Long-lasting disease stabilization in the absence of toxicity in metastatic lung cancer patients vaccinated with an epitope derived from indoleamine 2,3 dioxygenase

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None of the authors have conflicts of interests to declare. It should 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.
**Translational relevance**

Indoleamine 2.3-dioxygenase (IDO) potently inhibits T-cell immunity in cancer patients. Blocking of this negative regulation pathway might promote immune-mediated tumour regression. In this first in man clinical study (NCT01219348) we demonstrate clinical relevance of targeting IDO by a peptide vaccine strategy in 15 metastatic, stage III/IV non small-cell lung cancer (NSCLC) patients. We show that IDO is frequently expressed in lung adenocarcinomas hence representing an attractive target for cancer immunotherapy. Clinical evaluation demonstrated one patient with a partial response induced and another 6 patients eliciting long-lasting disease stabilization. The median OS was 25.9 months with no severe toxicities observed. Treg level was reduced during treatment and IDO-specific CD8⁺ T cells from treated patients were able to kill IDO-expressing cancer cells. Lysis of cancer cells while suppressing regulatory T cell function might be important for clinical benefit and therapeutic targeting of IDO may be an important vaccine strategy in metastatic NSCLC.
Abstract

**Purpose:** To investigate targeting of indoleamine 2.3 dioxygenase (IDO) enzyme using a synthetic peptide vaccine administered to patients with metastatic NSCLC.

**Experimental design:** In a clinical phase I study we treated 15 HLA-A2 positive patients with stage III-IV NSCLC in disease stabilization (SD) after standard chemotherapy. Patients were treated with Imiquimod ointment and subcutaneous vaccinations (100 µg IDO5 peptide, sequence ALLEIASCL, formulated in 900 µL Montanide). Primary end point was toxicity. Clinical benefit and immunity were assessed as secondary endpoints.

**Results:** No severe toxicity was observed. One patient developed a partial response (PR) after 1 year of vaccine treatment while long-lasting disease stabilization (SD≥8.5 months) was demonstrated in another 6 patients. The median overall survival (OS) was 25.9 months. Patients demonstrated significant improved OS (P=0.03) when compared to the group of patients excluded due to HLA-A2 negativity. IDO specific CD8+ T cell immunity was demonstrated by IFN-y Elispot and Tetramer staining. FACS analyses demonstrated a significant reduction of the Treg population (P=0.03) after the 6th vaccine (2.5 months) compared to pre-treatment levels. Furthermore, expression of IDO was detected in 9/10 tumour biopsies by immunohistochemistry. HPLC analyses of Kynurenine/Tryptophan (Kyn/Trp) ratio in sera were performed. In long term analyses of two clinical responding patients the ratio of Kyn/Trp remained stable.

**Conclusion:** The vaccine was well-tolerated with no severe toxicity occurring. A median OS of 25.9 months was demonstrated and long-lasting PR+SD were seen in 47% of the patients.
Introduction

Lung cancer is one of the leading causes of cancer deaths in both men and women worldwide, with NSCLC accounting up to 85% of the cases (1). 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%. 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. Nonetheless, therapeutic vaccines such as tumour cell vaccine 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 and metastatic settings in NSCLC patients (2). Immune checkpoint blockade such as anti-cytotoxic T-cell lymphocyte-4 (CTLA-4) antibody is an anticancer therapy of current interest in NSCLC due to the shown inductions of T-cell activation and clinical activity (3). Other checkpoint blockade treatments like the antibody against programmed death 1 (PD-1) and its ligand (PD-L1) have shown clinical responses in patients with advanced NSCLC in early phase I clinical trials (4,5). These data supports the idea that checkpoint blockade can enhance polyfunctional spontaneous pre-existing T cell responses. We have formerly shown spontaneous cytotoxic T cell reactivity against indoleamine 2.3 dioxygenase (IDO) (6,7) an immune regulatory enzyme that suppresses T-cell immunity. IDO catalyzes the initial and rate-limiting step in the degradation of tryptophan (Trp), which leads to local depletion of Trp and an increase in downstream metabolites e.g. kynurenine (Kyn). It has been demonstrated that patients with different tumour types have elevated Kyn/Trp ratio compared to healthy donors (HD), which suggests elevated IDO activity in cancer patients (8). Moreover, the ratio of Kyn/Trp in serum has been proposed by others as a non-invasive, in vivo biomarker for evaluating the efficacy of IDO.
inhibitors in the clinic (9). Clinical investigation of IDO inhibition in phase I dose-escalating trials has been initiated for patients with metastatic solid tumours (10,11).

It is well-known that cancer cells as well as dendritic cells may upregulate IDO. As IDO-specific CD8 T cells has been shown to boost immunity against tumor associated antigens by eliminating IDO+ regulatory cells, which, in turn, has lead to a decrease in regulatory T cells (Tregs) (7) this justified clinical testing of the safety and efficacy of IDO-targeting vaccination. Thus, we performed a phase I vaccination study in patients with stage III/IV NSCLC testing an IDO-derived peptide vaccine applied in Montanide adjuvant.

**Material and Methods**

**Patients**

Patients were enrolled at the Department of Oncology at Copenhagen University Hospital, Herlev, Denmark from June 2010 to May 2012. All patients provided written informed consent prior to inclusion and GCP monitoring of the study was conducted. The study was performed in accordance with the Helsinki II declaration and was initiated after approval from the National Board of Health and the local Ethics Committee at the Capital Region of Denmark. The study was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01219348). Primary end point was toxicity. In addition, clinical benefit and immunity were assessed. Tumour staging was performed according to the Union for International Cancer Control (UICC) classification on CT scans according to RECIST version 1.1. Clinical assessment by CT scans was performed at baseline and subsequently every 3rd month. Anti-neoplastic treatments prior to inclusion were administered at the Department of Oncology according to standards of care. HLA typing was performed at the Laboratory of Tissue Typing at the Copenhagen University Hospital at Rigshospitalet. Inclusion criteria were: Stage III/IV metastatic
NSCLC, ≥18 years of age, stable disease (SD) after chemotherapy/TKI inhibitor (any line) according to RECIST 1.1 and a WHO performance status of 0-1. In total, 34 patients were screened for inclusion, only patients harbouring the tissue type HLA-A2 were eligible (N=15). The HLA-A2 negative patients (N=10) were treated at the Department of Oncology with the standard of care for metastatic NSCLC disease. Patients defined as screening-failure (failure to meet inclusion criteria) due to (prednisone treatment N=3, progressive disease N=4, autoimmune disease N=1, diagnosis of prostate cancer <5 years prior to inclusion N=1), were excluded from the statistical analyses. Post trial, re-evaluation of CT scans revealed that 1 patient treated with the vaccine did not completely meet the RECIST 1.1 definition at baseline, since this patient’s (#17) target lesion was down-scaled from 1.5 to 1.3 cm. The patient is included in both the clinical and immunological analyses. Patients were treated every second week (induction) for 2.5 months and thereafter monthly (maintenance) with Imiquimod ointment (®Aldara, Meda AS, www.meda.se) and vaccine (100 µg IDO5 peptide, sequence ALLEIASCL, mixed with 900 µL Montanide) administered subcutaneously until progression. The Imiquimod (1 sachet 5%) was applied 8 hours before vaccination and covered by a patch until vaccine administration in the same area of the skin.

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. Briefly, the sections were pre-treated in PT Link from Dako® using high low pH target retrieval solution (Dako DM828). The staining was performed on the DakoLink 48 from Dako utilizing the EnVision Flex+ detection kit (Dako K8002). The primary antibody was diluted 1:25 using Antibody Diluent (Dako DM830) and was incubated for 20 minutes. The sections were
counterstained with haematoxylin. As control for IDO staining tissue samples from placenta (syncytiotrophoblasts cells) known to stain positive for IDO were used.

**Flow cytometry**

Flow cytometry analyses were carried out on a FACS Canto II cytometer (BD) Biosciences. 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.

For the CD4+/Treg assay PBMCs were thawed and incubated with mouse serum (Invitrogen) followed by labelling with fluorochrome–conjugated antibodies: PB-CD3 (DAKO), PerCP-CD4 (BD Bioscience), APC-CD25 (BD-Pharmgien), PE-CD27 (BD-Pharmgien) and the relevant isotypes were used for antigen surface staining. PE-FoxP3 (ebioscience) and PE-isotype PE rat IgG2a (ebioscience) were used for intracellular staining; cells were fixed and permeabilized using a fixation/permeabilization kit (ebioscience) according to the manufacturer’s instruction. As dead cell marker APC-Cy7 near IR (Invitrogen) fluorescent reactive dye was used. Antigen surface staining for CD8+/CD4+ Tcm, Tem, Temra and Tnaïve analyses were PB-CD3 (DAKO), PerCP-CD4 (BD Bioscience), APC-CD8 (BD Pharmingen), PE-Cy7-CCR7 (BD Pharmingen), Krom-Orange-CD45 (Ramcon), FITC-CD45RA (BD Bioscience). For analyses of myeloid-derived suppressor cells (MDSC) surface antigen labelling was used: FITC-HLA-DR- lineage negative (BD Pharmingen), PE-Cy7-CD3 lineage (BD Pharmingen), PE-Cy7-CD19 lineage (BD Pharmingen) and PE-Cy7-CD56 lineage (BD Pharmingen), PE-CD33 (DAKO), APC-CD11b (BD Pharmingen), PerCP-CD14 (BD Pharmingen) and PB-CD15 (Biolegend).
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 (12). The MHC tetramer complexes used were: HLA-A2/IDO5(ALLEIASCL)-PE, HLA-A2/IDO5(ALLEIASCL)-APC, HLA-A2/HIV(ILKEPVHGV)-PE and HLA-A2/HIV(ILKEPVHGV)-APC. The antibodies used for the stainings were: CD8-PerCP, CD4-FITC, CD14-FITC, CD16-FITC, CD19-FITC (BD Biosciences) and CD40-FITC (AbD SeroTec).

**Cytotoxicity assay**

Conventional 51Cr-release assays for CTL-mediated cytotoxicity were carried out as described (13). T2 cells were pre-loaded with 20 µM peptide and 51Cr for 1 hour, then washed 2 times in RPMI + 10% FCS. Target cells (5000/well) were T2 cells (American Type Culture Collection (ATCC) with HIV-1 pol476-484 (ILKEPVHGV) or T2 cells with IDO5199-207(ALLEIASCL). Target cells (5000/well) were used in a cytotoxicity assay with an IDO positive HLA-A2 cancer cell line (SW480). Cell lines included in the study were tested and authenticated by HLA genotyping. The cell lines were routinely confirmed with their HLA typing and antigen expression by flow cytometry and coculture assays, respectively.

**Elispot**

ELISPOT analyses were performed according to CIMT Immunoguiding Program (CIP) guidelines (http://cimt.eu/cimt/files/dl/cip_guidelines.pdf). PBMC were stimulated once in x-vivo medium +5% HS, 15 µM peptide + 120 U/ml IL2 in vitro with the IDO5 peptide prior to analysis to extend the sensitivity of the assay (14). Nitrocellulose bottomed 96-well plates (MultiScreen MSIPN4W; Millipore) were coated overnight with IFN-γ capture mAb (Mabtech). The wells were washed, blocked by X-vivo medium (no serum used) and the effector cells were added in duplicates at two
different cell concentrations, with or without 5 μM of the IDO5 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 Ab (Mabtech). The plates were incubated at room temperature (RT) for 2 hours, washed, and avidin-enzyme conjugate (AP-Avidin; Calbiochem/Invitrogen Life Technologies) was added to each well. Plates were incubated at RT for 1 hour and the enzyme substrate NBT/BCIP (Invitrogen Life Technologies) was added to each well and incubated at RT 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-y secreting cells were at least 2-fold above the negative control (medium) and with a minimum of 50 spots (per 6*10e5 PBMC) detected.

Establishment of IDO specific T-cell cultures

The culture was enriched for specific cells by staining PBMC with HLA-A2/IDO5-PE and HLA-A2/IDO5-APC tetramer followed by sorting of tetramer-binding cells on a FACS Aria. Specific cells were expanded by incubation with 0.6 μg anti-CD3 (eBioscience, clone OKT3) and high dose (6000 U/ml for at least 14 days) IL-2 (Proleukin, Novartis).

Assessment of IDO activity in patient serum

Sera from patients were obtained at pre-treatment, at the 4th, at the 6th vaccine and subsequently every 3rd month. 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° Celsius freezer.
IDO activity was estimated by quantifying tryptophan (Trp) and its metabolite kynurenin (Kyn) in patient sera, essentially as previously described (15,16). 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®, Dr Maisch 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. At the end of each run, the column was washed with methanol 90% and re-equilibrated with 3% ACN 0.05% TFA before injection of the next sample. Peaks were detected with a fluorescent detector (Exc. 285nm/Em 405nm) and a UV/Vis detector at 360nm for Trp and Kyn respectively (16). To quantify both products, a series of 8 standards from 5-100µM Trp and 1.25-20 µM Kyn (both Sigma) was generated by diluting frozen stock solutions in human serum albumin 70g/l (Biotest AG, Dreieich, Germany) in duplicates. Sera of three selected healthy donors were also added to each HPLC run in duplicate, showing an inter-assay coefficient of variation for Trp, Kyn and Kyn/Trp ratio <6%. Normalization was performed by spiking 3-nitro-L-tyrosinase at 0.1 mM (Sigma, detection 360nm) as internal calibrator in all samples. Results were calculated from peak areas and are expressed as Kyn µM / Trp mM ratios (mean of triplicate or duplicate measurements) (15).

Statistics
Statistical analyses were performed by GraphPad Prism software v. 5.0. Survival statistics for overall survival (OS) and progression free survival (PFS) were performed by the Kaplan Meier method. Survival comparisons between HLA-A2 positive and negative patients were calculated by the Log Rank test. Individual serial samples of T-lymphocyte subsets were assessed using paired Wilcoxon signed rank non parametric tests. For analyses of the pre-level of Kyn/Trp ratio for SD
versus progressive disease (PD) patients an unpaired t-test was applied. In all analyses a two-sided P value of <0.05 was considered to be significant.

Results

Demographics

Patients characteristics for both HLA-A2 positive patients (N=15) and HLA-A2 negative patients (N=10) otherwise eligible but not harbouring the necessary HLA-A2 allele for vaccination are presented in Fig.1A. According to protocol requirements CT scans for HLA-A2 positive patients was described using RECIST 1.1 whereas for HLA-A2 negative patients RECIST 1.0 according to local standard was used thus baseline characteristics were not completely comparable among the two groups. Patients were in SD after chemotherapy and/or TKI treatment (any line) at time of enrolment. Vaccine treatment was initiated at least 28 days after last dose of chemotherapy. Metastatic sites of tumour lesions as visualized by CT scan are shown in Fig.1B as expected the most frequent locations were in the lung and lymph nodes (LN). Screening for brain metastasis was not routinely performed at inclusion. Four patients (26%) included in the trial eventually developed brain metastases.

Toxicity

No grade 3-4 CTCAE toxicity was observed hence the CTCAE 1-2 events are shown in Fig.1C. High frequencies of fatigue, shortness of breath, coughing and haemoptysis were present at baseline and were probably related to the lung cancer. Nausea and headache were observed in relation to the development of brain metastases, while dyspepsia, abdominal pain and diarrhoea could be related to the vaccine treatment, as IDO is expressed in the epithelial cells in the gastrointestinal tract (17,18). In 90% of the patients development of grade 1-2, short term, local reactions at the vaccine site (i.e.
redness, swelling or itching) were observed. Local treatments with steroid ointments removed the symptoms.

**Clinical efficacy and survival estimates**

Duration of the vaccine treatment demonstrated a mean of 7.9 months (238 days) of vaccine treatment (with a 95% confidence interval (CI) of 4.6-11.3 months) as shown in Fig.1D. The mean number of vaccinations were 11 (range 6-29). Clinical benefit was defined as objective response or SD for a minimum of 8.5 months after vaccine initiation, corresponding to treatment with at least 9 vaccines and demonstration of SD on 3 consecutive CT-scans. Based on this definition 7 of 15 patients had clinical benefit; 1 PR and 6 SD. Tumour response to the vaccine treatment is depicted in Fig.2A. One patient (Pt. #18) developed PR of target lesions in the liver with continuous regression from the 2nd to the 5th evaluation (-14%, -22%, -31% and -31% respectively, confirmed PR) as shown in Fig.2B. This patient is still on-study (17 months+). Patients were divided into 2 groups: SD + PR (SD; N=6, PR; N=1) and PD (N=8) for the following analyses. PFS defined as date of first vaccine treatment to date of PD is provided in Fig.2C (top), median PFS was 5.2 month (157 days), 2 patients are still on-study (15-17 months+). Patients in the SD+PR group (N=7) had a trend towards longer OS (P=0.05) than patients in the PD group (N=8), as shown in Fig.2C (bottom). In the vaccination group 9 patients are still alive. Among these 9 patients 6 belonged to the SD+PR group. OS estimate defined as date of first vaccine to date of death is shown for both vaccinated HLA-A2 positive patients (dotted line) and unvaccinated HLA-A2 negative patients (solid line) in Fig.2D. Vaccinated patients had a median survival of 25.9 months (778 days) demonstrating significant longer OS (P=0.03) when compared to the vaccine-untreated group of patients demonstrating a median OS of 7.7 months (237 days).
**IDO expression in NSCLC**

Expression of IDO in the cytoplasm of the cancer cells in pre-treatment tumour biopsies was detected in 9 out of 10 patients. Grading of IHC staining (negative, 1+, 2+ or 3+ positive) of IDO expression was performed as suggested in a recent study on NSCLC (19). The grading of IDO expression in the tumour samples was defined as grade 1=0-30%; grade 2 > 30-50% and grade 3 > 50% of the tumour cell stained, the estimates were performed by an experienced pathologist blinded to clinical response. From 5 patients we did not have access to sufficient tumour biopsies and in 1 patient the IHC was inconclusive. Grading of IDO expression is shown in Fig.3A and examples of IHC (grade 3 in Pt. #04 and Pt. #06, respectively) are shown in Fig.3B. All accessible tumours expressed IDO and no correlation of grading in IDO expression and clinical response to vaccine treatment was found.

**Frequency of IDO-specific T cells in PBMC during vaccination**

Presence of IDO specific CD8+ T cells was demonstrated by IFNy Elispot. All assays were performed in duplicates at two different cell numbers (2.5*10e5 and 6.0*10e5 PBMCs/well). In Fig.4A Elispot responses +/- IDO peptide are exemplified for patient #02 who had prolonged SD.

IFNy Elispot IDO responses could be demonstrated in all patients at different time points as shown in Fig.4B (left panel). Thus, IFNy releasing cells were frequently detected both in SD+PR and PD patients during vaccinations. In Fig. 4B (right panel) mean IDO response for different time points are demonstrated. Only at baseline did the measurement of CD8+ IDO specific T cells trend towards differing (P=0.05) the SD+PR compared to PD patients, as shown in Fig.4C.

Low frequency-responses of tetramer positive IDO-specific T cells were detected after cell culturing and expansion in 3 SD patients post vaccine treatment, as exemplified for patient #03 in
Fig.4D (left panel). The tetramer positive cells were isolated by cell sorting and expanded in vitro. These T cells demonstrated effective, specific killing (~20% lysis) of TAP-deficient T2-cells pulsed with the IDO5 peptide and in addition effective killing (~30% lysis) of an IDO+ HLA-A2 positive cancer cell line (SW480) as shown in Fig.4D (right panel).

**Treg reduction after vaccine therapy**

Flow cytometry analyses of PBMCs were performed pre-treatment and after 4th and 6th vaccinations in all patients. Gating strategy (shown in supplementary) was based on living lymphocytes (single cells, living, CD45+) as being the reference population for subtypes of immune cells CD4+ and CD8+ including memory subtypes (TNaïve, TCM, TEM, TEMRA), regulatory T cells (Treg) and natural killer cells (NK) analysed. For the monocytic myeloid derived suppressor cells (Mo-MDSCs) monocytic cells were included in the gating on lymphocytes. Fig.5A (left panel) depicts the percentage CD8+ T cells during treatment, (right panel) the CD8+ TEM (CD3+CD8+CD45RA-CCR7-) and Fig.5B (left panel) the CD4+ T cell population. In general, no significant changes were seen during treatment in these cell populations. However, the SD+PR patients experienced an increase (though insignificant) in peripheral CD8+ and in CD8+ TEM cells which was not observed in the PD patients. Interestingly, a significant decreased level of the Tregs (P=0.03) was detected after 6 vaccinations in both SD+PR and PD patients, depicted in Fig. 5B (right panel). In addition, a trend towards an increase in the NK cell population (P=0.05) after 6 vaccinations demonstrated no difference between SD+PR and PD patients seen in Fig.5C. The monocytic MDSC population remained unchanged during treatment as shown in Fig 5D. Only a tendency in lower pre-treatment level of MDSC was seen in SD+PR patients compared to pre-treatment level in PD patients.

**Stabilization of Kyn/Trp ratio suggesting IDO blockade**
Elevated Kyn/Trp ratio has been suggested to mirror IDO activity hence HPLC measurements of Kyn and Trp levels from sera at baseline, 4th and 6th vaccines were performed. In Fig.6A the ratio of Kyn/Trp at the different time points are shown. In 8/11 patients the level at the 6th vaccine was stable or lower than at pre-treatment with no difference between SD+PR patients (4/5) and PD patients (4/6). However, at baseline the SD+PR patients had non-significantly higher Kyn/Trp levels (mean 71.1 95% CI 35.9-106.3) compared to PD patients (mean 57.8 95% CI 32.9-82.8) shown in Fig.6B. Overall no significant decrease in Kyn/Trp ratio was measurable after the 6th vaccine (mean 68.4 95% CI 44.2-92.2) compared to pre-treatment (mean 67.0 95% CI 42.8-91.2), as shown in Fig.6C (N=11, SD+PR; N=5, PD; N=6). However, only 2 patients (Pt. #04 (after 4th vacc) and #06 (after 6th vacc)), see Fig. 6A and 6C experienced a strong augmentation of Kyn/Trp, suggesting increased IDO activity. Interestingly, these two patients also demonstrated high IDO expressions (grade 3) in the IHC analyses and experienced early tumour progression. Measurements of long term Kyn/Trp ratio were performed from 4 patients with maintained SD as shown in Fig.6D. Long term stabilization was demonstrated in two clinical responders i.e. Pt. #03 (SD for 2 years) and Pt. #18 (PR ongoing). Regarding Pt. #03, #15 and #19 they went off study due to progression within 3 months after the latest time point of Kyn/Trp measurements.

Discussion
We recently described that IDO-reactive T cells are able to recognize and kill tumor cells as well as IDO-expressing dendritic cells, i.e. one of the major immune suppressive cell populations (6,7). Consequently, IDO may serve as an important and widely applicable target for anti-cancer immunotherapeutic strategies. We tested this hypothesis in a first-in-man clinical phase I vaccination trial, comprising 15 advanced NSCLC patients were vaccinated with an HLA-A2 restricted IDO peptide formulated in Montanide. The vaccine was well tolerated with manageable
side effects and no CTCAE grade 3/4 toxicities. Only local reactions at the vaccine site were frequently induced (90% of patients) most likely due to Montanide (20) and Imiquimod (21).

Based on an expected median PFS in this patient group of approximately 6-7 months (22) we defined clinical benefit as objective response or SD for at least 8.5 months which was demonstrated in 47% of the treated patients. The one patient with objective response (PR) had continuous tumour regression on vaccine treatment for 1 year before qualifying as a partial response. This pattern of long term disease stabilization which eventually can lead to objective responses has also been shown with ipilimumab treatment, and has lead to development of the immune related Response Criteria (irRC) (23). In our study the median OS was >2 years, which was higher than expected for this patient group and is emphasized by the fact that 6/7 of the SD patients are still alive. One explanation could be that T-cell priming by the IDO vaccine in some patients is of importance for response to succeeding anti-neoplastic treatments or induces long lasting anti-tumour effects (24). To this end, the recently FDA approved sipuleucel T vaccine for advanced prostate cancer patients demonstrated prolonged overall survival though no effect on time to progression was demonstrated (25).

The clinical significance of HLA phenotype in cancer patients has been widely investigated. Importantly, it was recently described in a large study that expression of HLA-A2 was an unfavourable prognostic factor in stage I NSCLC patients (26). This study underlines the potential importance of the significantly longer OS observed in vaccinated HLA-A2 patients compared to unvaccinated HLA-A2 negative NSCLC patients, though these data need confirmation in large clinical trials. To this end, it is important to stress that the difference in OS among the two groups might have been influenced by differences in baseline characteristics in terms of tumour load,
distribution of metastatic lesions and histological subtypes thus underscoring the need for a randomized trial. Also, it should be noted that OS is influenced by subsequent therapy. Available treatments other than the IDO vaccine have been identical among HLA-A2 positive and negative patients. Despite this, several of the vaccinated patients have obtained exceptional long responses to succeeding therapy; including radiation therapy e.g. whole brain radiation for multiple brain metastases and targeted therapy such as Tarceva (Erlotinib). Several cancer vaccine studies have previously indicated a possible positive influence on efficacy of subsequent therapy e.g. in a recent lung cancer study (27). Thus, it cannot be ruled out that the IDO vaccine have increased responsiveness to further therapy.

Tumour cells expressing IDO by IHC staining have been detected in many different carcinomas including NSCLC (28). The correlation between IDO expression (IHC) and negative clinical outcome has among other tumour types been shown in ovarian cancer (29), glioblastoma (30) and endometrial cancer (31). Our data demonstrate that IDO was frequently expressed in the NSCLC samples, indicating that the tumour tissue did indeed express the targeted antigen. In general, there was no correlation between the intensity of IDO expression and the clinical response to vaccine treatment. Furthermore, no clear correlation between IDO expression in tumour biopsies and Kyn/Trp ratio in sera could be demonstrated. However, in 2 of the PD patients with very short term survival grade 3+ expression of IDO was detected which might implicate an unfavourable prognosis. Also, these 2 patients experienced a strong increase in the Kyn/Trp ratio.

Immune monitoring was performed by IFN-y Elispot, which is a highly sensitive method to detect antigen specific T cells (magnitude and function) from peripheral blood mononuclear cells (PBMC). Our data demonstrate that IDO specific T cells were indeed detectable in all patients. This
observation indicates that IDO could be a suitable target for vaccination as immunity towards IDO seems to be a general characteristic of NSCLC patients independent of clinical status. In 2 SD patients an IDO-specific Elispot response was detected during 1 year of treatment, suggesting sustained long-term IDO reactivity. Noteworthy, we found a trend towards higher pre-treatment levels of IDO specific T cells in the SD+PR patients compared to the PD patients. Thus, our data indicate that the SD+PR patients had a higher level of T cells primed for an IDO response, which is in accordance with previously suggested IDO immunity (7). In addition, we utilized the MHC peptide tetramer technology to directly visualize IDO-specific T cells. In 3 patients low-frequent tetramer positive IDO specific T cells were detectable, which was fewer compared to the number of patients with Elispot responses. Interestingly, in 1 patient (#03) clinical benefit (SD in 2 years) and continuously high numbers of IFNγ producing IDO specific T (Elispot) cells were demonstrated. In addition, in the same patient IDO specific T cells were demonstrated by tetramer staining and these T cells exerted specific cytotoxicity towards IDO expressing tumour cells. Overall, although IDO-specific T-cell responses were detectable, the vaccine did in general not seem to induce strong responses (magnitude). Although we only measured specific responses in PBMCs and not at the site of the tumour, this suggests that IDO-vaccination might be more effective using other adjuvants that facilitate more powerful immune responses. Hence, the 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. Recently, it was recognized that persisting antigen delivery at vaccine sites using Montanide induced dysfunctional T cells (32) forming the basis for development of newer rapidly degradable adjuvants (33). Other aspects 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.
The boosting of IDO-specific immunity could have both direct and in-direct effects. Firstly, such T cells may directly kill IDO+ cancer cells. In addition, they may function by eliminating suppressive immune and stromal cells. Hence, we performed phenotypic characterization of PBMCs to quantitate different immune cells. Noteworthy, we observed a significant reduction of the Treg population after the 6th vaccine in all treated patients. Albeit that the observed decrease in Tregs was seen in both SD+PR and PD patients our results show that important pharmacodynamic changes in immune cells during vaccine treatment can occur. The decrease in Tregs is in accordance with our previous observation that the “supportive” effect of IDO-specific T cells in vitro is, in part, mediated through a decrease in the frequency of Tregs (7). The IDO pathway is linked to Treg biology, since IDO expressing dendritic cells induce the differentiation of naïve CD4+ cells towards a FoxP3+ phenotype (34,35). The observed trend of an increase in CD8+ T cells and TEM in patients with clinical benefit might also indicate reduced suppression.

The role of natural killer (NK) cells in regard of an anti-neoplastic effect in human cancer is controversial. NK cells may contribute to anti-tumour activity because they recognize tumor cells independent of MHC Ag expression (36). Recently, it has been suggested that IDO as part of an immune-evasion strategy does induce down-regulation of cell surface NK receptor expression (37). Interestingly, we found a trend towards an increase in the NK cell population in all treated (both SD+PR and PD) patients hence no clear correlation between clinical benefit and NK cell activity was demonstrated.

Many cancer patients have an elevated Kyn/Trp ratio compared to HD but it is still unclear whether elevation in Kyn/Trp ratio is indicative for disease progression. Moreover Kyn production might reflect IDO induction in different peripheral cells hence blurring the picture. Since the IDO gene is
sensitive for IFN\(\gamma\) response elevated \(\text{Kyn/Trp}\) ratio may also reflect unspecific inflammation (38). The presence of an "inflamed" microenvironment might be used to consider patients as candidates for immunotherapy likewise the expression of PD-L1, IDO or presence of Tregs might necessitate agents targeting negative regulation (39). We found 8/11 patients demonstrating stable or lower level of IDO activity after the 6\(^{th}\) vaccine. The SD+PR patients had a trend toward a higher rate of IDO specific T cells and higher Kyn/Trp ratio prior to treatment. Taken together, this point towards a higher pre-vaccination IDO activity in the SD+PR patients compared to the PD patients. Interestingly, in two patients with long-lasting clinical response stable Kyn/Trp ratio was maintained at long term pointing towards a possible role as marker of response.

Overall, regarding the induced Treg decrease, the possibly NK cell increase, the demonstration of IDO expression in tumour biopsies and the Kyn/Trp ratio measurements no clear correlation to clinical activity could be demonstrated. However, important immune changes during vaccine treatment have been demonstrated implying an immune modulating capacity of the IDO vaccine and potential fundamental immune parameters to monitor in future IDO based clinical trials.

The role of IDO as a novel mechanism whereby tumours evade immune mediated rejection and the original idea of targeting negative regulation by IDO inhibition were authored more than ten years ago (40). Results from the first clinical trials of IDO inhibitors such as 1-methyl-D-tryptophan (1-MDT) and INCB024360 in advanced cancer patient settings are still awaited (10,11). 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 cytotoxic T cells naturally differs in several ways. One important difference is the
potential risk of inductions of toxicities by systemic treatment with IDO inhibitors versus the
localized reactions induced by the IDO vaccine treatment. 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
suppressiv 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.

In conclusion we have shown that IDO inhibition mediated by a vaccine strategy is safe and might
lead to objective tumour response as well as sustained disease stabilization in NSCLC patients. To
further test our findings of an IDO based therapeutic anti-cancer vaccine in a larger patient
population we recently set up a phase II trial combining IDO vaccine and Temozolomide (41) for
metastatic melanoma patients (NCT01543464) at our institution.

Acknowledgement

We would like to thank Jane Andersen, Merete Jonassen and Kirsten Nikolajsen for excellent
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University Hospital, in particular the project nurses Birgitte Christiansen and Susanne Wehmeyer.
Hubert Kalbacher from the Interfaculty Institute of Biochemistry in Tübingen is thanked for providing access to the HPLC system and advices for establishing Kyn/Trp detection experiments.

Reference List


Figure legends

**Figure 1.** Patient characteristics, toxicity and duration of the vaccine treatment. (A) Baseline patient characteristics for both HLA-A2 positive (N=15) and HLA-A2 negative patients (N=10). According to protocol requirements CT scans for HLA-A2 positive patients was described using RECIST 1.1 whereas for HLA-A2 negative patients RECIST 1.0 according to local standard was used. (B) Localization of metastatic sites. LN; lymph node. (C) Type and frequency of CTCAE grade 1/2 toxicities. (D) Duration of vaccine treatment (days) in the individual patients.

**Figure 2.** Tumour responses to vaccine treatment and estimates of survival in NSCLC patients. (A) Overall tumour responses presented as % changes in target lesions. (B) Patient #18 with confirmed partial tumour regression (-31% = PR) in target lesions in the liver. (C) (Top panel) Kaplan Meier estimate of progression free survival (PFS), defined as date of first vaccine treatment to date of PD. (Bottom panel) Overall survival (OS) curves for SD+PR patients and PD patients. (D) Kaplan Meier estimate of OS, defined as date of first vaccine to date of death for HLA-A2 vaccinated patients (dotted line) and for HLA-A2 negative standard of care treated patients (solid line).

**Figure 3.** Estimates of survival and immunohistochemistry staining of IDO expression in NSCLC patients. (A) Grading of IDO expression in tumour biopsies by IHC method. (B) Examples of IHC imaging in NSCLC patients (Pt. #04 and #06, respectively). The blue colour is representing Hematoxylin and Eosin (HE) staining of tumour cell nuclei, and the brown colour is representing IDO expression in the cytoplasm of tumour cells.
Figure 4. Detection of IDO specific CD8+ T cells. (A) Example of IFNy Elispot response +/- IDO peptide from Pt. #02 (B) (left panel) IFNy Elispot responses in the individual patients (SD+PR and PD patients marked at the graph, respectively) at different time points (mean of duplets, 6.0*10e5 PBMC/well, background spot production is subtracted). (Right panel) mean IFNy Elispot response for different time points i.e. baseline (N=15), at 4th (N=15), at 6th (N=15), at 9th (N=8, mainly SD patients) and at 12th (N=6, only SD patients) (background spot production is subtracted) vaccine. (C) Frequency of IDO specific CD8+ T cells at baseline in SD versus PD patients (background spot production is subtracted). (D) (left panel) flow cytometry dot-plots of tetramer positive IDO specific T cells from Pt #03 compared to a control with an irrelevant HIV peptide. (Right panel) after T cell culturing, cell sorting and rapid expansion effective lysis (~20%) of IDO peptide loaded T2 cells was detected as well as effective killing (~30%) of an IDO positive HLA-A2 cancer cell line (SW480) by IDO specific CD8+ T cells.

Figure 5. Flow cytometry analyses performed at pre-treatment and after the 4th and the 6th vaccine in all patients (N=15) white bars SD+PR (N=7), black bars PD (N=8) grey bars. Gating strategy was based on lymphocytes (single cells, living, CD45+) as the reference population for subtypes of immune cells except for the Mo-MDSC in which monocytic cells were included in the gating. (A) (left panel) changes in percentage of CD8+ T cells. (Right panel) changes in percentage of CD8+ TEM cells (%CD3+CD8+CD45RA-CCR7-). (B) (left panel) changes in percentage of CD4+ T cells. (Right panel) changes in percentage of Treg cells (%CD4+CD25+CD127-FoxP3+) (C) Changes in percentage of natural killer (NK) cells (%CD3-CD19-CD56+CD16+). (D) Changes in percentage of monocytic myeloid derived suppressor (Mo-MDSC) cells (%HLA-DRlowLIN-CD33+CD11b+CD14+).
Figure 6. Ratios of kynurenine and tryptophan levels from patient sera as determined by HPLC. (A) Ratio of Kyn/Trp (μM/mM) from sera at baseline, at 4th and at 6th vaccine, SD+PR (N=7) white bars, PD (N=8) black bars. From Pt. #02 only baseline level was obtained; from Pt. #4, #5 and #11 only sera at pre-treatment and at the 4th vaccine were available, and from Pt. #03 only sera at pre-treatment and after the 6th vaccine were available. (B) Baseline levels of Kyn/Trp ratio in SD versus PD patients. (C) Ratio of Kyn/Trp at baseline compared to at 6th vaccine (N=11). (D) Long term analyses of Kyn/Trp levels in 3 patients with SD and 1 patient with PR. This graph combines experiments performed at 2 different time points.

Supplementary figures:

FACS plots
Fig. 1

A

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>HLA-A2 pos. (N=15)</th>
<th>HLA-A2 neg. (N=10)</th>
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<td>PS 0=9 (60%)</td>
<td>PS 0=6 (60%)</td>
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<td></td>
<td>PS 1=6 (40%)</td>
<td>PS 1=4 (40%)</td>
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<tr>
<td>Histology</td>
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<td>Adenocarcinoma = 70%</td>
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<tr>
<td>Anti-neoplastic treatment (chemo-duplet)</td>
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<td>1st line = 100% 2nd line = 40% 3rd line = 20%</td>
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<tr>
<td>Target lesions (Baseline – total diameter)</td>
<td>6.8 cm (1.3-12.6)</td>
<td>8.6 cm (2.9-21.1)</td>
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</table>

B

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<td>lung, LN</td>
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<td>#19</td>
<td>lung, LN</td>
</tr>
<tr>
<td>#20</td>
<td>lung, liver, adrenal gland</td>
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C

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<tr>
<th>Toxicity, CTCAE grade 1/2</th>
<th>Percentage (%)</th>
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<tbody>
<tr>
<td>Shortness of breath</td>
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<td>Coughing</td>
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<td>Haemoptysis</td>
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<td>Abdominal pain</td>
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<td>13</td>
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<td>Obstipation</td>
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</table>

D

IDO vaccine duration
Mean 7.9 months (95% 4.6 -11.3CI)

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<tr>
<th>Patient number</th>
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<tr>
<td>19</td>
<td></td>
</tr>
<tr>
<td>20</td>
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On study

Days
Fig. 2

A. Response to IDO vaccine treatment (N=15)

- Blue: New lesion
- Red: >20% PD
- Orange: On study

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<tr>
<th>Months</th>
<th>0</th>
<th>2.5</th>
<th>5.5</th>
<th>8.5</th>
<th>12</th>
<th>18</th>
<th>21</th>
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<tr>
<td>% Changes in target lesions</td>
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<td>-30</td>
<td>-20</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
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- A (liver) 2.9 2.8 2.5 2.6 2.2 2.2
- B (liver) 2.7 2.5 2.1 1.6 1.8 1.8
- C (liver) 1.8 1.8 1.8 1.8 1.1 1.1
- Sum (cm) 7.4 7.3 6.4 6.0 5.1 5.1

C. Progression Free Survival (N=15)

- Median PFS = 5.2 months

D. Overall survival (N=25)

- LogRank Test P=0.05
- SD+PR (N=7)
- PD (N=8)

E. Overall survival (N=25)

- N=15 HLA-A2 pos. (+vacc.)
  - Median OS=25.9 months
- N=10 HLA-A2 neg. (-vacc.)
  - Median OS=7.7 months

LogRank Test P=0.03
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<td>X</td>
<td></td>
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<td>X</td>
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</tr>
<tr>
<td># 06 (PD)</td>
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<td># 11 (PD)</td>
<td>X</td>
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<td># 14 (PD)</td>
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</tr>
<tr>
<td># 15 (SD)</td>
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<td></td>
</tr>
<tr>
<td># 17 (SD)</td>
<td>X</td>
<td></td>
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</tr>
<tr>
<td># 18 (PR)</td>
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<tr>
<td># 20 (PD)</td>
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Fig. 4

A

IFN$\gamma$ Elispot Pt #02

-IDO peptide +IDO peptide

13th + IDO
13th - IDO
9th + IDO
9th - IDO
6th + IDO
6th - IDO
4th + IDO
4th - IDO
Pre + IDO
Pre - IDO

IDO specific Tcells / 6*10^5 PBMC

B

IFN$\gamma$ Elispot N=15

IDO specific Tcells / 6*10^5 PBMC

Patient number

#1 #2 #3 #4 #5 #6 #11 #13 #14 #15 #16 #17 #18 #19 #20

Pre 4th 6th 9th 12th

C

IFN$\gamma$ Elispot

Pre treatment

P=0.05

IDO specific Tcells / 6*10^5 PBMC

SD PD

D

IDO$\gamma$ specific CD8+T-cells

HIV

T-cell response 0.01%

Tetramer-APC

Effector cells : Target cells

Specific killing of cancer cells

- SW480
- T2+IDO5
- T2+HIV

% Lysis

40:1 13:1 4.4:1 1.5:1 0.5:1 0.16:1
Fig. 6

A

Kyn/Trp ratio
Pre, 4th and 6th vaccine

B

Kyn/Trp Ratio
Pre treatment level

C

Kyn/Trp ratio
SD+PR (N=5) PD (N=6)

D

Kyn/Trp ratio (N=4)
Long term analyses

Patient numbers

MicroM/MilliM

Pt. #06

Pt. #03
Pt. #15
Pt. #18
Pt. #19

Pre 4thV. 5thV/ 6thV 9thV. 12thV. 15thV. 18thV. 26thV.
Long-lasting disease stabilization in the absence of toxicity in metastatic lung cancer patients vaccinated with an epitope derived from indoleamine 2,3 dioxygenase


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