The role of MAPK pathways in \textit{in vitro} maturation of dendritic cells, cultured from patients' peripheral blood-monocytes
Erfahrungen vererben sich nicht, 

doch muss sie alleine machen! 

(Kurt Tucholsky)

Widmung
Ich widme diese Diplomarbeit meiner Familie und meinen Freunden, die mich mit Verständnis und aufmunternden Worten stets tatkräftig unterstützt haben.

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This thesis aims to investigate the role of the most known MAPKs, i.e. ERK, JNK and p38 during the transformation of monocytes into dendritic cells (moDCs) and their subsequent maturation process, including antigen presentation in human-derived primary cultures. DCs play a crucial role in the antigen presentation during the immune response against different pathogens as well as in the activation of both CD8\(^+\) and CD4\(^+\) T-cells against cancer. In this particular case, it has been reported that patients suffering from different tumours show a common feature: a decreased number of moDCs, which bear a more immature phenotype. On these bases, we analysed moDCs derived both from healthy controls and cancer patients, in order to evaluate the eventual differences. According to our results and possibly due to the in vitro culture conditions, set to induce a normal maturation pathway in tumour patients’ derived moDCs, the response to the incubation in presence of selective MAPK inhibitors did not show paramount differences between cells derived from the healthy controls or from cancer patients. On the other site, we could show an interesting individual-dependent pattern of effects for each inhibitor, according to at least one of the analysed parameters.

The most striking finding is that all the three MAPKs are required for the differentiation of human DCs from monocytes. Indeed, also the normal pro-proliferative kinase, ERK, in this specific case, is rather required to induce DC maturation, along with JNK and p38. We observed as well that the inhibition of all the analysed kinases caused strong effects on the morphology of the “mature cells”, which were not able to become adherent and kept their “immature” phenotype, i.e. round shape and loss of adherence. Such morphology appeared to be the most relevant cause of the impaired functionality of these cells, although not the only one. Indeed, ERK, JNK and p38 seem to work through positive feedback loops in order to induce and maintain human DC differentiation, in both phenotype and functionality. Further studies are required in order to investigate in details the mechanisms involved.
Zusammenfassung


Andererseits konnten wir ein interessantes, personenabhängiges Muster von Effekten für jeden Inhibitor bei wenigstens einem der analysierten Parameter zeigen.


Weitere Studien sind erforderlich, um die involvierten Mechanismen im Detail zu untersuchen.
Introduction

1. Dendritic cells – the sentinels of the immune system

Dendritic cells (DCs) are the sentinels of the immune system, due to their function as potent antigen-presenting cells.

1.1 Types and subsets of dendritic cells in humans

DCs derive from hematopoietic stem cells and they can originate from both lymphoid and myeloid lineages. In humans, myeloid lineage DCs are considered as the “classical” DC. These cells originate from myeloid committed CD34+ progenitor cells. When this type of DC matures, it is known as an interstitial DC. In addition to be able to activate naïve CD4+ and CD8+ T-cells, interstitial DCs can induce differentiation of naïve B cells to antibody secreting plasma cells. Interstitial DCs are assumed to migrate to the lymphoid follicles and become follicular DCs. These DCs are activated by lipopolysaccharides (LPS) over toll-like-receptor 4 (TLR4) during inflammation and infection. IL-4, produced in this inflammatory process, leads in combination with GM-CSF, to trans-differentiation of monocytes (mos) into MoDCs. Beside their function in activating cytotoxic T-lymphocyte (CTL) responses, they are also capable of stimulating CD4+ helper T-cells to prime memory CD8+ T-cell responses in their mature state. Additionally, immature MoDCs can induce tolerance and are therefore important in avoidance of autoimmune-diseases. A less considered subtype of myeloid DCs are cord blood derived DCs, which can be compared to MoDCs, but are stronger inducers of tolerance and do not have the ability to mature.

Myeloid committed CD34+ cells, which are CD14-negative can mature to what is called a Langerhans DC, in the presence of TGF-β. Langerhans DC are also capable of activating naïve T-cells, but not B-cells. Langerhans DC capture antigens, migrate to lymphoid tissues and present antigen to T-cells. A further subtype of myeloid DCs is constituted by the interferon-producing killer DCs (IKDC). They are efficient in cross-presentation of antigens on MHC I and are able to induce Th1 helper T-cells to produce interferon gamma (IFN-γ).

The lymphoid DCs constitute to the DC subset that originates from CD34+ cells committed to the lymphoid lineage, are CD11c- and are driven to become DCs by IL-3. These DCs are referred to as plasmacytoid DCs. They have the capacity to produce IFN-α and reside in the T-cell compartment of lymphoid tissues. Plasmacytoid DCs received their name from
morphological similarities to antibody-producing plasma cells. Maturation of plasmacytoid DCs is induced by CpG and as a result, express Fc-Receptor, toll-like-receptor 7 (TLR7) and toll-like-receptor 9 (TLR9) through the activation of which, they produce type I interferons (interferon α; IFN-α)⁴.

1.2 Functions and properties of dendritic cells

Immature DCs (iDCs) are localized in peripheral tissues and are activated by stress events, e.g. ultraviolet irradiation, tissue damage or different kinds of pathogens. Furthermore, DCs can also be activated by tumour cells, which in turn are often invisible for the immune system either because of mimicking a physiological expression of surface markers or their production of molecules, such as interleukins (e.g. IL-10) which inhibit the maturation or the function of DCs.

In their immature state, DCs can take up particles and microbes via phagocytosis, but they can also form pinocytic vesicles and take up solutes and extracellular fluid via macropinocytosis or express receptors that mediate adsorptive endocytosis. After antigen-uptake and followed maturation, DCs cannot take up other antigens anymore, due to the downregulation of phagocytic receptors. Because of the capture of the antigen in the peripheral tissues, the iDCs are activated, undergo maturation and become highly motile. In consequence, semi-mature DCs migrate toward the draining lymph nodes for presenting the antigen to T-cells⁵. This process is shown graphically in Fig. 1: T-cells (in blue) and B-cells (in pink) from the blood stream move to their distinct areas in the lymph node. Activated DCs (orange stellate profiles) migrate to the T-cell area of the lymph node⁶,⁷ where they cause activation of T-helper cells and cytotoxic T-cells.

Fig. 1: Migration of dendritic cells into the draining lymph nodes (from: Nature reviews 449: 419-426; 2007)
Maturation of DCs is characterized by complex changes in phenotype and function, which are important for their activity in the innate immunity. The most obvious change in the phenotype is the morphological alteration: mature DCs (mDCs) possess branch-shape protrusion, as the name “dendritic (ancient greek for “tree”) cell” indicates. These dendrites are favourable for the cell-cell interactions, because due to the enlargement of the cell-surface a huge amount of cells can efficiently be activated from one DC at the same time. Beside the shape of the cell, also the expression pattern of the surface markers is changed. In iDCs only low expression of MHC molecules (HLA-DR), adhesion and co-stimulatory molecules (CD40, CD80, CD83, CXCR4, CCR4, CCR5) are found, whereas in mDCs these markers are expressed at a higher rate. Also the secretion of cytokines such as IL-10 or IL-12p70 is different according to the maturation stage; when DCs are fully mature the release of IL-10, which keeps DCs in their immature state is down-regulated, whereas IL-12p70, an important molecule for the development of T\textsubscript{H}1 type T-cell responses\textsuperscript{8,9} is up-regulated. Beside the inhibition of DC-maturation, IL-10 is also responsible for the activation of regulatory T-cells, which in turn mediate immunosuppression\textsuperscript{10}.

### 1.3 A three step mechanism for T-cell activation

To activate CD4\textsuperscript{+} as well as CD8\textsuperscript{+} T-cells, DCs have to present the antigen via MHC class I or class II receptor. This interaction is the first, preliminary contact between T-cells and DCs. As this signal is not enough to induce potent T-cell activation, another interaction has to be built. Therefore the receptors CD80 and CD86 on DCs can bind to CD28 on T-cells. If this second interaction is missing (e.g. if they are immature), DCs induce tolerance. The third bridge between DCs and T-cells is formed by CD40 on DCs and CD40L on CD4\textsuperscript{+} T-cells, which in turn can activate CD8\textsuperscript{+} T-cells. The interaction between CD40 and CD40L leads to IL-12 production of the DC, which triggers the T\textsubscript{H}1 type T-cell responses. It is important that this CD40 signalling is only of transient nature, because a persistent interaction would lead to a blocked migration of the DC.

### 1.4 Dendritic cells and cancer immunotherapy

#### 1.4.1 Work flow of in vitro culture of DCs for immunotherapy

DCs are crucial for orchestrating the immune activation against the tumour upon endocytosis of immunogenic portion of tumour protein (peptides). Such peptides are internalized, processed and subsequently expressed over MHC receptors, leading to activation of tumour-specific CTLs, which in turn attack the tumour. In cancer patients, this tumour recognition process through DCs is compromised. Indeed, cancer patients present less DCs, which are
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kept to an immature state by the tumour-induced immunosuppressive environment\textsuperscript{11,12}. An approach to stimulate DC activation through maturation in cancer patient is based on the \textit{in vitro} culture, stimulation, maturation and reinjection of autologous DCs, derived from patients’ monocytes. This process is graphically shown in Fig. 2. \textit{In vitro} cultured DCs have been shown to be resistant to immunosuppressive factors derived from the tumour and to be phenotypically and functionally stable in the absence of cytokines\textsuperscript{13,14}.

MoDCs can be generated by culture of peripheral blood mononuclear cells (PBMNCs), in presence of GM-CSF and IL-4\textsuperscript{15,16}. PBMNCs also contain CD34\textsuperscript{+} stem cells and CD14\textsuperscript{+} monocyte precursor cells, which give rise to DCs when cultured with these chemokines. With this method large quantities of DCs can be generated \textit{in vitro}. There are some important points, which have to be considered in \textit{in vitro} preparation of DCs. The application of mature DCs is crucial. There is clinical evidence, that administration of iDCs activate regulatory T-cells, leading to an undesired immunosuppression, instead of immune activation\textsuperscript{17}. Maturation is induced by exposure of iDCs to pro-inflammatory mediators, such as IL-1\textbeta, agonists of co-stimulatory molecules like CD40L, the chemokine CCL16 or TLR agonists, like LPS. In \textit{in vitro} culture of DCs, it is essential to use endotoxin-free materials, because even transient exposure of DC precursor monocytes to endotoxin impairs subsequent IL-12 production\textsuperscript{18}. Fully mature DCs, which lack IL-12 are unable to induce the favourable CD8\textsuperscript{+} T-cell response.
1.4.2 Loading strategies for DCs

For the *in vitro* preparation of DCs, the choice of the antigen to be used for the loading step as well as the choice of the loading method is of paramount importance. Indeed, the antigen choice is upstream of the specificity and the sensitivity of the immune-response activation through DCs, while the loading method, mRNA or proteic antigen, directs the immune response towards the short-term (CTL) or the long-lasting (T-memory and T\textsubscript{H}) response, respectively. The ideal antigen would be expressed exclusively by the tumour and entirely not by normal cells, in order to avoid side-effects due to the unspecificity of the immune response. Up to date, several approaches are used for the DC-loading, including pulsing DCs with peptides or protein, applying lysates of the whole tumour or using a genetic approach, where DCs are transfected with DNA or mRNA.

Using synthetic peptides for the DC-loading has the great advantage, that they can be produced synthetically, are therefore GMP-grade and there is no patient-derived tumour tissue needed, which quantity is often limited. Unfortunately, the use of synthetic peptides has some limitations: first, the peptides underlie MHC-restriction, which should be identified by MHC typing, next, the antigens of the tumour have to be known, to design an antigen peptide and at last, there is a lack of CD4\textsuperscript{+} helper T-cell activation\textsuperscript{19}.

Another method is the pulsing of DCs using tumour lysates, which contain all proteins of the tumour. Here the drawbacks are that there is a very high amount of tumour tissue needed and further, the lysates contain also peptides expressed from normal tissue, leading to immunoreactions against all cells\textsuperscript{20}.

Genetic approaches seems to be very promising, where either DNA or mRNA are presented to DCs using an active or passive transfection. One advantage is that the genetic constructs can easily be cloned and the genetic information of different antigens can be transferred to the DCs in one step, leading to a broader immune-response. Another advantage is that a passive transfection, where DNA or mRNA is just added as naked strands to the DCs, is very effortless. Until now, the loading with DNA was not very successful and due to the property to integrate into the host genome, loading of DNA is not very safe\textsuperscript{21}. The use of mRNA can circumvent the safety problem, because it cannot integrate into the host genome, due to its transient expression, and it has also a higher efficiency in triggering the immune-response. Another important question about loading mRNA is the source of the nucleic acids: they can
be derived either from the patient himself, using PCR-amplified whole tumour mRNA or can be in vitro amplificated from mRNA of special genes, which are present in the tumour, but not in the normal tissue. There are only very few genes known, which fulfil these criteria, among these are carcinoembryonic antigen (CEA), telomerase reverse transcriptase (TERT) or survivin\textsuperscript{22,23,24}. Due to the endogenous expression of the antigen, the peptides are expressed over the MHC class I. Immunization with mRNA-loaded DCs will activate CD8\textsuperscript{+} T-cells, but lacks CD4\textsuperscript{+} T-cell stimulation.

1.4.3 Loading of DCs with Survivin-mRNA

In the present diploma thesis, survivin mRNA was used for the loading of DCs because it is known to be overexpressed in common human cancers but absent in normal tissue. Survivin is also known as apoptosis inhibitor 4 (API4), due to its property to inhibit the programmed cell death and was discovered by Ambrosini et al in 1997 in a hybridization screening\textsuperscript{25}. The inhibitor of apoptosis (IAP) encodes a protein with a size of 142 amino acids, which lacks a signal peptide and has no hydrophobic domain for membrane insertion. Therefore, it is not expressed on the cell surface. In healthy, adult tissue there is only a rare expression in thymus and placenta but it is absent in all other tissues. In the foetus, survivin is expressed in liver, kidney and to a lesser extent in fetal lung and brain at least until week 21\textsuperscript{26}.

Survivin is expressed in the G2/M phase of the cell cycle, where it is connected to the microtubules of the mitotic spindle and regulated by microtubule dynamics. In case of distorting this microtubule-survivin interaction, the antiapoptotic function of survivin is lost and leads to activation of caspase-3, which in turn induces cell death during mitosis. It can be suggested, that survivin is important for blocking of default apoptosis in G2/M phase and that the overexpression in cancer may also inhibit indispensable apoptosis, leading to transformation of cells\textsuperscript{27}. Tumour suppressor p53 is important for the repression of survivin mRNA and protein and therefore has an important function in blocking of tumourigenesis\textsuperscript{28}.

The role of survivin in progression of tumour disease and in resistance to therapy was already shown. In diffuse large B-cell lymphomas and in soft tissue sarcoma, expression of survivin correlates with an unfavourable prognosis\textsuperscript{29,30}. 
2 Mitogen-activated protein kinases (MAPKs)

2.1 Involvement of MAPK-pathways in the maturation of human dendritic cells (DCs)

It has already been shown that the maturation of human monocyte-derived DCs (MoDCs) is induced by lipopolysaccharides (LPS), tumour necrosis factor α (TNFα) or interleukin-1β (IL-1β), which are signals that can also lead to activation of members of the MAPK-pathways\textsuperscript{31,32}. The role of MAPK-pathways in DC maturation is also demonstrated in a study where dose-dependent ultraviolet-B radiation mediates either maturation or apoptosis through p38-signalling in human MoDCs \textit{in vitro}\textsuperscript{33}.

2.2 A three-modular system involved in various biological responses

Mitogen-activated protein kinases (MAPKs) are main regulators of the cellular metabolism. Their function in multicellular organisms is important for stress response, apoptosis, proliferation, development and differentiation of cells. MAPKs are highly conserved serine/threonine kinases that can be found in eukaryotic organisms from yeast to human, such as \textit{Caenorhabditis elegans}, \textit{Drosophila melanogaster} and \textit{Xenopus laevis} or \textit{tobacco} or \textit{arabidopsis thaliana}\textsuperscript{34}.

In mammals three subfamilies of MAPKs were discovered. All of them are acting as three-modular systems. A MAPK kinase kinase (MAPKKK), which is activated by membrane associated events will lead to the activation of a MAPK kinase (MAPKK). Extracellular signals will lead to activation of one or more of the three different MAPK-pathways, depending on the nature of the signal from the outside of the cell. The MAPKKK has the ability to phosphorylate serine and threonine on the MAPKK. The MAPKK in turn can recognize a Thr-X-Tyr motif in the activation loop of a MAPK\textsuperscript{35}. Due to their property to phosphorylate threonine as well as tyrosine, they are called dual-specificity kinases. Finally, the MAPK phosphorylates a number of substrates, which are either transcription factors (TFs), phospholipases, cytoskeleton-associated proteins or other protein kinases leading to additional influences of other biological pathways.

Currently, the MAPK-pathways are supposed to act independently and it has been suggested that this independence is important to differentiate and control the three pathways. Furthermore, it is well known that there are connections between the pathways and that a
particular orchestration of activatory or inhibitory signals is important for the determination of cellular events. One example is the MAPKKK MEKK, which is activated by Rac and Cdc42, leading to a turn-on of the JNK-pathway, but which can be activated by Ras association too, and therefore stimulates both, the JNK- as well as the ERK-pathway\textsuperscript{36}.

![Schematic diagram of the three mammalian mitogen-activated protein kinase (MAPK) signalling pathways](From: Nature immunology reviews 7: 202-212; 2007)

2.2.1 Extracellular signal-regulated kinase pathway (ERK-pathway)

The first step in the stimulation of the ERK-pathway is the activation of the MAPKKK Raf-1 by Ras. Ras has in its active form a GTP in its binding pocket and is therefore able to bind to the NH\textsubscript{2}-terminal regulatory domain of Raf-1, which in turn will be bound to the plasma membrane and becomes activated\textsuperscript{37,38,39}. Therefore, the tyrosines 340 and 341 are phosphorylated by membrane-bound tyrosine kinases\textsuperscript{40}. B-Raf and A-Raf are homologs of Raf-1, which can be activated alternatively by Ras and result in the activation of the ERK-pathway. B-Raf is activated by oncogenic Ras and does not need any other stimuli, whereas
A-Raf and Raf-1 need other signals to be strongly activated. These inputs can come from other kinases upstream of Raf-1, like Src or PKC or other tyrosine kinases\textsuperscript{41}. Phosphorylation of Raf does not always lead to activation of the ERK-pathway. Indeed, phosphorylation of Raf-1 by its own downstream target ERK or cAMP-dependent protein kinase (PKC) leads to inhibition of Raf-1 activity\textsuperscript{42,43}.

The intermediate signal transducers of the ERK-pathway are two MAPKK, called MEK1 and MEK2. Both have similar amino acid sequences and can be activated by Raf-1 phosphorylation. MEK1/2 act as dual specificity kinases, phosphorylating threonine and tyrosine of the MAPK ERK1/2.

The active MAPK ERK1/2 itself can phosphorylate the serine or threonine amino acids from cytoplasmic substrates such as S6 kinase p90\textsuperscript{sk}, phospholipase A2, epidermal growth factor receptor (EGF-R) or microtubule-associated proteins (MAPs). ERK1/2 can translocate into the nucleus in its phosphorylated form and can activate a variety of TFs, again by phosphorylation. The most important TFs activated by ERK1/2 are Elk1, Ets1, c-Myc, STAT, SRF and TCF. The majority of the activated proteins and TFs correlate with cell growth and maturation, therefore it has been suggested that the ERK-pathway is responsible for maintenance and differentiation of cells.

2.2.2 c-Jun N-terminal kinase pathway (JNK-pathway)

JNK-pathway is mainly activated by cellular stress. Therefore, the MAPK JNK received the synonym stress-activated protein kinase (SAPK). The MAPKKK MEKK is activated by several stress events, such as heat shock, DNA-damage, oxidation events or hyperosmolarity. There is evidence, that the full length MEKK, which has a size of 196 kDa induces survival responses, whereas a 91kDa cleavage kinase fragment of MEKK serves as substrate for caspase-3 and therefore induces apoptosis\textsuperscript{44}.

Beside the stress events, the MAPKKK MEKK is also turned on by Rac and Cdc42, which are two members of the Rho family, both linked to cell differentiation and proliferation. MEKK itself phosphorylates MKK4 and MKK7. The active MKK4 and MKK7 are again dual-specificity kinases like MEK1/2 in the ERK-pathway and phosphorylate the threonine and tyrosine of the MAPK JNK. There are ten genes known encoding for JNK, leading to three splice variants named as JNK1 and JNK2 which are ubiquitously expressed and JNK3,
which expression is restricted to brain, heart and testis. The activated JNK leads to activation of SRF, TCF, ATF2, c-Jun, p53, Elk1 as well as to the homodimerization and activation of AP1.

The effects of the JNK-pathway are versatile: they can lead to growth, differentiation and survival of cells, but can as well drive cells into programmed cell death as a response to cellular stress. The important function of JNK-pathway in apoptosis was shown by a study inducing apoptosis in response to chemotherapeutic drugs. For the survival of the cells the DNA-repair function is of great importance. Studies with JNK-inhibitors in cells treated with DNA-damaging drugs showed that the repair function is altered when the JNK-pathway is switched off.

**2.2.3 p38-pathway**

In mammals four different p38 enzymes are known, p38α, p38β, p38γ and p38δ. These isoforms show an identity of about 60% within their sequence and an identity to other MAPK family members of about 40-45%. Like in JNK/SAPK signalling, p38-pathway can be activated by cellular stress events, such as UV-irradiation, osmotic shock, heat shock, lipopolysaccharides (LPS) or protein synthesis inhibitors and it is as well turned on by cytokines like IL-1 and TNF-α as well as G-protein coupled receptors (GPCR). Not only stress events and stress-related signals lead to activation of p38-pathway, but as well the constitutively active forms of Cdc42 and Rac may increase the activity of this MAPK-pathway.

Due to these activation events, the MAPKKKs TAK1, ASK1 or MLK3 are turned on and the MAPKKKs phosphorylate the MAPKKs M KK3 and M KK6 as well as M KK4 in few specific cell types. All of them are leading to induction of p38α and p38δ activation and in case of M KK4, the activation of the JNK-pathway takes place too.

M KK3 and M KK6 are dual specificity kinases like all of the MAPKK and therefore phosphorylate p38 on their threonine and tyrosine residues. The activated MAPK p38 itself phosphorylates a broad range of substrates, including cytoplasmic phospholipase A2, EGFR and Bcl-2. Beside these proteins, also a variety of TFs is activated by phosho-p38, the most important ones are ATF2, Elk1, TCF and Max. The TF Max can bind to the truncated
isoform of p38α, which phosphorylates Max, leading to heterodimerization with c-Myc, a substrate of the ERK-pathway

The activation of the p38-pathway leads to proliferation, development, differentiation, immune responses as well as apoptosis.

2.3 Inactivation of the MAPK-pathways in vivo and in vitro

Considering the huge influences of the MAPK-pathways in the cellular metabolism, it appears clear, that in contrast to the activation, a negative regulation on the molecular level of the pathways is essential. Among the desensitization of the receptors, dissociation of signalling complexes from receptors and deactivation of pathway mediators, the most efficient inactivation is the dephosphorylation of the kinases by MAPK phosphatases.

Dephosphorylation does not only lead to an inactivation of the MAPK-pathways in vivo; indeed, blocking of phosphorylation is also a very useful tool in vitro to investigate the role of MAPK-pathways in ex-vivo cultured cell-lines or primary cells, such as monocyte-derived DCs. For each of the three MAPK-pathways selective inhibitors are available. For the present thesis, four different synthetic inhibitors are used, two against the ERK-pathway, one for inhibition of JNK-pathway and one for the p38-pathway.

Inhibitors against ERK-signalling:

For the inhibition of the ERK-signalling pathway, two synthetic agents are used: PD 98059 and U 0126.

- PD 98059 is a potent, selective and cell-permeable inhibitor of MEK1/2. It inhibits the phosphorylation of the MAPK ERK by MAPKKs MEK1/2 but does not inhibit the MAPK itself. The chemical structure of the inhibitor is shown in Fig. 4.
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- The inhibitor U 0126 (Fig. 5) inhibits the MAPKKs MEK1/2 with a 100-fold higher potential than PD 98059. Beside the inhibition of MEK1/2 it is also a weak inhibitor of PKC, Raf, ERK, JNK, MEKK, MKK-3, MKK-4/SEK, MKK-6, Abl, Cdk2 and Cdk4.

![Chemical structure of the MEK1/2 inhibitor U 0126](image1)

**Fig. 5:** Chemical structure of the MEK1/2 inhibitor U 0126

**Inhibitors against JNK-signalling:**

- To reveal the function of JNK-signalling in maturation of DCs, the inhibitor SP 600125 is used. It blocks selectively the phosphorylation of JNK by MAPKKs MKK4/7. The chemical structure is given in Fig. 6.

![Chemical structure of the JNK inhibitor SP 600125](image2)

**Fig. 6:** Chemical structure of the JNK inhibitor SP 600125

**Inhibitors against p38-signalling:**

- For the investigations of the third MAPK-pathway, a potent, cell-permeable, selective inhibitor against the phosphorylation of p38 is used. Fig. 7 shows the chemical structure of the synthetic inhibitor PD 169316.

![Chemical structure of the p38 inhibitor PD 169316](image3)

**Fig. 7:** Chemical structure of the p38 inhibitor PD 169316
Structure of the project

In the following scheme, an overview of the project structure is shown. After the isolation, the PBMNCs derived from the healthy controls or the patients suffering from cancer are taken into culture in a special growth medium and after a selective enrichment of monocytes, the trans-differentiation of these cells into iDCs is performed. The next step is the loading of the iDCs with Survivin mRNA. Finally, the loaded iDCs are fully matured into mDCs. Afterwards the mDCs are morphologically, phenotypically and functionally characterized by several assays, along with iDCs.

In order to determine the exact time-points during DC maturation at which the analysed MAPK pathway play the most crucial roles and in order to investigate the effects of their relative inhibitors according to the time-point as well as to the duration of the administration, selective inhibitors of the ERK, JNK and p38 pathway were added to:

A) monocyte cultures,
B) iDCs cultured for 5 days,
C) iDCs cultured for 5 days and loaded with survivin.

The effects of the inhibitor addition were always compared with untreated cells cultured under the same conditions (D).

![Fig. 8: Structure of the project (black arrows A, B, C show points of inhibition; blue arrow D demonstrates untreated portion of cells)](image-url)
Material and Methods

1 Culture of dendritic cells

1.1 Monocyte enrichment

Peripheral blood mononuclear cells (PBMNCs) from patients suffering from cancer are collected through leukapheresis in an associated and certified center. The apheresate is transported in appropriate temperature-controlled boxes between 8-20°C to CELL MED, where it is eventually processed in order to obtain an enriched phase of monocytes (mos) with minimal contaminations by lymphocytes, erythrocytes and platelets.

Peripheral blood from healthy volunteers is collected directly at CELL MED by vein puncture, using Heparin-Vacutainer tubes and processed in the same way as the patients’ material, in order to enrich the mos phase.

Shortly, the leukapheresate/whole blood is diluted 6X and 2.5X, respectively, in ACD solution (acid citrate dextrose; Baxter) and mixed with Ficoll (Biochrome) in a final ratio of 1:1.5 before being centrifuged for 20 min at 2,000 rpm (break 0) at RT. Afterwards the interface is collected and centrifuged twice for 10 min at 1,200 rpm (break 9) at RT. Finally, the pellets are resuspended in plain medium for 2h at a final concentration of $2 \times 10^6$ mos/mL, after counting the number of mos with an automatical cell counter (CASY-1®, Schärfe System GmbH). Thereafter the non-adherent cells are washed out and the adherent ones are cultured as described in the following paragraph. The purity of the mos phase is measured by FACS analysis of CD14 and CD45. The eventual contaminations are evaluated by analysing the expression of CD3, CD16, CD19, CD41 and Glycophorine A (GPA).

1.2 Primary cell culture

To induce the switch from mos to immature dendritic cells (iDCs), the cells are cultured in CellGro DC medium (CellGenix), added up with 2,500U/mL GM-CSF (CellGenix) and 1,000U/mL IL-4 (R&D), in a concentration of $2 \times 10^6$ mos/mL, at 37°C and 5% CO₂ for 5 days. Thereafter the iDCs are loaded with human Survivin mRNA in a concentration of 150 ng/1*10⁷ cells for 2h. Finally maturation of the DCs is induced by culturing them in presence of 100µg/mL Ribomunyl (Pierre Fabre) and 1,000U/mL INF-γ (R&D) for 24h at 37°C and 5% CO₂.
Since a hallmark of mature dendritic cells (mDCs) is their switch from non-adherent to adherent phenotype, Accutase is used to dislodge the mDCs from the plastic dish after washing out the non-adherent cells, eventually present in the supernatant. Shortly, the mDCs are incubated with Accutase (PAA Laboratories) for 5 min at 37°C. The cells are washed at 1,200 rpm for 5 min at RT, resuspended in RPMI (Gibco) containing 2%FBS (Gibco) and used for further experiments. Alternatively the cells are frozen in Glycerol sterile solution (Mayrhofer).

2 Chemical inhibition

The following chemical inhibitors are used:
- MEK inhibitor (PD98059; LC), 20µM
- MEK inhibitor (U0126; LC), 40µM
- p38 inhibitor (PD169316; Sigma), 2.5µM
- JNK inhibitor (SP600125; Sigma), 5µM

The inhibitors are applied either from the start of the culture, from the loading step or from the maturation step and when needed, are replaced every second day.

3 Survivin mRNA preparation

3.1 Cloning of Survivin c-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 (see Fig. 9) is added. The reaction is mixed gently and incubated for 5 min at RT. To achieve a higher number of colonies, the incubation time can be extended from 30 sec to 3 min. After the incubation, the reaction is placed on ice until transformation into one shot competent E.Coli (Invitrogen) is performed.
Material and Methods

Transformation of one shot competent E.Coli

For the transformation, E.Coli bacteria are thawed on ice for 3 min. Then 2µL of the TOPO TA cloning reaction are added and incubated for 5 to 30 min. Next a heat shock for 30 sec at 42°C is performed and 250µL of SOC medium (Invitrogen) at RT are added. The transformed cells are incubated for 1h 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 18h 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 Biomedical)
15g Agar agar (Carl Roth GmbH)
The final volume is adjusted to 1L with water. The solution is autoclaved for 15-20 min and cooled down to 50°C. Finally it is added up with 100mg Ampicillin (Sigma).

LB liquid media
10g/L Tryptone (MP Biomedical)
5g/L Yeast Extract (MP Biomedical)
10g/L NaCl (Merck)
Finally, the pH is adjusted to 7.0 with 1N NaOH and then the solution is autoclaved for 15-20 min.
3.2 Plasmid isolation using Qiagen® plasmid isolation kit

1.5 mL of the cell suspension are transferred to a 2 mL tube and centrifuged for 5 min 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 solved in buffer P1. Each pellet is completely resuspended in 300 µL of buffer P1. This lysing step is the crucial step in the procedure. Afterwards 300 µL lysis buffer P2 are added and mixed by inverting the tube several times followed by an incubation step for 5 min at RT. This step is required to grant the degradation of genomic DNA of bacterial origin.

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

In the meantime, the Qiagen tips are equilibrated. Shortly, 1 mL of the equilibration buffer QBT is loaded on each column and let it completely flow through and dry 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 instead been bound by the resin, is washed twice with 1 mL of QC buffer.

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

3.3 Transcription of Survivin from a Miniprep-sample

pCMV-Survivin linearization from Miniprep

5µg plasmid are 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 2h at 37°C. Thereafter 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 ethanol (100%; Merck). The reaction is incubated for 15 min at -20°C and afterwards centrifuged for 15 min at top speed. Next 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 a final concentration of 200µg/mL to the linearized cDNA. The sample is thereafter incubated 30 min at 50°C.

Phenol/Chloroform extraction

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

To precipitate the DNA, 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. The reagents are mixed to the sample and incubated for 15-20 min 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 min at top speed. The supernatant is removed with a fine-tipped pipette and 10µL water are added to the air-dried pellet. The concentration is determined by using a photometer (Peqlab).
Material and Methods

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 of 10x reaction buffer, 2µL enzyme mix and 1µg linearized c-DNA 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 2h at 37°C. Afterwards 1µL TURBO DNase is added, mixed well and incubated for 15 min at 37°C.

Cleaning and recovery of RNA
Contents of the MEGAclear kit (Ambion) are brought 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. Next 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 min at 10,000xg. 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 are applied to the center of the filter, the cap is closed and the tube is incubated for 5 to 10 min at 65°C. The RNA is recovered by centrifugation for 1 min at RT. To maximize the RNA recovery the elution procedure is repeated with 50µL water. The RNA yield is determined by using a photometer and the quality is checked by real-time RT-PCR.

RT-PCR
First the RNA is transcribed into cDNA with superscript. Shortly 100ng random hexamers (1,250ng; Roche), 0.8mM dNTP Mix (10mM each dNTP; Amersham) are mixed with 1µg RNA and filled up to a volume of 12µL with water. The reaction is heated up to 65°C for 5 min and afterwards quick chilled on ice and spinned down. Thereafter 7µL of the master mix consisting of 4µL first-strand buffer (5x; Invitrogen), 2µL DTT (0.1M; Invitrogen) and 1µL Rnase inhibitor (40U/µL; Promega) are added. The reagents are incubated for 2 min at 25°C and then 1µL of SuperScript™ II reverse transcriptase (200U/µL; Invitrogen) is added and mixed by pipetting up and down. The mixture is incubated for 50 min at 42°C, followed by a heating step for 15 min at 70°C. The cDNA is diluted 1:5 with water and stored at -80°C until further use.
Material and Methods

For the RT-PCR the following intron-spanding primers are used. The Primer design has been done by “Primer3” free software.

**hSurvivin primers:**

- forward: 5’ – CTG GAC AGA GAA AGA GCC AAG – 3’
- reverse: 5’ – AGG GAA TAA ACC CTG GAA GTG – 3’

The master mix used in this reaction contains 2.5µL PCR buffer (10x; Invitrogen), 1.5mM MgCl₂ (50mM; Invitrogen), 0.8mM dNTP mixture (10mM each dNTP; Amersham), 0.3µM each primer (10µM each), 1U *Taq* DNA Polymerase (5U/µl; Invitrogen) filled up to 22.5µL with water. 2.5µL of the diluted cDNA or, in case of the negative control, 2.5µL water are added to each aliquot of the master mix and the PCR reaction is performed in a cycler (MJ Research).

The following conditions are used:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary denaturation</td>
<td>94°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

The PCR products are separated on a 2% agarose gel (Biozym), containing 10µg/mL Ethidium bromide (10mg/mL; Sigma), run for 45 min at 90 volt. As running buffer 1xTAE buffer is used. Each sample is mixed with 4µL loading dye (6x; Fermentas) and 7µL are loaded on the gel. As length marker, 2µg of a 100bp DNA ladder are used (0.5µg/µL; Fermentas). A picture of the gel is taken with a gel documentary system (Biozym).

**4 Analysis of the cells**

**4.1 Morphology**

Before the cells are harvested after maturation, pictures are taken from the cultures with the help of an inverted light microscope (Axiovert, Zeiss), combined with a photo-camera (AxioCam, Zeiss) and relative software (Axiovision, Zeiss). Pictures are taken using a 40x objective and an exposure time of 20 sec.
Material and Methods

4.2 Phenotype (FACS)

For each staining 1*10^6 cell/mL are resuspended in 50µL staining buffer (1x PBS supplemented with 1% BSA and 0.1% NaN₃). Each aliquot of cells is incubated for 15 min at RT with the appropriate amount of FACS antibody according to Table 1, Table 2 and Table 3. After incubation, the cells are washed with 1.5mL of PBS at 800rpm at RT, resuspended in 0.5mL staining buffer and further measured with a FACS device (Beckmann Coulter).

To determine the concentration of mos after enriching this phase by Ficoll, the resultant cell suspension is analysed by FACS for the following CD markers:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorescence dye</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>FITC</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>CD45</td>
<td>PC7</td>
<td>Beckmann Coulter</td>
</tr>
</tbody>
</table>

Table 1: monitoring antibodies

To determine the levels of eventual contaminations by leukocytes, erythrocytes, granulocytes and platelets, still present after Ficoll, the cell suspension is analysed by FACS for the following CD markers:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorescence dye</th>
<th>Expressed on</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>PC5</td>
<td>T-cells</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>CD16</td>
<td>PC5</td>
<td>Granulocytes</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>CD19</td>
<td>PE</td>
<td>B-cells</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>CD41</td>
<td>FITC</td>
<td>Platelets</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>Glycophorine A</td>
<td>PE</td>
<td>Erythrocytes</td>
<td>Beckmann Coulter</td>
</tr>
</tbody>
</table>

Table 2: antibodies for contamination control

The stage of DC maturation is monitored after 5 days culture in presence of IL-4 and GM-CSF and after inducing their maturation by Survivin-mRNA loading and addition of INF-γ and Ribomunyl, by analysing the expression of the following markers:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorescence dye</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>PC5</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>CXCR4</td>
<td>PE</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>CD80</td>
<td>FITC</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>CD83</td>
<td>PC5</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>CCR5</td>
<td>FITC</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>PC5</td>
<td>Beckmann Coulter</td>
</tr>
<tr>
<td>CCR4</td>
<td>PE</td>
<td>Becton Dickinson</td>
</tr>
</tbody>
</table>

Table 3: antibodies for phenotype determination
Material and Methods

The calculation is done by using the quadrant statistics and is given in percentage of positive cells relative to the total cell number.

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

The ELISA is performed starting from supernatant collected from each cell culture set after 5 days culture in presence of IL-4 and GM-CSF and after inducing DC maturation by Survivin-mRNA loading and addition of INF-γ and Ribonunyl. Human IL-10 and IL-12p70 secretion is analysed following OptEIA ELISA Sets (BD Pharmingen) protocol.

Shortly, the required number of wells for the analysis are coated with 100µL of diluted IL-10 or IL-12 antibody (dilution in coating buffer as recommended in the lot-specific analysis certificate) and incubated overnight at 4°C. Next day the plate is washed thrice and a blocking step is performed for 1 h at RT with 200µL of the supplied assay diluent. The plate is again washed for 3 times. Next 100µL of each sample (1:2 diluted) as well as 100µL of each standard are applied to the wells in duplicate and incubated for 2h at RT. Afterwards a washing step is performed for 5 times. Next 100µL of the working detector reagent are added to each well. The working detector reagent is a one-step Biotin/Streptavidin reagent and consists of the diluted, biotinylated IL-10 or IL-12 detection antibody and streptavidin horseradish peroxidase. After 30 min the plate is washed again for 7 times. Each washing step has a soaking time of 1 min. Next 100µL of substrate solution are added and incubated for 30 min at RT in the dark. To stop the reaction 50µL of a stop solution is pipetted to each well and the plate is read on a photometer (BioRad) at 450nm within 30 min with a wavelength correction of 570nm. The absorbance is directly related to the amount of detected interleukin and corresponding to the standard dilutions.

4.4 Proliferation Assay (Mixed lymphocyte reaction)

DCs are co-cultured at a final concentration of 1*10^6 cells/well in a 96-well U-bottom plate with the non-adherent fraction of Ficoll-separated PBMNCs from a healthy donor at a final concentration of 1*10^4 cells/well in RPMI with 2% FBS (Gibco), supplemented with 10% AlamarBlue® at a final volume of 150µL. Proliferation causes reduction of the dye changing from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form. For 2 to 7 days, every 4 to 12h, the absorbance is measured on a plate-reader (SLT-Tecan) at 595nm with reference wavelength at 620nm.
Material and Methods

4.5 Phagocytosis Assay (FITC-Dextran uptake)

For each sample 3 measurements are done: one control, one for spontaneous phagocytosis and one to check the induced phagocytosis. Therefore DCs are plated in a density of 1*10^6 cells on a 6-well-plate. Each well is filled with 1.5mL of RPMI 1640 (Gibco), supplemented with 2% FBS (Gibco) and 0.5µg/µL of FITC-dextran (Sigma) are added to well 2 (spontaneous phagocytosis) and well 3 (induced phagocytosis). 300ng/mL of CCL19 (Sigma) are added to the 3rd well only (induced phagocytosis). The cells are incubated for 2h with or without FITC-dextran at 37°C and washed twice in cold PBS, centrifuged for 5 min at 1,200rpm at RT, before resuspending them in 0.5mL of cold PBS and measuring with a FACS device (Becton Dickinson). The phagocytosis activity is given in percentage of phagocytizing cells relative to total cell number.

4.6 Migration Assay (Transwell assay)

To measure the migration potentiality of the cultured DCs, 1*10^6 cells are transferred to a 6-well plate. For each sample 3 wells are used: one well where the total cell number is determined, one for spontaneous migration and one for induced migration. The lower part of each well of the 6-well plate is filled with 1.5mL of RPMI supplemented with 2% FBS. 300ng/mL of CCL19 are added to the 3rd well only (induced migration). Thereafter the filter is inserted and 500µL of the cell suspension are added to the well and incubated for 2h at 37°C at 5% CO₂. Afterwards 1.5 mL and 500µL of 8% paraformaldehyde (PFA; Sigma) are added to the medium and to the filter, respectively, in order to reach a final concentration of 4% PFA and the plates are incubated for 10 min at RT. Afterwards the wells are washed twice with 1x PBS.

Finally the upper surface of the filter is scraped only in the second and third well (spontaneous and induced migration) with a cotton-tipped swab and the remained cells are stained with a solution made of 0.2% crystal violet (Sigma) in 20% methanol (Merck) for 5 min at RT. The cells in the first well are also stained, but not scraped before, because with this well the total cell number is determined. Thereafter the inserts are rinsed with water to get rid of the excess staining solution and air-dried. Afterwards the membranes are cut out of the plastic ring, placed on a glass slide and mounted with Entellan (Merck). Each membrane is photographed, the cells are counted and the cell number is given in percentage of total cells (well 1).
4.7 Viability assay

Viability test is used to determine the inhibitors toxicity depending either on their final concentration (unspecific) or on their effects upon the different MAPK-pathways (specific). The viability test is performed by and according to the protocol included in the Annexin V-FITC apoptosis detection kit (Biovision). Shortly $1 \times 10^5$ cells are resuspended in $500 \mu L$ of $1x$ binding buffer. Next $5 \mu L$ of Annexin V-FITC and $5 \mu L$ of propidium iodide (PI) are added and incubated for 5 min at RT in the dark. The Annexin V-FITC binding is analysed by flow cytometry using FITC signal detector and PI staining by the phycoerythrin emission signal detector.

4.8 Western Blot

Protein Extraction

To avoid protein degradation, all steps are performed on ice. First $2 \times 10^6$ cells are thawed in $100 \mu L$ Frackelton buffer (preparation see Table 4 and Table 5), vortexed and incubated for 20 min at 4°C to gain cytoplasmic proteins. After another vortexing step, the cells are centrifuged for 20 min at 20,000g at 4°C and the nuclear pellet is separated from the supernatant, which corresponds to the cytoplasmic fraction. At last 100µL of the cytoplasmic protein fractions are mixed with 25µL of 5x SDS sample buffer added up with DTT 100mM just before use. The samples are heated up to 75°C for 5 min before loading.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Amount added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>0.24g</td>
</tr>
<tr>
<td>Na₄P₂O₇</td>
<td>2.7g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.6g</td>
</tr>
<tr>
<td>Triton 100x</td>
<td>2mL</td>
</tr>
<tr>
<td>Deionised water</td>
<td>Fill up to 200mL</td>
</tr>
</tbody>
</table>

Table 4: Frackelton buffer concentrate, stored at 4°C until further use

Added just before use:

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Final concentration</th>
<th>Dilution</th>
<th>fV = 1.2mL Frackelton Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>50mM</td>
<td>1:20</td>
<td>60µL</td>
</tr>
<tr>
<td>Na₃VO₄</td>
<td>0.1mM</td>
<td>1:1000</td>
<td>1.2µL</td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>1:25</td>
<td>48µL</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>0.1mM</td>
<td>1:1000</td>
<td>1.2µL</td>
</tr>
<tr>
<td>PMSF</td>
<td>1mM</td>
<td>1:100</td>
<td>12µL</td>
</tr>
</tbody>
</table>

Table 5: Frackelton buffer working solution, prepared prior before use
Material and Methods

SDS-PAGE

For the electrophoresis a SDS-Polyacrylamidgel is prepared, therefore the miniprotean III system is used (Biorad). The 10% polyacrylamid separating gel is prepared according to the following formula:

<table>
<thead>
<tr>
<th></th>
<th>1 small gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 8.8 1.5 M</td>
<td>3mL</td>
</tr>
<tr>
<td>Bis/Acrylamide 30% (Biorad)</td>
<td>4mL</td>
</tr>
<tr>
<td>SDS 20% (Sigma)</td>
<td>60μL</td>
</tr>
<tr>
<td>Water</td>
<td>5mL</td>
</tr>
<tr>
<td>APS 10% (Sigma)</td>
<td>48μL</td>
</tr>
<tr>
<td>TEMED (Sigma)</td>
<td>24μL</td>
</tr>
</tbody>
</table>

Table 6: Running gel for a 10% SDS-Polyacrylamidgel

The gel is covered with isopropanol and 15 min time is given for polymerisation at RT. The isopropanol is discarded and the stacking gel solution is layered on top of the separating gel, which is prepared according to the following table:

<table>
<thead>
<tr>
<th></th>
<th>1 small gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 6.8 1.5 M</td>
<td>1mL</td>
</tr>
<tr>
<td>Bis/Acrylamide 30%</td>
<td>0.5mL</td>
</tr>
<tr>
<td>SDS 20%</td>
<td>20μL</td>
</tr>
<tr>
<td>Water</td>
<td>1.25mL</td>
</tr>
<tr>
<td>APS 10%</td>
<td>24μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12μL</td>
</tr>
</tbody>
</table>

Table 7: Stacking gel for a SDS-Polyacrylamidgel

Finally a comb is inserted in the stacking gel, which is let to polymerize for 15 min. After removing the comb, the slots are filled with running buffer and the gel is inserted into an electrophoresis chamber that is in turn filled with running buffer. Next 5μL of the prestained protein marker (Fermentas) and 5μg of each sample are loaded on the gel. The electrophoresis is performed for about 90 min at 160V.

Immunoblot and Ponceau Red staining

After SDS-PAGE, the proteins are transferred from the polyacrylamid gel to a nitrocellulose membrane using 1x transfer buffer. Blotting is performed for 1h at 30V using a semi-dry blotting apparatus. The immunoblotting sandwich is made up of three pre-wetted filter papers, cut to the size of the gel. Next the gel is placed on the filter papers and covered with the pre-equilibrated membrane. At last another three pre-wetted filter papers are placed on top. The sandwich is oriented in a way that the gel is at the cathode and the membrane at the anode.
Material and Methods

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 twice in TBST until all the red staining is completely removed.

Reagents:
- 5x SDS sample buffer: 1g Tris (Sigma), 12.5g SDS (Sigma), 12.5mL glycerol (Fluka), 6mg bromphenol blue (Biorad) dissolved in deionised water, brought to pH 6.8 and added up with 100mM DTT (Invitrogen) just before use
- 10% APS: 1g ammonium persulphate (Sigma) dissolved in 10mL of deionised water, stored at -20°C
- Ponceau Red 10x (stock solution): 2g Ponceau S (Serva), 30g Trichloracetic acid (Merck), 30g Sulphoalocyclic acid (Merck), deionised water to 100mL
- 10x Running buffer: 30 g. TRIS-base (Sigma); 144 g Glycine (Fluka); 50 mL SDS (Sigma) 20% and water to 1L Separation gel buffer (pH 8.8): 1.5M Tris-HCl, 0.4% SDS, deionised water to 1L
- Stacking gel buffer (pH 6.8): 0.5M Tris-HCl, 0.4% SDS, deionised water to 1L
- 10x Blotting buffer: 29g Glycine (Merck), 58g Tris-Base (Sigma), 3.7g SDS, deionised water to 1L
- 1x Blotting buffer: 10% 10X blotting buffer, 20% methanol (Merck), deionised to 1L
- 10x TBS: 250mM Tris, 1.5M NaCl (Merck); pH 8.0, deionised water to 1L
- TBST: 100mL 10x TBS, 500 µL Tween-20 (Promega), deionised water to 1L

Incubation with the antibodies

Non-specific binding is blocked by incubating the membrane for 1h in a solution of TBST added up with 5% powder milk, followed by two washing steps with TBST for 10 min. Finally the membrane is incubated overnight with the appropriate primary antibody diluted in TBST.

The day after the membrane is washed 3 times in TBST and subsequently incubated for 1h with the appropriate secondary antibody, diluted in TBST. Thereafter, the membrane is washed three times for 10 min with TBST.
Material and Methods

The following antibodies are used:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Raf C-terminal (Santa Cruz)</td>
<td>1:500</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>C-Raf (BD Transduction Laboratories)</td>
<td>1:1,000</td>
<td>Mouse monoclonal</td>
</tr>
<tr>
<td>MEK 1/2 (Cell Signalling Technology)</td>
<td>1:500</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>pMEK 1/2 (Cell Signalling Technology)</td>
<td>1:500</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>JNK (Cell Signalling Technology)</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>pJNK (Cell Signalling Technology)</td>
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</tr>
<tr>
<td>p38 (Santa Cruz)</td>
<td>1:1000</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>p-p38 (Santa Cruz)</td>
<td>1:200</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>Actin (Santa Cruz)</td>
<td>1:500</td>
<td>Goat polyclonal</td>
</tr>
</tbody>
</table>

Table 8: Primary antibodies and secondary antibodies used for western blots

ECL detection

After the immunoblotting the detection is performed by incubating the membrane for 3 min with 1mL of previously mixed Luminol/Enhancer Solution and Stable Peroxide-Solution (Pierce). Excess detection buffer is poured off and the membrane is positioned between two transparent foils 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 sec and 1h, depending on the strength of the signal.
Results

All the experiments described in this session are performed with human dendritic cells derived either from whole blood of healthy volunteers (controls 1-6) or from surplus from leukaphereses of patients suffering from different types of tumours. For details see Table 9.

<table>
<thead>
<tr>
<th>Name</th>
<th>sex</th>
<th>Age [in years]</th>
<th>type of cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>female</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Control 2</td>
<td>female</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>Control 3</td>
<td>female</td>
<td>34</td>
<td>-</td>
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<tr>
<td>Control 4</td>
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<tr>
<td>Control 5</td>
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</tr>
<tr>
<td>Control 6</td>
<td>male</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Patient A</td>
<td>male</td>
<td>52</td>
<td>colon</td>
</tr>
<tr>
<td>Patient B</td>
<td>female</td>
<td>50</td>
<td>breast</td>
</tr>
<tr>
<td>Patient C</td>
<td>female</td>
<td>59</td>
<td>ovarian</td>
</tr>
<tr>
<td>Patient D</td>
<td>female</td>
<td>51</td>
<td>breast</td>
</tr>
<tr>
<td>Patient E</td>
<td>female</td>
<td>47</td>
<td>ovarian</td>
</tr>
</tbody>
</table>

Table 9: Description of controls and patients

Due to a quantitative limitation of cells, not all the experiments could be carried out with the same patients.

1 Morphology

To determine whether the addition of different selective MAPK inhibitors affects the phenotype of human dendritic cells, we observed the morphology of the analysed DCs in presence and in absence of the following inhibitors: Control, PD = MEK-inhibitor PD 98095, U = MEK-inhibitor U 0126, JNK = JNK-inhibitor SP 600125, p38 = p38 inhibitor PD 169316. In Fig. 10 it is possible to compare the effects of different MAPK inhibitors, added from the start of the in vitro culture, on the morphology of loaded, fully mature DCs.

Fig. 10: Morphology of DCs cultured from the start in absence (control) or in presence of selective MAPK inhibitors (MEK-inhibitor PD 98059, MEK-inhibitor U 0126, JNK-inhibitor, p38-inhibitor) at the start of the culture
Untreated mDCs grew adherent and showed the typical stellate shape, whereas upon MEK-inhibition with either PD 98059 or U 0126, the cells did not attach to the plastic anymore and revealed DCs with only rare extensions, which is a hallmark of the immature phenotype. Such immature phenotype was even strengthened upon addition of either JNK- or p38-inhibitors. Indeed, in these specific cases, the DCs completely lost the adherent, mature phenotype in favour of the immature one, characterized by a fully non-adherent pattern accompanied by the complete loss of dendrites formation. Moreover, JNK- or p38-inhibition was characterized by a reduced viability, a fact that may be related to the well known pro-apoptotic features of these two pathways.

2 Phenotype (FACS)

Since the addition of different selective MAPK 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 the surface markers, which are known to be increased during human DC maturation. Shortly, we analysed by FACS DCs derived either from healthy controls or patients for the following markers: CD80, CD83, CD40, CXCR4, CCR5, CCR4 and HLA-DR after the first 5 days of culture in presence of IL-4 and GM-CSF (iDCs) and 24h after loading and maturation.

2.1 Addition of selective MAPK inhibitors from the start

We added the MAPK inhibitors from the very start of the culture of DCs derived from one healthy control (control 1) respectively one patient (patient A).

2.1.1 iDCs

After 5 days in culture, untreated iDCs derived from healthy control showed higher levels of CD40, CD80 and HLA-DR in comparison with untreated iDCs derived from the patient. Reversely, untreated iDCs derived from the patient showed higher levels of CCR5 and CCR4 in comparison with untreated iDCs derived from the healthy control.

MEK inhibition by PD 98059 strongly decreased the expression of CD40, CXCR4, CD80 and HLA-DR on the surface of iDCs derived from healthy control, without significantly affecting the expression of CD83, CCR4 and CCR5. On the other hand PD 98059 addition to iDCs derived from patients slightly increased the expression of CD40, slightly decreased the expression of HLA-DR but did not affect the expression of CD83 and CXCR4 and strongly decreased CD80, CCR5 and CCR4 expression.
MEK-inhibitor U 0126 led to a slightly decrease of CD40 and a stronger decrease of CXCR4 and CD80, but did not affect the expression of CD83, CCR5 and HLA-DR in the healthy control while strongly increasing CCR4 expression. Whereas on the patients’ iDCs the expression of CD40, CD80, CCR5, HLA-DR and CCR4 was decreased, the levels of CXCR4 and CD83 remained stable.

Treating iDCs with JNK-inhibitor SP 600125 did not affect CD83 expression rate, caused a strong decrease of CD40 and CD80 expression combined with a strong increase of CXCR4, CCR5 and CCR4 expression on the surface of DCs derived both from patient and from healthy control. While such inhibitor caused as well as a strong decrease of HLA-DR in iDCs derived from the healthy control, iDCs derived from the patient showed no change in the expression of HLA-DR compared to the untreated cells.

The results obtained upon SP 600125 addition to iDCs culture were completely mimicked by incubation with the p38-inhibitor PD 169316, with one exception: CD83 the expression of which greatly rose upon incubation in presence of PD 169316 on the surface of patient-derived iDCs, while being slightly increased on the surface of healthy control-derived iDCs.

These results showed that iDCs respond differently to MAPKs inhibition from DCs, according to their source: cancer patients or healthy control. Such discrepancy combined with the different phenotype regarding CD40, CD80, CCR5, HLA-DR and CCR4 showed by untreated iDCs derived from healthy people or from cancer patients is in agreement with the finding that DCs isolated from cancer patients retain a more immature phenotype, in comparison to that of iDCs derived from healthy people (ref 60). Indeed, patient-derived iDCs express less CD40, HLA-DR and CD80 in comparison to iDCs isolated from healthy controls. Interestingly, the inhibition of MEK either by PD 98059 or by U 0126 caused a more immature phenotype in comparison to untreated cells. This fact was particularly evident upon addition of PD 98059 and mostly in healthy control-derived DCs. The effects were indeed far reduced in patient-derived DCs. Since MEK-pathway usually correlates with cell proliferation and undifferentiated state (ref 61), it is very curious to see that such effects are not reproducible in human DCs, cultured in vitro. Possibly, this characteristic is due to the fact that iDCs are already “differentiated” cells, derived from monocytes, which do not show any in vitro proliferative capability. For such cells, the importance of the MEK-pathway may
Results

reside elsewhere from mere proliferation enhancement and maintenance of the undifferentiated state.

As expected, inhibition of JNK and of p38 pathway did correlate with a more immature phenotype, according to the finding that such MAPK pathways correlate with cell differentiation (ref.\textsuperscript{62,63}). In this case, too, the effects of the inhibitors were particularly evident on iDCs derived from healthy controls in comparison to those patient-derived. Such effects were stronger regarding CXCR4 and CD80 expression. Curiously, CD83 expression increased upon PD 169316 but not upon SP 600125, suggesting that the two MAPKs may affect different aspects of DCs maturation.

![Immature cells – Inhibition from the start of the culture](image)

**Fig. 11:** Phenotype of immature cells inhibited from the start of the culture (two different humans: control 1 and patient A)

### 2.1.2 mDCs

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.

Upon maturation, both patient- and healthy donor-derived, untreated DCs showed a completely new pattern of expression regarding the analysed surface markers in comparison with that shown by the respectively iDCs. Indeed, CD80, CD83, CCR5 and CCR4 expression strongly increased in comparison to that detectable on the respective iDCs. HLA-DR and
Results

CD40 expression remained high on mDC, derived from healthy donor and remarkably increased on the surface of mDCs, derived from cancer patient. These data suggest that, as reported in literature (ref 64), in vitro cultured patient-derived DC can be fully mature, to levels comparable to those shown by healthy donor-derived DCs. The only true difference in the marker expression patterns between mDCs derived either from healthy donor or patient regards CD83 expression, which remained lower on the latter. On the other side, slightly differences were detectable in CXCR4 expression, which was higher in patient-derived in comparison with healthy donor-derived DCs and in CD80 expression, which showed the opposite pattern.

Prolonged incubation with MEK-inhibitor PD 98059 led to a strong decrease of all the analysed markers on the surface of healthy control-derived DCs as well as of patient-derived DCs, with the exception of CD83, which expression on patient-derived DCs, opposite to healthy control-derived DCs, appeared to be only slightly, if ever, affected by the addition of PD 98059.

Treatment with the inhibitor U 0126 mimicked the effects of PD 98059 but as seen as well in the iDCs, to less extend. Indeed, U 0126 addition decreased the expression of CXCR4, CD83, CCR5 and CCR4 on the surface of healthy control-derived DCs as well as of all the analysed markers on the surface of against the MEK-pathway mainly impaired the levels of CXCR4, CD83, CCR5 expression of patient-derived DCs. According to these data, the effects of U 0126 were stronger on patient-derived DCs in comparison with healthy control-derived DCs, suggesting that this inhibitor block a part of the MEK-pathway, which has a more crucial role in patient-derived DCs. Again, the MEK-pathway seems to be involved in DC maturation rather than proliferation and maintenance of the immature state.

As expected, JNK-inhibition diminished expression of all surface markers in the healthy control as well as in the patient, compared to the cells grown without inhibitor, suggesting that this MAPK is deputed to induce the terminal maturation of human DCs.

As expected, the inhibition of the p38-pathway mimicked the effects showed upon addition of JNK-inhibitor. Interestingly, again, the inhibition of p38 was less effective on the expression of CD83 on patient-derived DCs, suggesting that cancer may uncouple the expression of this marker from the p38-pathway.
Results

In summary, these data suggest that during DCs maturation all three MAPKs (MEK, JNK and p38) played together a pivotal role towards cell differentiation, in contrast to the most common pattern of action, which sees MEK playing pro proliferation and maintenance of the undifferentiated state and versus the pro-differentiation kinases JNK and p38. The results showed as well that cancer impairs DCs differentiation both by actively affecting cell phenotype and by making DCs more prone to respond to MEK inhibition, especially through the pathway selectively blocked by U 0126.

![Mature cells – Inhibition from the start of the culture](image)

Fig. 12: Phenotype of mature cells inhibited from the start of the culture (two different humans: control 1 and patient A)

**2.2 Addition of selective MAPK inhibitors from the loading step**

In order to determine whether the effects shown upon selective inhibition of different MAPKs were due to the prolonged exposure to such substances or whether their effects upon the DC maturation depend as well or principally on the time point at which they are added, we tested the effects of selective inhibition of MEK, JNK and p38, upon addition of the respective inhibitor just before performing the loading step.

The addition of the selective MEK inhibitor PD 98059 just before the loading step mimicked the effects shown by the addition of the same inhibitor from the very start of the culture in both healthy donor- and patient-derived DCs, the only small detectable differences being the fact that in this case, the negative effects on HLA-DR were lower in both samples, whereas
Results

they increased on CCR4 in patient-derived DCs, in comparison to the prolonged incubation with the same inhibitor. These data suggest that the decreasing rate of HLA-DR expression directly correlates with the exposure length to such inhibitor, whereas the effects on CCR4, at least in patient-derived DCs are increased by a timely addition of PD 98059, suggesting that a short but precise exposure to the inhibitor may be more effective than a durable, not-time-aimed incubation.

More interestingly, the effects of the selective MEK inhibitor U 0126 on the surface of healthy control-derived DCs were far stronger upon addition just before the loading step than upon prolonged incubation from the start of the culture. This fact was particularly evident from the strong impairment of CD40, CD80, CD83 and CCR5. On the contrary, the addition of the selective MEK inhibitor U 0126 just before the loading step mimicked the effects shown by the addition of the same inhibitor from the very start of the culture in patient-derived DCs. Such effect may support the idea that U 0126 blocks a MEK-pathway, which is important for the switching towards maturation for healthy DCs and which becomes active during the antigen encountering. At the same time, it shows that timely inhibition through U 0126 may be more effective than a prolonged exposure, which in turn can make the cells refractory to its action. The fact that U 0126 timely and prolonged inhibitions gave the same effects on patient-derived DCs may suggest that MEK plays a role on the differentiation of these cells already at the stage of iDCs, before the antigen encountering due to cancer related effects.

JNK-inhibition just before the loading step completely mimicked the effects shown upon addition of the same inhibitor from the very start of cell culture, suggesting that the effects of this inhibition upon DC maturation do depend more on the check-point of administration rather than on its duration.

Treatment with p38-inhibitor reveals a similar result to JNK-inhibitor addition. In this case, too, both p38 and JNK seem to be involved as expected, in the differentiation of the analysed cells. Due to the minor differences showed upon addition of these two inhibitors in the effects on healthy donor-derived versus patient-derived DCs, it seems that such kinases are not really affected by the tumour establishment.
Results

Mature cells – Inhibition from the loading with Survivin mRNA

![Graph showing mature cell inhibition](image)

Fig. 13: Phenotype of mature cells inhibited from the loading with Survivin mRNA (two different humans: control 1 and patient A)

2.3 Addition of selective MAPK inhibitors from the maturation step

In order to further evaluate the role of MEK, JNK and p38 in different steps of DCs maturation, we tested the effects of selective inhibition of MEK, JNK and p38, upon addition of the respective inhibitor just before adding the maturation cocktail and after the loading step.

The addition of the selective MEK inhibitor PD 98059 just before maturing healthy donor-derived DCs mimicked the effects shown by the addition of the same inhibitor from the very start of the culture or just before the loading step. Therefore the data suggest that, at least in in vitro culture, MEK plays a pivotal role for the final DC maturation upon maturation induction and that the inhibition of such a pathway rather depends on the check-point of the administration than on the duration of the treatment. The addition of the selective MEK inhibitor PD 98059 just before maturing patient-derived DCs almost completely mimicked the effects shown by the addition of the same inhibitor just before the loading step, with the following exceptions. In this case, the inhibition of CD40 expression results weaker and more similar to that shown upon prolonged exposure to the inhibitor, whereas the opposite is true about the expression of CD80 and CD83. Again the phenotypical expression of such markers mimicked that shown upon prolonged exposure to the inhibitor. Therefore the data suggest
that, at least in \textit{in vitro} culture, MEK plays a pivotal role for the final DC maturation upon maturation induction and that the inhibition of such a pathway rather depends both on the check-point of the administration (CXCR4, CCR5, HLA-DR and CCR4) and/or on the duration of the treatment (CD40, CD80 and CD83). Our experiments cannot distinguish between the role played by duration and check-point of MEK inhibition by PD 98059.

Interestingly, the effects of the selective MEK inhibitor U 0126 on the surface of healthy control-derived DCs completely mimicked those shown by the addition of the same inhibitor just before the loading step, with the exception of CCR4, which expression resulted completely inhibited. These data strongly suggest that this MEK inhibitor action, too rather depends on the check-point of the administration than on the duration of the treatment. In the case of its effects upon CCR4, it seems that a prolonged exposure reverses the effects driven by a short, timely inhibitor addition. The addition of the selective MEK inhibitor PD 98059 just before maturing to patient-derived DCs mimicked the effects shown by the addition of the same inhibitor from the loading step. Therefore the data suggest that, at least in \textit{in vitro} culture, MEK plays a pivotal role for the final DC maturation upon maturation induction and that the inhibition of such a pathway rather depends on the check-point of the administration than on the duration of the treatment.

Interestingly, the use of the two different MEK inhibitors showed that the response of the DCs to such a treatment is mostly check-point exposure dependent and that the cancer establishment plays a pivotal role on the cell response pattern.

JNK-inhibition just maturing the cells completely mimicked the effects shown upon addition of the same inhibitor from the very start of cell culture or just before the loading step, suggesting that the effects of this inhibition upon DC maturation does depend more on the check-point of administration rather than on its duration. Since no significant differences were detectable between healthy donor- and patient-derived DCs, it seems that the cancer establishment does not affect in a relevant way this pathway.

Interestingly p38-inhibition of healthy-control derived DCs from the maturing step showed weaker effects in comparison to those shown upon exposure from the start and from the loading step regarding the impairment of CD80, CCR5 and HLA-DR; whereas the effects were similar concerning CD40, CXCR4 and CCR4 expression. These data suggest a different
Results

pathway of action of such an inhibitor upon different maturation markers. Indeed, the expression of CD80, CCR5 and HLA-DR seems to require the p38-pathway from the loading step on and/or that p38 inhibition must be prolonged in order to increase its effects. On the other hand, the expression of CD40, CXCR4 and CCR4 seems to become p38-dependent upon maturation.

![Graph](image.png)

**Fig. 14:** Phenotype of mature cells inhibited from the maturation step (two different humans: control 1 and patient A)

### 3 ELISA (IL-10 and IL-12 secretion)

Since our preliminary experiments about the effects of MAPK upon the morphology and the phenotype of DCs during their maturation showed that indeed, MEK, JNK and p38 play a pivotal role, we further investigated whether such effects mirrored the role of these kinases on DC functionality.

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.
3.1 Addition of selective MAPK inhibitors from the start

3.1.1 iDCs

As expected, untreated iDCs derived either from healthy controls or cancer patients showed a very slight, if any, secretion of IL-10 or IL-12. The lack of IL-10 expression even at the immature stage is characteristic of excellent conditions of in vitro cultures of human DC, which support the following maturation step by minimizing eventual tolerogenic stimuli. On the other side, the lack of IL-12 secretion is typical of iDCs.

Interestingly, treatment of iDCs with MEK inhibitor PD 98059 led to an IL-10 secretion in two healthy controls, but remained negative in the other healthy individual and in the patients. These results may indicate that the effects of such inhibitor are either/both dependent on the source (healthy controls vs cancer patients) or/and on the individual characteristics. Further experiments in this direction are required to solve this question. In any case, such inhibitor apparently may disturb the MEK-pathway in a way, which correlates with increased production of IL-10. As expected, no IL-12 secretion could be detected, either in the healthy controls or in the patients derived iDCs, reflecting their immature state.

In contrast, in the supernatants of patients and the healthy controls derived iDCs treated with MEK-inhibitor U 0126, neither IL-10 nor IL-12 secretion was detectable. These results, very similar to those obtained regarding untreated cells suggest that such MEK inhibitor is unable to affect the MEK-pathway at the same way PD 98059.

Such results suggest repeating the experiments with different concentration of PD 98059 and U 0126 in order to avoid artefacts and determine the real effects of such inhibitors on iDCs.

The incubation with chemical inhibitor against JNK revealed very low, if any, levels of secreted IL-10 or IL-12 by iDCs derived from both healthy controls and patients, suggesting that the JNK-pathway does not influence ILs secretion by iDCs.

IL-10 secretion was increased upon addition of p38 inhibitor in two controls, but was completely absent in all the other samples. As for PD 98059, further experiments are required to determine how p38-pathway is involved in the secretion of IL-10 by immature DCs. As expected, no IL-12 secretion could be detected, either in the healthy controls or in the patients’ derived iDCs, reflecting their immature state.
3.1.2 mDCs

As expected, untreated mDCs, regardless of their source, do not secrete IL-10 almost at all, while releasing a large amount of IL-12 in the supernatant of the cell culture. Such results support the fact that even cancer patients-derived DCs, cultured in vitro under the described conditions, become able to produce levels of IL-12 comparable to those shown by healthy controls-derived DCs and therefore that they can be safely used in autologous immune therapies.

Upon exposure to PD 98059 mDCs do not express almost at all IL-10, suggesting that the increased levels of IL-10 production at the immature state is either a transient effect or an artefact. The effects of this inhibitor on the production of IL-12 were minimal. These results suggest that the strong effects of PD 98059 on cell morphology and cell phenotype are uncoupled from the IL-12 production pathway. In one sentence, this inhibitor impairs the phenotypical but not the functional maturation of human DCs, independently of their source.

Interestingly, addition of MEK-inhibitor U 0126 led to an increased secretion of IL-10 in mDCs, an effect particularly evident in mDCs derived from healthy controls in comparison with mDCs derived from cancer patients. The effects of such an inhibitor showed as well a strong individual-dependent pattern, in contrast to that shown upon iDCs. Such data suggest that the effects of this inhibitor correlate either/both with treatment duration or/and maturation stage. Further experiments are required to answer to such a question. Interestingly, in contrast to PD 98059, U 0126 affects less the phenotype and more the functionality of mDCs, independently of their source. Moreover, the effects of such inhibitor are well detectable on both phenotype and functionality, possibly according to the fact that U 0126 is known to be a stronger MEK inhibitor in comparison to PD 98059. The levels of IL-12 secretion were comparable (or slightly decreased) to those observed in untreated cells, suggesting that the role of MEK may be to impair IL-10 production rather than increase the levels of IL-12. In any case, MEK function appears to be linked to cell differentiation.

JNK inhibition mildly correlated with increased secretion of IL-10, a characteristic, which appears to be individual-specific and independent from the DCs source, and with a strongly decreased production of IL-12, source- and individual-independent. Such pattern of effects correlates well with the results about cell morphology and phenotype observed upon JNK inhibition. Indeed, inhibition of JNK maintains an immature phenotype, which correlates with
the establishment of an impaired functionality of the mDCs. Curiously, if MEK inhibitions appear to increase the levels of IL-12, JNK inhibition correlates with decreased IL-12 secretion, suggesting that possible MEK impairs IL-10 production while JNK increases IL-12 secretion upon DCs maturation.

Treatment with p38-inhibitor correlated with mild to strong induced IL-10 secretion, which appears to be individual- but not source-dependent. As in the case of JNK inhibitor treatment, the addition of p38 inhibitor strongly impaired IL-12 secretion, suggesting that p38 is required for both phenotypical and functional maturation of human DCs. In comparison to JNK, p38 inhibition appears to be stronger in the effects and very similar if not identical in the pattern, according to the data reported in literature.

Fig. 15: IL-10 and IL-12 secretion of dendritic cells inhibited from the start of the culture (three patients and three controls)
3.2 Addition of selective MAPK inhibitors from the loading step

In order to determine:
- whether the effects shown upon selective inhibition of different MAPKs were due to the prolonged exposure to such substances or whether their effects upon the DC maturation depend as well or principally on the time point at which they are added;
- whether the addition of the inhibitors affects at the same time phenotype and cell functionality synergistically,

we tested the effects of selective inhibition of MEK, JNK and p38, upon addition of the respective inhibitor just before performing the loading step.

As expected, untreated mDCs derived from both healthy controls and patients did not secrete IL-10 while producing high amounts of IL-12, which levels of expression varied from individual to individual and which appear to be higher in patients’- rather than in healthy controls-derived mDCs.

Regarding IL-10 and IL-12 secretion, exposure to PD 98059 from the loading step completely mimicked the effects observed upon long-lasting incubation mDCs, confirming that the strong effects of PD 98059 on cell morphology and cell phenotype are uncoupled from the IL-12 production pathway. The secretion pattern of IL-12 showed a very individual specific phenotype.

As observed upon prolonged exposure, addition of MEK-inhibitor U 0126 led to an increased secretion of IL-10 in mDCs, an effect particularly evident in mDCs derived from healthy controls in comparison with mDCs derived from cancer patients. IL-10 secretion levels appear again to be individual-specific. Again, the effects of such inhibitor are well detectable on both, phenotype and functionality, in comparison to PD 98059. The levels of IL-12 secretion were again comparable (or slightly decreased) to those observed in untreated cells, strongly pointing out that the role of MEK may be to impair IL-10 production rather than increase the levels of IL-12. In any case, MEK function was confirmed to be linked to cell differentiation.

JNK inhibition correlated with increased secretion of IL-10, which differently from what observed upon prolonged exposure to this inhibitor, appears to be less individual-specific and more depending on the donor group. As already observed upon prolonged incubation, the levels of IL-12 were strongly down-regulated, again with a pattern, which is less individual-specific and more depending on the donor group. Such results suggest that the effects of JNK
Results

on DC maturation may be differently modulated according to the cell stage. Anyhow, inhibition of JNK maintains an immature phenotype, which correlates with the establishment of an impaired functionality of the mDCs, confirming the role of JNK in cell differentiation.

Treatment with p38-inhibitor correlated with mild to strong induced IL-10 secretion, which appears to be individual- and source-dependent. As in the case of JNK inhibitor treatment, the addition of p38 inhibitor strongly impaired IL-12 secretion, suggesting that p38 is required for both phenotypical and functional maturation of human DCs, and appeared to be somehow differently modulated according to the administration way. As already observed upon prolonged exposure, in comparison to JNK, p38 inhibition appears to be stronger in the effects and very similar if not identical in the pattern, according to the data reported in literature.

Fig. 16: IL-10 and IL-12 secretion of dendritic cells inhibited from the loading with Survivin mRNA (three patients and three controls)
3.3 Addition of selective MAPK inhibitors from the maturation step

IL-10 and IL-12 secretion levels were finally analysed upon addition of selective MAPK inhibitors from the maturation step, in order to get more insights about the effects of the analysed MAPK pathway during the different stage of DCs maturation.

As expected, untreated mDCs derived from both healthy controls and patients almost did not secrete IL-10 while producing high amounts of IL-12, which levels of expression varied from individual to individual.

Regarding IL-10 and IL-12 secretion, exposure to PD 98059 from the maturing step completely mimicked the effects observed upon long-lasting incubation or addition from loading step, confirming that the strong effects of PD 98059 on cell morphology and cell phenotype are uncoupled from the IL-12 production pathway. The secretion pattern of IL-12 showed again a very individual specific phenotype.

As observed upon prolonged exposure or addition at the loading step, addition of MEK-inhibitor U 0126 led to an increased secretion of IL-10 in mDCs, but differently from the previous experiments, such effects were individual- but not source-specific. The effects of such inhibitor were well detectable on both phenotype and functionality, in comparison to PD 98059. The levels of IL-12 secretion were almost always comparable to those observed in untreated cells, but showed again an individual- but not source-specificity. These results suggest that addition of U 0126 just before inducing maturation may modulate in a quite different way human DCs, suggesting that MEK-pathway is particularly crucial for such stage of DC maturation.

JNK inhibition before maturation strongly increased the effects on IL-10 and IL-12 expression, observed upon incubation from the loading point, suggesting that the JNK-pathway is strongly linked to ILs secretion by mature, probably by affecting the ratio between IL-12 and IL-10. The real mechanism below such effects requires further investigations.

Treatment with p38-inhibitor correlated with strongly induced IL-10 secretion, which appears to be both individual- and source-dependent, although such effect was particular evident in the patients-derived DCs. As in the case of JNK inhibitor treatment, the addition of p38 inhibitor strongly impaired IL-12 secretion, suggesting that p38 is required for both
Results

phenotypical and functional maturation of human DCs. Indeed, the results that the synergy between p38, JNK and MEK are required to induce the secretion of IL-12 and IL-10 at appropriate levels, probably through both, positive and negative feed-back loops.

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**Fig. 17:** IL-10 and IL-12 secretion of dendritic cells inhibited from the maturation step (three patients and three controls)
**4 Proliferation assay (mixed lymphocyte reaction)**

Since we showed that human DCs maturation, studied according to morphology, phenotype and ILs production, was strongly affected by chemical inhibition of MAPK, JNK and p38, we further investigate 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 lymphocytes induced by mature DCs is given as relative proliferation rate compared to that shown by the immature cells.

**4.1 Addition of selective MAPK inhibitors from the start**

As expected, non-inhibited mDCs derived from healthy controls as well as from patients stimulated the proliferation of lymphocytes at a very high rate, which was similar in mDCs of both sources. This characteristic to induce proliferation of T-cells, which is in agreement with the literature, showed again the quality of the culture conditions, which leads to fully mature and functional mDCs.

Prolonged exposure to the MEK-inhibitor PD 98059 caused a strong inhibition of the proliferative capability of DCs, independently of their source and very slightly individual-specific. Interestingly such inhibition caused as well morphology and phenotypical changes according to the impairment of DC maturation, while it did not really affect either the levels of secretion of IL-12 and IL-10 or their ratio. These data suggest that the induction of lymphocyte proliferation may be only partially dependent on IL-12 secretion, while being more dependent on the morphology and the phenotype of the cells. This finding would be in accordance with Redy et al., who showed the importance of DCs phenotype for the stochastic encounters with the lymphocytes, in order to stimulate the proliferation of the latter. In this case, IL-12 levels will activate the lymphocyte only once they are become in touch with the DCs, through the dendrits.

Both addition of MEK U 0126 and p38 inhibitors completely impaired the lymphocyte proliferation induced by DCs, while JNK inhibition had just slightly weaker effects. Whether such results depend on the morpho-phenotypical effects of these inhibitors or on their effects on the secretion of IL-12 and IL-10 or on a synergy of these two effects, it remains to be investigated.
Fig. 18: relative proliferation rate of lymphocytes induced by dendritic cells inhibited from the start of the culture (three patients and three controls)

4.2 Addition of selective MAPK inhibitors from the loading step

The addition of MEK inhibitors PD 98059 and U 0126, as well as of JNK and p38 selective inhibitors from the loading step instead of inhibition from the very beginning of the culture, completely mimicked the effects observed in the latter case, suggesting that the modulation in cell phenotype and in the pattern of IL-10 and IL-12 production, shown in the previous experiments according to the inhibitor administration window, do not affect the induction of lymphocyte proliferation, which in turn appears to be strongly dependent on the morphology, in comparison to the response to IL-12 secretion.
Inhibition from the loading with Survivin mRNA

Fig. 19: relative proliferation rate of lymphocytes induced by dendritic cells inhibited from the loading with Survivin mRNA (three patients and three controls)

4.3 Addition of selective MAPK inhibitors from the maturation step

The addition of MEK inhibitors PD 98059 and U 0126, as well as of JNK and p38 selective inhibitors from the maturing step instead of the inhibition from the very beginning of the culture or from the loading step, completely mimicked the effects observed in the latter cases, pointing out that the induction of lymphocyte proliferation by human DCs is strongly dependent on the morphology, in comparison to the response to IL-12 secretion, which appears however to be able to induce a weaker and possibly independent lymphocyte proliferation.

Further experiments about the exact mechanism below the release of IL-12 and IL-10 as well as their function upon DC-lymphocytes encounter are needed.
**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 have been loaded with an antigen, mDCs lose the capability to endocyte further. Since we observed that the inhibition of each MAPK pathway strongly impaired human DCs in vitro maturation, we investigated whether such morphological, phenotypical and functional characteristic correlated with 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 phagocytic capability, the cells were induced by addition of CCL19, the ligand of CCR7, a surface marker expressed exclusively by mDCs.
5.1 Addition of selective MAPK inhibitors from the start

5.1.1 iDCs
As expected, untreated iDCs, independently of their source, showed the highest phagocytosis capability, unable to respond to CCL19 induction. This was independent as well of the addition of any MAPK inhibitors, suggesting that these pathways are not crucial for phagocytosis, at least when the DCs are immature.

5.1.2 mDCs
As expected, upon maturation, DCs lost completely or almost completely their spontaneous and CCL19-induced phagocytosis capabilities, independently of their source.

In contrast, inhibition of MEK-pathway with PD 98059 correlated with a very slight decrease of the phagocytosis capabilities, a feature, which showed both, individual and to some extend source specific characteristics, above all regarding CCL19-induction effects. They appear indeed to be stronger in healthy controls’ than in patients’ derived DCs.

Inhibition of the MEK-pathway, using inhibitor U 0126, showed effects similar to those observed for PD 98059 regarding the pattern and the levels of phagocytosis capability. In this case the effects of CCL19 induction are less significant.

Both JNK and p38 inhibition caused the same effects obtained upon addition of both MEK inhibitors, but less strong in the levels. The major difference is that upon inhibition of JNK and p38-pathways, CCL19 induction is particularly effective to induce phagocytosis, especially in the patients’ derived DCs, while being more individual-specific and less significant in healthy controls’ derived DCs.

These results suggest that all the analysed pathways have paramount effects on DC maturation, but they reach their goal through different ways. Indeed, while JNK and p38 inhibition seems to be more dependent on CCL19 induction, showing a linkage to the expression of specific surface markers, MEK inhibition appears to be less dependent on the phenotype, at least for what concerns the phagocytosis properties. It can as well be that the morphology of the cells plays a major role regarding this feature. Further experiments in this direction are needed to clarify this point.
Results

Fig. 21: phagocytosis potential of dendritic cells inhibited from the start of the culture (three patients and three controls)

5.2 Addition of selective MAPK inhibitors from the loading step

As expected, upon maturation, DCs lost completely or almost completely their spontaneous and CCL19-induced phagocytosis capabilities, independently of their source.

Both PD 98059 and U 0126 driven inhibition of MEK correlated with increased levels of phagocytosis, comparable with those observed in the previous set of experiments. Such effects as well as those related to CCL19 exposure appeared extremely individual rather than source specific.

JNK inhibition increased the basal levels of phagocytosis in comparison with those shown by untreated cells and a strong positive induction upon exposure to CCL19 in patients’ derived DCs and an individual-depending pattern in healthy controls derived DCs.

The effects observed upon JNK inhibition were strengthened upon inhibition with p38.
Results

5.3 Addition of selective MAPK inhibitors from the maturation step

As expected, upon maturation, DCs lost completely or almost completely their spontaneous and CCL19-induced phagocytosis capabilities, independent of their source.

In contrast, inhibition of MEK-pathway with PD 98059 correlated with a more pronounced decrease of the phagocytosis capabilities, in comparison to the observations made for cultures added up with this inhibitor from the start. The effects of CCL19 were very individual-specific, more than source dependent.

Inhibition of the MEK-pathway, using inhibitor U 0126, showed effects similar to those observed for PD 98059 regarding the pattern and the levels of phagocytosis capability. In this case the effects of CCL19 induction were particular significant in inducing phagocytosis, in contrast with what was observed upon prolong incubation with such an inhibitor.

Fig. 22: phagocytosis potential of dendritic cells inhibited from the loading with Survivin mRNA (three patients and three controls)
These results showed that MEK inhibition efficacy depends on longer incubation and/or on addition of the inhibitor at the immature stage. Further experiments are required to monitor such effects.

JNK inhibition increased the basal levels of phagocytosis in comparison with those shown by untreated cells and a strong positive induction upon exposure to CCL19 especially in patients’ derived DCs and a more individual-depending pattern in healthy controls’ derived DCs.

The effects observed upon JNK inhibition were strengthened upon inhibition with p38.

Fig. 23: phagocytosis potential of dendritic cells inhibited from the maturation step (three patients and three controls)

6 Migration Assay (Transwell assay)

Finally, we investigated the effects of MAPKs on the migratory potentialities, which as well as the phagocytosis are 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.
6.1 Addition of selective MAPK inhibitors from the start

6.1.1 iDCs
As expected, immature DCs are particularly active in migration, do not respond to CCL19 exposure due to the absence of the respective receptor, CCR7 and as shown in the previous experiments as well, they almost do not respond to any MAPK inhibition.

6.1.2 mDCs
In contrast to iDCs, mDCs almost completely lost their migratory potentialities and almost did not respond to CCL19 exposure.

Upon inhibitor PD 98059 or with U 0126, the mDCs showed increased migration and a significant positive induction of this parameter upon incubation with CCL19.

The same effects observed upon MEK inhibition were as well observed upon inhibition of JNK and mostly upon p38-inhibition, supporting the theory that the three MAPKs work in a positive feedback loop. Both JNK and p38 inhibition showed a striking CCL19-dependent increased migration.

Fig. 24: migration of dendritic cells inhibited from the start of the culture (three patients and three controls)
6.2 Addition of selective MAPK inhibitors from the loading step

The same results observed during prolonged incubation with MAPK inhibitors were found if the respective inhibitors were added just before the loading step. In this case, however, MEK inhibition showed a more individual-like CCL19 specificity, while JNK and p38 inhibition caused increased migration upon CCL19 addition.

Fig. 25: migration of dendritic cells inhibited from the loading with Survivin mRNA (three patients and three controls)

6.3 Addition of selective MAPK inhibitors from the maturation step

The same results observed upon addition before the loading step with MAPK inhibitors were observed if the respective inhibitors were added just before maturation. In this case, the effects of CCL19 were less striking and more individual-specific.

These results showed that according to the different exposure time, MAPKs inhibition may show a different pattern of effects, suggesting that the three kinases may modulate DCs migration according to various mechanisms, which details remain presently unknown.
Results

Fig. 26: migration of dendritic cells inhibited from the maturation step
(three patients and three controls)

7 Viability assay

In order to finally investigate the role of the three MAPKs during DC maturation, we investigated as well their effects on cell viability, through 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.

Normal cell viability of untreated cells was around 75%. This rate was strongly decreased upon addition of specific inhibitors of the MAPKs. MEK inhibition through PD 98059 or U 0126 caused a reduction of cell viability of around 15-23%, with effects, which appear to have an individual-dependent component. JNK and p38 inhibition caused as well an impairment of cell viability of around 20 to 25%, respectively.

These results seem to indicate that the three analysed MAPK pathways are required for a normal cell viability of human DCs. However, due to the potential toxicity of such inhibitors,
further experiments regarding titration of the used inhibitors are needed to determine the real worth of this test.

<table>
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<th>Inhibition from the start of the culture</th>
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<td>Patients or Controls</td>
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Table 10: Percentage of viable cells (five patients and six controls)

Fig. 27: Viability assay from mature dendritic cells without or with inhibition from the start of the culture (five patients and six controls)

8 Western Blot

Due to the fact that an indirect analysis of the MAPKs pathways through cell morphology, phenotype and different functionalities showed that MEK, JNK and p38 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 are obtained by Frackelton lysis from mDCs derived from patient A, suffering from colon cancer. These cells are incubated without or with inhibitors from the start of the culture.
Results

8.1 MEK, C-Raf and B-Raf

MEK
The antibody against phospho-MEK 1/2 selectively detects phosphorylated Serine 217 and Serine 221. The size of this phosphorylated MEK 1/2 is identical to the unphosphorylated form, which is 45kDa. MEK 1/2 antibody recognizes endogenous levels of total MEK 1/2 protein with a size of 45kDa.

As expected, MEK expression did not change upon MAPK selective inhibition. On the contrary, the active forms of MEK 1/2 were strongly expressed by control cells, whereas they were strongly impaired upon inhibition with PD 98059 and absent upon inhibition with U 0126, according to the well known findings that the latter one is a stronger inhibitor of the MEK-pathway. Interestingly, also JNK and p38 inhibition caused the inhibition of MEK phosphorylation. Although the precise mechanism remains unknown, this fact further suggests the strong linkage among the three MAPKs in human DCs.

C-Raf
The C-Raf antibody is prepared from a human C-Raf immunogen of 216 amino acids length. This antibody detects the C-Raf protein, which has a size of 74kDa. Since C-Raf is strongly linked to the MEK-pathway, we investigated as well the effects of selective MAPK inhibition on the levels of such protein.

Differently from what expected, C-Raf was mildly expressed in untreated cells and in cells treated with PD 98059, while showing an increased expression upon inhibition with U 0126
Results

or JNK/p38 inhibition. The real significance of such result is unknown and requires more experiments to be either proven or rejected.

**B-Raf**

Due to the inconclusive results about C-Raf and in order to get further insights about the role of MEK in DC maturation, we investigated the expression of B-Raf upon MAPK inhibition with selective chemicals. B-Raf antibody (C-19) is raised against a peptide, which is mapped at the C-terminus of Raf-B of human origin on chromosome 7q34. It has molecular weights of 95kDa and 62kDa.

In our case, untreated DCs showed an exclusive presence of the 62 kDa form. Cells treated with PD 98059 showed a strong predominance of the 95 kDa form and a very slight presence of the 62 kDa. Cells treated with U 0126 showed levels of expression of both the 95 kDa and the 62 kDa form, comparable to those observed in untreated cells. JNK inhibition decreased the expression of the 62 kDa isoform and there was no expression of the 95 kDa. Inhibition of p38 did not affect the expression of the 95 kDa isoform and caused a minimal decrease of the expression of the 62 kDa isoform.

![Western blot analysis of Raf-B and actin](image)

MEK inhibition with U 0126 showed bands of balanced intensity at 62kDa as well as 95kDa. The JNK-inhibitor led to a decreased 62kDa band compared to the control and no B-Raf protein of 95kDa size could be detected. Cells grown with p38-inhibitor showed a band at 62kDa, which is almost equal to the control and a more intense 95kDa protein signal.

These results require further experiments in order to prove their reliability, but they seem to indicate that B-Raf expression may be modulated by all of the three analysed MAPK in different ways, which meaning is presently unknown.
Results

8.2 JNK

JNK 2/3

To detect the levels of active JNK, the phosphorylated JNK is measured by means of an antibody recognizing phosphorylation of Threonin 183 and Tyrosin 185.

SAPK or JNK antibody detects endogenous levels of total SAPK/JNK protein. The immunogen is a JNK2-GST-Fusion protein. The antibody recognizes the 46kDa protein JNK1 as well as JNK2/3 which has a size of 54kDa.

Fig. 31: Western blot analysis of pJNK vs JNK and Actin

Untreated cells showed the expression of pJNK as well as cells treated with PD 98059. On the contrary, cells treated with U 0126, JNK and p38 inhibitor showed a strongly diminished expression of the phosphorylated form of JNK. These results showed again that in human DCs all the three MAPK pathways are required for the maturation, that they work in the same direction, i.e. final cell maturation, and that they are probably linked by positive feedback loops.

8.3 p38

p38 antibody recognizes the C-terminus of human p38 protein.

The active form of p38 carries a phosphorylated Tyrosin at the position 182 in the p38 amino acid sequence.

Fig. 32: Western blot analysis of p-p38 vs p38 and Actin
Untreated cells showed an expression of p38 as well as cells treated with PD 98059, although at very decreased levels. On the contrary, cells treated with U 0126, JNK and p38 inhibitor showed almost no expression of the phosphorylated form of p38. Shortly, the expression pattern of p-p38 mimicked that of pJNK, but to increased levels. These results showed again that in human DCs all the three MAPK pathways are required for the maturation, that they work in the same direction, i.e. final cell maturation, and that they are linked probably by positive feedback loops, which seem to affect mostly p38 phosphorylation, pointing out a possible more critical role for this MAPK in comparison with the other two.
Discussion

The aim of this thesis has been the investigation of the role of three MAPKs pathways, i.e. ERK, JNK and p38 during the *in vitro* maturation of monocyte-derived DCs, derived from healthy controls’ versus cancer patients’, in order to determine their function according to the different stages of DC maturation and according to the health state of the single individual.

Our results showed that all three pathways are of paramount importance for the right maturation course. Indeed, once inhibited, each of them caused a striking impairment of DC maturation regarding both phenotype and functionality. Completely unexpected was the finding that inhibition of MEK by both, PD 98059 or U 0126, caused always a strong impairment of DC maturation, according to the results of analyses of cell morphology, surface markers and functionality. On the other side, as expected, the inhibition of either JNK or p38 led to the same effect.

It appears that the three analysed kinases, during MoDCs differentiation, work together and interact through positive feedback loops. Anyhow, further experiments in this direction are required to shed new light about these pathways in the human DC differentiation. We did not observe striking differences between the effects of the three MAPKs inhibition according to their source (healthy controls or cancer patients). These results are in agreement with the fact that DCs derived from different sources were always *in vitro* cultured under conditions, set to induce a normal DC maturation from cancer patients’ derived monocytes. Therefore, to determine the real influence of cancer on MAPKs activities, other cell culture conditions may be eventually tested.

On the other side, the effects of MAPKs inhibitions showed a clear individual-specific pattern in most cases. However, since the number of samples used for the analyses did not reach a statistical significance and patients with different types of cancer were investigated, the real significance of this parameter must be further ascertained.

In any case, inhibition of all three MAPK caused a complete inhibition of the morphological maturation of human DCs. Indeed, the cells fully lose the capability to develop dendrites and become adherent, while keeping the round shape and the non-adherent characteristic, typical for immature DCs. These observations were confirmed by the phenotypical analysis of cell
surface parameters. Again, the selective inhibition of any MAPKs correlated with a strongly decreased amount of mature markers on DCs membrane. These results about the phenotype were mirrored by those obtained about cell functionality. Indeed, if on one hand, DCs selectively treated with MAPK inhibitors showed a severe impairment in IL-12 secretion and in the induction of allogenic T-cell proliferation, two typical hallmark of DC maturation, on the other hand, they showed increased levels of migration and phagocytosis as well as higher IL-10 production, features, which correlate with the establishment of an immature state.

Cell viability was mildly affected by any selective MAPK inhibition. These data may suggest that in this particular case, the three analysed pathway play minor role in cell viability, but further experiments must confirm or refute this finding.

This work aimed to analyse as well the role of the three MAPKs according to different maturation stages, therefore the inhibitor were added at the start, before performing the loading step and just before inducing maturation. The effects of selective inhibition on iDCs was negligible, suggesting that the three pathways are mostly crucial during loading and upon maturation induction, possibly driving or at least modulating the response to such stimuli.

Interestingly, the effect of PD 98059 or U 0126 reached different intensity according to each parameter analysed. Indeed, if PD 98059 addition mostly affected the expression of mature surface markers on DC membrane, U 0126 mostly stimulated IL-10 production and inhibited the induction of allogenic T-cells proliferation. These data suggest that distinct ERK-linked pathways are differently involved in DC maturation. Again, further experiments are required to explore this possibility.

On the other side, both JNK and p38 appeared to be mostly involved in the stimulation of DC maturation from the phenotypical and functional prospective. In any case, p38 seems to induce stronger effects in comparison to JNK inhibition.

One interesting finding regards the levels of IL-10 and IL-12 secretion upon selective inhibition of the MAPKs. Indeed, it seems that MEK inhibition induced IL-10 production while JNK or p38 inhibition impaired IL-12 secretion. These data suggest a possible positive feedback loop supporting physiological DC maturation: while ERK pathway activation
impairs IL-10 production, the contemporaneous activation of JNK and p38 pathway induces the secretion of IL-12, which in turn stimulates DCs activity upon CTL response.

A further striking finding is that upon PD 98059 addition, although it was observed only a partial inhibition of IL-12 secretion in absence of IL-10 production, we could show a very strong effect on the impairment of the induction of an allogenic stimulus. These data suggest that the induction of lymphocyte proliferation may be only partially dependent on IL-12 secretion, while being more dependent on the morphology and the phenotype of the cells. This finding would be in accordance with Redy et al., who showed the importance of DCs phenotype for the stochastic encounters with the lymphocytes, in order to stimulate the proliferation of the latter ones. In this case, IL-12 levels will activate the lymphocytes only once they come in touch with the DCs, through the dendrits.

In conclusion, this work showed that ERK, JNK and p38 pathways play a paramount role in phenotypical and functional maturation of human DCs. The details of the mechanisms underlying the establishment of the mature state are presently unknown and further investigations are required in this direction. However, we could show preliminary results, suggesting new interesting trails which may lead to a better knowledge of a type of immune system cells, known to be particularly effective against cancer, if correctly activated against specific tumour antigens.
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CURRICULUM VITAE

PERSONAL HISTORY:
Name: Marianne Raith
Place and date of birth: Hollabrunn, 6th of April 1978
Address: A-2023 Nappersdorf 30
Mail: Marianne-Raith@gmx.at
Nationality: Austria

EDUCATION:
October 2000: Diploma „Medical Technician“ passed with distinction
1997-2000: School for medical technicians at the General Hospital of Vienna
1992-1997: Handelsakademie, Hollabrunn (commercial academy), passed with distinction
1984-1988: Volksschule Nappersdorf (primary school)

WORKING EXPERIENCE:
7/2000 to 10/2002: Medical University Vienna, Clinical Institute of Medical and Chemical Laboratory Diagnostics
“The role of E-selectin polymorphism in the pathogenesis of diabetes-associated microangiopathy”
Direction: Oswald Wagner, MD; Markus Exner, MD

10/2002-9/2004: Medical University Vienna, Clinical Institute of Medical and Chemical Laboratory Diagnostics
“Heme oxygenase-1 and atherosclerosis”
Direction: Markus Exner, MD

9/2004-4/2006: Medical University Vienna, Clinical Institute of Medical and Chemical Laboratory Diagnostics
“The role of Hedgehog signaling pathways in human and mouse”
Direction: Harald Esterbauer, MD, PhD

5/2006-2/2007: Medical University Vienna, Clinical Institute of Medical and Chemical Laboratory Diagnostics and CEMM, Center of Molecular Medicine
“The role of natural antibodies in atherosclerosis”
Direction: Christoph Binder, MD, PhD

“The role of MAPK pathways in in vitro maturation of dendritic cells, cultured from patients’ peripheral blood-monocytes”
Direction: Wolfgang J. Schneider, PhD; Cristina Rubiolo, PhD