Aus der Chirurgischen Klinik und Poliklinik der Universität Würzburg
Chirurgische Klinik I
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Charakterisierung unreifer dendritischer Zellen aus dem Knochenmark der Ratte: Untersuchungen zum Phänotyp und zur Immunmodulation *in vitro* und nach Organtransplantation

Characterization of immature rat bone marrow-derived dendritic cells: Evaluation of their phenotype and immunomodulatory properties *in vitro* and after organ transplantation

Inaugural-Dissertation
zur Erlangung der Doktorwürde
der Medizinischen Fakultät
der Bayerischen Julius-Maximilians-Universität zu Würzburg

vorgelegt von
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Würzburg, Juli 2006

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Tag der mündlichen Prüfung: 19.12.2006

Der Promovend ist Arzt

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1 Introduction

Transplantation of solid organs is a frequent procedure in modern medicine and represents the therapy of choice for end-stage organ failure. Transplantation between genetically different individuals induces a rapid and destructive immune response, which, in the absence of immunosuppression, leads to graft destruction. This type of immune response, called rejection, is primarily driven by the ability of alloreactive host T cells to recognize the polymorphism encoded within the molecules of the major histocompatibility complex or MHC.

Major improvements in surgical techniques, MHC matching and immuno-suppressive drugs have increased the one-year survival rate for most solid organ grafts to over 90% (Hariharan S et al., 2000). The success of clinical transplantation depends largely on the successful suppression of rejection. Although different immunosuppressive drugs found access into the clinic in the past two decades, most of them cause severe side-effects. Drug-related adverse effects (nephrotoxicity, diabetes and hyperlipidaemia) and a reduced immunity to infections and malignant diseases are caused by the life-long immunosuppression required to minimize the risk of allograft rejection (Dowling JN et al., 1976). However, an alternative to the immunosuppression does not exist presently in the clinic.

The experimental transplantation immunology searches for alternatives to the life-long immunosuppression (Strober S et al., 2000). Strategies for the suppression of the allograft rejection in an antigen-specific manner are preferred (Fändrich F et al., 2004). This would help to inhibit only the unwanted immune responses directed towards the transplant and maintain the beneficial immune responses towards infections and malignancies. The great perspective is that the patients will be independent of life-long immunosuppression. Dendritic cells, the most important antigen-presenting cells of the immune system which initiate and regulate immune responses, can be of great importance for tolerogenic

strategies after transplantation due to their ability to induce antigen-specific unresponsiveness (Banchereau J et al., 2000; Coates PT et al., 2002).

1.1 An overview of allorecognition and graft rejection

The reason for allograft rejection is that the immune system of the recipient is activated by allogeneic MHC molecules expressed on the allograft. Alloreactive T cells recognize the differences in the MHC molecules between donor and recipient and induce a complex immune response which includes all components of the innate and adaptive immune system. The capital importance of alloreactive T cells in this process was demonstrated in different T cell-deficient animal models (Rosenberg AS and Singer A, 1992). Alloreactive T cells recognize allogeneic MHC antigens via two distinct mechanisms (Rogers NJ and Lechler RI, 2001): the *direct pathway* and the *indirect pathway* of allorecognition (**Figure 1.1**).

The direct pathway of allorecognition

The direct recognition of allogeneic MHC class I and class II molecules on the surface of donor-derived, so-called passenger leukocytes, by host T cells results in the generation of cytotoxic and helper T cells (Lechler R and Batchelor JR, 1982). These passenger leukocytes are transferred together with the transplanted organ into the recipient where some of them, for example dendritic cells, act as professional antigen-presenting cells. In the case of allogeneic transplantation (transplantation between non-identical individuals) all these cells express MHC molecules which are foreign or allogeneic to the recipient's immune cells.

The importance of donor-derived dendritic cells for the direct activation of alloreactive T cells was demonstrated by different experiments, where their selective depletion increased allograft survival (Game DS and Lechler RI, 2002). The molecular reason for the direct recognition of allogeneic MHC molecules is interpreted as a cross-reaction of certain subtypes of T cell receptors. These receptors, specific for a certain combination of self-MHC and

peptide, also recognize allogeneic MHC molecules (Shirwan H et al., 1995). Recent structural analysis of an alloreactive T cell receptor confirmed that it interacts with an allogeneic MHC/peptide complex in a mode similar to its interaction with self-MHC molecules (Reiser JB et al., 2000). The conesquence of an allogeneic situation, where both presented peptides and presenting MHC molecules are foreign, is that all MHC/peptide combinations on the surface of a donor-derived dendritic cell (more than 10^5 molecules) are potential targets for alloreactive T cells independent of the presented peptide. Between 0.1% and 10% of an individual's T cell repertoire are capable of directly recognising allgeneic MHC molecules (Suchin EJ et al., 2001). This is a very high percentage when compared to the less than 0.001% of T cells responding to a "normal" non-allogeneic peptide (Karulin AY et al., 2000). The very high frequency of alloreactive T cells appears to be one reason for the extraordinary strength of the polyclonal alloimmune response mediated via the direct pathway of allorecognition.

The indirect pathway of allorecognition

In the *indirect pathway* of allorecognition, allogeneic MHC molecules are recognized as conventional peptide antigens by alloreactive T cells (**Figure 1.1**). This pathway became the focus of research early in the 1980s and it was shown to be the results of self-MHC-restricted presentation of donor MHC peptides. These peptides are processed and presented by antigen-presenting cells of the recipient, mainly by dendritic cells. The percentage of alloreactive T cells primed by the indirect pathway of allorecognition is about 100 times lower than that of T cells participating in the direct pathway (Liu Z et al., 1993; Benichou G, 1999). Although the number of responder T cells is much lower than in the direct allorecognition, this pathway is very important for the process of allograft rejection. It was clearly shown that the antigen recognition through this pathway induces allograft rejection (Auchincloss H et al., 1993; Preston EH et al., 2003). Recent studies have demonstrated that this pathway is implicated in the chronic rejection of transplanted organs (Shoskes DA and Wood KJ, 1994).

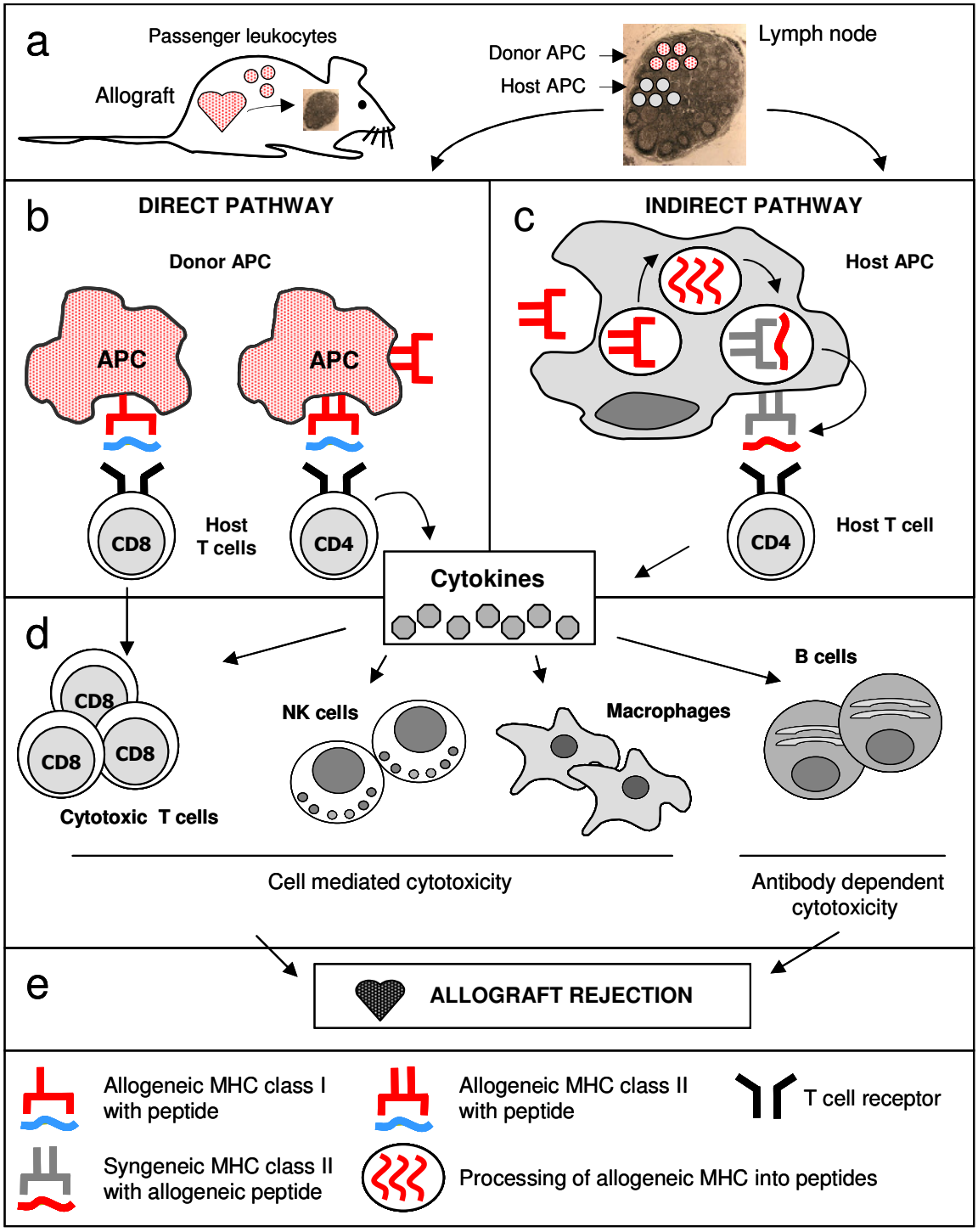


Figure 1.1: ***Characteristics of the alloimmune response induced by the allograft in local lymph nodes.*** (**a**) Host T cells can be activated in response to an allogeneic stimulus by the direct or indirect allorecognition. In the direct pathway, alloreactive host T cells recognize intact donor MHC molecules on the surface of donor-derived passenger leukocytes (antigen-presenting cells or APC). (**b**) In the indirect pathway, host T cells respond to processed donor-derived allogeneic peptides bound to syngeneic or self-MHC molecules. (**c**) Particularly CD4+ T cells activate different cells of the innate and adoptive immune system over cytokines (**d**) which participate in graft rejection (**e**).

The indirect pathway occurs after transplantation when host antigen-presenting cells (mainly dendritic cells), which permanently guard the environment, take up and process allogeneic molecules shed from donor tissue. The phagocytosis of these molecules by host dendritic cells results in the presentation of allo-peptides by self-MHC class II molecules. This leads to the activation of CD4+ T cells, in contrast to the direct pathway where both CD4+ and CD8+ T cells are activated (Popov IA et al., 1995; Benichou G, 1999). While the direct pathway depends on the limited presence of passenger leukocytes, the indirect pathway depends on the continuous supply of alloantigens from the graft. The activated CD4+ T cells provide further help for the activation of other effector cells such as B cells, natural killer cells and macrophages and together they lead to the allograft rejection (**Figure 1.1**).

Ways of influencing allograft rejection

The immunologic characteristics of the direct and indirect pathway may require individual strategies for immunosuppression. The direct pathway starting immediately after transplantation has to be controlled with a short-term immuno-suppression because of its extraordinary strength. Most of the passenger leukocytes, responsible for this pathway, will be destroyed by the immune system and therefore, this pathway seems to be time-limited. An experimental validation of the fact that their selective depletion reduces the graft's immunogenicity and prolongs graft survival was given by Laferty's group (Talmage DW et al., 1976). However, this strategy alone will not induce long-term allograft survival because firstly, the indirect pathway of allorecognition will not be influenced (**Figure 1.1**) and secondly, the direct alloreactivity may not necessarily be limited to the early phase of the allograft response. Donor endothelial cells express many of the same costimulatory and adhesion molecules found on dendritic cells to activate directly recipient CD8+ T cells (Kreisel D et al., 2002). In addition, the expression levels of MHC class I on donor endothelium are markedly up-regulated during allograft rejection.

The indirect pathway of allorecognition is a chronic process which remains active as long as the transplanted organ is kept in the recipient. Through this pathway alloreactive T cells recognize the allogeneic MHC molecules as conventional peptide antigens bound by self-MHC class II molecules. Therefore, it may be possible to influence specifically alloreactive T cells activated by this pathway with distinct type of peptides with subtle modifications in their sequence, termed altered peptide ligands (Sloan-Lancaster J and Allen PM, 1996). Altered peptide ligands have been studied in different murine models of autoimmune diseases because the dominant antigens are well characterized in most autoimmune diseases. Their use has highlighted the possibility of inducing a variety of biological activities, such as changes in the cytokine profile and induction of anergy (Sloan-Lancaster J et al., 1993). Although the use of altered peptide ligands has been well studied in experimental autoimmune diseases, very few data are yet available on the application of altered peptide ligands in transplantation. Present data show that such peptides can indeed inhibit the activation of alloreactive T cells *in vitro* by the indirect pathway of allorecognition (Frasca L et al., 2000; Sitaru AG et al., 2004).

In general, T cell-mediated immunity occurs if T cells encounter their specific antigen in the restricted form of a peptide/self-MHC complex on the surface of antigen-presenting cells. Since the donor MHC peptides are the principal antigens in the initiation and maintenance of the alloimmune response, it is not surprising that most of the experimental studies have focused on modulation of the alloimmune response using MHC allopeptides (Sayegh MH et al., 1992). Some allopeptides have been shown to have potential immunomodulatory capacities when administrated intrathymically (Oluwole SF et al., 1993), orally (Zavazava N et al., 2000), or as peptide-pulsed recipient dendritic cells (Oluwole OO et al., 2001; Ali A et al., 2001). This demonstrates that allogeneic peptides are able to influence the T cell population by mechanisms belonging to the central and peripheral tolerance. Presently, components of the peripheral tolerance, including tolerogenic dendritic cells (Hackstein H and Thomson AW, 2004) or regulatory T cells (Shevach EM, 2000), are preferred in order to

suppress the alloimmune response. With these cell subpopulations, the control of the alloimmune response is possible via the indirect pathway of allorecognition but they can also address the direct pathway. Some studies showed indeed that the control of the indirect pathway also enables the control of the direct pathway (Cote I et al., 2001).

1.2 The control of T cell activation by dendritic cells: immunity versus tolerance

In 1973, Ralph M. Steinman and Zanvil A. Cohn identified a novel cell type in peripheral lymphoid organs (Steinman RM and Cohn ZA, 1973). These dendritic cells, as termed by the authors due to their distinct morphology, represent a heterogeneous population of antigen-presenting cells and are important in immune responses (Banchereau J and Steinman RM, 1998; Wei C, 2005). After transplantation, donor dendritic cells migrate immediately from the allograft to the regional lymph nodes, where they initiate a robust immune response to the allograft leading to acute rejection (direct pathway of allorecognition). A few days after transplantation host dendritic cells will process allogeneic MHC molecules into peptides and present them in a self-restricted manner to host T cells. Besides their extremely powerful ability to initiate immunity, particularly dendritic cells are also able to induce antigen-specific unresponsiveness that includes deletion and induction of regulatory cells (Banchereau J et al., 2000). Therefore, their central role in immunity and tolerance seems to make them the ideal cellular candidate for the antigen-specific modulation of the alloimmune response after transplantation.

A closer look into the biology of dendritic cells reveals that these properties are dependent on their state of maturation (Banchereau J et al., 2000; Kuwana M, 2002; Zhu M et al., 2003; Nikolic T et al., 2003). Mature dendritic cells are extremely powerful activators of naïve T cells since they provide all the signals required for their fully activation. The most important costimulatory molecules expressed on the cell surface of the dendritic cells are the B7.1 (CD80), B7.2

(CD86) and CD40. In contrast, the so-called immature or semi-mature dendritic cells induce anergic, non-responsive or regulatory T cells instead of alloreactive T cells. The general characteristic of the immature dendritic cells is an intermediate expression of MHC class II coupled with a low or no expression of costimulatory molecules (Peche H et al., 2005; Nikolic T et al., 2003). Most of the authors described a suppressive or tolerogenic effect of these cells *in vitro* (Lutz MB et al., 2000 [1]; Beriou G et al., 2005). Some of them, for instance Grauer (Grauer O et al. 2002), found that immature rat BM-DCs were poor stimulators of mixed leukocyte cultures. Powell et al. discriminated two subtypes of immature rat BM-DCs, FcR-positive and FcR-negative cells (Powell TJ et al., 2003). The first ones were poor stimulators and the second ones were excellent stimulators of T cell activation. In addition, different studies have also shown the ability of immature DCs to induce the prolongation of allograft survival (DePaz HA et al., 2003; Garovillo M et al., 2001).

The granulocyte-macrophage colony stimulating factor or GM-CSF is the cytokine that appears constantly in most of the protocols used to generate dendritic cells. Initially, this cytokine was characterized for its ability to induce the *in vitro* formation of colonies of granulocyte-macrophage progenitors. It is produced by activated T cells, B cells, mast cells, macrophages, endothelial cells and fibroblasts in response to immune and inflammatory stimuli (Gala RR and Shevach EM, 1994; Channon JY et al., 2002). Later, it was demonstrated that GM-CSF is able to induce also the formation of dendritic cells. The ability of this cytokine to induce the formation of cell clusters seems to be vital for the *in vitro* development of dendritic cells from hematopoetic-lineage precursors. The enhancement of DC numbers generated *in vitro* by the addition of GM-CSF was described for the first time by the group of Steinman in 1987 (Witmer-Pack MD et al., 1987). Since then, different groups have developed different protocols based on the use of GM-CSF alone (DePaz HA et al., 2003) or in combination with other cytokines like IL-4 (Menges M et al., 2005) or IL-10 (Commeren DL et al., 2003) in order to induce DCs at different maturation stages.

2 Aims of the study

The present study analyses the importance of rat dendritic cells with immune inhibitory properties (Klinkert WE et al., 1982; Miranda de Carvalho et al., 2005). The rat is one of the most important animal models for experimental organ transplantation in a clinic-relevant procedure (Timmermann W et al., 1998). Therefore, the main purpose of this study was to characterize immature rat dendritic cells and to analyse their regulatory effect *in vitro* and *in vivo*.

In general, large numbers of dendritic cells can be propagated for therapeutic purposes (Lutz MB et al., 1999) due to the improvements in the isolation of dendritic cells and culture techniques. The generation of rat dendritic cells derived from bone marrow progenitor has been well documented (Grauer O et al., 2002). In the present study, the effect of two protocols, GM-CSF plus IL-4 and GM-CSF plus IL-10, on the generation of immature dendritic cells was analysed. To confirm their expected immature character, their phenotype (expression of MHC molecules and costimulatory molecules), their endocytosis capacity, and their inhibitory effect on the activation of naïve and antigen-specific T cells have been investigated.

The results of current clinical trials regarding the use of autologous dendritic cells for the induction of anti-tumor immune responses support the idea of using dendritic cells as a vehicle to deliver target antigens. In this work the possibility of preventing or delaying allograft rejection with autologous tolerogeneic dendritic cells pulsed with a relevant allopeptide was analysed. For a certain rat model of allograft rejection (**Fig. 10.1** in the appendix), the immunodominant allopeptide P1 that accelerated graft rejection was identified as an important antigen (Sitaru AG et al., 2004). Such a model offers an attractive and practical approach to analyse the potential of host immature dendritic cells pulsed with P1 to suppress the allograft-induced immune response in an antigen-specific manner without the need of chronic immunosuppression.

Questions

(1) What are the main phenotypic characteristics of immature bone marrow-derived dendritic cells?

(2) What is the effect of immature bone marrow-derived dendritic cells on naïve and antigen-specific T cells?

(3) Do P1-pulsed immature bone marrow-derived dendritic cells influence the allograft survival in an antigen-specific manner?

3 Materials and Methods

3.1 Animals

Inbred Lewis, Wistar Furth and Brown Norway rats, weighing between 200 and 350 g, were provided by Harlan Winkelmann GmbH (Borchen, Germany). Animal experiments were conducted in accordance with national and institutional animal care policies. Dendritic cells and antigen (P1)-specific T cells were habitually isolated from Lewis rats (3.5, 3.6, and 3.7). In addition, inbred male rats from Lewis, Wistar Furth and Brown Norway strains were used in the transplantation experiments (3.14).

3.2 The allogeneic peptide P1

The Wistar Furth (WF) MHC class I ($RT1.A^u$) peptide P1 was previously characterized (Sitaru AG et al., 2002). The immunization of Lewis rats with P1 previous to transplantation reduced the survival of Wistar Furth grafts from 7.3 ± 0.5 to 5.2 ± 0.4 days (**Figure 10.1**). P1 was synthesized by Jeriny AG (Berlin, Germany) based on published sequences (Joly E et al., 1995*;* Chowdhury NC et al., 1998) with a purity of 95%. The lyophilized peptide was dissolved in sterile PBS at 1.0 mg/ml and stored at -20°C. P1 is a 19-mer peptide located in the β-plate of the α1 domain of the $RT1.A^u$ (**Figure 10.1**). The different amino acids between P1 and the corresponding region of the MHC class I molecule of the Lewis rat are located in positions 5, 9 and 10 (**Table 3.1**). Subsets of alloreactive Lewis T cells recognize these amino acids (Sitaru AG et al., 2004).

Table 3.1: Comparison of the amino acid sequence between peptide P1 and the first 19 amino acids of MHC class I of Wistar Furth ($RT1.A^u$) and Lewis ($RT1.A^l$) in one-letter code. The identical amino acids are represented by bars, the different amino acids by letters. The numbers indicate the position of the amino acid residues in the molecule.

	Amino acid sequence																		
	1	2	3	4	**5**	6	7	8	**9**	**10**	11	12	13	14	15	16	17	18	19
P1	G	S	H	S	**L**	R	Y	F	**L**	**T**	A	V	S	R	P	G	L	G	E
$RT1.A^u$ (1-19)	–	–	–	–	**L**	–	–	–	**L**	**T**	–	–	–	–	–	–	–	–	–
$RT1.A^l$ (1-19)	–	–	–	–	**M**	–	–	–	**D**	**I**	–	–	–	–	–	–	–	–	–

3.3 Immunization

Lewis rats were immunized in the hind footpad with 12.5 µl (= 12.5 µg) of peptide P1 (working stock solution: 1 mg/ml) combined with an equal volume of the adjuvant TiterMax (Alexis GmbH, Grünberg, Germany). The animals were anaesthetized with Isofluran (Abbot GmbH, Wiesbaden, Germany) shortly before immunization.

3.4 Culture medium and buffers

Culture medium

Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (Gibco/ Invitrogen, Karlsruhe, Germany) was used as the standard culture medium supplemented with 20 mmol/L HEPES, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, $5x10^{-5}$ mol/L 2-mercaptoethanol, 1% non-essential amino acids and 10% fetal calf serum or FCS (all reagents provided by Gibco/Invitrogen, Karlsruhe, Germany). All concentrations are indicated as final concentrations.

Phosphate-buffered saline (PBS)

The phosphate-buffered saline (pH 7.2) contains a mixture of 140 mmol/L sodium chloride, 2.7 mmo/L potassium chloride, 7.2 mmol/L sodium dihydrogen phosphate and 1.47 mmol/L potassium hydrogen phosphate. The solution was sterilized and kept at 4°C.

Lyse buffer (10-fold concentrated)

The erythrocyte lyse buffer (diluted 1:10 with sterile aqua injectabila) contains 1.68 mol/L ammonium chloride, 99.88 mmol/L potassium hydrogen carbonate and 12.6 mmol/L EDTA.

Tris-NaCl buffer

The Tris-NaCl buffer was used for immunohistochemistry procedures (3.13). The buffer consists of 0.6 g/L Tris-HCl and 8.1 g/l NaCL. Bovine serum

albumine (BSA) was added to the solution in a final concentration of 2 g/L. The pH was adjusted to 7.4.

3.5 Generation of bone marrow-derived immature dendritic cells

Bone marrow was extracted from femur and tibia bones of young (8-10 weeks) Lewis rats. The bones, cleaned of muscular tissues with a scalpel, were disinfected for 3 minutes in 70% alcohol and washed 2 times in sterile PBS. Both ends of the bones were cut off with scissors. The bone marrow was flushed with culture medium through a 70 µm nylon cell strainer (Becton Dickinson Biosciences, Heidelberg, Germany) into a Falcon tube using a syringe with a 20-gauge needle. The bone marrow cells, washed three times without lysing the red blood cells, were cultured in Petri dishes (Falcon, Becton Dickinson Biosciences) with a diameter of 100 mm or in the cavities of 6 well-plates (Greiner bio-one, Frickenhausen, Germany) at a cell density of $5x10^5$ cells/ml. The following cytokines were added to the cultures (final concentration): 5 ng/ml recombinant rat granulocyte-macrophage colony stimulating factor (rGM-CSF, R&D Systems, Heidelberg, Germany or Biosource, Camarillo, California, USA); 5 ng/ml recombinant rat interleukin-4 (rIL-4, Strathmann Biotech AG, Hamburg, Germany) or 5 ng/ml interleukin-10 (rIL-10, Strathmann Biotech AG). On day 4, half of the medium was substituted with fresh medium without cytokines. On day 6 the non-adherent and light-adherent cells were collected and centrifuged over 14.5% metrizamide (Linaris Biologische Producte GmbH, Wertheim, Germany) at 4°C and 1,823 xg for 13 minutes. The immature DC were gently collected from the interface and counted. Maturation was induced with bacterial lipopolysaccharide or LPS (Sigma, Deisenhofen, Germany) in a final concentration of 20 ng/ml for 24 h.

Table 3.2: The cytokines used for the induction of immature BM-DCs and their corresponding name.

Cytokines	Corresponding name
GM-CSF+IL-4	IL-4 DC
GM-CSF+IL-10	IL-10 DC

3.6 Isolation of splenic dendritic cells

Splenic dendritic cells (= S-DCs) from naïve Lewis rats were used as mature antigen-presenting cells. The spleens were prepared mechanically through a 70 µm nylon cell strainer (Becton Dickinson Biosciences) and the erythrocytes were lysed with lyse buffer. The leukocytes were incubated overnight in culture medium at 37°C in a 5% humidified CO_2 atmosphere. The non-adherent cells were collected and centrifuged over a 14.5% metrizamide gradient in order to enrich the dendritic cells on the interface.

3.7 Isolation of antigen-specific lymph node cells

Popliteal lymph node cells of P1-immunized Lewis rats were isolated 7 days after immunization and prepared mechanically with a 10 ml syringe piston through a 70 µm nylon cell-strainer (Becton Dickinson Biosciences). After washing twice, the viable cells (they did not incorporate trypan blue) were counted in the Neubauer chamber.

3.8 *In vitro* assay systems

3.8.1 Proliferation assay for naïve T cells

Naïve T cells (10^5 cells/well) from lymph nodes of Wistar Furth or Brown Norway rats were incubated with irradiated (20 Gy) Lewis mature splenic DCs (10^4 cells/well) in a final volume of 150 µl. The 96-well round-bottom plates were incubated for 3 days at 37°C in a 5% humidified CO_2 atmosphere and pulsed with 0.5 µCi/well [^{3}H]-thymidine (Biomedicals Germany GmbH, Eschwege, Germany) for the last 6 hours of culture. Control wells consisted of naïve T cells cultured alone. The incorporation of [^{3}H]-thymidine was measured with the scintillation method (3.8.2). The ability of IL-4 DCs and IL-10 DCs to activate naïve T cells was tested in this assay (mixed leukocyte culture or MLC).

3.8.2 T cell proliferation assay for activated T cells

P1-specific Lewis lymph node cells (10^5 cells/well) were incubated with splenic DCs (10^4 cells/well) loaded with P1 (1.25 µg/well or 8.33 µg/ml) in 96-well round-bottom plates. The final volume was 150 µl in each well. The DCs were

irradiated with 20 Gray (Institut für Strahlenkunde, Würzburg). The plates were incubated for 3 days at 37°C in a 5% humidified CO_2 atmosphere and pulsed with 0.5 µCi/well [^{3}H]-thymidine for the last 6 hours of culture. The incorporation of [^{3}H]-thymidine by the proliferating T lymphocytes was measured with the scintillation procedure. For this, the cells were harvested with the MicroBeta Filtermat-96 cell harvester (Wallac, Turku, Finland) and their DNA was collected on special filter paper (1450-421 Filtermat A, Wallac, Perkin-Elmer Life and Analytical Sciences, Rodgau, Germany). The filter paper was dried for 1 hour and then sealed in special plastic bags (Wallac, PerkinElmer) with liquid scintillation. The [^{3}H]-thymidine incorporation was measured with a Wallac-MicroBeta TriLux β-radiation counter (Institut für Virologie und Immunbiologie, Würzburg). Control wells consisted of P1-specific T cells cultured alone. The experiments were set up in 6-12 replicates and the results (mean ± standard deviation) were expressed in counts per minute (cpm). The ability of IL-4 DCs and IL-10 DCs to restimulate antigen-specific T cells was tested in this assay.

3.8.3 Restimulation of antigen-specific T cells after incubation with immature BM-DCs (restimulation assay)

This assay proved the ability of P1-T cells, which were pre-incubated with IL-4 DCs or IL-10 DCs, to proliferate in the presence of P1-loaded mature splenic DCs. The P1-T cells were incubated with P1-loaded IL-4 DCs and IL-10 DCs, respectively, in a cell ratio of 1 DC to 2 T cells for 3 days. For depletion of DCs the cells were incubated with Ox-42 (**Table 3.3**) for 20 minutes on ice on the rocking platform. The cells, washed 2 times with PBS, were incubated with paramagnetic polymer beads (CELLection Pan Mouse IgG Kit; Dynal, Hamburg, Germany) for 20 minutes on ice and, afterwards, the bead-coated DCs were depleted with a magnet. The T cells remaining in the supernatant were collected (purity > 90%) and transferred to the T cell proliferation assay. After incubation for 3 days, the plates were pulsed with 0.5 µCi/well [^{3}H]-thymidine for the last 6 hours of the culture. The incorporation of radioactivity was measured using a beta-radiation counter as previously described (3.8.2).

3.8.4 T cell mediated inhibition after incubation with immature BM-DCs (inhibition assay)

P1-specific T cells were incubated with P1-pulsed IL-4 DCs or IL-10 DCs in a cell ratio of 1 DC to 2 T cells for 3 days in culture dishes with 10 mm diameter. After depletion of DCs the purified T cells (termed as DC-Ts) were transferred to the T cell proliferation assay (P1-specific T cells and P1-pulsed splenic DCs). Different cell numbers (from 10^1 to 10^5) of these DC-Ts were tested for their possible regulatory or inhibitory properties on freshly isolated P1-specific T cells in the proliferation assay. The cells were cultured for 72 h and pulsed with [^{3}H]-thymidine for the last 6 h of culture. The incorporation was measured as described before (3.8.2).

3.9 Flow cytometric analysis

3.9.1 Phenotyping

For the flow cytometric analysis, $5x10^5$ cells (in 50 µl PBS) were incubated for 20 minutes at 4°C with the optimal concentrations of specific antibodies. The antibodies (**Table 3.3**) were conjugated with fluorescein isothiocyanate (FITC) or phycoerythrine (PE). The non-conjugated antibody Ox-62 and the CTLA-4Ig fusion protein were detected with the FITC-conjugated secondary antibody donkey anti-mouse (Dianova, Germany). The nonviable cells were identified with 7-amino actinomycin D (7-AAD) (Becton Dickinson Biosciences). The non-specific binding was controlled with isotype control antibodies. The samples were measured using an argon-laser flow-cytometer FACSscan (Becton Dickinson Biosciences). The analysis was performed with WinMDI, Version 2.8.

3.9.2 Determination of antigen uptake by dendritic cells

The ability of IL-4 and IL-10 DCs to take up antigens was tested using FITC-conjugated Dextran (Sigma-Aldrich, Germany) at 1 µg/ml (diluted with PBS) and fixed cells as negative control. FITC-conjugated dextran was added to the wells and incubated at 37°C for 6 and 24 hours.

Table 3.3: Antibodies and fusion protein for phenotyping of rat dendritic cells by flow cytometry and immunohistochemistry (3.13).

Antibody	Specificity	Distributor
Ox-1	CD45	Linaris Biologische Produkte, Wertheim-Bettingen
Ox-6	MHC II (RT1B)	
Ox-22	CD45RC	
Ox-42	CD11b/c	
Ox-62	α-E2 Integrin	
ED-1	CD68	
CTLA-4Ig *)	CD80/CD86	Alexis Biochemicals Cat.-No: CHI-MF120A4-M001

*) nonlytic mouse CTLA-4Ig fusion protein [mCD152 (CTLA-4)Ig] consisting of the extracellular domain of mouse CD152 fused to the Fc portion of mutant mouse IgG2a.

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

Total RNA was isolated from 10^6 cells with 1 ml of the ready-to-use reagent Trizol Reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's recommendations. The addition of chloroform (Carl Roth GmbH, Karlsruhe, Germany) followed by centrifugation (15 minutes, 16,000 xg, at 4°C) separated the solution into an aqueous phase containing exclusively the RNA, and an organic phase. The RNA was recovered after precipitation with Isopropanol (Merck Eurolab, Nürnberg, Germany) overnight at -20°C. After centrifugation the pellet was washed with Ethanol (Merck Eurolab). The air-dried RNA was dissolved in 40 µl RNA storage solution (Ambion (Europe), Huntingdon, United Kingdom) and stored at -80°C. 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 (Applied Biosystems GmbH, Weiterstadt, Germany). Five µl cDNA was amplified using Gold AmpliTaq DNA-Polymerase (0.05 U/µl) and the specific primers (5 µmol/L each) (**Table 3.4**) mixed in nuclease free water (Promega GmbH, Mannheim, Germany) to an end volume of 50 µl per sample. Ten µl PCR product mixed with 1.5 µl Blue/Orange 6x loading dye (Promega GmbH) was transferred to an ethidium bromide loaded 2% agarose gel (Amresco, Solo, Ohio, USA). The gel

was run at 80 volt for 30 minutes and the separated PCR products were photographed in the gel with UV light illumination (ImageMaster workstation from Amersham Pharmacia Biotech, Piscataway, USA). The house-keeping gene Glycerinaldehyd-3-Phosphat-Dehydrogenase (GAPDH) was used as positive control.

3.11 Real time PCR

The SYBR Green kit from Applied Biosystems was used to quantify the expression of MHC class II mRNA. From the cDNA (3.10) probes were amplified in the Opticon 2 Cycler (M.J. Research, Bio-rad, USA). SYBR Green was used as a fluorescence reporter for the double stranded DNA and the signal increased in direct proportion to the amount of the PCR product. A melting curve was performed to analyse product homogeneity. For absolute quantification calibration curves of GAPDH and MHC II was performed. For this, both the Taq-amplified PCR products of GAPDH and MHC II (**Table 3.4**) were ligated into the PCR vector (TA cloning kit from Invitrogen GmbH, Karlsruhe, Germany) and the constructs were transformed into TOP10F´ chemically competent E. coli. Colonies from the plates were picked after 24 h and were grown overnight in liquid cultures with ampicillin as selection antibiotic. The extracted plasmid DNA (QIAprep Spin Minipreps from QIAGEN, Hilden, Germany) was checked for the inserts and the concentration was determined by measuring the absorbance at 260 nm. The calibration curves were performed in the range from 10^2 to 10^8 start molecules (start copy number against threshold cycle or C_t). The reactions were performed in special tubes (thin-wall 8-tube strips from Biozym, Hess. Oldendorf, Germany) and the process was repeated 3 times for each sample.

Table 3.4 (next page): ***The sequences of the primers used in the work.*** The forward and backward sequence is presented for each of the primers. All the primers were synthesized by MWG Biotech AG (Ebersberg, Germany) according to published sequences: GAPDH (Krusse JJ et al., Cytokine 1999); IL-2 (McKnight AJ et al., J Immunol Methods 1989); IL-4 (Siegling A et al., J Immunol Methods 1994); IL-10 (Siegling A et al., J Immunol Methods 1994); IL-12 (Stumbles P A et al., J Exp Med 1998); IL-13 (Gillespie KM et al., Eur J Immunol 1996); IFN-γ (Dijkema R et al. Meth Enzymol 1986); MHC class II (Syha-Jedeljauser J et al., Biochem Biophys Acta 1991); CD40 (Yutake Matsui et al., J Mol Cell Cardiol 2002); CD80 (Holowachuk E.W. et al., Biochem and Biophys Research Comm 2001); CD86 (self made with GeneFisher).

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
IL-2	351	62	for rev	GCG CAC CCA CTT CAA GCC CT CCA CCA CAG TTG CTG GCT CA
IL-4	547	60	for rev	AGT CAA CAC CCT AAC ATC AGG G GGC CTC AAA CAG CTC CAT ACA G
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
IL-13	280	55	for rev	CAG GGA GCT TAT CGA GGA GC AAG TTG CTT GGA GTA ATT GAG C
IFN-γ	419	62	for rev	CCC TCT CTG GCT GTT ACT GC CTC CTT TTC CGT TTC CTT AG
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.

3.12 ELISA

Purified IL-4 and IL-10 DCs from day 6 of culture were replated in 96-well plates (10^5 cells/well in a final volume of 150 µl) without exogenous cytokines and stimulated for 24 hours with 20 ng/ml LPS. The presence of IL-10 and IL-12 in the supernatant was checked using the BioSource kits (Ratingen, Germany): IL-12 (p40 and p70): KRC0121; IL-10: KRC0101.

3.13 Immunohistochemistry

Purified day 6 IL-4 DCs and IL-10 DCs ($5x10^5$ cells per slide (cytospin) or cultured on glass slides) were air-dried. After fixation in acetone for 10 minutes the preparations were incubated overnight with the following anti-rat monoclonal

antibodies and fusion protein (**Table 3.3**) diluted in background reducing buffer (DAKO Cytomation, Hamburg, Germany): OX-62 (rat-dendritic cell marker) 1:50; OX-6 (MHC class II) 1:200; ED1 (macrophage marker) 1:200; CTLA-4Ig fusion protein (CD80/CD86) 1:50. The next day the slides were washed and incubated with a mix of 1:50 peroxidase-conjugated rabbit anti-mouse antibody (DAKO) and rat serum in a final volume of 200 µl per slide for 1 hour and then washed again. The preparations were incubated with hydrogen peroxidase and diaminobenzidine (Liquid DAB, BioGenex, San Ramon, USA), counterstained with hematoxilin and eosin (H&E) and embedded with Entelan (Merk, Darmstadt, Germany).

3.14 *In vivo* transfer of bone marrow-derived immature dendritic cells and heterotopic heart transplantation

The possible influence of IL-4 DCs or IL-10 DC on allograft survival was tested. Therefore, $10x10^6$ cells (P1-pulsed and unpulsed cells) were transferred subcutaneously into Lewis rats one day before transplantation. They were incubated with 20 µg P1 for 30 minutes in 500 µl PBS and then washed 2 times with PBS. On "day 0" the animals received hearts from Wistar Furth donors in a heterotopic position according to the method of Ono and Lindsey (Ono K and Lindsey ES, 1969) using standard microsurgical techniques. The donor ascending aorta and the pulmonary trunk were anastomosed end-to-side to the recipient infrarenal abdominal aorta and inferior vena cava. The allograft survival was monitored by daily abdominal palpation of cardiac contractions. A complete loss of palpable ventricular contractions was considered to be rejected. No immunosuppression was administered.

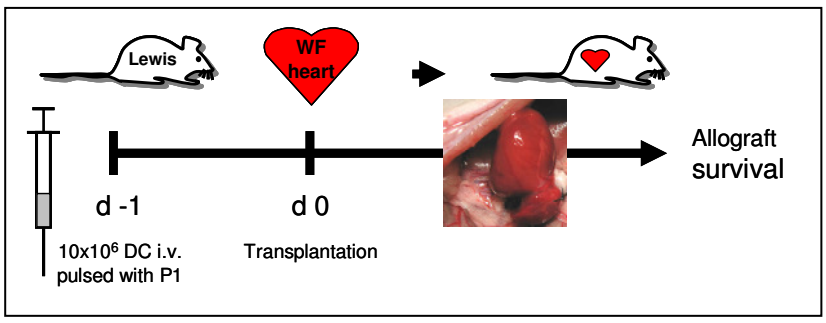


Figure 3.1: ***The dendritic cell transfer protocol.*** Ten million IL-4 or IL-10 DCs were pulsed with 20 µg peptide P1 and injected into a Lewis recipient one day before organ transplantation.

4 Results

4.1 Generation of bone marrow-derived immature dendritic cells

Femurs and tibias were extracted from 8 to 10 week old male Lewis rats and approximately 12 x 10^7 to 15 x 10^7 bone marrow cells (**Figure 4.1**) were flushed out from the four bones (n = 50 animals). The bone marrow cells were cultured in a cell density of at least 5 x 10^5 cells per ml (**Table 4.1**). With this starting cell number, approximately 2.5 x 10^6 to 4.0 x 10^6 bone marrow-derived dendritic cells were isolated from each petri-dish at the end of the 6-day culture period.

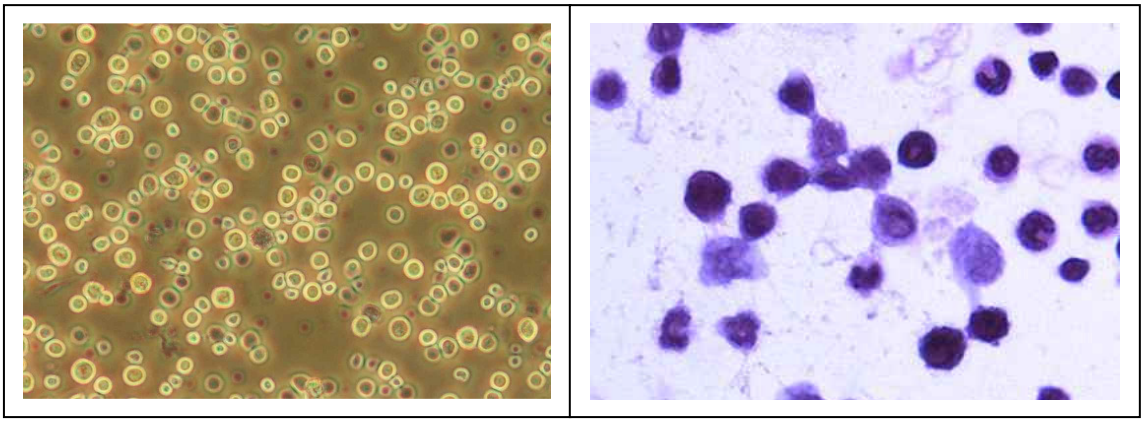


Figure 4.1: ***The morphology of bone marrow cells***. Freshly isolated bone marrow cells in culture (left, x200, phase contrast microscopy) and after H&E staining (right, x600 plus digital magnification with Corel Photo-Paint 11). For the H&E staining most of the red blood cells which appeared as dim cells in the culture (left) were lysed.

After 6 days in culture with GM-CSF plus IL-4 or GM-CSF plus IL-10 the recovered non-adherent cells were purified over a 14.5% metrizamide gradient and the dendritic cells were enriched at the interface (**Figure 4.2**). The flow cytometric analysis of these cells exhibited a large forward scatter or FSC and a low side scatter or SSC (**Figure 4.7** and **Table 10.6** in the appendix). In addition, these cells, stained by the monoclonal antibody Ox-1, were CD45-positive (not shown).

Table 4.1: ***The density of bone marrow precursor cells strongly influence the formation of cell clusters during the period of culture***. The best results were obtained with a cell density between 5.0 x 10^5 and 7.5 x 10^5 cells/ml. A lower cell density was not able to induce the formation of cell clusters and a density around 10^6 cells/ml did not increase the number of cell clusters and, in consequence, the yield of immature DCs. Data from 6 different experiments are shown.

Bone marrow cells [1)]	Cluster formation	Number of harvest cells [2)]
< 5 x 10^5	No	< 0.1 x 10^6
5 x 10^5 - 7.5 x 10^5	Yes	2.5 x 10^6 - 5 x 10^6
> 10^6	Yes	2.5 x 10^6 - 5 x 10^6

1) Number of seeded cells per ml.
2) Per culture dish (100 mm ∅ from Falcon, Becton Dickinson Biosciences) with 15 ml culture medium.

The cells purified from the cultures with GM-CSF and IL-4 (= IL-4 DCs) were separated into two layers: a low density cell population at the interface and a high density cell population at the bottom of the tube (**Figure 4.2** and **Table 4.2**). In contrast, all DCs cultured with GM-CSF and IL-10 (= IL-10 DCs) remained at the interface and no pellet was observed. This might indicate that a more homogenous cell population was obtained with GM-CSF and IL-4.

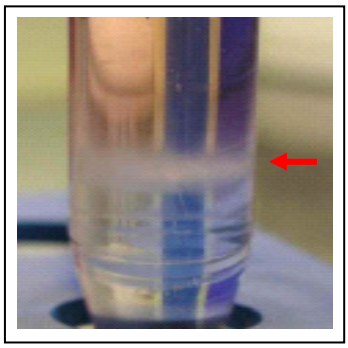


Figure 4.2: ***The metrizamide purification of day 6 immature BM-DCs***. The low-density cells accumulated at the interface between medium and metrizamide (arrow).

Table 4.2: ***Purification of immature BM-DCs over metrizamide.***(*) IL-4 DCs: culture medium with GM-CSF plus IL-4; IL-10 DCs: culture medium with GM-CSF plus IL-10 (Table 3.2). Some of the IL-4 DCs passed the metrizamide and were also found at the bottom of the tube, whereas all IL-10 DCs remained at the interface.

	Centrifugation over metrizamide	
	Interface	Pellet
IL-4 DCs *)	Yes	Yes
IL-10 DCs *)	Yes	No

Both the density of the seeded bone marrow cells and the cytokines added to the cultures seem to influence the yield of immature DCs. Therefore, different culture conditions were tested in order to maximize the yield of bone marrow-derived dendritic cells (**Table 4.3**). The granulocyte-macrophage colony stimulating factor (GM-CSF) alone was not able to induce the formation of high numbers of BM-DCs. The addition of IL-4 or IL-10 to the cultures was a key factor for the development of immature BM-DCs. It seems that IL-10 induces the formation of fewer cell colonies in comparison with IL-4. In addition, IL-10 DCs were not as adherent as IL-4 DCs. Higher numbers of immature BM-DCs were usually recovered from the IL-4 cultures (up to 5×10^6 cells per culture dish) in comparison to IL-10 DCs (up to 3.5×10^6 cells per culture dish). Both IL-4 DCs and IL-10 DCs showed a comparable phenotype on day 6 of culture (**Figure 4.3**).

Table 4.3: ***The effect of different concentrations of cytokines on the development of IL-4 DCs and IL-10 DCs***. The best results concerning the amount of harvested cells were obtained in the presence of 5 ng/ml GM-CSF plus 5 ng/ml IL-4 and GM-CSF plus IL-10 (each 5 ng/ml). Higher concentrations (around 10 ng/ml) did not increase the number of BM-DCs and lower concentrations (under 5 ng/ml) were unable to induce the formation of BM-DCs at all. Data from six different experiments are shown.

Cytokine concentration [1)]	Cell cluster formation	Number of harvest cells [2)]
GM-CSF 5 ng/ml	Yes	$< 0.1 \times 10^6$
GM-CSF+IL-4 < 5 ng/ml	No	$< 0.5 \times 10^6$
GM-CSF+IL-4 5 ng/ml	Yes	4.0×10^6 - 5.0×10^6
GM-CSF+IL-4 > 10 ng/ml	Yes	4.0×10^6 - 5.0×10^6
GM-CSF+IL-10 < 5 ng/ml	No	$< 0.1 \times 10^6$
GM-CSF+IL-10 5 ng/ml	Yes	2.5×10^6 - 3.5×10^6
GM-CSF+IL-10 > 10 ng/ml	Yes	2.5×10^6 - 3.5×10^6

1) Final concentration.
2) Per culture dish (100 mm ∅ from Falcon, BD Biosciences) with 15 ml culture medium.

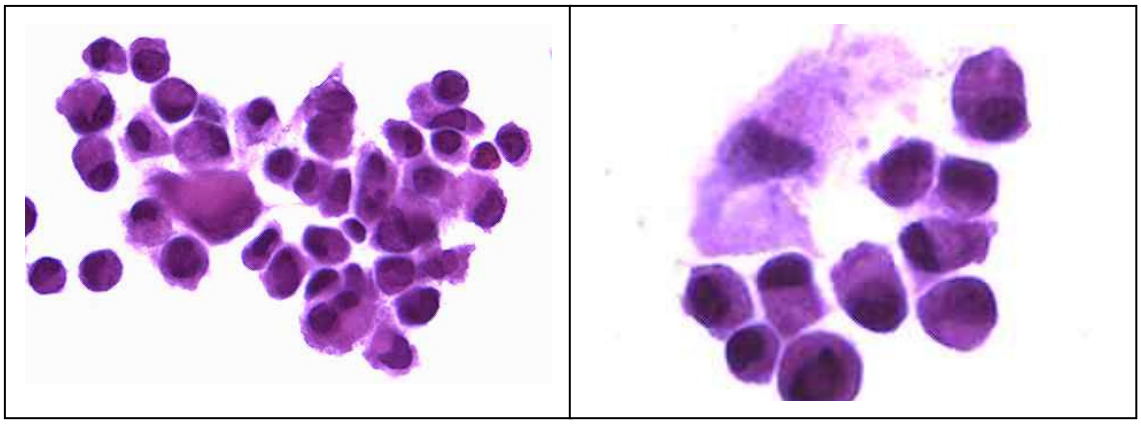


Figure 4.3: ***The morphology of IL-4 DCs and IL-10 DCs***. Typical morphology of IL-4 DCs (left) and IL-10 DCs (right) on day 6 of culture (H&E staining). Small- to medium-sized round cells represent the main morphology in the cell cluster (contaminating fibroblasts are not shown). Magnification: x600 plus a 2-fold digital magnification (IL-10 DCs) with Corel Photo-Paint 11.

Under the influence of the cytokine combinations GM-CSF and IL-4 or GM-CSF and IL-10 some of the bone marrow cells started to proliferate within 24 hours. These enlarged cells were characteristic for the first two days of culture. Some of them were still floating while others became adherent. No cell clusters were observed at this early time point (**Figure 4.4**). Around day 3-4, some of the cells started to form adherent cell clusters and on day 6 of culture, the cells developed large clusters (**Figure 4.4**). They became less adherent after day 8 and most of the cells were easily dislodged by pipetting. A longer period of culture (day 10) was not able to improve the yield of immature BM-DCs. Both IL-4 DCs and IL-10 DCs showed similar characteristics during their culture. In addition, these cells did not mature during the 10 days of culture.

Most of the cells isolated from the clusters had a very similar phenotype: they were small, round and mononucleated (**Figure 4.3**). The cells growing outside the clusters were large mononucleated cells with a round or irregular cytoplasm. Most of these very strong adherent cells were fibroblasts as demonstrated by the positive staining with the monoclonal antibody 6-9H6 with specificity to rat prolyl 4-hydroxylase (not shown). In general, these fibroblast-like cells could not be isolated just by pipetting.

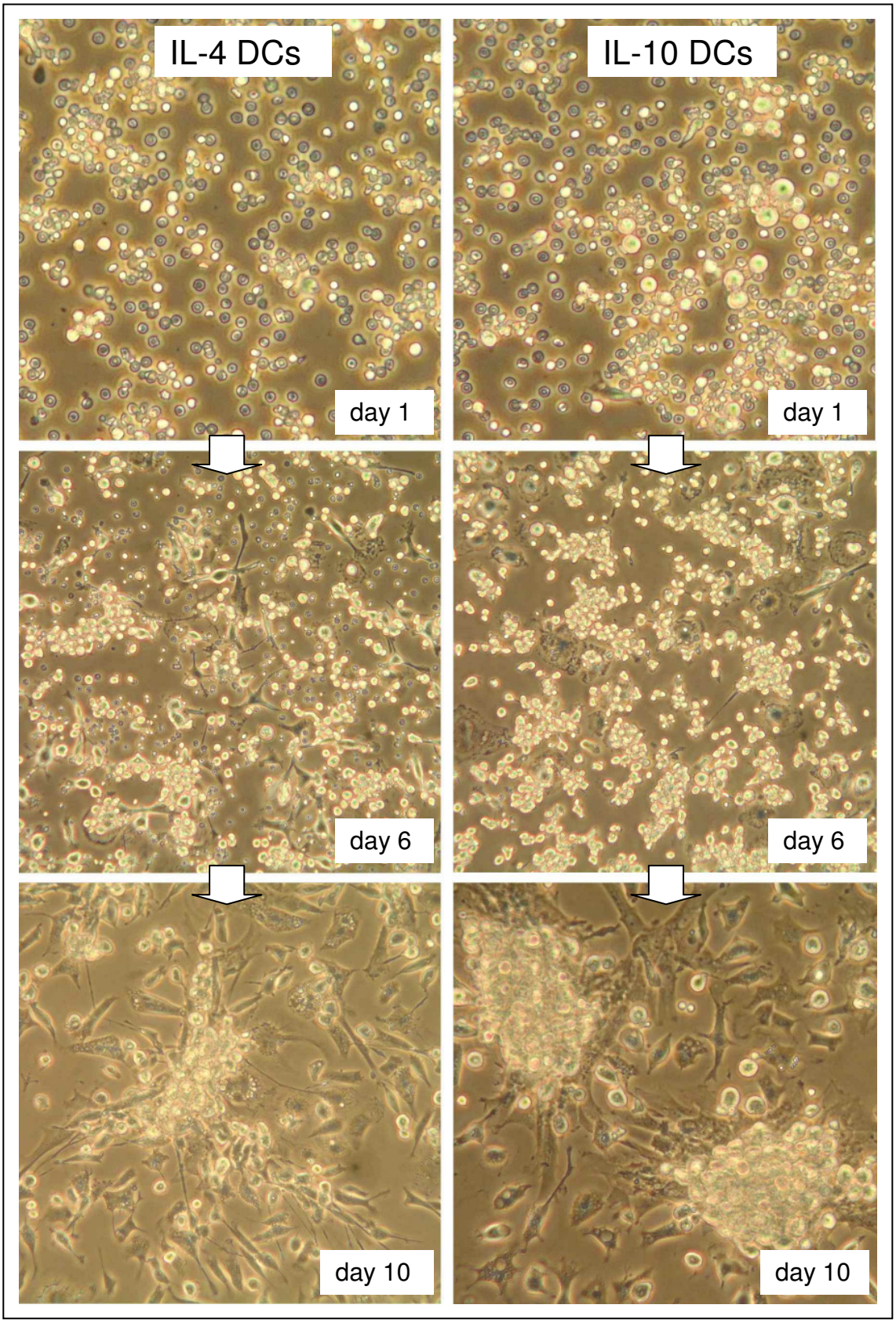


Figure 4.4: ***Representative changes in the culture morphology of immature IL-4 DCs and IL-10 DCs***. Between day 3 and 4 the first cell clusters were seen and most of the cells were already developing in clusters around day 6. Outside the clusters, the fibroblasts were forming

an adherent cell layer (magnification: x200 using an Olympus IMT-2 microscope. Images were captured using an Olympus digital camera C-5050 and processed with Corel Photo-Paint 11). Especially during the first days of culture, contaminating red blood cells were visible as dim cells (Figure 4.1).

The maturation of the bone marrow DCs was induced by incubation with bacterial LPS in a final concentration of 20 ng/ml for 24 hours. In contrast to immature BM-DCs, the mature BM-DCs were able to restimulate antigen (P1)-specific T cells in the T cell proliferation assay (**Figure 4.5**). The strength of the T cell proliferation with 16,000 ± 2,800 cpm was similar to the proliferation induced by mature S-DCs (18,000 ± 2,000 cpm). The RT-PCR analysis confirmed their mature phenotype by showing strong signals for the expression of MHC class II molecules and costimulatory molecules. However, as demonstrated in chapters 4.2 and 4.3, the presence of specific mRNA for costimulatory molecules was not an exclusive feature of the mature BM-DCs.

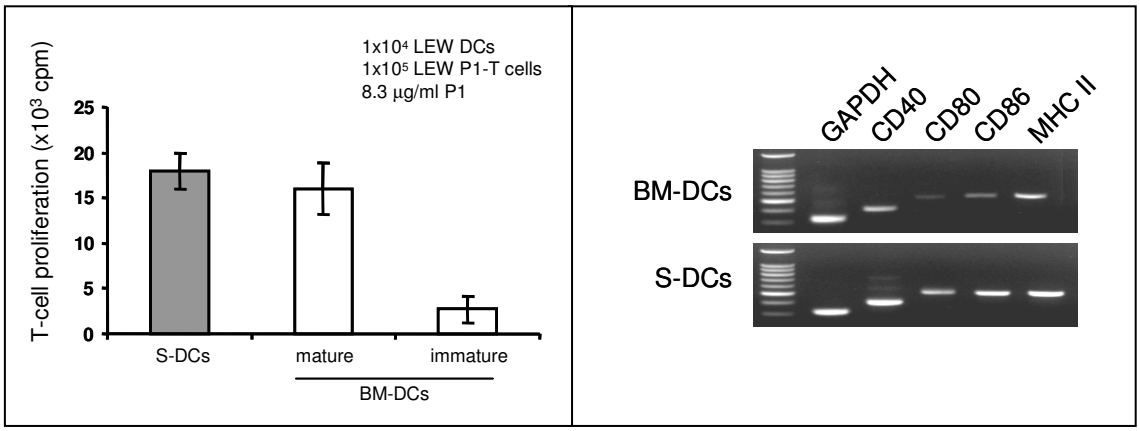


Figure 4.5: ***The comparison between mature splenic DCs and immature BM-DCs***. Day 8 BM-DCs treated for 24 hours with LPS (**Figure 4.7**) became excellent stimulators of T cell proliferation in the T cell proliferation assay (left diagram). Mature splenic DCs (S-DCs) and immature BM-DCs were used as controls. The T cell proliferation is represented as [^{3}H]-thymidine incorporation in counts per minutes (cpm). The diagram shows the results of one experiment (with mean and standard deviation) representative for a series of three. Both, IL-4 DCs and IL-10 DCs matured and acted similarly in the presence of LPS; therefore, only the results for IL-4 DCs are shown. The T cell proliferation assay is described in the chapter "Materials and Methods" (3.8.2). Specific mRNA for MHC class II molecules and costimulatory molecules was found in the mature BM-DCs. A representative RT-PCR analysis of three is shown on the right. Supplementary information on the PCR products can be found in the chapter "Material and Methods" (Table 3.4).

As shown in **Figure 4.5**, the immature dendritic cells are weak stimulators of antigen-specific T cells. To analyse whether this effect is really mediated by the immature dendritic cells and not by the fibroblasts and macrophages which are also components of the cell cultures (chapter 4.1), the different cell types were tested for their possible effect on antigen-specific T cell proliferation. The results in **Figure 4.6** clearly demonstrate that immature IL-4 DCs and IL-10 DCs were not able to restimulate antigen-specific T cells in the proliferation assay in comparison to mature S-DCs (51,500 ± 4,600 cpm): 2,900 ± 1,000 cpm for IL-4 DCs and 2,700 ± 1,100 cpm for IL-10 DCs. In contrast, it seems that macrophages and fibroblasts have almost no influence on the antigen-specific T cell proliferation (macrophages: 22,000 ± 7,100 cpm and fibroblasts: 14,000 ± 2,900 cpm). However, the comparison with the results of the autostimulation (activated T cells incubated with P1 but without DCs (∅); the grey bar in **Figure 4.6**) shows no or only slight differences for fibroblast and macrophages, respectively. Antigen-specific T cells are capable of autostimulation since they express MHC class II molecules and therefore they are able to present P1 and to restimulate each other. With 14,300 ± 4,400 cpm, the strength of the T cell-mediated autostimulation represented approximately ¼ of the DC-mediated T cell activation (51,500 ± 4,600 cpm). In contrast to macrophages and fibroblasts, the IL-4 DCs and IL-10 DCs do not restimulate the antigen-specific T cells and prevent their autostimulation (**Figure 4.6**).

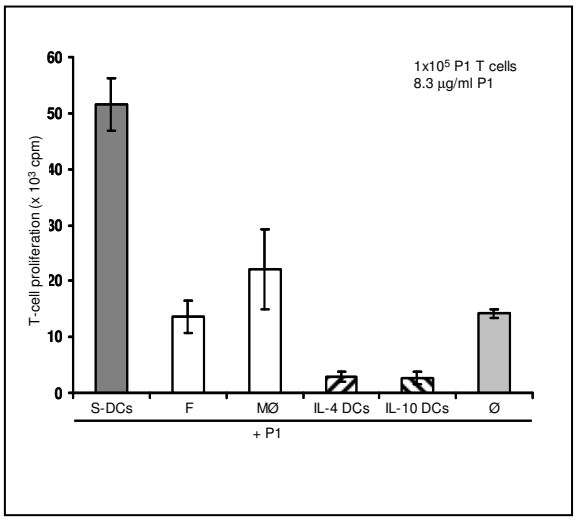


Figure 4.6: ***The comparison of different subsets of stimulator cells concerning their ability to induce T cell proliferation***. IL-4 DCs and IL-10 DCs were clearly not able to induce the restimulation of antigen-specific T cells, whereas the mature S-DCs induced a strong T cell restimulation. Fibroblasts (F) did not influence the T cell restimulation and macrophages had a moderate effect in comparison to the strength of autostimulation (∅; see also text). The value of non-restimulated antigen-specific T cell proliferation was 2,100 ± 500 cpm (not shown). The results are represented as mean ± standard deviation (n = 2 per group).

Both IL-4 and IL-10 DCs expressed mRNA encoding for the Toll-like receptor 4 or TLR4 (RT-PCR data not shown). TLR4 is a critical component of the heteromeric receptor complex that transduces signals delivered by lipopoly-saccharide (LPS) of gram-negative bacteria. On the other hand, the LPS treatment is a very stressful procedure for the rat BM-DCs. The use of a concentration higher than 20 ng/ml LPS (as final concentration) or an incubation over 24 hours induced apoptosis in most of the cells (**Figure 4.7**).

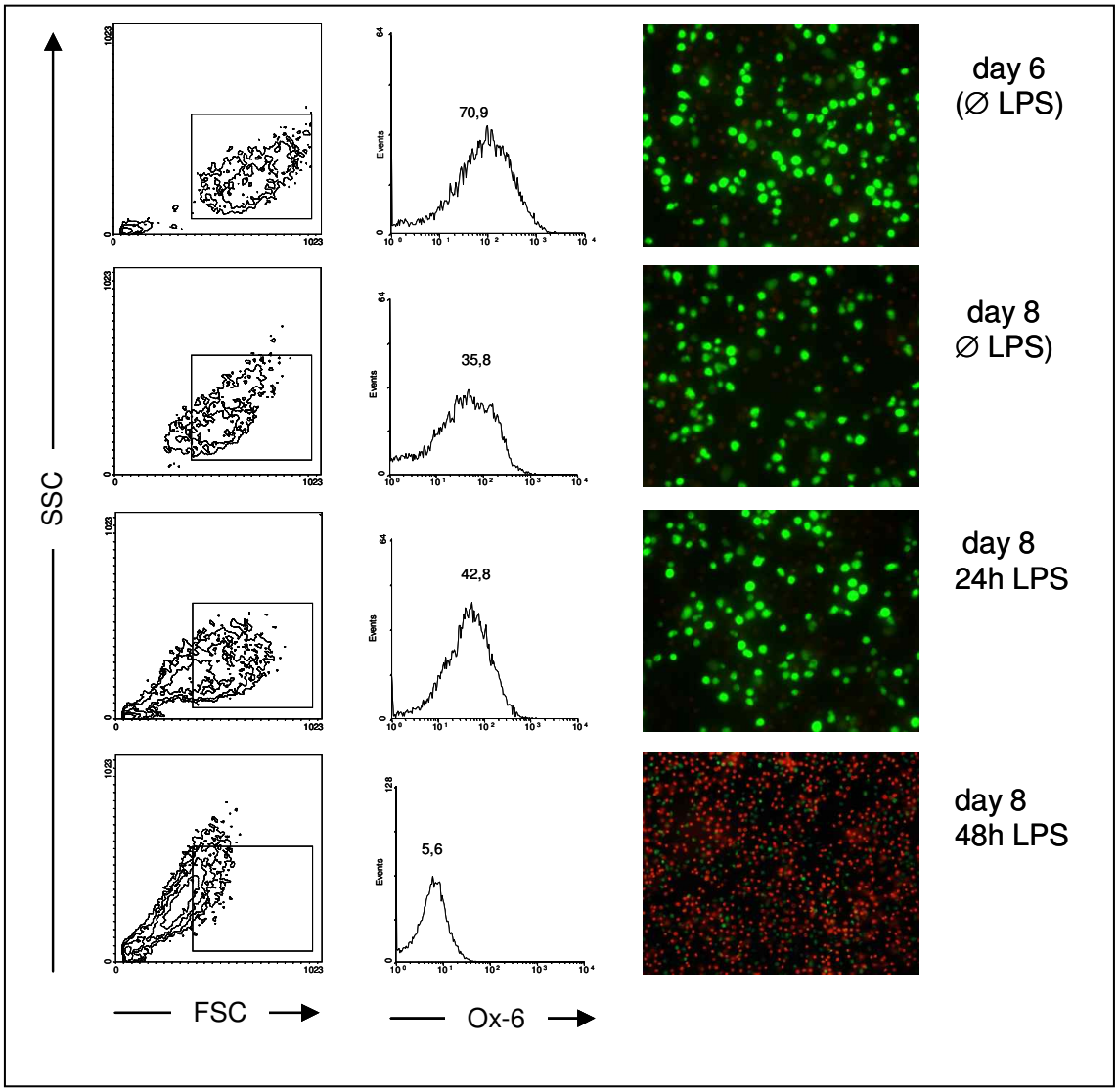


Figure 4.7: ***The effect of LPS on the vitality of immature BM-DCs***. In combination with a 24 hour incubation time 20 ng/ml LPS provided the best results. In contrast, a prolongation of the incubation time to 48 hours induced cell death in most of the cells. The picture shows the alterations in cell size and cell granularity (FSC vs. SSC), the strength of the cell surface expression of MHC class II (Ox-6) and the cell vitality determined by staining with the fluorogenic dyes fluorescein diacetate (FDA) and propidium iodide (PI): green cells are vital

cells and red cells are dead cells. The numbers on the top of the histograms indicate the mean fluorescence intensity of MHC class II molecules on the cell surface. Magnification: x200 using an Olympus BX 50 microscope. Images were captured using a digital camera and processed with Corel Photo-Paint 11. The data are representative for three independent experiments.

A further effect induced by the incubation with LPS on IL-4 DCs and IL-10 DCs was the induction of IL-10 and IL-12 production. Both cytokines were detected in the supernatant with specific ELISA kits from Biosource.

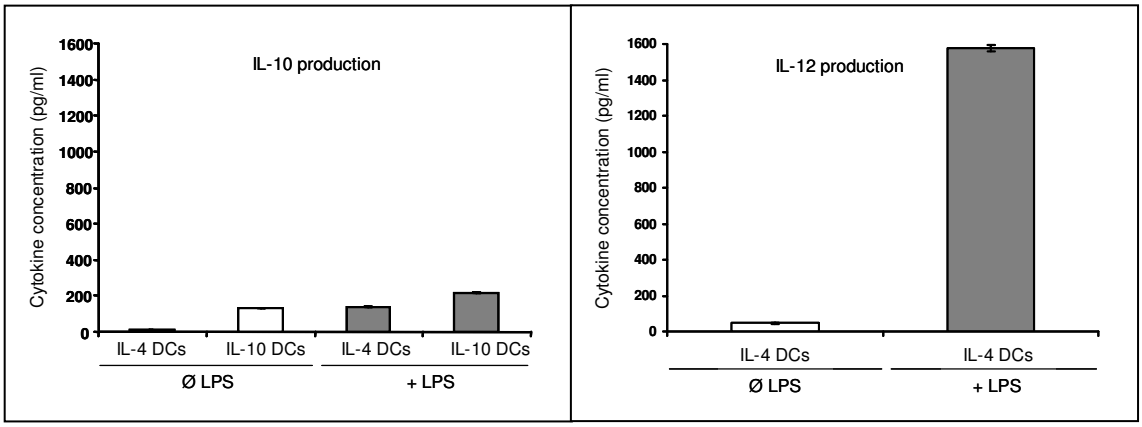


Figure 4.8: ***The LPS-induced production of IL-10 and IL-12 by IL-4 DCs and IL-10 DCs***. For this, 10^5 cells/well of a 96-well plate were stimulated for 24 hours in a final volume of 150 µl. The amount of IL-10 $^{(*)}$ and IL-12 were measured with specific ELISA. The results (mean ± standard deviation) shown are of one experiment in triplicate wells. The values (pg/ml) are: IL-4 DCs: 12.4 ± 0.4; IL-10 DCs: 130 ± 2; IL-4 DCs + LPS: 138 ± 1.9; IL-10 DCs + LPS: 216 ± 1.8.

The results demonstrate that immature BM-DCs produced IL-12 in response to the LPS treatment. The results for IL-4 DCs are shown in the right diagram of **Figure 4.8**: 45 ± 5 pg/ml IL-12 for non-treated IL-4 DCs and 1,576 ± 20 pg/ml IL-12 after the 24 hours treatment with LPS. IL-12, a heterodimeric cytokine, plays an important role in the induction of T-helper 1 (Th1) responses. The RT-PCR results in **Figure 4.11** revealed that the immature IL-4 DCs and IL-10 DCs expressed no mRNA encoding for IL-12.

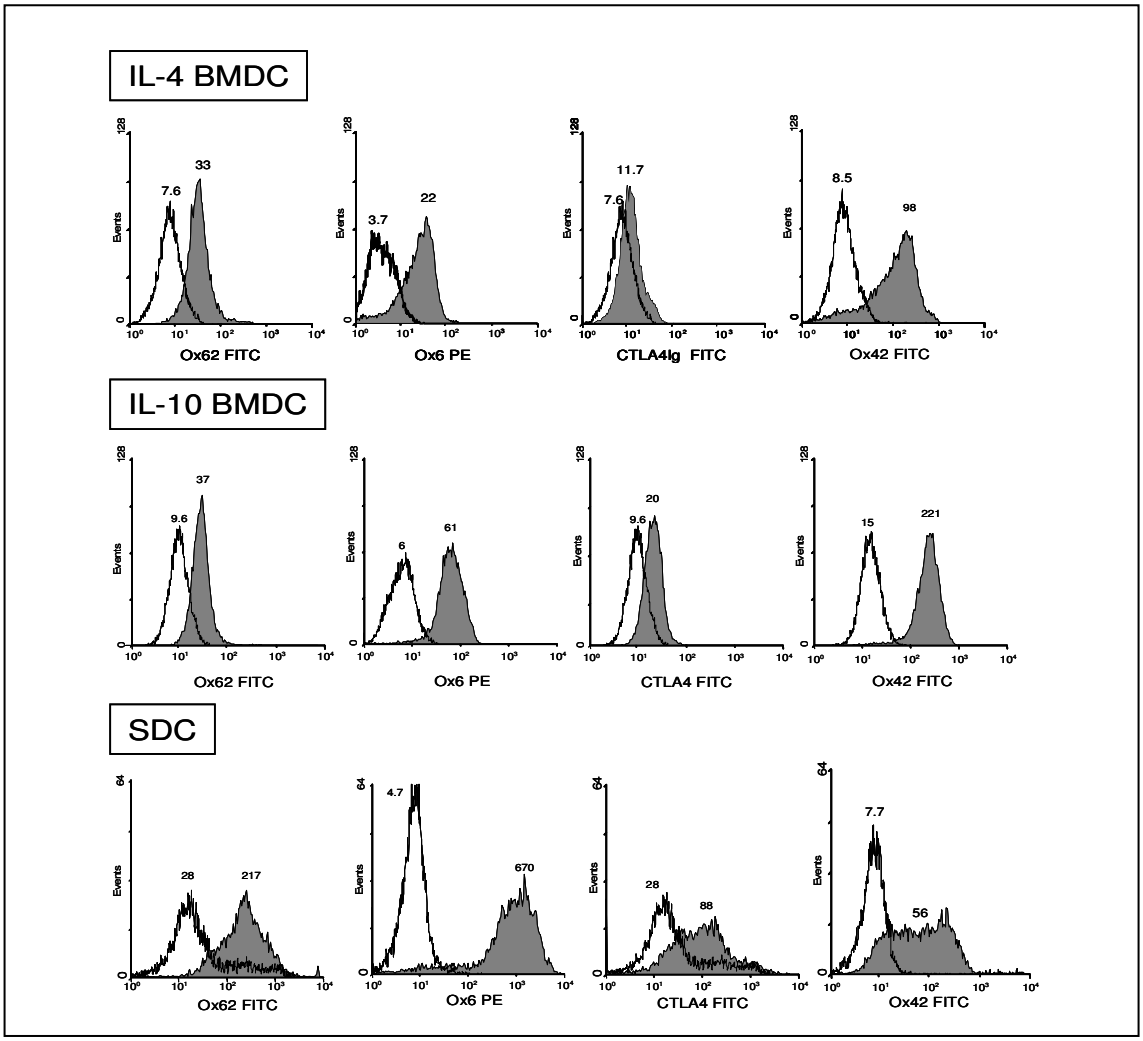
4.2 Characterization of bone marrow-derived immature dendritic cells

4.2.1 The flow cytometric analysis demonstrates single bone marrow-derived dendritic cells with low surface expression of costimulatory molecules

The phenotypes of immature BM-DCs and splenic DCs, as typical mature DCs, were compared. The flow cytometric analysis showed that both immature and mature DCs were positive for Ox-62 (an integrin-like molecule used as a marker for rat DCs) as well as for Ox-42 (binds CD11b/c molecule on the surface of dendritic cells and macrophages). The comparison of the fluorescence intensity for Ox-62 indicates that IL-4 DCs and IL-10 DCs have reduced expression levels of the integrin-like molecule.

Most of the IL-4 DCs and IL-10 DCs expressed MHC class II molecules on their cell surface as shown by staining with the monoclonal antibody Ox-6 (anti-MHC class II). However, in comparison to mature S-DCs their surface expression of MHC class II was decreased 10-fold (**Figure 4.9**). In addition, both subtypes had a low surface expression of the costimulatory molecules CD80 and CD86 as shown when stained with the CTLA-4Ig fusion protein (anti-CD80/86). The expression of costimulatory molecules was clearly lower than on mature splenic DCs. No difference in the surface expression of MHC class II or CD80 and CD86 was found between IL-4 DCs and IL-10 DCs. In summary, the phenotype of IL-4 DCs and IL-10 DCs was very similar.

Figure 4.9 (next page): ***Flow cytometric phenotyping of immature IL-4 DCs and IL-10 DCs in comparison to mature S-DCs***. The immature BM-DC subtypes did not demonstrate obvious differences in their phenotype. They were positive for the rat DC marker Ox-62, Ox-42 (monocytes/macrophages marker) and Ox-6 (anti-MHC class II). The cell surface expression of costimulatory molecules (demonstrated by the failure to stain with CTLA-4Ig fusion protein) on immature BM-DCs was lower than on mature S-DCs which confirms the immature phenotype of BM-DCs. The open curves represent the background staining and the numbers on the top of the histograms indicate the mean fluorescence intensity of different surface molecules (for more details see **Table 10.2** in the appendix). The results shown are representative for four flow cytometric analysis.



4.2.2 The immunohistochemistry demonstrates bone marrow-derived dendritic cells positive for MHC class II molecules

The monoclonal antibodies Ox-62, Ox-6, and ED1 (macrophage marker) were tested. Most of the cells (> 95%) were positive for the rat DC marker Ox-62 (**Figure 4.10**). High numbers of enlarged cells with an irregular shape were visible all of them being positive for MHC class II (Ox-6).

Cytospins of purified day 6 IL-4 DCs and IL-10 DCs were stained with CTLA-4Ig fusion protein to prove the surface expression of CD80 and CD86. More than 95% of the immature DCs demonstrated sometimes a low rate of surface

expression for these costimulatory molecules (results not shown). It seems that they have a mixed phenotype at this stage of development (day 6). This was similar for IL-4 DCs as well as for IL-10 DCs.

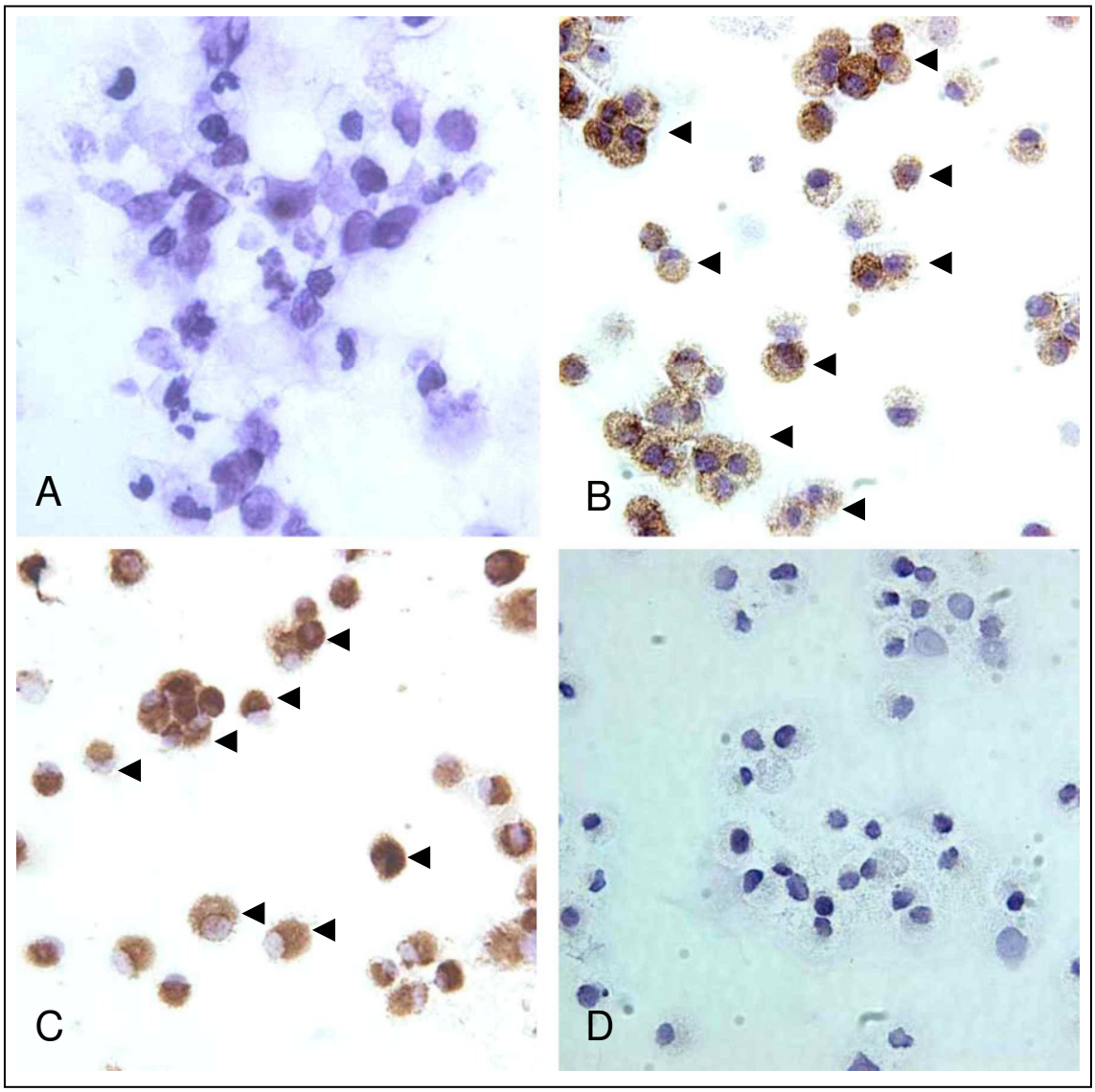


Figure 4.10: ***Cytospin preparations of IL-4 DCs from day 6 of culture***. (A) Negative control, (B) Ox-62, (C) Ox-6, (D) CTLA-4Ig. The staining is representative for four independent experiments. Magnification: x200 using an Olympus BX 50 microscope. Images were captured using a digital camera and processed with Corel Photo-Paint 11.

4.3 The RT-PCR analysis of bone marrow-derived dendritic cells demonstrates specific mRNA for MHC class II molecules, costimulatory molecules, and interleukin-10

Since the low expression of costimulatory molecules is considered a sign for the immaturity of the dendritic cells, the expression of mRNA for the costimulatory molecules CD80, CD86 and CD40 was also investigated (**Figure 4.11**). The results demonstrate that immature BM-DCs were positive for these mRNAs. However, as shown in **Figure 4.9** the bone marrow-derived dendritic cells did not demonstrate a strong surface expression of these costimulatory molecules.

It was also investigated whether IL-4 DCs and IL-10 DCs produced different cytokines which allow them to modulate the T cell response (**Figure 4.8**). Interleukin-12, for instance, which plays an important role in the allograft rejection, is able to direct the immune response towards a Th1 phenotype both *in vitro* and *in vivo*.

The RT-PCR analysis demonstrates that freshly isolated IL-4 DCs and IL-10 DCs did not express mRNA for IL-12 in contrast to the mature S-DCs. Interleukin-10 is another important cytokine with a suppressive effect on activated T cells as well as on the development of mature antigen-presenting cells. Both subtypes of BM-DCs expressed IL-10 mRNA (see **Table 10.3** in the appendix). The cytokine production of LPS-treated DCs is shown in **Figure 4.8**.

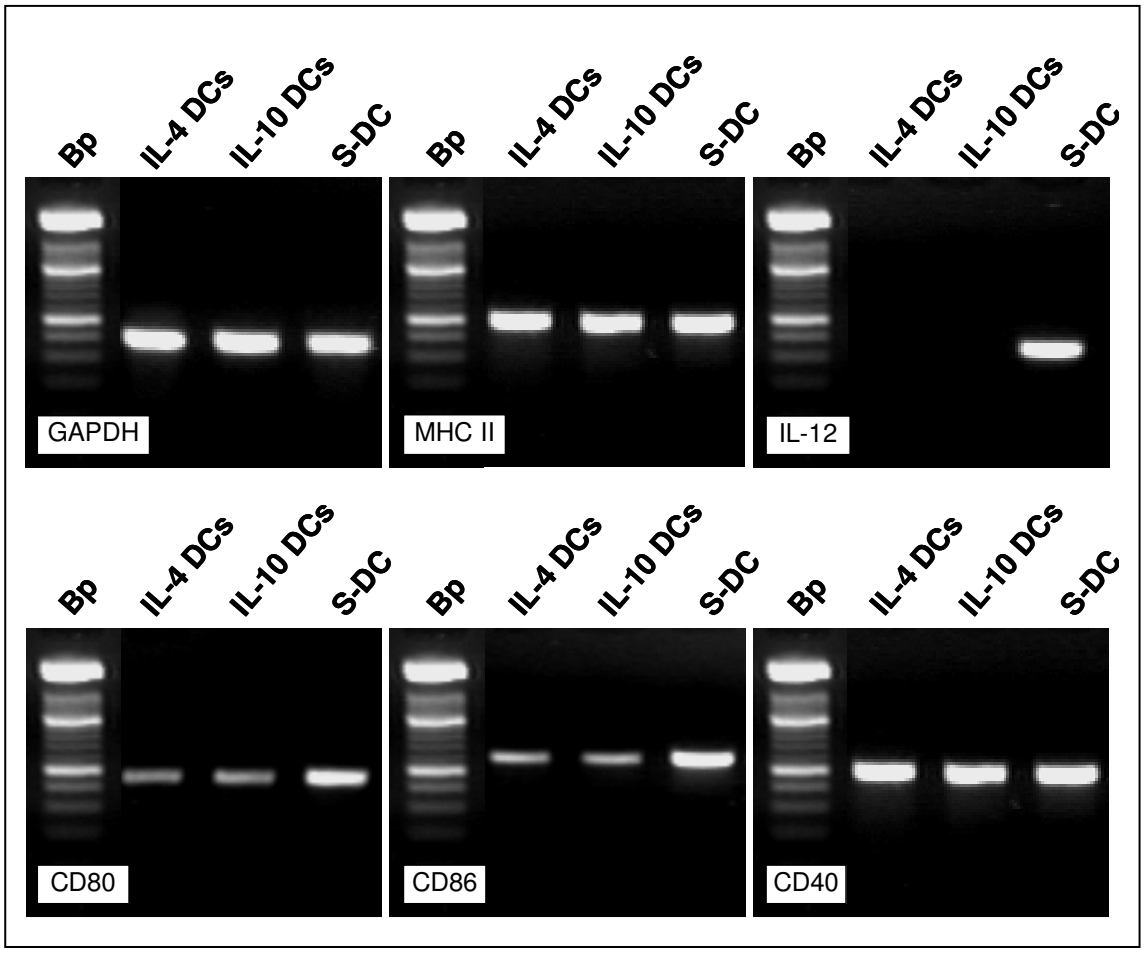


Figure 4.11: ***RT-PCR analysis of IL-4 DCs and IL-10 DCs***. Mature splenic DCs (S-DCs) were used as controls. All three cell subsets expressed mRNA for MHC class II and the costimulatory molecules CD40, CD80, and CD86. The S-DCs were positive for IL-12 mRNA (see the appendix also). The presented results are from a single experiment but they are representative for five different experiments that were performed. Additional information regarding the PCR products can be found in chapter Materials and Methods (**Table 3.4**). For more details see **Table 10.3** in the appendix.

In addition to the qualitative proof of MHC class II mRNA, a quantification step was performed. The real time RT-PCR quantification showed comparable levels of specific mRNA for MHC class II between mature S-DCs and immature BM-DCs (**Figure 4.12)**. The levels of copies in the mature S-DCs were approx. 0.317 ± 0.105

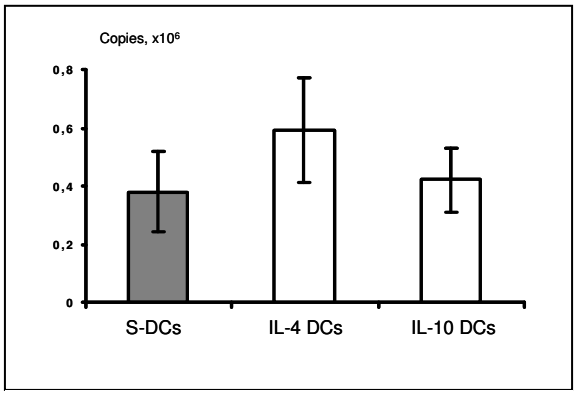


Figure 4.12: ***The real time RT-PCR quantification of MHC class II***. Results (mean ± standard deviation) from 3 different experiments are shown.

million copies. The same levels were also found for IL-4 DCs (0.410 ± 0.106 million copies) and IL-10 DCs (0.366 ± 0.107 million copies). With 0.018 ± 0.0003 million copies freshly isolated bone-marrow cells did not express MHC class II (not shown).

4.4 Bone marrow-derived immature dendritc cells take up antigen

The ability of the immature BM-DCs to take up antigen was assessed using fluorescein-isothyocyanate (FITC) conjugated Dextran. The FITC-Dextran incorporation was tested at different time points by flow cytometry. Purified IL-4 DCs and IL-10 DCs from day 6 of culture were incubated with FITC-conjugated Dextran for 6 and 16 hours. The analysis showed an increased FITC-Dextran uptake depending on the length of incubation time (**Figure 4.13**). Formalin-fixed BM-DCs were used as negative control to exclude the possibility that FITC-Dextran binds at the cell surface.

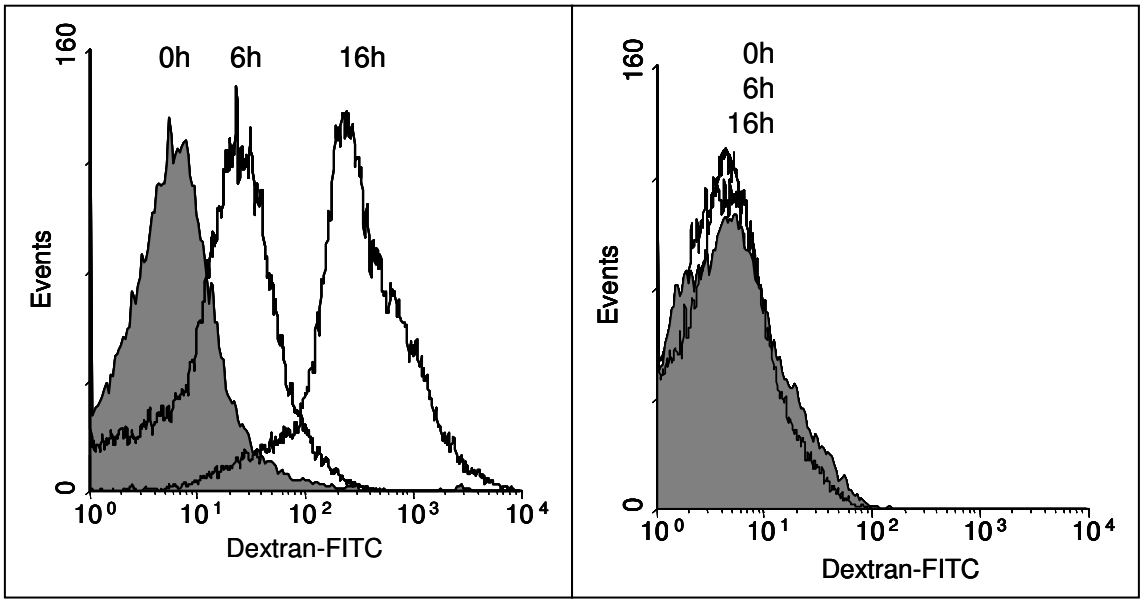


Figure 4.13: ***The flow cytometric analysis of the uptake of FITC-conjugated Dextran***. Both IL-4 DCs and IL-10 DCs were able to internalize antigens in a time-dependent manner (left side). The cells incubated with FITC-conjugated Dextran were measured after 6 and 16 hours. Formalin-fixed BM-DCs were used as negative controls (right side). They were not able to incorporate FITC-conjugated Dextran. Both, IL-4 DCs and IL-10 DCs were tested for their endocytosis capacity and the data shown here are representative for three different experiments with IL-4 DCs (IL-10 DCs showed similar effects).

4.5 Bone marrow-derived immature dendritic cells are weak stimulators for T cells

The ability of IL-4 and IL-10 DCs to activate naïve T cells was tested in the mixed leukocyte culture (MLC), an *in vitro* assay used to estimate the strength of the direct pathway of allorecognition (**Figure 1.1**). Naïve T cells from the popliteal lymph nodes of Wistar Furth rats did not proliferate in the presence of IL-4 DCs and IL-10 DCs as stimulator cells (**Figure 4.14 A**). The measured proliferation rates were 3,000 ± 500 cpm for IL-4 DCs and 2,600 ± 300 cpm for IL-10 DCs. In contrast, mature S-DCs induced a strong proliferation of naïve T cells from Wistar Furth, up to 27,000 ± 2,500 cpm. The same inabillity of IL-4 DCs and IL-10 DCs was observed for the restimulation of antigen-specific T cells in the T cell proliferation assay, an *in vitro* assay used to evaluate the potency of the indirect pathway of alloantigen recognition (**Figure 1.1**).

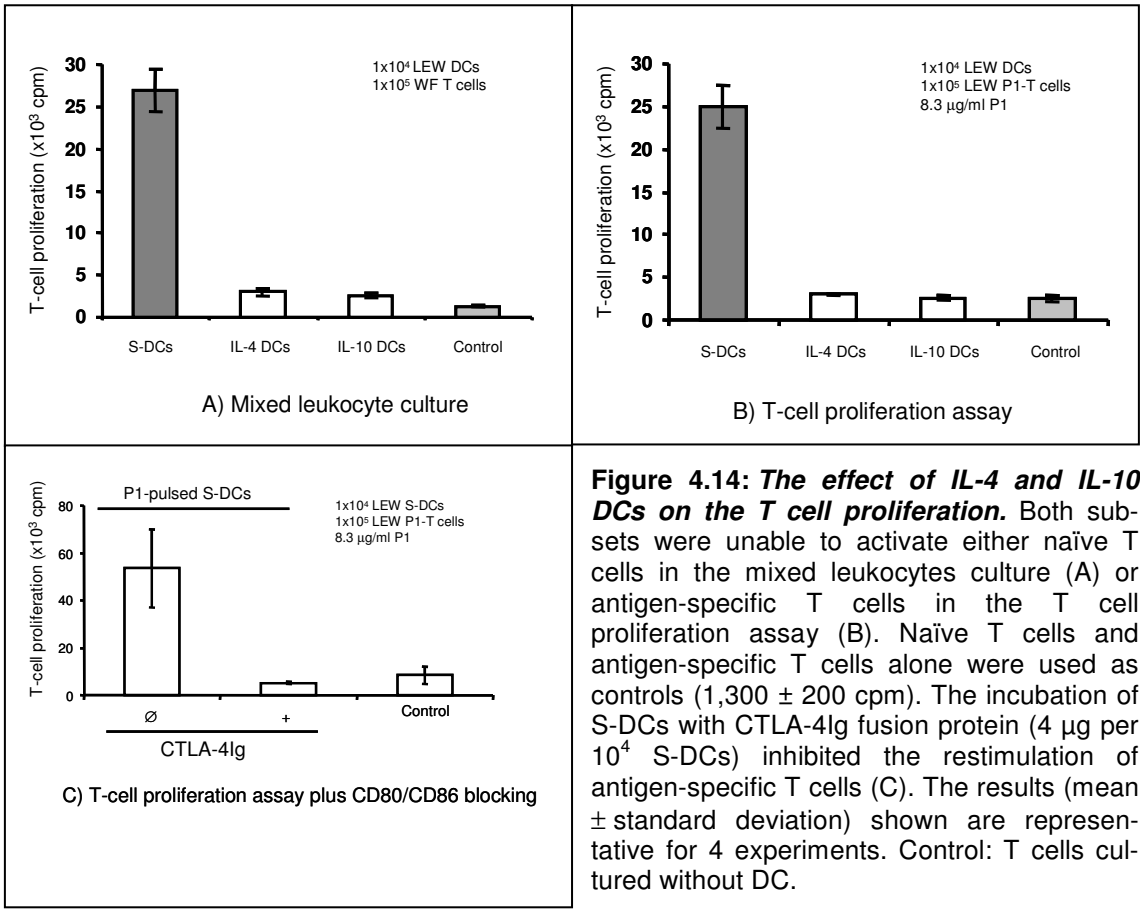


Figure 4.14: ***The effect of IL-4 and IL-10 DCs on the T cell proliferation.*** Both subsets were unable to activate either naïve T cells in the mixed leukocytes culture (A) or antigen-specific T cells in the T cell proliferation assay (B). Naïve T cells and antigen-specific T cells alone were used as controls (1,300 ± 200 cpm). The incubation of S-DCs with CTLA-4Ig fusion protein (4 µg per 10^4 S-DCs) inhibited the restimulation of antigen-specific T cells (C). The results (mean ± standard deviation) shown are representative for 4 experiments. Control: T cells cultured without DC.

In addition, the mature S-DCs incubated with CTLA-4Ig fusion protein failed as stimulators of the proliferation assay (**Figure 4.14 C**). This indicates the importance of costimulation mediated by CD80 and CD86 molecules.

4.6 Characterization of the inhibitory effect of bone marrow-derived immature dendritic cells

4.6.1 The inhibitory effect of IL-4 DCs and IL-10 DCs on T cell proliferation is not prevented by IL-2

Exogenous IL-2 was able to increase dramatically the proliferation of antigen-specific T cells in the proliferation assay up to 50,500 ± 4,500 cpm in a concentration-dependent manner as shown in **Table 4.4** and **Figure 4.15**. The exogenous IL-2 was also able to increase the autostimulation of P1-specific T cells (up to 28,000 ± 6,600 cpm). The autostimulation is a phenomenon which is characterized by the ability of activated T cells to express MHC class II and, therefore, to present peptides within those molecules (see also page 27). The addition of IL-2 delivers a supplementary activation signal to these T cells and should increase their proliferation. In contrast, this stimulatory effect of IL-2 was very low when the P1-specific T cells were cultured with IL-4 DCs and IL-10 DCs (**Table 4.4** and **Figure 4.15**).

Table 4.4: ***IL-2-dependent T cell proliferation in dependence of the DC subset used.*** The addition of IL-2 to antigen (P1)-specific T cells increased their proliferation rates in a concentration-dependent manner whether they were incubated with P1-loaded S-DC or not. In contrast, the addition of IL-2 to the cultures of P1-specific T cells with P1-loaded IL-4 DCs or IL-10 DCs did not increase the T cell proliferation (see also Figure 4.15).

	IL-2 concentration (ng/ml)		
DC subset	**0**	**133**	**266**
∅	3,100 ± 100 *)	22,800 ± 7,900	28,000 ± 6,600
S-DCs **)	25,000 ± 2,500	40,000 ± 3,400	50,500 ± 4,500
IL-4 DCs **)	3,100 ± 100	3,200 ± 500	3,500 ± 600
IL-10 DCs **)	2,600 ± 300	2,900 ± 800	4,100 ± 750

*) Results (mean ± standard deviation) of T cell proliferation in cpm.
**) Loaded with peptide P1.
∅ No DCs, P1-specific T cells were cultured in the presence of P1 without DCs.

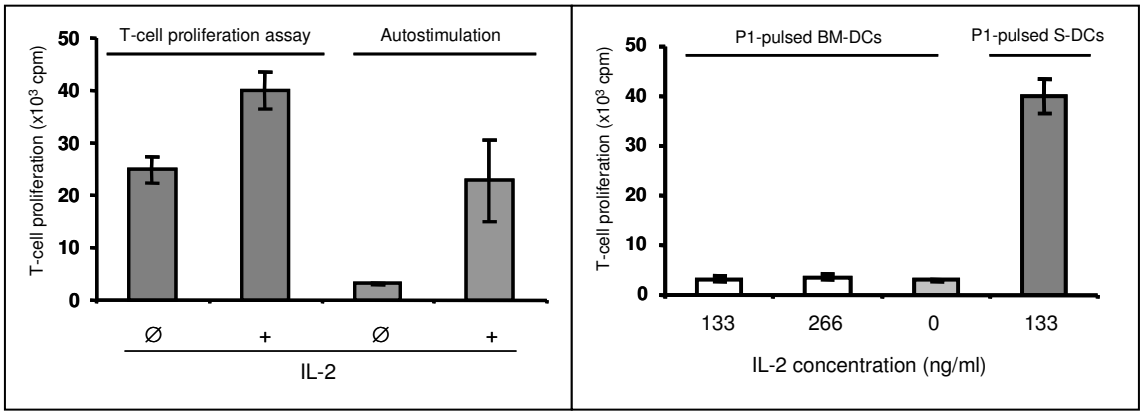


Figure 4.15: ***IL-2 dependent T cell proliferation***. The addition of IL-2 to P1-specific T cells alone (autostimulation) or to the T cell proliferation assay induced an increased T cell proliferation (diagram on the left). Exogenous IL-2 in different concentrations did not increase the antigen-specific T cell proliferation when these T cells were incubated with IL-4 DCs or IL-10 DCs. The diagram on the right shows the results for IL-4 DCs (IL-10 DCs showed the same effect). The results (mean ± standard deviation) are representative for 3 different experiments. (∅) = without IL-2 and (+) = 133 ng/ml IL-2. The values are shown in Table 4.4.

4.6.2 The supernatants from IL-4 DCs and IL-10 DCs inhibit the proliferation of antigen-specific T cells

The effect of supernatant from IL-4 DCs and IL-10 DCs on the T cell proliferation was tested in order to investigate the presence of soluble factors with a suppressive potential secreted by immature DCs. For this, purified immature DCs from day 6 of culture were transferred to 96-well culture plates (10^4 cells/well) and cultured without the addition of any cytokines in order to avoid their possible influence on the T cell proliferation. Twenty-four hours later, the supernatant from these cultures was gently collected and transferred in different volumes (10 µl, 25 µl, and 50 µl) to the proliferation assay (**Table 4.5** and **Figure 4.16**). The final volume was kept constant at 150 µl per well in each experiment. The results showed clearly a dose-dependent reduction of the T cell proliferation when supernatant from IL-4 DCs and IL-10 DCs was transferred. This effect was not observed when supernatant from cultures with S-DCs was tested (**Table 4.5**).

Table 4.5: ***The supernatant from IL-4 DCs and IL-10 DCs inhibited the antigen-specific T cell proliferation in the proliferation assay***.[1)] The strength of inhibition was increased with the volume of transferred supernatant. As control ("zero volume") 50 µl from cultures of S-DCs were used in order to test possible negative effects of day 2 supernatant, e.g. the exhaustion of essential nutrition components may influence the T cell proliferation. The results (median ± standard deviation) from 4 experiments for IL-4 respectively for IL-10 DCs are shown in the table (see also Figure 4.16).

	Volume of supernatant (µl) *)			
	10	**25**	**50**	**0**
IL-4 DCs	11,000 ± 1,000 **)	5,700 ± 1,400	3,100 ± 900	35,200 ± 5,100
IL-10 DCs	15,400 ± 800	8,600 ± 2,000	4,600 ± 900	

1) P1-specific T cells were incubated with P1-loaded S-DCs.
*) The final volume was kept constant at 150 µl per well.
**) Results (mean ± standard deviation) of T cell proliferation in cpm.

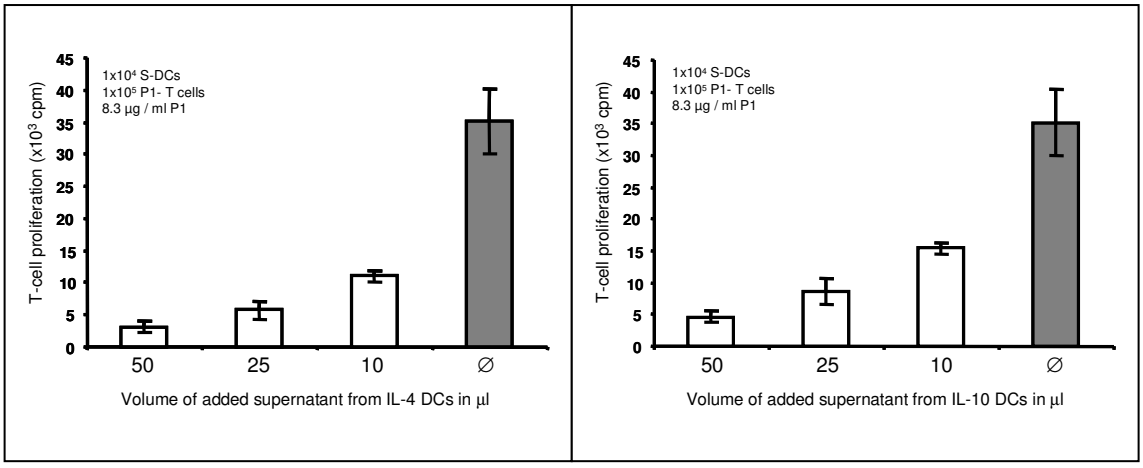


Figure 4.16: ***The inhibitory effect of supernatant from cultures of IL-4 DCs and IL-10 DCs***. Supernatant from both IL-4 and IL-10 DCs was able to reduce the T cell proliferation in a concentration-dependent manner. For this, different volumes of supernatant were transferred to the proliferation assay. The data (mean ± standard deviation) are representative for 4 different experiments, for both IL-4 DCs and IL-10 DCs. (∅) 50 µl from cultures of S-DCs were added to the proliferation assay. The values are shown in Table 4.5.

In the next series of experiments it was tested whether exogenous IL-2 is able to influence the inhibitory effect of supernatant from IL-4 DCs and IL-10 DCs in the T cell proliferation assay. As shown in **Table 4.6** and **Figure 4.17** different concentrations of IL-2 were not able to compensate the inhibitory effect transferred with the supernatant from IL-4 DCs or from IL-10 DCs.

Table 4.6: ***The effect of exogenous IL-2 on the suppressive effect of supernatant from IL-4 DCs and IL-10 DCs.*** The inhibitory effect of supernatant from IL-4 DCs on the proliferation of antigen-specific T cells was not reversed by the addition of exogenous IL-2. In contrast, the inhibitory effect of supernatant from IL-10 DCs was abolished with high doses of IL-2. Addition of IL-2 to the cultures containing supernatant from S-DCs increased the T cell proliferation.

	Transferred volume of supernatant (50 µl) from DC cultures		
IL-2 (ng/ml)	**IL-4 DCs**	**IL-10 DCs**	**S-DCs**
∅	3,100 ± 900 *)	4,600 ± 900	25,000 ± 2,500
133	2,600 ± 1,500	8,500 ± 750	40,000 ± 3,400
266	3,500 ± 600	27,800 ± 2,000	86,200 ± 9,900

*) Results (mean ± standard deviation) of T cell proliferation in cpm. (∅) = no IL-2 was added.

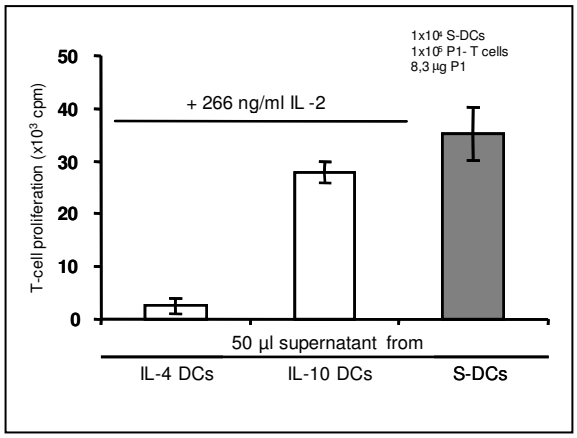


Figure 4.17: ***The effect of exogenous IL-2 on the suppressive effect of supernatant from IL-4 DCs and IL-10 DCs.*** Exogenous IL-2 was not able to induce the proliferation of antigen-specific T cells in the presence of supernatant from IL-4 DCs. In contrast, the proliferation was reconstituted in those wells containing supernatant from IL-10 DCs combined with 266 ng/ml IL-2. This proliferation was similar to control wells with supernatant from S-DCs and 133 ng/ml IL-2. The results (mean ± standard deviation) are representative for 3 different experiments. The values are shown in Table 4.6.

4.6.3 IL-4 DCs and IL-10 DCs inhibit the restimulation of antigen-specific T cells dependent on their number

The inhibitory effect of BM-DCs on the restimulation of antigen-specific T cells (**Figure 4.14 B**) was analysed in more detail. For this, the effect of different numbers of IL-4 DCs and IL-10 DCs on the T cell proliferation was tested (**Table 4.7** and **Figure 4.18**). The inhibitory effect was dependent on the number of BM-DCs transferred to the culture. The proliferation of P1-specific T cells in the presence of P1-pulsed S-DCs was 35,200 ± 5,100 cpm. The addition of 10^3 IL-4 DCs or IL-10 DCs to 10^4 mature S-DCs reduced the T cell proliferation and 10^5 IL-4 DCs or IL-10 DCs totally inhibited the proliferation of P1-T cells.

Table 4.7: ***The inhibitory effect of IL-4 DCs and IL-10 DCs is dependent on their cell number.*** Different numbers of IL-4 DCs and IL-10 DCs were transferred to the proliferation assay as described in "Materials and Methods" (3.8). Both subtypes of BM-DCs inhibited the T cell proliferation dependent on their number. (∅) = no BM-DCs were present in the proliferation assays (see also Figure 4.18).

	Number of BM-DCs			
	10^3	10^4	10^5	∅
Ratio BM-DCs:S-DCs	1:10	1:1	10:1	----
IL-4 DCs	24,600 ± 4,500 *)	21,000 ± 2,600	3,200 ± 1,300	35,200 ± 5,100
IL-10 DCs	26,900 ± 5,000	13,100 ± 1,500	2,000 ± 500	

*) Results (mean ± standard deviation) of T cell proliferation in cpm.

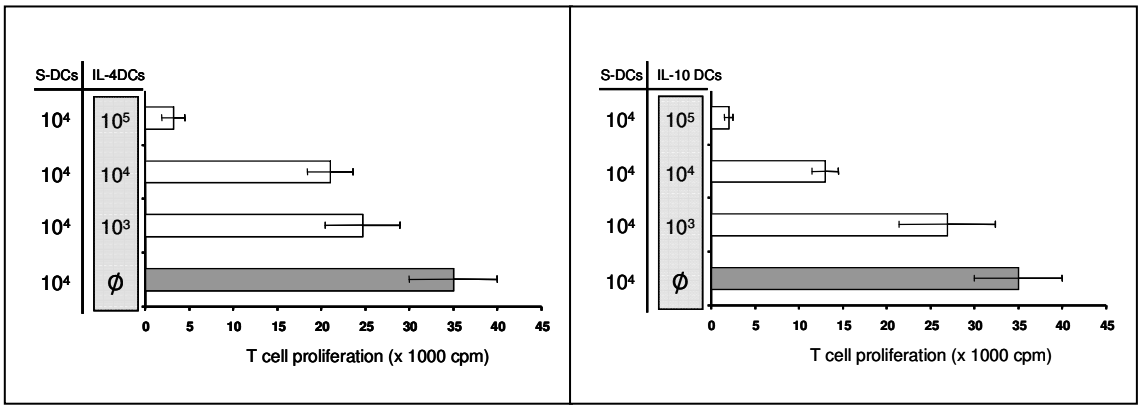


Figure 4.18: ***The inhibitory effect of IL-4 DCs and IL-10 DCs is dependent on their cell number.*** Different numbers of IL-4 DCs (left) or IL-10 DCs (right) influenced the strength of T cell proliferation when added to the cultures of 10^4 S-DCs and 10^5 antigen-specifc T cells. 10^4 IL-10 DCs seem to be more powerful in suppressing the T cell proliferation than 10^4 IL-4 DCs. The results (mean ± standard deviation) are representative for 3 different experiments. The values are shown in Table 4.4 (see also the commentary in the appendix).

4.6.4 IL-4 DCs and IL-10 DCs mediate a fast inhibitory effect

For understanding the next set of experiments it is necessary to know the proliferation rates in the T cell proliferation assay at different time points. After 1 day: below 10,000 cpm, after 2 days: 32,000 ± 5,000 cpm and after 3 days: 70,900 ± 11,000 cpm. The effect of the addition of different numbers of immature DCs to the proliferation assay at different time points was tested (**Table 4.8** and **Figure 4.19**). Immature bone marrow-derived dendritic cells were

added to the cultures between day 0 (the starting point of the culture) and day 3 (the end of the culture). Subsequently, the cells were pulsed with [^{3}H]-thymidine on day 3. In contrast to the uninfluenced proliferation rate on day 2 (32,000 ± 5,000 cpm; see above) no incorporation was measured under these conditions (**Table 4.8** and **Figure 4.19**). The reason for this is that the addition of immature BM-DCs to the proliferation assay on day 2 immediately inhibited the T cell proliferation and therefore, the "arrested" (non-proliferating) T cells could not incorporate [^{3}H]-thymidine when pulsed subsequently on day 3. This is the reason why no proliferation could be measured on day 3.

Table 4.8: ***The correlation between the T cell proliferation and the time point of addition of DCs.*** The addition of BM-DCs to the proliferation assay at different time points (day 0, 1, 2) was able to block the proliferation of antigen-specific T cells. For this, different times of cell addition were proved. All plates were pulsed on day 3 as described in material and methods. The results are representative for a set of 3 experiments for each dendritic cell type (see also Figure 4.19).

	Day of addition		
	0	**1**	**2**
IL-4 DCs	1,037 ± 610 *)	900 ± 400	1,500 ± 300
IL-10 DCs	1,130 ± 400	1,500 ± 100	1,700 ± 400

*) Results (mean ± standard deviation) of T cell proliferation in cpm.

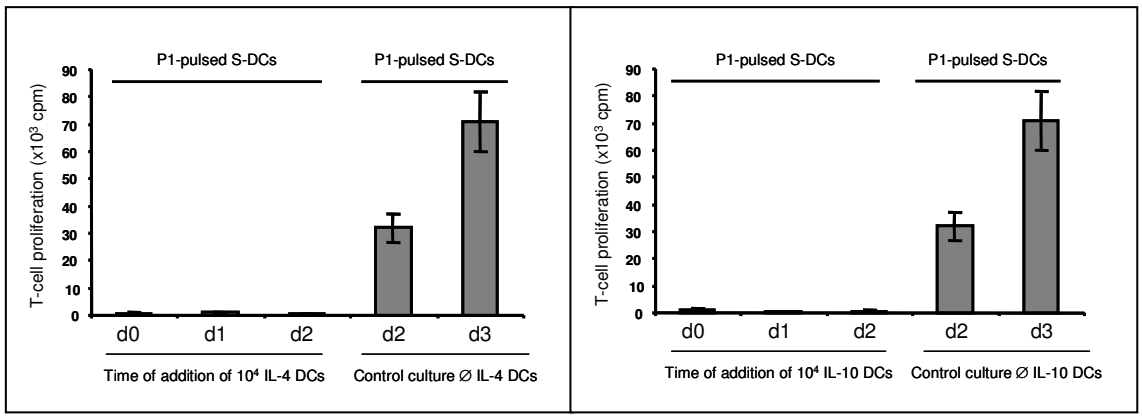


Figure 4.19: ***Immature BM-DCs inhibited the antigen-specific T cell proliferation***. No incorporation of [^{3}H]-thymidine was measured when IL-4 DCs and IL-10 DCs were added to the proliferation assay on day 0, 1 or 2. The cells were pulsed on day 3 as described in "Materials and Methods". The values are shown in Table 4.8. In contrast, a high rate of T cell proliferation was measured on day 2 (32,000 ± 5,000 cpm) and 3 (70,900 ± 11,000 cpm) in the presence of

P1-pulsed S-DCs. The control cultures were set up without BM-DCs (∅ IL-4 DCs or ∅ IL-10 DCs). The results (mean ± standard deviation) are representative for 3 different experiments (see also the commentary in the appendix).

For the next set of experiments, the time point where the cells were pulsed with [^{3}H]-thymidine was modified in order to determine the strength of T cell proliferation before the immature BM-DCs were added to the cultures. As described above, the presence of BM-DCs completely inhibited the cell division of activated T cells between day 2 and 3 and therefore, the incorporation of [^{3}H]-thymidine was not possible. In order to measure the real level of T cell proliferation before the addition of BM-DCs, the procedure of [^{3}H]-thymidine pulsing was modified so that the T cells were pulsed on day 2, 6 hours before the immature BM-DCs were added to the cultures (**Table 4.10** and **Figure 4.20**). The incorporated radioactivity was measured at the end of culture on day 3. The results showed indeed an antigen-specific T cell proliferation until day 2 (15,500 ± 1,500 cpm for IL-4 DCs and 17,000 ± 2,000 cpm for IL-10 DCs) before 10^4 immature BM-DCs were added. The addition of immature DCs suppressed the heavy increase of T cell proliferation between day 2 and day 3 observed in the normal proliferation assay. These results demonstrate the quick inhibitory effect mediated by IL-4 DCs and IL-10 DCs.

Table 4.10: ***Immature BM-DCs inhibited immediately the antigen-specific T cell proliferation.*** The modified pulsing protocol (see text) showed that the antigen-specific T cells proliferated until the BM-DCs were added to the culture. The addition of 10^3 or 10^4 IL-4 DCs or IL-10 DCs to the proliferation assay on day 2 suppressed the increase of T cell proliferation observed in the uninfluenced T cell proliferation assay (**Figure 4.20**). The results (mean ± standard deviation) are representative for a series of 3 experiments for each type of BM-DCs.

	Number of BM-DCs	
	10^3	**10^4**
IL-4 DCs	31,000 ± 3,200 *)	15,500 ± 1,500
IL-10 DCs	32,000 ± 5,300	17,000 ± 2,000

*) Results (mean ± standard deviation) of T cell proliferation in cpm.

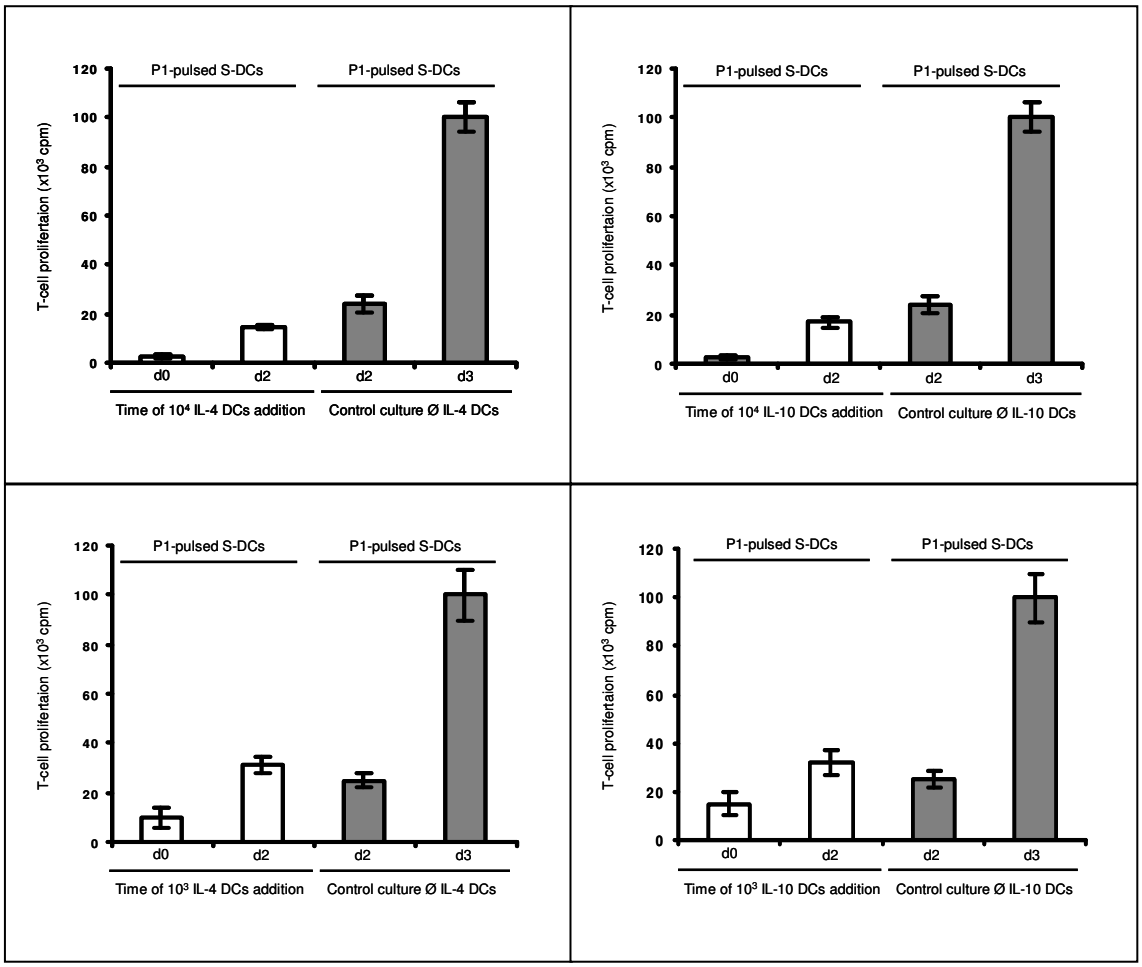


Figure 4.20: ***Immature BM-DCs inhibited immediately the antigen-specific T cell proliferation***. The addition of 10^4 IL-4 DCs or 10^4 IL-10 DCs to the proliferation assay on day 2 suppressed the extremely strong increase in T cell proliferation between day 2 (25,000 ± 3,200 cpm) and day 3 (100,000 ± 10,000 cpm) in the normal proliferation assay (grey columns). The same effect was also observed when 10^3 IL-4 DCs or 10^3 IL-10 DCs were added to the cultures on day 2. For these experiments the modified pulsing protocol was used (see page 43) The results (mean ± standard deviation) are representative for 3 different experiments. The values are shown in Table 4.10.

4.6.5 The incubation of P1-specific T cells with IL-4 DCs and IL-10 DCs induces an anergic state

Hundred thousand P1-specific T cells were incubated with 10^4 P1-pulsed IL-4 DCs and 10^4 IL-10 DCs for 72 hours. Afterwards, the DCs were depleted with the antibody Ox-42 and magnetic-beads and the resulting T cells (= DC-Ts) were transferred to the second culture consisting of 10^4 P1-pulsed mature S-DCs per well (**Figure 4.21**). After 72 hours the T cell proliferation was determined. The level of [^{3}H]-thymidine incorporation was 1,700 ± 560 cpm in

the case of IL-4 DC-Ts and 6,600 ± 1,000 cpm in the case of IL-10 DC-Ts. This demonstrates that P1-pulsed S-DCs are not able to induce the restimulation of T cells pre-incubated with BM-DCs (termed as IL-4 DC-Ts or IL-10 DC-Ts). In contrast, a very strong proliferation was found in the second cultures when antigen-specific T cells, which were not incubated with BM-DCs during the first culture, were pulsed with P1 (22,100 ± 3,400 cpm) (autostimulation) or incubated with P1-pulsed S-DCs (39,800 ± 4,200 cpm) during the first culture (**Figure 4.21**).

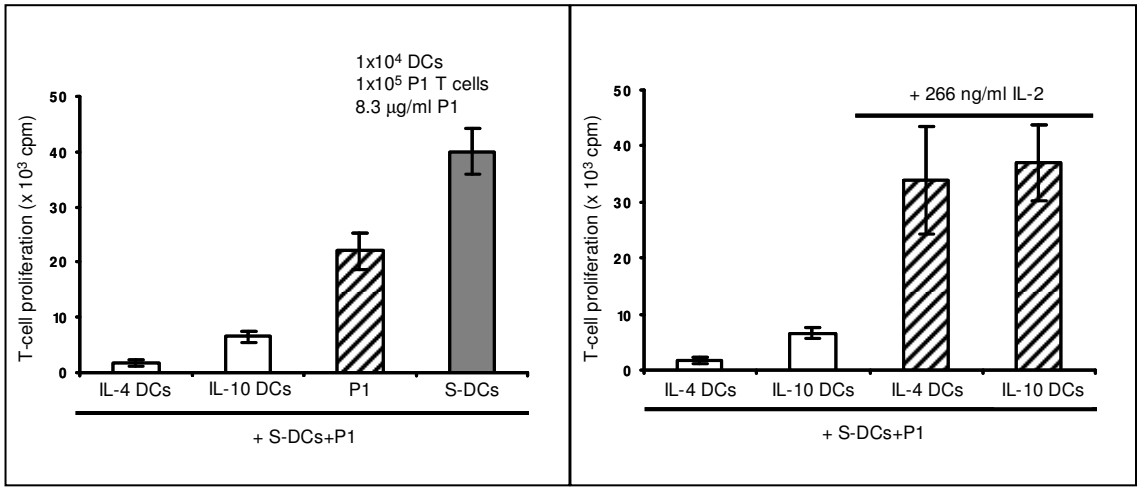


Figure 4.21: ***Antigen-specific T cells pre-incubated with IL-4 DCs and IL-10 DCs (first culture) did proliferate in the presence of P1-pulsed S-DCs after addition of IL-2***. Antigen-specific T cells were pre-incubated with IL-4 DCs and IL-10 DCs for 72 hours (first culture). Afterwards, the purified T cells were transferred to P1-pulsed S-DCs (second culture) shown on the left side. The conditions of the first culture for the antigen-specific T cells are indicated under each column of the diagram: IL-4 DCs: T cells incubated with IL-4 DCs; IL-10 DCs: T cells incubated with IL-10 DCs; T cells alone: T cells were incubated without antigen-presenting cells; S-DCs: T cells incubated with mature S-DCs. The diagram on the right side: Addition of IL-2 in a concentration of 266 ng/ml to the second culture rescued the T cells from the non-proliferative state induced by the BM-DCs. The columns represent the T cell proliferation before and after the addition of IL-2. The results (mean ± standard deviation) are representative for 3 different experiments.

In order to test the strength of the inhibition induced by IL-4 DCs and IL-10 DCs on antigen-specific T cells, exogenous IL-2 was added to the second culture. The results indicate that both subtypes of BM-DCs induced an anergic state in the antigen-specific T cells which can be reverted by the addition of exogenous IL-2. A concentration of IL-2 of 266 ng/ml was able to induce an increase of proliferation from 1,700 ± 560 cpm to 34,000 ± 9,600 cpm in the case of IL-4 DCs and from 6,600 ± 1,000 cpm to 36,900 ± 6,800 cpm in the case of IL-10 DCs (**Figure 4.21**).

4.6.6 P1-specific T cells incubated with IL-4 DCs but not with IL-10 DCs mediate an inhibitory effect

In the previous chapter, it was shown that P1-specific T cells which were cultured with IL-4 DCs and IL-10 DCs did not proliferate when they were transferred to P1-loaded mature splenic DCs. However, this anergic-like effect could be reverted by adding exogenous IL-2 (**Figure 4.21**, right diagram). In the following series of experiments, these T cells (termed as DC-Ts) were tested for their potential to suppress the proliferation of uninfluenced P1-specific T cells restimulated in the proliferation assay. The DC-Ts recovered from the first culture with IL-4 DCs and IL-10 DCs were transferred to the T cell proliferation assay in different numbers (from 10 cells up to 10^4 cells per well). These cultures were incubated for 3 days and the proliferation was measured afterwards. The results showed that the T cells incubated with IL-4 DCs in the first culture (= IL-4 DC-Ts) were able to inhibit the proliferation of the P1-T cells in the proliferation assay depending on their number (**Figure 4.22**). This effect was not seen for the IL-10 DC-Ts which were pre-incubated with IL-10 DCs.

Table 4.11: ***P1-specific T cells pre-incubated with IL-4 DCs had an inhibitory effect on T cell proliferation.*** These IL-4 DC-Ts were able to inhibit the proliferation of antigen-specific T cells in the proliferation assay depending on the added cell number. The transfer of T cells pre-incubated with IL-10 DCs (= IL-10 DC-Ts) had no effect irrespective of how many cells were transferred (see also Figure 4.22).

Number of IL-4 DC-Ts transferred to the proliferation assay *)		
10^3	**10^4**	**10^5**
36,500 ± 4,600 **)	23,600 ± 8,700	5,900 ± 3,300
Number of IL-10 DC-Ts transferred transferred to the proliferation assay *)		
10^3	**10^4**	**10^5**
41,000 ± 9,500 **)	43,900 ± 8,000	40,000 ± 9,600

*) IL-4 DC-Ts: pre-incubated with IL-4 DCs; IL-10 DC-Ts: pre-incubated with IL-10 DCs.
**) Results (mean ± standard deviation) of T cell proliferation in cpm.

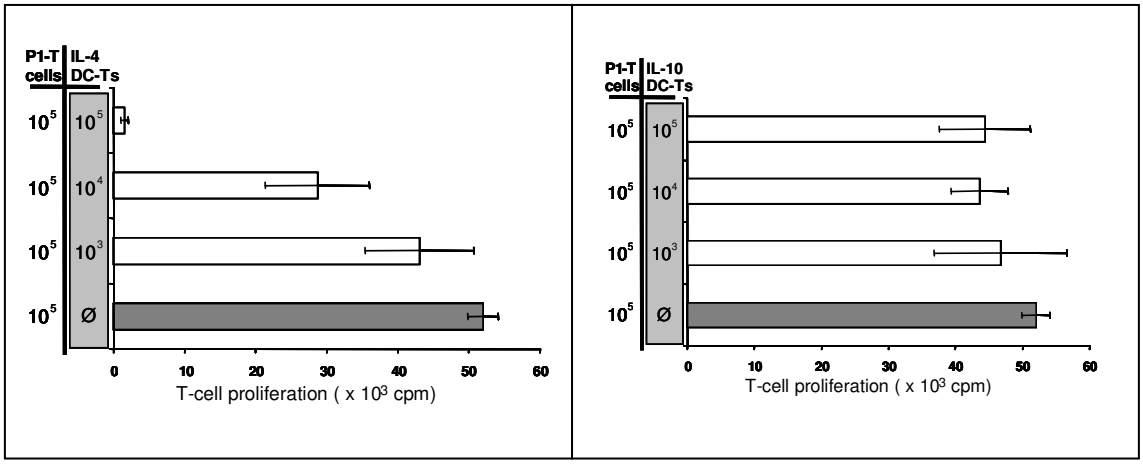


Figure 4.22: ***P1-specific T cells incubated with IL-4 DCs but not with IL-10 DCs seem to mediate an inhibitory effect on the proliferation of antigen-specific T cells***. P1-specific T cells were pre-incubated for 72 hours with P1-pulsed IL-4 DCs or IL-10 DCs. Afterwards, the dendritic cells were depleted and the purified T cells were transferred to a second culture, the T cell proliferation assay consisted of P1-pulsed S-DCs and antigen-specific T cells as described in "Materials and Methods". The inhibitory effect of IL-4 DC-Ts (antigen-specific T cells which were incubated with IL-4 DCs) was dependent on the number of transferred cells (left diagram and **Table 4.11**). In contrast, IL-10 DC-Ts (T cells which were pre-incubated with IL-10 DCs were not able to influence the proliferation of P1-T cells in the proliferation assay. The results (mean ± standard deviation) from 3 different experiments (each for IL-4 DC-Ts and IL-10 DC-Ts) are shown. DC-T cells: P1-specific T cells pre-incubated with the appropriate BM-DCs; P1-T cells: P1-specific T cells freshly isolated from P1-immunized Lewis rats.

In order to estimate whether the effect of IL-4 DCs on antigen-specific T cells will be impaired when they are separated by a membrane, both cell subsets were cultured in transwell plates from Greiner bio-one, Germany (**Figure 4.23**). The P1-pulsed IL-4 DCs were incubated in the upper chambers (the transwell insert) and the P1-T cells in the lower compartment. Direct contact between the IL-4 DCs and the T cells was avoided by a membrane with a pore size of 0.4 µm. After 3 days the T cells (= IL-4 DC-Ts) were transferred to the proliferation assay or second culture. The unaffected T cell proliferation in the second culture was 102,500 ± 15,500 cpm. However, the IL-4 DC-Ts, which were separated by a membrane from the IL-4 DCs during the first culture, did not show any inhibitory effects on the T cell proliferation even at high numbers (**Table 4.12**).

These results demonstrate that the direct contact between the IL-4 DCs and the antigen-specific T cells in the first culture is absolutely necessary in order to

induce regulatory properties in these T cells. However, these experiments could not elucidate whether the IL-4 DCs mediated their inhibitory effect through a soluble factor small enough to pass the membrane.

Table 4.12: ***IL-4 DC-Ts needed the direct contact with IL-4 DCs to demonstrate an inhibitory effect***. The transfer of antigen-specific T cells pre-incubated with IL-4 DCs (= IL-4 DC-Ts) in transwell plates had no effect on the proliferation of antigen-specific T cells in the proliferation assay (see also **Figure 4.23**). In contrast, antigen-specific T cells pre-incubated with IL-4 DCs (= IL-4 DC-Ts) without separation by a membrane demonstrated an effect on the proliferation of antigen-specific T cells (**Table 4.11**).

	Number of transferred IL-4 DC-Ts			
	10^2	10^3	10^4	10^5
T cell proliferation	116,000 ± 11,000 *)	127,000 ± 12,000	128,000 ± 14,000	111,000 ± 8,000

*) Results (mean ± standard deviation) of T cell proliferation in cpm.

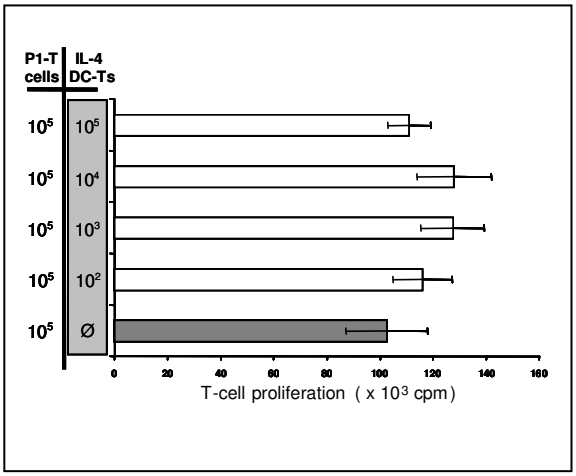


Figure 4.23: ***IL-4 DC-Ts needed the direct contact with IL-4 DCs to demonstrate an inhibitory effect.*** In the first culture the T cells were incubated in the upper chamber of a transwell plate. IL-4 DCs were pulsed with P1 and cultured in the lower chamber of the same transwell plate. After 72 hours the T cells from the first culture (IL-4 DC-Ts or IL-10 DC-Ts were transferred to a second culture or proliferation assay *) and incubated for another 72 hours. No inhibitory effect of the transferred cells was observed. The results (mean ± standard deviation) are representative for 3 different experiments.#)

*) The proliferation assay consists of 10^4 P1-loaded S-DCs and 10^5 P1-specific T cells.
#) The values are shown in **Table 4.12**.

4.7. The *in vivo* effects of bone marrow-derived immature dendritic cells

Seven days after subcutaneous immunization with the immunogeneic peptide P1, the popliteal lymph nodes were enlarged (**Figure 4.24**) and the cell number increased from $3.3 \times 10^7 \pm 1.4 \times 10^7$ in non-immunized animals to $12.3 \times 10^7 \pm 2.7 \times 10^7$ in P1-immunized animals (not shown).

The next set of experiments with IL-4 DCs and IL-10 DCs were performed *in vivo* in order to clarify whether these cells are able to influence the local immune response. Therefore, the effect of P1-pulsed S-DCs was compared to the effect mediated by IL-4 DCs and IL-10 DCs, respectively. The cells were injected subcutaneously into the hind foot pad of naïve Lewis rats (**Figure 4.24**). Peptide P1 mixed with the adjuvant TiterMax were used as control. The popliteal lymph nodes were collected 7 days after injection and the isolated lymphocytes were tested in the proliferation assay. The immunization with P1 induced a strong local immune response as indicated by the enlarged lymph nodes (**Figure 4.24**) and an increased T cell proliferation (90,000 ± 8,900 cpm) (**Figure 4.25**). The same T cell proliferation was observed after the injection of P1-loaded S-DCs (81,000 ± 8,900 cpm). In contrast, P1-loaded IL-4 DCs caused a decreased T cell proliferation of 33,900 ± 4,600 cpm (**Figure 4.14B**). P1-loaded IL-10 DCs induced an increased T cell proliferation of 70,900 ± 11,000 cpm (**Figure 4.14B**). From the results *in vitro* (**Figure 4.14**) it was expected that both types of BMDCs prevent the induction of antigen-specific T cells in the local lymph nodes (see also appendix).

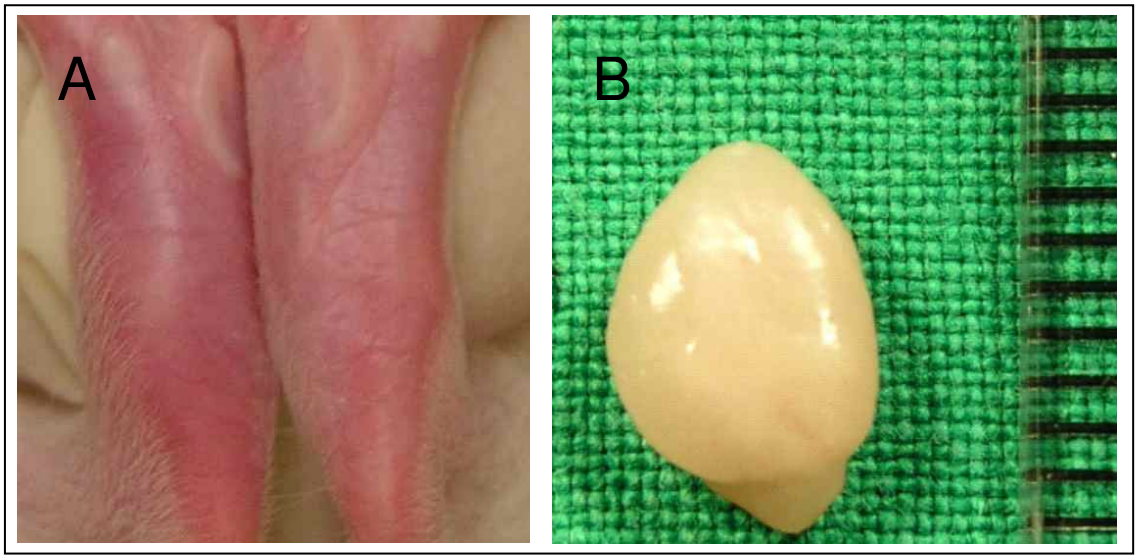


Figure 4.24: ***The subcutaneous immunization with peptide P1 and TiterMax lead to a strong local immune response***. This immune activation is not obviously reflected in swelling of the foot pad (A; left: non-immunized, right: immunized) but in the enlarged popliteal lymph nodes. They increased from 1-2 mm in non-immunized animals to 6-7 mm in P1-immunized animals (B). The immunization with TiterMax did not enlarge significantly the lymph nodes (2-3 mm). The distance between the lines represents 1 mm.

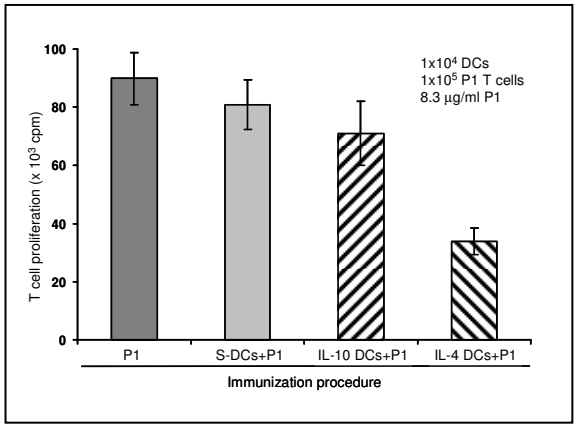


Figure 4.25: ***Subcutaneous immunization with P1 lead to a strong proliferation of T cells isolated from the popliteal lymph nodes***. Seven days after immunization the isolated lymphocytes were tested in the proliferation assay with P1-loaded S-DCs. P1-loaded S-DCs or P1-loaded IL-10 DCs led to a similar reaction in the local lymph node. P1-loaded IL-4 DCs induced a weaker local immune response. The results are representative for 3 different experiments for each immunization procedure.

The level of non-stimulated antigen-specific T cell proliferation was 17,900 ± 2,000 cpm (not shown).

The main question remained whether P1-loaded immature DCs influenced the survival time of Wistar Furth allografts in Lewis rats (**Figure 3.1**). Therefore, P1-loaded IL-4 DCs and IL-10 DCs were transferred into Lewis rats 7 days or 1 day prior to transplantation of a Wistar Furth heart (see also **Figure 10.1** in the appendix). Peptide P1 led to an accelerated rejection of Wistar Furth allografts but not of syngeneic Lewis grafts (**Table 4.13**, groups 2 and 4).

Table 4.13: ***The immunization of Lewis rats with the allogeneic peptide P1 seven days before transplantation influence the survival of heart allografts from Wistar Furth donors***. In the allogeneic control (Ctr) group, Lewis rats were not immunized with P1. Untreated Lewis rats in the syngeneic control (Ctr) group and P1-immunized Lewis rats in group 4 received syngeneic heart grafts. The immunization was performed 7 days before transplantation.

Group [#)]	Graft survival (days)	MST ± SD (days) [#,#)]	n
1 Allogeneic Ctr	7 (x4), 8 (x2)	7.3 ± 0.5	6
2 Allogeneic Ctr + P1 [*)]	5 (x2), 6	5.2 ± 0.4	3
3 Syngeneic Ctr [**)]	> 50 (x3)	> 50	3
4 Syngeneic Ctr + P1 [*) **)]	> 50 (x3)	> 50	3

[#)] Direction of transplantation: Group 1: Wistar Furth → Lewis
Group 2: Wistar Furth → P1-immunized Lewis
Group 3: Lewis → Lewis
Group 4: Lewis → P1-immunized Lewis

[#,#)] MST: mean survival time; SD: standard deviation.

[*)] Peptide P1 was mixed with TiterMax before injection. TiterMax does not influence the survival time of heart allografts: 7.7 ± 0.6 days (n=3); (single time points: 7 (x1), 8 (x2)).

[**)] The syngeneic transplantation led to indefinite graft survival (> 100 days); here the experiments were regularly terminated on day 50 p. op.

For the transplantation experiments, 10 million IL-4 DCs or IL-10 DCs were administered intravenously 7 days or 1 day before transplantation (1 million cells had no effect; see **Table 10.1** in the appendix). On day 0, the animals received a heart allograft in a heterotopic position and the allograft survival was estimated by palpation of the heart beating (**Figure 3.1**). The transfer of P1-loaded BM-DCs one day before transplantation led to better results than the cell transfer 7 days before transplantation (**Table 4.14** groups 6-9). Both IL-4 DCs and IL-10 DCs seem to have a slight protective effect on the allograft function. They prolonged the survival time for two days without immunosuppression in comparison to the allogeneic control group (**Table 4.13**, group 1 vs. groups 8 and 9 in **Table 4.14**).

Table 4.14: ***Ten million P1-pulsed IL-4 DCs and IL-10 DCs slightly prolong the survival of heart allografts of Wistar Furth donors in Lewis recipients***. P1-pulsed DCs and unpulsed control (Ctr) DCs were injected i.v. into Lewis rats 7 days (d -7) or 1 day (d -1) before the transplantation of WF heart grafts. Untreated Lewis rats did not receive DCs before transplantation. For more details see **Table 10.1** in the appendix.

Group [#)]		Graft survival (days)	MST ± SD (days) [#,#)]	n
5	Ctr-DC (d -7, d -1) [*)]	6, 7 (x6), 8	7.0 ± 0.8	8
6	IL-4 DC + P1 (d -7) [**)]	7 (x2), 8 (x4), 9	7.9 ± 0.7	7
7	IL-10 DC + P1 (d -7) [**)]	7 (x2), 9 (x3)	8.2 ± 1.1	5
8	IL-4 DC + P1 (d -1) [**)]	9 (x3), 11	9.5 ± 1.0	4
9	IL-10 DC + P1 (d -1) [**)]	8, 9 (x2), 10 (x5)	9.5 ± 0.8	8

[#)] Direction of transplantation: Wistar Furth → Lewis.
[#,#)] MST: mean survival time; SD: standard deviation.
[*)] Unpulsed IL-4 and IL-10 DC were used as controls (n=4 for each DC subtype.
[**)] Ten Million DCs were loaded with 20 µg P1 in a total volume of 500 µl PBS for 30 minutes at 4°C.

In order to prove the antigen specificity of P1-pulsed immature BM-DC in prolongation of WF allografts, their effect on third party allografts was measured. For this, Brown Norway (BN) rats were used as third party donors. Again, P1-loaded IL-4 or IL-10 DCs were transferred intravenously into Lewis rats one day before transplantation. As shown in **Table 10.1** (in the appendix)

heart allografts from Brown Norway donors were not protected by the administration of P1-loaded BM-DCs. This indicates that the prolongation of WF allograft survival induced by P1-pulsed BM-DCs is, indeed, an antigen-specific effect manifest only for Wistar Furth allografts.

Table 4.15: ***Ten million P1-pulsed IL-10 DCs do not influence the survival of heart allografts from third party donors (Brown Norway rats)***. The P1-pulsed IL-10 DCs were injected i.v. one day (d -1) before transplantation. The recipients in the untreated controls were not infused with DCs. For more detail see **Table 10.1** in the appendix.

Group [#)]		Graft survival (days)	MST ± SD (days) [#,#)]	n
10	Untreated	7 (x3)	7.0 ± 0.0	3
11	IL-10 DC + P1 (d -1) [*)]	7 (x3)	7.0 ± 0.0	3

[#)] Direction of transplantation: BN → Lewis.
[#,#)] MST: mean survival time; SD: standard deviation.
[*)] Ten million DCs were loaded with 20 µg P1 in a total volume of 500 µl PBS for 30 minutes at 4°C.

To prove whether the effect of P1-pulsed IL-10 DCs could be enhanced, the amount of transferred DCs was increased to 30 million cells. As demonstrated in **Table 4.16** (group 12) the protective effect was increased and the allograft survival was prolonged to day 10 (**Table 4.16**). The effect of 30 million P1-pulsed IL-4 DCs will be tested, too.

Table 4.16: ***Thirty million P1-pulsed IL-10 DCs prolong the survival of heart allografts of Wistar Furth donors in Lewis recipients (group 12)***.

Group [#)]		Graft survival (days)	MST ± SD (days) [#,#)]	n
1	Allogeneic Ctr [*)]	7 (x4), 8 (x2)	7.3 ± 0.5	6
9	IL-10 DC + P1 (d -1) [*)]	8, 9 (x2), 10 (x5)	9.5 ± 0.8	8
12	IL-10 DC + P1 (d -1) [**)]	10 (x3), 11 (x2), 12	10.6 ± 0.8	6

[#)] Direction of transplantation: Wistar Furth → Lewis.
[#,#)] MST: mean survival time; SD: standard deviation.
[*)] See tables 4.12 and 4.13.
[**)] Thirty million DCs were loaded with 20 µg P1 in a total volume of 500 µl PBS for 30 minutes at 4°C.

5 Conclusions

The present study demonstrates an immune inhibitory effect for IL-4 DCs and IL-10 DCs *in vitro* and *in vivo*. The results provide the answers to the following questions:

(1) What are the main phenotypic characteristics of immature bone marrow-derived dendritic cells?

A homogenous population of rat immature dendritic cells was generated from bone marrow precursors cultured with GM-CSF and IL-4 (= IL-4 DCs) or GM-CSF and IL-10 (= IL-10 DCs). These cells had a similar immature phenotype and showed no or a very low surface expression of costimulatory molecules such as CD80 and CD86. They also demonstrated a 10-fold reduced expression of MHC class II molecules on the cell surface in comparison to mature splenic DCs (**Figure 4.9**).

(2) What is the effect of immature bone marrow-derived dendritic cells on naïve and antigen-specific T cells?

Neither IL-4 DCs nor IL-10 DCs were able to activate naïve T cells or to restimulate antigen-specific T cells (**Figure 4.14**). This strong inhibitory effect, mediated within 24 hours (**Figure 4.20**), was dependent on the number of immature dendritic cells added to the proliferation assay (**Figure 4.18**). Antigen-specific T cells pre-incubated with IL-4 DCs and IL-10 DCs were not able to proliferate in the presence of P1-pulsed S-DCs. This anergic state was reversible with the addition of exogenous IL-2 (**Figure 4.21**).

(3) Do P1-pulsed immature bone marrow-derived dendritic cells influence the allograft survival in an antigen-specific manner?

The *in vivo* results demonstrate that these cells play an important role in the antigen-specific prolongation of allograft survival (**Tables 4.13** and **4.15**). Both IL-4 DCs and IL-10 DCs loaded with P1 were able to prolong the allograft survival in an antigen-specific manner.

6 Discussion

The use of immature dendritic cells with regulatory properties for the induction of graft acceptance instead of chronic immunosuppression appears to be very attractive. However, the diversity between humans and the common animal models mice and rats makes the development of a "universal" protocol for immunomodulation difficult. Presently, rat immature dendritic cells are only poorly characterized. Therefore, in the present study, the morphology, phenotype, and tolerogenic function of rat immature bone marrow-derived dendritic cells were characterized.

6.1 IL-4 or IL-10 in combination with GM-CSF influence the promotion of immature rat dendritic cells

Immature rat dendritic cells (DCs) were propagated from bone marrow cells isolated from big bones such as femurs and tibia (Chen-Woan M et al., 1995; MacDonald KP et al., 2005). In the present study it was shown that a cell density of 5.0×10^5 bone marrow precursors per ml is ideal to generate approximately 2.5×10^6 to 5.0×10^6 bone marrow DCs per culture dish (**Table 4.1**). These findings are in accordance with the results published by Grauer (Grauer O et al., 2002). The experiments performed in the present study showed that the yield of immature dendritic cells generated from up to 30×10^6 bone marrow cells (isolated from two femurs) was more than sufficient for different *in vitro* assays and adoptive transfer experiments (chapter 4.7).

In the present study, the rat bone marrow-derived immature dendritic cells generated in the presence of GM-CSF plus IL-4 were termed IL-4 DCs and those generated in the presence of GM-CSF plus IL-10 were termed IL-10 DCs.

Both cytokine combinations, GM-CSF plus IL-4 and GM-CSF plus IL-10, promote the differentiation of bone marrow precursor cells into immature dendritic cells as confirmed in the present study. Many publications describe the

successful generation of immature dendritic cells with these cytokine combinations in different species, e.g. human, mouse and rat (Kubsch S et al., 2003; Zheng Z et al., 2004; Lutz MB et al., 2000 [1,2]; DePaz HA et al. 2003). However, it is not always clearly defined how the different concentrations of cytokines influence the maturation state of dendritic cells in different animals. For example, GM-CSF (1 ng/ml and more as the final concentration) combined with IL-4 induces the formation of mature BM-DCs in mice (Lutz MB et al., 2000 [1]), whereas the same combination propagates immature dendritic cells in rats (Garrovillo M et al., 2001).

In the present study, 5 ng/ml GM-CSF combined with IL-4 (5 ng/ml) was used to generate immature DC. These results are opposed to the findings of other groups, where such high doses of GM-CSF generated potent immune stimulatory DCs (Lutz MB et al., 1999). The combination of GM-CSF with IL-10 was shown to be able to induce the formation of immature bone marrow dendritic cells in mice and rats (Duan RS et al., 2005).

Both the phenotype and the immature state of IL-4 DCs and IL-10 DCs seem to be identical: the cells demonstrate a very low surface expression of costimulatory molecules as well as a 10-fold reduced surface expression of MHC class II molecules (**Figure 4.9**). In addition, these cells were positive for the Ox-62 epitope, an integrin-like molecule that is characteristic for rat dendritic cells. The high endocytosis capacity, demonstrated by measuring the uptake of FITC-conjugated Dextran, is another characteristic pleading for the immaturity of these cells. It is well known that immature dendritic cells demonstrate a high capacity to take up antigens but an inability to present them, whereas mature dendritic cells are professional antigen presenting cells with a low capacity of endocytosis (Hotta C et al., 2006). The immature dendritic cells recovered from the cell clusters of a 6-day culture (**Figure 4.4**) were found to be negative for the fibroblast marker prolyl 4-hydroxylase in contrast to the strongly adherent cells with a fibroblast-like shape outside the clusters (not shown). In summary, these

data confirm that the cells propagated in the presence of GM-CSF plus IL-4 and GM-CSF plus IL-10 are dendritic cells in an immature stage of development.

6.2 The nature of the inhibitory effect mediated by immature dendritic cells: indications for soluble and non-soluble factors

None of the IL-4 DCs or IL-10 DCs have the capacity to stimulate naïve T cells (**Figure 4.14 A**). This was not surprising because these cells had a low or no expression of the costimulatory molecules CD80 and CD86 as demonstrated by flow cytometry (**Figure 4.9**). In addition, the RT-PCR analysis showed the presence of mRNA for CD80, CD86 and CD40.

In addition, the immature BM-DCs were also unable to restimulate antigen-specific T cells (**Figure 4.14 B**). The reason for this may be the noted inability of these cells to fully express costimulatory molecules and, in consequence, their inability to deliver optimal signals to the T cells. The immune inhibitory effect caused by the block of costimulation was confirmed by *in vitro* experiments with the CTLA-4Ig fusion protein (**Figure 4.14 C**). This protein, which blocks the interaction between CD28 on the surface of T cells and the costimulatory molecules CD80 and CD86 on the surface of DCs, inhibits the stimulatory effect of mature DCs in the T cell proliferation assay. The block of costimulation also has an inhibitory effect *in vivo*: for example, the combined application of anti-CD40 and anti-CD86 antibodies prolonged the allograft survival in primates (Haanstra KG et al., 2003; Guillot C et al., 2003; Bjorck P et al., 2005).

In a further set of experiments, BM-DCs were added in different cell numbers (10^3 - 10^5) to the proliferation assay with constant numbers (10^4) of splenic DCs (**Figure 4.18**). Immature and mature splenic DCs compete for the antigen-specific T cells and, as expected, the T cell proliferation was totally inhibited in the presence of high numbers of immature DCs (10^5 cells). In addition, the inhibitory effect mediated by the immature DCs was measurable within 24 hours (**Figure 4.20**). In most of the studies, immature DCs were incubated with T cells

for 3 or 4 days prior the proliferation rate was measured (DePaz HA et al., 2003). However, as demonstrated in this study, these types of experiments are not very insightful with regard to the efficacy of immature DCs. Therefore, the protocol was changed and the T cells were pulsed with [^{3}H]-Thymidine before the immature DCs were added. The results showed clearly that immature DCs dramatically inhibited the T cell proliferation within 24 hours. The effect, caused by already one million (10^3) immature IL-4 DCs, was strong enough to prevent the increase of T cell proliferation from 31,000 ± 3,200 cpm to 100,000 ± 10,000 which usually occur within the last 24 hours of culture in the proliferation assay (**Figure 4.20**).

Furthermore, the inhibitory effect of BM-DCs on the T cell proliferation was not reversible with exogenous IL-2. This result was unexpected because the activated T cells expressed the IL-2 receptor and, therefore, they should proliferate strongly in the presence of IL-2 (**Figure 4.15**). In addition, activated rat T cells expressed MHC class II molecules on their cell surface that enabled them to present antigens independently of dendritic cells and allowed them to stimulate each other (Kottenmeier S, 2005). This phenomenon, called auto-stimulation, should also occur in the presence of such costimulation-impaired BM-DCs. However, as demonstrated in this study, the presence of IL-4 DCs and IL-10 DCs completely inhibited the autostimulation (**Figure 4.6**).

The reduced T cell proliferation in the presence of supernatant from IL-4 DCs and IL-10 DCs may indicate the existence of a soluble factor. The inhibitory effect was dependent on the volume of transferred supernatant (**Figure 4.16**). The possibility that immature dendritic cells influence the proliferation of T cells by secreting a soluble factor was already described (Guillot C et al., 2003). In order to avoid any influence by the cytokines used for the DC culture on the T cell proliferation, purified dendritic cells from day 6 were cultured without cytokines. An anti-proliferative effect was found, when the supernatant from these cultures was tested. In contrast, the supernatant from mature S-DCs did not influence the T cell proliferation. In order to exclude the possibility that the

results are influenced by contaminated cells (macrophages, fibroblasts) these cell types were also tested for their possible effect on the T cell proliferation. The results showed that macrophages and fibroblasts are unable to restimulate antigen-specific T cells and they also do not stop the autostimulation in contrast to immature DCs (**Figure 4.6**).

The effect of purified IL-4 and IL-10 on the restimulation of antigen-specific T cells was totally different from the inhibitory effect of supernatant from IL-4 DCs and IL-10 DCs: IL-4 increased the T cell proliferation, whereas IL-10 had no effect (**Tables 10.4** and **10.5** in the appendix).

In summary, these results demonstrate that immature IL-4 DCs and IL-10 DCs are very potent inhibitors of the activation of naïve T cells and the restimulation of antigen-specific T cells. The main reason for their inhibitory effect may be the low expression of costimulatory molecules on their cell surface. However, different other mechanisms such as the secretion of a soluble factor may also be involved (**Figure 4.17**).

6.3 Immature dendritic cells are able to induce anergic T cells with immune inhibitory properties

A very important question addresses the possibility of turning antigen-specific T cells into regulatory T cells with immune inhibitory properties (Kuwana M, 2002). As demonstrated in this study, P1-specific T cells did not proliferate in the presence of IL-4 DCs and IL-10 DCs. A further result of this interaction is that these T cells demonstrated an anergic state (**Figure 4.21**) after the incubation with immature DCs.

For the human system, several groups have demonstrated that antigen-specific CD4+ T cells can be converted into an anergic state (Kuwana M et al., 2001; Steinbrink K et al., 2002). These cells shared similar characteristics with the so-called type I regulatory T cells (Tr1) and they act mainly through secretion of

soluble factors like IL-10 and TGF-β (Bacchetta R et al., 2005). In contrast, no information was found in the literature*) about the effect of rat immature dendritic cells on inducing antigen-specific anergy. Therefore, in the present study, the effect of IL-4 DCs or IL-10 DCs on P1-specific T cells was analysed in more detail. For this, P1-specific T cells were incubated with P1-pulsed IL-4 DCs or IL-10 DCs for 3 days. Afterwards, the purified T cells were transferred to the proliferation assay consisting of P1-pulsed mature S-DCs and antigen-specific T cells. T cells incubated with IL-4 DCs (called IL-4 DC-Ts) were able to inhibit the T cell proliferation in a cell number dependent manner (**Figure 4.22**). In contrast, antigen-specific T cells pre-incubated with P1-pulsed IL-10 DCs (= IL-10 DC-Ts) showed no effect on the proliferation assay. This was the unique difference between IL-4 DCs and IL-10 DCs found in the present study. The suppressive effect of IL-4 DC-Ts cells was confirmed in 3 independent experiments.

The regulatory effect of anergic T cells is well known (Kuwana M. et al., 2002; Jonuleit H et al., 2003; Kubsch S et al., 2003; Bashuda H et al., 2005). Most of these studies described the necessity of a cell-cell contact between the immature dendritic cells and activated T cells in order to induce anergy. The suboptimal presentation of the antigen in the absence of costimulation induces anergy. Both subsets of immature dendritic cells, the IL-4 DCs and the IL-10 DCs, demonstrate, besides the negligible cell surface expression of costimulatory molecules, a ten-fold decreased expression of MHC class II molecules. The need for IL-2 in order to reactivate antigen-specific T cells after the incubation with IL-4 DCs and IL-10 DCs (**Figure 4.21**) indicate their ability to induce anergy in antigen-specific T cells. The experiments in the present study, performed in transwell chamber plates, confirmed that the contact between the antigen-specific T cells and the P1-pulsed IL-4 DCs is essential in order to induce anergy (**Figure 4.23**).

* Medline research with the keywords: rat, antigen-specific T cells, anergy.

From the results presented here it is difficult to conclude whether the suppressive effect of IL-4 DCs or IL-10 DCs is mainly mediated by cell-cell contact or soluble factors. Presently, it is controversial whether soluble factors may be potent enough in order to influence T cell activation in an antigen-specific manner. Therefore, the cell-cell contact mechanism is more important. In addition, a combination of both mechanisms may be possible. The soluble factor might be sufficient to mediate the direct effect of the immature DCs on the proliferation of antigen-specific T cells while the cell-cell contact mechanism might be absolutely necessary in order to induce the regulatory properties of these T cells.

6.4 Immature dendritic cells and their potential to prevent allograft rejection

The immature IL-4 DCs and IL-10 DCs have a series of interesting *in vitro* effects and, therefore, they were also tested *in vivo*. In order to modulate the alloimmune response specifically, the strategy should include the antigen. For the animal model used in the present study the antigen was the allogeneic peptide P1 (**Figure 10.1**). The main idea was to present this antigen, which accelerates allograft rejection, to host T cells in the absence of costimulatory signals in order to turn alloreactive T cells into anergic or regulatory T cells instead of effector T cells. The presence of MHC class II on the cell surface of IL-4 Dcs and IL-10 DCs allows their loading with P1.

One characteristic of the *in vivo* situation is that it is very complex. Therefore, the first question was whether P1-loaded immature BM-DCs influence the local immune response. For this, one million P1-loaded IL-4 DCs and IL-10 DCs were administered subcutaneously into the foot-pad of Lewis rats. S-DCs were used as controls. Seven days later, lymphocytes isolated from the local lymph nodes were tested in the proliferation assay. Lymphocytes from animals immunized with P1-loaded S-DCs were sensitized and therefore showed a strong T cell proliferation in the proliferation assay (**Figure 4.25**). In contrast, after the

injection of P1-loaded IL-4, the T cell proliferation was strongly reduced, whereas the P1-loaded IL-10 DCs did not desensitize the lymphocytes *in vivo*. In this case, the T cells isolated from the local lymph nodes demonstrated a normal proliferation *in vitro*. These results showed that it is possible to influence the local T-cell immune response with P1-loaded dendritic cells.

Further on, the efficiency of P1-pulsed IL4-DCs and IL-10 DCs to prevent allograft survival was proved. The cell transfer performed one day before transplantation was quite efficient than the administration at day -7 (**Table 4.14**). The reason for this is presently unknown. One possible explanation could be that during the long time between cell transfer and transplantation the immature BM-DCs get different maturation stimuli from the environment. The matured DCs are able to activate effector T cells, which decrease the allograft survival time. The positive effect of P1-pulsed BM-DCs on the survival time can be improved with an increase in the number of transferred cells. With 30 million P1-pulsed BM-DCs the survival time was prolonged to a median of 10.6 ± 0.8 days (**Table 4.16**).

For the development of an efficient antigen-specific cell therapy it is necessary to understand which part of the host immune response is affected by such a protocol. Immediately after transplantation the direct pathway of allorecognition (**Figure 1.1**) dominates the immune response. On the other hand, there is evidence that the indirect pathway alone is sufficient in order to induce allograft rejection (Game DS et al., 2002; Nouri-Shirazi M et al., 2002). Therefore, in order to prolong allograft survival, it is important to control both pathways (Lutz MB et al., 2002; Ichim TE et al., 2003; Abe M et al., 2005). The results of the transplantation experiments indicate that it is possible to influence the activation of alloreactive T cells with P1-pulsed BM-DCs (**Tables 4.15** and **4.16**). Another very important aspect is the antigen-specificity of the effect of P1-loaded BM-DCs. This was clearly demonstrated in the present study in the transplantation model where P1-loaded BM-DCs had no protective effect on the survival of third party allografts in Lewis rats (**Figure 10.1** in the appendix).

Rat BM-DCs can be useful to influence the alloimmune response (**Table 6.1**). The *in vitro* results showed an extraordinary ability of these cells to impair the activation of naïve T cells as well as the restimulation of antigen-specific T cells. The present study also demonstrated their effects *in vivo*. Considering the strength of the alloimmune response, even a slight prolongation of the allograft survival of 2 to 4 days is a step forward. These data clearly show that immature DCs influence the complete immune system of immune competent animals. However, the data also demonstrate the necessity to support the cell-mediated inhibitory effect with a short-term immunosuppression. Therefore, different immunosuppressive agents used in the clinic should be tested to find the best one for synergizing the suppressive effect of the immature DCs (Taner T et al., 2005; Sheng Sun D et al., 2005). Beriou described an indefinite allograft survival for more than 100 days when they combined immature DCs with a suboptimal immunosuppressive treatment (Beriou G et al., 2005). In contrast, no immunosuppressive treatment was administered during the experiments described in the present study.

Presently, there is no alternative to the immunosuppressive treatment, yet, immature dendritic cells pulsed with alloantigens seem to be promising candidates for an antigen-specific therapy to eventually replace the unspecific chronic immunosuppression after transplantation (Jonuleit H et al., 2003; Schlichting CL et al., 2005).

Table 6.1: ***Properties of immature bone marrow-derived rat dendritic cells demonstrated in the present study.*** The properties for IL-4 DCs and IL-10 DCs are compared with data from the literature (listed in "References").

	IL-4 DCs	IL-10 DCs	Literature
No surface expression of CD80 and CD86	Yes	Yes	Yes (1, 2)
Reduced surface expression of MHC class II	Yes	Yes	Yes (1)
Inhibition of the activation of naïve T cells	Yes	Yes	Yes (1)
Inhibition of the restimulation of activated T cells	Yes	Yes	Yes (1)
Soluble factor (s) with inhibitory properties	Yes	Yes	Yes (3, 4)
Induction of T cell anergy	Yes	Yes	No data
Induction of anergic T cells with regulatory properties	Yes	No	No data

(1) DePaz HA et al. *Transplantation* 2003; 75 (4): 521-528
(2) Karulin AY et al. *J Immunol* 2000; 164 (4): 1862-1872
(3) Guillot C et al. *Blood* 2003; 15; 101 (8):3325-33
(4) Powell TJ et al. *Immunology* 2003; 109 (2): 197-208

7 Summary

Solid organ transplantation is an established therapeutic approach in modern medicine to extend and to improve the life of patients in the final stages of organ failure. Transplantation between genetically non-identical individuals leads to the activation of the transplant recipient's immune system. This alloimmune response is a consequence of the recognition of foreign MHC molecules by alloreactive host T cells. To prevent their activation and the subsequently induced activation of further cell subsets (e.g. B cells, cytotoxic T cells, macrophages) immunosuppressive drugs are absolutely necessary in the clinic. However, permanent immunosuppression leads to severe side effects such as nephrotoxicity, diabetes and hyperlipidaemia, and a reduced immunity to infections and malignant diseases. At the moment, there is no real alternative to immunosuppression.

The purpose of this study was to analyse the importance of rat dendritic cells with immune inhibitory properties to prevent the immune activation after experimental transplantation. The rat is one of the most important animal models for experimental organ transplantation in a clinic-relevant procedure. In order to modulate the immune response after transplantation in an antigen-specific manner, the strategy should include the alloantigens. These antigens have to be presented by immature dendritic cells in the absence of co-stimulatory signals in order to turn alloreactive T cells into anergic or regulatory T cells instead of effector T cells. For a certain rat model of allograft rejection, the immunodominant peptide P1 was identified as an important alloantigen which accelerates graft rejection. Such a model offers an attractive and practical approach to analyse the potential of host tolerogeneic dendritic cells pulsed with P1 to suppress the allograft-induced immune response in an antigen-specific manner without the need of chronic immunosuppression.

A homogenous population of rat immature dendritic cells was generated from bone marrow precursors cultured with GM-CSF and IL-4 (= IL-4 DCs) or GM-

CSF and IL-10 (= IL-10 DCs). These cells with an identical immature phenotype showed no or a very low surface expression of costimulatory molecules like CD80 and CD86 and a 10-fold reduced expression of MHC class II molecules in comparison to mature splenic DCs. No obvious difference was observed between the phenotype of the IL-4 DCs and the IL-10 DCs.

Neither IL-4 DCs nor IL-10 DCs were able to activate naïve T cells or to restimulate antigen-specific T cells. This strong inhibitory effect, mediated within 24 hours, was dependent on the number of immature dendritic cells added to the proliferation assay. Antigen-specific T cells pre-incubated with IL-4 DCs and IL-10 DCs, respectively, were not able to proliferate in the presence of P1-pulsed mature DCs. This anergic state was reversible with the addition of exogenous IL-2. T cells incubated with IL-4 DCs (= IL-4 DC-Ts) were able to inhibit the T cell proliferation in a cell number dependent manner. In contrast, antigen-specific T cells pre-incubated with P1-pulsed IL-10 DCs (= IL-10 DC-Ts) showed no effect on the proliferation assay. This was the unique difference between IL-4 DCs and IL-10 DCs found in the present study.

Immature DCs influenced also the immune response after transplantation. Different numbers of P1-loaded immature IL-4 DCs and IL-10 DCs were transferred intravenously into Lewis rats one day before transplantation. The best results were obtained with 30 million P1-pulsed immature DCs which prolonged the survival time to a median of 11.2 $\pm$ 1.6 days. In addition, the antigen specificity of this effect was demonstrated with a third-party graft from Brown Norway donors.

These findings suggest that an antigen-specific modulation of the immune response is possible using immature dendritic cells loaded with the allogeneic antigens. Even more, the protocols described in the present study show that the immune system can be, at least temporarily, controlled after transplantation without the use of immunosuppressive drugs.

8 Zusammenfassung

Die allogene Organtransplantation, d.h. die Übertragung zwischen genetisch nicht-identischen Individuen der gleichen Spezies, ist bei irreversiblen Organerkrankungen nach wie vor die Therapie der Wahl. Die Transplantatabstoßung ist eine zum Funktionsverlust von Organtransplantaten führende T-Zell-vermittelte Immunantwort. Ihre Ursache liegt in der Inkompatibilität von Organtransplantat und Transplantat-Empfänger hinsichtlich der Moleküle des Haupthistokompatibilitätskomplexes, die auch als Alloantigene bezeichnet werden. Zwar lässt sich die Transplantatabstoßung mit immunsuppressiven Medikamenten hemmen, doch vermindern diese die Immunabwehr und begünstigen die Entstehung von Infektionen und Tumorerkrankungen. Für die klinische Transplantation gibt es momentan keine Alternativen zur Immunsuppression.

Um das Transplantat ohne Immunsuppression dauerhaft zu schützen, müssen die regulatorischen Komponenten des Immunsystems gezielt gestärkt werden. Das Ziel dieser Arbeit war es deshalb, die inhibierende Wirksamkeit unreifer dendritischer Zellen auf die nach Transplantation einsetzende Alloimmunantwort zu überprüfen.

Charakteristisch für die Alloimmunantwort ist die Vielzahl der beteiligten Alloantigene. Doch ist es in den letzten Jahren gelungen, Peptidantigene mit einer nachweisbaren Funktion bei der Transplantatabstoßung (vermittelt über den indirekten Weg der Alloantigenerkennung) zu identifizieren. Für die in dieser Arbeit verwendete experimentelle Spender-Empfänger-Kombination ist die Bedeutung des Alloantigens P1, hierbei handelt es sich um ein aus 19 Aminosäuren bestehendes Peptid, für die Alloimmunantwort bekannt.

Autologe unreife dendritische Zellen lassen sich aus Knochenmarkvorläuferzellen mit GM-CSF und IL-4 (diese Zellen werden als IL-4 DCs bezeichnet) bzw. mit GM-CSF und IL-10 (IL-10 DCs) kultivieren. Sowohl für IL-4 DCs als auch IL-10 DCs wurde keine bzw. eine sehr geringe Expression der kostimula-

torischen Moleküle CD80 und CD86 auf ihrer Zelloberfläche nachgewiesen. Die Oberflächenexpression von MHC-Klasse II Molekülen war im Vergleich zu reifen, aus der Milz isolierten dendritischen Zellen, um den Faktor 10 reduziert.

In einem nächsten Schritt wurde die Wirkung von IL-4 DCs und IL-10 DCs auf T-Lymphozyten getestet. Sie können weder naive T-Lymphozyten aktivieren noch antigenspezifische T-Lymphozyten restimulieren. Der von diesen Zellen vermittelte suppressive Effekt wurde innerhalb von 24 Stunden wirksam und war eindeutig abhängig von der Zellzahl. Antigenspezifische T-Lymphozyten waren nach ihrer Inkubation mit IL-4 DCs oder IL-10 DCs nicht mehr mit P1-beladenen reifen DCs zu restimulieren. Dieser anergische Zustand ließ sich aber nach Zugabe von IL-2 aufheben. Anergische T-Lymphozyten, die mit IL-4 DCs kokultiviert wurden (= IL-4 DC-Ts), zeigten ihrerseits einen inhibierenden Effekt auf antigenspezifische T-Lymphozyten. Im Gegensatz dazu waren IL-10 DC-Ts hierzu nicht in der Lage. Dies ist der einzige Unterschied zwischen IL-4 DCs und IL-10 DCs, der in dieser Arbeit gefunden wurde.

Auch *in vivo* zeigten IL-4 DCs und IL-10 DCs sowohl eine inhibierende Wirkung auf die lokale T-Zellpopulation als auch einen protektiven Effekt auf die Transplantatfunktion. Diese ließ sich in Abhängigkeit von der Zellzahl um 4 Tage ohne jegliche Unterstützung mit Immunsuppressiva verlängern. Dabei wurden maximal 30 Millionen unreife DCs pro Lewis Ratte eingesetzt, was ca. 10 Millionen Zellen pro 100 g Körpergewicht entspricht. Ihr immunprotektiver Effekt war dabei eindeutig antigenspezifisch.

Insgesamt lassen die Ergebnisse den Schluss zu, dass autologe unreife dendritische Zellen, beladen mit Alloantigenen, eine hochattraktive Strategie zur antigenspezifischen Modulation der Alloimmunantwort nach Transplantation darstellen. In weiteren Studien soll die Effizienz dieser Zellen gesteigert werden.

9 References

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10 Appendix

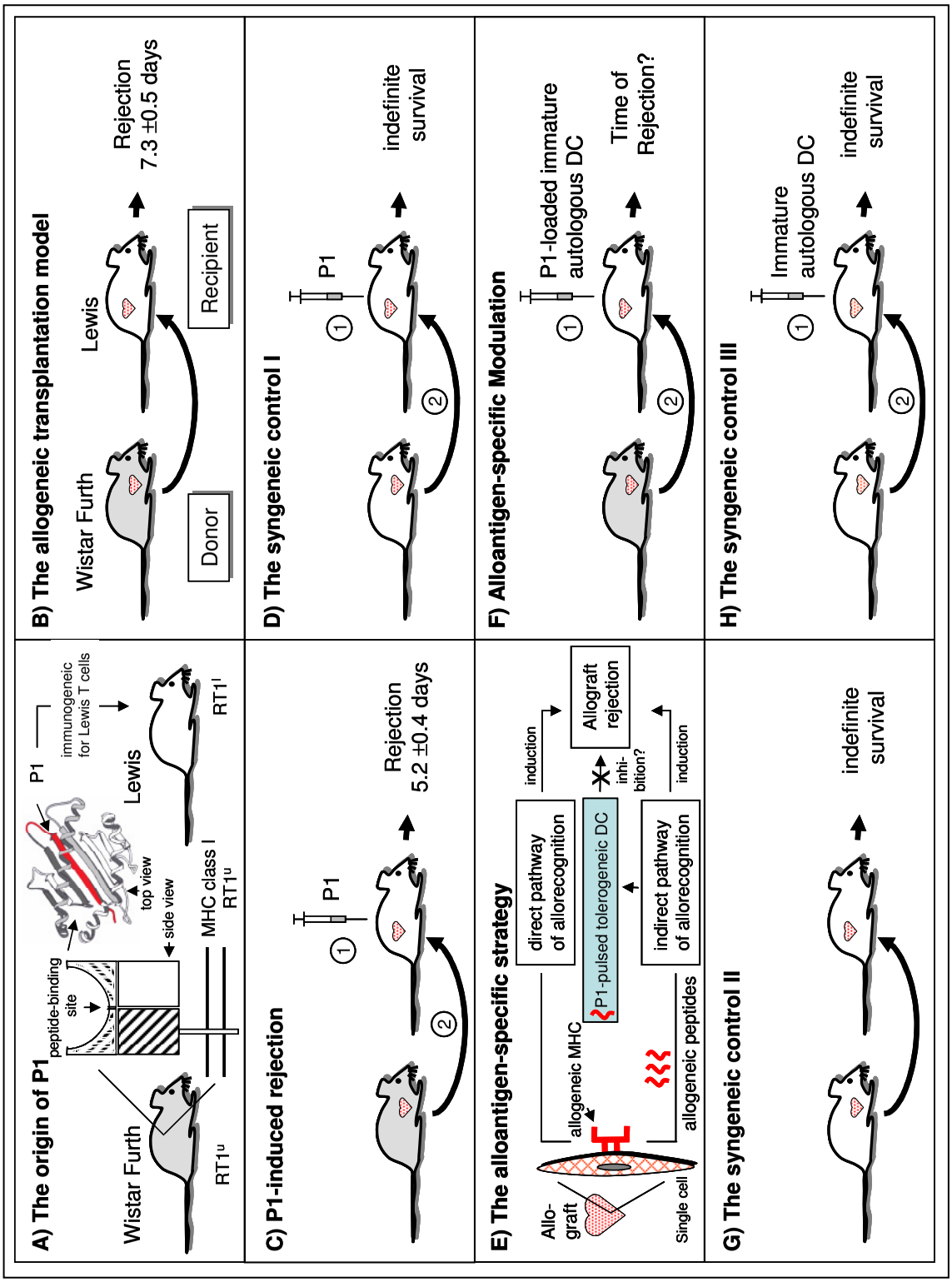

Figure 10.1: "The P1 story". Alloantigen P1 influences the survival of Wistar Furth allografts in Lewis rats. P1 is identical to a unique part of the MHC class I molecule of Wistar Furth ($RT1^u$ haplotype) and is immunogeneic for T cells from Lewis ($RT1^l$) rats (A). The unaffected survival time of Wistar Furth allografts in Lewis rats is 7.3 ± 0.5 day (B), whereas P1 accelerates the

rejection (C). In the syngeneic control group I the survival of syngeneic grafts was not influenced by P1 (D). The working hypothesis of this study is based on the assumption that P1-loaded immature autologous dendritic cells (DC) inhibit specifically the allograft rejection (E). This possible effect of P1-loaded immature autologous DC should be analysed *in vivo* (F). The indefinite survival of syngeneic grafts (G) is not influenced by non-pulsed immature autologous DC (H).

Commentaries:

Figure 4.18 and 4.19:

The strenght of the BM-DCs induced inhibition is different in Figure 4.18 and 4.19. One explanation for this could be the different quality between the BM-DCs recovered from the cultures at day 6. In later experiments we found that day 6 BM-DCs are expressing different levels of CD80 from very low up to a very strong expression.

	Number of DCs		Inhibition in %
Figure 4.18	$1x10^4$ SDCs	$1x10^4$ IL-4 DCs	40%
	$1x10^4$ SDCs	$1x10^4$ IL-10 DCs	63%
Figure 4.19	$1x10^4$ SDCs	$1x10^4$ IL-4 DCs	> 95%
	$1x10^4$ SDCs	$1x10^4$ IL-10 DCs	

Chapter 4.7:

The results shown on Figure 4.25 demonstrate that immature DCs are able to prime antigen-specific T cells in local lymph nodes. However, presently it is not clear wheather these antigen-specific T cells are effector or suppressor T cells. Therefore, the cytokine profile of these cells will be investigated. Both subsets of BM-DCs were able to prolong the allograft survival for few days in the transplantation model. This may indicate that the *in vivo* primed T cells are able to exert a suppressive effect.

Table 10.1: ***Summary of the transplantation experiments.*** The results clearly indicate an effect of IL-4 DCs and IL-10 DCs on the prolongation of allograft survival. For more information about the transplantation model see "Material and Methods" (**Figure 3.1**).

Group	Strain combination	P1	Day of Injection	DC	Route	Date	Day of Rejection
1 Allogeneic Control							
HTX 01/04	WF → LEW	–	–	–	–	10.11.04	8
HTX 02/04	WF → LEW	–	–	–	–	11.11.04	7
HTX 01/05	WF → LEW	–	–	–	–	10.02.05	8
HTX 02/05	WF → LEW	–	–	–	–	16.02.05	7
HTX 18/05	WF → LEW	–	–	–	–	08.06.05	7
HTX 19/05	WF → LEW	–	–	–	–	14.06.05	7
2 Allogeneic Control + P1							
HTX 01/02	WF → LEW	Yes	-7	–	s.c.	02.05.02	5
HTX 02/02	WF → LEW	Yes	-7	–	s.c.	02.05.02	5
HTX 03/02	WF → LEW	Yes	-7	–	s.c.	03.05.02	6
HTX 05/04	WF → LEW	Yes	-7	–	s.c.	24.11.04	5
HTX 06/04	WF → LEW	Yes	-7	–	s.c.	25.11.04	5
3 Syngeneic Control							
HTX 02/02	LEW → LEW	–	–	–	–	09.02.02	> 50
HTX 03/02	LEW → LEW	–	–	–	–	09.02.02	> 50
HTX 11/04	LEW → LEW	–	–	–	–	13.12.04	> 50
4 Syngeneic Control + P1							
HTX 01/02	LEW → LEW	Yes	-7	–	s.c.	05.11.02	> 50
HTX-N2/02	LEW → LEW	Yes	-7	–	s.c.	05.11.02	> 50
HTX 44/05	LEW → LEW	Yes	-7	–	s.c.	24.11.05	> 50
5 Control LEW DC (d -7, d -1)							
HTX 03/05	WF → LEW	–	-7	10^7 IL-4 DC	i.v.	18.04.05	7
HTX 04/05	WF → LEW	–	-7	10^7 IL-4 DC	i.v.	20.04.05	7
HTX 11/05	WF → LEW	–	-7	10^7 IL-4 DC	i.v.	18.04.05	7
HTX 12/05	WF → LEW	–	-7	10^7 IL-4 DC	i.v.	20.04.05	7
HTX 31/05	WF → LEW	–	-1	10^7 IL-4 DC	i.v.	20.09.05	7
HTX 32/05	WF → LEW	–	-1	10^7 IL-4 DC	i.v.	20.09.05	6
HTX 33/05	WF → LEW	–	-7	10^7 IL-4 DC	i.v.	27.09.05	7
HTX 34/05	WF → LEW	–	-7	10^7 IL-4 DC	i.v.	27.09.05	8

Table 10.1 (continued): ***Summary of the transplantation experiments.***

Group	Strain combination	P1	Day of Injection	DC	Route	Date	Day of Rejection
6 LEW IL-4 DC + P1 (d -7)							
HTX 09/04	WF → LEW	Yes	-7	10^7 IL-4 DC	i.v.	13.12.04	9
HTX 05/05	WF → LEW	Yes	-7	10^7 IL-4 DC	i.v.	03.05.05	8
HTX 06/05	WF → LEW	Yes	-7	10^7 IL-4 DC	i.v.	09.05.05	8
HTX 07/05	WF → LEW	Yes	-7	10^7 IL-4 DC	i.v.	10.05.05	7
HTX 13/05	WF → LEW	Yes	-7	10^7 IL-4 DC	i.v.	03.05.05	8
HTX 14/05	WF → LEW	Yes	-7	10^7 IL-4 DC	i.v.	09.05.05	8
HTX 15/05	WF → LEW	Yes	-7	10^7 IL-4 DC	i.v.	10.05.05	7
7 LEW IL-10 DC + P1 (d -7)							
HTX 10/04	WF → LEW	Yes	-7	10^7 IL-10 DC	i.v.	13.12.04	9
HTX 08/05	WF → LEW	Yes	-7	10^7 IL-10 DC	i.v.	11.05.05	9
HTX 09/05	WF → LEW	Yes	-7	10^7 IL-10 DC	i.v.	18.05.05	7
HTX 16/05	WF → LEW	Yes	-7	10^7 IL-10 DC	i.v.	11.05.05	9
HTX 17/05	WF → LEW	Yes	-7	10^7 IL-10 DC	i.v.	18.05.05	7
8 LEW IL-4 DC + P1 (d -1)							
HTX 22/05	WF → LEW	Yes	-1	10^7 IL-4 DC	i.v.	29.06.05	9
HTX 23/05	WF → LEW	Yes	-1	10^7 IL-4 DC	i.v.	30.06.05	11
HTX 24/05	WF → LEW	Yes	-1	10^7 IL-4 DC	i.v.	05.07.05	9
HTX 30/05	WF → LEW	Yes	-1	10^7 IL-4 DC	i.v.	04.08.05	9
9 LEW IL-10 DC +P1 (d -1)							
HTX 20/05	WF → LEW	Yes	-1	10^7 IL-10 DC	i.v.	15.06.05	9
HTX 21/05	WF → LEW	Yes	-1	10^7 IL-10 DC	i.v.	16.06.05	10
HTX 25/05	WF → LEW	Yes	-1	10^7 IL-10 DC	i.v.	08.07.05	10
HTX 26/05	WF → LEW	Yes	-1	10^7 IL-10 DC	i.v.	27.07.05	8
HTX 27/05	WF → LEW	Yes	-1	10^7 IL-10 DC	i.v.	27.07.05	10
HTX 28/05	WF → LEW	Yes	-1	10^7 IL-10 DC	i.v.	02.08.05	10
HTX 29/05	WF → LEW	Yes	-1	10^7 IL-10 DC	i.v.	02.08.05	9
HTX 39/05	WF → LEW	Yes	-1	10^7 IL-10 DC	i.v.	16.11.05	10
10 Third-party (BN) Control							
HTX 37/05	BN → LEW	–	–	–	–	07.11.05	7
HTX 38/05	BN → LEW	–	–	–	–	08.11.05	7
HTX 41/05	BN → LEW	–	–	–	–	21.11.05	7

Table 10.1 (continued): ***Summary of the transplantation experiments.***

Group	Strain combination	P1	Day of Injection	DC	Route	Date	Day of Rejection
11 Third-party Control + LEW IL-10 DC + P1							
HTX 40/05	BN → LEW	Yes	-1	10^7 IL-10 DC	i.v.	17.11.05	7
HTX 42/05	BN → LEW	Yes	-1	10^7 IL-10 DC	i.v.	22.11.05	7
HTX 43/05	BN → LEW	Yes	-1	10^7 IL-10 DC	i.v.	23.11.05	7
12 LEW IL-10 DC + P1 (d -1)							
HTX 39/05	WF → LEW	Yes	-1	$3x10^7$ IL-10 DC	i.v.	16.11.05	10
HTX 46/05	WF → LEW	Yes	-1	$3x10^7$ IL-10 DC	i.v.	08.12.05	12
HTX 47/05	WF → LEW	Yes	-1	$3x10^7$ IL-10 DC	i.v.	13.12.05	10
HTX 49/06	WF → LEW	Yes	-1	$3x10^7$ IL-10 DC	i.v.	24.01.06	12
HTX 50/06	WF → LEW	Yes	-1	$3x10^7$ IL-10 DC	i.v.	31.01.06	11
HTX 51/06	WF → LEW	Yes	-1	$3x10^7$ IL-10 DC	i.v.	01.02.06	10
Experiments not shown in the results							
HTX 03/04	WF → LEW	Yes	-7	10^7 IL4-DC	i.v.	17.11.04	8
HTX 04/04	WF → LEW	Yes	-7	10^7 IL10-DC	i.v.	17.11.04	8

Table 10.2: IL-4 DCs and IL-10 DCs demonstrated an immature phenotype in comparison to mature S-DCs. The flow cytometric results represent the mean fluorescence intensity of different surface molecules. The background staining of isotype antibodies are in brackets. *) not measured:

	Antibodies							
Cell Type	Ox-62	Ox-6	CTLA-4Ig	Ox-1	Ox-22	Ox-42	OX18	Date
	CD 103	MHC-II	CD80/86	CD45	CD45RC	CD11b/c	MHC-I	
IL-4 DC	43 (9,7)	58 (3,6)	15 (9,7)	255 (17)	11 (17)	181 (17)	–	13.04.05
IL-4 DC	25 (3,9)	24 (2,3)	7,8 (3,9)	– *)	–	–	–	19.04.05
IL-4 DC	34 (3,9)	36 (3,4)	11 (3,9)	–	–	–	–	19.04.05
IL-4 DC	33 (7,6)	22 (3,7)	11,7 (7,6)	196 (8,5)	–	98 (8,5)	–	25.04.05
IL-4 DC	28 (10,5)	27 (4,5)	30 (10,5)	328 (13)	11 (13)	182 (13)	–	25.04.05
IL-4 DC	29 (4,7)	24 (2,3)	10 (4,7)	278 (6,6)	–	106 (6,6)	24 (2,3)	09.05.05
IL-4 DC	19 (11)	16 (1,1)	14 (11)	274 (15)	–	116 (15)	8 (1,1)	25.05.05
IL-4 DC	21 (12,9)	32,2 (5,5)	11 (12,9)	–	–	–	–	14.09.05
IL-10 DC	71 (15)	78 (6)	38 (15)	354 (50)	50 (26)	249 (50)	–	13.04.05
IL-10 DC	59 (10,3)	100,6 (9)	29 (10,3)	–	–	–	–	19.04.05
IL-10 DC	37 (9,6)	61 (6)	20 (9,6)	321 (15)	14 (15)	221 (15)	–	25.04.05
IL-10 DC	40 (9)	53 (3,4)	16 (9)	323 (15)	–	188 (15)	55 (3,4)	04.05.05
IL-10 DC	35 (9,3)	58 (4,2)	22 (9,3)	298 (16)	–	187 (16)	57 (4,2)	09.05.05
IL-10 DC	20 (11)	15 (1,5)	24 (11)	212 (21)	–	153 (21)	7 (1,5)	25.05.05
S-DC	468 (220)	523 (5,1)	304 (220)	–	–	–	–	07.06.05
S-DC	274 (133)	113 (8,6)	260 (133)	–	–	–	–	09.06.05
S-DC	271 (28)	254 (3,5)	88 (28)	493 (7,7)	30,5 (7,7)	56 (7,7)	–	13.07.05
S-DC	164 (21)	460 (8,9)	89 (21)	–	–	–	–	21.07.05

Table 10.3: Qualitative RT-PCR analysis of different preparations of IL-4 DCs and IL-10 DCs in comparison to freshly isolated bone marrow cells (BM) and mature splenic DCs (S-DCs). As shown in Figure 4.11 specific mRNA for CD80 and CD86 was proved. (+) Signal; (–) No signal; (+/-) low signal

Cell Type	RNA Isolation	RT- PCR	GAPDH	MHC II	CD40	CD80	CD86	iNOS	IL-2	IL-12	IL-10
IL-4 DC	07.04.05	18.04.05	+	+	+	+	+	+	–	–	–
IL-4 DC	07.04.05	18.04.05	+	+	+	+	+	+	–	–	–
IL-4 DC	26.04.05	02.05.05	+	+	+	+	+	–	–	–	–
IL-4 DC	26.04.05	02.05.05	+	+	+	+	+	–	–	–	+
IL-4 DC	03.05.05	12.10.05	+	+	+	–	+	–	–	–	+
IL-4 DC	03.05.05	12.10.05	+	+	+	–	+	–	–	–	+
IL-4 DC	09.05.05	12.10.05	+	+	+	+	+	+	–	–	+
IL-4 DC	09.05.05	12.10.05	+	+	+	+	+	+	–	–	+
IL-10 DC	15.11.04	24.03.05	+	+	+	+	+	+	–	–	–
IL-10 DC	03.12.05	24.03.05	+	+	+	+	+	+	–	–	–
IL-10 DC	24.02.05	24.03.05	+	+	+	+	+	+	–	–	–
IL-10 DC	22.03.05	24.03.05	+	+	+	+	+	+	–	–	–
IL-10 DC	07.04.05	18.04.05	+	+	+	+	+	+	–	–	+
IL-10 DC	26.04.05	02.05.05	+	+	+	+	+	+	–	+	+
IL-10 DC	04.05.05	12.10.05	+	+	+	+	+	+	–	+	+
IL-10 DC	09.05.05	12.10.05	+	+	+	+	+	+	–	+	+
BM	07.04.05	02.05.05	+	+	+	–	+	+	–	+/-	–
BM	07.04.05	20.06.05	+	+	+	+	+	+	–	+/-	–
BM	08.04.05	18.04.05	+	+	+	+/-	+	+	–	+	–
BM	13.04.05	20.06.05	+	–	–	–	–	+	–	–	–
S-DC	27.02.04	24.03.05	+	+	–	–	+/-	–	–	–	–
S-DC	17.08.05	24.03.05	+	+	+	+	+	+	–	–	–
S-DC	12.11.05	24.03.05	+	+	+	+	+	+	–	–	–
S-DC	13.01.05	24.03.05	+	+	+	+	+	+	–	–	–
S-DC	16.06.05	20.06.05	+	+	+	+	+	+	+	+	+

Table 10.4: ***Different concentrations of IL-10 had no effect on the proliferation of antigen-specific T cells.****)** P1-specific T cells (P1-T) were tested in the proliferation assay in the presence of P1-loaded mature splenic DCs (P1-T + P1-S-DCs) and in the autostimulation assay in the presence of P1 (P1-T + P1). Antigen-specific T cells cultured alone (P1-T) were used as controls. As described in materials and methods the cells were cultured for three days and pulsed with 0.5 µCi/well [^{3}H]-thymidine for the last 6 hours of culture.

	Concentration of IL-10 (ng/ml)					
Culture conditions	∅	1600	800	400	200	100
P1-T + P1-S-DCs	35.0 ± 2.3 *)	33.7 ± 2.5	31.0 ± 2.0	31.5 ± 3.6	32.0 ± 2.3	34.0 ± 2.3
P1-T + P1	16.0 ± 3.0	35.0 ± 2.3	18.5 ± 2.5	20.0 ± 2.7	19.4 ± 2.3	18.8 ± 2.6
P1-T	3.90 ± 1.0	3.50 ± 2.3	4.20 ± 2.1	3.80 ± 2.0	4.50 ± 1.2	3.30 ± 1.6

*) Results (mean ± standard deviation) of T-cell proliferation in cpm x 10^3.
(∅) Without IL-10.

Table 10.5: ***Different concentrations of IL-4 increased slightly the proliferation of antigen-specific T cells.****)** P1-specific T cells (P1-T) were tested in the proliferation assay in the presence of P1-loaded mature splenic DCs (P1-T + P1-S-DCs) and in the autostimulation assay in the presence of P1 (P1-T + P1). Antigen-specific T cells cultured alone (P1-T) were used as controls. As described in materials and methods the cells were cultured for three days and pulsed with 0.5 µCi/well [^{3}H]-thymidine for the last 6 hours of culture.

	Concentration of IL-4 (ng/ml)		
Culture conditions	∅	6.66	13.2
P1-T + P1-S-DCs	23.0 ± 1.9 *)	24.0 ± 2.9	29.0 ± 1.9
P1-T + P1	6.50 ± 1.7	8.80 ± 1.3	10.0 ± 2.1
P1-T	2.40 ± 0.9	3.70 ± 0.6	5.20 ± 1.2

*) Results (mean ± standard deviation) of T-cell proliferation in cpm x 10^3.
(∅) Without IL-4

Table 10.6: The settings used for FACS analysis. The analysed cell types were BM-DCs (IL-4 DCs and IL-10 DCs), S-DCs (isolated from spleen) and lymphocytes. Amp. gain: Amplifier gain

Parameter	Detector	Voltage	Amp. gain	Mode	Compensation
BM-DCs					
P1	FSC	E00	1.00	Lin	FL1−2.4%FL2
P2	SSC	316	1.00	Lin	FL2-32.6%FL1
P3	FL1	469	1.00	Log	FL2-0.0%FL3
P4	FL2	469	1.00	Log	FL3-0.0%FL2
P5	FL3	628	1.00	Log	–
S-DCs					
P1	FSC	E-1	5.73	Lin	FL1-0.0%FL2
P2	SSC	294	1.00	Lin	FL2-65.3%FL1
P3	FL1	416	1.00	Log	FL2-0.0%FL3
P4	FL2	482	1.00	Log	FL3-0.0%FL2
P5	FL3	628	1.00	Log	–
Lymphocytes					
P1	FSC	E00	1.00	Lin	FL1-0.8%FL2
P2	SSC	368	1.00	Lin	FL2-18.0%FL1
P3	FL1	654	1.00	Log	FL2-0.0%FL3
P4	FL2	631	1.00	Log	FL3-18.1%FL2
P5	FL3	782	1.00	Log	–

Acknowledgments

The present study was performed in the Experimental Transplantation Immunology (ETI) Unit of the Department of Surgery of the University of Würzburg Hospital. The financial support was provided by the Deutsche Forschungsgemeinschaft (DFG) through the Graduate College "Immuno-modulation" (GK520).

I express my gratitude to Dr. Christoph Otto for the very interesting scientific discussions and excellent coordination during my work as a doctoral fellow in Würzburg.

I thank Professor Dr. Karin Ulrichs for her constant support and for the friendly working environment in the Experimental Transplantation Immunology Unit and Professor Dr. Arnulf Thiede for the modern working place in the Department of Surgery.

I express my gratitude to Professor Thomas Hünig for organizing an excellent scientific training in the context of the Graduate College "Immunomodulation".

I thank Manuela Schneider, Bianca Hein, Monika Koospal, and Christine Lesch for the very skilful technical support during my experimental work in the ETI Unit and Mrs. Lorraine Stevenson for helping me with the documents and correcting my thesis.

I also thank all the colleagues from the Experimental Transplantation Immunology Unit and from the Graduate College "Immunomodulation" for the friendly environment and for the helpful collaborations.

I express my gratitude to my family and to my wife for their support during my activity in Würzburg. Last, but not least, I would like to thank Dr. Sitaru's family for their help.

Lebenslauf

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Wissenschaftliche Publikationen

Timmermann W., Sitaru A.G., **Tiurbe G.C.**, Ulrichs K., Otto C. Alloantigen-specific modulation of the immune response after transplantation: immunodominant peptides contain amino acids important for MHC binding and T cell receptor recognition. *Deutsche Gesellschaft für Chirurgie, Forumband* 2004. 33: 367-369.

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Vorträge

Seminare des Graduiertenkollegs 520

Mai 2003	Strategies to modulate the allograft induced immune response
Dez. 2003	Modulation of the allograft induced immune response: first results
Mai 2004	Specific suppression of alloreactive Th1 responses
Okt. 2004	Modulation of the alloreactive immune response: Specific suppression of the alloreactive Th1 response
Mai 2005	The covalent structure of the immunoglobulines

Gemeinsame Seminare der Graduiertenkollegs 520 und 592

Juli 2003	Modulation of the transplant induced immune response: which conditions for the induction of regulatory CD4+ T cells by MHC class I peptides must be fulfilled?
Juli 2004	Modulation of the alloimmune response: specific suppression of the alloreactive Th1 response
Juli 2005	Characterization of rat bone marrow dendritic cells and their potential to modulate the activation of alloreactive T cells

Seminare der Experimentellen Transplantations-Immunologie

April 2003	The induction of tolerance versus immunity
Dez. 2003	Modulation of the alloreactive immune response by antibodies and cytokines
Juni 2004	Immunobiology of rat dendritic cells

Nationale und internationale Kongresse und Arbeitskreistreffen

März 2003 **Jährliches Treffen des Arbeitskreises "Transplantations-Immunologie" der Deutschen Gesellschaft für Immunologie in Berlin**

Vortrag mit dem Titel: "CD80/CD86 negative rat dendritic cells loaded with a synthetic allogeneic MHC class I peptide are potent inhibitors of the activation of alloantigen-specific CD4+ Tcells".

Sept. 2004 **20. Kongress der Internationalen Gesellschaft für Transplantation in Wien**

Posterpräsentation mit dem Titel: "CD80/CD86 negative rat dendritic cells loaded with a synthetic allogeneic MHC class I peptide are potent inhibitors of the activation of alloantigen specific CD4+ T cells".

Okt. 2004 **Jahrestagung der Deutschen Transplantationsgesellschaft in Kiel**

Posterpräsentation mit dem Titel: "Induction of hyporesponsiveness of alloreactive CD4+T cells by CD80/CD86 negative rat dendritic cells loaded with an important allogeneic MHC peptide".

März 2005 **Jährliches Treffen des Arbeitskreises "Transplantations-Immunologie" der Deutschen Gesellschaft für Immunologie in Würzburg**

Vortrag mit dem Titel: " Cell-mediated inhibition of the allogeneic immune response: Immature rat dendritic cells generated *in vitro* with IL-4 and IL-10 influence the activation of antigen-specific T cells".

Sept. 2005 **Jahrestagung der Vereinigung der Mittelrheinischen Chirurgen in Würzburg**

Posterpräsentation mit dem Titel: "Characterization of immature rat dendritic cells and their potential to modulate regulatory T cells"

Würzburg, den 20. Juli 2006