent on specific environmental conditions in order to improve the internalization process.

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Extracellular vesicles: From isolation to cancer therapy applications

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OBJECTIVES

The subjects of the study are extracellular vesicles (EVs) in terms of optimization of their isolation methods, morphological and functional characterization, stability and storage conditions for further engineered modification towards cancer therapy.

METHODS

A new hybrid smart device, called TrojaNanoHorse (TNH), is under development loading EVs with ZnO nanoparticles for a drug-free nanotherapeutic cancer treatment approach [1]. EVs were isolated by differential ultracentrifugation from B-lymphocyte and characterized at different storage conditions (-80, -20, 4, 25 and 37°C, for 1, 7 and 30 days) through electron microscopy (EM), nanoparticle tracking analysis (NTA), Bradford assay (BA) and flow cytometry (FC). The EVs' internalization in either healthy lymphocytes and cancer cells (i.e. Daudi and HL60 cell lines) was investigated with FC and fluorescence microscopy (FM) at 24 and 48 hours at 5, 10 and 20 μg/ml concentration.

RESULTS

EVs observed at EM display a fair percentage of exosomes with the typical cup-shaped morphology. EVs average dimension is around 110 nm with $1x10^{11} \pm 6x10^{10}$ particles/ml and 140 ± 36 μg/ml of surface proteins concentration. In absence of cryo preservation additives, we verify that ice crystal formation in subzero temperatures strongly damages EVs, thus, 4°C is the temperature

to prefer for few days of preservation, while, for more than one week, lower temperatures are recommended.

The presence of exosomes in the population of EVs is also confirmed at FC by the expression of two typical exosome surface markers, CD63 and CD81. Furthermore, CD20 surface presence is successfully quantified in lymphocytes EVs, as on the membrane of the three different cell lines. EVs internalization for the 10 μg/ml at 48h results statistically higher in Daudi (94.6±1.7%) if compared to lymphocytes (79±3%) and HL60 (67±3%).

CONCLUSIONS

EVs isolated with ultracentrifugation technique show a heterogeneous population, among which exosomes are surely present. Upon isolation, the storage temperature must be carefully chosen depending on the length of the preservation time to reduce the damages due to the ice crystals formation and to avoid the impairment of further applications. For the TNH optimization its surface functionalization with targeting ligands is scheduled with the aim of reducing its internalization by healthy cells enhancing its uptake by the cancerous ones.

ACKONOWLEDGMENTS

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Cancer-associated fibroblasts targeting and killing using FAP-selective ferritin nanodrugs

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OBJECTIVES

Cancer-associated fibroblasts (CAFs) are key actors in the context of tumor microenvironment. They are emerging as interesting stromal target in many solid tumors since they contribute to cancer progression, drug resistance and immune suppression. Our aim was to develop a nanosized delivery agent able to specifically target and kill protumorigenic CAFs, thus reshaping the tumor microenvironment and favoring cancer eradication.

METHODS

Bionanoparticles made of human H-ferritin chains were used as versatile and biocompatible protein nanocarrier for the pro-apoptotic drug navitoclax. To steer drug delivery in CAFs, ferritin nanocages were engineered by surface functionalization with antibody fragments specific for the fibroblasts activation protein (FAP), a recognized marker of protumorigenic CAFs. Targeting capability of functionalized versus bare nanoparticles was assessed by flow cytometry on primary murine CAFs and human activated myofibroblasts overexpressing FAP. The pro-apoptotic activity of the nanodrugs was assessed by confocal microscopy and viability assay in cell culture in vitro.

RESULTS

Functionalized nanocages showed enhanced binding to FAP-overexpressing CAFs than non-functionalized ferritins, while they only had limited binding to FAP-negative cancer cells. Navitoclax loading into H-ferritins exerted efficient

pro-apoptotic activity in sensitive cells; moreover, the nanoformulation improved drug stability in aqueous solutions. In vitro treatment of FAP-overexpressing cells with functionalized drug-loaded nanoparticles induced improved reduction of cell viability as compared to non-functionalized nanodrug, while no difference was observed in FAP-negative cells equally treated. Accordingly, a significantly higher uptake of navitoclax was observed with functionalized versus bare nanoparticles only in FAP-overexpressing cells.

CONCLUSIONS

Our data show that FAP targeting by drug-loaded ferritins could be a promising strategy to enhance specific drug delivery into CAFs, thus opening new therapeutic possibilities aimed at remodelling the tumor microenvironment.

Zinc oxide nanocrystals combined with ultrasound for the controlled generation of Reactive Oxygen Species

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OBJECTIVES

Reactive Oxigen Species (ROS) effects on living cells viability and proliferation are numerous. Due to their ability to react with different types of biological molecules, ROS are involved in many cell functions[1]. The ability of maintain the redox homeostasis is crucial and an imbalance can lead to a variety of possible diseases. Controlled generation of ROS can be exploited to generate oxidative stress in cells, leading to cell death, with the aim of developing drug and drug-free therapeutic tools for anticancer treatments. Aminopropyl-functionalized ZnO NCs (ZnO-NH_2 NCs) are proved able to produce ROS in a tunable and reproducible manner, when stimulated by ultrasound (US), using an already approved medical device, LipoZero G39. The generation of hydroxyl radicals is the result of inertial cavitation under the US exposure.

METHODS

Dynamic Light Scattering technique, Field Emission Scanning Electron Microscopy, Transmission Electron Microscopy, X-Ray Diffraction were used to characterize the ZnO-NH_2 NCs previously synthetized via microwave-assisted sol-gel synthesis. A large variety of parameters were evaluated: US frequency and power as well as ZnO-NH_2 NCs concentration. The generation of ROS was evaluated by Passive Cavitation Detection, Electron Paramagnetic Resonance and Ultrasound B-mode imaging.

RESULTS

When ZnO-NH_2 NCs were present in the aqueous solution a significative enhancement of ROS production, and an improved cavitation signal was measured compared with the water alone. The same behavior was verified in PBS, cell culture media and in presence of tissue mimicking materials[2], as ex vivo tissue and phantom.

CONCLUSIONS

It is verified that ZnO-NH_2 NCs are ultrasound responsive nano-agent due to their ability to induce inertial cavitation under pulsed US exposure, which consequently leads to a large ROS production. Controlled ROS production was also assessed in biological media as PBS and cell culture media. This result suggests the applicability of this technology in the biological field, as a possible tool to induce cancer cells death, predicting high potential healthcare applications.

ACKNOWLEDGMENTS

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The perivascular niche protects ALK+ lymphoma cells from ALK inhibition through the CCL19/21-CCR7 axis

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OBJECTIVES

The ALK inhibitor crizotinib showed promising therapeutic efficacy for relapsed/refractory Anaplastic Large Cell Lymphoma (R/R ALCL). However, in patients that achieve complete remission, crizotinib discontinuation causes rapid disease relapses due to the expansion of persister lymphoma cells that are never completely eradicated by the ALK inhibitor[1]. Growing evidence shows that ALK+ ALCL can persist for years in patients being undetectable. ALCL grows around blood and lymphatic vessels in the lymph node[2]. We hypothesize that this perivascular niche provides pro-survival signals contributing to ALK+ ALCL persistence and TKI resistance.

METHODS

We performed RNA-seq analysis on ALK+ ALCL cells and scRNA-seq analysis in one ALK+ ALCL primary sample to identify differentially expressed chemokines and chemokine-receptors and found the CCL19/21-CCR7 axis. To explore the involvement of CCL19/21 and CCR7 we knocked-out CCR7 in ALK+ALCL

cells using the CRISPR/Cas9 system. We developed a 3D microchip model of the perivascular niche where lymphoma cells interact with a blood vessel (Huvec cells) and treated CCR7^{wt} and CCR7^{ko} lymphoma cells with crizotinib. Cell viability was evaluated by CellTiter-Glo Luminescent Cell Viability Assay.

RESULTS

By RNA-seq analysis on ALK+ ALCL cells and scRNA-seq analysis in one ALK+ ALCL primary sample, we found that ALK+ cells expressed the chemokine receptor CCR7, while endothelial cells and fibroblasts expressed CCR7 ligands, CCL19 and CCL21. Therefore, we explored whether the CCL19/21-CCR7 chemokine-receptor signaling axis could be involved in the persistence of ALK+ ALCL cells during ALK inhibitor treatment. We show that treatment

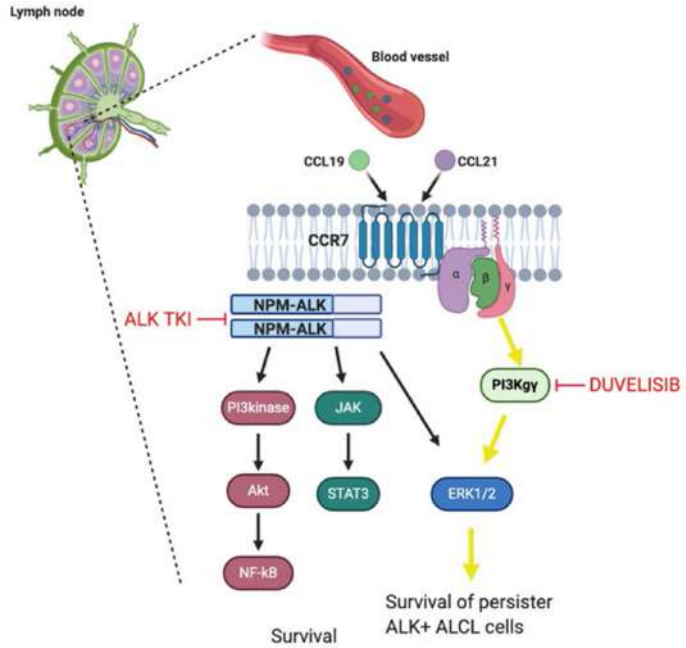

FIGURE 1: The CCL19/21-CCR7- PI3Kg axis protects ALK+ ALCL cells from crizotinib treatment and its disruption could contribute to MRD eradication.

with crizotinib caused upregulation of CCR7 in ALK+ ALCL cells via STAT3, as demonstrated by ChIP-seq data. Besides, stimulation of ALK+ ALCL cells with both CCL19/21 potently activated the MAPK signaling and sustained MAPK activation during ALK inhibition by crizotinib. Mechanistically, we demonstrate that this MAPK activation is mediated by PI3Kγ-dependent CCR7 signaling (Figure 1). Indeed, this effect was strongly reduced in murine lymphoma PI3Kγ^{KO} cells, generated from NPM-ALK transgenic mice crossed with PI3Kγ^{KO} mice. Treatment with the PI3Kγ/δ dual inhibitor duvelisib abrogated the MAPK phosphorylation induced by CCL19/21. When we knocked-out the CCR7 gene via CRISPR/Cas9, human ALK+ ALCL showed markedly reduced activation of the MAPK pathway upon stimulation with CCL19/21. Next, using a 3D microchip model of the perivascular niche we demonstrated that the presence of endothelial cells conferred resistance to crizotinib and sustained cell viability of CCR7^{WT} cells, whereas the protective effect was lost in CCR7^{KO} cells. In *in vivo* experiments CCR7 was required for lymphoma cell survival and diffusion to the brain during crizotinib treatment.

CONCLUSIONS

Overall, our results suggest that the perivascular niche could promote survival of ALK+ ALCL persister cells and protect them from the effect of ALK TKIs via the CCL19/21-CCR7 axis. The disruption of this survival axis could contribute to eradicating minimal residual disease (MRD) in combination with ALK TKI.

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PAMAM and PPI dendrimers as potential anti-cancer drug carriers: A computational investigation

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OBJECTIVES

Photodynamic therapy (PDT) is a promising technique for several types of anti-cancer therapy, exploiting a photosensitizer, a light source and oxygen. The present work computationally investigates the properties of poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) dendrimers of generation 3 and 4 as potential nanoscale drug delivery systems [1] for Rose Bengal (RB), a candidate photosensitizer for PDT.

METHODS

The starting configurations corresponding to neutral pH for G3 and G4 PPI and PAMAM dendrimers were generated using the Dendrimer Builder Toolkit [2]. Protonation of amines was checked and corrected according to the Ising model [3]. 200-ns Molecular Dynamics simulations of neat dendrimers were carried out in GROMACS 2020.4 after equilibration. Final dendrimer configurations were extracted and used as starting structures for further, 200-ns MD simulations with Rose Bengal at 10:1 ratio with respect to the dendrimers in the surrounding solvent. Dendrimer geometry and behaviour was analysed from the neat simulations in terms of radii of gyration (RoG) and Radial Distribution Functions (RDF) of external amines, negative Cl- ions and water. The formation of dendrimer-RB complexes was analysed in the simulations with RB.

RESULTS

Analysis of the RoG over the last 20 ns of neat MD simulations shows stable results, consistent with data from previous literature (Fig. 1). The RDF for negative Cl- ions showed the counterion penetration with peaks between 0.5 and 1.0 nm for both PPI G3 and G4, with more subdued penetration maxing at around 1.5 nm for PAMAM G3 and G4. The RDF for water showed penetration towards the dendrimer core, in agreement with previous computational and experimental results, in the case of PPI G3 and G4, with peaks at 0.5 nm. PAMAM showed comparably less water density, with RDFs gradually increasing from 0.3 nm from the core. Simulations in the presence of Rose Bengal show the formation of a stable dendrimer-drug complex after about 20-50 ns, with all RB molecules remaining bound to the dendrimers, which undergo conformational rearrangements that lock RB into position, as visible in the snapshot in Fig.2.

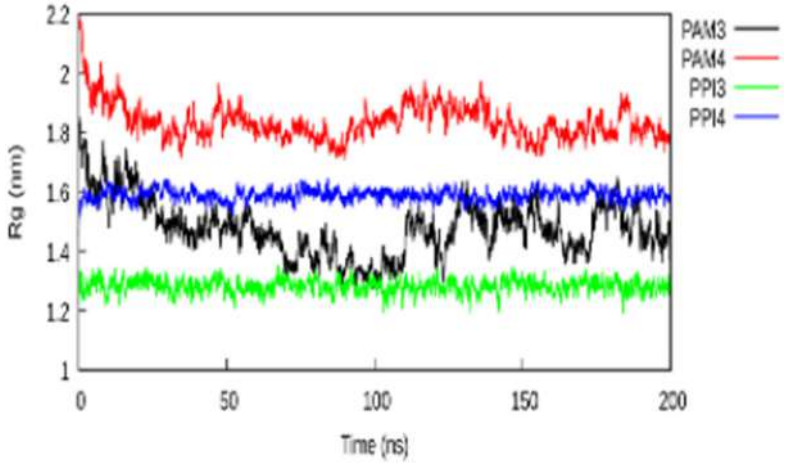

FIGURE 1: RoG of the simulated systems.

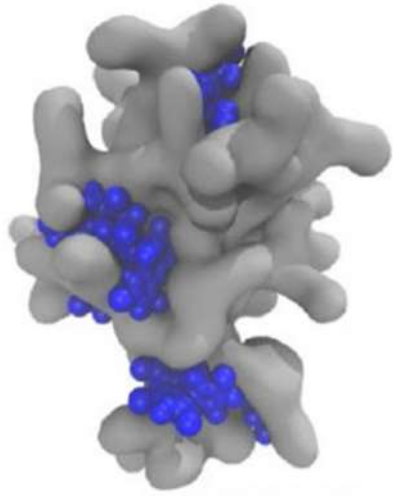

FIGURE 2: PAMAM G4 Dendrimer (grey) in complex with RB (blue).

CONCLUSIONS

MD simulations show that both PPI and PAMAM G3 and G4 dendrimers are promising drug delivery systems for Rose Bengal. Indeed, a single dendrimer is shown to be able to form a stable complex with up to 10 RB molecules, a might as such constitute a valid scaffold for enhanced RB delivery for PDT cancer therapy.

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