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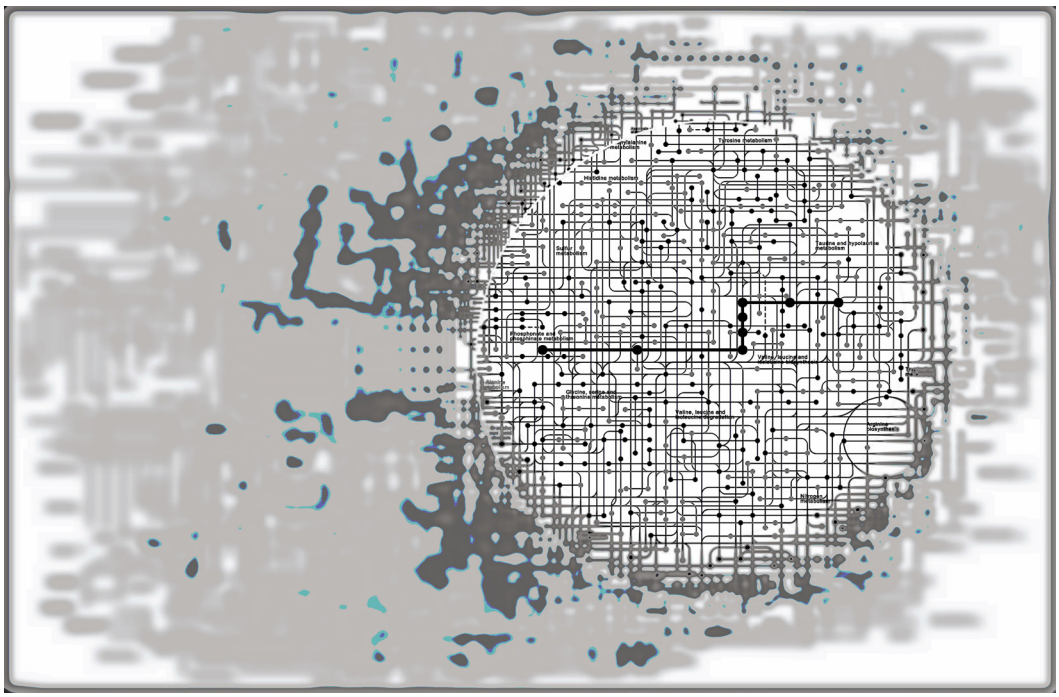
Immunometabolic network interactions of the kynurenine pathway in cutaneous malignant melanoma



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IMMUNOMETABOLIC NETWORK INTERACTIONS OF THE KYNURENINE PATHWAY IN CUTANEOUS MALIGNANT MELANOMA

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TO MY LOVELY FAMILY

KEY WORDS

*: The graph was created using an online word cloud tool (http://www.wordle.net).
It was based on the text content of this thesis, excluding acknowledgements, references and the constituent research articles.

ABSTRACT

Cancer is the second leading cause of mortality worldwide, and melanoma represents the most aggressive and deadliest form of skin cancer. Despite the various therapeutic approaches, metastatic melanoma is a disease with a poor prognosis. Recently, the evolution of immune checkpoint inhibitors (ICIs) led to a substantial improvement of the overall survival of patients. However, the long-term effectiveness of such treatments is restricted by the sometimes rapidly emerging resistance to treatment. Several molecular mechanisms underlying this resistant phenotype have begun to be elucidated. The Kynurenine pathway activity via indoleamine 2, 3-dioxygenase 1 (IDO1), is one such mediator of immunosuppression and resistance to ICIs.

Studies included in this thesis, therefore, aim to clarify the role of the kynurenine pathway (KP) in metastatic cutaneous melanoma. To this end, we established an *in vitro* co-culture model consisting of CD4 +T cells in culture with different melanoma cell lines (MCLs) to investigate the implication of KP modifications on CD4 + T-cell function. We found that in addition to IDO1, other KP enzymatic activities such as KMO may regulate CD4 + T-cell immunity **(Study I)**. Following this finding, we evaluated the immune-metabolic network interactions of KP in CMM patients to explore the link between KP metabolites (KPMs) and regulation of the anti-tumour immune response. Our data showed a significant association between MAPKIs treatments and alterations of 3-HK and 3HAA concentrations. These results suggest that KP is clinically relevant in CMM patients **(Study II)**. We further aimed to identify possible KP-related predictive biomarkers of response to ICIs treatment (Study III, IV). Our findings demonstrate the elevated S100A9+ monocytes among PBMCs of the CMM patients who are not responding to the PD-1 inhibition **(Study III)**. Subsequently, by using the PBMCs and plasma of CMM patients on ICI therapy, we observed that kynurinase (KYNU) and LGALS3 (Galectin-3) expression in protein and RNA levels are negatively linked to clinical outcomes. Moreover, we found that the KYNU-LGALS3 network in monocytes is connected to the CD74-MYC network in CD4+ T-cells. These results suggest that LGALS3, MYC, CD74, and KYNU are biologically connected, and perturbing their interaction will possibly modulate ICI efficacy in CMM patients **(Study IV)**.

In summary, this thesis provides insights into the induction of n immune-suppressive phenotype by KP activation in CD4+ T-cells and demonstrates the therapeutic potential of targeting KP in the treatment of malignant melanoma.

LIST OF SCIENTIFIC PAPERS

I. **Soudabeh Rad Pour**#, Hiromasa Morikawa, Narsis A. Kiani, Muyi Yang, Alireza Azimi, Gowhar Shafi, Mingmei Shang, Roland Baumgartner, Daniel FJ Ketelhuth, Muhammad Anas Kamleh, Craig E. Wheelock, Andreas Lundqvist, Johan Hansson, Jesper Tegnér.
Exhaustion of CD4+ T-cells mediated by the Kynurenine Pathway in Melanoma, Sci Rep. 2019 Aug 21;9(1):12150. doi: 10.1038/s41598-019-48635-x. PMID: 31434983

II. **Soudabeh Rad Pour**#, Hiromasa Morikawa, Narsis A. Kiani, David Gomez-Cabrero, Alistair Hayes, Xiaozhong Zheng, Maria Pernemalm, Janne Lehtiö, Damian J. Mole, Johan Hansson, Hanna Eriksson, Jesper Tegnér
Immunometabolic network interactions of the kynurenine pathway in cutaneous malignant melanoma, Front Oncol. 2020 Feb 3;10:51. doi: 10.3389/fonc.2020.00051. eCollection 2020.PMID: 32117720

III. **Soudabeh Rad Pour***, Yago Pico de Coaña*, Xabier Martinez De Morentin, Jeroen Melief , Manjula P. Thimma, Maria Wolodarski, David Gomez Cabrero, Johan Hansson, Rolf Kiessling, Jesper Tegnér
The Immune Cell Composition of PBMCs in melanoma Patients and their Association with Response to Nivolumab, Manuscript

IV. **Soudabeh Rad Pour**, Hanna Eriksson , Johan Hansson, Jesper Tegnér
Kynurenine pathway activity predict primary resistance to immune checkpoint blockade in cutaneous malignant melanoma, Manuscript

SUPPORTING RESULTS

Sunjay Fernandes*, **Soudabeh Rad Pour***, Manjula P. Thimma, Faiez Al Nimer, Alexsandra Gyllenberg, Fredrik Piehl, David Gomez Cabrero, Ingrid Kockum, Jesper Tegnér
Single cell transcriptomics of paired blood and cerebrospinal fluid of multiple sclerosis patients with special focus on the immune repertoire, Manuscript

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*, Equal contributions

PREFACE

My introduction to the world of tryptophan as essential amino acid and its metabolism by kynurenine pathway developed from my wish to find new ways to better grasp the biology of melanoma tumours and immunosuppression, hoping for finding new treatment strategies. As my research continued, I began to search the field of tryptophan for possible research collaborators, reagents, and supplies, and I was surprised to note that tryptophan catabolism plays an important role not only in health but also in a broad spectrum of human diseases. I was astonished to learn that the kynurenine pathway was acknowledged as a leading player in various diseases, including inflammation, cardiovascular disease, respiratory disease, psychiatric disorders, neurodegenerative diseases, and stem cell biology. Although investigation of the kynurenine pathway association with melanoma is still in its infancy, the picture for the field of tryptophan metabolism is quite mature. The research described in my thesis provides a link between the basic mechanistic understanding of the kynurenine pathway and clinically relevant translational applications. It investigates indications that tryptophan metabolism via the kynurenine pathway is a potential biomarker for disease activity, may subsidize to local and possibly systemic immune suppression in cancer, and is an attractive target in this field. Given the large number of people suffering from the disorders listed above, the potential clinical efficacy of drugs targeting the enzymes in the kynurenine pathway is truly promising.

This thesis could not have been completed without the contribution of my supervisor, professor Tegner, and my co-supervisors, professor Hansson, Dr. Lundqvist, and Dr. Morikawa. I am also incredibly grateful to my colleagues and lab-members for their support, assistance, and patience throughout my Ph.D. education. I hope that the readers will receive the same appreciation for the complexity in the field of kynurenine pathway that I did in the preparation of this thesis.

CONTENTS

LIST OF ABBREVIATIONS

AADAT	Aminoadipate aminotransferase
AP-1/AP-2a)	Activating protein 1 and 2α
BCR	B-cell receptor
CCBL1	KYAT1, kynurenine aminotransferase 1
CCBL2	KYAT3, kynurenine aminotransferase 3
CD274	Programmed death-ligand 1;B/-H1, PD-L1
CMM	Cutaneous malignant melanoma
CSF1R	Colony stimulating factor 1 receptor
CtBP1	C-terminal-binding protein
CTL	Cytotoxic T-lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated protein 4, CD156
DAMPs	Damage-associated molecular patterns
DTIC	Dacarbazine
FasL	Fas ligand
GOT2	Glutamic-oxaloacetic transaminase 2
HAAO	3-hydroxyanthranilate 3,4-dioxygenase
IDH1	Isocitrate dehydrogenase 1
IDO1	Indoleamine 2,3-dioxygenase 1
IDO2	Indoleamine 2,3-dioxygenase 2
ILT4	Leukocyte immunoglobulin-like receptor subfamily B member 2
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
KYNU	Kynureninase
LILRB2	Leukocyte immunoglobulin like receptor B2
LRRC23	Leucine rich repeat containing 23
MAPK	Mitogen-activated protein kinase
MDSCs	Myeloid-derived suppressor cells
MHCs	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
MYC	MYC proto-oncogene, bHLH transcription factor
NFκB	Nuclear factor-kappa-light-chain enhancer of activated B cells
NR	Non-responders
OXPHOS	Mitochondrial oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed cell death receptor-1, PDCD1, CD279
PD-L2	Programmed cell death ligand-2, CD273, B7DC, PDCD1LG2
PI3K	Phosphoinositide 3-kinases
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alfa
PIP3	Dephosphorylating phosphatidylinositol (3,4,5)- trisphosphate
PRE	Pre treatment
PTEN	phosphatase and tensin homolog

PTK	Protein tyrosine kinases
R	Responders
SARDH	Sarcosine dehydrogenase
SDH	Succinate dehydrogenase (SDH) enzyme.
SDHB	Succinate dehydrogenase complex iron sulfur subunit B
SDS	Serine dehydratase
TCRs	T cell receptors
TDO2	Tryptophan 2,3-dioxygenase
TME	Tumour microenvironment
TMZ	Temozolomide
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
TRM	During treatment
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A

1 INTRODUCTION

Cancer is the second foremost cause of mortality worldwide, and melanoma characterizes the most destructive and lethal form of skin cancer. Despite the various therapeutic approaches, which include surgical resection, chemotherapy, immunotherapy, and targeted therapy, metastatic melanoma is a disease with a poor prognosis. The evolution of immune checkpoint inhibitors (ICIs) led to a substantial reform in the overall survival of patients. Though, the long-term effectiveness of such treatments is restricted by the sometimes rapidly emerging resistance to treatment. Several molecular mechanisms mediating this resistant phenotype have already been explained. The Kynurenine pathway (KP) activity via indoleamine 2, 3-dioxygenase 1 (IDO1), is one mediator of immunosuppression and resistance to ICIs.

In order to facilitate the understanding of the different biological contexts of KP in malignant melanoma, this section is divided into four parts. Part I (1.1), addresses the immune response as a process and introduces critical elements involved in every significant step. Part II (1.2), delineates the dynamic dialogues between tumour cells and the immune system. Part III (1.3) focuses on melanoma, its development, and therapeutic potential. Finally, part IV (1.4) highlights the KP immunosuppressive effect and potential as an immune checkpoint target for cancer immunotherapy.

1.1 THE IMMUNE SYSTEM

The immune system is divided into innate and adaptive arms. The former includes a variety of cell types that respond rapidly to invading pathogens. In contrast, the latter is composed of highly specialized cells to eradicate pathogens or prevent their growth and involved in the establishment of immunological memory. We will have a closer look at these processes in the sections below.

1.1.1 The innate, or non-specific, immunity

The innate immunity refers to a non-specific defense mechanism that generally initiates within hours after infections and leads to migration of the antigen carrying antigen-presenting cells (APCs) to lymph nodes and activation fo the residing T and B lymphocytes.

The concept of " danger signal," which was first suggested by Burnet in 1949 and clarified by numerous subsequent studies (1), is the principle of immune protection. In other words, the immune system has been shaped by the evolutionary force to detect standard features of dangerous pathogens, known as the pathogen-associated molecular patterns (PAMPs). Infected

healthy cells express the 'kill me' signals, or damage-associated molecular patterns (DAMPs), to pledge immune recognitions. APCs receptors specifically bind to PAMPs or DAMPs. Once APCs capture the infected cells upon detection of PAMPs or DAMPs, promote further immune responses by extracting antigens.

NK cells also play a pivotal role in the immediate control of invading pathogens, constitute approximately 5 to 15% of the human peripheral blood (PBMCs) immune cells. They rapidly respond to cells lacking MHC class I surface molecules, which is often caused by viral infections (2,3). In addition, the complement system, which includes a variety of circulating or membrane-associated proteins with enzymatic activities, plays a rapid defensive role through the lysis of microbes. The secondary immunity in many cases will be recruited when rapidly responding immunity is not sufficient to eradicate invading pathogens.

1.1.1.1 The three signals

1.1.1.1.1 Antigen presentation to lymphocytes

The unique surface molecules which are called T cell receptors (TCRs) are essential for the activation and functions of T lymphocytes. TCRs have specific reactivity to a short peptide sequence, which is presented by MHC molecules on the cell surface. Ligation between peptide-containing MHCs on APCs and TCRs can induce intracellular signal transduction cascades required for activation and expansion of T cells. There are two forms of MHCs, MHC Class I and II, which are involved in antigen recognition. TCRs on CD8 + cytotoxic T cells (CTL) specifically bind to MHC class I peptide complexes, while MHC class II peptide complexes are responsible for the presentation of the foreign antigen to CD4 + helper T cells.

1.1.1.1.2 Co-stimulation

In order to reach full activation capacity for T cells, signal transduction mediated by co-stimulatory molecules on professional APCs must be engaged. B7 family members, such as B7.1 (CD80) and B7.2 (CD86), are an example of co-stimulatory molecules on the membrane of APCs and interact with CD28 on T cells. This ligation to co-stimulatory molecules can enhance T cell activation by stabilizing immune synapses between APCs and T cells. In contrast, co-inhibitory molecules that follow similar principles, negatively regulate T cell functions. This mechanism maintains immune homeostasis upon infection and is involved in tumour mediated immune suppression.

1.1.1.1.3 Cytokines

Cytokines are a group of proteins that bind to their matching receptors to control cell functions. A panel of cytokines released by APCs such as IL-12 potentiates various functions of T cells by stimulating the production of IFNγ, which is a crucial regulator for immune defense (4). Moreover, the cytokine milieu during antigen presentation could shape the fate of activated T cells, particularly in the CD4+ T-cell subset.

1.1.2 The secondary immunity

1.1.2.1 T lymphocytes

The three signals consequently lead to the clonal expansion of pathogen-reactive T cells and their migration to the site of infections to eliminate target cells. Once CD8+ CTLs identify cells presenting peptides by the MHC class I molecules, they induce apoptosis of target cells through a variety of mechanisms, such as perforin, granzymes, granulysin, or via membrane-bound molecules like FasL or TRAIL. On the other hand, CD4+ T-cells function mainly by producing cytokines. They are categorized into different subsets based on the cytokines that activate them and those released by these CD4+ T-cells. For example, Th1 cells release cytokines such as IFNγ, IL-2, and TNF-α, which promote immune functions of CTLs, macrophages, or NK cells. In contrast, cytokines released by Th2 cells such as IL-4, IL-5, IL-13 and IL-10, coordinate mainly humoral immune responses. It has been proposed that the balance between Th1 and Th2 cells is critical in autoimmunity and cancer (5).

1.1.2.2 Humoral responses

Humoral responses are mediated by the activation of B lymphocytes and antibody production. B cell receptors (BCRs) are membrane-bound immunoglobulins (IgG) that identify distinct antigens. In contrast to TCRs, the presence of MHC-peptide complexes is not required for BCR signaling and B cells can directly recognize microbial surfaces. Consequently, recognition of pathogen by BCRs leads to B cell proliferation and their maturation into antibody-producing plasma cells. This process leads to the induction of antibodies that bind and clear the pathogens through antibody-mediated cellular cytotoxicity. Besides, B cells are equipped with MHC and co-stimulatory machinery, which could activate and amplify antigen-specific T cells (6).

1.2 IMMUNE RESPONSES IN CONTROLLING CANCERS

1.2.1 Historical overview

The interplay between the immune system and cancer has been widely investigated for over 150 years (7). The underlying basis of this interaction has three basic principles to 1) detect antigen from pathogens, 2) function effectively to target and remove the pathogen, and 3) establish immunological memory through the adaptive immune responses (8). On the other hand, the ability of the immune system to eliminate tumor cells provides the platform for immunotherapy. These multifarious processes consist of the three primary phases: elimination, equilibrium, and escape, which contribute to the elimination of cancer, dormancy, and progression, respectively (9). Although the first use of immunotherapy for cancer treatments dates back to the early 1800s, by William B. Coley's work (7,10), recent scientific expansions have helped to develop advanced methods of enforcing immunotherapies to treat various cancers. These innovations have made the concept of immunotherapy for cancer more clinically relevant.

1.2.2 Barrier to anti-tumour immunity

Numerous mechanisms mediated by different cell types are involved in tumour-induced immune suppression. The molecular basis of some of these mechanisms is described below.

1.2.2.1 Regulatory T cells

Regulatory T cells (Tregs), which belong to the CD4 + helper T subunits, occur naturally in the thymus and are essential for maintaining self-tolerance under physiological conditions (11). Tregs can be induced in malignancies and inflammation in response to various inflammatory signals, such as IL-10 and TGF-β (12). Tregs express CD25 (IL-2Rα) on the surface and the transcription factor FoxP3 intracellularly. Besides, the low expression of CD127 (IL-7Rα) in humans was used to define Tregs (13). Tregs are identified as a significant barrier to the immune response to tumours in many in vivo studies. The higher expression of CD25 in Tregs inhibits effector T-cell activation (13). Furthermore, Tregs are potent producers of immune-regulating cytokines such as IL-10 or TGF-β (14,15). These factors have multifaceted effects and facilitate tumour growth and metastasis.

1.2.2.2 Immune checkpoints

Immune checkpoint proteins have attracted growing interest because of their association in cancer growth in recent years. Often, tumour cells can hijack checkpoint pathways to prevent

attacks from the immune system. Thus, many believe that disabling immune checkpoints would support re-engage the body's immune system to fight cancer.

One of the well-characterized immune checkpoints is CTLA-4 (16,17), which is expressed in high levels on activated T cells. CTLA4 is homologous to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on APCs. CTLA-4 binds CD80 and CD86 with higher affinity and avidity than CD28 and consequently enabling it to surpass CD28 in competition for its ligands (18,19). PD-1, similarly, can down-regulate T cell functions and induce T cell apoptosis by ligation to PD-L1 (20,21), or PD-L2 (22,23). B7-H3 (CD276), and B7-H4 (VTCN1), members of the B7 family were also identified; these proteins are expressed on the surface of APCs and interact with the ligands CD28 (24). Expression of these immune checkpoints on tumour or immunosuppressive cells is known to be critical protective mechanisms that facilitate tumour growth (25,26) and has been found to be some of the most promising therapeutic targets for the treatment of human cancer (section 3.1). Anti-cytotoxic T lymphocyte antigen 4 (anti-CTLA-4) and anti-PD1 monoclonal antibodies have already shown anti-tumour activity in patients. This finding led to increasing interest in looking into other immune checkpoint proteins to find better treatments for cancer. These immune checkpoints are also responsible for immune homeostasis and the maintenance of tolerance after eliminating pathogens (27).

1.2.2.3 Enzymes and metabolic machinery

Tumour tissues are characterized by high energy consumption levels and altered metabolic profiles, and cancer metabolism not only is crucial in cancer signaling for supporting tumourigenesis and survival, but it also has broader implications in the regulation of anti-tumour immune signaling. Lately, much attention has been devoted to the influence of tryptophan (TRP) metabolic pathways on both tumour cell growth and the host's immune anti-tumour response. Production of various enzymes such as indoleamine 2,3-dioxygenase (IDO) catalyzes TRP to N-formyl-kynurenine. IDO is an important regulatory channel for APCs to modulate T cell functions during antigen presentation through calibrating TRP levels (28,29). Tumour cells and many types of immunosuppressive cells also utilize this pathway to sabotage T cell responses (30).

Besides the direct effects, IDO activity could control other regulatory schemes in the tumour micro-environment, including COX-2/PGE2 pathway (31,32), TGF-β, or IL-10 production (33,34). IDO has been the target of small-molecule inhibitors in clinical development in combination with PD-1 checkpoint inhibitors (35).

1.2.3 Therapeutic outlook

Immunotherapy is proven to be clinically useful in improving the prognosis of many patients with a wide variety of tumours. The main drivers behind this success are immune checkpoint inhibitors (ICIs) and adoptive cell transfer. In this section, I will highlight the major advances in these fields.

1.2.3.1 'Check-point' inhibitors, ICIs

Immune checkpoint molecules negatively regulate immune effector cells by binding to the matching receptors. As discussed in section 2.2.2, CTLA-4 and PD-1 are two well-characterized receptors on T cells, and their therapeutic potentials have been evaluated in preclinical models and clinical studies. In preclinical animal models, blocking CTLA-4 signaling effectively limited tumour growth in mice through activation of T cells (36–39). Ipilimumab, an anti-human CTLA-4 blocking antibody, was approved by the FDA in 2011, which was motivated by results of the landmark phase III clinical trial, which has demonstrated durable survival in metastatic melanoma patients who had failed standard therapies (40–42).

The landmark development of the ipilimumab, have accelerated the research and approval of antibodies blocking against the PD-1 pathway. Apart from the sustained survival benefits that have been achieved in melanoma patients by PD-1 blockade antibodies (43,44), they enabled significant clinical responses in patients not responding to ipilimumab treatment (45–47). This result can be explained by the unique regulatory role of PD-1 on the immune system (48). Notably, PD-1–deficient mice experienced tolerable autoimmune responses (49,50), while mice lacking CTLA-4 proteins developed destructive autoimmunity (51,52). The CTLA-4 and PD-1 receptors serve as two T cell receptors with independent inhibitory mechanisms of action. Based on preclinical studies, CTLA-4 requires an activation threshold to be able to mitigate the proliferation of tumour-specific T lymphocytes (53), while PD-1 performs primarily as a stop signal that attenuates T cell effector function within a tumour. The distinct mechanisms and sites of action of these two inhibitory receptors led to investigations of combined blockade of both in recent years (54).

Additional therapeutic opportunities targeting two PD-1 ligands have also been described, PD-L1 also named as B7H1 (CD274), and PD-L2 also named B7-DC (CD273) which are often expressed on tumour cells or immunosuppressive cell types. A recent investigation of the PD-L1-blocking antibody revealed promising and well-tolerated clinical responses in patients with different solid cancers (55–57). It has also reported that PD-L1 expression in tumour tissues serves as a predictive marker for ICIs therapy (58). However, it should be

noted that this observation remains controversial as the expression of PD-L1 expression can be impacted by external factors such as IFNγ or by other PD-L1 producing cell types such as tumour cells, fibroblasts, endothelial cells, and immune cells (59–62). PD-L2 expression has observed on APCs as well as other immune or non-immune cell types (23,63,64). Similar to PD-L1, PD-L2 inhibits the proliferation of T cells by PD-1 signaling (23). Existing evidence from tumour models confirms utilizing PDL2 blockade as an adjunct to the anti-PD-1/PD-L1 antibodies (65,66). Although blocking of PD-L2 enhanced the anti-tumour effects of other checkpoint blocking agents such as anti-PD-1 / PD-L1 antibodies (67), but, PD-L2 deficient mice displayed more aggressive tumour progression (68). Although clinical approaches for PD-L2 blockade are currently limited due to unknown biological functions, combining anti-PD-1 and anti-PD-L1 strategies may be useful in order to obtain an efficient blockage of the PD-1 pathway. In summary, ICIs have generated encouraging clinical responses and elicited sustainable tumour control in patients with advanced solid tumours. However, current clinical studies have focused is on more immunogenic cancers such as melanoma or lung cancer, while the clinical efficacy of these agents in other cancers is still under investigation.

1.2.3.2 Adoptive cell transfer

Since immune responses can control tumour growth, it is reasonable to assume that the adoptive infusion of highly functional tumour-reactive immune cells may be useful as a therapeutic method. Many investigations have been conducted and have shown fantastic anti-tumour effects. This section is devoted to treatment strategies with activated T cells or NK cells in human solid and hematological malignancies.

1.2.3.3 Tumour-infiltrating lymphocytes (TILs)

T lymphocytes often infiltrate into solid tumour tissues, which is an independent prognostic factor for clinical outcome in various cancer types. Furthermore, it is commonly believed that the recruitment of T cells in tumour tissues is due to their tumour-targeting properties. TILs recovered from surgically removed tumour tissues treated by high-dose IL-2 have become an attractive treatment option in melanoma patients (69,70). Furthermore, lymphodepletion followed by transfusion of CD4 + and CD8 + T cells positive TILs were shown to be critical factors for clinical efficacy (71–73). Furthermore, melanoma patients treated with TIL achieved survival for longer than three years (74,75). Nevertheless, the major limitation of this approach is the generation of sufficient autologous TILs from individual patients. Therefore, alternative strategies for using genetically engineered T cells were developed to overcome this shortage.

1.2.3.4 Creating anti-tumour T cells through genetic modifications

Recognition of tumour-associated antigens (TAA) by TCR is required for their specific killing of tumour cells (76–78). Shrinkage of tumour burden in various cancer types has reported upon infusion of T cells with TAA-specific TCR (76,79,80).

T cells alternatively can be engineered to express chimeric antigen receptors (CAR-T). Typically, in this structure, the recognition domain of antigen specificity of a monoclonal antibody coupled to intracellular T cell-activating signaling domain with transmembrane spacer molecules. Since the first investigations, several improvements have been introduced, mainly by calibrating the content of intracellular signal domains (81,82). In contrary to TAA-specific T cell, MHC-peptide complexes on tumour cells are not required for the cytolytic function of CAR-T cells. The superior efficacy of CAR T cell therapy has been demonstrated when anti-CD19 CAR T cell treatments in B cell cancers have accomplished striking successes (83–86). Currently, CAR-T cell therapy is developing rapidly, and many ongoing clinical studies are investigating the therapeutic potential of CAR-expressing T cells as a treatment for solid and hematological malignancies (87).

1.3 CUTANEOUS MALIGNANT MELANOMA

Melanoma, a tumour originating from the melanocyte (the pigment producing cells), continues to be highly fatal. Although the majority of melanoma is cutaneous, it can also begin as ocular, mucosal or with unknown primary (88).

Cutaneous malignant melanoma (CMM) maintains a long-standing trend of rapidly uprising incidence and with a comparable trend between males and females (89,90). Figure 1 shows the incidence and mortality rate of skin melanoma in the Nordic countries. CMM is highly curable if discovered early (91), and most risk factors display a small augmented risk alone (box 1) except for genetic syndromes, such as familial malignant melanoma (germ-line mutations in the CDKN2A gene) (92,93).

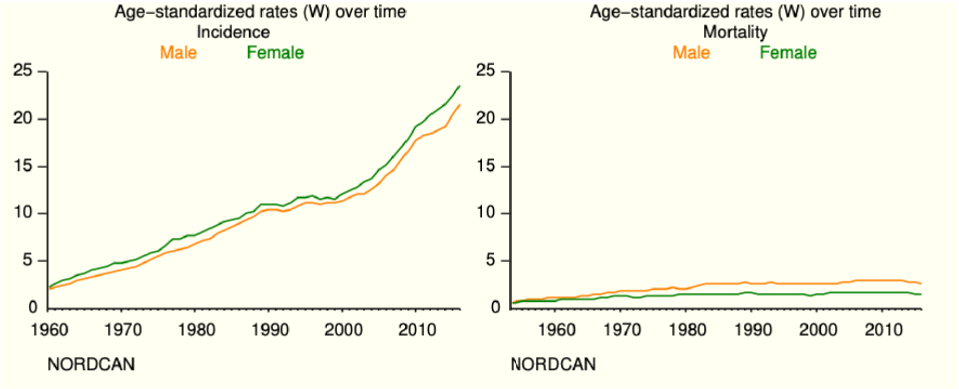

Figure 1. *statistics based on NORDCAN database showing the incidence and mortality of skin melanoma in Nordic countries: Number of new cases and related deaths in 100,000 individuals, age 0-85+ , between years 1950/60-2016.*

Box 1
Risk factors for melanoma development
Gender
Age
Family history of melanoma
Dysplastic nevi
Multiple Number of melanocytic nevi
Fair-skinned races
Sunburns
Indoor tanning
Skin cancer history
https://www.uspreventiveservicestaskforce.org/Page/Document/RecommendationStatementFinal/skin-cancer-screening2. Accessed April, 2020.

1.3.1 Common aberrations in CMM

Genetic alterations in MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) or in RAS/RAF/MEK/ERK pathways has a major role in melanomagenesis. The MAPK/ERK pathway regulates a broad range of fundamental cellular processes, including cell proliferation, differentiation, senescence, survival, transformation and migration (94–96). In CMM, a mutation in RAS or BRAF result in the activation of MAPK/ERK signaling (97). Alteration in the MAPK pathway contributes to the oncogenic transformation of melanoma which results in the uncontrolled growth of melanoma tumours (97–99). The majority of BRAF mutations appear in exon 15, among those, the most frequent BRAF activating mutation occurs in codon 600 (BRAFV600E) that substitutes valine by glutamic acid (100) while a majority of the RAS mutations occur in codon 61(101). In CMM, the prevalence of NRAS mutations is up to 30% (102,103), while approximately 50% of the

cases carry BRAF mutations (100,104). Moreover, NRAS and BRAF mutations in CMM are mutually exclusive (105).

Similar to the MAPK/ERK pathway, PI3K (phosphatidylinositol 3-kinase)-AKT pathway activation plays a significant role in melanoma. Upon activation of the PI3K pathway, AKT proteins (AKT1-3) are phosphorylated by PDK1 and mTORC2, which activate downstream targets. PI3K/AKT activation is controlled by the tumour suppressor PTEN (106). PTEN mutations lead to AKT activation (107). The tumour-suppressive PTEN activation through dephosphorylating phosphatidylinositol (3,4,5)- trisphosphate (PIP3) leads to inhibition of phosphorylation of serine/threonine AKT and inactivating the pathway (108). PTEN loss is correlated to increased invasiveness of the CMMs and decreased overall survival (OS) in patients with BRAF V600E mutated tumours (109).

Genomic alterations in CDKN2A, a tumour suppressor gene, is reported in 44% of CMM (TCGA database: http://cancergenome.nih.gov/) and is associated negatively with patient survival (110). Neurofibromin 1 (NF1, chromosome 17q11.2) is a negative regulator of the RAS signaling pathway, and around 26% of CMMs with wild type BRAF or NRAS carry NF1 mutations (95), while co-occurrence of loss of function mutation in NF1 and BRAF or NRAS mutation is less frequent (96).

Protein kinases (PK) compose of a large family of regulatory proteins that phosphorylates other proteins and usually leads to their activation, has two major subfamilies: serine/threonine kinases (STK) and protein tyrosine kinases (PTK). The most commonly modified serine/threonine kinase in melanoma is the BRAF protein, which harbors V600 mutations in about 50% of CMM patients (100). Similarly, overexpression of the RTKs has been associated with CMM progression (111).

A group of transcription factors has been shown to play pivotal roles during the progression of CMM such as the microphthalmia-associated transcription factor (MITF), nuclear factor-kappa-light-chain enhancer of activated B cells (NFκB), activating protein 1 and 2α (AP-1/AP-2a) and C-terminal-binding protein (CtBP1) (112,113). Genetic variants in DNA repair genes may be especially relevant since their transformed function in response to sun exposure-related DNA damage may be related to risk for CMM (114).

1.3.2 Treatment of CMM

Surgical excision of the primary tumour for early-stage CMM is the standard treatment with a favorable prognosis. Surgery of advanced metastatic disease often has a poor prognosis and, if performed, usually has a palliative role (115). Radiotherapy is usually considered as palliative therapy in metastatic disease. Therapy with chemotherapeutic agents such as

dacarbazine (DTIC) and temozolomide (TMZ) has been the conventional treatment for CMM. However, the response rates were only 5-12%, with a median overall survival of less than one year (116,117). Moreover, immunostimulants such as IL-2 and IFNα have been used to stimulate the immune response against cancer. On the other hand, targeted therapy is defined as small therapeutic molecules that are intended to inhibit specific molecules responsible for uncontrolled proliferation and growth in cancer cells; therefore, they may be more efficient with fewer side effects compared to chemotherapy. Vemurafenib (Zelboraf®) and dabrafenib (Tafinlar®) are two inhibitors for BRAF mutated CMM, which have been approved by the FDA in 2011 and 2013, respectively, with improved PFS and OS (118,119). Today, combination treatment with BRAF-inhibitors and MEK-inhibitor is standard of care since it is associated with better outcome than therapy with BRAF-inhibitors alone (120).

Novel immune therapies, in parallel with targeted drugs, have been developed for CMM. In 2011, ipilimumab (Yervoy®), a monoclonal blocking antibody against CTLA-4, was approved for the treatment of unresectable CMMs. Pembrolizumab (KEYTRUDA®), and nivolumab (OPDIVO®) PD-1 inhibitors, were approved for the treatment of CMM, in 2014 and 2017, respectively. Finally, autologous adoptive T-cell transfer, which refers to an approach of collection, ex vivo expansion, and reinfusion of tumour-infiltrating T-cells (TILs), targets the cancer cell antigen precisely and has shown anti-tumour activity in advanced CMM patients (74).

1.3.3 Therapy resistance in CMM

Drug Resistance poses a major challenge for CMM treatment. For several decades, chemotherapy with DTIC and TMZ has been mostly inefficient due to innate and acquired resistance to treatment (121). Drug resistance in solid tumours has been investigated mainly with regard to epigenetic and genetic modifications. Other factors, such as changes in drug uptake and metabolism and tumour microenvironment (TME), are also suggested to play roles in drug resistance (122).

1.3.4 Biomarker-based prediction of response to therapy for CMM

Despite the tremendous efforts that have been made to enhance outcomes using checkpoint inhibitors (ICIs) or targeted therapy, still only a fraction of patient response to the treatment. Therefore, the identification of reliable biological markers that can predict a sustainable therapy response would enable oncologists to identify patients that would benefit from the procedure. This approach may help to personalize the treatments based on a prediction of the therapy response. Moreover, biomarker-based identification of possible non-responders enables the use of alternative treatment options such as a combination of two targeted drugs

or a combination of immunotherapeutic agents. High levels of calcium-binding S100 protein, an example of these group of biomarkers, was shown to correlate to increased tumour angiogenesis, metastatic capacity, and immune evasion in different cancers (123). For example, the importance of S100A9 in cancer is highlighted by a substantial body of evidence. For example, a high density of S100A9 positive immune cells in the tumour stroma of prostate cancer patients was associated with poor clinical outcome (124). Moreover, several studies in murine and human cells have provided strong evidence that S100A9 represents a novel marker for MDSC (125,126). In tumour cells, expression of S100A9 leads explicitly to the more aggressive outcome and metastasis (127). Moreover, S100A9 in myeloid cells is connected with hampered DC differentiation and intensified MDSC formation (126,128). Lastly, increased kynurenine and kynurenine/tryptophan ratios, and consequently, the activity of IDO which is associated with more inferior OS, can be used as predictive markers for the future course in melanoma patients (129).

1.4 METABOLIC INTERVENTIONS IN THE CANCER IMMUNE RESPONSE

The question in the therapeutic dilemma for cancer is how to produce more effective treatments that distinguish between healthy and cancerous tissues. Despite the absence of globally applicable principles, there are two well-accepted theories: first, that malignant conversion goes hand in hand with apparent changes in cellular metabolism; second, that the immune system is crucial for clearance and tumour control. Combining our understanding of immune cell function with tumour metabolism may substantiate to be a powerful approach in the construction of efficient cancer therapies. This section is devoted to describing how nutrient accessibility in the tumor microenvironment (TME) forms immune responses and identifies intervention areas to modulate the metabolic limitations placed on immune cells in this setting.

1.4.1 General overview of metabolism

The word "metabolism" represents a series of connected chemical reactions that begin with one particular molecule and transforms it into other forms through anabolic and catabolic reactions. Anabolism attributes to the synthesis of molecules from its precursors, whereas catabolism refers to the breakdown of molecules and the production of free energy, which is essential to maintain cells and organisms alive (130). The most abundant source of free energy in metabolism is carbohydrates (e.g., glucose), which can be catabolized via glycolysis and the citric acid cycle. Fatty acids can also be metabolized via the citric acid cycle and produce free energy. Amino acids, the third most critical class of small molecules, are the least rich source of free energy (130). Nonetheless, amino acids serve for protein

synthesis and therefore are required for living organisms. The term "metabolic regulation" refers to processes that serve to maintain homeostasis at the molecular level (131), by controlling the amounts of enzymes, their catalytic activities, and the substrate accessibility (130).

In mammals, unlike fatty acids and glucose, excess amino acids cannot be stored in the body. Thus, excess amino acids undergo oxidative degradation. The aminotransferases catalyze this reaction by converting amino acid into urea through the urea cycle. In contrast, fatty acids, ketone bodies, and glucose can be constructed from the carbon skeleton of the amino acid (132).

1.4.1 Metabolic diversity in tumours

As previously discussed, cancer progression arises when the immune system cannot destroy or control the growth of nascent tumours due to limited antigen recognition and impaired immune cell function which is caused by nutrient restriction or production of specific metabolites and molecules within TME. The neoplastic cells metabolic flexibility which allows them to adapt to different environments (Box 1), supports their growth and colonization, exacerbate the problem (133). Thus, the etiology of cancer is closely related to both the malfunction of the immune system and metabolism to destroy emerging tumours (133–135).

Metabolic adaptations in cancer can be developed by mutation of oncogenes such as MYC, PTEN and PI3K or changes in specific metabolic enzymes such as IDO, IDH1 or SDH (135–139). Thus, these factors cause a different landscape of cancer cell metabolism and nutrient heterogeneity in TME of various tumour types (139,140), as well as within a single tumour (141). Although malignant transformation usually extend aerobic glycolysis (also known as Warburg metabolism), which mediate an oxygen-independent process of ATP production (139), mitochondrial oxidative phosphorylation (OXPHOS) and other mitochondrial metabolic consequences may also have noticeable roles in cancer metabolism (141,142). The increases in OXPHOS in some tumours, may be supported by oxidation of glucose (143), proteins, and amino acids or fatty acids (144–147). In addition, some tumours may use other sources of energy, such as metabolites or ammonia and lactate which traditionally considered as waste products (141,148–150). Despite the massive effort on exploring the tumour metabolic heterogeneity as an essential aspect of developing immunofocused therapeutics, our understanding of the metabolism of different tumour types at different tissue locations is still limited. Analysis of large bioinformatics databases providing sequence data from thousands of human cancers has shown a correlation between oncogenes and metabolic

reprogramming (151–153). Alternatively, the metabolic changes in plasma could be a reflection of changes in immune response and disturbed hematopoiesis, which corresponded to the tumour growth and metastasis formation. Therefore, metabolic profiling of plasma is considered a powerful tool for the identification of biomarkers and altered metabolic pathways in cancer.

1.4.2 Targeting Metabolism for Cancer Therapy

Targeting cancer or immune cell metabolism when combined with existing therapeutic strategies may have the potential to improve cancer treatment. Therefore, selecting critical metabolic pathways (Figure 2) that are differentially engaged in cancer and effector immune cells could be useful.

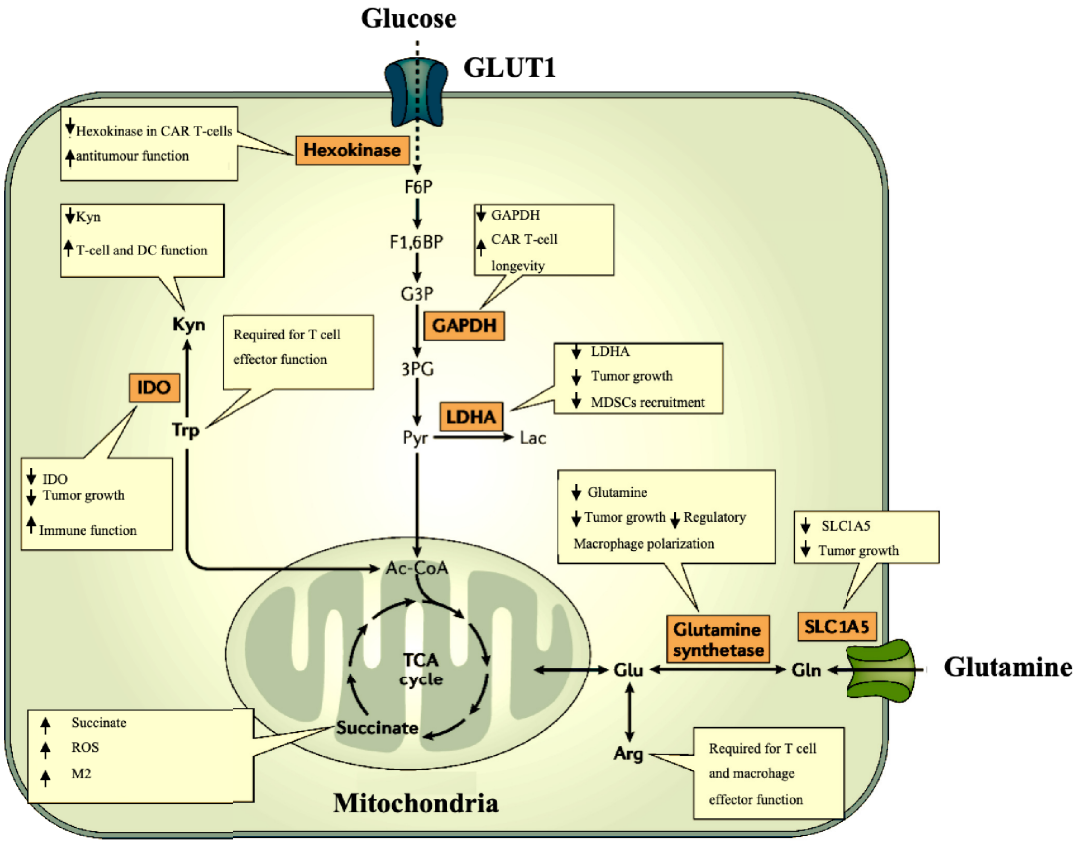

Figure 2. Potential metabolic targets for combination therapies (154), *modified.*
The scheme is the result of inhibiting key enzymes of diverse pathways that encompass cellular metabolism or regulating the concentration of critical metabolites to fine-tune immune cell function. 3PG, 3-phosphoglyceric acid; Ac-CoA, acetyl-CoA; CAR, chimeric

antigen receptor; DC, dendritic cell; F1,6BP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde 3-phosphate; IDO, indoleamine 2,3-dioxygenase; MDSC, myeloid-derived suppressor cell; ROS, reactive oxygen species; TCA, tricarboxylic acid.

However, broadly targeting metabolic pathways could be argued that targeting them in tumour cells may impair the anti-tumour activity of the immune cells due to the complex interconnectivity of the metabolism of the tumour and immune cells. Interventions intended to redirecting tumour metabolism rather than just killing the cancer cell or repairing metabolite homeostasis could be one strategy to limit the adverse effects on the immune compartment.

Nutrient availability in TME shapes the composition of infiltrated immune cells into tumours (155,156). Infiltrating of the immune cells themselves can decrease the nutritional level in TME, which can potentially contribute to an immunosuppressive milieu. Glucose is an example which is widely used by human tolerogenic DCs, MDSCs, and tumour endothelial cells (157–159), which have been part of the immune-suppressive environment that is allowed for tumour growth and metastasis.

The limitation of glutamine in TME might also support the buildup of Treg cells (160,161). Cytotoxic cells, for example, CD8 + T cells and NK cells, are also susceptible to limitation of amino acid and under conditions of glutamine, serine or glycine deprivation exhibit impaired function (162–165). Essential amino acid TRP depletion in cancer is increasingly being identified as an essential microenvironmental factor that suppresses anti-tumour immune responses. It has been reported that the TRP is catabolized in the tumour tissue by IDO which is expressed in tumour cells or APCs. Depletion of TRP and accumulation of downstream metabolites mediate the immunosuppressive milieu in tumours and tumour-draining lymph nodes by stimulating T-cell anergy and apoptosis (166), and impaired priming capacity of DCs (167). Besides, variations in systemic metabolite concentrations in patients plasma may display metabolic alterations urged by tumours (140) and could present clinically related information. For example, depletion of TRP and elevation of the kynurenine (KYN) level in plasma was detected in various cancer types (168).

Following identification of the kynurenine pathway (KP) as a critical metabolic pathway contributing to immune escape and the central role of IDO in this pathway, an active effort both clinically and preclinically was devoted to the strategies of inhibiting immunosuppressive mechanisms mediated by IDO. Despite promising results in early phase clinical trials in a range of tumour types, a recent clinical trials on investigating the IDO-selective inhibitor epacadostat in combination with pembrolizumab displayed no difference between the epacadostat-treated groups versus placebo in patients with metastatic melanoma

(169). These seatbacks not only refer to the complexity of metabolism but also suggest that the absence of IDO causes TRP to be shunted into other pathways to produce serotonin and melatonin, which may mediate immunosuppression (170,171). Thus, a thorough analysis of TRP pathway metabolic fluctuations or enzymatic activities in patient samples treated with IDO blockade can not only explain the breakdown of IDO inhibitors but it also specifies a way forward in promoting other approaches to inhibit this pathway. Moreover, higher KYN production and elevated KYN to TRP ratios have been introduced as lung cancer biomarkers (172)

1.4.3 The kynurenine pathway metabolism

TRP is an essential amino acid that can only be acquired through the diet, and it serves as a precursor in the manufacture of several important biologically active metabolites, including melatonin, serotonin, and kynurenines (173).

It is estimated that up to 99% of the metabolism of TRP is metabolized via the KP (174,175), with the remaining 1% being converted into serotonin. The KP generates a set of biologically active indole-derived metabolites, termed 'kynurenines (Figure 3). Tryptophan metabolism via the KP leads to the production of nicotinamide adenine dinucleotide (NAD+), which is a crucial component of metabolic processes occurring in all cells. The KP pathway is likely to be involved in a range of diseases, including tumour immune resistance (168,176).

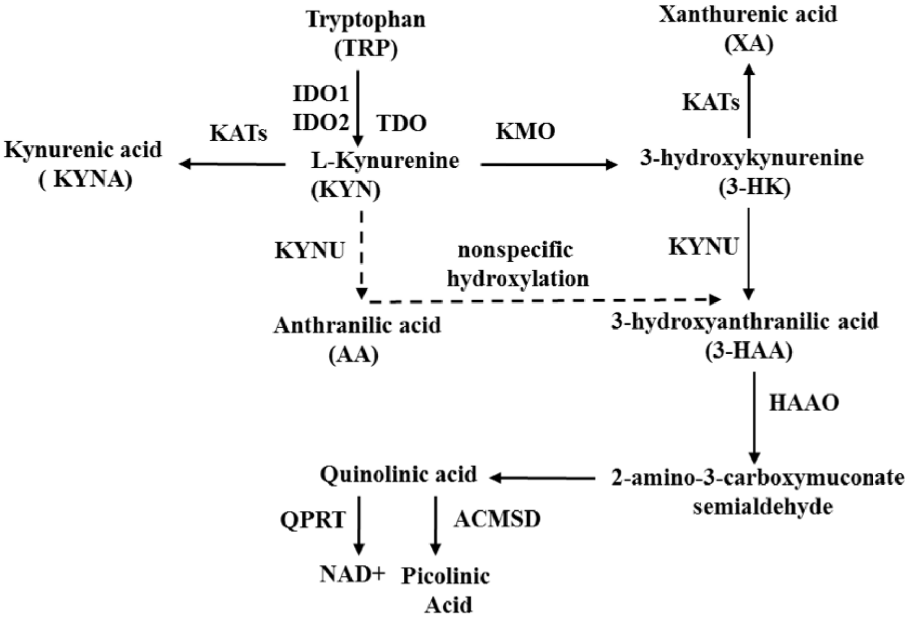

Figure 3. The kynurenine pathway of tryptophan catabolism
Abbreviations: 3-HAAO, 3-hydroxyanthranilic acid oxygenase; IDO, indoleamine 2,3-dioxygenase; KATs, kynurenine aminotransferases; KMO, kynurenine monooxygenase or kynurenine hydroxylase; KYNU kynureninase; NAD+, Nicotinamide adenine dinucleotide; QPRT, quinolinate phosphoribosyltransferase;; TDO, Trp 2,3-dioxygenase.

1.4.4 The kynurenines and therapeutic potential of KYN catabolites

TRP is initially metabolized by IDO and tryptophan 2,3-dioxygenase (TDO2) into kynurenine (KYN) (177). KYN, as an intermediate metabolite, provides the substrate to three different enzymes producing different metabolites that exert diverse physiological effects. KMO converts KYN into the toxic 3-hydroxykynurenine (3HK) and kynureninase (KYNU) hydrolysis 3-HK to 3-HAA. Kynurenine aminotransferase (KAT; KAT I, II, III, and IV) converts KYN and 3-HK into KYNA and xanthurenic acid (XA). Anthranilic acid (AA) is produced from KYN by kynureninase (KYNU) (178,179). 3,4-dioxygenase (oxidase: 3-HAAO) is the most active among KP enzymes which converts 3HAA into croleyl aminofumarate (2-amino-3-carboxymuconate-6-semialdehyde [ACMS]) and consequently leads to the production of quinolinic acid (QA) and NAD+ (180,181). The creation of these metabolites has generated interest in the KP since these metabolites have crucial roles in inflammation, immunity, and the pathogenesis of neurological disorders (182).

Disturbed KP has clinical and therapeutic implications, and targeting the enzymes involved in this pathway is now used as a strategy to address a variety of immune and neurorological diseases (183–185) (Table 1). The disturbance of TDO2, IDO or accelerated TRP degradation through their induction in some types of cancers and their ability to suppress anti-tumour immune responses, suggests that their inhibition could form a therapeutic strategy (186,187). Many inhibitors have been developed targeting IDO as a modulator of immune responses (188). Among TRP metabolites KYNA, XA, and KYN have the highest ligand activity for the AhR. Activation of the AhR by KYNA may allow specific tumour cells to escape immune surveillance mechanisms by IL-6 secretion (189). Targeting KMO, KYNU, or 3-HAAO has been reported to limit the formation of QA, which involves a variety of neurological conditions (190,191). Theoretically, KMO inhibition should result in an elevation of KYN, KYNA, AA and a decrease in 3-HK and subsequent metabolites (192). KAT inhibition is vigorously used as a strategy to treat schizophrenia (183,184,193). As the KP enzymes perform equally essential role in health and disease and are consequently targeted for therapeutic intervention. Therefore, the KP of TRP degradation is an essential topic for basic and clinical research. Through a wide range of KP enzymes and intermediates, KP outstandingly regulates many critical physiological processes throughout the body and renders multiple opportunities to address multiple disease states.

Table 1. KP enzymes as a therapeutic target, (modified).

Enzyme	Potential clinical condition t otarget the KP enzymes
TDO2	Cancer, major depressive disorder, porphyria
IDO	Cancer, immune diseases, neurological and neurodegenerative disorders
KAT	Schizophrenia,
KMO	Schizophrenia, drug dependence, infectious diseases
KYNU	Neurodegenerative conditions
3-HAAO	Neurological diseases
QPRT	Inflammatory disorders
NMPRT	Cancer
NNMT	Cancer, diabetes, schizophrenia
NADase	Infectious diseases
PARP	Cancer, inflammatory, metabolic, and neurological disorders

Abbreviations: 3-HAAO, 3-hydroxyanthranilic acid oxygenase; IDO, indoleamine 2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine monooxygenase or kynurenine hydroxylase; Kynase, kynureninase; NADase, NAD^+ glycohydrolase; NMPRT, nicotinamide phosphoribosyltransferase; NNMT, nicotinamide *N*-methyltransferase; QPRT, quinolinate phosphoribosyltransferase; PARP, poly-(ADP-ribose) polymerase; TDO, Trp 2,3-dioxygenas (177).

1.5 SUMMARY

Immunotherapy modalities such as ICI therapies have revealed a new era of immuno-oncology by targeting the patient's immune system (194). It is more likely these treatments are affected by the metabolism of the targeted cells which highlights an intimate relationship between checkpoint pathways, and consequently ICIs therapy, with cellular metabolism (195–199). Intensifying the potential of these new strategies will provide a new opportunity for the further assessment of the tumours and the immune system metabolic interaction.

2 AIM OF THE THESIS

The research projects presented in the current thesis aimed to elucidate the therapeutic potential of targeting KP for the treatment of malignant melanoma.

Specific aims were:

- To explore the implication of KP change on CD4 + T-cell function in an *in vitro* co-culture model
- To outline immune-metabolic network interactions of KP in CMM patients to explore the link between KP metabolites and regulation of the anti-tumour immune response

- To explore possible KP-related predictive biomarkers of response to ICIs treatment in CMM patients

3 MATERIALS AND METHODS

Below is a summary of the main methods were employed to reach the aims described earlier. Please refer to the individual articles Of note, for a more detailed description.

3.1 CD4+ AND CANCER CELL CO-CULTURE ASSAYS, AND TREATMENT

To study the implication of KP alteration on CD4+ T-cell function, we established an *in vitro* co-culture model consisting of CD4+CD25- T-cells obtained from healthy volunteers in culture with four different MCLs (BRAF wt and BRAF V600E).

In short, freshly isolated T-cells (CD4+ CD25-) primed overnight with cross-linked anti-CD3/anti-CD28 antibodies. The cellular interactions *in vitro* were assessed by co-culture models using melanoma cell lines (MCLs: BE, DFB, A375, and SK-MEL-28). MCLs were first incubated for 24 hours, and on the following day, MCLs cultured with activated CD4+ T-cells for up to 5 days, in the presence or absence of IFNγ cytokine and IFNγ blocking antibody or Epacadostat (INCB024360: an IDO1 inhibitor). Following quick magnetic, CD4+ T-cells and MCLs were processed for further downstream analysis. For the functional assay, responder T cells were labeled with CFSE and stimulated alone or with pre-conditioned T-cells for 3-5 days, and proliferation was measured by flow cytometry. Specific details regarding stimulations and co-cultures are specifically given in each publication (Paper I).

Furthermore, to facilitate the study of KP metabolic fluctuation and acquired resistance to MAPKIs, parental A375 BRAF V600E-mutated human melanoma cell line and daughter cell line with induced BRAFi resistance [vemurafenibR4 resistant subline (A375R)] were cultured for a short time (48 h) to measure the kynurenine pathway metabolites in the presence or absence of IFNγ (and TNFα (Paper-II).

3.2 PLASMA SAMPLING AND SAMPLE COLLECTION

To explore the role of KP alteration in CMM patients, plasma samples of CMM patients were collected at two-time points (before and during the first treatment with MAPKIs (n=5, paper I) and with ICIs (n=24, paper II). Furthermore, plasma samples of healthy volunteers (n=5) were included as controls (Paper-II).

3.3 PLASMA PROTEIN DETECTION

3.3.1 LC-MS instrumentation

In the paper I, the TPR and KYN concentration in the medium derived from the *in vitro* co-culture of CD4+ CD25− T-cells from three healthy donors and human MCLs were measured by HPLC (high-performance liquid chromatography). However, the analysis of KP metabolites by HPLC is a challenge due to its poor derivative stability, and side reactions. Therefore, HILIC–MS/MS used for the screening of the KP in the co-cultured derived medium.

In paper II, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) was used for the analysis of KP metabolites in plasma samples due to the outstanding selectivity and sensitivity of this techniques.

In paper IV, high-resolution isoelectric focusing of peptides-liquid chromatography-mass spectrometry (HiRIEFLC-MS/MS)-based method was used to analyze the change in protein levels during treatment. Details were described previously (200).

3.3.2 Proximity extension assay (PEA)

In paper II, PEA measurements were performed using 1 μL of each sample. Briefly, 92 antibody pairs, which are labeled with DNA oligonucleotides, bind to target antigen in the samples. Oligonucleotides will hybridize when the two probes are in close proximity and expand with a DNA polymerase and generate a unique PCR target sequence. This newly created DNA barcode is detected and quantified by PCR; details were described previously (201).

3.3.3 Single-cell RNA sequencing of PBMCs populations

For study III and IV, blood samples were collected from the melanoma patients (n=8), before the first nivolumab infusion and before the third infusion (6 weeks). PBMCs were isolated and cryopreserved until use. 10X Genomics 3' v2 reagent kit was performed in order to capture single cells and library preparation. Single-cell barcoded cDNA libraries were quantified with Qubit™ dsDNA BR Assay Kit and sequenced on Hiseq 2500 (Illumina, San Diego CA). The Cell Ranger Single Cell Software Suite was used to perform sample de-multiplexing, barcode processing, and single-cell 3′ gene counting, and reads were aligned to Ensemble human reference genome GRCh38, using STAR aligner. The Seurat R package Version 2.0 was used for analyses of graph-based cell clustering, dimensionality reduction, and data visualization. The R packages ggplot2 was used for generating the graphics.

3.3.4 Network analysis

In the correlation network analysis, a node represents a kynurenine pathway metabolite or CD4+ T-cell related genes (Paper I, II), genes enriched in TRL and c-MYC signaling pathways (Paper III) and differentially expressed gene in 4 different groups (Paper IV). An edge is determined by statistically significant correlations between genes in two groups. Values are the absolute value of Spearman correlation ≥0.5, which is the highest level of correlation where the network is not fragmented. These values were used to reconstruct networks (202). A network analysis was conducted using a cystoscope (3.4.0).

3.3.5 Additional methods

Flow cytometry: For paper I, the purity of CD4+ T-cells was validated by using a cell surface staining protocol consisting of anti-CD45RA, CD4, CD25, CD8A, CD14, and CD19. Viability and proliferation stainings were performed using a fixable viability dye, eFluor 780, and a cell proliferation dye, carboxyfluorescein diacetate succinimidyl ester (CFSE), respectively. Recording the flow cytometry was performed on a CyAn ADP 9-Colour Analyser (Beckman Coulter), and single-stained compensation beads (BD CompBeads, BD Biosciences) was used to create the compensation settings. Data of flow cytometry were performed on FlowJo® software (Tree Star).

For paper III, PBMCs were stained directly after purification, and dead cells were excluded using the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific). Stained PBMCs were acquired using an LSRII (BD Biosciences) flow cytometer, followed by analysis in the FlowJo v10.x platform (Treestar). CV values and signal to noise ratios were monitored using CS&T beads (BD Biosciences) to ensure consistent flow cytometer performance on a day to day basis. A nonstained control was acquired for each sample, and fluorescence-minus-one (FMO) controls were used to validate critical stainings.

Gene expression analysis (qPCR): TaqMan real-time PCR (Applied Biosystems) was used to quantify mRNA expression of selected genes in cell lines versus drug treatment.

RNA extraction: RNA extraction: RNA was extracted from cell lines and primary CD4+ T-cells with and without treatments using the extraction kits according to the product manual; RNeasy Mini kit from Qiagen.

Luminex: A multiplex bead array assay was performed on a culture medium to measure the concentration of the cytokine. Briefly, individual bead sets (Luminex) were coupled to cytokine-specific capture antibodies following the manufacturer's instructions. the data was

recorded on the Bio-Plex-200 platform and analyzed using the Bioplex Manager® software (version 6.1; Bio-Rad) with a 5-parameter logistic regression algorithm.

Gene silencing and Immonobloting: GenElute Plasmid Miniprep Kit prepared empty vector pcDNA3.1 and wild-type BRAF expression plasmid. SK-MEL-28 and DFB cell lines transfected for 72 h with vectors expressing wild type BRAF or empty control vectors following the standard protocol from Lipofectamine. The whole protein lysates were extracted and used to validate the candidate proteins' expression. The protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific). The same concentration of the samples was loaded on NuPAGE Novex Bis-Tris Gel (Life Technologies, Carlsbad, CA) and then transferred to PVDF membranes (Thermo Scientific, Rockford), according to the manufacturer's standard protocol. Finally, the Chemiluminescent method was used to visualize protein expression.

4 RESULT AND DISCUSSION

In order to achieve the aim described in the earlier section, the role of the kynurenine pathway in the immunobiology of CMM was characterized using the public databases as well as an experimental set-up (paper I). Furthermore, the immune-metabolic network interactions of the Kynurenine Pathway in CMM patients were evaluated to validate the clinical relevance of immune tolerance, which is mediated by KP fluctuation. In this respect, first, this metabolic pathway activity was targeted in CMM patients undergoing MAPKIs therapy (paper II). Next, this evaluation extended into a group of CMM patients with ICIs therapy (paper III and IV). Below is a summary of the main results. Of note, for a more detailed description, please refer to the individual articles.

4.1 EXHAUSTION OF CD4+ T-CELLS MEDIATED BY THE KYNURENINE PATHWAY IN MELANOMA (PAPER I)

In recent years, complementary immune metabolism targeting IDO has been used to expand the response rate of ICIs. However, the blockade of IDO1 and treatment with PD-1 inhibitors in metastatic melanoma have displayed no benefit in survival compared to treatment with a single PD-1 inhibitor. Therefore, it is of interest to investigate an alternative regulatory path in KP, which contributes to the regulation of the CD4+T-cell subset.

In this study, we identified the link between KP with T-cell status in the TME. The TCGA cohort of cutaneous skin melanoma patients (SKCM) gene expression data have been utilized. Based on the observed correlations, KYN, 3-HK, and KYNA production *in vitro* were characterized using melanoma-derived BRAF wild type (wt) and BRAF V600E mutant

cell lines in culture with CD4+ CD25− T-cells. Moreover, correlation networks for CD4+ T-cells and KP-related genes in BRAF V600E compared with BRAF wt has generated to investigate the link between CD4+ T cell status and KP profile.

4.1.1 Kynurenine pathway-related genes are associated with T-cell status in the tumour microenvironment.

Tumour infiltrating lymphocytes (TILs) is considered to be a positive prognostic marker for various malignancies, and elevated levels of TILs have been linked to better clinical outcomes and longer-term survival for melanoma patients. To investigate whether the kynurenine metabolic pathway and T cell status are associated in the TME, SKCM in the TCGA cohort were separated into groups with low and high T cell signature gene expressions. Spearman correlation coefficient analyses were conducted on KP-related and T-cell status-related genes showing that the expression of *IDO1, IDO2, KYNU, and KMO* and T cell status-related genes are connected.

4.1.2 Inhibition of CD4 + CD25 T cell proliferation by melanoma cell lines (MCL) associated with KP enzymatic alteration

To illustrate how melanoma tumours affect CD4 + CD25 T cells, pre-activated primary CD4 + CD25 T cells (obtained from healthy donors) were cultured with MCLs (V600E and V600 wild type), for five days. Although CD4 + T-cells had a higher expression of CTLA4 and FOXP3 in culture with MCLs, their proliferation and IFNγ production were considerably reduced. Besides, CD4 + T-cells had a higher expression of CTLA4 and FOXP3 in culture with MCLs. These observations collectively may suggest the evolution of an exhausted phenotype of CD4 + T-cells. To assess whether CD4+ T-cells exhaustion and KP reprogramming are associated, KP metabolite concentration was measured with HILIC - MS/MS in cultured derived medium (cell type alone or cultured with CD4+ T cells) after 48 hours. This analysis revealed that co-cultured derived medium had a notable drop in TRP, 3-HK level, and higher level of KYN, KYNA when compared to the medium of MCLs and CD4 + T-cells alone.

Thus, we asked if KYN and KYNA elevated levels stimulate an exhausted phenotype of CD4+ T-cells upon exposure to MCLs. For this purpose, mRNA and protein expression of IDO1, IDO2, TDO2 mediating the KYN production, were compared in both CD4 + T-cells and MCLs. Only greater expression of IDO1 was identified in co-cultures compared to MCL alone, suggesting a higher activity of IDO1 in a co-culture setting.

4.1.3 IDO1 inhibition reconstruct CD4+ T-cells proliferation in culture with MCLs

To further examine whether IDO1 inhibition and KYN depletion can resume CD4+ T-cell proliferation, MCLs were cultured for 48 hours with CD4+ T-cells with or without IDO1 inhibitor INCB024360 (Epacadostat). CD4+ T-cells proliferation was retrieved in culture with MCLs, while the IDO1 inhibition in co-culture set-up resulted in lower KYN production. IDO1 blockade also led to inferior CTLA4 protein expression from CD4+ T-cells in culture with MCLs. CD4+ T-cells in culture with BRAF wt, interestingly, were more proliferative when treated with IDO1 blockade, which may suggest that mutation background of MCLs, KP metabolites profile and distinct CD4+ T-cell behavior in co-culture set-up are linked.

4.1.4 CD4+ T-cell behavior in co-culture set-up and KYNA production are linked

To further explore KP metabolite's role in CD4+ T-cell behavior, CD4+ T-cell proliferation and KP metabolites were assessed independently. We found CD4+ T-cells cultured with BRAF wt MCLs were less proliferative. On the other hand, no notable differences were detected in KYN production among different MCLs. We then explored the role of TRP depletion on CD4+ T-cell proliferation by adding TRP to the culture which was not able to rescue the CD4+ T cell proliferation. However, KYNA production, as well as PD-L1 and AHR mRNA expression, were higher in V600 wt MCLs cultured with CD4+ T-cells. On the This remark suggests that PD-L1 and AHR expression may lead to elevated KYNA and CD4+ T-cell exhaustion. We, therefore, evaluated the effect of KYNA production on CD4 + T-cell proliferation.

To explore the link between KYNA and CD4 + T-cell exhaustion, CD4 + T-cells were treated with various concentrations of KYNA. CD4 + T-cell proliferation and IFNγ production were measured on day five which reveals the CD4 + T-cell proliferation and IFNγ production may be affected negatively by KYNA.

In an effort to verify the link between BRAF status to CD4 + T-cell exhaustion, V600E cell lines (SK-MEL-28 and DFB) were transiently transfected with an expression vector encoding BRAF wild type and co-cultured with CD4 + T-cell for five days. The measurement of proliferation and IFNγ production of CD4+ T-cells on day five supports that BRAF wt phenotype are more prone to induce the exhausted phenotype in CD4+ T-cells compared to BRAF V600E.

4.1.5 IFNγ triggers KYN and KYNA but not 3-HK production in melanoma tumours

Due to the close association between KP activation and inflammatory stimuli, Th1 cytokine profile such as IFNγ, TNFα, IL-2 and Th2 cytokine profile such as IL-4, IL 2 and IL-10 were implemented on culture medium and measured using a multiplex Luminex assay. IFNγ and TNFα were the main cytokines that were secreted by CD4 + CD25− T cell activation. IL-2 production in the co-culture setting was not altered. Additionally, MCLs were treated with recombinant human IFNγ and TNFα for 48 hours in order to explore their role in KP activation. While no changes were observed in IDO1 expression or KYN production following TNFα treatment, IFNγ treatment was able to induce KYN, KYNA, and AA production, however, 3-HK production remained very low. In addition, CD4 + T-cells in culture with conditioned medium (CM) derived from IFNγ-treated MCL were less proliferative compared to CM derived from MCLS + CD4 + T-cell co-culture. This may partially explain by the KYNA induction. In addition, KYNA production in BRAF wt was higher than BRAF V600E MCLs. Interestingly, CD4 + T-cells, when co-cultured with MCLs, had lower *KMO* mRAN expression, suggesting that KMO regulates the levels of KYNA and may also be an attractive candidate that controls the KP in tumour immunity. On the other hand, KYN production was induced in MCLs after IFNγ treatment, but there was no meaningful change between KYN productions in control group. This result suggests that CD4 + T-cell exhaustion in the co-culture system may be due to elevated KYNA production.

4.1.6 Profiling of the correlation network for CD4 + T-cell and KP-related genes in BRAF V600E compared to BRAF wt with SKCM

Our previous results suggest that the mutation status of MCLs and KP metabolites concentration in the co-culture setting may mediate the distinct CD4+ T cell phenotype. We, therefore, evaluated whether KP retains different anti-tumour CD4 + T-cell immune responses in BRAF V600E compared to BRAF wt in a larger cohort. The CD4 + T-cells and KP related genes were extracted from PubMed. The correlation network between CD4 + T-cells and KP-related genes were reconstructed for each group (BRAF V600E & BRAF wt) using these Spearman correlation values. The differences between these two networks were visualized by DyNet which led to the identification of structural differences between two networks. Collectively, these results support our earlier finding in the co-culture set-up and suggest that KMO and KYNU pivotal role in the distinct CD4 + T-cell behavior in tumour immunity. Taken together, KP and CD4 + T-cell interactions in the BRAF wt and V600E networks suggest that kynurenine metabolism may play a vital role in the dysregulation of the immune response of the anti-tumour CD4 + T-cell.

Discussion:

Here, to facilitate our investigation on how KP changes were implemented on CD4 + T-cell function, we established an *in vitro* co-culture set- up consisting of CD4 + CD25 T cells and different MCLs (BRAF wt and BRAF V600E). The analysis of the SKCM-TCGA database, in support of our results, revealed a robust correlation between KP enzymes and T cell exhaustion. Furthermore, higher KYN and KYNA were closely connected to anergic phenotype in CD4 + T-cells, with defects in proliferation. Collectively, our data propose that in addition to IDO1 and KYN, other kynurenine metabolites and enzymatic activity may regulate CD4 + T-cell immunity. Besides, KMO may serve as an attractive candidate for regulating the KP in tumour immunity.

4.2 IMMUNOMETABOLIC NETWORK INTERACTIONS OF THE KYNURENINE PATHWAY IN CUTANEOUS MALIGNANT MELANOMA (PAPER II)

It has been reported that patients in many types of cancer have elevated KP activity and IDO expression by tumour cells can suppresse T-cell activity (203), and its expression has been connected to poor prognosis in CMM and other cancers (203). It was also described that there is a connection between higher KYN, 3-HK, and KYNA plasma levels and more aggressive forms of breast cancer and glioblastoma (204,205). However, not only KP metabolic dysregulation has not yet been entirely described in CMM patients, but also our understanding of the impact of treatment such as MAPKIs on KP machinery in these groups of patients is limited. Therefore, this study aimed to assess immune-metabolic network interactions of KP in CMM to explore the link between KP metabolites (KPMs) and regulation of the anti-tumour's immune response. The LC-MS/MS and proximity extension analysis (PEA), respectively were utilized to measure the plasma levels of KPMs and immune-related proteins in healthy volunteers and CMM patients before and during treatment with MAPKI.

4.2.1 Differential expression of enzymes with KP is associated with poor survival in patients with CMM.

TCGA database were utilized to gain further insight into the mutations, copy number changes, and expression profiles of KP enzymes. The TCGA melanoma cohort is consisting of 479 samples (<70% CMM; stage II, and III) from patients without neoadjuvant therapy before tumour resection were analyzed for this purpose. Our analysis revealed that metastatic

melanoma and breast cancer are the two types of cancer with the highest burden of gene changes in KP related enzymes. The most considerable proportion of KP gene mutations (> 20%) was found in melanoma patients. And KAT members have the highest mutation rate within the KP. In addition, we found that genetic changes in KAT responsible for KYNA production from KYN were connected to the poor survival of melanoma patients in the TCGA cohort. Further studies with Human Protein Atlas, CBio portal in healthy skin and CMM lesion (steps III and IV), show more extensive mRNA/protein expression of CCBL1 and CCBL2, among other KATs. It is important that CCBL1 change is correlated with poor prognosis in CMM patients, while KYNU is linked to better outcomes ($p < 0.05$), which may indicate that the KP enzymes have another inherent mechanism of action. KMO expression is not detected in healthy skin, as reported earlier, or melanoma tumours. In contrast, the protein expression of KYNU is inferior compared to CCBL1 and CCBL2 in both healthy skin and melanoma tumours. Further analyses using a TCGA cohort showed that BRAF mutation background and distinct KP profile might be connected.

4.2.2 KP Metabolite Profiles in PRE and TRM Metastatic CMM Patients Compared to Healthy Controls

In this study, we aimed to validate our previous observation and explore KP change in plasma to describe the role of KP in CMM patients. Therefore, LC-MS/MS was performed on plasma samples, which were collected from CMM patients, before (PRE) and during the first treatment (TRM) with MAPKIs as well as five healthy individuals (as controls). The downstream metabolites of the KP, including TRP, KYN, KYNA, 3-HK, AA, 3HAA, and XA, were analyzed. We found that CMM patients have lower levels of 3-HK and 3HAA compared to healthy controls. Therapeutic intervention with MAPKIs led to a higher level of 3-HK and 3HAA in TRM plasma samples. These results suggest that the 3-HK and 3HAA may serve as predictable metabolites in CMM. Moreover, a higher 3-HK/KYN ratio in healthy individuals and a higher XA/3-HK ratio was in melanoma patients were observed. In summary, this result suggests that melanoma patients have a higher buildup of KYN and a lower concentration of 3-HK and 3HAA in plasma compared to healthy individuals.

4.2.3 MAPKIs treatment and its association with distinct kynurenine pathway activities: Comparison of the correlation network in PRE and TRM groups

In the patient group, we assessed whether there is a relationship between treatment and altered KPMs concentrations in human plasma proteins in PRE and TRM samples with MAPKIs therapy. Correlation network analyses were executed on plasma immune-related

proteins and KPMs in PRE and TRM groups. Our result indicates the distinct characteristics of the PRE and TRM networks.

Moreover, therapeutic intervention with MAPKIs led to considerable changes in 3-HK levels in CMM plasma samples. Additionally, 3-HK and KYNA in PRE network interacted with a group of proteins that were enriched in the angiopoietin receptor Tie2 and tyrosine phosphatase SHP2 signaling, which negatively regulate Th1 differentiation.

On the other hand, The TRM network identified a set of proteins which mainly enriched in processes involved in Th1/Th2 differentiation, IL12-mediated signaling events, and TLR signaling.

Besides, analyses of mass spectrometry-based proteome analysis, which was performed on parental A375 and MAPKI-resistant sublines (A375R), displayed that the KYNU expression was higher in the A375 cell line. KYNU was also correlated with a group of proteins that is enriched in the activation of the mTORC1 signaling pathway in these data sets. It has previously reported that TRP depletion can suppress the mTORC1 pathway and, therefore, cell cycle arrest and T-cell energy (206,207). Thus, we suggested that KYNU expression in A375R may contribute to the acquisition of resistance to MAPKIs. Taken together, these results support that therapeutic intervention of MAPKIs leads to different KP metabolic trajectories in PRE and TRM CMM groups.

Discussion:

Collectively, Correlation network analyses of data resulting from PEA and LC/MS-MS characterized a group of proteins that modify the differentiation of Th1 cells, which is related to 3-HK levels. Besides, MAPKIs treatments and alteration of 3-HK and 3HAA concentrations which linked T and NK cell activation. These results suggest that KP is pathologically relevant in CMM patients.

4.3 THE IMMUNE CELL COMPOSITION OF PBMCS IN MELANOMA PATIENTS AND THEIR ASSOCIATION WITH RESPONSE TO NIVOLUMAB (PAPER III)

Despite these encouraging results, the clinical outcomes remain very variable; only a small fraction of patients show sustainable responses. Therefore, there is a need for predictive biomarkers and a more in-depth mechanistic examination of the cellular populations is required in clinical response. Here, PBMCs of patients diagnosed with CMM were collected before and during the treatment to examine immune signatures associated with clinical response to anti-PD-1 immunotherapy. We then conducted single-cell RNA seq analysis together with an interactive bioinformatics pipeline to produce a thorough analysis of

peripheral blood immune cells to identify response associated predictive signatures. We further validated our observation in an independent cohort by flow cytometry.

4.3.1 Stratification of therapy response using single-cell profiling of immune cells in melanoma patients treated with nivolumab

In order to study the association of peripheral blood immune cells with clinical response to checkpoint therapies, scRNA-seq analyses were performed on cells isolated from PBMC obtained from eight metastatic melanoma patients treated with first-line PD-1 inhibitor therapy (nivolumab), either sampled at baseline or during treatment. We used the following patient response categories defined by RECIST criteria: complete response (CR) and partial response (PR) for responders and progressive disease (PD) for non-responders. To relate molecular and cellular factors with the response to therapy, we classified each of the eight samples based on radiological assessments in progression/non-responders (NR; n = 4, including PD samples) or regression/responders. To determine the immune landscape in an unbiased manner, unsupervised clustering of 50,000 cells were used to identify 11 cell clusters, with two B cell clusters (clusters 7 and 10), two myeloid clusters (1 and 6), two CD4 + T-cell clusters (cluster 0 and 2) and five clusters enriched for CD8 + T / NK / NKT cells. While each patient presented changes in cluster frequencies between baseline and posttreatment samples, there were no significant changes when collecting all samples. When considering therapy response versus non-response for each patient, cluster 1 (monocytes) frequencies were higher in non-responders. Interestingly, a higher ratio of CD4 + T-cells to monocytes was associated with a better response. Our analysis thus identified distinct cell populations associated with the clinical outcome of nivolumab therapy.

4.3.2 Characterization of monocytic cell heterogeneity and its association with therapy response

Following our observation regarding the composition of the frequency of monocytes and the relationship between CD4 + T-cells and monocytes with clinical results, we aimed to look closer into these two subtypes. We found that most responders had a cell count for CD4 + T-cells/monocytes>2, and most non-responders had a ratio<1 for both baseline and posttreatment samples.

Furthermore, immune cell type-specific gene markers were used to annotate monocytic subtypes showing that cluster 1 and 6 cluster have distinct gene expression patterns; we called them monocyte and monocyte-1, respectively. Despite the insignificant statistical power, the frequency of monocyte-1 was inversely associated with clinical response. We further

identified markers in monocytes associated with insufficient response. These analyses revealed monocyte-related markers associated with a lack of response to checkpoint therapy.

On the other hand, CD4 + T-cells in the responding patient group had higher expression of a set of genes, which is enriched for leukocyte activation, T-cell differentiation, and T-follicular helper cell differentiation, gene ontology (GO) terms, using Gene Set Enrichment assay (GSEA). Furthermore, after the first treatment in the responding patient group, CD4 + T-cells had higher expression of a set of genes that are enriched in the regulation of adaptive immune response, T-cell mediated cytotoxicity, and interferon-gamma-mediated signaling pathway, GO terms. In contrast, CD4 + T-cells in the non-responders had higher expression of *PPP2R2D, KPNA6, MYCBP, TRMT61B, RAB18, ARID3B, CSTB, EDEM3, IWS1, C8orf44, PIGL, and PDK3* enriched in the mTOR and VEGF signaling pathway. Similarly, GSEA assay performed on DE genes identified a set of genes involved in TLR signaling cascade, which is upregulated in NR group monocytes compared to the R group. Parallel analyses of the DE gene in CD4 + T-cells identified a set of genes enriched in the c-MYC signaling pathway. This observation may support the link between TLR and the c-MYC signaling cascade. Finally, protein-protein interaction (PPI) network analyses of genes involved in the TLR and c-MYC signaling pathways show that these two networks are connected.

4.3.3 S100A9 expression in monocytes and response to PD-1 blockade

Our results generated by scRNA-seq analysis of PBMC in an initial detection group of melanoma patients indicated that *S100A9* expression with cluster 1 (monocytes) was one of the most differentially expressed genes between responding and not responding to anti-PD1 therapy. Most importantly, this demonstrated that low S100A9 levels in CD14+ cells were strongly associated with clinical responsiveness to anti-PD1 therapy. This raised the possibility that S100A9 expression in monocytes could serve as a predictive biomarker for a clinical outcome for this type of treatment. This is in line with existing evidence that S100A9 can indeed act as a prognostic marker for cancer, as well as a new marker for MDSC (124,125). Therefore, we decided to validate our results with scRNA-seq analysis in an independent cohort of patients with metastatic melanoma (n = 20) undergoing anti-PD1 therapy. Parameters studied were frequencies for all monocytes and S100A9 + monocytes within PBMC, while the ratio of CD4 + T-cells to monocyte frequencies and the ratio of CD4 + T-cells to S100A9 + monocyte frequencies within the total PBMC population were investigated. Group-wide comparisons studied each of these parameters after stratification

according to progression-free survival (PFS) in patients either shorter (n = 9) or longer than six months (n = 11). No differences were found between patient groups with long and short PFS in the frequency of monocytes. After determining a cut-off value of 20.1%, there are no differences in survival between groups with high or low frequencies of monocytes. Interestingly, when analyzing S100A9 + monocytes, we found that their frequencies were significantly higher in patients with short PFS. In addition, patients with S100A9 + monocyte frequencies higher than 15.3% showed a significantly lower overall survival compared to patients with frequencies lower than 15.3%. The relevance of monocyte populations was further confirmed when comparing the CD4 + T-cell/monocyte ratio between long and short PFS patients. In this case, patients with prolonged PFS showed a significantly higher CD4 + T-cell/monocyte ratio, which also resulted in an OS benefit. These differences showed higher statistical significance when considering the CD4 + T-cell/S100A9+ monocyte ratio. A similar analysis was also done for ratios of all and S100A9 + monocytes with CD8 + T cells, but there were no differences. Taken together, while scRNA-seq data indicate that the frequency of monocytes is inversely correlated with overall survival, results from multicolor cytometric analysis in a more extended patient group indicate that it is the relative size of the S100A9+ monocyte subgroup within the total PBMC population that is the strongest determinant for survival after anti-PD1 therapy.

Discussion:

Our findings demonstrate the elevated monocytes+S100A9 in the PBMCs of the CMM patients who are not responding to the PD-1 inhibition and highlights the therapeutic potential of S100A9. Moreover, a higher CD4+T-cells/monocyte ratio was associated with a better response to this therapy. Detailed knowledge of the functionality of S100A9+ monocytes is of high translational relevance. Therefore, the monocytic population play pivotal role in the outcome of the PD-1 blockade treatment, and the expression of S100A9 proteins is possible predictive biomarkers.

4.4 KYNURENINE PATHWAY ACTIVITY PREDICT PRIMARY RESISTANCE TO IMMUNE CHECKPOINT BLOCKADE IN CUTANEOUS MALIGNANT MELANOMA (PAPER IV)

Despite the tremendous success of ICIs in the treatment of CMM, still the vast majority display primary or acquired resistance. Therefore, there is a need for predictive biomarkers as well as more in-depth mechanistic insight into a clinical response. The field of immune metabolism is an attractive alternative strategy, and several studies have introduced

numerous metabolic players that serve as critical points for immune homeostasis and immunity against tumours. KP activity via IDO and tryptophan degradation has been characterized as a possible mechanism of resistance to immune checkpoint inhibitors. KP suppresses immunity by several different mechanisms including the direct immunosuppressive role of KYN or downstream metabolic mediators of KYN metabolism. Therefore, we aimed to explore the KP alteration in CMM patients undergoing therapy with ICIs, trying to deconvolute its potential contribution to resistance to ICIs therapy. In this respect, HiRIEF LC-MS/MS and scRNA-seq were performed on plasma samples, and PBMCs collected from CMM patients.

4.4.1 Elevated plasma concentration of kynurinase (KYNU) in CMM patient and its association with clinical response

To characterize the KPs fluctuations and their role in CMM patients, we performed LC-MS / MS analysis on plasma samples derived from (n = 24) CMM patients, before (PRE) and during the first treatment (TRM) with ICIs. All KP-related enzymes were analyzed, and only elevated KYNU expression in PRE-CMM patients was associated with poor clinical outcomes. Furthermore, in PRE-CMM patients in the non-responsive (NR) group was KYNU correlated with 48 proteins (DE) with a high representation of genes involved in the immune system and metabolism. However, when comparing KYNU expression in PRE-CMM patients with the TRM group, no notable changes were reported. These results suggest that KYNU levels may be linked to CMM patient responsiveness to ICIs.

4.4.2 KYNU cell-type-specific induction in CMM patients

Following our observation of elevated plasma KYNU level in NR-CMM patients in response to ICIs, we then aimed to explore the KP enzyme expression specificity in various immune cell types within PBMCs. To this end, scRNA-seq analysis were performed on CMM patients PBMC (n = 8) before and during the first treatment (TRM) with ICIs. Our study showed that clusters 1 and cluster 6, which have been identified as monocytes, have the highest expression of KYNU in NR, PRE CMM groups. On the other hand, from 49 plasma proteins which differentially expressed in NR groups, 12 of these markers were explicitly expressed by monocytes (Cluster 1) such as BLVR4, CAPN2, GSTO1, GUSB, LAP3, LMNB1, NQO2, PSMB8, PSME1, PYGL and LGALS3 were also associated with unfavorable clinical outcome. The other remaining two genes (HLA-B, HLA-DPB1), were expressed by several

other cell types. This result suggests that monocytic specific expression of KYNU in CMM patients.

4.4.3 KYNU-LGALS3 network in monocytes is connected to CD74-MYC network in CD4 + associated with clinical outcome in CMM patients

It has been previously reported that KP metabolic alteration and consequently, KP enzymatic activities linked to the exhausted phenotype of CD4 + T-cells. In our recent work, we reported that the ration of monocytes to CD4 + T-cell was an indication of inadequate response to ICIs (Unpublished, Rad Pour S., et al., 2020). Therefore, we evaluated whether KYNU-high expression in monocytes has any suppressive effect in CD4 + T-cells in a group of patients who do not respond to ICI treatment. Therefore, we first examined any association between highly connected plasma proteins to KYNU and their expression in PBMCs of CMM patients. We found that except HLA-B and, HLA-DPB1, other markers expressed by monocytes (cluster1 and 6) and their elevated expression are associated with inadequate response. HLA-B, and HLA-DPB1 were expressed by various cell types (Supplemental Fig. 3A-B). On the other hand, higher expression of *MYC* and *CD74* were detected in the NR group in CD4+ T-cells.

Spearman correlation analysis between KYNU, LGALS3 in Monocytic subtypes, and MYC and CD74 in CD4+ T-cell subtypes in PRE CMM-identifies a set of genes in each group, using GSEA analyses in the Molecular Signatures Database. Finally, to examine the possible role of the KYNU induction in regulating the CD4+ T-cell, a PPI network was generated by merging all identified DE genes in each group (total node= 171). MYC has the highest number of connections with (n=24) compared with CD74 (n=8), LGAS3 (n=6) and KYNU (n=2). More interestingly, they are connected either directly, such as LGALS3 and MYC or indirectly via intermediary proteins such as CD74 and KYNU. These results support that LGALS3, MYC, CD74, and KYNU are biologically connected, and perturbing their interaction will possibly modulate ICIs efficacy in CMM patients.

Discussion:

In this study, we found that KYNU and LGALS3 expression in protein and RNA levels negatively linked to clinical outcomes of CMM patients treated with ICIs therapy. Moreover, PPI analyses revealed that the KYNU-LGALS3 network in monocytes is connected to the CD74-MYC network in CD4+ T-cell. These results suggest that LGALS3, MYC, CD74, and

KYNU are biologically connected, and perturbing their interaction will possibly modulate ICIs efficacy in CMM patients.

5 CONCLUSIONS

(PAPER I) In this study, the association between the KP with T-cell status in the TME was first, identified in SKCM- TCGA dataset. We then established an *in vitro* co-culture set-up consisting of CD4+CD25- T-cells in culture with MCLs to investigate the outcome of KP alteration on CD4+ T-cell function. We then characterized the production of KYN, 3-HK, and KYNA *in vitro* using MCLs and primary CD4+ CD25− T-cells. We also found that frequency of IFNγ producing CD4+ T-cells associated with elevated levels of KYN and KYNA. Simultaneously, the proliferation of CD4+ T-cells and KMO expression were reduced, while exhaustion markers such as PD-L1, AHR, FOXP3, and CTLA4 were augmented. Our results conclude that there is an alternative immune regulatory mechanism in addition to IDO1 which is associated with the lower KMO expression and the higher KYNA production, which contributes to dysfunctional effector CD4+ T-cell response.

(PAPER II) By following up on the immune regulatory role of KP discussed in paper I, we aimed to investigate the KP alteration in CMM patients tumour environment. Therefore, plasma samples of the CMM patients were collected before (PRE) and during treatment (TRM) with MAPKIs. Proximity extension assay (PEA) and LC/MS-MS were performed on these samples. Correlation network analyses of the PRE CMM patients samples revealed that lower 3-HK concentration might negatively contribute to the differentiation of Th1 cells. On the other hand, CMM patients treated with MAPKIs have shown a higher concentration of 3-HK and 3HAA as well as higher “CXCL11” and “KLRD1” protein expression in their plasma. This result proposes that melanoma patients may have a higher accumulation of KYN and a lower concentration of 3-HK and 3HAA in plasma. Therefore, KP holds a different trajectory and path in healthy individuals compared with CMM patients.

(PAPER III) In order to find predictive markers for PD-1 checkpoint -based immunotherapy, scRNA-seq's analyses of PBMCs (n=8) as well as an in-depth immune monitoring study (n=24) were carried in CMM patients treated with nivolumab. Blood samples were collected before treatment and at the time of second doses. A lower ratio of two distinct cellular populations, CD4+ T-cells to monocytes and a higher level of monocytes, were inversely associated with overall survival. Our results produced by scRNA-seq analysis of PBMC in an initial discovery cohort of melanoma patients showed that S100A9 expression by

monocytes in cluster 1 was one of the most differentially expressed genes between responders and non-responders to anti-PD1 therapy. Most importantly, this revealed that low S100A9 levels in CD14+ cells were considerably associated with clinical responsiveness to anti-PD1. Furthermore, analyzing S100A9+ monocytes indicated that their frequencies were significantly higher in patients with short PFS. The relevance of monocytic populations was further confirmed when the CD4+ T-cell/monocyte ratio was compared between long and short PFS patients. Our result suggests that the frequency of monocytes is inversely correlated with survival and clinical benefit. Therefore, the monocytic population can be critical in the outcome of the PD-1 blockade treatment and the expression of S100A9 proteins are possible predictive biomarkers.

(PAPER IV) In this study, we found that KYNU and LGALS3 expression in protein and mRNA levels negatively linked to clinical outcomes of CMM patients treated with ICIs. Additionally, KYNU and LGAlS3 have shown a cell-type-specific pattern in PBMCs, in which monocytes have significantly higher expression of KYNU and LGALS3 compared with other cell types.

Moreover, differential network and protein-protein interaction analyses revealed that the KYNU-LGALS3 system in monocytes is connected to the CD74-MYC network in CD4+ T-cell. These results support that LGALS3, MYC, CD74, and KYNU are biologically associated, and perturbing their interaction will possibly modulate ICIs efficacy in CMM patients. Our work suggests that the expression of KYNU in monocytes is inversely correlated with survival and clinical benefit. Therefore, not only can the monocytic population can be crucial in the outcome of the ICIs, but also the KP activity serves as an influential factor in the ICIs outcome.

6 REMARKS AND FUTURE PERSPECTIVES

While the KP is responsible for the production of the necessary cofactor NAD+, many of the pathway catabolites play roles in a variety of disease states. The engagement of the KP enzymes and catabolites in cancer arises via both immune and nonimmune machinery. Much attention has been dedicated to determining the role played by IDO in enabling tumour immune escape via TRP depletion. While the role played by other enzymes, such as KYNU, KMO, or KATs—which modulate the immune response by producing 3-HAA, 3-HK, and KYNA, respectively, is not yet described. Further to this, the involvement of KP downstream enzymes and catabolites in tumour progression is not yet well discussed.

Here, we have explicitly reported that elevated plasma level of KYNU in CMM patients is associated with poor response to the ICIs therapy. This result basically demonstrates the clinical relevance of KYNU, the downstream enzymes of KP, which mediate the production of AA and eventually 3HAA. Further validation of this observation by scRNA-seq profiling of CMM patients immune cell type (PBMCs) treated with ICIs, not only supports that elevated KYNU expression is linked to the clinical outcome but also reported that only monocytes have shown the differential expression of KYNU. However, we need to develop more efficient ways of analyzing and evaluating the role of the KYNU in a clinical setting by in-depth metabolic analysis on the KP as a complex system.

Further efforts must be applied for the implementation of state-of-the-art analytical tools to assess the KUNU enzymatic function in the tumour microenvironment. Finally, additional attempts should be made to assess the druggability of KYNU as strategy to enhance the treatment response in CMM patients.

What is clear from this study is that the KP is of great importance in CMM and therefore characterizes as a crucial metabolic checkpoint for the development of future cancer immunotherapy methods. Therefore, it is likely that we will soon witness not only the discovery of additional physiological and pathological roles for KP activity but also an increasing interest in drug development based on these roles. Specifically, by targeting the KP with novel pharmacological or genetic manipulation, it may be possible to enhance the treatment response in CMM patients.

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