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A translational exploration of melanoma immunology focused on indoleamine 2,3-dioxygenase

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Lessons for immunoprofiling and immunotherapy

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Voor mijn familie

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◆ List of abbreviations

1-D-MT	1-methyl-D-tryptophan
1-L-MT	1-methyl-L-tryptophan
1MT	1-methyl-tryptophan
ABC	Avidin-biotin-peroxidase
AEC	3-amino-9-ethylcarbazole
AhR	Aryl hydrocarbon receptor
APC	Allophycocyanin
ASCO	American Society of Clinical Oncology
BADO	Belgian Association for Dermato-Oncology
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CFA	Complete Freund's adjuvant
CLA	Cutaneous lymphocyte-associated antigen
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non integrin
DMSO	Dimethylsulfoxyde
DRU	Dermatology Research Unit
EDTA	Ethylenediaminetetraacetic acid
EORTC	European Organisation for Research and Treatment of Cancer

FBS	Foetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
GAS	gamma-activated sequence
GCN2	General control nonderepressible 2
G-CSF	Granulocyte colony-stimulating factor
GITR	Glucocorticoid-induced TNF receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
Gp100	Glycoprotein 100
H&E	Haematoxilin and eosin
HIF-1a	Hypoxia-inducible factor 1alpha
HLA	Human leukocyte antigen
HPF	High power field
HUVEC	Human umbilical vein endothelial cells
ICQ	Interquartile range
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IL	Interleukin
IMCs	Immature myeloid cells
iNOS	Inducible nitric oxide synthase

ISRE	Interferon-stimulated response element
LCs	Langherhans cells
MACS	Magnetic-activated cell sorting
MAGE	Melanocyte-associated antigen
MART-1	Melanoma Antigen Recognized by T-cells
M-CSF	Macrophage colony-stimulating factor
mDCs	Myeloid dendritic cells
MDSCs	Myeloid-derived suppressor cells
MelanA	Melanocyte Antigen
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mMDSCs	Monocytic myeloid-derived suppressor cells
mTOR	mammalian target of rapamycin
NF-κB	Nuclear factor-κB
NK cells	Natural killer cells
NY-ESO-1	New York oesophageal squamous cell carcinoma 1
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid dendritic cells
PD-L1	Programmed death ligand 1
PE	Phycoerythrin
PerCP	Peridin-chlorophyll-protein complex

pmnMDSC	polymorphonuclear myeloid-derived suppressor cells
RFS	Relapse-free survival
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCF	Stem cell factor
STAT	Signal transducer and activator of transcription
TAA	Tumour associated antigen
TCR	T-cell receptor
TDLNs	Tumour-draining lymph nodes
TDO	Tryptophan dioxygenase
TGF-β	Transforming growth factor-beta
Th	T helper
TILs	Tumour infiltrating lymphocytes
TNF-α	Tumour necrosis factor-α
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor

SUMMARY

◆ Summary

In this thesis, we performed a comparative evaluation of adaptive melanoma immunity focusing on the immunosuppressive network surrounding indoleamine 2,3-dioxygenase (IDO), an immunosuppressive enzyme known to have a negative prognostic value in melanoma. To do so, we have chosen for a translational approach, aiming to clarify the in vivo relevance of the investigated immunosuppressive mechanisms in melanoma patients.

In the first part of this thesis, we set out to identify IDO expression in the primary melanoma by immunohistochemistry. The role and pattern of IDO expression by tumour cells versus host cells was compared, and found to be different. Sentinel IDO-positivity was inversely correlated with CD8+ lymphocytes and tumour-infiltrating lymphocytes (TILs) in the primary melanoma, which are both markers of an active anti-tumour response. Remarkably, endothelial IDO expression was found to be highly consistent in the primary, the sentinel and metastatic tissues of melanoma patients, indicating that immune suppression in melanoma is determined very early in the disease course. These results show that IDO expression in melanoma is a marker of the patients' anti-tumour immune response with an independent prognostic value.

In the second part of this thesis, we used multi-colour flow cytometry to comparatively assess the clinical relevance of six circulating cell types with a role in anti-tumour adaptive immunity. We demonstrated that circulating plasmacytoid dendritic cells (pDCs) and myeloid-derived suppressor cells (MDSCs) represent a single immunologic system and have prognostic value in melanoma patients, independent of disease stage. Both were correlated with cytotoxic and regulatory T-cell frequencies, indicating that they are key players in the systemic immunological climate of melanoma patients.

In the third part of this thesis, IDO expression could be identified in pDCs and mMDSCs using multi-colour flow cytometry. This IDO expression increased in advanced disease stage and its functional activity was confirmed by concomitant tryptophan breakdown, which was measured by ultra-performance liquid chromatography (UPLC) in the patients' serum. Systemic IDO expression was associated with increased circulating programmed death ligand 1 (PD-L1+) cytotoxic T-cells, and high levels of circulating PD-L1+ cytotoxic T-cells in turn correlated with increased cytotoxic T-lymphocyte antigen-4 (CTLA-4) expression by Tregs. This illustrates that negative feedback immune mechanisms in melanoma should be considered as one interrelated signaling network. Moreover, both increased PD-L1+ T-cells and CTLA-4 expression in Tregs conferred a negative prognosis, indicating their in vivo relevance.

In the fourth part of this thesis, we wanted to test whether these systemic immunosuppressive mechanisms are relevant during immunotherapy. We chose adjuvant IFN-α2b therapy for patients with high-risk melanoma as a model. IDO expression was upregulated by circulating pDCs in melanoma patients treated with adjuvant IFN-α2b. This was associated with tryptophan consumption in the patients' serum and higher Treg and PD-L1+ cytotoxic T-cell frequencies in the blood. We hypothesize that in IFN-α2b treated patients, IDO activity could act as a negative feedback mechanism and might thus limit the clinical efficacy of IFN-α2b by failing to overcome T-cell anergy.

In conclusion, we believe that this work has contributed to clarifying the in vivo role of IDO expression in various compartments of the immune system of stage I to IV melanoma patients. By placing these data in the context of current immunotherapeutic strategies, they could have implications for both immunoprofiling and immunotherapy.

◆ Samenvatting

In deze thesis werd een vergelijkende evaluatie van het adaptieve immuunsysteem in melanoompatiënten uitgevoerd. Hierbij lag de klemtoon op indoleamine 2,3-dioxygenase (IDO), een immunosuppressief enzym waarvan de negatieve impact op de prognose in melanoom gekend is. Er werd gekozen voor een translationele benadering, om zo de in vivo relevantie van de onderzochte immunosuppressieve mechanismen in melanoompatiënten te verduidelijken.

In het eerste deel van deze thesis werd immuunhistochemie gebruikt om IDO expressie in het primaire melanoom te identificeren. De rol en het patroon van IDO expressie door tumorcellen enerzijds en gastheercellen anderzijds werd vergeleken en bleek verschillend te zijn. IDO expressie in de sentinelklier was omgekeerd gecorreleerd met een CD8+ T-cel infiltraat in het primaire melanoom, dit laatste is een gekende merker van een actieve antitumorale immuunrespons. IDO expressie door endotheelcellen in het primaire melanoom, de sentinelklier en in metastasen bleek ook opmerkelijk consistent. Dit alles geeft aan dat immuunsuppressie al vroeg in het verloop van een melanoom kan ontstaan. Onze resultaten tonen dat IDO expressie in melanoompatiënten mogelijk een merker is van de antitumorale immuunrespons, met een onafhankelijke prognostische waarde.

In het tweede deel van deze thesis hebben we acht-kleuren flow cytometrie gebruikt om een vergelijkende evaluatie uit te voeren van zes verschillende circulerende celtypes die een rol hebben in het adaptieve immuunsysteem. We konden aantonen dat circulerende plasmacytoïde dendritische cellen (pDCs) en myeloid-derived suppressor cellen (MDSCs) deel uitmaken van eenzelfde immunologisch systeem. Deze celtypes hadden bovendien een prognostische waarde in melanoom, ongeacht het ziektestadium waarin de patiënt zich bevond.

In het derde deel van deze thesis hebben we IDO expressie kunnen vaststellen in circulerende pDCs en mMDSCs door middel van acht-kleuren flow cytometrie. Deze IDO expressie was hoger in patiënten met gevorderde ziekte, en ging gepaard met afbraak van tryptofaan in het serum van de patiënten. De concomitante tryptofaanafbraak onderbouwt dat de gemeten IDO expressie ook functioneel actief is, ze werd gemeten met “ultra-performance liquid chromatography” (UPLC). Systemische IDO expressie was gecorreleerd met verhoogde niveaus van circulerende programmed-death ligand 1 (PD-L1)-positieve cytotoxische T-cellen. Deze laatste waren op hun beurt gecorreleerd met een toegenomen cytotoxic T-lymphocyte antigen-4 (CTLA-4) expressie door regulatoire T-cellen (Treg). Dit alles illustreert dat verschillende negatieve feedback mechanismen in melanoom als een signaalnetwerk met elkaar in verbinding staan. Bovendien gingen verhoogde niveaus van circulerende PD-L1+

cytotoxische T-cellen en CTLA-4 expressie in Tregs gepaard met een slechtere prognose, wat de in vivo relevantie van deze celtypes onderstreept.

Tenslotte wilden we in het vierde deel van deze thesis nagaan of de geïdentificeerde systemische immunosuppressieve mechanismen ook relevant zijn tijdens immunotherapie. Hiervoor werd de adjuvante behandeling met interferon-alfa (IFN-α2b) voor melanoompatiënten met een hoog risico op herval als model gekozen. Patiënten die behandeld werden met IFN-α2b hadden een verhoogde IDO expressie in circulerende pDCs. Dit ging gepaard met tryptofaan verbruik in het serum, alsook met hogere frequenties van Tregs en PD-L1+ cytotoxische T-cellen in het bloed. We vermoeden daarom dat in deze patiëntengroep, IDO activiteit deel zou kunnen uitmaken van een negatief feedback mechanisme. Hierdoor zou dan de capaciteit van IFN-α2b om T-cel anergie te overwinnen kunnen beperkt worden, wat de werkzaamheid ervan mogelijk limiteert.

Als conclusie kunnen we stellen dat we geloven dat dit werk heeft bijgedragen tot het verhelderen van de in vivo rol van IDO expressie in verschillende delen van het immuunsysteem van stadium I tot IV melanoompatiënten. Door deze data te interpreteren in de context van de immunotherapieën die momenteel gebruikt worden, kunnen hieruit gevolgen getrokken worden voor zowel immunoprofilering als immunotherapie.

CHAPTER 1:

Introduction

1. Melanoma

Melanoma is a malignant tumour that arises from melanocytes and is most commonly cutaneous in origin. It can also occur on mucosal surfaces and within the uveal tract of the eye. Four major subtypes of melanoma have been described (superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma and acral lentiginous melanoma), but more rare variants also exist. While the majority of melanomas are brown-black in colour due to melanin deposition, some are skin-coloured to pink-red (amelanotic melanomas). Clinical observations such as incomplete or complete regression of melanoma, melanoma-associated development of vitiligo-like depigmentations and a higher incidence of melanoma in immunosuppressed patients point to the fact that melanoma is an immunogenic tumour.[1] Over the last decades, the incidence rates of cutaneous melanoma have significantly increased. The annual rise in melanoma incidence varies depending on the population (Caucasians are most at risk), but is estimated to be between 3% and 7%. These estimates predict a doubling of melanoma incidence rates every 10-20 years.[2] Mortality rates from melanoma peaked around the 1990s'. Subsequently trends have been less uniform, but tend to remain more or less stable.[3] This is probably due to improved detection, as there is an ongoing trend for diagnosis of thinner melanomas in Western Europe.[4] The vertical tumour thickness (Breslow thickness) is indeed the most important local prognostic factor in primary cutaneous melanoma. Other independent prognostic markers are ulceration and the number of mitoses present in the dermis. The importance of tumour thickness reflects the biology of a primary melanoma, that encompasses several phases. In the radial growth phase, melanoma cells grow within the epidermis. Next, individual melanoma cells start infiltrating the superficial dermis. When the vertical growth phase fully develops, tumour cell nests can be found in the dermis, where further expansion will take place. When the melanoma expands in the dermis, tumour cell nests can also develop at a certain distance from the primary lesion. These are called microsatellites (arising at 0.5 to 20 mm from the primary melanoma), and their presence immediately classifies a melanoma as stage III. The current American Joint Committee on Cancer (AJCC) classification for staging of melanoma takes all these factors into account, an overview can be found in addendum 1.[5, 6]

2. Current management of melanoma

The guidelines and principles that are outlined here are based on the recommendations of the Belgian Association for Dermato-Oncology (BADO) that we published in the Belgian journal 'OncoHemato'. This article can be found in addendum I.[7]

❖ Local disease (stage I&II)

The treatment of a primary melanoma starts with the diagnostic excision, using a 2 mm margin. If the diagnosis of melanoma is confirmed, a thorough physical examination with total skin inspection is necessary. After all, patients with a history of melanoma have a 0.2-8% risk of developing a second primary melanoma, and 30 to 40% of these will occur synchronously.[8] At the same time, the presence of cutaneous or lymphoid metastases has to be checked. There is no consensus on which imaging should be done, but BADO has made recommendations for each tumour stage. If there is no evidence for metastasis, a wide local excision is the next therapeutic step, aiming to decrease the risk of local recurrence. The margins for this second excision are based on the Breslow thickness and range from 0.5 to 2 cm. In melanomas of stage pT1b or more, a sentinel lymph node biopsy can be performed at the same time. This procedure allows for a more careful staging, but does not affect survival.[9] If (micro)metastases are detected in the sentinel lymph node, the patient has stage III disease.

❖ Regional disease (stage III)

Patients who are diagnosed with stage III melanoma due to lymph node involvement or in-transit metastasis, should be surgically treated. Both micro- and macro-metastases necessitate a complete lymph node dissection. Adjuvant treatment with interferon-alpha (IFN-α) can also be proposed, because these patients have a high risk of relapse. Currently, IFN-α is the only therapy approved in Europe for adjuvant use in patients with resected melanoma at high risk of recurrence. In Belgium, intermediate-dose IFN-α2b is used with an induction phase of $10x10^6$ U/m² IV 5d/week, followed by a maintenance phase of $5x10^6$ U subcutaneously 3x/week for 2 years. Significant relapse-free survival (RFS) benefits have been demonstrated, but impact on overall survival (OS) is modest.[10] An important hiatus in the use of IFN-α is the lack of knowledge on which patient subgroup benefits most from this treatment. A recent post-hoc analysis of EORTC trials 18952 and 18991 suggests that adjuvant IFN-α has the greatest impact in patients with microscopic regional disease and/or an ulcerated primary

melanoma.[11] This interesting idea is now being investigated in a prospective clinical trial (EORTC 18081) for patients with ulcerated primary melanomas (T2b-4b) who have no regional or systemic disease.[12]

❖ Systemic disease (stage IV)

Melanoma that has disseminated to distant sites is regarded as incurable, with 5-year survival rates of around 10% and a median overall survival of 9 months (Fig. 1).[13] Until 2010, DTIC (dacarbazine) was the standard therapy for stage IV melanoma patients, with response rates of around 20% and a median duration of response of 5-6 months. Nevertheless, treatment with DTIC never produced a significant survival benefit.[14]

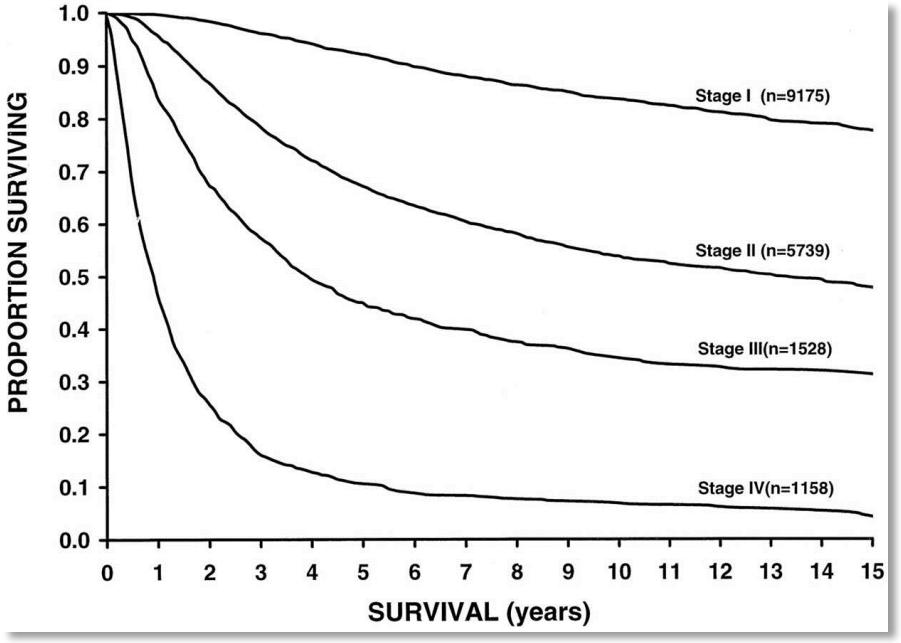

Figure 1: Stage-specific overall survival in melanoma.
Fifteen-year survival curves comparing localized melanoma (stages I and II), regional disease (stage III), and systemic disease (stage IV). The numbers in parentheses are patients from the AJCC melanoma staging database used to calculate the survival rates. The differences between the curves are significant ($P < 0.0001$). Adapted from Balch et al., 2001.[13]

Since 2010, this landscape has changed spectacularly, as two distinct treatment approaches have shown survival benefit for patients with metastatic melanoma: targeting mutations in the tumour cells and immunomodulation.

Targeted therapy

Melanoma is a molecularly heterogeneous disease, and the discovery of driver mutations in various melanoma subtypes has led to the development of inhibitors that target the mutated signal transduction cascade.[15] A V600 BRAF mutation causes constitutive activation of the MAPK signalling pathway, resulting in increased cell proliferation. On average, 41% of all melanomas is BRAF-positive, but this percentage increases to 49% for melanomas arising in younger individuals, on non-chronically sun-damaged skin and melanomas of the superficial spreading type.[16] Vemurafenib (Zelboraf®) was the first BRAF-inhibitor to be approved as a first-line treatment for V600-mutant melanomas, and a second BRAF-inhibitor (dabrafenib - Tafinlar®) has recently followed. Treatment with vemurafenib produces median progression-free survival rates of 6.9 months, compared to 1.7 months with DTIC. Similarly, median overall survival rises from 9.7 months with DTIC to 13.6 months with vemurafenib. The most remarkable features of treatment with vemurafenib are the high response rates (48%), which occur fast (1.45 months) and a relatively good tolerance. The most common side-effects are arthralgia (59%), fatigue (42%) and cutaneous manifestations such as rash (52%), photosensitivity (52%) and squamous cell carcinomas or kerato-acanthomas (26%). Data on dabrafenib are less mature, but similar clinical responses are seen and good responses have been registered with this compound in patients with brain metastases.[17] Dabrafenib's toxicity profile seems to be more favourable compared to vemurafenib, with less photosensitivity and fewer squamous-cell carcinomas (7% of patients versus 20-30%).[18] Unfortunately, even though the clinical responses with BRAF-inhibitors can be spectacular, they are usually short-lived (median 6 months).[19] Possible mechanisms of resistance are an area of active research, and combining BRAF inhibition with MEK (a molecule downstream in the MAPK pathway) inhibition seems a promising approach.[20]

Immunotherapy

Ipilimumab (Yervoy®) is a fully human monoclonal antibody that binds to and blocks CTLA-4, which is an immune checkpoint molecule that downregulates T-cell activation in physiological circumstances. The mechanism of action will be discussed in more detail later on in this thesis. In 2010 the results of a phase III clinical trial were published, demonstrating a significant survival benefit with ipilimumab compared to a gp100 vaccine. Median overall survival was 10.1 months, which is a 34% increase compared to the control group. One year after treatment, 46% of patients was still alive, compared to 25% in the control population.[21] Even though the response rate is limited to 15-20%, an important and

previously unseen phenomenon occurs in the so-called "tail-of-the-curve"; about 15% of all patients treated with ipilimumab are still alive 4 years after treatment.[22] An important limitation to treatment with ipilimumab is that responses are delayed up to 3 months or more. Adverse events occur in 40% of patients and comprise mostly auto-immune phenomena affecting the skin (dermatitis[23], pruritus), gastro-intestinal organs (colitis, hepatitis) and the endocrine system (hypophysitis, adrenal insufficiency). Grade 3–4 adverse events occur in less than 10% of patients, but can be fatal. Adverse events usually resolve spontaneously or after steroid treatment, but endocrine failure frequently needs permanent hormonal supplementation. In Belgium, ipilimumab is approved as a second-line therapy in stage IV melanoma patients.

3. Immune response in melanoma: basic principles & concepts

❖ Cancer immunoediting: from elimination over equilibrium to escape

The idea that the immune system plays an important role in cancer biology is not new. The first milestone in the discovery of tumour immunology occurred in 1863, when Rudolph Wirchow perceived that tumours are commonly infiltrated with leukocytes. In the early 20th century, Paul Ehrlich predicted the existence of immune surveillance and in the 1950's Burnet and Thomas laid the foundations for the concept of immune tolerance.[24] The role of the immune system in cancer remained controversial until the development of genetically-modified mouse models for immunodeficiency in the 1990's. Ever since, there has been a growing recognition that the immune system can also play a tumour-promoting role, eventually leading to the immunoediting hypothesis.[23, 25] The process of cancer immunoediting consists of 3 phases, as outlined here (and reviewed in detail by Dunn et al. in 2002).[23]

Elimination (=immune surveillance)

The origin of an anti-tumour immune response lies in signals derived from apoptotic or necrotic tumour cells and their surroundings, such as damage-associated proteins (DAMP). These are internalised by immature dendritic cells (DCs), which are potent antigen presenting cells (APCs) that subsequently process the tumour-associated antigens (TAA) and present them on their cell surface. After migration to the lymphoid tissues the activated mature DCs stimulate naïve T-cells to become skin-homing effector cells that should eliminate the causal stimulus (Fig. 2).[26] Apart from lymphocytes, cells from the innate immunity (such as natural killer cells and macrophages) can also kill tumour cells.

Equilibrium

If some tumour cells are not killed, the process can progress to the second phase in which the tumour persists but is prevented from expanding by immune pressure. In this way, tumours can be controlled by the immune system for long periods of time. T-cells, IL-12 and IFN-γ are known to sustain this dormant state.[27] Cancer cells in equilibrium proliferate poorly, but when they eventually become edited, an escape from immune control can occur.

Escape

The third phase begins as the delicate balance between tumour growth and immune control turns over to the former.[28] Continuous pressure from the immune system on genetically unstable cells can lead to the generation of tumour variants that are no longer recognized by the immune system, have become insensitive to the effector mechanisms or induce an immunosuppressive, tolerant microenvironment. Tumours that were non-immunogenic from the start will directly go into the escape phase. In this phase, regulatory T-cells, myeloid-derived suppressor cells and many other immunosuppressive mechanisms play a role.[29] The result of the escape phase is the formation of clinically detectable, progressively growing tumours.

In the following paragraphs, the role of the different immune cells of adaptive immunity that play a leading role in the (failing) anti-melanoma immune response will be described. This discourse will be limited to those cell subsets that are further investigated in this thesis.

❖ Antigen presentation: dendritic cells

Dendritic cells originate from hematopoietic progenitor cells in the bone marrow upon stimulation by various soluble factors such as granulocyte/macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF) and IL-3.[30] Most DCs develop along the myeloid-lineage pathway that also produces macrophages and granulocytes as mature end-products.

Pathogens are internalized by DCs upon encounter and degraded by enzymes to produce small antigenic peptides to be presented to naïve T-cells via major histocompatibility complex (MHC) class I and II molecules. This recognition of an MHC-bound antigen on APCs by the T-cell receptor (TCR) constitutes the first step of T-cell activation. Secondly a co-stimulatory signal is required, in which CD28 on the T-cell interacts with CD80 or CD86 (also known as B7-1 and B7-2) on the DC. Together, these two signals will stimulate T-cell proliferation. A third signal, which is either a DC-derived cytokine (IL-12 and type I IFNs) or CD40 ligation, will drive the polarization of the T-cell into a specific functional

subset.[31] Before encounter of a pathogen, DCs are immature and reside in the skin as epidermal Langerhans cells or dermal DCs. Immature DCs have a low expression of MHC and co-stimulatory molecules, but they are not entirely passive. Immature DC are not only capable of inducing regulatory T-cells to preserve tolerance to foreign and self-antigens, they also cause anergy of effector T-cells.[32]

Two important subsets of DCs circulate in the blood: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Both subsets are competent stimulators of T-cell function and share the lineage (CD3, 14, 15, 19, 20, 56) negative and MHC class II (HLA-DR) positive phenotype. Myeloid DCs are CD11c+ CD123- and require GM-CSF for growth and function, they primarily secrete IL-12 and IL-18. Plasmacytoid DCs are CD11c-CD123+, depend on IL-3 for survival in vitro and produce high levels of IFN-α.[31, 33] These DC subsets have complementary functions, but accumulating evidence indicates that to generate an effective anti-tumour immune response, extensive cross-talking between both subsets is required.[34, 35]

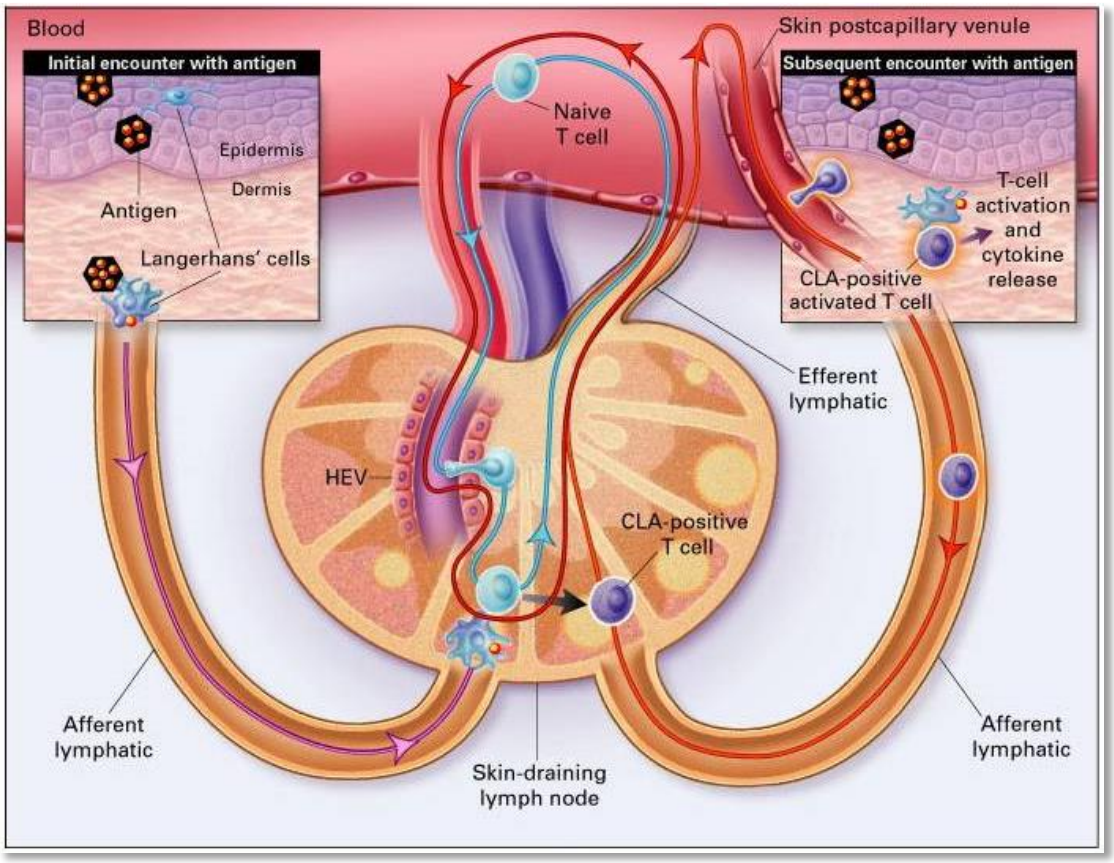

Figure 2: Movement of dendritic cells and T-cells in the skin.
Left: antigen presenting cells (APC) that reside in the skin, such as Langerhans' cells and dermal dendritic cells, can internalize foreign antigens (such as tumour-associated antigens [TAA] in melanoma patients) in the skin and migrate through the afferent lymphatics to the sentinel skin-draining lymph nodes, where they present the TAA to naïve T-cells. Centre: these naïve T-cells continuously screen the lymphoid organs via high endothelial venules (HEV) until their corresponding peptide is presented to them in the context of the MHC (blue loop). When a TAA is presented by an APC to its specific naïve T-cell, the T-cell will become activated. Right: it will then proliferate and acquire "antigen memory" and a distinct set of adhesion molecules that will allow it to navigate outside of the blood vessels and the lymphoid organs, to the peripheral tissues and organs (like the melanoma metastases) where it will be able to be reactivated upon re-presentation of the same TAA (red loops). Adapted from Robert and Kupper, 1999.[26]

Dendritic cells have a central role in anti-tumour immunity. They constitute the crucial link between innate and adaptive immunity, and their capacity to induce either immune activation or tolerance makes them powerful players in the maintenance of this delicate balance. Consistent with their central role, both local and systemic DC dysfunction often occur in cancer. One contributing factor is the deficient maturation of immature myeloid cells to mature DCs, a process that will be discussed in the chapter on myeloid-derived suppressor cells. Secondly, the migration and function of normal DCs are also impaired in the tumour-microenvironment, which is characterized by hypoxia.[36] In the hypoxic microenvironment, DCs upregulate the transcription factor hypoxia-inducible factor 1 alpha (HIF-1α). This ultimately results in increased expression of vascular endothelial growth factor (VEGF) and of the pro-inflammatory cytokines IL6 and IL8. However, expression of immunosuppressive mediators such as IL-10, transforming growth factor beta2 (TGF-β2) and indoleamine 2,3-dioxygenase (IDO) will also be increased. DCs primed by hypoxia can cause increased tumour vascularisation and promote tumour growth in mice.[37]

The presence of mDCs and pDCs in the tumour microenvironment has been correlated with poor clinical outcome. In melanoma patients, circulating DC frequencies have been reported to be unchanged in stage I-III,[38, 39] and reduced in stage IV.[39-41] Similar patterns in circulating DC alterations have been described in breast, liver, head and neck and lung cancer.[42-45]

❖ Effector function: cytotoxic T-cells

After appropriate activation by APCs, antigen-primed T-cells start to proliferate and differentiate. When they arrive at the site of immune attack, effector T-cells will exert their function by releasing cytokines to mediate local inflammation. The most important cytokine in cytotoxic T-cell function is IFN-γ, which mediates its effects via activation of STAT1 (signal transducer and activator of transcription 1).[28] Effector T-cells will also secrete cytotoxic granules (containing granzymes and perforin) by exocytosis in the vicinity of tumour cell membranes. Granzymes are a family of structurally related serine proteases with various substrate specificities and perforin is a membrane-disrupting protein, both will induce apoptosis of the target (tumour) cell.[46]

However, in the tumour microenvironment, cytotoxic T-cells are often in an immunotolerant state that interferes with their cytotoxic function. This results in a reduced IFN-γ and perforin expression.[47] Tumour cells secrete factors (such as Galectin-3) that can induce apoptosis of cytotoxic T-cells.[48] Furthermore, the function of cytotoxic T-cells will also be affected by regulatory cells, as is outlined in the paragraphs below. The functional state of tumour-specific T-cells in the peripheral blood and

metastatic melanoma tissues has been compared, showing that tumour-infiltrating lymphocytes (TILs) expressed lower levels of IFN-γ and perforin than peripheral T-cells, indicating a local state of tolerance. However, cytotoxic activity could be restored when these CD8+ TILs were stimulated in culture, indicating that the local induction of tolerance can be reversible.[47]

Infiltration of a primary melanoma by immune cells is an early event in malignant transformation, and several studies in melanoma patients have correlated the quantity, quality and distribution of TILs with patient survival.[49, 50] Both TILs and CD8+ lymphocytes in the primary melanoma are established positive prognostic markers.[49, 51]

❖ Regulatory function: Tregs and MDSCs

Regulatory T-cells

Regulatory T-cells (CD3+CD4+CD25+FoxP3+ cells) are naturally present in the immune system, being necessary for the maintenance of self-tolerance and immune homeostasis. They are produced in the thymus as a functionally mature T-cell subpopulation, but can also be induced in the periphery from naïve T-cells under certain conditions, such as antigenic stimulation in the presence of high amounts of TGF-β.[52] Tregs are characterized by the expression of the transcription factor forkhead box P3 (FoxP3), a master regulator in their development and function. Treg dysfunction, for example due to mutations in the FoxP3 gene, causes fatal autoimmune disease and immunopathology in mice.[53] FoxP3 controls the genes encoding proteins that are necessary for the suppressive function of Tregs. These include CD25, glucocorticoid-induced TNF receptor (GITR) and cytotoxic T-lymphocyte antigen-4 (CTLA-4). Moreover, FoxP3 also inhibits the production of effector cytokines caused by TCR activation, such as IL-2 and IFN-γ.[54, 55]

Regulatory T-cells can suppress the activation, proliferation and effector function of a wide range of cells including CD8+ cytotoxic T-cells and APCs, both in vitro and in vivo, but the exact mechanisms are incompletely understood (Fig. 3).[56] Several ways by which Tregs can directly suppress effector T-cells have been described. One is by inhibiting the transcription of cytokines such as IL-2 in the responder cells.[57] Several soluble factors (such as IL-10, TGF-β and galectin-1) secreted by Tregs have also been postulated to suppress T-cell function, by causing cell cycle arrest and inhibiting the production of pro-inflammatory cytokines. Galectin-1 could also mediate this effect via direct cell contact.[58] Another mechanism is by cytolysis of target cells through the perforin/granzyme pathway that is usually associated with cytotoxic T-cell function.[59] Apart from directly targeting effector T-cells, Tregs can also suppress DC function which will then indirectly cause defective effector T-cell function. An important

indirect mechanism acts through CTLA-4, which is constitutively expressed by Tregs and will bind to CD80/CD86 on DCs. This abrogates the capacity of the DCs to co-stimulate naïve T-cells through CD28.[60]

There are several hypotheses regarding why and how Tregs infiltrate in tumours. One putative mechanism is that tumour cells and tumour-associated macrophages secrete the C-C motif chemokine ligand 22 (CCL22), which chemo-attracts Tregs that express the C-C chemokine receptor 4 (CCR4).[61] After migration from the circulation to the tumour site, Tregs become activated and expand after recognizing TAA or self-antigens released from dying tumour cells. Alternatively, tumour cells are known to secrete IL-10 and TGF-β, which can induce FoxP3+ Tregs from non-Tregs directly or indirectly by inducing tolerogenic DC that subsequently induce Tregs.[62, 63]

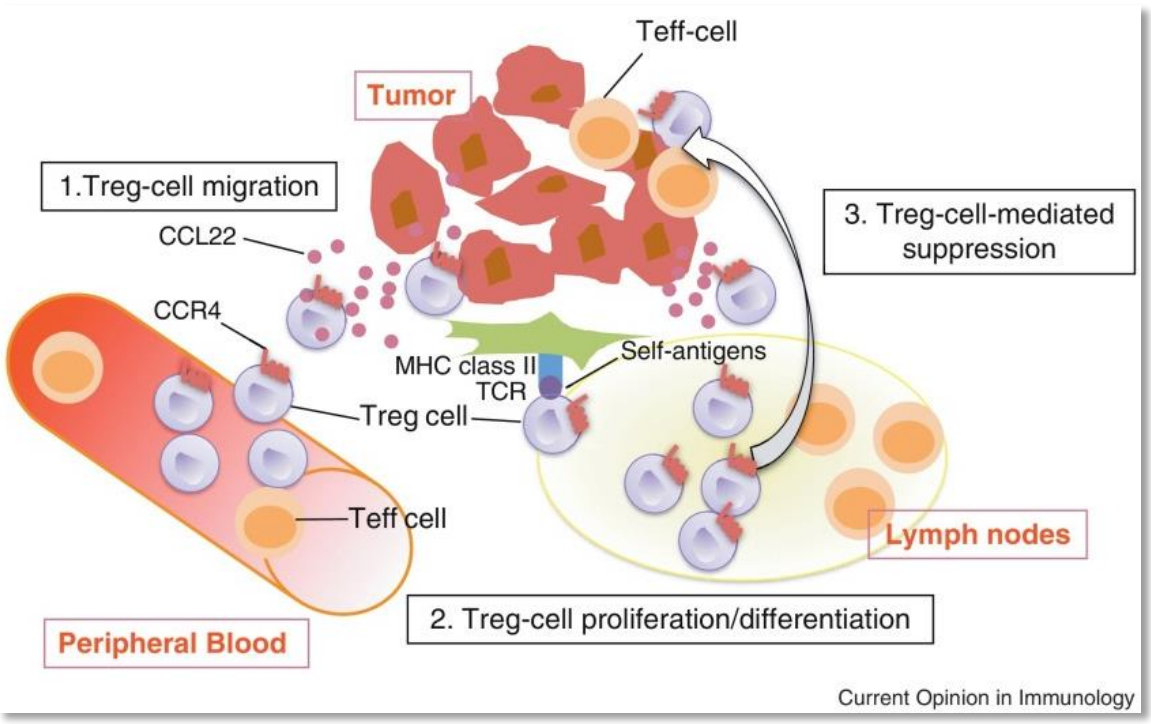

Figure 3: Migration and action of Tregs.

FOXP3+ Tregs (purple cells) infiltrate into tumour tissues through chemo-attraction (e.g. via CCR4–CCL22) and recognize self-antigens -including tumour antigens- present in tumour tissues. Proliferated/differentiated Tregs efficiently suppress the activation of tumour antigen-specific effector T-cells. Adapted from Nishikawa, 2014.[64]

Evidence for the pathways and mechanisms mentioned above was largely obtained in vitro. The Tregs' central role in suppressing T-cells and APCs has made them an attractive target for therapy, which could provide more in vivo evidence for their function in cancer patients in the future.

The powerful regulatory function of Tregs acts as a double-edged sword; on one hand it is vital to prevent auto-immune disease and tolerance in non-pathological conditions such as pregnancy, but on the other hand it enables Tregs to suppress anti-tumour immune responses and favour tumour progression. Large numbers of Tregs have been found in tumours and tumour-draining lymph nodes (TDLN) in patients with different malignancies, correlating with poor prognosis in patients with breast, gastric and ovarian cancer.[65-67] Treg infiltrations also occur in primary melanomas and sentinel lymph nodes. An association with disease progression or vertical growth phase in melanoma has been described, but the impact on the patients' prognosis seems to be limited.[68-70]

Myeloid-derived suppressor cells

Myeloid cells with a suppressive function were first described more than 20 years ago, but their importance in the immune system was underestimated until the last decade.[71] In healthy individuals, immature myeloid cells (IMC) quickly differentiate into mature granulocytes, macrophages and dendritic cells. By contrast, in pathological conditions that affect the immune system (such as cancer, sepsis, autoimmune disorders,…), a partial block in the differentiation of IMCs occurs, leading to their accumulation. When IMCs become activated in pathological conditions, immunosuppressive mechanisms are initiated that result in the expansion of an immunosuppressive IMC population that is defined as "myeloid-derived suppressor cells" (MDSCs).[72] In humans, MDSCs are usually defined as CD14-CD11b+ cells that express the common myeloid marker CD33 but lack the expression of markers of mature myeloid and lymphoid cells (CD3, CD16, CD19, CD20, and CD56) and of the MHC class II molecule HLA-DR. In healthy subjects, IMCs represent +/- 0.5% of peripheral blood mononuclear cells (PBMCs) but tenfold higher levels can be found in the circulation of cancer patients.[73, 74] Current data suggest that these MDSCs are not a defined subset of cells, but rather a phenotypically heterogeneous group of myeloid cells that share a common biologic activity. Two subsets have been defined in humans; CD14- polymorphonuclear MDSCs (pmnMDSCs) and CD14+ monocytic MDSCs (mMDSCs). In some studies, the CD14- MDSCs are further subdivided in promyelocytic MDSC and pmnMDSCs, the first being CD15- and the latter CD15+.[75]

The consequences of the failed maturation of IMCs to DCs in cancer patients are depicted in figure 4.

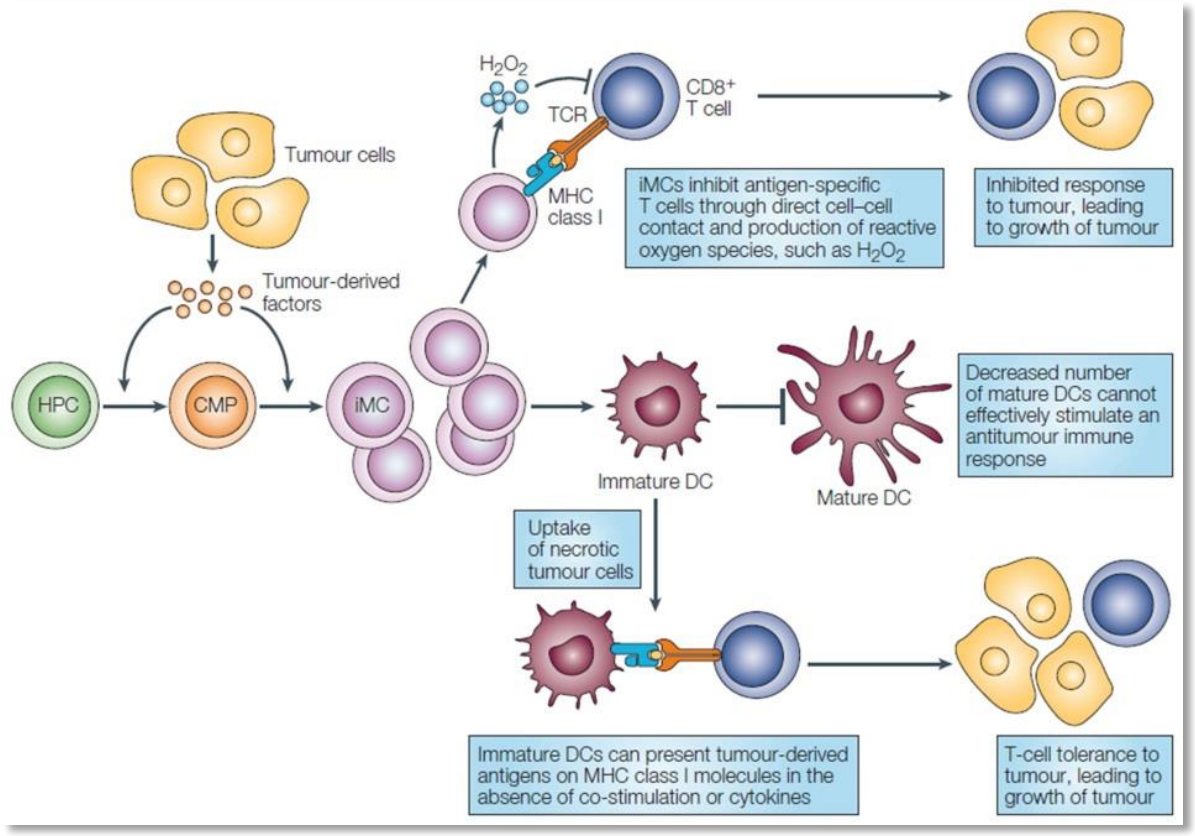

Figure 4: Tumour-derived factors affect normal dendritic cell differentiation in the bone marrow. This abnormal differentiation has at least three main effects: decreased production of mature, functionally competent DCs; accumulation of immature DCs (iDCs); and an increased production of immature myeloid cells (iMCs). iDCs can process and present antigen in the context of MHC class I molecules. However, these cells do not express co-stimulatory molecules or adequate levels of MHC class II molecules. Together with the lack of production of cytokines that are required for T-cell stimulation, antigen presentation by iDCs might result in the induction of tolerance. iMCs can also process and present antigens in the context of MHC class I molecules. They inhibit T cells through direct cell-cell contact and the production of reactive oxygen species (ROS), mainly H_2O_2. Adapted from Gabrilovich, 2004.[32]

The process of MDSC function in cancer patients can be divided in their accumulation and activation. Accumulation of MDSCs is caused by cytokines (such as GM-CSF, G-CSF, M-CSF, VEGF, IL-3, TGF-β) which promote myelopoiesis and are chiefly tumour-derived. Most of these factors will converge in signalling pathways that trigger persistent STAT3 phosphorylation and activation in myeloid progenitor cells. STAT3 is argued to be the main transcription factor responsible for MDSC expansion in cancer patients.[76] Next, activation of MDSCs is initiated by pro-inflammatory factors derived from the tumour stroma (such as IL-1β, IL-6, S100-A8 and S100-A9) and cytokines released by activated T-cells (IFN-γ, IL-4, IL-10 and IL-13). These factors activate several different pathways that involve STAT1, STAT6 and NF-kB, ultimately conferring immunosuppressive properties to the MDSCs.[77]

STAT1 is a prototypical pathway that is activated by IFN-γ signalling and will lead to inducible nitric oxide synthase (iNOS) expression by MDSCs, one of the mediators of the suppressive function of MDSCs.[78] Other immunosuppressive MDSC characteristics, such as arginase1 expression, are mediated by the STAT6 pathway which is activated by IL4 and IL13.[79] iNOS and arginase1 are two different but related enzymes that catabolise L-arginine to generate urea and NO respectively, depleting this non-essential amino-acid from the tumour microenvironment. Thirdly, MDSCs also produce reactive-oxygen species (ROS). The detailed molecular mechanisms underlying the immunosuppressive effects of MDSCs are beyond the scope of this thesis, but have been comprehensively reviewed elsewhere.[80]

This two-tiered arrangement that requires both accumulation and activation to generate immunosuppressive MDSCs allows for careful regulation of this system. This also explains why in certain conditions (such as for example infection) an accumulation of IMCs can be seen that does not lead to an immunosuppressive effect. One should note that the available evidence on MDSC function and regulation has mostly been obtained in murine tumour models, and might therefore not be fully relevant or complete for humans. In human cancer patients, some data on MDSC function and clinical significance do exist. Significant increases in circulating MDSC frequencies have been detected in the blood of patients with a.o. glioblastoma, breast, colon, lung and kidney cancer. A correlation with disease burden and prognosis was described in breast and colon cancer.[72, 77]

4. Immunotherapy for melanoma

The immunogenicity of melanoma has inspired the medical community for decades as a possible means to fight this aggressive malignancy. One of the first immunotherapies to become a standard in melanoma treatment was IFN-α2b. Even though clinical success rates with this compound are modest, it is currently the best/only approved adjuvant therapy for disease-free melanoma patients who are at a high risk of relapse. Over the last 5 years, a revolution has taken place as immune checkpoints have arisen as therapeutic targets in stage IV melanoma patients. The first of these was an anti-CTLA-4 antibody, but in the near future anti-PD1 antibodies are expected to become standard treatments for melanoma too. In the following paragraphs, the theoretical framework supporting the three immunotherapies for which clinical data are available will be outlined.

❖ Adjuvant interferon-alpha-2b

Interferons are classically grouped as type I (IFN-α and IFN-β) and type II (IFN-γ) interferon. The IFN-α family is composed of 13 IFN subtypes which all share the IFN-α receptor system and have similar biologic activities.[81] With over 50 years of research as a foundation, there still is relatively limited certainty on the exact mechanism of action of IFN-α as an anti-tumour therapy.[82] For a long time, its anti-tumour properties were attributed to direct inhibitory effects on tumour cell growth and function. These effects do exist, as IFN-α can directly inhibit the proliferation of tumour cells in vitro and in vivo. Tumour cells genetically modified to express IFN-α are also more sensitive to apoptosis induced by cytotoxic agents.[83] However, IFN-α also exerts several effects via host immune cells that can play an important role in the anti-tumour immune response. IFN-α enhances proliferation and survival of CD8+ T cells in response to antigens.[84] It also provides a bridge between innate and adaptive immunity through pDCs, which were originally even designated as "naturally IFN-producing cells" based on their high IFN production upon microbial challenge.[85] Conversely, DCs also respond to IFN-α with increased maturation and differentiation.[86] Nowadays the anti-tumour mechanism of action of IFN-α is believed to be mainly immunomodulatory, but results of previous research to completely elucidate it have been inconclusive so far.[10, 87] Nevertheless, a systemic immunomodulatory effect can be inferred from the observed correlation between clinical response to IFN-α therapy and the occurrence of auto-immune phenomena such as vitiligo and circulating auto-antibodies.[88]

Interferons, like most cytokines, are produced by the body to act locally. When used as a systemic pharmacologic compound, non-negligible side effects can be observed. These include weight and appetite loss, fatigue, fever, exacerbation of auto-immune diseases and neurological effects such as depression. The combination of considerable side-effects and limited clinical responses has fuelled the search for predictive biomarkers to identify those patients who would benefit the most from adjuvant IFN-α therapy. Some have suggested that only a subset of patients is sensitive to IFN-α.[89, 90] This hypothesis is substantiated by the fact that there is no clear association between the outcome of treatment with adjuvant IFN-α and dose or duration of the treatment regimen. Currently, ulceration of the primary melanoma seems to be a promising predictive biomarker to pre-identify responders. However this remains to be validated in (ongoing) prospective trials,[12] and the molecular mechanisms behind this effect are unknown to date.[10]

❖ Immune checkpoint inhibitors

Immune checkpoints and adaptive immune resistance

The term “immune checkpoint” refers to the abundance of receptor/ligand pairs that have a biologic role as regulators of T-cell activation, generally because they provide co-stimulatory signals that are required for full T-cell activation in addition to the antigen-specific signal mediated by the T-cell receptor (TCR). These pathways are inherent to the immune system and are crucial to maintain self-tolerance. In addition, they cover the modulation of the duration and magnitude of physiological immune responses, which is necessary to minimize collateral tissue damage in peripheral tissues. Tumours can make use of immune checkpoint pathways as a mechanism of immune resistance, particularly against TAA-specific T-cells. Because many of the immune checkpoints are initiated by ligand-receptor interactions, they can be targeted by antibodies. The anti-CTLA-4 antibody was the first of this class of immunotherapeutic compounds to achieve approval by the US Food and Drug Administration (FDA). Preliminary findings with another checkpoint-inhibitor, programmed cell death protein 1 (PD-1), are promising and indicate broad and diverse opportunities to enhance anti-tumour immunity with the potential to produce durable clinical responses.[91, 92]

Both CTLA-4 and PD-1 depend on the principle of adaptive resistance, which can succinctly be defined as the induction of inhibitory ligands in response to immune attack. When the immune system is activated, it positions itself for self-regulation by making T-cells susceptible to inhibitory signals that will occur later on in the immune response. This homeostatic mechanism is the theoretical basis for the two most promising immunotherapies currently available in melanoma, as will be discussed in the following paragraphs.

Cytotoxic T-lymphocyte antigen-4 (CTLA-4)

CD28 is a widely recognized co-stimulatory molecule that is expressed exclusively on T-cells and binds with the ligands B7-1 (CD80) and B7-2 (CD86) on APCs.[93] Apart from supporting T-cell activation, ligation of CD28 will also induce an upregulation of CTLA-4 expression on the T-cell surface.[94] CTLA-4 shares the ligands CD80 and CD86 with CD28, but has a much higher affinity for them. Ligation of CTLA-4 with CD80/86 will antagonise early T-cell activation, causing a decreased IL-2 production, inhibition of cell-cycle progression and modulation of TCR signalling.[95] This inhibitory signal is caused by the competition with CD28, resulting in reduced T-cell costimulation. When CTLA-4 binds with CD80 or CD86, it can also remove CD80 and CD86 from the APC, resulting in a reduced co-stimulatory capacity.[96] Apart from these cell-extrinsic mechanisms, CTLA4 ligation will also actively deliver inhibitory signals to the T-cell. The specific pathways have not been elucidated yet, but the activation of phosphatases that counteract the kinase signals induced by TCR and CD28 activation has been proposed.[97] CTLA-4

signalling will thus down-modulate CD8+ effector T-cells and CD4+ helper T-cells, but it can also enhance regulatory T-cell activity. Tregs constitutively express CTLA-4, since this is a target gene of the transcription factor FoxP3 that determines the Treg lineage.[98] Exactly how CTLA-4 mediates the immunosuppressive function of Tregs is unknown, but Treg-specific knock-out of CTLA-4 abrogates the Tregs' immunosuppressive function.[60]

Taken together, the following (simplified) mechanism of action is proposed for CTLA-4 blockade (Fig. 5): CTLA-4 outcompetes the stimulatory receptor pair CD28/CD80-CD86, thereby inhibiting the activation and expansion of naïve T-cells by APCs. Additional inhibition of CTLA-4 on Tregs is probably also in part responsible for the effects of CTLA-4 blockade.

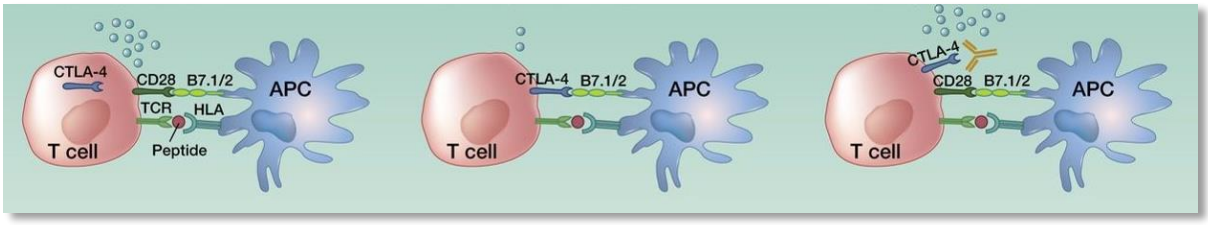

Figure 5: Proposed mechanism for CTLA-4 blockade.
After antigen-specific activation of a naïve or memory T-cell in lymphatic tissue (left), CTLA-4 is upregulated leading to decreased effector function (middle). CTLA-4 blockade will reconstitute T-cell activation via CD28-CD80/86 ligation (right). Adapted from Ott et al., 2013.[92]

The clinical results of CTLA-4 blockade are discussed above. The most remarkable feat of ipilimumab is certainly the ability to induce long-term survival in a subset of patients, leading to speculations that these patients could be clinically cured.[22, 99] The logic behind this is that the immune system, once appropriately activated, has the plasticity that is needed to keep a continuously transforming tumour under control. This contrasts with targeted drugs, which will sooner or later become subject to resistance caused by newly acquired mutations. In this small subset of responding patients, CTLA-4 is presumably the dominant inhibitory driver of pre-existing tumour-specific T-cells. The 3-month delay that is typically seen before a clinical response to ipilimumab occurs is thought to reflect the time needed for these tumour-specific T-cells to become activated, expand and infiltrate into the tumour.[92]

Programmed cell death protein 1 (PD-1)

Similar to CTLA-4, PD-1 is a cell-surface molecule that is expressed by activated T and B-cells, as well as monocytes and DCs. It has two ligands, PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also known as B7-H2 or CD273), which have a different expression pattern. In mice, PD-L1 is broadly expressed by leukocytes, non-hematopoietic cells and non-lymphoid tissues. In humans, data on PD-L1 expression are more limited but indicate expression by activated T-cells, pDCs, mDCs and monocytes. Expression is also found in keratinocytes, liver, lung and placental tissues. PD-L2 expression by immune cells is limited to DCs and monocytes, both in mice and humans, but it can also be found in human lung and placental tissues.[100] Signalling through PD-1 limits the function of activated T-cells by reducing proliferation and IFN-γ production and increasing T-cell apoptosis.[101] Similar to CTLA-4, the exact mechanisms remain to be elucidated but have been suggested to imply the inhibition of kinases that induce T-cell activation.[102] PD-1 is also expressed on Tregs, and binding with its ligand enhances Treg development and FoxP3 expression.[103] This could implicate that tumour-infiltrating Tregs, known to inhibit effector immune responses, are also possible targets for blockade of the anti-PD1 pathway.

Simply put, the rationale for the current use of PD-1 blocking antibodies in melanoma is the observation of a dual expression pattern (Fig. 6). On one hand, PD-1 expression by CD8+ TILs is associated with an anergic state and reduced cytokine secretion.[104] On the other hand, PD-L1 expression by melanoma cells has been reported, and ligation with PD-1 can inhibit local anti-tumour T-cell responses.[105] Blocking either PD-1 or PD-L1 should therefore enhance anti-tumour immune effector functions in the tumour microenvironment.

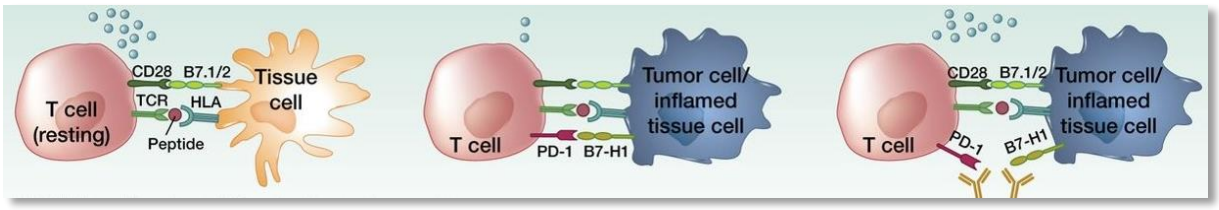

Figure 6: Proposed mechanism for PD-1/PD-L1 blockade.
T-cells become antigen-experienced after adequate stimulation by an APC (left). PD-1 is mainly expressed on antigen-experienced memory T cells in peripheral tissues. The immune modulation mediated by this pathway ensures protection of the tissue from collateral damage during an inflammatory response. Tumour cells can also use this regulatory mechanism to evade a tumour-directed T-cell response by upregulating the PD-1 ligand B7-H1 (middle). Antibodies directed against either PD-1 or B7-H1 will prevent this immunomodulation from taking place (right). Adapted from Ott et al., 2013.[92]

How PD-L1 expression by malignant cells arises is not yet completely clear, but two mechanisms could play a role. Firstly, the occurrence of an oncogenic mutation could induce PD-L1 expression. This was demonstrated in gliomas, where loss of PTEN function lead to an increased PD-L1 expression.[106] Secondly, the principle of adaptive immune resistance also applies to tumour cells. Expression of PD-L1 can be induced in response to adaptive anti-tumour immunity, such as the presence of IFN-γ.[105]

Clinical results with a PD-1 blocking antibody (nivolumab, BMS) are very promising, to the extent that a recent randomized controlled phase III trial in melanoma patients (going by the name of Checkmate-066) was ended prematurely because an improved survival compared to dacarbazine could already be demonstrated.[107] With nivolumab, the median progression-free survival was 5.1 months, with a 1-year survival rate of 72.9%. Objective responses were seen in 40.0% of patients, with an additional 16.7% experiencing stable disease.[108] Median overall survival was not reached in Checkmate-066, but in previous reports the median duration of response was 2 years, in the subgroup of patients with an objective response.[109] Treatment with nivolumab had an acceptable safety profile, the most common adverse events in melanoma patients were fatigue (19.9%), pruritus (17%) and nausea (16.5%). Grade 3 to 4 adverse events occurred in 11.7% of patients, but no drug-related deaths were seen in melanoma patients.[108] Another PD-1 inhibitor (lambrolizumab) is also being tested in early phase clinical trials, as well as PD-L1 inhibitors.

Differences between CTLA-4 and PD-1 blockade

CTLA-4 and PD-1 have in common that they are both immune checkpoints working by the principle of adaptive resistance, but significant differences between them also exist. If their roles were overlapping, the absence of either CTLA-4 or PD-1 would not be expected to result in autoimmunity. However, mice deficient for either molecule both develop autoimmune phenotypes, but the severity and timing is significantly different. CTLA-4-deficient mice develop lymphoproliferative disorders and die within 3-4 weeks after birth.[110] In contrast, PD-1 deficient mice take several months to develop autoimmunity, and the disease is targeted at specific organs depending on the genetic background of the mice.[111] As outlined above, blockade of these two pathways not only results in different response rates, but also in a distinct safety profile. Several theories exist that help to explain the differences between the two compounds.

The major role of PD-1 is to limit the activity of T-cells upon re-presentation of antigens in peripheral tissues at the time of an inflammatory response, limiting autoimmunity. In the tumour micro-environment, this translates into a mechanism of immune resistance.[100] In contrast, the physiological role of CTLA-4 lies in limiting the initial activation of naïve T-cells in lymphoid tissues (such as the tumour-draining lymph node). An important draw-back of anti-CTLA-4 therapy therefore is its lack of

specificity; replacement of CD28 by CTLA-4 downregulates T-cell activation within 24 to 48 hours. The theory that CTLA-4 blockade could therefore lead to more important, diffuse and non-specific T-cell activation is indeed in concordance with the clinically observed immune-related adverse events. PD-1 blockade acts at a much more local level, in peripheral tissues where an inflammatory response is already ongoing. These differences in time and space help to explain (1) why CTLA-4 blockade is associated with more severe immune-related adverse events and (2) why PD-1 blockade produces more early clinical responses (Fig. 7).[92]

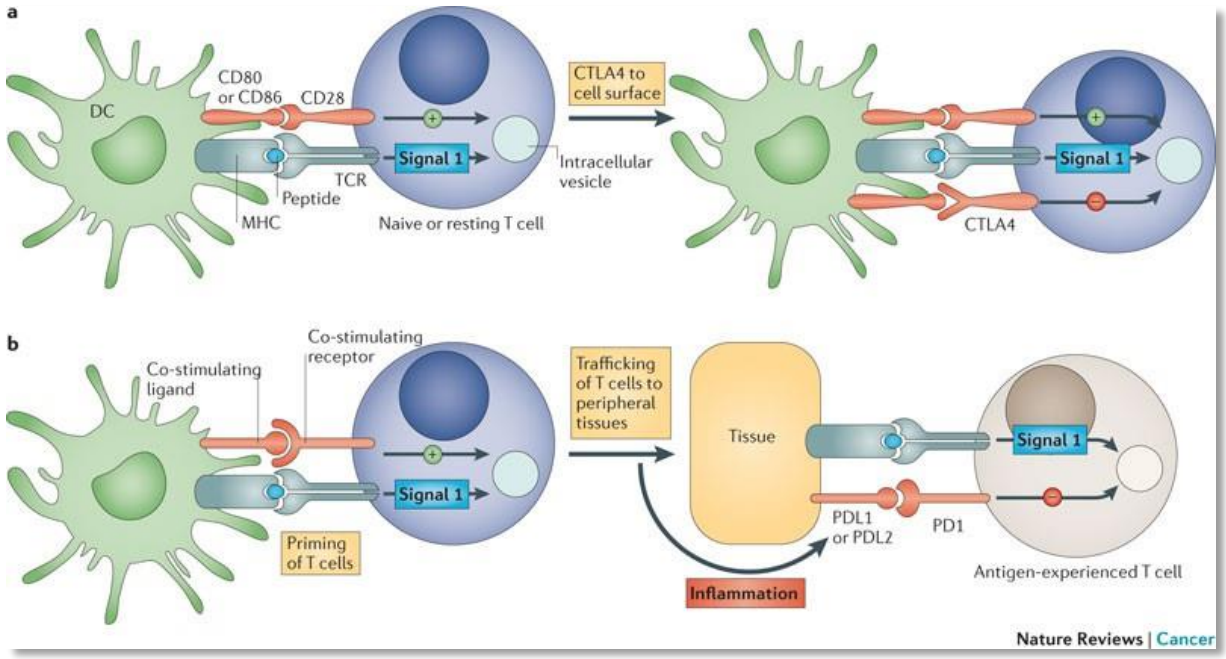

Figure 7: Differences between CTLA-4 and PD-1.
Upper panel (a): CTLA-4 is induced in T-cells at the time of their initial response to antigen. Naive and memory T-cells express high levels of cell surface CD28 but do not express CTLA-4 on their surface. Instead, CTLA-4 is sequestered in intracellular vesicles. After the TCR is triggered by antigen encounter, CTLA-4 is transported to the cell surface. The stronger the stimulation through the TCR (and CD28), the greater the amount of CTLA-4 that is deposited on the T-cell surface. Lower panel (b): By contrast, the PD-1 pathway will regulate inflammatory responses by effector T-cells recognizing an antigen in peripheral tissues. Activated T-cells upregulate PD1 and continue to express it in tissues. Inflammatory signals in the tissues induce the expression of PD1 ligands, which downregulate the activity of T-cells and thus limit collateral tissue damage in response to inflammation. Excessive induction of PD1 on T-cells in the setting of chronic antigen exposure can induce an exhausted or anergic state in T-cells. Adapted from Pardoll, 2012.[91]

Compared to CTLA-4, PD-1 is more broadly expressed. Both are found on activated T-cells, but PD-1 is additionally expressed by other activated non-T-cell populations, such as B-cells and NK cells.[112, 113] Thus, in addition to enhancing the activity of effector T-cells, PD-1 blockade might also enhance NK and B-cell activity. In theory, these observations could help explain the greater clinical effect of PD-1 blockade, as observed in the first clinical trials, compared to CTLA-4 inhibition.

Even though both of these immunotherapeutic strategies are very promising, combinatorial strategies will likely be necessary to increase the proportion of melanoma patients who can benefit from the long-lasting clinical responses that appear to be within reach. In this context, both combinations of multiple immunotherapies and of immunotherapies with targeted therapies are possible and even actively investigated. Even though this subject is beyond the scope of this thesis, it will be touched upon in the discussion.

5. Indoleamine 2,3-dioxygenase (IDO)

❖ The IDO family and function

IDO1 is a cytosolic enzyme encoded by the *INDO* gene located on human chromosome 8p12. The principal effect of IDO1 is the catabolism of tryptophan to kynurenine and its downstream metabolites. Tryptophan depletion is a mechanism of immunoregulation, but tryptophan metabolites such as kynurenine, kynurenic acid, 3-hydroxy-kynurenine and 3-hydroxy-anthralinic acid can also actively suppress T-cell function (Fig. 8).

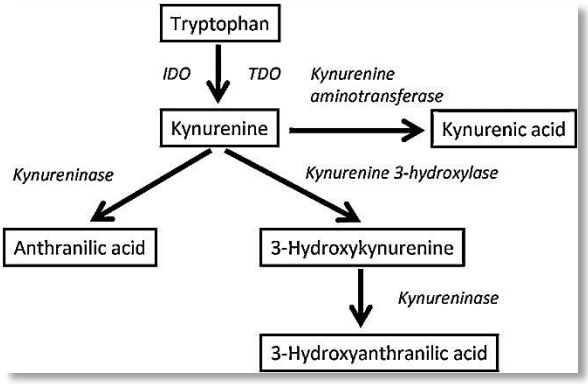

Figure 8: Tryptophan catabolism by IDO or TDO and downstream enzymes.

Tryptophan can be converted either by IDO or by TDO in the liver. Kynurenine is then converted into secondary metabolites.[114]

Tryptophan is an essential amino acid that is necessary for vital cellular functions and its degradation by IDO can cause a tryptophan deficiency. This deficiency can lead to "death by starvation" by inducing an accumulation of uncharged tryptophan-tRNA. Uncharged tRNAs (as a proxy for cellular deficiencies in amino acids) are sensed by the stress-response kinase general control nonderepressible 2 (GCN2), which then prevents T-cell activation. T-cells in which GCN2 is genetically disrupted are not susceptible to IDO-mediated proliferation suppression in vivo, and these T-cells are not anergized by IDO-expressing DCs.[115] Apart from the GCN2-pathway, amino acid withdrawal can also affect the nutrient-sensing mammalian target of rapamycin (mTOR) pathway. In mammalian cells, TOR links nutrition to cellular functions. Inhibition of mTOR can be caused by amino acid withdrawal, leading to the promotion of Treg differentiation and induction of tolerogenic DCs.[116] The mTOR pathway has been implicated in IDO-mediated immunosuppression, and is proposed to act independent of GCN2.[117] Another effect of IDO-mediated tryptophan depletion is the production of kynurenine, that can bind the aryl hydrocarbon receptor (AhR). The biological effects of AhR activation include inhibition of T-cell activation, induction of Treg activation and IDO expression by DCs.[118-120]

Apart from IDO1 (designated as "IDO" in this thesis), two other enzymes exist that catabolize tryptophan along the same pathway; indoleamine 2,3-dioxygenase 2 (IDO2) and tryptophan dioxygenase (TDO). IDO2 is encoded by the *INDOL1* gene, located just downstream of *INDO* and is structurally very similar to IDO. It is mainly expressed in the kidney, liver and reproductive organs, which is a more limited expression pattern than IDO's. Important genetic polymorphisms exist for IDO2, abolishing the enzyme's function in up to 50% of Caucasians.[121] IDO2 can also be expressed by DCs, albeit without exerting enzymatic activity.[122] Taken together, these data imply that the clinical relevance of IDO2 in cancer patients remains debated. TDO (encoded by the gene *TDO2*) is expressed

in high levels in the liver, and was long thought to solely mediate tryptophan homeostasis in the body by metabolising dietary tryptophan intake. More recently, TDO expression was also found in human tumour cell lines -including melanoma- and was likewise capable of suppressing anti-tumour immune responses.[123] No TDO expression in host immune cells has been documented so far, so the clinical relevance of this enzyme in immunomodulation remains to be clarified.

❖ IDO is a mechanism of acquired tolerance

Acquired tolerance of tumours is undesirable and unfavourable to the host. Then again, acquired tolerance for other types of antigens is necessary and often to the host's advantage. IDO has been demonstrated to be a normal endogenous mechanism of acquired peripheral tolerance in vivo, in a variety of settings. IDO is expressed in the placenta, which is the site of one of its most compelling tolerogenic effects. Pregnant mice treated with the pharmacologic IDO-inhibitor 1-methyl-trypophan (1MT) developed T-cell responses against paternal allo-antigens, leading to the rejection of their concepti.[124] Many other examples of IDO-mediated acquired tolerance exist, such as at mucosal interfaces in gastrointestinal and pulmonary tissues.[125-127] One of the most telling observations is that murine tissue allografts designed to overexpress IDO are not rejected, even in cases with full MHC haplotype mismatches, without the need for any additional immunosuppression.[128-130] As powerful as IDO may be in inducing acquired systemic tolerance to foreign antigens, it does not seem to play a role in the constitutive maintenance of tolerance to self-antigens. This can be deduced from the fact that mice genetically modified to lack IDO (*IDO*$^{-/-}$ mice) do not develop spontaneous autoimmune or lymphoproliferative disorders.[131]

❖ IDO expression patterns

IDO expression and regulation

Many tumour types express IDO, as discussed below. However, IDO expression can also be found in non-tumour tissues such as the placenta, mucosal and lymphoid tissues. Literature reports on IDO expression in human tissues have not always been consistent, which can probably be explained by the variety of different antibodies, concentrations and detection systems that have been used. IDO expression is also context-dependent; constitutive expression is rare, and IDO is upregulated upon various inflammatory stimuli.

In placental tissues, IDO expression is found in CD31-positive endothelial cells at the feto-maternal interface.[132, 133] IDO is also expressed in normal lymphoid tissues, such as lymph nodes and spleen,

predominantly by mature interdigitating dendritic cells.[134, 135] Constitutive IDO expression also exists in interstitial cells of the duodenum, small bowel and colon and in epithelial cells from the female reproductive tract.[125, 133, 136] In lung parenchyma, IDO expression was also found in endothelial cells.[133]

The functional consequences of these constitutive IDO expression patterns in normal human tissues remain debated, and there is no consensus on how it is regulated. Dendritic cells are the most extensively studied non-malignant cell type in this context. The promotor of the *INDO* gene contains 2 gamma-activated sequences (GAS-2 and GAS-3) and 2 IFN-γ-stimulated response elements (ISRE-1 and ISRE-2), which are involved in activating IDO gene expression.[137] These are implicated in IFN-γ-induced, STAT-1 mediated IDO expression in dendritic cells. Other inflammatory signals known to induce IDO expression are amongst others type I IFNs, IL-1, IL-6 and TNF-α. Additional T-cell-derived signals can also regulate IDO expression by DCs, including reverse signalling via CD80/86 and CTLA-4 or CD40 and CD40L.[138] Apart from these mechanisms that induce IDO as part of a negative feedback signal accompanying inflammation, immunosuppressive cytokines such as TGF-β have also been reported to induce IDO expression.[139]

IDO expression in the tumour microenvironment

Tumour cells can express IDO, as has been demonstrated in many different malignancies including melanoma.[140, 141] The relevance of IDO expression by tumour cells is illustrated by the correlations that have been found with variables such as poor survival, increasing disease stage, presence of metastatic disease, decreased tumour-infiltrating lymphocytes and increased FoxP3+ Tregs.[142-145] IDO expression by tumour cells can be part of genetic changes involved in malignant transformation, through loss of Bin1.[146] Alternatively, IDO is known to be inducible in tumour cells by stimulation with IFN-γ or other inflammatory mediators.[147] IDO is also expressed by host cells in the tumour microenvironment. The mechanisms that play a role here are thought to be similar to those in the TDLNs, as discussed in the next paragraph.

The tumour-associated IDO expression patterns are summarized in Figure 9.

IDO expression in the tumour-draining lymph node

The sentinel lymph node is the key site for immunological interactions between the hosts' resting naïve T-cells and TAAs presented by APCs. This is classically thought to lead to T-cell activation, but it can also lead to antigen-specific tolerance induction. The TDLN is therefore crucial in the orientation of the anti-tumour immune response.[148] IDO is expressed in sentinel lymph nodes, predominantly by host cells rather than by metastatic tumour cells.[135, 149] It remains to be indisputably proven which cell type is responsible for host IDO expression, but plasmacytoid dendritic cells seem plausible.[70, 135] The IDO-

competent subtype of DCs in TDLNs has been shown to suppress T-cell responses and induce antigen-specific immune tolerance.[115, 134] Moreover, the effect of IDO+ DCs is mediated by bystander suppression, meaning that IDO+ DCs can suppress T-cell responses to antigens presented by neighbouring IDO- DCs.[134] This explains how even small populations of IDO+ DCs can have a powerful effect in vivo.

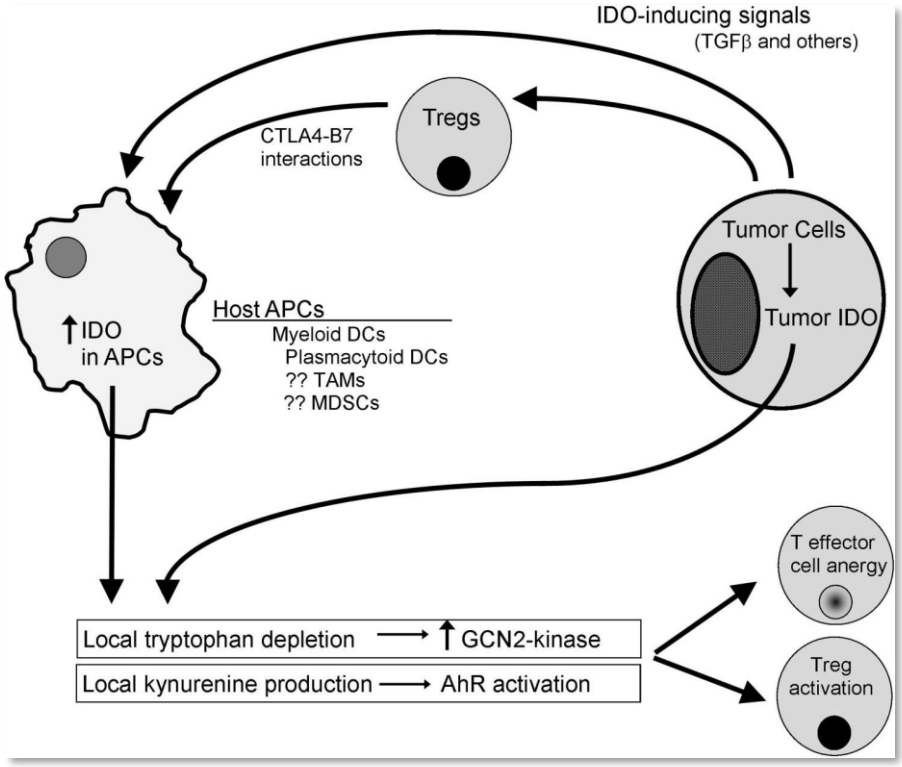

Figure 9: Expression of IDO in host immune cells and tumour cells.
Antigen-presenting cells (APCs) can be induced to express IDO by tumour cells themselves (via factors such as TGF-β) or by Tregs recruited by the tumour (via interactions between B7 molecules on APCs and CTLA4 on Tregs). Both host APCs and tumours cells can upregulate IDO expression and lead to: (1) local tryptophan depletion that activates the GCN2-kinase pathway; and/or (2) local production of bioactive kynurenine pathway compounds, which activate the transcription factor AhR. Both of these pathways are capable of driving anergy of effector T-cells and activation of Tregs. Figure adapted from Johnson and Munn, 2012.[150]

❖ Clinical relevance of IDO: impact on prognosis

The expression of IDO in the tumour microenvironment has been associated with the outcome of many different malignancies (Table 1). In most cases, IDO expression conferred a worse prognosis, which is compatible with its proposed mechanism of action as outlined in the previous paragraphs. However, a seemingly paradoxical positive prognostic effect has also been observed. There are two hypotheses that explain this putative contradiction. In the first place, local tryptophan deprivation with inherent accumulation of toxic metabolites has been reported to also decrease tumour cell proliferation.[147, 151] Secondly, the most powerful cytokine to induce IDO expression is IFN-γ.[151] During an anti-tumour immune response, large quantities of pro-inflammatory cytokines (including IFN-γ) are secreted. IDO expression could therefore also be a marker of an ongoing immune response, which is beneficial for the patient.

Table 1: Prognostic data for IDO expression

Type of cancer	Authors	Tissue	Number of patients	Prognosis
AML	Fukuno et al., 2004[152]	Leukemic blasts	n= 62	Worse prognosis
	Chamuleau et al., 2008[153]	Leukemic blasts	n = 286	Worse prognosis
	Folgiero et al., 2014[154]	Leukemic blasts	N=37	Worse prognosis (children)
Breast cancer	Specht et al., 2009[155]	Primary tumour	n = 70	Better prognosis
	Jacquemier et al., 2012[156]	Primary tumour	n = 1749	Better prognosis (basal-like breast cancer)
	Chen et al., 2014[157]	Primary tumour stroma	N=202	Worse prognosis
	Sakurai et al., 2013[158]	Tryp/Kyn ratio	N=32	Worse prognosis (for low Tryp/Kyn ratio)
	Soliman et al., 2013[159]	Primary tumour	N=203	Better prognosis (only in node-positive patients)
Cervical cancer	Inaba et al., 2010[160]	Primary tumour	n = 112	Worse prognosis
Colorectal cancer	Huang et al., 2002[161]	Serum tryptophan	n = 66	Worse prognosis
	Brandacher et al., 2006[143]	Primary tumour	n = 143	Worse prognosis
	Gao et al., 2009[162]	Primary tumour Sentinel nodes	n = 60 n = 60	Worse prognosis Worse prognosis
	Ferdinande et al., 2012[163]	Primary tumour	n = 265	Worse prognosis
DLBCL	Yoshikawa et al., 2010[164]	L-kynurenine in serum	n = 73	Worse prognosis
	Ninomiya et al., 2011[165]	Tumour cells	n = 119	Worse prognosis
Endometrial cancer	Ino et al., 2006[144]	Primary tumour	n = 80	Worse prognosis

	De Jong et al., 2012[166]	Primary tumour	N=355	Worse prognosis
Glioma	Wainwright et al., 2012[167]	Primary tumour	N=343	Worse prognosis
	Mitsuka et al., 2013[168]	Primary tumour	N=68	Worse prognosis
Hepatocellular carcinoma	Pan et al., 2008[169]	Primary tumour	n = 138	Better prognosis
	Lin et al., 2013[170]	Primary tumour	N=89	Worse prognosis
Hodgkin lymphoma	Choe et al., 2014[171]	Tumour-associated macrophages, DC, EC	N=121	Worse prognosis
Lung	Astigiano et al., 2005[172]	Primary tumour	n = 25	Worse prognosis
	Karanikas et al., 2007[173]	Primary tumour	N=28	No impact on prognosis
Melanoma	Munn et al., 2004[134]	Sentinel nodes	n = 40	Worse prognosis
	Weinlich et al., 2007[174]	Trytophan in serum	n = 87	Worse prognosis (of lower tryptophan concentration)
	Brody et al., 2009[140]	Melanoma metastases	n = 15	Worse prognosis
	Speeckaert et al., 2011[70]	Sentinel nodes	n = 116	Worse prognosis
	Chevolet et al., 2014[175]	Primary melanoma (ECs)	N=87	Worse prognosis
	Ryan et al., 2014[176]	Sentinel nodes	N=56	Worse prognosis
Non-Hodgkin lymphoma	Liu et al., 2014[177]	Tumour (lymph node)	N=57	Worse prognosis
Osteosarcoma	Urakawa et al., 2009[178]	Primary tumour	n = 139	Worse prognosis
Ovarian cancer	Okamoto et al., 2005[179]	Primary tumour	n = 24	Worse prognosis (serous type)
	Takao et al., 2007[180]	Primary tumour	n = 122	Worse prognosis (serous type)

	Inaba et al., 2009[181]	Primary tumour	N=60	Worse prognosis (all types)
Renal cell carcinoma	Riesenberg et al., 2007[182]	Primary tumour and metastases (ECs)	n = 107	Better prognosis
	Yuan et al., 2012[183]	Primary tumour	N=40	Better prognosis
SCC (laryngeal)	Ye et al., 2013[184]	Primary tumour	N=187	Worse prognosis
SCC (esophageal)	Zhang et al., 2011[185]	Primary tumour	N=135	Worse prognosis
SCC (oral)	Laimer et al., 2011[186]	Primary tumours	n = 88	Worse prognosis
SCC (vulvar)	Sznurkowski et al., 2011[187]	Primary tumours Lymph node metastases	n = 76 n = 35	Worse prognosis
	De Jong et al., 2012[188]	Primary tumour	N=286	No impact on prognosis

AML: acute myeloid leukemia, DLBCL: diffuse large cell B-cell lymphoma, SCC: squamous cell carcinoma, DC: dendritic cell, EC: endothelial cell

❖ IDO-inhibitors

In 1991, Cady and Sono reported that 1-methyl tryptophan (1MT) could inhibit IDO activity in rabbits by competition with tryptophan.[189] In this study, the racemic mixture of 1MT was used (i.e. containing both the L-1MT and D-1MT isomers). Later on, in vivo data in various mouse tumour models showed that 1MT can induce an immune-mediated delay in tumour development but cannot prevent tumour growth.[141, 146, 190] Its efficacy does improve when combined with chemotherapy, such as paclitaxel.[191] In 2007, the D-isomer was demonstrated to be a superior anti-cancer agent compared to L-1MT in chemo-immunotherapy regimens.[191] Remarkably, in the same year Metz and co-workers demonstrated that D-1MT selectively inhibits IDO2, whereas L-1MT inhibits IDO1.[121] This is important, as IDO1 (and not IDO2) has been shown to mediate tryptophan degradation in tumour cells and DCs.[122, 192] The observation that D-1MT can even upregulate IDO1 transcription in tumour cells in vitro further fuelled the controversy about this compound.[193] Several hypotheses have been formulated to explain the clinical efficacy of D-1MT, such as an in vivo racemization process leading to the formation of L-1MT, a possible underestimation of IDO2 activity in cancer or the modulation of yet unknown targets. Supported by the preclinical efficacy data, D-1MT (Indoximod®, Incyte corp.) is currently being evaluated in phase II clinical trials for patients with breast and prostate cancer, combined with either taxotere or a DC vaccine. No data on the results in humans have been published so far.

Concerns about the inhibitory effects of D-1MT fuelled the search for pharmacologically superior IDO inhibitors. Two IDO inhibitors have entered clinical trials, INCB024360 and the less studied NLG919. Some promising preclinical data on both have been reported, albeit all by investigators who have reported conflicts of interest with Incyte, the developing corporation.[194, 195] Interestingly, INCB024360 was recently reported to show cross-reactivity with TDO as well.[196] This compound is currently being tested in a phase II trial for melanoma patients in combination with a peptide vaccine (clinicaltrials.gov identifier NCT01961115) as well as in patients with ovarian cancer as a monotherapy before surgery (clinicaltrials.gov identifier NCT02042430) or in combination with a peptide vaccine (phase I, clinicaltrials.gov identifier NCT02166905).

The discovery of circulating IDO-specific CD8+ T-cells that could recognize IDO1 and IDO2-positive tumour cells as well as IDO1+ DCs, and are associated with pro-inflammatory cytokine profile, has paved the way for IDO-based vaccines.[197-199] Results of a phase I trial with an IDO-based peptide vaccine in patients with non-small cell lung carcinoma showed disease stabilisation without significant toxicity.[200]

As results obtained with the various IDO inhibitors are trickling in, the question arises as to what will be the best way to use them. Several large research groups are working on this issue, and they tend to have a somewhat different view. The group of Mellor and Munn (who are involved in Incyte) seem to hold on to D-1MT apart from the enzyme inhibitors mentioned above, and envisage combinations with chemotherapy. Platten's group (who placed TDO on the map) is screening for small-molecule inhibitors of the interaction between kynunerine and AhR, which could theoretically inhibit the downstream effects of both IDO and TDO. A Belgian group lead by Van Den Eynde (co-founder of ITEOS, a spin-off company also working on an IDO inhibitor) is working on a novel compound that should inhibit both IDO and TDO, aiming to use it in combination with a cancer vaccine.[201]

Even though research on the possible clinical applications for IDO as a therapeutic target or biomarker is moving forward, we are still far from understanding its precise function in the immune system.

Addendum I: Verslag van de BADO launch meeting[7]

CONGRES

Peer-reviewed article

THE BELGIAN ASSOCIATION OF DERMATO-ONCOLOGY LAUNCH MEETING, BRUSSEL, DECEMBER 2013

Verslag van de vergadering

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Op 7 december hield de *Belgian Association of Dermato-Oncology* (BADO) haar launch meeting in het Pullman hotel Brussel. Er waren een 200-tal enthousiaste aanwezigen waaronder dermatologen, oncologen en een aantal chirurgen en verpleegkundigen.

Het BADO kwam tot stand als een initiatief dat het belang van een multidisciplinaire benadering in dermato-oncologie wil beklemtonen op nationaal en internationaal vlak. Het bestuur bestaat uit 4 dermatologen, 3 oncologen en 2 andere specialiteiten en zal elke 3 jaar opnieuw verkozen worden tijdens een jaarlijkse BADO-meeting. Medici en paramedici kunnen lid worden door zich in te schrijven voor de jaarlijkse meeting.

BADO stelt zich als doel de wetenschappelijke kennis i.v.m. dermato-oncologie te verspreiden, zowel onder medici en paramedici als bij patiënten. Verder is het de bedoeling beleidsaanbevelingen op te stellen in samenspraak met alle BADO-leden. Daarnaast wil BADO fungeren als aanspreekpunt voor de overheidsinstanties en de farmaceutische industrie. Tot slot wil BADO het onderzoek op het vlak van dermato-oncologie promoten.

In het afgelopen jaar werd een website ontwikkeld (http://www.huidkanker-bado.be of http://www.cancerdela-peau-bado.be). Er werd een eerste aanzet gegeven tot consensus guidelines en er werd meegewerkt aan de arts- en patiëntinformatiebrochures i.v.m. vemurafenib.

Met deze launch meeting nam BADO in elk geval een enthousiaste officiële start. Hieronder vindt u feedback van wat tijdens de launch meeting aan bod kwam. Het BADO-bestuur hoopt met zijn leden de komende jaren even enthousiast verder te werken aan de hoger beschreven doelstellingen.

Impact van huidkanker op de gezondheidszorg in België

Mia Slabbaert verving haar collega Liesbet van Eycken van het Nationaal Kankerregister en vertelde iets meer over de demografische kenmerken van melanoom en de belangrijke impact van huidkanker op de volksgezondheid in België.

De niet-melanoomhuidkankers (waaronder basocellulair carcinoom (BCC) en spinocellulair carcinoom (SCC)) zijn samen het meest frequent voorkomende kankertype: er wordt geschat dat het levenslange risico om een dergelijke huidkanker te ontwikkelen 1 op 6 bedraagt. Alhoewel deze niet-melanoomhuidkankers doorgaans een uitstekende prognose hebben, kunnen zij wegen op het gezondheidsbudget door een belangrijke directe kost (gerelateerd aan behandeling).

Er is een duidelijk stijgende trend in de incidentie van melanoom bij zowel mannen als vrouwen. De grootste toename in de incidentie werd gezien in de periode van 2004-2011 voor de stadium I melanomen bij zowel mannen als vrouwen. De incidentie van melanoom is hoger bij vrouwen dan bij mannen. Momenteel is melanoom de 5de meest incidente kanker bij vrouwen in België. Bij mannen is de voorkeurslokalisatie hoofd/hals en romp, bij vrouwen is dit de ledematen.

Hoewel de melanoomincidentie fors is gestegen, is er voor de gehele groep een relatief gunstige prognose: er is een 5 jaarsoverleving rond de 88%. De overleving is duidelijk hoger voor vrouwen dan voor mannen. De incidentie van melanoom neemt toe met de leeftijd en de gemiddelde leeftijd bij diagnose is 55 jaar. Nochtans komt melanoom in vergelijking tot andere kankers voor bij een relatief jonge populatie bv. bij vrouwen in de leeftijdsgroep 15-29 jaar is melanoom de meest frequent gediagnosticeerde kanker. Op die manier kan melanoom naast een directe kost (gerelateerd aan behandeling) ook een belangrijke indirecte kost (ten gevolge van arbeidsongeschiktheid en evt. vroegtijdige sterfte) betekenen voor het gezondheidsbudget.

Detectie van huidkanker

Arjen Nikkels (Dienst Dermatologie, CHU de Liège) gaf een voordracht over de diagnostiek van huidkanker.

Het basocellulair carcinoom (BCC) is de meest frequente vorm van huidkanker, gevolgd door het spinocellulair carcinoom (SCC). Beide ontstaan uit de keratinocyten (epitheelcellen) van de huid, i.t.t. de melanomen die ontstaan ter hoogte van de melanocyten (pigmentcellen) in de huid. Meer zeldzame huidtumoren zijn o.m. de primaire cutane lymfomen (T- en B-cel-subtype), het Merkelcelcarcinoom, het dermatofibrosarcoma protuberans, Kaposisarcoom.

ONC565N

Tabel 1: AJCC TNM staging system 7th edition (2010).

Classification	Thickness (mm)	Ulceration status/mitoses
T		
Tis	NA	NA
T1	≤ 1.00	a: without ulceration and mitosis < 1/mm²
		b: with ulceration or mitoses ≥ 1/mm²
T2	1.01-2.00	a: without ulceration
		b: with ulceration
T3	2.01-4.00	a: without ulceration
		b: with ulceration
T4	> 4.00	a: without ulceration
		b: with ulceration
N	N° of metastatic nodes	Nodal metastatic burden
N0	0	NA
N1	1	a: micrometastasis*
		b: macrometastasis+
N2	2-3	a: micrometastasis*
		b: macrometastasis+
		c: in transit mestastases/satellites without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes	
M	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral mestastases	Normal
	Any distant metastasis	Elevated

Abbreviations
NA: not applicable; LDH: lactate dehydrogenase
* Micrometastases are diagnosed after sentinel lumph node biopsy
+ Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically

	Clinical staging*				Pathological staging+		
	T	N	M		T	N	M
0	Tis	N0	M0	0	Tis	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	Any T	N > N0	M0	IIIA	T1-4a	N1a	M0
					T1-4a	N2a	M0
				IIIB	T1-4b	N1a	M0
					T1-4b	N2a	M0
					T1-4a	N1b	M0
					T1-4a	N2b	M0
					T1-4a	N2c	M0
				IIIC	T1-4b	N1b	M0
					T1-4b	N2b	M0
					T1-4b	N2c	M0
					Any T	N3	M0
IV	Any T	Any N	M1	IV	Any T	Any N	M1

* Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant mestastases.
+ Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial (i.e. sentinel node biopsy) or complete lymphadenectomy. Pathological stage 0 or stage IA patients are the exception; they do not require pathologic evaluation of their lymph nodes.

Er zijn verschillende subtypes BCC met elk hun eigen klinisch beeld en histologie. Ongeveer 60% van de BCC zijn van het nodulaire subtype, 20% van het superficiële subtype, de overige hebben een plexiforme of sclerodermiforme histologie. BCC is in het bijzonder een klinisch-dermoscopische diagnose met hoge sensitiviteit en positief predictieve waarde (1). Histologie kan deze diagnose bevestigen. Nieuwere technieken zoals de 20MHz-echografie of de confocale reflectie microscopie moeten hun exacte toegevoegde waarde nog bewijzen. Het SCC ontstaat in een aantal gevallen uit een precancereus letsel (voorloperletsel) zoals een actinische keratose, actinische cheilitis of de ziekte van Bowen. De detectie van een invasief SCC of zijn voorloperletsels gebeurt voornamelijk klinisch, met aanvulling van dermoscopie en histologie ter bevestiging.

Melanomen kunnen onderverdeeld worden in 4 clinico-pathologische subtypes, waarvan het superficieel spreidend melanoom het meest frequente type is (70%). In 10-25% van de gevallen presenteert een melanoom zich als een nodulair letsel. Andere subtypes zijn het lentigo maligna type (vnl. op chronisch zon-blootgestelde delen bij een oudere populatie) en de acrolentigineuse melanomen (vnl. t.h.v. handpalmen/voetzolen en subunguaal). In de diagnostiek van melanoom zijn de anamnese en het klinisch onderzoek (visuele inspectie) belangrijk.
De ABCD-regel heeft een sensitiviteit van 77-98% en een specificiteit van 80-100%. De dermoscoop is een toestel dat - in ervaren handen - de diagnostische accuraatheid van pigmentletsels kan verhogen (2). Daarnaast kan de klinische fotografie voor opvolging van hoogrisicopatiënten met dysplastisch nevus syndroom een meerwaarde bieden voor de vroege detectie van melanomen (3). Ondanks de belangrijke inspanningen voor vroegtijdige detectie van melanoom, is er een subgroep van snelgroeiende melanomen die ondanks snelle detectie toch een belangrijke diktegroei vertonen op het moment dat ze gediagnosticeerd worden.

Biologie van het primair melanoom

Joost Van Den Oord (Dienst Pathologie, UZ Leuven) sprak over de groei en progressie van het primair melanoom.

Er kan onderscheid worden gemaakt tussen 3 belangrijke fasen bij de groei van melanoom:

- in de radiale groeifase verloopt de groei radiaal/intra-epidermaal; in deze fase is de overleving 100%;
- in een volgende fase zijn er individuele tumorcellen zichtbaar in de superficiële dermis;
- in de verticale groeifase zijn er nesten van melanoomcellen in de dermis.

Een nodulair melanoom begint quasi meteen in de verticale groeifase i.t.t. de andere vormen (superficieel spreidend melanoom, lentigo maligna en het acrolentigineus melanoom) waar de radiale groeifase een wisselende duur kan vertonen.
De prognostische factoren bij stadium I- en II-melanomen zijn Breslowdikte, ulceratie en mitosen. De Breslowdikte wordt door de patholoog bepaald als zijnde de afstand in millimeter tussen de granulaire cellaag van de epidermis en de meest invasieve melanoomcel. Deze afstand varieert van minder dan 1mm bij T1- tot meer dan 4mm bij een T4-melanoom. Dit is de meest belangrijke onafhankelijke prognostische factor en bepaalt ook het verdere beleid. Aanwezigheid van ulceratie van de primaire tumor is een belangrijke negatieve prognostische factor onafhankelijk van de Breslowdikte. Ook de aanwezigheid van dermale mitosen heeft een onafhankelijk negatief prognostisch effect en wordt in de TNM-classificatie vermeld bij de T1-tumoren (4) (**Tabel I**).
Het aantal mitosen wordt uitgedrukt in aantal/mm^2. Om dit te meten zal de patholoog de zone in de tumor bepalen met het grootste aantal mitosen en zal dan het aantal mitosen in een vierkante millimeter van deze zone tellen. Tumorkarakteristieken die een positieve invloed hebben op de overleving zijn de aanwezigheid van tumorinfiltrerende lymfocyten in het stroma en tumorregressie.
De aanwezigheid van microsatellieten, dit zijn nesten van melanoomcellen gelokaliseerd in de nabije omgeving (0,5-20mm) van het primair melanoom, klasseert een melanoom onmiddellijk als stadium III (**Tabel I**). Macrosatellieten zijn vergelijkbaar met de microsatellieten maar dan op meer dan 20mm afstand van het primaire letsel. De TNM voor stadium III houdt verder rekening met micro- versus macroscopische aantasting van regionale lymfeklieren en het aantal ingenomen lymfeklieren.

Behandeling van stadium I, II en III melanoom

François Salès (Dienst Chirurgie, Bordet Instituut, ULB) vatte de richtlijnen voor het chirurgisch beleid zoals voorgesteld door BADO bij stadium I, II en III melanoom samen. Deze zijn samengevat in tabellen 2 en 3.

De behandeling van een primair melanoom begint bij de diagnostische excisie, waarbij een marge van 2mm gehanteerd wordt. Deze excisie gebeurt volgens de lymfebanen eerder dan volgens de lijnen van Langer d.w.z. dat op de ledematen de grootste as van de excisie in de lengterichting georiënteerd wordt.
Bij bevestiging van de diagnose van melanoom is een zorgvuldige stagering de eerste stap. In eerste instantie is een grondig klinisch onderzoek met totale huidinspectie nodig. Patiënten

Tabel 2: Recommendation management primary cutaneous melanoma.

	T in situ	T1a	T1b	T2a	T2b	T3a	T3b	T4a	T4b
Preoperative	/	None*	Medical imaging**	Medical imaging**	Medical imaging**	Medical imaging**	Medical imaging**	Medical imaging**	Medical imaging**
		If staging negative:	If staging negative:	If staging negative:	If staging negative:	If staging negative:	If staging negative:	If staging negative:	If staging negative:
Wide excision	0,5cm	1cm	1cm	1-2cm	1-2cm	2cm	2cm	2cm	2cm
Sentinel node biopsy	/	/	possible	possible	possible	possible	possible	possible	possible
Clinical trial		?	?	?	?	?	?	?	?

* potentially: ultrasonography draining lymph nodes; optional: ultrasonography abdomen, chest radiograph
** ultrasonography draining lymph nodes (strongly advised) AND ultrasonography abdomen/chest radiograph OR CT thorax/CT abdomen/MR brain; PET-CT only reimbursed from stage IIC (pT4b)

These recommendations may serve as a guidance but need to be tuned according to the specific situation.

met voorgeschiedenis van melanoom hebben immers een verhoogd risico (3-6%) om een 2e melanoom te ontwikkelen en in 2 op 5 gevallen doet het 2e melanoom zich synchroon voor met het eerste. Daarnaast wordt gezocht naar argumenten voor eventuele huid- of kliermetastasering.
Bij melanoma *in situ* zijn geen beeldvormingsonderzoeken nodig. Voor de andere tumorstadia is er in de literatuur geen consensus over welke onderzoeken hier op hun plaats zijn. BADO doet een voorstel over de mogelijke stagingonderzoeken bij de verschillende tumorstadia (**Tabel 2**). Bij negatieve stadiëring wordt een therapeutische brede excisie tot op de fascia uitgevoerd met als doel het risico op lokaal recidief door occulte tumorhaarden te verlagen. Hierbij wordt een vaste marge gehanteerd afhankelijk van het tumorstadium. Een sentinelklierbiopsie kan gecombineerd worden met de brede resectie vanaf een melanoom pT1b-stadium. Deze procedure laat een nauwkeuriger stadiëring, maar er is geen bewezen overlevingsvoordeel (6).

Gemiddeld is slechts 1% van de sentinelklierprocedures vals-negatief. Een uitzondering hierop zijn melanomen in de hoofd/halsregio, waar tot 10% van de sentinelklieren vals-negatief kan zijn. Bij positieve sentinelklier komt de patiënt in stadium III terecht. In geval van microscopische bevestiging van regionale ziekte wordt een meer doorgedreven staging geadviseerd (**Tabel 3**). In afwezigheid van systeemmetastasen is chirurgie de eerste behandeling van stadium III-ziekte. Zowel micro- als macrometastasen in de lymfeklieren noodzaken een totale lymfeklierexcisie.

Voor patiënten met zeer uitgebreide in-transitmetastasen is geïsoleerde lidmaatinfusie/-perfusie ook een mogelijke behandeling. Zo'n 50% van de patiënten bereikt hiermee lokaal een complete respons. Deze therapie heeft echter geen effect op metastasen op afstand en verhoogt dus de algemene overleving niet. Het gebruik van deze behandeling neemt langzaamaan af gezien de nieuwe systeembehandelingen (zie verder).

Adjuvante behandeling van melanoom

Pol Specenier (Dienst Medische Oncologie, UZ Antwerpen) gaf een literatuuroverzicht van de adjuvante behandelingsopties bij melanoom.

Een adjuvante behandeling is een bijkomende behandeling die gegeven wordt na de primaire behandeling op het moment dat de patiënt ziektevrij is en die de bedoeling heeft om het risico op herval te verkleinen. Bij melanoompatiënten is de belangrijkste indicatie hiervoor gereseceerde stadium III-ziekte, gezien deze subgroep na de standaardbehandeling een hoge recidiefkans heeft. BADO concludeert dat er momenteel geen standaard adjuvante behandeling is bij melanoom.

Interferon-alfa is ongetwijfeld de meest gedocumenteerde adjuvante behandelingsmodaliteit bij melanoom, al bestaat er internationaal discussie over de waarde ervan. In Nederland wordt deze behandeling bijvoorbeeld niet gegeven, terwijl in Frankrijk bv. zowel stadium II- als -III-patiënten ervoor in aanmerking komen. De bron van de controverse rond de waarde van adjuvant interferon zijn de inconsistente resultaten

Tabel 3: Recommendation management stage III melanoma.

	In-transit metastasis	Microscopically invaded lymph node	Macroscopically invaded lymph node
Pre-operative	Confirm with biopsy Staging upgrade*	Staging upgrade*	Confirm with FNAC** or tru-cut biopsy*** Staging upgrade*
	Staging excludes stage IV	Staging excludes stage IV	Staging excludes stage IV
Treatment	Excision if possible; if excision is not possible consider isolated limb infusion/perfusion, systemic treatment or clinical trial	Total lymph node excision	Total lymph node excision
Adjuvant treatment	NOT STANDARD clinical trial?	NOT STANDARD clinical trial?	NOT STANDARD clinical trial?

* CT thorax/abdomen or PET-CT; possible: CT/MR brain
** FNAC: fine needle aspiration cytology usually ultrasound-guided
*** tru-cut biopsy: percutaneous biopsy prelevating tissue cylinder, usually ultrasound-guided

These recommendations may serve as a guidance but need to be tuned according to the specific situation

Tabel 4: Recommendation management stage IV melanoma.

	1° line	2° line	3° line
BRAF negative	for solitary or few metastases: consider surgery* or gamma knife** chemotherapy (DTIC) c-kit inhibitor c-kit mutation clinical trial?	ipilimumab clinical trial?	clinical trial?
BRAF positive	for solitary or few metastases: consider surgery* or gamma knife** BRAF inhibitor clinical trial?	ipilimumab BRAF inhibitor clinical trial?	clinical trial?

* mostly for one or few metastases of the brain, lung; sometimes for metastases of GI tractus, skin/soft tissue, other
** mostly for one or few metastases of the brain

These recommendations may serve as a guidance but need to be tuned according to the specific situation, among which the type of clinical trial, the tumor kinetics…

van kleinere studies. De recent gepubliceerde Cochrane review was daarom een mijlpaal: de auteurs konden voor het eerst met een grondige meta-analyse aantonen dat adjuvant interferon-alfa voor hoogrisicomelanoompatiënten zowel de ziektevrije als de algemene overleving positief beïnvloedt (resp. HR = 0,83; P = 0,00001 en HR = 0,91; P = 0,03) (7).

Nochtans blijft de vraag in welke subgroepen van patiënten met melanoom men een dergelijke adjuvante behandeling dan best aanbiedt, en welk doseringsschema best gebruikt wordt. Een post-hocanalyse van de EORTC-trials 18952 en 18991 suggereert dat de subgroep patiënten met microscopische regionale ziekte en/of een geülcereerd primair melanoom een groter voordeel kunnen ondervinden van een adjuvante behandeling met interferon (8). Deze observatie wordt momenteel verder onderzocht in prospectieve klinische trials. Zo loopt er momenteel een EORTC-studie 18081 die de impact van interferon-alfa bij patiënten met geulcereerde primaire tumorT2b,T3b,T4b in afwezigheid van regionale en systemische metastasen wil onderzoeken.

Mogelijk zal het landschap van de adjuvante behandelingen in de komende jaren nog veranderen gezien de systemische opties bij stadium IV-melanoom momenteel in klinische studies geëxploreerd worden als adjuvante behandeling bij hoogrisicopatiënten.

De behandeling van stadium IV-melanoom

Jean-François Baurain (Dienst Medische Oncologie St-Luc, UCL) en Pascal Wolter (Dienst Medische Oncologie, UZ Leuven) spraken over behandeling van stadium IV-melanoom en lichten het voorstel van BADO hieromtrent toe (Tabel 4, Figuur 1). Ze hadden het ook over nieuwe behandelingsmodaliteiten die in de nabije toekomst verwacht kunnen worden.

Sinds 2010 heeft in het domein van de behandeling van stadium IV-melanoom een grote omwenteling plaatsgevonden. Voordien was klassieke chemotherapie met DTIC de standaardbehandeling met een respons van niet meer dan 5-10% en een 1 jaarsoverleving rond 25%.

- **Targeted therapieën**

Het melanoom is een moleculair heterogene ziekte, waarin verschillende drivermutaties kunnen voorkomen afhankelijk van het type melanoom en de locatie ervan (9). De ontdekking van deze mutaties heeft geleid tot een revolutie in de behandeling van gemetastaseerd melanoom, waarbij de BRAF-mutaties op de voorgrond staan. Een V600 BRAF-mutatie leidt tot constitutieve activatie van de MAPK-signaalweg, met een verhoogde celproliferatie als gevolg. Gemiddeld is 41% van alle melanomen BRAF-positief. Dit percentage stijgt tot 49% voor melanomen die op niet-zonbeschadigde huid voorkomen en voor superficieel spreidende melanomen (10).

Vemurafenib, een BRAF-inhibitor, is momenteel goedgekeurd als eerstelijnsbehandeling voor V600-mutante melanomen in België. Een andere BRAF-inhibitor, met name dabrafenib, is momenteel reeds beschikbaar in *medical need* en zal snel volgen. De BRAF-mutatieanalyse gebeurt idealiter op metastatisch weefsel, maar kan ook uitgevoerd worden op primaire melanomen. De progressievrije overleving van melanoompatiënten stijgt van 1,7 maanden na behandeling met DTIC naar 6,9 maanden na behandeling met vemurafenib. De *overall survival* stijgt van 9,7 maanden met DTIC naar 13,6 maanden met vemurafenib.

Opvallend aan de behandeling met vemurafenib is de hoge *response rate* (48%), de korte tijd tot respons (1,45 maand) en de relatief beperkte toxiciteit. De belangrijkste bijwerkingen zijn arthralgie (59%), huiduitslag (52%), fotosensitiviteit (52%) en vermoeidheid (42%). Daarnaast ziet men ook het ontstaan van huidpapillomen (29%) en spinocellulaire carcinomen of keratoacanthomen (26%) (11). Hoewel vemurafenib vaak spectaculaire en snelle resultaten geeft, is de duur van de respons gewoonlijk relatief beperkt tot ongeveer 6 maanden. De mogelijke resistentiemechanismen zijn het onderwerp van uitgebreid onderzoek. De combinatie van BRAF-inhibitie met MEK-inhibitie, een molecule die verder downstream in de MAPK-signaalweg ligt, lijkt op dit vlak een veelbelovende piste (zie verder).

- **Immunotherapie**

Het concept van 'immunosurveillance' in kanker dateert reeds van midden vorige eeuw en was gebaseerd op de observatie dat immuungedeprimeerde patiënten vaker maligniteiten ontwikkelen. Decennia lang was interferon-alfa de enige vorm van immunotherapie voor melanoom die goedgekeurd was voor gebruik in Europa, zij het met bescheiden resultaten. Een groot aantal therapeutische vaccins en verschillende vormen van autologe T-celtherapieën werd in de tussentijd getest, maar tot op heden zijn ook hier de resultaten relatief beperkt. Globaal wordt met de verschillende onderzochte vaccintypes in patiënten met gemetastaseerde maligniteiten een objectieve respons gezien van 3,8% (12).

De ontdekking van de therapeutische mogelijkheden van anti-CTLA-4-antilichamen (ipilimumab) heeft eveneens voor een revolutie in de behandeling van gemetastaseerd melanoom gezorgd. CTLA-4 is een immuuncheckpointmolecule die in fysiologische omstandigheden de T-celactivatie downreguleert. In 2010 werden de resultaten van een fase III-trial gepubliceerd waarin het anti-CTLA4-antilichaam ipilimumab een significant overlevingsvoordeel aantoonde t.o.v. de klassieke chemotherapie bij patiënten met gemetastaseerd melanoom (13). Er werd een mediane overleving gezien van 10,1 maanden, een winst met 34% t.o.v. de controlegroep. Na 1 jaar blijkt nog 46% van de behandelde patiënten in leven te zijn, t.o.v. 25% in de controlegroep.

Hoewel de *response rate* met 15-20% objectief gezien laag is, vindt er een belangrijk en tot voorheen ongezien fenomeen plaats in de zogenaamde 'staart van de curve': een kleine 20% van de patiënten die met ipilimumab behandeld werden, lijken een lange overleving te hebben, tot meer dan 5 jaar na de behandeling. Een belangrijke beperking is dat de werking van ipilimumab zich pas voordoet zo'n 3-4 maanden na start van de

behandeling. Patiënten met een snelle achteruitgang of een levensverwachting van minder dan 3 maanden komen daardoor minder in aanmerking om een goede respons te vertonen op het product.

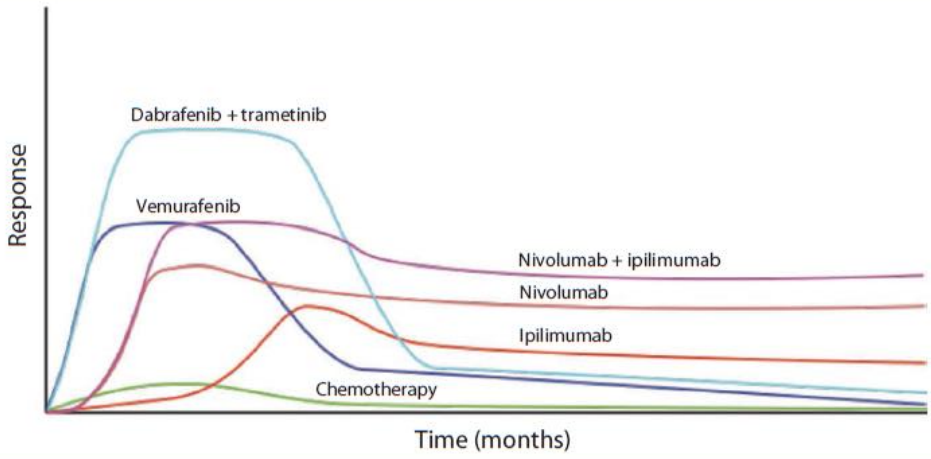
Het spectrum van bijwerkingen van ipilimumab is ook nieuw t.o.v. de tot dan gekende producten en wordt gecategoriseerd onder de '*immune-related adverse events*' (irAE). Het gaat om variabele auto-immune fenomenen die vooral de huid (pruritus, 'rash'), het gastro-intestinale stelsel (diarree, nausea) en het endocriene systeem (hypofysitis, bijnierinsufficiëntie) blijken te treffen. De beste indicatiestelling voor ipilimumab blijft voorlopig de veeleer traag-progressieve patiënten, die in voldoende goede algemene toestand zijn om de soms zware bijwerkingen te dragen. In België is ipilimumab momenteel pas terugbetaald als 2e lijn bij stadium IV-patiënten.

- **BRAF- + MEK-inhibitoren**

De rationale voor de combinatiebehandeling van BRAF- met MEK-inhibitoren is het omzeilen van de resistentie die optreedt tegen BRAF-inhibitoren in monotherapie met ziekteprogressie. De resultaten van een fase I/II-trial met dabrafenib en trametinib in V600-mutante melanoompatiënten lijken veelbelovend (14). De *response rate* stijgt tot 76% met deze combinatiebehandeling, de progressievrije overleving neemt toe tot 9,4 maanden en de duur van de respons bedraagt gemiddeld 10 maanden. De combinatie heeft als bijkomend voordeel dat de ontwikkeling van hyperkeratotische huidletsels (waaronder spinocellulaire carcinomen) hiermee verminderd (2-7%) in vergelijking tot een BRAF-inhibitor in monotherapie. Er lijkt wel een toename te zijn van algemene huidtoxiciteit (42%), met vooral een stijging van de acneïforme huiderupties. De andere voornaamste bijwerkingen zijn koorts (58%), rillingen (37%) en vermoeidheid (38%).

De MEK-inhibitoren hebben nog een andere troef, met name lijken ze ook effectief te zijn tegen NRAS-mutante melanomen (zo'n 15% van de melanomen). Dit verbreedt de therapeutische mogelijkheden, aangezien NRAS- en BRAF-mutaties mutueel exclusief zijn (15).

Figuur 1: Overzicht van kinetiek en grootte van respons op de diverse systeembehandelingen voor stadium IV-melanoom (dia uit presentatie JF Baurain).



Nieuwe behandelingen die verwacht worden voor huidkanker

- **Nivolumab**

Een nieuwe behandelingsmodaliteit in de categorie immunotherapie zijn de anti-PD-1-antilichamen (bv. nivolumab). De interactie tussen PD1 en PD-L1 is net als die tussen CTLA-4 en B-7 een immuun checkpoint. Een belangrijk verschil is dat PD-L1 tot expressie gebracht wordt door de tumor in respons op inflammatie. De interactie tussen PD1 en PD-L1 vindt dus plaats in de tumorsite (perifeer), eerder dan in de lymfoïde organen (centraal) zoals voor CTLA-4 het geval is. Uit de eerste resultaten blijkt nivolumab een gunstiger toxiciteitsprofiel te hebben dan ipilimumab en zowel betere als snellere responsen te geven. Na 1 jaar is nog 61% van de behandelde patiënten in leven en na 2 jaar 44%. Voorlopig is nivolumab enkel in studieverband verkrijgbaar, maar dat zal vermoedelijk in de komende jaren veranderen.

- **cKIT**

Mutaties van c-KIT komen voor in 2-3% van alle melanomen, dit percentage stijgt naar 15-20% in acrale en mucosale melanomen (8). Imatinib, een c-KIT inhibitor, toonde in het verleden geen duidelijke winst in ongeselecteerde melanoompatiënten. Een recente fase II-trial waarbij alleen c-KIT-gemuteerde melanomen ermee behandeld werden, kon ziektecontrole bekomen in 50% van de patiënten (16).

- **Combinatietrials**

De toekomst lijkt vooral schijnbaar eindeloze mogelijkheden in combinatietrials te brengen, een handgreep ervan wordt hier overlopen. De combinatie van ipilimumab met DTIC toonde een winst in overleving, maar was sterk hepatotoxisch (17). Hepatotoxiciteit lijkt ook de beperkende factor te zijn in de combinatie van vemurafenib en ipilimumab, een fase I-trial met de combinatie van beide werd om deze reden stopgezet (18). De voor de hand liggende combinatie van ipilimumab en nivolumab werd reeds in fase I getest. Deze combinatie blijkt een acceptabel toxiciteitsprofiel te hebben en induceerde zowel snellere als betere responsen dan beide therapieën apart (19).

- **Nieuwe systeembehandelingen in epitheliale tumoren**

- Vismodegib is een Hegdehog-inhibitor die bestudeerd werd voor lokaal uitgebreid inoperabel of metastatisch basocellulair carcinoom. Deze behandeling biedt ook mogelijkheden voor patiënten met een Gorlin-syndroom (syndroom o.b.v. een PTCH-mutatie in de Hedgehog pathway) die multipele basocellulaire carcinomen kunnen ontwikkelen. De eerste resultaten tonen respons in 30% van de metastatische en in 43% van de lokaal uitgebreide basocellulaire carcinomen (20, 21).
- *Epidermal growth factor-receptor* (EGFR)-inhibitoren worden verder uitgewerkt voor de behandeling van niet-reseceerbare of gemetastaseerde spinocellulaire carcinomen. In fase II-trials werden *response rates* van 28% gezien (22).

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CHAPTER 2:

Research aims

Aims

Melanoma is one of the most immunogenic tumours. This is reflected by the recent progress in immunotherapeutic strategies. Nonetheless, clinical responses are only obtained in a minority of patients.[1] The patients' immune response appears to play an important role in determining response rates and survival, but many gaps exist in the existing knowledge on this topic. Our research group was previously able to show that expression of IDO in tumour-free sentinel lymph nodes of melanoma patients is associated with a worse prognosis, independent of other prognostic markers.[2]

The current research project was initiated starting from this striking observation, aiming to broadly explore where and when IDO expression occurs in melanoma patients, and what the clinical relevance of the expression patterns is. Research on local or systemic factors that skew the immune system into an immunosuppressed state may reveal new biomarkers, or identify subsets of patients with a different prognostic profile. Furthermore, new therapeutic targets may be found. Our first aim was to identify and characterise IDO expression in the primary melanoma. Secondly, circulating immune subsets were assessed for their prognostic relevance and IDO expression. Thirdly, we wanted to test if IDO expression is altered in patients treated with adjuvant interferon-alpha.

Outlines

❖ Evaluation of IDO expression in the primary melanoma

Starting from our previous observations on IDO in tumour-free sentinel lymph nodes, the question of whether IDO is also expressed in primary and metastatic melanoma tissues suggested itself. As IDO can be expressed by tumour cells but also by host cells, we carefully assessed both expression patterns separately. To gain insight in the immune contexture in which IDO expression is found, FoxP3 (as a marker for immunosuppression by Tregs) and CD8 (indicating the presence of cytotoxic T-cells) expression were added.

❖ Identification of possible candidates for immunoprofiling in the blood

Before examining the presence of immunosuppressive mechanisms in the peripheral blood of melanoma patients, we wanted to gain more overview on their systemic immunity. In melanoma, it is currently unclear which circulating immune cell types confer the most powerful prognostic information. Besides T-cells, myeloid-derived suppressor cells (MDSCs) and dendritic cells (DCs) are

the most elaborately studied circulating cell types. However, these have mostly been studied separately, making direct comparisons difficult. Hence, we decided to comparatively examine a panel of 6 different circulating immune cell types for their clinical relevance.

❖ Expression of immunosuppressive markers by the identified circulating cell types

After confirming the clinical importance of the frequencies of circulating DCs and MDSCs in melanoma patients, we sought to assess their expression of immunosuppressive markers. For this purpose two immune checkpoint molecules were chosen in addition to IDO. Even though IDO, CTLA-4 and PD-L1 are promising targets for immunotherapy, data on their expression by immune cells in the peripheral blood of melanoma patients are lacking. In this study, we therefore investigated the in vivo expression of IDO, PD-L1 and CTLA-4 by immune cells of the lymphoid and myeloid lineage in the peripheral blood of stage I to IV melanoma patients. To ascertain the functional relevance of the detected IDO, tryptophan metabolism was also measured.

❖ Presence of systemic negative feedback mechanisms during IFN-α2b immunotherapy

Finally, we wanted to test whether our previous observations could be relevant in melanoma patients who are actively treated with immunotherapy. To this end, IFN-α2b was chosen as this is the immunotherapy with which dermatologists have most experience. The mechanism of action of adjuvant IFN-α2b in high-risk melanoma patients is believed to be mainly immunomodulatory, but results of previous research to elucidate it have been inconclusive so far. The effect of adjuvant treatment with IFN-α2b on OS in melanoma patients is modest and currently limited data exist on possible predictors of response. Only few studies have focused on alterations in circulating immune cells during adjuvant IFN-α2b treatment. We therefore decided to test the relevance of our results in IFN-α2b treated melanoma patients.

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CHAPTER 3:

Methods

Patients and biobank

In our dermatology clinic, a specialised consultation for melanoma patients takes place on a weekly basis. Patients attending this consultation were invited to take part in this research project. The minimum requirement was that a sentinel lymph node procedure had been done. After informed consent was given, a blood sample was drawn. Whenever possible, the archival primary melanoma and sentinel lymph node were retrieved. Clinical data were acquired from the patients' medical file and registered in an anonymized database.

Protocols

❖ Immunohistochemistry

Immunohistochemistry was performed on archival formalin-fixed paraffin-embedded (FFPE) tissues. Four to five µm sections were cut. For all immunohistochemical stainings, the sections were placed in a Dako PT Link (Dako, Heverlee, Belgium) for deparaffinisation and heat-mediated antigen retrieval at 97°C for 20 minutes. Subsequently, the sections were stained in a DAKO Autostainer device (Dako). CD8 (Dako), indoleamine 2,3 dioxygenase (IDO, clone 10.1, Millipore) and Forkhead Box P3 (Foxp3, eBioscience, Hatfield, UK) were used as primary antibodies. For all stainings, an incubation time of 30 minutes was used, except for IDO and Foxp3 which required an incubation time of 1 hour. As Foxp3 was a biotin-linked antibody, the standard avidin biotin complex (ABC) method was used. All other stainings were carried out using the Envision Flex (Dako) substrates. For IDO staining in primary and metastatic melanomas, a mouse-linker was added to the protocol (Dako).

❖ PBMC isolation

Venous blood was drawn using 9 mL EDTA tubes. PBMCs were isolated within 4 hours after sample procurement. The blood was diluted in RPMI 1640 and layered on top of 15 mL Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). After 30 minutes of centrifugation, the buffy coat containing PBMCs was collected. After 3 wash steps with RPMI 1640, the cells were frozen at -80°C in freezing medium which consisted of heat-inactivated foetal bovine serum (FBS) supplemented with 10% dimethylsulfoxyde (DMSO). Frozen PBMC samples were stored in liquid nitrogen until analysis.

At the same time, a serum sample was also obtained. These were centrifuged for 10 min, and stored at -80°C until analysis.

❖ PBMC culture & stimulation

PBMCs were cultured overnight in a medium containing Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS and 1% glutamine. PBMCs were also stimulated to induce IDO expression by adding either 1000U/ml recombinant human IFN-γ (Imukin, Boehringer Ingelheim) or 5µg/ml recombinant human CTLA-4 (R&D Systems) to the same medium for 12h at 37°C in 10% CO2. After incubation, adherent and non-adherent cells were collected for flow cytometry.

❖ Flow cytometry

Controls

Isotype controls were used to assess the staining quality. Fluorescence-minus-one (FMO) controls were used to set the gates. The FMO controls contained every colour in the panel except the one being controlled for in that sample, with the addition of an isotype for the omitted colour.

To provide a representative sample, a median amount of 500 000 cells was analysed per cell mix. Samples with less than 100 000 cells were excluded to ensure that only samples of adequate quality were used for interpretation.

Antibodies

The following antibody combinations were used to define the different cell subsets:

	Treg	Tcyt	pDC	mDC	mMDSC	pmnMDSC
CD3 - BV421	+	+				
CD4 - APC-Cy7	+					
CD25 - FITC	+					
FoxP3 - PerCP-Cy5.5	+					
CD8 - APC		+				
HLA-DR - PerCP-Cy5.5			+	+	-	-
Lineage - FITC			-	-	-	-
CD123 - BV421			+	-		
CD11c - APC			-	+		
CD33 - BV421					+	+
CD11b - APC-Cy7					+	+
CD14 - APC					+	-
CTLA-4 - APC	?					
IDO - PE	?	?	?	?	?	?
PD-L1 - PE-Cy7	?	?	?	?	?	?

BV421: Brilliant Violet 421, Tcyt: cytotoxic T-cell.

The antibodies in the lower part of the table (marked with '?') were used undiluted to allow for mean-fluorescence intensity (MFI) calculation. Intracellular stainings were added after surface staining of the PBMCs. To do so, PBMCs were fixed and permeabilized with fixation⁄permeabilization solution (BD Biosciences), and then stained with antihuman IDO PE (R&D Systems), CTLA-4 APC (BD Biosciences) and FoxP3 PerCP-Cy5.5 (eBioscience) antibodies. The gating strategies that were used are illustrated in addendum II.

In some studies, the CD14- MDSCs have been subdivided in promyelocytic MDSC and pmnMDSCs, the first being CD15- and the latter CD15+.[1] In our own work a CD15 staining could not be included, we will use the term pmnMDSC to encompass both.

❖ Ultra-performance liquid chromatography (UPLC)

UPLC-MS/MS (Waters Acquity TQD) was used to quantify tryptophan and its metabolites on stored, frozen patient sera. These analyses were done in a collaboration with the Laboratoire de Toxicologie, CHU Lille (Lille, France). The following tryptophan metabolites were quantified: kynurenine, kynurenic acid, quinaldic acid, 3-OH-kynurenine, 5-OH-tryptophan and 5-HT (serotonin).

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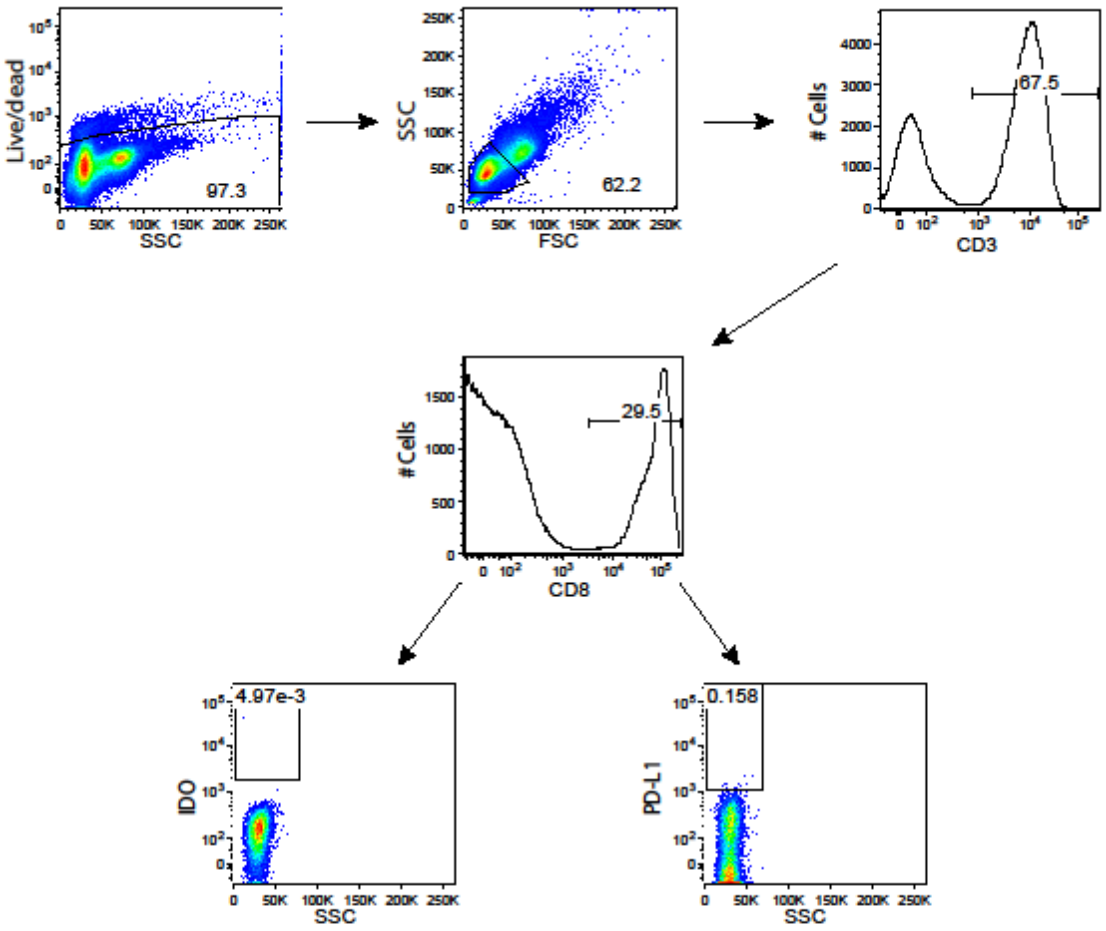
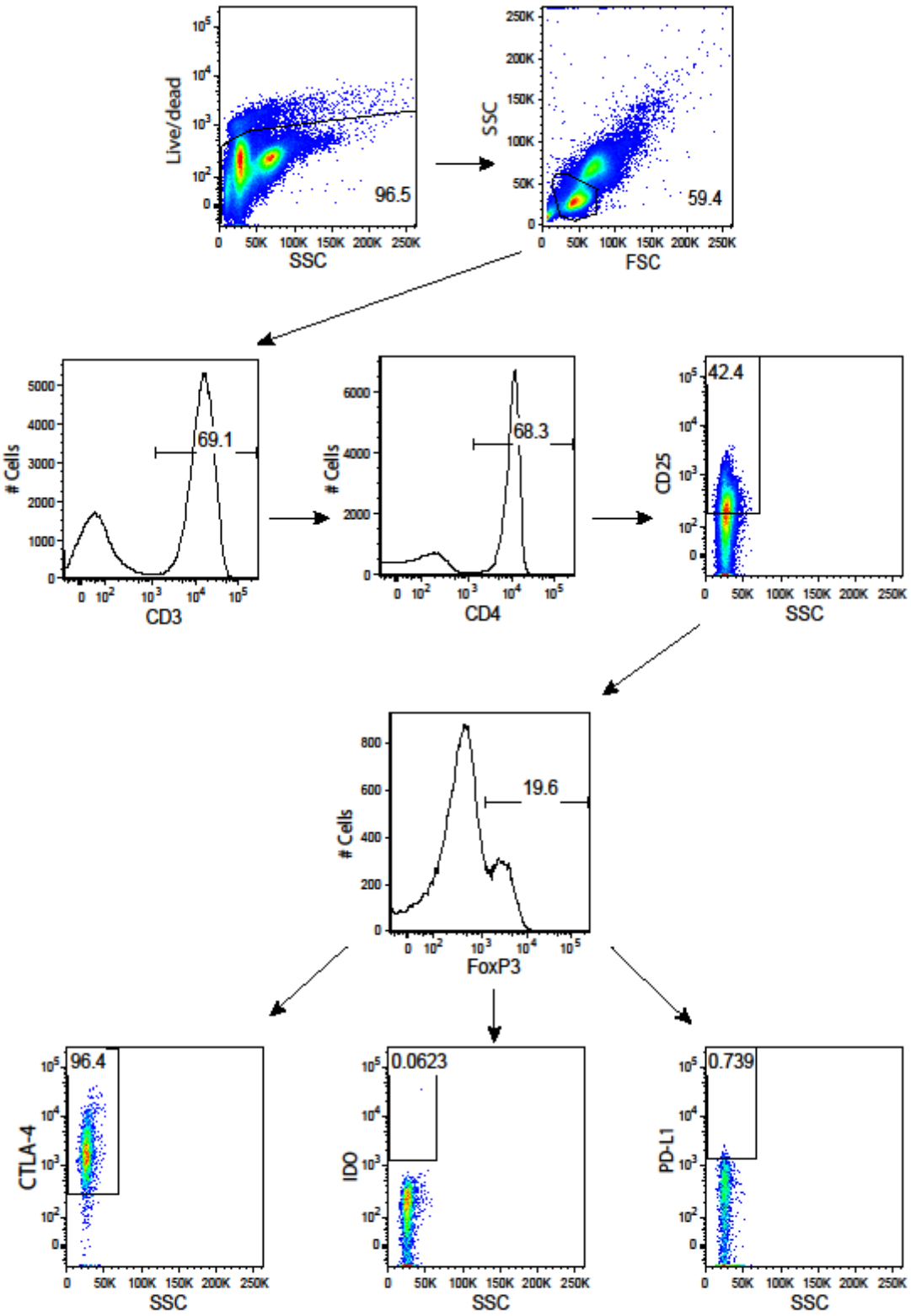
Addendum II: gating strategies

In the flow cytometry experiments that were performed, up to 8 colours were used to identify specific cell populations. Each monoclonal antibody is linked to a different fluorochrome, therefore the different cell types can be distinguished from each other by the combination of colours they emit when they pass through the laser beam in the flow cytometer. Gates are set for each fluorochrome, to separate positive from negative cells. To quantify the number of cells present in a certain cell population, the sample is gated by progressively selecting a more defined subpopulation in a step-wise manner. This procedure is known as a "gating strategy". The gating strategies for the cell types that were investigated in this thesis are outlined here. Live cells are selected based upon a live/dead staining, this is often the first gate. The forward/side scatter (FSC/SSC) is used to select a population of cells based on their size and granularity respectively, thereby also excluding any debris that could be present in the sample.

❖ Cytotoxic T-cells

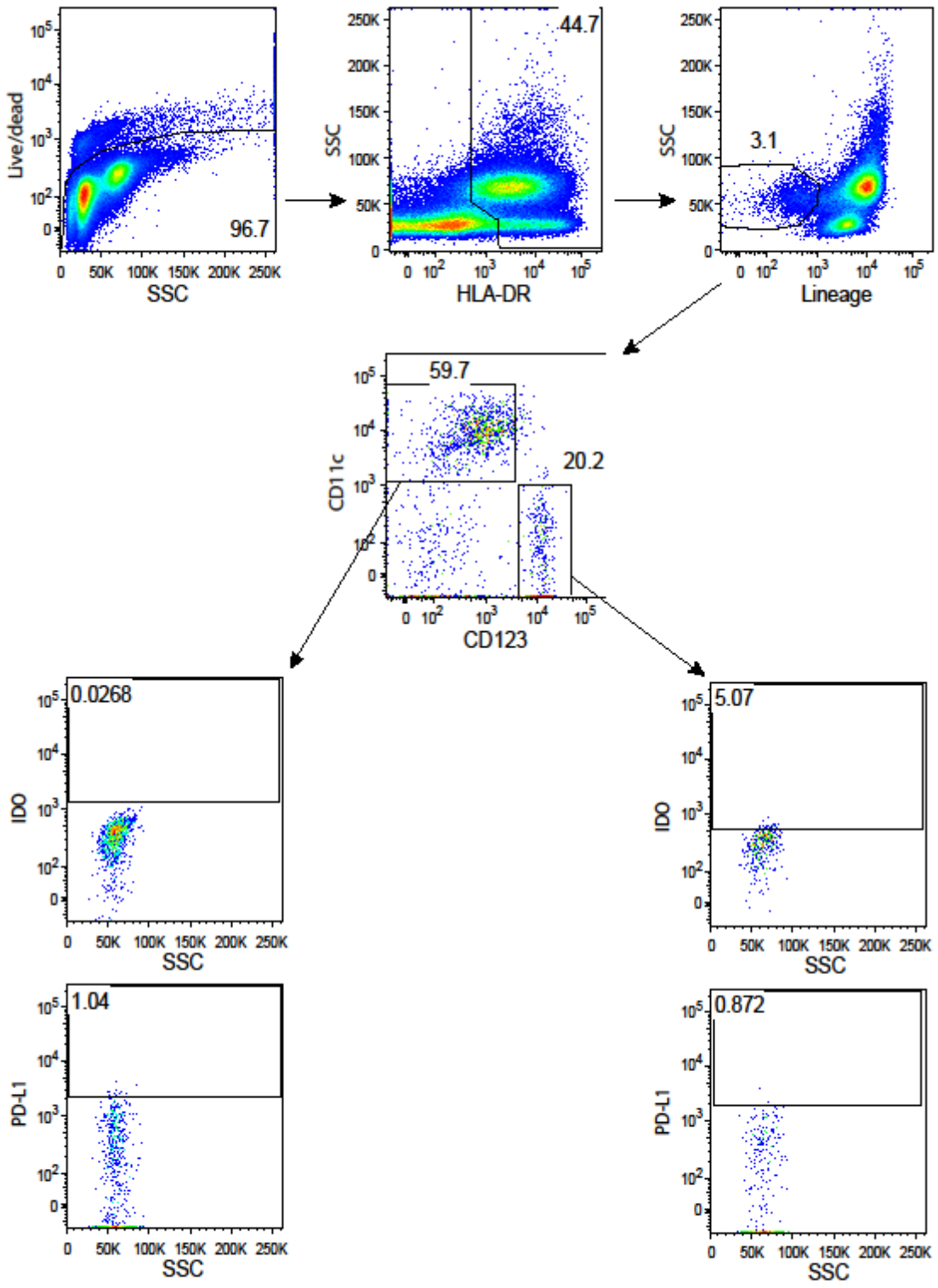


❖ Regulatory T-cells



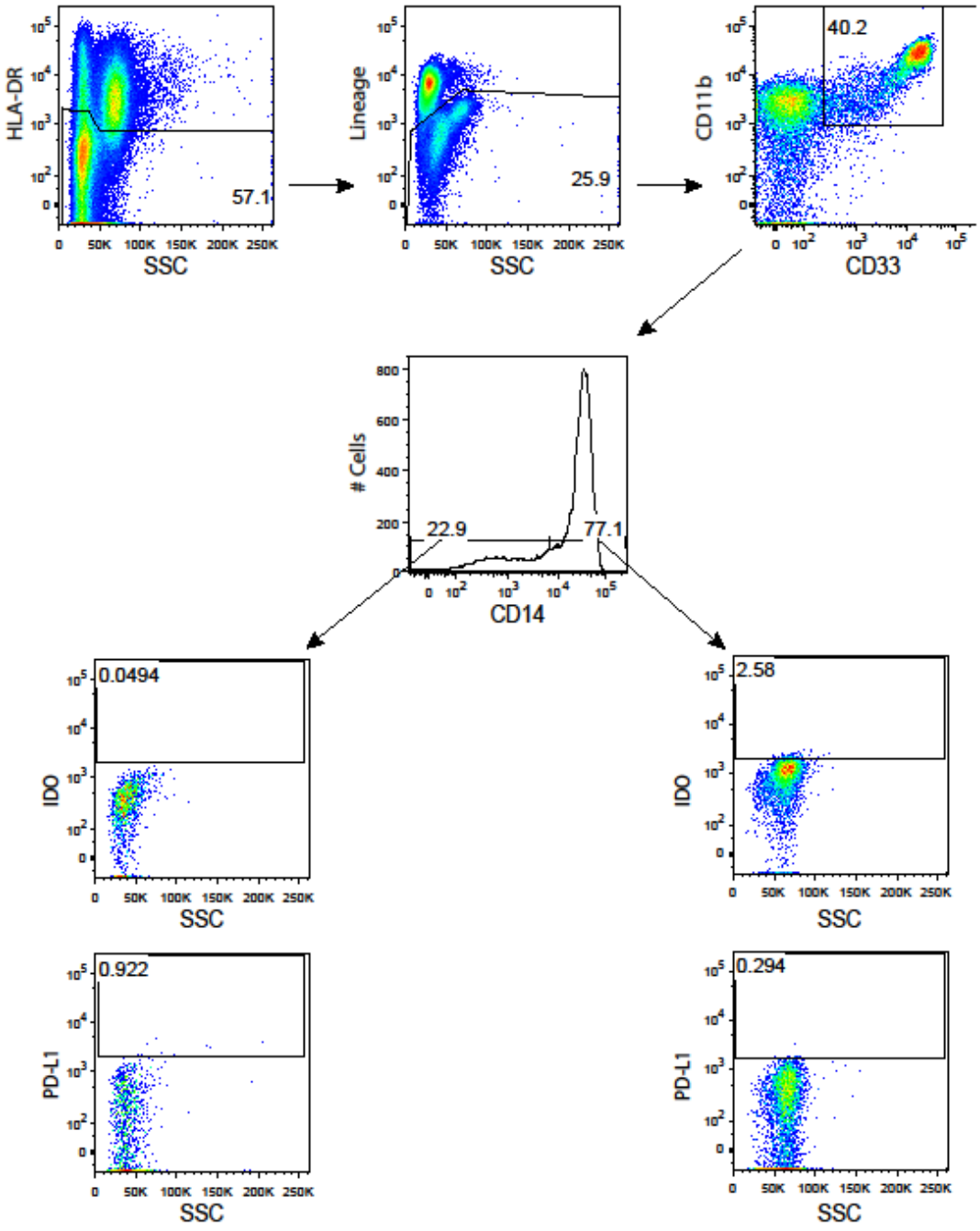
❖ Dendritic cells

Gate to exclude debris is not shown.



❖ Myeloid-derived suppressor cells

Gates to exclude dead cells and debris are not shown.



CHAPTER 4:

Results

PERI-TUMORAL INDOLEAMINE 2,3-DIOXYGENASE EXPRESSION IN MELANOMA: AN EARLY MARKER OF RESISTANCE TO IMMUNE CONTROL?

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Abstract

Background: The immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) is an emerging immunomodulating factor in cancer. IDO expression in tumor-negative sentinel lymph nodes of melanoma patients has a negative prognostic value.

Objectives: To analyse the IDO-expression pattern and the associated immunological changes in corresponding primary melanomas, sentinel lymph nodes and metastases.

Methods: In a cohort of 120 melanoma patients, primary melanomas with corresponding sentinel lymph nodes (n=85) and metastases (n=18) were analysed by immunohistochemical staining for IDO, CD8 and FoxP3. Tumor-infiltrating lymphocytes (TILs) were scored. In 27 patients, IDO expression in stimulated PBMCs was analysed by flow cytometry.

Results: IDO expression in the sentinel node strongly correlated with endothelial IDO expression in the peritumoral stroma of the corresponding primary ($P<0.001$) and metastatic melanoma ($P=0.048$). Sentinel IDO-positivity was inversely correlated with CD8+ lymphocytes ($P=0.005$) and TILs ($P=0.047$) in the primary melanoma. Both IDO expression in the sentinel and the primary melanoma had a negative prognostic effect on overall survival (Cox regression analysis, $P=0.001$ and $P=0.035$ respectively), independent of Breslow thickness, gender, age, ulceration and sentinel invasion. IDO expression by PBMCs after stimulation with CTLA-4 was not correlated with sentinel IDO expression but tended to correlate with disease stage ($P=0.044$).

Conclusion: Endothelial IDO expression is highly consistent in the primary, the sentinel and metastatic tissues of melanoma patients, indicating that immune suppression in melanoma is determined very early in the disease course. These results provide evidence that IDO expression in melanoma is a marker of the patients' antitumor immune response with an independent prognostic value.

1. Introduction

Indoleamine 2, 3-dioxygenase (IDO) is the rate-limiting enzyme in the pathway that catabolises tryptophan to kynurenines. It was first discovered for its beneficial inhibitory effect in various infectious diseases, based on a reduction of the availability of the essential amino-acid tryptophan in the inflammatory environment.[1] Later, interest shifted towards the central role that IDO plays in immune tolerance.[2-4] In recent years, IDO-research has focused on its role in the induction of immune resistance in malignant tumours.[5, 6] We previously demonstrated an independent negative prognostic effect of IDO expression in the sentinel lymph node (SLN) on both relapse free (RFS) and overall survival (OS) in patients with stage I and II melanoma. IDO expression in the SLN was also correlated with changes in systemic immunity, such as a higher expression of CTLA-4 in circulating regulatory T-cells (Tregs).[7] IDO has been studied in many human cancer types and its expression by cancer cells has commonly been associated with a worse prognosis.[8-13] These results however have not been consistent and in some cancer types IDO expression has even been associated with improved patient survival.[14, 15] Apart from its expression in malignant cells, IDO has also been found in a variety of normal human cells such as antigen presenting cells and endothelial cells.[3, 4, 16, 17] This has led to the hypothesis that the biological effect of IDO expression depends on the cell type it is expressed in, which could explain the inconsistent results reported in literature. Evidence for a possible predictive role of IDO expression is also emerging as pre-treatment IDO expression in tumour biopsies has been associated with response to anti-CTLA4 immunotherapy in melanoma, and endothelial IDO expression has been observed in this context.[18, 19] IDO clearly has an important role in melanoma-associated immunomodulation, but whether this is exerted mainly by the tumoral cells or by the surrounding host cells is still unknown. Therefore in the present study the primary endpoint was firstly to investigate if IDO expression in the sentinel lymph node of melanoma patients is associated with tumoral and peri-tumoral IDO expression, tumour-infiltrating lymphocytes (TILs) and FoxP3 in the corresponding primary tumour. Secondly, the consistency of IDO expression in the primary tumour and the sentinel was also analysed. As a consequence of the observations that were made, metastatic tissues of a subgroup of patients were also included, and the impact of IDO expression by circulating immune cells was assessed.

2. Materials and methods

Table 1: database patient characteristics

Characteristic	
Patient characteristics	
Number of patients	120
Follow-up time (median)	88 months (IQR 47.2-110)
Age diagnosis (median)	49.5 years (IQR 35.7-61.2)
Gender (%)	62% female - 38% male
Location of primary melanoma	
Head & neck	7.5% (9/120)
Trunk	31.7% (38/120)
Extremities	60.8% (73/120)
Clinical stage at diagnosis	
Stage I	45% (54/120)
Stage II	30% (36/120)
Stage III	25% (30/120)
Melanoma characteristics	
Breslow (median)	1.73 (IQR 1,20-2,52)
Ulceration (%)	27.1% (32/118)
Regression (%)	3.0% (3/101)
Sentinel node invasion (%)	22.5% (27/120)
Evolution during follow-up	
Disease progression (%)	31.0% (36/116)
Melanoma related death (%)	21.2% (25/118)

❖ Patients

One-hundred-and-twenty melanoma patients who underwent a sentinel lymph node procedure were enrolled in this study, with a median follow-up time of 88 months. The SLN was available in 116 patients, the primary melanoma (PM) was available in 87 patients and both tissues were retrieved in 85 patients. Detailed patient characteristics of the whole patient cohort (n=120) can be found in Table 1, a flow chart of the excluded patients can be found in Figure 1. In 27 additional patients venous blood samples were drawn, with a median interval of 33 months (IQR 23-151) after progression. In 9 patients one or more corresponding in-transit metastases (n=14) were present, with a median delay of 17 months (IQR 12-72 months) after the first melanoma surgery. In 9 other patients one or more corresponding stage IV metastases were present (n=15), with a median delay of 42 months (IQR 11-90 months) after the first melanoma surgery. This study was approved by the local medical ethical committee, all included patients signed written informed consent.

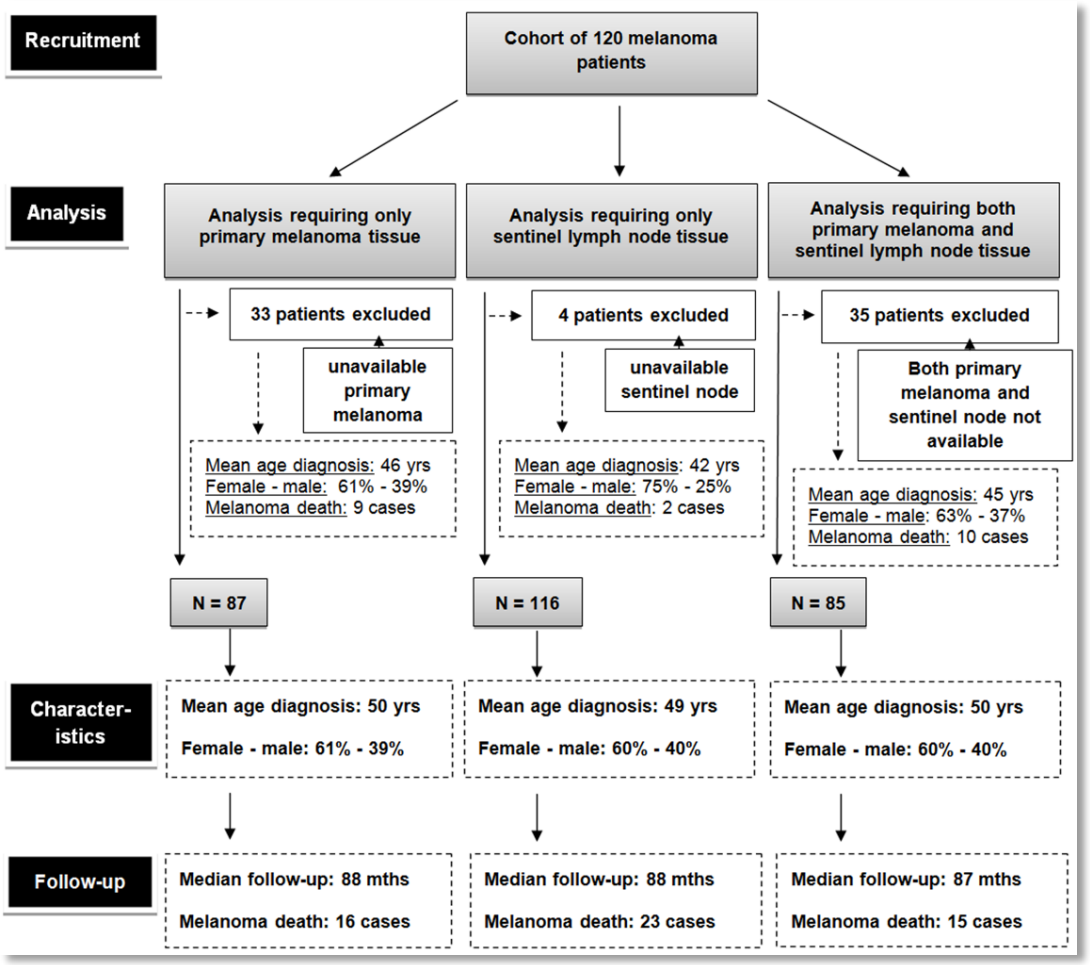

Figure 1: Flow chart depicting the characteristics and follow-up of the in- and excluded patients for each analysis.

❖ Immunohistochemistry

All samples of PMs, SLNs and metastases were formalin-fixed paraffin-embedded archival tissues. Immunohistochemical staining of 4-5 µm sections was performed according to standard avidin-biotin-peroxidase protocols (Envision Flex+, Dako, Denmark). The signal was visualized by incubation with 3-amino-9-ethylcarbazole (AEC) substrate (Dako). Sections were counterstained with hematoxylin. For antigen retrieval, slides were boiled (97°C) for 20 min (PT Link, Dako). Serial sections were incubated with a monoclonal anti-FoxP3 (1/100, eBioscience, San Diego, USA) and a monoclonal anti-IDO antibody (clone 10.1, 1/200, Millipore, Billerica, USA) for 1h. For IDO staining of PMs and metastases, a mouse linker was added to the protocol (Dako). For staining with CD3 (RTU, Dako), CD8 (RTU, Dako) and CD31 (RTU, Dako) antibodies, an incubation time of 30 min was used.

Tumor-infiltrating lymphocytes (TILs) were scored as “brisk”, “non-brisk” and “absent”.[20] CD8 expression in the PM was scored as “present” or “absent”. IDO expression in SLN and peri-tumoral stromal tissue of the primary and metastatic melanoma could be dichotomized as “present” or “absent”. Total IDO expression by the melanoma cells in the primary was calculated as the product of a proportion score and an intensity score, resulting in a total score ranging from 0 to 9, as previously described.[8] We defined IDO expression as a total score > 6.5. The percentage of FoxP3 positive cells was assessed with ImageJ software. Three hotspots (400x magnification) were photographed and the mean percentage of positively stained cells over the total number of counted nuclei was calculated. More than 10% and 5% of FoxP3 positive cells was regarded as over-expression in the SLN and the PM, respectively. All slides were assessed blinded with regard to the results of other immunostainings and clinical patient data.

❖ Isolation and stimulation of PBMCs

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by centrifugation on a Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden) within 4h of venepuncture. The PBMCs were cryopreserved in liquid nitrogen in heat-inactivated foetal bovine serum (FBS) supplemented with 10% dimethyl sulphoxide (DMSO) until analysis.

To induce IDO expression, PBMCs were stimulated for 12h at 37°C in 10% CO2 with 1000U/ml human recombinant interferon-gamma (R&D systems, Minneapolis, USA) or 5µg/ml recombinant Human CTLA-4 (R&D Systems) in a medium containing Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 20% FBS and 1% glutamine. After incubation, adherent and non-adherent cells were collected for flow cytometry.[21]

❖ Flow cytometry analysis

Flow cytometry for IDO expression was performed on stimulated PBMCs. For intracellular IDO staining, PBMCs were fixed and permeabilized with fixation/permeabilization solution (BD Biosciences), and then stained with antihuman IDO (ab55305, Abcam, Cambridge, UK) and a secondary goat-anti-mouse antibody (Dylight650, Abcam). Live/dead staining was performed using an aqua Dead Cell Stain kit (Life Technologies Europe, Ghent, Belgium). Patients with less than 50% living cells were excluded (n=4). Cells were analysed on a FACSCanto™ II flow cytometer (BD Bioscience, Erembodegem, Belgium) using FlowJo software (Tree Star Inc, Ashland, OR, USA).

❖ Statistical analysis

All multivariate Cox- and logistic regression analyses were corrected for sex, age at time of diagnosis, Breslow index, ulceration, sentinel invasion and location of the PM. Median values were compared by the Mann-Whitney U-test. To compare proportions of categorical variables, Pearson's Chi^2 test or Fisher's Exact test were used. All statistical analyses were performed using SPSS 21.0 (SPSS Inc, Chicago, IL, USA), a P-value less than 0.05 was considered statistically significant.

3. Results

❖ Sentinel IDO expression is strongly correlated with IDO expression in the corresponding primary melanoma and metastases.

In the sentinel lymph nodes (n=116), IDO expression could be found in the cytoplasm of perisinusoidal immune cells but was most prominent in CD31+ high endothelial venules (HEV) in the paracortex, in 30.2% of patients. In the primary melanomas (n=87), 2 distinct IDO expression patterns were identified. On one hand, the malignant melanoma cells showed a dusty cytoplasmatic IDO staining in a clonal distribution in 30.3% of patients. On the other hand, endothelial cells in the peri-tumoral stroma closely surrounding the melanoma showed a paranuclear dot-like IDO positivity in 39.1% of patients (Fig. 2). Some IDO positivity in stromal cells could also be observed, but this was not included in the further analyses. The 2 different expression patterns (tumoral versus peri-tumoral endothelial) were not correlated (P=0.069). For an overview of patient characteristics per IDO expression pattern, see Table 2.

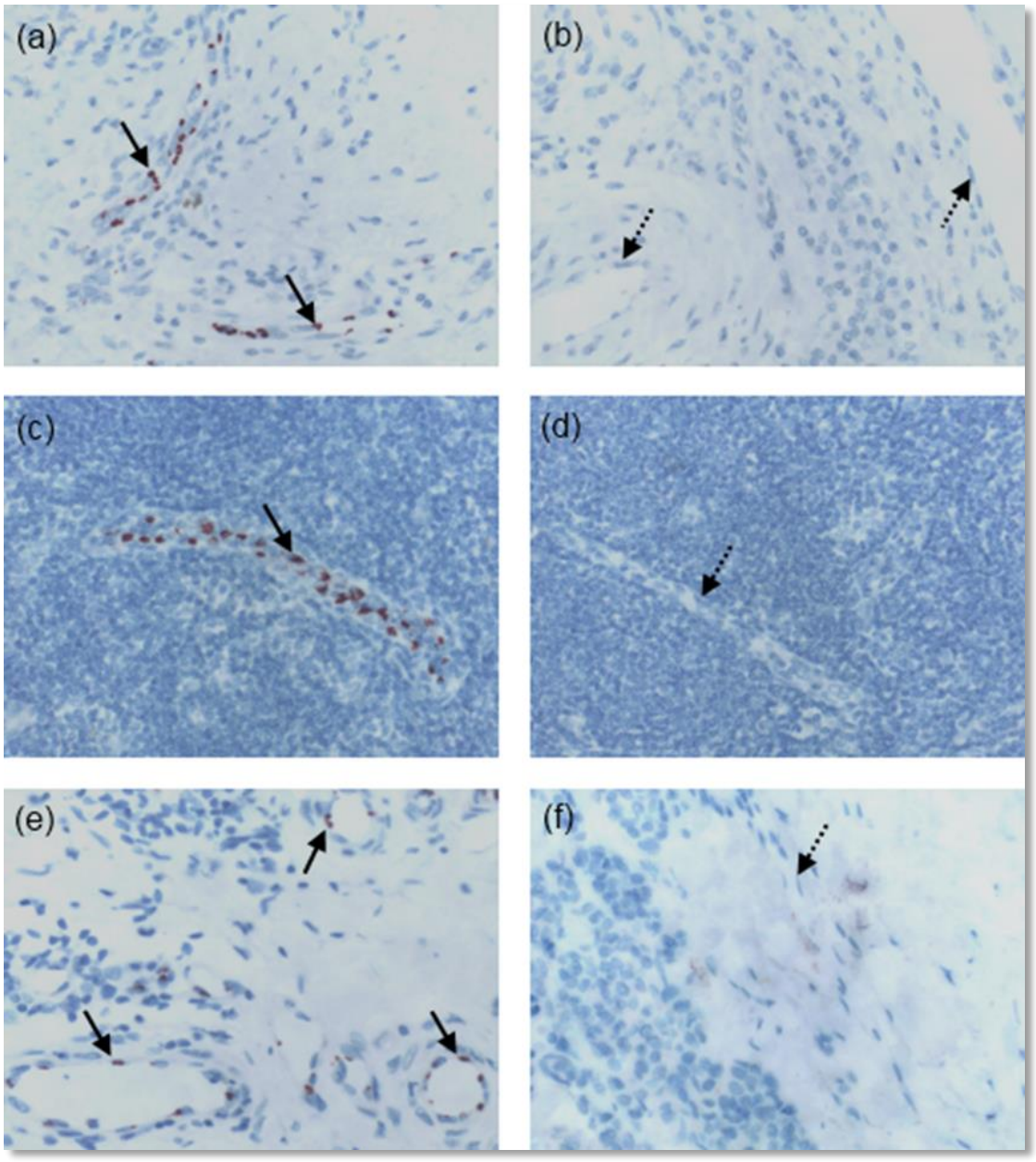

Figure 2: Immunohistochemical pictures showing presence (arrow) and absence (dotted arrow) of expression of indoleamine 2, 3-dioxygenase (IDO) in different melanoma tissues: (a) present and (b) absent IDO expression in peri-tumoral endothelium of a primary melanoma, (c) present and (d) absent IDO expression in the sentinel node and (e) present and (f) absent IDO expression in peri-tumoral endothelium of an in-transit metastasis.

Table 2: patient characteristics per IDO expression pattern

Characteristic	IDO PM MC positive	IDO PM END positive	IDO SLN positive	TOTAL
Number of patients	30.3% (27/89)	39.1% (34/89)	30.2% (35/116)	100% (n=120)
Breslow thickness (median – IQR)	1.40 (1.12-2.00)	1.54 (1.15-2.10)	1.82 (1.13-2.54)	1.73 (1.20-2.52)
Evolution during follow-up				
Melanoma related death	22.2% (6/27)	14.7% (5/34)	26.5% (9/35)	21.2% (25/118)
New stage IV metastases	26.9% (7/26)	21.2% (7/34)	35.3% (12/34)	31.0% (36/116)
New in-transit metastases	4.8% (1/21)	10.7% (3/34)	10.3% (3/29)	11.3% (11/97)
Clinical stage at diagnosis				
Stage I	59.3% (16/27)	55.9% (19/34)	42.9% (15/35)	45% (54/120)
Stage II	18.5% (5/27)	29.4% (10/34)	42.9% (15/35)	30% (36/120)
Stage III	22.2% (6/27)	14.5% (5/34)	14.3% (5/35)	25% (30/120)
Tumor-infiltrating lymphocytes				
Absent	42.3% (11/26)	53.1% (17/34)	68% (17/25)	46.6% (41/88)
Non-brisk	57.7% (15/26)	40.6% (13/34)	28% (7/25)	47.7% (42/88)
Brisk	0% (0/26)	6.3% (2/34)	4% (1/25)	5.7% (5/88)
PM immune infiltrate				
FoxP3 (median – IQR)	3.11% (1.29-9.09)	1.83% (0-4.52)	1.74% (0-3.89)	2.19% (0-5.15)
CD4	44.4% (12/27)	34.4% (11/34)	32% (8/25)	39.1% (34/87)
CD8	88.9% (24/27)	78.8% (26/34)	74% (20/27)	85.6% (77/90)
SLN FoxP3 positivity (median – IQR)	7.69% (5.18-12.36)	7.40% (5.03-10.81)	8.19% (6.11-13.23)	7.60% (5.30-11.47)

IDO, indoleamine 2,3-dioxygenase; PM, primary melanoma; MC, melanoma cells; END,peri-tumoral endothelium; SLN, sentinel lymph node; IQR, interquartile range.

Peri-tumoral endothelial IDO expression in the PM was strongly correlated with IDO positivity in the SLN (n=85, P<0.001). In patients without peri-tumoral IDO expression in the PM, the corresponding SLN was also IDO-negative in 92.3%. In patients with peri-tumoral IDO expression, the corresponding SLN was also IDO-positive in 66.7%. This correlation was confirmed by multivariate logistic regression analysis (P<0.001, Fig. 2). There was no correlation between IDO expression in the melanoma cells and in the sentinel (P=0.516).

Similar to PMs, in-transit metastases (n=9) also showed these two distinct expression patterns (tumoral and peri-tumoral endothelial) of IDO positivity (Fig. 2). In 9 patients with in-transit metastases, endothelial IDO-positivity in the peri-tumoral stroma of the metastasis showed a trend towards correlation with prior IDO expression in the SLN (P=0.048). In 8/9 patients, there was complete accordance of peri-tumoral endothelial IDO expression in the 3 tissues (PM, SLN and metastasis). Peri-tumoral IDO expression was consistent in metachronous metastases per patient (Table 3). In 9 other patients with distant metastases, endothelial IDO positivity could also be distinguished from its expression in other (both malignant and inflammatory) cell types. In 8/9 patients, IDO expression in the SLN and in the peri-tumoral endothelium of the metastasis were congruent (P=0.083, Table 3). Although this sample is too small to reach a solid conclusion, prior treatment with DTIC or interferon-alpha did not seem to alter IDO expression (Table 3).

Table 3: IDO expression in metastases

	Case n°	IDO SLN	IDO PM	Tissue type META 1	IDO META 1	Tissue type META 2	IDO META 2	Tissue type META 3	IDO META 3
IN TRANSIT METASTASES	1.	?	-	In-transit *,°	-				
	2.	+	+	In-transit	+				
	3.	-	-	In-transit	+				
	4.	-	?	In-transit *	-				
	5.	-	?	In-transit	-				
	6.	-	?	In-transit *	-	In-transit *	-		
	7.	-	-	In-transit	-	In-transit	-		
	8.	+	+	In-transit	+	In-transit	+	In-transit	+
	9.	+	?	Lymph node	+	In-transit	+		
STAGE IV METASTASES	10.	+	+	Peritoneal °	+				
	11.	+	+	Liver *	-				
	12.	-	-	Lung	-				
	13.	-	-	Lymph node *	-				
	14.	+	+	Lymph node	+	Lung *	+		
	15.	-	-	Lymph node	-	Subcutaneous	-		
	16.	-	-	Lung *	-	Colon *	-		
	17.	-	-	Lung *	-	Gastric *,°	-		
	18.	-	-	Subcutaneous *	-	Lung *	-	Colon *,°	-

IDO, indoleamine 2,3-dioxygenase; SLN, sentinel lymph node; PM, primary melanoma; META, metastatic melanoma."?", tissue not available. * indicates patient was treated with adjuvant interferon before the metastasis was removed. ° indicates patient was treated with DTIC before metastasis was removed.

❖ Endothelial IDO expression is prognostic both in the PM and the SLN

We have previously reported the independent negative prognostic effect of IDO expression in the tumor-negative SLN. In the present study, with a longer follow-up time (median 88 months), this prognostic effect on both RFS (P=0.006, HR 3.75) and OS (P=0.001, HR 6.40) was confirmed (Fig. 3).[7] The impact on OS was present in both invaded (P=0.012, HR 20.13) and non-invaded SLNs (P=0.015, HR 6.80). Endothelial IDO expression in the PM also had an independent negative prognostic effect on OS (P=0.035, HR 5.65, Fig. 3) but not on RFS (P=0.177). This prognostic effect on OS was strongest in patients without TILs (P=0.018).

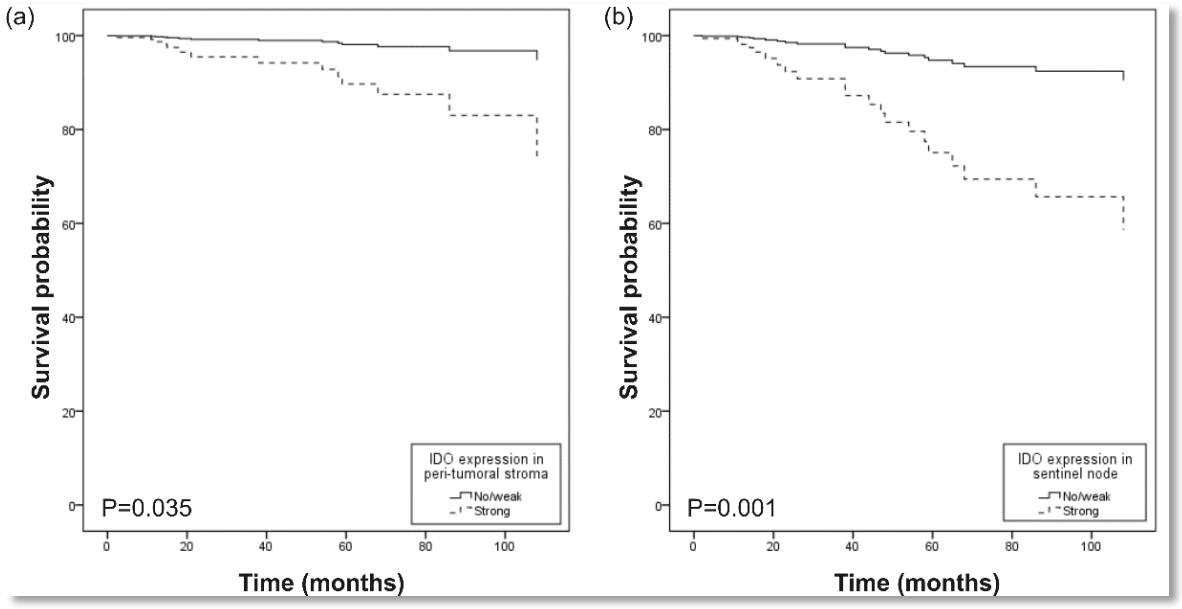

Figure 3: Kaplan Meier plots for melanoma-specific survival according to (a) IDO expression in the peri-tumoral endothelial cells of the primary melanoma and (b) IDO expression in the sentinel lymph node of melanoma patients.

❖ Sentinel IDO expression is related to the immune micro-environment in the primary melanoma

Both TILs and CD8+ T-lymphocytes are established prognostic immunological markers in primary melanomas.[20, 22] A CD8+ infiltrate was present in 51.7% of PMs. TILs were absent in 46.6%, non-brisk in 47.7% and brisk in 5.7% of patients. TILs were dichotomized into present/absent for subsequent analyses. The independent prognostic effect of both CD8+ lymphocytes infiltrating or close to the PM (P=0.003) and of the presence of TILs (P=0.047) was confirmed in our dataset (Fig. 4). Strikingly, the presence of TILs and mainly CD8+ lymphocytes in the PM was inversely related to IDO expression in the SLN (P=0.017 and P=0.003, Fig. 5). These observations were confirmed in multivariate logistic regression analysis (P=0.004; OR 0.188 and P=0.003; OR 0.048).

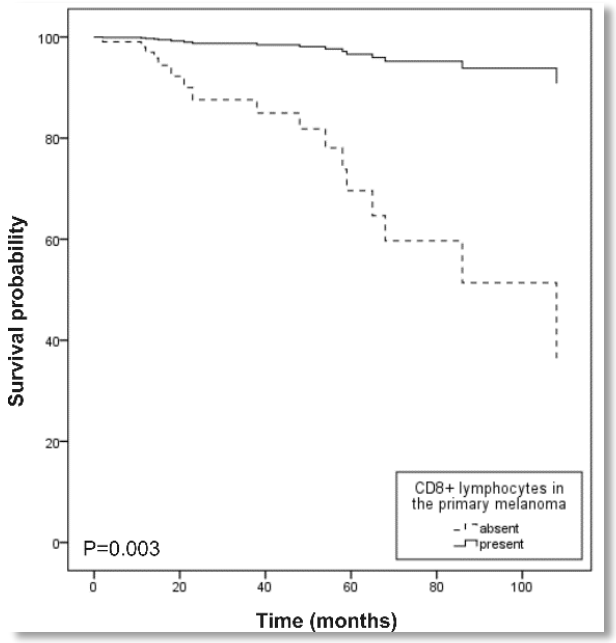

Figure 4: Kaplan Meier plots for melanoma-specific survival according to the presence or absence of CD8+ lymphocytes in the primary melanoma.

Tregs are highly immunosuppressive cells known to be induced by IDO. Overexpression of the Treg marker FoxP3 was present in 26.3% of PMs and in 29.1% of SLNs. The percentage of FoxP3 positive cells was lower in tumor-negative SLNs (P=0.002) and a positive association with IDO expression was found in patients with uninvolved sentinels (P=0.029). IDO expression by the melanoma cells showed a trend towards correlation with a local rise in FoxP3 expression (P=0.054).

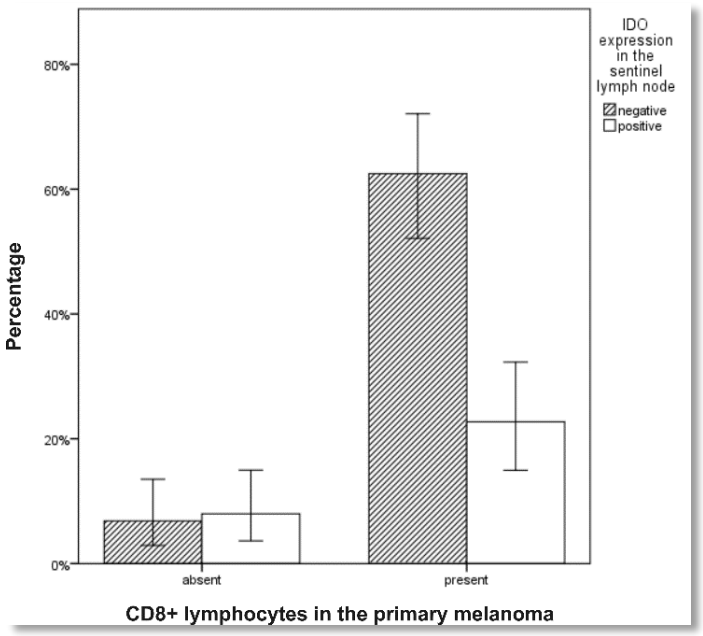

Figure 5: The frequency of IDO expression in the sentinel is shown, according to the presence or absence of CD8+ lymphocytes in the primary melanoma. Error bars indicate 95% confidence intervals.

❖ IDO expression by circulating lymphocytes tends to correlate with disease stage

Upon stimulation with either IFN-γ or CTLA-4, PBMCs (n=23) expressed variable levels of IDO. The mean fluorescence intensity (MFI) for IDO expression was higher after CTLA-4 stimulation. The MFI for IDO after CTLA-4 stimulation tended to be higher in increased disease stage (P=0.044) and in patients with in-transit metastases (P=0.012) (Fig. 6). There was also a tendency to a higher MFI for IDO in patients progressing after diagnosis (P=0.051). IDO expression after stimulation with IFN-γ did not correlate with clinical variables. IDO expression in PBMCs and IDO expression of the SLN or the PM did not correlate.

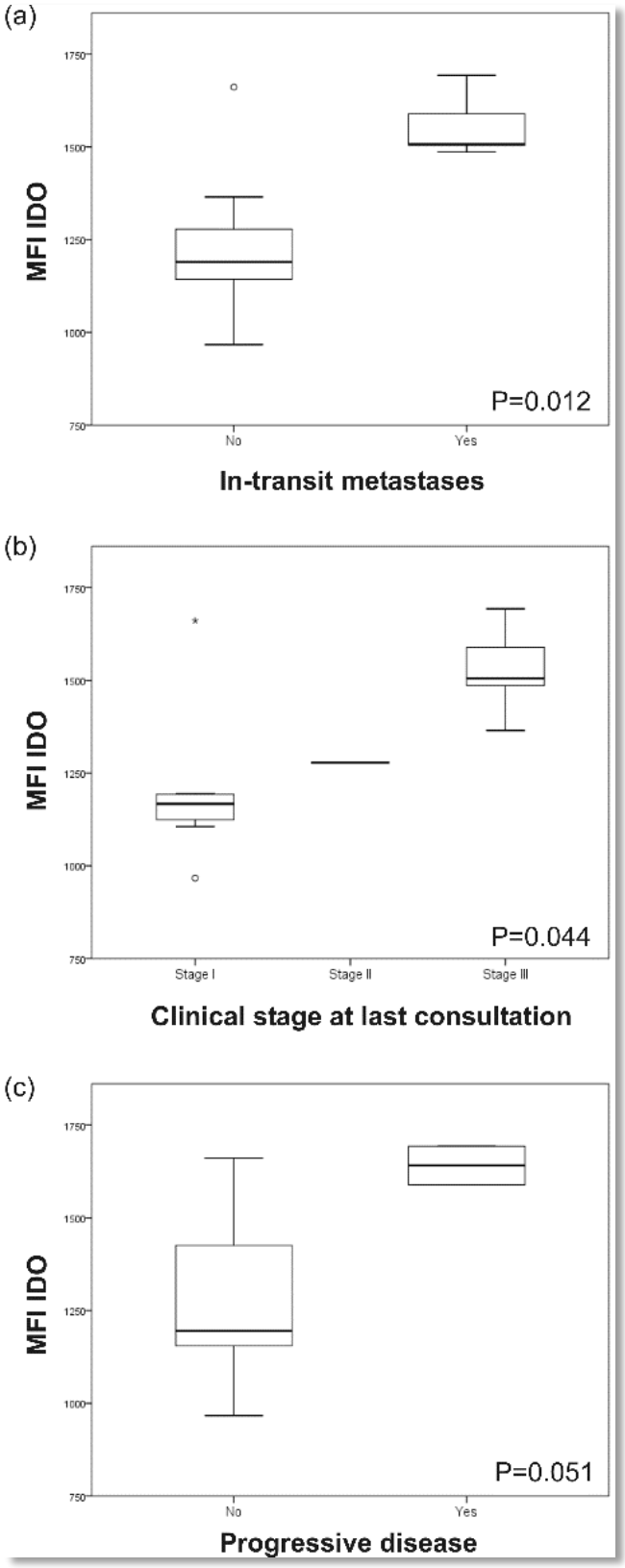

Figure 6: Box-and-whisker plots showing the mean fluorescence intensity (MFI) for IDO in peripheral blood mononuclear cells of melanoma patients after stimulation with CTLA-4 according to:

(A) the presence of in-transit metastases, (B) clinical stage at last consultation and (C) progression after the first melanoma diagnosis.

4. Discussion

Melanoma is known to be a highly immunogenic tumor. In the primary melanoma, this is illustrated by the positive prognostic value of both TILs and CD8+ lymphocytes.[20, 22, 23] However, the initial anti-tumoral immune response is often skewed towards immunosuppression, causing T-cell anergy and an accumulation of Tregs. High expression of FoxP3 in primary melanomas has been associated with a worse prognosis.[24] The immunosuppressive enzyme IDO plays a central role in this process, as it can potently suppress antitumoral immune responses when it is expressed by host immune cells.[6, 16, 17] The functional consequences of IDO expression by dendritic cells include both the inhibition of effector T-cell proliferation and the induction of differentiation of FoxP3+ regulatory T-cells in vitro.[16, 17, 25] The role of IDO as a mechanism of immune escape in cancer has been explored over the last years, with increasing reports on its prognostic properties and even as a possible target for future therapies.[6, 7, 19, 26, 27] Mainly IDO expression by host cells seems to be crucial for the induction of systemic tumor tolerance, a topic that was recently thoroughly reviewed by Johnson and Munn.[6] To date, early markers to pre-identify patients with a failing anti-tumor immune response are lacking. In this study, we investigated the pattern of IDO expression in different stages of melanoma, and its impact on prognosis.

We report a strong and independent correlation between peri-tumoral IDO expression in the primary tumor and the corresponding sentinel lymph node. IDO expression even seems to persists in metastatic tissues that occurred with a median delay of 41.5 months after the first melanoma surgery. Both IDO expression by HEVs in the sentinel and by peri-tumoral endothelial cells in the primary melanoma had an independent negative prognostic effect on overall survival. This effect is independent of the vicinity of tumor cells, as it was equally strong in patients with tumor-negative and tumor-invaded SLNs. Moreover, in our patient population IDO expression by melanoma cells failed to show an effect on progression-free or overall survival. Hence, we hypothesize that IDO expression by the malignant melanoma cells and IDO expression by the host endothelial cells can have different biologic effects. Bearing in mind that IDO modulates complex immune responses also in non-tumor bearing hosts, it is expected that IDO expression produces different effects depending on timing and site. Our data suggest that endothelial IDO expression could be a valuable marker in melanoma, as its expression is not only prognostic but also consistent throughout different tumor stages. The prognostic effect of IDO is most likely immune-mediated, as there is a correlation with local and systemic regulatory T-cells and an inverse correlation with a cytotoxic TIL response in the primary tumor.[7] These observations could also explain the sometimes conflicting results on the prognostic effect of IDO published in literature.[8-15]

Indoleamine 2,3-dioxygenase activity has been most extensively studied in dendritic cells, but there is also evidence that IDO expression by endothelial cells can play an important role in immune regulation. The importance of endothelial IDO has been demonstrated by experiments in mice, where IDO-expression by endothelial cells could prevent the rejection of cardiac allografts.[3, 4] Moreover, IDO expression in monocyte-derived endothelial-like cells has also been described in infantile haemangioma.[28] In renal cell cancer, IDO expression in tumor-associated endothelial cells was found to have a positive prognostic effect, but this concerned mainly intra- and not peri-tumoral vessels and the prognostic results were based on qRT-PCR, and not on immunohistochemistry.[14] A recent clinical study also demonstrated that IDO expression, partly by endothelial cells, could be a predictor for response in melanoma patients treated with anti-CTLA-4 therapy.[19] Based on all the above results, endothelial IDO could be used to pre-identify stage I to III melanoma patients with more aggressive disease, caused by a skewed immune response. The clinical relevance of IDO expression in melanoma is further supported by the observed increase in IDO expression by PBMCs in patients with higher disease stage, after stimulation with CTLA-4.

Both TILs and CD8+ lymphocytes in the primary melanoma are established prognostic markers.[20, 22] In patients with a CD8+ infiltrate in their primary melanoma, we detected a decreased IDO-positivity in the sentinel lymph node. This suggests that an initial cytotoxic response in the primary tumor can prevent future immune suppression. These results are in line with previous studies that have associated IDO expression with increased FoxP3 expression and decreased (CD8+) TILs in other tumor types.[8, 9, 12, 29] Different results have been described in melanoma metastases, where the combined expression of markers for active immune response (CD8) and multiple immunosuppressive mechanisms (IDO, FoxP3 and PD-L1) has been reported. These immunosuppressive mechanisms act as a negative feedback mechanism to T-cell infiltration and hence immune activation.[30, 31] As IDO is known to be induced by IFN-γ and IDO-negative tumor cells have been reported to start expressing IDO when exposed to an inflammatory immune environment, the combined expression of CD8, FoxP3, IDO and PD-L1 in a metastatic tumor is not surprising.[32]

In the current era of immunotherapy for melanoma, the immune micro-environment has been of increasing interest to researchers looking for markers to predict or interfere with the susceptibility of patients for different immunotherapeutic strategies.[18, 30, 33] The response to immunotherapy might be increased by reversing mechanisms of immune resistance. In a mouse melanoma model, the addition of an IDO inhibitor to anti-CTLA-4 therapy significantly increased responses when compared to anti-CTLA-4 therapy alone.[27] Our data suggest that susceptibility to immunotherapy might be determined right from the first diagnosis of melanoma and long before metastatic disease occurs. This could open the possibility to use the patient's primary tumor or sentinel lymph node to evaluate his immunological

susceptibility. Predictive immunoprofiling may not only provide therapeutic strategies to stimulate the immune response, but possibly even to have a preventive impact on the development of metastatic disease. The latter is supported by experiments where IDO inhibition by means of skin administration of IDO short hairpinRNA not only delayed tumor growth in a subcutaneous hepatoma model in mice, but also suppressed tumor growth in distant metastases.[34]

In conclusion, in contrast to tumoral IDO expression, peri-tumoral IDO expression in melanoma is not only prognostic, but also consistent over time in different disease stages of the patient and related to the immune response at the primary tumor. Further studies are needed to establish if IDO can be part of prognostic and predictive immunoprofiling efforts in melanoma clinical practice.

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CLINICAL SIGNIFICANCE OF PLASMACYTOID DENDRITIC CELLS AND MYELOID-DERIVED SUPPRESSOR CELLS IN MELANOMA

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Abstract

Background: Immune markers in the peripheral blood of melanoma patients could provide prognostic information. However, there is currently no consensus on which circulating cell types have more clinical impact. We therefore evaluated myeloid-derived suppressor cells (MDSC), dendritic cells (DC), cytotoxic T-cells and regulatory T-cells (Treg) in a series of blood samples of melanoma patients in different stages of disease.

Methods: Flow cytometry was performed on peripheral blood mononuclear cells of 69 stage I to IV melanoma patients with a median follow-up of 39 months after diagnosis to measure the percentage of monocytic MDSCs (mMDSCs), polymorphonuclear MDSCs (pmnMDSCs), myeloid DCs (mDCs), plasmacytoid DCs (pDCs), cytotoxic T-cells and Tregs. We also assessed the expression of PD-L1 and CTLA-4 in cytotoxic T-cells and Tregs respectively. The impact of cell frequencies on prognosis was tested with multivariate Cox regression modelling.

Results: Circulating pDC levels were decreased in patients with advanced (P=0.001) or active (P=0.002) disease. Low pDC levels conferred an independent negative impact on overall (P=0.025) and progression-free survival (P=0.036). Even before relapse, a decrease in pDC levels was observed (P=0.002, correlation coefficient 0.898). High levels of circulating MDSCs (>4.13%) have an independent negative prognostic impact on OS (P=0.012). High MDSC levels were associated with decreased CD3+ (P<0.001) and CD3+CD8+ (P=0.017) T-cell levels. Conversely, patients with high MDSC levels had more PD-L1+ T-cells (P=0.033) and more CTLA-4 expression by Tregs (P=0.003). pDCs and MDSCs were inversely correlated (P=0.004). The impact of pDC levels on prognosis and prediction of the presence of systemic disease was stronger than that of MDSC levels.

Conclusion: We demonstrated that circulating pDC and MDSC levels are inversely correlated but have an independent prognostic value in melanoma patients. These cell types represent a single immunologic system and should be evaluated together. Both are key players in the immunological climate in melanoma patients, as they are correlated with cytotoxic and regulatory T-cells. Circulating pDC and MDSC levels should be considered in future immunoprofiling efforts as they could impact disease management.

1. Introduction

Melanoma is a highly immunogenic tumour that is capable of successfully evading the patients' immune response. Evidence for an anti-tumoral immune reaction, as well as concomitant immunosuppressive mechanisms, can already be observed in the primary tumour and in tumour-free sentinel lymph nodes.[1, 2] The possibility to integrate immune markers in the existing TNM-classification is currently being investigated in melanoma. The primary objective is to increase prognostic accuracy but it could also become a strategy to pre-select patients for adjuvant therapies.[3] Immunoprofiling initiatives such as the "Immunoscore" focus on markers in the primary and metastatic tumour site, mainly assessed by immunohistochemistry [4]. However, options to evaluate the immune status in a tumour-free patient during clinical follow-up are currently lacking. In this context, circulating biomarkers could be a practical approach.

In melanoma, it is currently unclear which circulating immune cell types confer the most powerful prognostic information. Besides T-cells, myeloid-derived suppressor cells (MDSCs) and dendritic cells (DCs) are the most elaborately studied circulating cell types, but research is often focused on the tumour microenvironment.

MDSCs are HLA-DR- lineage- CD33+ CD11b+ cells that do not constitute a defined subset of cells but rather a group of phenotypically heterogeneous myeloid cells that have a common biological activity.[5] Two clinically relevant subsets have been defined, monocytic (CD14+) and polymorphonuclear (CD14-CD15+) MDSCs (resp. mMDSCs and pmnMDSCs). An expanding body of evidence shows increased levels of MDSCs in almost all cancer types, correlating with advanced clinical cancer stage and a worse prognosis.[6, 7] Myeloid differentiation is often disturbed in cancer patients, leading to an accumulation of immunosuppressive immature myeloid cells such as MDSCs and reduced frequencies of mature, immunostimulatory dendritic cells (DCs).[8, 9] Tumour-derived factors are thought to inhibit the natural differentiation of immature myeloid cells, resulting in the accumulation of MDSCs.[5] This concomitant increase in MDSCs and decrease in mature DCs in the peripheral blood has been described in several cancer types.[10] High MDSC frequencies in the peripheral blood of melanoma patients have also been reported to have a negative impact on prognosis, but their relation to dendritic cells or lymphocytes is not well documented.

DCs are potent antigen-presenting cells that play a central role in developing anti-tumour immune responses. Two subsets of DCs have been defined in the blood and in lymphoid tissues, myeloid (CD11c+) and plasmacytoid (CD123+ CD11c-) DCs (resp. mDCs and pDCs) [9, 11]. Additional surface markers for blood DCs exist; BDCA-1 and -3 define two distinct subsets of mDCs and that BDCA-2 and

BDCA-4 are present on pDCs. Many other surface markers further characterize these cells, as recently reviewed elsewhere [11, 12]. The differentiation capacity of DCs is diminished in many cancers, resulting in lower frequencies of circulating mature DCs in patients with higher tumour stages or active disease [9, 13, 14]. In melanoma, circulating DC frequencies have been reported to be unchanged in stage I-III patients [13, 14], and reduced in stage IV [14-16]. Similar patterns in DC alterations have been described in breast, liver, head and neck and lung cancer [10, 17-19]. However, data on circulating DCs frequencies in untreated melanoma patients are limited and their prognostic relevance is unknown. The in vivo clinical relevance of these circulating subsets in melanoma therefore remains subject to debate [8].

Despite evidence that lymphoid cell types such as cytotoxic and regulatory T-cells are important in melanoma and are modulated by current immunotherapeutic strategies,[20] many recent studies on circulating cell types in melanoma have focused on MDSCs alone and have left their relation to lymphoid cell types largely unexplored. The presence of regulatory T-cells (Tregs) in the melanoma tumour microenvironment confers a negative prognosis.[21] However, the prognostic relevance of circulating Tregs in untreated melanoma patients is unclear. Cytotoxic T-cells are powerful allies in the anti-tumoral immune response and their presence in the melanoma micro-environment is protective,[1] but data on circulating cell frequencies in untreated patients are scarce.

Even in stage I and II melanoma a shift in systemic immune activity can be present and these systemic immune alterations have been reported to increase as patients develop metastatic disease. Many different cell types have separately been described in this context, but there is no consensus on which alterations have the predominant immunosuppressive effect. We therefore performed a comparative evaluation of the presence of different circulating immune subsets in untreated melanoma patients in different stages of disease, with a focus on DCs and MDSCs. The clinical relevance of circulating DC and MDSC subsets and their relation with regulatory and cytotoxic T-cells was assessed.

2. Methods

❖ Patients

Sixty-nine patients with melanoma were included in this study, with a median follow-up time of 39 months after diagnosis and of 15 months after inclusion (inclusion was defined as the time of sample procurement). Venous blood samples were drawn during clinical follow-up, with a median interval of 21 months after diagnosis. Disease staging was done according to the 2009 American Joint Committee on Cancer system (AJCC). Local disease was defined as AJCC stage I and II, regional disease as AJCC stage III and systemic disease as AJCC stage IV. The local medical ethical committee approved this study; all included patients gave written informed consent. Detailed patient characteristics can be found in Table 1.

Table 1: Patient characteristics

Number of patients, n	69
Follow-up time since inclusion, months (median - IQR)	15 (6 - 35)
Follow-up time since diagnosis, months (median - IQR)	39 (20.5-108)
Age at diagnosis, years (median - IQR)	53 (41.5 - 60)
Female sex, % (n)	53.6 (37/69)
Stage at inclusion, % (n)	
Local (AJCC stage I & II)	46.4 (32/69)
Regional (AJCC stage III)	37.7 (26/69)
Systemic (AJCC stage IV)	15.9 (11/69)
Active disease at inclusion	20.3 (14/69) (3 stage III, 11 stage IV)
Melanoma characteristics	
Breslow (median, IQR)	1.60 (1.08 - 2.60)
Ulceration, % (n)	35.7 (20/56)
Sentinel invasion, % (n)	27.1 (16/59)
Location of primary melanoma	
Head & neck, % (n)	10.6 (7/69)
Trunk, % (n)	39.4 (26/69)
Extremities, % (n)	50 (33/69)
Unknown primary	3 (3/69)

IQR, interquartile range; AJCC, American Joint Committee on Cancer

❖ PBMC isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by centrifugation on a Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden) within 4h of venepuncture. The PBMCs were cryopreserved in liquid nitrogen in heat-inactivated foetal bovine serum (FBS) supplemented with 10% dimethyl sulphoxide (DMSO) until analysis. Cells were thawed by submersion at 37° for 1-2 minutes and resuspended in a medium containing Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS and 1% glutamine.

❖ Flow cytometry

MDSCs were characterized by the HLA-DR- lineage- (CD3, CD19, CD56) CD33+ CD11b+ phenotype, mMDSCs are CD14+, pmnMDSCs are CD14-. Dendritic cells were characterized by the HLA-DR+ lineage- (CD3, CD14, CD16, CD19, CD20, and CD56) phenotype, pDCs are CD123+ CD11c- and mDCs are CD123- CD11c+. Tregs were defined as CD3+ CD4+ CD25+ FoxP3+ and cytotoxic T-cells as CD3+ CD8+ cells. All antibodies used in this study were fluorescently conjugated mouse anti-human monoclonal antibodies. The following antibodies were purchased from BD Biosciences; CD3 BV421 (563797), CD4 APC-Cy7 (561839), CD25 FITC (560990), CD33 BV421 (562854), CD11b APC-Cy7 (560914), CD123 BV421 (562517). The following antibodies were purchased from eBioscience; B7-H1 (PD-L1) PE-Cy7 (25-5983-42), CD8 APC (9017-0087-025), CD3 FITC (11-0038-41), CD19 FITC (11-0199-41), CD56 FITC (11-0569-41), CD14 APC (17-0149-41), CD11c APC (17-0116-41), HLA-DR PerCP-Cy5.5 (45-9956-42). For intracellular stainings, after surface staining PBMCs were fixed and permeabilized using Live/dead® fixable aqua dead cell stain (BD Biosciences), and then stained with antihuman CTLA-4 APC (BD Biosciences, 560938) and FoxP3 PerCP-Cy5.5 (eBioscience, 45-4776-42) antibodies. Live/dead staining was performed using an aqua Dead Cell Stain kit (Life Technologies Europe, Ghent, Belgium). Patients with less than 75% living cells were excluded (n=4). Cells were analyzed on a FACS Canto™ II flow cytometer (BD Bioscience, Erembodegem, Belgium) using FlowJo software (Tree Star Inc, Ashland, OR, USA). For setting the gates, isotype and fluorescence-minus-one (FMO) controls were used. To provide a representative sample a median amount of 500000 cells were analysed (min 261000 – max 569750). Absolute cell counts were corrected for the number of acquired events during flow cytometry. Samples with less than 100000 cells were excluded (n=1).

❖ Statistical analysis

Median values between 2 groups were compared by the Mann-Whitney U-test, between >2 groups with Kruskall-Wallis testing. To compare proportions of categorical variables, the Pearson's Chi² test or Fisher's Exact test were used. To evaluate correlations, Spearman correlation coefficients (CC) were calculated. To assess the prognostic relevance of continuous variables, ROC curve analysis was used to dichotomize them with the aid of the online tool "cut-off finder".[22] All statistical analyses were performed using SPSS 21.0 (SPSS Inc, Chicago, IL, USA), a P-value (double-sided) less than 0.05 was considered statistically significant.

3. Results

Flow cytometry was performed to quantify MDSC and DC subsets, Tregs and cytotoxic T-cells in PBMCs from melanoma patients (Table 1). Table 2 summarizes the mean detected cell frequencies for all cell types, and the cut-off points for the dichotomized cell frequencies that were used in Cox regression models. As a first step, all immune subsets were compared for their relevance with regard to clinical variables such as disease stage and outcome. This showed us that both pDCs and MDSCs are associated with disease stage and have an impact on prognosis. Therefore further analyses were focused on these cell types, as outlined below.

Table 2: Frequencies of circulating immune subsets in melanoma patients

Cell type	Cell frequency (mean % - SD)*	Dichotomization cut-off (%)*	Patients with "high" cell levels (%)
Dendritic cells (DC)	1.54 (0.54)		
Plasmacytoid DCs	0.34 (0.18)	0.2515	66.7
Myeloid DCs	0.96 (0.40)		
Myeloid-derived suppressor cells (MDSC)	4.00 (2.29)	4.13	33.3
Monocytic MDSC	2.94 (2.81)		
Polymorphonuclear MDSC	1.04 (0.64)		
CD3+ cells	43.60 (9.32)	40.25	68.2
Cytotoxic T-cell	14.79 (6.06)		
Regulatory T-cell	4.77 (1.35)		
CTLA-4 expression by Tregs	92.32 (4.01)	93.7	37.3

DC, dendritic cell; MDSC, myeloid-derived suppressor cell; Treg, regulatory T-cell; SD, standard deviation. "*" Percentage of live cells, except for Tregs (percentage of CD4+ cells).

❖ Myeloid-derived suppressor cells (MDSCs)

Melanoma patients with systemic disease have significantly higher frequencies of circulating MDSCs (P=0.046). There was a trend towards higher MDSC frequencies in patients with active disease at time of inclusion, but this did not reach significance (Fig 1).

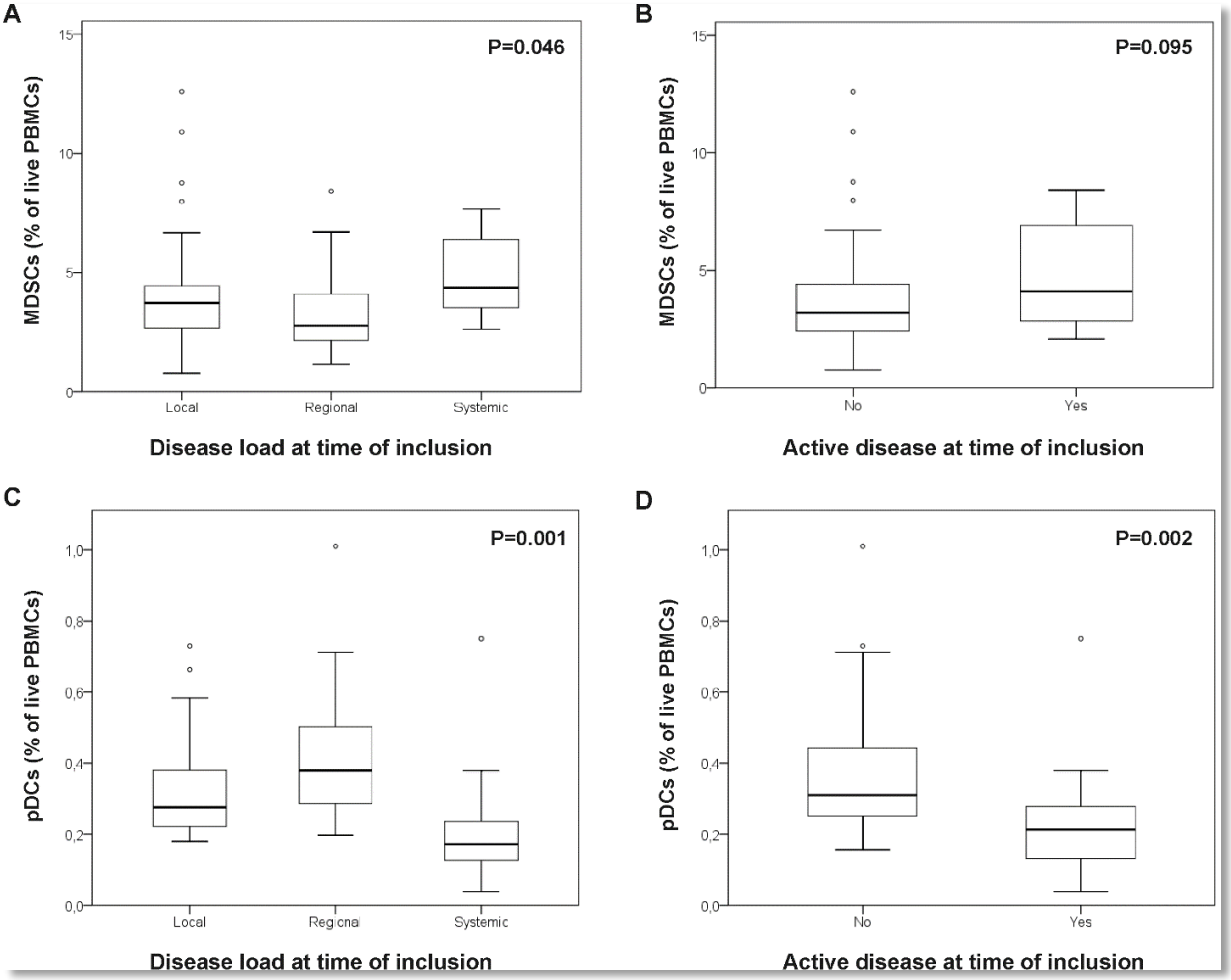

Figure 1: pDC and MDSC frequencies according to melanoma stage and activity.
Box-and-whisker plots showing variations in the levels of circulating myeloid-derived suppressor cells (MDSCs; A and B) and plasmacytoid dendritic cells (pDCs; C and D) according to the presence of systemic or active disease at the time of inclusion. Circulating cell frequencies are expressed as a percentage of live peripheral blood mononuclear cells (PBMCs).

High levels of circulating MDSCs (> 4.13%) conferred a negative impact on OS (Log Rank test, P=0.002). This effect was independent of disease stage (P=0.012, HR 4.77, CI 1.42-16.04). There was no impact of MDSC frequency on progression-free survival (PFS). Patients who died of melanoma after inclusion showed a trend towards more circulating MDSCs at the time of inclusion (P=0.073).

❖ Link between MDSCs and other immunosuppressive markers

To further evaluate how MDSCs are related to systemic immunity, their relation to cytotoxic Tcells and Tregs was also assessed (Table 3).

Table 3: Association of myeloid-derived suppressor cells (MDSCs) and plasmacytoid dendritic cells (pDCs) with melanoma disease course and circulating immune markers.

	MDSCs	pDCs
Melanoma activity and prognosis		
Systemic disease	Increased (P=0.046)	Decreased (P=0.001)
Active disease	Increased (P=0.095)	Decreased (P=0.002)
Death	Increased (P=0.073)	Decreased (P=0.009)
Other immune markers		
CD3 cells	Inverse (P<0.001)	None
CD8 cells	Inverse (P=0.017)	None
PD-L1+ CD8 cells	Positive (P=0.033)	Inverse (P=0.044)
Regulatory T-cells	Inverse (P=0.007)	None
CTLA-4 expression in Tregs	Positive (P=0.003)	None

PD-L1, Programmed-Death Ligand 1; CTLA-4, Cytotoxic T Lymphocyte-Associated Antigen 4; Treg, regulatory T-cell.

The frequency of CD3+ cells was inversely correlated with MDSCs (P<0.001, CC -0.519), mMDSCs (P<0.001, CC -0.454) and pmnMDSCs (P=0.004, CC -0.349). The cytotoxic T-cell frequency was also inversely correlated with MDSCs (P=0.017, CC -0.294). On the other hand, there was a positive correlation between PD-L1+ cytotoxic Tcells and MDSCs (P=0.033, CC 0.263), mainly observed in pmnMDSCs (P=0.008, CC 0.323). These data suggest that high MDSC frequencies are associated with relative lymphopenia and with an immune climate that is unfavourable for cytotoxic T-cells.

The percentage of MDSCs was inversely correlated with the percentage of Tregs (P=0.007, CC -0.327). However, the proportion of highly CTLA-4-positive Tregs was positively correlated with the percentage of MDSCs (P=0.002, CC 0.365) and mMDSCs (P=0.008, CC 0.321). This was also the case for the mean fluorescence intensity (MFI) of CTLA-4 and MDSCs (P=0.029, CC 0.266). These data suggest that high MDSC frequencies are associated with a higher suppressive capacity of circulating Tregs, but not with higher Treg frequencies.

Patients who died of melanoma during follow-up had a trend towards more circulating highly CTLA-4+ Tregs (P=0.081). A high percentage of CTLA-4 positivity in circulating Tregs (>93.7%) conferred a negative prognosis (Log Rank test, P=0.003), independent of disease stage (P=0.040, HR 3.80, CI 1.06-13.70).

❖ Plasmacytoid dendritic cells (pDCs)

Melanoma patients with systemic disease have significantly lower frequencies of circulating pDCs (P=0.001). This decrease in pDCs was also seen in patients with active disease at the time of inclusion (P=0.002) (Fig1). In patients who were disease-free at the time of inclusion but who presented with disease relapse or progression in the months after inclusion, a decline in pDCs frequency could already be seen. The shorter the time frame between inclusion and relapse, the lower the observed frequency of pDCs (P=0.002, CC 0.898).

A low amount of circulating pDCs (<0.2515%) had a negative prognostic impact on OS, independent of disease stage (Fig 2; P=0.025, HR 4.17, CI 1.20-14.52). In patients who were disease-free at the time of inclusion, low frequencies of circulating pDCs were also associated with a shorter PFS, independent of disease stage (Fig 2; P=0.036, HR 10.29, CI 1.162-90.90).

❖ Link between pDCs and other immunosuppressive markers

To further evaluate the impact of pDCs on systemic immunity, their relation to Tregs and cytotoxic Tcells was also assessed by flow cytometry. An inverse correlation between pDC frequency and the percentage of PD-L1+ cytotoxic T-cells was found (P=0.044, CC -0.249). No correlation with Treg frequency or CTLA-4 expression was detected.

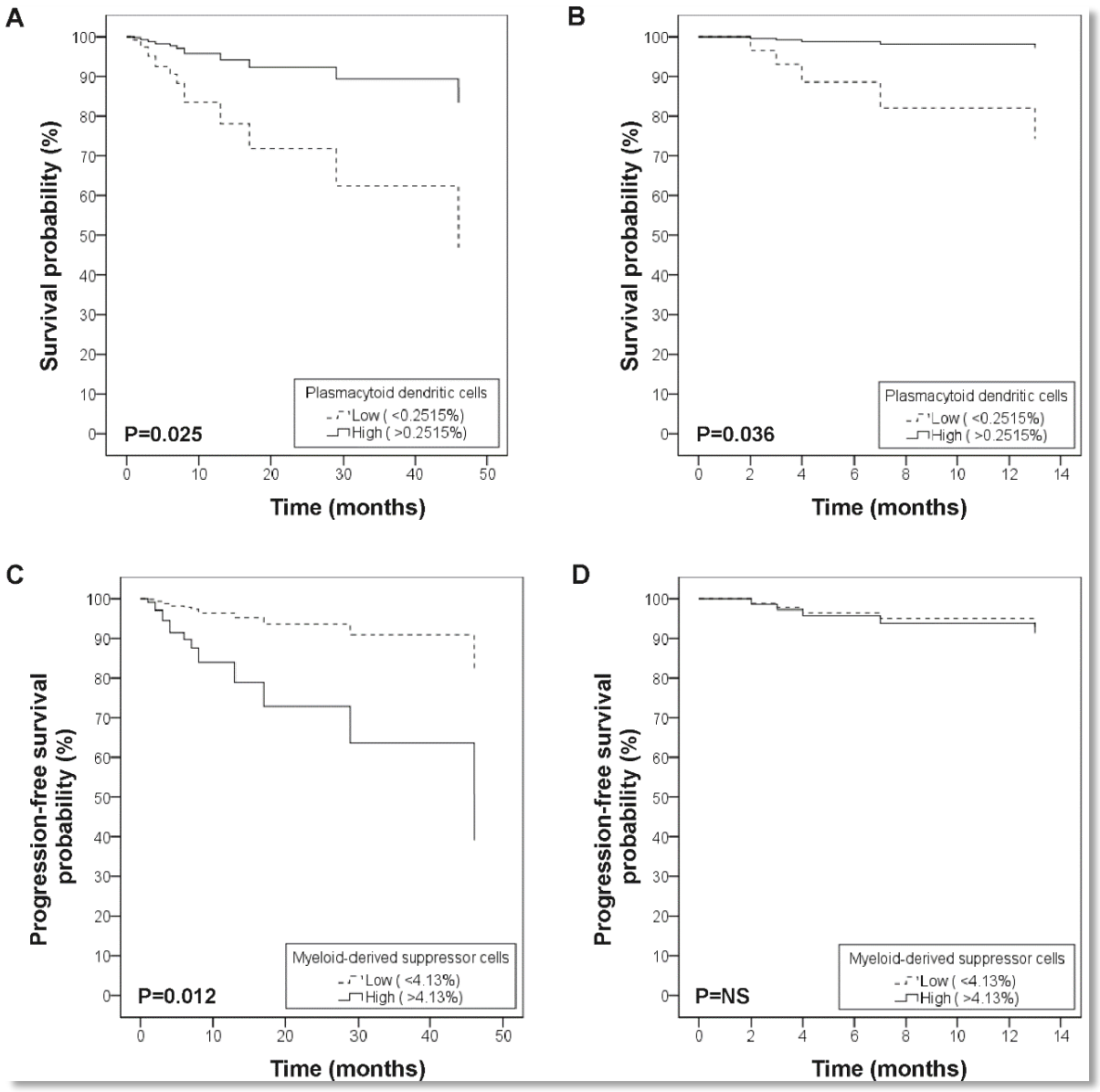

Figure 2: Impact of pDC and MDSC frequency on overall and progression-free survival.
Cox regression analysis of overall (A and C) and progression-free (B and D) survival according to the levels of circulating plasmaytoid dendritic cells (A and B) or myeloid-derived suppressor cells (C and D), after adjustment for disease stage. Percentages should be interpreted as percentages of live peripheral blood mononuclear cells (PBMCs).

❖ Relative importance of different immune subsets

There was no inverse relation between the frequency of DCs and MDSCs in general. However, when evaluating the relationship between MDSC and DC subtypes, a significant inverse correlation was found between pDCs and MDSCs (P=0.004, CC -0.338). This was due to an increase in the absolute number of mMDSCs (P=0.010, CC -0.307), not pmnMDSCs. The observed changes in pDC and MDSC frequencies in patients with systemic disease were also reflected by an alteration of the MDSC/DC ratio (P=0.048). These data suggest that low pDC frequencies are associated with a defective maturation of myeloid cells, and an accumulation of MDSCs.

To assess the relative importance of pDCs and MDSCs with regard to prognosis (impact on OS), a combined Cox regression model was made (Table 4, upper part). MDSC and pDC frequencies had a similar impact on OS, independent of each other and of disease stage. When CTLA-4+ Treg levels or CD3+ T-cell frequencies were separately added to this model, both lost their effect on prognosis, whereas pDCs did not (P=0.008 for adding CTLA-4, P=0.019 for adding CD3). These results indicate that circulating myeloid cells (principally pDCs) have a superior impact on prognosis (OS) compared to T-cells.

Table 4: Combined Cox (upper part) and logistic (lower part) regression models comparing circulating myeloid-derived suppressor cells and plasmacytoid dendritic cells in melanoma patients

Combined Cox regression model: pDC vs MDSC & overall survival					
	Coefficient	P-value	HR	95% Confidence Interval for HR	
				Lower	Upper
Stage at inclusion	1.87	<0.001	6.46	2.39	17.46
MDSC frequency	-1.61	0.009	4.98	1.50	16.67
pDC frequency	1.49	0.019	4.42	1.27	15.33
Logistic regression model: pDC and MDSC & systemic disease					
	Coefficient	P-Value	OR	95% Confidence Interval for OR	
				Lower	Upper
Stage at diagnosis	-0.702	0.407	0.496	0.095	2.601
MDSC frequency	-0.248	0.753	0.78	0.167	3.655
pDC frequency	1.741	0.032	5.705	1.159	28.091

pDC, plasmacytoid dendritic cell; MDSC, myeloid-derived suppressor cell; HR, hazard ratio; OR, odds ratio

As pDCs and MDSCs emerge as the most prognostically relevant cell types, we next assessed their relative importance to each other in predicting the presence of systemic disease. As indicated in Table 4 (lower part), a logistic regression model was made. A low pDC frequency at the moment of inclusion could predict the presence of systemic disease, independent of the patients' stage at the time of inclusion and independent of MDSC frequency. MDSC frequency itself had no significant predictive quality.

4. Discussion

Various systemic immune alterations have been described in melanoma patients, but a direct comparison of their clinical relevance is often lacking. In this study, we demonstrated that an increased amount of circulating MDSCs and a decreased number of pDCs were associated with systemic disease and had both an independent negative prognostic value, although they were inversely correlated. Both MDSCs and pDCs were linked with T-cell anergy. Nonetheless, pDCs and MDSCs were the most prognostically relevant cell types.

In this patient cohort, elevated MDSC frequencies were seen in stage IV melanoma patients, which is in accordance with previous publications.[23-25] The accumulation of MDSCs in melanoma is most prominent in stage IV patients, but has been reported as early as stage I.[26] Most authors agree that MDSCs are upregulated in melanoma patients compared to healthy controls.[23, 25, 26] Whether pmnMDSCs or mMDSCs dominate depends on the cancer type. Based on literature and the observations made in this patient set, mMDSCs appear to have a higher impact in melanoma.[27, 28] A negative impact of high CD14- and CD14+ MDSCs on OS and PFS was previously described in stage IV melanoma patients.[25, 29] Significant increases in circulating MDSC frequencies have also been detected in the blood of patients with a.o. glioblastoma, breast, colon, lung and kidney cancer.[5, 30]

We observed significantly lower circulating pDC frequencies in stage IV melanoma patients. The changes in total DC frequencies in advanced melanoma have been attributed to pDCs rather than mDCs.[14, 15] Accordingly, we did not observe significant changes in mDC frequencies, contrary to reduced pDC levels in stage IV patients. Remarkably, we observed that a decline in pDC levels had a negative prognostic effect on both OS and PFS, independent of disease stage or other cell frequencies. Previous studies have reported a negative prognostic effect of the presence of pDCs within the tumour site,[31, 32] but to our knowledge this is the first study to report an independent negative prognostic effect of low circulating pDC frequencies in untreated melanoma patients. Moreover, low pDC frequencies were not only associated with a decreased PFS, but were also already reduced up to a year before

relapse was clinically diagnosed, suggesting that low pDC frequencies could have a predictive value in disease-free patients. A possible explanation is that pDC frequencies are regulated by the tumour itself and that this regulation takes place very early in the metastatic process. Alternatively, the observed low pDC frequencies could also be an indicator of a failing anti-tumoral immune response, making the patient more susceptible to relapse. The relevance of pDC frequencies in detecting early disease progression should be further investigated in prospective trials with longitudinal follow-up samples.

Our data demonstrate a disturbed myeloid differentiation in melanoma, resulting in an accumulation of MDSCs and a decline in pDCs. To our knowledge, there are no studies directly reporting this effect in melanoma patients yet. The independent prognostic effect of both pDCs and MDSCs suggests that even a partial dysfunction of myeloid differentiation is sufficient to negatively influence melanoma disease course. In our dataset, the effect of pDCs on OS and PFS outweighs that of MDSCs (table 4). An alternative interpretation could be that MDSC frequency is related to the actual tumour load, whereas adequate pDCs levels could also have a protective effect on disease progression. Regardless of which myeloid cell type dominates over the other during the course of the disease, it is clear that the myeloid lineage is globally altered in melanoma as a single system involving both differentiated myeloid cells and their pathologically activated immature progenitors.[30] Additionally, our combined Cox regression model showed that the prognostic importance of circulating myeloid cells dominates over circulating cytotoxic T-cells and to a lesser extent regulatory T-cells, as the latter two lose their impact on prognosis when adjusting for pDC or MDSC frequencies.

Over the last few years, immunoprofiling efforts are increasing as immunotherapeutic strategies are gaining importance in the management of melanoma and other cancers. Several (combinations of) markers with predictive or prognostic quality have been suggested, mainly focusing on the tumour microenvironment.[4, 33] Based on our data, we conclude that circulating pDCs and MDSCs should be considered in future prognostic profiling studies. One limitation of our study in this respect is the use of cryopreserved PBMC samples, which impedes a direct comparison with studies using fresh whole blood samples. MDSCs have already been investigated as possible predictive biomarkers for immunotherapy in melanoma and other malignancies. Low mMDSC levels were associated with clinical response and improved OS in melanoma patients treated with ipilimumab.[34, 35] Moreover, mMDSC levels have been reported to be inversely correlated with the presence of tumour-specific T-cells and with a CD8+ T-cell rise on ipilimumab therapy.[29, 36] The inverse correlation between mMDSC and (antigen-specific) cytotoxic T-cell levels that has been observed in independent studies including ours,[37] also raises the question whether mMDSC-related immune suppression could be limiting the therapeutic benefit of ipilimumab. Alternatively, high mMDSC levels could also be a marker for

deficient myeloid cell maturation leading to a pDC deficit that hampers efficient immune activation after ipilimumab therapy.

Recently, Schilling and colleagues reported that mMDSC frequencies decline in patients who have a response to vemurafenib and rise again when progressive disease occurs. The inhibitory effect of vemurafenib on mMDSCs was present in vitro as well as in vivo.[38] Finkelstein and colleagues reported that high DC/MDSC ratios and low pretreatment MDSC levels could predict response to high-dose IL-2 therapy in patients with melanoma and renal cell carcinoma.[39] In our patient cohort, pDC frequencies had the highest impact on prognosis. Furthermore a gradual decline in pDCs levels seemed to occur before relapse, but as this was a cross-sectional study these data need to be confirmed in a longitudinal study with multiple follow-up samples during disease course. However, our data do suggest that pDCs might also be valuable candidates in predictive immune profiles. The prominent prognostic role of circulating pDCs also warrants further research into possible therapeutic strategies, for example with Toll-like receptor stimulating drugs which have been shown to enhance pDC activation in the skin [40].

5. Conclusion

Plasmacytoid dendritic cells (pDC) and myeloid-derived suppressor cells (MDSC) in the peripheral blood should be regarded as one system and thus be evaluated together. Both are key players in the immunological climate in melanoma patients, as they are correlated with circulating cytotoxic and regulatory T-cells. The independent prognostic impact of pDCs and MDSCs makes these cell types attractive for future prognostic and possibly even predictive immunoprofiling efforts.

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CHARACTERIZATION OF THE IN VIVO IMMUNE NETWORK OF IDO, TRYPTOPHAN METABOLISM, PD-L1 AND CTLA-4 IN CIRCULATING IMMUNE CELLS IN MELANOMA

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Abstract

In melanoma, both the induction of immunosuppression by tumour cells and the inflammatory anti-tumour response can induce an upregulation of counterregulatory mechanisms such as indoleamine 2,3-dioxygenase (IDO), programmed death-ligand 1 (PD-L1) and CTLA-4+ regulatory T-cells (Tregs) in the tumour microenvironment. Even though these immunosuppressive mediators are targets for immunotherapy, research investigating their expression in the peripheral blood is lacking. We therefore performed flow cytometry on PBMCs of stage I-IV melanoma patients. IDO expression was detected in plasmacytoid dendritic cells (pDC) and monocytic myeloid-derived suppressor cells (mMDSC), and increased in advanced disease stage (P=0.027). Tryptophan breakdown confirmed the functional activity of IDO and was linked with increased PD-L1+ cytotoxic T-cells (P=0.009), relative lymphopenia (P=0.036), and a higher mDC/pDC ratio (P=0.002). High levels of circulating PD-L1+ cytotoxic T-cells were associated with increased CTLA-4 expression by Tregs (P=0.005) and MDSC levels (P=0.033). This illustrates that counterregulatory immune mechanisms in melanoma should be considered as one interrelated signalling network. Moreover, both increased PD-L1+ T-cells and CTLA-4 expression in Tregs conferred a negative prognosis, indicating their in vivo relevance. Remarkably, circulating CTLA-4, IDO and pDC levels were altered according to prior invasion of the sentinel lymph node and IDO expression in the sentinel was associated with more IDO+ PBMCs. We conclude that the expression of IDO, PD-L1 and CTLA-4 in the peripheral blood of melanoma patients is strongly interconnected, associated with advanced disease and negative outcome, independent of disease stage. Combination treatments targeting several of these markers are therefore likely to exert a synergistic response.

Introduction

Malignant melanoma has become the prototype of an immunogenic tumour that can be successfully treated with immune checkpoint inhibitors such as anti-CTLA-4 and anti-PD-1/PD-L1 antibodies.[1, 2] Clinical responses are more frequent in the subgroup with T-cell inflamed tumours,[3] but still less than half of this patient subset is estimated to respond. This suggests that counter-regulatory immune mechanisms also play a role. Indeed, evidence emerges that anti-tumour immune responses are often accompanied by multiple immunosuppressive mechanisms, probably acting as negative feedback mechanisms. In this context, Spranger et al. were able to show that in metastatic melanoma tissue, the presence of indoleamine 2,3-dioxygenase (IDO), programmed death-ligand 1 (PD-L1) and Forkhead box P3 (FoxP3+) regulatory T-cells (Tregs) coincides and is dependent on preceding IFN-γ secretion by CD8+ lymphocytes.[4]

Indoleamine 2,3-dioxygenase is an immunosuppressive intracellular enzyme that initiates the catabolism of the essential amino acid tryptophan to kynurenine and its derivatives.[5] IDO expression has been described in a variety of immune and stromal cells, but is best characterized in dendritic cells.[6] The function of IDO is the regulation of adaptive immune responses, consequently contributing to tumour-protective immune suppression.[7] We were previously able to show that IDO expression in the primary tumour or sentinel lymph node has an independent negative prognostic effect on overall and relapse-free survival in melanoma.[8, 9]

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is a target gene of FoxP3 and is constitutively expressed by Tregs. It has a major physiological role in enhancing Treg activity and down-modulating T helper cell activity, by competing with CD28 for binding with CD80.[10] CTLA-4 was the first immune-checkpoint to be clinically targeted, and CTLA-4 inhibition was the first treatment to induce a significant improvement of overall survival (OS) in stage IV melanoma patients.[11] This success has paved the way for other checkpoints inhibitors, such as those targeting the PD1/PD-L1 axis. Engagement of PD1 by PD-L1 limits T-cell activity in peripheral tissues at the time of an inflammatory response to infection, leading to immune resistance in the tumour micro-environment.[10]

These immunosuppressive mechanisms are probably more complex than is currently understood. It has for example been shown that PD-L1 can also be a ligand for CD80 in vitro, and that this interaction inhibits T-cell proliferation and cytokine production.[12] Furthermore, CTLA-4 can induce IDO activity in plasmacytoid dendritic cells (pDCs) via reverse signalling with CD80. Adding more complexity to this issue, several different cell types have been reported to be implicated in these feedback loops, the most important being Tregs and myeloid-derived suppressor cells (MDSC).[13]

Even though IDO, CTLA-4 and PD-L1 are promising targets for immunotherapy, data on their expression by immune cells in the peripheral blood of melanoma patients are lacking. We previously demonstrated an increased mean-fluorescence intensity (MFI) for IDO in peripheral blood mononuclear cells (PBMCs) of melanoma patients with advanced disease after stimulation with CTLA-4.[8] In the present study, we investigated the in vivo expression of IDO, PD-L1 and CTLA-4 by immune cells of the lymphoid and myeloid lineage in the peripheral blood of stage I to IV melanoma patients. To ascertain the functional relevance of the detected IDO, tryptophan metabolism was assessed by ultra-performance liquid chromatography (UPLC).

1. Methods

❖ Patients

Seventy-two melanoma patients were enrolled in this study, with a median follow-up time of 16 months after inclusion (i.e. the time of venepuncture for blood collection) and of 41.5 months after diagnosis. For 69 patients both peripheral blood mononuclear cells (PBMCs) and a serum sample were available, in an additional 3 patients only a serum sample was present. Only patients who were without treatment for their melanoma at the time of venepuncture were included, to avoid any possible influence on marker expression. Of all included patients (n=72), 14 had active disease at the time of venepuncture. Disease activity was defined as the presence of metastatic melanoma; 4 patients had stage IIIc disease and 10 patients stage IV disease. The sentinel lymph node could be retrieved for additional immunohistochemistry in 51 patients, 27.7% of these lymph nodes were invaded and 72.3% were tumour-free. Detailed patient characteristics can be found in Table 1. The local medical ethical committee approved this study; all included patients signed written informed consent.

❖ PBMC isolation

PBMCs were isolated from heparinized venous blood by centrifugation on a Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden) within 4h of venepuncture. The PBMCs were cryopreserved in liquid nitrogen in heat-inactivated foetal bovine serum (FBS) supplemented with 10% dimethyl sulphoxide (DMSO) until analysis. Cells were thawed by submersion at 37° for 1-2 minutes and resuspended in a medium containing Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS and 1% glutamine.

Table 1: Patient characteristics

Number of patients, n	72
Follow-up time, months (median - IQR)	16 (21-104.5)
Age at diagnosis, years (median - IQR)	53 (40.5-65)
Female sex, % (n)	52.9 (36)
Stage at inclusion, % (n)	
Local (stage I & II)	50 (36)
Regional (stage III)	34.7 (25)
Systemic (IV)	15.3 (11)
Active disease at inclusion	16.7 (12)
Melanoma characteristics	
Breslow (median, IQR)	1.81 (1.10-2.80)
Ulceration, % (n)	36.1 (22)
Sentinel invasion, % (n)	22.2 (14)
Location of primary melanoma	
Head & neck, % (n)	13 (9)
Trunk, % (n)	42 (29)
Extremities, % (n)	44.9 (31)
Unknown primary	3

❖ PBMC culture and stimulation

PBMCs were cultured overnight in a medium containing Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS and 1% glutamine. When sufficient cells were available, PBMCs were also stimulated to induce IDO expression by adding 1000U/ml human recombinant interferon-gamma (Imukin) to the same medium for 12h at 37°C in 10% CO2. After incubation, adherent and non-adherent cells were collected for flow cytometry.

❖ Flow cytometry

MDSCs were characterized by the HLA-DR- lineage- (CD3, CD19 and CD56) CD33+ CD11b+ phenotype, mMDSCs are CD14+, pmnMDSCs are CD14-. Dendritic cells were characterized by the HLA-DR+ lineage- (CD3, CD14, CD16, CD19, CD20, and CD56) phenotype, pDCs are CD123+ CD11c- and mDCs are CD123- CD11c+. Tregs were defined as CD3+ CD4+ CD25+ FoxP3+ and cytotoxic T-cells as CD3+ CD8+ cells. All antibodies used in this study were fluorescently conjugated mouse anti-human monoclonal antibodies. The following antibodies were purchased from BD Biosciences; CD3 BV421 (563797), CD4 APC-Cy7 (561839), CD25 FITC (560990), CD33 BV421 (562854), CD11b APC-Cy7 (560914), CD123 BV421 (562517). The following antibodies were purchased from eBioscience; B7-H1 PE-Cy7 (25-5983-42), CD8 APC (9017-0087-025), CD3 FITC (11-0038-41), CD19 FITC (11-0199-41), CD56 FITC (11-0569-41), CD14 APC (17-0149-41), CD11c APC (17-0116-41), HLA-DR PerCP-Cy5.5 (45-9956-42). For intracellular stainings, after surface staining PBMCs were fixed and permeabilized with fixation⁄permeabilization solution (BD Biosciences), and then stained with antihuman IDO PE (R&D Systems, IC6030P), CTLA-4 APC (BD Biosciences, 560938) and FoxP3 PerCP-Cy5.5 (eBioscience, 45-4776-42) antibodies. Live/dead staining was performed using Live/dead® fixable aqua dead cell stain (Life Technologies Europe). Patient samples with less than 75% living cells were excluded (n=4). Cells were analyzed on a FACSCanto™ II flow cytometer (BD Bioscience, Erembodegem, Belgium) using FlowJo software (Tree Star Inc, Ashland, OR, USA). For setting the gates, isotype and fluorescence-minus-one (FMO) controls were used. To provide a representative sample a median amount of 500000 cells was analysed per cell type (min 261000 – max 569750). Samples with less than 100000 cells were excluded (n=1). The reported frequencies of circulating cell types are percentages of live PBMCs, except for Treg frequencies which are percentages of CD4+ cells.

❖ Immunohistochemistry for IDO

All sentinel lymph nodes (n=51) were formalin-fixed paraffin-embedded (FFPE) archival tissues. Immunohistochemical staining of 4-5 µm sections was performed according to standard avidin-biotin-peroxidase protocols (Envision Flex+, Dako, Denmark), a mouse linker was added to the protocol (Dako). The signal was visualized by incubation with 3-amino-9-ethylcarbazole (AEC) substrate (Dako). Sections were counterstained with hematoxylin. For antigen retrieval, slides were boiled (97°C) for 20 min (PT Link, Dako). Sections were incubated with a monoclonal anti-IDO antibody (clone 10.1, 1/200, Millipore) for 1h.

❖ Statistical analysis

Median values between 2 groups were compared by the Mann-Whitney U-test, between >2 groups with Kruskal-Wallis testing. To compare proportions of categorical variables, the Pearson's Chi² test or Fisher's Exact test was used. To evaluate correlations, Spearman correlation coefficients (CC) were calculated. All statistical analyses were performed using SPSS 21.0 (SPSS Inc, Chicago, IL, USA), a P-value less than 0.05 was considered statistically significant (double-sided).

❖ Ultra-performance liquid chromatography (UPLC)

Tryptophan, kynurenine and its other downstream metabolites in patient sera were quantified by UPLC-MS/MS (Waters Acquity TQD), according to previously published methods, with slight modifications.[14, 15] An overview of the mean detected serum concentrations for the different molecules can be found in table 2.

Table2: Mean serum concentrations (nM) of tryptophan and tryptophan catabolites

Molecule	Mean (SD) concentration (nM)
Tryptophan (Trp)	49586.11 (14926.746)
Kynurenine (Kyn)	2095.94 (764.201)
Kyn/Trp ratio	4.43 (1.516)
Kynurenic acid	179.67 (104.775)
3-OH-kynurenine	36.47 (40.656)
5-OH-tryptophan	64.63 (18.823)
5-HT (serotonin)	841.92 (550.497)

2. Results

Flow cytometry was performed on peripheral blood mononuclear cell (PBMC) samples of 72 American Joint Committee on Cancer (AJCC) stage I to IV melanoma patients who were not under active therapy for their disease at the time of sample procurement. Detailed patient characteristics can be found in table 1.

❖ Indoleamine 2,3-dioxygenase

IDO expression by PBMCs was 3.8-fold upregulated after stimulation with IFN-γ overnight. We compared the clinical relevance of IDO expression with and without stimulation with IFN-γ, and found no correlation between stimulated IDO expression and any of the clinical variables. All further experiments are therefore based on spontaneous, unstimulated IDO expression values.

IDO expression was predominantly detected in pDCs and monocytic MDSCs (mMDSCs). A lower degree of IDO expression was also detected in Tregs, myeloid dendritic cells (mDCs) and polymorphonuclear MDSCs (pmnMDSC). There was no IDO expression in cytotoxic T-cells. For a complete overview of IDO expression in these cell types, we refer to table 3. IDO expression by pDCs and mMDSCs was strongly correlated ($P<0.001$, CC 0.621).

To check the clinical relevance of the cell types with the strongest IDO-positivity, their relation to disease stage/activity and prognosis was assessed. IDO expression by MDSCs did not correlate with disease activity. In patients with stage IV disease, higher frequencies of IDO+ mMDSCs ($P=0.027$) and IDO+ pmnMDSCs ($P=0.043$) were found compared to patients in stage I-III. However, this had no impact on overall- or progression-free survival.

The percentage of circulating pDCs was dichotomized by ROC analysis into “low” (<0.2515%) and “high” (>0.2515%). Patients with high levels of pDCs had a positive prognosis on OS, independent of disease stage ($P=0.021$, HR 4.16, CI 1.24-13.94 – Table 3, part 1). The frequency of IDO+ pDCs had no impact on prognosis in a model that corrects for disease stage and the percentage of circulating pDCs.

Table 3: IDO and PD-L1 expression by different circulating cell types in AJCC stage I-IV melanoma patients

Cell type	Observed cell frequency (mean % - SD)*	Cell frequency with IDO expression (mean % - SD)	Cell frequency with PD-L1 expression (mean % - SD)
All PBMCs	NA	0.55 (0.58)	1.22 (0.97)
Dendritic cells (DC)	1.54 (0.54)		
Plasmacytoid DCs	0.34 (0.18)	1.66 (2.27)	0.71 (0.37)
Myeloid DCs	0.96 (0.40)	0.015 (0.028)	0.91 (0.38)
Myeloid-derived suppressor cells (MDSC)	4.00 (2.29)		
Monocytic MDSC	2.94 (2.81)	0.96 (1.95)	0.23 (0.61)
Polymorphonuclear MDSC	1.04 (0.64)	0.13 (0.26)	1.10 (1.94)
CD3+ cells	43.60 (9.32)		
Cytotoxic T-cell	14.79 (6.06)	0.0030 (0.0046)	0.17 (0.11)
Regulatory T-cell	4.77 (1.35)	0.083 (0.19)	0.57 (0.24)

"*" percentage of live cells, except for Tregs (percentage of CD4+ cells)

❖ IDO and tryptophan metabolism

In order to confirm that the measurement of IDO by flow cytometry has functional relevance, we assessed tryptophan metabolism by UPLC-MS/MS. A higher MFI for IDO in PBMCs was correlated with a higher Kyn/Trp ratio (P=0.008, CC 0.389) and with lower tryptophan levels (P=0.014, CC -0.364). This pattern matches tryptophan consumption, confirming metabolic activity of the IDO expression measured by flow cytometry.

There was no impact on prognosis of tryptophan levels or any of the IDO catabolites. However, patients with active disease at the time of diagnosis had lower serum tryptophan levels (P=0.041). In parallel, higher serum anthralinic acid levels were found in stage IV patients compared to stage I-III (P=0.012). For the other metabolites, trends were observed in the same direction. These results confirm that IDO activity is more prominent in patients with higher grade or active disease.

Next we evaluated whether tryptophan catabolism in the serum influences circulating cell frequencies. Mean serum concentrations for tryptophan and its catabolites are summarized in table 2. Patients with a high serum Kyn/Tryp ratio had lower levels of circulating CD3+ cells (P=0.036, CC -0.267), increased PD-L1+ cytotoxic T-cells (P=0.009, CC -0.328) and a higher MFI for IDO in Tregs (P= 0.009, CC 0.371).

Moreover, in patients with elevated serum kynurenine levels a shift in DC subset frequencies was observed toward a higher mDC/pDC ratio (P=0.002, CC 0.373).

❖ PD-L1

PD-L1 expression was most prominent in pmnMDSCs, but could also be found in other circulating cell types (Table 2). Despite its low frequency, PD-L1 expression by cytotoxic T-cells was clinically most relevant. Patients who died of melanoma had higher levels of PD-L1+ cytotoxic T-cells at the time of inclusion (P=0.004). To further assess the impact of PD-L1+ cytotoxic T-cells on prognosis, the frequency of PD-L1+ cytotoxic T-cells was dichotomized by ROC analysis into “low” (<0.1505%) and “high” (>0.1505%). High PD-L1+ cytotoxic T-cell levels were present in 42.4%, these patients had a negative prognosis on OS (Log Rank test, P<0.001). This effect was independent of disease stage (P=0.035, HR 5.82, CI 1.13-29.95) (Fig. 1A). Moreover, in patients who were disease-free at inclusion but had disease progression in the following 2 years, a rise in PD-L1+ cytotoxic T-cell frequency could already be seen. The closer to relapse, the higher the PD-L1+ cytotoxic T-cell frequencies were (P=0.021, CC 0.829), suggesting that PD-L1+ cytotoxic T-cells could be relevant to detect impending or subclinical relapse.

Next, the relation of PD-L1 to IDO and Tregs was assessed. A high MFI for IDO correlated with a high MFI for PD-L1 in unstimulated, cultured cells (P<0.001, CC 0.506), indicating that both immunosuppressive mechanisms occur together. PD-L1+ cytotoxic T-cells were also correlated with the percentage of Tregs (P=0.048, CC 0.247), the levels of CTLA-4 expression by Tregs (P=0.005, CC 0.346) and with MDSCs (P=0.033, CC 0.263). Furthermore, levels of PD-L1+ cytotoxic T-cells were inversely correlated with pDC levels (P=0.044, CC -0.249). These results indicate that PD-L1 expression by cytotoxic T-cells is part of an immunosuppressive environment in the peripheral blood.

❖ CTLA-4 expression by Tregs

CTLA-4 expression was only assessed in regulatory T-cells, which were all to some extent CTLA-4-positive. The level of CTLA-4 expression by Tregs was dichotomized by ROC analysis into “low” (<93.7%) and “high” (>93.7%). The mean percentage of patients with high CTLA-4 expression was 37,3%. Patients with high levels of CTLA-4 expression by Tregs had a negative prognosis on OS (Log Rank test, P=0.003), independent of disease stage (P=0.040, HR 3.80, CI 1.06-13.64) (Fig. 1B).

The link between Tregs, IDO and PD-L1 was described in the previous paragraphs. We also observed that the subgroup of patients with head and neck melanoma had increased frequencies of Tregs with high CTLA-4 expression (P=0.026).

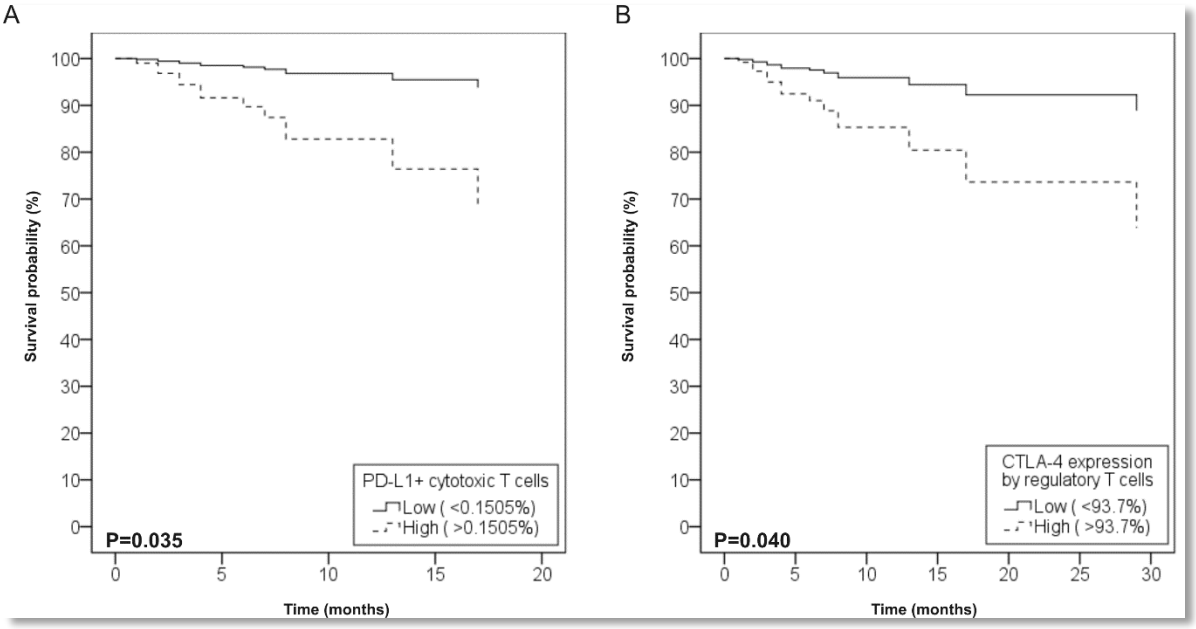

Figure 1: Impact of PD-L1+ cytotoxic T-cells and CTLA-4+ Tregs on overall survival

Cox regression analysis of overall survival according to the levels of circulating PD-L1+ cytotoxic T-cells (A) and CTLA-4 expression level of regulatory T-cells (B), after adjustment for disease stage. Data are presented as percentages of live peripheral blood mononuclear cells (PBMCs).

❖ Link between the sentinel lymph node and circulating immune cells

As clinically relevant immune changes can even occur in tumour-free sentinel lymph nodes, we also evaluated whether invasion of the sentinel lymph node had an impact on circulating immune cells. The mean interval between surgical removal of the sentinel and venepuncture for PBMC isolation was 21 months (IQR 6.5-79.5 months). Patients with an invaded sentinel lymph node had increased frequencies of circulating highly CTLA-4+ Tregs (P=0.045) and a higher MFI for CTLA-4 (P=0.036). Invasion of the sentinel lymph node was also associated with higher frequencies of circulating pDCs (P=0.034) (Fig. 2).

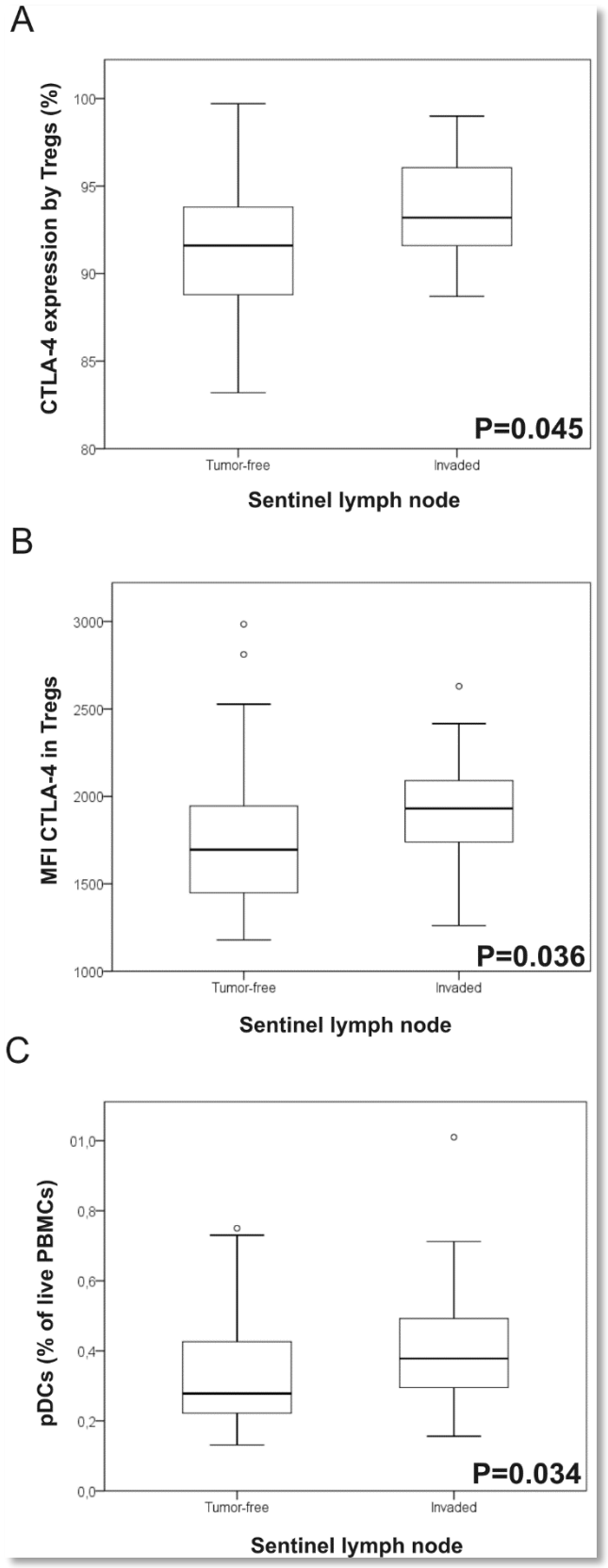

Figure 2: Systemic immune changes in melanoma patients according to sentinel invasion

Box-and-whisker plots showing variations in the level of CTLA-4 expression by regulatory T-cells (Tregs, A), mean-fluorescence intensity of CTLA-4 in Tregs (B) and plasmacytoid dendritic cells (pDCs; C) according to tumour-invasion of the sentinel lymph node. Circulating cell frequencies are expressed as a percentage of live peripheral blood mononuclear cells (PBMCs).

We previously demonstrated that IDO expression in the sentinel is an early marker of immune suppression, irrespective of sentinel invasion. Therefore, the sentinel lymph nodes of the included patients were immunohistochemically stained for IDO and assessed as previously reported.[8] Remarkably, patients with an IDO-positive sentinel had more circulating IDO-positive PBMCs (cultured, unstimulated cells, P=0.016). This could be attributed to higher levels of IDO+ mMDSCs (Fig. 3), which were found to be associated with IDO status of the sentinel (P=0.018). IDO-positivity of the sentinel was also associated with higher frequencies of circulating dendritic cells (P=0.037, data not shown).

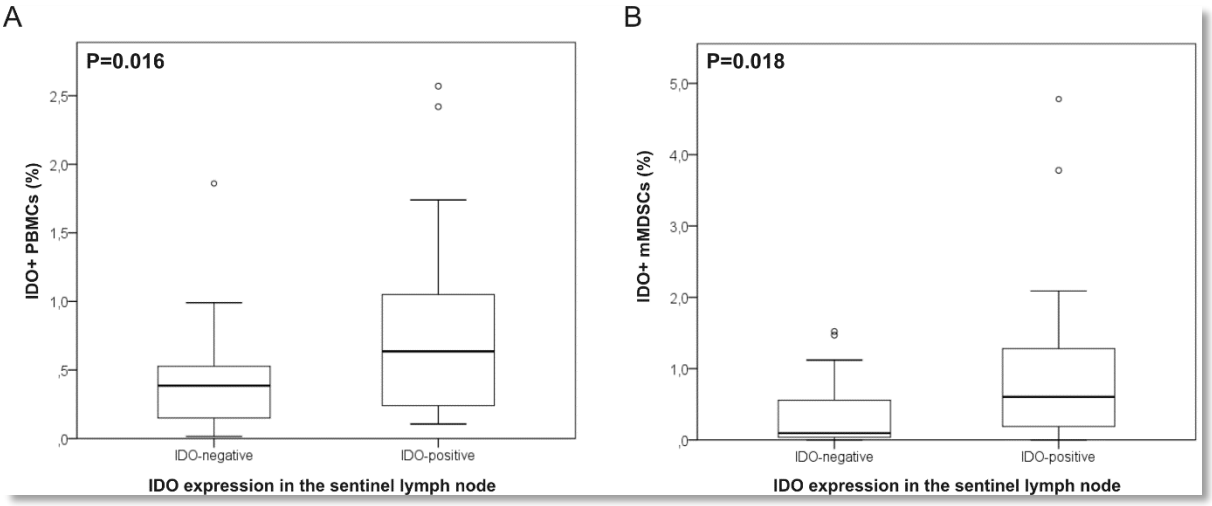

Figure 3: Systemic IDO expression according to IDO expression in the sentinel lymph node

Box-and-whisker plots showing variations in the level of IDO-positive PBMCs (A) and IDO-positive monocytic myeloid-derived suppressor cells (mMDSCs, B) according to IDO expression in the sentinel lymph node. Circulating cell frequencies are expressed as a percentage of live peripheral blood mononuclear cells (PBMC).

3. Discussion

The presence of a cytotoxic, inflammatory tumour micro-environment has been demonstrated to cause a concomitant upregulation of negative feedback mechanisms such as IDO, PD-L1 and FoxP3+ Tregs.[4] Even though these immunosuppressive mediators are targets for current immunotherapies in melanoma, there is insufficient data on their expression by the different immune cells in the peripheral blood. In this paper we demonstrated that IDO, PD-L1 and CTLA-4 expression in the peripheral blood of melanoma patients is not only interconnected but also associated with advanced disease and a negative prognosis independent of disease stage.

With regard to IDO, we demonstrated that spontaneous expression can be detected in the peripheral blood of melanoma patients, predominantly in pDCs and mMDSCs. IDO expression in mMDSCs is higher in patients with advanced disease. Up till now, systemic IDO expression was unreported in melanoma patients, but it had already been detected in other conditions. During pregnancy, circulating DCs express more IDO, contributing to foetal tolerance.[16] IDO+ pDCs have been proposed to be a regulatory DC subset.[6] In breast cancer patients, IDO expression by MDSCs in the tumour stroma was recently demonstrated to suppress immune responses.[17] Circulating IDO+ cells could not be detected, but this might be due to insufficient numbers of recorded events during flow cytometry. As can be deduced from table 2, absolute numbers of IDO+ cells in the peripheral blood are low, so analysing a large sample (500.000 cells) is necessary for detection. The IDO expression we detected is metabolically active, as it is associated with tryptophan consumption in the serum. Tryptophan degradation by IDO was more prominent in patients with high grade or active disease, as previously reported.[18] Tryptophan degradation was also associated with relative lymphopenia, a phenomenon that has also been observed in patients with myelodysplastic syndromes.[19] Low tryptophan levels were associated with increased PD-L1+ cytotoxic T-cells and a higher mDC/pDC ratio. Taken together, our data suggest that IDO expression in the peripheral blood of melanoma patients is clinically relevant and is associated with tryptophan catabolism that seems to affect immune cell maturation and function.

Regarding PD-L1, we demonstrated that this marker is expressed by multiple circulating immune cells. The current paradigm on the PD1/PD-L1 axis states that PD-L1 expression by malignant cells will cause anergy by binding with PD-1 on T-cells.[10] We demonstrated that clinically relevant PD-L1 expression also occurs in circulating cytotoxic T-cells, conferring a worse prognosis on OS, independent of tumour stage. To the best of our knowledge, these data have not been reported so far. Moreover, PD-L1+ cytotoxic T-cell cell counts were higher in disease-free patients who relapsed within a year after sample procurement, suggesting that PD-L1+ cytotoxic T-cells could already increase before relapse is clinically detected.

In our study, the levels of circulating Tregs were not related to disease stage or prognosis, but high CTLA-4 expression by Tregs did have a negative effect on OS independent of disease stage. Tregs have been reported to be overrepresented in the peripheral blood of melanoma patients, but contrary to their increased presence in the tumour micro-environment, the impact of circulating Tregs on prognosis had not been established yet.[20]

In this study we demonstrated that IDO, PD-L1 and CTLA-4 positivity of circulating immune cells are all individually of clinical importance. However, the expression of these three markers is also significantly intertwined. There was a strong correlation between the expression of IDO and PD-L1 in PBMCs. Furthermore the level of PD-L1+ cytotoxic T-cells was associated with increased CTLA-4 expression by Tregs. The combined expression of these three immunosuppressive markers was previously reported in the metastatic melanoma microenvironment.[4] Both the interactions between PD-L1 and PD-1 and between CTLA-4 and CD80/CD86 have been abundantly described and provide the rationale for current melanoma immunotherapies.[10] Nevertheless, our results support previous studies demonstrating that these markers have a much more complex, network-like interplay. In vitro experiments showed that when CTLA-4 on Tregs binds CD80/CD86 on dendritic cells, an IFN-γ dependent induction of IDO is elicited with subsequent tryptophan catabolism.[21] However, we could not withhold a correlation between CTLA-4 expression by Tregs and IDO in pDCs, suggesting that this might not be a dominant mechanism of IDO induction in the peripheral blood. Tregs have also been reported to functionally crosstalk with MDSCs through the PD-L1 pathway during melanoma development in mice.[22] We did find a correlation between Treg and MDSC frequencies and PD-L1 expression in our patients, justifying further research on this interaction in the peripheral blood of melanoma patients.

Our results also illustrate that important immune changes can occur in melanoma patients with invaded and even with tumour-free sentinel lymph nodes. This is of importance, as about half of all melanoma-related deaths occur in those patients who had local stage disease (AJCC stage I and II) at the time of diagnosis.[23] Sentinel node invasion was associated with systemic immune changes with a median delay of 19 months (Fig. 2). Nevertheless we observed that irrespective of invasion, IDO expression in the sentinel also conferred systemic immunological changes. In patients with an IDO+ sentinel, a higher frequency of circulating DCs was seen (implying immune activation) but higher frequencies of IDO+ mMDSCs were also observed (suggesting concomitant immunosuppression). This clustering of IDO expression in the sentinel and in PBMCs is remarkable, as we previously showed that IDO expression is also consistent in the primary, sentinel and even metastatic tissue of melanoma patients.[8] Our results indicate that IDO expression by host cells is important in the pathogenesis of melanoma, but the question remains how this IDO expression is induced. Soluble tumour-derived

factors could be responsible, but alternatively this clustering could also indicate a certain patient-related predisposition. Gene polymorphisms of both the IDO and IFN-γ genes have been demonstrated to be associated with an upregulation of IDO expression and tryptophan metabolism in healthy individuals.[24, 25] If this would also be the case in melanoma patients, it could help explain a patient-dependent IDO expression pattern.

There is a solid basis for investigating the effect of IDO inhibitors as part of combination strategies with other immunotherapies in melanoma patients. If IDO expression is associated with the spontaneous host anti-tumour immune response, it could also be induced by immunotherapy, hence limiting the effectiveness of the treatment.[4] In a B16 mouse melanoma model, Holmgaard et al. have demonstrated that host IDO expression has an inhibitory role in both anti-CTLA-4 and anti-PD1/PD-L1 therapy and that pharmacological inhibition of IDO combined with CTLA-4 blockade gives superior effects.[26] IDO expression analysis could also be useful for predictive immunoprofiling, as immunohistochemical IDO-positivity in the tumour microenvironment has been shown to predict response to anti-CTLA-4 therapy in patients with metastatic melanoma.[27] Others have also demonstrated that lower levels of circulating mMDSCs before initiation of anti-CTLA-4 therapy are associated with increased response.[28, 29] The relevance of the IDO+ mMDSCs we observed should therefore be evaluated in melanoma patients, as it could not only provide predictive information, but also help elucidate the counter-regulatory mechanisms that hamper the efficacy of current immunological checkpoint inhibitors.

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SYSTEMIC IMMUNE CHANGES ASSOCIATED WITH ADJUVANT IFN-A2B-THERAPY IN STAGE III MELANOMA PATIENTS: FAILURE AT THE EFFECTOR PHASE?

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Abstract

Objectives: Interferon-alpha (IFN-α) is the only approved adjuvant treatment for high-risk melanoma patients in Europe, but impact on overall survival is low. Although it is believed that IFN-α exerts its effects through immunomodulation, data on its impact on circulating immune cells are scarce.

Methods: Flow cytometry was performed on peripheral blood mononuclear cells of 8 IFN-α2b-treated stage III melanoma patients and 26 untreated stage III melanoma patients as controls, to enumerate myeloid and plasmacytoid dendritic cells (mDC and pDC), monocytic and polymorphonuclear myeloid-derived suppressor cells (mMDSC and pmnMDSC) and cytotoxic and regulatory T-cells (Tregs). The expression of several immunosuppressive markers [indoleamine 2,3-dioxygenase (IDO), programmed-death ligand-1 (PD-L1) and CTLA4] was explored. IDO activity in the blood was confirmed by ultra-performance liquid chromatography (UPLC).

Results: Compared to controls, IFN-α2b treatment was associated with increased IDO expression by pDCs (P=0.021) and an increased kynurenine/tryptophan ratio in the serum (P=0.004), compatible with IDO enzyme activity. Furthermore, IFN-α2b-treated patients had a decreased mDC/DC ratio (P=0.002), decreased CD3+ lymphocytes (P=0.034) and increased circulating Treg (P<0.001) and PD-L1+ cytotoxic T-cell (P=0.001) frequencies.

Conclusion: IDO expression is upregulated in circulating pDCs of high-risk melanoma patients treated with adjuvant IFN-α2b. This is associated with tryptophan consumption in the patients' serum and higher Treg and PD-L1+ cytotoxic T-cell frequencies. We hypothesize that in IFN-α2b-treated patients, IDO activity acts as a negative feedback mechanism and might limit the clinical efficacy of IFN-α2b therapy. The underlying mechanism should be explored as this could lead to more efficient immunotherapies.

1. Introduction

Melanoma incidence and mortality have been increasing worldwide over the past decades. Currently, interferon-alpha (IFN-α) is the only therapy approved in Europe for adjuvant use in patients with resected melanoma at high risk of recurrence. Significant relapse-free survival (RFS) benefits have been demonstrated, but impact on overall survival (OS) is modest.[1] Nonetheless, adjuvant treatment options remain important as treatment of stage IV melanoma only produces long-term clinical responses in a minority of patients.[2]

Some studies have suggested that only a subset of patients is sensitive to IFN-α.[2, 3] This hypothesis is substantiated by the fact that there is no association between the outcome of treatment with adjuvant IFN-α and dose or duration of the treatment regimen. Currently, ulceration of the primary melanoma seems to be a promising predictive biomarker to pre-identify responders. However this remains to be validated in (ongoing) prospective trials,[4] and the molecular mechanisms behind this effect are unknown to date.[1]

The mechanism of action of IFN-α is believed to be mainly immunomodulatory, but results of previous research to elucidate it have been inconclusive so far.[1, 5] Nevertheless, a systemic immunomodulatory effect can be inferred from the observed correlation between clinical response to IFN-α therapy and the occurrence of auto-immune phenomena such as vitiligo and circulating auto-antibodies.[6]

IDO is the enzyme that catalyses the initial rate-limiting step in the degradation of the essential amino acid tryptophan into kynurenine metabolites. We were previously able to show that IDO expression in the sentinel lymph node is an independent negative prognostic marker that is inversely related to a cytotoxic immune response in the primary tumour.[7, 8] IDO, together with programmed-death ligand 1 (PD-L1) and FoxP3+ regulatory T-cells (Tregs), was also recently shown to be upregulated in the T-cell inflamed melanoma microenvironment.[9]

In this study, we analysed circulating DC, MDSC, cytotoxic and regulatory T-cell populations, and the expression of IDO, PD-L1 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in the relevant subpopulations. The enzymatic activity of IDO was measured by the kynurenine (kyn) to tryptophan (trp) ratio in the patient's serum.

2. Methods

❖ Patients

Eight American Joint Committee on Cancer (AJCC) stage III melanoma patients under adjuvant treatment with intermediate-dose IFN-α2b (IntronA, induction phase of $10x10^6$ U/m² IV 5d/week, followed by maintenance phase of $5x10^6$ U subcutaneously 3x/week for 2 years) were enrolled in this retrospective study. As a control population, 21 untreated melanoma patients with comparable AJCC stage III disease were included. Some differences in prognostic factors did exist between both patient groups, these are outlined in Table 1. Blood samples were prospectively collected during clinical follow-up in the maintenance phase (mean interval of 175 days after start of therapy). This study was approved by the local medical ethical committee; all included patients signed written informed consent.

Table 1: Patient characteristics

	Stage III IFN-α2b-treated	Stage III untreated
Number of patients, n	8	21
Follow-up time since inclusion, months (median - IQR)	13.0 (8.0-30.5)	29.0 (15.0-45.5)
Follow-up time since diagnosis, months (median – IQR)	36.0 (18.5-58.5)	67.0 (27.5-147.5)
Age at diagnosis, years (median - IQR)	46.5 (32.0-57.0)	53.0 (31.5-67.5)
Female sex, % (n)	37.5 (3)	52.4 (11)
Stage at inclusion, % (n)		
Stage IIIa	/	/
Stage IIIb	75.0 (6)	61.9 (13)
Stage IIIc	25.0 (2)	38.1 (8)
Stage IV	/	/
Melanoma characteristics		
Breslow (median, IQR)	2.74 (1.83-5.85)	2.10 (1.09-4.00)
Ulceration, % (n)	50.0 (3)	33.3 (7)
Location of primary melanoma		
Head & neck, % (n)	12.5 (1)	20.0 (4)
Trunk, % (n)	62.5 (5)	25.0 (5)
Extremities, % (n)	25.0 (2)	55.0 (11)
Unknown primary	/	1

IFN-α: interferon-alpha. IQR: interquartile range.

❖ PBMC isolation, culture and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated as previously described [7]. PBMCs were thawed by submersion at 37° for 1-2 minutes and resuspended in a medium containing Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS and 1% glutamine. Two separate samples were cultured overnight at 37°C in 10% CO2 in the same medium, one without stimulation and one with 1000 U/ml human recombinant interferon-gamma (IFN-γ, Imukin). Patient serum was collected at the time of PBMC isolation and stored at -80°C.

❖ Flow cytometry

Flow cytometry was performed on freshly thawed PBMCs. MDSCs were characterized by the HLA-DR- lineage- (CD3, CD19, CD56) CD33+ CD11b+ phenotype, mMDSCs are CD14+, pmnMDSCs are CD14-. Dendritic cells were characterized by the HLA-DR+ lineage- (CD3, CD14, CD16, CD19, CD20, and CD56) phenotype, pDCs are CD123+ CD11c- and mDCs are CD123- CD11c+. Tregs were defined as CD3+ CD4+ CD25+ FoxP3+ and cytotoxic T-cells as CD3+ CD8+ cells. All antibodies used in this study were fluorescently conjugated mouse anti-human monoclonal antibodies. The following antibodies were purchased from BD Biosciences; CD3 BV421, CD4 APC-Cy7, CD25 FITC, CD33 BV421, CD11b APC-Cy7, CD123 BV421. The following antibodies were purchased from eBioscience; B7-H1 PE-Cy7, CD8 APC, CD3 FITC, CD19 FITC, CD56 FITC, CD14 APC, CD11c APC, HLA-DR PerCP-Cy5.5. For intracellular stainings, PBMCs were fixed and permeabilized with fixation⁄permeabilization solution (BD Biosciences), and then stained with antihuman IDO and FoxP3 antibodies. Live/dead staining was performed using Live/dead® fixable aqua dead cell stain (Life Technologies Europe). Cells were analysed on a FACSCanto™ II flow cytometer (BD Bioscience, Erembodegem, Belgium) using FlowJo software (Tree Star Inc, Ashland, OR, USA). For setting the gates, isotype and fluorescence-minus-one (FMO) controls were used. To provide a representative sample a median amount of 500000 cells were analysed per cell type. The reported frequencies of circulating cell types are percentages of live PBMCs, except for Treg frequencies which are percentages of CD4+ cells.

❖ Ultra-performance liquid chromatography (UPLC)

Tryptophan, kynurenine and its other downstream metabolites in patient sera were quantified by UPLC-MS/MS (Waters Acquity TQD), according to previously published methods, with slight modifications.[10, 11]

❖ Statistical analysis

Median values between 2 groups were compared by the Mann-Whitney U-test, between >2 groups with Kruskall-Wallis testing. All statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA), a P-value less than 0.05 was considered statistically significant (double-sided), without correction for multiple testing.

3. Results

❖ Shifts in circulating cell frequencies

In patients treated with IFN-α2b, we observed lower frequencies of circulating CD3+ lymphocytes (P=0.034) and a lower mDC/DC ratio (P=0.002) resulting in a decreased mDC/pDC ratio (P=0.025) compared to untreated stage III patients. There was no difference in circulating MDSC (subset) frequencies between the patient groups. Compared to untreated stage III patients, the IFN-α2b treated patients had a higher circulating Treg/CD4 ratio (P<0.001, Fig. 1a).

❖ Expression of IDO, PD-L1 and CTLA-4 by circulating immune cells

Compared to untreated stage III patients, the IFN-α2b treated patients had higher frequencies of circulating PD-L1+ cytotoxic T-cells (P=0.001, Fig. 1b) and a trend towards higher CTLA-4 expression by Tregs (P=0.078).

We have previously described IDO expression by pDCs and mMDSCs in melanoma patients (data submitted for publication), so IDO levels in all circulating cell subsets were compared. IFN-α2b treated patients had significantly higher levels of IDO-expression by pDCs (P=0.021, Fig. 1c), and a trend towards higher IDO expression by mMDSCs (P=0.118).

❖ IDO metabolism and response to stimulation with IFN-γ

To analyse whether the observed IDO expression corresponded with metabolic enzyme activity, UPLC for tryptophan and kynurenin was performed on the corresponding patients' serum. When comparing patients treated with IFN-α2b with untreated stage III patients, we observed a significant rise in kyn/trp ratio (P=0.004, Fig. 1d) with an underlying decrease of Trp levels (P=0.011) and a trend toward increased levels of the different kynurenine metabolites. IFN-α2b-treated patients had lower 5-HT levels (P=0.046). This pattern is in accordance with tryptophan consumption, and thus with IDO activity.

Subsequently, to assess whether IFN-α2b treated patients respond differently to an inflammatory stimulus, PBMCs were stimulated with IFN-γ. In patients in the IFN-α2b treatment group, the absolute number of PBMCs that was capable of upregulating IDO or PD-L1 was lower compared to the untreated stage III patients (P=0.002 for both).

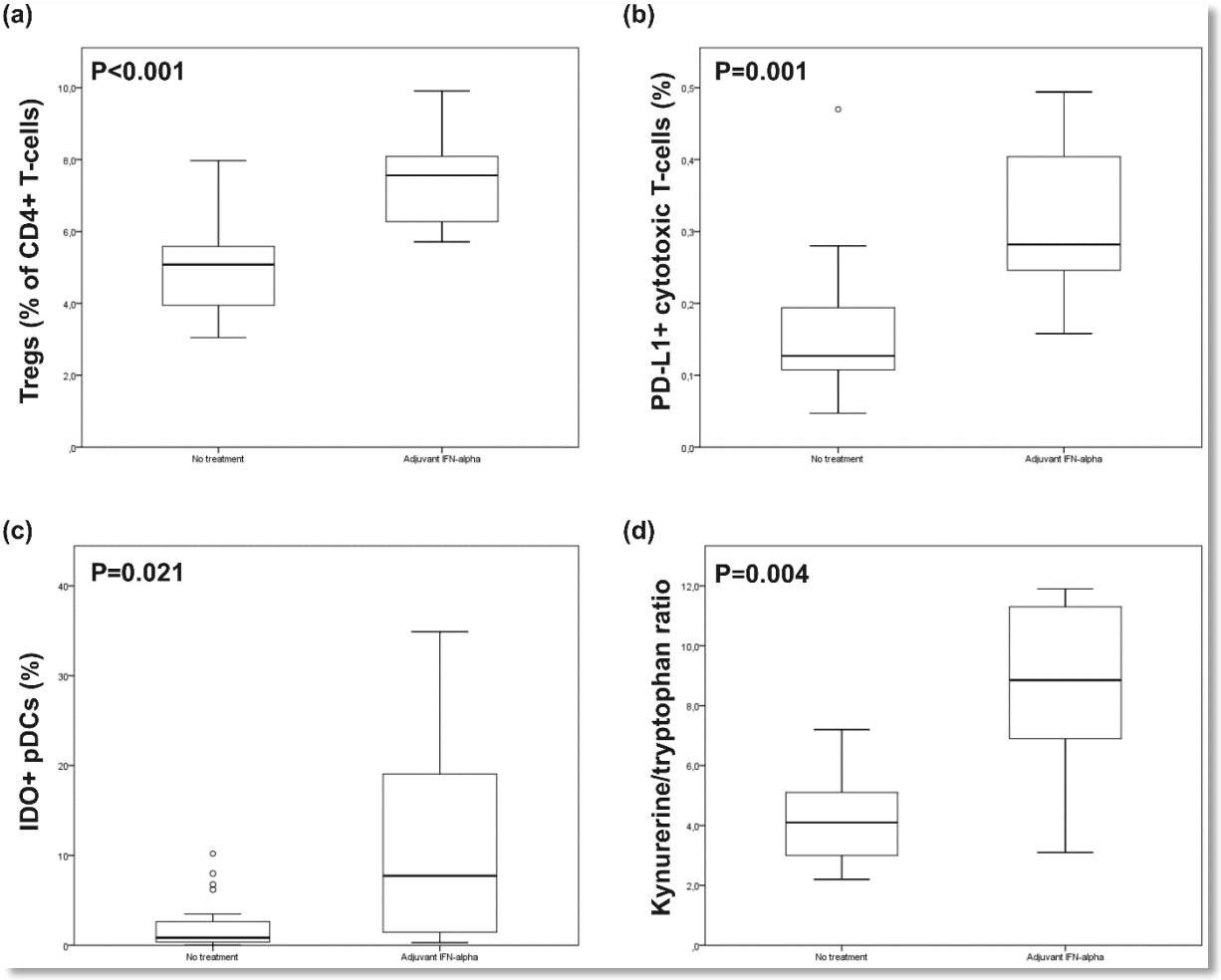

Figure 1

Box- and whisker plots showing that in our cohort of stage III melanoma patients, patients treated with adjuvant IFN-α2b had higher circulating levels of (a) regulatory T-cells (Tregs), (b) programmed-death ligand 1 (PD-L1)+ cytotoxic T-cells, (c) indoleamine 2,3-dioxygenase (IDO)+ plasmacytoid dendritic cells (pDCs) and (d) a higher kynurenine/tryptophan ratio compared to untreated controls.

❖ Immunological changes in patients with ulcerated melanoma

As ulceration is being investigated as a possible biomarker for response to IFN-α therapy, we explored whether patients with an ulcerated primary melanoma had a different circulating immune profile. No differences were observed with regard to lymphoid cells or MDSC subsets. However, patients with an ulcerated primary had lower frequencies of circulating DCs (P=0.037), due to both lower mDC (P=0.012) and lower pDC (P=0.034) frequencies.

4. Discussion

The effect of adjuvant treatment with IFN-α on OS in melanoma patients is modest and currently limited data exist on its mechanism of action or predictors of response. Only few studies have focused on changes in circulating immune cells during adjuvant IFN-α treatment.

In our patient population, significantly increased circulating Treg frequencies were observed, as well as a decrease in circulating CD3+ lymphocytes. Decreases in both CD4+ and CD8+ T-cell subsets have been previously described in IFN-α-treated patients, and one trial reported reduced Treg frequencies during high-dose IFN-α therapy, but this did not reach significance.[12, 13] However, our data also point towards a role for circulating DCs during IFN-α treatment. Treatment with adjuvant IFN-α2b was associated with a decrease of the mDC/pDC ratio in the peripheral blood, while in untreated stage III melanoma patients circulating DC frequencies are unaltered (data submitted for publication).[14] Taken together, these data show systemic alterations of adaptive immune cells during treatment with IFN-α2b, but the consequences remain to be elucidated.

We observed a remarkable overexpression of IDO by pDCs and to a lesser extent mMDSCs in stage III melanoma patients treated with IFN-α2b compared to untreated controls. Moreover, this overexpression was correlated with concomitant tryptophan consumption, corroborating that IDO expression by pDCs is associated with enzymatic IDO activity in the blood. The prototypical cytokine known to induce IDO expression directly by transcriptional activation is IFN-γ, but IFN-α can also induce IDO expression indirectly via downstream cytokines such as TNF-α and IFN-γ.[15] Overactivation of IDO in response to IFN-α treatment in melanoma patients has been demonstrated, it is a key event in the pathogenesis of IFN-α-related depression.[16] Indeed, in patients with melanoma and renal cell carcinoma receiving IFN-α therapy, serum tryptophan concentrations are negatively correlated with depressive symptoms, further supporting the presence of systemic IDO-activity.[17]

Not only was IDO upregulated in our IFN-α2b treated patients, we also found higher levels of PD-L1+ cytotoxic T-cells and FoxP3+ Tregs. To the best of our knowledge, this has not been reported in literature before. The combined upregulation of IDO, PD-L1 and Tregs was recently demonstrated to occur in response to IFN-γ secretion in the melanoma microenvironment.[9] In theory, treatment with IFN-α could unwantedly contribute to the parallel induction of immunosuppressive negative feedback mechanisms. The development of a negative feedback response is indeed a logic sign of an induced immune activation. Dendritic cells have been shown to express IDO upon activation, resulting in the inhibition of T-cell proliferation.[18] Moreover, IDO expression by pDCs has also been proposed as a negative factor for other immunotherapies such as DC vaccination.[19] However, as the treatment scheme of intermediate-dose IFN-α2b takes 2 years, these mechanisms could persist and limit the efficacy of the treatment. The existence of both an initial immune recognition and a resulting immune effector phase is believed to be beneficial for the success of checkpoint inhibitors such as ipilimumab.[9] Therefore, one could also hypothesize that treatment with IFN-α contributes to this necessary immune recognition, but is insufficient to overcome the negative feedback loops, which would be essential for a longstanding final effector phase.

We also observed borderline increased IDO expression in mMDSCs in IFN-α2b-treated patients. Even though MDSCs are not the typical IDO+ cell type in cancer patients, IDO+ MDSCs mediating immunosuppression have been described in breast cancer.[20] Low MDSC levels before anti-CTLA4 therapy in melanoma patients could predict response to anti-CTLA4 therapy, and MDSC frequencies remained stable during the treatment course.[21] In combination with our current observations, it could thus be interesting to assess IDO expression by MDSCs in other immunotherapies.

As ulceration is currently tested as a possible predictive biomarker, we evaluated the associated systemic immune changes and found decreased circulating DC frequencies in patients with an ulcerated primary melanoma. A correlation between ulceration and reduced DCs in the sentinel lymph node has been described,[22] but as far as we know lower DC frequencies in the peripheral blood were unreported. These observations suggest that future research should explore the role of (p)DCs to work out the mechanism behind the possible predictive value of ulceration of the primary melanoma.

Our study is the first to observe increased systemic IDO expression by pDCs and to a lesser extent mMDSCs in melanoma patients treated with adjuvant IFN-α2b, compared to untreated controls. Moreover, a concomitant increase in Tregs and PD-L1+ cytotoxic T-cells was present, suggesting the existence of negative feedback mechanisms in IFN-α2b-treated patients. We hypothesize that these could represent a persistent counterregulatory mechanism, possibly limiting the clinical effects of this

therapy. The underlying mechanism should be explored as this could lead to further optimization of IFN-α2b and other immunotherapies.

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CHAPTER 5:
Discussion & future perspectives

◆ Discussion

1. Lessons for immunoprofiling

The need for local and systemic immunological biomarkers is increasingly recognized and subject of intensive investigation.[1-3] These immunoprofiling efforts are often focussed on providing prognostic information, trying to improve the current TNM classification. In the current era of immunotherapy for melanoma, predictive immunoprofiling is also gaining interest. This domain chiefly focuses on the immune micro-environment, looking for markers to predict the susceptibility of patients for different immunotherapeutic strategies.[4-6]

❖ IDO expression in melanoma patient tissues

In the first part of this thesis, we demonstrated that IDO-positivity in peri-tumoral endothelium is consistent in corresponding primary melanomas, sentinel lymph nodes and metastases. Sentinel IDO-positivity was also inversely correlated with CD8+ lymphocytes and TILs in the primary melanoma. We hypothesized that IDO expression in the sentinel node of melanoma patients could be used as an early marker that describes the patients' anti-tumour immune response.

In our patients, we detected IDO expression in both host and tumour cells in the primary melanoma. Whereas the host IDO expression had an independent negative impact on overall survival, the IDO expression by melanoma cells did not. IDO expression by melanoma cells was scored for intensity on a 4-tiered scale (Fig. 1). Amelanotic melanomas also stained positive for IDO, making interference from underlying melanin in the staining less likely. We had the impression that the intensity of IDO expression by tumour cells was heterogeneous, as this could vary per tumour cell nest. Remarkably, there was no correlation between IDO expression by tumour cells and IDO expression by host cells.

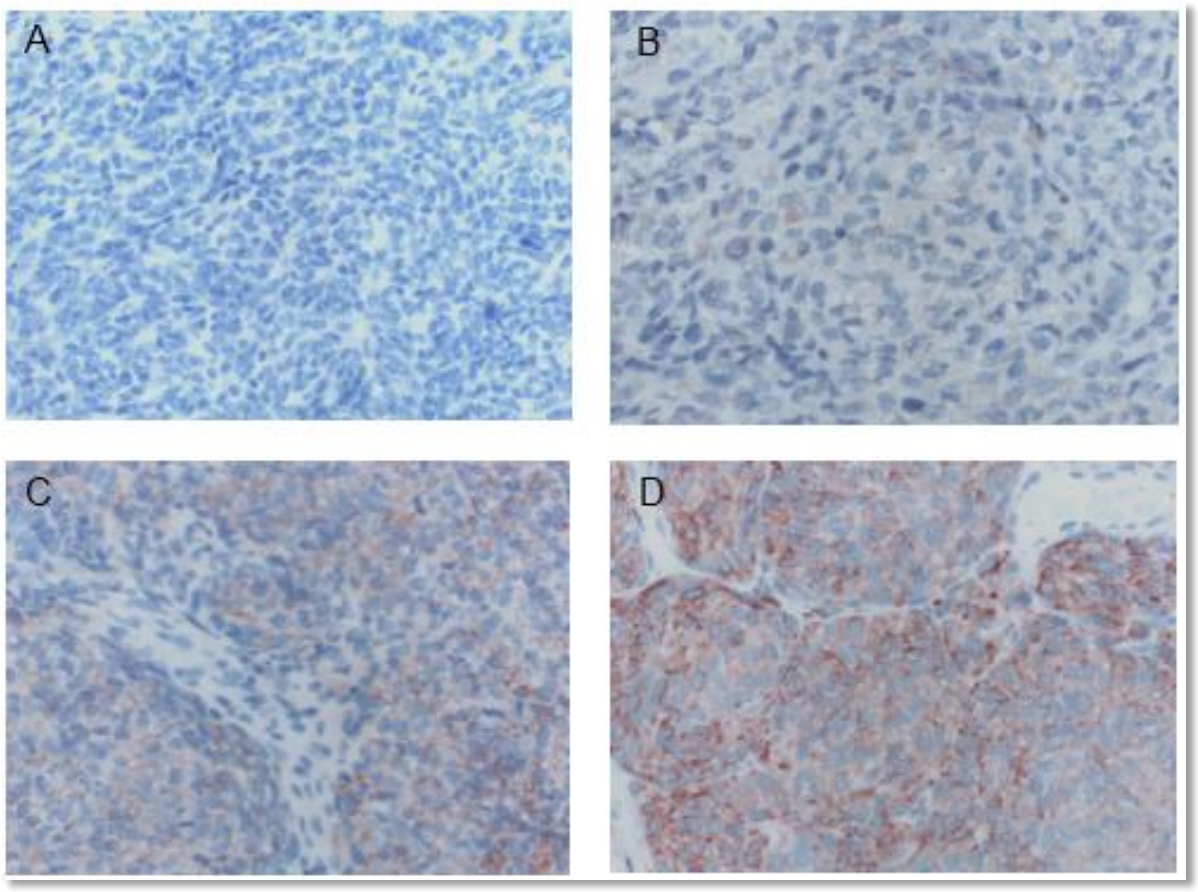

Figure 1: IDO expression by melanoma cells in the primary melanoma.
Slides were scored on a four-tiered scale showing IDO expression as (A) absent, (B) weak, (C) moderate and (D) strong.

IDO expression by tumour cells can exert a biologic effect in cancer patients, which is illustrated by the prognostic impact in different malignancies. However, the fact that IDO expression by tumour cells has also been reported to lead to tumour cell apoptosis, suggests that the tumour could somehow need to "lose" its IDO positivity to be able to grow. We did observe an inverse correlation between the intensity of IDO expression by melanoma cells and Breslow tumour thickness. This could indirectly support the hypothesis that continued IDO expression might somehow also impede expansive tumour growth.

It goes beyond doubt that IDO can be expressed by both tumour and host cells, but why some patients in our study remained either IDO-positive or IDO-negative in their host cells throughout the disease course is unclear. This relative consistency of IDO expression per patient could be in favour both of a tumour-derived IDO-induction and of a host-dependent IDO-expression. Tumours could be associated with a particular signature of tumour-derived soluble factors, leading to a similar IDO induction throughout different disease stages. TGF-β, a regulatory cytokine that inhibits T-cell differentiation and

promotes Treg induction, has for example been proposed to be an inducer of IDO expression.[7] One of the TGF-β family members, TGF-β2, was identified in primary melanoma cells and suggested to induce tolerogenic DCs.[8] However, our attempts to immunohistochemically stain for TGF-β2 in primary melanomas succeeded in replicating the published images, but did not convince us that TGF-β2 was truly identified. The staining significantly overlapped with melanin expression by the tumour cells, and was most prominent in macrophages.

The observation that IDO expression in metastases and peripheral blood of melanoma patients was consistent with IDO expression in the sentinel lymph node (resp. median 41.5 and 19 months earlier) does indicate that a certain host predisposition should be considered as well. It would indeed be striking if IDO expression in the absence of a detectable tumour load would still be orchestrated by the tumour cells. The host-related paradigm does not preclude the influence of the tumour, on the contrary. It is the presence of a tumour that elicits a certain host immune response, but the adequacy of this response is likely to co-depend on the host. This idea is supported by the existence of IDO and IFN-γ gene polymorphisms. Gene polymorphisms of both *INDO* and *IFNG* have been demonstrated to be associated with an upregulation of IDO expression and tryptophan metabolism in healthy individuals.[9, 10] The role of these polymorphisms in melanoma patients remains to be elucidated, but it is not impossible that they could help explain a patient-dependent IDO expression pattern.

❖ Circulating immune cell subsets in melanoma patients

In melanoma, it is currently unclear which circulating immune cell types confer the most powerful prognostic information. Besides T-cells, myeloid-derived suppressor cells and dendritic cells are the most elaborately studied cell types, but research is often focused on the tumour microenvironment. We therefore performed a comparative evaluation of the presence of different circulating immune subsets in untreated melanoma patients in different stages of disease. Additionally, the expression of immunosuppressive molecules was also assessed. Although our data are observational and therefore cannot provide mechanistic insight, several patterns can be discerned when all data are analysed together (Table 1, in which clusters of significant observations are highlighted).

Table 1: Association of MDSCs and pDCs with disease course and other immune markers

	MDSCs	pDCs
Association with disease state and prognosis		
Systemic disease	Increased (P=0.046)	Decreased (P=0.001)
Active disease	Increased (P=0.095)	Decreased (P=0.002)
Death	Increased (P=0.073)	Decreased (P=0.009)
Correlation with other immune markers		
CD3 cells	Inverse (P<0.001)	None
CD8 cells	Inverse (P=0.017)	None
PD-L1+ CD8 cells	Positive (P=0.033)	Inverse (P=0.044)
Tregs	Inverse (P=0.007)	None
CTLA-4 expression in Tregs	Positive (P=0.003)	None

MDSC: myeloid-derived suppressor cell; pDC: plasmacytoid dendritic cell; PD-L1: Programmed death ligand 1; Tregs: regulatory T-cells; CTLA-4: Cytotoxic T-lymphocyte antigen-4.

In our patient population, circulating MDSC frequencies were strongly correlated with alterations in various other circulating cell types. In contrast, we observed a much weaker correlation with disease course and prognosis. This pattern is compatible with the profile of a principal regulatory function, in which case MDSCs are anticipated to influence multiple other cell types that have a downstream effector function. With regard to circulating pDCs, we observed that a high frequency was strongly correlated with disease activity, the presence of systemic disease and an evolution towards melanoma-related death. Their impact on prognosis surpassed the MDSCs'. However, pDC frequencies were not consistently correlated with alterations in the other circulating cell types. Taken together, these observations suggest that compared to MDSCs, the regulatory impact of pDCs is weaker. The pDCs might be relevant more downstream, closer to the effector cells. One would expect a loss of effector function to be primarily related to disease severity, with a minor effect on other immune cells. Another pattern could be observed for circulating Tregs and cytotoxic T-cells. The frequencies of these cell types had no prognostic impact as such, but their expression of immunosuppressive markers (resp. CTLA-4 and PD-L1) did ensue a negative prognostic power. We therefore hypothesize that this might indicate an intermediate profile, in which regulatory properties could be enforced in secondary effector cells.

The combination of these propositions confers a central role to MDSCs with regard to the various alterations in circulating cell frequencies in our melanoma patient population.

The impact of a defective myeloid differentiation on mature DC levels has been extensively discussed in the second research paper and will therefore not be discussed in detail here. In line with our observations, an accumulation of immunosuppressive immature myeloid cells such as MDSCs combined with reduced frequencies of mature DCs is often seen in cancer patients.[11, 12] Our data also corroborate those of other groups who observed that pDC defects seem to outweigh mDC defects in clinical importance for melanoma patients.[13, 14]

2. Lessons for immunotherapy

❖ Targets for therapy: network of IDO, PD-L1 and CTLA-4

In this thesis we demonstrated that IDO, PD-L1 and CTLA-4 expression by circulating immune cells are not only individually clinically important, but that the expression of these three markers is also significantly intertwined. Our results support previous studies demonstrating that these markers have a complex, network-like interplay.[15, 16] The pattern of combined expression of these three immunosuppressive markers was previously reported in the metastatic melanoma microenvironment, it is part of what is called 'adaptive immune resistance'.[17] This process starts with host type I IFN signals, which are required for the priming of antitumor CD8+ T-cells.[18] However, even tumours that are infiltrated with CD8+ T-cells are not necessarily rejected, and this has been proposed to be due to a CD8 and IFN-γ-dependent induction of IDO, PD-L1 and Tregs in the melanoma microenvironment. In this study, the authors proposed that the IDO and PD-L1 expression that were detected by RT-PCR were attributable to tumour cells, but immunohistochemical staining of a small set of human melanoma metastases showed that both molecules were also expressed by stromal cells.[17]

Our data in patients who are under adjuvant treatment with the type I interferon IFN-α2b suggest that IDO, PD-L1 and Tregs could also be relevant in patients receiving immunotherapy. Figure 2 summarizes our observations in a hypothetical model.

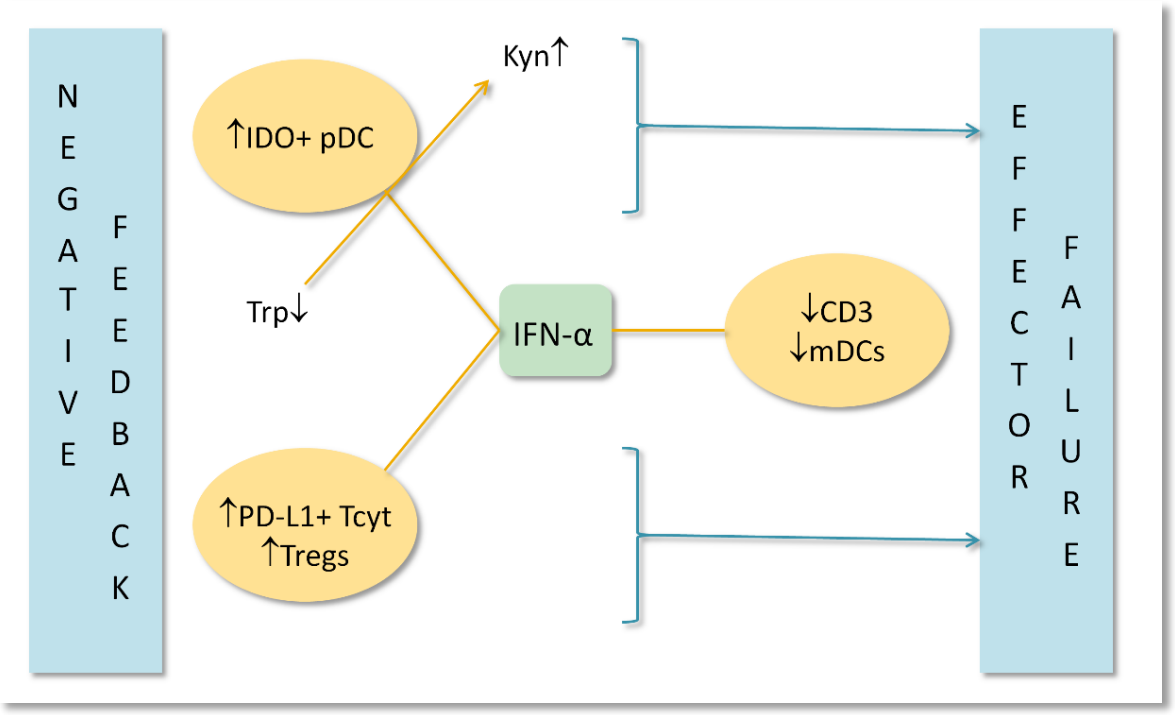

Figure 2: Summary of the systemic immune alterations observed in IFN-α2b-treated patients. Adjuvant IFN-α2b therapy was associated with higher levels of circulating IDO+ pDCs, Tregs and PD-L1+ cytotoxic T-cells (Tcyt). A concomitant tryptophan consumption in the patients' serum was also observed. We hypothesize that these alterations represent negative feedback mechanisms that could be induced by the immune stimulus provided by IFN-α, which is possibly insufficient to overcome the resulting effector cell failure.

At first sight, our approach of assessing PD-L1 instead of PD-1 expression in immune cells might seem to be in contradiction with the current paradigm on this pathway. However, PD-L1 expression has been shown to occur on almost all murine lymphohematopoietic cells at varying levels, and could strongly be induced in T-cells after stimulation with IFN-γ.[19] It is not merely a T-cell activation marker, as PD-L1 deficient mice have enhanced T-cell responses and DC function. Moreover, PD-L1 had a role in limiting the expansion and survival of murine CD8+ T-cells in vivo.[20] In our study, we also detected relatively high levels of PD-L1 expression on pmnMDSCs, but this was not related to disease course or prognosis. Similarly, PD-L1 expression has been detected in normal murine immature granulocytic progenitor cells,[19] as well as in melanoma-bearing mice where it was necessary for the immunosuppressive effect of MDSCs.[21] The functional relevance of this expression pattern in humans is unknown.

Taken together, both literature and our data suggest that in melanoma patients multiple immunosuppressive mechanisms appear to operate in concert, and not just in pairs. Therefore, one could imagine that unless two or more of them are targeted together, resistance could emerge.

❖ Therapeutic potential

Combination strategies

To optimize immunotherapy for melanoma, combination strategies with the currently available compounds could be a successful approach. Clinical trials evaluating the concurrent use of anti-CTLA4 and anti-PD-1 antibodies are ongoing, and preliminary results suggest excellent response rates (objective response rate of 40%) and manageable toxicity.[22] The use of anti-PD-L1 antibodies could also be interesting, as the first phase I trials showed clinical results comparable to anti-PD-1 therapy.[23] PD-L1 blockade is not only expected to interrupt the PD-1/PD-L1 axis, but could also impact on the inhibitory signal to T-cells via CD80.[24] The role of circulating PD-L1+ cytotoxic T-cells is unknown, but certainly worth investigating as these cells might also be affected by PD-L1 blockade.

Even though the checkpoint inhibitors are promising as mono- or combination therapies, there seem to be limitations to their effects. Combining several immune checkpoint inhibitors is mainly expected to improve response rates in the subgroup of metastatic melanoma patients who already have a T-cell inflamed tumour microenvironment.[25] To truly target the melanoma from different angles, a broader view on combination strategies could apply, as illustrated in figure 3.[26] In theory, optimal combination strategies should be designed to not only break pre-existing tolerance but also provide the immune system with a set of tumour antigens. These antigens should be presented in an activated immune milieu, with sufficient co-stimulatory signals to drive a potent anti-tumour response.[27]

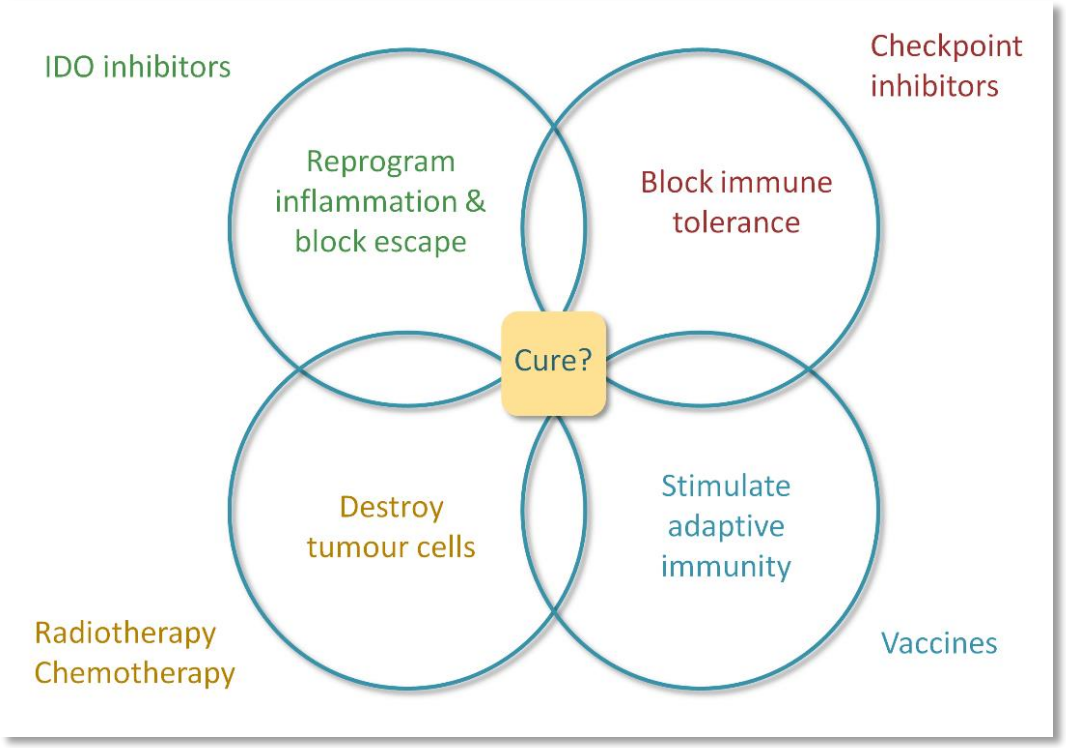

Figure 3: Proposed model for combination therapy in the future, including IDO inhibitors.
Multiple applications are suggested in various combinations that include tolerance blockade (immune checkpoint inhibitors), active immunotherapeutic interventions (vaccines), and classical/targeted chemotherapy or radiotherapy. Adapted from Prendergast, 2014.[26]

In mouse models, the administration of an IDO inhibitor as a monotherapy could retard but not stop tumour growth,[28, 29] so combination strategies are a logical approach. In mice, an upregulation of IDO mRNA was observed after treatment with a PD-L1 inhibitor.[25] Adding an IDO-inhibitor to either checkpoint inhibitors or vaccines might prevent the tumour from escaping the induced immune response by reprogramming the inflammatory climate. Holmgaard et al. elegantly showed that IDO impedes the function of anti-CTLA-4 and anti-PD-1/PD-L1 therapies. Adding an IDO-inhibitor to anti-CTLA-4 therapy significantly increased response rates in B16 melanoma-bearing mice.[30] However, improved tumour control with combinations of immunotherapies has been reported to depend on an increased proliferation and IL-2 secretion of tumour-infiltrating CD8+ T-cells.[25] This indicates that recognition of the tumour by the immune system is required to achieve the full therapeutic effect.

Even though melanoma is a highly immunogenic tumour, not all melanomas elicit a spontaneous immune response. Strategies to first induce an immune response could therefore be promising, and might even be found in currently available treatment options. The mechanism of cell death following treatment with a BRAF inhibitor is not known, but animal models suggest apoptosis over necrosis.

Apoptotic cell death can cause the release of tumour antigens, which in turn could set the stage for more effective immunotherapy.[31] Indeed, BRAF-targeted therapy can increase the expression of melanocyte differentiation antigens, leading to enhanced recognition by antigen-specific cytotoxic T-lymphocytes without compromising lymphocyte function in vitro.[32] Hypothetically, this could reinforce an existing or launch an absent immune response by the host, resulting in more effective immunotherapy afterwards. The combined use of ipilimumab and a BRAF-inhibitor unfortunately caused unacceptable liver toxicity,[33] but sequential treatment might resolve this issue.[34] The same theory might also apply to chemotherapy. The combination of 1MT with paclitaxel did produce improved responses in a murine breast cancer model, compared to paclitaxel or 1MT alone.[35] Yet another possibility is radiotherapy, which is widely believed to act by inducing lethal DNA damage in tumour cells. However, in a B16 melanoma model, mice receiving local tumour irradiation had increased TILs that secreted IFN-γ and lysed tumour cell targets.[36] Even more, the induction of type I IFN by local radiotherapy was reported to be necessary for the therapeutic effect, by enhancing the cross-priming ability of tumour-infiltrating DCs.[37]

Vaccination as a strategy to stimulate the adaptive immune system in the context of tumours has often been tried, but the various vaccination approaches that have been tested in melanoma so far have at best generated moderate effects. It could be worthwhile to investigate the role of IDO in this type of treatment, as IDO-mediated immune suppression can be induced when CpG-rich oligonucleotides (a commonly used vaccine adjuvant) are delivered systemically.[38, 39] Moreover, mature DCs generated for therapeutic vaccination have been described to become IDO-positive and be able to induce FoxP3+ cells at the injection site.[40] It could therefore be interesting to investigate the addition of an IDO-inhibitor. However, IDO-deficient mice were reported to develop a more severe acute pancreatitis upon administration of complete Freund's adjuvant (CFA) compared to wild-type mice.[41] Some caution is thus warranted in future clinical trials assessing IDO inhibition combined with vaccine administration.

Theoretically speaking, IDO inhibitors could also be used in the adjuvant setting. There are no data available from mouse models in this context, but combination therapy of anti-PD-L1 and anti-CTLA-4 was less effective when murine melanomas were allowed to grow larger before therapy was initiated.[25] Our data suggest the continued presence of IDO-based negative feedback mechanisms in high-risk melanoma patients treated with IFN-α2b. Adding an IDO inhibitor might help to counteract this.

Controversies surrounding the clinical potential of IDO inhibition

As outlined in the introduction, IDO is a powerful mechanism of acquired systemic tolerance to foreign antigens, but is not essential for the maintenance of self-tolerance. The effects of IDO appear to be selective, and focused on specific forms of acquired peripheral tolerance. This specificity is a potential advantage in case of pharmacological IDO inhibition, as severe autoimmunity is not expected to be a limiting side-effect.[42] On the other hand, the relative redundancy of IDO raises questions on the efficacy of IDO inhibition.

When we view the idea of IDO blockade in the light of our new data, some remarks have to be made. For one, the in vivo role of IDO+ pDCs in melanoma patients remains unclear. IDO+ DCs with immunosuppressive properties were identified as CD123+ CCR6+ cells, generated from blood monocytes.[43] Neither the IDO expression nor the immunosuppressive effects of these CCR6+ pDCs could be confirmed by another group.[44] One proposed factor that could account for these discrepancies is that the studies identifying IDO expression in DCs used a highly cross-reactive polyclonal rabbit antibody that has been reported to also display non-specific staining.[43, 45, 46] In our research, IDO expression was detected in CD123+ pDCs, but this was not associated with disease progression or survival. Monocytic MDSCs also expressed IDO, but the functional implications are equally unclear. The role of IDO in pDCs and mMDSCs in melanoma should therefore be further investigated.

Some authors have reported positive prognostic effects of IDO expression, causing concern about the consequences of inhibition. Based on the available evidence, IDO seems to mainly play a role in chronic inflammation, and not so much in acute. Depending on the context, chronic inflammation may either promote or suppress neoplastic transformation. Hence, it is conceivable IDO could play a dual role in tumorigenesis by restricting the development of cancers that are driven by chronic inflammation, while promoting the development of tumours that are kept under control by chronic inflammation. This theory is supported by the observation that IDO-knock-out mice were reported to develop more colon carcinomas in a model of induced colitis.[41] In contrast, IDO-deficient mice were more resistant to tumour formation in a chemically-induced skin carcinogenesis model.[47] An alternative hypothesis is that IDO's tumour-promoting role is only evident in patients who already have an active immune response against the tumour, in which IDO can act as a negative feedback mechanism. In patients in whom the tumour is not recognized by the immune system, IDO cannot impede the immune response and might then act as a tumour cell growth inhibitor.[44] The eventual effect of IDO thus seems to depend on the context of its expression, but how this is determined remains subject to debate.

Even though these arguments illustrate that a certain degree of caution regarding the use of IDO inhibition in cancer patients is warranted, history has also taught that a thorough understanding of the mechanism of action of a compound often lags behind its therapeutic utility. The first clinical trials using IDO inhibitors are indeed already ongoing, and results are eagerly awaited.

◆ Future perspectives

1. IDO as a possible predictive marker for response to immunotherapy?

Prognostic markers are useful to make a risk-assessment for an individual patient and can provide insights in the biology of a certain tumour. However, contrary to predictive biomarkers, they do not confer information on the likelihood of therapeutic effectiveness.[48] Over the last few years, immunoprofiling efforts have increased as immunotherapeutic strategies gain importance in the management of melanoma and other cancers. Several (combinations of) markers with predictive or prognostic quality have been suggested, mainly focusing on the tumour microenvironment.[49, 50] Apart from its prognostic properties, IDO expression analysis could also be useful for predictive immunoprofiling, as immunohistochemical IDO-positivity in the tumour microenvironment has been shown to predict response to anti-CTLA-4 therapy in patients with metastatic melanoma.[51] Similarly, overexpression of the IDO1 gene correlated with the effectiveness of ipilimumab in another trial.[52] We have therefore started to collect melanoma tissue samples (FFPE, primary melanoma, sentinel lymph nodes and metastatic melanoma) with a known response to ipilimumab to assess the relevance of IDO expression as a possible marker for response. Others have also demonstrated that lower levels of circulating mMDSCs before initiation of anti-CTLA-4 therapy are associated with increased response.[53, 54] The relevance of the IDO+ mMDSCs we observed could therefore also be evaluated in melanoma patients. These projects might not only provide predictive information, but also help to elucidate the counter-regulatory mechanisms that seem to hamper the efficacy of current checkpoint inhibitors.

2. Endothelial IDO expression

IDO activity has been most extensively studied in dendritic cells, but there is also evidence that IDO expression by endothelial cells plays an important role in immune regulation. The most mature data on this topic come from transplantation experiments, because the first cells that are encountered by a T-cell when moving into a solid organ transplant are endothelial cells. Spontaneous IDO expression has been found in human umbilical vein endothelial cells (HUVECs) and overexpression of IDO by endothelial cells could prevent T-cell activation in vitro.[55] IDO expression in the placenta protects the foetus from attack by maternal lymphocytes, so one could deduce that IDO upregulation in tumour-surrounding endothelium may likewise form a barrier that protects malignant cells from attack by circulating immune cells.

The in vivo importance of endothelial IDO has been demonstrated by experiments in mice, where IDO-expression by endothelial cells could prevent the rejection of cardiac allografts.[56, 57] In humans, IDO expression in monocyte-derived endothelial-like cells has also been described in infantile haemangioma.[58] Moreover, IDO expression in tumour-associated endothelial cells was found to have a positive prognostic effect in patients with renal cell cancer, but the prognostic data were based on qRT-PCR, and not on immunohistochemistry.[59] A recent clinical study also demonstrated that IDO expression, partly by endothelial cells, is an independent risk factor for poor response in melanoma patients treated with anti-CTLA-4 therapy.[51]

In our studies, we have found a remarkable correlation between peri-tumoral endothelial IDO expression in the primary melanoma and IDO expression by high-endothelial venules in the corresponding sentinel lymph node. This raises the question of whether the origin of the peri-tumoral IDO-expressing endothelial cells is vascular or lymphatic. We will therefore perform immunohistochemistry on serial sections of primary melanomas for IDO, CD31 (a vascular marker) and podoplanin (a lymphatic marker).

3. Prospective bio-banking essential in future of translational research

In our studies we observed that in disease-free patients who relapse shortly after sample procurement, a decline in pDCs and a rise in PD-L1+ cytotoxic T-cells can be seen before relapse is clinically detected (Fig. 4). These observations suggest that pDCs and PD-L1+ cytotoxic T-cells might be interesting biomarkers for follow-up of high-risk, disease-free melanoma patients. However, as these data were obtained in a cross-sectional study, confirmation in a prospective longitudinal study with multiple follow-up samples during disease course is necessary before any conclusions can be drawn.

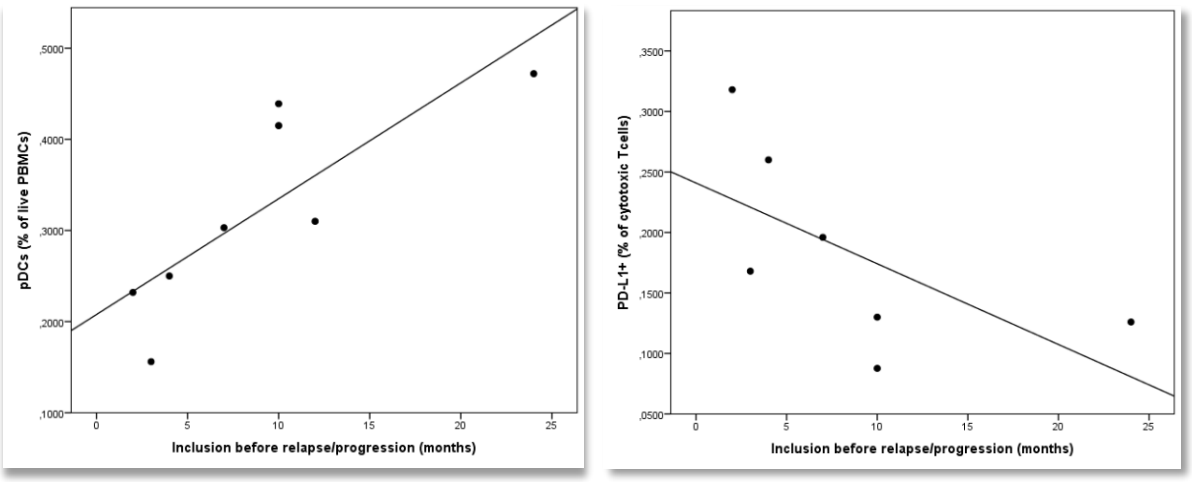

Figure 4: Circulating pDC and PD-L1+ cytotoxic T-cell levels before relapse.
Scatter plots showing a decline in pDCs (left) and a rise in PD-L1+ cytotoxic T-cells (right) in disease-free melanoma patients. The 'zero' time point indicates the moment that disease relapse was diagnosed.

To assess the validity of these observations, we will start to prospectively recruit all high-risk (stage II-III) melanoma patients who are disease-free after surgery for the primary tumour. Blood samples for PBMC isolation will be collected every 3 months, to enable the detection of alterations in cell subset frequencies over time. Functional assessment of systemic immunity by CFSE suppression assays will be done to compare patients who relapse to those who remain disease-free. This will be focussed on pDCs and PD-L1+ cytotoxic T-cells. Plasmacytoid DCs will be isolated by MACS and tested for their expression of markers for activation vs tolerogenicity, as well as their IDO expression. This could identify new targets to stimulate the DC populations that are failing in patients with relapsing disease. The function of PD-L1+ cytotoxic T-cells is unknown. These cells will likewise be separated, to allow for further characterisation. Mart-1 tetramer+ CD8+ TILs have previously been reported to be PD-1+.[60] One hypothesis is that these circulating PD-L1+ cytotoxic T-cells might thus be tumour-specific T-cells, so a tetramer analysis will also be performed.

Similarly, prospective longitudinal PBMC sample collection will be initiated in stage III and IV melanoma patients who start on IFN-α2b or ipilimumab therapy, to further evaluate the role of systemic IDO expression by pDCs and mMDSCs during immunotherapy.

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◆ Dankwoord

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Als derde speler in dit verhaal verscheen Reinhart ten tonele. Hem slechts ‘mijn voorganger’ noemen zou zijn rol oneer aandoen. Je hebt talloze nieuwe ideeën aangereikt, waar nodig de mijne bijgestuurd en met de glimlach mijn vele vragen beantwoord. Ik prijs me gelukkig dat ik van je voorbeeld heb mogen leren.

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Eens de bezetting compleet was, kon de uitvoering beginnen. Dit werk rust sterk op een grote biobank. Het verzamelen van de bloed- en weefselstalen die de bron voor dit translationele onderzoek vormen is een werk zonder einde. Ik ben al het personeel van de polikliniek dermatologie dan ook zeer dankbaar voor de ondersteuning die zij hierin boden. De inzet van de verpleegkundigen, de supervisoren en de collega’s assistenten is en blijft een conditio sine qua non voor dit soort onderzoek.

Onze oncologisch verpleegkundige speelde hierin een centrale rol. An, het plezier en de efficiëntie waarmee je de rekrutering in handen hebt genomen werkten aanstekelijk. Ik heb je inspirerende positivisme erg gewaardeerd. Een bijzonder woord van dank gaat ook naar de melanoompatiënten. Zij waren nagenoeg altijd bereid om deel te nemen aan deze studie, ondanks de vaak moeilijke omstandigheden. Deze mensen verdienen hiervoor een warm applaus.

Met enig vallen en opstaan werden resultaten behaald, papers geschreven en kon tenslotte deze thesis worden samengesteld. Nazicht en deliberatie doen elke tekst wel varen, voor een proefschrift is het niet anders. Dit werk heeft in elk stadium kunnen profiteren van opbouwende kritiek; eerst tijdens de onderzoeksvergaderingen van de DRU, vervolgens door mijn trouwe co-auteurs en tenslotte door de juryleden. Ik ben iedereen die inbreng heeft gehad zeer erkentelijk voor hun bijdrage aan de kwaliteit van mijn thesis.

Zonder financiering blijven ideeën slechts ideeën, en zou van een doctoraat nu geen sprake zijn. Mijn aspirantschap bij het FWO was de aanzet, de fondsen van het Kankerplan via Prof. Bart Neyns waren het vervolg. Ik dank jullie voor jullie vertrouwen en interesse in dit project.

Men denkt wel eens dat het leven van een onderzoeker eenzaam is, opgesloten tussen cellen en toestellen in een labo. Wie in het MRB gewerkt heeft, weet dat het tegendeel waar is. De laboranten, post-docs en doctoraatsassistenten vormen een goed team, met ploeggeest en wederzijdse sympathie. De sfeer waarin ik heb kunnen werken was fantastisch. Ik wens de andere doctoraatsstudenten Ana-Karina, Anesh, Eline en Isabelle heel veel succes bij hun projecten, met als beloning een welverdiend doctoraat. Isabelle, je bent de voorbije 2 jaar uitgegroeid tot meer dan een collega. Ik hoop dat we in de toekomst samen kunnen blijven lachen, en alle grote en kleine problemen op aarde oplossen tijdens de lunch.

Ik heb werkelijk genoten van mijn werk als doctorandus, maar niets is te vergelijken met het plezier dat ik heb met mijn vrienden. De steun, het begrip en het geluk waarvan jullie de bron zijn, geeft mijn leven kleur. We zien elkaar thuis en op restaurant, op weekend en op reis, en telkens weer doet jullie gezelschap me lachen. Mijn nakende meterschap van Ingeborgs' Baby Pluis maakt me trotser dan wat dan ook.

Een ereplaats in dit dankwoord komt toe aan de regisseurs van mijn bestaan. Mama en papa, jullie liggen aan de basis van alle kleine en grote successen in mijn leven. Het warme gezin waarin ik ben opgegroeid heeft me gemaakt tot wie ik ben. Jullie leerden me doorzetten en steeds rustig te blijven. Dankzij jullie heb ik zowel vertrouwen als plezier in alles wat ik onderneem. Ook mijn zus Aude verdient

een medaille. Zusje, zonder het te beseffen leer je me creatiever denken en buiten mijn comfortzone treden. Je onvoorwaardelijke steun sterkt me enorm.

Doorheen de voorbije tien jaar heb ik in de Schreuers een tweede familie gevonden. Ik had me geen betere schoonouders dan Tania en Rodolphe kunnen wensen. Bovendien zijn mijn schoonbroers en –zussen echte vrienden geworden. Mijn eigen grootouders kunnen dit niet meer meemaken, maar dankzij Bonnemaman en opa en oma Bril voelt het alsof ik nog steeds grootouders heb.

Zo kom ik bij de apotheose van dit dankwoord, en die wil ik wijden aan mijn echtgenoot Max. Je kritische oog heeft menigeen van mijn ideeën en teksten verbeterd, je bent een klankbord voor alle beslissingen die ik neem. Maar boven alles ben je mijn beste vriend, mijn partner voor het leven en de papa van onze kleine Roxane. Mijn echte thuis is waar jij bent, met jouw steun kan ik alles aan.

Inès

20 augustus 2015

CURRICULUM VITAE

◆ Curriculum Vitae

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EDUCATION

2012-2015	PhD fellow (FWO fellowship, Ghent University)
2011-2017	Residency Dermatology (Dept. of Dermatology, Ghent University Hospital)
2004-2011	Medical doctor, summa cum laude (Ghent University)
1998-2004	Latin-Mathematics, magna cum laude (Sint-Pietersinstituut Ghent)

POSTGRADUATE COURSES

2013	EADO School “Fundamentals of cutaneous oncology” (EADO, Berlin)
2012	Statistical analysis, advanced course (Ghent University)
2012	Statistical analysis, basic principles (Ghent University)
2008	Basic principles of electrocardiography (Ghent University)

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ORAL PRESENTATIONS

2014	Oncopoint: "Peri-tumoral indoleamine 2,3-dioxygenase expression in melanoma: an early marker of immune resistance?" (University Hospital Ghent)
2014	Wetenschapsdag: "Endothelial indoleamine 2,3-dioxygenase is consistently expressed in the immune micro-environment and influences prognosis." (Het Pand, Gent)
2014	Wetenschapsdag: "Yield of a lesion-directed screening initiative in Flanders, Belgium." (Het Pand, Gent)
2013	8th World Congress of Melanoma: "CD8+ TILs and sentinel lymph node: indications for altered loco-regional immunity" (EADO, Hamburg)
2013	PEARLS Centre of excellence: "Introduction to Ghent University Hospital" (University Hospital Ghent)
2013	PEARLS Centre of excellence: "Outlines of melanoma and pigment cell research in the DRU" (University Hospital Ghent)
2012	AGL Gent: presentation on a facial variant of frontal fibrosing alopecia (Aula, Gent)

MISCELLANEOUS

2012-2015	FWO aspirant

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