

# Dendritic cell based antitumor vaccination: impact of functional indoleamine 2,3-dioxygenase expression

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## Abstract

**Background** Recent reports have demonstrated that the enzyme indoleamine 2,3-dioxygenase (IDO) is upregulated in human dendritic cells (DCs) upon in vitro maturation. IDO is supposed to convey immunosuppressive effects by degrading the essential amino acid tryptophan, thereby downregulating T-cell functions. Hence, we evaluated IDO expression in DC preparations used for therapeutic DC vaccination and its in vivo effects.

**Patients, methods and results** IDO expression was detected by real-time-PCR in a series of human clinical grade DCs ( $n = 28$ ) prior to vaccination of advanced melanoma patients ( $n = 11$ ). These analyses revealed an intra- and interpersonal variation in IDO mRNA levels. IDO was strongly upregulated in human DCs on RNA and on protein level upon in vitro maturation by

Interleukin-1 $\beta$  (IL-1 $\beta$ ), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin-6 (IL-6) and Prostaglandin E2 (PGE2) over a time course of 24 h. The enzymatic activity of induced IDO was demonstrated by measuring tryptophan degradation. Moreover, in biopsies obtained 24 h after application of the DC vaccine a prominent infiltrate of IDO-positive cells was observed by immunohistochemistry. The inflammatory infiltrate of these sites stained positive for the transcription factor Forkhead box P3 (FoxP3), suggesting an IDO-mediated induction of regulatory T-cells. All analysed melanoma patients ( $n = 11$ ) receiving DC based immunotherapy exhibited rapid disease progression with a short overall survival due to advanced tumour stage.

**Conclusion** The presented observations suggest a potential clinical relevance of IDO expression in DC-based therapeutic vaccines via the attraction or induction of FoxP3+ T-cells.

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**Keywords** Indoleamine 2,3-dioxygenase (IDO) ·  
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## Abbreviations

APC	Antigen presenting cell
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
FoxP3	Forkhead box P3
GM-CSF	Granulocyte monocyte colony stimulating factor
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
PBMCs	Peripheral blood mononuclear cells
PGE2	Prostaglandin E2
Treg	Regulatory T-cell
TGF- $\beta$	Transforming growth factor- $\beta$

TNF- $\alpha$  Tumour necrosis factor- $\alpha$   
 1MT 1-Methyl-tryptophan

## Introduction

Immunological strategies to fight cancer have demonstrated less clinical efficacy than anticipated. Several factors contribute to this status quo, including immune escape mechanisms of tumours or the limited immunogenicity of the antigen delivery systems [1]. One potential key mechanism, leading to immunological tolerance, is the recently recognised immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) [2]. IDO is a heme-containing enzyme which catalyses the initial, rate limiting-step in the degradation of the essential amino acid tryptophan into distinct kynurenine metabolites [3]. Depletion of tryptophan renders T-cells more susceptible to apoptosis [4]. Moreover, various tryptophan downstream metabolites, e.g. kynurenine and quinolinate, themselves are directly toxic for T-cells [5]. IDO-expressing, tolerizing antigen presenting cells (APCs) are furthermore supposed to induce regulatory T-cells (Tregs) [6]. Clinically relevant immunoregulatory functions of IDO include the protection from intracellular pathogens [7], maintenance of maternal tolerance towards the foetus during pregnancy [8], suppression of T-cell responses to MHC-mismatched allografts [9], protection from autoimmune diseases [10] and—most important—tumour resistance to cytotoxic CD8+ T-lymphocytes [11, 12]. In this respect, IDO is expressed by two complementary constituents both in the tumour microenvironment as well as in the regional draining lymph nodes: the malignant cells themselves and subsets of APCs [13]. Thereby, both

local and systemic tolerance to neoplastic cells may be generated and maintained.

IDO-expression in APCs and its complex modulation by various cytokines and direct cellular interactions may become an issue for dendritic cell (DC)-based vaccine therapies. Indeed, previous reports have demonstrated that functional IDO is induced upon in vitro generation of mature DCs [14, 15]. Thus, we analysed IDO expression, its activity as well as its in vivo relevance for melanoma patients receiving DC-based vaccinations [16, 17].

## Patients, materials and methods

### Patient characteristics

Eleven patients suffering from metastasised melanoma, which were treated by DC-based vaccinations, were included into this advanced immunomonitoring. Informed consent was obtained from each patient prior to study inclusion and any subsequent measures. All procedures were approved by the local ethics committee. The study details have been described before [17]; patients received autologous peptide-pulsed monocyte-derived DCs administered subcutaneously every 2 weeks for the first five vaccinations, followed by vaccinations in 4-weekly intervals [16]. Detailed patient characteristics are summarised in Table 1.

### Dendritic cells

Peptide-pulsed dendritic cells were generated from leukapheresis samples as previously described [17, 18].

Briefly, peripheral blood mononuclear cells (PBMCs) were plated in 85 mm culture dishes (Falcon,

**Table 1** Patient characteristics of 11 melanoma patient treated with peptide-loaded dendritic cells

P	Sex	Age	AJCC	Primary tumour	Metastatic sites	Objective response	TTP (months)
1	f	40	M1c	Uvea melanoma	Liver	PD	3
2	m	52	M1c	NM, CLIV, 2.5 mm	Lung, kidney, soft tissue	PD	1
3	m	51	M0	NM, CL III, 0.42 mm			
4	m	71	M1c	SSM, CL IV, 1.3 mm	Liver, lung, LN	PD	2
5	m	38	M1c	SSM, CL III, 0.9 mm	LN, kidney	PD	3
6	m	69	M1c	NM, CL IV, 6.0 mm	LN, liver	PD	3
7	m	72	M1c	NM, CLIV, 3.2 mm	Liver, LN, kidney	PD	2
8	m	72	M1c	Soft tissue melanoma	Soft tissue, skin	PD	1
9	m	40	M1a	SSM, CL III, 0.6 mm	LN, soft tissue	PD	4
10	m	52	M1c	Uvea melanoma	Liver, kidney, muscle, soft tissue	PD	3
11	w	36	M1c	SSM, CL III, 0.6 mm	Liver, spleen, LN, lung	PD	Immediately

Patient 3 was treated with DC vaccine in an adjuvant setting exhibiting no evidence of metastatic disease

Clark level *CL*, nodular melanoma *NM*, progressive disease *PD*, superficial spreading melanoma *SSM*, time to progression *TTP*

Becton Dickinson, Hershey, USA) at a density of  $5 \times 10^7$  cells per dish in 10 ml of complete culture medium [RPMI-1640 (Cambrex, Vervier, Belgium) with 10% FCS] and incubated at 37°C and 5% CO<sub>2</sub> for 1 h. After a microscopic control of adherence, the non-adherent fraction was removed and 10 ml of fresh, prewarmed medium was added (day 0). All adherent fractions were cultured until day 1, then culture medium was taken off carefully so that loosely adherent cells were not removed, and fresh culture medium containing granulocyte monocyte colony stimulating factor (GM-CSF) (800 U/ml final concentration) and Interleukin-4 (IL-4) (1,000 U/ml final concentration) was added. Cytokines were added again on day 5 in 5 ml fresh medium (containing 800 U GM-CSF and 1,000 U IL-4) per dish. On day 6, different stimuli to induce maturation of DCs were added or not [Interleukin-1 $\beta$  (IL-1 $\beta$ ) 2 ng/ml, Interleukin-6 (IL-6) 1,000 U/ml, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) 10 ng/ml (all of R&D Systems, Minneapolis, USA), Prostaglandin E2 (PGE2) 1  $\mu$ g/ml (Sigma)]. Cells or supernatant were harvested at different time points for further analysis. On day 7, DCs were loaded with different peptides and subsequently injected subcutaneously into the extremities close to the inguinal lymph nodes [16, 17].

#### Real-time-PCR of IDO

DNase-treated total RNA was isolated from DCs ( $1 \times 10^5$  cells) by means of RNeasy Micro Kit (Stratagene, La Jolla, CA, USA). The cDNA was synthesised from mRNA with poly(dT) primers, dNTPs, random primer and Superscript II reverse transcriptase (Invitrogen, Rockville, MD, USA). Quantification of IDO-expression in dendritic cells was performed by real-time PCR with Taqman technology (Applied Biosystems, Weiterstadt, Germany). Primers and probe for IDO were designed with Primer Express software (Applied Biosystems) and are 5'-TTG-GAG-AAA-GCC-CTT-CAA-GTG-T-3' (forward primer), 5'-TGC-CTT-TCC-AGC-CAG-ACA-A-3' (reverse primer) and 5'-CAC-CAA-ATC-CAC-GAT-CAT-GTG-AAC-CC A-3' (probe) (Eurogentec, Seraing, Belgium). The relative expression of IDO was calculated in relation to the expression of mRNA coding for the housekeeping gene GAPDH (Applied Biosystems). Measurements were performed in triplicates.

#### Western Blot analysis

For Western blot analysis cell lysates ( $1 \times 10^5$  cells) were prepared by suspending cultured dendritic cells at indicated time points in Laemmli lysis buffer. Lysates

were incubated for 5 min at 96°C and were then loaded on a 10% polyacrylamid gel (Cambrex). Gels were run under reducing conditions. Following electrophoresis, samples were electroblotted on nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Blots were blocked for 30 min in blocking solution (3% milk powder in phosphate-buffered saline (PBS)/0.05% Tween) at room temperature and then incubated overnight with primary monoclonal IDO antibody (1:1,000, 636931A, Chemicon, CA, USA) in blocking solution at 4°C. After washing with PBS/0.05% Tween, membranes were incubated for 2 h at room temperature with secondary antibody rabbit anti mouse peroxidase-labeled immunoglobulin G (1:1,000 dilution) (Dako, Hamburg, Germany) in blocking solution. After additional washings, binding of the peroxidase-labeled antibody was visualised by chemiluminescence (Boehringer Mannheim, Germany). Analysis was performed at least twice for each sample. As loading control  $\beta$ -actin was detected using a monoclonal antibody (Dako).

#### Chromatography of supernatants for tryptophan concentration

To analyse tryptophan catabolism at day 6 of in vitro maturation  $1 \times 10^5$  cells/well were plated in 96-well plates (Falcon, Becton Dickinson, Hershey, USA) in Hanks buffered salt solution (HBSS) (Gibco BRL, Gaithersburg, MD, USA) containing 100  $\mu$ M L-tryptophan (Sigma, Deisenhofen, Germany). Supernatants of matured or immature dendritic cells were harvested at different time points and were analysed for tryptophan concentration by thin layer chromatography (TLC). Chromatography was performed on 20  $\times$  20 cm silica gel 60 pre-coated plates (Merck, Darmstadt, Germany) with mobile phase containing 20% methanol and 10% Tris-glycine for 12 h. After developing and drying TLC layers, spots on chromatograms were detected by reflectance densitometry at wavelength of 280 nm using a CAMAG TLC Scanner 3 (CAMAG, Muttenz, Switzerland). Data collection and integration were accomplished using CATS Software Version 4.06.

#### Immunohistochemical analysis of IDO, CD83 and FoxP3

Briefly, cryopreserved specimen of vaccination sites and normal skin were fixed in 4% formalin for 10 min at 4°C and washed in PBS afterwards. Endogenous peroxidase was blocked with peroxidase blocking solution (S2023, Dako) and slides were washed twice with

buffer. The tissue was then incubated for 40 min at room temperature with anti-IDO antibody (AB 5969 Rabbit polyclonal, Chemicon), with anti-CD83 antibody (74841E, BD Biosciences, Heidelberg, Germany) or anti-FoxP3 antibody (236A/E7, Chemicon). After washing with PBS, sections were subjected to secondary antibody anti-rabbit HRP (Envision K4003, Dako) for 30 min. After washing in PBS, the sections were placed in AEC-substrate (Multi link Biotin Kit, K5003, Dako). Sections were washed in Aqua bidestilata, mounted and cover-slipped. Evaluation was performed by two independent histologists.

## Results

### IDO mRNA expression in mature human DCs

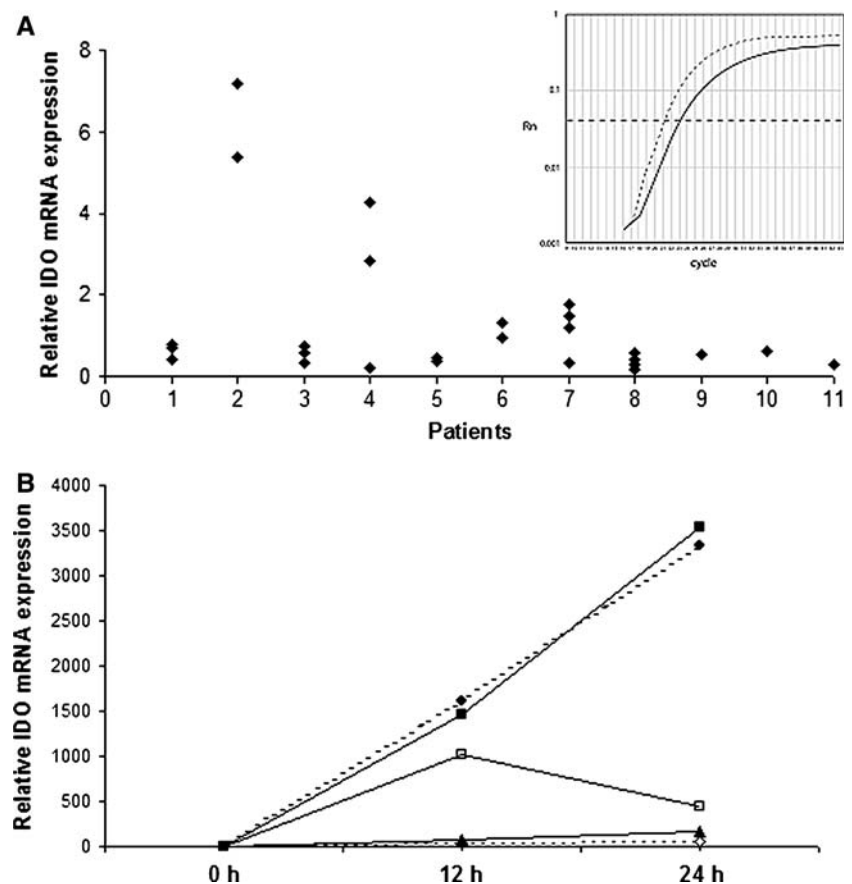
DCs are known to possess both immunostimulatory as well as immunomodulatory properties. Indeed, upregulation of IDO in human DCs under certain culture conditions has already been demonstrated [14, 15]. Consequently, we scrutinised IDO expression in clinical grade DCs generated for therapeutic vaccination of melanoma patients. Leukaphereses were repeatedly

performed for individual patients at different time points during immunotherapy for therapeutic and diagnostic purposes; thus a total of 28 DC preparations of 11 patients were analysed. IDO mRNA expression was analysed by real-time-PCR at day 7 of *in vitro* maturation, i.e. directly prior to vaccine application. A generally strong IDO mRNA expression as compared to the housekeeping gene GAPDH was observed in the majority of the patients (Fig. 1a) with inter- and intrapersonal variation.

Expression of IDO mRNA and protein is upregulated in human DCs during *in vitro* maturation

In order to study if the observed IDO mRNA expression in mature DCs at day 7 is a result of the maturation process or a sign for an incomplete maturation, a kinetic expression profile of IDO mRNA was performed in five of the patients. The time course covered 24 h ( $n = 5$ ) and 48 h ( $n = 2$ ) after induction of maturation at day 6. This kinetic study revealed, that in all specimen IDO mRNA expression was increased within 12–24 h by stimulation with IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and PGE-2 (Fig. 1b). This elevation in IDO mRNA expression was absent in DCs generated from the same

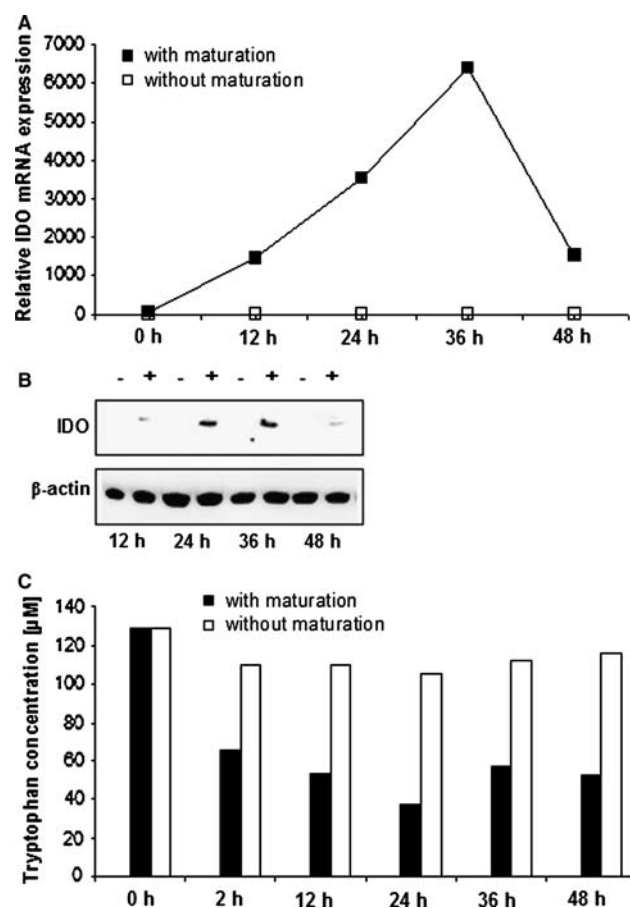
**Fig. 1** **a** Relative IDO mRNA expression profile in multiple DC preparations of 11 melanoma patients at day 7 of *in vitro* generation, prior to DC vaccine application. An inter- and intrapersonal variation of IDO expression relative to GAPDH is demonstrated. All data are normalized to IDO mRNA expression level of patient 1. *Insert* Representative example of mRNA levels of IDO (solid line) and GAPDH (interrupted line) of one patient. **b** IDO mRNA expression in DCs obtained from 5 melanoma patients (filled square, filled circle, thick line, diamond) after *in vitro* maturation over a time course of 24 h



leukapheresis sample if no maturation cocktail was added (Fig. 2a). However, maximal expression levels varied substantially between the different DC preparations, and the time course of maximal IDO expression was heterogeneous. Notably, induced IDO gene expression correlated with an enhanced protein expression, as analysed by Western blot using  $\beta$ -actin for normalization of protein loading (Fig. 2b).

Overexpression of IDO in mature human DCs is associated with tryptophan catabolism in vitro

IDO exerts its immunoregulatory functions via degradation of the essential amino acid tryptophan together with the concomitant production of directly toxic metabolites. To test the notion that upregulation of



**Fig. 2** **a** IDO expression in correlation to the maturing process with IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and PGE-2 of human DCs (representative example). Expression of IDO mRNA relative to the house-keeping-gene GAPDH is depicted. *Filled square* Mature DCs, *open square* Immature DCs. **b** Western blot analysis of protein lysates of immature (-) and mature (+) human dendritic cells. **c** Parallel measurement of tryptophan concentration in supernatant, obtained at the given time points after the induction of maturation (*filled bars*) or not (*open bars*). Similar results were obtained on repetitive experiments

IDO correlates with enzymatic activity, we measured tryptophan catabolism in supernatant of immature and matured DCs at different time points during the maturation process. Indeed, this analysis revealed that in parallel with the observed increase in IDO mRNA and protein levels, tryptophan concentration decreased. In contrast, in supernatants of immature DCs, which did not upregulate IDO, no such decline in the tryptophan concentration was observed (Fig. 2c).

DCs maintain IDO expression at vaccination sites in situ

A crucial question is, if the observed IDO expression in human DCs is merely an in vitro culture phenomenon or may have indeed in vivo implications. Therefore, we obtained biopsies from vaccination sites of melanoma patients 24 h after DCs had been injected subcutaneously. These vaccination sites contained mature DCs as visualised by CD83 reactivity of the inflammatory infiltrate (Fig. 3a). Immunohistochemical analysis for IDO expression revealed a diffuse infiltrate of IDO-positive cells present in the subcutaneous compartment at the vaccination site (Fig. 3b). These IDO-expressing cells were detected with different frequencies among individual patients. In normal skin, however, no CD83 positive cells were detected (Fig. 3c) and only rare, single IDO-positive dendritic cells were scattered in the dermal compartment (Fig. 3d).

FoxP3+ cells are present at the vaccination sites in situ

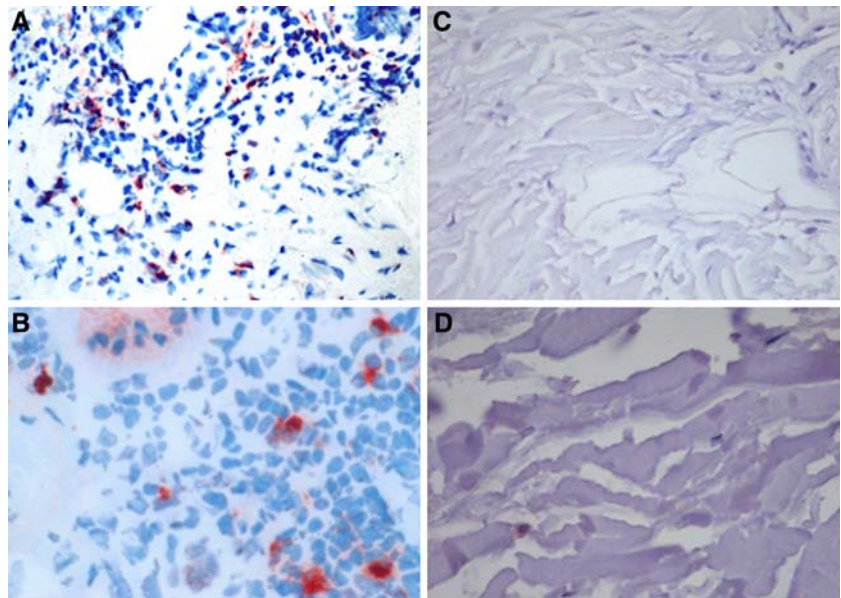
As there is strong evidence that IDO-competent APCs are able to induce regulatory T-cells in a self-reinforcing network, we additionally scrutinised vaccination sites for Forkhead box P3 (FoxP3) expressing cells (Fig. 4). To this end, FoxP3+ cells were observed at higher frequencies at DC vaccination sites (Fig. 4a, b) as compared to normal skin (Fig. 4c). The amount of FoxP3 positive cells varied between the different patients (Fig. 4a, b, d).

Clinical course of melanoma patients receiving DC based cancer vaccine

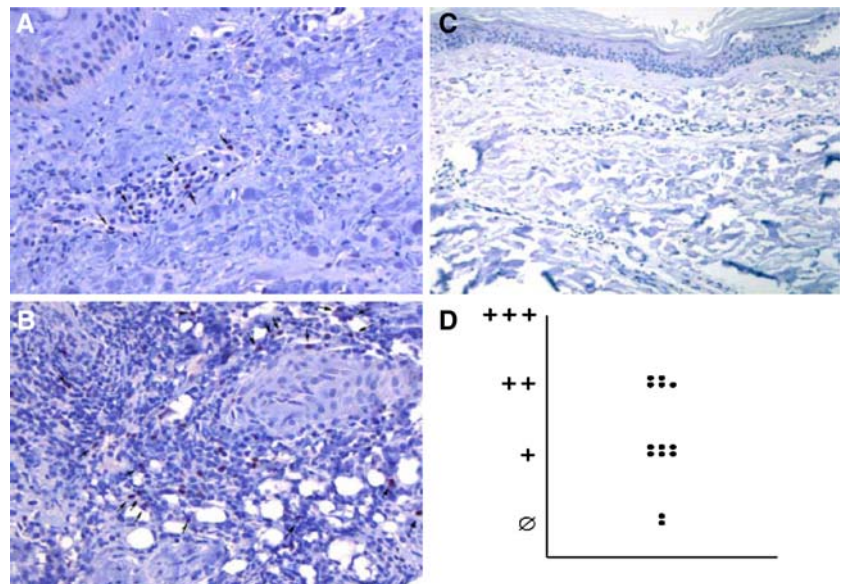
All patients ( $n = 11$ ) included in this analysis demonstrated rapid disease progression with a median time to progression of only 3 months (Table 1). Due to the collective lack of therapeutic activity—with respect to objective responses or even disease stabilization—no correlation between IDO expression or FoxP3 induction with the clinical course was drawn.



**Fig. 3** Immunohistochemical detection of CD83 ( $\times 20$ ) (a) and IDO ( $\times 40$ ) (b) in representative biopsies of DC vaccination sites. CD83 ( $\times 20$ ) (c) and IDO ( $\times 40$ ) (d) expression in normal skin are shown as negative control



**Fig. 4** a, b Immunohistochemical analysis of FoxP3 expression on vaccination site infiltrating cells (indicated by arrows) ( $\times 20$ ). Representative examples of specimen with different frequencies of FoxP3-positive cells are shown. c Normal skin as negative control ( $\times 20$ ). d Graph demonstrating a low (+), medium (++) and high (+++) frequency of FoxP3-expressing cells at 13 analysed vaccination sites of 8 different patients



## Discussion

The clinical efficacy of DC based vaccines for cancer immunotherapy is influenced by various interplaying, immunomodulating factors. Interleukin-10 (IL-10) and transforming growth factor- $\beta$  (TGF- $\beta$ ) are well known to counteract the inherent, immunostimulatory capacity of APCs allowing the generation of immunosuppressive regulatory T-cells. A recently recognised additional immunoregulatory determinant is indoleamine 2,3-dioxygenase, which attenuates cytotoxic T-lymphocyte responses inter alia by promoting tryptophan degradation.

Here, we demonstrate that functionally active IDO is expressed in DC preparations used for therapeutic vac-

cination. IDO mRNA and functional protein was induced upon in vitro maturation of human DCs with standard cytokine cocktail containing IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and PGE-2, but was absent in immature DCs. Previous reports have suggested that immature and especially fully mature antigen presenting cells gain the capacity to express IDO [14, 15]. Indeed, a subtype of IDO-positive dendritic cells, i.e. CD123+ CCR6+ cells, was defined [19]. Prostaglandins, together with additional signalling through TNF receptor or toll like receptors, have been identified as critical determinants for IDO induction and its functional activation [14, 15]. To scrutinise the in vivo relevance of IDO for DC vaccinations, we extended our in vitro observations to in situ immunohistochemical studies of DC injection sites.

Biopsies of subcutaneous vaccination sites, i.e. after injection of antigen-pulsed, in vitro matured DCs, revealed a diffuse, dermal infiltrate of CD83+ and IDO+ cells. Notably, a pronounced infiltrate of FoxP3+ cells were present at the vaccination sites. FoxP3 is a lineage specific transcription factor, bearing crucial roles for Treg development and function [20]. It is possible, that FoxP3+ Tregs traffick from the thymus, bone marrow, lymph nodes and peripheral blood under the influence of cytokines to DC vaccination sites [21, 22]. Alternatively, IDO+ DCs may promote Treg-development conveying both local and systemic immunosuppressive properties beyond tryptophan catabolism. The latter scenario has been suggested by several studies [6, 23, 24]. In this context, it has been very recently demonstrated that human DCs, which have undergone maturation induced by inflammatory stimuli, are able to efficiently expand Tregs both in vitro and in vivo in cancer patients [25]. All patients in our cohort, receiving DC vaccination therapy for advanced melanoma, were characterised by a rapid tumour progression. The detailed immune monitoring of these patients is currently performed within the clinical trial and will provide additional information in this respect. With regard to clinical relevance, several lines of evidence suggest an impact of IDO-expression on the clinical course and a possible role for modulating IDO-activity in cancer therapy: (I) Inhibition of IDO activity by competitive antagonists, e.g. 1-methyl-tryptophan (1MT) [19] or antioxidants [26, 27], results in rejection of allogeneic foetus [8] and—most important—(II) a modified tumour growth in murine models [28]. In addition, constitutive IDO expression in mouse tumour draining lymph nodes by plasmacytoid dendritic cells has been suggested as one mechanism of inducing systemic immunological unresponsiveness towards the tumour; this unresponsiveness can be reverted by administration of 1MT [29]. Consequently, IDO-inhibitors are considered as novel complements in DC maturation cocktails or adjuvants for therapeutic vaccinations [14]. It should be noted, however, that immunostimulatory cytokines induced in DCs upon maturation may abrogate tolerogenic effects of IDO upregulation [30]. We observed a decline in IDO expression at 24 h after induction of maturation, implying that IDO expression is a complex, regulated process. Interestingly, IDO enzyme activity and tryptophan metabolism may be also essential for DC maturation, activation [31] and chemotactic responsiveness [32].

In summary, our present data corroborate with former studies, which have raised concern about IDO upregulation in mature human DCs by confirming the in situ relevance of IDO induction in DCs applied for

cancer immunotherapy. The presence of FoxP3 positive cells at the site of DC injection suggests possible immunoregulatory effects of IDO expressing DCs. This may contribute to systemic immunological unresponsiveness and thus to therapeutic failure of DC based cancer immune therapies. Future studies will have to evaluate if modulating IDO activity may help to improve the clinical outcome of therapeutic vaccinations.

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