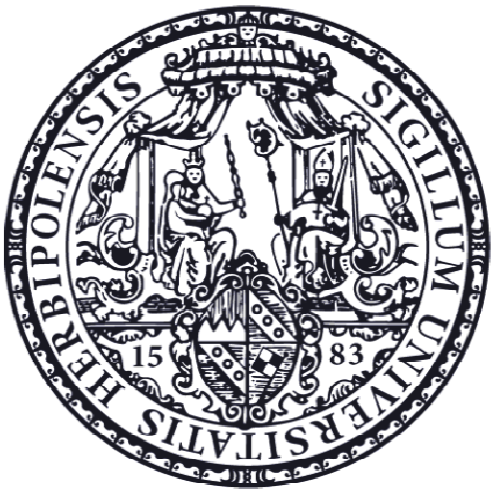

Characterization of tolerogenic rat bone marrow-derived dendritic cells and regulatory T cells

Charakterisierung tolerogener dendritischer Knochenmarkszellen und regulatorischer T-Lymphozyten aus der Ratte

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
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Section Infection and Immunity

submitted by
Anja Matuschek
from
Lauchhammer

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Submitted on:

Members of the *Promotionskomitee*:

Chairperson:	
Primary Supervisor:	Priv.-Doz. Dr. rer. nat. Christoph Otto
Supervisor (Second):	Priv.-Doz. Dr. rer. hum. biol. Ulrike Kämmerer
Supervisor (Third):	Prof. Dr. rer. nat. Roland Benz

Date of Public Defence:

Date of Receipt of Certificates:

Für meine

Eltern, Großeltern und Clemens

Die größte Tragödie in der Wissenschaft überhaupt
ist der Tod einer wunderschönen Hypothese
durch die Hand einer hässlichen Tatsache.

T. H. Huxley (1825 - 1895)

Table of Contents

1 Abbrevations

APC	Antigen-presenting cell
BM-DC	Bone marrow-derived dendritic cell
BN	Brown Norway
CD	Cluster of differentiation
cpm	Counts per minute
CTL	Cytotoxic T cell
DC	Dendritic cell
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein Isothiocyanate
FSC	Forward scatter
IFN-γ	Interferon-gamma
IL-2	Interleukine-2
IL-12	Interleukine-12
LCs	Langerhans cells
LEW	Lewis
LN	Lymph node
MDC	Myeloid-derived DC
MF	Mean fluorescence
MHC	Major histocompatibility complex
MLC	Mixed leukocyte culture
MLR	Mixed leukocyte reaction
NK cell	Natural killer cell
NMMA	N^G-methyl-L-arginine acetate
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PDC	Plasmacytoid-derived DC
PD-L1	Programmed cell death 1
PD-L2	Programmed cell death 2
PRRs	Pattern recognition receptors
RBCs	Red Blood Cells

rGM-CSF	Rat granulocyte-macrophage colony stimulating factor
rIL-4	Rat Interleukine-4
SSC	Side scatter
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
T_H cell	T-helper cell
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
T_{reg} cell	Regulatory T cell
RPE	R-Phycoerythrin
S.D.	Standard deviation
S-DC	Spleen dendritic cells
S.E.M.	Standard errow of the mean
WF	Wistar Furth

2 Summary

Tolerogenic dendritic cells (DC) and regulatory T (T_{reg}) cells are able to prevent destructive immune responses. There is reason to hope that it may soon be possible to use DC and T_{reg} cells to suppress immune responses antigen-specific, not only after transplantation, but also in the case of autoimmunity and allergy. At the moment, the generation of such cell types is very time-consuming and not suitable for clinical routine. In addition, it is not yet fully understood how these cells elicit a desired protective immune response *in vivo* and how the risks of an excessive immune suppression can be managed.

The rat is one of the most important animal models in biomedical research. It is therefore surprising that tolerogenic DC and T_{reg} cells in particular have not been more thoroughly investigated in this model. Thus, the aim of the present study was to systematically characterize these immune cells and investigate their impact on the immune system.

Tolerogenic DC were generated from bone marrow precursors cultured with GM-CSF and IL-4 (= IL-4 DC). The proportion of naturally occurring T_{reg} cells with a $CD4^{pos}CD25^{pos}Foxp3^{pos}$ phenotype comprises approximately 5-8% of the peripheral $CD4^{pos}$ T cells.

The characterization of IL-4 DC revealed an up to 26-fold reduced expression of surface molecules such as MHC class II molecules, CD80, CD86, ICAM-1 and CD25 in comparison to mature splenic DC (S-DC). This low expression did not change when the cells where stimulated with different maturation-inducing signals such as replating, LPS, TNF-α and CD40L. Thus, these cells possess a robust phenotype resistant to maturation-inducing stimuli.

IL-4 DC take up antigen via endocytosis and are not able to activate naïve T cells or to restimulate antigen-specific T cells. Furthermore, they are able to inhibit and prolongate mature S-DC induced T cell proliferation as well as mature S-DC induced restimulation of antigen-specific T cells, respectively. Thereby, the T cell proliferation was reduced up to 95%. This strong inhibitory effect was mediated within 24 hours in

association with a reduced cytokine production (IL-2 about 49% and IFN-γ about 92%).

The inhibitory properties of IL-4 DC don´t seem to be caused exclusively by the reduced expression of co-stimulatory molecules. In this study, the detection of the inhibitory molecules PD-L1 and PD-L2 on IL-4 DC suggests they have an impact on mediating inhibitory signals to the T cells.

In addition, a suppressive effect of soluble factors was shown. The supernatant of one million IL-4 DC, collected after a 24 hour culture, suppressed mature S-DC induced proliferation of naïve T cells by about 90%. TGF-β, which was detected in the supernatant (up to 300 pg/ml), appears to be the causing soluble factor for this immune inhibition. By contrast, the supernatants of mature S-DC, which did not inhibit the activation of T cells, showed a TGF-β concentration of only about 100 pg/ml. The cytotoxic nitric oxide does not contribute to the IL-4 DC-mediated inhibition of T cell proliferation. The NO synthase inhibitor NMMA reduced the amount of NO by about 50%, but the decreased NO levels did not influence T cell proliferation.

Indeed, IL-4 DC are not able to induce T cell proliferation, but this doesn´t mean that there is no change on the molecular level. For instance, T cells co-cultured with IL-4 DC during a first culture are not able to proliferate in the presence of mature S-DC during a second culture. This anergic-like state, however, could be abolished by adding exogenous IL-2. In addition, T cells co-cultured with IL-4 DC are able to inhibit the activation of naïve T cells. Naïve and activated T cells were not able to inhibit the mature S-DC induced T cell proliferation. This observation suggests the induction of T_{reg} cells and was investigated in more detail. Indeed, flow cytometric analysis showed a 1.6-fold expansion of $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T cells from naturally occurring T_{reg} cells in the presence of IL-4 DC. Thereby, the expansion of $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T cells occurs independently of the maturation state of DC. Both immature IL-4 DC as well as mature S-DC were able to expand the percentage of naturally occurring T_{reg} cells. However, T_{reg} cells pre-incubated with mature S-DC demonstrated a diminished inhibitory effect compared to T_{reg} cells pre-incubated with

IL-4 DC. T_{reg} cells pre-incubated with IL-4 DC were able to inhibit the activation of naïve T cells.

In this study it was shown that the regulatory potential of DC cannot be deduced solely by their phenotype or maturation state. Other factors, such as functional properties, need to taken into consideration, too. The induction of T_{reg} cells with suppressive properties induced by *in vitro* generated tolerogenic IL-4 DC might provide an important mechanism for the maintenance of peripheral tolerance. However, for clinical application further investigation is necessary, not only to understand the interactions between tolerogenic DC and T_{reg} cells, but also to investigate the impact of the transfer of a larger quantity of regulatory cells on the immune system of the recipient.

3 Zusammenfassung

Tolerogene Dendritische Zellen (DZ) und regulatorische T-Lymphozyten (T_{reg}) verfügen über die Fähigkeit, destruktive Immunantworten zu verhindern. Die Hoffnung besteht, solche Zellen in naher Zukunft für therapeutische Zwecke einzusetzen, um z. B. Immunantworten nach Transplantation, aber auch bei Autoimmunität und Allergie antigenspezifisch zu supprimieren. Zum jetzigen Zeitpunkt ist die Generierung solcher Zellen aufwendig und noch nicht für die klinische Routine geeignet. Zudem sind die Mechanismen noch wenig verstanden, wie diese Zellen eine gewünschte Immunhemmung *in vivo* auszulösen und wie der möglichen Gefahr einer zu starken Immunhemmung zu begegnen ist.

Das Kleinnagermodell Ratte ist für die biomedizinische Forschung noch immer von großer Bedeutung, umso überraschender ist es, dass insbesondere tolerogene DZ und T_{reg} in diesem Modell bisher nur unzureichend untersucht wurden. Das Ziel der Arbeit war deshalb, diese Immunzellen umfassend zu charakterisieren und ihre Funktion auf das Immunsystem zu untersuchen.

Tolerogene DZ wurden mit GM-CSF und IL-4 aus Knochenmarkvorläuferzellen generiert (= IL-4 DC). Der Anteil an natürlich vorkommenden T_{reg} mit einem Phänotyp $CD4^{pos}CD25^{pos}Foxp3^{pos}$ umfasst ca. 5-8% der peripheren naiven $CD4^{pos}$ T-Lymphozyten.

Die Charakterisierung der IL-4 DC zeigte im Vergleich zu reifen DZ der Milz eine bis zu 26-fach geringere Expression von Oberflächenmolekülen wie MHC-Klasse II Molekül, CD80, CD86, ICAM-1 und CD25. Diese geringe Expression änderte sich auch nicht, wenn die Zellen verschiedensten Reifungssignalen wie das Replattieren, LPS, TNF-α und CD40L ausgesetzt wurden. IL-4 DC verfügen somit über einen robusten und gegenüber Reifungssignalen überaus resistenten Phänotyp.

IL-4 DC nehmen Antigene durch Endozytose auf und sind unfähig, sowohl naive T-Lymphozyten zu aktivieren, als auch antigenspezifische T-Lymphozyten zu restimulieren. Zudem sind sie in der Lage, die Aktivierung naiver T-Lymphozyten und die Restimulierung antigenspezifischer T-Lymphozyten durch reife Milz-DZ zu

verhindern bzw. zu verzögern. Dabei verringerte sich die Proliferation der T-Lymphozyten um bis zu 95%. Diese Beeinflussung der Proliferation ist nach Zugabe der IL-4 DC bereits innerhalb von 24 Stunden zu messen. Die verringerte Aktivierung geht zu dem mit einer verringerten Zytokinausschüttung (IL-2 um 49% und IFN-γ um 92%) einher.

Die inhibitorischen Eigenschaften der IL-4 DC scheinen aber nicht ausschließlich auf der verringerten Expression kostimulatorischer Moleküle zu beruhen. Der Nachweis der beiden inhibitorischen Oberflächenmoleküle PD-L1 und PD-L2 auf IL-4 DC lässt ebenfalls eine Bedeutung dieser Moleküle bei der Vermittlung inhibierender Signale vermuten.

Auch die suppressive Wirkung löslicher Faktoren wurde in der vorliegenden Arbeit gezeigt. Überstände einer 24-stündigen Kultur mit einer Million IL-4 DC hemmten die Aktivierung naiver T-Lymphozyten durch reife Milz-DZ um etwa 90%. Für diese Immunhemmung scheint das in diesen Überständen nachgewiesene Zytokin TGF-β (bis 300 pg/ml) verantwortlich zu sein. Im Vergleich dazu wiesen Überstände reifer Milz-DZ, die nicht die Aktivierung von T-Lymphozyten hemmten, eine TGF-β Konzentrationen von bis 100 pg/ml auf. Im Gegensatz dazu scheint zelltoxisches Stickstoffmonoxid nur eine geringe Rolle bei der Inhibierung der T-Zellproliferation zu spielen. Die Zugabe des NO Synthase-Inhibitors NMMA verringerte zwar den Anteil an NO um ca. 50%, doch führte dies nicht zu einer Steigerung der Proliferation von T-Lymphozyten.

IL-4 DC sind zwar nicht in der Lage, T-Lymphozyten zur Proliferation zu bringen, doch bedeutet dies nicht, dass keinerlei Veränderungen auf molekularer Ebene festzustellen wären. So sind T-Lymphozyten nach ihrer Inkubation mit IL-4 DC nicht in der Lage, in Gegenwart von reifen Milz-DZ zu proliferieren. Dieser anergische Zustand wurde nach Zugabe von IL-2 aufgehoben. Zudem können diese T-Lymphozyten nach ihrer Inkubation mit IL-4 DC die Aktivierung naïver T-Lymphozyten hemmen. Naïve und aktivierte T-Lymphozyten können dies nicht. Diese Beobachtung, die auf eine Induktion von T_{reg} schließen lässt, wurde genauer untersucht. In der Tat zeigten durchflusszytometrische Analysen eine 1,6-fach verstärkte Expansion von $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T-Lymphozyten aus natürlich

vorkommenden T_{reg} in Gegenwart von IL-4 DC. Dabei erfolgte die Expansion von $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T-Lymphozyten unabhängig vom Reifegrad der DZ. So waren auch reife Milz-DZ dazu in der Lage, die Zahl der natürlich vorkommenden T_{reg} zu erhöhen. Doch wiesen diese mit Milz-DZ inkubierten T_{reg} einen verminderten inhibitorischen Effekt auf. Im Gegensatz dazu waren die mit IL-4 DC inkubierten T_{reg} in der Lage die Aktivierung naiver T-Lymphozyten zu hemmen.

In dieser Arbeit wurde gezeigt, dass sich das regulatorische Potential von DZ nicht ausschließlich vom Phänotyp bzw. ihrem Reifegrad ableiten lässt, sondern dass hierzu auch ihre funktionellen Eigenschaften zu untersuchen sind. Die Induktion von T_{reg} mit suppressiven Eigenschaften durch *in vitro* generierte tolerogene IL-4 DC könnte ein wichtiger Mechanismus zur Aufrechterhaltung der peripheren Toleranz darstellen. Vor einer klinischen Umsetzung sind aber noch weitergehende Untersuchungen notwendig, um das Zusammenspiel zwischen tolerogenen DZ und T_{reg} zu verstehen, aber auch um die Auswirkungen eines Transfers großer Mengen regulatorischer Zellen auf das Immunsystem des Empfängers zu untersuchen.

4 Introduction

4.1 The immune system

The immune system, that is divided into the innate immune system and the adaptive immune system, is a complex network of many interdependent cell types that protect the body against foreign substances such as bacteria, parasites, fungi, viruses, tumor cells and even transplanted organs and tissues. Thereby, one of the most important functions of the immune system is the discrimination between self and non-self. The distinction between self and non-self is considered to involve a series of interactions between various cells of the immune system, most notably the extensive bidirectional interactions between dendritic cells (DC) and T cells. DC serve as a link between the innate and adaptive immune system, as they present antigen to T cells, one type of key cell of the adaptive immune system (Guermonprez P *et al*., 2002). Recent evidence has shown that DC and also a certain type of T cells or regulatory T cells (T_{reg}), play a key role in the maintenance of central and peripheral tolerance which is critical to prevent autoimmunity, allergy and allograft rejection (Fadilah SA *et al*., 2007; Oh JW *et al*., 2002; Sakaguchi S *et al*., 2005; Shevach EM *et al*., 2002; Steinman RM and Nussenzweig MC, 2002; Suciu-Foca N *et al*., 2009). A major challenge in immunology and medicine is to determine how immunological self-tolerance is established, maintained and controlled to avoid damage to the host. An understanding of the mechanisms of immunological self-tolerance and therefore, an understanding of the biology of tolerogenic DC and T_{reg} cells is important as a basis for new therapeutic tools to prevent autoimmune diseases and transplant rejection.

This study is focussing on the characterization of tolerogenic bone marrow-derived dendritic cells (BM-DC) and T_{reg} cells from rat. The following chapters of this introduction will provide some background information on the importance of DC and T cells in general. Additional information is given on potential roles of different DC subsets in tolerance, their interaction with T_{reg} cells and their roles in health and disease. Finally, the aims and the experimental approach of this study are explained.

4.1.1 The innate immune system

The innate or “non-specific” immune system, which presents the first line of defence against any pathogenic agent, is capable of responding immediately or within several hours to pathogenic agents. Innate immunity comprises four types of defensive barriers: anatomic (skin, mucous membrane), physiologic (chemical mediators, low pH), phagocytic (e.g. eosinophils, neutrophils, monocytes and macrophages), and inflammatory (acute phase proteins, cytokines and complement proteins). Most of the cells of the innate immune system are able to identify specific components of microbial organisms, the so called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) peptidoglycan, lipoteichoic, and other components of microbial cell walls (Janeway CA Jr and Medzhitov R, 2002) via pattern-recognition receptors (PRRs) on their surface, which can bind PAMPs on bacteria, fungi, and viruses. The most important PRRs are the Toll-like receptors (TLR) that are expressed on the surface of various effector cells (Medzhitov R and Janeway CA Jr, 2000). The engagement of TLRs elicits a significant cytokine release, which in turn leads to the recruitment and activation of cells of the innate immune defence system, thus activating the adaptive immune response (Pasare C *et al*., 2004).

However, innate immunity is not antigen specific in its response and does not exhibit a memory response, meaning that this system does not confer long-lasting immunity against a pathogen. Therefore, if innate immunity was not able to clear the infection and to initiate long-term protection, cellular mediators of the adaptive immunity move into action.

4.1.2 The adaptive immune system

While the innate immune system is the first line of defense against invading microbes, the adaptive or “specific” immune system acts as a second line of defense against any "foreign" substance. The four characteristic attributes of adaptive immunity are antigen specificity, diversity, immunologic memory and discrimination between “self and non-self” (Jiang H and Chess L, 2009). The adaptive immune response involves three major groups of cells: B cells (humoral immunity), T cells (cell-mediated immunity) and antigen-presenting cells (APC). The adaptive immune

system is based on the total repertoire of lymphocytes bearing clonally diverse antigen-specific receptors facilitating the recognition of almost any foreign antigen (diversity). In contrast to the innate immune system, the adaptive immune system uses somatically generated antigen receptors which are clonally distributed on T and B cells. B and T cell specificity is created by random recombination of gene segments that encode the antigen-binding receptor (antibody on B cells and T-cell receptor on T cells) with a single specificity on the cell surface on B cells. Once antigen receptor gene rearrangement occurs, lymphocytes are sorted for “self/non-self” distinction within the thymus. This includes the elimination of lymphocytes whose receptors are specific for self antigens in order to prevent autoimmunity (Eason DD *et al*., 2004).

The induction of adaptive immunity requires antigens that can be captured, processed and presented to specific lymphocytes by APC. Among APC, dendritic cells are the most highly specialized. They are able to encounter microbial agents from the external environment and transport them to the lymphoid organs where they present antigens on either MHC class I or MHC class II major histocompatibility complex (MHC) molecules to naïve T cells. This in turn leads to the activation, proliferation and differentiation of naïve T cells into effector T cells and memory T cells. Effector T cells function to eliminate the antigen. Memory cells show enhanced potential of activation allowing a more rapid and effective secondary response upon repetitive exposure to the antigen (Cellular and Molecular Immunology, Lichtman, 5th edition).

4.2 Dendritic cells

Dendritic cells are highly specialized APC that provide a direct link between innate and adaptive immunity (Steinman RM *et al*., 2003). They were first described as dendritically shaped cells of the epidermis by Paul Langerhans, a medical student in Berlin, in 1868 (Langerhans P *et al*., 1868; Wolff K *et al*., 1991). Today we know that Langerhans cells (LC) are a subpopulation of epithelial-tissue-specific non-lymphoid DC (Steinman RM *et al*., 1991). One century later, in 1973, the term “dendritic cell” was coined by Ralph Steinman and Zanvil Cohn, for a novel cell type they identified in adherent cell populations prepared from mouse peripheral lymphoid organs

(spleen, lymph node, Peyer's patch) on the basis of morphological criteria such as a large nucleus, long and cytoplasmic processes after adherence and spreading on glass or plastic surfaces (**Figure 4.1**; Steinman and Cohn, 1973).

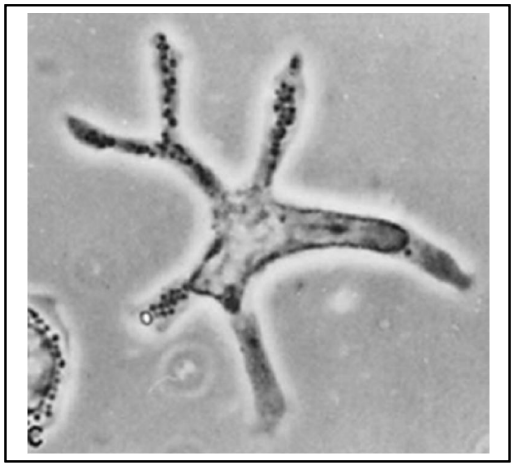


Figure 4.1: Phase-contrast micrograph of a dendritic cell isolated from the spleen (Steinman RM and Cohn ZA, 1973).

In the following years mouse dendritic cell research with respect to DC isolation, *in vitro* culture, as well as functional properties *in vitro*, started to emerge (Steinman RM and Cohn ZA, 1974; Steinman *et al.*, 1974, Steinman RM *et al*., 1975). Steinman's group demonstrated for the first time their unique capacity to induce activation of naïve T cells in response to antigen presentation (Steinman and Witmer MD, 1978; Romani N *et al*., 1989).

4.2.1 Dendritic cells: differentiation from precursor populations

Dendritic cells develop from bone-marrow derived $CD34^{pos}$ hematopoietic precursors (Banchereau J *et al.,* 2000; de Heer HJ *et al.,* 2004). Although they share many common features, DC can be subdivided into a number of sub-populations based on their expression of cell surface markers and functional properties under the influence of different cytokines and growth-factors (Quah BJ and O'Neill HC, 2005). Two major subsets of DC have been described: myeloid-derived DC (MDC) and plasmacytoid-derived DC (PDC) (Liu YJ *et al*., 2001).

4.2.1.1 Plasmacytoid-derived DC (PDC)

PDC, which constitute the rarer cell type, comprise approximately < 1% of the total blood mononuclear cells. Based on their plasma cell-like morphology, these cells have been termed plasmacytoid DC (Nakano H *et al*., 2001) while others have referred to them as natural interferon-producing cells (Asselin-Paturel C *et al*., 2001). In contrast to MDC, PDC play an essential role in the innate immune system by secreting various cytokines such as type I interferon after stimulation with TLR7 and

TLR9 agonists. In addition, they have only a low capacity to activate naïve T cells (Cella M *et al.,* 1999; Gibson SJ *et al*., 2002; Kadowaki N and Liu YJ, 2002; Kerkmann M *et al*., 2003).

PDC develop from haematopoietic stem cells in the bone marrow. FLT-3 ligand (FLT-3L) has been identified as an important key differentiation and growth factor for these cells (Gilliet M *et al*., 2002; Maraskovsky E *et al*., 2000; Pulendran B *et al*., 2000; Pulendran B *et al*., 1997; Shurin MR *et al*., 1997). Activation with virus or CD40-ligand together with IL-3 promotes the differentiation of PDC into mature DC which can effectively stimulate T cells (Grouard G *et al*., 1997). Depending on the way of activation these PDC induced T cells, are able to produce different types of cytokines such as interleukine-10 (IL-10) and interferon-gamma (IFN-γ) after activation of the PDC with virus or interleukine-4 (IL-4), and interleukine-5 (IL-5) after activation of the PDC with interleukine-3 (IL-3) and CD40L (Cella M *et al*., 2000; Kadowaki N, 2000; Rissoan MC *et al*., 1999).

4.2.1.2 Myeloid-derived DC (MDC)

The major DC populations that develop from bone marrow hematopoitic stem cells are the MDC. They are considered the “classical” DC, which are potent APC typically associated with the ability to activate naïve T cells and to initiate an adaptive immune response (Zuniga EI *et al*., 2004). In comparison to PDC, MDC express a complementary yet distinct set of TLRs (e.g. TLR2, 3, 4, 5, 6 and 7) and thus, respond to different PAMPs from a variety of pathogens (Reis e Sousa C, 2004; Ito T *et al*., 2002; Mazzoni A and Segal DM, 2004). MDC, just like PDC, originate from myeloid committed $CD34^{pos}$ progenitor cells, and monocytes can be driven to become DC in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF), c-kit ligand and tumor necrosis factor-alpha (TNF-α) (Caux C *et al*., 1997), or with GM-CSF and IL-4/IL-13 (Bender A *et al*., 1996; Sallusto F *et al*., 1994).

The growth factor GM-CSF, first isolated and characterized by Gough *et al*. (1984), is used in most of the protocols to generate DC. *In vivo* it is produced by lymphocytes and monocytes, as well as by cells not belonging to the immune system (e.g.

fibroblasts) in response to immune and inflammatory stimuli (Banchereau and Steinman RM, 1998; Channon JY *et al*., 2002; Gala RR and Shevach EM, 1994; Kaplan G *et al*., 1992). *In vitro*, GM-CSF stimulates the differentiation and proliferation of progenitor cells of granulocytes, macrophages and DC (Burges AW and Metcalf D, 1980; Metcalf D *et al.,* 1986; Inaba K *et al*., 1992; DePaz HA *et al*., 2003). In addition, GM-CSF is essential for the development, survival and function of DC, including the development of specific DC surface markers (e.g. MHC class II expression) (Witmer-Pack MD *et al*., 1987; Alvaro-Gracia JM *et al*., 1989; Steinman RM, 1991). In combination with the cytokine IL-4, GM-CSF enhances the expression of MHC class II molecules on the surface of monocytes, therefore enhancing their ability to present antigen (te Velde AA *et al*., 1988; Paul WE, 1991). However, IL-4 also enhances the DC numbers generated *in vitro* (Talmor M *et al*., 1998), inhibits the differentiation of macrophages (Jansen JH *et al*., 1989) and reduces the amount of $CD14^{pos}$ cells (Lauener RP *et al.,* 1990).

4.2.2 Dendritic cell maturation stages

The induction of $CD4^{pos}$ T helper (T_H) subset responses is a distinct function of DC, depending on different stages of DC development (Jonuleit H *et al*., 2000). DC can exist in three different developmental stages: immature, semi-mature and mature (**Figure 4.2**). These different maturational stages seem to be critical for the tolerogenic or immunogenic capacity of DC (Hackstein H, 2006).

4.2.2.1 Immature DC

DC arising from myeloid- or lymphoid precursors exhibit an immature phenotype characterized by a high phagocytic capacity and low expression of MHC class II and co-stimulatory molecules such as CD80, CD86 and CD40, and the inability to stimulate T cells (Mahnke K *et al*., 2002). These cells, residing in non-lymphoid peripheral tissues in the immune steady-state, constitutively take up antigens, both self and foreign by phagocytosis, macropinocytosis or endocytosis (Banchereau J *et al*., 2000; Hawiger D *et al*., 2001; Sallusto F *et al.,* 1995). Once they have acquired and processed the foreign antigens, they migrate to the T cell areas of lymph nodes and the spleen, and undergo maturation to stimulate an immune response.

However, immature DC are reported to be impaired in their antigen processing and presenting capacity (Wilson NS *et al*., 2004). It is believed that immature DC induce a negative selection of autoreactive cells mediating central tolerance to self when presenting self antigens to developing T cells in the thymus. During migration to the regional lymph nodes immature DC can also mediate Ag-specific peripheral tolerance, inducing T cell deletion or induction of T_{reg} cells (Steinman RM and Nussenzweig MC, 2002).

4.2.2.2 Semi-mature DC

Semi-mature DC, an intermediate stage of DC maturation, was described for the first time by Menges and colleagues (Menges M *et al*., 2002). This dendritic cell subgroup is produced *in vitro* by stimulating immature DC with TNF-α. These *in vitro* generated semi-mature DC, when injected intravenously into mice, prevented experimental autoimmune encephalomyelitis (EAE) by inducing IL-10-secreting T_{reg} cells (Menges M *et al*., 2002). *In vivo* these cells remain in a “steady state” after internalization of apoptotic cells, vesicles and/or soluble molecules which enables these cells to induce efficient and antigen-specific tolerance (Lutz BL and Schuler G, 2002). Semi-mature DC express high levels of MHC class II and medium levels of cell surface markers such as CD40, CD80, and CD86, but do not secrete inflammation-inducing cytokines such as IL-1, IL-6, and IL-12. Semi-mature DC can be further matured with LPS alone or combined with an enhancing CD40 signal (Lutz MB and Schuler G, 2002; Verginis P *et al*., 2005).

4.2.2.3 Mature DC

To fully activate naïve T cells, DC residing in non-lymphoid tissue need to undergo a process of functional maturation (Quah BJC and O´Neill HC, 2005). DC maturation factors are LPS (de Smedt T *et al*., 1996), dsRNA and bacterial DNA (Aderem A and Ulevitch RJ, 2000; Sparwasser T *et al.,* 1998; Bauer M *et al*., 2001). DC maturation also occurs in the absence of infection when T cells express CD40L following transplantation, contact allergy, and autoimmunity (Abrams JR *et al.,* 2000; Enk A and Katz SI, 1992; Larsen CP *et al.,* 1990). This maturation of immature DC is characterized by changes in their capacity to migrate from non-lymphoid peripheral

organs to T cell rich areas of the secondary lymphoid tissue (Fossum S, 1988; Cumerbatch M and Kimber I, 1995), and reduced antigen capture capacity (Banchereau J and Steinman RM, 1998). In addition, maturing DC upregulate MHC class II, co-stimulatory molecules such as CD80, CD86 and CD40 as well as adhesion molecule ICAM-1 (CD54) to enhance adhesion and signalling to T cells, and increase the secretion of cytokines and chemokines (Dietz BA *et al*., 2000; Romani M *et al*., 1989; Witmer-Pack MD *et al*., 1988).

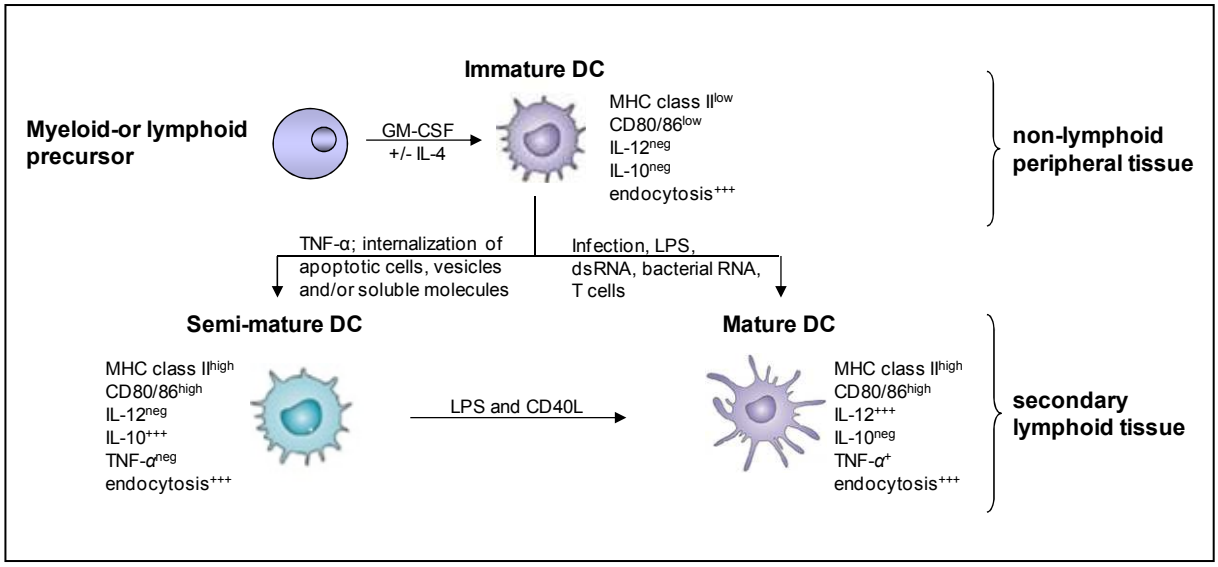


Figure 4.2: Different dendritic cell maturation stages. Immature DC differentiate from myeloid- or lymphoid progenitors in response to certain cytokines such as GM-CSF and IL-4. They are characterized by a high phagocytic capacity, express low levels of MHC class II and co-stimulatory molecules like CD80/CD86, and are not able to secrete inflammation-inducing cytokines such as IL-12 and IL-10. Upon activation DC travel from non-lymphoid peripheral tissues to the lymphoid organs such as the spleen and lymph nodes to efficiently induce primary T cell responses. DC maturation is associated with up-regulation of MHC class II and co-stimulatory CD80/CD86 molecules and by production of IL-12. However, the existence of a transitional stage of DC maturation between immature DC and mature DC has been shown. This subgroup of cells (which are referred to as semi-mature DC) is produced *in vitro* by stimulating immature DC with TNF-α. *In vivo* these cells remain in a "steady state" after internalization of apoptotic cells, vesicles and/or soluble molecules. Semi-mature DC express high levels of MHC class II and medium levels of cell surface markers such as CD80 and CD86. Semi-mature DC do not secrete inflammation-inducing cytokines such as TNF-α and IL-12 coupled with enhanced secretion of IL-10. Semi-mature DC can be further matured with LPS alone or combined with an enhancing CD40 signal (Hackstein H, 2006; Lutz MB and Schuler G, 2002).

4.2.3 Dendritic cell-mediated T cell activation

As mentioned before, immature DC undergo phenotypic and functional changes after contact with different maturation factors. DC maturation is initiated in the peripheral tissue, followed by migration of maturing DC carrying antigen into the T cell area of

lymphoid organs. Here they interact with T cells to initiate a specific immune response (Lipscomb MF and Masten BJ, 2002). Thereby, DC maturation and subsequent migration regulate the outcome of a T cell response: immunity or tolerance. DC provide three signals to naïve T cells for T cell activation, proliferation and differentiation: T-cell receptor (TCR) binding to MHC-bound antigen, co-stimulation, and secretion or lack of secretion of factors such as IL-12 by DC (Lipscomb MF and Masten BJ, 2002) (**Figure 4.3**).

DC are able to process and present antigen to activate both naïve $CD4^{pos}$ T_H cells and naïve $CD8^{pos}$ T cells. Thereby, a cellular contact between a T cell and a DC is necessary for antigen recognition. This contact occurs through the TCR that recognizes the peptide - MHC complex present at the DC surface (referred to as signal 1). To stimulate naïve $CD4^{pos}$ T cells antigenic peptides are loaded on MHC class II molecules. In contrast, naïve $CD8^{pos}$ T cells recognize peptides presented by MHC class I molecules on the cell surface of APC (Pamer E and Cresswell P, 1998; Rock KL and Goldberg AL, 1999).

	DC	$CD4^{pos}$ T cell
signal 1	MHC class II/peptide	TCR
signal 2	CD40	CD40L
	PD-L1	PD-1
	ICOS-L	ICOS
	CD80/86	CTLA-4
	CD80/86	CD28
	PD-L2	PD-1
signal 3	IL-2 secretion	T_H1 response
	no IL-2 secretion	T_H2 response

Figure 4.3: T cell activation. T cell activation depends on signal 1, signal 2 and signal 3 delivered by an antigen-presenting cell through the antigen-specific T cell receptor (TCR), co-stimulatory molecules and cytokine secretion such as IL-12 and others. Signal 1 is delivered through the TCR-MHC engagement. Signal 2 is provided by co-stimulatory molecules to stimulate T cell proliferation. Secretion or lack of secretion of cytokines such as IL-12 promotes the differentiation of naïve T cells into T_H1 or T_H2 cells (referred as to signal 3) (from Libscomb MF and Masten BJ, 2002; with slide modifications).

Ligation of TCR alone is insufficient to induce full T cell activation. Signal 2, referred to as “co-stimulation”, is required together with signal 1 to induce T cell proliferation and differentiation into effector T cells. This signal is antigen non-specific and not restricted by MHC. The absence of sufficient co-stimulation leads to the induction of

T cell anergy or T cell apoptosis, thereby inducing T cell tolerance (Reis e Sousa C, 2006). The most important co-stimulatory molecules are CD28 on naïve T cells and the corresponding ligands, CD80 (B7-1) and CD86 (B7-2), which are upregulated on maturing APC (Coyle AJ and Gutierrez-Ramos JC, 2001; Schwartz RH, 2001). Other co-stimulator molecules that have been implicated include CD54 (ICAM-1), CD102 (ICAM-2) and CD50 (ICAM-3) interacting with CD11a/18 (LFA-1), CD58 (LFA-3) with CD2, and CD40 with CD154 (CD40 ligand) (Damle NK *et al.,* 1992; McLallan AD *et al.,* 1996; Starling GC *et al.,* 1995). However, not all co-stimulatory molecules provide a “positive” signal leading to T cell proliferation, cytokine production, and further differentiation into effector cells. Some co-stimulatory signals can also provide “negative” signals leading to cellular anergy, loss of proliferative capacity, and reduction of cytokine production (Pawelec G, 2000; Yamada A *et al.,* 2002). For example, cytotoxic T-lymphocyte antigen-4 (CTLA-4) functions to provide such a "negative" signal. CTLA-4 has a strong homology to CD28, is upregulated on activated T cells, binds with a higher affinity to CD80 and CD86 than CD28, and down regulates the immune response (Walunas TL *et al.,* 1994).

Another B-7 family member, PD-L1 (B7-HI), is constitutively expressed on resting T cells, B cells, macrophages and DC. It is further upregulated upon activation and binds to the programmed cell death 1 (PD-1) receptor on T cells (Coyle AJ and Gutierrez-Ramos JC, 2001). This interaction results in a negative regulatory effect with inhibition of T cell proliferation and cytokine production. Furthermore, PD-1 deficiency in PD-1-deficient (PD-$1^{-/-}$) mice results in a variety of autoimmune pathologies, including lupus-like disease in B6-mice, indicating its important role in the maintenance of tolerance (Keir ME *et al.,* 2006).

PD-L2 (B7-DC), a second ligand of PD-1, plays a similar role in inhibition and also binds to PD-1 but seems to have a distinct function in regulating T_H2 responses, whereas PD-L1 participates in T_H1 inflammation (Coyle AJ and Gutierrez-Ramos JC, 2001). In contrast to PD-L1 expression, PD-L2 expression is much more restricted. PD-L2 is inducibly expressed on DC and macrophages (Latchman Y *et al.,* 2001; Tseng SY *et al.,* 2001). PD-L2 can be induced by IFN-γ, IL-4 and GM-CSF (Loke P and Allison JP*,* 2003; Yamazaki T *et al.,* 2002).

Signal 3, with either no or secretion of IL-12 by DC, is important for the final differentiation of $CD4^{pos}$ T cells into T helper 2 (T_H2) or T helper 1 (T_H1) effector T cells, respectively (Libscomb MF and Masten BJ, 2002; Trinchirie G, 2003).

4.3 T cells

4.3.1 Differentiation of effector T cells

T cells can be divided into two major populations, $CD4^{pos}$ and $CD8^{pos}$ cells. $CD4^{pos}$ T cells are able to secrete cytokines, which in turn activate other cells of the immune sytem like B cells and $CD8^{pos}$ T cells or macrophages. That is why $CD4^{pos}$ T cells are also referred as to T_H cells. Thereby, the local cytokine environment, antigen dose and the source of co-stimulation determine differentiation into T_H1, T_H2, or T_H17 cells (Constant SL and Bottomly K, 1997) **(Figure 4.4**). In contrast, activated $CD8^{pos}$ T cells acquire the ability to lyse their target cells by producing cytotoxic molecules such as perforin and granzym B.

4.3.1.1 T_H1 cells

The T_H1 cells, which provide the so called cell-mediated immune response, secrete IL-2, tumor necrosis factor-β (TNF-β) and INF-γ under the influence of IL-12 and IFN-γ, thereby activating phagocytes, NK cells and macrophages. IL-12 is secreted by DC whereas IFN-γ is initially secreted by NK cells and by T_H1 cells themselves. The main transcription factors that control the transition of a T_H1 are T-bet and STAT-4 (Ouyang W *et al.,* 1999; Szabo SJ *et al.,* 2000).

4.3.1.2 T_H2 cells

In contrast to T_H1 cells, T_H2 cells express IL-4, IL-5 IL-6, IL-9, IL-10 and IL-13 under the influence of IL-4, which mediates B cell activation and differentiation and initiates a humoral immune response (Mosmann TR *et al.,* 1986; Paul WE and Seder RA, 1994; Purkerson J and Isakson P, 1992; Yang J *et al.,* 1999). The main transcription factors that control the transition of a T_H2 are GATA-3, c-maf and STAT-6 (Aggarwal S and Rao A, 1998; Hodge MR *et al.,* 1996; Zhang DH *et al.,* 1997; Zheng WP and Flavell RA, 1997).

4.3.1.3 T_H17 cells

In 2003 Aggarwal and colleagues showed that $CD4^{pos}$ T cells are able to differentiate into a T_H cell type other than T_H1 and T_H2 under the influence of IL-23. These so called T_H17 cells are able to secrete the cytokine IL-17 under the influence of IL-6 and TGF-β in addition to IL-23 (Bettelli E *et al.,* 2006). IL-17 exerts strong pro-inflammatory effects, ensures the differentiation, maturation, and activation of epithelial, endothelial, and fibroblastic cells, stimulates the production of pro-inflammatory cytokines and chemokines including IL-6, IL-8, G-CSF, and MCP-1 by these cells and activates DC (Cai XY *et al.,* 1998; Chabaud M *et al.,* 1999; Fossiez F *et al.,* 1996).

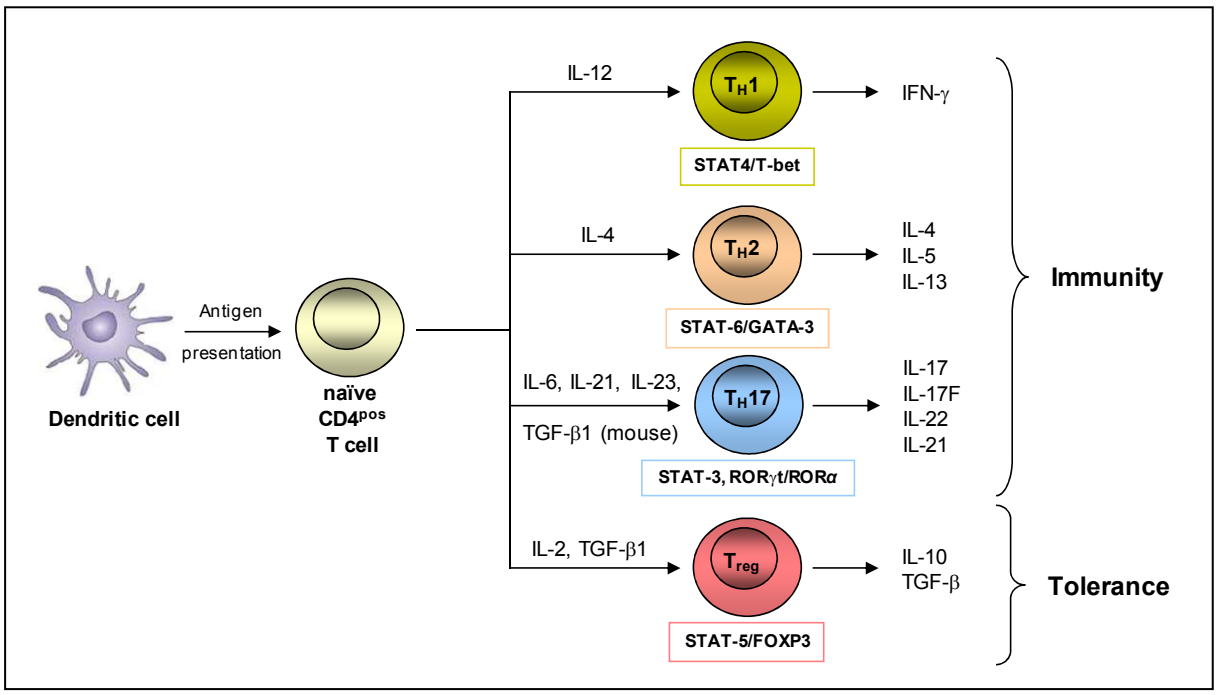


Figure 4.4: Differentiation of $CD4^{pos}$ T helper cells. Through the interaction of DC with naïve $CD4^{pos}$ T helper cells and associated TCR activation, naïve $CD4^{pos}$ T cells can differentiate into one of several lineages of T helper (T_H) cells, including T_H1, T_H2, T_H17, and T_{reg}. These different subpopulations vary in their profiles of cytokine production and in effector functions. Each linieage of $CD4^{pos}$ T cells is controlled by a unique main transcription factor (T-bet, STAT-3, STAT-4, STAT-6, GATA-3, c-maf, RORγt/RORα, STAT-5 and Foxp3, respectively). T_H1, T_H2, T_{reg}, and T_H17 cells are characterized by their synthesis of specific cytokines and their immuno-regulatory functions. T_H1 cells are characterized by their production of IFN-γ and are involved in cellular immunity against intracellular microorganisms. IL-12 produced by DC polarizes T cells toward the T_H1 cells through action of transcription factors STAT-4/T-bet). IL-4, IL-5 and IL-13 are major cytokines produced by T_H2 cells. IL-4 produced by DC polarizes T cells toward T_H2 cells through action of transcription factors STAT-6/GATA-3. The recently discovered T_H17 cells produce IL-17A (IL-17), IL-17F, IL-22, and IL-21 as major cytokines and play important roles in clearance of extracellular bacteria and fungi, especially at mucosal surfaces. IL-6, IL-21, IL-23 and TGF-β1 produced by mouse DC polarize T cells toward T_H17 cells through action of transcription factors STAT-3 and RORγt/RORα. T_{reg} cells are characterized by the expression of IL-10,

TGF-β1 and transcription factor Foxp3. T_{reg} cells may participate in maintenance of peripheral tolerance and prevention of autoimmunity (from Jetten AM, 2009; with slight modifications).

The main transcription factor involved in the development of T_H17 cells is the orphan nuclear receptor RORγt. Maintenance of a T_H17 response primarily depends on IL-23 (Aggarwal S *et al.,* 2003). Binding of IL-23 to its receptor triggers downstream activation of STAT-3 transcription factor and subsequent upregulation of ROR-γ and production of IL-17A. Since IL-17A leads to the induction of many pro-inflammatory factors it has been suggested that T_H17 cells might play a crucial role in the induction of autoimmune diseases (Weaver CT *et al.,* 2006).

4.4 Central and peripheral T cell tolerance

Immunological tolerance can be defined as unresponsiveness to a self- or foreign antigen. The main mechanisms of T cell tolerance are central tolerance which occurs in the thymus during early T cell activation and peripheral tolerance which occurs in the secondary lymphoid tissue after the migration of mature self-reactive T cells from the thymus (Sprent J and Kishimoto H, 2001). Unresponsiveness of T cells involves different mechanisms such as clonal deletion (death), functional inactivation without cell death (anergy) and inhibition of function by regulatory lymphocytes (immunoregulation) (Abbas AK and Lichtmann AH, 2005).

4.4.1 Central tolerance

Central tolerance can be regarded as a form of self-tolerance induced in the central lymphoid organs as a consequence of immature self-reactive lymphocytes recognizing self-antigens leading to apoptotic cell death (negative selection, or deletion). Clonal deletion is suicide of T-cell progenitors that have high affinity for self-antigens (Palmer E, 2003). The main factors leading to negative selection are the concentration of self-antigen and the affinity of the TCR that recognizes the antigen. But not all mechanisms of central tolerance are able to delete self-reactive T cells. In contrast, T cells that can recognize self-determinants with substantial but low affinity further progress to maturity. This process is termed positive selection that includes

the development of mature T_{reg} cells that enter the peripheral tissue to inhibit immune responses that are recognized as self (Abbas AK and Lichtmann AH, 2005; Fink PJ and Bevan MJ, 1995).

4.4.2 Peripheral tolerance

Although central tolerance is the major mechanism to eliminate self-reactive T cells, their deletion is incomplete. Self-reactive T cells with low affinity for self-antigens are able to escape negative selection (Bouneaud C *et al.,* 2000). The immune system prevents autoimmunity with the mechanisms of peripheral tolerance. Peripheral tolerance can be regarded as the maintenance of unresponsiveness of mature T cells to self-antigens that are expressed in the peripheral tissue and not abundant in the thymus (Hogquist KA *et al.,* 2005). The main mechanisms of peripheral T cell tolerance are deletion, T cell anergy and suppression.

When mature T cells are rapidly stimulated by self-antigen, the T cells undergo apoptosis, a process called “Activation induced cell death” (AICD) which occurs through cross linking and active signalling to lymphocytes through the death receptor Fas (CD95) and its ligand, FasL (CD95L) (Brunner T *et al.,* 1995; Combadiere B *et al.,* 1996). Surprisingly the Fas pathway mediated AICD is enhanced by IL-2 since IL-2 is traditionally thought to be a survival and growth-promoting cytokine (Leonardo MJ, 1991).

As mentioned previously (**section 4.2.3**), full T cell activation requires the recognition of antigen by the TCR (signal 1) and recognition of co-stimulatory molecules such as CD80 and CD86 by CD28 (signal 2). Induction of functional T cell anergy, the functional unresponsiveness of T cells, is associated with a block of T cell proliferation and IL-2 production (Schwartz RH, 2003). Anergy is induced by antigen recognition through the TCR without adequate co-stimulation or with inhibitory signals such as CTLA-4 binding. Thereby, CTLA-4 engagement may be necessary in addition to the absence of co-stimulation, to induce anergy in naïve $CD4^{pos}$ T cells *in vivo.* CTLA-4 binds to the *B7* molecules with higher affinity than CD28, which might explain why CTLA-4 may be the predominant receptor used when self-antigens are recognized by T cells (Greenwald RJ *et al.,* 2001; Macian F *et al.,* 2004).

Some immune responses are inhibited by cells that block the activation and function of effector T cells. Since the 1970s studies have suggested that suppression of development and activation of self-reactive T cells can occur through regulatory T cells (T_{reg}) (Waldmann H and Munro AJ, 1974; Basten A *et al.,* 1975; see also **section 4.4.3**). Different studies indicate that T_{reg} cells induce tolerance by secreting immunosuppressive cytokines such as IL-10 and transforming growth factor-β (TGF-β) (Weiner HL, 2001; Sundstedt A *et al.,* 2003). IL-10 is produced by T_H2 cells and monocytes and inhibits macrophage activation and the generation of T_H1 cytokines. TGF-β inhibits T- and B cell proliferation.

4.4.3 Regulatory T (T_{reg}) cells

T_{reg} cells play an important role in silencing self-reactive T cells and are able to inhibit autoimmunity and protect against tissue injury (Sakaguchi S *et al.,* 1995). Distinct populations of T_{reg} cells have been identified. They can be subdivided into two major cell populations by their production of cytokines, expression of cell surface markers and distinct suppressive mechanisms: naturally occurring T_{reg} cells and induced T_{reg} cells (Jonuleit H and Schmitt E, 2003).

4.4.3.1 Naturally occurring T_{reg} cells

Naturally occurring T_{reg} cells were first described by Gershon in 1975 (Gershon RK, 1975). They arise during the normal process of maturation in the thymus and represent 5-10% of all peripheral $CD4^{pos}$ T cells. It wasn´t until the nineties that more focus was put on these cells by Sakaguchi and colleagues. They were able to show that a certain type of naturally occurring T cells exhibits a regulatory $CD4^{pos}CD25^{pos}$ -like phenotype (Sakaguchi S *et al.,* 1995). Thereby, one of their substantial functions is to maintain natural self-tolerance and to prevent autoimmune disease by controlling potential auto-reactive T cells.

During the following years the phenotype and regulatory function of these cells was confirmed by several research groups. Naturally occurring T_{reg} cells are characterized by high levels of CD25 and the forkhead/winged helix transcription factor Foxp3 (Takahashi T *et al.,* 1998; Thornton AM and Shevach EM, 1998; Papiernik M *et al.,*

1998; Yi H *et al.,* 2006). Additionally they are characterized by the constitutive expression of several accessory molecules including GITR (TNFRSF18), CD152 (CTLA-4) and CD134 (OX40) (Shimizu JS *et al.,* 2002; Read S *et al.,* 2000; McHugh RS *et al.,* 2002). However, none of these molecules, including CD25, is exclusive for $CD4^{pos}CD25^{pos}$ T_{reg} cells since conventional effector $CD4^{pos}$ T cells without regulatory function do express CD25 after activation. Currently, the transcription factor Foxp3 appears to be the most specific and critical marker regarding the development and function of T_{reg} cells (Fontenot JD *et al.,* 2003; Hori S *et al.,* 2003; Khattri R *et al.,* 2003; Picca CC and Caton AJ, 2005; Sakaguhi S, 2005).

$CD4^{pos}CD25^{pos}$ T_{reg} cells are non-proliferative (anergic) themselves and suppress the activation/proliferation of other T cells when they are co-cultured with APC and stimulated with antigen. This anergic/suppressive state can be abrogated by TCR stimulation and IL-2 (Sakaguchi S *et al.,* 2001). The suppressor effector function of T_{reg} cells is antigen non-specific (Thornton AM and Shevach EM, 2000). $CD4^{pos}CD25^{pos}$ T_{reg} cells exert their suppressive effects in a contact-dependent manner by membrane-bound molecules that in some models seems to be independent of the production of cytokines like IL-10 and TGF-β (Jonuleit H *et al.,* 2001; Thornton AM and Shevach EM, 1998). However, some studies show that the suppressive function of $CD4^{pos}CD25^{pos}$ T_{reg} cells might be dependent on the production of immunoregulatory cytokines. The role of these cytokines during the induction of regulatory function is still controversy discussed. For example, *in vivo* administration of IL-10 and TGF-β antibodies resulted in abrogation of the T cell-mediated suppression in some experimental autoimmune and inflammatory models (Asseman C *et al.,* 1999; Powrie F *et al.,* 2000). However, this could not be confirmed *in vitro* (Thornton A *et al.*, 1998).

The suppression of immune responses seems to occur only when the $CD4^{pos}CD25^{pos}$ T_{reg} cells are stimulated through their TCR and seems to be related to their constitutive expression of CTLA-4 (Takahashi T *et al.,* 2000; Read S *et al.,* 2000). Thereby, not only polyclonal but also allogenic stimulation of $CD4^{pos}CD25^{pos}$ T_{reg} cells results in suppression of both $CD4^{pos}$ T cells as well as $CD8^{pos}$ T cells (Itoh M *et al.,* 1999; Takahashi T *et al.,* 1998). The result of this suppression is the direct or indirect

inhibition of IL-2 production in the responder cell population. This suppression can be overcome by the addition of exogenous IL-2 or enhancement of endogenous IL-2 production (Thornton AM and Shevach EM, 1998). However, the mechanisms of the suppressive functions of $CD4^{pos}CD25^{pos}$ T_{reg} cells are still incompletely understood and are still under intensive investigation. Presently, human and murine T_{reg} cells are well characterized whereas only few studies have focused on the characterization of rat T_{reg} cells. Since the rat is one of the most important animal models for experimental organ transplantation in a clinic-relevant procedure (Timmermann W *et al.,* 1998), intensive investigation of rat T_{reg} cells might open up exciting opportunities for new therapies, not only in transplantation but also in the prevention of autoimmune diseases.

4.4.3.2 Induced T_{reg} cells

Besides thymus-derived, naturally occurring $CD4^{pos}CD25^{pos}$ T_{reg} cells, there are various types of induced T_{reg} cells. Three types of secondary suppressive T cells have been described so far: T helper 3 (T_H3) cells, type 1 T regulatory (T_r1) cells and $CD8^{pos}$ regulatory T cells (Mills KGH, 2004; Roncarolo MG *et al.,* 2001; Weiner HL *et al.,* 2001). Induced T_{reg} cells have acquired their regulatory activity during activation *in vitro* or *in vivo*. These populations of mainly antigen-specific T_{reg} cells can be induced from naïve $CD4^{pos}CD25^{neg}$ or $CD8^{pos}CD25^{neg}$ T cells in the periphery under the influence of IL-10, TGF-β, semi-mature DC. The suppressive capacity of these cell populations is in contrast to $CD4^{pos}CD25^{pos}$ T_{reg} cells contact-independent and mainly mediated through suppressive cytokines (Buer J *et al.,* 2005). *In vitro* these cells demonstrate limited proliferation (Mittrücker HW and Kaufmann SHE, 2004).

The development of T_r1 cells *in vitro* was first shown by Groux and colleagues in 1997. They showed that chronic activation of $CD4^{pos}$ T cells by an IL-10-dependent process leads to the development of IL-10 producing T_r1 cells which are able to prevent the induction of colitis. T_r1 cells are characterized by a unique pattern of cytokine production distinct from that of T_H1 and T_H2 cells. Upon TCR mediated activation these cells secrete high levels of IL-10 and/or TGF-β, low amounts of IL-2 and no IL-4 (Asseman C *et al.,* 1999; Jonuleit H *et al.,* 2000; Powrie F *et al.,* 1996).

Depending on the experimental conditions T_r1 cells produce either low levels of IFN-γ and IL-5 or none at all (Bacchetta R *et al.,* 1990). T_r1 cells proliferate poorly after activation with antigen *in vitro* and do show immunosuppressive functions by preventing the development of T cell mediated immune responses (Levings MK *et al.,* 2001). In addition, supernatants of activated T_r1 cells are able to impair the ability of DC to induce alloantigen mediated T cell proliferation (Lecart S *et al.,* 2001; Cavani A *et al.,* 2000).

T_H3 cells were identified during the investigation of mechanisms associated with oral tolerance. Oral tolerance is a natural immunogenic event induced following oral antigen administration. The development of T_H3 cells was first described by Chen Y and colleagues in 1994 after oral tolerance induction to myelin basic protein (MBP). T_H3 cells in contrast to T_r1 cells primarily secrete high levels of TGF-β. They support IgA production and have suppressive properties for T_H1 and T_H2 cells in an antigen non-specific fashion (Weiner HL, 2001).

In addition to T_r1 and T_H3 cells there is also evidence for a subtype of $CD8^{pos}$ cells with regulatory functions. These cells are able to secrete IL-10 or TGF-β and have been therefore called $CD8^{pos}$ regulatory T cells (Garba M *et al.,* 2002; Haynes LM *et al.,* 2000). They can be generated *in vitro* by stimulation of naïve $CD8^{pos}$ T cells with CD40L-activated plasmacytoid DC or with IL-10 treated myeloid DC (Gilliet M and Liu YJ, 2002; Steinbrink K *et al.,* 1999). Recently it has been demonstrated that an anti-CD45RO/RB monoclonal antibody also induces $CD8^{pos}$ cells with suppressive activity (Gregori S *et al.,* 2005).

4.5 Tolerogenic DC and T_{reg} cells

As mentioned earlier the ability of the immune system to distinguish between self and non-self is controlled by different mechanisms of central and peripheral tolerance (**section 4.4**). These mechanisms involve deletion of self-reactive T cells through the interaction with DC followed by the induction of cell death or T cell anergy or the active suppression by T_{reg} cells. However, the mechanisms of T_{reg} cell development

and how they exert their immune regulatory properties are still under intensive investigation.

Besides their well-known immunostimulatory properties, DC have been found to play a crucial role in induction/maintenance of peripheral T-cell tolerance by acting on T_{reg} cells. There is growing evidence that interactions between DC and T_{reg} cells play an important role in the balance between immunity and tolerance. Thereby, the communication between these two cell types appears to be very complex and mediated by soluble or cell surface molecules (**Figure 4.5**). The regulatory properties of DC include induction of T cell anergy, apoptosis, and the generation of T cells with regulatory capacities as well as the generation of T cells that secrete immunomodulatory cytokines.

Direct killing of T cells by the induction of T cell apoptosis was first described by Süss and Shortman (Süss G and Shortman K, 1996). They showed that a subclass of DC is able to kill $CD4^{pos}$ T cells via Fas/Fas-ligand-induced apoptosis. In 2008 Vassiliou EK and colleagues showed that BM-DC generated in the presence of resolvin E1, a strong anti-inflammatory lipid, induce apoptosis of activated $CD4^{pos}$ T cells (Vassilio EK *et al.,* 2008).

The immuneregulatory functions of DC seem to be strictly dependent on their different stages of maturation and activation. It has been shown that immature DC are more likely to actively exert tolerogenic function than be inactive (Mahnke K *et al.,* 2002; Mahnke K *et al.,* 2003). In general immature tolerogenic DC are derived from processing and presentation of apoptotic cells (Steiman RM *et al.,* 2000). In 2002 Stuart and colleagues demonstrated that DC that ingested apoptotic cells have diminished capacity to stimulate naïve T cells. In addition, Marguti *et al.* showed that expansion of suppressive T_{reg} cells is favoured when immature DC were loaded with allogenic apoptotic thymocytes (Marguti I *et al.,* 2008).

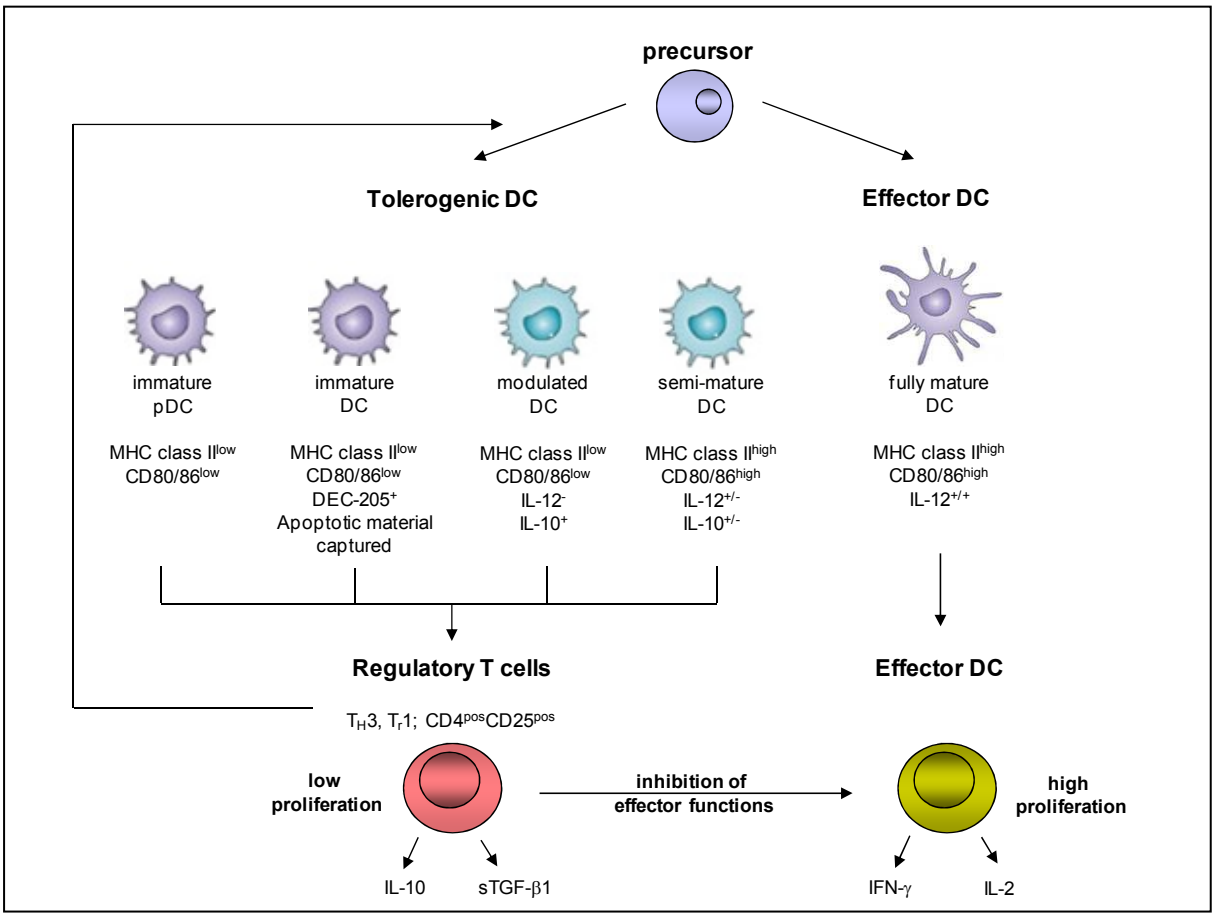


Figure 4.5: Bidirectional interplay between different DC subsets and T_{reg} cells. Different subsets of tolerogenic DC with distinct phenotypic profiles induce T_{reg} cells with a low ability to proliferate. T_{reg} cells in turn inhibit effector cells but also program the generation of tolerogenic DC from DC progenitors. In contrast, fully mature DC induce effector T cells with a high ability to proliferate (from Hubert P *et al.*, 2007; with slight modifications).

There is a rapid expansion of literature regarding the effects of DC on T_{reg} cells. The first evidence that immature DC are able to induce tolerance came from studies by Jonuleit and colleagues in 2000 (Jonuleit H *et al.,* 2000). They showed that repeated stimulation with *ex vivo* prepared immature DC induce IL-2 receptor expression on T cells which is an indicative marker for T_{reg} cells. These T cells were not able to proliferate and did suppress proliferation of $CD4^{pos}$ and $CD8^{pos}$ effector T cells. This suppression was mediated by cell-cell contact, independent of soluble factors. Steinman and colleagues (Steinman RM and Nussenzweig MC, 2002; Steinman RM *et al.,* 2003) have proposed that under steady-state conditions (in the absence of acute infection and inflammation), antigen-loaded immature DC with low cell-surface expression of MHC class II and co-stimulatory molecules may have the capacity to induce peripheral tolerance to those peptides presented to Ag-specific T cells by

silencing T cells either by deletion (induction of apoptosis) of effector T cells or by expanding T_{reg} cells that suppress effector immune responses. Two papers, one by Jenkins MK *et al.,* 1990 and one by Jonuliet H *et al.,* 2000, strongly suggest that this is due to TCR binding to the MHC class II complex, either in the absence of co-stimulatory molecules or with low levels, leading to anergy or apoptosis of the antigen-specific T cells or to the generation or regulatory antigen-specific T cells. In contrast, T_{reg} cells that produce IL-10 and TGF-β are able to program the generation of tolerogenic DC from DC progenitor cells (Cederbom L *et al.,* 2000; Frasca L *et al.,* 2002; Min WP *et al.,* 2003).

4.5.1 Tolerogenic DC and T_{reg} cells in autoimmunity, transplantation and tumorimmunity

Tolerogenic DC and T_{reg} cells have been shown to be attractive cell types for therapeutic manipulation of the immune system in order to enhance the insufficient immune responses during infectious disease and cancer or to inhibit excessive immune responses in autoimmune disorders and transplant rejection (Trabell KV *et al.,* 2004; Zhang X *et al.,* 2008; Pardoll DM, 1999). The following sections highlight the therapeutic potential of tolerogenic DC and T_{reg} cells.

4.5.1.1 Autoimmunity

A key feature in the development of autoimmune diseases is a failure of immunological self-tolerance leading to aberrant immune responses to self-antigen. In addition to the potential of DC to induce autoimmune responses, their role in controlling autoimmune responses was examined. Under certain conditions antigen loaded DC are able to induce T cell tolerance, either through induction of T cell anergy or deletion of T cells, or by induction of T_{reg} cells. It has been shown that both immature and mature DC can prime T_{reg} cells that prevent autoimmunity (Trabell KV *et al.,* 2004; Yamazaki S *et al.,* 2003). Some reports also describe a regulatory capacity for semi-mature DC. For example in 2002 Menges and colleagues showed that repeated injections of DC matured with TNF-α are able to induce IL-10

producing peptide-specific T cells *in vivo* and protect mice from autoimmune encephalomyelitis.

Evidence is accumulating that $CD4^{pos}Foxp3^{pos}$ T_{reg} cells contribute to maintaining self-tolerance by down-regulating immune response to self and non-self antigens in an antigen-nonspecific manner in which they actively suppress self-reactive T cells. Recently Kim and colleagues (Kim JM *et al.,* 2007) showed that T_{reg} cells prevent catastrophic autoimmunity throughout the life span of mice. The frequency and function of naturally occurring T_{reg} cells have been examined in a number of human autoimmune diseases like systemic lupus erythematosus (SLE) (Valencia X *et al.,* 2007), rheumatoid arthritis (Flores-Borja F *et al.,* 2008), type 1 diabetes (Lindley S *et al.,* 2005), and multiple sclerosis (MS) (Hafler DA *et al.,* 2005). Depletion or functional abrogation of naturally occurring T_{reg} cells from the periphery of normal mice leads to the spontaneous development of various autoimmune diseases. Inoculation of normal $CD4^{pos}CD25^{pos}$ T cells prevented these autoimmune developments in a dose-dependent manner (Sakaguchi S *et al.,* 1995; Asano M *et al.,* 1996).

4.5.1.2 Transplantation

Continued advances in surgical techniques and immunosuppressive therapy have allowed transplantation of organs, tissues and cells to become the therapy of choice for patients with end-stage organ failure. However, despite the success of today's immunosuppression strategies clinical transplantation is still limited by the side effects associated with long-term immunosuppression. At the present time no alternative to immunosuppression exists in the clinic. Immunosuppressive agents are non-specific and target different sites in the T cell activation cascade, usually by inhibiting activation of alloreactive T cells or via depletion of alloreactive T cells. Strategies aimed at specific depletion of alloreactive T cells are needed for new therapeutic roles in reducing this barrier to transplantation tolerance. Crucial to finding new ways of influencing allograft rejection is our understanding of alloactivation which occurs by two major pathways, the direct and indirect pathway of allorecognition (Gokmen MR *et al.,* 2008) (**Figure 4.6**).

The direct pathway of alloactivation results from the direct interaction between the TCR on recipient T cells and allogenic MHC class I and class II molecules on donor APC, resulting in the generation of cytotoxic $CD8^{pos}$ T cells and $CD4^{pos}$ T_H cells (Lechler R and Batchelor JR, 1982). The indirect pathway of allorecognition involves processed peptides of allo-MHC molecules presented by antigen-presenting cells of the recipient, mainly by DC, to effector T cells in the context of self-MHC (**Figure 4.6**).

Our knowledge of the role of tolerogenic DC and T_{reg} cells in the generation and maintenance of T-cell tolerance has expanded rapidly and is now a key area of research in transplant rejection. Therefore, attention has been focused on the utilization of T_{reg} cells and tolerogenic DC in adoptive cell-based therapy to induce allograft tolerance without the need for additional immunosuppression. A number of studies have investigated the use of *in vitro* generated tolerogenic DC and T_{reg} cells to promote allograft acceptance. Recently, Zhang and colleagues (Zhang X *et al.,* 2008) showed that tolerogenic DC generated *in vitro* by treatment with LF 15-0195 are capable of augmenting $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T_{reg} cell numbers and activity resulting in the prevention of allograft rejection. Lutz and colleagues (Lutz MB *et al.,* 2000) were able to show that pre-treatment of CBA mice with allogenic immature DC 7 days before cardiac transplantation resulted in prolonged survival of allografts. However, data demonstrating prolonged graft survival without immunosuppression are rare (Horibe EK *et al.,* 2008; Ikeguchi R *et al.,* 2008).

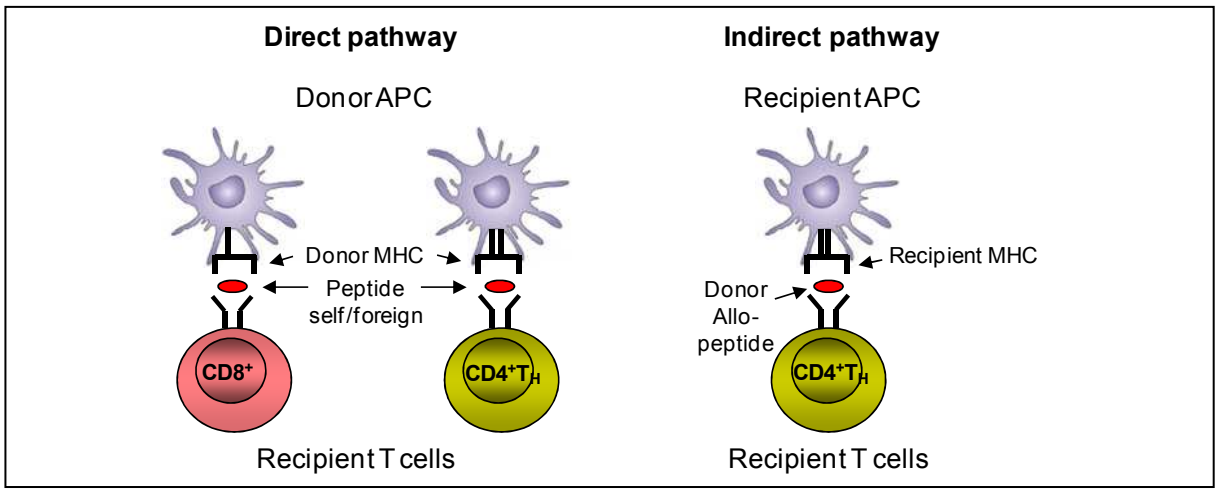


Figure 4.6: Direct and indirect antigen recognition. MHC alloantigens can be recognized by T cells via two distinct pathways: the direct and the indirect pathway. In the direct pathway it comes to ligation

of the TCR on alloreactive T cells (recipient T cells) to intact MHC molecules on donor APC. The indirect pathway results from the presentation of processed peptides of allo-peptide molecules by recipient APC to the recipient effector T cells in the context of self-MHC.

4.5.1.3 Tumorimmunity

Although T_{reg} cells are able to prevent autoimmune diseases through the induction of immune tolerance by suppressing host immune responses against self- or non-self-antigens, emerging evidence suggests that they are also able to inhibit the immune response of immune cells against tumor cells, thus promoting tumor growth and progression. Already during the 1970s and 1980s it was suggested that suppressor T cells contributed to tumor growth (Rotter V and Trainin N, 1975; Berendt MJ and North RJ, 1980). These studies have shown that depletion of suppressor T cells is associated with delayed tumor growth and prolonged survival in murine cancer models. Removal of $CD4^{pos}CD25^{pos}$ T_{reg} cells can abrogate immunological unresponsiveness to syngenic tumors *in vitro* and *in vivo*, thus leading to tumor-specific or non-specific effector T cells (Shimizu J *et al.,* 1999; Onizuka S *et al.,* 1999). However, since T_{reg} cells are engaged in suppressing immune responses toward both self and non-self, one challenge would be to find ways to distinguish between T_{reg} cells required for immune homeostasis and those involved in pathological processes such as tumor immune evasion. This could lead to novel immunomodulatory strategies for the treatment of cancer.

Clinical and experimental studies have demonstrated that tumor growth is closely associated with defects in DC function. These dysfunctions of DC have been associated with impaired differentiation and maturation of DC caused by tumor produced cytokines such as IL-10, TGF-β and VEGF that downregulate the effectiveness of DC (Kobie JJ *et al.,* 2003; Sharma S *et al.,* 1999; Gabrilovich DI *et al.,* 1996). *In vitro* maturation of DC is inhibited by IL-10 and VEGF and immature DC convert into tolerogenic DC by sensing IL-10 and TGF-β, making them unable to respond to inflammatory stimuli and inducing the development of anergic or suppressor T cells instead of effector T cells. However, different studies have shown that DC-based anti-tumor immunotherapy is able to generate protective anti-tumor

immune responses *in vivo*. Several researches have focused on the possibility of inducing autoimmune diseases to treat cancer (Pardoll DM, 1999). Previous studies have shown that vaccination with DC loaded with tumor-antigen in animal tumor models can inhibit tumor growth and induce autoantibodies transiently without clinical or histological features of autoimmunity (Tsai BY *et al.,* 2009).

In summary, all these findings support the importance of the development of therapeutic approaches concurrently promoting tolerogenic DC and T_{reg} cell generation for clinical conditions, ranging from organ transplantation over autoimmunity to treatment of cancer. A very careful characterization, especially of the different subsets of T_{reg} cells and tolerogenic DC and their respective immunosuppressive properties, will be an indispensable prerequisite for future studies. Thereby, a better understanding of the interactions between T_{reg} cells and tolerogenic DC will lead to improved tools for the treatment of different diseases such as autoimmunity, transplant rejection and cancer.

5 Aims of the study

In addition to the key role of DC in the induction of immunity, DC also play a role in the induction/maintenance of peripheral T cell tolerance by generating T cells with regulatory properties. These capacities have raised great interest in their therapeutic manipulation of the immune system in order to enhance the insufficient immune responses during infectious disease and cancer or to inhibit excessive immune responses in autoimmune disorders and transplant rejection.

The rat is widely used as an experimental animal system in a variety of areas such as immune responses to pathogens, tumors, self-antigens, or allografts. However, their DC and T_{reg} cells have not been as well characterized as research has mainly focused on the characterization of DC and T_{reg} cells of the mice system. Interestingly, the rat immune system offers a close analogy to that of humans, for example, the expression of MHC class II by activated T cells (Reizis B *et al.,* 1994; Ko HS *et al.,* 1979) that could make the rat model more attractive than the mouse model for some specific immunological studies.

The present study analyses the importance of rat tolerogenic immature DC and T_{reg} cells with immune regulatory properties. Since only few studies have focused on the generation of a defined population of immature DC from rat bone marrow, information on the culture conditions and the influence of different culture conditions on generating rat tolerogenic immature BM-DC still requires clarification to develop new strategies for the treatment or prevention of different diseases. Therefore, one aim of this study was to generate and characterize immature rat BM-DC. Cytokines such as GM-CSF and IL-4 seem to influence the yield of these cells *in vitro* (Lutz MB *et al.,* 1999; Grauer O *et al.,* 2002). Thus, the optimal growth conditions required for the generation of a defined population of immature BM-DC was systematically evaluated. In addition, these cells were phenotypically and functionally characterized to confirm their expected immature tolerogenic character. Furthermore, their ability to generate T_{reg} cells was investigated in addition to functional characterization of possibly induced T_{reg} cells.

5.1 Questions

(A) What is the optimal culture condition required for the generation of immature IL-4 DC?

(B) What are the main phenotypic characteristics of IL-4 DC?

(C) What is the effect of IL-4 DC on naïve T cells *in vitro*?

(D) Do IL-4 DC exhibit a stable immature phenotype?

(E) Are IL-4 DC able to generate T_{reg} cells from naïve T cells *in vitro*?

6 Materials

6.1 Animals

Inbred male Lewis (LEW), Wistar Furth (WF) and Brown Norway (BN) rats (8-10 weeks old, 200-350g) were purchased by Harlan Winkelmann GmbH (Borchen, Germany; **Table 6.1**).

Table 6.1: Rat Strains used in this study for cell isolation. The haplotype and reference are shown.

Rat Strain (inbreed)	MHC-Haplotype	Reference
Lewis (LEW/Han^{TM}Hsd)	*RT ^{l}*	Harlan Winkelmann GmbH (Borchen, Germany)
Brown Norway (BN/RijHsd)	*RT1^{n}*	Harlan Winkelmann GmbH (Borchen, Germany)
Wistar Furth (WF/NHsd)	*RT1^{u}*	Harlan Winkelmann GmbH (Borchen, Germany)

Femurs, spleen and lymph nodes for the isolation of dendritic cell progenitor cells, mature dendritic cells and naïve T cells were regulary isolated from Lewis rats. In addition, naïve T cells were isolated from lymph nodes of Wistar Furth or Brown Norway rats. All animals were handled according to national and institutional animal care policies.

6.2 Antibodies

The following antibodies were used in this study for flow cytometry and western Blotting (**Table 6.2** and **6.3**):

Table 6.2 (see next page): Antibodies for phenotyping of rat dendritic cells and rat T cells by flow cytometry. The name (clone) specificity and source of antibodies are shown.

Name (clone)	Specificity	Source
donkey anti-mouse IgG (H+L):RPE	whole molecule mouse IgG	Dianova, Germany
CD80[*)]	CD80	Linaris Biologische Produkte, Wertheim-Bettingen, Germany
CD86[*)]	CD86	
mouse anti-rat CD25:RPE (MRC Ox39)	rat CD25 cell surface antigen	
mouse anti-rat CD28, monoclonal (JJ319)	rat CD28 cell surface antigen	
mouse anti-rat RT1B:RPE, monoclonal (Ox6)	rat MHC II (RT1B)	
mouse anti-rat TCR alpha/beta, monoclonal (R73)	constant determinant on the beta chain of the rat alpha/beta T cell receptor	
mouse anti-rat CD4:FITC (W3/25)	rat CD4 cell surface glycoprotein	
mouse anti-rat CD54:FITC, Monoclonal (1A29)	rat cell surface CD54 (ICAM-1) antigen	
mouse anti-rat CD45 (LCA):RPE, monoclonal (MRC Ox1)	rat leukocyte common antigen	
mouse anti-rat CD25 (NDS61)	rat CD25 cell surface antigen	
mouse anti-rat CD11b/c (Ox-42)	rat CD11b/c (dendritic cells)	
FITC mouse IgG1, κ isotype control (MOPC-31C)	unknown specificity	BD Biosciences, Germany
PE mouse IgG1, κ isotype control (MOPC-21)	unknown specificity	
FITC hamster anti-mouse CD40 (HM40-3)	rat CD40 glycoprotein	
PE-Cy^{TM}5 mouse anti-rat CD4 (Ox-35)	CD4 antigen	
mouse anti-rat Ox62, monoclonal (MRC Ox-62) [*)]	rat α-E2 Integrin	Serotec, Germany
7-amino actinomycin D (7-AAD)	staining dye for dead cell exclusion	BD Via-Probe^{TM}, Germany

[*)] stained with secondary donkey anti-mouse IgG (H+L): RPE

Table 6.3: Antibodies for western blotting used in this study. The name (clone) specificity and source of antibodies are shown.

Name (clone)	Specificity	Source
anti-PD-L1	17 amino acid peptide from near the center of human PD-L1	ANASPEC, San Jose, Canada
anti-PD-L2	16 amino acid peptide from near the center of human PD-L2	
monoclonal antibody to GAPDH-loading control (GAPDH 1D4)	GAPDH	IMGENEX, San Diego, Canada
polyclonal goat anti-rabbit immunoglobulins/HRP*)	rabbit immunoglobulin	DakoCytomation, Denmark
polyclonal rabbit anti-mouse immunoglobulins/HRP **)	mouse immunoglobulin	
rabbit anti-nitric oxide synthase II (iNOS) polyclonal antibody	iNOS protein	Stressgen Bioreagents Corp., Victoria, Canada
phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody	endogenous levels of p44 and p42 MAP Kinase (Erk1 and Erk2) when phosphorylated	Cell Signalling, USA
p44/42 MAPK (Erk1/2) antibody	endogenous levels of total p44/42 MAP Kinase (Erk1 and Erk2) protein	

*) secondary antibody for anti-PD-L1, anti-PD-L2, anti-iNOS, anti-phosphop44/42 MAPK, anti-p44/42 MAPK

**) secondary antibody for monoclonal antibody to GAPDH-loading control

6.3 Primer for Reverse transcriptase-polymerase chain reaction (RT-PCR)

The following primers were used in this study for RT-PCR:

Table 6.4 (see next page): The sequences of the primers used in this work. The forward and reverse sequence, PCR product size (bp), and the annealing temperature are presented. All the primers were synthesized by MWG Biotech AG (Ebersberg, Germany) according to published sequences: GAPDH (Kruse *et al.,* 1999); MHC class II (Syha-Jedelhauser *et al.,* 1991); CD40 (Matsui *et al.,* 2002); CD80 (Holowachuk and Ruhoff, 2001); CD86 (self made with GeneFisher), IL-10 (Siegling et al., 1994), iNOS (Sterin-Borda *et al.,* 2003); IL-12 (Stumbles PA *et al.,* 1998); TGF-β (Siegling A *et al.,* 1994). In addition, the related nucleotide sequences are listed in PubMed.

Primer	bp	Temp		Sequence 5´→ 3´
GAPDH	319	62	for rev	GGT CGG TGT GAA CGG ATT TG GTG AGC CCC AGC CTT CTC CAT
TGF-β	396	60	for rev	GCC TCC GCA TCC CAC CTT TG GCG GGT GAC TTC TTT GGC GT
iNOS	578	60	for rev	GAT CAA TAA CCT GAA GCC CG GCC CTT TTT TGC TCC ATA AGG
IL-10	371	55	for rev	CTC GCT TCA CAG TGG ATG AA TAA ATA CGG TGG TGC GTG AA
IL-12	484	58	for rev	TGG AGT CAT AGG CTC TGG A GAT GAA GAA GCT GGT GCT G
MHC II	517	55	for rev	CAG GAT CTG GAA GGT CCA AGC TGT GGT TGT GCT GA
CD40	401	58	for rev	CGC TAT GGG GCT GCT TGT TGA CAG GAC GGT ATC AGT GGT CTC AGT GGC
CD80	517	50	for rev	TGG TGA AAC ACC TGA CCA GTT TCT CTG CTT GCC TCA
CD86	518	53	for rev	TGG GAA ACA GAG CTC TCA AGG TTG ATC GAC TCG TCA

bp = base pair; Temp = annealing temperature of the primer in °C; for = forward, rev = reverse.

6.4 Chemicals, reagents, cytokines and commercial kits

The following chemicals, reagents, cytokines and commercial kits were used throughout this study:

Table 6.5 (see also next page): Chemicals, reagents and commercial kits. Chemicals, Reagents and commercial kits are shown together with the source of supply.

Chemicals, reagent, cytokines and commercial kits	Source	Chemicals, reagent, cytokines and commercial kits	Source
Annexin V Apoptosis Detection Kit I	BD Pharmingen, Germany	**Griess reagent (modified)**	Sigma, Deisenhofen, Germany
Blue/Orange 6x loading dye	Promega GmbH, Mannheim, Germany	**Isopropanol**	Merck Eurolab, Nürnberg, Germany

Chemicals, reagent, cytokines and commercial kits	Source	Chemicals, reagent, cytokines and commercial kits	Source
CD40L	ALEXIS Biochemicals, USA	**Lipopolysaccharide (LPS)**	Sigma-Aldrich, Germany
Cellection™ Pan Mouse IgG Kit	Invitrogen, Karlsruhe, Germany	**M-PER® Mammalian Protein Extraction Reagent**	Thermo Scientific
Chloroform	Carl Roth GmbH, Karlsruhe, Germany	**N^G-methyl-L-arginine acetate (NMMA)**	Sigma-Aldrich, Germany
Rat IL-2 and rat IL-4	Miltenyi Biotec, Bergisch Gladbach, Germany	**nuclease free water**	Promega GmbH, Mannheim, Germany
Dextran-Alexa488	kindly provided by Prof. Lutz, Institute for Virology and Immunobiology, University of Würzburg	**rat IL-2, rat IFN-γ and rat IL-10 ELISA**	eBioscience, Canada
DMSO	Sigma-Aldrich, Germany	**Ready-to-use reagent Trizol Reagent**	Invitrogen GmbH, Karlsruhe, Germany
ECL Plus Western Blotting Detection System	GE Healthcare, UK	**RNA storage solution**	Ambion (Europe), Huntingdon, United Kingdom
ethidium bromide	Amresco, Solo, Ohio, USA	**SYBR Green PCR Master Mix**	Applied Biosystems GmbH, Weiterstadt, Germany
Ethanol	Merck Eurolab	**[^{3}H]-thymidine**	Biomedicals Germany GmbH, Eschwege, Germany
Fetal calf serum (FCS)	Cell Concepts, Germany	**TNF-α**	R&D Systems, Heidelberg, Germany
GeneAmp RNA-PCR-Kit	Applied Biosystems GmbH, Weiterstadt, Germany	**UO126 MEK1/2 inhibitor**	Cell Signaling Technology, INC., USA
GM-CSF	R&D Systems, Heidelberg, Germany		

6.5 Culture medium, buffer und stock solutions

All buffers and stock solutions (**Table 6.6** and **6.7**) were prepared with deionised water. Heat-insensitive solutions were autoclaved (135 °C, 2 bar, 20 min) for use in the cell culture, while heat-sensitive solutions were sterile-filtered (pore size 0.2 µm). To inactivate the complement factors fetal calf serum (FCS) was heated for 30 min to 56 °C and stored until needed at -20 °C.

Table 6.6: Composition of culture medium, buffer and stock solutions. The name, components and concentration are presented (see also next page).

Name	Components	Concentration
2% Agarose gel	agarose	2 g
	1xTAE buffer	200 ml
Culture medium *)	RPMI 1640	500 ml
	HEPES	20 mmol/L
	L-glutamine	2 mmol/L
	penicillin	100 U/ml
	streptomycin	100 µg/ml
	2-mercaptoethanol	$5x10^{-5}$ mol/L
	non-essential amino acids	1 %
	fetal calf serum (FCS)	10 %
Phosphate-buffered saline (PBS) **)	sodium chloride	140 mmol/L
	potassium chloride	2.7 mmo/L
	sodium dihydrogen phosphate	7.2 mmol/L
	potassium hydrogen phosphate	1.47 mmol/L
	pH was adjusted to 7.2	
RBCs-lyse-buffer (10-fold concentrated)	ammonium chloride	1.68 mol/L
	potassium hydrogen carbonate	99.88 mmol/L
	EDTA	12.6 mmol/L
TAE buffer (50x)	Tris (ultrapure)	242 g
	acetic acid	57.1 ml
	0.5M EDTA (pH 8.0)	100 ml
	add 1,000 ml ddH_2O	
Tris-NaCl buffer	Tris-HCl	0.6 g/L
	NaCl	8.1 g/L
	bovine serum albumine (BSA)	2 g/L
	pH was adjusted to 7.4	

*) Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (Gibco/ Invitrogen, Karlsruhe, Germany) was used as the standard culture medium supplemented with reagents listed above.

**) The solution was sterilized and kept at 4°C.

The following buffers and stock solutions were used for western blotting:

Table 6.7: Composition of buffer and stock solutions for western blotting. The name, components and concentration are presented.

Name	Components	Concentration
dry milk	dry milk powder	5 % (disolved in 1xTBS)
Reduction mix	Tris-HCl SDS ß-mercaptoethanol Glycerol bromphenolblue	130 mM 4.2 % 10 % 21 % 0.01 %
Running buffer	Tris (ultrapure) SDS Glycine add 2,000 ml ddH_2O pH was adjusted to 8.3	6.2 g 2 g 28.8 g
10% SDS polyacrylamide gel	30% acrylamide mix 1.5M Tris (ultrapure, pH 8.8) 10% SDS 10% amonium persulfate TEMED ddH_2O	3.3 ml 2.5 ml 0.1 ml 0.05 ml 0.004 ml 4 ml
10xTBS	Tris (ultrapure) NaCl HCl add 1,000 ml ddH_2O	24.2 g 80 g 38 ml
Towbin buffer	Tris (ultrapure) Glycin MetOH add 1,000ml ddH_2O pH was adjusted to 8.3	25 mM 152 mM 20 %

6.6 Equipment and aids

Table 6.8: Equipment and aids. The item and source of supply are shown.

Item	Source of supply
70 μm nylon cell strainer	Becton Dickinson Biosciences, Germany
5, 10, 20 ml syringe piston	Becton Dickinson Biosciences, Germany
1450-421 Filtermat A	Wallac, Perkin-Elmer Life and Analytical Sciences, Rodgau, Germany
96-well round-bottom plates	NUNC, Denmark
argon-laser flow-cytometer FACSscan	Becton Dickinson Biosciences
FlowJo	Tree Star, Inc., USA
ImageMaster workstation	Amersham Pharmacia Biotech, Piscataway, USA
MicroBeta Filtermat-96 cell harvester	Wallac, Turku, Finland
Neubauer chamber	Optik Labor, Germany
Opticon 2 Cycler	M.J. Research, Bio-rad, USA
plastic bags	Wallac, PerkinElmer
Wallac-MicroBeta TriLux β-radiation counter	Institut für Virologie und Immunbiologie, Würzburg
PROTRAN® Nitrocellulose Transfer Membrane	Whatman® Schleicher & Schnell
X-Ray Medical Film	Konica Minolta
6-well, 24-well plate	Greiner Bio-One, Germany
petri dishes (Ø 100 mm)	Becton Dickinson Biosciences
microplate reader	Dynatech laboratories, USA

7 Methods

7.1 Preparation of bone marrow-derived immature dendritic cells

Femur bones of Lewis rats were removed from the surrounding muscle tissue and purified by scraping with a scalpel. Thereafter intact bones were left in 70% ethanol for 3 minutes for disinfection and washed 2 times in sterile PBS. Then, both ends of the bone were cut off with surgical scissors and marrow flushed with 20 ml PBS for each bone through a 70 μm nylon cell using a syringe with a 20-gauge needle. The bone marrow cells, after lysis with 1-fold concentrated red blood cell (RBC) lyse buffer, were plated in petri dishes with a diameter of 100 mm at a cell density of $5x10^5$ cells/ml in medium supplemented with rat rGM-CSF (5 ng/ml) and rat rIL-4 (5 ng/ml) for the generation of IL-4 DC. The cells were cultured at 37°C in a 5% humidified CO_2 atmosphere and on day 3 or 4, half of the culture was removed, spun down, and replaced with fresh medium and cytokines. Non-adherent single cells and loose adherent cell clusters were harvested for use on day 6 or 7.

For evaluation of optimal growth conditions as mentioned above, freshly isolated bone marrow cells were plated on 24-well plates in 1 ml culture medium per well at a density of $1x10^5$ cells per ml with varying GM-CSF concentrations ranging from 0.2 to 60 ng/ml, either alone or in combination with varying IL-4 concentrations ranging from 1 to 20 ng/ml. On day 3 or 4, culture medium was replaced by fresh medium and cytokines as mentioned above.

For maturation experiments non-adherent single cells and loose adherent cell clusters were replated on day 6 at a density of $5x10^5$ cells/ml on 6-well plates in 3 ml medium per well and cultured until day 8 with 2.5 ng/ml GM-CSF only. In a second round, on day 8, cells were replated again at a density of $5x10^5$ cells/ml on new 6-well plates in 3 ml fresh medium supplemented with LPS (1 µg/ml), TNF-α (20 ng/ml) or CD40L (1 µg/ml) until day 10.

7.2 Isolation of mature splenic dendritic cell population

Harvested spleens of Lewis rats were passed through a 70 μm nylon cell strainer with a 5 ml syringe piston to generate a single-cell suspension and RBCs were lysed for 5 minutes using 1-fold concentrated RBC lyse buffer. Then, the cells from the

spleen of one rat were plated in two petri dishes with a diameter of 100 mm overnight in culture medium at 37°C in a 5% humidified CO_2 atmosphere. The next day, the non-adherent cells were collected and centrifuged over a 14.5% Metrizamide - or 10.5% OptiPrep - gradient at 4°C and 1,823 x g for 13 minutes for density gradient separation. Mature spleen DC (S-DC) were collected from the interface and counted in a Neubauer chamber.

7.3 Isolation of lymph node T cells

Lymph node (LN) cells from Wistar Furth or Brown Norway rats were prepared from popliteal and mesenteric LN by gentle homogenization with a 5 ml syringe piston through a 70 µm nylon cell strainer. After washing and lysing the RBCs with 1-fold concentrated RBC lyse buffer for 5 minutes, the viable cells (cells that did not incorporate trypan blue) were counted in a Neubauer chamber.

7.4 Depletion of $CD25^{pos}$ T cells from lymph node cell suspension

The Cellection^{TM} Pan Mouse IgG Kit was used for the depletion of $CD25^{pos}$ T cells from lymph node cell suspension. The depletion was performed following the manufacturer's instructions using anti-CD25-antibody NDS61. Alternatively, $CD4^{pos}CD25^{neg}$ and $CD4^{pos}CD25^{pos}$ T cells were sorted using CD4 and CD25 expression with a FACS DIVA sorter (Becton Dickinson).

7.5 Cell yield evaluation

Cultured cells were washed once and an aliquot volume mixed 1:10 in Trypan Blue solution. Trypan Blue negative cells were counted as viable under the microscope in a Neubauer chamber.

7.6 Flow cytometric analysis

7.6.1 Phenotyping

Surface antigen expression by IL-4 DC was determined by two-colour flow cytometry. $5x10^5$ cells were suspended in in 50 µl PBS and incubated for 20 minutes at 4°C with

the optimal concentrations of specific fluorescein isothiocyanate (FITC) - or phycoerythrine (RPE) - conjugated antibodies (**Table 6.2**). The non-conjugated antibodies Ox-62, CD80 and CD86 were detected with the RPE-conjugated, affinity-purified F(ab´)2 fragments of donkey anti-mouse IgG. The nonviable cells were identified with 7-amino actinomycin D (7-AAD). The non-specific binding was controlled with appropriate isotype-matched, non-reactive negative control antibodies. The samples were measured using an argon-laser flow-cytometer FACSscan. The analysis was performed with the FLOWJO program.

7.6.2 Apoptosis assay

After co-culture of S-DC or IL-4 DC with T lymphocytes for 72 hours, apoptosis of T cells was measured using the Annexin V Apoptosis Detection Kit I, and the cells were then analyzed on FACSscan. Staining with FITC Annexin V allows the detection of early apoptotic cells, relying on the property of cells to lose membrane asymmetry. Thereby, phosphatidylserine (PS) is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet. The Annexin V protein has a high affinity for PS which provides the basis for identifying apoptotic cells.

7.6.3 Quantitation of antigen uptake by dendritic cells

The capacity of day 6 or 7 IL-4 DC to take up antigen was tested using Dextran-Alexa 488. $2x10^5$ cells diluted with 200 µl PBS were incubated with 5 µl of Dextran-Alexa 488 stock solution for 30 minutes in the dark at 37°C, or at 4°C for determination of background staining. The samples were measured using an argon-laser flow-cytometer FACSscan. The analysis was performed with the FLOWJO program.

7.7 Cytokine analysis by ELISA (enzyme-linked immunosorbent assay)

Cytokines were measured in the supernatants of IL-4 DC replated on day 6 and in the supernatants of MLR, respectively. Rat IL-2, rat IFN-γ and rat IL-10 were measured following the instructions provided by the supplier.

7.8 Nitric Oxide (NO) assay

For measurement of NO production by DC in general, cells were re-cultured at $5x10^5$ cells/150 µl in 96-well round-bottom plates in the presence or absence of LPS (1 µg/ml). For measurement of NO production during 72 hour MLR, culture was set up as described under **section 7.9** or **7.10.1**. Cell-free culture supernatants were collected after 24 hours and the formation of stable oxidative end product of NO, inorganic nitride (NO_2-), was assessed as a quantitative indicator of NO production by using Greiss reagent. Briefly, 100 µl of Greiss reagent were added to each well of a 96-well flat-bottom plate containing 100 µl of culture supernatant. After 10 minutes of incubation, absorbance was measured at 575 nm in a microplate reader. For each sample, a negative control was included, either without LPS or containing 250 µg/ml NMMA. The values were referred to a standard curve with defined sodium nitride concentrations (7.8-500 µM).

7.9 Proliferation assay for naïve T cells

The allo-stimulatory potential of IL-4 DC was evaluated in an allogenic mixed leukocyte reaction (MLR). Day 6 or 7 Lewis IL-4 DC (10^4 cells/well) or irradiated (20 Gy) Lewis S-DC as stimulators, and naïve T cells (10^5 cells/well) from lymph nodes of Wistar Furth or Brown Norway rats as responders were co-cultured in a round-bottomed 96-well plate in a total volume of 150 µl/well. After 72 hours at 37°C in a 5% humidified CO_2 atmosphere, cells were pulsed with 0.5 µCi/well [^{3}H]-thymidine for 6 hours. Control wells consisted of naïve T cells cultured alone. The cells were harvested with the MicroBeta Filtermat-96 cell and their DNA was collected on special filter paper. The filter paper was dried for 1 hour and then sealed in special plastic bags with liquid scintillation. The level of [^{3}H]-thymidine incorporation was measured with a Wallac-MicroBeta TriLux β-radiation counter and the results were expressed as counts per minute (cpm). All experiments were set up in 6 replicates.

7.10 Inhibition assay

7.10.1 IL-4 DC mediated inhibition of T cell proliferation assay

With this assay the ability of IL-4 DC to inhibit T cell proliferation induced by S-DC was tested. 10^4 IL-4 DC from day 6 or 7 were added to the culture of 10^4 S-DC and 10^5 T lymphocytes for 6 (the end of the culture), 24 or 72 (the starting point of the culture) hours. After 72 hours cells were pulsed with 0.5 µCi/well [^{3}H]-thymidine for 6 hours. Subsequently, after 72 hours, the cells were pulsed with [^{3}H]-thymidine for 6 hours. The incorporation of radioactivity was measured using a beta-radiation counter as previously described (**section 7.9**).

7.10.2 T cell-mediated inhibition after incubation with immature IL-4 DC

Possible regulatory or inhibitory properties of T cells, which were pre-incubated with IL-4 DC, on freshly isolated T cells in the proliferation assay were tested with this. 10^5 T lymphocytes were incubated with 10^4 IL-4 DC for 72 hours. The Cellection™ Pan Mouse IgG Kit was used for depletion of IL-4 DC. The depletion was performed following the manufacturer's instructions using anti-CD11c/b-antibody Ox-42 (**Table 6.2**). After depletion of IL-4 DC, T cells were collected (purity > 90%) and transferred to the T cell proliferation assay (freshly isolated T cells and S-DC). The cells were cultured for 72 hours and pulsed with [^{3}H]-thymidine for the last 6 hours of culture. The incorporation was measured as described before (**section 7.9**).

7.11 Restimulation assay

The ability of T cells, which were pre-incubated with IL-4 DC, to proliferate in the presence of mature splenic DC was tested. 10^5 T cells were incubated with 10^4 IL-4 DC for 72 hours. Depletion of IL-4 DC with Ox-42 was performed as described before (see **section 7.10.2**). After depletion of IL-4 DC, T cells were collected (purity > 90%) and transferred to the T cell proliferation assay. 10^5 purified T cells, which were pre-incubated with IL-4 DC, were incubated with freshly isolated 10^4 S-DC for 72 hours. After 72 hours, cells were pulsed with 0.5 µCi/well [^{3}H]-thymidine for 6 hours. The incorporation of radioactivity was measured using a beta-radiation counter as previously described (**section 7.9**).

7.12 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Extraction of total mRNA from $1x10^6$ cells was performed using ready-to-use Trizol reagent according to the manufacturer's instructions. After homogenization of cells with Trizol reagent, the cells were separated into an aqueous phase containing the RNA and an organic phase by adding chloroform followed by centrifugation (15 minutes, 16,000xg, at 4°C). The RNA, recovered from the aqueous phase, was precipitated with isopropanol overnight at -20°C. After centrifugation at 13,000 rpm for 10 minutes, the pellet was washed with ethanol, air-dried, dissolved in 40 µl RNA storage solution and stored at -80°C until use. The cDNA synthesis was performed by reverse transcription of 5 µl RNA with reverse-transcriptase (2.5 U/µl) and Oligo $d(T)_{16}$ primer (2.5 µmol/L), using the GeneAmp RNA-PCR-Kit. Amplification of 5 µl cDNA was performed by using Gold AmpliTaq DNA-Polymerase (0.05 U/µl) and the specific primers (5 µmol/L each) (**Table 6.4**) mixed in nuclease free water to an end volume of 50 µl per sample. Ten µl of PCR product were mixed with 1.5 µl Blue/Orange 6x loading dye and transferred to an ethidium bromide loaded 2% agarose gel. The gel was run at 80 volt for 30 minutes. The separated PCR products were photographed in the gel with UV light illumination. The house-keeping gene Glycerinaldehyd-3-Phosphat-Dehydrogenase (GAPDH) was used as positive control.

7.13 Real time PCR

Real time PCR is a method used to quantitate differences in mRNA expression. All real time PCR reactions were performed in a 25 µl mixture containing 1 µL of sample cDNA, specific primers (**Table 6.4**) and 2-fold concentrated solution of the SYBR Green PCR Master Mix containing Ampli Taq Gold polymerase and dNTPs. The real time quantitation was performed using Opticon 2 Cycler. The reactions were run online at 95°C for 15 minutes, and 35 cycles [95°C 15 seconds, annealing (temperatures see **Table 6.4**) 30 seconds, 72°C 30 sec]. The housekeeping gene GAPDH and nuclease free water were used as positive controls. The threshold cycle (Ct) data was calculated using default threshold settings. The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold (for more details see appendix **Figure 12.1**). A melting curve was recorded to analyse product homogeneity. To assess the quality of PCR products by agarose gel, 10 µL of each PCR product were mixed with 1.5 µL Blue/Orange 6x loading dye and

transferred to a 2% agarose gel loaded with ethidium bromide. The gel was run at 80 volt for 30 minutes. The separated PCR products were photographed in the gel with UV light illumination. The house-keeping gene GAPDH was used as positive control.

7.14 Western Blotting

For determination of iNOS or PD-L1 and PD-L2 protein expression $1.5x10^6$ cells were resuspended in 10 µl M-PER® Mammalian Protein Extraction Reagent and ultrasonicated for 10 minutes. Then lysates were gently shacked at room temperature for 10 minutes and clarified by centrifugation at 20,442 x g at 4°C for 30 minutes. Cell-free culture supernatants were collected. After a short spinning, addition of reduction mix and subsequent boiling at 99°C for 10 minutes, whole cell lysates were loaded and separated by electrophoresis in 10% SDS-polyacrylamide gels and transferred to a nitrocellulose transfer membrane. The membrane was blocked in 5% dry milk for 1 hour. Washes with 1xTBS buffer were performed 3 times for 10 minutes. Then the membrane was blotted with the corresponding antibody: rabbit anti-nitric oxide synthase II (iNOS) polyclonal antibody (1:10,000), anti-PD-L1 (0.5 µg/ml), anti-PD-L2 (1 µg/ml), phospho-p44/42 MAPK (Erk1/2) antibody (1:1,000), p44/42 MAPK (Erk1/2) antibody (1:1,000) and monoclonal antibody to GAPDH (1:1,000) over night at 4°C in the dark. After washing, as described above, the membrane was incubated with a secondary, HRP-conjugated goat anti-rabbit IgG or HRP-conjugated rabbit anti-mouse IgG antibody at 1:10,000 for 1 hour at 4°C in the dark. After additional washings as mentioned above, the membrane was treated with ECL reagents according to the manufacturer's instruction. The blotted bands were detected with X-Ray Medical Film.

7.15 Signal transduction analysis

To study the signal transduction pathway involved in PD-L1 and PD-L2 expression, day 6 IL-4 DC were plated on 6-well plates in 3 ml culture medium per well at a density of $1.5x10^6$ cells with UO126 MEK1/2 inhibitor (10 µM or 50 µM) for 1, 24, 48 and 72 hours. Incubation with DMSO (50 µM) for 72 hours served as a control. For the analysis of PD-L1 and PD-L2 expression after incubation with the UO126

MEK1/2 inhibitor at the indicated time points, the IL-4 DC were collected and prepared for western blot analysis (see **section 7.14**).

7.16 Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.) or ± standard deviation (S.D.). Differences among groups were determined by the Student *t*-test for paired data. Differences between groups were considered significant when $P < 0.05$.

8 Results

In the present study, a comprehensive characterization of immature rat DC called IL-4 DC is described. The effect of GM-CSF plus IL-4 on the generation of IL-4 DC was analysed (**section 8.1**). An extensive phenotypic analysis of IL-4 DC was performed to confirm their maturation status and their endocytic activity (**section 8.2**). Since immature DC are known to be unable to stimulate T cells, the inhibitory effect of IL-4 DC on the activation of naïve T cells in both proliferation and inhibition assays was investigated (**section 8.3**). In addition, the "origin" of the IL-4 DC-mediated inhibitory effect was determined. For this, different properties such as the secretion of soluble factor(s), the induction of T cell apoptosis and the expression of inhibitory molecules such as PD-L1 and PD-L2 were analysed (**section 8.4**). A further interesting property of IL-4 DC was analysed in **section 8.5**: the induction of regulatory T cells with an immune inhibitory effect.

8.1 Generation of bone marrow-derived immature IL-4 DC

8.1.2 Evaluation of growth conditions required for the generation of IL-4 DC

One major part of this study was the characterization of IL-4 DC, a type of DC with tolerogenic properties (Tiurbe C *et al.,* 2009). In order to explore the importance of such a DC population in immunomodulation, large numbers of these cells are required (Freudenthal PS and Steinman RM, 1990; O'Doherty U *et al.,* 1993; O'Doherty U *et al.,* 1994). In this study, the differentiation of unfractionated bone marrow precursor cells into IL-4 DC in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukine-4 (IL-4) was examined. Cells with high surface expression of Ox-62, an integrin-like molecule used as a marker for rat DC (Brenan M and Puklavec M, 1992), were considered as DC and in combination with low surface expression of MHC class II they were considered as immature rat DC.

In order to optimise the culture conditions with an acceptable amount of cytokines and to generate high yields of an IL-4 DC population on day 6, culture conditions were tested under the influence of different cytokine combinations (**Figure 8.1**).

Femur bones of 8-10 week old male Lewis rats were removed and approximately 1.2×10^8 to 1.5×10^8 bone marrow cells were flushed out from 2 femur bones. Freshly isolated bone marrow cells were transferred into 24-well plates with 1 ml culture medium per well. The cells were seeded at a density of 1×10^5 cells per ml and the GM-CSF concentrations ranged from 0.2 to 60 ng/ml. The effect of GM-CSF was tested either alone or in combination with diverse IL-4 concentrations ranging from 1 to 20 ng/ml. Cell yield, formation of cell clusters and number of MHC class II-positive as well as Ox-62 positive cells were determined under different culture conditions on day 6 of cell culture (**Figure 8.1**; **Table 8.1**).

Bone marrow cells did not proliferate when cultured without GM-CSF (**Table 8.1**), whereas under the influence of even low concentrations of GM-CSF the cells started to proliferate (**Table 8.1**), confirming the indispensability of GM-CSF for DC generation (Inaba K *et al.,* 1992; Lu L *et al.,* 1995; Masurier *et al.,* 1999). With increasing GM-CSF concentrations the number of proliferating cells increased (**Table 8.1**). Cell yield could be further increased by adding IL-4 to the cultures, however, high concentrations (20-60 ng/ml) of both cytokines resulted in decreasing cell yields (**Table 8.1**).

It is well described that murine DC are continuously released from cell aggregates formed by growing bone marrow cells cultured in the presence of GM-CSF (Inaba K *et al.,* 1992). As shown in **Figure 8.1** rat bone marrow progenitor cells developed loosely adherent cell clusters only under the influence of IL-4 (5 ng/ml, indicated by arrows). Within these cell clusters proliferating rat immature IL-4 DC were found (Talmor *et al.,* 1998). The combination of GM-CSF and IL-4 resulted, as mentioned above, in an increased cell yield (**Table 8.1**). This suggests an accessory role of IL-4 for generation and proliferation of immature IL-4 DC.

Flow cytometric analysis clearly confirmed the generation of immature IL-4 DC. More than 85% of the cells isolated on day 6 of culture expressed Ox-62 molecules on their cell surface regardless of which cytokine combination was used (**Table 8.1**). In addition, flow cytometric analysis also revealed the expression of MHC class II on Ox-62 positive DC. Thereby, the percentage of MHC class II positive cells decreased

with increasing GM-CSF and IL-4 levels. Under these conditions primarily DC with an immature phenotype were found (**Table 8.1**).

Table 8.1: Combinations of different levels of GM-CSF and IL-4 strongly influence the yield of IL-4 DC, formation of IL-4 DC clusters and IL-4 DC phenotype during a 6-day culture period. Bone marrow cells were cultured with varying GM-CSF concentrations (0.2 to 60 ng/ml) either alone or in combination with varying IL-4 concentrations (1 to 20 ng/ml). Prior to cell harvesting on day 6, the cultures were optically analysed for cell cluster formation. Non-adherent single cells and loosely adherent cell clusters were dislodged and the cells were counted and analysed for their MHC class II and Ox-62 expression by flow cytometry. The cell yield (%) corresponds to the number of initially plated bone marrow cells ($1x10^5$ cells per well). Data are presented as means ± S.E.M. in four independent experiments. See also **Figure 8.1**.

Cytokines (ng/ml)		Cell yield (%)	Cell cluster formation	MHC class II expression (%)	Ox-62 expression (%)
GM-CSF	**IL-4**	**24-well plate**			
0	0	no proliferation	No	0	0
0.2	0	61±8	No	87±3	96±2
2	0	87±19	No	66±7	89±5
5	0	132±19	No	65±5	87±5
20	0	128±29	No	65±6	91±4
60	0	103±15	No	66±5	93±3
0	1	no proliferation	No	0	0
0.2	1	67±7	No	83±4	93±3
2	1	139±18	No	50±5	93±3
5	1	143±23	No	45±5	94±3
20	1	136±28	No	45±5	93±4
60	1	114±16	No	51±5	94±3
0	5	no proliferation	No	0	0
0.2	5	97±14	No	65±10	97±1
2	5	159±26	Yes	45±13	94±2
5	5	193±35	Yes	38±14	95±2
20	5	139±31	Yes	41±12	93±2
60	5	133±29	Yes	43±13	94±2
0	20	no proliferation	No	0	0
0.2	20	124±26	Yes	60±10	94±3
2	20	159±32	Yes	43±7	95±1
5	20	213±46	Yes	39±7	94±2
20	20	124±21	Yes	39±7	91±4
60	20	72±8	Yes	37±8	94±3

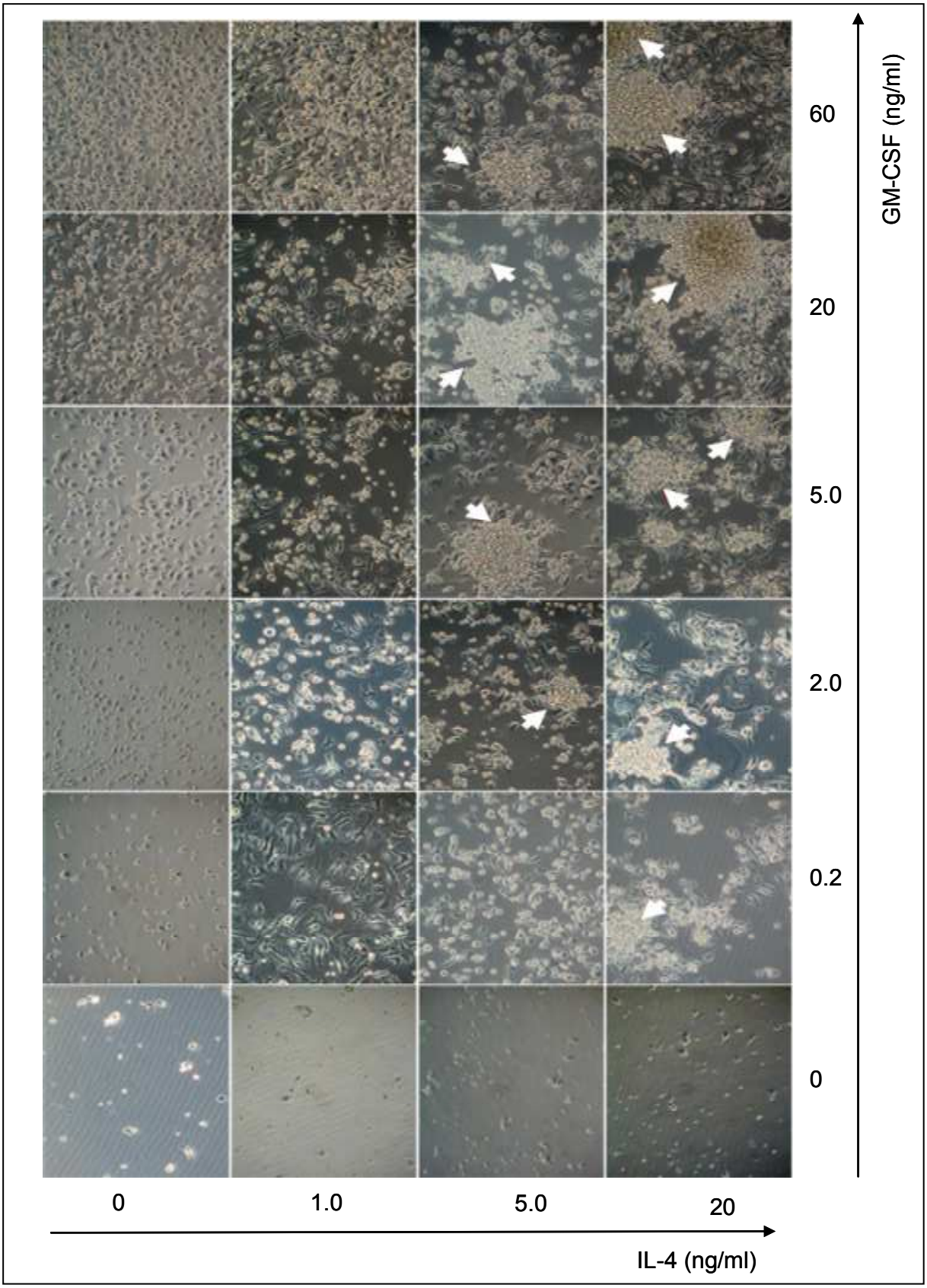


Figure 8.1: Combinations of different levels of GM-CSF and IL-4 strongly influence the yield of IL-4 DC and formation of IL-4 DC clusters during a 6-day culture period. Bone marrow cells were cultured with varying GM-CSF concentrations (0.2 to 60 ng/ml) either alone or in combination with varying IL-4 concentrations (1 to 20 ng/ml) on a 24-well plate at a density of $1x10^5$ cells per well. On day 6 of culture cells were analysed for cluster formation by eye. One example of at least 3 different experiments is shown. See also **Table 8.1** for more information.

Altogether, the results revealed that GM-CSF appears to be a key factor necessary for the induction of IL-4 DC proliferation and differentiation, whereas bone marrow progenitor cells cultured without GM-CSF did not proliferate. IL-4 seems to play an accessory role for the generation of cell clusters from which high numbers of proliferating IL-4 DC are released. With respect to the generation of high cell yield of immature IL-4 DC, addition of 5 ng/ml GM-CSF and 5 ng/ml IL-4 produced the best results at the end of the culture period with high cell yield of 193 ± 35% cells with 38 ± 14% $MHCII^{low}$ cells and 95 ± 2% $Ox\text{-}62^{pos}$ cells. Similar results were observed at a concentration of 5 ng/ml GM-CSF and 20 ng/ml IL-4 with a high cell yield of 213 ± 46% cells ($MHCII^{low}$: 39 ± 7%; $Ox\text{-}62^{pos}$: 94 ± 2%). However, since one major aim of the present study was in keeping the amount of cytokines as low as possible, a cytokine combination of 5 ng/ml GM-CSF and 5 ng/ml IL-4 provided the best results and was chosen for further experiments. For high yield of IL-4 DC, bone marrow cells were cultured in petri dishes with a diameter of 100 mm at a cell density of $5x10^5$ cells/ml and the medium was supplemented with 5 ng/ml GM-CSF and 5 ng/ml IL-4. The phenotypic and functional characterization of the IL-4 DC is shown in the following sections.

8.2 Characterization of the phenotype of immature IL-4 DC generated with 5 ng/ml GM-CSF and 5 ng/ml IL-4

8.2.1 Cluster formation and changes in cell size during the development of IL-4 DC from bone marrow progenitors

Under the influence of 5 ng/ml GM-CSF and 5 ng/ml IL-4 bone marrow cells started to proliferate within 48 hours indicated by an increase in cell size (**Figure 8.2**: right hand panel). Around day 2 to day 4 some of these enlarged cells were still floating and started to form cell clusters while others became adherent (**Figure 8.2:** left hand panel). In association with proliferation and cluster formation an increase in cell size and cell granularity could be seen. On day 2 the proportion of the cell population of interest was about 52.6% and increased up to 75.3% on day 4 (**Figure 8.2**: FACS diagrams, right hand panel). On day 6 of culture, the cells developed large clusters loosely attached to the plate which could easily be dislodged with a pipette. These

cells were identified as a homogenous cell population of IL-4 DC (**Figure 8.2**: day 6, 79.1%) as indicated by a large Forward Scatter (FSC) and a low Side Scatter (SSC).

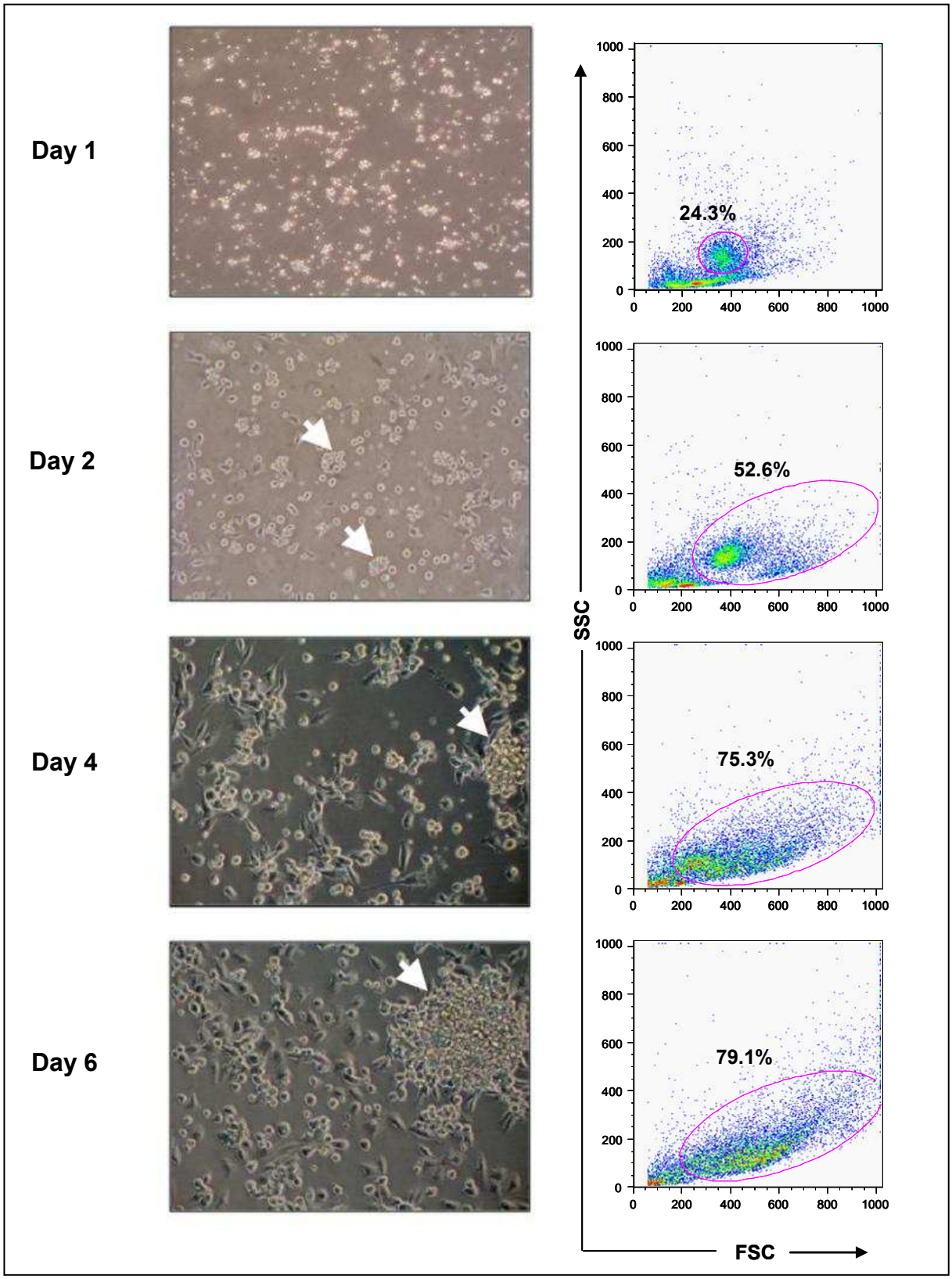


Figure 8.2: Cluster formation and changes in cell granularity and cell size of immature IL-4 DC during a 6-day culture period. Between day 2 and day 4 bone marrow progenitors started to form growing cell aggregates. On day 6 most of the cells were developing in clusters. Outside the clusters,

cells were forming an adherent cell layer (left hand panel; magnification: x100 or x200, phase contrast microscopy. Images were captured using an Olympus digital camera C-5050 and processed with Microsoft Office Picture Manager). An increase in cell size and cell granularity could be seen as indicated by Forward Scatter (FSC) and Side Scatter (SSC) by using flow cytometry shown on the right hand panel. One representative example of three different experiments is shown.

8.2.2 Phenotypic changes during the development of immature IL-4 DC from bone marrow progenitors

The phenotype of IL-4 DC at different developmental stages during their generation from bone marrow precursor cells was characterized. An increase in cell size and cell granularity was observed and in addition, an up-regulation of the co-stimulatory molecules CD80, CD86 and CD40 as well as MHC class II and Ox-62 was measured over time (day 0 to day 6) by flow cytometry (**Figure 8.3** and **Table 8.2**).

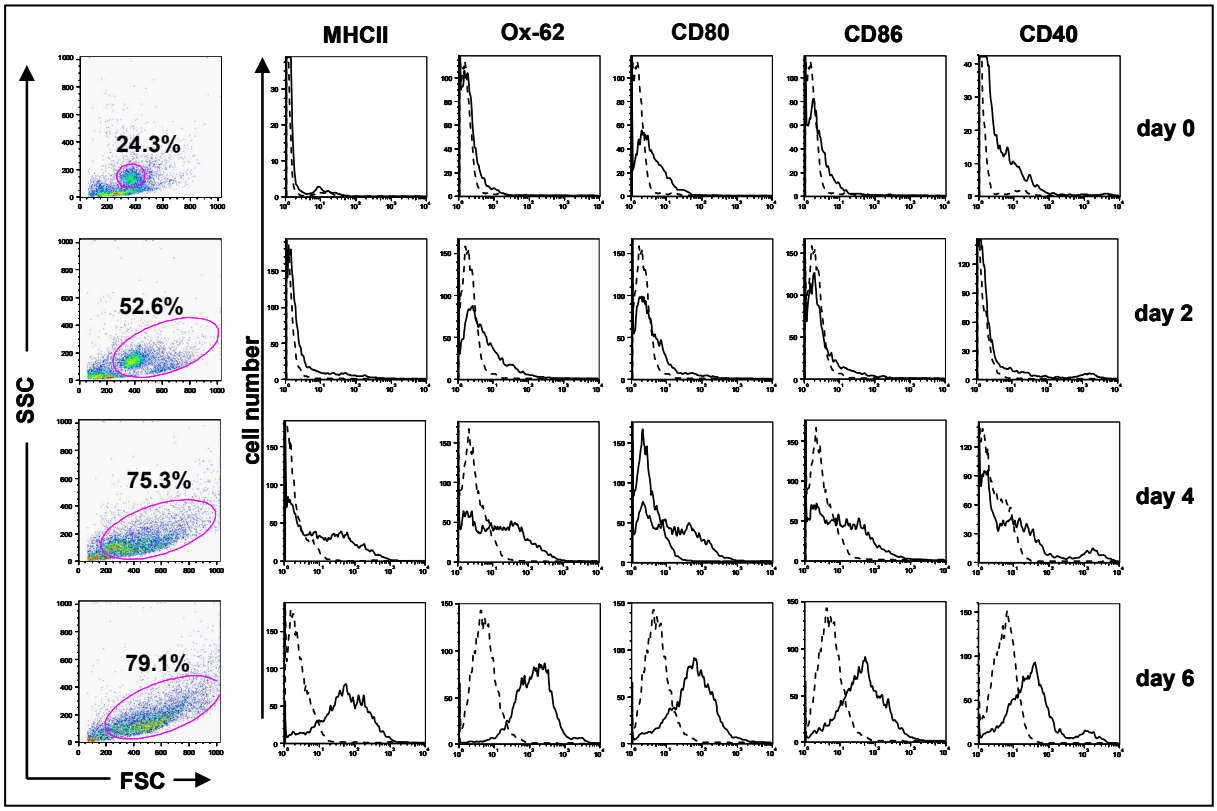


Figure 8.3: Changes in surface antigen expression during culture indicates the generation of immature IL-4 DC from bone marrow precursor cells. Fresh isolated bone marrow cells were seeded in culture dishes (100 mm ∅) and cells were collected at different time points (day 0, 2, 4 and 6). They were analysed for different cell surface markers [Ox6 (MHC class II), Ox-62 (α-E2 integrin), CD80, CD86 and CD40] by flow cytometry. Over time (day 0-6), an increase in cell size and cell granularity as indicated by Forward Scatter (FSC) and Side Scatter (SSC) on the left hand panel could be seen in association with an up-regulation of cell surface antigens (solid histograms) mentioned above. Isotype control antibodies were used for background staining (dashed histograms). One representative example of at least 3 different experiments is shown (see also appendix **Table 12.1**). The values of mean fluorescence and percentage of positive cells are shown in **Table 8.2**.

The bone marrow cells were clearly negative for most of the investigated surface markers (**Figure 8.3, Table 8.2**). Nearly half (10.9%) of the investigated cell population on day 0 showed a low expression of CD40 molecule with a mean fluorescence value (MF) of 28.1 (**Table 8.2**). On day 2 the amount of proliferating cells increased to 52.6% of the total precursor cell population (**Figure 8.3:** left hand panel). The percentage of these cells expressing the surface molecules of interest increased (**Table 8.2**) as well as the number of these molecules (**Table 8.2**).

Table 8.2: Changes in the expression of different surface molecules during culture indicates the generation of immature IL-4 DC from bone marrow precursor cells (see also Figure 8.3). Cells were collected at different developmental stages (day 0, 2, 4 and 6). Differences in the cell surface expression were quantified by mean fluorescence (MF) values of antibody binding. One representative example of at least 3 different experiments is shown (see also appendix **Table 12.1**). In addition, percentage of positive cells (P= Proportion) is shown.

Antigen	day 0		day 2		day 4		day 6	
	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)
MHC II	1.39	1.34	11.1	10.3	43.3	40.7	119	84.6
Ox-62	8.23	2.74	29.7	23.4	60.7	51.8	119	73.4
CD80	5.89	12.0	11.6	12.8	53.9	48.6	114	70.7
CD86	7.14	5.22	10.2	11.5	39.2	45.5	133	77.7
CD40	28.1	10.9	68	10.7	190	37.5	172	61.9

On day 4, after culture medium was changed and “fresh” cytokines were added on day 3 (see materials and methods), the investigated cell population constituted 75.3% of the total precursor cell population and these proliferating cells expanded into immature IL-4 DC (**Figure 8.3**: left hand panel) with an increased expression of surface molecules (**Table 8.2**).

On day 6 the flow cytometric analysis revealed the development of IL-4 DC constituting 79.1% of the total cell population (**Figure 8.3**: left hand panel). These cells were positive for the co-stimulatory molecules CD80, CD86, CD40 as well as MHC class II and Ox-62 indicated by elevated mean fluorescence (MF) values (**Table 8.2**).

Although the cell surface expression of these molecules on IL-4 DC increased during the 6 day culture, it should be considered that this expression was clearly reduced in comparison to the expression of these molecules on mature S-DC (see **section 8.2.3; Figure 8.4**). The expression of surface molecules of both immature IL-4 DC and mature S-DC is shown in the following **section 8.2.3**.

8.2.3 IL-4 DC demonstrate an immature phenotype: A comparison of the surface expression of different molecules by immature IL-4 DC and mature S-DC

High expression of MHC class II and co-stimulatory molecules such as CD80, CD86, and CD40 characterizes mature DC and is needed for full T cell activation (Reis e Sousa C, 2004; McLellan AD *et al.,* 1996). Other co-stimulatory molecules such as ICAM-1 have also been implicated in co-stimulatory signalling for T cell receptor-initiated activation (Damle NK *et al.,* 1992). In addition, CD45 molecule helps to regulate T cell proliferation and differentiation (Montoya M *et al.,* 2006; Pricket TCR and Hart DN, 1990). CD25 molecule was found to be a clear distinguishing feature of DC maturity correlating with the ability these CD25 expressing mature DC to direct the immune response toward T_H1 (Mnasria K *et al.,* 2008; Pilon C *et al.,* 2009).

In the next set of experiments the “status of immaturity” of IL-4 DC on day 6 of culture was analysed in more detail by comparing their phenotype to that of mature S-DC. For this, IL-4 DC and S-DC were labelled with several antibodies recognizing different surface molecules and analysed by flow cytometry.

Figure 8.4 compares the expression of Ox-62, ICAM-1, CD40, CD45, MHC class II, CD80, CD86, and CD25 by IL-4 DC and mature S-DC on day 6.

Table 8.3 shows both the corresponding mean fluorescence (MF) values of antibody binding and the percentage of positive cells.

Figure 8.5 shows that differences in the surface expression between IL-4 DC and S-DC are statistically significant.

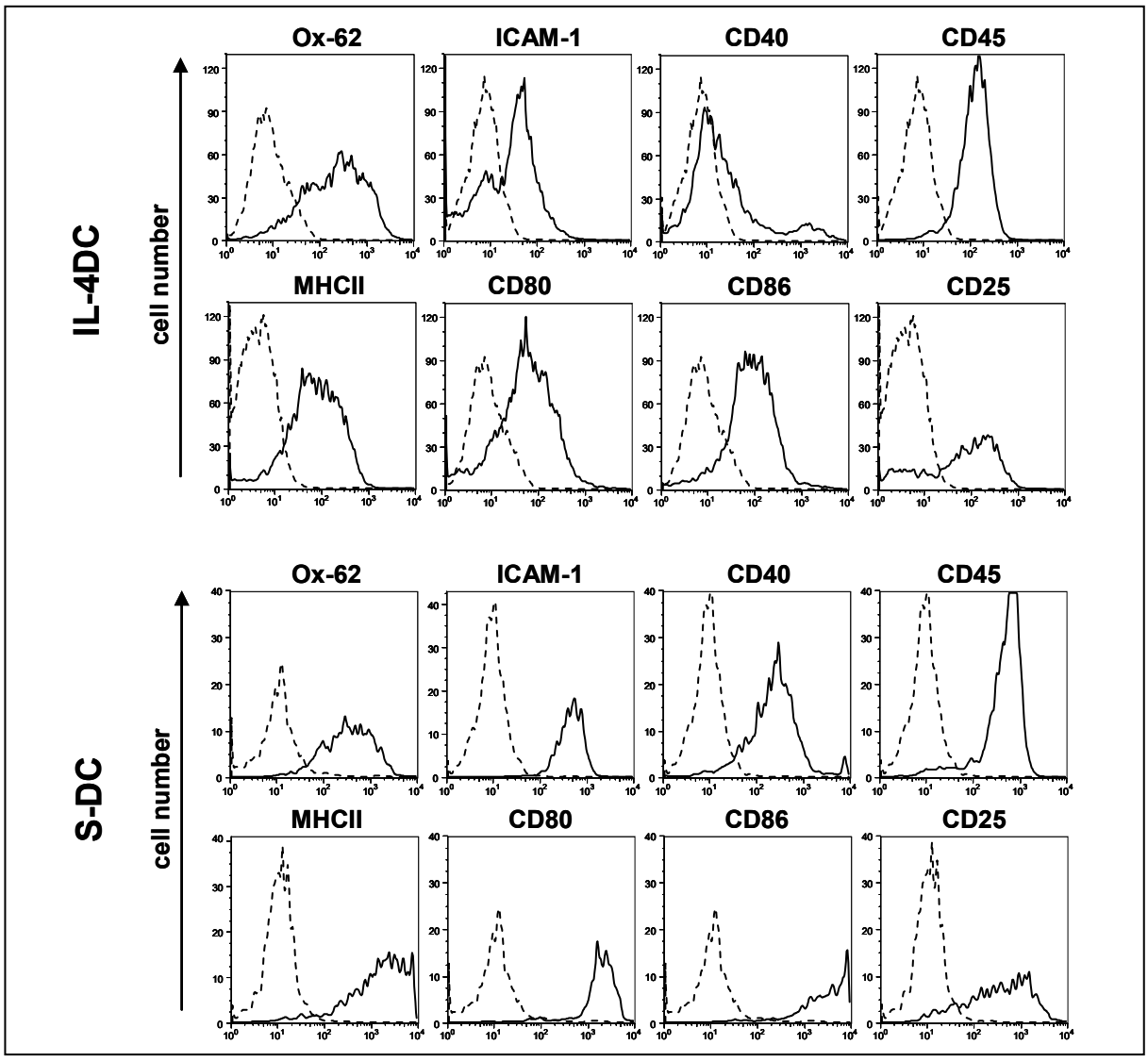


Figure 8.4: Comparison of surface expression of different molecules by immature IL-4 DC and mature S-DC. IL-4 DC were cultured with 5 ng/ml GM-CSF and 5 ng/ml IL-4 and were collected on day 6 of culture. S-DC were isolated from spleen cells and collected on day 1 of culture (see materials and methods). Cells were analysed by flow cytometry for the following cell surface markers (solid histograms): Ox-62, MHC class II, CD80, CD86, CD40, ICAM-1, CD45 and CD25. Isotype control antibodies were used for background staining (dashed histograms). In comparison to S-DC, IL-4 DC demonstrated a clear reduced expression of most surface molecules analysed in this study. One representative example of at least 3 different experiments is shown (see also **Figure 8.5**). The values of mean fluorescence and percentage of positive cells are shown in **Table 8.3**.

The flow cytometric analysis revealed both considerable surface expression of Ox-62 and comparable amounts of Ox-62 positive cells for IL-4 DC and mature S-DC (**Table 8.3**). Most of the IL-4 DC (between 62% and 87%, **Figure 8.4** and **Table 8.3**) showed a moderate expression of the following surface molecules: ICAM-1, CD45, MHC class II, CD80, CD86, and CD25 with mean fluorescence (MF) values between 48 and 469 (**Table 8.3**). The lowest MF value was found for ICAM-1 surface

expression with a MF of 48.4 (**Table 8.3**). A small proportion of IL-4 DC (42.9%, **Table 8.3**) expressed CD40 at higher levels (MF: 200) on their surface (**Table 8.3**).

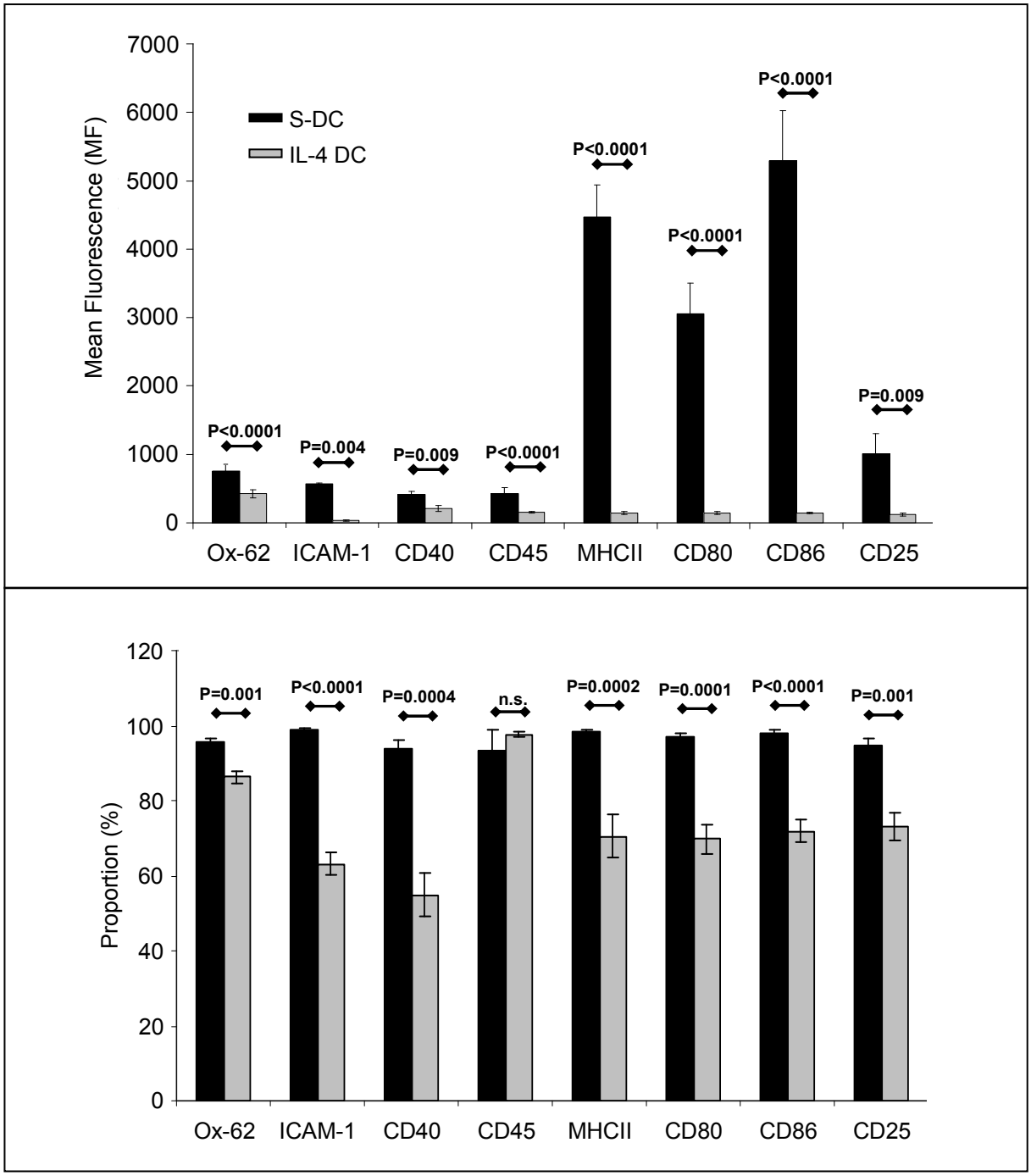
However, the expression of all investigated molecules was significantly lower in comparison to mature S-DC (**Figure 8.5** upper panel). The most striking difference was observed for MHC class II with a nearly 20-fold reduced expression on IL-4 DC (**Table 8.3** and **Figure 8.5**), and the co-stimulatory molecules CD80, CD86 and ICAM-1. Their surface expression was reduced between 11- and 26-fold (**Table 8.3** and **Figure 8.5**).

Table 8.3: Comparison of surface expression of different molecules by immature IL-4 DC and mature S-DC (see Figure 8.4). Differences in the cell surface expression were quantified by mean fluorescence (MF) values of antibody binding. In addition, percentage of positive cells (P= Proportion) is shown. One representative of at least 3 different experiments is shown (see also **Figure 8.5**).

	Surface molecules															
	Ox-62		**ICAM-1**		**CD40**		**CD45**		**MHCII**		**CD80**		**CD86**		**CD25**	
	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)
IL-4 DC	469	87.4	48.4	61.9	200	42.9	152	65.7	130	82.7	121	71.0	156	84.9	149	72.2
S-DC	608	95.8	532	99.8	520	93.5	592	95.2	2537	96.8	2295	99.5	4024	98.6	763	88.7
Fold exp.*)	**1.2**	**1.1**	**10.9**	**1.6**	**2.6**	**2.1**	**3.8**	**1.0**	**19.5**	**1.2**	**18.9**	**1.4**	**25.7**	**1.2**	**5.1**	**1.2**

***) Fold expression (S-DC over IL-4 DC)**

Figure 8.5 (see next page): Comparison of immature IL-4 DC and mature S-DC for the surface expression of Ox-62, ICAM-1, CD40, CD45, MHC class II, CD80, CD86 and CD25. IL-4 DC were collected at day 6 of culture and S-DC were freshly isolated and collected on day 1 of culture (see materials and methods). Cells were analysed for different cell surface markers as mentioned above by flow cytometry. The upper panel shows the intensity of expression (MF). In the lower panel the proportion of DC expressing the given antigens is shown. Immature IL-4 DC (grey bars) did demonstrate significantly reduced surface antigen expression of all antigens investigated in comparison to mature S-DC (black bars). The proportion of immature IL-4 DC expressing Ox-62, ICAM-1, CD40, MHC class II, CD80, CD86 and CD25 is significantly lower compared to mature S-DC. Data are presented as means ± S.E.M. in a minimum of five independent experiments. Statistical significance of antigen expression by immature IL-4 DC and mature S-DC is indicated using the paired Student *t*-test (n.s. = not significant).



In summary, flow cytometric analysis revealed that a cytokine combination of 5 ng/ml GM-CSF plus 5 ng/ml IL-4 promotes the differentiation of rat bone marrow progenitor cells into immature IL-4 DC. Their immature phenotype was characterized by significantly reduced levels of surface expression of MHC class II, the co-stimulatory molecules CD80, CD86, CD40 as well as ICAM-1, CD45 and CD25 compared to mature S-DC (**Figure 8.5**). Thereby, the most striking differences were observed for MHC class II, CD80, CD86, ICAM-1 and CD25 (**Table 8.3**). The increased

expression of these molecules on the cell surface of S-DC is typical for fully mature DC.

The immature stage of IL-4 DC verified by flow cytometry was also confirmed by RT-PCR analysis (**section 8.2.4**) and endocytosis assay (**section 8.2.5**).

8.2.4 RT-PCR analysis revealed specific mRNA for MHC class II molecules, co-stimulatory molecules, immune regulatory and suppressive cytokines and iNOS

The analysis of the expression of different surface molecules by IL-4 DC and mature S-DC (**Figure 8.4**) was completed by the detection of specific mRNA for MHC class II molecules and the co-stimulatory molecules CD40, CD80 and CD86. As shown in **Figure 8.6** specific mRNA for these molecules was proven, although IL-4 DC did not show a strong surface expression of these molecules in comparison to mature S-DC (see **section 8.2.3**).

In addition, the expression of the immune regulatory cytokine interleukin-12 (IL-12) by IL-4 DC was also investigated on mRNA level. IL-12 plays an important role in inducing the development of particular T_H cell subsets directing T_H1 and T_H2 responses, respectively. As shown in **Figure 8.6** IL-4 DC did not express mRNA specific for IL-12 in contrast to mature S-DC.

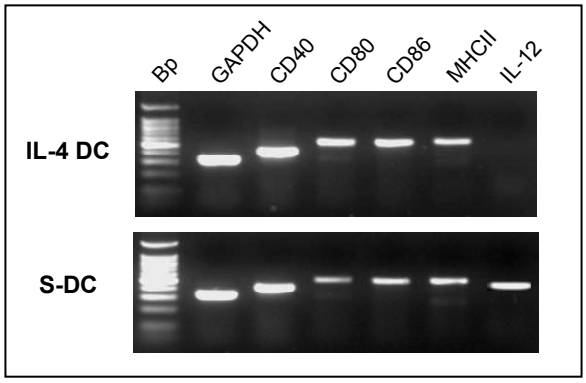


Figure 8.6: RT-PCR gene expression array of different molecules (MHC class II, CD40, CD80, CD86, and IL-12 for IL-4 DC and S-DC. Both IL-4 DC and mature S-DC expressed mRNA specific for co-stimulatory molecules CD40, CD80, CD86 and MHC class II. IL-4 DC do not express IL-12 molecules in comparison to mature S-DC. GAPDH was used as a control. The results shown are representative for at least four different experiments (see also appendix **Table 12.6**).

In addition, IL-4 DC were analysed for the presence of the immune inhibitory cytokines transforming growth factor-β (TGF-β) and interleukine-10 (IL-10) (**Figure 8.7**). Both cytokines are potent inhibitors of T cell activation and demonstrate tolerizing effects on these cells. As shown in **Figure 8.7** IL-4 DC expressed mRNA specific for TGF-β and IL-10.

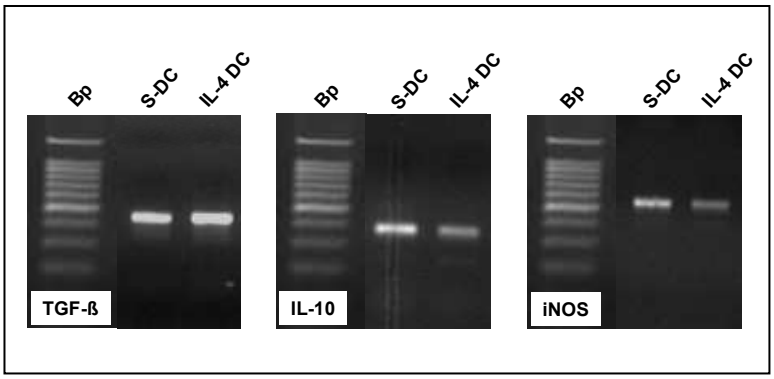


Figure 8.7: RT-PCR gene expression array of immune regulatory cytokines TGF-β and IL-10 and cytotoxic enzyme iNOS. Mature S-DC were used as controls. RT-PCR analysis shows that both IL-4 and mature S-DC do express TGF-β, IL-10 and iNOS mRNA. The results shown are representative for at least three different experiments (see also appendix **Table 12.5** and **12.6**).

The enzyme nitric oxide synthase (iNOS) mediates the production of cytotoxic nitric oxide (NO), which is known to be involved in the killing of allogenic cells during destruction of organ grafts; known as transplant rejection. The presence of iNOS specific mRNA was proven (**Figure 8.7**).

In addition to the qualitative analysis of gene expression by "traditional" PCR, the relative quantification of mRNA levels for the cell surface molecules CD80, CD86, CD40, and MHC class II, IL-12 and TGF-β was investigated using quantitative ("real time") RT-PCR (**Figure 8.8**). Quantitative RT-PCR allows the detection of amplicons during the exponential phase of enzyme reaction, whereas traditional PCR detects PCR amplicons at the final phase of the PCR reaction (end-point analysis). Therefore, qualitative analysis of mRNA levels by "traditional" PCR is not as precise as relative quantitation by real time PCR in detecting differences in expression levels of certain mRNA.

The relative quantification was done with the $2^{-\Delta\Delta Ct}$ method (Livak KJ and Schmittgen TD, 2001); for details see appendix **Table 12.7** and **Figure 12.1.** The results revealed that immature IL-4 DC demonstrate reduced levels of IL-12 and co-stimulatory molecules CD80, CD86 and CD40. This is in accordance with the flow cytometric results (**section 8.2.3**).

The following results are in contradiction to results revealed from flow cytometric analysis: the real time PCR quantification showed comparable levels of specific mRNA for MHC class II in mature S-DC and immature IL-4 DC (**Figure 8.8** and appendix **Table 12.7**), whereas the surface expression was clearly reduced in IL-4 DC (**Figure 8.5**).

In addition, the real time PCR quantification demonstrated comparable levels of specific mRNA for TGF-β in immature IL-4 DC and mature S-DC (**Figure 8.8** and appendix **Table 12.7**) which was not apparent from "traditional" PCR (**Figure 8.7**). However, expression of TGF-β specific mRNA does not necessarily imply TGF-β expression and secretion. The levels of secreted TGF-β by IL-4 DC and S-DC after a culture period of 24 hours is shown in **section 8.4.2**, **Figure 8.19**.

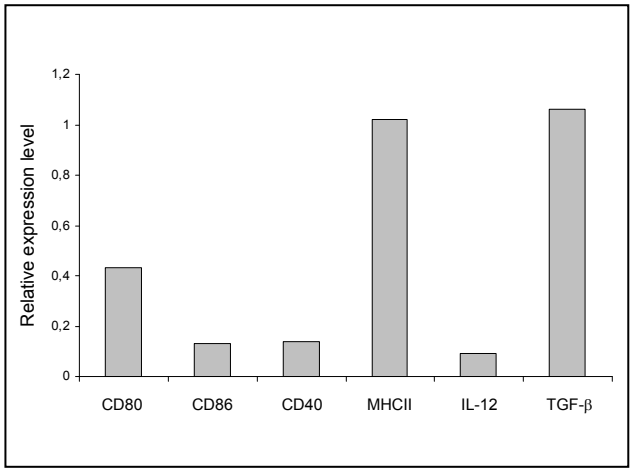


Figure 8.8: Real time RT-PCR quantification: the relative expression level of different target mRNA in immature IL-4 DC compared to mature S-DC. The relative expression level of certain mRNA (CD80, CD86, CD40 MHC class II, IL-12 and TGF-β) in immature IL-4 DC in comparison to mature S-DC is shown. With the exception of MHC class II and TGF-β mRNA, IL-4 DC show reduced levels of m RNA for IL-12 and co-stimulatory molecules (CD80, CD86 and CD40) compared to mature S-DC. GAPDH mRNA served as endogenous control. Data are presented as means ± S.D. of 3 independent experiments (see also appendix **Table 12.7** and **Figure 12.1**)

8.2.5 Bone marrow-derived IL-4 DC demonstrate endocytic activity

Another characteristic of immature DC is their ability to take-up antigens by a process called endocytosis (Quah BJC and O'Neill HC, 2005; Hotta C *et al.,* 2006). Endocytosis is the process by which cells absorb molecules (such as proteins) from outside the cell by engulfing it with their cell membrane. In the next set of experiments the ability of IL-4 DC to take up antigens from outside was assessed using Alexa 488 conjugated Dextran. The incorporation of Dextran-Alexa 488 was tested by flow cytometry. Therefore, purified day 6 IL-4 DC or mature S-DC were incubated with Dextran-Alexa 488 for 30 min at 37°C. Incubation for 30 min at 4°C

was used as negative control for determination of possible background staining. The uptake was calculated as the change in mean fluorescence (MF) between cell samples incubated at 37°C and 4°C. FACS analysis showed a clear up-take of Dextran-Alexa 488 by IL-4 DC compared to mature S-DC when mean of background staining is subtracted (**Figure 8.9**).

The result of the endocytosis assay and the reduced expression of co-stimulatory molecules measured by flow cytometry confirm the immaturity of IL-4 DC.

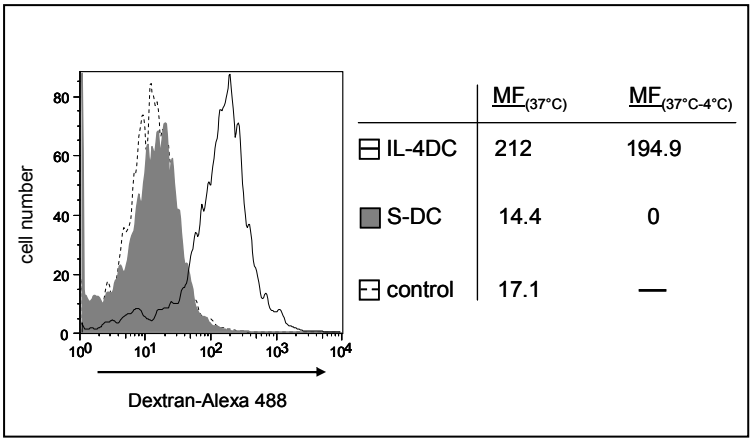


Figure 8.9: Uptake of Dextran-Alexa 488 by IL-4 DC versus S-DC. IL-4 DC (open histogram, constant line) show increased Dextran-Alexa 488 up-take compared to mature S-DC (closed histogram). The histogram shows IL-4 DC and mature S-DC uptake at 37°C versus uptake at 4°C (open histogram, dashed line). The results depict $MF_{(37–4°C)}$. The histogram shown here is representative for three different experiments (see also appendix **Table 12.2**).

Altogether, the phenotypical characterization of IL-4 DC shows an immature phenotype for them. Their immaturity is confirmed by significantly reduced surface expression of molecules such as ICAM-1, CD40, CD45, MHC class II, CD80, CD86 and CD25 compared to mature S-DC (**Figure 8.5**).

This is in accordance with reduced expression levels of mRNA specific for CD80, CD86 and CD40 as shown by real time RT-PCR quantification (**Figure 8.8**). In addition, IL-4 DC do not express mRNA specific for IL-12 (**Figure 8.6**) and demonstrate endocytotic activity (**Figure 8.9**).

The functional properties of these IL-4 DC are described in the following sections.

8.3 Characterization of the IL-4 DC-mediated inhibition of T cell proliferation

8.3.1 IL-4 DC are unable to activate naïve T cells

The ability to activate T cells is a major feature of DC, and DC maturation increases this effect. Immature IL-4 DC and mature S-DC were compared for their capacity to induce the proliferation of naïve T cells as a sign of full T cell activation. To evaluate the possible stimulatory or suppressive capacity of IL-4 DC on naïve T cells, the mixed leukocyte culture (MLC) was used to estimate the strength of the direct pathway of allorecognition (see **section 4.5.1.2**). Thereby, strong elevated radioactive signals of [^{3}H]-thymidine incorporation expressed in counts per minute (cpm), demonstrate a high rate of T cell proliferation.

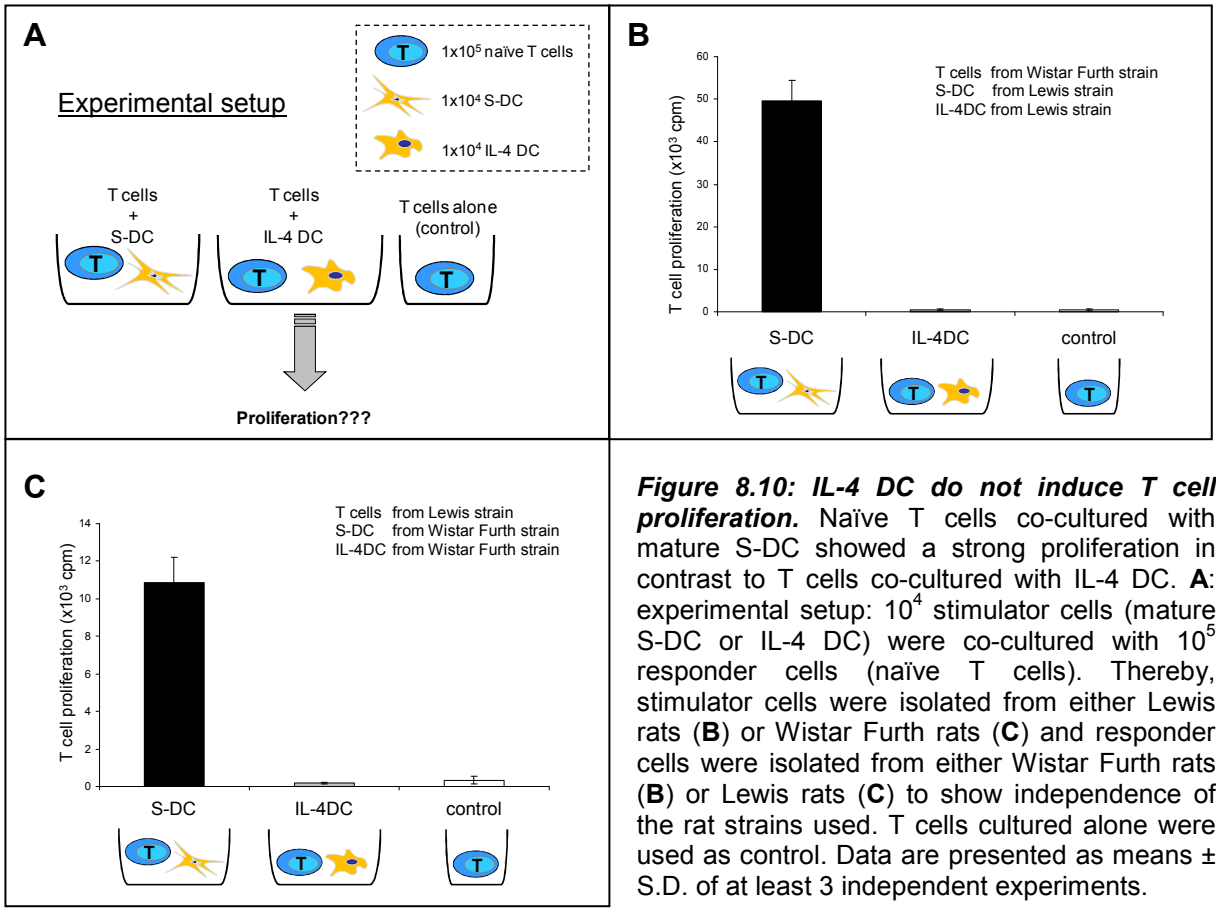


Figure 8.10: IL-4 DC do not induce T cell proliferation. Naïve T cells co-cultured with mature S-DC showed a strong proliferation in contrast to T cells co-cultured with IL-4 DC. **A**: experimental setup: 10^4 stimulator cells (mature S-DC or IL-4 DC) were co-cultured with 10^5 responder cells (naïve T cells). Thereby, stimulator cells were isolated from either Lewis rats (**B**) or Wistar Furth rats (**C**) and responder cells were isolated from either Wistar Furth rats (**B**) or Lewis rats (**C**) to show independence of the rat strains used. T cells cultured alone were used as control. Data are presented as means ± S.D. of at least 3 independent experiments.

Immature IL-4 DC or mature S-DC from Lewis rats were co-cultured with naïve lymph node T cells isolated from Wistar Furth rats at a DC:T cell ratio of 1:10 (**Figure 8.10 A** or see materials and methods). T cells cultured alone served as control. **Figure**

8.10 B shows that immature IL-4 DC did not stimulate naïve T cells in contrast to mature S-DC. In detail, the measured proliferation rates were 483 ± 284 cpm for IL-4 DC and 49,620 ± 4,929 cpm for mature S-DC. The inability of IL-4 DC to activate naïve T cells was independent of the rat strain used for DC generation. The same result was observed when IL-4 DC and S-DC, isolated from Wistar Furth rats instead of Lewis rats, were co-cultured with naïve lymph node T cells isolated from Lewis rats instead of Wistar Furth rats (**Figure 8.10 C**). The measured proliferation rates were 175 ± 56 cpm for IL-4 DC and 10,845 ± 1,367 cpm for mature S-DC.

Immature IL-4 DC do not activate naïve T cells. The reason for this may be their inability to express levels of co-stimulatory molecules on their cell surface comparable to those of mature S-DC (see **section 8.2**). In consequence, they are unable to deliver optimal signals to naïve T cells necessary for their full activation (see **section 4.2.3**).

8.3.2 IL-4 DC-mediated inhibition of T cell activation is associated with increased levels of TGF-β and reduced levels of IFN-γ and IL-2

Mature DC activate naïve T cells and induce their development to certain T cell subsets. T cells of these subsets can be distinguished according to the cytokine profile produced by them (see **section 4.3**; **Figure 4.4**). As shown above, naïve T cells co-cultured with S-DC were fully activated and demonstrated proliferation. IL-4 DC were unable to induce T cell proliferation. However, T cells co-cultured with IL-4 DC that do not proliferate are not necessarily naïve T cells; they are different from naïve T cells. To investigate the possibility that immature IL-4 DC influence naïve T cells, their cytokine profile was analysed. Supernatants of T cells co-culture with S-DC and IL-4 DC, respectively, were analysed for Interferon-γ (IFN-γ), interleukine-2 (IL-2), TGF-β and IL-10 using ELISA assay.

IL-2 is a cytokine produced by activated T cells and essential for their clonal expansion. IL-2 is important for T cells leaving the G1 phase and starting DNA synthesis. Therefore, IL-2 levels can be measured before T cells start to proliferate. IFN-γ is predominantly produced by T_H1 (effector) cells which differentiate from naïve

T cells under the influence of IL-12. The immunomodulatory cytokines IL-10 and TGF-β inhibit the release of proinflammatory cytokines by innate immune cells. They are also produced by T cells with immunoregulatory properties, e.g. T_{reg} cells.

As shown in **Figure 8.11**, mature S-DC induced a strong differentiation of naïve T cells into T_H1 cells. These T cells produced high levels of IFN-γ (917.3 ± 539.6 pg/ml) and IL-2 (228.3 ± 46.4 pg/ml). In contrast, naïve T cells cultured with immature IL-4 DC showed a 23-fold reduced IFN-γ production (39.1 ± 6.4 pg/ml) and 2.5-fold reduced IL-2 levels (88.9 ± 23.1 pg/ml). These T cells demonstrated an enhanced production of TGF-β (225.8 ± 147.2 pg/ml) in comparison to T cells cultured with mature S-DC (78.7 ± 13.5 pg/ml). This indicates that naïve T cells differentiate under the influence of IL-4 DC into a type of T cell with possible regulatory functions[1]. Naïve T cells co-cultured with S-DC differentiated into T cells with effector functions, indicated by the secretion of IFN-γ. A secretion of IL-10 was not observed for T cells cultured with mature S-DC (37.6 ± 35.8) or immature IL-4 DC (38.4 ± 32.9).

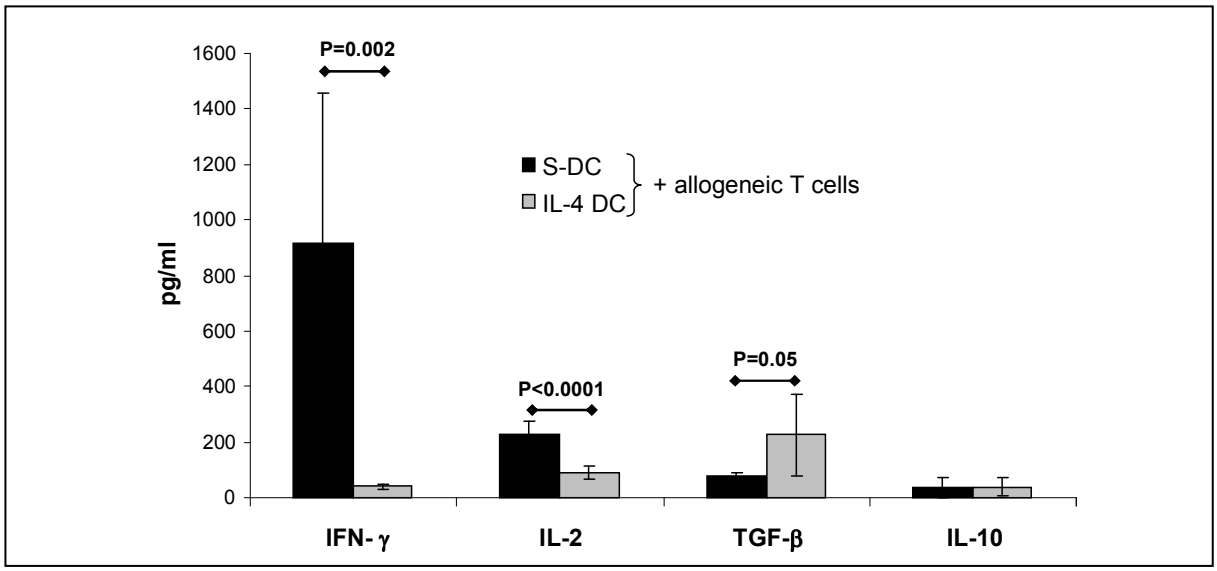


Figure 8.11: Naïve T cells co-cultured with IL-4 DC showed a TGF-β dominated cytokine profile. 10^4 mature S-DC or 10^4 immature IL-4 DC were cultured with 10^5 naïve T cells for 3 days. The co-culture with IL-4 DC did not influence the expression of IFN-γ by naïve T cells in contrast to T cells co-cultured with S-DC. An IL-10 production was not observed for both T cells co-cultured with S-DC and IL-4 DC, respectively. Data are means ± S.D. of a minimum of 3 independent experiments.

[1] Please note that IL-4 DC are able to produce TGF-β themselves (**section 8.4.2**, **Figure 8.19**). This observation is in accordance with results described by Morelli and colleagues (Morelli AE *et al.,* 2001).

8.3.3 IL-4 DC-meditated inhibition of pre-activated T cell proliferation

As shown in **section 8.3.1**, immature IL-4 DC were not able to induce the proliferation of naïve T cells. In the next set of experiments their capacity to further increase or inhibit the proliferation of activated T cells was analysed. Therefore, naïve T cells were incubated with JJ319 and R73 antibodies for 24 hours and these pre-activated T cells were cultured with immature IL-4 DC or mature S-DC. Pre-activated T cells and non-preactivated T cells cultured without any stimulation served as controls. The T cell proliferation was evaluated after 72 hours.

Figure 8.12 shows a significantly increased proliferation of pre-activated T cells compared to non-preactivated T cells (first two bars on the left side of **Figure 8.12**). The proliferation rates were 3,605 ± 632 cpm for pre-activated T cells and 444 ± 312 cpm for non-preactivated T cells. Pre-activated T cells cultured with S-DC increased their proliferation up to 6-fold (20,990 ± 3,179 cpm). In contrast, pre-activated T cells co-cultured with IL-4 DC demonstrated with 1,480 ± 243 cpm a strongly reduced proliferation. This proliferation rate was significantly reduced in comparison to the proliferation rate of pre-activated T cells cultured without any stimulation (1,480 ± 243 cpm versus 3,605 ± 632 cpm). This is a reduction in T cell proliferation of more than 50%. Therefore, this result underlines that IL-4 DC are able to inhibit actively T cell proliferation.

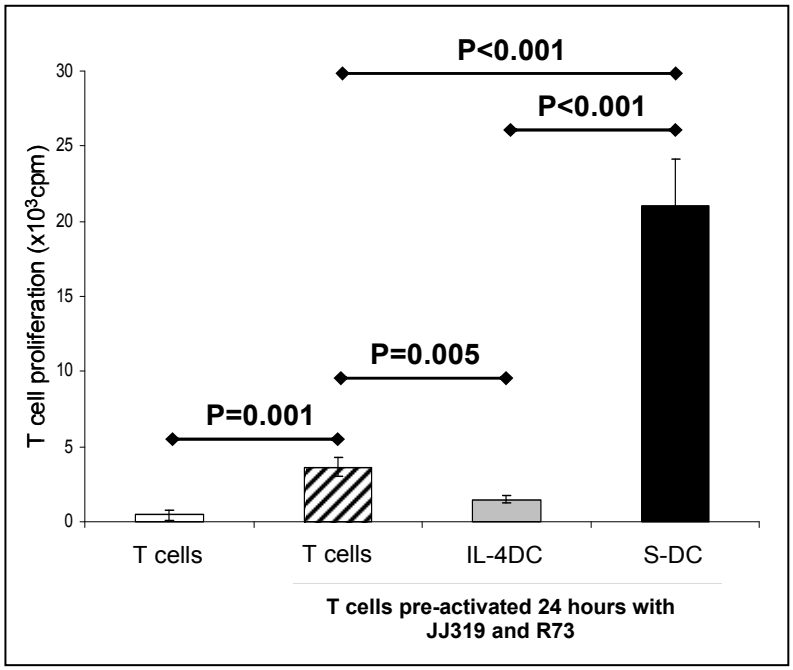


Figure 8.12: Activated T cells stopped proliferation during co-culture with immature IL-4 DC. Naïve T cells were pre-activated antigen-independent with the monoclonal antibodies R73 and JJ 314 for 24 hours. Subsequently, these T cells were co-cultured with IL-4 DC and S-DC for 72 hours. S-DC increased the proliferation of these pre-activated T cells, whereas IL-4 DC inhibited significantly the proliferation of pre-activated T cells. Untreated or pre-activated T cells cultured alone were used as controls. Data are means ± S.D. of three independent experiments. Statistical significance is indicated using the paired Student *t*-test.

8.3.4 IL-4 DC-mediated inhibition of mature S-DC-induced T cell proliferation in co-cultures

In the next set of experiments the inhibitory effect of immature IL-4 DC on T cell stimulation was analysed in more detail. Previous experiments showed (**section 8.3.3)** that immature IL-4 DC have a suppressive effect on the proliferation of pre-activated T cells (**Figure 8.12**). Here, the effect of IL-4 DC on inhibiting S-DC induced T cell proliferation was analysed. Since S-DC and IL-4 DC were co-cultured with naïve T cells, it was investigated whether IL-4 DC were able to compete with the stimulatory effect of mature S-DC. Different numbers of competitor IL-4 DC (10^3, 10^4 or 10^5) were transferred to the proliferation assay consisting of a constant number of stimulator cells (10^4 mature S-DC) and a constant number of responder cells (10^5 allogenic T cells) (**Figure 8.13 A**).

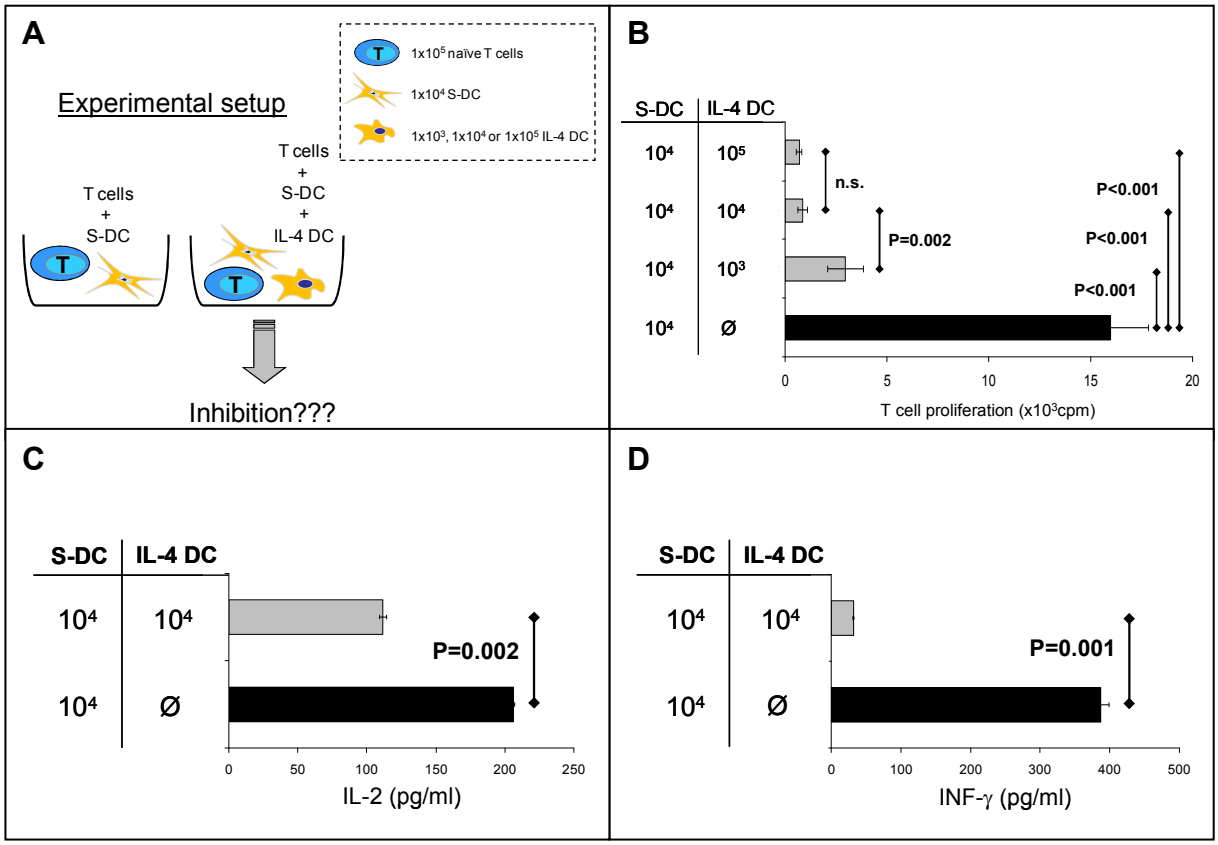


Figure 8.13: Immature IL-4 DC inhibit the proliferation of naïve T cells induced by mature S-DC in co-cultures. **A**: experimental setup: Different numbers of competitor cells (10^3, 10^4 and 10^5 immature IL-4 DC) were added to the assay containing 10^4 stimulator cells (mature S-DC) and 10^5 naïve T cells. **B:** results: Immature IL-4 DC demonstrated a suppressive effect on mature S-DC induced T cell proliferation dependent on their cell number. The results observed in the competition assay at a ratio of 1:1 correlated with the IL-2 and IFN-γ production by naïve T cells. Allogenic T cells co-cultured with mature S-DC and immature IL-4 DC produced significantly lower amounts of IL-2 **(C)** and IFN-γ **(D)** compared to the proliferation assay without the addition of immature IL-4 DC. Data are presented as means ± S.D. of 3 independent experiments. The values are shown in **Table 6.3**. Statistical significance is indicated using the paired Student *t*-test (n.s. = not significant).

As shown in **Figure 8.13 B**, immature IL-4 DC in co-cultures with S-DC demonstrated a suppressive effect on S-DC induced T cell proliferation in a dose-dependent manner. The activation of naïve T cells in the presence of S-DC was measured as T cell proliferation and was 15,994 ± 1,857 cpm (**Table 8.4**). This proliferation was significantly reduced when 10^3, 10^4 or 10^5 immature IL-4 DC were added to the proliferation assay (**Figure 8.13 B**). Already a cell ratio of competitor to stimulator cell of 1:10 suppressed mature S-DC induced T cell proliferation about 5.3-fold. The most potent effect was observed with a competitor : stimulator cell ratio of 1:1 and 10:1 (**Figure 8.13 B** and **Table 8.4**).

In addition to measuring T cell proliferation, the cytokine profile was determined. For this, supernatants of the competition assays were analysed for IL-2 and IFN-γ secretion using ELISA assay (**Figure 8.13 C** and **D**). As shown in **Figure 8.13 C** and **Table 8.4** naïve T cells co-cultured only with mature S-DC produced high levels of IL-2 (206.2 ± 2.52 pg/ml) and IFN-γ (387.4 ± 11.78 pg/ml), whereas the addition of immature IL-4 DC to the proliferation assay reduced both IL-2 production (111.6 ± 1.01 pg/ml) and IFN-γ production (31.9 ± 0.39 pg/ml). The reduction was about 49% and 92%, respectively, in comparison to the S-DC induced T cell proliferation.

Taken together these results show that IL-4 DC suppress the S-DC induced T cell proliferation as well as IL-2 and IFN-γ production. This inhibition might be due to IL-4 DC produced TGF-β (see **section 8.4.2**, **Figure 8.19**). The immunomodulatory cytokine TGF-β has been shown to suppress T cell receptor (TCR)-induced proliferation of effector T cells by inhibiting IL-2 production (Das L and Levine AD, 2008). In addition, TGF-β has also been shown to inhibit the ability of mature DC to stimulate IFN-γ secretion by T cells (Kobie JJ *et al.,* 2003).

Table 8.4 (see next page) Immature IL-4 DC inhibit the proliferation of naïve T cells induced by mature S-DC. Different numbers of IL-4 DC ranging from 10^3 - 10^5 were added to the proliferation assay (10^4 S-DC and 10^5 naïve T cells). IL-4 DC inhibited the mature S-DC induced T cell proliferation in a dose-dependent manner. The strongest inhibition of T cell proliferation was found at a ratio 1:1 of stimulator cells (mature S-DC) and competitor cells (IL-4 DC) (results of T cell proliferation in cpm). The results observed in the competition assay at a ratio of 1:1 correlate with the production of IL-2 and IFN-γ (results of produced IL-2 and IFN-γ in pg/ml). The IL-2 and IFN-γ production of allogenic T cells in the presence of mature S-DC was significantly higher compared to the culture in which 10^4 IL-4 DC were added to 10^4 mature S-DC. Data are presented as means ± S.D. of 3 independent experiments.

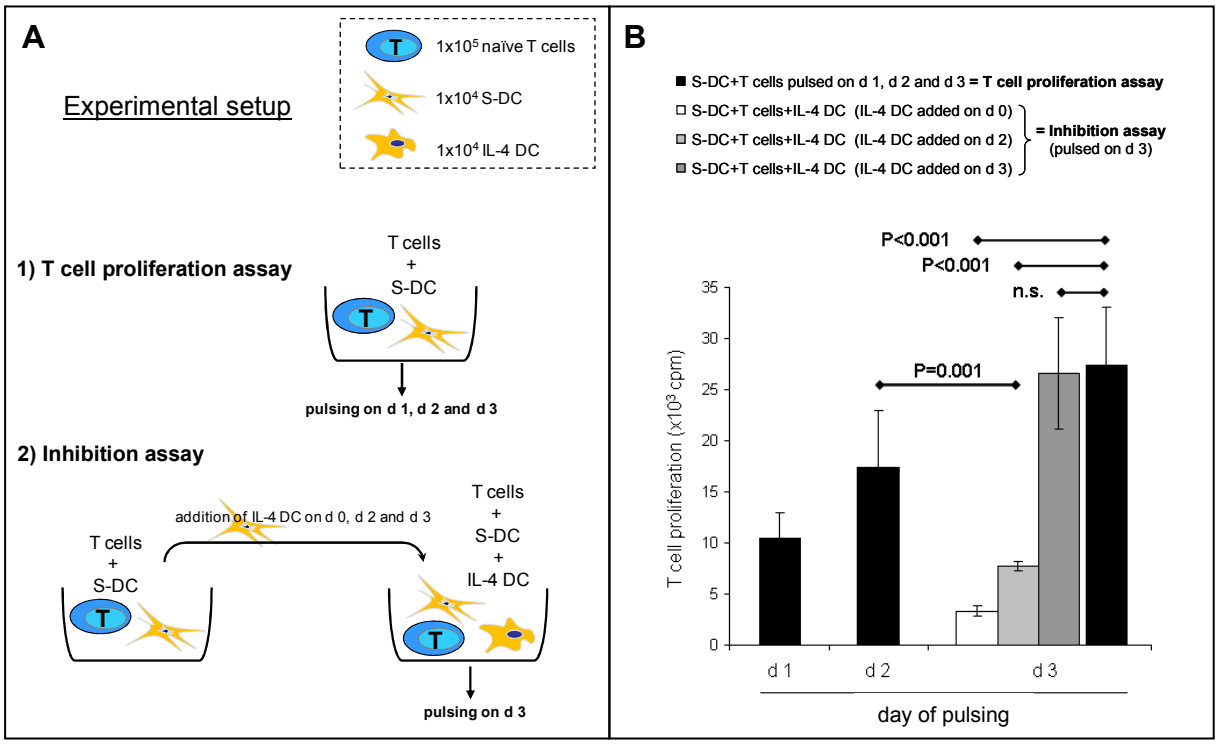
Number of IL-4 DC	10^3	10^4	10^5	∅
Ratio IL-4 DC : S-DC	1:10	1:1	10:1	S-DC alone
T cell proliferation (cpm)	2,971 ± 875	870 ± 240	702 ± 143	15,994 ± 1,857
IL-2 (pg/ml)	----	111.6 ± 1.0	----	206.2 ± 2.5
IFN-γ (pg/ml)	----	31.9 ± 0.4	----	387.4 ± 11.8

(∅) = no IL-4 DC were present in the proliferation assays

8.3.5 T cell proliferation stopped in the presence of IL-4 DC within 24 hours

As described in **section 8.3.4**, the presence of immature IL-4 DC mediates a suppressive effect on the S-DC induced T cell proliferation. In order to determine the speed of IL-4 DC mediated suppression in more detail, they were added to the proliferation assays at different time points (**Figure 8.14** and **Table 8.5**). Ten thousand immature IL-4 DC were added to the proliferation assay (consisting of 10^4 S-DC and 10^5 naïve T cells) on day 0 (culture starting point), day 2 (24 hours before the end of culture), and day 3 (6 hours before the end of culture). Finally, the incorporated radioactivity was measured at the end of culture on day 3 (after 72 hours) (**Figure 8.14 A**).

Figure 8.14 (see next page): Immature IL-4 DC inhibit mature S-DC-induced T cell proliferation within 24 hours. **A:** Experimental setup: 1) T cell proliferation assay: 10^4 stimulator cells (mature S-DC) were co-cultured with 10^5 responder cells (T cells). The cells were pulsed on day 1, day 2 and 3 of the culture 2) Inhibition assay: 10^4 IL-4 DC were added to the proliferation assay (10^4 S-DC and 10^5 naïve T cells) on day 0, day 2 and day 3 of the culture. The cells were pulsed on day 3 as described in "Materials and Methods". **B:** Results: The addition of immature IL-4 DC to the proliferation assay on day 0 and 2 (white and light grey bar) suppressed the increase of T cell proliferation observed in the uninfluenced T cell proliferation assay (black bars). Addition of immature IL-4 DC on day 3, 6 hours before the end of the culture, had no effect on T cell proliferation (dark gray bar Data are presented as means ± S.D. of 3 independent experiments. Statistical significance is indicated using the paired Student *t*-test (n.s. = not significant). The values are shown in **Table 8.5**.



In the T cell proliferation assay, the strongest increase in T cell proliferation occurred between day 2 and 3 of the 3-day culture. On day 1: 10,472 ± 2,534 cpm, day 2: 17,367 ± 5,610 cpm and day 3: 27,369 ± 5,678 cpm (**Table 8.5**).

As shown in **Figure 8.14 B**, the addition of immature IL-4 DC on day 2 of culture suppressed the strong increase in T cell proliferation between day 2 and day 3. The measured T cell proliferation was reduced 4-fold: 7,740 ± 467 cpm. The strongest inhibition of T cell proliferation (8-fold) was seen when immature IL-4 DC were added at the beginning of culture or, with other words, 72 hours before the end of culture. The addition of immature IL-4 DC 6 hours before the end of culture on day 3 had no effect on T cell proliferation (**Figure 8.14** and **Table 8.5**).

Altogether, the results show that immature IL-4 DC suppress the increase of T cell proliferation between day 2 and day 3 of the 3-day culture. The suppressive effect of IL-4 DC on mature S-DC induced T cell proliferation is obvious within 24 hours. The strength of IL-4 DC mediated suppression is dose-dependent, meaning that the amount of IL-4 DC added to the cultures determines the level of S-DC induced T cell proliferation. T cell proliferation was already strongly inhibited with low numbers ($1x10^3$) of immature IL-4 DC (**Figure 8.13 B**).

Table 8.5: Immature IL-4 DC inhibit mature S-DC-induced T cell proliferation within 24 hours. **A:** The proliferation rates at different time points in the T cell proliferation assay are shown. **B:** The effect of the addition of 10^4 immature IL-4 DC on T cell proliferation was tested in an inhibition assay. Immature IL-4 DC were added to the cultures on day 0 (the starting point of culture), day 2 (24 hours before the end of the culture) and on day 3 (6 hours before the end of culture). Subsequently, the cells were pulsed with [^{3}H]-thymidine on day 3. The addition of immature IL-4 DC to the proliferation assay on day 0 and day 2 of the 3-day culture prevented the marked increase in the T cell proliferation that had been induced by mature S-DC between day 2 and day 3. Addition of immature IL-4 DC on day 3, 6 hours before the end of culture, had no significant effect on T cell proliferation. The results (mean ± S.D.) of T cell proliferation in cpm of 3 different experiments.

(A) Proliferation assay			
Day of pulsing	**1**	**2**	**3**
	10,472 ± 2,354	17,367 ± 5,610	27,369 ± 5,678
(B) Inhibition assay			
Day of addition of IL-4 DC (pulsing on day 3)	**0**	**2**	**3**
	3,349 ± 526	7,740 ± 467	26,620 ± 5,471

8.3.6 IL-4 DC are phenotypically and functionally resistant to maturation induced by TNF-α, LPS and CD40L

One prerequisite for the clinical application of tolerogenic immature DC, for example after organ transplantation, is that therapeutic DC should be extensively resistant to different maturation stimuli. How IL-4 DC mature *in vitro* and acquire full DC function in the presence of different maturation stimuli such as replating, LPS, TNF-α and CD40L was investigated.

IL-4 DC isolated from day 6 culture were transferred to 6-well plates and cultured in medium supplemented with GM-CSF (2.5 ng/ml) until day 8. On day 8 non-adherent cells were transferred onto new 6-well plates and stimulated with TNF-α (20 ng/ml), LPS (1 µg/ml) or CD40L (1 µg/ml). The cells were collected at different time points (day 6, day 8 and day 10) and analysed by flow cytometry. **Figure 8.15** shows one representative flow cytometric analysis comparing the expression of different surface molecules on days 6, 8 and 10. **Table 8.6** shows the corresponding MF values of antibody binding and the percentage of positive cells. **Figure 8.16** shows the statistical significance of three independent experiments comparing the results of day 6, day 8 and day 10 of culture.

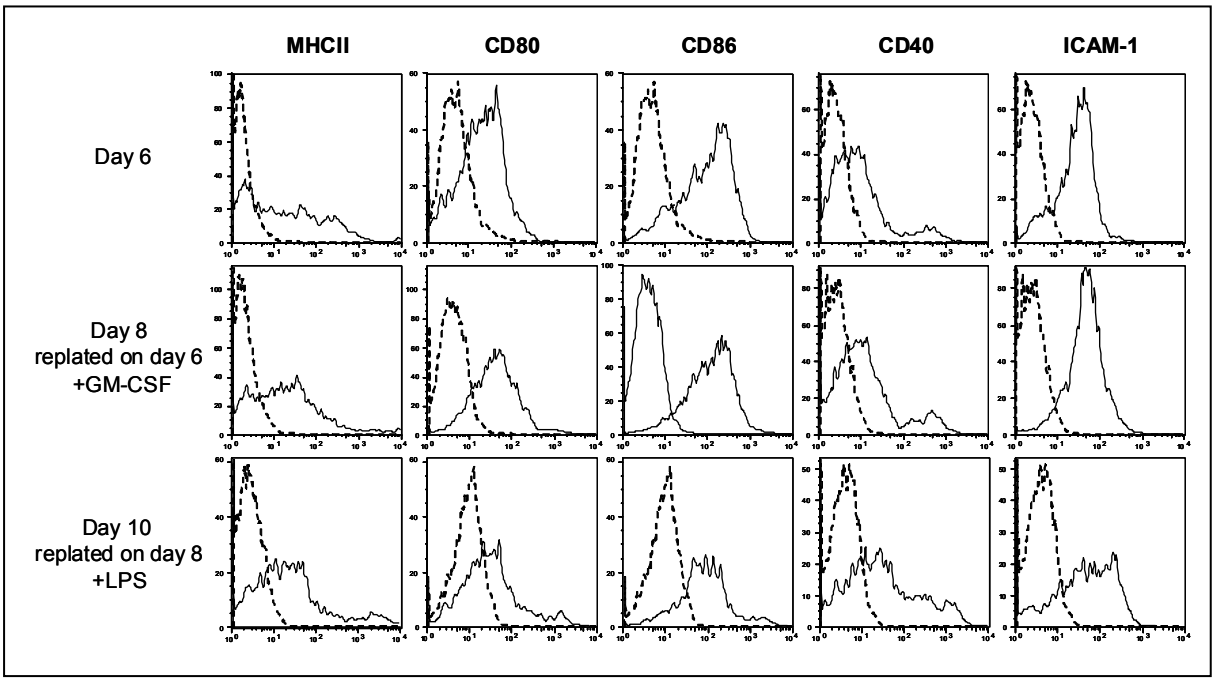


Figure 8.15: Marginal phenotypic changes of immature IL-4 DC in response to maturation stimuli. Immature IL-4 DC were generated from a 6-day culture. The cells were replated on 6-well plates in 3 ml medium per well at a density of $5x10^5$/ml supplemented with GM-CSF only (2.5 ng/ml). On day 8 cells were replated on new 6-well plates in 3 ml fresh medium per well at a density of $5x10^5$/ml supplemented with 20 ng/ml TNF-α, 1 µg/ml LPS or 1 µg/ml CD40L until day 10. Immature IL-4 DC were collected at different maturation stages (day 6, 8 and 10) and were analysed for different cell surface markers by flow cytometry. One representative example of three different experiments for LPS stimulation is shown (see also appendix **Table 12.4**). The values of mean fluorescence and percentage of positive cells are shown in **Table 8.6**.

Table 8.6: Analysis of phenotypic changes of immature IL-4 DC in response to maturation stimulis (see Figure 8.15). Cells were collected on day 6, day 8 and day 10 of culture and analysed by flow cytometry. Differences in the cell surface expression were quantified by mean fluorescence (MF) values of antibody binding. In addition, percentage of positive cells (P=Proportion) is shown. One representative example for LPS stimulation of at least 3 different experiments is shown.

	Surface molecules									
	MHCII		CD80		CD86		CD40		ICAM-1	
	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)
Day 6	134	51.6	37.2	51.7	181	82.9	53	38.5	39.8	82.9
Day 8[*)]	173	59.6	88	87.3	216	95.4	73.3	48.2	69.9	93
Day 10 + LPS[**)]	295	63.9	139	77.8	228	92.4	207	62.9	104	75.4

[*)] Day 8: replated on day 6 + GM-CSF; [**)] Day 10: replated on day 8 + LPS

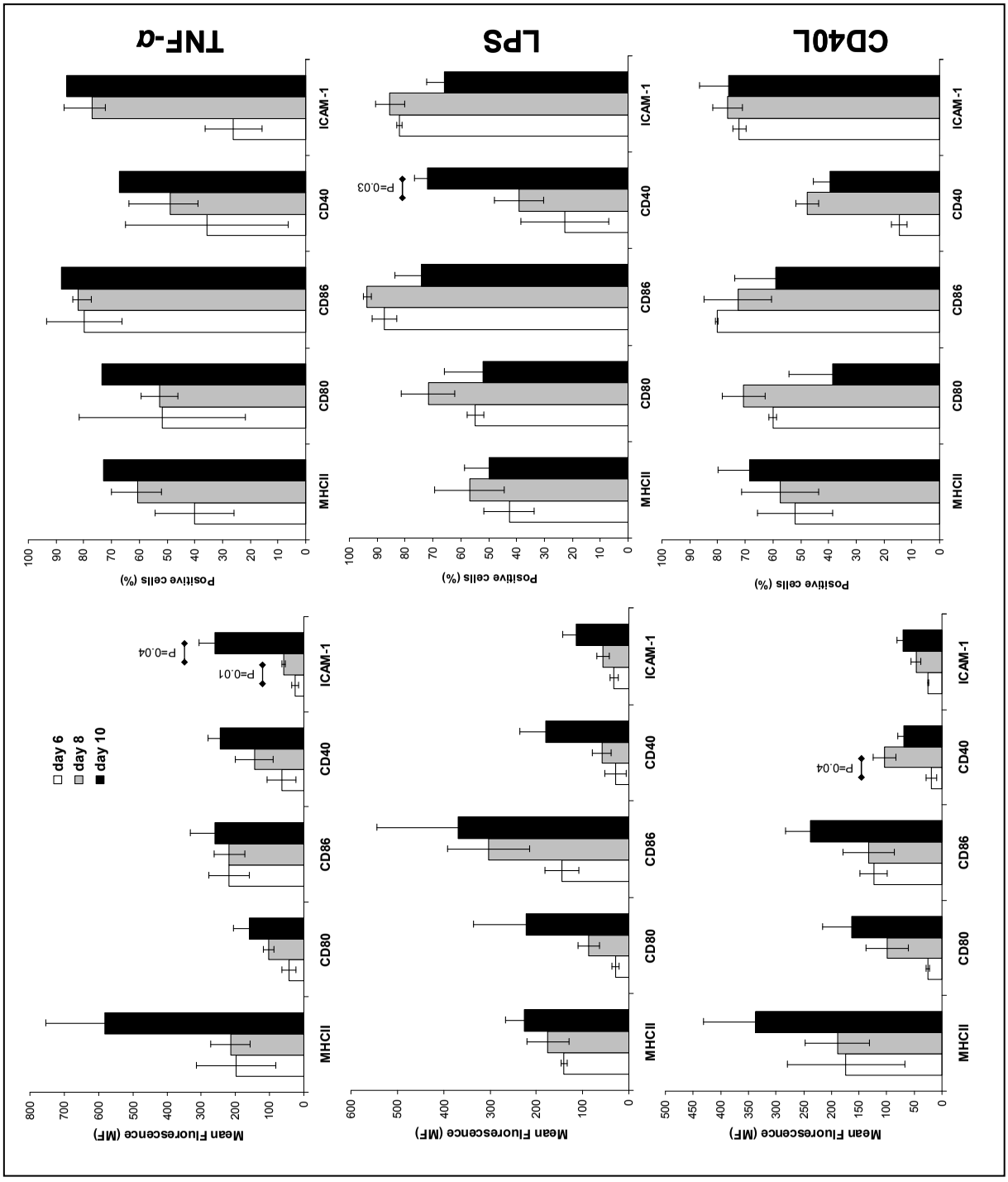


Figure 8.16: Marginal phenotypic changes of immature IL-4 DC after stimulation with TNF-α, LPS or CD40L. Immature IL-4 DC were generated from a 6-day culture. The cells were replated on 6-well plates in 3 ml medium per well at a density of $5x10^5$/ml supplemented with GM-CSF only (2.5 ng/ml). On day 8 cells were replated on new 6-well plates in 3 ml fresh medium per well at a density of $5x10^5$/ml supplemented with 20 ng/ml TNFα, 1 µg/ml LPS or 1 µg/ml CD40L until day 10. Immature IL-4 DC were collected at different maturation stages (day 6, 8 and 10) and were analysed for different cell surface markers by flow cytometry. Differences in the cell surface expression were quantified by mean fluorescence (MF) values of antibody binding. In addition, percentage of positive cells (P= Proportion) is shown. Data are presented as means ± S.D. of 3 independent experiments (see also appendix **Table 12.4**).

In general, replating of IL-4 DC and subsequent addition of cytokines resulted in a slight up-regulation of the co-stimulatory molecules CD80, CD86 and CD40 as well as MHC class II and ICAM-1 on the surface of immature IL-4 DC (**Figure 8.15**, **Table 8.6** and **Figure 8.16**).

Only the surface expression of ICAM-1 was significantly increased on IL-4 DC on day 8 after replating them on day 6 and day 10 in the presence of TNF-α: MF of day 6: 25.5 ± 10.1; MF of day 8: 58.7 ± 4.4; MF of day 10: 259.7 ± 45.9 (**Figure 8.16**). The same result was observed for the amount of cells expressing these molecules. The increase of positive cells was statistically insignificant (**Figure 8.16**). Only the amount of IL-4 DC expressing CD40 after exposure to LPS between days 8 and 10 was significantly increased: day 8: 39.2 ± 8.8%; day 10: 71.8 ± 4.9%.

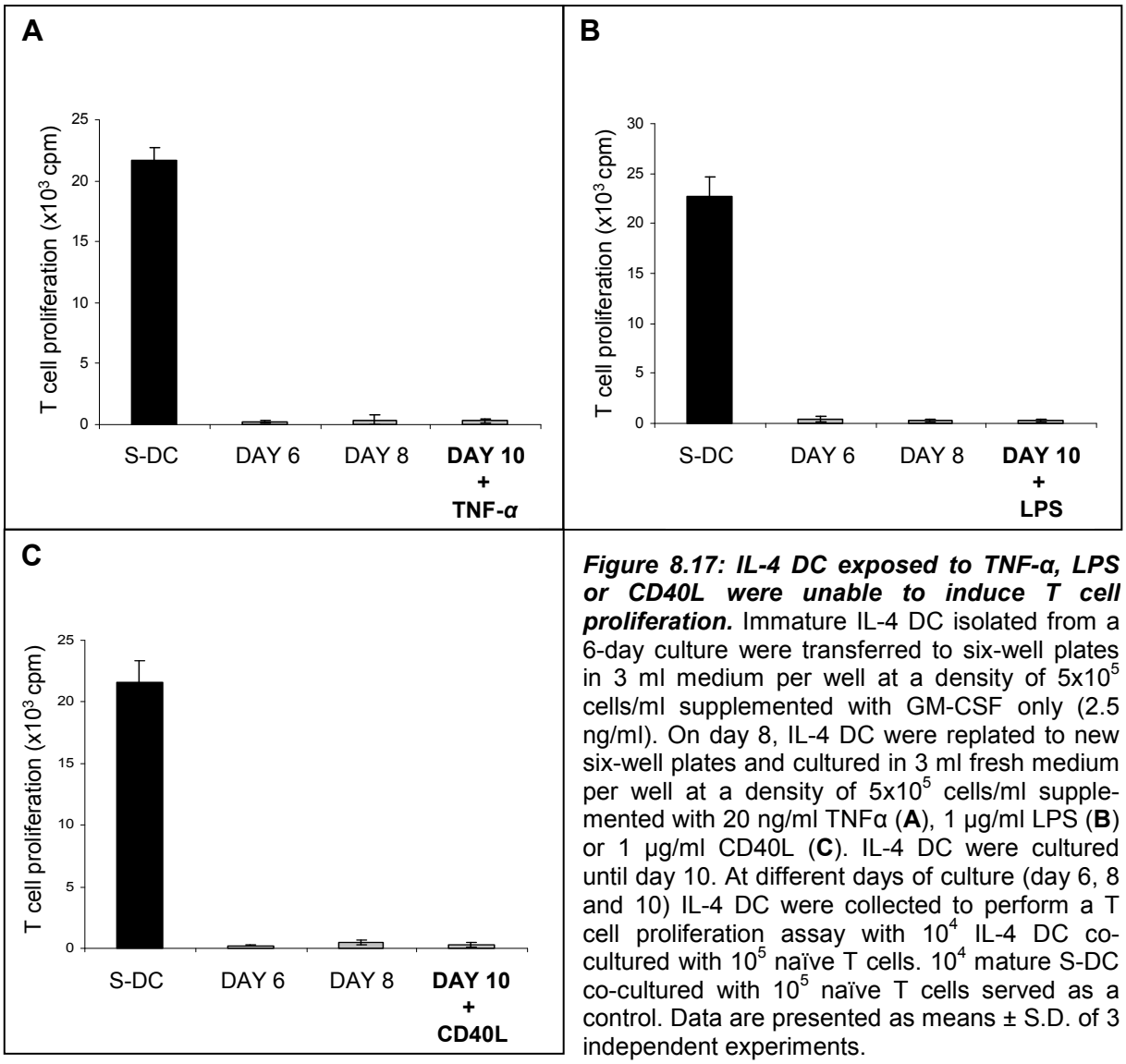


Figure 8.17: IL-4 DC exposed to TNF-α, LPS or CD40L were unable to induce T cell proliferation. Immature IL-4 DC isolated from a 6-day culture were transferred to six-well plates in 3 ml medium per well at a density of $5x10^5$ cells/ml supplemented with GM-CSF only (2.5 ng/ml). On day 8, IL-4 DC were replated to new six-well plates and cultured in 3 ml fresh medium per well at a density of $5x10^5$ cells/ml supplemented with 20 ng/ml TNFα (**A**), 1 µg/ml LPS (**B**) or 1 µg/ml CD40L (**C**). IL-4 DC were cultured until day 10. At different days of culture (day 6, 8 and 10) IL-4 DC were collected to perform a T cell proliferation assay with 10^4 IL-4 DC co-cultured with 10^5 naïve T cells. 10^4 mature S-DC co-cultured with 10^5 naïve T cells served as a control. Data are presented as means ± S.D. of 3 independent experiments.

The effect of different maturation stimuli on IL-4 DC maturation was measured as phenotypic changes by flow cytometry and as functional changes in the T cell proliferation assay. For this, IL-4 DC incubated with different maturation stimuli were isolated at different time points and tested in T cell proliferation assay to proof their ability to stimulate naïve T cells. **Figure 8.17** shows that the stimulatory capacity of IL-4 DC was not increased after replating them on day 6, as well as after exposure to TNF-α, LPS and CD40L (**Figure 8.16**).

It is generally accepted that DC maturation is induced by exposing them to different maturation stimuli, e.g. TNF-α, LPS and CD40L. In this study it was shown that immature IL-4 DC were resistant to maturation stimuli tested in this work, shown by their failure to up-regulate the co-stimulatory molecules CD80, CD86 and CD40 as well as MHC class II and ICAM-1. This maturation resistance has no effect on the inability of IL-4 DC to activate naïve T cells.

8.4 Why IL-4 DC inhibit T cell proliferation so fast? Some experiments for explanation

As shown in **section 8.3.5** immature IL-4 DC inhibit T cell proliferation within 24 hours (**Figure 8.14**). The reason for this strong effect is unclear. Therefore, in the next set of experiments the “origin” of this inhibitory effect was analysed on cellular and molecular levels in more detail. For this, immature IL-4 DC were characterized for possible regulatory properties such as the secretion of a soluble factor, the induction of T cell apoptosis and anergy and the expression of inhibitory molecules such as PD-L1 and PD-L2.

8.4.1 Supernatant of IL-4 DC cultures inhibits S-DC-induced T cell proliferation

Several mechanisms of suppression have been described so far, such as the secretion of TGF-β and TNF (Angulo I *et al.,* 2000; Meisel R *et al.,* 2004; Terabe M *et al.,* 2003). Another candidate known for inhibiting T cell proliferation is nitric oxide or NO (Mazzoni A *et al.,* 2002). To investigate the presence of a possible soluble factor

secreted by IL-4 DC and its effect on T cell proliferation, different volumes (25 µl, 50 µl and 100 µl) of supernatant from cultures with immature IL-4 DC were added to the T cell proliferation assay (10^4 mature S-DC and 10^5 T cells). For these experiments, IL-4 DC isolated on day 6, were cultured for further 24 hours with a cell density of 10^6 IL-4 DC in 150 µl culture medium. The supernatant of 24-hour cultured 10^6 mature S-DC or T cells served as a control. The final volume was kept constant at 150 µl per well and the cells were pulsed with [^{3}H]-thymidine on day 3.

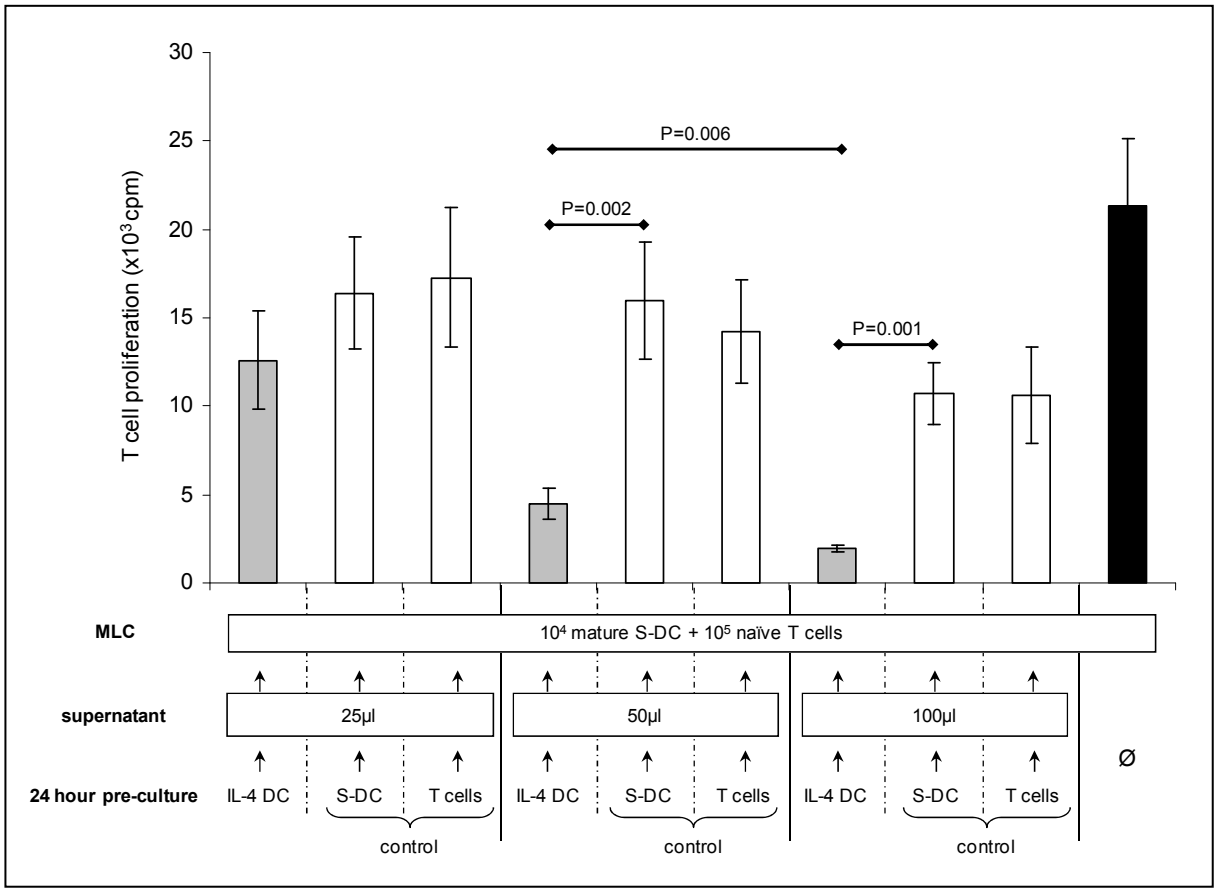


Figure 8.18: Supernatant from IL-4 DC cultures inhibits T cell proliferation in a dose-dependent manner. The supernatant of immature IL-4 DC had a suppressive effect on mature S-DC-induced T cell proliferation. Purified immature IL-4 DC from day 6 of culture were transferred to 96-well culture plates (10^6 cells/well). 24 hours later the supernatant was collected and transferred in different volumes (25 µl, 50 µl, and 100 µl) to the proliferation assay (10^5 naïve T cells cultured with 10^4 mature S-DC). The final volume was kept constant at 150 µl/well. The supernatant of mature S-DC or T cells served as a control (white bars). Data are presented as means ± S.D. of 3 independent experiments. Statistical significance is indicated using the paired Student *t*-test (n.s. = not significant).

As shown in **Figure 8.18** and **Table 8.7**, the supernatant from 10^6 IL-4 DC transferred to T cell proliferation assays was able to inhibit mature S-DC induced T cell proliferation in a dose-dependent manner. Interestingly, supernatant from cultures with only 10^4 IL-4 DC (instead of 10^6 cells) showed no suppressive effect (**Table**

12.10 appendix). The same effect was observed, when only 25 µl supernatant from 10^6 IL-4 DC cultures were tested. In contrast, 50 µl supernatant reduced mature S-DC induced T cell proliferation about 79%: from 21,341 ± 3,778 cpm for uninfluenced T cell proliferation to 4,456 ± 875 cpm for IL-4 DC supernatant influenced T cell proliferation (**Table 8.7**). The suppressive effect of IL-4 DC supernatant increased with the volume transferred. With 100 µl supernatant the T cell proliferation was reduced by more than 90%: from 21,341 ± 3,778 cpm for uninfluenced T cell proliferation to 1,962 ± 182 cpm for IL-4 supernatant influenced T cell proliferation (**Table 8.7**).

One hundred µl supernatant from 10^6 cultured mature S-DC suppressed T cell proliferation, also (**Figure 8.18**), but the inhibitory effect of this S-DC supernatant was 5-fold lower compared to that of IL-4-DC (**Table 8.7**). From these results it is possible to conclude the existence of a soluble factor or soluble factors responsible for the inhibitory effect of immature IL-4 DC on mature DC induced T cell proliferation.

Table 8.7: Supernatant from IL-4 DC cultures inhibits T cell proliferation. The strength of suppressed T cell proliferation increased with increasing volume of transferred supernatant. The supernatant of mature S-DC or naïve T cells served as a control to exclude possible negative effects of day 2 supernatant. Data are presented as means ± S.D. of 3 independent experiments.

	Volume of supernatant (µl) *)			
	25	**50**	**100**	**0**
IL-4 DC	12,586 ± 2,783**)	4,456 ± 875	1,962 ± 182	21,341 ± 3,778
S-DC	16,387 ± 3,182	15,972 ± 3,335	10,733 ± 1770	
T cells	17,255 ± 3,957	14,238 ± 2,910	10,625 ± 2,737	

*) The final volume was kept constant at 150 µl per well.
**) Results (mean ± S.D.) of T cell proliferation in cpm.

8.4.2 IL-4 DC secrete TGF-β, not IL-10 and NO

Since the supernatant of 10^6 immature IL-4 DC inhibits the mature S-DC-induced activation of T cell proliferation, the presence of a soluble inhibitory factor was assumed. The immune inhibitory cytokines TGF-β and IL-10, as well as the cell toxic effector molecule nitric oxide (NO) have been implicated in the inhibition of T cell

proliferation (Ding L and Shevach EM, 1992; Lu L *et al.,* 1996; Bonham CA *et al.,* 1996; Zeller JC *et al.,* 1999). Therefore, the presence of TGF-β, IL-10 and NO was investigated in the supernatant of 10^6 immature IL-4 DC in comparison to 10^6 mature S-DC. For these experiments, the DC were cultured for 24 and 48 hours. In contrast to IL-10 and NO (appendix **Table 12.9**), TGF-β was detected in the supernatant of 10^6 immature IL-4 DC (**Figure 8.19**). This amount of TGF-β (24 hours: 346.77 ± 103.05 pg/ml; 48 hours: 2,792 ± 1,292 pg/ml) was significantly increased compared to TGF-β in the supernatant of mature S-DC (24 hours: 105.77 ± 55.57 pg/ml; 48 hours: 93.6 ± 38.07 pg/ml).

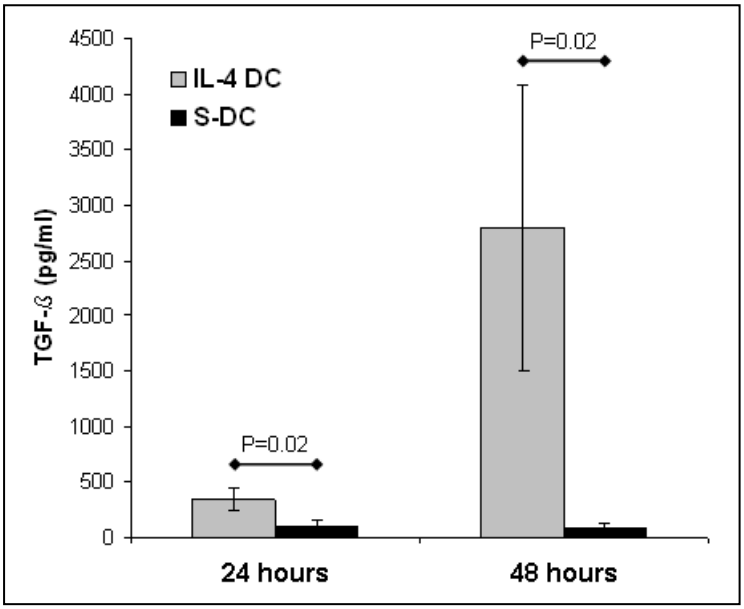


Figure 8.19: IL-4 DC produce more TGF-β than mature S-DC. Immature IL-4 DC from day 6 of culture and purified mature S-DC were transferred to 96-well culture plates (10^6 cells/well). After 24 and 48 hours of culture, the supernatant was collected and measured with specific ELISA. Data are presented as means ± S.D. of 3 independent experiments. Statistical significance is indicated using the paired Student *t*-test.

Altogether IL-10 and NO do not contribute to the inhibitory effect of supernatant from IL-4 DC cultures. TGF-β appears to be a promising candidate responsible for the IL-4 DC mediated inhibition of T cell proliferation. This assumption can be proven with a TGF-β neutralising antibody. Such an antibody added to T cell proliferation assay neutralises the effect of TGF-β transferred with the supernatant. However, unfortunately, a rat TGF-β neutralising antibody is not available yet. Preleminary experiments, testing neutralizing monoclonal anti-TGF-β1, -β2, -β3 antibody (specific for bovine, mouse and human TGF-β1 and TGF-β2; R&D Systems), showed no effect on T cell proliferation (data not shown). At the moment it is not clear whether and how strong TGF-β contributes to the suppressive effect of IL-4 DC supernatant on mature S-DC induced T cell proliferation.

8.4.3 TGF-β, not IL-10, is detectable in competition assays with IL-4 DC

In **section 8.4.1** it was shown that the supernatant of 10^6 immature IL-4 DC inhibited the mature S-DC induced T cell proliferation and TGF-β, but not IL-10, was detected in these supernatant (**section 8.4.2**). In this section the presence of TGF-β was analysed in competition assays. The reason for this is, that the number of IL-4 DC added to the T cell proliferation assay ranged from 10^3 to 10^5 IL-4 DC and it is not clear whether the amount of TGF-β produced by these cell numbers is really effective to inhibit mature S-DC induced T cell proliferation (see **section 8.3.4**, **Figure 8.13 B**). Therefore, the possible presence of TGF-β and IL-10 was investigated in the supernatant of competition assays. In **Table 8.8** the competition assay consists of 10^4 S-DC and 10^5 naïve T cells and a constant number of IL-4 DC.

Table 8.8: Detection of TGF-β in competition assays in the presence of IL-4 DC. 10^4 competitor cells (immature IL-4 DC) were added to the proliferation assay containing 10^4 stimulator cells (= mature S-DC) and 10^5 naïve T cells. The T cell proliferation assay is called competition assay when IL-4 DC are present. The idea is that IL-4 DC compete with S-DC for the contact with T cells. T cells cultured alone served as a control. The detection of IL-10 served as a control. The supernatant was collected after 72 hours. Three different experiments are shown (each experiment in duplicate wells).

	Control	Competition assay
Experiment	**TGF-β (pg/ml)**	
#1	73.2 ± 2.91	89.6 ± 6.65
#2	82.0 ± 0.00	360.2 ± 0.04
#3	56.6 ± 0.02	96.7 ± 0.11
	IL-10 (pg/ml)	
#1	3. 6 ± 0.13	4.8 ± 0.02
#2	0.01 ± 0.01	0.02 ± 0.01
#3	0	0

Table 8.8 shows the results of 3 different experiments. It is possible to detect levels of TGF-β in the supernatant of the competition assay compared to supernatant of control T cells. The levels of TGF-β increased 4-fold in experiment #2. However, the other two experiments showed only a slight increase in TGF-β levels. These experiments do not show beyond a doubt that TGF-β contributes to the direct

inhibitory effect of IL-4 DC on mature S-DC induced T cell proliferation. Therefore, as mentioned in **section 8.4.2**, the effect of a TGF-β neutralising antibody, which is not available yet, needs to be tested.

8.4.4 LPS-stimulated IL-4 DC secrete NO

In **section 8.4.2** it is described that untreated IL-4 DC did not synthesize cell toxic NO. In **section 8.3.6** it is also shown that immature IL-4 DC did not mature in the presence of LPS, since LPS treated IL-4 DC did not show an increased expression of co-stimulatory molecules on their surface and they did not activate naïve T cells (**Figures 8.15** - **8.17**). However, to date it cannot be shown that LPS induced the secretion of different effector molecules by IL-4 DC. For cytotoxic NO it was shown that immature BM-DC can produce NO in response to LPS and/or IFN-γ (Lu L *et al.,* 1996).

Therefore, in a next set of experiments it was examined whether LPS-treated IL-4 DC can be stimulated to synthesise inducible nitric oxide synthase (iNOS) necessary for the production of large amounts of NO. Therefore, 10^6 IL-4 DC or 10^6 mature S-DC were exposed to LPS (1 µg/ml) for 24 hours. NO production was measured as the accumulation of its stable end product NO_2^-.

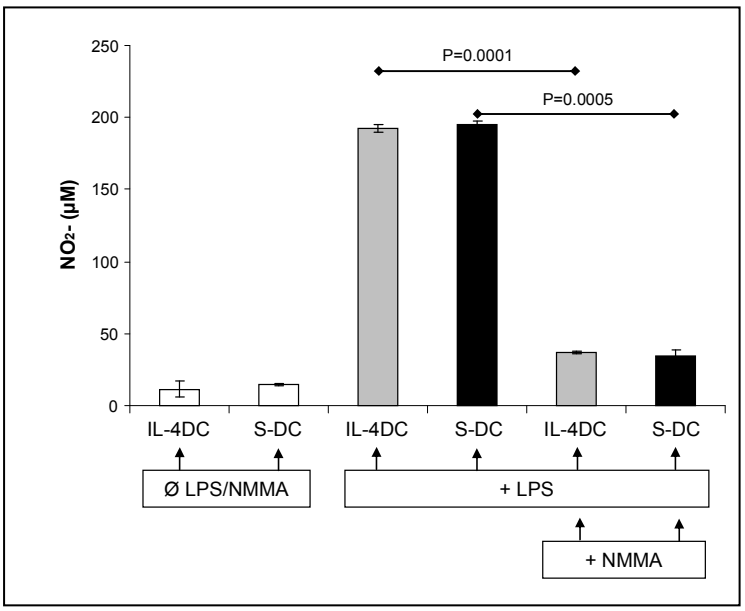


Figure 8.20: LPS-stimulated IL-4 DC synthesise iNOS and produce large amounts of NO. 10^6 immature IL-4 DC (harvested on day 6) and 10^6 mature S-DC were cultured with LPS with or without NMMA or with medium alone for 24 hours. The NO release was measured as the accumulation of the stable end product NO_2^- (see methods and materials). Both immature IL-4 DC and mature S-DC can be stimulated to secrete NO. Data are presented as means ± S.D. of 3 independent experiments. Statistical significance is indicated using the paired Student *t*-test (n.s. = not significant).

NO was strongly induced in both IL-4 DC and mature S-DC (**Figure 8.20**). The addition of the NO synthase inhibitor NMMA (250 µM) at the start of culture significantly inhibited the LPS-induced NO production, shown in **Figure 8.20** by low detection of NO_2^-. To confirm the induction of iNOS protein expression, LPS stimulated IL-4 DC were lysed and cell proteins were analysed by immunoblot using a rabbit anti-nitric oxide synthase II (iNOS) polyclonal antibody. As shown in **Figure 8.21**, iNOS expression was confirmed in LPS stimulated IL-4 DC, but not in unstimulated cells.

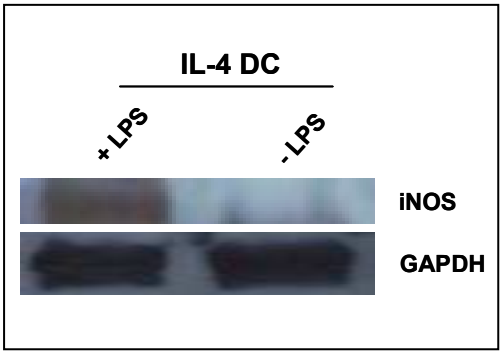


Figure 8.21 Western blot analysis of iNOS expression in IL-4 DC. iNOS expression was assessed by Western blotting in untreated IL-4 DC and in IL-4 DC incubated with LPS for 24 hours. GAPDH was used as a loading control. The results shown are representative for at least 3 different experiments.

Altogether these results demonstrate that LPS stimulation induced the production of NO by IL-4 DC (**Figure 8.20**). In addition, the presence of iNOS was successfully detected in LPS-stimulated IL-4 DC (**Figure 8.21**). This NO production can be suppressed by the NO synthase inhibitor NMMA (**Figure 8.20**).

8.4.5 Inhibition of LPS-induced NO secretion does not influence IL-4 DC mediated-inhibition of T cell proliferation

IL-4 DC produce NO by LPS stimulation and the presence of the NO synthase inhibitor NMMA inhibited NO production (**Figure 8.20**). In this section the impact of NO was analysed for the IL-4 DC-mediated inhibition of both naïve T cell activation and mature S-DC induced T cell proliferation. Therefore, NO production was measured in the supernatant of T cell proliferation assays and inhibition assays (for more detail see **Figure 8.22**).

The basal level of NO secretion was 14.4 ± 0.7 µM for IL-4 DC and 12.7 ± 2.5 µM for S-DC. The NO production in cultures of IL-4 DC and naïve T cells was low (10.25 ± 2.1 µM), but in the presence of the NO synthase inhibitor NMMA NO levels were significantly lowered to 4.6 ± 0.8 µM (**Figure 8.22 A**). The same result was observed for supernatant of the inhibition assay (S-DC plus naïve T cells plus IL-4 DC): NO production did not cross the NO basal level (11.3 ± 0.8 µM) and, again, NMMA significantly reduced NO production (5.6 ± 0.6 µM).

Although the levels of NO did not cross the basal level, nevertheless it was analysed whether these low NO levels may be responsible for both IL-4 DC mediated inhibition of T cell activation and mature S-DC induced T cell proliferation. Therefore, NMMA was added at the start of the culture and proliferation response was evaluated after 72 hours. As shown in **Figure 8.22 A** (diagram on the right, T cell proliferation assay), addition of NMMA did not increase the proliferation of naïve T cells in the presence of IL-4 DC: without NMMA the T cell proliferation was 528.5 ± 154 cpm and with NMMA: 513.5 ± 57 cpm. In addition, no dramatic effect on T cell proliferation in the presence of NMMA was observed in the inhibition assay. In the presence of NMMA the IL-4 DC-mediated inhibition of S-DC-induced T cell proliferation also took place. The T cell proliferation increased only slightly from 635.17 ± 211 cpm to 1,918 ± 499 cpm. This T cell proliferation was still reduced 10-fold compared to mature S-DC-induced T cell proliferation (19,454 ± 7,365 cpm).

To exclude that the amount of NMMA (250 µM) used in all experiments was not sufficient to restore proper T cell proliferation, NMMA was added at different concentrations (125, 250 and 500 µM) to the T cell proliferation assay and inhibition assay. As shown in **Figure 8.22 B**, also a higher NMMA concentration (500 µM) had no significant effect on T cell proliferation in comparison to lower amounts of NMMA (250 µM).

Interestingly, NO also accumulated in cultures with mature S-DC and naïve T cells (35.6 ± 2.6 µM) and the in presence of NMMA the NO production was reduced: 5.6 ± 0.7 µM (streaked bar). A consequence of this reduction of NO was a dramatic increase in T cell proliferation from 19,454 ± 7,365 cpm without NMMA to 49,948 ±

7,723 cpm with NMMA. This result clearly shows that a reduction of NO production increases T cell proliferation.

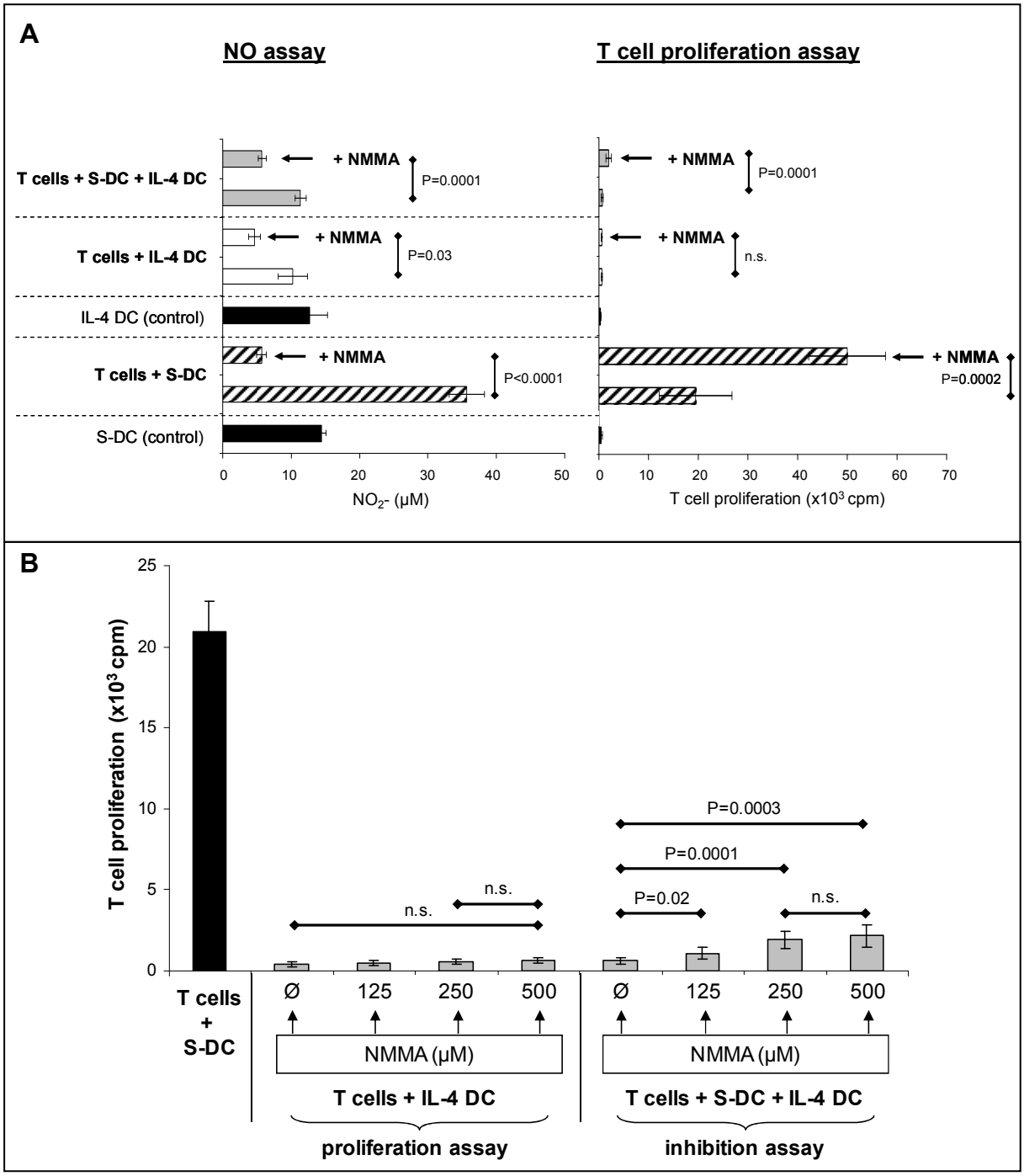


Figure 8.22: The inhibitory effect of immature IL-4 DC is not abrogated by NO inhibitor N^G-methyl-L-arginine acetate (NMMA). **A:** NO_2^- accumulation in supernatant of T cell proliferation assay with S-DC (streaked bars; 10^4 S-DC and 10^5 naïve T cells) and IL-4 DC (white bars; 10^4 IL-4 DC and 10^5 naïve T cells) and inhibition assay (grey bars; 10^4 IL-4 DC, 10^4 mature S-DC and 10^5 naïve T cells) with or without NMMA. IL-4 DC and mature S-DC cultured alone served as controls (=basal level). Mature S-DC are able to produce NO during the interaction with naïve T cells. NMMA was able to inhibit NO production. **B:** Addition of NMMA at different concentrations (125, 250 and 500 µM) to the culture of 10^4 IL-4 DC and 10^5 naïve T cells and inhibition assay. NMMA at a high concentration (500 µM) had only a marginal effect on T cell proliferation. Data are presented as means ± S.D. of 3

independent experiments. Statistical significance is indicated using the paired Student *t*-test (n.s. = not significant).

Altogether, the results demonstrate that NO does not contribute to the IL-4 DC-mediated inhibition of T cell activation in proliferation assays and S-DC induced T cell proliferation in inhibition assays (**Figure 8.22 A,** diagram on the right). Increased levels of NO are secreted by S-DC in T cell proliferation assays (**Figure 8.22 A**).

8.4.6 IL-4 DC do not induce T cell apoptosis

As demonstrated in **chapter 8.4.4**, LPS induces NO secretion by IL-4 DC. However, the results of T cell proliferation assay and inhibitory assay lead to the assumption that NO is not involved in the IL-4 DC-mediated inhibition of T cell proliferation (**Figure 8.22**). Therefore, the impact of NO for IL-4 DC mediated suppression of T cell activation was analysed in more detail. It has been shown for immature DC that they are able to induce T cell apoptosis (Lu L *et al.,* 1999; Zhu M *et al.,* 2004) and, in addition, NO induces apoptosis in many kinds of immune cells, including T cells (Fehsel K *et al.,* 1995). Flow cytometry, a method to analyse the percentage of apoptotic T cells after incubation with IL-4 DC, was performed to investigate a possible effect of NO on T cell viability.

As shown in **Figure 8.23**, T cells cultured with both mature S-DC or immature IL-4 DC showed a low percentage of apoptosis (18.2% and 12.1%). The same result was observed for T cells cultured in the inhibition assay (13.2%). In the presence of the NO synthase inhibitor NMMA, the percentage of apoptotic T cells cultured with mature S-DC was reduced from 18.2% to 9.38% apoptotic cells (**Figure 8.23**). In contrast, the presence of NMMA in T cell cultures with IL-4 DC did not influence the viability of T cells (**Figure 8.23**).

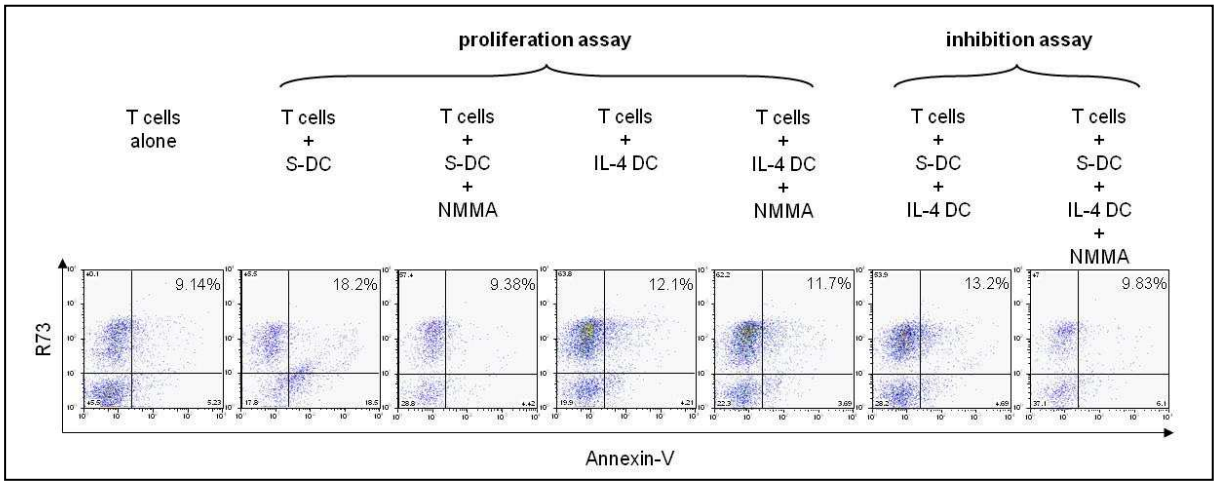


Figure 8.23: T cell apoptosis is not relevant for the IL-4 DC mediated suppressive effect on T cell proliferation. Naïve T cells were co-cultured with 10^4 mature S-DC or 10^4 immature IL-4 DC. The effect on T cell apoptosis was also tested in the inhibition assay in the presence or absence of NMMA (250 µg/ml). Controls are T cells cultured alone. After 3 days of culture, apoptotic T cells were identified by double staining with anti -R73 and Annexin-V. Compared to T cell cultures with immature IL-4 DC, mature S-DC induced the highest T cell apoptosis, which was partially inhibited by addition of NMMA in both proliferation and inhibition assay. Data are representative of three separate experiments (see appendix **Table 12.3**).

Altogether, the results show that IL-4 DC mediated inhibition of T cell proliferation is not based on induction of T cell apoptosis.

8.4.7 IL-4 DC express the inhibitory molecules PD-L1 and PD-L2

Despite low surface expression of MHC class II and the co-stimulatory molecules CD80 and CD86 on immature IL-4 DC (see **section 8.2.3**), immature IL-4 DC are able to stimulate the development of anergic T_{reg} cells with suppressive function *in vitro* (see **sections 8.5.1**, **8.5.2** and **8.5.3**). It is known that the balance between co-stimulatory and co-inhibitory signals mediated by DC determines the final outcome of T cell activation (del Rio ML *et al.,* 2008). The ability of the co-stimulatory molecules of the *B7* family CD80 and CD86 to provide co-stimulatory signals for full T cell activation has been well characterized. Recently, it has been shown that two additional *B7* family members, PD-L1 and PD-L2, can deliver inhibitory signals, resulting in down-regulation of T cell activation (Keir ME *et al.,* 2008). Moreover, blocking PD-L1 and PD-L2 on immature DC of the human system enhances T cell proliferation, suggesting an important role of PD-L1 and PD-L2 in induction and maintenance of T cell unresponsiveness (Brown JA *et al.,* 2003).

In a next set of experiments, the possible expression of PD-L1 and PD-L2 on IL-4 DC was assessed by western blotting. For this, the IL-4 DC were cultured for 4 and 6 days with GM-CSF and IL-4. As shown in **Figure 8.24 A** immature IL-4 DC expressed PD-L1 and PD-L2 after 4 and 6 days of culture, suggesting that GM-CSF and/or IL-4 induces and maintains PD-L1 and PD-L2 expression. The investigation of PD-L1 and PD-L2 expression after culture of progenitor cells with IL-4 only was not possible since progenitor cells were not able to proliferate and differentiate without the addition of GM-CSF (**section 8.1.2**, **Figure 8.1**).

To investigate whether GM-CSF alone is sufficient to induce PD-L1 and PD-L2 expression, bone marrow progenitor cells were cultured with GM-CSF alone instead of GM-CSF plus IL-4 for 4 and 6 days. Again a clear PD-L1 and PD-L2 expression was assessed by Western blot analysis. This leads to the conclusion that GM-CSF alone is sufficient to induce and maintain PD-L1 and PD-L2 expression on BM-DC. In addition, treatment of day 6 IL-4 DC with the maturation stimuli LPS for 24 and 48 hours resulted in an increase of PD-L1 expression in comparison to untreated IL-4 DC (**Figure 8.28 B**).

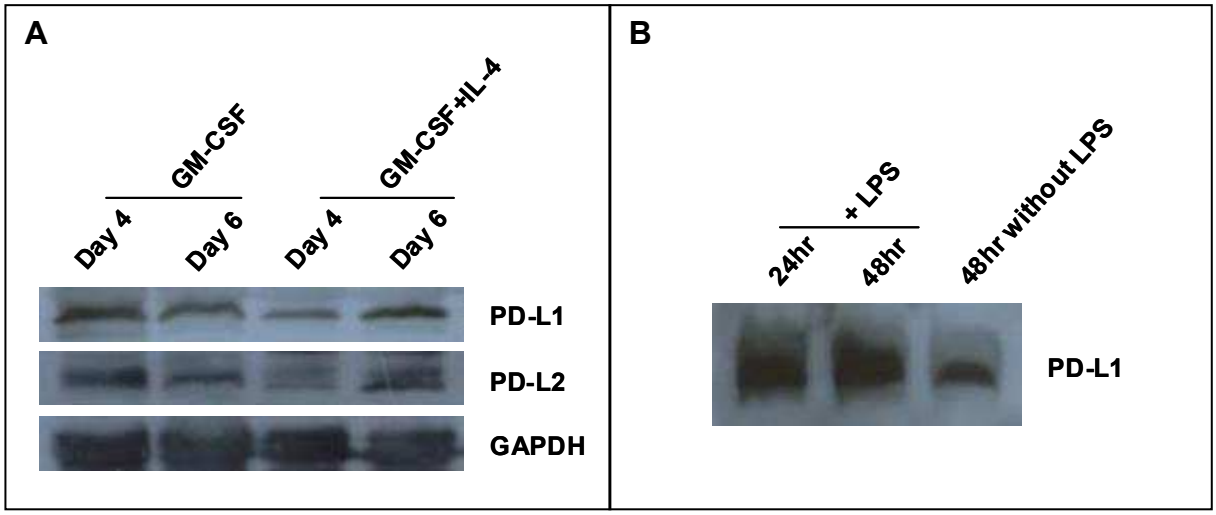


Figure 8.24: Western blot analysis of PD-L1 and PD-L2 expression in immature IL-4 DC. Protein extracted from $1.5x10^6$ IL-4 DC harvested on day 4 and day 6 of culture (**A**) and from $1.5x10^6$ day 6 IL-4 DC stimulated with LPS (1 µg/ml) for 24 and 48 hours (**B**) was loaded and separated by electrophoresis in 10% SDS-polyacrylamide gels. Western blot analysis was performed with antibodies against PD-L1 and PD-L2. GAPDH served as loading control. The results shown are representative for 3 different experiments.

Altogether the results show that immature IL-4 DC express the co-inhibitory molecules PD-L1 and PD-L2 on their surface and GM-CSF seems to induce and maintain PD-L1 and PD-L2 expression.

8.4.8 PD-L1 and PD-L2 surface expression is decreased by inhibition of MEK-pathway but does not influence IL-4 DC-mediated inhibition of T cell proliferation

As shown in **section 8.4.7**, immature IL-4 DC expressed the co-inhibitory molecules PD-L1 and PD-L2 on their cell surface. Brown and colleagues showed that the blockade of PD-L1 and PD-L2 expression by human DC with monoclonal antibodies results in enhanced T cell activation (Brown JA *et al.,* 2003). Therefore, the PD-L1 and PD-L2 blockade on immature IL-4 DC should also enhance T cell proliferation. Unfortunately, rat monoclonal antibodies for blocking PD-L1 and PD-L2 on rat DC are not available yet. However, several studies with pharmacological inhibitors have examined signalling pathways that are required for the expression of PD-L1. The group of Liu showed that PD-L1 expression in cell lines is decreased when MEK1/2-pathway is blocked by MEK inhibitor UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) (Liu J *et al.,* 2007).

In this study the importance of MEK-pathway for the expression of PD-L1 and PD-L2 on IL-4 DC was investigated. For this, the MEK-pathway was blocked by the MEK inhibitor UO126 and the outcome on PD-L1 and PD-L2 expression was analysed by western blot.

Figure 8.25 shows that the expression of PD-L1 and PD-L2 was clearly reduced in immature IL-4 DC when Erk1/2 phosphorylation was blocked by 10 µM UO126. This reduction was observed for 1 hour and 24 hours after the addition of UO126 to IL-4 DC.

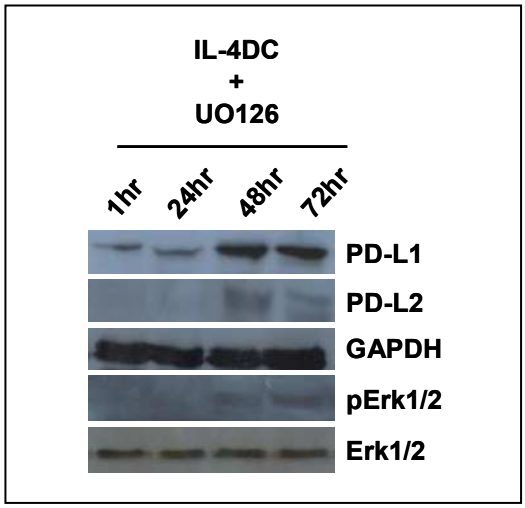


***Figure 8.25: Blocking the ERK1/2 phosphorylation with UO126 reduces the expression of* PD-L1 and PD-L2.** Proteins were extracted from $1.5x10^6$ IL-4 DC harvested on day 6 of culture and separated by electrophoresis in 10% SDS-polyacrylamide gels. Western blot analysis was performed with indicated antibodies. GAPDH served as a loading control. The detection of Erk1/2 phosphorylation was also analysed in IL-4 DC harvested on day 6 of culture. IL-4 DC were replated on 6-well plates ($1.5x10^6$/well) and incubated with 10 µM UO126 at different time points. Western blot analysis was performed with indicated antibodies. Unphosphorylated p43/44 served as a loading control. The results shown are representative for 3 different experiments.

In the next set of experiments it was analysed whether a decreased protein expression of PD-L1 and PD-L2 on immature IL-4 DC could overcome the IL-4 DC-mediated inhibition of naïve T cell activation. Therefore, 10^4 immature IL-4 DC were co-cultured with 10^5 naïve T cells for 72 hours with or without 10 µM UO126. Since UO126 was of limited stability in medium, UO126 was added to the cultures every 24 hours.

Figure 8.26 shows that reduced levels of PD-L1 and PD-L2 expression on immature IL-4 DC induced by MEK-inhibitor UO126 did not result in an increased T cell proliferation. This might be due to the fact that UO126 influence the expression of newly synthesized PD-L1 and PD-L2 proteins in immature IL-4 DC and not PD-L1 and PD-L2 molecules, which are already present on the surface of immature IL-4 DC. In this experiment IL-4 DC were used with a clear PD-L1 and PD-L2 surface expression (**Figure 8.24**). Therefore, blocking PD-L1 and PD-L2 with monoclonal antibodies would be necessary. Unfortunately antibodies recognizing rat PD-L1 or PD-L2 are not available yet. Antibodies specific for mouse or human PD-L1 or PD-L2 have not been tested in this study.

Altogether the results show that IL-4 DC express the inhibitory molecules PD-L1 and PD-L2 on their cell surface. Their expression is regulated by GM-CSF induced MEK/ERK-pathway. The importance of PD-L1 and PD-L2 surface expression for the IL-4 DC-mediated inhibition of T cell activation remains unclear.

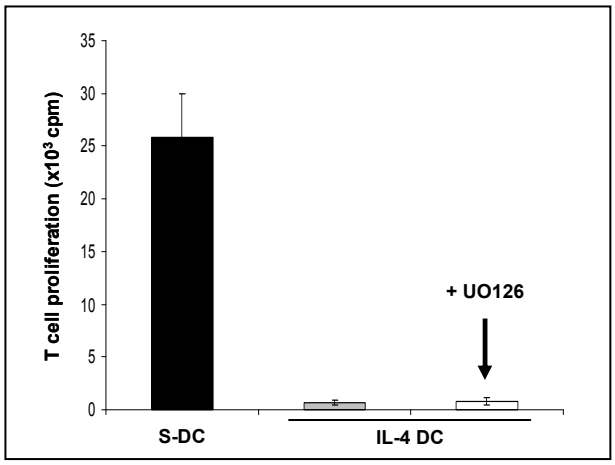


Figure 8.26: Impact of MEK inhibitor UO126 on T cell proliferation in co-cultures with immature IL-4 DC. 10^4 immature IL-4 DC were co-cultured with 10^5 naïve T cells for 72 hours, with or without the addition of 10 µM UO126 every 24 hours to the culture. Proliferation assay (10^4 S-DC co-cultured with 10^5 naïve T cells) served as a control. Data are presented as means ± S.D. of 3 independent experiments.

8.5 Immature IL-4 DC induce T cells with regulatory properties and T_{reg} cell-like phenotype

8.5.1 IL-4 DC-induced expansion of naturally occurring T_{reg} cells

Initially, it was believed that immature DC induced T cell unresponsiveness because of the absence of both inflammatory signals and low expression of co-stimulatory molecules on their surface. However, to date, a growing body of evidence indicates that immature DC induce T cell unresponsiveness by induction and / or stimulation of T_{reg} cells (Morelli AE and Thomson AW, 2003).

The ability of immature IL-4 DC to generate T_{reg} cells was investigated in a next set of experiments. For this, lymph node cells were co-cultured with immature IL-4 DC or mature S-DC. Lymph node cells cultured alone served as control. On day 3, DC were depleted from the cultures and DC-free lymph node cells, dominated by T cells, were analysed by flow cytometry with monoclonal antibodies representing T_{reg} cell-specific marker consisting of CD4, CD25, MHC class II, and Foxp3. **Figure 8.27** shows one representative example of flow cytometric analsyis. **Figure 8.28** summarises the results of 3 independent experiments.

The percentage of so-called naturally occurring $CD4^{pos}Foxp3^{pos}$ T_{reg} cells within unpurified total lymph node cells ranged between 5 and 7% (**Figure 8.27**). Their amount increased after co-culture with mature S-DC to 11% and after co-culture with IL-4 DC to 16%.

In 2006 the group of Baecher-Allan *et al.* demonstrated that MHC class II expression by human $CD4^{pos}CD25^{high}$ T cells indicates a functionally distinct population of mature T_{reg} cells inhibiting T cell proliferation and cytokine production via an early contact-dependent mechanism associated with induction of Foxp3 (Baecher-Allan C *et al.,* 2006). Based on these observations the MHC class II expression of rat T_{reg} cells was analysed. As shown in **Figure 8.27** all $Foxp3^{pos}$ T cells were also positive for MHC class II molecules. Therefore, MHC class II surface expression may also be a useful marker for the identification of rat T_{reg} cells.

The number of T_{reg} cells increased during the co-culture of unpurified lymph node cells with IL-4 DC. T_{reg} cells can be induced from naïve $CD4^{pos}CD25^{neg}$ T cells (Groux H *et al.,* 1997; Kemper C *et al.,* 2003) or expanded from naturally occurring $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T_{reg} cells (Marguti I *et al.,* 2008).

The induction of T_{reg} cells from naïve $CD4^{pos}CD25^{neg}$ T cells was analysed by the following experiment: naturally occurring $CD4^{pos}Foxp3^{pos}$ T_{reg} cells were depleted from total lymph node cells and the T_{reg} cell-free lymph node cells, most of them naïve T cells, were cultured with IL-4 DC for 3 days (**Figure 8.27,** middle panel). Corresponding T cell co-culture experiments were done with mature S-DC. Flow cytometric analysis revealed that the number of T_{reg} cells increased slightly (up to 8%) during the co-culture with IL-4 DC. Since the naïve T cells were contaminated with approximately 3% T_{reg} cells, it is not clear whether this increase was actually caused by naïve T cells or by the remaining T_{reg} cells.

In further experiments the naturally occurring $CD4^{pos}Foxp3^{pos}$ T_{reg} cells within lymph node cells were enriched (**Figure 8.27,** lower panel) and subsequently co-cultured with immature IL-4 DC. The T_{reg} cell enrichment provided a T_{reg} cell population contaminated with naïve T cells but the flow cytometric analysis revealed that enriched $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T_{reg} cells were expanded intensively by both S-DC and IL-4 DC.

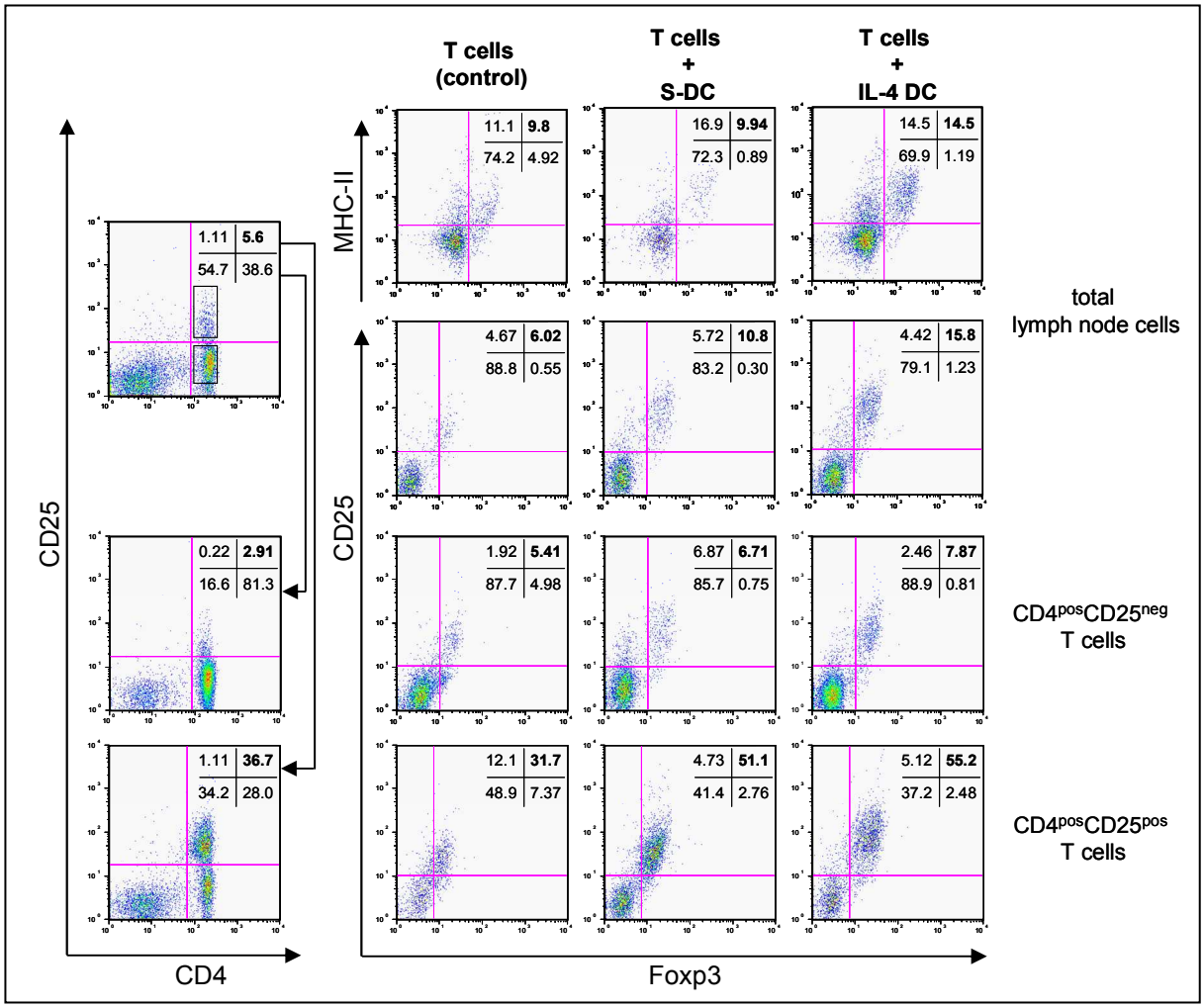


Figure 8.27: Naturally occurring T_{reg} cells are expanded by IL-4 DC and S-DC in vitro. 10^4 immature IL-4 DC or mature S-DC were co-cultured with 10^5 total lymph node T cells (upper panel), 10^5 naïve T cells depleted of $CD4^{pos}CD25^{pos}$ T cells (middle panel) or with purified 10^5 $CD4^{pos}CD25^{pos}$ T cells (lower panel). After 3 days the cells were collected and, after depletion of DC, labelled with fluorescent antibodies for CD4, CD25 and Foxp3. All cells are gated on $CD4^{pos}$ cells. One representative example of at least 3 different experiments is shown (see appendix **Table 12.8**).

These results show that immature IL-4 DC as well as mature S-DC are able to induce the proliferation of $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T_{reg} cells to nearly the same extent. They are not able to induce the generation of $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T_{reg} cells from naïve T cells. Since T_{reg} cells demonstrate a low rate of proliferation, the expansion of T_{reg} cells by IL-4 DC might correspond to the very low T cell proliferation by IL-4 DC (**Figure 8.10 B**). In contrast, activation and proliferation of effector T cells was not observed in the presence of IL-4 DC (**Figure 8.10 B** and **Figure 8.11**, **section 8.32**). High T cell proliferation observed in the proliferation assay (T cells + S-DC, **Figure 8.10 B**) might be due more to the preferential activation / expansion of effector T cells than to the activation/expansion of T_{reg} cells.

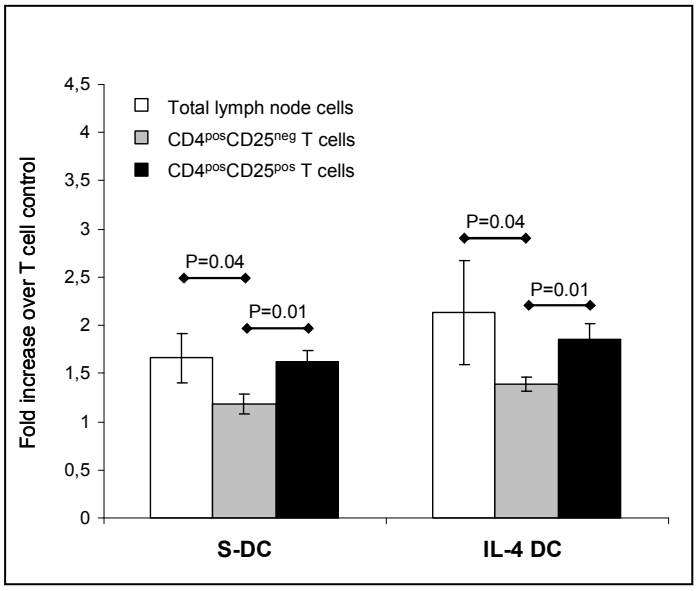


Figure 8.28: IL-4 DC and S-DC induce the expansion of T_{reg} cells in vitro. 10^4 immature IL-4 DC or mature S-DC were co-cultured with 10^5 total lymph node T cells (white bars), 10^5 naïve T cells depleted of naturally occurring T_{reg} cells ($CD4^{pos}CD25^{neg}$ T cells) or with enriched 10^5 naturally occurring T_{reg} cells ($CD4^{pos}$ $CD25^{pos}$ T cells) for 3 days. Subsequently, DC-depleted cells were labelled with fluorescent antibodies for CD4, CD25 and Foxp3. The fold increase of $CD4^{pos}$ $CD25^{pos}Foxp3^{pos}$ T cells after co-culture with S-DC or IL-4 DC over the T cell control was analysed. Data are presented as means ± S.D. of 3 independent experiments. Statistical significance is indicated using the paired Student *t*-test.

8.5.2 IL-4 DC-mediated induction of anergic T cells

In 2000 Jonuleit *et al.* showed that repetitive stimulation of naïve cord blood–derived T cells with allogenic immature DC resulted in the induction of suppressive, nonexpanding (anergic), IL-10-producing T_{reg}-like cells (Jonuleit H *et al.,* 2000). Anergy appears when co-stimulatory signals are absent and describes a state of functional unresponsiveness of T cells that are unable to initiate a productive response to secondary stimulation, even if this includes full co-stimulation (Sebille F *et al.,* 2003; Wekerle T *et al.,* 2002). In addition, anergy is characterized by an inhibition of IL-2 secretion (Lechler R *et al.,* 2001).

To determine whether immature IL-4 DC are able to induce an anergic state in naïve T cells, $1x10^5$ naïve T cells were incubated with $1x10^4$ immature IL-4 DC. T cells cultured alone or together with mature S-DC served as control. At the end of culture, IL-4 DC or S-DC were depleted with the antibody Ox-42 and magnetic-beads. The resulting T cells of the first culture were transferred to the second culture consisting of $1x10^4$ fresh mature S-DC per well (**Figure 8.29 A**). Finally, the incorporated radioactivity was measured at the end of the second culture on day 3 (**Figure 8.29 B**).

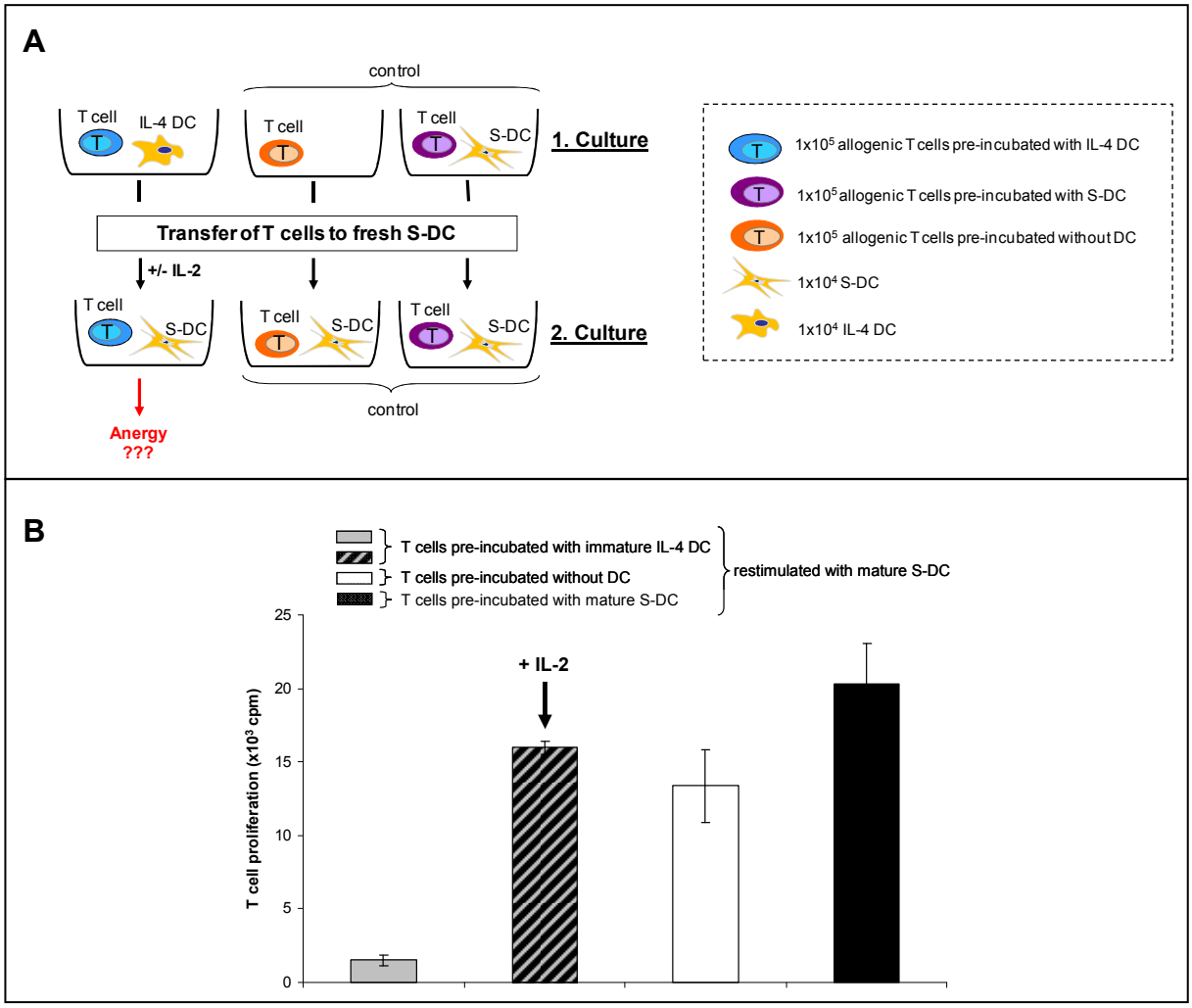


Figure 8.29: IL-4 DC induce anergic T cells. **A:** Experimental setup: 10^5 naïve T cells were pre-incubated for 3 days with 10^4 immature IL-4 DC. T cells cultured alone or with mature S-DC served as control. After depletion of dendritic cells, T cells were transferred to mature S-DC. 72 hours later the T cell proliferation was determined. **B:** Results: Mature S-DC are not able to induce a restimulation of T cells pre-incubated with IL-4 DC. The suppressed capacity of T cell proliferation could be restored by addition of exogenous IL-2 (266 ng/ml). The data shown are represented as means ± S.D. and represent the results from representative experiments repeated three times.

As shown in **Figure 8.29 B,** S-DC activated T cells from the first co-culture could expand in the presence of fresh, isolated S-DC during the second culture: 20,324 ± 2,761 cpm. In contrast, T cells co-cultured with IL-4 DC during the first culture could not expand in the presence of mature S-DC during the second culture: 1,503 ± 335 cpm. This unresponsiveness, however, could be abolished by adding of 266 ng/ml exogenous IL-2. The measured proliferation was then 15,952 ± 467 cpm (**Figure 8.29 B**).

Naïve T cells cultured without S-DC (and therefore without proliferation signals) during the first culture could be activated during the second culture with S-DC: 13,352 ± 2,437 cpm. This result shows that most of the naïve T cells survived without proliferation signals during the first culture period. Therefore, immature IL-4 DC induce a reversible state of functional unresponsiveness in co-cultured T cells and these T cells show features of anergic T cells (**Figure 8.29**).

8.5.3 IL-4 DC-induced anergic T cells demonstrate immune inhibitory properties

The regulatory effect of anergic T cells is well known. For humans and mice, several groups have demonstrated that T cells generated in co-culture with immature DC are able to inhibit T cell responses *in vitro (*Bashuda H *et al.,* 2005; Cools N *et al., 2008;* Gad M *et al.,* 2004; Jonuleit H *et al.,* 2003; Kubsch S *et al.,* 2003; Kuwana M, 2002).

In this study we further analysed the function of T cells pre-incubated with immature IL-4 DC. These T cells were tested for their ability to suppress mature S-DC induced T cell proliferation. The possible suppressor T cells were recovered from the first co-culture with IL-4 DC and transferred to the second assay containing fresh isolated naïve “indicator” T cells and mature S-DC. T cells incubated with S-DC during the first culture were used as control cells. The viability of “indicator” T cells was tested in a control proliferation assay. After 3 days the [^{3}H]- thymidine incorporation was measured (**Figure 8.30 A**).

The results show that T cells co-cultured with IL-4 DC reduced the S-DC induced proliferation of “indicator” T cells in the second culture about 92% from 22,494 ± 469 cpm to 1,768 ± 325 cpm (**Figure 8.30 B**).

T cells activated by S-DC during the first culture were tested in the second culture. These activated T cells were not able to inhibit the S-DC induced proliferation of “indicator” T cells, although proliferation was reduced in comparison to the control proliferation assay. The reason for this is the different number of cells. For the control proliferation assay 10^4 S-DC und 10^5 naïve “indicator” T cells were co-cultured. The

inhibitory effect of T cells cultured with IL-4 DC or S-DC was shown in a second proliferation assay consisting of 10^4 S-DC und 10^5 naïve "indicator" T cells and 10^5 T cells transferred from the first culture with IL-4 DC or S-DC (**Figure 8.30**).

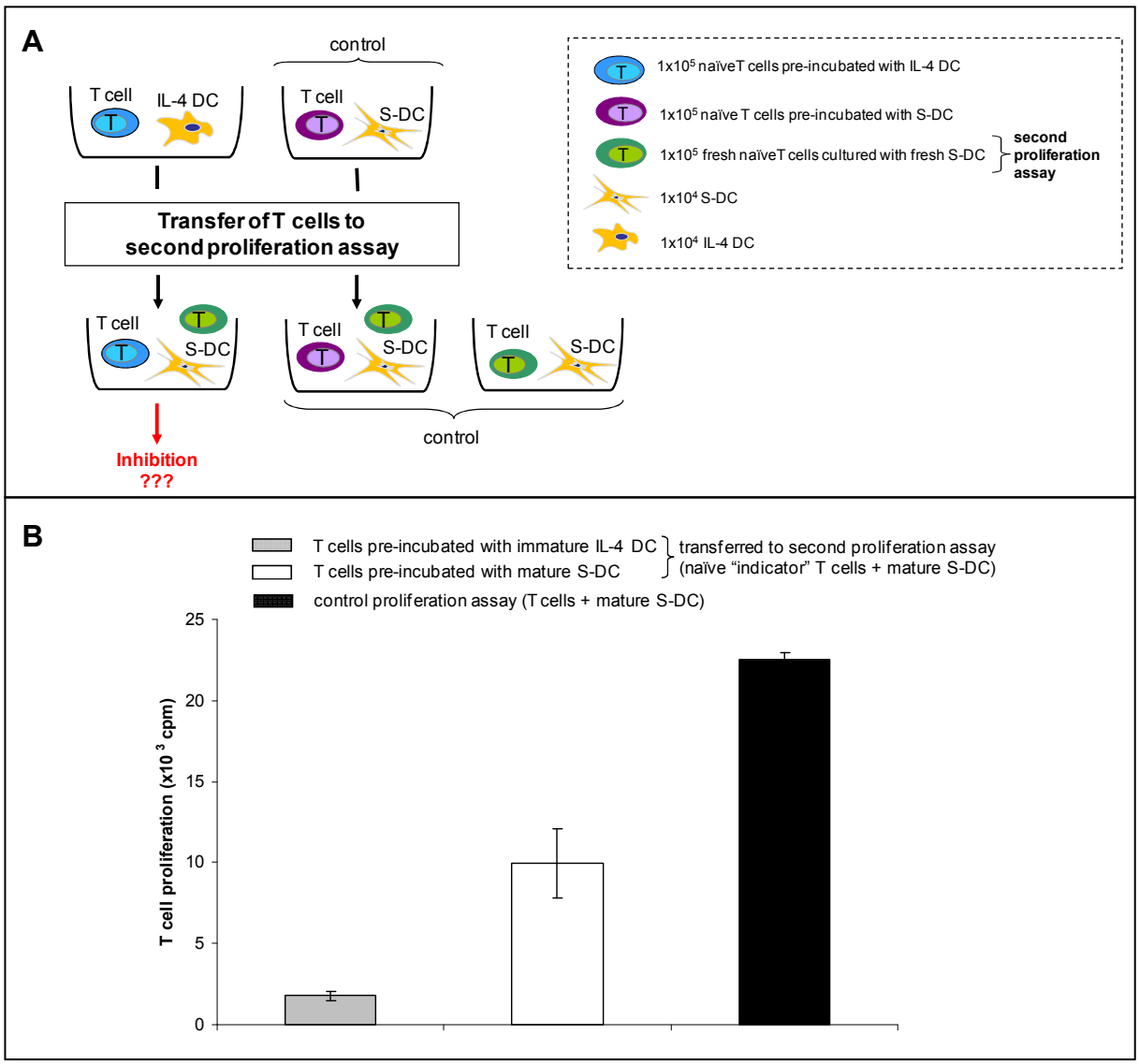


Figure 8.30: IL-4 DC-induced anergic T cells inhibit S-DC induced T cell proliferation. **A:** Experimental setup: 10^5 naïve T cells were pre-incubated for 3 days with 10^4 immature IL-4 DC. Naïve T cells cultured with mature S-DC served as control. After depletion of dendritic cells, T cells were transferred to a second T cell proliferation assay containing fresh mature S-DC and naïve T cells. 72 hours later the T cell proliferation was determined. **B:** Results: T cells pre-incubated with immature IL-4 DC inhibit mature S-DC induced T cell proliferation. The data shown are represented as means ± S.D. and represent the results from representative experiments repeated three times.

The results of **Figure 8.30** demonstrate that naïve T cells co-cultured with immature IL-4 DC demonstrate immune inhibitory effects and a phenotyp characteristic for T_{reg} cells (**Figure 8.27**).

S-DC primarily induce a population of effector T cells that is unable to inhibit the activation of "indicator" T cells. In addition, S-DC are also able to restimulate T_{reg} cells (**Figure 8.27**).

Altogether IL-4 DC, phenotypically characterized by reduced surface expression of MHC class II and co-stimulatroy molecules, are unable to activate naïve T cells or suppress proliferation of activated T cells. The characterization of IL-4 DC does not lead to the identification of one mechanism that fully explain all features of IL-4 DC, but evidence was found that TGF-β as soluble factor may be strongly involved in IL-4 DC-mediated inhibition of T cell proliferation. Two exciting properties of the IL-4 DC are their inability to mature and the induction of anergic T cells with regulatory features.

IL-4 DC are characterized by the following properties:

- Growth in cell clusters (**Figure 8.1, 8.2**)
- Low surface expression of co-stimulatory molecules and MHC II (**Figure 8.3**)
- Deficient production of IL-12 (**Figure 8.6, 8.8**)
- Endocytosis (**Figure 8.9**)
- Resistance to different maturation stimuli (**Figure 8.15, 8.17**)
- Expression of inhibitory molecules PD-L1 and PD-L2 (**Figure 8.24-8.26**)

IL-4 DC demonstrate the following effects:

- Inability to activate naïve T cells (**Figure 8.10**)
- Inhibition of S-DC induced T cell proliferation (**Figure 8.13, 8.29**)
- Inhibition of antibody induced T cell proliferation (**Figure 8.12**)
- Inhibition effect within 24 hours (**Figure 8.14**)
- Secretion of soluble factors suppressing T cell proliferation (**Figure 8.18**)
- Secretion of TGF-β was successfully proofed (**Figure 8.19**)

- Expression of iNOS and secretion of NO; probably not involved in IL-4 DC-mediated inhibition of T cell proliferation (**Figure 8.20-8.22**)

T cells co-cultured with IL-4 DC are characterized by:

- TGF-β dominated cytokine profile (**Figure 8.11**)
- Inhibition of S-DC-induced T cell proliferation (**Figure 8.29**)
- Reduced ability to respond to full activation by S-DC (**Figure 8.30**)
- A phenotype typical for T_{reg} cells (**Figure 8.27**)

9 Discussion

DC are the main key regulators of the immune system. They are known as inducers of destructive immune responses but they can also exert tolerogenic functions (Steinman RM *et al.,* 2003). The ability to induce tolerance makes them interesting for clinical applications to inhibit excessive immune responses in autoimmune disorders and transplant rejection (Tarbell KV *et al.,* 2004; Zhang X *et al.,* 2008). This characteristic is the reason why DC have been the focus of numerous studies over the last years investigating their clinical relevance to modulate the outcome of unwanted immune responses (van Duivenvoorde LM *et al.,* 2006; Steinman RM and Banchereau J, 2007).

It has become obvious over the past years that not a single type of tolerogenic DC is able to control the diversity of mechanisms involved in T cell tolerance. Therefore, it is important to generate and select the optimal cell type suitable for DC-based therapies. However, the differences existing between humans and the most popular small animal models mice and rat, makes it difficult to develop a common protocol suitable for the generation of immature DC for the 3 species. *In vitro* characterization of DC mechanisms underlying their suppressive effect has mainly been focused on humans and mice and less on rat. Since the rat is widely used as an experimental animal model in a variety of scientific areas such as immune responses to pathogens, tumors, self-antigens, or allografts, the present study analyses the importance of rat tolerogenic immature DC and their interaction with immunoregulatory T_{reg} cells.

9.1 The outcome of a specific IL-4 DC population depends on the combination of the cytokines GM-CSF and IL-4

The immune regulatory functions of DC seem to be mainly related to an immature developmental stage. Mature DC can efficiently induce the development of effector T cells, whereas immature DC are involved in maintenance of peripheral tolerance (Mahnke K *et al.,* 2002; Mahnke K *et al.,* 2003). The low number of DC found in tissues or in circulation (1-3%) limited their characterization (Banchereau J and

Steinman R, 1998; Jenkins DE *et al.,* 1998), since for this purpose large numbers of isolated DC are necessary (Freudenthal PS and Steinman RM, 1990; O'Doherty U *et al.,* 1993; O'Doherty U *et al.,* 1994). In contrast to tissues or blood, large numbers of DC can be generated from bone marrow precursor cells, when cultured with appropriate cytokines (Inaba K *et al.,* 1992; Romani N *et al.,* 1994; Szabolcs P *et al.,* 1997; Lutz MB *et al.,* 1999).

One of the first protocols for the generation of rat immmature DC was published by Kliniker *et al.* (Klinkert WE *et al.,* 1982). They cultured low density bone marrow progenitor cells with supernatant from ConA-treated spleen cells. In 1986 a protocol followed that described DC expansion from rat bone marrow progenitor cells in a serum-free medium (Bowers WE and Berkowitz MR, 1986). Both protocols revealed a cell yield of about 1% or less of the starting bone marrow cultures. Thereafter, Inaba K *et al.* was one of the first using purified GM-CSF for the generation of mice DC from bone marrow cells. They showed that GM-CSF improves the cell yield of rat BMDC, but the numbers were still low (Inaba K *et al.,* 1992). Ten years later the group of Bai L *et al.* published their experiences in generating human DC in the presence of GM-CSF (Bai L *et al.,* 2002). Further cytokines, like IL-4 or TNF-α, combined with GM-CSF have been shown to be necessary for the induction of high cell numbers of DC (Szabolcs P *et al.* 1995; Romani N *et al.* 1996; Talmor M *et al.,* 1998; Young JW, 1999; Labeur MS *et al.,* 1999; Lu L *et al.,* 1999).

In this study the optimal growth conditions required for the generation of large numbers of immature rat IL-4 DC from bone marrow cells was systematically evaluated; only few studies have been done on this. The cultivation protocols of these studies differ concerning cytokine concentration, end point of culture and the type of bone marrow progenitor cells (Talmor M *et al.,* 1998; Grauer O *et al.,* 2002). Bone marrow is an extremly complex mixture of different cell types; it also contains three types of stem cells: hematopoietic stem cells, mesenchymal stem cells and endothelial stem cells. Most of these cells adhere to plastic surfaces and are used for the generation of immature DC (Xiao BG *et al.,* 2001; Muthana M *et al.,* 2004). During culture some cells loose their adherence and move into the supernatant. These non-adherent cells also can be used for the generation of immature DC as

well (Chen-Woan M *et al.,* 1995; Lutz MB *et al.,* 1999; Grauer O *et al.,* 2002; Powell TJ *et al.,* 2003; Muthana M *et al.,* 2004; Berger TG *et al.,* 2009).

The influence of GM-CSF alone and in combination with IL-4 to induce immature rat DC was analysed. It was found that GM-CSF was a key factor in promoting both DC proliferation and differentiation *in vitro* (**Table 8.1, Figure 8.1**) but resulted in a poor DC yield (**Table 8.1, Figure 8.1**). This observation, which is in accordance with the results of other studies, seems to be unique for the generation of rat DC (Chen-Woan M *et al.,* 1995; Talmor M *et al.*, 1998; Grauer O *et al.,* 2000; Muthana M *et al.,* 2004). The presence of GM-CSF alone is sufficient for the generation of mice and human bone marrow-derived DC (Romani N *et al.* 1994; Lutz MB *et al.,* 1999). In this study it is shown, that IL-4 in combination with increasing concentrations of GM-CSF further increases the cell yields (**Table 8.1, Figure 8.1**). However, the percentage of cell yield peaked at a concentration of 5 ng/ml of GM-CSF, with or without IL-4, but only under the influence of IL-4 did bone marrow progenitor cells develop loosely adherent cell clusters which increased the cell yield (**Table 8.1, Figure 8.1**). The formation of cell clusters indicates the development of bone marrow progenitor cells along the LC pathway (Riedl E *et al.,* 2000). Cellular aggregates were formed between day 2 and day 4 in the present study (**Figure 8.2**).

Rat IL-4 DC recovered from cultures revealed the surface expression of the rat DC marker Ox-62 and MHC class II, confirming the development of immature DC. Ox-62 recognizes the rat homolog of the 150 kDa integrin-α_{E2} subunit and was originally described as being a useful marker for rat veiled cells (Brenan M and Puklavec M, 1992). High surface expression of MHC class II can be considered as an indicator for mature DC (Santambrogio L *et al.,* 2006). In this study, the flow cytometric analysis clearly demonstrated more than 85% Ox-62-positive cells, regardless of the cytokine combination used (**Table 8.1**). These results differ from those shown by Brisette-Starkus *et al.* and Muthana *et al.* The authors did not observe such high levels of Ox-62-positive cells generated in the presence of GM-CSF and IL-4 (Brisette-Starkus CS *et al.,* 2002; Muthana M *et al.,* 2004). The reason for this is not clear, since both publications used GM-CSF and IL-4 concentrations investigated in this study. The protocol of Brisette-Starkus *et al.* only differs in that they removed and discarded non-adherent cells on day 4 of culture. Previous studies have employed different

treatments to improve the purity of BM-DC precursors for the outcome of highly pure DC populations. These protocols include the removal of contaminating non-DC progenitors (Inaba K *et al.,* 1992; Talmor M *et al.,* 1998 and Grauer O *et al.,* 2002). However, these depletion steps may also remove vital and expanding DC precursor subsets (Maroof A, 2001; Hart DN, 1997). For this reason the culture protocol described in this study avoids procedures such as removing non-adherent precursor cells during culture (**section 7.1**).

Literature on the generation of immature DC is heterogeneous and often controversial. Thereby, it is not always clear how different cytokine combinations influence DC maturation. For example, GM-CSF at a concentration of 1 ng/ml combined with IL-4 generates phenotypically immature DC in rats (Garrovillo M *et al.,* 2001), whereas the same cytokine combination induces the generation of phenotypically mature BM-DC in mice (Lutz MB *et al.,* 2000). All these aspects e.g. different progenitor cell populations, cytokine combination as well as different species need to be taken into consideration for the final outcome of phenotypic and functional characteristics of DC (**Table 9.1**).

In the present study, DC growth conditions were optimised to increase the yield of immature (MHC class II^{low}) Ox-62 positive DC from bone marrow progenitor cells. A cytokine combination of 5 ng/ml GM-CSF plus 5 ng/ml IL-4 produces one of the best results concerning cell yield of Ox-62 positive IL-4 DC, cluster formation, and expression of MHC class II (**Table 8.1**). The results show that GM-CSF is an essential key factor for the generation / proliferation of bone marrow-derived DC. IL-4 seems to play a secondary / accessory role as an “enhancer” for the generation of cell clusters with proliferating DC demonstrating an immature phenotype (MHC class II^{low}).

Table 9.1: Different culture techniques for the generation of dendritic cells.

	Present study	Literature			
		(1)	(2)	(3)	(4)
Species	rat	rat	rat	mice	human
Progenitor cells	BM cells	BM cells	fractionated BM r cells	BM cells	PBMC
Culture conditions					
Starting cell culture density	$5x10^5$ cells/ml; petri dish (Ø 100 mm)	$5x10^5$ cells/ml; 6-well plate	$1x10^6$ cells/ml; 24-well plate	$2x10^5$ cells/ml; petri dish (Ø 100 mm)	$2x10^7$/ml
Cytokines	GM-CSF (5 ng/ml); IL-4 (5 ng/ml)	GM-CSF (5 ng/ml); IL-4 (5 ng/ml)	GM-CSF (30 ng/ml); IL-4 (2000 U/ml)	GM-CSF (20 ng/ml)	GM-CSF (30 ng/ml); IL-4 (20 ng/ml)
Depletion of contaminating cells	no	lymphocytes and granulocytes on day 5-6	lymohocytes and MHC class II^{pos} cells on day 0	no	lymphocytes after 2 hr
Exchange of Medium	on day 3 or day 4 with fresh cytokines	on day 3	every 2-3 days	on day 3, 6, 8 and 10	on day 3 with fresh cytokines
Outcoming DC population					
Days of culture	6 days	replating on day 7-8; harvesting on day 9-10	6 or 7 days	6, 8, 10 and 12 days	6 days
Investigated DC population	loosely and non-adherent cells	non-adherent cells	semi- and non-adherent FcR^{pos} and FcR^{neg} cells	non-adherent cells	non-adherent cells
Ox-62^{pos} cells	> 85%	no information	negative for Ox-62	–	–
MHC class II^{pos} cells	~ 38%	~ 80%	MHC class II^{pos}	MHC class II^{low} and MHC class II^{high}	no information
Cluster formation	yes	yes	no information	yes	yes
Cell yield	~ 193%	~ 38%	no information	$1\text{-}3x10^8$	$8.2x10^6$/ml
State of maturation	immature IL-4 DC	mature DC	FcR^{pos}: immature DC FcR^{neg}: mature DC	mixture of immature and mature DC	immature

(1) Grauer O *et al., Histochem Cell Biol* 2002; 117 (4): 351-362 ; **(2)** Powell TJ *et al., Immunology* 2003; 109 (2): 197-208; **(3)** Lutz MB et al., *Journal of Immunological Methods* 1999; 223 (1): 77-92; **(4)** Tang L *et al., J Zhejiang Univ SCIENCE B* 2005; 6(12): 1176-1181

9.2 Immature IL-4 DC are resistant to different maturation stimuli

Immature DC are able to take up antigen, but they are unable to present antigens, whereas mature DC are professional antigen presenting cells with a low capacity of endocytosis (Quah BJC and O'Neill HC, 2005; Hotta C *et al.,* 2006). The immature phenotype of IL-4 DC was demonstrated by a very low expression of co-stimulatory molecules CD80, CD86 and CD40 as well as significantly reduced levels of CD45, MHC class II, CD25 and adhesion molecule ICAM-1 compared to that of mature S-DC (**Figure 8.5**). In addition, their immature phenotype was confirmed by their pronounced endocytic activity, demonstrated by measuring the uptake of Dextran-Alexa 488 (**Figure 8.9)**. Furthermore, the immaturity of IL-4 DC was confirmed by their inability to activate naïve T cells (**Figure 8.10**). It is well known that according to the “two-signal” hypothesis naïve T cells need engagement of the T cell receptor with MHC molecule (signal 1) in addition to a second stimulus through co-stimulation by CD80 and CD86 (signal two) to become fully activated (Libscomb MF and Matsen BJ, 2002). This hypothesis may explain the inability of IL-4 DC to activate naïve T cells because they demonstrate a low surface expression of both MHC class II as well as co-stimulatory molecules (**Figure 8.5**). It is also possible to inhibit T cell proliferation by blocking the co-stimulation with e.g. the CTLA-4Ig fusion protein (Balzevic V *et al.,* 2001).

IL-4 DC also demonstrate low expression of ICAM-1, CD25 and CD45 compared to mature S-DC (**Figure 8.5**). Different studies suggest that adhesion molecules such as ICAM-1 on the DC surface are required for optimal activation of T cells through interaction of LFA-1 on T cells with ICAM-1 (Van Seventer GA *et al.,* 1990; Ybarrondo B *et al.,* 1994). It has been shown that blocking ICAM-1 with a monoclonal antibody prolonged skin and heart allografts (Isobe M *et al.,* 1992; Isobe M *et al.,* 1996). CD45 molecule has been shown to help regulation of T cell proliferation and differentiation (Montoya M *et al.,* 2006; Pricket TCR and Hart DN *et al.,* 1990). Several studies demonstrated that a monoclonal antibody specific for the CD45RB

isoform is a potent immunomodulator that prolongs allograft survival in several murine transplantation models (Lazarovits AI *et al.,* 1996; Auersvald LA *et al.,* 1997; Gregori S *et al.,* 2005). CD25 expression by human and pig DC is described and it is suggested they have an important role in driving the immune response towards a T_H1 response (Mnasria K *et al.,* 2008; Pilon C *et al.,* 2009). The immune inhibitory effect caused by blocking CD25 with an anti-CD25 antibody was confirmed by *in vitro* experiments (Mnasria K *et al.,* 2008). Presently, anti-CD25 monoclonal antibodies are widely used in clinical transplantation to prevent acute allograft rejection (Vincenti F *et al.,* 1998; Ter Meulen CG *et al.,* 2004).

Mature DC are required for full activation of naïve T cells (Quah BJC and O´Neill HC, 2005). DC maturation can be triggered by a response to microbial components such as LPS, double-stranded RNA, or CpG-DNA, or by inflammatory cytokines such as CD40L and TNF-α (de Smedt T *et al*., 1996; Sparwasser T *et al.,* 1998; Ridge JP *et al.,* 1998; Caux C *et al.,* 1992). However, maturation-resistance of tolerogenic DC is one prerequisite for a possible clinical application. To our knowledge the maturation-resistance of rat IL-4 DC has not been described yet. In this study we show that low-adherent immature rat IL-4 DC did not respond to maturation stimuli such as LPS, TNF-α or CD40L. These treated IL-4 DC still expressed low levels of MHC class II and co-stimulatory molecules (**Table 8.6**) compared to the higher surface expression of these molecules on mature S-DC (**Table 8.3**). However, several studies exist showing that rat BM-DC cultured at either low or high GM-CSF concentration are able to mature in response to different maturation stimuli (Brissette-Storkus CS *et al.,* 2002; Grauer O *et al.,* 2002; DePaz HA *et al.,* 2003; Janelidze S *et al.,* 2005). In contrast, Muthana and colleagues showed that LPS had no effect on CD80 and MHC class II expression on adherent rat BM-DC (Muthana M *et al.,* 2004).

The maturation-resistance of immature IL-4 DC reflects their inability to stimulate T cells (**Figure 8.17**). These results are in contrast to other studies on rat (Grauer O *et al.,* 2002) and murine bone marrow-derived DC (Lutz MB *et al.,* 1999), although the phenomenon of maturation-resistance of mice DC has also been shown by Lutz and colleagues (Lutz MB *et al.,* 2000). They reported that mice bone marrow cells cultured with low doses of GM-CSF generate immature DC that do not respond to maturation stimuli. However, the presence of IL-4 abrogated the maturation-resistant

phenotype of these BM-DC. The same effect of IL-4 on DC maturation was observed for human DC (Berger TG *et al.,* 2009). The reason for numerous conflicting results may be the use of different DC populations, such as adherent and non-adherent cells (Powell TJ *et al.,* 2001; Powell TJ *et al.,* 2002), the use of DC at different maturation stages and/or different treatments of the DC cultures, such as different cytokine combinations and/or concentrations, time point of cell harvesting and re-plating (Lutz MB *et al.,* 1999).

The generation of maturation-resistant rat IL-4 DC for tolerance induction *in vivo* might be a better strategy than the generation of maturation-sensitive immature DC. The important goal, however, is to indentify such maturation-resistant immature DC for humans.

9.3 Implication for a soluble factor responsible for the IL-4 DC-mediated suppressive effect

Inhibition of T cell responses by DC secreted inhibitory molecules is possible (Guillot C *et al.,* 2003). IL-4 DC demonstrate a suppressive effect on mature S-DC-induced T cell proliferation in a dose-dependent manner (**Figure 8.13**). Similar results have been observed by Powell and colleagues (Powell TJ *et al.,* 2003). They reported that immature BM-DC, separated by expression of the Fc receptor, inhibited T cell proliferation. In addition, we were able to show that the suppressive effect of immature IL-4 DC was detectable within 24 hours after the addition of IL-4 DC on day 2 to the culture with naïve T cells and mature S-DC (**Figure 8.14**). To our knowledge this is the first description of the time course immature BM-DC needed to suppress T cell proliferation. The suppressive effect of IL-4 DC on mature S-DC-induced T cell proliferation seems to be mediated by their ability to inhibit IL-2 and IFN-γ production and to induce TGF-β production (**Figure 8.11** and **8.13**).

IL-4 DC are able to produce TGF-β themselves (**Figure 8.19**). This has also been demonstrated by Morelli and colleagues (Morelli AE *et al.,* 2001). They detected high levels of TGF-β in the supernatant of immature $CD11c^{pos}CD86^{neg}$ but not in the

supernatant of mature $CD11c^{pos}CD86^{pos}$ DC cultured for 24 hours. Supernatant of IL-4 DC demonstrated a dose-dependent inhibitory effect on mature S-DC-induced T cell proliferation (**Figure 8.18**). TGF-β is a potent immunoregulatory cytokine known to have potent effects on inhibiting the differentiation and proliferation of both $CD4^{pos}$ and $CD8^{pos}$ T cells (Li MO *et al.,* 2006). Das and Levine, for example, showed that TGF-β suppresses TCR-induced proliferation of effector T cells by inhibiting IL-2 production (Das L and Levine AD, 2008). In addition, it has also been shown that TGF-β inhibits the ability of mature DC to stimulate IFN-γ secretion by T cells (Kobie JJ *et al.,* 2003). Zang *et al.* demonstrated that prolongation of graft survival is TGF-β dependent and associated with the generation of TGF-β secreting T_{reg} cells (Zang W *et al.,* 2008). In addition, T_{reg} mediated TGF-β production was suggested as an important mechanism of suppression (Kariminia A *et al.,* 2005; McGeachy MJ *et al.,* 2007). IL-4 DC producing TGF-β were able to expand T_{reg} cells with inhibitory functions. The presence of T_{reg} cells after co-culture with immature IL-4 DC may account for increased TGF-β levels (**Figure 8.11)**. In addition to TGF-β produced by IL-4 DC themselves (**Figure 8.19**), it is known that T_{reg} cells can also produce TGF-β (Simon JC *et al.,* 2002; Wan YY *et al.,* 2007). However, whether T cells incubated with IL-4 DC really produce TGF-β producing cells has not been analysed so far and will be the focus of a future study.

Mature S-DC induced the activation of T_H1 cells secreting IFN-γ and IL-2, whereas IL-4 DC favoured the development of T cells producing low levels of IFN-γ and IL-2, and significantly increased levels of TGF-β (**Figure 8.11**). Secretion of IL-10 was not detected in either situation. Different studies have shown that T_r1 cells, a subtype of T_{reg} cells, are characterized by a unique pattern of cytokine production distinct from that of T_H1 and T_H2 cells. They produce high levels of IL-10 and/or TGF-β, and low amounts of IL-2 (Asseman C *et al.,* 1999; Jonuleit H *et al.,* 2000; Powrie F *et al.,* 1996).

In addition to TGF-β, toxic NO has been reported to act as a suppressive mediator of DC (Bonham CA *et al.,* 1996; Yamamoto K *et al.,* 1998; Powell TJ *et al.,* 2003; Rößner S *et al.,* 2005). Elevated NO levels have also been implicated in rejection of organ or tissue allografts (Krulová M *et al.,* 2002; Holán V *et al.,* 2006; Matuschek A

et al., 2009). In contrast, the inhibition of NO production promotes graft acceptance (Winlaw DS *et al.,* 1995). Immature IL-4 DC have been shown to produce NO after appropriate stimulation with LPS (**Figure 8.20**). Induction of iNOS protein expression in immature IL-4 DC could be confirmed by western blotting (**Figure 8.21**). It has been shown that NO production of DC results from the induction of iNOS enzyme by LPS, proinflammatory cytokines such as exogenous IFN-γ produced in interaction with allogenic T cells (Lu L *et al.,* 1996). A clear association between NO production and the presence of mature S-DC, but not immature IL-4 DC, was observed in the present study. The presence of the NO inhibitor NMMA increased the S-DC-induced T cell proliferation from 19,454 ± 7,365 cpm to 49,948 ± 7,723 cpm (**Figure 8.22**). The high levels of NO detectable in S-DC-induced T cell proliferation might be due to IFN-γ produced by activated T cells (Nadeau KC *et al.,* 1995; Melter M *et al.,* 1999; Rocha PN *et al.,* 2003) (**Figure 8.11**). IFN-γ is mainly produced by activated T cells and stimulates the NO production by DC and others (Lu L *et al.,* 1996). The presence of NMMA had little effect on the IL-4 DC-mediated inhibition of S-DC-induced T cell proliferation (**Figure 8.22**) and no evidence was found for NO-mediated T cell apoptosis (**Figure 8.23**).

Altogether, the results of this study demonstrate that immature IL-4 DC are very potent inhibitors of the activation of naïve T cells. The main reason for their inhibitory effect may be the low expression of co-stimulatory molecules on their cell surface. However, other mechanisms such as the secretion of soluble immune inhibitory cytokines such as TGF-β may also be involved. Therefore, the detection of TGF-β in the supernatant of immature IL-4 DC described in this study is an interesting finding that needs to be further investigated to clarify its role in the inhibition of T cell proliferation.

9.4 PDL-1 and PD-L2 signalling on IL-4 DC: a possible role in tolerance induction?

The ability of co-stimulatory molecules of the *B7* family, such as CD80 and CD86, to provide co-stimulatory signals for full T cell activation has been well characterized. Recently, two additional *B7* family members, PD-L1 and PD-L2, have been shown to deliver inhibitory signals resulting in down-regulation of T cell activation (Keir ME *et al.,* 2008); thereby playing an essential role in peripheral tolerance (Keir ME *et al.,* 2006; Habicht A *et al.,* 2007).

To our knowledge PD-L1 and PD-L2 expression on rat DC and its role in T cell tolerance has not been previously investigated. Therefore, in the present study, the expression of PD-L1 and PD-L2 by immature IL-4 DC was examined. Both PD-L1 and PD-L2 protein are expressed in immature IL-4 DC via a MEK/ERK-dependent pathway, possibly induced by GM-CSF (**Figure 8.24 A**). Thereby, LPS was able to up-regulate PD-L1 expression (**Figure 8.24 B**; PD-L2 antibody was not available at this time). PD-L1 expression on plasma cells from multiple myeloma patients via a MEK-dependent pathway has been reported by Lui and colleagues (Liu J *et al.,* 2007). The role of IL-4 in induction of PD-L1 and/or PD-L2 on immature IL-4 DC remains to be investigated. A study by Yamazaki *et al.* showed that PD-L1 is further up-regulated on macrophages by LPS, IFN-γ, GM-CSF and IL-4, whereas PD-L2 is induced on macrophages by IFN-γ, GM-CSF and IL-4 (Yamazaki T *et al.,* 2002). However, it is not clear whether and to what extent the expression of PD-L1 and/or PD-L2 on the surface of immature IL-4 DC contributes to their ability to inhibit S-DC-induced T cell proliferation.

In this study UO126, an inhibitor of MEK-pathway, was tested to investigate the role of PD-L1 and PD-L2 on immature IL-4 DC interacting with T cells. Indeed, we observed reduced protein levels of both PD-L1 and PD-L2 protein expression. However, UO126 had no effect on the low capacity of IL-4 DC to stimulate naïve T cells (**Figure 8.26**). This might be due to the fact that UO126 only decreases the expression of both molecules on the protein level. However, previous studies revealed that PD-L1 and PD-L2 are already present on the surface of immature IL-4 DC on day 4 and day 6 of DC culture. To better understand the function of both

molecules in the system of the present study one would have to develop anti-rat PD-L1 and PD-L2 monoclonal antibodies (both are not commercially available) which block engagement with PD-1. Both anti-PD-L1 and PD-L1 antibody have been shown to enhance the capacity of human immature DC to stimulate T cell activation and cytokine production (Brown JA *et al.,* 2003).

The study by Brown and colleagues showed that in addition to immature DC, PD-L1 and PD-L2 are also expressed on mature DC. This study did not investigate the expression of both molecules on the surface of mature S-DC. A possible expression of PD-L1 on mature DC might appear questionable at first glance, since these cells are able to induce full T cell proliferation rather than T cell inhibition. But high levels of co-stimulatory molecules and MHC class II might provide strong stimulatory signals to overcome the negative signals delivered via PD-L1:PD-1 pathway, resulting in full T cell activation. Recently, the group of Wang *et al.* provided evidence that signaling through PD-L1 expressed on DC is required for the induction of T_{reg} cells (Wang L *et al.,* 2008). The extent of PD-L1 and/or PD-L2 expression for the expansion of T_{reg} cells by immature IL-4 DC and mature S-DC is presently not clear.

9.5 IL-4 DC are able to induce anergic T cells with immune inhibitory properties: implications for the generation of T_{reg} cells

Immunological tolerance is the ultimate goal for the treatment of different immunological diseases such as autoimmunity and transplant rejection. DC, with their well-known immunostimulatory properties, have been found to play a crucial role in induction/maintenance of peripheral T-cell tolerance (Steinman RM *et al.,* 2003). There is growing evidence that interactions between DC and T cells play an important role in the balance between immunity and tolerance. Various mechanisms causing T cell tolerance include induction of T cell apoptosis, anergy and the generation of T_{reg} cells (Abbas AK *et al.,* 2004). It is generally believed that T cell tolerance induced by immature DC is caused by “intermediate” levels of MHC class II and the absence or low expression levels of co-stimulatory molecules on immature DC (Lutz MB *et al.,* 2000; Schwartz RH *et al.,* 2003). Therefore, low expression of co-

stimulatory molecules on immature IL-4 DC (**Figure 8.5**) may allow and contribute to the induction of T cell anergy by immature IL-4 DC.

Several recent studies indicate that immature DC can also promote peripheral T cell tolerance by the induction of regulatory T cell populations (Jonuleit H *et al.,* 2000; Jonuleit H *et al.,* 2001; Min WP *et al.,* 2003; Yamazaki S *et al.,* 2003; Trabell KV *et al.,* 2006; Zhang X *et al.,* 2008). In the present study, the capacity of IL-4 DC to generate or expand T_{reg} cells with the phenotype $CD4^{pos}CD25^{pos}Foxp3^{pos}$ *in vitro* was investigated. IL-4 DC are able to induce the proliferation and therefore the expansion of occurring T_{reg} cells (**Figure 8.27** and **Figure 8.28**), but are unable to generate T_{reg} cells from naïve T cells. This observation is in accordance with results published by Marguti *et al.* showing that an increase in T_{reg} cells by immature DC is mainly attributable to the expansion of naturally occurring T_{reg} cells in lymph nodes (Marguti I *et al.,* 2008).

In addition to DC it is well known that MHC class II is also expressed on activated T cells, enabling them to present peptide antigens to other T cells (Holling TM *et al.,* 2002). This T-T cell interaction results in tolerance (LaSalle JM *et al.,* 1992). In 2006, Baecher-Allan *et al.* demonstrated that MHC class II expression by human $CD4^{pos}CD25^{high}$ T cells identifies a functionally distinct population of mature T_{reg} cells. These cells inhibit T cell proliferation and cytokine production via an early contact-dependent mechanism that is associated with induction of Foxp3 (Baecher-Allan C *et al.,* 2006). In contrast, MHC-$II^{neg}CD4^{pos}CD25^{high}$ T cells did not induce early contact-dependent suppression. Based on these observations the MHC class II expression of T_{reg} cells induced by immature IL-4 DC was examined. This study clearly demonstrates that naturally occurring T_{reg} cells are positive for MHC class II molecule (**Figure 8.27**). Therefore, the MHC class II molecule might be a useful marker for the identification and selection of rat T_{reg} cells inducing early contact-dependent suppression.

There is accumulating evidence that DC maturation can no longer be used to distinguish tolerogenic and immunogenic DC, since the ability of mature DC to expand T_{reg} cells in human and mice has already been demonstrated in several studies (Banerjee DK *et al.,* 2006; Marguti I *et al.,* 2008; Verhasselt V *et al.,* 2004;

Yamazaki S *et al.,* 2003; Yamazaki S *et al.,* 2006). In this study it was also shown that the expansion of T_{reg} cells occurred independently, regardless of the maturation state of rat DC. Both immature IL-4 DC as well as mature S-DC are able to expand T_{reg} cells from naturally occurring T_{reg} cells (**Figure 8.27; Figure 8.28**). It seems that co-stimulatory molecules such as CD80, CD86 and CD40 might not be involved in the expansion of T_{reg} cells. Therefore other factors, possibly soluble factors, might be involved. Yamazaki *et al.* suggested that the ability of mature BM-DC to expand T_{reg} cells by induction of proliferation is dependent on exogenous IL-2 (Yamazaki S *et al.,* 2003). In the periphery, IL-2 signalling seems to be critical for maintaining the survival of T_{reg} cells (Fontenot JD *et al.,* 2005). The results of this study show that the expansion of T_{reg} cells occurs independently of the presence of exogenous IL-2. This has also been observed by Marguti and colleagues (Marguti I *et al.,* 2008). In addition, T cells co-cultured with IL-4 DC secreted reduced levels of IL-2 (**Figure 8.11**). The extent to which IL-2 contributes to the expansion of T_{reg} cells was not analysed in this study and still needs to be investigated.

Purified T cells collected from cultures with immature IL-4 DC were not able to fully respond to stimulatory signals of mature S-DC, whereas T cells isolated from co-cultures with mature S-DC were able to expand in response to mature S-DC (**Figure 8.29 B**). This unresponsiveness of T cells pre-incubated with immature IL-4 DC, however, could be reversed by external addition of IL-2 (**Figure 8.29 B**). These results allow the assumption that immature IL-4 DC induce a reversible state of functional T cell unresponsiveness (T cell anergy). Indeed, Sakaguchi *et al.* reported that the anergic state of T_{reg} cells can be abrogated by IL-2 (Sakaguchi S *et al.,* 2001). DC treated with IL-10 have also been shown to induce anergy in CD4 and CD8 T cells (Steinbrinck K *et al.,* 1999; Jonuleit H *et al.,* 2000).

It has been shown that T_{reg} cells are non-proliferative (anergic) and are able to suppress the activation/proliferation of other T cells when they are co-cultured with antigen presenting cells (Sakaguchi S *et al.,* 2001). For humans and mice, several groups have demonstrated that T cells generated in co-culture with immature DC are able to inhibit T cell responses *in vitro (*Bashuda H *et al.,* 2005; Cools N *et al., 2008;* Gad M *et al.,* 2004; Jonuleit H *et al.,* 2003; Kubsch S *et al.,* 2003; Kuwana M, 2002). Thereby, the suppressor effector function of T_{reg} cells seems to be antigen non-

specific (Thornton AM and Shevach EM, 2000). No information was found in literature that rat immature DC are able to induce anergic T cells with suppressive functions. Therefore, in the present study, the effect of immature IL-4 DC on naive T cells was analysed in more detail. Naive T cells were incubated with immature IL-4 DC for 3 days. Afterwards, the purified T cells were transferred to the proliferation assay consisting of mature S-DC and naïve T cells. T cells pre-incubated with IL-4 DC were able to inhibit mature S-DC-induced T cell proliferation (**Figure 8.30**). In contrast, T cells pre-incubated with mature S-DC inhibited slightly T cell proliferation. This is not unexpected since mature S-DC are able to expand T_{reg} cells; also shown in this study. However, T_{reg} cells expanded by S-DC are not as suppressive as T_{reg} cells expanded by IL-4 DC.

10 Conclusions

The usage of *in vitro* generated autologous DC for immunomodulation is one of the most promising approaches to initiating peripheral tolerance, especially after organ transplantation. In the present study rat bone marrow-derived immature IL-4 DC were generated in the presence of GM-CSF plus IL-4, displaying suppressive properties (inhibition of T cell activation) and tolerogenic functions (expansion of T_{reg} cells). IL-4 DC demonstrate an immature phenotype (MHC class II^{low}, $CD80^{low}$/$CD86^{low}$, IL-12^{neg}) and characteristic effects, e.g. inhibition of S-DC-induced T cell proliferation and expansion of T_{reg} cells (**Table 10.1**).

Table 10.1: Comparison of phenotype and properties of tolerogenic immature IL-4 DC with mature S-DC.

DC properties	IL-4 DC	S-DC
Immature phenotype		
$MHCII^{low}$, $CD80/86^{low}$, ICAM-1^{low}, IL-12^{neg}	Yes	No
Endocytic activity	Yes	No
***In vitro* functions**		
Activation of naïve T cells	No	Yes
Inhibition of proliferation of pre-activated T cells	Yes	No
Inhibition of S-DC induced T cell proliferation	Yes	No
Maturation resistant	Yes	No
Soluble factor(s) with inhibitory properties (TGF-β; NO)	Yes[1)]	Yes[2)]
Expansion of T_{reg} cells	Yes	Yes
Induction of T cell anergy	Yes	No
Induction of T cells with regulatory properties	Yes	No
Expression of inhibitory molecules (PD-L1, PD-L2)	Yes	No data
***In vivo* functions** [*)]		
Prevention of allograft rejection	Yes	No

[1)] presumably TGF-β; [2)] partially NO; [*)] Tiurbe GC *et al.,* 2009

IL-4 DC may mediate these functions by a passive process, e.g. low expression of co-stimulatory molecules, and/or through an active process such as inhibitory

signalling (e.g. through PD-L1 and/or PD-L2), and/or secretion of immune inhibitory cytokine TGF-β.

The results of the present study provide answers to the following questions:

(A) What is the optimal culture condition required for the generation of immature IL-4 DC?

The prerequisite for phenotypic and functional characterization was the high yield generation of IL-4 DC from bone marrow progenitors in the presence of GM-CSF and IL-4. Testing the influence of different cytokine combinations on the yield of Ox-62 positive cells at the end of a 6-day culture period reveals that the addition of 5 ng/ml GM-CSF and 5 ng/ml IL-4 produced the best result with an accept amount of cytokines (**Table 8.1; Figure 8.2**). The results showed that GM-CSF is absolutely necessary for the generation of DC from progenitor cells. However, GM-CSF alone was not able to induce the formation of high cell numbers. The addition of IL-4 to the culture was the key factor for the development of high cell yields and cluster formation. Under the influence of 5 ng/ml GM-CSF and 5 ng/ml IL-4 bone marrow cells started to proliferate and to form cell clusters within 48 hours. On day 6, the end of the culture, the cells developed large clusters loosely attached to the plate. These cells were identified as a homogenous cell population of IL-4 DC (**Figure 8.2**).

(B) What are the main phenotypic characteristics of IL-4 DC?

IL-4 DC demonstrate a phenotype different from that of mature S-DC. At different developmental stages (day 0, 2, 4 and 6) an up-regulation of co-stimulatory molecules CD80, CD86 and CD40, MHC class II and OX62 could be seen (**Figure 8.3**), in addition to an expression of CD45 and CD25 on day 6 (**Figure 8.4**). However, the expression of all investigated molecules was significantly lower compared to their expression on mature S-DC (**Figure 8.4** and **Figure 8.5**). The reduced expression of CD80, CD86 and CD40 was confirmed by real time RT-PCR (**Figure 8.8**). In addition, IL-4 DC did not express mRNA specific for IL-12 and demonstrated endocytic activity (**Figure 8.6** and **Figure 8.9**). In summary, these results confirm an immature phenotype of IL-4 DC on day 6 of the culture.

(C) What is the effect of IL-4 DC on naïve T cells *in vitro*?

The present study shows that immature IL-4 DC were not able to activate naïve T cells (**Figure 8.10**). This induction of hyporesponsiveness was associated with increased levels of secreted TGF-β and reduced levels of IFN-γ and IL-2 (**Figure 8.11**). Immature IL-4 DC were able to inhibit S-DC-induced T cell proliferation dependent on the number of IL-4 DC added to the proliferation assay (**Figure 8.13**). This strong inhibitory effect was mediated within 24 hours (**Figure 8.14**). The inhibition of mature S-DC-induced T cell proliferation by immature IL-4 DC might be partly caused by soluble factors (**Table 8.8; Figure 8.22**), since the supernatant of immature IL-4 DC showed a clear suppressive effect (**Figure 8.18**). The presence of the immune regulatory cytokine TGF-β was proofed in these supernatants (**Figure 8.19**).

Naïve T cells pre-incubated with immature IL-4 DC were not able to proliferate in the presence of mature S-DC (induction of an anergic state). This anergic state was reversible by the addition of exogenous IL-2 (**Figure 8.29**). In addition, T cells pre-incubated with IL-4 DC inhibited the mature S-DC induced T cell proliferation, demonstrating that immature IL-4 DC are able to induce T cells with immune inhibitory functions (**Figure 8.30**).

(D) Do IL-4 DC exhibit a stable immature phenotype?

To achieve full activation of T cells, DC need to undergo a process of functional maturation. DC maturation can be triggered by LPS, CD40L and TNF-α, for example. In this study immature IL-4 DC were found to be resistant to maturation. They kept their inability to activate naïve T cells (**Figure 8.17**) and failed to up-regulate the co-stimulatory molecules CD80, CD86 and CD40, MHC class II and ICAM-1 (**Figure 8.15** and **8.16**).

(E) Are IL-4 DC able to generate T_{reg} cells from naïve T cells *in vitro*?

Immature IL-4 DC were able to increase the percentage of T_{reg} cells with the phenotype $CD4^{pos}CD25^{pos}Foxp3^{pos}$ (**Figure 8.27**). This increase came from the expansion of naturally occurring T_{reg} cells rather than from the generation of naïve T cells. In addition, immature IL-4 DC induced an anergic state in T cells that could be reversed by adding exogenous IL-2 (**Figure 8.29**). Furthermore, these T cells were able to inhibit S-DC-induced T cell proliferation *in vitro,* demonstrating a regulatory effect of IL-4 DC on naïve T cells during co-culture (**Figure 8.30**).

In this study we demonstrated that not only immature IL-4 DC but also mature S-DC are able to expand T_{reg} cells *in vitro*. Based on these results, we conclude that the induction of tolerance and immunity can no longer be distinguished by the phenotype of DC.

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12 Appendix

Table 12.1: Increase of different surface antigens over time. Cells were collected at different developmental stages (day 0, 2, 4 and 6). Differences in the cell surface expression were quantified by mean fluorescence (MF) values of antibody binding. In addition, percentage (%) of positive cells (P= Proportion) is shown. Data of three different experiments are shown. (x) not analysed.

Antigen	day 0		day 2		day 4		day 6	
#1	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)
MHC II	1.39	1.34	11.1	10.3	43.3	40.4	119	84.6
OX62	8.23	2.74	29.7	23.4	60.7	51.8	119	73.4
CD80	5.89	12.0	11.6	12.8	53.9	48.6	114	70.7
CD86	7.14	5.22	10.2	11.5	39.2	45.5	133	77.7
CD40	28.1	10.9	68	10.7	190	37.5	172	61.9

Antigen	day 0		day 2		day 4		day 6	
#2	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)
MHC II	1.76	0.31	31.7	13.2	49.6	39.6	115	79.7
OX62	20.3	5.68	23.9	15	51.4	45.4	212	85.3
CD80	4.61	0.69	18.8	19.7	93.8	57.3	156	77.5
CD86	6.42	1.92	21	18.9	59.7	46.5	129	81.5
CD40	27.7	2.45	97	16.8	157	42.3	188	66.8

Antigen	day 0		day 2		day 4		day 6	
#3	MF	P (%)	MF	P (%)	MF	P (%)	MF	P (%)
MHC II	1.21	0.47	18.2	15	57.6	60.9	123	73.1
OX62	30.5	2.49	110	23.5	237	72.6	X	X
CD80	3.51	4.45	20.6	20.8	98	63.1	126	56.7
CD86	4.58	2.62	10.9	14.9	66.4	52.3	108	54.1
CD40	8.13	3.21	65.6	13.1	196	47.6	231	52.8

Table 12.2: IL-4 DC demonstrate endocytic activity. IL-4 DC show increased Dextran-Alexa 488 up-take compared to mature S-DC. The uptake at 37 °C versus uptake at °4 C (control). The results depict $MF_{(37-4°C)}$. Data of three different experiments are shown.

IL-4 DC		S-DC		control
$MF_{(37°C)}$	$MF_{(37°C-4°C)}$	$MF_{(37°C)}$	$MF_{(37°C-4°C)}$	$MF_{(4°C)}$
351	314	37	6.4	30.6
212	194.9	14.4	-2.7	17.1
101	92.8	11.8	3.61	8.19

Table 12.3: Analysis of T cell apoptosis by staining with Annexin V. T cells cultured with mature S-DC alone showed the highest rate of apoptosis. Data of three different experiments are shown.

	T cells alone	T cells + S-DC	T cells + S-DC + NMMA	T cells + IL-4 DC	T cells + IL-4 DC + NMMA	cells + S-DC + IL-4 DC	T cells + S-DC + IL-4 DC + NMMA
apoptotic T cells (%)	9.14	18.2	9.38	12.1	1.7	13.2	9.83
	3.6	8.26	6.06	5.72	4.93	6.23	4.82
	8.44	14.3	11.6	10.8	7.38	13.6	7.75

Table 12.4: Phenotypic changes of IL-4 DC after stimulation with LPS, TNF-α or CD40L. IL-4 DC were collected at different developmental stages [day 6, day 8 and day 10 (with addition of TNF-α, LPS or CD40L on day 8)]. Differences in the cell surface expression were quantified by mean fluorescence (MF) values of antibody binding. In addition percentage (%) of positive cells (P= Proportion) is shown. Data of three different experiments for each cytokine are shown.

Developmental stage		MHCII		CD80		CD86		CD40		ICAM-1	
		MF	P(%)	MF	P(%)	MF	P(%)	MF	P(%)	MF	P(%)
Day 6	#1	315	26	22.5	21.7	159	66.1	108	64.9	15.5	15.8
	#2	82.1	54.4	63.1	81.8	278	93.5	22.1	6.38	35.6	36.3
	#3	-	-	-	-	-	-	-	-	-	-
Day 8 *)	#1	135	51	75.6	48.5	274	80.3	80.9	20.6	50	59.7
	#2	327	79.7	103	65.6	129	85.5	97.5	70.6	63.6	95.2
	#3	182	51	131	43.7	250	80.5	255	55.7	62.7	75.8
Day 10 **) (+TNF-α)	#1	888	59.8	122	68.4	292	81.6	215	47.4	341	84.3
	#2	568	88.6	103	86.5	123	97.2	206	80.4	256	95
	#3	288	70.5	253	65.9	366	85.7	315	73.7	182	79.5
Day 6	#1	147	33.8	22.1	57.8	108	91.9	6.03	7.02	24	81.2
	#2	134	51.6	37.2	51.7	181	82.9	53	38.5	39.8	82.9
	#3	407	87	93.3	79.5	250	91.5	74.5	61	63.8	85.9
Day 8	#1	97.5	34.1	45.6	73.9	214	94.5	17.3	21.6	29.9	75.4
	#2	173	59.6	88	87.3	216	95.4	73.6	48.2	69.9	93
	#3	254	77.1	126	54	479	90.7	84.6	47.8	67.7	87.7
Day 10 (+LPS)	#1	158	33.7	78.7	31.4	144	68.3	259	72.8	68.7	54.7
	#2	295	63.9	139	77.8	248	92.4	207	62.9	104	75.4
	#3	227	51.7	446	47.4	715	62	73.6	79.8	168	68.1
Day 6	#1	280	65.6	28.9	61.4	148	80.6	9.98	17.5	25.8	74.5
	#2	67	38.6	22.3	58.6	99.1	79.8	28.9	11.6	24.7	69.8
	#3	220	78.7	69	59	127	72.5	54.9	48.6	58.7	78.8
Day 8	#1	153	47.4	158	85.6	39.2	51.6	142	46.4	38.8	69.8
	#2	111	40.2	28.2	59.6	187	93.4	68.9	41.4	37	72.2
	#3	303	84.9	110	66.4	171	73.1	101	55.1	65.5	87
Day 10 (+CD40L)	#1	486	80.5	195	23.5	224	30.8	47.3	48.2	69.2	91.2
	#2	161	45.9	62.8	22.3	170	64.2	86.1	27.4	52	56.3
	#3	363	79.3	233	70	321	81.5	73.7	42.4	89.6	80.6

*) IL-4 DC were replated on day 6 and cultured until day 8 in the presence of 2.5 ng/ml GM-CSF (see materials and methods)
**) IL-4 DC were replated on day 8 and cultured until day 10 in the presence of TNF-α (20 ng/ml), LPS (1µg/ml) or CD40L (1µg/ml) (see materials and methods).

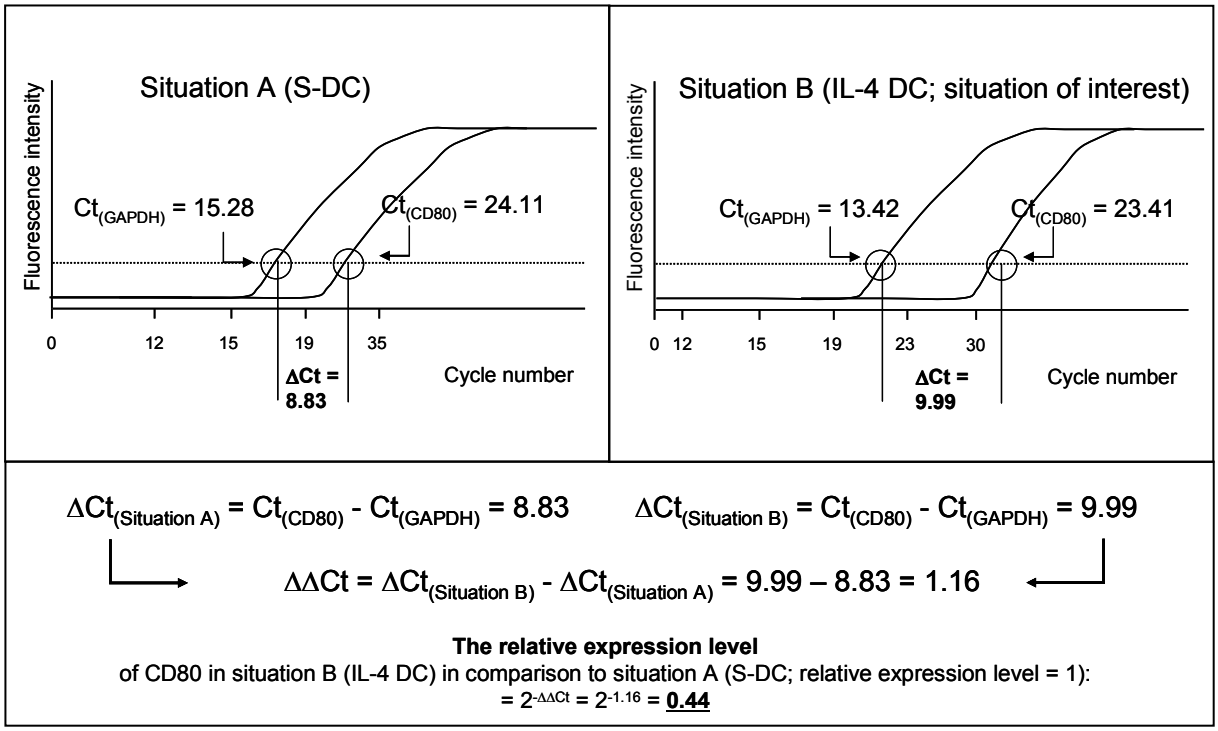

Figure 12.1: Calculation of the relative expression level of CD80 in immature IL-4 DC compared to mature S-DC: an example for calculation of relative expression levels by using the $2^{-\Delta\Delta Ct}$ method. Calculation steps: ΔCt = Ct(CD80) - Ct(GAPDH), ΔΔCt = ΔCt(IL-4 DC) - ΔCt(S-DC), relative expression level = $2^{-\Delta\Delta Ct}$. Expression level of CD80 mRNA is 2.3-fold reduced compared to expression level of CD80 mRNA in mature S-DC.

Cell type	RT-PCR	GAPDH	CD40	CD80	CD86	MHC II	IL-12	TGF-β	IL-10	iNOS	IL-6	TNF-α	TLR-4	IL-18
IL-4 DC	06.06.06	+	+	–	–	+	X	X	X	X	X	X	X	X
IL-4 DC	16.08.06	+	+	–	+	+	X	X	X	X	X	X	X	X
IL-4 DC	28.08.06	+	+	+/-	+	–	X	X	X	X	X	X	X	X
IL-4 DC	21.09.06 *)	+	+	+	+	+	X	X	X	X	X	X	X	X
IL-4 DC	21.09.06 *)	+	+	+	+	+	X	X	X	X	X	X	X	X
IL-4 DC	12.10.06 *)	+	+	+	+	+	X	X	X	X	X	X	X	X
IL-4 DC	12.10.06 *)	+	+	+	+	+	X	X	X	X	X	X	X	X
IL-4 DC	07.11.06	+	+	+	+	+	X	X	X	X	X	X	X	X
IL-4 DC	09.11.06	+	+	+	+	+	X	X	X	X	X	X	X	X
IL-4 DC	17.04.07	+	+	+	+	+	–	X	X	X	X	X	X	X
IL-4 DC	28.06.07	+	+	+/-	+	+	–	X	X	X	X	X	X	X
IL-4 DC	03.07.07	+	X	X	X	X	X	X	X	X	+/-	+/-	+	X
IL-4 DC	12.07.07	+	+	–	+	+	X	+	+	+/-	X	X	X	+
IL-4 DC	06.08.07	+	X	X	X	X	–	X	+/-	+/-	X	X	X	+
IL-4 DC	13.08.07	+	X	X	X	X	–	X	+	–	X	X	X	+/-
IL-4 DC	17.08.07	+	+	–	+/-	X	X	+	+/-	X	X	X	X	X
IL-4 DC	29.08.07	+	+/-	+/-	+/-	+	X	+	+/-	X	X	X	X	X

Table 12.5: RT-PCR analysis of IL-4 DC. As shown in Figure 8.6 specific mRNA for of co-stimulatory molecules CD40, CD80 and CD86 and MHC class II was proved by agarose gel analysis. (+) Signal; (–) No signal; (+/-) low signal; (x) not analysed.

*) RT-PCR of different cell preparations was performed on the same day

Cell type	RT-PCR	GAPDH	CD40	CD80	CD86	MHC II	IL-12	TGF-β	IL-10	iNOS	IL-6	TNF-α	TLR-4	IL-18
S-DC	01.08.06	+	+	+	+	+	X	X	X	X	X	X	X	X
S-DC	21.08.06	+	+	+/-	+	+	X	X	X	X	X	X	X	X
S-DC	21.09.06	+	+	+	+	+	X	X	X	X	X	X	X	X
S-DC	04.1006	+	+	+	+	+/-	X	X	X	X	X	X	X	X
S-DC	19.04.07	+	+	+/-	+/-	+	+/-	X	X	X	X	X	X	X
S-DC	28.06.07 *)	+	+	+/-	+	+	+/-	X	X	X	X	X	X	X
S-DC	28.06.07 *)	+	+	+/-	+	+/-	+/-	X	X	X	X	X	X	X
S-DC	28.06.07 *)	+	+	+	+	+	+/-	X	X	X	X	X	X	X
S-DC	28.06.07 *)	+	+	–	+	+	+/-	X	X	X	X	X	X	X
S-DC	03.07.07 *)	+	X	X	X	X	X	X	X	X	+	+	+/-	X
S-DC	03.07.07 *)	+	X	X	X	X	X	X	X	X	+	+/-	+	X
S-DC	03.07.07 *)	+	X	X	X	X	X	X	X	X	+	+	+	X
S-DC	08.08.07	+	+	+	+	+	+	+	+/-	+/-	X	X	X	+/-
S-DC	14.08.07	+	X	X	X	X	+	X	–	+	X	X	X	+/-
S-DC	17.08.07	+	+	–	+	X	X	+/-	–	X	X	X	X	X
S-DC	29.08.07	+	+/-	+/-	+	+	X	+	–	X	X	X	X	X
S-DC	15.11.07	+	X	+	+	+	X	+	X	X	X	X	X	+/-

Table 12.6: RT-PCR analysis of S-DC. As shown in Figure 8.6 specific mRNA for of co-stimulatory molecules CD40, CD80 and CD86 and MHC class II was proved by agarose gel analysis. (+) Signal; (–) No signal; (+/-) low signal; (x) not analysed.

*) RT-PCR of different cell preparations was performed on the same day

	CD80 Ct			GAPDH Ct			CD80 ΔCt			CD80 mean Δ Ct	CD80 ΔΔCt	CD80 relative expression level
S-DC	24.11	26.79	25.66	15.28	17.37	15.66	8.83	9.42	10.00	9.41	---	1
IL-4 DC	23.41	32.05	26.50	13.42	22.98	13.62	10.00	9.07	12.88	10.65	1.23	0.43
	CD86 Ct			**GAPDH Ct**			**CD86 ΔCt**			**CD86 mean Δ Ct**	**CD86 ΔΔCt**	**CD86 relative expression level**
S-DC	13.748	21.80	18.67	15.28	17.37	15.66	-1.54	4.43	3.01	1.97	---	1
IL-4 DC	15.05	---	21.93	13.42	22.98	13.62	1.63	---	8.31	4.97	3.00	0.13
	CD40 Ct			**GAPDH Ct**			**CD40 ΔCt**			**CD40 mean Δ Ct**	**CD40 ΔΔCt**	**CD40 relative expression level**
S-DC	19.41	21.89	18.92	15.28	17.37	15.66	4.13	4.52	3.27	3.97	---	1
IL-4 DC	20.10	30.28	19.90	13.42	22.98	13.62	6.68	7.30	6.28	6.76	2.79	0.14
	MHC II Ct			**GAPDH Ct**			**MHC II ΔCt**			**MHC II mean Δ Ct**	**MHC II ΔΔCt**	**MHC II relative expression level**
S-DC	17.70	23.25	22.06	15.28	17.37	15.66	2.42	5.88	6.40	4.90	---	1
IL-4 DC	14.10	33.49	17.05	13.42	22.98	13.62	0.68	10.51	3.43	4.87	-0.003	1.02

	IL-12 Ct			GAPDH Ct			IL-12 ΔCt			IL-12 mean Δ Ct	IL-12 ΔΔCt	IL-12 relative expression level
S-DC	27.71	29.93	26.68	16.29	17.29	16.54	11.41	12.64	10.15	11.40	---	1
IL-4 DC	31.71	31.67	33.11	14.21	15.04	22.54	17.50	16.62	10.57	14.90	3.50	0.009
	TGF-β Ct			**TGF-β Ct**			**TGF-β ΔCt**			**TGF-β mean Δ Ct**	**TGF-β ΔΔCt**	**TGF-β relative expression level**
S-DC	17.33	21.31	19.22	15.28	17.37	15.66	2.05	3.94	3.56	3.18	---	1
IL-4 DC	15.41	27.57	16.32	13.42	22.98	13.62	1.99	4.95	2.70	3.09	-0.09	1,06

Table 12.7 (see also next page): The real time RT-PCR quantification of MHC class II, IL-12, TGF-β and co-stimulatory molecules CD80, CD86 and CD40. Total RNA, cDNA, and Real-Time PCR amplification were performed as described in methods and materials. Relative expression level of target mRNA in immature IL-4 DC was compared to that of mature S-DC. Shown are the Ct values (of three different experiments), the Δ Ct values, the mean of the ΔCt values, the ΔΔCt values and the relative expression level ($2^{-\Delta\Delta Ct}$). GAPDH mRNA served as endogenous control. For calculation details see also **Figure 12.1**.

Table 12.8: Expansion of $CD4^{pos}CD25^{pos}Foxp3^{pos}$ T cells after co-culture with DC. **Upper rows:** T cell populations before and after depletion or purification of $CD4^{pos}CD25^{pos}$ T cells are shown. **Lower rows:** 10^4 immature IL-4 DC or mature S-DC were co-cultured with 10^5 total lymph node T cells, 10^5 naïve T cells depleted of $CD4^{pos}CD25^{pos}$ T cells or with purified 10^5 $CD4^{pos}CD25^{pos}$ T cells. After 3 days the cells were collected and after depletion of DC labelled with fluorescent antibodies for CD4, CD25 and Foxp3. All cells are gated on $CD4^{pos}$ cells. 3 different experiments are shown.

	T cells cultured with DC								
	Total lymph node cells			$CD4^{pos}CD25^{neg}$ (after depletion of $CD4^{pos}CD25^{pos}$ T cells)			$CD4^{pos}CD25^{pos}$ (after purification of $CD4^{pos}CD25^{pos}$ T cells)		
Experiment No.[*)]	**1**[**)]	**2**[***)]	**3**[**)]	**1**	**2**	**3**	**1**	**2**	**3**
$CD4^{pos}CD25^{pos}$ (%)	6.02	5.6	9.87	1.3	2.91	5.08	35.3	36.7	62.3
$CD4^{pos}CD25^{neg}$ (%)	38.9	38.6	56.8	39.5	81.3	49	51.2	28	33
MLR condition	**$CD4^{pos}CD25^{pos}Foxp3^{pos}$ T cells (%)**								
T cells alone (control)	7.84	6.02	7.02	5.22	5.41	5.33	17.2	31.7	35.8
T cells + S-DC	10.7	10.8	12.9	6.46	6.71	5.68	26	51.1	62.5
T cells + IL-4 DC	17.6	15.8	16.5	7.26	7.87	7.08	34.1	55.2	63.1

*) repetition of three independent experiments
**) Cellection™ Pan Mouse IgG Kit (see methods and materials)
***) depletion and purification by FACS sorting (see methods and materials)

Table 12.9: Release of NO and IL-10 into the supernatant of mature S-DC and immature IL-4 DC. Immature IL-4 DC from day 6 of culture and purified mature S-DC were transferred to 96-well culture plates (10^6 cells/well). The supernatant was collected 24 and 48 hours later. Data are presented as means ± S.D. of 3 independent experiments.

	S-DC		IL-4 DC	
NO (NO_2^-)[*)]	**24hr**	**48hr**	**24hr**	**48hr**
concentration (µM)	3.09 ± 1.98	3.80 ± 2.30	1.55 ± 1.05	5.32 ± 4.67
IL-10	**24hr**	**48hr**	**24hr**	**48hr**
concentration (pg/ml)	10.37 ± 6.03	9.39 ± 8.61	2.17 ± 1.98	4.18 ± 3.23

*) The NO release was measured as the accumulation of the stable end product NO_2^- (see methods and materials).

Table 12.10: The supernatant from 10^4 IL-4 DC does not inhibit T cell proliferation in the proliferation assay. Different volumes (25 µl, 50 µl and 100 µl) the supernatant of 24 hour cultured IL-4 DC (10^4) had no effect on mature S-DC induced T cell proliferation. The supernatant of mature S-DC or media cultured without DC served as a control to exclude possible negative effects of day 2 supernatant. Data are presented as means ± S.D. of 3 independent experiments.

	Volume of supernatant (µl) *)			
	25	**50**	**100**	**0**
IL-4 DC	39,172 ± 3977 **)	41,872 ± 3572	34,667 ± 3507	42,984 ± 4,991
S-DC	-	-	36,353 ± 7476	
media*)**	-	-	43,754 ± 5551	

*) The final volume was kept constant at 150 µl per well.
**) Results (mean ± S.D.) of T cell proliferation in cpm.
***) media cultured for 24 hours without the addition of DC.

13 Index of Figures

14 Index of Tables

Original scientific publications

Tiurbe G.C., **Matuschek A.,** Kämmerer U., Schneider M., Ulrichs K., Thiede A., Otto C. Inhibitory properties of rat bone marrow derived dendritic cells on naïve and alloantigen-specific CD4+ T cells *in vitro* and *in vivo*: a comparison of dendritic cells generated with GM-CSF plus IL-4 and GM-CSF plus IL-10. *BMC Res Note.* 2009; 23;2:12.

Matuschek A., Ulbrich M., Timm S., Schneider M., Thomas Germer C., Ulrichs K., Otto C. Analysis of parathyroid graft rejection suggests alloantigen-specific production of nitric oxide by iNOS-positive intragraft macrophages. *Transpl Immunol.* 2009; 21(4):183-91.

Poster presentations

24-26/07/2006 **6th Joint Retreat Graduiertenkolleg 520/1 „Immunmodulation“ in Markt Taschendorf**

Matuschek A., Otto C. “The use of tolerogenic dendritic cells in transplantation medicine: Useful for tolerance induction?”

19-20/10/2006 **15th Annual Meeting of the “Deutsche Transplantationsgesellschaft e.V.” (DTG), Munich, Germany**

Matuschek A., Tiurbe G.C., Ulrichs K. , Otto C. “ Allo-antigen presenting dendritic cells (DC) of the transplant recipient regulate the allo-immune response and prolongate transplant rejection”

Matuschek A., Otto C., Cross J., Johnson P. „Untersuchungen zur Rolle von CD45 auf die Funktion von Makrophagen”

11-13/10/2007 **16th Annual Meeting of the “Deutsche Transplantationsgesellschaft e.V.” (DTG), Mainz, Germany**

Matuschek A., Ulbrich M., Timm S., Schneider M., Thomas Thiede A., Ulrichs K., Otto C. “Evidence for a cross-talk between CD4+ T lymphocytes and macrophages inducing nitric oxide production by macrophages during parathyroid allograft rejection”

26-27/06/2008 **14th N.A.T. - Innate immunity and inflammation in transplantation, Nantes, France**

Matuschek A., Tiurbe G.C., Thiede A., Ulrichs K., Otto C. “Inhibitory properties of rat bone marrow-derived dendritic cells: evidence that reduced surface expression of co-stimulatory molecules and not the release of inhibitory molecules are responsible for T-cell inhibition”

03-06/09/2008 **Joint Annual Meeting of the Austrian and German Immunology Societies (ÖGAI, DGfI), Wien, Austria**

Matuschek A., Tiurbe G.C., Thiede A., Ulrichs K., Otto C. “Inhibitory properties of rat bone marrow-derived dendritic cells: evidence that reduced surface expression of co-stimulatory molecules and not the release of inhibitory molecules are responsible for T-cell inhibition”

20-22/11/2008 **17th Annual Meeting of the "Deutsche Transplantationsgesellschaft e.V." (DTG), Bochum, Germany**

Matuschek A., Tiurbe G.C., Thiede A., Ulrichs K., Otto C. "Evidence that tolerogenic bone marrow-derived rat dendritic cells promote T cell hyperresponsiveness by suboptimal co-stimulation and not by release of inhibitory molecules"

Oral scientific talks

19-20/10/2006 **15th Annual Meeting of the "Deutsche Transplantationsgesellschaft e.V." (DTG), Munich, Germany**

Matuschek A., Otto C., Cross J., Johnson P. "Investigations to the role of CD45 on macrophages: Determining the effect of the loss of CD45 on macrophage function"

20-21/04/2007 **Annual Meeting of the study group "Transplantation Immunology"; part of German Society of Immunology (DGfI), Leipzig, Germany**

Matuschek A., Otto C., Cross J., Johnson P. "Investigations to the role of CD45 on macrophages: Determining the effect of the loss of CD45 on macrophage function"

14-16/05/2007 **1st Graduate College-Network Meeting, Kloster Banz, Germany**

Matuschek A., Otto C. "Characterization of bone marrow-derived rat dendritic cells and their potential to modulate the allogenic immune response"

11-13/10/2007 **16th Annual Meeting of the "Deutsche Transplantationsgesellschaft e.V." (DTG), Mainz, Germany**

Matuschek A., Ulbrich M., Timm S., Schneider M., Thomas Thiede A., Ulrichs K., Otto C. "Evidence for a cross-talk between CD4+ T lymphocytes and macrophages inducing nitric oxide production by macrophages during parathyroid allograft rejection"

16-17/05/2008 **Annual Meeting of the study group "Transplantation Immunology"; part of German Society of Immunology (DGfI), Hannover, Germany**

Matuschek A., Ulbrich M., Timm S., Ulrichs K., Thiede A., Otto C. "Evidence for a cross-talk between CD4+ T lymphocytes and macrophages inducing nitric oxide production by macrophages during parathyroid allograft rejection"

06-08/07/2008 **2nd Graduate College-Network Meeting Wildbad, Rothenburg ob der Tauber, Germany**

Matuschek A., Otto C. "Tolerogenic rat dendritic cells and regulatory T cells: their potential to modulate the allogenic immune response"

National and international congresses and workshops

24-26/07/2006	6th Joint Retreat of the Graduate College 520/1 „Immunmodulation“, Markt Taschendorf, Germany
19-20/10/2006	15th Annual Meeting of the “Deutsche Transplantationsgesellschaft e.V.” (DTG), Munich, Germany
20-21/04/2007	Annual Meeting of the study group “Transplantation Immunology“, Leipzig, Germany
14-16/05/2007	1st Graduate College-Network Meeting, Kloster Banz, Germany
11-13/10/2007	16th Annual Meeting of the “Deutsche Transplantationsgesellschaft e.V.” (DTG), Mainz, Germany
16-17/05/2008	Annual Meeting of the study group “Transplantation Immunology“, Hannover, Germany
26-27/06/2008	14th N.A.T. - Innate immunity and inflammation in transplantation, Nantes, France
06-08/07/2008	2nd Graduiertenkolleg-Network Meeting Wildbad, Rothenburg ob der Tauber, Germany
03-06/09/2008	Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), Wien, Austria

Awards and scholarships

19-20/10/2006	Poster Award of the “Deutsche Transplantationsgesellschaft” (DTG) in Munich “Investigations to the role of CD45 on macrophages: Determining the effect of the loss of CD45 on macrophage function”
12/2007	Young Scientist Award of the University of Würzburg
07/2006-06/2009	Scholarship of the “Deutsche Forschungsgemeinschaft” (DFG) within the Graduate College “Immunomodulation” (GK520)
10/2009-02/2010	Scholarship of the University of Würzburg for funding the final phase of the conferral of a doctorate

Curriculum Vitae

Name:	Anja Matuschek
Date of birth:	19.03.1980
Place of birth:	Lauchhammer
Address:	Zeppelinstr. 1, Potsdam
Family status:	single
Citizenship:	German
Current academic rank:	Master of sciences (MSc.) in biotechnology

Education

1986-1993	Primary school Schwarzheide
1993-1999	Secondary school (Emil-Fischer), Schwarzheide qualification: Abitur
1999-2000	Au-pair (USA)
2000-2006	Fachhochschule Lausitz - Lausitz University of Applied Sciences, Senftenberg
09/2000-02/2004	Bachelor of Science program: Biotechnology
09/2002-02/2003	Internship at the ”BioAssays-Diagnostica-Entwicklungsgesellschaft mbH“ (BRAHMS AG), Hennigsdorf (Berlin)
09/2003-02/2004	Bachelor Thesis at the ”Institut für Biotechnologie“, Thurgau (Switzerland), University of Konstanz
28/02/2004	Bachelor´s degree: Bachelor of Science (BSc.)
03/2004-01/2006	Master of Science program: Biotechnology
03/2005-12/2005	Master Thesis at the Department of Microbiology & Immunology, University of British Columbia, Vancouver, Canada
23/01/2006	Master´s degree: Master of Science (MSc.)
06/2006-03/2010	Dissertation: research group Experimental Surgery, Experimental Transplantation Immunology, Clinic of General, Visceral, Vascular, and Paediatric Surgery (Surgical Clinic I), University of Würzburg Hospital

Würzburg, 2010

Anja Matuschek

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Affidavit
(Eidesstattliche Erklärung)

I hereby declare that my thesis entitled:

“Characteriztion of tolerogenic rat bone marrow-derived dendritic cells and regulatory T cells“

„Charakterisierung tolerogener dendritischer Knochenmarkszellen und regulatorischer T-Zellen aus der Ratte“

is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as a part of another examination process, neither in identical nor in similar form.

Würzburg,