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DISSERTATION

Titel der Dissertation

Hematopoietic stem cell-derived dendritic cells express inactive indoleamine 2,3-dioxygenase *in vitro*

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Table of contents

Figures

Abbreviations

1-MT	1-methyl-tryptophan
AA	amino acid
Ab	antibody
A.d.	aqua destillata
APC	allophycocyanin
APCs	antigen presenting cells
BSA	bovine serum albumin
CD40L	CD40 ligand
cDNA	complementary deoxyribonucleic acid
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA
CLIP	class II-associated Ii peptide
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cell
dsRNA	double stranded ribonucleic acid
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
hr	hour
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
HSC-derived DC	hematopoietic stem cell-derived dendritic cell
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase

int	intermediate
IRF1	interferon regulatory factor 1
kDa	kilodalton
Kyn	kynurenine
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC class I/II	major histocompatibility complex class I/II
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NCM	normal cell culture medium
NO	nitric oxide
OVA	ovalbumin
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
pDC	plasmacytoid DC
PE	phycoerythrin
PRRs	pattern-recognition receptors
RT	room temperature (22°C)
RT-PCR	reverse transcription-polymerase chain reaction
STAT1	signal transducer and activator of transcription 1
TCR	T cell receptor
TDO	tryptophan 2,3-dioxygenase
Th cell	T helper cell
TLR	toll like receptor
TNF	tumor-necrosis factor
TRP	tryptophan
UV	ultraviolet

Abstract

Expression of the tryptophan (TRP)-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) in various types of dendritic cells (DCs) has been associated with suppression of T cell mediated immune responses, including suppression of immunological cancer control. In this project, we studied whether the T cell stimulatory capacity of murine hematopoietic stem cell-derived DCs (HSC-derived DCs) is affected by IDO activity.

We show that activation of HSC-derived DCs from C57BL/6 mice with lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) up-regulated CD80, CD86, major histocompatibility complexes class II (MHC class II), and CD40 expression, as well as T helper cell 1 (Th1) response cytokine secretion, compatible with an activated phenotype. While showing IDO activity in human DCs, activation up-regulates IDO messenger ribonucleic acid (mRNA), but does not induce IDO enzymatic activity in murine DCs. To exclude insufficient gene transcription that would lead to attenuated IDO protein expression and hence to enzymatic activity below detection limit, HSC-derived DCs were transfected with an IDO-encoding vector by electroporation technique. Despite significant and stable over-expression of both, IDO mRNA and protein, IDO activity was still undetectable in these cells. To study the stimulatory capacity of these DCs co-cultures with syngeneic T cell receptor (TCR) transgenic ovalbumin (OVA) specific CD4 (OT-II) or CD8 (OT-I) T cells were performed. As expected, we found vigorous T cell responses that were proportional to the number of OVA-peptide pulsed activated DCs. LPS and IFN-γ stimulation of HSC-derived DCs also activated inducible nitric oxide synthase (iNOS) and hence the production of NO which has been reported to be a powerful inhibitor of IDO activity. We found levels of NO in our cultures comparable to those found in other studies that inhibited IDO activity. Interestingly, iNOS activity was undetectable in various murine (Raw264.7 and J558) and human (Phoenix and HeLa) cell lines that exhibited effective IDO activity after transfection with the equivalent IDO-encoding vector.

In summary, we suggest that murine HSC-derived DCs maintain IDO in an enzymatically inactive form. IDO expression in murine HSC-derived DCs, when not

displaying enzymatic activity, does obviously not interfere with their potent T cell stimulatory capacity.

Zusammenfassung

Die Expression des Tryptophan (TRP)-abbauenden Enzyms Indolamin 2,3-Dioxygenase (IDO) in bestimmten Typen von dendritischen Zellen (DCs) wurde mit Suppression T-Zell-vermittelter Immunantworten und damit auch mit der Suppression der immunologischen Kontrolle von Krebszellen in Verbindung gebracht. In dieser Studie haben wir untersucht, ob die T-Zell-stimulierende Kapazität von DCs, die aus murinen hämatopoietischen Stammzellen generiert wurden (HSC-derived DCs), durch die Aktivität von IDO beeinflusst ist.

Wir zeigen, dass HSC-derived DCs von C57BL/6 Mäusen nach Aktivierung mit Lipopolysacchariden (LPS) und Interferon-gamma (IFN-γ) sowohl die Expression von CD80, CD86, Haupthistokompatibilitätskomplex Klasse II (MHC class II) und CD40, als auch die Sekretion von T Helferzellen 1 (Th1) induzierenden Zytokinen hochregulierten und damit einen aktivierten Phänotyp aufwiesen. Während LPS und IFN-γ stimulierte humane DCs IDO Aktivität aufzeigten, zeigten murine HSC-derived DCs keinen Tryptophan-Abbau, obwohl die Boten-Ribonukleinsäure (mRNA) des IDO-Gens erhöht vorlag. Um auszuschließen, dass die vermehrte Gen Transkription immer noch in einer für die Detektion der enzymatische Aktivität zu geringen Protein-Expression resultierte, wurden HSC-derived DCs mit einem IDO-Expressionsvektor transfektiert. Trotz signifikanter und stabiler Überexpression von IDO mRNA und Protein zeigten diese Zellen keine IDO Aktivität. Um die immunstimulierende Kapazität dieser DCs zu untersuchen wurden Ko-Kulturen mit T Zellrezeptor-transgenen, Ovalbumin (OVA) spezifischen CD4 (OT-2) oder CD8 (OT-1) T Zellen durchgeführt. Erwartungsgemäß zeigten diese Experimente kräftige T-Zell-Antworten, die proportional mit der Anzahl der eingesetzten OVA-Peptid beladenen DCs zusammenhängten. LPS und IFN-γ Stimulierung von HSC-derived DCs aktivierte auch die induzierbare Stickstoffmonoxid –Synthase (iNOS) und damit die Produktion von NO, das als starker Inhibitor der IDO Aktivität beschrieben worden ist. NO Konzentrationen in unseren Kulturen waren mit jenen Werten vergleichbar, die die IDO Aktivität in anderen Studien inhibierten. Interessanterweise konnte keine iNOS Aktivität in verschiedenen murinen (Raw264.7 und J558) und humanen (Phoenix und HeLa) Zelllinien nachgewiesen werden, die nach Transfektion mit dem äquivalenten IDO-Gen effiziente IDO Aktivität aufwiesen.

Zusammenfassend deuten diese Daten darauf hin, dass murine HSC-derived DCs IDO in einer enzymatisch inaktiven Form halten. Die IDO Expression in seiner inaktiven Form scheint die potente T-Zell-stimulierende Kapazität von murinen HSC-derived DCs nicht zu beeinträchtigen.

Introduction

Induction of T cell tolerance and immune evasion are essential mechanisms that counteract effective immunity which would otherwise result in dangerous collateral damage to the host (Munn 2006). In recent years, increasing evidence has been accumulated that antigen presenting cells (APCs) such as DCs play a major role in induction, but also in inhibition of immune responses (Banchereau and Steinman 1998; Steinman 2003). Converting DCs from stimulatory to regulatory cells involves a variety of molecular mechanisms. The TRP catabolizing enzyme IDO has been proposed as a key molecule for immuno-suppressive DCs. Its potential responsibility for impaired T cell responses has positive and negative implications (Rutella, Danese et al. 2006). On the one hand IDO is essential for successful allogeneic pregnancy in mice (Munn, Zhou et al. 1998) and inhibition of proliferation of tryptophan sensitive pathogens (Yoshida, Urade et al. 1979; Rottenberg, Gigliotti Rothfuchs et al. 2000; Hayashi, Rao et al. 2001; Fujigaki, Saito et al. 2002), but on the other hand the enzyme is thought to impede immunological cancer control (Friberg, Jennings et al. 2002; Uyttenhove, Pilotte et al. 2003). Anti-tumor DC vaccines that prime anti-tumor specific T cells are intensively tested for their potential to supplement conventional chemotherapy. Early clinical trials in humans showed that DC vaccination appeared to be safe, but that only occasionally significant tumor regression was yielded (Banchereau and Palucka 2005; Zou 2005; Steinman and Banchereau 2007). Given its proposed role as a key molecule in immuno-suppression, recent reports focused on evaluation of IDO in these immunotherapeutic strategies (Wobser, Voigt et al. 2007; Ou, Cai et al. 2008).

Biology of IDO

IDO mechanism

TRP belongs to the least abundant of the essential amino acids (AAs) and as a consequence, TRP deficiency is easily attained. In the mammalian system several pathways exist that metabolize TRP (Figure 1). Most commonly known is the conversion of TRP into serotonin which involves 1% of the total body TRP content. More than 95% of TRP are converted through the KYN pathway (Takikawa 2005). In mammals, two known enzymes are involved in the catalysis of the first and rate limiting step in the Kynurenine (Kyn) pathway. The first, termed tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11), was identified in the liver in 1937 (Kotake and Masayama 1937). Decades later, the second TRP-degrading enzyme, IDO (EC 1.13.11.52), was identified in rabbit intestine (Yamamoto and Hayaishi 1967).

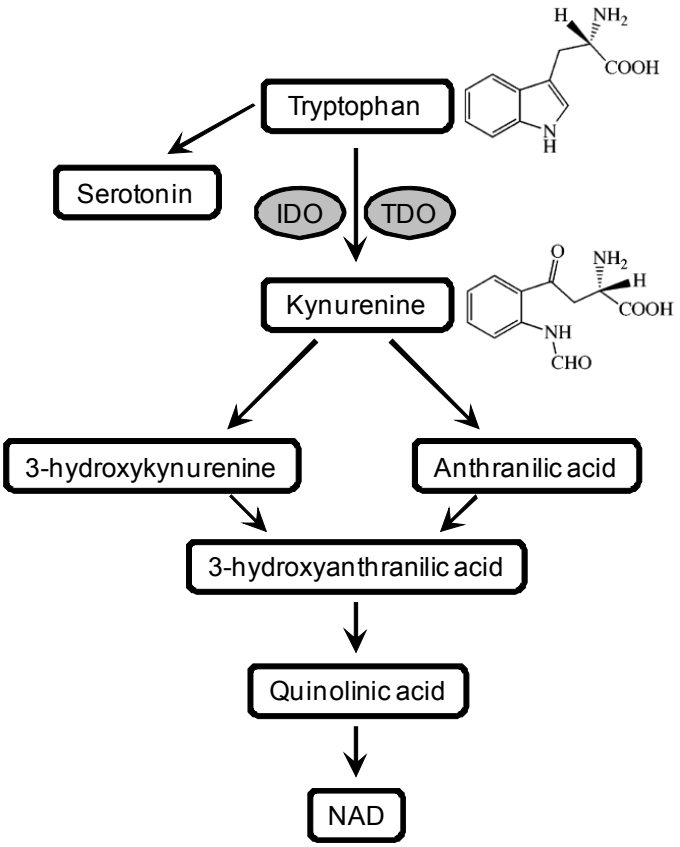


Figure 1: The major metabolic pathway of TRP.
IDO catalyzes the initial and rate-limiting step in the degradation of TRP along the Kyn pathway. Chemical structures taken from (Zhang, Kang et al. 2007).

IDO and TDO are cytosolic monomeric heme-containing dioxygenases that oxidatively cleave the 2,3 double bond in the indole ring of TRP upon incorporation of two oxygen atoms of O_2 which results in the production of N-formylkynurenine (Shimizu, Nomiyama et al. 1978). N-formylkynurenine is subsequently metabolized into other downstream molecules, including 3-hydroxyanthranilic acid, kynurenate and quinolinate, to predominantly generate nicotinamide adenine dinucleotide (NAD) (Takikawa, Yoshida et al. 1986).

Although both enzymes are responsible for the initial degradation step in the TRP metabolism, they are distinct. In mammals, TDO is only found in the liver, whereas IDO is distributed in various nonhepatic organs, e.g. lung, placenta or brain (Yoshida, Nukiwa et al. 1980). IDO can also be found in a variety of murine tumor cells (Habara-Ohkubo, Takikawa et al. 1991). Most importantly, IDO can be expressed in different APCs, e.g. DCs (Alberati-Giani, Ricciardi-Castagnoli et al. 1996; Fallarino, Vacca et al. 2002). IDO has a wider substrate spectrum, including many indoleamine derivatives like L- and D-tryptophan, tryptamine, 5-hydroxytryptophan, and serotonin (Taylor and Feng 1991). Moreover and of crucial importance, TDO steady-state activity is observed in homeostatic conditions, whereas IDO activity appears to be regulated in a dynamic fashion, i.e. it is enhanced in an inflammatory environment (Yamamoto and Hayaishi 1967).

The early literature documented that IDO is part of the cellular host defense against certain pathogens that depend on exogenous TRP and are thus sensitive to TRP depletion by IDO activity. The induction of IDO activity and hence depletion of TRP was implicated in the suppression of various pathogens. These included viruses (Yoshida, Urade et al. 1979) and pathogens which invaded cells or remained in intimate proximity to a host cell, such as Chlamydia pneumonia (Rottenberg, Gigliotti Rothfuchs et al. 2000), Toxoplasma gondii (Fujigaki, Saito et al. 2002) or mycobacteria (Hayashi, Rao et al. 2001). The role of IDO was profoundly reconsidered after Munn et al. reported that placental IDO activity was crucial to protect the fetus by suppressing T cell–driven local inflammatory responses to fetal alloantigens and that pharmacologic inhibition of IDO induces fetal rejection (Munn, Zhou et al. 1998). In the past years, the potential immuno-suppressive role of IDO was supported by *in vitro* and *in vivo* studies which suggested that IDO induced TRP depletion by APCs suppressed T cell responses (Mellor and Munn 2004) and that

IDO expressing islets inhibited T cell mediated rejection of allografted pancreas islets in mice (Alexander, Crawford et al. 2002)

Regulation of IDO protein expression

Murine IDO is a 407 AA long protein (MW 45,639 kilodalton (kDa)) encoded by a single gene, termed Indo, located on the chromosome 8. The cDNA was first isolated in 1991 from different cell lines upon exposure to IFN-γ and showed 62.5% homology to human IDO (Habara-Ohkubo, Takikawa et al. 1991). Various studies have demonstrated that IDO induction occurs in response to toll like receptor (TLR) ligands, e.g. bacterial endotoxin (lipopolysaccharide, LPS) (Yoshida and Hayaishi 1978) or viruses (Yoshida, Urade et al. 1979), as well as by pro-inflammatory cytokines, including IFN-γ or tumor necrosis factor (TNF) (Bianchi, Bertini et al. 1988). It is generally accepted that IFN-γ is one of the most important inducers of IDO (Fujigaki, Saito et al. 2002) and that induction is mediated through the JAK/STAT, in an IFN regulatory factor 1 (IRF1)- and signal transducer and activator of transcription 1 (STAT1)-dependent pathway (Du, Sotero-Esteva et al. 2000; Silva, Rodrigues et al. 2002).

Regulation of IDO activity

It is crucial to mention that IDO expression does not necessarily mean activity. Constitutive IDO expression is found in various types of DCs, but for enzyme activity further stimulatory signals are needed (Grohmann, Bianchi et al. 2000). Mechanisms that enable enzyme activity are poorly understood, but protein modifications on posttranslational level are suggested.

IDO contains a protoheme IX as its sole prosthetic group that seems to be essential for enzyme activity. Normally, inactive IDO contains the heme present as ferric-iron (Fe^{3+}) that needs to be reduced to the ferrous state (Fe^{2+}) for enzyme activation. When the ferric enzyme is reduced by co-factors to the ferrous state it binds TRP and O_2 to form the ternary complex (TRP-Fe^{2+}-O_2) (Sono, Taniguchi et al. 1980; Sono, Roach et al. 1996). The intracellular co-factors required for IDO heme-iron reduction are unknown. Putative candidates include superoxide anion radical, dihydroflavin mononucleotide, tetrahydrobiopterin and cytochrome reductases (King and Thomas

2007). *In vitro*, a combination of methylene blue (a redox dye) with ascorbic acid was suggested for activating IDO, whereby methylene blue reduces the molecular oxygen to superoxide anion (O_2^-) which, in turn, reduces the ferric form of IDO to the ferrous form (Takikawa 2005).

Additionally, negative regulation of IDO on the posttranslational level by anti-oxidants (Thomas, Salahifar et al. 2001) or nitric oxide (NO) generators was reported. Whereas anti-oxidant pyrrolidine dithiocarbamate and heme biosynthesis inhibitors limit the availability of heme, NO generated by iNOS blocks the heme site to O_2 binding. NO induces conformational changes by breaking the Fe-N bond resulting in enzyme inactivity without reduction of protein quantity (Oh, Pae et al. 2004; Wood and Sawitzki 2006; Hara, Ogasawara et al. 2008).

Implication of IDO activity

There is evidence that IDO activity influences a variety of physiological and pathological conditions *in vivo*. Whereas IDO induced immuno-suppression is desirable in situations like murine pregnancy (Munn, Zhou et al. 1998), transplantation (Miki, Sun et al. 2001; Alexander, Crawford et al. 2002) or autoimmune syndromes (Sakurai, Zou et al. 2002), enzyme activity in others, e.g. malignancies (Friberg, Jennings et al. 2002; Uyttenhove, Pilotte et al. 2003) that implicates a worse clinical outcome, is unwanted.

Two theories have been proposed that explain the immunological influence of enhanced TRP metabolism by IDO activity (Moffett and Namboodiri 2003). The *TRP depletion theory* postulates that TRP breakdown suppresses T cell proliferation by markedly reducing the supply of this critical AA. This theory was supported by studies that induced tolerance in activated T cells *in vitro* after TRP decrease below 0.5–1 µM from the culture medium by IDO expressing DCs (Munn, Shafizadeh et al. 1999). It has been reported that in these TRP-free culture media activated T cells entered the cell cycle, but halted proliferation at the mid-G1 phase (Lee, Park et al. 2002). The second theory (*TRP utilization theory*) posits that the downstream metabolites of the TRP metabolism suppress certain immune cells, as shown in an *in vitro* study where TRP metabolites induce T cell apoptosis (Fallarino, Grohmann et al. 2002).

IDO in DCs

It is widely accepted that the DCs (Figure 2) are the most potent and versatile APCs that are capable to induce adaptive immune responses (Steinman and Witmer 1978; Kapsenberg 2003; Reis e Sousa 2006).

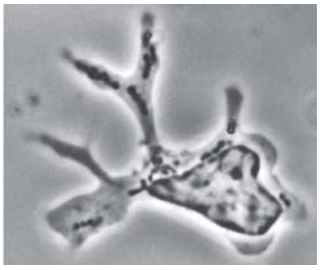


Figure 2: Dendritic cell.
First visualized as Langerhans cells in the skin in 1868 (Langerhans 1868), the characterization of DCs began only 35 years ago (Steinman and Cohn 1973).One of the first images ever made of a DC was published by Steinman in 1974, who dubbed them dendritic cells, derived from the Greek word for tree. Picture taken from (Steinman 2007).

DCs are generated in the bone marrow and migrate as precursor cells to sites of potential entry of pathogens (Steinman, Lustig et al. 1974; Kapsenberg 2003). This not only includes nonlymphoid organs like epithelia, dermis and the interstitial of vascularised organs such as heart and kidneys, but also lymphoid organs or the blood and lymph (Reis e Sousa, Sher et al. 1999).

It was first claimed by Steinman and colleagues that DCs exist in two functional states, immature and activated. In a quiescent immature state, DCs play a critical role in establishing tolerance to self antigen (Steinman and Nussenzweig 2002). In the periphery, they induce deletion or anergy in self-reactive T cells or expansion of regulatory T cells. Only in the activated state DCs have the ability to drive clonal T cell expansion and prime immune responses. These DCs are referred to as immunogenic DCs. Upon their activation a complex activation program is triggered that results in up-regulation of MHC class I and class II and co-stimulatory molecules, and production of cytokines. Moreover, they migrate to lymphoid organs, such as spleen and lymph nodes, where processed antigens are presented to activate antigen specific T cells (Cella, Sallusto et al. 1997; Banchereau and Steinman 1998).

Recently, it was proposed that DC activation and the IDO pathway, as one of the negative feedback mechanisms, are initiated simultaneously (Hainz, Jurgens et al. 2007), rendering DCs tolerogenic. On the one hand, negative regulatory pathways are important in the immune system to limit intensive and strong immune responses, which would otherwise result in dangerous collateral damage to the host. Some studies have shown that blocking of IDO led to a significant exacerbated inflammation and sometimes lethality during local inflammation (Gurtner, Newberry et al. 2003). On the other hand, negative regulatory responses in an immunological tumor environment would result in tumor progression. Whether IDO expression in tumor cells or in tumor associated DCs account for tumor progression is still under debate.

Murine DC subtypes

A variety of DCs described in lymphoid and non-lymphoid organs can be classified into at least six mouse DC subtypes (Table 1). DCs in the spleen can be divided into $CD8^-$ and $CD8^+$ subsets, whereas $CD8^-$ DCs can further be subdivided into $CD4^-$ and $CD4^+$ DCs. In the thymus, $CD8^+$ DCs are supposed to be the main DC subtype. The lymph node additionally harbors DCs that express CD205. In non-lymphoid organs like the skin Langerhans cells have been observed. Recently, plasmacytoid DCs (pDCs) were found in all lymphoid organs (Shortman and Liu 2002; Ardavin 2003).

	'Lymphoid' DCs $CD4^-CD8^{hi}$ $CD205^{hi}CD11b^-$	'Myeloid' DCs $CD4^+CD8^-$ $CD205^-CD11b^+$	'Myeloid' DCs $CD4^-CD8^-$ $CD205^-CD11b^+$	'Myeloid' DCs $CD4^-CD8^-$ $CD205^+CD11b^+$	'Langerhans' DCs $CD4^-CD8^{lo}$ $CD205^{hi}CD11b^+$
Percentage total DCs in					
Spleen	23	56	19	< 4	< 1
Thymus	70				
Mesenteric lymph nodes	19	4	37	26	< 4
Skin-draining lymph nodes	17	4	17	20	33

Table 1 Lymphoid tissues distribution of mouse DC subtypes.
The terms "lymphoid" and "myeloid" DCs no longer denote hematopoietic origin, but are still commonly used. The table and values for steady-state, uninfected laboratory mice are based on the publication of (Shortman and Liu 2002).

Originally, it was assumed that DCs derive from common myeloid precursors due to their functional, phenotypic and morphological similarities to macrophages (van Furth and Cohn 1968). This concept has been confirmed by DCs generated from bone marrow proliferating MHC class II negative precursors in presence of granulocyte-

macrophage colony-stimulating factor (GM-CSF) (Inaba, Inaba et al. 1992) and additionally strengthened by studies with human (Sallusto and Lanzavecchia 1994) and murine monocytes (Randolph, Inaba et al. 1999). The first assumption that DCs might be related to the lymphoid lineage came from the finding that the typical lymphoid marker CD8 was expressed on mouse thymic DCs and on a subset of splenic DCs (Vremec, Zorbas et al. 1992). Transfer experiments with thymic lymphoid precursors that fully reconstituted the thymic $CD8\alpha^+$ DC population (Ardavin, Wu et al. 1993; Ardavin 1997) supported the hypothesis that $CD8\alpha^-$ DCs are of myeloid origin and $CD8\alpha^+$ DCs of lymphoid origin (Wu, Li et al. 1996). Today it is known that both subsets can be generated from common lymphoid or myeloid precursors (Martin, del Hoyo et al. 2000; Traver, Akashi et al. 2000). The last DC subtype completing the list is murine pDCs. The first evidence for these cells came from DC-studies that solely secreted IFN-alpha and IL-12 upon virus challenge, but not upon treatment with bacterial products (Asselin-Paturel, Boonstra et al. 2001).

Even though different DC subtypes share the common capacity to present antigen to T cells and to promote their proliferation, none of them is a product of the other and all are short-lived products (Kamath, Pooley et al. 2000). *In vitro* studies indicate that any of the DC subtypes can functionally divide into either immunogenic or tolerogenic DCs. Factors determining the DC phenotype include the activation state of the DCs, type and concentration of antigen/microorganism invading a host thereby restricting the DC subtype available in the specific tissue, the type of receptor responsible for antigen uptake and the genetic background of the host (Abbas, Murphy et al. 1996; Moser and Murphy 2000; Ardavin 2003). For instance, $CD8^+$ DCs have the ability to induce strong Th1 response, representing immunogenic DCs (Maldonado-Lopez, De Smedt et al. 1999). Interestingly, IDO activity in the same DC subtype was implicated in enhanced apoptosis of CD4 T cells. Consequently, they might also belong to a specialized subset of tolerogenic or regulatory DCs (Kronin, Winkel et al. 1996; Grohmann, Fallarino et al. 2001).

Immunogenic DCs

Pathogen primed activated DCs can readily prime naïve T cells (Figure 3). Their fate is determined by three signals that are provided by the DCs. Signal 1 stimulation results from the ligation of TCRs with pathogen-derived peptides bound to respective MHC on the DC surface and defines the antigen specificity of the response. Induction of clonal T cell expansion, development into effector T cells and thus protective immunity, requires stimulation through signal 1 and co-stimulation through signal 2 (CD80/CD86). Typically, these two signals are accompanied by high level secretion of selective sets of cytokines – signal 3 – determining effector cell differentiation (CD4 Th1, CD4 Th2 and cytotoxic CD8 T-lymphocyte (CTL)) (Kapsenberg 2003; Reis e Sousa 2006).

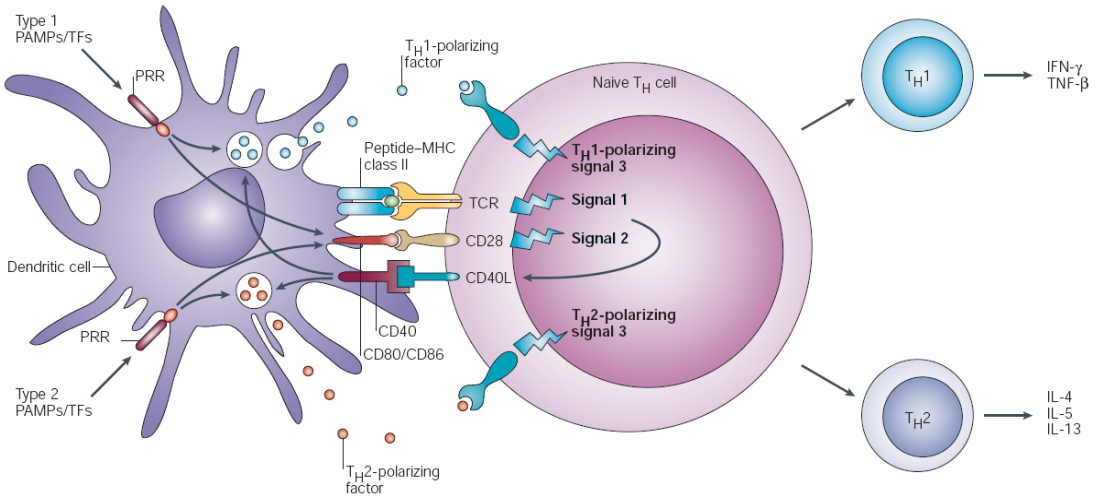


Figure 3: For T cell activation and thus polarization 3 signals are mediated by the DC, including the antigen-specific signal (Signal 1), the co-stimulatory signal (Signal 2) and the polarizing signal (Signal 3). IFN-γ, interferon-gamma; PAMPs, pathogen-associated molecular patterns; PRR, pattern-recognition receptors; T cell receptor, TCR; TFs, inflammatory tissue factors; T_H1/2, T helper cell 1/2; TNF-β, tumor-necrosis factor-beta (Kapsenberg 2003).

Activation of DCs and initiation of the immune system

More than 15 years ago, two major models proposed that resting DCs can be activated by signals from their immediate environment. In Janeway`s Infectious-Nonself model, DC activation was suggested to be induced by direct recognition of exogenous signals (produced by different organisms), known as pathogen-associated molecular patterns (PAMPs) through a series of germline-encoded

pattern-recognition receptors (PRRs) (Janeway 1989). As a result, DCs are able to discriminate between “infectious-nonself” and “noninfectious-self” (Janeway 1992). An important group of PRRs, Toll, was first identified in Drosophila as an essential molecule for embryonic patterning (Hashimoto, Hudson et al. 1988) and in antifungal immunity (Lemaitre, Nicolas et al. 1996). Later, the homologous family of Toll receptors, the so called TLRs were found in mammals (Medzhitov, Preston-Hurlburt et al. 1997). The TLRs are type I transmembrane glycoproteins (Akira and Takeda 2004) and signal through a pathway that mostly leads to activation of NFkB, a family of transcription factors that seems to be implicated in many inflammatory responses (Gay and Keith 1991). Currently, twelve members of the TLR family in mice are known (TLR1-TLR9, TLR11-TLR13). Each of them recognizes specific patterns of microbial components (Takeda and Akira 2007). For example, TLR4 is triggered by LPS, a cell wall component of gram-negative bacteria (Medzhitov, Preston-Hurlburt et al. 1997); TLR3, by PolyIC, a synthetic double stranded ribonucleic acid (dsRNA) (Alexopoulou, Holt et al. 2001); and TLR7, by imidazoquinoline resiquimod (R848) (Heil, Ahmad-Nejad et al. 2003).

In contrast, Matzinger`s Danger model suggested that DCs can be activated through endogenous danger signals (produced by the same organism) normally sequestered in intracellular compartments (Matzinger 1994). A study by Gallucci and colleagues revealed that in absence of foreign substances DCs could neither be activated by healthy cells nor by those dying of apoptosis, but through signals from stressed or virally infected or necrotic cells instead (Gallucci, Lolkema et al. 1999). This response includes the production of IFNs by virally infected cells in response to dsRNA; TNF-α, interleukin-1β (IL1β) and IL18 by keratinocytes (Winzler, Rovere et al. 1997); heat shock proteins by necrotic cells (Basu, Binder et al. 2000); and also CD40L expressed on activated T- and many other cells (Caux, Massacrier et al. 1994; Sallusto and Lanzavecchia 1994; Cella, Scheidegger et al. 1996).

Antigen presentation by DCs – Signal 1

The initiation of antigen-specific immune responses requires the conversion of proteins into peptides that bind to MHC molecules (Germain 1994). Intracellularly synthesized antigens (of viral or tumor origin) are presented by MHC class I molecules and activate CTLs. The generation of activated, peptide-loaded MHC

class I molecules at the cell surface requires the co-ordination of three essential processes (Lehner and Cresswell 1996). Firstly, proteins need to be degraded in the cytosol into antigenic peptides that are 8-11 AA long (Groettrup, Soza et al. 1996). This is a continuous process in all eukaryotes to eliminate damaged and unfolded or incomplete protein (Glickman and Ciechanover 2002), mainly by the ubiquitin-proteasome pathway (Wilkinson, Urban et al. 1980). Secondly, antigens need to be translocated across the endoplasmic reticulum membrane and thirdly, they need to be assembled with MHC class I heterotrimers for their subsequent transport to the cell surface. Extracellular antigens are presented by MHC class II molecules to activate CD4 Th cells (Cresswell 1996). They enter the endocytic route of APCs by a variety of mechanisms: pinocytosis, phagocytosis, receptor-mediated endocytosis or autophagocytosis (Watts 1997). The generation of antigenic bound MHC class II molecules also involves three essential processes. Firstly, the MHC heterodimers need to be associated to the invariant chain. Secondly, they are transported to specialized lysosme compartments where they, thirdly, bind to lysosomally generated antigens that are 9-30 AA long (Miyazaki, Wolf et al. 1996). Subsequently, the MHC class II-peptide complexes are transported to the cell surface. A further mechanism that has been studied extensively is termed cross-presentation which permits extracellular antigen to stimulate CTLs via the MHC class I pathway (Mellman and Steinman 2001). Tumor antigens are exogenous antigens which would otherwise fail to induce CTL activity. Therefore, cross-presentation, hence presentation of exogenous tumor antigens to CD8 T cells, is an essential mechanism for cancer treatment with anti-tumor DC vaccines (Bhardwaj 2001).

Co-stimulatory signals – Signals 2

Signal 1 in absence of signal 2 delivered by immature DCs promotes T cell deletion or anergy which might lead to tolerance (Steinman and Nussenzweig 2002). To induce immunity also signal 2 – the co-stimulation signal molecules expressed on cell surface of APCs – must be provided together with signal 1 (Reis e Sousa 2006).

The B7-1/B7-2 – CD28/cytotoxic T-lymphocyte antigen-4 (CTLA-4) co-stimulatory pathway is best characterized and includes two B7 family members, B7-1 (CD80) (Freeman, Gray et al. 1991; Larsen, Ritchie et al. 1992) and B7-2 (CD86) (Freeman, Borriello et al. 1993) expressed on DCs. They have dual specificity for two CD28

family members, the stimulatory receptor CD28 (Gross, St John et al. 1990; June, Ledbetter et al. 1990) and the inhibitory receptor CTLA-4 (CD152) (Brunet, Denizot et al. 1987). Whereas CTLA-4 expression is up-regulated upon T cell activation (Linsley, Bradshaw et al. 1996), CD28 is constitutively expressed on the surface of T cells (Gross, Callas et al. 1992). B7-1 and B7-2 interaction with CD28 transmits a signal that synergizes with the TCR signal to augment and sustain T cell activation initiated by antigen-receptor signaling (Shahinian, Pfeffer et al. 1993). In contrast, B7-1 and B7-2 interaction with CTLA-4 in various DC subtypes was implicated with an IDO dependent tolerogenic DC phenotype (Grohmann, Bianchi et al. 2003).

T cell polarization – Signal 3

Migrating DCs not only carry antigenic (Signal 1) and co-stimulatory signals (Signal 2), but they are also well equipped to transmit an additional signal 3 to contribute to the polarization of naïve Th cells towards Th1 or Th2, supporting cell-mediated and humoral immunity respectively (Kalinski, Hilkens et al. 1999).

The classification of these two cell populations on the basis of their patterns of cytokine production was first shown by Mosmann and colleagues. By analyzing Th cell clones they characterized Th1 clones by their production of IL2, IFN-γ and TNF-β, and Th2 clones by IL4, IL5, IL6 and IL13 (Mosmann, Cherwinski et al. 1986). Shortly after, it was realized that the function of Th1 and Th2 cells correlated with their distinctive cytokines. Whereas Th1 cytokines promote cell-mediated immunity by activating cytotoxic functions in effector cells such as CTLs, natural killer cells and macrophages, Th2 cytokines support humoral immunity by inducing B cell production of AG-specific antibodies (Abs). These secreted cytokines also serve as autocrine growth factors that promote differentiation of naïve T cells to the same subset and also cross-regulate each other`s development and activity, e.g. inhibited Th2 cell proliferation by IFN-γ or Th1 by IL10 (Mosmann and Sad 1996). The key Th1 and Th2 inducing cytokines secreted by APCs were defined through experiments with IL-12 (Magram, Connaughton et al. 1996) and IL4 (Kopf, Le Gros et al. 1993) knockout mice respectively.

IL-12 cytokine

IL-12 secretion by DCs represents highly immunogenic Th1 polarizing cells and detection of IL-12 in our study is used to determine this DC phenotype. The cytokine was first discovered in human B lymphoblastoid cells (Kobayashi, Fitz et al. 1989) and a few years later in murine DCs (Trinchieri 1994; Macatonia, Hosken et al. 1995; Heufler, Koch et al. 1996). The bioactive form of IL-12 is a 70 kDa heterodimeric protein composed of two disulfide-linked subunits designed p35 (35-kDa) and p40 (40-kDa) which reside at independent loci in the murine genome on chromosome 3 and 11, respectively (Schoenhaut, Chua et al. 1992). The 40 kDa fragment is formed exclusively in DCs, macrophages and monocytes and produced in excess of the 35 kDa subunit. To generate the bioactive form, both subunits need to undergo post-translational modification and dimerization within the same cell (Wolf, Temple et al. 1991; Babik, Adams et al. 1999). IL-12 secretion by DCs can be induced in response to conventional stimuli such as LPS or with IFN-γ (Abdi, Singh et al. 2006), PolyIC (Heufler, Koch et al. 1996) or by ligation of CD40 (Koch, Stanzl et al. 1996). Its secretion, however, is only a transient process that induces strong Th1 polarization at early time points after DC activation, and that primes Th2 or nonpolarized cells at later times points (Langenkamp, Messi et al. 2000).

Licensing signal – for effector function of CTLs

CTLs are responsible for the recognition of peptide antigens presented by MHC class I molecules and subsequent lysis of these antigen-bearing target cells, e.g. tumor cells. To enable priming of CTLs for extracellular tumor antigens, interaction of Th cells with DCs is required (Guerder and Matzinger 1992). CD40 and CD40 ligand (CD40L) were identified as responsible molecules for the proposed mechanism. In these *in vivo* and *in vitro* studies it was reported that mice lacking Th cells could only regain CTL response upon substitution of an agonistic Ab to CD40 and that blocking CD40L signaling inhibits CTL priming (Bennett, Carbone et al. 1998; Ridge, Di Rosa et al. 1998; Schoenberger, Toes et al. 1998).

CD40L is a 39 kDa type II membrane protein that is preferentially expressed on activated CD4 T cells. The receptor for CD40L, CD40 (40 kDa), is a phosphorylated 50 kDa glycoprotein that is expressed on mature B cells, vascular endothelial cells, monocytes/macrophages, Langerhans cells and DCs (Armitage, Fanslow et al. 1992;

Caux, Massacrier et al. 1994; Stout and Suttles 1996; Grewal and Flavell 1998). Interaction of CD40L with CD40 on DCs induces up-regulation of T cell co-stimulatory molecules and release of cytokines that favor Th1 development which consecutively stimulate generation of CD8 CTLs (Cella, Scheidegger et al. 1996).

Tolerogenic DCs in malignancies

During tumor development, referred to as cancer immunoediting, the surviving tumor variants acquire insensitivity to immunologic detection and/or elimination and thus can escape immune surveillance. In the elimination phase, cells of the innate and adaptive immune system help to destroy the developing tumor cells. In the equilibrium and escape phases, on the contrary, tumor cells that carry different mutations arise, which are more resistant to immune attack and thus begin to expand in an uncontrolled manner (Dunn, Bruce et al. 2002).

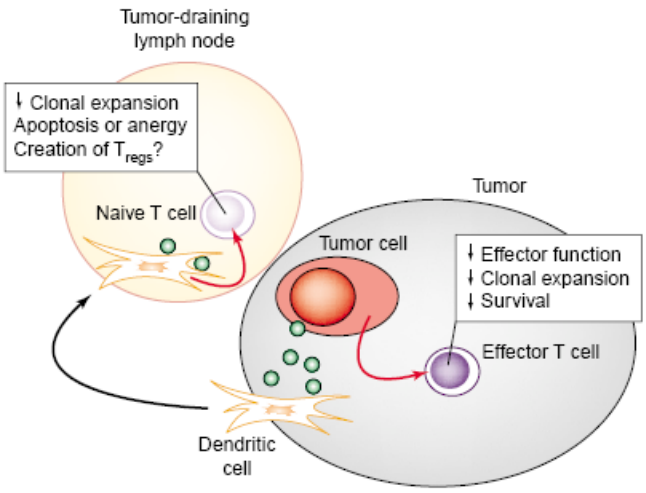


Figure 4: Two possible sites for the immuno-suppressive action of IDO in tumor-bearing hosts.
IDO (red arrows) expressed by tumor cells themselves could create a localized milieu of immuno-suppression in the tumor (right). Alternatively, DC expressing IDO could pick up tumor-derived AG (green spheres) and migrate to tumor-draining lymph nodes, in which they would present antigen to naïve T cells (left) (Munn and Mellor 2004).

Tumor progression and escape in patients was (among other mechanisms) shown to be associated with secretion of immuno-suppressive factors by the tumor itself that contribute to proliferation and survival of malignant cells (Yu and Jove 2004) or secretion of tumor-derived factors that alter immunogenic DCs to become tolerogenic (Pinzon-Charry, Maxwell et al. 2005). These emerging tolerogenic factors may contribute to block the initial response to tumor antigens (Staveley-O'Carroll, Sotomayor et al. 1998), inhibit the ability of activated T cells to kill tumor cells (Gajewski, Meng et al. 2006) and enhance the suppressive activity of regulatory T cells (Zou 2006). As a consequence, the immune system is no longer optimally

activated and fails to reject the tumor (Gilboa 2004; Gilboa 2007; Steinman and Banchereau 2007).

One of the immuno-suppressive factors expressed in both, tumor cells and DCs, and that might contribute to this tolerance is the immuno-regulatory enzyme IDO (Figure 4). The immuno-suppressive role of IDO expression in tumor cells was supported by human and murine studies. In primary human tumors it was observed that expression of IDO (e.g. malignant melanoma, ovarian carcinoma, endometrial cancer and colon carcionma) was associated with a markedly worse clinical outcome (Lee, Torisu-Itakara et al. 2005; Okamoto, Nikaido et al. 2005; Brandacher, Perathoner et al. 2006; Ino, Yoshida et al. 2006). In pre-immunized mice IDO transfection in IDO-negative tumor cells protected the tumor from immune-mediated rejection (Uyttenhove, Pilotte et al. 2003) and IDO silencing in IDO-positive murine tumor cells resulted in recovery of T cell response and enhanced tumor-specific killing (Zheng, Koropatnick et al. 2006; Muller, Sharma et al. 2008). However, it is not clear whether local TRP depletion by tumor cells could suppress systemic immune responses to tumor AGs. In addition, TRP depletion would tend to have an anti-proliferative effect on the dividing tumor cells (Taylor and Feng 1991). Interestingly, a recent publication suggested that TRP metabolize dependent DC self-starvation is protected by simultaneous expression of tryptophanyl-tRNA-synthetase that accumulates TRP in human DCs (Boasso, Herbeuval et al. 2005). Based on these data, tumor-associated DCs expressing IDO in addition to tumor cells would be ideally positioned to control responses to tumor AG (Grohmann, Fallarino et al. 2001).

IDO in DC subtypes

IDO does not appear to be critical for maintaining constitutive tolerance, as IDO deficient mice lack the generation of a spontaneous autoimmune phenotype. However, involvement in certain types of immune regulation, e.g. in certain organs (Chen, Liu et al. 2006), tolerance after transplantation (Grohmann, Orabona et al. 2002), or during pregnancy (Munn, Zhou et al. 1998) appear to be the case. The first hint for a role of IDO in DCs was shown in a study with human cells that suppressed T cell proliferation *in vitro* (Hwu, Du et al. 2000). Recent *in vivo* and *in vitro* reports support a general immuno-suppressive role of IDO expressing cells at least in distinct subtypes of murine DCs (Mellor and Munn 2004; Popov and Schultze 2008).

Extensive studies have been carried out on the potential immuno-suppressive role of IDO in $CD8^+$ and $CD8^-$ splenic DCs. It was reported that IFN-γ treatment of $CD8^+$ or $CD8^-$ splenic DCs up-regulated IDO protein and mRNA *in vitro*. Only in the $CD8^+$ splenic DCs subset, active IDO expression and enhanced apoptosis in CD4 T cells was observed (Fallarino, Vacca et al. 2002). Another publication by the same authors suggested that $CD8^+$ splenic DCs mediated suppression of a delayed-type hypersensitivity response to tumor-antigens was dependent on IDO activity *in vivo*. IDO activity could be implicated in the immuno-suppressive properties of $CD8^+$ splenic DCs, because Kyn accumulation was only seen with IFN-γ treated $CD8^+$ splenic DCs, but not with IFN-γ treated $CD8^-$ splenic DCs or with any of the untreated splenic DCs *in vitro*. Additionally, treatment with 1-methyl-tryptophan (1-MT) (Cady and Sono 1991), a pharmacologic agent that blocks IDO activity, reversed apoptosis in CD4 T cells and suppression of delayed-type hypersensitivity responses (Grohmann, Bianchi et al. 2000; Grohmann, Fallarino et al. 2001; Grohmann, Fallarino et al. 2001). Reversion of the immuno-suppressive properties of $CD8^+$ DCs into immunogenic DCs was also obtained after engagement of B7 with CD28-Ig or after CD40 cross-linking with αCD40 mAb (Fallarino, Grohmann et al. 2002; Fallarino, Asselin-Paturel et al. 2004). Interestingly, the otherwise immunogenic $CD8^-$ DC subset has been reported to become tolerogenic upon B7-1 ligation of CLTA-4-Immunoglobulin (Ig) (Grohmann, Bianchi et al. 2003).

Studies with tumor-bearing mice showed that $CD11c^+$ $B220^+$ $CD19^+$ pDCs, isolated from tumor-draining lymph nodes, but not from contralateral lymph nodes, actively suppressed T cell proliferation *in vitro* and induced antigen specific T cell anergy *in vivo*. None of these effects were seen when pDCs were treated with 1-MT or taken from IDO knock-out mice. Moreover, OT1 T cells from GCN2 knock-out mice, lacking a stress-response kinase that is activated by elevations in uncharged tRNA and suggested as a molecular sensor in T cells were refractory to IDO-induced anergy in a mixed lymphocyte reaction with IDO active pDCs from tumor draining lymph nodes (Munn, Sharma et al. 2004; Munn, Sharma et al. 2005). Delayed-type hypersensitivity studies with otherwise nontolerogenic $CD11c^+$ $B220^+$ $120G8^+$ splenic pDCs expressed active IDO and initiated immuno-suppressive responses after B7-1 engagement with CLTA-4-Ig or after CD200-Receptor-1 engagement with CD200-Ig. Again, 1-MT was able to reverse the immuno-suppressive property of these pDCs. In the same studies it was suggested that CD200-Receptor-1 engagement with CD200-

Ig contingent upon autocrine effects of type I IFNs and that B7-1 engagement with CLTA-4-Ig was IFN-γ dependent (Fallarino, Asselin-Paturel et al. 2004; Fallarino, Orabona et al. 2005).

Up-regulated IDO protein and acquired potent T cell regulatory function was also detected in $CD11c^+$ splenic DCs that co-expressed the marker CD19, but not in $CD19^-$ splenic DCs after injection of CLTA-4-Ig or CpG *in vivo*. *In vitro* mixed lymphocyte reactions of these *in vivo* CpG treated $CD19^+$ DCs with T cells could be reversed by addition of 1-MT and the same DCs from IDO knock-out mice never acquired suppression. IDO expression in these cells was suggested to be type I IFN dependent (Baban, Hansen et al. 2005; Mellor, Baban et al. 2005).

Only sparse information about IDO activity in HSC-derived DCs is available. In a study that examined this specific DC subtype in context of IDO mediated immuno-suppression, up-regulation of the enzyme after treatment with LPS or IFN-γ was measured (Jung, Lee et al. 2007). A subsequent study revealed that the IFN-γ induced IDO expression and activity was associated with a suppressed proliferation of OT1 T cells *in vitro*. 1-MT was as well sufficient to enhance T cell proliferation. Interestingly, rosmarinic acid seemed to have the same effect as 1-MT in enhancing T cell proliferation (Lee, Jeong et al. 2007).

DCs and their potential as anti-tumor vaccines

The idea that the immune system can be activated to recognize and respond to tumors was formed in the late 19th century when William Coley noticed that rare events of spontaneous tumor regression were often preceded by infections. However, attempts to stimulate systemic immunity directed against tumors with bacterial extracts had limited success (Nauts 1989). The finding that the immune system can respond to defined tumor-specific antigens (Van den Eynde and van der Bruggen 1997) together with the discovery of *in vitro* DC generation from murine hematopoietic stem cells (HSCs) (Inaba, Inaba et al. 1992), human $CD34^+$ hematopoietic progenitors (Santiago-Schwarz, Belilos et al. 1992) and peripheral blood-derived monocytes (Romani, Gruner et al. 1994) introduced the era of *ex vivo* anti-tumor DC vaccines.

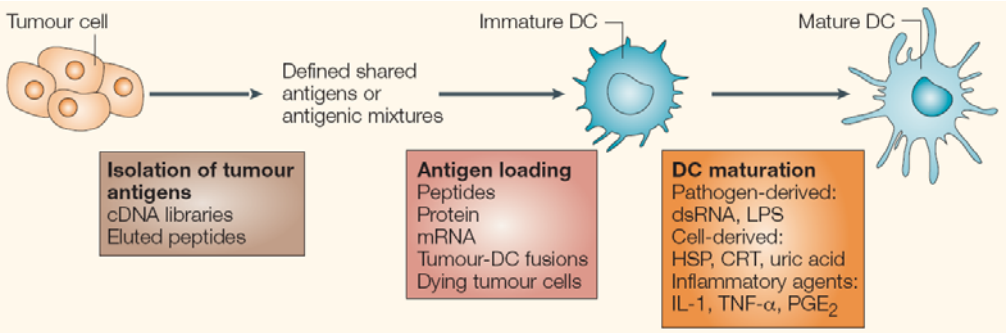


Figure 5: *Ex vivo* loading of DCs with tumor antigens.
DCs are loaded with defined tumor antigens or whole tumor lysat using various techniques at the immature stage to allow for efficient processing of the loaded antigen. Subsequently, DCs are activated *ex vivo* by incubation with various agents. CRT, calreticulin; ds, double stranded; HSP, heat-shock proteins; IL-1, interleukin-1; LPS, lipopolysaccharide; PGE2, prostaglandin E_2; TNF-α, tumour-necrosis factor-alpha (Gilboa 2004).

Today, anti-tumor DC vaccines are one of the strategies to supplement conventional chemotherapy (Banchereau and Palucka 2005; Zou 2005; Steinman and Banchereau 2007). For most studies *ex vivo* generated DCs are loaded with tumor-specific antigens and activated with various stimuli. Subsequently, these cells are re-injected (Figure 5), with the objective to induce a robust and long-lasting CD4 and CD8 T cell response against the tumor in cancer-bearing patients (Gilboa 2007). Studies in mice that effectively inhibited tumor growth in both prophylactic and therapeutic contexts using DCs loaded with tumor antigens endorsed the idea for anti-tumor DC vaccines (Celluzzi, Mayordomo et al. 1996; Zitvogel, Mayordomo et al. 1996). Nevertheless, early clinical trials in humans showed that DC vaccination appeared to be safe, but that only occasionally significant tumor regression was yielded. As a consequence, improvements of therapeutic outcomes invoke the need to develop increasingly potent cancer vaccination strategies, e.g. optimal generation and activation of DCs, choice of antigen (peptides, proteins or whole tumor lysate), antigen-dose, -route and -frequency of DC delivery (Schuler, Schuler-Thurner et al. 2003; Steinman and Banchereau 2007; Koski, Cohen et al. 2008).

IDO as a possible target to improve anti-tumor DC vaccines

It is assumed that immunization with anti-tumor DC vaccines loaded with tumor antigens, against which tolerance has been established, could intensify antigen-specific immuno-suppression (Zhou, Drake et al. 2006). Therefore, one of the most promising strategies to enhance DC immunogenicity is the down-regulation of

negative feedback mechanisms in these cells that would otherwise dampen the immune response of a DC vaccine. It seems that IDO emerges as a potentially important immuno-suppressive factor in patients with cancer (Munn and Mellor 2007). Studies with IDO-deficient mice that exhibit a robust tumor-resistant phenotype in comparison to IDO-positive-wild type hosts IDO related immune-suppression is also suggested (Muller, Sharma et al. 2008). For this reason, conventional chemotherapy with concomitant inhibition of IDO in tumor bearing hosts should allow disruption of tolerance toward established tumors and trigger anti-tumor immune responses (Muller, DuHadaway et al. 2005; Hou, Muller et al. 2007).

We are currently conducting a clinical trial where treatment with an anti-tumor DC vaccine loaded with tumor cell lysate from patients suffering from advanced solid pediatric malignancies are tested (Dohnal, Witt et al. 2007). At the same time, functionality of anti-tumor DC vaccines has been investigated using murine HSC-derived DCs in prophylactic and therapeutic tumor models *in vivo* (Huttner, Breuer et al. 2005).

In the current study we wanted to investigate whether IDO is actively expressed in murine HSC-derived DCs and potentially dampens an otherwise enhanced T cell stimulatory capacity of the anti-tumor DC vaccine.

Results

Inactive IDO in murine HSC-derived and splenic DCs

Studies have shown that the activation of DCs with selected stimuli results in IDO activity in certain DC subtypes (Popov and Schultze 2008). To investigate whether IDO activity in different DC subtypes can be induced, we used different stimuli to trigger IDO activity. First, we established the conditions to generate murine HSC-derived DCs and in addition to enrich murine $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DC subsets from splenic cells.

Generation of murine HSC-derived DCs

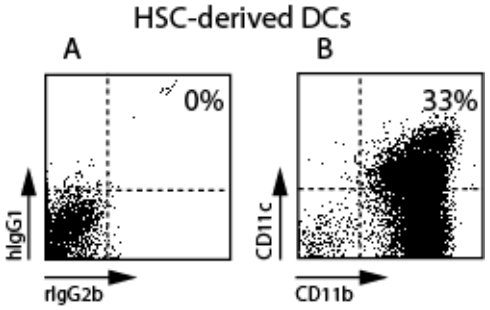


Figure 6: CD11b/c expression by HSC-derived DCs.
HSC were cultured with 5 ng/ml IL4 and 5 ng/ml GM-CSF. On day 6 of culture non adherent cells were stained with isotype-matched (A) or anti-CD11b and CD11c mAb (B) and analyzed by flow cytometry. The diagram shows the analysis of viable cells.

For the generation of murine HSC-derived DCs, HSCs were differentiated with IL4 and GM-CSF. After 6 days of culture, non adherent cells were harvested and tested by flow cytometry for expression of characteristic DC surface markers, hereafter referred to as day 6 HSC-derived DCs. Based on dose response curves 5 ng/ml IL4 and 5 ng/ml, GM-CSF resulted in stable generation of HSC-derived DCs as identified by CD11b/c expression (data not shown). In several individual experiments, approximately 33±4% (mean±SD) of viable cells were positive for CD11b/c expression (Figure 6).

Enrichment of CD8α⁺ and CD8α⁻ splenic DCs

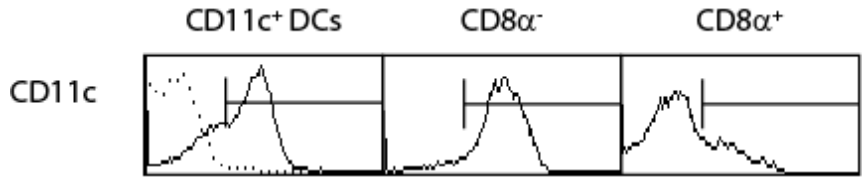


Figure 7: CD11c expression by splenic DC subsets.
DC purity was evaluated by flow cytometry analysis after CD11c MACS enrichment ($CD11c^+$ DCs) and subsequent separation into $CD8^-$ ($CD8\alpha^-$) and $CD8^+$ splenic DCs ($CD8\alpha^+$). Cells were stained with isotype-matched (dotted line) or anti-CD11c mAb (black lines). The diagram shows the analysis of viable cells and illustrates one representative experiment out of three. The bar indicates percentage of CD11c positive cells.

As splenic DCs share expression of CD11c, this marker was used to enrich the cells by magnetic cell sorting. Typically, 3.4 x 10^6 cells per spleen were collected from which approximately 64±4.5% were positive for CD11c (Figure 7). $CD11c^+$ splenic DCs were MACS separated into $CD8\alpha^+$ and $CD8\alpha^-$ splenic DC subsets. The separated subsets typically contained 23±2.6% and 94±4% $CD11c^+$ DCs, respectively.

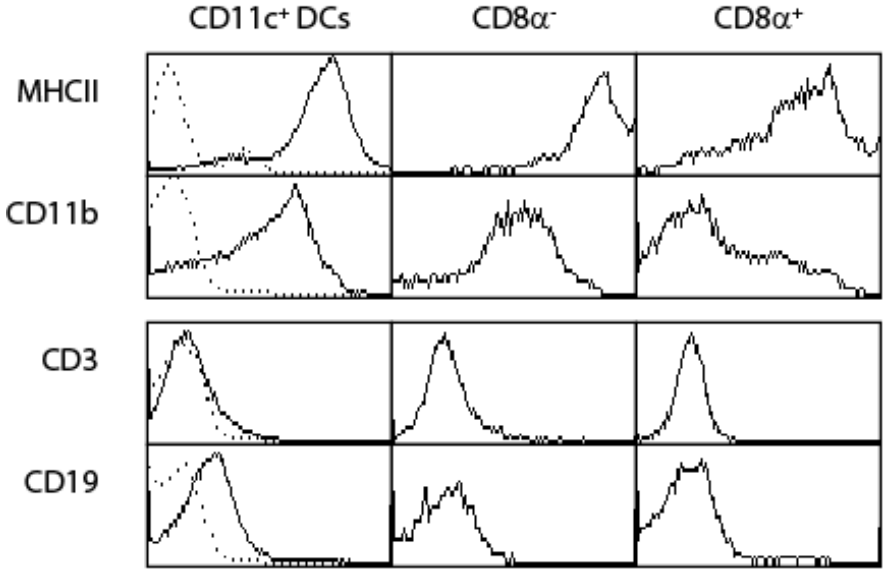


Figure 8: Surface marker expression by splenic DC subsets.
The same splenic DCs as described in Figure 7 were tested for the expression of additional cells surface markers. Cells were stained with isotype-matched (dotted lines) or anti-MHC class II, CD11b, CD3 and CD19 mAb (black lines). The diagram shows the analysis of viable cells and illustrates one representative experiment out of three.

The surface phenotype of $CD11c^+$ splenic DCs and the $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets thereof were compared using different DC markers. In accordance with the literature, $CD8\alpha^+$ splenic DCs expressed high levels of MHC class II and displayed low levels of CD11b (Figure 8), whereas $CD8\alpha^-$ splenic DCs expressed high levels of MHC class II and CD11b. About 25±3% of the cells in the $CD8\alpha^+$ cell fraction showed CD8 expression. All splenic DC subsets were negative for CD3 and CD19 expression, which are common T -and B-cell markers.

Activation of HSC-derived and splenic DCs

For IDO induction, day 6 HSC-derived DCs were sorted according to CD11c expression (day 6 $CD11c^+$ HSC-derived DCs) and activated for 24 hours. Cells were activated with IFN-γ in combination with LPS or R848 or PolyIC. Alternatively, cells were exposed to IFN-γ in combination with CD40L transgenic J558 (J558L) cells or αCD40 monoclonal antibody (mAb) for cross linking CD40 surface molecules. The purified $CD8\alpha^+$ and $CD8\alpha^-$ splenic DC subsets were treated with IFN-γ alone for 24 hours (Grohmann, Bianchi et al. 2000).

Surface marker expression by activated HSC-derived DCs

Flow cytometry of CD11b/c positive HSC-derived DCs was used to analyze the expression density of CD80, CD86, CD40 and MHC class II before and 24 hours after exposure to the respective activation stimulus (Figure 9).

Immature DCs were negative for CD40 and CD80 expression and displayed basal levels of CD86 and MHC class II. Activated DCs demonstrated low and strong expression of CD40 and CD80, respectively, and enhanced expression of CD86 and MHC class II. Expression was most pronounced in LPS/IFN-γ and R848/IFN-γ stimulated DCs.

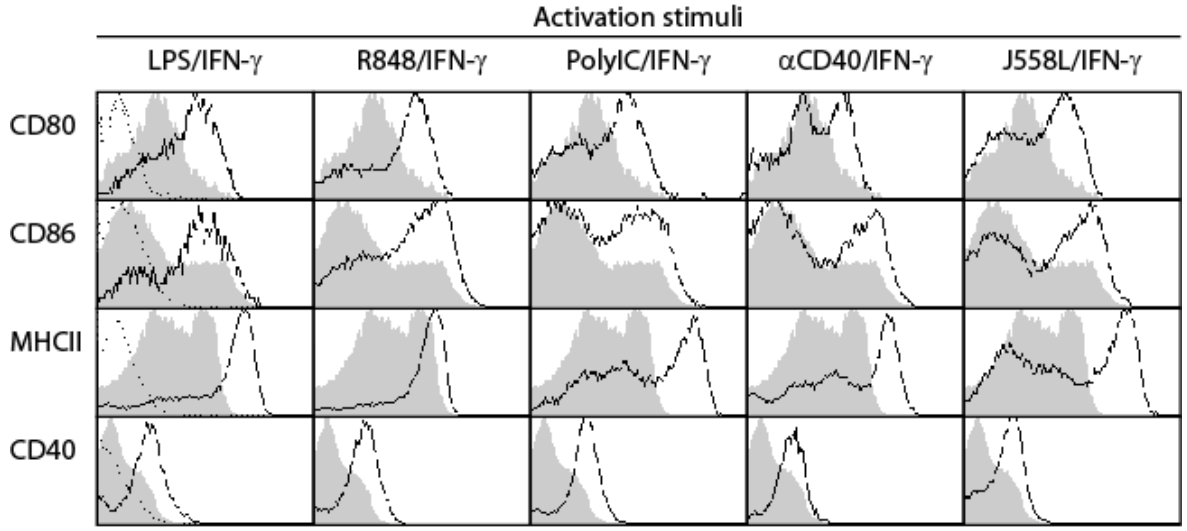


Figure 9: Surface marker expression by activated HSC-derived DCs.
Day 6 CD11c$^+$ HSC-derived DCs were triple stained with either isotype-controls (dotted lines) or anti-CD80, CD86, MHC class II or CD40 mAb in addition to anti-CD11b and CD11c mAb. Surface marker expression was analyzed 24 hrs after activation with (black lines) or without (grey) the respective stimulus. The diagram shows viable CD11b and CD11c positive cells and illustrates one representative experiment out of five.

Cytokine production by activated HSC-derived and splenic DCs

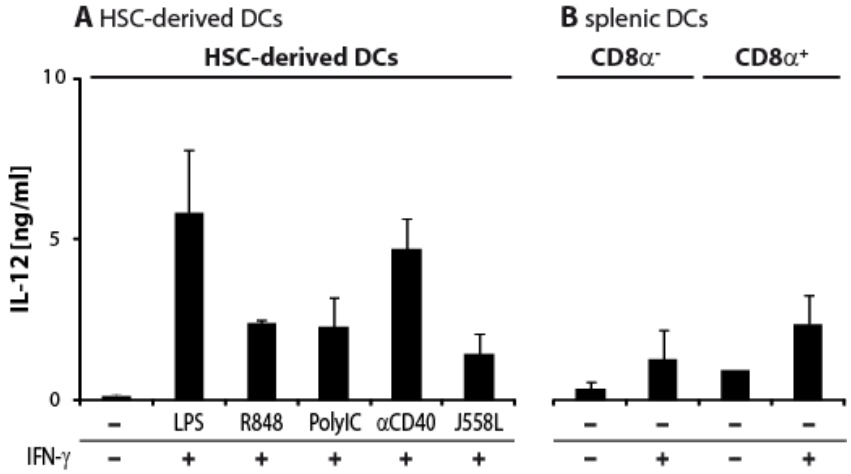


Figure 10: IL-12 secretion by HSC-derived DCs and splenic DC subsets.
0.25 x 10^6 day 6 CD11c$^+$ HSC-derived DCs/ml were activated with the indicated stimuli in combination with IFN-γ (A) and 0.5 x 10^6 splenic DC subsets/ml were activated with IFN-γ alone (B). Accumulated IL-12p70 levels in the supernatants were measured by ELISA assay 24 hrs later and compared to DCs cultured in medium alone (-). The values represent mean±SD of three experiments.

The ability of DCs to produce the Th1 cytokine IL-12p70 (mean±SD of three experiments) upon stimulation was determined by enzyme linked immunosorbent assay (ELISA) in the supernatants of the different DC cultures. No IL-12p70 was

detected in immature CD11c$^+$ HSC-derived DCs (Figure 10A). Upon activation with R848/IFN-γ, PolyIC/IFN-γ or J558L/IFN-γ, concentrations of 2.3±0.9 ng/ml, 1.4±0.7 ng/ml or 2.4±2.3 ng/ml IL-12p70 were detected, respectively. More IL-12p70 was secreted in CD11c$^+$ HSC-derived DC cultures stimulated with αCD40 mAb/IFN-γ (4.7±0.9 ng/ml). The treatment with LPS/IFN-γ induced highest secretion of IL-12p70 (5.8±1.9 ng/ml).

Without additional stimulation, CD8α$^-$ (0.4±0.2 ng/ml) and CD8 α$^+$ (1.0±0.1 ng/ml) splenic DCs secreted basal IL-12p70 levels (Figure 10B). IFN-γ stimulation enhanced IL-12p70 production by CD8α$^-$ splenic DCs (1.3±1.0 ng/ml) and CD8α$^+$ splenic DCs (2.4±1.0 ng/ml). IL-12p70 production tended to be superior in CD8α$^+$ splenic DCs when compared to CD8α$^-$ splenic DCs.

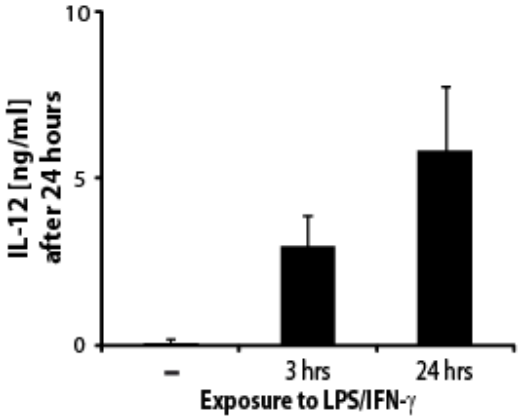


Figure 11: IL-12 secretion by 3 hrs activated HSC-derived DCs.
0.25 x 10^6 day 6 CD11c$^+$ HSC-derived DCs/ml were activated with LPS/IFN-γ for 24 hrs (24 hrs) or for 3 hrs with subsequent re-culture in stimulus-free medium for additional 21 hrs (3 hrs). Accumulated IL-12p70 levels in the supernatants were measured by ELISA assay and compared to DCs cultured in medium without activation stimulus (-). The values represent mean±SD of three.

We investigated whether a reduced stimulation time period would still be sufficient to induce IL-12p70 secretion by CD11c$^+$ HSC-derived DCs and analyzed the supernatants after 3 hrs LPS/IFN-γ stimulation and subsequent re-cultivation in medium alone for additional 21 hrs (Figure 11). As shown previously, immature CD11c$^+$ HSC-derived DCs did not secrete IL-12p70. Activation with LPS/IFN-γ for 3 hrs slightly reduced IL-12p70 concentrations (2.9±0.9 ng/ml) in comparison to 24 hrs stimulated CD11c$^+$ HSC-derived DCs (6.5±1.8 ng/ml), suggesting that similarly to the human system a short stimulation is sufficient for IL-12 induction.

IDO expression and activity in activated HSC-derived and splenic DCs

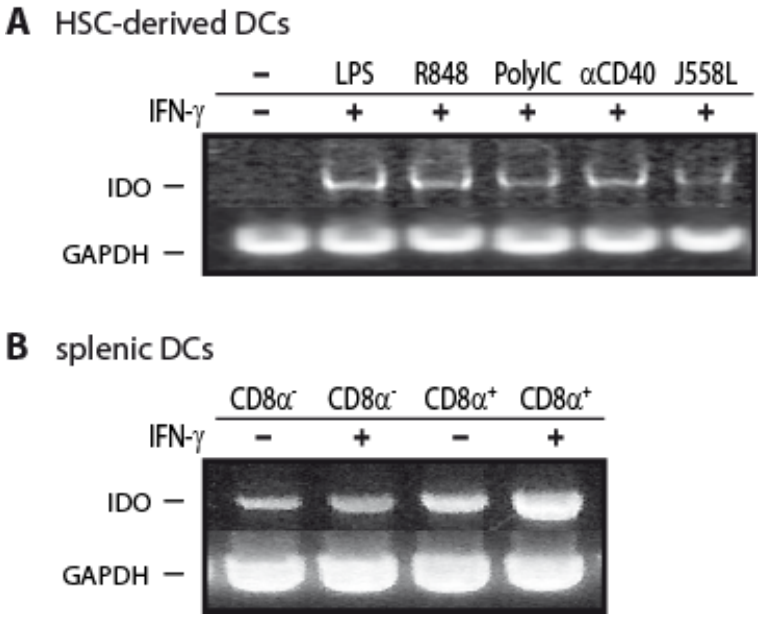


Figure 12: IDO mRNA expression of HSC-derived and splenic DCs.
0.25 x 10^6 day 6 $CD11c^+$ HSC-derived DCs/ml were activated with the indicated stimuli in combination with IFN-γ (A) and 0.5 x 10^6 splenic DC subsets/ml were activated with IFN-γ alone (B) for 24 hrs. Expression of IDO mRNA by these cells and by cells cultured in medium alone (-) was analyzed by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA levels were used to control equal gel loading. The diagram illustrates one representative experiment out of three.

Immature $CD11c^+$ HSC-derived DCs did not express IDO at levels detectable by standard RT-PCR methods (Figure 12A). Upon activation, IDO-gene transcription was observed. IDO mRNA in $CD8\alpha^+$ and $CD8\alpha^-$ splenic DCs was constitutive expressed and enhanced by IFN-γ treatment (Figure 12B).

Levels of TRP and Kyn in the supernatant of DC cultures were examined by high performance liquid chromatography (HPLC, in generous collaboration with D. Fuchs) to detect constitutive or induced IDO activity, whereas lack of TRP depletion and Kyn accumulation indicate no enzymatic activity.

Although IDO mRNA expression was detectable upon activation of $CD11c^+$ HSC-derived DCs and constitutively in splenic DCs, TRP and Kyn levels in the supernatants of $CD11c^+$ HSC-derived DCs (Figure 13A) and splenic DCs (Figure 13B) were similar to concentrations in medium alone (Figure 13C). Even one week after cultivation of HSC-derived and splenic DCs, IDO activity was lacking (data not shown). Interestingly, IDO activity in human monocyte derived DCs activated with

LPS and IFN-γ could be routinely detected (Figure 13C), indicating a potential difference in the regulation of IDO activity.

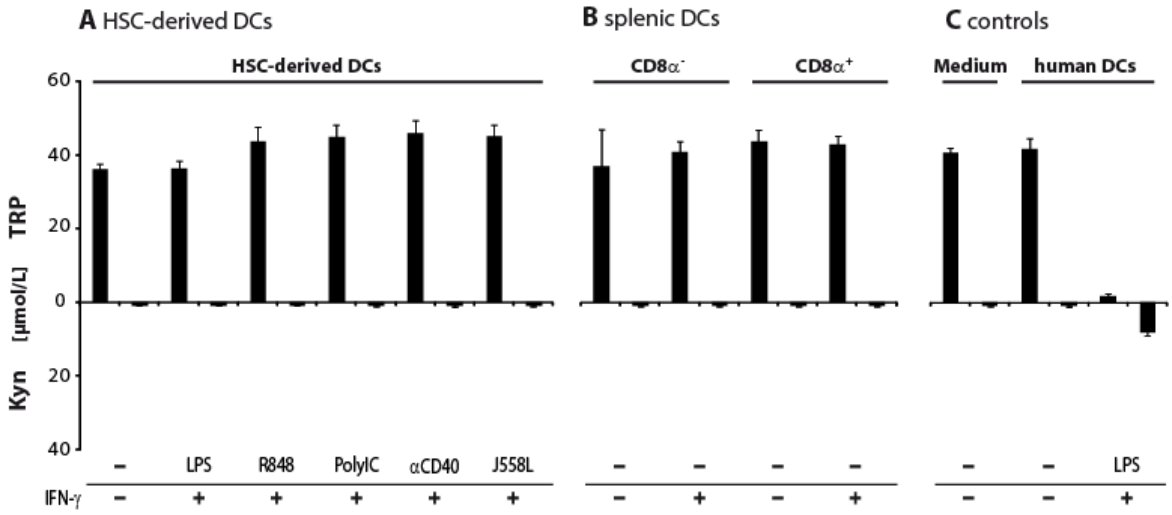


Figure 13: IDO activity in DC subtypes.
0.25 x 10^6 day 6 $CD11c^+$ HSC-derived DCs (A) or 0.5 x 10^6 splenic DC subsets (B)/ml medium were treated as described in Figure 12. TRP and Kyn levels in the supernatants were determined by HPLC analysis. Lack of TRP depletion and Kyn accumulation indicate no enzymatic activity. The values represent mean±SD of three experiments. I acknoledge B. Jürgens for providing data on human DCs.

Inactive IDO over-expressed in HSC-derived DCs

In contrast to the literature, activation of HSC-derived DCs or $CD8^+$ or $CD8^-$ splenic DC subsets did not induce IDO activity. Therefore, it was of substantial interest whether lack of IDO activity was caused by low IDO protein quantity. For this reason, we compared different transfection methods for HSC-derived DCs to subsequently use the most optimal one to over-express IDO.

Comparison of transfection methods for HSC-derived DCs

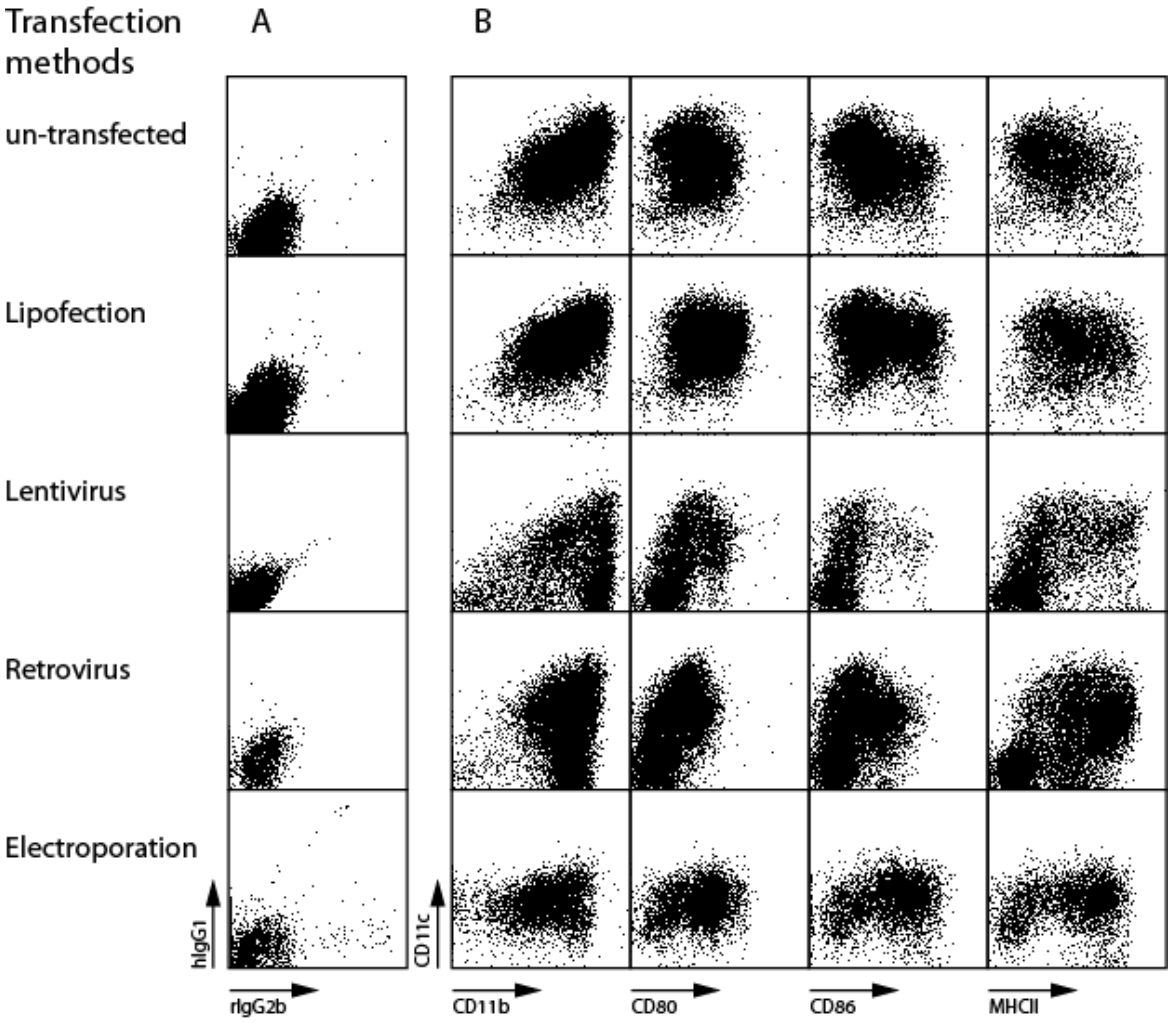


Figure 14: Surface marker expression by transfected HSC-derived DCs.
HSC-derived DCs were double stained with isotype-matched mAb (A) or with anti-CD11c in addition to CD11b, CD80, CD86 or MHC class II mAb (B). Marker expression was analyzed 24 hrs (lipofection, lentivirus, electroporation) or 6 days after transfection (retrovirus) and compared to un-transfected cells. The diagram shows viable cells and illustrates one representative experiment out of three.

HSCs during DC generation or day 6 HSC-derived DCs with or without enrichment according to the expression of CD11c were transfected with the indicated method using compatible green fluorescence protein- (GFP-) encoding vectors. Cells were tested by flow cytometry regarding GFP expression, representing transfection efficiency, possibly altered cell viability and unwanted induction of activity by the particular method in comparison to DCs left un-transfected 24 hrs later. The different transfection methods used are described in detail in materials and methods.

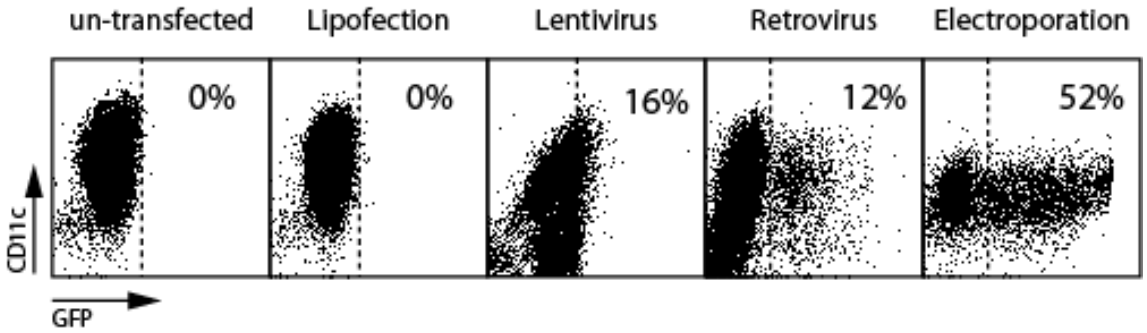


Figure 15: GFP expression by transfected HSC-derived DCs.
GFP transfected or un-transfected HSC-derived DCs were stained with anti-CD11c mAb. The percentage of CD11c and GFP co-expressing cells was determined either 24 hrs (lipofection, lentivirus, electroporation) or 6 days post transfection (retrovirus). The diagram shows viable cells and illustrates one representative experiment out of three.

Lipofectamine transfection of HSC-derived DCs

To examine the transfection efficiency of lipofectamine in HSC-derived DCs, cells were transfected with pMax-GFP at different time points of DC generation and analyzed 24 hrs post transfection.

Transfection of day 6 HSC-derived DCs with lipofectamine did not up-regulate CD80, CD86 and MHC class II expression 24 hrs after manipulation (Figure 14B, 2nd row) in comparison to un-transfected day 7 CD11c^{+} DCs (Figure 14B, 1st row). To evaluate whether transfection of precursor cells or DCs was more effective, cells were additionally transfected on day 1, 4 and 5 of DC generation. GFP expression was never detected 24 hrs later (Figure 15, 2nd dot plot and Figure 16A). 7AAD, a nucleic acid dye that stains dead cells, was used to determine cell viability. However, transfection with lipofectamine did not substantially reduce the viability of the cells (Figure 16A).

The method and the reagent itself were efficient for transfection strategies in cells other than DCs. When HeLa cells were transfected with lipofectamine, 68% of the cells were positive for GFP expression after 24 hrs (Figure 16B), whereas un-transfected HeLa cells never expressed GFP. HSC-derived DCs were not able to express GFP; transfection with lipofectamine was concluded not to be suitable for DCs.

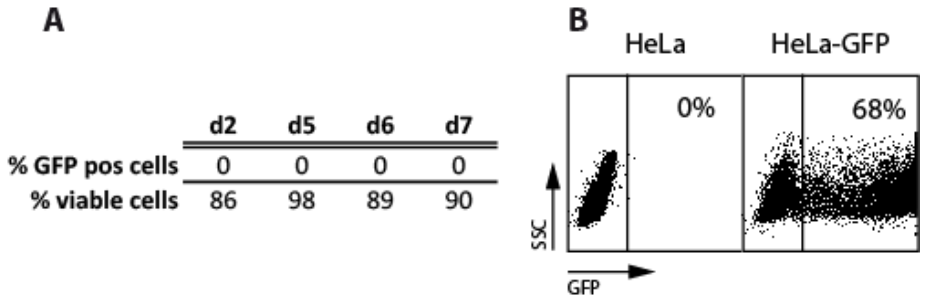


Figure 16: HSC-derived DCs and HeLa cell transfection with lipofectamine. HSC-derived DCs were stained with anti-CD11c mAb and 7-AAD. GFP expression and viability of $CD11c^+$ DCs was analyzed by flow cytometry 24 hrs after transfection with lipofectamine at different time points of DC generation (A). HeLa cells were stained with 7-AAD (B). GFP expression of lipofectamine transfected cells (HeLa-GFP) was analyzed by flow cytometry and compared to untreated cells (HeLa). The diagram shows 7-AAD negative populations representing viable cells and illustrates one representative experiment out of three.

Lentivirus transfected HSC-derived DCs

Next, the efficiency of lentiviral transfection of HSC-derived DCs was evaluated. The generated lentiviral particles were titered as described in materials and methods and subsequently used to transfect day 6 HSC-derived DC populations at a multiplicity of infection of one.

Expression of CD80, CD86, MHC class II (Figure 14, 3rd row), and viability (data not shown) was not altered 24 hrs post lentiviral transfection when compared to un-transfected DCs (Figure 14, 1st row). CD11c negative cells could be detected as HSC-derived DCs were not CD11c MACS enriched. Although lentivirally transfected HSC-derived DCs lacked an altered DC phenotype and decreased viability, only 16% of the cells expressed GFP (Figure 15, 3rd dot plot), which excluded this method for further experiments.

Retroviral transfection of HSCs and differentiation into HSC-derived DCs

Further, the efficiency of retroviral transfection of dividing HSCs was investigated. For the production of GFP-encoding retroviral particles, ecotropic packaging Phoenix cells were transfected with MSCV-MigR1-GFP using lipofectamine. 24 hrs later cells were irradiated. Another 24 hrs later, the un-transfected and GFP-transfected Phoenix cells were analyzed for the expression of GFP by flow cytometry and subsequently used for co-culture experiments with freshly isolated HSCs. After two days of co-culture, HSCs were recovered and re-cultured alone for additional four days. Further testing was done, but without sorting according to CD11c expression.

97% and 86% of un-transfected and GFP-transfected Phoenix cells, respectively, were viable (data not shown) and 68% of the transfected cells were GFP positive (Figure 17).

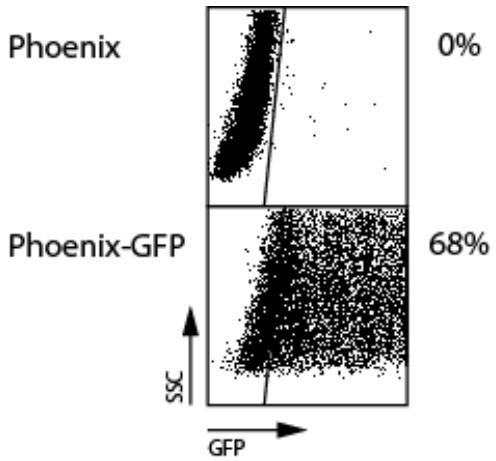


Figure 17: Phoenix cell transfection with lipofectamine. GFP expression was analyzed 24 hrs after lipofectamine transfection (Phoenix-GFP) and compared to cells without treatment (Phoenix). The diagram shows viable cells and illustrates one representative experiment out of three.

The presence of retrovirus producing Phoenix cells did no influence the generation of HSC-derived DCs (Figure 14, 4th row). The percentage of CD11b/c positive DCs and the expression density of CD80, CD86 and MCHII were comparable to that of DCs generated under control conditions (Figure 14, 1st row). GFP expressing cells were also positive for CD11c, the most prominent DC marker (Figure 15, 4th dot plot). However, GFP expression was not observed when co-cultures were conducted with un-transfected Phoenix cells. Usually, DC cultures were more than 90% viable.

Phoenix cells did not express CD11b and CD11c thus demonstrating that GFP expressing cells were solely of DC origin (data not shown). Our experiments provide evidence that HSC can be transfected by retroviral particles to some extent and that further differentiation into DCs is not altered.

Transfection of HSC-derived DCs by electroporation technique

The final transfection method evaluated was the electroporation technique (Dullaers, Breckpot et al. 2004). Day 6 $CD11c^+$ HSC-derived DCs were either left un-transfected, or transfected with pMax-GFP vector and analyzed 3 to 72 hrs later.

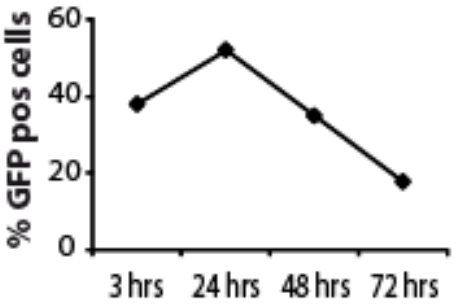


Figure 18: Transfection of HSC-derived DCs by electroporation technique.
Day 6 $CD11c^+$ HSC-derived DCs were stained with anti-CD11c mAb. The percentage of GFP expressing $CD11c^+$ cells was analyzed up to 72 hrs after transfection. Diagram shows viable $CD11c^+$ cells.

In comparison to un-transfected DCs (Figure 14, 1st row), GFP transfected DCs showed slight up-regulation of DC surface markers (Figure 14, 5th row) and minimal reduction of cell viability from 96% to 86% (data not shown). GFP expression by DCs was tested at various time points after manipulation. While un-transfected DCs never showed GFP expression (data not shown) more than 50% of the GFP transfected cells were tested positive after 24 hrs (Figure 15, 5th dot plot). Already 3 hrs and up to 72 hrs after treatment, GFP expression was detectable (Figure 18). Transfection of 24 hrs LPS/IFN-γ stimulated DCs did not increase the percentage of GFP positive DCs (data not shown). Electroporation emerged as the most efficient method to transfect $CD11c^+$ HSC-derived DCs and was subsequently used for all experiments.

Over-expression of IDO

IDO transfected mammalian cell lines

To evaluate whether IDO produced by IDO transfected cells is active, different mammalian cell lines were transfected by electroporation technique. Several murine and human cell lines were either transfected with pMax-GFP (GFP), co-transfected with pMax-IDO and pMax-GFP (IDO/GFP) or left un-transfected. 24 to 72 hrs later, cells were tested for viability and GFP expression by flow cytometry. In addition, IDO mRNA expression and activity were determined by RT-PCR and HPLC analysis, respectively.

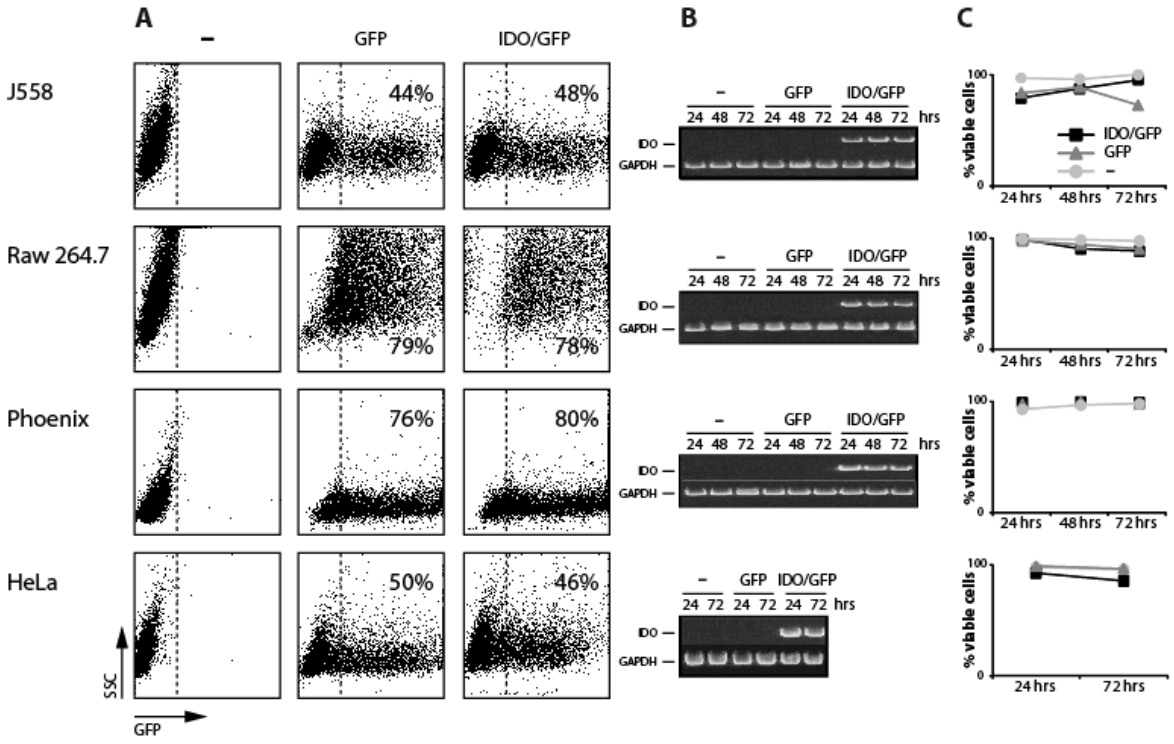


Figure 19: IDO expression by murine and human cell lines.
0.25 x 10^6 cells/ml medium were either transfected with pMax-GFP (GFP), co-transfected with pMax-IDO and pMax-GFP (IDO/GFP) by electroporation technique or left un-transfected (-). GFP expression was analyzed by flow cytometry 24 hrs later. Dot plots show viable cells (A). The expression of IDO-specific mRNA by the different cell types was analyzed by RT-PCR up to 72 hrs later. GAPDH cDNA levels were used to control equal gel loading (B). Cells were stained with 7-AAD and analyzed by flow cytometry to evaluate viability up to 72 hrs later (C).

We found GFP expression in all cell lines which were transfected with GFP 24 hrs later (Figure 19A). While approximately 50% of the murine J558 and the human Phoenix cells were GFP positive, up to 80% of the murine Raw264.7 and human

HeLa cells expressed GFP. Un-transfected cells never expressed GFP. IDO/GFP co-transfection did not alter the percentage of GFP positive cells. IDO mRNA expression was solely detected in co-transfected cells; irrespective of the time point tested (Figure 19B). Staining with 7AAD to determine viable cells showed that viability was not affected by electroporation treatment 24 to 72 hrs later (Figure 19C).

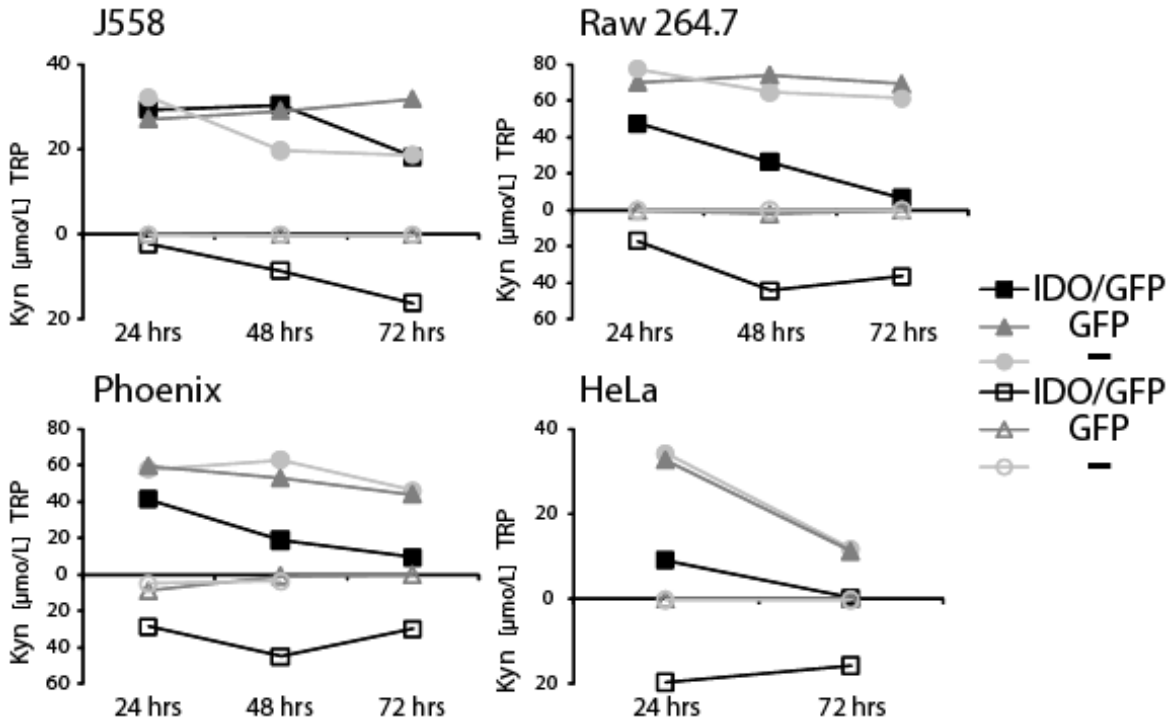


Figure 20: IDO activity in murine and human cell lines.
0.25 x 10^6 cells/ml medium were treated as described in Figure 19. TRP (full symbols) and Kyn (empty symbols) levels in the supernatants were determined by HPLC analysis up to 72 hrs later.

Supernatants of these cell cultures were tested for IDO activity 24 to 72 hrs after treatment (Figure 20). Exclusively co-transfected cell lines showed TRP depletion and Kyn accumulation. Whereas murine J558 cells depleted TRP and accumulated Kyn to a minor extent, complete TRP depletion and massive Kyn accumulation was detected in the other cell lines 72 hrs after treatment. We suggest that TRP depletion without accumulation of Kyn in un-transfected and GFP transfected cell lines was most probably due to nutrient consumption, caused by rapid cell growth.

Electroporation was shown to be an efficient method to transfect a variety of murine and human cell lines. IDO/GFP co-transfection resulted in active IDO expression, indicating that the artificially over-expressed IDO was able to work as the naturally described enzyme.

IDO transfected HSC-derived DCs

To investigate the effect of over-expressed IDO in HSC-derived DCs, cells were transfected with an IDO-encoding vector using the electroporation technique. Day 7 $CD11c^+$ HSC-derived DCs were either transfected with pMax-GFP (GFP), co-transfected with pMax-IDO and pMax-GFP (IDO/GFP) or left un-transfected. Subsequently, these cells were cultured with and without LPS/IFN-γ for 3 hrs and re-cultured in fresh medium for additional 21 hrs. To verify that DC activation and cytokine secretion was independent of electroporation treatment, cells were analyzed for the expression of DC surface markers and viability by flow cytometry, and IL-12p70 secretion by ELISA assay. The same cells were consecutively tested for the expression of IDO mRNA by RT-PCR, of IDO protein by western blot and for IDO activity by HPLC analysis.

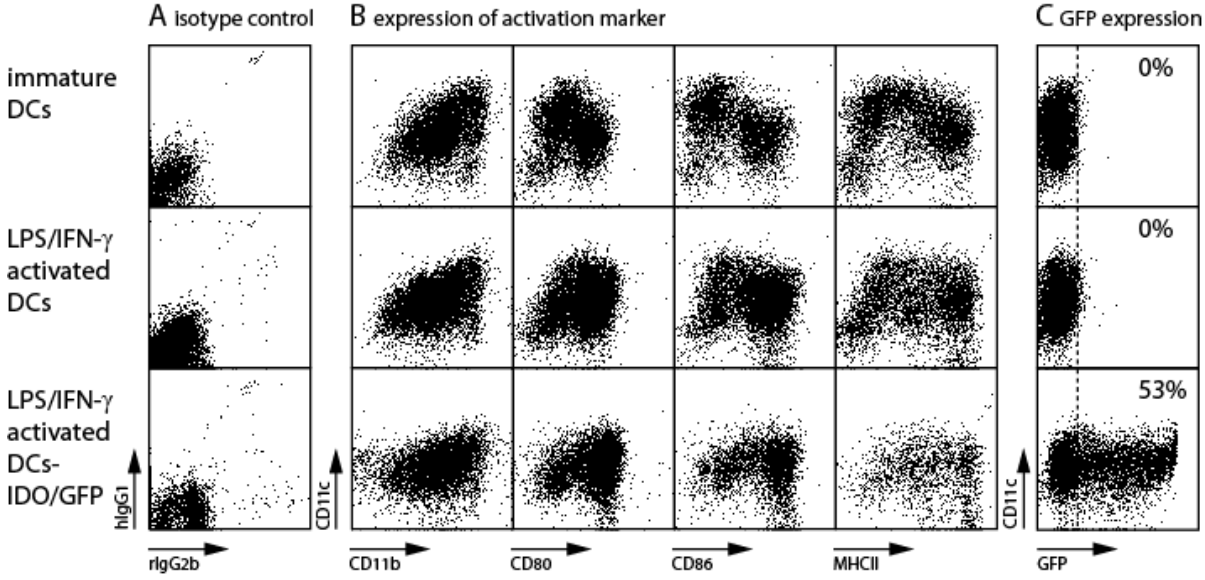


Figure 21: Surface marker expression by transfected and activated HSC-derived DCs.
DCs were tripled stained with either isotype-control mAb (A) or anti-CD80, CD86 or MHC class II mAb in addition to anti-CD11b and CD11c mAb (B). Day 7 $CD11c^+$ HSC-derived DCs were co-transfected with pMax-IDO and pMax-GFP by electroporation technique, activated with LPS/IFN-γ for 3 hrs and subsequently re-cultured in medium alone (LPS/IFN-γ activated DCs-IDO/GFP). Marker (B) and GFP expression (C) was analyzed by flow cytometry 24 hrs later and compared to un-transfected DCs with (LPS/IFN-γ activated DCs) or without (immature DCs) equal activation. The diagram shows viable cells and illustrates one representative experiment out of three.

Flow cytometry was done using activated DCs with (LPS/IFN-γ activated DCs-IDO/GFP) and without IDO/GFP co-transfection (LPS/IFN-γ activated DCs) in comparison to DCs left immature (immature DCs). DCs typically consisted of two cell populations expressing DC surface markers CD80, 86 and MHC class II at a low or

high expression density (Figure 21B). Most activated DCs, independent of the electroporation treatment, expressed the markers to a higher level, whereas most of the immature DCs expressed these markers at a lower level. GFP expression was solely observed in co-transfected DCs with intensity equal to earlier experiments where DCs were transfected with GFP only (Figure 21C and Figure 15). Cell viability was minimally decreased from 92% to 88% upon activation and to 74% upon additional co-transfected (data not shown).

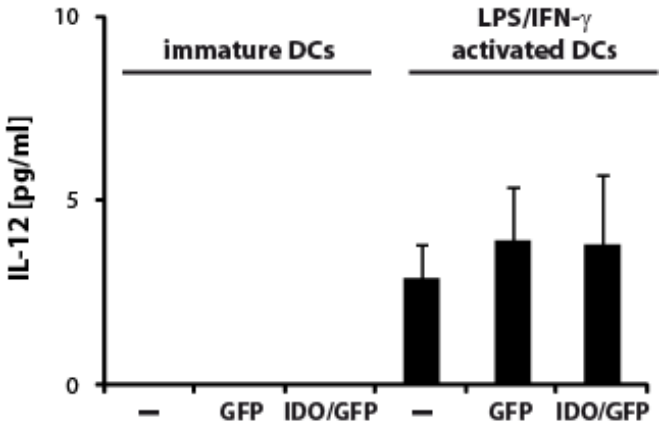


Figure 22: IL-12 secretion by transfected and activated HSC-derived DCs.
0.25 x 10^6 day 7 $CD11c^+$ HSC-derived DCs/ml were either transfected with pMax-GFP (GFP), co-transfected with pMax-IDO and pMax-GFP (IDO/GFP) by electroporation techique or left un-transfected (-). Subsequently, these cells were cultured with and without LPS/IFN-γ for 3 hrs and re-cultured in fresh medium for additional 21 hrs. Accumulated IL-12p70 levels in the supernatants were measured by ELISA assay. The values represent mean±SD of three experiments.

IL-12p70 was solely detected in supernatants of activated DCs, independent of electroporation treatment (Figure 22). Un-transfected, GFP transfected and IDO/GFP co-transfected DCs produced 2.9±0.9 ng/ml, 3.9±1.5 ng/ml, and 3.8±1.9 ng/ml, respectively. DCs without activation stimulus never secreted IL-12p70.

In un-transfected and GFP transfected DCs, IDO mRNA expression was induced upon activation (Figure 23A). Usually, the expression of IDO mRNA in IDO/GFP co-transfected DCs was most prominent and irrespective of cell activation. IDO protein expression was usually detectable in all DC cultures, independent of stimulation or electroporation treatment (Figure 23B). Activation of un-transfected and GFP transfected DCs provoked increased IDO protein expression in comparison to immature cells. IDO protein expression by IDO/GFP co-transfected DCs was always superior to the other DCs, independent of cell activation.

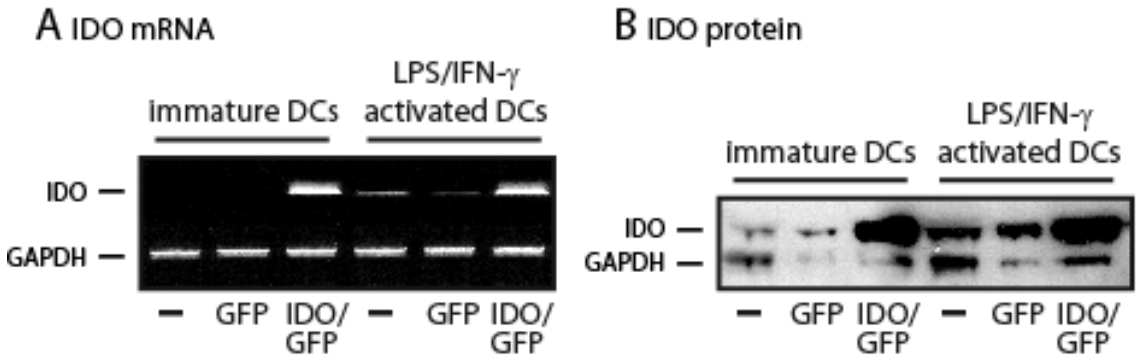


Figure 23: IDO mRNA/protein expression by transfected and activated HSC-derived DCs.
0.25 x 10^6 day 7 $CD11c^+$ HSC-derived DCs/ml medium were treated as described in Figure 22. Expression of IDO- mRNA (A) and proteins (B) was analyzed by RT-PCR and western blotting, respectively. GAPDH cDNA and protein levels were used to control gel loading. The diagram illustrates one representative experiment out of three.

Supernatants of the above tested DC cultures were tested for IDO activity by HPLC analysis. As shown in previous experiments (Figure 13), un-transfected and GFP transfected DCs, irrespective of their activation status, did not express active IDO (Figure 24). Surprisingly, IDO over-expressing DCs, with and without activation stimuli, were also negative for IDO activity, as TRP and Kyn levels in the cultures were comparable to medium alone (data not shown).

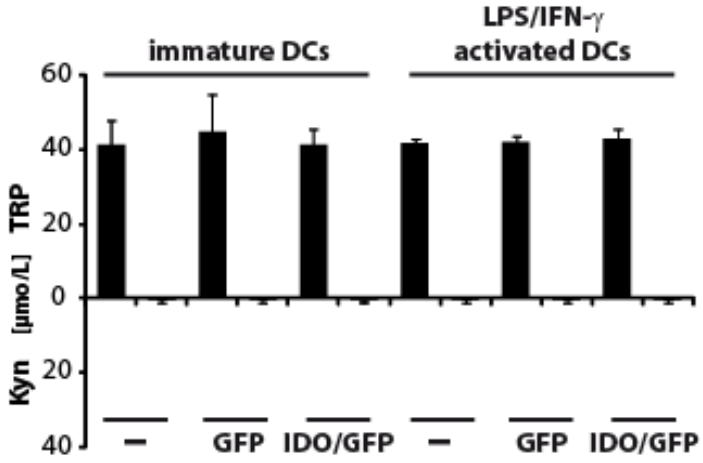


Figure 24: IDO activity in transfected and activated HSC-derived DCs.
0.25 x 10^6 day 7 $CD11c^+$ HSC-derived DCs/ml medium were treated as described in Figure 22. TRP and Kyn levels in the supernatants were determined by HPLC analysis. The values represent mean±SD of three experiments.

IDO transfected HSC-derived DCs stimulate T cell proliferation *in vitro*

Reports suggested that IDO activation in DCs could be induced by certain signals, normally mediated by T cells (Hwu, Du et al. 2000; Mellor, Baban et al. 2003). We questioned whether DCs, co-cultured with T cells, induce IDO activity. Day 7 $CD11c^+$ HSC-derived DCs were either transfected with pMax-GFP (GFP), co-transfected with pMax-IDO and pMax-GFP (IDO/GFP), or left un-transfected. After subsequent stimulation with LPS/IFN-γ and loading with OVA-peptide or SIINFEKL for 3 hrs, cells were co-cultured with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA (CFSE) mixed isomers labeled TCR transgenic OT2 (CD4) or OT1 (CD8) T cells, respectively. Co-cultures were conducted in ratios from 1:1 to $1:10^5$ DCs to T cells. After three days the percentage and total number of proliferating T cells were analyzed by flow cytometry. Additionally, supernatants were analyzed for enzyme activity by HPLC analysis.

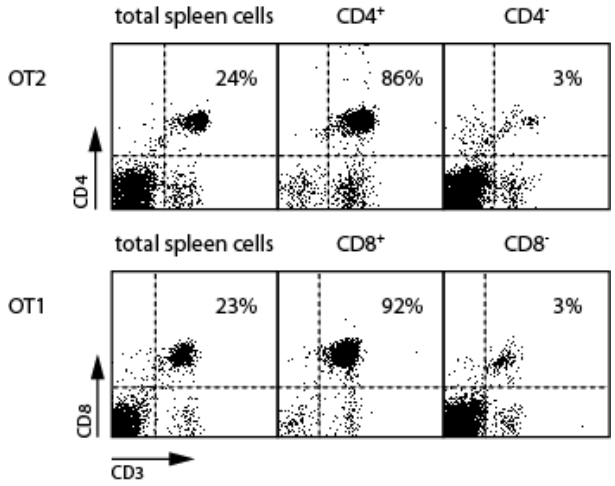


Figure 25: CD4 and CD8 T cell purification.
CD4 and CD8 T cell purification from OT2 and OT1 C57BL/6 mice respectively, was evaluated by flow cytometry analysis. Total spleen cells and the positive ($CD4^+$ and $CD8^+$) and negative ($CD4^-$ and $CD8^-$) MACS sorted cell fractions were stained with anti-CD4 or CD8 mAb in addition to anti-CD3 mAb. The diagram shows analysis of viable cells and illustrates one representative experiment out of three.

Typically, 85% CD4 positive and 90% CD8 positive T cells were obtained with MACS enrichment procedures (Figure 25).

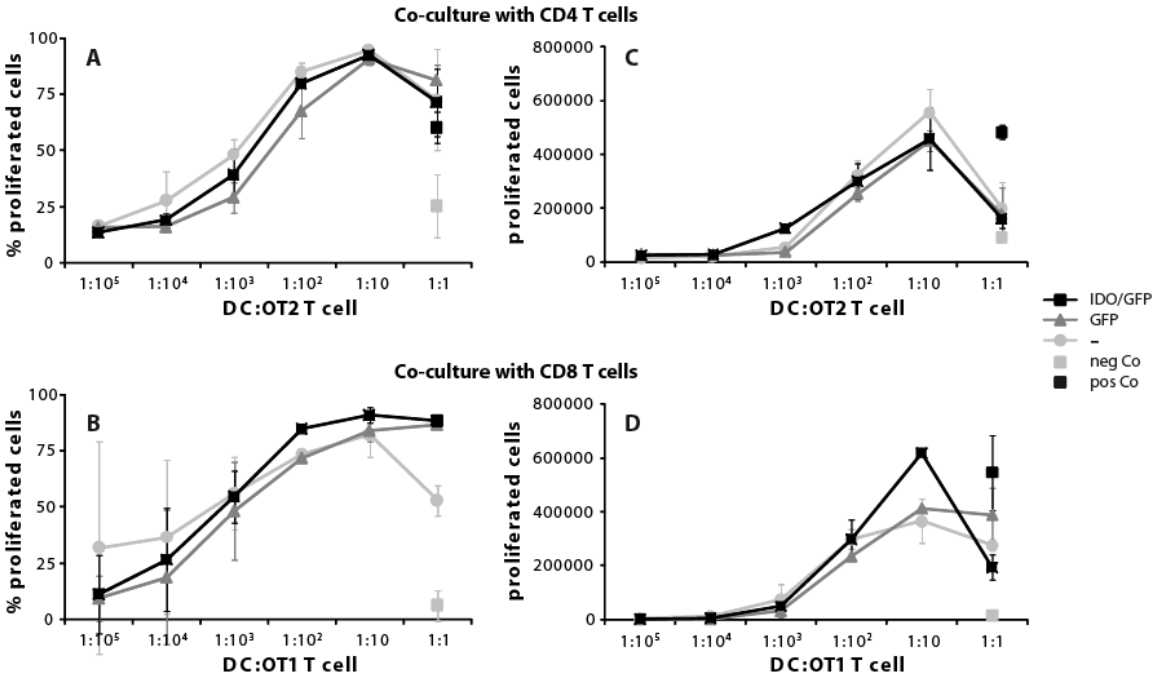


Figure 26: Percentages and total numbers of proliferated CD4 or CD8 T cell.
Day 7 $CD11c^+$ HSC-derived DCs were either transfected with pMax-GFP (GFP), co-transfected with pMax-IDO and pMax-GFP (IDO/GFP) or left un-transfected (-). Subsequently, cells were activated with LPS/IFN-γ and loaded with10 µg/ml OVA-peptide for co-cultures with OT2 T cells (A and C) or with 10 ng/ml SIINFEKL for co-cultures with OT1 T cells (B and D) for 3 hrs. Cells were co-cultured with CFSE labeled T cells at indicated ratios. Control T cells were cultured alone (negative control) or in presence of 1 µg/ml αCD3 mAb (positive control). After 3 days cells were stained with anti-CD4 or anti-CD8 mAb and analyzed by flow cytometry. The diagram shows the percentage (A and B) and the total number (C and D) of proliferated T cells. Proliferated T cells were defined by their loss of green fluorescence due to CFSE dilution compared to maximum green fluorescence in un-proliferated T cells. The values represent mean±SD of three experiments.

In Figure 26A and B, the percentage of proliferated CD4 and CD8 T cells in co-culture settings was analyzed, respectively. The low or un-existing proliferation in cultures with CD4 or CD8 T cells alone (negative control) could be enhanced to 60% or 88%, respectively, upon stimulation with αCD3 mAb (positive control). CD4 T cells proliferated optimal in co-culture with 10^4 and 10^3 DCs. 10^5, 10^4 and 10^3 DCs were optimal to induce proliferation of CD8 T cells. We found a general decreased T cell proliferation with reduced DC numbers. Irrespective of IDO over-expression by DCs, the percentage of proliferating CD4 or CD8 T cells was akin.

TruCount technique enabled the determination of total numbers of proliferated CD4 and CD8 T cells as shown in Figure 26C and D, respectively. However, no significant difference in the total number of proliferated CD4 or CD8 T cells was measured, irrespective of the over-expressed IDO protein by DCs.

Supernatants of the above described co-cultures were tested for IDO activity by HPLC analysis. Analyses showed that co-culture of DCs with CD4 (Figure 27A), as well as with CD8 (Figure 27B) T cells did not induce IDO activity, irrespective of whether IDO was over-expressed or not.

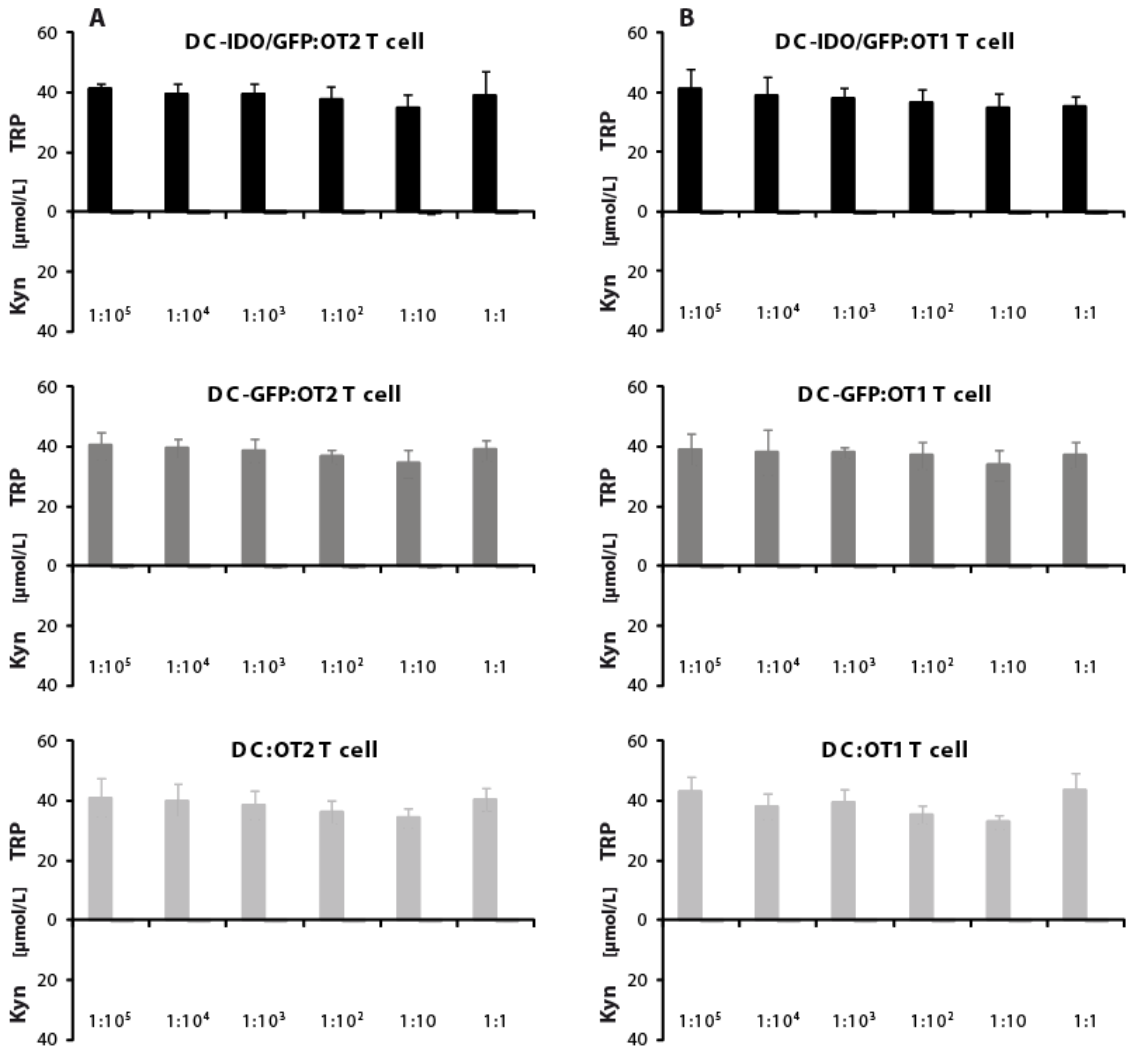
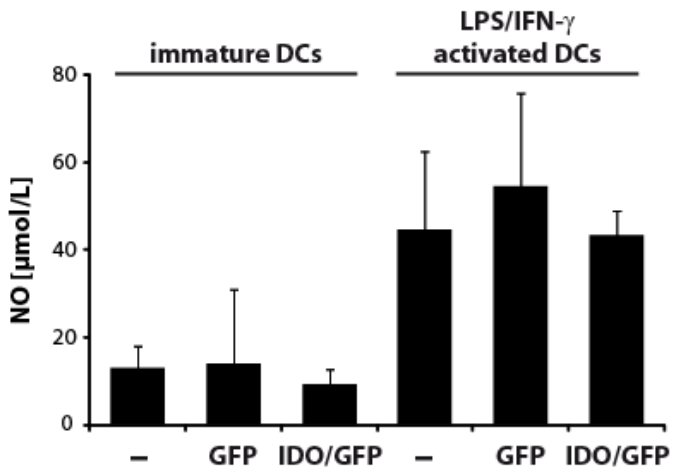


Figure 27: IDO activity in HSC-derived DC co-cultures with CD4 or CD8 T cells.
The supernatants of co-cultures described in Figure 26 with either OT2 (CD4) (A and C) or OT1 (CD8) (B and D) T cells, respectively, were tested for TRP depletion (full symbols) and Kyn accumulation (empty symbols) by HPLC analysis. The values represent mean±SD of three experiments. DC-IDO/GFP, pMax-IDO and pMax-GFP co-transfected DCs; DC-GFP, pMax-GFP transfected DCs; DC, un-transfected DCs.

Enhanced NO production in activated HSC-derived DCs

To investigate, whether NO production inhibits IDO activity (Wood and Sawitzki 2006), we tested supernatants of HSC-derived DC, cultured alone, or in co-cultures with CD4 or CD8 T cells at ratios of 1:1 for accumulated NO. Levels of NO were examined by Griess reaction in generous collaboration with D. Fuchs.



DC	Enhancement of NO after LPS/IFN-γ activation
–	p<0.05
GFP	not significant
IDO/GFP	p<0.001

Figure 28: iNOS activity in HSC-derived DCs.
0.25 x 10^6 day 7 $CD11c^+$ HSC-derived DCs/ml medium were treated as described in Figure 22. To determine iNOS activity, NO levels in the supernatants were measured. The values represent mean±SD of three experiments. -, un-transfected; GFP, pMax-GFP transfected DCs; IDO/GFP, pMax-IDO and pMax-GFP co-transfected DCs.

Un-transfected, GFP transfected, and IDO/GFP co-transfected DCs without further stimulation produced 13.1±4.9 µmol/L, 14.0±17.1 µmol/L and 9.2±3.5 µmol/L NO, respectively (Figure 28). Activation with LPS/IFN-γ usually enhanced NO production. After activation, un-transfected DCs produced 44.6±17.9 µmol/L, GFP transfected produced 54.6±21.2 µmol/L and IDO/GFP co-transfected produced 43.3±5.6 µmol/L NO. Enhancement of NO after activation with LPS/IFN-γ was significant for un-transfected and IDO/GFP co-transfected DCs. GFP transfected DCs had an un-significant tendency for enhanced NO production.

Supernatants of co-cultures additionally provided evidence for the presence iNOS activity (Figure 29). Un-transfected, GFP transfected and IDO/GFP co- transfected DCs produced 38.8±0.2 µmol/L, 23.2±2.1 µmol/L and 19.6±2.9 µmol/L NO in co-cultures with CD4 T cells, respectively, or 55.3±0.8 µmo/L, 18.6±7.2 µmol/L and 17.4±9.3 µmol/L NO in co-cultures with CD8 T cells, respectively. T cells cultured

alone, even after stimulation with αCD3 mAb did not show any iNOS activity, thus confirming that iNOS is solely active in DCs (data not shown).

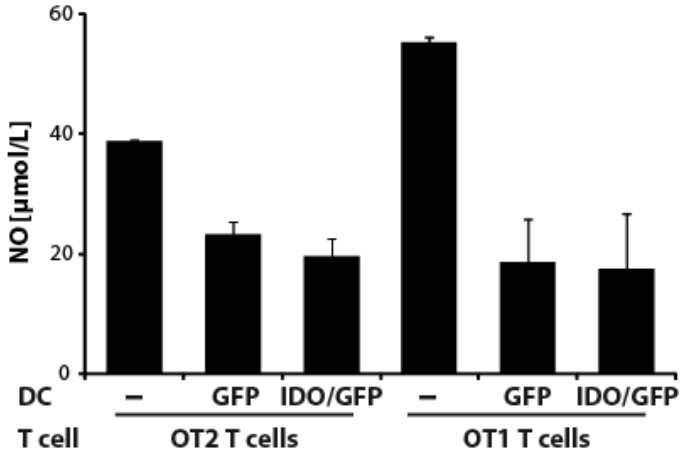


Figure 29: iNOS activity in HSC-derived DC co-cultured with CD4 or CD8 T cells.
The supernatants of co-cultures described in Figure 26 of 1:1 ratios with either OT2 (CD4) (A and C) or OT1 (CD8) (B and D) T cells respectively, were tested for NO accumulation. The values represent mean±SD of three experiments. -, un-transfected DCs; GFP, pMax-GFP transfected DCs; IDO/GFP, pMax-GFP and pMax-IDO co-transfected DCs.

Supernatants of IDO/GFP co-transfected murine and human cell lines that expressed active IDO were tested negative for iNOS activity (Figure 30). J558 cells produced 0.7±0.5 µmol/L, Raw 264.7 cells 2.7±1.8 µmol/L, Phoenix cells 1.8±0.5 µmol/L and HeLa cells 0.1±0.0 µmol/L NO. These experiments provide evidence that iNOS activity is possibly involved in the inhibition of IDO activity. Usually, iNOS activity was solely found in cultures (mainly HSC-derived DCs) that expressed inactive IDO, but not in those (murine and human cell lines) that expressed active IDO.

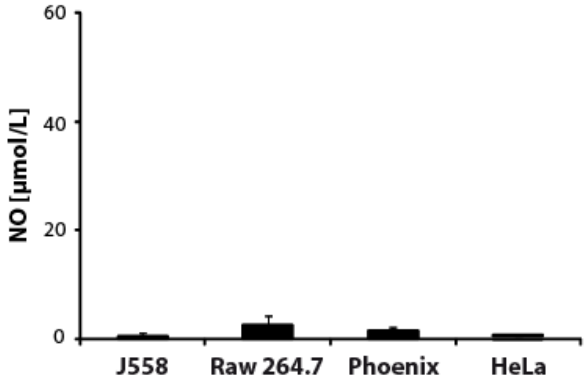


Figure 30: iNOS activity in IDO/GFP co-transfected murine and human cell lines.
0.25 x 10^6 cells/ml medium were co-transfected with pMax-GFP and pMax-IDO. To determine iNOS activity, NO levels in the supernatants were measured. The values represent mean±SD of three experiments.

Discussion

Anti-tumor DC vaccines for cancer immunotherapy are intensively tested for their potential to support conventional chemotherapy (Banchereau and Palucka 2005; Zou 2005; Steinman and Banchereau 2007). However, their modest success might be explained by the increased resistance of malignant cells to immune attacks. Malignant cells may acquire resistance during cancer immunoediting through several tolerogenic mechanisms (Dunn, Bruce et al. 2002; Zhou, Drake et al. 2006). Among them, expression of active IDO by DCs has recently been identified as a potential impediment for successful cancer immunotherapy (Munn and Mellor 2004; Munn and Mellor 2007). We hypothesize that a down-regulation of IDO in DCs used as anti-tumor vaccines may increase their power in promoting tumor immunity (Muller, DuHadaway et al. 2005; Hou, Muller et al. 2007).

The functionality of DCs used in an anti-tumor DC vaccine trial conducted by our group was previously substantiated in prophylactic and therapeutic tumor mouse models (Huttner, Breuer et al. 2005). In this study it was shown that prophylactic immunization with activated HSC-derived DCs of BALB/c mice mediated complete or partial protection of tumor development, depending on the antigen used. Therapeutic immunization with activated HSC-derived DCs of this inbred strain was successful within 12 days after tumor cell challenge. In the current study we used HSC-derived DCs from C57BL/6 mice, because this allowed us to study any T cell inhibition in the syngeneic OT1 and OT2 system. The main purpose was to analyze whether the T cell stimulatory capacity of activated HSC-derived DCs of C57BL/6 is potentially impeded by expression of enzymatically active IDO *in vitro*.

While IDO mRNA and protein expression was readily detectable in various types of activated murine DCs, enzymatic activity was never detected. The causes for this observation may be manifold. We performed the following attempts to explore mechanisms that may prevent the onset of IDO enzymatic activity in the murine system, (i) insufficient DC activation, (ii) unsuitable mouse strain, (iii) insufficient IDO expression, (iv) insufficient IDO protein quantity or (v) inhibition by iNOS activity.

(i) Studies have shown that different types of DCs that constitutively express IDO protein need to be sufficiently activated by certain stimuli to express enzymatically active IDO (Fallarino, Vacca et al. 2002; Grohmann, Orabona et al. 2002; Fallarino, Grohmann et al. 2003). We assume that inactive IDO in our DC cultures is due to insufficient DC activation. In previous studies we were able to show that sufficiently activated human and murine DCs are characterized by up-regulation of cell surface markers such as MHC class II (Signal 1), CD80 and CD86 (Signal 2), as well as by secretion of Th1 polarizing cytokine IL-12 (Signal 3). The concerted activation of all three signals was shown to be important to allow generation of CTL that trigger effective anti-tumor immunity (Felzmann, Huttner et al. 2005; Huttner, Breuer et al. 2005). We also found up-regulation of DC surface markers and secretion of IL-12 in activated HSC-derived, as well as in $CD8^+$ and $CD8^-$ splenic DC-cultures. We conclude that insufficient activation of the different investigated types of DCs can be neglected as a reason for inactive IDO.

(ii) Studies have shown that genetic variances of different inbred strains of mice determine, among others, the outcome of certain infections, e.g. susceptibility to induced colitis (Mahler, Bristol et al. 1998; Houpt, Glembocki et al. 2002; Suzuki, Kohno et al. 2006). We asked whether the genetic background of C57BL/6 mice, which were exclusively used in our study, is appropriate to induce enzymatic activity in IDO protein. The original observation of IDO enzymatic activity to prevent fetus rejection in allogeneic pregnancies by suppressing T cell responses was found in CBA/CaJ mice (Munn, Zhou et al. 1998; Mellor and Munn 2004). IDO activity in DCs enriched from different organs that were activated *in vitro* was mostly found in mouse strains other than C57BL/6 mice, e.g. DBA/2J mice for investigating IDO activity in $CD8^+$ and $CD8^-$ splenic DCs (Fallarino, Vacca et al. 2002; Belladonna, Grohmann et al. 2006). However, basal and induced IDO enzymatic activity was also found in splenic DCs from C57BL/6 mice (Grohmann, Orabona et al. 2002; Orabona, Tomasello et al. 2005). Mostly, C57BL/6 mice were used to investigate IDO activity in DCs enriched from different organs after *in vivo* manipulation. In these studies, IDO activity was found after implantation of tumor cells (Munn, Sharma et al. 2004), elicitation of local inflammation (Muller, Sharma et al. 2008), exposure to CpG oligodeoxynucleotides (Mellor, Baban et al. 2005), or exposure to CLTA-4-Ig (Grohmann, Orabona et al. 2002). Little evidence exists that HSC-derived DCs of C57BL/6 mice are also competent to express enzymatically active IDO (Lee, Jeong

et al. 2007). Subsuming, IDO activity has been demonstrated in various mouse strains. Among them C57BL/6 mice have been used in some of the crucial studies delineating mechanisms of IDO activity. We conclude that C57BL/6 mice are suitable to investigate IDO enzymatic activity in HSC-derived DCs.

(iii) We previously determined sufficient DC activation, featuring one of the factors crucial for IDO enzymatic activity. In addition, sufficient induction or else augmentation of IDO mRNA and protein expression are essential. Studies have shown that in HSC-derived DCs IDO expression needs to be induced (Jung, Lee et al. 2007; Hara, Ogasawara et al. 2008), whereas $CD8^+$ and $CD8^-$ splenic DCs constitutively express IDO (Fallarino, Vacca et al. 2002). As expected, we found IDO mRNA and protein expression in HSC-derived DCs after activation with different stimuli (LPS, R848, PolyIC or CD40 cross-linking) in combination with IFN-γ. $CD8^+$ as well as $CD8^-$ splenic DCs constitutively expressed IDO mRNA, which could be amplified after treatment with IFN-γ.

(iv) Although IDO expression was induced or else enhanced in our DC cultures, it remains unclear whether this level of transcription is sufficient to produce adequate amounts of IDO protein. Attenuated IDO protein expression might lead to enzymatic activity below detection limit. We co- transfected HSC-derived DCs with an IDO- and GFP-encoding vector using electroporation technique and induced GFP expression in more than 50% of DCs 24 hours after treatment. In the same cells we also found significant over-expression of IDO protein. However, even with this abundant protein expression, no evidence for IDO enzymatic activity was found. We conclude that lack of IDO activity is most likely not due to low protein quantities.

(v) In the mammalian system IDO is a highly conserved protein. However, while IDO activity in human monocyte derived DCs activated with LPS and IFN-γ could be routinely detected (Jurgens, Hainz et al. 2009), we never detected IDO enzymatic activity in murine DCs from C57BL/6 mice. Several studies provide evidence that immunological differences are not the exception within human and murine organisms (Mestas and Hughes 2004). While a potential difference in regulating IDO activity still remains unclear, differences in the regulation of iNOS activity associated with the inhibition of IDO activity was shown (Thomas, Mohr et al. 1994). Additional studies provide evidence that stimulated HSC-derived DCs express inactive IDO due to simultaneous induction of iNOS (Hara, Ogasawara et al. 2008). In our study, levels of

NO in HSC-derived DCs that expressed inactive IDO were constitutively present and enhanced after treatment with LPS and IFN-γ. Interestingly, IDO transgenic human (Phoenix and HeLa) and murine cell lines (Raw264.7 and J558) that express active IDO lack iNOS activity. These results might provide evidence for the influence of functional iNOS on the onset of IDO enzymatic activity.

In a single report it has been suggested that HSC-derived DCs of C57BL/6 express enzymatically active IDO after treatment with LPS and IFN-γ. In addition, these cells were associated with IDO-dependent suppression of syngeneic OT1 T cell proliferation *in vitro* (Lee, Jeong et al. 2007). The key characteristics of these murine HSC-derived, active IDO expressing DCs, are described by nonadherence and expression of CD11c. In our study, DCs which were characterized by the same origin and phenotype never expressed enzymatically active IDO. Moreover, in our co-cultures with OT1 or OT2 T cells, vigorous T cell responses were induced which were proportional to the number of OVA-peptide pulsed and LPS and IFN-γ activated HSC-derived DCs. We further suggest that these DCs mediate a pro-inflammatory effect on a per cell basis.

Two parameters are used to determine IDO activity: the reduction of TRP and the augmentation of Kyn concentration. In our study, we measured TRP and Kyn concentrations in cell culture supernatants by HPLC technique, a widespread method to investigate IDO activity. With this technique we can directly draw conclusion from the extent of IDO activity in the cell cultures. In contrast to this method the group of Lee, Jeong et al. 2007 measured the Kyn formation of cellular extracts. Thereby, the cellular extracts are resuspended in an “IDO buffer”, including methylene blue and ascorbate that were previously described as redox factors for IDO activation *in vitro* (Takikawa 2005). Interestingly, a study using HSC-derived DCs from C57BL/6 mice showed that IDO activity could solely be detected by the enzyme assay as described in the study of Lee, Jeong et al. 2007. In contrast, no IDO activity could be detected in the supernatants of IDO expressing DC cultures by HPLC analysis directly (Hara, Ogasawara et al. 2008). However, the authors assume that iNOS activity has solely weak impact on the inhibition of IDO activity, as increased Kyn concentrations were far below those found in other studies (Grohmann, Orabona et al. 2002; Belladonna, Grohmann et al. 2006).

Whether HSC-derived DCs represent a suppressive subset of IDO-producing DCs is of pivotal importance for the regulation of immune responses, particularly for an improved design of anti-tumor DC vaccines. In the current study, we have shown that under experimental conditions used, nonadherent $CD11c^+$ HSC-derived DCs neither express active IDO nor suppress syngeneic T cell response *in vitro*. It appears that the experimental conditions are favorable to generate a strong immuno-stimulatory phenotype in activated HSC-derived DCs. Regulation of IDO activity seems to involve a complex array of molecules that are still poorly understood. Our experiments suggest that iNOS activity may contribute to the inhibition of IDO activity in activated HSC-derived DCs, however, it may not be the only and not the most dominant factor. It would be of crucial importance to evaluate whether other inhibitory mechanisms counteract the otherwise potent T cell stimulatory capacity of HSC-derived DCs.

In summary, our data suggest that apparently murine HSC-derived DCs maintain IDO in an enzymatically inactive form. IDO protein expression without enzymatic activity in murine HSC-derived DCs does obviously not interfere with their potent T cell stimulatory capacity *in vitro*.

Materials and Methods

Cell culture

Mice

C57BL/6, OT-1 TCR transgenic ($OVA^{257-264}$/H-$2K^b$–restricted) and OT-2 TCR transgenic ($OVA^{323-339}$/I-A^b–restricted) mice were bred in the animal facility of the Institute of Pharmacology (Medical University of Vienna, Austria). All mice were maintained under specific pathogen-free conditions following a protocol approved by the Institutional Animal Care and Use Committee and in accordance with the ethical guidelines of the Federal Ministry of Science and Culture. All experiments were conducted with 6–8 week-old female mice.

Media and cell lines

The DC culture medium (subsequently termed normal cell culture medium, NCM) was RPMI 1640 supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential AA and 50 µM 2-mercaptoethanol (all from Invitrogen, Grand Island, N.Y.). If not stated otherwise, NCM was supplemented with 10% (v/v) heat inactivated fetal calf serum (subsequently termed FCS; PAA Laboratories GmbH, Pasching, Austria).

HeLa cells, a human carcinoma cell line, were cultured in RPMI 1640 supplemented with 2 mM L-glutamine and 10% FCS. 293FT cells, a primary embryonal human kidney cell line that stably expresses the SV40 large T antigen, were cultured in D-MEM supplemented with 2 mM L-glutamine, 0.1 mM nonessential aminoacids, 1 mMs sodium pyruvate, 10% FCS and 500 µg/ml geneticin (Invitrogen). Phoenix cells, a cell line based on the 293T cells, were cultured in D-MEM supplemented with 2 mM L-glutamine and 10% FCS. J558, a murine BALB/c myeloma cell line and J558L, a modified J558 cell line transfected with murine CD40L cDNA, were cultured in NCM without streptomycin and penicillin. Raw 264.7, a murine macrophage cell line, were cultured in D-MEM supplemented with 10% FCS.

Opti-MEM® I medium was used for transfection experiments with Lipofectamine™ 2000 (both from Invitrogen).

General incubation conditions and treatment of adherent cells

Cells were cultured at 37°C in a humidified incubator containing 5% CO_2. Centrifugation, if not stated otherwise, was done for 7 minutes at 400 g at 4°C. Adherent cell lines were detached using either Accutase (PAA Laboratories GmbH, Pasching, Austria) or 0.25% Trypsin-EDTA (Invitrogen). Briefly, after washing the adherent cells with 1x phosphate-buffered saline (PBS) (Invitrogen), the reagent was applied for 5 minutes at 37°C. Detached cells were collected, washed twice and re-cultured in the respective medium.

DC subtypes

Generation of HSC-derived DCs

DCs were generated from mouse HSCs according to an established protocol (Inaba, Inaba et al. 1992; Lutz, Kukutsch et al. 1999) with minor modifications. Briefly, femurs and tibias of female C57BL/6 mice were dislodged and remaining tissue and muscles were removed with sterile gauze. The bones were sterilized with 70% (v/v) EtOH in a 60-mm petri dish for 1 minute, air-dried and placed into NCM. Both ends of the bones were cut with sterile scissors, and then the bone marrow was flushed with 10 ml of NCM (Invitrogen, Carlsbad, USA) using a syringe and a 27G¾ needle (BD Microlances). The HSCs were centrifuged and the pellet resuspended in NCM. After counting, the cells were cultured in 12-well plates (Iwaki, Japan) at a concentration of 1×10^6 cells per well in 4 ml NCM in the presence of 5 ng/ml recombinant murine IL4 and 5 ng/ml recombinant murine GM-CSF (BD Pharmingen). Half of the medium (including all supplements) was replaced every second day. This process removed nonadherent granulocytes, whereas clusters of developing DCs remained loosely attached. On day 6, non-adherent and loosely attached DCs were harvested (day 6 HSC-derived DCs). The number of DCs averaged 0.4×10^6 per well stained with anti-CD11c mAb. For some experiments, DCs were further positively selected according to CD11c expression (day 6 $CD11c^+$ HSC-derived DCs) using standard MACS

isolation with a MACS LS separation column using CD11c beads. Usually, >70±4.5% (mean±SD) of the cells expressed CD11c (clone N418, Miltenyi Biotec, Germany). These cells were either activated immediately or re-cultivated in NCM, including cytokines, for 24 hours (day 7 $CD11c^+$ HSC-derived DCs).

Isolation of splenic DCs

Isolation of splenic DCs was done similar to previously described protocols (Steinman, Van Voorhis et al. 1986; Vremec, Zorbas et al. 1992; Grohmann, Belladonna et al. 1998) with minor modifications. Briefly, 8-10 spleens of C57BL/6 mice were cut into small fragments and then digested in 10 ml RPMI with 5% FCS containing collagenase (1 mg/ml; type IV; Worthington, USA) and DNase I (50 µg/ml, Sigma-Aldrich) for 25 minutes at room temperature (RT) with continuous agitation. To disrupt DC-T cell complexes, EDTA (1 ml, 0.1 M, pH 7.2) was added and mixing continued for 5 minutes. For further disruption and removal of undigested stromal fragments, the suspension was rapidly pipetted up and down with 25, 10 and 5 ml pipettes, followed by a filtration through a Nylon cell strainer (70 µm, BD Falcon). The cells were recovered by centrifugation, and then the pellet was immediately re-suspended in 10 ml 1.077 g/cm³ NycoPrep (Axis-Shield PoC AS, Oslo, Norway) and overlaid with 5 ml RPMI containing 5% FCS. Centrifugation was done at 800 g for 25 minutes at RT. All remaining procedures were performed at 0-4°C. Low-density cells were collected and washed twice in PBS with 5% FCS and 5 mmol/L EDTA. The recovered cells were further enriched using magnetic microbeads ($CD8^+$ Dendritic Cell Isolation Kit, MACS, Miltenyi Biotec, Germany) to deplete T cells, NK cells, and B cells with a cocktail of biotin-conjugated Abs against CD90 (Thy1.2), CD49b (DX5), and CD45R (B220) as well as Anti-Biotin MicroBeads. The negative fraction containing $CD11c^+$ splenic DCs was eluted on a LD column, washed and resuspended with RPMI containing 10% FCS and allowed to adhere in 60-mm dish for 2 hrs, followed by an additional incubation to allow DCs to detach at 37°C for 18 hrs. The recovered cells were routinely 60±3.2% (mean±SD) positive for $CD11c^+$ expression. Subsequently, splenic DCs were positively selected according to CD8 expression using CD8α (Ly-2) MicroBeads and positive selection columns.

Activation of HSC-derived and splenic DCs

For activation, cells were incubated with different reagents for 24 hrs. Day 6 $CD11c^+$ HSC-derived DCs were exposed to 500 ng/ml LPS from *E. coli* O111:B4 (Calbiochem). Alternatively, 2 µg/ml PolyIC (Sigma-Aldrich, Germany) or 2.5 µg/ml R848 (Axxora Platform) was used for the induction of activation. To deliver a CD40/CD40L-mediated activation signal, HSC-derived DCs were co-cultured with J558L (Lane, Burdet et al. 1995); a kind gift from Dr. H. Strobl) at a ratio of 2 x 10^6 HSC-derived DCs/1 x 10^6 J558L. Alternatively, mAbs directed against CD40 were used to trigger CD40 cross-linking as described previously by (Ridge, Di Rosa et al. 1998; Bianchi, Grohmann et al. 1999) with minor modifications. Briefly, HSC-derived DCs were incubated on ice, for 20 minutes with hamster anti-mouse CD40 mAb (5 µg/ml, clone HM40-3, BD Pharmingen), and then overnight at 37°C with polyclonal goat anti-hamster IgG (H+L) (3.3 µg/ml, Pierce Biotechnology) in NCM including cytokines. Activation signals, if not stated otherwise, were always applied in combination with 10 ng/ml IFN-γ (R&D Systems, UK). The $CD8\alpha^+$ and $CD8\alpha^-$ splenic DCs were activated with l0 ng/ml IFN-γ alone for 24 hrs.

Transfection methods for HSC-derived DCs

Construction of recombinant plasmids

For cloning IDO and GFP, primers were designed using available mouse sequences from databases (MWG Biotech AG), and RT-PCR was used for amplification from cDNA-templates. PCR-fragments of expected sizes were purified from 1% agarose-gels using the "GFXTM PCR" DNA and Gel BandPurification Kit (Amersham Biosciences) and ligated into different expression vectors using T4-ligase (Fermentas). Chemically competent *E. coli* (Top 10F`) were transformed with the ligation mixture and plated on LB-IPTG/X-Gal (1.6 mg each, Blue-White Select Screening Reagent, Sigma) including 100 µg/ml Ampicillin or Kanamycin to select for transformants and to enable blue/white-screening. Plasmid DNA was prepared from white colonies, digested with appropriate restriction endonucleases (BioLabs Inc. New England) and checked for inserts with correct sizes. All cloned fragments were sequenced to confirm insert identity. For expression of the different recombinant plasmids, TOP 10F' *E.coli* were transformed with the relevant plasmid, a colony was picked and cells were grown over night in 6 ml LB-medium including the appropriate antibiotic. Plasmids were eluted from the overnight culture with the Fast Plasmid® Mini Kit (Eppendorf) or with the EndoFree® Plasmid Maxi Kit (Qiagen) in H_2O or TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) and either used immediately or stored at -20°C.

Primer sequences

pLenti6/V5-primer

5`IDO Topo	5`-CAC CAT GGC ACT CAG TAA AAT ATC TCC TAC-3`
3`IDO Topo	5`-TTG CTA CAC TAA GGC CAA CTC AGA AGA-3`
5`GFP Topo	5`-CAC CCA CTT TGC CTT TCT CTC CAC AGG-3`
3`GFP Topo	5`-TGC ATT CTA GTT GTG GTT TGT CCA-3`

pMax-primer

5`IDO KpnI	5`-CCG GTA CCA TGG CAC TCA GTA AAA TAT CTC CTA CAG-3`
3`IDO XhoI	5`-CCC TCG AGC TAA GGC CAA CTC AGA AGA GCT TT-3`

Plasmids

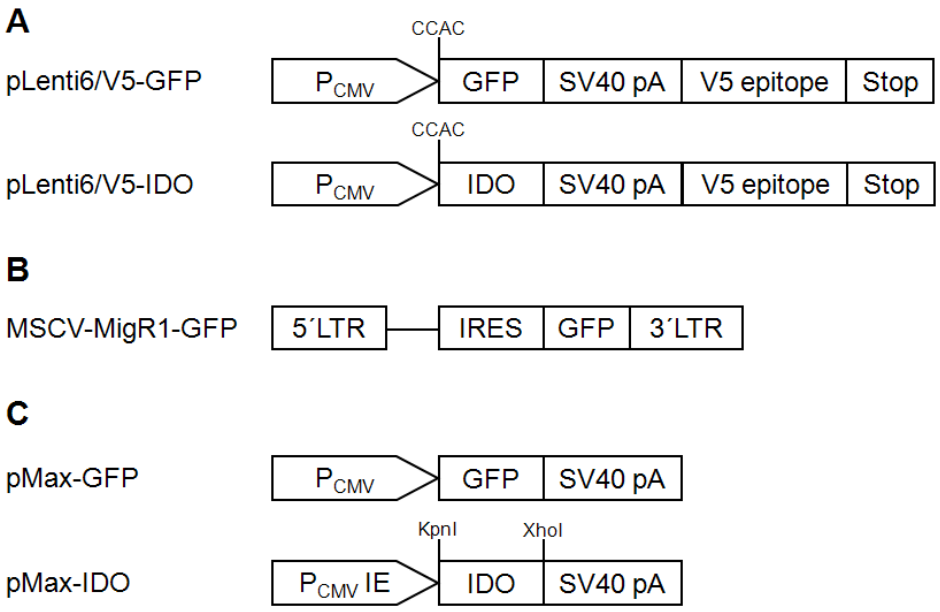


Figure 31: Schematic representation of the different vectors.
The lentiviral vectors expressed GFP or IDO under the control of the CMV promoter (A). In the retroviral bicistronic vector the GFP was linked to the IRES sequence (B). The vectors for lipofectamine transfection and electroporation expressed GFP and IDO under the control of the CMV promoter (C). P_{CMV}, promoter of the cytomegalovirus; SV40pA, polyadenylation signal; IRES, internal ribosomal entry site of the encephalomyocarditis virus; P_{CMV} IE, immediate early promoter of the cytomegalovirus

For the generation of pLenti6/V5-constructs (Invitrogen), the IDO and GFP sequences were amplified from 24 hrs LPS/IFN-γ activated HSC-derived DCs using the 5´ and 3´IDO Topo primers and from the pMax-GFP construct (Amaxa AG) using the 5´ and 3´GFP Topo primers, respectively. All amplified fragments were cloned directly into the pLenti6/V5 vector using the TOPO® Cloning technology for insertion of blunt-end PCR products.

MSC-MigR1-GFP (provided by Dr. W. Ellmeier) contains an internal ribosome entry site allowing translation of two open reading frames from one mRNA and a GFP gene for testing transfection efficiency.

pMax-GFP was provided with the Mouse Dendritic Cell Nucleofector® Kit (Amaxa AG) and used as a positive control for transfection efficiency with different cell types. For the generation of the pMax-IDO construct, the IDO sequence was amplified from 24 hrs LPS/IFN-γ activated HSC-derived DCs using primers including a KpnI restriction site at the 5´end and a XhoI restriction site at the 3´end (5´mIDO KpnI and 3´mIDO XhoI primers). The fragment was cloned into pCR®2.1 using the TA Cloning® method (Invitrogen) which uses a PCR product with adenosine triphosphate at the

3´ends that can be cloned into a linearized vector with complementary 3´T overhangs. After identification of the sequence identity the gene was extracted from pCR®2.1 by KpnI and XhoI restriction enzymes (NEW ENGLAND BioLabs) and ligated into the expression vector pMax-Cloning (Amaxa) that was linearized with the same enzymes.

Lipofectamine transfection

HSCs or HeLa cells were transfected with pMax-GFP (GFP) using Lipofectamine™ 2000 according to the manufacturers' recommendations (Figure 31C). In detail, for transfection of HSC-derived DCs 1.6 µg plasmid DNA and 4 µl Lipofectamine™ 2000 were diluted in 100 µl Opti-MEM® respectively. For HeLa cells 4 µg plasmid DNA and 10 µl Lipofectamine™ 2000 were diluted in 250 µl Opti-MEM®, respectively. After 5 minutes incubation at RT both preparations were combined and incubated for additional 25 minutes. These lipofectamine transfection solutions were added to 1 x 10^6 stem cells in 500 µl NCM supplemented with IL4 and GM-CSF cultured in 12-well plates or to 1 x 10^6 HeLa in 2 ml medium cultured in 6-well plates. Incubation was conducted for different time periods.

Lentiviral transfection

Production of lentiviral particles

Lentiviral particles were prepared using the ViraPower™ Lentiviral Expression Systems (Invitrogen), according to the manufacturers' manual. Briefly, the 293FT cells were cultured avoiding overgrow and early cell passages (< cell passage 6) were taken for production. Replication-defective lentiviral particles pseudotyped with the VSV-G envelope were produced by a four plasmid transient transfection of 293FT cells with 5.7 µg of pLP1, 5.7 µg of pLP2, 7.5 µg of VSVG and 5.7 µg of pLenti6/V5-D-Topo containing the gene of interest under the control of the CMV promoter (pLenti6/V5-IDO or pLenti6/V5-GFP; Figure 31A). The four plasmids and 68.4 µl Lipofectamine™ 2000 were diluted in 2.85 ml Opti-MEM®, respectively. After 5 minutes incubation at RT both preparations were combined and incubated for additional 25 minutes. While DNA-lipid complexes were formed, 293FT cells were

trypsinized and 12 x 10^6 293FT cells were re-plated in 19 ml Opti-MEM with 10% FCS using a 150 cm² flask (Iwaki, Japan). After the addition of the DNA-lipid complexes the cells were incubated at 37°C for 6 hrs. Then the medium was carefully removed and replaced by 30 ml D-MEM fully supplemented, without geneticin. The supernatant containing the viral particles was harvested 72 hrs after transfection and sterile filtrated through a 45 µm filter to remove contaminating 293FT cells. Supernatants were concentrated by ultracentrifugation (28.000 rpm, 14°C, 90 minutes), resuspended in 300 µl NCM including cytokines (100 times concentrated) and either used immediately to transfect HSC-derived DCs or stored frozen at -80°C until use.

Titering the lentiviral stock solution

In order to obtain the transducing unit of each lentiviral stock, the viral titer was determined. On day 0, 2 x 10^5 HeLa cells were plated in 4 ml RPMI with 2 mM L-glutamine and 10% FCS in 6 well plates. On day 1, dilutions from 10^{-2} up to 10^{-7} of the lentiviral stock and 6 µg/ml Polybrene were applied onto the cells in a total volume of 1 ml medium. On day 2, the culture supernatant was replaced by 2 ml fully supplemented NCM. Transfected Hela cells were selected by replacing NCM supplemented with 3 µg/ml Blasticidin (Invitrogen) every second day. Approximately 14-16 days after transfection no viable cells were left in mock transfected cultures. Then the colonies of lentiviral transfected cells were stained by crystal violet and counted.

Transfection of HSC-derived DCs

Transfection of day 6 HSC-derived DCs was performed in 48-well plates at a multiplicity of infection of one. 1 x 10^6 DCs were centrifuged and cultured in 100 µl NCM containing viral supernatant and supplemented with 5 ng/ml IL4, 5 ng/ml GM-CSF and 8 µg/ml Polybrene for 18 hrs. Then the transfection medium was replaced by NCM including cytokines and incubation continued for different periods of time.

Retroviral transfection of HSCs and differentiation into DCs

Retroviral transfection was done according to an established protocol (Specht, Wang et al. 1997) with minor modifications. Briefly, 24 hrs previous to transfection, 1 x 10^6

Phoenix-Eco retrovirus packaging cells (kind gift from Dr. W. Ellmeier) were cultured in 6 well plates in 2 ml medium. The cell line transiently expresses the retroviral gag, pol and env proteins for ecotropic retroviruses. Sub-confluent Phoenix cells were transfected with MSCV-MigR1-GFP retroviral vector (Figure 31B), using Lipofectamine™ 2000 reagent, according to manufacturers' recommendations. Briefly, 4 µg retroviral vector and 10 µl Lipofectamine™ 2000 were diluted in 250 µl Opti-MEM®, respectively. After 5 minutes incubation at RT both preparations were combined and incubated for additional 25 minutes. This lipofectamine transfection solution was added to Phoenix cells in 2 ml medium for 24 hrs before irradiation (30 Gy). Then, 2 x 10^6 cells were re-cultured in 2 ml medium in 6 well plates for additional 24 hrs. Freshly prepared HSCs (7.5 x 10^5 cells/ml) were added to the irradiated Phoenix cells (1.5 x 10^6 cells/well) and cultured with 2 ml NCM in the presence of 5 ng/ml IL4, 5 ng/ml GM-CSF and 8 µg/ml Polybrene (Sigma). On day 2, co-cultures were terminated upon removal of target cells by gentle swirling and pipetting to avoid detachment of the viral packaging cell line. DCs were re-plated at a concentration of 7.5 x 10^5 cells/ml in 1 ml NCM including cytokines in 24 well plates. On day 3, one ml of NCM including cytokines was added to each well. On day 6, HSC-derived DCs were harvested by gentle pipetting and analyzed.

Electroporation

1 x 10^6 of day 7 $CD11c^+$ HSC-derived DCs or 1 x 10^6 cells of each cell line were resuspended in 100 µl Mouse DC Nucleofector® Solution (Mouse Dendritic Cell Nucleofector® Kit, Amaxa AG) containing either 2 µg pMax-GFP (GFP) or a combination of 1 µg pMax-GFP and 1 µg pMax-IDO vector (IDO/GFP) (Figure 31C). DCs were transfected with the Y-001 Nucleofector® program on the Nucleofector™ and then resuspended in 800 µl pre-warmed NCM including 5 ng/ml IL4 and 5 ng/ml GM-CSF and transferred into 24 well plates. The different cell lines were transfected with the appropriate program and then resuspended in the respective medium and transferred into 6 well plates. At different time points after treatment cells and supernatant were analyzed.

Analyzing methods

Flow cytometry

Cells were centrifuged and surface staining was performed by blocking Fcγ receptors with anti-CD16/CD32 (mouse, clone 2.4G2, BD Pharmingen) mAb followed by staining with fluorochrome- or biotin- conjugated mAbs (CD11b-PE (clone M1/70), CD11c-APC (clone HL3), CD80/B7-1-Biotin (clone 16-10 A1), CD86/B7-2-Biotin (clone GL1), MHC class II/I-A/I-E-FITC (clone 2G9), CD40-FITC (clone 3/23), CD8α/Ly-2-PerCP (clone 53-6.7); all obtained from BD Pharmingen, San Diego, CA) using 1 x 10^5 cells per staining. CD4 or CD8 T cells subsets were stained with a cocktail including CD3ε-FITC (clone 145-2C11), CD4/L3T4-PerCP (clone RM4-5) or CD8α/Ly-2-PerCP (clone 53-6.7). Cells were stained with labeled mAb for 30 minutes at 4°C. After washing with PBS, Streptavidin PerCP was added to cells labeled with Biotin conjugated mAbs for 15 minutes at 4°C. After an additional washing step with PBS, the supernatant was discarded and the cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The viability of the cells was measured by addition of 7-AAD (BD, Pharmingen) shortly before measurement. For exact cell numbers the Trucount system (BD Bioscience) was used. Appropriate isotype control Abs were included in the analyses. Data analyses were performed with CellQuest software (Becton Dickinson).

IL-12 ELISA

Levels of IL-12 p40/p35 (IL-12p70) heterodimer were determined in the supernatants of DC cultures by ELISA (OptEIA, BD Biosciences). ELISA plates were coated with anti-mouse IL-12 (p70) mAb (BD Biosciences) in PBS overnight at 4°C. On the following day, the plates were washed with PBS and incubated with 250 µl blocking solution (2% bovine serum albumin (BSA) in PBS) at RT for 3 hrs. After washing the plate, standards and samples were added at the appropriate dilution and incubated at RT overnight. After an additional washing step, the biotinylated anti-mouse IL-12 (p70) mAb (BD Biosciences) was added in PBS with 1% BSA and incubated at RT for 4 hrs. After the next washing step, incubation with streptavidin-alkaline phosphatase in PBS with 1% BSA (Chemicon, Temecula, CA, USA) at RT followed.

After 1 hr ,the solution was replaced by 100 µl phosphatase substrate (Sigma-Aldrich) in diethanolamine buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, 120 mM HCl, pH 9.8), the plates were incubated in the dark at RT for 10 minutes and then measured with an ELISA plate reader (Anthos, Salzburg, Austria) at 405 nm using 690 nm as reference wavelength. The cytokine content was calculated using recombinant standards.

Protein expression studies by western blot analysis

For the preparation of protein extracts from different cell cultures, cells were harvested, washed twice with 1x PBS and lysed by addition of 50 µl per 1 x 10^6 cells radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche) for 10 minutes at 4°C. After centrifugation, 4x NuPAGE LDS Sample Buffer was added to the supernatant containing the whole cell lysate and proteins were denaturated for 5 minutes at 95°C. After cooling on ice, the samples were either used immediately for analyses or stored at -80°C for extended periods. Marker (BenchMark™ Pre-Stained Protein Ladder (6-180 kDa); Invitrogen) and protein samples were fractionated on a NuPAGE 4-12% Bis-Tris gel (Xcell SureLock Mini-Cell; Invitrogen, Carlsbad, CA) for 90 minutes with 170 Volt. To visualize proteins separated by gel electrophoresis, the gels could be stained with Coomassie staining solution for 1 hr up to overnight, and destained with destain solution (5% methanol, 7% acetic acid, 88% A.d.) until sufficient contrast was reached to detect the bands of interest. Proteins on gels used for immunoblot analysis were transferred to a polyvinylidene difluoride membrane (Immobilon Transfer Membrane, 0.45 µm, Millipore) by electroblotting for 60 minutes with 30 Volt. In order to control the efficiency of blotting, and for quantifying the amount of protein in a given lane, membranes were reversibly stained with 1x PonceauS Red (Sigma). After destaining with A.d., membranes were used for immunodetection. The membrane was immersed in 1x blocking reagent (Roche Diagnostics GmbH, Germany) at RT for 1 hr. Incubation with the appropriate dilution of primary Abs (diluted in 0.5% blocking reagent) overnight at 4°C was followed by washing three times for 5 minutes with 0.05% PBS-tween. The horseradish peroxidase (HRP) conjugated secondary Abs were applied (diluted in 0.5% blocking

reagent) at RT for 1 hr and membranes were washed afterwards as described previously. Immobilized proteins were visualized by chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrate; Pierce), according to the manufacturers recommendations. Detection was performed by exposing the Saran Wrap wrapped membrane briefly (5 seconds to 15 minutes) to an X-ray film.

Primary Abs

- rabbit anti-mouse IDO polyclonal Ab (1:1000, kindly provided by O. Takikawa)
- mouse anti-mouse GAPDH mAb (1:1.000.000, IgG_1, Ambion)

Secondary Abs

- goat anti-mouse IgG (H+L), HRP conjugated (1:10.000, Pierce)
- goat anti-rabbit IgG (H+L), HRP conjugated (1:10.000, Pierce)

Gene expression studies by RT-PCR

Total mRNA was isolated from cell populations with the RNeasy Mini kit (Qiagen) and stored at -80°C or immediately used for cDNA preparation. First-strand cDNA synthesis was started by heating 2 µg of total mRNA with 2 µg random hexamer $pd(N)_6$ (GE Healthcare) in a total volume of 27.4 µl to 70°C for 5 minutes, followed by a short incubation on ice. After the addition of 40 U Recombinant Rnasin® Ribonuclease Inhibitor, 200 U M-MLV Reverse Transcriptase and 1x M-MLV Reverse Transcriptase Reaction Buffer (Promega) in a total volume of 40 µl, the sample was incubated at 37°C for 1 hr. cDNA preparations were stored at -80°C or immediately used for experiments. Primers (MWG Biotech AG) used for amplification of the murine IDO or of the murine GAPDH as internal control and PCR product sizes are listed in Table 2. Cycling variables on the Gene Amp® PCR System 9700 (Applied Biosystems) were as followed: 10 minutes at 95°C and 35 cycles of 15 seconds at 95°C, 30 seconds at 58°C and 1 minute at 72°C, followed by 7 minutes at 72°C and then 4°C. Usually 10 µl of the preparation was loaded on a 1% agarose gel.

Gene	Sense primer	Antisense primer	Amplicon size (bp)
IDO	CGA CAT AGC TAC CAG TCT GGA GAA AG	GCG AGG TGG AAC TTT CTC ACA GAG	441
GAPDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	452

Table 2: Oligonucleotides and PCR product sizes for the determination of IDO and control gene expression.

Quantification of Kyn, TRP and NO

The IDO enzymatic activity was determined by measuring the levels of TRP and Kyn in the cell culture supernatants in cooperation with D. Fuchs (Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Austria) as previously described (Hainz, Obexer et al. 2005). Briefly, TRP was detected by its natural fluorescence at 286 nm excitation and 366 nm emission wavelengths. 3-nitro-L-tyrosine, used as an internal standard, and Kyn were determined by ultraviolet (UV) absorption at 360 nm. An albumin-based external standard mix was prepared that included 50 µM TRP (Serva, Heidelberg, Germany), 10 µM Kyn (Sigma) and a frozen serum-pool. Upon the addition of 25 µl 2 M trichloroacetic acid (Merck, Darmstadt, Germany), reaction vials were immediately vortexed and centrifuged at 12000 g for 6 minutes at RT to precipitate protein. The concentration of the components was calculated according to peak heights and was compared to 3-nitro-L-tyrosine as a reference standard.

Measuring levels of nitrate and nitrite in the cell culture supernatants are used for indirect evaluation of NO synthesis by iNOS activity. The iNOS activity was determined in cooperation with D. Fuchs (Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Austria) as previously described (Maloney, St Claire Morgan et al. 2000). Briefly, cell supernatant concentrations of total nitrite and nitrate were measured by the Griess reaction: 75 µl A.d. and 125 µl Griess reagent were added to 50 µl of samples and the mixture is incubated for 10 min at RT. A 96-well-reader/photometer was used for determination of the absorption at 560nm.

Proliferation assay

Day 7 $CD11c^+$ HSC-derived DCs were either transfected with pMax-GFP (GFP), co-transfected with pMax-IDO and pMax-GFP (IDO/GFP) or left un-transfected. Subsequently, cells were stimulated with 500 ng/ml LPS and 10 ng/ml IFN-γ and simultaneously either loaded with 10 ng/ml SIINFEKL-peptide ($OVA^{257-264}$/H-$2K^b$) or 10 µg/ml OVA-peptide ($OVA^{323-339}$/I-A^b, genXpress Service). During the 3 hrs incubation period of DCs, CD4 or CD8 T cells were enriched from OT2 and OT1 transgenic mice, respectively. T cells of these mice express a transgenic TCR specific for OVA-$peptide_{323–339}$ in the context of MHC class II H-$2K^b$ (OT2 CD4 T

cells) or OVA-peptide $_{257-264}$ (SIINFEKL), presented by MHC class I H-2K^{b} (OT1 CD8 T cells). Spleens from these TCR transgenic mice were washed in PBS and then filtrated through a nylon cell strainer (70 µm, BD Falcon). Cells were collected, centrifuged and resuspended in 1 ml of a hypotonic buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM, Na_2EDTA, pH 7.2-7.4) to lyse red blood cells. After 5 minutes incubation, 20 ml NCM were applied and cells were centrifuged twice to remove the hypotonic buffer. The OT2/CD4 and OT1/CD8 T cells were further enriched using magnetic microbeads (CD4$^+$ T Cell Isolation Kit for OT2/CD4$^+$ T cells and CD8α$^+$ T Cell Isolation Kit for OT1/CD8$^+$ T cells, MACS, Miltenyi Biotec, Germany) to deplete NK, monocytes, B cells and erythrocytes with a cocktail of biotin-conjugated Abs against CD11b (Mac-1), CD45R (B220), DX5 and Ter-119. Further, CD8 T cells were depleted from CD4 T cell enrichment with a biotin-conjugated Ab against CD8α$^+$ and CD4α T cells from CD8 T cells enrichment with a biotin-conjugated Ab against CD4 (L3T4). Enriched OT-2/OT-1 T cells were labeled with 10 nM CFSE (Invitrogen, Eugene, Oregon, USA) for 5 minutes at 37°C in PBS, washed twice and cultured at a concentration of 1 x 10^5 T cells per well in a 96 well plate (round bottom) in 100 µl NCM. After incubation the differently treated DCs were harvested and the exact cell number of viable DCs was assessed by staining dead cells with 7-AAD using the Trucount system (BD Pharmingen, according to the manufacturers' recommendations). DCs were co-cultured with 10^5 T cells in graded DC-T cell ratios as indicated. Resting T cells served as negative control and CD3 cross-linked T cells (1 µg/ml αCD3 mAb) served as positive control. DCs cultured alone were analyzed in addition 24 hrs later. Proliferation was determined after 3 days when T cells and supernatants were used for different analyses. Proliferating T cells were defined by their loss of green fluorescence due to CFSE dilution compared to maximum green fluorescence in un-proliferated T cells.

Statistical analysis

Data are presented as mean values±standard deviations of at least three separate experiments. The two-tailed Student`s t-test was used to determine statistical significance between two differentially treated cultures. Differences were considered significant if $p \leq 0.05$.

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