Molecular Characterization of the Immune Modulatory Function of Matrix Metalloproteinase-7, a Factor in the Tumour Micromilieu

Dissertation

Zur Erlangung des Grades

Doktor der Naturwissenschaften

Am Fachbereich Biologie

der Johannes Gutenberg-Universität Mainz

Vijay Alla

geb. am 28. August 1978 in Indien

Mainz, June 2007

Table of Contents

1 Introduction

1.1 Tumourigenesis

In normal organism, the growth and differentiation of cells are strictly controlled at a proper level suitable for each stages of ontogenesis. If the cell growth gets out of control, tumours may emerge. Tumours can be classified as either benign or malignant. The malignant tumours or cancers are characterized by their ability to spread by invasion and metastasis in contrast to benign tumours. According to their tissue origin, cancers can be classified as carcinomas which are derived from endodermal or ectodermal tissues (e.g. dermatomas and adenomas), leukemia and lymphomas derived from hematopoietic cells, and sarcomas derived from mesodermal tissues such as osteomas. Cancers are one of the most potentially life-threatening diseases today.

In general, the uncontrolled, mostly rapid cell division is caused by damage to DNA, resulting in mutations to genes that encode for proteins controlling cell division. These mutations can be caused by environmental factors such as radiation, chemicals or physical agents that cause cancer (carcinogens), or by certain viruses that can insert their DNA into the human genome. Mutations occur also spontaneously with aging of the cells. The altered or mutated cells are normally eliminated by different mechanisms including apoptosis and immunesurveillance. However, cancer cells often triumph easily over the body's defenses. Gene fusion, gene amplification and gene silencing promote tumour survival, proliferation, and angiogenesis.

1.2 Apoptosis

Apoptosis or programmed cell death is a tightly controlled process of cellular self destruction characterized by a well choreographed sequence of morphological and biochemical events. The term apoptosis was coined by Kerr to describe a morphologically distinguished form of cell death compared to necrosis, in which uncontrolled cell death leads to lysis of cells, inflammatory responses and potentially to serious health problems (Kerr *et al*., 1972). The dying cells undergo chromatin condensation and cell shrinkage

with blebbing of the plasma membrane and breaks up eventually into membrane-enclosed particles termed apoptotic bodies containing intact organelles, as well as portions of the nucleus. These apoptotic bodies are then rapidly recognized, ingested and degraded by professional phagocytes or neighbouring cells. As apoptosis typically does not araise inflammation or tissue scarring, it is well suited for a role in normal cell turnover during embryogenesis and for maintaining homeostasis in adult tissues (Jacobson *et al*., 1997; Steller, 1995; Wyllie, 1980). In some cases, the induction of Apoptosis by cytotoxic T lymphocytes (CTL) or natural killer (NK) cells is also one of the main mechanisms for eliminating virus infected cells, aged cells, genetically mutated cells, or posing a risk of cancer (Pinkoski *et al*., 2001; Shi, 2004; Sutton *et al*., 2000).

1.2.1 Signalling pathways of apoptosis

Two main pathways of caspase-mediated cell death have been described in mammals. The extrinsic or death receptor mediated pathway plays a fundamental role in the maintenance of tissue homeostasis, especially in the immune system, whereas the intrinsic, mitochondria-dependent pathway is used extensively in response to extracellular cues and internal insults such as DNA damage (Danial and Korsmeyer, 2004). In addition, a caspase independent pathway in cells killed by CTLs was reported (Pinkoski *et al*., 2001; Sutton *et al*., 2000).

1.2.1.1 Death receptor mediated pathway

Death receptors belong to tumour necrosis factor receptor (TNFR) super family. Till now five death receptors are identified: CD95 (Fas/Apo1), TNF receptor-1 (TNF-R1/Apo2), TNF related apoptosis inducing ligand receptor-1 (TRAIL-R1 or DR4), TNF related apoptosis inducing ligand receptor-2 (TRAIL-R2), and TNF receptor related apoptosis mediated protein (TRAMP). Among these, CD95 and TNFR are the best characterized death receptors.

Death receptors consist of one or more extracellular cysteine rich domains (CRDs) for

binding of ligand, and a distinct set of modular protein motifs capable of homotypic interaction, including death domains (DD) and death effector domains (DED).

The death receptor mediated pathway is triggered by binding of specific ligands to their receptors, followed by activation of a caspase cascade. Caspases are cysteine proteases that cleave after certain aspartate residues. All caspases are maintained as a zymogen and activated by proteolytic cleavage (Shi, 2004). The extracellular part of CD95 e.g. contains

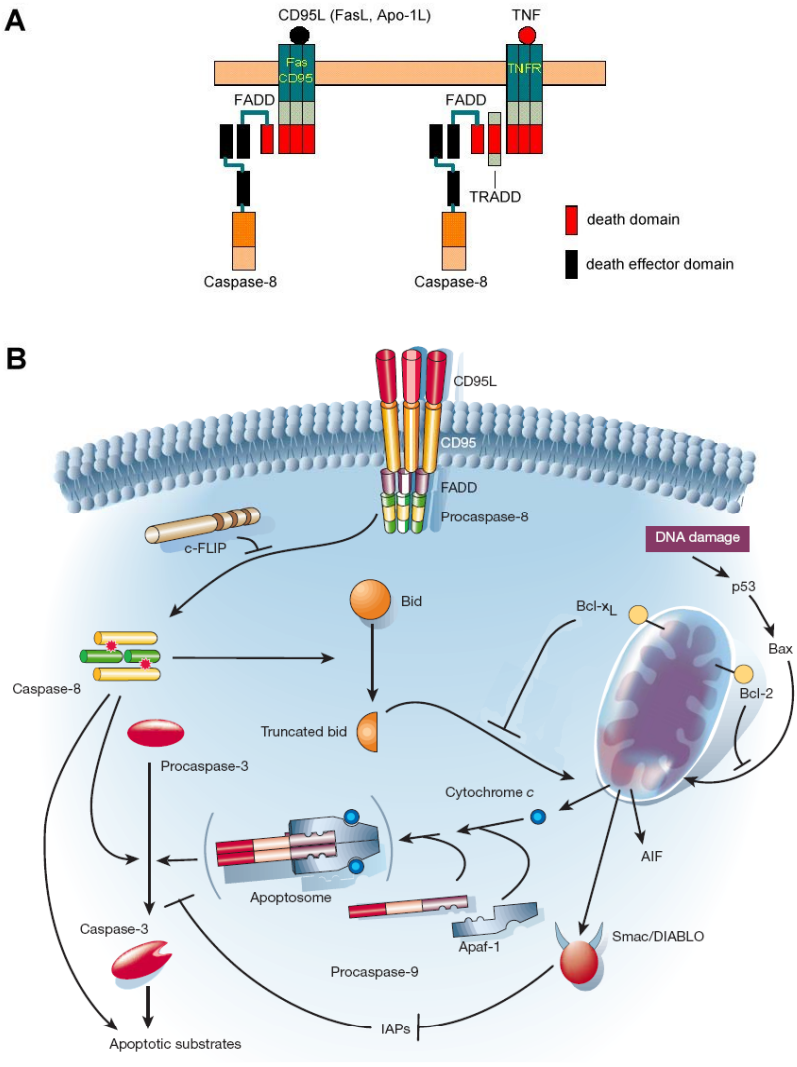


Figure 1: The main signalling pathways of apoptosis

A: Structure of death inducing signalling complex (DISC, adapted from *Web-Books.Com*).

B: Death receptor-mediated (left) and mitochondrial (right) pathway of apoptosis (adapted from (Hengartner, 2000).

three CRDs. The binding of CD95 ligand (CD95L) to CD95 induces trimerization of CD95 which recruit an adaptor named FADD (Fas associated death domain) via their death domains. FADD recruits then procaspase-8 via their death effector domains to form a complex termed death inducing signalling complex (DISC), resulting in cleavage of procaspase-8 to release active caspase-8 (Kischkel *et al*., 1995). For TNFR, the formation of DISC needs one more adaptor called TNF-R associated death domain (TRADD, Figure 1A). Some caspases like caspase-8 are involved in the initiation phase and thus called initiator caspases. Initiator caspases have a long prodomain, and their autocatalytic activation is triggered by dimerization of the zymogen on a dedicated adaptor or scaffold protein (Boatright *et al*., 2003). The active initiator caspases cleave then the "executioner" caspases such as caspase-3, which in turn activates caspase-6 and caspase-7. Finally, caspase-6 and caspase-7 cleave their cellular substrates inducing the cell death (Figure 1B, left).

1.2.1.2 Mitochondrial pathway

Mitochondria are not only required for ATP production, but also play a central role in apoptosis (Danial and Korsmeyer, 2004). The mitochondrial pathway (Figure 1B, right) is used extensively in response to various extracellular cues and internal insults such as DNA damage (Decker and Muller, 2002), caused by irradiation (Zhan *et al*., 2002) or drugs used for cancer chemotherapy. According to current understanding, such stress stimuli promote binding of the proapoptotic Bcl-2 family member Bid and possibly other cytosolic BH3-only polypeptides (Gross *et al*., 1999) to the mitochondrial proapoptotic Bcl-2 family members Bax and Bak, which in turn facilitate release of cytochrome c and other loosely bound proteins of the mitochondrial intermembrane space. Once released to the cytosol, cytochrome c binds to the cytoplasmic scaffolding protein Apaf-1, causing an ATP- or dATP induced conformational change in Apaf-1 (Budihardjo *et al*., 1999; Earnshaw *et al*., 1999). Apaf-1 then allosterically activates procaspase-9 forming a complex known as apoptosome (Rodriguez and Lazebnik, 1999). Finally, the activated caspase-9 proteolytically activates procaspase-3 and procaspase-7.

Furthermore, the mitochondrial apoptosis signalling can also be operated through caspase independent pathways, e.g. by release of apoptosis inducing factor (AIF) or endonuclease EndoG from mitochondria. AIF and EndoG translocate to the nucleus where they participate in DNA degradation (Li *et al*., 2001; Susin *et al*., 1999).

The death receptor mediated and mitochondrial pathways converge at the level of caspase-3 activation. Crosstalk and integration between these two pathways is provided by Bid. Caspase-8 mediated cleavage of Bid greatly increases its pro-death activity, and results in its translocation to mitochondria, where it promotes cytochrome c exit. However, under most conditions, this crosstalk is minimal, and the two pathways operate largely independently of each other (Gross *et al*., 1999; Yin *et al*., 1999).

1.2.1.3 Other pathways

In addition to the two major pathways involving caspase activation, several other pathways for activation of apoptosis in mammalian cells have been described. For instance, cytotoxic T cells (CTLs) trigger apoptosis of susceptible target cells either through a CD95 mediated or perforin/granzyme B dependent pathway. The latter pathway involves a pore forming protein, perforin and a serine protease, granzyme B, which is injected into the cytoplasm of the target cell upon degranulation of the activated CTLs (Sutton *et al*., 2000; Trapani and Smyth, 2002). Granzyme B can directly cleave and activate procaspase-3, thereby initiating the apoptosis cascade in the target cell, or cleave other substrates such as the Bcl-2 family member Bid, resulting in cell death (Barry *et al*., 2000; Goping *et al*., 2003). This pathway operates mainly in viral defense and immune surveillance against cancer, but recent studies in mice and in humans with inherited perforin mutations indicate that perforin dependent killing may also play a crucial role in the maintenance of immune homeostasis (Jordan *et al*., 2004; Stepp *et al*., 1999).

Furthermore, recent studies show that CTLs also utilize other serine proteases to trigger apoptosis of target cells. Granzyme A cleaves components of the so called SET complex to release and activate granzyme A activated DNase (GAAD), which then translocates to the nucleus to initiate DNA damage, whereas granzyme C acts through yet another caspase

independent pathway (Johnson *et al*., 2003; Martinvalet *et al*., 2005). CTLs thus employ several auxiliary pathways of apoptosis induction, to ensure that harmful cells are eliminated.

Finally, a novel cell-to-cell transmission of a lethal (proapoptotic) signal has been proposed to occur in acquired immune deficiency syndrome (AIDS). Early studies suggested that inappropriate induction of apoptosis could account for both qualitative and quantitative helper T cell defects of human immunodeficiency virus (HIV) infected patients (Ameisen, 1992; Ameisen and Capron, 1991); however, whether apoptosis is a direct effect of viral infection or is due to bystander killing of noninfected cells has remained a matter of debate. Andreau and his associates have recently proposed that the HIV envelope glycoprotein (Env) triggers a 'contagious' form of apoptosis in which cells expressing the Env protein fuse with CD4 expressing target cells resulting in syncytium formation and caspase dependent nuclear alterations affecting both the donor and acceptor cell (Andreau *et al*., 2004). These data thus point to a novel mechanism through which HIV could induce bystander killing in AIDS patients.

1.2.2 Regulation of apoptosis

Under normal conditions, apoptosis is tightly regulated to meet the needs of development or homeostasis. Numerous factors are known to affect apoptosis. The output depends therefore on the balance between the pro- and anti-apoptotic factors.

1.2.2.1 CD95 and CD95 ligand

CD95 ligand (CD95L) is a member of the tumour necrosis factor (TNF)/nerve growth factor family which induces apoptosis when binds with its CD95 receptor. CD95L is expressed by a variety of cells but the expression is abundant in the lymphatic cells. When compared to the CD95 the expression of CD95L is restricted to the specific tissues like the activated T cells, testis and in the eye. CD95L expression is highly regulated at the transcription level. Transcription factors such as NF-κB, c-Myc and forkhead transcriptional regulator are involved in the expression of CD95L (Kavurma and

Khachigian, 2003). In classical lpr mutant mice, a point mutation in the C terminal of the CD95 abolishes to bind to FADD and leads to the inhibition of apoptosis. CD95 null mice showed lymphoadenopathy and splenomegaly which shows that the CD95 system is involved in the death of T cells. CD95L is involved in the immune privilege of certain tissues by killing the T cells through binding to CD95 and graft rejection studies demonstrated that CD95L is important for the killing of the donor cells by CD95 mediated apoptosis (Griffith *et al.,* 1995). CD95L mutated gld/gld mice are incapable of inducing apoptosis in the immune privileged sites. The expression of the CD95L is high in the T cells and plays an important role in clonal selection of the T cells (Green *et al.,* 2003). CD95L is crucial in the execution of the target cell killing by the Cytotoxic T cells, in which CD95L binds to the CD95 of the target cells and induces apoptosis. Mutation in the CD95L leads to the changes in apoptosis and subsequently to autoimmune diseases. CD95 is important in the regulation of the T cell proliferation after the activation and mutations or loss of CD95 leads to the autoimmune diseases. The expression of CD95 is increased in the viral infected cells and other damaged cells which leads to the apoptosis. Many tumour cells exhibit the loss of CD95 resulting in escape from CD95 mediated apoptosis.

1.2.2.2 Caspase activity

The death receptor mediated and mitochondrial pathways for caspase activation converge on downstream effector caspases, which ultimately results in apoptotic cell death. Since caspases play a central role in regulation and execution of cell death, they must be tightly regulated. Regulation can occur by either inhibiting caspase activity, or by blocking its activation. Caspases are subject to regulation by a family of proteins termed inhibitor of apoptosis proteins (IAP) including XIAP, cIAP1, cIAP2, hILP-2, ML-IAP, NAIP, survivin and apollon.

A key domain present in all members is a baculovirus IAP repeat (BIR), a 65 residue domain rich in histidine and cysteine residues, which acts in concert with the flexible linker N-terminal to the BIR domain binds to the substrate groove of caspases -3 and -7, thus blocking access of the protein substrate to the active caspase. Inhibition of caspase-9 has a

distinct molecular basis relying on direct interaction of the BIR3 domain of X linked IAP (XIAP). Mammalian IAPs are in turn, subject to regulation in several ways, including binding to Smac (second mitochondrial activator of caspases)/DIABLO (direct IAP binding protein with low pI) or Omi/HtrA2, two intermembrane space proteins that are released from the mitochondrion into the cytosol during mitochondrial apoptosis signalling (Du *et al*., 2000; Suzuki *et al*., 2001; Verhagen *et al*., 2000). These molecules antagonize IAP inhibition of caspases, thereby promoting caspase activation, while cytochrome c acts as a direct cofactor for caspase activation; Smac is an indirect facilitator of caspase activation, by alleviating caspase inhibition. Also, present in some IAPs is a RING domain located at the carboxy terminus, which functions as an E3 ubiquitin ligase to provide specificity of transfer of ubiquitin moieties to the target protein.

Caspases can also be regulated by blocking their activation. For example, the c-FLIP protein (cellular FLICE inhibitory protein), a caspase-8 homolog lacking proteolytic activity, can block caspase-8 activation. C-FLIP possess DEDs at their N-termini and can be recruited to the DISC, and under conditions of overexpression, can prevent caspase-8 activation (Irmler *et al*., 1997).

Heat shock proteins act at multiple steps in the pathway to modulate apoptosis, Hsp70 interacts with the Apaf-1 and prevents the apoptosome formation (Takayama *et al*., 2003). Studies also revealed that heat shock proteins are involved in the activation of caspases for example recombinant Hsp60 and Hsp10 accelerated the activation of procaspase-3 by cytochrome c and dATP in an ATP dependent manner which suggests that the Hsp may accelerate caspase activation (Samali *et al*., 1999). Hsp70 and Hsp40 are responsible for the proper folding of DNase CAD (caspase activated DNase) also known as DEF 40 (DNA fragmentation factor 40) which digests the chromosomal DNA which is an important event during apoptosis (Sakahira and Nagata, 2002).

1.2.2.3 Bcl-2 protein family

The Bcl-2 protein family constitutes a critical checkpoint in the mitochondrial pathway of apoptosis. The Bcl-2 family includes anti-apoptotic as well as pro-apoptotic proteins and

can be divided into three main subgroups based on the homology shared within four conserved regions termed Bcl-2 homology (BH) domains. The anti-apoptotic members include Bcl-2, Bcl-X_L, Mcl-1 and many others, and display conservation in all four BH domains, whereas the multidomain pro-apoptotic members (such as Bax and Bak) posses BH1–3 domains. The third group includes those having only the BH3 domain, such as Bid (Gross *et al*., 1999).

Recently, Bax and Bak were shown to constitute a requisite gateway to the mitochondria dependent pathway of apoptosis (Wei *et al*., 2001). The anti-apoptotic Bcl-2 proteins (e.g. Bcl-2 and Bcl-X_L) are often found in the cytosol where they act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria and inhibit the effect of pro-apoptotic proteins like Bax, probably by formation of heterodimers with the latter (Adams and Cory, 1998; Antonsson and Martinou, 2000; Reed, 1997). In contrast, members of the 'BH3-only' subgroup appear to serve as upstream sensors that respond to specific death signals. For instance, the BH3-only protein Bid is cleaved by caspase-8 during death mediated apoptosis signalling and serves to engage a mitochondrial amplification loop in certain cell types (Danial and Korsmeyer, 2004). BH3-only molecules appear to require Bax and/or Bak for execution of cell death, whereas anti-apoptotic Bcl-2 family members serve mainly to sequester BH3-only molecules, thereby preventing Bax/Bak activation and release of apoptogenic factors from mitochondria. Therefore, the BH3-only proteins are believed to play the initiating role in signalling pathways involving Bcl-2 proteins.

1.2.2.4 p53 protein

The tumour suppressor protein p53 is the first identified and the most important gene which regulates apoptosis (Fridman and Lowe, 2003). p53 serves as a switch in the mitochondrial pathway to decide the fate of the damaged cells. p53 is a sequence specific nuclear transcription factor that binds to defined consensus sites within DNA as a tetramer and affects the transcription of its target genes (el-Deiry, 1998).

In normal unstressed cells, p53 protein is kept in a latent form via the binding of a negative

regulator protein named MDM2, which hinders the nuclear export and promoting the degradation of p53 (Haupt *et al*., 1997). Upon DNA damage or other stress, various pathways, will lead to the dissociation of the p53/MDM2 complex (Stommel and Wahl, 2005). On the other hand MDM2 gene itself is a transcriptional target of p53 which suggests a self regulation of p53 by negative feedback (Barak *et al*., 1994). Stress activated protein kinases (e.g. the DNA dependent protein kinase activated by DNA damage) phosphorylates p53, protecting it from degradation and activating its function as a transcripton factor. Once activated, p53 will either induce a cell cycle arrest at G1 phase to allow DNA repair and survival of the cell, or, if the damage should no more be repairable, apoptosis will be triggered to eliminate the damage cell, preventing the fixation of DNA damage as mutations. Therefore, p53 has been called the ‘‘guardian of the genome’’ because of its function in ensuring the genomic integrity (Lane, 1992).

However, how p53 make this choice is currently still not fully understood. Several factors influencing the response of the cell have been suggested: cell type, oncogenic cell composition, intensity of stress, level of p53 expression, interaction of p53 with specific proteins and affinity of p53 for promoters (Vousden and Lu, 2002).

A large number of genes are known to be downstream target of p53 which are involved in cell cycle control, DNA repair, apoptosis, angiogenesis, and in cellular stress response. The protein p21 is a cyclin dependent kinase (CDK) which has multiple functions including mediation of cell cycle arrest, inhibition of other CDKs and inhibition of DNA replication. Studies also suggest that p53 is involved in the angiogenesis inhibition by the transcription of thrombospondin. Pro-apoptotic protein Bax is a transcriptional target of p53 and by promoting the expression of Bax, p53 contributes to the apoptosis. The expression of Noxa, a BH3 only member of the Bcl-2 family is also upregulated by p53 leading to apoptosis (Oda *et al*., 2000). Forced expression of wild type p53 leads to the upregulation of CD95 receptor which suggests the role of p53 in enhancing CD95 expression.

1.2.3 Apoptosis in cancer and other diseases

Many diseases have been found to be related to abnormality in the rate of apoptosis. For

example, the mutation of p53 leads to partial or complete loss of apoptosis is the main cause of cancer development in human (Vogelstein *et al*., 2000). In addition, many factors decreasing apoptosis via cleavage of death receptors or increasing the expression of anti-apoptotic genes such as FLIP are involved in the carcinogesis. Alzheimer's disease is due to the excessive apoptosis. Studies show that the upregulation of apoptosis is associated with HELLP syndrome (Strand *et al*., 2004a). Apoptosis plays an important role in the T cell development, alteration or loss of apoptosis leads to the lymphoproliferative diseases. Furthermore, enhanced apoptosis mediated by CD95 was found in many liver diseases like Wilson's disease (Strand *et al*., 1998), the alcoholic liver damage and viral hepatitis (Galle *et al*., 1994; Galle and Krammer, 1998) and the primary hepatocytes show a high apoptosis sensitivity after *ex vivo* stimuation with agonistic anti-CD95 antibody (anti-APO-1) (Galle *et al*., 1995).

1.3 Extracellular matrix and MMPs

1.3.1 Extracellular matrix

In multicellular organisms, the cells in a tissue or organ are surrounded by a complex network of proteins termed "Extra cellular matrix" (ECM). ECM consists of different kinds of proteins including collagen, fibrin, laminin, elastin, gelatin etc. ECM provides the mechanical support and structural integrity for tissues or organs and plays an important role in cell differentiation and tissue development. Matrix proteins bind to the integrin receptors and alters the cell function by transmitting extracellular signals (Hynes, 1987; Hynes, 1992). ECM helps in the formation of focal adhesions by tether actin stress fibers to the cytoplasmic face of the plasma membrane and helps in the process of cell motility (Burridge *et al.,* 1988; Horwitz and Parsons, 1999). Different cytoskeletal reorganization is the result of interaction between various ECM proteins with cell surface integrins which influence the rate of endocytosis of some surface receptors (Boura-Halfon *et al.,* 2003). The form of the ECM varies based on the role of the tissue, for example the ECM is hard in the bone and teeth because of the presence of the mineral components in the ECM. The morphology and cellular functions are greatly influenced by the ECM, for example

morphogenesis takes place based on the changes in the ECM. During embryogenesis, changes takes place in the ECM which helps in cell differentiation and ultimately leads to the tissue development (Martin-Bermudo and Brown, 2000).

Many changes in the ECM takes place during pathological situations like cancer and modifications in the ECM takes place during angiogenesis also (Davis and Senger, 2005). Metastasis is one of the crucial steps in the progression of cancer, normally each organ is compartmentalized from the others through ECM and breakdown of ECM is necessary for the cancer cells to invade to other tissues. Matrix metalloproteinases are one of the important groups of enzymes involved in the dissociation of ECM by cleavage of extracellular matrix proteins.

1.3.2 Matrix metalloproteinases (MMPs)

MMPs are a family of zinc dependent metalloproteinases which cleave the matrix proteins as the name indicates (McCawley and Matrisian, 2000). Till date there are 28 members identified which belongs to this family. Based on their structure, substrate specificity and distribution, MMPs are divided into different subclasses like collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9) and stromeolysins (MMP-3, -10, -11). Membrane bound MMPs build up an another class in contrast to the secreted MMPs (Goffin *et al.,* 2003).

ECM consists of many proteins which helps in the cell binding and by cleaving the ECM, MMPs helps in the migration of the cells to the surrounding tissues. MMPs are expressed in normal physiological as well as pathological conditions (Galis and Khatri, 2002). Over expression of MMPs were observed in many types of tumours like mammary, colon, ovarian, liver etc (Izawa *et al.,* 2000). MMPs play an important role in cellular functions like differentiation, tissue remodeling, embryogenesis and wound healing. These proteinases have some common substrates, mostly the matrix proteins like collagen but they act on other substrates more specifically. In healthy situation the expression of the MMPs is tightly regulated and most of these genes are inducible by growth factors, cellular stress etc.

1.3.3 Structure of MMPs

Most of the MMPs are synthesized as inactive zymogens (or proMMPs). The most common domains of MMPs are signal peptide, propeptide domain (prodomain), catalytic domain, hinge and hemopexin like domain (Figure 2). The catalytic domain of MMPs is responsible for the cleavage of substrates and its structure is conserved in most of the MMPs (Massova *et al.,* 1998). The prodomain located at the N-terminus is responsible for the latency of MMPs. The cysteine residue present in the prodomain interacts with the zinc atom in the catalytic domain and keeps the enzyme inactive. Other domains of MMPs are important for their substrate specificity or affinity to TIMPs (tissue inhibitors of metalloproteinases). For example, collagenases lacking the hemopexin domain are incapable to cleave collagens but can still cleave other substrates (Chung *et al.,* 2004), and truncated stromeolysins which lacks the hemopexin domains binds less efficiently to the TIMPs than the full length stromeolysins (Baragi *et al.,* 1994).

MMP-7 (Matrilysin), consisting of 267 amino acids, belongs to the "minimal domain containing MMPs" because of the lack of the hemopexin like domain at the C-terminal end. MMP-7 is also called "putative matrix metalloproteinase-1" (PUMP-1) due to the absence of hemopexin domain which is present in all other MMPs. Because of lacking the hemopexin domain, MMP-7 binds less efficiently to TIMPs in comparison to other MMPs (Halpert *et al.,* 1996).

1.3.4 Transcriptional regulation of MMPs

The expression of the MMPs is tightly controlled to low level under normal physiological conditions and increased by various growth factors as well as in situations like the tissue repair, embryogenesis etc.

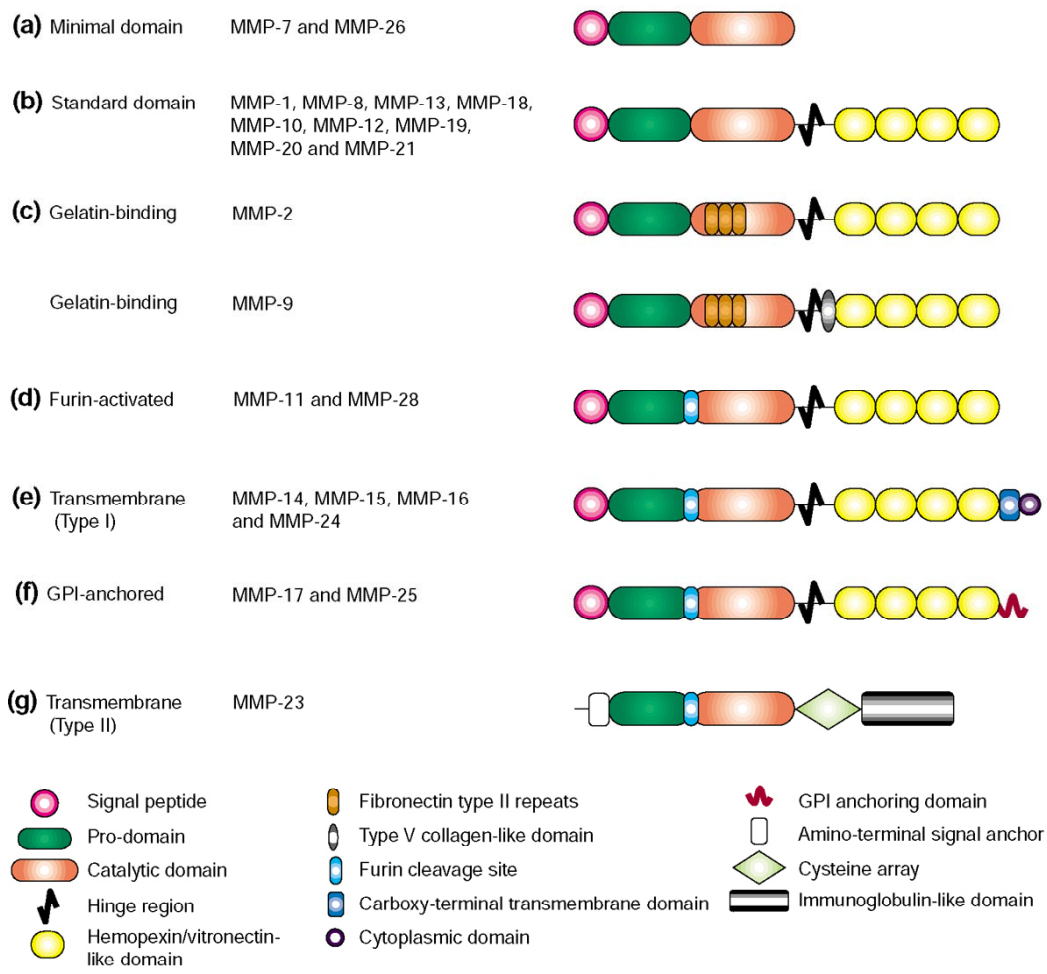
Previous studies show that the promoter regions of the MMPs contain cis acting elements such as NF-κB site, Sp-1 and activator protein-1 (AP-1) sites (Jones *et al.,* 1999). AP-1 are the strongly involved and most studied transcriptional factors regarding to the expression of MMPs. AP-1 binding sites are found in the promoter regions of MMP-1, -3, -7, -10, -11,

-12 (Westermarck and Kahari, 1999). The expression of MMPs is regulated by the AP-1 transcriptional factors such as c-fos, c-jun, c-myc (Benbow and Brinckerhoff, 1997; Hu *et al.,* 1994). Growth factors such as epidermal growth factor and platelet derived growth factor influence the expression of MMPs (Hu et al., 1994; Shima et al., 1993). Previous studies suggest that the expression of MMPs is also regulated by the tumour microenvironment which is a great source for stromal and infiltrating inflammatory cells (Uria *et al.,* 1997). Ets transcription factors also regulate the expression of MMPs (Bolon *et al.,* 1995; Ozaki *et al.,* 2003). Previous studies show that E1AF, Ets related transcription factor is also involved in the regulation of the MMPs (Yamamoto *et al.,* 2004). The binding sites for Ets and its related transcription factor, PEA3 elements are present in the promoter region of some MMPs. The PEA3 promotes the expression of MMPs through the β-catenin dependent pathway. The AP-1 and Ets binding sites are present in the same region of the MMPs promoters (Crawford and Matrisian, 1996).

The expression of the MMP-7 is regulated by many factors like the inflammatory cytokines and growth factors such as the fibroblast growth factor (Udayakumar *et al.,* 2002). Studies indicated that the *Helicobacter pylori* can also induce the expression of MMP-7, mediated by the small GTPase of Rho family through the activation of transcription factors NF-κB and AP-1 (Wroblewski *et al.,* 2003). PEA3, an Ets related transcription factor also regulates the expression of MMP-7. The expression of MMP-7 is also mediated through the transcription factor STAT3. The most important transcriptional regulation of the MMP-7 is by the Wnt or β-catenin pathway. The promoter region of the MMP-7 contains two TCF binding sites which can be activated by β-catenin (Brabletz *et al.,* 1999). Mutations in the adenomatosis polyposis (APC) occur in most of the MMP-7 producing tumours which gives the clear indication that the APC and MMP-7 are interlinked. β-catenin is a transcriptional coactivator which binds with the transcription factors like TCF and helps in the transcription of the regulated genes. In normal cells APC regulates the expression of MMP-7 by degrading the β-catenin, in most of the intestinal cancers mutations in APC occurs and leads to the inability of the APC to degrade β-catenin. The regulation of MMP-7 by β-catenin is also dependent on the type of tissue for example in the pancreatic cancer tissues where the over expression of MMP-7 has no correlation with the expression of

β-catenin.

MMP-7 is constitutely expressed in some epithelial tissues and also helps in the tissue repair. Over expression of MMP-7 is observed in colon, gastric, pancreatic, breast, hepatocellular and renal cell carcinoma (Kitoh *et al*., 2004; Miyata *et al*., 2006; Yamamoto *et al*., 1997). Early production of the MMP-7 by tumour cells provides the evidence for the



Figuer 2 Diagrammatic structure of MMPs (Massova et al., 1998)

role of MMP-7 in the early stage tumour development (Fingleton *et al.,* 1999). MMP-7 has a unique importance as it is produced by the tumour cells but not by the mast cells. Knockout studies suggest that 67% reduction in the tumour formation in the MMP-7 null mice (Wilson *et al.,* 1997). Besides the cancer, MMP-7 is also expressed in the

inflammatory diseases like the ulcerative colitis and the degree of expression of MMP-7 shows the severity of the disease (Matsuno *et al.,* 2003; Newell *et al.,* 2002). MMP-7 also plays a role in the formation of artherosclerotic lesions.

1.3.5 Activation of MMPs

The other important level for the upregulation of MMPs is the activation of MMPs. Only a few MMPs are already active after *de novo* synthesis. Most of the MMPs, including MMP-7, are activated by proteolytic cleavage of the prodomain by different proteases after secretion. Some MMPs are activated through cleavage by other MMPs, for example MMP-9 cleaves and activates MMP-2, and some MMPs can be activated autolytically.

MMP-7 is secreted in an inactive form. Like other MMPs, the prodomain present in the MMP-7 causes its latency. The cysteine amino acid present in the prodomain makes the MMP-7 inactive. Modification of this bond by the chemical treatment leads to the activation of MMP-7 (Fu *et al*., 2001). Mutational studies at the cysteine aminoacids present in the prodomain of MMP-7 showed the activation because of the inability of the mutated protein to form cystine bond. After secretion MMP-7 becomes active due to the cleavage at prodomain by proteases and remove it from the catalytic domain. Once activated, MMP-7 has the ability to activate by its own, using its autolytic property.

In vitro experiment shows that proMMP-7 can be cleaved and activated by the trypsin releasing the active MMP-7 with a MW of 19 kDa. Furthermore, MMPs can be activated *in vitro* by chemical compounds which modifies the cysteine residue in the prodomain, such as APMA, SIN (a NO-donor) and H_2O_2 (Imai *et al.,* 1995).

1.3.6 Inhibition of MMPs by TIMPs

The most important natural inhibitors of MMPs are the tissue inhibitors of metalloproteinases (TIMPs) and the tissue factor pathway inhibitor-2. TIMPs are a protein family with 4 members (TIMP-1, -2, -3, and -4) (Jones *et al.,* 1999). TIMP-1, -2 and -4 are the secretory proteins where as TIMP-3 is membrane bound (Brew *et al.,* 2000). TIMPs

bind to the hemopexin domain at the C-terminus and act on the catalytic domain of MMPs. All TIMPs share the same mechanism of inhibition, but they show different specificity. TIMP-1 is a poor inhibitor of MMP-14, -16 and -17 (Lee and Murphy, 2004). Some of the MMPs, for example MMP-13 as well as MMP-7 can be inactivated by all TIMPs (Crabbe *et al.,* 1992; Liu *et al.,* 1997).

The disruption of the balance between TIMPs and MMPs can lead to the pathological conditions. It has been demonstrated that the decrease in TIMPs expression leads to the enhancement of MMPs activity which ultimately promotes the cancer progression (Ponton *et al.,* 1991). Besides the inactivation of MMPs, TIMPs have some other roles like angiogenesis inhibition, steroidogenesis and germ cell development (Gomez *et al.,* 1997). Studies also indicated that MMP-7 is inhibited by the estrogen treatment (Gorodeski, 2006).

1.3.7 Substrates of MMPs

MMPs have a very broad range of substrate. Most of them are components of ECM such as collagen, fibrin, laminin, elastin, gelatin and so on. MMP-7 has broad range of substrates like fibronectin, laminin-I, type IV collagen, gelatin, insoluble elastin (Imai *et al*., 1995). Besides matrix proteins MMP-7 has many other non matrix protein substrates like the alpha defensins, syndecan-1, E-cadherin, β-4 integrin and connexin-43 (Agnihotri *et al*., 2001; Egeblad and Werb, 2002; Lindsey *et al*., 2006; McCawley and Matrisian, 2001; Mitsiades *et al*., 2001; Powell *et al*., 1999). Besides matrix proteins MMP-7 also cleaves CD95 and CD95 ligand revealing the role of MMP-7 in modulation of apoptosis (Powell *et al*., 1999; Strand *et al*., 2004b).

1.3.8 Function of MMPs

A major task of MMPs is disruption of ECM and thus by ECM degradation MMPs play an important role migration and invasion of tumour cells. MMPs are also known to be involved in many cellular functions like differentiation, tissue remodeling during embryogenesis and wound healing.

The most important function of MMP-7 is the recruitment of the neutrophils by creating the chemokine gradient, which is necessary for migration of neutrophils through endothelium to the desired sites. Many steps are involved in neutrophil migration like the rolling, adhesion and migration to the specific sites. KC is a chemokine which is the ligand for CXCR2 and plays an important role in the migration of neutrophils. Experiments indicated that the expression of KC in specific tissues leads to the recruitment of neutrophils to those tissues (Lira *et al.,* 1994). *In vivo* experiments revealed that MMP-7 plays an important role in the process of neutrophil migration together with the syndecan-1 and KC. MMP-7 knockout mice can survive longer than wild type mice after the bleomycin induced lung injury because of the protection against bleomycin induced fibrosis. Forcing the neutrophils to the injured sites by the coinstallation of bacterial peptide leads to the mortality of the mice which indicates that the survival is due to the impairment of neutrophil migration. Immunohistology shows that the neutrophils are strucked in the epithelial matrix interface. Syndecan-1 is a heparan sulfate proteoglycan and is the major source of the heparin sulfate which is produced by the epithelial cells (Hayashida *et al.,* 2006). Syndecan-1 influences many cellular functions by binding with chemokines, growth factors, ligands and extracellular matrix (Elenius *et al.,* 1990; Stanley *et al.,* 1995). KC secreted from the cells and binds to the ectodomain of the syndecan-1 and forms a complex known as syndecan-KC. This complex forms the chemokine gradient and promotes the chemotaxis of the neutrophils. MMP-7 which also binds to the heparin sulfate cleaves the syndecan-KC complex and helps in migration of this complex to the apical surface creating a chemotactic gradient and thus helps in the recruitment of neutrophils. In MMP-7 knockout mice, the cleavage of the syndecan-KC complex can not takes place and leads to defect in the formation of chemokine gradient, resulting in loss of neutrophil migration (Li *et al.,* 2002).

MMP-7 is involved in the tissue remodeling and differentiation. In mice, MMP-7 is expressed in the epithelial tissues of different organs like colon, stomach, kidney, lung, and mammary gland. MMP-7 has the positive role in controlling bacterial infections by producing the mature alfa defensins (Wilson *et al.,* 1999). Generally the paneth cells of the mammalian intestine secretes alfa defensins in response to bacterial infection in pro-form (inactive form), activation of these defensins is necessary for their action to kill the bacteria. The propeptide present in the defensins makes them inactive; MMP-7 cleaves the

propeptide and makes them to active form. This is the evidence for the involvement of MMP-7 either directly or indirectly in the primary defense mechanism.

ECM acts as a natural barrier for the tumour invasion and metastasis. MMP-7 helps in the tumour progression by cleaving the ECM. E-cadherin is an adhesion molecule which helps in the formation of tight junctions between the cells and also plays an important role in the signalling pathway by interacting with the catenins through its cytoplasmic tail which acts as a structural and functional integrity of the cells. In some cancers the function of the E-cadherin is altered and cancer invasion correlates with the loss of E-cadherin (Potter *et al.,* 1999). E-cadherin helps in the cell adhesion by its homopholic calcium dependent binding. The N-terminal end of the E-cadherin from the adjacent cells binds together and acts as cell adhesion molecules. MMP-7 is one of the proteins which is involved in the ectodomain shedding of the E-cadherin (Noe *et al.,* 2001). The ectodomain of E-cadherin was reported in the media of the human breast cancer cells (MCF-7 cells) which is called as soluble E-cadherin (sE-cadherin) (Pece and Gutkind, 2000). This sE-cadherin helps the tumour cells become more scatter and the effect could be neutralized by treating with E-cadherin antibodies. sE-cadherin acts in a paracrine way to inhibit the E-cadherin functions by disturbing the cell contacts and increases the invasive potential of the cells. MMP-7 makes the shedding of E-cadherin and increases the invasion of the tumour cells which is important for the progression of cancer (Noe *et al.,* 2001). MMP-7 also plays a role in the artherosclerosis, MMP-7 cleaves versican, a high molecular chrondrictin sulafate proteoglycan which is a component of the normal blood vessels and leads to formation of artherosclerotic lesions (Halpert *et al.,* 1996).

MMP-7 also cleaves the CD95L which is important for the apoptosis. Many groups studied the effect of MMP-7 on the CD95L and CD95 mediated apoptosis. Contradictory results were obtained, increase or decrease in the apoptosis after the cleavage of the CD95L by MMP-7 (Powell *et al.,* 1999; Schneider *et al.,* 1998). Cell culture experiments shows that MMP-7 cleaves the extracellular region of the CD95L and makes it soluble (sCD95L) which can be readily detected in the cell culture supernatants.

1.4 Immunosurveillance against cancers

1.4.1 Tumour microenvironment and immune modulation

One further important factor that influences the tumour development is the tumour microenvironment, which plays an important role in the host–tumour interactions. Normally the host cytotoxic T lymphocytes (CTLs) kill the tumour cells and helps in the elimination of the tumours. Tumour establishes the microenvironment so that they can escape from the cytotoxic action of the CTLs.

The tumour microenvironment is characterized by regions of fluctuating hypoxia, low pH, and nutrient deprivation (Reynolds *et al.,* 1996). Nonmalignant cells like inflammatory cells, fibroblasts, endothelial cells interact with tumours and maintains an situation which helps for tumour growth and ECM modified by the proteases also contributes to the tumour microenvironment (DeClerck *et al.,* 2004). MMPs are involved in the tumour microenvironment and help the tumour progression. Tumour microenvironment also influences the immune actions against tumours; changing tumour microenvironment is one of the strategies in the tumour therapy. Previous studies stated that tumours inflammatory cytokines released from the immune cells also involved in enhancing the tumour growth (Saijo *et al.,* 2002). Tumour cells release some immune suppressive cytokines which inhibits the action of the cytotoxic T cells. Tumour microenvironment is considered as an obstacle for the treatment of cancer and focused as a target during cancer therapy (Postovit *et al.,* 2006). The inability of the immune system to naturally control tumour progression in cancer patients is generally due to $CD8^+$ T cell dysfunction, or deletion of tumour specific $CD8^+$ T cells (Dhodapkar *et al*., 2002). It was reported that, because of lack of access of tumours to DCs or defective DC activation leads to the attenuation of activation of tumour specific T cells *in vivo* (Brown *et al*., 2001).

1.4.2 Cytotoxic T lymphocytes (CTLs)

Immunesurveillance of the tumour cells from the effector cells has a great impact on the tumour progression. CTLs are characterized by the surface expression of CD8 and an

antigen specific T cell receptor, which recognizes antigen in context of MHC-I (Major Histocompactability Complex Class I). Normally, CTLs recognize the tumour cells and other transformed cells, for example viral infected cells, and kill them by their cytolytic activity. CTLs kill the tumour cells by two different mechanisms: Perforin/granzyme–B and the CD95 mediated apoptosis. In the perforin dependent killing, CTLs secretes perforin into the target cells which forms pores on the cell membrane, the granzyme–B which enters into the cells through these pores and induces apoptosis. Studies demonstrated that the expression of CD95 varies in the malignant cells , which means that the tumour cells express low levels of CD95 and escapes from the CTL mediated apoptosis (Keane *et al.,* 1996). CTLs also contain other costimulatory receptors like CD3, CD28 and leukocyte function associated antigen-1 (LFA-1) which also plays an essential role in the tumour killing. Studies show that the tumours expressing CD80 (B7.1) were more susceptible for the CTLs killing (Townsend and Allison, 1993). Antigen presentation plays a pivotal role in the induction of immune response against the tumours. Dendritic cells (DCs) are the major source for the antigen presentation. The antigen cross presentation by the dendritic cells plays a crucial role in the stimulation of CTLs. Tumours affect the antigen presenting capacity by interfering with the essential process of antigen processing (Johnsen *et al.,* 1998).

1.4.3 T cell activation

T cell activation is an important step in the immune response. Upon binding with the antigen presenting cells (APCs), T cells are activated, proliferated and differentiate based on the specific stimuli. T cell receptors (TCRs) and other T cell surface molecules which play an important role in the activation. TCRs bind with the Major histocompatibility complex (MHC) proteins which presents the processed antigen. Only the TCR binding is not enough to induce the T cell signalling, other molecules on the T cells also participates in the adhesion to the APCs, especially the coreceptors such as CD2, CD3, and CD28 (Boussiotis *et al.,* 1997; Mueller *et al.,* 1989). Integrin lymphocyte function associated antigen LFA-1 (CD18/11a) binds to the intracellular adhesion molecules (ICAMs) on the DCs. CD28 binds to the CD80 (B7.1) and CD86 (B7.2) which are highly expressed on the

mature dendritic cells (Gimmi *et al.,* 1991). T cell activation leads to the production of the diacyl glycerol (DAG) by the hydrolysis of the membrane phosphotidyl inositol (1, 4) diphosphate which ultimately leads to the increased levels of the intracellular free calcium levels, important for the induction of cytokine gene expression (Acuto and Cantrell, 2000).

T cell anergy resulted by the activation of TCR in the absence of coreceptors stimulation can be overcome by activation of the CD28 by monoclonal antibodies. CD28 stimulation is an important mechanism in the activation of the T cells, competitive binding experiments indicated that the inhibition of the CD28 binding to B7 or B7.1 leads to the abrogation of IL-2 production which results for non proliferation of T cells. Regulatory T cells which are involved in the control of autoimmune diseases express a CD28 homologous CTLA-4 (CD152) receptor. CTLA-4 also binds with the B7 and B7.1 of the dendritic cells and competes with the CD28 (Thompson and Allison, 1997). The stimulation of T cells does not take place after the CTLA-4 binding with the B7 or B7.1 because of the absence of the down stream signalling events. In some types of cancers the regulatory T cells regulates the activation of the T cells which leads to the immune system less effective against cancer (Lau *et al.,* 2007; Nishikawa *et al.,* 2005). CTLA-4 knock out experiment shows that the CTLA-4 is important for the regulation of the autoimmune diseases. CTLA-4 regulates the T cell activation by two different mechanisms (Carreno *et al.,* 2000). CTLA-4 binds with the SHP-2 tyrosine phosphatase and dephosphorylates the key components of the TCR signalling complex molecules like the ζ chain (Lee *et al.,* 1998). In the other mechanism, CTLA-4 competes with the CD28 for binding to their same ligands, CD80 and CD86. CTLA-4 has 10-100 times higher affinity for their ligands than the CD28 so even in the low expression CTLA-4 strongly competes with CD28 and attenuates the CD28 signalling pathway (Thompson and Allison, 1997).

1.4.4 Dendritic cells and antigen presentation

Dendritic cells (DCs) got the name because of its appearance like the neural cell dendrites. DCs are derived from the hematopoietic stem cells in the bone marrow and migrate to the peripheral tissues to take up the antigen which acts as sentinels for the immune system and

plays an important role in inducing immune response. DCs constitute only 0.1-1% of the total blood cells, but are the most important antigen presenting cells. DCs are the most efficient in antigen uptake. Many steps are involved in the induction of T cells by DCs, antigen uptake, antigen processing and antigen presentation. DCs capture the antigen through different mechanisms like the phagocytosis, macropinocytosis and endocytosis. Clathrin dependent endocytosis is an important mechanism by which the DCs take up the antigen. Defect or blocking of the clathrin mediated endocytosis leads to the decrease in antigen uptake by DCs. After capturing the antigen, DCs migrate to the regional lymph nodes where the T cells are available in large numbers and present the processed antigen along with the MHC-I molecules. In some types of cancer, functional defects in the DCs were detected but the exact mechanism of how the tumour cells make the DCs less effective is still under investigation (Lenahan and Avigan, 2006). Studies show that the functions of DCs are altered in some types of cancer, for example in the HNSCC (Head and neck squamous cell carcinoma) and in breast cancer patients the overall DCs number is decreased when compared to the healthy persons (Almand et al., 2000); (Wojas et al., 2004). Abnormal differentiation of the myeloid cells may be the cause for the low turnover of the DCs in the cancer patients (Gabrilovich, 2004). Cancer cells produce cytokines which affects the maturation of the DCs (Beissert *et al.,* 1995). Gangliosides which are produced by the tumour cells also influence the differentiation and functionality of the dendritic cells (Shurin *et al.,* 2001; Wolfl *et al.,* 2002). Thus, tumours evade from the CTLs by altering the antigen presenting efficacy of the DCs by limiting their differentiation.

1.4.5 Clathrin mediated endocytosis

Clathrin mediated endocytosis is a mechanism in which the receptors are endocytosed by forming the clathrin coated pits. Clathrin mediated endocytosis is an important mechanism to execute several physiological process like the taking of essential nutrients, induction and regulation of cell signalling pathways (Di Fiore and De Camilli, 2001). Endocytosis occurs through the formation of vesicles by the membrane invagination and then membrane fusion which results in the formation of clathrin baskets (Heymann *et al.,* 2005). Besides clathrin many other proteins are involved in this mechanism, adaptor proteins are the important

proteins which are involved in the incorporation of cargo proteins into the coated pits (Gonzalez-Gaitan and Stenmark, 2003). Clathrin forms a triskelion which consists of three heavy and three light chains where the C-terminus of heavy chains forms a hub. Besides clathrin, adaptor proteins (AP) are the major proteins involved in the pit formation. AP-2 is a large protein complex composed of four subunits α, β, μ2 and δ2. After formation of the coated pits, the coated pits pinched off from the cell membrane and then fuses to the endosomes. The adaptor protein AP180, also called as AP-3 and its isoform CALM (clathrin assembly lymphoid leukemia) contains the binding site for both clathrin and AP-2. AP180 and AP-2 interacts to form a complex that assembles clathrin more efficiently than either protein alone (Hao *et al*., 1999). AP-2 consists of two high affinity phosphoinositide binding sites in particular with PtdIns (4,5)P2. AP-2 lacking functional phosphoinositide binding sites acts as an inhibitor of coated pit formation (Gaidarov and Keen, 1999). After formation of the clathrin pits, provide the driving force for membrane invagination. Clathrin releases from the coated pits before the fusion of the coated pits into the endosomes. Many factors are involved in the formation and dissociation of the coated pits. Dynamin is involved in the pinching off process of the clathrin coated vesicles, GTPase activity of dynamin is important in the formation of the coated pits (Muhlberg *et al.,* 1997; Warnock *et al.,* 1996; Warnock and Schmid, 1996). Mutant of the dynamin which lacks the GTPase activity interferes the clathrin mediated endocytosis shows the importance of dynamin in this mechanism (van der Bliek *et al.,* 1993). The protein amphiphysin acts as a linker between dynamin and clathrin coats. Receptors are internalized based on the sequence at the cytosolic domains, normally these receptors contain Tyr-X-X-O, and where X can be any aminoacid where as O is a hydrophobic aminoacids like Phe, Leu or Met. Studies suggest that mutation in one of the aminoacids aborts the internalization of the receptors. Ligand binding to the receptors triggers the recruitment of receptors to the clathrin coated pits followed by internalization of the receptor-ligand complex and subjects to lysosomal degradation (Vieira *et al*., 1996). Hsc70 is an important protein, which is involved in the dissociation of the clathrin coated vesicles. Hsc70 does not bind to the clathrin directly but binds to the auxilin, which interacts with the clathrin. By binding to the clathrin complex Hsc70 disassemble the clathrin vesicles and makes the way to form the new vesicles, which is important for the continuous execution of this process (Schlossman

et al., 1984).

1.5 Aim of the study

The expression of MMP-7 has been reported in various types of tumours and its expression correlates with poor prognosis. Thus inhibition of MMP-7 may be a useful adjuvant therapy in these kinds of tumours. At present other than TIMPs there is no such available candidate which can specifically inhibit MMP-7. Therefore this study was focused to identify the natural inhibitor for MMP-7. Stable cell line expressing MMP-7 was established by cloning of MMP-7 cDNA into the ecdysone inducible plasmid and transfected into the HEK293 EcR cells. By using these cell lines, cleavage analysis of MMP-7 was performed after treating with different candidates and the activity of MMP-7 was analysed by using MMP-7 specific fluorogenic substrate. This study suggests HLE produced by neutrophils as an inactivator of MMP-7. Further study was focused to analyse the effect of HLE on MMP-7 mediated apoptosis resistance and on CTL killing.

MMPs are present in the tumour microenvironment so can potentially influence antitumour immune reactions. Therefore the present study also focuses on studying the immunomodulatory functions of MMP-7. This study involves identifying the new immune relevant substrate for MMP-7 using Jurkat cell line. Experiments were carried out to analyse the effect of MMP-7 on clathrin mediated endocytosis. Clathrin staining after treating with MMP-7 was performed and the effect of MMP-7 on transferrin uptake was analysed. The influence of MMP-7 on the surface expression of the cellular receptors was also studied. The impact of MMP-7 on dendritic cell differentiation and cellular uptake of transferrin was studied.

2 Materials and Methods

2.1 Commonly used instruments and equipments

Instruments	Manufacturer
-20°C freezer	Bosch, Germany
-80°C freezer	Ultra-Low technology of laboratory equipment, USA
Binder cell culture incubator	Tuttlingen, Germany
Cell culture laminar air flow	Allerod, Denmark
Cell culture flowbank laminar air (type S-2000 1.5)	Heto-Holten, Danmark
Cell culture microscope wilovert 30 standard	Hund, Wetzlar, Germany
Centrifuge 5405 R	Eppendorf, Germany
Confocal laser scanning microscope (CLSM) 510-UV	Zeiss, Oberkochen,Germany
Digital camera system cybertech CSI	Hitachi, Japan
Eppendorf centrifuge 5840	Eppendorf, Germany
Electrophoresis apparatus (agarose gel)	OWL scientific for Labotec, Wiesbaden, Germany
Electrophoresis power supply EPS 601	Amersham Pharmacia Biotech, Sweden
Electrophoresis power supply	Micro Bio Tec Brand, Gießen, Germany
Fluorometer Spectra FluorPlus	Tecan, Salzburg, Austria
Film developer machine	Fujitsu, Japan
GFL waterbath, type 1002 and 1004	Germany
Innova 4230 refrigerator incubator shaker	New Brunswick Scientific, USA
Leiva light microscope	Leica, Portugal
Megafuge 1.0R	Laborgeräte Vetter GmbH, Germany
Microcentrifuge	Eppendorf, Hamburg, Germany
PCR machine	Oldendorf, Germany
pH-Meter Toledo 320	Mettler Toledo, Gießen, Germany
Shaker	IKA-Labortechnik, Germany
UV-Transilluminator	Renner, Damstadt, Germany

UV/Visible spectrophotometer ultrospec 3000	Amersham Pharmacia Biotech, Sweden

2.2 Commonly used chemicals

Chemicals	Supplier
Acrylamide	Merck Bioscience, Darmstadt, Germany
Acetic acid	Roth, Karlsruhe, Germany
Agarose	Sigma Aldrich, Corp, USA
Ammonium hydroxide	Roth, Karlsruhe, Germany
APS	Sigma Aldrich, Corp, USA
Bacto agar	Calbiochem, Darmstadt, Germany
Bromophenol blue	Merck Bioscience, Darmstadt, Germany
Bovine serum albumin (BSA)	Sigma Aldrich, Corp, USA
Butanol	Roth, Karlsruhe, Germany
Citric acid	Sigma Aldrich, Corp, USA
CDP Star	Tropix, Bedford, USA
DEA	Tropix, Bedford, USA
DTT	Sigma Aldrich, Corp, USA
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Sigma Aldrich, Corp, USA
Glycine	Sigma Aldrich, Corp, USA
Hydrochloric acid	Merck Bioscience, Darmstadt, Germany
IGEPAL	Sigma Aldrich, Corp, USA
I-Block	Tropix, Bedford, USA
KCl	Merck Bioscience, Darmstadt, Germany
Methanol	Roth, Karlsruhe, Germany
$MgCl_2$	Merck Bioscience, Darmstadt, Germany
NaCl	Roth, Karlsruhe, Germany
NaOH	Merck Bioscience, Darmstadt, Germany
Na_2HPO_4	Merck Bioscience, Darmstadt, Germany
Nitroblock II	Tropix, Bedford, USA

SDS	Sigma Aldrich, Corp, USA
Silver nitrate ($AgNO_3$)	Merck Bioscience, Darmstadt, Germany
TEMED	Applichem, Darmstadt, Germany
Triton X-100	Merck Bioscience, Darmstadt, Germany
Tris	Sigma Aldrich, Corp, USA
Tryptone	Sigma Aldrich, Corp, USA
Tween 20	Merck Bioscience, Darmstadt, Germany
Yeast extract	Sigma Aldrich, Corp, USA

2.3 Antibodies

Primary antibodies (all of the following antibodies are raised against human antigens.)

Name	Supplier
anti-Apo-1	Provided by Dr. P. H. Krammer
anti-clathrin	Sigma Aldrich,Corp,USA
anti-CTLA-4	eBioscience, Frankfurt,Germany
anti-CD3	BD Bioscience, Heidelberg, Germany
anti-CD14	Serotec, Dusseldorf, Germany
anti-CD28	B.D.Bioscience, Heidelberg, Germany
anti-CD47	B.D.Bioscience, Heidelberg, Germany
anti-CD71	Ancell, Bayport, USA
anti-CD80	Immunotools, Friesoythe, Germany
anti-CD83	Biozol, Eching, Germany
anti-CD86	Immunotools, Friesoythe, Germany
anti-HLA-DR	Immunotools, Friesoythe, Germany
anti-Hsc70	Stressgen, Victoria, Canada
anti-HLE	Neomarkers,Fremont, USA
anti-MMP-7	Oncogene Research Laboratories
anti-TGF-β receptor II	Provided by Dr. Theobald

Secondary antibodies for Western blot

Name	Supplier
Alkaline phosphotase conjugated anti-rabbit immunoglobulin	Sigma-Aldrich, Corp, USA
Alkaline phosphotase conjugated anti-goat immunoglobulin	Sigma-Aldrich, Corp, USA

Fluorescence labelled secondary antibodies and fluorescence staining dye

Name	Supplier
Hoechst 33342	Molecular Probes, Leiden, NL
Alexa-Flour-488 conjugated transferrin	Molecular Probes, Leiden, NL
Alexa-Flour-488 conjugated mouse-anti-rabbit IgG	Molecular Probes, Leiden, NL
Alexa-Flour-488 conjugated donkey-anti-goat IgG	Molecular Probes, Leiden, NL
Alexa-PE conjugated goat-anti-mouse IgG	Molecular Probes, Leiden, NL

2.4 Software

Microsoft Office 2000	Macromedia Freehand 8.0.1	Zeiss LSM 510
Adobe Photoshop 5.0	BD CellQuest Pro	Tecan X FLUOR4.5
MagicScan V 4.5	Endnote 7.0	Adobe Acrobat 6.0

2.5 Molecular biological and biochemical methods

2.5.1 Cloning of target gene

Enzymes

Restriction endonucleases	Promega, Mannheim, Germany
T4-Ligase	MBI Fermentas, St. Leon-Rot
CIAP	Promega, Mannheim, Germany
DNA polymerase	Qiagen, Hilden, Germany
Oligonucleotide	Qiagen, Hilden, Germany

Plasmids

Inducible mammalian expression system Invitrogen, Karlsruhe, Germany

The system contains a vector pIND (SP1) which allows gene expression induced by ponasterone A and selection with G418.

Isolation of plasmid DNA

According to the protocol from Qiagen Company, 2 ml LB medium containing *E. coli* was cultured overnight for obtaining small amount plasmid DNA, and the isolation was performed with QIAprep Miniprep Kit (Qiagen, Hilden, Germany). For obtaining large-scale plasmid DNA, *E. coli* was inoculated in 25 ml of LB medium and incubated overnight. The plasmid DNA was isolated with Qiagen Plasmid Midi Kit according to manufacturer instructions.

The DNA isolation is based on alkaline lysis of bacteria, and DNA was absorbed and purified through the silicium matrix cylinder, the isolation process was followed according to the manual of Qiagen Company.

Phenol/chloroform extraction

The DNA solution was mixed with an equal volume of phenol/chloroform (1:1) (adjusted with 10 mM Tris to pH 8.0). The solution was vortexed for short time until the solution become viscous. Afterwards the mixture was centrifuged at 5,000 g for 1 minute. The upper phase was carefully transferred into a new tube, and the lower phase was discarded.

The above purification step was repeated once. Finally an equal volume of chloroform was added to the supernatant to remove the rest of phenol. After vortexing the mixture, the solution was centrifuged at 5,000 g for 1 minute, and the supernatant containing purified DNA was carefully transferred into a new tube.

DNA precipitation

The DNA solution was added with 0.5 volume 7.5 M ammonium acetate pH 7.5 and addition with 2.5 volumes 100% ethanol. Then the solution was stored at -20°C for 2 hours. Afterwards it was centrifuged at 4°C at 12,000 g for 20 minutes, and DNA pellet

was washed once by ice cold 70% ethanol, air dried, dissolved in appropriate volume of TE buffer and stored at -20°C.

2.5.2 Agarose gel electrophoresis

- DNA marker: Gene Ruler 100 bp plus DNA ladder (MBI Fermentas, St. Leon-Rot)
- TBE (Tris-Borate-EDTA-buffer)

Tris/HCl, pH 8.0	90.0	mM
Boric acid	90.0	mM
EDTA pH 8.0	2.5	mM

- Loading buffer for DNA agarose gel

Bromophenol blue	0.2	%
Glycerol	30.0	%
EDTA, pH 8.0	100.0	mM

The digestion of DNA was performed with 5-10 units endonuclease per µg of DNA in the recommended reaction buffer and temperature for 1-2 hours according to the instructions of the manufacturer.

The electrophoretic separation of DNA fragments were performed by using 1% agarose gels which contains 0.1 µg/ml ethidium bromide (from a stock solution of 10 mg/ml in water). Before electrophoresis, DNA was mixed with 1/6 volume DNA loading buffer. The electrophoresis buffer was 1x TBE buffer, and molecular weight marker was used for checking the size of the DNA fragments. The electrophoretic results were documented with digital camera after observation under the UV-Transilluminator.

2.5.3 Subcloning of DNA fragments

For cloning, the vector was cleaved by the appropriate restriction enzyme, and dephosphorylated by calf intestinal alkaline phosphatase (CIAP) to remove 5'-phosphate groups from linear DNA, and this process prevents religation by itself. DNA was resuspended in purified H_2O; afterwards CIAP and the appropriate buffer were added, incubated at 37°C. For the inactivation of the CIAP enzyme, EDTA (pH 8.0) and SDS were added to a final concentration of 20 mM and 0.5% respectively after dephosphorylation,

and the solution was incubated at 70°C for 10 minutes. For purification of the vector, the phenol/chloroform method was performed, and precipitated by ethanol. Afterwards the ligation of the vector and target insert fragment was completed as following:
The ratio of insert fragment and vector is from 3:1 to 10:1. The maximum amount of the vector is 100 ng and the amount of insert fragment was variable. The responsible amount of T4 DNA ligase (1 U/µl) and 10x ligase buffer is 1 µl each. The reaction of ligation was performed in a final volume of 10 µl.

Preparation and conservation of competent bacteria

LB medium (1 L)

Trypton	10	g/L
Yeast extract	5	g/L
NaCl	10	g/L

Adjust pH with NaOH to 7.0, then autoclave.

LB agar plates

Add 15 g of agar to 1 liter LB medium, autoclave and add antibiotic after cooling down to 50-60°C

Antibiotics

	Stock solution	Final concentration
Ampicillin	100 mg/ml in H_2O, stored at -20°C	100 µg/ml
Kanamycin	10 mg/ml in H_2O, stored at -20°C	30 µg/ml

E. coli (strain XL-1 Blue) was directly picked from a frozen stock onto the surface of the LB agar plate, and the plate was incubated overnight at 37°C. Well isolated colonies were picked up, put into 15 ml sterile LB medium and incubated overnight at 37°C (LB medium without antibiotic). The cells were transferred to 500 ml sterile LB medium and incubated at 37°C until the OD_{600} reaches 0.3. Then the cells were centrifuged at 6,000 g for 2 minutes at 4°C, the pellet was resuspended by gentle vortexing in 125 ml ice cold 50 mM $CaCl_2$ solution. Afterwards the cells were incubated on ice for 20 minutes and centrifuged again. The pellet was carefully transferred into 25 ml ice cold 50 mM $CaCl_2$ containing

10% glycerol solution by gentle vortexing. Ultimately the suspension was dispensed into small tubes at 100 µl aliquots, shock frozen in liquid nitrogen and stored at -70°C.

2.5.4 Transformation

One aliquot of the competent bacteria was carefully thawed on ice and the ligated DNA was added to the tube containing competent bacteria. The tube was swirled very gently to mix the contents, and incubated on ice for 20 minutes. Afterwards the tube was placed in a 42°C preheated waterbath for exactly 90 seconds, and then returned to ice for 5 minutes.
400 µl prewarmed (37°C) LB medium was added in the tube and incubated at 37°C for 1 h to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. Finally the bacteria were carefully resuspended, and an appropriate volume (about 100 µl per plate) of transformed competent cells was plated onto the LB agar plates containing the appropriate antibiotic. The plates were inverted and incubated at 37°C overnight. Single colonies were picked with sterilized toothpicks and put in a culture tube containing LB medium with appropriate antibiotic for overnight incubation.

2.5.5 RT-PCR

RNA isolation

The total RNA was isolated using the RNAeasy kit (Qiagen) and all steps were performed with filtered pipette and centrifuged at room temperature. Cells culture in 10 cm petri dishes were lysed in 600 µl of RLT buffer containing 6 µl β-mercaptoethanol. The lysate was carefully drawn through a 2 ml Syringe with 20-gauge needle for several times to shear the genomic DNA. Then 600 µl of 70% ethanol was added. After mixing by carefully pipetting up and down, 700 µl of the solution were transferred to a column and centrifuged for 15 seconds at 10,000 rpm. The flow through solution was discarded; the rest of 500 µl mixture were transferred to the same RNAeasy column and centrifuged for 15 seconds at 10,000 rpm, the flow through solution was discarded. Afterwards 350 µl of RW1 buffer was added in column and the samples were centrifuged again and the flow through solution was discarded. 500 µl wash buffer RPE was added into the column and the samples were centrifuged for 15 seconds at 10,000 rpm, the flow through solution was discarded, the wash step was repeated once. New 1.5 ml tube was used to collect the RNA solution, while

30 µl RNase free water was added into the column and centrifuged for 1 minute at 10,000 rpm. The RNA samples were immediately frozen at -80°C.

cDNA synthesis

Total RNA content was estimated by measuring absorbance at 260 nm (A_{260}), purity of RNA was measured by calculating ratio of A_{260} to A_{280}. 1 µg of total RNA was transcribed to cDNA in a 20 µl reaction volume using the kit Omniscript (Qiagen). Oligo d(T) primer was used for cDNA synthesis. The reaction mixture was incubated sequencially at 25°C for 5 minutes, at 37°C for 45 minutes, at 85°C for 5 minutes and at 4°C for 10 minutes.

Polymerase Chain Reaction (PCR)

1 µl of cDNA was used for 25 µl PCR reaction. PCR was performed using Taq PCR core kit (Qiagen). PCR reaction mixture was prepared according to the manufacturer's instructions. After an initial denaturation for 4 minutes at 94°C, the reaction runs for 28 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds.

The specific primers were:

MMP-7 forward:	5'-AGTGCCAGATGTTGCAGAATACTC-3'
MMP-7 reverse:	5'-GTGAGCATCTCCTCCGAGACCTGT-3'
β-Actin forward:	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'
β-Actin reverse:	5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'

2.6 Ecdysone mammalian expression system

The ecdysone inducible mammalian expression system (Invitrogen, Karlsruhe, Germany) is based on the ecdysone system from *Drosophila*. In this expression system both subunits of a heterodimeric ecdysone receptor are constitutively expressed in the regulator vector pVgRXR. The ecdysone responsive promoter, which finally drives expression of the gene of interest, is located on the pIND expression vector. The ecdysone system expresses the gene of interest by using the ecdysone analog ponasterone A. The target gene was first cloned into the inducible expression plasmid pIND (SP1), and this construct was stably transfected into HEK293 EcR cell lines by Lipofectmine 2000 (Invitrogen, Karlsruhe,

Germany) according to the standard transfection protocol. These HEK293 EcR cell lines expressed stably the heteromeric ecdysone receptor. The cDNA of MMP-7 containing the entire open reading frame was cloned into an ecdysone inducible mammalian expression vector, pIND (SP1). The stable transfected cells were selected in medium containing both G418 (400 ng/ml) and Zeocin (400 µg/ml). The clones growing up after about 4 weeks of selection were picked up and further analysed.

2.7 Cell biological methods

2.7.1 Cell culture

Medium

DMEM (Dulbecco's modified Eagle's medium, Gibco BRL/Invitrogen, Karlsruhe)
Without sodium pyruvate
With 4500 mg/ml glucose
With pyroxidine HCl

Solutions or reagents used in cell culture

Hepes Buffer 1 M in 0.85% NaCl	BioWhittaker Europe, Vervies Belgien
Fetal Bovine Serum (FBS)	BiochromKG, Berlin
Penicillin/Streptomycin	GibcoBRL/Invitrogen, Karlsruhe
10,000 U/ ml penicillin and 10,000 µg/ml streptomycinsulfate in 0.85% NaCl	
L-Glutamine (200 mM)	PAA, Cölbe
Trypsin/EDTA	PAA, Cölbe
0.1% Trypsin for HEK293 EcR and HEK293-MMP-7 cell lines	
G418 (Genticin G418 sulfate, 10 mg/ml)	BibcoBRL/ Invitrogen, Karlsruhe
Zeocin (100mg/ml)	Invitrogen, Karlsruhe

Cell lines

HEK293 (human embryonic kidney cells)

- HEK293 cell line was developed from human embryo kidney cells and immortalized by adenovirus transfection.
- This cell line is adherent and showed fibroblastic morphology.

- This cell line has high transfection efficiency and is suitable both for transient and stable expression of protein to analyse.

HEK293 EcR

- From Invitrogen, Karlsruhe, Germany, developed from HEK293
- This transgene cell line expresses stably the modified ecdysone receptor and was used in ecdysone system for the pIND (SP1) regulatable stable expression.
- Under Zeocin selection (400 µg/ml).

HEK293-MMP-7 cells

- This cell line was developed using HEK293 EcR as parent cells by stable transfection with the recombinant plasmid pIND-MMP-7.
- Selection medium: Zeocin 400 µg/ml and G418 400 µg/ml.

HepG2 cells

- Human hepatocellular liver carcinoma cell line.
- These cells grow adherently.

Jurkat 16 cells

- Jurkat cells are an immortalized line of T lymphocytes orignally established from the peripheral blood of a boy with T cell leukemia.

Culture of the cells

HEK293 EcR, HEK293-MMP-7 cells as well as HepG2 cells were maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM Hepes buffer, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO_2 atmosphere. For passage the cells, confluent cultures were washed once with PBS and treated with 1 ml 0.1% trypsin/EDTA solution at room temperature for 3-5 minutes. The trypsin was then inactivated by addition of medium containing FBS. A tenth of the resuspended was seeded into a new culture plate in 10 ml medium. In case of selection of tansfectants, zeocin (400 µg/ml) and G418 (400 µg/ml) were included in culture medium.

Jurkat cells were maintained in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM Hepes buffer, 100 units/ml penicillin and 100 µg/ml streptomycin under the same conditions as described above. For passaging, cells were resuspended and splitted in appropriate dilution in fresh medium.

2.7.2 Preservation of cells

Adherent cells were allowed to grow to 70% confluency, washed once with PBS and then trypsinized with 0.1% trypsin/EDTA solution. The cells were then resuspended in 10 ml culture medium and recovered by centrifugation at 1,000 g for 5 minutes. The cell pellet was resuspended in 5 ml of culture medium containing 20% FBS and 10% DMSO. Aliquots of 1 ml cell suspension were dispensed into cryotubes. The tubes were transferred into a styropor box which was placed into -80°C refrigerator overnight. Finally, the tubes were transferred into liquid nitrogen.

For the reculture of the cells, the frozen cells was removed from liquid nitrogen and immediately thawed at 37°C in water bath, washed with 10 ml culture medium by centrifuged at 1,000 g for 5 minutes to remove DMSO. The cell pellet was resuspended in 10 ml medium and was seeded in cell culture dishes and incubated at 37°C.

2.7.3 Transfection

One day before the transfection, the cells were seeded in 6-well plate at about 50% confluency, and they were cultured in DMEM medium containing 10% FBS, 10 mM Hepes buffer and 2 mM L-glutamine without any antibiotics. For each well, 5 µg of plasmid DNA and 5 µl of Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) were diluted respectively into 250 µl of serumfree DMEM medium, and incubated at room temperature for 5 minutes. The diluted DNA and lipofectamine solution from the above step were carefully mixed together and incubated at room temperature for further 20 minutes. After incubation 500 µl of the complexes were carefully added directly into each well, and mixed very gently by rocking the plate. After 8-10 hours, the medium was replaced with culture medium containing serum and antibiotics. The cells were incubated at 37°C for 48 hours until analysis of the target gene expression was performed.

2.7.4 Western blot

Reagents

Lysis buffer: PBS containing 1% NP-40 and protease inhibitors (*C*omplete, Roche)

2x sample buffer

Tris/HCl	120.0	mM
Glycerin	10.0	%
SDS	4.0	%
β-mercaptoethanol	4.0	%
Bromphenolblue	0.02	%
pH6.8		

Resolving gel composition

	Gel concentration			
	10%	**12%**	**15%**	
30% Acrylamide*	13.3	16.0	20.0	ml
1.8 M Tris/HCl, pH8.8	8.0	8.0	8.0	ml
H_2O	18.1	15.5	11.5	ml
10% SDS	400.0	400.0	400.0	µl
10% APS	200.0	200.0	200.0	µl
TEMED	8.0	8.0	8.0	µl

*: Acrylamide/Bis-acrylamide (37.5:1), purchased from Merck

Stacking gel composition

30% Acrylamide	3.0	ml
0.8 M Tris/HCl pH (6.8)	4	ml
H_2O	12.7	ml
10% SDS	200.0	µl
10% APS	100.0	µl
TEMED	10.0	µl

10x Running buffer (Laemmli buffer)

Tris	250.0	mM
Glycine	2.0	M
SDS	1.0	%

Blot buffer I:

1 M Tris/HCl, pH10.4	300.0	ml
Distilled H_2O	700.0	ml

Blot buffer II:

1 M Tris/HCl, pH10.4	25.0	ml
Distilled H_2O	975.0	ml

Blot buffer III:

E-amino-n-corporic acid	5.24	g
Distilled H_2O add to	1.0	l

Block solution: 1g of I-Block reagent in 500 ml of PBS and incubated at 60°C for 2 hours, afterwards 500 µl of Tween 20 was added.

Wash buffer: PBS containing 0.1% Tween 20

Assay buffer: 0.1 M Diethanolamine, 1 mM $MgCl_2$

CDP-Star luminescent detection system (Tropix):

Nitro-Block:	1:20 in assay buffer
CDP-Star:	1:1,000 in assay buffer

Procedure

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate the proteins into their individual polypeptide subunits and minimize their aggregation in an electrophoresis field. After the protein was treated by the ionic detergent SDS, reduced with β-mercaptoethanol and denatured by heating at 99°C, the proteins were separated by gel electrophoresis based on the different molecule mass of the polypeptide. Using proteins of known molecular mass, it was possible to elevate the

molecule mass of the polypeptide chains. The SDS-PAGE was performed with a discontinuous buffer system (Laemmli, 1970). 10%, 12% or 15% acrylamide gels were used. The gels were used directly or stored under humid conditions at 4°C.

Preparation of samples

Cells were washed once with PBS and then lysed by adding 100 µl of lysis buffer per well of 6-well plate, and incubated on ice for 15 minutes. The lysates were then recovered and centrifuged at 10,000 rpm for 10 minutes to remove the cell debris. The protein concentration was determined using Nanoquant reagent (Roth). 30 µg of protein were mixed with 2x sample buffer and heated at 99°C for 5 minutes. Then the samples were kept on ice.

The samples and prestained molecular mass marker (SDS7B2, Sigma) were loaded on the SDS-PAGE gel. The gel was run at 25 mA/gel in stacking gel and 30 mA/gel for separation under room temperature.

Blotting

The gel containing proteins was transferred on the PVDF membrane using the semidry method as follows:

- Anode
- 3 pieces of Whatman papers, soaked with buffer I
- 3 pieces of Whatman papers, soaked with buffer II
- PVDF membrane, soaked first in methanol, then in water, finally in buffer II
- gel
- 6 pieces of Whatman papers, soaked with buffer III
- Cathode

The PVDF membrane (Millipore, Schwalbach) was activated in methanol, washed in H_2O and finally soaked in blot buffer II. The transfer was carried out in a Multiphor II-Kammer (Millipore, Schwalbach) at 0.8 mA/cm^2 gel area at room temperature for 90 minutes.

Immune detection of the protein in Western blot

After blotting, the PVDF membrane was washed in wash buffer for 5 minutes, blocked with the I-block buffer for 1 hour at room temperature and incubated with the primary antibody in I-block buffer at 4°C overnight. The blot was washed 3 times with wash buffer for 5 min each, followed by incubation with secondary antibody at room temperature for 1 hour. Then it was washed 3 times for 20 min each with wash buffer. After equilibration in assay buffer (2 times for 10 minutes each), the membrane was incubated with Nitro-Block (2 ml/blot) buffer for 5 minutes. Finally the CDP-Star buffer containing substrate was added. The membrane was carefully covered with a transparent plastic film and exposed to X-ray film for different time (from 1 minute to 1 hour) in an autoradiography box.

2.7.5 Immunofluorescence staining

Reagents

Fixation solution:

1) 4% paraformaldehyd (PFA) in PBS
2) Methanol/acetone mixture (1:1)

Wash buffer:	PBS containing 0.1% Triton-X 100
Block solution:	PBS containing 3% BSA (bovine serum albumin fraction V) and 0.1% Tween 20
Dye:	Hoechst dye

Procedure

Cells were fixed with PFA solution at room temperature for 10 min, or alternatively with methanol/acetone mixture at -20°C for 5 minutes and then allowed to dry at -20°C. The cells were washed three times with wash buffer, and afterwards the cells were incubated in block solution at room temperature for 1 h. The first antibody was diluted in block solution at 1:200 and the cells were incubated with first antibody for 2 h in a humid chamber. The cells were washed again with wash buffer 3 times, and they were treated with fluorescence conjugated secondary antibody (which was diluted in block solution at 1:200) at room temperature for 2 hours. The cells were washed 3 times with wash buffer, and washed 2

times with PBS; afterwards the cells were incubated with Hoechst dye (diluted 1:10,000 in PBS) at room temperature for 5 minutes in the dark. The excess dye was removed by washing twice with PBS. The slides were mounted with permafluor medium and dried overnight. Finally the staining was analysed by confocal laser scanning microscope (Zeiss 510-UV, Zeisse, Germany).

2.7.6 CellTiter-Glo® Luminescent Cell Viability Assay

After the induction of apoptosis or cytotoxic killing, the cell viability was measured in 96-well plates using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany). The assay is based on a luminescent reaction, in which the mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen, resulting in emission of light. The luminescent signal correlates highly to the number of living cells which supply the metabolically active ATP.

Cells were seeded at a density of 15,000 cells/well in 96-well plates. After treatment of cells with the substances to analyse, 25 µl of CellTiter-Glo® reagent were added directly to the wells containing 100 µl culture medium. The plate was shaked gently for 2 minutes to induce lysis and incubated at room temperature for 10 minutes to stablize luminescent signal. 85 µl of lysate were then transferred to opaque walled plate and the luminescence was recorded using a luminometer.

2.7.7 Induction of HEK293-MMP-7 cells

HEK293-MMP-7 cells were plated a day prior to induction in DMEM media supplemented with 10% FBS. Next day the media was aspirated out from the plate and fresh DMEM media (without serum for Western blot analysis) was added to the cells. Ponasterone A was used to induce the cells at different concentrations and for different time points as mentioned in results. Ponasterone A was dissolved in 100% ethanol to a concentration of 1 mM as stock solution. Equal amount of ethanol was used to treat the control cells.

Acetone Precipitation

2.5 volume of ice cold acetone was added to the sample and incubated at -20°C for 3 hours. Samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. After discarding the supernatant, pellet was air dried and resuspended in desired amount of distilled water and processed as required.

2.7.8 FACS (fluorescence activated cell sorting) analysis

FACS buffer: PBS containing 0.4 % BSA, 0.03 % NaN_3, 10 mM EDTA and 20 mM HEPES, Sterile filtered

FACS analysis is a method to quantify and characterize the cells. Through fluorescent markers (fluorescently labelled monoclonal antibodies) bound on cells, FACS machine can recognize and sort the cells. FACS machine possess the capacity of performing multiparameter analysis on a single cell, such as size, volume, viscosity, DNA or RNA content and surface antigens. Information obtained from FACS analysis is variable, depending on the properties which are analysed. Normally the data is primarily displayed as histogram or plot. The x-axis of the histogram shows the fluorescent intensity and y-axis displays the number of cells found within each parameter. When analysing more parameters, the histogram is displayed in the multidimensional colour coordinated display.

2.7.8.1 Analysis of apoptosis by FACS (PI staining)

For the apoptosis analysis by FACS, cells were stained by propidium iodide (PI) also known as Nicoletti staining. Propidium iodide interacted with DNA in prefixed cells. It was activated to emit fluorescence under the 480 nm laser source, and produced an emission signal at 650 nm. In flow cytometry analysis, the normal growing cells show a typical DNA profile from G1, G2 and S phase after propidium iodide staining. When the cell cycle is influenced or disrupted (for example during apoptosis), and the change in DNA profiles will be observed according to the measurement of the peaks from G1, G2 and S populations.

DNA will become fragmented during the apoptosis process. Reduction in the DNA content of the stained cells reveals the apoptotic cells in the sub-G1 population determined by FACS analysis.

Preparation of cells for determining apoptosis by FACS

Staining solution: PBS containing 50 µg/ml propidium iodide

Cells were seeded at a density of 150,000 cells/well in 12-well plates, and treated as indicated in the results. For analysis of CD95 or doxorubicin mediated apoptosis, the cells were treated with anti-Apo-1 or doxorubicin respectively. After 48 hours culture, the cells were harvested by trypsinization, washed with PBS and centrifuged at 1,000 rpm for 5 minutes. The cell pellet was resuspended in PBS and fixed with prechilled 100% ethanol at -20°C for 30 minutes. The fixed samples were centrifuged again and the pellet was resuspended in 500 µl of staining solution. The stained samples were kept at 4°C overnight.

FACS measurement for apoptosis

The analysis of fluorescence can be carried out by FACSCalibur instrument (Becton-Dickinson, Heidelberg, Germany). The 488 nm argon-laser was used and the fluorescence was measured. The data were directly shown on the screen in a histogram form, and 10,000 cells were measured. All data were registered, saved and analysed under the CellQuestPro-software.

2.7.8.2 Measurement of receptor staining by FACS

For detection of receptor on the cell membrane, cells were incubated sequentially with the specific primary antibody and R-Phycoerythrin or Alexa Flour 488 labelled secondary antibody. The measuring process was carried out same as the steps mentioned above. R-Phycoerythrin has absorption at 488 nm and fluoresced at 575 nm. Alexa 488 has absorption at 495 nm and emission at 519 nm. The intensity of the fluorescence of the stained cells corresponds to the expression of the receptor on the cell membrane. When the expression of the receptors is increased, the fluorescent peak of the FACS analysis will responsibly move towards right on the histogram comparing to control. In parallel, cells stained only with secondary antibody served as control for nonspecific binding of the secondary antibody.

2.7.8.3 Clathrin staining

To perform the clathrin staining for FACS analysis, cells were collected in a FACS tube. 100 µl of methanol/acetone (1:1) mixture was added to the cell pellet. The sample was incubated at room temperature for 2 minutes and then washed twice with PBS. The cells were then blocked in 100 µl of PBS containing 3% BSA and 0.1% Tween 20 for 30 minutes at room temperature. 2 µl of anti-clathrin antibody was added to the each tube to get final dilution of 1:50. The samples were incubated at room temperature for 30 minutes and then washed twice with PBS. The cells were then resuspended in 100 µl of 1:200 diluted (in blocking solution) donkey anti-goat FITC labelled antibody. The samples were incubated at room temperature for 30 minutes in dark. After incubation with secondary antibody the cells were washed with PBS and analysed by FACS.

2.7.9 Transferrin uptake

The dynamics of receptor mediated endocytosis can be readily demonstrated using fluorescently labelled transferrin. Transferrin first binds to the transferrin receptor on the cell surface and is taken up by the cells through clathrin míiated endocytosis. Endocytosis only occurs to an appreciable extent when the cells are above the phase transition temperature of the plasma membrane. For this reason the experiment was carried out at 37°C. However, the lack of endocytosis at low temperature was exploited to accumulate a relatively high level of fluorescently labelled transferrin on the cell surface (at 4°C), before warming to 37°C and following the uptake of the transferrin.

2.7.9.1 Transferrin uptake analysis by FACS

1x 10^5 cells were incubated in a FACS tube on ice for 15-20 minutes to stop all endocytotic processes. FITC labelled transferrin (Molecular Probes, Eugene) was added to the cells at a final concentration of 25 µg/ml followed by further incubation on ice for another 30 minutes. The cells were washed twice with ice cold culture medium to remove unbound transferrin. The cell pellet was resuspended in 500 µl of warm (37°C) culture medium and the samples were incubated at 37°C in the cell incubator witn 5% CO_2. The uptake was

stopped at indicated time point by washing the cells with ice cold solution of 0.2 M acetic acid and 0.5 M sodium chloride. The cells were recovered by centrifugation and fixed in 500 µl of PBS containing 4% PFA. The uptake was analysed after measuring the fluorescence by using FACS.

2.7.9.2 Transferrin uptake analysis by CLSM

For analysing transferrin uptake by CLSM (confocal laser scanning microscope), the cells were grown on chamber slides. Prior to the assay, the chamber slide was cooled on ice to 4°C. FITC labelled transferrin was added to the cells at a final concentration of 25 µg/ml and allowed to bind to the cell surface on ice for 30 minutes. The cells were washed twice with ice cold culture medium and incubated at 37°C to allow the transferrin uptake. The uptake was stopped at the indicated time points by washing the cells with ice cold solution of 0.2M acetic acid and 0.5M of sodium chloride. Cells were fixed with 4% PFA and imaged by CLSM.

2.7.10 Isolation of $CD4^+CD25^+$ cells by MACS

The $CD4^+CD25^+$ Regulatory T cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany) was used for the isolation of $CD4^+CD25^+$ cells from PBMCs. First, $CD4^+$ cells were enriched by negative selection using a cocktail of biotinylated antibodies and MicroBeads coated with anti-biotin antibody. Then $CD4^+CD25^+$ cells were purified by positive selection using MicroBeads coated with anti-CD25 antibody.

Isolation of $CD4^+$ cells

MACS Buffer: 3% BSA in PBS

$2x10^8$ PBMCs cells isolated from the buffycoat were centrifuged at 300 g for 10 minutes. The cell pellet was resuspended in 800 µl of MACS buffer, followed by addition of 100 µl of biotinylated antibody cocktail containing anti-CD8, -CD14, -CD16, -CD19, -CD36, -CD56, -CD123, -TCRγ/δ and glycophorin A. The sample was mixed and incubated at 4°C for 10 minutes. Then 600 µl of MACS buffer and 400 µl of anti-biotin MicroBeads were added. After incubation at 4°C for 15 min, cells were washed by adding 20 ml of MACS

buffer and centrifuged at 300 g for 10 minutes. The supernatant was completely removed and the cell pellet was resuspended in 500 µl of MACS buffer.

LS column was placed in the magnetic field of a suitable MACS separator. The column was rinsed with 3 ml of MACS buffer; before the cell suspension was loaded on the column. The effluent was collected in a 15 ml tube and the column was washed three times with 3 ml of MACS buffer. The effluents from this step were also collected in the same tube. The cells present in the effluent contain $CD4^+$ cells. After centrifugation at 300 g, pellet was resuspended in MACS buffer and proceeded for isolation of $CD25^+$ cells.

Isolation of $CD4^+CD25^+$ cells

$CD4^+$ cells were counted and $3x10^7$ cells were centrifuged at 300 g for 10 minutes. The cell pellet was resuspended in 270 µl of MACS buffer and 30 µl of anti-CD25 coated MicroBeads was added. After incubation at 4°C for 15 minutes, cells were washed in 5 ml of MACS buffer and centrifuged at 300 g for 10 minutes and the pellet was resuspended in 500 µl of MACS buffer.

LS Column was placed in the magnetic field of a suitable MACS separator. The column was rinsed with 3 ml of MACS buffer; before the cell suspension was loaded on the column. The column was washed three times with 3 ml of MACS buffer. The column was removed from the separator and placed in 15 ml tube. 5 ml of MACS buffer was added to the column and the retained cells were eluted by using plunger. The cells were counted and used for further experiments.

2.7.11 Dendritic cell culture

PBMCs were isolated from buffy coat using ficoll method. 35 ml of 1:2 diluted buffy coat (in PBS) was carefully layered on the top of 15 ml ficoll in a 50 ml falcon tube. The tube was centrifuged at 2,000 rpm for 15 min without break. The white interphase was removed without disturbance and collected in a new 15 ml centrifuge tube. The cells were washed twice with PBS and $3x10^7$ PBMCs were plated in a 6 well plate containing 3 ml of X-vivo medium (Invitrogen, Karlsruhe, Germany) in each well. Plates were incubated at 37°C for one hour. Medium was removed and the cells were washed twice with PBS. 2 ml of medium which contains 800 IU/ml of GM-CSF and 500 IU/ml of IL-4 was added to each

well. On third day one ml of X-vivo medium containing 1,000 IU/ml of GM-CSF and 1,000 IU/ml of IL-4 was added in each well without removing the old medium. On day 7, floating cells were collected and plated in the new plates for the preparation of the immature and mature dendritic cells. For immature dendritic cells, cells were treated with 1600 IU/ml of GM-CSF and 1,000 IU/ml of IL-4. To obtain mature dendritic cells, cells were treated with 800 IU/ml of GM-CSF, 500 IU/ml of IL-4, 100 ng/ml of IL-1, 1,000 IU/ml of IL-6, 10 ng/ml of TNF-α. After 2 days the cells were harvested and used for further experiments.

2.7.12 Chrome release assay for T cell cyotoxicity

An alloreactive HLA-A2.1 retricted murine polyclonal cytotoxic T lymphocyte (CTL) line (named CTL CD8 Allo-A2) was established previously (Theobald *et al*., 1995). From these cells a monoclonal CTL line specific for the peptide Flu M1 58-66 (named CTL CD8xA2K^{b}FluM1) has been generated (Drexler *et al*., 1999). These CTLs were cocultured with ^{51}Cr labelled, peptide loaed HepG2 cells at different effectors to target (E:T) ratios. ^{51}Cr release was measured after 4-5 hours. The peptide specificity of the CD8xA2K^{b}FluM1 CTLs was controlled by their failure to the lysis of the target cells loaded with an irrelevant peptide.

2.8 MMP-7 activity assay

The activity of MMP-7 was determined using the MMP-7 specific fluorogenic peptide substrate (Calbiochem). The sequence of this substrate was DNP-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser-OH. In the uncleaved form, the fluoroscence emitted by tryptophan residue is quenched by the DNP. MMP-7 cleaves the substrate at Ala-Leu and makes the DNP unable to absorb the fluorescence, so that the emitted fluorescence can be measured. The fluorescence intensity correlates to the amount of the cleaved substrate, which reflects the activity of MMP-7. To perform the assay, samples were placed in a 96 well, flat bottomed black wall plate and the final volume was adjusted to 100 µl using buffer containing 150 mM NaCl, 10 mM HEPES, 5 mM $CaCl_2$, pH 7.4. After addition of the fluorogenic peptide to a final concentration of 5 µM, the samples were incubated at 37°C in dark for 20

minutes. Fluorescence was measured at an excitation wavelength of 328 nm and emission wavelength of 392 nm using a spectrofluorometer (Tecan, Salzburg Austria).

2.9 Detection of MMP-7 by ELISA

MMP-7 ELISA was performed using Quantikine Human MMP-7 Immunoassay kit according to the manufacturer's instruction (R&D Systems, Wiesbaden-Nordenstadt, Germany). The assay is based on the principle of sandwich immunoassay. MMP-7 specific monoclonal antibody coated plate was used to immobilize MMP-7. After washing off the unbound substances, an enzyme linked polyclonal MMP-7 specific antibody (HRP conjugated MMP-7 antibody) was added to the wells. Further washing removes the unbound conjugated antibody and the addition of HRP substrate to the wells results in colour development. The intensity of colour developed is directly proportional to the amount of MMP-7 present in the sample. The amount of MMP-7 can be quantified by using known amount of MMP-7 standards in a parallel assay. To perform the MMP-7 ELISA from the HEK293-MMP-7 cell line, 50 µl of cell supernatant was used. 5 ng/ml and 10 ng/ml of MMP-7 were used as standards. Cell culture medium was taken as background control.

2.10 2-Dimensional electrophoresis (2-D electrophoresis)

In 2-Dimensional gel electrophoresis the separation of proteins is based on their charge and molecular weight. In first step, proteins are separated on the basis of isoelectric focusing (IEF). In IEF, the proteins migrate electrophoretically in a stationary pH gradient until they reach the pH point where their net charge equals to zero (point, PI). In second step the gel strip is loaded on a SDS-PAGE gel in which the isoelectrically focused proteins are separated based on their molecular mass. Therefore, this method employs both the PI point and molecular mass to get better separation of proteins.

2.10.1 First dimensional electrophoresis

Protein loading and rehydration of the IPG strips

Rehydration stock solution:

Urea	8	M
CHAPS	2	%
IPG buffer	2	%
Bromophenol blue	0.02	%

50 µg of precipitated sample protein were dissolved in 340 µl of the rehydration buffer and placed in a clean reswelling tray. IPG strips (G.E. Healthcare,) are placed gel side down on the sample and care was taken to ensure that there were no air bubbles between the buffer and the gel. To minimize the evaporation and urea crystallization, IPG strips were overlaid with dry strip cover fluid.

Preparation for Iso Electric Focusing (IEF)

The temperature on Multitemp III thermostat circulation was set to 10°C. The cooling plate was placed on Multiphor II unit and ensured that the surface was level. 4 ml of dry strip cover fluid was placed on the cooling plate. The immobilized dry strip tray was aligned on the cooling plate so that the anodic (red) electrode connection of the tray is positioned at the top of the plate near the cooling tubes. Large bubbles between the tray and the cooling plate was removed. 10 ml of dry strip cover fluid was placed into the immobilized dry strip tray and placed the immobiline dry strip aligner 12 groove side up in to the tray on top of the dry strip cover fluid. The electrode strips were cut to a length of 110 mm and soaked with distilled water on a clean surface and the excess water was removed. The rehydrated IPG strips were transferred to the adjacent grooves of the aligner in the immobilized dry strip tray. The strips were placed at the top of the tray with the pointed (acidic) end near the anode (red). The IPG strips were aligned so that the anodic gel edges were lined up. The moistened electrode strips were placed across the cathodic and anodic ends of the aligned IPG strips. Electrodes were aligned over an electrode strip and pressed them down to contact the electrode strips and proceeded for running.

Running condition

The running conditions used for Multiphore II unit was as follows:

For 7 cm IPG strips	500	V	1	minute

	3500	V	90 minutes
	3500	V	90 minutes
For 18 cm strips	500	V	1 minute
	3500	V	90 minutes
	3500	V	6 hours

Equilibration of IPG strips

Equilibration buffer:

Tris/HCl, pH8.8	50	mM
Urea	6	M
Glycerol	30	% (v/v)
SDS	2	%
Bromophenol blue	0.002	%

10 mg/ml of DTT was added to the equilibration buffer prior to use.

IPG strips were placed in the pipette which contains 10 ml of equilibration solution, the plug end of the 10 ml cell culture pipette was removed by cutting and the other pointed end was blocked by heating, after placing the strips in the pipette, the pipette was sealed by using parafilm and placed it on rocker for 15 minutes.

2.10.2 Second dimensional electrophoresis

After completion of the equilibration step, the IPG strips were placed on the top of the SDS-PAGE. The entire lower edge of the IPG strip was put in contact with the top surface of the SDS-gels by gently pressing the strips with a thin plastic ruler. It should be ensured that there is no air bubbles between the strip and the gel. The sealing gel was slowly pipetted onto the IPG strip to seal the narrow gaps between the strip and the gel edges, preventing the IPG strip from moving or floating in the electrophoresis buffer. Gels were kept in the Ettan DALT six gel separation unit and the remaining space was filled by blank cassettes. The lower chamber was filled with 1x running buffer (as described for Western blot) and the upper chamber with 2x running buffer. The run was performed at a constant power of 7 watt per gel for first 30 minutes and 14 watt per gel for further 16-18 hours until

the dye reaches the bottom. The temperature was maintained at 25°C with water circulation through the tank in order to minimize heating.

2.10.3 Silver staining

To visualize the spots, alkaline silver nitrate staining method was chosen because it is compatible for MALDI-TOF sequencing.

Solutions:

Fixing solution:	50% Methanol
Staining solution:	0.2% $AgNO_3$ in 0.06% NaOH containing NH_4OH
Developing solution:	0.037% HCHO in 0.001% CH_3COOH
Stop solution:	5% CH_3COOH in 20% Methanol

Procedure

Gels were transferred in clean trays containing fixing solution and kept on a shaker for overnight at room temperature. On next day the fixing solution was changed twice at an interval of 15 minutes. After fixation the gel was incubated in the staining solution for 20 minutes, followed by washing three times with distilled water for 15 minutes. Developing solution was added and after the appearance of the intense spots, developing was stopped by adding the stop solution.

3 Results

3.1 Establishing MMP-7 expressing HEK293 cell lines

The first part of this study aims to characterize the activation mechanism of MMP-7. It is therefore necessary to produce large amounts of biologically active MMP-7. For establishing a cell line expressing MMP-7, the ecdysone expression system, an inducible mammalian expression system was chosen. This system allows the cells to express MMP-7 when induced by ecdysone or its analog ponasterone A. Furthermore, it offers the possibility to study the dose dependent effects in contrast to constitutive expression systems. HEK293 EcR, a modified human embryonic kidney cell line, was chosen as parent cells. This cell line was established in earlier investigation projects and express stably the receptor for ecdysone.

3.1.1 Construction of the expression vector

The main step in constructing the ecdysone inducible expression system was cloning the MMP-7 cDNA into the plasmid vector pIND (SP1), in which the activity of the promoter is

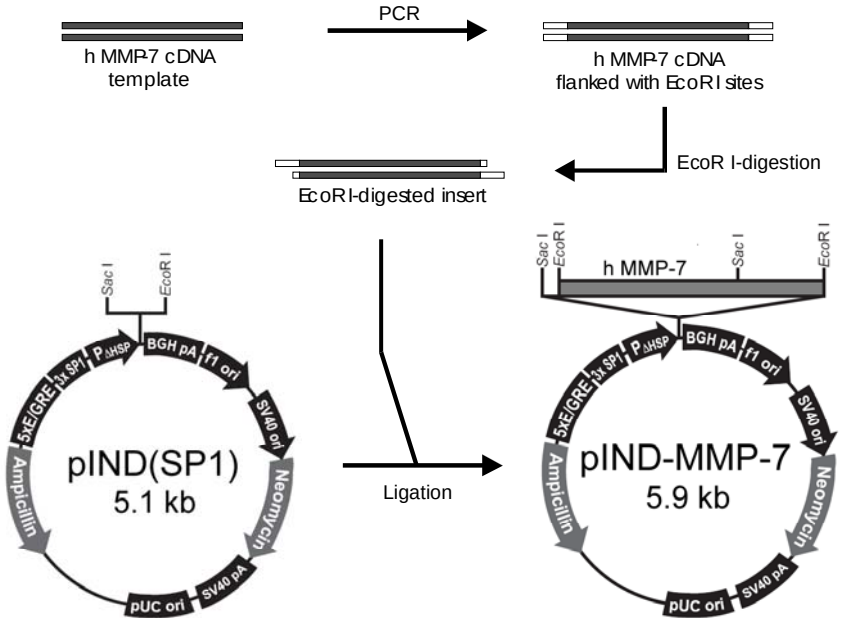


Figure 3: Strategy of constructing an ecdysone inducible expression vector for human MMP-7

controlled by ecdysone response elements. The strategy for cloning MMP-7 into pIND is shown in Figure 3. The full length human MMP-7 cDNA (clone pOTB7-MMP-7), was used as template and amplified by PCR using specific primers containing an EcoR I restriction site. As shown in Figure 4A, the full length human MMP-7 cDNA (800 bp) was successfully amplified as analysed on an agarose gel. The PCR product was then purified using a PCR purification kit and digested with EcoR I. The recovered fragment was ligated with the EcoR I digested pIND (SP1) vector (Figure 4B). The XL-1 Blue *E. coli* cells were transformed with the ligation product. Clones containing full length human MMP-7 cDNA were screened by DNA minipreparations and digestion with EcoR I. As shown in Figure 5A, 6 of the 24 clones tested (clone 11, 12, 13, 15, 17 and 18) were proved to contain the insert, which can be released by digesting with EcoR I.

Since the EcoR I digested fragments could be inserted into the vector in different orientations, the clones containing insert were further digested with Sac I, which releases a fragment of 510 bp in case of the correct orientation and a fragment of 350 bp, if the cDNA is wrongly inserted. This orientation analysis revealed that 4 clones contain MMP-7 cDNA in the correct orientation (clone 13, 15, 17 and 18, Figure 5B). Finally, clones 13 and 15

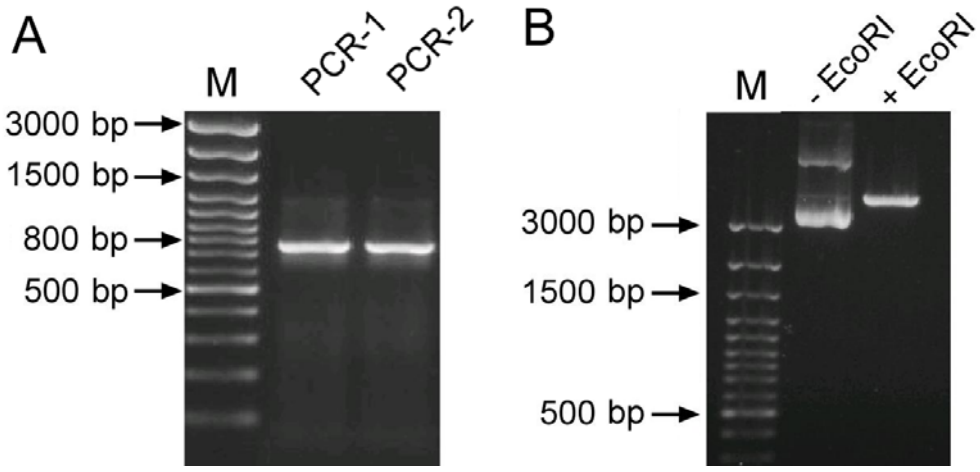


Figure 4: Amplification of full length MMP-7 cDNA by PCR

A full length human cDNA was used as template for amplification with specific primers containing an EcoR I sites in two separate reactions. PCR was performed with an annealing temperature of 60°C for 35 cycles (A). After purification the 810 bp PCR product was digested with EcoR I and ligated to EcoR I digested pIND (SP1) vector fragment (B).
M: DNA molecular weight marker.

were sequenced and it was confirmed that the coding sequence contained no error. The pIND plasmid containing human MMP-7 cDNA was designated as pIND-MMP-7.

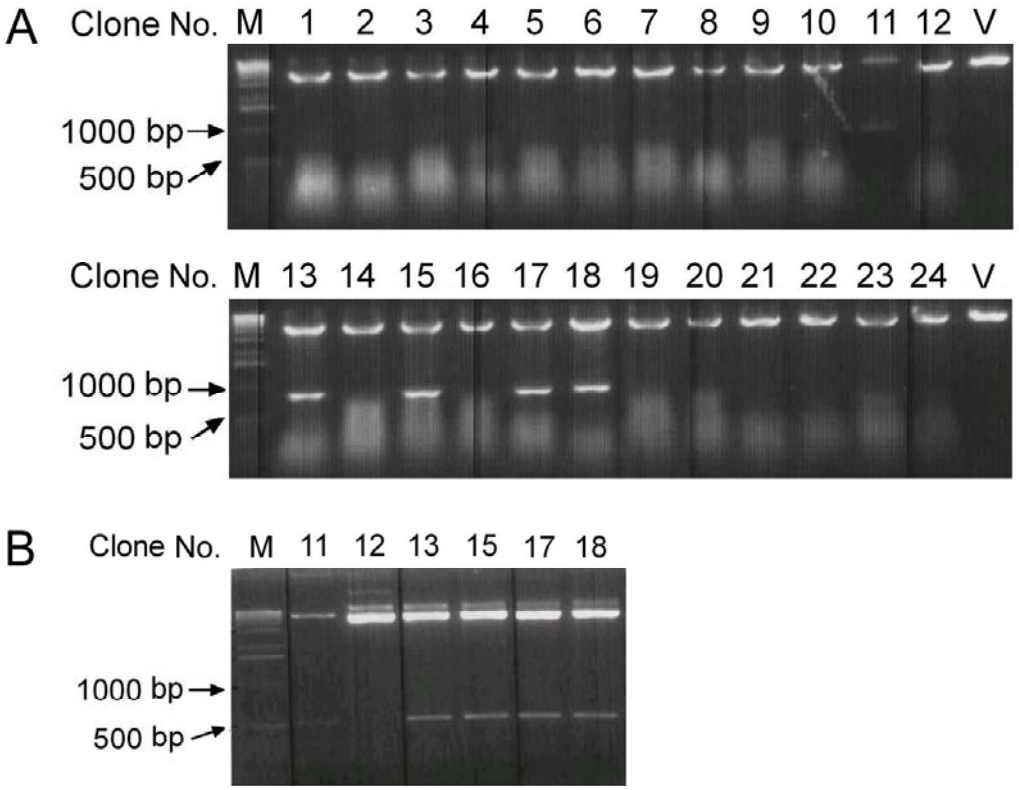


Figure 5: Screen of pIND-MMP-7 expression vector by DNA minipreparation.

24 colonies were picked after transforming the *E. coli* with the ligation product. The isolated plasmid DNA samples were digested with EcoR I to release the insert (A). Six clones containing the insert were analysed on the orientation of their insert by digesting the plasmid with Sac I (B).
M: DNA molecular weight marker.
V: parent vector digested in the same way as control.

3.1.2 Establishment of stable cell lines

As mentioned above, the ecdysone responsive cell line HEK293 EcR was chosen as parent cells. These cells have a stably integrated pVgRXR vector carrying both the retinoid X receptor and the ecdysone receptor genes. Furthermore, the zeocin resistance gene in the pVgRXR vector enables the selection by zeocin.

The HEK293 EcR cells were transfected with pIND-MMP-7 plasmid using lipofectamine.

Transfections were performed in 6 well plates. 5x 10^4 cells per well were incubated with 500 µl DNA/lipofectamine mixture containing 4 µg of pIND-MMP-7 plasmid for 48 hours. The cells were then trypsinized and seeded on 10 cm culture dishes at a density of 3-5x 10^3 cells per dish. The cells were selected by medium supplemented with 400 µg/ml of zeocin, and 400 µg/ml of G418 which selects the pIND-MMP-7 containing cells. Single colonies were picked using a column cylinder and plated in 24 well plates. After growing to 70-80% cell confluency, cells were transferred into 6 well plates and then to 25 cm^2 flasks.

The expanded clones were screened by ELISA for MMP-7 in culture supernatants after induction with 5 µM of ponasterone A. Figure 6 shows that all the 4 tested clones (clone 2, 3, 4 and 5) could be induced to secrete large amounts of MMP-7. The clone 5 was further expanded and analysed by Western blot using a MMP-7 specific antibody. As shown in Figure 7, in the total lysate of untreated cells MMP-7 was not detectable, where as the lysate of cells incubated with ponasterone A (5 µM) contained MMP-7 protein of about 27 kDa molecular weight. MMP-7 expressed in the transfected cells is larger than the

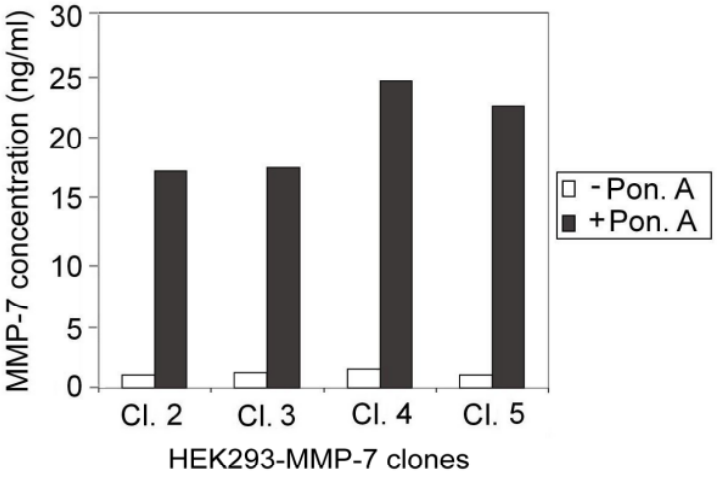


Figure 6: Induction of MMP-7 expression in selected HEK293 EcR MMP-7 clones

Cells of selected clones were allowed to grow to 70-80% confluency in 25 cm^2 culture flasks. 5 µM of ponasterone A was then added. The concentration of MMP-7 in culture supernatants was determined after 24 hours using ELISA specific for MMP-7.
-Pon. A: untreated control cells.
+Pon. A: cells treated with ponasterone A.

commercially available recombinant MMP-7, because the former carries a prodomain in comparison to the recombinant MMP-7, which lacks this domain. Clone 5 was designated as "HEK293-MMP-7" and used for further study.

3.2 Analysis of HEK293-MMP-7 cells

After establishing the HEK293-MMP-7 cell line, the inducible expression of MMP-7 in these cells should be further characterized at different levels.

3.2.1 Transcription of MMP-7 mRNA upon induction

To study the transcription of MMP-7 mRNA upon induction by ponasterone A, the HEK293-MMP-7 cells were incubated with ponasterone A at different concentrations (from 0.5 to 10 µM) for 48 hours. 1 µg of the isolated total RNA was transcribed to cDNA in a reaction volume of 20 µl using a cDNA synthesis kit. 1 µl of cDNA was then amplified by PCR in a volume of 25 µl for 28 cycles using primers specific for MMP-7 and actin,

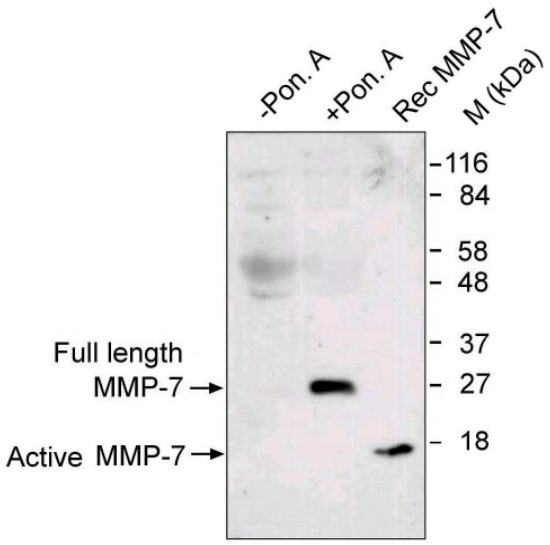


Figure 7: Induction of MMP-7 expression in cells of HEK293-MMP-7 clone 5

Cells of HEK293-MMP-7 clone 5 were incubated with 5 µM ponasterone A for 24 hours. Total cell lysate was analysed for MMP-7 by Western blot using MMP-7 specific antibody.
-Pon. A: untreated control cells.
+Pon. A: cells treated with ponasterone A.
Rec MMP-7: Recombinant active MMP-7 serving as positive control.
M: Protein molecular weight marker

respectively. The annealing temperature was 60°C for both primer pairs. 5 µl of PCR product was loaded on a 1% agarose gel. As shown in Figure 8, the MMP-7 mRNA was not detectable in the absence of ponasterone A but was detected after ponasterone A induction. The expression increases with the dose of ponasterone A (0.5-1.0 µM) and reached the maximum at higher concentrations. These results indicate that the transcription of MMP-7 mRNA is dependent on the presence of ponasterone A.

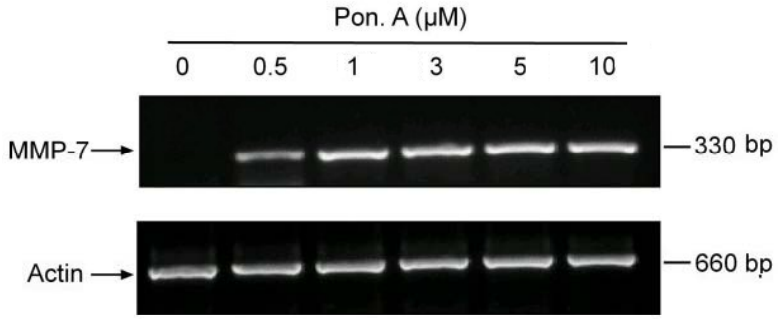


Figure 8: Induction of transcription of MMP-7 mRNA

HEK293-MMP-7 cells were treated with different concentrations of ponasterone A (Pon. A) as indicated for 48 hours. 1 µg isolated total RNA was transcribed to cDNA. A 1/20 of the cDNA was then amplified using primers for MMP-7 and β-actin, respectively. PCR was run at 60°C for annealing for 28 cycles for both genes. The PCR products were resolved on 1% agarose gel stained with ethidium bromide.

3.2.2 Translation of MMP-7 protein upon induction

To analyse induction of MMP-7 expression at the protein level, HEK293-MMP-7 cells were incubated with different concentrations of ponasterone A (0.5-10 µM) for 48 hours, before being lysed in PBS containing 1% NP-40. The lysates were analysed by Western blot using a polyclonal anti-human MMP-7 antibody. As shown in Figure 9, MMP-7 protein was absent in untreated cells, but could be detected at all concentrations of ponasterone A tested. Increased expression of MMP-7 was observed with increasing concentrations of ponasterone A, indicating that the translation of MMP-7 in these cells is also ponasterone A dose dependent.

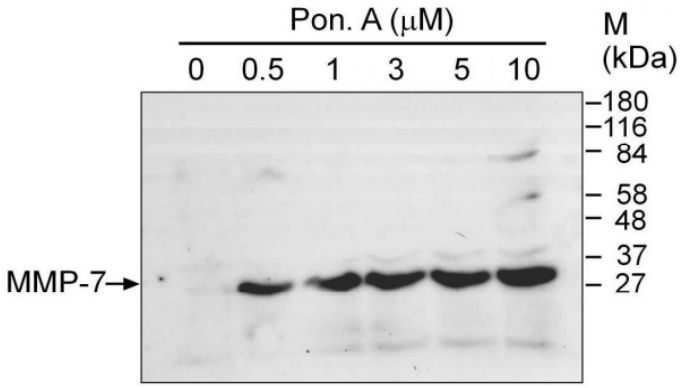


Figure 9: Induction of MMP-7 protein

The expression of full length MMP-7 in HEK293-MMP-7 cells was analysed after induction with different concentrations of ponasterone A (Pon. A) as indicated for 48 hours. The MMP-7 content in total cell lysates was analysed by Western blot using MMP-7 specific antibody.
M: Protein molecular weight marker

3.2.3 Secretion of MMP-7 upon induction

Under physiological conditions, MMP-7 is secreted as a precursor (proMMP-7). The ELISA results shown in Figure 6 demonstrated that MMP-7 was secreted into cell culture supernatant after induction with ponasterone A. In this experiment the secretion of MMP-7 by HEK293-MMP-7 cells was further characterized by Western blot. The dose dependent response was investigated after expansion of HEK293-MMP-7 cells. First the cells were

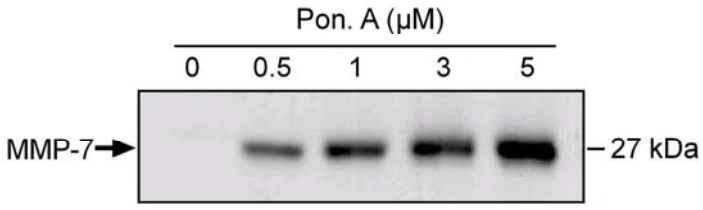


Figure 10: Secretion of MMP-7 by HEK293-MMP-7 cells

HEK293-MMP-7 cells were induced with different concentrations of ponasterone A (Pon. A) in serum free media as indicated for 48 hours. 500µl of supernatant was subjected to acetone precipitation to enrich MMP-7, which was then analysed by Western blot using specific antibody.

seeded in serum containing medium allowing the cells to attach to bottom. Thereafter, the cells were incubated in serum free medium with different concentrations of ponasterone A (0.5-5 μM) for 48 hours. 500 μl supernatants were subjected to acetone precipitation to enrich protein. The precipitated protein was detected by Western blot for MMP-7 expression. As shown in Figure 10, the amount of MMP-7 secreted into culture supernatants upon induction by ponasterone A and increased in a dose dependent manner. This result indicates that the secretion of MMP-7 in HEK293-MMP-7 cells correlates with induction.

3.2.4 Kinetics of MMP-7 expression

The expression level of MMP-7 at different time points after induction was analysed. After attached to the bottom in serum containing medium, HEK293-MMP-7 cells were incubated with 5 μM of ponasterone A in serum free medium for different times (0, 12, 36, 48 and 72 hours). The secreted MMP-7 was acetone precipitated from 500 μl of supernatant and analysed by Western blot. The results shown in Figure 11A demonstrate that the secreted MMP-7 was already detectable by 12 hours after induction, and accumulated over a time period of 72 hours.

The secretion kinetics of MMP-7 by HEK293-MMP-7 cells was further quantified by ELISA. In a separate experiment with the same setting, 50 μl of supernatant was used to determine the concentration of secreted MMP-7 with the MMP-7 Quantikine ELISA Kit. The concentration of MMP-7 in the supernatants was calculated according to a standard curve made by known amounts of recombinant MMP-7. As shown in Figure 11B, the concentration of MMP-7 increased over a period from 12 to 72 hours. These results correspond to those obtained by Western blot analysis, indicating that the induced MMP-7 can be effectively secreted.

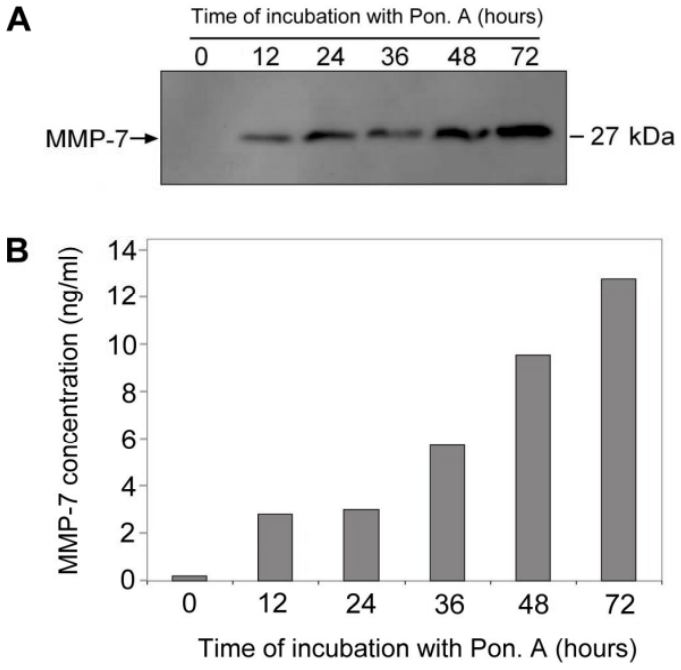


Figure 11: Time kinetics of MMP-7 expression

HEK293-MMP-7 cells were induced with 5 µM of ponasterone A (Pon. A). Supernatants were collected at different time points after induction as indicated. 500 µl of culture supernatants were subjected to acetone precipitation to enrich MMP-7, which was then detected by specific antibody on Western blot (A). For further quantification, 50 µl supernatants were collected from a separate experiment with the same setting and the concentration of MMP-7 was determined by a MMP-7 specific ELISA using commercially available recombinant MMP-7 to produce the standard curve (B).

3.3 Cleavage of MMP-7

Many studies have shown that full length MMP-7 (proMMP-7) is functionally inactive. It is activated by removal of the prodomain. Several substances have been reported to serve as an activator, such as the 3-morpholinosydnonimine hydrochloride (SIN, a NO-donor), the protease trypsin, human leukocyte elastase (HLE) and hydrogen peroxide (H_2O_2) (Fu *et al*., 2001; Imai *et al*., 1995). An autocleavage of proMMP-7 at the temperature of 37°C has been also described (Crabbe *et al*., 1992).

In the following experiments, the cleavage and activation of MMP-7 produced by HEK293-MMP-7 cells were investigated.

3.3.1 Cleavage of MMP-7 by the NO-donor SIN

To analyse the cleavage of proMMP-7 by SIN, HEK293-MMP-7 cells were allowed to attach to culture plate in serum containing medium and then incubated with 5 µM of ponasterone A for 24 hours in serum free medium. Different concentrations of SIN (25, 50 and 100 µM) were added. After 24 hours the supernatants were collected and precipitated with acetone. The cleavage of MMP-7 was analysed by Western blot using a specific antibody recognizing both proMMP-7 and cleaved active MMP-7. Results shown in Figure 12 indicate that the 27 kDa MMP-7 produced by the HEK293-MMP-7 cells could be cleaved by SIN, releasing a protein fragment of 18 kDa, which is of the same size as the recombinant active MMP-7. The cleavage efficiency seemed not to be very high, but dependent on the concentration of SIN.

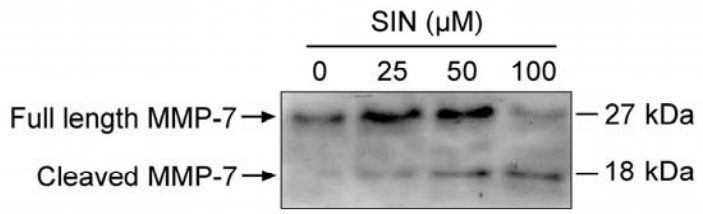


Figure 12: Cleavage of proMMP-7 by SIN

HEK293-MMP-7 cells were induced with 5 µM of ponasterone A for 24 hours. SIN was then added to the cells at different concentrations as indicated. 500 µl supernatants were collected and subjected to acetone precipitation. Enriched proMMP-7 and its cleavage product were analysed by Western blot using a specific antibody recognizing both forms of MMP-7.

3.3.2 Cleavage of MMP-7 by trypsin

Imai *et al*. reported that trypsin cleaves MMP-7 inside its prodomain releasing the active MMP-7 (Imai *et al*., 1995). To test the effect of trypsin on MMP-7 produced by the HEK293-MMP-7 cells, these cells were incubated with 5 µM of ponasterone A in serum free medium for 24 hours to induce the MMP-7 expression. Trypsin was then added at

different concentrations (0, 5, 10 and 50 µg/ml) for further 24 hours. 500 µl supernatants were subjected to acetone precipitation. The enriched MMP-7 and its cleavage product were detected by Western blot using a specific antibody recognizing both proMMP-7 and cleaved active MMP-7. Results shown in Figure 13 indicate that the proMMP-7 produced by HEK293-MMP-7 cells upon induction could also be cleaved by trypsin, as revealed by the bands of 18 kDa. Furthermore, it was observed in this experiment that trace amount of active MMP-7 was also present in supernatant of untreated cells. This could be explained by the mechanism of autocleavage at 37°C as reported (Crabbe *et al*., 1992). That this autocleavage was not detectable in the experiments shown before in this work, was probably due to the amount of samples tested was too low.

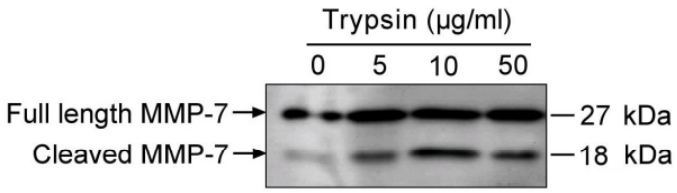


Figure 13: Cleavage of proMMP-7 by trypsin

HEK293-MMP-7 cells were induced with 5 µM of ponasterone A for 24 hours. Trypsin was then added to the cells at different concentrations as indicated. 500 µl supernatants were collected 24 hours later and subjected to acetone precipitation. Enriched proMMP-7 and its cleavage product were analysed by Western blot using a specific antibody recognizing both forms of MMP-7.

3.3.3 Cleavage of MMP-7 by H_2O_2

H_2O_2 is also reported to be capable to activate MMP-7 by cleaving its prodomain. To analyse the effect of H_2O_2 on MMP-7, HEK293-MMP-7 cells were induced to secret MMP-7 with ponasterone A as described in §3.3.1. The cells were then treated with different concentrations of H_2O_2 (5, 10 and 50 µM) for 24 hours. The supernatants were collected and proteins were concentrated by acetone precipitation. proMMP-7 and its cleavage product were analysed by Western blot using a specific antibody. The data shown in Figure 14 demonstrated, similar to the experiments with SIN and trypsin, that the proMMP-7 could be cleaved by H_2O_2, although the efficiency was relatively low. In

supernatant of untreated cells, only trace amount of cleaved MMP-7 could be noticed.

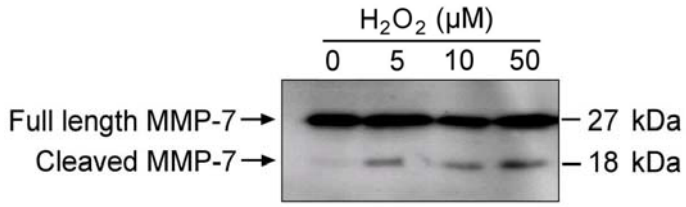


Figure 14: Cleavage of proMMP-7 by H_2O_2

HEK293-MMP-7 cells were induced with 5 µM of ponasterone A for 24 hours. H_2O_2 was then added to the cells at different concentrations. After 24 hours, 500 µl supernatants were collected and the proteins concentrated by acetone precipitation. Enriched proMMP-7 and its cleaved product were detected by Western blot using specific antibody recognizing both forms of MMP-7.

3.3.4 Cleavage of MMP-7 by human leukocyte elastase (HLE)

Imai *et al*. have reported that HLE also cleaves and activates full length MMP-7 isolated from human rectal carcinoma cells. Nevertheless, even using high concentration of HLE (10 µg/ml), an activation rate of about 50% of the full activity could be reached. The authors speculated that there must be some other mechanisms necessary for proMMP-7 activation *in vivo* (Imai *et al*. 1995). Efforts were made in the following experiments to find out the role of HLE in cleavage and activation of MMP-7.

At first the capability of HLE to cleave the proMMP-7 was examined. The expression of proMMP-7 in HEK293-MMP-7 cells were induced with 5 µM of ponasterone A as described in §3.3.1. HLE was added to cells at different concentrations (25, 50 and 100 ng/ml). The culture supernatants were collected 24 hours later. The proMMP-7 and its cleavage product were concentrated by acetone precipitation and analysed by Western blot using a specific antibody. As shown in Figure 15, HLE cleaved the proMMP-7 at a much higher efficiency than the other activators tested before.

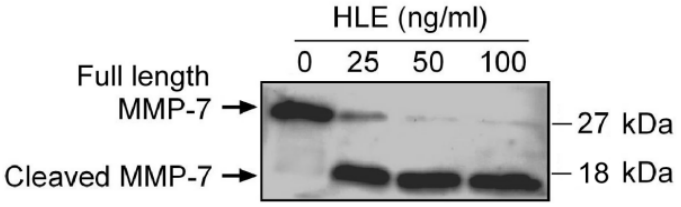


Figure 15: Cleavage of proMMP-7 by HLE

HEK293-MMP-7 cells were induced with 5 µM of ponasterone A for 24 hours and treated with different concentrations of HLE as indicated. The supernatants were collected 24 hours later. proMMP-7 and its cleavage product were analysed by Western blot using a specific antibody.

3.4 Influence of HLE on MMP-7 activity

3.4.1 Enzymatic activity of the cleaved MMP-7

Based on the findings that HLE can very effectively cleave proMMP-7, it was possible to examine if the released MMP-7 was functionally active. The expression of MMP-7 in HEK293-MMP-7 cells was induced with 5 µM of ponasterone A for 24 hours in the same way as described before. Especially for this functional assay, equal amount of ethanol (solvent of ponasterone A) was added to the control cells. The secreted proMMP-7 was recovered from culture supernatants by precipitation with acetone and dissolved in water. The samples were then treated with 50 ng of HLE for 3 hours at 37°C. The activity of MMP-7 was measured using a MMP-7 specific fluorogenic substrate. This substrate is a small peptide consisting of 8 amino acid residues with the following sequence: DNP-Arg -Pro-Leu-Ala-Leu-Trp-Arg-Ser-OH. In the uncleaved form, the fluorophore Trp is quenched by DNP so that it emits no fluorescence. MMP-7 cleaves this peptide by breaking the bond between alanine and leucine, resulting in emission of fluorescence. The intensity of fluorescence correlates with the activity of the MMP-7. The results shown in Figure 16 demonstrate that only very low MMP-7 activity could be detected in the uninduced samples, which presented the background of the assay. But unexpectedly, the induced sample exhibited relatively high activity without cleavage by HLE. This may be explained by the temperature induced autocleavage mechanism described previously (Crabbe *et al.*,

1992). Moreover, it was surprising to find out that the cleavage by HLE lead even to a reduction of more than 80% of the MMP-7 activity compared to the uncleaved sample.

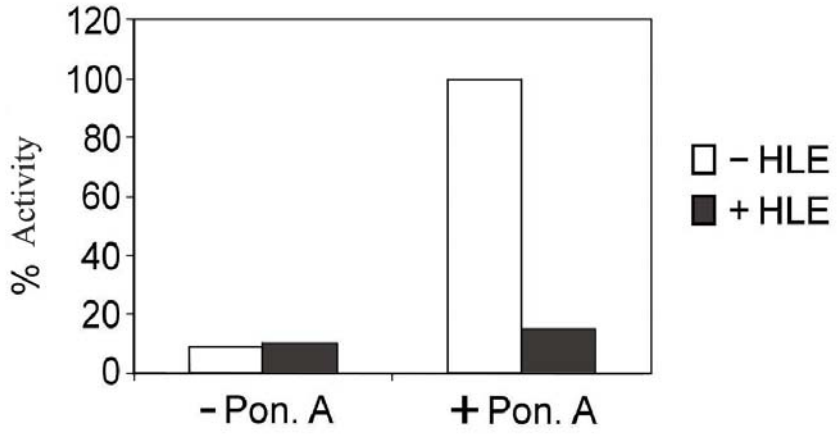


Figure 16: Enzymatic activity of MMP-7 after cleavage by HLE

HEK293-MMP-7 cells were induced with 5 µM of ponasterone A for 24 hours. 500 µl supernatants were used to precipitate secreted proMMP-7. The recovered precipitates were dissolved in water and incubated without or with 50 ng of HLE per reaction for 3 hours. MMP-7 activity was measured using a MMP-7 specific fluorogenic substrate. The cleaved substrate emits fluorescence which is detected using a spectrofluorometer. The relative activity was calculated, taken the fluorescence intensity of uncleaved sample from induced cells as 100%.
-Pon. A: uninduced control cells; **-Pon. A:** cells induced with ponasterone A
-HLE: untreated samples; **+HLE:** HLE treated samples

3.4.2 HLE cleaves the catalytic protein domain of MMP-7

The finding that HLE cleaves proMMP-7 but reduces its activity suggests that HLE may cleave MMP-7 within its catalytic domain. To examine this possibility, experiments were performed to map the cleavage site of MMP-7. HEK293-MMP-7 cells were induced and treated with APMA and HLE. Recombinant active MMP-7 which consists of the catalytic MMP-7 domain and lacks the prodomain was also treated with HLE. Cell supernatants were acetone precipitated and subjected to Western blot for MMP-7. Figure 17A shows that the full length MMP-7 was cleaved by APMA and HLE. The cleaved products from APMA and HLE treatment runs differently which indicates the difference in their cleavage sites. To analyse the dose dependent effect of HLE on MMP-7, active recombinant MMP-7 was

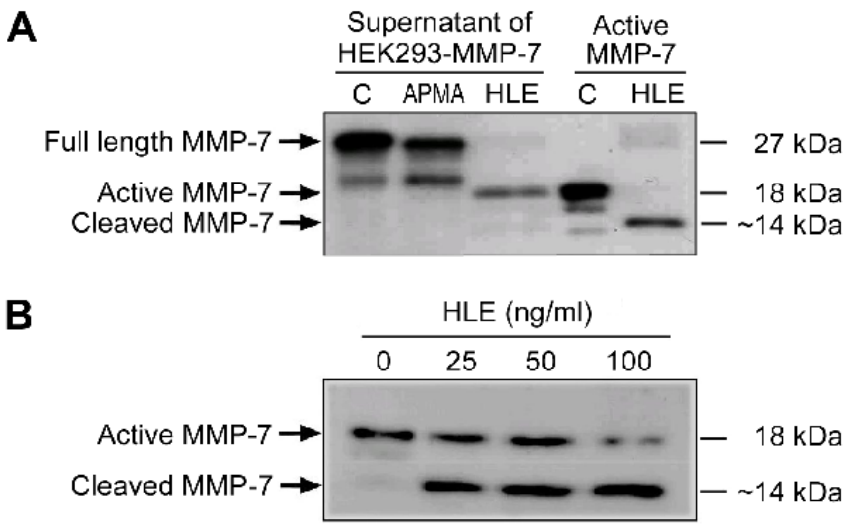


Figure 17: Cleavage of active MMP-7 by HLE

HEK293-MMP-7 cells were induced with 5 M of ponasterone A for 24 hours. Cell supernatants were collected and acetone precipitated 100 ng of recombinant active MMP-7 were incubated with different concentrations of HLE as indicated at 37°C for 3 hours. The cleavage products were analysed by Western blot using a specific antibody recognizing the N-terminus of active MMP-7.

treated with HLE at different concentrations (25, 50 and 100 ng/ml) at 37°C for 3 hours. The cleavage product was detected by Western blot using a MMP-7 specific antibody. As shown in Figure 17B, the active MMP-7 could be further cleaved by HLE, releasing a shorter fragment. The cleavage efficiency was also HLE dose dependent. At the concentration of 100 ng HLE/ml a cleavage rate of approximately 90% was reached. Because the epitope recognized by the antibody is located near the N-terminus, the detected fragment must contain the N-terminal part, whereas the cleaved C-terminal fragment(s) cannot be detected by Western blot. These results have confirmed the presence of at least one HLE cleavage site inside the catalytic domain of MMP-7.

3.4.3 Mapping of the HLE cleavage site within the catalytic domain of MMP-7

The results from the Western analysis demonstrated that active MMP-7 lacking the prodomain is cleaved by HLE. Further experiments were performed to identify the HLE

cleavage site in active MMP-7. For this purpose, 2 µg of recombinant MMP-7 was treated with 500 ng of HLE for 3 hours at 37°C. Cleavage products were separated by SDS-PAGE and stained by silver nitrate to visualize all the cleavage products. As shown in Figure 18A, HLE released from active MMP-7 one more fragment in addition to the fragment, which could be detected by Western blot. Therefore, it seems to be only a single cleavage site in the active MMP-7. To sequence these two fragments, the cleavage products were transferred to PVDF membrane after being resolved on SDS-PAGE. The membrane was reversibly stained with Ponceau-S to visualize protein bands. The two bands corresponding to the cleaved fragments were eluted and used for sequencing. Sequencing from amino termini was performed by Dr. Hans Heid at German Cancer Research Center (DKFZ, Heidelberg). The amino terminal sequences, as shown in Figure 18B, revealed that the cleavage site is located towards the C-terminus of MMP-7, between amino acid residues valine^{231} and methonine^{232}. The band I with predicted molecular mass of 14 kDa is proved to be the N-terminal part and the band II with deduced molecular mass of 4.1 kDa the C-terminal part. Furthermore, the HLE cleavage site is located near the active center containing the zinc binding domain. This explains the finding that cleavage of MMP-7 by HLE lead to reduction of the MMP-7 activity.

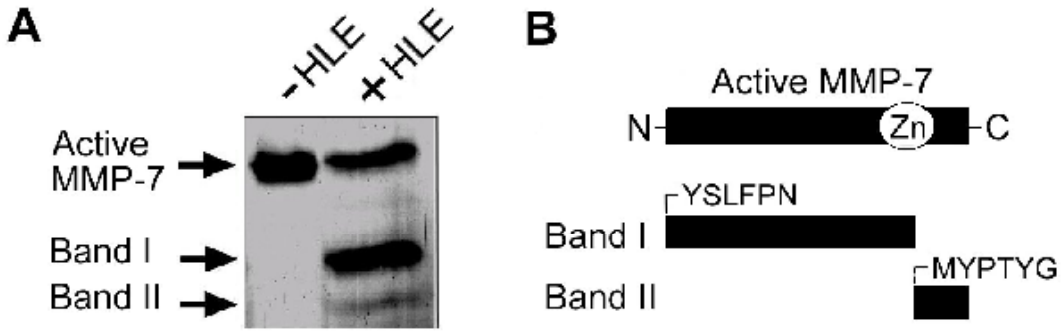


Figure 18: Mapping of HLE cleavage site within the catalytic domain of MMP-7

2 µg of recombinant MMP-7 were treated with 500 ng HLE per reaction. The cleaved products were separated on a SDS-PAGE. Protein bands were visualized by silver staining (A). The two cleaved fragments (bands I and II) were sequenced after being resolved on SDS-PAGE, transferred to membrane, visualized by Ponseau-S staining and eluted from the membrane. Sequencing was performed from the N-terminus. The obtained sequences were compared to the published MMP-7 sequence from PubMed database (accession number: NP_002414).

3.4.4 Dose dependent inactivation of MMP-7 by HLE

Previous experiments in this study showed that HLE cleaves the proMMP-7 produced by HEK293-MMP-7 cells resulting in loss of MMP-7 activity. Sequencing of fragments cleaved by HLE from recombinant active MMP-7 reveals a cleavage site of HLE near the active center in the catalytic domain and cleavage at this site should lead to the inactivation of the enzyme. In the following experiment the inactivation of active MMP-7 by HLE was analysed. 100 ng of recombinant MMP-7 was incubated with different amounts of HLE (25, 50, 75 and 100 ng per reaction) at 37°C for 3 hours. 100 ng of MMP-7 alone and 100 ng of HLE alone served as positive and negative controls respectively. Activity of MMP-7 was determined using a MMP-7 specific fluorogenic substrate as described in §3.4.2. The data shown in Figure 19 demonstrate the dose dependent reduction of MMP-7 activity in correlation to HLE concentration. A 30% reduction in MMP-7 activity was observed with 25 ng of HLE compared to positive control, and 50 ng of HLE reduced MMP-7 activity almost to the background level as shown by negative control. These results confirm that cleavage within the active domain of MMP-7 by HLE indeed abolishes the proteolytic activity of MMP-7.

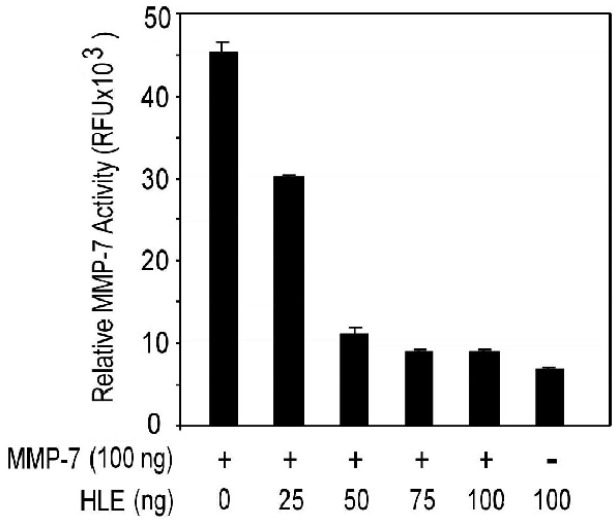


Figure 19: Dose dependent inactivation of MMP-7 by HLE

100 ng of recombinant MMP-7 were incubated with different amount of HLE as indicated for 3 hours. Activity of MMP-7 was measured using a MMP-7 specific fluorogenic substrate. The intensity of fluorescence was measured using a spectrofluorometer and expressed as relative fluorescence unit (RFU).

3.4.5 Time dependent inactivation of MMP-7 by HLE

The time course of the cleavage inactivation of MMP-7 by HLE was analysed in this experiment. 200 ng of recombinant active MMP-7 was incubated with 50 ng of HLE at 37°C for different times (30, 60, 120 and 180 minutes). MMP-7 without HLE was also incubated for same time periods as controls. MMP-7 activity was measured using a MMP-7 specific fluorogenic substrate as described previously (§3.4.1). The results shown in Figure 20 demonstrate that HLE reduces the activity of MMP-7 time dependently, whereas the activity of MMP-7 in controls remained constant.

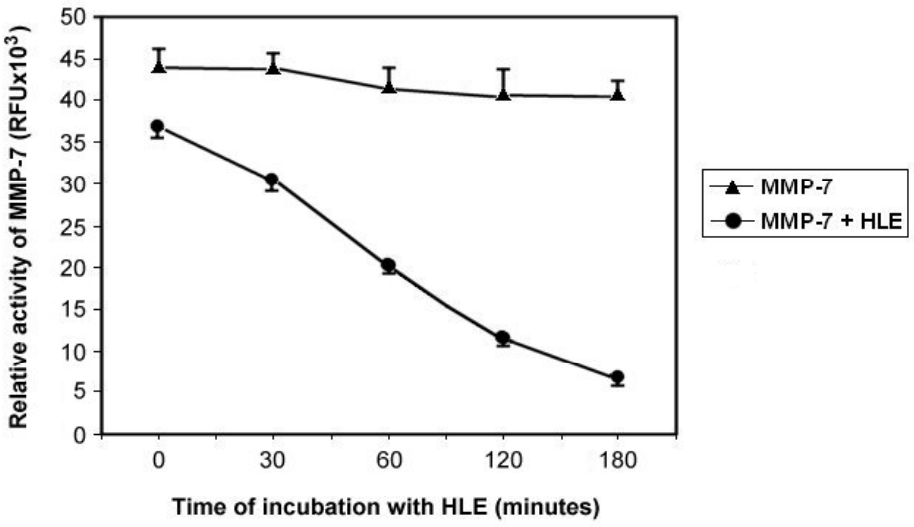


Figure 20: Time dependent inactivation of MMP-7 by HLE

200 ng of recombinant MMP-7 was incubated without or with 50 ng of HLE at 37°C for indicated times. Activity of MMP-7 was measured using a specific fluorogenic substrate. The intensity of fluorescence was measured by a spectrofluorometer. MMP-7 activity was expressed as relative fluorescence unit (RFU).

3.5 Modulation of the MMP-7 induced apoptosis resistance by HLE

MMP-7 is known to play important roles in tumourigenesis (Zeng *et al*., 2002). For example, it has been reported previously that MMP-7 decreases the apoptosis sensitivity of tumour cells (Strand *et al*., 2004b). Therefore, great efforts have been made in seeking for modulators which inhibit or eliminate MMP-7 activity and may be used for tumour therapy. The finding in this study that HLE cleaves active MMP-7 to abolish its enzymatic activity

suggests that HLE may be a hopeful candidate. In the following experiments the effects of HLE on MMP-7 mediated apoptosis resistance were investigated.

3.5.1 Effects of MMP-7 and HLE on CD95 mediated apoptosis

To test the roles of HLE in modulation of MMP-7 mediated apoptosis resistance, a CD95 mediated apoptosis model was chosen. In this experiment model the apoptosis is induced by binding of anti-Apo-1, an agonistic antibody to the apoptosis inducing receptor CD95. In this experiment HepG2 cells were seeded in 12 well plates and treated with 200 ng/ml of recombinant MMP-7, 50 ng/ml of HLE or both MMP-7 and HLE for 12 hours, before apoptosis was induced by adding anti-Apo-1 to cells at the concentration of 200 ng/ml. 48 hours later, the apoptotic cells were detected and quantified by FACS analysis after fixation and staining with propidium iodide, according to Nicoletti *et al.* (Nicoletti *et al*., 1991). As shown in Figure 21, the anti-Apo-1 induced apoptosis in 51% of cells. If the cells were pretreated with MMP-7 the rate of apoptotic cells was strongly decreased to 31%. As expected, if cells were pretreated with MMP-7 in the presence of HLE, the rate of apoptotic cells increased to 41%. As the pretreatment with HLE alone did not change the apoptotic rate compared to control (47%), it could be concluded that HLE can abolish the ability of MMP-7 to increase apoptosis resistance. In other words, HLE can enhance the apoptosis sensitivity of cells by inactivating MMP-7.

In further experiments, cell death was measured by cytotoxicity assays using “Cell Titer Glo® Luminescent Cell Viability Assay” to confirm the results obtained by FACS. With this kit, cell viability is measured based on the ability of live cells to provide metabolically active ATP which facilitates the generation of luminescent signal by luciferase. HepG2 cells were treated as described above. The cell lysates were prepared according to manufacturer’s instructions. The intensity of luminescence was recorded using a luminometer.

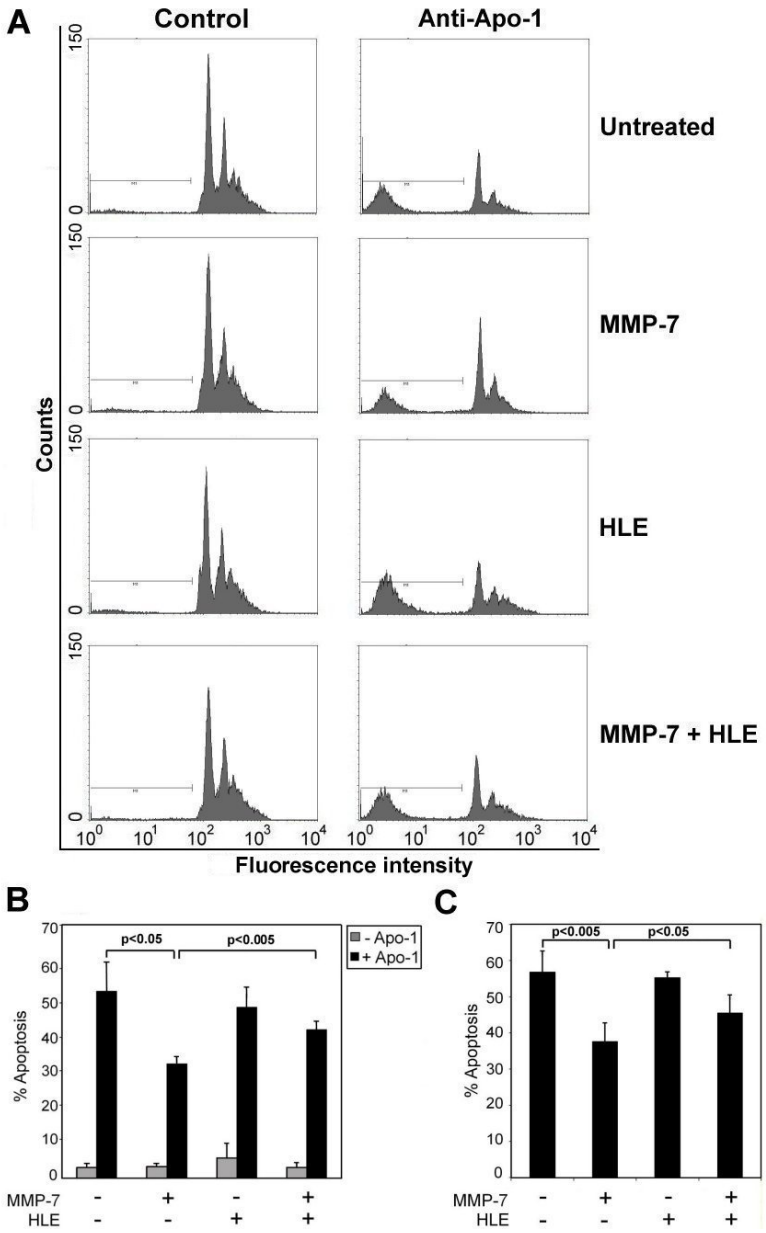


Figure 21: Effect of HLE on MMP-7 induced apoptosis resistance in HepG2 cells

HepG2 cells were pretreated with MMP-7 (200 ng/ml), HLE (50 ng/ml) or both MMP-7 and HLE for 12 hours. Apoptosis was then induced by adding 200 ng/ml of anti-Apo-1 antibody. After 48 hours cells were fixed and stained with propidium iodide. The number of apoptotic cells was measured by FACS (A). The quantitative evaluation of the apoptosis rates was summarized in a graphic (B). In the parallel treatments, the cell viability was measured using Cell Titer Glo® Luminescent Cell Viability Assay (C). The rate of apoptosis for treated samples was calculated taken the %apoptosis for control cells not treated with anti-Apo-1 as 0%.

The results shown in Figure 21C were similar to those of FACS analysis (Figure 21A and B). MMP-7 increased significantly the apoptosis resistance of cells and this effect was almost completely abolished by HLE. These results suggest that HLE inactivates MMP-7 and thus restores apoptosis sensitivity. Similarly experiment was performed in HEK293 cells and the results obtained were in agreement with HepG2 cells as shown in Figure 22A and B.

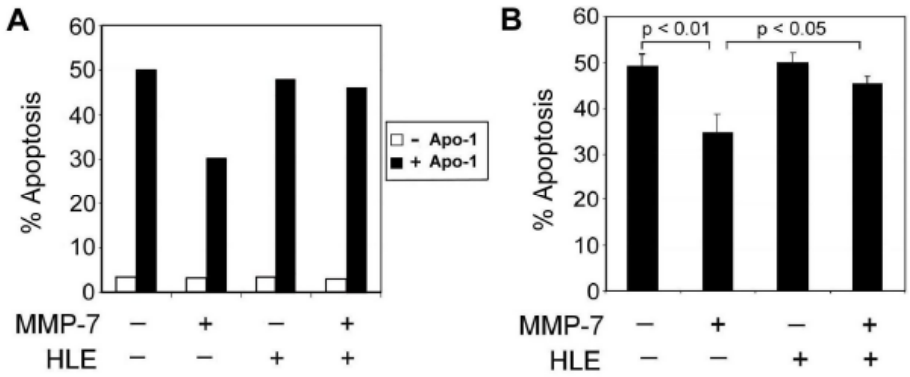


Figure 22: Effect of HLE on MMP-7 induced apoptosis resistance in HEK293 cells

HEK293 cells were pretreated with MMP-7 (200 ng/ml), HLE (50 ng/ml) or both MMP-7 and HLE for 12 hours. Apoptosis was then induced by adding 200 ng/ml of anti-Apo-1 antibody. After 48 hours cells were fixed and stained with propidium iodide. The number of apoptotic cells was measured by FACS. The quantitative evaluation of the apoptosis rates was summarized in a graphic (A). In the parallel treatments, the cell viability was measured using Cell Titer Glo® Luminescent Cell Viability Assay (B). The rate of apoptosis for treated samples was calculated taken the %apoptosis for control cells not treated with anti-Apo-1 as 0%.

3.5.2 Effects of MMP-7 and HLE on cytotoxicity mediated by Doxorubicin

Doxorubicin (Dox) is widely used for cancer chemotherapy. Dox activates the tumour suppressor p53 by DNA damage resulting in increased CD95 expression and induction of apoptosis (Muller *et al*., 1997; Poulaki *et al*., 2001). It was reported that MMP-7 could reduce the cytotoxicity of Dox by cleavage of the CD95 ligand (Poulaki *et al*., 2001). It is thus of interest to analyse the effect of HLE on Dox mediated cytotoxicity in the presence of MMP-7.

Because Dox can induce both apoptosis and necrosis, a specific method for detecting apoptosis, such as Nicoletti assay is used (Nicoletti *et al*., 1991). Otherwise the term

"cytotoxicity" is used.

Before the effect of HLE was tested, it was necessary to optimize the concentrations of Dox and MMP-7, so that about 50% cell death would be reached, allowing the detection of further up or down modulation by HLE. For this purpose, HEK293 cells were incubated with MMP-7 at different concentrations (0, 100 and 200 ng/ml) in the presence of Dox (250 and 500 ng/ml) for 24 hours. The viable cells were quantified using the Cell Titer Glo® Luminescent Cell Viability Assay. As shown in Figure 23, Dox induced cytotoxicity dose dependently, which could be overcome by MMP-7 also in a dose dependent manner.

These results were in agreement with the previous report (Mitsiades *et al*., 2001). The combination of 200 ng/ml of MMP-7 and 500 ng/ml of doxorubicin was chosen as optimal, because it yielded about 50% cytotoxicity allowing further modulation by HLE both upwards and downwards.

After the optimal concentrations of Dox and MMP-7 were optimized, the effect of HLE on the MMP-7 mediated apoptosis resistance was examined using the Nicoletti assay (Nicoletti *et al*., 1991). HepG2 and HEK293 cells were treated additionally with 50 ng/ml of HLE in the presence or absence of MMP-7. Dox was added to 500 ng/ml. Cells were

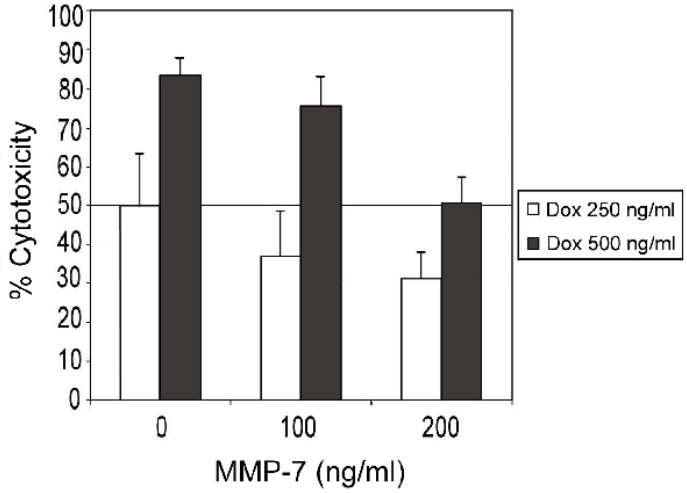


Figure 23: Effect of MMP-7 on doxorubicin induced cytotoxicity

HEK293 cells were treated with different combinations of MMP-7 and Dox as indicated for 24 hours to induce cytotoxicity. The survival cells were quantified using the Luminescent Cell Viability Assay. The percentage of cytotoxicity of treated samples was calculated, taken the cytotoxicity rate of untreated control cells as 0%.

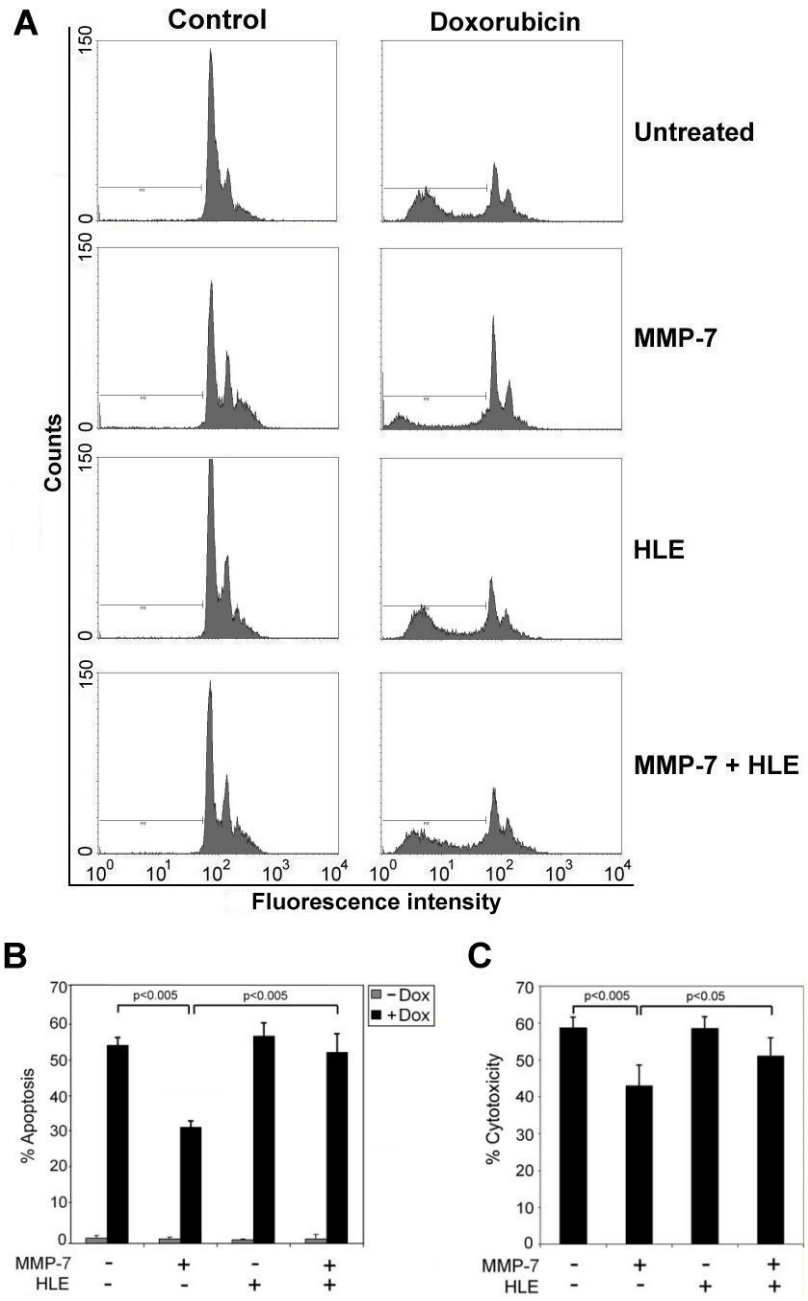


Figure 24: Effect of MMP-7 and HLE on Dox mediated apoptosis in HepG2 cells

HepG2 cells were treated with MMP-7 (200 ng/ml), HLE (50 ng/ml) or both HLE and MMP-7 for 24 hours. Dox (500 ng/ml) was then added and cells were cultured for further 48 hours. Apoptotic cells were analysed by FACS after fixation and staining with propidium iodide. The quantitative evaluation of the apoptosis rates was summarized in a graphic (A). In the parallel treatments, the cell viability was measured using the Cell Titer Glo® Luminescent Cell Viability Assay (B). The percentage of cytotoxicity of treated samples was calculated taken the cytotoxicity rate of untreated control cells as 0%.

harvested 48 hours later and apoptotic cells were quantified by FACS after fixation and staining with propidium iodide. The FACS analysis from HepG2 cells (Figure 24A and B) shows a decrease in the percentage of apoptotic cells in the MMP-7 treated cells (31%) compared to control cells (53%). If HLE was present, the rate of apoptosis was increased nearly to the level of control (51%). Results from the HEK293 cells also show the similar trend as in HepG2 cells (Figure 25A). This effect was solely due to the inactivation of MMP-7 by HLE, since HLE alone did not affect the apoptosis rate. These results were further confirmed by the Cell Viability Assay (Figure 24C). Similar results were obtained with HEK293 cells as demonstrated in Figure 25B. MMP-7 decreased the percentage of cell death caused by Dox significantly ($p<0.05$). HLE diminished the effect of MMP-7 ($p<0.05$), without influencing the cytotoxicity of Dox by itself.

In summary, in both CD95 mediated apoptosis and Dox mediated apoptosis, HLE can abolish the MMP-7 induced apoptosis resistance.

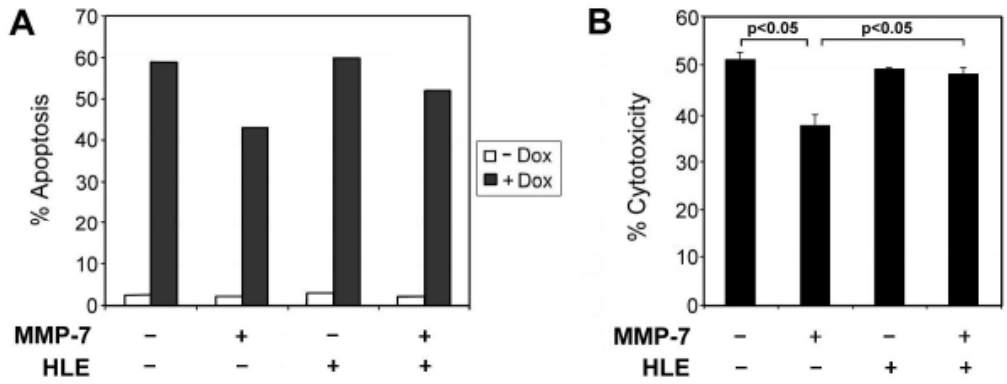


Figure 25: Effect of MMP-7 and HLE on Dox mediated apoptosis in HEK293 cells

HEK293 cells were treated with MMP-7 (200 ng/ml), HLE (50 ng/ml) or both HLE and MMP-7 for 24 hours. Dox (500 ng/ml) was then added and cells were cultured for further 48 hours. Apoptotic cells were analysed by FACS after fixation and staining with propidium iodide. The quantitative evaluation of the apoptosis rates was summarized in a graphic (A). In the parallel treatments, the cell viability was measured using the Cell Titer Glo® Luminescent Cell Viability Assay (B). The percentage of cytotoxicity of treated samples was calculated taken the cytotoxicity rate of untreated control cells as 0%.

3.5.3 Effect MMP-7 and HLE on CTL functions-

It is well known that tumour cells can be eliminated by cytotoxic T cells (CTLs) via CD95 mediated apoptosis. Tumour cells can escape from the CTL mediated killing. Tumour caused immunsuppression is an important mechanisms for tumour evasion from cytotoxic effects of host CTLs. MMP-7 facilitates tumour escape by cleaving both CD95 and CD95L. Previous studies have shown that specific cytotoxic T cell killing of their target cells was reduced in the presence of MMP-7 (Strand *et al.*, 2004b). After having shown that HLE inactivates MMP-7, the effect of HLE on the MMP-7 mediated ablation of CTL killing was analysed in coculture experiments using an A2.1 restricted CTL clone specific for a p53 peptide (CD8xA2k^b p53). A2.1 restricted HepG2 hepatoblastoma cells were pulsed with the p53 peptide and labelled with ^{51}Cr after being treated with MMP-7 (200 ng/ml),

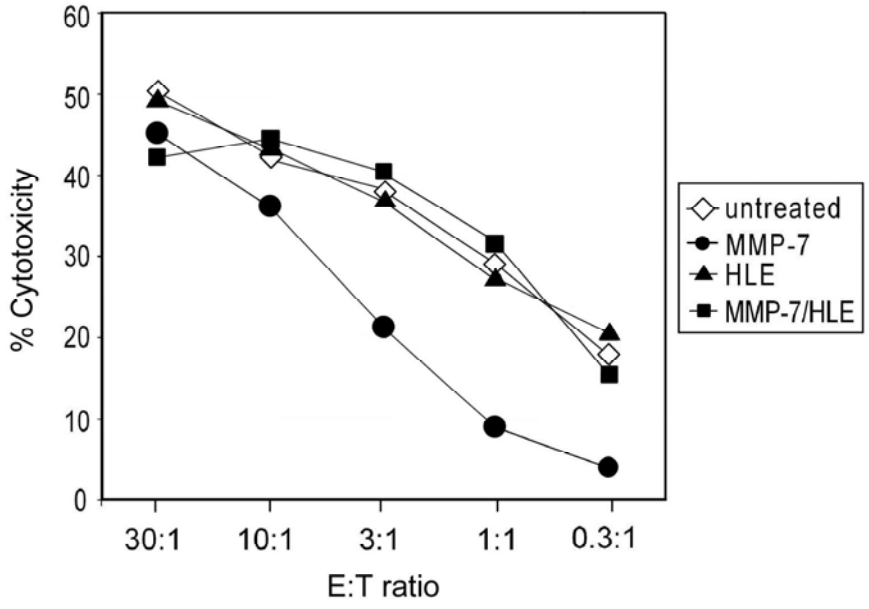


Figure 26: Modulation of resistance against CTL-killing by MMP-7 and HLE

HepG2 cells were treated with MMP-7 (200 ng/ml), HLE (50 ng/ml) or both MMP-7 and HLE. After treatment, HepG2 cells were pulsed with a p53 peptide and labelled with ^{51}Cr. These cells were then cocultured with a CTL clone specific for the p53 peptide (CD8xA2k^b p53) at the indicated effector to target ratios (E:T ratio). ^{51}Cr release was measured after 4-5 hours. The percentage of cytotoxicity was calculated taken the cytotoxicity value of control coculture as 0%, in which the target cells were not pulsed with any peptide. Maximal lysis of target cells was taken as 100%.

HLE (50 ng/ml) or both MMP-7 and HLE. The CTL (CD8xA2k^b p53) and treated HepG2 cells were then cocultured at different effector to target ratios (E:T ratio). ^{51}Cr release was measured after 4-5 hours. The peptide specificity of the CTLs was controlled by their inability to lyse target cells loaded with an irrelevant peptide.

As shown in Figure 26, in the presence of MMP-7, substantial reduction in CTL killing of targets was observed whereas in the presence of both HLE and MMP-7, the cytotoxic effects of T cells were kept at control levels. HLE alone did not affect cytotoxicity. This result indicates that HLE can also counteract the MMP-7 mediated apoptosis resistance of target cells during cytotoxic T cell killing.

3.5.4 Colocalization of MMP-7 and HLE in tumour tissues

The results of this study have revealed the role of HLE to counteract the anti-apoptotic effects of MMP-7 *in vitro*, it became interesting to examine, whether such events could also happen *in vivo*. Because HLE is produced only by polymorphonuclear neutrophils (PMNs), colocalization of PMNs and MMP-7 producing tumour cells is necessary for counteracting

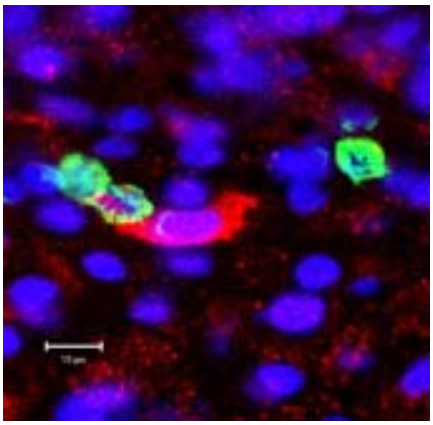


Figure 27: Colocalization of MMP-7 and HLE in tumour tissues

Tumour cells expressing MMP-7 and PMNs producing HLE were detected on cryosections of colorectal tumour biopsies by immunofluorescence staining. MMP-7 was detected by a mouse anti-MMP-7 antibody and FITC-conjugated goat anti-mouse antibody (green). HLE was detected by rabbit anti-HLE antibody and Cy3-conjugated goat anti-rabbit antibody (red). Cell nuclei (blue) were visualized by counterstaining with Hoechst. Images were taken using a confocal laser scanning microscope. Bar = 10 μm.

MMP-7 by HLE. The colocalization of PMNs and MMP-7 producing tumour cells was identified by immunostaining of colorectal tumours which are reported to express MMP-7. Cryosections of MMP-7 positive colorectal tumour tissue samples were stained for MMP-7 and HLE. Figure 27 shows a presentative overlay image, demonstrating the colocalized MMP-7 positive and HLE positive, neutrophil cells.

3.6 Identification of new substrate(s) for MMP-7

The second main task of this study is to identify new immune relevant substrates of MMP-7 for better understanding the mechanism of immune modulations involving MMP-7.

3.6.1. Screening of MMP-7 substrates by using 2-D electrophoresis

Since MMP-7 acts on the extracellular proteins, so it was assumed that cleavage products might be detected in supernatants of MMP-7 treated cells. The 2-D electrophoresis technology was chosen for analysing the cleavage product, because small differences in proteins can be detected due to the high resolution of protein separation using this technique. As a model system the T lymphoblastic cell line Jurkat (J16) cells were selected for this study.

Two million Jurkat cells were treated with 200 ng of recombinant active MMP-7 in 1 ml of serum free medium for 1 hour at 37°C. Untreated cells served as control. After treatment, the supernatants were collected by centrifugation at 3,000 rpm for 5minutes. The proteins were precipitated with acetone and dissolved in distilled water. The protein concentration was estimated using Bradford method. For first dimensional electrophoresis, 100 µg of protein was added to IPG buffer and loaded on 18 cm IEF strips which had a pH gradient from 3 to 10. Samples were separated in the first dimensional electrophoresis by following the conditions described in chapter *Materials and Methods*. For the second dimensional electrophoresis, strips were loaded on SDS-PAGE gels. After running the 2-D electrophoresis, gels were developed by silver staining. The spots on gels with MMP-7 treated and untreated samples were compared by overlaying the gels. This experiment was repeated three times and one spot found to be strongly differentially detected in all three

tests was excised and stored at -80°C for further sequence analysis.

Figure 28 shows a representative gel pair. The spot marked with the arrow was in all three experiments strongly differentially displayed. Using the MALDI-TOF technology, it was identified as heat shock cognate protein 70 (Hsc70).

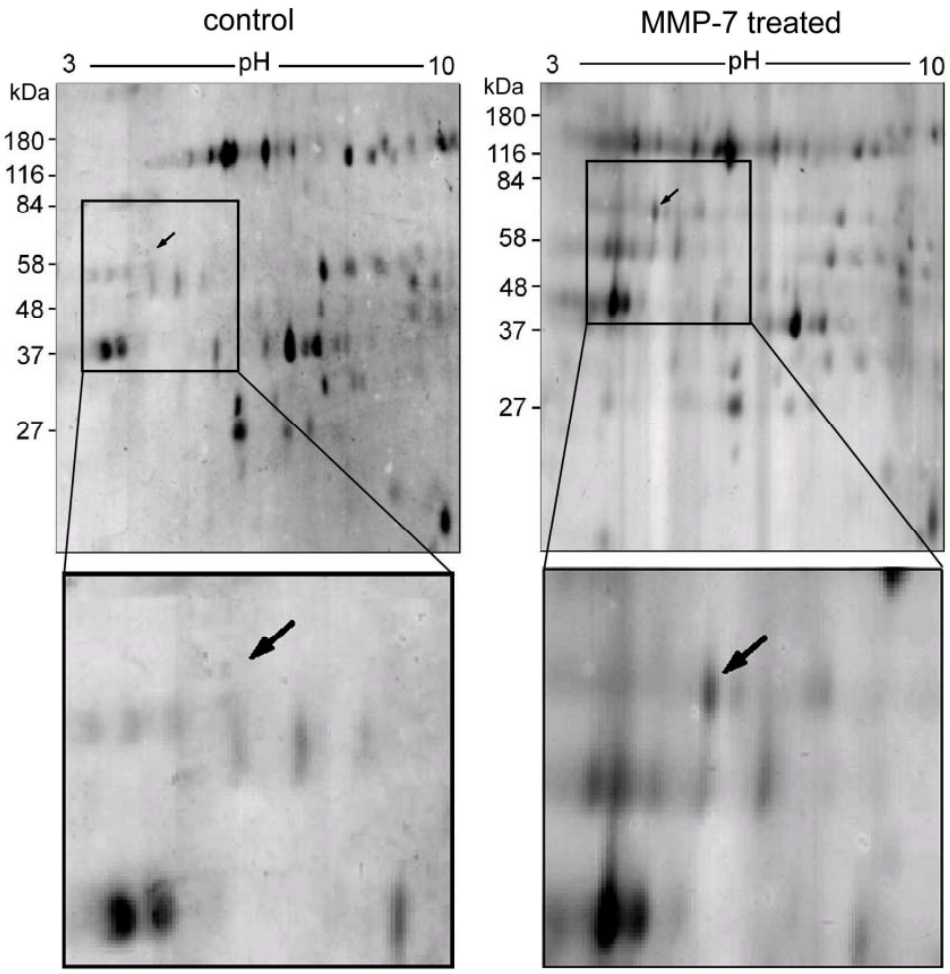


Figure 28: Identification of new MMP-7 substrates by 2-D gel electrophoresis.

Two million Jurkat cells were treated with 200ng/ml of MMP-7 in one ml of serum free media for one hour. Untreated cells served as negative control. Proteins in supernatants were collected, acetone precipitated and separated on 2-D electrophoresis. Spots were visualized by silver staining. The experiment was repeated 3 times to ensure the reproducibility. Spots which were differentially detected in all the three experiments such as that marked with arrow, was excised and identified using MALDI-TOF. The upper picture represents the full 2-D gels. The lower panel shows the enlarged view of cutouts.

3.6.2 Verification of Hsc70 as MMP-7 substrate

In the next experiment the protein identified as Hsc70 should be confirmed by Western blot analysis. Jurkat cells were treated with 200 ng/ml of MMP-7 for 3 hours. Untreated cells served as negative control. Proteins were concentrated from the supernatants by precipitation with acetone. Hsc70 was detected by Western blot using a specific antibody. As shown in Figure 29A, Hsc70 could not be detected in supernatants of untreated cells, but was abundantly present in supernatant of MMP-7 treated cells as well as in total lysate of untreated cells, which served as a positive control for Hsc70 detection. These results confirmed that the spot isolated from the 2-D gels was Hsc70. Nevertheless, it was noticed that the Hsc70 detected in supernatant and total lysate of untreated cells exhibited the same molecular weight (73 kDa according to literature). This suggests that the Hsc70 was in fact

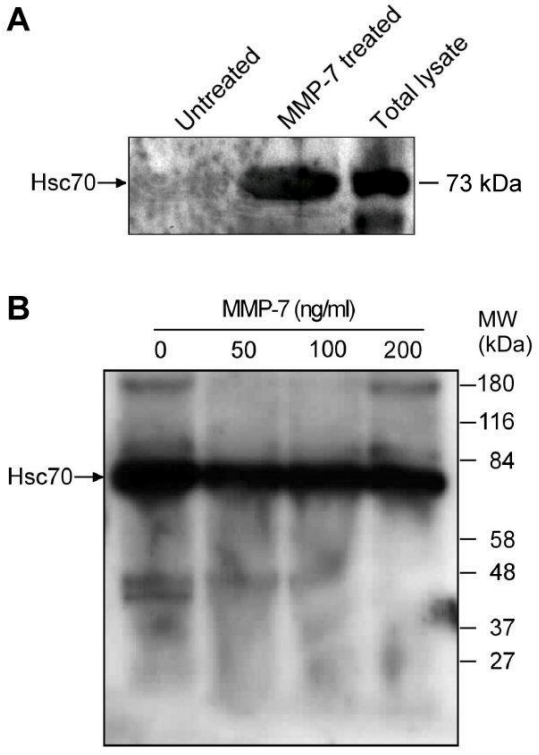


Figure 29: Cleavage analysis of Hsc70 by MMP-7

Jurkat cells were treated with 200 ng/ml of MMP-7 for 3 hours. Untreated cells served as negative control. Proteins were precipitated from the supernatants with acetone. Hsc70 was detected by Western blot using a Hsc70 specific antibody. The total cell lysate was loaded as positive control for Hsc70 (A). Proteins were precipitated from total lysates with acetone, after Jurkat cells were treated with different concentrations of MMP-7 as indicated. 30 µg recovered protein was analysed by Western blot for Hsc70 using a specific antibody (B).

not cleaved by MMP-7.

To further examine whether Hsc70 is a direct substrate of MMP-7, the Western blot analysis presented in Figure 29A was repeated with some modifications. Jurkat cells were treated with MMP-7 at different concentrations (0, 50, 100 and 200 ng/ml) for 3 hours. Proteins were enriched from total lysates by acetone precipitation. For Western blot analysis, up to 30 μg of total protein was used to increase the chance, that trace amount of cleavage products might also be detected. The blot was probed with an anti-Hsc70 antibody. As demonstrated in Figure 29B, even treated with high concentrations of MMP-7, no additional bands could be detected. Moreover, the single full length Hsc70 bands detected in all the treatments appeared to have the same migration rate and the same intensity. Based on these results, it can be concluded that Hsc70 is not a substrate of MMP-7.

3.7 Modulation of clathrin mediated endocytosis by MMP-7

Since Hsc70 is proved not to be a substrate of MMP-7, the presence of Hsc70 in the supernatants of MMP-7 treated samples may be due to defect in internalization of Hsc70. As Hsc70 is known to be involved in clathrin mediated endocytosis, in which clathrin coated vesicles are formed (Prasad *et al*., 1993). After the pinching off, the clathrin coated vesicles are uncoated and the released clathrin molecules are recruited to cell surface. Hsc70 has been reported to play an important role in disassemble of the clathrin coated vesicles. These finding suppose that clathrin may be a potential substrate of MMP-7.

3.7.1 Modulation of clathrin level in cells by MMP-7

To explore if clathrin can be cleaved by MMP-7, Jurkat cells were treated with 200 ng/ml of recombinant MMP-7 for 2, 4 and 18 hours. Cells were then fixed with methanol/acetone and stained with a goat anti-human clathrin antibody, followed by staining with Alexa -Fluor-488 conjugated donkey anti-goat antibody. Untreated cells served as negative control for MMP-7 effect. As control for staining specificity, cells were stained with secondary antibody only. Samples were measured by FACS and analysed for staining

intensities. For imaging using the confocal laser scanning microscope, stained cells were transferred to 8 well chamber slides. Pictures were taken under the same settings for

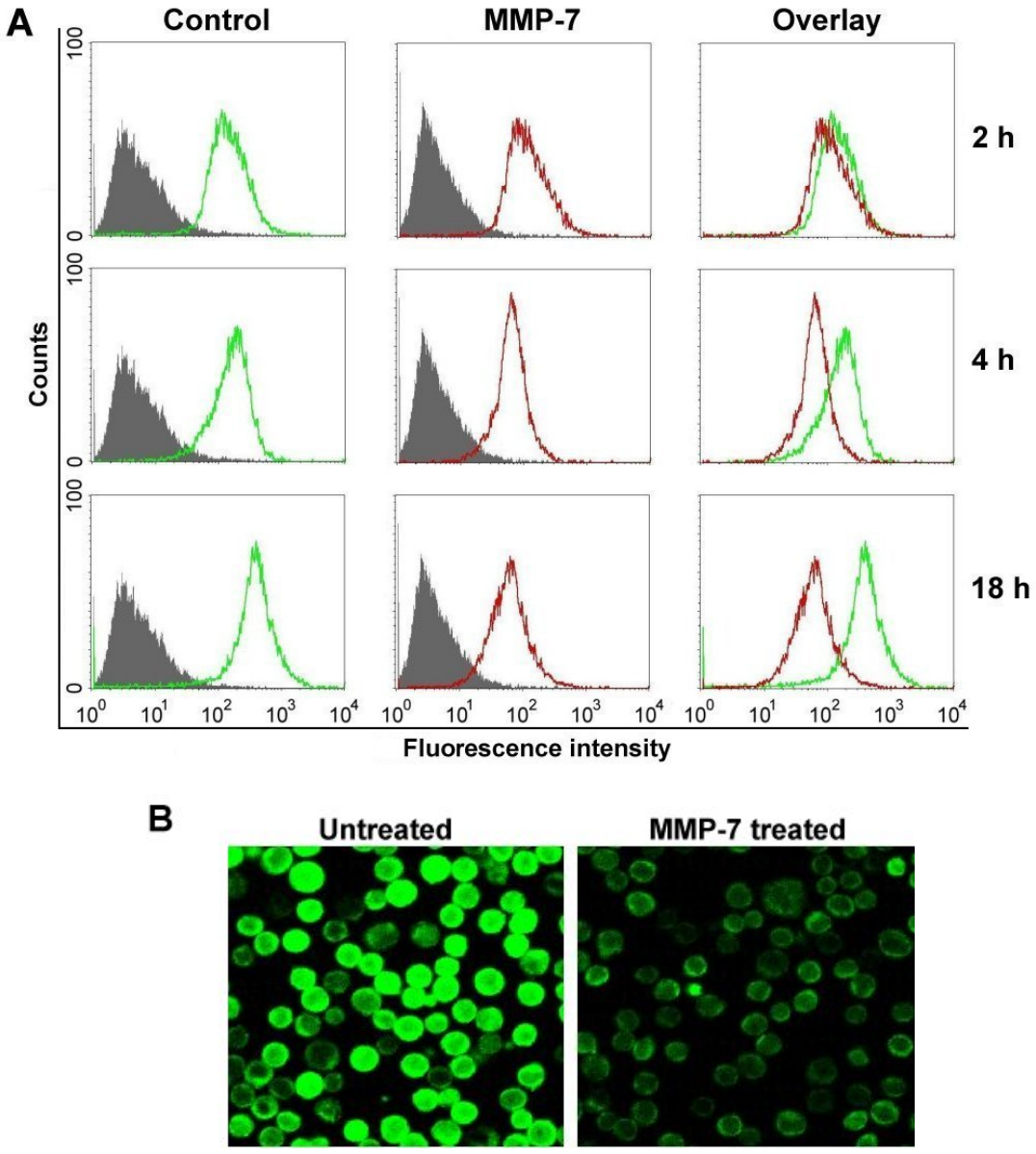


Figure 30: Modulation of clathrin level by MMP-7 in Jurkat cells

10^6 Jurkat cells were treated with 200 ng/ml of MMP-7 for different times as indicated. Clathrin positive cells were measured by FACS using a specific primary antibody and Alexa-Fluor-488 conjugated secondary antibody. Histogram plots were displayed in green for untreated control and red for MMP-7 treated samples to distinguish them in overlays. Cells stained only with the secondary antibody were shown as filled histogram plots. (A) For imaging using confocal laser scanning microscope (B), stained cells were transferred to chamber slides.

MMP-7 treated and control samples. Data shown in Figure 30 demonstrate a progressive decrease of staining intensity for clathrin in the MMP-7 treated samples compared to control samples, indicating that the clathrin level was reduced by MMP-7.

To investigate whether the MMP-7 caused downmodulation of clathirn is restricted to Jurkat cells, two other cell lines were examined. HEK293 and HepG2 cells were treated with MMP-7 for 3 hours. After treatment, cells were trypsinized, stained for clathrin and measured by FACS. Cells stained only with secondary antibody served as background control. As shown in Figure 31, MMP-7 caused decrease of clathrin level can also be observed both in HEK293 and HepG2 cells. Thus, the reduction of clathrin by MMP-7 is not limited to Jurkat cells.

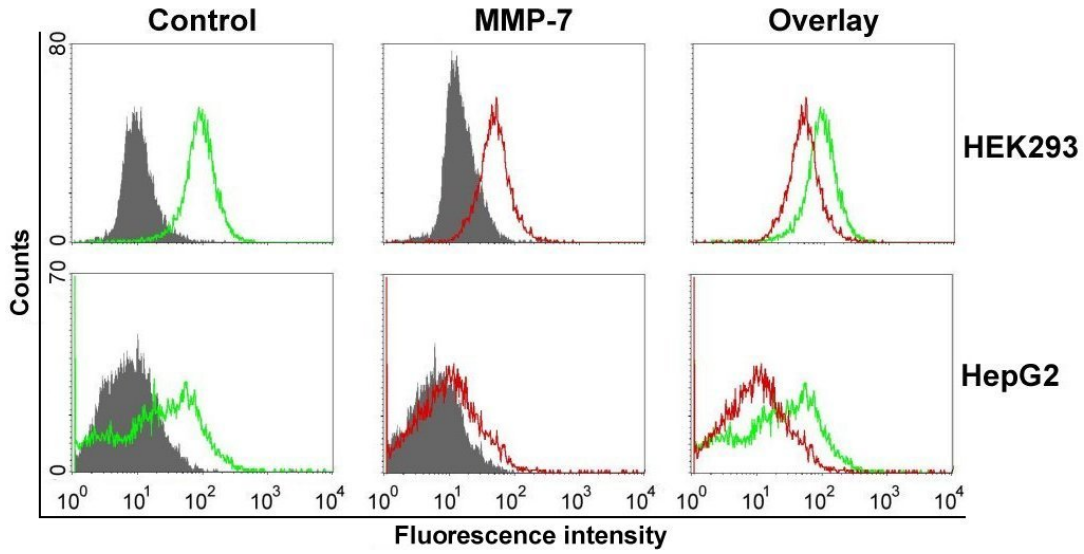


Figure 31: Modulation of clathrin level by MMP-7 in HEK293 and HepG2 cells

HEK293 and HepG2 cells were treated with 200 ng/ml of MMP-7 for 3 hours. Untreated cells were taken as control. Clathrin positive cells were measured by FACS using a specific primary antibody and Alexa-Fluor-488 conjugated secondary antibody. Histogram plots were displayed in green for untreated control and red for MMP-7 treated samples to distinguish them in overlays. Cells stained only with the secondary antibody were shown as filled histogram plots.

3.7.2 Analysis of clathrin cleavage by MMP-7

As the effect of MMP-7 to reduce clathrin level in cells was verified, the cleavage of clathrin by MMP-7 was analysed. Jurkat cells were treated with 200 ng/ml of MMP-7 for 3 hours. Total lysates were analysed by Western blot for clathrin using specific antibody. As shown in Figure 32, no cleavage product could be detected as extra band in MMP-7 treated sample. Thus, clathrin seems impossible to be substrate for MMP-7.

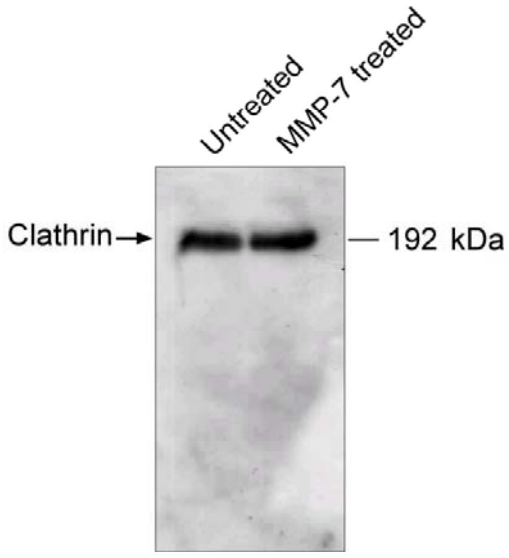


Figure 32: Verification of clathrin cleavage by MMP-7

Jurkat cells were treated with 200 ng/ml of MMP-7 for 3 hours. Untreated cells served as negative control. Total lysates were analysed by Western blot using a clathrin specific antibody.

3.7.3 Role of MMP-7 on receptors regulated by clathrin mediated endocytosis

Many receptors are regulated by clathrin mediated endocytosis. Upon binding of extracellular signal molecules such as hormone or specific ligand to their receptor, the formed complex is internalized by endocytosis, in which clathrin coated pits are formed. After the signalling pathway is activated within the cell, the vesicles are uncoated leading to disassemble of the receptor/ligand complexes. The receptors are recruited to cell surface again, while the released clathrin is utilized for coating new pit (Hannan *et al*., 1998).

3.7.3.1 Influence of MMP-7 on immune relevant receptors

Based on the finding that MMP-7 reduces the clathrin level in cells, it can be assumed that the clathrin mediated endocytosis could be impaired by MMP-7. To examine this assumption, several receptors known to be regulated via clathrin mediated endocytosis were chosen to study the influence of MMP-7. The transferrin receptor (CD71) is a well studied model receptor for studying clathrin mediated endocytosis as well as receptor recycling (van Dam and Stoorvogel, 2002). Further selected receptors include Type II TGF-β receptor (TGF-βRII), CD47, CD28 and CD3. TGF-βRII, which forms a heteromeric complex with TGF-βRI, binds TGF-β and phosphorylates and thereby activates the TGF-βRI to signal downstream pathways (Oshima *et al*., 1996). CD28, expressed on most T cells, is the receptor of a membrane glycoprotein named B7 with 2 isoforms (B7-1 and B7-2) which are expressed on many immune cells. CD28 plays an important role in T cell activation (Linsley *et al*., 1993). CD3, a complex consisting of 6 chains, is associated to T cell receptor and necessary for signal transduction. CD47 is one of the receptors for thrombospondin (thrombospondin 1-5). The thrombospondins are known to interact with blood coagulation and anticoagulant factors. They are involved in inhibition of tumour growth and angiogenesis (Streit *et al*., 1999). Except CD47, other selected receptors are all known to be internalized via clathrin mediated endocytosis (Cefai *et al*., 1998; San Jose and Alarcon, 1999).

To investigate the effect of MMP-7 on internalization of these receptors by FACS, Jurkat cells were treated with 200 ng/ml of MMP-7 for 3 hours. Except for clathrin, cells were stained without fixation with specific antibodies against TGF-βRII, CD71, CD47, CD28, CD3 and clathrin, followed by staining with FITC or phycoerythrin conjugated secondary antibodies. As negative controls, parallel sets of cells with or without MMP-7 treatment were stained with secondary antibody only. Results of this experiment have confirmed the reduction of clathrin level by MMP-7 treatment, but no changes in surface expression of all tested receptors was observed as a result of MMP-7 treatment (Figure 33).

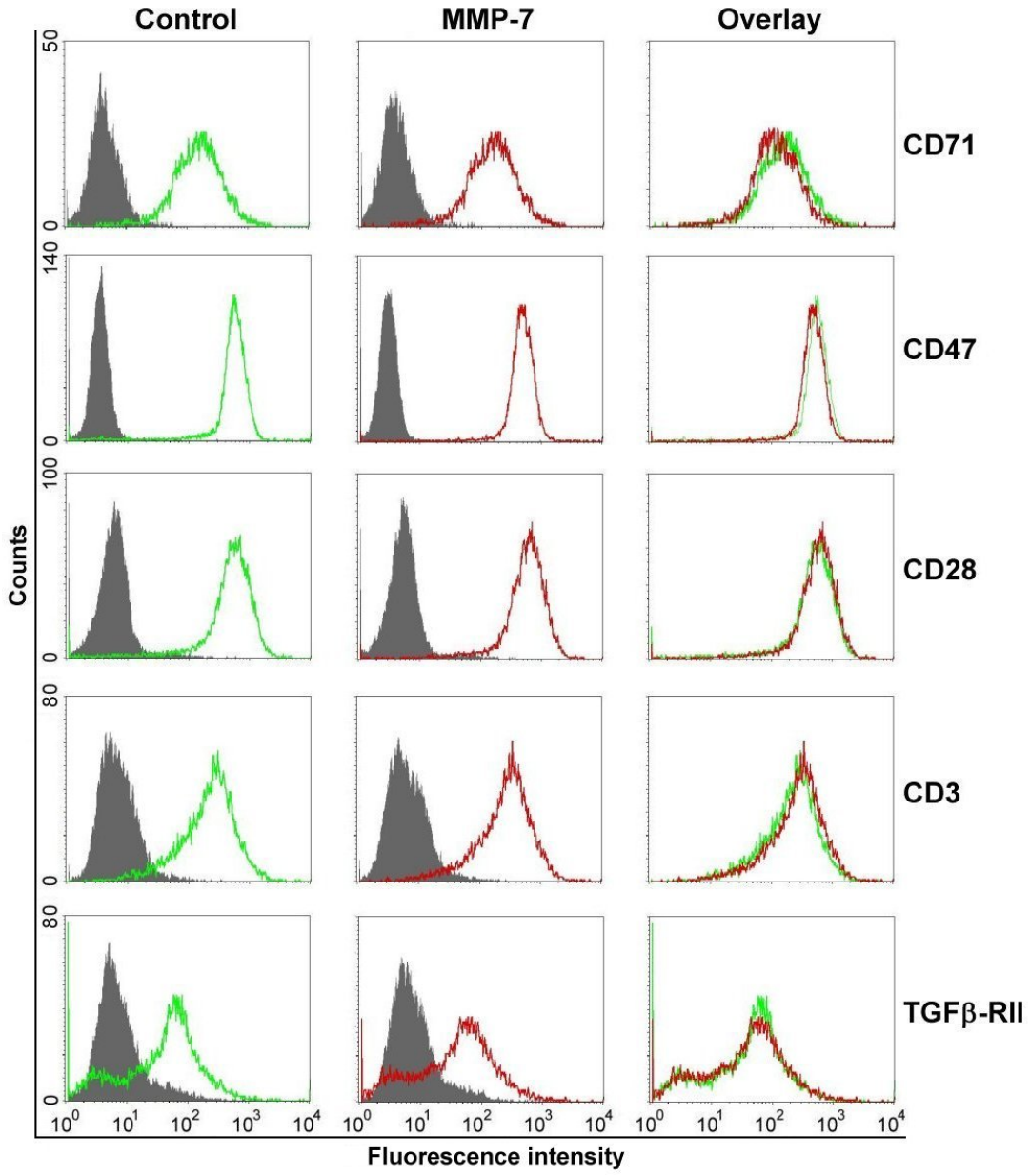


Figure 33: Influence of MMP-7 on some receptor density in Jurkat cells

Jurkat cells were treated with 200 ng/ml of MMP-7 for 3 hours. Untreated cells were taken as control. Without fixation, cells were stained with specific antibodies against CD28, CD47, CD71, CD3 and TGF-βRII, respectively, followed by staining with FITC or phycoerythrin conjugated secondary antibodies. Stained cells were analysed by FACS. Histogram plots were displayed in green for untreated control and red for MMP-7 treated samples to distinguish them in overlays. Cells stained only with secondary antibody were shown as filled histogram plots.

3.7.3.2 Influence of MMP-7 on transferrin uptake by Jurkat cells

A transferrin uptake assay was chosen, because transferrin is one of the proteins which is rapidly internalized via clathrin mediated endocytosis. In a preliminary experiment, the rate of transferrin uptake by Jurkat cells was analysed to optimize the test duration for further experiments with MMP-7. First, Jurkat cells were incubated with 25 ng/ml of FITC labelled transferrin on ice for 30 minutes allowing transferrin to bind to its receptor without being internalized. After washing off the unbound transferrin with ice cold PBS, cells were incubated at 37°C for 0, 5 and 25 minutes. Endocytosis was stopped by immediately returning the cells on ice. The transferrin which was not internalized was removed from surface by washing with ice cold acidic high salt solution (0.2 M acetic acid/0.5 M NaCl). Cells were then fixed with PFA and measured by FACS. Figure 34 demonstrate that the uptake of transferrin increased with the incubation time at 37°C and the uptake is not linear but time dependent.

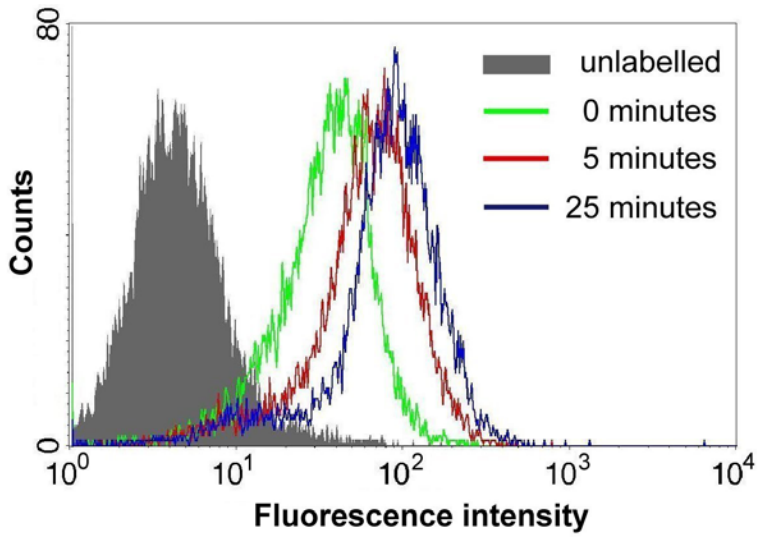


Figure 34: Time course of transferrin uptake by Jurkat cells

Jurkat cells were incubated with FITC labelled transferrin at 37°C for different time as indicated. Cells incubated without labelled transferrin served as negative control. After washing and fixation of the cells, the uptaken transferrin was measured by FACS. Histogram plots were displayed in green for uptake time 0, red for 5 and blue for 25 min. The transferrin uptake of the control cells without incubation with labelled transferrin was displayed as filled histogram plot.

Jurkat cells were then treated with 200 ng/ml of MMP-7 for 3 hours, before they were proceeded for transferrin uptake assay as described above. Endocytosis was allowed to continue for 15 minutes. As negative control, both untreated and MMP-7 treated cells were incubated in the absence of labelled transferrin. The results shown in Figure 35 demonstrate the decrease in transferrin uptake in MMP-7 treated cells. As demonstrated in Figures 30, 31 and 35, MMP-7 was found to decrease the clathrin level in 3 cell lines and reduced the rate of transferrin uptake by Jurkat cells without influencing the amount of tansferrin receptor CD71 on cell surface. This finding suggests that MMP-7 can interfere with the clathrin mediated endocytosis in the tested cells.

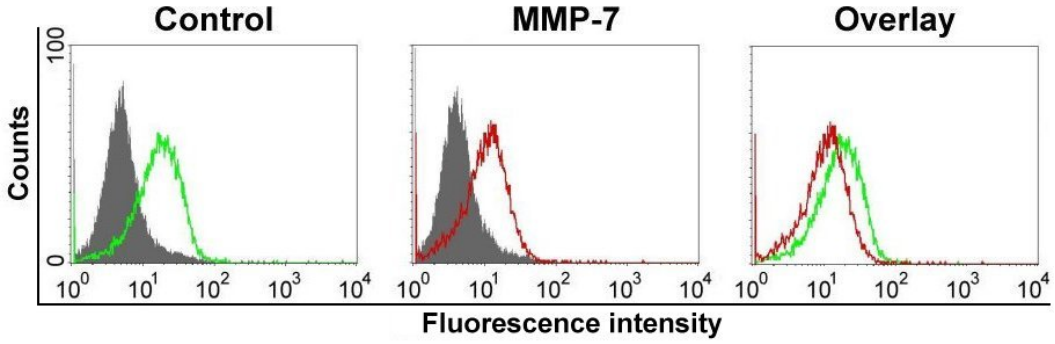


Figure 35: Influence of MMP-7 on transferrin uptake by Jurkat cells

Jurkat cells were treated with 200 ng/ml of MMP-7 and incubated with FITC labelled transferrin at 37°C for 15 min. As negative controls, both untreated and MMP-7 treated cells were incubated without labelled transferrin. The transferrin uptake was measured by FACS. Histogram plots were displayed in green for untreated control and red for MMP-7 treated samples to distinguish them in overlays. The transferrin uptake of control cells incubated in the absence of labelled transferrin was displayed as filled histogram plots.

3.8 Effect of MMP-7 on internalization of surface receptor CTLA-4 in $CD4^+CD25^+$ cells

CTLA-4 (cytotoxic T-lymphocyte-associated molecule-4) is expressed by the regulatory T cells and is important for the immune suppression function. Regulation of surface expression of CTLA-4 is an example of clathrin mediated endocytosis. Inhibition of the clathrin mediated endocytosis leads to the increased surface expression of CTLA-4. $CD4^+CD25^+$ regulatory T cells (T_{regs}) actively suppress harmful immune responses and prevent pathological self reactivity, e.g. autoimmune disease. Regulatory T cells are defined by expression of the forkhead family transcription factor FOXP3 (forkhead box p3). In addition, $CD4^+CD25^+$ T_{reg} cells are typically characterized by expressing high levels of CTLA-4 which has been reported to be strongly regulated through clathrin mediated endocytosis (Chuang *et al*., 1997). To further confirm this effect of MMP-7 and to explore possible role of MMP-7 in immune modulation, the expression of CTLA-4 in $CD4^+CD25^+$ T_{reg} cells after MMP-7 treatment was analysed in following experiments. T_{regs} are a good model system to connect MMP-7 to clathrin mediated endocytosis of CTLA-4 and immune suppression.

3.8.1 Effect of MMP-7 on expression of clathrin

At first the effect of MMP-7 on clathrin level in $CD4^+CD25^+$ T_{reg} cells was examined. $CD4^+CD25^+$ T_{reg} cells were isolated from PBMC's by magnetic cell sorting (MACS). In the first step, non-$CD4^+$ T cell were depleted using a cocktail of biotin labelled antibodies and anti-biotin conjugated magnetic beads. $CD4^+CD25^+$ cells were then isolated in a positive selection step using anti-CD25 conjugated magnetic beads. The isolated cells were treated with 200 ng/ml of MMP-7 for 3 hours, while the control cells were incubated in the absence of MMP-7. After treatment, cells were stained with a clathrin specific antibody followed by staining with FITC labelled secondary antibody. As negative controls, both untreated and MMP-7 treated cells were stained with secondary antibody. The staining of clathrin was analysed by FACS. As shown in Figure 36, a strong reduction of clathrin was observed in the MMP-7 treated samples.

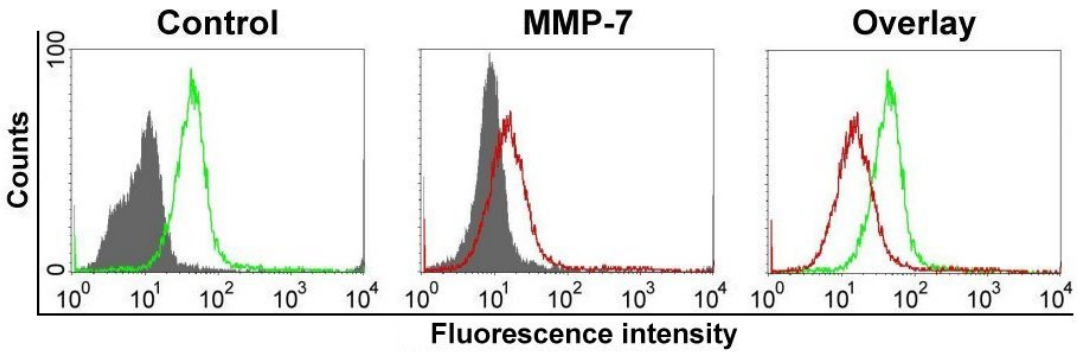


Figure 36: Effect of MMP-7 on clathrin level in $CD4^+CD25^+$ T_{reg} cells

$CD4^+CD25^+$ T_{reg} cells were isolated from PBMC using MACS technology in two steps. First, non-$CD4^+$ cells were depleted and then $CD25^+$ cells were selected. The isolated cells were treated with 200 ng/ml of MMP-7 for 3 hours. Clathrin level was analysed by FACS after staining the cells with a clathrin specific antibody and FITC labelled secondary antibody. Histogram plots were displayed in green for untreated control and red for MMP-7 treated samples to distinguish them in overlays. The clathrin level of control cells incubated only with secondary antibody was displayed as filled histogram plots.

3.8.2 Effect of MMP-7 on surface expression of CTLA-4

As mentioned above, a typical mark of $CD4^+CD25^+$ T_{reg} cells is the high expression of CTLA-4. Iida *et al* (Iida *et al.*, 2000) have reported that CTLA-4 is rapidly internalized via clathrin mediated endocytosis and degraded in lysosomes, so that the surface level of CTLA-4 is kept low. On stimulation, the surface expression of CTLA-4 is increased by enhanced *de novo* synthesis (Linsley *et al*., 1992). Other researchers have demonstrated that inhibition of the clathrin mediated endocytosis leads to an increased surface expression of the CTLA-4. After confirming that MMP-7 reduces the clathrin level in $CD4^+CD25^+$ T_{reg} cells, the effect of MMP-7 on CTLA-4 expression was analysed.

For this, $CD4^+CD25^+$ cells were isolated by MACS and treated with MMP-7 as described in §3.8.1. CTLA-4 level was measured by FACS after the cells were stained with a CTLA-4 specific antibody and FITC conjugated secondary antibody consecutively. Both untreated and MMP-7 treated cells stained with secondary antibody only served as negative controls. Data in Figure 37 demonstrate an increase in CTLA-4 level in MMP-7 treated

sample. This result supports the effect of MMP-7 in interfering with clathrin mediated endocytosis as found in Jurkat cells.

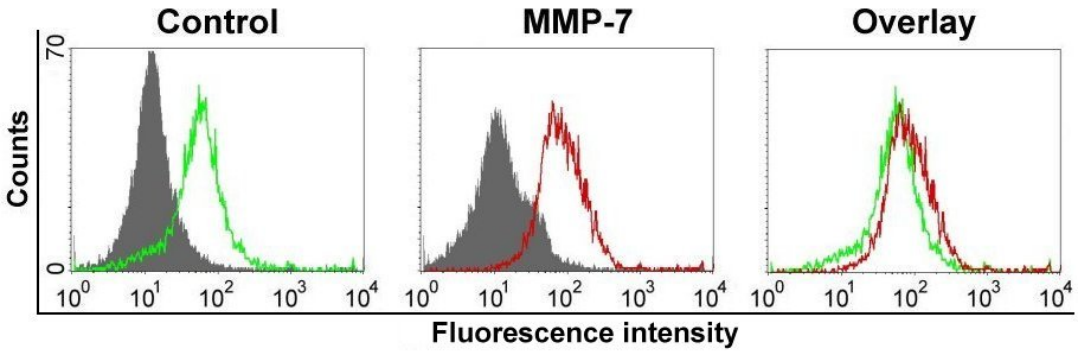


Figure 37: Effect of MMP-7 on CTLA-4 level in $CD4^+CD25^+$ T_{reg} cells

$CD4^+CD25^+$ T_{reg} cells were isolated from PBMC and treated with MMP-7 as described in Figure 36. CTLA-4 level was analysed by FACS after staining the cells with a CTLA-4 specific antibody and FITC labelled secondary antibody. Histogram plots were displayed in green for untreated control and red for MMP-7 treated samples to distinguish them in overlays. The CTLA-4 level of control cells incubated only with the secondary antibody was displayed as filled histogram plots.

3.8.3 Effect of MMP-7 on transferrin uptake by $CD4^+$ cells:

In the next experiment the effect of MMP-7 on transferrin uptake by T cells was analysed. Since the expression of transferrin receptor is not restricted to $CD4^+CD25^+$ T_{reg} cells, total $CD4^+$ T cell population was used. $CD4^+$ T cells were isolated by negative selection using MACS and treated with MMP-7 as described in §3.8.1. The transferrin uptake assay was performed as described in §3.7.3.2. The assay was allowed to run for 0 and 30 minutes and evaluated by FACS. As shown in Figure 38, decreased transferrin uptake was observed in MMP-7 treated cells. This result reveals that the endocytosis in $CD4^+$ cells was reduced in presence of MMP-7.

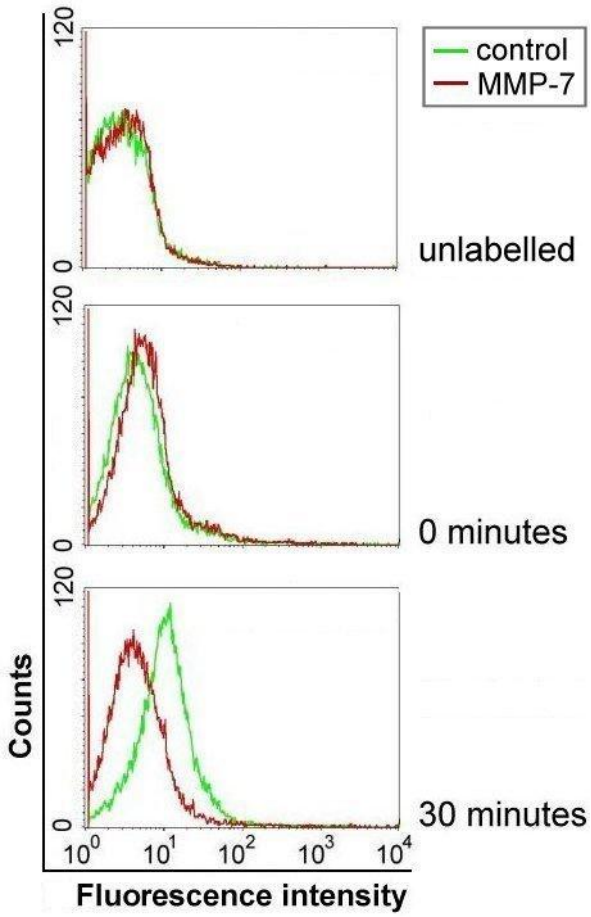


Figure 38: Effect of MMP-7 on transferrin uptake by $CD4^+$ T cells

$CD4^+$ cells were isolated by negative selection using MACS and treated with 200 ng/ml of MMP-7 for 3 hours. Transferrin uptake assay was performed for different times as indicated, and measured by FACS. As negative controls, cells were treated in the same way except no labelled transferrin was added (unlabelled). Histogram plots were displayed in green for untreated and red for MMP-7 treated cells.

3.9 Influence of MMP-7 on differentiation and endocytosis of dendritic cells

After demonstrating the effect of MMP-7 to reduce clathrin expression and to interfere with clathrin mediated endocytosis both in Jurkat cells and $CD4^+$ T cells (including $CD4^+CD25^+$ T_{reg} cells), the analysis was expanded to dendritic cells (DCs), which also plays an important role in immune functions. Antigen presentation is one of the most important functions of DCs where endocytosis plays an important role.

3.9.1 Morphological analysis

DCs necessary for performing experiments can be obtained by inducing the differentiation of monocytes present in peripheral blood to DCs. In addition, this procedure enables not only the study of mature DCs but also the differentiation process of DC.

First, PBMCs were isolated from buffy coat. Then monocytes were purified by MACS using anti-CD14 conjugated microbeads and cultured in the presence of the cytokines GM-CSF and IL-4, which promote the differentiation of monocytes to immature DCs (Liu, 2001). Under these conditions, typical immature DCs appear after 3-4 days of culture and after 6-7 days 60-80% cells are immature DCs. To test the effect of MMP-7 on differentiation of immature DCs, 100 ng/ml of MMP-7 was added along with GM-CSF and IL-4 at the beginning of culture. Morphological changes were judged daily and pictures were collected on day 2, 4 and 6 using differential interference contrast (DIC) microscopy. As shown in Figure 39, differences in cell morphology could be observed in the MMP-7

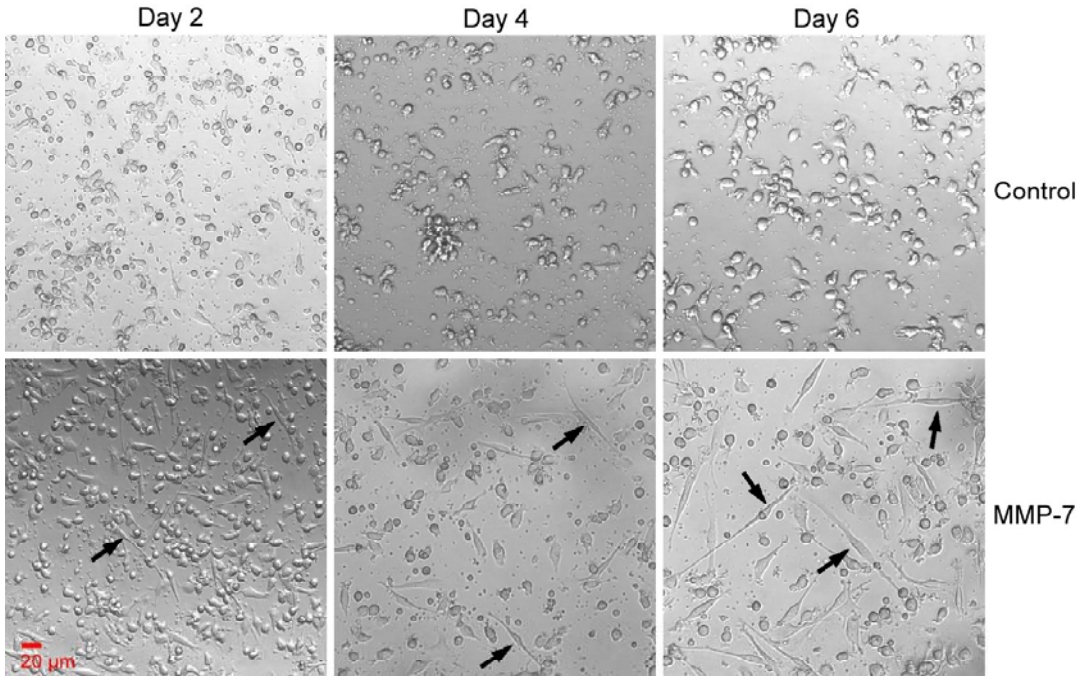


Figure 39: Effect of MMP-7 on morphological development of immature DCs

Monocytes were isolated from PBMCs and treated with MMP-7 along with cytokines GM-CSF and IL-4. Images were taken at days 2, 4 and 6 of culture using differential interference contrast microscopy. Arrows indicate the MMP-7 induced long spindle-shaped cells. Length of the bar = 20 µm.

treated cells after 2 days, in which some cells appeared to take the form of a long spindle and attached much strongly to the bottom of the culture plate. The number of such cells increased with culture time and reached over 50% in average at day 6. In contrast, such cells could hardly be observed in the absence of MMP-7. Thus MMP-7 seems to affect the morphological development of immature DCs.

Next, MMP-7 was tested to see if it can also affect the morphological development of mature DCs. For this purpose, monocytes were at first induced to differentiate to immature DCs as described above, except for adding no MMP-7. The non-adherent cells were recovered and further cultured in the presence of cytokines IL-6, IL-2, TNFα and low concentration of GM-CSF for 2 days to induce maturation. 100 ng/ml of MMP-7 was added at the beginning along with the maturation cytokines. As result, the morphological differences between MMP-7 treated and untreated cells became more prominent than that observed in immature DCs (Figure 40). On the one hand, the spindle shaped cells were

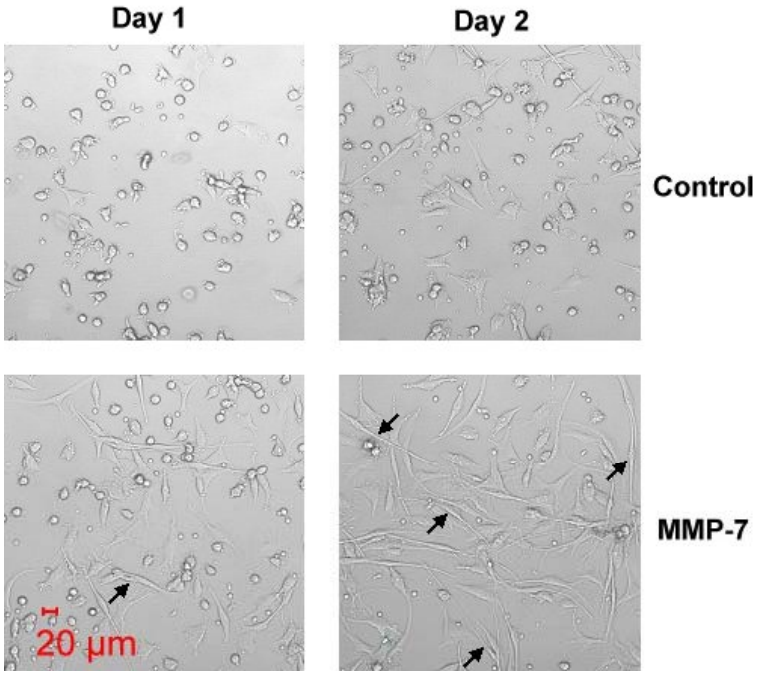


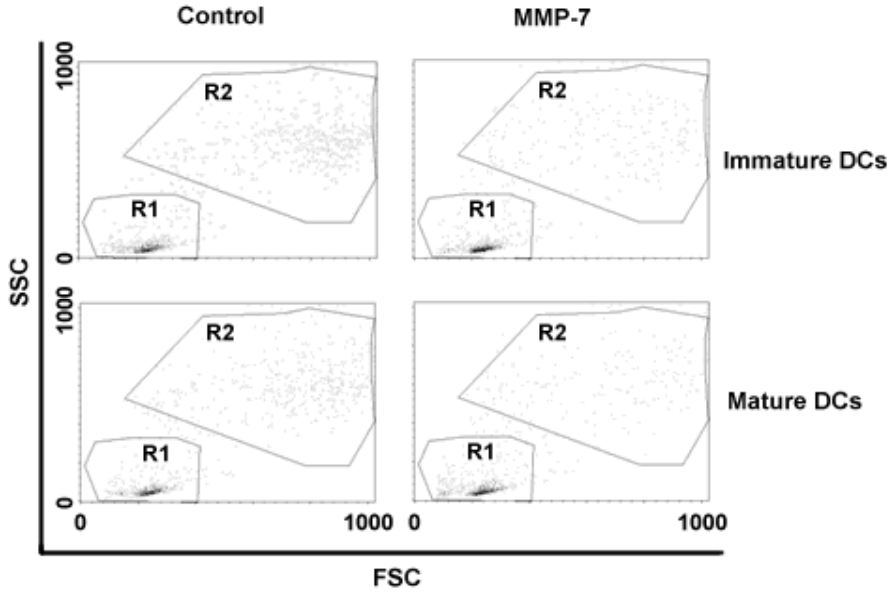
Figure 40: Effect of MMP-7 on morphological development of mature DCs

Immature DCs were treated with cytokines to induce maturation along with MMP-7 for 2 days. Pictures were taken on day 1 and day 2 after maturation induction using differential interference contrast microscopy. Arrows indicate the MMP-7 induced long spindle shaped cells. Length of the bar = 20 µm.

much longer and on the other hand, over 90% of the cells were such "deformed" cells. These results suggest that the morphological development of mature DCs continued.

To quantify the morphologically different subpopulations caused by MMP-7, cells were analysed by FACS. As revealed by dot plots, cells could be clearly divided into two subpopulations based on their SSC/FSC (side scatter to forward scatter) properties (Figure 41A). One subpopulation was characterized by low SSC and low FSC (R1), whereas the other exhibited high SSC and high FSC (R2). Furthermore, the data demonstrate that the

A



B

		R1	R2
Immature DCs	**Control**	56%	38%
	MMP-7	74%	19%
Mature DCs	**Control**	55%	39%
	MMP-7	79%	15%

Figure 41: Characterization of the subpopulations induced by MMP-7

Immature and mature DCs were allowed to develop in the absence or presence of MMP-7. The cells were harvested and analysed by FACS on the SSC/FSC property. Dot plots were used to identify the 2 subpopulations. Dot plot data from immature and mature DCs of control and MMP-7 treated cells (A). The ratios between the two subpopulations were listed in the table (B).

presence of MMP-7 during differentiation of immature DCs as well as mature DCs strikingly increased the percentage of R1 subpopulations (Figure 41B). Taken the optical observations into consideration, the R1 subpopulation should be the spindle shaped, strong adherent cells, induced by MMP-7; while R2 represents the normal DCs (see Figures 39 and 40).

3.9.2 Analysis of DC maturation markers

To further characterize the two DC subpopulations, their surface markers were analysed. DCs express a variety of surface markers including CD83, CD86 and HLA-DR. The expression level of these markers varies during stages of differentiation, for example the DCs precursors monocytes are characterized by the expression of CD14, immature DCs are known to express CD86 and HLA-DR whereas mature DCs expresses CD83 along with CD86 and HLA-DR (Palucka *et al*., 1998).

Immature DCs which were allowed to differentiate in the absence of MMP-7 as described above. Cells were stained by antibodies specific for CD83, CD86 and HLA-DR followed by staining with FITC conjugated secondary antibody. After acquiring SSC/FSC data, two gates were set to define the subpopulations (R1 and R2, Figure 42A), which were then separately measured for the three surface markers. As shown in Figure 42B, the subpopulation R2 was CD86 positive, HLA-DR positive but CD83 negative, confirming that they were immature DCs. In contrast to R2 cells, the R1 subpopulation was negative for all three markers, indicating that they were not immature DCs. These results suggest that MMP-7 can probably interfere with the differentiation and development of DCs, leading to development of cells of a non-DC phenotype.

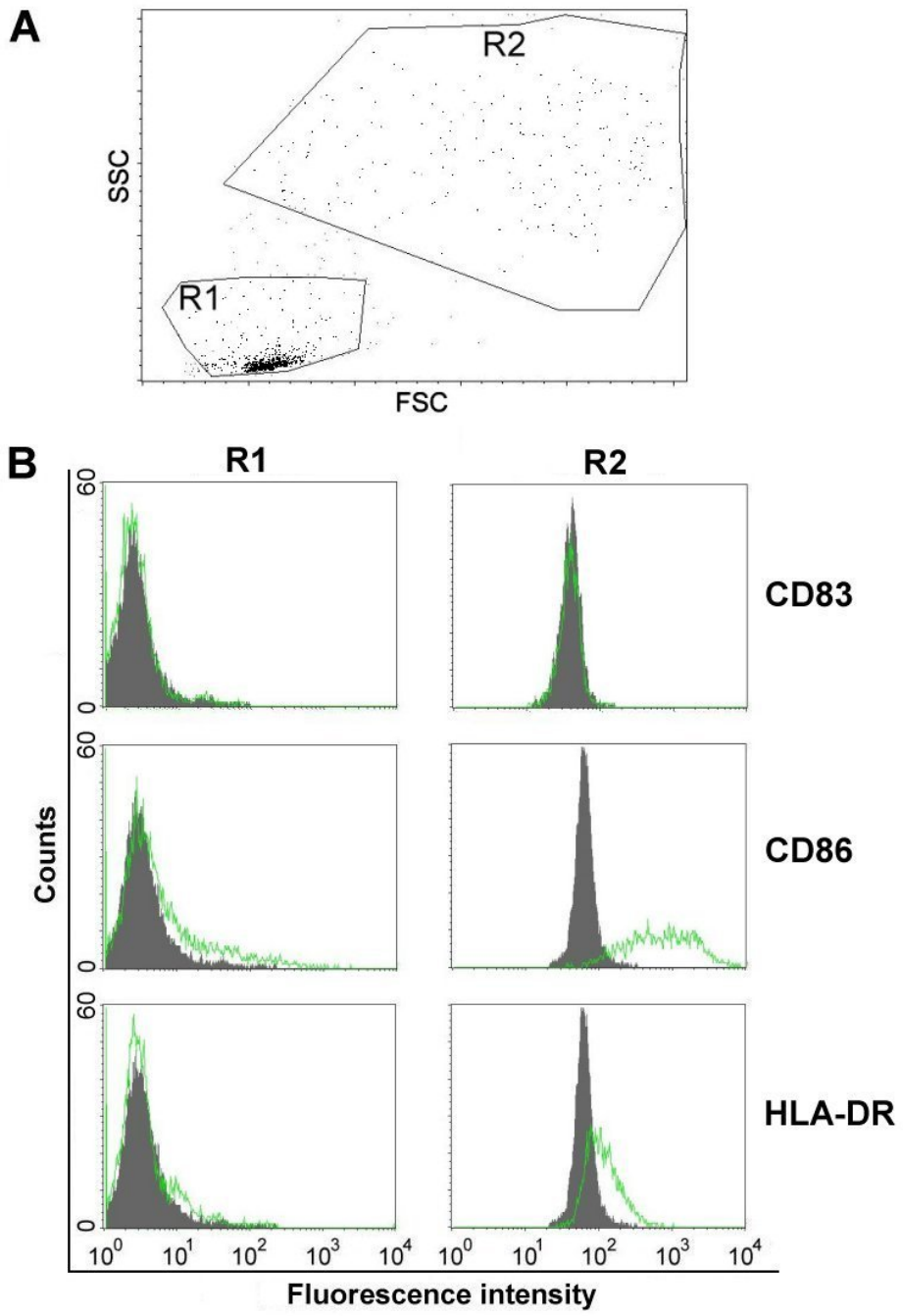


Figure 42: Surface markers of the subpopulations immature DCs

Immature DCs were prepared in the absence of MMP-7 as described. Expression levels of CD86, HLA-DR and CD83 were analysed by FACS using specific antibodies. Two gates were set to define the subpopulations which were then separately measured. Cells stained only with secondary antibody were displayed as filled histogram plots.

In the following experiments the surface marker expression of the R2 subpopulation (DCs as characterize in Figure 42) under influence of MMP-7 was further characterized. Immature and mature DCs were prepared in the absence and presence of MMP-7 as described and analysed by FACS to detect the expression of CD86, CD83 and HLA-DR. Additionally, the monocyte marker CD14 was also included. As negative control, cells were stained with secondary antibody only.

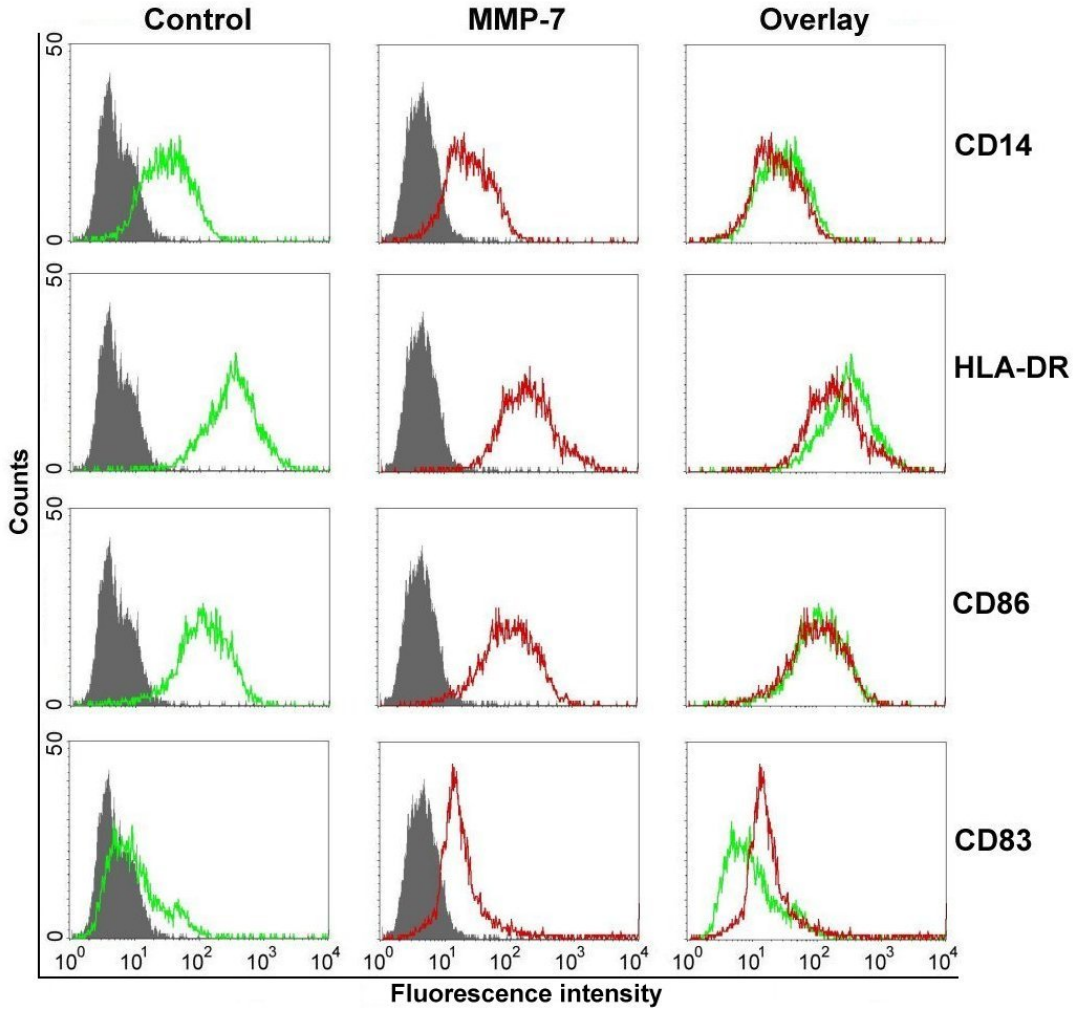


Figure 43: Surface marker expression of immature DCs in R2 subpopulation

Immature DCs were prepared in the absence or presence of MMP-7 as described. Expression levels of CD14, CD86, HLA-DR and CD83 were analysed by FACS using specific antibodies. The R2 subpopulation was gated similarly as shown in Figure 42. Histogram plots were displayed in green for untreated cells and red for MMP-7 treated cells to distinguish them in overlays. Cells stained only with secondary antibody were displayed as filled histogram plots.

Results from Figure 43 and 44 demonstrate that MMP-7 does not influence the expression of surface marker in both immature and mature DCs, except that a slight increase in the CD83 expression in the immature DCs. Results indicate that MMP-7 did not dramatically alter the maturation markers but it changes the ratio of R1/R2 population which suggests the decrease in the total turnover of the DCs.

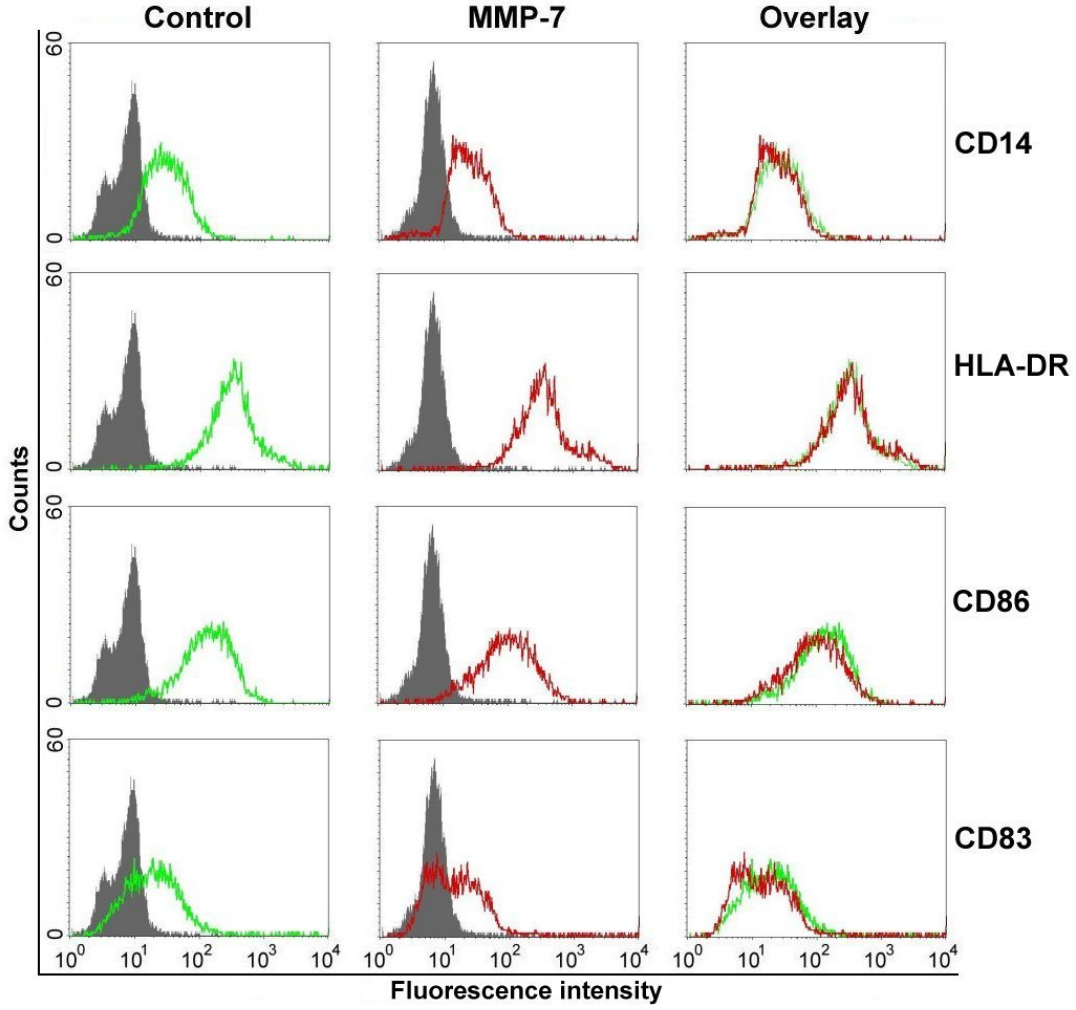


Figure 44: Surface marker expression of mature DCs in R2 subpopulation

Mature DCs were prepared in the absence or presence of MMP-7 as described. Expression levels of CD14, CD86, HLA-DR and CD83 were analysed by FACS using specific antibodies. The R2 subpopulation was gated similarly as shown in Figure 42. Histogram plots were displayed in green for untreated cells and red for MMP-7 treated cells to distinguish them in overlays. Cells stained only with secondary antibody were displayed as filled histogram plots.

3.9.3 Modulation of endocytosis in DCs by MMP-7

The influence of MMP-7 on DCs was studied in the following experiment. As mentioned above, one of the most important functions of DCs is the antigen uptake via clathrin mediated endocytosis. Since immature DCs were reported to show higher ability of endocytosis (Garrett *et al*., 2000), immature DCs were examined concerning clathrin level and transferrrin uptake in following experiments.

3.9.3.1 Clathrin level in DCs

Immature DCs were prepared from PBMCs and allowed to differentiate in the absence or presence of MMP-7 as described. After staining with a clathrin specific antibody and FITC labelled secondary antibody, cells were analysed by FACS. Cells stained only with secondary antibody were used as negative controls. As shown in Figure 45, the level of clathrin in MMP-7 treated cells was strongly reduced in comparison to control cells.

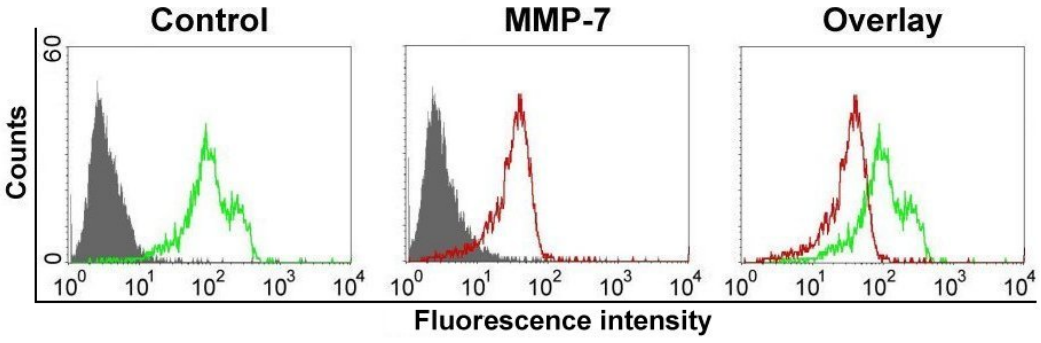


Figure 45: Effect of MMP-7 on clathrin level in immature DC's

Immature DCs were prepared in the absence or presence of MMP-7 as described. The whole cell population was stained with a clathrin specific antibody and FITC labelled secondary antibody. Fluorescence intensities were measured by FACS. Histogram plots were displayed in green for untreated cells and red for MMP-7 treated cells to distinguish them in overlays. Cells stained only with secondary antibody were displayed as filled histogram plots.

3.9.3.2 Transferrin uptake by DCs

In parallel, the effect of MMP-7 on endocytosis of DCs was examined using the transferrin uptake assay. First the transferrin uptake was analysed in immature DCs acquiring the live cell imaging using CLSM (Confocal Laser Scanning Microscopy). For this purpose monocytes were allowed to differentiate into immature DCs in presence or absence of MMP-7. Prior to image acquirement, temperature was brought down to 4°C by incubating DCs on ice. Transferrin was added to the cells and continuous images were taken at an interval of 30 seconds. Figure 46 demonstrates decreased transferrin uptake in immature DCs in presence of MMP-7 when compared to control cells at an indicated time points. For quantification by CLSM, both immature and mature DCs were allowed to differentiate in the absence or presence of MMP-7. The transferrin uptake assay was then performed in chamber slides. The incubation time at 37°C was 15 minutes. After the acidic wash, cells were fixed with 4% PFA. Images of single cells were collected by CLSM (Zeiss 510) using a Plan-Apochromat 63x oil immersion objective under scan zoom setting of 2 and pinhole setting of 81 µm. All the images were acquired using 488 laser under the similar settings.

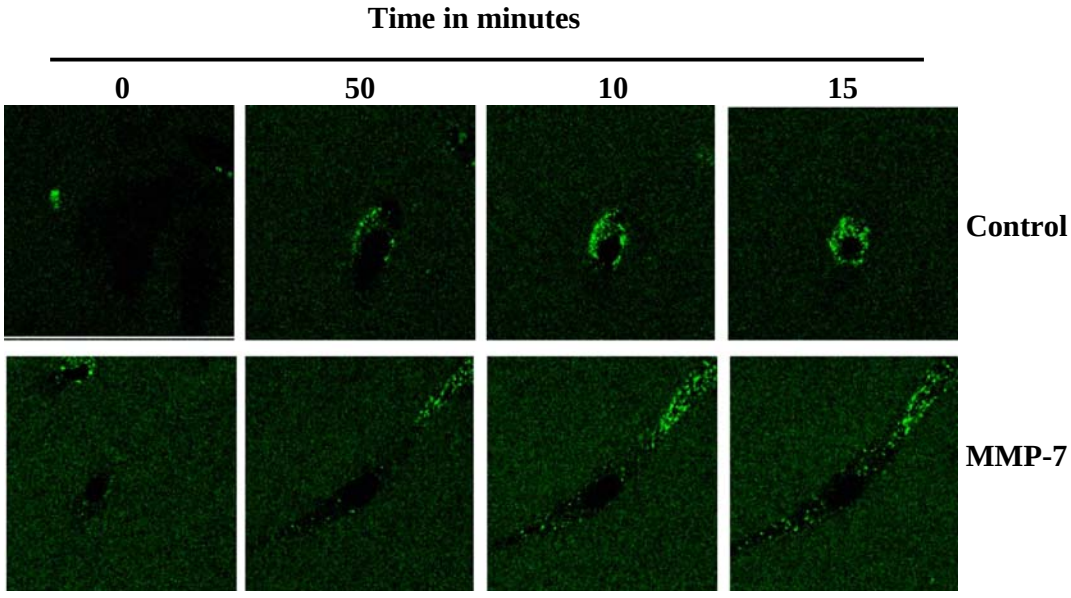


Figure 46: Transferrin uptake in immature DCs at different time point in presence or absence of MMP-7.

Immature DCs were prepared in the absence or presence of MMP-7. Cells were allowed to takeup the transferrin and continuous images were acquired immediately at a time interval of 30 seconds.

The transferrin uptake by single cells was semiquantitatively evaluated by manually counting the number of peaks displayed on the topographical charts (right panel in Figure 47A and B) delivered by the LSM 5 PASCAL Software (Zeiss). For each test set, 20 cells were evaluated and the average number of peaks was calculated. The results summarized in Figure 47C demonstrate a significant decrease in transferrin uptake in immature DCs when they were exposed to MMP-7 during differentiation. Mature DCs were also analysed in similar way to study the influence of MMP-7 on transferrin uptake. Results obtained from mature DCs are shown in Figure 48A and B. Number of peaks displayed on the topographical charts from MMP-7 treated and untreated cells were counted (20 cells for each set) Result from Figure 48C indicates significant decrease in transferrin uptake in mature DCs in presence of MMP-7.

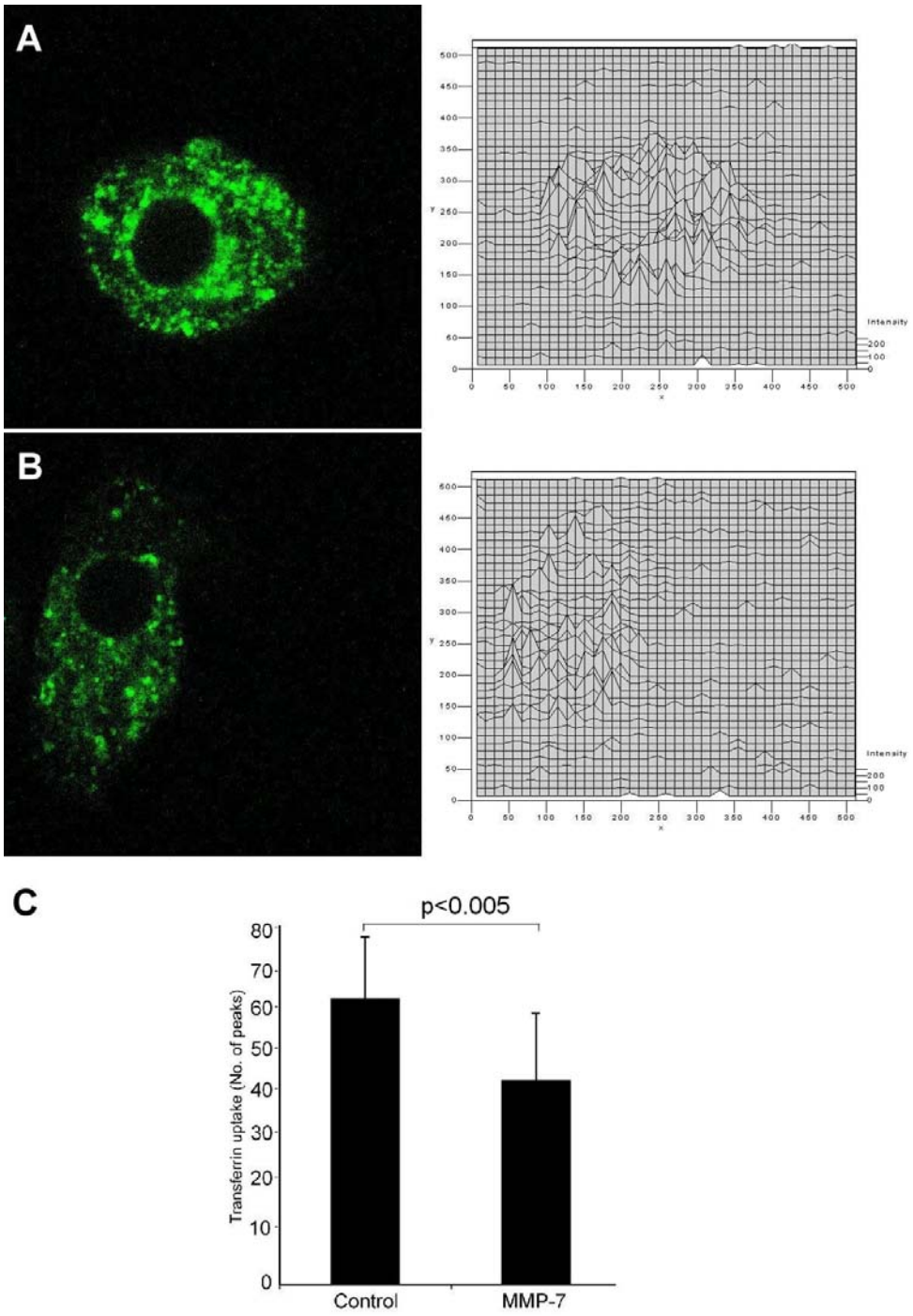


Figure 47: Trasferrin uptake by immature DCs

Immature DCs were prepared in the absence or presence of MMP-7. Cells were incubated with FITC labelled transferrin and uptake was performed at 37°C for 15 min. Images of single cells were collected by CLSM (left panel in A-B). The semiquantitative evaluation was achieved by manually counting the number of peaks displayed on the topographical charts delivered by the LSM Software (right panel in A-B). For each test set, 20 cells were evaluated and the average number of peaks were calculated (C).
A: Untreated immature DCs; **B:** MMP-7 treated immature DCs.

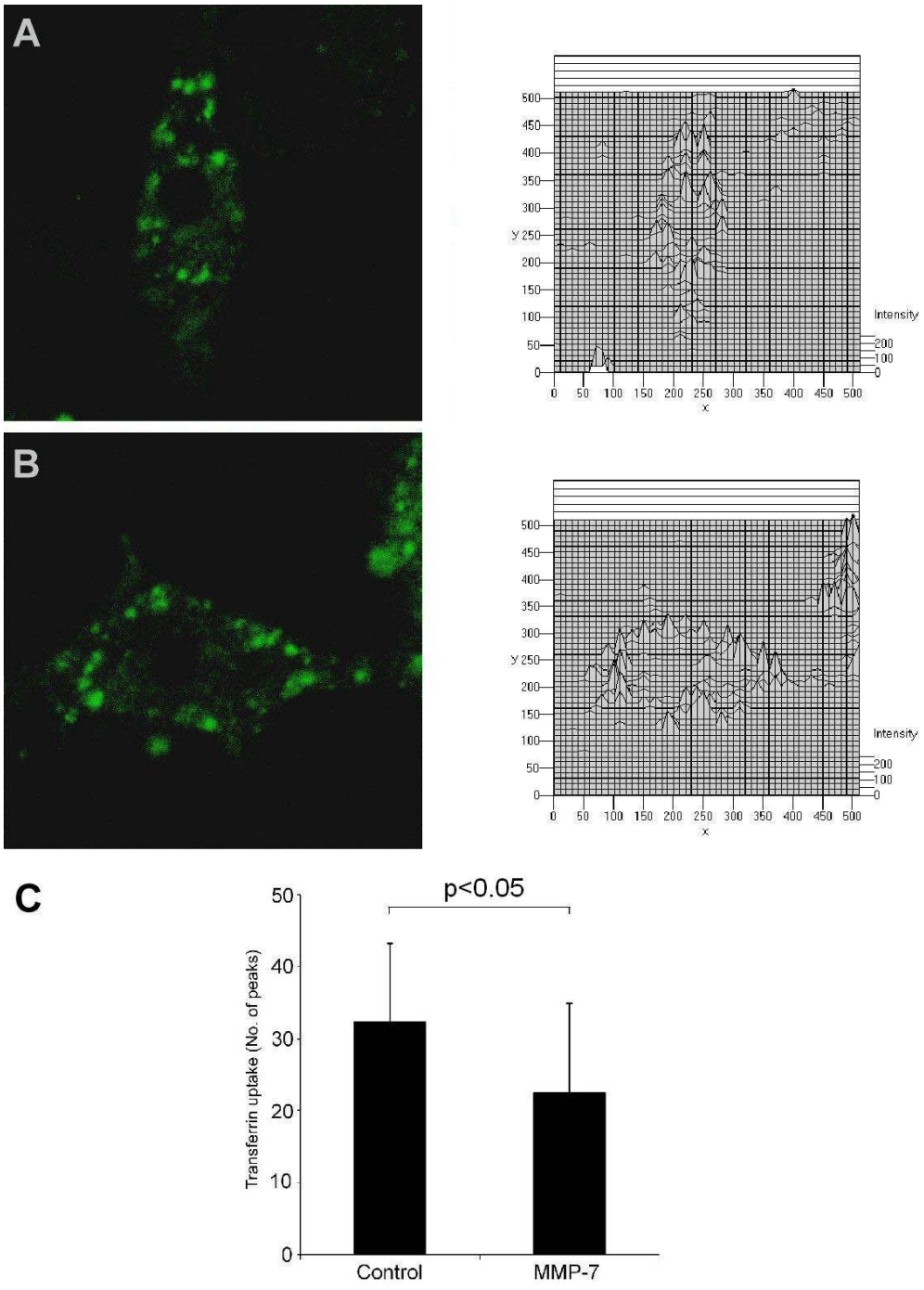


Figure 48: Trasferrin uptake by mature DCs

Mature DCs were prepared in the absence or presence of MMP-7. Cells were incubated with FITC labelled transferrin and uptake was performed at 37°C for 15 minutes. Images of single cells were collected by CLSM (left panel in A-B). The semiquantitative evaluation was achieved by manually counting the number of peaks displayed on the topographical charts delivered by the LSM Software (right panel in A-B). For each test set, 20 cells were evaluated and the average number of peaks were calculated (C).
A: Untreated mature DCs; **B:** MMP-7 treated mature DCs.

4 Discussion

Matrix metalloproteinases (MMPs) are a family of structurally related zinc dependent endopeptidases collectively capable of degrading essentially all components of extracellular matrix (ECM). MMPs play an important role in the physiologic degradation of ECM, e.g., in tissue morphogenesis, tissue repair and angiogenesis. MMPs also have important functions in pathologic conditions characterized by excessive degradation of ECM, particularly in many autoimmune diseases and in tumour invasion and metastasis (Nagase and Woessner, 1999; Shapiro, 1998; Westermarck and Kahari, 1999). MMPs are mainly expressed in epithelial cells in different normal glandular structures, but in general, the level of expression of MMPs by unstimulated cells in culture and in intact tissues *in vivo* is low (Saarialho-Kere *et al*., 1995). In many types of cancers, MMPs are upregulated and become components of the tumour microenvironment involved in the alteration of many cellular events such as migration, invasion and apoptosis (Jodele *et al*., 2006; Sheu *et al*., 2003).

The tumour promoting effects of MMPs are supported by the findings, that MMPs help cancer cells to invade to other tissues or organs and the level of MMPs has been linked to the severity of cancer (Jones *et al*., 2004). Direct evidence for the role of distinct MMPs in tumour growth and invasion has been provided by studies with knockout mice for specific MMPs. The rate of tumour formation and metastasis were found to be considerably lower in certain MMP knockout mice (Itoh *et al*., 1998; Masson *et al*., 1998; Wilson *et al*., 1997), e.g. mice lacking MMP-7 show a strong 67% reduction in intestinal tumourigenesis (Wilson *et al*., 1997). Due to the relationship of high expression levels of certain MMPs to tumours, MMPs become important criteria for cancer severity and prognosis. Thus intensive studies are performed to develop natural inhibitors and chemical drugs to target MMPs for cancer therapy (Zucker *et al*., 2000).

MMP-7 belongs to the minimal domain structural class of MMPs whose enhanced expression in tumours has been demonstrated (Adachi *et al*., 1999; Yoshimoto *et al*., 1993). Expression of MMP-7 in tumours is associated with an aggressive malignant phenotype and poor prognosis (Ishikawa *et al*., 1996; Masaki *et al*., 2001; Witty *et al*., 1994). Mouse

tumour models have shown that MMP-7 plays a role in early tumour progression (Kioi *et al*., 2003). Ablation of MMP-7 in a mouse model for intestinal cancer reduced tumour formation (Hasegawa *et al*., 1998; Wilson *et al*., 1997). On the other hand, overexpression of MMP-7 in MMTV-neu mice, a mouse model for mammary tumourigenesis, significantly promoted tumour development in mammary glands (Rudolph-Owen *et al*., 1998). MMP-7 is also found to be associated with recurrence in hepatocellular cancer (Yamamoto *et al*., 1999) and lymph node metastasis in gastric carcinomas (Yamashita *et al*., 1998). MMP-7 is exceptionally interesting, because it can modulate CD95 induced apoptosis by cleaving CD95 and CD95L (Strand *et al*., 2004b). Furthermore, MMP-7 can severely reduce the apoptosis sensitivity induced by cytotoxic T cells.

In the present study, efforts were made to clarify the role of MMP-7 in tumour progression, with particular attention focused on activation/inactivation and the immune modulatory functions of MMP-7.

4.1 HLE as an effective inactivator of MMP-7

Natural inhibitors of MMP-7 such as tissue inhibitory metalloproteases (TIMPs) are well characterized inactivators of MMPs and have been taken to clinical trials but have proved unsatisfactory (Brew *et al*., 2000; Overall and Lopez-Otin, 2002). Broad-band synthetic MMP inhibitors have also proved disappointing in clinical trials (Folgueras *et al*., 2004). Furthermore, no specific MMP-7 inhibitors are available. Thus successful therapy or combination therapies using MMP-7 inhibitors have not been achieved to date. New strategies to specifically inactivate selected MMPs such as MMP-7 are thus needed. One approach is to identify physiological inhibitors of MMP-7 and study their action. An advantage of this approach is that it may be possible to recruit cells producing these inhibitors to the site they are needed such as the tumour microenvironment. The feasibility of recruiting cells to tumours has been shown. Cytokine gene transfer strategies in animal models have provided tools to increase intratumoural cytokine availability and elevate the anti-neoplastic potential of locally recruited PMNs (Morgan *et al*., 2005).

Like most MMPs, MMP-7 is secreted by cells as an inactive proenzyme (proMMP-7), which are proteolytically activated in the pericellular space by tissue or plasma proteinases, bacterial proteinases or other MMPs. The proteolytical activation leads to removal of the prodomain, which quenches the catalytic activity. The catalytic domain is important for the activity of MMP-7. Therefore inhibition of MMP-7 activation through cleavage of the catalytic domain may be an effective means to abrogate the function of MMP-7.

For this purpose, a cell line was established, which express full length MMP-7 (proMMP-7) upon induction. The proMMP-7 secreted by this cell line was tested for the cleavage by some known activators including NO-donor SIN, trypsin and H_2O_2. Nevertheless, these activators are shown to cleave proMMP-7 only at very low efficiency. HLE, a serine protease produced by polymorphonuclear neutrophils (PMNs), is also known to be able to cleave MMP-7, releasing the active MMP-7 (Imai *et al*., 1995). In the present study, HLE is found not only to cleave the prodomain but also to cleave MMP-7 additionally within the catalytic domain, resulting in great loss of MMP-7 activity as measured using a specific fluorogenic peptide substrate. Because the inactivation of MMP-7 by HLE is based on the proteolytic cleavage near the zinc finger motif in the active domain, HLE should be a more effective inactivator than those which inactivate MMP-7 by competitively binding to the active domain of MMP-7.

However, an effective inactivation of MMP-7 by HLE in tumours depends on the local concentration of HLE in the tumour microenvironment. Since HLE is mainly produced by PMNs, the infiltration of PMNs is crucial. In a preliminary experiment of this study, the colocalization of MMP-7 expressing tumour cells and PMNs producing HLE was observed in a human colorectal tumour biopsy. This finding suggests that there may be an interaction between these two cell types in the tumour. A connection between MMP-7 and PMNs has been previously described. In a murine lung injury model, MMP-7 mobilizes PMNs to the sites of injury by cleaving the syndecan-1/KC complex thus setting up a chemo attractant gradient (Li *et al*., 2002). Though whether such mechanism exists also in tumours is yet unknown, the inactivation of MMP-7 in tumour might be achieved by increasing the expression level of HLE in PMNs and by stimulating the infiltration of PMNs producing HLE into tumour sites.

4.2 Modulation of MMP-7 mediated apoptosis resistance by HLE

The recent advances in cancer immunotherapy have repeatedly shown their efficacy in animal models but have achieved limited success in Phase I and II trials. It is clear from these studies that immune responses to tumours can be mounted, but eradication of human tumours has proved daunting (Coussens *et al*., 2002). Additional factors such as the tumour microenvironment must be considered to optimize future immune based cancer therapies. As described previously, the tumour microenvironment plays a crucial role in tumour progression not only by producing growth factors which can stimulate the growth of tumour cells but also by influencing immune reactions against tumours (Kaufman and Disis, 2004).

Apoptosis is an important mechanism for the maintenance of tissue homeostasis (Siegel *et al*., 2000) and plays a crucial role in many pathological changes including cancer and organ failure (Lowe and Lin, 2000; Strand *et al*., 1998; Strand *et al*., 1996; Strand *et al*., 2004a). Reduced apoptosis sensitivity is often observed in cancer cells. Alteration of the expression of death receptors such as CD95, leads to a change in the rate of apoptosis and consequently to pathological changes. Downregulation of CD95 in tumour cells can evade from the immune cells and expression of CD95L leads to the killing of the lymphocytes (Strand *et al*., 1996). It was previously reported that MMP-7, which is abundant in the tumour microenvironment, cleaves immune relevant targets like CD95 and CD95L, leading to decrease in apoptosis (Strand *et al*., 2004b; Tanaka *et al*., 1998). Thus the MMP-7 produced by tumour cells can facilitate tumours to escape from the CD95 mediated apoptosis.

The present study has demonstrated that the MMP-7 mediated resistance against apoptosis and cytotoxicity induced by CD95L and/or doxorubicin as well as the resistance against specific CTL killing can be abrogated by HLE via cleavage of the catalytic domain of MMP-7. This finding supports strongly the potential of HLE as a pro-apoptotic reagent in MMP-7 mediated apoptosis resistance. In case of tumour therapy, if it could succeed in increasing the expression of HLE in PMNs as well as stimulating their infiltration rate, HLE may exert anti-tumour effects via inactivation of MMP-7.

4.3 Searching for immune relevant substrates of MMP-7

Besides the mechanisms of MMP-7 activation and inactivation, more detailed knowledge will help to understand the effect of MMP-7 and provide new possibilities to interfere with its functions. MMP-7 also affects the CTL killing of CD95 negative target cells, shows that mechanism other than cleaving of CD95 and CD95L is involved in the suppression of CTL killing. To understand the mechanism involved in the modulation of CTL functions, study was focused to identify the immune relevant substrates of MMP-7 that could inhibit the CTL functions.

Up to date, substrate specificity for the MMPs is not yet fully characterized. Known substrates include some ECM components (fibronectin, vitronectin, aggrecan). Among the targets of MMP-7 are collagen IV, elastin, fibronectin, laminin, nidogen, tenascin, 1PI, osteopontin, E-cadherin, pro-TNF-α, β4 integrin (Agnihotri *et al*., 2001; Egeblad and Werb, 2002; McCawley and Matrisian, 2001; Mitsiades *et al*., 2001; Powell *et al*., 1999), α-defensin, syndecan and versican (Halpert *et al*., 1996). Recently connexin-43 has been shown to be specifically cleaved by MMP-7 (Lindsey *et al*., 2006). Of special interest is the finding that CD95 and CD95L are also the substrates of MMP-7 revealing the role of MMP-7 in modulation of apoptosis.

In the present study, experiments were performed to find immune relevant substrates of MMP-7 by using 2-D electrophoresis. The heat shock protein Hsc70 was found in the supernatants of the MMP-7 treated cells. However, MMP-7 failed to cleave Hsc70, indicating that Hsc70 is not a direct substrate of MMP-7. Secretion of Hsc70 was reported during the stress conditions, it may be possible that the Hsc70 secreted by untreated cells was internalized where as in the MMP-7 treated cells internalization of Hsc70 was inhibited. As Hsc70 is involved in the uncoating of clathrin coated vesicles, inhibition of Hsc70 internalization would leads to the drastic effect on clathrin mediated endocytosis. Further work was focused on clathrin mediated endocytosis to examine the affect of MMP-7 on this mechanism.

4.4 Modulation of clathrin mediated endocytosis by MMP-7

Clathrin mediated endocytosis plays an important role in receptor mediated internalization of antigens and extracellular proteins such as growth factors and hormones. Clathrin mediated endocytosis is triggered by binding of ligands to their receptors. The process begins then at the inner surface of the plasma membrane with the sequential assembly of coat components to form a clathrin coated pit (Mousavi *et al*., 2004). Internalized receptors interact with a class of adaptors like AP-2 and become clustered into the growing coated pit. The adaptors link the membrane proteins with clathrin to form the outer layer of the coat. Together with accessory and regulatory molecules (so called cargo proteins), adaptors and clathrin co-assemble, and the growing coated pit invaginates. In the final stage, the membrane neck is severed to form a closed coated vesicle (Takei and Haucke, 2001). The clathrin coated vesicles (CCVs) bud off from the membrane and deliver their cargo to the endosomal system for recycling or degradation. Following detachment from the plasma membrane, the clathrin coat is quickly dissembled by the combined action of the ATPase Hsc70 and the coat component auxilin.

To clarify the effect of MMP-7 on clathrin mediated endocytosis, different cells were tested in the present study on their ability to uptake transferrin, a well established model for clathrin mediated endocytosis. In Jurkat cells, HEK293 cells, HepG2 cells, $CD4^+CD25^+$ T_{reg} cells and DCs, less clathrin staining was observed when the cells were treated with MMP-7. In parallel, reduced transferrin uptake has been found in Jurkat cells, $CD4^+CD25^+$ T_{reg} cells and DCs treated with MMP-7, inspite that surface expression of transferrin receptor CD71 remains unchanged. Since of all the proteins involved in clathrin mediated endocytosis, the receptor is the only one which is located at the outer surface of plasma membrane, the effect of MMP-7 must have been mediated by a known or unknown membrane bound substrate of MMP-7. These results suggest that MMP-7 has indirectly affected the clathrin level in cells and consequently the transferrin uptake.

4.5 Influence of MMP-7 on differentiation of dendritic cells (DCs)

In the immune system, DCs are one cell type that is very dependent on endocytosis for their biological functions. Especially the immature DCs posses high endocytotic capacity and

can more efficiently uptake antigens than the mature DCs. Mature DCs are most efficient antigen presenting cells and potent inducers of immune responses and play a pivotal role in eliciting immune response against tumours (Vicari *et al*., 2002).

DCs capture the antigens mainly by receptor mediated endocytosis (Sallusto *et al*., 1995). DCs are capable of evolving from immature, antigen capturing cells to mature, antigen presenting, T cell priming cells. The maturation process *in vivo* is triggered by internalization of foreign antigens. During their conversion from immature to mature cells, DCs undergo a number of phenotypical and functional changes. The process of DC maturation, in general, involves a redistribution of major histocompatibility complex (MHC) molecules from intracellular endocytic compartments to the DC surface, down regulation of antigen internalization, an increase in the surface expression of costimulatory molecules, morphological changes (e.g. formation of dendrites), cytoskeleton reorganization, secretion of chemokines, cytokines and proteases, and surface expression of adhesion molecules and chemokine receptors.

*In vitro***,** immature and mature DCs can be obtained by culture of mononuclear precursor cells isolated form peripheral blood or bone marrow in the presence of certain cytokines. In the present study, the presence of MMP-7 during the differentiation process influenced the development of DCs. Additional treatment of monocytes with MMP-7 during cytokine induced differentiation to DCs induces a subpopulation of cells with typical morphology and becomes even dominant in mature DCs cultures. These cells were long spindle shaped and strongly adherent. They showed low SSC and low FSC in FACS analysis and were negative for all DC maturation markers. The low SSC and low FSC property reveals that these cells are poorly granulated and small after detachment. This subpopulation could evidently not defined as DCs. Cells of the other subpopulation were characterized by their typical DC morphology, high SSC and high FSC in FACS analysis, normal expression of DC maturation markers, indicating that they are DCs. The percentage of the non-DC subpopulation ranges from 50-60% in cultures without MMP-7 and 70-80% in MMP-7 treated cultures, as measured by FACS based on SSC/FSC dot plots. Comparison of maturation markers of MMP-7 treated or untreated DCs revealed no difference in differentiated subpopulations, suggesting that MMP-7 has interfered with the

differentiation of DCs at very early stages. The mechanism, how MMP-7 affect the DC differentiation, is yet unknown. Also the influence of MMP-7 on DC differentiation *in vivo* remains unidentified. Anyway, it can be speculated, that this effect of MMP-7 may contribute to a suppression of DCs immune functions in the tumour milieu where MMP-7 is present.

4.6 Future perspectives

One of the most interesting findings of the present study is that HLE can act as an effective natural inactivator of MMP-7 due to the presence of an HLE cleavage site in the catalytic domain of MMP-7. Furthermore, it has been shown in this study that HLE can abrogate the increased apoptosis resistance of cells mediated by MMP-7. To validate the effects of HLE in MMP-7 producing tumours, *in vitro* experiments should be at first performed using appropriate tumour cell lines. If the inhibition of tumour cell growth could be confirmed, *in vivo* experiments employing appropriate animal models can follow. Finally, how to increase the expression of HLE in PMNs and to promote the infiltration of such cells should be investigated.

Another important result of this study is that MMP-7 interferes with the clathrin mediated endocytosis, supported by the findings that the cellular clathrin level and the uptake of transferrin were reduced by MMP-7 in parallel. But in these experiments MMP-7 acts only indirectly, because neither the transferrin receptor nor clathrin is a direct substrate of MMP-7. To clarify the mechanism of endocytosis reduction caused by MMP-7, known membrane bound substrates of MMP-7 should be examined concerning clathrin mediated endocytosis. The identification of new MMP-7 substrates which are located on cell membranes should connect the MMP-7 activity and its effect on clathrin mediated endocytosis.

To point out the mechanism how MMP-7 affects DC differentiation, a more precise characterization of the non-DC subpopulation may help. For example, the surface marker profile may give information, if MMP-7 has blocked the differentiation of DCs or it has shifted the differentiation to other cell type. On the other hand, it is also very important to examine, if other functions of DCs, such as the antigen presentation and receptor independent endocytosis, are influenced by MMP-7.

5 Summary

Matrix metalloproteinases are the components of the tumour microenvironment which play a crucial role in tumour progression. Matrix metalloproteinase-7 (MMP-7) is expressed in a variety of tumours and the expression is associated with an aggressive malignant phenotype and poor prognosis. A role for MMP-7 in the immune escape of tumours has been postulated, but the mechanisms are not clearly understood. The present study was focused on identifying physiological inactivators of MMP-7 and also to unravel the mechanisms involved in MMP-7 mediated immune escape.

This study shows that human leukocyte elastase (HLE), secreted by polymorphonuclear leukocytes cleaves MMP-7 in the catalytic domain as revealed by N-terminal sequencing. Further analysis demonstrates that the activity of MMP-7 was drastically decreased after HLE treatment in a time and dose dependent manner.

MMP-7 induces apoptosis resistance in tumour cells by cleaving CD95 and CD95L. The effect of HLE on MMP-7 mediated apoptosis resistance was analysed. *In vitro* stimulation of apoptosis by anti-Apo-1 (anti-CD95 antibody) and the chemotherapeutic drug doxorubicin is reduced by MMP-7. Also tumour specific cytotoxic T cells do not effectively kill tumour cells in the presence of MMP-7. This study revealed that HLE abrogates the negative effect of MMP-7 on apoptosis induced by CD95 stimulation, doxorubicin or cytotoxic T cells and restores apoptosis sensitivity of tumour cells.

To gain insight into the possible immune modulatory functions of MMP-7, experiments were performed to identify new immune relevant substrates. The human T cell line, Jurkat, was selected for these studies. Hsc70 which is involved in uncoating of clathrin vesicles was found in the supernatants of the MMP-7 treated cells indicating a modulatory role of MMP-7 on endocytosis. Further studies demonstrated that MMP-7 leads to decreased clathrin staining in HEK293, HepG2, Jurkat, $CD4^+$ T cells and dendritic cells. Results also show MMP-7 treatment increased surface expression of cytotoxic T lymphocyte associated protein-4 (CTLA-4) which accumulated due to inhibition of the clathrin mediated internalization in $CD4^+CD25^+$ cells.

6 References

Acuto, O., and Cantrell, D. (2000). T cell activation and the cytoskeleton. Annu Rev Immunol *18*, 165-184.

Adachi, Y., Yamamoto, H., Itoh, F., Hinoda, Y., Okada, Y., and Imai, K. (1999). Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. Gut *45*, 252-258.

Adams, J. M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.

Agnihotri, R., Crawford, H. C., Haro, H., Matrisian, L. M., Havrda, M. C., and Liaw, L. (2001). Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). J Biol Chem *276*, 28261-28267.

Almand, B., Resser, J. R., Lindman, B., Nadaf, S., Clark, J. I., Kwon, E. D., Carbone, D. P., and Gabrilovich, D. I. (2000). Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res *6*, 1755-1766.

Ameisen, J. C. (1992). Programmed cell death and AIDS: from hypothesis to experiment. Immunol Today *13*, 388-391.

Ameisen, J. C., and Capron, A. (1991). Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. Immunol Today *12*, 102-105.

Andreau, K., Perfettini, J. L., Castedo, M., Metivier, D., Scott, V., Pierron, G., and Kroemer, G. (2004). Contagious apoptosis facilitated by the HIV-1 envelope: fusion-induced cell-to-cell transmission of a lethal signal. J Cell Sci *117*, 5643-5653.

Antonsson, B., and Martinou, J. C. (2000). The Bcl-2 protein family. Exp Cell Res *256*, 50-57.

Baragi, V. M., Fliszar, C. J., Conroy, M. C., Ye, Q. Z., Shipley, J. M., and Welgus, H. G. (1994). Contribution of the C-terminal domain of metalloproteinases to binding by tissue inhibitor of metalloproteinases. C-terminal truncated stromelysin and matrilysin exhibit equally compromised binding affinities as compared to full-length stromelysin. J Biol Chem *269*, 12692-12697.

Barak, Y., Gottlieb, E., Juven-Gershon, T., and Oren, M. (1994). Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. Genes Dev *8*, 1739-1749.

Barry, M., Heibein, J. A., Pinkoski, M. J., Lee, S. F., Moyer, R. W., Green, D. R., and Bleackley, R. C. (2000). Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Mol Cell Biol *20*, 3781-3794.

Beissert, S., Ullrich, S. E., Hosoi, J., and Granstein, R. D. (1995). Supernatants from UVB radiation-exposed keratinocytes inhibit Langerhans cell presentation of tumor-associated antigens via IL-10 content. J Leukoc Biol *58*, 234-240.

Benbow, U., and Brinckerhoff, C. E. (1997). The AP-1 site and MMP gene regulation: what is all the fuss about? Matrix Biol *15*, 519-526.

Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, I. M., Ricci, J. E., Edris, W. A., Sutherlin, D. P., Green, D. R., and Salvesen, G. S. (2003). A unified model for apical caspase activation. Mol Cell *11*, 529-541.

Bolon, I., Gouyer, V., Devouassoux, M., Vandenbunder, B., Wernert, N., Moro, D., Brambilla, C., and Brambilla, E. (1995). Expression of c-ets-1, collagenase 1, and urokinase-type plasminogen activator genes in lung carcinomas. Am J Pathol *147*, 1298-1310.

Boura-Halfon, S., Voliovitch, H., Feinstein, R., Paz, K., and Zick, Y. (2003). Extracellular matrix proteins modulate endocytosis of the insulin receptor. J Biol Chem *278*, 16397-16404.

Boussiotis, V. A., Freeman, G. J., Berezovskaya, A., Barber, D. L., and Nadler, L. M. (1997). Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. Science *278*, 124-128.

Brabletz, T., Jung, A., Dag, S., Hlubek, F., and Kirchner, T. (1999). beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol *155*, 1033-1038.

Brew, K., Dinakarpandian, D., and Nagase, H. (2000). Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta *1477*, 267-283.

Brown, R. D., Pope, B., Murray, A., Esdale, W., Sze, D. M., Gibson, J., Ho, P. J., Hart, D., and Joshua, D. (2001). Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10. Blood *98*, 2992-2998.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol *15*, 269-290.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu Rev Cell Biol *4*, 487-525.

Carreno, B. M., Bennett, F., Chau, T. A., Ling, V., Luxenberg, D., Jussif, J., Baroja, M. L., and Madrenas, J. (2000). CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. J Immunol *165*, 1352-1356.

Cefai, D., Schneider, H., Matangkasombut, O., Kang, H., Brody, J., and Rudd, C. E. (1998). CD28 receptor endocytosis is targeted by mutations that disrupt phosphatidylinositol 3-kinase binding and costimulation. J Immunol *160*, 2223-2230.

Chuang, E., Alegre, M. L., Duckett, C. S., Noel, P. J., Vander Heiden, M. G., and Thompson, C. B. (1997). Interaction of CTLA-4 with the clathrin-associated protein AP50

results in ligand-independent endocytosis that limits cell surface expression. J Immunol *159*, 144-151.

Chung, L., Dinakarpandian, D., Yoshida, N., Lauer-Fields, J. L., Fields, G. B., Visse, R., and Nagase, H. (2004). Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. Embo J *23*, 3020-3030.

Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002). Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science *295*, 2387-2392.

Crabbe, T., Willenbrock, F., Eaton, D., Hynds, P., Carne, A. F., Murphy, G., and Docherty, A. J. (1992). Biochemical characterization of matrilysin. Activation conforms to the stepwise mechanisms proposed for other matrix metalloproteinases. Biochemistry *31*, 8500-8507.

Crawford, H. C., and Matrisian, L. M. (1996). Mechanisms controlling the transcription of matrix metalloproteinase genes in normal and neoplastic cells. Enzyme Protein *49*, 20-37.

Danial, N. N., and Korsmeyer, S. J. (2004). Cell death: critical control points. Cell *116*, 205-219.

Davis, G. E., and Senger, D. R. (2005). Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res *97*, 1093-1107.

Decker, P., and Muller, S. (2002). Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. Curr Pharm Biotechnol *3*, 275-283.

DeClerck, Y. A., Mercurio, A. M., Stack, M. S., Chapman, H. A., Zutter, M. M., Muschel, R. J., Raz, A., Matrisian, L. M., Sloane, B. F., Noel, A.*, et al.* (2004). Proteases, extracellular matrix, and cancer: a workshop of the path B study section. Am J Pathol *164*, 1131-1139.

Dhodapkar, M. V., Krasovsky, J., and Olson, K. (2002). T cells from the tumor microenvironment of patients with progressive myeloma can generate strong, tumor-specific cytolytic responses to autologous, tumor-loaded dendritic cells. Proc Natl Acad Sci U S A *99*, 13009-13013.

Di Fiore, P. P., and De Camilli, P. (2001). Endocytosis and signaling. an inseparable partnership. Cell *106*, 1-4.

Drexler, I., Antunes, E., Schmitz, M., Wolfel, T., Huber, C., Erfle, V., Rieber, P., Theobald, M., and Sutter, G. (1999). Modified vaccinia virus Ankara for delivery of human tyrosinase as melanoma-associated antigen: induction of tyrosinase- and melanoma-specific human leukocyte antigen A*0201-restricted cytotoxic T cells in vitro and in vivo. Cancer Res *59*, 4955-4963.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell *102*, 33-42.

Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem *68*, 383-424.

Egeblad, M., and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer *2*, 161-174.

el-Deiry, W. S. (1998). Regulation of p53 downstream genes. Semin Cancer Biol *8*, 345-357.

Elenius, K., Salmivirta, M., Inki, P., Mali, M., and Jalkanen, M. (1990). Binding of human syndecan to extracellular matrix proteins. J Biol Chem *265*, 17837-17843.

Fingleton, B. M., Heppner Goss, K. J., Crawford, H. C., and Matrisian, L. M. (1999). Matrilysin in early stage intestinal tumorigenesis. Apmis *107*, 102-110.

Folgueras, A. R., Pendas, A. M., Sanchez, L. M., and Lopez-Otin, C. (2004). Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. Int J Dev Biol *48*, 411-424.

Fridman, J. S., and Lowe, S. W. (2003). Control of apoptosis by p53. Oncogene *22*, 9030-9040.

Fu, X., Kassim, S. Y., Parks, W. C., and Heinecke, J. W. (2001). Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase. J Biol Chem *276*, 41279-41287.

Gabrilovich, D. (2004). Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol *4*, 941-952.

Gaidarov, I., and Keen, J. H. (1999). Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. J Cell Biol *146*, 755-764.

Galis, Z. S., and Khatri, J. J. (2002). Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res *90*, 251-262.

Galle, P. R., Hagelstein, J., Kommerell, B., Volkmann, M., Schranz, P., and Zentgraf, H. (1994). In vitro experimental infection of primary human hepatocytes with hepatitis B virus. Gastroenterology *106*, 664-673.

Galle, P. R., Hofmann, W. J., Walczak, H., Schaller, H., Otto, G., Stremmel, W., Krammer, P. H., and Runkel, L. (1995). Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J Exp Med *182*, 1223-1230.

Galle, P. R., and Krammer, P. H. (1998). CD95-induced apoptosis in human liver disease. Semin Liver Dis *18*, 141-151.

Garrett, W. S., Chen, L. M., Kroschewski, R., Ebersold, M., Turley, S., Trombetta, S., Galan, J. E., and Mellman, I. (2000). Developmental control of endocytosis in dendritic cells by Cdc42. Cell *102*, 325-334.

Gimmi, C. D., Freeman, G. J., Gribben, J. G., Sugita, K., Freedman, A. S., Morimoto, C., and Nadler, L. M. (1991). B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. Proc Natl Acad Sci U S A *88*, 6575-6579.

Goffin, F., Munaut, C., Frankenne, F., Perrier D'Hauterive, S., Beliard, A., Fridman, V., Nervo, P., Colige, A., and Foidart, J. M. (2003). Expression pattern of metalloproteinases and tissue inhibitors of matrix-metalloproteinases in cycling human endometrium. Biol Reprod *69*, 976-984.

Gomez, D. E., Alonso, D. F., Yoshiji, H., and Thorgeirsson, U. P. (1997). Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol *74*, 111-122.

Gonzalez-Gaitan, M., and Stenmark, H. (2003). Endocytosis and signaling: a relationship under development. Cell *115*, 513-521.

Goping, I. S., Barry, M., Liston, P., Sawchuk, T., Constantinescu, G., Michalak, K. M., Shostak, I., Roberts, D. L., Hunter, A. M., Korneluk, R., and Bleackley, R. C. (2003). Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. Immunity *18*, 355-365.

Gorodeski, G. I. (2006). Estrogen decrease in tight junctional resistance involves MMP-7 - mediated remodeling of occludin. Endocrinology.

Green, D. R., Droin, N., and Pinkoski, M. (2003). Activation-induced cell death in T cells. Immunol Rev *193*, 70-81.

Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R., and Ferguson, T. A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. Science *270*, 1189-1192.

Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. (1999). Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem *274*, 1156-1163.

Halpert, I., Sires, U. I., Roby, J. D., Potter-Perigo, S., Wight, T. N., Shapiro, S. D., Welgus, H. G., Wickline, S. A., and Parks, W. C. (1996). Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. Proc Natl Acad Sci U S A *93*, 9748-9753.

Hannan, L. A., Newmyer, S. L., and Schmid, S. L. (1998). ATP- and cytosol-dependent release of adaptor proteins from clathrin-coated vesicles: A dual role for Hsc70. Mol Biol Cell *9*, 2217-2229.

Hao, W., Luo, Z., Zheng, L., Prasad, K., and Lafer, E. M. (1999). AP180 and AP-2 interact directly in a complex that cooperatively assembles clathrin. J Biol Chem *274*, 22785-22794.

Hasegawa, S., Koshikawa, N., Momiyama, N., Moriyama, K., Ichikawa, Y., Ishikawa, T., Mitsuhashi, M., Shimada, H., and Miyazaki, K. (1998). Matrilysin-specific antisense

oligonucleotide inhibits liver metastasis of human colon cancer cells in a nude mouse model. Int J Cancer *76*, 812-816.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Hayashida, K., Johnston, D. R., Goldberger, O., and Park, P. W. (2006). Syndecan-1 Expression in Epithelial Cells Is Induced by Transforming Growth Factor beta through a PKA-dependent Pathway. J Biol Chem *281*, 24365-24374.

Hengartner, M. O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.

Heymann, J. B., Iwasaki, K., Yim, Y. I., Cheng, N., Belnap, D. M., Greene, L. E., Eisenberg, E., and Steven, A. C. (2005). Visualization of the binding of Hsc70 ATPase to clathrin baskets: implications for an uncoating mechanism. J Biol Chem *280*, 7156-7161.

Horwitz, A. R., and Parsons, J. T. (1999). Cell migration--movin' on. Science *286*, 1102-1103.

Hu, E., Mueller, E., Oliviero, S., Papaioannou, V. E., Johnson, R., and Spiegelman, B. M. (1994). Targeted disruption of the c-fos gene demonstrates c-fos-dependent and -independent pathways for gene expression stimulated by growth factors or oncogenes. Embo J *13*, 3094-3103.

Hynes, R. O. (1987). Integrins: a family of cell surface receptors. Cell *48*, 549-554.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11-25.

Iida, T., Ohno, H., Nakaseko, C., Sakuma, M., Takeda-Ezaki, M., Arase, H., Kominami, E., Fujisawa, T., and Saito, T. (2000). Regulation of cell surface expression of CTLA-4 by secretion of CTLA-4-containing lysosomes upon activation of CD4+ T cells. J Immunol *165*, 5062-5068.

Imai, K., Yokohama, Y., Nakanishi, I., Ohuchi, E., Fujii, Y., Nakai, N., and Okada, Y. (1995). Matrix metalloproteinase 7 (matrilysin) from human rectal carcinoma cells. Activation of the precursor, interaction with other matrix metalloproteinases and enzymic properties. J Biol Chem *270*, 6691-6697.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., *et al.* (1997). Inhibition of death receptor signals by cellular FLIP. Nature *388*, 190-195.

Ishikawa, T., Ichikawa, Y., Mitsuhashi, M., Momiyama, N., Chishima, T., Tanaka, K., Yamaoka, H., Miyazakic, K., Nagashima, Y., Akitaya, T., and Shimada, H. (1996). Matrilysin is associated with progression of colorectal tumor. Cancer Lett *107*, 5-10.

Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H., and Itohara, S. (1998). Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res *58*, 1048-1051.

Izawa, M., Kumamoto, K., Mitsuoka, C., Kanamori, C., Kanamori, A., Ohmori, K., Ishida, H., Nakamura, S., Kurata-Miura, K., Sasaki, K., *et al.* (2000). Expression of sialyl 6-sulfo

Lewis X is inversely correlated with conventional sialyl Lewis X expression in human colorectal cancer. Cancer Res *60*, 1410-1416.

Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. Cell *88*, 347-354.

Jodele, S., Blavier, L., Yoon, J. M., and DeClerck, Y. A. (2006). Modifying the soil to affect the seed: role of stromal-derived matrix metalloproteinases in cancer progression. Cancer Metastasis Rev *25*, 35-43.

Johnsen, A., France, J., Sy, M. S., and Harding, C. V. (1998). Down-regulation of the transporter for antigen presentation, proteasome subunits, and class I major histocompatibility complex in tumor cell lines. Cancer Res *58*, 3660-3667.

Johnson, H., Scorrano, L., Korsmeyer, S. J., and Ley, T. J. (2003). Cell death induced by granzyme C. Blood *101*, 3093-3101.

Jones, L., Ghaneh, P., Humphreys, M., and Neoptolemos, J. P. (1999). The matrix metalloproteinases and their inhibitors in the treatment of pancreatic cancer. Ann N Y Acad Sci *880*, 288-307.

Jones, L. E., Humphreys, M. J., Campbell, F., Neoptolemos, J. P., and Boyd, M. T. (2004). Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival. Clin Cancer Res *10*, 2832-2845.

Jordan, M. B., Hildeman, D., Kappler, J., and Marrack, P. (2004). An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. Blood *104*, 735-743.

Kaufman, H. L., and Disis, M. L. (2004). Immune system versus tumor: shifting the balance in favor of DCs and effective immunity. J Clin Invest *113*, 664-667.

Kavurma, M. M., and Khachigian, L. M. (2003). Signaling and transcriptional control of Fas ligand gene expression. Cell Death Differ *10*, 36-44.

Keane, M. M., Ettenberg, S. A., Lowrey, G. A., Russell, E. K., and Lipkowitz, S. (1996). Fas expression and function in normal and malignant breast cell lines. Cancer Res *56*, 4791-4798.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Kioi, M., Yamamoto, K., Higashi, S., Koshikawa, N., Fujita, K., and Miyazaki, K. (2003). Matrilysin (MMP-7) induces homotypic adhesion of human colon cancer cells and enhances their metastatic potential in nude mouse model. Oncogene *22*, 8662-8670.

Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. Embo J *14*, 5579-5588.

Kitoh, T., Yanai, H., Saitoh, Y., Nakamura, Y., Matsubara, Y., Kitoh, H., Yoshida, T., and Okita, K. (2004). Increased expression of matrix metalloproteinase-7 in invasive early gastric cancer. J Gastroenterol *39*, 434-440.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lane, D. P. (1992). Worrying about p53. Curr Biol *2*, 581-583.

Lau, K. M., Cheng, S. H., Lo, K. W., Lee, S. A., Woo, J. K., van Hasselt, C. A., Lee, S. P., Rickinson, A. B., and Ng, M. H. (2007). Increase in circulating Foxp3+CD4+CD25(high) regulatory T cells in nasopharyngeal carcinoma patients. Br J Cancer *96*, 617-622.

Lee, K. M., Chuang, E., Griffin, M., Khattri, R., Hong, D. K., Zhang, W., Straus, D., Samelson, L. E., Thompson, C. B., and Bluestone, J. A. (1998). Molecular basis of T cell inactivation by CTLA-4. Science *282*, 2263-2266.

Lee, M. H., and Murphy, G. (2004). Matrix metalloproteinases at a glance. J Cell Sci *117*, 4015-4016.

Lenahan, C., and Avigan, D. (2006). Dendritic cell defects in patients with cancer: mechanisms and significance. Breast Cancer Res *8*, 101.

Li, L. Y., Luo, X., and Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. Nature *412*, 95-99.

Li, Q., Park, P. W., Wilson, C. L., and Parks, W. C. (2002). Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell *111*, 635-646.

Lindsey, M. L., Escobar, G. P., Mukherjee, R., Goshorn, D. K., Sheats, N. J., Bruce, J. A., Mains, I. M., Hendrick, J. K., Hewett, K. W., Gourdie, R. G.*, et al.* (2006). Matrix metalloproteinase-7 affects connexin-43 levels, electrical conduction, and survival after myocardial infarction. Circulation *113*, 2919-2928.

Linsley, P. S., Bradshaw, J., Urnes, M., Grosmaire, L., and Ledbetter, J. A. (1993). CD28 engagement by B7/BB-1 induces transient down-regulation of CD28 synthesis and prolonged unresponsiveness to CD28 signaling. J Immunol *150*, 3161-3169.

Linsley, P. S., Greene, J. L., Tan, P., Bradshaw, J., Ledbetter, J. A., Anasetti, C., and Damle, N. K. (1992). Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. J Exp Med *176*, 1595-1604.

Lira, S. A., Zalamea, P., Heinrich, J. N., Fuentes, M. E., Carrasco, D., Lewin, A. C., Barton, D. S., Durham, S., and Bravo, R. (1994). Expression of the chemokine N51/KC in the thymus and epidermis of transgenic mice results in marked infiltration of a single class of inflammatory cells. J Exp Med *180*, 2039-2048.

Liu, Y. E., Wang, M., Greene, J., Su, J., Ullrich, S., Li, H., Sheng, S., Alexander, P., Sang, Q. A., and Shi, Y. E. (1997). Preparation and characterization of recombinant tissue inhibitor of metalloproteinase 4 (TIMP-4). J Biol Chem *272*, 20479-20483.

Liu, Y. J. (2001). Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell *106*, 259-262.

Lowe, S. W., and Lin, A. W. (2000). Apoptosis in cancer. Carcinogenesis *21*, 485-495.

Martin-Bermudo, M. D., and Brown, N. H. (2000). The localized assembly of extracellular matrix integrin ligands requires cell-cell contact. J Cell Sci *113 Pt 21*, 3715-3723.

Martinvalet, D., Zhu, P., and Lieberman, J. (2005). Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. Immunity *22*, 355-370.

Masaki, T., Matsuoka, H., Sugiyama, M., Abe, N., Goto, A., Sakamoto, A., and Atomi, Y. (2001). Matrilysin (MMP-7) as a significant determinant of malignant potential of early invasive colorectal carcinomas. Br J Cancer *84*, 1317-1321.

Masson, R., Lefebvre, O., Noel, A., Fahime, M. E., Chenard, M. P., Wendling, C., Kebers, F., LeMeur, M., Dierich, A., Foidart, J. M.*, et al.* (1998). In vivo evidence that the stromelysin-3 metalloproteinase contributes in a paracrine manner to epithelial cell malignancy. J Cell Biol *140*, 1535-1541.

Massova, I., Kotra, L. P., Fridman, R., and Mobashery, S. (1998). Matrix metalloproteinases: structures, evolution, and diversification. Faseb J *12*, 1075-1095.

Matsuno, K., Adachi, Y., Yamamoto, H., Goto, A., Arimura, Y., Endo, T., Itoh, F., and Imai, K. (2003). The expression of matrix metalloproteinase matrilysin indicates the degree of inflammation in ulcerative colitis. J Gastroenterol *38*, 348-354.

McCawley, L. J., and Matrisian, L. M. (2000). Matrix metalloproteinases: multifunctional contributors to tumor progression. Mol Med Today *6*, 149-156.

McCawley, L. J., and Matrisian, L. M. (2001). Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol *13*, 534-540.

Mitsiades, N., Yu, W. H., Poulaki, V., Tsokos, M., and Stamenkovic, I. (2001). Matrix metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity. Cancer Res *61*, 577-581.

Miyata, Y., Iwata, T., Ohba, K., Kanda, S., Nishikido, M., and Kanetake, H. (2006). Expression of matrix metalloproteinase-7 on cancer cells and tissue endothelial cells in renal cell carcinoma: prognostic implications and clinical significance for invasion and metastasis. Clin Cancer Res *12*, 6998-7003.

Morgan, M. D., Harper, L., Lu, X., Nash, G., Williams, J., and Savage, C. O. (2005). Can neutrophils be manipulated in vivo? Rheumatology (Oxford) *44*, 597-601.

Mousavi, S. A., Malerod, L., Berg, T., and Kjeken, R. (2004). Clathrin-dependent endocytosis. Biochem J *377*, 1-16.

Mueller, D. L., Jenkins, M. K., and Schwartz, R. H. (1989). Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. Annu Rev Immunol *7*, 445-480.

Muhlberg, A. B., Warnock, D. E., and Schmid, S. L. (1997). Domain structure and intramolecular regulation of dynamin GTPase. Embo J *16*, 6676-6683.

Muller, M., Strand, S., Hug, H., Heinemann, E. M., Walczak, H., Hofmann, W. J., Stremmel, W., Krammer, P. H., and Galle, P. R. (1997). Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J Clin Invest *99*, 403-413.

Nagase, H., and Woessner, J. F., Jr. (1999). Matrix metalloproteinases. J Biol Chem *274*, 21491-21494.

Newell, K. J., Matrisian, L. M., and Driman, D. K. (2002). Matrilysin (matrix metalloproteinase-7) expression in ulcerative colitis-related tumorigenesis. Mol Carcinog *34*, 59-63.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods *139*, 271-279.

Nishikawa, H., Jager, E., Ritter, G., Old, L. J., and Gnjatic, S. (2005). CD4+ CD25+ regulatory T cells control the induction of antigen-specific CD4+ helper T cell responses in cancer patients. Blood *106*, 1008-1011.

Noe, V., Fingleton, B., Jacobs, K., Crawford, H. C., Vermeulen, S., Steelant, W., Bruyneel, E., Matrisian, L. M., and Mareel, M. (2001). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. J Cell Sci *114*, 111-118.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053-1058.

Oshima, M., Oshima, H., and Taketo, M. M. (1996). TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev Biol *179*, 297-302.

Overall, C. M., and Lopez-Otin, C. (2002). Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer *2*, 657-672.

Ozaki, I., Mizuta, T., Zhao, G., Zhang, H., Yoshimura, T., Kawazoe, S., Eguchi, Y., Yasutake, T., Hisatomi, A., Sakai, T., and Yamamoto, K. (2003). Induction of multiple matrix metalloproteinase genes in human hepatocellular carcinoma by hepatocyte growth factor via a transcription factor Ets-1. Hepatol Res *27*, 289-301.

Palucka, K. A., Taquet, N., Sanchez-Chapuis, F., and Gluckman, J. C. (1998). Dendritic cells as the terminal stage of monocyte differentiation. J Immunol *160*, 4587-4595.

Pece, S., and Gutkind, J. S. (2000). Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. J Biol Chem *275*, 41227-41233.

Pinkoski, M. J., Waterhouse, N. J., Heibein, J. A., Wolf, B. B., Kuwana, T., Goldstein, J. C., Newmeyer, D. D., Bleackley, R. C., and Green, D. R. (2001). Granzyme B-mediated

apoptosis proceeds predominantly through a Bcl-2-inhibitable mitochondrial pathway. J Biol Chem *276*, 12060-12067.

Ponton, A., Coulombe, B., and Skup, D. (1991). Decreased expression of tissue inhibitor of metalloproteinases in metastatic tumor cells leading to increased levels of collagenase activity. Cancer Res *51*, 2138-2143.

Postovit, L. M., Seftor, E. A., Seftor, R. E., and Hendrix, M. J. (2006). Influence of the microenvironment on melanoma cell fate determination and phenotype. Cancer Res *66*, 7833-7836.

Potter, E., Bergwitz, C., and Brabant, G. (1999). The cadherin-catenin system: implications for growth and differentiation of endocrine tissues. Endocr Rev *20*, 207-239.

Poulaki, V., Mitsiades, C. S., and Mitsiades, N. (2001). The role of Fas and FasL as mediators of anticancer chemotherapy. Drug Resist Updat *4*, 233-242.

Powell, W. C., Fingleton, B., Wilson, C. L., Boothby, M., and Matrisian, L. M. (1999). The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol *9*, 1441-1447.

Prasad, K., Barouch, W., Greene, L., and Eisenberg, E. (1993). A protein cofactor is required for uncoating of clathrin baskets by uncoating ATPase. J Biol Chem *268*, 23758-23761.

Reed, J. C. (1997). Double identity for proteins of the Bcl-2 family. Nature *387*, 773-776.

Reynolds, T. Y., Rockwell, S., and Glazer, P. M. (1996). Genetic instability induced by the tumor microenvironment. Cancer Res *56*, 5754-5757.

Rodriguez, J., and Lazebnik, Y. (1999). Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev *13*, 3179-3184.

Rudolph-Owen, L. A., Chan, R., Muller, W. J., and Matrisian, L. M. (1998). The matrix metalloproteinase matrilysin influences early-stage mammary tumorigenesis. Cancer Res *58*, 5500-5506.

Saarialho-Kere, U. K., Crouch, E. C., and Parks, W. C. (1995). Matrix metalloproteinase matrilysin is constitutively expressed in adult human exocrine epithelium. J Invest Dermatol *105*, 190-196.

Saijo, Y., Tanaka, M., Miki, M., Usui, K., Suzuki, T., Maemondo, M., Hong, X., Tazawa, R., Kikuchi, T., Matsushima, K., and Nukiwa, T. (2002). Proinflammatory cytokine IL-1 beta promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: in vivo analysis of tumor-stromal interaction. J Immunol *169*, 469-475.

Sakahira, H., and Nagata, S. (2002). Co-translational folding of caspase-activated DNase with Hsp70, Hsp40, and inhibitor of caspase-activated DNase. J Biol Chem *277*, 3364-3370.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major

histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med *182*, 389-400.

Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., and Orrenius, S. (1999). Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells. Embo J *18*, 2040-2048.

San Jose, E., and Alarcon, B. (1999). Receptor engagement transiently diverts the T cell receptor heterodimer from a constitutive degradation pathway. J Biol Chem *274*, 33740-33746.

Schlossman, D. M., Schmid, S. L., Braell, W. A., and Rothman, J. E. (1984). An enzyme that removes clathrin coats: purification of an uncoating ATPase. J Cell Biol *99*, 723-733.

Schneider, P., Holler, N., Bodmer, J. L., Hahne, M., Frei, K., Fontana, A., and Tschopp, J. (1998). Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. J Exp Med *187*, 1205-1213.

Shapiro, S. D. (1998). Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol *10*, 602-608.

Sheu, B. C., Lien, H. C., Ho, H. N., Lin, H. H., Chow, S. N., Huang, S. C., and Hsu, S. M. (2003). Increased expression and activation of gelatinolytic matrix metalloproteinases is associated with the progression and recurrence of human cervical cancer. Cancer Res *63*, 6537-6542.

Shi, Y. (2004). Caspase activation: revisiting the induced proximity model. Cell *117*, 855-858.

Shima, I., Sasaguri, Y., Kusukawa, J., Nakano, R., Yamana, H., Fujita, H., Kakegawa, T., and Morimatsu, M. (1993). Production of matrix metalloproteinase 9 (92-kDa gelatinase) by human oesophageal squamous cell carcinoma in response to epidermal growth factor. Br J Cancer *67*, 721-727.

Shurin, G. V., Shurin, M. R., Bykovskaia, S., Shogan, J., Lotze, M. T., and Barksdale, E. M., Jr. (2001). Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function. Cancer Res *61*, 363-369.

Siegel, R. M., Chan, F. K., Chun, H. J., and Lenardo, M. J. (2000). The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. Nat Immunol *1*, 469-474.

Stanley, M. J., Liebersbach, B. F., Liu, W., Anhalt, D. J., and Sanderson, R. D. (1995). Heparan sulfate-mediated cell aggregation. Syndecans-1 and -4 mediate intercellular adhesion following their transfection into human B lymphoid cells. J Biol Chem *270*, 5077-5083.

Steller, H. (1995). Mechanisms and genes of cellular suicide. Science *267*, 1445-1449.

Stepp, S. E., Dufourcq-Lagelouse, R., Le Deist, F., Bhawan, S., Certain, S., Mathew, P. A., Henter, J. I., Bennett, M., Fischer, A., de Saint Basile, G., and Kumar, V. (1999). Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science *286*, 1957-1959.

Stommel, J. M., and Wahl, G. M. (2005). A new twist in the feedback loop: stress-activated MDM2 destabilization is required for p53 activation. Cell Cycle *4*, 411-417.

Strand, S., Hofmann, W. J., Grambihler, A., Hug, H., Volkmann, M., Otto, G., Wesch, H., Mariani, S. M., Hack, V., Stremmel, W., *et al.* (1998). Hepatic failure and liver cell damage in acute Wilson's disease involve CD95 (APO-1/Fas) mediated apoptosis. Nat Med *4*, 588-593.

Strand, S., Hofmann, W. J., Hug, H., Muller, M., Otto, G., Strand, D., Mariani, S. M., Stremmel, W., Krammer, P. H., and Galle, P. R. (1996). Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells--a mechanism of immune evasion? Nat Med *2*, 1361-1366.

Strand, S., Strand, D., Seufert, R., Mann, A., Lotz, J., Blessing, M., Lahn, M., Wunsch, A., Broering, D. C., Hahn, U., *et al.* (2004a). Placenta-derived CD95 ligand causes liver damage in hemolysis, elevated liver enzymes, and low platelet count syndrome. Gastroenterology *126*, 849-858.

Strand, S., Vollmer, P., van den Abeelen, L., Gottfried, D., Alla, V., Heid, H., Kuball, J., Theobald, M., Galle, P. R., and Strand, D. (2004b). Cleavage of CD95 by matrix metalloproteinase-7 induces apoptosis resistance in tumour cells. Oncogene *23*, 3732-3736.

Streit, M., Riccardi, L., Velasco, P., Brown, L. F., Hawighorst, T., Bornstein, P., and Detmar, M. (1999). Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis. Proc Natl Acad Sci U S A *96*, 14888-14893.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., *et al.* (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. Nature *397*, 441-446.

Sutton, V. R., Davis, J. E., Cancilla, M., Johnstone, R. W., Ruefli, A. A., Sedelies, K., Browne, K. A., and Trapani, J. A. (2000). Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. J Exp Med *192*, 1403-1414.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell *8*, 613-621.

Takayama, S., Reed, J. C., and Homma, S. (2003). Heat-shock proteins as regulators of apoptosis. Oncogene *22*, 9041-9047.

Takei, K., and Haucke, V. (2001). Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends Cell Biol *11*, 385-391.

Tanaka, M., Itai, T., Adachi, M., and Nagata, S. (1998). Downregulation of Fas ligand by shedding. Nat Med *4*, 31-36.

Theobald, M., Biggs, J., Dittmer, D., Levine, A. J., and Sherman, L. A. (1995). Targeting p53 as a general tumor antigen. Proc Natl Acad Sci U S A *92*, 11993-11997.

Thompson, C. B., and Allison, J. P. (1997). The emerging role of CTLA-4 as an immune attenuator. Immunity *7*, 445-450.

Townsend, S. E., and Allison, J. P. (1993). Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science *259*, 368-370.

Trapani, J. A., and Smyth, M. J. (2002). Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol *2*, 735-747.

Udayakumar, T. S., Stratton, M. S., Nagle, R. B., and Bowden, G. T. (2002). Fibroblast growth factor-1 induced promatrilysin expression through the activation of extracellular-regulated kinases and STAT3. Neoplasia *4*, 60-67.

Uria, J. A., Stahle-Backdahl, M., Seiki, M., Fueyo, A., and Lopez-Otin, C. (1997). Regulation of collagenase-3 expression in human breast carcinomas is mediated by stromal-epithelial cell interactions. Cancer Res *57*, 4882-4888.

van Dam, E. M., and Stoorvogel, W. (2002). Dynamin-dependent transferrin receptor recycling by endosome-derived clathrin-coated vesicles. Mol Biol Cell *13*, 169-182.

van der Bliek, A. M., Redelmeier, T. E., Damke, H., Tisdale, E. J., Meyerowitz, E. M., and Schmid, S. L. (1993). Mutations in human dynamin block an intermediate stage in coated vesicle formation. J Cell Biol *122*, 553-563.

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell *102*, 43-53.

Vicari, A. P., Caux, C., and Trinchieri, G. (2002). Tumour escape from immune surveillance through dendritic cell inactivation. Semin Cancer Biol *12*, 33-42.

Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996). Control of EGF receptor signaling by clathrin-mediated endocytosis. Science *274*, 2086-2089.

Vogelstein, B., Lane, D., and Levine, A. J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Vousden, K. H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nat Rev Cancer *2*, 594-604.

Warnock, D. E., Hinshaw, J. E., and Schmid, S. L. (1996). Dynamin self-assembly stimulates its GTPase activity. J Biol Chem *271*, 22310-22314.

Warnock, D. E., and Schmid, S. L. (1996). Dynamin GTPase, a force-generating molecular switch. Bioessays *18*, 885-893.

Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727-730.

Westermarck, J., and Kahari, V. M. (1999). Regulation of matrix metalloproteinase expression in tumor invasion. Faseb J *13*, 781-792.

Wilson, C. L., Heppner, K. J., Labosky, P. A., Hogan, B. L., and Matrisian, L. M. (1997). Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Natl Acad Sci U S A *94*, 1402-1407.

Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., Lopez-Boado, Y. S., Stratman, J. L., Hultgren, S. J., Matrisian, L. M., and Parks, W. C. (1999). Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science *286*, 113-117.

Witty, J. P., McDonnell, S., Newell, K. J., Cannon, P., Navre, M., Tressler, R. J., and Matrisian, L. M. (1994). Modulation of matrilysin levels in colon carcinoma cell lines affects tumorigenicity in vivo. Cancer Res *54*, 4805-4812.

Wojas, K., Tabarkiewicz, J., Jankiewicz, M., and Rolinski, J. (2004). Dendritic cells in peripheral blood of patients with breast and lung cancer--a pilot study. Folia Histochem Cytobiol *42*, 45-48.

Wolfl, M., Batten, W. Y., Posovszky, C., Bernhard, H., and Berthold, F. (2002). Gangliosides inhibit the development from monocytes to dendritic cells. Clin Exp Immunol *130*, 441-448.

Wroblewski, L. E., Noble, P. J., Pagliocca, A., Pritchard, D. M., Hart, C. A., Campbell, F., Dodson, A. R., Dockray, G. J., and Varro, A. (2003). Stimulation of MMP-7 (matrilysin) by Helicobacter pylori in human gastric epithelial cells: role in epithelial cell migration. J Cell Sci *116*, 3017-3026.

Wyllie, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature *284*, 555-556.

Yamamoto, H., Horiuchi, S., Adachi, Y., Taniguchi, H., Nosho, K., Min, Y., and Imai, K. (2004). Expression of ets-related transcriptional factor E1AF is associated with tumor progression and over-expression of matrilysin in human gastric cancer. Carcinogenesis *25*, 325-332.

Yamamoto, H., Itoh, F., Adachi, Y., Fukushima, H., Itoh, H., Sasaki, S., Hinoda, Y., and Imai, K. (1999). Messenger RNA expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human hepatocellular carcinoma. Jpn J Clin Oncol *29*, 58-62.

Yamamoto, H., Itoh, F., Adachi, Y., Sakamoto, H., Adachi, M., Hinoda, Y., and Imai, K. (1997). Relation of enhanced secretion of active matrix metalloproteinases with tumor spread in human hepatocellular carcinoma. Gastroenterology *112*, 1290-1296.

Yamashita, K., Azumano, I., Mai, M., and Okada, Y. (1998). Expression and tissue localization of matrix metalloproteinase 7 (matrilysin) in human gastric carcinomas. Implications for vessel invasion and metastasis. Int J Cancer *79*, 187-194.

Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature *400*, 886-891.

Yoshimoto, M., Itoh, F., Yamamoto, H., Hinoda, Y., Imai, K., and Yachi, A. (1993). Expression of MMP-7(PUMP-1) mRNA in human colorectal cancers. Int J Cancer *54*, 614-618.

Zeng, Z. S., Shu, W. P., Cohen, A. M., and Guillem, J. G. (2002). Matrix metalloproteinase-7 expression in colorectal cancer liver metastases: evidence for involvement of MMP-7 activation in human cancer metastases. Clin Cancer Res *8*, 144-148.

Zhan, Q., Jin, S., Ng, B., Plisket, J., Shangary, S., Rathi, A., Brown, K. D., and Baskaran, R. (2002). Caspase-3 mediated cleavage of BRCA1 during UV-induced apoptosis. Oncogene *21*, 5335-5345.

Zucker, S., Cao, J., and Chen, W. T. (2000). Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment. Oncogene *19*, 6642-6650.

7 Abbreviations

2-D	2-dimensional
APS	ammonium persulphate
AP	adaptor protein/activating protein
APMA	4-aminophenyl mercuric acetate
CLSM	confocal laser scanning microscope
CTLA-4	cytotoxic T-lymphocyte-associated protein-4
CD	cluster of differentiation
CRD	cysteine rich domain
CTLs	cytotoxic T lymphocytes
DCs	dendritic cells
DD	death domain
DED	death effector domain
DISC	death inducing signalling complex
DMEM	Dulbecco's minimal essential medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
GM-CSF	granulocyte macrophage-colony stimulating factor
ECM	extracellular matrix
FACS	fluorescence activated cell sorting
FADD	fas associated death domain
FBS	fetal bovine serum
Hsc70	heat shock cognate protein 70
HLE	human leukocyte elastase
HEK	human embryonic kidney
IEF	isoelectric focusing
IL	interleukin
IPG	immobilized pH gradient
MACS	magnetic associated cell sorting
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MMP	matrix metalloproteinase

NE	neutrophil elastase
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood monocytes
PBS	phosphate buffered saline
PFA	paraformaldehyde
PMNs	polymorphonuclear neutrophils
PVDF	polyvinyl diflouride
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction.
SIN	3-morphol inosydnonimine, hydrochloride
SDS	sodium dodecyl sulphate
TEMED	tetramethylethylenediamine
TIMPs	tissue inhibitors of metalloproteinases
TNF-α	tumour necrosis factor alpha
TRAIL	TNF receptor apoptosis induced ligand

Publications

Strand, S., Vollmer, P., van den Abeelen, L., Gottfried, D., **Alla, V**., Heid, H., Kuball, J., Theobald, M., Galle, P. R., and Strand, D. (2004). Cleavage of CD95 by matrix metalloproteinase-7 induces apoptosis resistance in tumour cells. *Oncogene 23, 3732-3736.*

Vijay Alla, Anubha Kashyap, Sebastian Gregor, Matthias Theobald, Hans Heid, Peter R. Galle, Dennis Strand and Susanne Strand. Human neutrophil elastase is an inactivator of MMP-7 and counteracts MMP-7 induced apoptosis resistance of tumor cells (submitted).

Anubha Kashyap, Tim Zimmerman, Anja Bosserhoff , Urs Hartman, **Vijay Alla**, Shenchu Jin, Frauke Bataille, Stefan Biesterfeld, Peter R. Galle, Susanne Strand and Dennis Strand. The human Lgl polarity gene, Hugl-2, is regulated by Snail and can suppress Snail-induced EMT (submitted**).**

Abstract and Poster Presentation

Vijay Alla, Daniela Lange, Ralf Wagner, Klaus Überla. Differential requirements for transfer of primate lentiviral vectors by HIV-1 and SIV particles. Annual Conference of the German Society of Virology, March 2003, Berlin, Germany.

Vijay Alla, A. Kashyap, H. Heid, M. Theobald, P. R. Galle, D. Strand, S. Strand. Neutrophil elastase is an inactivator of MMP-7 and counteracts MMP-7 induced apoptosis resistance of tumor cells. Cancer Immunotherapy (CIMT), 4th Annual Meeting May 2006 Mainz, Germany.

Curriculum Vitae

Alla Vijay Bhaskar Reddy
Scheffel strasse-9
55118 Mainz

Personal Details

Family name	Alla
First name	Vijay Bhaskar Reddy
Date of birth	28.08.1978
Gender	Male
Nationality	Indian

Education

09.2003 – 05.2007	PhD. Johannes Gutenberg University, Mainz, Germany.
08.1998 – 08.2000	M.Sc. Guru Ghasidas University, Bilaspur (C.G.) India.
07.1995 – 04.1998	B.Sc. Nagarjuna University, Guntur (A.P.) India.
07.1993 – 04.1995	Intermediate, Board of intermediate education (A.P.) India.
07.1988 – 04.1993	Secondary School, Board of secondary education (A.P.) India.
07.1981 – 04.1988	Primary School, Little citizens, Tenali (A.P.) India.

Work Experience

07.2002 – 08.2003	Research Co-worker, Ruhr University, Bochum, Germany.
01.2002 – 06.2002	Junior Research Associate, Virchow Biotech, Hyderabad, India.
09.2000 – 04.2001	Project Trainee, Dabur Pharmaceuticals, Delhi, India.

May 2007, Mainz