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Charakterisierung immunmodulatorischer Effekte von Zellen monozytären Ursprungs: Bedeutung für die Hemmung ungewollter Immunantworten?

Characterisation of immunomodulatory effects of monocyte-derived cells: Relevance for suppressing unwanted immune responses?

Master Thesis
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1 Introduction

1.1 Clinical transplantation and the immunology of allograft rejection

Transplantation of organs, tissues and cells represents a life saving procedure for patients suffering from incurable diseases. Despite advances in surgical techniques and medical therapies over the past few decades, allograft rejection remains the major obstacle to long-term survival of the allograft (Starzl, 2000).

After transplantation, the recipient's immune system recognises donor-derived proteins as foreign resulting in a strong immunological reaction where donor cells are specifically killed and thus, the graft destroyed (Denton et al., 1999). This phenomenon can be explained by a set of specific antigens, termed major histocompatibility complex (MHC) antigens, which are expressed on all somatic cells and which normally differ greatly between non-related individuals (Benichou, 1999; Sekine et al., 1997).

Host T cells that recognize foreign (allogeneic) MHC molecules on donor cells via the direct and indirect pathway are the mediators of allograft rejection (Batchelor and Lechler, 1982; Benichou, 1999). The direct pathway involves the activation of naïve T cells by contact of the T cell receptor (TCR) with the allogeneic MHC/peptipes molecules expressed on the surface of donor antigen-presenting cells (APC) together with costimulatory signals provided by B7 molecules on APC (Bingaman and Farber, 2004; Chandok and Farber, 2004).

In contrast, recipient T cells involved in the indirect pathway recognise peptides of allogeneic MHC molecules that were processed and then presented by self (recipient) APC. Afterwards, the T cells proliferate and differentiate into effector cytotoxic or helper T cells that secrete cytokines such as IFN-γ and IL-2 (Benichou, 1999). As a consequence, further immune cells, for example cytoxotxic T lymphocytes, B lymphocytes for antibody production and macrophages for antigen clearance are recruited and activated (Bingaman and Farber, 2004; Chandok and Farber, 2004; Pandiyan et al., 2007; Sekine et al.,

1997). If the recipient possesses a fully functional immune system, the transplantation almost unavoidably ends in allograft rejection.

The strategies used to avoid or delay allograft rejection are pre-operative MHC matching between donor and host and general immunosuppression (Hariharan et al., 2000). MHC matching can only partly contribute to a successful acceptance of the graft. Therefore, allograft recipients have to undergo life-long immunosuppression to minimise the possibility of a rejection (Buckley, 2003). However, even with enormous progress in the development of immunosuppressive strategies over the last years, a non-specific down-regulation of the patients' immune system is still inevitable bearing strong side effects: the host's capacity to respond appropriately to infectious, fungal and carcinogenic threats is restricted to a large extent (Buckley, 2003). Therefore, an important goal in transplantation medicine would be to induce donor-specific tolerance, which would allow recipients to live without pharmacological immunosuppression.

1.2 The tolerogenic potential of APC

T lymphocytes are mediators of allograft rejection but their activation is under control of APC (Heeger, 2003). However, besides their role in T cell immunity, APC, namely dendritic cells and macrophages, present regulatory and tolerogenic properties because they are also involved in the induction and maintenance of T cell tolerance, respectively (Hoves et al., 2006; Morelli and Thomson, 2003).

The ability of DC to induce T cell tolerance is related to their state of maturation (Banchereau et al., 2000; Lutz and Schuler, 2002; Morelli and Thomson, 2003; Nouri-Shirazi and Guinet, 2002). Mature DC with high expression levels of MHC class II molecules and the costimulatory molecules CD80 and CD86 represent potent APC in activating naïve T cells. In contrast, immature DC with only a moderate expression of MHC class II molecules and a low level of costimulatory molecules are attributed tolerogenic properties (Nouri-Shirazi and Guinet, 2002;

Li et al., 2005; Vanclee et al., 2006). Without costimulatory signals, antigen-presentation leads to anergy in antigen-specific T cells (Li et al., 2005; Morelli and Thomson, 2003; Nouri-Shirazi and Guinet, 2002). Furthermore, immature DC have been shown to induce tolerance by activating regulatory T cells (Jonuleit et al., 2000). Lutz and Schuler reviewed a new subset of DC, which is distinguished as mature by their surface expression, however, they do not release elevated levels of proinflammatory cytokines, such as IL-1β or IL-12p40 (Lutz and Schuler, 2002).

As shown for DC, tolerance-inducing subpopulations also exist within the macrophages. For this group of APC, derived from the same haematopoietic precursors as DC, the type of activation seems to be responsible for the development of a tolerogenic or immunogenic phenotype (Gordon, 2003; Schebesch et al., 1997). Classical activation by IFN-γ and lipopolysaccharides leads to immunostimulatory and proinflammatory properties of macrophages, whereas so-called alternative activation by agents such as IL-4, IL-10 or glucocorticoids produces an immunosuppressive phenotype (Goerdt and Orfanos, 1999; Mosser, 2003). *In vitro* assays have shown that alternatively activated macrophages are able to inhibit proliferation of peripheral blood lymphocytes and $CD4^+$ T lymphocytes in an active manner (Schebesch et al., 1997). The anti-inflammatory IL-4 induces the development of macrophages with the alternatively activated phenotype (Schebesch et al., 1997). In this study macrophages from bone marrow precursor cells of the Lewis rat are generated with the macrophage colony-stimulating factor (M-CSF) alone and in combination with IL-4.

1.3 Experimental settings

Previous studies of the group focused on the generation of immunomodulatory DC from haematopoietic bone marrow precursor cells isolated from Lewis rats. The cultivation of haematopoietic precursors with the granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 leads to the development of so-called IL-4 DC. These cells showed an immature phenotype and

immunomodulatory properties (Tiurbe GC, 2006). For the present study, macrophage colony stimulating factor (M-CSF) was used to stimulate the differentiation of haematopoietic precursors into monocytes/macrophages (M-CSF MΦ); an additional stimulation with IL-4 generated so-called IL-4 MΦ. The study focused on the characterisation of the effect of bone marrow derived M-CSF MΦ and IL-4 MΦ on naïve, allogeneic T lymphocytes, whether they act as activators or inhibitors of T cell activation.

To evaluate the stimulatory or suppressive capacities of APC on naïve T lymphocytes, the allogeneic mixed leukocyte culture (MLC) is used in this study (Fig. 1.1). In addition, MLC serves as a predictive test of T cell-mediated graft rejection, since the T cell receptor (TCR) of the alloreactive T lymphocytes recognises allogeneic MHC molecules on the surface of allogeneic (foreign) APC.

According to the “two-signal” hypothesis of T cell activation, naïve T cells need two signals for complete activation. The first signal needed is the engagement of the TCR with the peptide antigen presented by self major histocompatibility complex (MHC) molecules. This first signal must be accompanied by a second stimulus mediated via coreceptors (signal two), namely the transmembrane protein CD28 on T cells with its ligands B7-1 (CD80) and B7-2 (CD86) on APC (Schwartz, 1992). Consequently, APC presenting both signals will be strong T cell activators in MLC (Fig. 1.1 A).

A high rate of T cell proliferation in the MLC is characterised by a strong incorporation of [^{3}H]-thymidine resulting in elevated radioactive signals (Fig. 1.1 E). In addition, activated T lymphocytes are visible under the light microscope as cell clusters of proliferating T lymphocytes. Both costimulator- and MHC-deficient APC are unable to activate T cell proliferation. These T lymphocytes demonstrate a normal cell size under the microscope and no incorporation of radioactivity is measurable (Fig. 1.1 B, C and F). Therefore, mature DC are the most potent stimulators of naïve T cells. In this study, mature DC isolated from

the spleen of Lewis rats were used as stimulator cells. Their interaction with T lymphocytes, however, can be impaired by so-called competitor cells. These cells inhibit the APC-induced T cell proliferation in a dose dependent manner (Fig. 1.1 D and F). The effects of the IL-4 MΦ and M-CSF MΦ as competitor cells are proven in this study.

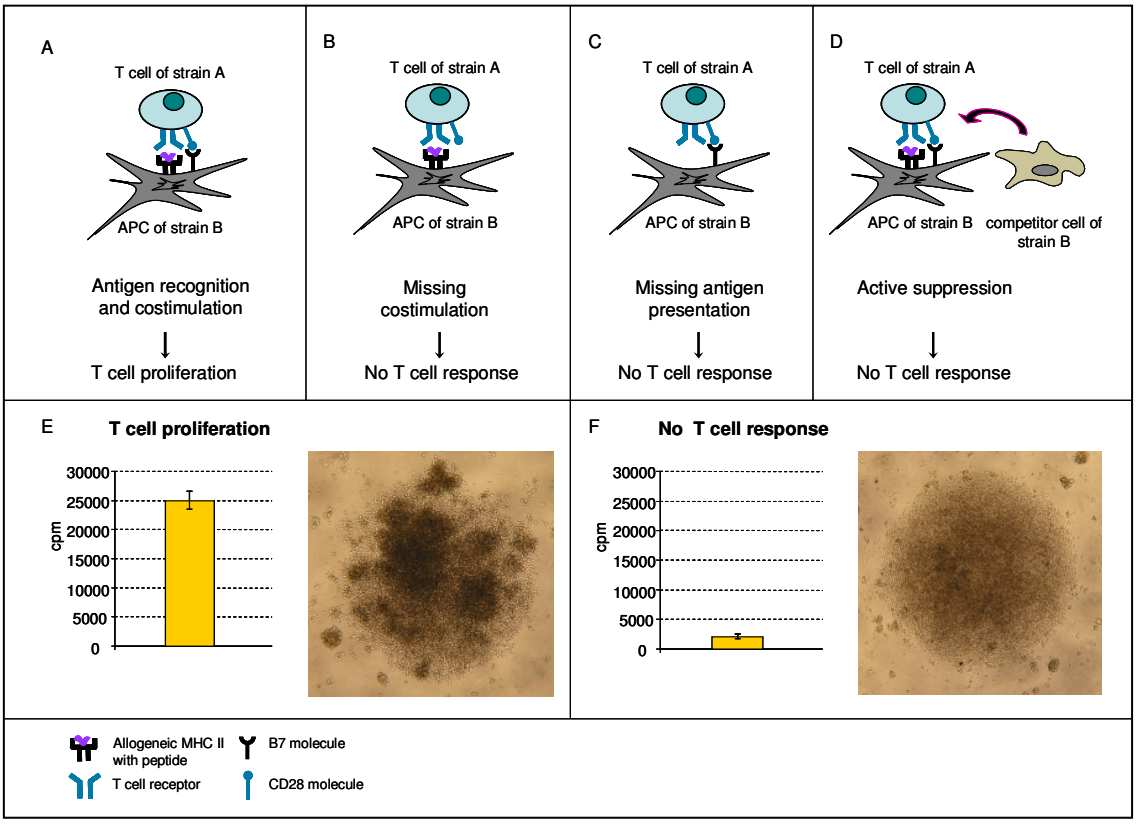


Fig. 1.1. T cell activation and proliferation in mixed leukocyte cultures. For full T cell activation, antigen recognition and costimulation is necessary (A). Increased T cell proliferation is characterised by a strong uptake of [^{3}H]-thymidine presented in the medium (E). Under the light microscope, these proliferating cells are obvious as cell clusters. APC lacking a surface expression of costimulatory molecules as well as MHC molecules are not able to activate T lymphocytes (B and C). The consequence is no proliferation and no activated (normal-sized) T lymphocytes under the light microscope (F). The activation of T lymphocytes by fully functional APC, however, can be prevented by competitor cells (D).

2 Aims of the study

The aim of the study was to analyse the biological effect of bone marrow derived macrophages. They are generated from bone marrow precursor cells cultured with M-CSF and IL-4 (IL-4 MΦ) and with M-CSF alone (M-CSF MΦ). Previous studies of the group (Tiurbe GC, 2006) demonstrated a suppressive effect of bone marrow derived dendritic cells (DC) cultured with GM-CSF and IL-4 (IL-4 DC). In this study the possible immune regulatory effect of IL-4 MΦ and M-CSF MΦ are evaluated.

Cell type	**Effect on T cell proliferation**
Rat macrophages (M-CSF MΦ)	Unknown (will be investigated in this study)
Rat macrophages (IL-4 MΦ)	Unknown (will be investigated in this study)
Rat dendritic cells (IL-4 DC)	Suppression (Tiurbe GC, 2006)

Questions

(1) IL-4 combined with GM-CSF induces bone marrow derived DC with immune regulatory effects. Does IL-4 combined with M-CSF also induce the development of bone marrow derived immune regulatory macrophages?

(2) IL-4 is an anti-inflammatory cytokine. How does IL-4 influence the expression of certain surface molecules on IL-4 MΦ, which are involved in T cell activation?

(3) IL-4 DC suppress the activation of naïve T lymphocytes. What are the immune regulatory effects of IL-4 derived macrophages in comparison to IL-4 DC?

3 Materials and Methods

3.1 Animals

Inbred Lewis and Wistar Furth rats were provided by Harlan Winkelmann GmbH (Borchen, Germany). The animals weighed about 250-350g prior to the removal of femurs, spleen and lymph nodes for the isolation of macrophage / dendritic cell progenitor cells, dendritic cells and T cells.

3.2 Culture medium and buffers

Culture medium

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

Phosphate-buffered saline (PBS)

PBS contains 140 mmol/L sodium chloride, 2.7 mmol/L potassium chloride, 7.2 mmol/L sodium dihydrogen phosphate and 1.47 mmol/L potassium dihydrogen phosphate and the pH was adjusted to 7.2. The solution was kept at 4°C after sterilization.

Erythrocyte lysis buffer I and II

Lysis buffer I (10-fold concentration) contains 1.68 mol/L ammonium chloride, 99.88 mmol/L potassium hydrogen carbonate and 12.6 mmol/L EDTA. The buffer was diluted 1:10 with sterile aqua injectabila before use. Lysis buffer II consists of 154.95 mmol/L ammonium chloride, 9.99 mmol/L potassium hydrogen carbonate and 1.27 mol/L EDTA.

Tris Acetat (TA) buffer

TA buffer contains 40 mmol/L Tris(hydroxymethyl)aminomethane hydrochloride, 20 mmol/L sodium acetate and 20 mmol/L EDTA.

Macrophage detaching buffer (2-fold concentrated)

Macrophage detaching buffer contains 1.6 mmol/L EDTA, 240 mmol/L sodium chloride, 5.4 mmol/L potassium chloride, 16 mmol/L sodium hydrogen phosphate, 3 mmol/L potassium dihydrogen phosphate. The pH was adjusted to 7.4.

3.3 Isolation of bone marrow cells

Bone marrow cells were isolated from femur bones of Lewis rats. The extracted bones were cleaned of muscular and sinewy tissues with a scalpel and washed in 70% ethanol and in sterile PBS afterwards. Both ends were cut off with surgical scissors. The bone marrow was flushed with 20 mL sterile PBS for each bone using a syringe and a 20-gauge needle through a 70 µm nylon cell strainer (Becton Dickinson Biosciences, Heidelberg, Germany) to receive a single cell suspension. The contaminating erythrocytes were lysed with 10 mL lysis buffer II for 5 min at room temperature. Subsequently, the bone marrow cells were cultured in culture dishes (Falcon, Becton Dickinson Biosciences, Heidelberg, Germany) at a cell density of approximately $0.6x10^6$ cells per mL. Then, cytokines were added to the culture medium: 5 ng/mL murine M-CSF (macrophages colony stimulating factor; R&D Systems, Heidelberg, Germany) for the induction of M-CSF-derived macrophages (M-CSF MΦ); 5 ng/mL murine M-CSF plus 5 ng/mL rat IL-4 (interleukin-4, Strathmann Biotech AG, Hamburg, Germany) for the induction of IL-4 MΦ; 5 ng/mL rat GM-CSF (granulocyte macrophage colony stimulating factor, R&D Systems) plus 5 ng/mL rat IL-4 (for the induction of IL-4 DC. The cells were cultured at 37°C in a 5% humidified CO_2 atmosphere and after 3 days of culture, medium and cytokines were renewed. On day +6, the M-CSF/IL-4-derived, adherent IL-4 MΦ were detached from the culture dishes by incubation with 1xHyQTase (HyClone, Perbio Science Deutschland GmbH, Bonn, Germany) for 25 min at 37°C and the gentle

use of a cell scraper. M-CSF MΦ were incubated with 2x macrophage detaching buffer for 10 min at 37°C and then detached by moderate pipetting. IL-4 DC were collected from the medium. After washing, the cells were counted in a Neubauer chamber.

3.4 Isolation of mature splenic DC

In order to receive a single cell suspension of mature DC (mDC), the spleen of a Lewis rat was homogenized with a 5 ml syringe piston through a 70 µm nylon cell strainer (Becton Dickinson Biosciences). After washing and lysing the erythrocytes with 10 mL 1x lysis buffer I for 3 min, the cells were disseminated in Petri dishes (Falcon, Becton Dickinson Biosciences). The next day, non-adherent and slightly adherent cells were centrifuged over 14.5% metrizamide (Linaris Biologische Produkte GmbH, Wertheim, Germany) at 4°C and 1,823 x g for 13 minutes. Mature DC were collected from the interface and counted in a Neubauer chamber.

3.5 Isolation of T lymphocytes from lymph nodes

In order to get naïve T lymphocytes, cervical lymph nodes were isolated from Wistar Furth rats and homogenized with a 5 mL syringe piston through a 70 µm nylon cell strainer (Becton Dickinson Biosciences). After washing and lysing the erythrocytes with 5 mL 1x lysis buffer I for 3 min, the viable cells were counted in a Neubauer chamber.

3.6 Activation of naïve T lymphocytes

The effect of IL-4 MΦ, M-CSF MΦ or IL-4 DC on the activation of naïve T lymphocytes were investigated in mixed lymphocyte cultures (MLC). For this, naïve T lymphocytes isolated from lymph nodes of Wistar Futh rats were incubated with various allogeneic stimulator cells or culture supernatant from these stimulator cells in 96-well round-bottom plates at 37°C in a 5% humidified CO_2 atmosphere for 3 days. Assays of T cells ($1x10^5$ cells/well) together with

mDC ($1x10^4$ cells/well) isolated from spleens of Lewis rats served as positive control for antigen-dependent T cell stimulation. IL-4 MΦ, M-CSF MΦ or IL-4 DC ($1x10^4$ cells/well) were incubated with T lymphocytes ($1x10^5$ cells/well). Furthermore, the inhibitory effect of IL-4 MΦ, M-CSF MΦ and IL-4 DC on the T cell activation mediated by mature DC was measured. In such competition assays $1x10^5$ allogeneic T lymphocytes cells were incubated with $1x10^4$ mDC and different amounts (10^4-10^1) of IL-4 MΦ, M-CSF MΦ or IL-4 DC. In addition, the possible effect of soluble factors secreted by IL-4 MΦ, M-CSF MΦ or IL-4 DC was proven. Culture supernatant from these cells (up to 10^6 cells per 96-well plate) were collected after 24h and 48h incubation time. Cancer cells (MDA-MB 231) served as negative control cells, T lymphocytes incubating alone served as negative control for T cell proliferation and T lymphocytes incubating with mature splenic DC as positive control. After 3 days of culture, the cells were pulsed with 0,5 µCi/well [^{3}H]-thymidine (Biomedicals Germany GmbH, Eschwege, Germany) for the last six hours of culture. To quantify [^{3}H]-thymidine incorporation, the cells were harvested with the MicroBeta Filtermat-96 cell harvester (Wallac, Turku, Finland) and their DNA blotted on special filter paper (1450-421 Filtermat A, Wallac, Perkin-Elmer Life and Analytical Sciences, Rodgau, Germany). The paper was dried for 45 min and then sealed in special plastic bags (Wallac, Perkin-Elmer) with scintillation liquid. The measurement of [^{3}H]-thymidine incorporation was performed with a Wallac-MicroBeta TriLux radiation counter (Institute für Virologie und Immunologie, Würzburg).

3.7 Immunohistochemistry

Cells (IL-4 MΦ, M-CSF MΦ or IL-4 DC) from day 6 of culture were isolated, counted and centrifuged on cytospins ($5x10^5$ cells/slide). The cytospins were then air-dried and after fixation in acetone for 10 min, the cells were incubated overnight with monoclonal antibodies (Tab. 3.1) diluted in background reducing buffer (DAKO Cytomation, Hamburg, Germany). 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 1h and then washed again. The preparations were incubated with diamino-

benzidine (Liquid DAB, BioGenex, San Ramon, USA) afterwards, counter-stained with hematoxilin and embedded with Entelan (Merk, Darmstadt, Germany).

Tab. 3.1. Primary antibodies used for immunohistochemical staining. All antibodies were purchased from Linaris Biologische Produkte, Wertheim-Bettingen, Germany.

Antibody	Dilution	Specifity	Secondary antibody	Dilution
ED1	1:100	CD68	rabbit-anti-mouse	1:50
Ox6	1:100	MHCII	rabbit-anti-mouse	1:50
Ox18	1:100	MHCI	rabbit-anti-mouse	1:50
CD80	1:100	CD80	rabbit-anti-mouse	1:50
CD86	1:100	CD86	rabbit-anti-mouse	1:50
Ox62	1:100	α-E2 Integrin	rabbit-anti-mouse	1:50

3.8 Flow cytometric analysis

For the flow cytometric analysis, various specific antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrine (PE) (Tab. 3.2) were incubated with $5x10^5$ cells each for 20 min at room temperature. Non-conjugated primary antibodies were detected with PE-conjugated secondary antibodies (“indirect staining”). Isotype antibodies conjugated with FITC and PE, respectively, served as isotyp controls for controling non-specific binding. Non-viable cells were detected with 7-amino actinomycin D (7-AAD) (Becton Dickinson Biosciences). The flow cytometric analysis was performed using an argon-laser flow-cytometer FACSscan (Becton Dickinson Biosciences) and evaluated with the program FlowJo. Detection of apoptosis was performed using the Annexin V Apoptosis Detection Kit I (Becton Dickinson Biosciences). Annexin V provides a simple and effective method to detect apoptosis at a very early stage. Soon after the induction of apoptosis the phosphatidylserine is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet. The annexin V protein has a strong, specific affinity

for phosphatidylserine and the binding by labeled annexin V provided the basis for this staining assay.

Tab. 3.2. Primary and secondary antibodies used in flow cytometric analysis. All antibodies were purchased from Linaris Biologische Produkte, Wertheim-Bettingen, Germany.

Antibody	Dilution	Specifity	Secondary antibody	Dilution
ED1	1,5:50	CD68	DAM-PE	1:16
Ox6-PE	3:50	MHC II	-	undiluted
Ox18-PE	5:50	MHC I	-	undiluted
CD80	2:50	CD80	DAM-PE	1:16
CD86	2:50	CD86	DAM-PE	1:16
CD40-FITC	1,5:50	CD40	-	undiluted
Ox62	3:50	αE2 Integrin	DAM-PE	1:16
Ox1-FITC	5:50	CD45	-	undiluted
R73-PE	5:50	αβ TCR	-	undiluted

3.9 RNA isolation and reverse transcriptase

Total RNA from $1x10^6$ cells were isolated with 1 mL of the ready-to-use Trizol reagent (Invitrogen GmbH, Karlsruhe, Germany). The cell samples were homogenized in the Trizol reagent and chloroform (Carl Roth GmbH, Karlsruhe, Germany) was added to the homogenous lysate. The mixture was separated by centrifugation (15 min, 16060 x g at 4° C) into two phases. The aqueous phase with RNA was then transferred into a new tube and precipitated with isopropanol (Merck Eurolab, Nürnberg, Germany) at -20°C overnight. After centrifugation at 13.000 rpm for 10 min, the pellet was washed with ethanol (Merck Eurolab), air-dried, dissolved in 40 µL RNA storage solution (Ambion, Huntingdon, United Kingkom) 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).

3.10 Real time polymerase chain reaction (PCR)

Real time PCR was applied to monitor the gene expression levels of different cell surface molecules. The housekeeping gene GAPDH was used as positive control. A 2x concentrated solution of the SYBR Green PCR Master Mix (Applied Biosystems) containing Ampli Taq Gold polymerase and dNTPs was added to 1 µL of sample cDNA to a final volume of 25 µL. Specific primers (Tab. 3.3) were added to the mix and the amplification was performed by Opticon 2 Cycler (M.J. Research, Bio-rad, USA). The thermal cycling conditions were: 95°C 15 min, and 35x (95°C 15 sec, annealing (temperatures see Tab. 3.3) 30 sec, 72°C 30 sec). Positive controls were 10^8 copies of PCR vector with a certain cloned PCR fragment (TA Cloning Kit from Invitrogen) and nuclease free water (Promega) was used as negative control. A melting curve was performed to analyse product homogeneity. Afterwards, the PCR products were proven in agarose gel. For this, 10 µL of each PCR product were mixed with 1.5 µL Blue/Orange 6x loading dye (Promega GmbH) and transferred to a 2% agarose gel loaded with ethidium bromide (Amresco, Solo, Ohio, USA). The gel was run at 75 volt for approximately 30 minutes. The separated PCR products were photographed in the gel with UV light illumination (ImageMaster workstation from Amersham Pharmacia Biotech, Piscataway, USA).

3.11 ELISA

IL-4 MΦ, M-CSF MΦ and IL-4-DC were replated in 96-well flat bottom plates after 6 days of culture ($1x10^6$ - 10^4 cells/well in a final volume of 150 µL) without exogenous cytokines. The presence of IL-10 and TGF-β1 in the supernatant was detected using ELISA kits from Biosource, Ratingen, Germany, for rat IL-10 (KRC0101) and TGF-β1 (KAC1688).

Tab. 3.3. Sequences of the primers used for this study. The forward and reverse sequence, PCR product size (in bp), and the annealing temperature (Temp) are presented for each primer. All the primers were synthesized by MWG Biotech AG (Ebersberg, Germany) according to published sequences: GAPDH (Kruse et al., 1999); MHC class II (Syha-Jedelhauser et al., 1991); MHC class I (Sourial-Bassillious N et al., 2006), CD40 (Matsui et al., 2002); CD80 (Holowachuk and Ruhoff, 2001); CD86 (self made with GeneFisher), IL-10 (Siegling et al., 1994), iNOS (Sterin-Borda et al., 2003). In addition, the related nucleotide sequences are listed in PubMed Nukleotide. bp = base pair; Temp = annealing temperature of the primers, for = forward, rev = reverse.

Primer	**bp**	**Temp**		**Sequence 5´→ 3´**
GAPDH [1)]	319	62°C	for	GGT CGG TGT GAA CGG ATT TG
			rev	GTG AGC CCC AGC CTT CTC CAT
MHC II [2)]	517	55°C	for	CAG GAT CTG GAA GGT CCA
			rev	AGC TGT GGT TGT GCT GA
MHC I [3)]	530	58°C	for	CCT CCT CCT CCT CAC AAC AAC CAC
			rev	AGG GCG GCT CTC ACA CCA TCC
CD40 [4)]	401	58°C	for	CGC TAT GGG GCT GCT TGT TGA CAG
			rev	GAC GGT ATC AGT GGT CTC AGT GGC
CD80	517	50°C	for	TGG TGA AAC ACC TGA CCA
			rev	GTT TCT CTG CTT GCC TCA
CD86 [5)]	518	53°C	for	TGG GAA ACA GAG CTC TCA
			rev	AGG TTG ATC GAC TCG TCA
IL-10 [6)]	371	55°C	for	TCC ATC CGG GGT GAC AAT AAC
			rev	AAT CAT TCT TCA CCT GCT CC
iNOS	578	60°C	for	GAT CAA TAA CCT GAA GCC CG
			rev	GCC CTT TTT TGC TCC ATA AGG

1) Sequence X02231
2) Sequence X56596
3) Sequence NM_012645
4) Sequence AF241231
5) Sequence NM_020081
6) Sequence AJ305049

4 Results

4.1 Generation of bone marrow derived macrophages and DC

Bone marrow cells, isolated from femurs of Lewis rats, were cultured at a cell density of about $1x10^7$ cells per 15 mL culture medium and incubated in presence of the cytokines M-CSF, M-CSF + IL-4, and GM-CSF + IL-4 (5 ng/mL each) for six days to induce macrophages and dendritic cells (DC). Freshly isolated bone marrow cells are a mix of lymphocytes and granular cells (Fig. 4.1) and after three days of culture first morphological changes occurred (Fig. 4.2). Within the granular cell fraction, progenitor cells started to differentiate under the influence of the cytokines.

In presence of M-CSF, the progenitor cells differentiated into adherent, spindle-shaped macrophages (M-CSF MΦ) with considerable projections (Fig. 4.2 A). Until day 6, the end of culture, these cells were enlarged and showed more distinct processes (Fig 4.2 B). The incubation with M-CSF + IL-4 also led to the development of large, adherent macrophages (IL-4 MΦ) with prominent projections (Fig. 4.2 C). On day 6, the plastic surface of the culture dishes was nearly completely covered by a cell layer of enlarged IL-4 MΦ (Fig. 4.2 D).

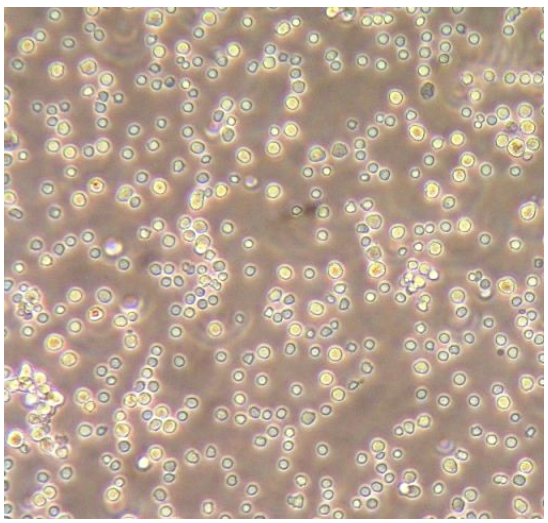


Fig. 4.1. Cell morphology of freshly isolated bone marrow cells in culture. Freshly isolated bone marrow cells appeared round and were a mixture of lymphocytes and granular cells. Phase contrast microscope Olympus IMT-2, 100x magnified.

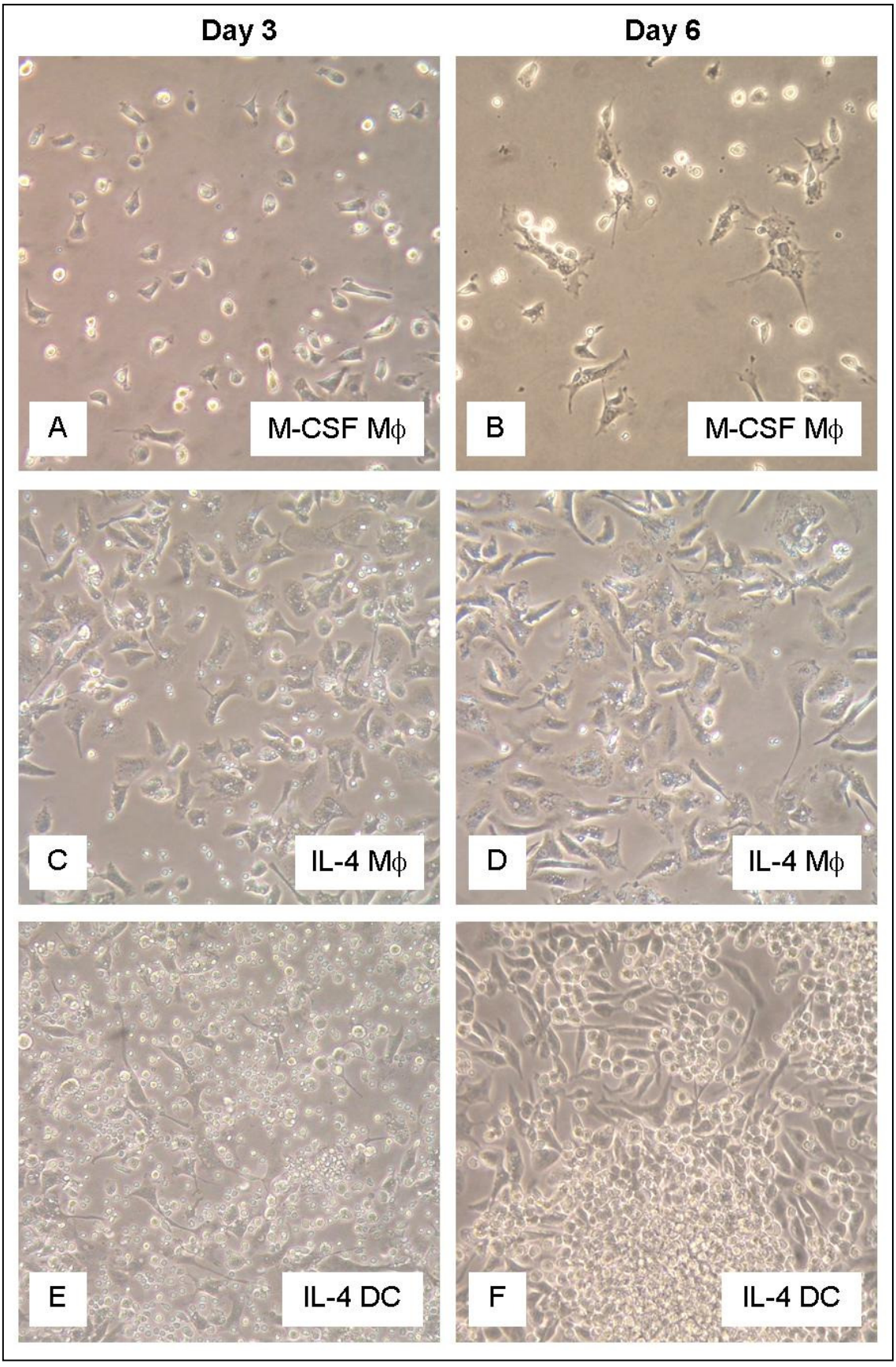

Day 3
Day 6
A
M-CSF Mϕ
B
M-CSF Mϕ
C
IL-4 Mϕ
D
IL-4 Mϕ
E
IL-4 DC
F
IL-4 DC

Fig. 4.2 (page 16). ***Representative changes in the cell morphology of bone marrow cells after 3 and 6 days of culture.*** Bone marrow cells developed into spindle-shaped, adherent macrophages with characteristic processes after three days of culture with M-CSF and M-CSF + IL-4, respectively (A and C). After six days, most of the cells were enlarged and showed definite processes (B and D). Large, round DC floating in the culture medium developed after three days of culture with GM-CSF + IL-4 (E). On day 6, major clusters consisting of large, round IL-4 DC slightly attached to the plastic surface were observed. Contaminating fibroblasts were also present (F). Phase contrast microscope Olympus IMT-2, 200x magnified.

The incubation of bone marrow precursor cells with GM-CSF + IL-4 led to the development of dendritic cells (IL-4 DC). After 3 days of culture, numerous large, round IL-4 DC floating in the medium were seen and some spindle-shaped fibroblasts which were adherent to the plastic surface of culture dishes (Fig. 4.2 E). Until day 6, large, round IL-4 DC grew in compact clusters slightly attached to the plastic surface. The amount of fibroblasts increased, too (Fig. 4.2 F).

4.2 The effect of IL-4 MΦ, M-CSF MΦ and IL-4 DC on allogenic T lymphocytes

4.2.1 IL-4 MΦ, M-CSF MΦ and IL-4 DC are not T cell stimulators

IL-4 MΦ, M-CSF MΦ and IL-4 DC were tested *in vitro* for their stimulatory effects on allogenic T lymphocytes in mixed leukocyte cultures (MLC). For this, allogeneic T lymphocytes from Wistar Furth rats were cultured with different types of stimulator cells propagated from bone marrow precursor cells of Lewis rats (Fig. 1.1 displays the principles of the assay). Compared to mDC, IL-4 MΦ, M-CSF MΦ and IL-4 DC were unable to induce T cell proliferation (Fig. 4.3 and Tab. 9.1 of the appendix). In detail, in presence of IL-4 MΦ the proliferation of T lymphocytes was limited to 3% of the proliferation rate reached after stimulation by mDC; in the presence of M-CSF MΦ to 2% and in presence of IL-4 DC to 4% (Tab. 9.1 of the appendix).

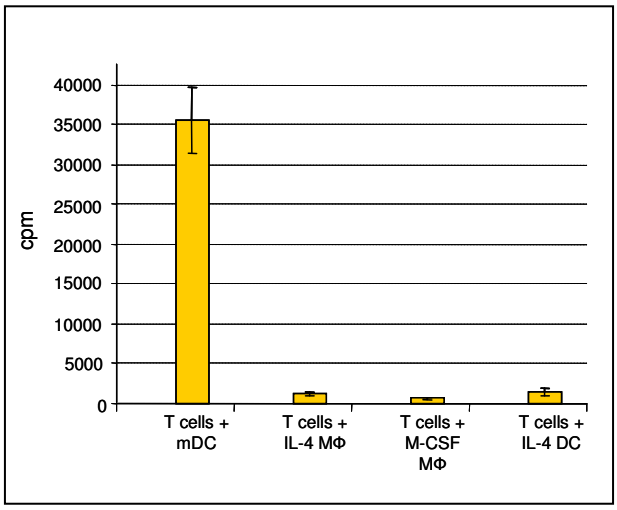


Fig. 4.3. IL-4 MΦ, M-CSF MΦ and IL-4 DC did not activate naïve T cells in the MLC. In co-culture with mDC the allogeneic T lymphocytes showed a strong proliferation but not with IL-4 *Mϕ, M-CSF Mϕ and IL-4 DC*. The stimulator cells were isolated from the spleen (mDC) or generated from bone marrow cells (IL-4 Mϕ, M-CSF Mϕ and IL-4 DC) of Lewis rats. The naïve responder T lymphocytes were isolated from Wistar Furth rats. 10^4 stimulator cells were co-cultured with 10^5 responder cells. The results are presented as mean ± standard deviation (n = 3 per group).

4.2.2 IL-4 MΦ, M-CSF MΦ and IL-4 DC suppress the mDC-mediated activation of naïve T lymphocytes in competition assays

In competition assays (Fig. 1.1 displays the principles of the assay), the effect of IL-4 MΦ, M-CSF MΦ and IL-4 DC as competitor cells on the stimulation of naïve T lymphocytes (10^5 cells/well) mediated by mDC (10^4 cells/well) was analysed (Fig. 4.4). The ratio of mDC to T lymphocytes was constant (1:10) for each experiment and the ratio of mDC to competitor cells ranged from 1:1 to 10:1, 100:1 and 1000:1. As shown in Fig. 4.4, all three types of competitor cells demonstrated a suppressive effect dependent on their cell number. The strongest suppressive effect was observed on a stimulator cell ratio of 1:1 and this effect rapidly nearly disappeared at a lower ratio of mDC to competitor cells. The slight effect on T cell proliferation at a ratio of 1000:1 seems not to be the effect of the 10 stimulator cells on T cell proliferation but rather a problem of the readout system (Fig. 4.4 A, B, C and Tab. 9.2 of the appendix). In Table 4.1, the percentages of suppression (Δ%) were calculated for the experiments in Fig. 4.4. In order to estimate the cut-off level, different numbers of mDC as pseudo competitor cells were added to the MLC (Fig. 4.4 D, Tab. 9.4 of the appendix). This strategy allows setting the cut-off level for active cell-mediated suppression to 33% (Tab. 4.1, Tab. 9.4 of the appendix).

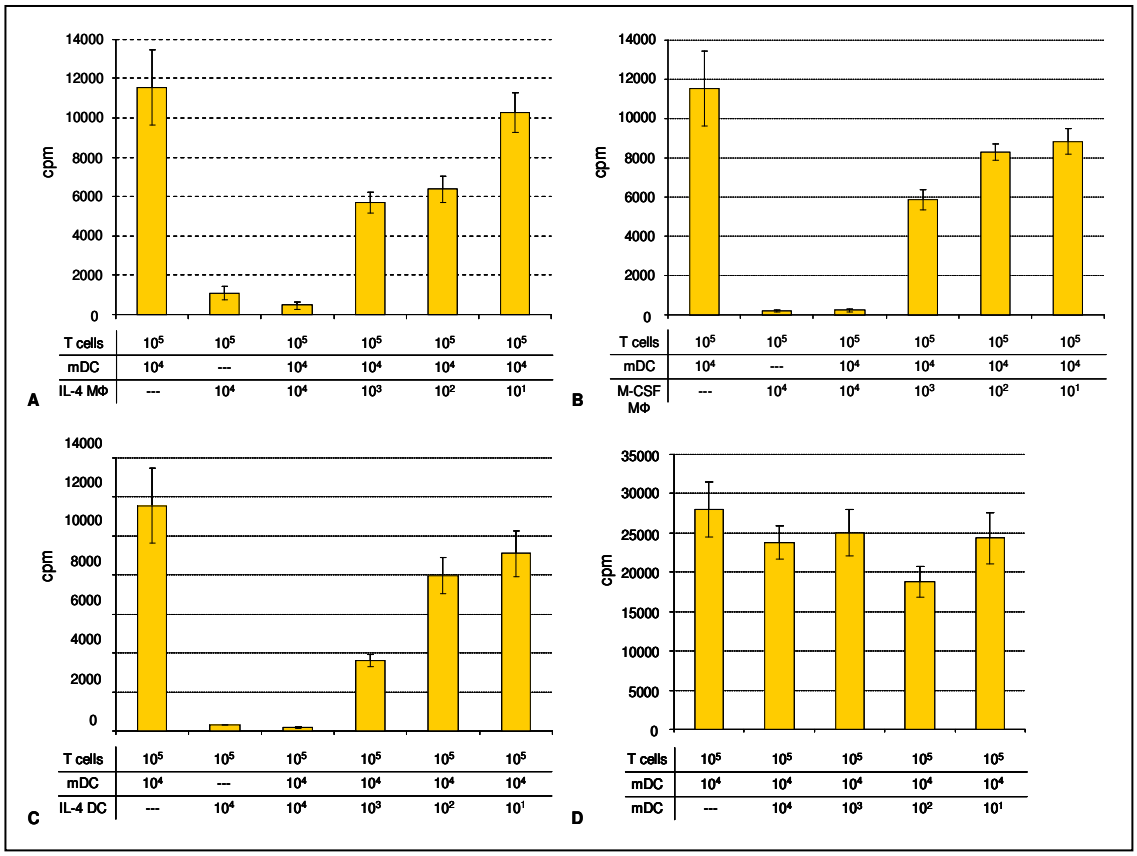


Fig. 4.4. IL-4 MΦ, M-CSF MΦ and IL-4 DC inhibited the mDC-induced proliferation of naïve T lymphocytes in competition assays. The strongest inhibition of T cell proliferation was found in those experiments where the ratio of stimulator cells and competitor cells was 1:1 (see also Tab. 4.1). With the stepwise reduction of the competitor cells IL-4 MΦ (A), M-CSF MΦ (B) and IL-4 DC (C), the mDC-induced proliferation of naïve T cells increased. The addition of different amounts of mDC as pseudo competitor cells to the MLC did not influence the proliferation rate as expected (D). This allows the calculation of the cut-off level for the suppression rate (Tab. 4.1). The obvious reduction of T cell proliferation at the ratio of 100:1 (the fourth bar from left in D) do not reflect a real suppression but demonstrate the variability of the *in vitro* system. The results (mean ± standard deviation) shown are representative for three experiments.

The results of the competition assays (Fig. 4.4, Tab. 4.1) indicated an active suppressive effect mediated by IL-4 MΦ, M-CSF MΦ and IL-4 DC for the cell ratios 1:1 and 10:1 (ratio of mDC to competitor cells). In addition, IL-4 MΦ also demonstrates a suppressive effect of 45% at the cell ratio 100:1 (Tab. 9.3 of the appendix). The values for the other competitor cells are below the cut-off value of 33% (Tab. 9.3 of the appendix).

In summary, IL-4 MΦ, M-CSF MΦ and IL-4 DC did not induce the proliferation of naïve T lymphocytes (Fig. 4.3) but they did demonstrate competitor qualities indicating a cell mediated suppressive effect (Fig. 4.4).

Tab. 4.1. Calculation of the suppressive effect mediated by IL-4 MΦ, M-CSF MΦand IL-4 DC (Fig. 4.4). The uninfluenced T cell proliferation in MLC (10^4 mDC co-cultured with 10^5 allogeneic T cells) is set as 100% proliferation or 0% suppression. The suppression rate Δ% induced by IL-4 MΦ, M-CSF MΦ and IL-4 DC in the competitor assays is calculated according to the following formula: $\Delta\% = (cpm_{\text{mDC induced T cell proliferation}} - cpm_{\text{T cell proliferation in the co-cultures}} / cpm_{\text{mDC induced T cell proliferation}}) \times 100$.

	Competition assays (Fig. 4.4)			
	10^5 T cells + 10^4 mDC + 10^4 IL-4 MΦ	**10^5 T cells + 10^4 mDC + 10^3 IL-4 MΦ**	**10^5 T cells + 10^4 mDC + 10^2 IL-4 MΦ**	**10^5 T cells + 10^4 mDC + 10^1 IL-4 MΦ**
Suppression (Δ%)	96	51	45	11
	10^5 T cells + 10^4 mDC + 10^4 M-CSF MΦ	**10^5 T cells + 10^4 mDC + 10^3 M-CSF MΦ**	**10^5 T cells + 10^4 mDC + 10^2 M-CSF MΦ**	**10^5 T cells + 10^4 mDC+ 10^1 M-CSF MΦ**
Suppression (Δ%)	98	49	28	23
	10^5 T cells + 10^4 mDC + 10^4 IL-4 DC	**10^5 T cells + 10^4 mDC + 10^3 IL-4 DC**	**10^5 T cells + 10^4 mDC + 10^2 IL-4 DC**	**10^5 T cells + 10^4 mDC + 10^1 IL-4 DC**
Suppression (Δ%)	99	69	31	21
	10^5 T cells + 10^4 mDC + 10^4 mDC	**10^5 T cells + 10^4 mDC + 10^3 mDC**	**10^5 T cells + 10^4 mDC + 10^2 mDC**	**10^5 T cells + 10^4 mDC + 10^1 mDC**
Suppression (Δ%)	15	11	33	13

The specificity of the observed suppressive effect mediated by IL-4 MΦ, M-CSF MΦ and IL-4 DC was proven by varying the ratio of mDC to competitor cells (Fig. 4.4). In addition, human cells of the mammarian carcinoma cell line MDA-MB 231 were also used to control the specificity of the suppressive effect mediated by IL-4 MΦ, M-CSF MΦ and IL-4 DC. Cells of this cell line did not activate T lymphocytes and did not act as competitor cells in the competition assay (Fig. 4.5 and Tab. 4.1).

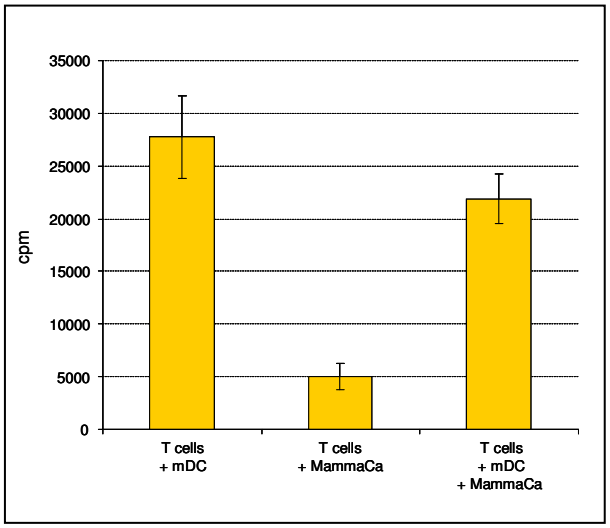


Fig. 4.5. Control cells did not stimulate naïve T lymphocytes and did not suppress the mDC-mediated T cell proliferation. Cells of the human mammarian carcinoma cell line MDA-MB 231 ("MammaCa") served as control cells in MLC and competition assay. With a suppression rate of 21%, no active suppressive effect was observed (Tab. 9.5 in appendix). 10^4 MammaCa cells were co-cultured with 10^5 T lymphocytes in the MLC and with 10^4 mDC and 10^5 T lymphocytes in the competition assay. The results (mean ± standard deviation) shown are representative for two experiments.

Figure 4.6 summarises the results of the chapter. It shows that the competitor cells IL-4 MΦ, M-CSF MΦ and IL-4 DC demonstrate a suppressive effect on the mDC-mediated T cell proliferation. Their suppressive effect was most obvious at a ratio of mDC to competitor cells of 1:1 as shown in Fig. 4.4. The pseudo competitor cells of the human carcinoma cell line MDA-MB 231 did not show a suppressive effect at the same cell ratio (Fig. 4.5).

In Fig. 4.6, the ratio of T lymphocytes to mDC is shown. In the MLC (Fig. 4.3), this ratio is 10:1 (10^5 T lymphocytes : 10^4 mDC) as demonstrated in Fig. 4.6 A. In contrast, the ratio of T lymphocytes to mDC is reduced in the competition assays depending on the amount of competitor cells. With 10^4 competitor cells added to the competition assays, the ratio of T lymphocytes to mDC is with 5:1 as small as possible: 10^5 T lymphocytes : (10^4 mDC + 10^4 competitor cells). In Fig.4.6, three different situations are shown to achieve this ratio of 5:1 (Fig. B-D). First, by reducing the amount of T lymphocytes to $0.5x10^5$; second, by adding 10^4 pseudo competitor cells ("invalid cells") and third, by adding 10^4 competitor cells. Only the competitor cells IL-4 MΦ, M-CSF MΦ and IL-4 DC suppress significantly the mDC-induced T cell proliferation at this ratio 5:1 (Fig. 4.6 D).

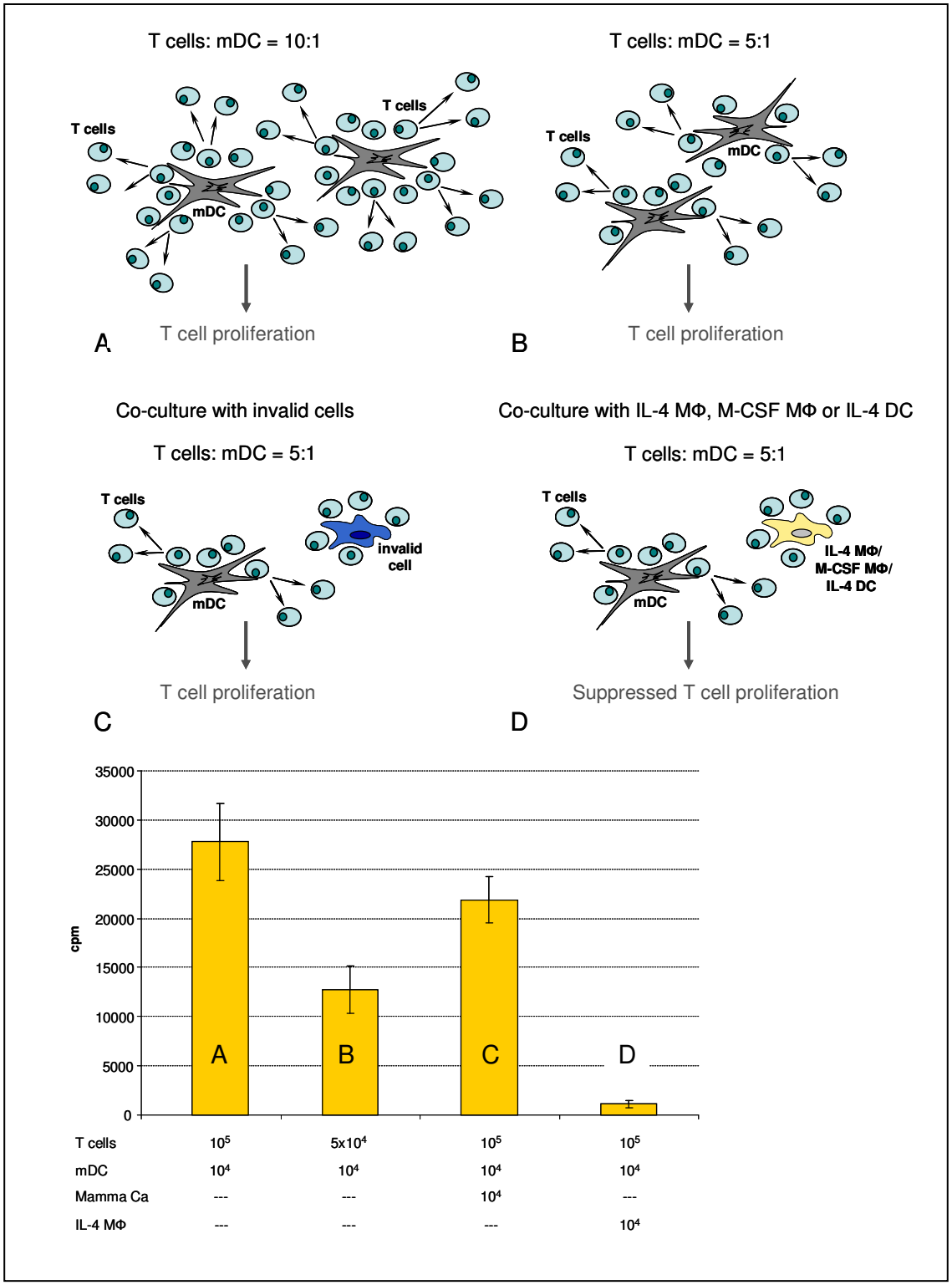


Fig. 4.6. IL-4 MΦ, M-CSF MΦ, and IL-4 DC suppressed the mDC-induced proliferation. Different situations are displayed where T cell proliferation may be reduced (B and C). However, only the presence of the competitor cells IL-4 MΦ, M-CSF MΦ and IL-4 DC (D) significantly suppressed the T cell proliferation. Results (mean ± standard deviation) of T cell proliferation in cpm.

4.2.3 Supernatant from IL-4 MΦ, M-CSF MΦ and IL-4 DC suppresses the activation of naïve T lymphocytes

To investigate the role of soluble factors secreted by IL-4 MΦ, M-CSF MΦ and IL-4 DC in the suppression of T cell proliferation, supernatant was collected after 24h and 48h of culture of IL-4 MΦ, M-CSF MΦ and IL-4 DC (10^6 cells/150 µL culture medium) and added to MLC (10^5 T lymphocytes and 10^4 mDC).

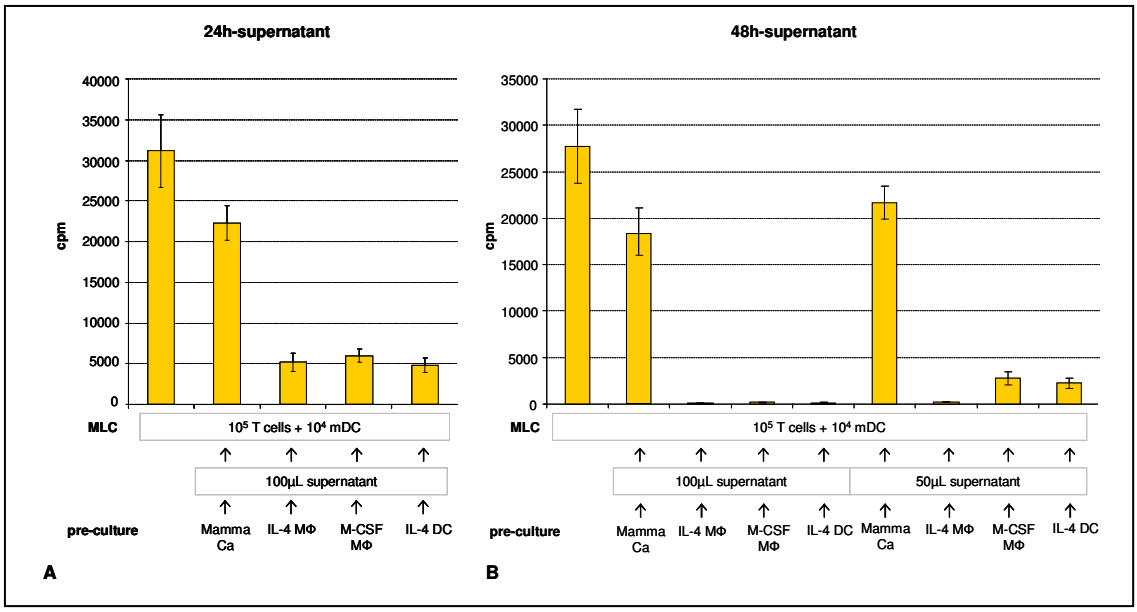


Fig. 4.7. Supernatant of IL-4 MΦ, M-CSF MΦ and IL-4 DC cultured for 24h/48h suppressed the mDC-stimulated T cell proliferation in a dose-dependent manner. After six days of culture, IL-4 MΦ, M-CSF MΦ and IL-4 DC were replated in 96-well plates (10^6 cells/150 µL culture medium) and cultured for 24h or 48h (pre-culture). The supernatant was collected and 50 µL and 100 µL, respectively, were transferred to the MLC (10^5 T lymphocytes cultured with 10^4 mDC). The supernatant of 10^6 MammaCa cells served as negative control. MammaCa: Cells of the cell line MDA-MB 231. The results (mean ± standard deviation) shown are representative for two experiments.

The results obtained with the supernatant from 24h- and 48h- cultures of IL-4 MΦ, M-CSF MΦ, and IL-4 DC demonstrate a suppressive effect (Fig. 4.7, Tab. 9.6 of the appendix). Therefore, the presence of IL-4 MΦ, M-CSF MΦ, and IL-4 DC in the MLC was not obligatory to induce suppression on T cell proliferation and the secretion of a soluble factor into the culture medium can be assumed. This factor was not identified in this study; the immunoregulatory cytokine TGF-β, however, was detected in supernatant from cultures with IL-4 MΦ, M-CSF

MΦ, and IL-4 DC (Fig. 4.18). Supernatant of 1x10^6 cells collected after 24h suppressed T cell proliferation with a suppression rate of 81-85% (Tab. 4.2). The suppressive effect of the 48h supernatant was even stronger with a suppression of 90-100% (Tab. 4.2). The values for the supernatant of cells from the mammarian carcinoma cell line MDA-MB 231 ("MammaCa") were below the cut-off value of 33%.

Tab. 4.2. ***Calculation of the suppressive effect mediated by 24h- and 48h-supernatant of IL-4 MΦ, M-CSF MΦ and IL-4 (Fig. 4.7).*** The T cell proliferation in MLC (10^4 mDC co-cultured with 10^5 allogeneic T cells) without addition of supernatant is set as 100% proliferation or 0% suppression. The suppression rate Δ% induced by supernatant of 10^6 IL-4 MΦ, M-CSF MΦ and IL-4 cultured for 24h or 48h in the MLC is calculated according to the following formula: Δ% = (cpm $_{\text{mDC induced T cell proliferation}}$ - cpm $_{\text{T cell proliferation with supernatant}}$ / cpm $_{\text{mDC induced T cell proliferation}}$) x 100. Supernatant of 10^6 Mamma Ca cells served as negative control. MammaCa: Cells of the cell line MDA-MB 231. See also Table 9.6 in the appendix. SN: supernatant.

	Assay with 24h- and 48h supernatant (Fig. 4.7)				
	10^5 T cells+ 10^4 mDC	**SN: MammaCa (24h, 100µL) + 10^5 T cells+ 10^4 mDC**	**SN: IL-4 MΦ (24h, 100µL) + 10^5 T cells+ 10^4 mDC**	**SN: MCSF MΦ (24h, 100µL) + 10^5 T cells+ 10^4 mDC**	**SN: IL-4 DC (24h, 100µL) + 10^5 T cells+ 10^4 mDC**
Suppression (Δ%)	---	28	83	81	85
	10^5 T cells+ 10^4 mDC	**SN: MammaCa (48h, 100µL) + 10^5 T cells+ 10^4 mDC+**	**SN: IL-4 MΦ (48h, 100µL) + 10^5 T cells+ 10^4 mDC**	**SN: MCSF MΦ (48h, 100µL) + 10^5 T cells+ 10^4 mDC**	**SN: IL-4 DC (48h, 100µL) + 10^5 T cells+ 10^4 mDC**
Suppression (Δ%)	---	33	100	99	100
	10^5 T cells+ 10^4 mDC	**SN: MammaCa (48h, 50µL) + 10^5 T cells^5+ 10^4 mDC**	**SN: IL-4 MΦ (48h, 50µL) + 10^5 T cells+ 10^4 mDC**	**SN: MCSF MΦ (48h, 50µL) + 10^5 T cells+ 10^4 mDC**	**SN: IL-4 DC (48h, 50µL) + 10^5 T cells+ 10^4 mDC**
Suppression (Δ%)	---	22	99	90	92

4.3 Characterisation of the suppressive effect on T cell proliferation mediated by IL-4 MΦ, M-CSF MΦ, and IL-4 DC

4.3.1 Possible reason for T cell hyporesponsiveness: T cell apoptosis

As described in the previous chapter, IL-4 MΦ, M-CSF MΦ and IL-4 DC did not activate naïve T lymphocytes and in addition, they acted as competitor cells indicating an active suppressive effect. To investigate the "nature" of this suppressive effect, first the rate of apoptotic T cells after the incubation with IL-4 MΦ, M-CSF MΦ and IL-4 DC was calculated. The programmed cell death (apoptosis) of T lymphocytes is an important mechanism to regulate T cell activation *in vivo* and *in vitro*. T lymphocytes cultured alone and or with mDC showed an average rate of apoptosis between 9-10% (Fig. 4.8).

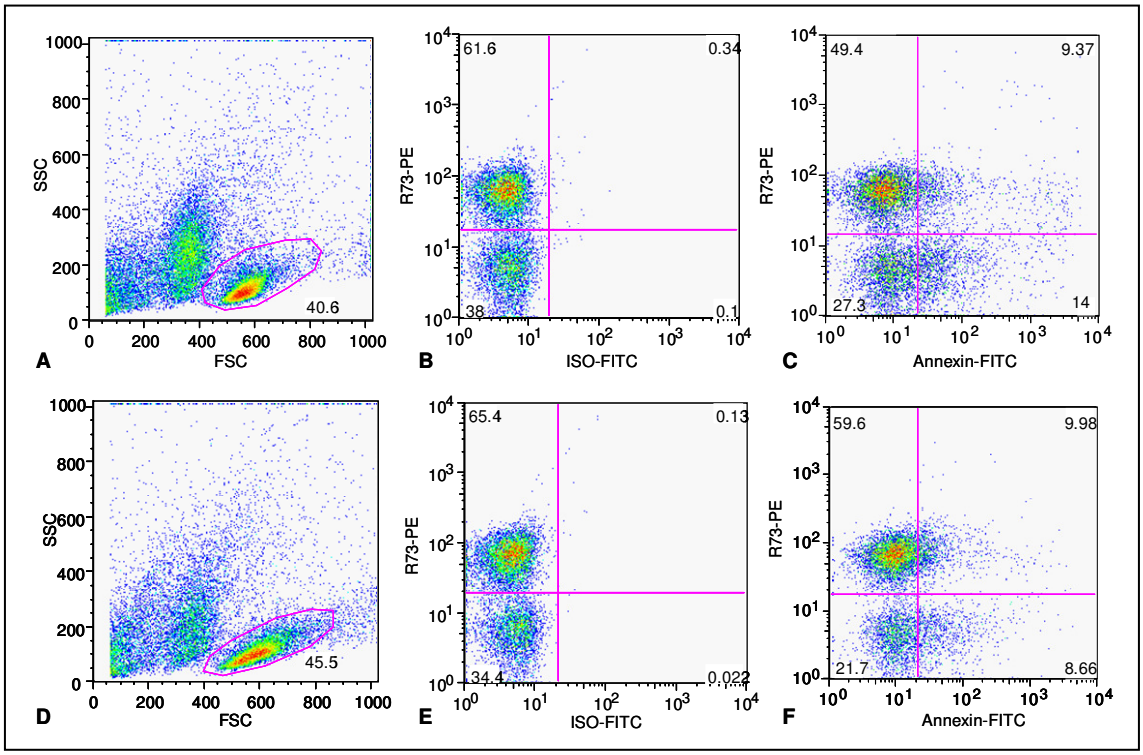


Fig. 4.8. Detection of early T cell apoptosis by flow cytometry. The FSC and SSC characteristics are shown for T lymphocytes cultured alone (A) and with mDC (D) after three days of culture in 96-well plates. Forward Scatter (FSC) and Side Scatter (SSC) are parameters related to cell size and cell granularity, respectively. The region indicates the analysed lymphocyte population. T lymphocytes were detected with the antibody R73 (B, E) and T cell apoptosis were detected with a combination of R73 and Annexin-V (C, F). The results shown are representative for two independent experiments.

T lymphocytes collected from co-cultures with IL-4 MΦ, M-CSF MΦ and IL-4 DC, respectively, did not show a significant increase of apoptosis (Fig. 4.9 A, Tab. 9.7 of the appendix). In contrast, increased rates of apoptosis between 20-30% were measured for T lymphocytes cultured in competition assays (Fig. 4.9 B, C and Tab. 9.7 of the appendix). Interestingly, these rates of apoptosis were found in those competition assays with only 100 IL-4 MΦ and M-CSF MΦ competitor cells, respectively.

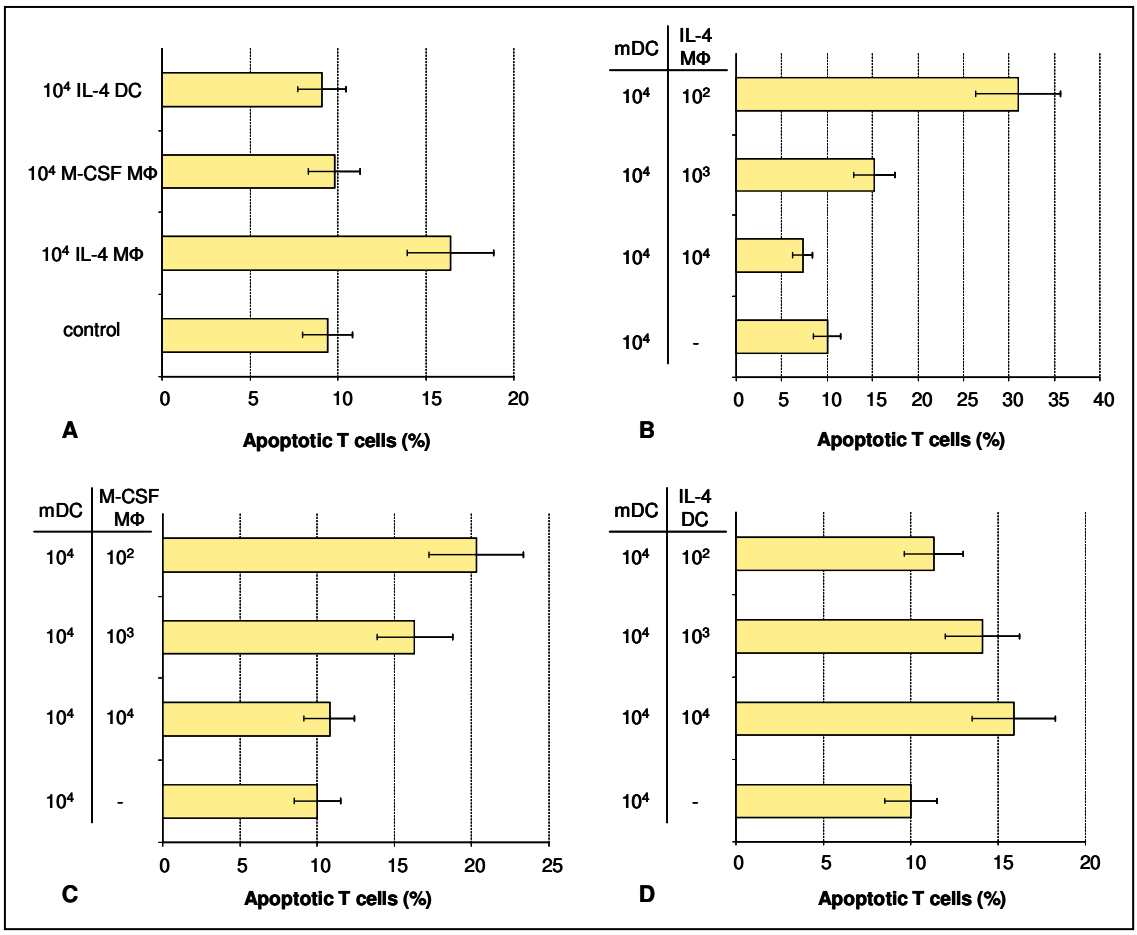


Fig. 4.9. IL-4 MΦ and M-CSF MΦ induced T cell apoptosis. Shown are the results for the MLC (A) and competition assays (B-D). IL-4 MΦ were of low effect and M-CSF MΦ and IL-4 DC were without any effects in the MLC (A). In contrast to IL-4 MΦ and M-CSF MΦ, which showed the strongest effects at a cell ratio of mDC to competitor cells of 1:100 (B, C), IL-4 DC did not considerably induce T cell apoptosis in the competition assays (D). Detection of apoptosis in T lymphocytes was performed by flow cytometry (Fig. 4.8). The results (mean ± standard deviation) shown are representative for two independent experiments.

4.3.2 Possible reason for T cell hyporesponsiveness: T cell anergy

Another possible effect of IL-4 MΦ, M-CSF MΦ and IL-4 DC on allogenic T lymphocytes leading to non- or reduced proliferation is the induction of T cell anergy. T cell anergy is characterised by reduced proliferation and cytokine production (Fathman and Lineberry, 2007). This state is distinct from apoptosis and anergic T cells do not proliferate in response to appropriate antigenic stimulation (Appleman and Boussiotis, 2003). To investigate whether IL-4 MΦ, M-CSF MΦ and IL-4 DC induced an anergic state in T lymphocytes, they were collected from the first MLC and restimulated by mDC in a second MLC.

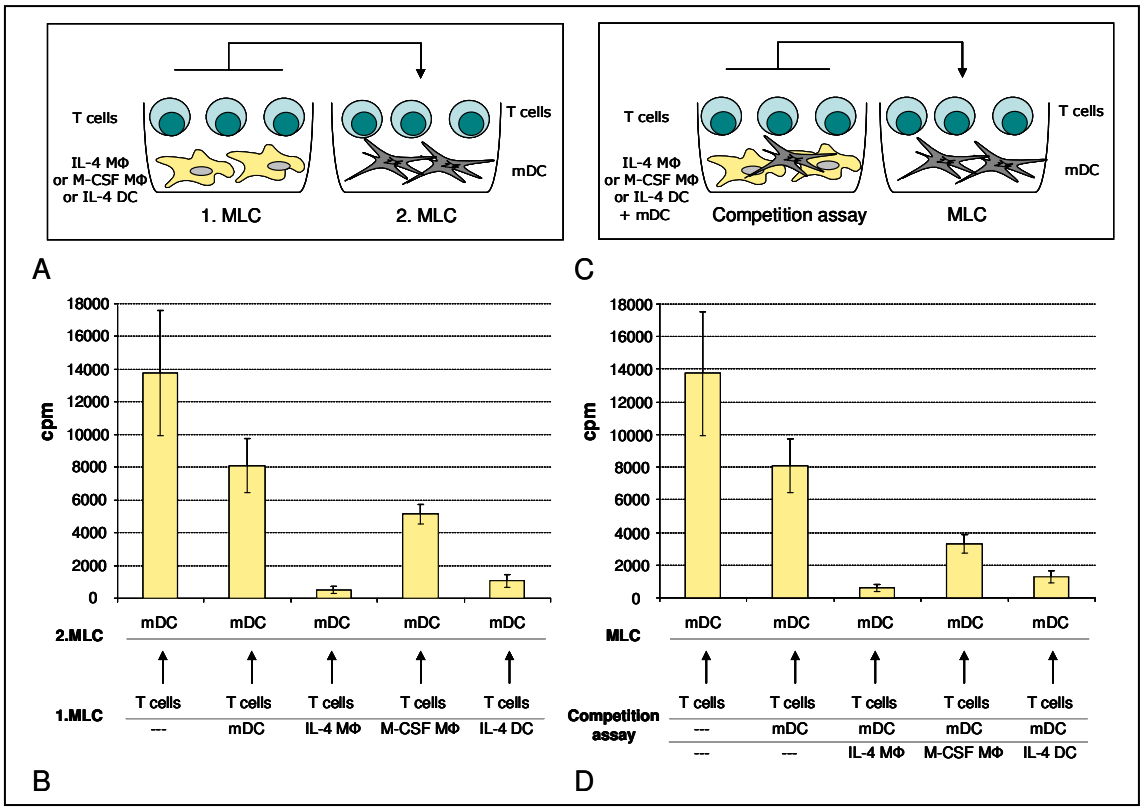


Fig. 4.10. T lymphocytes from co-cultures with IL-4 MΦ, M-CSF MΦ and IL-4 DC showed a reduced proliferation when incubated with mDC. T lymphocytes were cultured with IL-4 MΦ, M-CSF MΦ, and IL-4 DC (first MLC in B) and after three days of culture they were transferred to the second MLC with mDC. In addition, T lymphocytes were cultured in the competition assay (D) first and then incubated with mDC. The proliferation of these T lymphocytes was clearly reduced in comparison to the control T lymphocytes which were cultured alone or with mDC during the first MLC or competition assay. The results (mean ± standard deviation) shown are representative for two independent experiments.

T lymphocytes cultured with IL-4 MΦ, M-CSF MΦ or IL-4 DC alone or in competition assays showed a strongly reduced proliferation when subsequently transferred to mDC (Fig. 4.10 B and D, Tab. 9.8 of the appendix). T lymphocytes cultured for three days without stimulation demonstrated a clear proliferation in the second culture with mDC. The same effect was observed for T lymphocytes, which were stimulated and restimulated with mDC.

4.4 Characterisation of the phenotype of IL-4 MΦ, M-CSF MΦ, and IL-4 DC

4.4.1 The costimulatory molecules CD80 and CD86 are not detected on the surface of IL-4 MΦ and M-CSF MΦ but on IL-4 DC by immunohistochemistry

IL-4 MΦ, M-CSF MΦ and IL-4 DC did not activate naïve T lymphocytes and, used as competitor cells, inhibited their activation by mDC (Fig. 4.3 and 4.4). For full T cell activation, antigens and costimulatory molecules are necessary; therefore, the surface expression of MHC class I, MHC class II, CD40, CD80, and CD86 was proven by immunohistochemistry and flow cytometry with the antibodies listed in Tables 3.1 and 3.2.

With the monoclonal antibodies ED1 (CD68, macrophage marker) and Ox62 (Ox62 antigen, DC marker) the origin of IL-4 MΦ, M-CSF MΦ and IL-4 DC was validated. More than 95% of IL-4 MΦ and M-CSF MΦ were positive for CD68 (Fig. 4.11 B and D) and negative for Ox62 antigen (data not shown). IL-4 DC expressed both CD68 and Ox62 (Fig. 4.12 B and C). The expression of MHC class I and class II molecules was proven with the monoclonal antibodies Ox6 and Ox18, respectively. Whereas MHC I molecules were expressed on all three cell types (data not shown), MHC II molecules were only detected on IL-4 MΦ and IL-4 DC, not on M-CSF MΦ (Fig. 4.11 A and C, Fig. 4.12 A).

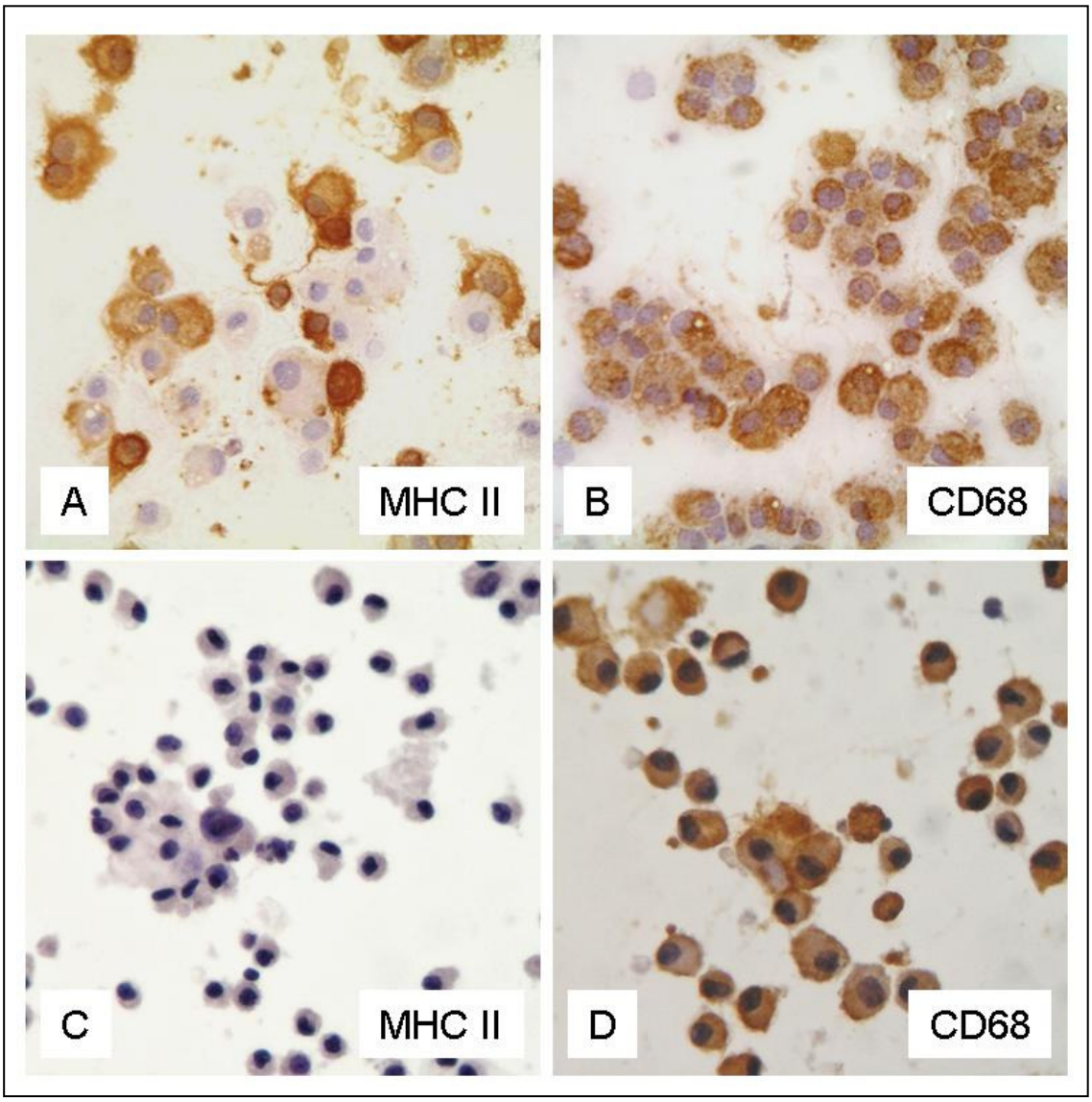


Fig. 4.11. IL-4 MΦ and M-CSF MΦ expressed CD68, but only IL-4 MΦ expressed MHC II molecules. Immunohistochemical staining of IL-4 MΦ (A, B) and M-CSF MΦ (C, D) with the monoclonal antibodies ED1 (CD68) and Ox6 (MHC class II). Nuclear staining was performed with hematoxylin. The staining is representative for three independent experiments. Olympus BH-2 light microscope, 400x magnification. Images were captured using a digital camera and processed with Corel Photo-Paint 11.

IL-4 MΦ, M-CSF MΦ and IL-4 DC demonstrated different expression of costimulatory molecules. IL-4 MΦ and M-CSF MΦ did not show any expression of CD80 or CD86 molecules (data not shown), whereas approximately 75% of IL-4 DC were positive for these costimulatory molecules (Fig. 4.12 D, no data shown for CD86).

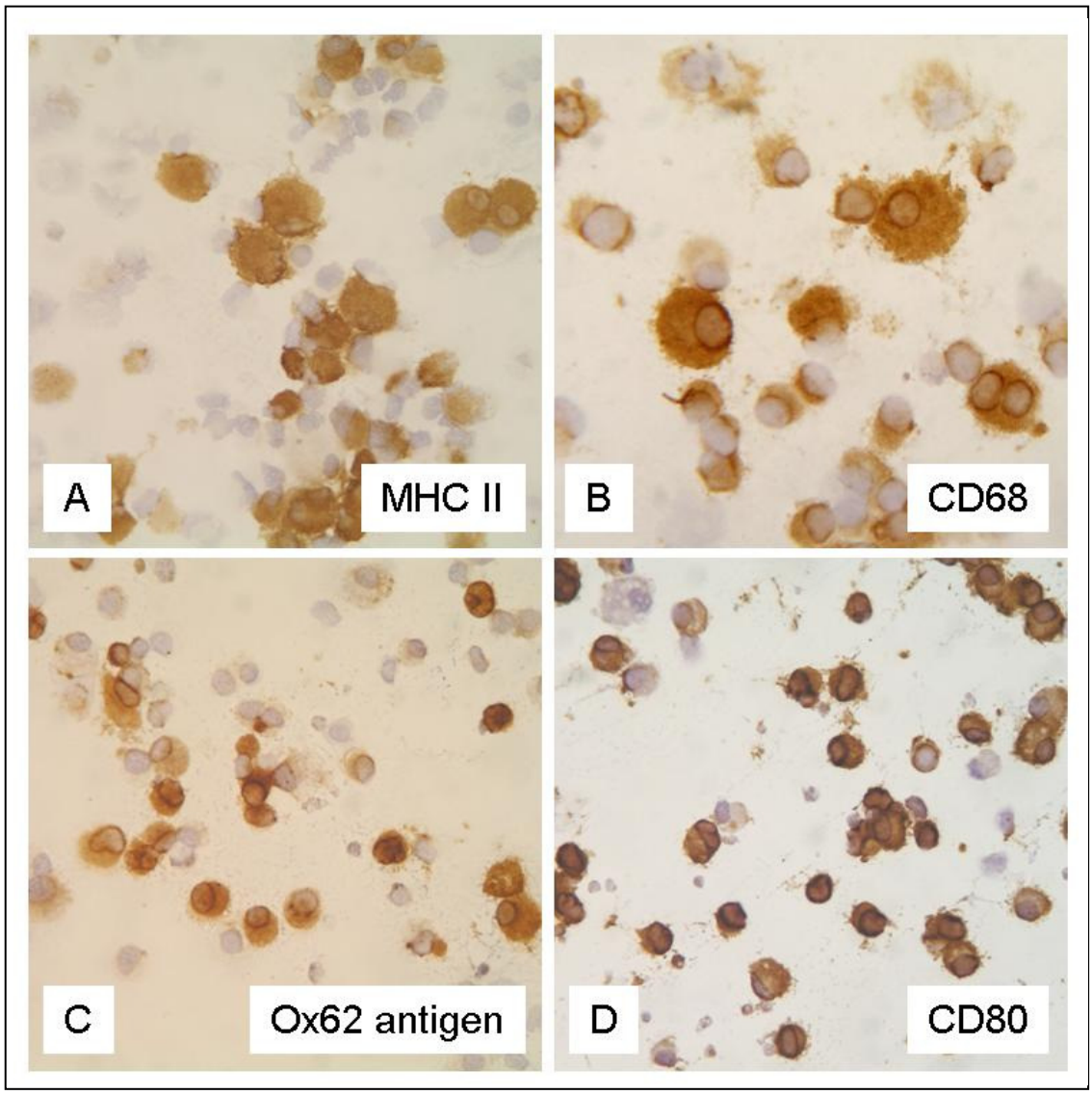


Fig. 4.12. IL-4 DC expressed CD68, Ox62 antigen, CD80, CD86, MHC I and II molecules. Immunohistochemical staining of IL-4 DC revealed various cell surface molecules. Shown are the results for MHC II, CD68, Ox62 antigen and CD80. Nuclear staining was performed with hematoxylin. The staining is representative for three independent experiments. Olympus BH-2 light microscope, 400x magnification. Images were captured using a digital camera and processed with Corel Photo-Paint 11.

4.4.2 The costimulatory molecules CD80, CD86 and MHC class I and class II are expressed differently on the surface of IL-4 MΦ, M-CSF MΦ and IL-4 DC shown by flow cytometry

In addition to the immunohistochemistry, the phenotypes of IL-4 MΦ, M-CSF MΦ and IL-4 DC were characterised by flow cytometry.

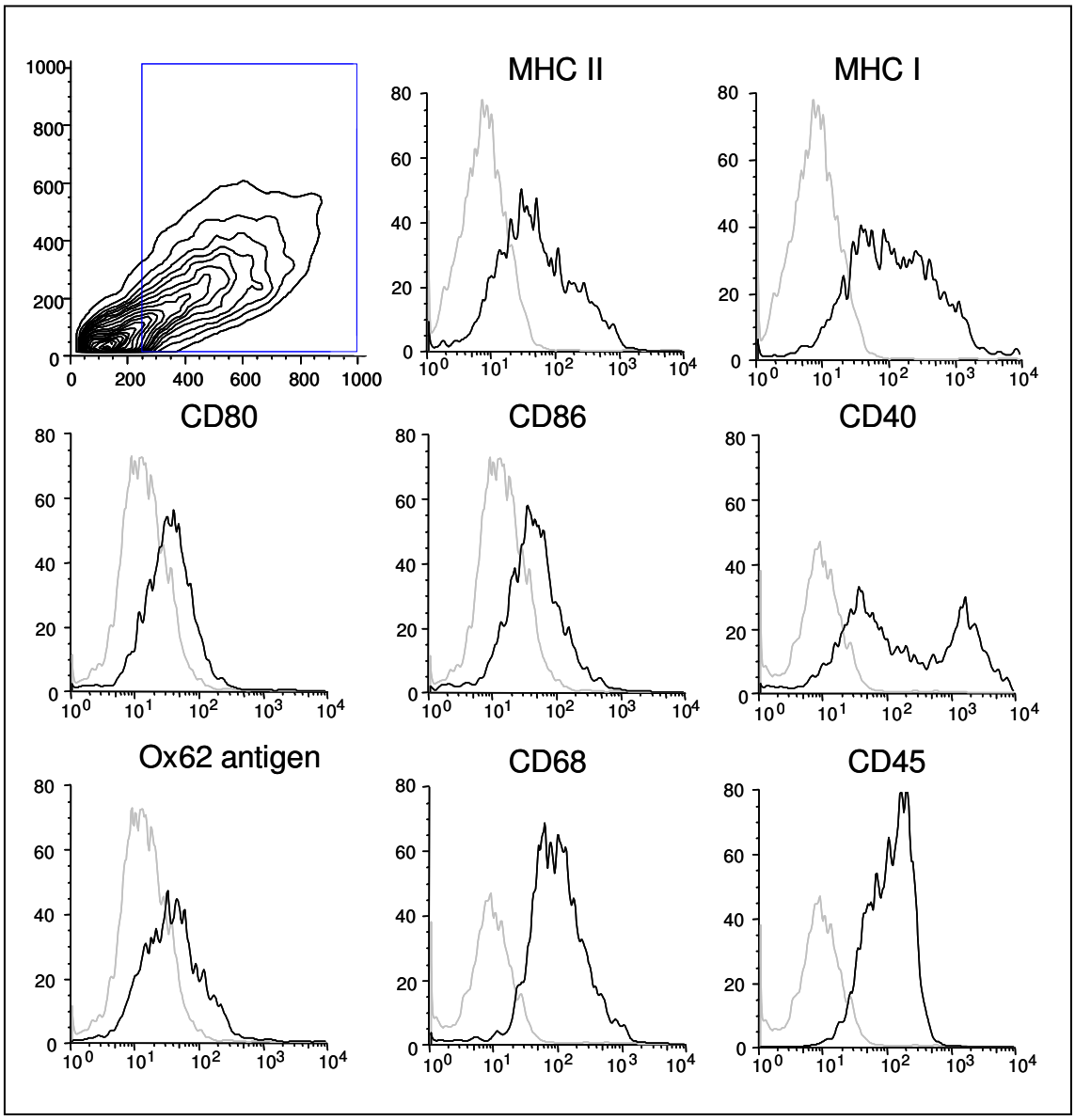


Fig. 4.13. Flow cytometry profiles of IL-4 MΦ. *The expression* of different cell surface molecules was examined by flow cytometry. FSC/SSC profile is shown in the top left corner. Forward Scatter (FSC) and Side Scatter (SSC) are parameters related to cell size and cell granularity, respectively. The isotype control is shown in grey. The data are representative for eight independent experiments.

L-4 MΦ showed a considerable expression of the leukocyte cell marker CD45 and the macrophage cell marker CD68 but no or a very low expression of the DC marker Ox62 (Fig. 4.13). Simultaneously, no or a very low expression of the costimulatory molecules CD80 and CD86, but a considerable expression of the costimulatory molecule CD40 was detected. In view of MHC molecules class I and class II, IL-4 MΦ were clearly positive (Fig. 4.13, Tab. 4.3).

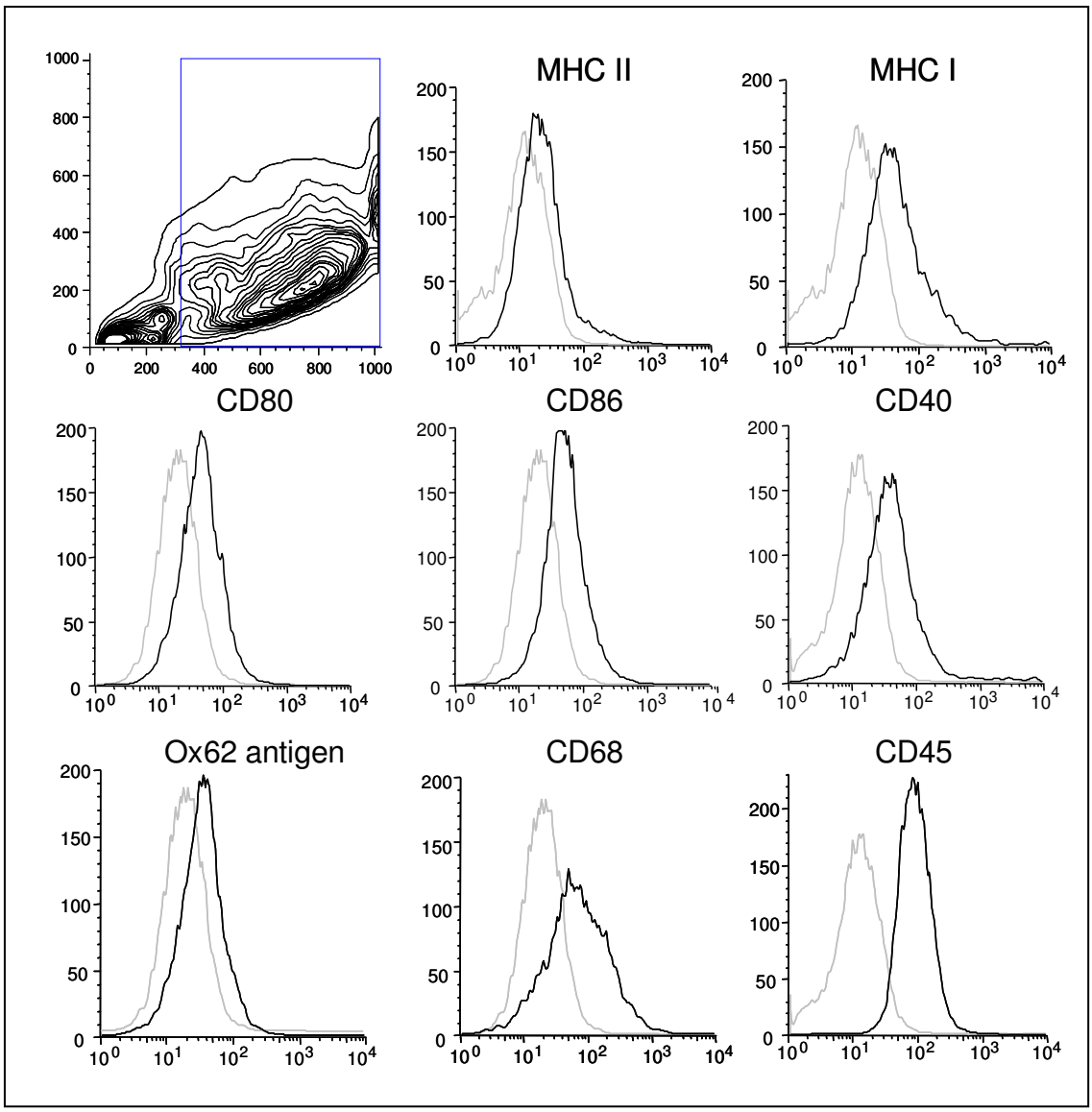


Fig. 4.14. Flow cytometry profiles of M-CSF MΦ. *The expression* of different cell surface molecules was examined by flow cytometry. FSC/SSC profile is shown in the top left corner. Forward Scatter (FSC) and Side Scatter (SSC) are parameters related to cell size and cell granularity, respectively. Isotype control is shown in grey. The data are representative for four independent experiments.

M-CSF MΦ also showed a clear expression of both the leukocyte marker CD45 and the macrophage marker CD68. These cells were also negative for the DC marker Ox62 and the costimulatory molecules CD80 and CD86. A moderate expression of CD40 and MHC class I molecules but no expression of MHC II molecules can be assumed according to the FACS profiles (Fig. 4.14, Tab. 4.3).

The comparison of IL-4 MΦ and M-CSF MΦ shows that IL-4 induced the expression of both MHC class I and class II as well as of the costimulatory molecule CD40. This may indicate that IL-4 MΦ are activated macrophages in contrast to M-CSF MΦ.

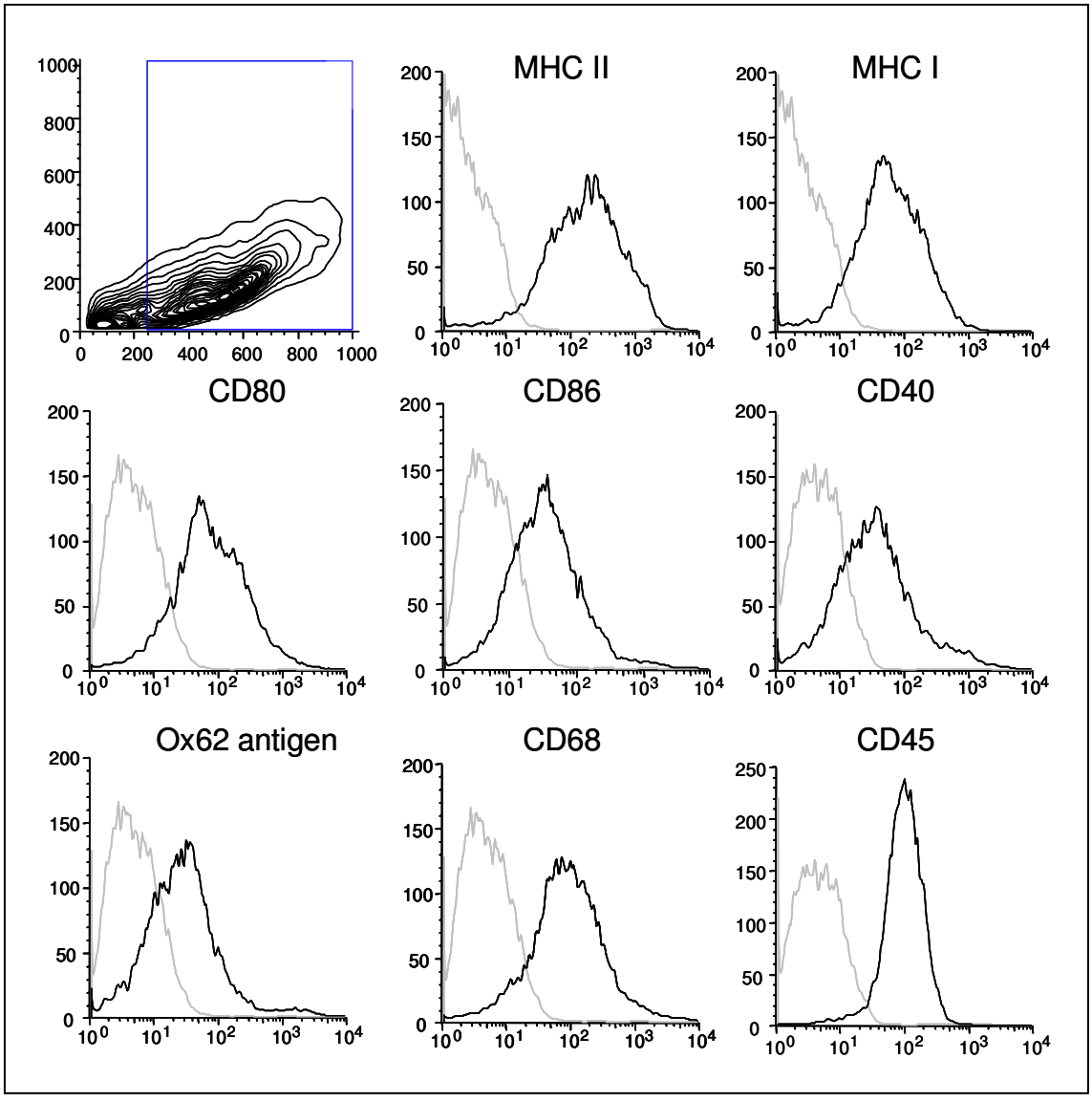


Fig. 4.15 (page 33). Flow cytometry profiles of IL-4 DC. The expression of different cell surface molecules was examined by flow cytometry. FSC/SSC profile is shown in the top left corner. Forward Scatter (FSC) and Side Scatter (SSC) are parameters related to cell size and cell granularity, respectively. Isotype control is shown in grey. The data are representative for four independent experiments.

According to the flow cytometry profiles in Fig. 4.15, IL-4 DC expressed the leukocyte marker CD45, the macrophage cell marker CD68 and the DC marker Ox62. In contrast to M-CSF and IL-4 MΦ, IL-4-DC showed a clear expression of the costimulatory molecules CD40, CD80 and CD86. Moreover, these cells were evidently positive for MHC class I and II molecules (Fig. 4.15, Tab. 4.3).

Tab. 4.3. Analysis of the flow cytometry profiles (Fig. 4.13-4.15). The fluorescence intensity of antibody binding was calculated to discriminate differences in the cell surface expression. IL-4, M-CSF MΦ and IL-4-DC generated from bone marrow derived precursor cells were harvested after 6 days of culture. Mean fluorescence (MF) values and percentage of positive cells (P) are shown. MF = F (protein of interest) - F (isotype control).

Antigen	IL-4 MΦ		M-CSF MΦ		IL-4 DC	
	MF	**P (%)**	**MF**	**P (%)**	**MF**	**P (%)**
MHC II	103	58	20	16	335	96
MHC I	346	78	80	47	102	92
CD40	831	80	101	37	113	70
CD80	29	25	33	34	169	86
CD86	53	36	51	42	72	67
CD45	120	95	90	89	112	98
OX62 antigen	56	32	29	21	81	60
CD68	139	92	104	55	215	87

4.4.3 The Real time PCR revealed a lower expression of MHC class I and class II, CD80 and CD86 in IL-4 MΦ and M-CSF MΦ than in IL-4 DC

The Real time PCR was used to investigate the expression of specific mRNA for the cell surface molecules MHC I and MHC II, CD40, CD80 and CD86. In addition, the presence of specific RNA for the cytokine TGF-β and for the enzyme iNOS was proven. The housekeeping gene GAPDH served as internal positive control, 10^8 copies of the TA vector with a specific cloned PCR fragment or mRNA isolated from mDC served as positive and Nuclease-free water as negative control (Fig. 9.1 of the appendix).

Real time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle. For the relative quantitation of the expression of certain mRNA, the Ct ("threshold cycle") is used. Ct is the cycle number where the fluorescence signal caused by the increased amounts of PCR amplicon is above background fluorescence and the PCR amplification is in the exponential phase. The sample with the highest amount of the mRNA template, reaches this point first (Fig. 9.2 A and B). In a relative quantitation, the expression of the gene of interest (GOI) is set in relation to the expression of a housekeeping gene, here GAPDH: $\Delta Ct = Ct_{(GOI)} - Ct_{(GAPDH)}$ (Pfaffl, 2001; Tichopad et al., 2003). As shown in Table 9.9 of the appendix, a relative quantitation is performed for the macrophages IL-4 MΦ, M-CSF MΦ and for IL-4 DC. The $\Delta\Delta Ct$ calculation involves the subtraction as follows: $\Delta\Delta Ct = \Delta Ct_{(x)} - \Delta Ct_{(IL\text{-}4\ DC)}$ (x = IL-4 MΦ or M-CSF MΦ), whereby the IL-4 DC served as reference. Fig. 9.2 C and D of the appendix illustrates the interpretation of the comparative threshold (Ct) method. The last step in quantitation is to transform these values to absolute values. The formula for calculating the comparative expression level is: $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001). The relative quantitation revealed that IL-4 MΦ and M-CFS MΦ demonstrated reduced levels for MHC class I and class II, CD80 and CD86 in comparison to IL-4 DC. However, the relative expression levels were more strongly reduced for M-CSF MΦ than for IL-4 MΦ. For CD40, only IL-4 MΦ showed a comparable expression to IL-4 DC (Tab. 4.4).

Tab. 4.4. Comparative expression levels. The relative expression of MHC class I and class II, CD40, CD80 and CD86 for IL-4 MΦ and M-CSF MΦ in comparison to IL-4 DC is shown. These data and the results from immunohistochemistry (Fig. 4.11, 4.12) and flow cytometry (4.13-4.15) demonstrate strongly reduced expression levels for the M-CSF MΦ.

	MHC I	MHC II	CD40	CD80	CD86
IL-4 MΦ	0.63	0.77	1.42	0.15	0.45
M-CSF MΦ	0.18	0.003	0.25	0.06	0.01

In accordance with the results of the flow cytometric analysis (Fig. 4.14), the M-CSF MΦ were scarce in the surface molecules MHC class II, CD80 and CD86 (Fig. 4.16). For these molecules, the relative expression levels of the certain mRNA were below 0.1 (Tab. 4.4). The IL-4 MΦ expressed MHC I and II and CD40 and low amounts of CD86. The relative expression level of CD86 was 0.45 (Tab. 4.4). The lack of costimulation may explain why these cells are not able to activate T lymphocytes (Fig. 4.3). Nonetheless, these results do not explain why these cells inhibit the mDC-induced proliferation of naïve T lymphocytes in competition assays (Fig. 4.4). IL-4 DC express all these molecules and are poor stimulator cells, too.

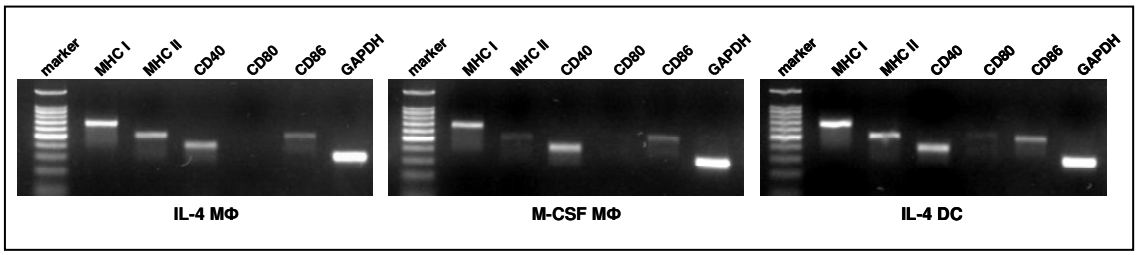


Fig. 4.16. Agarose gel electrophoresis of the amplified PCR products. Additional information regarding the PCR products can be found in Table 2.3. Marker: 100 bp DNA Ladder (Promega).

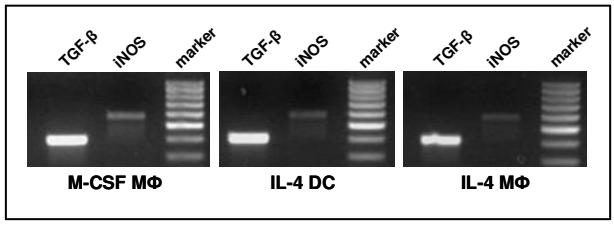
In addition to MHC class I and class II, CD40, CD80 and CD86, comparative expression levels for TGF-β and iNOS were also calculated (Tab. 4.5). IL-4 MΦ demonstrated the same expression level for TGF-β as IL-4 DC; the relative expression level of M-CSF MΦ was 0.65 (Tab. 4.5). TGF-β is an immunosuppressive cytokine that inhibits T cell proliferation (Kehrl et al., 1986). In addition, supernatant from these cells was positive for soluble TGF-β (Fig.

4.18). Both macrophage subtypes expressed reduced levels of specific mRNA coding for the enzyme inducible nitric oxide synthase (iNOS) that produce nitric oxide (NO) and reactive oxygen species involved in T cell apoptosis (Sade and Sarin, 2004).

Tab. 4.5. Comparative expression levels. The relative expression of TGF-β and iNOS for IL-4 MΦ and M-CSF MΦ in comparison to IL-4 DC.

	TGF-β	iNOS
IL-4 MΦ	0,99	0,63
M-CSF MΦ	0,65	0,25

Fig. 4.17. Agarose gel electrophoresis of the amplified PCR products. Additional information regarding the PCR products can be found in Table 2.3. Marker: 100 bp DNA Ladder (Promega).



4.4.4 Testing supernatant with ELISA: IL-4 MΦ, M-CSF MΦ and IL-4 DC secrete the immunoregulatory cytokine TGF-β but not IL-10

The presence of IL-10 and TGF-β, both immunoinhibitory cytokines (Wahl et al., 2006), in the supernatant of replated 10^4 and 10^6 M-CSF MΦ, IL-4 MΦ and IL-4 DC was investigated after 48h of incubation. In contrast to IL-10, TGF-β was detected in the supernatant (Fig. 4.18, Tab.9.10 of the appendix).

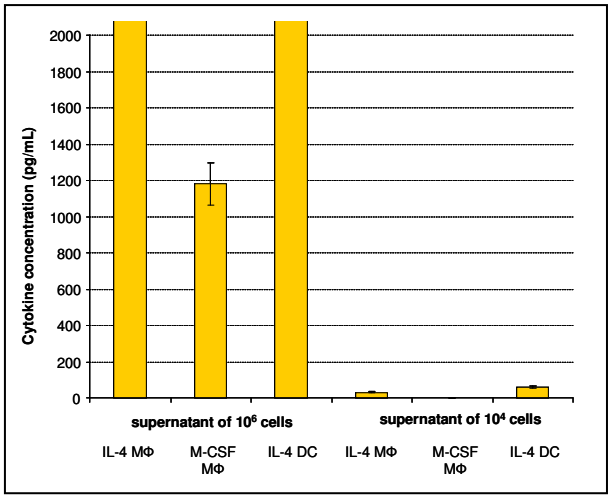


Fig. 4.18. TGF-β production of M-CSF MΦ, IL-4 MΦ and IL-4 DC. The results (mean ± standard deviation) shown are from one experiment in triplicate wells.

5 Discussion

The growth and differentiation of macrophages and dendritic cells from haematopoietic stem cells of the bone marrow depend on lineage-determining cytokines (Gordon, 2003). Granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) are related growth factors participating in macrophage development (Santin et al., 1999). M-CSF is considered more specific to the macrophage lineage, whereas GM-CSF promotes dendritic cell differentiation, particularly when coupled to the Th2 cytokines IL-4 or IL-13 (Mueller et al., 2007). Dependent on the environmental cytokines, a common myeloid precursor differentiates into monocytes/ macrophages, dendritic cells and osteoklasts (Gordon, 2003). Monocytes are distributed through the blood stream, enter tissue compartments and undergo activation due to present, local stimuli (Gordon, 2003). Macrophages share many properties with dendritic cells and are of great importance in the biology of allograft rejection (Wyburn et al., 2005).

5.1 Influence of IL-4 on the phenotype of IL-4 MΦ

In this study the biological effect of bone marrow derived macrophages was analysed. They were generated from bone marrow precursor cells cultured with M-CSF and IL-4 (IL-4 MΦ) and with M-CSF (M-CSF MΦ). IL-4 MΦ demonstrated a unique phenotype different from M-CSF MΦ. They are positive for MHC class I and class II molecules as well as for CD40 and negative for CD80 and CD86 as shown by flow cytometry (Fig. 4.13). The relative quantitation with real time PCR demonstrates the presence of specific mRNA for MHC class I and class II as well as for CD40 and CD86 (Tab. 4.4, Fig. 5.1). The results of flow cytometry and real time PCR indicate the presence of CD86 mRNA but not of CD86 protein. The M-CSF MΦ, with a clear surface expression of CD40 and a low expression of MHC I, seem to be negative for MHC class II, CD80 and CD86 in the flow cytometric profiles (Fig. 4.14 and 5.1).

However, on mRNA level, signals for MHC class II and CD86 was detected (Fig. 4.16).

The engagement of IL-4 with its receptor can regulate a wide variety of biological responses. IL-4 stimulates *in vitro* the growth and viability of T and B lymphocytes, monocytes and myeloid progenitors, deactivates inflammatory macrophages and regulates the induction of $CD4^+$ T-helper 2 (Th2) cells (Paul, 1991). Furthermore, IL-4 inhibits the release of inflammatory molecules such as IL-1, TNF-α, and IL-8 (Rocken and Shevach, 1996).

Macrophages are well-known immunostimulatory cells of the innate immune system with a proinflammatory phenotype, but immunomodulatory activities are also described (Mantovani et al., 2004; Gordon, 2003). The anti-inflammatory cytokine IL-4 is characterised as one of the agents inducing a so-called alternative activation in macrophages (Gordon, 2003; Goerdt and Orfanos, 1999). These macrophages which exert immunoregulatory functions, are particularly found in the placenta and lungs of healthy individuals where they protect from unwanted immune responses (Chang et al., 1993; Holt et al., 1988; Miyazaki et al., 2003; Mues et al., 1989). In addition, they also accumulate in wound tissue during the healing phase where they down-regulate inflammation (Song et al., 2000).

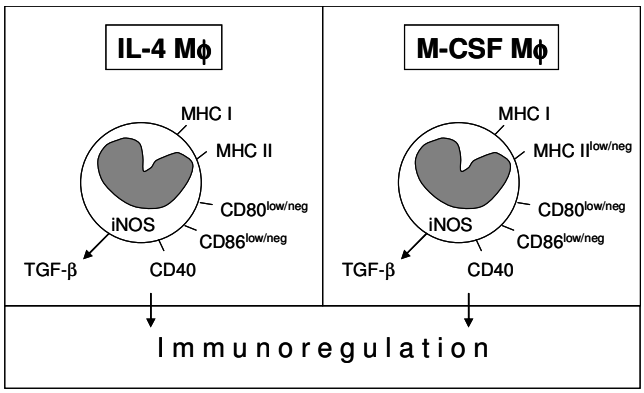


Fig. 5.1. The phenotype of IL-4 Mϕ and M-CSF Mϕ. The results based on analysis with flow cytometry (Fig. 4.13 and 4.14), real time PCR (Tab. 4.4 and 4.5, Fig. 4.16 and 4.17) and ELISA (Fig. 4.18).

In previous studies of the *Experimental Transplantation Immunology* of the Department of Surgery, immunosuppressive properties for so-called IL-4 DC

during the alloimmune response were described (Tiurbe GC, 2006). These cells were derived from bone marrow precursor cells by granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4. In this study, the effect of IL-4 on the induction of bone marrow derived macrophages and their proposed immunoregulatory properties was analysed. The results of this study show that M-CSF MΦ and IL-4 MΦ do not activate allogeneic T lymphocytes in mixed leukocyte cultures (Fig. 4.3). The phenotypes of these cells lack the costimulatory molecules CD80 and CD86 and this may explain these results (Fig. 4.11, 4.13, 4.14). According to the “two-signal” hypothesis of T cell activation, naïve alloreactive T lymphocytes need two signals for their complete activation (Fig. 1.1). The first signal is induced by the engagement of the T cell receptor with the MHC molecules plus the presented peptide. This signal must be accompanied by a second stimulus through the coreceptor CD80 and CD86 (signal two). IL-4 DC, however, analysed as control cells in this study, demonstrated surface expression of CD80 and CD86 and are poor T cell stimulators, too. Nevertheless, in comparison to mature dendritic cells the expression levels for CD80 and CD86 on IL-4 DC are reduced (data not shown). Consequently, the costimulation of IL-4 DC as well as of M-CSF MΦ and IL-4 MΦ seems not to be effective enough to activate naïve T lymphocytes. The induction of hyporesponsiveness in allogeneic T lymphocytes with so-called resting (non-activated) macrophages was described by Hoves et al. (Hoves et al., 2006). This effect was independent of the lack of costimulatory molecules and even with costimulation, provided by the addition of anti-CD28 monoclonal antibodies, allogeneic T lymphocytes still did not proliferate adequately.

In the present study, competition assays show a suppressive effect on mDC-mediated T cell proliferation for the M-CSF MΦ, IL-4 MΦ and IL-4 DC (Fig. 4.4). This suppressive effect was dose-dependent indicating an active, inhibitory mechanism. This result is in accordance with Schebesch et al. who showed that mitogen-induced proliferation of peripheral blood lymphocytes and purified $CD4^+$ T cells was strongly inhibited by human IL-4 MΦ. Again, this effect did not depend on expression of costimulatory molecules (Schebesch et al., 1997).

5.2 Characterisation of the suppressive effect mediated by IL-4 MΦ

T cell tolerance can be caused by diverse mechanisms including the induction of apoptosis, T cell anergy or differentiation and activation of T regulatory cells (Abbas et al., 2004). Low rates of apoptosis of T lymphocytes were detected after the incubation with M-CSF MΦ, IL-4 MΦ and IL-4 DC but the highest rates were found in those competition assays with only 100 IL-4 MΦ and M-CSF MΦ competitor cells, respectively. Therefore, it is not clear whether this is a relevant biological effect (Fig. 4.9).

Viable T lymphocytes collected from competition assays with M-CSF MΦ, IL-4 MΦ and IL-4 DC could not fully respond to maximal stimulatory signals provided by mDC (Fig. 4.10). This result supports the assumption of anergy induction by M-CSF MΦ, IL-4 MΦ and IL-4 DC. Since anergic T lymphocytes occur after activation in absence of sufficient costimulation (Schwartz, 1992), the low expression levels of costimulatory molecules on the surface of M-CSF MΦ and IL-4 MΦ and IL-4 DC (Fig. 4.11, 4.13, 4.14, 4.15, Tab. 4.4) may allow anergy induction by these cells.

The possible role of soluble factors secreted by IL-4 MΦ for the suppression of T cell proliferation was investigated and the results obtained in this study demonstrate an inhibitory effect. Therefore, the direct presence of IL-4 MΦ was not obligatory to suppress T cell activation (Fig. 4.7). Whether this soluble factor induces anergy in T lymphocytes, as observed in T lymphocytes co-cultured with IL-4 MΦ, was not investigated. The nature of this factor was not idntified but the immunoregulatory cytokine TGF-β was detected in the supernatant of cultures with IL-4 MΦ as well as of M-CSF MΦ and IL-4 DC (Fig. 4.18).

TGF-β (and IL-10) are important immunoregulatory cytokines which can regulate alloreactive T cell responses after organ transplantation (Bickerstaff et al., 2001). TGF-β suppresses allospecific delayed-type hypersensitivity (DTH) responses primarily mediated by alloantigen-specific $CD4^+$ T-helper 1 (Th1) cells (Bickerstaff et al., 2000). Th1 cells triggered by alloantigens secrete

proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor nekrose factor (TNF). IFN-γ promotes the MHC class II expression and has effects on the generation of cytotoxic T lymphocytes (“killer T cells”), which are capable of killing cells. After transplantation, they are involved in the destruction of the allograft. The presence of TNF has been associated with acute rejection and blocking the action of TNF-α with neutralising antibodies can prolong experimental cardiac allograft survival (Imagawa et al., 1991). Similar blunting effects of TGF-β on the DTH response was observed in mouse recipients of spontaneously accepted kidney allografts (Bickerstaff et al., 2001). TGF-β, a multifunctional peptide, is a part of a superfamily of proteins controlling proliferation, differentiation, and other function in most cell types (Rubtsov and Rudensky, 2007). In immunology, TGF-β is known as an important immunoregulatory cytokine, secreted by so-called regulatory T cells (Sakaguchi and Powrie, 2007), which suppress T cell proliferation (Barker et al., 2002). Therefore, the detection of TGF-β in the supernatant of IL-4 MΦ (M-CSF MΦ and IL-4 DC) described in this study is an important finding but further investigations are necessary to clarify its role in the suppression of T cell activation in this system.

6 Conclusions

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

(1) IL-4 combined with GM-CSF induces bone marrow derived DC with immune regulatory effects. Does IL-4 combined with M-CSF also induce the development of bone marrow derived immune regulatory macrophages (IL-4 MΦ)?

IL-4 combined with M-CSF induced the development of bone marrow derived macrophages (IL-4 MΦ). The IL-4 MΦ showed a suppressive effect on the activation of allogenic T lymphocytes when used as competitor cells (Fig. 4.4). This effect was comparable with that of the IL-4 DC. However, the effect of IL-4 on the development of haematopoietic stem cells into IL-4 MΦ and IL-4 DC, respectively, does not seem to be unique because the M-CSF MΦ also show a suppressive effect.

(2) IL-4 is an anti-inflammatory cytokine. How does IL-4 influence the expression of certain surface molecules on IL-4 MΦ, which are involved in T cell activation?

IL-4 MΦ demonstrated a unique phenotype different from M-CSF MΦ and IL-4 DC. They are positive for MHC class I and class II molecules as well as for CD40 and negative for CD80 and CD86 as shown by flow cytometry (Fig. 4.13). Results from real time PCR, however, demonstrate the presence of specific mRNA for CD86 (Tab. 4.4). The M-CSF MΦ, clearly positive for CD40 and MHC I, seem not to express MHC II, CD80 and CD86 or only in low amounts (Fig. 4.14). According to the “two-signal” hypothesis of T cell activation, naïve alloreactive T lymphocytes need two signals for their complete activation (Fig. 1.1). The first signal is induced by the engagement of the T cell receptor with

the MHC molecules and must be accompanied by a second stimulus through the coreceptor CD80 and CD86 (signal two). The loss or reduced surface expression of CD80 and CD86 might explain why IL-4 MΦ and M-CSF MΦ are poor stimulator cells for T cell activation as shown in this study (Fig. 4.3).

(3) IL-4 DC suppress the activation of naïve T lymphocytes. What are the immune regulatory effects of IL-4 derived macrophages in comparison to IL-4 DC?

Until now, it is not clear whether the suppressive effect of IL-4 MΦ, M-CSF MΦ and IL-4 DC is mediated via cell-cell contact or soluble factors. This study presents results for both mechanisms. The three subsets demonstrate a suppressive effect on the activation of alloreactive T lymphocytes when used as competitor cells. T lymphocytes co-cultured with IL-4 MΦ, M-CSF MΦ or IL-4 DC were not restimulated by mature DC. This indicates that IL-4 MΦ, M-CSF MΦ and IL-4 DC induce a permanent state of hyporesponsiveness (T cell anergy). Since a suppressive effect could be attributed to the supernatant from cultures of IL-4 MΦ, M-CSF MΦ and IL-4 DC, the presence of a soluble inhibitory factor is assumed (Fig. 4.7). The immunoregulatory cytokine TGF-β was found in those supernatants (Fig. 4.18). In addition, specific mRNA for iNOS was proven in all three subsets. However, we have no evidence that the iNOS product NO is the pursued soluble inhibitory factor. iNOS-positive macrophages are generally known to induce T cell apoptosis (van der Veen et al., 2000). The highest rates of apoptosis were found in those competition assays with only 100 IL-4 MΦ and M-CSF MΦ competitor cells, respectively. It is not clear whether this is a relevant biological effect.

7 Summary

Immune cells with suppressive or regulatory properties appear very attractive for selective suppression of unwanted immune responses, occurring for example after transplantation.

In previous studies, we showed that immunoregulatory dendritic cells can be generated from rat bone marrow haematopoietic precursor cells with GM-CSF and IL-4 (so-called IL-4 DC). In the present study, bone marrow derived macrophages generated in the presence of M-CSF plus IL-4 (IL-4 MΦ) were compared to IL-4 DC in view of their phenotype and immunological functions. M-CSF MΦ generated with M-CSF alone were used as controls. In contrast to mature splenic dendritic cells (mDC), M-CSF MΦ, IL-4 MΦ and IL-4 DC were not able to activate naïve T lymphocytes *in vitro*. Moreover, used as competitor cells, a dose-dependent suppression of mDC-stimulated allogeneic T cell activation was observed. At a cell ratio of 1:1 (IL-4 MΦ:mDC), IL-4 MΦ induced a suppression rate of 96%. M-CSF MΦ and IL-4 DC demonstrated similar effects at the same cell ratio. It is not clear how the cells mediated the suppressive effect, but this study presents evidence for both a cell surface mediated mechanism and a mechanism mediated by soluble factors.

Flow cytometric, immunohistochemical and real time PCR analysis demonstrated a unique phenotype for IL-4 MΦ and M-CSF MΦ. Their surface expression of CD80 and CD86 was lower in comparison to the expression found on IL-4 DC whose surface expression of costimulatory molecules was reduced in comparison to mDC as shown in previous studies. Therefore, insufficient costimulation could be the reason for the observed inability of the three subsets to activate naïve T lymphocytes. All three subsets were positive for MHC I and CD40, and additionally, IL-4 MΦ and IL-4 DC were clearly positive for MHC II. T lymphocytes co-cultured with IL-4 MΦ, M-CSF MΦ or IL-4 DC, demonstrated a minimal proliferation when subsequently restimulated with mDC. This indicates that IL-4 MΦ, M-CSF MΦ and IL4-DC induced a stable

hyporesponsiveness (anergy) in these T lymphocytes, which is probably due to the insufficient costimulation. Moreover, a marginally increased rate of apoptosis was detected in T lymphocytes collected from the competition assays with IL-4 MΦ, M-CSF MΦ and IL4-DC in contrast to T lymphocytes cultured alone or with mDC during the competition assays.

Soluble inhibitory factors secreted by IL-4 MΦ, M-CSF MΦ or IL-4 DC could also play a role in T cell suppression. Supernatant of 10^6 M-CSF MΦ, IL-4 MΦ and IL-4 DC collected after 24h of culture suppressed mDC-induced T cell proliferation with a suppression rate of 81-85%. The suppressive effect of the supernatant collected after 48h was even stronger with a suppression rate of 90-100%. As shown in ELISA and real time PCR analysis, IL-4 MΦ, M-CSF MΦ and IL4-DC expressed the immunoinhibitory cytokine TGF-β that might be involved in T cell suppression.

In conclusion, these results show that the bone-marrow derived IL-4 MΦ and M-CSF MΦ suppress the activation and proliferation of naïve T lymphocytes. They share this feature with the bone marrow derived IL-4 DC despite different phenotypes. The results of this study indicate that dendritic cells and macrophages derived from haematopoietic stem cells of the bone marrow share the same suppressive properties. The mechanism how these cells mediate their suppressive effect is still unknown and should be analysed in further studies. In this context, these cells should be able to suppress unwanted immune responses in an antigen specific manner.

8 Zusammenfassung

Immunzellen mit hemmenden bzw. regulatorischen Eigenschaften erscheinen sehr attraktiv, um ungewollte Immunantworten, wie sie z.B. nach Transplantationen auftreten, selektiv zu hemmen.

In früheren Arbeiten konnten wir zeigen, dass sich mit GM-CSF und IL-4 immunregulatorische Dendritische Zellen (IL-4 DC) aus hämatopoetischen Vorläuferzellen des Knochenmarks der Lewis-Ratte generieren lassen. In der vorliegenden Arbeit wurden Makrophagen aus Knochenmarkvorläuferzellen mit M-CSF und IL-4 (IL-4 MΦ) generiert und mit den IL-4 DC hinsichtlich ihres Phänotyps und ihrer immunologischen Eigenschaften verglichen. M-CSF MΦ, die nur mit M-CSF generiert wurden, dienten hierbei als Kontrolle. Im Gegensatz zu reifen DC (mDC), die aus der Milz isoliert wurden, sind weder M-CSF MΦ noch IL-4 MΦ bzw. IL-4 DC in der Lage, naive T Lymphozyten *in vitro* zu aktivieren. Zudem wurde eine Zellzahl-abhängige Hemmung der mDC induzierten T Zell-Aktivierung beobachtet. So hemmten IL-4 MΦ die mDC-induzierte T-Zellproliferation nahezu vollständig (bis zu 96%), wenn sie in einem Zellverhältnis von 1:1 (IL-4 MΦ:mDC) als Kompetitorzellen eingesetzt wurden. Ähnliche Effekte waren auch für M-CSF MΦ und IL-4 DC zu beobachten. Zwar ist nicht geklärt, wie die Zellen diesen hemmenden Effekt vermitteln, doch lassen die Daten der vorliegenden Arbeit sowohl einen durch Oberflächenmoleküle als auch lösliche Mediatoren vermittelten Mechanismus für möglich erscheinen.

Durchflusszytometrische Analysen, ergänzt durch Immunhistochemie und Real time PCR, zeigten für IL-4 MΦ und M-CSF MΦ einen Phänotyp, der gegenüber IL-4 DC, eine geringere Expression von CD80 und CD86 auf der Zelloberfläche aufwies. Die IL-4 DC wiesen ihrerseits eine geringe Expression von CD80 und CD86 in Vergleich zu mDC auf, wie in vorangegangenen Arbeiten gezeigt wurde. Alle drei Subpopulationen waren positiv für MHC I und CD40, während IL-4 MΦ und IL-4 DC zusätzlich positiv für MHC II waren. T Lymphozyten in

Kokultur mit IL-4 MΦ bzw. mit M-CSF MΦ oder IL-4 DC wiesen nur eine geringe Proliferation auf, wenn sie anschließend mit mDC restimuliert wurden. Diese Daten deuten darauf hin, dass IL-4 MΦ, M-CSF MΦ und IL-4 DC naive T-Lymphozyten dauerhaft in ihrer Restimulierung hemmen. Dieser anergische Zustand ist möglicherweise auf die unzureichende Kostimulation zurückzuführen. Zudem wurde bei den mit IL-4 MΦ, M-CSF MΦ und IL-4 DC kokultivierten T Lymphozyten eine geringfügig erhöhte Rate an apoptotischen Zellen gemessen als bei T Lymphozyten, die zuvor alleine oder mit mDC kultiviert wurden.

Auch von IL-4 MΦ, M-CSF MΦ und IL-4 DC sezernierte lösliche Faktoren können eine Rolle bei der Hemmung der T-Zellaktivierung spielen. Überstände einer 24-stündigen Kultur mit 10^6 IL-4 MΦ, M-CSF MΦ und IL-4 DC, hemmten die mDC-induzierte T-Zellaktivierung mit einer Suppressionsrate von 81-85%. Mit einer nahezu vollständigen Hemmung der T-Zellaktivierung war der Effekt von Überständen aus 48-stündigen Kulturen sogar noch stärker. Wie anhand von ELISA und real time PCR gezeigt wurde, exprimierten IL-4 MΦ, M-CSF MΦ und IL-4 DC das immuninhibitorische Zytokin TGF-β, das für die beobachtete Hemmung der T-Zellproliferation verantwortlich sein könnte.

Die Ergebnisse zeigen somit, dass die aus Knochenmarkvorläuferzellen generierten IL-4 MΦ und M-CSF MΦ die Aktivierung naiver T-Lymphozyten hemmen. Diese Eigenschaft teilen sie sich mit IL-4 DC trotz deutlicher Unterschiede bei den Phänotypen dieser Zellen. Die Ergebnisse dieser Arbeit lassen den Schluss zu, dass die aus hämatopoetischen Stammzellen des Knochenmarks hergestellten Dendritische Zellen und Makrophagen die Aktivierung naiver T-Lymphozyten effektiv hemmen. Der Mechanismus, wie dieser hemmende Effekt vermittelt wird, ist zurzeit noch unbekannt und sollte in weiteren Arbeiten analysiert werden. Von grundlegender Bedeutung wäre der erfolgreiche Nachweis, dass diese Zellen ungewollte Immunantworten antigen-spezifisch hemmen.

9 Abbreviations

APC	Antigen-presenting cells
BMDC	Bone marrow derived cells
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter Channel
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
IFN-γ	Interferon-γ
IL-4	Interleukin-4
IL-10	Interleukin-10
M-CSF	Macrophage Colony Stimulating Factor
mDC	Mature dendritic cells
MΦ	Macrophages
PBS	Phosphate-buffered saline
PE	Phycoerythrine
rpm	Rounds per minute
SSC	Side Scatter Channel
TCR	T cell receptor
TGF-β	Transforming Growth Factor-β
WF	Wistar Furth

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Tab. 9.1. M-CSF MΦ, IL-4 MΦ and IL-4 DC are not stimulators of T cell proliferation. Representative MLC of n=4. The proliferation rate in the MLC is calculated according the following formula: % = (cpm $_{\text{T cell proliferation in the co-cultures}}$ / cpm $_{\text{mDC induced T cell proliferation}}$) x 100

	10^5 T cells	10^4 mDC	10^4 IL-4 MΦ	10^4 M-CSF MΦ	10^4 IL-4 DC	10^5 T cells + 10^4 mDC	10^5 T cells + 10^4 IL-4 MΦ	10^5 T cells + 10^4 M-CSF MΦ	10^5 T cells + 10^4 IL-4 DC
Mean value (cpm)	279	83	2037	450	282	35485	1197	643	1556
Standard deviation (cpm)	64	28	586	64	121	4200	159	79	507
Proliferation (%)	---	---	---	---	---	100	3	2	4

Tab. 9.2. M-CSF MΦ, IL-4 MΦ and IL-4 DC act as competitor cells and inhibit the mDC-induced proliferation of naïve T lymphocytes. Representative competition assay of n=4. mDC served as control cells.

	10^5 T cells	10^4 mDC	10^5 T cells + 10^4 mDC	10^4 IL-4 MΦ	10^5 T cells + 10^4 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^4 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^3 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^2 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^1 IL-4 MΦ
Mean value (cpm)	329	178	11542	291	1084	470	5682	6363	10251
Standard deviation (cpm)	26	36	1915	64	328	183	539	677	994

	10^4 M-CSF MΦ	10^5 T cells + 10^4 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^4 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^3 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^2 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^1 M-CSF MΦ
Mean value (cpm)	113	199	212	5859	8272	8838
Standard deviation (cpm)	18	46	56	508	405	640

	10^4 IL-4 MΦ	10^5 T cells + 10^4 IL-4 DC	10^5 T cells + 10^4 mDC + 10^4 IL-4 DC	10^5 T cells + 10^4 mDC + 10^3 IL-4 DC	10^5 T cells + 10^4 mDC + 10^2 IL-4 DC	10^5 T cells + 10^4 mDC + 10^1 IL-4 DC
Mean value (cpm)	157	307	177	3601	7963	9094
Standard deviation (cpm)	35	20	34	314	929	1152

	10^5 T cells + 10^4 mDC	10^5 T cells + 10^4 mDC + 10^4 mDC	10^5 T cells + 10^4 mDC + 10^3 mDC	10^5 T cells + 10^4 mDC + 10^2 mDC	10^5 T cells + 10^4 mDC + 10^1 mDC
Mean value (cpm)	27940	23783	25018	18773	24331
Standard deviation (cpm)	3517	2083	2959	1952	3272

Tab. 9.3. Calculation of the suppressive effect mediated by IL-4 MΦ, M-CSF MΦ and IL-4 DC (based on values of Tab. 9.2). The T cell proliferation in MLC (10^4 mDC co-cultured with 10^5 allogeneic T cells) is setting as 100% proliferation or 0% suppression. The suppression rate Δ% induced by IL-4 MΦ, M-CSF MΦ and IL-4 in the competition assays (Tab. 9.2) is calculated according the following formula: Δ% = (cpm $_{\text{mDC induced T cell proliferation}}$ - cpm $_{\text{T cell proliferation in the co-cultures}}$ / cpm $_{\text{mDC induced T cell proliferation}}$) x 100. Proliferation is calculated as follows: % = (cpm $_{\text{T cell proliferation in the co-cultures}}$ / cpm $_{\text{mDC induced T cell proliferation}}$) x 100.

	10^5 T cells + 10^4 mDC	10^5 T cells + 10^4 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^4 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^3 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^2 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^1 IL-4 MΦ
Proliferation (%)	100	9	4	49	55,	89
Suppression (Δ%)	---	---	96	51	45	11
	10^5 T cells + 10^4 mDC	10^5 T cells + 10^4 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^4 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^3 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^2 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^1 IL-4 MΦ
Proliferation (%)	100	2	2	51	72	77
Suppression (Δ%)	---	---	98	49	28	23
	10^5 T cells + 10^4 mDC	10^5 T cells + 10^4 IL-4 DC	10^5 T cells + 10^4 mDC + 10^4 IL-4 DC	10^5 T cells + 10^4 mDC + 10^3 IL-4 DC	10^5 T cells + 10^4 mDC + 10^2 IL-4 DC	10^5 T cells + 10^4 mDC + 10^1 IL-4 DC
Proliferation (%)	100	3	2	31	69	79
Suppression (Δ%)	---	---	99	69	31	21

Tab. 9.4. Calculation of the suppressive effect mediated by mDC as pseudo competitor cells (Tab. 9.3). SD= Standard deviation. N=2.

	10^5 T cells + 10^4 mDC	10^5 T cells + 10^4 mDC + 10^4 mDC	10^5 T cells + 10^4 mDC + 10^3 mDC	10^5 T cells + 10^4 mDC + 10^2 mDC	10^5 T cells + 10^4 mDC + 10^1 mDC
Mean (cpm)	27940	23783	25018	18773	24331
SD (cpm)	3517	2083	2959	1952	3272
Proliferation (%)	100	85	90	67	87
Suppression (Δ%)	---	15	11	33	13

Tab. 9.5. Calculation of the suppressive effect mediated by cells of the mammarian cell line MDA-MB 231 ("MammaCa") as pseudo competitor cells. SD= Standard deviation. N=2.

	10^5 T cells + 10^4 mDC	10^5 T cells + 10^4 MammaCa	10^5 T cells + 10^4 mDC + 10^4 MammaCa
Mean (cpm)	27758	5021	21885
SD (cpm)	3947	1229	2314
Proliferation (%)	100	18	79
Suppression (Δ%)	---	---	21

Tab. 9.6. ***Proof of soluble factors secreted by IL-4 MΦ, M-CSF MΦ and IL-4 DC, which mediate suppression.*** 10^6 cells (IL-4 MΦ, M-CSF MΦ and IL-4 DC and cells of the cell line MDA-MB 231 as control) were cultured in 96 well plates in 150 µL culture medium. Supernatant (50 µL, 100 µL) of 24h- and 48h- cultures were transferred to MLC. MammaCa: Cells of the mammarian carcinoma cell line MDA-MB 231. SN= supernatant. SD= Standard deviation.

	10^5 T cells + 10^4 mDC	SN: Mamma Ca (24h, 100µL) + 10^5 T cells + 10^4 mDC +	SN: IL-4 MΦ (24h, 100µL) + 10^5 T cells + 10^4 mDC	SN: MCSF MΦ (24h, 100µL) + 10^5 T cells + 10^4 mDC	SN: IL-4 DC (24h, 100µL) + 10^5 T cells + 10^4 mDC
Mean (cpm)	31170	22306	5146	5939	4776
SD (cpm)	4481	2157	1178	795	875
Proliferation (%)	100%	72	17	19	15
Suppression (∆%)	---	28	83	81	85
	10^5 T cells + 10^4 mDC	SN: Mamma Ca (48h, 100µL) + 10^5 T cells + 10^4 mDC	SN: IL-4 MΦ (48h, 100µL) + 10^5 T cells + 10^4 mDC	SN: MCSF MΦ (48h, 100µL) + 10^5 T cells + 10^4 mDC	SN: IL-4 DC (48h, 100µL) + 10^5 T cells + 10^4 mDC
Mean value (cpm)	27758	18598	92	150	121
SD (cpm)	3947	2458	28	51	40
Proliferation (%)	100	67	0	1	0
Suppression (∆%)	---	33	100	99	100
	10^5 T cells + 10^4 mDC	SN: Mamma Ca (48h, 50µL) + 10^5 T cells + 10^4 mDC	SN: IL-4 MΦ (48h, 50µL) + 10^5 T cells + 10^4 mDC	SN: MCSF MΦ (48h, 50µL) + 10^5 T cells + 10^4 mDC	SN: IL-4 DC (48h, 50µL) + 10^5 T cells + 10^4 mDC
Mean (cpm)	27758	21659	222	2781	2272
SD (cpm)	3947	1798	63	670	532
Proliferation (%)	100	78	1	10	8
Suppression (∆%)	---	22	99	90	92

Tab. 9.7. Flow cytometric analysis of T cell apoptosis by staining with Annexin V. T lymphocytes was proven in MLC and competition assays. Controls are T lymphocytes cultured alone and with mDC. Representative results from n=2 experiments.

	10^5 T cells + 10^4 IL-4 MΦ	10^5 T cells + 10^4 mDC +10^4 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^3 IL-4 MΦ	10^5 T cells + 10^4 mDC + 10^2 IL-4 MΦ
Rate of apoptosis (%)	15-17	15-17	13-15	10-12
	10^5 T cells + 10^4 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^4 M-CSF MΦ	10^5 T cells + 10^4 mDC +10^3 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^2 IL-4 MΦ
Rate of apoptosis (%)	9-11	10-13	15-17	19-21
	10^5 T cells + 10^4 IL-4 DC	10^5 T cells + 10^4 mDC + 10^4 IL-4 DC	10^5 T cells + 10^4 mDC + 10^3 IL-4 DC	10^5 T cells + 10^4 mDC + 10^2 IL-4 DC
Rate of apoptosis (%)	8-10	6-8	14-16	30-32
	10^5 T cells	10^5 T cells + 10^4 mDC		
Rate of apoptosis (%)	8-10	9-11		

Tab. 9.8. Naïve T lymphocytes pre-incubated with IL-4 MΦ, M-CSF MΦ and IL-4-DC showed low proliferation in the presence of mDC (=second culture). Shown are the results of second culture. T lymphocytes cultured alone were used as controls for the second culture. SD= Standard deviation. Representative results from n=2 experiments.

	10^5 T cells	10^5 T cells + 10^4 mDC	10^5 T cells + 10^4 IL-4 MΦ	10^5 T cells + 10^4 mDC +10^4 IL-4 MΦ
Mean (cpm)	13730	8075	507	599
SD (cpm)	3794	1635	338	197
	10^5 T cells + 10^4 M-CSF MΦ	10^5 T cells + 10^4 mDC + 10^4 M-CSF MΦ	10^5 T cells + 10^4 IL-4 DC	10^5 T cells + 10^4 mDC + 10^4 IL-4 DC
Mean (cpm)	5140	3299	1050	1293
SD (cpm)	1757	579	333	387

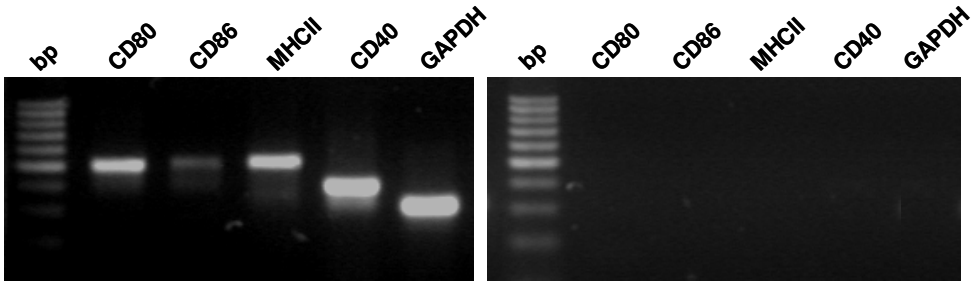

Fig. 9.1. Controls of the polymerase chain reaction (PCR). Positive control: 10^8 copies of PCR vector (TA vector from Invitrogen) with a certain cloned PCR fragment. Negative control: PCR grade water (Nuclease-free) from Promega.

Tab. 9.9. Analysis of real time PCR data for relative quantitation of different target mRNA in IL-4 MΦ and M-CSF MΦ. Targets are MHC I and II mRNA, CD80, CD86 and CD40 mRNA, IL-10 mRNA, TGF-β mRNA and iNOS mRNA; endogenous control is GAPDH mRNA. Shown are the Ct values, the calculated ΔCt and ΔΔCt values and the relative expression ($2^{-\Delta\Delta Ct}$) of certain mRNA of IL-4 MΦ and M-CSF MΦ in comparison to IL-4 DC.

	Ct MHC I		**Ct** GAPDH		**MHC I** Mean Ct	**GAPDH** Mean Ct	**ΔCt**	**ΔΔCt**	$2^{-\Delta\Delta Ct}$
IL-4 DC	12.28	12.49	14.51	13.14	12.39	13.83	-1.44	---	1.00
IL-4 MΦ	14.44	15.82	15.24	16.55	15.13	15.89	-0.76	0.68	0.63
M-CSF MΦ	18.52	14.65	16.55	14.53	16.58	15.54	1.04	2.48	0.18

$\Delta Ct = Ct_{(MHC\ I)} - Ct_{(GAPDH)}$, $\Delta\Delta Ct = \Delta Ct_{(x)} - \Delta Ct_{(IL\text{-}4DC)}$ (x = IL-4 MΦ or M-CSF MΦ)

	Ct MHC II		**Ct** GAPDH		**MHC II** Mean Ct	**GAPDH** Mean Ct	**ΔCt**	**ΔΔCt**	$2^{-\Delta\Delta Ct}$
IL-4 DC	17.34	17.46	14.51	13.14	17.40	13.83	3.58	---	1.00
IL-4 MΦ	19.36	20.33	15.24	16.55	19.85	15.89	3.95	0.38	0.77
M-CSF MΦ	28.39	26.80	16.55	14.53	27.59	15.54	12.05	8.48	0.00

$\Delta Ct = Ct_{(MHC\ II)} - Ct_{(GAPDH)}$, $\Delta\Delta Ct = \Delta Ct_{(x)} - \Delta Ct_{(IL\text{-}4DC)}$ (x = IL-4 MΦ or M-CSF MΦ)

	Ct CD80		**Ct** GAPDH		**CD80** Mean Ct	**GAPDH** Mean Ct	**ΔCt**	**ΔΔCt**	$2^{-\Delta\Delta Ct}$
IL-4 DC	27.37	26.91	14.51	13.14	27.14	13.83	13.31	---	1.00
IL-4 MΦ	32.12	31.76	15.24	16.55	31.94	15.89	16.05	2.74	0.15
M-CSF MΦ	35.00	30.99	16.55	14.53	32.99	15.54	17.45	4.14	0.06

$\Delta Ct = Ct_{(CD80)} - Ct_{(GAPDH)}$, $\Delta\Delta Ct = \Delta Ct_{(x)} - \Delta Ct_{(IL\text{-}4DC)}$ (x = IL-4 MΦ or M-CSF MΦ)

	Ct CD86		**Ct** GAPDH		**CD86** Mean Ct	**GAPDH** Mean Ct	**ΔCt**	**ΔΔCt**	$2^{-\Delta\Delta Ct}$
IL-4 DC	22.00	23.16	14.51	13.14	22.58	13.83	8.75	---	1.00
IL-4 MΦ	25.88	25.70	15.24	16.55	25.79	15.89	9.89	1.14	0.45
M-CSF MΦ	35.00	28.55	16.55	14.53	31.78	15.54	16.24	7.49	0.01

$\Delta Ct = Ct_{(CD86)} - Ct_{(GAPDH)}$, $\Delta\Delta Ct = \Delta Ct_{(x)} - \Delta Ct_{(IL\text{-}4DC)}$ (x = IL-4 MΦ or M-CSF MΦ)

	Ct CD40		**Ct** GAPDH		**CD40** Mean Ct	**GAPDH** Mean Ct	**ΔCt**	**ΔΔCt**	$2^{-\Delta\Delta Ct}$
IL-4 DC	20.98	21.43	14.51	13.14	21.20	13.83	7.38	---	1.00
IL-4 MΦ	22.95	22.59	15.24	16.55	22.77	15.89	6.87	-0.51	1.42
M-CSF MΦ	24.70	25.18	16.55	14.53	24.94	15.54	9.40	2.02	0.25

$\Delta Ct = Ct_{(CD40)} - Ct_{(GAPDH)}$, $\Delta\Delta Ct = \Delta Ct_{(x)} - \Delta Ct_{(IL\text{-}4DC)}$ (x = IL-4 MΦ or M-CSF MΦ)

	Ct TGF-β		**Ct** GAPDH		**TGF-β** Mean Ct	**GAPDH** Mean Ct	**ΔCt**	**ΔΔCt**	$2^{-\Delta\Delta Ct}$
IL-4 DC	18.74	17.75	14.51	13.14	18.24	13.83	4.42	---	1
IL-4 MΦ	19.90	20.75	15.24	16.55	20.33	15.89	4.44	0.02	0.99
M-CSF MΦ	22.02	19.12	16.55	14.53	20.57	15.54	5.03	0.61	0.65

$\Delta Ct = Ct_{(TGF\text{-}\beta)} - Ct_{(GAPDH)}$, $\Delta\Delta Ct = \Delta Ct_{(x)} - \Delta Ct_{(IL\text{-}4DC)}$ (x = IL-4 MΦ or M-CSF MΦ)

	Ct iNOS		**Ct** GAPDH		**iNOS** Mean Ct	**GAPDH** Mean Ct	**ΔCt**	**ΔΔCt**	$2^{-\Delta\Delta Ct}$
IL-4 DC	27.34	25.82	14.51	13.14	26.58	13.83	12.75	---	1
IL-4 MΦ	29.81	28.81	15.24	16.55	29.31	15.89	13.42	0.66	0.63
M-CSF MΦ	33.39	27.23	16.55	14.53	30.31	15.54	14.77	2.02	0.25

$\Delta Ct = Ct_{(iNOS)} - Ct_{(GAPDH)}$, $\Delta\Delta Ct = \Delta Ct_{(x)} - \Delta Ct_{(IL\text{-}4DC)}$ (x = IL-4 MΦ or M-CSF MΦ)

Tab. 9.10. Proof of the production of IL-10 and TGF-β by IL-4 MΦ, M-CSF MΦ and IL-4 DC with specific ELISA kits (Biosource). Cells were harvested on day +6 of culture and replated in 96 well plates (10^4 and 10^6 cells/well) in 150 µL medium. Supernatant was collected after 24h and 48h.

IL-10	**IL-4 MΦ 10^6, 24h**	**M-CSF MΦ 10^6, 24h**	**IL-4 DC 10^6, 24h**	**IL-4 MΦ 10^4, 24h**	**M-CSF MΦ 10^4, 24h**	**IL-4 DC 10^4, 24h**
Cytokine concentration (pg/mL)	---	7	---	---	---	---
TGF-β	**IL-4 MΦ 10^6, 48h**	**M-CSF MΦ 10^6, 48h**	**IL-4 DC 10^6, 48h**	**IL-4 MΦ 10^4, 24h**	**M-CSF MΦ 10^4, 24h**	**IL-4 DC 10^4, 24h**
Cytokine concentration (pg/mL)	over 2000	1180	over 2000	31	---	62

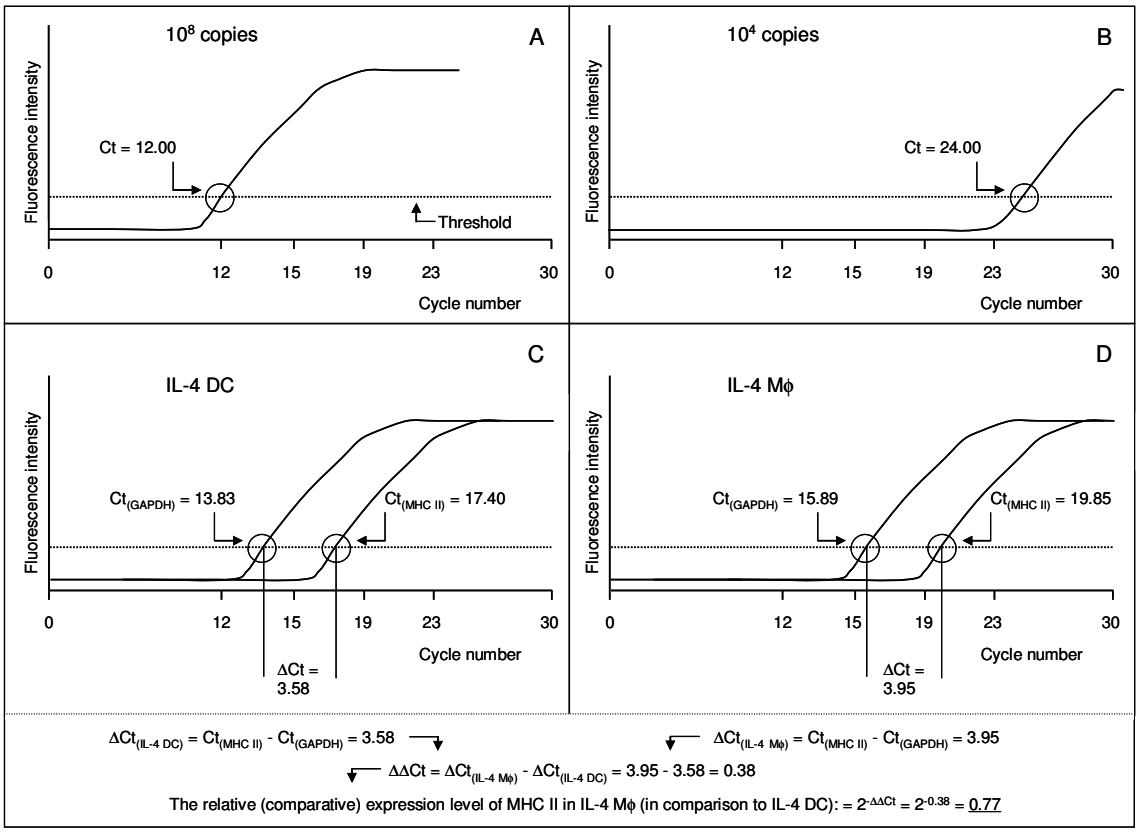

Fig. 9.2. Interpretation of real time PCR results and the comparative threshold (Ct) method as an approach to calculate relative (comparative) expression levels. The advantage of using the comparative Ct method is that the need of a standard curve is eliminated.

12 Danksagung

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13 Erklärung

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Ich versichere hiermit, dass ich die vorliegende Master Thesis selbständig verfasst und keine anderen als die angegebenen Quellen als Hilfsmittel benutzt habe.

Würzburg, den .....

.....

(Unterschrift)