Molecular mechanisms involved in induction and function of IDO^+ $CD25^+$ regulatory dendritic cells

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1 Summary

The main focus of this study was the characterization of the molecular mechanisms that are involved in the induction and function of regulatory dendritic cells (DCreg) which can be found in the environment of different tumors as well as in chronic infections. It was shown that co-expression of the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO) and the α-chain of the IL-2 receptor (CD25) is one of the major hallmarks of DCreg, induced either by treatment with the tumor-derived factor PGE_2 in combination with the maturation stimulus TNF or by infection with the Gram-positive bacteria *Listeria monocytogenes*.

Induction of IDO in DCreg is functional and leads to the deprivation of the essential amino acid tryptophan as well as an accumulation of its toxic metabolites, namely kynurenine. CD25 is also secreted in a soluble form (soluble CD25) and acts as a IL-2 scavenger, resulting in reduced amounts of accessible IL-2 for T cells. It was demonstrated that IDO^+ $CD25^+$ DCreg are able to suppress T-cell proliferation and that this inhibition is dependent on the effects of a variety of inhibitory molecules including IDO, CD25, IL-10 and COX-2 which act in concert to mediate the suppressive function of DCreg . Only the simultaneous blockade of all these inhibitory factors could reverse T-cell suppression. Notably, knockdown of IL2RA, the gene encoding CD25, restored the proliferative capacity of T cells co-incubated with DCreg pointing towards an important role of CD25 expression and subsequent IL-2 deprivation in DCreg mediated T-cell suppression.

Furthermore, the responsible receptors and signaling pathways leading to the induction of IDO^+ $CD25^+$ DCreg were investigated. PGE_2 mediated induction of IDO and CD25 was dependent on EP2 and EP4 as well as TNFRI. Similarly, induction of DCreg by infection with Listeria monocytogenes is dependent on TNF, yet both TNF receptors are responsible for induction of the regulatory molecules. The analysis of downstream signaling events revealed that PI3K as well as Jak molecules were necessary for induction of IDO and CD25 while inhibition of Gsk-3 differentially affected IDO and CD25 expression. These results suggest a shared Jak-PI3K signaling pathway but different downstream mediators control the induction of IDO and CD25 in DCreg. The work we have established so far in this regard will form the basis for future research on further dissecting the different signaling components required for the induction of regulatory DC.

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2.1 Figure legend

3 Abbreviations

17-PT-PGE_2	17-phenyl trinor PGE_2
aAPC	artificial antigen-presenting cell
AC	adenylate cyclase
AP-1	activator protein 1
BCA	bicinchoninic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
cIAP1	cellular inhibitor of apoptosis 1
COX	cyclooxygenase
CTLA-4	cytotoxic T-lymphocyte antigen 4
DAG	diacylglycerol
DC	dendritic cell(s)
DD	death-domain
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP	E prostanoid receptor
FITC	fluorescein isothiocyanate
Flt	FMS-like tyrosine kinase
Gsk-3	glycogen synthase kinase 3

HLA	human leukocyte antigen
HRP-Strep	horseradish peroxidase coupled streptavidin
HSP	heat shock protein
IC	inhibitory concentration
ICAM	intercellular adhesion molecule
IDO	indoleamine-2,3-dioxygenase
IFN	interferon
IκB	inhibitor of nuclear factor κ light chain gene enhancer in B cells
IKK	inhibitor of NFκB kinase
IL	interleukin
IL2R	interleukin-2 receptor
ILT	immunoglobulin-like transcript
immDC	immature dendritic cells
IP_3	inositol triphosphate
IRF-1	interferon regulatory factor-1
ITIM	immunoreceptor tyrosine-based inhibitory motif
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
LC	Langerhans cells
LFA	lymphocyte function-associated antigen
L.m.	Listeria monocytogenes
LPS	lipopolysaccaride
LTA	lipoteichoic acid
MIIC	MHC class II rich compartment
MACS	magnetic-activated cell sorting
matDC	mature dendritic cells
mDC	myeloid dendritic cells

MEKK1	MEK kinase
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
mo-DC	monocyte-derived dendritic cells
MyD88	myeloid differentiation primary response gene (88)
NFκB	nuclear factor kappa light chain enhancer of activated B cells
NIK	NFκB inducing kinase
NOD	nucleotide-binding oligomerization domain
Pam_3	Pam_3Cys-Ser-$(Lys)_4$trihydrochlorid (Pam_3CSK4)
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cells
PE	phycoerythrin
PerCP	Peridinin Chlorophyll Protein Complex
PGE_2	prostaglandin E_2
PI	propidium iodide
PI3K	phosphoinosite 3-kinase
PIP_2	phosphatidylinositol 4,5-biphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PK	protein kinase
PLC	phospholipase C
PRR	pattern recognition receptor
RIG-I	retinoic acid inducible gene 1
RIP1	receptor interacting protein 1
RISC	RNA induced silencing complex
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
TBS	Tris buffered saline
TGF-β	transforming growth factor-β
TICAM	TIR-containing adapter molecule
TIR	Toll/interleukin-1 receptor domain
TIRAP/MAL	TIR domain-containing adaptor protein
TLR	toll like receptor
TMB	tetramethylbenzidine
TNF	tumor necrosis factor α
TNFR	TNF receptor
Tr1 cells	T-regulatory 1-like cells
TRADD	TNFR type I-associated death domain protein
TRAF	TNFR associated factor
TRAM	Trif related adapter molecule
Tregs	regulatory T cells
TRIF	TIR domain containing adapter inducing IFN-β
VEGF	vascular endothelial growth factor

4 Introduction

4.1 Balance of inflammation and tolerance in the immune system

It is the major challenge of the immune system to maintain a perfect balance between inflammation and tolerance. Pro-inflammatory factors that initiate immune responses against pathogens or neoplasia and anti-inflammatory factors that limit the inflammatory response and prevent autoimmunity have to be strictly controlled. The predominance of one side would result in pathogen spreading or autoimmune disorders, respectively. To prevent this imbalance, the immune system has evolved complex strategies involving the precise regulation of soluble factors as well as interactions between cells of the innate and adaptive immune system. Interactions between pathogens, antigen-presenting cells (APC) and lymphocytes are critical in this balancing act (Bachmann and Kopf, 2002). Due to their ability to specifically distinguish between self and foreign in the periphery and their unique capacity to activate antigen-specific T cells, APC are the major cellular components of the immune system that connect innate and adaptive immune responses. The innate immune response initiates local or systemic responses, which, unless properly resolved, could result in acute or chronic inflammatory disease. Thus, there are multiple cellular responses that help to initiate events that lead to resolution of the inflammatory response. Many places along the pathways are involved, including factors that reduce receptor function by interfering with ligand binding or assembly of downstream signaling complexes. Other pathways that limit the host response activate degradative pathways that rely on intracellular proteolysis mediated by ubiquitination and targeting to proteasome pathways. When these pathways act in concert, host inflammatory responses are reduced. In other settings, there are dysfunctional responses that lead to human disease (Han and Ulevitch, 2005).

Dendritic cells (DC), macrophages and B cells are APC whereby DC have been repeatedly described to be the most potent APC type. Besides their ability to stimulate an effective T-cell mediated immune response against foreign antigens, DC are also able to transmit T-cell tolerance to self antigens. In the past years, extensive studies on DC function revealed a very complex regulation of stimulatory or tolerogenic mechanisms depending on various exogenous factors.

4.2 Dendritic cells as potent antigen-presenting cells

4.2.1 DC subsets

Dendritic cells were first visualized as Langerhans cells in the skin in 1868. However, until now it has become evident that DC can be divided into several subpopulations according to their phenotype, tissue localization and function. Plasmacytoid and myeloid DC describe the first major division of DC subsets. This subdivision has first been described in humans (Grouard et al., 1997) and was later also extended to the mouse system (Asselin-Paturel et al., 2001; Nakano et al., 2001; O'Keeffe et al., 2002). The two subsets can be phenotypically distinguished by expression of CD123 and CD11c. The key property of $CD123^{high}$ $CD11c^{-}$ plasmacytoid DC (pDC) is the production of type I interferons that block viral replication and the promotion of T_H2 immune reactions (Cella et al., 1999; Jego et al., 2003). In mice, pDC can be distinguished from conventional DC by expression of CD45RA and lower expression of CD11c (O'Keeffe et al., 2002). The $CD123^{low}$ $CD11c^{high}$ myeloid DC (mDC) can be further subdivided into the main subset of BDCA-1^{+} mDC and a smaller subset of BDCA-3^{+} mDC (Dzionek et al., 2000) while pDC express BDCA-4. In humans, the two subpopulations of mDC are the Langerhans´cells (LC) which reside in the skin epithelia and the dermal or interstitial DC (Shortman and Liu, 2002) which are characterized by high or intermediate expression of DEC-205 (CD205) (Henri et al., 2001), respectively, as well as expression of CD11b. Langerhans´cells additionally express high amounts of intracellular langerin (CD207) which induces the formation of a unique endocytic compartment of LC, Birbeck granules (Valladeau et al., 2000). Interstitial DC most probably represent the tissue stadium of blood myeloid DC in humans (Shortman and Liu, 2002).

In mice, conventional blood-derived DC in the spleen and lymph nodes can be separated into three subgroups based on the expression of CD4 and CD8α ($CD4^{+}$, $CD8^{+}$, $CD4^{-}$ $CD8^{-}$) (Vremec et al., 2000; Vremec et al., 1992). Under steady state conditions $CD4^{+}$ and $CD4^{-}$ $CD8^{-}$ DC are found in the marginal zone of the spleen while $CD8^{+}$ DC predominate in the T-cell areas of lymphoid organs (Pulendran et al., 1997; Steinman et al., 1997) and are the main producers of IL-12 (Steinman et al., 1997), an important signal in T-cell priming (Curtsinger et al., 2003).

4.2.2 Development of DC

A small subset of hematopoietic progenitors in the bone marrow that is characterized by expression of CD34 gives rise to all blood cells and DC (Banchereau and Steinman, 1998). Several cytokines contribute to the growth and differentiation of these progenitors while transmembrane proteins on stromal cells like c-Kit ligand and Flt-3 ligand sustain DC progenitors (Young et al., 1995). Granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-3, products of activated T cells and other cells, enhance DC differentiation while macrophage-colony stimulating factor (M-CSF) favours differentiation of progenitors to macrophages. Developing DC precursors are thought to migrate from bone marrow to blood (del Hoyo et al., 2002) and circulate in the blood as immature precursors prior to migration into the peripheral tissues (Robinson et al., 1998) where they supply the interstitial DC that can be observed throughout the non-lymphoid peripheral organs of the body (Banchereau et al., 2000). In tissue, DC reside in an immature state and wait at the portal of pathogen entry like the skin, the airways or the gastrointestinal mucosa.

4.2.3 in vitro DC models

In vitro generated DC are mostly used as model system to study DC biology since both subpopulations of DC represent rare cells in vivo and are therefore difficult to isolate (Van Voorhis et al., 1982). Until 1994, human DC lines were solely generated from $CD34^+$ precursors that were isolated from cord blood (Caux et al., 1992) or bone marrow (Reid et al., 1992) and differentiated using GM-CSF and tumor necrosis factor-α (TNF). Sallusto and Lanzavecchia then established a method to culture DC from peripheral blood which maintain the phenotypic and functional characteristics of immature DC (Sallusto and Lanzavecchia, 1994). Criteria for the acceptance of DC identification were typical morphology, high expression of CD1, MHC class I and II molecules, FCγRII, B7, CD40, ICAM-1, LFA-3 and CD11c as well as a high stimulatory capacity for naïve T cells. Adherent cells were isolated from peripheral blood mononuclear cells (PBMC), depleted of $CD19^+$ B cells and $CD2^+$ T cells and cultured in medium containing GM-CSF and IL-4 instead of TNF. The generated immature DC (immDC) satisfied the above mentioned criteria. They were capable of efficient antigen uptake and could be matured by treatment with TNF which reduced their ability to take up antigen but strongly increased their T-cell stimulatory

capacities. Until now, different methods have been established to enrich $CD14^+$ monocytes from the fraction of PBMC including the above mentioned plastic adherence (Thurner et al., 1999b) and immunomagnetic sorting (Padley et al., 2001) followed by differentiation of immature DC with GM-CSF and IL-4. Especially the presentation of soluble antigens, a feature of immature DC, was considerably improved by stimulation with GM-CSF in combination with IL-4 instead of TNF. These monocyte-derived immature DC (mo-DC) rapidly respond to various maturation stimuli such as TNF (Zhou and Tedder, 1996), TNF and prostaglandin E_2 (PGE_2) (Rieser et al., 1997), lipopolysaccaride (LPS) and poly:IC (Rouas et al., 2004) with upregulation of adhesion and co-stimulatory molecules (Sallusto and Lanzavecchia, 1994; von Bergwelt-Baildon et al., 2006). Mo-DC thus possess many features of primary myeloid blood DC and are even more potent in stimulating T cells (Osugi et al., 2002). Stimulated with monocyte conditioned medium, mo-DC cultured in the presence of GM-CSF and IL-4 are the most potent antigen-presenting cells known (Reddy et al., 1997; Romani et al., 1996). Yet, these results also indicate functional differences between circulating DC *in vivo* and *in vitro* cultured DC which are not yet fully unterstood.

4.2.4 Role of DC in pathogen recognition and antigen uptake

Dendritic cells are specialized in recognizing microbial products of Gram-positive and Gram-negative bacteria, mycobacteria, viruses, yeast and parasites and initiate innate and adaptive immune responses to clear the infection. In most tissues, DC reside in an immature state. Langerhans cells in the epidermis are the most frequent immature DC (immDC) while some immDC can also be isolated from blood, spleen, heart, lung, kidney and the B- and T-cell areas of tonsils (Banchereau and Steinman, 1998; Rao et al., 1993; Robinson et al., 1998). These immature DC lack the expression of co-stimulatory molecules, e.g. CD80, CD86 and CD40, and are therefore unable to stimulate T-cell activation. Immature DC are specialized in the capturing and processing of antigens to form antigen-MHC (major histocompatibility complex) complexes. There are at least three different mechanisms of antigen uptake by immDC. Particles and microbes can be taken up by phagocytosis (Inaba et al., 1993; Moll et al., 1993; Reis e Sousa et al., 1993; Svensson et al., 1997), whereas extracellular fluids are collected by micropinocytosis, e.g. uptake of small vesicles via clathrin-coated pits or by macropinocytosis of large vesicles mediated by membrane

ruffling which is driven by the actin sceleton (Sallusto et al., 1995). These mechanisms also play an important role in the capture of membrane-bound ligands. Receptor-mediated endocytosis is the third possible mechanism of antigen uptake. These receptors evolved to recognize conserved products of microbial pathogens that are not produced by the host (Janeway and Medzhitov, 2002). ImmDC express C-type lectin receptors like the macrophage mannose-receptor (Sallusto et al., 1995) and DEC-205 (Jiang et al., 1995) as well as Fcγ and Fcε receptors. The mannose receptor binds to various types of sugars such as mannose, fucose, N-acetyl-glucosamine as well as hydrophobic molecules and mediates their delivery into the lysosomal compartment by phagocytosis where they are destroyed by lysosomal enzymes (Fraser et al., 1998).

Through expression of pattern-recognition receptors (PRRs), dendritic cells are well equipped to recognize specific patterns of microbial components, so called pathogen associated molecular patterns (PAMPs) that are conserved among pathogens but not found in mammals. Due to the essential function of most PAMPs in the survival of the microorganism, alterations of these patterns are not possible, accounting for their conserved expression. A highly conserved family of transmembrane PRRs is the toll-like receptor family (TLRs). Toll receptors were first identified in drosophila to control the dorso-ventral polarity in the embryonic development (Hashimoto et al., 1988) and the later identified mammalian homologues were termed toll-like receptors. In mammals the family of toll-like receptors (TLRs) consists of more than 13 members (Kawai and Akira, 2005) while until now 10 different TLRs have been identified in humans. Various PAMPs are recognized by TLRs. Peptidoglycan (Schwandner et al., 1999), lipoteichoic acid, lipoarabinomanan, the yeast cell wall component zymosan (Underhill et al., 1999) as well as bacterial lipopeptide and lipoproteins (Aliprantis et al., 1999; Brightbill et al., 1999; Takeuchi et al., 2000) are recognized by TLR2 whereby heteromer formation with TLR1 or TLR6 is required for recognition (Ozinsky et al., 2000; Takeuchi et al., 2001). This consequently increases the repertoire of ligand specificities and accounts for the large number of ligands that are recognized by TLR2. The current model suggests that triacylated and diacylated lipoproteins are recognized by TLR2/TLR1 or TLR2/TLR6 heteromers, respectively. Following reports suggest a more complex art of recognition with the lipid and the N-terminal peptide contributing to the specificity of TLR2 heteromer recognition (Buwitt-Beckmann et al., 2005, 2006). Lipopolysaccharide (LPS), a cell wall component of gram-negative

bacteria, is recognized by TLR4. However, TLR4 alone is not sufficient for LPS recognition but requires a complex of TLR4 with CD14 and MD-2 (da Silva Correia et al., 2001; Lien et al., 2000). The LPS-binding protein (LBP) mediates the transport of LPS in the serum and is thought to transfer LPS monomers to the GPI-linked cell surface protein CD14. The requirement of both proteins, CD14 and MD-2, in LPS recognition has been demonstrated in CD14 deficient mice (Haziot et al., 1996; Moore et al., 2000) and a cell line with mutated MD-2 (Schromm et al., 2001; Shimazu et al., 1999), respectively. In both cases a critical role for cell responsiveness to LPS was demonstrated. TLR5 detects flagellin while double- or single-stranded RNA is recognized by TLR3 or TLR7 and TLR8, respectively. TLR9 recognizes unmethylated CpG motifs present in bacterial DNA (Hemmi et al., 2000) while most of the mammalian genome is methylated. While some TLRs are expressed on the cell surface (TLR1, 2, 4, 5, 6), others can be found in intracellular compartments, e.g. endosomes (TLR3, 7, 8, 9). Each TLR can exhibit a specific response and uses specific signaling molecules to manifest the specific response (Takeda and Akira, 2004). Yet, all TLRs share a common cytoplasmic signaling domain, the toll-interleukin 1 receptor domain (TIR domain) which mediates association between TLRs and the family of TIR-domain containing adaptors, e.g. MyD88. Upon ligand binding, TLRs dimerize and undergo conformational changes, thereby recruiting adaptor molecules to the TIR domain. Four adaptor molecules have been identified so far, including MyD88, TIRAP/MAL, TRIF/TICAM1 (Oshiumi et al., 2003) and TRAM. The selective usage of one of the adaptor molecules contributes to the differential response of the TLRs.

The second class of PRRs are the cytosolic PRRs which include the RIG-I family, IFN-inducible double-stranded RNA dependent protein kinase (PKR) and NOD proteins (Akira et al., 2006; Janeway and Medzhitov, 2002). These recognition systems are differentially activated depending on the pathogen and the cell type thereby initiating different immune responses. Since the TLRs recognize PAMPs either at the cell surface or at lysosomal or endosomal membranes, pathogens that have invaded the cytosol are not recognized by the TLR system but require cytoplasmic PRRs.

4.2.5 Antigen processing by DC

After encountering a pathogen, DC proceed with antigen processing and lose their ability to efficiently capture antigens. In contrast, DC enter the process of maturation, including induction of co-stimulatory activity, antigen processing, increased MHC molecule expression, migration to the lymph node and subsequent priming of naïve T cells (Banchereau and Steinman, 1998).

The inflammatory stimuli that induce DC maturation boost MHC class II synthesis and prolong the half-life of class II molecules (Cella et al., 1997). The antigens are loaded on MHC class II molecules in specialized MHC class II rich compartments (MIICs) that convert to non-lysosomal vesicles that discharge their MHC-peptide complexes to the cell surface. Peptide-MHC-II complexes remain stable on the DC surface for days (Cella et al., 1997; Pierre et al., 1997).

Presentation of antigens on MHC class I molecules is usually restricted to antigens that are present in the cytoplasm, processed by the proteasome and loaded to MHC class I molecules in the ER. Most cells have the capacity to process antigens in the MHC class I pathway while only antigen-presenting cells like DC, B cells and macrophages also process and present antigens on MHC class II molecules. Peptides loaded on MHC-I molecules mostly originate from self-antigens or viruses that have direct access to the cytoplasm. More recently there has been evidence that also exogenous antigens that do not have access to the cytoplasm can be processed into the MHC class I pathway, a mechanism which is referred to as “cross-presentation”. DC can therefore phagocytose apoptotic cell material and present relevant antigens on MHC class I molecules (Albert et al., 1998) and this process also accounts for the presentation of antigens derived from tumor cells (Huang et al., 1994) or transplants. Nevertheless, apoptosis is not required since there is also evidence that live cellular material can be processed in the cross-presentation pathway (Harshyne et al., 2001; Harshyne et al., 2003). Further mechanisms of antigen access to cross-presentation are receptor-mediated capture and internalization of heat shock proteins (HSPs) by DC (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000) as well as uptake of exosomes that are released by many cell types (Stoorvogel et al., 2002) and might be the source of antigens for the cross-presentation pathway (Wolfers et al., 2001; Zitvogel et al., 1998). Besides cellular antigens, also soluble antigens (Pooley et al., 2001; Staerz et al., 1987) and immune

complexes (den Haan and Bevan, 2002) can enter the cross-presentation pathway, the latter far more efficient.

4.2.6 DC migration

The process of DC maturation is initiated within one day after contact with the foreign antigen, stress or danger signals and includes antigen presentation, secretion of pro-inflammatory cytokines like IL-12 and expression of co-stimulatory molecules.
Of course migration of DC from the place of antigen encounter to the T-cell areas of lymphoid tissues like spleen and lymph nodes is required for the initiation of adaptive immunity. DC travel in response to chemokines that stimulate the respective chemokine receptors on the DC surface. Subsequently, the DC enter the blood flow and travel along the chemokine gradient to lymphoid tissues where they leave at high endothelial venules. Antigens that induce strong T-cell responses are also capable of inducing maturation and migration of DC, e.g. inhaled viruses or bacteria mobilize DC to the airway epithelium. Upon maturation, DC secrete large amounts of pro-inflammatory cytokines and chemokines like CCL2, CCL3, CCL4 and CCL5 thereby recruiting further immature DC, monocytes and macrophages to the inflamed tissue (Sallusto et al., 1999). At the same time these DC lose their responsiveness towards the mentioned inflammatory chemokines but upregulate the homing chemokine receptor CCR7 thereby migrating via afferent lymphatic vessels from the site of infection towards CCL19 and CCL21 (Dieu et al., 1998; Sallusto et al., 1998; Sozzani et al., 1998) which are expressed in the T-cell rich areas of the sentinel lymph nodes. CCR7 deficiency in mice demonstrated the mandatory role of CCR7 and its ligands for DC homing to lymphnodes (Forster et al., 1999; Gunn et al., 1999; Ohl et al., 2004). Depending on the localization of the infection, DC from different origins are attracted. While DC along liver sinusoids migrate to celiac lymph nodes, DC from the intestine move to mesenteric lymph nodes.

4.2.7 Antigen presentation on the DC surface

When DC have entered T-cell rich areas of the lymph node, the process of maturation is completed and the mature DC present MHC:peptide complexes as well as costimulatory molecules, CD80 and CD86, on their surface. They now possess the full repertoire which is necessary to activate T cells. Expression of MHC:peptide

complexes is 10-100 fold higher on DC than on other antigen-presenting cells like monocytes and B cells (Inaba et al., 1997). DC were therefore declared as the most potent antigenpresenting cells.

$CD8^+$ and $CD4^+$ T cells express clonally distributed receptors that recognize fragments of antigens (peptides) associated with MHC class I and II molecules, respectively. Specific binding of a T-cell receptor to the MHC:peptide complex presented on the DC surface combined with co-stimulation by binding of CD80 and CD86 to CD28 on T cells is then sufficient to activate the T-cell mediated immune response and to induce clonal expansion of T cells that specifically identify the respective peptide. Furthermore, engagement of CD40 by T cells triggers IL-12 production by DC, which further activates T cells (Guermonprez et al., 2002).

Mature DC also express accessory molecules, e.g. LFA-3, ICAM-1 or CD86 that interact with respective receptors on T cells to enhance adhesion and signaling.

The crosstalk between DC and T cells does not solely affect T-cell function but also increases DC survival (Wong et al., 1997) and induces secretion of cytokines and chemokines by DC (Anderson et al., 1997; Caux et al., 1994).

4.3 Immune regulation by DC

4.3.1 Immunoregulatory DC subsets

In the past it became more and more evident that DC are not only the most potent stimulatory cells for T-cell activation but that they are also capable of suppressing T-cell mediated immune responses (Munn et al., 2002). Dependent on the localization and maturation status, DC can exert various regulatory immune functions including secretion of inhibitory cytokines, suppression of naïve T cells and induction of regulatory T cells. DC can induce T-cell tolerance in central lymphoid organs and in the periphery (Steinman et al., 2003). While self-reactive T cells that recognize self-antigens presented on DC are eliminated in the thymus, DC generate tolerance in the periphery against captured antigens in the absence of maturation signals. Tumors can induce a tolerogenic environment by secretion of soluble factors such as IL-10 (Steinbrink et al., 1997), VEGF or PGE_2 thereby reducing the number of DC in the tumor microenvironment (Fiore et al., 2006; Gabrilovich et al., 1996b; Troy et al., 1998) or altering their maturation status (Gabrilovich et al., 1996a; Pardoll, 2003).

There is increasing evidence that maturation of the DC can no longer be considered as a distinguishing feature of stimulatory versus regulatory DC. In contrast, regulatory DC can be subdivided into immature, semi-mature and full mature DC that each use different mechanisms to induce immune tolerance.

4.3.1.1 Immature regulatory DC

The majority of resident DC in peripheral tissues but also in lymphoid organs like the spleen (Inaba et al., 1994; Leenen et al., 1998) and mesenteric lymph nodes (Henri et al., 2001) has an immature phenotype. In the absence of costimulatory signals, these immDC induce T-cell anergy or apoptosis (Hawiger et al., 2001). This mechanism accounts for the deletion of auto-reactive T cells in the body that recognize self-peptides presented on immDC. ImmDC can maintain peripheral tolerance not only by induction of anergic T cells but also by induction of T cells with regulatory properties or T cells that secrete immunomodulatory cytokines (Mahnke et al., 2002). Jonuleit et al. reported that T-cell priming with immature DC suppressed the proliferation of alloreactive T cells which was associated with constitutive upregulation of CTLA-4, the high-affinity counterregulator of co-stimulatory molecules, on immDC primed T cells. In contrast to mature DC which induced T_H1 polarization of alloreactive T cells, stimulation with immDC induced IL-10 producing T cells with regulatory function (Jonuleit et al., 2000). Similarly, Levings et al. showed that immature DC mediate the induction of IL-10 producing T-regulatory 1-like cells (Tr1) that suppress proliferation and cytokine secretion of autologous $CD4^+$ T cells (Levings et al., 2005). Furthermore, DC with an immature phenotype can be converted by tumor cells to express transforming growth factor β (TGF-β), thereby stimulating the proliferation of $CD4^+CD25^+$ regulatory T cells (Tregs) (Ghiringhelli et al., 2005).

4.3.1.2 Semi-mature regulatory DC

Maturation of DC includes upregulation of MHC class II and costimulatory molecules as well as secretion of proinflammatory cytokines. Dendritic cells that express high numbers of MHC class II and costimulatory molecules but do not secrete inflammatory cytokines like IL-1β, IL-6, TNF and especially IL-12 display tolerogenic rather than stimulatory properties and were therefore referred to as semi-mature DC (Lutz and Schuler, 2002). Furthermore, the study of Akbari et al. showed that IL-10

producing DC with a mature phenotype could initiate $CD4^+$ T-cell unresponsiveness after respiratory exposure to antigen and induce Tr1 cells that also produce high amounts of IL-10 (Akbari et al., 2001). The $IL\text{-}10^+$ $IL\text{-}12^-$ cytokine production profile of DC along with low grade maturation distinguishes semi-mature DC with regulatory properties from full mature stimulatory DC.

4.3.1.3 Mature regulatory DC

Dendritic-cell (DC) maturation has traditionally been viewed as a linear process leading from an immature (tolerogenic) to a mature (immunogenic) phenotype. However, some DC seem to be both mature and tolerogenic. Recently, several publications described that mature DCs can induce $CD4^+$ T-cell tolerance (Mellor and Munn, 2004; Menges et al., 2002; von Bergwelt-Baildon et al., 2006). These data conflict with the bimodal concept of immature versus mature DCs and raised the question of which DC maturation stage induces what type of T-cell tolerance mechanism. It was hypothesized that a specialized subset of mature DC might actively divert T-cell responses towards tolerance (Shortman and Heath, 2001) and indeed, Munn et al. induced DC by treatment with IFN-γ that were characterized by a morphology consistent with mature DC ($CD80^+$ $CD83^+$ $CD86^{high}$ $HLA\text{-}DR^{high}$) but exerted tolerogenic properties by additional expression of IDO (Munn et al., 2002). These mature regulatory DC were effective stimulators of T-cell proliferation if IDO was blocked with the specific inhibitor 1- methyl tryptophan (1-MT) which suggests that these cells could act as competent APC. Menges et al. showed that in contrast to mature tolerogenic DC, mature immunogenic DC produce large amounts of the pro-inflammatory cytokines IL-12, TNF, IL-1 and IL-6 (Lutz and Schuler, 2002; Menges et al., 2002). These results point towards a functional plasticity of mature DCs, allowing them to adopt either suppressive/tolerogenic or activating/immunogenic phenotypes, depending on the signals received (Grohmann, 2003, 2581). Antigen-presenting cells, especially dendritic cells, are most often the focus of studies concerning IDO expression as immunoregulatory property (Mellor and Munn, 2004). Induction of IDO in DC results in inhibition of T-cell proliferation (Hwu et al., 2000). Furthermore, IDO-expressing DC have been recently reported to expand autologous Tregs (Chung et al., 2009; Hill et al., 2007).

4.3.2 Mechanisms of immune regulation

4.3.2.1 Soluble factors

The capacity of DC to orchestrate the immune response is likely not an intrinsic quality of the cell but is rather the result of environmental stimulation which includes the local cytokine milieu. Soluble, e.g. tumor-derived factors like IL-10, TGF-β and PGE_2 can render DC to aquire regulatory instead of stimulatory capacities. IL-10 which is produced by many immune cells like T cells, B cells, macrophages, mast cells but also by tumor cells inhibits maturation of antigen-presenting cells including upregulation of MHC class II and co-stimulatory molecules as well as inflammatory cytokine production (de Waal Malefyt et al., 1991; Fiorentino et al., 1991; Willems et al., 1994). CCR-7 dependent migration of DC to secondary lymphoid tissue is also impaired by IL-10 (Takayama et al., 2001). Furthermore, these IL-10 treated DC induce T-cell anergy (Steinbrink et al., 1997). IL-10 production by tumor cells, e.g. melanoma cells, might therefore cause tolerance induction against tumor tissue by changing phenotypic and functional properties of DC in the tumor microenvironment (Enk et al., 1997). TGF-β is another soluble factor produced in the tumor microenvironment (Fischer et al., 1994; Rodeck et al., 1994) which has been associated with tolerance induction. Similar to IL-10, exposure of DC to TGF-β inhibits their ability to present antigen, stimulate tumor-sensitized T cells and migrate to draining lymph nodes (Kobie et al., 2003). Recently, it has been shown that the tumor-derived factor PGE_2 induces DC-mediated T-cell tolerance. PGE_2 levels are increased in many different solid and hematological tumors, especially in tumors associated with chronic inflammatory responses including colon (Rigas et al., 1993), breast cancer (Benoit et al., 2004; Pockaj et al., 2004) and lymphoma (Chemnitz et al., 2006). These elevated PGE_2 concentrations can directly suppress $CD4^+$ T-cell proliferation by inhibition of the TCR associated tyrosine kinase Lck (Chemnitz et al., 2006). Furthermore, the induction of regulatory T cells has been linked to the effects of PGE_2 (Akasaki et al., 2004; Baratelli et al., 2005; Sharma et al., 2005). In addition to these direct effects on T cells, PGE_2 has also been shown to influence APC, especially DC, by downregulation of IL-12 (Kalinski et al., 2001) and induction of regulatory molecules (von Bergwelt-Baildon et al., 2006) resulting in impaired T-cell stimulatory capacities.

An inhibitory microenvironment which is characterized by the existence of different soluble inhibitory factors, such as TGF-β, VEGF, IL-10 and PGE_2 can therefore direct dendritic cells to aquire regulatory properties.

4.3.2.2 Inhibitory receptors

Dendritic cells express a number of inhibitory receptors which are characterized by the presence of cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Upon receptor engagement, ITIMs are phosphorylated and subsequently activate phosphatases that downregulate the process of cell activation. Recently, two inhibitory receptors, namely ILT3 and ILT4, were associated with the induction of regulatory DC. Upregulation of ILT3 and ILT4 was associated with decreased expression of costimulatory molecules and induction of T-cell anergy (Chang et al., 2002). HLA-G, a non-classical MHC class I molecule, is a natural ligand of inhibitory receptors and was described to induce tolerogenic DC characterized by low-level expression of MHC class II and costimulatory molecules on the cell surface. Additionally, binding of HLA-G reduces the potential of DC to stimulate allogenic T cells but leads to the induction of anergic and immunosuppressive T cells (Ristich et al., 2005). In addition to soluble factors, activation of inhibitory receptors can therefore render DC tolerogenic as well. Furthermore DC can upregulate ligands that bind to inhibitory receptors on T cells, thereby providing inhibitory signals to interacting T cells. For example, DC stimulated with dsRNA or poly(I:C) upregulate PD-L1 and CD86, both ligands for the inhibitory receptor PD-1 on T cells, and thereby suppress proliferation of interacting T cells (Groschel et al., 2008).

4.3.2.3 Deprivation of essential factors

Indoleamine-2,3,dioxygenase (IDO) is the rate-limiting enzyme in the tryptophan catabolism, also known as the kynurenine pathway. It degrades the essential amino acid tryptophan thereby leading to an accumulation of its metabolites, the kynurenine (Grohmann et al., 2003). The amino acid tryptophan is required by all forms of life for protein synthesis and other metabolic functions. Animals must ingest tryptophan in the form of proteins which are then hydrolysed into the constituent amino acids in the digestive system. After digestion, tryptophan can be distributed to the blood stream to be used for protein synthesis or it can be degraded in the liver through the kynurenine pathway. In addition, tryptophan is required for the synthesis of serotonin

in the nervous system and the gut as well as for melatonin synthesis in the pineal gland. Recently, there is increasing interest in the extrahepatic kynurenine metabolism occurring in the immune system rather than in the liver. The first enzyme of the kynurenine pathway, IDO, was first reported to be implicated in the inhibition of viruses (Yoshida et al., 1979) and other intracellular pathogens like Toxoplasma gondii (Pfefferkorn, 1984) and Chlamydia psittaci (Byrne et al., 1986) whose survival is dependent on host tryptophan by starving these pathogens of tryptophan. In these settings, induction of IDO and subsequent tryptophan deprivation were dependent on IFN-γ. More recently, IDO has been shown to not only directly affect the growth of pathogens but to be associated with immune tolerance. There are two theories that might explain how IDO facilitates immune tolerance. First, the dramatic reduction of tryptophan might prevent the supply of T cells with this critical amino acid and second, downstream metabolites of the tryptophan catabolism might exert toxic effects on immune cells, probably by pro-apoptotic mechanisms (Moffett and Namboodiri, 2003) and were shown to block T-cell proliferation (MacKenzie et al., 2007). The intriguing work of Munn and Mellor demonstrated that IDO plays an important role in the prevention of fetal rejection by suppression of T-cell activation at the maternal-fetal interface. Consistent with this idea, inhibition of tryptophan catabolism by blockade of IDO during pregnancy allowed maternal lymphocytes to mediate rejection of the fetus in mice (Munn et al., 1998). In human placenta IDO is expressed by syncytiotrophoblasts (Kamimura et al., 1991) while maternal serum tryptophan levels decrease from the first to the third trimester of pregnancy according to the development and growth of the placenta (Schrocksnadel et al., 1996). T cells on the other hand possess a specific cell-cycle regulatory checkpoint that is sensitive to the level of free tryptophan. Tryptophan depletion therefore has anti-proliferative effects on T cells in the local microenvironment. Since then, numerous reports demonstrated a role of IDO activation and subsequent tryptophan depletion in the regulation of immune responses during infectious processes and also in tumorigenesis (Mellor et al., 2002; Muller et al., 2005; Popov et al., 2006). Various inflammatory mediators can act as potent IDO inducers. Among these, especially IFN-γ and LPS have been shown to induce IDO (Braun et al., 2005; Jung et al., 2007; Ozaki et al., 1988; Yasui et al., 1986; Yoshida and Hayaishi, 1978). Pathogens have evolved mechanisms to evade the host immune attack including induction of IDO in the infected cell. During HIV infection, the serum tryptophan level is markedly

decreased while kynurenine levels are increased (Huengsberg et al., 1998). This mechanism might contribute to the survival of the pathogen through inhibition of the local T-cell response due to the lack of tryptophan in the microenvironment of the infected cell. Starvation of the pathogen is therefore not the only cause of tryptophan deprivation but immune evasion of the pathogen by local suppression of T-cell proliferation might be another function of IDO induction which is in turn more favorable to the pathogen.

Besides the well-defined role of tryptophan deprivation in the maternal immune response during pregnancy, the deprivation of another amino acid, namely arginine, mediated by the enzyme arginase has been attributed to temporary suppression of T-cell proliferation (Kropf et al., 2007). In mice, arginine deprivation mediated by tumor-infiltrating DC contributes to the suppression of protective T-cell-based anti-tumor immunity (Norian et al., 2009).

IL-2 is a cytokine which is primarily produced by activated T cells (Paliard et al., 1988). In contrast to observations made in human myeloid DC that do not produce IL-2 during maturation (Velten et al., 2004; von Bergwelt-Baildon et al., 2006), it was reported by Granucci et al. that murine DC matured by exposure to Gram-negative bacteria could also be the source of IL-2 (Granucci et al., 2001). Although IL-2 mediates multiple biological processes in various cell populations, e.g. proliferation and differentiation of B cells (Jung et al., 1984; Waldmann et al., 1984) and NK cells (Fehniger et al., 2003), T cells remain the major target of IL-2 (Seder and Paul, 1994). IL-2 binds autocrine to the high-affinity IL-2 receptor on the T-cell surface and thereby allows entrance into the cell cycle and clonal expansion of activated T cells (Nelson and Willerford, 1998). CD25 is the α-chain of the IL-2 receptor which is composed of three different subunit complexes, the α- (CD25), β- (CD122) and γ-chains (CD132). The high-affinity IL-2R consists of all three chains and their concomitant expression is indispensable for IL-2 signaling to be executed (Minami et al., 1993). Due to its short cytoplasmic tail, isolated expression of CD25 is not sufficient to trigger the IL-2 signaling pathway (Minami et al., 1993) but might be sufficient to capture IL-2 thereby making it unaccessible for T cells.

4.3.3 IDO$^+$ CD25$^+$ regulatory dendritic cells

In the majority of cases, CD25 expression on DC has been linked to maturation (Bell et al., 2001; Mackensen et al., 1995). DC that concomitantly express CD25, CD83

and co-stimulatory molecules on their cell surface were generally considered as mature stimulatory DC (Velten et al., 2007). However, recently it was demonstrated that CD25 might not be a function of maturation but is rather associated with the induction of a regulatory DC phenotype. Von Bergwelt-Baildon et al. showed that sequestration of IL-2 might be a function of CD25 expressing regulatory DC (von Bergwelt-Baildon et al., 2006). These regulatory DC express the whole set of maturation markers but are additionally characterized by expression of the immune-inhibitory enzyme IDO (Popov and Schultze, 2008) and will further be referred to as IDO^+ $CD25^+$ DCreg.

4.4 Objectives

Regulatory DC have been implicated in the development and maintenance of tumors and chronic infectious diseases by inducing T-cell tolerance and suppressing T-cell mediated immune responses. Yet, the mechanisms how regulatory DC are induced in these settings and how they transmit T-cell unresponsiveness, are not completely understood.

This study was conducted to rule out the molecular mechanisms that are involved in induction and function of regulatory dendritic cells. Two models of regulatory DC will be analyzed, the first model representing DC in the tumor microenvironment which will be induced by combined stimulation with the tumor derived factor PGE_2 and the inflammatory maturation stimulus TNF and the second model representing DC in infections is analyzed by infection of DC with the Gram-positive bacteria *Listeria monocytogenes*. Since there is no molecular marker which allows to distinguish between stimulatory and regulatory DC, first the specific phenotype of PGE_2-treated and listeria-infected DC will be characterized to possibly find hallmarks that allow the identification of DCreg in both models. Furthermore, it is planned to analyze functional effects of DCreg on T cells with a special emphasis on the molecular mechanisms behind T-cell inhibition. In this regard, it will be tried to establish a link between the phenotypic markers and the functional capacity of DCreg. If the phenotypic and functional characteristics of DCreg are revealed, it is planned to focus on the signaling mechanisms which are responsible for the induction of DCreg. First, the inducing soluble factors and their associated receptors will be analyzed in detail, with special regard to TNF and its associated receptors, TNFRI and TNFRII, as well as PGE_2 and its associated receptors EP1 to EP4. Depending on these results,

downstream signaling pathways that are known to be associated with the respective receptors found to be responsible for induction of the regulatory DC phenotype will be further analyzed. In conclusion, the results of this study could help to aquire a deeper knowledge of regulatory DC, especially of the molecular mechanisms behind their induction and function and furthermore provide a tool for the identification of DC with regulatory properties in other than the mentioned disease models.

5 Material and Methods

5.1 Materials

5.1.1 Chemicals and Reagents

1-methyl-DL-tryptophan	Sigma Aldrich, Taufkirchen, DE
17-phenyl trinor PGE2	Cayman Chemical, Ann Arbor, US
BCA protein assay kit	Thermo Scientific, Rockford, US
Boric acid	Merck, Darmstadt, DE
BSA	Sigma, St Louis, US
Butaprost	Cayman Chemical, Ann Arbor, US
CFSE	Sigma-Aldrich, München, DE
Cytofix/Cytoperm kit with GolgiStop	BD Biosciences, Heidelberg, DE
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, München, DE
Dithiothreitol (DTT)	Sigma Aldrich, München, DE
Dynabeads® M-450	Invitrogen LifeTechnologies,Karlsruhe, DE
ECL™ Western blotting detection reagent	GE healthcare, Piscataway, US
Ethylendiamintetraacetat (EDTA)	Sigma, St Louis, US
Ethanol	Roth, Karlsruhe, DE
Ficoll-Paque™ PLUS	GE healthcare, Piscataway, US
Fetal calf serum (FCS)	Invitrogen LifeTechnologies,Karlsruhe, DE
Forskolin	Sigma Aldrich, Taufkirchen, DE
Glacial acetic acid	Roth, Karlsruhe, DE
Gentamycin	Invitrogen Life Technologies, Karlsruhe, DE
Glacial acetic acid	Roth, Karlsruhe, DE
Glutamax	Invitrogen Life Technologies, Karlsruhe, DE
Interleukin 2 (IL-2)	Immunotools, Friesoythe, DE
Interleukin 4 (IL-4)	Immunotools, Friesoythe, DE
L000902688	Merck Frosst, Kirkland, CAN
L-kynurenine	Sigma Aldrich, Taufkirchen, DE
LPS, commercial *E.coli* O55:B5	Sigma Aldrich, Taufkirchen, DE
LPS, ultrapure *E.coli* K12	InvivoGen, San Diego, US

Methanol	Roth, Karlsruhe, DE
MicroBeads $CD14^+$	Miltenyi Biotech, Bergisch Gladbach, DE
Natriumchlorid (NaCl)	Roth, Karlsruhe, DE
NuPAGE® Transferpuffer 20x	Invitrogen Life Technologies, Karlsruhe, DE
Odyssey® Blocking Buffer	Licor Biosciences, Bad Homburg, DE
Odyssey® Two-Color molecular weight marker (10-250 kDa)	Licor Biosciences, Bad Homburg, DE
Optimem Reduced Serum Medium	Invitrogen LifeTechnologies,Karlsruhe, DE
Pam_3Cys-Ser-$(Lys)_4$trihydrochlorid	Alexis Biochemicals, Lausen, CH
PBS	PAA Laboratories GmbH, Pasching, AT
P-dimethylbenzaldehyde	Roth, Karlsruhe, DE
Penicillin	PAA Laboratories GmbH, Pasching, AT
Phosphatase inhibitors cocktail I+II	Sigma Aldrich, Taufkirchen, DE
Propidium Iodide (PI)	Sigma Aldrich, Taufkirchen, DE
Prostaglandin E_2 (PGE_2)	Sigma Aldrich, Taufkirchen, DE
Protease Inhibitor cocktail tablets	Roche Diagnostics, Basel, CH
Rofecoxib	a kind gift of Drs. K. Schrör and J. Meyer-Kirchrath, Institute for Pharmacology and Clinical Pharmacology, University of Düsseldorf, DE
Re-Blot plus mild solution	Chemicon, Temecula, US
RosetteSep $CD4^+$ T cell enrichment kit	Stem Cell Technologies, London, GB
Running buffer 20x	Invitrogen Life Technologies, Karlsruhe, DE
SDS	AppliChem, Darmstadt, DE
Skim milk powder	Heirler Cenovis GmbH, Radolfzell, DE
Sodium chloride (NaCl)	Roth, Karlsruhe, DE
Sodium hydroxide (NaOH) (32 %)	Merck, Darmstadt, DE
Streptomycin	PAA Laboratories GmbH, Pasching, AT
Trichloroacetic acid	Merck, Darmstadt, DE
TRIS (hydroxymethyl)-aminomethane	Roth, Karlsruhe, DE
Triton X-100	Promega Corporation, Madison, US
TRIzol®	Invitrogen Life Technologies,Karlsruhe, DE
Trypanblue	Merck, Darmstadt, DE
Tween 20	Merck, Darmstadt, DE

Trypan blue	Merck, Darmstadt, DE

5.1.2 Antibodies for Western blotting and functional assays

Antigen	Species	Distributor
β-actin	mouse	Chemicon, Temecula, US
CD3	mouse	Janssen-Cilag, Neuss, DE
CD28	mouse	a kind gift of Dr. Carl June, Abramson Cancer Research Center, University of Pennsylvania, Philadelphia
COX-2	rabbit	Sigma Aldrich, Taufkirchen, DE
EP1-EP4	rabbit	Cayman Chemical, Ann Arbor, US
IDO	mouse	a kind gift of Prof. Takikawa, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan
IDO	mouse	Chemicon, Hofheim, DE
IL-10	goat	R&D Systems, Abingdon, UK
MHC-I	mouse	own hybridoma
TNFRI and TNFRII	mouse	R&D Systems, Abingdon, UK

5.1.2.1 Secondary antibodies for Western blotting

anti-mouse IgG, HRP-conjugated	DakoCytomation, Glostrup, DK
anti-mouse IgG, IRDye 680	Licor Biosciences, Bad Homburg, DE
anti-mouse IgG, IRDye 800CW	Licor Biosciences, Bad Homburg, DE
anti-rabbit IgG, IRDye 680	Licor Biosciences, Bad Homburg, DE
anti-rabbit IgG, IRDye 800CW	Licor Biosciences, Bad Homburg, DE

5.1.3 Antibodies for flow cytometry

Antigen	Conjugate	Distributor
CD11c	PE	BD Biosciences, Heidelberg, DE
CD25	PE	BD Biosciences, Heidelberg, DE
CD80	FITC	BD Biosciences, Heidelberg, DE
CD83	FITC	BD Biosciences, Heidelberg, DE
CD86	PE	BD Biosciences, Heidelberg, DE
CD206	FITC	BD Biosciences, Heidelberg, DE
HLA-DR	PerCP	BD Biosciences, Heidelberg, DE

5.1.4 Agonists and Inhibitors

Name	Alternate Name
EP1/EP3 agonist	**17-phenyl trinor Prostaglandin E_2**
EP2 agonist	**Butaprost**
EP4 agonist	**L000902688**
cAMP agonist	**Forskolin**
Akt inhibitor	**Akt inhibitor IV**
Gsk-3 inhibitor	**GSK-3 inhibitor IX** (2'Z,3'E)-6-Bromoindirubin-3'-oxime (BIO)
Jak/STAT3 inhibitor	**Cucurbitacin I, Cucumis sativus L.**
NFκB inhibitor	**NFκB activation inhibitor** 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline
p38 inhibitor	**p38 MAP kinase inhibitor III** (RS)-{4-[5-(4-Fluorophenyl)-2-methylsulfanyl-3H-imidazol-4-yl]pyridine-2-yl}-(1-phenylethyl)amine]
PI3K inhibitor	**LY 294002** 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
PKA inhibitor	**4-Cyano-3-methylisoquinoline**

PKC inhibitor	**Calphostin C, Cladosporium cladosporioides**
STAT3 inhibitor	**STAT3 inhibitor peptide** (PpYLKTK-mts)

5.1.5 siRNA

Target Gene	Description	Target Sequence
INDO	**IDO siRNA - 1**	**UCACCAAAUCCACGAUCAU**
INDO	**IDO siRNA - 2**	**UUUCAGUGUUCUUCGCAUA**
INDO	**IDO siRNA - 3**	**GUAUGAAGGGUUCUGGGAA**
INDO	**IDO siRNA - 4**	**GAACGGGACACUUUGCUAA**
IL2RA	**CD25 siRNA - 1**	**GAGAGAAUUUAUCAUUUCG**
IL2RA	**CD25 siRNA - 2**	**GGGCAGAUGGUUUAUUAUC**
IL2RA	**CD25 siRNA - 3**	**AGAGGUUUCCGCAGAAUAA**
IL2RA	**CD25 siRNA - 4**	**AUACAGGGCUCUACACAGA**
Renilla	**control siRNA**	**AAAAACATGCAGAAAATGCTGTT**

5.1.6 Plastic ware

96-well tissue culture plate	Sarstedt, Nürnbrecht, DE
0,2 - 2 ml Eppendorf tubes	Eppendorf GmbH, Hamburg, DE
Hyperfilm™ ECL	GE healthcare, Piscataway, US
LS columns	Miltenyi Biotech, Bergisch Gladbach, DE
Nitrocellulose-Membrane, Hybond-C Extra	GE healthcare, Piscataway, US
Nunclon™ 6-well tissue culture plate	Thermo Scientific, Rockford, US
Nunclon™ 24-well tissue culture plate	Thermo Scientific, Rockford, US
Nunclon™ 48-well tissue culture plate	Thermo Scientific, Rockford, US
NuPAGE® Novex Bis-Tris Gels, 10 %	Invitrogen, Carlsbad, US
Parafilm	Pechiney, Chicago, US
Pipettes 2, 5, 10 and 25 ml	Sarstedt, Nürnbrecht, DE
Pipette tips, 10, 200, 1000 µl	Sarstedt, Nürnbrecht, DE
Pre-Separation Filters	Miltenyi Biotech, Bergisch Gladbach, DE
Safe Seal Tips	BIOzym Diagnostik GmbH, DE
Falcon 15 ml	Sarstedt, Nürnbrecht, DE

Falcon 50 ml	Sarstedt, Nürnbrecht, DE
Syringe 50 ml	Braun, Melsungen, DE
Sterile filter ∅ 2 µm	Sartorius, Hannover, DE

5.1.7 Equipment

Centrifuges	
Type 5402	Eppendorf GmbH, Hamburg, D
Biofuge pico	Heraeus Christ Instruments, Düsseldorf, D
Varifuge RF	Heraeus Christ Instruments, Düsseldorf, D
Incubator	Heraeus Christ Istruments, Düsseldorf, DE
FACSCanto™	BD Biosciences, Heidelberg, DE
Gene pulser Xcell™	BioRad Laboratories, München, DE
Magnet MPC-S	Dynal Biotech, Oslo, NO
Magnet MACS Multi Stand	Miltenyi Biotech, Bergisch Gladbach, DE
Medgenix 400 AT microplate reader	SLT Instruments, Salzburg, A
Mikroskope SM-LUX	Leitz, Wetzlar, DE
Mini-Protean Electrophoresis System	Bio-Rad Laboratories, München, DE
Neubauer chamber	Neubauer, DE
Odyssey® Infrared Imaging System	LI-COR Biosciences, Bad Homburg, DE
pH-meter	Knick, DE
Pipette boy	IBS Integra Biosciences, CH
PowerPac HC Power Supply	Bio-Rad Laboratories, München, DE
Roller Mixer SRT 1	Stuart, DE
Scale	Vern
Shaker (type 3011)	GFL, Burgwedel, DE
Trans-Blot Semi-Dry Transfer Cell	Bio-Rad Laboratories, München, DE
Vortex Genie2	Bender&Hobein AG, Zürich, CH

5.1.8 Software

CorelDRAW X3	Corel Corporation, CA
Cyflogic	free license, http://www.cyflogic.com/
FACSDiva™	BD Biosciences, Heidelberg, DE

Mayday 2.0	free license, http://www.zbit.uni-tuebingen.de/pas/mayday
Mircosoft Office	Microsoft Deutschland GmbH, DE
ImageJ	free license, http://rsb.info.nih.gov/ij/
Odyssey V3.0	Licor Biosciences, Bad Homburg, DE
SigmaPlot 10.0	Systat Software GmbH, DE

5.2 Methods

5.2.1 Peripheral blood samples

Blood samples from healthy blood donors were collected at the Center for Transfusion Medicine of the University hospital Cologne or at the Institute for Experimental Hematology and Transfusion Medicine of the University hospital Bonn, after written consent was obtained. The blood samples in form of buffy coats were provided immediately after processing.

5.2.2 Isolation of PBMC

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation. Blood derived from buffy coats was diluted 1:2 with PBS and carefully overlaid on Ficoll solution before centrifugation at 2100 rpm for 25 minutes without break. The interphase consisting of PBMC was removed afterwards and used for the enrichement of $CD14^+$ monocytes by magnetic assorted cell sorting (MACS).

5.2.3 Isolation of $CD14^+$ monocytes

PBMC were isolated as described in 5.2.2. After 2 wash cycles of PBMC with PBS, cells were resuspended in MACS buffer (PBS with 0.5 % BSA and 20 mM EDTA) and incubated with 250 µl CD14 microbeads for 17 minutes at 4°C. Cells were washed with MACS buffer and applied to a LS separation column which was assembled on a MACS multistand with a MidiMACS magnet and a pre-seperation filter (30 µm). The column was rinsed with 3 x 3 ml MACS buffer and removed from the magnet. $CD14^+$ cells were immediately eluated from the column with 5 ml MACS buffer and counted. The purity of $CD14^+$ cells was determined by flow cytometry and was regularly above 98 %.

5.2.4 Isolation of $CD4^+$ T cells

Human RosetteSep $CD4^+$ enrichment cocktail was added to blood derived from buffy coats (360 µl/7.5 ml blood) and incubated for 20 minutes at room temperature. After Ficoll density gradient centrifugation, the small interphase consisting of $CD4^+$ T cells was removed and cells were washed and counted before further processing. The

purity of $CD4^+$ cells was analyzed by flow cytometry and only cell preparations with a purity above 90 % were further processed.

5.2.5 Cell counting

Cells were counted in a Neubauer counting chamber. Depending on the estimated cell density in the solution, cells were diluted 1:1, 1:10 or 1:100 with trypan blue which enters dead cells through little holes in the cell membrane and therefore allows the exclusion of dead cells during counting. The cell number was calculated using the following formula:

cell number = viable cells x dilution x volume x 10^4

5.2.6 Generation of monocyte-derived dendritic cells

`DC culture media´ was prepared with serum-free CellGro® media containing 5 % Glutamax and supplemented with 500 IU/ml interleukin-4 (IL-4) and 800 IU/ml GM-CSF. $CD14^+$ monocytes were resuspended in DC culture media at a concentration of 2 x 10^6/ml and seeded on 6-well plates (5 ml/well). The plates were placed in an incubator which maintains a temperature of 37°C, an air atmosphere of 95 % and a constant level of 5 % CO_2. Monocyte-derived dendritic cells (mo-DC) were harvested after a three to seven day culture period. If cells were cultured for seven days, the culture media was renewed after three and five days.

5.2.7 Maturation/Stimulation of dendritic cells

Immature mo-DC were harvested, counted and transferred to 24- or 48-well plates at a concentration of 2 x 10^6/ml in DC culture media. For maturation, the DC culture media was supplemented with 20 ng/ml TNF. Mo-DC were stimulated with 1 µg/ml PGE_2, 1 µg/ml ultrapure or commercial LPS or 1 µg/ml Pam_3CSK4 (Pam_3) separately or in combination with TNF. Depending on the conducted assay, DC were stimulated for 24 to 72 hours. In some experiments, DC were stimulated with TNF in combination with agonists of the four known EP receptors: the EP2 receptor agonist Butaprost, the EP1/EP3 receptor agonist 17-PT-PGE_2 and the EP4 agonist L000902688. Additionally, an agonist of the adenylate cyclase (Forskolin) which increases intracellular cAMP levels was used to stimulate DC in combination with TNF. Mo-DC were also cultured in supernatants of differentially treated DC to

analyze the effect of DC-derived soluble factors on the phenotype of unstimulated DC.

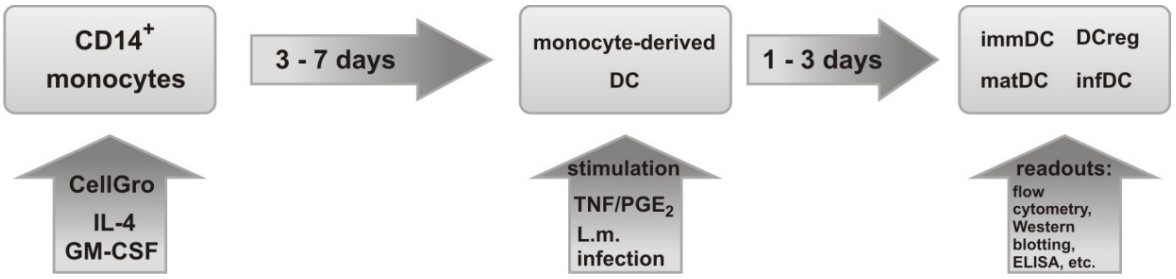


Figure 1: Differentiation and stimulation of mo-DC
$CD14^+$ monocytes were cultured for 3-7 days in CellGro® supplemented with IL-4 and GM-CSF. The differentiated mo-DC were stimulated with soluble factors, e.g. PGE_2 and TNF, or infected with *Listeria monocytogenes*. After one to three days of stimulation, the differentially treated DC were harvested and readouts were performed.

5.2.8 Infection of dendritic cells with *Listeria monocytogenes*

Infection and all post-infection cell culture experiments with virulent wild-type *Listeria monocytogenes* were performed in the S2 facility of the Institute of Medical Microbiology of the University of Cologne. Mo-DC were harvested after three to seven days and infected with wild-type *Listeria monocytogens* (strain EGD) at a multiplicity of infection (MOI) of five in HBSS buffer for 30 min at 37°C. Infected DC (infDC) were washed four times in HBSS buffer to remove extracellular bacteria that were not phagocytosed by the DC. Additionally, infected DC had to pass through a 30 % sucrose layer to avoid contamination of the subsequent cell culture. DC culture media was prepared without cytokines but with addition of gentamycin (50 µg/ml) if infected DC were cultured for longer than 24 hours. Listeria-infected DC were cultured at a density of 2×10^6/ml. In some experiments, infected DC were stimulated with anti-TNF (Infliximab), anti-TNF receptor I, anti-TNF receptor II or anti-IFN-γ neutralizing antibodies immediately after infection. Analog to the above described protocol, DC were infected with heat-killed *Listeria monocytogenes*. Supernatants of these infected DC were used in assays with CTLL-2 cells.

5.2.9 CTLL-2 assay

The murine IL-2 dependent T-cell line CTLL-2 was obtained from ATCC (TIB-214) and cultured in RPMI 1649 medium containing 0.1 mg/ml streptomycin, 100 U/ml

penicillin, 10 % FCS and recombinant IL-2. For functional assays, CTLL-2 cells were washed and cultured in supernatants of differentially stimulated DC in a ratio of 1:1 to CTLL-2 culture medium. Proliferation of CTLL-2 cells was assessed by cell counting, cell viability was determined by staining with propidium iodide (PI) and subsequent flow cytometric analysis.

5.2.10 Generation of artificial antigen-presenting cells (aAPC)

Artificial antigen-presenting cells (aAPC) were generated in form of magnetic beads coated with antibodies against CD3 to deliver only signal 1 or with antibodies against CD3 and CD28 to deliver signal 1 and signal 2 and thereby induce T-cell activation and proliferation. To achieve a physiological degree of T-cell activation, the beads were not completely covered with anti-CD3 and CD28 but the bead surface was filled with a non-signaling anti-MHC class I antibody (W6/32). 5 % of the bead surface was coated with anti-CD3, the rest either completely with anti-MHC-I (anti-CD3 beads) or with anti-CD28 and anti-MHC-I in a ratio of 1:7 (anti-CD3/CD28 beads). Dynal beads were incubated with the respective antibodies diluted in Bead-binding buffer (0.1 M boric acid) overnight at 4°C with gentle rotation. Following coating, beads were washed four times with Bead-wash buffer (PBS with 3 % BSA and 0.1 % sodium acide) at 37°C and overnight at 4°C. Buffers were renewed after coating and during washing steps by placing the reaction tube on a magnet and removing the buffer while the magnetic beads stick to the tube along the magnet. Afterwards, `dry´beads can be easily resuspended in fresh washing buffer. After overnight rotation, the wash buffer was removed and the beads were resuspended in fresh buffer. The number of beads was determined with a Neubauer counting chamber identical to the counting of cells. The method was described by Chemnitz et al. (Chemnitz et al., 2004).

5.2.11 T-cell proliferation assay

To determine the proliferative response, CFSE (carboxyfluorescein diacetate succinimidyl ester) dilution in proliferating T cells was measured by flow cytometry. CFSE is a membrane-permeable dye which is rendered fluorescent after entering the cell and by this change cannot diffuse out of the cell again. Upon cell division, the fluorescence intensity of the CFSE labeled cell is halved which can be detected by

flow cytometry. This method also allows to distinguish the number of divisions a cell has undergone.

$CD4^+$ T cells were isolated as described in 5.2.3 and washed twice with PBS. 0.5 mM CFSE was added to 1.5 x 10^7 $CD4^+$ T cells in 1 ml PBS and cells were incubated for eight minutes at room temperature with constant shaking. The reaction was stopped by addition of 10 ml heat-inactivated FCS. CFSE-labeled cells were washed twice with RPMI media, counted and cultured in RPMI media at a concentration of 1 x 10^6/ml.

In order to induce proliferation, T cells were stimulated with aAPC in form of magnetic beads coated with anti-CD3 and anti-CD28 antibodies to deliver signal 1 and signal 2. To analyze the role of DC-derived soluble factors on T-cell proliferation, T-cell media was replaced by DC supernatants or T cells were cultured in tryptophan-containing or tryptophan-free RPMI media supplemented with L-kynurenine (5 µg/ml), IL-10 (100 ng/ml) or PGE_2 (1 µg/ml).

5.2.12 Mixed lymphocyte reaction (MLR)

Differentially treated dendritic cells were harvested, intensively washed and resuspended in DC culture media without cytokines at a concentration of 2 x 10^4 DC/150 µl. $CD4^+$ T cells were labeled with CFSE for proliferation assays and left unstained for intracellular IFN-γ measurement and resuspendend in the same media at a concentration of 2 x 10^5 T cells/50 µl. The ratio of DC to T cells was 1:10, the ratio of beads to T cells 1:1, in one experiment also 1:10. CFSE labeled $CD4^+$ T cells and beads were either added directly to DC or after 24 h of DC pre-incubation. After 72 h of co-culture T-cell proliferation and IFN-γ production were assessed. To block infDC-derived inhibitory factors, the IDO inhibitor 1-MT (10 µM), the COX-2 inhibitor Rofecoxib (10 µM), anti-IL-10 (10 µg/ml) or rhIL-2 (20 U/ml) were added to the co-culture separately or in combination.

5.2.13 Transfection of dendritic cells

Small interfering RNAs (siRNAs) are double-stranded RNA molecules with a length of about 21 bp that mediate a post-transcriptional mechanism of gene silencing. By this mechanism, called RNA interference (RNAi), which is catalyzed by the RISC complex (RNA induced silencing complex), siRNAs mediate the degradation of specific

complementary and usually fully processed mRNA without an alteration in the rate of transcription of the target gene itself (Agrawal et al., 2003; Tuschl, 2001). DC were transfected with siRNA by electroporation, a method based on the oberservation that electrical pulses can shortly increase the permeability of biological membranes for DNA, RNA or other macromolecules by reversible pore formation without sustained damage of the membrane structure (Sugar and Neumann, 1984). In contrast to exponential decay, square wave electroporation allows the controlled application of a pulse of fixed amplitude for a precisely fixed period of time (Golzio et al., 1998) and was identified as optimal method for delivery into hematopoietic cells (Liu and Bergan, 2001). siRNA was resuspended in 1 x siRNA buffer to a final concentration of 20 µM. Four different siRNAs targeting IDO or CD25 mRNA were used separately or in combination with a total amount of 10 µg siRNA per 4 x 10^6 DC. Mo-DC were harvested, washed with 50 ml PBS and counted. 4 x 10^6 DC were resuspended in 100 µl Optimem, transferred on top of the siRNA (total 10 µg) in a 4mm-cuvette and incubated for exactly three minutes at room temperature. Electroporation was performed with a BioRad GenePulser XCell II using a square wave protocol with two pulses of 1000 V and 0.5 ms pulse length. After transfection, DC were immediately transferred in fresh DC culture media and cultured for two days before additional stimulation.

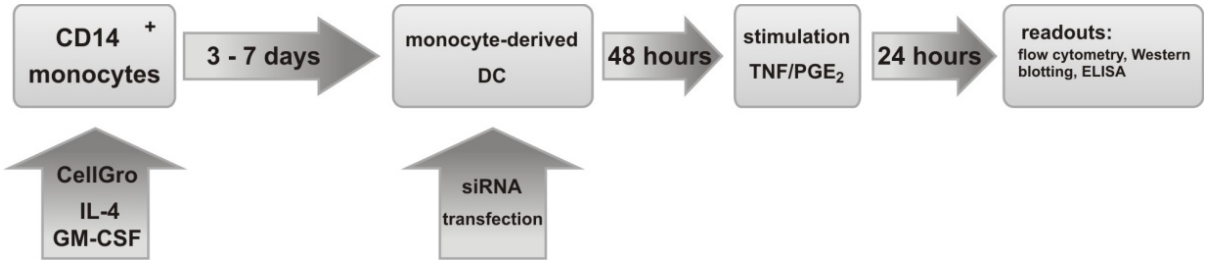


Figure 2: Transfection of mo-DC with siRNA
$CD14^+$ monocytes were cultured for 3-7 days in CellGro® supplemented with IL-4 and GM-CSF. The differentiated mo-DC were transfected with siRNA and cultured for additional 48 hours. Transfected DC were then stimulated with PGE_2 and TNF for another 24 hours before DC were harvested and readouts were performed.

5.2.14 Inhibitor assays

For biochemical inhibition of intracellular signaling molecules, DC were treated with cell-permeable chemical compounds or peptides that specifically interfere with the effector molecules of interest. The inhibitors were resolved in DMSO according to the instructions of the manufacturer. Dendritic cells were harvested, counted and

transferred to 24-, 48- or 96-well plates in a concentration of 2 x 10^6 DC/ml. Inhibitors were added in different concentrations depending on the IC_{50} concentration (concentration where the inhibitor exerts 50 % of its inhibitory function) declared by the manufacturer. As a control, DC were stimulated with DMSO alone. After one hour of pre-incubation with the inhibitors, DC were additionally stimulated with PGE_2 and TNF for 24 hours. Then, DC were harvested and processed for analysis of CD25 and CD83 expression by flow cytometry, IDO protein expression by Western blotting or sCD25 secretion by ELISA.

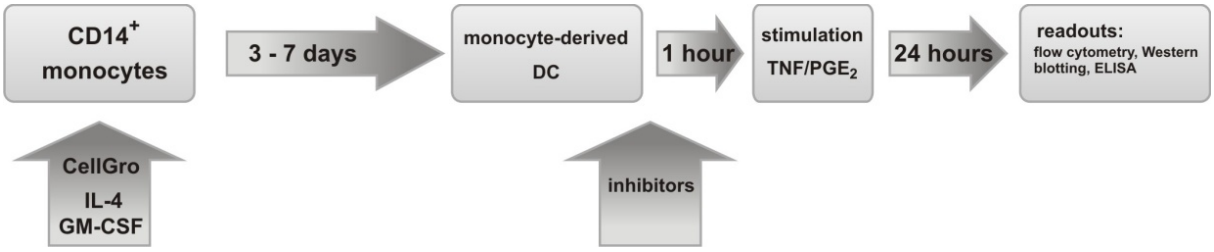


Figure 3: Inhibitor assays with mo-DC
$CD14^+$ monocytes were cultured for 3-7 days in CellGro® supplemented with IL-4 and GM-CSF. The differentiated mo-DC were treated with different inhibitors and cultured for one hour before additional stimulation with PGE_2 and TNF for 24 hours. DC were then harvested and readouts were performed.

5.2.15 Flow cytometry

In a flow cytometer fluorescent dyes are excited by lasers and collection optics direct light scatter and fluorescent signals through spectral filters to the detectors. While cells that were stained with fluorescent dyes or with antibodies coupled to fluorescent dyes pass a small capillary, the fluorescent signal can be detected on the single cell level. DC were harvested after stimulation and a small aliquot with about 100,000 to 200,000 cells was stained with 2-3 µl of antibodies labeled with the following different fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrine (PE) and Peridinin Chlorophyll Protein Complex (PerCP). After 20 minutes of incubation at 4°C, cells were washed twice with CellWash™ buffer and resuspended in CellWash™ buffer before acquisition with the flow cytometer. Living cells corresponding to dendritic cells in size and granularity were gated with the FACSDiva software and further analyzed for fluorescent signal intensity of stained cells. To determine cell viability, DC were stained with 1 µl propidium iodide (PI) immediately before analysis. PI is a fluorescent molecule which stains DNA but is generally not able to enter viable cells

and can therefore be used to distinguish between viable and necrotic or apoptotic cells.

5.2.16 Intracellular staining

Cells were stained for intracellular expression of IDO (DC) or IFN-γ (T cells) using the BD Cytofix/Cytoperm™ kit. To stop secretion of IFN-γ by T cells, BD GolgiStop™ was added 12 hours before assessment of intracellular IFN-γ. Addition of BD GolgiStop™ to cell activation cultures blocks intracellular transport processes, thereby resulting in the accumulation of most cytokine proteins in the endoplasmic reticulum and enhancing cytokine staining signal. Cells were harvested, washed with PBS and resuspended in Cytofix/Cytoperm solution. After 20 minutes of permeabilization, cells were washed and stained with anti-IDO or anti-IFN-γ antibodies diluted in BD Perm/Wash buffer. Following intracellular staining, DC were additionally stained for cell surface expression of CD25, CD83 and HLA-DR. Flow cytometric analysis was performed within 30 minutes. According to size and granularity, living dendritic cells and lymphocytes were gated with the FACSDiva software, respectively.

5.2.17 Cell lysis and protein measurement

Cells were harvested and centrifuged in 1.5 ml tubes at 3500 rpm for 5 minutes. The supernatant was removed and for some experiments stored at -20°C. The resulting cell pellets were pipetted completely dry and resuspended in 20-30 µl Lysis buffer per 1 x 10^6 DC.

Lysis buffer:
20 mM Tris-HCl, pH 8
10 % Triton X-100
100 mM NaCl
1 mM EDTA
1 M DTT
CompleteMini protease inhibitor tablet

For detection of phosphorylated proteins, phosphatase inhibitor cocktail I and II were added to the Lysis buffer. During incubation on ice for 30 minutes, the cell membranes are destroyed and intracellular proteins are solubilized. With a final centrifugation at 13,000 rpm and 4°C, cell debris is collected in the pellet while the

supernatant is further used as protein lysate. The amount of protein in the cell lysates was determined using the BCA protein assay kit. This assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid (BCA). 1 µl of the cell lysate was mixed with 100 µl of Working reagent and incubated at 37°C for 30 minutes. The absorbance was measured at 562 nm and afterwards compared to the absorbance of a standard concentration range to calculate the amount of protein.

5.2.18 SDS-PAGE and Western Blot

To separate the proteins in cell lysates according to their size, a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. 20-50 µg of total protein was loaded on 10 % separating gels covered by a collecting gel. After passing the collection gel at constant 100 V, the proteins were separated at 180 V for 1-1.5 hours. After the gel run, proteins were transferred on a nitrocellulose membrane with a wet or semi-dry blotting system. Transfer was conducted with 100 V for 1 hour or with 20 V for 25 minutes, respectively. To block unspecific binding sites, the membrane was incubated for 30 minutes in 5 % BSA/TBS buffer or 5 % milk/TBS buffer for the conventional chemiluminescent protocol and in Licor Blocking Solution for the infrared protocol. For immunodetection, the membrane was incubated overnight with the primary antibody diluted in BSA buffer or Licor Blocking Solution at 4°C. After washing the membrane four times with TBS containing 0.1 % Tween (Wash buffer) for 5 minutes, the secondary antibody was applied for 1 hour at room temperature. For chemiluminescent signal detection, horseradish-peroxidase coupled secondary antibodies were used in a dilution of 1:2,000. For infrared detection, secondary antibodies coupled to infrared dyes (red or green) were used in a dilution of 1:20,000. After secondary antibody incubation, the membranes were washed again four times in TBST, in case of the infrared protocol all steps from secondary antibody incubation to detection were performed in a black box.

Chemiluminescent signal detection:

For chemiluminescent signal detection, the membrane was covered with ECL™ detection reagent (Solution 1 and 2 in equal volumes) for one minute. Subsequently, the membrane was fixed in an x-ray cassette. In a dark room, the membrane was

exposed to an x-ray film for 2-120 minutes depending on the signal strength and developed.

Infrared signal detection:
For infrared signal detection, the membrane was rinsed with PBS without Tween and scanned on an Odyssey Imager. Beams from two separate 685 and 785 nm lasers are focused to form an excitation spot on the scanning surface of the Odyssey Imager. The light from both fluorescing dyes is collected by a microscope objective and passed through a dichroic mirror that splits the light into two fluorescent signals which are detected on separate photodiodes. Therefore, two colors can be imaged simultaneously in a single scan.
Membranes were stripped with 1 x Re-blot mild solution for 10 minutes followed by blocking for additional 10-20 minutes before re-probing with primary antibodies. Differences in specific protein expression were analyzed densitometrically with the house-keeping protein β-actin as equal loading control.

5.2.19 Kynurenine measurement

The kynurenine amount in DC supernatants was determined with a spectrophotometric assay (Braun et al., 2005). Supernatants were mixed with 30 % trichloracetic acid in a ratio of 2:1, vortexed and centrifuged at 10,000 rpm for 5 minutes. 75 µl of the upper phase was removed and added to an equal volume of Ehrlich reagent (100 mg P-dimethylbenzaldehyde and 5 ml glacial acetic acid) in a 96-well plate. Samples were run in triplicates against a standard curve of L-kynurenine (0-100 µg/ml). Optical density was measured with a Medgenix 400 AT microplate reader at 492 nm.

5.2.20 ELISA

The concentration of different cytokines (IL-2, IL-10, TNF, IFN-γ) and soluble CD25 (sCD25) in DC supernatants was assessed by enzyme-linked immunosorbent assays (ELISA) using the human IL-2, IL-10, TNF, IFN-γ and sCD25 Eli-pair kits. 96-well plates were coated with capture antibody overnight at 4°C. After two washes with ELISA wash buffer (PBS with 0.05 % Tween), wells were blocked with 250 µl of ELISA saturation buffer (PBS with 5 % BSA) for two hours at room temperature. The plate was used after drying overnight. The DC derived supernatants were diluted with

ELISA dilution buffer (PBS with 1 % BSA) in a ratio of 1:1 to 1:20 depending on the respective cytokine. The respective standards were reconstituted in ELISA dilution buffer and a two steps fold dilution was made to run the standard curve. 100 µl of the dilution was distributed in each well followed by addition of biotinylated detection antibody (50 µl/well) and subsequent co-incubation for one to three hours at room temperature. Afterwards, plates were washed three to four times with ELISA wash buffer and incubated 20 minutes with horseradish-peroxidase conjugated streptavidin (HRP-Strep). For color development 100 µl ready-to-use tetramethylbenzidine (TMB) was added per well and incubated for 10 minutes in the dark. The reaction was stopped by addition of 100 µl 1 M H_2SO_4. Absorbance was read at 450 nm with a Medgenix 400 AT microplate reader.

5.2.21 Statistics

Statistical analysis was performed using R software package (version 2.9.2). Since the student´s t-test assumes normal distribution of the samples, the Shapiro test was applied to analyze if the values belonging to one group were normally distributed. The student´s t-test was then used if the values were normally distributed. If the values did not match normal distribution, the Wilcoxon signed-rank test was applied to calculate if significant differences exist between two groups of samples. In both tests a p-value below 0.05 proves a significant difference.

6 Results

6.1 The regulatory phenotype of human dendritic cells

6.1.1 PGE_2-treated and listeria-infected DC are characterized by co-expression of IDO and CD25

Regulatory DC can be induced in different conditions where an effective stimulatory capacity of the DC would be useful for the host, e.g. during malignant cell transformation to prevent tumor progression or to avoid persisting chronic infections by elimination of the infectious agent. Regulatory DC might account for the unresponsiveness of the adaptive immune system by blockade of effective T-cell mediated immune responses. To analyze the molecular mechanisms that are involved in the induction of regulatory DC, two different models of regulatory DC were studied.

The first model is based on the finding that the level of PGE_2 is increased in the microenvironment of different tumor entities, e.g. in breast and colon cancer and that stimulation with the tumor-derived factor prostaglandin E_2 induces DC with a rather tolerogenic phenotype (von Bergwelt-Baildon et al., 2006).

In the second model, we analyzed phenotypic and functional changes of DC upon infection with *Listeria monocytogenes* (*L.monocytogenes*, L.m.). *L.monocytogenes* is a gram-positive ubiquitary bacteria which tolerates low temperatures (Gray and Killinger, 1966). Infection with *L.monocytogenes* mostly occurs via the gastro-intestinal tract, mainly through consumption of raw milk products. Therefore listeriosis is a food-borne disease. The immune system is able to clear the infection in healthy individuals with little or no clinical symptons while predisposing conditions, e.g. immune suppression, Diabetes mellitus or older age can result in a listerial infection with severe life-threatning clinical symptons. In these patients the mortality can reach 30 % (Southwick and Purich, 1996). Especially pregnant women are at risk of a fatal maternal or fetal infection. During chronic listeriosis, the formation of granuloma prevents the dissemination of the bacteria, yet the listeria remain in the granulomatous structure and are not completely eliminated by the immune system. The role of DC in listerial infection is the phagocytosis of the bacteria in the small intestine after they crossed the intestinal barrier as well as the transportation to the

draining mesenterical lymph nodes. After phagocytosis, listeria escape out of the phagolysosome and start their replication in the cytosol which accounts for the processing and presentation of listerial antigens on MHC class I molecules and subsequent elimination of listeria-infected cells by cytotoxic T cells. The role of DC in advanced-staged listeriosis especially in granuloma formation remains unclear.

PGE_2 has been previously described to enhance the migratory and stimulatory capacity of dendritic cells (Krause et al., 2007; Luft et al., 2002) and was therefore used in combination with a maturation cocktail of IL-1, IL-6 and TNF to generate DC for implementation in cancer vaccines (Jonuleit et al., 1997). Recent studies suggest that DC under specific conditions aquire a regulatory phenotype in the presence of PGE_2 (von Bergwelt-Baildon et al., 2006). Similar opposing roles were described for dendritic cells infected with Listeria monocytogenes. Listerial infection has been associated with the induction of a stimulatory (Kolb-Maurer et al., 2000; Paschen et al., 2000) as well as a regulatory DC phenotype (Popov et al., 2006; Popov et al., 2008).

To clarify this divergence, panels of genes associated with either stimulatory or inhibitory function were analyzed for their expression pattern in differentially stimulated dendritic cells. Expression values of differentially expressed transcripts associated with stimulatory or regulatory DC function were obtained at NCBI GEO, dataset GSE9946, standardized using Z score transformation at the probeset level (Popov et al., 2008) and visualized in a heat map with Mayday 2.0 (Dietzsch et al., 2006). Immature dendritic cells (immDC), DC matured by stimulation with TNF (matDC) or a combination of TNF and PGE_2 (PGE-DC) as well as listeria-infected DC (infDC) were included in the comprehensive analysis. As shown in Figure 4, there is a similar expression pattern of the analyzed molecules in immature, mature and PGE_2-treated DC while infected DC present a rather specific transcriptional signature, which clearly distinguishes them from the other DC types. Listeria-infected DC simultaneously express numerous stimulatory as well as inhibitory molecules including *IL2RA* (CD25), *PTGS2* (COX-2), *IL10* (IL-10) and inhibitory immunoglobulin-like transcripts among the inhibitory genes. Multiple co-stimulatory and cell adhesion molecules as well as chemokines were among the upregulated stimulatory transcripts in infected DC. On the other hand, several genes with stimulatory function were specifically upregulated in mature DC including *CCL19* (ELC), TNF superfamily members and CD1 family molecules. The expression pattern

of PGE_2-treated DC is comparable to that of mature DC. Yet, two inhibitory transcripts, namely INDO and IL2RA are contrarily regulated in PGE_2-treated and listeria-infected DC compared to immature and mature dendritic cells. Both transcripts are highly upregulated in dendritic cells during Listeria infection.

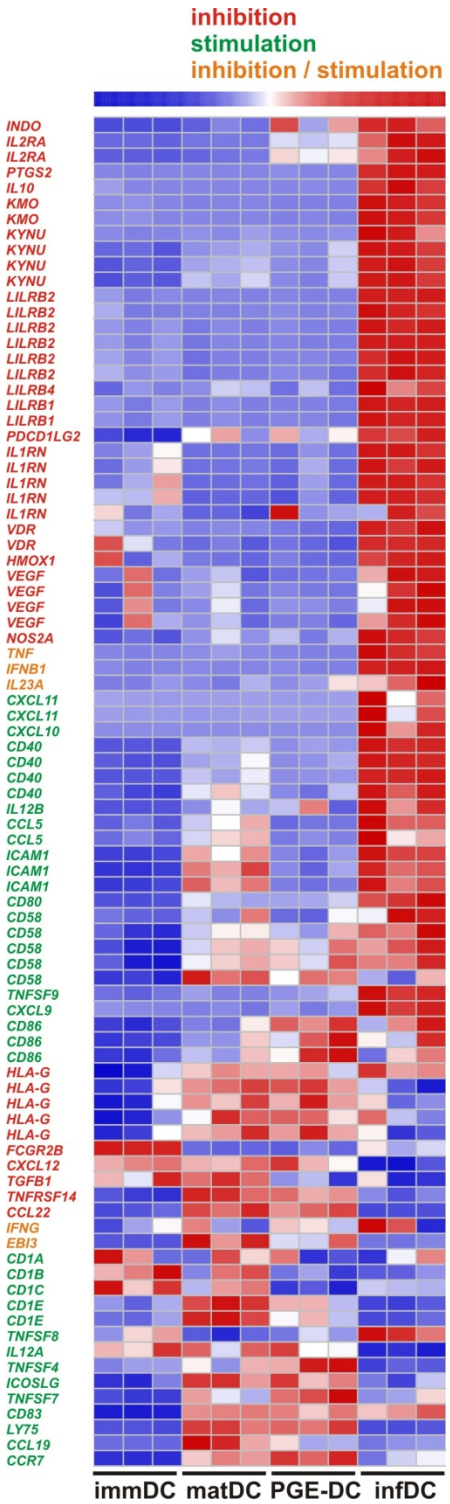


Figure 4: Expression of genes associated with stimulatory and regulatory DC function

Heat map displaying expression of genes that were previously associated with stimulatory or regulatory DC function. Average expression signals were standardized using Z score transformation. The comparative analysis included immature dendritic cells (immDC), TNF-treated dendritic cells with and without PGE_2 (matDC and PGE-DC) and DC infected with *Listeria monocytogenes* (infDC). Expression values of up- and downregulated genes are color-coded; genes with low expression are shown in blue and genes with high expression in red, respectively. Gene symbols for transcripts with stimulatory or regulatory function are differentially color-coded.

Expression values were obtained at NCBI GEO, dataset GSE9946 „Comparison of stimulatory and inhibitory dendritic cell subsets reveals new role of DC in granulomatous infection";

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9946

Transcriptional upregulation of a gene does not ultimately have to result in expression of the respective protein. Therefore, expression of IDO and CD25 in

PGE_2-treated and listeria-infected dendritic cells was analyzed on protein level. Immature and TNF-stimulated mature dendritic cells do not express IDO or CD25. In contrast, the majority of PGE_2-treated and infected DC co-expresses intracellular IDO and surface CD25 (Figure 5A). Comparable to mature DC and despite expression of the regulatory molecules IDO and CD25, PGE_2 treated and infected DC additionally express the well known maturation marker CD83 (Figure 5B).

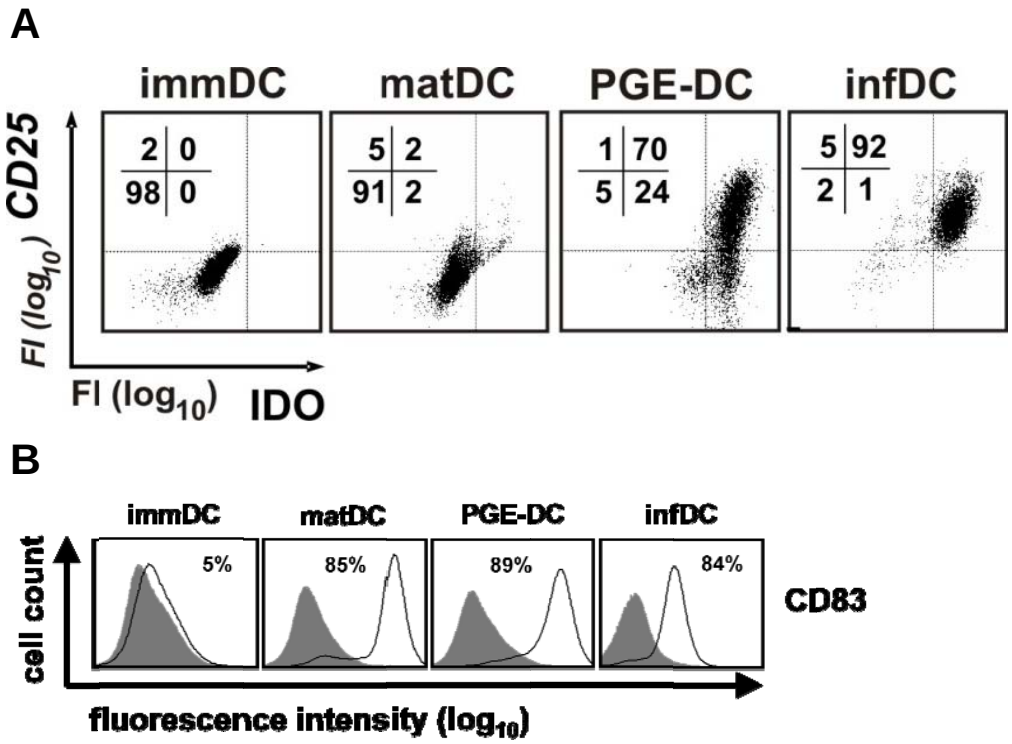


Figure 5: Co-expression of IDO and CD25 by PGE_2-treated and listeria-infected DC is accompanied by maturation

Dendritic cells were either left untreated (immDC), stimulated with TNF (matDC), TNF and PGE_2 (PGE-DC) or infected with *Listeria monocytogenes* (infDC). (**A**) Intracellular expression of IDO and surface expression of CD25 were analyzed by flow cytometry. The fluorescence intensity (FI) of IDO and CD25 is shown. The percentage of cells in each quadrant is indicated in the respective dot plot. One representative experiment (n=4) is shown. (**B**) Surface expression of CD83 was analyzed by flow cytometry and is depicted in a histogram (black line) in comparison to the isotype control staining (grey area). One representative experiment out of five is shown.

6.1.2 PGE_2-treated and infected DC express functional active IDO

Enzymatically active IDO degrades the amino acid tryptophan and leads to the accumulation of its metabolites, the kynurenine. Yet, expression of the protein does not necessarily result in functional IDO enzymatic activity. To prove that IDO is functional in PGE_2-treated and listeria-infected dendritic cells, the level of kynurenine in the supernatants of these cells was measured. High amounts of kynurenine were

found in the supernatants of IDO-expressing PGE_2-treated and listeria-infected DC whereas the supernatants of immature and mature did not contain kynurenine (Figure 6).

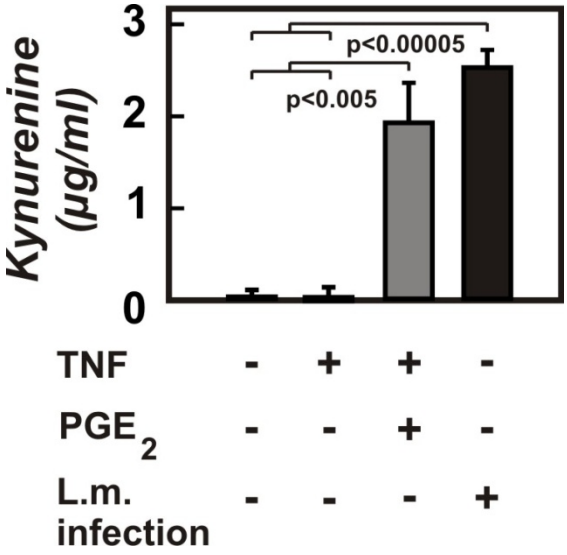


Figure 6: Kynurenine production by PGE_2-treated and listeria-infected DC
Dendritic cells were either left untreated, stimulated with TNF, PGE_2 and TNF or were infected with *Listeria monocytogenes*. The amount of kynurenine was measured in the supernatants of these diferentially treated dendritic cells in a colorimetric assay. Mean values and standard deviations of at least four independent experiments are shown. Statistical significance is indicated.

6.1.3 PGE_2-treated and infected DC secrete soluble CD25

CD25 also exists in a soluble form that is not the product of a unique posttranscriptional splicing event (Rubin and Nelson, 1990) but is most likely derived from proteolytic cleavage of surface CD25 (Robb and Kutny, 1987) mediated by metalloproteinase-9 (Sheu et al., 2001). Soluble CD25 (sCD25) binds IL-2 efficiently with a dissociation constant similar to that of surface CD25 (Jacques et al., 1987). High amounts of soluble CD25 were measured in the supernatants of PGE_2-treated and listeria-infected dendritic cells while immature and mature DC do not secrete measurable amounts of sCD25 (Figure 7). The expression of CD25 on the surface is therefore accompanied by the secretion of soluble CD25.

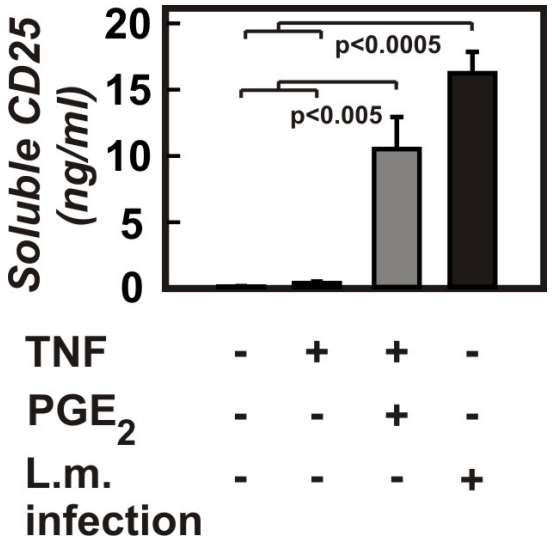


Figure 7: Secretion of soluble CD25 by PGE_2-treated and listeria-infected DC
Dendritic cells were either left untreated, stimulated with TNF, PGE_2 and TNF or were infected with *Listeria monocytogenes*. The amount of sCD25 was measured in the supernatants of the differentially treated dendritic cells by ELISA. Mean values and standard deviations of at least four independent experiments are shown. Statistical significance is indicated.

6.1.4 Infection of DC induces additional inhibitory molecules

In contrast to PGE_2-treated dendritic cells, infected DC express additional transcripts that are associated with inhibitory function (Figure 4). For IL-10 and PTGS2 (COX-2), which are differentially regulated between PGE_2-treated and infected DC, expression of the respective proteins was verified. COX-2 is an inducible enzyme, catalyzing two sequential enzymatic reactions, namely oxygenation and reduction of arachidonic acid, leading to PGH_2 synthesis and finally, by terminal prostaglandine synthases, to the production of PGE_2 (Park et al., 2006). Multiple pathways involved in tumorigenesis, including angiogenesis, invasion and tumor-induced immune suppression are affected by COX-2 expression (Dannenberg and Subbaramaiah, 2003; Zha et al., 2004) while many of these effects are induced by PGE_2 (Okuno et al., 1995; Pockaj et al., 2004). COX-2 expression can be induced by multiple cytokines and growth factors (Morita, 2002) and is responsible for the control of PGE_2 production in response to inflammation. Since it was shown that PGE_2 induces expression of the regulatory molecules IDO and CD25 (Figure 5A), it would be of interest to analyze if infected DC express COX-2 protein, thereby synthesize PGE_2, which in an autocrine fashion might be responsible for IDO and CD25 induction in infected DC.

Listeria-infected DC secrete up to 100 ng/ml IL-10 whereas immature, mature and PGE_2-treated DC do not produce measurable amounts of IL-10 (Figure 8A). Similarly, the enzyme COX-2 is only expressed in infected dendritic cells (Figure 8B).

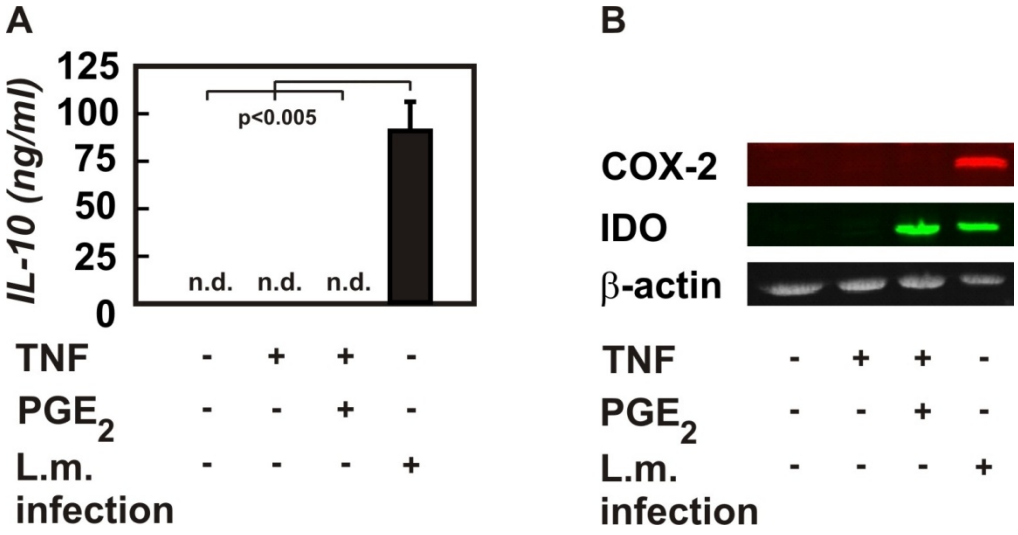


Figure 8: IL-10 and COX-2 are expressed by infected DC
Dendritic cells were either left untreated, stimulated with TNF or PGE_2 and TNF or were infected with Listeria monocytogenes. (**A**) The amount of IL-10 was measured in supernatants of the differentially treated dendritic cells by ELISA. Mean values and standard deviations of at least three independent experiments are shown. Statistical significance is indicated. n.d.: not determinable (**B**) The expression of COX-2 and IDO protein was measured by Western Blot analysis. β-Actin was used as loading control. One representative experiment out of four is shown.

6.1.5 IDO^+ $CD25^+$ DC are the major component of listerial granuloma *in vivo*

To prove that IDO^+ $CD25^+$ infected dendritic cells are also induced during listeria infection *in vivo*, immunohistochemical and -fluorescent stainings of a lymph node section of a patient with serologically confirmed cervicoglandular-type listeriosis with suppurative granuloma was performed by Dr. Claudia Wickenhauser and colleagues at the Institute of Pathology at the University hospital of Cologne. Dendritic cells expressing the DC marker S100 form a ringwall around the bacteria in the center of the granuloma. These cells express IDO, CD25 and COX-2, the above mentioned hallmarks of Listeria infected dendritic cells. Furthermore $CD4^+$ and $CD8^+$ T cells are located primarily outside of the granuloma, separated from the pathogen by the ringwall of IDO^+ $CD25^+$ $COX\text{-}2^+$ cells (Figure 9).

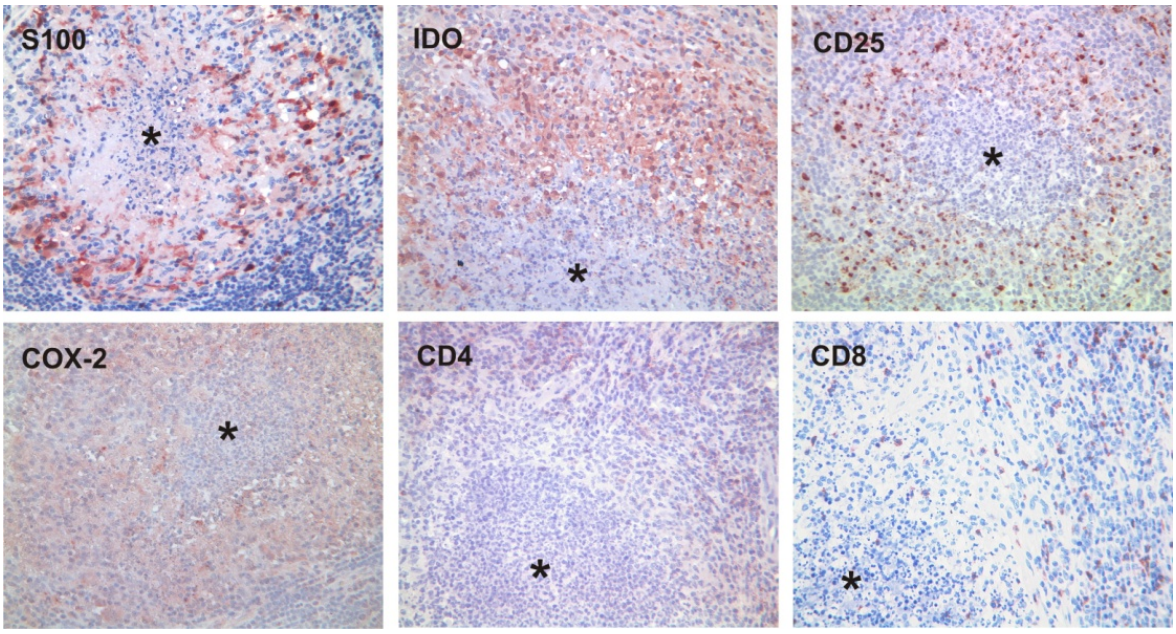


Figure 9: Dendritic cells in listerial granuloma
Immunohistochemical staining of a granuloma section of a patient with chronic listeriosis. From upper left to lower right, stainings were performed with antibodies against the dendritic cell marker S100, the hallmarks of listeria-infected dendritic cells, IDO, CD25 and COX-2 as well as the T-cell markers CD4 and CD8. The center of the granuloma are marked by asterisks.

Co-staining of IDO and CD25 in listerial granuloma sections revealed that the majority of cells in the ringwall co-express IDO and CD25 as it was shown for infected dendritic cells *in vitro* (Figure 10).

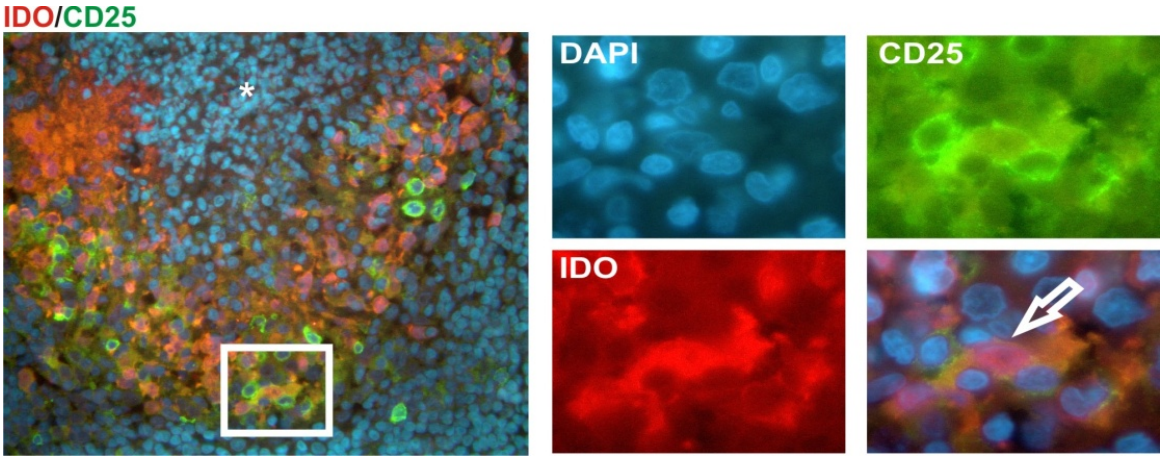


Figure 10: Co-expression of IDO and CD25 by dendritic cells in listerial granuloma
Immunofluorescent staining of a granuloma section of a patient with chronic listeriosis. IDO is shown in red, CD25 in green, the center of the granuloma is marked by an asterisk. The small pictures display a magnification of the area marked with the white box. Single stainings for DAPI, CD25 and IDO as well as an overlay are shown. The arrow points at a double-positive cell.

6.2 Regulatory function of IDO$^+$ CD25$^+$ dendritic cells

While the inhibitory function of IDO concerning T-cell activation is generally accepted and has been proven several times (Mellor et al., 2002; Terness et al., 2002), the functional role of CD25 expression by dendritic cells is still controversially discussed (Driesen et al., 2008; Velten et al., 2007; von Bergwelt-Baildon et al., 2006). It was shown that PGE_2-treated and listeria-infected dendritic cells share the co-expression of IDO and CD25 and that infected dendritic cells express additional molecules that are associated with regulatory functions. To address the functional role of IDO$^+$ CD25$^+$ DC, we performed mixed lymphocyte reactions (MLR) by co-incubation of dendritic cells with CD4$^+$ T cells and assessed T-cell proliferation by CFSE labeling. Only a small number of T cells proliferated after three days of co-incubation with mature dendritic cells compared to anti-CD3/CD28 treated T cells (Figure 8). Stimulation of T cells with anti-CD3 alone does not induce proliferation but anergy of CD4$^+$ T cells (Driesen, 2005). Yet, co-incubation of mature DC and additional delivery of Signal 1 by stimulation with low-dose anti-CD3 increased T-cell proliferation to an extent comparable to anti-CD3/CD28 stimulation.

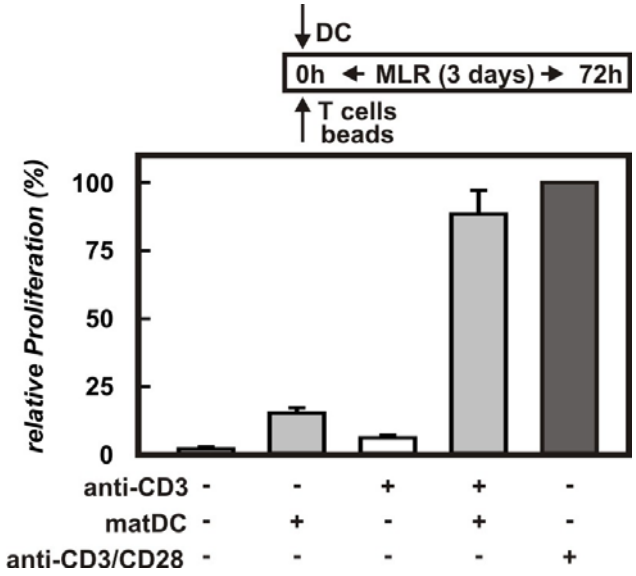


Figure 11: Setup of T-cell stimulation for MLR
CD4$^+$ T cells were co-incubated with mature dendritic cells (matDC) and anti-CD3 separately or in combination. Unstimulated T cells and anti-CD3/CD28 stimulated T cells served as negative and postivie controls for proliferation, respectively. T-cell proliferation was assessed by CFSE dilution. The percentage of proliferating T cells is shown relative to the positive control.

6.2.1 IDO$^+$ CD25$^+$ DC suppress CD4$^+$ T-cell proliferation

Since dendritic cells in the in-vitro culture system do not provide signal 1 but express costimulatory molecules upon maturation, co-incubation of dendritic cells and T cells is not sufficient to induce effective T-cell proliferation which is measurable by CFSE

dilution. The missing T-cell receptor signal which is usually provided by the specific antigen presented on MHC molecules has to be compensated by addition of anti-CD3 coated beads that deliver equal signal 1 in all conditions. To analyze the capacity of IDO^+ $CD25^+$ DC to stimulate T-cell proliferation, PGE_2-treated and listeria-infected DC were co-incubated with CFSE-labeled $CD4^+$ T cells and anti-CD3 for 72 hours.

PGE_2-treated DC significantly suppressed the proliferation of T cells compared to mature dendritic cells if they were pre-incubated for 24 hours before addition of T cells (Figure 12B). Pre-incubation of PGE_2-treated DC was required to reach a significant reduction of T-cell proliferation.

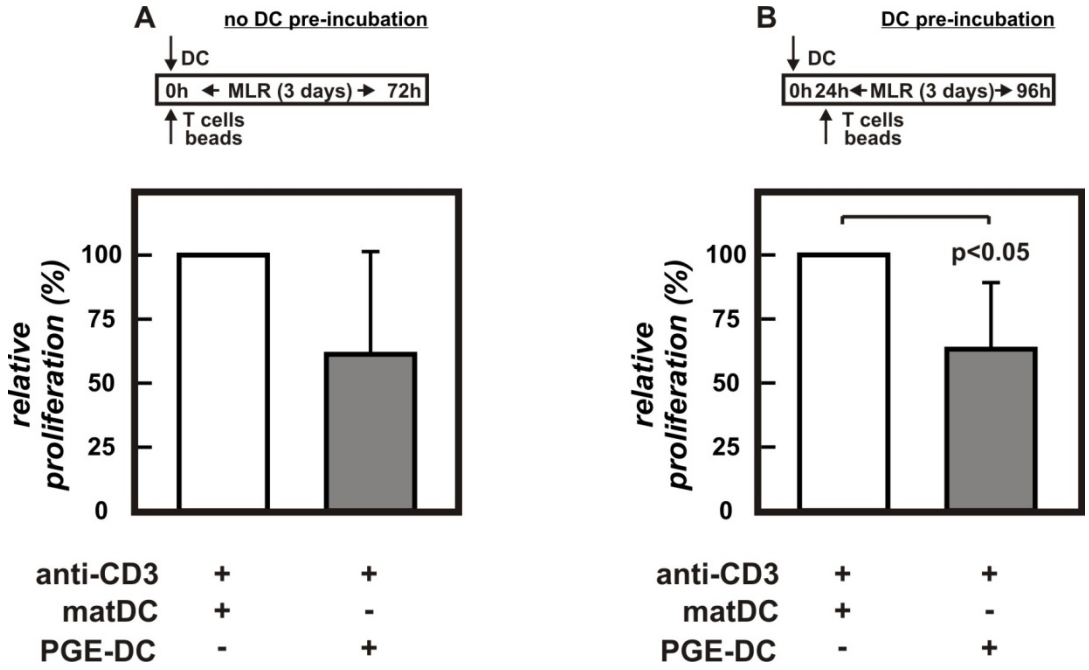


Figure 12: PGE_2-treated DC suppress $CD4^+$ T-cell proliferation
Dendritic cells stimulated with TNF (matDC) or TNF in combination with PGE_2 (PGE-DC) were either directly (**A**) or after 24h of pre-incubation (**B**) co-incubated with $CD4^+$ T cells in a ratio of 1:10. Anti-CD3 coated beads were added in a ratio of 1:1 to T cells. T-cell proliferation was analyzed after 72h by CFSE labeling. T-cell proliferation is shown relative to T cells stimulated with matDC. Mean values and standard deviations of at least four independent experiments are shown. Statistical significance is indicated.

Co-incubation of T cells with listeria-infected DC resulted in even more pronounced suppression compared to co-incubation with PGE_2-treated DC and did not require DC pre-incubation to reach significance (Figure 13).

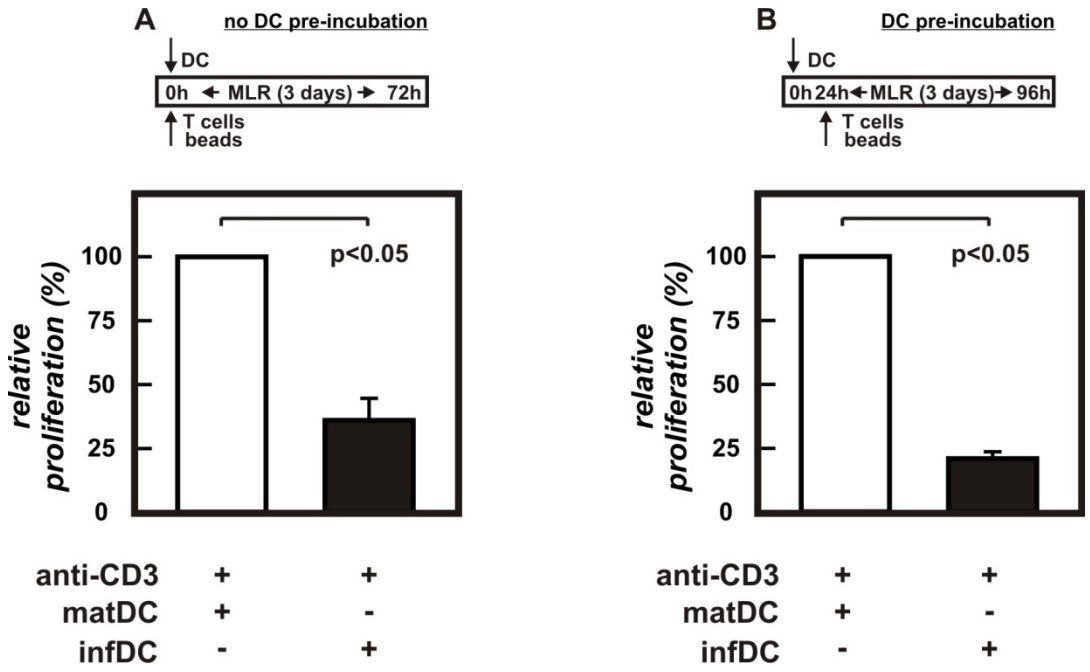


Figure 13: Suppression of $CD4^+$ T-cell proliferation by infected DC does not require pre-incubation

Dendritic cells stimulated with TNF (matDC) or infected with *Listeria monocytogenes* (infDC) were either directly (**A**) or after 24h of pre-incubation (**B**) co-incubated with $CD4^+$ T cells in a ratio of 1:10. Anti-CD3 coated beads were added in a ratio of 1:1 to T cells. T-cell proliferation was analyzed after 72h by CFSE labeling. T-cell proliferation is shown relative to T cells stimulated with matDC. Mean values and standard deviations of at least three independent experiments are shown. Statistical significance is indicated.

6.2.2 IDO^+ $CD25^+$ DC suppress IFN-γ production of $CD4^+$ T cells

Besides entry of the cell cycle and subsequent proliferation, effective stimulation of $CD4^+$ T cells also results in the expression of several pro-inflammatory cytokines, including IFN-γ. The production of IFN-γ by $CD4^+$ T cells was analyzed after stimulation with mature, PGE_2-treated or listeria-infected DC. In contrast to mature DC which induce IFN-γ expression, PGE_2-treated and infected DC significantly suppress IFN-γ production of $CD4^+$ T cells (Figure 14).

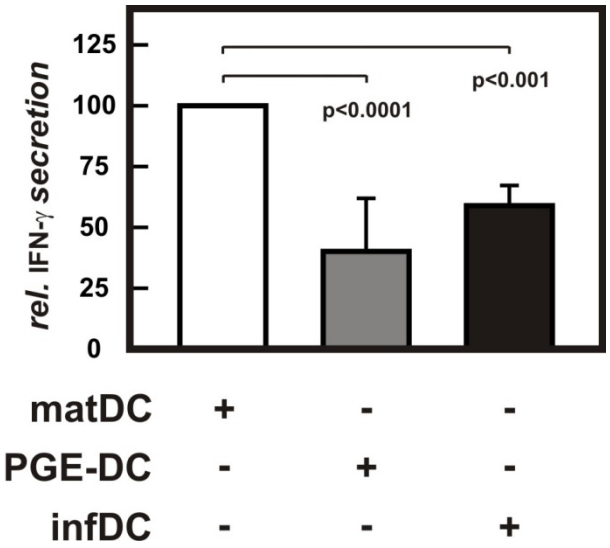


Figure 14: PGE_2-treated and infected DC suppress IFN-γ production of $CD4^+$ T cells
Dendritic cells stimulated with TNF (matDC), TNF and PGE_2 (PGE-DC) or infected with *Listeria monocytogenes* (infDC) were co-incubated with $CD4^+$ T cells in a ratio of 1:10. Anti-CD3 coated beads were added in a ratio of 1:1 to T-cells. IFN-γ was measured by intracellular staining of T cells and flow cytometric analysis. IFN-γ production is shown relative to T cells stimulated with matDC. Mean values and standard deviations of at least three independent experiments are shown. Statistical significance is indicated.

Compared to mature DC, PGE_2-treated and infected DC show an impaired ability to stimulate T-cell proliferation (Figures 12 and 13). To analyze whether PGE_2-treated and infected DC can suppress activated T cells that were provided with a strong stimulatory signal, $CD4^+$ T cells were co-incubated with dendritic cells and anti-CD3/CD28 beads. Stimulation with anti-CD3/CD28 was sufficient to induce strong T-cell proliferation (Figure 15). Additional treatment with mature dendritic cells slightly increases T-cell proliferation. In contrast, PGE_2-treated and infected DC suppress anti-CD3/CD28 induced T-cell proliferation (Figure 15). Again, infected DC exert a stronger inhibitory effect than PGE_2-treated DC, reaching a significant level.

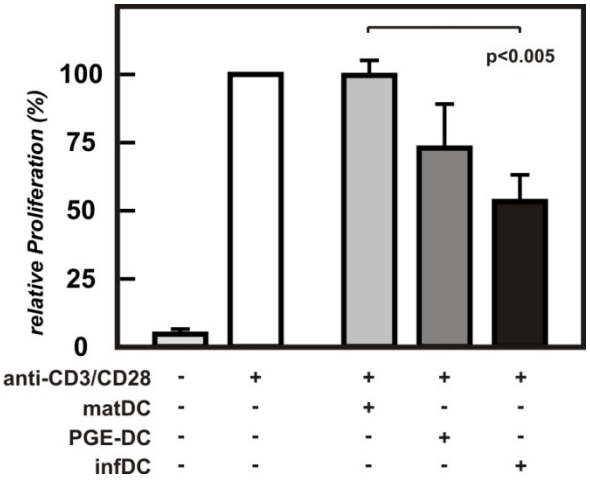


Figure 15: PGE_2-treated and infected DC suppress activated $CD4^+$ T cells
Dendritic cells stimulated with TNF (matDC), PGE_2+TNF (PGE-DC) or infected with *Listeria monocytogenes* (infDC) were co-incubated with $CD4^+$ T cells in a ratio of 1:10. Anti-CD3/CD28 coated beads were added in a ratio of 1:1 to T cells. T-cell proliferation was analyzed after 72h by CFSE labeling and is depicted relative to anti-CD3/CD28 stimulated T-cell proliferation. Mean values and standard deviations of at least three independent experiments are shown. Statistical significance is indicated.

Timing of co-incubation has an important impact on T-cell activation. So far it was shown that pre-incubation of dendritic cells for 24 hours before addition of T cells and aAPC resulted in more pronounced suppressive effects of PGE_2-treated and listeria-

infected DC than co-incubation without pre-incubation of DC (Figures 12 and 13). In line with these results, 24 hour T-cell pre-treatment before addition of dendritic cells abolishes the suppressive capacity of infected DC (Figure 16). $CD4^+$ T cells were pre-treated with anti-CD3/CD28 for 24 hours before addition of mature or listeria-infected DC. There was no significant change in the proliferation rate after 72 hours of co-incubation if T cells were co-incubated with mature or infected DC even if T cells were provided with a suboptimal stimulus (ratio beads/T cells 1:10). Timing and pre-activation status of T cells are therefore important parameters for the outcome of interaction between IDO^+ $CD25^+$ DC and T cells.

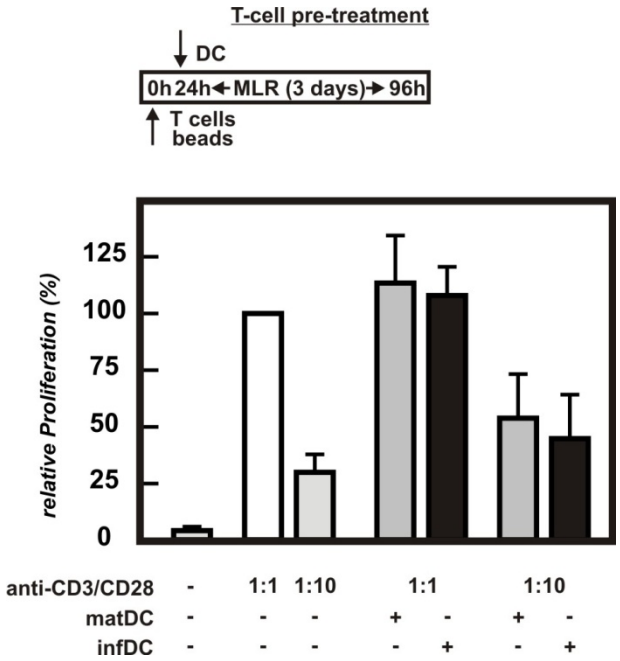


Figure 16: T-cell suppression by infected DC is reversed by T-cell pre-treatment
$CD4^+$ T cells were stimulated for 24h with anti-CD3/CD28 in a ratio of 1:1 or 10:1 before addition of mature or listeria-infected dendritic cells to the co-culture. T-cell proliferation was analyzed by CFSE labeling and is depicted relative to anti-CD3/CD28 activated T cells. Mean values and standard deviations of at least three independent experiments are shown.

6.3 Mechanisms of T-cell suppression

6.3.1 Supernatants of infected DC can induce a regulatory phenotype in uninfected cells

Dendritic cells express the enzyme COX-2 upon infection with *Listeria monocytogenes*. Our group has previously shown that the induction of COX-2 in course of listerial infection leads to the secretion of prostaglandins (Popov et al., 2006) which in turn induce expression of the regulatory molecules IDO and CD25 (Figure 5). To analyze the influence of infDC-secreted factors on non-infected dendritic cells, we incubated immature dendritic cells with supernatants of mature or infected DC and subsequently analyzed hallmark expression of infDC, namely CD25, IDO and COX-2. Mo-DC stimulated with supernatants of immature or mature DC do not express CD25, IDO or COX-2 (Figure 17). In contrast, dendritic cells treated with supernatants of infected DC express high amounts of the regulatory hallmarks CD25, IDO and COX-2.

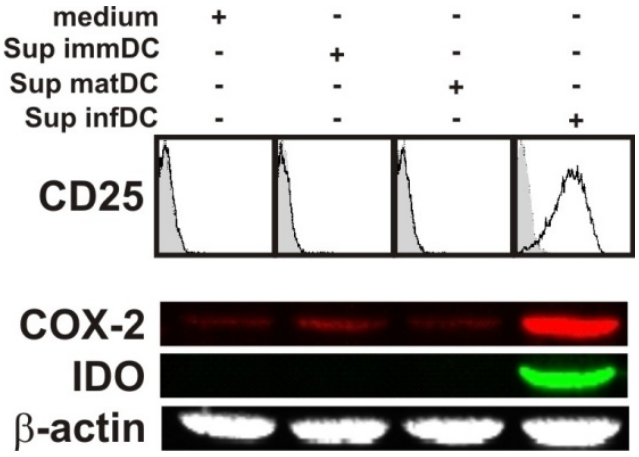


Figure 17: Supernatants of infected DC induce IDO and COX-2 expression
Immature dendritic cells were stimulated with supernatants of immature (Sup immDC), mature (Sup matDC) or listeria-infected DC (Sup infDC). CD25 expression was analyzed by flow cytometry and is displayed in a histogramm as black line. The isotype control staining is shown as grey area. COX-2 and IDO protein expression were analyzed by Western blotting. β-Actin served as loading control.

6.3.2 Supernatants of infected DC suppress CD4$^+$ T-cell proliferation

It was shown so far that IDO$^+$ CD25$^+$ DC are able to inhibit T-cell activation in co-culture and create an environment which leads to the induction of further IDO$^+$CD25$^+$ DC. To demonstrate a possible effect of solube DC-derived factors on T cells without direct cell-cell contact between T cells and dendritic cells, CD4$^+$ T cells were stimulated with anti-CD3/CD28 and increasing concentrations of supernatants of mature or infected DC before proliferation was analyzed after 72 hours. T cells incubated with supernatants of infected DC show a significantly decreased proliferation compared to T cells incubated with supernatants of mature DC (Figure 18).

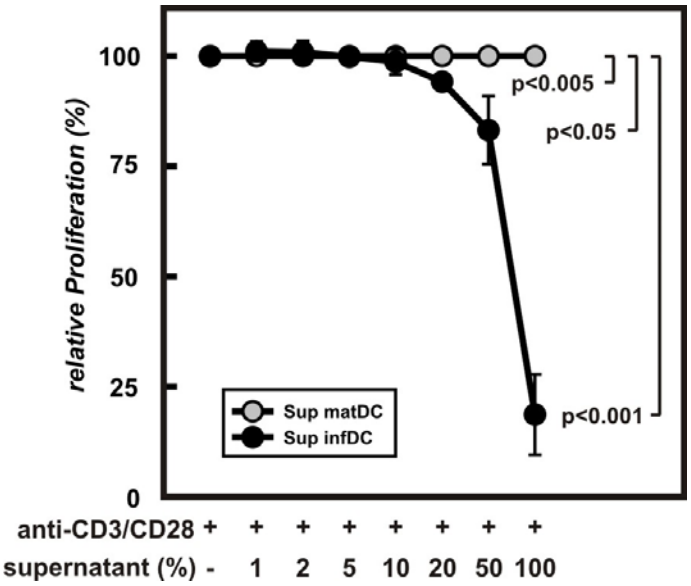


Figure 18: Supernatants of infected DC decrease CD4$^+$ T-cell proliferation
CD4$^+$ T cells were stimulated with supernatants of mature DC (grey circles) or listeria-infected DC (black circles) and proliferation was measured by CFSE dilution after 72h. The proliferation of T cells stimulated with supernatants of infected DC is depicted relative to the proliferation with supernatants of mature DC. Statistical significant differences are indicated.

6.3.3 Single DC-derived factors suppress CD4$^+$ T-cell proliferation

The supernatants of infected dendritic cells contain a number of inhibitory factors, namely soluble CD25, IL-10 and PGE_2, and additionally are deprived of factors which are indispensable for the activation of T cells like tryptophan and IL-2. To clarify the contribution of each factor to the suppressive effects of DC-derived supernatants, T cells were stimulated with anti-CD3/CD28 to induce proliferation and additionally with single DC-derived factors. Stimulation of T cells with anti-CD3/CD28 in tryptophan-containing media strongly induced T-cell proliferation compared to tryptophan-free

media and was further decreased by addition of kynurenine, IL-10 or PGE_2 (Figure 19).

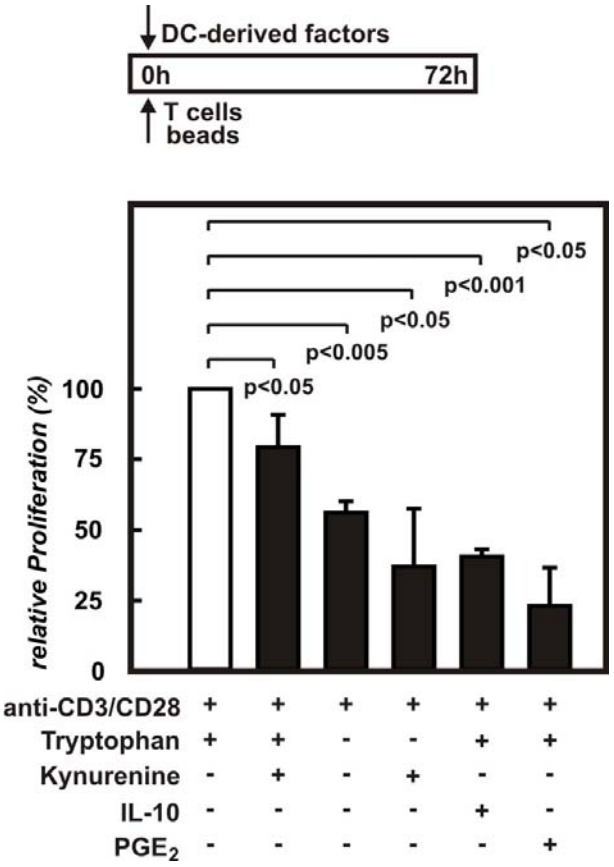


Figure 19: Soluble DC-derived factors suppress $CD4^+$ T-cell proliferation
$CD4^+$ T cells were stimulated with anti-CD3/CD28 in tryptophan-containing or tryptophan-free media with addition of 5 µg/ml kynurenine, 100 ng/ml IL-10 and 1 µg/ml PGE_2 for 72h. T-cell proliferation was assessed by CFSE labeling and is depicted relative to T cells stimulated with anti-CD3/CD28 in tryptophan-containing media (n=3). Mean values and standard deviations of at least three independent experiments are shown.Statistical significant differences are indicated.

6.3.4 Supernatants of infected DC suppress proliferation of an IL-2 dependent cell line

IDO, COX-2 and IL-10 were previously associated with inhibitory functions while the role of surface and soluble CD25 is less understood. To prove that CD25 expression of dendritic cells has a functional relevance by means of capturing IL-2 in their environment, the IL-2 dependent cell-line CTLL-2 and heat-killed Listeria monocytogenes were used. Heat killed L.m. induce CD25 but not IDO or COX-2 in infected DC (Popov et al., 2008) and could therefore be used to analyze the influence of CD25 expression separately from other inhibitory factors. CTLL-2 cells were cultured in supernatants of mature or listeria-infected DC and the number of viable and dead cells was determined. Compared to mature DC, the proliferation of CTLL-2 cells was significantly reduced by supernatants of infected DC (Figure 20). Consequently, the number of dead CTLL-2 cells increased significantly with infected

DC supernatants. $CD25^+$ DC are therefore able to reduce the amount of IL-2 in their environment.

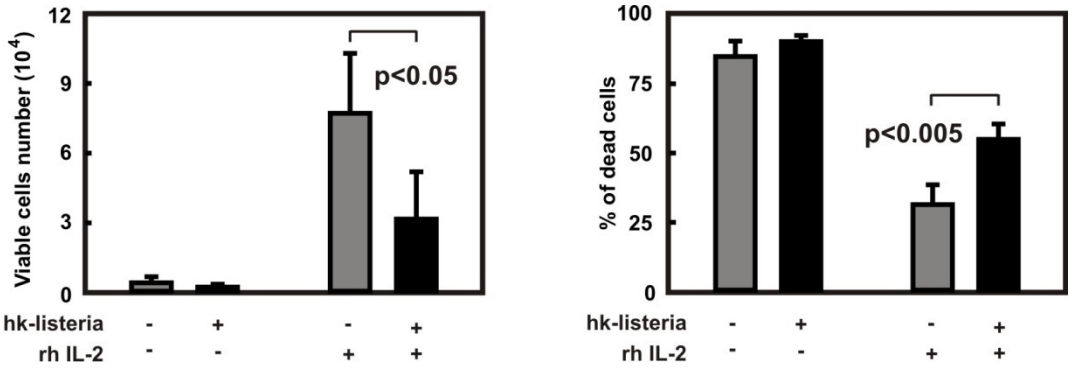


Figure 20: Supernatants of infected DC impair IL-2 dependent CTLL-2 cells
Analysis of proliferation and viability of the IL-2 dependent cell-line CTLL-2. CTLL-2 cells were cultured in supernatants of DC infected with heat-killed *Listeria monocytogenes* (black bars) or supernatants of non-infected DC (grey bars). Cultures were supplemented with rhIl-2 as displayed. Mean values and standard deviations of four independent experiments are shown. Statistical significance is indicated.

6.3.5 IDO and CD25 in DCreg can be specifically silenced by siRNA mediated gene knockdown

To further investigate the influence of IDO and CD25 in the regulatory capacity of dendritic cells, siRNA mediated knockdown of the respective genes in dendritic cells was performed before induction of the regulatory phenotype with PGE_2 and TNF. Electroporation is a suitable and efficient method to deliver siRNA into monocyte-derived dendritric cells (Prechtel et al., 2006). Furthermore, square wave electroporation was identified as the optimal method for delivering oligonucleotides into hematopoietic cells (Liu and Bergan, 2001). The combination of different siRNAs targeting various regions of the same gene could inhibit expression in a more efficient way than the use of single siRNA at the same final concentration (Chen et al., 2005). Hence, immature dendritic cells were transfected with a total concentration of 10 µg IDO or CD25 specific siRNAs (each four different siRNAs) using square wave electroporation with two electric pulses of 1000 V lasting 0.5 ms followed by treatment with PGE_2 and TNF for 48 hours after transfection. Dendritic cells do not express CD25 after PGE_2 treatment if siRNA-mediated knockdown of IL2RA (CD25) was performed in the DC whereas expression of IDO and maturation (CD83) are unchanged (Figure 21). In the same way, DC do not express IDO after PGE_2

treatment if knockdown of INDO (IDO) was performed but expression of CD25 and CD83 remains unchanged.

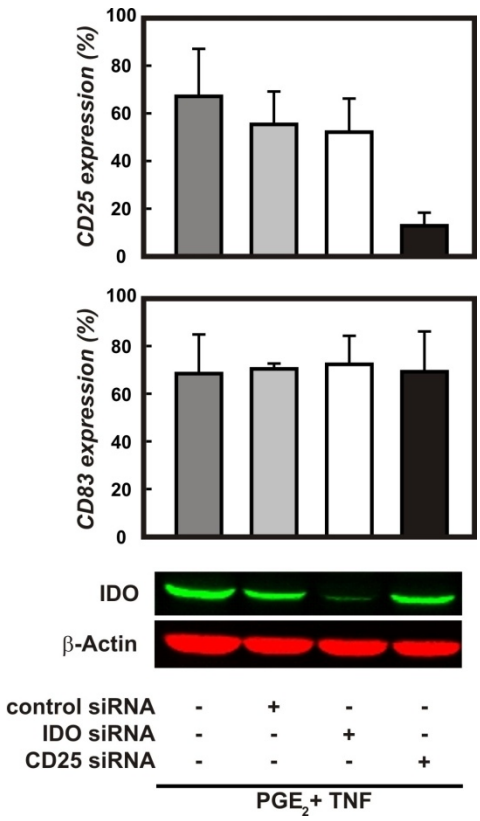


Figure 21: Specific knockdown of CD25 and IDO
Dendritic cells were electroparated using square wave electroporation with 10µg control siRNA, IDO- (INDO) or CD25- (IL2RA) specific siRNA. After 48 hours of incubation, DC were stimulated with PGE_2 and TNF for additional 24 hours before CD25 and CD83 surface expression was assessed by flow cytometry. Mean values and standard deviations of at least four independent experiments are shown. IDO protein expression was analyzed by Western blotting with β-actin as loading control.

Knockdown of CD25 (IL2RA) was performed using square wave electroporation with 10µg total siRNA. Four different CD25 specific siRNAs (1-4) were used together (each 2.5µg) to improve outcome efficiency as it has been described above and was recommended by the manufacturer. To analyze CD25 knockdown efficiency of each siRNA, electroporation of DC was performed with 10µg of the single siRNAs and CD25 as well as CD83 expression was assessed. Of the four siRNAs, only siRNA-3 reduced expression of CD25 on the cell surface to a comparable degree as the combination of all four siRNAs (Figure 22). CD83 expression stayed unaffected. Due to this result the following CD25 knockdown experiments were performed with CD25 siRNA-3.

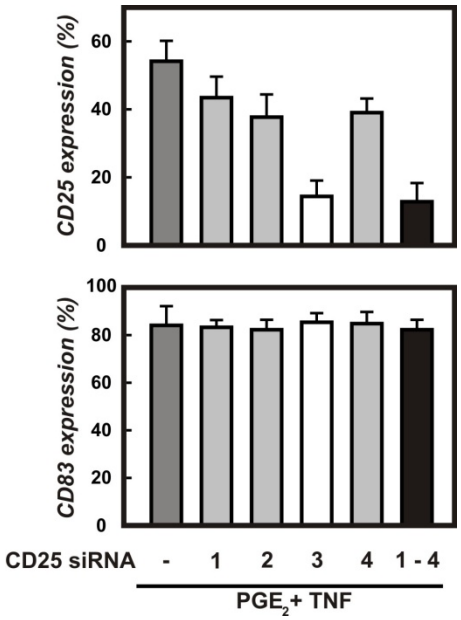


Figure 22: Single and combinatory use of four different CD25 specific siRNAs
Dendritic cells were electroparated using square wave electroporation with a total amount of 10 µg CD25-(IL2RA) specific siRNA 1, 2, 3 and 4, single or in combination. After 48 hours of incubation, DC were stimulated with PGE_2 and TNF for additional 24 hours before CD25 and CD83 surface expression was assessed by flow cytometry. Mean values and standard deviations of at least four independent experiments are shown.

Comparable to the knockdown of CD25, IDO knockdown was also performed with 4 different IDO specific siRNAs (1-4). Knockdown efficiency of each siRNA was analyzed and compared to knockdown efficiency of the combination of all four siRNAs. After knockdown with the single siRNAs followed by PGE_2 treatment, IDO expression is reduced comparable with all four single siRNAs (Figure 23). Knockdown of IDO using the combination of all four siRNAs reduces IDO expression more efficiently to an amount of protein which is not measurable by Western blotting. Due to this result, the following IDO knockdown experiments were performed with the combination of all four IDO siRNAs.

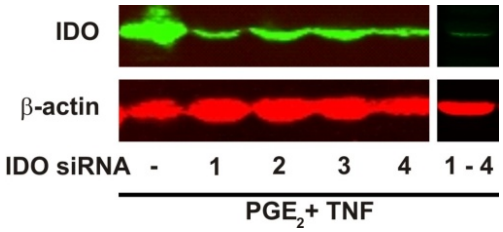


Figure 23: Single and combinatory use of four different IDO specific siRNAs
Dendritic cells were electroporated using square wave electroporation with a total amount of 10 µg IDO-specific siRNA 1, 2, 3 and 4, single or in combination. After 48 ours of incubation DC were stimulated with PGE_2 and TNF for additional 24 hours before IDO protein expression was assessed by Western Blotting. β-actin served as loading control. One representative experiment out of three is shown.

6.3.5.1 CD25 is responsible for the suppressive capacity of DCreg

To analyze the contribution of IDO and CD25 to the suppressive capacity of PGE_2-treated DC, $CD4^+$ T cells were co-incubated with anti-CD3 or anti-CD3/CD28 and PGE_2-treated dendritic cells with single or combinatory knockdown of IDO and CD25. DC were either pre-incubated for 24 hours or directly co-incubated with $CD4^+$ T cells for 72 hours before T-cell proliferation was assessed. Interestingly, knockdown of CD25 restored the stimulatory capacity of dendritic cells while knockdown of IDO only showed moderate effects (Figure 24). Without DC pre-incubation the same tendency is given but is not reaching significance. As shown before, PGE_2-treated DC had to be pre-incubated for 24 hours to be able to significantly inhibit induction of T-cell proliferation (Figure 12). The same is true for the suppression of anti-CD3/CD28 activated T cells which shows the same tendency if DC are pre-incubated but is not significant. Still the slightly lower proliferation of T cells co-incubated with PGE_2-treated DC can be overcome by knockdown of CD25 but not IDO (Figure 24). The suppressive effect of PGE_2-treated DC on anti-CD3/CD28 stimulated T cells is completely lost if DC were not pre-incubated.

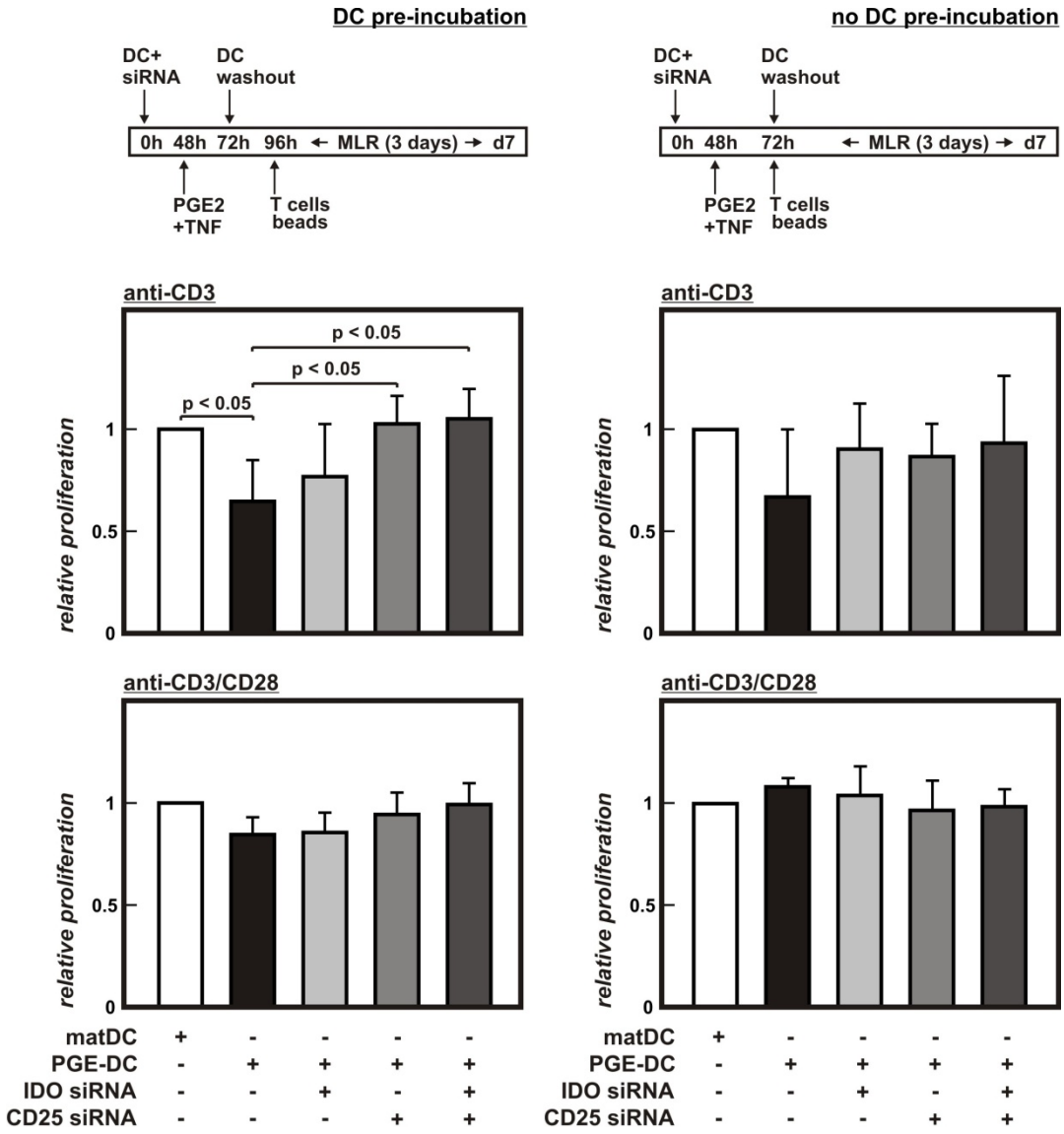


Figure 24: Knockdown of CD25 reverses PGE-DC mediated CD4$^+$ T-cell suppression
Dendritic cells were electroparated using square wave electroporation with 10µg IDO- (INDO) or CD25- (IL2RA) specific siRNA, single and in combination. After 48 hours DC were stimulated with PGE_2 and TNF for 24 hours followed by washout and pre-incubation for 24h (left part) or without pre-incubation (right part). CD4$^+$ T cells and anti-CD3 (upper part) or anti-CD3/CD28 beads (lower part) were added in a ratio of 1:1. T-cell proliferation was analyzed after 72 hours by CFSE dilution and is depicted relative to T cells stimulated with mature DC (n=4). Statistical significance is indicated.

6.3.6 T-cell proliferation is restored by combined blockade of soluble DC-derived factors

Obviously, IDO$^+$ CD25$^+$ DC create an inhibitory environment which influences T-cell activation independent of cell-cell interactions. We have shown that infected dendritic cells express IDO, CD25, soluble CD25, IL-10 and COX-2 resulting in decreased tryptophan and IL-2 concentrations and increased levels of kynurenine, IL-10 and

PGE_2 in their microenvironment. To show the influence of the single factors on T-cell activation, we reversed the inhibitory effects of each factor by blockade of IDO, IL-10 and COX-2 and add-back of IL-2 during a mixed lymphocyte reaction with T cells and infected DC. Blockade of the single inhibitory factors had no effect on infDC suppressed T-cell proliferation, only the combined blockade of IDO, CD25, IL-10 and COX-2 restored T-cell proliferation (Figure 25).

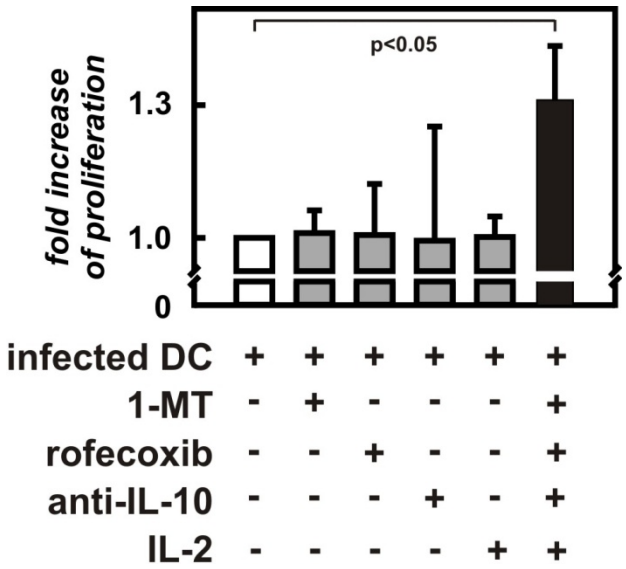


Figure 25: Combined blockade of soluble DC-derived factors restores T-cell proliferation
T cells were co-incubated with infected DC and 1-MT, rofecoxib, anti-IL-10 and IL-2 separately or in combination. T-cell proliferation was measured by CFSE dilution after 72 hours and is depicted relative to T cells co-incubated with infected DC. Mean values and standard deviations of at least three independent experiments are shown. Statistical significance is indicated.

6.3.7 $CD25^+$ DC do not express the high affinity IL-2 receptor

To address the question if dendritic cells are influenced by the binding of IL-2 to CD25, first the expression of the IL-2 receptor subunits was analyzed on differentially treated dendritic cells. In order to execute IL-2 signaling all three subunits have to be expressed on the target cell and form the high-affinity IL-2R. While the α- and β-chains are inducible, the γ-chain is constitutively expressed on DC. The majority of known IL-2 signal transduction pathways are linked to the receptor via the β-chain (CD122), while the α-chain (CD25) is necessary for the formation of the high-affinity IL-2R and is mainly involved in IL-2 binding due to its short cytoplasmic tail. Although Fukao and Koyasu reported the expression of the β-chain in murine splenic DC (Fukao and Koyasu, 2000) and Naranjo-Gomez et al. showed its expression on human pDC (Naranjo-Gomez et al., 2007), expression of the β-chain was not detected on human monocyte-derived DC (Figure 26). CD25 and CD122 are not present on the surface of immature dendritic cells and maturation of the DC is also not sufficient to induce expression of the respective molecules. In contrast, treatment

with PGE_2 in combination with TNF strongly induces CD25 expression while CD122 is not altered.

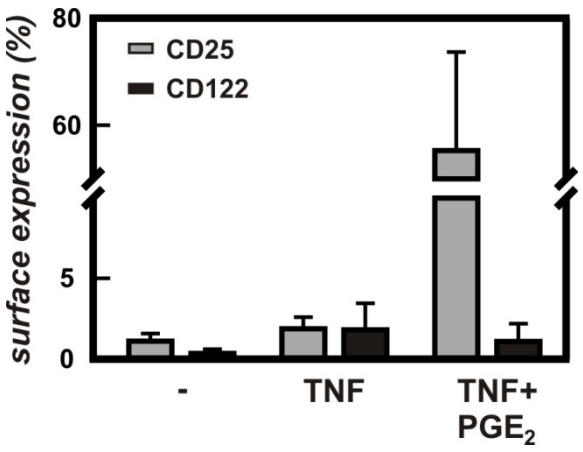


Figure 26: The IL-2 receptor β-chain (CD122) is not expressed on DC
Expression of CD25 (grey bars) and CD122 (black bars) was analyzed on differentially treated dendritic cells. DC were cultured for 72 hours without stimulation, with TNF or TNF in combination with PGE_2. Surface expression of CD25 and CD122 was analyzed by flow cytometry. Mean values and standard deviations of at least three independent experiments are shown.

6.3.8 IL-2 does not influence the phenotype of $CD25^+$ DC

To rule out any possible effects of IL-2 on the DC phenotype, analysis of a variety of different surface molecules that were described to characterize immature, mature or regulatory dendritic cells was performed. Previous results showed that CD25 can serve as a marker for regulatory dendritic cells and is therefore only expressed in PGE_2-treated DC, while CD80, CD83 and CD86 are induced during DC maturation (Figure 27). In contrast to HLA-DR and CD11c which are expressed by all differentially treated DC, only immature DC express CD206. Nevertheless, addition of IL-2 to the DC culture does not alter the expression of any of the mentioned phenotypic markers of immature, mature or PGE_2-treated DC.

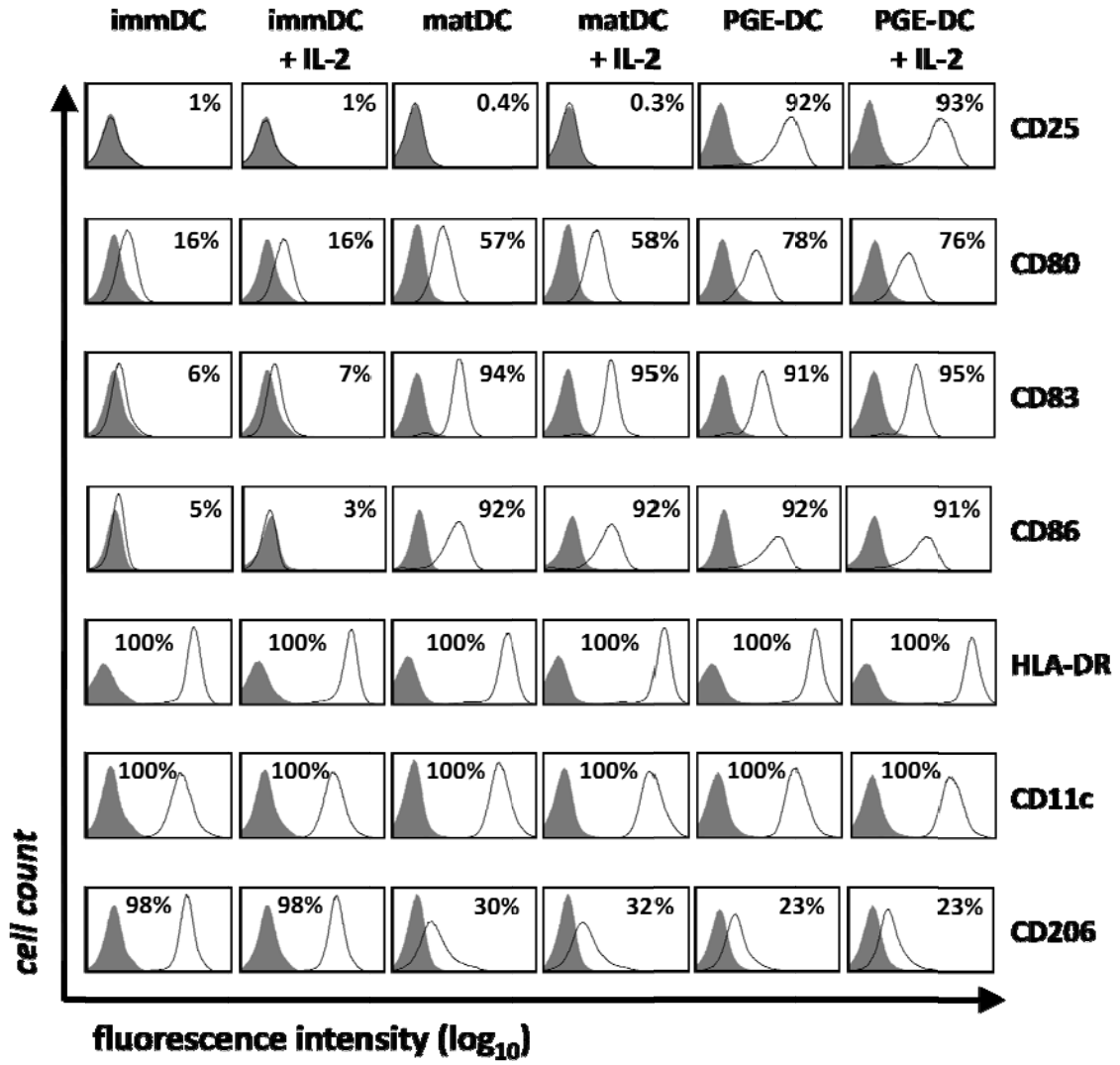


Figure 27: IL-2 does not affect the phenotype of dendritic cells
Cell surface expression of CD25, CD80, CD83, CD86, HLA-DR, CD11c and CD206 was analyzed on immature, mature and PGE_2-treated DC that were additionaly treated with or without 20IU/ml IL-2. Analysis was performed by flow cytometry. Expression of the respective surface markers is depicted in a histogram (black line) in comparison to the isotype control staining (grey area). One representative experiment out of three is shown.

6.3.9 CD25 expressed by DCreg is responsible for IL-2 deprivation

To demonstrate that IL-2 deprivation in the supernatants of $CD25^+$ dendritic cells is actually attributed to CD25 expression, the amount of IL-2 was measured in the supernatants of PGE_2-treated DC with or without knockdown of CD25. Indeed, the amount of IL-2 was decreased in supernatants of PGE_2-treated DC and restored if a knockdown of CD25 was performed in these DC (Figure 28).

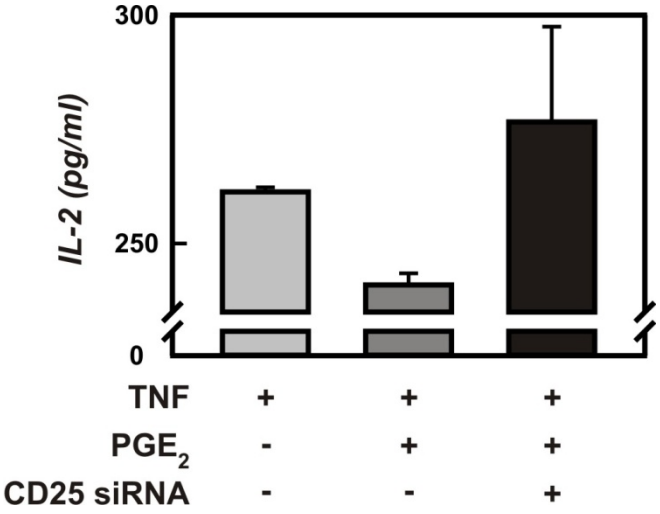


Figure 28: CD25 is responsible for IL-2 deprivation
IL-2 was measured in the supernatant of mature as well as PGE_2-treated DC with or without previous electroporation with CD25 specific siRNA. IL-2 was added in a concentration of 20IU/ml to every culture condition. After 24 hours of stimulation the amount of IL-2 was determined by ELISA. Mean values and standard deviations of at least three independent experiments are shown.

6.3.10 Proposed model of DCreg function

In conclusion, IDO^+ $CD25^+$ DCreg create an inhibitory microenvironment which is deprived of tryptophan and IL-2 and enriched with kynurenine thereby inhibiting T-cell activation and clonal expansion. Listeria-infected DCreg additionally increase the level of IL-10 and PGE_2 which directly suppress T-cell activation (not shown).

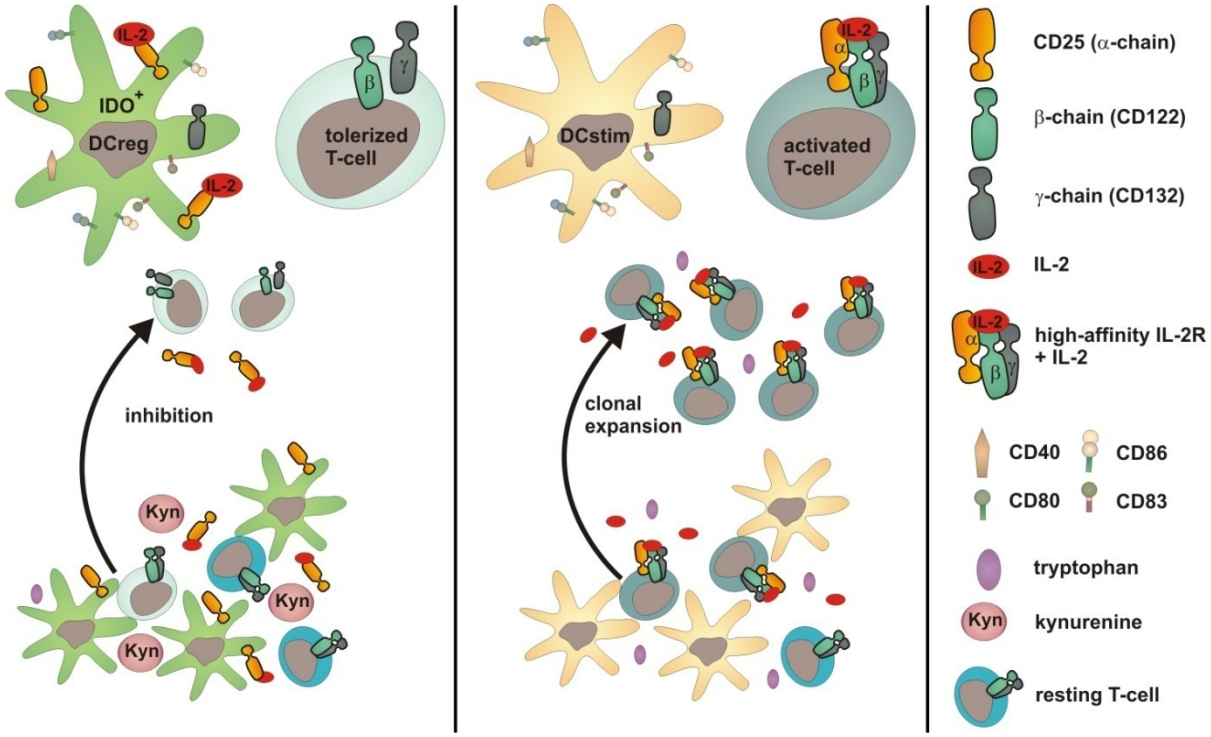


Figure 29: Schematic microenvironment of regulatory DC versus stimulatory DC
DCreg express IDO thereby decreasing the level of tryptophan and increasing the level of kynurenine in their environment. Additionally, DCreg express CD25 and secrete sCD25 which functions as a decoy receptor and captures IL-2. The inhibitory microenvironment, induced by $CD25^+$ IDO^+ DCreg `tolerizes´ T cells. In contrast, stimulatory DC (DCstim) induce T-cell activation and clonal expansion.

6.4 Factors and receptors associated with the induction of a regulatory DC phenotype

6.4.1 Induction of DCreg requires TNF in a combination with a second signal

IDO^+ $CD25^+$ DC can be induced by PGE_2 treatment in combination with the maturation stimulus TNF or by infection with *Listeria monocytogenes* (Figure 5A). Yet, the responsible signal for DCreg induction during Listeria infection has not been identified. Dendritic cells can detect microbial products by a family of pattern-recognition receptors, called Toll-like receptors. Until now 10 different toll-like receptors have been identified in humans that mediate recognition of bacterial antigens, DNA or RNA. *Listeria monocytogenes* is a Gram-positive bacteria, characterized by the presence of lipoteichoic acid (LTA) in the bacterial cell membrane while gram-negative bacteria express lipopolysaccharide (LPS). LTA and LPS are recognized by TLR2 or TLR4, respectively, To figure out if activation of TLR2 or TLR4 could mimick DC infection and might have similar effects on the DC phenotype than Listeria infection, DC were stimulated with TLR2 or TLR4 agonists and maturation status as well as CD25 expression were analyzed. At the same time, the role of maturation in DCreg induction was further investigated by combined treatment of DC with TNF and TLR agonists. Pam_3CSK4 (Pam_3) is a synthetic analog of the triacylated N-terminal part of bacterial lipoproteins. Comparable to LTA, a cell wall component of *Listeria monocytogens*, Pam_3 binds to TLR2 associated with TLR1. Treatment of DC with TNF, PGE_2, the TLR2 agonist Pam_3 or the TLR4 agonist LPS alone did not induce CD25 expression while only TNF as single agent was sufficient to induce the maturation marker CD83 (Figure 30). Interestingly, combination of TNF with PGE_2, Pam_3 or LPS results in strong induction of CD25 combined with maturation (CD83). According to the surface expression, soluble CD25 was only secreted in significant amounts if DC were treated with TNF and a second signal in form of PGE_2 or Pam_3. Treatment of DC with LPS and TNF only slightly increased sCD25 secretion.

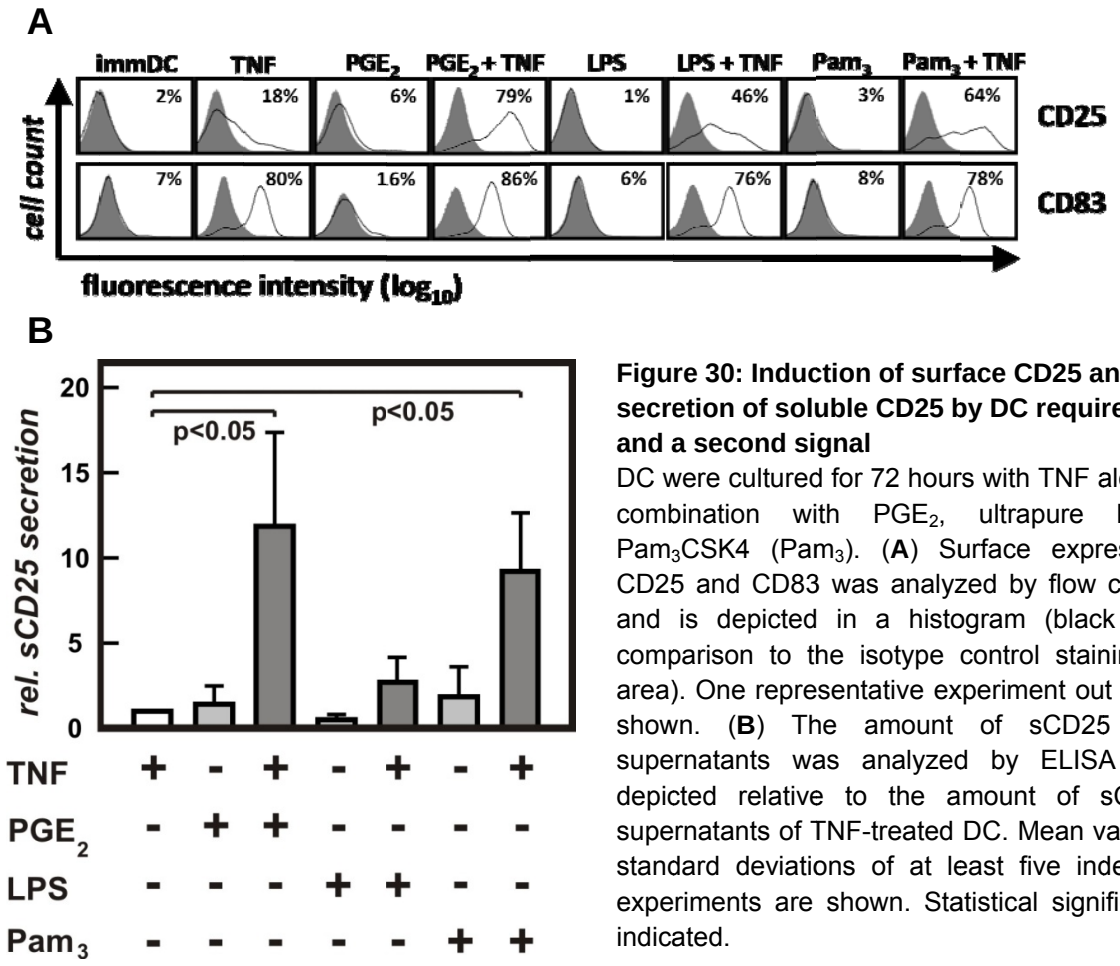


Figure 30: Induction of surface CD25 and secretion of soluble CD25 by DC require TNF and a second signal

DC were cultured for 72 hours with TNF alone or in combination with PGE_2, ultrapure LPS or Pam_3CSK4 (Pam_3). (**A**) Surface expression of CD25 and CD83 was analyzed by flow cytometry and is depicted in a histogram (black line) in comparison to the isotype control staining (grey area). One representative experiment out of five is shown. (**B**) The amount of sCD25 in DC supernatants was analyzed by ELISA and is depicted relative to the amount of sCD25 in supernatants of TNF-treated DC. Mean values and standard deviations of at least five independent experiments are shown. Statistical significance is indicated.

Consistent with these results, induction of IDO, the second hallmark molecule of regulatory DC was also dependent on additional TNF stimulation. TNF, PGE_2, Pam_3 or ultrapure LPS alone were not sufficient to induce expression of IDO in DC. Only the combination of PGE_2, Pam_3 or LPS with TNF resulted in IDO induction. Strongest IDO expression was detected in DC treated with PGE_2 and TNF, slightly weaker expression in DC treated with Pam_3 or LPS in combination with TNF, respectively.

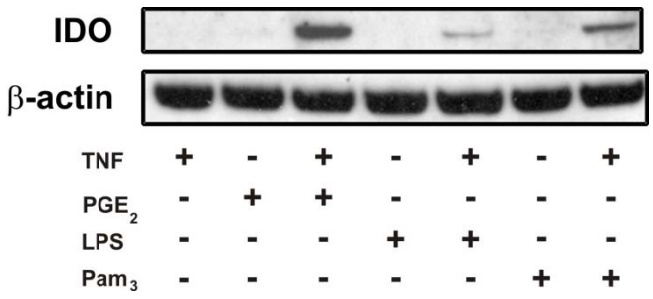


Figure 31: Induction of IDO requires TNF and a second signal

DC were cultured for 72 hours with TNF alone or in combination with PGE_2, ultrapure LPS or Pam_3CSK4 (Pam_3). IDO expression was analyzed by Western blotting, β-actin served as loading control. One representative experiment out of four is shown.

6.4.2 Ultrapure and commercial LPS differ in their ability to induce DCreg

The data presented before clearly shows that induction of DCreg requires two signals. The first signal is provided by the maturation stimulus while the second signal can be provided by different stimuli, e.g. PGE_2, TLR2 or TLR4 activation. In contrast to previously published studies, LPS alone was not sufficient for induction of IDO. This difference in the activity of LPS might be due to the here used ultrapure LPS in contrast to the commercial LPS which is generally more often used for analysis of LPS mediated effects. Most commercial preparations of LPS contain low concentrations of highly bioactive contaminants like bacterial lipoproteins (Hirschfeld et al., 2000). Indeed, comparison of ultrapure and commercial LPS revealed clearly different activities regarding CD25 and IDO induction. While CD25 and CD83 are only induced by treatment of DC with ultrapure LPS in combination with TNF, commercial LPS alone is sufficient to induce high expression of CD25 and CD83 (Figure 32A). According to the surface expression of CD25, soluble CD25 is only secreted by DC treated with ultrapure LPS in combination with TNF but not after ultrapure LPS treatment alone (Figure 32B). In contrast, stimulation of DC with commercial LPS alone resulted in strong secretion of sCD25.

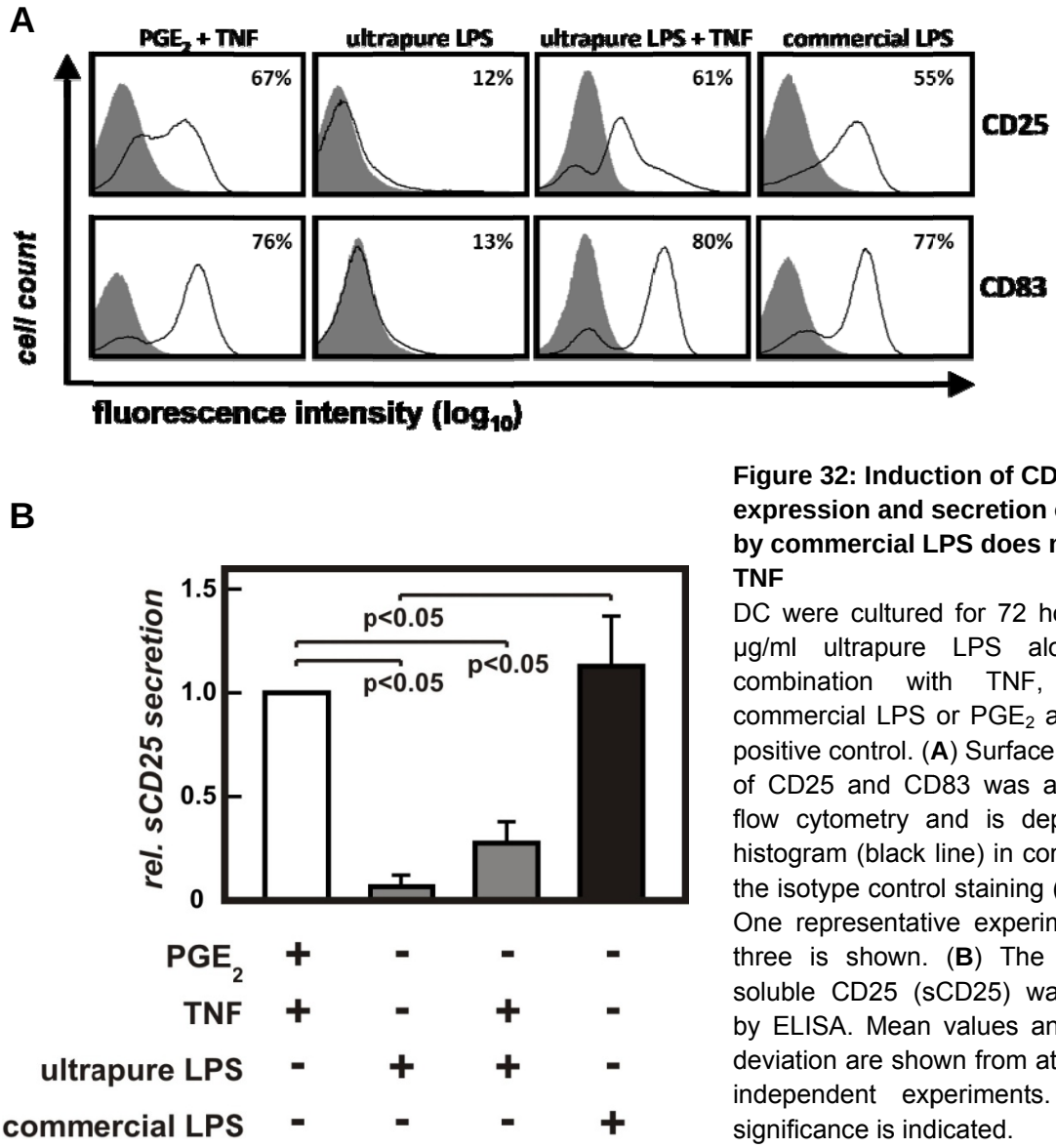


Figure 32: Induction of CD25 surface expression and secretion of sCD25 by commercial LPS does not require TNF

DC were cultured for 72 hours with 1 µg/ml ultrapure LPS alone or in combination with TNF, 1 µg/ml commercial LPS or PGE_2 and TNF as positive control. (**A**) Surface expression of CD25 and CD83 was analyzed by flow cytometry and is depicted in a histogram (black line) in comparison to the isotype control staining (grey area). One representative experiment out of three is shown. (**B**) The amount of soluble CD25 (sCD25) was detected by ELISA. Mean values and standard deviation are shown from at least three independent experiments. Statistical significance is indicated.

TNF mediates its effect through binding to one of the two TNF receptors, TNFRI (p55 TNF receptor) and TNFRII (p75 TNF receptor) (Wallach et al., 1999). Analysis of TNF receptor expression values of immature, mature, PGE_2-treated and infected DC revealed that TNFRI is constantly expressed in immature, mature and PGE_2-treated DC while it is upregulated during infection with *Listeria monocytogenes* (Figure 33). In contrast TNFRII expression is upregulated upon maturation and PGE_2 treatment and is highly expressed in infected DC.

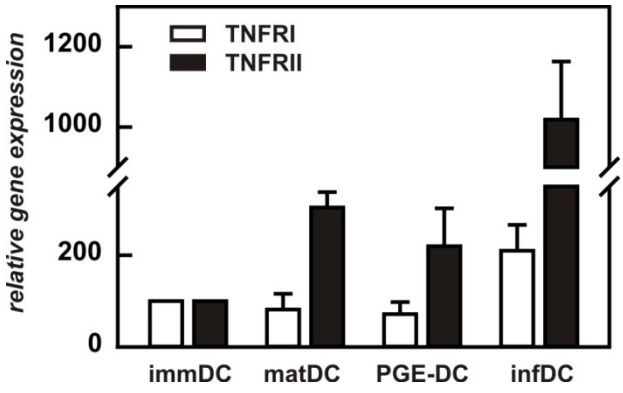


Figure 33: TNF receptor expression by DC
Gene expression values of TNFR1A (white bars) and TNFR1B (black bars) were obtained at NCBI GEO, dataset GSE9946 „Comparison of stimulatory and inhibitory dendritic cell subsets reveals new role of DC in granulomatous infection". Expression values of matDC, PGE-DC and infDC are depicted relative to immDC.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9946

6.4.3 TNFRI-mediated signaling in combination with PGE_2 is necessary to induce IDO^+ $CD25^+$ DCreg

To clarify the role of each TNF receptor in DCreg induction, TNFRI and TNFRII were blocked with respective neutralizing antibodies during DC stimulation with PGE_2 and TNF. Analysis of CD25 and CD83 surface expression clearly shows decreased expression of both molecules only if TNFRI is blocked while blockade of TNFRII has no significant effect on CD25 or CD83 expression (Figure 34A). Blockade of both receptors yields slightly higher reduction of CD25 and CD83 compared to blockade of TNFRI alone. The secretion of soluble CD25 matches the expression of CD25 on the surface after TNF receptor blockade (Figure 34B). Only blockade of TNFRI reduces the amount of sCD25 while TNFRII is not involved.

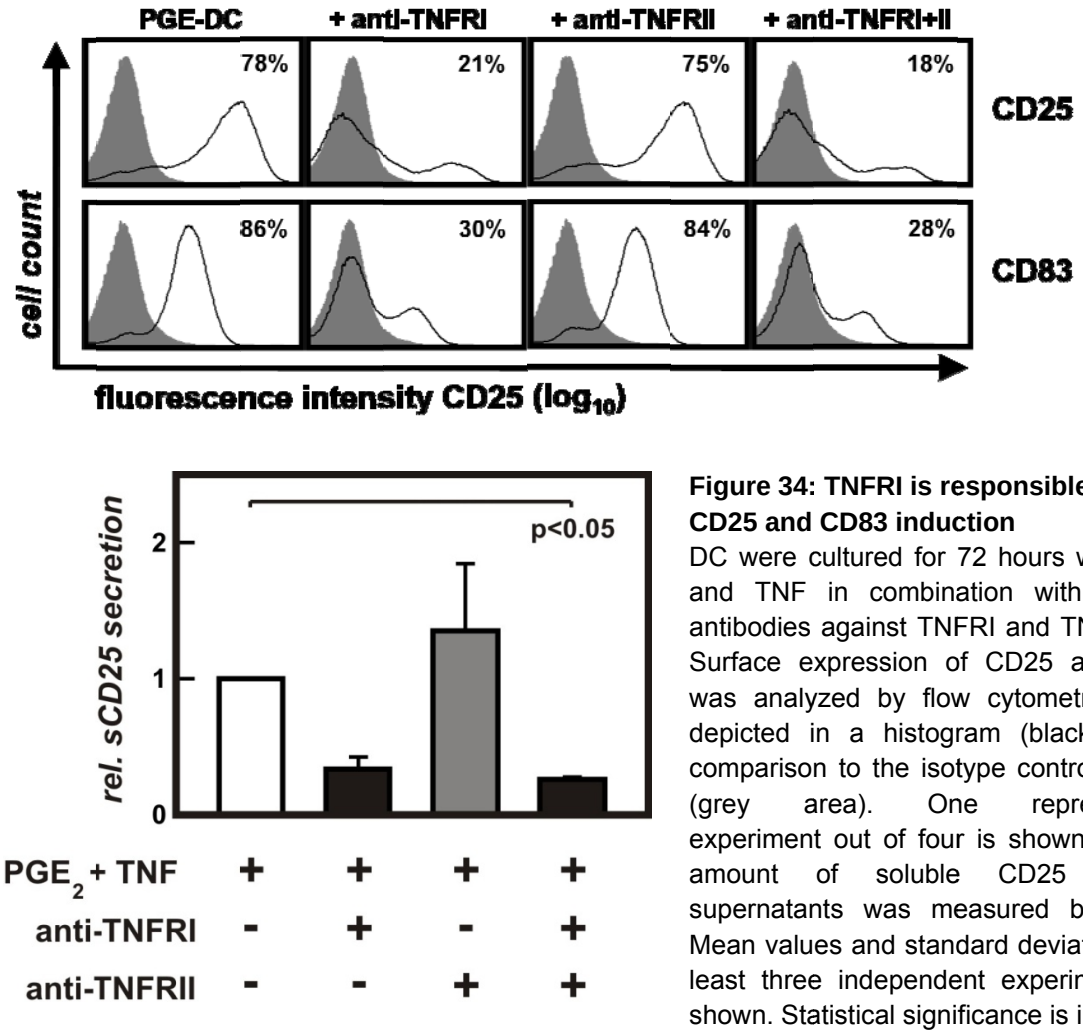


Figure 34: TNFRI is responsible for CD25 and CD83 induction
DC were cultured for 72 hours with PGE_2 and TNF in combination with blocking antibodies against TNFRI and TNFRII. (**A**) Surface expression of CD25 and CD83 was analyzed by flow cytometry and is depicted in a histogram (black line) in comparison to the isotype control staining (grey area). One representative experiment out of four is shown. (**B**) The amount of soluble CD25 in DC supernatants was measured by ELISA. Mean values and standard deviations of at least three independent experiments are shown. Statistical significance is indicated.

The same expression pattern that was observed for CD25, sCD25 and CD83 during blockade of the TNF receptors is also detectable for IDO. Blockade of TNFRI clearly reduced expression of IDO whereas blockade of TNFRII had no effect on PGE_2 and TNF induced IDO expression.

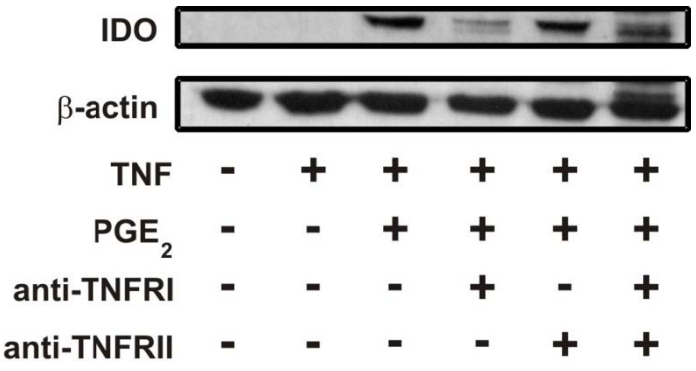


Figure 35: TNFRI is responsible for PGE_2-mediated IDO induction
DC were cultured for 72 hours with PGE_2 and TNF in combination with blocking antibodies against TNFRI and TNFRII. IDO expression was analyzed by Western blotting with β-actin as loading control. One representative experiment out of three is shown.

6.4.4 TNF signal during infection is upstream of IFN-γ

It was previously demonstrated that the infection of dendritic cells with *Listeria monocytogenes* leads to the production and secretion of IFN-γ by the DC (Popov et al., 2006). To rule out that the induction of regulatory DC during infection is solely dependent on autocrine IFN-γ effects, the amount of IFN-γ as well as TNF was determined in supernatants of infected DC. While inhibition of IFN-γ has no effect on TNF secretion by infected DC, blockade of TNF as well as both TNF receptors decreases IFN-γ production (Figure 36).

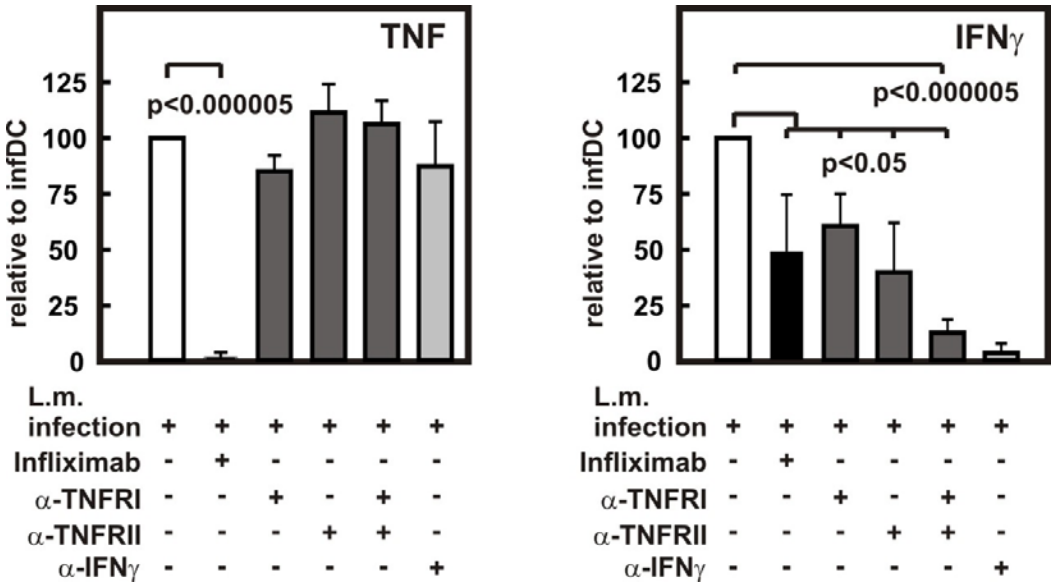


Figure 36: IFN-γ production by infected DC is dependent on TNF
Dendritic cells were infected with *Listeria monocytogenes* and subsequently stimulated with anti-TNF (Infliximab), anti-TNFRI and anti-TNFRII, single and in combination, or anti-IFN-γ neutralizing antibodies for 24 hours. The amount of TNF and IFN-γ was measured by ELISA. Mean values and standard deviations are shown of at least four independent experiments. Statistical significance is indicated.

6.4.5 DCreg induction during infection is dependent on TNF and requires both TNF receptors

To figure out if the induction of regulatory DC during Listeria infection is similarly dependent on TNF and TNF receptor activation, infected DC were co-incubated with anti-TNF or anti-TNF receptor antibodies before induction of the typical regulatory molecules expressed by infected DC was assessed. On the other hand, the role of IFN-γ in DCreg induction was further elucidated by analysis of regulatory molecules after blockade of IFN-γ. Blockade of TNF resulted in significantly reduced IDO

expression accompanied by diminished kynurenine secretion (Figure 37). Similarly, blockade of TNFRII as well as blockade of both TNF receptors resulted in decreased IDO expression and kynurenine secretion while blockade of TNFRI alone did not have significant effects. Interestingly, inhibition of IFN-γ which is secreted later during infection compared to TNF, also significantly decreased IDO expression and subsequent kynurenine production.

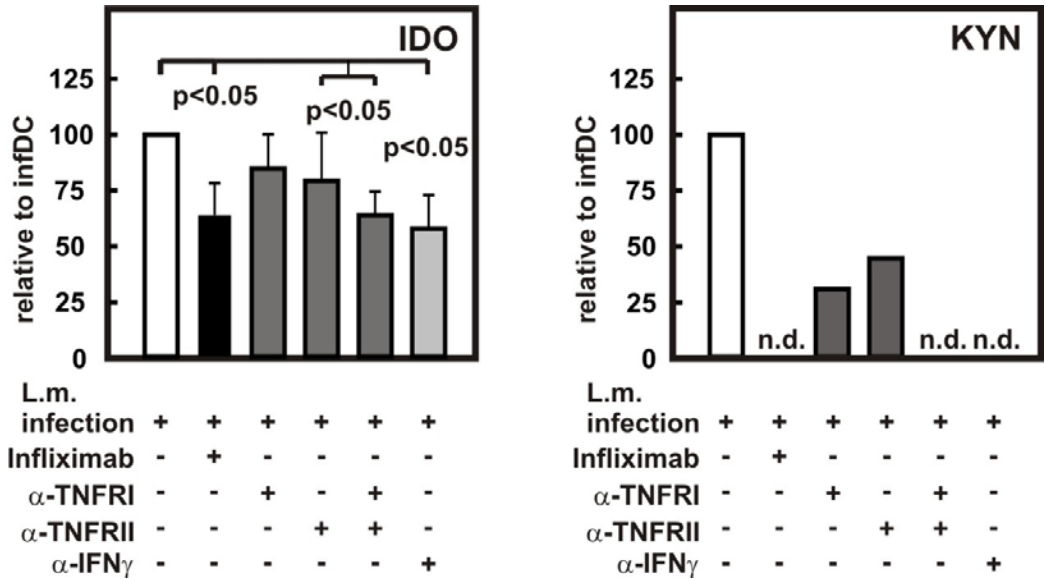


Figure 37: TNF signaling is necessary to induce IDO and kynurenine in infected DC
Dendritic cells were infected with *Listeria monocytogenes* and subsequently stimulated with anti-TNF (Infliximab), anti-TNFRI and anti-TNFRII, single and in combination, or anti-IFN-γ for 24 hours. Expression of IDO protein was measured by Western blotting and densitometrically analyzed relative to β-actin expression. The amount of kynurenine in DC supernatants was determined in a colorimetric assay. Mean values and standard deviations of at least three independent experiments are shown relative to listeria-infected DC. Statistical significance is indicated.

Comparable to IDO induction and kynurenine secretion, expression of CD25 on the cell surface and secretion of soluble CD25 are dependent on TNF (Figure 38). Both TNF receptors are involved in CD25/sCD25 induction. Only blockade of both TNF receptors significantly reduces CD25 surface expression while blockade of the single receptors is sufficient to significantly decrease sCD25 but only slightly decreases surface expression. In contrast to IDO and kynurenine induction, blockade of IFN-γ has no effect on CD25 expression or sCD25 secretion.

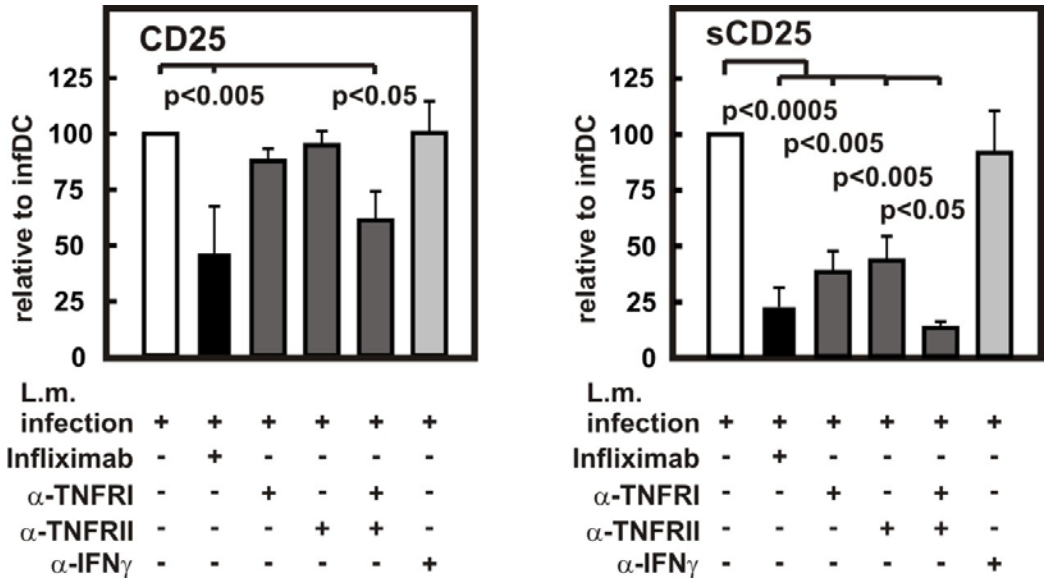


Figure 38: TNF signaling is necessary to induce CD25 and soluble CD25 by infected DC
Dendritic cells were infected with *Listeria monocytogenes* and subsequently stimulated with anti-TNF (Infliximab), anti-TNFRI and anti-TNFRII, single and in combination, or anti-IFN-γ for 24 hours. Expression CD25 was measured by flow cytometry. The amount of soluble CD25 in DC supernatants was determined by ELISA. Mean values and standard deviations are shown of at least three independent experiments relative to listeria-infected DC. Statistical significance is indicated.

Contrary to PGE_2 treatment, DC secrete significant amounts of IL-10 during Listeria infection (Figure 8A). Similarly to the induction of the hallmark molecules IDO and CD25, the production of IL-10 is dependent on TNF since blockade of TNF with infliximab significantly decreases the amount of IL-10 in the supernatants of infected DC (Figure 39). Furthermore, only blockade of both TNF receptors clearly reduces IL-10 secretion while this effect is less pronounced when the single receptors are blocked. Inhibition of IFN-γ has no effect on IL-10 secretion by infected DC.

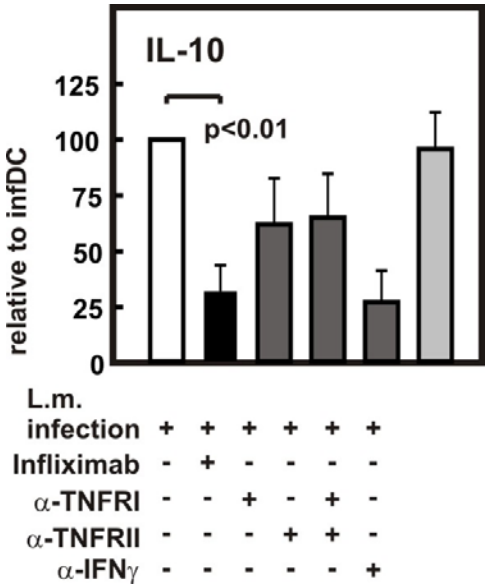


Figure 39: TNF signaling is necessary to induce IL-10 secretion by infected DC
Dendritic cells were infected with *Listeria monocytogenes* and subsequently stimulated with anti-TNF mAb (Infliximab), anti-TNFRI and anti-TNFRII, single and in combination, or anti-IFN-γ for 24 hours. The amount of IL-10 in DC supernatants was determined by ELISA. Mean values and standard deviations are shown of at least three independent experiments relative to listeria-infected DC. Statistical significance is indicated.

6.4.6 PGE_2 mediates induction of IDO^+ $CD25^+$ DCreg via EP2 and EP4 receptors

PGE_2 exerts its function by binding to one of four known G-protein coupled receptors (GPCRs), termed E prostanoid receptors 1,2,3 and 4 (EP1-EP4), thereby activating different signaling cascades depending on the respective receptor. All four EP receptors are composed of seven transmembrane domains coupled to G-proteins that transfer the signal of receptor activation to downstream effector molecules. The EP subtypes exhibit differences in associated signal transduction but they also differ in tissue localization and regulation of expression. Therefore it is necessary to know the expression pattern of EP1-EP4 on dendritic cells in order to analyze downstream signaling pathways that are involved in DCreg induction. Analysis of EP receptor expression revealed that DC do not express EP1 while EP2 is expressed by immature, mature and PGE_2-treated DC at a constant level with slightly lower expression in mature DC (Figure 40). EP3 is expressed upon DC maturation and EP4 expression is increased by PGE_2 treatment.

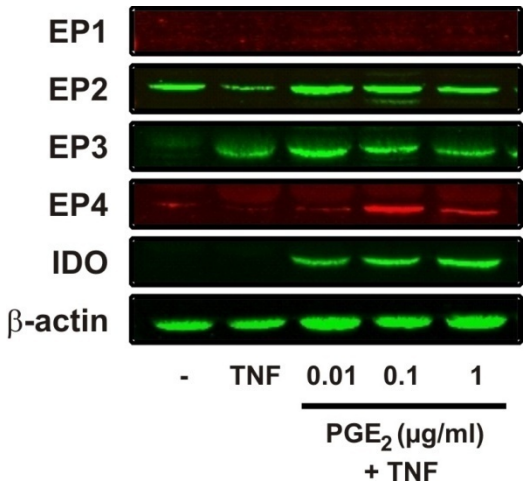


Figure 40: EP receptor expression on human DC
Dendritic cells were left untreated, stimulated with TNF alone or in combination with 0.01, 0.1 or 1µg/ml PGE_2. Expression of EP receptors 1-4, IDO and β-actin as loading control was assessed by Western blotting. One representative experiment out of four is shown.

To analyze the role of each EP receptor in DCreg induction, dendritic cells were stimulated with different EP receptor agonists instead of PGE_2 in combination with TNF which is indispensable for DCreg induction. Additionally, the role of cAMP activation which is mediated by EP2 and EP4 was analyzed by Forskolin stimulation. After 72 hours of stimulation with EP receptor or adenylate cyclase/cAMP agonists, the morphology of the dendritic cells was rather comparable to PGE_2-treated regulatory DC than immature or mature DC. Immature DC were of round shape and uniformly distributed in the cell culture plate. Mature dendritic cells were stellate and

displayed fine dendrites and can therefore easily be distinguished from immature DC. In contrast, PGE_2-treated DC, though they also express maturation markers, lose the dendritic shape and are rather characterized by the formation of cell clusters. Stimulation of DC with EP2 or EP4 agonists resulted in similar shape and cell cluster formation compared to PGE_2-treated DC and this effect was remarkably enhanced by stimulation with the adenylate cyclase/cAMP agonist Forskolin (Figure 41). Most of these DC present a rather round shape combined with cell aggragate formation. DC stimulated with the EP1/EP3 agonist 17-PT-PGE_2 did not clearly change their morphology compared to mature dendritic cells.

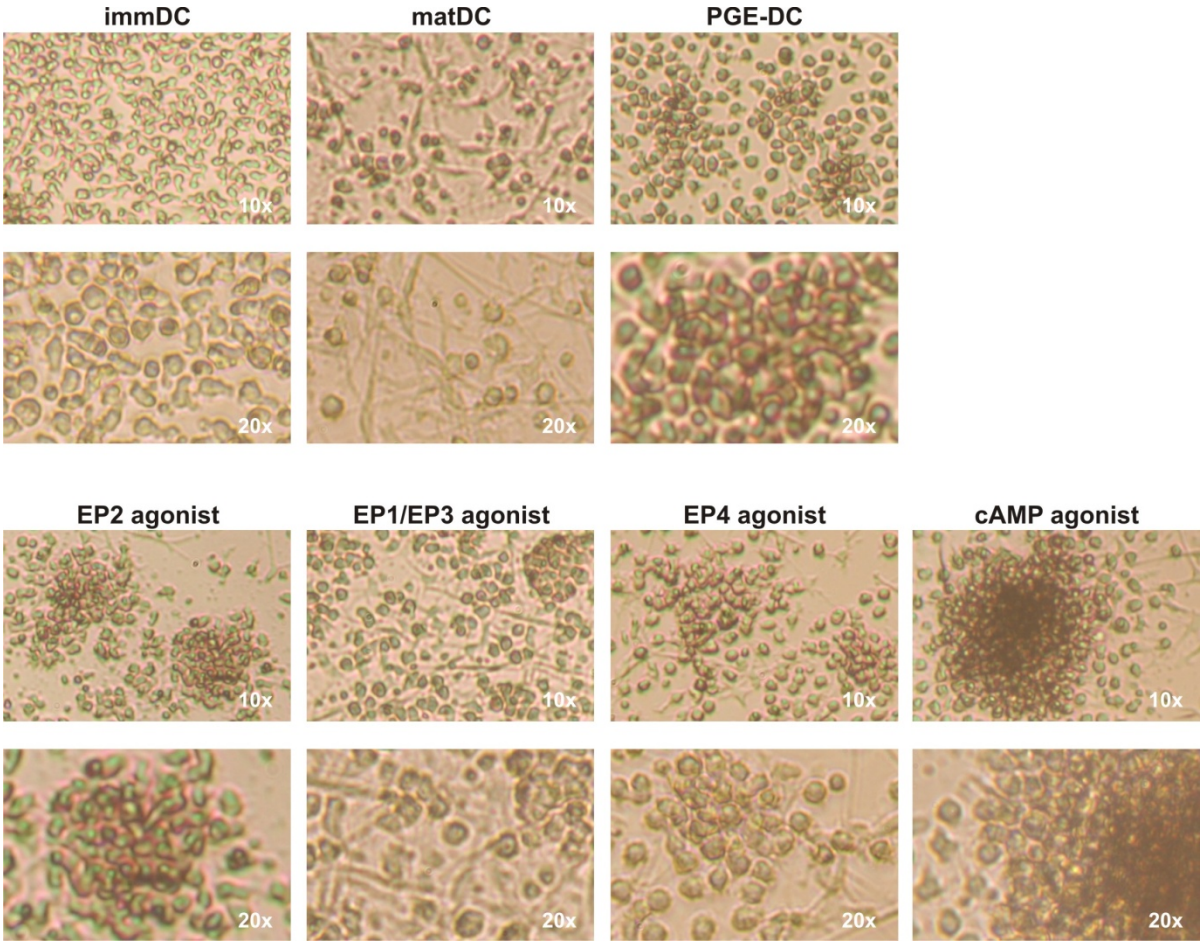


Figure 41: Morphology of DC treated with EP receptor agonists
Dendritic cells were stimulated with TNF alone or in combination with PGE_2, the EP2 agonist Butaprost, the EP1/EP3 agonist 17-phenyl trinor PGE_2 (17-PT-PGE_2), the EP4 agonist L000902688 or the adenylate cyclase/cAMP agonist Forskolin. The microscopic picture was taken with 10x and 20x magnification of DC after 72 hours. One representative experiment out of three is shown.

The morphology of the DC might be an indication but is surely not sufficient to draw any conclusions about the stimulatory or regulatory capacity of the DC. Therefore

expression of the established regulatory hallmark molecules, IDO and CD25, was analyzed. Comparable to PGE_2 treatment, IDO and CD25 are upregulated upon EP2, EP4 and adenylate cyclase activation while activation of EP1 and EP3 has no effect on IDO or CD25 expression.

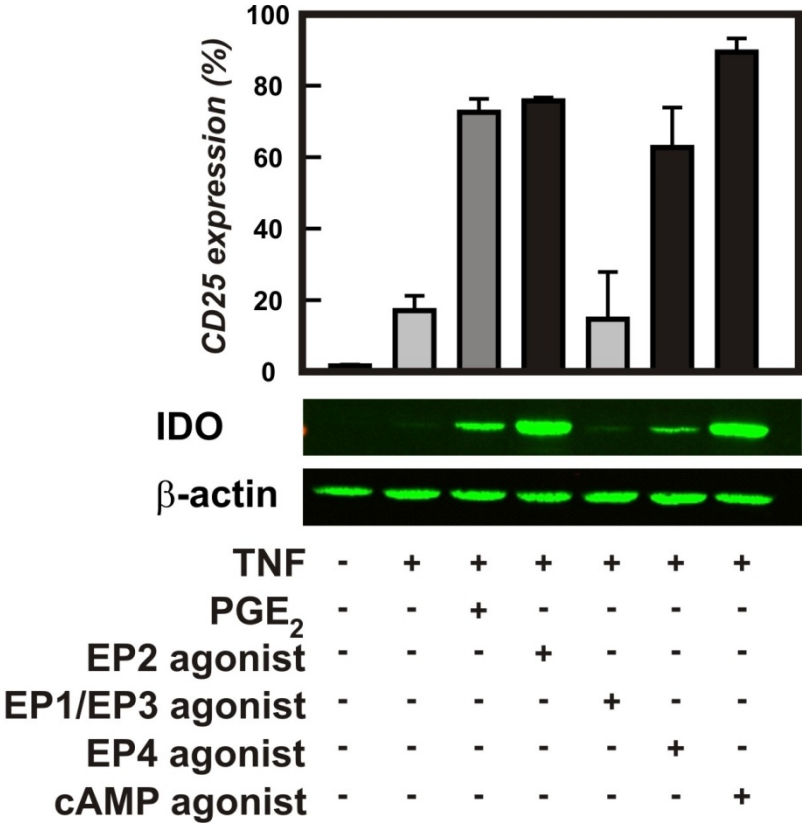


Figure 42: CD25 and IDO are induced by EP2 and EP4 receptor activation
Dendritic cells were treated with TNF alone or in combination with PGE_2, Butaprost (EP2 agonist), 17-PT-PGE_2 (EP1/EP3 agonist), L000902688 (EP4 agonist) or Forskolin (adenylate cyclase/cAMP agonist) for 72 hours. CD25 expression was analyzed by flow cytometry. Mean values and standard deviations of three independent experiments are shown. IDO protein expression was assessed by Western blotting. β-Actin served as loading control. One representative experiment out of three is shown.

6.5 Identification of downstream signaling molecules responsible for the induction of DCreg

6.5.1 Setup of biochemical pathway inhibition

To analyze the signaling pathways that are responsible for DCreg induction, specific cell-permeable inhibitors of downstream signaling molecules were used. Depending on the half-maximal inhibitory concentration (IC_{50}) as declared by the manufacturer, a concentration range of each inhibitor was used to stimulate dendritic cells in order to identify the right inhibitor concentration. To avoid that the specific signaling pathway is already activated by DC stimulation before the inhibitory effect is attained, DC were pre-incubated for one hour with each inhibitor before stimulation with PGE_2 and TNF.

To avoid prolonged exposure of DC to the inhibitors, readouts were performed after 24 hours of stimulation which appeared to be sufficient to measure induced expression of CD25, CD83 and IDO. Assessment of sCD25 amounts in supernatants of DC by ELISA was used to screen a wide range of inhibitor concentrations in order to find the most effective concentration without toxic effects. Since sCD25 is only secreted by $CD25^+$ DC and IL-2 deprivation by CD25/sCD25 is an essential inhibitory mechanism of DCreg, assessment of sCD25 appeared to be the most effective readout system.

6.5.2 Signaling events downstream of EP2 and EP4

EP1, EP2 and EP4 are coupled to stimulatory G-proteins (G_s-proteins) while EP3 is coupled to inhibitory G-proteins (G_i-protein). Binding of PGE_2 to EP1 is followed by activation of calcium-dependent signaling including activation of PIP_3 and release of intracellular Ca^{2+} as well as activation of PKC by DAG. In contrast, binding of PGE_2 to EP2 or EP4 results in activation of adenlate cyclase followed by increased cAMP levels and subsequent activation of protein kinase A (PKA). Binding of PGE_2 to the respective receptors also provokes the release of $G\beta$ and $G\gamma$ subunits, which stimulate Akt (protein kinase B) through phosphatidylinositol 3-kinase (PI3K). Gsk-3 is phosphorylated and inactivated by Akt, which leads to the stabilization, nuclear translocation and transcriptional activation of β-catenin.

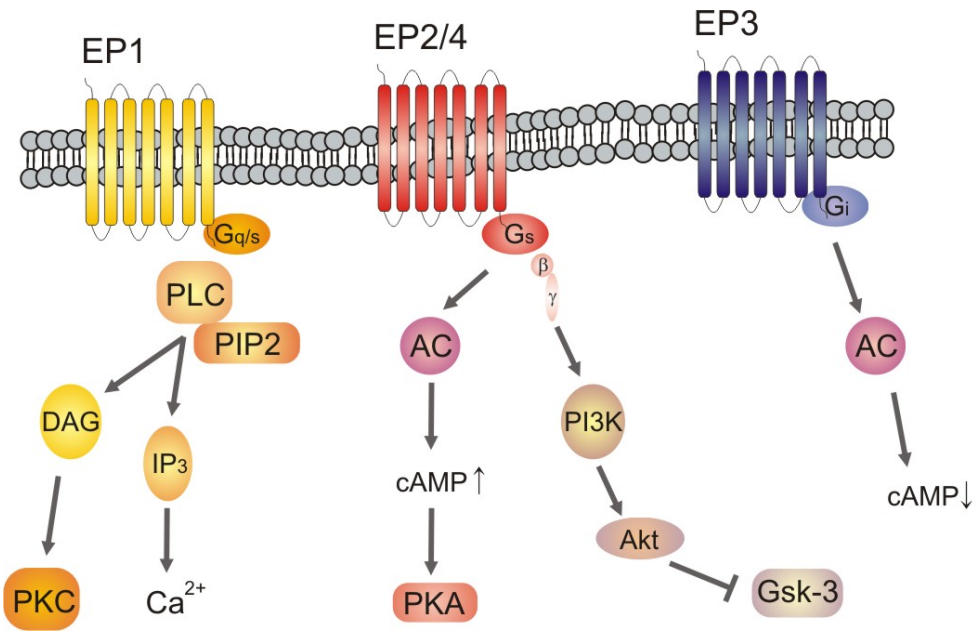


Figure 43: Known signaling events following EP receptor activation
EP receptors have seven transmembrane domains and couple to stimulatory (G_s) or inhibitory (G_i) G proteins. Binding of PGE_2 to EP1 leads to activation of PLC and PIP_2 resulting in release of Ca^{2+} and activation of PKC. Binding to EP2 results in activation of adenylate cyclase (AC) followed by increased cAMP levels and activation of PKA. The release of Gβ-and γ-subunits stimulates Akt through PI3K while Akt inhibits Gsk-3 functional activity. Binding of PGE_2 to EP3 activates inhibitory G proteins thereby decreasing the intracellular level of cAMP.

6.5.2.1 PKA is not involved in DCreg induction

Since induction of a regulatory DC phenotype could be mimicked by stimulation of EP2, EP4 and adenylate cyclase with specific agonists, it was obvious to analyze the role of the well-known downstream effector molecule protein kinase A (PKA) in DCreg induction.

For this purpose 4-Cyano-3-methylisoquinoline, a PKA specific cell-permeable inhibitor with an IC_{50} concentration of 30 nM was used. This inhibitor shows only weak inhibitory activity against Ca^{2+}-dependent protein kinase (CDPK), MLCK, PKC and cyclic nucleotide-binding phosphatase (Pase). Dendritic cells were pre-incubated with the PKA inhibitor in three different concentrations for one hour before stimulation with PGE_2 and TNF to induce the regulatory DC phenotype. Analysis of the DC phenotype revealed that inhibition of PKA has no influence on the induction of DC maturation (CD83), the induction of regulatory DC (CD25/sCD25) or DC viability (PI) (Figure 44).

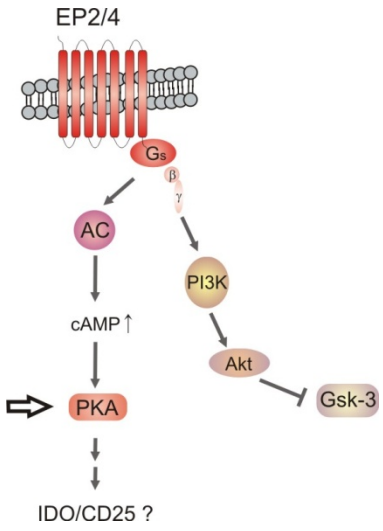
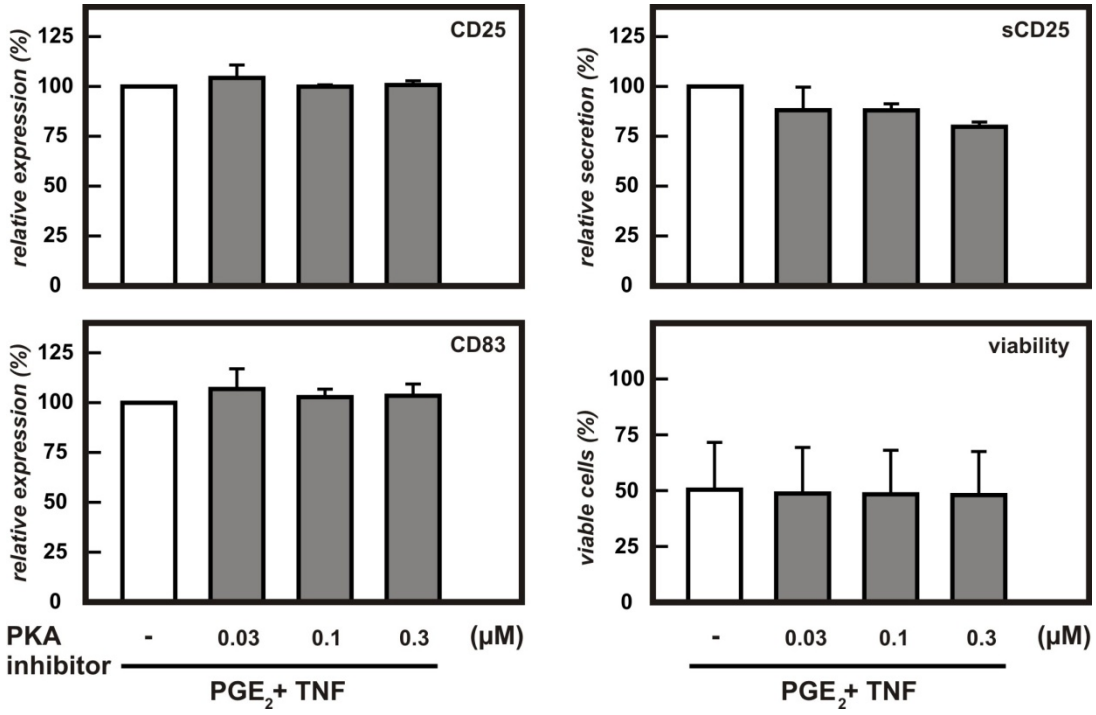


Figure 44: Inhibition of PKA does not alter DCreg phenotype
Dendritic cells were treated with different concentrations of the PKA inhibitior for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 was analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. Soluble CD25 was measured by ELISA. Mean expression values and standard deviations are shown from at least three indenpendent experiments relative to PGE_2-treated DC.



To rule out the possibility that the concentration of the PKA inhibitor was too low to significantly reduce PKA activity, higher concentrations of the inhibitor were used to stimulate DC before treatment with PGE_2 and TNF. Even more than 10-fold increase in the concentration of the PKA inhibitor did not significantly change the viability of DC (Figure 45A). Despite the high concentrations of the PKA inhibitor, there was only a slight but not a significant decrease in sCD25 secretion with a concentration up to 5 µM (Figure 45B).

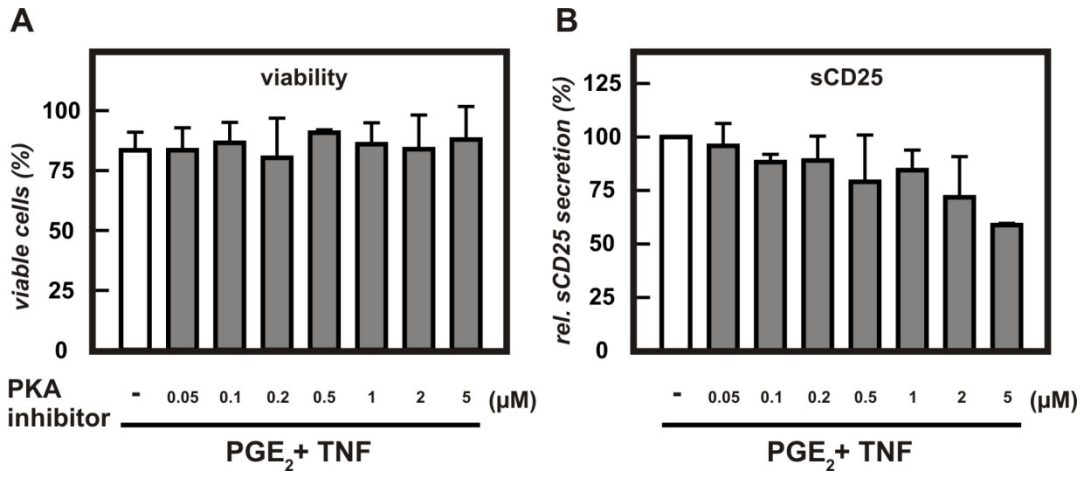


Figure 45: High concentrations of the PKA inhibitor do not affect DC viability or sCD25 secretion

Dendritic cells were treated with seven different concentrations of the PKA inhibitior for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. (**A**) The percentage of dead cells was analyzed by propidium iodide (PI) staining. Mean values and standard deviation s are shown of at least three indenpendent experiments. PGE_2 and TNF treated DC without inhibitor stimulation were used as control (white bar) (**B**) The amount of sCD25 in DC supernatants was measured by ELISA. Mean values and standard deviations are shown from at least three independent experiments relative to stimulation without inhibitor (white bar).

6.5.2.2 PKC is not involved in DCreg induction

Binding of PGE_2 to EP1 as well as activation of PKA can both result in the activation of protein kinase C (PKC). Therefore, DC viability and induction of a regulatory DC phenotype exemplified by sCD25 secretion were assessed after inhibition of PKC and subsequent stimulation with PGE_2 and TNF. The viability of the DC did not change significantly after stimulation with 5 to 100 nM of the PKC inhibitor compared to PGE_2 treatment alone (Figure 46A). As expected from the results with the EP1/EP3 agonist and the PKA inhibitor, inhibition of PKC did not have any influence on sCD25 secretion (Figure 46B). A concentration range of 5 nM to 100 nM did not significantly change the amount of sCD25 in the supernatants of DC compared to PGE_2 stimulation without addition of the PKC inhibitor.

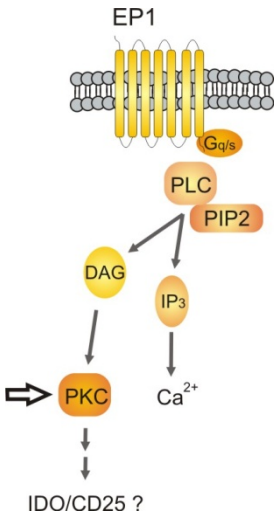
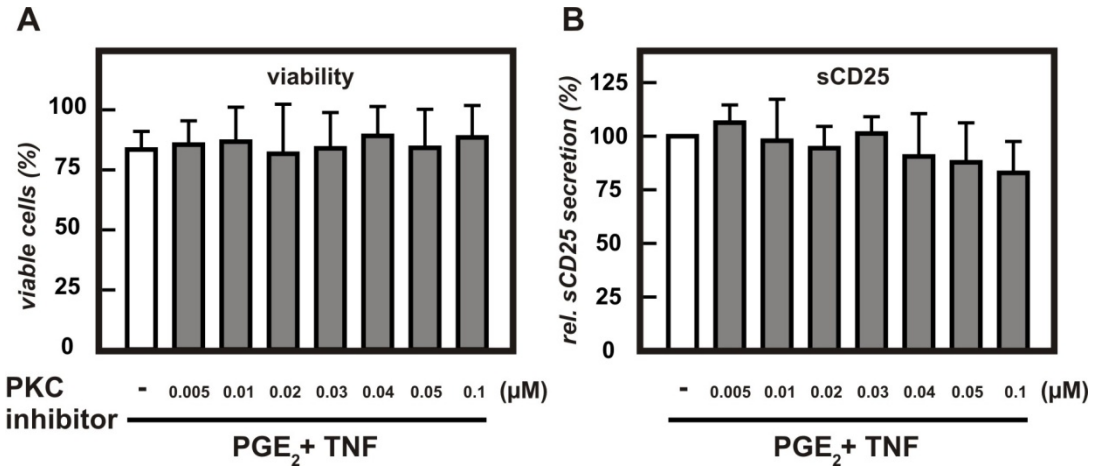


Figure 46: PKC inhibition does not influence DC viability or sCD25 secretion
Dendritic cells were treated with seven different concentrations of the PKC inhibitor for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours (grey bars). (**A**) The percentage of viable cells was calculated after propidium iodide (PI) staining. Mean values and standard deviations are shown from at least three indenpendent experiments. PGE_2 and TNF treated DC without inhibitor stimulation were used as control (white bar). (**B**) The amount of sCD25 in supernatants was measured by ELISA. Mean values and standard deviations from at least three independent experiments are shown relative to stimulation without inhibitor (white bar).



6.5.2.3 Inhibition of PI3K significantly decreases induction of IDO^+ $CD25^+$ DCreg by PGE_2

None of the so far analyzed major signaling molecules that are known to play a role in EP2/EP4 receptor mediated signaling are essential for DCreg induction since inhibition of these molecules did not significantly influence the expression of the established regulatory hallmark molecules. Besides, also maturation of the DC was not affected by inhibition of the respective signaling molecules.

Therefore we concentrated on analyzing the PI3K-Akt signaling pathway. First, the involvement of PI3K was analyzed by inhibition of PI3K signaling with the inhibitor LY294002. DC were pre-incubated with the PI3K inhibitor for one hour before stimulation with PGE_2 and TNF for additional 24 hours. Inhibition of PI3K significantly decreased expression of the regulatory molecules IDO and CD25 as well as expression of the maturation marker CD83 (Figure 47).

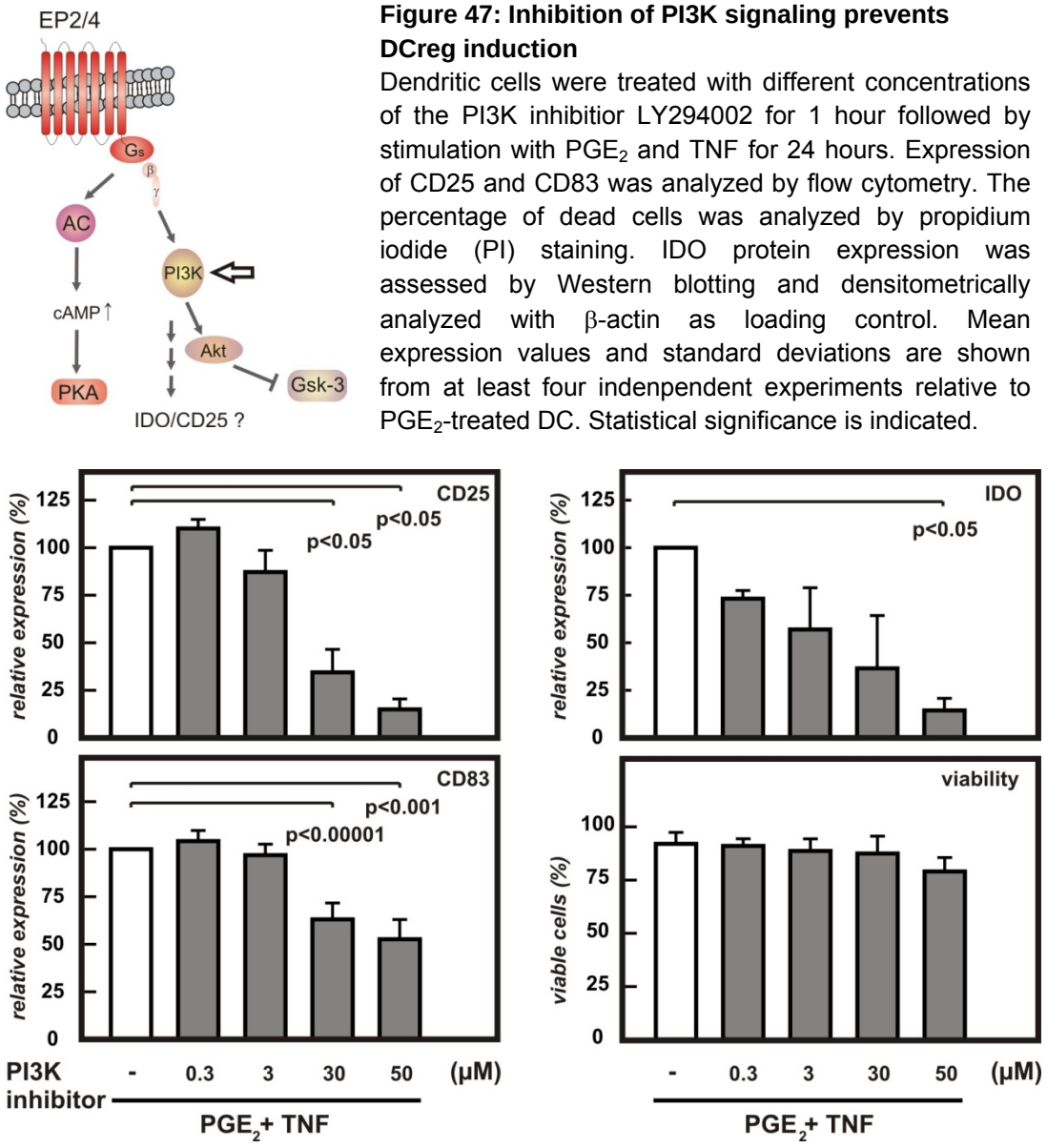


Figure 47: Inhibition of PI3K signaling prevents DCreg induction
Dendritic cells were treated with different concentrations of the PI3K inhibitior LY294002 for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 was analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. IDO protein expression was assessed by Western blotting and densitometrically analyzed with β-actin as loading control. Mean expression values and standard deviations are shown from at least four indenpendent experiments relative to PGE_2-treated DC. Statistical significance is indicated.

PI3K phosphorylates PIP_2 to generate PIP_3 as a second messenger to recruit and activate downstream targets including the protein kinase Akt (PKB) (Weichhart and Saemann, 2009) which is involved in regulating a wide array of cellular processes, including metabolism, growth, proliferation and apoptosis (Brazil et al., 2004). Because a role of PI3K in DCreg induction has been demonstrated, it was reasonable to evaluate the involvement of Akt, one of its main targets. Inhibition of Akt reduced the expression of CD25 and CD83 as well as the secretion of sCD25 (Figure 48). At the same time starting at a concentration of 2 µM, the Akt inhibitor exerted toxic effects thereby doubling the number of dead cells during the 24 hours of stimulation.

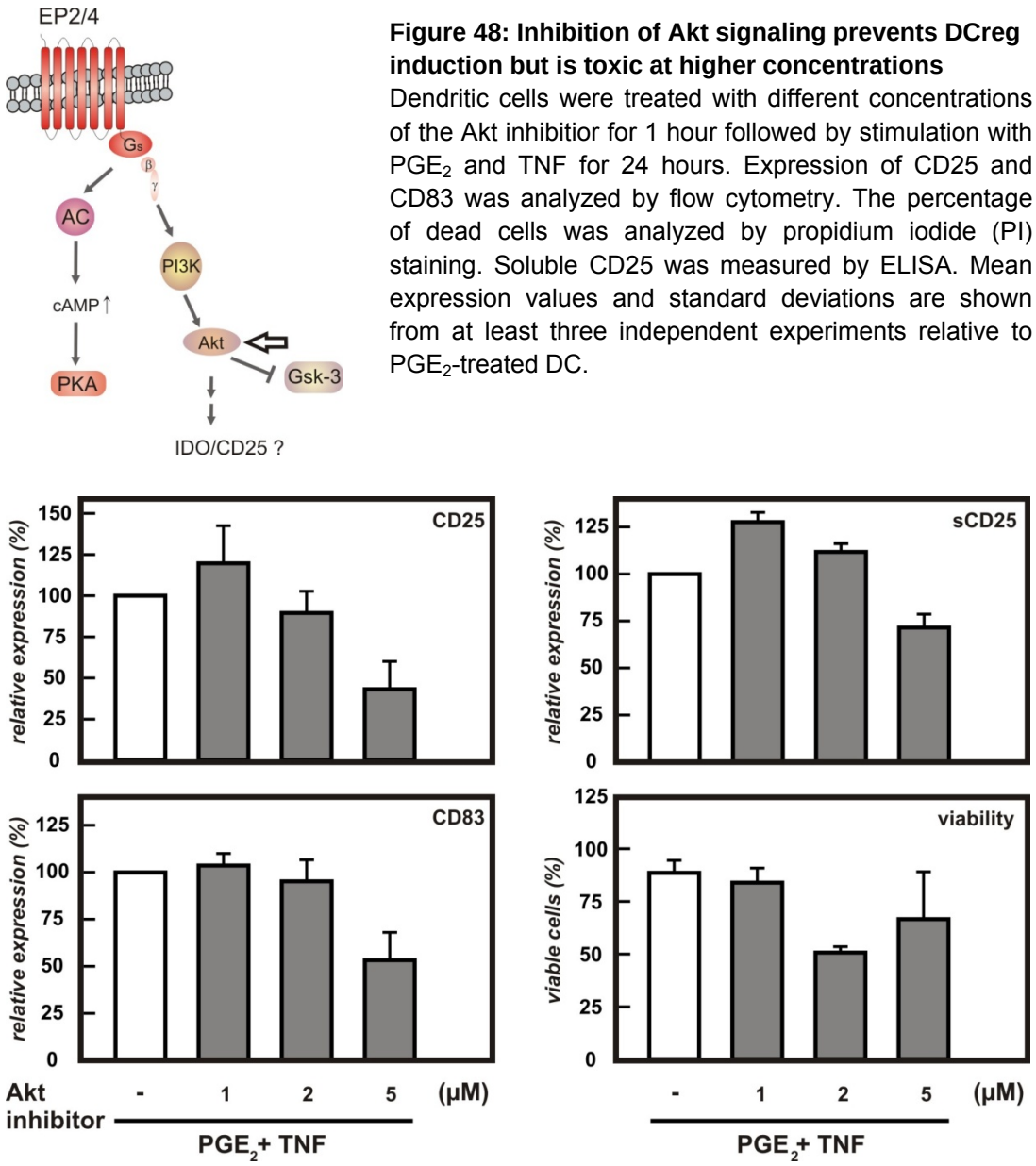


Figure 48: Inhibition of Akt signaling prevents DCreg induction but is toxic at higher concentrations
Dendritic cells were treated with different concentrations of the Akt inhibitior for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 was analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. Soluble CD25 was measured by ELISA. Mean expression values and standard deviations are shown from at least three independent experiments relative to PGE_2-treated DC.

Since the Akt inhibitor decreased the number of viable cells by half starting with a concentration of 2 µM, lower concentrations of the inhibitor were used to stimulate DC before treatment with PGE_2 and TNF. In the second set of experiments the Akt inhibitor exerted toxic effects already at a concentration of 0.5 µM (Figure 49A). Using the same concentration range of the Akt inhibitor, the secretion of sCD25 was decreased only if DC were treated with the toxic concentration of 1 µM while lower concentrations did not affect sCD25 secretion (Figure 49B).

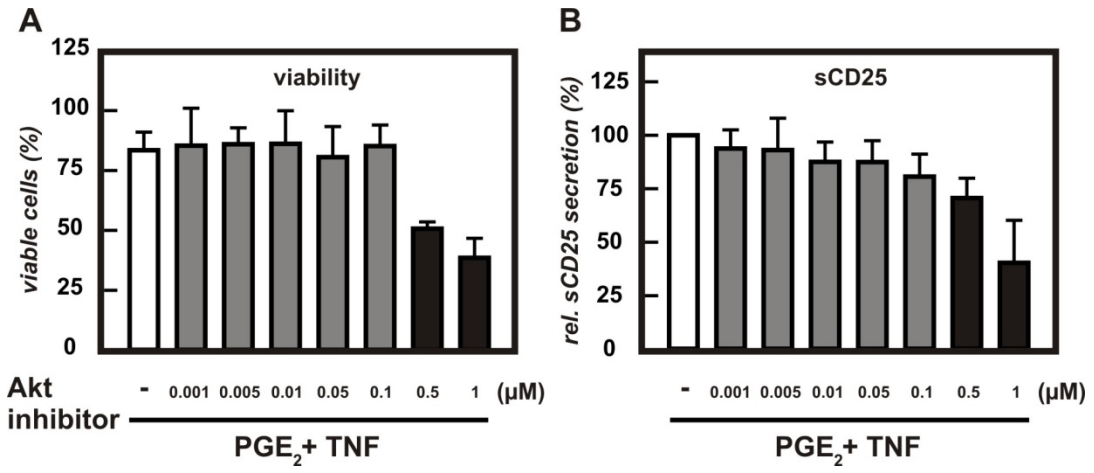


Figure 49: Inhibition of Akt signaling has toxic effects
Dendritic cells were treated with seven different concentrations of the Akt inhibitior for 1 hour followed by stimulation with PGE_2 and TNF for 24h. (**A**) The percentage of viable cells was calculated after propidium iodide (PI) staining. Mean values and standard deviations are shown from at least three indenpendent experiments. PGE_2 and TNF treated DC without inhibitor stimulation were used as control (white bar). (**B**) The amount of sCD25 in supernatants was measured by ELISA. Mean values and standard deviations from at least three independent experiments are shown relative to stimulation without inhibitor (white bar).

6.5.2.4 Inhibition of Gsk-3 increases CD25 but decreases IDO expression by DCreg

Previous publications suggested a role of Gsk-3 in EP2 and EP4 mediated signal transduction. Fujino et al. showed that PGE_2 via the EP2 and EP4 receptor facilitates Gsk-3 phosphorylation, yet both receptors are using different downstream signaling molecules. EP2 mediates Gsk-3 phosphorylation via activation of PKA while EP4 employs the PI3K-Akt pathway (Fujino et al., 2002). Phosphorylation of Gsk-3 and thereby inhibition of Gsk-3 kinase activity prevents phosphorylation and subsequent degradation of cytoplasmic β-catenin which is then stabilized and translocated to the nucleus where it can interact with members of the Tcf/Lef family of transcription factors to alter gene expression (Peifer and Polakis, 2000). To establish a possible link between the induction of regulatory DC and EP2/EP4 mediated Gsk-3 phosphorylation, we blocked Gsk-3 during DCreg induction with PGE_2 and TNF. Interestingly, inhibition of Gsk-3 significantly increased the expression of CD25 by PGE_2 treated DC while decreasing IDO and CD83 expression (Figure 49). At the same time there was no significant effect of the Gsk-3 inhibitor on DC viability.

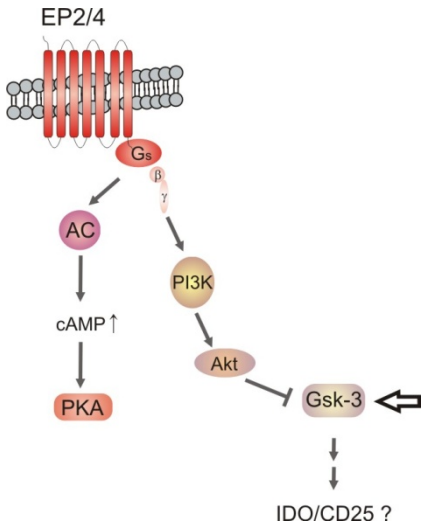
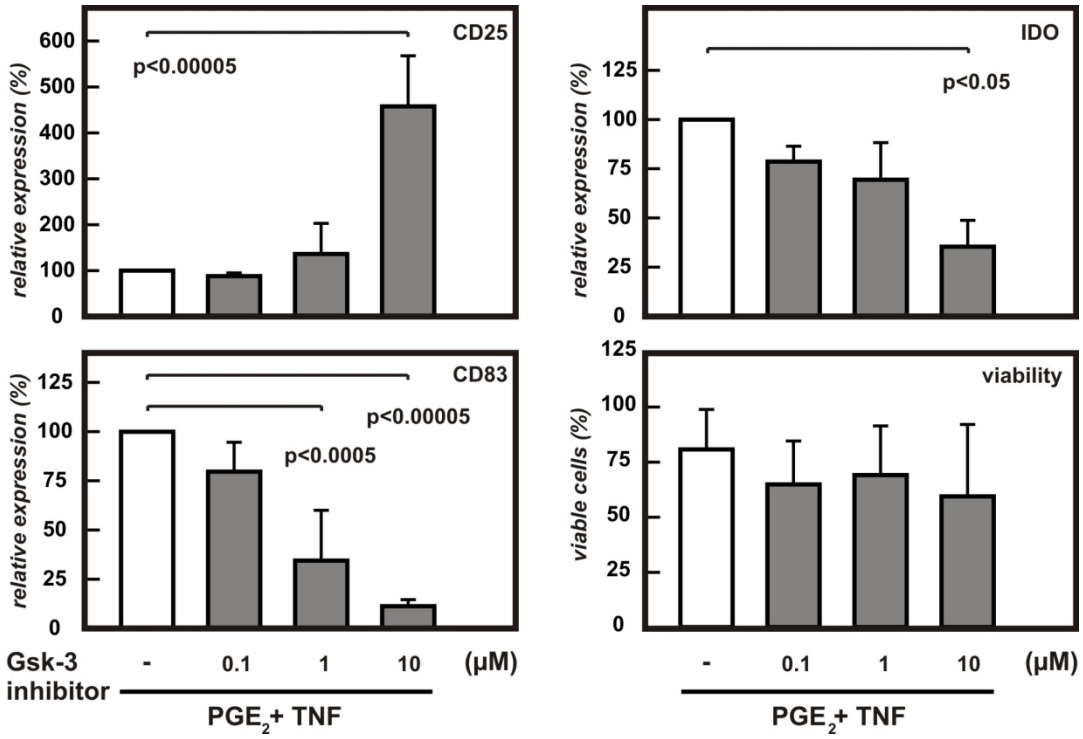


Figure 50: Inhibition of Gsk-3 increases expression of CD25 while suppressing IDO induction and maturation

Dendritic cells were treated with different concentrations of the Gsk-3 inhibitior for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 was analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. IDO protein expression was assessed by Western blotting and densitometrically analyzed with β-actin as loading control. Mean expression values and standard deviations are shown from at least four indenpendent experiments relative to PGE_2-treated DC.



Another signaling molecule which is downstream of Akt is the MAP kinase p38. Acitvation of Akt results in the blockade of MAP kinases upstream of p38. Since Akt is positively regulated by PI3K (Toker and Cantley, 1997), inhibition or lack of PI3K upregulates p38 activity (Fukao et al., 2002). If the PI3K-Akt pathway is involved in the induction of DCreg by PGE_2 and TNF, inhibition of p38 should therefore not result in decreased expression of regulatory molecules. The role of p38 in DCreg induction was analyzed with a specific p38 inhibitor. Induction of the regulatory molecules IDO and CD25 was slightly decreased by p38 inhibition while there was no effect on CD83 surface expression (Figure 51). In contrast to treatment with the Akt inhibitor, DC viability was not impaired by p38 inhibition.

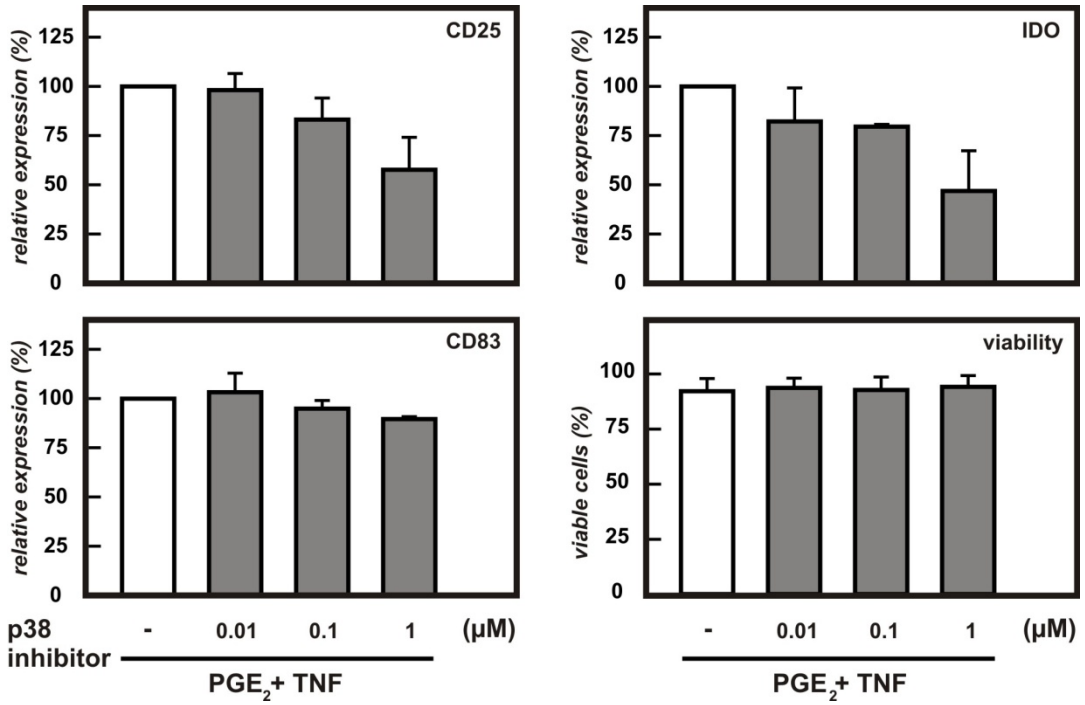


Figure 51: Inhibition of p38 slightly decreases the number of IDO$^+$ CD25$^+$ DCreg
Dendritic cells were treated with different concentrations of the p38 inhibitor for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 was analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. IDO protein expression was assessed by Western blotting and densitometrically analyzed with β-actin as loading control. Mean expression values and standard deviations are shown from at least three independent experiments relative to PGE_2-treated DC.

6.5.3 Signaling events downstream of TNFRI

Induction of IDO and CD25 by treatment with PGE_2 and TNF was dependent on TNF receptor I. TNFRI is a death-domain (DD) containing receptor which can activate caspase cascades via DD-containing signaling intermediates, leading to apoptosis. TNFRII does not contain a cytoplasmic DD and cannot directly engage the apoptotic machinery, yet both TNF receptors can independently activate nuclear translocation of the transcription factor NFκB (Thommesen and Laegreid, 2005) which is associated with cellular activation, differentiation, cytokine production and survival signaling (Aggarwal, 2003; Hehlgans and Pfeffer, 2005; Mocellin et al., 2005). Binding of TNF to TNFRI allows binding of TRADD (TNFRI-associated death domain protein) to the DD and recruits additional adapter proteins such as RIP1 (receptor interacting protein), TRAF2 (TNF receptor associated factor 2) and cIAP1 (cellular inhibitor of apoptosis 1) to form a complex which signals via NIK (NFκB inducing kinase) and MEKK1 (MAPK kinase-1) resulting in the phosphorylation of IκB by IKK-α/IKK-β and subsequent proteolytic degradation of IκB followed by translocation of

NFκB to the nucleus (Karin and Ben-Neriah, 2000). JNK participates in the regulation of gene expression by TNF by enhancing the function of transcription factors, of which AP-1 and c-Jun are the most thoroughly studied (Wallach et al., 1999).

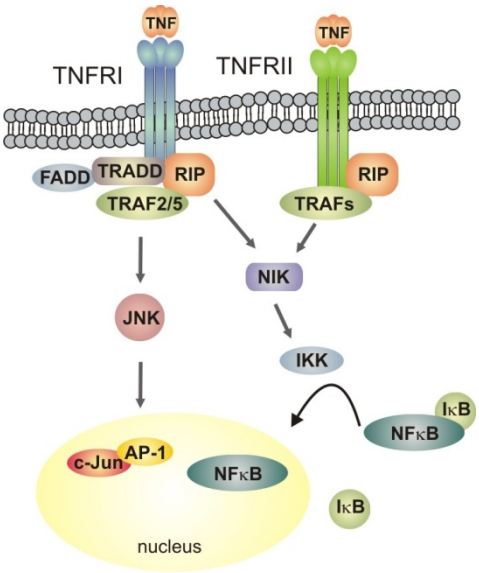


Figure 52: Known signaling events following TNF receptor activation
Binding of TNF to TNFRI allows binding of TRADD to the receptor associated death-domain which recruits additional adapter proteins such as RIP and TRAFs. Binding of TNF to TNFRII also results in the formation of a receptor-associated complex containing TRAFs and RIP which signals via NIK and MEKK1 resulting in the phosphorylation of IκB by IKKs and subsequent proteolytic degradation of IκB followed by translocation of NFκB to the nucleus. JNK participates in TNFRI associated signaling by enhancing the function of transcriptions factors AP-1 and c-Jun.

6.5.3.1 NFκB is not involved in DCreg induction

First, the role of NFκB, the major transcription factor of TNF associated signaling, in DCreg induction by PGE_2 and TNF was analyzed. Inhibition of NFκB does not alter PGE_2 and TNF induced CD25 and CD83 surface expression (Figure 53). In contrast, with increasing concentrations of the NFκB activation inhibitor the amount of soluble CD25 slightly decreased compared to stimulation without the inhibitor. Furthermore, the number of dead cells after PGE_2 and TNF stimulation slightly decreases by additional NFκB inhibition.

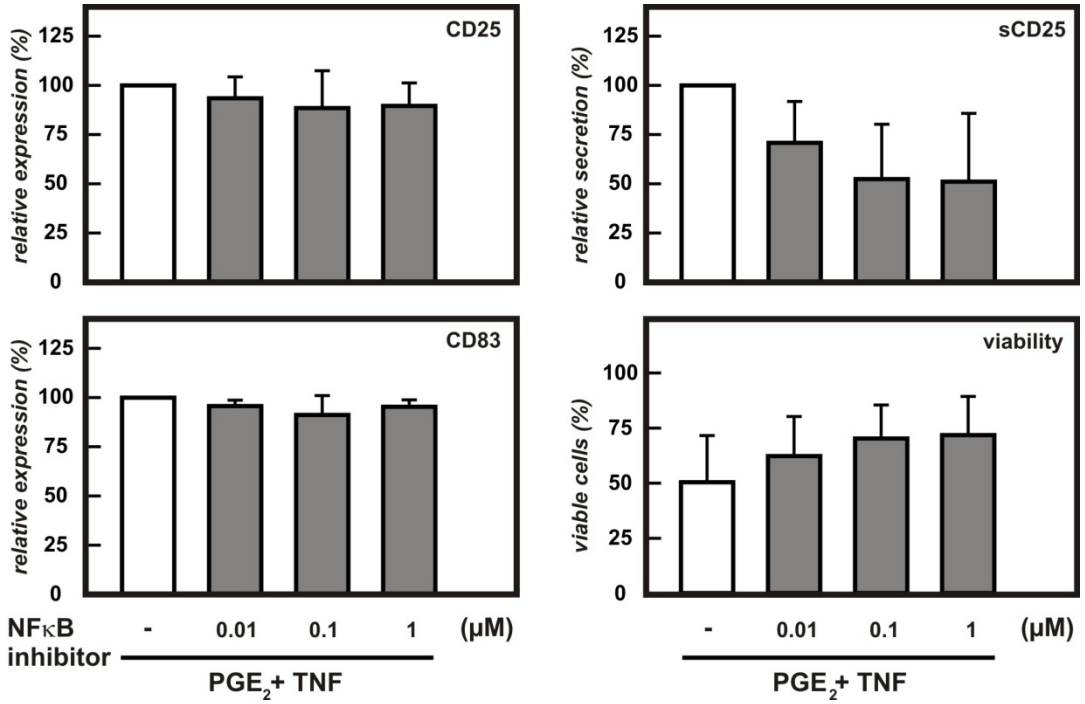


Figure 53: Inhibition of NFκB does not alter DCreg phenotype
Dendritic cells were treated with different concentrations of the NFκB activation inhibitor for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 was analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. Soluble CD25 was measured by ELISA. Mean expression values and standard deviations are shown from at least three independent experiments relative to PGE_2-treated DC.

To figure out if the reduction of sCD25 secretion might reach a significant level when using higher concentrations of the NFκB inhibitor, DC were pre-stimulated with seven different concentrations ranging from 10 nM to 50 μM. The survival rate of DC was not significantly changed after treatment with 10 nM to 10 μM of the NFκB inhibitor compared to PGE_2 treatment alone while 50 μM of the inhibitor were not compatible with cell survival (Figure 54A). The secretion of sCD25 is slightly decreased with an NFκB inhibitor concentration of 100 nM but is not further decreased by higher

concentrations of the inhibitor (Figure 54B). The amount of sCD25 in DC supernatants remains at the same level with an NFκB inhibitor concentration ranging from 100 nM to 10 µM which is slightly lower than with PGE_2 treatment alone. DC stimulated with a concentration of 50 µM NFκB inhibitor which is highly toxic for the DC certainly also terminates CD25 expression and secretion.

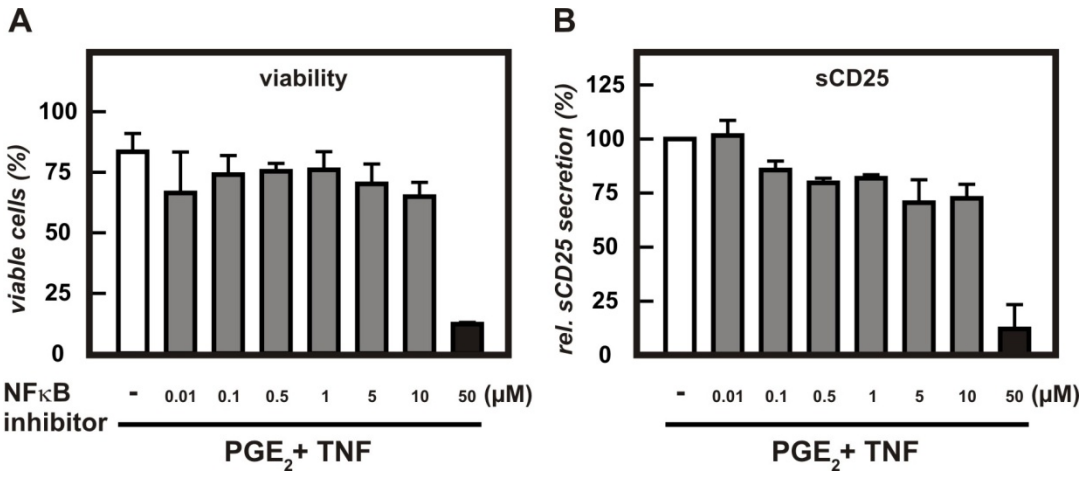


Figure 54: NFκB inhibition does not influence DC viability or sCD25 secretion
Dendritic cells were treated with seven different concentrations of the NFκB inhibitor for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. (**A**) The percentage of viable cells was calculated after propidium iodide (PI) staining. Mean values and standard deviations are shown from at least three independent experiments. PGE_2 and TNF treated DC without inhibitor stimulation were used as control (white bar). (**B**) The amount of sCD25 in supernatants was measured by ELISA. Mean values and standard deviations from at least three indenpendent experiments are shown relative to stimulation without inhibitor (white bar).

6.5.3.2 JNK is not involved in DCreg induction

To investigate if inhibition of JNK would affect the induction of regulatory DC, DC were stimulated with PGE_2 and TNF after blockade of JNK with a specific inhibitor. None of the used inhibitor concentrations significantly influenced expression of CD25, sCD25 or CD83 and cell viability was not affected (Figure 55).

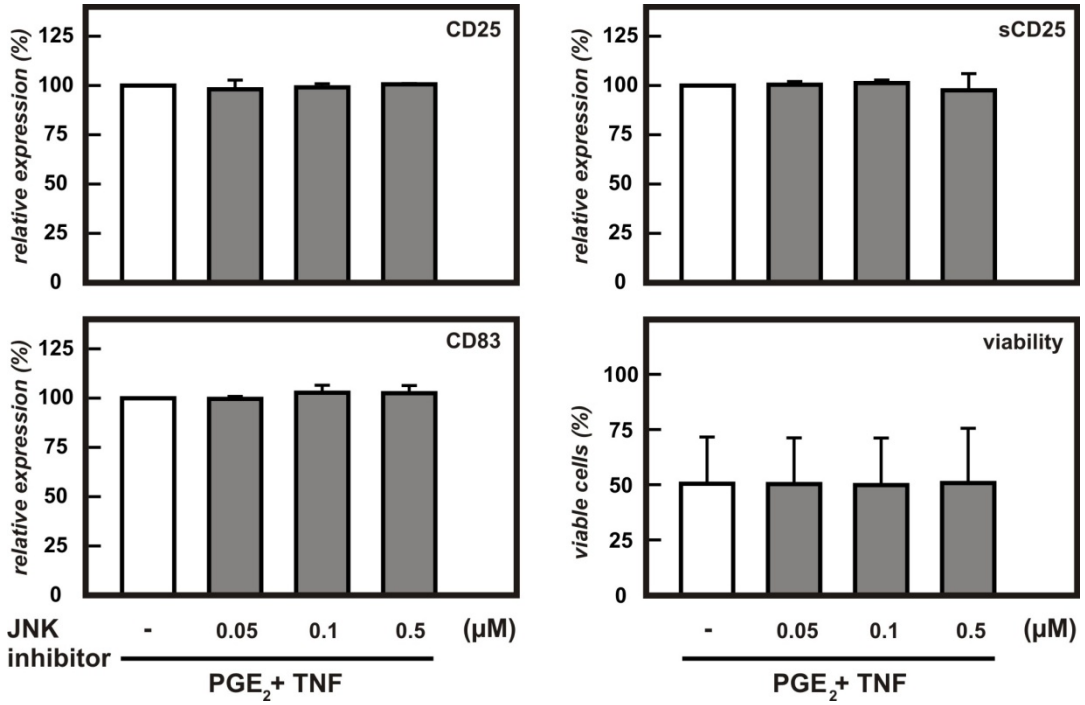


Figure 55: Inhibition of JNK does not alter DCreg phenotype
Dendritic cells were treated with different concentrations of the JNK inhibitor for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 was analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. Soluble CD25 was measured by ELISA. Mean expression values and standard deviations are shown from at least three independent experiments relative to PGE_2-treated DC.

If using higher concentrations of the JNK inhibitor, DC viability is affected starting at a concentration of 50 µM (Figure 56A). With the same concentration range of the JNK inhibitor, sCD25 secretion was only decreased using toxic inhibitor concentrations (Figure 56B).

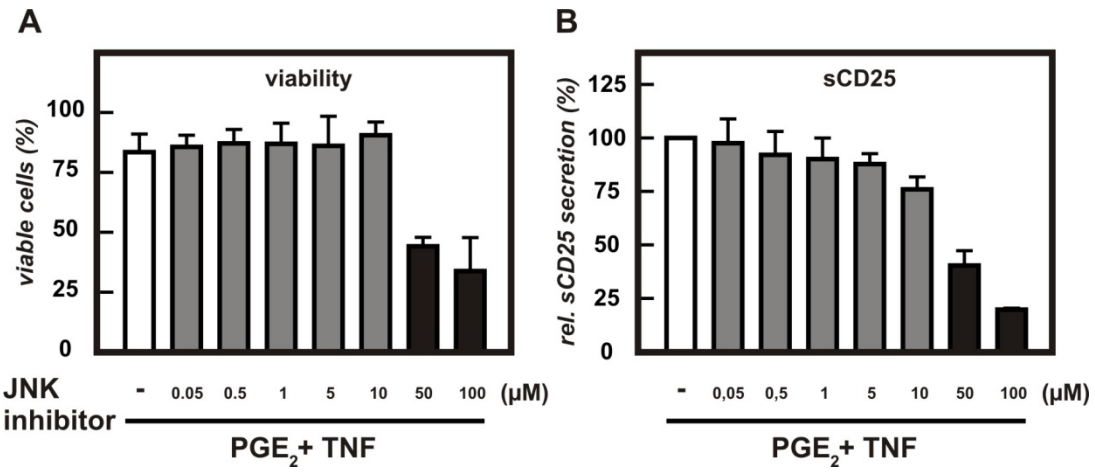


Figure 56: JNK inhibition influences DC viability at 50 µM
Dendritic cells were treated with seven different concentrations of the JNK inhibitor for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. (**A**) The percentage of viable cells was calculated after propidium iodide (PI) staining. Mean values and standard deviations are shown from at least three independent experiments. PGE_2 and TNF treated DC without inhibitor stimulation were used as control (white bar). (**B**) The amount of sCD25 in supernatants was measured by ELISA. Mean values and standard deviations from at least three indenpendent experiments are shown relative to stimulation without inhibitor (white bar).

6.5.3.3 Inhibition of Jak molecules but not STAT3 significantly decreases induction of DCreg

Many previous studies emphasized the role of IFN-γ in DCreg induction (Hwu et al., 2000; Munn et al., 2002). Yet, Popov et al. demonstrated that IFN-γ expression is induced later during infection compared to TNF, which was induced within the first 2 hours after infection (Popov et al., 2006). Similarly, IDO and CD25 gene expression were induced before IFN-γ expresssion. Therefore, induction of the respective regulatory molecules is in the first line independent of IFN-γ while it cannot be excluded that IFN-γ influences DCreg induction at later points in time. Also, we could show that IDO expression by listeria-infected DC and subsequent kynurenine accumulation were significantly decreased following DC treatment with neutralizing anti-IFN-γ antibodies.

The Jak/STAT pathway is commonly used by various cytokine receptors and plays an important role in the regulation of immune responses. Until now, four members of the Jak family (Stark et al., 1998), a subgroup of the non-receptor associated tyrosine kinases and seven STAT family members were identified (Copeland et al., 1995; Darnell, 1997). The receptor associated Jaks are activated upon cytokine binding and subsequently phosphorylate the STAT transcriptions factors. Phosphorylated STATs dimerize and translocate to the nucleus where they regulate gene transcription. Recent publications linked the Jak/STAT pathway also with TNF receptor associated signaling (Kumar et al., 1997).

To clarify if Jak/STAT signaling pathways might also play a role in DCreg induction, expression of IDO and CD25 was analyzed after inhibition of Jaks and STAT3 with the Jak/STAT3 inhibitor Cucurbitacin I. Surprisingly, inhibition of Jaks and STAT3 significantly decreased IDO and CD25 expression (Figure 57). At the same time, induction of maturation as measured by surface expression of CD83 was significantly decreased while none of the used inhibitor concentrations significantly affected DC viability.

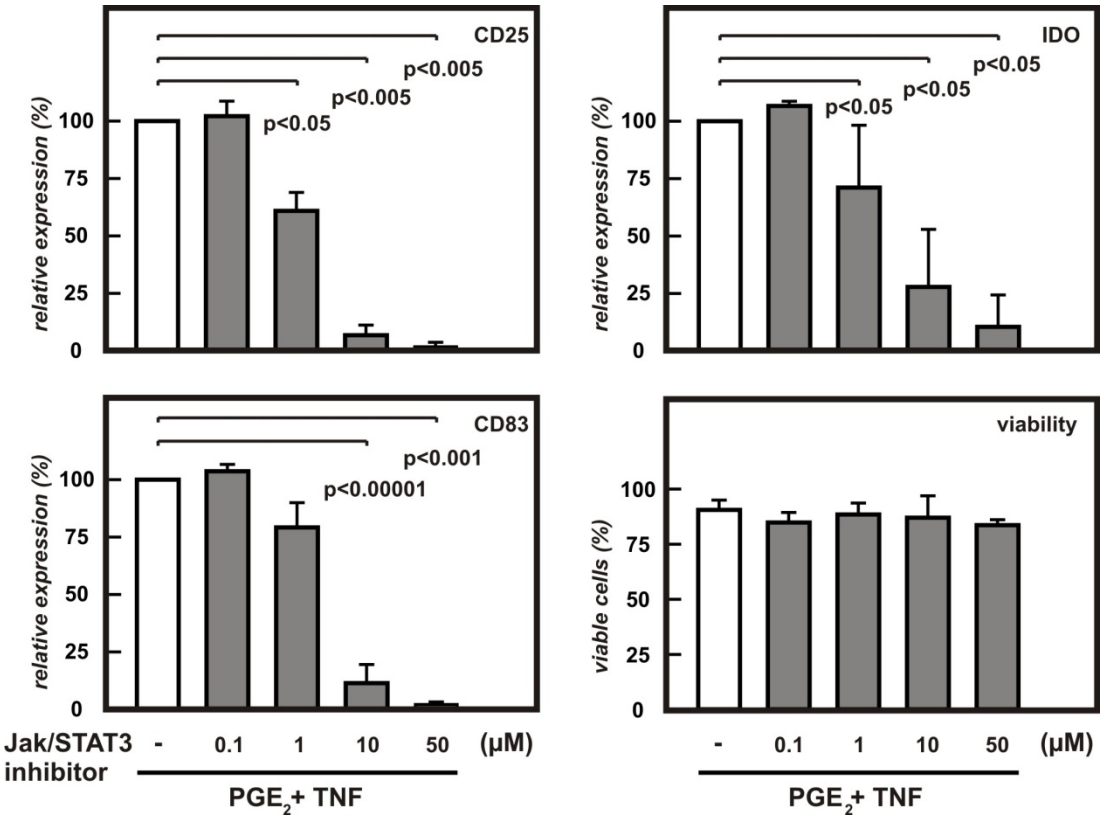


Figure 57: Inhibition of Jak/STAT3 signaling prevents DCreg induction
Dendritic cells were treated with different concentrations of the Jak/STAT3 inhibitor Cucurbitacin I for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 was analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. IDO protein expression was assessed by Western blotting and densitometrically analyzed with β-actin as loading control. Mean expression values and standard deviations are shown from at least four independent experiments relative to PGE_2-treated DC. Statistical significance is indicated.

Activation of associated STATs is not the exclusive effect of Jak activation. Jak proteins also play a role in further STAT-independent pathways, e.g. upstream of the PI3K signaling pathway (Uddin et al., 1997; Uddin et al., 2000). It was clearly shown that Jak/STAT3 signaling as well as PI3K signaling play a role in DCreg induction. To elaborate if inhibition of Jaks or STAT3 is responsible for the effect of the Jak/STAT3 inhibitor, DC were stimulated with a STAT3 specific inhibitor before PGE_2 treatment without additional inhibition of Jaks. Inhibition of STAT3 does not change expression of IDO, CD25, CD83 or DC viability while STAT3 tyrosine phosphorylation at position 705 is decreased with increasing concentrations of the inhibitor.

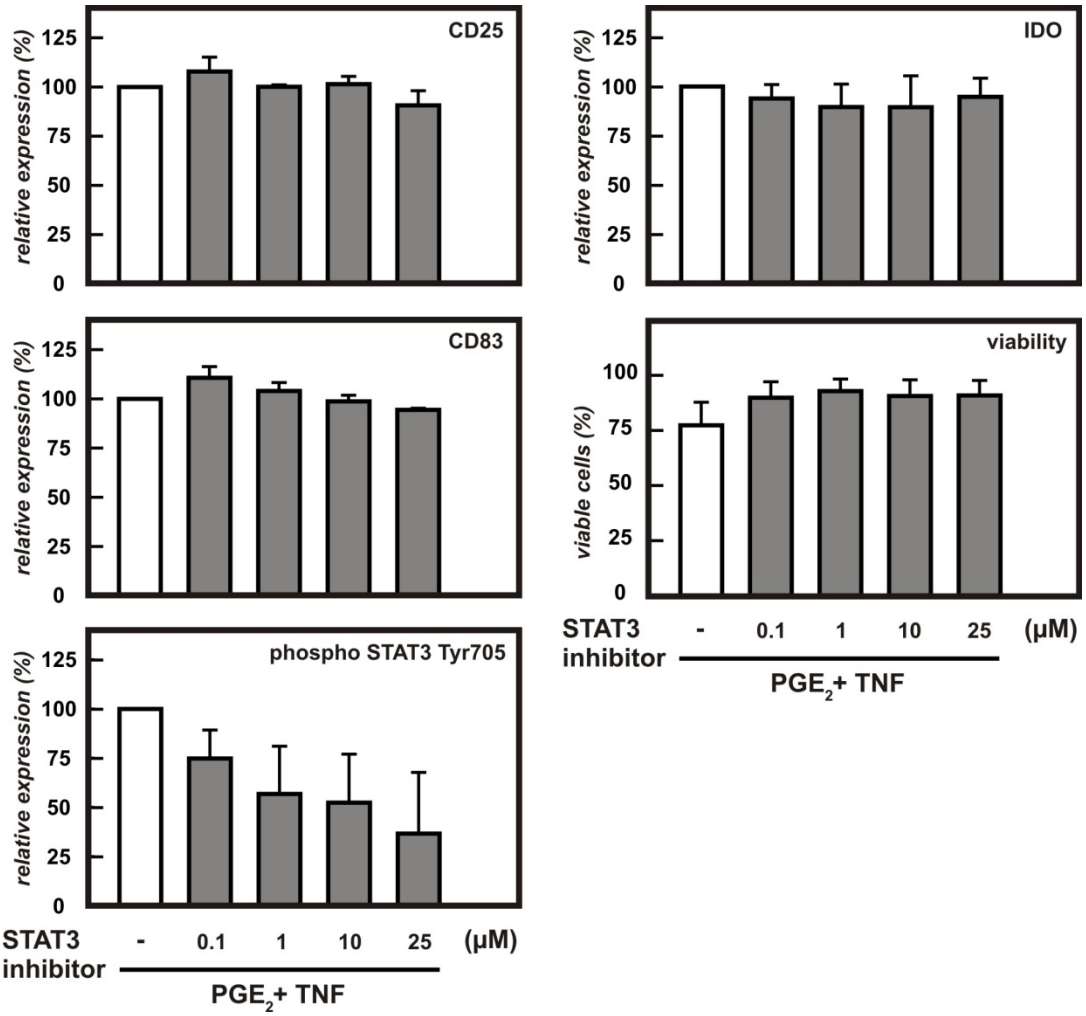


Figure 58: Inhibition of STAT3 has no effect on DCreg induction
Dendritic cells were treated with different concentrations of the STAT3 inhibitor for 1 hour followed by stimulation with PGE_2 and TNF for 24 hours. Expression of CD25 and CD83 were analyzed by flow cytometry. The percentage of dead cells was analyzed by propidium iodide (PI) staining. IDO and phosphorylated STAT3 (Tyr705) protein expression was measured by Western blotting and densitometrically analyzed with β-actin as loading control. Mean expression values and standard deviations from at least three independent experiments are shown relative to PGE_2-treated DC. Statistical significance is indicated.

7 Discussion

7.1 The regulatory DC phenotype

Regulatory or tolerogenic DC have been implicated to play an important role in the induction of tolerance to tumor associated antigens (Steinman et al., 2003) and during infections (Popov et al., 2006). Yet, no molecular marker existed so far which allowed to distinguish between DC with stimulatory or regulatory function in different disease settings.

PGE_2 is a soluble factor produced by tumor cells and cells of the immune system, e.g. dendritc cells, and its concentration is increased in the microenvironment of different tumor entities. Besides its direct suppressive effects on T cells by early interference with T-cell receptor mediated signaling (Chemnitz et al., 2006), it was recently shown that PGE_2 also induces DC with regulatory properties which are characterized by expression of the immunomodulatory enzyme IDO (von Bergwelt-Baildon et al., 2006). This is in contrast to multiple reports that linked enhanced antigen presentation and migration to secondary lymphoid organs with PGE_2 (Jonuleit et al., 1997; Luft et al., 2002; Scandella et al., 2004). In order to clarify this divergence, the influence of PGE_2 on DC phenotype and functional properties was further analyzed.

In this study it was shown that stimulation of mo-DC with PGE_2 alone is not sufficient to induce DC maturation or expression of regulatory molecules. Only in combination with the inflammatory stimulus TNF, PGE_2 induced co-expression of IDO and CD25 by DC (Figures 30 and 31). These IDO^+ $CD25^+$ DC at the same time present a full mature phenotype including expression of the maturation marker CD83 (Figure 5B) and the co-stimulatory molecules CD80 and CD86 (Figure 27). TNF treatment alone resulted in comparable expression of the respective maturation markers, yet IDO and CD25 were not induced. Additional PGE_2 treatment therefore seems to account for additional expression of IDO and CD25 in TNF-matured DC while both factors are not sufficient separately. The previously described enhanced co-stimulatory and migratory capacity of DC stimulated with PGE_2 is therefore supported by the mature phenotype of PGE_2-treated DC in this study. Yet, the additional expression of regulatory molecules might have an influence on the T-cell stimulatory capacity of DC despite enhanced co-stimulation and migration. Thus, IDO^+ $CD25^+$ mature DC were

compared to IDO^- $CD25^-$ mature DC in their ability to induce T-cell proliferation. Mature IDO^+ $CD25^+$ DC clearly decreased T-cell proliferation in comparison to mature IDO^- $CD25^-$ DC (Figures 12 and 13) and were therefore termed mature regulatory DC (DCreg). In contrast to regulatory DC with an immature or semi-mature phenotype which can be separated from stimulatory DC by the lack of maturation and co-stimulatory molecules or additional secretion of inhibitory cytokines, respectively, the discrimination between mature regulatory and mature stimulatory DC is much more difficult. Yet, several other studies reported the induction of regulatory DC which express a full mature phenotype which is consistent with the results of this study (Grohmann et al., 2003; Munn et al., 2002).

A comparison of gene expression including a variety of genes that were previously associated with stimulatory or inhibitory DC function revealed that TNF stimulated DC (mature DC) and TNF plus PGE_2 stimulated DC (PGE_2-treated DC) show a similar expression pattern of the analyzed genes which clearly distinguishes these mature DC from immature DC (Figure 4). On the other hand, only a few genes are differentially regulated through additional PGE_2 treatment, among these INDO and IL2RA, the genes encoding IDO and CD25, are upregulated compared to mature DC (von Bergwelt-Baildon et al., 2006). Despite substantial differences in the transcriptional profile, PGE_2-treated and listeria-infected DC share upregulation of IDO and CD25 genes (Figure 4). Transcriptional analysis was confirmed on the protein level and revealed co-expression of the enzyme IDO and the α-chain of the IL-2 receptor, CD25, in PGE_2-treated as well as listeria-infected DC. Both molecules could therefore serve as hallmarks for DCreg independent of the inducing factor and allowed to distinguish between stimulatory and regulatory DC in this study. PGE_2-treated as well as listeria-infected DC are therefore further termed IDO^+ $CD25^+$ DCreg unless markable differences between both models of DCreg account for their separate mentioning. Compatible to the expression of CD25 on the surface, substantial amounts of sCD25 were detected only in supernatants of DCreg (Figure 7). Likewise, toxic tryptophan metabolites were detected only in supernatants of DCreg resembling the enzymatic activity of IDO and proving IDO´s functional activation in DCreg (Figure 6).

The expression of additional inhibitory or stimulatory molecules like IL-10 and COX-2 was dependent on the inducing stimuli and varied between the two different models of regulatory DC. Corinti et al. reported low levels of IL-10 in supernatants of

unstimulated, immature DC (Corinti et al., 2001). In contrast, IL-10 was not measurable in supernatants of immature DC in this study (Figure 9A), therefore autocrine IL-10 effects on DC maturation can be excluded. This observed difference might be due to the different culture media used in both studies. While all experiments involving DC in this study were performed in serum-free CellGro® media supplemented only with IL-4 and GM-CSF, Corinti et al. cultured mo-DC in RPMI media supplemented with FCS and the respective cytokines. Royer et al. evidenced significant differences in terms of cytotoxicity as well as phenotypic and functional differentiation of DC under different culture conditions including different protein supplements (Royer et al., 2006). Interestingly, DC grown in serum-free media display a higher expression of activation markers upon stimulation (Kim and Diamond, 2007). Moreover, FCS-containing culture conditions are inappropriate for human applications (Jonuleit et al., 1997) and bovine serum-containing media was shown to decrease IDO expression (Munn et al., 2002) while the use of serum-free media enhanced IDO-mediated suppression (Munn et al., 2005). If the cell culture conditions play a role especially concerning IL-10 secretion needs further evaluation. IL-10 was also not detectable in supernatants of mature or PGE_2-treated DC (Figure 9A) which is opposed to findings of Harizi et al. that PGE2-treated murine BM-DC produce IL-10 (Harizi et al., 2002). Furthermore, Harizi et al. showed that IL-10 which was produced by murine DC in response to PGE2 downregulates the production of COX-2 derived PGE2 (Harizi and Norbert, 2004). Differences between human and murine DC might account for these controversary results.

Yet, infected regulatory DC produced substantial amounts of IL-10 and could thereby be separated from PGE_2-treated regulatory DC. It was previously shown that activation of at least two different TLR receptors is necessary to induce IL-10 production by DC while combination of TLR4 with TLR7 or TLR9 agonists was most effective whereas activation of single TLRs was not sufficient to induce significant IL-10 production by DC (Hirata et al., 2009). During infection of DC with *Listeria monocytogenes* a variety of different signals is integrated thereby inducing a specific transcriptional profile consisting of genes associated with stimulatory as well as inhibitory functions. The stimulation of a variety of different surface and intracellular receptors during the process of DC infection with Listeria, among those also different TLRs, could therefore be responsible for the additional expression of inhibitory molecules in infected DC compared to PGE_2-treated DC. Stimulation of EP and TNF

receptors by combined treatment of DC with PGE_2 and TNF is apparently not sufficient to induce IL-10. The infection of DC therefore seems to create a stronger inhibitory phenotype which is further supported by additional expression of COX-2 only in infected DC. Ishikawa et al. reported that COX-2 expression is regulated by cell-type specific feedback mechanisms. While a positive COX-2 feedback regulation by PGE_2 occurred in macrophages, exogenous PGE_2 suppressed COX-2 transcription in embryonic fibroblasts suggesting a possible negative feedback regulation (Ishikawa et al., 2009). Moreover, Jozefowski et al. demonstrated differences of exogenous and endogenous PGE2 in the regulation of cytokine secretion by murine DC and suggested that PGE2 might rather act in a paracrine than in an autocrine manner and affect neighboring cells (Jozefowski et al., 2003). In this study, exogenous PGE_2 did not induce COX-2 expression, therefore a positive feedback mechanism can be excluded. Moreover, endogenous PGE_2 produced by infected DC (Popov et al., 2006) did not block COX-2 expression in these cells which is therefore not negatively regulated by endogenous PGE_2. Although phenotypic hallmarks that characterize DCreg in different settings were established, it was not yet clear if the respective molecules also account for the inhibitory functions of DCreg.

7.2 The regulatory function of IDO^+ $CD25^+$ DC

Although expression of stimulatory molecules like CD40, CD80 and CD86 is induced in DC upon PGE_2 treatment or Listeria infection, the simultaneous induction of inhibitory molecules like IDO, CD25, COX-2 and IL-10 seems to prevail leading to T-cell inhibition rather than activation. Compared to TNF-treated mature DC, IDO^+ $CD25^+$ DCreg decreased T-cell proliferation and IFN-γ production. Contrarily to Krause et al., who suggested an enhanced stimulatory capacity of PGE_2-treated DC despite expression of IDO (Krause et al., 2007), additional treatment of DC with PGE_2 lowered their potential to stimulate T cells compared to TNF treatment alone (Figure 12). Since mature DCreg possess all characteristics of stimulatory DC, it was difficult to setup a system which reveals the regulatory capacity of mature DCreg. Direct co-incubation of DC and T cells without pre-conditioning of the media by DCreg as well as co-incubation without additional stimulation of the T-cell receptor with anti-CD3 coated aAPC did not reveal clear differences between IDO^+ $CD25^+$ and

IDO^- $CD25^-$ mature DC (Figure 11 and 12). PGE_2-treated DC could only exert significant inhibitory functions if DC were pre-incubated before co-incubation with T cells and additionally this inhibition was dependent on effective T-cell receptor stimulation, the duration of co-incubation and the method chosen as readout for T-cell proliferation. Velten et al. demonstrated that $CD25^{high}$ mature DC exhibit a higher stimulatory capacity than $CD25^{low}$ mature DC (Velten et al., 2007). Yet, expression of maturation and activation markers on $CD25^+$ DC was also increased compared to $CD25^{low}$ DC which might also account for their higher stimulatory capacity. Both reports of Velten et al. and Krause et al. support the idea of combined induction of maturation as well as CD25 and IDO expression, respectively, yet in both studies T-cell proliferation was assessed after direct co-incubation of DC and T cells (without DC pre-incubation) which might account for their enhanced T-cell stimulatory capacity.

As already mentioned, the most significant reduction of T-cell proliferation was achieved if DCreg were pre-incubated before addition of T cells (Figures 12 and 13). This preconditioned environment seems to be necessary to block rapid T-cell activation. A strong stimulatory signal for T cells (anti-CD3/CD28) as well as the pre-activation of T cells before co-stimulation with DC significantly lowers the suppressive capacity of IDO^+ $CD25^+$ DCreg (Figures 15 and 16). As a result of the stronger regulatory phenotype of infected DC (additional IL-10 and COX-2 expression, Figure 9), inhibition of T-cell proliferation is more pronounced with infected DC and in contrast to PGE_2-treated DC, infected DC are able to suppress already activated T cells.

Two mechanisms might account for the suppression of T-cell proliferation by IDO-expressing DC: first, depletion of the essential amino acid tryptophan was shown to inhibit T-cell proliferation (Mellor and Munn, 1999; Munn et al., 1999) and second, toxic tryptophan metabolites, mainly kynurenine, can suppress T-cell proliferation and induce T-cell death (Frumento et al., 2002; Terness et al., 2002). In this study it was shown that both mechanisms contribute to T-cell inhibition by IDO^+ $CD25^+$ DCreg. T-cell proliferation was clearly suppressed in tryptophan-free cell culture media or by addition of kynurenine to the T-cell culture with both effects being additive as proliferation was even more decreased in tryptophan-free media containing kynurenine (Figure 19). This is consistent with observations of Fallarino et al. who demonstrated that combined effects of tryptophan starvation and tryptophan

catabolites impair T-cell activation in mice (Fallarino et al., 2006). Although Munn et al. stated that tryptophan starvation is exclusively responsible for IDO-mediated T-cell suppression, the presence of tryptophan metabolites was not disproved in their study (Munn et al., 1999) and therefore it cannot be ruled out that both mechanisms contributed to T-cell suppression.

Additional inhibitory molecules secreted by DCreg such as IL-10 and PGE_2 are able to decrease T-cell activation separately while the combination of all mentioned inhibitory mechanisms (low tryptophan, high kynurenine, IL-10, PGE_2) suppressed T-cell proliferation to a degree which was not measurable by CFSE dilution (Figure 19). Opposing results demonstrating an increased capacity of IDO-expressing DCreg in T-cell stimulation can therefore be explained by the missing inhibitory microenvironment in fresh in vitro culture conditions (Krause et al., 2007). Although proliferation of T cells stimulated in the presence of the single inhibitory factors (low tryptophan, high kynurenine, IL-10, PGE_2) was significantly decreased, blockade of the single factors was not sufficient to restore T-cell proliferation (Figure 25). Only the simultaneous blockade of all analyzed inhibitory factors restored proliferation which supports the hypothesis that different regulatory mechanisms of DC act in concert to suppress T-cell activation and that the inhibitory microenvironment created by DCreg plays a major role in T-cell suppression. Furthermore, T cells stimulated in the presence of DC supernatants were significantly impaired (Figure 18), pointing out that cell-cell contact between T cells and DCreg is not necessarily required for the suppressive effects of DCreg.

Although the role of tryptophan depletion in mediating T-cell tolerance was recently questioned because of the generally rather low frequencies of DC in vivo (Terness et al., 2006), the dense localization of IDO^+ $CD25^+$ DC within the granuloma wall might result in significant tryptophan depletion and kynurenine accumulation leading to T-cell suppression. Moreover, the inhibitory microenvironment created by DCreg induces a regulatory phenotype in former unstimulated DC (Figure 17). This mechanism might lead to a locally concentrated accumulation of DCreg and inhibitory factors which is sufficient to influence T cells in the microenvironment. Moreover, Belladonna et al. showed that IDO-competent DC are able to transfer the tolerogenic potential on otherwise immunogenic DC in a transwell system (Belladonna et al., 2006) which confirms that the conversion of DC to DCreg is not dependent on cell-cell contact but can be induced by soluble factors secreted by DCreg.

It was demonstrated that different inhibitory factors cooperate to suppress T cells and therefore silencing of a single factor, namely IDO, in PGE_2-treated DC was not sufficient to restore T-cell proliferation (Figure 24). The same result was expected for the silencing of CD25. Notably, knockdown of CD25 in DC stimulated with PGE_2 and TNF which did not affect IDO expression or maturation, clearly restored their stimulatory capacity pointing towards an important role of CD25 expression and subsequent IL-2 deprivation in DCreg mediated T-cell suppression (Figure 24). Nevertheless, the role of CD25 expression on DC is still controversially discussed. DC do not express the β-chain of the IL-2 receptor (Figure 26) which is consistent with previous results concerning β-chain expression on human or murine DC (Kronin et al., 1998; Velten et al., 2007). Although Fukao and Koyasu reported the expression of the β-chain in murine splenic DC at mRNA level, expression at protein level was not demonstrated (Fukao and Koyasu, 2000). Naranjo-Gomez et al. showed expression of the β-chain on human pDC (Naranjo-Gomez et al., 2007), though these data may not apply to human mDC (von Bergwelt-Baildon et al., 2006) or mo-DC (Figure 26). This finding already indicates that IL-2 does not induce IL-2 dependent signaling in DC because expression of the β-chain is indispensable for IL-2 signaling to be executed. Moreover, it was shown that the phenotype of immature, mature and regulatory DC is not affected by IL-2 (Figure 27) and our group previously showed that the expression of genes of the IL-2 signaling pathway, e.g. p56Lck, as well as the expression of IL-2 target genes was not changed by IL-2 treatment of DC (Driesen et al., 2008; von Bergwelt-Baildon et al., 2006). Possible functions of isolated CD25 expression that are independent of IL-2 signaling therefore have to be considered. Since CD25 is mainly responsible for binding of IL-2, it seemed possible that the sequestration of IL-2 might be the function of DC-derived CD25. Indeed, sCD25-containing supernatants of DCreg could suppress the proliferation and decrease the viability of the IL-2 dependent T-cell line CTLL-2 indicating the deprivation of IL-2 in the cell culture media. Still, in various reports CD25 expression by DC was regarded as a function of maturation and $CD25^+$ DC were even thought to be more effective in stimulating T cells while blocking of CD25 led to impaired T-cell stimulatory activity and cytokine secretion and to an inhibition of IFN-γ and IL-12 production by DC (Fukao and Koyasu, 2000; Mnasria et al., 2008). Nonetheless, the mechanisms behind these outcomes and particularly the role of CD25 during these

processes was not assessed. This study provides evidence that CD25 functions as a decoy receptor for IL-2 (Figure 28) thereby reducing T-cell proliferation (Figure 24).
Still, it cannot be ignored that regulatory DC also exerted T-cell stimulatory capacities, yet to a lesser extent than mature DC since the induction of T-cell proliferation was clearly reduced but not completely blocked by stimulation with DCreg (Figures 12 and 13). Additionally, it is likely that DCreg not only impair the activation and proliferation of $CD4^+$ T_H1 cells as it was shown in this study but are also able to induce the conversion of naïve $CD4^+$ T cells into regulatory $CD25^+$ $Foxp3^+$ T cells as it has been demonstrated in mice (Fallarino et al., 2006). Moreover, IDO^+ mature DC were shown to expand regulatory T cells by an IDO-dependent mechanism (Chung et al., 2009). Inhibition of this counter-regulatory pathway should therefore prove useful in sustaining responses stimulated by DC-based immunotherapy.
Induction of IDO and CD25 in murine DC is at least in part differentially regulated compared to human DC. Not only the factors contributing to IDO and CD25 induction differ in murine DC but also the affected DC subsets (Popov and Schultze, 2008). Moreover, previous work pointed to significant interspecies differences in tryptophan catabolism (Terness et al., 2006) indicating that the concentration of immunosuppressive tryptophan metabolites in different tissues depends on the species (Allegri et al., 2003). The transfer of results obtained in mice to the human system therefore has to be questioned. Contrarily to humans, enteral infection of mice with *Listeria monocytogenes* is not efficient because of a single amino acid substitution in murine E-cadherin (Lecuit et al., 1999; Lecuit et al., 2001). The process of infection can therefore hardly be analyzed in mice making it difficult to reveal the immune inhibitory mechanisms of Listeria that enable encapsulation of the bacteria in a granuloma, an immune-privileged site. Genetically modified animal models, e.g. transgenic mice expressing human E-Cadherin might be useful to solve the problems of studying human infectious diseases with animal models (Lecuit and Cossart, 2002).

7.3 Signaling events responsible for the induction of IDO$^+$ CD25$^+$ DCreg

A variety of different soluble factors has been shown to induce IDO$^+$ CD25$^+$ DCreg. However, the responsible signaling mechanisms have not yet been revealed. The induction of IDO$^+$ CD25$^+$ DCreg was strictly dependent on TNF. Treatment of DC with PGE_2 or the TLR ligands Pam_3 and LPS separately was not sufficient to induce expression of the regulatory molecules. Only combination of the respective soluble factors with TNF resulted in IDO and CD25 expression (Figures 30 and 31). Similarly, listerial infection of DC was not sufficient to induce a regulatory phenotype if TNF was neutralized by the clinically applied blocking antibody Infliximab (Figures 37, 38 and 39). Altogether, TNF seems to be a central mediator of inhibitory molecules during Listeria infection as it is required for induction of IFN-γ, COX-2, IL-10, CD25 and IDO.
Commercial LPS was the only soluble factor tested which was able to solely induce IDO$^+$ CD25$^+$ DC without additional TNF (Figure 32). Yet, most of the commercially available TLR ligands are reported to be contaminated with bacterial lipoproteins (Hirschfeld et al., 2000) and stimulation of DC with commercially available LPS results in TNF and IFN-γ secretion (Popov and Schultze, 2008), which, in turn, can be responsible for IDO expression. Since stimulation of DC with ultrapure LPS is not sufficient to induce IDO and CD25 without TNF (Figure 32), it is likely that the induction of IDO$^+$ CD25$^+$ DCreg by commercial LPS is due to the contaminants.
To delineate signaling pathways that lead to the induction of IDO or CD25 in DC, first the responsible receptors used by PGE_2 and TNF were analyzed for their capacity to transfer the IDO- or CD25-inducing signal. Induction of IDO$^+$ CD25$^+$ DCreg by PGE_2 was clearly associated with activation of EP2 and EP4 receptors and subsequent increase in cAMP levels by activation of adenylate cyclase (Figure 42). It was previously reported that both stimulatory and inhibitory actions of PGE_2 on DC are mediated via EP2 and EP4 (Harizi et al., 2003; Scandella et al., 2002). Since EP2 is in contrast to EP4 already expressed by immature DC (Figure 40) and stimulation of DC with the EP2 receptor agonist was more effective in inducing the regulatory molecules than stimulation with the EP4 agonist (Figure 42), a dominant role of EP2 in the induction of DCreg by PGE_2 is proposed. Braun et al. observed that concomitant triggering of EP2 and TLR2 or TLR4 provides an adequate stimulus for IDO induction (Braun et al., 2005). Whether TLR2 and TLR4 ligands are indeed able

to induce IDO in combination with PGE_2 needs further clarification since in the mentioned study, commercially available lipoprotein-contaminated TLR ligands, such as LPS, were used. The function of EP1 and EP3 expression on DC remains unknown, yet it was speculated that these receptors might be required for counteracting PGE_2 induced effects mediated by EP2 and EP4 and may represent a possible mean by which expression of EP2 and EP4 returns to basal levels (Harizi et al., 2003).

Further analysis of EP2 associated signaling revealed that activation of PKA by elevated cAMP levels is apparently not involved in the induction of IDO^+ $CD25^+$ DCreg (Figures 44 and 45) while activation of PI3K which is independent of adenylate cyclase is indispensable for DCreg induction (Figure 47). Several studies in mice suggested that the PI3K pathway is a negative inflammatory feedback regulator for cytokines and its disruption resulted in excessive production of the pro-inflammatory cytokine IL-12 by murine splenic DC (Fukao et al., 2002) as well as increased IL-12 but suppressed IL-10 synthesis by human monocytes stimulated with LPS (Martin et al., 2003).

Since inhibition of PI3K had a considerable effect on the induction of regulatory molecules by DC, we expected similar results during inhibition of the downstream effector molecule of PI3K, Akt. Yet, reduction of IDO and CD25 expression by the Akt inhibitor was accompanied by cell death (Figure 48 and 49A). Thus, one cannot dissect between inhibitory or toxic effects of the Akt inhibitor. Additionally, it remains unclear if Akt inhibition is generally opposed to DC survival. Other methods than biochemical inhibition are therefore necessary to evaluate the influence of Akt in DCreg induction, e.g. siRNA mediated knockdown of Akt which would at least clarify the role of Akt in cell survival.

Recently, the mammalian target of rapamycin (mTOR) was shown to restrict pro-inflammatory and promote anti-inflammatory responses in monocytes/macrophages and peripheral mDC. The best documented pathway leading to activation of mTOR involves activation of Akt after its recruitment to the cell membrane by PI3K and subsequent relieve of the inhibitory activity of the TSC1-TSC2 complex on mTOR (Weichhart and Saemann, 2009; Yang and Guan, 2007). It is therefore possible that EP2/EP4 mediated activation of PI3K finally results in activation of mTOR, yet this hypothesis needs further evaluation.

Fujino et al. already suggested a role of Gsk-3 in EP2 and EP4 mediated signal transduction. It was demonstrated that PGE_2 via the EP2 and EP4 receptors facilitates Gsk-3 phosphorylation, yet both receptors are using different downstream signaling molecules. While EP2 mediates Gsk-3 phosphorylation via activation of PKA, EP4 employs the PI3K-Akt pathway (Fujino et al., 2002). If PGE_2 by binding to EP2 or EP4 and subsequent activation of PI3K and Akt would lead to inhibition of Gsk-3 by phosphorylation, the Gsk-3 specific inhibitor should mimick PGE_2 mediated effects and increase the expression of IDO and CD25. Interestingly, Gsk-3 inhibition actually increased expression of CD25 while IDO expression and maturation were significantly reduced (Figure 50). Yet, only PI3K and not PKA seems to be involved in induction of IDO and CD25 via EP2 and EP4. This difference might be due to the different cell types used in both studies since Fujino et al. analyzed a cell line stable transfected with EP2 or EP4 while in this study only human mo-DC were analyzed. These results also suggest that different downstream signaling molecules mediate the induction of IDO and CD25 and that Gsk-3 might function as a `switch molecule´ which enables to distinguish between PGE_2 mediated IDO or CD25 induction. Furthermore, induction of IDO seems to be closer connected to DC maturation than CD25 expression.

It was previously demonstrated that activation of PI3K blocks the p38 activation pathway in DC (Fukao et al., 2002) and suppresses MAPK and NFkB pathways in monocytes (Guha and Mackman, 2002). Since PI3K is involved in DCreg induction, it was expected that inhibition of p38 would not affect the regulatory phenotype. Yet, although the reduction of regulatory molecules did not reach a significant level, slight decreases of CD25 and IDO expression were observed after p38 inhibition while expression of the maturation marker CD83 remained unaltered (Figure 51). Due to the complex phenotype of DCreg consisting of different stimulatory as well as inhibitory molecules (Mellor and Munn, 2004; Munn et al., 2002), it is likely that not only anti-inflammatory but also pro-inflammatory pathways contribute to the regulatory phenotype.

Interestingly, it was demonstrated in this study that both TNF receptors are responsible for transmitting the signal which mediates induction of regulatory molecules during *Listeria monocytogenes* infection. Blockade of either one receptor was compensated by the second, not blocked receptor and therefore did not reach a significant decrease of regulatory molecules (Figures 37, 38 and 39). Contrarily, only

TNFRI was responsible for induction of IDO and CD25 in DC treated with PGE_2 and TNF and blockade of TNFRII did not have any effects (Figures 34 and 35). Obviously, a strong upregulation of TNFRII accounts for the involvement of this receptor in DCreg induction during listerial infection (Figure 33).

If DC were stimulated with neutralizing anti-TNF receptor antibodies and IDO protein expression was assessed by Western blotting with the purchased Chemicon antibody, a second band near below the IDO signal appeared. This additional signal might be generated by interactions between the anti-IDO and the anti-TNFR antibodies. The monoclonal mouse anti-human IDO antibody that was a kind gift of Prof. Takikawa was used in most of the performed Western blots and specifically reacts with human IDO. Similarly, the monoclonal anti-IDO antibody that was purchased from Chemicon generates a specific band with a size of 45 kDa when analyzing human IDO. Yet, the monoclonal anti-IDO antibody from Chemicon cross-reacts with mouse IDO. To avoid such an unspecific signal, the more specific anti-IDO antibody could be used for Western blotting while immunoprecipitation of IDO would presumably be the more successful method to prevent this experimental problem.

In contrast to TNF, CD25, sCD25 and IL-10, the expression of IDO protein by listeria-infected DC was dependent on IFN-γ (Figure 37). Although Popov et al. showed that IDO gene expression is induced before IFN-γ is secreted by the infected DC (Popov et al., 2006), it cannot be excluded that IFN-γ might play a role in post-transcriptional regulation or protein stabilization of IDO. Silencing of IFN-γ or its receptor during Listeria infection would be useful to address this question in the future.

It was previously shown that IFN-γ by activation of Jak2 induces IDO expression in human islets while at the same time several genes of the Jak/STAT pathway, Jak2, IRF-1 and STAT1 were upregulated (Sarkar et al., 2007). Similarly, the Jak/STAT pathway is essential for IFN-γ mediated IDO induction in a human fibrosarcoma cell line (Ceravolo et al., 1999). Although Jak/STAT signaling is primarily known to be activated by interferons, various cytokine receptors can induce Jak/STAT signaling upon activation, including TLRs. It is dependent on the receptor which of the four known Jak molecules, namely Jak1, Jak2, Jak3 or Tyk2 is associated and therefore phosphorylated upon receptor activation. Hu et al. recently reported phosphorylation of STAT1 and STAT4 and subsequent activating expression of the STAT1 target gene IRF-1 in DC treated with PGE_2 and TNF (Hu et al., 2008). Moreover, it was

recently shown that activation of Jak2/STAT3 signaling by tumor derived factors is directly involved in abnormal DC differentiation in cancer (Nefedova et al., 2004; Wang et al., 2004) while inhibition of Jak2/STAT3 overcomes the differentiation block induced by tumor-derived factors and promotes the differentiation of mature DC and macrophages (Nefedova et al., 2005). Interestingly, so far Jaks have not been described to be associated with EP or TNF receptors. Still, inhibition of Jak/STAT3 but not STAT3 signaling alone clearly influenced the PGE_2 and TNF induced DC phenotype (Figures 57 and 58). Either STAT3 is especially not involved in induction of the here analyzed regulatory molecules or differences in the human and mouse system account for the different results concerning STAT3 in this study and the previously mentioned studies of Nefedova et al. Similarly to inhibition of Jaks, inhibition of PI3K reduced hallmark expression of PGE_2-treated DC (Figure 47). These results support the hypothesis that the Jak-PI3K pathway plays a role in DCreg induction and that Jaks and PI3K belong to one signaling pathway that leads to the induction of the regulatory hallmarks IDO and CD25. Although the activation of the PI3K signaling pathway downstream of Jaks but STAT-independent was previously only described for type I interferons (Uddin et al., 1997; Uddin et al., 2000), this study suggests a similar mechanism for PGE_2 and TNF induced expression of IDO and CD25. It remains to be elucidaded which of the four known Jak molecules participates in DCreg induction. However, activating mutations of all the different Jaks have been associated with malignant transformation, e.g. gain-of-function mutations of Jak2 in polycythemia vera (James et al., 2005) and other myeloproliferative disorders (Baxter et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Permanent activation of Jaks might therefore result in the induction of regulatory DC that prevent effective T-cell mediated immune surveillance of malignant cell transformation.

Since DC stimulation with PGE_2 and TNF facilitates at least three different receptors, namely EP2, EP4 and TNFRI, different signaling pathways are activated which appear to interact and therefore only the simultaneous activation of the respective receptor-associated signaling molecules is sufficient to induce DCreg. Therefore, it is a true challenge to delineate the signaling pathway which is induced by different signals which are integrated to form a common pathway.

Although the involvement of PKA, PKC, NFκB or JNK in the induction of a regulatory DC phenotype was not proven (Figures 44, 45, 46, 53, 55, 56), a role of these

signaling molecules in DCreg induction cannot be excluded only by biochemical inhibition. Silencing of the respective molecules by siRNA mediated knockdown, if compatible with DC survival, would be a tool to delineate their role in DCreg induction. The work that was established so far in this regard will form the basis for future research on further dissecting the different signaling components required for the induction of regulatory DC.

7.4 Clinical implications

PGE_2 has been used several times as a component of DC maturation cocktails for DC-based immunotherapy protocols (Jonuleit et al., 1997), mostly due to the role of PGE_2 in sensitization of DC to CCR7 ligands and the enhanced migration of PGE_2-treated DC to secondary lymphoid organs (Luft et al., 2002; Scandella et al., 2004). With the results of this study it cannot be ruled out that DC treated with a maturation cocktail in combination with PGE_2 migrate to secondary lymphoid organs and interact with T cells specific for the respective tumor antigen. Furthermore, Thurner et al. reported that vaccination with tumor-peptide pulsed, PGE_2-treated DC expanded specific cytotoxic T cells and induced regression of metastases in advanced stage IV melanoma (Thurner et al., 1999a). However, cell infiltrates of IDO^+ cells were found in melanoma patients after vaccination with PGE_2-treated DC and these patients exhibited rapid disease progression with a shorter overall survival (Wobser et al., 2007). Therefore, the improved stimulatory capacity of such DC has to be questioned. Although PGE_2 and TNF treated DC express a mature phenotype, the functional outcome of PGE_2 treatment was rather T-cell suppressive (Figure 12), hence it would be important to address the use of PGE_2 in DC vaccination cocktails. Replacement of PGE_2 in DC vaccination protocols might be a possibility to increase the T-cell stimulatory capacity of DC and improve clinical outcome. Instead of PGE_2, agonists of specific EP receptors or PGE_2-induced signaling molecules could be used which maintain the enhanced migratory capacity of PGE_2-treated DC but circumvent induction of regulatory molecules such as IDO and CD25. As shown in this study (Figures 41 and 42) as well as in a study of Braun et al., the EP2 receptor seems to have a dominant role in the induction of regulatory properties (Braun et al., 2005). Therefore, the replacement of PGE_2 with e.g. EP4 specific agonists might be an opportunity to increase the efficiency of DC vaccinations. Otherwise, PGE_2 could be used in combination with an IDO inhibitor, e.g. 1-MT, to avoid negative effects of

PGE_2 on the stimulatory capacity of DC since 1-MT has been reported to converse the inhibitory function of IDO^+ DC (Hwu et al., 2000; Munn et al., 2002).

Interestingly, expression of IDO and CD25 by DCreg might also be favorable to the host, especially in the case of chronic infections. Induction of IDO during infection has been frequently described to be associated with inhibition of pathogen growth as a protective host response (Carlin et al., 1989; Grant et al., 2000; Silva et al., 2002). Boasso et al. then reported that HIV-1 inhibits $CD4^+$ T-cell proliferation by induction of IDO in pDC (Boasso et al., 2007) suggesting an opposite role of IDO induction during infection. Here, it was shown that in sections of a human listerial granuloma, IDO^+CD25^+ DC were the major component of the ringwall surrounding the encapsulated bacteria (Figures 9 and 10). This concentrated localization of IDO^+ $CD25^+$ DCreg facilitates the generation of an inhibitory microenvironment which is strong enough to keep the majority of T cells ($CD4^+$ and $CD8^+$) beyond the ringwall and spatially separated from the bacteria. The destruction of the granuloma by cytotoxic T cells would be counterproductive to the mostly immunosuppressed host and result in uncontrolled pathogen spreading (Kaufmann, 2002). The number and local distribution of DC might play a significant role and local accumulation of high numbers of IDO^+ $CD25^+$ DCreg might be sufficient to decrease the level of tryptophan and IL-2 and increase the level of kynurenine to an extend that can be sensed by T cells and influences their activation. While Terness et al. expected that the largest proportion of IDO is produced not by the thinly scattered DC but by the bulk of other cells at the side of infection (Terness et al., 2006), granuloma might present a contrary model where most of the enzyme is produced by antigenpresenting cells such as DC or macrophages (Popov et al., 2006).

Popov et al. could further show that other pathogens associated with the formation of granuloma like Mycobacterium tuberculosis and Leishmania major are characterized by a gene expression pattern which closely resembled the expression pattern of listeria-infected DC including expression of IDO and CD25 (Popov et al., 2008). Furthermore, immunohistochemistry of early stage granuloma in tuberculosis revealed prominent expression of IDO and CD25 by macrophages and fewer DC. It can only be hypothesized that a tumor might represent a comparable spatial structure which is able to create an inhibitory microenvironment for T cells. Supporting this hypothesis, the existence of peritumoral IDO-expressing DC in the border area of a gastric carcinoma associated with increased PGE_2 concentrations, has already been

described (Popov and Schultze, 2008; von Bergwelt-Baildon et al., 2006). Yet, these peritiumoral DC were found in much lower frequencies within the carcinoma or the tumor-draining lymph node than in the listerial granuloma.

The exacerbation of tuberculosis, listeriosis and other granulomatous infections has recently been recognized as severe adverse event in patients with rheumatoid arthritis treated with TNF-neutralizing agents such as infliximab (Remicade®) or etanercept (Enbrel®) (Keane et al., 2001; Slifman et al., 2003; Wallis et al., 2005). It was shown that the induction of a regulatory phenotype in listeria-infected DC including expression of IDO and CD25 is strictly dependent on TNF (Figures 36, 37, 38 and 39). The breakdown of the granuloma structure during anti-TNF therapy is therefore likely due to the blockade of regulatory DC that maintain the granulomatous structure by spatial separation of bacteria and T cells (Popov and Schultze, 2008).

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9 Zusammenfassung

Das Hauptziel dieser Arbeit war die Charakterisierung der molekularen Mechanismen, die für die Induktion und Funktion von regulatorischen denritischen Zellen (DCreg) verantwortlich sind. DCreg konnten sowohl in der Umgebung verschiedener Tumoren als auch bei chronischen Infektionskrankheiten nachgewiesen werden. DCreg, die durch eine Kombination des tumor-assoziierten Faktors PGE_2 mit TNF oder durch Infektion mit dem Gram-positiven Bakterium *Listeria monocytogenes* induziert wurden, co-exprimieren das Tryptophan katabolisierende Enzym IDO und die α-Kette des IL-2 Rezeptors, CD25. Die Anhäufung von toxischen Metaboliten (Kynureninen) bewies die Funktionalität von IDO in DCreg. Außerdem sezernieren DCreg große Mengen von löslichem CD25 (sCD25). CD25 und sCD25 binden IL-2 und verringern somit die für T-Zellen verfügbare Konzentration an IL-2. Weiterhin wurde gezeigt, dass IDO^+ $CD25^+$ DCreg die Proliferation von $CD4^+$ T-Zellen supprimieren. Diese Suppression war abhängig von einer Vielzahl inhibitorischer Faktoren, unter anderem IDO, CD25, IL10 und COX-2, wobei alle genannten Faktoren zusammwirken, um die inhibitorische Funktion der DCreg zu vermitteln. Nur die gleichzeitige Blockade aller inhibitorischer Faktoren hob die DCreg vermittelte T-Zellsuppression auf. Interessanterweise konnte durch den spezifischen “knockdown” von CD25 die stimulatorische Funktion von DCreg ebenfalls wiederhergestellt werden. Dies deutet auf eine wichtige Rolle von CD25-Expression in DCreg vermittelter T-Zell Suppression hin. Außerdem wurden die für die Induktion von IDO^+ $CD25^+$ DCreg verantwortlichen Rezeptoren und Signalwege untersucht. Die PGE_2-vermittelte Induktion von IDO und CD25 war abhängig von EP2 und EP4 sowie von TNFRI. Auch die Induktion von DCreg durch Infektion mit Listerien war abhängig von TNF, jedoch waren beide TNF Rezeptoren an der Induktion von infizierten DCreg beteiligt. Die Analyse der Rezeptor-assoziierten Signalwege zeigte, dass sowohl PI3K als auch Jak Signalmoleküle an der Induktion von IDO und CD25 beteiligt sind, während die Inhibition von Gsk-3 unterschiedliche Auswirkungen auf IDO und CD25 hat. Diese Ergebnisse weisen auf einen gemeinsamen Jak-PI3K Signalweg hin, wobei jedoch unterschiedliche nachgeschaltete Signalelemente die Expression von IDO und CD25 induzieren. Diese Arbeit kann demnach als Grundlage für die weitere Entschlüsselung von Signalwegen dienen, die für die Induktion von DCreg verantwortlich sind.

10 List of publications

Driesen J, Popov A**,** Schultze JL
“CD25 as an immune regulatory molecule expressed on myeloid dendritic cells.”
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