Immune modulations during chemoimmunotherapy & novel vaccine strategies - In metastatic melanoma and non small-cell lung cancer

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This review has been accepted as a thesis together with four clinical studies by University of Copenhagen 28th of June 2013 and defended on 10th of October 2013.

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Dan Med J 2013;60:(12) B4774

This thesis is based on the following studies

I) Trine Zeeberg Iversen, Marie Klinge Brimnes, Kirsten Nikolajsen, Rikke Sick Andersen, Sine Reker Hadrup, Mads Hald Andersen, Lars Bastholt and Inge Marie Svane. T-Lymphocyte depletion is correlated to treatment response of Temozolomide in melanoma patients. OncoImmunology 2:2,1-10 February 2013

II) Trine Zeeberg Iversen, Maria Therese Rasmussen, Jon Bjoern, Stine Kiaer Larsen, Sine Reker Hadrup, Mads Hald Andersen, Henrik Schmidt and Inge Marie Svane. Induced lymphocytosis during treatment with Interferon alfa-2b and high dose Interleukin 2 is correlated with treatment outcome in melanoma patients. Manuscript.

III) Trine Zeeberg Iversen, Lotte Engell-Noerregaard, Eva Ellebaek, Rikke Andersen, Stine Kiaer Larsen, Jon Bjoern, Claus Zeyher, Cécile Gouttefangeas, Birte Moerk Thomsen, Bente Holm, Anders Mellemgaard, Per thor Straten, Mads Hald Andersen, Inge Marie Svane (www.clinicaltrial.gov NCT 01219348) Long-lasting disease stabilization in the absence of toxicity in metastatic lung cancer patients vaccinated with an epitope derived from indoleamine 2.3 dioxygenase. Clinical Cancer Research, Tracking Nº CCR-13-1560, In press.

IV) Trine Zeeberg Iversen, Jon Bjoern, Rikke Andersen, Per Kongsted, Per thor Straten, Mads Hald Andersen and Inge Marie Svane (www.clinicaltrial.gov NCT 01543464) Combination of IDO and Survivin peptide vaccine with Temozolomide chemotherapy in patients with stage IV metastatic melanoma. Ongoing phase II clinical study – preliminary clinical data provided only (no manuscript).

INTRODUCTION

Metastatic melanoma remains a significant medical challenge with a rapidly increasing incidence worldwide (1). During the last decade an increasing number of new cases have been reported also in Denmark (1021 new cases in 2001 to 1789 new cases in 2010) (2). The 5-year survival rate for patients with metatatic melanoma is 10-15% with a median overall survival of less than 1 year (3).

For decades, metastatic melanoma has been one of the solid tumours with the most severe lack of therapies to show improvement in overall survival. Available treatments remain insufficient although new drugs demonstrating prolonged survival have emerged recently. The standard chemotherapeutic agent Dacarbazine (DTIC) was approved in 1976 (4), in Denmark replaced by Temozolomide (TMZ) due to the advantage of oral administration (5). In 1998, FDA approval of the first immunotherapeutic agent Interleukin 2 (IL2) for metastatic melanoma patients emerged and was based on a small fraction of patients (5-8%) obtaining durable responses (6). Randomized clinical trials combining different chemotherapy regimens have not proven better than single agent DTIC/TMZ (7). Moreover, combination of chemotherapy and IL2-based regimens has shown important antitumour activity nevertheless, no demonstration of additional survival benefit when compared to single agent chemotherapy (8).

In 2010 advances in the field of immunotherapy lead to the approval by FDA of an anti-CTLA-4 antibody, Ipilimumab, which has demonstrated prolonged overall survival in metastatic melanoma patients (9). Moreover, newly progress within targeted therapies have resulted in FDA approval (in 2011 and June 2013 respectively) of the BRAF inhibitors Vemurafenib and Dabrafenib for patients harbouring a V600 BRAF mutation (10,11). Furthermore, as

of June 2013 the first MEK inhibitor Trametinib was FDA approved for MM patients harbouring a BRAF mutation. These successes have paved the way for both immunotherapy and targeted therapy in melanoma and intense research for further improvements is ongoing. Currently, the development of immune checkpoint blockade such as antibody against programmed death 1 (anti-PD-1) Nivolumab (12) and its ligand (anti-PD-L1) (13) has shown impressing overall response rates (RR) in phase I/II trials. Furthermore, the engineered IgG4 PD-1 antibody Lambrolizumab has been tested in a multicenter phase 1 study demonstrating RR of 38% across all evaluated patients raising to RR of 52% in the subgroup of patients receiving the highest dose (10 mg/kg every 2nd week) of Lambrolizumab (14). Moreover, the combination of anti-CTLA-4 and anti-PD1 antibody treatment in melanoma seems promising in terms of toxicity profiling, RR and durability of response (15-17). In the era of targeted therapies combination of BRAF and MEK inhibitors are exciting and are currently under clinical investigation (18). Widespread mutational status of RAS-RAF-MEK-MAPK and CKIT pathways in melanoma patients appears equally important for future trials. A different approach in treating melanoma patients is the adoptive cell therapy (ACT) where large numbers of autologous tumour-specific T cells in combination with non-myeloablative chemotherapy and IL2 are infused to the patients (19,20) an experimental design in which we similarly at CCIT have gathered promising clinical experience (21).

Immunological mechanisms are of importance in melanoma pathogenesis since the tumour expresses antigens recognized by T cells. Activation of immune cascades can lead to spontaneous tumour regression which has been evidenced by invasion of T cells into tumour tissue (22). New insight in melanoma biology within the last few years has lead to recent impressing clinical advances and may importantly segregate the treatment for different subtypes of melanoma i.e. cutaneous, mucosal and ocular melanoma. However, the majority of patients will eventually die of metastatic disease and new strategies to defeat melanoma are therefore still needed.

Non small-cell lung cancer

Lung cancer is one of the leading causes of cancer deaths in both men and women worldwide, with non small-cell lung cancer (NSCLC) accounting for up to 85% of the cases (23). At time of diagnosis, most patients present with inoperable, advanced stage III-IV disease, with poor prognosis and a 5-year survival rate of less than 5% (24,25). Furthermore, lung cancer is still increasing both in prevalence and in mortality worldwide (26). Chemotherapy and/or radiation are effective treatments in most NSCLC patients due to the often fast growth rates of the tumours (27). Recently, tyrosine-kinase inhibitors (TKI) for patients with tumours harbouring either activated EGFR or EML4-ALK translocation have emerged. Despite progress in personalized treatment modalities, acquired resistance to targeted therapy is a huge clinical challenge. Thus, to date available anti-neoplastic treatments for metastatic NSCLC offer only temporary disease control (28,29).

Immunotherapy has set a new paradigm for the treatment of MM and recent research hold the promise of immunotherapy to show similar clinical efficacy in NSCLC (30). Phase III studies of different vaccines strategies in both adjuvant and metastatic settings are underway (31). In early clinical trials of immune checkpoint blockade with Ipilimumab (30) and PD-1/PD-L1 antibodies have shown clinical responses in patients with advanced NSCLC both squamous and non-squamous (12,13). A recent update on Nivolumab treatment in NSCLC patients has shown durable response rates and large phase III clinical studies investigating PD1/PDL1 antibodies are being set up (32,33). Furthermore, recent data suggests that tumour specific CTLs are crucial for efficacy of immunomodulatory antibodies in patients with lung cancer (34). This leads to a new way of approaching NSCLC in terms of response evaluation (RECIST vs. irRC), the necessity of implementing immune monitoring assays and management of toxicity profiles.

The immune system

An enormous variety of cells and molecules form the complex dynamic network of the immune system. In general, immune responses can be divided into the innate and the adaptive response. Innate immunity serves as first line defence against pathogens and is mediated by phagocytic cells (monocytes, macrophages and neutrophils), natural killer (NK) cells, dendritic cells (DC) and cells releasing inflammatory signals (basophils, mast cells and eosinophils). Adaptive immunity consists of a humoral branch mediated by B cells and a cellular branch mediated by T cells (35).

Antigen presentation

The dendritic cell (DC) is the most powerful antigen presenting cell (APC) in the immune system. To initiate an adaptive immune response the DC process and present the antigen on its cell surface combined with major histocompatibility complex (MHC) molecules, known as human leucocyte antigen (HLA) in humans. MHC molecules come in two distinct types, class I and class II. The MHC I present short peptides (8-10 amino acids) from mainly endogenous derived antigens whereas the MHC II binds longer peptides (15-24 amino acids) from mainly foreign derived peptides (36). The DCs take up local antigens and migrate to the lymph nodes to present the antigen to the naïve T cells. Since the DCs can activate both CD8+ T cells through MHC class I expression and CD4+ T cells through MHC class II expression, they are capable of cross-presentation (37). Both co-stimulatory signals (i.e. B7, ICOS) and co-inhibitory signals (i.e. CTLA-4, BTLA, LAG-3, PD-1) are of importance to balance between T cell activation and tolerance(38).

T lymphocytes

T cells arise in the bone marrow and migrate to thymus for maturation where they differentiate to αβ CD4+ and CD8+ (~95%) and to γδ (~5%) T cells (39). T cells are commonly divided into four groups; T Naïve (CCR7+CD45RA+), central memory (TCM) (CCR7+CD45RA-), effector memory (TEM) (CCR7-CD45RA-) and an intermediate effector memory population (TEMRA) (CCR7-CD45RA+) each group representing distinct differentiation status (40).

Cytotoxic CD8+ T cells (CTL) are able of killing target cells directly when forming a complex comprising the T cell receptor (TCR) and the specific peptide-MHC complex. As a consequence, lytic granules containing cytotoxic compounds (perforin and granzymes) are released thus killing the target cells. CTL also produces a number of cytokines including tumour necrosis factor α (TNFα) and interferon gamma (IFNγ) triggering apoptosis and leading to T cell mediated killing. To this end, IFNγ enhances the expression of cell death surface receptor (Fas) in target cells resulting in increased lysis via Fas - Fas ligand interactions (35).

Natural killer cells

Like DCs, NK cells are linked to both the innate and the adaptive immune system. NK cells are characterised by expression of CD45+CD3-CD19-CD56+CD16+ and represent a unique lymphocyte population. NK cells are able to recognize tumour cells (independent of MHC antigen expression) and kill these either directly or by IFNy release. However, the exact role of NK cells in regard of the anti-neoplastic effects in human cancer is debated (41). As recently described, effective tumour rejection is dependent on a two-way immune cross-talk of the innate and the adaptive immune system, firstly by direct killing mediated by T- and NK cells and secondary by other immune cells, i.e. DCs, macrophages and neutrophilic granulocytes in the tumour microenvironment (42).
Tumour associated antigens

Tumour associated antigens (TAAs) are proteins expressed by tumours and recognized by CTLs. Malignant transformation generates an altered protein repertoire and enormous effort has been spent on identification and characterization of TAAs. Spontaneous CTL responses against TAAs have been demonstrated in both peripheral blood and in tumour lesions from cancer patients (43). The identification of TAAs has led to development of several new strategies for immunotherapy of cancer in an attempt to elicit or boost CTL responses against TAAs (44). TAAs are divided into four groups (mutation-, cancer testis-, tissue differentiation- and overexpressed antigens) based on their expression profile and origin (35).

Immune suppressive cells

Immunosuppressive mechanisms are important to evade self-destruction and autoimmune diseases. A complex network of regulatory myeloid- and lymphoid derived cells are well-described. Regulatory cells are believed to be part of the limited success of currently applied immunotherapeutic strategies due to their key role in suppression of anti-tumour immunity (45).

Regulatory T cells

Regulatory T cells (Tregs) are a distinct population of CD4+ T cells characterized by the expression of CD4+CD25highCD127-FoxP3+ (transcription factor forkhead box P3). In tumour settings Tregs recognize tumour antigens as self-antigens and provide immune tolerance towards the cancer cells (46). Anti-tumour immune responses can be suppressed by Tregs (47) and the impact of immune suppression mediated by Tregs in advanced melanoma and NSCLC has been reported previously (48,49). Strategies to deplete Tregs have been explored with the IL2 diphtheria toxin conjugate suggesting that short term decreases in Tregs were associated with increased T-cell responses (50). Yet another study using diphtheria conjugate showed a decrease of both Tregs and T effector cells which might be a matter of different dosing regimens applied (51). Furthermore, an anti-IL-2R monocloncal antibody (Daclizumab) has the potential of Treg suppression and is currently tested in a clinical set-up (52).

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of immature cells comprised of myeloid progenitor cells and immature macrophages, monocytic and granulocytic cells. Human MDSCs are characterised by the common myeloid surface marker CD33+ and the lack of mature markers of myeloid and lymfoid cells. A monocytic MDSC (Mo-MDSC) population has been well defined and can be distinguished by expression of CD3-CD19-CD56-HLA-DRlowCD33+CD11b+CD14+ (53). Studies have demonstrated a higher frequency of Mo-MDSC in the peripheral blood of metastatic melanoma patients when compared to healthy donors (HD) (54,55). Similarly, MDSCs are also found elevated in NSCLC patients and a high level of MDSCs are associated with a decreased number of CD8+ T cells as compared to HD (56). As a strategy to inhibit the function of MDSCs in vivo a blockade of IL4Ralfa signaling has been suggested. As a consequence cell mechanisms of tumoral immune escape are inhibited (57). Furthermore, some anti-neoplastic drugs like the TKI Sunitinib has been shown to down-regulate the level of MDSCs in peripheral blood (58).

Immune escape mechanisms

Immune escape is one of the hallmarks in cancer progression and development of metastases. The immune escape phase is characterized by the lack of the immune system to eliminate malignantly transformed cells. The tumour cells uses a variety of strategies to avoid elimination and a full understanding of this complex interplay within tumour and the host immune system is far from reached (59). Within the tumour cell down regulation of “self” antigen and/or MHC molecule expression is a mechanism of defence (60,61). In addition, tumour cells have several counterattack methods to defeat immunity which include secretion of cytokines e.g. Interleukin10 (IL10) and TGF-ß. These cytokines are associated with poor prognosis and lack of response to immune therapy partly because TGF-ß expression is known to facilitate expansion of Tregs (62). Moreover, tumour cells are able to up regulate Fas ligand which facilitates cancer cells being resistant to Fas-induced cell death mediated by CTLs (63). Yet another mechanism by which tumour cells escape immunity is by inhibition of effector cells by up-regulation of inhibitory ligands including PD-L1, CTLA-4 and LAG-3 (64,65). Finally, the abundance of suppressive factors may also foster the recruitment and differentiation of various immune suppressive cells. Overall, better understanding of immune escape mechanism and hence limiting tumour cells development of cascade inhibitory signalling and immune suppression may lead to more effective immunotherapy in the near future.

Indoleamine 2.3 dioxygenase (IDO) mediated T cell suppression

A newly discovered option for the cancer cells to avoid CTL mediated killing is by over-expression of IDO, which is a tryptophan (Trp) catabolizing enzyme. Trp is an amino acid essential for T cell activation and proliferation. Thus depletion of Trp by up-regulation of IDO in the local tumour micro-environment result in T cell anergy and apoptosis (66). Both the Trp depletion and the development of kynurenine (Kyn) metabolites have direct and indirect inhibitory effects on T cells. In healthy conditions, it has been shown that IDO is crucial for creating maternal tolerance during pregnancy and in maintaining tolerance towards transplanted tissue. Furthermore IDO is important in protection against development of autoimmunity and allergic reactions. In contrast, IDO has undesirable effects in the context of metastatic cancer by suppressing T cell immunity. It has been demonstrated that patients with different tumour types have elevated Kyn/Trp ratio compared to HD suggesting that IDO activity is increased in cancer patients (67). Moreover, the ratio of Kyn/Trp in serum has been proposed by others as a non-invasive, in vivo biomarker for evaluating IDO inhibitors in the clinic (68).

IDO expression in primary tumour

Enhanced expression of IDO is seen in primary tumour lesions of different cancer types. IDO expression can be detected by immunohistochemistry in both the cytoplasm of tumour cells and in the tumour stromal cells. At the site of primary cancers IDO is believed to inhibit the effector phase of the immune response by directly suppressing the T cells (69) since tumours expressing IDO have been correlated with impaired lymphocyte infiltration (70). Negative correlation of IDO expression and clinical outcome has been demonstrated in different cancer types e.g. ovarian cancer (71), glioblastoma (72), colorectal cancer (73) and endometrial cancer (74). Moreover, it has been demonstrated that IDO expressing tumours had an elevated frequency of metastases (73). Furthermore, expression of IDO has been demonstrated in both melanoma and NSCLC (69) suggesting IDO as a relevant target in a broad spectrum of different solid tumours.

IDO expression in tumour draining lymph nodes

Tumour draining lymph nodes (TDLN) are a site of contact between TAA and the adaptive immune system, since APC migrate to TDLN after antigen uptake. In melanoma as well as other solid tumours TDLN also normally represents the initial site of metastases (75). In TDLN it has been demonstrated that IDO can be expressed by APC. IDO expression by regulatory cells drives the TDLN towards a tolerogenic microenvironment instead of a site of active immunization processes (66). Furthermore, IDO expressing APC in TDLN are believed to suppress the priming phase of the immune response to TAA and maybe even create systemic tolerance (66). Accumulation of IDO expressing cells in TDLN (76) has been correlated to decreased long term survival in melanoma patients (77). Of importance, only few cells constitutively express IDO in normal lymphoid tissue except in the gastrointestinal tract where IDO is expressed in the epithelial cells (78,79).

IDO specific T cell response, Treg and NK

The IDO pathway is linked to Treg biology, since IDO expressing DCs induce the differentiation of naïve CD4+ cells towards a FoxP3+ phenotype (80,81) Moreover, resting Tregs have been shown to elicit suppressive behaviour (82). Previously, it has been demonstrated that cancer patients do possess spontaneous IDO peptide specific T cell responses, which are able to recognize and kill both IDO positive tumour- and DCs (83,84). In addition, IDO specific CD8 T cells were shown to boost immunity against TAAs by eliminating IDO regulatory cells, which, in turn, lead to a decrease in Tregs (84). The boosting of IDO specific immunity could have both direct and indirect effects. Firstly, these T cells may directly kill IDO cancer cells. In addition, they may function by eliminating suppressive immune cells. Recently, it has been suggested that IDO as part of an immune-evasion strategy induces down-regulation of cell surface NK receptor expression (41,85). The interplay of various cells in the tumour microenvironment, i.e. IDO tumour cells, IDO Tregs, stromal cells, NK cells and the associated immune responses mediated by CD8+ and CD4+ T cells is complex. Better understanding of these mechanisms might facilitate therapeutic strategies of targeting IDO.

IDO as an anti-neoplastic target

Clinical investigation of IDO inhibition in phase I dose-escalating trials have been initiated for patients with metastatic solid tumours. Results from these clinical trials of IDO inhibitors such as 1-methyl-D-tryptophan (1-MDT) and INCB024360 are still awaited (86,87). Lately, combination studies of 1-DMT and Docetaxel for patients with solid tumours (NCT01191216) and the combination of INCB024360 and Ipilimumab for melanoma patients (NCT01604889) have started patient recruitment. The targeting of IDO through small molecule inhibitors versus the induction of CTLs naturally differs. The benefit of a vaccine strategy may be the induction of long-lasting IDO specific memory T cells. Hence, in theory these specific memory cells might possibly become re-activated and recruited to tumour site when needed.

Therapeutic vaccination

In spite of all therapeutic advances made recently in melanoma and NSCLC, there is still a lack of adequate disease control using conventional therapies (88). Immunotherapy has the ability to activate the host's cytotoxic CD8+ T cells and these immune cells might infiltrate the tumour and mediate elimination of cancer cells. Thus, therapeutic cancer vaccines have the potential to induce long-lasting, tumour specific immune memory although in terms of treating metastatic cancer results have been somewhat disappointing. Promising pre-clinical data still remains translated into large randomized vaccine trials showing innovative, safe and effective therapeutic gain.

The simplest vaccine strategy i.e. targeting only one or few antigens can be done by peptide vaccines. Effectiveness relies on sufficient antigen uptake and presentation, which is potentially enhanced by the use of immunogenic adjuvants. Hence, targeting universal tumour antigens combined with effective adjuvants and suitable agents to counteract regulatory mechanisms might improve the outcome of peptide vaccines (89). Some of the most frequently applied adjuvants are low dose IL2, Thymalfasin, Interferon, Montanide and GM-CSF (90). Historically, the use of chemotherapy in combination with immunotherapy was avoided due to the risk of immune inhibition. However, recent evidence states that chemotherapy-induced mechanisms such as enhanced antigen presentation, increased sensitivity in killing of tumours cells and the depletion of suppressor cells appears to be a promising method of enhancing therapeutic efficacy (91,92). Recently, Rosenberg et al. have shown that the addition of lymphodepleting cytotoxic regimens in adoptive T-cell transfer trials for melanoma patients have lead to impressive clinical responses (93) implying that chemotherapy may provide a window of enhanced responsiveness to immunotherapy.

Objectives

This thesis comprises two studies in metastatic melanoma (MM) patients in which blood samples have been obtained during standard treatments; Temozolomide (TMZ) chemotherapy and Interferon-α2b/Interleukin2 (IFNα/IL2) immune therapy.

Furthermore, the thesis contains a finalized clinical study of peptide vaccination with an HLA-A2 restricted epitope derived from indoleamine 2.3 dioxygenase (IDO); a phase I trial in metastatic non small-cell lung cancer (NSCLC) patients and presentation of preliminary data from an ongoing phase II trial in metastatic melanoma patients.

THE AIMS OF THE THESIS HAVE BEEN TO:

Investigate changes in immune parameters during standard treatments and their possible correlation with clinical efficacy by assessing changes in frequency and absolute counts of different immune cells before and after treatment with TMZ chemotherapy and by evaluating changes in different immune cells before and after treatment with IFNα/IL2 immune therapy and by correlating

changes in immune cells to clinical benefit of abovementioned anti-neoplastic treatments

Evaluate the feasibility of IDO as an anticancer vaccine target in cancer patients by investigating the targeting of IDO by a synthetic peptide vaccineand assessing safety and tolerability of an IDO derived peptide vaccine and evaluating clinical response and immunity in metastatic NSCLC and MM patients after treatment with an IDO peptide vaccine.

CONFLICTS OF INTEREST TO DECLARE

None of the authors have conflicts of interests to declare. It should however be noted that Mads Hald Andersen and Per thor Straten have filed a patent application based on the use of IDO in peptide vaccination. The rights of the patent application have been transferred to the University Hospital at Herlev according to Danish Law of Publich Inventions at Public Research Institutions.

Study overview
In this section, each study is presented with a description of the patients enrolled, the methods used for evaluation of endpoints and the different treatments applied

Study I – TMZ treatment in MM patients
Evaluable patients: 40
Treatment: 150 mg/m2 TMZ day 1-7 and 15-21 in a 28 day cycle
Acquisition of blood samples: 30 ml at pre-treatment, after the 1st and the 2nd cycles of TMZ
Methods used for access of clinical responses: CT scan (RECIST 1.0)
Methods used for immunological responses: Flow cytometry and MHC multimer encoding

Study II – IFNα/IL2 treatment in MM patients
Evaluable patients: 35
Treatment: Week 1: 300 µg IFNα, Week 2: IL2 (decrescendo regimen) Week 3: Recovery
Acquisition of blood samples: 100 ml at pre-treatment, after the 1st and the 2nd cycles of IFNα/IL2
Methods used for access of clinical responses: CT scan (RECIST 1.0)
Methods used for immunological responses: Flow cytometry and MHC multimer encoding

Study III – IDO peptide vaccinations in NSCLC patients
Evaluable patients: 15 HLA-A2 positive
Treatment: 1 sachet Aldara and vaccines containing 100µg IDO peptide mixed in 900 µl Montanide
Acquisition of blood samples: 100 ml at pre-treatment and subsequently every 3rd months until PD
Acquisition of sera: 8 ml at pre-treatment and subsequently every 3rd months until PD
Methods used for access of clinical responses: CT scan (RECIST 1.1)
Methods used for immunological responses: HLA tissue typing, Flow cytometry, Elispot, T cell culturing, cell sorting, cytotoxicity assay, tetramer staining, immunohistochemistry and HPLC.

Study IV – IDO/Survivin peptide vaccinations combined with TMZ in MM patients
Ongoing patient recruitment: 31 patients have been screened
Evaluable patients: 7 HLA-A2 positive out of 30 planned
Treatment: 1 sachet Aldara, 75µg Leukine sc and vaccines containing 250µg IDO and 250µg survivin peptide mixed in 500 µl Montanide, alternating with TMZ 150 mg/m2 every 2nd week
Acquisition of blood samples: 100 ml at pre-treatment and subsequently every 3rd months until PD
Acquisition of sera: 8 ml at pre-treatment and subsequently every 3rd months until PD
Methods used for access of clinical responses: PET/CT scans (PERCIST 1.0 / RECISIT 1.1)
Methods used for immunological responses: HLA tissue typing. No other immune analyses have been performed yet.

HLA restriction
Tissue typing was performed at the Laboratory for Tissue Typing at Copenhagen University Hospital at Rigshospitalet prior to inclusion. In the peptide vaccination trials only patients harbouring the tissue type HLA-A2 were eligible, due to the HLA restriction of the peptide sequences used for vaccine generation:

IDO-5 peptide, A - 9 – L	HLA-A2:	ALLEIASCL
Sur1M2 peptide, L - 9 - L	HLA-A2:	LMLGEFLKL

The clinical significance of HLA phenotype in cancer patients has been widely investigated. In NSCLC patients (stage I), it was recently described that expression of HLA-A2 was an unfavourable prognostic factor (N=695) (94), which was supported in another smaller NSCLC study (N=204) (95). In melanoma, treatment efficacy is thought to be HLA independent. In a retrospective analysis of HLA subtyping in patients treated with Ipilimumab, the hypothesis that Ipilimumab-treated patients with advanced melanoma have similar outcomes regardless of their HLA-A*0201 status was supported (96). Similarly, in a recent vaccine study it was demonstrated that clinical outcome of the vaccine was independent of HLA-A2 allele type compared to a control group (N=553) (97). More knowledge and better standardization of the methods used for tissue typing (serological typing/genotyping) and the relation of specific tissue types and clinical impact in cancer patients are warranted.

Treatments

Temozolomide

Temozolomide (TMZ) is a cytotoxic alkylating chemotherapy used in the treatment of metastatic MM. TMZ has the advantage of oral administration and penetration of the blood-brain barrier with comparable efficacy to DTIC (5,98). TMZ monotherapy is associated with an objective response rate of 4-20% in this patient group (7,99-101). In Denmark TMZ is used as systemic therapy for metastatic MM in selected patients. The treatment schedule of TMZ is 150 mg/m2 given at day 1-7 and day 15-21 in a 28 day cycle which is the standard dosing.

Interferon-α/Interleukin2
Immunotherapy with Interferon-α-2b/Interleukin-2 (IFNα/IL2) has in Denmark, among other countries, been the preferred immunotherapy for the last two and a half decades. The objective response rate of IL2 treatment is 15% with durable complete responses in 5-8% (6,102). The standard treatment of high dose IL2 (intravenously (iv) administered) and interferon alfa-2b (IFNα) (subcutaneously (sc) administered) used in Denmark is the "decrescendo" regime which is given as 1st line systemic treatment

in selected stage IV MM patients in fit medical condition (103). The treatment consists of 300 µg sc administered pegylated (PEG) IFNα on day 1, iv IL2 18 MU/m2 in 6, 12 and 24 hours on day 8-9 respectively, and with iv IL2 4.5 MU/m2 in 24 hours on day 10-12 respectively.

Imiquimod
Imiquimod (Aldara®) is a cream used for topical treatment of non-melanoma skin cancer (e.g. basal cell carcinoma) where it induces tumour regression. Aldara is widely used as an immune response modifier due to the ability of activating APCs through binding to toll-like receptor 7 (104). Since Aldara is known to cause activation of APCs in the dermal layers of the skin thus trigger antigen presentation and cytokine release it serves as an immunologic adjuvant. 1 sachet containing 5% Imiquimod was applied and covered by a patch in 8 hours prior to sc vaccine administration in the same area of the skin.
Montanide

The immune system will often be non-responsive to any antigen administered in a soluble "naked" form. Conversely, the same antigen may initiate a strong immune response if administered with an immunostimulating agent (90). An adjuvant is not considered a real drug but is important for activating the innate immune system whereas the peptide is used for generating an antigen specific immune response i.e. activating the adoptive immune system. A common adjuvant for peptide vaccines has been Montanide (Seppic, Inc., Paris, France). Repeated multipeptide vaccination mixed in Montanide has been shown to induce dermal lymphoid aggregates with a predominant infiltration of T cells (105). The peptides applied within our studies were formulated in Montanide.

IDO5 peptide
Preclinical toxicology analyses in mice were performed prior to the use of IDO5 peptide for humans. The toxicology assays were designed in order to analyse potential side-effects of a peptide given as repeated subcutaneous injections. The toxicology report "Preclinical study of vaccination with human peptide mixes in C57B16/J mice" is attached (Appendix A). The results showed no impairment of health observed in any of the animals over the experimental period exceeding 2 months. At injection site was found local reactions e.g. redness and swelling in some of the animals.

Survivin peptide
Survivin antigen is over-expressed in most human neoplasms but not expressed in normal differentiated tissues. Survivin acts as an inhibitor of the apoptosis protein family. Molecules involved in apoptosis represent potential diagnostic markers and therapeutic targets. Studies have shown that cancer patients elicit spontaneous T cell reactivity against survivin, hence survivin is considered as a universal target antigen for cancer immunotherapy (106-108). Since survivin is over-expressed in most human tumours including 70-100% of malignant melanomas, this antigen is of particular interest as immunotherapeutic target for MM patients (109).

Peptide deliverance
The IDO5 peptide (HLA-A2 sequence: ALLEIASCL) and survivin peptide (Sur1M2) (HLA-A2 sequence: LMLGEFLKL) were synthesized by chemical synthesis (the preparation involved no materials of human or animal origin) for a purity of >97% and was delivered in dry vials (Polypeptide Laboratories, Strasbourg, France). The proceeding manufacturing of the vaccine product was performed in haematological laboratory according to §39 approval from the National Board of Health and following GMP acquirements. The mixing of the peptides with Montanide was done at the outpatient clinic at the Department of Oncology shortly before administering the vaccine to the patients.

Granulocyte-Macraphage colony stimulating factor
Granulocyte-Macrophage colony stimulating factor (GM-CSF) (Leukine®) is a cytokine that functions as a growth factor for white blood cells. GM-CSF stimulates the bone-marrow stem cells to produce granulocytes and macrophages. GM-CSF is approved for the stimulation and production of white blood cells. Simultaneous application of GM-CSF as an adjuvant in clinical oncology studies of cancer vaccines have shown long term survival of patients with solid tumours correlated to immune responses (110-112). Recently, a randomized phase II trial in advanced MM patients ipilimumab +/- GM-CSF has shown improved OS in favour of the Ipilimumab and GM-CSF arm with no significant differences in toxicity among the two arms (113). Moreover, new immune therapeutic strategies might evolve such as oncolytic virotherapy mediating an anti-neoplastic effect through infecting and killing cancer cells while stimulating tumour specific immune responses (114). A recent phase III melanoma (stage IIB/C, IV1Ma) study (OPTIM trial NCT00769704) has exploited the cancer-killing virus talimogene laherparepvec (T-VEC) engineered to replicate in tumour tissue when injected directly into cutaneous/subcutaneous lesions. The T-VEC is encoding a gene for GM-CSF production thus promoting local immune reactivity towards the tumour, in fact, responding patients had regression in both injected and non-injected lesions opening up for a possible secondary (abscopal) immune-mediated anti-tumour effect (115,116).

Clinical evaluation

Toxicity

Patients were systematically evaluated according to common terminology criteria adverse events (CTCAE) version 3.0 (117). Serious adverse events (SAEs) were reported to the National Board of Health and the Ethics committee at the Copenhagen Capital Region, Denmark according to Danish law requirements. The toxicity registration was part of the monitoring plan assessed in collaboration with the GCP Unit, University Hospital at Bisbebjerg, Denmark.

Response evaluation criteria in solid tumours
Response evaluation criteria in solid tumours (RECIST) and PET (positron emission tomography) response evaluation criteria in solid tumours (PERCIST) are the golden standards for evaluation of response in clinical trials using computed tomography (CT) and/or PET/CT. Patients enrolled in the trials were scanned prior to inclusion (baseline scan) and succeeding every 3rd month.

Patients included in study III were monitored by the use of CT scan evaluated according to RECIST version 1.1 (118). The patients included in study IV were monitored by the use of PET-CT scans evaluated according to PERCIST and RECIST version 1.0/1.1 respectively (119). In study IV a magnetic resonance (MR) scan of the brain was performed at baseline in order to diagnose brain metastases.

Immune evaluation

Peripheral blood monocytic cell

Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by gradient centrifugation by Lymphoprep technique. Isolated cells were frozen immediately with 10% DMSO and 90% humanised AB-serum and stored at -140º Celsius.

Flow cytometri
Flow cytometry analyses were carried out on a FACS Canto II cytometer (BD) Biosciences. Briefly, PBMCs were thawed and then labelled for surface staining with fluorchrome–conjugated antibodies and the relevant isotypes as matched control antibodies. Fox-P3 and isotype controls were used for intracellular staining in which cells were fixed and permeabilized using a fixation/permeabilisation kit according to manufacturer's instruction. For dead cell marker we used near IR fluorescent reactive dye. In general 100,000 – 150,000 lymphocytes were collected and gated on forward and side scatter profiles for analyses.

Elispot
PBMCs were used to perform either directly Elispot analyses or in-direct analyses after 1 week of pre-stimulation with the peptide. Nitrocellulose bottomed 96-well plates were coated overnight with IFNy capture mAb. The wells were washed, blocked by X-vivo medium and the effector cells were added in duplicates at two different cell concentrations, with or without the peptide. The plates were incubated overnight. The following day, medium was discarded and the wells were washed prior to addition of the relevant biotinylated secondary antibody. The plates were incubated at room temperature, washed, and avidin-enzyme conjugate was added to each well. Plates were incubated and the enzyme substrate NBT/BCIP was added to each well and incubated at room temperature for 5–10 min. Upon the emergence of dark purple spots, the reaction was terminated by washing with tap water. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers). Elispot responses were considered positive when the numbers of IFN-γ secreting cells were at least 2-fold above the negative control (medium) and with a minimum of 50 spots detected.

Combinatorial encoding with MHC multimers
Cells were thawed in Pulmozyme buffer and washed twice, then re-suspended in FACS-PBS buffer and distributed into 96-well plates before centrifugation. A panel of MHC multimers (with a specific two-colour combination for each antigen-peptide specificity) was applied. 2uL per specificity produced were mixed with cells and incubated for 10 min at 37ºC, relevant fluorochrome–conjugated antibodies and dead cell marker (Near IR) we added for further incubation in 30 min at 4ºC. Analyses were recorded on an LSRII SORP flow cytometer and data were analyzed with FACS Diva Version 6.1.3, BD bioscience.

Immunohistochemistry
Available formalin fixed paraffin-embedded samples of NSCLC tumour specimens were collected for immunohistochemical (IHC) studies. IHC evaluation, on 3 µm thick sections, was performed using the IDO antibody (Anti-IDO, clone 1F8.2, Millipore) following the manufacturers instructions. The sections were counterstained with haematoxylin. As control for IDO staining tissue samples from placenta (syncytiotrophoblasts cells) known to stain positive for IDO were applied.

Cytotoxicity assay
Tetramer staining was performed in PBS +2% FCS for 15 minutes at 37°C, followed by antibody staining for 30 minutes on ice. The tetramers were prepared using the MHC-peptide exchange technology as described (120). Conventional 51Cr-release assays for CTL-mediated cytotoxicity were carried out as described (121).

High performance liquid chromatography
Sera from patients were obtained to perform high performance liquid chromatography (HPLC) analyses. Nine ml of blood were drawn in a dry vial and spun down at 3000 rpm in 10 minutes. Sera were aliquoted in 1.8 ml Nunc cryo-preservation vials and stored at -80° C freezer. IDO activity was estimated by quantifying tryptophan (Trp) and its metabolite kynurenin (Kyn) in patient sera, essentially as previously described (122,123). Briefly, 100 µL thawed serum were diluted 1:2 with 0.05M KOP4 buffer PH 6.0, followed by protein precipitation with TCA 2M. Trp and Kyn were then identified in 100 µL supernatant by high-performance liquid chromatography (HPLC) (LC 10 AvP system, Shimadzu, Duisberg, Germany) using a C18 column (ReproSil-Pur Basic®, GmbH, Entringen, Germany) and a 3% Acetonitrile (ACN) 0.05% trifluoroacetic acid (TFA) isocratic gradient over 30 min at a flow rate of 0.25ml/min. Results were calculated from peak areas and expressed as Kyn µM / Trp mM ratios (mean of triplicate or duplicate measurements) (122).

Logistics and Contributions
Authoring and legal approval of the protocols were performed by Professor, MD Inge Marie Svane (Study I) and by the author (Study II, III and IV). The task as principal investigator was performed by the author whereas the task as sponsor was performed by Professor Inge Marie Svane. Clinical assessment, toxicity registration and vaccine treatment in the studies III and IV was done by the author and PhDs, MDs Lotte Engell-Noerregaard and Eva Ellebaek plus fellow PhD students, MDs Rikke Andersen and Per Kongsted. The case report forms and GCP requirements in general were performed by the author in collaboration with head of research nurses Birgitte Christiansen. The evaluation of PET/CT scan and clinical assessment were done by the author in collaboration with MD, PhD Helle Hendel at the Department of Nuclear medicine and the Department of Radiology, and by MDs, PhDs Bente Holm, Anders Mellemgaard and Inge Marie Svane.

All clinical trials described in this thesis were accepted by the Danish Legal Authorities. The studies have been conducted in accordance with the Helsinki declaration and monitored according to GCP requirements. All patients have provided written informed consent prior to inclusion. The studies were accepted by the local Ethics committee at the Capital Region of Denmark (Study I: H-A-2007-0124 and study II: H-4-2010-092), by the Danish Data Protection Agency and by the National Board of Health. The vaccination studies were registered at www.ClincalTrials.gov (Study III: NCT01219348 and study IV: NCT01543464).

Patients enrolled in study I were treated at the Department of Oncology, University Hospitals at Herlev (supervised by Professor Inge Marie Svane) and at Department of Oncology at Odense (supervised by MD Lars Bastholt). Patients enrolled in study II were treated at the Department of Oncology, University Hospitals at Herlev (supervised by Professor Inge Marie Svane) and at De-

partment of Oncology at Aarhus (supervised by PhD, MD Henrik Schmidt and MD Kirsten Fode).

Patients enrolled in study III and IV were treated at the Department of Oncology, University Hospitals at Herlev (supervised by Professor Inge Marie Svane, MDs, PhDs Bente Holm and Anders Mellemgaard). All vaccine treatments have been generated at the GMP facilities at the Department of Haematology, Herlev supervised by head technician Eva Gaarsdal and the technicians Charlotte Vajhøj, Signe Møllebæk Larsen and Zanne Henriksen.

Statistical work (described in each manuscript separately) in this thesis was done by the author and was supervised by the statistician Tobias Wirenfeldt Klausen.

For the immune monitoring part many important contributions have been made. The PBMC harvesting was done by technicians at the Haematological Laboratory and fellow PhD students at the CCIT. The FACS analyses were performed by the author in collaboration with student Maria Therese Rasmussen, PhD Marie Klinge Brimnes, PhD student Jon Bjoern, PhD Sine Reker Hadrup and Professor Inge Marie Svane. The T cell culture and ELIspot assays analyses were performed by technicians Charlotte Vajhøj and Merete Jonassen. The analyses of Elispot data were performed by the author, PhD student Stine Kiaer Larsen and Professor Mads Hald Andersen. The MHC multimer assays were performed by technician Kirsten Nikolajsen, PhD Rikke Sick Andersen and PhD Sine Reker Hadrup. The IHC staining was performed at the Department of Pathology, University Hospital at Bisbebjerg, Denmark, by MD Inger Birte Moerck Thomsen. The cytoxicity assays were performed by technician Merete Jonassen, PhD Stine Kiaer Larsen and Professor Mads Hald Andersen. The HPLC analyses were performed at the Interfaculty Institute of Biochemistry and Cell Biology, University of Tübingen, Germany, by Msc Claus Zeyher and PhD Cécile Gouttefangeas.

The daily work at the laboratory and in the clinic was supervised by Professor Mads Hald Andersen and Professor Inge Marie Svane, whom also co-authored all manuscripts included in the thesis.

Ethical considerations

Overall, there was no therapeutic benefit of participating in the study I and II for the individual patients. In these studies 30 ml and 100 ml of blood respectively were drawn at different time points (at time of routine blood sampling) for the overall purpose of monitoring induced immune changes.
Ultimately, a better understanding of the immune mechanisms underlying TMZ and IFNα/IL2 treatment is essential for the continued development of treatments in MM patients.

In general, patients with metastatic cancer (NSCLC and MM) have a poor prognosis. Currently, very few treatment options with curative potential exist. Regarding study III and IV there was a potential therapeutic benefit in participating. In these studies, 100 ml of blood were drawn every 3rd month (at time of routine blood sampling) to gather knowledge of immune changes during vaccine treatment. On the basis of current know-how of peptide vaccines and the toxicology study performed in advance of the clinical studies no unacceptable risks were associated with the vaccines. Participation in the studies is voluntary and the treatment is stopped if unreasonable side effects occur or according to the patient's wish.

RESULTS

Four studies are presented in this thesis. A brief summary describing the main findings of each study is provided below. Study IV is ongoing thus only preliminary clinical data are provided (no manuscript).

Study I – TMZ treatment in MM patients

Forty patients with stage IV melanoma were prospectively enrolled and were included in the statistical analyses. All patients were treated with a minimum of 3 cycles of TMZ and were evaluated for clinical response. Six patients were excluded from evaluation of immunological response due to concomitant treatment with prednisone. The median age was 67 years (range 39-85), disease stage distribution; M1a = 2, M1b = 9 and M1c = 29 patients and an average number of completed TMZ cycles of 5.3 (range 3-13). The overall response rate was 12.5% including 2 patients with a complete response (CR) and 3 patients with a partial response (PR) (short term). Median progression free survival (PFS) was 8.7 months. All lymphocyte count (ALC) (N=34) were assessed from routine sampling of white leucocytes before treatment and after each TMZ cycle. Lymphopenia (<0.7*109 cells/L ~ CTCAE grade 2) developed in 71% of the patients after 3 TMZ cycles (~100 days). The lymphocyte reduction was highly significant ($p<0.001$). Moreover, we demonstrated that lymphopenia ≥grade 2 after 3 TMZ cycles correlated with treatment response.

In fifteen patients alterations in the frequency of different T cell subsets were evaluated by thawed PBMCs using multicolour flow cytometry. Overall, a significant increase in the frequency of CD8+ T cell population were observed after 2 TMZ cycles paralleled by a significant decrease in the frequency of the CD4+ T cells. The same tendencies were observed in absolute cell counts. Regarding the Tregs no significant changes in the frequency of cells were observed but a significant decrease in the absolute count of Tregs after 2 TMZ cycles was demonstrated. In general Treg numbers were low.

The CD8+/CD4+ T cell ratio before and after 2 completed TMZ cycles indicated a weighted CD8+ T cell profile among clinical responders compared to the non-responders. Moreover, distinct phenotypic characterizations of CD8+ T cells including memory subsets revealed a significant decrease in the frequency of CD8+ naïve T cells counteracted by an increase in the frequency of differentiated CD8+ T cells. In 7 patients, we were able to evaluate tumour- and viral associated antigen specific T cell responses by the MHC multimer encoding method. In 5/7 patients we demonstrated enhanced T cell responses against TAAs while preserved T cell responses against selected viral epitopes were observed.

Study II – IFNα/IL2 treatment in MM patients

Thirty-five patients were enrolled in this study and all patients were included in the statistical analyses. All patients received IFNα/IL2 as 1st line therapy and were evaluable for clinical and immunological response. Twenty-five patients progressed rapidly (PD patients) whereas 10 patients (17%) had clinical benefit (PR+SD≥6 months). None of the patients had a complete response

to treatment however, 3 patients achieved ongoing "no evidence of disease" (NED) after post therapy metastatectomy of remaining lesions. Patients with clinical benefit had a lower tumour burden (M1a disease) and received more treatment cycles compared to PD patients. Estimated median TTP was 3.3 months and median OS 20.3 months. A trend towards longer survival for PR+SD compared to PD patients was seen. Furthermore, we demonstrated a significant correlation between LDH >UNL at pre-treatment to both impaired treatment response and inferior survival hence confirming increased LDH as a negative prognostic factor.

Highly significant increases in ALC were demonstrated during treatment. Furthermore, we found a positive correlation between percentage increase of ALC and achievement of clinical benefit. Levels of different immune cells were further scrutinized by flow cytometry. No significant changes during therapy in the CD8+ or CD4+ T cells were observed, however preliminary data suggested a decreasing tendency of inhibitory receptors as BTLA, CTLA and PD-1. Furthermore, within the lymphocyte population we found an increase in both the CD8+ TEM as well as the Tregs during therapy in both clinical benefit and PD patients. Further analyses of immune cells demonstrated a significant increase in the NK cells in clinical benefit and PD patients, where as the MDSCs remained unchanged during therapy. Surprisingly, only very low-frequent TAA responses was demonstrated pre- and post treatment in a small fraction of patients.

Study III – IDO vaccinations in NSCLC patients (NCT01219348)

Fifteen HLA-A2 positive patients with stage III/IV NSCLC and SD after standard chemotherapy were enrolled. Clinical trial registration is provided (Appendix B). All treated patients were included in the statistical analyses. The vaccine treatment was initiated at least 28 days after last dose of chemotherapy. No grade 3-4 CTCAE toxicity due to vaccine treatment was observed and no serious adverse events (SAEs) were reported. In the majority of patients' induction of grade 1-2, short term, local reactions (redness, swelling) was observed. The mean number of vaccinations were 11 (range 6-29) and mean duration of vaccine treatment was 7.9 months. Seven patients had clinical benefit; 1 PR and 6 SD. The estimates of median PFS were 5.2 months and median OS was 25.9 months. Vaccinated patients demonstrated longer survival compared to the vaccine untreated patients (otherwise eligible, but HLA-A2 negative). Furthermore, patients in the PR+SD group had longer survival compared to PD patients. Expression of IDO in the cytoplasm of cancer cells in pre-treatment tumour biopsies was detected in 9/10 patients. All accessible tumours expressed IDO and grading of IHC staining (negative, 1+, 2+, or 3+ positive) was performed. No correlation of IDO expression and clinical response to vaccine treatment was found.

Presence of IDO specific CD8+ T cells was demonstrated by IFNγ Elispot. IFNγ releasing cells were frequently detected in PR+SD and PD patients. At pre-treatment, the measurement of IDO specific CD8+ T cells was significant higher in PR+SD compared to PD patients. Low-frequency responses of tetramer positive IDO specific T cells were detected after cell culturing and expansion in 3 SD patients post vaccine treatment. In 1 patient, the tetramer positive CTLs demonstrated effective, specific killing of an IDO+, HLA-A2 positive cancer cell line (SW480).

Flow cytometry analyses were performed in all patients. In general, no significant changes in the CD8+ and CD4+ T cells were seen. However, the PR+SD patients demonstrated a trend towards increased peripheral CD8+ and CD8+ TEM which was not observed in the PD patients. Moreover, a decreased level of Tregs and an increased level of NK cells were seen after 6 vaccinations. No difference in Treg- or NK cells between PR+SD and PD patients was observed.

Elevated Kyn/Trp ratio has been suggested to mirror IDO activity hence HPLC measurements of Kyn and Trp were performed. In 8/11 patients the level at the 6th vaccine was stable compared to pre-treatment with no difference between SD+PR patients (4/5) and PD patients (4/6). Two patients experienced an augmentation of Kyn/Trp and also had high IDO expression (grade 3) in the IHC analyses. Measurements of long term Kyn/Trp ratio were accessible from 4 SD patients showing stabilization of Kyn/Trp in two clinical responders. Poster presentation is provided (Appendix C).

Study IV – IDO/Survivin peptide vaccine combined with TMZ in MM patients (NCT01543464)

Patient enrolment was initiated in June 2012. Thirty HLA-A2 positive patients with stage IV melanoma are planned for inclusion. To augment immune responses the vaccine comprises IDO and Survivin peptides plus Montanide, Imiquimod and GM-CSF as adjuvants. To improve clinical responses the vaccine is combined with TMZ as an anti-neoplastic agent with immune modulator properties demonstrated in study I. Clinical trial registration is provided (Appendix D).

To date, a total of 31 patients have been screened for HLA-A2 tissue type of whom 15 patients were positive. Two patients progressed prior to inclusion thus 13 patients have started vaccine treatment. Five patients have been excluded from the protocol prior to evaluation (thus not evaluable patients) due to progression in the brain (known CNS lesions – 3 patients), grade 4 haematological toxicity (1 patient) and bilateral pleural effusion (known lung lesions - 1 patient). 1 patient is ongoing in trial but awaits evaluation after 3 cycles of treatment. Overall, 7 patients have been evaluated for clinical response hence these preliminary data are presented.

Patients were evaluated by PET-CT scan at baseline and subsequently every 3rd month. Two patients (pt.#04 and #09) have demonstrated a partial response with 57% and 45% tumour reduction for 10 and 6 month+ (PR not confirmed in the latter patient) respectively. Three patients had SD for 4.5, 3.0 and 4 month+ respectively. Two patients had PD with development of new lesions. Thus the very preliminary objective response rate in evaluable patients is 29% (2 PR), short term SD seen in 42% of patients (3 SD) and rapid PD observed in 29% of the patients (2 PD). PET-CT scan demonstration of pt. #04 and #09 is provided (Appendix E). Immune analyses have not yet been initiated.

Toxicities have been safe and manageable. Two SAEs of severe dizziness (probably not related to treatment) and widespread rash (probably related) respectively, have been reported.

DISCUSSION

In the studies described in this thesis, we have aimed to treat cancer patients through an immunotherapeutic approach. Melanoma is well-known to be linked to the immune system and other cancer types as NSCLC might likewise benefit from immunothera-

py (16). The aim of immunotherapy is to ameliorate the anti tumour responses generated by the immune system. Immunotherapy has the ability to activate cytotoxic CD8+ T cells infiltrating the tumour and potentially mediate elimination of cancer cells. Thus, therapeutic cancer vaccines have the potential to induce long-lasting, tumour specific immune memory, although in terms of treating metastatic cancer results have been somewhat disappointing. Nonetheless, therapeutic vaccines such as tumour cell- and antigen-specific vaccines have shown clinical efficacy in early trials and are currently under clinical investigation in large, phase III randomized trials for adjuvant (MAGRIT study, NCT00480025) and metastatic settings (START study, NCT00676507 and STOP study, NCT00676507) in NSCLC patients (31). Peptide vaccines can play a role when combined with other immunotherapies. To this end, a clinical trial combining Nivolumab (escalating doses of anti-PD1) with a multipeptide vaccine targeting MART1, NY-ESO-1, gp100 emulsified in Montanide for metastatic solid tumours (MM and NSCLC) is currently being conducted (NCT01176461). This trial design allows for facilitating and optimizing T cell response monitoring (124). Reliable immune biomarkers of response either derived from peripheral blood or from tumour lesions are being intensively investigated simultaneously with the implementation of these new checkpoint inhibitors (125,126). This thesis has aimed at characterizing potential immune cell changes derived from peripheral blood during standard chemo- and immunotherapy treatments. Furthermore, we have investigated the feasibility of IDO as an anticancer vaccine in a `First in man` trial in NSCLC patients. The IDO peptide vaccine has proven to be well-tolerated and long-lasting SD+PR in the treated patients were observed. Based on this, we recently initiated a phase II clinical trial targeting IDO and Survivin cancer proteins in MM patients aiming at assessing clinical benefit rate.

Lymphocyte count as immune correlate

Treatment induced changes in immune cells such as changes in specific T cell populations have been proposed as immune biomarkers. Recent data have indicated a prognostic value of peripheral blood count of ALC in MM patients (127). The association between clinical activity of Ipilimumab and changes in ALC from baseline has been demonstrated (128). Likewise, two recent studies in MM have suggested that an increase in ALC is correlated to response to Ipilimumab and to overall survival (129,130). Regarding immune markers, we demonstrated (study I) that CTC ≥ grade 2 lymphopenia after 3 treatment cycles of TMZ was correlated to treatment response. Previously, we have likewise demonstrated a correlation between lymphopenia and treatment response to TMZ and Thalidomide in stage IV MM patients (131). No correlation between lymphopenia and overall survival was seen. The individual turnover of TMZ was not measured, thus potential individual differences on the impact of how TMZ affected the bone marrow function is unknown, however, all patients were treated with TMZ dosage according to body surface. Thus, we do not fully understand the importance of the changes in lymphocyte level in MM patients e.g. lymphopenia in connection with TMZ might not be directly involved in the clinical response but merely an indicator of high TMZ sensitivity. Hence, more knowledge is necessary to elicit if ALC count can be used in this setting as a surrogate immune marker.

In contrast to TMZ-induced lymphopenia we demonstrated a significantly increased level of ALC during IFNα/IL2 treatment. Furthermore, we found a positive correlation between percentage increase of ALC and achievement of clinical benefit in a well described patient cohort (study II). Consistent with our data, it has formerly been demonstrated that clinical responders to high dose IL2 had higher maximum as well as higher change in ALC after therapy compared to non-responders (132). In our study, no correlation between increased ALC and overall survival in the IFNα/IL2 treated patients was seen. The correlation between ALC rise and response to IFNα/IL2 is indicative that lymphocyte level might serve as a surrogate marker. However, this is probably not a universal phenomenon in that MM patients treated with the PD-1 antibody (Nivolumab) did not have increasing ALC levels during therapy. Moreover, a low level of ALC was not precluding response to Nivolumab. Interestingly, phenotypic changes in peripheral T cell subsets were detected during Nivolumab treatment included an increase in activated CD8+ cells expressing HLA-DR, ICOS and/or KI67 (125).

Peripheral immune cell subsets as immune correlates

By the use of multicolour flow cytometry thawed PBMCs revealed a selective decrease in CD4+ T cell counts within the lymphocyte population during TMZ therapy (study I). Furthermore, we demonstrated a reduction in peripheral Tregs during TMZ however in general Treg numbers were low. The impact of the lowered Treg level in terms of clinical efficacy is unclear. In contrast, we demonstrated an increase in Treg level within the peripheral lymphocyte population during IFNα/IL2. The bimodal role of IL2 in stimulating both clinically important effector T cells and Tregs is complex and the clinical impact of Treg induction in this setting remains unclear. Another potent cell type with immunosuppressive properties is the MDSCs. No change in the MDSC population during treatment with either TMZ or IFNα/IL2 was demonstrated.

NK cells are known to bridge innate and adoptive immunity however, the anti-tumour effects of NK cells are debated. During TMZ treatment no changes in NK cells were seen. In contrast, during IFNα/IL2 therapy there was a significant increase in the NK cell subpopulation which was expected, since IFNα is a well-known inducer of NK cells. The clinical efficacy of IFNα/IL2 migth be partly due to the NK induction although, we did not see any difference in NK induction among responders and non-responders.

Since CTLs are believed to be the most important cells for anti-tumour regression intense assessment of peripheral T cells was mediated. Surprisingly, in the TMZ study (study I), an increase in the CD8+ T cell population was demonstrated. Moreover, the ratio of CD8+/CD4+ T cells relative to pre-treatment levels indicated a weigthed CD8+ T cell profile in clinical responders to TMZ. In contrast, during IFNα/IL2 treatment (study II) no significant changes in the peripheral CD8+ or CD4+ T cells were seen despite the induced lymphocytosis. Moreover, no difference were found among responders and non-responders however, final interpretation of CD4+, CD8+ and CD4+/CD8+ ratio awaits completion of analyses. To this end, we also performed characterization of inhibitory (BTLA, CTLA-4 and PD-1) and stimulatory (CD3zeta) receptors on T cells during IFNα/IL2. Preliminary data suggested a decreasing tendency of expression (decreased MFI) of inhibitory molecules. Potentially, CD8+ T cells with less inhibitory functionality might increase clinical efficacy, however, more analyses will have to be performed. For further characterization of differentiation properties of T cells, distinct populations of CD8+ T cells including naïve and antigen-experienced cells were measured. During both TMZ and IFNα/IL2 treatment we demonstrated a general increase in differentiated CD8+ T cells. Thus, analyses of

viral- and TAA specific T cell responses as correlates to clinical response was investigated.

TAA specific T cell responses as immune correlates

Genetic alterations in tumour cells results in antigen expression which is recognized by the TCR on host T cells. The recognition is mediated by peptide-HLA complexes and leads to killing of cancer cells by host cells. To identify the antigen recognition pattern a high-throughput technique was applied using a panel of well-described viral- and TAAs in MM patients (133). In study I we found that CD8+ T cell responses against TAAs were enhanced after TMZ therapy whereas virus-targeting CD8+ T cell responses remained stable. This suggests that chemotherapy mediated killing of cancer cells might release TAAs that can be presented by APC hence eliciting T cell responses. Classical chemotherapy may stimulate tumour specific immune responses by inducing immunogenic cell death, a particular form of apoptosis involving the release of ATP and activating numerous immune effector mechanisms (134). Recently, it was shown that DTIC treatment prior to peptide vaccine strategy in melanoma enhances the T cell repertoire (135). The increased TAA responses mediated by TMZ chemotherapy indicate that firstly TMZ is not destroying the immune responses and secondly that in fact TMZ seem to enhance T cell mediated tumour regression. Accordingly, we observed an increase in peripheral CD8+ T cells corresponding to the possible impact of enhanced TAA immune responses.

In study II, we focused on a larger panel of peptides described as targets for tumour infiltrating lymphocytes (TILs) presented on HLA-A1, A2, A3 or B7 alleles. Also, viral epitopes was monitored pre- and post therapy as a general status of the immune system. Surprisingly, only very low-frequent TAA responses in a small fraction of patients was demonstrated pre- and post treatment and with no correlation to clinical efficacy. Previously, it has been demonstrated that IL2 administration causes a decline in anti-tumour CTL responses in peripheral blood due to migration of specific T cells to tumour site (136). Also, a lower frequency of CTLs in peripheral blood may still elicit functional antitumour activity if the levels of inhibitory molecules on the T cells are decreased. Furthermore, a decreased frequency of specific CTLs in peripheral blood could also be relative due to the demonstrated lymphocytosis after IL2 therapy including induction in Treg and NK cells.

Overall, we have demonstrated immune modulating properties of chemo-immunotherapy in melanoma patients which may contribute to the anti-neoplastic efficacy (134). A drawback of the studies is that only PBMCs and not tumour tissue have been available for analyses.

Safety

Patient safety is the most important issue when new treatment modalities are being tested and is accordingly the primary endpoint in first in man trials. We have implemented the first peptide vaccine trials for cancer patients in Denmark (studies III & IV) which demanded registration of all emerging side effects during the study periods. In contrast to conventional anti-neoplastic drugs the toxicities of the vaccine is related directly to the peptides and adjuvants applied. In general, toxicities reported world wide from peptide vaccine studies are modest and manageable (88,137). In our two clinical studies we have not seen any unexpected or severe side effects in patients treated with IDO/Survivin vaccine emulsified in Montanide including the use of the adjuvants Imiquimod and GM-CSF. The use of TMZ has resulted in nausea, fatigue and mild myelosuppression as expected. Topical applied steroids have been sufficient to treat indurations at the vaccine site. In case of more severe reactions due to the vaccine treatments the use of systemic steroids would be 1st choice of management. Overall, the IDO/Survivin peptide vaccines have been safe as monotherapy as well as in combination with TMZ.

Peptide vaccines in metastatic MM and NSCLC

Within melanoma several kinds of peptide vaccines have been tested (138). Spontaneous CTL activity against selected melanoma antigens such as Melan A / MART-1 / MAGE, tyrosinase and Gp100 are seen and boosting of tumour response have been demonstrated by specific vaccination strategies (139-141). Most peptide vaccine studies have been conducted without induction of severe toxicity thus peptide vaccines are easily applied in clinical settings. Peptide vaccines can be used to induce accurate T cell responses, which are easily reproducible in comparison to more complex vaccine types. Cytotoxic CTLs with specificity for HLA class I bound TAAs can inhibibit tumour growth thus active immunization have been assesed to activate CTL responses (90,142). In MM settings, a promising clinical efficacy was demonstrated in a study of IL2+/- Gp100 in which objective RR and and OS was improved in favour of IL2 + Gp100 (143). In NSCLC, a peptide vaccine targeting telomerase with a 16 mer long peptide sequence combined with GM-CSF as adjuvant has shown strong correlation between induced immune response and survival (110). In an updated report from this trial durable responses and long term survival were correlated to patients with induced immune responses (111).

Improvement of peptides and adjuvants

Formerly, it has been demonstrated in cancer patients that spontaneous T cell reactivity occur against survivin thus viewed as an universal target antigen for immunotherapy (106,108). Experience with this peptide in particular has formerly been obtained in DC vaccine trials performed in MM patients (144). Formerly performed vaccine studies at CCIT have used Survivin as target (145-147). To this end, the survivin peptide was applied in study IV to optimize T cell responses and improve clinical efficacy.

In MM, candidate cancer proteins recognized by the most frequent HLA alleles have been described whereas in NSCLC new relevant targets may be discovered. Another approach, applying long peptide sequences thus aiming at inducing highly diverse specific T cell responses integrating both T helper and CTL responses may be essential for tumour regression and generation of long term T cell memory (148).

Recent data indicate that administration of a short IDO peptide formulated in Montanide is most likely not optimal and research into the generation of enhanced CTL stimulation needs further attention. Thus, it has been recognized that persisting antigen delivery at vaccine sites using Montanide induced dysfunctional T cells (149), and has lead to suggesting development of newer rapidly degradable adjuvants (150). Thus, new adjuvants based on liposome technology capable of inducing CD8+ T cell aggregates at vaccine site are under current investigations (151,152) and may lead to better priming of immune responses. Although immune responses were detected in study III they were of lower magnitude than hoped for. To this end, improvement of adjuvants and peptide administration are being tested in study IV. Other aspects of generating powerful CTLs include route of administration,

homing of T cells to the tumor site, entry into the tumor microenvironment, and maintenance of function systemically, as well as at the tumor site.

Small molecule IDO inhibitors

Metastatic cancers can exploit the IDO pathway in dampening the immune response thus promoting increased tumour growth and survival. IDO pathway inhibitors may be seen as checkpoint inhibitors akin to CTLA-4 and PD-1 antibodies. Preclinical studies have found that Indoximod, an orally administered IDO inhibitor, increased anti-tumour T cell responses and reduced Tregs thereby slowing down tumour growth. Determining the importance of Treg reduction in terms of clinical efficacy is crucial. Interestingly, we also demonstrated Treg reduction after IDO vaccine administration (study III). In murine models the combination of Indoximod and Paclitaxel has promoted 30% tumour regression and an initial phase I trial of this combination has shown to be safe (86). A second phase I trial in metastatic solid tumours combining Indoximod and Docetaxel report encouraging clinical activity with tumour regression (>30%) in 4/22 patients (18%) (153). A phase II trial with this combination is now accruing patients (NCT01792050).

Another oral inhibitor of IDO, INCB024360 (Incyte Corporation), has proven to be generally well-tolerated at doses up to 700 mg BID (twice daily) in a clinical phase I study of metastatic solid tumours (87). Doses of ≥300 mg BID were capable of >90% inhibition of IDO activity and was found to normalize Kyn plasma concentrations. The dosing applied with INCB024360 in a phase II monotherapy study in ovarian cancer is 600 mg BID (NCT01685255). INCB024360 is also being studied in MM patients combined with Ipilimumab (NCT01604889) (154). The ratio of Kyn/Trp measurements in sera as potential biomarker is interesting. In our study we found long term stabilization in Kyn/tro ratio in two clinical responding patients.

The targeting of IDO through small molecule inhibitors versus the induction of cytotoxic T cells by a vaccine strategy differs naturally in several ways. One difference is related to the toxicity profile of systemic treatment with IDO inhibitors in contrast to IDO inhibition by a vaccine strategy. Moreover, the benefit of a vaccine strategy may be the induction of long-lasting IDO specific memory T cells. Hence, in theory these specific memory cells might possibly become re-activated and attracted to tumour site when needed. The direct killing of IDO-expressing cells may diminish IDO-mediated immune suppression hence boosting tumour reactivity targeting other tumour antigens. Furthermore, IDO positive cells may be suppressive by other means than IDO, e.g. arginase, PD-L1 or HLA-G. Hence, IDO-specific T cells might not only reduce IDO-mediated suppression but also additional immune suppression mediated by IDO positive regulatory cells.

IDO combination strategies

The identification and understanding of resistance mechanism in metastatic cancer settings is important for improving clinical outcome in immunotherapy. In melanoma in particular, clinical vaccine trials have resulted in induction of TAA specific T cell responses circulating in the blood yet not necessarily mimicking the clinical outcome. Within the tumour microenvironment inhibitory ligands such as PD-L1 and IDO may contribute to immune escape in different settings (65). To identify resistance mechanisms a recent murine study has shown that IDO knockout mice treated with either anti-CTLA4 antibody or anti-PD-1 antibody demonstrated a striking delay in tumour growth and increased overall survival when compared to wild-type mice (155). Furthermore, the effect was demonstrated to be T cell dependent due to an increase effector/Treg ratio in the tumours (155). To this end, combination therapies of checkpoint blockade and IDO inhibition in metastatic cancer settings are warranted.

Limitations

Study I: Although blood sampling was processed quickly by skilled laboratory employees and the thawing procedure was performed gently according to standard operating procedures low viability of cells in some of the frozen samples were evident. Transporting the frozen cells from Odense to Herlev unfortunately led to cells of too low a quality for immune cell analyses. Thus, of 40 eligible patients only thawed PBMCs from 15 patients treated at Herlev were used.

Study II: Learning from the experience of study I, we increased the amount of blood sampling pr time point (from 30 ml to 100 ml) to ensure enough viable cells for immune analyses. Analyses of immune cells from fresh material may possible make results more reliable in regard to the exact numbers and the relation between different immune cell subsets. However such a setup includes a number of logistically difficulties. Patient recruitment has been slower than expected. Hence, for the time being we have performed immune analyses from approximately half of the patients, thus only a manuscript describing data from this study is provided.

Study III: HLA tissue typing was initially performed at our laboratory at the Department of Haematology, 54P4. In 4 patients the tissue typing was not performed correctly due to a failure in a fluorochrome-conjugated antibody. Subsequently, all tissue typing was performed at the Laboratory of Tissue Typing at the Copenhagen University Hospital at Rigshospitalet.

Study IV: Patient recruitment is ongoing thus only preliminary data are provided.

Future perspectives and concluding remarks

The objectives of this thesis were to investigate changes in immune parameters during chemo-immunotherapy as well as evaluating the feasibility of IDO as an anticancer vaccine target. Four clinical studies have been performed (1 ongoing) and results have been analysed and discussed according to the litterature in the field. Immune changes are seen during chemo-immunotherapies hence understanding these treatments as immune modifiers become evident. Other treatment modalities might also assist immune therapy. Thus, the hypothesis, that also radiation therapy (RT) induces tumour antigen release thus potentially acts synergistically with conventional immunotherapy is under investigation (156-158). In a phase 1 trial combining high dose IL2 and stereotactic body radiation therapy (SBRT) in MM patients the combination seemed to be safe, to induce important clinical efficacy and moreover to induce activated, proliferating T cells in peripheral blood (159,160). The potential ascopal effect of RT and immunotherapy e.g. Ipilimumab in MM patients has been demonstrated in case report form and is under current clinical assessment (NCT01449279) (161).

Peptide vaccine strategies are still far from being standard therapies in metastatic cancer settings yet a number of novel epitopes described might be candidates for a second generation of peptide vaccines. Furthermore, broadening and in-depth immunological analyses of T cell response repertoire are crucial. The combination of long peptides containing several epitopes and improved

adjuvants might induce widespread immune responses leading to improved clinical outcome. Recently, a tumour biopsy study in melanoma patients before and after treatment with BRAF inhibitors showed an increased expression of melanoma antigens and CD8+ T cell infiltrate post treatment revealing targeted therapies as potential immune modulators (162). At our institution a clinical trial for stage IV MM patients (EudraCT nummer: 2013-000365-37) combining long peptide sequences (see below) with standard treatments of either Ipilimumab or Vemurafenib is being set up.

IDO-long/IDO(194-214) peptide sequence: DTLLKALLEIASCLEKALQVF
hTERT(611-626) peptide sequence: EARPALLTSRLRFIPK
Survivin/Surv(95-111) peptide sequence: ELTLGEFLKLDRERAKN
MART-1(51-73) peptide sequence: RNGYRALMDKSLHVGTQCALTRR

In terms of both melanoma and NSCLC very recent promising data of checkpoint inhibition by blocking antibodies against CTLA-4, PD-1 and PD-L1 including combination trials of checkpoint blockade are now emerging in randomized phase III clinical testing (17). Enthusiasm, that combination of checkpoint blockade will generate highly effective anti-tumour responses not just for melanoma but also for NSCLC patients is real, thus broadening immunotherapy more widely into oncology settings (16). Furthermore, recent data underscores that both number and functions of tumour-specific CTLs are crucial for efficacy of immunomodulatory antibodies in NSCLC patients (34).

Bringing melanoma immunotherapy into other solid tumours such as NSCLC is a new paradigm. Exploiting the knowledge gathered from immunotherapy in melanoma is crucial to fastened development of immune therapeutics in general since multiple challenges still exist. Firstly, determining the relevant endpoints and the best comparison treatment in randomized trials are important. Secondly, evaluating the clinical efficacy correctly by the use of RECIST, PERCIST or irRC are necessary to design and conduct clinical trials. Thirdly, an in-depth understanding of immune responses demands the collection of PBMCs and multiple tumour biopsies to detect immunological and genetic changes in the peripheral blood and tumour microenvironment.

Overall, this thesis has correlated immune changes to clinical efficacy during standard chemo-immunotherapy. Furthermore, we have been bridging the translational idea of IDO as being a relevant target in cancer thus demonstrating IDO expression in NSCLC tumours. We have performed the clinical set-up and immune analyses to understand the induced changes during IDO vaccine therapy. Finally, the use of IDO peptide vaccines to complement standard cancer treatment is under current investigation.

SUMMARY

This thesis describes the treatment of metastatic melanoma (MM) and non small-cell lung cancer (NSCLC) from an immunotherapeutic approach.

The purpose of the first part of the thesis was to assess how treatment with Temozolomide (TMZ) chemotherapy affects the immune system in patients with metastatic MM. Our results showed that the number of T lymphocytes was significantly reduced after 3 treatment cycles. Furthermore, the induced lymphopenia was positive correlated to achievement of clinical benefit. We demonstrated that the proportion of CD4+ and Treg lymphocytes decreased whereas the CD8+ T cells increased. In particular, we demonstrated that mature CD8+ T cells increased during treatment. Analyses of peripheral blood before and after treatment showed that T cell responses against common viral epitopes were conserved despite chemotherapy. Surprisingly, we found a significant increase in T cell responses against well-known MM tumour specific antigens. Overall, we have verified that TMZ in addition to being an alkylating and cytotoxic chemotherapy, also posess immune modulatory effect in MM patients treated with standard dosage of TMZ.

In the second part of the thesis we examined how treatment with Interferon alfa-2b and Interleukin 2 (IFNα/IL2) affects the immune system. We demonstrated a significant induced lymphocytosis during treatment. Furthermore, we showed that the percentage increase in lymphocytes was positively correlated to clinical outcome. Moreover, we have seen that IFNα/IL2 leads to significant increase in NK and Treg cells in both patients with and without clincal effect. In general, T cell responses against common viral epitopes and well-known melanoma tumour specific antigens were low. Furthermore, the study confirmed that elevated LDH is negatively correlated with both treatment response and median overall survival. Overall, we have characterized changes of immune cells and correlated them with clinical efficacy during the couse of IFNα/IL2 used in standard dosage.

In the third part we investigated if vaccination with a peptide derived from IDO was feasible in patients with metastatic NSCLC. This “First in Man” trial was safe and showed modest side effects only. Since IDO was expressed in NSCLC tissues it was found to be a relevant target. One patient achieved significant regression of liver metastases (confirmed partial response) and another 6/15 patients achieved prolonged disease stabilization. Furthermore, median overall survival was 25.9 months demonstrating a better survival in vaccinated compared to non-vaccinated comparable NSCLC patients. The presence of IDO specific CD8+ T cells were detected by IFNγ Elispot. In patients with clinical effect of the vaccine IDO-specific CD8+ T cells at pre-treatment was significanctly increased. Moreover, low-frequent IDO positive tetramer CD8+ T cells were detected and led to effective killing of an IDO+ HLA-A2 positive cancer cell line (SW480) in 1 patient. Moreover, flow cytometry was performed and in general no significant changes in CD8+ and CD4+ T cells were seen, although patients with clinical response showed a trend towards increased mature CD8+ T cells during treatment. In addition, we found lower levels of Tregs as well as an increased level of NK cells after 6 vaccinations.

Elevated Kyn/Trp ratio is suggested to mirror IDO activity. In 8/11 patients the level after the 6th vaccine was stable compared to baseline. No differences between patients with clinical benefit (4/5) and patients with progressive disease (4/6) were demonstrated. Two patients had an increase in Kyn/Trp ration meanwhile demonstrating a high expression of IDO. In 2 patients with clinical response long-term stabilization of Kyn/Trp was observed. Overall, the vaccine was well tolerated with no adverse toxicity. Median overall survival was 25.9 months with long term disease stabilization achieved in 47% of the treated patients. Based on the promising clinical results achieved in the vaccine trial for NSCLC patients, we launched a new clinical trial for MM patients (ongoing patient recruitment) in June 2012. In order to enhance the immune response the vaccine comprises IDO plus Survivin

peptide as well as the adjuvants Montanide, Aldara and GM-CSF. Finally, the vaccine is given in combination with TMZ. Patients are evaluated every 3rd month with PET-CT scan. Preliminary clinical data from the first 7/30 evaluable patients are presented. Two patients demonstrated a patial response with 57% and 45% tumour regression lasting for 10 months and 6+ months respectively, corresponding to a preliminary objective response rate of 29%. The vaccine has been manageable and without significant side effects.

LIST OF ABBREVIATIONS

ALK = Anaplastic lymphoma kinase	IV = Intravenously
ACT = Adoptive cell transfer	Kyn = Kynurenin
APC = Antigen presenting cells	LAG-3 = Lymphocyte activation gene 3
BID = Bis-In-Die ~ two times daily	MDSC = Myeloid derived suppressor cells
BRAF = Proto-oncogene B-raf	MEK = Mitogen-activated extracellular kinase
BTLA = B and T lymphocyte attenuator	MHC = Major histocompatibility complex
CKIT = Proto-oncogene c-kit	MM = Malignant melanoma
CR = Complete remission	NE = Not evaluable
CTCAE = Common terminology criteria adverse events	NED = No evidence of disease
CTL = Cytotoxic CD8+ T cells	NK = Natural killer cells
CTLA4 = Cytotoxic T lymphocyte antigen 4	NSCLC = Non small-cell lung cancer
DTIC = Dacarbacine	PBMC = Peripheral blood mononuclear cell
DC = Dendritic cells	PR = Partial response
SD = Disease stabilization	PD-1= Programmed death 1
EGFR = Epidermal growth factor receptor	PD-L1= Programmed death ligand 1
EML4= Echinoderm microtubule associated protein-like 4	RT = Radiotherapy
FAS = Cell death surface receptor	RAS-RAF-MEK-MAPK = Mitogen-activated, extracellular regulated protein kinases
FoxP3 = Forkhead box P3	RCC = Renal cell carcinoma
GCP = Good clinical practice	RR = Response rate
GMP = Good manufacturing practice	SAE = Serious adverse event
GM-CSF = Granulocyt/Macrophage colony stimulating factor	SC = Subcutaneously
HD = Healthy donors	SBRT = Stereotactic Body Radiotherapy
HPLC = High performance liquid chromatography	TAA = Tumour associated antigen
HLA = Human leucocyte antigen	TCR = T cell receptor
ICOS = Inducible T cell co-stimulator	TDLN = Tumour draining lymph nodes
IDO = Indoleamine 2.3 dioxygenase	TGF-β = Transforming growth factor beta
IFNα/IL2 = Interferon-α2b/Interleukin2	TKI = Tyrosine kinase inhibitor
IFNγ = Interferon gamma	TMZ = Temozolomide
IHC = Immunohistochemistry	Tregs = Regulatory T cells
1-MDT = 1-methyl-D-tryptophan	Trp = Tryptophan

REFERENCE LIST

1. Aamdal, S. 2011. Current approaches to adjuvant therapy of melanoma. Eur. J. Cancer 47 Suppl 3: S336-S337.

2. Danish Board of Health. the Danish Cancer Registry 2010. Copenhagen, Danish Board of Health, 2010-Http://www.sst.dk/publ/Publ2011/DAF/Cancer/Cancerregisteret2010.pdf. Accessed May 15, 2012.

3. Froslev, T., A. F. Grann, M. Olsen, A. B. Olesen, H. Schmidt, S. Friis, and M. Sogaard. 2012. Completeness of TNM cancer staging for melanoma in the Danish Cancer Registry, 2004-2009. Clin. Epidemiol. 4 Suppl 2: 5-10.

4. Loo, T. L., G. E. Housholder, A. H. Gerulath, P. H. Saunders, and D. Farquhar. 1976. Mechanism of action and pharmacology studies with DTIC (NSC-45388). Cancer Treat. Rep. 60: 149-152.

5. Middleton, M. R., J. J. Grob, N. Aaronson, G. Fierlbeck, W. Tilgen, S. Seiter, M. Gore, S. Aamdal, J. Cebon, A. Coates, B. Dreno, M. Henz, D. Schadendorf, A. Kapp, J. Weiss, U. Fraass, P. Statkevich, M. Muller, and N. Thatcher. 2000. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. J. Clin. Oncol. 18: 158-166.

6. Atkins, M. B., M. T. Lotze, J. P. Dutcher, R. I. Fisher, G. Weiss, K. Margolin, J. Abrams, M. Sznol, D. Parkinson, M. Hawkins, C. Paradise, L. Kunkel, and S. A. Rosenberg. 1999. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. J. Clin. Oncol. 17: 2105-2116.

7. Gogas, H. J., J. M. Kirkwood, and V. K. Sondak. 2007. Chemotherapy for metastatic melanoma: time for a change? Cancer 109: 455-464.

8. Atkins, M. B., J. Hsu, S. Lee, G. I. Cohen, L. E. Flaherty, J. A. Sosman, V. K. Sondak, and J. M. Kirkwood. 2008. Phase III trial comparing concurrent biochemotherapy with cisplatin, vinblastine, dacarbazine, interleukin-2, and interferon alfa-2b with cisplatin, vinblastine, and dacarbazine alone in patients with metastatic malignant melanoma (E3695): a trial coordinated by the Eastern Cooperative Oncology Group. J. Clin. Oncol. 26: 5748-5754.

9. Hodi, F. S., S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbe, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos, and W. J. Urba. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. N. Engl. J. Med. 363: 711-723.

10. Chapman, P. B., A. Hauschild, C. Robert, J. B. Haanen, P. Ascierto, J. Larkin, R. Dummer, C. Garbe, A. Testori, M. Maio, D. Hogg, P. Lorigan, C. Lebbe, T. Jouary, D. Schadendorf, A. Ribas, S. J. O'Day, J. A. Sosman, J. M. Kirkwood, A. M. Eggermont, B. Dreno, K. Nolop, J. Li, B. Nelson, J. Hou, R. J. Lee, K. T. Flaherty, and G. A. McArthur. 2011. Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation. N. Engl. J. Med.

11. Hauschild, A., J. J. Grob, L. V. Demidov, T. Jouary, R. Gutzmer, M. Millward, P. Rutkowski, C. U. Blank, W. H. Miller, Jr., E. Kaempgen, S. Martin-Algarra, B. Karaszewska, C. Mauch, V. Chiarion-Sileni, A. M. Martin, S. Swann, P. Haney, B. Mirakhur, M. E. Guckert, V. Goodman, and P. B. Chapman. 2012. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. Lancet 380: 358-365.

12. Topalian, S. L., F. S. Hodi, J. R. Brahmer, S. N. Gettinger, D. C. Smith, D. F. McDermott, J. D. Powderly, R. D. Carvajal, J. A. Sosman, M. B. Atkins, P. D. Leming, D. R. Spigel, S. J. Antonia, L. Horn, C. G. Drake, D. M. Pardoll, L. Chen, W. H. Sharfman, R. A. Anders, J. M. Taube, T. L. McMiller, H. Xu, A. J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G. D. Kollia, A. Gupta, J. M. Wigginton, and M. Sznol. 2012. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N. Engl. J. Med. 366: 2443-2454.

13. Brahmer, J. R., S. S. Tykodi, L. Q. Chow, W. J. Hwu, S. L. Topalian, P. Hwu, C. G. Drake, L. H. Camacho, J. Kauh, K. Odunsi, H. C. Pitot, O. Hamid, S. Bhatia, R. Martins, K. Eaton, S. Chen, T. M. Salay, S. Alaparthy, J. F. Grosso, A. J. Korman, S. M. Parker, S. Agrawal, S. M. Goldberg, D. M. Pardoll, A. Gupta, and J. M. Wigginton. 2012. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N. Engl. J. Med. 366: 2455-2465.

14. Hamid, O., C. Robert, A. Daud, F. S. Hodi, W. J. Hwu, R. Kefford, J. D. Wolchok, P. Hersey, R. W. Joseph, J. S. Weber, R. Dronca, T. C. Gangadhar, A. Patnaik, H. Zarour, A. M. Joshua, K. Gergich, J. Elassaiss-Schaap, A. Algazi, C. Mateus, P. Boasberg, P. C. Tumeh, B. Chmielowski, S. W. Ebbinghaus, X. N. Li, S. P. Kang, and A. Ribas. 2013. Safety and Tumor Responses with Lambrolizumab (Anti-PD-1) in Melanoma. N. Engl. J. Med.

15. Jedd D Wolchok, Harriet M Kluger, and Margaret K Callahan et al. 2013. Safety and clinical activity of nivolumab (anti-PD-1, BMS-936558, ONO-4538) in combination with ipilimumab in patients (pts) with advanced melanoma (MEL)., 2013 ASCO Annual Meeting, Abstract No 9012 ed.

16. Riley James L. 2013. Combination Checkpoint Blockade - Taking Melanoma Immunotherapy to the next level., The New England Journal of Medicine ed.

17. Wolchok, J. D., H. Kluger, M. K. Callahan, M. A. Postow, N. A. Rizvi, A. M. Lesokhin, N. H. Segal, C. E. Ariyan, R. A. Gordon, K. Reed, M. M. Burke, A. Caldwell, S. A. Kronenberg, B. U. Agunwamba, X. Zhang, I. Lowy, H. D. Inzunza, W. Feely, C. E. Horak, Q. Hong, A. J. Korman, J. M. Wigginton, A. Gupta, and M. Sznol. 2013. Nivolumab plus Ipilimumab in Advanced Melanoma. N. Engl. J. Med.

18. Flaherty, K. T., J. R. Infante, A. Daud, R. Gonzalez, R. F. Kefford, J. Sosman, O. Hamid, L. Schuchter, J. Cebon, N. Ibrahim, R. Kudchadkar, H. A. Burris, III, G. Falchook, A. Algazi, K. Lewis, G. V. Long, I. Puzanov, P. Lebowitz, A. Singh, S. Little, P. Sun, A. Allred, D. Ouellet, K. B. Kim, K. Patel, and J. Weber. 2012. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. N. Engl. J. Med. 367: 1694-1703.

19. Dudley, M. E., J. R. Wunderlich, J. C. Yang, R. M. Sherry, S. L. Topalian, N. P. Restifo, R. E. Royal, U. Kammula, D. E. White, S. A. Mavroukakis, L. J. Rogers, G. J. Gracia, S. A. Jones, D. P. Mangiameli, M. M. Pelletier, J. Gea-Banacloche, M. R. Robinson, D. M. Berman, A. C. Filie, A. Abati, and S. A. Rosenberg. 2005. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J. Clin. Oncol. 23: 2346-2357.

20. Dudley, M. E., J. C. Yang, R. Sherry, M. S. Hughes, R. Royal, U. Kammula, P. F. Robbins, J. Huang, D. E. Citrin, S. F. Leitman, J. Wunderlich, N. P. Restifo, A. Thomasian, S. G. Downey, F. O. Smith, J. Klapper, K. Morton, C. Laurencot, D. E. White, and S. A. Rosenberg. 2008. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J. Clin. Oncol. 26: 5233-5239.

21. Ellebaek, E., T. Z. Iversen, N. Junker, M. Donia, L. Engell-Noerregaard, O. Met, L. R. Holmich, R. S. Andersen, S. R. Hadrup, M. H. Andersen, S. P. thor, and I. M. Svane. 2012. Adoptive cell therapy with autologous tumor infiltrating lymphocytes and low-dose Interleukin-2 in metastatic melanoma patients. J. Transl. Med. 10: 169.

22. Sznol, M. 2009. Betting on immunotherapy for melanoma. Curr. Oncol. Rep. 11: 397-404.

23. Rami-Porta, R., J. J. Crowley, and P. Goldstraw. 2009. The revised TNM staging system for lung cancer. Ann. Thorac. Cardiovasc. Surg. 15: 4-9.

24. Ferlay, J., H. R. Shin, F. Bray, D. Forman, C. Mathers, and D. M. Parkin. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int. J. Cancer 127: 2893-2917.

25. Molina, J. R., P. Yang, S. D. Cassivi, S. E. Schild, and A. A. Adjei. 2008. Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin. Proc. 83: 584-594.

26. Jadus, M. R., J. Natividad, A. Mai, Y. Ouyang, N. Lambrecht, S. Szabo, L. Ge, N. Hoa, and M. G. Dacosta-Iyer. 2012. Lung cancer: a classic example of tumor escape and progression while providing opportunities for immunological intervention. Clin. Dev. Immunol. 2012: 160724.

27. Azzoli, C. G., S. Temin, T. Aliff, Baker S Jr, J. Brahmer, D. H. Johnson, J. L. Laskin, G. Masters, D. Milton, L. Nordquist, W. Pao, D. G. Pfister, S. Piantadosi, J. H. Schiller, R. Smith, T. J. Smith, J. R. Strawn, D. Trent, and G. Giaccone. 2011. 2011 Focused Update of 2009 American Society of Clinical Oncology Clinical Practice Guideline Update on Chemotherapy for Stage IV Non-Small-Cell Lung Cancer. J. Clin. Oncol. 29: 3825-3831.

28. Peters, S., A. A. Adjei, C. Gridelli, M. Reck, K. Kerr, and E. Felip. 2012. Metastatic non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann. Oncol. 23 Suppl 7: vii56-vii64.

29. Reck, M. 2012. What future opportunities may immuno-oncology provide for improving the treatment of patients with lung cancer? Ann. Oncol. 23 Suppl 8: viii28-viii34.

30. Lynch, T. J., I. Bondarenko, A. Luft, P. Serwatowski, F. Barlesi, R. Chacko, M. Sebastian, J. Neal, H. Lu, J. M. Cuillerot, and M. Reck. 2012. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. J. Clin. Oncol. 30: 2046-2054.

31. Brahmer, J. 2013. Harnessing the Immune System for the Treatment of Non Small-Cell Lung Cancer., Juornal of Clinical Oncology 31, February 11, 2013 ed.

32. Brahmer, J., and et al. 2013. ASCO 2013 Annual Meeting, Abstract # 8030. The immune checkpoint receptor programmed death-1 (PD-1) negatively regulates T-cell activation. Nivolumab, a PD-1 receptor blocking antibody, was evaluated in a phase 1 trial in patients with various tumours including NSCLC.

33. Suzanne L Topalian et al. 2013. ASCO 2013 Annual Meeting, Abstract # 3002. Blockade of programmed cell death-1 (PD-1) in advanced solid tumours.

34. Aerts, J. G., and J. P. Hegmans. 2013. Tumor-specific cytotoxic T cells are crucial for efficacy of immunomodulatory antibodies in patients with lung cancer. Cancer Res. 73: 2381-2388.

35. Andersen, M. H., D. Schrama, S. P. thor, and J. C. Becker. 2006. Cytotoxic T cells. J. Invest Dermatol. 126: 32-41.

36. Ting, J. P., and J. Trowsdale. 2002. Genetic control of MHC class II expression. Cell 109 Suppl: S21-S33.

37. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392: 245-252.

38. Pardoll, D. M. 2012. The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer 12: 252-264.

39. Ferreira L.M.R. 2013. Gammadelta T cells: Innately Adaptive Immune Cells., Basic Immunology; International Reviews og Immunology, 32:223-248, 2013 ed.

40. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol. 22: 745-763.

41. Fruci, D., M. E. Lo, L. Cifaldi, F. Locatelli, E. Tremante, M. Benevolo, and P. Giacomini. 2013. T and NK cells: two sides of tumor immunoevasion. J. Transl. Med. 11: 30.

42. Shanker, A., and F. M. Marincola. 2011. Cooperativity of adaptive and innate immunity: implications for cancer therapy. Cancer Immunol. Immunother. 60: 1061-1074.

43. Van den Eynde, B. J., and S. Morel. 2001. Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. Curr. Opin. Immunol. 13: 147-153.

44. Castelli, C., L. Rivoltini, G. Andreola, M. Carrabba, N. Renkvist, and G. Parmiani. 2000. T-cell recognition of melanoma-associated antigens. J. Cell Physiol 182: 323-331.

45. Ostrand-Rosenberg, S., P. Sinha, D. W. Beury, and V. K. Clements. 2012. Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression. Semin. Cancer Biol. 22: 275-281.

46. Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. Nat. Rev. Immunol. 6: 295-307.

47. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? Immunity. 30: 626-635.

48. Correll, A., A. Tuettenberg, C. Becker, and H. Jonuleit. 2010. Increased regulatory T-cell frequencies in patients with advanced melanoma correlate with a generally impaired T-cell responsiveness and are restored after dendritic cell-based vaccination. Exp. Dermatol. 19: e213-e221.

49. Karagoz, B., O. Bilgi, M. Gumus, A. A. Erikci, O. Sayan, O. Turken, E. G. Kandemir, A. Ozturk, and M. Yaylaci. 2010. CD8+CD28- cells and CD4+CD25+ regulatory T-cells in pheripheral blood of advanced stage lung cancer patients. Med. Oncol. 27: 29-33.

50. Dannull, J., Z. Su, D. Rizzieri, B. K. Yang, D. Coleman, D. Yancey, A. Zhang, P. Dahm, N. Chao, E. Gilboa, and J. Vieweg. 2005. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J. Clin. Invest 115: 3623-3633.

51. Attia, P., A. V. Maker, L. R. Haworth, L. Rogers-Freezer, and S. A. Rosenberg. 2005. Inability of a fusion protein of IL-2 and diphtheria toxin (Denileukin Diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma. J. Immunother. 28: 582-592.

52. Jacobs, J. F., C. J. Punt, W. J. Lesterhuis, R. P. Sutmuller, H. M. Brouwer, N. M. Scharenborg, I. S. Klasen, L. B. Hilbrands, C. G. Figdor, I. J. de Vries, and G. J. Adema. 2010. Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: a phase I/II study in metastatic melanoma patients. Clin. Cancer Res. 16: 5067-5078.

53. Bronte, V., and P. Zanovello. 2005. Regulation of immune responses by L-arginine metabolism. Nat. Rev. Immunol. 5: 641-654.

54. Daud, A. I., N. Mirza, B. Lenox, S. Andrews, P. Urbas, G. X. Gao, J. H. Lee, V. K. Sondak, A. I. Riker, R. C. Deconti, and D. Gabrilovich. 2008. Phenotypic and functional analysis of dendritic cells and clinical outcome in patients with high-risk melanoma treated with adjuvant granulocyte macrophage colony-stimulating factor. J. Clin. Oncol. 26: 3235-3241.

55. Filipazzi, P., R. Valenti, V. Huber, L. Pilla, P. Canese, M. Iero, C. Castelli, L. Mariani, G. Parmiani, and L. Rivoltini. 2007. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. J. Clin. Oncol. 25: 2546-2553.

56. Liu, C. Y., Y. M. Wang, C. L. Wang, P. H. Feng, H. W. Ko, Y. H. Liu, Y. C. Wu, Y. Chu, F. T. Chung, C. H. Kuo, K. Y. Lee, S. M. Lin, H. C. Lin, C. H. Wang, C. T. Yu, and H. P. Kuo. 2010. Population alterations of L-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14(-)/CD15+/CD33+ myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer. J. Cancer Res. Clin. Oncol. 136: 35-45.

57. Roth, F., A. C. De La Fuente, J. L. Vella, A. Zoso, L. Inverardi, and P. Serafini. 2012. Aptamer-mediated blockade of IL4Ralpha triggers apoptosis of MDSCs and limits tumor progression. Cancer Res. 72: 1373-1383.

58. Finke, J., J. Ko, B. Rini, P. Rayman, J. Ireland, and P. Cohen. 2011. MDSC as a mechanism of tumor escape from sunitinib

mediated anti-angiogenic therapy. Int. Immunopharmacol. 11: 856-861.

59. Dunn, G. P., L. J. Old, and R. D. Schreiber. 2004. The immunobiology of cancer immunosurveillance and immunoediting. Immunity. 21: 137-148.

60. Marincola, F. M., E. M. Jaffee, D. J. Hicklin, and S. Ferrone. 2000. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Adv. Immunol. 74: 181-273.

61. Seliger, B. 2008. Molecular mechanisms of MHC class I abnormalities and APM components in human tumors. Cancer Immunol. Immunother. 57: 1719-1726.

62. Lang, K., F. Entschladen, C. Weidt, and K. S. Zaenker. 2006. Tumor immune escape mechanisms: impact of the neuroendocrine system. Cancer Immunol. Immunother. 55: 749-760.

63. Rivoltini, L., P. Canese, V. Huber, M. Iero, L. Pilla, R. Valenti, S. Fais, F. Lozupone, C. Casati, C. Castelli, and G. Parmiani. 2005. Escape strategies and reasons for failure in the interaction between tumour cells and the immune system: how can we tilt the balance towards immune-mediated cancer control? Expert. Opin. Biol. Ther. 5: 463-476.

64. , A., and D. H. Raulet. 2002. The innate immune response to tumors and its role in the induction of T-cell immunity. Immunol. Rev. 188: 9-21.

65. Gajewski, T. F., Y. Meng, and H. Harlin. 2006. Immune suppression in the tumor microenvironment. J. Immunother. 29: 233-240.

66. Munn, D. H., and A. L. Mellor. 2007. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J. Clin. Invest 117: 1147-1154.

67. Liu, X., N. Shin, H. K. Koblish, G. Yang, Q. Wang, K. Wang, L. Leffet, M. J. Hansbury, B. Thomas, M. Rupar, P. Waeltz, K. J. Bowman, P. Polam, R. B. Sparks, E. W. Yue, Y. Li, R. Wynn, J. S. Fridman, T. C. Burn, A. P. Combs, R. C. Newton, and P. A. Scherle. 2010. Selective inhibition of IDO1 effectively regulates mediators of antitumor immunity. Blood 115: 3520-3530.

68. Muller, A. J., W. P. Malachowski, and G. C. Prendergast. 2005. Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors. Expert. Opin. Ther. Targets. 9: 831-849.

69. Uyttenhove, C., L. Pilotte, I. Theate, V. Stroobant, D. Colau, N. Parmentier, T. Boon, and B. J. Van den Eynde. 2003. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat. Med. 9: 1269-1274.

70. Ino, K., E. Yamamoto, K. Shibata, H. Kajiyama, N. Yoshida, M. Terauchi, A. Nawa, T. Nagasaka, O. Takikawa, and F. Kikkawa. 2008. Inverse correlation between tumoral indoleamine 2,3-dioxygenase expression and tumor-infiltrating lymphocytes in endometrial cancer: its association with disease progression and survival. Clin. Cancer Res. 14: 2310-2317.

71. Okamoto, A., T. Nikaido, K. Ochiai, S. Takakura, M. Saito, Y. Aoki, N. Ishii, N. Yanaihara, K. Yamada, O. Takikawa, R. Kawaguchi, S. Isonishi, T. Tanaka, and M. Urashima. 2005. Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. Clin. Cancer Res. 11: 6030-6039.

72. Wainwright, D. A., I. V. Balyasnikova, A. L. Chang, A. U. Ahmed, K. S. Moon, B. Auffinger, A. L. Tobias, Y. Han, and M. S. Lesniak. 2012. IDO expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival. Clin. Cancer Res. 18: 6110-6121.

73. Brandacher, G., A. Perathoner, R. Ladurner, S. Schneeberger, P. Obrist, C. Winkler, E. R. Werner, G. Werner-Felmayer, H. G. Weiss, G. Gobel, R. Margreiter, A. Konigsrainer, D. Fuchs, and A. Amberger. 2006. Prognostic value of indoleamine 2,3-dioxygenase expression in colorectal cancer: effect on tumor-infiltrating T cells. Clin. Cancer Res. 12: 1144-1151.

74. Ino, K., N. Yoshida, H. Kajiyama, K. Shibata, E. Yamamoto, K. Kidokoro, N. Takahashi, M. Terauchi, A. Nawa, S. Nomura, T. Nagasaka, O. Takikawa, and F. Kikkawa. 2006. Indoleamine 2,3-dioxygenase is a novel prognostic indicator for endometrial cancer. Br. J. Cancer 95: 1555-1561.

75. Holtan, S. G., R. S. Dronca, W. K. Nevala, L. F. Porrata, A. S. Mansfield, M. S. Block, A. A. Leontovich, T. E. Grotz, J. D. Turner, H. P. Frisch, and S. N. Markovic. 2011. The dynamic human immune response to cancer: it might just be rocket science. Immunotherapy. 3: 1021-1024.

76. Lee, J. R., R. R. Dalton, J. L. Messina, M. D. Sharma, D. M. Smith, R. E. Burgess, F. Mazzella, S. J. Antonia, A. L. Mellor, and D. H. Munn. 2003. Pattern of recruitment of immunoregulatory antigen-presenting cells in malignant melanoma. Lab Invest 83: 1457-1466.

77. Munn, D. H., M. D. Sharma, D. Hou, B. Baban, J. R. Lee, S. J. Antonia, J. L. Messina, P. Chandler, P. A. Koni, and A. L. Mellor. 2004. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J. Clin. Invest 114: 280-290.

78. Andersen, M. H. 2012. The specific targeting of immune regulation: T-cell responses against Indoleamine 2,3-dioxygenase. Cancer Immunol. Immunother. 61: 1289-1297.

79. Cherayil, B. J. 2009. Indoleamine 2,3-dioxygenase in intestinal immunity and inflammation. Inflamm. Bowel. Dis. 15: 1391-1396.

80. Fallarino, F., and U. Grohmann. 2011. Using an ancient tool for igniting and propagating immune tolerance: IDO as an inducer and amplifier of regulatory T cell functions. Curr. Med. Chem. 18: 2215-2221.

81. Munn, D. H. 2011. Indoleamine 2,3-dioxygenase, Tregs and cancer. Curr. Med. Chem. 18: 2240-2246.

82. Sharma, M. D., D. Y. Hou, Y. Liu, P. A. Koni, R. Metz, P. Chandler, A. L. Mellor, Y. He, and D. H. Munn. 2009. Indoleamine 2,3-

dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. Blood 113: 6102-6111.

83. Sorensen, R. B., L. Berge-Hansen, N. Junker, C. A. Hansen, S. R. Hadrup, T. N. Schumacher, I. M. Svane, J. C. Becker, S. P. thor, and M. H. Andersen. 2009. The immune system strikes back: cellular immune responses against indoleamine 2,3-dioxygenase. PLoS. One. 4: e6910.

84. Sorensen, R. B., S. R. Hadrup, I. M. Svane, M. C. Hjortso, S. P. thor, and M. H. Andersen. 2011. Indoleamine 2,3-dioxygenase specific, cytotoxic T cells as immune regulators. Blood 117: 2200-2210.

85. Pietra, G., C. Manzini, S. Rivara, M. Vitale, C. Cantoni, A. Petretto, M. Balsamo, R. Conte, R. Benelli, S. Minghelli, N. Solari, M. Gualco, P. Queirolo, L. Moretta, and M. C. Mingari. 2012. Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. Cancer Res. 72: 1407-1415.

86. Hatem Hussein Soliman. 2013. A phase I study of 1-methyl-D-tryptophan in patients with advanced malignancies.

87. Robert Charles Newton, 2013. Pharmacodynamic assessment of INCB024360 an inhibitor of indeolamine 2.3 dioxygenase 1 (IDO1), in advanced cancer patients.
88. Cancer Vaccines, Challenges and Opportunities in Translation, Adrian Bot & Mihail Obrocea. 2011. Book chapter no. 6. "Peptide-based Active ImmuneTherapy in Cancer", ISBN 978-142005467-5.

89. Walter, S., T. Weinschenk, A. Stenzl, R. Zdrojowy, A. Pluzanska, C. Szczylik, M. Staehler, W. Brugger, P. Y. Dietrich, R. Mendrzyk, N. Hilf, O. Schoor, J. Fritsche, A. Mahr, D. Maurer, V. Vass, C. Trautwein, P. Lewandrowski, C. Flohr, H. Pohla, J. J. Stanczak, V. Bronte, S. Mandruzzato, T. Biedermann, G. Pawelec, E. Derhovanessian, H. Yamagishi, T. Miki, F. Hongo, N. Takaha, K. Hirakawa, H. Tanaka, S. Stevanovic, J. Frisch, A. Mayer-Mokler, A. Kirner, H. G. Rammensee, C. Reinhardt, and H. Singh-Jasuja. 2012. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat. Med.

90. Jager, E., D. Jager, and A. Knuth. 2003. Antigen-specific immunotherapy and cancer vaccines. Int. J. Cancer 106: 817-820.

91. Andersen, M. H., N. Junker, E. Ellebaek, I. M. Svane, and S. P. thor. 2010. Therapeutic cancer vaccines in combination with conventional therapy. J. Biomed. Biotechnol. 2010: 237623.

92. Emens, L. A. 2010. Chemoimmunotherapy. Cancer J. 16: 295-303.

93. Rosenberg, S. A., J. C. Yang, R. M. Sherry, U. S. Kammula, M. S. Hughes, G. Q. Phan, D. E. Citrin, N. P. Restifo, P. F. Robbins, J. R. Wunderlich, K. E. Morton, C. M. Laurencot, S. M. Steinberg, D. E. White, and M. E. Dudley. 2011. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin. Cancer Res. 17: 4550-4557.

94. Nagata, Y., T. Hanagiri, M. Mizukami, K. Kuroda, Y. Shigematsu, T. Baba, Y. Ichiki, M. Yasuda, T. So, M. Takenoyama, K. Sugio, A. Nagashima, and K. Yasumoto. 2009. Clinical significance of HLA class I alleles on postoperative prognosis of lung cancer patients in Japan. Lung Cancer 65: 91-97.

95. So, T., M. Takenoyama, M. Sugaya, M. Yasuda, R. Eifuku, T. Yoshimatsu, T. Osaki, and K. Yasumoto. 2001. Unfavorable prognosis of patients with non-small cell lung carcinoma associated with HLA-A2. Lung Cancer 32: 39-46.

96. Wolchok, J. D., J. S. Weber, O. Hamid, C. Lebbe, M. Maio, D. Schadendorf, P. de, V, K. Heller, T. T. Chen, R. Ibrahim, A. Hoos, and S. J. O'Day. 2010. Ipilimumab efficacy and safety in patients with advanced melanoma: a retrospective analysis of HLA subtype from four trials. Cancer Immun. 10: 9.

97. Sosman, J. A., J. M. Unger, P. Y. Liu, L. E. Flaherty, M. S. Park, R. A. Kempf, J. A. Thompson, P. I. Terasaki, and V. K. Sondak. 2002. Adjuvant immunotherapy of resected, intermediate-thickness, node-negative melanoma with an allogeneic tumor vaccine: impact of HLA class I antigen expression on outcome. J. Clin. Oncol. 20: 2067-2075.

98. Kim, C., C. W. Lee, L. Kovacic, A. Shah, R. Klasa, and K. J. Savage. 2010. Long-term survival in patients with metastatic melanoma treated with DTIC or temozolomide. Oncologist. 15: 765-771.

99. Amaravadi, R. K., L. M. Schuchter, D. F. McDermott, A. Kramer, L. Giles, K. Gramlich, M. Carberry, A. B. Troxel, R. Letrero, K. L. Nathanson, M. B. Atkins, P. J. O'Dwyer, and K. T. Flaherty. 2009. Phase II Trial of Temozolomide and Sorafenib in Advanced Melanoma Patients with or without Brain Metastases. Clin. Cancer Res. 15: 7711-7718.

100. Spieth, K., R. Kaufmann, R. Dummer, C. Garbe, J. C. Becker, A. Hauschild, W. Tilgen, S. Ugurel, M. Beyeler, E. B. Brocker, K. C. Kaehler, C. Pfohler, J. Gille, U. Leiter, and D. Schadendorf. 2008. Temozolomide plus pegylated interferon alfa-2b as first-line treatment for stage IV melanoma: a multicenter phase II trial of the Dermatologic Cooperative Oncology Group (DeCOG). Ann. Oncol. 19: 801-806.

101. Tarhini, A. A., J. M. Kirkwood, W. E. Gooding, S. Moschos, and S. S. Agarwala. 2008. A phase 2 trial of sequential temozolomide chemotherapy followed by high-dose interleukin 2 immunotherapy for metastatic melanoma. Cancer 113: 1632-1640.

102. Atkins, M. B., M. Regan, and D. McDermott. 2004. Update on the role of interleukin 2 and other cytokines in the treatment of patients with stage IV renal carcinoma. Clin. Cancer Res. 10: 6342S-6346S.

103. Keilholz, U., C. Scheibenbogen, W. Tilgen, L. Bergmann, E. Weidmann, E. Seither, M. Richter, B. Brado, P. S. Mitrou, and W. Hunstein. 1993. Interferon-alpha and interleukin-2 in the treatment of metastatic melanoma. Comparison of two phase II trials. Cancer 72: 607-614.

104. Gaspari, A. A. 2007. Mechanism of action and other potential roles of an immune response modifier. Cutis 79: 36-45.

105. Harris, R. C., K. A. Chianese-Bullock, G. R. Petroni, J. T. Schaefer, L. B. Brill, K. R. Molhoek, D. H. Deacon, J. W. Patterson,

and C. L. Slingluff, Jr. 2012. The vaccine-site microenvironment induced by injection of incomplete Freund's adjuvant, with or without melanoma peptides. J. Immunother. 35: 78-88.

106. Andersen, M. H., L. O. Pedersen, J. C. Becker, and P. T. Straten. 2001. Identification of a cytotoxic T lymphocyte response to the apoptosis inhibitor protein survivin in cancer patients. Cancer Res. 61: 869-872.

107. Andersen, M. H., L. O. Pedersen, B. Capeller, E. B. Brocker, J. C. Becker, and S. P. thor. 2001. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. Cancer Res. 61: 5964-5968.

108. Andersen, M. H., and S. P. thor. 2002. Survivin--a universal tumor antigen. Histol. Histopathol. 17: 669-675.

109. Grossman, D., J. M. McNiff, F. Li, and D. C. Altieri. 1999. Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. J. Invest Dermatol. 113: 1076-1081.

110. Brunsvig, P. F., S. Aamdal, M. K. Gjertsen, G. Kvalheim, C. J. Markowski-Grimsrud, I. Sve, M. Dyrhaug, S. Trachsel, M. Moller, J. A. Eriksen, and G. Gaudernack. 2006. Telomerase peptide vaccination: a phase I/II study in patients with non-small cell lung cancer. Cancer Immunol. Immunother. 55: 1553-1564.

111. Brunsvig, P. F., J. A. Kyte, C. Kersten, S. Sundstroem, M. Moeller, G. L. Hansen, M. Nyakas, G. Gaudernack, and S. Aamdal. 2011. Telomerase Peptide Vaccination in NSCLC: A Phase II Trial in Stage III Patients Vaccinated after Chemo-radiotherapy and an 8-years Update on a Phase I/II Trial. Clin. Cancer Res.

112. Kyte, J. A., G. Gaudernack, S. Dueland, S. Trachsel, L. Julsrud, and S. Aamdal. 2011. Telomerase peptide vaccination combined with temozolomide: A clinical trial in stage IV melanoma patients. Clin. Cancer Res.

113. Hodi F.S., Lee S.J., McDermott D.F Rao U.N., Butterfield L.H., Tarhini A.A., Leming P.D., Puzanov I., and Kirkwood J.M. 2013. Multicenter, randomized phase II trial of GM-CSF (GM) plus ipilimumab (Ipi) versus ipi alone in metastatic melanoma., ASCO Annual Meeting 2013, Abstract # CRA9007 ed.

114. Galluzzi, L., and E. Lugli. 2013. Cancer immunotherapy turns viral. Oncoimmunology. 2: e24802.

115. Andtbacka R.H.I, Collichio F.A., Amatruda T, Senzer N.N., Chesney J, Delman K.A., Spitler L.E., Puzanov I, Doleman S, Ye Y, Vanderwalde A.M., Coffin R, and Kaufman H. 2013. OPTIM trial: A randomized phase III trial of taligmogene laherparepvec (T-VEC) versus subcutaneous (SC) granulocyte-macrophage colony-stimulating factor (GM-CSF) for the treatment (tx) of unresected stage IIIB/C and IV melanoma., J Clin Oncol 31, 2013 (supll; abstract LBA9008) ed.

116. Kaufman, H. L., and S. D. Bines. 2010. OPTIM trial: a Phase III trial of an oncolytic herpes virus encoding GM-CSF for unresectable stage III or IV melanoma. Future. Oncol. 6: 941-949.

117. Therapy Evaluation Program, Common Terminology Criteria Adverse Events (CTCAE), V. 3.0., August 9, 2006 http: ctep.cancer.gov. 2013

118. Eisenhauer, E. A., P. Therasse, J. Bogaerts, L. H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, R. Kaplan, D. Lacombe, and J. Verweij. 2009. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur. J. Cancer 45: 228-247.

119. Wahl, R. L., H. Jacene, Y. Kasamon, and M. A. Lodge. 2009. From RECIST to PERCIST: Evolving Considerations for PET response criteria in solid tumors. J. Nucl. Med. 50 Suppl 1: 122S-150S.

120. Toebes, M., M. Coccoris, A. Bins, B. Rodenko, R. Gomez, N. J. Nieuwkoop, K. W. van de, G. F. Rimmelzwaan, J. B. Haanen, H. Ovaa, and T. N. Schumacher. 2006. Design and use of conditional MHC class I ligands. Nat. Med. 12: 246-251.

121. Andersen, M. H., J. E. Bonfill, A. Neisig, G. Arsequell, I. Sondergaard, G. Valencia, J. Neefjes, J. Zeuthen, T. Elliott, and J. S. Haurum. 1999. Phosphorylated peptides can be transported by TAP molecules, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL. J. Immunol. 163: 3812-3818.

122. Sperner-Unterweger, B., G. Neurauter, M. Klieber, K. Kurz, V. Meraner, A. Zeimet, and D. Fuchs. 2011. Enhanced tryptophan degradation in patients with ovarian carcinoma correlates with several serum soluble immune activation markers. Immunobiology 216: 296-301.

123. Widner, B., E. R. Werner, H. Schennach, H. Wachter, and D. Fuchs. 1997. Simultaneous measurement of serum tryptophan and kynurenine by HPLC. Clin. Chem. 43: 2424-2426.

124. Weber Jeffrey S, and et al. 2013. Phase I/II trial of PD-1 antibody nivolumab with peptide vaccine in patients naive to or that failed ipilimumab., J Clin Oncol 31, 2013 (Suppl; abstr 9011) ed.

125. Callahan M.K., Horak E., Curran M.A., Hollman T., Schaer D.A., Yuan J., Lesokhin A.M., Kitano S., Hong Q., Ariyan C.E, Busam K-J., Feely W., Jure-Kunkel M., Grosso J., Simon J.S., Korman J., Wigginton J.M., Gupta A.K., Sznol M, and Wolchok J.D. 2013. Peripheral and tumour immune correlates in patients with advanced melanoma treated with the comnbination nivolumab (anti-PD-1, BMS-936558, ONO-4538) and ipilimumab., J Clin Oncol 31, 2013 (suppl; Abstract 3003^) ed.

126. Powderly, J. D., Koeppen H, Hodi F.S., Sosman J.A., D. R. Gettinger S.N., Tabernero J, Soria J, Hamid O, Fine G.D., Xiao Y, Mokatrin A, Wu J, Anderson M, Irving B.A., Chen D.S, and Kowanetz M. 2013. Biomarkers and association with the clinical activity of PD-L1 blockade in a MPDL3280A study., J Clin Oncol 31, 2013 (suppl; abstract 3001) ed.

127. Hamid, O., H. Schmidt, A. Nissan, L. Ridolfi, S. Aamdal, J. Hansson, M. Guida, D. M. Hyams, H. Gomez, L. Bastholt, S. D. Chasalow, and D. Berman. 2011. A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma. J. Transl. Med. 9: 204.

128. Berman DM, W. J. W. J. H. O. O. D. S. C. S. 2013. Association of peripheral blood absolute lymphocyte count (ALC) and clinical activity in patietns (pts) with advanced melanoma treated with ipilimumab., J Clin Oncol 2009, 27(Supll 15s): 3020 ed.
129. Ku, G. Y., J. Yuan, D. B. Page, S. E. Schroeder, K. S. Panageas, R. D. Carvajal, P. B. Chapman, G. K. Schwartz, J. P. Allison, and J. D. Wolchok. 2010. Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting: lymphocyte count after 2 doses correlates with survival. Cancer 116: 1767-1775.

130. Wilgenhof, S., F. S. Du, F. Vandenbroucke, H. Everaert, I. Salmon, D. Lienard, V. D. Marmol, and B. Neyns. 2013. Single-center experience with ipilimumab in an expanded access program for patients with pretreated advanced melanoma. J. Immunother. 36: 215-222.

131. Vestermark, Bastholt et. al. ecancermeidcalscience, 2008, 2:91 2011. A phase 2 study of thalidomide and temozolamide in patients with brain metastases from malignant melanoma: lymphopenia correlates to response.

132. Phan, G. Q., P. Attia, S. M. Steinberg, D. E. White, and S. A. Rosenberg. 2001. Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. J. Clin. Oncol. 19: 3477-3482.

133. Andersen, R. S., C. A. Thrue, N. Junker, R. Lyngaa, M. Donia, E. Ellebaek, I. M. Svane, T. N. Schumacher, S. P. thor, and S. R. Hadrup. 2012. Dissection of T-cell antigen specificity in human melanoma. Cancer Res. 72: 1642-1650.

134. Galluzzi, L., L. Senovilla, L. Zitvogel, and G. Kroemer. 2012. The secret ally: immunostimulation by anticancer drugs. Nat. Rev. Drug Discov. 11: 215-233.

135. Palermo, B., B. D. Del, A. Sottini, F. Serana, C. Ghidini, N. Gualtieri, V. Ferraresi, C. Catricala, F. Belardelli, E. Proietti, P. G. Natali, L. Imberti, and P. Nistico. 2010. Dacarbazine treatment before peptide vaccination enlarges T-cell repertoire diversity of melan-a-specific, tumor-reactive CTL in melanoma patients. Cancer Res. 70: 7084-7092.

136. Andersen, M. H., J. Gehl, S. Reker, L. O. Pedersen, J. C. Becker, P. Geertsen, and S. P. thor. 2003. Dynamic changes of specific T cell responses to melanoma correlate with IL-2 administration. Semin. Cancer Biol. 13: 449-459.

137. Thomas F Gajewski, and F Stephen Hodi. 2013. Targeted Therapeutics in Melanoma., Current Clinical Oncology, Series Editor: Maurie Markman, MD, Humana Press ed.

138. Sarnaik, A. A., B. Yu, D. Yu, D. Morelli, M. Hall, D. Bogle, L. Yan, S. Targan, J. Solomon, G. Nichol, M. Yellin, and J. S. Weber. 2011. Extended dose ipilimumab with a peptide vaccine: immune correlates associated with clinical benefit in patients with resected high-risk stage IIIc/IV melanoma. Clin. Cancer Res. 17: 896-906.

139. Ribas, A., J. S. Weber, B. Chmielowski, B. Comin-Anduix, D. Lu, M. Douek, N. Ragavendra, S. Raman, E. Seja, D. Rosario, S. Miles, D. C. Diamond, Z. Qiu, M. Obrocea, and A. Bot. 2011. Intralymph node prime-boost vaccination against Melan A and tyrosinase for the treatment of metastatic melanoma: results of a phase 1 clinical trial. Clin. Cancer Res. 17: 2987-2996.

140. Romero, P., D. Valmori, M. J. Pittet, A. Zippelius, D. Rimoldi, F. Levy, V. Dutoit, M. Ayyoub, V. Rubio-Godoy, O. Michielin, P. Guillaume, P. Batard, I. F. Luescher, F. Lejeune, D. Lienard, N. Rufer, P. Y. Dietrich, D. E. Speiser, and J. C. Cerottini. 2002. Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. Immunol. Rev. 188: 81-96.

141. Walker, E. B., D. Haley, W. Miller, K. Floyd, K. P. Wisner, N. Sanjuan, H. Maecker, P. Romero, H. M. Hu, W. G. Alvord, J. W. Smith, B. A. Fox, and W. J. Urba. 2004. gp100(209-2M) peptide immunization of human lymphocyte antigen-A2+ stage I-III melanoma patients induces significant increase in antigen-specific effector and long-term memory CD8+ T cells. Clin. Cancer Res. 10: 668-680.

142. Andersen, M. H., J. C. Becker, and P. Straten. 2005. Regulators of apoptosis: suitable targets for immune therapy of cancer. Nat. Rev. Drug Discov. 4: 399-409.

143. Schwartzentruber, D. J., D. H. Lawson, J. M. Richards, R. M. Conry, D. M. Miller, J. Treisman, F. Gailani, L. Riley, K. Conlon, B. Pockaj, K. L. Kendra, R. L. White, R. Gonzalez, T. M. Kuzel, B. Curti, P. D. Leming, E. D. Whitman, J. Balkissoon, D. S. Reintgen, H. Kaufman, F. M. Marincola, M. J. Merino, S. A. Rosenberg, P. Choyke, D. Vena, and P. Hwu. 2011. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N. Engl. J. Med. 364: 2119-2127.

144. Lotte Engell-Noerregaard, Eva Ellebæk, Trine Zeeberg Iversen, Troels Holz Hansen, Marie K Brimnes, Özcan Met, Jon Bjoern, Mads Hald Andersen, Per thor Straten, Inge Marie Svane. "Analysis of regulatory T cell phenotype, including CD49d, during treatment of melanoma patients with dendritic cell vaccine, Interleukine-2 and/or metronomic Cyclophosphamide". Clinical and Cellular immunology, 2011. In press.

145. , A., R. Trepiakas, L. Wenandy, P. F. Geertsen, S. P. thor, M. H. Andersen, A. E. Pedersen, M. H. Claesson, T. Lorentzen, J. S. Johansen, and I. M. Svane. 2008. Therapeutic dendritic cell vaccination of patients with metastatic renal cell carcinoma: a clinical phase 1/2 trial. J. Immunother. 31: 771-780.

146. Ellebaek, E., L. Engell-Noerregaard, T. Z. Iversen, T. M. Froesig, S. Munir, S. R. Hadrup, M. H. Andersen, and I. M. Svane. 2012. Metastatic melanoma patients treated with dendritic cell vaccination, Interleukin-2 and metronomic cyclophosphamide: results from a phase II trial. Cancer Immunol. Immunother. 61: 1791-1804

147. Trepiakas, R., A. Berntsen, S. R. Hadrup, J. Bjorn, P. F. Geertsen, P. T. Straten, M. H. Andersen, A. E. Pedersen, A. Soleimani, T. Lorentzen, J. S. Johansen, and I. M. Svane. 2010. Vaccination with autologous dendritic cells pulsed with multiple tumor antigens for treatment of patients with malignant melanoma: results from a phase I/II trial. Cytotherapy. 12: 721-734.

148. Inderberg-Suso, E. M., S. Trachsel, K. Lislerud, A. M. Rasmussen, and G. Gaudernack. 2012. Widespread CD4+ T-cell reactivity to novel hTERT epitopes following vaccination of cancer

patients with a single hTERT peptide GV1001. Oncoimmunology. 1: 670-686.

149. EP Salermo, SM Shea, WC olson, GR Petroni, ME Smolkin, C McSkimming, Ka Chianse-Bullock, and CL Slingluff. 2013. Activation, dysfunction and retention of T cells in vaccine sites after injection of incomplete Freund´s adjuvant, with or witout peptide., Cancer immunology, immunotherapy: CII 2013 ed.

150. Hailemichael, Y., Z. Dai, N. Jaffarzad, Y. Ye, M. A. Medina, X. F. Huang, S. M. Dorta-Estremera, N. R. Greeley, G. Nitti, W. Peng, C. Liu, Y. Lou, Z. Wang, W. Ma, B. Rabinovich, K. S. Schluns, R. E. Davis, P. Hwu, and W. W. Overwijk. 2013. Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and deletion. Nat. Med. 19: 465-472.

151. Hansen, J., T. Lindenstrom, J. Lindberg-Levin, C. Aagaard, P. Andersen, and E. M. Agger. 2012. CAF05: cationic liposomes that incorporate synthetic cord factor and poly(I:C) induce CTL immunity and reduce tumor burden in mice. Cancer Immunol. Immunother. 61: 893-903.

152. Nordly, P., F. Rose, D. Christensen, H. M. Nielsen, P. Andersen, E. M. Agger, and C. Foged. 2011. Immunity by formulation design: induction of high CD8+ T-cell responses by poly(I:C) incorporated into the CAF01 adjuvant via a double emulsion method. J. Control Release 150: 307-317.

153. Jackson E, Dees EC, Kauh JS, Harvey RD, Neuger A, Lush R, Minton SE, Ismail-Khan R, Han HS, Vahanian NN, Ramsey WJ, Link CJ, Streicher H, Sullivan D, Antonia S, and Soliman HH. 2013. A phase I study of indoximod in combination with docetaxel in metastatic solid tumours., ASCO Annual Meeting 2013 (Abstract 3026) ed.

154. Beatty GL, O´Dwyer PJ, Clark J, Shi JG, Newton RC, Schaub R, Maleski J, Leopold L, and Gajewski T. 2013. Phase I Study of the Safety, Pharmacokinetics, and Pharmacodynamic of the Oral Inhibitor of Indoleamine 2,3-dioxygenase (IDO1) INCB024360 in patients with Advanced Malignancies., ASCO Annual Meeting 2013 (Abstract 3025) ed.

155. Holmgaard RB, Zamarin D, Munn DH, Wolchok JD, and Allison JP. 2013. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumour T cell immunotherapy targeting CTLA-4., The Journal of Experimental Medicin (JEM) 2013 ed.

156. Demaria, S., and S. C. Formenti. 2012. Radiation as an immunological adjuvant: current evidence on dose and fractionation. Front Oncol. 2: 153.

157. Demaria, S., and S. C. Formenti. 2013. Radiotherapy effects on anti-tumor immunity: implications for cancer treatment. Front Oncol. 3: 128.

158. Formenti, S. C., and S. Demaria. 2013. Combining radiotherapy and cancer immunotherapy: a paradigm shift. J. Natl. Cancer Inst. 105: 256-265.

159. Seung, S. K., B. Curti, M. Crittenden, and W. Urba. 2012. Radiation and immunotherapy: Renewed allies in the war on cancer. Oncoimmunology. 1: 1645-1647.

160. Seung, S. K., B. D. Curti, M. Crittenden, E. Walker, T. Coffey, J. C. Siebert, W. Miller, R. Payne, L. Glenn, A. Bageac, and W. J. Urba. 2012. Phase 1 study of stereotactic body radiotherapy and interleukin-2--tumor and immunological responses. Sci. Transl. Med. 4: 137ra74.

161. Postow, M. A., M. K. Callahan, C. A. Barker, Y. Yamada, J. Yuan, S. Kitano, Z. Mu, T. Rasalan, M. Adamow, E. Ritter, C. Sedrak, A. A. Jungbluth, R. Chua, A. S. Yang, R. A. Roman, S. Rosner, B. Benson, J. P. Allison, A. M. Lesokhin, S. Gnjatic, and J. D. Wolchok. 2012. Immunologic correlates of the abscopal effect in a patient with melanoma. N. Engl. J. Med. 366: 925-931.

162. Frederick, D. T., A. Piris, A. P. Cogdill, Z. A. Cooper, C. Lezcano, C. R. Ferrone, D. Mitra, A. Boni, L. P. Newton, C. Liu, W. Peng, R. J. Sullivan, D. P. Lawrence, F. S. Hodi, W. W. Overwijk, G. Lizee, G. F. Murphy, P. Hwu, K. T. Flaherty, D. E. Fisher, and J. A. Wargo. 2013. BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. Clin. Cancer Res. 19: 1225-1231.