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Universidade do Minho
Escola de Medicina

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Tackling metabolism in cancer cells: implications for gastrointestinal cancer therapy

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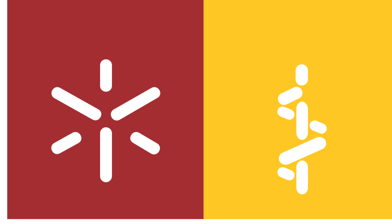

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Tackling metabolism in cancer cells: implications for gastrointestinal cancer therapy

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Trabalho efetuado sob a orientação da
Professora Doutora Fátima Baltazar
e da
Professora Doutora Ana Preto

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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Combater o metabolismo nas células cancerígenas: implicações para a terapia do cancro gastrointestinal

Para atender às suas necessidades catabólicas e anabólicas, necessárias para manter a homeostasia e a proliferação celular, as células cancerígenas reprogramam o seu metabolismo. Otto Warburg introduziu o conceito de que as células cancerígenas alteram as fontes energéticas para auxiliar a sua proliferação, uma alteração específica dos tumores conhecida como "efeito de Warburg". Este comportamento confere uma vantagem metabólica às células cancerígenas, uma vez que fornece energia e compostos intermediários necessários à síntese de novos componentes celulares. O metabolismo das células cancerígenas cria também um microambiente tumoral (TME) limitado em nutrientes e imunossupressor, que diminui a capacidade das células imunes de montar uma resposta adequada contra o cancro. Assim, o metabolismo aberrante das células cancerígenas apresenta oportunidades terapêuticas atrativas para combater o cancro. O trabalho apresentado foca-se na compreensão do papel do metabolismo das células cancerígenas na tumorigénese e nos benefícios terapêuticos em inibir proteínas-chave nestas vias para superar a resistência à quimioterapia convencional e ao bloqueio de checkpoints imunológicos (ICB).

Neste sentido, investigamos o papel dos transportadores de monocarboxilatos (MCTs), proteínas responsáveis pelo transporte transmembranar de lactato e protões, na sobrevivência das células do cancro colorretal (CRC) e explorá-los como alvos terapêuticos, isoladamente ou em combinação com 5-fluorouracilo (5-FU). A inibição da atividade ou silenciamento da expressão dos MCTs diminuíram o crescimento celular, inibiram o metabolismo glicolítico e proliferação, aumentaram a morte celular e potenciaram a citotoxicidade do 5-FU nas células de CRC.

Na segunda parte desta tese identificamos a citidina desaminase (CDA), uma enzima da via de recuperação das pirimidinas, como um potencial alvo envolvido na falta de resposta ao ICB. A inativação genética e a inibição farmacológica da CDA nas células cancerígenas sensibilizou os tumores de adenocarcinoma ductal pancreático (PDAC) para o ICB e aumentou o recrutamento de células T citotóxicas ativadas e macrófagos associados a tumores do tipo M1 nestes tumores.

Em suma, o trabalho apresentado nesta tese contribui para um melhor entendimento do papel do metabolismo das células cancerígena na tumorigénese e resistência à terapia farmacológica.

Palavras-chave: 5-fluorouracilo, bloqueio de checkpoints imunológicos, citidina desaminase, transportadores de monocarboxilatos.

Tackling metabolism in cancer cells: implications for gastrointestinal cancer therapy

In order to match their catabolic and anabolic requirements, necessary to maintain cellular homeostasis and cell proliferation, cancer cells reprogram their metabolism. Otto Warburg introduced the concept that cancer cells switch their energy sources to support their proliferation, a tumour-specific alteration known as "Warburg effect". This metabolic behaviour confers a metabolic advantage to cancer cells as it provides energy and intermediates necessary for the synthesis of cell building blocks. The metabolism of cancer cells also creates a nutrient deprived and immunosuppressive tumour microenvironment (TME) that dampens the capacity of immune cells to mount a proper immune response against cancer. Therefore, the aberrant metabolism of cancer cells presents attractive therapeutic opportunities to fight cancer. The work presented here focus on understanding the role of cancer cell metabolism in tumorigenesis and the therapeutic benefits of tackling key players in metabolic pathways to overcome resistance to conventional chemotherapy and immune checkpoint blockade (ICB).

Here we investigated the role of monocarboxylate transporters (MCTs), proteins responsible for the transmembrane transport of lactate and protons, in the survival of colorectal cancer (CRC) cells and explored them as therapeutic targets alone, or in combination with 5-fluorouracil (5-FU). MCT activity inhibition or expression silencing decreased cell growth, disrupted the glycolytic metabolism, inhibited proliferation, enhanced cell death and potentiated 5-FU cytotoxicity in CRC cells.

In the second part of this thesis we identified cytidine deaminase (CDA), an enzyme of the pyrimidine salvage pathway, as a potential target involved in unresponsiveness to ICB. Genetic inactivation or pharmacological inhibition of CDA in cancer cells sensitized pancreatic ductal adenocarcinoma (PDAC) tumours to ICB and increased the recruitment of activated cytotoxic T cells and M1-like anti-tumour associated macrophages into these tumours.

In summary, the work presented in this thesis contributes to our understanding of the role of cancer cell metabolism in tumorigenesis and resistance to pharmacological therapy.

Keywords: 5-fluorouracil, cytidine deaminase, immune checkpoint blockade, monocarboxylate transporters.

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List of Abbreviations

5’-NT - 5’-nucleotidase

5-FU - 5-Fluorouracil

7ACC2 - 7-(N-benzyl-N-methylamino)- 2-oxo-2H-chromene-3-carboxylic acid

ACT - Adoptive T Cell Transfer

AID - Activation-induced Deaminase

AMP - Adenosine Monophosphate

APC - Adenomatous Polyposis Coli

APCs - Antigen-presenting Cells

APOBECs - Apolipoprotein B mRNA Editing Catalytic Polypeptide-like

ATCC - American Type Culture Collection

ATM - Ataxia Telangiectasia Mutated

BAX - BCL2 Associated X, Apoptosis Regulator

BCR - B Cell Receptor

BIOMEX - Biological Interpretation of Metabolomics Experiments

BMDCs - Bone Marrow-derived Dendritic Cells

BMDMs - Bone Marrow-derived Macrophages

BrdU - Bromodeoxyuridine

CA - Carbonic Anhydrases

CAFs - Cancer-associated Fibroblasts

CDA - Cytidine Deaminase

CDC4 - Cell Division Control Protein 4

cDCs - Conventional DCs

CDK - Cyclin-dependent Kinase

CDKN2A - CDK Inhibitor 2A

CFSE - Carboxyfluorescein Succinimidyl Ester

CGI - CpG Island

CHC - α-cyano-4-hydroxycinnamate

CI - Combination Index

CIMP - CpG Island Methylator Phenotype

CIMP-H - CIMP-High

CIN - Chromosomal Instability

CMV - Cytomegalovirus

CRC - Colorectal Cancer

CSF1R - Colony-stimulating Factor 1 Receptor

CTLA-4 - Cytotoxic T-Lymphocyte Associated Protein 4

CTLs - Cytotoxic T lymphocytes

DAC - 5-aza-2′-deoxycytidine

DAMPs - Danger-associated Molecular Patterns

DBDS - 4,4'-dibenzamidostilbene-2,2'-disulfonate

DCC - Deleted in Colorectal Cancer

dCK - Deoxycytidine Kinase

DCs - Dendritic Cells

DCTD - Deoxycytidine Monophosphate Deaminase

dFdC - Gemcitabine

dFdCDP - Gemcitabine Diphosphate

dFdCMP - Gemcitabine Monophosphate

dFdCTP - 2',2'-difluoro-2'-deoxycytidine triphosphate /Gemcitabine Triphosphate

dFdU - 2'-deoxy-2',2'-difluorouridine

dFdUMP - 2'-deoxy-2',2'-difluoro-uridine monophosphate

DHAP - Dihydroxyacetone Phosphate

DHFU - Dihydrofluorouracil

DIDS - 4,4'-diisothiocyanostilbene-2,2'-disulfonate

DMSO - Dimethyl Sulfoxide

DPD - Dihydropyrimidine Dehydrogenase

ECL - Enhanced Chemiluminescent

EDTA - Ethylenediamine Tetraacetic Acid

EGFR - Epidermal Growth Factor Receptor

EMMPRIN - Extracellular Matrix Metalloproteinase Inducer

FACS - Fluorescence-activated Cell Sorting

FAO - Fatty Acid β-oxidation

FAP - Familial Adenomatous Polyposis

F-BAL - Fluoro-b-alanine

FBS - Fetal Bovine Serum

FDG - 2-(^{18}F)-fluoro-2-deoxy-D-glucose

FdUDP - Fluorodeoxyuridine Diphosphate

FdUMP - Fluorodeoxyuridine Monophosphate

FdUTP - Fluorodeoxyuridine Triphosphate

FMO - Fluorescence Minus One

FoxP3 - Forkhead Box P3

fTHF - Formyl-tetrahydrofolate

FUDP - Fluorouridine Diphosphate

FUDR - Fluorodeoxyuridine

FUMP - Fluouroxyuridine Monophosphate

FUPA - Fluoroureidopropionate

FUR - Fluorouridine

FUTP - Fluorouridine Triphosphate

G418 - Geneticin

GI - Gastrointestinal

GM-CSF - Granulocyte-Macrophage Colony-Stimulating-Factor

GMP - Guanosine Monophosphate

GZMB - Granzyme B

HDI - Human Development Index

HGPRT - Hypoxanthine Guanine Phosphoribosyl Transferase

HIF-1 - Hypoxia-inducible Factor

HNPCC - Hereditary Nonpolyposis Colorectal Cancer

HPF - High-power Fields

I.P. - Intraperitoneally

ICB - Immune Checkpoint Blockade

IDO1 - Indoleamine 2,3-dioxygenase 1

IFN-γ - Interferon-γ

IGF2R - Insulin-like Growth Factor 2 Receptor

ILCs - Innate Lymphoid Cells

IMP - Inosine Monophosphate

LAG-3 - Lymphocyte-activation Gene 3

LC-MS - Liquid Chromatography-mass Spectrometry

LOH - Loss of Heterozygosity

MAPK - Mitogen-activated Protein Kinase

MCTs - Monocarboxylate Transporters

MDSCs - Myeloid Derived Suppressive Cells

MHC - Major Histocompatibility Complex

MINT1 - Methylated in Tumors 1

MMPs - Matrix Metalloproteinases

MMR - Mismatch Repair

MSI - Microsatellite Instability

MSI-H - Microsatellite Instability-High

MSI-L - Microsatellite Instability-Low

MSS - MSI Stable

NEAA - Nonessential Amino Acids

NHE1 - Na+/H+ Exchanger 1

NK - Natural Killer

NR5A2 - Nuclear Receptor Subfamily 5, Group A, Member 2

NTPs - Ribonucleoside Triphosphates

OPRT - Orotate Phosphoribosyltransferase

OVA - Ovalbumin

OXPHOS - Oxidative Phosphorylation

PALB2 - Partner and Localizer of BRCA2

PanINs - Pancreatic Intraepithelial Neoplasias

PARP-1 - poly(ADP-ribose) Polymerase 1

PCA - Principle Component Analysis

PD-1 - Programmed Cell Death Protein 1

PDAC - Pancreatic Ductal Adenocarcinoma

pDCs - Plasmacytoid DCs

PDK-1 - Pyruvate Dehydrogenase Kinase-1

Pen/Strep - Penicillin-Streptomycin

PET - Positron Emission Tomography

PFA - Paraformaldehyde

PGC-1α - Peroxisome Proliferator-activated Receptor-γ Coactivator

PID - Pre-immune Donkey Serum

PIK3CA - Phosphatidylinositol 4,5-bisphosphate 3-kinase Catalytic Subunit Alpha

PKM2 - Pyruvate Kinase M2

PMA - Phorbol 12-myristate 13-acetate

PPP - Pentose Phosphate Pathway

PPRs - Pattern Recognition Receptors

PRPP - 5-phosphoribose-1-pyrophosphate

PRSS1 - Protease, Serine 1

RNA-seq - RNA-sequencing

RNR - Ribonucleotide Reductase

RT - Room Temperature

S.C. - Subcutaneously

SCFA - Short Chain Fatty Acids

SEM - Standard Error of the Mean

SLC - Solute Carrier

SMAD4 - SMAD Family Member 4

SPINK1 - Serine Peptidase Inhibitor, Kazal type 1

SRB - Sulforhodamine B

STK11 - Serine/Threonine Kinase 11

TAMs - Tumour-associated Macrophages

TANs - Tumour-associated Neutrophils

TBS - Tris Buffered Saline

TCA - Tricarboxylic Acid

TCR - T Cell Receptor

Tfh - T Follicular Helper

TGFBR2 - TGFβ Receptor 2

TGF-β - Transforming Growth Factor-β

THU - Tetrahydrouridine

TIGIT - T-cell Immunoreceptor with Ig and ITIM Domains

TK - Thymidine Kinase

TKTL1 - Transketolase 1

TMDs - Transmembrane Domains

TME - Tumour Microenvironment

TNB - Tris-NaCl-blocking

TNF-α - Tumour Necrosis Factor α

TNM - Tumour, Nodes, and Metastasis

Treg - Regulatory T Cells

T_{RM} - Tissue-resident memory T Cells

TS - Thymidylate Synthase

UK - Uridine Kinase

UMP - Uridine Monophosphate

UP - Uridine Phosphorylase

VEGF - Vascular Endothelial Growth Factor

Aims and Thesis Layout

Cancer cells reprogram their metabolic pathways in order to fulfil their catabolic and anabolic requirements, necessary to maintain a chronic and uncontrolled cell growth and division. By rewiring their metabolism, cancer cells are able to survive and proliferate while also influencing the capacity of immune cells to mount a proper and efficient immune response against cancer. Despite the fact that the aberrant metabolism of cancer cells presents an attractive therapeutic opportunity to fight cancer, it is necessary to understand the biological role and therapeutic value of cancer metabolism in different steps of carcinogenic progression. The main **aim** of the work presented in this thesis is to understand the role of metabolism in cancer survival and progression, and immune surveillance escape, by providing evidence for the therapeutic potential of targeting different metabolic pathways. This thesis is organized in four individual chapters.

Chapter 1 contains a general introduction to the thesis subject, aiming to provide sufficient and solid information on the "state of the art" in order to prepare the reader for the research topics addressed in the following chapters. In the beginning of this chapter, a brief introduction is given on colorectal and pancreatic cancer epidemiology and risk factors, staging, carcinogenesis and current treatment options. Following this there will be a focus on two hallmarks of cancer: reprogramming of energy metabolism and avoiding immune destruction, as they will be the main focus of **Chapters 2** and **3**.

Chapter 2 focuses on the role of monocarboxylate transporters (MCTs) on the survival of colorectal cancer and the potential use of these transporters, alone or in combination with 5-fluorouracil (5-FU), the standard of care for colorectal cancer, as targets for cancer therapy.

Chapter 3 attempts to uncover the role of cytidine deaminase (CDA) in resistance to cancer immunotherapy, namely the immune checkpoint blocker α-PD-1. Specifically, we addressed how CDA contributes to immunosuppression, namely the consequences of its genetic inactivation and pharmacological inhibition in combination with α-PD-1 on tumour growth and remodelling of the pancreatic cancer immune landscape.

In **Chapter 4**, the main conclusions of **Chapters 2** and **3** are summarised by highlighting the major findings of the research work presented. It includes an overall discussion and some important future directions are suggested to complement the research results presented in this thesis.

CHAPTER 1. General Introduction

1.1 Cancer

Cancer is a term used to define a group of related diseases that can affect any part of the body. All types of cancer share a common feature: an abnormal growth of a clonal population of cells beyond their usual boundaries and consequent capacity to invade adjoining parts of its host and spread into surrounding tissues or distant organs, the latter process known as metastization [1].

Cancer is a major global public health problem, despite consistent medical development and scientific research. The global cancer burden has more than doubled during the last 30 years, representing the second leading cause of death globally. It was estimated that in 2018, 18.1 million new cases occurred and this malignancy was responsible for 9.6 million deaths. The most commonly diagnosed cancer in both sexes combined are lung and breast (2 million cases, 11.6% of total, each), with lung cancer being the leading cause of cancer-related death (18.4% of the total cancer deaths) [2]. Therefore, it is critical to develop new and efficient anti-cancer therapies to revert this dramatic scenario.

1.1.1. Gastrointestinal cancers

The human digestive system is made up of the gastrointestinal (GI) tract, a series of hollow organs comprised by the mouth, oesophagus, stomach, small intestine, large intestine and anus, and additional solid organs including the pancreas, liver, gallbladder and biliary ducts. Due to the substantial cellular mass and rapid turnover of cells in these organs, GI cancers are among the most frequent malignancies [2].

Among GI cancers, colorectal cancer (CRC) is the most frequent (1.8 million new cases, 10% of all sites), followed by stomach (1 million, 5.7%), liver (0.84 million, 4.7%), oesophagus (0.57 million, 3.2%) and pancreas (0.46 million, 2.5%). Concerning the number of cancer related-deaths, CRC is the most lethal (9% of all sites), followed by stomach and liver cancer (8.2% each), oesophagus (5.3%) and pancreas (4.5%) [2].

1.2. Epidemiology and risk factors

1.2.1 Colorectal cancer

Accounting for nearly 1 in 10 cancer cases and deaths, CRC is the third most frequent malignancy in terms of incidence but the second most lethal when comparing with all cancer sites,

and the first when considering only GI cancers. CRC cancer affects men and women almost equally, with an incidence of 10.9% in men and 9.5% in women and a mortality of 9.0% in men and 9.5% in women. However, this malignancy is not uniformly common throughout the world and shows a large geographic discrepancy in the global distribution. The highest incidence rate of CRC is observed in regions with high Human Development Index (HDI) scores, namely parts of Europe, Australia/New Zealand, Northern America and Eastern Asia. On the other hand, most regions of Southern Asia and Africa show lower incidence rates of CRC cancer. Therefore, CRC can be considered as a marker of socioeconomic development [2-4].

CRC is a result of complex networks between genetic and environmental risk factors [5]. They fall into two major categories: non-modifiable and environmental (modifiable). Non-modifiable are those risk factors that an individual cannot control, such as: age, personal history of adenomatous polyps, inflammatory bowel disease and family history of CRC or adenomatous polyps. In this context, inherited genetic risk accounts for approximately 5 to 10% of CRC, being familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) the most common inherited conditions. However, CRC is widely considered to be an environmental disease, including cultural, social, and lifestyle factors. Of those, dietary patterns, physical activity and sedentary lifestyle, obesity, cigarette smoking and heavy alcohol consumption are well established environmental risk factors [5].

1.2.2 Pancreatic cancer

According to GLOBOCAN 2018, pancreatic cancer was the 11th most common cancer worldwide with almost 460,000 new cases, and responsible for more than 432,000 cancer-related deaths in 2018, being the 7th leading cause of global cancer death in industrialized countries in both men and women. The incidence and mortality toll worldwide correlates with increasing age (almost 90% of all deaths occur after the age of 55 years) and is slightly more common in men than in women [5]. By 2040, 355,317 new cases are estimated, despite advances in newly available diagnostic tools and increased knowledge of potential risk factors. As for CRC cancer, incidence rates are 3- to 4-fold higher in countries with higher HDI ranking, especially in Europe, North America and Australia/ New Zealand. Eastern Africa and Southern Asia show the lowest incidence (less than 1.0 per 100,000) of pancreatic cancer, in particular women.

The risk factors that contribute to the development of pancreatic cancer can be grouped into two categories: non-modifiable and modifiable risk factors [6]. Modifiable risk factors include

smoking, alcohol, obesity, dietary factors and occupational exposure. Non-modifiable risk factors include gender, age, ethnicity, diabetes mellitus, family history of pancreatic cancer, genetic risk factors, chronic infections, blood group and chronic pancreatitis. Pancreatic cancer is more common in older individuals, in particular men and African-Americans. Moreover, an association between ABO blood groups and the risk of pancreatic cancer development has been described [7], with people with A, B, or AB blood groups showing a higher risk of developing pancreatic cancer than people with blood group O [8]. Pancreatic cancer is a genetic disease caused by inherited and acquired genetic mutations. Inherited genetic mutations account for 5-10% of all patients and mutations in *BRCA2* are the most common inherited mutations observed in familial pancreatic cancer. Moreover, several familial cancer syndromes, such as familial atypical multiple-mole melanoma syndrome, hereditary pancreatitis, Peutz-Jeghers syndrome, cystic fibrosis, hereditary breast and ovarian cancer, Fanconi anaemia, FAP, Li-Fraumeni syndrome, and Lynch syndrome were found to be associated with a higher risk of developing pancreatic cancer. These syndromes are associated with germline mutations in genes like *BRCA2*, *p16*, ataxia-telangiectasia mutated gene (*ATM*), serine/threonine kinase 11 (*STK11*), protease, serine 1 (*PRSS1*), serine peptidase inhibitor, Kazal type 1 (*SPINK1*), and partner and localizer of BRCA2 (*PALB2*) [6, 9]. The increased risk of pancreatic cancer has been linked to some single nucleotide polymorphism, such as ABO, sonic hedgehog, telomerase reverse transcriptase and nuclear receptor subfamily 5, group A, member 2 (*NR5A2*) [8, 10]. Acquired or somatic mutations account for more than 80% of pancreatic cancer cases, with *KRAS* (95% of tumours), cyclin-dependent kinase Inhibitor 2A (*CDKN2A*) (p16) (90%), *TP53* (75%), and SMAD family member 4 (*SMAD4*) (55%) the most prevalent genes affected [9, 11, 12].

1.3 Cancer staging

1.3.1 Colorectal cancer

CRC staging system has undergone significant improvements since the original classifications were proposed by Dukes *et al*. The initial Dukes classification was based on the extent of the disease and was evaluated by the degree of tumour infiltration through the bowel wall and presence/absence of lymph node involvement. Later two new features were added, the nature of the expanding front of the tumour and the presence/absence of lymphocytic infiltration at the advancing edge [13].

The Dukes CRC staging was largely replaced by the Tumour, Nodes, and Metastasis (TNM) staging system (**Table 1.1**). This system, initially developed to predict cancer prognosis, is also used to define treatment regimens and entry into clinical trials. The TNM system is used to plan treatment, gives some indication of prognosis, assists in assessing the effects of treatment, helps with the exchange of information between treatment centres and contributes to the continuing investigation of human cancers [14].

1.3.2 Pancreatic cancer

As for CRC, the TNM staging system has been widely applied worldwide as the most recognized tool for pancreatic cancer assessment (**Table 1.1**).

Table 1.1: TNM Staging for CRC and Pancreatic cancer (Adapted from [15, 16]).

Tumour Definition			
CRC		**Pancreatic cancer**	
Primary tumour			
TX	Primary tumour cannot be assessed	**TX**	Primary tumour cannot be assessed
T0	No evidence of primary tumour	**T0**	No evidence of primary tumour
Tis	Carcinoma *in situ*	**Tis**	Carcinoma *in situ*
T1	Tumour invades submucosa	**T1a**	Tumour ≤ 0.5cm in greatest dimension
T2	Tumour invades muscularis propria	**T1b**	Tumour > 0.5cm and < 1cm in greatest dimension
T3	Tumour invades subserosal or nonperitonealized tissue	**T1c**	Tumour 1-2cm in greatest dimension
T4a	Tumour invades the surface of the visceral peritoneum	**T2**	Tumour > 2cm and ≤ 4cm in greatest dimension
T4b	Tumour directly invades other organs or structures	**T3**	Tumour > 4cm in greatest dimension
		T4	Tumour involves celiac axis, superior mesenteric artery, and/or common hepatic artery, regardless of size
Regional lymph nodes			
NX	Regional lymph nodes cannot be assessed	**NX**	Regional lymph nodes cannot be evaluated
N0	No lymph node metastasis and no tumour deposits	**N0**	No spread to regional lymph nodes
N1a	1 regional lymph node metastases	**N1**	Tumour cells found in 1-3 regional lymph nodes
N1b	2-3 regional lymph nodes metastases	**N2**	Tumour cells found in 4 or more regional lymph nodes
N1c	Nodules made up of tumour cells found in structures near the colon that do not appear to be lymph nodes		

(continued on next page)

N2a	4-6 regional lymph nodes metastases		
N2b	7 or more regional lymph nodes metastases		
Distant metastasis			
M0	No distant metastasis	**M0**	No distant metastasis
M1a	Distant metastasis in one organ or site without peritoneal metastasis	**M1**	Distant metastasis
M1b	Distant metastasis in more than one organ or site without peritoneal metastasis		
M1c	Metastasis in the peritoneal surface with or without other site or organ metastases		

Stage grouping							
CRC				**Pancreatic cancer**			
Stage	**T**	**N**	**M**	**Stage**	**T**	**N**	**M**
0	Tis	N0	M0	**IA**	T1	N0	M0
I	T1-T2	N0	M0	**IB**	T2	N0	M0
IIA	T3	N0	M0	**IIA**	T3	N0	M0
IIB	T4a	N0	M0	**IIB**	T1-T3	N1	M0
IIC	T4b	N0	M0	**III**	T1-T3 T4	N2 Any N	M0 M0
IIIA	T1-T2 T1	N1/N1c N2a	M0 M0	**IV**	Any T	Any N	M1
IIIB	T3-T4a T2-T3 T1-T2	N1/N1c N2a N2b	M0 M0 M0				
IIIC	T4a T3-T4a T4b	N2a N2b N1-N2	M0 M0 M0				
IVA	Any T	Any N	M1a				
IVB	Any T	Any N	M1b				
IVC	Any T	Any N	M1c				

1.4 Carcinogenesis

1.4.1 Colorectal cancer

Development of cancer requires multiple oncogenic mutations which confer phenotypic characteristics essential for malignancy, including self-sufficiency with respect to positive growth

signals, insensitivity to growth-inhibitory signals, evasion of cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [17].

CRC can arise from one or a combination of three different mechanisms, namely chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) (**Figure 1.1**).

1.4.1.1 Chromosomal instability

The classical CIN pathway follows the model proposed by Fearon [18], where carcinomas develop from premalignant polyps (adenomas). This model suggests that well-defined genetic events drive linear morphological alterations in the tissue, from normal mucosa to a small polyp, to a large polyp and finally to an invasive cancer. It begins with the acquisition of inactivating mutations in the tumour suppressor adenomatous polyposis coli (*APC*) gene (5q21), followed by activating mutations in the *KRAS* oncogene (12p12) and mutational inactivation of the tumour suppressor gene *TP53* (17q13). Mutations in other genes such as deleted in colorectal cancer (*DCC*) (18q21.1), phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) (3q26) and transforming growth factor beta (*TGF-β*) (3p22) are also acquired [19]. The main events in CIN tumours, accounting for the majority of the sporadic tumours (85%), are aneuploidy and loss of heterozygosity (LOH) [20].

1.4.1.1.1 *APC*

The tumour suppressor gene *APC* is located on the long arm of chromosome 5 in band q21 (5q21) and contains 15 exons. *APC* inactivation can occur through germline and somatic mutations or hypermethylation of its promoter and around 75% of CRCs have mutations or LOH in the APC gene [21]. Mutations in *APC* activate the Wnt signalling pathway by increasing the β-catenin levels. When β-catenin is not directed towards degradation, as in case of mutations in *APC*, it is translocated into the nucleus and enhances the transcription of several oncogenes with T-cell factor transcription factors [22]. *APC* is also described to play Wnt-independent roles, with consequences in carcinogenesis [23].

1.4.1.1.2 *KRAS*

As aforementioned, mutations in the *KRAS* oncogene constitute an early event in the development of CRC. They induce a more invasive phenotype of the tumour cells, with a higher

frequency of mutations observed in lymph node metastasis when compared to primary tumours [24]. *KRAS* mutations are found in 30-40% of CRC cases and 60-90% of hyperplastic or non-dysplastic aberrant crypt foci. *KRAS* point mutations in codons 12, 13, and 61 of exons 2 and 3 activate the enzyme and increase RAS signalling, namely the Raf-MEK-ERK and PI3K/AKT/PKB pathways or Ral small GTPases [25-27]. In particular, mutations in codon 12 are associated with more aggressive tumours, as shown by the higher probability of lymph node metastasis [28]. RAS triggers Raf activation, initiating a cascade involving MEK and ERK activation [29] that culminates in the phosphorylation of transcription factors responsible for the regulation of genes involved in cell proliferation and apoptosis [30]. PI3K further activates AKT1 and AKT2, which enhances tumour growth by promoting epithelial to mesenchymal transition (EMT) [31]. Loss-of-function mutations in the tumour suppressor gene and PI3K/AKT pathway antagonist *PTEN* induce AKT-regulated metastasis in CRC [32].

1.4.1.1.3 *TP53*

The tumour suppressor *TP53* is located on chromosome 17p13.1 and is activated under stress conditions. *TP53* targets inhibitors of cell cycle and pro-apoptotic factors [33]. p53 expression is abnormal in more than 50% of human tumours, where certain tumours show a gain-of-function mutation in p53. The mutated isoform mutp53 causes chronic activation of transcription factor NF-κB thus enhancing inflammation and tumorigenesis that ultimately results in an invasive carcinoma [34].

1.4.1.1.4 *DCC*

The *DCC* gene has been proposed as a tumour suppressor gene. *DCC* is located at 18q21.1 and allelic losses of this gene are observed in about 70% of CRCs [35]. The protein codified by *DCC* is a transmembrane receptor of the Ig superfamily for netrins, factors involved in axon guidance in the developing nervous system. Moreover, DCC has a role in intracellular signalling, apoptosis, cell cycle, actin organization and cell motility [36, 37].

1.4.1.2 Microsatellite instability

15% of CRCs show MSI, a result of inactivating mutations in genes involved in the correction of DNA replication errors, the DNA mismatch repair (*MMR*) genes. The important components of this machinery are ATPases hMSH2, hMSH6, hMSH3, hMLH1, hPMS2, hPMS1, and hMLH3 [38].

Tumours can be classified as MSI-high (MSI-H), MSI-low (MSI-L) and MSI stable (MSS), depending on the level of instability [39]. Genetic and epigenetic alterations of hMLH1 (mainly silencing caused by methylation) and hMSH2 are the most common causes of MSI-H in sporadic CRC and HNPCC. *hMSH6* mutations are less frequent and alterations in the other MMR genes are very rare [40], thus enforcing the idea that loss of *hMLH1* and *hMSH2* is associated with complete inactivation of MMR [41]

MSI-H sporadic CRCs present reduced frequency, or absence, of mutation or allelic losses at the genes usually altered in the CIN pathway, *APC*, *KRAS* and *TP53*, and loss of heterozygosity at 5q, 17p, and 18q [42].

1.4.1.3 CpG island methylator phenotype

Global DNA hypomethylation and localized promoter hypermethylation are epigenetic events that occur commonly in cancer. Hypomethylation in repetitive DNA sequences (e.g. satellite regions) can lead to genomic instability. On the other hand, and as a consequence of promoter region CpG island hypermethylation, genes involved in tumour suppression or cell cycle are transcriptionally inactivated [43]. The CIMP pathway is therefore characterized by hypermethylation of the promoter of several tumour suppressor genes, the most important ones the DNA repair *MGMT* and *MLH1*, and is often associated with MSI and *BRAF* mutations [44]. In fact, *BRAF* mutations are believed to be an early event in CIMP tumours, with the V600E being strongly correlated with *MLH1* hypermethylation [45]. Tumours can be classified as CIMP-high (CIMP-H) if two or more of the following promoters are hypermethylated: *MLH1*, *CDKN2A*, methylated in tumours 1 (*MINT1*), *MINT2*, and *MINT31* [44].

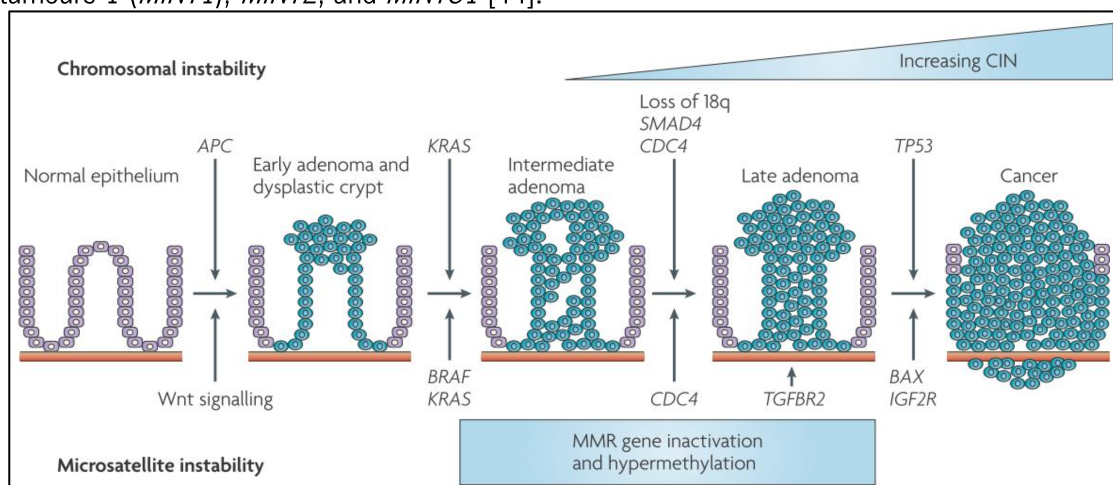


Figure 1.1. Adenoma–carcinoma sequence model of chromosomal instability in CRC. The initial step in chromosomal instability (CIN)+ colorectal cancer (CRC) tumorigenesis is the formation of an adenoma, associated with loss of adenomatous polyposis coli (*APC*). Larger adenomas/early carcinomas acquire mutations in *KRAS*, followed

by loss of chromosome 18q with SMAD family member 4 (*SMAD4*), and mutations in *TP53*. Microsatellite instability (MSI+) CRCs, present reduced frequency, or absence, of mutation or allelic losses at the genes previously mentioned. MSI is uncommon in adenomata, and the initial step involves alterations in the Wnt signalling. Mutations in *BRAF*, common in MSI+ CRC, are likely to occur in the place of *KRAS* mutations, although the latter do occur in a minority of cases. Mismatch repair (MMR) deficiency in sporadic CRC occurs predominantly by downregulation of MLH1 through promoter methylation. Further positive selection occurs for mutations affecting microsatellites in TGFβ receptor 2 (*TGFBR2*), insulin-like growth factor 2 receptor (*IGF2R*) and BCL2 associated X, apoptosis regulator (*BAX*), which in turn provides a TP53-independent mechanism of progression to carcinoma. Cell division control protein 4 (*CDC4*). Figure used with permission from authors [46].

1.4.2 Pancreatic cancer

The evolution of pancreatic ductal adenocarcinoma (PDAC, hereafter referred to as pancreatic cancer) can be defined by three broad stages [47]: tumour initiation by the acquisition of a driver gene mutation, clonal expansion into a multicellular neoplasm and spread of neoplastic cells into both local and distant microenvironments.

The adult pancreas has a relatively low proliferation rate [48] where it is exceptionally rare for an initiating driver gene mutation to occur solely by chance. In fact, the appearance of a driver gene mutation in the first cell occurs at least 20 years before diagnosis of sporadic pancreatic cancer [49]. The relative contribution of both intrinsic and extrinsic factors for the development of pancreatic cancer is far from being defined. It is believed that stochastic mistakes during DNA replication largely contribute to the risk of developing cancer [50] but extrinsic factors such as radiation and carcinogens were found to outweigh the influence of intrinsic factors in a follow-up study [51].

Germline mutations in components of the DNA double-strand break repair machinery, namely *BRCA1*, *BRCA2*, *PALB2*, the Fanconi anaemia genes *FANCC* and *FANCG*, and *ATM* [52, 53], are linked to risk of developing pancreatic cancer by increasing genomic instability and consequently the rate at which somatic mutations occur [54]. Germline mutations in *CDKN2A*, which encodes pl6INK4A and p19ARF, are also strongly associated with a higher risk of developing pancreatic cancer [52]. Moreover, chronic pancreatitis due to *PRSS1* or *SPINK1* mutations is also linked to an increased risk of pancreatic cancer development [55]. The initiating event, survival and expansion of clonal populations and accumulation of additional mutations can also be due to inflammatory processes [56], smoking [57], obesity [58] and type II diabetes [59].

In order to trigger the carcinogenic process, the initiating driver gene mutation must become fixed in the epithelial cell population. The mutated cell then undergoes additional cell divisions,

boosting the accumulation of somatic alterations over time (stepwise progression) or rapidly over a limited number of cell cycles (punctuated). The stepwise progression model (**Figure 1.2**) is characterized by waves of clonal expansion, associated with accumulation of driver gene mutations in the oncogene *KRAS* and the tumour suppressors *CDKN2A*, *TP53* and *SMAD4* with increasing atypia of pancreatic intraepithelial neoplasias (PanINs), known precursor lesions of pancreatic cancer [60-63]. On the other hand, the less frequent, punctuated evolution model is characterized by the acquisition of catastrophic genome-wide events in a single cell cycle, resulting in widespread structural damage and simultaneous acquisition of multiple driver gene alterations [64]. Regardless of the evolution model, pancreatic cancer is the result of only a few evolutionary paths and the genetic landscape of pancreatic cancer is dominated by somatic alterations in *KRAS*, *CDKN2A*, *TP53* and *SMAD4*, all of which have been shown to arise in PanINs [65].

1.4.2.1 *KRAS*

More than 90% of pancreatic tumours show activating mutations of the *KRAS* oncogene on chromosome 12p [66]. It encodes a member of the RAS family of GTP-binding proteins responsible for regulation of cell proliferation, survival and cytoskeletal remodelling [67]. The majority of *KRAS* mutations result in a constitutively active protein. *KRAS* amplification, along with the oncogenic mutation, occurs in approximately 4% of pancreatic cancer cases. 3-4% of cases show mutually exclusive *BRAF* mutations or amplifications [68].

1.4.2.2 *CDKN2A*

CDKN2A is inactively mutated in more than 90% of pancreatic cases. *CDKN2A* is a tumour suppressor gene that encodes p16INK4A and p19ARF through a common locus on chromosome 9p52 [69]. p16INK4A arrests cell cycle through the G1/S checkpoint mediated by cyclin-dependent kinases such as CDK4 and CDK6 [70] and its loss creates an environment that favours chromosome instability and accumulation of structural rearrangements [71]. On the other hand, p19ARF inhibits cell cycle progression independently of CDKs by binding to the E3 ubiquitin ligase MDM2 to inhibit p53 degradation. Consequently, loss of p19ARF hinders p53-induced apoptosis and cell cycle arrest [72].

1.4.2.3 *TP53*

Upon activation by stimuli such as DNA damage or stress, the transcription factor p53 is responsible for regulation of the G1/S checkpoint, maintenance of G2/M arrest to enable DNA repair, and apoptosis [73]. Somatic mutations in *TP53* are found in around 85% of pancreatic cancers and up to 66% are missense mutations that affect the DNA binding domain [74].

1.4.2.4 *SMAD4*

SMAD4 inactivating mutations are found in approximately 55% of pancreatic cancer cases, 30% by homozygous deletion and 25% by inactivation [75]. *SMAD4* encodes a co-transcription factor and mediator of the TGF-β canonical signalling pathway, crucial for cell growth, differentiation and maintenance of tissue homeostasis [76]. During the early stages of clonal expansion, the TGF-β pathway restrains neoplastic cell growth, whereas it promotes it in later stages, in part due to the loss of SMAD4 and the canonical arm of the pathway [77].

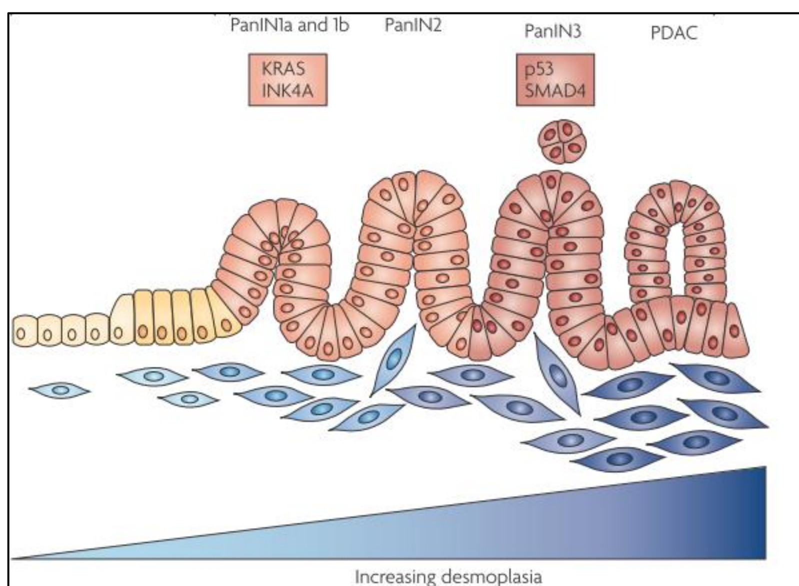


Figure 1.2. The stepwise progression model for pancreatic cancer. Constitutively active *KRAS* is sufficient to initiate the development of pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma (PDAC). Accumulation of driver gene mutations in the tumour suppressors *CDKN2A*, *TP53* and *SMAD4* are found in PanINs with increasing cellular atypia. Changes in the epithelium are matched by desmoplastic changes in the stroma. Figure adapted from [78] and used with permission from authors.

1.5 Treatment

1.5.1 Colorectal cancer

The treatment, and consequently, survival of CRC patients depends largely on the disease stage at diagnosis. Approximately 35% of patients are diagnosed with metastatic disease and around 50% of those with nonmetastatic CRC ultimately develop metastasis [79].

Surgery and chemotherapy are the standard therapeutic regimens for the majority of patients diagnosed with CRC. Surgery is the main approach when detected at an early stage and it is usually performed on all patients with a localized tumour mass. However, when the disease is diagnosed at an advanced stage, surgery is usually combined with chemotherapy and/or radiation. Chemotherapy can be applied before (neo-adjuvant) and after (adjuvant) surgery, or as the primary approach (palliative). Radiotherapy treatment can be applied as a preoperative, intraoperative, or postoperative treatment. In addition, chemotherapy can be given in combination with radiotherapy in order to sensitize cells to radiation. Chemotherapy is used to treat all patients with stages III and IV of the disease and occasionally for some stage II patients [80, 81]. It is frequently used in patients with metastatic disease, either as an adjuvant treatment, or to control the size and growth of the metastases in order to be eligible for surgical [82].

In the last decades, chemotherapeutic treatment of CRC had multiple revolutionary breakthroughs with new regimens and agents being approved or under investigation, with a particular focus on compounds targeting specific signalling pathways and promoting the action of the immune system [83, 84].

1.5.1.1 5-Fluorouracil

Although several chemotherapeutic drugs have been developed to improve the survival and life quality of CRC patients, 5-Fluorouracil (5-FU) is still recommended as one of the first line chemotherapeutic agent for the treatment of CRC and it was the first compound with demonstrable activity against this malignancy [85]. This antimetabolite drug is a uracil analogue and uses the same facilitated transport mechanism to enter the cell. Inside the cell, it is preferentially used and consequently a fluorinated analogue of this base can selectively alter the cell metabolism [86].

5-FU is metabolized into the active metabolite fluouroxyuridine monophosphate (FUMP), and this uracil replacer is incorporated into RNA, arresting its processing and consequently cell growth. Fluorodeoxyuridine monophosphate (FdUMP), another 5-FU metabolite, combines with methylenetetra-hydrofolate to form a ternary complex with thymidylate synthase (TS) that inhibits the conversion of deoxyuridylate to thymidylate and thus interferes with *de novo* synthesis of thymine and DNA synthesis. 5-FU can also cause cytotoxic effects through direct incorporation into RNA and, to a lesser extent, into DNA [87] (**Figure 1.3**).

Despite being applied as a first line chemotherapeutic agent for CRC, there is a large body of evidence demonstrating resistance to 5-FU. The majority of the compound is catabolised in the

liver by dihydropyrimidine dehydrogenase (DPD) upon administration. As a result, inactive dihydrofluorouracil (DHFU) is formed and further degraded to fluoroureidopropionate (FUPA) and inactive fluoro-b-alanine (F-BAL), which is excreted in urine [86, 88, 89]. Moreover, TS inhibition can be alleviated by the action of thymidine kinase (TK), salvaging TS from thymidine [86] (**Figure 1.3**).

Aiming to enhance the low therapeutic effect of 5-FU when administrated as a single agent and to reduce side-effects, new combinatory modalities were developed. Based on *in vitro* studies which showed that leucovorin had a modulating effect on 5-FU, it was added to the therapeutic regimen and shown to improve the efficacy of 5-FU with minor toxicity. 5-FU plus leucovorin became a standard systemic chemotherapy for metastatic colorectal cancer [90]. Oxaliplatin, a third-generation platinum with a 1,2-diaminocyclohexane carrier ligand which forms DNA adducts and resultant strand breaks, was shown to interact favourably with infusional fluorouracil, leading to the current standard combination FOLFOX. Irinotecan, a semi-synthetic derivative of the natural alkaloid camptothecin which targets the activity of topoisomerase I, was combined with infusional 5-FU and folinic acid (leucovirin) (FOLFIRI). The combination therapies FOLFOX and FOLFIRI were shown to be efficacious cytotoxic regimens for the treatment of metastatic CRC, improving the survival of patients in approximately 2 years [91]. All the combination therapies where found to be superior to 5-FU and leucovorin alone [92, 93] and to have similar efficacies [94, 95]. Capecitabine is an orally administrated prodrug of 5-FU that was shown to be rapidly absorbed by the GI tract with nearly 100% bioavailability [89, 96, 97].

Figure 1.3. Cellular metabolism and mechanism 5-Fluorouracil. Once inside the cell, 5-Fluorouracil (5-FU) is metabolized into three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). 5-FU is activated by conversion into fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT), or indirectly via fluorouridine (FUR) through an enzymatic cascade involving uridine phosphorylase (UP) and uridine kinase (UK). FUDP can be either phosphorylated to fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate FdUTP and FdUMP, respectively. Alternatively, 5-FU can be activated into fluorodeoxyuridine (FUDR) by thymidine phosphorylase (TP), and then phosphorylated by thymidine kinase (TK) to FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated

conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism. PRPP: phosphoribosyl pyrophosphate. Figure used with permission from authors [86].

1.5.1.2 Targeted therapy

In order to attenuate off-target effects, such as bone marrow and epithelial cell damage observed with compounds that target all proliferating cells, agents that tackle unique biological features of cancers were developed.

Angiogenesis, the physiological process through which new blood vessels are formed from pre-existing ones, promotes the growth, proliferation and survival of both normal cells and cancer cells and eases the dissemination of metastases [98]. It is orchestrated by a balance between pro-angiogenic and anti-angiogenic factors and receptors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, and fibroblast growth factor [99]. Bevacizumab, a monoclonal antibody against VEGF-A, is one of the anti-angiogenic drugs developed. It acts by depleting and normalizing tumour vasculature, thus enhancing the delivery of chemotherapeutic agents, with some encouraging results in CRC [100].

Epidermal growth factor receptor (EGFR) ligand binding ultimately leads to activation of signalling pathways involved in cell proliferation, angiogenesis, migration, survival and adhesion [101]. Cetuximab and panitumumab are monoclonal antibodies approved for metastatic CRC that bind to the extracellular domain of EGFR and downregulate its pro-oncogenic signalling [102]. However, several pathways are clinically validated to trigger resistance to EGFR monoclonal antibodies in CRC, including activation of KRAS and BRAF. Despite EGFR inhibition, these pathways trigger a constant activation of downstream signalling and block the apoptosis or decreased cellular proliferation [103].

8 to 12% of metastatic CRC cases are BRAF mutated and the V600E mutation confers a worse prognosis [104]. Since single-agent therapy against BRAF was shown to induce resistance [105], current treatment strategies for BRAF V600E-mutated metastatic CRC rely on combinations of targeted inhibition [106, 107].

1.5.2 Pancreatic cancer

Although tremendous advances have been made to better understand the genetic, epigenetic, and metabolic complexity, as well as the interplay of cancer cells with the remaining populations of the tumour microenvironment (TME), they have not reflected in a dramatic change in the overall

outcome of pancreatic cancer patients [108]. In addition, there are no clinically validated screening methods for pancreatic cancer in the curative stage.

Treatment options for pancreatic cancer are selected in multidisciplinary and stage-dependent approaches, and include surgery, chemotherapy, radiotherapy and palliative care. Surgical resection remains the only treatment option that offers a potential cure of pancreatic cancer, however, only 10–15% of newly diagnosed patients are deemed eligible and more than 90% of patients relapse and die after surgery without additional therapy [108, 109]. Most patients die with liver, lung and/or peritoneum metastases, the most common sites of spread [110]. The limitations of treatment strategies in pancreatic cancer led to the development of adjuvant treatment strategies during the past decades. The administration of chemo-radiotherapy in a neo-adjuvant setting has resulted, in some cases, in a further improvement in survival, but still far from changing the pancreatic cancer paradigm.

1.5.2.1 Chemotherapy

The backbone of pancreatic cancer chemotherapy is the deoxycytidine analogue gemcitabine and, to lesser extent, 5-FU. A trial carried out in the 1970s paved the way for the use of 5-FU as a standard chemotherapeutic option to treat pancreatic cancer. In this study, 5-FU-based radiation surgery followed by weekly 5-FU for 2 years or until recurrence proved to be favourable in comparison with surgery alone [111]. Later studies further supported the use of 5-FU alone or in combination with other chemotherapeutic agents [112-114].

Adjuvant chemotherapy with gemcitabine is usually administrated upon surgery for resectable pancreatic cancer if the patient can tolerate chemotherapy. The use of gemcitabine as adjuvant chemotherapy for the treatment of pancreatic cancer was supported by the CONKO-001 study, where the administration of gemcitabine after complete surgical resection was compared with surgery alone. The results showed a significantly improved median disease free survival and overall survival with the use of adjuvant gemcitabine [115-117]. Further clinical trials were carried out during the past decades seeking to identify the best gemcitabine-based chemotherapy regimen [115, 118-122].

The administration of adjuvant therapy has been shown beneficial for pancreatic cancer patients. However, between 71 to 76% end up relapsing within two years and up the adjuvant therapy is discontinued in 40% due to surgery-related complications [123]. Therefore, the role of neo-adjuvant chemo(radio) therapy has been investigated in several studies in patients with

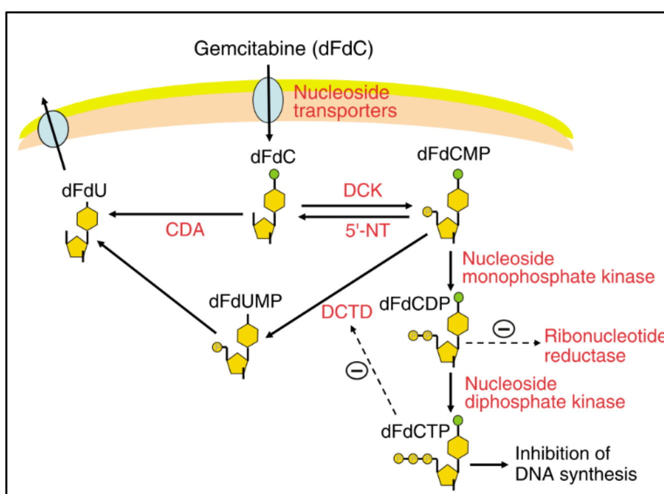
resectable or borderline resectable disease [124]. The advantage of administering neo-adjuvant therapy relies on the shrinking of the primary tumour and elimination of micro-metastases, factors that might reflect in decreased incidence of tumour recurrence and patient survival [125]. The poor drug delivery and low sensitivity to radiation due to decreased oxygenation [126], support the administration of preoperative therapy.

1.5.2.1.1 Gemcitabine

Gemcitabine is a nucleoside analogue that has been used as the standard treatment regimen for locally advanced and metastatic pancreatic cancer. It is also a therapeutic option, especially when in combination with platinum-based drugs cisplatin and carboplatin, to treat solid tumours such as breast, ovarian and non-small cell lung cancer [127-129]. Once inside the cell, gemcitabine is sequentially phosphorylated to the active triphosphate form (2’,2’-difluoro-2’-deoxycytidine triphosphate; dFdCTP) by deoxycytidine kinase (dCK) and exerts its cytotoxic effects by inhibiting DNA synthesis [130], enzymes involved in deoxycytidine metabolism and ribonucleotide reductase (RNR) [131, 132] (**Figure 1.4**). Another mechanism of gemcitabine cytotoxicity is the induction of apoptosis, through activation of p38 mitogen-activated protein kinase (MAPK), in response to cellular stress [133, 134].

Among the mechanisms known to cause gemcitabine resistance are loss of membrane transporters, dCK deficiency, competition with *de novo* CTP, and upregulation of cytidine deaminase (CDA), an enzyme that inactivates more than 90% of administrated gemcitabine by converting dFdCytidine to dFdUridine, its inactive form, which is passively excreted out of the cell [135].

Figure 1.4. Cellular metabolism and mechanism of gemcitabine. Inside the cells, gemcitabine (dFdC) is subsequently phosphorylated into gemcitabine triphosphate (dFdCTP), by the action of deoxycytidine kinase (DCK), nucleoside monophosphate and diphosphate kinase. dFdCTP is then incorporated into the DNA strand and inhibits DNA synthesis. Gemcitabine diphosphate (dFdCDP) potently inhibits ribonucleotide reductase, resulting in a decrease of competing deoxyribonucleotide pools necessary for DNA synthesis. dFdCTP suppresses inactivation of gemcitabine



monophosphate (dFdCMP) by inhibiting deoxycytidine monophosphate deaminase (DCTD). On the other hand, cytidine deaminase (CDA) converts, and consequently inhibits, more than 90% of administered gemcitabine into 2'-deoxy-2',2'-difluorouridine (dFdU). Phosphorylated metabolites of gemcitabine are reduced by cellular 5'-nucleotidase (5'-NT), and dFdCMP is also converted, and inactivated, by DCTD into 2'-deoxy-2',2'-difluorouridine monophosphate (dFdUMP). Figure used with permission from authors [136].

1.5.2.2 Targeted therapy

As for CRC, several targeted agents have been studied, alone or in combination with chemotherapeutic drugs, in metastatic pancreatic cancer. A special focus has been made to tackle angiogenesis, with the use of antiangiogenic VEGF inhibitors bevacizumab and aflibercept [137, 138] and multikinase inhibitors with antiangiogenic activity [139-142]. However, the hypovascular nature of the tumour stroma was proven be a major obstacle for the antiangiogenic approaches tested so far. With the exception of the EGFR inhibitor erlotinib [143], the remaining strategies which target key signalling pathways, in combination with gemcitabine, failed to produce any overall benefit for patients, namely the anti-insulin-like growth factor 1 receptor antibodies ganitumab and cixutumumab, the multi-kinase inhibitor masitinib and the PI3K inhibitor rigosertib [144].

1.6 The hallmarks of cancer

Cancer manifests in disconcertingly complex and diverse ways affecting different organs with distinct genetic backgrounds, histopathology, effects on systemic physiology, prognosis, and response to therapeutic intervention. Although these neoplastic diseases are masked by complex genetic and phenotypic backgrounds, they all share a common set of unique capabilities that enable uncontrolled cell proliferation, ultimately leading to focal or disseminated growth of cancer cells: the hallmarks of cancer.

The hallmarks of cancer model describes eight characteristics, shared by most forms of human cancer, that serve unique functional roles in supporting the development, progression, and persistence of tumours: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics and metabolism, and avoiding immune destruction. Fostering these hallmarks are two characteristic traits of neoplastic growths: genome instability,

which generates the genetic diversity that expedites their acquisition, and inflammation [17] (**Figure 1.5**).

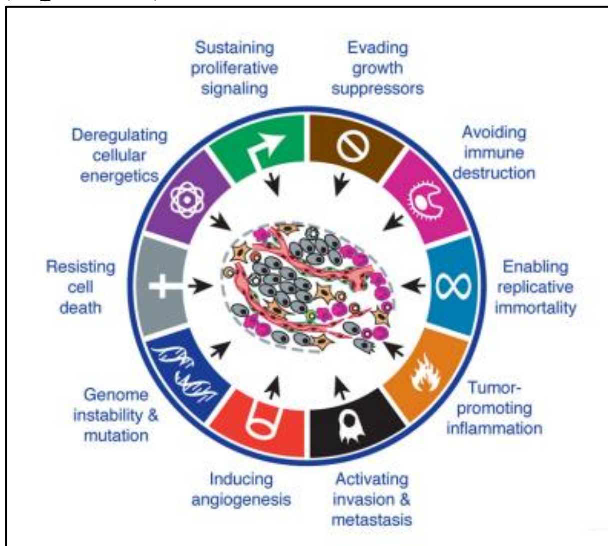


Figure 1.5. The Hallmarks of cancer. Illustrations representing the hallmarks of cancer model. Most forms of human cancer share eight characteristics, sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics and metabolism, and avoiding immune destruction, that serve unique functional roles in supporting the development, progression, and persistence of tumours. Fostering these hallmarks are two characteristic traits of neoplastic growths: genome instability and inflammation. Figure adapted from [17] and used with permission from authors.

1.6.1 Reprogramming of energy metabolism

1.6.1.1 The Warburg effect

In order to support cancer initiation, progression and aggressiveness, transformed cells reprogram their cellular metabolism. During the carcinogenic process, malignant cells have to compete for nutrients with neighbouring cells in a metabolite- and oxygen-deprived microenvironment, leading to a necessary but evolutionary favourable metabolic plasticity [145].

Almost one century ago, the Nobel Prize laureate Otto Warburg described the first tumour-specific alteration, altered metabolism. Warburg observed that even in the presence of adequate oxygen supply, certain cultured-cancer cells showed enhanced glucose uptake and a preference to metabolize it via the less energetically efficient pathway glycolysis [146]. In the presence of oxygen, non-transformed cells completely oxidize glucose via cytoplasmic glycolysis, mitochondrial citric acid cycle and electron transport chain/oxidative phosphorylation (OXPHOS), yielding the maximum possible energy. When oxygen supply is disrupted, the mitochondrial function is suppressed and normal cells shift their metabolism to anaerobic glycolysis. Surprisingly, even in the presence of oxygen, tumour cells strongly rely on this metabolic pathway (“Aerobic glycolysis” or “Warburg effect”) (**Figure 1.6**). Since most tumour cells demand a higher energetic input to maintain their proliferation rates, opting for this metabolic pathway is counterintuitive, as glycolysis is far less efficient at producing ATP. Warburg hypothesized that this metabolic shift was a

consequence of an impairment of mitochondrial respiration [146]. However, mitochondrial respiration has been shown to be intact in several tumours and the suppression of OXPHOS by glycolysis is an adaptation to hypoxic conditions during tumour development.

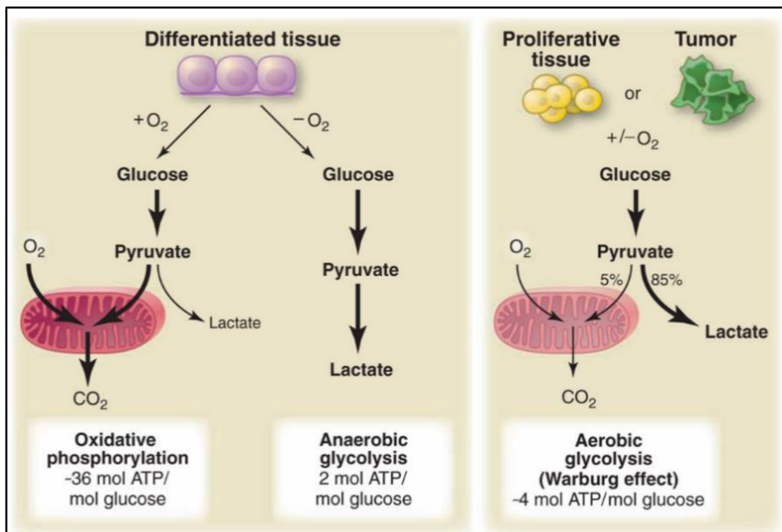


Figure 1.6. Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis ("Warburg effect"). In the presence of oxygen, differentiated tissues metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO_2 during the process of oxidative phosphorylation. When oxygen availability is limited, cells can redirect pyruvate away from oxidative phosphorylation by generating lactate (anaerobic glycolysis). This results in minimal ATP production when compared with oxidative phosphorylation. Cancer cells and normal proliferative tissues tend to convert most glucose to lactate, regardless of oxygen levels (aerobic glycolysis). Aerobic glycolysis is less efficient than oxidative phosphorylation for generating ATP. Figure used with permission from authors [147].

In order to fulfil the high rate of "aerobic glycolysis", cancer cells increase glucose uptake and consumption. This metabolic phenomenon has proven to be useful for the clinical detection and treatment monitoring of tumours by using positron emission tomography (PET) with radiolabelled analogues as tracers. The glucose analogue 2-(^{18}F)-fluoro-2-deoxy-D-glucose (FDG) is used as a PET probe for the visualization of glycolytic tumours due to their elevated expression of glucose transporters and consequently increased glucose uptake [148].

Although "aerobic glycolysis" is far less efficient at producing ATP, the primary currency of intracellular energy, the rewiring of cellular metabolism towards this pathway is advantageous for tumour growth [149]. The theoretical energetic inefficiency of glycolysis is balanced by the faster rates of ATP production when compared with OXPHOS [150]. The accelerated glycolytic flux also provides proliferating cells with glycolytic intermediates that are crucial to fuel other biosynthetic pathways [151]. For example, glucose 6-phosphate, can be oxidized through the pentose phosphate pathway (PPP) to generate the nucleotide precursor ribose 5-phosphate and NADPH, the last used for lipid biosynthesis and scavenging of reactive oxygen species that are produced

during the enhanced cell proliferation. Additionally, dihydroxyacetone phosphate and 3-phosphoglycerate can be channelled for phospholipid and serine production, respectively [152, 153]. Pyruvate can be used for the biosynthesis of alanine and malate and enter a truncated tricarboxylic acid cycle (TCA) where the resultant acetyl-CoA is exported from the mitochondrial matrix and becomes available for the synthesis of fatty acids, cholesterol, and isoprenoids [154, 155]. Lactate was long considered to merely be toxic waste secreted by cells undergoing aerobic and anaerobic glycolysis. However, it is now appreciated to have several tumour-promoting capabilities [154]. Extracellular lactate can be imported via specific transporters and used as fuel for ATP and biomaterial generation, particularly in cancer cells undergoing glucose deprivation. In addition, lactate can be taken up by stromal cells such as cancer-associated fibroblasts (CAFs) to regenerate pyruvate that either can be extruded to refuel the cancer cell or can be used for OXPHOS [156]. This metabolic symbioses established by glucose-importing/lactate-exporting cells and lactate-importing cells allows buffering and recycling of products of anaerobic metabolism to sustain cancer cell survival and growth [157]. The upregulation of glycolysis triggers the extracellular accumulation of lactate and consequent acidification of the microenvironment, which is fatal for normal cells but harmless for cancer cells [158, 159]. Consequently, the released lactate boosts the carcinogenic cascade, influencing a wide range of biological processes, such as cancer cell migration, invasion and metastasis and immune surveillance escape [159].

Contrary to what was initially postulated, cancer cells do not switch from OXPHOS to aerobic glycolysis in cancer cells, but rather continue to utilize OXPHOS in addition to incorporating fluctuating rates of glycolysis. This dynamic metabolic flow is time dependent, varies among cancer cells within a tumour and is tissue microenvironment specific. Additionally, cancer cells do not rely solely on glucose and use additional sources of energy, including TCA and fatty acid β-oxidation (FAO) during metabolically adverse conditions [152].

1.6.1.2 Monocarboxylate transporters

The increased production of lactate via aerobic glycolysis can ultimately lead to cellular acidosis, thus compromising cancer cell survival. Intracellular acidification has been shown to be a trigger in the early phases of apoptosis and can lead to DNA fragmentation via activation of endonucleases. Cancer cells counteract cytoplasmic acidification by induction of specific forms of membrane-bound carbonic anhydrases (CA), especially CAIX, that remove protons generated by the enhanced glycolytic metabolism of malignant cells [160]. The Na^+/H^+ exchanger 1 (NHE1) is also a significant

contributor to pH regulation in tumour cells, by pumping out protons, coupled to a transmembrane Na^+ gradient. The H^+-coupled lactate transporters, namely the monocarboxylate transporters (MCTs), belong to another important class of plasma membrane transporters. These transporters perform the efflux of lactate/H^+ from cells, preventing intracellular acidification, but also the influx.

The 14 members of the MCT family belong to the solute carrier (SLC) transporter superfamily and are encoded by the members of the SLC16 gene family [161]. The predicted topology comprises 12 transmembrane domains (TMDs) and less conserved intracellular N- and C-termini. The TMDs are highly conserved among isoforms with the greatest sequence variations observed in the C-terminus and the large intracellular loop between TMDs 6 and 7. These sequence variations are predicted to be related to substrate specificity or regulation of transport activity [162]. Four MCT isoforms MCT1-MCT4 are responsible for the H^+-coupled transport of short chain monocarboxylates, primarily L-lactate, pyruvate, or ketone bodies, with varying substrate affinities. Besides the previously mentioned monocarboxylates, these isoforms also transport branched-chain oxoacids derived from leucine, valine and isoleucine, and the ketone bodies acetoacetate, β-hydroxybutyrate and acetate [162]. Other members of the MCT family have been shown to mediate H^+-uncoupled translocation of more hydrophobic monocarboxylates, such as thyroid hormones (MCT8), carnitine (MCT9), aromatic amino acids (MCT10), or creatine (MCT12) [163]. Consequently, MCTs play a pivotal role in mammalian metabolism and are critical for metabolic communication between cells.

MCT1 is encoded by the *SLC16A1* gene located on chromosome 1 (1p13.2-p12) [164]. This isoform has a ubiquitous tissue distribution, however, localization within specific tissues varies. It is especially prominent in heart and red muscle where it is upregulated in response to increased stress, suggesting an important role in lactic acid oxidation [165, 166]. MCT1 is a proton-dependent cotransporter/exchanger which is mainly responsible for uptake of substances, however transport can occur bidirectionally [165]. It transports a variety of substrates including short chain (C2-C5) unbranched aliphatic monocarboxylates (e.g. acetate and propionate), monocarboxylates with C2 or C3 substitutions (e.g. pyruvate, L-lactate, acetoacetate and β-hydroxybutyrate) and branched oxoacids, with a greater affinity than lactate [167].

MCT4 is encoded by the *SLC16A3* gene, which is located in chromosome 17 (17q25.3). The distribution of MCT4 in the tissue, as well as its regulation and substrate/inhibitor specificity is remarkably similar to MCT1. The main differences between the isoforms arise in their substrate affinity as well as the tissue specific localisation. MCT4 is predominantly expressed in highly

glycolytic cells such as white muscle and white blood cells, suggesting a physiological role in lactate efflux [165, 168]. This isoform has a lower affinity for substrates than MCT1, with mower K_m values for L-lactate [169, 170] and pyruvate [170].

MCT2 catalyses the proton-linked transport of a range of monocarboxylates, but with a considerably higher affinity than the other isoforms. Its expression is more restricted and highly species dependent. It is mainly expressed in neurons, testis, liver, kidneys, adipose tissue, and tissues that rely on substrate uptake as fuel for OXPHOS or as substrate for gluconeogenesis or lipogenesis. MCT2 is also expressed in pancreas, heart, colon, and stomach [171]. MCT3 has a unique distribution, being confined to the basal membrane of the retinal pigment epithelium and choroid plexus epithelia [172]. MCT3 expression was also found in vascular smooth muscle cell lines, human aorta and human kidney [173, 174].

1.6.1.2.1 Regulation of MCT activity

Besides genetic variation, non-genetic factors like age, gender, or disease state, regulatory factors such as transcription factors, or epigenetic mechanisms affect the expression and function of transport proteins [175]. In order to adapt to changes in activity or different metabolic states, MCT gene expression regulation has to be rapid. MCT1 expression is regulated by transcription factors such as c-MYC, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), or p53, and by lactate itself, or post-transcriptionally by miRNA-29 [176-181]. Due to its expression in highly glycolytic and hypoxic tissues, MCT4 possesses hypoxia response elements within its promoter region and its transcription is upregulated by the hypoxia-inducible factor 1 (HIF-1) [182]. Epigenetically, MCT4 has been shown to be regulated by DNA methylation in clear cell renal cell carcinoma [183] and temporal lobe epilepsy [184]. Additionally, the MCT1/*SLC16A1* promoter can be methylated in breast cancer, reducing gene expression [185]

Besides being regulated at transcriptional, translational and post-transcriptional levels, MCTs are also regulated by interactions with other proteins. MCT1 and MCT4 proper membrane expression and function are dependent on their association with the mature glycosylated form of the chaperone CD147/basigin (also known as extracellular matrix metalloproteinase inducer (EMMPRIN)) [186]. Conversely, MCT1 and MCT4 also regulate CD147 maturation and trafficking to the plasma membrane [187, 188]. CD147 is ubiquitously expressed on the cell surface, with metabolically active cells such as lymphoblasts and malignant tumour cells expressing the highest levels [189, 190]. The increased expression of CD147 on tumour cells stimulates the production

of multiple matrix metalloproteinases (MMPs) by surrounding tumour cells, stromal fibroblasts and endothelial cells, promoting the degradation of the extracellular matrix and consequently tumour growth and tumour cell mobility [190-193]. CD147 also stimulates the expression of VEGF through the PI3K/AKT pathway [194], potentially contributing to tumour aggressiveness by stimulating angiogenesis. CD44, a widely distributed transmembrane glycoprotein, is also believed to be a MCT chaperone as it was shown to co-immunoprecipitate with MCT1 and MCT4 and regulate their intracellular trafficking [195].

1.6.1.2.2 MCT expression in tumours

As previously mentioned, MCTs mediate the efflux of lactate coupled to a proton from cancer cells, allowing enhanced glycolytic metabolism and the maintenance of intracellular pH. Additionally, these transporters are gatekeepers of a lactate shuttle between cells with distinct energetic demands: between hypoxic glycolytic and oxygenated oxidative tumour cells, between hypoxic tumour and endothelial cells, or between oxidative tumour and glycolytic stromal cells. This intratumoral symbiosis has been described for several solid tumours [196] and, accordingly, aberrant expression of MCT1 and/or MCT4 has been shown in colon cancer, glioblastoma, breast cancer, prostate cancer, pancreatic cancer, or clear cell renal cell carcinoma [197, 198].

Anaerobic glycolysis is a crucial pathway in CRC metabolism, as proven by the expression patterns of several glycolytic enzymes [156]. Results published concerning the expression of MCTs in CRC showed an increased plasma membrane expression of MCT1 [156, 199, 200] and MCT4 [199-202] in cancer cells as compared to adjacent stroma or in normal tissue. In particular, MCT4 was found to be also overexpressed in lymph nodes and hepatic metastasis, when compared with non-neoplastic tissue [200]. In addition, the high expression of MCT1 and MCT4 has been associated with poor prognosis features in CRC. Due to their important role in lactate metabolism, MCT1 and MCT4 have been proposed as diagnostic biomarkers or prognostic factors for cancer outcome and survival [199, 200, 202, 203].

Due to the specific tumour architecture which is characterized by poor vascularization, the concomitant lack of oxygen and nutrient diffusion leads to a cellular metabolic reprogramming in pancreatic cancer, namely addiction of cancer cells to glucose [204-207]. Concerning the expression of MCT1 and MCT4, evidence points to a role of MCT4 on the biology of pancreatic cancer. GuemHee and collaborators [208] assessed the expression of the genes encoding MCT1 and MCT4 and saw that SLC16A3 (which encodes MCT4) was overexpressed in PDAC samples

and associated with a poor outcome. Additionally, it was observed that MCT4 was highly expressed in the epithelial compartment and in the stromal compartment. Cases with high stromal MCT4 expression were associated with cases that demonstrated high MCT4 expression in cancer cells, and this pattern correlated with poor survival. Instead, MCT1 expression was restricted to epithelial cells and no association with outcome was observed. More recently, Sukeda *et al.* [209] showed that expression of MCT1 in PDAC was associated with extended overall and progression-free survival and decreased nodal metastasis and MCT4 expression in CAFs was associated with shortened survival.

1.6.1.2.3 MCT Targeting in Cancer

In view of the role of MCTs in cancer lactate shuttle and pH homeostasis and the fact that MCT isoforms are frequently overexpressed in cancer cells, their inhibition has been studied as a therapeutic approach to tackle cancer. In addition, MCTs are transmembrane proteins exposed to the extracellular environment, which allows targeting of these transporters by systemic administration of small-molecule inhibitors. Several agents have been shown to inhibit lactate transport via MCTs, with different affinities and specificities for each MCT isoform. They can be organized into three major groups: i) Bulky or aromatic monocarboxylates such as α-cyano-4-hydroxycinnamate (CHC); ii) amphiphilic compounds with widely divergent structures like bioflavonoids (e.g. quercetin and phloretin); and iii) stilbene-derived compounds such as 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) and 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS) [162]. However, these compounds are unspecific and cannot be used to determine the individual role of MCT isoforms in cancer. For example, CHC is a strong inhibitor of the mitochondrial pyruvate carrier [210], and both DIDS and DBDS also inhibit the activity of the chloride/bicarbonate exchanger AE1 [162]. Nevertheless, they have been demonstrated to exert therapeutic effects. CHC was proven to be effective *in vitro* by reducing lactate transport, cell proliferation, invasion and migration and increasing cell death in models of glioma [211], colorectal [212], cervix [213] and breast cancer [214]. *In vivo*, CHC was shown to reduce tumour growth [211, 215], sensitize cells to radiation [213], induce tumour necrosis and decrease tumour invasion [215]. The anticancer drug lonidamine, by impairing lactate transport through unspecific inhibition of MCT1 and MCT4, re-sensitizes tumour cells to other chemotherapeutic agents [216].

In CRC, several studies have shown that pharmacological inhibition of lactate transport decreases cell viability, induces apoptosis [217], delays *in vivo* tumour growth [213, 218] and

improves the efficacy of chemotherapeutic agents such as cisplatin [217]. The inhibition of MCT1 and MCT4 chaperone CD147 resulted in an accumulation of intracellular lactate [219-222] and also reflected in a reduction of cell proliferation and invasion [221]. In addition, CD147 inhibition re-sensitizes cancer cells to cisplatin [221] and the mitochondrial complex I inhibitor, phenformin [219].

MCTs also appear to be attractive therapeutic targets in pancreatic cancer with several *in vitro* and *in vivo* studies dissecting the roles of MCT1 and MCT4 in the malignant process of pancreatic cancer. MCT4 knockdown was shown to compromise pancreatic cell survival and growth, and disrupted cell lactate pools which led to metabolic reprogramming [208]. Additionally, CD147 silencing reduced intracellular pH, proliferation, invasion and metastatic potential of pancreatic cancer cells and sensitized them to the chemotherapeutic drug gemcitabine. It was also shown that MCT disruption decreased pancreatic tumour cell invasion in the chorioallantoic membrane of chick embryos and inhibited tumour formation and tumorigenicity in a xenograft mouse model [223, 224]. More recently, MCT1 and MCT4 chemical inhibition or genetic deletion confirmed that these two isoforms regulate the migration and invasion of pancreatic cancer cells [225]. In addition, MCT1 inhibition disrupted the “tumour-stromal” metabolic symbiosis between cancer cells and CAFs, decreasing the migration and invasion capacities of pancreatic cancer cells [226].

Several specific inhibitors with higher affinity for MCTs have been developed and their efficacy has been assessed in the preclinical setting. AstraZeneca’s MCT1 and MCT2 but not MCT4 inhibitor AR-C155858 [227] was shown to influence lactate transport [220]. AZD3965, a selective MCT1/2 inhibitor, was shown to interfere with intracellular lactate levels in small cell lung carcinoma cell lines and xenografts and reduced tumour growth [228]. AZD3965 is currently being evaluated as an anticancer agent in Phase I clinical trial for patients with solid tumours, prostate cancer, gastric cancer, and diffuse large cell B lymphoma (NCT01791595). More recently, bindarit, known as an anti-inflammatory agent, was described as a potent and highly selective non-competitive MCT4 inhibitor [229].

Due to the functional redundancy of MCT1 and MCT4, knockdown or selective inhibition of either one of the isoforms does not lead to tumour cell death despite inducing tumour growth arrest or increasing chemosensitivity and radiosensitivity [196]. Additionally, simultaneous inhibition of both isoforms can induce metabolic plasticity of cancer, resulting in a shift towards OXPHOS. Consequently, rational drug combinations including MCT1 and MCT4 inhibition as well as OXPHOS blockade with metformin or phenformin are currently the most promising approaches to induce a

metabolic catastrophe, leading to tumour cell death [196, 230]. In line with this approach, several new compounds belonging to the 7-aminocarboxycoumarine family that potently inhibit MCTs have been recently identified [218]. One of the compounds, 7-(N-benzyl-N-methylamino)- 2-oxo-2H-chromene-3-carboxylic acid (7ACC2) was shown to block both the extracellular uptake of lactate but also mitochondrial pyruvate transport, thus preventing compensatory glucose oxidation [231].

1.6.1.3 Nucleotide metabolism

The oncogenic cascade is generally accompanied by continuous cellular metabolic reprogramming in order to allow cancer cells to adapt and sustain the energetic requirements necessary to support growth, proliferation and survival. As previously mentioned, cancer cells are characterized by enhanced rates of aerobic glycolysis when compared with normal cells. The adoption of this metabolic pathway allows the accumulation of metabolic intermediates that are crucial for anabolic reactions, thus increasing the biomass essential for sustaining cancer cell growth and proliferation [157].

The maintenance of nucleotide pools necessary for DNA and RNA synthesis is essential to withstand continuous cancer cell division. Cells can produce nucleotides via *de novo* synthesis pathways, using amino acids and small molecules to build the purine and pyrimidine rings, or through salvage pathways, by recycling existing nucleosides and nucleobases. Unlike nonproliferating cells, neoplastic cells are highly dependent on the *de novo* synthesis of nucleotides to maintain DNA replication and RNA production, and this shift from a normal to a high rate of *de novo* nucleotide synthesis involves coordinated input from metabolic and signalling pathways [145]. The metabolic pathways that support the production of nucleotides are dependent on intermediates provided by glycolysis and the TCA cycle. Cancer cells are able to control the glycolytic flux at multiple points in order to allow the build-up of metabolic intermediates that are needed to fuel the nucleotide biosynthesis. These metabolites are channelled into the PPP and used to produce the 5-phosphoribose-1-pyrophosphate (PRPP), the activated form of ribose derived from ribose-5-phosphate [232, 233]. PRPP can be produced through the two branches of PPP, the irreversible oxidative branch that produces ribose-5-phosphate and NADPH-reducing equivalents, and the reversible non-oxidative branch which produces ribose-5-phosphate.

In order to sustain ribose-5-phosphate synthesis, cancer cells need to maintain high levels of the glycolytic intermediates glucose-6-phosphate, glyceraldehyde-3-phosphate and fructose-6-phosphate. Cancer cells do so by, for example, expressing the inactive, dimeric form of pyruvate

kinase M2 (PKM2). This enzyme catalyses the formation of pyruvate in the final step of glycolysis and its decreased activity results in the accumulation of upstream metabolic intermediates that can be pushed into other biosynthetic pathways [234]. Additionally, several transformed cells upregulate the activity and expression of the non-oxidative PPP branch enzyme transketolase 1 (TKTL1), which is associated with poor prognosis [232, 235].

Briefly, in the *de novo* pyrimidine synthesis, the pyrimidine ring is first assembled from glutamine, bicarbonate, and aspartate and is then condensed with PRPP over six reactions. The initial steps of pyrimidine synthesis are carried out in the cytosol and culminate with the production of dihydroorotate. Dihydroorotate then enters mitochondria and is oxidized to orotate that in the cytosol is converted into uridine monophosphate (UMP). Contrary to pyrimidine synthesis, all of the reactions that lead to purine synthesis occur in the cytosol and the purine ring is directly built onto PRPP. Synthesis of the purine ring requires glutamine, glycine, aspartate, formyl-tetrahydrofolate (fTHF) and CO_2 and, after a 10-step enzymatic reaction, the common precursor to all purine nucleotides inosine monophosphate (IMP) is produced and converted into guanosine monophosphate (GMP) or adenosine monophosphate (AMP). The key regulated enzyme in *de novo* purine biosynthesis is glutamine PRPP amidotransferase. The *de novo* synthetized ribonucleoside triphosphates (NTPs) can be then utilized for RNA synthesis. For DNA synthesis, the NAPDH-dependent enzyme RNR catalyses the cytoplasmic reduction of NTPs to deoxy-NTPs [233]. The synthesis of purines and pyrimidines is also upregulated in cancer cells and enzymes involved in nucleotide synthesis are regulated by both oncogenes and tumour suppressor genes. Inactivation of tumour suppressors such as *TP53* and *STK11* and hyperactivation of the mTOR pathway and of oncogenes such as *MYC*, *RAS*, and *AKT* have been shown to fuel nucleotide synthesis in tumour cells [236].

Due to the high energetic cost of the *de novo* nucleotide synthesis pathway, cells have developed a more energy-efficient alternative route to synthetize nucleotides, the salvage pathway [233, 237]. This pathway allows the recycling of purine and pyrimidine nucleobases and nucleosides arising from the diet and nucleic acid breakdown [233]. Turnover of RNA and other nucleotides occurs regularly in order to maintain cell homeostasis. In particular, RNA is constantly recycled during the production of mature RNAs from longer precursors and to regulate the amounts of mRNA. The nucleotides released from polymeric RNA and DNA breakdown can be recycled by nucleotide kinases. In addition, cells can maintain the nucleotide pools by transporting nucleobases from the external environment and adding the suitable sugar. For purines, the nitrogen base is added to

ribose and is accomplished via the PRPP step. Briefly, the free purine bases hypoxanthine and guanine can be salvaged by hypoxanthine guanine phosphoribosyl transferase (HGPRT) to PRPP amidotransferase allosteric inhibitors IMP, GMP, AMP. Consequently, HGPRT salvages purines and reduces *de novo* synthesis of purine nucleotides by increasing the concentration of inhibitory nucleotides and decreasing the concentration of PRPP. The salvage pathway of pyrimidines requires the addition of the base to ribose-1-phosphate, and it is catalysed by a specific pyrimidine phosphorylase [238]. (deoxy)Cytidine can be salvaged either by CDA to produce (deoxy)Uridine [239], or more directly via uridine-cytidine kinase producing (deoxy)cytidine monophosphate [240]. The degradation of nucleotides is extremely important to maintain their pools [241]. Pyrimidine nucleotides are generally degraded by nucleotidases and nucleoside phosphorylases, which cleave the base from the sugar. Uracil and thymidine rings can be completely degraded to β-alanine and β-aminoisobutyrate, respectively, and both metabolites can be either excreted or transformed into intermediates of the TCA cycle [242]. Conversely, purine bases cannot be completely degraded and are instead stripped from phosphates and sugar to finally become oxidized to the end product uric acid, which is excreted into the urine. Adenine and guanine nucleotides are degraded to hypoxanthine and xanthine, respectively, and these oxidized by xanthine oxidase to hydrogen peroxide and uric acid [243].

1.6.1.3.1 Cytidine deaminase

The deamination of cytidines is performed by two families of cytidine deaminases, CDA catalyses the hydrolytic deamination of free cytidine and deoxycytidine to uridine and deoxyuridine, respectively, while the deamination of cytidines incorporated within the DNA or RNA polymers is performed by the AID/APOBECs (activation-induced deaminase/apolipoprotein B mRNA editing catalytic polypeptide-like) proteins. The human APOBEC family consists in 11 primary gene products and alternatively spliced variants that include AID, APOBEC2 (A2), APOBEC3A-H (A3A-H) and APOBEC4 (A4) proteins [244, 245]. The deamination of cytidine by specific APOBEC proteins has well-regulated physiological roles in restriction of endogenous and exogenous retroviruses, innate and adaptive immunity, epigenetics, and lipid metabolism [246].

Cytotoxic compounds remain the pillar of most anticancer treatments, despite the increasing number of targeted therapies and biotherapies approved. The class of nucleoside analogues such as gemcitabine, capecitabine, cytarabine or azacytidine act as antimetabolites, either by interfering directly with DNA or RNA synthesis or by blocking the capacity of the cancer cell to synthesize

precursors of nucleic acids required to ensure sustained growth. These compounds are widely used to treat both solid and haematological disorders in infants and adults and share similar structures and a common metabolic pathway where CDA plays a major role [247]. Besides catalysing the hydrolytic deamination of free (deoxy)cytidine to (deoxy)uridine, this enzyme catalyses deamination of pyrimidine analogues, thus allowing either their deactivation (gemcitabine, cytarabine and azacytidine) or activation (capecitabine).

Human CDA is encoded by the 4-exon gene *CDA* located on the first pair of chromosomes (1p36.2-p35) and consists of 4 identical subunits, all containing an essential zinc atom in the active site. This enzyme is mainly produced in the liver and placenta but it is also highly expressed in other tissues, such as neutrophils and monocyte/macrophages [248, 249]. CDA is subject to a wide inter-individual variability in terms of activity and 1000 variations affecting *CDA* have been evidenced (Ensembl). The most described polymorphisms in coding regions are the two non-synonymous 79A>C (rs2072671) and 208G>A (rs60369023) substitutions and the synonymous 435C>T (rs1048977) variant gene. Additionally, -31delCdeletion (rs3215400) or -92A>G (rs602950) in the promoter region and 154 + 37G>A polymorphism (rs12059454) in intronic regions have been described [118]. Besides the gene polymorphisms, little is known about the transcriptional and posttranscriptional regulation of *CDA*. Recently, it was reported that CDA is regulated by miR-484 through direct targeting of its 3'-UTR in a gemcitabine-resistant model of breast cancer survival [250]. Additionally, CDA was shown to be also induced by miR-365 which is transferred to pancreatic cancer cells by macrophage-derived exosomes [251]. *CDA* is mostly inactivated by DNA methylation [252] and increased levels of NTPs upregulate CDA [251, 253].

Since CDA acts as a key modulator in the conversion of cytidine/deoxycytidine to uridine/deoxyuridine, this enzyme might affect the balance of the nucleotide pools and jeopardize genome stability by affecting the rate of fork progression. Bloom syndrome is a rare genetic disease characterized by high levels of chromosomal instability, an increase in cancer risk and CDA downregulation. In this context, it was observed that CDA deficiency induces a pyrimidine imbalance that inhibits the activity of poly(ADP-ribose) polymerase 1 (PARP-1), a multifunctional enzyme involved in many cellular processes, including the response to DNA damage. The decrease in PARP-1 activity leads to the accumulation, during mitosis, of unreplicated DNA at "difficult-to-replicate" loci, such as centromeres and fragile sites, resulting in ultrafine anaphase bridge formation. Moreover, the attenuated PARP-1 activity due to CDA deficiency also impairs sister chromatid disjunction during mitosis [254-256]. Besides inducing genomic instability, silencing of

CDA arrests the proliferation of cancer cells, induces apoptosis and inhibits tumour growth [257]. Alternately, CDA overexpression has recently been identified as a potential target for anticancer treatment. CDA was found to be involved in the deamination of oxidized and epigenetically modified cytidine nucleosides. This enzyme converts these cytidine analogues into variants of uridine that are incorporated into DNA, resulting in accumulation of DNA damage, and ultimately, cell death [258, 259].

The levels of CDA expression are tumour-specific: pancreas, stomach, thyroid, bladder and leukaemia cancers have high levels of CDA expression but in liver, cervix, colon, oesophagus and breast cancers CDA is under-expressed [250, 252, 257, 258, 260, 261]. Mameri *et al.* studied the CDA expression levels in several tumours and proposed the use of this criteria to define two new subgroups: CDA-deficient tumours and CDA-proficient tumours. In addition, they provided proof-of-concept that the subgroup of cancers not expressing CDA were susceptible to the specific toxic effects of drugs such as aminoflavone. Thus, CDA deficiency might be a new predictive marker of susceptibility to antitumor drugs that could be used as a new target for anticancer therapies [252].

Besides catalysing the hydrolytic deamination of cytidine and deoxycytidine, CDA is a representative detoxification enzyme of anticancer cytidine analogues such as gemcitabine, cytarabine, 5-aza-2′-deoxycytidine (DAC) and 5-azacytidine, and CDA activity limits their therapeutic efficacy. With the intention of overcoming cytidine analogue resistance and increasing their bioavailability, CDA inhibition has been extensively studied. The uridine analogue tetrahydrouridine (THU) is a competitive inhibitor of CDA that has been used as a CDA inhibitor in combination with cytidine analogues. Both preclinical and clinical studies show that CDA inhibition with THU increases the chemosensitivity of cancer cells to cytidine analogues without any reported toxic side effects [262-269]. Due to the instability of THU, cedazuridine, a novel CDA inhibitor was recently developed. Cedazuridine has shown to be safely orally bioavailable in preclinical studies [267, 270, 271].

1.6.2 Avoiding immune destruction

Tumours are complex and dynamic masses of heterogeneous proliferating neoplastic cells that interact with a variety of resident and infiltrating host cells, secreted factors and extracellular matrix proteins, collectively known as the TME. Besides the extracellular matrix and a variety of different cytokines and metabolites, the TME comprises recruited genetically stable normal cells (tumour-

infiltrating immune cells, CAFs and blood and lymphatic endothelial cells) that form the tumour-associated stroma [272]. The interaction of cancer cells with their environment, namely with immune cells, actively influences tumour progression, as it dictates whether the primary tumour is eradicated, spreads or establishes dormant micrometastases, but it also shapes therapeutic response and resistance [273, 274].

The immune system acts as an active and important barrier during the lengthy multistage development of many forms of human cancer, however emerging neoplasias are able to avoid active surveillance by the immune system.

1.6.2.1 The immune system in cancer

The effective killing of cancer cells by the immune system requires several steps that must be initiated and allowed to proceed and expand iteratively, the anti-tumour immune cycle (**Figure 1.7**). Briefly, neo-antigens released from dead or dying cancer cells are captured by antigen-presenting cells (APCs), namely dendritic cells (DCs), for processing. Next, APCs migrate to the draining lymph nodes and present the captured antigens on major histocompatibility complex (MHC)-I (MHCI) (cross-presentation) and MHC-II (presentation) molecules to T cells, leading to the priming and activation of effector T cell responses against the cancer-specific antigens. Finally, activated effector T cells exit the lymph node, migrate to and infiltrate the tumour bed. Tumour antigen-specific $CD8^+$ T cells can recognize and bind specific cancer cells through the interaction between its T cell receptor (TCR) and its cognate antigen bound to MHC-I, and kill the target cancer cell. Additionally, tumour antigen-specific $CD4^+$ cells can also communicate with APCs residing in the tumours and exert immune responses. The killing of cancer cells releases additional tumour-associated antigens to enhance the anti-tumour immune responses and amplify the anti-tumour immune cycle [275].

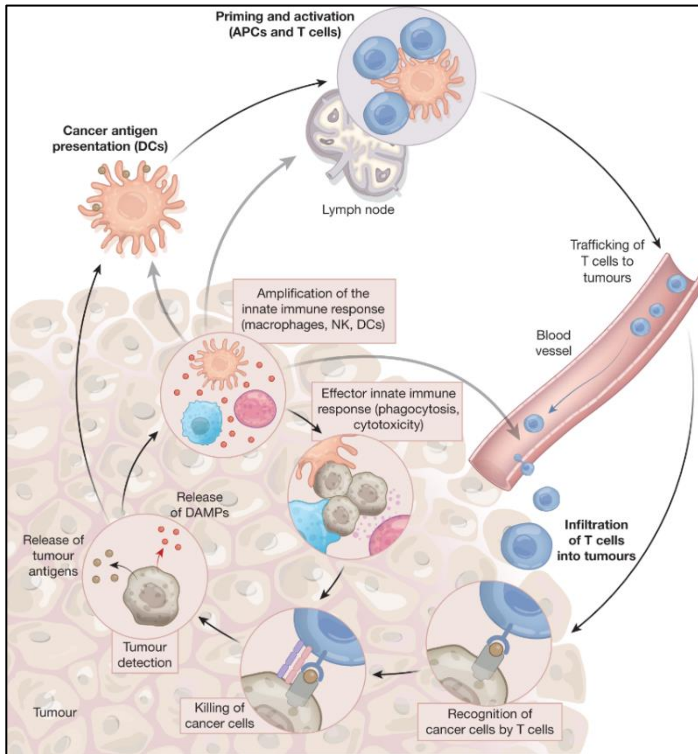


Figure 1.7. The cancer-immunity cycle. The cancer-immunity cycle drives T-cell response against tumours. Briefly, tumour antigens are captured by antigen-presenting cells (APCs), for processing. In the draining lymph nodes, APCs present the captured antigens to T cells, leading to the priming and activation of effector T cell responses against the cancer-specific antigens. Lastly, activated effector T cells migrate and infiltrate the tumour, and kill the cancer cells. Figure used with permission from authors [276].

Depending on the tumour and immune context, different subsets of immune cells have different roles in the TME, including pro-tumorigenic and anti-tumorigenic functions. Consequently, the immune system can act as a double-edged sword by promoting or inhibiting tumour growth. Additionally, the immune system can be conceptually divided into two major arms, the innate arm and the adaptive/acquired arm, however they are functionally interconnected and influence each other [277]. The key distinction between the two immunities is the capacity of each cell to recognize and respond to a single specific molecular entity, or antigen.

1.6.2.1.1 The innate immune system in cancer

One of the primary functions of the innate immune system is to regulate the initiation of an immune response and in the context of cancer, it is rapidly recruited to the forming tumour. Upon recognition of a common array of molecular patterns called danger-associated molecular patterns (DAMPs), through pattern recognition receptors (PPRs), the innate immune system initiates an early immune response but also launches a full and robust immune response by transmitting critical signals to activate the adaptive immune system [278]. Besides its critical role in the initiation of an immune response, the innate immune system is also responsible for its maintenance and resolution [279]. Despite being critical for launching an effective immune response against cancer, the innate immune system can also promote tumour initiation by inducing inflammatory responses [280].

The innate immune system is composed of macrophages, DCs, neutrophils, myeloid derived suppressive cells (MDSCs) and innate lymphoid cells (ILCs). Myeloid cells, including macrophages and neutrophils, are the most abundant immune cells in the TME [281]. Tumour-associated macrophages (TAMs), together with MDSCs, are responsible for skewing and suppressing adaptive immunity, orchestration of tissue repair and damage regulation, promotion of immunosuppression, modulation of the response to immunogenic cell death, effector functionality against tumour cells and the mediation of abscopal effects [282]. The wide range of roles played by TAMs is a result of their plasticity. Depending on the environmental stimuli they receive, TAMs can undergo classical activation and exhibit an anti-tumour M1 phenotype upon stimulation with LPS and IFN-γ. On the other hand, IL-4 stimulation can lead to alternative activation where TAMs display an immunosuppressive M2 phenotype [283]. The high number of TAMs in tumours is normally associated with a poor survival in human cancer patients. However, in some cancers such as CRC, high TAM density is correlated with good prognosis [284-286].

As for other myeloid cells, tumour-associated neutrophils (TANs) exhibit both anti-tumoral and pro-tumoral functions. It is believed that TANs can fight tumour progression in early stages but tend to support tumour growth and metastasis as the tumour progresses [287]. TANs can be divided in anti-tumour "N1-like" and pro-tumour "N2-like" neutrophils, depending on the stimuli they receive from cancer cells and stromal cells within the TME [288]. The presence of circulating neutrophils is associated with poor prognosis in several cancer types including pancreatic cancer [289, 290].

MDSCs are a group pathologically activated immature myeloid cells with the ability to exert potent immunosuppressive effects and are only found in pathological conditions such as chronic infection, autoimmunity and cancer. They express markers of monocytes, macrophages, and neutrophils, making it difficult to distinguish their immunosuppressive activities [291, 292]. MDSCs promote the formation of regulatory T (Treg) cells, cells which are part of the adaptive immune system crucial to the development of immune tolerance, and promote differentiation of fibroblasts to CAFs [293, 294]. The presence of circulating MDSCs in cancer patients is correlated with higher staging and worse survival rates [295, 296].

As previously mentioned, DCs are the main type of professional APCs and play a critical role in the generation of protective antitumor immunity by providing antigens and co-stimulatory signals to cells of the adaptive immune system. Tumour-infiltrating dendritic cells capture and process tumour-associated antigens and then migrate to draining lymph nodes, where they prime and activate T cells [275]. There are two major subsets of DCs, plasmacytoid DCs (pDCs) and

conventional DCs (cDCs), and the latter can be further divided into cDC1 and cDC2 based on the expression pattern of surface markers and their functions. pDCs are considered to be immunosuppressive and promote tumour progression and metastasis as they support Tregs function [297]. cDC1s excel in the activation of cytotoxic lymphocytes including $CD8^+$ T cells (CTLs), natural killer (NK) cells, and NKT cells, and its gene signature positively correlates with survival of cancer patients with different tumour types. cDC2 seems to play a role on the MHC-II-mediated activation of $CD4^+$ T cells [298-300].

ILCs are a heterogeneous immune cell population and are one of the first lines of defence against a threat. ILCs play an important role in shaping both innate and adaptive immune responses as a consequence of the cocktail of cytokines produced. Additionally, the cytokines produced and the expression of master regulator transcription factors resemble the T lymphocyte subpopulations, being therefore considered their innate counterparts. However, unlike T cells, ILCs are activated by cytokines and as a result of a balance between activating and inhibitory cues in an antigen-independent manner since they lack antigen specific receptors (TCR-/CD3-) and their development is independent from rearrangement genes [301]. ILCs are divided into 3 main groups, NK/ILC1s, ILC2s, and ILC3s, based on the expression of surface markers, transcription factors and cytokines, as well as functional features [301, 302]. NK cells constitute the innate equivalent of cytotoxic $CD8^+$ T cells, whereas ILC1s, ILC2s, and ILC3s are the counterparts of $CD4^+$ T helper Th1, Th2, and Th17, respectively. Group 1 ILCs, like Th1, are characterized by constitutive expression of the transcription factor T-bet and can produce Interferon γ (IFN-γ), Tumour Necrosis Factor α (TNF-α), Granulocyte-Macrophage Colony-Stimulating-Factor (GM-CSF), granzyme and perforin in response to IL-12, IL-15 and IL-18. ILC1s cooperate with Th1 and activate macrophages and some can exert direct cytotoxicity [303, 304]. Although NK cells are capable of killing tumour cells without requiring further 'education' by other immune cells, it is now clear that regulatory mechanisms within the TME can suppress their activity [305, 306]. Mirroring Th2, ILC2s constitutively express GATA3 and secrete IL-4, IL-5, IL-13, IL-9, and amphiregulin in response to IL-25, IL-33, and Thymic Stromal LymphoPoietin. Since type 2 cytokines create an immunosuppressive TME, ILC2s are considered to be pro-tumorigenic. Similar to Th17, ILC3s express RORγt, the lymphotoxins α and β, IL-17 and IL-22, GM-CSF, and TNF-α and can be activated by IL-23, IL-1β, or by Natural Cytotoxicity Receptors ligands. As for Th17, the role of ILC3s in cancer progression is controversial. They were shown to have both pro- and anti-tumour functions, most likely due to their heterogeneity [307].

1.6.2.1.2 The adaptive immune system in cancer

The adaptive immune system, which is comprised of T and B lymphocytes, recognizes tumour antigens presented on cancer cells or APCs and triggers an antigen-specific immune response. Depending on their TCR chain, co-receptor and cytokine expression profile, T cells can be divided into $\alpha\beta$ T cells, further subdivided into $CD4^+$ T cells and $CD8^+$ cytotoxic T cells or CTLs according to their co-receptor expression, or $\gamma\delta$ T cells. As previously mentioned, upon TCR binding to antigens presented on the MHC-II of professional APCs or MHC-I molecules of all nucleated cells, $CD4^+$ and $CD8^+$ T cells are activated. $CD4^+$ T cells can further be differentiated into Th1, Th2, Th17 and Tregs subsets depending on the cytokine environment. These different subsets display distinct functions and cytokine expression profiles and are controlled by different molecular mechanisms. Upon the primary immune response, the majority of T cells undergo apoptosis to allow the resolution phase of the immune response, whereas a small fraction remain and constitute the memory T cells [308]. These memory T cells are crucial in case of a exposure to the antigen for a second time as they respond faster and in a more efficient fashion [309]. In particular, tissue-resident memory $CD8^+$ T cells (T_{RM}) are a subtype of the $CD8^+$ memory T cell population that is generated and persists in the tissue where they originated. Upon exposure to the same cancer antigen, T_{RM} cells provide a first line of adaptive cellular defence, therefore showing a promising anti-tumour capacity [310].

Unlike other lymphocytes, B lymphocytes synthetize and display the B-cell receptor (BCR), a transmembrane receptor located on the cell surface that binds to antigen. The BCR plays a key role in B cell development and adaptive immune responses [311]. Peripheral B cells can be divided into various subsets according to an array of phenotypic surface markers: transitional, ‘immature,’ B cells; mature naïve B cells; germinal centre B cells; memory B cells; and antibody-secreting cells plasmablasts and plasma cells [312]. In the cancer context, B cells have been shown to display both pro- and anti-tumour activities. For example, the presence of tumour-infiltrating B cells and tumour-reactive antibodies correlates with extended patient survival [313, 314]. However, recently an emerging role for B cells with immunosuppressive and/or regulatory functions in modulating anti-tumour immune responses and in carcinogenesis has been described [315, 316]. Hence, subsets of B cells with dissimilar phenotypes and functions may play different roles in relation to anti-tumour responses.

$\gamma\delta$ T cells are one of three immune cell types that express antigen receptors that undergo somatic recombination and are defined by the expression of heterodimeric TCRs composed of γ

and δ chains [317]. Human γδ thymocytes are highly poised to become anti-tumoral type 1 cytotoxic effector cells mainly due to IFN-γ production [318]. However, a pro-tumoral role for γδ T cells has been recently shown, where IL-17-producing γδ T cells recruited immunosuppressive MDSCs into the malignant microenvironment, further driving pro-tumoral inflammation [319].

T helper cells or $CD4^+$ T cells play a critical role in regulating health and disease by coordinating the immune system to contend with danger induced by foreign antigens, such as cancer formation. Upon antigen-dependent activation via the MHC-II-peptide complex found on APCs, T helper cells can target tumour cells in several ways, either directly by eliminating tumour cells through cytolytic mechanisms or indirectly by activating B cells, CTLs, macrophages and other cells that mount an immune response [320]. Although the strength of TCR signalling and the quality of co-stimulation can influence the polarization of naïve $CD4^+$ T cells, it is the cytokine milieu that mainly dictates T helper cell differentiation. $CD4^+$ T cells differentiate in response to particular combinations of cytokines into subsets such as Th1, Th2, Th17, Treg, Th9, Th22, and T follicular helper cells (Tfh) [321]. These subsets of $CD4^+$ T cells are controlled by different transcription factors to produce unique cocktails of cytokines and exert an effector function against self and foreign antigens. $CD4^+$ T cell division was initially hypothesized to be dominated solely by Th1 and Th2 helper subsets. However, recent evidence showed that Th17 cells and Tregs also play important roles in regulating health and exacerbating autoimmunity and cancer. Although distinct helper T cell subsets (i.e. Th9, Th22, and Tfh) have been recently described, knowledge of the programming cytokines and master transcription factors and their role in tumour biology for these subsets is still under debate.

Th1 cells are involved in pro-inflammatory immune responses and rely on the expression of T-bet and produce high amounts of IFN-γ, IL-2 and TNF-α in response to IL-12. IFN-γ, and TNF-α, secreted by Th1 cells directly kill cancer cells, activate macrophages and are required for cytokine-mediated activation and regulation of tumour-specific CTLs and induce cancer cell senescence [322-324].

Th2 cells support humoral immune responses, play a role in the presentation of allergens and promote immunity against extracellular pathogens through production of IL-4, IL-5, and IL-13. Th2 cells are regulated by transcription factor GATA3 and their differentiation is also mediated by IL-4, thus creating a positive feedback loop that boosts proliferation. Contrary to Th1 cells, cytokines secreted by Th2 are pro-tumoral, inducing T cell anergy, inhibiting T cell mediated cytotoxicity and promoting the immunosuppressive functions of other immune cells, such as TAM polarization into the M2 pro-tumour phenotype [325, 326].

Th17 cells secrete IL-17A, IL-17F, IL-21, IL-22, and GM-CSF and their differentiation is induced by IL-6, IL-22, IL-23 and TGF-β and regulated by the transcription factors STAT3 and orphan receptor RORγt [327]. The role of Th17 cells in cancer is controversial as they have been reported to promote both carcinogenesis and antitumor immunity [328].

Tregs are a subset of $CD4^+$ T cells that a play a crucial role in maintaining immune tolerance and have been shown to dampen autoimmunity and antitumor immunity. Tregs suppress effector function through secretion of inhibitory cytokines such as IL-10 and TGF-β or through cell-mediated engagement of inhibitory checkpoint molecules such as T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) [329]. These subset of T helper cells express the transcription factor forkhead box P3 (FoxP3) and unlike other $CD4^+$ helper T cells, Tregs are highly immunosuppressive [330] and their circulating or intratumoral levels correlate with a poor clinical prognosis in several human cancer types [331-334].

CTLs express the CD8 co-receptor (one CD8α and one CD8β chain) and are considered to be frontline defensive cell types for fighting cancer progression. Naïve $CD8^+$ T cells are committed to detecting antigenic peptides presented by MHC-I molecules expressed by APCs. Upon priming in the lymphatic organs, they become activated, proliferate and differentiate to form effector CTLs, which are then able to eliminate infected or malignant cells. Once activated, effector $CD8^+$ T cells traffic back to the TME and tackle cancer cells through granule exocytosis and Fas ligand-mediated apoptosis induction. In the granule exocytosis pathway, CTLs first produce perforin to form pores in the plasma membrane of cancer cells. These pores allow the serine proteases granzymes A and B to enter the target cells, cleave their intracellular substrates and induce programmed cell death. In addition, CTLs activate caspases through activation of Fas ligand and the subsequent cytochrome c release in the target cells, stimulating engagement of apoptosis in Fas-expressing target cells [335, 336]. Alternately, CTLs also release IFN-γ and TNF-α to induce cytotoxicity in the cancer cells. IFN-γ produced by both $CD8^+$ T cells and $CD4^+$ T cells stimulates M1 macrophages to exert anti-tumoral effects [337]. Therefore, the number of CTLs within the TME is a pivotal prognostic factor for cancer, including prolonged survival and higher response rates to cancer immunotherapy, in many different tumour types [338].

1.6.2.2 Immunotherapy: immune checkpoint blockade

The tumour immune microenvironment encompasses a wide range of complex interactions between tumour cells, immune cells and tumour stroma, and maintaining immune homeostasis is

critical for host survival. To prevent inflammatory tissue damage and autoimmune diseases, the extent and magnitude of the immune response is tightly regulated by a balance between co-stimulatory and inhibitory signals, or immune checkpoints. Under normal circumstances, immune checkpoints control the type, magnitude, and duration of the immune response, allowing the immune system to respond to a threat while protecting tissues from harm. However, transformed cells dampen the immune surveillance by expressing some of these immune-checkpoint proteins, thus promoting an immunosuppressive TME, as well as growth and expansion of tumours [339].

Activated T cells express multiple co-inhibitory receptors such as lymphocyte-activation gene 3 (LAG-3), programmed cell death protein 1 (PD-1) and CTLA-4. T cell activation requires binding of antigen-loaded MHC to the TCR and a co-stimulatory signal transduced by the binding of T cell co-stimulatory surface receptor CD28 to its ligand CD80 (B7-1) or CD86 (B7-2) on the surface of professional APCs. Upon activation, an inhibitory signal prevents continued T cell activation, and T cells start to express co-inhibitory cell surface receptors, such as CTLA-4 and PD-1. Since CTLA-4 binds with higher affinity to CD80 and CD86 than the co-activating receptor CD28, it is able to deliver an inhibitory signal to restrain T cell immune responses [323]. Besides being expressed on activated T cells, CTLA-4 is constitutively expressed on Tregs [340]. Activated T cells upregulate and continue to express PD-1 in tissues where inflammatory signals induce the expression of its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) on the surface of APCs, myeloid cells, and cancer cells. The binding of PD-1 to its ligands inhibits signalling downstream of the TCR, cytokine production and T cell proliferation, thus limiting collateral tissue damage [341]. Contrary to CTLA-4-mediated immune checkpoint, which is induced in T cells at the time of their initial response to antigen and occurs in secondary lymphoid organs, PD-1 inhibitory signalling occurs in order to regulate inflammatory responses in tissues by effector T cells which recognize antigen in peripheral tissues, including tumours (**Figure 1.8**) [339].

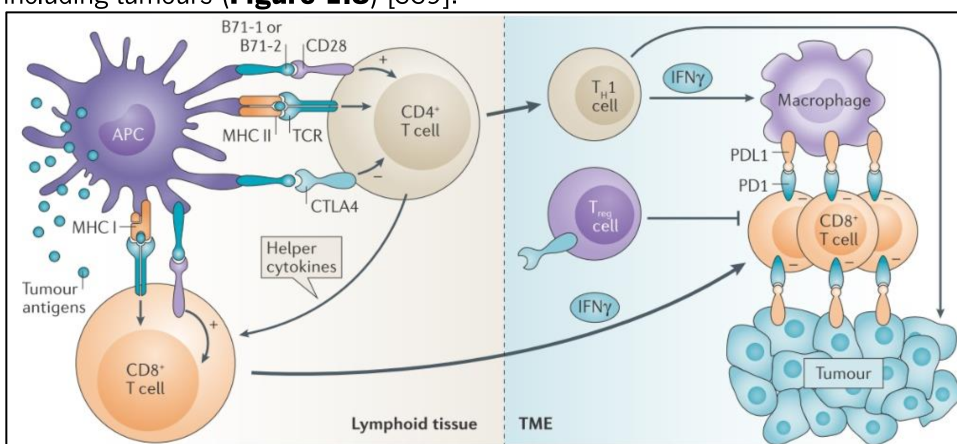


Figure 1.8. The immune checkpoint CTLA-4 and PD-1 pathways. Upon priming, both CD4+ and CD8+ T cells are activated through co-stimulatory pathways, leading to their proliferation, secretion of inflammatory cytokines,

acquisition of cytolytic properties and migration to sites of antigen display. The immune checkpoint cytotoxic T-lymphocyte associated protein 4 (CTLA-4), regulates the amplitude of $CD4^+$ T cell priming and help for $CD8^+$ T cell priming in lymphoid tissue. Within hours to days, activated T cells also begin to express the co-inhibitory receptor and immune checkpoint programmed cell death protein 1 (PD-1). $CD4^+$ T helper 1 (TH1) cells and $CD8^+$ T cells produce interferon-γ (IFN-γ), which, on the one hand, activates tumour killing by macrophages and antigen display by tumour cells, but, on the other hand, induces programmed death-ligand 1 (PD-L1) expression by these same macrophages and tumour cells. Tumour-specific $PD1^+$ $CD8^+$ T cells encountering $PDL1^+$ cells within the TME will be functionally disabled. Additionally, CTLA-4 expressed by Treg cells enhances their ability to suppress $CD8^+$ T cell-dependent cytokine production and direct tumour cell killing. Figure used with permission from authors [342].

Binding of CTLA-4 and PD-1/PD-L1 to cancer cells or TME ligands dampens the actions of T cells, which enables tumour cells to avoid immune surveillance and destruction [339]. Therefore, strategies such as immune checkpoint blocking antibodies or recombinant forms of ligands, to target these regulatory pathways in order to reinvigorate exhausted T cells at the tumour site and enhance immune activity against tumour cells have been developed. Blockade of CTLA-4 with ipilimumab, PD-1 with nivolumab, pembrolizumab and cemiplimab or its ligand PD-L1 with atezolizumab, avelumab and durvalumab have already been approved (**Table 1.2**) [343] and have shown to enhance T cell fitness and led to clinical benefits with high response rates of prolonged duration in patients with a wide range of solid and liquid cancers such as melanoma, renal cancer, lung cancer, lymphoma, head and neck squamous cell cancer, bladder cancer, liver cancer, gastro-oesophageal cancer and MSI-H CRC [344-355]. Currently, hundreds of phase I and II clinical trials and phase III/IV clinical trials are being carried out to assess the efficacy of multiple immune checkpoint inhibitors as monotherapy or in combination.

Table 1.2: FDA-approved immune checkpoint inhibitors for cancer

Drug name	Target
Ipilimumab (Yervoy®)	CTLA-4
Nivolumab (Opdivo®)	PD-1
Ipilimumab + nivolumab	CTLA-4 + PD-1
Pembrolizumab (Keytruda®)	PD-1
Atezolizumab (Tecentriq®)	PD-L1
Avelumab (Bavencio®)	PD-L1
Durvalumab (Imfinzi®)	PD-L1
Cemiplimab (Libtayo®)	PD-1

Despite the success of anti-CTLA-4 and anti-PD-1/PDL1 therapies, the percentage of patients who respond to immune checkpoint blockers regimens remains around 30% for the most common solid tumours [356], and in tumours such as MSS and MSI-L CRC and pancreatic cancer, immunotherapy failed to show any clinical benefit [357, 358]. Moreover, due to moderate-to-severe immunological toxicities, treatment requires discontinuation and/or active management [359] and has an estimated cost of 100,000 to 250,000 USD per patient [360]. There are several factors that influence the priming, activation and recruitment of T cells into the TME and therefore determine the efficacy of immune checkpoint blockade: composition, localization and fitness of the immunological tumour infiltrate, mutational landscape, PD-L1 expression, function of interferon signalling pathways, expression of antigen-presenting molecules and immune-evasive oncogenic signalling pathways.

1.6.2.2.1 Immunological tumour infiltrate: from “cold” to “hot” tumours

The success of anticancer therapy (e.g. immunotherapy) depends largely on three aspects of the immunological tumour infiltrate: its composition, localization and functionality (**Figure 1.9**).

The composition of the immunological tumour infiltrate will determinate the disease outcome and response to immune checkpoint blockers and the presence of a high number of tumour-infiltrating anti-tumoral CTLs, Th1 T cells, and cDC1 over immunosuppressive and pro-tumoral Tregs and M2-polarized macrophages have been associated with a better response to immunotherapy [361]. The TME is segregated into three major types based on the localization of the immune infiltrate: immune desert, immune excluded and immune inflamed. The so-called immune desert or “cold” phenotype is characterized by a global absence of T cells either in the parenchyma or the stroma of the tumour and the lack of suitable T cell priming or activation, and these tumours rarely respond to anti-PD-L1/PD-1 therapy [362]. As opposed to immune desert tumours, the immune-excluded phenotype is characterized by the presence of multiple chemokines, vascular factors or mediators and stromal-based inhibition, and high levels of T cells and other immune cells. However, these immune cells cannot penetrate into the tumour and accumulate at the tumour border. Upon immune checkpoint blockade, T cells can show evidence of activation and proliferation but not infiltration, and clinical responses are uncommon. The immune desert and immune excluded phenotypes can be considered as non-inflamed tumours. In the immune inflamed or “hot” phenotype, intratumoral $CD4^{+}$ and $CD8^{+}$ T cells, myeloid and

monocytic cells are abundant and get into direct opposition with neoplastic cells. The best clinical responses to immune checkpoint inhibitors occurs most often in patients with this phenotype [362, 363].

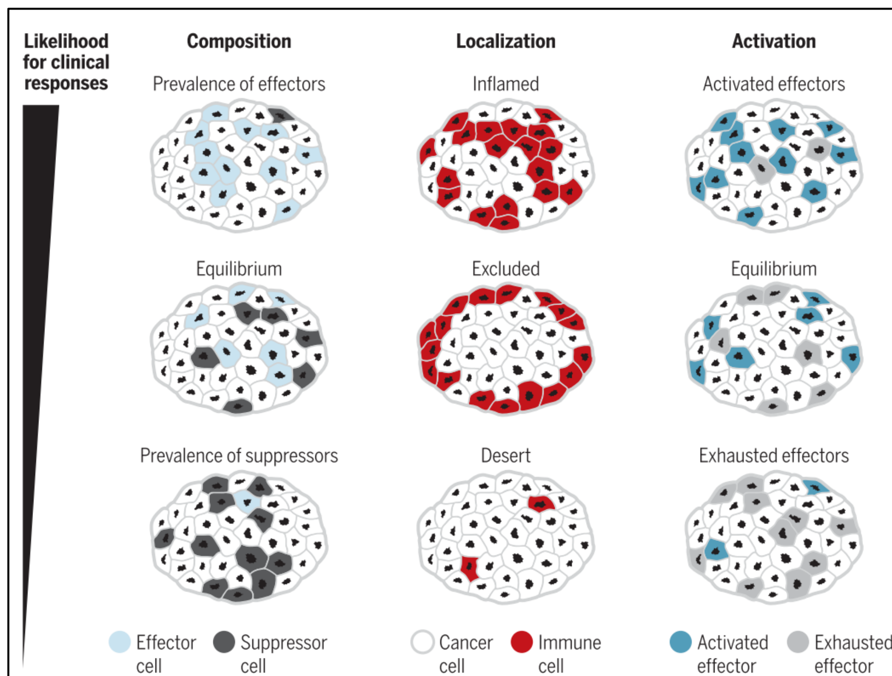


Figure 1.9. Impact of the tumour infiltrate on clinical responses to immunotherapy according to its composition, localization and activation. The likelihood of cancer patients to respond to immunotherapy is dictated by three features of the immunological tumour infiltrate: composition (left); localization (middle); and activation (right). Adapted from [363] and used with permission from authors.

Lastly, the functional status of the immune infiltrate, such as diversified TCR repertoire, Ki67 positivity, CD69 expression, and CD45RO-to-CD45RA switch in T cells have been positively associated with successful immunotherapy response in preclinical and clinical settings [363]. In opposition, increased expression of CD39, involved in extracellular ATP degradation in Tregs, and elevated levels of indoleamine 2,3-dioxygenase 1 (IDO1) and arginase 1 in tumour-infiltrating DCs have been associated with increased resistance to immunotherapy [364-366].

Therefore, one can claim that altering the recruitment and the location of specific immune cells within different tumour niches is an appealing strategy to boost anti-tumour immune responses.

1.6.2.2.2 TME metabolism and immunological tumour infiltrate

In recent years, studies have highlighted the lack of correlation between tumour T cell infiltration, response to immunotherapy (i.e., immune checkpoint blockade) and immunogenic antigen landscape [341, 356, 367], and raised the question whether antigen-independent mechanisms were responsible for limiting anti-tumour responses. In fact, evidence shows that unsatisfactory immunotherapeutic interventions fail to promote T cell expansion and anti-tumour

activity due to specific metabolic traces of the TME. A link between tumour metabolism and immune response has long been suspected [368] and while tumour and stromal cells adjust their metabolic pathways to adverse conditions in the TME, they simultaneously dampen tumour-infiltrating immune cell functions and fate, thus allowing immunological tolerance and reduce anti-tumour effector functions (**Figure 1.10**). For instance, glycolysis-driven acidification and lactate accumulation, hypoxia, metabolic competition for limiting nutrients, certain immunosuppressive metabolites, macrophage-driven arginine depletion, nitric oxide production as well as tryptophan catabolism by IDO hinder T cell functions and allow tumour immune evasion and growth [369-375].

Malignant cells have high glucose demands and can deprive the TME of this limiting nutrient [376]. T cells, NK cells, macrophages and DCs use glucose to support their effector functions, thus this competition can ultimately block effective anticancer immune responses [370, 377, 378]. Mechanistically, glycolysis regulates IFN-γ production in T cells, and glucose can blunt Ca^{2+} signalling, glycolytic capacity and cytokine production in tumour-infiltrating lymphocytes [370, 377, 379]. In fact, tumours with increased expression of glycolytic enzymes are resistant to adoptive T cell therapy [380]. Therefore, blocking of PD-L1 expressed by cancer cells can decrease their glycolytic flux, thus harnessing glucose competition and further increasing T cell cytotoxicity [370]. Glucose is also highly utilized by human tolerogenic DCs, MDSCs and tumour-adjacent endothelial cells, all of which have been shown to contribute to a permissive immune environment for tumour growth and metastasis [381-383].

Besides competition for glucose, high glycolytic flux in cancer results in the increased secretion of immunosuppressive metabolites, such as lactate. Lactate accumulates in the microenvironment, resulting in local acidification or acidosis, which also blunts CTL and NK immunosurveillance capabilities [384, 385]. Acidic conditions resulting from high lactate decrease the cytotoxic fitness of CTLs by decreasing T cell secretion of IL-2, TNF and IFN-γ and upregulation of CTLA-4 expression [386]. Lactate can also inhibit DC cytokine release and monocyte-derived dendritic cell differentiation and activation and sustain the pro-tumour M2-like phenotype of TAMs [387, 388]. Contrary to the inhibition of the function of effector T cells, lactate does not impair Treg cell function [389].

Restriction of glutamine in the TME may also favour the differentiation of naïve T cells into Tregs and the accumulation of Treg cells over the emergence of effector Th1 cells [390, 391]. Cytotoxic

cells are also sensitive to amino acid restriction and show impaired function under glutamine deprivation [392].

Lastly, immunosuppressive metabolites such as adenosine and kynurenine are abundant in tumours. Tregs convert ATP to adenosine in the TME and in turn this metabolite can suppress the activity of other immune cells in the tumour [393]. IDO catabolizes the essential amino acid tryptophan into the immunosuppressive metabolite kynurenine, resulting in T-cell inhibition [394]. Additionally, expression of IDO in APCs is associated with tumour progression and poor responses to immunotherapies and kynurenine induces Treg differentiation and suppress DCs immunogenicity [395, 396].

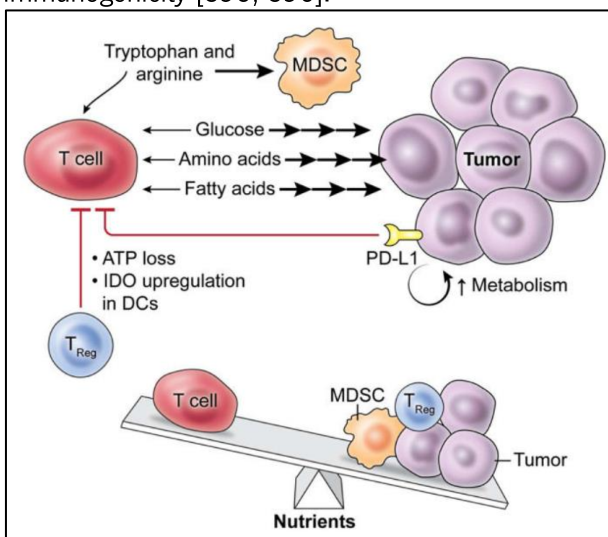


Figure 1.10. The effect of a metabolically deprived and immunosuppressive TME in T cells. Anti-tumour T cells must cope with, and overcome, a challenging metabolic microenvironment in order to successfully mount an immune response against tumours. First, tumour cells take up large amounts of key nutrients including glucose, amino acids, and fatty acid that required for optimal T cell activity. Second, besides directly inhibiting T cell metabolism, tumour expression of programmed cell death protein 1 (PD-1) also promotes the metabolic activity of the tumour. Anti-tumour T cells must also deal with suppressive cell subsets, such as myeloid-derived suppressor cells (MDSCs) and Tregs, which deplete key nutrients such as arginine and tryptophan. Figure used with permission from authors [397].

While nutrient availability can hinder the activity of immune cells in immunologically 'cold' or poorly controlled tumours, the accumulation of immunosuppressive metabolites generated by immunosuppressive cells can also prevent efficient immune cell function. Therefore, the metabolic competition and crosstalk between different cell populations within the TME determines the pro- or anti-inflammatory function of immune cells and the inherent efficiency of immunotherapies.

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CHAPTER 2. Monocarboxylate transport inhibition potentiates the cytotoxic effect of 5-fluorouracil in colorectal cancer cells

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Monocarboxylate transport inhibition potentiates the cytotoxic effect of 5-fluorouracil in colorectal cancer cells

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Abstract

Cancer cells rely mostly on glycolysis to meet their energetic demands, producing large amounts of lactate that are extruded to the tumour microenvironment by monocarboxylate transporters (MCTs). The role of MCTs in the survival of colorectal cancer (CRC) cells is scarce and poorly understood. In this study, we aimed at better understand this issue and exploit these transporters as novel therapeutic targets alone or in combination with CRC classical chemotherapeutic drugs.

For that purpose, we characterized the effects of MCT activity inhibition in normal and CRC derived cell lines and assessed the effect of MCT inhibition in combination with 5-fluorouracil (5-FU), a CRC classical chemotherapeutic drug.

Here, we demonstrated that MCT inhibition using CHC, DIDS and quercetin decreased cell growth, disrupted the glycolytic phenotype, inhibited proliferation and enhanced cell death in CRC cells. These results were confirmed by specific inhibition of MCT1/4 by RNA interference. Notably, we showed that 5-FU cytotoxicity was potentiated by lactate transport inhibition in CRC cells, either by activity inhibition or expression silencing.

These findings provide novel evidence for the pivotal role of MCTs in CRC maintenance and survival, as well as for the use of these transporters as potential new therapeutic targets in combination with CRC conventional therapy.

Keywords

Colorectal cancer, monocarboxylate transporters, lactate transport, glycolytic metabolism, 5-fluorouracil.

2.1 Introduction

The distinct metabolic behaviour observed in tumour cells was recently recognized as a hallmark of cancer [1]. To support their energy demands, cancer cells increase the rates of glycolysis, leading to an overload of lactic acid, which must be exported to the extracellular milieu, decreasing extracellular pH. This acidification contributes to the malignant phenotype of tumour cells, being associated with increased invasion [2], suppression of anti-cancer immune response [3], tumour proliferation, angiogenesis and metastasis [4, 5]. Also, high extracellular lactate has been associated with poor prognosis in cancer patients [5, 6].

Monocarboxylate transporters (MCTs) are essential players in the maintenance of the glycolytic metabolism having a dual role, both as lactate transporters and pH regulators [7]. The MCT family presently comprises 14 members; however, only the first four (MCT1-4) are known to mediate the proton-coupled transport of monocarboxylic acids across the plasma membrane [8-14]. CD147 is a chaperone for both MCT1 and MCT4, promoting their correct plasma membrane expression and activity [15-18]. MCT inhibition disrupts both cellular and extracellular balance, namely affecting pH homeostasis, inducing apoptosis [19] and reducing tumour angiogenesis, invasion [20], and metastasis [21]. Several agents are known to inhibit MCT activity like α-cyano-4-hydroxycinnamic acid (CHC), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and quercetin [22]. MCTs are currently seen as promising therapeutic targets in cancer, with encouraging results in various *in vitro* and *in vivo* studies [13, 23-30].

Data on the expression of MCTs in colorectal cancer (CRC) are scarce and contradictory [31-34]. Koukoukaris *et al.* [31] found expression of MCT1 in cancer cells and in tumour-associated fibroblasts, while MCT4 was weakly expressed in the tumour environment. Conversely, our group [32], detected a significant gain in MCT1 and MCT4 membrane expression, comparing with the adjacent normal epithelium. More recently [33, 34], 50% MCT4 plasma membrane positive staining in two cohorts of CRC patients was described, supporting the role of these MCT isoforms in CRC malignancy.

In the last years, chemotherapeutic treatment of CRC suffered revolutionary changes, with new compounds and regiments approved or under investigation, namely the development of compounds targeting specific alterations in cell signalling pathways [35]. One of the mostly used chemotherapeutic agents for the treatment of CRC is 5-fluorouracil (5-FU), however, there is growing evidence for 5-FU resistance [36]. When administered as a single agent, 5-FU activity is

modest, with response rates of less than 10-15% [37]. Efforts have been made to unravel new combinatory therapies aiming to enhance the therapeutic efficacy of 5-FU and reduce its side effects. Addition of leucovorin was shown to improve the efficacy of 5-FU with little toxicity [38] and, more recently, capecitabine (an orally administered prodrug of 5-FU) and newer monoclonal antibodies targeting the epidermal growth factor receptor (cetuximab and panitumumab) and the vascular endothelial growth factor (bevacizumab) have been introduced in CRC therapeutics [39-41].

The need for more effective therapeutic approaches led us to try to understand the role of MCTs in CRC cells and explore these transporters as therapeutic targets. Here, we assessed the role of MCTs on the viability, proliferation and energetic metabolism of CRC derived cell lines, and explored the potential of combining MCT inhibition with 5-FU. We observed that MCT activity inhibition inhibited cell growth and proliferation, disrupted the glycolytic phenotype, and enhanced cell death in CRC cells. These results were corroborated by MCT expression inhibition. Moreover, we showed that MCT inhibition potentiated the cytotoxic effect of 5-FU.

2.2 Materials and Methods

Cell lines and culture conditions

The human colon carcinoma-derived cell lines HCT-15 and RKO were kindly provided by Dr. Raquel Seruca (IPATIMUP, Porto, Portugal). HCT-15 cells were cultured in RPMI 1640 medium (Gibco, Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Invitrogen, USA) and 1% (v/v) penicillin-streptomycin solution (Pen/Strep, Invitrogen, USA). RKO cell line was grown in DMEM medium (Gibco, Invitrogen, USA) supplemented with 10% FBS and 1% Pen/Strep. The normal-derived colon mucosa cell line NCM460 was obtained from INCELL Corporation upon MTA approval (LLC, San Antonio, USA). NCM460 cells were maintained in INCELL's enriched M3™ Base medium supplemented with 10% FBS and 1% Pen/Strep. All cell lines were incubated at 37°C in a 5% CO_2 humidified atmosphere.

Paraffin Cytoblock Preparation and Immunocytochemistry

Paraffin cytoblocks of HCT-15, RKO and NCM460 cells were prepared and MCT1, MCT4, CD147 and GLUT1 protein expression in cytoblocks was evaluated by immunocytochemistry, as previously described [30]. Detailed information is given in **Table 2.1**.

Western blot

MCT1, MCT4, CD147 and GLUT1 protein expression was evaluated by Western blotting, according to the conditions described in **Table 2.2**, as previously described [30].

Chemicals

Stock solutions of α-cyano-4-hydroxycinnamic acid (CHC), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), quercetin and 5-fluorouracil (5-FU) (Sigma-Aldrich, St. Louis, USA) were obtained by dissolution in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA). Working concentrations were obtained through dilutions with culture medium. The final concentration of DMSO was maintained at a maximum of 1%. All controls were performed using DMSO alone (vehicle).

Cell Biomass and Proliferation Assays

Cell biomass was assessed by the Sulforhodamine B (SRB) based *In Vitro* Toxicology Assay Kit (Sigma-Aldrich, St. Louis, USA) and cell proliferation was measured with the Cell Proliferation ELISA, BrdU (colorimetric) assay (Roche, Mannheim, Germany), as previously described [30].

Table 2.1. Details on the immunocytochemical procedure used to evaluate the expression the different proteins.

Protein	Antigen retrieval	Positive Control	Detection system	Antibody		
				Company	Dilution	Incubation
MCT1	Citrate buffer (10mM, pH=6.0) 98°C; 20 min.	Colon carcinoma	R.T.U. VECTASTAIN® Elite® ABC Kit (Vector laboratories)	Chemicon Ref. AB3538P	1:200	Overnight, 4°C
MCT4	Citrate buffer (10mM, pH=6.0) 98°C; 20 min.	Colon carcinoma	Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation)	Santa Cruz Biotechnology Ref. sc-50329	1:500	2 hours, RT
CD147	EDTA (1mM,pH=8) 98°C; 15 min.	Colon carcinoma	Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation)	Zymed Ref. 18-7344	1:500	2 hours, RT
GLUT1	Citrate buffer (10mM, pH=6.0) 98°C; 10 min.	Skin	Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation)	Abcam Ref. ab15309-500	1:500	2 hours, RT

*RT- room temperature

Table 2.2. Western-blot conditions to evaluate the expression of the different proteins.

Protein	Primary polyclonal antibody			Secondary antibody		
	Company	Dilution	Incubation	Reactivity	Dilution	Incubation
MCT1	Santa Cruz Biotechnology Ref. sc-365501	1:500	Overnight, 4°C	Anti-mouse	1:5000	45 min, RT*
MCT4	Santa Cruz Biotechnology Ref. sc-50329	1:2000	Overnight, 4°C	Anti-rabbit	1:5000	45 min, RT
CD147	Santa Cruz Biotechnology Ref. sc-71038	1:200	Overnight, 4°C	Anti-mouse	1:5000	45 min, RT
GLUT1	Abcam Ref. ab15309-500	1:800	Overnight, 4°C	Anti-rabbit	1:5000	45 min, RT

*RT- room temperature

Assessment of glucose and lactate levels

Extracellular levels of glucose (Roche, Germany) and lactate (SpinReact, Spain) were assessed by the enzymatic colorimetric kits, following the manufacturer's instructions.

Cell death assay

Cell death (apoptosis/necrosis) was determined by Annexin V-FLOUS Apoptosis Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions and as previously described [30]. The percentage of apoptosis/necrosis in the cell population was analysed by flow cytometry (LSRII model, BD Biosciences).

Downregulation of MCT1 and MCT4 expression

Silencing of MCT1 and MCT4 expression was performed with siRNA (s580 and s17417, respectively, Ambion, Foster City, CA, USA), using an adequate control (scramble siRNA, #4390843, Ambion, Foster City, CA, USA). Lipofectamine (RNAiMAX 13778-075, Invitrogen, Carlsbad, CA, USA) was used as permeabilization agent, according to the manufacturer's instructions.

Drug dose-effect analyses

The combined effect of 5-FU and lactate transport inhibitors (CHC, DIDS and quercetin) was analysed by calculating the combination index (CI) using the CalcuSyn software (Biosoft, Cambridge, UK). When CI <1, the effect is considered as synergistic, CI$=1$ is additive, and CI >1 antagonistic.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 software. Statistical significance was assessed by unpaired *t*-test or one-way ANOVA, followed by Tukey or Dunnett post-test. The threshold for significance was considered $p \leq 0.05$.

2.3 Results

2.3.1 CRC and normal colon-derived cell lines express MCT1, MCT4, CD147 and GLUT1 and CRC cells show a more glycolytic profile

Immunocytochemical (**Figure 2.1A**) and Western blot (**Figure 2.1B**) characterization of MCT1, MCT4, CD147 and GLUT1 revealed that all proteins are expressed, mainly at the plasma membrane, in CRC (HCT15, RKO) and normal colon-derived (NCM460) cell lines.

By analysing glucose consumption and lactate production of the different cell lines (**Figure 2.1C**), we observed that the CRC derived cells consume more glucose and produce more lactate over time than NCM460 cells. RKO cells appear to be more glycolytic than HCT-15 since they exhibited a higher consumption of glucose and lactate production up to 24 hours.

2.3.2 CHC, DIDS and quercetin impair growth and disrupt the glycolytic phenotype of colorectal carcinoma cells but not of normal colon

CRC and NCM460 cells were treated with the known lactate transport inhibitors CHC, DIDS and quercetin for 24 hours. All tested compounds inhibited in a similar way HCT-15 and RKO cell growth, in a dose dependent-manner, which was not observed for NCM460 cells, within the range of the concentrations used (**Figure 2.2A**).

To better understand the inhibitory effect of CHC, DIDS and quercetin on HCT-15 and RKO cells, the effect on energetic metabolism was assessed. Cells were treated with the correspondent IC_{50} values determined at 24 hours (HCT-15: CHC=10.55 mM, DIDS=0.80 mM, quercetin=142.7 µM; RKO: CHC=9.42 mM, DIDS=0.88 mM, quercetin=121.9 µM), and the extracellular amounts of glucose and lactate were estimated over time. Treatment with CHC and quercetin significantly decreased glucose consumption and lactate production in HCT-15 cells (**Figure 2.2B**), with reduction in glucose consumption and lactate production already after 4 hours of incubation. In RKO cells, only the treatment with CHC significantly affected glucose consumption (**Figure 2.2B**). Regarding lactate production, CHC and DIDS treatment showed a significant inhibitory effect in the first 4 hours, with no differences for the remaining incubation periods. Finally, quercetin was the only compound able to inhibit lactate production along time in RKO cells (**Figure 2.2B)**.

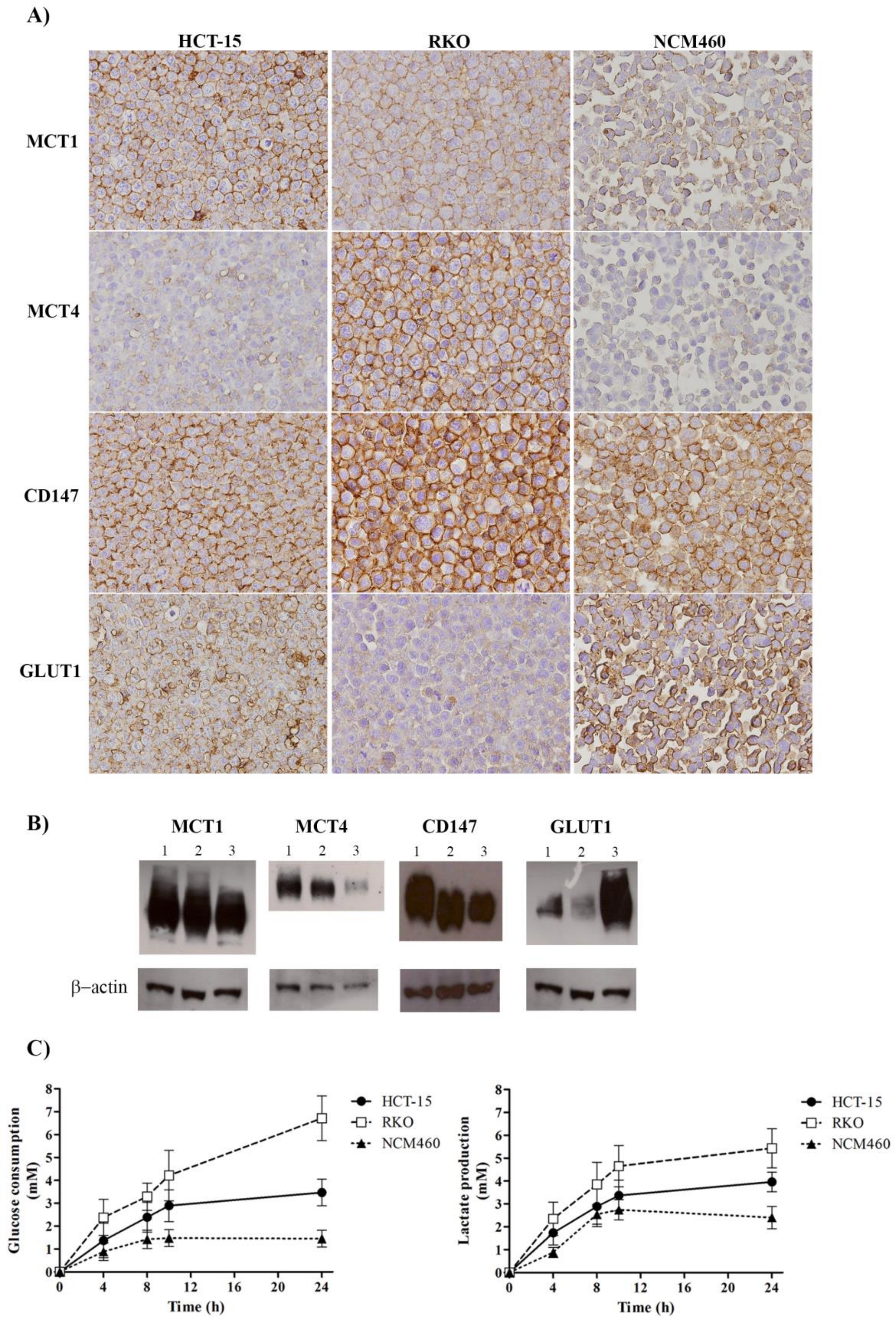

Figure 2.1. **Protein expression and metabolic profile of human colorectal and normal colon cell lines.** **A)** Immunocytochemical expression of MCT1, MCT4, CD147 and GLUT1 in human colorectal (HCT-15 and RKO) and normal colon (NCM460) cell lines (400x magnification); **B)** Western blotting of 1) HCT-15, 2) RKO and 3) NCM460

cell lines for MCT1 (50 kDa), MCT4 (52 kDa), CD147 (50-60 kDa) and GLUT1 (55 kDa). β-actin was used as internal loading control; **C)** CRC-derived (HCT-15 and RKO) and normal colon (NCM460) cell lines extracellular amounts of glucose and lactate, overtime (4, 8, 10 and 24 hours). Values are expressed as mean ± SD of at least 3 independent experiments, each in triplicate.

2.3.3 CHC, DIDS and quercetin impair CRC cells proliferation and induce cell death

As shown in **Figure 2.2C**, all compounds significantly inhibited the proliferation of both cell lines. CHC appears to have a stronger effect on RKO than HCT-15 cells, whereas DIDS and quercetin showed a similar capacity to inhibit proliferation.

Since the effect of the lactate transport inhibitors studied could be attributed not only to inhibition of the proliferative capacity of the cells but also to induction of cell death (apoptosis/necrosis), annexin V/PI staining was assessed. As shown in **Figure 2.2D**, cell death significantly increased after treatment with DIDS for both cell lines, while quercetin significantly increased RKO cell death, with no significant effect on HCT-15 cells. CHC did not increase the percentage of cell death in both cell lines. Among the three compounds tested, DIDS was the most potent in inducing cell death in both CRC cell lines. Moreover, HCT-15 appears to be more sensitive to these lactate transport inhibitors, since a higher overall percentage of dead cells were observed (**Figure 2.2D**).

2.3.4 Downregulation of MCT1 or MCT4 mimics the effects of lactate transport inhibition

In order to confirm if the results previously observed in CRC cells were a consequence of MCT1/4 activity inhibition, downregulation of MCT1 or MCT4 expression was performed using specific siRNAs in HCT-15 and RKO cells. As shown in **Figure 2.3A**, an effective reduction of MCT1 or MCT4 expression was observed upon MCT1 or MCT4 targeting by siRNAs in both cell lines.

As observed for MCT activity inhibition with CHC, DIDS and quercetin for both CRC cell lines, MCT1 or MCT4 downregulation decreased HCT-15 and RKO cell growth after 24 hours of silencing (**Figure 2.3B**). Likewise, a reduction of the proliferative capacity of these cells was obtained upon silencing of MCT1 or MCT4 (**Figure 2.3C**). Similarly, to the results obtained with MCT activity inhibition, MCT1 or MCT4 downregulation induced a significant decrease in glucose consumption

and lactate production (siMCT1) in HCT-15 cells, while MCT4 silencing resulted in a significant inhibition of lactate production in RKO cells (**Figure 2.3D**).

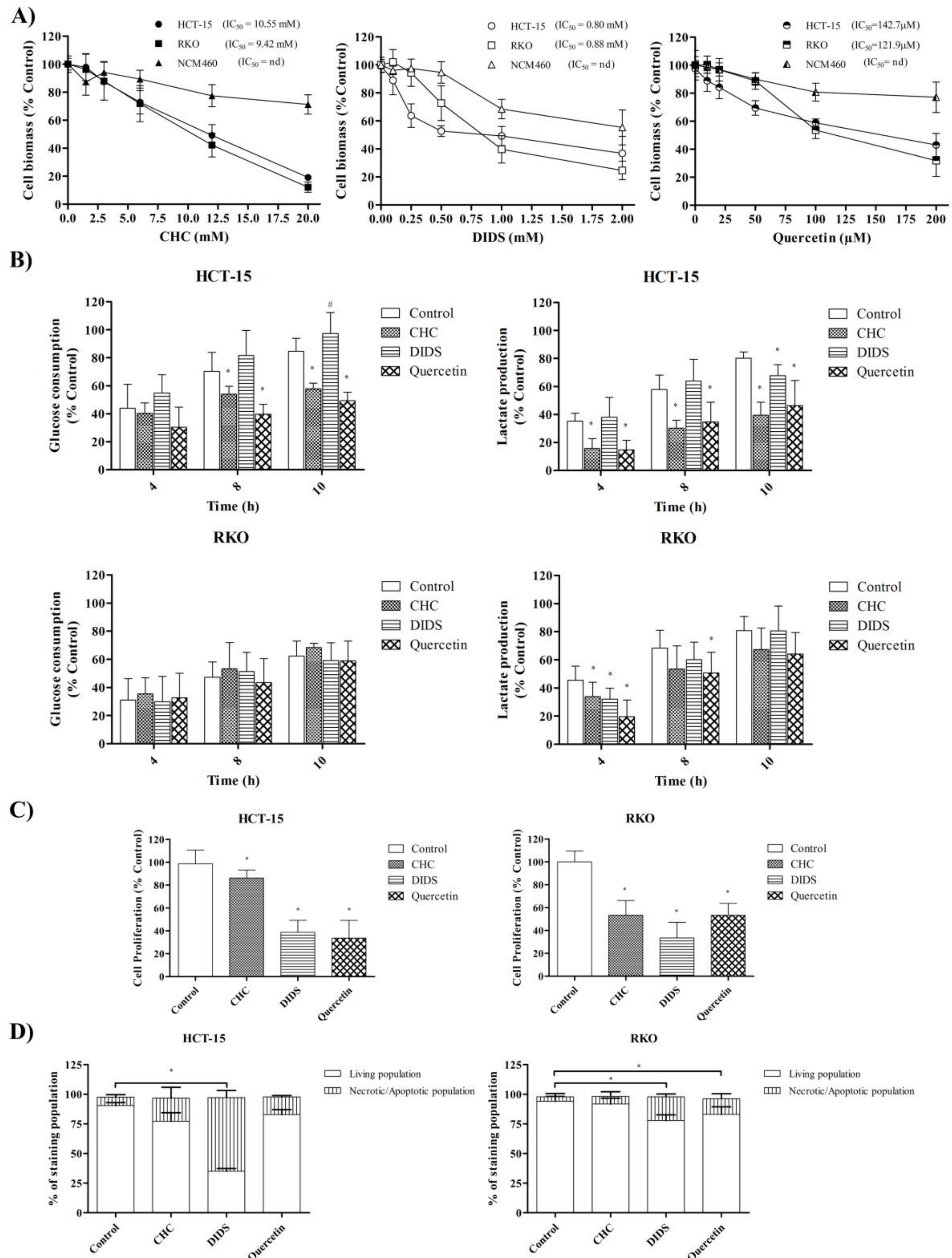

Figure 2.2. **Effect of lactate transport inhibition on cell biomass, metabolism, proliferation and death.** **A)** Effect of CHC, DIDS and quercetin on HCT-15, RKO and NCM460 total cell biomass (Sulphorhodamine B assay). Cell lines were incubated with increasing concentrations of CHC, DIDS and quercetin for 24 hours; **B)** Effect of CHC, DIDS and quercetin on HCT-15 and RKO glucose consumption and lactate production. Cells were incubated with the

calculated IC_{50} values for CHC, DIDS and quercetin for 12 h, and glucose and lactate were quantified over time (4, 8 and 10 hours); **C)** Effect of CHC, DIDS and quercetin in HCT-15 and RKO cell proliferation (bromodeoxyuridine (BrdU) incorporation). Cells were incubated with the calculated IC_{50} values for CHC, DIDS and quercetin for 24 hours; **D)** Effect of CHC, DIDS and quercetin on HCT-15 and RKO cell death (annexin-V/PI (flow cytometry)); *$p\leq0.05$, when compared to control (DMSO 1%). Results are expressed as the mean ± SD of at least 3 independent experiments, each in triplicate.

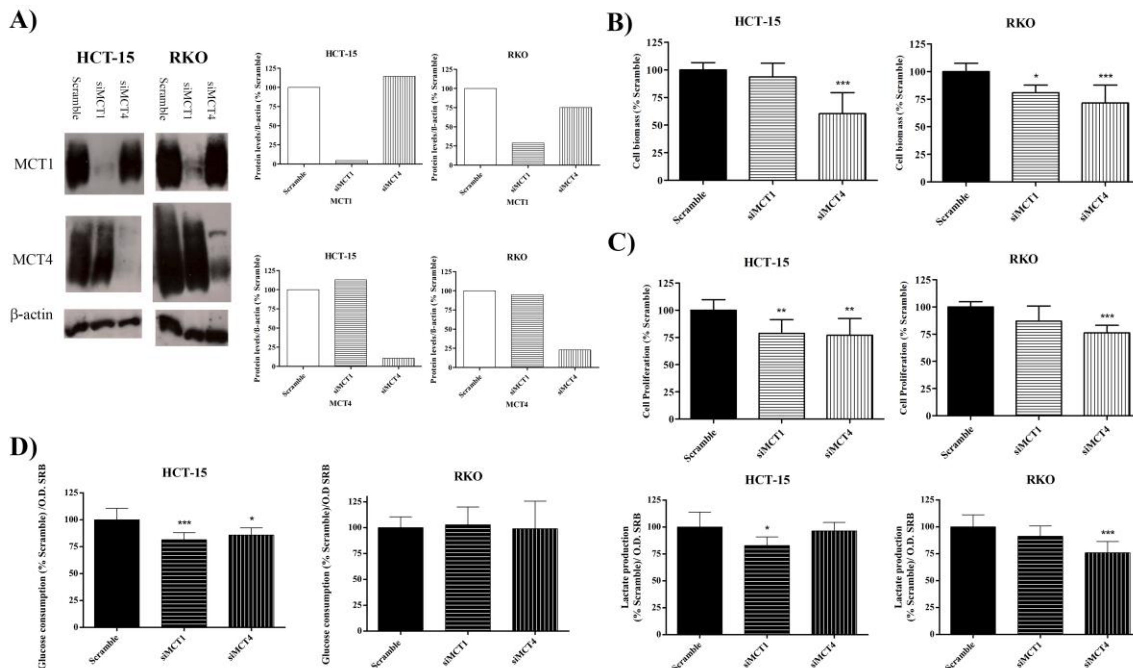

Figure 2.3. Effect of MCT1 and MCT4 downregulation on cell growth, proliferation and metabolism. **A)** Western blot analysis of MCT1 (50kDa) and MCT4 (52kDa) expression in siMCT1 and siMCT4 HCT-15 and RKO cells. Cells were transfected with scramble, siMCT1 or siMCT4 and expression of MCT isoforms was evaluated after 48 hours; Effect of MCT1 and MCT4 downregulation on **B)** cell growth, **C)** cell proliferation and **D)** glucose consumption/lactate production; ***$p\leq0.001$, **$p\leq0.01$, *$p\leq0.05$, siMCT1 and siMCT4 cells compared with scramble. Results represent the mean ± SD of at least 3 independent experiments, each in triplicate.

2.3.5 MCT activity inhibition enhances 5-FU cytotoxic effect in CRC cells

5-FU decreased HCT-15 and RKO cell biomass in a dose-dependent manner, while no cytotoxic effect was observed on the normal colon cell line, as shown in **Figure 2.4A**. To test if monocarboxylate transport inhibition could potentiate the cytotoxic effect of 5-FU in CRC cells, two approaches were followed: combination and pre-treatment assays. For the combination assay, CRC cells were incubated simultaneously with the lactate transport inhibitors and increasing concentrations of 5-FU during 24 hours. The combination of either CHC or DIDS with 5-FU resulted in an increase of 5-FU cytotoxic effect in CRC cell lines (**Figure 2.4B**). For HCT-15 cells, a

synergistic effect was consistently observed only when combining DIDS with 5-FU. For RKO cells, a synergism was observed with the combination of 5-FU with either CHC or DIDS (only for 5-FU higher doses) (**Table 2.3**).

Table 2.3. Combinatory interaction between 5-FU and lactate transport inhibitors (CHC, DIDS and Quercetin). CI was calculated using CalcuSyn 2.0 software.

5-FU + CHC (mM)	CI	5-FU + DIDS (mM)	CI	5-FU (mM) + Quercetin (µM)	CI
		HCT-15			
0.01 + 10.55	0.938	0.01 + 0.80	0.699	0.01 + 142.7	>2
0.1 + 10.55	1.024	0.1 + 0.80	0.538	0.1 + 142.7	>2
1 + 10.55	0.983	1 + 0.80	0.440	1 + 142.7	>2
10 + 10.55	1.604	10 + 0.80	0.847	10 + 142.7	>2
		RKO			
0.01 + 9.42	0.981	0.01 + 0.88	1.045	0.01 + 121.9	>2
0.1 + 9.42	0.986	0.1 + 0.88	1.042	0.1 + 121.9	>2
1 + 9.42	0.640	1 + 0.88	0.779	1 + 121.9	>2
10 + 9.42	0.455	10 + 0.88	0.561	10 + 121.9	>2

CI - combination index

To assess the effect of lactate transport inhibitors as pre-treatment, CRC cells were pre-incubated with the lactate transport inhibitors (for 24 hours) and then treated with increasing concentrations of 5-FU during 48 hours. As observed in **Figure 2.4C,** pre-incubation of both CRC cell lines with CHC, DIDS and quercetin sensitized cells to 5-FU.

In order to confirm if treatment of CRC cells with 5-FU could enhance the inhibition of glycolytic metabolism observed upon MCT activity inhibition with CHC, DIDS and quercetin, cells were incubated simultaneously with the correspondent IC_{50} of 5-FU and the MCT inhibitors for 12 hours and the effects on glucose consumption and lactate production was assessed. As observed in **Figure 2.4D**, with the exception of glucose consumption in HCT-15 cells, 5-FU *per se* inhibited the glycolytic metabolism of CRC cells, as a statistically significant decrease in glucose consumption and lactate production was obtained. Importantly, the combination with 5-FU enhanced significantly the inhibitory effect of MCT activity inhibitors on CRC glycolytic metabolism (**Figure 2.4D**).

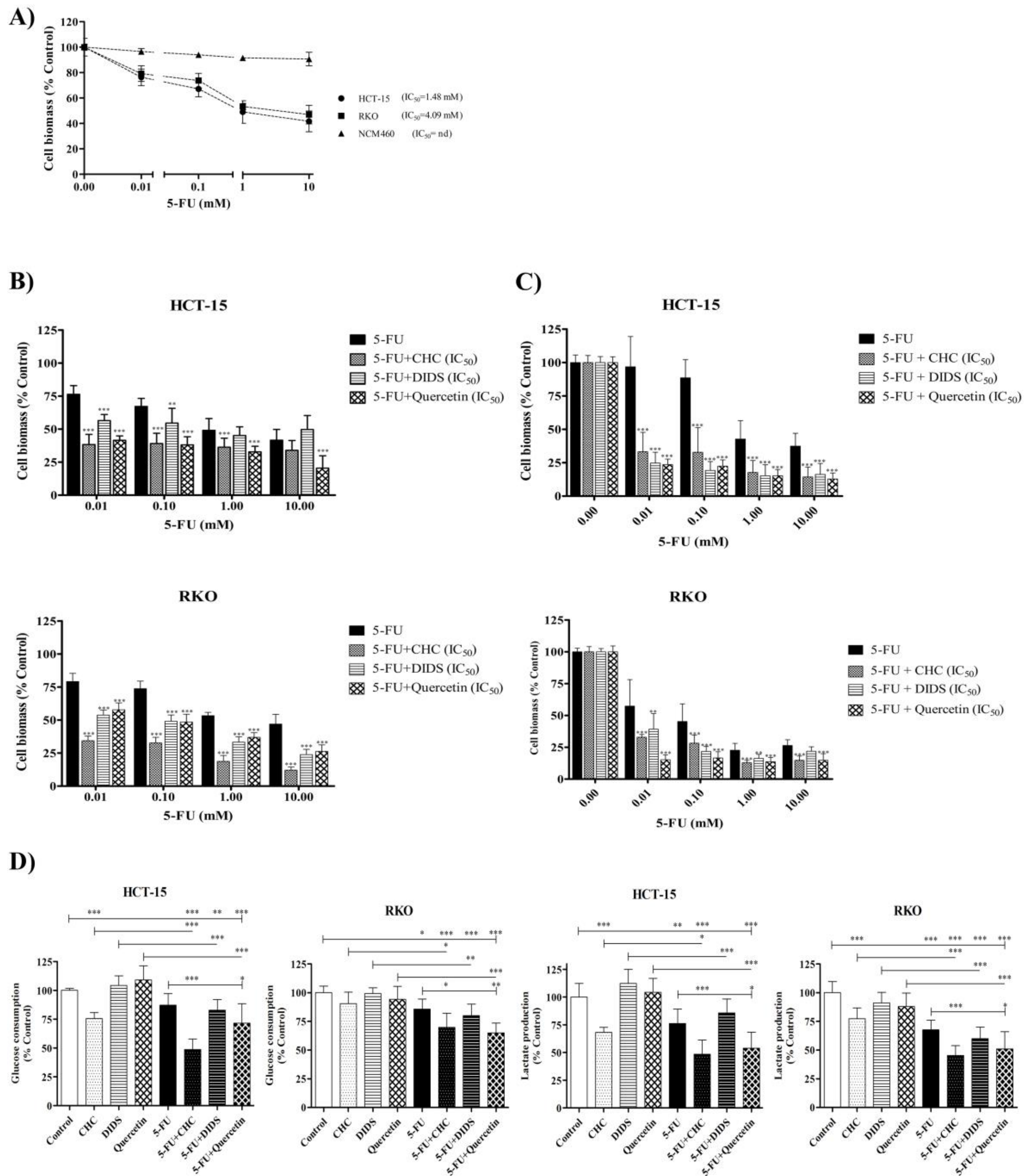

Figure 2.4. **Effect of 5-FU, 5-FU plus lactate transport inhibition combination and pre-treatment on cell biomass and metabolism. A)** Effect of 5-FU treatment on HCT-15, RKO and NCM460 on total cell biomass (Sulphorhodamine B assay). Cell lines were incubated with increasing concentrations of 5-FU for 24 hours; **B)** Effect of 5-FU + CHC, DIDS and quercetin combination on HCT-15 and RKO total cell biomass. (Sulphorhodamine B assay). Cell lines were incubated with increasing concentrations of 5-FU along with the correspondent IC_{50} values for CHC, DIDS and quercetin for 24 hours. ****p*≤0.001, ***p*≤0.01, **p*≤0.05, when compared to 5-FU alone; **C)** Effect of pre-treatment with lactate transport inhibitors on sensitization of cells to 5-FU (Sulphorhodamine B assay). CRC cells were pre-incubated with the correspondent IC_{50} values for the lactate transport inhibitors (for 24 hours) and then treated with increasing concentrations of 5-FU during 48 hours. ****p*≤0.001, ***p*≤0.01, **p*≤0.05, when compared to 5-FU alone; **D)** Effect of 5-FU plus CHC, DIDS and quercetin combination on HCT-15 and RKO cell metabolism. CRC cells

were incubated simultaneously with the correspondent IC_{50} of 5-FU and MCT inhibitors for 12 hours and the effects on glucose consumption and lactate production were assessed. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. Results represent the mean ± SD of at least 3 independent experiments, each in triplicate.

2.3.6 MCT1/MCT4 expression silencing support the potentiation of 5-FU cytotoxic effect by lactate transport inhibitors

Aiming to confirm the potentiation of 5-FU cytotoxic effect obtained with MCT activity inhibitors, we combined specific MCT1 or MCT4 downregulation by RNA interference with this chemotherapeutic drug in CRC cell lines. The treatment of HCT-15 and RKO cells, with MCT1 or MCT4 siRNA oligos, in combination with 5-FU, resulted in an evident decrease of 5-FU IC_{50} values when compared with the control cells (scramble siRNA) (**Figure 2.5A**). For HCT-15 cell line, the 5-FU IC_{50} value decreased from 5.34 mM in control cells to 2.94 mM and 1.65 mM in cells treated with siMCT1 and siMCT4, respectively. Concerning RKO cells, the IC_{50} value of 5-FU for control cells treated with scramble siRNA, decreased from 2.11 mM to 0.66 mM and 0.62 mM upon silencing of MCT1 or MCT4, respectively (**Figure 2.5A**).

Furthermore, 5-FU *per se* led to a significant reduction in lactate production for both CRC-derived cell lines in cells treated with scramble (**Figure 2.5B1**), and upon MCT1 (**Figure 2.5B2**) and MCT4 (**Figure 2.5B3**) silencing. Concerning glucose consumption, only the treatment with 5-FU in HCT-15 silenced for MCT4 led to a significant decrease (**Figure 2.5B3**). Moreover, treatment of CRC cells with MCT1 or MCT4 siRNAs in combination with 5-FU led to a statistically significant reduction in glucose consumption for both cell lines and a decrease in lactate production in HCT-15 cells upon MCT1 silencing, when comparing to control cells (scramble siRNA) treated with 5-FU (**Figure 2.5C).**

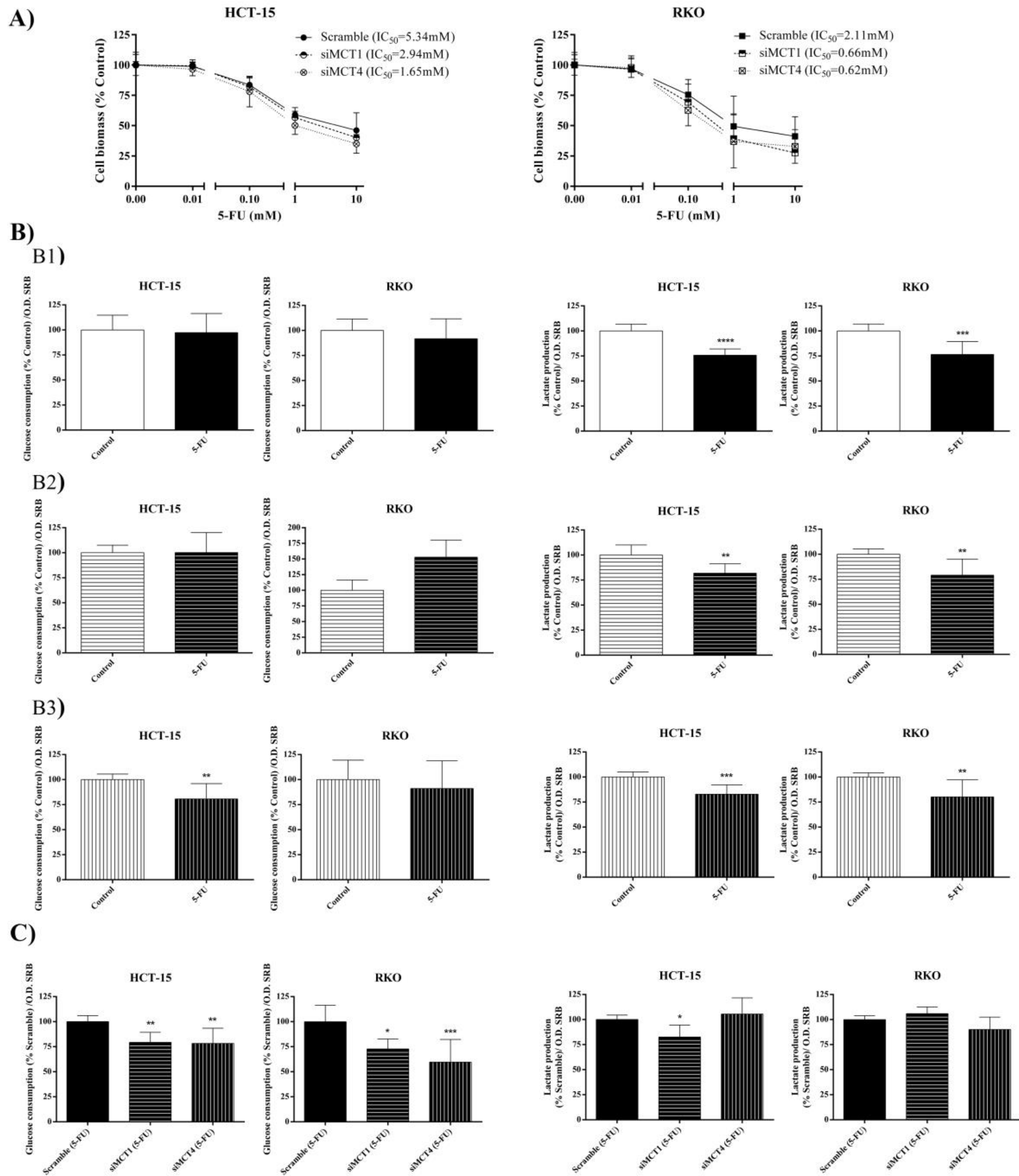

Figure 2.5. **Effect of MCT1 and MCT4 downregulation combined with 5-FU on cell biomass and metabolism. A)** Effect of 5-FU treatment on HCT-15 and RKO cells, with MCT1 or MCT4 silencing, on total cell biomass (Sulphorhodamine B assay). Cell lines were incubated with increasing concentrations of 5-FU for 24 hours; **B)** Effect of 5-FU on HCT-15 and RKO cells, with **B1)** scramble, **B2)** MCT1 and **B3)** MCT4 silencing, on cell metabolism, when comparing with control condition (DMSO 1%); **C)** Effect of 5-FU on HCT-15 and RKO cells, with MCT1 or MCT4 silencing, on cell metabolism, when comparing with scramble condition treated with 5-FU. CRC cells were incubated with the IC_{50} values of 5-FU obtained in Figure 4A for 12 hours and the effects on glucose consumption and lactate production were assessed. ***p≤0.001, **p≤0.01, *p≤0.05. Results represent the mean ± SD of at least 3 independent experiments, each in triplicate.

2.4 Discussion

MCTs are essential players in the maintenance of cancer cell metabolism, being promising therapeutic targets [7, 13, 23-28]; however, the role of MCTs in CRC cell survival and metabolism is still poorly understood. Here, we aimed to characterize the dependence of CRC cells on MCT activity for survival, proliferation and maintenance of energetic metabolism as well as test if MCT inhibition could potentiate the cytotoxic effect of 5-FU, a classical chemotherapeutic agent.

Following our previous findings in human CRC primary tumours [32], we aimed to further dissect the expression of MCTs in CRC derived cells. For that, we evaluated the expression of MCT1, MCT4, CD147 (MCT1/4 chaperone) and GLUT1 (glucose transporter) in CRC (HCT-15 and RKO) and in a normal human colon epithelium (NCM460) derived cell lines. The positive expression of both MCT isoforms, CD147 and GLUT1 in the CRC cell lines HCT-15 and RKO support the adoption of a glycolytic phenotype. On the other hand, we demonstrated for the first time, the expression of MCT1, MCT4 and CD147 in the normal colon cell line NCM460. The expression of MCT1 and its chaperone in normal colon cells was expected [42] since MCT1 is important in the transport of short chain fatty acids (SCFA) in colon [43]. SCFA were demonstrated to protect normal colon mucosa and induce apoptosis of CRC cells *in vitro* [43, 44].

In this study, we also addressed the effects of lactate transport inhibition in human CRC cell lines in comparison to normal colon derived cells, using the compounds CHC, DIDS and quercetin, which are known to inhibit lactate transport [11, 13, 25, 45, 46]. We demonstrated that MCT activity inhibition with these compounds inhibited CRC cells biomass, in a dose-dependent manner and with similar IC_{50} values, increased cell death and decreased cell proliferation. There was an overall decrease in both glucose consumption and lactate production in HCT-15 cells after treatment with all the compounds. In opposition, no significant alterations in the metabolic rates were found for RKO cells. The reduction of glucose consumption observed is probably due to a negative feedback upon inhibition of lactate transport. Upon MCT activity inhibition, lactate levels within the cell increase and this overload will negatively signal for glucose entrance into the cells. Importantly, the normal colon cell line was less glycolytic and less sensitive to MCT inhibition than CRC cell lines, which is probably related with the lower expression of MCT1 and MCT4 in normal colon cells. This selectivity for CRC cells could constitute a valuable approach to be further exploited in the use of MCT targeting in CRC therapy.

Our results support the hypothesis that MCTs could be promising targets in future CRC therapies and corroborate previous *in vitro* and *in vivo* studies in various tumour models using these MCT activity inhibitors [47]. In CRC cells, other authors observed an inhibition of cell survival along with an increase of apoptosis upon treatment with CHC [48]. The authors linked the alterations observed in cell survival with disruption of lactate efflux and glucose uptake, pH homeostasis, expression of glucose transporters and HIF-1α, and generation of nitric oxide [48]. In order to investigate a novel method to enhance radiosensitivity of gliomas, Colen *et al.* successfully disrupted cell metabolic balance and survival with CHC [25]. Moreover, it was also shown that, when applied *in situ*, CHC is nontoxic at the concentrations up to 20 mmol/L, in an orthotopic nude rat brain model. More recently, in two glioma cell lines and in an organotypic (brain) slice culture, glioma cell invasion impairment was shown upon lactate efflux inhibition with CHC, with no adverse neurologic effects on control animals [49]. Using both CHC and MCT1 downregulation, antitumor effects were documented without evident toxicity in three different models of animal and human tumours [13]. More recent studies from our group showed an overall decrease in glycolytic metabolism, cell proliferation, migration and invasion, as well as an increase in cell death in glioma [30] and breast cancer cells [29], upon lactate transport inhibition with CHC [29, 30] and quercetin [29]. Using oocytes transfected with rat MCT1 or MCT4, Dimmer *et al.* [46] demonstrated that 500 µM DIDS reduced lactate transport by 60% in rat MCT4, while with superior concentrations (up to 2 mM) the transporter remained insensitive. In contrast, lactate uptake via MCT1 was completely blocked by DIDS [46].

Taking into account that CHC, DIDS and quercetin are not MCT specific inhibitors [50], it cannot be excluded that the results observed with these compounds are due to inhibition of other cell targets. Thus, we performed downregulation of MCT1 and MCT4 expression with specific siRNAs. Overall, the effects of MCT1 or MCT4 expression inhibition were similar to inhibition of MCT activity, confirming the pivotal role of MCT isoforms 1 and 4 in the maintenance of CRC survival and glycolytic metabolism. As observed in this tumour model, the effects of MCT activity inhibition with CHC and quercetin were corroborated by MCT1 silencing in glioma and breast tumour models [29, 30]. Additionally, siRNA specific for MCT1 and MCT2 reduced lactate efflux in glioma cells, with concomitant decrease in intracellular pH, and reduction of cell viability with prolonged silencing [24]. Le Floch *et al.* [51] showed that MCT1/2 inhibition with AR-C155858 (specific MCT1/2 inhibitor) in Ras-transformed fibroblasts led to suppression lactate export, glycolytic rates, and tumour growth. When MCT4 expression was restored, cells became resistant to MCT1/2 inhibition

and reestablishment of tumorigenicity was observed. Moreover, in this same study, using human colon adenocarcinoma cells, CD147 gene silencing, alone or in combination with MCT1/MCT4 silencing, reduced glycolytic flux as well as tumour growth [51].

The classical chemotherapeutic drug 5-FU has been largely used in CRC treatment, although there is growing evidence for 5-FU resistance [36] and low efficacy [37]. Several efforts have been made to explore new combination therapies, aiming to enhance the efficacy of 5-FU and reduce side effects. In the present work, we showed for the first time in a CRC model that the use of CHC, DIDS and quercetin potentiates the cytotoxic effect of 5-FU, and this effect was even more evident when cells were pre-treated with the lactate transport inhibitors. These results led us to conclude that these MCT activity inhibitors, by arresting the glycolytic flux through inhibition of lactate transport, turn CRC cells more sensitive to standard therapy. Consequently, pre-treatment of CRC cells with glycolytic inhibitors, namely lactate transport inhibitors, might be a promising strategy for patients with this malignancy. Moreover, we also demonstrated that 5-FU *per se* arrested glycolytic flux of CRC cells and potentiated the inhibitory effect on glycolysis obtained with MCT activity inhibitors. Importantly, and since these compounds are not MCT specific inhibitors, we downregulated MCT1 and MCT4 expression with specific siRNAs and assessed the effects of combining MCT1 or MCT4 silencing with 5-FU on cell biomass and metabolism. The results obtained with MCT1 or MCT4 silencing corroborated and supported the potentiation of 5-FU cytotoxic effect obtained with lactate transport activity inhibition, namely, we could observe a reduction of 5-FU IC_{50} values and an impairment in the glycolytic metabolism.

The beneficial use of metabolic inhibitors, namely MCT inhibitors, in combination with gold-standard therapy was already described in other studies. Colen *et al.* [25] observed that pre-treatment of glioma cells with CHC enhanced the sensitivity of these cells to radiotherapy. Moreover, in a cell line derived from colon adenocarcinoma, the authors described an enhanced cytotoxicity of cisplatin together with decreased expression of multidrug resistance regulating genes, when cells were pre-treated with CHC [48]. Recently, Miranda-Gonçalves *et al.* showed that CHC potentiated the effect of temozolomide, the gold standard anti-glioblastoma chemotherapeutic agent, with an important synergistic effect [30].

The mechanism by which 5-FU ultimately benefits from tumour cell glycolytic metabolism arrest, namely lactate transport inhibition, remains unclear. However, recent studies demonstrated an association between 5-FU sensitivity and glucose uptake. In human liver cancer cells, it was observed that 5-FU resistant cells showed higher glucose uptake and lactate production when

compared with cells sensitive to 5-FU [52]. By establishing a 5-FU-resistant human colon cancer cell line, Liu *et al.* [53] demonstrated that resistance to 5-FU was associated with overexpression of GLUT1 and specific inhibition of this glycolytic marker increased the sensitivity of these 5-FU insensitive cells to the chemotherapeutic drug [53]. Moreover, in a study using *PIK3CA* mutant and wild-type gastric cancer cells, the authors described higher resistance to 5-FU when cells were cultured with lower concentrations of glucose [54]. A recent study also reported that inhibition of the glycolytic metabolism by targeting pyruvate dehydrogenase kinase-1 (PDK-1) with the specific inhibitor dichloroacetate was able to re-sensitize gastric cancer cells to 5-FU [55]. Taken together, these studies support the use of glycolytic inhibitors as a pre-treatment or in combination with 5-FU for novel therapeutic protocols to overcome chemotherapeutic resistance.

Overall, our findings showed that MCT activity is important in the survival of CRC and support the use of MCTs as new molecular targets for CRC treatment. Our results also suggest that inhibition of these transporters alone or in combination with 5-FU should be further explored as a novel therapeutic approach for this malignancy in the clinical context.

Authors' contributions

RA performed the *in vitro* studies and wrote the first draft of the manuscript; CP, VMG and HP performed part of the *in vitro* studies; MPM provided the normal colon-derived cell line and assisted the development of the *in vitro* studies; AP helped in the design of the study and revised the manuscript; FB conceived and supervised the study and revised the manuscript. All authors read and approved the final manuscript.

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CHAPTER 3. Cytidine deaminase: A novel metabolic target in resistance to immunotherapy

The results presented in this chapter are related to an ongoing work:

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Cytidine deaminase: A novel metabolic target in resistance to immunotherapy

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Abstract

Cancer immunotherapy, namely immune checkpoint blockade (ICB) with α-PD-1, α-PD-L1 or α-CTLA-4 antibodies, has provided patients with a promising treatment option. However, tumour types such as pancreatic ductal adenocarcinoma (PDAC) fail to show any clinical benefit. PDAC tumours have low infiltration of cytotoxic T cells, in addition these tumours are nutrient deprived and have a harsh tumour microenvironment (TME) which may hinder their ability to survive, proliferate and attack the tumour, thus contributing to immunological tolerance and limited success of this therapy option.

Here, we set-up and performed a meta-analysis in order to identify metabolic pathways within the TME, affecting immunosuppression and resistance to immunotherapy. Cytidine deaminase (CDA), an enzyme of the pyrimidine salvage pathway, was identified as a potential target involved in unresponsiveness to ICB. We addressed how CDA contributes to immunosuppression, namely the consequences of genetic inactivation of CDA in combination with ICB on tumour growth and the remodelling of the immune landscape. Our results showed that genetic inactivation or pharmacological inhibition of CDA in cancer cells decreased tumour growth, synergized with α-PD-1 treatment and increased the recruitment of cytotoxic T cells and M1-like anti-tumour tumour-associated macrophages into the tumours. Moreover, we observed an inverse correlation between CDA expression and cytotoxic T cell infiltration in a human PDAC series.

These findings provide novel evidence that CDA expression in pancreatic tumours can modulate the TME, in particular by blocking the recruitment and effector functions of anti-tumorigenic immune cells.

Keywords

CDA, Cytotoxic T cells, ICB, Immunotherapy, Tumour microenvironment,

3.1 Introduction

Cancer immunotherapy is a promising new approach for the treatment of cancer patients. Therapeutic regimens such as adoptive T cell transfer (ACT), cancer vaccines and immune checkpoint inhibitors (e.g. α- PD-1 or α-CTLA-4 antibodies), harness the ability of the immune system to recognize and reject cancer [1]. Immune checkpoint inhibitors have only shown high response rates, with prolonged duration, in a subset of melanoma [2-4], renal [5, 6] and lung cancer patients [7-9]. In addition, the percentage of patients who respond to immune checkpoint blockade (ICB) regimens is only around 30% for the most common solid tumours [10], and recent clinical trials for many other cancer types [11, 12] revealed that several tumours such as mismatch repair (MMR)-proficient and microsatellite instability-low (MSI-L) colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC), rarely exhibit robust responses to these therapies [13-17]. In addition, regardless of the density of immunogenic antigens [18], tumours use different approaches to escape the immune response.

$CD8^+$ cytotoxic T cells can be present either within the tumour but in a dysfunctional state (generally referred to as T cell-inflamed or "hot" tumours) or they can be prevented from entering the tumour via T cell exclusion mechanisms (generally referred to as non-T cell-inflamed or "cold" tumours) [1, 19-21]. The latter represents an even more potent mechanism of immune escape, typically observed in specific tumour types such as PDAC where the high hopes of immunotherapies were not be fulfilled, mainly because PDAC shows a low infiltration of cytotoxic T cells [22]. The limited treatment options and the high mortality of PDAC [23, 24] clearly shows the urgent need for further immunotherapeutic approaches. Therefore, the next challenge is to identify novel mechanisms and therapeutic targets able to promote both T cell fitness and T cell influx within tumours thus converting ''cold'' tumours into immunologically proficient, ''hot'' T cell-rich environments. This represents a novel frontier of cancer immunotherapy and is a promising strategy to overcome resistance to ICB and improve clinical outcomes [1, 22, 25, 26].

The harsh tumour microenvironment (TME), along with the cellular and metabolic crosstalk between cancer and stromal cells, is a key player in orchestrating these immunosuppressive mechanisms [19, 27-29]. For instance, within the TME, low pH, hypoxia, metabolic competition for limiting nutrients (*e.g.,* glucose and glutamine), macrophage-driven arginine depletion, nitric oxide production as well as tryptophan catabolism by indoleamine-2,3-dioxygenase (IDO) can greatly impair T cell fitness [30-39]. The metabolic remodelling and environmental cues that occur

in the harsh TME can affect chemotaxis, differentiation, immune recognition and effector functions of innate and adaptive immune cells [40, 41]. As a consequence, tumour metabolism is responsible for mounting an immunosuppressive environment in all those cases where immune intervention does not offer a durable response, or in the worst case, when the tumour is completely refractory to these treatments.

Herein, we set-up and performed a meta-analysis in order to identify metabolic pathways within the TME, which are affecting immunosuppression and resistance to immunotherapy. Among the metabolic pathways that were consistently deregulated we have identified cytidine deaminase (CDA) as a potential target involved in unresponsiveness to ICB. Besides free nucleotides, CDA also deaminates and inactivates cytidine analogues such as gemcitabine, cytosine arabinoside and 5-azacytidine, agents used to treat cancer. Consequently, CDA plays a crucial role in the resistance of cancer cells to treatment with cytidine analogues, with several studies showing a link between its overexpression and resistance to treatment [42-44].

Therefore, to fully assess the key role of CDA in mounting immunosuppressive responses in tumours, we addressed how this enzyme promotes the action of the immune system and responsiveness to ICB *in vivo*, by pinpointing the crucial players within the immune cell compartment responsible for the observed increase in anti-tumour immune response.

3.2 Materials and Methods

Animals

C57BL/6 and NMRI-*Foxn1*^{nu} mice were purchased from Envigo. OT-I mice were purchased from Taconic. All mice used were females between 8 and 10 weeks old. Housing and all experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the KU Leuven (ECD 226/2017).

Cell lines

The murine pancreatic ductal adenocarcinoma Panc02 cell line was kindly provided by Prof. B. Wiedenmann (Charité, Berlin). Panc02 cells were cultured in DMEM medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Gibco) and 1% (v/v) penicillin-streptomycin solution (Pen/Strep, Gibco). The murine pancreatic KPC1245 cell line, generated from the KPC murine model (*Kras*$^{LSL.G12D/+}$; *p53*$^{R172H/+}$; *Pdx:Cre*$^{Tg/+}$), was kindly provided by Prof. D. Tuveson (New York, USA). KPC1245 cells were cultured in DMEM medium supplemented with 10% FBS, 1mM sodium pyruvate (Gibco) and 1% Pen/Strep.

The murine colon carcinoma MC38 cell line was obtained from Kerafast and cultured in DMEM medium supplemented with 10% FBS, 2mM glutamine (Gibco), 0.1mM nonessential amino acids (NEAA, Gibco), 1mM sodium pyruvate, 10mM Hepes (Gibco) and 1% Pen/Strep. CT26 murine colon carcinoma cells were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI medium (Gibco) supplemented with 10% FBS and 1% Pen/Strep. B16-F10 melanoma cells were obtained from the ATCC and cultured in RPMI medium supplemented with 10% FBS and 1% Pen/Strep. The melanoma YUMM 1.7 cell line was a kind gift from Prof. R. Marais (Manchester, UK) and cultured in DMEM/F-12 medium supplemented with 10% FBS and 1% Pen/Strep.

The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere and subcultured approximately every three days and maintained in a log growth phase.

Tumour models

$4x10^6$ Panc02, $1x10^6$ MC38 or $2x10^6$ CT26 cells were injected subcutaneously (s.c.) in the right flank of the mouse in a final suspension of 200µl PBS. $0.1x10^6$ B16-F10 or $1x10^6$ YUMM 1.7 cells were injected intradermally in a final volume of 50µl PBS. Tumour volumes were measured at least

three times per week with a calliper and calculated using the formula: Volume (mm^3): ((Width(mm))^2 × Length(mm))/2. 0.1x10^6 KPC1245 cells were injected orthotopically into the head of the pancreas in 20µl of PBS.

Mice were randomized and treated intraperitoneally (i.p.) at indicated time points with 10 mg/kg of α-PD-1 (BioXcell) or control IgG from rat serum (Sigma-Aldrich), 5 mg/kg α-CD8 (BioXcell) or control IgG from rat serum, 50 mg/kg clodronate liposomes or PBS liposomes (Liposoma research). Treatment was continued until the end of the experiment unless otherwise stated. In the tetrahydrouridine (THU) experiment, mice were randomized and treated i.p. with 120mg/kg/day THU (MolPort) or vehicle (PBS) twice-daily at indicated time points. At the end stage, tumour weight was measured and in the orthotopic KPC1245 model, mesenteric metastatic metastases were assessed under a stereomicroscope. For the *in vivo* T cell memory assay, mice with Panc02 CDA KO tumours which had regressed after treatment with α-PD-1 were re-challenged with 4x10^6 wild-type Panc02 cancer cells.

Mice were monitored and weighed continuously during the experiments. Mice showing symptoms of illness, that lost 20% of initial body weight, peritoneal leakage or with ulcerated tumours were sacrificed and excluded from the experiments. At the end stage, tumour weight was registered and samples were collected for histological examination or FACS/sorter analysis.

Meta-analysis

Murine bulk tumour transcriptomics and metabolomics were integrated with publicly available pre-treatment transcriptomic data of patients responding and non-responding to immune checkpoint inhibitors α-CTLA-4 and α-PD-1 [2, 45, 46]. The datasets were integrated and interpreted using the Biological Interpretation of Metabolomics Experiments (BIOMEX) platform [47].

The general analysis was divided in: i) exploratory analysis which included principle component analysis (PCA), heatmap analysis and hierarchical clustering; ii) differential analysis to calculate fold changes and *p*-values; iii) bioinformatics analysis which included pathway mapping, competitive and self-contained enrichment analysis; and iv) multi-omics visualization where differential gene expression data were mapped together with differential metabolite level data onto (metabolic) maps. The exploratory statistical analysis assessed whether the global transcriptomics and metabolomics profiles were distinct. The enrichment analysis indicated which pathways were deregulated while differential analysis showed which individual genes or metabolites are changed.

Heatmap analysis was performed using the D3heatmap package and hierarchical clustering was performed on auto-scaled data using the Euclidean distance and complete linkage as implemented in the R-package Stats, significant clusters were calculated via multiscale bootstrap analysis (R, Pvclust package).

To increase the confidence with which metabolic targets were identified, the murine findings were compared with previously published human transcriptomics datasets. To this end Pubmed, ArrayExpress and the GEO database were screened for transcriptomics studies investigating CTLA-4 or PD-1 resistance. The datasets were first analysed separately, and if a dataset contained three or more groups, all relevant comparisons were made. A rank-based meta-analysis approach was then used where all 1,289 genes detectable in all datasets based on log2 fold-change were sorted, the most upexpressed gene received index number 1, and the most decreased gene received index number 1,289. The ranks of all genes in all datasets were combined in three different approaches which all emphasized different aspects of the data: i) sum; ii) median; and iii) product. To validate the meta-analysis performed on the metabolic genes, the meta-analysis was re-performed by including all the genes. A rank-based meta-analysis approach was used where all 10,789 genes detectable in all datasets based on log2 fold-change were sorted, the most upexpressed gene received index number 1, and the most decreased gene received index number 10,789.

Human single-cell RNA-sequencing (RNA-seq)

Publically available human PDAC single-cell RNA-sequencing (RNA-seq) data deposited in the Genome Sequence Archive under the project PRJCA001063 with the access number GSA: CRA001160 was used [48]. The differential expressed genes were identified by edgeR package and filtered by $|\text{fold change}| > 2$ and $FDR < 0.05$ (Bonferroni adjust).

Lentiviral Knockdown and Overexpression Strategies

Panc02, KPC1245, B16-F10 and YUMM1.7 CDA genetic inactivated (hereinafter referred to as KO) and scramble cells were engineered genetically using a doxycycline inducible CRISPR/Cas9 platform. Briefly, cells were transduced with a doxycycline-inducible Cas9 nuclease (Edit-R Inducible Lentiviral Cas9, Dharmacom) and selected with blasticidin (Bio-Connect). A multiplicity of infection reaching approximately 30% of transduction was used to guarantee that each cancer cell was infected with a single copy of the plasmid. Cells expressing the doxycycline-inducible Cas9 nuclease were transduced with a target specific gRNA for CDA and a control non-targeting scramble

gRNA and selected with puromycin (Sigma-Aldrich). After selection, cells were treated for seven days with doxycycline (Sigma-Aldrich) to induce Cas9 expression and grown for seven more days without doxycycline before being used in functional assays. Silencing efficiency was assessed by qRT-PCR.

CDA and Ovalbumin (OVA) overexpression in cells was driven under the control of a Cytomegalovirus (CMV) promoter. Control cells were transduced with empty vectors. Transduced MC38 cells were selected with puromycin and Panc02 cells with geneticin (G418, Invivogen).

Gene name, sgRNA number and sequence were as follows: CDA sgRNA1, CGCTCGGCACACACACCTAG; CDA sgRNA2, TCCAGAAGGCCATCTCCGAA.

RNA extraction, cDNA synthesis and qRT-PCR

To assess gene expression, RNA from cells was extracted with a RNeasy Minikit (Qiagen) according to the manufacturer's instructions and resuspended in 30μL RNase-free water. RNA concentration was measured with the Nanodrop 2000 (Thermo Scientific). Reverse transcription of cDNA was performed with a QuantiTect Reverse Transcription Kit (Qiagen) or a SuperScript™ III First-Strand Synthesis System (Invitrogen), according to manufacturer's protocol. cDNA, primers mix and PowerUp™ SYBR Green Mix (Applied Biosystems) or TaqMan™ Fast Universal PCR Master Mix (Applied Biosystems) were prepared according to manufacturer's instructions. A total volume of 12μL was pipetted into a 96-well MicroAmp plate (Applied Biosystems) and analysed using the QuantStudioTM 12K Flex Real-Time PCR System (Applied Biosystems). Gene transcription was presented as number *gene* mRNA copies relative to the housekeeping gene.

Target: GADPH, Mm.PT.39a.1 (IDT); CDA, #1: *In-house*, forward: AAGGCCATCTCCGAAGGGTA, reverse: CAGTCGGTCCCAAACTCTCT; CDA, #2: *In-house*, forward: CTCGTGAGGCCAAGAAGTCA, reverse: CACACCTAGTGGGTAGCAGG); IL-10, Mm.PT.58.13531087 (IDT); TGF-β, Mm.PT.58.11254750 (IDT); TNF-α, Mm.PT.58.12575861 (IDT); CXCL10, Mm.PT.58.43575827 (IDT); IL-12, Mm.PT.58.12409997 (IDT); Arginase, Mm.PT.58.12409997 (IDT).

Western Blot

Protein was extracted with RIPA lysis buffer (50mM Tris HCl pH 8, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche). After a 30 minutes incubation period on ice, cell lysates were centrifuged for 15 minutes at 4°C and supernatants were collected

and protein concentration was determined by using bicinchoninic acid reagent (Pierce). Protein samples were incubated for 5 minutes at 95°C with loading buffer 6X (β-mercaptoethanol 0.6M; SDS 8%; Tris-HCl 0.25M pH 6.8; glycerol 40%; Bromophenol Blue 0.2%) to denature the proteins. After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Bio-Rad) using the Bio-Rad Trans-Blot® Turbo™ Transfer System (Bio-Rad). The membranes were blocked for non-specific binding in 5% non-fatty dry milk in Tris Buffered Saline-Tween 0.1% (50mM Tris HCl ph 7.6, 150mM NaCl, 0.1% Tween; TBS-T) for 1h at room temperature (RT) and incubated with CDA (1:200 rabbit anti-mouse, Sigma-Aldrich) and Tubulin (1:3000 HRP-conjugated anti-beta-tubulin, Abcam) in 5% non-fatty dry milk in TBS-T overnight at 4°C. Membranes were washed with TBS-T for 15 minutes and incubated with goat anti-rabbit (1/5000 in 5% non-fatty dry milk in TBS-T, Santa Cruz biotechnology) for 50 minutes at RT. The signal was visualized with Enhanced Chemiluminescent Reagents (ECL; Invitrogen) or SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) with a digital imager (ImageQuant LAS 4000, GE Health Care Life Science Technologies)

Cell growth analysis

CDA KO and Scramble Panc02 or KPC1245 cells were seeded in 6-well plates ($5x10^4$ cells/2mL/well) and incubated at 37°C in a 5% CO_2 humidified atmosphere until cells attached (T0), for 24 (T24), 48 (T48) and 72h (T72). At these time points, cells were counted using a haemocytometer. Cell growth rate was defined as the number of cell divisions normalized for T0.

Histology and immunostaining

After dissection, murine tumour samples were fixed in 2% paraformaldehyde (PFA) overnight at 4°C, dehydrated and embedded in paraffin. Serial sections of 7µm were cut using a Microm HM360 Microtome, deparaffinised and immersed in target retrieval solution (Dako) for 20 minutes at 100°C. After cooling down, the sections were washed with TBS and kept in 100% MeOH with 0.3% H_2O_2 for 20 minutes to inactivate endogenous peroxidases. After washing, the samples were blocked with pre-immune donkey serum (PID, Sigma-Aldrich) diluted 1:10 in Tris-NaCl-blocking buffer (TNB). Afterwards, the sections were incubated with rabbit anti-mouse CD8 (Cell Signaling, 1:100) or rat anti-mouse F4/80 (Serotec, 1:100) + 10% PID in TNB overnight. After washing with TNT buffer (100mM Tris pH 7.5, 150mM NaCl, 0.05% Tween-20), the sections were incubated with the secondary antibody donkey anti-rabbit (Jackson Immunoresearch, 1:200) or donkey anti-

rat Biotin (Jackson Immunoresearch, 1:500) in TNB for 45 minutes. CD8 immune complex was then amplified with Cyanine 3 (Perkin Elmer) according to the manufacturer's instructions. F4/80 immune complex was amplified with Streptavidin-HRP Conjugate (Perkin Elmer) and TSA Fluorescein kit (Perkin Elmer) according to the manufacturer's instructions. The endogenous peroxidases were inactivated with 100% MeOH with 0.3% H_2O_2, samples were blocked with PID and incubated with goat anti-mouse MMR/CD206 (R&D Systems, 1:100) + 10% PID in TNB overnight. Sections were incubated with the secondary antibody donkey anti-goat biotin (Bio-Connect Life Sciences, 1:500) and amplified with TSA Fluorescein kit and Cyanine 3 according to the manufacturer's instructions. Hoechst solution (Life Technologies, 1:1000) was utilized in order to visualize nuclei. Mounting of slides was done with ProLong Gold mounting medium without DAPI (Invitrogen). Imaging and microscopic analysis was performed with an Olympus BX41 microscope and CellSense imaging software.

4 µm paraffin-embedded tumour samples from 34 patients without preoperative chemo/radio-therapy who received a pancreaticoduodenectomy after a pancreas adenocarcinoma (pN0; pM0; pR0; pRM > 1mm) were analysed histologically for the presence of CDA and CD8. The clinical protocol was approved by the Ethical Committee of the University Hospitals KU Leuven (Leuven, Belgium) with the reference number ML3452, and all subjects consented prior to study participation. All immunohistochemical stains were performed on the Bond-III Fully Automated IHC and ISH Stainer (Leica Biosystems). Primary antibodies against CDA (Abcam, 1:500) and CD8 (Agilent, 1:200) were used. Bond Polymer Refine Red Detection and Bond Polymer Refine Detection kits (Leica Biosystems) were used following manufactures' instructions. Slides were scanned with the IntelliSite Ultra-Fast Scanner (Philips). Digital images were analysed by an expert pathologist. Two groups were selected based on a high or low expression of CDA in the tumour cells. CD8 positive cells were counted in 10 random high-power fields (HPF). 5 HPFs were randomly assigned in the border and 5 HPFs in the centre of the tumour.

Murine bulk RNA-seq

RNA from snap frozen Panc02 tumours was prepared using TRIzol reagent (Invitrogen™) according to the manufacturer's instruction. Starting from 1 mg total RNA, poly-adenylated fragments were isolated, reverse transcribed, and converted into indexed sequencing libraries using the KAPA stranded mRNA-seq kit (Sopachem). The first 50 bases of these libraries were sequenced on a HiSeq4000 system (Illumina). The raw sequenced reads were mapped to the mouse reference

transcriptome and genome (GRCm38/mm10) using the Bowtie TopHat pipeline. Mapped reads were assigned to Ensembl gene IDs by HTSeq.

Metagene signatures were calculated using gene set variation analysis, with human gene sets being derived from the PanCancer immune metagenes as described in [49], and lifted over to their murine orthologues using ENSEMBL BioMart.

FACS analysis

Tumour-bearing mice were sacrificed by cervical dislocation and perfused with saline to remove circulating immune cells. Tumours were harvested and minced in αMEM medium (Lonza) supplemented with 5% FBS, 1% Pen/Strep, 50μM β-mercaptoethanol (Gibco), 5U/mL DNase I (Qiagen), 0.85mg/mL Collagenase V (Collagenase from *Clostridium histolyticum*, Sigma-Aldrich), 1.25mg/mL Collagenase D (Collagenase from *Clostridium histolyticum*, Roche) and 1mg/mL Dispase II (Gibco) and incubated for 30 minutes at 37°C. The digested tissue was filtered using a 70μm pore sized mesh strainer and cells were centrifuged 5 minutes at 300 xg. The samples were resuspended in Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich) for 30 seconds, inactivated with FACS buffer (PBS containing 2% FBS and 2 mM EDTA) and centrifuged. The cell pellets were resuspended in FACS buffer and filtered with a 40μm pore sized mesh strainer. Cells were resuspended in FACS buffer and for the intracellular measurement of interferon γ (IFN-γ) and granzyme B (GZMB), single-cell suspensions were culture in RPMI medium supplemented with 10% FBS, 1% glutamine, 1% Pen/Strep and stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin Cell Stimulation Cocktail (eBioscience™, 1:500) in the presence of Brefeldin A (BioLegend®, 1:1000) or Monensin (eBioscience™, 1:1000) for 4 h at 37°C. Cells were then incubated for 15 minutes at 4°C with Mouse BD Fc Block purified Rat Anti-Mouse CD16/CD32 mAb (BD Pharmingen™) and stained with the following antibodies for 30 minutes at 4°C: Fixable viability dye (eFluor™ 506, eBioscience™), anti-CD45 (30-F11, APC-Cy7 or FITC, BioLegend®), anti-CD11b (M1/70, PerCP-Cy5.5, BioLegend®; or eFluor™ 450, eBioscience™), anti-TCR-β chain (H57-597, BV421, BD Biosciences), anti-CD4 (RM4-5, PE, BioLegend®), anti-CD8 (53-6.7, PE-Cy7, eBioscience™ or 53-6.7, APC-Cy7, BioLegend®), anti-CD69 (H1.2F3, APC, eBioscience™), anti-F4/80 (BM8, Alexa Fluor 488, eBioscience™; PerCP-Cy5.5, BioLegend®), anti-IFN-γ (XMG1.2, PE-Cy7, eBioscience™), anti-GZMB (GB11, Alexa Fluor 647, BioLegend®), anti-MHC-II (M5/114.15.2, APC, eBioscience™) and anti-CD11c (N418, PE-Cy7, eBioscience™). Cells were subsequently

washed and resuspended in cold FACS buffer before FACS analysis or flow sorting by a FACS Verse or FACS Aria (BD Biosciences), respectively.

Alternatively, 100µl of blood from tumour-bearing mice were resuspended in Red Blood Cell Lysing Buffer Hybri-Max™ for 60 seconds, centrifuged and resuspended in FACS buffer. Cells were stained with the following cocktail of antibodies for 30 minutes at 4°C: Zombie NIR™ Fixable Viability Dye (APC-Cy7, BioLegend®), anti-TCR-β chain (H57-597, BV421, BD Biosciences), anti-CD4 (RM4-5, PerCP-Cy5.5, BioLegend®), anti-CD8 (53-6.7, PE-Cy7, eBioscience™), anti-CD44 (IM7, BV510, BioLegend®) and anti-CD62L (MEL-14, APC, eBioscience™). Cells were resuspended in Red Blood Cell Lysing Buffer Hybri-Max™, centrifuged and resuspended in FACS buffer before FACS analysis by a FACS Verse.

FACS quantification of SIINFEKL-H2K(b) and MHC-I expression in Panc02 cancer cells was performed as previously described. Cells were stained with the following cocktail of antibodies for 30 minutes at 4°C: Fixable viability dye (eFluor™ 450, eBioscience™), anti-MHC-I (AF6-88.5.5.3, PE-Cy, eBioscience™) and anti-H-2K^b bound to SIINFEKL (25-D1.16, PE, BioLegend®).

Fluorescence minus one (FMO) controls, unstained control and single staining controls were also performed in order to ensure proper gating of populations. Data were analysed by FlowJo (TreeStar).

Flow sorting

4x10^6 Panc02 cells genetically engineered to express CD90.1 by lentiviral transduction, were injected s.c. in the right flank of the mouse and tumours were harvested for FACS sorting as previously mentioned. Cells were stained with the following cocktail of antibodies for 30 minutes at 4°C: Fixable viability dye (eFluor™ 506 or eFluor™ 450), anti-CD45 (30-F11, APC-Cy7), anti-CD11b (M1/70, PE, BD Biosciences), anti-F4/80 (BM8, APC, eBioscience™), anti-TCR-β chain (H57-597, eFluor™ 450, eBioscience™) and anti-CD90.1 (OX-7, Alexa Fluor 488, BioLegend®). Cells were subsequently washed and resuspended in cold FACS buffer before flow sorting.

Bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs)

Murine bone marrow-derived macrophages (BMDMs) were derived from bone marrow precursors as described before [31]. Briefly, bone marrow cells (1x10^7) were cultured in a volume of 5 ml in a 6-well plate in RPMI supplemented with 20% FBS, 30% L929 conditioned medium as source of M-CSF, 1% glutamine, 1mM HEPES and 1% Pen/Strep. After 3 days of culture, additional

3 ml of differentiation medium were added. At day 7, BMDMs were harvested with ice cold Ca^{2+}- and Mg^{2+}-free PBS. The cells obtained were uniformly macrophages as assessed by FACS, using the myeloid cell-specific marker CD11b and the pan-macrophage-specific marker F4/80.

For murine bone marrow-derived dendritic cells (BMDCs), $1x10^7$ bone marrow cells were cultured during 8 days without disturbing in a volume of 6 ml in a 10 cm Petri dish (non-tissue culture treated, bacterial grade) in RPMI supplemented with 10% FBS, 1% Glutamine, 25mM HEPES, 1% NEAA, 1% Sodium Pyruvate, 1% Pen/Strep, 55μM β-Mercaptoethanol and 100ng/ml recombinant human Flt3L-Fc (BioXcell). At day 8, BMDCs were harvested with ice cold Ca^{2+}- and Mg^{2+}-free PBS. The cells obtained were uniformly dendritic cells as assessed by FACS, using the myeloid cell-specific marker CD11b and the specific markers CD11c and MHC-II.

OT-I T cell isolation and activation

Naïve T cells were isolated from the spleens of OT-I T cell receptor transgenic mice. Briefly, spleens were processed on a 40μM pore cell strainer in sterile PBS and centrifuged at 350 xg for 7 minutes. Red blood cells were lysed using Hybri-Max™ (Sigma-Aldrich) buffer. Total splenocytes ($5x10^6$) were cultured in 1ml in a 24-well plate in in T cell medium (RPMI medium supplemented with 10% FBS, 1% Pen/Strep, 1% NEAA, 1% sodium pyruvate, 25μM β-Mercaptoethanol). Depending on the experimental setup, OT-I T cells were activated for 3 days with 1μg/ml soluble anti-mouse CD28 (BD Biosciences) and 1μg/ml "SIINFEKL" peptide (IBA Lifesciences) and 10ng/ml recombinant human IL-2 (PeproTech). After 72h, activated OT-I T (2 $x10^6$) cells were transferred into fresh media containing IL-2 and allowed to expand for 5–7 days.

BMDMs and BMDCs phagocytosis

CDA KO and Scramble Panc02 cells genetically engineered to express mCherry by lentiviral transduction, were FACS sorted and $mCherry^+$ cells were seeded together with BMDMs or BMDCs in a 1:4 ratio for 24 hours. Cells were harvested as previously mentioned, span down, resuspended in FACS buffer and incubated for 15 minutes at 4°C with Mouse BD Fc Block purified Rat Anti-Mouse CD16/CD32 mAb (BD Pharmingen™). Cells were stained with the following cocktail of antibodies for 30 minutes at 4°C: Fixable viability dye (eFluor™ 450, eBioscience™), anti-CD45 (30-F11, APC-Cy7, BioLegend®), anti-F4/80 (BM8, APC or FITC, eBioscience™) anti-MHC-I (AF6-88.5.5.3, PE-Cy, eBioscience™), anti-CD11c (N418, APC, eBioscience™) and anti-MHC-II

(M5/114.15.2, PerCP-eFluor 710, eBioscience™). Cells were subsequently washed and resuspended in cold FACS buffer before FACS analysis by a FACS Aria (BD Biosciences).

FMO controls, unstained control and single staining controls were also performed in order to ensure proper gating of populations.

BMDMs polarization

The conditioned medium of Scramble and CDA KO Panc02 cells cultured in DMEM containing 5.5 mM glucose (Sigma-Aldrich) and 2 mM glutamine (Sigma-Aldrich) for 32 hours, was collected and transferred to BMDMs. After 16 hours, RNA was extracted.

OT-I CD8$^+$ T cell killing

Scramble and CDA KO OVA-expressing Panc02 cells were labelled with 1 μM carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific) for 10 minutes at RT and scramble and CDA KO non-OVA Panc02 cells were labelled with 3.5 μM violet cell tracer (Thermo Fisher Scientific) at 37°C for 20 minutes. Mixed populations of OVA-expressing CFSE labelled CDA KO or Scramble Panc02 cells and non-OVA expressing Violet labelled CDA KO or Scramble Panc02 cells were seeded at 1:1 ratio and co-cultured with pre-activated OT-I CD8$^+$ T cells for 24 hours at the indicated ratios. Cells were stained with Zombie NIR™ Fixable Viability Dye, washed and resuspended in cold FACS buffer before analysed by flow cytometry for changes in the ratio of CFSE$^+$:Violet$^+$ cells.

OT-I CD8$^+$ T cell activation

OVA-expressing CDA-null or control cells were co-cultured with or without BMDMs at 1:4 ratio for 24 hours and afterwards total OT-I splenocytes were added at a 1:15 ratio (cancer cell:splenocyte) for 36 hours in T cell medium with 10ng/ml recombinant human IL-2. Cells were then stained with the following cocktail of antibodies for 30 minutes at 4°C: Fixable viability dye (eFluor™ 506), anti-TCR-β chain (H57-597, BV421), anti-CD4 (RM4-5, PE), anti-CD8 (53-6.7, APC-Cy7), anti-CD69 (H1.2F3, PerCP-Cy5.5, BioLegend®), anti-IFN-γ (XMG1.2, PE-Cy7) and anti-GZMB (GB11, Alexa Fluor 647). Cells were subsequently washed and resuspended in cold FACS buffer before FACS analysis.

Liquid chromatography-mass spectrometry (LC-MS) analysis

Cancer cells were seeded at a density of 30,000 cells/well in DMEM complete in 6-well plates. On day 1, the cells and empty wells (for background noise control), were washed once with PBS, and then replenished with DMEM containing 5.5 mM glucose, 2 mM glutamine with or without 0.1mM Cytidine (Sigma-Aldrich). On day 0, 1 and 2, 100 µl of medium were collected and 900 µl of ice-cold extraction buffer (80% methanol and 2 µM d27 myristic acid) were added. Samples were centrifuged for 15 minutes at 4°C using 20.000 xg and 250 µl of the supernatant were transferred to fresh vials. On day 2, cells were harvested on ice by removing the media and washing them once in ice-cold saline solution (9 g/L NaCl). Cells were immersed in 250 µL of ice-cold extraction buffer for 2 minutes, scraped, transferred to fresh vials and stored overnight at -80°C. The cellular debris were then pelleted at 20,000 xg for 15 minutes at 4°C and the supernatant was transferred to a new vial. The cell pellet was dissolved in 100 µL of 200 mM NaOH (for 20 min at 95°C), and the protein concentration was determined by using bicinchoninic acid reagent method.

Small pieces of Panc02 tumours were inserted in collection tubes (2ml eppendorf with 5 holes at the bottom in a 15 ml falcon) and 20 to 40 µl of 9 g/L NaCl pH 7.4 were added to the samples. Samples were centrifuged at 110 xg for 10 minutes at 4°C, the interstitial fluid was collected in new vials and 800 µl of ice-cold extraction buffer (Methanol:Water, 5:3) were added. After vortexing and centrifuging at 20,000 xg for 5 minutes at 4°C, the supernatant was transferred to a new vial.

10 µL of sample were used for LC-MS and the metabolites were resolved on a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific).

Statistical analysis

Data entry and all analyses were performed in a blinded fashion. All statistical analyses were performed using GraphPad 7 Prism software on mean values, calculated from the averages of technical replicates. Statistical significance was calculated by two-tailed unpaired t-test on two experimental conditions or two-way ANOVA when repeated measures were compared, with $p< 0.05$ considered statistically significant. Detection of mathematical outliers was performed using the Grubbs' test in GraphPad. Sample sizes for all experiments were chosen based on previous experience and material availability. Independent experiments were pooled and analysed together whenever possible as detailed in figure's legends. All graphs show mean values ± standard error of the mean (SEM).

3.3 Results

3.3.1 Cytidine deaminase is upregulated in immune checkpoint blockade resistant tumours

In order to identify metabolic pathways within the TME which affect immunosuppression and resistance to immunotherapy, we set-up and performed a tailored meta-analysis. Briefly, we integrated the first publicly available pre-treatment transcriptomic data of patients responding and non-responding to immune checkpoint inhibitors (*i.e.*, α-CTLA-4 and α-PD-1) [2, 45, 46] (**Table 3.1**) with bulk tumour transcriptomic and metabolomic profiling of responsive (MC38), low responsive (CT26) and non-responsive (Panc02) murine tumour models (**Figure 3.1A-C**). Snap frozen tumour samples were analysed by LC-MS for untargeted metabolomics and by RNA-seq for transcriptomics (**Table 3.1**). Endpoint tumour weight was included as a covariate throughout analyses. The datasets were integrated and interpreted using the BIOMEX platform [47].

Table 3.1. Datasets used in the meta-analysis

NAME	ORGANISM	CANCER TYPE	OMICS TYPE	REGIMENT
PRM	murine	multiple	metabolomics	**α**-PD-1, **α**-CTLA-4
PRT	murine	multiple	transcriptomics	**α**-PD-1, **α**-CTLA-4
Hugo	human	melanoma	transcriptomics	**α**-PD-1
Ascierto	human	renal	transcriptomics	**α**-PD-1
Van Allen	human	melanoma	transcriptomics	**α**-CTLA-4

An exploratory analysis performed on 235 annotated metabolites showed that CT26 tumours have a clearly distinct metabolic profile whereas MC38 and Panc02 were clustered closer together but were nevertheless distinct (**Figure 3.1D**). Immunotherapy did not affect the overall metabolic profile of these cells (data not shown). In line with the metabolome data, exploratory transcriptome analysis of the three murine tumour models showed highly distinct metabolic gene profiles (**Figure 3.1E**). This was also reflected in the differential analysis which showed a large number of differentially expressed genes when comparing the ICB resistant Panc02 tumours with the low responsive CT26 and the responsive MC38 (**Figure 3.1F**).

In order to prioritize genes that were causally related with therapy resistance, we combined the murine datasets with human gene expression studies that assessed immunotherapy resistance (**Table 3.1**). After analysing each of the transcriptomics datasets separately, we then combined all the results, excluding the genes that were not expressed in each of the studies. The outcome of

the gene set enrichment was then processed through median-based meta-analysis to prioritize up-regulated genes as well as with product-based meta-analysis to prioritize down-regulated genes, yielding genes involved in the treatment response in both human and murine tumours. This meta-analysis revealed metabolic pathways that were consistently deregulated in immune checkpoint resistance across the datasets and highlighted all the metabolic genes that were present in all datasets (~1300).

Among the metabolic pathways that were consistently deregulated we have identified CDA as a potential target that is involved in the lack of response to ICB (**Figure 3.1G**). Besides deamination of free nucleotides, CDA also deaminates and inactivates cytidine analogues such as gemcitabine, cytosine arabinoside and 5-azacytidine, agents used to treat cancer. Consequently, CDA plays a crucial role in the resistance of cancer cells to treatment with cytidine analogues, with several studies showing a link between its overexpression and treatment resistance [42-44]. Pancreatic cancer patient survival data extracted from The Human Protein Atlas showed that patients with high CDA expression have a statistically significant lower overall survival **(Figure 3.1H)** [50]**.** Interestingly, our single cell RNA-seq analysis of human PDAC revealed that the tumour cell type with the highest expression of CDA corresponds to ductal cell type 2 cancer cells **(Figure 3.1I)**, that according to Peng *et al* [48] show malignant features. This indicates that this metabolic alteration stems mostly from the cancer cell compartment**.** This was also corroborated in our murine tumour model, where tumour sorted Panc02 cancer cells were the compartment with the highest expression of CDA, with similar levels of the *in vitro* condition (**Figure 3.1J**).

3.3.2 Loss of cytidine deaminase in cancer cells sensitizes pancreatic tumours to immunotherapy

To validate the observation that CDA overexpression in cancer cells is responsible for resistance to ICB in PDAC (a tumour type where CDA overexpression is strongly correlated with poor disease outcome as seen above), we genetically engineered mouse pancreatic cancer cells (Panc02 and KPC1245) deficient for CDA using the doxycycline-inducible CRISPR/Cas9 system (**Figure S3.1A-D**). CDA KO and Scramble cells were injected s.c into the flank (Panc02) or orthotopically into the pancreas (KPC1245) of immunocompetent C57BL/6 mice and treated i.p. with control IgG or the α-PD-1. Interestingly, we found that the depletion of CDA in Panc02 cancer cells significantly reduced the tumour growth and weight *per se* (**Figure 3.2A**), and synergized with the immune checkpoint inhibitor α-PD-1, resulting in a complete regression of the tumours (**Figure 3.2A**).

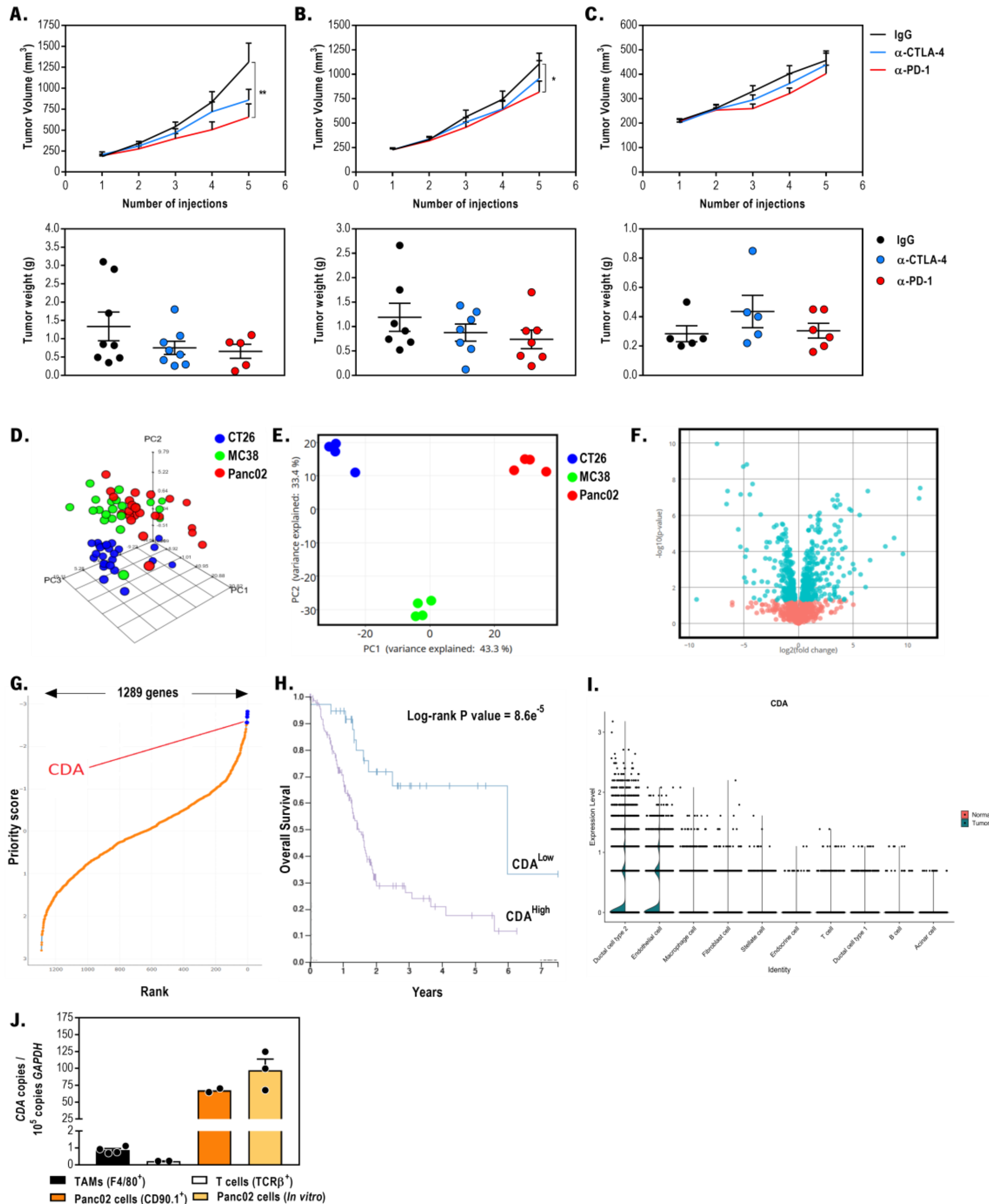

Figure 3.1. CDA is upregulated in ICB resistant tumours. (**A-C**) Subcutaneous MC38 (**A**), CT26 (**B**) and Panc02 (**C**) tumour growth and weight treated with IgG, α-CTLA-4 or α-PD-1; (**D-F**) PCA on CT26, MC38 and Panc02 metabolomics (**D**) and metabolic gene expression (**E**) and volcano plot showing differentially expressed metabolic genes when comparing immune checkpoint resistant Panc02 tumours with responsive MC38+CT26 tumours (**F**); (**G**) Ranking of the 1289 genes identified in the meta-analysis; (**H**) Kaplan-Meier overall survival curve for pancreatic cancer patients with high and low CDA expression; (**I**) Violin plots displaying the expression of CDA across the cell types identified in human PDAC; (**J**) *CDA* transcript abundance in different murine tumour compartments sorted from subcutaneous Panc02 tumours. n=5-8 (**A-C**) and n=2-4 (**J**). All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

We also observed a significant reduction of tumour weight in the clinically relevant and ICB resistant pancreatic tumour model KPC1245, when CDA depleted tumours were treated with α-PD-1 (**Figure 3.2B** and **Figure S3.2A-F**). Moreover, a dramatic reduction of the number of mesenteric metastases was obtained when we combined CDA KO with PD-1 blockade (**Figure 3.3C-D**). Importantly, the genetic deletion of CDA did not impair pancreatic cancer cell growth *in vitro* (**Figure S3.1F-G**) and mutational burden (data not shown).

Conversely, the re-introduction of CDA in Panc02 CDA KO cancer cells (**Figure S3.1H**) promoted resistance to ICB, with the overexpression of CDA resulting in larger and unresponsive tumours (**Figure 3.2E**). Similarly, the overexpression of CDA in a MC38 colorectal inflamed and α-PD1 responsive model (**Figure S3.1I**) conferred a striking acquisition of resistance towards ICB (**Figure 3.2F**).

In our meta-analysis, we integrated pre-treatment transcriptomic data of melanoma patients responding and non-responding to immune checkpoint inhibitors. As observed in the pancreatic models, the deletion of CDA in ICB resistant orthotopic melanoma models (YUMM 1.7 and B16-F10) sensitized cells to α-PD-1 (**Figure S3.2G-J**), suggesting that the effect observed might not be tumour type-specific but rather related with the role of CDA in immunotherapy resistance.

3.3.3 Cytidine deaminase depletion in pancreatic tumours modulates the recruitment of activated cytotoxic T cells and induces a $CD8^+$ T cell central memory phenotype

The previous results show that the loss of CDA in cancer cells promotes the activation of the immune system and consequently sensitizes resistant tumours to immune checkpoint inhibitors. In order to determine the crucial players within the immune cell compartment underlying the increased anti-tumour immune response observed, we started by using a simple and straight forward approach. We compared the growth patterns of scramble and CDA KO Panc02 tumours grafted onto immunocompetent C57BL/6 and immunodeficient NMRI-*Foxn1*^{nu} mice, which are unable to mount an adaptive immune response. While the deletion of CDA in Panc02 cells resulted in a significant decrease of tumour growth and weight in immunocompetent mice, the same metabolic alteration did not affect tumour growth and weight when tumours were s.c. implanted in immunodeficient mice (**Figure 3.3A**). This observation proves that the anti-tumour effect observed upon CDA KO is T cell dependent and that the modulation of the TME, namely the activation of the immune system, is downstream to CDA inactivation in cancer cells.

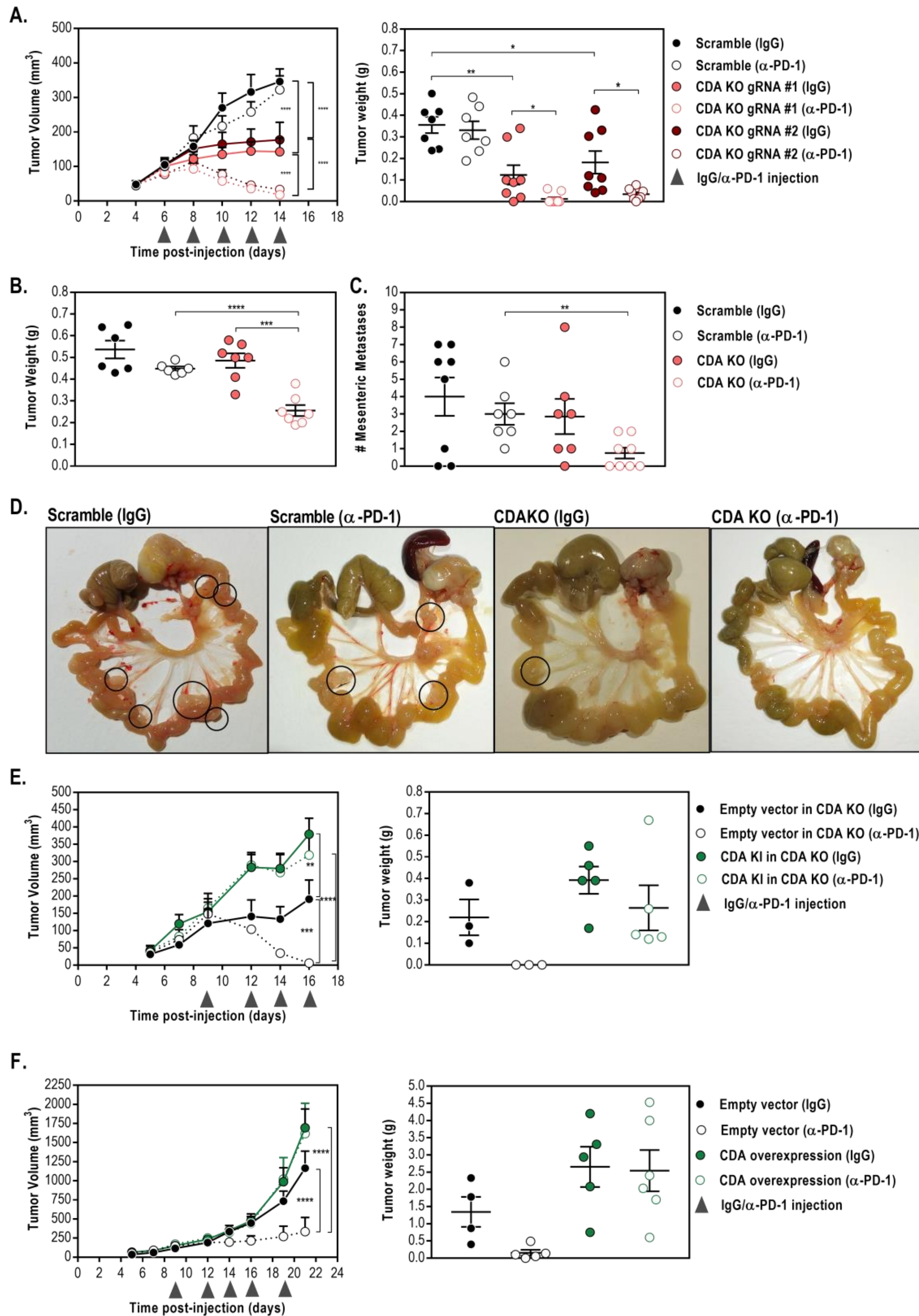

Figure 3.2. CDA depletion in cancer cells sensitizes tumours to immunotherapy. (**A**) Subcutaneous Panc02 Scramble and CDA KO tumour growth and weight treated with IgG or α-PD-1; (**B**-**D**) Orthotopic KPC1245 Scramble and CDA KO tumour weight (**B**), quantification (**C**) and representative pictures (**D**) of mesenteric metastases of mice treated with IgG or α-PD-1; (**E**) Subcutaneous Panc02 tumour growth and weight of CDA KO or CDA KI tumours treated with IgG or α-PD-1; (**F**) Subcutaneous MC38 tumour growth and weight of control (empty vector) or CDA overexpressing

tumours treated with IgG or α-PD-1. n=7-9 (**A**), n=6-8 (**B**, **C**), n=3-5 (**E**) and n=4-6 (**F**). Circles, mesenteric metastases (**D**). All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

In order to support our previous observation, CDA KO and scramble Panc02 tumours treated with IgG were immunostained for CD8, a marker of cytotoxic T cells. We observed that, while in the outer tumour rim there are no differences in the number of $CD8^+$ cells, there is a significant infiltration of cytotoxic T cells into the core of CDA KO Panc02 tumours when compared with the scramble control (**Figure 3.3B-C**).

To further characterize the link between CDA loss and immune populations present in the tumour niche, we performed a qualitative and quantitative characterization of tumour-infiltrating immune cells in whole tumours by FACS. While no differences were observed for total number of tumour-infiltrating CD4 positive lymphocytes in CDA KO Panc02 tumours, there was a significant increase of total ($CD8^+$) and activated, ($CD69^+CD8^+$; $IFN\gamma^+CD8^+$) cytotoxic T cells as compared with scramble tumours. Furthermore, the ratio between $CD8^+$ and $CD4^+$ cells increased when comparing CDA depleted tumours with the control condition (**Figure 3.3D-E**). These results are further supported by our Panc02 whole tumour RNA-seq, where we observed a significant upregulation of cytotoxic T cell markers such as *Gzmb* and *Prf1* in CDA depleted tumours (**Figure 3.3F**).

Concerning the KPC1245 tumour-infiltrating immune populations, the FACS analysis revealed an increase of total ($CD8^+$) and activated, ($IFN\gamma^+CD8^+$) cytotoxic T cells in CDA depleted tumours treated with α-PD-1, as compared to both scramble tumours treated with α-PD-1 and CDA KO tumours treated with IgG (**Figure 3.3G**). On the other hand, higher infiltration of activated cytotoxic T cells upon α-PD-1 therapy was reversed when CDA was overexpressed in MC38 cells (**Figure 3.3H**).

In addition to inducing infiltration of activated $CD8^+$ T cells into the tumour niche, the deletion of CDA in cancer cells also promoted a central memory phenotype in tumour-infiltrating cytotoxic T cells. This was shown by the RNA-seq analysis, where CDA KO Panc02 tumours displayed a higher $CD8^+$ T cell central memory metagene signature (**Figure 3.4A**). FACS analysis in the blood of Panc02 (**Figure 3.4B**) and KPC1245 (**Figure 3.4C**) tumour-bearing mice revealed a higher percentage of circulating $CD8^+$ central memory T cells ($CD44^+CD62L^{high}$) in mice challenged with KO cells. Importantly, we performed an *in vivo* T cell memory assay where mice which had regressed Panc02 tumours after treatment with α-PD-1 were re-challenged with wild-type Panc02 cancer cells. Tumour re-challenge experiment demonstrated that mice cured with α-PD-1

generated a memory immune response and subsequently rejected re-challenge with wild-type cells (**Figure 3.4D**).

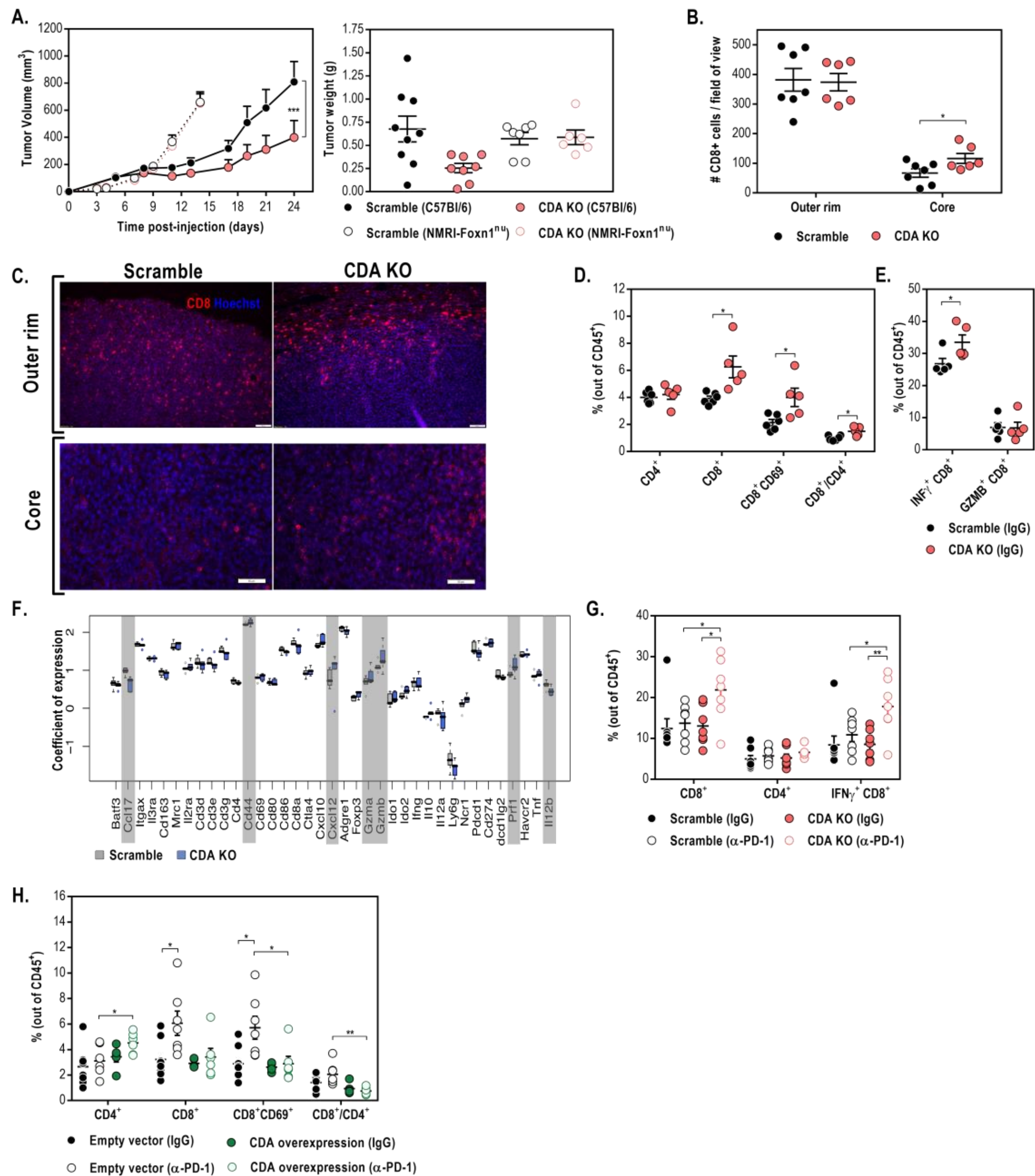

Figure 3.3. CDA depletion in cancer cells increases the recruitment of activated cytotoxic T cells. (**A**) Subcutaneous Panc02 Scramble and CDA KO tumour growth and weight in C57Bl/6 and NMRI-*Foxn1*^{nu} mice; (**B-C**) Histological quantification (**B**) and representative micrographs (**C**) of CD8-stained cells in the core (lower panel) and outer rim (upper panel) of Panc02 Scramble and CDA KO tumour sections; (**D**) FACS quantification of tumour-infiltrating CD4 T cells ($CD4^+$), cytotoxic T cells ($CD8^+$), activated cytotoxic T cells ($CD8^+CD69^+$) and $CD8^+/CD4^+$ ratio in CDA KO and Scramble Panc02 tumours; (**E**) FACS quantification of tumour-infiltrating activated cytotoxic T cells ($IFN\gamma^+CD8^+$) and ($GZMB^+CD8^+$); **(F)** Log-transformed expression values (reads per million) of activation markers in Panc02 Scramble and CDA KO tumour (**G**) FACS quantification of cytotoxic T cells ($CD8^+$), CD4 T cells ($CD4^+$) and activated cytotoxic T cells ($IFN\gamma^+CD8^+$ in CDA KO and Scramble KPC1245 tumours treated with IgG or α-PD-1; (**H**)

FACS quantification of tumour-infiltrating CD4 T cells (CD4+), cytotoxic T cells (CD8+), activated cytotoxic T cells (CD8+CD69+) and CD8+/CD4+ ratio in CDA overexpressing and control empty vector MC38 tumours. n=6-9 (**A**), n=5-6 (**D**), n=5 (**E**), n=5 (**F**), n=7-8 (**G**) and n=5-8 (**H**). 100x magnification, lower panel: cropped images (**C**). Grey boxes, *p<0.05 (**F**). All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

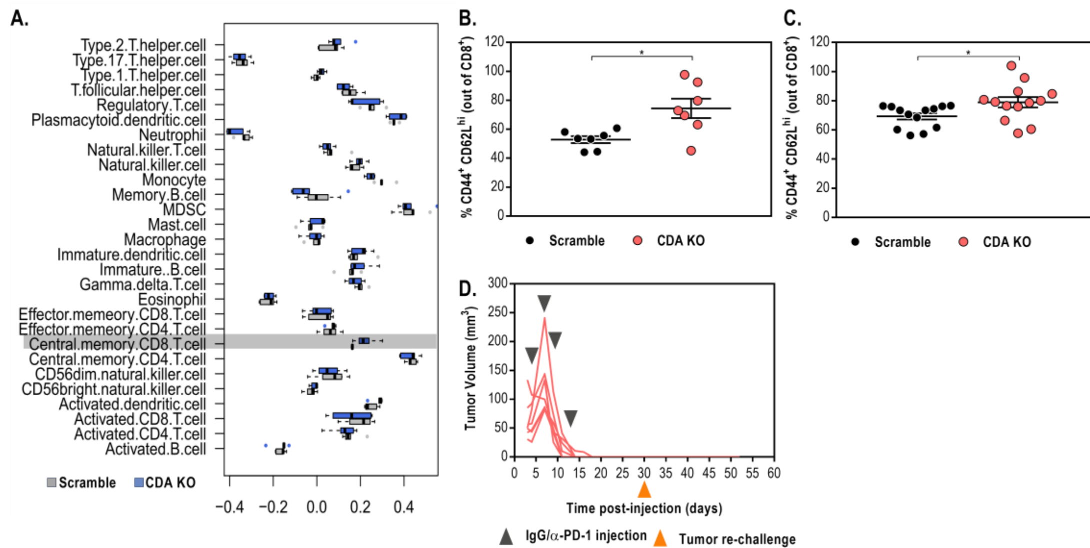

Figure 3.4. CDA KO in cancer cells induces a central memory phenotype in CD8+ T cell. (**A**) Metagene signatures in Panc02 Scramble and CDA KO tumour (**B-C**) FACS quantification of CD8+ central memory (CD44+CD62Lhigh) in the blood of CDA KO and Scramble Panc02 (**B**) and KCP1245 (**C**) tumour-bearing mice treated with IgG; (**D**) Subcutaneous Panc02 CDA KO tumour growth treated with α-PD-1. n=5 (**A**), n= 7 (**B**, **D**) and n=13 (**C**, two independent experiments pooled). All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

3.3.4 Loss of cytidine deaminase in tumour cells skews "tumour-associated macrophages" polarization away from the immunosuppressive phenotype to immunostimulatory phenotype

We reasoned that the previously observed increase in activated cytotoxic T cells in CDA KO tumours could be due to an improved recognition of tumour cells through increased antigen recognition. To confirm this, we expressed full-length OVA in CDA-null or control Panc02 cells and FACS stained with a monoclonal antibody specific to the SIINFEKL epitope from OVA in the context of H2K(b). As shown in **Figure S3.3A**, CDA depleted cells showed significantly higher levels of expression. Similarly, an increase was also observed in total MHC-I in CDA-deficient cells (**Figure S3.3A**), suggesting that the deletion of CDA from cancer cells increased their levels on the surface of antigen-loaded MHC-I.

To verify whether the depletion of CDA made tumour cells more recognizable to T cells, we seeded mixed populations of OVA-expressing CFSE labelled CDA KO or Scramble Panc02 cells with

non-OVA expressing Violet labelled CDA KO or Scramble Panc02 cells and co-cultured with OT-I $CD8^+$ T cells that recognize the SIINFEKL epitope. After 24 hours, we observed that both OVA-expressing populations were equally depleted from the co-culture, suggesting that the deletion of CDA does not induce a direct $CD8^+$ cytotoxic response (**Figure S3.3B**). Since CDA depletion induces the recruitment of activated cytotoxic T cells (**Figure 3.3**) we assessed a possible role of antigen presenting cells (APCs) on cross-priming naïve $CD8^+$ T cells. We started by determining the capacity of APCs to phagocyte cancer cells and cross-activate naïve $CD8^+$ T cell. BMDMs and BMDCs were co-cultured with mCherry-expressing CDA KO or control cells for 24 hours and quantified by FACS the numbers of phagocyted cancer cells. Both BMDMs and BMDCs (**Figure S3.3C**) showed an increased phagocytosis of CDA-null cells. Next, we assessed the capacity of APCs to activate naïve $CD8^+$ T cells. To investigate this, OVA-expressing CDA-null or control cells were co-cultured with or without BMDMs for 24 hours and afterwards total OT-I splenocytes were added for 36 hours. OT-1 $CD8^+$ T cells cultured with CDA-null OVA-Panc02 cells showed significantly more activation as measured by intracellular GZMB and IFN-γ staining only when BMDMs were present (**Figure 3.5A**). *In vivo*, CDA KO tumours displayed lower overall infiltration of tumour-associated macrophages (TAMs) (**Figure 3.5B-D**) and these TAMs switched their phenotype from immunosuppressive to immunostimulatory, as shown by an increase of anti-tumour M1-like TAMs (**Figure 3.5B**) and a decrease of pro-tumour M2-like TAMs' infiltration and polarization (**Figure 3.5C-D**). To prove the relevance of TAMs and $CD8^+$ T cells in CDA KO tumours, we depleted these immune populations in α-PD-1 treated scramble and CDA KO Panc02 tumours with clodronate liposomes and a depleting antibody, respectively. As previously shown (**Figure 3.2**), the deletion of CDA in cancer cells sensitized tumours to immunotherapy as CDA KO tumours were significantly smaller when compared with α-PD-1 treated scramble tumours. The deletion of TAMs with clodronate and cytotoxic T cells with α-CD8 abolished the difference in tumour growth between scramble and CDA KO tumours (**Figure 3.5E**, left panel). Interestingly, compared with IgG-treated tumours, the deletion of $CD8^+$ T cells resulted in larger tumours and the co-deletion of TAMs rendered similar tumour growth curves (**Figure 3.5E**, lower panel). On the other hand, TAM and $CD8^+$ T cell depletion resulted in larger tumours as compared to clodronate treatment only (**Figure 3.5E**, upper panel). Altogether, this data suggests that even though CDA deletion sensitizes tumours to immunotherapy by promoting the recruitment of activated cytotoxic T cells, this phenotype is mediated by immunostimulatory TAMs.

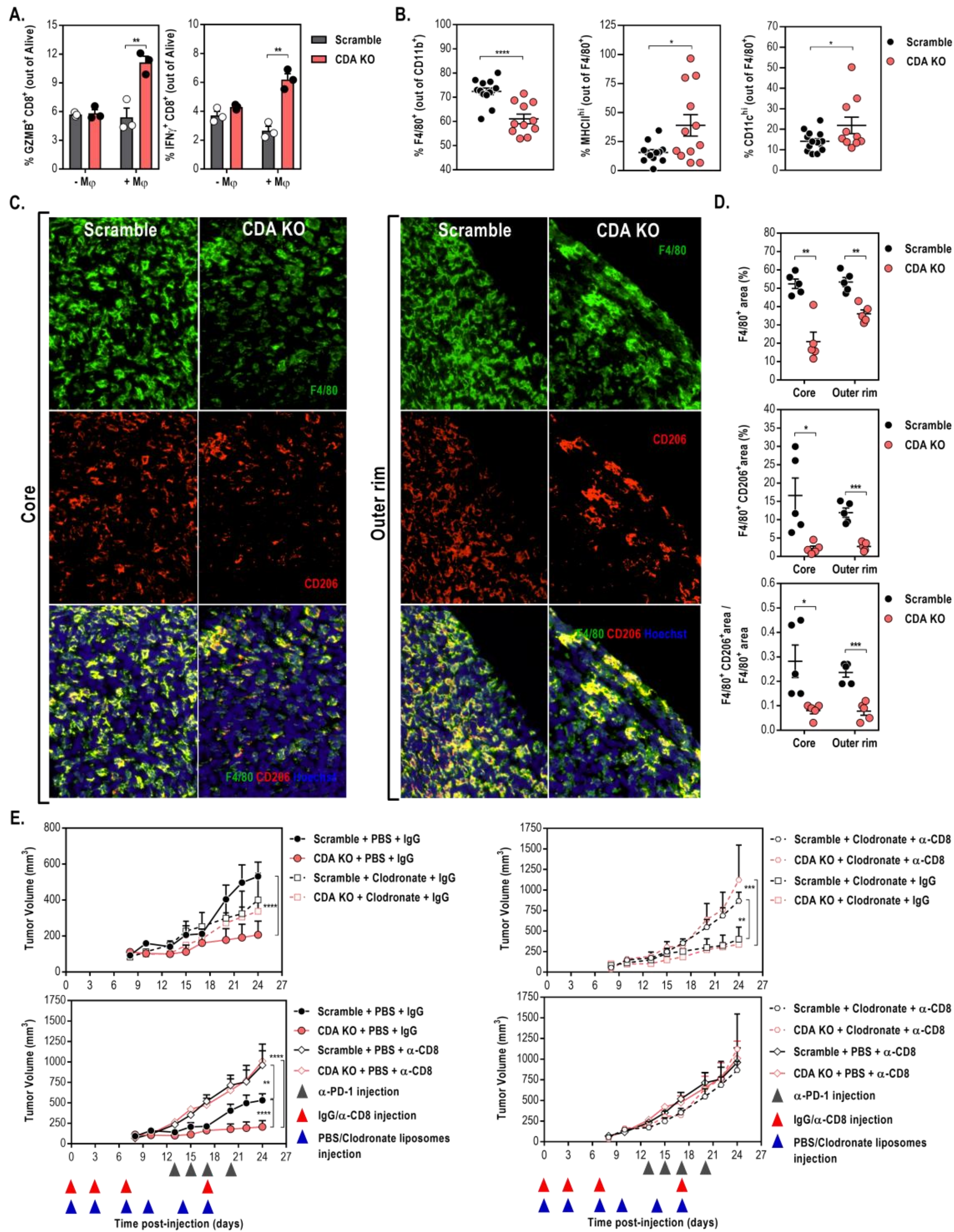

Figure 3.5. CDA depletion skews TAMs polarization to a T cell stimulatory phenotype (**A**) FACS quantification of activated $CD8^+$ T cells ($GZMB^+$ $CD8^+$; ($INFg^+$ $CD8^+$) in Scramble and CDA KO Panc02 cancer cells co-cultured in the presence or absence of BMDMs (Mφ); (**B**) FACS quantification of tumour-infiltrating tumour-associated macrophages (TAMs, $F4/80^+$) and M1-like TAMs ($MHC\text{-}II^{high}$; $CD11c^{high}$) in CDA KO and Scramble Panc02 tumours; Representative micrographs (**C**) and histological quantification (**D**) of F4/80- and M2-like CD206-stained cells in the core (left panel) and outer rim (right panel) of Panc02 Scramble and CDA KO tumour sections; (**E**) Subcutaneous α-PD-1-treated Panc02 Scramble and CDA KO tumour growth treated with PBS or clodronate liposomes and/or IgG or

α-CD8; n=10-14 (**B**, three independent experiments pooled), n= 5 (**C**, **D**) and n=3-7 (**E**). 100x magnification (**C**) All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

3.3.5 Cytidine deaminase depletion in cancer cells disrupts intracellular and extracellular pools of pyrimidines

As previously mentioned, CDA plays a pivotal role in the pyrimidine salvage pathway by performing the deamination of free cytidine and deoxycytidine to uridine and deoxyuridine, respectively. Therefore we reasoned that the promotion of polarization towards a M1-like anti-tumour phenotype in TAMs along with CD8+ T cell infiltration and antitumor effector functions which were observed, could be a consequence of an imbalance of the intracellular pools of uridine, and subsequently, UXP. To confirm this, we used LC-MS-based untargeted metabolomics and observed, in CDA depleted Panc02 and KPC1245 cells, a dramatic increase in the intracellular levels of cytidine and a concomitant decrease in the uridine pools (**Figure 3.6A,C**). Moreover, our metabolomic analysis also revealed that CDA KO Panc02 cells showed a significant reduction in the intracellular levels of uridine derivatives UDP, UTP (**Figure 3.6A**) and less uptake of cytidine and less release of uridine in the extracellular medium (**Figure 3.6B**). Importantly, the interstitial fluid of CDA KO tumours showed a tendency to accumulate cytidine and was found to be deprived of UDP (**Figure 3.6D**). Next, we assessed if the different compositions in pyrimidines of scramble and CDA KO cells media could dictate the polarization status of BMDMs. As shown in **Figure S3.3D**, CDA KO cancer cell conditioned medium switched the signatures of BMDMs to an M1-like anti-tumour phenotype.

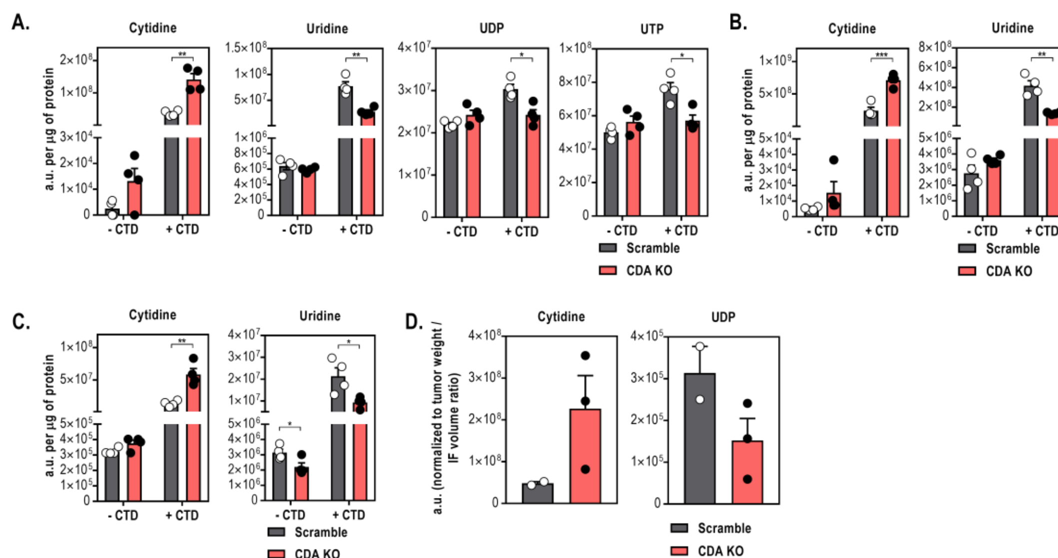

Figure 3.6. Loss of CDA in pancreatic cancer cells decreases uridine and UXP nucleosides. (**A**)

Intracellular cytidine, uridine, UDP and UTP levels in Scramble and CDA KO Panc02 cancer cells upon 48 hours; (**B**) Extracellular cytidine and uridine levels in Scramble and CDA KO Panc02 cancer cells upon 48 hours; (**C**) Intracellular cytidine and uridine in Scramble and CDA KO KPC1245 cancer cells upon 48 hours; (**D**) Cytidine and UDP levels in interstitial fluid of Scramble and CDA KO Panc02 tumours. All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001. CYT, cytidine.

3.3.6 THU and α-PD-1 combinatory treatment prevents mesenteric metastases formation and stimulates recruitment of activated cytotoxic T cells

Since CDA catalyses and inactivates cytidine analogues, ultimately contributing to increased gemcitabine resistance in pancreatic cancer [42, 43, 51], CDA inhibitors, mostly THU-based compounds, are currently under clinical evaluation in combination with gemcitabine. However, no prior art was found covering the use of CDA inhibitors to overcome immunosuppression, so as in combination with immunotherapy.

To determine whether CDA could be exploited for therapeutic purposes in the treatment of cancer and overcome resistance to immunotherapy, KPC1245 orthotopic tumour-bearing mice were treated with the CDA inhibitor THU in combination with either IgG or α-PD-1. The THU regimen did not cause obvious toxic side-effects in recipient mice (**Figure S3.4A-E**). Co-administration of THU and α-PD-1 resulted in a striking and statistically significant decrease in tumour weight (**Figure 3.7A**) and in the number of mesenteric metastases (**Figure 3.7B-C**).

Similar to what we observed with the genetic depletion of CDA, the competitive inhibition of this enzyme with THU, in combination with ICB, increased the infiltration of total ($CD8^+$) and activated, ($IFN\gamma^+CD8^+$) cytotoxic T cells as compared to IgG treated tumours (**Figure 3.7D**). Moreover, inhibition of CDA resulted in an increase of central memory $CD8^+$ T cells in circulation (**Figure 3.7E**).

3.3.7 Cytidine deaminase expression is inversely correlated with cytotoxic T cell infiltration in PDAC patients

From our initial meta-analysis, CDA was revealed to be a potential target involved in unresponsiveness to ICB in human and mouse datasets and the results obtained confirmed that CDA expression correlates with ICB resistance in murine pancreatic tumour models. Finally, we addressed if T cell exclusion could be assessed based on CDA expression in PDAC patients. A series of 34 PDAC without preoperative chemo/radio-therapy were co-immunostained for CDA and CD8 and, while no differences in the number of $CD8^+$ cells were found in the outer tumour rim,

tumours with low expression of CDA showed a significant infiltration of cytotoxic T cells into the tumour core (**Figure 3.8A-B**).

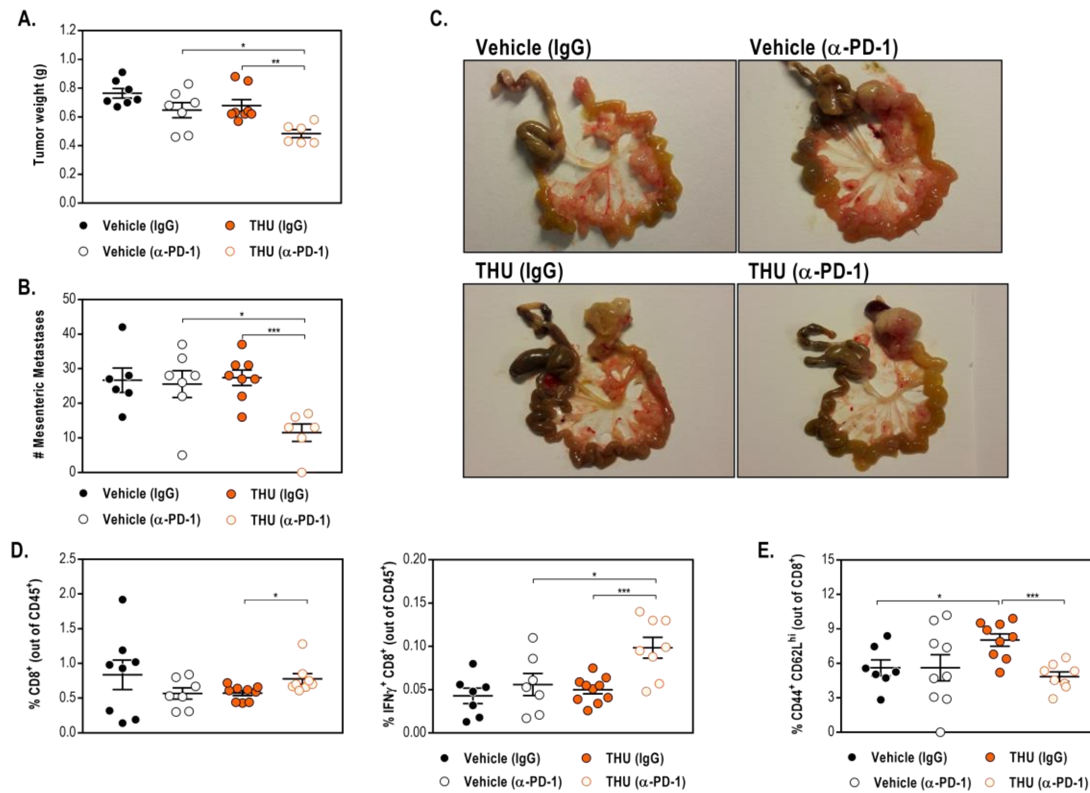

Figure 3.7. THU and α-PD-1 combinatory treatment inhibits mesenteric metastases formation and stimulates the recruitment of activated cytotoxic T cells in KPC1245 tumour-bearing mice. (**A-C**) Orthotopic KPC1245 tumour weight (**A**), quantification (**B**) and representative pictures (**C**) of mesenteric metastases of mice co-treated with Vehicle or THU and IgG or α-PD-1; (**D-E**) FACS quantification of tumour-infiltrating cytotoxic T cells ($CD8^+$) and activated cytotoxic T cells ($IFN\gamma^+CD8^+$) in KPC1245 tumours (**D**) and circulating central memory $CD8^+$ T cells ($CD44^+CD62L^{high}$) in the blood of KPC1245 tumour-bearing mice co-treated with Vehicle or THU and IgG or α-PD-1 (**F**). n= 6-8 (**A-C**), n=7-9 (**D-E**). All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

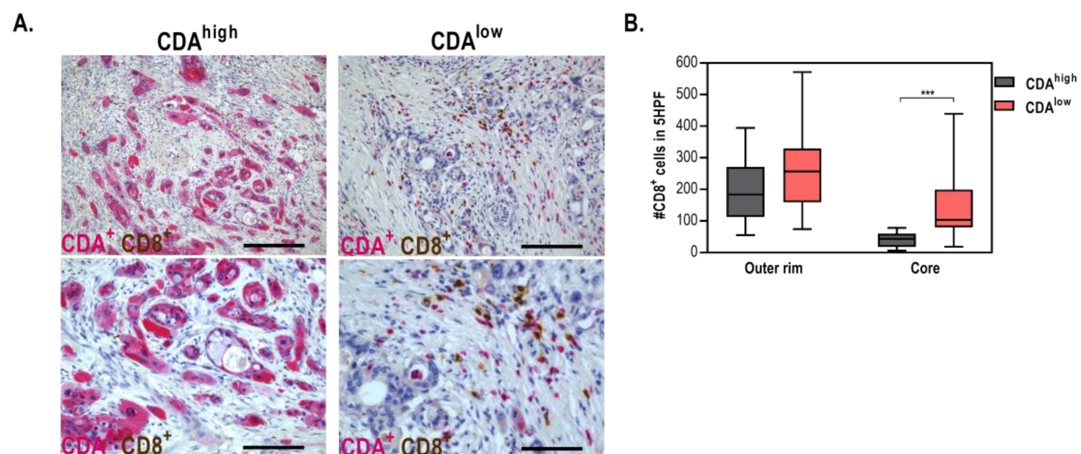

Figure 3.8. Low-CDA expressing PDAC tumours have a higher infiltration of $CD8^+$ T cells in the tumour

core. Representative micrographs (**A**) and histological quantification (**B**) of CD8-stained cells in the core and outer rim of human PDAC tumour sections with high and low expression of CDA. 5HPFs, 5 high-power fields. n=14-18. 40x magnification, upper panel; 100x magnification, lower panel (**A**). Graph shows Min to Max. $^{*}p<0.05$; $^{**}p< 0.01$; $^{***}p<0.001$, $^{****}p<0.0001$.

3.4 Discussion

Cancer immunotherapy is emerging as a revolutionary and promising treatment approach, namely the use of human antibodies against immune checkpoint proteins such as CTLA-4, PD-1 and PD-L1. The blockade of these immune checkpoints harnesses the ability of the immune system to recognize and reject cancer by stimulating anti-tumour T cell responses [15, 52, 53]. As a consequence, therapeutic regimens with these inhibitors, alone or in combination with chemotherapy, have shown high response rates with prolonged duration in subsets of melanoma [2-4], renal [5, 6] and lung cancer patients [7-9]. However, for several tumours such as MMR-proficient CRC and PDAC, and for the majority of the aforementioned tumour types, immunotherapy fails to show any clinical benefit [14-17]. In fact, one of the main reasons why immunotherapy rarely exhibits robust responses in PDAC is due to the fact that these tumours are characterized by the low number of infiltrating cytotoxic T cells (generally referred to as non-T cell-inflamed or "cold" tumours). Moreover, growing evidence shows that, even in the presence of an immunotherapeutic intervention, the TME can compromise the functions and fate of tumour-infiltrating immune cells in such a way, as to favour immunological tolerance and reduce anti-tumour effector functions. Indeed, T cells have to compete for limited nutrients within the TME, which undermines their capacity to survive and proliferate [30-39].

The identification of new metabolic pathways and therapeutic targets able to promote both T cell fitness and influx within PDAC is crucial. To do so, we performed a meta-analysis of pre-treatment transcriptomic datasets of patients who were either responsive or non-responsive to ICB with bulk tumour transcriptomic and metabolomic profiling of ICB responsive, low responsive and non-responsive murine tumour models (**Table 1** and **Figure 3.1A-C**). By applying a whole tumour analysis, we were able to highlight metabolic networks that are deregulated in conditions that are as close as possible to the *in vivo* state. This is not the case when isolated or sorted cells are used, since metabolites are highly unstable. Among the altered and clinically relevant metabolic pathways that correlated with resistance to ICB, we have identified CDA as potential target involved in the lack of response to ICB (**Figure 3.1G**). CDA is an evolutionarily conserved enzyme of the pyrimidine salvage pathway responsible for the hydrolytic deamination of free cytidine and deoxycytidine to uridine and deoxyuridine, respectively. In PDAC patients, high expression of CDA at diagnosis is a bad prognostic factor [50] (**Figure 3.1H)**, further supporting the idea that CDA might be a good therapeutic target in PDAC treatment. Since we used whole tumours for the meta-

analysis, we aimed at identifying which tumour compartment accounted for the highest expression of CDA. Both in human pancreatic cancer patients and murine pancreatic tumour model, cancer cells were the cell population with the highest expression of CDA (**Figure 3.1I-J**).

We therefore targeted CDA (genetic deletion and overexpression) in cancer cells to validate that its overexpression is responsible for resistance to ICB in pancreatic cancer. CDA was firstly deleted by an inducible CRISPR/Cas9 system in murine pancreatic cell lines to determine how this enzyme promotes the action of the immune system and responsiveness to immune checkpoint inhibitors *in vivo*. Our results showed that genetic inactivation of CDA sensitized tumours to immunotherapy in a cytotoxic T cell dependent fashion. In particular, deletion of CDA in cancer cells increased the efficacy of ICB in s.c. and orthotopic pancreatic primary tumours (**Figure 3.2A-B**) and metastases (**Figure 3.2C-D**). This decrease in tumour growth and metastases formation is T cell dependent (**Figure 3.3A**), with CDA KO tumours showing a drastic infiltration of activated cytotoxic T cells (**Figure 3.3B-G**). However, when CDA was overexpressed in α-PD-1-sensitive models, a reversion of the phenotype was observed (**Figure 3.2E-F** and **Figure 3.3H**), proving that the activation of the immune system is downstream to CDA inactivation in cancer cells. Our data suggests that the deletion of CDA in cancer cells is able to unleash $CD8^+$ T cell effector functions *per se* or in combination with ICB, depending on the tumour model, and induce the central memory phenotype in circulating and tumour-infiltrating cytotoxic T cells (**Figure 3.4A-D**).

The increase in activated cytotoxic T cells in CDA-null tumours was a consequence of an improvement in their ability to recognize tumours cells, as shown by their higher antigen presentation capacity (**Figure S3.3A**), and consequent phagocytosis by APCs that are then capable of cross-priming and activating naïve $CD8^+$ T cells (**Figures S3.3C** and **3.5A**). The KO of CDA also led to a lower infiltration of TAMs and a switch of their phenotype from immunosuppressive to immunostimulatory (**Figure 3.5B-D**). The depletion of TAMs and cytotoxic T cells confirmed the involvement of these immune populations in the downstream CDA immune response (**Figure 3.5E**).

Mechanistically, we observed that the skewing of TAMs' polarization to a M1-like anti-tumour phenotype, $CD8^+$ T cell infiltration and antitumor effector functions observed upon the deletion of CDA, could be a consequence of an imbalance in the intracellular pools of uridine, and subsequently, UDP and UTP (**Figure 3.6A-D**). Consistent with this observation, differences in pyrimidine pools in the conditioned media might explain the tendency of CDA KO cells to promote the polarization of BMDMs to a M1-like anti-tumour phenotype (**Figure S3.3D**). Although CDA is

an enzyme of the pyrimidine savaging pathway, cancer cells upregulate CDA for uridine production and secretion rather than for DNA synthesis. Besides being building blocks for nucleic acids and essential components of cellular metabolism, nucleotides are also potent signalling molecules when released into the extracellular milieu. The purinergic signalling triggered by the extracellular release of nucleotides is a fundamental immunoregulatory mechanism that can affect chemotaxis, differentiation, immune recognition and effector functions of innate and adaptive immune cells [40]. The harsh TME can hijack this mechanism to orchestrate potent immunosuppressive crosstalk via the engagement of specific purinergic receptors (*i.e.*, adenosine receptors) expressed on tumour-infiltrating immune cells [40, 54]. In particular, the UDP-activated metabotropic receptor P2Y6 is actively involved in the inflammatory regulation of monocytes, macrophages, human plasmacytoid dendritic cells, by modulation of their function and IFN-α production, as well as the activation of Tregs as shown in Graves' disease [40, 55-58]. One can then speculate that a possible purinergic receptor differentially expressed on immune cells (*e.g.* TAMs) can be modulated by the unbalanced uridine derivatives pool and be responsible for the immunostimulatory properties observed in CDA deficient tumours.

Next, we explored the therapeutic potential of CDA in the treatment of cancer, namely in the reactivation of T cell effector functions *per se* and its capacity to overcome resistance to immunotherapy in a clinically relevant model by validating a single agent regimen. We showed that CDA inhibition with THU in combination with immunotherapy drastically reduced the tumour weight and number of mesenteric metastasis in an orthotopic model of pancreatic cancer and restored $CD8^+$ T cell activation (**Figure 3.7A-E**). Although the use of an orthotopic model increases the clinical relevance, in the future we plan to validate our findings in spontaneous pancreatic cancer models, namely the KPC model (*LSL-Kras*$^{G12D/+}$*;LSL-Trp53*$^{R172H/+}$*;Pdx-1-Cre*) to have better histological and genetic translation to human cancer [59]. Finally, we showed an inversed correlation between CDA expression in cancer cells and $CD8^+$ T cell infiltration in PDAC patients (**Figure 3.8A-B)**.

Altogether, the data obtained proves that CDA inhibition in pancreatic tumours can alter the TME in such a way as to favour the recruitment of M1-like anti-tumour TAMs and cytotoxic T cells while also promoting anti-tumour effector functions. Moreover, our bench to bedside approach shows that CDA can be used as a prognostic and therapeutic target in the treatment of cancer, namely to sensitize "cold" and resistant tumours to immunotherapy.

Authors' contributions

The results presented in this chapter are derived from an ongoing project that involves the contribution of other co-authors. Mathieu Pecqueux, Chiara Varamo and I performed the experimental design, all the experiments, data acquisition and interpretation. Fátima Baltazar and Massimiliano Mazzone performed experimental design, helped in the data analysis and conducted scientific direction.

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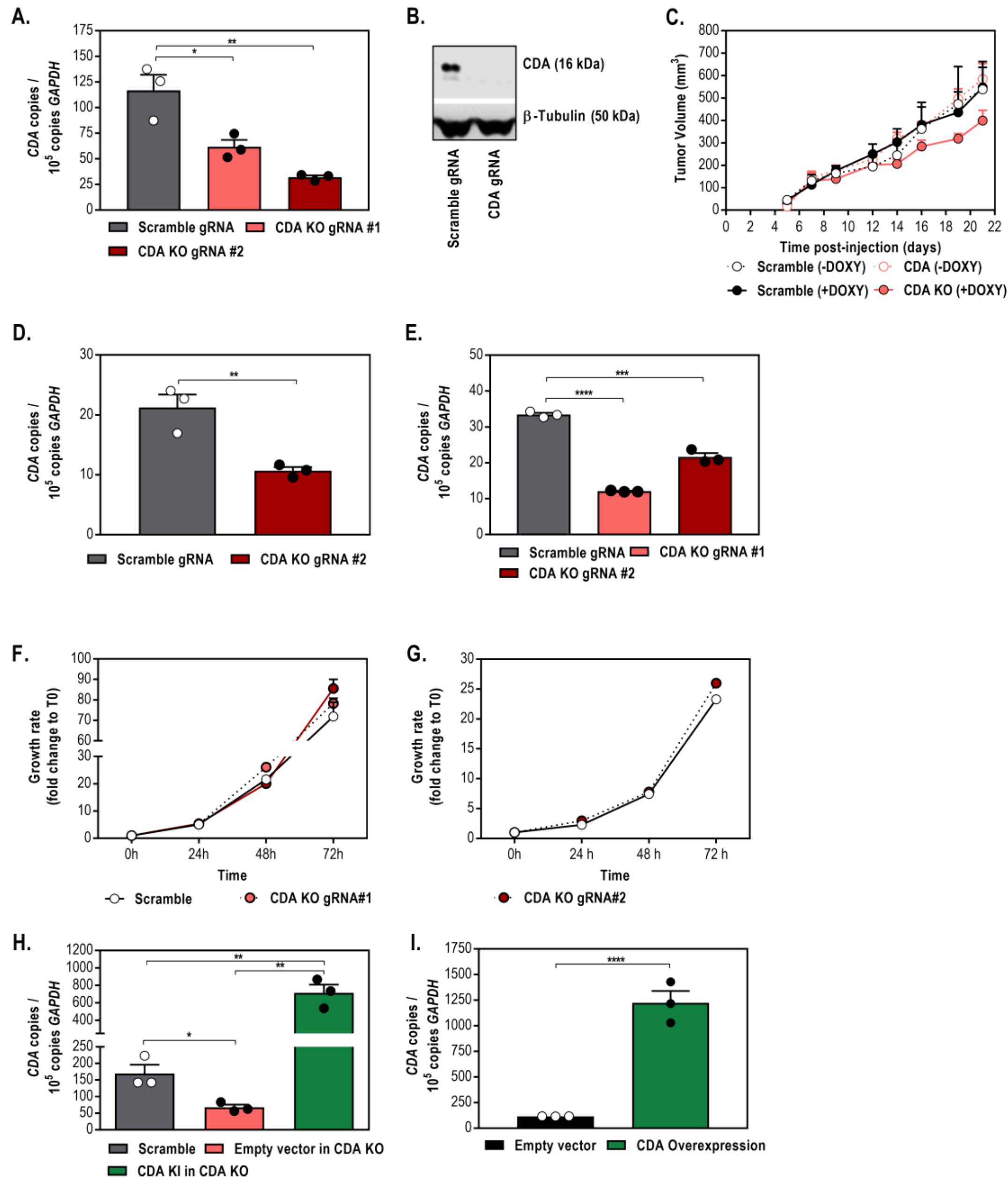
3.6 Supplementary information

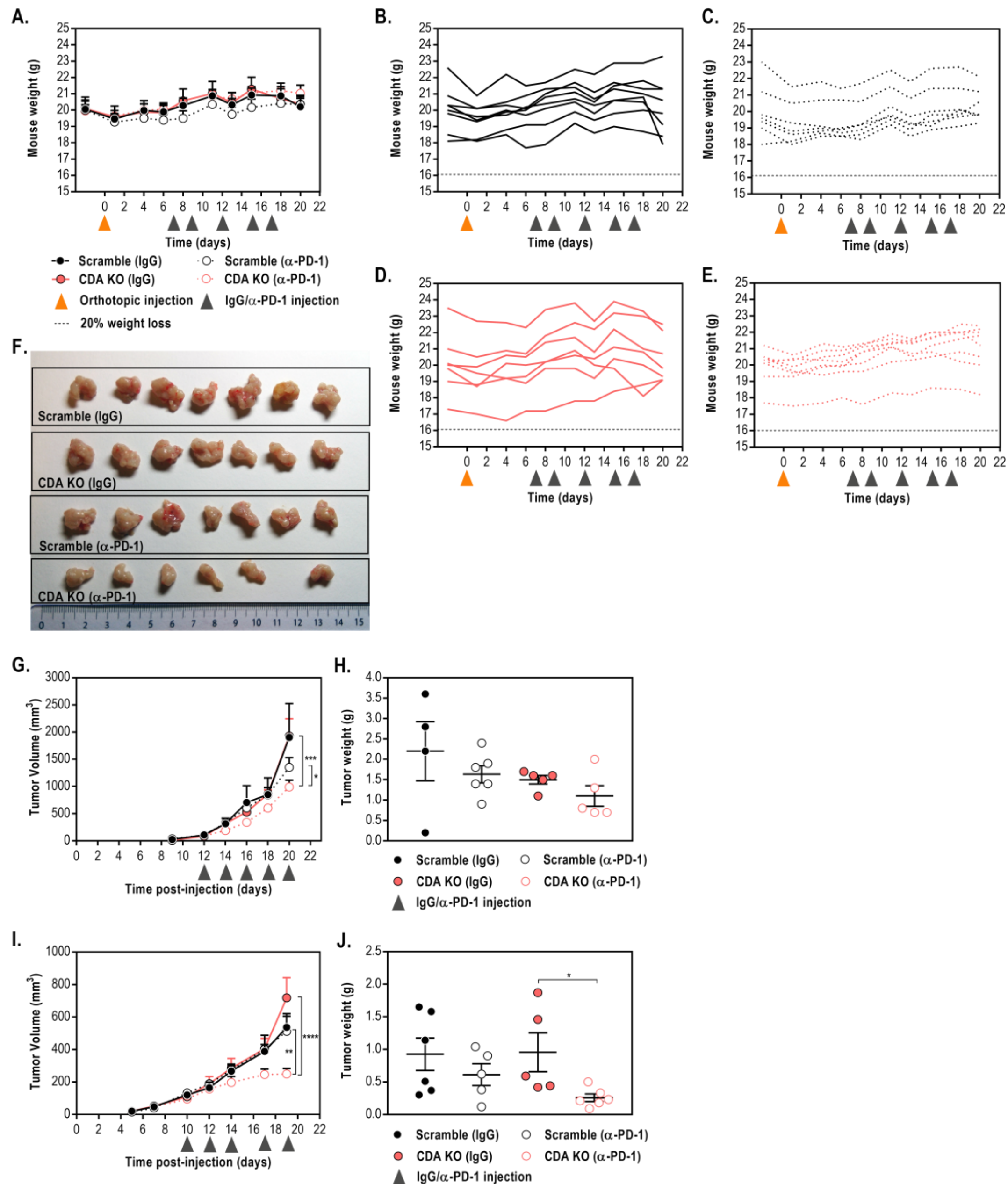
Figure S3.1, related to Figure 3.2.



Doxycycline-inducible CRISPR/Cas9 platform successfully reduces CDA mRNA and protein levels, and tumour growth without affecting the proliferative capacity of cancer cells. (**A**, **B**, **E**, **F**, **I**, **J**) *CDA* transcript abundance in Panc02 cells after transduction with control or two CDA-targeting gRNAs (**A**); in KPC1245 cells after transduction with control or CDA-targeting gRNA (**D**); in YUMM 1.7 cells after transduction with control or two CDA-targeting gRNAs (**E**); and in CDA KO Panc02 (**H**) and MC38 (**I**) cells after transduction with an empty or a CDA-overexpressing vector; (**B**) Western blot of Panc02 cell lysates for CDA and β-Tubulin upon

transduction with control or a CDA-targeting gRNA; (**C**) Subcutaneous Panc02 tumour growth of mice injected with control or CDA-targeting gRNA cancer cells with or without doxycycline induction; (**F**, **G**) Effect of CDA KO on Panc02 (**F**) and KPC1245 (**G**) cell growth rate upon 72h. DOXY, Doxycycline. All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

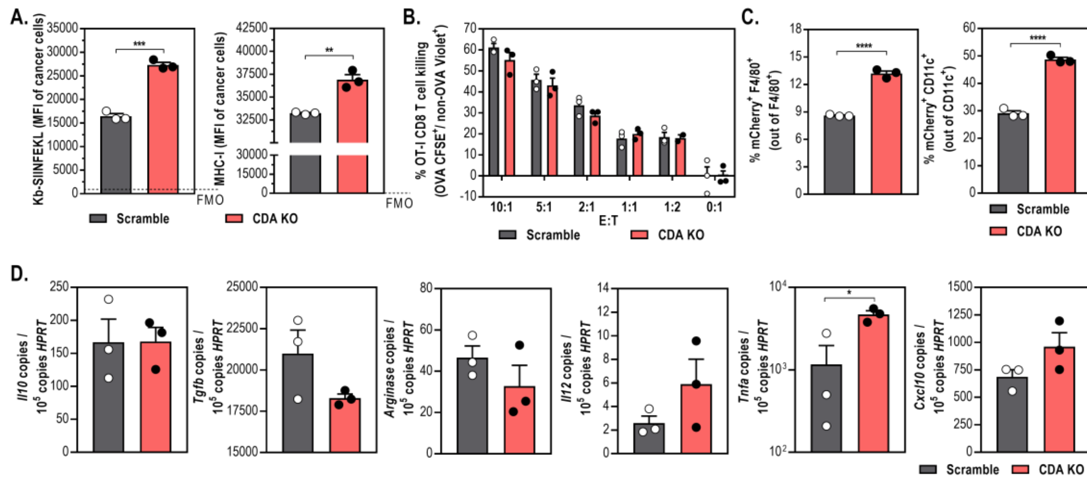
Figure S3.2, related to Figure 3.2



Loss of CDA in cancer cells increases the efficacy of immunotherapy in pancreatic orthotopic and melanomas models. (**A-F**) Orthotopic KPC1245 Scramble and CDA KO mice weight (**A**) and end-stage tumours (**F**) treated with IgG or α-PD-1; Individual weight progression of KPC1245-Scramble (**B**, **C**) and –CDA KO (**D**, **E**) tumour bearing mice treated with IgG (**B**, **D**) or

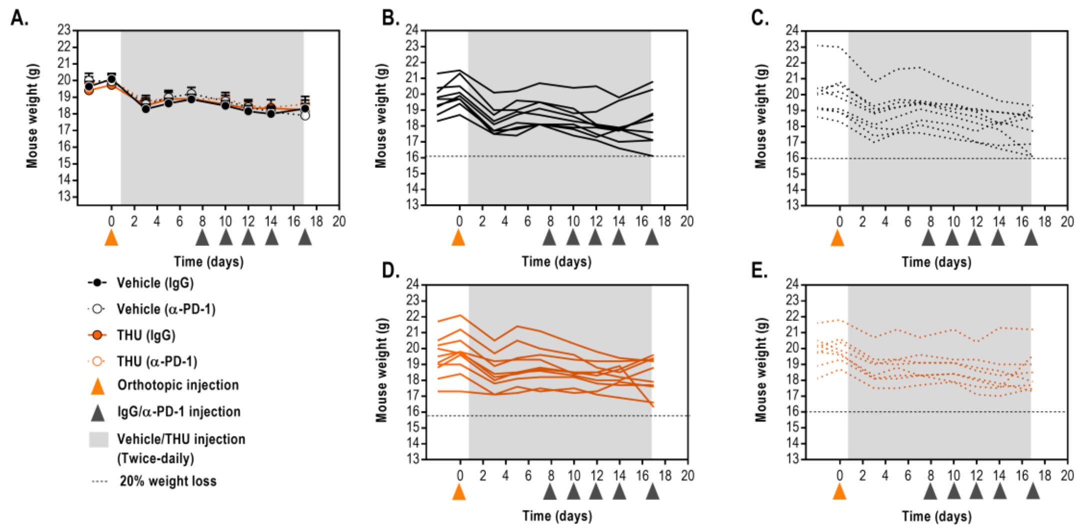
αPD-1 (**C**, **E**); (**G-J**) Orthotopic B16-F10 (**G**, **H**) and YUMM 1.7 (**I**, **J**) Scramble and CDA KO tumour growth (**G**, **I**) and weight (**H**, **J**) treated with IgG or α-PD-1. n=6-8 (**A-F**), n=4-6 (**G**, **H**), n=5-6 (**I**, **J**). Graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

Figure S3.3, related to Figures 3.5 and 3.6



CDA depletion increases cancer cell antigen cell presentation, APC phagocytosis and switches BMDMs signatures towards an anti-tumour phenotype. (**A**) FACS quantification of SIINFEKL-H2K(b) expression in OVA- Scramble and CDA KO Panc02 cancer cells stimulated with SIINFEKL peptide and of MHC-I expression in Scramble and CDA KO Panc02 cancer cells; (**B**) OT-I CD8 T cell killing of Scramble and CDA KO Panc02 cancer cells; (**C**) FACS quantification of BMDMs (Mφ) and BMDCs (DCs) phagocyted mCherry+ Scramble and CDA KO Panc02 cancer cells; (**D**) *IL-10*, *TGF-β*, *Arginase*, *IL-12*, *TNF-α* and *CXCL10* transcript abundance in Mφ cultured with scramble or CDA KO Panc02 conditioned medium. E:T, Effector:Target (**B**). Dashed lines represent FMO controls. All graphs show mean ± SEM. *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

Figure S3.4, related to Figure 3.7



CDA inhibitor treated mice do not show weight loss. (**A**-**E**) Orthotopic KPC1245 mice weight co-treated with Vehicle or THU and IgG or α-PD-1 (**A**); Individual weight progression of KPC1245-tumour bearing mice treated with Vehicle and IgG (**B**), Vehicle and α-PD-1 (**C**), THU and IgG (**D**) and THU and α-PD-1 (**E**). n=8-10 (**A-E**). Graph shows mean ± SEM (**A**). *p<0.05; **p< 0.01; ***p<0.001, ****p<0.0001.

CHAPTER 4. General Discussion

Cancer is a group of more than 100 different diseases characterized by the abnormal growth of a clonal population of cells and their capacity to invade and spread into surrounding tissues and organs [1]. Cancer is a major global socio-economic and public health problem and is expected to be the most important barrier to increasing life expectancy, and the leading cause of death worldwide in the current century [2]. In order to overcome these challenges, new therapeutic options are urgently needed.

Largely dependent on the organ that is affected, the multitude of cancer types and forms display a disconcerting diversity and complexity that is reflected in their genetics, histopathology, effects on systemic physiology, prognosis, and responses to therapeutic intervention. However, they all share a common set of acquired distinctive and complementary functional capabilities, or hallmarks, that support tumour growth and metastatic dissemination [3, 4]. The chronic and uncontrolled cell proliferation that characterizes neoplastic diseases demands a concomitant reprogramming of glycolytic metabolism, a hallmark of cancer, in order to fuel and sustain cell growth and division. Therefore, the influence of energy metabolism on cancer initiation, progression and spread is now widely appreciated and considered an attractive target as proliferating cells show different metabolic requirements from non-proliferating cells [5-7]. In fact, while non-proliferating cells primarily have catabolic demands, proliferating cells engage in distinct metabolic pathways from the tissue of origin in order to balance the divergent catabolic and anabolic requirements crucial for maintaining cellular homeostasis while duplicating cell mass.

The Nobel Prize laureate Otto Warburg introduced the idea of metabolic alterations in cancer cells nearly one century ago, where he observed that even in the presence of adequate oxygen supply, cancer cells showed enhanced glucose uptake and relied on the cytoplasmic fermentation of glucose to lactic acid for energy production, rather than oxidative phosphorylation (OXPHOS) [8]. Known as the “Warburg effect”, this tumour-specific alteration confers a metabolic advantage to the tumour mass compared to normal somatic cells and is a consequence of the necessity to fuel anabolic and catabolic metabolic pathways simultaneously. In fact, the increased glycolytic flux provides intermediate compounds that are diverted and used as the primary source for new building blocks synthesis (lipids, proteins and nucleic acids), necessary for assembling new cells and sustaining cell growth [9, 10]. Thus, the aberrant metabolic behaviour of proliferating cells

presents potential therapeutic opportunities, and there has been growing enthusiasm to study how to best tackle cancer metabolism [11-13].

Understanding not only the metabolic diversity of the tumour but also the metabolic profiles of different tumour types in different tissue locations will be crucial for cancer comprehension and for the development of novel metabolism-focused therapeutics.

The research presented throughout this thesis focused on understanding the influence of cancer metabolic pathways in tumorigenesis and the beneficial role of targeting key players of these pathways to overcome resistance to conventional chemotherapy and immunotherapy in gastrointestinal (GI) cancers, namely in colorectal cancer (CRC) and pancreatic ductal adenocarcinoma cancer (PDAC). CRC is the third most frequent malignancy in terms of incidence and the second most lethal [2]. In spite of the large body of evidence which demonstrate widespread resistance to 5-Fluorouracil (5-FU), this antimetabolite is still recommended as a first line chemotherapeutic agent for the treatment of CRC. Therefore, one of our studies focused on understanding the effect of disruption to the glycolytic flux in CRC cells, namely by inhibition of monocarboxylate transporters (MCTs), in combination with 5-FU. In the second part of the work presented here, and with the focus remaining on therapy resistance, we studied the influence of metabolism in resistance to immune checkpoint blockade (ICB) in PDAC. In particular, we investigated the effect of cytidine deaminase (CDA) deletion, an enzyme involved in the pyrimidine salvage pathway, in the immune system and anti-tumour immunity.

4.1 Monocarboxylate transporters as mediators of 5-fluorouracil cytotoxicity in colorectal cancer

The increased production of lactate through aerobic glycolysis can ultimately lead to cellular acidosis, thus compromising cancer cell survival. Cancer cells counteract cytoplasmic acidification and maintain glycolytic metabolism by the upregulation of specific forms of MCTs, plasma membrane transporters that mediate the efflux of lactate/H^+ from cells but can also be involved in its uptake [14]. MCTs are promising therapeutic targets [14-21]; however, the role of MCTs in CRC cell survival and metabolism is still poorly understood.

In chapter 2 of this thesis, we characterized the dependence of CRC cells on MCT activity for survival, proliferation and maintenance of energetic metabolism and assessed if MCT inhibition could potentiate the cytotoxic effect of 5-FU. The results obtained support the hypothesis that these

lactate transporters are promising targets in future CRC therapies, especially since the isoforms MCT1 and MCT4 are found to be overexpressed in CRC and associated with poor prognosis features [22-27]. The data obtained further corroborates previous *in vitro* and *in vivo* studies in various tumour models using these MCT activity inhibitors [28].

The MCT inhibitors α-cyano-4-hydroxycinnamate (CHC), 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) and quercetin used in this research have been shown to inhibit MCT activity, and throughout the execution of this thesis, other studies corroborated this observation. For example, in human and murine PDAC cells, CHC was shown to inhibit lactate influx and export, cell proliferation, migration and invasion and spheroid outgrowth, induced cell death, inhibited the ERK pathway and stimulated the p38 signalling pathway [29, 30]. In addition, CHC as a monotherapy or in combination with metformin, blocked the autophagic flux and slightly impaired murine pancreatic cancer development [31]. In melanoma cells, CHC inhibited lactate-induced motility and invasiveness [32]. Yao Hu and collaborators demonstrated that quercetin effectively suppressed cell proliferation and promoted apoptosis via a significant decrease in the activity of MCT1 in bladder cancer [33]. More recently, quercetin was shown to induce intracellular acidification in a mouse model of glioblastoma multiforme [34]. Despite the use of these compounds as MCT inhibitors, they inhibit MCT activity with different affinities and specificities [35, 36]. For example, CHC, DIDS and Quercetin inhibit MCT2 with a higher affinity than MCT1, and MCT4 exhibits a much lower affinity for most substrates and inhibitors than MCT1 [37-40]. Additionally, these compounds can have other targets besides MCTs: for example, CHC is a strong inhibitor of the mitochondrial pyruvate carrier [41], and DIDS inhibits the activity of the chloride/bicarbonate exchanger AE1 [36]. Therefore, it cannot be excluded that the results observed with these compounds are due to inhibition of other cell targets and the individual role of MCT isoforms in cancer cannot be determined by using these drugs. Thus, we performed downregulation of MCT1 and MCT4 expression with specific siRNAs. Overall, the effects of MCT1 or MCT4 expression inhibition were similar to inhibition of MCT activity, confirming the pivotal role of MCT isoforms 1 and 4 in the maintenance of CRC survival and glycolytic metabolism.

Several specific inhibitors with higher affinity for MCTs have been developed and their efficacy has been assessed in the preclinical setting. The anti-inflammatory agent bindarit was described as a potent and highly selective non-competitive MCT4 inhibitor [42]. AstraZeneca's MCT1 and MCT2 inhibitors AR-C155858 and AZD3965 were shown to influence lactate transport and reduce lung tumour growth [43-45]. AZD3965, a MCT1 specific inhibitor, is currently being evaluated as

an anticancer agent in Phase I/II clinical trials for patients with solid tumours, prostate cancer, gastric cancer, and diffuse large cell B lymphoma (NCT01791595). Unfortunately, knockdown or selective inhibition of MCT1 or MCT4 isoforms does not lead to tumour cell death despite inducing tumour growth arrest or increasing chemosensitivity and radiosensitivity [46]. This is a consequence of their functional redundancy. Additionally, simultaneous targeting of MCT1 and MCT4 can induce metabolic plasticity of cancer leads, resulting in a shift from aerobic glycolysis to OXPHOS. Due to the simultaneous usage of anabolism and catabolism in cancer cells, targeting one pathway is not sufficient to stop tumour growth [47]. The metabolic plasticity and dichotomy of CRC cells upon MCT genetic and pharmacological inhibition was not fully addressed in this study. One can assume that the low induction of cell death observed might be a consequence of their capacity to shift between metabolic pathways. In order to identify differences between the metabolic profiles of control and MCT inhibited cancer cells, untargeted metabolomics and stable isotope labelling (e.g. $^{13}C_6$-glucose) should be performed. Moreover, rational drug combinations including MCT1/4 inhibition and OXPHOS blockade (e.g. metformin or phenformin), or the use 7-(N-benzyl-N-methylamino)-2-oxo-2H-chromene-3-carboxylic acid (7ACC2), which was shown to block both the extracellular uptake of lactate and mitochondrial pyruvate transport, could also be used since they are the most promising approaches to induce metabolic catastrophe, leading to tumour cell death [46, 48, 49].

5-FU is an antimetabolite drug used to treat several malignant tumours, including breast, pancreatic, skin, stomach, oesophageal, and head and neck cancers. Despite the progress made with the development of novel cancer therapies, 5-FU is one of the most effective and most commonly used chemotherapeutic agents in CRC treatment and the main constituent of chemotherapy combination regimens [50]. However, the efficacy of 5-FU-based therapy is frequently compromised by the development of chemoresistance. For instance, almost half of the patients diagnosed with metastatic CRC are resistant to 5-FU-based chemotherapy. Up to 40% of patients receiving 5-FU-based adjuvant chemotherapy following the resection of stage II and III colon cancer experience recurrence or die within 8 years of follow-up [50-53]. In this chapter, we assessed the possibility of using MCT inhibition as a gateway to overcome 5-FU resistance. We showed for the first time in a CRC model that MCT activity and expression inhibition potentiates the cytotoxic effect of 5-FU. These results suggest that MCT activity inhibitors sensitize CRC cells to conventional therapy by arresting the glycolytic flux through inhibition of lactate transport. The mechanism by which the efficacy of 5-FU is increased via glycolytic metabolism arrest in tumour

cells, namely lactate transport inhibition, remains poorly understood. However, several studies have demonstrated an association between 5-FU resistance and exacerbated aerobic glycolysis. 5-FU resistant cells show higher glucose uptake and lactate production, and overexpression of glycolytic enzymes in human hepatic [54], colon [55] and cervical cancer cells [56]. Moreover, in a study using *PIK3CA* mutant and wild-type gastric cancer cells, the authors described higher resistance to 5-FU when cells were cultured with lower concentrations of glucose [57]. Consequently, targeting of enzymes involved in this metabolic pathway has been proven to sensitize 5-FU-resistant cancer cells to this chemotherapeutic agent. Specific inhibition of glucose transporter GLUT-1 [55], targeting of pyruvate dehydrogenase kinase-1 with the specific inhibitor dichloroacetate [58], MCT1 inhibition [59], pyruvate kinase isoenzyme M2 suppression [60], inhibition of glycolysis with 3-bromopyruvate and 2-Deoxy-D-glucose and use of methylglyoxal, an anti-carcinogenic that affects glycolysis and mitochondrial respiration [61], sensitized different cancer types to 5-FU both *in vitro* and *in vivo*. Taken together, these studies support the use of glycolytic inhibitors as a pre-treatment or in combination with 5-FU for novel therapeutic protocols to overcome chemotherapy resistance.

Metabolism offers a unique opportunity to identify druggable enzymes to sustain anti-tumour responses [13]. However, the translation from *in vitro* to *in vivo* has limited the success of target identification in cancer metabolism. One factor that helps to explain this unsuccessful translation is the fact that metabolism cannot be addressed as single cell behaviour but rather a network and cross-talk between different cells within the tumour mass. Cell-cell lactate shuttle is a well-documented example of metabolic symbiosis and an expression of tumour flexibility. Despite its early portrayal as metabolic waste, lactate is pivotal in a complex feedback loop and can be used by other cells to obtain the remaining bioenergetic/biosynthetic potential contained in it [62]. To address the influence of MCT inhibition in this complex metabolic symbiosis, advanced cell models that recapitulate all of the features of tissues *in vivo*, including tissue organization, stiffness, and metabolite and oxygen gradients should be considered. For example, three-dimensional culture models such as spheroids, biomimetic 3D models or organoids [63-65] and CRC mouse models, coupled with stable isotope labelling of glucose and lactate can be valuable tools to investigate how MCT inhibition affects cancer metabolism.

4.2 Cytidine deaminase as a mediator of immune checkpoint therapy resistance in pancreatic adenocarcinoma cancer

In order to prevent inflammatory tissue damage and autoimmune diseases, the extent and magnitude of immune responses have to be tightly regulated by a fine-tuned balance between co-stimulatory and inhibitory signals. Immune checkpoints are inhibitory signals that allow the immune system to respond to a threat while protecting tissues from any unintended harm that may derive from this action by controlling the type, magnitude, and duration of the immune response. Activated T cells express multiple co-inhibitory receptors such as PD-1 and CTLA-4. While CTLA-4-mediated immune checkpoint is induced in T cells at the time of their initial response to antigen and occurs in secondary lymphoid organs, PD-1 inhibitory signalling occurs to regulate inflammatory responses in tissues by effector T cells recognizing antigen in peripheral tissues, including tumours. However, transformed cells dampen the T cell response by expressing some of these immune-checkpoint proteins (e.g. PD-1 ligand, PD-L1), thus promoting a permissive immune surveillance allowing tumour growth and expansion [66]. Immune checkpoint blocking antibodies or recombinant forms of ligands reinvigorate exhausted T cells at the tumour site and enhance immunity activity against tumour cells, respectively. Consequently, blockade of CTLA-4, PD-1 or PD-L1 has shown clinical potential in melanoma, renal cancer, lung cancer, lymphoma, head and neck squamous cell cancer, bladder cancer, liver cancer, gastro-oesophageal cancer and microsatellite instability high (MSI-H) CRC patients [67-78]. However, in tumours such as microsatellite stable (MSS) and microsatellite instability low (MSI-L) CRC and pancreatic cancer, immunotherapy failed to show any clinical benefit [79, 80]. In non-inflamed tumours such as PDAC, this lack of response can be explained by the absence of pre-existing cancer-specific T cells [81, 82]. However, the absence of immune infiltration in solid tumours is unlikely due to lack of antigens [83]. The proliferative and energetically-deregulated nature of tumour cells results in a nutrient deprived tumour microenvironment (TME) that creates "metabolic checkpoints" for tumour-infiltrating T cells, impacting their capacity to survive, proliferate and function properly [84]. This metabolic competition, and the crosstalk between different cell populations within the TME, determines the pro- or anti-inflammatory function of immune cells and the inherent efficiency of immunotherapies. Therefore, it is necessary to identify metabolic pathways within the TME that are responsible for T cell exclusion, immunosuppression and resistance to immunotherapy.

In chapter 3 of this thesis, we set-up and performed a meta-analysis where we integrated pre-treatment transcriptomic data of patients who responded and did not respond to immune checkpoint inhibitors with bulk tumour transcriptomic and metabolomic profiling of responsive, low responsive and non-responsive murine tumour models. The integration of human transcriptomics with murine transcriptomic and metabolomic datasets from multiple tumours allowed the identification of metabolic fluctuations and networks in the TME that are conserved and can be studied in animal models, are independent of a variety of genetic and environmental confounders (which could be tumour type specific), and that functionally affect metabolite concentrations (an aspect that is not ascertainable in human samples). In addition, the inclusion of multiple human and murine cancer types allows for the identification of targets that are consistently associated with clinical resistance to immunotherapy across tumour types, resulting in mechanisms that are tumour-type independent. With this approach we moved far beyond the current concept of targeting the metabolism of cancer cells [85] but rather tried to hinder metabolic crosstalk within the TME in order to trigger the activation of the immune system. Our approach allows for the identification of new immunotherapy targets in novel or unexpected pathways. A recent study by Manguso *et al.* [86] identified new immunotherapy targets involved in resistance to ICB. By using functional genomics screening to interrogate genes that regulate the sensitivity (or resistance) of tumour cells to PD-1 blockade, the authors identified genes such as the phosphatase *Ptpn2*, as well as genes involved in TNFα/NF-kB signalling, antigen processing, the ubiquitin proteasome pathway, and double-stranded RNA sensing as playing a role in the adaptive antitumor response.

Among the altered and clinically relevant metabolic pathways that correlated with resistance to ICB, we have identified CDA as potential target. CDA is an evolutionarily conserved enzyme of the pyrimidine salvage pathway responsible for maintaining the cellular pyrimidine pool. Besides catalysing the irreversible hydrolytic deamination of free cytidine and deoxycytidine to uridine and deoxyuridine, respectively, this enzyme also catalyses deamination of pyrimidine analogues such as gemcitabine, capecitabine, cytarabine or azacytidine, used to treat both solid and liquid cancers. These antimetabolites interfere directly with DNA or RNA synthesis and with cancer cell proliferation by blocking their capacity to synthesize precursors of nucleic acids [87]. Our results show that genetic inactivation of CDA in cancer cells with an inducible CRISPR/Cas9 system or pharmacological inhibition with tetrahydrouridine (THU), sensitized ICB resistant tumours to this immunotherapy in subcutaneous and orthotopic pancreatic primary tumours and metastases as well as in orthotopic melanoma models. The phenotypic corroboration in different models

anticipates a broad application as a novel combinatorial therapy. Although induction of Cas9 nuclease with doxycycline should have been performed in *vivo* in order to represent a more clinically relevant setting, the results obtained with the CDA inhibitor THU show the therapeutic potential of this enzyme in the treatment of cancer, namely to revert resistance to ICB.

Conversely, the re-introduction or overexpression of CDA in α-PD-1-responsive models reverted this phenotype. This proves that the action of the immune system is downstream to CDA inactivation in cancer cells. The reduction in tumour growth and metastases formation is T-cell dependent, since we observed in different models that CDA inactivation is able to unleash activated cytotoxic T cell infiltration. This was translated into human PDAC patients, where we observed a higher infiltration of $CD8^+$ T cells in tumours with low CDA expression. Our data suggests that the inactivation of CDA in cancer cells is able to turn excluded or “cold” tumours into “hot” or inflamed tumours. This is particularly exciting in PDAC, since most cases display low levels of T cell infiltration along with low PD-L1 expression and consequently poor objective responses to single-agent immune therapies [88-90]. Since the patients did not receive preoperative chemo/radio-therapy, the question that remains to be answered is whether those cases with low CDA expression and high $CD8^+$ T cell infiltration will benefit from ICB therapy. Nonetheless, high CDA expression at diagnosis is a factor of poor prognosis in PDAC [91].

In addition to an increase in cytotoxic T lymphocyte infiltration, we observed a skewing of tumour-associated macrophages (TAMs) towards a M1-like anti-tumour phenotype that were capable of cross-priming and activating naïve $CD8^+$ T cells. In fact, accumulating evidence indicate that TAMs are one of the major immune suppressor cell populations in solid tumours and pharmacological interventions of TAM accumulation and/or function are promising strategies to improve the outcomes of checkpoint therapies. For example, monoclonal antibodies or small molecule inhibitors against colony-stimulating factor 1 receptor (CSF1R), which is essential for the recruitment, differentiation, and survival of TAMs [92], was shown to impair tumour development and progression by reducing the number of TAMs and/or changing the phenotype of TAMs towards a M1-like polarization state [93-96]. In particular, CSF1R inhibition in a pancreatic cancer model improved checkpoint therapies, not only by depletion of TAMs, but also by reducing their expression of suppressive molecules [96]. TAM depletion with PLX3397, an inhibitor of CSF1R, was shown to enhance $CD8^+$ T cell migration and infiltration into tumour islets, and to boost α-PD-1 therapy, as observed by an increase of $CD8^+$ T cells in close contact with tumour cells and arrest of tumour growth [97]. Similarly, inhibition of TAM’s immunosuppressive arginase-1 molecule combined with

ICB suppressed tumour growth and metastases formation in different tumour models [98]. Additionally, inhibition of class IIa histone deacetylases, enzymes that regulate the activity of many transcription factors [99], interferes with the immune suppressive features of macrophages within the tumour and synergizes with ICB [100]. Cytokines such as IL-10 regulate macrophage polarization to an immunosuppressive phenotype and induce key macrophage receptors including toll-like receptors, Fc receptors (e.g., FcγR), and macrophage receptor with collagenous domain [101]. Recent published data pointed to these receptors as targets for the improvement of checkpoint therapies [102-104].

We also observed an induction of a central memory phenotype in circulating and tumour-infiltrating cytotoxic T cells in inactivated CDA tumours. Consensus is emerging that effector memory T cells only provide short-lived protection compared with central memory T cells in cancer models [105-107]. Therefore, CDA targeting, by enhancing the longevity of T cells, has the potential to broaden its applicability in the clinic. These data also prove that CDA knockdown is not causing the generation of neoantigens as re-boost and growth of WT Panc02 cells in mice that have carried CDA-deficient tumours and thus have developed a T cell memory was equally inhibited.

Mechanistically, we observed that skewing of TAM polarization to a M1-like anti-tumour phenotype upon the knockdown of CDA could be a consequence of an imbalance in the intracellular pools of uridine, and subsequently, UDP and UTP. Nucleic acids are potent signalling molecules when released into the extracellular milieu and the purinergic signalling triggered is a fundamental immunoregulatory mechanism that can affect chemotaxis, differentiation, immune recognition and effector functions of innate and adaptive immune cells [108]. The TME can hijack this mechanism to orchestrate potent immunosuppressive crosstalk via engagement of specific purinergic receptors expressed on tumour-infiltrating immune cells [108, 109]. UDP is a high affinity ligand for the metabotropic receptor P2Y6 that is expressed at very high levels in macrophages. Binding of UDP to P2Y6 leads to macrophage activation [110]. It is described that P2Y6 receptor activation by UDP elicits intracellular calcium responses [111-116] and Tedesco *et al.* [117] recently demonstrated that calcium uptake in mitochondria is instrumental for M2-like macrophage polarization. The fact that we observed lower release of UDP into the extracellular milieu of CDA KO tumours can explain the lower M2-like TAM infiltration and polarization and consequently the improved immune surveillance in CDA deficient tumours. To confirm this hypothesis, one has to assess the expression levels of P2Y6 in M1- and M2-like sorted TAMs and confirm the polarization of TAMs to a M2-like

phenotype in the presence of UDP supplementation, or alternatively assess if P2Y6 receptor blockade sensitizes PDAC tumours to α-PD-1 in a similar fashion as CDA targeting.

Overall, our findings show that cancer cell metabolism is an attractive target to overcome resistance to conventional chemotherapy and immunotherapy. In this thesis we have shown that MCT activity is instrumental for CRC survival and MCTs can be used as molecular targets for CRC treatment, alone or in combination with 5-FU. We also proved that CDA is a novel metabolic enzyme involved in ICB resistance. Its inhibition in pancreatic tumours altered the TME in a way to favour the recruitment of M1-like anti-tumour TAMs and cytotoxic T cells and promoted anti-tumour effector functions. Moreover, our translational approach showed that CDA can be used as a prognostic and therapeutic target in the treatment of cancer, namely to sensitize “cold” and resistant tumours to immunotherapy.

4.3 References

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