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Preclinical tumor-immune modeling

For the identification of immunomodulatory drugs

TOVE SELVIN



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Abstract

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For a long time, the field of cancer research was dominated by a tumor cell-centric view. That, however, changed once it became recognized that medical cancer treatment is largely influenced by the combined effect exerted on both cancer and immune cells. In this work, we aimed to develop and apply preclinical model systems for the identification and evaluation of immunomodulatory anti-cancer agents. In Paper I, we employed single-cell RNA sequencing (scRNA-seq) to investigate immunological effects of trifluridine (FTD), a nucleoside analogue used for the treatment of colorectal cancer (CRC). The study revealed that while FTD induces immunogenic cell death (ICD), it may also attenuate T cell-mediated antitumor responses. In paper II and III, we developed and applied a phenotypic screening platform based on a miniaturized tumor-immune model. In paper II, aiming to identify immunological effects of clinical relevance and provide a reference point for screening novel compound libraries, the model system was used to assess a broad panel of standard anticancer agents. In paper III, the platform was used to screen a drug library containing 1280 small molecule drugs, all approved by the FDA or other agencies. Using this approach, statins were identified as enhancers of immune cell-mediated cancer cell killing. Finally, in paper IV, we developed the immunology hollow fiber assay (HFA) with the goal of bridging the gap between cell based in vitro assays and more complex mouse models for evaluation of immuno-oncological agents. The HFA is an in vivo assay in which semipermeable fibers are filled with cancer cells and implanted in rodents. We further developed the HFA to incorporate both cancer and immune cells. This novel assay demonstrated the potential to capture immune-mediated cancer cell killing in vivo within a matter of days. Collectively, this work provides a research approach for immunology drug screening, in vitro validation, and initial in vivo evaluation.

Keywords: Immuno-oncology, anti-cancer drugs, preclinical modeling, drug screening, repurposing

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*“If you thought that science was certain?
Well, that is just an error on your part.”*

Richard Feynman

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.

- I **Selvin T**, Fasterius E, Jarvius M, Fryknäs M, Larsson R, Andersson CR. Single-cell transcriptional pharmacodynamics of trifluridine in a tumor-immune model. *Scientific Reports*. 2022;12(1):11960
- II **Selvin T**, Berglund M, Lenhammar L, Lindskog M, Jarvius M, Larsson R, Nygren P, Fryknäs M and Andersson CR. Immuno-oncological effects of standard anticancer agents and commonly used concomitant drugs: an in vitro assessment. *Submitted (2023)*.
- III **Selvin T**, Berglund M, Lenhammar L, Jarvius M, Fryknäs M, Nygren P, Larsson R, Andersson CR. Phenotypic screening platform identifies statins as enhancers of immune cell-induced cancer cell death. *BMC Cancer*.2023;23(164)
- IV **Selvin T**, Andersson C.R Berglund M, Jarvius M, Larsson R and Fryknäs M. The Immuno-oncology Hollow Fiber Assay. *Manuscript*.

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Other contributions, not included in the thesis

- I Andersson CR, **Selvin T**, Blom K, Rubin J, Berglund M, Jarvius M, Lenhammar L, Parrow V, Loskog A, Fryknäs M, Nygren P, Larsson R. Mebendazole is unique among tubulin-active drugs in activating the MEK–ERK pathway. *Scientific Reports*. 2020;10(1).
- II Ek F, Blom K, **Selvin T**, Rudfeldt J, Andersson CR, Senkowski W, Brechot C, Nygren P, Larsson R, Jarvius M, Fryknäs M. Sorafenib and nitazoxanide disrupt mitochondrial function and inhibit regrowth capacity in three-dimensional models of hepatocellular and colorectal carcinoma. *Scientific Reports*. 2022;12(1).

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Abbreviations

ANXA1	annexin A1
APC	antigen presenting cell
B2M	β -2-microglobulin
CAR	chimeric antigen receptor
CALR	calreticulin
CCC	Concordance Correlation Coefficient
CRC	colorectal cancer
CTL	cytotoxic T lymphocyte
CSF1R	colony stimulating factor-1 receptor
CTLA-4	cytotoxic T lymphocyte antigen 4
DAMP	damage-associated molecular pattern
DCs	dendritic cell
DEG	differentially expressed gen
dMMR	mismatch repair deficient
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
VEGF	vascular endothelial growth factor
FDA	food and drug administration
FTD	trifluridine
GFP	green fluorescent protein
HF	hollow fiber
HFA	hollow fiber assay
HCC	hepatocellular carcinoma
HLA-I	human leukocyte antigen
HMGB1	high-mobility group box 1
HTS	high-throughput screening
ICB	immune checkpoint blockade
ICD	immunogenic cell death
ICI	immune checkpoint inhibitor
IDO1	idoleamine 2,3-dioxygenase 1
IL	interleukin
I.P.	intraperitoneally
KRAS	kirsten rat sarcoma 2 viral oncogene homolog
NSCLC	non-small-cell lung cancer

mAbs	monoclonal antibody
mCRC	metastatic colorectal cancer
MBZ	mebendazole
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MHC-I	major histocompatibility complex class I
MHC-II	major histocompatibility complex class II
MMR-p	mismatch repair proficient
MSI-H	microsatellite instability-high
MSS	microsatellite stable
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
RCD	regulated cell death
scRNA-seq	single-cell RNA sequencing
SLE	systemic lupus erythematosus
S.C.	subcutaneously
SIRPα	signal regulatory protein α
TAM	tumor-associated macrophage
TCR	T cell receptor
TKI	tyrosine kinase inhibitor
TME	tumor microenvironment
Treg	T regulatory cell

Introduction

For a long time, the field of cancer research was dominated by a tumor cell-centric view. That however changed once it became recognized that the non-malignant cells in the tumor microenvironment (TME) have a profound impact on tumor biology and treatment response¹. The TME-composition varies between tumor types but generally includes stromal cells, blood vessels, extracellular matrix (ECM), and immune cells². Conventional chemotherapeutics as well as targeted anticancer drugs have been shown to modulate the immune contexture in the TME and thus affect disease outcome³. As it has become evident that medical cancer treatment is largely influenced by the combined effect exerted on both cancer and immune cells, it is essential that we further increase our knowledge regarding immunological effects of standard anticancer drugs. Furthermore, over the last decades, we have learned to exploit the immune system to combat cancer. The many advances in immuno-oncology have placed immunotherapy at the forefront of cancer research. To facilitate the translation of novel immunomodulators from bench to bedside, continued development of predictive preclinical models is essential⁴. In the work presented herein, we set out to further develop and apply preclinical model systems for the identification of drugs with repurposing potential for use in immuno-oncology.

Basic immunology

The immune system is broadly classified into two interconnected arms: innate immunity and adaptive immunity. Innate immunity is a fast and non-specific first line of defense that includes cellular components such as neutrophils, dendritic cells (DCs), macrophages, and natural killer (NK) cells, while adaptive immunity, mediated by T cells and B cells, is a slower but highly specific defense.⁵

T cell activation in the lymph nodes. A critical part of our immunity is maintained by antigen presenting cells (APCs) such as DCs, macrophages, and B cells. APCs have the ability to recognize “non-self” antigens, for example bacterial proteins or mutated self-antigens, and present them on major histocompatibility complex (MHC) molecules to T cells. There are two types of MHC molecules: MHC class I (MHC-I) and MHC class II (MHC-II). Almost all nucleated cells produce MHC-I through which they can present endogenous antigens to CD8⁺ T cells while only professional APCs produce MHC-II, enabling them to also present exogenous antigens to CD4⁺ T cells. The process through which APCs display antigens on MHC-I or MHC-II to activate T cells is known as antigen presentation and takes place in the lymph nodes. Optimal priming and activation of T cells, turning naïve T cells into effector T cells, requires a minimum of three signals: (1) recognition of the presented antigen through a T cell receptor (TCR), defining the specificity of the T cell, (2) co-stimulation through co-receptors that stabilize the interaction between the T cell and the APC, and (3) cytokines that promote proliferation and enhances the function of the T cells.⁶

Antigen presentation in the periphery. In humans, the MHC system is referred to as Human Leukocyte Antigen (HLA). Corresponding to MHC- I, there are three classical HLA type I molecules, HLA-A, HLA-B, and HLA-C, and their main function is to present peptides derived from intracellular pathogens and tumor cells to cytotoxic T cells (CTLs). A naïve T cell is incapable of killing target cells, a CTL that has undergone activation in the lymph nodes can however recognize antigens presented on HLA-I in the periphery and kill the target cell. Thus, HLA-I molecules play an essential role in immunosurveillance and in cell-mediated antitumor immune responses⁷.

Antitumor immunity

The tumor immune microenvironment. Together the cells of the tumor immune microenvironment (TIME) (Fig. 1) greatly influence tumor progression, immunosurveillance, cell-mediated antitumor responses, and treatment outcome. Due to their ability to recognize and kill tumor cells, the presence of CTLs in the TME is often associated with a positive prognosis. Another cell type associated with a positive prognosis is T helper 1 (Th1) cells, which can support CTLs by secreting proinflammatory cytokines². NK cells are very efficient at killing tumor cells in the circulation. However, immunosuppressive cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) interfere with NK-cell efficiency in the TME^{2,8}. Besides inhibiting NK cell function, MDSCs can suppress T cell infiltration and stimulate T cell apoptosis⁹. Other innate immune cells that play a critical role are macrophages. In the TME, macrophages are often referred to as tumor-associated macrophages (TAMs) and depending on their polarization they can be either tumor suppressive or tumor supportive. Monocytes migrate from the circulation into the TME where they, depending on signaling molecules secreted by cells already residing in the TME, differentiate into either pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages^{2,9}.

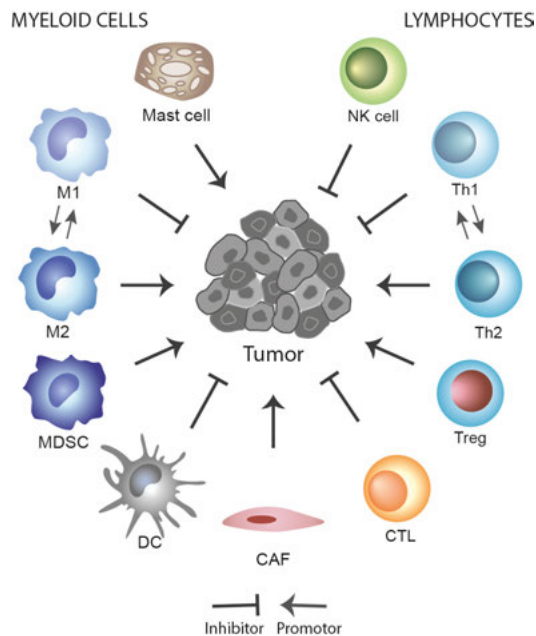


Figure 1. Illustration of the tumor immune microenvironment. An overview of the tumor immune microenvironment. Cell types with tumor suppressing properties indicated by T and cell types with tumor supporting properties indicated by ↑.

The Cancer-Immunity Cycle. T cells possess the ability to recognize and eliminate cancer cells, however, a series of events must proceed successfully for T cell-mediated tumor cell killing to occur. This seven-step process, described in 2013 by Chen and Mellman¹⁰, is called the Cancer-Immunity Cycle. Firstly, APCs must capture and process neoantigens released by cancer cells. Next, APCs must travel to the lymph node where the antigen can be presented to T cells, resulting in priming and activation of the T cells. Once activated, CTLs must travel back to and infiltrate the tumor site. Now, equipped with tumor antigen-specific TCRs, they can recognize and eliminate cancer cells. Unfortunately, tumors have developed immune escape mechanisms challenging all of these steps¹¹.

Immunoediting. Tumors can render themselves immunoevasive through a process known as cancer immunoediting. This process proceeds through three phases: elimination, equilibrium, and escape. Genetic instability of cancer cells and pressure from the adaptive immune system together drive selection of less immunogenic subclones that can evade the immune system and enter the escape phase. Generally, this results in higher abundance of immunosuppressive cell types such as TAMs, MDSCs, and Tregs, dysregulated secretion of signaling molecules, and higher expression of inhibitory immune checkpoint molecules such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and Programmed death-ligand 1 (PD-L1)¹². This immunosuppressive environment often leads to DC dysfunction and thus to impaired antigen presentation. T cell activation in the lymph node is also hampered by upregulation of CTLA-4 as it blocks an essential co-stimulatory signaling pathway. Additionally, even if T cell-activation is achieved, trafficking back to the tumor site can be impaired by overexpression of vascular endothelial growth factor (VEGF) causing formation of abnormal blood vessels. Finally, activated T cells that do make it back to the tumor site still have great obstacles to overcome, including inhibition by Programmed cell death protein 1 (PD-1)/ PD-L1 interactions and tumor cell downregulation of HLA-I molecules required for immunorecognition¹¹. How to counteract these escape mechanisms and reinitiate a functional cancer-immunity cycle without generating severe adverse inflammatory responses remain a major challenge.

Cancer treatments over the ages

For centuries surgery was the only available treatment option, until 1903 when two cancer patients for the first time were cured using radiation therapy¹³. The field was then dominated by surgery and radiotherapy until the 1960s¹⁴. The first modern oncology chemotherapeutic drugs to be discovered and used in clinical practice were nitrogen mustards: alkylating agents that inhibit DNA

replication. Although some of these first-generation alkylating agents are no longer used, modern day alkylating agents such as dacarbazine and oxaliplatin are still widely used as first- and second line treatment of various tumors. To date, a wide range of chemotherapeutics have been developed, including anti-metabolites (e.g., 5-fluorouracil), topoisomerase inhibitors (e.g., irinotecan), and mitotic inhibitors (e.g., vincristine). Additionally, over the last few decades, increased knowledge regarding the molecular mechanisms behind neoplastic transformation has fueled a revolution of targeted cancer therapy based on tyrosine and serine/threonine protein kinase inhibitors and monoclonal antibodies (mAbs)^{13,14}.

Another ongoing revolution in oncology is the development of various immunotherapies. The first documented proof of concept for treating cancer with immunotherapy was provided by William Coley in 1891 as he discovered that injecting a mixture of *Streptococcus pyogenes* and *Serratia marcescens* bacteria could achieve complete and durable remissions in sarcoma patients. However, as the mechanisms of action were unknown and the risk of infection high, the general attitude towards the concept was initially hostile. Almost a century passed before the strategy resurfaced again in 1976 with the use of the tuberculosis vaccine Bacille Calmette-Guérin (BCG) as a treatment for bladder cancer; a therapy that was proven very effective and still is used today. Another great discovery in 1976 was the identification of interleukin 2 (IL-2). This T cell growth factor was later approved by the Food and Drug Administration (FDA) for treatment of metastatic kidney cancer. Clinical use of cytokines to promote anti-tumor immune responses marked a milestone in immuno-oncology. However, due to high toxicity and low overall response rates, cytokine therapies have been phased out of the clinic in favor of targeted therapies and immune checkpoint inhibitors (ICIs)¹⁵.

Immunological effects of chemotherapy

Conventional chemotherapeutics as well as targeted anticancer drugs have been shown to modulate the immune contexture in the TME and thus affect disease outcome³. Some chemotherapeutics used in the clinic today have severe immunosuppressive adverse effects¹⁶ while others have the ability to enhance anti-tumor immunity^{17,18}. For example, the alkylating agent oxaliplatin has been shown to induce and maintain immunogenic cell death (ICD) in patients¹⁹. ICD is a particular form of apoptosis that can activate an adaptive immune response by provoking the release of damage-associated molecular patterns (DAMPs)²⁰. The concept of ICD induced by chemotherapeutics has gained increasing interest as it is of great clinical importance; not only can inducing ICD shift the immune balance in the TME from suppression to

activation but it can also create a specific and long-lasting immunological memory²¹. Improved knowledge regarding immunological effects of chemotherapy is necessary, especially now that we have entered an era where increasing pre-clinical and clinical efforts are put into combining chemotherapy and immunotherapy to combat cancer.

The era of single-cell sequencing. Since RNA-sequencing was developed over a decade ago it has revolutionized our molecular understanding of cells. In the past, each cell type of interest had to be sequenced separately and with bulk expression profiles as the read-out. The advancement of technologies now allows for sequencing with single-cell resolution, enabling evaluation of heterogeneous responses across multiple cell types and subsets of cell populations simultaneously²²⁻²⁴. In the field of immuno-oncology, single-cell RNA sequencing (scRNA-seq) has been applied to study the heterogeneity of cancer cell populations, the TME composition, and immune infiltrates of various tumors, which has led to the identification of novel factors that influence tumor progression and patient outcomes²⁵. In Paper I, we explored the feasibility of using scRNA-seq to study immunological effects of the chemotherapeutic agent trifluridine (FTD).

Immuno-oncology

Since the first ICI, ipilimumab, was approved by the FDA in 2011, immunotherapy has become one of the cornerstones of cancer treatment. Immunotherapy aims to boost the immune system's innate ability to fight cancer by promoting immune-mediated killing and/or limiting immune-escape²⁶. Different classes of immunotherapies include cell-based therapies (e.g., chimeric antigen receptor (CAR) T-cell therapy), oncolytic viruses (e.g., T-VEC), and the most widely used class; ICIs based on mAbs. Despite great clinical success for some cancer subtypes, limited overall response rates, safety issues, and complex pharmacokinetics remain major limitations for many current immunotherapies^{26,27}.

Immune checkpoint inhibitors. Immune checkpoints are key regulators of the immune system that are essential for self-tolerance, but they can also promote cancer progression. Tumors commonly downregulate stimulatory immune checkpoints while upregulating the expression of inhibitory immune checkpoints, resulting in dysfunctional T cell responses²⁷. The principle of immune checkpoint blockade (ICB) therapy is to restore T cell activity by blocking these inhibitory immune checkpoints with mAbs. To date, FDA has approved mAbs blocking CTLA-4, PD-1, PD-L1²⁸, and most recently LAG-3²⁹, for cancer therapy. Thus far, the greatest clinical success has been observed

for melanoma and non-small-cell lung cancer (NSCLC). For these indications ICB is approved as the first line of treatment²⁸. ICB treatment has also been approved for other solid tumor indications such as hepatocellular carcinoma (HCC) and microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer (mCRC) that has progressed following standard treatment²⁸. The overall response rates are however low and there is still an unmet need for immunotherapy, especially for cancers with a low mutational burden²⁷.

While T cell checkpoints are obvious targets for developing immunotherapies, it is important to note that not only T cells can eliminate cancer cells. The critical role of TAMs in the TME is widely accepted; however, they are perhaps most recognized for their tumor supporting properties. In addition to promoting cancer cell proliferation, metastasis, and immunosuppression, TAMs are known to increase resistance against chemotherapy and ICB treatment. Nonetheless, macrophages can also mediate phagocytosis and direct cytotoxic tumor killing when appropriately activated³⁰. Data from preclinical and early clinical studies suggest that targeting phagocytosis checkpoints, such as CD47, could be a promising treatment strategy³¹. CD47 is overexpressed in most human cancers and has been described as one of the most important anti-phagocytic signals. Thus, blocking signaling through the CD47-signal regulatory protein α (SIRP α) axis has the potential to restore phagocytosis of cancer cells³².

Small molecule drugs. Immunotherapies that target extracellular checkpoint proteins focus directly on adaptive immunity and address only the final step of the Cancer-Immunity Cycle: killing of cancer cells^{10,33}. The remaining steps of the cycle are dominated by cellular signaling processes and chemokine gradients that have not yet been effectively targeted using therapies based on large-molecule drugs³³. There is an increasing effort to exploit intracellular targets to engage the immune system. Immunotherapeutic small molecule drugs have emerged as a promising complement to current immunotherapies as they may offer advantages such as enhanced tissue penetration, improved bioavailability, and ability to reach intracellular targets³⁴.

An increasing number of clinical trials are evaluating combination treatments with ICIs and various small molecule drugs. As an example, clinical trials aim to improve the treatment outcome for metastatic melanoma by combining ICIs with drugs targeting various modalities within the TME such as colony stimulating factor-1 receptor (CSF1R), chemokine receptor CXCR1 and 2, and the JAK/STAT pathway³⁵. Furthermore, clinical trials have obtained promising results combining ICIs with small-molecule inhibitors targeting for example VEGF receptors, epidermal growth factor receptors (EGFRs), and idoleamine

2,3-dioxygenase 1 (IDO1)³⁶. Combining ICIs with small molecule drugs is a promising treatment strategy, but major challenges such as managing toxicities remain. There is a need to identify novel small molecule drugs for use in immuno-oncology. The fact that immunomodulators aiming to increase immune responses have, by their very nature, the potential to cause severe adverse immune reactions provides a rationale for using a repurposing approach.

The concept of drug repurposing

Drug repurposing, that is identifying new indications for already approved drugs that are outside the scope of the original medical indication, provides several advantages; the safety, pharmacokinetics, and pharmacodynamics of the drugs have already been established in humans³⁷. This greatly reduces the time frame and cost of drug development along with the risk of failure due to safety issues³⁸. A poster child for drug repurposing in oncology is thalidomide: a drug developed in the 1950s as a sedative for pregnant women and today is used as the first-line treatment for multiple myeloma. When the FDA approved thalidomide for treatment of plasma cell myeloma in 2006, it was the first new drug approved for the indication in over a decade and it has significantly improved the overall survival rate³⁹. To facilitate the identification of drugs with repurposing potential for use also in immuno-oncology, continued development of preclinical immuno-oncology model systems is imperative.

High throughput immuno-oncology modeling

Phenotypic high-throughput drug screening. High-throughput screening (HTS) is a common approach in early preclinical drug development. Phenotypic HTS has greatly facilitated the discovery of new cancer drugs and has given rise to several FDA-approved chemotherapeutics that are currently used in the clinic⁴⁰. However, evaluating large panels of drugs in an immuno-oncology setting in vitro is a challenging task; most phenotypic HTS platforms are based solely on cancer cell populations and do not allow for the identification of immunomodulatory drugs. Mo et al. addressed this in 2019 by developing the High-throughput immunomodulator phenotypic screening platform (HTiP), which integrates cancer and immune cells⁴¹. Using a similar approach, we have developed a miniaturized in vitro co-culture model system comprised of fluorescently labeled cancer cells and human peripheral blood mononuclear cells (PBMCs) (Fig. 2).

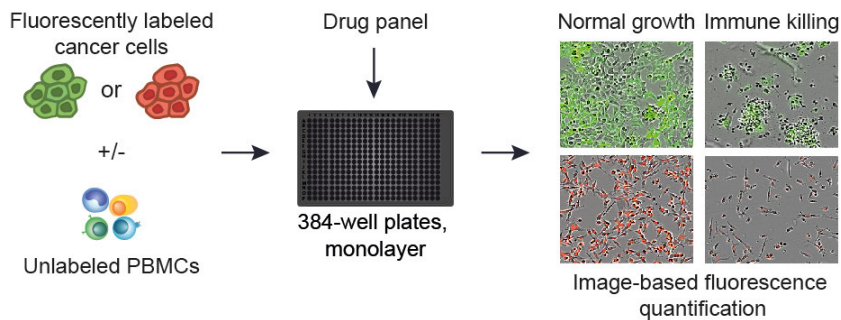


Figure 2. Schematic representation of in vitro co-culture model system. Cancer cell lines stably expressing GFP (green) or mKate2 (red) grown as monocultures or co-cultured with PBMCs in 384-well monolayer plates. Scanned in live-cell analysis system following treatment with drug panel of interest.

Fluorescence as an indirect marker for viability. Selecting a reliable method for evaluation of cancer cell viability is critical when assessing drug effects. Quantification of stably expressed green fluorescent protein (GFP) has previously been established as a robust assay; a direct relationship has been demonstrated between the loss of fluorescence and cell death induced by various apoptotic stimuli⁴². In paper II, we verified the validity of using image-based quantification of GFP and mKate2 as indirect measures of viability. After confirming the robustness of the assay, the model system was used to evaluate a broad panel of conventional anticancer drugs to identify immuno-modulatory effects and to serve as a point of reference for screens of novel compound libraries. In paper III, aiming to identify small molecule drugs with repurposing potential for use in immuno-oncology, we utilized the model system to screen 1280 FDA approved drugs.

Allogeneic models – strengths and caveats. The many advances in the development of refined in vitro models have contributed to an increased understanding of antitumor immunity. For example, the use of primary autologous models comprised of cancer and immune cells from the same patient has opened up for more personalized research approaches⁴³. Autologous models may provide a superior representation of patient tumor-immune interactions. However, they are still unattainable to many researchers, mainly due to poor access and short experimental windows⁴⁴. Furthermore, in the context of HTS, obtaining a sufficient cell yield may not be feasible even when accessing patient matched cells.

In contrast to autologous models, using allogeneic PBMCs offers a more straightforward approach. The PBMCs provide cellular components of innate (monocytes, NK cells, and DCs) and adaptive (B and T cells) immunity⁴⁵. However, the adaptive immune response will not be tumor antigen-specific, it

will be alloreactive. Therefore, while these models can detect intracellular interference with TCR signaling, they are not suitable for investigating immunomodulation caused by changes in the peptides presented on MHC. Moreover, immuno-oncology models based on PBMCs, whether autologous or allogeneic, omits other important modulators of anti-tumor immune responses, for example MDSCs and CAFs⁴⁶. Thus, they represent a simplified reflection of the immune interactions in the TME.

The impact of cellular configurations. There are undeniable advantages associated with the use of 2D model systems, such as the level of reproducibility and standardization, the compatibility with high throughput assays, and a more straightforward interpretation of the obtained results. However, the cost of simplicity is an inferior clinical translatability. It is widely acknowledged that 3D cell cultures, such as tumor organoids, better mimic the genotypic and phenotypic characteristics of original tumors⁴⁷. However, these benefits are also accompanied by challenges. For example, the growth rate is often slow, very specific culture conditions are required, and it is not yet fully elucidated how the many growth factors required to maintain organoid growth may affect non-cancer cells in these cultures⁴⁸. Spheroids, i.e., less complex primary cell or cell line derived 3D cultures, have long been widely used to perform oncology drug screens. Recently, spheroid models have also found their way into the field of immuno-oncology⁴⁹. In a study published in 2019, Varesano et al. used spheroid-based heterotypic co-cultures comprised of colorectal cancer (CRC) cells and $\gamma\delta$ T cells to assess the anti-tumoral activity of these effector lymphocytes⁵⁰. More recently, in a similar manner, Courau et al. co-cultured CRC spheroids with T cells and NK cells isolated from PBMCs to evaluate immunomodulatory Abs targeting the NKG2A axis, a novel immune checkpoint⁵¹. In paper IV, we established a 3D co-culture model system using CRC cells and complete PBMCs (Fig. 3).

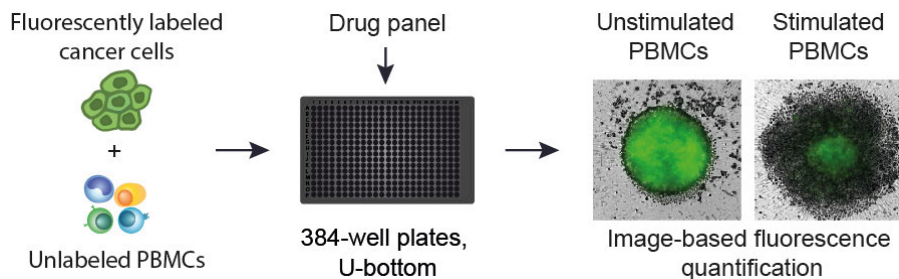


Figure 3. Schematic illustration of the 3D co-culture model system. Fluorescently labeled CRC cells co-cultured with PBMCs in 384-well U-bottom ultra-low attachment plates.

In vivo immuno-oncology modeling

Preclinical mouse models are key tools in cancer research. Commonly used in vivo models in the field of immuno-oncology includes syngeneic mouse models with immune-competent hosts, chemically induced mouse models, and genetically engineered mouse models⁵². Although these models undeniably are useful, one major shortage is that they all rely on the murine immune system. The development of humanized mouse models, such as the Hu-PBL model in which human PBMCs are engrafted into immune-deficient mice⁵³, allows for evaluation of potential immunotherapies on human tumor and immune cells. However, these models also have their shortages as engrafting human immune cells into mice rapidly results in xenograft-versus-host disease. Furthermore, most humanized mouse models are both cost and time consuming^{52,53}.

The immuno-oncology hollow fiber assay. The hollow fiber assay (HFA) is an in vivo assay where semipermeable hollow fibers (HF) are filled with cancer cells, heat-sealed, and surgically implanted intraperitoneally (i.p.) and/or subcutaneously (s.c.) in rodents. The National Cancer Institute (NCI) originally developed the assay to reduce the time and cost associated with early preclinical evaluation of novel anti-cancer drugs from drug screens⁵⁴. In paper IV, to provide a quicker and more cost-effective method for initial in vivo evaluation of immunomodulators, we modified the HFA to include both cancer and immune cells (Fig. 4).

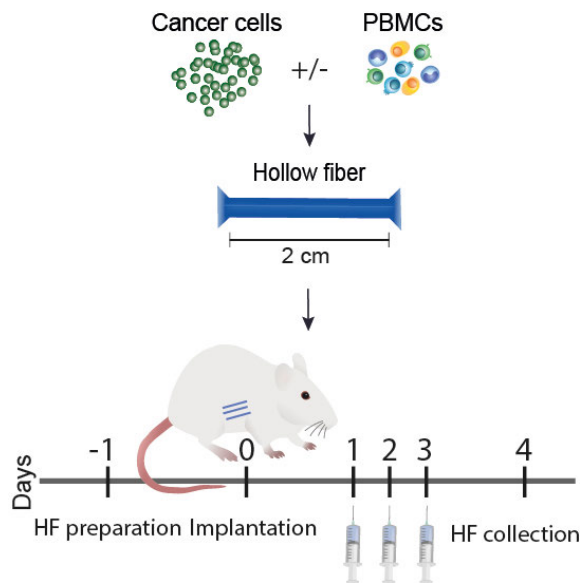


Figure 4. The immuno-oncology HFA.
Schematic illustration of the immuno-oncology HFA.

Aims of the thesis

The overall aim of this thesis was to further develop and apply preclinical model systems for the identification of drugs with immuno-oncological activity. The specific aims were:

- To evaluate immunomodulatory effects of conventional anticancer agents (paper I and paper II)
- To develop and apply a phenotypic screening platform for in vitro identification of small molecule drugs with repurposing potential in immuno-oncology (paper III).
- To develop a model system for initial in vivo evaluation of small molecule drugs with immuno-oncological activity (paper IV).

Methods

This is a brief summary of the methods used in this thesis, for more detail see individual papers.

Cell lines and immune cells

HCT116-GFP, a human CRC cell line stably expressing GFP, was used in all paper. In paper I and IV, we also used HCT116wt. In paper II, we used A549 NucLight Red (NLR), a lung cancer cell line stably expressing the fluorescent protein mKate2. All cell lines were cultured at 37°C in 5% CO₂ in their recommended medium supplemented with heat-inactivated fetal bovine serum (FBS). The medium was also supplemented with either L-glutamine and Penicillin/ Streptomycin (HCT116wt and GFP) or Penicillin/ Streptomycin and Puromycin (A549-NLR). Additionally, PBMCs from anonymous, healthy donors were used in all papers.

Cell cultures

Monolayer. Cells grown in monolayer, i.e., grown as a single layer in flat-bottom, cell culture-treated 384-, 96-, 12- or 6-well plates, were used in all papers. To establish monocultures, cancer cells were seeded in cell culture plates. To establish co-cultures, cancer cells were seeded in cell culture plates and precultured for 24 h before PBMCs were added at a 1:1, 1:4, or 1:8 ratio (paper I-III). To establish co-cultures in paper IV, cancer cells and PBMCs were seeded simultaneously at a 1:4 ratio.

U-bottom plates. In paper IV, 384-well ultra-low attachment U-bottom plates were used to promote a 3D configuration of the cells and thus increase the proximity of the cells. Monocultures were established by seeding HCT116-GFP alone. To establish co-cultures, PBMCs were added at a 1:4 ratio. The plates were then centrifuged at 200 x g for 2 min.

Hollow fibers. In paper IV, polyvinylidene fluoride (PVDF) hollow fibers were used. To establish hollow fiber monocultures, a single cell suspension of

cancer cells was injected into hollow fibers using a syringe. Subsequently, the fibers were heat-sealed at 2 cm intervals and cut into individual fibers. To establish hollow fiber co-cultures, the same procedure was performed using a single cell suspension containing cancer cells and PBMCs at a 1:4 ratio.

Drugs and reagents

In paper I, the chemotherapeutic agents trifluridine and oxaliplatin were used. In paper II, a drug panel comprised of 46 conventional anticancer agents and 22 commonly prescribed concomitant non-cancer drugs were used (see paper II, Table 1 in the Methods section). In paper III, we used the Prestwick Chemical Library containing 1280 small molecule drugs. Additionally, a panel of eight statins, including mevastatin, simvastatin, and pitavastatin, was used in paper III. Recombinant Human IL-2 and anti-human CD3 (aCD3) Monoclonal Antibody were used in all papers.

Drug handling

All drugs were kept as 10 mM stock solutions in DMSO or sterile water and diluted to desired final concentrations with culture medium. Drugs were added to experimental plates either using the acoustic liquid dispenser Echo Liquid handler 550 (all 384-well plates) or manually (96-, 24-, 12- and 6-well plates).

Viability and apoptosis measurements

Image-based fluorescence quantification. Cancer cells stably expressing fluorescent reporters (GFP or mKate2) were used in all papers. The viability of the cancer cells was indirectly monitored over time by image-based quantification of fluorescence using the Live-Cell Analysis System IncuCyte S3 or IncuCyte SX5.

Annexin V. In paper I, apoptosis was measured by detection of phosphatidylserine exposed on the extracellular surface using Annexin V Red Dye, quantified using the IncuCyte S3.

Fluorometric microculture cytotoxicity assay. In paper II, the viability of cancer cells and PBMCs was measured using the fluorometric microculture cytotoxicity assay (FMCA). Briefly, the cells were incubated with fluorescein diacetate (FDA) for 50 min, generating fluorescein signals in cells with intact

plasma membranes. The fluorescein signals were then measured using a microplate reader.

Clonogenic assay. In paper IV, clonogenic assay was used to measure the reproductive viability of the cancer cells. Briefly, harvested cells were re-seeded at low density in fresh medium in 6-well plates and allowed to re-grow for 10 days. Subsequently, colonies were fixed using methanol, stained with Giemsa dye and counted manually.

Detection of ICD-markers

In paper I, two gold standard in vitro ICD markers were measured, the secretion of ATP and the release of HMGB1. Extracellular ATP was measured in cell culture supernatants using CellTiter-Glo (CTG). Briefly, CTG solution was added to 96-well plates with supernatants at a 1:1 ratio and placed on a shaker for 2 minutes before the luminescence intensity, proportional to the ATP concentration, was measured using a FLUOstar Omega. HMGB1 levels in cell culture supernatants were measured using ELISA. Supernatant was added to a 96-well plate pre-coated with capture antibody and ELISA was performed according to the manufacturer's instructions. Absorbance was measured at 450 nm using a FLUOstar Omega.

Measurement of cytokines and Granzyme B

In paper I and IV, cytokine levels in cell culture supernatants were measured using a Luminex MAGPIX system and commercially available kits for analytes of interest. In paper IV, the concentration of Granzyme B was also measured in cell culture supernatants using the same system. Briefly, the analytes of interest were bound to magnetic beads via antibodies and subsequently detected using biotinylated antibodies with a fluorescent reporter.

Drug-immune interaction analysis

In paper II and III, the Bliss Independence Model⁵⁵ was used to detect synergy and antagonism between drugs and activated PBMCs. We adapted the Bliss model which states that the product of the reduced cell viability induced by two single drugs with independent effects is expected to be equal to the reduced cell viability induced by the combination of the two drugs. Defining a Bliss score as $B = (\text{Viability Drug 1}) \times (\text{Viability Drug 2}) - (\text{Viability Drug 1+2})$, a positive Bliss score thus indicates synergy while a negative Bliss score

indicates antagonism. In this thesis, the Bliss model was used to identify synergy and antagonism between drugs and aCD3/IL-2 activated PBMCs, i.e., $B = (\text{Cancer cell viability with drug alone}) \times (\text{Cancer cell viability with PBMCs alone}) - (\text{Cancer cell viability with drug} + \text{PBMCs combined})$.

Gene expression analysis

Single-cell RNA-sequencing. In paper I, we performed scRNA-seq on HCT116wt cells and PBMCs. Co-cultures were treated with trifluridine or DMSO vehicle for 12 h or 72 h before Chromium Next GEM scRNA-seq was performed, generating the transcriptomes of 21 256 individual cancer and immune cells after quality control and filtering.

RNA microarray. In paper III, we performed transcriptome-wide gene expression profiling on HCT116-GFP cells and PBMCs. Monocultured and co-cultured cells were treated with pitavastatin or DMSO vehicle for 24 h before gene expression was analyzed using a Human Clariom S array.

In vivo experiments

In paper IV, Hsd:Athymic Nude-Foxn1tm mice were used to perform a pilot in vivo evaluation of the immuno-oncology HFA. Briefly, hollow fiber mono- and co-cultures were prepared on day -1 and cultured in vitro for 18 h before they were surgically implanted i.p. in the mice. The mice received local aCD3 at the site of hollow fiber implantation and/or systemic IL-2 for three consecutive days. On day 4, the hollow fibers were explanted and the reproductive viability of the cancer cells was measured using clonogenic assay (see Viability and apoptosis measurements). The animal experiment was performed in accordance with institutional guidelines and approved by the local animal ethics committee.

Summary of the papers

Paper I

Selvin T, et al. Single-cell transcriptional pharmacodynamics of trifluridine in a tumor-immune model. *Scientific Reports*. 2022;12(1):11960

Increasing efforts are being put into combining chemotherapy and immunotherapy to combat cancer. To facilitate the development of combinatorial treatment regimens with improved clinical efficacy, increased knowledge regarding the immunological effects of chemotherapy is essential. In Paper I, we explored the feasibility of using scRNA-seq to study immunological effects of the nucleoside analogue trifluridine (FTD).

Trifluridine induces apoptosis and release of ICD markers in vitro. FTD, the active component of TAS-102, is a relatively novel anti-tumor drug approved for the treatment of mCRC^{56,57}. FTD has been shown to induce ICD in various human CRC cell lines in vitro⁵⁸, suggesting a favorable immunological effect. We assessed the treatment response and ICD induction of FTD and the positive control oxaliplatin (OXP) in the CRC cell line HCT116-GFP, cultured as monoculture or co-cultured with human PBMCs. Treatment with FTD and OXP for 72 h effectively decreased the viability (Fig. 5A) and increased apoptosis (Fig. 5B) in both mono- and co-cultures. Furthermore, FTD treatment significantly and dose-dependently increased the levels of extracellular ATP (Fig. 5C) and HMGB1 (Fig. 5D); two gold standard in vitro ICD markers. These data suggest induction of ICD also in our model system.

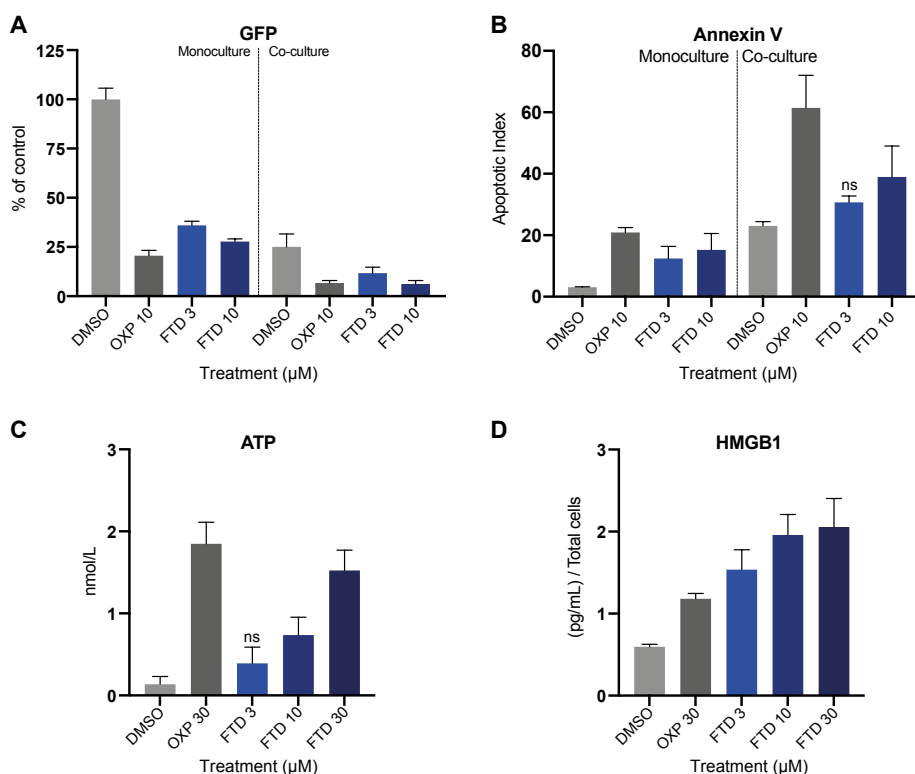


Figure 5. Trifluridine induces apoptosis and release of ICD markers in vitro. **A**) Viability indirectly measured by image based GFP quantification (n=9) and **B**) Apoptotic Index determined by Annexin V (number of Annexin V positive cells/ number of GFP expressing cells) in HCT116-GFP monoculture and co-culture, treated with DMSO vehicle (0.1%), oxaliplatin (OXP) (10 μM), or trifluridine (FTD) (3, 10 μM) for 72 h (n=6). **C**) ATP measured by CellTiter Glo in supernatants of HCT116-GFP monoculture treated for 48 h (n=6) and **D**) HMGB1 measured by ELISA in supernatants of HCT116-GFP monoculture treated for 72 h. Data normalized against total number of cells per sample. Results are shown as mean ± SD from two (B-D) or three (A) independent experiments. Compared to DMSO vehicle, $P \leq 0.05$ (One-way Anova with Dunnett's multiple comparison test) for all treatment data sets except the ones marked ns, not significant.

ScRNA-seq suggests that trifluridine treatment dampens T cell-mediated antitumor responses. ScRNA-seq was used to analyze posttreatment gene expression profiles in thousands of individual cancer and immune cells concurrently (Fig. 6A-B). The FTD-induced effects observed on HCT116 gene expression were concordant with the literature. For example, FTD has been shown to exert at least part of its antitumor effect by activation of the p53 pathway, resulting in p21 induction and cell cycle arrest⁵⁹, a mechanism recapitulated by scRNA-seq in our study (Fig. 6C).

Apart from capturing major mechanisms of action previously described for FTD, scRNA-seq demonstrated that FTD treatment might also induce immunosuppressive effects. Differential gene expression analysis of HCT116 cells treated with DMSO vehicle or FTD for 12 h revealed 52 differentially expressed genes (DEGs). Pathway enrichment analysis using these DEGs identified pathways involved in cancer immune escape as the top three enriched terms (Fig. 6D). Furthermore, HCT116 cells treated with FTD had a lower expression of all three classical HLA- I molecules, B2M, and TAP1 (Fig. 5E), all of which are involved in antigen presentation and commonly downregulated in cancers as a mechanism of immune escape^{11,60-62}. Finally, T cells treated with FTD exhibited higher expression of genes encoding negative regulators of the cell cycle and TCR signaling, further supporting that FTD could dampen T cell-mediated antitumor responses.

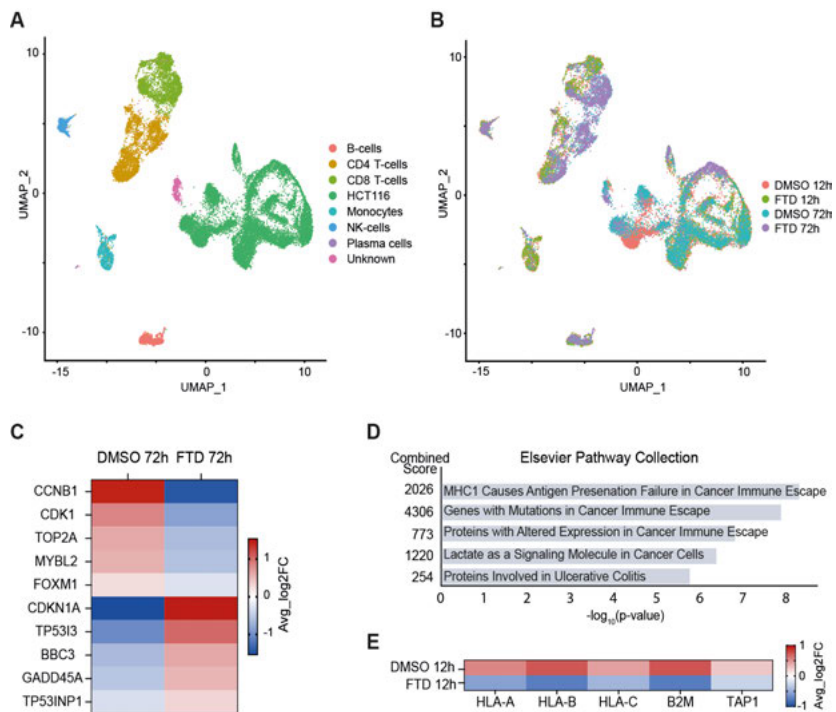


Figure 6. ScRNA-seq suggests that trifluridine treatment dampens T cell-mediated antitumor responses. UMAP projection of all cells colored by **A)** cell type and **B)** sample identity. **C)** Heatmap showing average Log2 fold change expression of genes of interest in HCT116 treated with DMSO vehicle (0.1%) or FTD (3 μ M) for 72 h. **D)** Top 5 enriched pathways obtained from pathway enrichment analysis using the “Elsevier Pathway Collection” database. The analysis was performed with 52 DEGs (Adjusted P-value<0.001) identified for HCT116 treated with DMSO vehicle or FTD for 12 h. **E)** Heatmap showing average Log2 fold change expression of genes of interest in HCT116 treated with DMSO vehicle or FTD for 12 h.

Summary and conclusion. Data generated by us and others suggest that FTD induces ICD, suggesting a favorable immunological effect. However, our scRNA-seq results indicate that FTD could dampen T cell-mediated antitumor responses. Apart from providing new insight into possible treatment-induced effects on T-cell mediated antitumor responses, scRNA-seq recapitulated mechanisms of action previously described for FTD, suggesting that using single-cell transcriptomics to study immunological effects of chemotherapy is a feasible approach.

Paper II

Selvin T, et al. Immuno-oncological effects of standard anticancer agents and commonly used concomitant drugs: an in vitro assessment. *Submitted.*

It has become evident that the outcome of medical cancer treatment is influenced by the combined effect exerted on both cancer- and immune cells. Furthermore, oncology patients often do not receive solely anticancer drugs; concomitant drugs such as corticosteroids, statins, painkillers, and antibiotics are commonly administered during the course of cancer treatment. Therefore, we evaluated potential immunological effects of 46 standard anticancer agents and 22 commonly administered concomitant non-cancer drugs. To enable time and cost-effective evaluation of a broad panel of drugs in an immunoncology setting in vitro, we utilized a miniaturized in vitro model system comprised of fluorescently labeled human colon and lung cancer cell lines grown as monocultures and co-cultured with activated PBMCs.

Quality assessment of the assay. To verify the validity of using image-based quantification of GFP and mKate2 as indirect measures of viability, we initially performed viability measures using FMCA⁶³ in parallel. Applying Lin's Concordance Correlation Coefficient (CCC) analysis to assess the correlation between the two assays confirmed the feasibility of using image-based fluorescence quantification to measure cancer cell viability (Fig. 7a-b). Furthermore, using CCC analysis, we demonstrated that the reproducibility of the results was not affected by the use of PBMCs from different donors (Fig. 7c-d). Taken together, these data demonstrate that our miniaturized tumor-immune model provides a robust platform for in vitro drug evaluation.

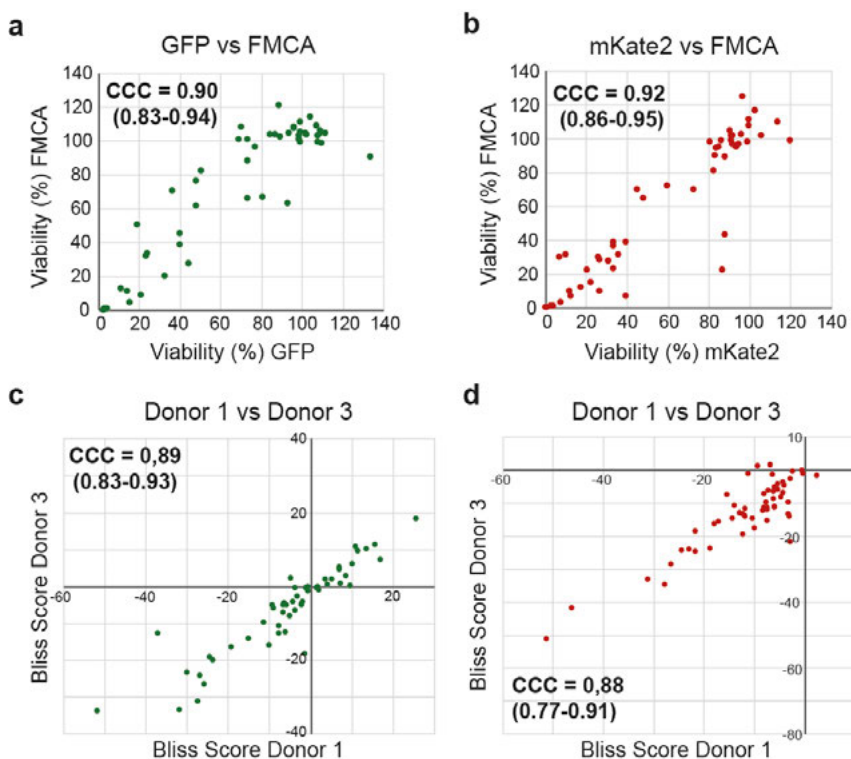


Figure 7. Quality assessment of the assay. (a-b) Viability of HCT116-GFP (a) and A549-NLR (b) cells, cultured as monocultures and treated with a validation drug panel comprised of 16 drugs at 1, 10, and 30 μM for 72 h. Viability measured by FMCA and by image-based quantification of fluorescence. Correlation between the two assays determined by calculating CCC. One representative experiment shown with data presented as means from three technical replicates. (c-d) Correlation between Bliss scores obtained with PBMCs from different donors determined by calculating CCC. Bliss Scores were calculated for HCT116-GFP (c) and A549-NLR (d) after treatment with the validation drug panel at 1, 10, and 30 μM for 72 h.

Evaluation of standard anticancer drugs and concomitant drugs. The CRC cell line HCT116-GFP and the lung cancer cell line A549-NLR were grown as monocultures and co-cultures with aCD3/IL-2 activated PBMCs. The drug panel was screened at 1 and 10 μM and the viability of the cancer cells was indirectly measured by image-based quantification of fluorescence intensity (Fig. 8a-d). A Bliss score was calculated for each drug and 16 drugs with Bliss scores ranging from the highest to the lowest were selected for validation experiments. Among the standard anticancer agents, tyrosine kinase inhibitors (TKIs) stood out as the top inducers of both antagonism and synergy. Ruxolitinib and dasatinib emerged as the most notably antagonistic substances, exhibiting the lowest Bliss scores. In contrast, sorafenib was shown to synergize with activated PBMCs (Fig. 8e). In patients, sorafenib reaches a maximum plasma concentration of around 20 μM at standard dosing⁶⁴.

Pharmacologic doses of sorafenib have been shown to decrease the activation of effector T cells, however, at sub-pharmacologic doses sorafenib has been described to selectively increase the activation of effector T cells and suppress regulatory T cells⁶⁵. Concordantly, we observed a synergistic effect with PBMCs at 1 μ M (Fig. 8e) while treatment with 30 μ M had an antagonistic effect (data not shown).

Next, a panel comprised of 22 commonly administered concomitant drugs was tested (Fig. 8g-h). Most concomitant drugs did not induce either antagonism nor synergy. However, the statins mevastatin and simvastatin were uniquely shown to synergize with activated PBMC in the colon cancer model (Fig. 8g). Interestingly, increasing data supports the notion that statins may exert pro-inflammatory effects in patients; several recent studies suggest that statin treatment is associated with improved clinical outcomes for cancer patients receiving therapy with ICIs⁶⁶⁻⁶⁹.

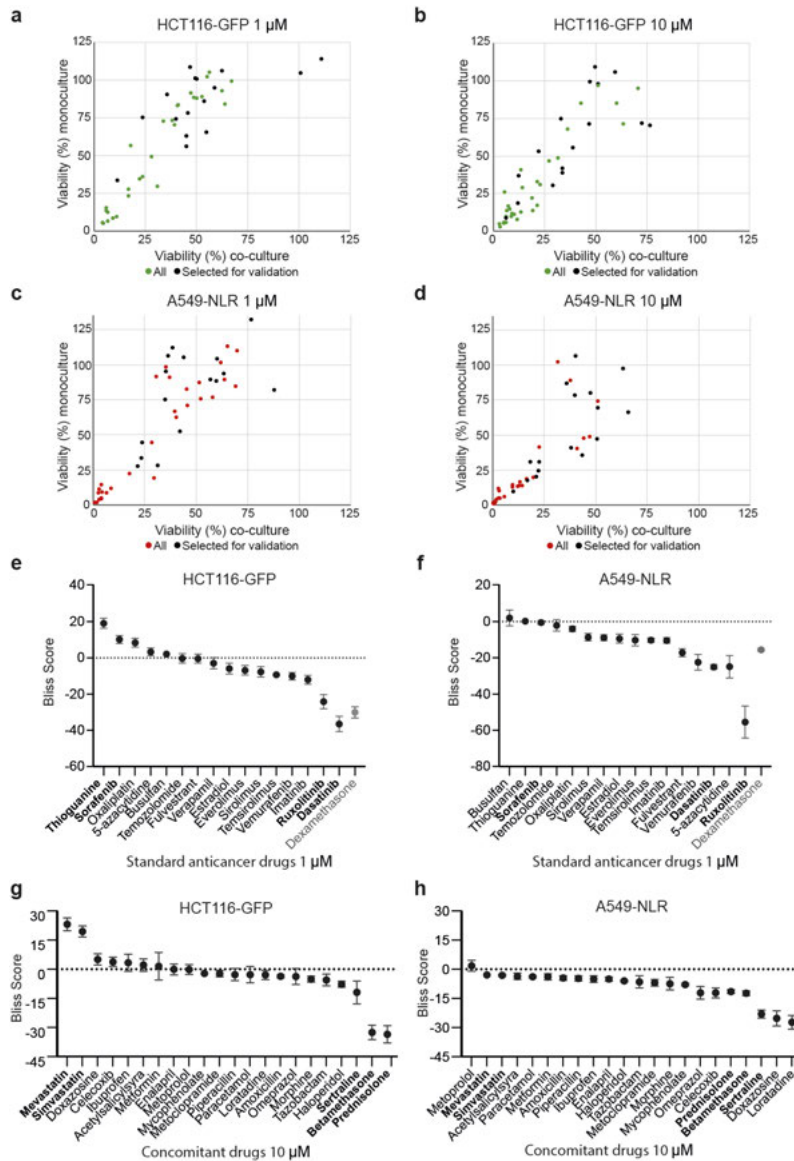


Figure 8. Evaluation of standard anticancer agents and concomitant drugs. The viability (expressed as % of control) in (a-b) HCT116-GFP and (c-d) A549-NLR mono- and co-cultures after treatment with the drug panel at 1 μ M and 10 μ M for 72 h (data shown as means from three technical replicates). (e-f) Bliss scores calculated after treatment with the validation drug panel and dexamethasone at 1 μ M for 72 h in HCT116-GFP (e) and A549-NLR (f). Data shown as mean \pm SEM from three independent experiments. (g-h) Bliss scores calculated after treatment with concomitant drugs at 10 μ M for 72 h in HCT116-GFP (g) and A549-NLR (h). Data shown as mean \pm SEM from three independent experiments.

Summary and conclusions. In this study, we co-cultured human colon and lung cancer cell lines with human PBMCs sourced from different donors. Firstly, we demonstrated the robustness and the reproducibility of the assay. Secondly, we utilized the assay to evaluate drugs commonly used by cancer patients to identify immunological effects of potential clinical relevance and to serve as a point of reference for screens of novel compound libraries. Using this approach, immunomodulatory effects exerted by TKIs and statins were identified. In summary, while not exhaustive, our model system has demonstrated the ability to capture previously described immunological effects of anticancer agents, such as the dose-dependent characteristic of sorafenib. Thus, it offers a valuable starting point for identifying potential drug-cell interactions that warrant further, more detailed investigation.

Paper III

Selvin T, et al. Phenotypic screening platform identifies statins as enhancers of immune cell-induced cancer cell death. *BMC Cancer*. 2023;23(164)

Immunomodulatory small molecules have emerged as a promising complement to current immunotherapies as they may provide enhanced tissue penetration, improved bioavailability, and the ability to reach intracellular targets³⁴. There is a need to identify novel small molecule drugs for this application; however, most phenotypic screening platforms used in the field of oncology do not allow for the identification of immunomodulatory agents. In Paper III, we addressed this by utilizing our miniaturized tumor-immune model system to screen a drug library containing 1280 small molecule drugs in an immuno-oncology setting.

Phenotypic screening platform identifies statins as immunomodulators. HCT116-GFP was cultured as monoculture and co-cultured with aCD3/IL2 activated PBMCs in a 384-well plate format (Fig. 9a). The Prestwick Chemical Library was screened in both mono- and co-cultures and the viability of the cancer cells was measured by quantification of GFP expression. The Bliss Independence Model was then used to identify small molecule drugs with the ability to potentiate immune cell-induced cancer cell death. Using this approach, 25 hit compounds were selected (Fig. 9b). Following hit validation, the statin mevastatin, together with a few additional molecules, was identified as an enhancer of immune cell-dependent cancer cell killing (Fig. 9c). Evaluation of additional statins demonstrated that the lipophilic statins mevastatin, simvastatin, pitavastatin, lovastatin and fluvastatin all synergized with aCD3/IL2 activated PBMCs (Fig. 9d).

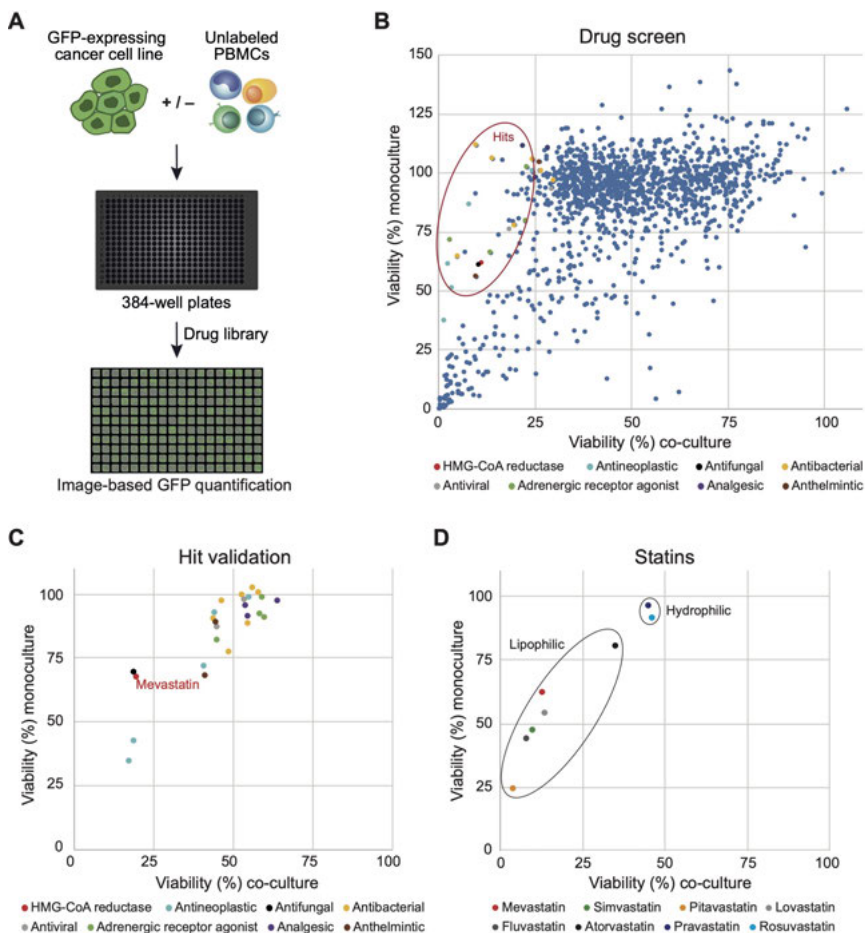


Figure 9. Phenotypic screening platform identifies statins as immunomodulators.

a) Schematic of the miniaturized 384-well plate-based model system used for drug screening: HCT116-GFP cells cultured as monoculture or co-cultured with aCD3/IL2 activated PBMCs at a 1:1 ratio. **b)** Viability of HCT116-GFP cells in monoculture (y-axis) and co-culture (x-axis) 72 h after addition of the Prestwick Chemical Library at a final drug concentration of 10 μ M. Hit compounds selected for validation experiments are encircled in red. **c)** Viability of HCT116-GFP cells, cultured as monoculture or co-cultured with PBMCs at a 1:4 ratio, 72 h after treatment with newly purchased hit compounds at a final concentration of 10 μ M (n=3). **d)** Viability of HCT116-GFP cells in mono- and co-cultures 72 h after treatment with statins at a final concentration of 10 μ M (n=3).

Pitavastatin treatment induces a pro-inflammatory gene expression profile in vitro. Pitavastatin exhibited the most potent anti-cancer effect and was selected for further analysis. Transcriptome-wide gene expression profiling was performed in HCT116-GFP monocultures, PBMC monocultures, and co-cultures. In HCT116-GFP monoculture and co-culture, pitavastatin treatment was shown to increase the expression of tumor suppressor genes such as *SI00A14*, krüppel-like factor 2 (*KLF2*) and *KLF 6* (Fig. 10a). Furthermore, pitavastatin treatment was shown to induce an overall pro-inflammatory gene expression profile. After 24 h treatment with pitavastatin (10 μ M), 158 DEGs were uniquely upregulated in co-culture (Fig. 10b). Enrichment analysis of these genes identified processes such as positive regulation of apoptotic cell clearance and regulation of immune effector processes among the top 10 enriched terms (adjusted P-value < 0.05) (Fig. 10c).

Additionally, the inflammatory mediators *CCL20* and *BIRC3* stood out in co-cultures treated with 1 μ M and 10 μ M pitavastatin as the two genes with the highest gene expression fold change increases (Fig. 10d). When analyzing the transcriptome of epithelial cells, *CCL20* is typically found among the most highly induced genes following pro-inflammatory stimuli⁷⁰, suggesting a pro-inflammatory effect of pitavastatin treatment.

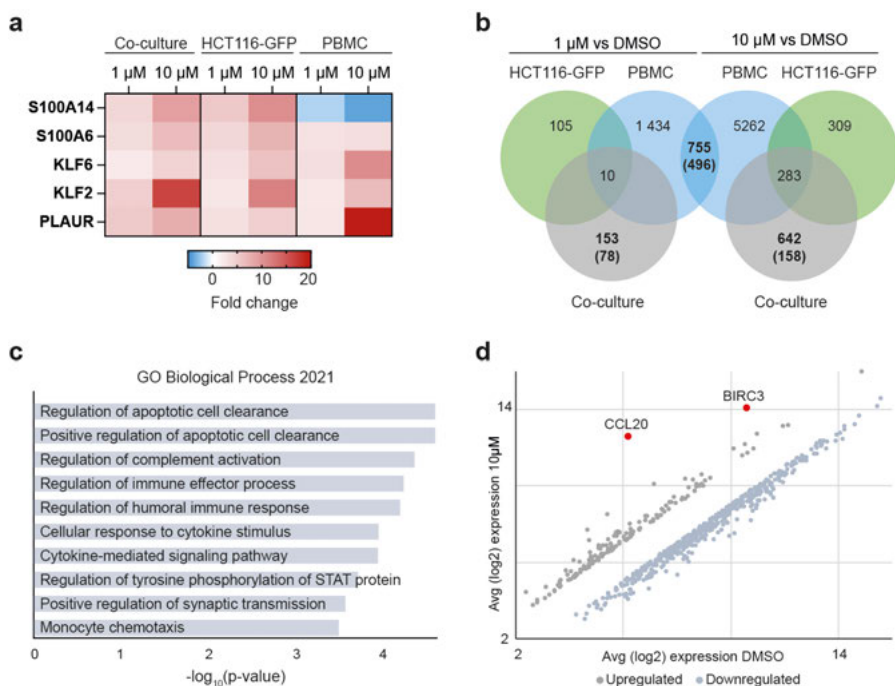


Figure 10. Pitavastatin treatment induces a pro-inflammatory gene expression profile in vitro. **a)** Gene expression fold change for DEGs common to all cultures (HCT116-GFP monoculture, PBMC-monoculture, and co-culture) after 24 h treatment with pitavastatin (1 μ M and 10 μ M) compared to DMSO vehicle. **b)** Venn diagram showing the number of DEGs (fold change > 2), and for selected cultures the number of upregulated DEGs within brackets, 24 h post treatment with pitavastatin (1 μ M and 10 μ M) compared to DMSO vehicle. **c)** Enriched terms (adjusted P-value < 0.05) obtained from enrichment analysis using the GO Biological Process 2021 database. The enrichment analysis was performed using the 158 DEGs (fold change > 2) that were uniquely upregulated in co-culture treated with 10 μ M pitavastatin. **d)** Average log₂ expression of DEGs found in co-culture treated with 10 μ M pitavastatin but not in either of the monocultures.

Summary and conclusion. In paper III, we developed a phenotypic screening platform. A pilot screen was performed which identified statins, a drug family gaining increasing interest as repurposing candidates for cancer treatment, as enhancers of immune cell-induced cancer cell death. Statins are widely prescribed to manage high cholesterol and are generally considered anti-inflammatory⁷¹. However, the data presented herein supports the growing notion that the variety of pleiotropic effects exerted by statins also include enhancement of pro-inflammatory responses. Several recent studies have reported a clinical benefit for cancer patients that receive statin treatment^{67-69,72,73}; we speculate that this results not only from statin induced tumor-cell apoptosis⁷⁴⁻⁷⁷ but rather is dependent on the combined effect exerted by statins on both cancer- and immune cells.

Paper IV

Selvin T, et al. The immuno-oncology Hollow Fiber Assay. *Manuscript*.

To facilitate the translation of novel immunotherapies from bench to bedside, continued development of predictive preclinical models is essential. Currently available model systems for evaluating immuno-oncological agents on human cancer and immune cells are labor intense and often costly. In paper IV, we developed the immuno-oncology HFA to bridge the gap between cell based in vitro assays and more complex mouse models for evaluation of immuno-oncological agents.

Development of the immuno-oncology HFA. HCT116-GFP cells were cultured as monocultures or co-cultured with PBMCs at a 1:4 ratio inside semi-permeable HF. The HF was scanned every 12 h for 4 days using the Live Cell Analysis system IncuCyte S3 (Fig. 11a). A steady cancer cell growth was observed and the formation of 3D structures could be visualized inside the HF. During initial in vitro characterization of the assay, monocultures and co-cultures were grown as conventional monolayers in parallel with the HF. IL-2 treatment was administered, with and without simultaneous T-cell activation using aCD3, to induce immune cell-mediated cancer cell death. After 4 days, the cells were harvested and clonogenic assay was performed to assess the reproductive viability of the cancer cells in the two models (Fig. 11b). As demonstrated by the number of colonies formed during clonogenic assay, the effect of immune cell stimulation was enhanced in the HF compared to in conventional monolayers (Fig. 11c-d).

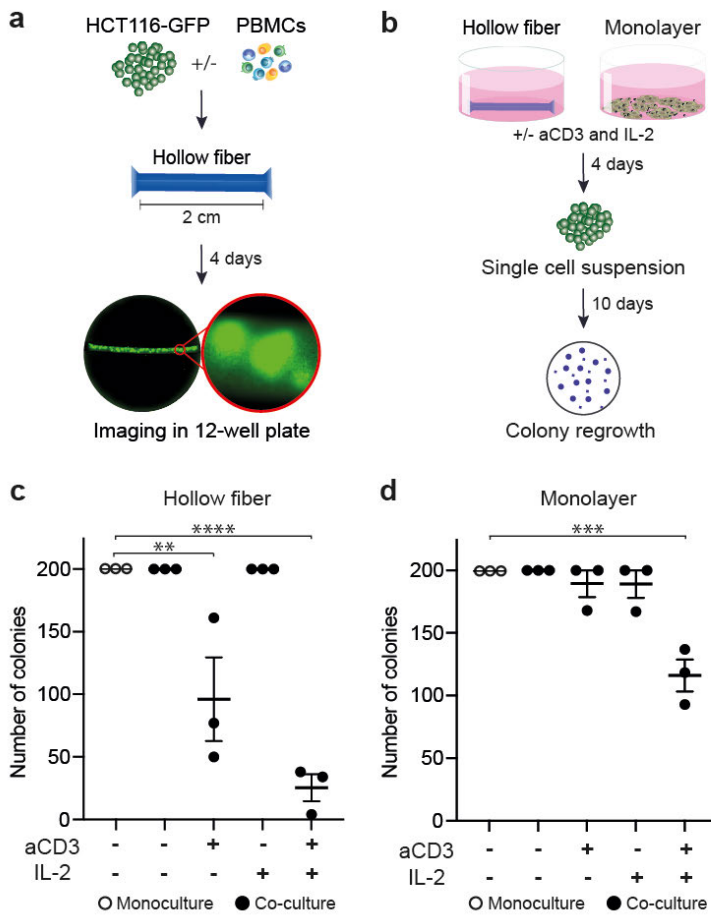


Figure 11. Development of the immuno-oncology HFA. Schematic of **a)** the immuno-oncology HFA and **b)** clonogenic assay. **c-d)** Monocultures and co-cultures grown in hollow fibers and as monolayers for 96 h with and without aCD3 stimulation and IL-2 treatment. Clonogenic assay colony count after 10 days of regrowth. Data shown as mean \pm SEM from three independent experiments. ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$ compared to untreated monoculture, determined by Ordinary one-way ANOVA with Dunnett's multiple comparison test.

Increased cell proximity augments immune cell activation and effector function. We hypothesized that the increased efficacy in the HF model could be attributed to the increased proximity of the cells. Cells cultured in HFs will be in closer proximity to each other compared to cells spread out in a monolayer which may result in improved cell-cell interactions, higher local cytokine concentrations, and a more efficient transfer of cytotoxic proteases from effector to target cells. To test the hypothesis, we employed 384-well ultra-low attachment U-bottom plates. The ultra-low attachment surface of the plates prevents cell adhesion and promotes a 3D configuration of the cells that mimic the spatial organization inside the hollow fibers. Supporting the hypothesis, the results obtained using U-bottom plates had a striking resemblance to the results obtained in the hollow fiber model. Increasing the cell proximity alone resulted in decreased cancer cell viability (Fig. 12b), increased secretion of pro-inflammatory cytokines (Fig. 12 d-g), and accelerated release of granzyme B (Fig. 12h-i).

Furthermore, as it has been suggested that GFP-labeling can exert cytotoxic effects and increase the immunogenicity of the labeled cancer cells⁷⁸, experiments in U-bottom plates were also performed with HCT116wt. In our model system, no indications of cytotoxicity or increased immunogenicity in GFP-labeled cells were observed as equivalent results were obtained with HCT116-GFP and HCT116wt (Fig. 12b-i).

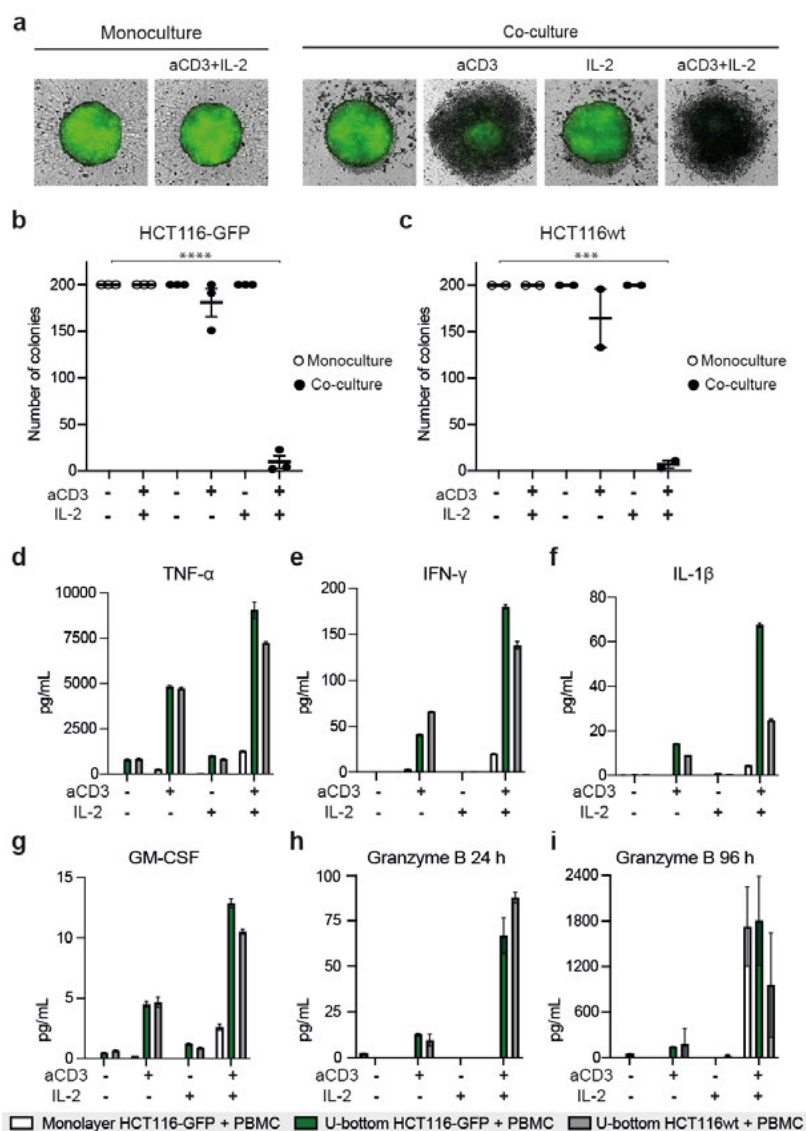


Figure 12. Increased cell proximity augments immune cell activation and effector function. **a**) Example images of HCT116-GFP monocultures and co-cultures grown in U-bottom plates for 96 h with and without aCD3 stimulation and IL-2 treatment. **b**) Clonogenic assay performed on HCT116-GFP and **c**) HCT116wt monocultures and co-cultures grown in U-bottom plates for 96 h with and without aCD3 stimulation and IL-2 treatment. Colony count performed after 10 days of regrowth in 6-well plates. Data shown as mean \pm SEM from three independent experiments for HCT116-GFP and two independent experiments for HCT116wt. *** = $P \leq 0.001$ and **** = $P \leq 0.0001$ compared to untreated monoculture, determined by Ordinary one-way ANOVA with Dunnett's multiple comparison test. **d**) TNF- α , **e**) IFN- γ , **f**) IL-1 β , **g**) CM-CSF and **h**) granzyme B concentrations measured in co-culture supernatants collected after 24 h and **i**) granzyme B after 96 h using a Luminex MAGPIX system. Data shown as mean \pm CV% from technical duplicates.

The immuno-oncology HFA captures immune cell-mediated cancer cell killing in vivo. To assess the functionality of the assay in vivo, a pilot study was performed using Hsd:Athymic Nude-Foxn1^{tm1} mice. The HFs were prepared on day -1 and cultured in vitro for 18 h before three fibers per mouse were surgically implanted i.p. The mice received local injections of 150 μ L aCD3 (100 μ g/mL) at the time of implantation and/ or systemic IL-2 via i.p. injections of 200 μ g/kg IL-2 once daily for 3 consecutive days. On day 4, the fibers were explanted and the viability of the cancer cells was assessed using clonogenic assay (Fig. 13a-b). For mice implanted with HCT116-GFP monocultures, a large variation was observed within the untreated group (Fig. 13b). However, the remaining control groups generated robust data. As expected, no effect was observed when mice implanted with HF-monocultures received the combination of aCD3 and IL-2 (Fig. 13b). In mice implanted with HF-co-cultures, the combination of aCD3 and IL-2 resulted in a significant decrease in cancer cell viability compared to untreated mice and mice receiving IL-2 alone (Fig. 13b). These data suggest that the assay is applicable also in vivo, although the variation in the monoculture control group warrants further investigation.

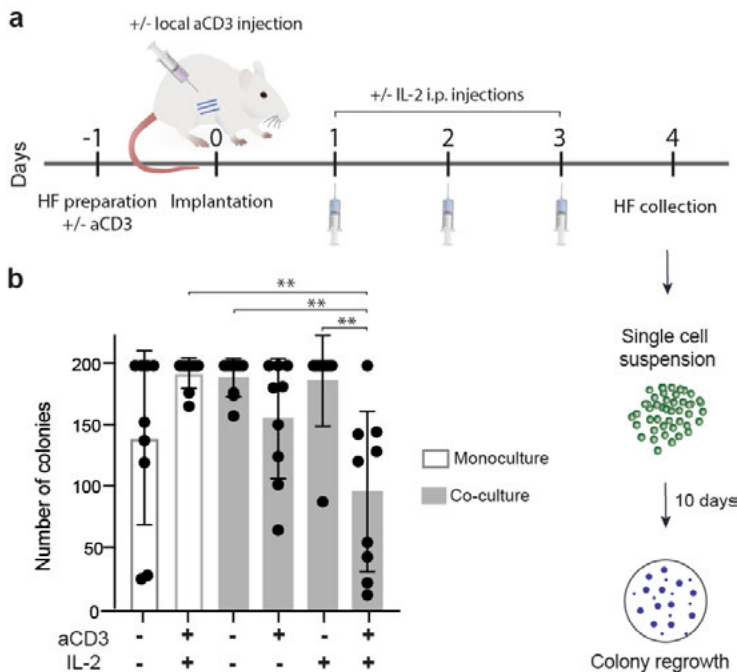


Figure 13. In vivo evaluation. **a)** Schematic illustration of the in vivo experimental layout. **b)** Clonogenic assay. Number of colonies counted after 10 days of regrowth. Each fiber (9 fibers/ treatment group) shown as an individual data point. Bars indicating means and error bars indicating SD. Ordinary one-way ANOVA with Turkey's multiple comparisons test used to compare the mean of each group to the mean of every other group, ** = $P \leq 0.01$.

Summary and conclusions. We developed the immuno-oncology HFA to serve as a tool for initial in vivo evaluation of small molecule drugs with immuno-oncological activity. The pilot study suggest that the assay can capture immune-mediated cancer cell killing in vivo, although the large variation in the monoculture control group warrants further in vivo validation. In summary, the assay currently represents a simplified model of the in vivo TME, yet, it allows for rapid assessment of immune-mediated killing within a matter of days.

Summary and Future perspectives

The overall aim of this thesis was to advance and apply preclinical model systems for the identification and evaluation of immunomodulatory drugs. In paper I, we co-cultured CRC cells with PBMCs and used scRNA-seq to study immunological effects of FTD in the co-culture system. To date, scRNA-seq is the most widely used single-cell omics in cancer research⁷⁹. Although few studies have utilized scRNA-seq to investigate mechanisms underlying immune-modulating therapies, the method has undeniably enriched the field of immuno-oncology. For example, recent scRNA-seq studies have revealed previously unknown subpopulations of immune cells, for instance CD8⁺ T cells that primarily express LAG3 rather than PD-1 or CTLA-4⁸⁰. Findings of this nature, that enhance our comprehension of the intricate TIME, will support the development of more effective treatments. However, it is worth noting that the explosion of articles featuring scRNA-seq in the last years has sparked an ongoing debate; to what extent can we derive conclusions about tumor biology solely from the analysis of transcript abundance? In light of this, I think it is safe to assume that the emerging trend of single-cell multi-omics is here to stay. By combining scRNA-seq with other omics like proteomics and epigenomics, now feasible at the single-cell level with spatial information, investigations with an unprecedented resolution are now attainable. The emergence of spatial multi-omics, and the integration of artificial intelligence-driven machine learning to manage expanding datasets, hold promise for intriguing revelations in the tumor biology field.

In recent years, we have witnessed advancements not only in omics approaches but also in the development of *in vitro* and *ex vivo* model systems. However, there is still a shortage of model systems that can capture aspects of the TIME while being compatible with high-throughput phenotypic drug screening. In paper II-III, we scaled up our tumor-immune model system to enable evaluation of large drug libraries. In paper II, we demonstrated the robustness of the assay and used it to evaluate a broad panel of standard anti-cancer agents. In paper III, it was utilized to screen 1280 FDA approved drugs. In this pilot screen, statins, a drug family gaining increasing interest as repurposing candidates for cancer treatment, were identified as enhancers of immune cell-induced cancer cell death. The literature regarding pleiotropic effects of statins is continuously growing. For instance, in a recent study by Jarr

et al., they utilized an unbiased approach to investigate gene expression patterns triggered by CD47-SIRP α blockade and revealed an unexpected link between statins and efferocytosis⁸¹. The study demonstrated that statins can enhance the phagocytic capacity of macrophages by suppressing the “don’t eat me” molecule CD47. This study focused on atherosclerosis but, as targeting phagocytosis checkpoints has emerged as a promising strategy for cancer treatment, an intriguing future direction could be to explore statin-induced CD47 suppression also in a cancer context.

The model system used to identify statins as repurposing candidates is focused on elements provided by PBMCs and their short-term interaction with each other and the target cells. While not exhaustive, the screening platform has demonstrated the ability to capture previously described immunological effects of anticancer agents and identify repurposing candidates. This underscores the feasibility of using simple means in the initial stages of drug development. Although more complex model system may offer enhanced clinical translatability, increased complexity also introduces challenges related to experimental designs, informative readouts, and data interpretation. As the drug screening field is progressively transitioning from 2D to 3D, finding a balance between reproducibility and physiological relevance is essential.

Along this line, as described in paper IV, we have established a co-culture system utilizing 384-well U-bottom ultra-low attachment plates. These plates prevent cell adhesion and thus facilitates a 3D cell configuration. The model remains robust with a straightforward readout while increasing physiological relevance in comparison to 2D models. In paper IV, the model system was only used to examine the influence of cellular proximity when characterizing the immuno-oncology HFA. However, given its characteristics, future applications will surely include immuno-oncology drug screening.

The immuno-oncology HFA assay was developed to bridge the gap between cell based in vitro assays and more complex mouse models. Although currently representing a simplified model of the TME due to its utilization of a cancer cell line and allogeneic immune cells, its clinical translatability could be enhanced by the incorporation of primary patient-derived cells. Importantly, it is not intended to replace more advanced models but rather to serve as an initial tool for in vivo evaluation, guiding the selection of compounds for further investigation. It also provides ethical advantages as shorter experimental timelines reduces the suffering of the animals. Additionally, given the complexity and timeframe associated with studies in e.g., humanized mouse models, we believe that our model system, when positioned upstream of such models, can serve as a valuable tool to decrease the number of animals used and increase the success rate in more complex models.

In summary, the work presented in this thesis provides a research approach that covers immuno-oncology drug screening, in vitro validation, and initial in vivo evaluation. As George E.P. Box once said, “All models are wrong, but some are useful”. No immuno-oncology models are perfect, but when used according to their strengths they can still provide answers to specific research questions. It is my hope that the methodologies presented herein can be of support to other researchers in their pursuit for answers.

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References

1. Klemm F, Joyce JA. Microenvironmental regulation of therapeutic response in cancer. *Trends Cell Biol.* 2015;25(4):198-213. doi:10.1016/j.tcb.2014.11.006
2. Anderson NM, Simon MC. The tumor microenvironment. *Current Biology.* 2020;30(16):R921-R925. doi:10.1016/j.cub.2020.06.081
3. Galluzzi L, Buqué A, Kepp O, Zitvogel L, Kroemer G. Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents. *Cancer Cell.* 2015;28(6):690-714. doi:10.1016/j.ccell.2015.10.012
4. Hegde PS, Chen DS. Top 10 Challenges in Cancer Immunotherapy. *Immunity.* 2020;52(1):17-35. doi:10.1016/j.immuni.2019.12.011
5. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. *Nat Rev Cancer.* 2004;4(1):11-22. doi:10.1038/nrc1252
6. Gaudino SJ, Kumar P. Cross-talk between antigen presenting cells and T cells impacts intestinal homeostasis, bacterial infections, and tumorigenesis. *Front Immunol.* 2019;10(MAR). doi:10.3389/fimmu.2019.00360
7. Aptsiauri N, Cabrera T, Garcia-Lora A, Lopez-Nevot MA, Ruiz-Cabello F, Garrido F. MHC Class I Antigens and Immune Surveillance in Transformed Cells. *Int Rev Cytol.* 2007;256:139-189. doi:10.1016/S0074-7696(07)56005-5
8. Goubran HA, Kotb RR, Stakiw J, Emara ME, Burnouf T. Regulation of Tumor Growth and Metastasis: The Role of Tumor Microenvironment. *Cancer Growth Metastasis.* 2014;7:CGM.S11285. doi:10.4137/cgm.s11285
9. Labani-Motlagh A, Ashja-Mahdavi M, Loskog A. The Tumor Microenvironment: A Milieu Hindering and Obstructing Antitumor Immune Responses. *Front Immunol.* 2020;11. doi:10.3389/fimmu.2020.00940
10. Chen DS, Mellman I. Oncology meets immunology: The cancer-immunity cycle. *Immunity.* 2013;39(1):1-10. doi:10.1016/j.immuni.2013.07.012
11. Tang S, Ning Q, Yang L, Mo Z, Tang S. Mechanisms of immune escape in the cancer immune cycle. *Int Immunopharmacol.* 2020;86. doi:10.1016/j.intimp.2020.106700
12. O'Donnell JS, Teng MWL, Smyth MJ. Cancer immunoediting and resistance to T cell-based immunotherapy. *Nat Rev Clin Oncol.* 2019;16(3):151-167. doi:10.1038/s41571-018-0142-8
13. Falzone L, Salomone S, Libra M. Evolution of cancer pharmacological treatments at the turn of the third millennium. *Front Pharmacol.* 2018;9(NOV). doi:10.3389/fphar.2018.01300

14. DeVita VT, Chu E. A history of cancer chemotherapy. *Cancer Res.* 2008;68(21):8643-8653. doi:10.1158/0008-5472.CAN-07-6611
15. Dobosz P, Dzieciatkowski T. The Intriguing History of Cancer Immunotherapy. *Front Immunol.* 2019;10. doi:10.3389/fimmu.2019.02965
16. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. *Nat Rev Immunol.* 2008;8(1):59-73. doi:10.1038/nri2216
17. Zitvogel L, Kepp O, Kroemer G. Immune parameters affecting the efficacy of chemotherapeutic regimens. *Nat Rev Clin Oncol.* 2011;8(3):151-160. doi:10.1038/nrclinonc.2010.223
18. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol.* 2013;31:51-72. doi:10.1146/annurev-immunol-032712-100008
19. Bains SJ, Abrahamsson H, Flatmark K, et al. Immunogenic cell death by neoadjuvant oxaliplatin and radiation protects against metastatic failure in high-risk rectal cancer. *Cancer Immunology, Immunotherapy.* 2020;69(3):355-364. doi:10.1007/s00262-019-02458-x
20. Kepp O, Tartour E, Vitale I, et al. Consensus guidelines for the detection of immunogenic cell death. *Oncoimmunology.* 2014;3(9). doi:10.4161/21624011.2014.955691
21. Bedognetti D, Ceccarelli M, Galluzzi L, et al. Toward a comprehensive view of cancer immune responsiveness: A synopsis from the SITC workshop. *J Immunother Cancer.* 2019;7(1). doi:10.1186/s40425-019-0602-4
22. McFarland JM, Paoletta BR, Warren A, et al. Multiplexed single-cell transcriptional response profiling to define cancer vulnerabilities and therapeutic mechanism of action. *Nat Commun.* 2020;11(1). doi:10.1038/s41467-020-17440-w
23. Villani AC, Satija R, Reynolds G, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science (1979).* 2017;356(6335). doi:10.1126/science.aah4573
24. Soneson C, Robinson MD. Bias, robustness and scalability in single-cell differential expression analysis. *Nat Methods.* 2018;15(4):255-261. doi:10.1038/nmeth.4612
25. Guruprasad P, Lee YG, Kim KH, Ruella M. The current landscape of single-cell transcriptomics for cancer immunotherapy. *Journal of Experimental Medicine.* 2021;218(1). doi:10.1084/JEM.20201574
26. Emens LA, Ascierto PA, Darcy PK, et al. Cancer immunotherapy: Opportunities and challenges in the rapidly evolving clinical landscape. *Eur J Cancer.* 2017;81:116-129. doi:10.1016/j.ejca.2017.01.035
27. He X, Xu C. Immune checkpoint signaling and cancer immunotherapy. *Cell Res.* 2020;30(8):660-669. doi:10.1038/s41422-020-0343-4
28. Vaddepally RK, Kharel P, Pandey R, Garje R, Chandra AB. Review of indications of FDA-approved immune checkpoint inhibitors per NCCN guidelines with the level of evidence. *Cancers (Basel).* 2020;12(3). doi:10.3390/cancers12030738
29. Aggarwal V, Workman CJ, Vignali DAA. LAG-3 as the third checkpoint inhibitor. *Nat Immunol.* Published online 2023. doi:10.1038/s41590-023-01569-z

30. Mantovani A, Allavena P, Marchesi F, Garlanda C. Macrophages as tools and targets in cancer therapy. *Nat Rev Drug Discov.* 2022;21(11):799-820. doi:10.1038/s41573-022-00520-5
31. Feng M, Jiang W, Kim BYS, Zhang CC, Fu YX, Weissman IL. Phagocytosis checkpoints as new targets for cancer immunotherapy. *Nat Rev Cancer.* 2019;19(10):568-586. doi:10.1038/s41568-019-0183-z
32. Chen J, Cao X, Li B, et al. Warburg Effect Is a Cancer Immune Evasion Mechanism Against Macrophage Immunosurveillance. *Front Immunol.* 2021;11. doi:10.3389/fimmu.2020.621757
33. Dhanak D, Edwards JP, Nguyen A, Tummino PJ. Small-Molecule Targets in Immuno-Oncology. *Cell Chem Biol.* 2017;24(9):1148-1160. doi:10.1016/j.chembiol.2017.08.019
34. Cheng B, Yuan WE, Su J, Liu Y, Chen J. Recent advances in small molecule based cancer immunotherapy. *Eur J Med Chem.* 2018;157:582-598. doi:10.1016/j.ejmech.2018.08.028
35. Chacon AC, Melucci AD, Qin SS, Prieto PA. Thinking small: Small molecules as potential synergistic adjuncts to checkpoint inhibition in melanoma. *Int J Mol Sci.* 2021;22(6):1-26. doi:10.3390/ijms22063228
36. Wang M, Liu Y, Cheng Y, Wei Y, Wei X. Immune checkpoint blockade and its combination therapy with small-molecule inhibitors for cancer treatment. *Biochim Biophys Acta Rev Cancer.* 2019;1871(2):199-224. doi:10.1016/j.bbcan.2018.12.002
37. Zhang Z, Zhou L, Xie N, et al. Overcoming cancer therapeutic bottleneck by drug repurposing. *Signal Transduct Target Ther.* 2020;5(1). doi:10.1038/s41392-020-00213-8
38. Pushpakom S, Iorio F, Eyers PA, et al. Drug repurposing: Progress, challenges and recommendations. *Nat Rev Drug Discov.* 2018;18(1):41-58. doi:10.1038/nrd.2018.168
39. Rehman W, Arfons LM, Lazarus HM. The rise, fall and subsequent triumph of thalidomide: Lessons learned in drug development. *Ther Adv Hematol.* 2011;2(5):291-308. doi:10.1177/2040620711413165
40. Coussens NP, Braisted JC, Peryea T, Sittampalam GS, Simeonov A, Hall MD. Small-molecule screens: A gateway to cancer therapeutic agents with case studies of food and drug administration-approved drugs. *Pharmacol Rev.* 2017;69(4):479-496. doi:10.1124/pr.117.013755
41. Mo X, Tang C, Niu Q, Ma T, Du Y, Fu H. HTiP: High-Throughput Immunomodulator Phenotypic Screening Platform to Reveal IAP Antagonists as Anti-cancer Immune Enhancers. *Cell Chem Biol.* 2019;26(3):331-339.e3. doi:10.1016/j.chembiol.2018.11.011
42. Steff AM, Ne Fortin M, Arguin C, Hugo P. Detection of a Decrease in Green Fluorescent Protein Fluorescence for the Monitoring of Cell Death: An Assay Amenable to High-Throughput Screening Technologies. Published online 2001. doi:10.1002/cyto.10024
43. McCarthy CE, Zahir N, Eljanne M, Sharon E, Voest EE, Palucka K. Developing and validating model systems for immuno-oncology. *Cancer Cell.* 2021;39(8):1018-1022. doi:10.1016/j.ccell.2021.05.017

44. Bareham B, Georgakopoulos N, Matas-Céspedes A, Curran M, Saeb-Parsy K. Modeling human tumor-immune environments in vivo for the preclinical assessment of immunotherapies. *Cancer Immunology, Immunotherapy*. 2021;70(10):2737-2750. doi:10.1007/s00262-021-02897-5
45. Kleiveland C. Peripheral blood mononuclear cells. In: *The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models*. Springer International Publishing; 2015:161-167. doi:10.1007/978-3-319-16104-4_15
46. Anderson NM, Simon MC. The tumor microenvironment. *Current Biology*. 2020;30(16):R921-R925. doi:10.1016/j.cub.2020.06.081
47. Barbosa MAG, Xavier CPR, Pereira RF, Petrikaitė V, Vasconcelos MH. 3D Cell Culture Models as Recapitulators of the Tumor Microenvironment for the Screening of Anti-Cancer Drugs. *Cancers (Basel)*. 2022;14(1). doi:10.3390/cancers14010190
48. Models for Immuno-oncology Research. *Cancer Cell*. 2020;38(2):145-147. doi:10.1016/j.ccell.2020.07.010
49. Boucherit N, Gorvel L, Olive D. 3D Tumor Models and Their Use for the Testing of Immunotherapies. *Front Immunol*. 2020;11. doi:10.3389/fimmu.2020.603640
50. Varesano S, Zocchi MR, Poggi A. Zoledronate triggers Vδ2 T cells to destroy and kill spheroids of colon carcinoma: Quantitative image analysis of three-dimensional cultures. *Front Immunol*. 2018;9(MAY). doi:10.3389/fimmu.2018.00998
51. Courau T, Bonnereau J, Chicoteau J, et al. Cocultures of human colorectal tumor spheroids with immune cells reveal the therapeutic potential of MICA/B and NKG2A targeting for cancer treatment. *J Immunother Cancer*. 2019;7(1). doi:10.1186/s40425-019-0553-9
52. Saito R, Kobayashi T, Kashima S, Matsumoto K, Ogawa O. Faithful preclinical mouse models for better translation to bedside in the field of immuno-oncology. *Int J Clin Oncol*. 2020;25(5):831-841. doi:10.1007/s10147-019-01520-z
53. de La Rochere P, Guil-Luna S, Decaudin D, Azar G, Sidhu SS, Piaggio E. Humanized Mice for the Study of Immuno-Oncology. *Trends Immunol*. 2018;39(9):748-763. doi:10.1016/j.it.2018.07.001
54. Decker S, Hollingshead M, Bonomi CA, Carter JP, Sausville EA. The hollow fibre model in cancer drug screening: The NCI experience. *Eur J Cancer*. 2004;40(6):821-826. doi:10.1016/j.ejca.2003.11.029
55. Berenbaum MC. What is Synergy? *Pharmalogical Revs*. 1989;(41).
56. Yoshino T, Mizunuma N, Yamazaki K, et al. TAS-102 monotherapy for pretreated metastatic colorectal cancer: A double-blind, randomised, placebo-controlled phase 2 trial. *Lancet Oncol*. 2012;13(10):993-1001. doi:10.1016/S1470-2045(12)70345-5
57. Chan BM, Hochster HS, Lenz HJ. The safety and efficacy of trifluridine-tipiracil for metastatic colorectal cancer: A pharmacy perspective. *American Journal of Health-System Pharmacy*. 2019;76(6):339-348. doi:10.1093/ajhp/zxy006

58. Limagne E, Thibaudin M, Nuttin L, et al. Trifluridine/tipiracil plus oxaliplatin improves PD-1 blockade in colorectal cancer by inducing immunogenic cell death and depleting macrophages. *Cancer Immunol Res.* 2019;7(12):1958-1969. doi:10.1158/2326-6066.CIR-19-0228
59. Matsuoka K, Iimori M, Niimi S, et al. Trifluridine induces p53-dependent sustained G2 phase arrest with its massive misincorporation into DNA and few DNA strand breaks. *Mol Cancer Ther.* 2015;14(4):1004-1013. doi:10.1158/1535-7163.MCT-14-0236
60. Restifo NP, Marincola FM, Kawakami Y, et al. *Loss of Functional Beta 2-Microglobulin in Metastatic Melanomas From Five Patients Receiving Immunotherapy.*
61. Zaretsky JM, Garcia-Diaz A, Shin DS, et al. Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *New England Journal of Medicine.* 2016;375(9):819-829. doi:10.1056/nejmoa1604958
62. Anderson P, Aptsiauri N, Ruiz-Cabello F, Garrido F. HLA class I loss in colorectal cancer: implications for immune escape and immunotherapy. *Cell Mol Immunol.* 2021;18(3):556-565. doi:10.1038/s41423-021-00634-7
63. Lindhagen E, Nygren P, Larsson R. The fluorometric microculture cytotoxicity assay. *Nat Protoc.* 2008;3(8):1364-1369. doi:10.1038/nprot.2008.114
64. Liston DR, Davis M. Clinically relevant concentrations of anticancer drugs: A guide for nonclinical studies. *Clinical Cancer Research.* 2017;23(14):3489-3498. doi:10.1158/1078-0432.CCR-16-3083
65. Cabrera R, Ararat M, Xu Y, et al. Immune modulation of effector CD4+ and regulatory T cell function by sorafenib in patients with hepatocellular carcinoma. *Cancer Immunology, Immunotherapy.* 2013;62(4):737-746. doi:10.1007/s00262-012-1380-8
66. Zhang Y, Chen H, Chen S, Li Z, Chen J, Li W. The effect of concomitant use of statins, NSAIDs, low-dose aspirin, metformin and beta-blockers on outcomes in patients receiving immune checkpoint inhibitors: a systematic review and meta-analysis. *Oncoimmunology.* 2021;10(1):1957605. doi:10.1080/2162402x.2021.1957605
67. Rossi A, Filetti M, Taurelli Salimbeni B, et al. Statins and immunotherapy: Togetherness makes strength The potential effect of statins on immunotherapy for NSCLC. *Cancer Rep.* 2021;4(4). doi:10.1002/cnr2.1368
68. Omori M, Okuma Y, Hakozaki T, Hosomi Y. Statins improve survival in patients previously treated with nivolumab for advanced non-small cell lung cancer: An observational study. *Mol Clin Oncol.* Published online November 13, 2018. doi:10.3892/mco.2018.1765
69. Cantini L, Pecci F, Hurkmans DP, et al. High-intensity statins are associated with improved clinical activity of PD-1 inhibitors in malignant pleural mesothelioma and advanced non-small cell lung cancer patients. *Eur J Cancer.* 2021;144:41-48. doi:10.1016/j.ejca.2020.10.031
70. Williams IR. CCR6 and CCL20: Partners in intestinal immunity and lymphorganogenesis. In: *Annals of the New York Academy of Sciences.* Vol 1072. Blackwell Publishing Inc.; 2006:52-61. doi:10.1196/annals.1326.036

71. Jain MK, Ridker PM. Anti-inflammatory effects of statins: Clinical evidence and basic mechanisms. *Nat Rev Drug Discov.* 2005;4(12):977-987. doi:10.1038/nrd1901
72. Ren QW, Yu SY, Teng THK, Li X. Statin associated lower cancer risk and related mortality in patients with heart failure. *Eur Heart J.* 2021;42(32):3060-3062. doi:10.1093/eurheartj/ehab482
73. Kotti A, Holmqvist A, Albertsson M, Sun XF. Survival benefit of statins in older patients with rectal cancer: A Swedish population-based cohort study. *J Geriatr Oncol.* 2019;10(5):690-697. doi:10.1016/j.jgo.2019.01.011
74. Alizadeh J, Zeki AA, Mirzaei N, et al. Mevalonate Cascade Inhibition by Simvastatin Induces the Intrinsic Apoptosis Pathway via Depletion of Isoprenoids in Tumor Cells. *Sci Rep.* 2017;7. doi:10.1038/srep44841
75. Jiang P, Mukthavaram R, Chao Y, et al. In vitro and in vivo anti-cancer effects of mevalonate pathway modulation on human cancer cells. *Br J Cancer.* 2014;111(8):1562-1571. doi:10.1038/bjc.2014.431
76. Newman A, Clutterbuck RD, Powles RL, Catovsky D, Millar JL. A comparison of the effect of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors simvastatin, lovastatin and pravastatin on leukaemic and normal bone marrow progenitors. *Leuk Lymphoma.* 1997;24(5-6):533-537. doi:10.3109/10428199709055590
77. Garwood ER, Kumar AS, Baehner FL, et al. Fluvastatin reduces proliferation and increases apoptosis in women with high grade breast cancer. *Breast Cancer Res Treat.* 2010;119(1):137-144. doi:10.1007/s10549-009-0507-x
78. Ansari AM, Ahmed AK, Matsangos AE, et al. Cellular GFP Toxicity and Immunogenicity: Potential Confounders in in Vivo Cell Tracking Experiments. *Stem Cell Rev Rep.* 2016;12(5):553-559. doi:10.1007/s12015-016-9670-8
79. Huang D, Ma N, Li X, et al. Advances in single-cell RNA sequencing and its applications in cancer research. *J Hematol Oncol.* 2023;16(1):98. doi:10.1186/s13045-023-01494-6
80. Li PH, Kong XY, He YZ, et al. Recent developments in application of single-cell RNA sequencing in the tumour immune microenvironment and cancer therapy. *Mil Med Res.* 2022;9(1). doi:10.1186/s40779-022-00414-y
81. Jarr KU, Ye J, Kojima Y, et al. The pleiotropic benefits of statins include the ability to reduce CD47 and amplify the effect of pro-ferocytic therapies in atherosclerosis. *Nature Cardiovascular Research.* 2022;1(3):253-262. doi:10.1038/s44161-022-00023-x

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