Tiago Roberto Gomes Pestana

Licenciado em Ciências Biomédicas

Methionine supplementation improves the efficacy of breast cancer immunotherapy

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Dr. ª Karine Marie Serre, Investigadora Pós Doutoral, Instituto de Medicina Molecular, FMUL

Co-orientador: Mestre Sofia Mensurado Santos, Investigadora, Instituto de Medicina Molecular, FMUL

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TABLE OF CONTENTS

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RESUMO

O cancro de mama triplo negativo (TNBC) é um subtipo agressivo de cancro de mama clinicamente difícil de tratar, devido à falta de terapias direcionadas. A baixa especificidade e as altas taxas de recidiva associadas à quimioterapia têm incentivado o uso da imunoterapia como um tratamento alternativo, no entanto, as taxas de resposta à imunoterapia com o anti-PD-1 ainda são baixas. A fim de melhorar a eficácia atual da imunoterapia em TNBC, a suplementação de metionina - um aminoácido com propriedades antioxidantes – foi proposta para diminuir a imunossupressão tumoral e aumentar a resposta anti tumoral das células T. Para testar esta hipótese, a suplementação *in vivo* de metionina foi combinada com a imunoterapia anti-PD-1 para tratar ratinhos com TNBC. Nesta tese, mostramos que a suplementação de metionina melhora a eficácia do tratamento anti-PD-1 no modelo tumoral E0771 de TNBC, através do atraso do crescimento tumoral. Além disso, foi observado uma acumulação de linfócitos infiltrantes de tumor (TILs), tais como células T CD8 e T γδ em ratinhos tratados com anti-PD-1 e metionina. A análise de ratinhos TCRδKO com tumor revelou um possível papel das células T γδ na indução da acumulação de células T CD8 no tumor, pois o número de células T CD8 deixou de estar aumentado após o tratamento combinado nesta estirpe de ratinhos. Além disso, observámos uma diminuição de células T CD8 produtoras de TNFα e IFNγ em ratinhos TCRδKO tratados com anti-PD-1 e metionina, sugerindo um papel fundamental das células T γδ na potencialização de respostas de células T CD8 anti tumorais na presença de metionina e do inibidor de PD-1. Coletivamente, esta tese mostrou, pela primeira vez, que a suplementação de metionina em combinação com o anticorpo anti-PD-1 pode exercer um papel protetor na imunidade tumoral. Mais estudos são necessários para entender os mecanismos moleculares por detrás deste efeito positivo da suplementação de metionina, e para avaliar uma potencial aplicação desta combinação em pacientes com TNBC.

Palavras-chave: Imunoterapia, metionina, PD-1, terapia de combinação, stress oxidativo, TNBC

ABSTRACT

Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype which is clinically difficult to treat, due the lack of targeted therapies. The low specificity and high relapse rates associated to cytotoxic chemotherapy have encouraged the use of immunotherapy as an alternative treatment option, however, overall response rates to anti-PD-1 immunotherapy are still low. In order to improve the current TNBC immunotherapy efficacy, we thought to use supplementation of methionine - an amino acid with antioxidant properties - to decrease the tumour immunosuppression and increase anti-tumour T cell response. To test our hypothesis, *in vivo* supplementation of methionine was combined with anti-PD-1 immunotherapy to treat TNBC-bearing mice. In this master thesis, we show that methionine supplementation improves anti-PD-1 treatment efficacy in E0771 TNBC tumour model by delaying the tumour growth. Furthermore, an accumulation of tumour-infiltrating lymphocytes (TILs), such as CD8 and γδ T cells was observed in anti-PD-1 and methionine treated mice. Analysis of tumour-bearing TCRδKO mice revealed a possible role for γδ T cells in inducing CD8 T cell accumulation within the tumour, since CD8 T cell number was no longer increased upon combined treatment in this mouse strain. Moreover, we observed a decreased frequency of TNFα and IFNγ producing CD8 T cells in anti-PD-1 + methionine treated TCRδKO mice, thus suggesting a key role of γδ T cells in potentiating anti-tumour CD8 T cell responses in the presence of PD-1 inhibitor and methionine. Collectively, the work presented in this thesis showed, for the first time, that methionine supplementation in combination with anti-PD-1 antibody can exert a protective role in tumour immunity. Further studies are required to understand the molecular mechanisms behind this positive effect of methionine supplementation, and to evaluate a potential application of this combination in TNBC patients.

Keywords: Immunotherapy, methionine, PD-1, combination therapy, oxidative stress, TNBC

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION LIST

ACT	Adoptive T cell therapy
AP-1	Activator protein 1
APCs	Antigen presenting cells
Arg2	Arginase-2
ATP	Adenosine triphosphate molecule
BATF	Basic leucine zipper transcriptional factor ATF-like
BHMT	Betaine-homocysteine methyltransferase
CAR-T	Chimeric antigen receptor T cell
CBS	Cystathionine b-synthase
CD	Cluster of differentiation
CGL	Cystathionine g-lyase
CR	Complete response
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCL	CXC chemokine ligand
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DMG	Dimethylglycine
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyltransferases
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
Foxp3	Forkhead box P3
FSC	Forward scatter
GCN2	General control nonderepressible 2
GSH	Glutathione
HER2	Human epidermal growth factor 2
HMTs	Histone methyltransferases
i.p.	Intraperitoneal
i.t.	Intra-tumour
iCOS	Inducible T-cell costimulator
IDO	indoleamine-2,3-dioxygenase
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
Iono	Ionomycin

ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
LAG3	Lymphocyte-activation gene 3
mAb	Monoclonal antibody
MAT	Methionine adenosyltransferase enzyme
mCRPC	Metastatic castrate-resistant prostate cancer
MDSC	Myeloid-derived suppressor cells
Met	Methionine
MHC	Major histocompatibility complex
MMP9	Matrix metallopeptidase 9
MS	Methionine synthase
NCR	Natural cytotoxic receptor
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
NK	Natural killer
NKG2D	Natural killer group 2 member D
NKT	Natural killer T cells
ORR	Overall response rate
PBS	Phosphate buffered salt
PCR	Polymerase chain reaction
pCR	Pathological complete response
PD-1	Programmed cell death-1
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PR	Progesterone receptor
PTPs	Protein tyrosine phosphatases
RAG2	Recombinant activating gene 2
ROS	Reactive oxygen species
rpm	Rotations per minute
RPMI	Roswell park memorial institute medium
s.c.	Subcutaneous
SAH	Adenosylhomocysteine
SAHH	Adenosylhomocysteine hydrolase
SAM	S-Adenosylmethionine
SLC	Solute carrier
TAA	Tumour-associated antigens
T_{CM}	Central memory T cells
TCR	T cell receptor
TDSFs	Tumour-derived soluble factors

T_{EM}	Effector memory T cells
TGF-β	Transforming growth factor β
Th	T helper
THF	Tetrahydrofolate
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TILs	Tumour-infiltrating lymphocytes
TIM3	T cell immunoglobulin and mucin domain-containing protein 3
TNBC	Triple negative breast cancer
TNFα	Tumour necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor
WT	Wild-type
ZAP70	Zeta-chain-associated protein kinase 70

INTRODUCTION

1. Cancer immunoediting

Tumours derive from transformed cells as a consequence of genetic modifications mostly caused by radiation exposure, viral infections, chronic inflammation or even inherited genetic mutations (Schreiber, Old, & Smyth, 2011). Fortunately, not all transformed cells lead to tumour development. There is an immune mechanism that prevents malignant cell outgrow which is called **tumour immune surveillance**. This mechanism consists in the ability of the immune system to specifically recognize or even eradicate tumour cells on the basis of their expression of tumour-specific or tumour-associated antigens or soluble molecules released within the tumour microenvironment (Swann & Smyth, 2007). Nonetheless, the immune system can promote or select tumour variants with low immunogenicity thereby providing a mechanism of **tumour immune escape**, which facilitates the tumour growth (Beatty & Gladney, 2014). Thus, immune cells play a dual role in cancer: preventing/controlling as well as shaping/promoting cancer by a process called **cancer immunoediting** which emphasizes the dual host-protective and tumour-promoting actions of immunity during the course of cancer development/progression.

Cancer immunoediting is a dynamic process that, in its most complex form, proceeds sequentially through three different phases: elimination, equilibrium and escape, as described in Figure 1. The **elimination** phase is the same process described by the tumour immune surveillance, in which innate and adaptive immune cells work together in order to eradicate emerging tumours. Indeed, there are some studies with genetically modified mice that support this theory. Immunodeficient mice (deficient for anti-tumoral effector molecules or immune cell types) displayed increased spontaneous tumour development compared with that seen in wild-type mice (Shankaran et al., 2001; Smyth et al., 2000; S. E. A. Street, Trapani, Macgregor, & Smyth, 2002). Importantly, the elimination phase can either lead to complete or to partial tumour clearance. If tumour cells are not completely eliminated by the immune system, they may then enter the **equilibrium** phase that is characterized by a balance between the tumour immune responses and the tumour outgrowth. During this phase, residual tumour cells can either stay dormant or accumulate further genetic alterations that ultimately can impair tumour recognition by immune cells through the modulation of tumour-specific or tumour associated antigen expression. Indeed, if immune cells fail to recognize and eliminate the tumour, a selection of low immunogenic tumour cells is generated, and these cells are more able to resist, avoid or supress the anti-tumour immune response, leading to the **escape** phase. In this phase, the lack of immune recognition, increased tumour-induced immunosuppression and dysfunction of the immune system leads to cancer immune evasion. Thus, when tumours reach the escape phase, the

immune system can no longer contain the tumour overgrowth (Schreiber et al., 2011; Swann & Smyth, 2007; Teng, Galon, Fridman, & Smyth, 2015).

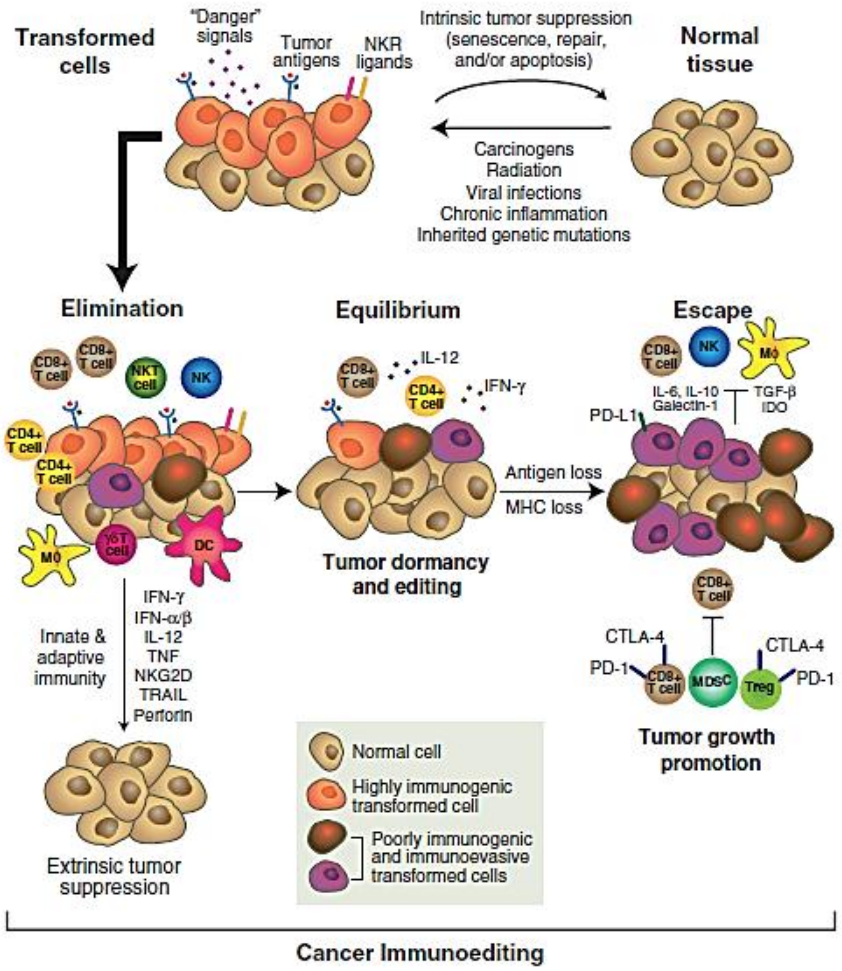


Figure 1. Cancer immunoediting. From Schreiber, 2014

1.1. Tumour-infiltrating lymphocytes (TILs) and their function in tumour growth

In several solid tumours, **tumour-infiltrating lymphocytes (TILs)** are commonly observed in the tumour microenvironment at early stages of development, reflecting the dynamic process of "cancer immunoediting". Presence of TILs in the tumour site reflects the tumour immunogenicity and is closely related to the patient's ability to generate an immune response against cancer (Lechner et al., 2013). Thus, a quantification of lymphocyte infiltration can have a potential prognostic and predictive significance in some solid tumours, suggesting that TILs might be involved in tumour progression, either promoting or preventing tumour growth (Barnes & Amir, 2017). In fact, different types of lymphocytes, displaying diverse effector functions, infiltrate tumours. The immunostimulatory or immunosuppressive effects of these lymphocytes in the tumour context will be individually described below.

On one hand, some lymphocytes display **anti-tumour effector functions** and therefore are responsible for a strong anti-tumour immune response that leads to tumour elimination. Most anti-tumour responses of lymphocytes are mediated by the production of several molecules, such as IFNγ, perforin and granzyme which endow TILs with the ability to kill cancer cells. **IFNγ** production occurs at the early stages of immune responses by innate immune cells like NK, NKT and γδ T cells (Frucht et al., 2001; Schroder, Hertzog, Ravasi, & Hume, 2004). IFNγ is a potent anti-tumour cytokine, and its presence in the draining lymph nodes facilitates the differentiation of T helper type 1 lymphocytes (Th1 cells) which in turn further increases the levels of IFNγ, thus contributing to potentiate even more the CD8 T cell cytotoxicity. Moreover, IFNγ is known to induce the production of several chemoattractants, such as CXCL9, CXCL10 and CXCL11, that are responsible, in the tumour microenvironment, to recruit other immune effector cells, such as Th1 and NK cells (Kursunel & Esendagli, 2016; Nagarsheth, Wicha, & Zou, 2017). The tumour killing capacity of CD8, NK, NKT and γδ T cells is mediated by the release of cytotoxic granules that enter into the cytoplasm of target cells and induce cell death. The major lytic proteins of those granules are perforin and granzymes. **Perforin** is a protein that forms multiple pores in the target cell's plasma membrane and facilitates the movement of granzymes into the target cell cytoplasm. **Granzymes** are serine proteases with the capacity to target cellular substrates that lead to apoptosis. Granzyme A promotes the degradation of the single-strand DNA by increasing DNase activity and weakens the structural integrity of the nucleus by targeting lamins A-C. Otherwise, granzyme B directly activates the effector caspases (3 and 7) that leads to proteolysis of several caspase substrates which result in an efficient programmed death of the target cell (Boivin, Cooper, Hiebert, & Granville, 2009; Cullen, Brunet, & Martin, 2010). **$CD8^+$ cytotoxic T lymphocytes (CTLs)** are the main anti-tumour immune cells that efficiently kill tumour cells through the production of cytotoxic molecules such as perforins and granzymes but also through the extracellular expression of FasL which induces apoptosis in target cells expressing the Fas receptor. (Hassin, Garber, Meiraz, Schiffenbauer, & Berke, 2011). Moreover, the increased IFNγ production by CTLs further increase the expression of MHC class I molecules by tumour cells (D. Street et al., 1997), thus turning them more susceptible to be eliminated by cytotoxic CD8 T cells, since the mechanism of tumour recognition by CTLs is mediated by the interaction between MHC-I and TCRαβ (Schirrmacher, Schild, Gückel, & Von Hoegen, 1993). $CD4^+$ T helper lymphocytes (Th cells) are heterogeneous cytokine-secreting lymphocytes, where each CD4 Th subtype modulates the immune response differently through the production of specific cytokines. The most studied ones are Th1, Th2 and Th17 cells of which the **Th1 cells** are the main participants in anti-tumour immune responses. Upon MHC class II-mediated tumour antigen encounter, Th1 cells can potentially produce large amounts of IFNγ and TNFα (tumour necrosis factor a) that promote CD8 T cell activation and growth, thus contributing to increase anti-tumour responses (Kim & Cantor, 2014). The role of **Th17 CD4 T cells** in tumour immunity is controversial, as there are evidences supporting both a tumour-supporting and anti-tumour roles. Regarding their anti-tumour role, Muranski et al showed that adoptively transferred Th17 cells were more efficient at eliminating tumours than Th1 cells. Th17 cell advantage over Th1 cells was associated with their increased survival *in vivo*. However, the anti-tumour function of Th17 is linked to their heterogeneity

and ability to differentiate into Th1-like cells, as their anti-tumour role was abolished upon IFNγ, but not IL-17, neutralization. (Muranski et al., 2008, 2011). Another study demonstrated that tumour-associated Th17 lymphocytes in ovarian cancer patients are inversely correlated with immunosuppressive Treg cell numbers and positively correlated with IFNγ-producing CD8 T cell and CD4 T cell numbers, suggesting that they potentiate an anti-tumour response (Kryczek et al., 2009). **NK cells** are another type of lymphocytes whose anti-tumour function is regulated by their cell surface activatory and inhibitory receptors. The downregulation of MHC class I expression by tumour cells, used to prevent its elimination by CTLs, leads to an increase in NK activation. MHC class I downregulation decreases the interaction between MHC-I and inhibitory receptors expressed by NK cells, resulting in their activation. Furthermore, NK cells have some activating receptors, such as NKG2D, that recognize stress-induced molecules expressed by tumour cells. Both events contribute to NK cell activation and an increase in cytolytic properties. Thus, activated NK cells are able to produce perforins and granzymes and also express FasL or TRAIL that induce tumour cell death (Nicholson, Keating, & Belz, 2017). Another constituent of TILs that exerts anti-tumour functions is natural killer T lymphocytes (NKT cells). Unlike conventional T cells, NKT cells can quickly react to several glycolipid antigens presented by CD1d. In the cancer context, **type I NKT cells** produce abundant amounts of IFNγ, which helps to activate both $CD8^+$ T cells and dendritic cells, thus augmenting anti-tumour immunity (Robertson, Berzofsky, & Terabe, 2014). **γδ T cells** are well known to act as anti-tumour immune cells in several types of mouse cancer models (Lança & Silva-Santos, 2012; Silva-Santos, Serre, & Norell, 2015). The cellular cytotoxicity of this unconventional T lymphocyte is not MHC-restricted, i.e., these cells can directly recognize stress molecules in dysfunctional cells, such as tumour cells and exert a strong cytolytic activity (Hayday, 2009) which turn them as attractive cells for cancer immunotherapy. In fact, melanoma tumour-bearing mice lacking γδ T cells display reduced IFNγ production and impaired cytotoxic CD8 T cell responses in the early stages of tumour progression, thus suggesting the importance γδ T cells in tumour immunosurveillance as an early source of IFNγ. (Gao et al., 2003). Furthermore, γδ T cells can recognize cell-surface molecules that are expressed only on tumour cells via TCR or through natural cytotoxic receptors (NCR) such as NKG2D. Upon NKG2D ligand binding, the cytotoxic activity of γδ T cells is increased and they release perforin, granzymes towards the target cell, as well as IFNγ and TNFα into the tumour environment. Moreover, γδ T cells also express some apoptosis-related ligands, such as TRAIL and FasL, that engage death receptors (TRAIL-R and Fas, respectively) on tumour cell surface (Chitadze, Oberg, Wesch, & Kabelitz, 2017).

On the other hand, there are some immune cell types displaying **pro-tumour features** in the tumour microenvironment and responsible for promotion of tumour growth. **$CD4^+$ regulatory T lymphocytes (Treg cells)** specifically express the transcriptional factor Foxp3 and are one of the most immunosuppressive cells in the body. Indeed, these cells are known to suppress the activation and proliferation of effector immune cells, such as αβ and γδ T cells, NK, NKT, B cells, macrophages and dendritic cells (Wan, 2010). The immunosuppressive functions of Tregs are mediated by contact-dependent or -independent mechanisms. Extracellular expression of inhibitory molecules, such as

cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1) and lymphocyte-activation gene 3 (LAG3) on Tregs allow contact-dependent immunosuppression (Huang et al., 2004; Takahashi et al., 2000; B. Zhang, Chikuma, Hori, Fagarasan, & Honjo, 2016). Alternatively, the production of inhibitory cytokines, such as IL-10 and TGF-β is the basis of the cytokine-mediated Treg immunosuppression (Liyanage et al., 2002). In addition to Tregs, activated **type II NKT cells** also display pro-tumour characteristics through the production of IL-13 that leads to TGF-β expression by myeloid cells, which ultimately suppress CD8 T cell anti-tumour responses (Robertson et al., 2014). Although **Th17 cells** may exhibit anti-tumour properties, their major role in tumour immunity is strongly associated to the production of the pro-inflammatory cytokine IL-17 (Gulubova, Ananiev, Ignatova, & Halacheva, 2016). The presence of this cytokine in the tumour microenvironment prevents anti-tumour immune responses and promotes tumour angiogenesis, a critical process to maintain the oxygen and nutrient supply of proliferating tumour cells (Fabre et al., 2016). Hayata and his colleagues demonstrated that *in vivo* inhibition of IL-17 at tumour sites significantly increases the cytotoxic activity of tumour-infiltrating CD8 T cells and suppresses the expression of the pro-angiogenic factors MMP9 and VEGF in tumour tissues from colon and melanoma-bearing mice (Hayata et al., 2013). These data demonstrate that angiogenesis and prevention of anti-tumour immunity are the main pro-tumour functions of Th17 cells. In the last few years, pro-tumour roles of **γδ T cells** have also been reported in several tumour models (Rei, Pennington, & Silva-Santos, 2015). Indeed, there is a study that demonstrates the immunosuppressive capacity of isolated γδ T cells from human breast cancer biopsies in vitro. Those cells were able to inhibit the proliferation of CD4 and CD8 T cells and consequently impair their anti-tumour responses (Peng et al., 2007). Although γδ T cells are able to suppress anti-tumour immune responses, their main pro-tumour feature is the capacity to produce large amounts of IL-17. In fact, in several cases, γδ T cells are considered to be the main producers of IL-17. Some studies suggest that the pro-tumour functions of IL-17-producing γδ T cells (γδ17) are mainly due their ability to promote angiogenesis (Hao et al., 2011; Wakita et al., 2010). Moreover, IL-17 production by γδ T cells also leads to the mobilization of myeloid -derived suppressor cells (MDSC) which impair cytotoxic CD8 T cell responses in a hepatocellular carcinoma mouse model (Ma et al., 2014).

The role of TILs in tumour growth is shown schematically in **Figure 2**.

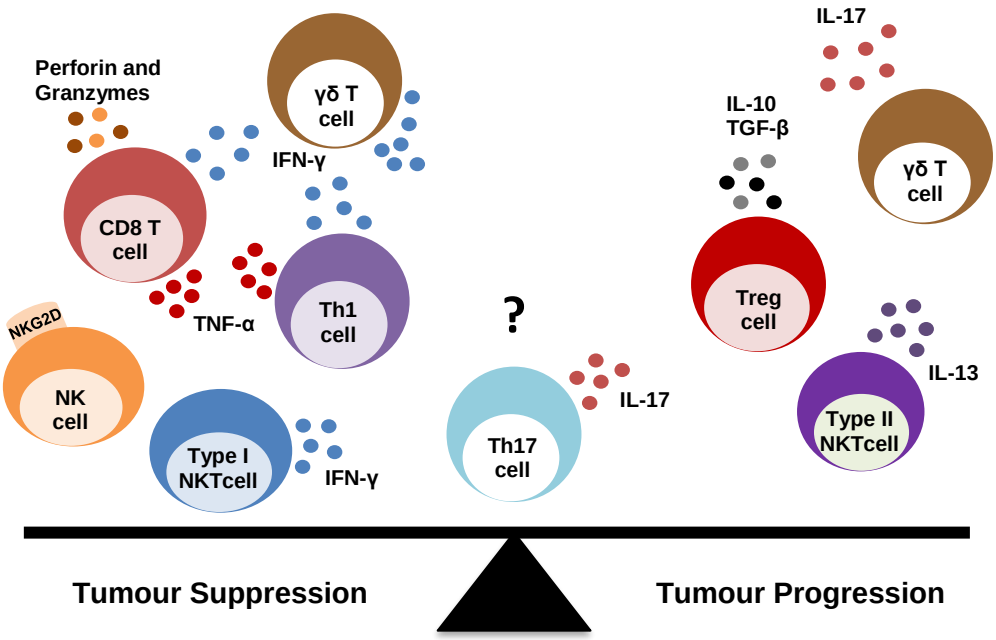


Figure 2. Role of TILs in tumour development.

1.2. Mechanisms of tumour escape from immune surveillance

The tumour-infiltrating immune cells displaying both anti-tumour and pro-tumour features interact dynamically with the tumour cells. Therefore, the balance between the anti-tumour/pro-tumour activity of immune cells influences the outcome of tumour growth. In addition to tumour growth being supported by the pro-tumour activity of the immune cells, several **escape mechanisms** from the immune system were developed by tumour cells in order to avoid the tumour immune surveillance (Lisiecka & Kostro, 2016).

One of those tumour escape mechanisms is the **decreased recognition of tumour cells by the host immune system**. This mechanism is mediated by the downregulation or loss of tumour associated antigens (TAA), MHC molecules, particularly MHC-I, and costimulatory molecules such as CD80 or CD86 which make impossible the priming/activation of T cells (Wang et al., 2017).

Furthermore, some tumours have the ability to **induce apoptosis of immunocompetent cells** through FasL-cell surface expression or secretion of FasL-expressing exosomes that cause death of T cells due to interaction with the Fas molecule expressed on the surface of T cells (Abusamra et al., 2005).

The **immunoregulatory effect of tumour cells** is another escape mechanism. Tumour cells frequently produce some molecules known as tumour-derived soluble factors (TDSFs) that contribute to establish an immunosuppressive tumour microenvironment. Some of those substances produced by tumour cells, such as IL-10, TGF-β, VEGF, and prostaglandin E2 (PGE2) are able to recruit Tregs

and MDSCs which comprise immature myeloid cells with immunosuppressive functions (Lisiecka & Kostro, 2016). Thus, tumours can modulate the proportion of effector immune cells by increasing the immunosuppressive environment in the tumour bed.

Moreover, tumour cells can avoid elimination by the immune system through the induction of **adaptive immune resistance**. This resistance is acquired through ligation of inhibitory receptors, such as the immune checkpoints programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), which are commonly expressed on some activated T cells (Chikuma, 2017). The interaction between PD-L1 or PD-L2 ligands expressed on tumour cells and PD-1 expressed on T cells induces T cell inhibition, leading to an anergic state of effector T cells, thus reducing their anti-tumour responses. CTLA-4 is another inhibitory receptor that is overexpressed in tumour-specific T cells that has higher binding affinity than the co-stimulatory T cell receptor CD28 to the co-stimulatory receptor B7, expressed on antigen-presenting cells (APCs). When inhibitory signals (CTLA-4 and B7) are higher than activator signals (CD28 and B7), T cells can no longer be activated, and the anti-tumour immune response becomes compromised (Lisiecka & Kostro, 2016; Teng et al., 2015).

Additionally, the production of interferons by tumour-specific T cells may help tumour cells to protect themselves from the T-cell attack. Besides inducing the expression of the ligand of PD-1 (PD-L1) which contributes for the T cell inhibition, the presence of interferons also induces the expression of the enzyme indoleamine 2,3-dioxygenase (**IDO**) in tumour cells. IDO is considered a rate-limiting enzyme in tryptophan metabolism and plays an immunosuppressive role by limiting the tryptophan availability in the tumour microenvironment, thus affecting the T cell functionality (Ribas, 2015).

In addition to IDO, tumour cells also overexpress the arginase 2 (**Arg2**) enzyme in order to metabolize arginine to ornithine, a metabolite needed to sustain rapid tumour growth (Tate et al., 2008). Thus, in addition to contributing to tumour growth, the production of Arg2 also causes a depletion of arginine in the tumour microenvironment, resulting in a decrease in CD3ζ expression, a key element for T cell function (Rodriguez et al., 2002). Therefore, Arg2-expressing tumour cells can modulate the arginine metabolic pathway in order to promote their cell growth and also to dampen T cell function, thus contributing for their escape from the immune system.

2. Cancer immunotherapy

Immunotherapy is a biological way to treat cancer, in which the anti-tumour functions of patient's immune cells are improved to effectively eliminate cancer cells. Remarkably, immunotherapy for cancer treatment has become a clinical success in many types of cancer in the last years (Emens et al., 2017).

Most immunotherapeutic strategies include cytokine administration, cancer vaccines, adoptive cell transfer of tumour-specific T cells and immune checkpoint inhibitors. IL-2 and IFN-α **cytokines** are

given to cancer patients in order to stimulate some immune cells and increase their cytotoxicity against cancer (Floros & Tarhini, 2015). Another strategy is the administration of therapeutic anti-cancer **vaccines,** which contain a tumour specific antigen, in an attempt to break the tolerance acquired by the tumour cells and to induce anti-tumour immune responses (Guo et al., 2013). **Adoptive T cell therapy (ACT)** aims to transfer autologous or allogenic *ex vivo* expanded TILs into the cancer patients. Those tumour-reactive T cells can be either naturally tumour-isolated T cells or engineered chimeric antigen receptor T cells (CAR-T). In addition to intracellular T cell receptor constant domain, CAR-Ts have an extracellular immunoglobulin variable domain which allows them to directly target tumour cells that downregulate the antigen-presenting machinery (Perica, Varela, Oelke, & Schneck, 2015). Moreover, **immune checkpoint inhibitors** prevent the ligation of PD-1 or CTLA-4 to its ligands which leads to increased T cell activation, proliferation and cytotoxicity against tumours (Dine, Gordon, Shames, Kasler, & Barton-Burke, 2017).

2.1. Immune Checkpoint Inhibitors

Immune checkpoints are a class of inhibitory receptors expressed on T cells that are responsible to maintain self-tolerance and to control the intensity of an immune response in peripheral tissues, to limit tissue damage. However, tumour cells have developed ways to escape from the host immune system by taking advantage of this peripheral tolerance. Tumour cells play an important immunosuppressive role through overexpression of the PD-1 ligand, which in turn interacts with the PD-1 molecules on TILs, thus leading to a diminished T cell response (Zarour, 2016). Therefore, blocking immune checkpoints like PD-1 or PD-L1 with antagonist monoclonal antibodies is a solution to achieve long-lasting T cell anti-tumour responses and to limit tumour growth (Teng et al., 2015).

Tumour-infiltrating lymphocytes can express several inhibitory receptors, such as PD-1, CTLA-4, T-cell immunoreceptor with Ig and ITIM domains (TIGIT), lymphocyte activation gene 3 (LAG3) and T cell membrane protein 3 (TIM3). However, **CTLA-4 and PD-1 inhibitors** are those that have been approved for use in clinical treatments for they displayed the most promising results regarding patient survival in numerous studies of several cancer types when compared to conventional chemotherapies (Sharpe & Pauken, 2018).

2.1.1. Anti-CTLA-4 therapy

CTLA-4 is an inhibitory receptor commonly upregulated on CD4 and CD8 T cells during their initial stage of activation. Thus, when a tumour antigen is presented by an APC to a T cell in the tumour-draining lymph node, CTLA-4 receptors are upregulated on the T cell surface and they strongly bind to the co-stimulatory molecules CD80 and CD86 (B7-1 and B7-2 in mouse) expressed on APCs (Figure 3). Since the co-stimulatory CD28 molecule expressed on T cells is crucial to amplify TCR signalling, the existence of inhibitory receptors like CTLA-4 that compete to the same ligands (CD80/CD86) leads to a diminished T cell activation and a decrease in cytokine production, such as

IL-2 and IFNγ. Therefore, the blockade of CTLA-4 inhibitory receptors occurs in the tumour-draining lymph node and frequently leads to an improvement of T cell activation and consequently an increase in tumour immune surveillance and elimination (Ott, Hodi, & Robert, 2013). The inhibitory function of CTLA-4 is mainly mediated by Tregs and effector CD4 T cells. *In vitro* studies showed a decreased regulatory capacity of Tregs lacking the CTLA-4 receptor to suppress effector CD4 T cells (Peggs, Quezada, Chambers, Korman, & Allison, 2009). Also, *in vivo* studies demonstrated that Treg-specific CTLA-4 deficiency impaired the suppressive functions of Tregs and promoted tumour immunity (Wing et al., 2008). On the other hand, anti-CTLA-4-treated patients showed an increased expression of inducible costimulator (ICOS), an activator receptor of T cells, and IFNγ in CD4 T cells from blood and tumour samples (Peggs et al., 2009). Consistent with this data, Wei et al found an increased expansion of tumour-infiltrating $iCOS^{+}$Th1-like CD4 T cells in MC38 colorectal tumours from mice treated with anti-CTLA-4 (S. C. Wei et al., 2017). All these findings support the idea that the anti–CTLA-4 therapy antitumor activity is mediated by both enhancement of effector CD4 T cell activity and inhibition of Treg cell-dependent immunosuppression. Although it is clear that CTLA-4 blockade generally decreases the Treg cell-mediated immunosuppressive capacity, the immunological contexts in which $CTLA\text{-}4^{+}$ Treg cells suppress T cell responses are not well defined. Some *in vitro* and *in vivo* studies support the idea that B7-1/B7-2 molecules expressed on APCs may be removed and captured by CTLA-4 on Tregs by a process of trans-endocytosis (T. Z. Hou et al., 2015; Qureshi et al., 2011). Thus, the lack of B7 molecules on APCs can impair the costimulation via CD28 of TILs, thus reducing the T cell proliferation and effector functions.

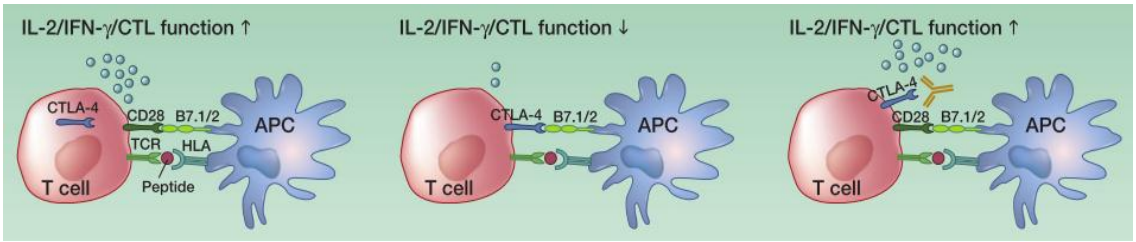


Figure 3. Modulation of T cell activation and anti-tumour activity by CTLA-4 immune checkpoint. From Ott et al., 2013.

The anti-CTLA-4 blocking antibody **ipilimumab** was the first approved immune checkpoint inhibitor by the Food and Drug Administration (FDA) for cancer treatment (2011) to treat unresectable or metastatic melanoma cancer patients (Emens et al., 2017). Moreover, this immunotherapeutic strategy is on clinical trials for several cancer types, such as prostate cancer and small-cell lung cancer (Beer et al., 2016; Reck et al., 2016). In the CA184 095 (ClinicalTrials.gov, NCT01057810) phase 3 study, ipilimumab was tested in 400 patients with metastatic castrate-resistant prostate cancer (mCRPC). Despite treatment have caused many grades 3-4 adverse effects in 31 patients, the ipilimumab-treated patients achieved a higher response rate (23%) when compared to non-treated mCRPC patients (8%).

2.1.2. Anti-PD-1/PD-L1 therapy

Mechanism of PD-1/PD-L1 signalling

Contrary to the CTLA-4-mediated immune responses that occur in the early induction phase in a lymphoid organ, the upregulation of **PD-1** on activated T cells commonly occurs in the peripheral tissues, thus regulating late effector-phase immune responses. The engagement-induced expression of PD-1, frequently transmits inhibitory signals that limit the activation and effector functions of T cells within the tumour. Of note, PD-1-induced inhibitory signals are triggered upon ligation to its ligands PD-L1 or PD-L2. Although tumour cells can express both ligands, PD-L1 expression is more frequently found in those cells since its upregulation is positively induced by pro-inflammatory cytokines typically found in the tumour microenvironment. In addition to tumour cells, PD-L1 can also be expressed by other immune cells, such as T and B cells and mainly by antigen-presenting cells (dendritic cells and macrophages). Therefore, when a T cell is activated via TCR (interacting with peptide-MHC complex), PD-1 is upregulated and the PD-1 inhibitory signalling mechanism is triggered upon engagement with its ligands (PD-L1 or PD-L2), as depicted in Figure 4. Thus, PD-1 engagement leads to phosphorylation of its two cytoplasmic tyrosine motifs, the immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM), which leads to binding of protein tyrosine phosphatases (PTPs), including SHP2, to the PD-1 cytoplasmic region. The SHP2 phosphatase can suppress T cell proliferation and effector functions by two different ways. On one hand, SHP2 can upregulate transcriptional factors, such as basic leucine zipper transcription factor ATF-like (BATF) that positively regulates genes involved in the inhibition of T cell function. On the other hand, SHP2-mediated T cell suppression can act through TCR and CD28 signalling attenuation. Indeed, SHP2 has the ability to dephosphorylate and inactivate some signalling proteins pre-induced by the TCR-CD8 signalling, such as Zeta-chain-associated protein kinase 70 (ZAP70) and phosphoinositide 3-kinase (PI3K) as well as proteins involved in the RAS signalling pathway. As a result, SHP2 causes a decreased activation of several transcriptional factors related to T cell proliferation, survival and cytokine production, such as activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB). Although the molecular mechanisms of the PD-L1/PD-L2 signalling in the PD-L1/PD-L2 expressing cells are not well known, it seems to alter their metabolism and increase its IL-10 production (Seidel, Otsuka, & Kabashima, 2018; Sharpe & Pauken, 2018)

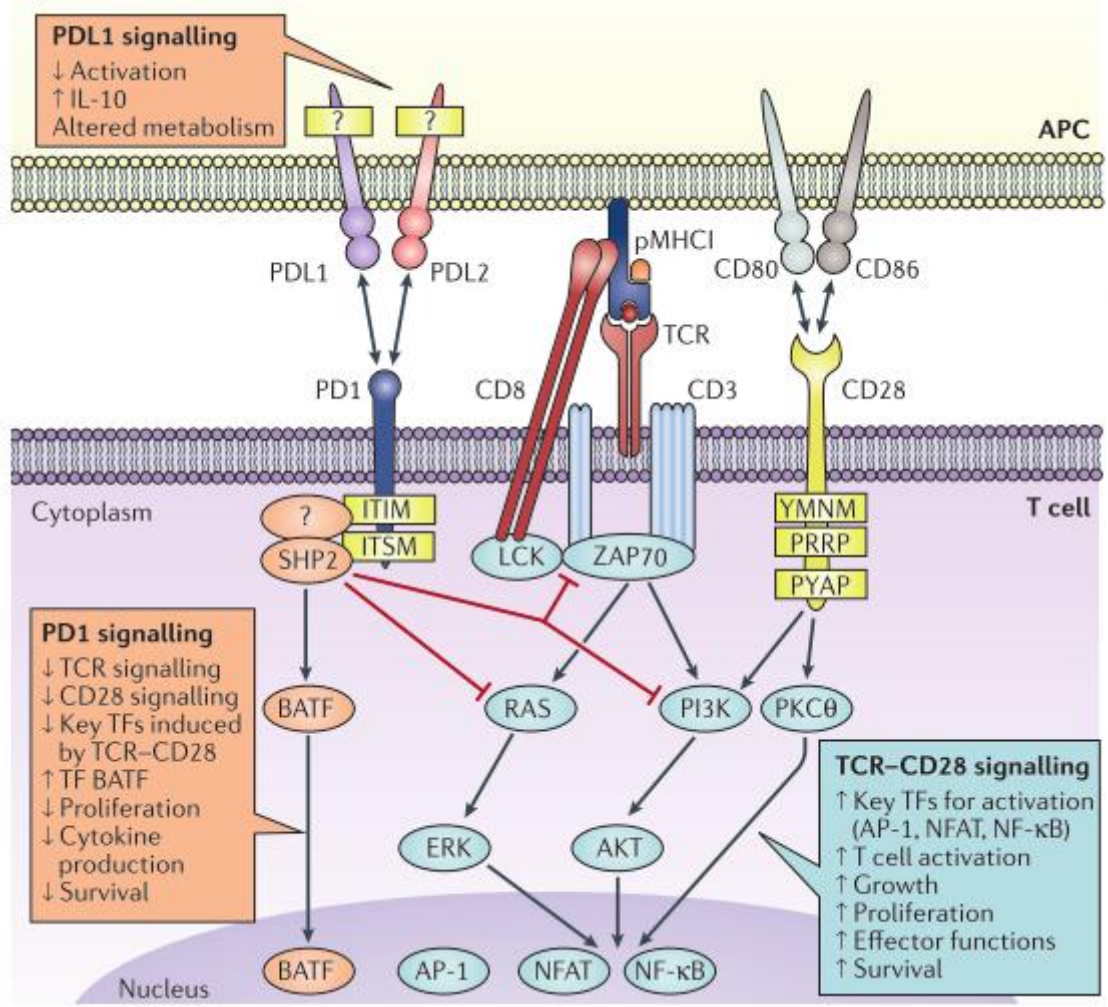


Figure 4. PD-1 signalling pathway in T cells. From Sharpe & Pauken, 2018

Clinical evidences of PD-1 blockade in breast cancer

Actually, two human anti-PD-1 monoclonal antibodies (mAbs) **pembrolizumab** and **nivolumab** are approved for the treatment of several malignancies, including metastatic melanoma, non-small cell lung cancer, Hodgkin lymphoma, renal cell cancer, head and neck squamous cell carcinoma and locally advanced or metastatic urothelial carcinoma. Moreover, pembrolizumab is also approved for advanced gastroesophageal cancer and nivolumab is approved in metastatic colorectal cancer and advanced hepatocellular carcinoma (Gong, Chehrazi-Raffle, Reddi, & Salgia, 2018). Results from the CheckMate 037 (NCT01721746) phase 3 trial demonstrated an overall response rate **(ORR)** of 31.7% in the nivolumab group and 10.6% in the chemotherapy group in patients with unresectable stage IIIC or IV melanoma (Weber et al., 2015). Also, pembrolizumab administration had shown to be effective in patients with classical Hodgkin lymphoma. In fact, in the KEYNOTE-087 (NCT02453594) phase 2 study, patients with relapsed or refractory classic Hodgkin lymphoma showed an ORR of 69% with pembrolizumab and an incredible complete response (CR) rate of 22.4% (R. Chen et al., 2017).

The increasing success of anti-PD-1 immunotherapy in numerous cancer types, together with the growing evidence on the prognostic and predictive value of TIL in breast cancer (Würfel et al., 2018), encouraged the use of anti-PD-1 inhibitors as an alternative treatment for breast cancer. Indeed, there are already some clinical trials showing the impact of this immune checkpoint inhibitor on the treatment of patients with breast cancer. In the KEYNOTE-028 (ClinicalTrials.gov, NCT02054806) phase 1b study, pembrolizumab was tested in 25 breast cancer patients with luminal subtype (ER^+HER2^-), which is characterized by the overexpression of the estrogen receptor (ER) and by the absence of mutation in human epidermal growth factor 2 (HER2). Although only 16% of patients have shown adverse effects, the overall response rate did not exceed 12%. More satisfactory response rates were observed in the KEYNOTE-012 (NCT01848834) phase 1b study, wherein 32 women with metastatic triple negative breast cancer (TNBC), a subtype of breast cancer that has no mutations in HER2, ER neither progesterone receptor (PR), were treated with pembrolizumab. In this subset, an ORR of 18.5% was observed in 27 evaluable patients and only about 15% of patients experienced grade 3-5 adverse effects. Regrettably, the results of anti-PD-1 immunotherapy in breast cancer obtained from clinical trials still have low response rates when compared to other types of cancer (Carretero-González et al., 2018).

Among the various subtypes of breast cancer, TNBC is the one that has presented the most promising results receiving the single-agent anti-PD-1 immune checkpoint inhibitor, and therefore is the subtype that has received a greater number of further clinical investigations which are still ongoing (Kwa & Adams, 2018). It has been reported that its greater capacity to respond to anti-PD-1 immunotherapy is due to the fact that this type of tumours display higher levels of PD-L1 expression in the tumour compared to other breast cancer subtypes (Voutsadakis, 2016). Moreover, the higher incidence of mutations in the tumour cells of TNBC contributes to the increased immunogenicity and frequency of TILs, thus correlating with response to anti-PD-1 therapy (Anders, Abramson, Tan, & Dent, 2016). Furthermore, the combination of PD-1 blockade with other standard therapies, such as chemotherapy has also been shown to be effective in TNBC patients. In the I-SPY2 (NCT01042379) phase 2 study, pembrolizumab was administrated with the combination of standard neoadjuvant chemotherapy (paclitaxel followed by doxorubicin and cyclophosphamide) in women with high risk TNBC. The addition of pembrolizumab to standard chemotherapy improved the pathological complete response (pCR) from 20% (control arm) to 60% (pembrolizumab-containing arm) for the subset of women with TNBC (Nanda et al., 2017).

Several clinical trials are currently on going to study the efficacy of anti-PD-1 immunotherapy in combination with conventional cancer treatments, such as radiotherapy and chemotherapy for the treatment of TNBC (Z. Li, Qiu, Lu, Jiang, & Wang, 2018; Solinas et al., 2017).

3. Amino acid metabolism in the tumour microenvironment

3.1. Amino acid and T cell response to tumours

Tumour-reactive T cells are activated lymphocytes that have high rates of cell proliferation and special metabolic needs. In fact, proliferating T cells display increased bioenergetic needs to support their rapid proliferation, differentiation and effector function. Therefore, upon activation, T cells frequently increase their nutrient uptake (i.e. glucose, amino acids) by upregulating some glucose and amino acid transporters in the cell surface in order to meet the increased energy requirements (J. Wei, Raynor, Nguyen, & Chi, 2017). It is already known that glucose uptake by TILs is extremely important to increase their glycolytic capacity and IFNγ production, thus allowing an efficient anti-tumour response and consequently tumour regression (Chang et al., 2015). In addition to glucose, T cells also require amino acid uptake to accommodate their bioenergetic requirements and effector functions. The SLC7 amino acid transporter family contributes to the uptake of amino acids by T cells (Sinclair, Neyens, Ramsay, Taylor, & Cantrell, 2018). Of note, amino acids are not only important sources of metabolic intermediates but also represent the building blocks of protein synthesis which play a crucial role during T cell division (J. Wei et al., 2017).

However, the most well-studied amino acids known to impact tumour immune responses are **glutamine**, **arginine** and **tryptophan**. Glutamine is a neutral amino acid that enters into the T cells through the transporter protein ASCT2 (encoded by SLC1A5) and seems to be involved in the balance between effector T cells and Treg cells. Indeed, a study demonstrated an impaired Th1 and Th17 CD4 T cell differentiation and function in the absence of the glutamine transporter, ASCT2. On the other hand, the absence of ASCT2 did not alter Treg generation, thus suggesting that glutamine is important to generate effector T cells and dispensable for Treg differentiation (Nakaya et al., 2014). Arginine is rapidly metabolized by T cells upon their activation and play a positive role in the survival and proliferation of CD4 and CD8 T cells. Furthermore, arginine-supplemented CD8 T cells displayed an increased IFNγ production *in vitro* and the injection of these cells in B16 melanoma-tumour bearing mice induced a significant tumour regression (Geiger et al., 2016). These findings demonstrate the beneficial effect of arginine in the T cell anti-tumour activity. However, the extracellular availability of this amino acid in the tumour site can be further modulated by the overexpression of the arginase enzyme by tumour cells and myeloid cells (Rodríguez & Ochoa, 2006). Thus, arginase-expressing cells in the tumour microenvironment may limit the protective effect of arginine in modulating anti-tumour T cell responses. Additionally, sustained extracellular tryptophan levels are by themselves important to maintain the T cell survival and to regulate its responsiveness against tumour cells. In fact, low tryptophan levels in the extracellular space are known to trigger amino acid-sensing transduction pathways in T cells which leads to GCN2 kinase activation and subsequent growth arrest and apoptosis of tumour-infiltrating T cells (Platten, Wick, & Eynde, 2012). However, in the tumour site, the tryptophan is quickly degraded by tumour cells and some immune cells, such as dendritic cells through overexpression of the indoleamine-2,3-dioxygenase (IDO) enzyme (Löb, Königsrainer, Rammensee, Opelz, & Terness, 2009; Uyttenhove

et al., 2003), thus limiting the positive effect of tryptophan on the activation and function of T cells. Moreover, the main metabolic product of tryptophan catabolism from IDO-expressing cells, kynurenine, can both strongly promote the differentiation of CD4 T cells into Treg cells and suppress effector functions of CD4 and CD8 T cells (Routy, Routy, Graziani, & Mehraj, 2016). Thus, both the accumulation of kynurenines and depletion of tryptophan in the tumour microenvironment are important mechanisms employed by tumour cells to dampen T cell responses.

3.1.1. Sulphur-containing amino acids and their anti-tumour properties

In addition to the previously mentioned amino acids and their effects on anti-tumour T cell responses, there are some other amino acids that may modulate immune cell responses and play a role in tumour immunity. Sulphur-containing amino acids such as **methionine** and **cysteine** are known to be involved in the cellular redox maintenance, a process intrinsically mediated by the thiol group present in the molecular structure of these amino acids (Collin, Seiliez, Tesseraud, & Me, 2008). Although these amino acids are not known to directly alter T cell functions, their metabolites, such as S-Adenosylmethionine (SAM), taurine and glutathione (GSH) may influence the cellular metabolism of T cells and thereby increasing the strength of antitumor T cell responses. (Grimble, 2006).

Methionine and Cysteine metabolism

Methionine is considered an essential amino acid since it cannot be produced in mammals and therefore must be obtained through diet. On the other hand, cysteine is a semi-essential amino acid that is synthesized in mammalian cells through the catabolism of intracellular methionine (Y. Hou, Yin, & Wu, 2015). Thus, methionine uptake is extremely important to maintain cellular and molecular processes mediated by its metabolites (Shiraki et al., 2014). Of note, methionine is transferred into the cell through several transporters belonging to SLC7 amino acid transporter family, such as SLC7A5, SLC7A6, SLC7A7 and SLC7A8 (Fotiadis, Kanai, & Palacín, 2013). Once inside the cell, methionine can be recruited by initiator tRNAs to initiate the translation process and protein synthesis or otherwise can be converted to SAM through the catalytic action of methionine adenosyltransferase enzymes (MATs) and ATP hydrolyses (Figure 5). SAM is the universal methyl donor that participates in the majority of cellular methylation reactions through specific methyltransferases that target several biomolecules, including DNA, RNA, lipids, proteins and some metabolites. Thus, after the transfer of the methyl group, SAM is converted to S-Adenosylhomocysteine (SAH). Then, SAH metabolite is further hydrolysed to homocysteine and adenosine in a reversible reaction by SAH hydrolase (SAHH). All these reactions, since methionine uptake to its transformation into homocysteine, belong to the transmethylation pathway. Importantly, homocysteine is considered a metabolic branch point since it can be remethylated and regenerate methionine or irreversibly degraded to cysteine through the transsulfuration pathway. Remethylation of homocysteine to regenerate methionine is strongly promoted in low intracellular methionine level conditions and occurs by two different metabolic pathways. In the kidney and liver, betaine acts as methyl donor

and transfers its methyl group to homocysteine in a reaction catalysed by betaine-homocysteine methyltransferase (BHMT), thus producing dimethylglycine (DMG) and methionine. In an alternative way, homocysteine can be remethylated by the transfer of a methyl group from 5-methyltetrahydrofolate through the catalytic action of vitamin B12-dependent methionine synthase (MS), producing tetrahydrofolate (THF) as a by-product. In the transsulfuration pathway, the conversion of homocysteine to cysteine is mediated by the catalytic action of two enzymes: cystathionine b-synthase (CBS) and cystathionine g-lyase (CGL) (Barroso, Handy, & Castro, 2017; Brosnan & Brosnan, 2006).

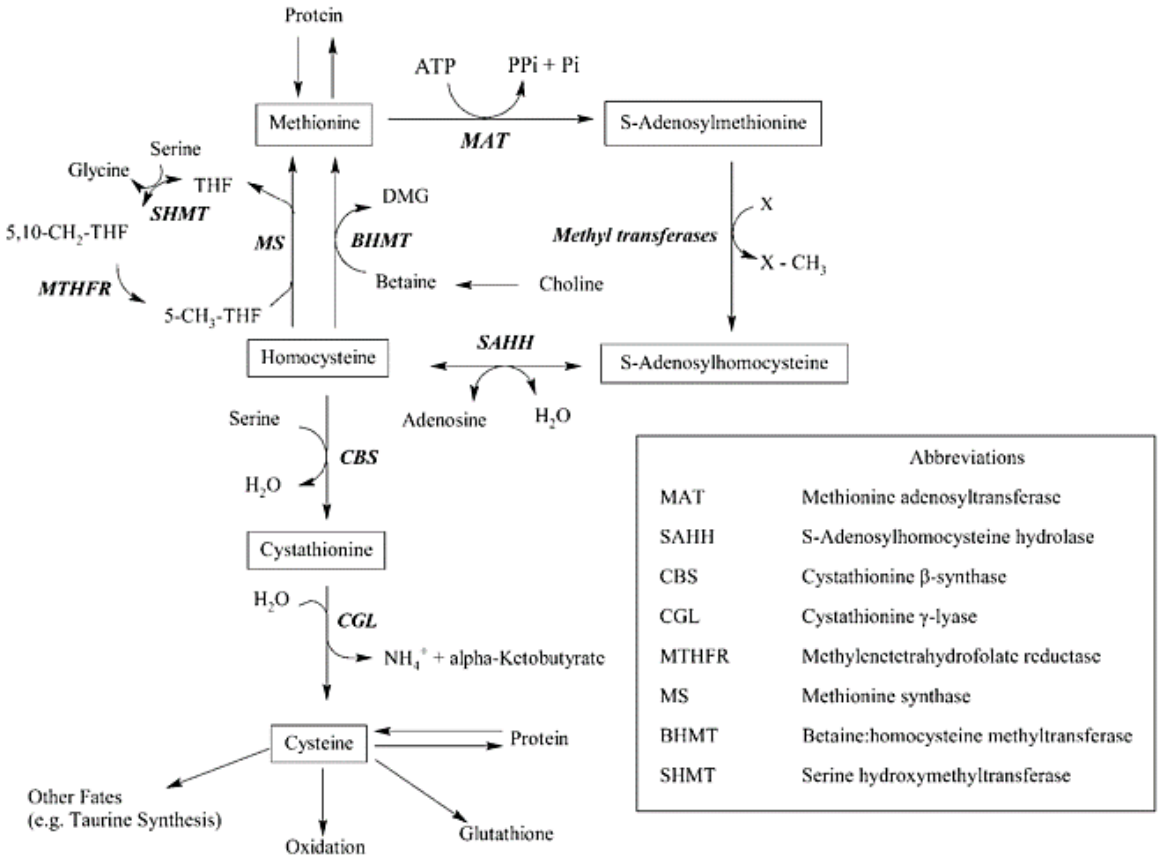


Figure 5. Methionine and cysteine amino acid metabolism. From Brosnan & Brosnan, 2006

SAM and epigenetic modifications

In addition to its role in protein synthesis and normal cell growth, methionine is also associated to most of the methylation reactions that occur within the cell. Indeed, its metabolite SAM is the main methyl donor of the cell and is required for methylation of nucleic acids (DNA and RNA), proteins, lipids and other metabolites through the action of methyltransferases. Therefore, SAM has the ability to enter to the cell nucleus and to regulate the expression of certain genes by DNA and histone methylations. Thus, the intracellular availability of SAM is essential for a normal epigenetic control of gene expression (N. Zhang, 2018).

SAM-mediated **DNA methylation** is a process that leads to the methylation of cytosines in CpG-rich regions located in or near gene promoters, commonly resulting in the suppression of gene transcription. DNA methyltransferases (DNMTs) are the enzymes responsible for that DNA methylation and gene silencing. In fact, increased DNA methylation levels are normally negatively correlated to anti-tumour immune responses. Ghoneim et al reported that de novo DNA methylation acquired in effector CD8 T cells during an anti-tumour immune response is strongly related to the establishment of T-cell exhaustion and worse response to immune checkpoint inhibitors (Ghoneim et al., 2017).

Besides DNA methylation, SAM metabolite can influence gene expression through the methylation of histone proteins, thus positively or negatively altering the gene transcription process. **Histone methylation** is catalysed by histone methyltransferases (HMTs) and frequently occurs at lysine residues (K) of histone H3, which can be monomethylated, dimethylated (me2) or trimethylated (me3). Gene transcriptional activation or repression induced by histone methylation only depends on the site and degree of methylation. Generally, trimethylation of lysine 4 and 36 on H3 (H3K4me3; H3K36me3) leads to the activation of the gene and otherwise trimethylation of lysine 9, 27 and 79 on H3 (H3K9me3; H3K27me3; H3K79me3) is strongly associated with gene repression (Schleithoff, Voelter-Mahlknecht, Dahmke, & Mahlknecht, 2012). Thus, SAM-mediated histone methylation reactions can have a role in establishing cell type-specific gene-expression patterns (Schmidl et al., 2009). In fact, high levels of active H3K4me3 epigenetic modifications are usually present at the IFNγ locus in Th1 CD4 T cells, whereas repressive H3K27me3 modifications are reduced in these cells. Furthermore, cytotoxic CD8 T cells also display increased H3K4me3 in the IFNγ and Granzyme B locus (S. He, Tong, Bishop, & Zhang, 2013). Therefore, histone methylation pattern of a cell can be crucial to mediate the activation of genes encoding effector molecules, thus modulating anti-tumour T cell responses.

Generation of antioxidant molecules (oxidative stress/ROS elimination)

The sulphur-containing amino acid metabolism gives rise to metabolites involved in the fight against oxidative stress, thereby making them important regulators of the antioxidant status of the cell. In fact, cysteine, produced in the transsulfuration pathway, is a potent precursor for several antioxidant molecules, such as **glutathione**, **hydrogen sulphide** (H_2S) and **taurine**. These molecules are extremely important to control ROS intracellular levels in effector T lymphocytes, since their overproduction of ATP in the mitochondria normally generates high levels of reactive oxygen species (Jones & Thompson, 2007). Indeed, high levels of intracellular ROS in T cells are known to cause DNA damage and to induce T cell apoptosis (Kesarwani, Murali, Al-Khami, & Mehrotra, 2013). Therefore, the balance of cellular oxidative stress mediated by antioxidant molecules seems to be critical for T cell proliferation and effector functions.

In the tumour microenvironment, increased levels of exogenous reactive oxygen species (ROS) produced mainly by tumour cells and immunosuppressive cells can negatively impact the survival and effector functions of anti-tumour T cells (X. Chen, Song, Zhang, & Zhang, 2016). Thus, high antioxidant property is fundamental to potentiate anti-tumour effector T cells. It is reported that effector memory T cells (T_{EM}) are not able to suppress tumour cell growth and ultimately die early due to decreased capacity to quench ROS. On the other hand, central memory T cells (T_{CM}) showed higher ability to survive and increased CTL activity in an oxidative tumour microenvironment. The improved anti-tumour activity of T_{CM} cells was mediated by the increase of glutathionylation-derived c-SH groups in the T cell surface that have the ability to quench extracellular ROS (Kesarwani, Thyagarajan, Chatterjee, Palanisamy, & Mehrotra, 2015). Indeed, antioxidant molecules produced by the sulphur-containing amino acid metabolism are crucial to enhance anti-tumour T cell responses.

HYPOTHESIS AND OBJECTIVES OF THIS THESIS

Reactive oxygen species (ROS) elevated in the tumour microenvironment contribute to increase local immunosuppression, thus limiting the efficacy of immunotherapeutic strategies to treat cancer (Arina, Corrales, & Bronte, 2016; Ghirelli & Hagemann, 2013). In fact, it has been reported that ROS have the ability to induce T cell unresponsiveness and apoptosis in cancer patients (Cemerski, Cantagrel, Van Meerwijk, & Romagnoli, 2002). ROS can be regulated by methionine metabolism since methionine is an important precursor of glutathione (GSH), a molecule known to increase the antioxidant capacity and the resistance to oxidative stress (Martínez et al., 2017). Thus, modulating the level of ROS may be important to extend T cell survival and enhance their anti-tumour effector functions.

We hypothesized that methionine supplementation could limit oxidative stress and thereby promote a sustained anti-tumour T cell response upon anti-PD-1 therapy, thus limiting the tumour growth in TNBC-bearing mice. We believed that TNBC model could be a good therapeutic target to benefit from our treatment, since this is the subtype of breast cancer that has the highest immune infiltrate as well as the higher intra-tumour expression of PD-L1 (Voutsadakis, 2016).

Building on this, the main objectives of this thesis were:

1. To determine the impact of methionine supplementation and anti-PD-1 inhibitor combination on tumour growth in TNBC-bearing mice;

2. To test if the potential effect on tumour growth mediated by methionine supplementation is immune cell-dependent;

3. To dissect the cellular and molecular players involved in the effect of the combined treatment.

MATERIALS AND METHODS

1. Mice

C57BL/6J wild type (WT) mice were purchased from Charles River Laboratories. TCRδ-/- mice were purchased from The Jackson Laboratory. Rag2γc-/- mice were bred in house. All animals were females with 5 to 16 weeks of age, which were age-matched within 2 weeks. Mice were maintained in a specific pathogen-free facility at Instituto de Medicina Molecular (Lisboa, Portugal). All experimental procedures were performed according to guidelines approved by the local and national ethics committees.

2. Cell culture

E0771 triple negative mammary adenocarcinoma tumour cell line was a kind gift from Drº Sergio Dias (iMM)**.** Cells were maintained in Dulbecco's modified Eagles' medium (DMEM; Life Technologies) with glutamine, sodium pyruvate, 10% fetal calf serum (FCS; Life Technologies) and 1% penicillin-streptomycin (P/S), at 37ºC and 5% CO_2. When around 90% confluency was reached cells were washed with phosphate buffer saline (PBS; Life Technologies), incubated with Triple Express (Life Technologies) for 2 min at 37ºC and resuspended in 3 times the volume of complete media. Cells were split in a proportion of 1:20 every 2 to 3 days. For long term storage, cells were resuspended in FCS with 10% dimethyl sulfoxide (DMSO).

3. *In vivo* transplantable E0771 tumour model

E0771 tumour cells were harvested in the exponential growth phase. After one wash with phosphate buffer saline (PBS) (Gibco; Life Technologies) and incubation with Triple Express for 3/5min at 37ºC (Gibco; Life Technologies), tumour cells were resuspended in PBS at a concentration of $20x10^6$ / ml. One million tumour cells were injected in the mammary fat pad of female C57BL/6J mice, in a volume of 50µl of PBS. Tumour volume was measured using a calliper and calculated as $\frac{\text{length} \times \text{width} \times \text{width}}{2}$ (mm^3).

For anti-PD-1 treatment, mice were injected i.p. with 200µg of purified anti-mouse PD-1 antibody (Clone 29F1A12; BioXCell) 7 or 10 days post-tumour inoculation until the end of the experiment and anti-PD-1 injections were given every 3 to 4 days.

For *in vivo* methionine supplementation, mice were supplemented with 1.65% DL-methionine (Sigma) in the drinking water. The water bottle supplemented with methionine was replaced every week.

For CD8+ T cell depletion, 150µg and 20 µg of monoclonal antibody anti-CD8 (Clone YTS156/YTS169) was injected i.p and i.t., respectively,. at days 8, 14 and 21 post-tumour inoculation. Antibody kindly provided by Dr Luis Graça (iMM).

4. Flow cytometry analysis and cell sorting

In order to monitor the tumour immune infiltrate, mice were sacrificed, and tumours were resected and weighed. Tumours were cut and digested for 30 minutes at 37ºC with shaker at 1000 rotation per minute (rpm) in 1.5ml DMEM plus collagenase I (0.4mg/ml; Wortington), collagenase IV (1 mg/ml; Wortington) and DNase (10µg/ml; Sigma) per tissue. After that time, erythrocytes were osmotically lysed using 500µl of Red Blood Cell Lysis Buffer (Biolegend) and the digested sample was filtered through a 100µm cell strainer and stained.

For fluorescence-activated cell sorting (FACS) analysis of tumour samples, surface staining was done in 96 well plates. Tumour or blood cells were incubated with anti-CD16/32 (clone 93, eBioscience), 2% of mouse serum and the respective antibodies in RPMI-1640 medium (Gibco; Life Technologies) supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin/streptomycin, 1% (vol/vol) and nonessential amino acids, 10 mM HEPES, 50 uM 2-mercaptoethanol, 1% (vol/vol) pyruvate and gentamycin (10µg/mL), for 1h at room temperature and in the dark. After surface staining, cells were treated with Zombie Violet (BioLegend) or LIVE/DEAD Fixable Near-IR stain to exclude dead cells, for 15min at 4ºC. Antibodies were purchased from eBioscience and Biolegend, as summarized in Table 1.

Table 1. List of antibody clones used in flow cytometry and manufacturers.

Marker	Clone	Manufacturer
CD45	30-F11	Biolegend
CD3	17A2	Biolegend
TCRδ	GL3	Biolegend
MHC-I	28-8-6	Biolegend
CD8	53-6.7	Biolegend
PD-L1	10F.9G2	Biolegend
IFNγ	XMG1.2	Biolegend
TNFα	MP6-XT22	eBioscience
IL-17	TC11-18H10.1	Biolegend

For intracellular cytokine staining, cells were stimulated with 50ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma), 1µg/mL ionomycin (Iono; Sigma), in the presence of 10µg/mL of brefeldin-A (Sigma) and 5µM of monensin (eBioscience) for 4h at 37ºC. Cells were stained for surface markers, fixed and permeabilized using BD Pharmingen cytofix/cytoperm kit, following the manufacturer's instructions, and then incubated for 1h with monoclonal antibodies to detect cytokine production.

Cells were either analysed on LSRFortessa (BD Bioscience) or sorted on Aria I (BD Bioscience) for *in vitro* polarization assays or posterior mRNA extraction. Compensation was performed using single-stained samples. Data was analysed using FACSDiva and FlowJo software (TreeStar). Cell gating strategy was performed as described in Supplementary Figure 3.

5. Gene expression analysis

RNA was extracted from sorted cells using High Pure RNA Isolation Kit (Roche) following manufacturer's instructions. RNA was reverse-transcribed into cDNA using random oligonucleotides (Invitrogen) and MMLV reverse transcriptase (Promega). Quantification of specific cDNA species was assessed by real-time-PCR on ViiA7 Real-Time PCR System (Applied Biosystems) with SYBR, relatively to endogenous references (β2-microglobulin, HPRT or β- actin). Target gene CT was subtracted from CT of the endogenous reference and the relative amount calculated as $2^{-\Delta CT}$.

6. Statistical Analysis

In tumour growth graphs means and standard error of mean are plotted, in all other graphs presented in this thesis each individual value is plotted as well as the mean of each group. GraphPad Prism software was used to perform statistical analysis.

RESULTS

1. Methionine supplementation delays tumour growth in combination with anti-PD-1 treatment

It has been shown that combination of arginine supplementation and PD-L1 immune checkpoint blocker can boost immune response against osteosarcoma in tumour-bearing mice (X. He, Lin, Yuan, & Li, 2017). In agreement with this finding and with the apparent anti-oxidant properties of methionine in modulating the oxidative tumour microenvironment (Martínez et al., 2017), we have decided to combine methionine supplementation and anti-PD-1 immunotherapy in order to improve treatment efficacy in TNBC-tumour bearing mice. Since TNBC is the breast tumour subtype that has the highest PD-1 expression in TILs and the highest PD-L1 expression in tumour cells (Voutsadakis, 2016), the anti-PD-1 therapy seems to be an alternative treatment option (Son, Budhathoki, Dhakal, & Opyrchal, 2018). In fact, there is already a study demonstrating the efficacy of αPD-1 antibody therapy by causing a significant delay on tumour growth in mice transplanted with E0771 tumour cells, a cancer model of TNBC (Gray et al., 2016). Aiming at testing our hypothesis, we supplemented E0771 tumour-bearing WT mice with methionine 21 days before tumour transplantation (prophylactic regimen) or 7 days after tumour implantation (therapeutic regimen) in combination with anti-PD-1 injections that were given every 2-3 days from day 7 after tumour inoculation (Figure 6A). Interestingly, this showed an enhanced anti-PD-1 treatment efficacy in both mice supplemented with methionine 21 days before and 7 days after tumour implantation as shown in Figures 6B and 6C. Taken together, these findings suggest that methionine supplementation improves anti-PD-1 treatment in both approaches, acting prophylactically or as a therapeutic agent. Thereafter, we decided to focus on the therapeutic regimen since this will be the regimen with greater relevance to be translated to the clinic.

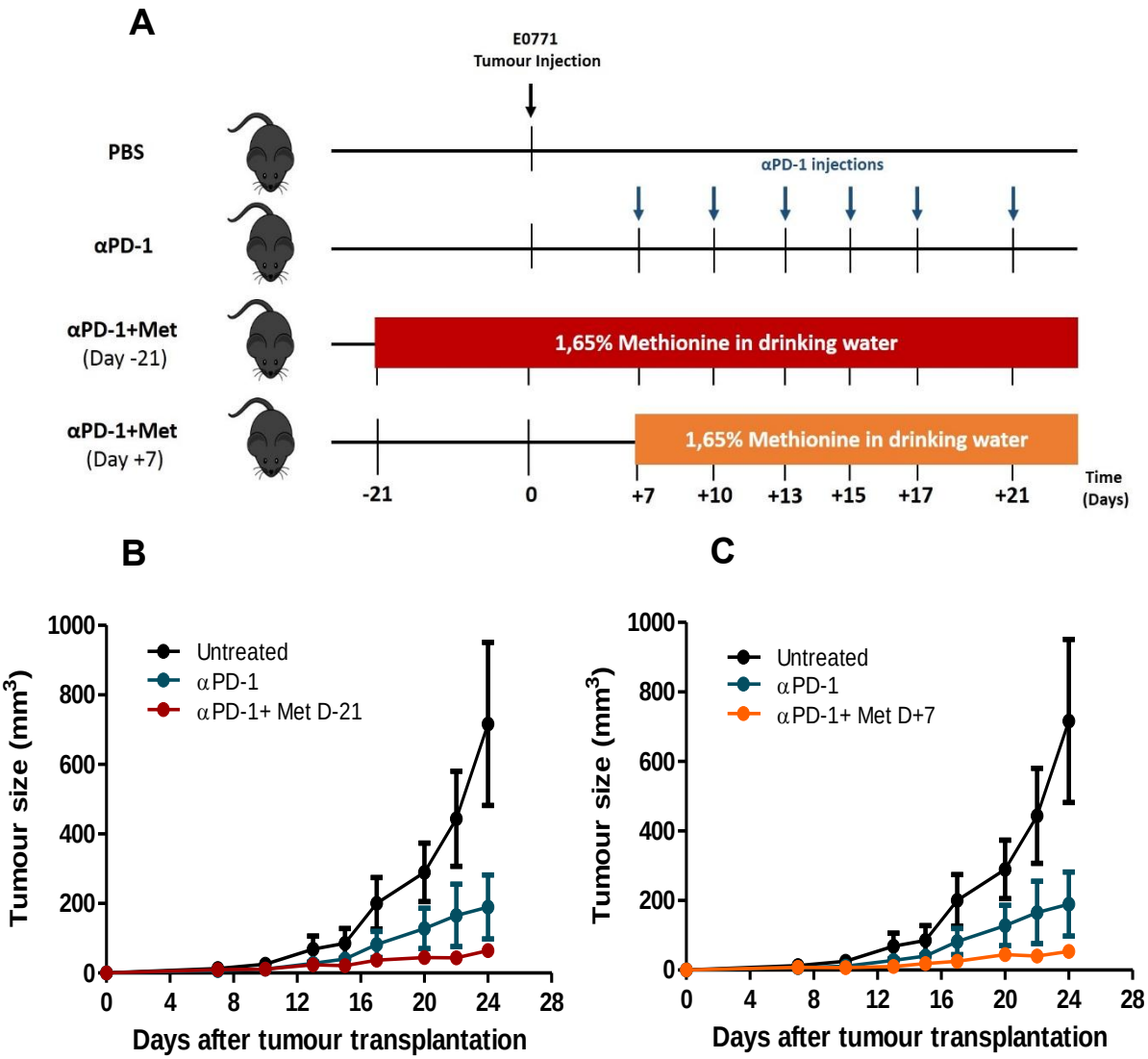


Figure 6. Methionine supplementation improves anti-PD-1 treatment efficacy in E0771 breast cancer tumour model. (A) In vivo experimental design: tumour transplantation (black arrow), αPD-1 injections (blue arrows) and methionine supplementation (21 days before or 7 days after tumour injection - red or orange bar, respectively). E0771 tumour growth in untreated C57BL/6J WT mice (black line; n=3) and αPD-1 treated C57BL/6J WT mice (Blue line; n=4). **(B)** E0771 tumour growth in αPD-1 treated and methionine supplemented C57BL/6J WT mice (dark red line; n=3) 21 days before tumour transplantation. **(C)** E0771 tumour growth in αPD-1 treated and methionine supplemented C57BL/6J WT mice (orange line; n=3) 7 days after tumour transplantation.

In order to understand whether methionine supplementation alone could have an impact on tumour growth, we supplemented WT mice with methionine 7 days after tumour transplantation. As shown in Figure 7, tumour growth was similar between the methionine supplemented and unsupplemented mice, suggesting that methionine alone failed to have a significant impact on tumour growth in E0771-bearing mice. Taken together these results suggest that the protective role of methionine is dependent on combination with anti-PD-1 monoclonal antibody.

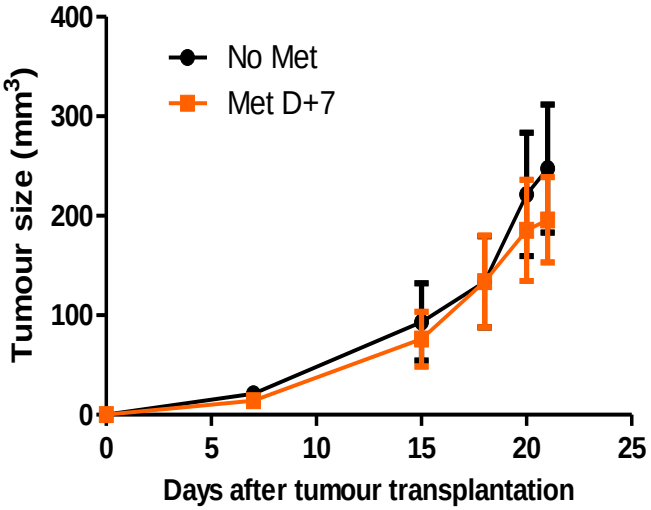


Figure 7. Methionine supplementation alone fails to inhibit E0771 breast cancer tumour growth. E0771 tumour growth in unsupplemented C57BL/6 WT mice (black line; n=5) and in methionine supplemented C57BL/6 WT mice (orange line, n=4) 7 days after tumour transplantation.

2. Anti-PD-1 plus methionine effect is dependent on lymphoid immune cells

In order to understand if the immune system is important for the anti-PD-1 + methionine combinatory effect on tumour growth, we tested the efficacy of the treatment in an immunosuppressed mouse strain. This strain, Rag2γcKO, is knock out for RAG2 and γ common chain genes. RAG2 gene is involved in the development of mature T and B cells and γ common chain is a component of several cytokine receptors and it is important in activation and maturation of NK cells (Nishana & Raghavan, 2012) (Pulliam, Uzhachenko, Adunyah, & Shanker, 2016). Our results suggest that anti-PD-1 + methionine treatment efficacy is dependent on the immune system, more particularly on lymphocytes because the effect of anti-PD-1 + methionine treatment on tumour growth was completely lost in this strain (Figure 8).

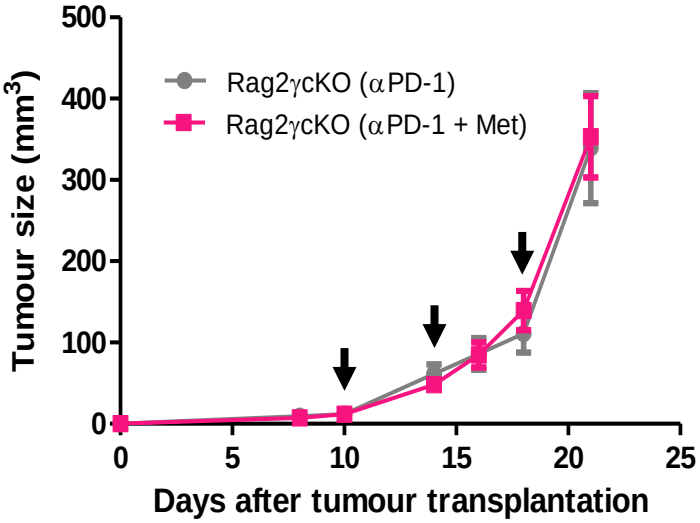


Figure 8. Anti-PD-1 + methionine effect is Lymphocyte-dependent. E0771 tumour growth in αPD-1 treated Rag2γcKO mice (grey line; n=4) and in αPD-1 treated and methionine supplemented Rag2γcKO mice (pink line; n=5). Methionine supplementation started at day 10 after tumour transplantation. αPD-1 injections were made every 4 days from day 10 to day 22 after tumour transplantation (black arrows).

3. Anti-PD-1 and methionine combination boosts anti-tumour immune response

Aiming at proving our hypothesis that methionine in combination with anti-PD-1 therapy can modulate the anti-tumour immune response, tumours from non-treated, anti-PD-1-treated and anti-PD-1- and methionine- treated mice were harvested 24 days after tumour inoculation and tumour cell suspension was analysed by flow cytometry. We found an accumulation of tumour-infiltrating CD8 T cells anti-PD-1- and methionine- treated mice in comparison to the non-treated or anti-PD-1-treated mice (Figure 9A). Furthermore, we also observed that these tumour-specific T cell subtype showed an increased production of IFNγ in anti-PD-1- and methionine treated mice compared to untreated or anti-PD-1 treated groups (Figure 10A). In fact, this could be an explanation for the combinatorial effect of anti-PD-1 and methionine in delaying the tumour growth, since CD8 T cells are reported to be the major anti-tumour immune cells contributing to tumour delay in anti-PD-1 therapies (S. A. Patel & Minn, 2018). Moreover, an increased frequency of tumour-infiltrating γδ T cells (Figure 9B) was observed in anti-PD-1- and methionine treated mice compared to untreated or anti-PD-1 treated mice. Tumour-infiltrating γδ T cells can play a critical role in promoting tumour growth by producing IL-17, a pro-tumour cytokine involved in angiogenesis (Wakita et al., 2010). Here, we observed a reduced accumulation of IL-17-producing γδ T cells in tumours from anti-PD-1- and methionine-treated mice (Figure 10B), which can contribute to limit the tumour growth. Consistent with what we were expecting, the total number of tumour immune infiltrate cells ($CD45^+$ cells) increased in anti-PD-1- and methionine- treated mice in comparison to the non-treated or anti-PD-1-treated mice (Figure 9C). All together, these data corroborate our previous finding in which we have shown that the combinatorial effect of anti-PD-1 and methionine is immune cell dependent, more specifically on T cells.

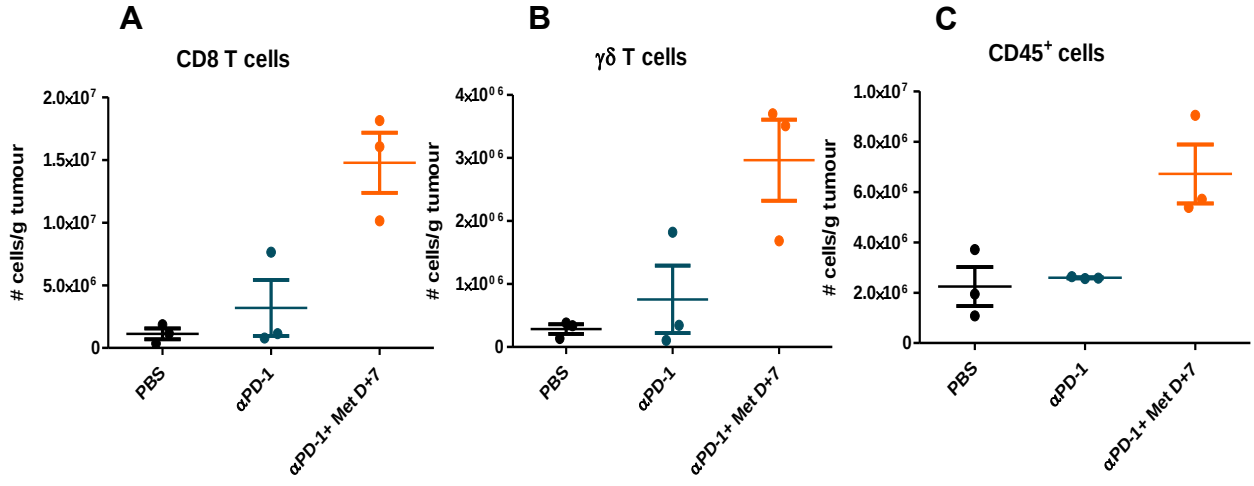


Figure 9. Combination of anti-PD-1 + methionine induced an increase in tumour-infiltrating immune cells. Graphs displaying the total number of **(A)** tumour-infiltrating CD8 T cells, **(B)** tumour-infiltrating γδ T cells and **(C)** immune cells infiltrating the tumour ($CD45^+$ cells) per gram of tumour in non-treated (black circles), αPD-1 treated (blue circles) or αPD-1 treated and methionine supplemented (orange circles) C57BL/6J WT mice.

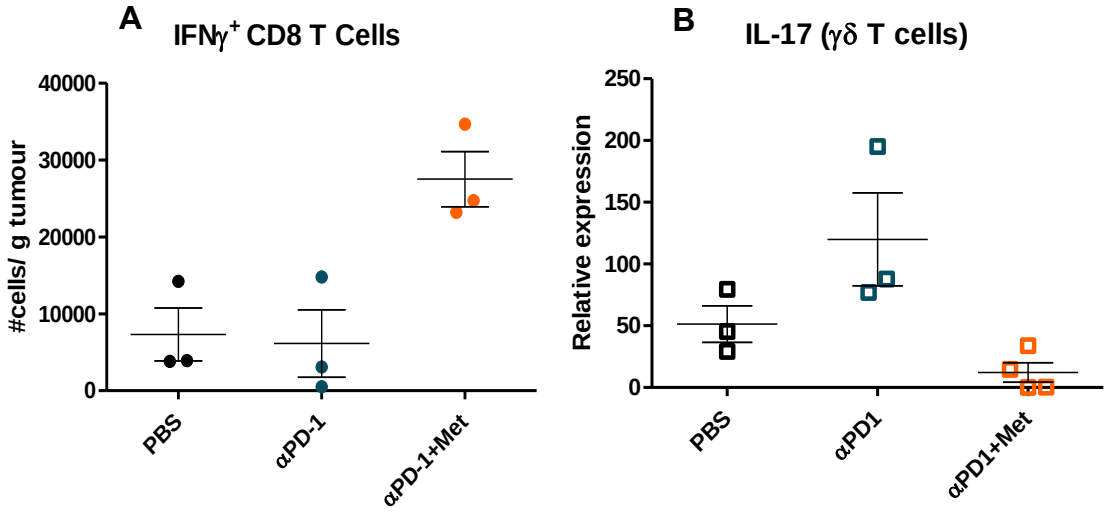


Figure 10. Anti-PD-1 and methionine supplementation enhances anti-tumour functions of CD8 T cells and limits pro-tumour functions of γδ T cells in the tumour bed. (A) Graph displaying the total number of IFNγ-producing tumour-infiltrating CD8 T cells per gram of tumour in non-treated (black circles), αPD-1 treated (blue circles) or αPD-1 treated and methionine supplemented (orange circles) C57BL/6J WT mice. **(B)** Relative mRNA expression of Interleukin 17 (IL-17) in FACS-sorted γδ T cells from tumour samples from non-treated (black squares), αPD-1 treated (blue squares) or αPD-1 treated and methionine supplemented (orange squares) C57BL/6J WT mice. Results were normalized to the housekeeping genes *b2microglobulin (b2m)* and *beta-actin (β-actin).*

4. Anti-PD-1 therapy favours methionine transport in tumour-infiltrating CD8 T cells

It has been shown that naïve T cells do not express the cystine transporter, however its expression is upregulated upon T cell activation (Garg et al, 2011). We hypothesized that the same could happen to methionine transporters. As anti-PD-1 antibodies led to an increased activation of T cells, this treatment could lead to increased expression of methionine transporters. This hypothesis would be in agreement with the previous results showing that methionine supplementation only leads to a delay in tumour growth when combined with anti-PD-1 therapy. Aiming at testing our hypothesis, we collected tumour samples from non-treated and anti-PD-1 treated WT mice to assess the relative expression of some genes involved in methionine uptake, i.e., amino acid transporters, in tumour-infiltrating T cells. Since methionine does not have a specific transporter, we analysed several amino acid transporters responsible for the uptake of large neutral amino acids, such as SLC7A5, SLC7A6, SLC7A7 and SLC7A8 in order to know if anti-PD-1 treatment could improve the methionine uptake on T cells (Fotiadis et al., 2013). Interestingly, tumour-infiltrating CD8 T cells showed an increased expression of SLC7A5 in anti-PD-1 treated mice compared to non-treated mice (Figure 11). Since SLC7A5 is an amino acid transporter that exhibits greater affinity for essential amino acids, more

precisely for histidine, isoleucine and methionine (Scalise, Galluccio, Console, Pochini, & Indiveri, 2018), our data indicates that CD8 T cells are favoured with the methionine supplementation because PD-1 blockade seems to enhance its methionine uptake. Indeed, our finding is in agreement with what is published, since it is reported that SLC7A5 is an amino acid transporter required for the full activation of T cells (Hayashi, Jutabha, Endou, Sagara, & Anzai, 2013). This data suggests that anti-PD-1 treated mice induce an increase of methionine uptake in tumour-infiltrating CD8 T cells which turn them potential immune cell targets to benefit from methionine supplementation.

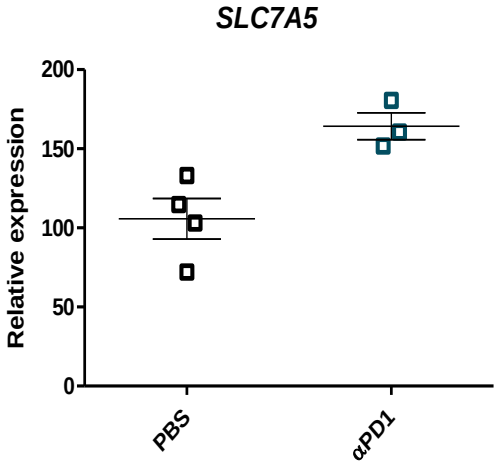


Figure 11. Tumour-infiltrating CD8 T cells from anti-PD-1 treated mice display a higher ability to uptake the methionine amino acid. Relative mRNA expression of SLC7A5 amino acid transporter in FACS-sorted CD8 T cells from tumour samples from non-treated (black squares) and αPD-1 treated (blue squares) C57BL/6J WT mice. Results were normalized to the housekeeping genes *b2microglobulin (b2m)* and *beta-actin (β-actin).*

5. Immune cell contribution for anti-PD-1 plus methionine treatment efficacy

As we have shown previously, anti-PD-1 treated and methionine supplemented mice showed an increase in tumour immune infiltrate and an accumulation of certain T lymphocytes. However, it remained unclear which immune cell types are indispensable or dispensable for the delay in tumour growth observed upon anti-PD-1 + methionine treatment Moreover, the phenotype does not stand in Rag2gc-/-, which suggests that T, B or NK cells are important for the effect of anti-PD-1 and methionine. Although we cannot discriminate between T, B and NK cells role, most likely B and NK cells are not involved because we did not see an accumulation of these cells in the tumour and tumour-draining lymph node from anti-PD-1 + methionine treated WT mice in comparison to anti-PD-1-treated mice (data not shown).

Our first candidate was CD8 T cell, since it is one of the most effective killer immune cells in mediating anti-tumour immune responses (Hadrup, Donia, & Thor Straten, 2013) and we have seen an accumulation of IFNγ-producing CD8 T cells (Figure 10B). To address this question, we have chosen the cell depletion approach through injection of depleting-antibodies (i.p and i.t.). Therefore, CD8 T cells from WT mice were depleted using anti-CD8 monoclonal antibodies aiming to deplete this subset and to understand its contribution to the anti-PD-1 + methionine effect. Thus, when we depleted CD8 T cells from anti-PD-1 + methionine treated mice the treatment no longer had a beneficial effect (Figure 12). This finding suggests that the effect of anti-PD-1 and methionine treatment is CD8 T cell-dependent.

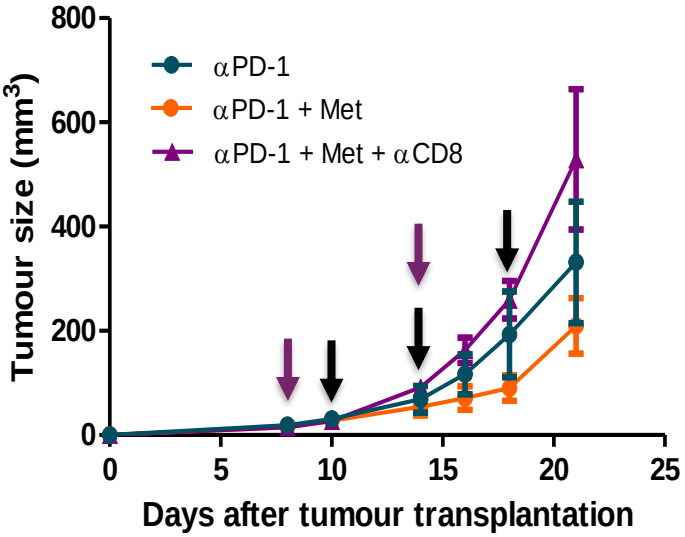


Figure 12. Anti-PD-1 + methionine effect is CD8 T cell-dependent. E0771 tumour growth in αPD-1 treated C57BL/6J WT mice (dark blue line; n=4), αPD-1 treated and methionine supplemented C57BL/6J WT mice (orange line; n=4) and αPD-1 treated, methionine supplemented and CD8 T Cell depleted C57BL/6J WT mice (purple line; n=5). The purple arrows represent the αCD8 antagonist antibody injections and the black arrows indicate the αPD-1 injections. Methionine supplementation (1,65% in drinking water) started at day 10 after tumour transplantation.

Next, we wanted to know if γδ T cells could have a key role in the tumour growth delay observed in anti-PD-1 + methionine treated mice. Aiming at testing our hypothesis, TCRδKO mice were anti-PD-1-treated or anti-PD-1- and methionine-treated. These specific mice are knock out for the T cell receptor δ chain gene, which compromises the development and maturation of γδ T Cells (Itohara et al., 1993). As shown in Figure 13, the tumour growth curve of anti-PD-1 + methionine treated TCRδKO mice suggests not only that γδ T cells are necessary for the effect of methionine supplementation to occur in anti-PD-1-treated mice but also that methionine supplementation in the absence of γδ T cells may become detrimental.

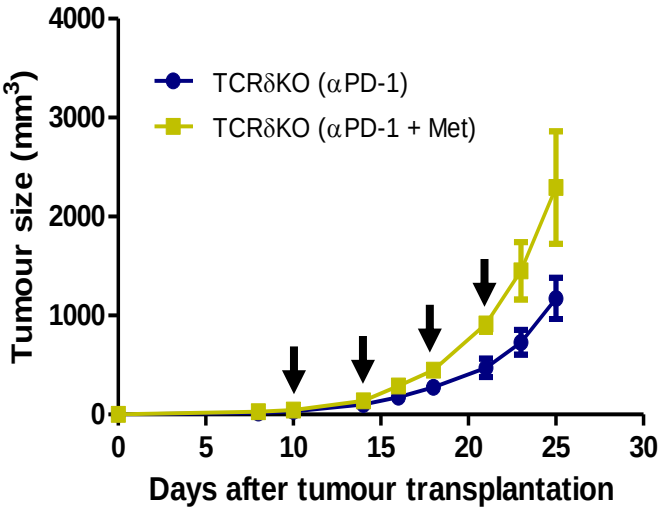


Figure 13. γδ T Cells seem to play a role in the αPD-1 + Met combination effect. E0771 tumour growth in αPD-1 treated TCR**δ**KO mice (dark blue line; n=5) and in αPD-1 treated and methionine supplemented TCR**δ**KO mice (light green line; n=4). Methionine supplementation started at day 10 after tumour transplantation. αPD-1 injections were made every 4 days from day 10 to day 22 after tumour transplantation (black arrows).

Furthermore, our data showed a decreased frequency of TNFα and IFNγ producing CD8 T cells in anti-PD-1 + methionine treated TCRδKO mice (Figure 14), which may help to explain the increased tumour growth in methionine supplemented mice (Figure 13).

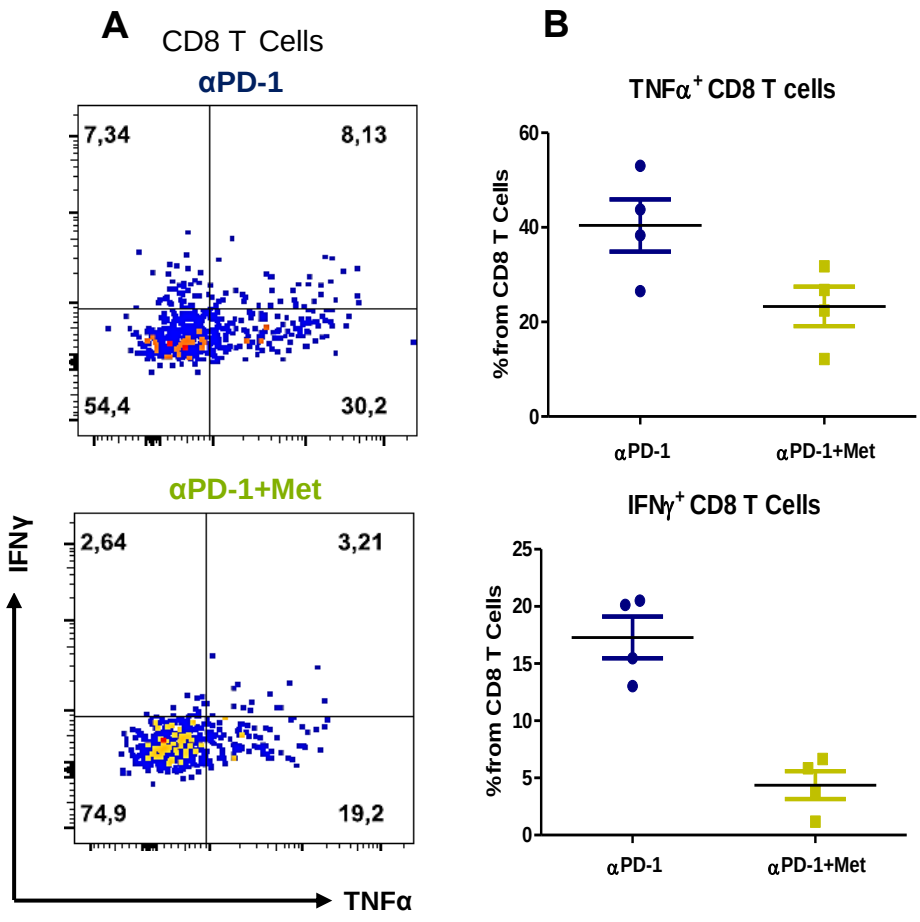


Figure 14. Pro-inflammatory cytokine production by CD8 T cells in the tumour is decreased in αPD-1- and methionine- treated TCRδKO mice. (A) Representative FACS plots for cytokine production by CD8 T cells. Pre-gated on CD45+ cells, lymphocytes (based on side scatter/forward scatter analysis) and $CD3^{+}CD8^{+}$ cells. **(B)** Frequency of TNFα-producing CD8 T cells or IFNγ-producing CD8 T cells in the tumour of αPD-1 treated or αPD-1 treated and methionine supplemented TCR**δ**KO mice.

Interestingly, the increased number of tumour-infiltrating CD8 T cells upon anti-PD-1 + methionine treatment in WT mice failed to occur in anti-PD-1 + methionine treated TCRδKO mice (Figure 15). These findings suggest a possible role for γδ T cells in inducing CD8 T cell accumulation and therefore suggest a key anti-tumour role for γδ T cells, promoted by anti-PD-1 and methionine supplementation, through potentiation of anti-tumour CD8 T cell responses.

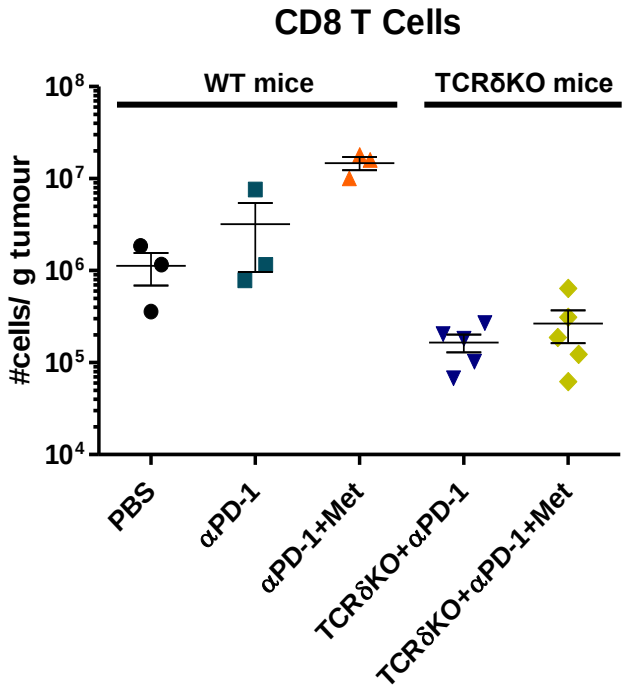


Figure 15. Tumour-infiltrating CD8 T cell number is no longer increased upon treatment, in the absence of γδ T cells. Graph displaying the total number of CD8 T cells infiltrating the tumour per gram of tumour in non-treated (black circles), αPD-1 treated (blue circles) or αPD-1 treated and methionine supplemented (orange circles) C57BL/6J WT mice and in αPD-1 treated (dark blue circles) or αPD-1 treated and methionine supplemented (light green circles) TCRδKO mice.

DISCUSSION

In this thesis we show, for the first time, the protective role of methionine in T cell-mediated responses. Here we demonstrate that *in vivo* methionine supplementation increases the efficacy of anti-PD-1 immunotherapy in TNBC-bearing mice and may potentially lead to a crosstalk between γδ and CD8 T cells. The accumulation of tumour-infiltrating γδ T cells induced by anti-PD-1 therapy and methionine supplementation seems to induce the accumulation of IFNγ-producing CD8 T cells, thus enhancing their anti-tumour properties and ability to kill tumour cells (Figure 16). Moreover, anti-PD-1 single therapy increases the SLC7A5 amino acid transporter expression on CD8 T cells, which potentially renders them more proficient to uptake methionine and benefit from the effects of this amino acid. The mechanism that γδ T cells employ to promote CD8 T cell accumulation and anti-tumour function is still to be determined, as well as the role of methionine in potentiating anti-tumour functions within T cells.

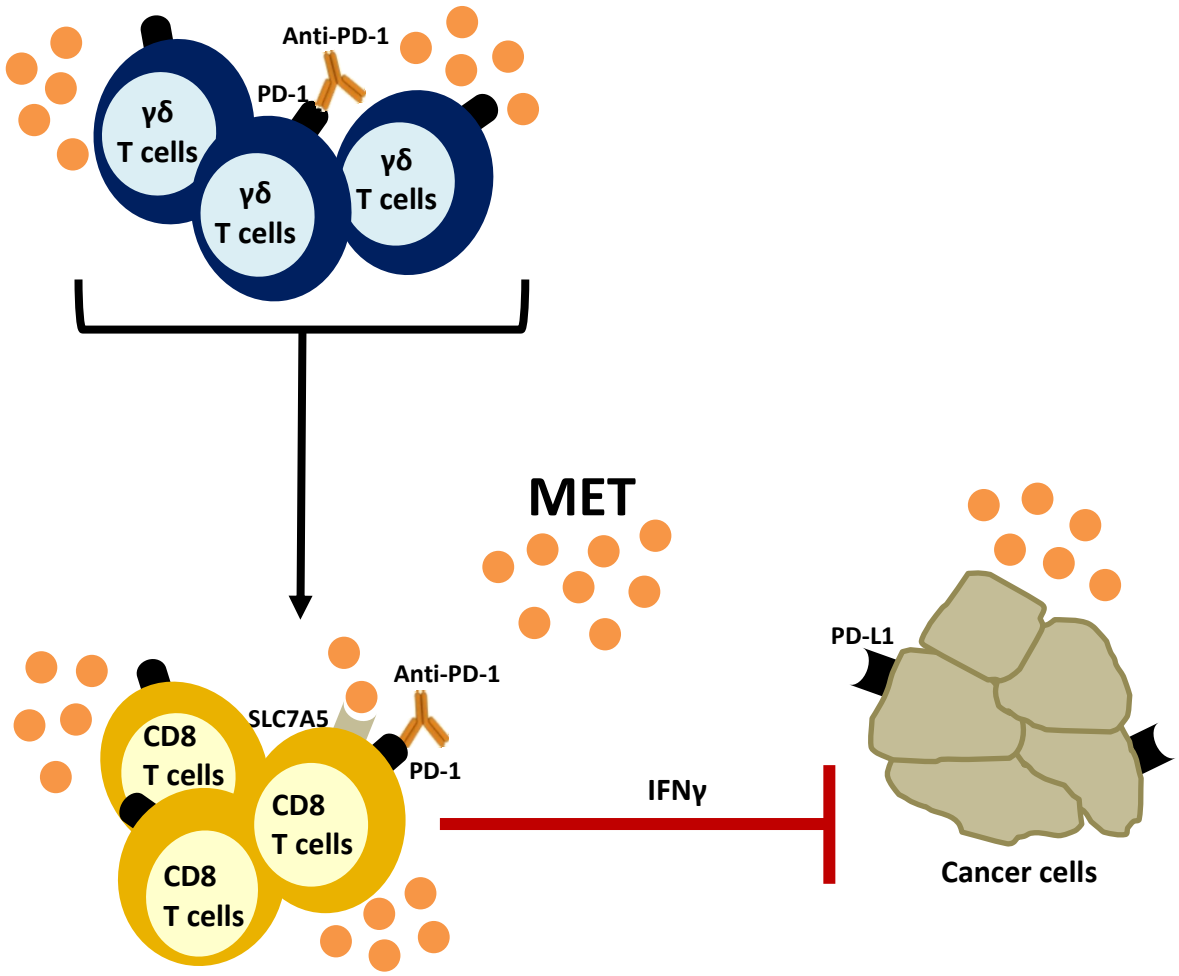


Figure 16. Schematic representation of the proposed working model in this thesis

1. Amino acid metabolism and T cell anti-tumour responses

It is known that individual dietary supplementation of some amino acids has an impact in leucocyte metabolism, including gluconeogenesis, generation of antioxidant molecules, cell activation status and even in the production of some cytokines involved in an infection challenge (P. Li, Yin, Li, Woo Kim, & Wu, 2007). Furthermore, there are specific amino acids known to positively impact anti-tumour immune responses by modulating T cell metabolism in the tumour microenvironment. Low arginine levels, for instance, are known to induce CD3ζ chain downregulation, decreased cytokine production and inhibition of T cell proliferation (Rodriguez et al., 2002). In addition, the lack of tryptophan caused by IDO-expressing cells in the tumour site results in a decrease of tumour-infiltrating immune cells and negatively affects the proliferation, effector function and survival of T lymphocytes (Godin-Ethier, Hanafi, Piccirillo, & Lapointe, 2011). Similarly, glutamine deprivation by tumour-derived glutaminase is strongly related to inhibition of T cell activity by decreasing IL-2 production and IL-2 receptor expression in T lymphocytes (Gottfried, Kreutz, & Mackensen, 2012; Newsholme, 2001). Therefore, a balanced extracellular amino acid content is needed to ensure proper performance during T cell-mediated immunity against cancer cells.

Currently, *in vivo* supplementation of the arginine amino acid is a strategy that is being used in combination with other anti-tumour agents in order to generate strong anti-tumour T cell responses in tumour-bearing mice. Cao et al demonstrated that L-arginine supplementation reduces tumour growth and increases the survival in breast tumour-bearing mice when combined with the chemotherapeutic agent docetaxel. These anti-tumour effects were associated with an improved proportion of systemic $CD8^+$ CTL and $CD4^+$ Th1 effector cells, as well as increased intra-tumour levels of IFNγ (Cao et al., 2016). Another study showed the improved efficacy of anti-PD-L1 immunotherapy when osteosarcoma-tumour bearing mice are orally administrated with L-arginine for 4 weeks. Of note, L-arginine supplementation significantly elevated the number of tumour-infiltrating CD8 T cells and these T lymphocytes displayed a higher cytotoxic profile due the anti-PD-L1-mediated protection from exhaustion (X. He et al., 2017). However, the molecular mechanisms of L-arginine supplementation that leads to an improved anti-tumour immune response is still unknown as well as the supplementation impact of other amino acids in the context of tumour immune surveillance.

Methionine is an amino acid that can impact the antioxidant capacity of immune cells, thus protecting them from reactive oxygen species commonly produced by tumour cells and some myeloid subsets present in the tumour side (Martínez et al., 2017). Thus, tumour-infiltrating immune cells, particularly T cells, may become less susceptible and more capable to proliferate, differentiate and to exert anti-tumour functions, upon increased methionine availability.

Here, in this thesis, we show that methionine supplementation improves the efficacy of anti-PD-1 immunotherapy in TNBC tumour-bearing mice. Thus, our work demonstrates for the first time the potential role of methionine supplementation in mounting anti-tumour immune responses. However,

the molecular mechanisms responsible for this increased treatment efficacy are still to be determined.

2. Impact of PD-1-inhibitor and methionine supplementation in cellular metabolism of T lymphocytes

It is reported that anti-PD-1 therapy frequently increases the proliferation and effector functions of tumour-infiltrating T lymphocytes that normally trigger anti-tumour immune responses and consequently a reduction of tumour size from melanoma cancer patients (Tumeh et al., 2014). Surprisingly, we only observed an accumulation of tumour-infiltrating immune cells, particularly infiltration of CD8 and γδ T lymphocytes in mice treated with anti-PD-1 antibody and supplemented with methionine. Indeed, anti-PD-1 is shown to accelerate γδ T cell clonal proliferation during prolonged Hodgkin lymphoma-associated inflammation (Ono et al., 2018). Also, an increased proliferation of CD8 T cells in peripheral blood can be detected in advanced stage non–small cell lung cancer patients after PD-1 therapy (Kamphorst et al., 2017). We postulate that the reason we did not see a significant increase of effector γδ T and CD8 T cells with the anti-PD-1 single treatment is due to the fact that breast cancer is less immunogenic and with a lower PD-L1 expression compared with other tumour types like the malignant melanoma (S. P. Patel & Kurzrock, 2015). Thus, the use of anti-PD-1 alone in TNBC may not be enough to induce a strong activation, proliferation and cytokine production by tumour-infiltrating T cells, since PD-1/PD-L1-mediated inhibition is not so strong as it is in melanoma patients. Also, we postulate that the decreased tumour growth observed in the anti-PD-1 treated group compared to non-treated group is a result of a partial activation of TILs which ultimately causes a delay on tumour growth. Despite having an impact on tumour growth, the anti-PD-1 single treatment is not sufficient to induce a significant activation and accumulation of anti-tumour T cells, which is only achieved when methionine is combined with anti-PD-1 antibody. Thus, when we combine methionine supplementation with anti-PD-1 therapy the frequency of γδ and IFN-g-producing CD8 T cells increases. These findings show the potential ability of methionine to modulate anti-tumour immune responses by interfering with proliferation and/or recruitment as well as function of tumour-infiltrating T cells. In addition to increase CD8 effector functions, methionine seems to upregulate MHC-I expression on tumour cells which renders them more susceptible to be killed by CD8 T cells (Supplementary Figure 1). Furthermore, methionine has the ability to revert the upregulation of IL-17 in tumour-infiltrating γδ T cells caused by anti-PD-1 single treatment. Curiously, the relative expression of IL-17 in γδ T cells from anti-PD-1 treated and methionine supplemented mice is even lower than untreated mice, thus suggesting that the combined treatment has a major negative impact in the IL-17 production by γδ T cells. Since IL-17 is a cytokine associated to angiogenesis, tumour promotion and inhibition of cytotoxic T lymphocytes (Hayata et al., 2013), its downregulation by the combined treatment in the tumour microenvironment favours anti-tumour T cell responses and limits the tumour growth.

It is reported that some cancer types exhibit an excessive requirement for methionine for the survival and proliferation of cancer cells, a phenomenon known as “methionine dependence” (Cavuoto & Fenech, 2012). A pioneer study about methionine dependency analysed human fresh patient tumours from 21 different tumour types and revealed a dependence on methionine in 5 human tumours, including tumours of the colon, breast, ovary, prostate, and melanoma (Hoffman, Herrera, Groce, & Hoffman, 1993). Moreover, *in vivo* studies have demonstrated that intra-tumour methionine level highly correlates with tumour volume in both the pancreatic cancer and melanoma patient-derived orthotopic xenograft nude mouse models (Kawaguchi et al., 2018). All together, these results contradict our findings about the supposed protective role of methionine supplementation shown in this work. We hypothesize that anti-PD-1 therapy in the E0771 model increases methionine consumption by tumour-infiltrating T cells thereby decreasing intra-tumour methionine availability and limiting tumour growth. The reason why methionine could be preferentially consumed by TILs is the fact that we believe that TNBC is a less methionine-dependent cancer type, which could also explain why methionine supplementation alone did not favour the tumour growth (Figure 7). Therefore, we postulate that this kind of treatment may only work in tumours that are not dependent on methionine.

However, it still remains unclear how methionine acts in the tumour-associated lymphocytes when combined with an anti-PD-1 inhibitor. It is known that the blockade of PD-1 induces an enhanced T cell activation and proliferation (Buchbinder & Desai, 2016) because tumour immunosuppression through PD-L1 engagement becomes less effective, thus making T lymphocytes more active and effective. In addition to the overexpression of glucose transporters, activated T cells also increase the expression of some amino acid transporters in order to sustain their metabolic needs (J. Wei et al., 2017). Indeed, we show here an increase in the relative expression of the SLC7A5 amino acid transporter in tumour-infiltrating CD8 T cells upon anti-PD-1 single treatment. This finding suggests that blockade of PD-1 signalling pathway in CD8 T cells increases their capacity to uptake extracellular amino acids, such as methionine. Therefore, we propose that PD-1 blockade favours methionine metabolization in tumour-infiltrating CD8 T cells, thus making these T lymphocytes potential cellular targets of the combined treatment. Most likely, the PD-1/PD-L1 interaction inhibits the transport of methionine on T cells, as it happens with the glutamine and branched-chain amino acids (Patsoukis et al., 2015). Thus, we hypothesize that methionine supplementation can only play a protective role when the PD-1 inhibitory signal is inhibited on tumour-infiltrating T cells.

Although we have not found evident differences in the enzymes related to the methionine metabolism nor in the methionine transporters in γδ T cells upon anti-PD-1 treatment, their accumulation in the tumour upon anti-PD-1 and methionine treatment is important to regulate anti-tumour CD8 T cell responses and crucial for the combined treatment to work. A recent study showed that the adoptive transfer of an autologous gamma delta subtype, Vγ9Vδ2 T cells, demonstrated low levels of PD-1 expression in that γδ T cell subset compared with CD8 T cells in the spleen of B-cell lymphoma-bearing mice (Zumwalde et al., 2017). This finding suggests that tumour-infiltrating γδ T cells may

display higher activation and proliferation rates compared to CD8 T cells prior treatment. Therefore, the supposed state of “low proliferation inhibition” of γδ T cells reflected by a low PD-1 expression may help to explain the lack of an increase in frequency of tumour-infiltrating γδ T cells upon anti-PD-1 single treatment (Figure 9B). However, γδ T cells seems to benefit with the methionine supplementation when it is combined with anti-PD-1 antibody, thus suggesting that methionine has a strong influence on potentiating the proliferation of this T cell subset, although we did not verify alterations in the expression of the methionine transporters in these cells. The protective role of γδ T cells and its impact on regulation of effector CD8 T cell responses upon anti-PD-1 treatment and methionine supplementation will be covered in detail in the next section.

3. Role of γδ/CD8 T cell crosstalk in tumour regression

We have shown that upon anti-PD-1 treatment and methionine supplementation, CD8 T cell anti-tumour phenotype is increased in the tumour site which correlates with the tumour growth delay. Moreover, CD8 T cells were shown to be crucial for the combined treatment to work, since their in vivo depletion resulted in the loss of the anti-PD-1 + methionine effect in tumour-bearing mice. Furthermore, the accumulation of γδ T cells in the tumour upon anti-PD-1 and methionine treatment seems to be important for the regulation of the CD8 T cell-mediated anti-tumour immune responses induced by the combined treatment. Our *in vivo* experiments in mice lacking γδ T cells demonstrate that the combination of anti-PD-1 and methionine does not lead to increased anti-tumour properties of CD8 T cells. This suggests that upon combined treatment γδ T cells may promote the accumulation and anti-tumour function of CD8 T cells, presumably through the production of a cytokine and/or chemokine. Besides, lack of γδ T cells upon combined treatment even impairs CD8 T cell anti-tumour effector functions, as evidenced by the decrease in TNFα and IFNγ-producing cells. We postulate that in the absence of γδ T cells, the decrease in CD8 T cell effector function is accompanied by a reduction in CD8 T cell methionine consumption. This probably results in excessive consumption of the extracellular methionine by tumour cells which favours its growth.

However, we did not dissect the mechanism by which γδ T cells could potentiate CD8 T cell-mediated anti-tumour responses upon anti-PD-1 and methionine treatment. Several mechanisms could potentially explain this phenomenon. It is known that mouse γδ T cells are an early source of IFNγ within the tumour microenvironment (Gao et al., 2003) and this is a known cytokine to recruit and activate CD8 T cells and enhance their responsiveness to tumours (Nakajima, Uekusa, & Iwasaki, 2001). However, we did not observe an upregulation of IFNγ in tumour-infiltrating-γδ T cells upon treatment with PD-1 inhibitor and methionine supplementation. Since γδ T cells are an important source of IFNγ in the early stage of the tumour development, we may have missed the overproduction of this cytokine in γδ T cells because we analyse tumours in a later stage of its development. Moreover, there are some subsets of γδ T cells that can produce chemoattractant molecules -chemokines- which are able to recruit effector immune cells into the tumour microenvironment (Latha et al., 2014). CXCL10 has been shown to be associated with the infiltration of tumour-associated CD8 T cells (Kryczek et al., 2009) and is commonly produced by human Vγ9Vδ2^{+} T cells and mouse

lung-resident γδ T cells (Vantourout & Hayday, 2013). Indeed, in our model, the combined effect of PD-1 blockade and methionine supplementation may have induced the CXCL10 production by γδ T cells which in turn could further recruit effector CD8 T cells into the tumour site, thus contributing for the tumour inhibition. Lastly, it is reported that activated human Vγ9Vδ2^{+} T cells have the ability to cross-present tumour antigens to CD8 T cells and are termed as γδ T-APCs (Brandes et al., 2009). After the tumour antigen processing, γδ T-APCs were shown to induce proliferation, target cell killing and cytokine production in both naïve and effector CD8 T cells. Nevertheless, all these possible mechanisms need to be further examined in our model in order to understand better the cellular crosstalk between γδ and CD8 T cells that results in a tumour growth delay upon combined therapy.

4. Limitations and future directions

In order to further consolidate our work, some relevant aspects need to be elucidated and new approaches should be explored. First of all, we need to quantify the methionine concentration within tumours from non-treated, anti-PD-1-treated and anti-PD-1 plus methionine treated mice, in order to correlate with our tumour growth results. Indeed, the water intake variability between mice can possibly interfere with our conclusions because we have considered that mice from the same group consume all the same amount of water and subsequently the same quantity of methionine. At least, the amount of water supplemented with methionine ingested by the mice belonging to the same group was similar, which provides greater veracity to our results. (Supplementary Figure 2). However, the lack of information about the amount of methionine that reaches the tumour of each mouse is a limitation of our study, which can be overcomed by the use of high-performance liquid chromatography (HPLC) techniques which are commonly used to precisely determine and quantify amino acids and its metabolites in several biological samples (Da Silva et al., 2016; Weaving, Rocks, Iversen, & Titheradge, 2006).

It may also be interesting to study the impact of cysteine supplementation on breast cancer anti-PD-1 immunotherapy efficacy. Although both amino acids can generate GSH molecules which are important to regulate the cellular redox and to fight the tumour-associated oxidative stress cysteine is a closer precursor of glutathione (GSH) compared to methionine (Bin, Huang, & Zhou, 2017; X. Chen et al., 2016). Thus, if cysteine has the same effect as methionine in delaying the tumour growth, we could more strongly postulate that anti-tumour immune responses mediated by the anti-PD-1 and methionine combination could be through the increased antioxidant properties of T cells. To formally confirm if the effect of methionine is through the induction of antioxidant properties the concentration of metabolites of methionine metabolism, including SAM, SAH, homocysteine, cystathionine and glutathione, needs to be evaluated by ELISA in both γδ and CD8 T cells. This analysis will determine if one subset relies more on methionine metabolism and if the anti-PD-1 and methionine combination can, in fact, increase the intracellular GSH levels which are correlated to anti-tumour T cell functions. In addition to analyse GSH production by γδ and CD8 T cells, it may be necessary to investigate the thiol distribution in the membrane of these T cells because it is directly correlated with a high ability to quench ROS and control tumour growth (Kesarwani et al., 2015).

The last and long-term goal is to try to reproduce the effect of our combined treatment in a genetic breast tumour model. These experimental models resemble sporadic human cancers more accurately by specifically controlling timing and location of mutations (Walrath, Hawes, Van Dyke, & Reilly, 2010). On the other hand, the tumour model used in this thesis is an orthotopic triple negative mammary adenocarcinoma (E0771) that is transplanted into the mammary fat pad of female mice. This is a tumour model characterized by very rapid tumour growth which does not allow normal interactions between tumour cells and the tumour microenvironment that normally occur in human tumours that grow slower. Thus, the elimination and equilibrium phase of cancer immunoediting do not occur and gradual stages of tumour development cannot be observed as well as in human tumours. Therefore, the oncogene-driven tumour mouse models would be the ideal tumour model to mimic the highly heterogenous tumours that we find in many human cancers (Eruslanov, Singhal, & Albelda, 2017).

5. Translation and implications of our findings

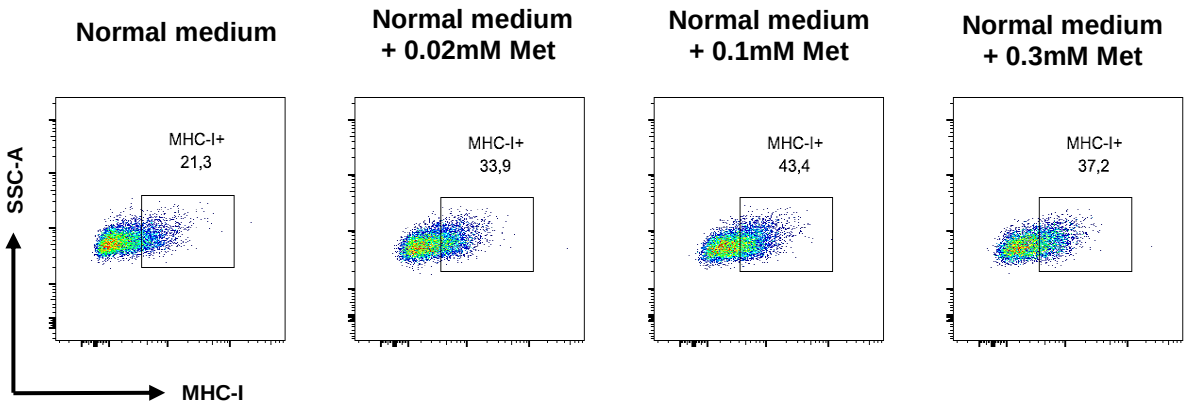
The absence of any specific targeted therapy for triple negative breast cancer (TNBC) limits the therapeutic options to chemotherapy, demonstrating the need for new therapeutic approaches (Wahba & El-Hadaad, 2015). Interestingly, TNBC is the breast cancer subtype that exhibits the higher PD-L1 expression on tumour cells as well as the higher level of tumour-infiltrating lymphocytes (Voutsadakis, 2016). All these tumour characteristics make TNBC attractive for immunotherapy, in particular for anti-PD-L1/anti-PD-1 therapy in this patient population. Although the number of clinical trials testing the PD-L1/PD-1 checkpoint blockade in TNBC is rapidly increasing, the overall response rate of single-agent anti-PD-1 treatment is lower than 20% (Emens, Kok, & Ojalvo, 2016; Nanda et al., 2016). One possible explanation for the low success rate could be a strong oxidative stress present in the tumour microenvironment that limits the anti-tumor immune response. Indeed, clinical studies demonstrated that breast tumour tissues display high levels of lipid peroxidation and protein oxidation likely due to a strong oxidative stress present in the tumour microenvironment of breast cancer women (Mannello et al., 2007; Mannello, Tonti, & Medda, 2009). Therefore, therapeutic strategies that aim to reduce the tumour oxidative stress may be a viable approach to improve the outcome of breast cancer patients.

In our study we successfully demonstrated that *in vivo* supplementation of methionine, an amino acid known to increase the cellular antioxidant capacity (Martínez et al., 2017), was able to improve the efficacy of anti-PD-1 immunotherapy in TNBC. Thus, our work may represent an alternative treatment strategy for TNBC which potentiates the anti-tumour immune response through a cellular crosstalk between γδ and CD8 T cells. Furthermore, because methionine can improve the responsiveness to PD-1 blockade and subsequently increases the T cell infiltrate, anti-PD-1 and methionine combination may be able to convert “cold” breast tumours to “hot” tumours, i.e., low-response to high-response tumours to immune checkpoint inhibitors (Tolba & Omar, 2018).

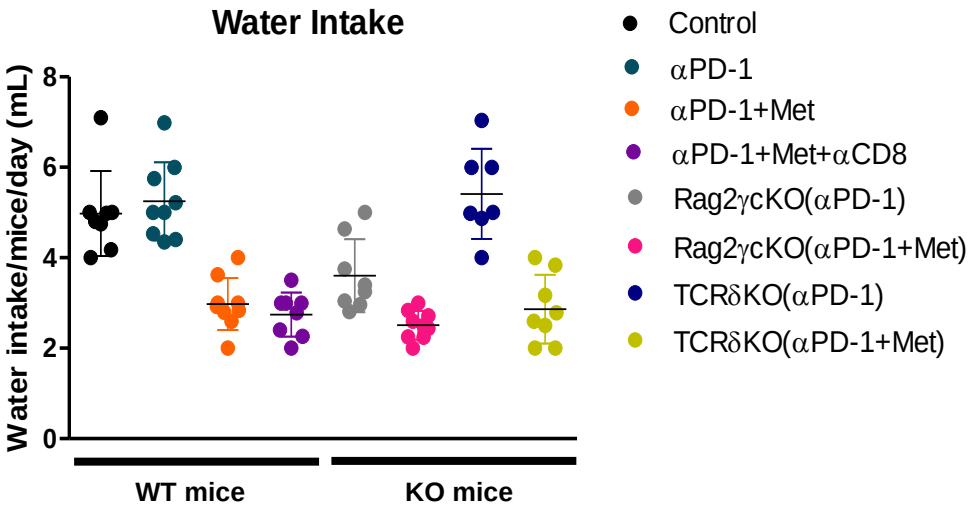
The tumour resistance to immune checkpoint blockade therapy is frequently associated to a high immunosuppressive tumour environment, where ROS are generally in abundance (Zhao & Subramanian, 2017). So, since methionine is involved in the synthesis of the main intracellular antioxidant, GSH, its combination with anti-PD-1 therapy may thus be a promising strategy to overcome treatment resistance in breast cancer patients by reducing immunosuppression mediated by ROS.

In conclusion, our work demonstrates promising results in the search for more effective treatments for TNBC which is very hard to treat with the conventional therapies. Moreover, the protective role of methionine supplementation was described for the first time in this thesis. This means that methionine may have the ability to improve anti-tumour immunity and the supplementation of this amino acid should be considered in other immunotherapeutic combination strategies. Further studies about the toxicity profiles of the investigated combination therapy are needed to confirm adequate safety and tolerability. Also, studies to generalize the findings to other models, even other types of tumours are needed.

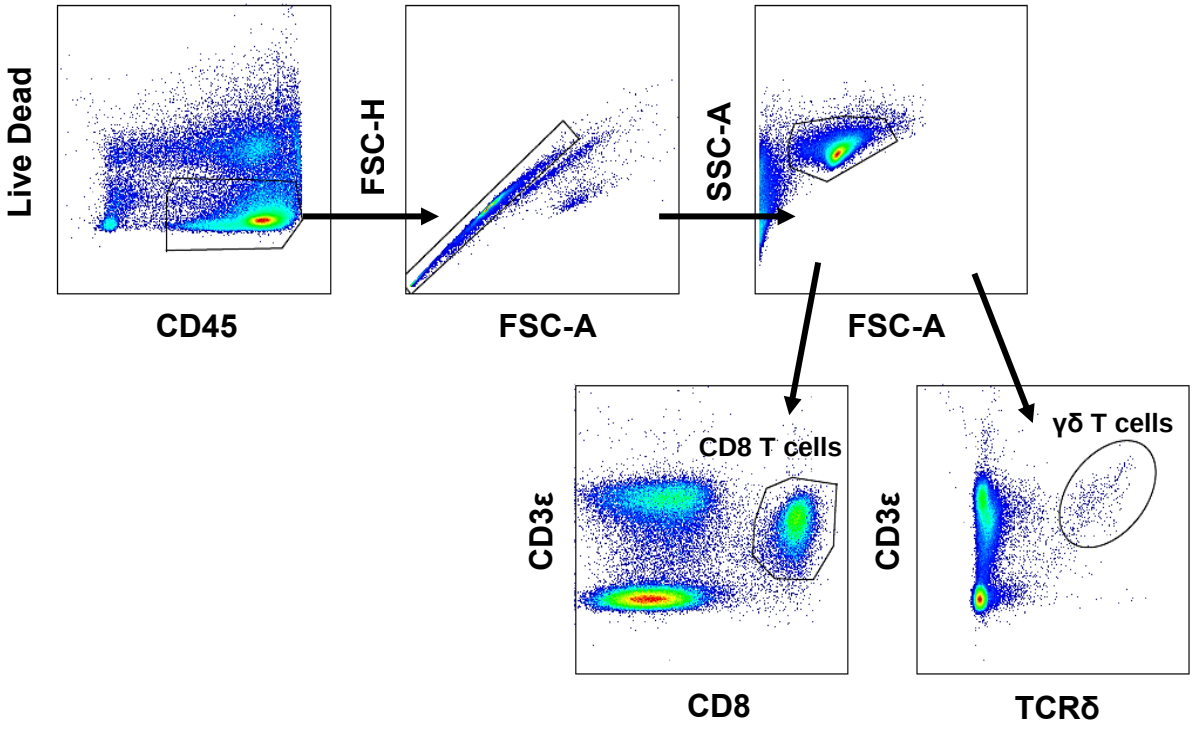
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Methionine supplementation increases the frequency of apoptotic E0771 tumour cells and MHC-I expression in vitro. Representative FACS plots of MHC-I expression in E0771 tumour cells supplemented with different methionine concentrations for 3 days (37ºC).



Supplementary Figure 2. All methionine supplemented mice drink the same water quantity. Graph displaying the water intake (mL) per mice and per day in C57BL/6J WT (black, blue, orange and green circles), Rag2γcKO (grey and pink circles) and TCRδKO (dark blue and light green) mice, untreated, αPD-1 treated or αPD-1 treated and methionine supplemented. Each dot on the graph represents a mean of water intake by each group of mice.



Supplementary Figure 3. Gating strategy used for FACS analysis of CD8 and γδ T lymphocytes.

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