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DIPLOMARBEIT

THE ROLE OF PI3K AND ROCKII PATHWAYS UPON *IN VITRO* MATURATION OF DENDRITIC CELLS, CULTURED FROM PBMNCS DERIVED EITHER FROM CANCER PATIENTS OR HEALTHY CONTROLS.

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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WIDMUNG

Für Andrea Pecina (1960-1993)

„Auch wenn der schönste Stern erlischt, sein Licht erstrahlt Millionen Jahre...wenn man weit genug Abstand nimmt."

DANKSAGUNG

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1 TABLE OF CONTENT

2 ABSTRACT

Dendritic cells (DCs) have been shown being pivotal in the immune system by recognizing antigens and presenting them to leukocytes inducing a proper and specific immune response. The DCs are the most powerful Antigen Presenting Cells (APCs) and recently have been identified as one of the most important tools for immune therapies against different types of cancer. Until now the molecular mechanisms underlying the maturation of monocyte-derived dendritic cells are still mostly unknown. The aim of this thesis is to start an analysis about the role of PI3K and ROCKII pathways during the maturation of monocyte-derived dendritic cells and to investigate whether and how these pathways are affected by tumors.

The preliminary results obtained during this thesis showed that both PI3K and ROCKII strongly affect the maturation process of human DCs, at least under *in vitro* culture conditions.

PI3K positively acts upon both morphological and functional maturation. Indeed, the chemical specific inhibition of PI3K correlates with a strong down regulation of the expression pattern of surface markers, typical of mature DCs as well as with the absence of down regulation of characteristics typical of immature DCs, such as high migration and phagocytosis. These changes are additionally accompanied by altered endogenous protein levels of B-Raf, JNK1, MEK and ERM, which strengthens the assumption of PI3K being a regulator of parallel molecular pathways such as the MAPK-pathway. PI3K does not appear to alter the secretion of IL-10 and IL-12. For the preliminary results obtained in this thesis, PI3K appears to be particularly relevant only at specific time-windows during the DC maturation, rather than through the entire differentiation. Anyhow, such findings require further investigation in a statistical-relevant study.

The effects observed upon ROCKII chemical inhibition were predominantly shown on cytoskeleton-related functions such as migration and phagocytosis, which were accompanied by changes in the endogenous ERM protein levels. Moreover, ROCKII inhibition affected the levels of B-Raf and JNK1 protein as well. Strikingly, ROCKII markedly altered the secretion of IL-10 and IL-12 without lowering the potential to induce a T cell response suggesting the existence of an alternative interleukin-independent mechanism for the activation of T cells by DCs, possibly through direct contact between the two types of cells. ROCKII inhibition mostly affected DCs by increasing the number of dendrites and by powerfully enhancing cell adherence. The effects of ROCKII inhibition on the expression of maturity-associated mark-

ers on the DC surface were minor, although such issues require further investigation in a statistical-relevant study.

Although some differences in the response to PI3K or ROCKII chemical inhibition were observed between DCs derived from healthy controls and patients, further studies are required to exclude individual-related bias.

3 ZUSAMMENFASSUNG

Es wurde gezeigt, dass dendritische Zellen eine entscheidende Rolle im Immunsystem spielen, da sie Antigene erkennen und an Leukozyten präsentieren können um eine passende Immunantwort auszulösen. Daher stellen DCs die stärksten Antigen-präsentierenden Zellen dar und wurden vor kurzem als eines der wichtigsten Werkzeuge zur Immuntherapie gegen unterschiedliche Krebsarten identifiziert. Die der Reifung von Monozyten-abstammenden dendritischen Zellen zugrunde liegenden molekularen Mechanismen sind bis heute größtenteils unbekannt. Ziel dieser Diplomarbeit war es eine Analyse der Rolle der PI3K und ROCKII Signalwege während der Reifung von dendritischen Zellen zu beginnen und zu untersuchen, ob und wie diese Signalwege durch Tumore beeinflusst werden.

Die vorläufigen Ergebnisse dieser Diplomarbeit zeigten, dass PI3K und ROCKII den Reifungsprozess von humanen DCs, zumindest unter *in vitro* Bedingungen, stark beeinflussen.

PI3K beeinflusst die morphologische und funktionelle Reifung positiv. Tatsächlich korreliert die chemische Inhibierung von PI3K mit einer starken Verminderung der Expresssionsmuster von Oberflächenmarkern, die typisch für reife DCs sind und auch mit einer fehlenden Verminderung von Charakteristiken typisch für unreife DCs, wie zum Beispiel ausgeprägte Migration und Phagozytose. Diese Veränderungen werden außerdem von modifizierten endogenen Proteinlevels von B-Raf, JNK1, MEK und ERM begleitet, was die Vermutung verstärkt, dass PI3K ein Regulator paralleler molekularer Signalwege, wie dem MAPK-Signalweg, ist. PI3K scheint die Sekretion von IL-10 und IL-12 nicht zu beeinflussen. PI3K scheint nur zu bestimmten Zeitfenstern relevant zu sein, statt während der gesamten Differenzierung. Diese Erkenntnisse erfordern weitere Untersuchungen in einer statistisch relevanten Studie.

Die Effekte einer chemischen Inhibierung von ROCKII waren hauptsächlich bei Zytoskelett-bezogenen Funktionen, wie Migration und Phagozytose feststellbar und wurden von Veränderungen in den endogenen ERM Proteinlevels begleitet. Außerdem beeinflusste die ROCKII Inhibierung die Levels von B-Raf und JNK1. Interessanterweise veränderte ROCKII die Sekretion von IL-10 und IL-12 dramatisch, ohne dabei das Potential eine T Zellantwort auszulösen zu verringern, was die Existenz eines alternativen, Interleukin-unabhängigen Mechanismus zur Aktivierung von T Zellen durch DCs nahe legt, möglicherweise durch direkten Kontakt dieser beider Zelltypen. ROCKII Inhibierung beeinflusste DCs am Meisten

durch die Erhöhung der Anzahl an Dendriten und durch die Verstärkung der Zelladherenz. Die Effekte von ROCKII auf die Expression von Reifungs-assoziierten Markern auf der Oberfläche von DCs waren gering, obwohl solche Ergebnisse weitere Untersuchungen in einer statistisch relevanten Studie benötigen.

Obwohl einige Unterschiede in den Auswirkungen der chemischen Inhibierung von PI3K und ROCKII zwischen gesunden Kontrollen und Patienten gefunden wurden, sind weitere Untersuchungen notwendig um Individuums-spezifische Tendenzen auszuschließen.

4 INTRODUCTION

4.1 The immune system

The human immune system consists of three lines of defense against invading pathogens. At first, physical barriers prevent pathogens from entering the host. If pathogens overcome this barrier, the innate immune system provides an immediate but non-specific response. The third line of defense is the adaptive immune system which, upon activation by the innate immune system, attacks pathogens specifically and also possesses the ability to memorize a certain invader for future attacks. It consists of two components: the cell-mediated and humoral immunity.

4.1.1 Dendritic cells play a pivotal role in immune surveillance

Dendritic cells were first visualized in the skin by Paul Langerhans in 1868 and in response were called Langerhans cells, but their further characterization started not until the 70ies of the last century. Until that time, it was assumed that a proper immune response needs some kind of 'accessory cells' to trigger a proper primary antibody response *in vitro*, but first with the identification and purification from contaminating lymphocytes, their role as antigen-presenting cells (APCs) became evident and further research was targeted upon them to identify their full spectrum of functions and effects. Until now, a broad understanding of dendritic cell biology has been unravelled and their pivotal role in the immune system slowly becomes clear, although there are still major questions unsolved and new arise every day.

Some of their fundamental properties are as following:

- Sentinels *in vivo*; *in situ* distribution to optimize antigen capture; migration into lymphoid organs to optimize clonal selection of rare $CD4^+$ and $CD8^+$ T cells
- Initiators of immune responses: stimulation of quiescent, naïve and memory B and T lymphocytes
- Potency in stimulating T cells: capacity of small numbers of DCs and low levels of antigen to induce strong T cell responses
- Inducers of tolerance: deletion of self-reactive thymocytes, and anergy to mature T cells

Table 1: Dendritic cells and the control of immunity (from Nature 392, 245-252 (1998)).

The ability to capture antigens, so-called 'danger' signals, which are subsequently processed and presented on the DC's surface via MHC class I/class II molecules, and to migrate to draining lymph nodes, with the goal to induce a cellular and a humoral response or to induce tolerance respectively, entitles DCs to be useful in medical fields as cancer therapy, graft rejection or autoimmune disease.

4.1.2 Subsets of DC: properties and development

DCs are able to react upon different types of danger signals and to induce an appropriate immune response. This is caused by different subtypes of dendritic cells with distinct properties and selective sensitivity to various antigens. The underlying mechanisms and the versatile lineage development are under investigation and so far crucial aspects have been discovered.

Subtypes and plasticity

It seems that two main pathways are the basement of DC differentiation[1,2]. The first is the myeloid pathway which generates two subsets, known as the Langerhans cells, predominately found in the skin, and the intestinal DCs, which distribute into all other tissues[3]. Secondly, the lymphoid pathway gives rise to plasmacytoid dendritic cells (pDCs), which characteristically secrete high levels of type I INFs upon viral infections[4,5].

Not only lineage segregation influences specificity of DCs, also the exposure to various cytokines *in vitro* markedly alters phenotype and functions. Monocytes for example, can differentiate in both, macrophages (G-CSF) and dendritic cells (GM-CSF) depending on cytokines encountered, and further in IL-4 DCs or INF-α DCs, if subsequently treated with IL-4 or INF-α[6,7,8,9,10,11,12]. These functional plasticity enables DCs to activate distinct immune responses corresponding to the encountered antigens although it has not yet become clear to which extent DC subsets or their plasticity determine a specific type of response. Monocyte-derived DCs, activated with CD40L, respond in an IL-12-dependend mechanism to induce a T_H1 response. In contrast, pDCs treated with IL-3 and CD40L prime a T_H2 response secreting low amounts of IL-12[13]. A mixture of both, the type of DC subset and the cytokine signals encountered affects the polarization of T cells.

Isolation of DC subtypes

The investigation of DC subtypes has mostly not been performed by direct isolation of mature dendritic cells as their isolation and purification are difficult because the main available source for DCs is the blood. So, predominately pDCs and immature DCs have been isolated and further matured in culture.

Three different precursor-cell starting points are preferentially used to generate mDCs. The earliest precursor cells used are $CD34^+$ fractions, isolated from bone marrow or umbilical-cord blood, giving rise to Langerhans cells, intestinal DCs or DCs from $CD34^+$ lymphoid precursors, depending on the combination of cytokines added[14,15,16,17,18].

The second and most commonly used precursor cells are isolated $CD14^+$ peripheral-blood monocytes which are treated with GM-CSF and IL-4 to differentiate them towards immature dendritic cells. Finally, incubation with microbial products as LPS and Ribomunyl, or with proinflammatory cytokines as TNF-α produces fully mature DCs.

The third precursors used, are interferon-α/β-producing plasmacytoid cells which are cultured with IL-3 and CD40L, or alternatively with microbial stimuli like the human herpes simplex virus[19,20,21].

DC properties

Immature DCs are present in most tissues and scan their environment for 'danger' signals. They are able to perform phagocytosis, endocytosis and pinocytosis to capture invading pathogens such as bacteria and viruses, proteins, immune complexes and dead or dying cells. Further, iDCs possess a wide array of cell surface receptors to distinguish between the types of antigens, for cell-cell interactions and signalling respectively. These receptors originate from diverse receptor classes, as Integrins, C-type lectin-like receptors or Fc receptors. Antigens recognized by an iDC, are processed, loaded onto MHC class molecules and presented on the cell surface.

Upon antigen uptake and processing, iDCs become activated and undergo terminal differentiation, which transforms them into cells specialized for T cell stimulation. The maturation process is characterized by an array of complex functional and phenotypical alterations. The potential of phagocytic uptake is strongly reduced, cytoplasmic extension, called dendrites, are formed, migration towards lymph nodes occurs and their potential to activate T cells is enhanced. Characteristic markers, including CD80, CD86, CD40, are higher expressed on the cell surface. Cytokines, including IL-12, are released to attract T and B cells and to skew an immune response into a specific direction[22,23,24].

Interactions with T cells and its consequences

mDCs which encounter naïve T cells in the lymph nodes, require three consecutive signals between T cells and mDCs to effectively prime an immune response. The first signal is through antigen presentation via MHC class I and class II molecules by which they can activate both, $CD4^+$ and $CD8^+$ T cells respectively. The second signal required, is interaction between CD80 and CD86 on the surface of DCs and CD28 on T cells. If this costimulatory signal is absent, T cells are tolerized. The third signal occurs through reciprocal interactions between CD40 on DCs and CD40L on T cells, priming IL-12 secretion by DCs[25,26,27] which skews the immune system towards a T_H1 response. The strength of the T cell response upon priming depends on various factors, like the state of DC maturation, the concentration of antigen on the DC and the specific type of maturation stimulus[28]. If the T cell is effectively primed, it leads to clonal expansion and differentiation into memory cells and cytokine-secreting effector cells to activate a full response. On the other side, improper activation due to diverse reasons, such as an immature state or profound expression of IL-10 can lead to the induction of tolerance through anergy of antigen-specific T cells, abortive proliferation or the induction of regulatory T cells[29,30,31].

4.2 Dendritic cells in cancer immunotherapy

Dendritic cells are used as a relatively novel approach to vaccination against cancer. They are loaded *ex vivo* with tumor-associated antigens (TAA) and reintroduced into the patient to induce tumor-specific killer and helper T cell responses directly in the patient. The ideal TAA would only be expressed in tumor cells and should be crucial for tumor growth and survival.

4.2.1 Different approaches to initiate an immune response

Several types of antigens and loading methods have been tried. The most common used strategy is to load MHC class I and class II molecules with defined antigens[32,33]. But this technique has limitation like the restriction of the used peptide to a given HLA type or the limited number of well-characterized tumor-associated antigens. Further techniques employed are loading with total tumor lysate, recombinant proteins or exosomes, transducing DCs with viral vectors, transfection with DNA, RNA or plasmid DNA, targeting DCs with antibodies specific for DC cell surface molecules or loading with immune complexes.

In this project, mRNA passive transfection is used to load iDCs with Survivin. This technique has some interesting advantages: mRNA is only transiently expressed in the target DCs and it

does not integrate into the host genome. Further, mRNA corresponding to gene products with known sequence can easily be generated *in vitro* by using appropriate primers and RT-PCR, which is coupled to a transcription reaction. The method is cost-effective and GMP-conform. There exist two possibilities, the amplification of whole-tumor mRNA and the amplification of defined genes, which are at best only expressed in tumors. Up to date, only a few genes, mostly fulfilling this criterion, could be identified, such as telomerase reverse transcriptase (TERT), oncofetal antigen (OFA), prostate-specific antigen (PSA), carcinoembryonic antigen (CEA) and Survivin[34,35,36,37,38]. Additionally, mRNA transfected DCs appear to be deficient in stimulating a $CD4^+$ immune response, as transfected mRNA is preferentially channelled into the class I processing pathway activating $CD8^+$ but not $CD4^+$ T cells.

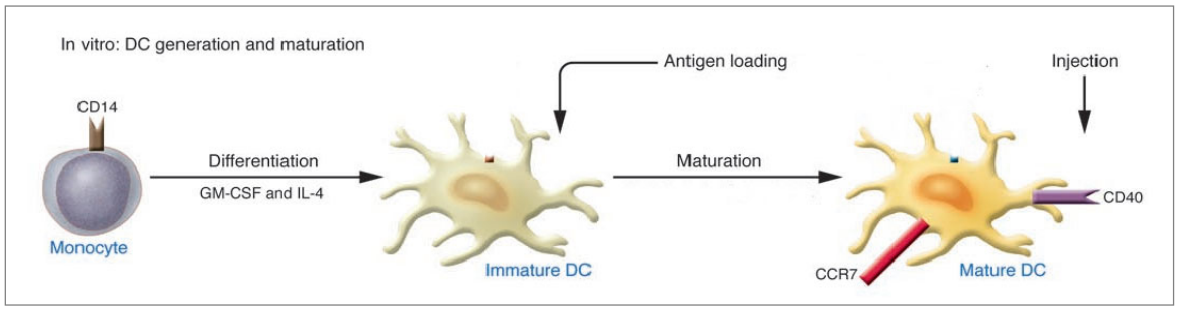

Fig. 1: Generation and maturation of DCs *in vitro* (modified; J. Clin. Invest. 117, 1195-1203 (2007)).

4.2.2 Survivin mRNA as tumor antigen

Survivin mRNA, which is used to transfect DC in the experiments described in this thesis, has a restricted expression pattern, limited to embryonic and fetal development and is re-expressed in certain cancer cells at a frequency of 34-100%[39,40], including ovarian, breast, prostate and colorectal cancer[41]. The expression in normal tissues seems to be very low or absent. Survivin functions as an inhibitor of apoptosis protein (IAP) and regulates cell cycle and apoptosis, increases tumor resistance to antiapoptotic stimuli, can block apoptosis and antagonizing Survivin in tumor cells induces apoptosis[42,43,44], which is further associated with resistance to chemotherapy[45,46], increased rate of tumor recurrence[47] and an enhanced proliferation index[48,49,50,51]. Due to the strong effects upon tumor progression and its restricted expression, Survivin appears to be a promising target in cancer immunotherapy.

4.3 Cellular mechanisms regulate DC maturation

The maturation process of dendritic cells is associated with fundamental alterations of morphology, cytokine secretion, capability of antigen engulfment, migratory potential and marker expression. These characteristics are regulated by extracellular factors, depending upon strength, persistence and type of encountered signals, which subsequently alter intracellular signalling pathways. Recent studies revealed functional roles of members of the MAPK- and PI3K-pathway[52], and RhoA and its downstream target ROCKII[53] in the regulation of cellular processes associated with maturation.

4.3.1 The involvement of PI3K-pathway in DC maturation

Phosphoinosite-3-kinases (PI3K) are responsible for the phosphorylation of the D3 position of phosphatidyl inositols (PtdIns) family members, which are interacting with a high number of proteins via special binding domains. Three classes of PI3K have been identified so far depending on their mechanism of activation and substrate specificity[54].

Class I PI3Ks are divided in two subgroups: Class IA PI3Kα, β and δ consist of a catalytic (p110) and an adaptor/regulator subunit (p85 family) associated in a heterodimeric fashion. Upon activation by tyrosine kinase receptors and G protein-coupled receptors (GPCR)[55], the p85 subunit dissociates and localizes next to the plasma membrane in the proximity of its substrate PtdIns[56]. Class IB PI3Kγ does not interact with p85 but with the p101[57] and p84/87 adaptor/regulators[58,59] and is uniquely activated by GPCR[60]. The interaction of all class I PI3Ks with GTP-loaded Ras contributes to its activation[61] and they all phosphorylate PtdIns(4,5)P_2 (PIP2) to produce PtdIns(3,4,5)P_3 (PIP3) to forward extracellular signals.

Class II and class III PI3K are poorly characterized and they might be involved in vesicular trafficking[62,63]. Class II PI3K is probably involved in the production of PIP3 and PtdIns3P; class III PI3K produces PtdIns3P from PtdIns[64].

Class I PI3K activity influences a wide array of cellular functions because many proteins possess lipid-binding domains such as the pleckstrin homology (PH) domain. Important examples are PKB/Akt or phospholipase C (PLC) which bind PIP3 and PIP2 respectively. PKB/Akt is at the center stage of PI3K signalling and influences many fundamental cellular functions such as survival, proliferation, cell growth, protein synthesis and metabolism. In addition, most activators of small GTPases of the Rho family possess PH domains[54], explaining the effects of PI3K on cytoskeletal remodelling, migration and membrane trafficking[65] (Fig. 2).

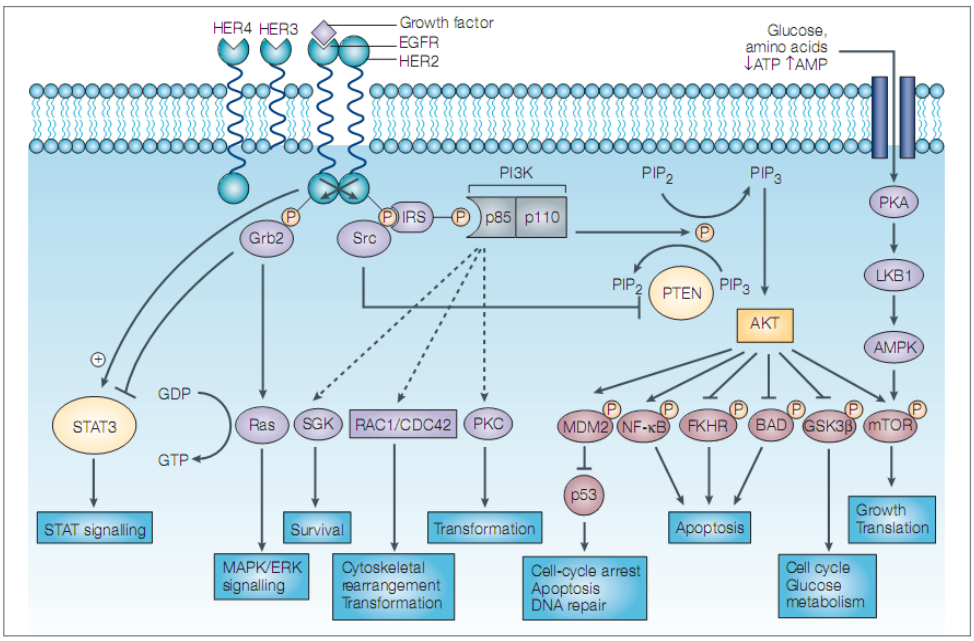

Fig. 2: Schematic of multiple signalling through PI3K (Nat. Rev. Drug Discov. 5, 988-1004 (2005)).

4.3.2 The RhoA-Rock-Ezrin-Pathway

RhoA is a monomeric G protein and forms, together with Rac, CDC42 and about 20 other proteins the Rho GTPases, a subgroup of the Ras superfamily. Like all Ras-related proteins, RhoA cycles between an inactive GDP- and an active GTP-bound state[66]. Upon an upstream activation signal, RhoA releases GDP, facilitated by guanosine nucleotide exchange factors (GEFs), and binds GTP which sets RhoA to the active state. On the other side, GTPase-activating proteins (GAPs) significantly enhance the intrinsic GTPase activity of RhoA[67,68], which leads to the hydrolysis of the bound GTP and returns RhoA into an inactive GDP-bound state. In addition, Rho GTPases are regulated by guanine nucleotide dissociation inhibitors (GDIs), which can inhibit hydrolysis of bound GTP and exchange of GTP[69]. Rho GTPases have been shown to play a crucial role in cellular processes such as cytoskeletal organization, cell growth, differentiation and apoptosis[70,71].

ROCKII is one of the best characterized downstream effectors of RhoA. Together with ROCK1, it forms the Rho-associated coiled-coil-containing protein kinase (ROCK) family. It consists of an amino-terminal coiled-coil domain, a central Rho-binding domain (RBD) and a carboxy-terminal PH domain. ROCKII also possesses an auto-inhibitory activity[72], which can be disrupted by binding of RhoA[73,74,75]. Up to date, several substrates of ROCKII have been identified such as myosin light chain (MLC)[76,77] stimulating actomyosin contractility[78,79],

LIM kinases[80,81,82] and ezrin/radixin/moesin[83] modulating the actin skeleton and promoting apoptosis[84], and PTEN[85], which negatively regulates the PI3K-Akt pathway (Fig. 3).

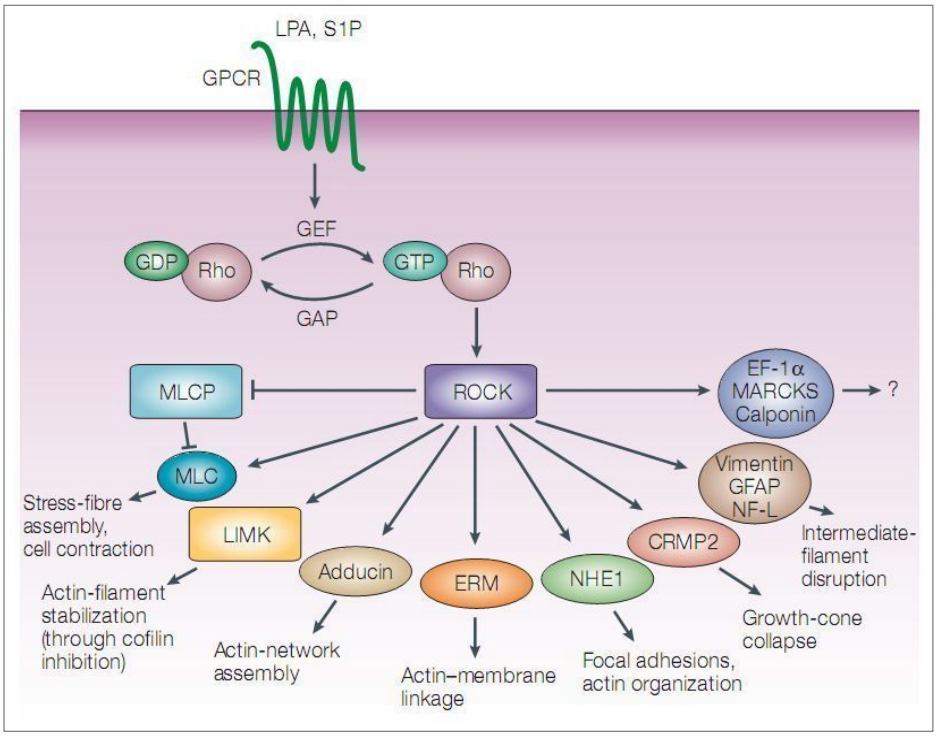

Fig. 3: The Rho-ROCK-pathway (from Nat. Rev. Mol. Cell Biol. 4, 446-456 (2003)).

4.3.3 Kinase inhibitors abrogate phosphorylation of downstream targets

As presented before; PI3K and ROCKII are essential for diverse cellular processes. The functional inactivation of these proteins in the maturation of DCs possibly sheds light on their importance in the production of fully mature dendritic cells, necessary for inducing a proper immune response in cancer immunotherapy.

The method of choice is the use of synthetic inhibitors which abrogate the phosphorylation of PI3K and ROCKII respectively without inhibiting the proteins itself.

PI3K inhibitors

- Wortmannin is an antifungal antibiotic isolated from *Penicillium funiculosum.* It inhibits PI3K of all classes and further is a potent inhibitor of myosin light chain kinase[86] and FMPL-stimulated processes[87,88] (Fig. 4).

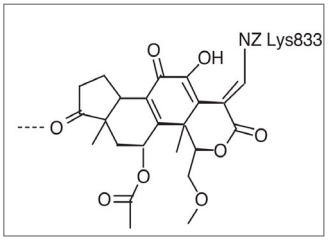

Fig. 4: chemical structure of Wortmannin.

- LY294002 is a specific cell-permeable inhibitor against PI3K[89] (Fig. 5).

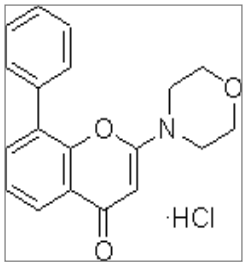

Fig. 5: chemical structure of LY294002.

ROCKII inhibitor

- Y27632 is a cell-permeable, potent inhibitor of both ROCK isoforms; ROCK1 and ROCKII. Studies suggest that it acts competitively with respect to ATP (Fig. 6).

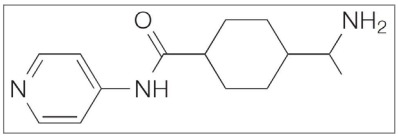

Fig. 6: chemical structure of Y27632.

5 MATERIAL AND METHODS

5.1 Culture of dendritic cells

5.1.1 Monocyte enrichment

Peripheral blood mononuclear cells (PBMNCs) are obtained from cancer patients by leukapheresis in an associated and certified center. The apheresate is immediately transported to CELL MED in an appropriate temperature-controlled box at 8°-20°C. It is eventually processed in order to gain an enriched phase of monocytes (mos) devoid of contaminating lymphocytes, erythrocytes and platelets.

PBMNCs are also obtained from healthy volunteers. This is done directly at CELL MED by vein puncture using Heparin-Vacutainer tubes. The cells are processed in the same manner as the cells from patients to enrich the mos phase.

The leukapheresate and whole blood are diluted in acid citrate dextrose solution (ACD; Baxter) 2.5 times, subsequently mixed with Ficoll (Biochrom) in a ratio of 1:1.5 and centrifuged for 20' at 2000 rpm (break 0) at RT. In the next step the interface is collected with a pipette and further centrifuged twice for 10´ at 1200rpm at RT to enrich the mos. After counting the cells with an automatic cell counter (CASY-1®, Schärfe System GmbH), the pellets are resuspended in CellGro DC (CellGenix) for 2 hours at a final concentration of $2x10^6$ cells/mL and subsequently the non-adherent cells are washed out and the adherent are cultured as described underneath. The grade of purity is measured by FACS analysis of the markers CD14 and CD45; possible contaminations are detected by measuring CD3, CD16, CD19, CD41 and Glycophorine A (GPA) expression.

5.1.2 Primary cell culture

The mos are cultured for 5 days in ventable $50cm^2$ tissue culture flasks (Falcon®) at 37°C with 5% CO_2. The medium, CellGro DC (CellGenix) supplemented with 2500U/mL GM-CSF (CellGenix) and 1000U/mL IL-4 (R&D), is used to induce the trans-differentiation to iDCs. Afterwards, the immature dendritic cells are loaded with human Survivin mRNA (150ng/10^7cells) for two hours, washed for 5 ´ at 1200 rpm, and finally matured in CellGro DC supplemented with 100µg/mL Ribomunyl (Pierre Fabre) and 1000µL/mL INF-γ (R&D) for 24 hours at 37°C with 5% CO_2 saturation. The attachment to the plastic dish is a hallmark of mature dendritic cells. The non-adherent iDCs are washed away with the super-

natant; the mDCs are detached by incubating the cells with Accutase (PAA Laboratories) for approximately 5 ´ at 37°C. The mDCs are centrifuged at 1200 rpm for 5 ´ at RT, then resuspended in RPMI (Gibco) supplemented with 2% FBS, and finally used for further experiments. As an alternative, the dried pellets are resuspended in Glycerol sterile solution (Mayrhofer) and frozen at -80°C.

5.2 Chemical inhibition

The DCs are treated with the following chemical inhibitors:

- 40µM of ROCK inhibitor (Y27632; Biosource),
- 10µM of PI3K inhibitor (LY294002; Sigma),
- 10µM of PI3K inhibitor (Wortmannin W1628; Sigma),

which are added at three different time points: either from the start of cell culture, before loading or before inducing DC maturation. If necessary, the inhibitors are replaced during cultivation every 2nd day.

5.3 Survivin mRNA preparation

5.3.1 Cloning of Survivin DNA using Topo TA cloning kit (Invitrogen)

TOPO TA cloning reaction

0.5 to 4µL of TrueClone pCMV-DNA (100ng/µL), which contains the Survivin full-length cDNA (OriGene) is mixed with 1µL salt solution and the reaction is filled up to 5µL with water. Finally, 1µL TOPO vector (Fig. 7) is added. The reaction is mixed gently and incubated for 5' at RT. The incubation time can be extended from 30'' to 3' to reach a higher number of colonies, After the incubation, the reaction is placed on ice until transforming into one shot competent E. coli (Invitrogen) is performed.

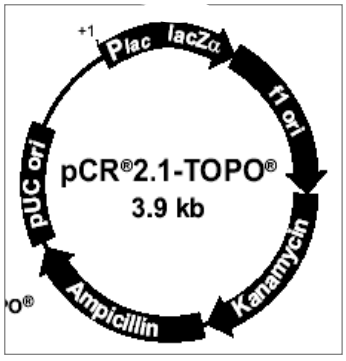

Fig. 7: pCR 2.1 TOPO cloning vector (Invitrogen).

Transformation of one shot competent E. coli

E. coli bacteria are thawed on ice for 3' for the transformation. Then 2µL of the TOPO TA cloning reaction are added and incubated for 5'-30'. Next, a heat shock for 30'' at 42°C is performed and 250µL of SOC medium (Invitrogen) are added at RT. The transformed cells are incubated for 1 h at 37°C and 250µL from the transformation is spread on pre-warmed LB agar plates, containing Ampicillin in a concentration of 100µg/mL. Plates are incubated at maximum for 18 hours at 37°C and checked for positive clones.

Liquid culture

Each clone is transferred to a sterile tube containing 3mL of LB liquid media, supplemented with Ampicillin. The liquid culture is incubated overnight at 37°C on an orbital shaker at 200 rpm.

Reagents

LB agar + Ampicillin plates

- 25g LB medium (MP Biomedicals)
- 15g Agar agar (Carl Roth GmbH)

The final volume is adjusted to 1L with water. The solution is autoclaved for 15'-20' and cooled down to 50°C. Finally, it is added up with 100mg Ampicillin (Sigma).

LB liquid media

- 10g/L Tryptone (MP Biomedicals)
- g/L Yeast Extract (MP Biomedicals)
- 10 g/L NaCl (Merck)

Finally, the pH is adjusted to 7.0 with 1N NaOH.

The final solution is autoclaved.

5.3.2 Plasmid isolation using Qiagen® plasmid isolation kit

1.5mL of the cell suspension are transferred to a 2mL tube and centrifuged for 5' at 10,000 rpm. Then the supernatant is discarded and the rest of the E. coli suspension is pipetted into the tube containing the bacteria pellet. This is again centrifuged under the same conditions.

The Kit contains Rnase A, which has to be dissolved in buffer P1. Each pellet is completely resuspended in 300µL of buffer P1. This lysing step is the crucial step in the procedure. Afterwards, 300µL lysis buffer P2 are added and mixed by inverting the tube several times followed by an incubation step for 5' at RT. This step is required to grant the degradation of genomic DNA of bacterial origin.

300µL pre-cooled neutralization buffer P3 are added to the tube after 5' and mixed again by inverting the tube several times before performing an incubation step of 5' on ice. The precipitation step is accelerated by the low temperature. Samples are centrifuged at 12,000 rpm for 10' to remove cell debris.

In the meantime, the Qiagen tips are equilibrated. Shortly, 1mL equilibration buffer QBT is loaded on each column, let be flown through completely and dried at RT. After centrifugation, the clear supernatant containing the plasmid-DNA is collected and transferred to the Qiagen tips. The solution enters the column and after the whole supernatant is flown through it, the plasmid-DNA, which has been bound by the resin instead, is washed twice with 1mL of QC buffer.

After the washing step, the plasmid-DNA is eluted with QF buffer. Shortly, a fresh 1.5mL tube is positioned under the column and 800µL QF buffer are pipetted to each column. 560µL isopropanol (7 times volume) are added to the eluted plasmid-DNA and mixed by inverting the tube. Afterwards, a centrifugation step at 12,000 rpm for 30' is performed at RT. It is important to collect the DNA at RT because at lower temperature salts can also be collected and can therefore contaminate the DNA. The supernatant is discarded and 1mL ethanol (70%) is added to each DNA-pellet before performing a second centrifugation step for 7' at 10,000 rpm. This procedure is repeated twice. Then the pellet is air-dried for 5' at RT and dissolved in 100µL 1xTE buffer. The plasmid DNA is stored at -20°C until further use.

5.3.3 Transcription of Survivin from a miniprep-sample

pCMV-Survivin linearization from Miniprep

5µg plasmid is digested with XbaI (Fermentas). Shortly, the plasmid DNA is diluted in water to obtain a final concentration of 5µg plasmid in 50µL volume. Next, 15µL of 10x buffer Tango (Fermentas) and 30 units XbaI restriction enzyme are added and the reaction is filled up to 153µL with water. The linearization is performed for 2 hours at 37°C. Subsequently, the reaction is stopped and the cDNA is precipitated by adding 1/20 EDTA (0.5M; Pharmacia

Biotech), 1/10 Sodium acetate (3M; Sigma) and 2 volumes of ethanol (100%; Merck). The reaction is incubated for 15' at -20°C and afterwards centrifuged for 15' at top speed. Finally, the supernatant is removed and the tube is re-spinned. The residual supernatant is removed with a fine-tipped pipette and the pellet is resuspended in 45µL water.

Proteinase K treatment

SDS (Sigma) is added to a final concentration of 0.5% and proteinase K (1mg/mL; Roche) is added to the linearized cDNA to a final concentration of 200µg/mL. The sample is thereafter incubated for 30' at 50°C.

Phenol/Chloroform extraction

DNA sample is added up to 100µL with water. Further 50µL phenol (Fluka) and 50µL chloroform (Fluka) are added. The sample is mixed and centrifuged for 3' at top speed. The aqueous phase is transferred into a fresh tube and centrifuged for 3' at top speed to remove the remaining phenol. Next, the aqueous phase is again transferred into a fresh tube and the equal volume of chloroform is added, centrifuged for 3' at high speed and again the aqueous phase is transferred into a fresh tube.

1/20 volume of EDTA (0.5M; Pharmacia biotech), 1/10 volume of sodium acetate (3M; Sigma) and 2 volumes of ethanol (100%; Merck) are added to the extracted DNA to precipitate the DNA. The reagents are mixed with the sample and incubated for 15'-20' at -20°C. The supernatant is carefully removed and 700µL ethanol (70%; Merck) are added to wash the precipitate, followed by a centrifugation step for 10' at top speed. The supernatant is removed with a fine-tipped pipette and 10µL water is added to the air-dried pellet. The concentration is determined by using a photometer (Peqlab).

Transcription reaction

For the transcription reaction the reagents included in the mMessage mMachine kit (Ambion) are thawed. 10µL 2x NTP/CAP are mixed with 2µL 10x reaction buffer, 2µl enzyme mix and 1µg linearized cDNA template and the reaction is filled up to a final volume of 20µL with nuclease-free water. The reaction is mixed by short centrifugation and incubated for 2 hours at 37°C. Afterwards, 1µL TURBO DNase is added, mixed well and incubated for 15' at 37°C.

Cleaning and recovery of RNA

The contents of the MEGAclear kit (Ambion) are warmed to RT. The volume of the RNA sample is adjusted to 100µL with elution solution and mixed gently. 350µL binding solution

concentrate are added and the reagents are mixed by pipetting. In the next step, 250µL ethanol (100%; Merck) are added and again mixed by pipetting. The RNA mixture is then applied onto a filter cartridge and centrifuged for 1' at 10,000g. Afterwards, the flow through is discarded and the filter cartridge is washed twice with 500µL washing solution, containing ethanol. Next, the RNA is diluted with 50µL water. Therefore, the filter cartridge is placed into a new collection tube and 50µL water is applied to the center of the filter, the cap is closed and the tube is incubated for 5'-10' at 65°C. The RNA is recovered by centrifugation for 1' at RT. The elution procedure is repeated with 50µL water to maximize the RNA recovery. The RNA yield is determined by using a photometer and the quality is checked by real-time RT-PCR.

5.4 Analysis of the cells

5.4.1 Morphology

An inverted microscope (Axiovert; Zeiss) and a digital photo camera (Axiocam; Zeiss) with the appropriate software (Axiovision; Zeiss) are used to take pictures for subsequent analysis of the morphology of the cell before the cells are harvested after maturation. The following settings are preset: 40x magnification and 20'' exposure time.

5.4.2 Phenotype (FACS)

Three aliquots of 10^6 cells each are necessary for this analysis. Each aliquot is resuspended in 50µL of the appropriate mix of antibodies or isotype controls, diluted in PBS, supplemented with 1% BSA and 0.1% NaN_3, and incubated in the dark for 15′ at RT. The cells are washed in 1.5 mL PBS at 800 rpm at RT and finally resuspended in 0.5 mL staining buffer to measure the signal strength with a FACS device (Beckmann Coulter).

As mentioned before, the mos are enriched by Ficoll purification. FACS analysis is used to measure the concentration of the mos obtained. The following markers are analysed:

Antibody	Fluorescence dye	Company
CD14	FITC	Beckmann Coulter
CD45	PC7	Beckmann Coulter

Table 2: FACS antibodies against mos.

Leukocytes, erythrocytes, platelets and granulocytes can still be present as contaminants after Ficoll purification. So, the cell suspensions are analysed via FACS to determine the level of the following CD markers:

Antibody	Fluorescence dye	Expressed on	Company
CD3	PC5	T-cells	Beckmann Coulter
CD16	PC5	Granulocytes	Beckmann Coulter
CD19	PE	B-cells	Beckmann Coulter
CD41	FITC	Platelets	Beckmann Coulter
Glycophorine A	PE	Erythrocytes	Beckmann Coulter

Table 3: FACS antibodies used to determine contaminating cells.

At last, the maturation stage of the dendritic cells is measured at two distinct time points: after 5 days of trans-differentiation and after 24 hours of maturation, respectively. The expression of the following markers is analysed:

Antibody	Fluorescence dye	Company
CD40	PC5	Beckmann Coulter
CXCR4	PE	Becton Dickinson
CD80	FITC	Beckmann Coulter
CD83	PC5	Beckmann Coulter
CCR5	FITC	Becton Dickinson
HLA-DR	PC5	Beckmann Coulter
CCR4	PE	Becton Dickinson

Table 4: FACS antibodies used to determine maturation stage.

5.4.3 ELISA (IL-10 and IL-12 secretion)

IL-10 and IL-12p70 secretion into the supernatant is measured after trans-differentiation and maturation, respectively, following the OptEIA ELISA sets protocol (BD Pharmington). At first the required wells are coated with IL-10 or IL-12p70 antibody, diluted in coating buffer as recommended in the lot-specific analysis certificate, and incubated at 4°C overnight. The next day, the plate is washed thrice and 200µL of the supplied assay diluent are added for 1h incubation at RT. After successive washing for three times, 100µL of 1:2 diluted samples and 100µL of each standard are filled into the wells in duplicate and incubated for 2h at RT. The samples are washed 5 times, 100µL of working detector reagent (consists of diluted, biotinylated IL-10 and IL-12p70 detection antibody and streptavidin horseradish peroxidase) are added for 30´ followed by 7 times washing with a soaking time of one minute per washing.

100µL of substrate solution are added and the wells are kept in a dark room for 30'. Finally, 50µL stop solution is pipetted into the wells and within 30' the plate is read out with a photometer (BioRad) at 450nm with a wavelength correction at 570nm. The measured absorbance directly relates to the detected interleukins and corresponds to the standard dilution.

5.4.4 Proliferation assay (mixed leukocyte reaction)

Dendritic cells (at a final concentration of $1x10^6$ cells/well) are co-cultured with the non-adherent fraction of Ficoll-separated PBMNCs from a healthy donor ($1x10^4$ cells/well) diluted in RPMI with 2% FBS (Gibco) supplemented with 10% AlamarBlue® in a 96-well U-bottom plate at a final volume of 150µL/well. AlamarBlue® changes from its oxidized (blue) to the reduced form (red). The absorbance is measured with a plate reader (SLT-Tecan) at 595nm with a reference wavelength at 620nm every 4-12h over a period of 2-7 days.

5.4.5 Phagocytosis assay (FITC-dextran uptake)

Three aliquots of each sample, with $1x10^6$ cells per aliquot, are resuspended in 1.5mL RPMI 1640 (Gibco), admixed with 2% FBS, in a 6-well plate. Two aliquots (spontaneous and induced phagocytosis) are supplemented with 0.5µg/ml FITC-dextran. 300ng/mL CCL19 (Sigma) are further added to one of them to induce phagocytosis. The cells are incubated for 2 hours at 37°C, washed twice in cold PBS and centrifuged for 5' at 1200 rpm at RT. Thereafter, the cells are resuspended in 0.5mL cold PBS and their phagocytic activity is measured with a FACS® device (Becton Dickinson).

5.4.6 Migration assay (Transwell assay)

3 wells of a 6-well plate are used for each sample. 1.5mL RPMI 1640 (Gibco), mixed with 2% FBS, are filled into the lower part of the well. 300ng/mL CCL19 are added to the third well to induce migration. In the next steps, the filter is inserted, 500µL of cell suspension ($1x10^6$ cells) are pipetted into the well and the plate is incubated for 2h at 37°C with 5% CO_2 saturation. The incubation is stopped by adding 1.5mL 8% paraformaldehyde (PFA, Sigma) to the lower part of the well, respectively 500µL to the upper part at a final concentration of 4% PFA, for 10' at RT. Afterwards, the wells are washed once with PBS.

In the next step, the upper surface of the filter of the second and third well is scraped with a cotton-tipped swab and the remaining cells are stained with a 0.2% crystal violet (Sigma) in 20% methanol (Merck) solution for 5' at RT. The filters are rinsed with water to remove ex-

cess staining solution and are air-dried. Finally, the membranes are cut out with a scalpel, mounted on a glass slide with Entellan (Merck) and photographed for successive counting.

5.4.7 Viability assay

$1x10^5$ cells are resuspended in 500µL binding buffer following the Annexin V FITC apoptosis detection kit (Biovision). Then, the suspension is incubated with 5µL Annexin V FITC and 5µL propidium iodide (PI) for 5' at RT in a dark room. Annexin V binding is measured by flow cytometry using FITC signal detector, PI staining with the phycoerythrin emission signal detector.

5.4.8 Western blot

Protein extraction

All steps are performed on ice to avoid protein degradation. $2x10^6$ cells are thawed in 100µL Frackelton buffer (Table 5, 6), vortexed and incubated at 4°C for 20' to gain cytoplasmatic proteins. After further vortexing, the cells are centrifuged for 20' at 4°C at 14000 rpm. So, the pellet (nuclear) and supernatant (cytoplasmatic) are separated from each other. 100µL of the supernatant are resuspended in 25µL 5x SDS sample buffer with 100mM DTT and stored at -20°C. The samples are heated up to 75°C before being loaded on the SDS-Page gel.

Reagents

Frackelton Buffer

Component	Stock solution	Amount added
Tris-Cl	10mM	0.24g
$Na_4P_20_7$	30mM	2.7g
NaCl	30mM	0.6g
Triton 100x		2mL
MilliQ water		Fill up to 200mL

Table 5: Frackelton buffer stock solution components.

stored at +4°C

Added just before use:

Component	Stock solution	Final concentration	Dilution	fV = 1.2mL Frackelton Buffer
NaF	1M	50mM	1:20	60µL
Na_3VO_4	100mM	0.1mM	1:1000	1.2µL
Protease Inhibitor cocktail			1:25	48µL
OA	100mM	0.1mM	1:1000	1.2µL
PMSF	0.1M	1mM	1:100	12µL

Table 6: Frackelton buffer components.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-Page gel is prepared as following described:

Two clean glass-plates and two clean spacers are used to prepare a gel chamber, which is inserted into a casting module (BioRad). A solution of 10% polyacrylamide separating gel (Table 7) is prepared and poured into the chamber with a pipette to fill approximately 75% of the chamber. The gel is covered with isopropanol and let polymerise for 5' at RT. Isopropanol is discarded after polymerisation and the 4% polyacrylamide stacking gel solution (Table 8) is poured on the top of the separating gel; finally a comb is inserted in the stacking gel that is let polymerise for 10'.

Running gel 10%	**1 small gel**
Tris-Cl, pH8.8 1.5M	3mL
Bis/Acrylamide 30% (Biorad)	4mL
SDS 20%	60µL
milliQ water	5mL
APS 10% (Sigma)	48µL
TEMED (Sigma)	24µL

Table 7: SDS-page 10% running gel.

Stacking gel 10%	
Tris-Cl, pH6.8 1.5M	1mL
Bis/Acrylamide 30%	0.5mL
SDS 20% (Sigma)	20µL
milliQ water	1.25mL
APS 10%	24µL
TEMED	12µL

Table 8: SDS-page stacking gel.

After removing the comb, the slots are filled with milliQ water and the gel is inserted in the electrophoresis chamber that is in turn filled with the running buffer. Finally 5µL of the marker (prestained SDS PAGE standard; Fermentas) and a volume corresponding to 5µg of each sample are loaded. The electrophoresis is performed for approximately 75' at 160V.

Immunoblot and Ponceau Red staining

After SDS-PAGE, the proteins are transferred from the PAA gel to a nitrocellulose membrane in 1x transfer buffer for 1h at 30V in a semi-dry blotting apparatus (Mini-PROTEAN Tetra system; Bio-Rad). The immunoblotting sandwich is made up of two pre-wetted filter papers, cut to reach the size of the gel, the previously pre-equilibrated membrane, the gel, and further two pre-wetted filter papers. The sandwich is placed in a way that the gel is at the cathode and the membrane at the anode.

After the transfer, the membrane is stained with Ponceau Red in order to determine the quality of the blotting. Afterwards, the membrane is rinsed once in water and washed with TTBS until all the red staining is completely removed.

Reagents

- 10% APS: 1g ammonium persulphate (Sigma) dissolved in 10mL milliQ water; 4°C.
- Ponceau Red 10x (stock solution): 2g Ponceau S (Serva), 30g trichloracetic acid (Merck), 30g sulphoalocyclic acid (Merck), MilliQ water to 100mL.
- 10x Running buffer: 0.25M Tris (Sigma), 1.92M glycine (Fluka), milliQ water to 1L.
- Separation gel buffer (pH 8.8): 1.5M Tris-Cl, 0.4% SDS, milliQ water to 1L.
- Stacking gel buffer (pH 6.8): 0.5M Tris-Cl, 0.4% SDS (Sigma), milliQ water to 1L.

- 10x Blotting buffer: 29g glycine (Merck), 58g Tris-Base, 3.7g SDS, water to 1L; 4°C.
- 1x Blotting buffer: 10% 10X Blotting buffer, 20% methanol, milliQ water to 1L.
- 10x TBS: 250mM Tris-Base, 1.5M NaCl (Merck); pH 8.0, milliQ water to 1L.
- TTBS: 100mL 10x TBS, 500µL Tween-20 (Promega), milliQ water to 1L; 4°C.

Incubation with the antibodies

Non-specific binding is blocked by incubating the membrane one hour in a solution of TTBS added up with 5% powder milk. Thereafter, the membrane is washed twice with TTBS for 10'. Finally it is incubated overnight with the appropriate primary antibody (Table 9) diluted in TTBS and 0.1mM NaN_3.

1st antibody	Company	Dilution	2nd antibody
Actin	Santa Cruz	1:1000	Goat
Ezrin/Moesin/Radixin	Cell Signaling	1:1000	Rabbit
pEzrin/pMoesin/pRadixin	Cell Signaling	1:1000	Rabbit
B-Raf C-terminal	Santa Cruz	1:500	Rabbit
B-Raf N-terminal	Santa Cruz	1:500	Rabbit
C-Raf	BD Transduction Laboratories	1:1000	Mouse
MEK1/2	Cell Signaling	1:500	Rabbit
pMEK	Cell Signaling	1:500	Rabbit
p38	Santa Cruz	1:500	Rabbit
pp38	Santa Cruz	1:200	Rabbit
pJNK	Cell Signaling	1:500	Rabbit
JNK	Cell Signaling	1:500	Rabbit

Table 9: Western blot antibodies used against specific endogenous proteins.

The day after, the membrane is washed three times for 10' in TTBS and subsequently incubated for 30' with the appropriate secondary antibody, diluted in TTBS.

Thereafter, the membrane is washed three times with TTBS for about 30' and finally rinsed in TTBS.

ECL detection

After the incubation with the primary and the secondary antibody, the membrane is incubated with 0.5mL of previously mixed Luminol/Enhancer Solution and Stable Peroxyde-Solution (Pierce) for 5'. The excess of the detection buffer is removed; the membrane is positioned between two clean transparent papers and thereafter placed in a film cassette. Finally, it is exposed to an ECL-hyperfilm (Amersham) in the dark room for a time comprised between 10'' and 1h, depending on the strength of the signal.

6 RESULTS

Dendritic cells were either obtained from whole blood of healthy volunteers or from surplus of leukaphereses of cancer patients (Table 10). Quantitative limitations made it impossible to perform all experiments with the same patients.

Name	sex	Age [years]	Cancer type
Patient A	male	52	Colon
Patient B	female	50	Breast
Patient C	female	59	Ovarian
Patient D	female	51	Breast
Patient E	female	47	Ovarian
Control 1	female	30	-
Control 2	female	29	-
Control 3	female	34	-
Control 4	female	24	-
Control 5	female	36	-
Control 6	male	23	-

Table 10: Cancer patients and healthy volunteers.

6.1 Morphology

In the first step, the morphology of non- and inhibitor-treated cells was analysed by light microscopy to assess the effects caused by LY294002, Wortmannin and Y27632 upon adherence, shape and viability (Fig. 8).

Dendritic cells derived from Patient B upon loading and maturation became adherent and formed big clusters. On the contrary, dendritic cells from the same patients but treated from the start with LY294002 or Wortmannin tended to remain in the supernatant and did not aggregate. Cells treated with Wortmannin showed an even less trend towards adherence than cells treated with LY294002. Cell treated with Y27632 showed a combined phenotype of star-shaped and strongly adherent cells and clusters of round cells, which tended to slightly adhere to the flask surface.

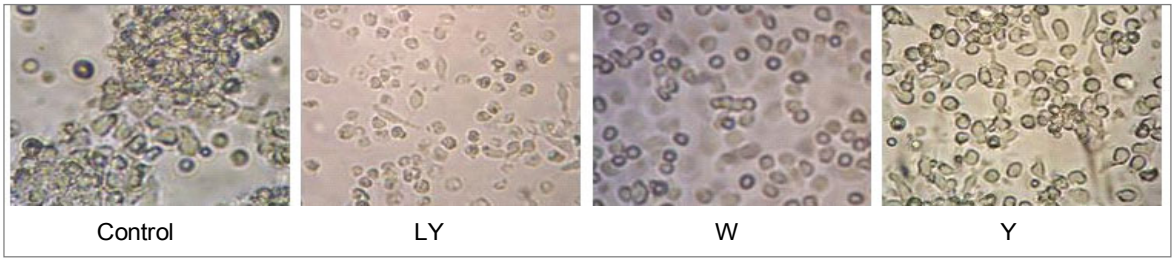

Fig. 8: DC morphology of untreated control and upon treatment with selective inhibitors from the start of culture.

6.2 Phenotype (FACS)

Since the addition of selective PI3K as well as ROCK inhibitors significantly modified the normal phenotype of mature DCs, we further investigated whether such a difference correlates as well with a modification in the expression of surface markers, which are known to be increased during human DCs maturation. Shortly, we analysed by FACS DCs derived either from healthy controls or patients for the following markers: CD80, CD83, CD40, CXCR4, CCR5, CCR4, HLA-DR and CD95L after the first 5 days of culture in presence of IL-4 and GM-CSF (iDCs) and 24h after loading and maturation (mDCs).

6.2.1 Addition of PI3K and ROCK inhibitors from start

The analysis of the phenotype was performed with a healthy control (Control 1) and a patient suffering from colon cancer (patient A). In this setting, the inhibitors were added at the start of cell culture and the expression of relevant surface markers was measured after 5 days (iDC), and after further 24 h of maturation mDC), respectively.

Immature DCs

Untreated cells derived from the healthy control showed high surface expression of HLA-DR and CD40, mild expression of CD80 and low CXCR4 expression. The other analyzed markers were almost absent on iDC surface. In comparison, immature DCs derived from the patient displayed a high HLA-DR expression, although not reaching the levels observed on iDCs derived from the healthy control; a mild-low expression of CD40, CXCR4, CD80, CCR5, CCR4 and CD95L, while CD83 was almost undetectable.

Addition of the PI3K inhibitor LY294002 did not affect the expression of CD40, CD83, CCR4 and HLA-DR on iDCs derived both from the healthy control and the patient; it slightly decreased the expression of CD80 on healthy control derived iDCs and strongly the expression of CD80 on patient derived iDCs. It almost did not affect CCR4 expression on healthy

control derived iDCs and slightly increased the expression of such a marker on patient derived iDCs. It did not affect CCR5 and CD95L expression on healthy control derived iDCs while strongly decreasing the expression of such a marker on patient derived iDCs.

Wortmannin addition caused different effects on iDCs phenotype in comparison to those observed upon LY294002 addition, suggesting that these two inhibitors affect parts of the PI3K pathway, which are pivotal for different aspects of the DC phenotype that are strongly affected upon cancer development as well. Indeed, upon Wortmannin addition, CD40 and HLA-DR expression were strongly down-regulated on healthy control derived iDCs but not on patient derived iDCs. Such an inhibitor strongly impaired the expression of CXCR4 and CD80 on healthy control only and on healthy control as well as patient derived iDCs, respectively. It did not affect the expression (already very low) of CD83, CCR5, CCR4 and CD95L on healthy control derived iDCs, while strongly increasing CXCR4 and CD83 expression and strongly decreasing the expression of CCR5 and CD95L on patient derived iDCs.

Inhibition of the Rho-associated kinase with Y27632 had minor effects on any analysed markers regarding iDCs derived from healthy control. On the contrary such an inhibitor strongly decreased the expression of CD80, CCR5, CCR4 and CD95L and mildly that of HLA-DR on iDCs derived from patient. These findings suggest that ROCK function on human DCs may be affected by tumor development.

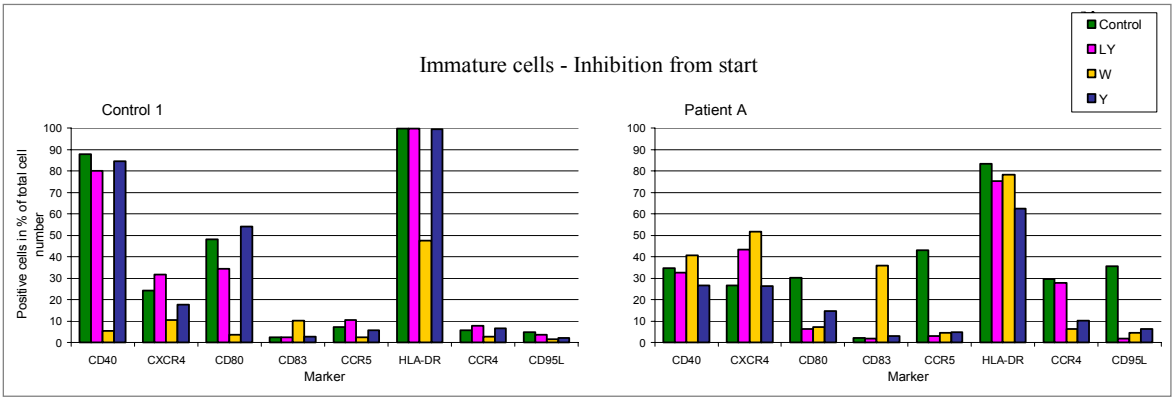

Fig. 9: Marker expression profile of iDCs of control 1 and patient A inhibited from start.

Mature DCs

After 5 days in culture, cells were loaded with Survivin by passive transfection (6h) and after maturation for 24h upon addition of the maturing cocktail, fully mature cells were harvested and tested for surface markers expression.

As expected, the expression of surface markers of both healthy control and patient derived mDCs, increased markedly upon maturation. Anyhow, the expression of CD83 was strongly reduced in mDCs derived from patient in comparison to that observed on healthy control derived mDCs.

Upon addition of LY294002 the expression of CXCR4, CCR4 and CD83 was slightly decreased while that of CCR5 and CD95L was strongly decreased on mDCs derived from healthy control. Differently, mDCs derived from patient showed a very strong impairment of the expression of all the analysed markers beside CD83 upon incubation with LY294002. Such results showed that the DC response to LY294002 is affected by the tumor. Indeed the PI3K pathway sensible to LY294002 seems to be more important for the establishment of the right phenotype of the mDCs in patient- in comparison to healthy control derived DCs.

Wortmannin addition strongly decreased the expression of all analysed markers on mDCs derived from healthy control, showing a more powerful effect on such DCs in comparison to LY294002. A strong impairment of CD40, CD80, CCR5, CCR4 and CD95L was detectable on mDCs derived from patient as well. In this case, anyhow, Wortmannin has only a mild down-regulative effect on the expression of CXCR4 and HLA-DR while slightly increasing the expression of CD83. Interestingly, the effects of Wortmannin on CXCR4 and HLA-DR expression were far weaker than those caused by the addition of LY294002. These results seem to suggest that Wortmannin effects are more important in healthy control derived DCs while LY294002 effects mostly interest patient derived DCs, possibly supporting the idea that tumor development causes a modification of the PI3K pathway involved in the phenotypical maturation of human DCs. More experiments in this direction are required to confirm or confute such a theory.

ROCK inhibition did not affect the surface markers expression on mDCs derived from healthy control and mildly affected that of CD80, CCR5, HLA-DR and CD95L on patient derived mDCs. The strongest effect was detectable regarding CCR4 expression on patient derived mDCs. In this case, too, ROCK pathway seems to be more related to the establishment of the right phenotype in mDCs derived from patients than in those derived from healthy controls, suggesting a possible correlation between ROCK function and tumor progression, regarding human DC phenotype.

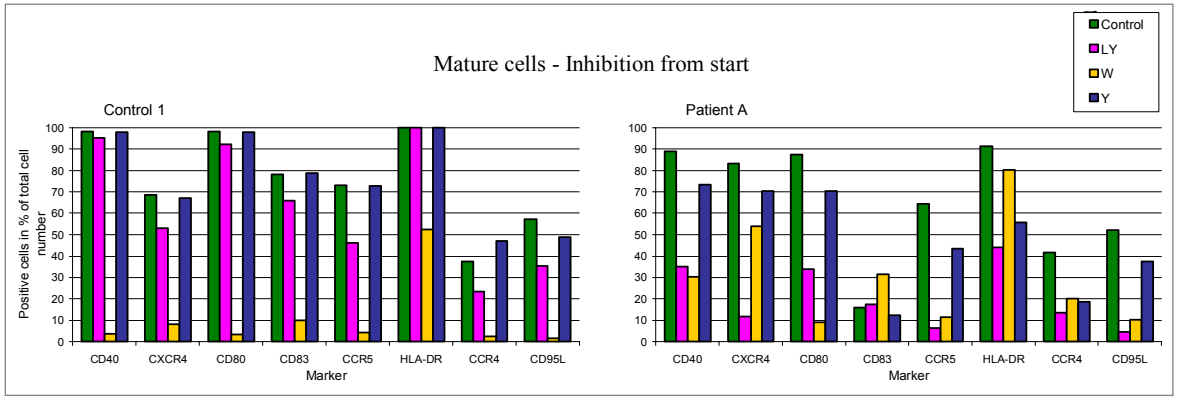

Fig. 10: Marker expression profile of mDCs of control 1 and patient A inhibited from start.

6.2.2 Addition of PI3K and ROCK inhibitors from loading

LY294002 treatment markedly lowered the expression of all markers of the healthy control besides CCR4, which was slightly up regulated, compared to the cells treated from the very start of culture. The patient's derived DC showed only reduction in CXCR4 expression and a higher expression level of CCR4 and HLA-DR. The strongest differences could be seen upon direct comparison between Control 1 and Patient A. Here, patient's derived DCs expressed lower levels of CD40, CD80 and CCR4 and higher levels of CD83 and HLA-DR. The production of CXCR4, CCR5 and CD95L was almost completely abrogated. LY294002 negatively influences the expression of maturity-associated markers, especially in the healthy control, the only exception being CCR4. These results give a primary indication of a crucial role of PI3K at least regarding the phenotypical maturation of human DCs.

The effects of incubation with Wortmannin significantly differed from those observed from the very start of culture in the healthy control and from the effects of LY. The expression, especially of CD40, CXCR4, CD83, HLA-DR and CCR4, was profoundly increased. Differently, patient's derived mDC did not reflect the same results. CD80, CCR5, HLA-DR, CCR4 and CD95L levels were comparable to those after inhibition from the start; only CD40 and CD83 were up-regulated and CXCR4 expression was actually diminished. Further comparing the healthy control with the patient showed almost completely identical expression levels of all markers. Wortmannin inhibition reflects the findings of LY, as marker expression decreased in comparison to untreated cells, denoting PI3K as promoter of phenotypical maturation. More controversial are the findings concerning exposure time, as a long exposure time enhances effects of LY in the healthy controls but not in the patients.

ROCK inhibition slightly reduced CD40, CD80 and CCR5 expression in the healthy control, respectively CD83 and HLA-DR more profoundly relative to the inhibition from start. CXCR4 and CCR5 expression levels were increased whereas CD95L remained stable. In the patient, the levels of CD40, CCR4 and CD95L were unaffected by the different time points of inhibition but CXCR4, CD80, CD83, CCR5 and HLA-DR showed reduced expression. Patient and control presented distinct differences if marker expression was analysed upon ROCK inhibition. So, only CD95L was expressed at the same level as in Control 1, the remaining markers were down-regulated in patient's derived DCs. ROCK inhibition decreased marker expression more strongly, if administered at a later time point and seemed to have a higher impact upon the phenotype of patient's derived DCs. The influence of ROCK, although significant, is not as pronounced as that of PI3K inhibitors.

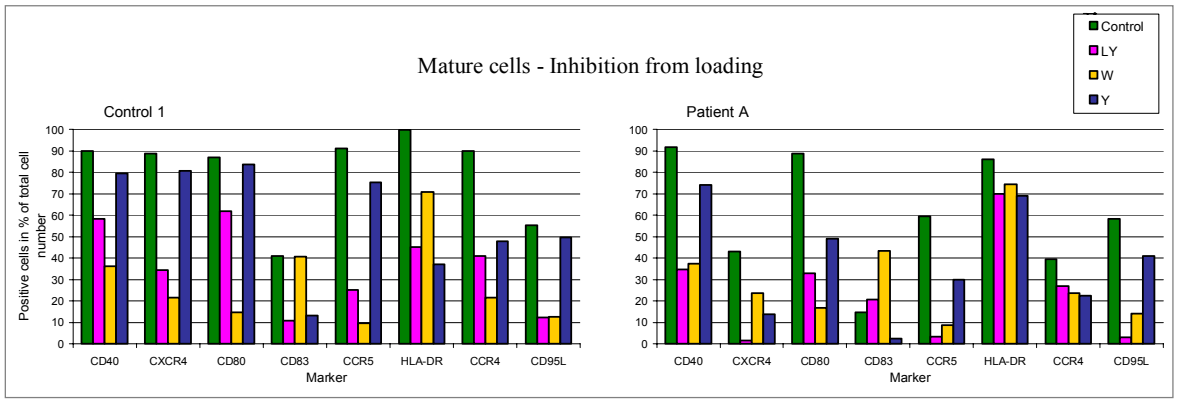

Fig. 11: Marker expression profile of mDCs of control 1 and patient A inhibited from loading.

6.2.3 Addition of PI3K and ROCK inhibitors from maturation

Upon PI3K inhibition with LY294002 significant differences could be observed between inhibition from maturation and from earlier time points in the healthy control. The expression of CXCR4, CCR5 and CD95L was decreased and the expression of the other markers levelled between or at the level of former time points. In the patient's derived DCs the expression levels of the investigated markers were more similar to those from earlier time points, although CD40, CD80 and CD83 were higher and HLA-DR lower expressed. Direct comparison between control's and patient's derived DCs could not elicit any greater differences in any of the markers. In conclusion, the presented results show that LY negatively influences the expression of surface markers and denote PI3K as crucial in maintaining phenotypical maturation.

Incubation with Wortmannin increased the expression of every marker in the healthy control similarly to inhibition from start, which reflected the same results obtained upon inhibition from loading besides a slight enhancement in production of CXCR4 and HLA-DR as well as a minor reduction in CD95L expression. These similarities could also be observed between Control 1 and Patient A, where all markers were expressed equally. Concerning Patient A, in comparison with inhibition from the loading, Wortmannin had the highest impact upon surface marker expression especially if administered from the very start of cell culture apart from a reduction in CD95L production. Interestingly, the effects of Wortmannin were partially different to those of LY, which might be caused by the different pathways, which are selectively blocked by each of the two PI3K inhibitors.

Finally, ROCK inhibitor Y27632 strongly reduced the expression of CXCR4 and CD80, impaired CCR5 production, slightly diminished the expression of CD83 and CCR4 and only increased the level of HLA-DR in the healthy control regarding inhibition from loading. In comparison, the markers in Patient A were almost equally expressed with a slight increase in HLA-DR, CCR4 and CD95L production but not in parallel with earlier time points of inhibitor addition, where CD40, HLA-DR, CCR4 and CD95L levels were up regulated, CCR5 production impaired and CD83 decreased. Taken together, the effects of ROCK seem to be smaller than those of PI3K with the exception of CD83, which was down to a basal level in mature DCs in five of six cases. The time of inhibitor administration appears not to be crucial for optimal effects.

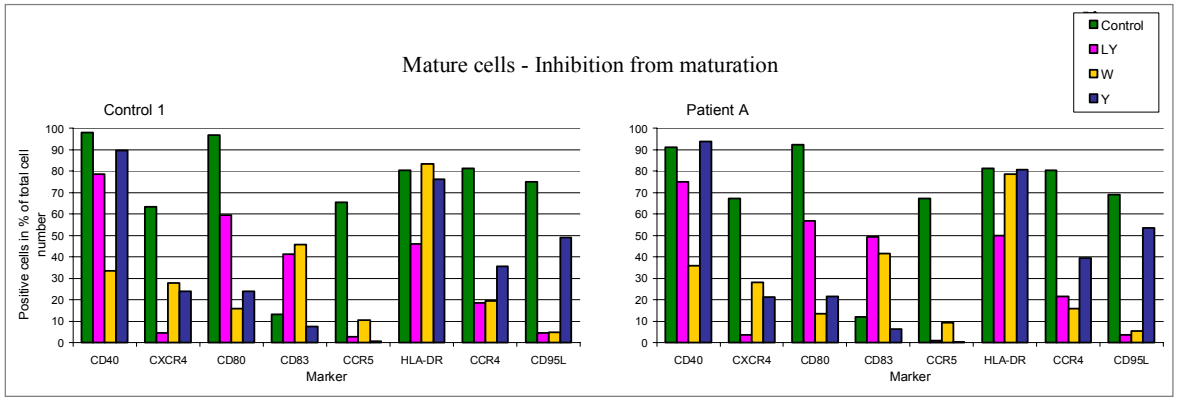

Fig. 12: Marker expression profile of mDCs of control 1 and patient A inhibited from maturation.

6.3 ELISA (IL-10 and IL-12 secretion)

6.3.1 Addition of PI3K and ROCK inhibitors from start

Since our preliminary experiments about the effects of PI3K and ROCK inhibition upon the morphology and the phenotype of DCs during their maturation showed that indeed, such pathways play crucial roles, we further investigated whether these phenotypical effects mirrored functional consequences as well.

IL-10 expression correlates with the induction of a tolerogenic environment, which strongly impairs the maturation of human DCs. Such IL is normally expressed at very low levels by *in vitro* cultured iDCs. On the contrary, IL-12 is highly secreted by mDCs according to their maturation stage and it increases the CTL response.

Immature DCs

The secretion levels of IL-10 and IL-12 in untreated iDCs were according to the literature reports, as expected. IL-12 was not produced by any of the samples and IL-10 only slightly in two controls. This reflects the immature state of the DCs, proper *in vitro* conditions and reduces probability for tolerogenic stimulation, which is typically mediated by excessive IL-10 secretion.

Inhibition of the PI3K pathway with LY294002 showed similar characteristics as in untreated DCs: very low IL-10 and IL-12 secretion. This result points to the assumption that the PI3K pathway does not influence the secretion of these interleukins if added from the start of culture, at least relative to the immature DCs.

Also Wortmannin, the second PI3K inhibitor, provided the same results as obtained before. IL-10 was only slightly expressed by one healthy control and no IL-12 was secreted by any iDC inhibited from the start of culture. This indicates, accompanied by the results provided by inhibition with LY, that PI3K is not involved in the molecular events leading to the secretion of IL-10 and IL-12 in iDCs.

The most significant results were obtained from DCs inhibited by Y27632 where the secretion of IL-10 was elevated to low-medium levels in a patient and a healthy control but as in the former cases, IL-10 was not measurable in the other samples and there was no IL-12 at all. Anyway, this result is not as significant as to suggest that ROCK pathway is involved in the secretion of these cytokines. The investigation of later time points will perhaps shed light on this possibility.

Mature DCs

Also untreated DCs in their matured status reflected the expected findings: IL-10 is not produced by any cell regardless of its source and IL-12 is secreted at a high level in all DCs. This shows that dendritic cells, even obtained from cancer patients, secrete IL-12 in large quantities and can subsequently promote the desired T_H1-type immune response. The impaired IL-10 secretion further excludes tolerogenic stimulation.

The inhibition with LY mimicked the effects in untreated cells: IL-12 secretion is comparably high as in untreated cells and no IL-10 could be detected, strengthening the suggestion that PI3K is not involved in the secretion of these interleukins. This result is somewhat puzzling regarding the findings provided by literature, which point to the involvement of this PI3K inhibitor in the up regulation of IL-12 in mature dendritic cells[52] and the down regulation of IL-10[90].

The secretion of IL-10 was impaired after Wortmannin inhibition as in untreated cells. Interestingly, interleukin-12 secretion was slightly to medium diminished in four of the six DC samples. This observation is contradictory to that seen after LY treatment, is stochastic regarding the different sources and maybe due to individual characteristics or to the known differing effector functions of Wortmannin compared to LY294002. Further, the concentration of used PI3K inhibitors possibly needs to be adjusted to access full effects on interleukin production. Also here, the up regulation of IL-12 after PI3K treatment, as presented in the literature, cannot be reproduced. On the other side, it was shown that a high concentration of Wortmannin (50mM) reduces IL-12 levels in the murine model[91].

The most interesting results could be observed from inhibition of the Rho-associated kinase, ROCK, which resulted in a high secretion of IL-10 in every sample, regardless of the source. Surprisingly, IL-12 could only be detected at a basal level and not at all in one of the patients. This observation clearly indicates a pivotal role of ROCK in the regulation of both IL-10 and IL-12 due to its strong effect upon differential interleukin secretion. The high level of IL-10 typically induces a T_H2-type of immune reaction, which is not desirable due to the promotion of tolerance.

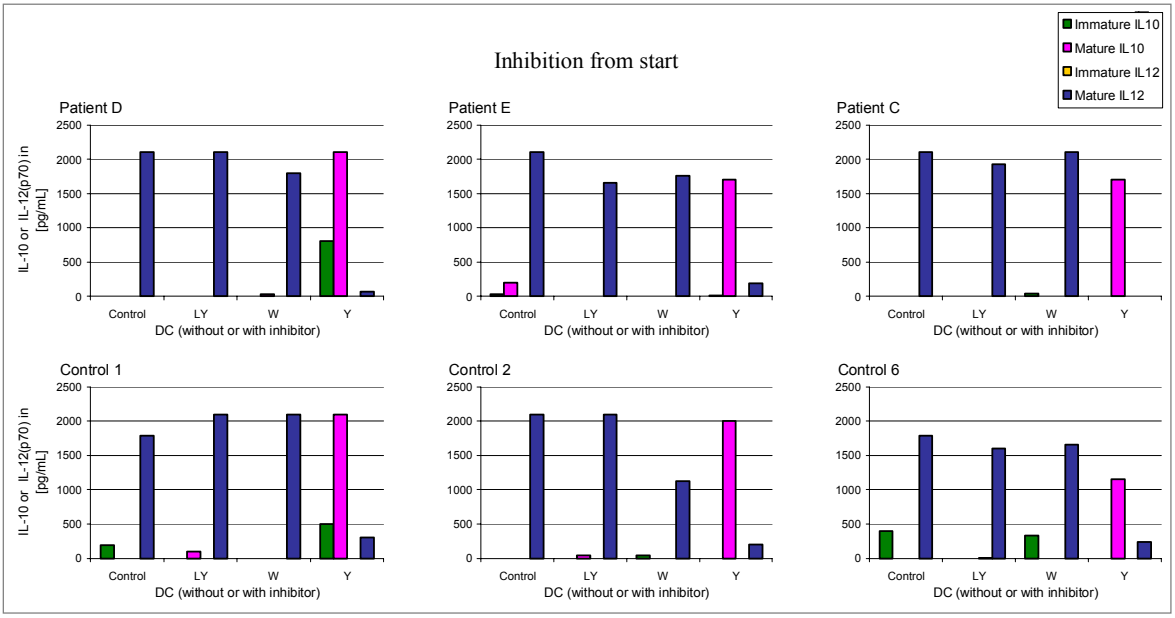

Fig. 13: Secretion of IL-10 and IL-12 upon inhibition from start.

6.3.2 Addition of PI3K and ROCK inhibitors from loading

Untreated mDCs mimicked in principle the results obtained above: the secretion of IL-10 was impaired and IL-12 was produced in high amounts although, beside by one patient, not at the same extent. This seems to be independent from the source of DCs. It can be assumed that, somehow, the culture conditions were not optimal, which led to a reduced IL-12 secretion.

Cells treated with LY294002 also had an impaired IL-10 and a pronounced IL-12 secretion, whose level was, with one exception, slightly lower than in untreated cells, regardless of its source. This finding strengthens the assumption that culture conditions reduced the IL-12 production and further that the influence of LY294002 upon interleukin secretion is negligibly small although this finding is not according to other study reports.

Also Wortmannin had no effect upon IL-10 and only a minor effect on IL-12 secretion, slightly increasing IL-12 production in four samples and decreasing it in one. Interestingly, a source-dependent effect could be seen, since the IL-12 level of all healthy controls was up regulated. Furthermore, this result is contradictory to the addition of the inhibitor from the very start of the culture, where the secretion of IL-12 was reduced or stable in each sample. Investigating the reasons for the different secretion patterns, leads to the conclusion that either the time point of Wortmannin addition or its effector function is causal for these observations. Finally the up regulation of IL-12 is consistent with findings presented in literature.

Once again, ROCK inhibition offered completely altered secretion of IL-10 and IL-12, with a medium-high to high production of the first and almost completely impaired secretion of the latter. This is in accordance to the secretion levels present after inhibition from the start and is not source-dependent. On the other side, the assumption that ROCK is important in mediating signals for the regulation of interleukin 10 and 12 is further strengthened and that impaired ROCK signaling promotes the activation of a tolerogenic immune response in both, healthy controls and cancer patients.

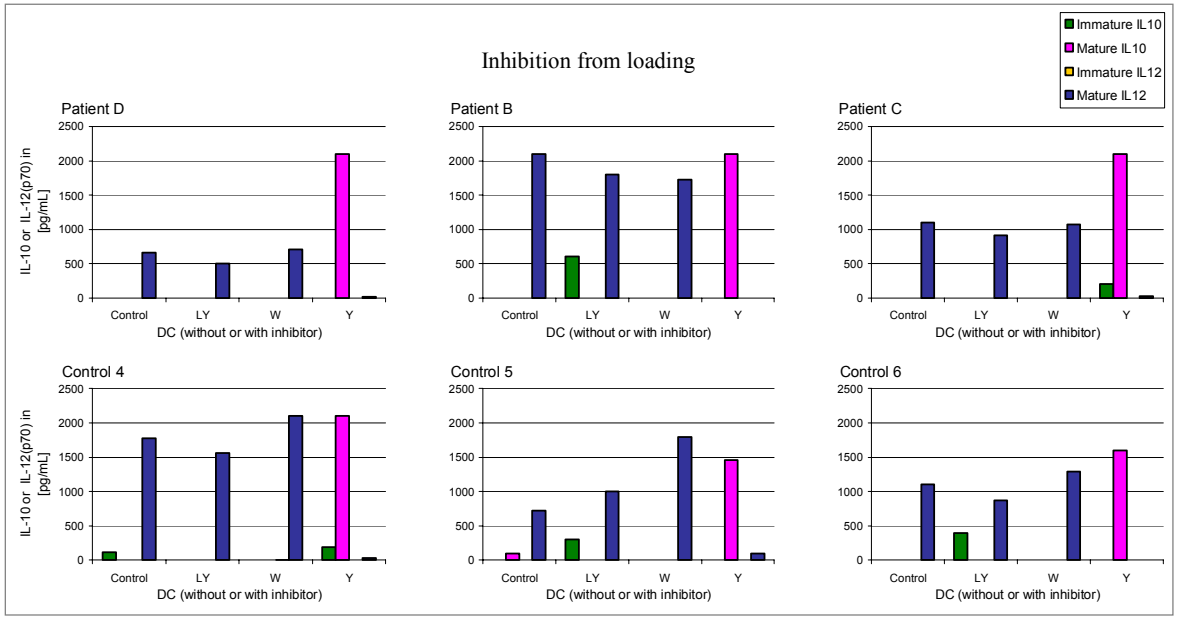

Fig. 14: Secretion of IL-10 and IL-12 upon inhibition from loading.

6.3.3 Addition of PI3K and ROCK inhibitors from maturation

The investigation of untreated DCs showed that IL-10 was almost absent in all samples and, curiously, IL-12 secreted at low to medium levels beside one healthy control and one patient, where it was highly expressed. In comparison to earlier time points, the IL-12 secretion markedly diminished and so strengthens the assumptions of problems in culture conditions.

Cells treated with LY294002 also had an impaired IL-10 and a pronounced IL-12 secretion, whose level was slightly lower than in untreated cells, regardless of its source. One healthy control's derived DCs showed a more profound reduction in IL-12 secretion but considering that in untreated cells the IL-12 level of this control was far higher than in the other samples some caution is advised. In conclusion, the results are according to former findings, which indicate that PI3K inhibition with LY294002 does only minor, if any, affect the secretion of these cytokines; also not if applied at different time points.

mDCs treated with Wortmannin from the maturation step on, did not secret any IL-10 and low to medium amounts of IL-12 comparable to untreated cells. The effect of enhanced IL-12 production, as in DCs inhibited from the loading on and as in literature mentioned, could not be reproduced. A time dependence of Wortmannin addition can be argued to be the cause for this discrepancy but also the effect of enhanced IL-12 secretion, as seen before, is only slight and can be due to culture conditions, too.

ROCK inhibition had the highest impact on IL secretion with almost total absence of IL-12 but a high production of IL-10. Mentionable, the Interleukin 10 level was slightly diminished in one patient and one healthy control, presumably due to individual characteristics. Also the inhibition from loading on elicits a pivotal role of ROCK in cytokine secretion, as seen before, and justifies further investigation of ROCK in the maturation of dendritic cells.

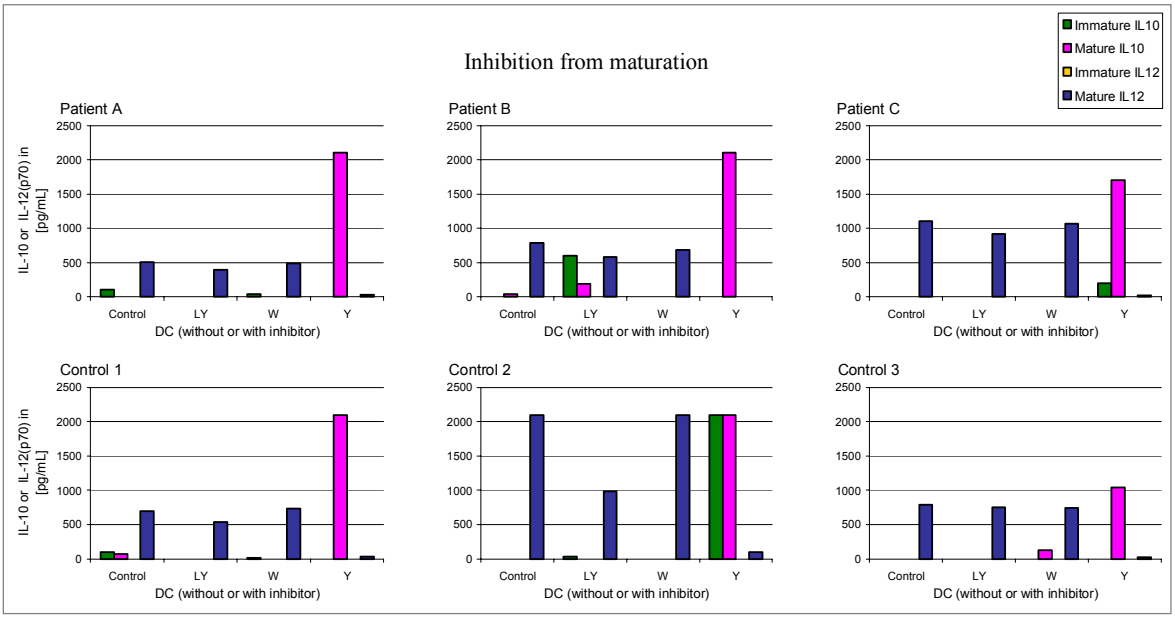

Fig. 15: Secretion of IL-10 and IL-12 upon inhibition from maturation.

6.4 Proliferation assay (mixed-leukocyte-reaction)

Since we showed that human DCs maturation, studied according to morphology, phenotype and IL production, was strongly affected by chemical inhibition of PI3K and ROCK, we further investigated whether such effects correlated with impaired capability to stimulate lymphocyte proliferation, a hallmark of mature and fully active DCs, measured by means of a mixed lymphocyte reaction (MLR). The proliferation rate is normalized to the proliferative potential of immature cells, which is set to the value of 1. The proliferation rate of the lym-

phocytes induced by mature DCs is given as a relative proliferation rate compared to that shown by immature cells.

6.4.1 Addition of PI3K and ROCK inhibitors from start

Untreated mDCs, both from healthy controls and patients, showed a high rate in the stimulation of proliferation of lymphocytes. This is a characteristical trait of fully mature dendritic cells and reflects good culture conditions.

LY-inhibited mDCs markedly reduced the potential to induce T cell proliferation, which is neither source-dependent nor individual-specific.

The effect of Wortmannin was stronger than that of LY and led to a reduction in T cell proliferation to the level of iDCs or slightly above. Both, LY and Wortmannin inhibition indicate an important function of PI3K in the induction of a proper T cell activation, corresponding to phenotypical analysis where the expression of relevant surface markers was reduced.

Y27632 inhibition from the start of culture significantly increased the potential to induce T cell proliferation in four samples. In the remaining two samples, one patient and one healthy control, the rate was equal or slightly lower than in the untreated cells. Interestingly, this is contradictory to the absence of IL-12 which usually promotes T cell activation and points toward an IL-12 independent mechanism.

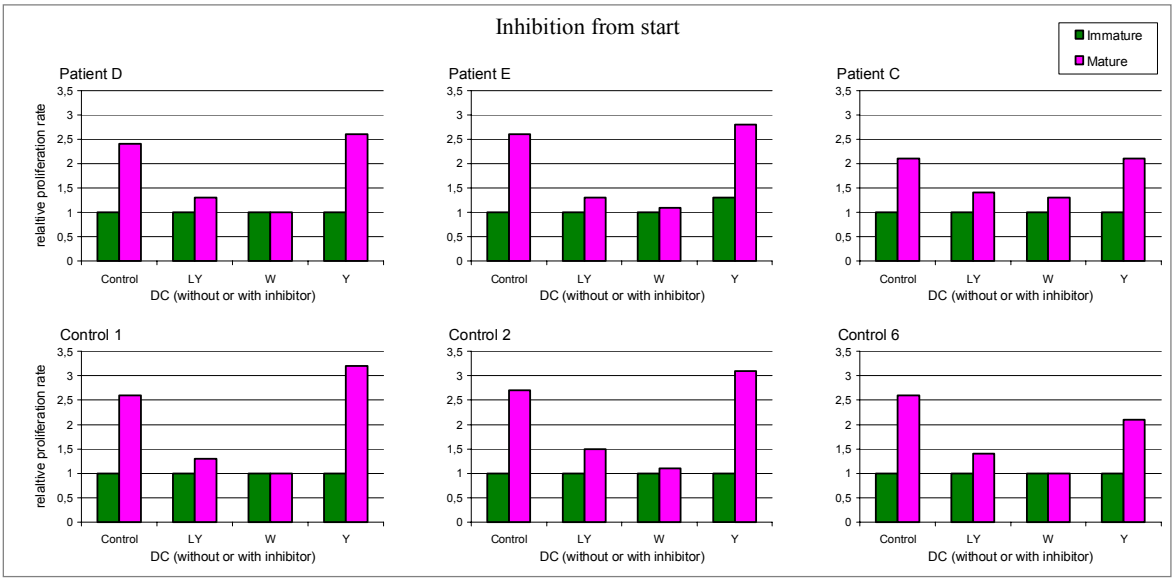

Fig. 16: relative proliferation rate of lymphocytes upon DC inhibition from start.

6.4.2 Addition of PI3K and ROCK inhibitors from loading

The addition of selective inhibitors from the loading on almost totally mimicked the results obtained from longer exposure, leading to the conclusion that the capability to induce T cell proliferation is not depending on prolonged exposure to the inhibitors.

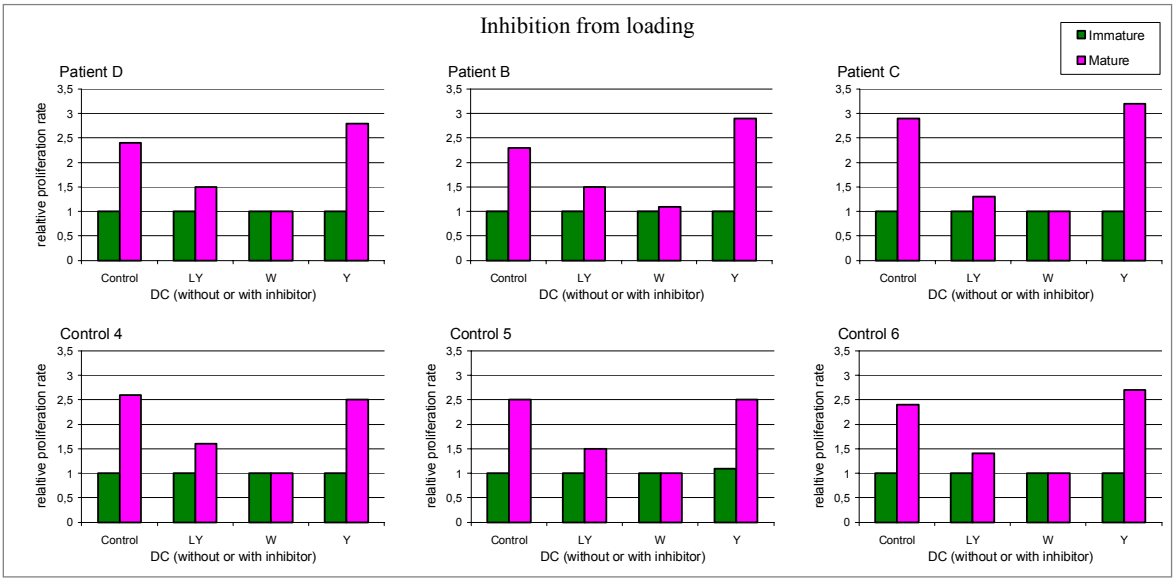

Fig. 17: relative proliferation rate of lymphocytes upon DC inhibition from loading.

6.4.3 Addition of PI3K and ROCK inhibitors from maturation

If the inhibitors were added on from the maturation of DCs, LY, Wortmannin and Y27632 mimicked the former results. So, the time-window of inhibitor addition does not influence the strength of T cell activation and administration from maturation on is sufficient to provoke these effects.

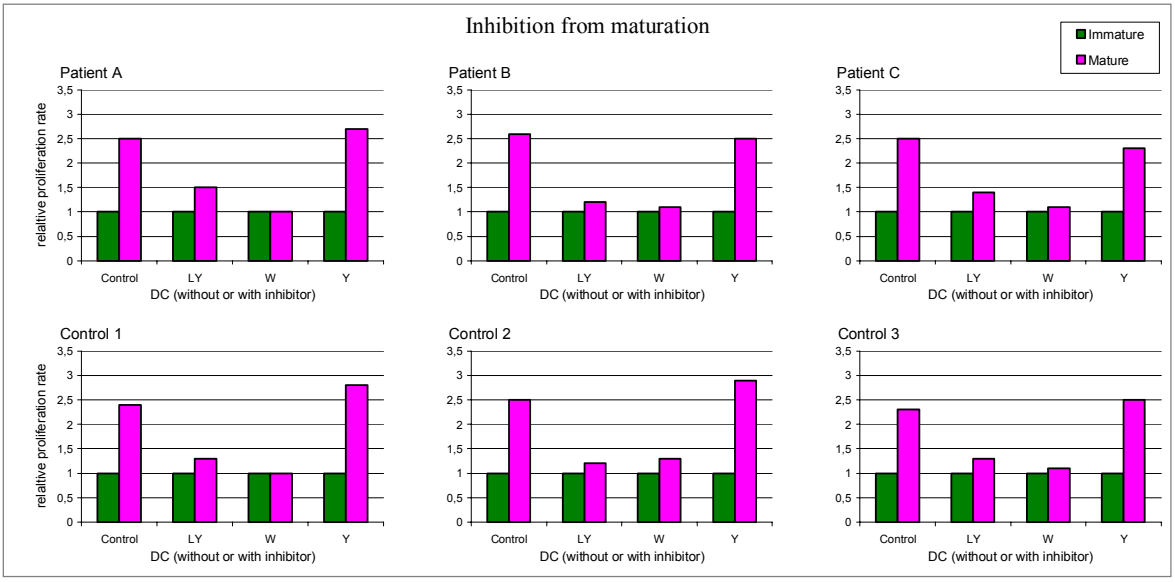

Fig. 18: relative proliferation rate of lymphocytes upon DC inhibition from maturation.

6.5 *Phagocytosis assay (FITC-dextran uptake)*

In order to be able to induce proliferation of lymphocytes, DCs need to phagocyte exogenous antigens. Therefore, such characteristic is a typical feature of iDCs, while once they are loaded with an antigen, mDCs loose the capability to endocyte further. Since we observed that the inhibition of PI3K and ROCK pathways impaired human DCs *in vitro* maturation, we investigated whether such morphological, phenotypical and functional characteristic correlated with the increased amount of activities, typical for iDCs. The phagocytosis property of DCs is determined through the rate of FITC-dextran uptake, which is given in percentage of cells, which take up the labelled dextran. In order to determine the effects of maturation on the phagocytotic capability, the cells were induced by addition of CCL19, the ligand of CCR7, a surface marker expressed exclusively by mDCs.

6.5.1 Addition of PI3K and ROCK inhibitors from start

Immature DCs

Immature dendritic cells, regardless of untreated or supplemented with selective inhibitors, showed a very high potential to phagocyte spontaneously and were unable to respond to CCL19 activation, according to results from literature. This suggests that neither PI3K nor ROCK is crucial for phagocytosis relative to the immature stage of human DCs.

Mature DCs

Typically, untreated and matured DCs were unable or only slightly able to perform spontaneous phagocytosis. Also upon CCL19 induction, hardly any improvement could be observed, which corresponds to the results reported in literature.

Inhibition by LY294002 from the start on showed an improved phagocytic capability of mDCs in spontaneous phagocytosis to a medium-high level and a somewhat higher capacity of patents' derived DCs than of their healthy counterparts. Upon induction with CCL19 the phagocytosis was further enhanced.

Upon Wortmannin treatment, spontaneous phagocytosis was comparably high like after LY294002 inhibition, with one minor individual–specific exception where it was slightly increased. Induced phagocytosis improved the capacity to phagocyte, regardless of its source. PI3K inhibition from the start on, leads to an improved phagocytosis in mDCs, which points to an important function of PI3K in this property of dendritic cells, by mediating maturation at all or, possibly, by directly influencing phagocytosis-related pathways.

ROCK inhibition elicited the highest impact on phagocytosis capacity increasing it to the rate of immature DCs, independent of CCL19 induction. This could be observed in both, the healthy controls and the patients. Interestingly, the high rate of phagocytosis is a typical hallmark of iDCs. Therefore, ROCK signalling appears to be important in functional maturation of DCs, possibly by impairing phagocytosis which is not surprising, as ROCK is known to be involved in the regulation of the cytoskeleton.

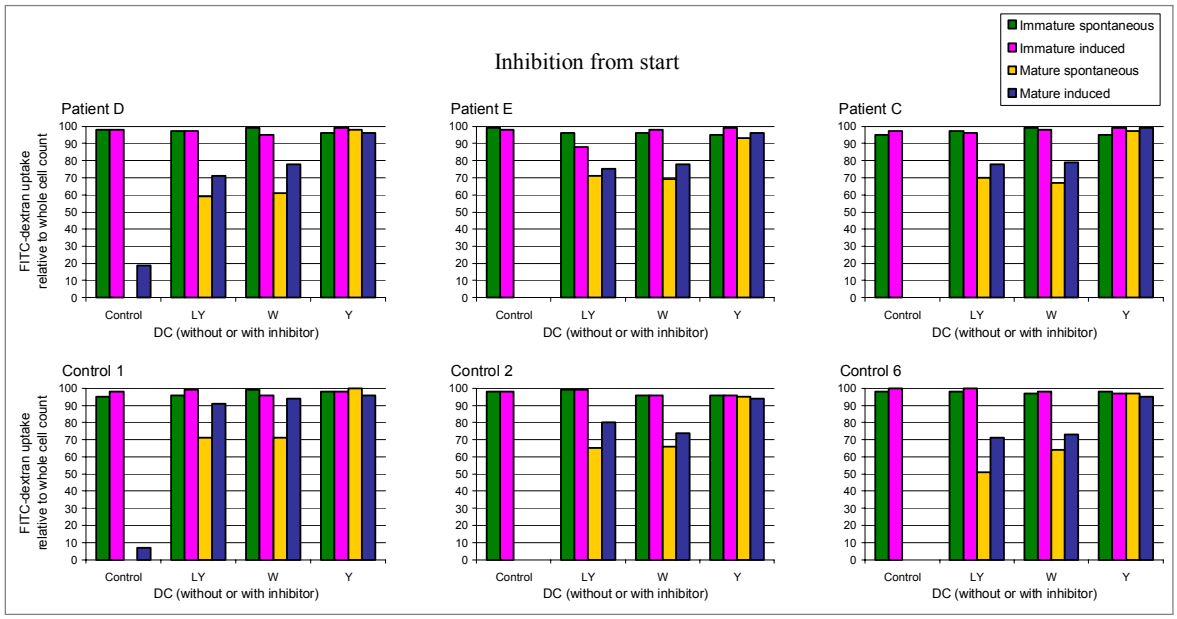

Fig. 19: relative FITC-dextran uptake upon inhibition from start.

6.5.2 Addition of PI3K and ROCK inhibitors from loading

Typically, untreated and matured DCs were unable or only slightly able to perform spontaneous phagocytosis. Also upon CCL19 induction, hardly any improvement could be observed which corresponds to the results shown in literature.

The inhibition of PI3K with LY294002 from the loading on had similar effects as the inhibition from start: improved spontaneous phagocytic capability, which was even more pronounced upon addition of CCL19. The only difference was that the phagocytosis was slightly lower compared to the earlier time point. This effect seems to be due to the shorter time of incubation with LY294002.

The effects caused by Wortmannin mimicked in general those of LY294002 although spontaneous phagocytosis was higher in cancer patients' derived DCs compared to LY294002 and also to the healthy controls. CCL19 enhanced the phagocytic potential further, apart from one healthy control in which induced phagocytosis led to a reduced potential. The assumptions made upon PI3K function from the start of culture were confirmed and time-dependence was additionally noticed.

Y27632 increased the phagocytic capacity almost to the rate of immature dendritic cells. This could be strengthened by the addition of CCL19, which was different from the former series of experiments, where also the spontaneous phagocytosis had equal rates compared to immature cells. It seems that the time point at which ROCK inhibitor is administered is crucial for a total induction of phagocytosis. Anyway, the assumption that ROCK is an important mediator in phagocytosis is confirmed.

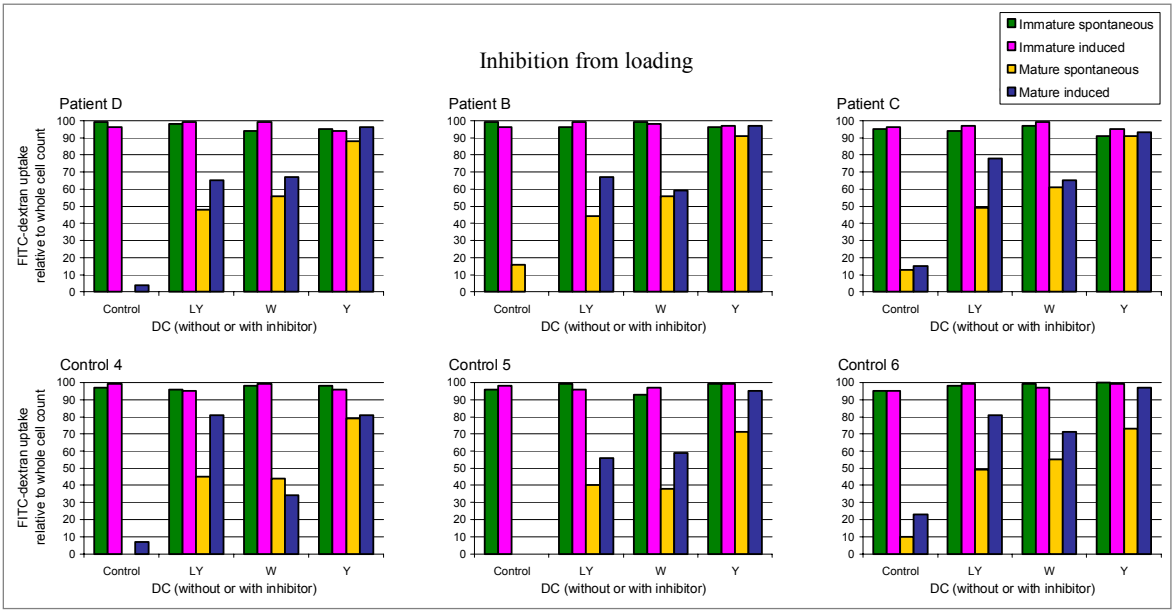

Fig. 20: relative FITC-dextran uptake upon inhibition from loading.

6.5.3 Addition of PI3K and ROCK inhibitors from maturation

Characteristically, untreated and matured DCs were unable or only slightly able to perform spontaneous phagocytosis. Also upon CCL19 induction, hardly any improvement could be observed which corresponds to findings presented in literature.

PI3K inhibition with LY correlated with increased spontaneous phagocytosis to a medium level, which is similar to the inhibition from loading and therefore also lower than after prolonged exposure. CCL19 addition caused a higher phagocytotic potential.

Exposure to Wortmannin from maturation on improved the potential to phagocyte to the level of inhibitor addition from loading on, spontaneously and induced. Again, this effect seems to be source-dependent as the phagocytic capability is slightly but significantly higher in DCs derived from cancer patients. Taken together, former findings are approved and an administration of LY and Wortmannin from the start of culture on brings about the highest impact on phagocytosis

In contrast, ROCK inhibition increased the level of spontaneous phagocytosis in nearly all samples to that of iDCs. CCL19 induced phagocytosis further enhanced the capability of the cells. This affirms the crucial role of ROCK in phagocytosis and shows that Y27632 addition is not time-dependent.

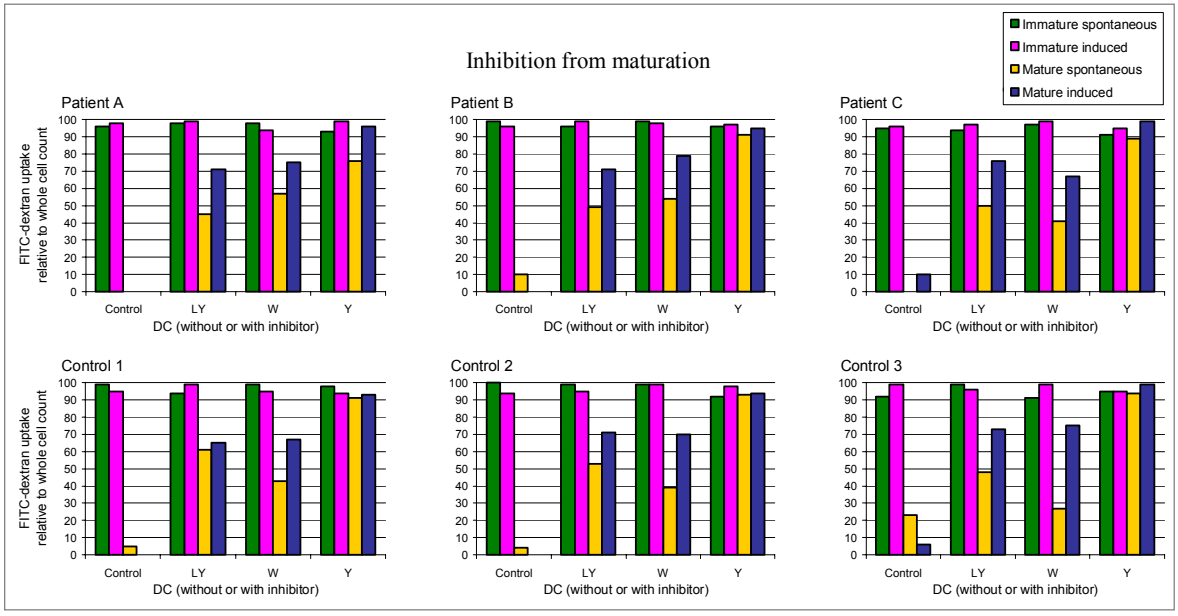

Fig. 21: relative FITC-dextran uptake upon inhibition from maturation.

6.6 *Migration assay (Transwell assay)*

Finally, we investigated the effects of PI3K and ROCK inhibition on the migratory potential, which as well as the phagocytosis is a hallmark of immature DCs.

The migration assay is carried out as a transwell assay. The results are given in percentage of migrated cells (spontaneous or induced) relative to the total cell number.

6.6.1 Addition of PI3K and ROCK inhibitors from start

Immature DCs

iDCs, regardless of their source and of the used inhibitors, possessed a very high potential in spontaneous and induced migration, which is in accordance to findings from literature.

Mature DCs

According to literature, the potential of untreated DCs to spontaneously migrate was down regulated to a basal level upon maturation and the addition of CCL19 reduced the capability further in two patients and two healthy controls.

Under the influence of LY294002 spontaneous migration was enhanced in five samples but only to a minor extend. Interestingly, this effect was completely different if the cells were

supplied with CCL19 because in this case the rate of migrating cells increased to a very high level, comparable to that of immature DCs.

Wortmannin administration mimicked upon CCL19-induced migration the effects of LY. The capability to migrate spontaneously in cancer patients' derived DCs was at the level of untreated cells or underneath. In the healthy control this was somewhat higher. Overall, LY stronger induced the potential to migrate spontaneously, especially in patients' cells. LY294002 and Wortmannin treatment influences spontaneous migration only minor but supplementation with CCL19 significantly increases the ability to migrate, which indicates a function of PI3K in CCL19-CCR7 mediated migration.

In contrast, inhibition with Y27632 increased the spontaneous migratory potential of DCs to a high level independent of its source. CCL19 further enhanced migration to a comparable level like in immature DCs in two patients and two controls. In the remaining samples CCL19 led to a slight decrease in parallel to spontaneous migration. In contrast to inhibition of PI3K, also the ability to spontaneously migrate is significantly enhanced upon depletion of ROCK, which entitles ROCK to be a negative regulator of migration. This is not surprising, as ROCK is known to be a pivotal player in the regulation of the cytoskeleton.

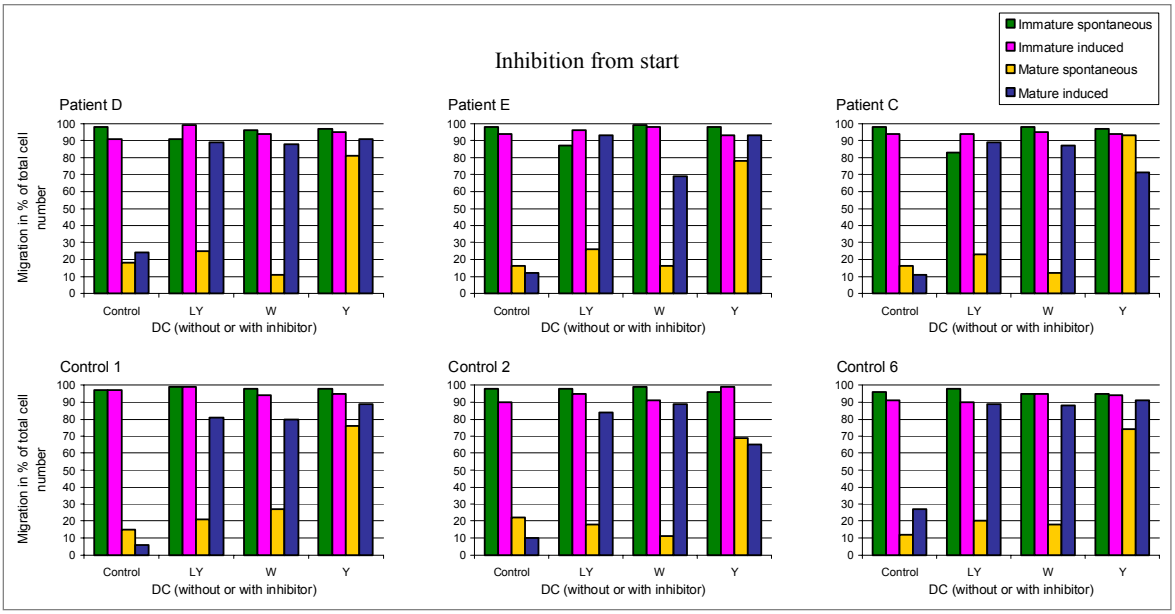

Fig. 22: migratory rate of DCs upon inhibition from start.

6.6.2 Addition of PI3K and ROCK inhibitors from loading

Untreated cells mimicked the effect seen before with the minor exception that in every case CCL19 further enhanced migration.

Upon treatment with LY former results can be affirmed as well except that the induction with CCL19 did not raise the migratory potential at the same extend any more, indicating a dependence on the time of LY administration.

Wortmannin treatment mimicked the results from prolonged exposure as seen before although the effect of CCL19 induction was not as pronounced, delivering evidence for time dependent effects of Wortmannin. Further, a source-dependence was no longer evident.

The later addition of the ROCK inhibitor even exceeded the capability to migrate seen after prolonged exposure but was less dependent upon CCL19 supplementation. This result is in accordance with former findings and indicates that the time point of inhibitor administration is crucial for optimal modulation of migration.

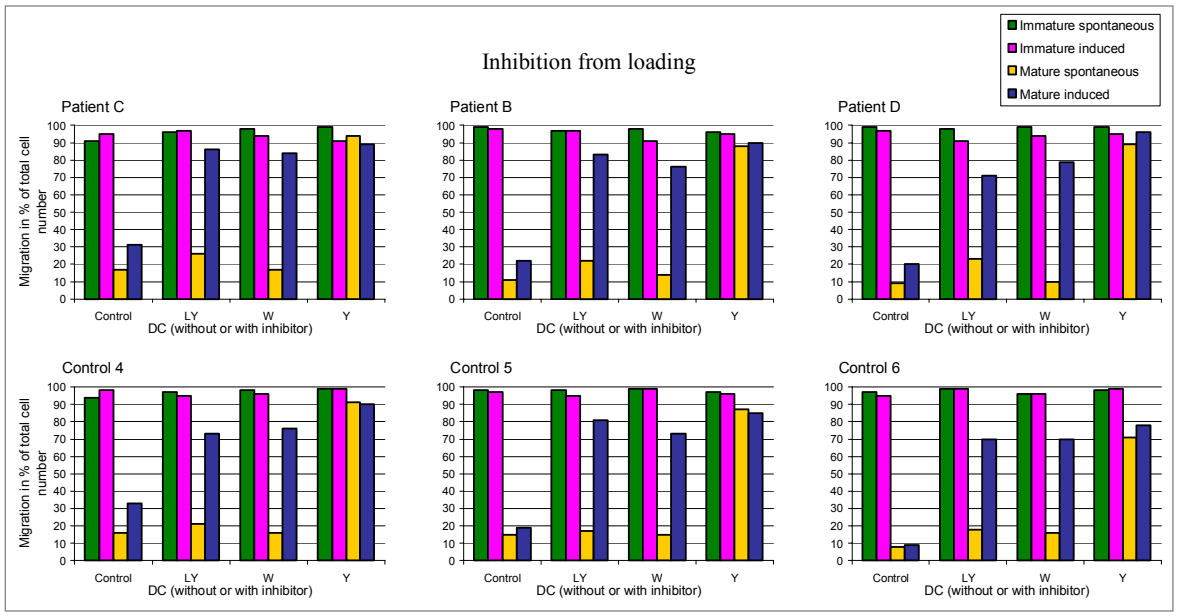

Fig. 23: migratory rate of DCs upon inhibition from loading.

6.6.3 Addition of PI3K and ROCK inhibitors from maturation

As expected, untreated cells mimicked the effects seen after inhibition from loading with one minor exception: DCs derived from one patient had a slightly reduced potential to migrate upon CCL19 induction.

Also, LY treatment revealed similar results like with earlier time points of inhibitor administration. Interestingly, the later addition of the PI3K inhibitor decreased the induced migratory potential further and leveled at a medium rate strengthening the importance of PI3K in induced migration.

Time-dependence could also be detected in Wortmannin treated cells, where the level of spontaneous migration was lower or equal to the rate of untreated cells. The potential of induced migration further decreased in comparison to longer exposure times, although to a lower extend in the healthy controls. Also Wortmannin inhibition showed the importance of PI3K in induced migration.

In the case of Y27632 treatment previous findings were strengthened, revealing a very high capability to migrate spontaneously and induced by CCL19. The effect upon migration was especially high if the inhibitor was added from loading or maturation on, leading to the conclusion that a short incubation with Y27632 is sufficient to optimally enhance migration in DCs.

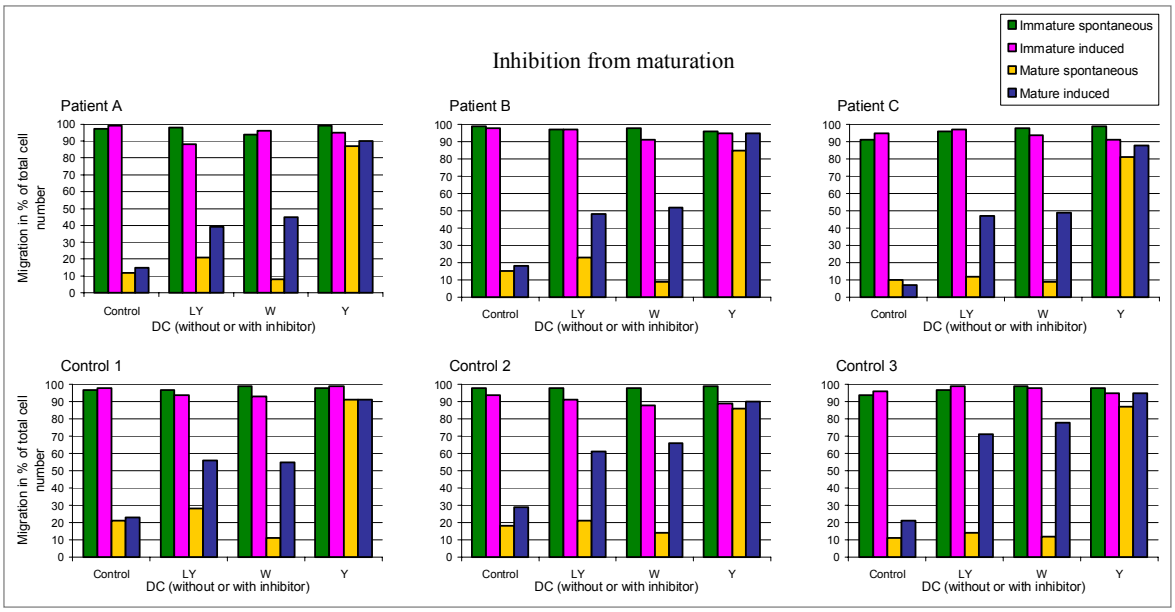

Fig. 24: migratory rate of DCs upon inhibition from maturation.

6.7 Viability assay

In order to finally investigate the role of PI3K and ROCK during DC maturation, we investigated their effects on cell viability as well, using a viability assay based on Annexin-V-FITC detection. The viability of mDCs was decreased to a rate of about one quarter of the total cell

number independent of their source. Cells were cultured in presence of the relative inhibitors from the start of the culture.

6.7.1 Addition of PI3K and ROCK inhibitors from start

The viability of untreated mDCs levelled between approximately 70% and 81% and was markedly reduced if the PI3K inhibitors LY or Wortmannin were added from the very start of the culture. LY-treated mDCs showed a decrease of viability to 50%-60%, Wortmannin treated cells to 42%-55% with one exception where viability was at 60% and even higher than upon LY treatment. Interestingly, ROCK inhibition with Y27632 only marginally reduced the viability of mDCs to 65%-80%, in spite of its high impact on functional and phenotypical properties, especially of mature dendritic cells and the administration of a relatively high dose (40mM), regarding literature. These observations were independent from the source of DCs but showed individual variations.

In conclusion, it has been shown that the inhibition of PI3K reduced the viability of mDCs with both inhibitors although stronger upon Wortmannin, which strengthens the assumption of PI3K as a pivotal player in the survival of mDCs. Y27632 does not alter the viability as much as with PI3K inhibitors and therefore ROCK does not seem to be a key component for DC survival. On the other side, the high level of the apoptosis-related surface marker CD95L in Y27632-treated cells could be important in mediating cell survival, as CD95L is strongly down regulated upon PI3K inhibition, accompanied by increased phagocytosis (Fig. 25).

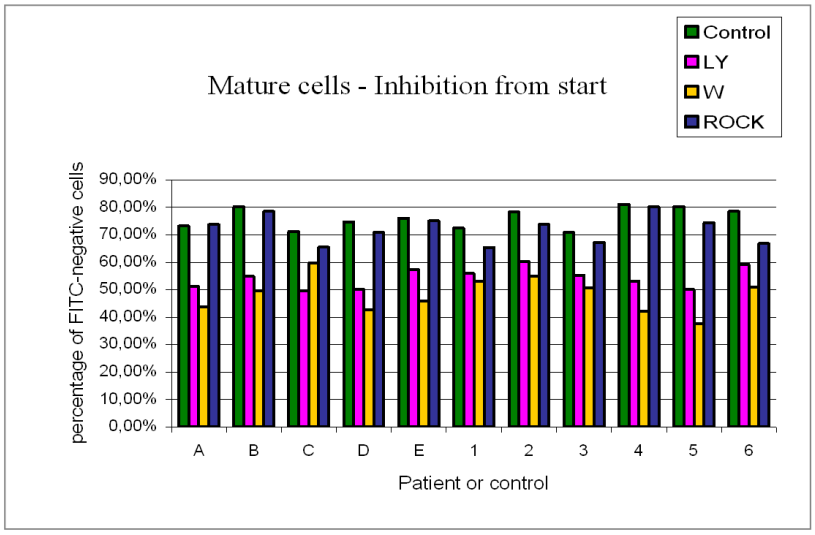

Fig. 25: percentage of viable DCs upon inhibition from start.

6.8 Western blot

Due to the fact that an indirect analysis of the PI3K and ROCK pathways through cell morphology, phenotype and different functionalities showed that they play a crucial role for the final maturation of human DCs and that they may be dependent to different extends on individual characteristics as well as from their source, i.e. healthy people or cancer patients, we investigated the expression of such kinases in untreated as well as inhibited cell cultures of human DCs by WB analysis. The cytoplasmatic fractions of the protein lysates were obtained by Frackelton lysis from mDCs derived from patient A, suffering from colon cancer. These cells were incubated without or with inhibitors from the start of the culture.

6.8.1 Ezrin/Moesin/Radixin

Ezrin and Moesin, members of the ERM protein family, are crucial for linking cell surface and cytosolic proteins to the actin skeleton. One of the most prominent activators of Ezrin is ROCK, whose inhibition is expected to alter expression levels of Ezrin.

Ezrin/Moesin/Radixin

The polyconal rabbit antibody against Ezrin/Moesin/Radixin detects endogenous levels of the ERM proteins Ezrin, Radixin (80kDa) and Moesin (80kDa) and binds to residues surrounding Thr567.

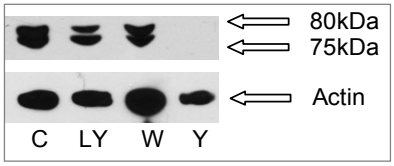

Fig. 26: Western blot of ERM proteins and actin.

Analysis of ERM protein expression levels showed no influence of Wortmannin and only a minor of LY. On the contrary, Y27632 totally impaired the expression of ERM proteins in the cytoplasm, indicating either no expression at all or an altered localization (Fig. 26). This is not surprising as, at least Ezrin, a crucial downstream target of ROCK is.

pEzrin/pMoesin/pRadixin

The presence of phosphorylated ERM proteins is detected with a polyclonal Ab raised against phosphorylation at Thr567 (Ezrin), Thr564 (Radixin) and Thr558 (Moesin) only, which disrupts the amino- and carboxy-terminal association and may alter conformation and function of ERM proteins.

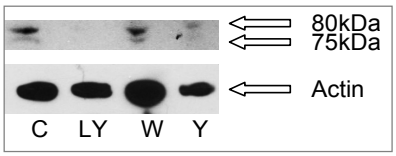

Fig. 27: Western blot of ERM protein phosphorylation and actin.

The phosphorylation of ERM proteins at specific residues significantly decreased upon LY and Y27632 treatment. The effect of Y27632 is not surprising, as total ERM proteins could not be detected (Fig. 27). The contradictory results showed upon PI3K inhibition either with LY or Wortmannin, may reside either in the different effects driven by the 2 inhibitors or in the differences observed in the loading. Further experiments in this direction are required in order to find out the real effects of PI3K upon ERM phosphorylation pathway in human DCs.

6.8.2 p38, MEK and JNK

To assess the impact upon MAPK pathways, which are known to diversely affect cellular processes like proliferation and differentiation, the expression levels of total and phosphorylated p38, MEK and JNK were measured. Further, MEK and JNK pathways have been shown to be modified by PI3K signalling.

p38

The p38 antibody binds to amino acids 213-360 at the C-terminus of p38. It is a polyclonal rabbit antibody.

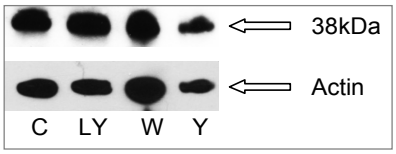

Fig. 28: Western blot of p38 and actin.

The expression of p38 did not seem to be affected by addition of PI3K or ROCK inhibitors (Fig 28).

MEK1/2

Polyclonal MEK1/2 antibody detects endogenous levels of total MEK1/2 protein with a size of 45kDa and was produced in rabbit.

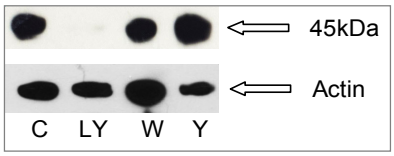

Fig. 29: Western blot of MEK1/2 and actin.

The expression of MEK 1/2 did not seem to be affected by addition of Wortmannin or ROCK inhibitors while upon inhibition with LY, the expression of MEK was abrogated (Fig. 29).

pMEK

The presence of phospho-MEK1/2 (45kDa) was investigated using a polyclonal rabbit antibody raised against the phosphorylation sites at Ser217/221.

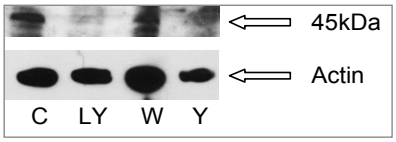

Fig. 30: Western blot of MEK phosphorylation and actin.

The phosphorylation pattern of MEK1/2 did mirror that observed for MEK1/2 expression, suggesting that the only effect observed upon LY addition (Fig. 30), drive the expression pattern of MEK1/2 and not its phosphorylation, suggesting a mechanism involved in long-term expression control, possibly a transcriptional one. Further experiments in this direction are required to determine the real significance of such hypothesis.

JNK

Endogenous level of total JNK was assessed with a polyclonal rabbit antibody detecting isoforms of JNK at the size of 46kDa (JNK1) and 54kDa (JNK2/3).

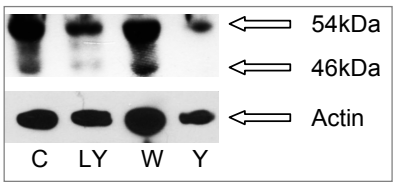

Fig. 31: Western blot of JNK and actin.

Both, LY294002 and Y27632 abrogated the expression of JNK1 in the cytoplasm. JNK 2/3 expression seemed unaffected by any inhibition (Fig. 31). Further experiments are required in order to determine the significance of such findings.

6.8.3 B-Raf and C-Raf

B-Raf and C-Raf are known to be closely linked to the MAPK pathway, of which the MEK and JNK pathway can be modulated by PI3K signalling, and were investigated to assess the effects of selective PI3K and ROCK inhibitors.

B-Raf (C19)

The polyclonal rabbit antibody, binding the C-terminal region, detects endogenous levels of B-Raf at the size of 62kDa and 95kDa.

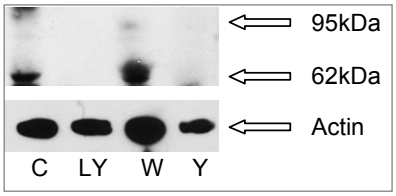

Fig. 32: Western blot of B-Raf (C-19) and actin.

Apparently both LY and ROCK inhibitions abrogated the expression of B-RAF (C-19), while Wortmannin has no effects on the 62kDa form and abrogated the expression of the 95kDa one (Fig. 32). Further experiments are required in order to determine the significance of such findings.

B-Raf (H145)

The B-Raf (H-145) polyclonal rabbit antibody binds to amino acids 12-156 of human B-Raf at the size of 62kDa and 95kDa.

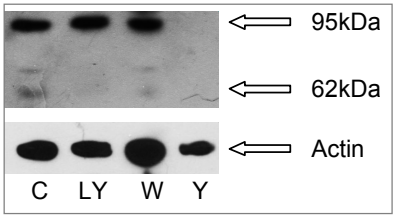

Fig. 33: Western blot of B-Raf (H145) and actin.

Interestingly, only ROCK inhibition impaired B-Raf expression in mature DCs at a size of 62kDa and 95kDa. LY294002 and Wortmannin only reduced the level at 62kDa (Fig. 33), which is contradictory to the results presented by detection with the C-terminal binding antibody and has to be investigated further.

C-Raf

Monoclonal mouse C-Raf antibody detects total C-Raf levels at 74kDa.

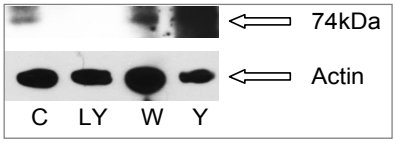

Fig. 34: Western blot of C-Raf and actin.

LY294002 had a high impact upon C-Raf, impairing its expression completely. The same influence could not be detected for Wortmannin or Y27632, although the high background possibly disguised the expression after treatment with the ROCK inhibitor (Fig. 34). Further experiments are required in order to determine the significance of such findings.

7 DISCUSSION

The main goal of this diploma thesis was to investigate the involvement of PI3K and ROCKII pathways in the *in vitro* maturation of dendritic cells, cultured from peripheral-blood monocytes. Additionally, this thesis aimed to show the potential effects of tumor establishment upon DC maturation according to PI3K and ROCKII. For these reasons all experiments were performed on DCs derived from healthy volunteers and from patients suffering from different types of cancer. The preliminary results showed that both PI3K and ROCKII clearly affected different aspects of DC maturation. In the experiments described in this thesis, PI3K kinase activity was inhibited by the use of two distinct and specific chemical compounds: LY294002 and Wortmannin, which affected the PI3K pathway mainly in different ways.

Both the PI3K inhibitors showed similar effects on DC morphology: the addition of either LY294002 or Wortmannin impaired the establishment of the typical mature morphology, since the treated cells remained round and free in the supernatant, unable to develop the typical dendrites and adherence showed by normal, mature DCs. These changes in DC morphology were accompanied by an impaired expression of mature markers on DC surface. The effect on the surface markers expression was different according to the type of inhibitor used, suggesting that the two chemical substances specifically affect different arms of the PI3K pattern. Further statistical significant studies in this direction are required. From the functional point of view, the cells treated with both PI3K inhibitors showed impaired ability to trigger T cell proliferation, suggesting that both PI3K pathways, alternatively impaired by the two inhibitors, are required for an efficient T cell activation.

Nevertheless, surprisingly, secretion of interleukin 10 and 12 appeared to be only slightly influenced by LY and Wortmannin, indicating that PI3K has minor if any effects on this process, although other groups showed that PI3K may affect the regulation of IL-12[52]. These differences might be explained with different ways of culturing the cells and/or cell source.

From our results, it appears that an active PI3K is required by human DCs during the maturation, upstream of the loss of phagocytosis upon loading and maturation. Indeed, cells treated with PI3K inhibitors maintain the capability to phagocyte even after being loaded and matured. On the contrary, induction by CCL19 increased this process. Interestingly, it appears that exposure to the inhibitor from the start of the culture increased the effects. This may indicate that a prolonged exposure is required or that the exposure effects are stronger when applied to immature cells. Further studies are required to answer this question. The same effects

although even stronger were observed regarding migration potentialities, pointing out that PI3K seems to be required by human DCs to acquire the mature, functional characteristics. PI3K inhibition was always accompanied by a decrease in cell viability. Although further studies regarding a defined titration of the inhibitors may be advisable, it appears that PI3K strongly affects DC viability, in accordance with its strong effects on cell metabolism. Western blot analyses indicates that PI3K inhibition through addition of LY294002 but not of Wortmannin affects the protein levels of C-Raf, B-Raf, JNK1, MEK and the ERM proteins, as well as their phosphorylation. These findings require more investigation in this direction to determine the real significance of PI3K inhibition on MAPK and ERM signalling pathways. Although new experiments are required, the preliminary results showed that PI3K is required to trigger the maturation of human DCs and that it acts differently according to morphological, phenotypical and functional processes. PI3K appears to be involved in at least two different pathways, which respond alternatively to PI3K inhibition through LY294002 or Wortmannin.

Only the combination of the two pathways triggers the proper maturation of human DCs, at least in *in vitro* culture conditions.

During this thesis, the role of ROCKII upstream of DC maturation was observed as well by analysing the effects of a selective, chemical inhibition of this pathway through the addition of Y27632 to the cell culture.

Morphological analysis revealed an important function of ROCKII in the regulation of the formation of dendrites as well as of the development of the adherent phenotype, a typical feature of mature DCs.

Although the effect of ROCKII inhibition on phenotype of DCs was not as pronounced as that of PI3K, this feature appears to be as well affected by tumor establishment, since ROCKII inhibition exerted different effects according to the source of the DCs (healthy controls or patients), regarding the expression of mature, surface markers.

Interestingly, ROCKII inhibition very strongly impaired the secretion of IL-12, while powerfully up regulating that of IL-10. This fact points out that ROCKII seems to act principally on cytokine secretion. The analysis of T cell proliferation capability showed that although IL-12 secretion is depleted, mDCs possess a high potential to induce T cells. This is somewhat surprising, as it points to an interleukin 10/12 totally independent mechanism in T cell activation.

ROCKII is also necessary in the regulation of phagocytosis as its depletion leads to a high phagocytosis rate, comparable to immature DCs. This feature may be explained by the known role as regulator of the cytoskeleton, played by ROCKII.

Also an enhanced migratory potential could be observed if ROCKII was inhibited, strengthening its important function in the regulation of the cytoskeleton. Further a short incubation with Y27632 is sufficient to strongly promote migration.

Interestingly, ROCKII inhibition had the lowest impact upon cell death. This is accompanied by a high expression rate of the apoptosis-related surface marker CD95L, possibly mediating cell survival.

Western blotting showed that ROCKII appears to have a positive effect on the levels of ERM proteins, which is not surprising as at least Ezrin is a known downstream target of ROCKII signalling. Interestingly, also JNK1 and B-Raf appear to be influenced by the activity of ROCKII.

In conclusion, these results show an important function of ROCKII in the phenotypical and functional maturation of DCs. Specific characteristics associated with the cytoskeleton are affected by ROCKII signalling. One interesting finding is that Y27632-treated cells produce high amounts of IL-10 and low levels of IL-12 but can still effectively stimulate T cell proliferation and it can be assumed that this fact is due to an interleukin 10/12 independent mechanism. Further, it appears that ROCKII activity is differently modulated in DCs derived from healthy controls in comparison to those derived from patients suffering from different types of cancer. This suggests that the establishment of the tumor may affect specific cell pathways of the APC, possibly in order to diminish their ability to induce the immune response against cancer-specific antigens.

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