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Expression and Function of Protease-activated Receptors in Human Monocyte-derived Dendritic Cells

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Abbreviations

AB	acrylamide bisacrylamide
AM	acetoxymethylester
AP-1	activating protein–1 (transcription factor)
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CCL-17	chemokine (CC motif) ligand 17
CCL-22	chemokine (CC motif) ligand 22
CD	cluster of differentiation
CDNA	complementary DNA
CIP	calf intestinal phosphatase
cpm	counts per minute
CTL	cytotoxic T lymphocyte
DAG	1,2-diacylsnglycerol
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
DNTP	deoxynucleoside triphosphates
DTT	ditiothreitol
EB	elution buffer
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GB	gel buffer
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage colony stimulating factor
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate

HEK	human embryonic kidney (cell line)
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HLA	human leukocyte antigen
HUVEC	human umbilical vein endothelial cells
IL	interleukin
iDC	immature dendritic cells
IP_3	inositol trisphosphate
LB-medium	Luria-Bretani medium
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
mDC	mature dendritic cells
MHC	major histocompatibility complex
MIP-3α	macrophage inflammatory protein-3 α
MMLV-RT	moloney murine leukaemia virus reverse transcriptase
NF-κB	nuclear factor κ-B
NK	natural killer cells
Oligo (dT)	oligo-deoxythymidine
PAR	protease-activated receptor
PAR1-AP	protease-activated receptor 1-activating peptide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PLC	phospholipase C
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethanesulfonyl fluoride
RNase	ribonuclease
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SLB	sample lysis buffer
TBE	tris-borate-EDTA buffer

TBS	tris-buffered saline
TCR	T cell receptor
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF-α	tumor necrosis factor-α
Tricine	N-tris(hydroxymethyl)methylglycine
TSR	template suppression reagent
Tween 20	polyoxyethylene-20-sorbitan monolaurate

Abstract

Protease-activated receptors (PARs) are activated by proteolytic cleavage of their extracellular domain, unmasking a new N-terminus acting as a tethered ligand. PAR1 and PAR3 were investigated at the promoter level. The proinflammatory stimuli PMA and TNF-α, and the NF-κB heterodimer p52/RelB, activated both the PAR1 and PAR3 promoter. Using DNase I footprinting, binding of the AP-1 transcription factor to the PAR1 promoter was localized. This indicates that NF-κB does not activate the promoter directly, but interacts with AP-1, which then occupies its binding site on the promoter. The role of PARs in human monocyte-derived dendritic cells was further investigated. Three different inducers of dendritic cell maturation were used, LPS, TNF-α, and CD40L. LPS maturation elicited enhanced expression of PAR1 and PAR3, while the two other stimuli slightly up-regulated PAR1 and PAR3 expression. PAR4 expression remained undetectable in all three maturation types. Thrombin stimulation of LPS-matured dendritic cells resulted in a chemotactic response. The peptide agonists, PAR1-AP and PAR3-AP, produced thrombin-like effects on chemotaxis. PAR1 and PAR3 antibodies were effective in blocking the thrombin responses. Subsequently we investigated the thrombin signalling pathway in dendritic cells, and detected that thrombin activates the Rho-kinase (ROCK), which phosphorylates the myosin light chain (MLC) 2. Thrombin stimulation also induced an increase in actin polymerisation. Both of these events are important for the cell migration. Finally, the treatment of dendritic cells with the specific pharmacological inhibitor of ROCK (Y-27632), completely abolished the MLC phosphorylation, suggesting that thrombin stimulates the dendritic cell migration through Rho/ROCK pathway. The major finding of this work is that dendritic cells are activated by thrombin. Since both thrombin and dendritic cells are present in atherosclerotic plaques, targeting this mechanism in the endothelium could prevent plaque destabilisation and become a novel therapeutic strategy for the treatment of atherosclerosis.

1. Introduction

1.1. Dendritic cells

The immune system is a network of cells, tissues, and organs that work together to defend the body against attacks by "foreign" invaders. The cells of the immune system are produced in the bone marrow. They are normally present as circulating cells in the blood, in lymphoid organs, and as scattered cells virtually in all tissues except the central nervous system. Lymphocytes are the cells that specifically recognise and respond to foreign antigens. However, the recognition and activation phases of immune response depend on non-lymphoid cells, called accessory cells, which are not specific for different antigens[1]. Mononuclear phagocytes and dendritic cells function as accessory cells in the induction of immune response.

Dendritic cells are the major antigen-presenting cells[2]. They are located in the skin[3] (where they are called Langerhans cells), airways[4], stomach and intestines[5], blood[6]. Once dendritic cells are activated, they migrate to the lymphoid tissues where they interact with lymphocytes to initiate the immune response[7].

They were first discovered in the spleen of mice by Ralph Steinman in 1975[8], and since then, much work has been done to understand their role in the regulation of the immune response.

1.1.1. Origin and heterogeneity of dendritic cells

The dendritic cells progenitor is a bone marrow haematopoietic stem cell[9]. Two different lineages originate from this stem cell. One is the myeloid lineage – whose precursor is a $CD34^+$ myeloid progenitor[10], and the second is the lymphoid lineage – with $CD34^-$ lymphoid progenitor[11]. Both lineages give rise to functional dendritic cells *in vitro* and *in vivo*[12].

In most tissues, dendritic cells are present in an immature state. They are equipped to capture the protein antigens, process them to peptides, and to present these peptides together with a major histocompatibility complex

(MHC). Once the antigen is displayed on the surface, dendritic cells become less phagocytic, and up-regulate the expression of co-stimulatory molecules (CD80, CD86). After that, dendritic cells migrate into secondary lymphoid organs, where they activate naïve T cells.

Activated T cells, in turn, home to the peripheral sites where the antigen was detected, to start the effector phase of immune response. It is believed that after interaction with lymphocytes, dendritic cells die by apoptosis[9]. Elimination of dendritic cells is important for the downregulation of primary immune response. The most common mechanism is MHC class II-mediated apoptosis[13]. This process makes way for the next wave of dendritic cells migrating from the local tissues into the afferent lymph.

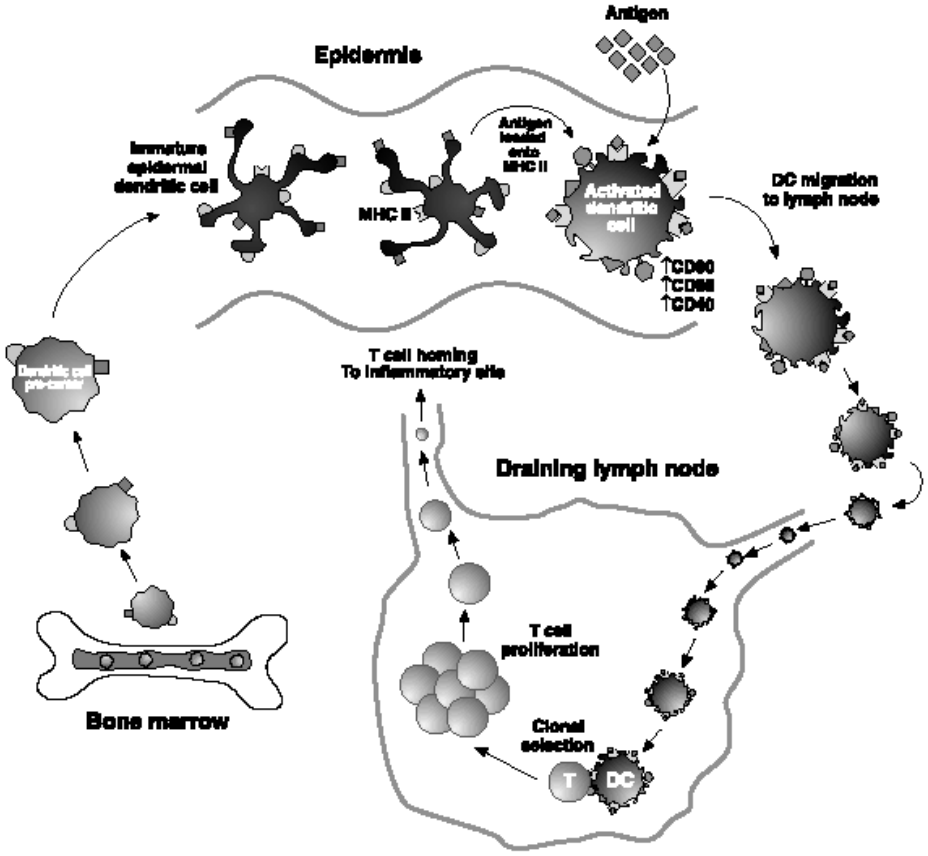


Figure 1: The life cycle of dendritic cells (Byrne S, Immunol Cell Biol 2002; 80:520-530)

Dendritic cells are not a single cell type, but a heterogeneous collection of cells that have arisen from distinct, bone marrow-derived, haematopoietic lineages.

This heterogeneity in humans is reflected at different levels[14]. Concerning the anatomical localisation, one can distinguish: skin epidermal LC (Langerhans

cells), dermal (interstitial) dendritic cells, splenic, thymic, liver, and blood dendritic cells.

The second level of heterogeneity comprises dendritic cells interaction with B cells, T cells or NK cells. They can regulate the B cell proliferation and the production of antibodies in the lymphoid follicles[15] or lead to differentiation of T and NK cells[16].

The third level of heterogeneity concerns the final outcome of immune response - induction of tolerance or immunity. Dendritic cells activation does not always include an immune response as a final result, it is also sometimes necessary to induce tolerance to self-antigens[17].

1.1.2. Maturation of dendritic cells

Dendritic cell maturation is a transition from immature antigen-capturing cells to mature antigen-presenting cells. This process is initiated in the periphery upon antigen encounter and completed during the dendritic cell-T cell interaction.

Numerous factors induce dendritic cell maturation, such as LPS, a bacterial product, signaling through Toll-like receptors[18], both bacterial DNA[19] and double-stranded RNA[20], endogenous mediators released locally in response to inflammatory stimuli, such as TNF-α, IFNγ and IL-1β, as well as prostaglandins[21]. Calcium ionophore A23187 activates dendritic cells to mature, through phospholipase C (PLC)[22]. CD40 ligation by T cell derived CD40L, induces transition from immature to mature dendritic cells[23].

Immature dendritic cells express low levels of MHC II on their surface (Figure 2) and very low level of T-cell co-stimulatory molecules such as CD40 (recognized by CD40L on T cells), CD80 and CD86 (co-stimulators for T cell activation); as the most important characteristic, they cannot activate naïve T cells.

Immature dendritic cells can capture antigens by endocytosis[24]. There are three different type of endocytosis: i) first, particles and microbes can be taken up by phagocytosis; ii) dendritic cells can form large pinocytic vesicles in which extracellular fluid and solutes are sampled. This involves the actin-

dependent formation of lamellipodia, sheet-like plasma membrane extensions supported by a web of actin filaments, a process called macropinocytosis[25]; iii) they express receptors that mediate adsorptive endocytosis, including lectin receptors like the macrophage mannose receptor and DEC-205[26]. Once a dendritic cell has captured an antigen, it becomes processed to peptides, and assembled in a peptide-MHC complex.

MHC class II is expressed on both immature and mature dendritic cells. Immature dendritic cells have a fast turnover (half-life less then an hour). Upon maturation, MHC II synthesis is down-regulated, and the turnover is slower. The parameter that best defines the maturational state of dendritic cells is the rate of turnover of MHC II-peptide complexes, rather that the level of the complex expression on the surface[27].

MHC class I is also upregulated with maturation, possibly reaching the surface in part together with the class II molecules[28]. The MHC I pathway enables the presentation of endogenous cellular antigens. In virus-infected cells, viral proteins expressed in the cytosol are subject to proteosomal lysis and the resulted peptides are loaded onto MHC I molecules.

Once dendritic cells have acquired and processed the foreign antigens, they migrate to the lymph nodes and the spleen. In lymphoid tissues, antigen-loaded dendritic cells present MHC I/II-peptide complexes and prime naïve and mature $CD4^+$ and $CD8^+$ T cells[14,29] or transfer antigens to naïve B cells to initiate a specific antibody response[30].

Expression of one or both of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) on the dendritic cells are essential for the effective activation of T lymphocytes[31]. These co-stimulatory molecules bind the CD28 molecules on T lymphocytes. If this fails to occur at the time of antigen recognition by the TCR, an alternative T lymphocyte function may result, namely induction of tolerance. Another CD80/CD86 ligand, CTLA-4, induced on activated T lymphocytes, may contribute to a negative regulatory signal[32].

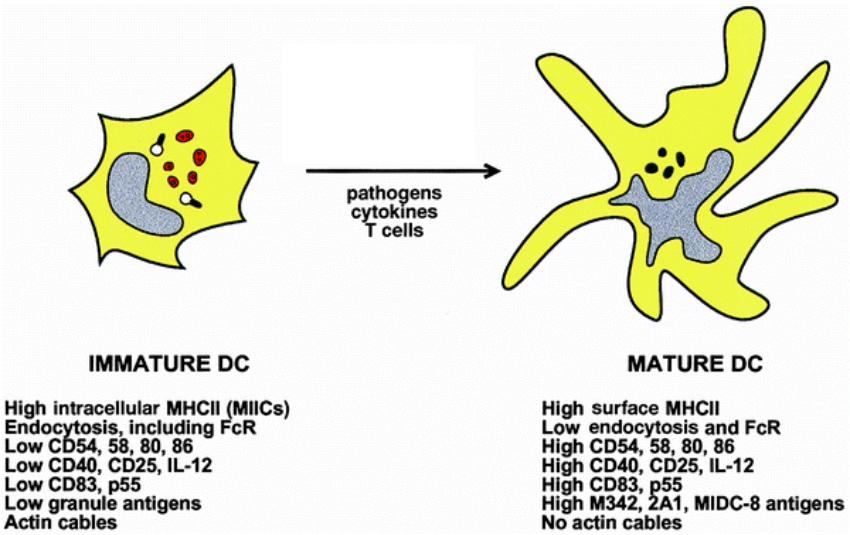


Figure 2: The phenotypic differences between immature and mature dendritic cells (Banchereau J, Nature 1998; 392:245-252)

Morphological changes during maturation involve translocation of F-actin in the areas below the cell membrane. Cofilin dephosphorylation was correlated with an increase in F-actin, suggesting a role of cofilin in rearrangements in the actin cytoskeleton[33]. The actin cytoskeleton is vital for the stability of the cell membrane and different process such as migration[34], phagocytosis[35] and the formation of the immunological synapse (the contact with T cells)[36].

At the molecular level, maturation of dendritic cells induces nuclear translocation of NF-κB, and as a result of this activation, MHC II and CD86 are upregulated[37]. LPS, used as a maturation stimulus, activates p38 stress-activated protein kinase (p38SARK), extracellular signal-regulated protein kinase (ERK), and the phosphoinositide 3-OH kinase (PI3 kinase)/Akt pathway[38]. The up-regulation of CD80, CD83 and CD86 by LPS is controlled by both the NF-κB and p38 pathway. Maturation of dendritic cells by MCM (monocyte conditioned medium) results in nuclear accumulation of p50, p52 and RelB, whereas the strongest upregulation was observed for c-Rel[39]. Mice deficient for RelB and p50/RelA double deficient mutants, had a severe impairment of dendritic cell function[40,41].

1.1.3. Dendritic cell immunobiology

Interaction of activated dendritic cells with T cells is not the only outcome of the immune response, as illustrated in figure 3. Upon returning to the lymph nodes, dendritic cells can interact with B cells, leading to differentiation of B cells in the antibody-producing plasma cells[42 9,43]. Upon exposure to CD40L, INF-γ, LPS of IFN-α, dendritic cells induce IgG isotype switching in B cells[44].

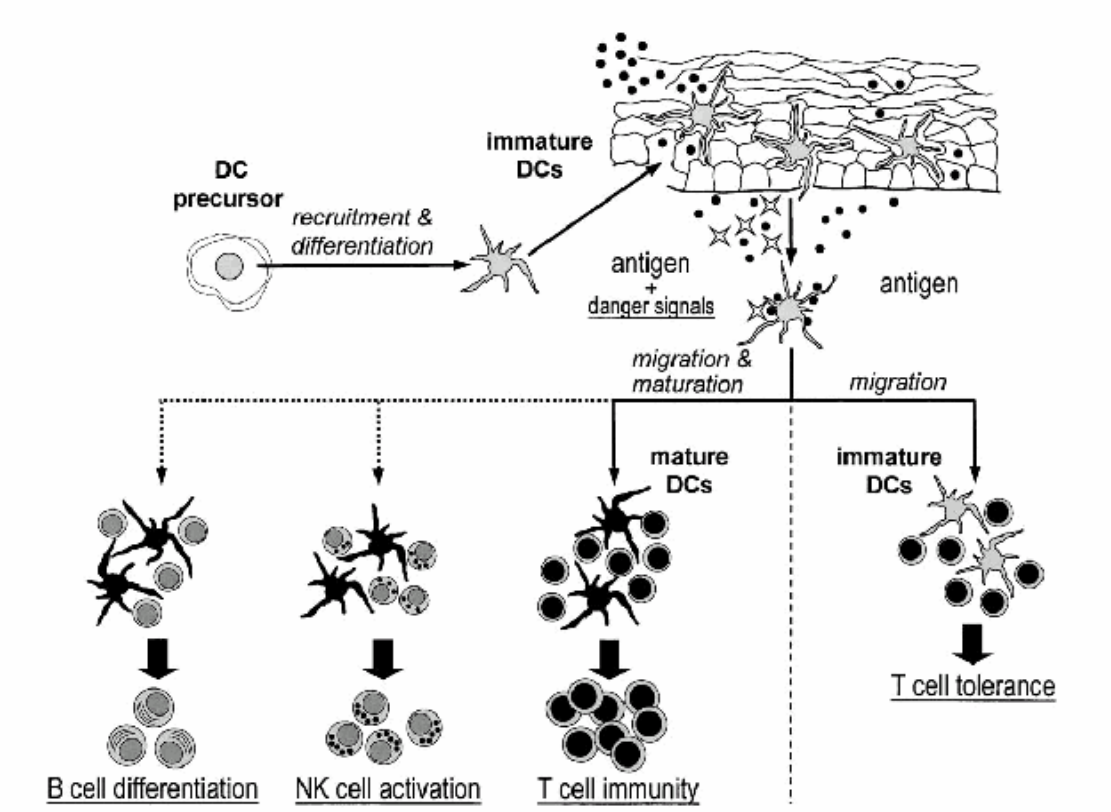


Figure 3: An integrated view of the dendritic cell immunobiology (Ardavin C, Immunity 2004; 20:17-23)

Natural killer cells are a subtype of lymphocytes, capable of lysing a variety of virus-infected and tumor cells. Activated dendritic cells produce IL-2 and prime NK cell cytolytic activity[45,46]. On the other hand, when IL-2 activated NK cells are co-cultivated with immature dendritic cell *in vitro*, they induce dendritic cells maturation[47]. NK cells can lead to dendritic cell apoptosis, in the peripheral tissues, as well as in the lymph nodes[45].

Activation of T cells, leads to differentiation of naïve T helper cells to Th1 or Th2 effector cells. Dendritic cells that secrete IL-12 and IFN-γ induce Th1 differentiation, and IL-4 secreting dendritic cells induce Th2 differentiation[48]

(figure 4). Both Th cell types cannot be differentiated at the same time, because IL-12 release inhibits Th2 development, and *vice versa*, IL-4 secretion inhibits Th1 differentiation.

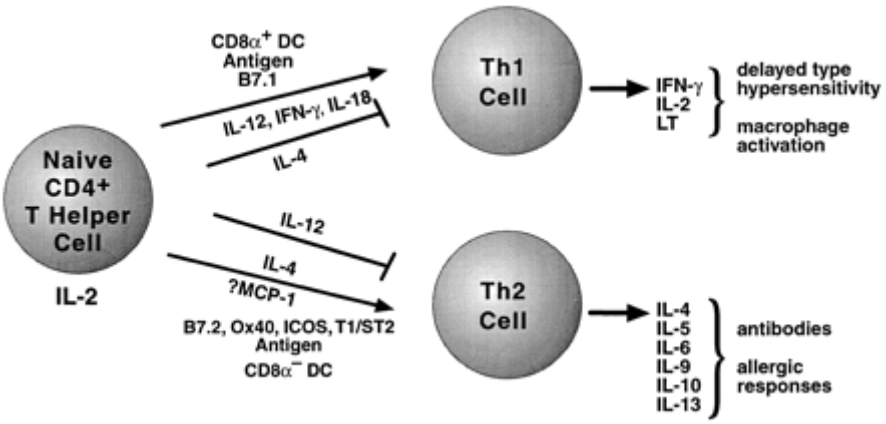


Figure 4: Signals that influence T helper cell differentiation (Glimcher LH et al, Genes Dev. 2000; 14:1693-1711)

Th1 cells secrete IL-2 that is important for the differentiation of cytotolytic T lymphocytes. Th1 cells also produce a macrophage activator IFN-γ, and lymphotoxin, important for lymphoid organogenesis[49]. Th2 secrete IL-4, a cytokine involved in class switching of B cells and the production of IgE antibodies, and IL-5 that attracts and activates eosinophils. IL-10, as a Th2 cytokine, downregulates IL-12 production and prevents entering of T cells into the Th1 pathway, whereas IL-13 promotes the synthesis of IgE and therefore is essential for antibody-mediated immunity[50].

1.1.4. Clinical implications of dendritic cells

Dendritic cells have an important applications in oncology, transplantation, autoimmune diseases and alergy[51].

Tumours express antigens that can be recognised by T cells and are potential targets for cancer immunotherapy. Isolated dendritic cells, loaded with tumor antigen *ex vivo*, and administered as a cellular vaccine, have been found to induce anti-tumor immunity in experimental animals.

In pilot clinical trials of dendritic cell vaccination of patients with non-Hodgkin's lymphoma, melanoma, multiple myeloma, prostate and renal cell carcinoma, induced an anti-tumor immune responses and tumor regressions with little or no side effects[52].

Additional trials with dendritic cell vaccination for a variety of human cancers are underway, and methods for targeting tumor antigens to dendritic cells *in vivo* are also being explored.

In transplantation, dendritic cells become immediately involved. Donor dendritic cells migrate to the draining lymph nodes of the recipient and initiate either graft rejection or graft tolerance. Infusions of dendritic cells lacking cell surface co-stimulatory molecules are able to prolong allograft survival[53].

In vitro manipulation of dendritic cells, by exposure to IL-10 and transforming growth factor-β (TGF-β), can induce tolerogenic properties of these cells and lead to anergy[54]. Corticosteroids can *in vitro* initiate an immunosuppression by inhibiting dendritic cell maturation and therefore strongly reduce the dendritic cell ability to induce a T cell response[55].

Autoimmune diseases are erroneous immune reactions to self-antigen. Altered function of dendritic cells is known to play a major role in autoimmune diseases like systemic lupus erythematosus (SLE). Therefore, preventing dendritic cell activation by IFN-α may be useful for treatment of SLE[56]. Another attractive approach to treat autoimmune disorders such as diabetes, is genetic engineering of dendritic cells that express immunosuppressive molecules, and thereby to reduce editing of self-reactive T cells [57].

Allergy is a pathological reaction to an outside allergen. Here, dendritic cells are responsible for the induction of Th2 sensitization to inhaled allergens, leading to allergic reaction. Interfering with dendritic cells function, in a way that they induce tolerance or Th1 response to the same antigen, could be used as a therapy[58].

1.1.5. Dendritic cells in atherosclerosis

Small numbers of dendritic cells are localized in the intima of apparently normal, non-diseased arteries[59]. In atherosclerotic arteries, the number of

dendritic cells increases, suggesting that some of them do not migrate to the lymph nodes, but stay in the arterial tissue, and activate T cells directly within the intima[60]. Here, in atherosclerotic lesions, dendritic cells might contribute to plaque destabilization through activation of T cells[61].

Active thrombin appears to be present in atherosclerotic plaque, where it causes fibrin deposition and acts as a mitogen for arterial smooth muscle cells[62]. *In situ* analysis of human atherosclerotic vessels showed that thrombin generation takes place in lesions[63].

We investigated the effect of the serine protease-thrombin on the development and function of dendritic cells.

1.2. Protease-activated receptors (PARs)

1.2.1. The roles of thrombin

Serine protease thrombin is a main effector of the coagulation cascade. The generation of thrombin from its precursor, prothrombin occurs in the case of vessel damage. The cells that are normally not in the contact with blood, express tissue factor. When tissue factor comes into direct contact with blood, it activates factor X. Activated factor X becomes Xa, and together with factor Va, converts prothrombin to thrombin. Thrombin cleaves fibrinogen to fibrin, which has the ability to create polymers, leading to the formation of the fibrin clot and to blood coagulation[64].

Thrombin is synthesized exclusively in the liver as the inactive zymogen, prothrombin. Conversion of prothrombin to thrombin involves the cleavage of peptide bonds on the C-terminal. Thus, thrombin is about half the size of prothrombin and comprises light and heavy chain joined by a disulphide bond[65].

Besides its role in blood coagulation, thrombin exhibits important “cell-activating” functions. It is the most potent stimulator of platelet aggregation[66] and induces platelet adhesion to endothelial cells[67].

Thrombin causes endothelial cells to express the leukocyte adhesion molecule P-selectin on their surfaces, and to release growth factors and cytokines[68,69]. Endothelial cells also change shape and show an increased

permeability in response to thrombin[70]. Thrombin stimulates endothelial cells to produce prostacyclin, platelet-activating factor, induces neutrophil adherence to the vessel wall[71].

Chemotactic activity of thrombin on monocytes, T lymphocytes[72] and eosinophils[73] has also been shown.

The cellular activity of thrombin is mediated, in a part, through the protease-activated receptors (PARs). Protease-activated receptors belong to a family of G protein coupled receptors. The first thrombin receptor, PAR1, was cloned in 1991, from a human megakaryoblastic cell line[74]. Since then, other members of the PAR family have been cloned, PAR2, PAR3 and PAR4. PAR1, PAR3 and PAR4 are considered as thrombin receptors, while PAR2 can be activated by trypsin, mast cell tryptase or factor Xa, but not by thrombin[75]. The genes encoding all four family members have a similar structure with a single intron interrupting the sequence encoding the receptor N-terminus. In humans, PAR1, PAR2 and PAR3 are tightly clustered at chromosome 5q13[76], whereas PAR4 is located separately at 19p12[77]. The receptors have a high sequence homology: PAR3 has 27% of amino acid homology with PAR1 and 28% homology with PAR2[78].

1.2.2. Protease-activated receptor 1

The activation mechanism of the PARs is very distinct from that of the other G-protein coupled-receptors. Thrombin cleaves the NH_2-terminal peptide of the receptor molecule at the specific cleavage site (figure 5), releasing a short receptor fragment, thereby unmasking the cryptic, receptor-activating sequence. On PAR1, thrombin cleaves the first extracellular domain between Arg^{41} and Ser^{42} (cleavage site LDPR ^{41}/S^{42}FLLRN)[79]. The exposed new NH_2-terminus, which usually comprises six or more amino acids, serves as a “tethered” ligand, and binds to the second extracellular loop of the receptor, resulting in the receptor autoactivation. The second step in receptor activation is a conformational change, leading to interaction with a heterotrimeric G protein in the plasma membrane, which transduces the signal. Mutation of the amino acid serine at the position 42 to proline, abolishes cleavage and activation by thrombin[80].

Synthetic peptides that correspond to the tethered ligand can activate PAR1 without cleavage of the receptor. Analogs of the tethered ligand are called PAR-activating peptides (PAR-AP), and they are of great interest since they are able to selectively activate the receptors. PAR-AP may be used to probe the function of PARs without the use of proteases, which cleave other proteins and thus exert effects by several mechanisms. The hexapeptide SFLLRN mimicked the actions of thrombin, and activated PAR1 in U937 monocytic cells[81]. Unfortunately, peptides corresponding to the tethered ligand domain are relatively weak agonists compared to proteases. The differences in potency are probably due to the inefficient presentation of these soluble peptides to the binding domains of the receptor, compared with the tethered ligand, and they are easily inactivated by proteolysis[80].

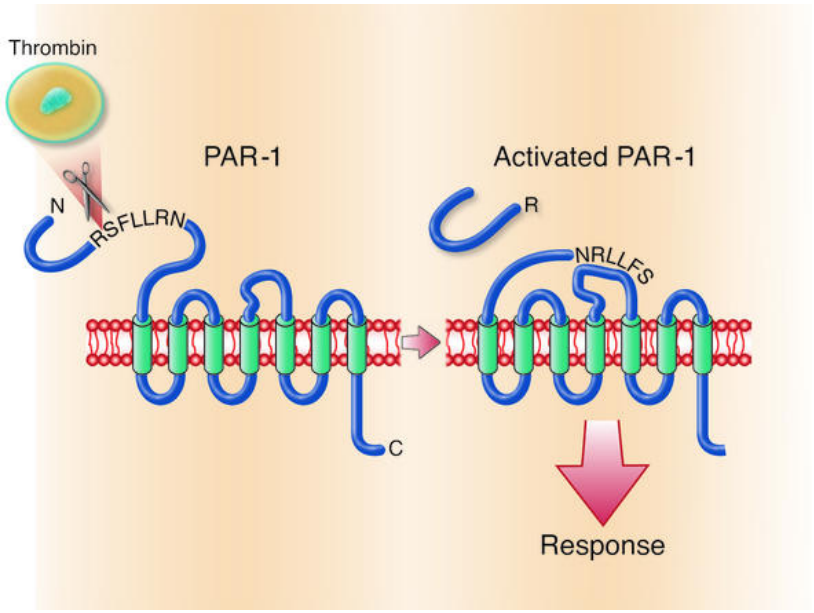


Figure 5: The mechanism of PAR1 activation (Coughlin SR, J. Clin. Invest. 2003; 111:25–27).

The mechanism of PAR1 activation is irreversible. Once the receptor is cleaved, it cannot be activated again. The tethered ligand cannot diffuse away from the receptor, so the receptor is internalised and degraded (figure 6). PAR1 is rapidly phosphorylated and uncoupled from the G protein after activation[82]. However, instead of efficiently recycling after internalisation, activated PAR1 sorts predominantly to lysosomes.

As PAR1 is cleaved and targeted to lysosomes for degradation, it is necessary to re-synthesize and replenish the cell surface with the functional

receptor. Termination of PAR1 signalling occurs at several levels: phosphorylation, internalisation, lysosome sorting of activated receptors, and degradation. An intracellular pool of thrombin receptors refreshes the cell surface with new receptors, maintaining thrombin responsiveness. This intracellular reservoir of intact receptors was does not exist in human platelets[83], being an evolutionary adaptation to their short circulatory half-life. On the other hand, when the receptor is activated by PAR-AP, it is not cleaved, and could be retained on the surface, or internalised and recycled.

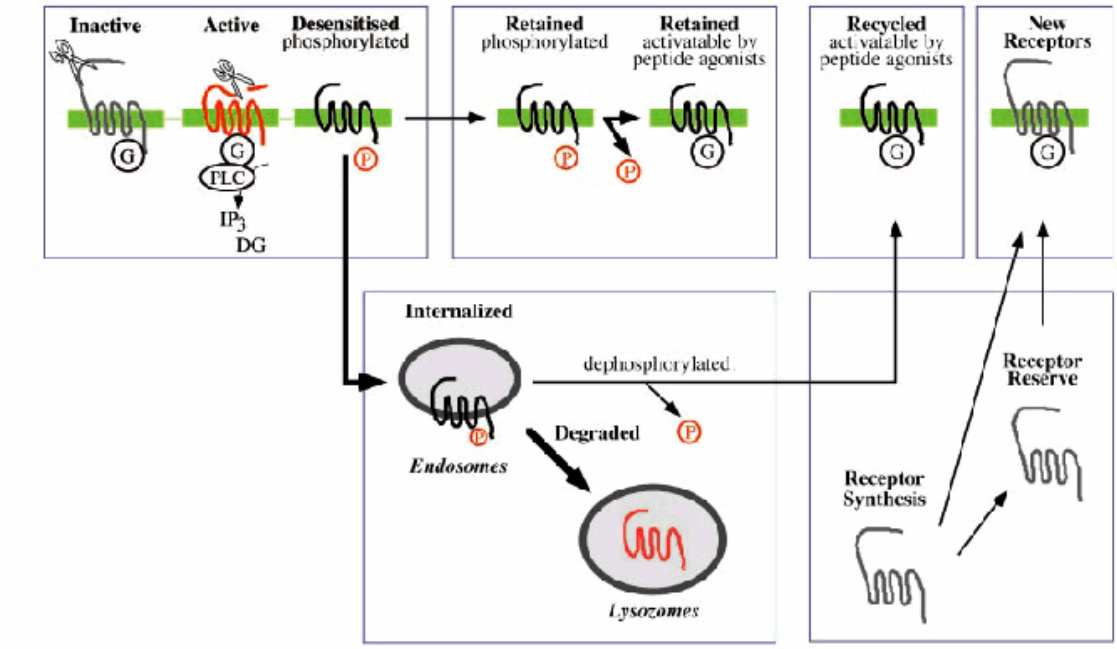


Figure 6: Trafficking and replacement of PAR1 (O'Brien et al. Oncogene 2001; 20:1570-1581)

PAR1 is expressed in almost all tissues: endothelium, intestine, kidneys, in the central nervous system (on astrocytes and neurones), skeletal muscles, bone (osteoclasts), in the cardiovascular system, blood (platelets), epidermis, and the immune system (monocytes, mast cells, T cells)[84]. Colognato et al. investigated PAR1 expression on dendritic cells. A very low level of this receptor was detected on the surface of immature dendritic cells by flow cytometry.

1.2.3. Protease-activated receptor 2

PAR2 was cloned in 1994[85] as a G protein-coupled receptor that responds to trypsin. Analysis of the PAR2 N-terminal amino acid sequence revealed a trypsin cleavage site between Arg^{42} and Asp^{43}.
PAR2 can be activated by trypsin, mast cell tryptase[85,86], and factor Xa. Since trypsin is the most abundant gastrointestinal protease, PAR2 is expressed in numerous cell types within the gastrointestinal tract[84], but also in smooth muscles[87,88] and in the epidermis[89]. A peptide agonist that activate preferentially PAR2 is SLIGRL[90]. Blackahrt et al. demonstrated that the PAR1-activating peptide (sequence SFLLRN), is an agonist for PAR2 as well[91].
Dendritic cells express PAR2, as reported by Fields at al[92]. The serine protease trypsin can activate PAR2 on dendritic cells, leading to up-regulation of co-stimulatory molecules and to the initiation of the immune response.

1.2.3. Protease-activated receptor 3

The finding that the platelets derived from mice, in which the PAR1 gene was deleted by homologous recombination, responded to thrombin, but not to PAR1-activating peptide, led to the conclusion that a second thrombin receptor exists[93]. Molecular cloning identified a clone with ~28% amino acid sequence similarity to human PAR1 and PAR2, which was named in analogy to the previously identified receptors, PAR3.
The molecular mechanism of PAR3 activation is similar to that of PAR1. Cleavage by thrombin exposes a new NH_2-terminus that interacts with the receptor as a tethered ligand. Thrombin cleaves PAR3 between Lys^{38} and Thr^{39}, and mutation of the cleavage site prevents activation of the receptor. The corresponding peptide agonist has a sequence: TFRGAP[94].
PAR3 is expressed in different tissues: heart, small intestine, bone marrow, airway smooth muscle and vascular endothelium[95-97].
In spite of the small amounts of PAR3 mRNA detected in dendritic cells, there was no protein expression on the surface of immature dendritic cells[98].

1.2.4. Protease-activated receptor 4

The cloning of the fourth protease-activated receptor was carried out simultaneously by two laboratories[99,100]. The human receptor protein was found to be 385 amino acids in length, and possessed both a signal peptide and a putative serine protease cleavage site at Arg^{47}/Gly^{48} in the N-terminal sequence.

PAR4 can be activated by thrombin and trypsin. It is less sensitive to thrombin than PAR1, with EC_{50} for thrombin approximately 50 fold higher than the corresponding value for PAR1[100]. The peptide agonist of PAR4 has the following sequence: GYPGQV. The tissue distribution of PAR4 was found to be distinct from the other PAR family members, with the highest levels of receptor mRNA detected in lung, pancreas, thyroid, testis, and small intestine. PAR4 mRNA was detected in platelets and vascular smooth muscle cells[100,101].

So far, there is no evidence of PAR4 expression on dendritic cells.

1.2.5. Signal transduction of protease-activated receptors

As mentioned before, activation of PARs leads to interaction with one or more guanine nucleotide binding proteins (G proteins) acting as signal transducers and amplifiers. G proteins modulate the activity of one or more effector systems. The inactive form of the G protein consists of α, β, and γ subunits with a molecule of GDP bound to the α subunit. When a ligand-bound receptor interacts with the G protein, it catalyses the exchange of GDP for GTP and activates the G protein. The G protein is then released from the receptor and it dissociates into separate β-γ and α-GTP (active) subunits. Active G proteins are returned to their inactive state upon the hydrolysis of GTP to GDP by the intrinsic GTPase activity of the α-subunit and the α-GDP and β-γ subunits can recombine.

PAR1 is able to couple effectively to following Gα subunits: $G\alpha_{i}$, $G\alpha_{q}$, and $G\alpha_{12/13}$[102].

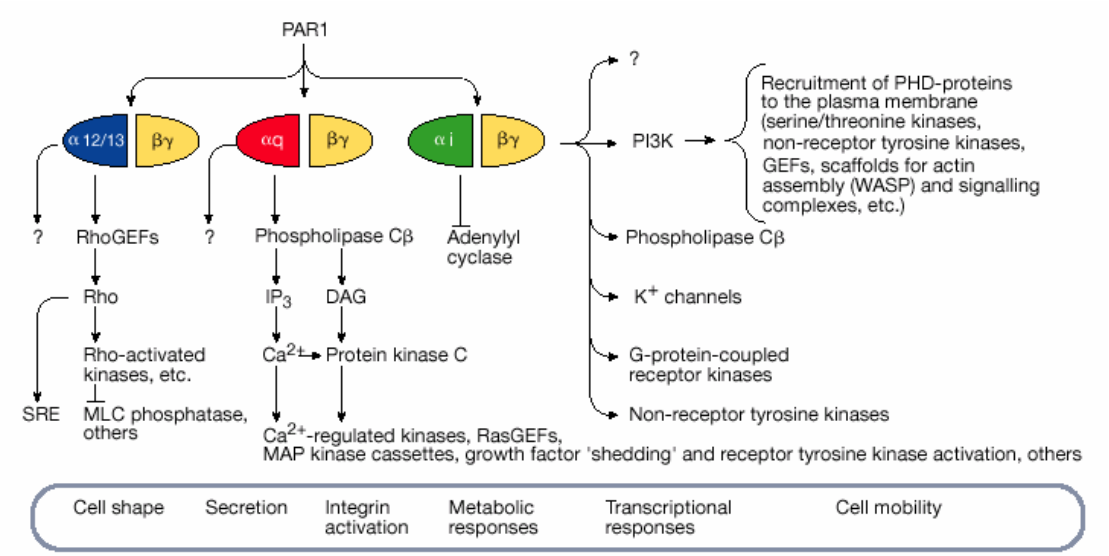


Figure 7: Signalling of the thrombin receptor (Coughlin SR, Nature, 2000; 407:258-264)

The α-subunit of G_{12} and G_{13} binds RhoGEFs (guanine-nucleotide exchange factors, which activate small G proteins such as Rho), providing the pathway to Rho-dependent cytoskeletal response, involved in shape changes in platelets and permeability and migration in endothelial cells.

$G\alpha_q$ activates phospholipase Cβ (PLCβ). Phospholipase Cβ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) and generates 1,4,5-inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 leads to release of calcium to the cytosol; and DAG activates protein kinase C. This provides a pathway to calcium-regulated kinases and phosphatases, guanine-nucleotide exchange factors (GEFs), mitogen-activated protein (MAP) kinases, and other proteins that mediate cellular responses ranging from granule secretion, integrin activation and aggregation in platelets, to transcriptional responses in endothelial cells.

$G\alpha_i$ inhibits the adenylate cyclase, resulting in diminished levels of cAMP and enhanced cellular responsiveness. Gβγ subunits can activate phosphoinositide 3-kinase (PI(3)K) and other lipid-modifying enzymes, protein kinases and ion channels. PI(3)K modifies the inner leaflet of the plasma membrane to provide the attachment sites for a host of signalling proteins.

1.2.6. PAR1 and PAR3 promoters

A promoter is a DNA sequence located upstream of the transcription start site. The promoter contains binding sites for transcription factors, whose binding results in either facilitating or inhibiting of transcription[103]. Transcriptional factors contain two essential functional regions: a DNA-binding domain and an activator domain. The DNA binding domain consists of amino acids that recognise specific DNA sequences. Transcription factors are typically classified according to the structure of their DNA-binding domain: zinc finger proteins, helix-turn-helix proteins, leucine zipper proteins, helix-loop-helix proteins, and high mobility group proteins. The activator domains of transcription factors interact with the components of the transcriptional machinery (RNA polymerase) and with other regulatory proteins, thus affecting the efficiency of DNA binding. Two transcription factor families: NF-κB and AP-1, have been previously shown to be activated as a result of thrombin signaling[104,105].

NF-κB proteins are a family of transcription factors consisting of homo and heterodimers. They possess a highly conserved DNA-binding/dimerisation domain called the Rel homology region[106]. The family includes five members: p50, p52, p65, c-Rel and RelB[107]. In most cells, NF-κB is present in the cytoplasm in an inactive form bound to inhibitor protein (IκB). IκB masks the NF-κB's nuclear binding domain via a non-covalent binding association[108]. NF-κB activation starts by phosphorylation of the IκB kinase inhibitory subunit. The inhibitory subunit is then targeted by ubiquitin for subsequent degradation by the proteasome. Once this occurs, NF-κB is released and is able to translocate into the nucleus of the cell, where it binds to specific DNA sequences triggering transcription of genes whose products are involved in NF-κB mediated response.

Activating protein-1 (AP-1) transcription factors consist of homodimers and heterodimers that belong to the Jun (c-Jun, v-Jun, JunB, JunD), Fos (c-Fos, v-Fos, Fos B, Fra1, Fra2) and the activating transcription factor (ATF2, ATF3/LRF1, B-ATF) subfamilies[109]. The exact subunit composition is influenced by the nature of the extracellular stimulus. Upon stimulation,

regulation of AP-1 is activated on two levels: transcriptional (activating transcription of these genes) and post-translational (through the phosphorylation of existing Jun and Fos proteins at specific serine and threonine sites)[110]. Jun proteins have the ability to form dimers (Jun-Jun) that are stable and capable of recognising and binding to the AP-1 DNA consensus sequences[111]. Fos proteins, on the other hand, cannot form stable homodimers. Instead, they mediate gene expression by forming heterodimers with various Jun proteins[112]. These heterodimers are more stable that the Jun-Jun heterodimers and possess higher DNA binding activity. Thus, the composition of the AP-1 subunits influences and modulates the binding activity of AP-1 complex. For example, while heterodimerisation of c-Jun with c-Fos is more stable and transcriptionally active, formation of c-Jun-JunB heterodimer decreases DNA binding activity[113].

We investigated the PAR1 and PAR3 promoter activity. The regulatory region of the PAR1 gene has been cloned, and the sequence analysis indicates that the PAR1 has a GC rich sequence, but no conventional TATA box[114] (figure 8). The TATA box represents a core promoter, that is, the minimal DNA sequence that is sufficient to direct the initiation of transcription by the RNA polymerase II. Promoters that are TATA-less, do not have a single, but multiple transcription start sites[115].

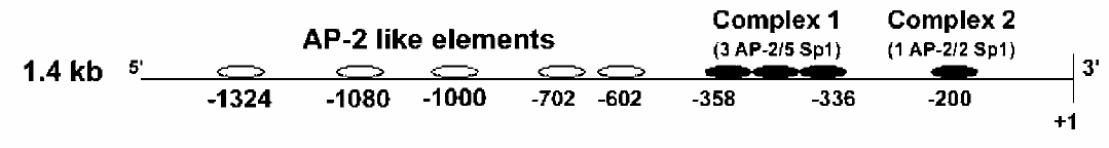


Figure 8: Putative transcriptional regulatory sequences of the PAR1 gene (Tellez C, Oncogene 2003; 22:3130-3137).

The presence of the following transcription factor binding elements in PAR1 promoter are described: Sp1 (stimulating protein) family[116], AP-2 (activator protein 2)[114], ARE (androgen response element)[117].

Sp1 transcription factor is binding to GC rich sequences (GC boxes), found in large numbers of mammalian promoters, suggesting that Sp1 is a transcriptional factor for housekeeping genes[118]. PAR1 promoter contains more than one Sp1 binding site, suggesting that this transcription factor can

interact with other factors to modulate the transcription in a different ways[119]. Sp1 is essential for the transcription regulation in TATA-less promoters[120]. AP-2 putative elements can be activated as a result of phorbol ester signalling[121]. In human melanoma cells, a decrease in AP-2 level is correlated with the overexpression of PAR1, suggesting a tumor suppressor role of these transcription factors[122].

Stimulation of human prostate cancer cells (LNCaP) with androgens leads to the activation of androgen response element (ARE), which binds to the PAR1 promoter and increases endogenous mRNA levels. This suggests that the PAR1 gene is regulated transcriptionally by androgens[123].

Sequence analysis of the PAR3 promoter region, from HUVEC cells, demonstrates that this promoter is also TATA-less, with the potential AP-1 and GATA transcription factors and octameric sequences[124].

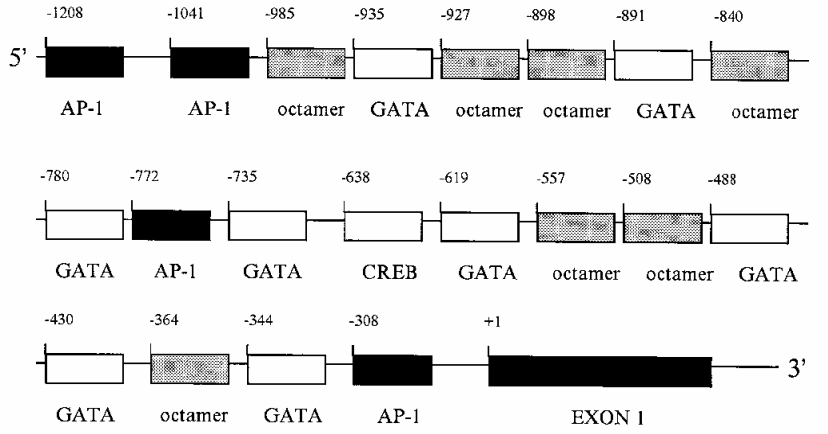


Figure 9: Putative transcriptional regulatory sequences of the PAR3 gene (Schmidt VA, J. Biol. Chem. 1998; 273:15061-15068).

GATA transcription factor has a major role in haematopoiesis, cell proliferation, organ morphogenesis[125]. It is expressed in megakaryocytes, mast cells, eosinophils[126]. Octamer sequences are recognised by Oct transcription factors, important for cell differentiation and development[127]. However, GC rich regions (Sp1 binding sites) found in PAR1 are absent in PAR3 promoter. This suggests that the PAR3 gene regulation has more restricted nuclear binding elements.

1.3. The aim of the work

The effect of proinflammatory agents on PAR1 and PAR3 promoter regulation was investigated by transfecting HEK 293 cells with PAR1 and PAR3 promoter constructs.

Using immature and mature dendritic cells as a model, we analysed the expression of PAR1 and PAR3 at the mRNA and protein level.

The main intent of this work was to determine whether thrombin can activate dendritic cells and to elucidate the molecular mechanism of thrombin-induced effects on dendritic cells.

2. Materials and Methods

2.1. Materials

2.1.1. Cell lines

HEK 293 cell line was purchased from the American Tissue Culture Collection (ATCC)

2.1.2. Bacteria

Chemically competent E. coli DH5-α, (Invitrogen, Karlsruhe, Germany)

2.1.3. Antibodies

Actin (Chemicon International, Temecula, NY, USA)
CD11c (DAKO Cytomation, Hamburg, Germany)
CD14 (Dianova, Hamburg, Germany)
CD1a (Pharmingen, Heildelberg, Germany)
CD40 (Pharmingen, Heildelberg, Germany)
CD83 (BD Biosciences, Heidelberg, Germany)
CD86 (BD Biosciences, Heidelberg, Germany)
HLA-DR (Pharmingen, Heildelberg, Germany)
Horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Sigma-Aldrich, Steinheim, Germany)
Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
IgG mouse (Sigma-Aldrich, Steinheim, Germany)
IgG rabbit (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
PAR3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
Phycoerythrin (PE)-conjugated donkey anti-mouse and IgG_2 $F(ab)_2$ (Dianova, Hamburg, Germany)
Phycoerythrin (PE)-conjugated donkey anti-rabbit IgG_2 $F(ab)_2$ (Dianova Hamburg, Germany)
p-MLC2 (Cell signalling, Beverly, MA, USA)
p-MYPT (Upstate, Lake Placid, NY, USA)

RelB (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
ROCK-2 (BD Biosciences, Heidelberg, Germany)
ROCK-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
WEDE 15 (Immunotech, Marseille, France)

2.1.4. Chemicals and kits
[^{3}H]-thymidine (Amersham Biosciences, Freiburg, Germany)
A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
Advantage GC Genomic PCR Kit (Clontech, Heidelberg, Germany)
Agarose (Invitrogen, Karlsruhe, Germany)
Ampicillin (Invitrogen, Karlsruhe, Germany)
Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany)
BSA endotoxin-free (Sigma-Aldrich, Steinheim, Germany)
CD40L (PJK Industrievertretungen, Kleinblittersdorf, Germany)
DNase I (Worthington Biochemical Corporation, St Katharinen, Germany)
dNTPs (Invitrogen, Karlsruhe, Germany)
Dulbecco's modified Eagle's medium (DMEM) (Gibco, Karlsruhe, Germany)
EDTA (Titriplex III) (Merck KG, Darmstadt, Germany)
FCS - foetal calf serum (Seromed, Berlin, Germany)
Film Developer (Kodak Industries, Chalon, France)
Film Fixative (Kodak Industries, Chalon, France)
Fura-2AM (Molecular Probes, Karlsruhe, Germany)
GM-CSF (Leukine) (Berlex Laboratories, Richmond, USA)
Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany)
Human α-thrombin (Enzyme Research Laboratories, Swansea, England)
IL-4 (Pierce, Rockford, IL, USA)
LPS from *E. coli* K-235 (Sigma-Aldrich, Steinheim, Germany)
Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA)
Luciferin (PJK Industrievertretungen, Kleinblittersdorf, Germany)
Micro BSA Assay Kit (Pierce, Rockford, IL, USA)
M-MLV (Moloney-Murine leukaemia virus) reverse transcriptase (Invitrogen, Karlsruhe, Germany)

mRNA Direct Kit (Dynal, Oslo, Norway)

MYPT1 – recombinant protein expressed in *E. coli*, a substrate in a ROCK kinase assay (Upstate, Lake Placid, USA)

Naïve $CD4^+$ T cell isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany)

Oligo $(dT)_{12\text{-}18}$, (Amersham Biosciences, Freiburg, Germany)

PAR1-AP (TFLLRNPNDK) (Interactiva, Ulm, Germany)

PAR3-AP (TFRGAP) (Interactiva, Ulm, Germany)

PAR4-AP (GYPGQV) (Bachem, Heidelberg, Germany)

PBS (w/o Ca^{2+}, Mg^{2+}) (Gibco, Karlsruhe, Germany)

Penicillin/Streptomycin (Invitrogen, Karlsruhe, Germany)

Percoll (Sigma-Aldrich, Steinheim, Germany)

pGL3 enhancer luciferase reporter (Promega, Manheim, Germany)

Plasmid Purification Kit (Qiagen, Hilden, Germany)

Poly (I:C) (Amersham Pharmacia, Piscataway, NJ, USA)

Protease inhibitor cocktail Set III (Calbiochem, La Jolla, CA, USA)

QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany)

QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)

Rainbow marker RPN800 (Amersham Biosciences, Freiburg, Germany)

Rapid ligation Kit (Roche Diagnostics, Mannheim, Germany)

RPMI 1640 (Gibco, Karlsruhe, Germany)

Scintilation liquid Ultima Gold XR (Packard, Groningen, Netherlands)

Skim milk powder, blotting grade (Carl Roth, Karlsruhe, Germany)

Super Script II RT (Invitrogen, Karlsruhe, Germany)

Super Signal West Pico Chemiluminiscent Substrate (Pierce, Rockford, IL, USA)

SuperFect Transfection Reagent (Qiagen, Hilden, Germany)

Taq DNA Polymerase Recombinant (Invitrogen, Karlsruhe, Germany)

Texas-Red Phalloidin (Molecular Probes, Karlsruhe, Germany)

Thrombin (Enzyme Research Laboratories, Swansea, UK)

TNF-α (Peprotech, London, UK)

Trizol Reagent (Invitrogen, Karlsruhe, Germany)

Tween 20 (Merck KG, Darmstadt, Germany)

2.1.5. Buffers and solutions

a) Western blot

3x GB (Gel Buffer)

3 M Tris-HCl (pH 8.45)

0.3 %SDS

Cathode buffer

100 mM Tris (pH 8.25)

100 mM Tricine

0.1% SDS

Anode buffer

100 mM Tris-HCl (pH 8.9)

Acrylamide/Bisacrylamide

48% acrylamide

1.5% bisacrylamide

3x SLB (sample lysis buffer)

150 mM Tris-HCl (pH 7.0)

12% SDS

6% β-mercaptoethanol

30% glycerol

0.05% Coomassie Brilliant Blue

TBS (Tris buffered saline)

0.1 M NaCl

0.01 M Tris-HCl (pH 7.6)

TBST

TBS

0.1% Tween 20

Blotting buffer

250 mM glycine

25 mM Tris (pH 8.3)

10% methanol

Stripping solution

30 mM Tris-HCl (pH 7.6)

1% SDS

100mM β-mercaptoethanol

b) Luciferase assay

Luciferase assay buffer (4x)

15 mM NaH_2PO_4/Na_2HPO_4 (pH 7.8)

25 mM glycylglycine

4 mM EGTA

2 mM ATP

1 mM DTT

2 mM $MgCl_2$

c) Buffers for isolation of mRNA with Dynabeads mRNA Direct Kit (Dynal)

Lysis/Binding buffer

100 mM Tris- HCl (pH 7.5)

500 mM LiCl

10 mM EDTA

1% LiDS

5 mM DTT

Washing buffer A

10 mM Tris-HCl (pH 7.5)

0.15 mM LiCl

1 mM EDTA

0.1% LiDS

Washing buffer B

10 mM Tis- HCl (pH 7.5)

0.15 mM LiCl

1 mM EDTA

d) Buffers for Reverse Transcriptase reaction and PCR

5x First-Strand Buffer

250 mM Tris-HCl (pH 8.3)

375 mM KCl

15 mM $MgCl_2$

0.1 M DTT

Taq polymerase 10x PCR buffer

200 mM Tris-HCl (pH 8.4)

500 mM KCl

50 mM $MgCl_2$

TE buffer

10 mM Tris-HCl (pH 7.5)

1 mM EDTA

e) Buffers for Agarose Gel Electrophoresis

10x TBE (Tris-Borate-EDTA) buffer

880 mM Tris (pH 8.3)

880 mM Boric acid

25 mM EDTA.

10x TAE (Tris-Acetate-EDTA) buffer

400 mM Tris (pH 8.3)

1.1% (v/v) Acetic acid

10 mM EDTA

Loading solution

50% Glycerol

0.1% bromphenol blue

0.1% xylene cianol

f) Intracellular Ca^{2+} measurement experiments

HEPES Tyrode buffer

25 mM HEPES (pH 7.4)

3.5 mM KCl

125 mM NaCl

1.2 mM $MgCl_2$

5 mM $NaHCO_3$

5 mM Glucose

5 mM Na-pyruvat

0.1% BSA

Fura-2AM

5 mM dissolved in DMSO

g) *In vitro* ROCK assay buffers

ROCK assay lysis buffer

50 mM Tris-HCl (pH 7.4)

100 mM NaCl

0.5% Triton X-100

10 mM $MgCl_2$

10% glycerol

10 mM NaF

1 mM Na_3VO_4

1 mM PMSF

1% protease inhibitor cocktail (Calbiochem)

ROCK assay kinase buffer

50 mM Tris-HCl (pH 7.4)

100 mM NaCl

0.05% Triton X-100

2 mM $MgCl_2$

2 mM $MnCl_2$

10% glycerol

10 mM NaF

1 mM Na_3VO_4

1 mM PMSF

1% protease inhibitor cocktail (Calbiochem)

2.1.6. Media

RPMI 1640 containing

1% L-Glutamine

100 U/ml Penicillin

100 µg/ml Streptomycin

10% FCS

Dulbecco's modified eagle medium containing

10% FCS

100 U/ml Penicillin

100 µg/ml Streptomycin

Medium for the bacterial culture - LB (Luria-Bertani)

10 g Bacto-Trypton

5 g Yeast Extract

10 g NaCl

Dissolved in 1l H_2O

2.1.7. Equipment

Centrifuges

Sigma 4K15 (Sigma laboratory centrifuges, Osterode im Harz, Germany)

Routina 48RS (Hettich Zentrifugen, Tuttlingen, Germany)

Eppendorf centrifuge 5415 (Eppendorf, Hamburg, Germany)

Spectrophotometer

Gene Quant Pro RNA/DNA calculator (Amersham, Freiburg, Germany)

Cell culture Incubator

Queue (Nunc, Wiesbaden, Germany)

Electrophoresis apparatus

Mini Protean 3 Electrophoresis Cell (BioRad Laboratories, Munich, Germany)

Agagel Mini (Biometra, Goettingen, Germany)

Agagel Maxi (Biometra, Goettingen, Germany)

Microplate reader

Dynatech MR7000 (Dynex Technologies, Berlin, Germany)

PCR machines

GeneAmp PCR System 2400 PE (Perkin Elmer, Rodgau - Jügesheim, Germany)

GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA)

Illuminometer

Plate Lumina (Started Biomedicine Systems AG, Birkenfeld, Germany)

Sonicator

Sonopuls GM70 (Bandelin Electronics, Berlin, Germany)

Semidry protein transfer apparatus

TransBlot SD (BioRad)

Fluorescence activated cell sorter (FACS)

FACScan (Becton Dickinson, Franklin Lakes, NY, USA)

Microscope

Olympus CK2 (Olympus, Japan)

Sequencer

ABI Prism 310 Gene Analyser (Applied Biosytems, Foster City, CA, USA)

Scintillation counters

Beckman LS6000TA (Beckman Coulter, Krefeld, Germany)

Liquid Scintillation and Luminescence Counter 1450 Micro Beta Trilux (Perkin Elmer, Rodgau - Jügesheim, Germany)

Gel dryer

MaxyDry Gel Drier (Biometra, Göttingen, Germany)

Scanner for autoradiography

FLA-3000 (Fuji Film, Düsseldorf, Germany)

Vacuum centrifuge

SpeedVac Plus SC210A (Thermo Savant, NY, USA)

UV Transilluminator

Herolab E.A.S.Y. 429 K (Herolab, Wiesloch, Germany)

Cell Harvester

Standard cell harvester (Inotech, Dottikon, Switzerland)

2.2. Methods

2.2.1. Mammalian cell cultures

HEK 293

The HEK 293 (human embryonic kidney) cell line, obtained from the American Type Culture Collection, was maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, in 5% CO_2 humidified atmosphere at 37^{o}C. For passaging, 70%-80% confluent cells were washed with PBS and treated with trypsin/EDTA (0.05%/0.02% w/v) for 5 min at 37^{o}C. After trypsinization, cells were diluted 6 times with DMEM and transferred to a new flask. The cells were splitted in the ratio 1:6, every two to three days.

Dendritic cells

Dendritic cells were obtained by differentiation of monocytes[128,129]. For the monocyte preparation we used human peripheral blood mononuclear cells (PBMC) isolated from buffy coats (provided by the German Red Cross, Ulm).
The buffy coat was diluted 2 times with washing buffer (PBS with 0.5% endotoxin-free BSA and 2 mM EDTA, sterilised by filtration through a 0.2 µm filter), and 20 ml of blood was carefully overlaid onto 15 ml Histopaque-1077 gradient. After 20 min centrifugation at 700 g, the PBMC containing ring on the top of Histopaque gradient, was collected, and the cells were washed three times in the washing buffer. The pellet was resuspended in cell culture media (RPMI 1640 with Glutamax I supplemented with 10% FCS, penicillin 100 U/ml, and streptomycin 100 µg/ml) and plated into four 10 cm tissue culture dishes. After 45 min incubation at 37^{o}C in a 5% CO_2 atmosphere, non-adherent cells were washed away with PBS. Adherent cells were 94% or higher, monocytes (the cells were stained with an anti-CD14 antibody). To obtain immature dendritic cells, monocytes were cultivated for 6 days in the presence of 100 ng/ml GM-CSF and 50 ng/ml IL-4. Every two days, one half of the medium was changed, and supplemented with only half the amount of cytokines. On day 6, 0.5 µg/ml LPS, or 50 ng/ml TNF-α, or 0.5 µg/ml CD40L,

were added for 48 hours, to produce different subtypes of matured dendritic cells[130].

2.2.2. Cloning of the PAR1 promoter with 5' deletions

The PAR1 sequence (Gene Bank accession number U35634) was used in cloning experiments. The 1.2 kbp long promoter region, as depicted in figure 10, was used for the promoter analysis.

```
   1 ctatacaact aagaccccaa agcatccttt taagctttat cgttttgtaa aatggttaaa
  61 aataacattt tccaccaatg acacagataa aattttaaag tgtggtggta ttattccatt
 121 cgtattttaa ggatatacat atatgcaatt tgtatgtgga tatgattttt tgaatggtga
 181 acagaaactt agtggtcagg tagctggatg cggtggctca tgcctgtaat cccagcactt
 241 tgggaggcta aggcgggtgg atcacctgag ttcaggagtt tgaggccaac ctggccaata
 301 tggtgaaacc cagtctctac taaaaataca aaaaattagc caggcgcggt ggtaggcgcc
 361 tgtaatccca agtactcggg aggctgaggc aggagaatcg cttgaaccca ggagacggag
 421 gttgctgtga gccgagtccg cgccattgca ctccagcctg ggcaacaaga gtgaaactcc
 481 gcctcaggaa aaaaaagaaa gaaagaaaat aagaaagaca gagagagaga gagaggaggaa
 541 ggaaggaaga gaggaaggaa ggaaggaaga gagggaggga ggaaggaagg aaggaaggaa
 601 ggaaggaagg aaggagggga aacttagtgg tcagatggca actaggggag gggaggcaga
 661 aggtaattta tttttcattt catatccttc aactatcaga aattttgtca tatgtgttta
 721 tattttccac tgtgtttcat tgattctaaa atgccttttc ttcataaccc tgaaattgga
 781 gtgtttctta caatcattgg tatgtcacat ttttttctgg cttgtggtaa tgggttaaaa
 841 tgataaattg taaaatcaat gacgtcttag gaataatgaa aaatagttta atagtgaatg
 901 aagaactatg taattttaac tgttcacatt tactcttggg tatgtttcca gaggataact
 961 gaacggggga tagattttaa a aagctttat ttaactgggt acttccgcaa tttagtgatc
1021 aacttctatg tacaacaagg tactgtcctt tgaggatgat gggagaatac agggaagaac
1081 gaaatcgcct ctgatcgtac tttctccacg gatgtaagtg tccgggctct agtgggggaa
1141 tgatactctt cgtgcgaaat tcacttttaa aaaaggctta gaaaactgac caccggctct
1201 cagctgcagc ttatcaacca cagaactccg aatatgcccg cacatcagtg cgctgggtta
1261 agaggagaag gggctgcggc tgagctttcc tagaaacagc tatttgggga cccatttcct
1321 gttggagtct gaaaggcgca cgcaccagag ↓ccagtggcaa agcaacttaa gctgcaggct
1381 cctcctgccc acccccagcc gcgacgcccc tgggggccct tagcagactg ccaagcgagt
1441 gtccccgccc tgtgccttga atttagccaa actcctttaa tatcaggcct ggcgcggtga
1501 ttcacgcctg taatccccgc actttaggag gctgaggcag gaggatcgct tgaggccagc
1561 agtgaaatta gccggacatg gtggcgcgcg cct ↓ttctgtg gtcccaggta atccggaggc
1621 tggggcgaaa gggtggcctg agccagggag atcgaggctg taagtgagcc gtgttggcgc
1681 cactgcactc cagcctgggc gacagggcaa gaccctgcct caaaaacaaa acaaactgta
1741 atatcgttca agaacgtgtg aaggacactt ggaaagttac caggccattt ctctcctggg
1801 cgcccgcggc cctagaacgc cggcctcacc ggcagacgcg cgccctcctc ccagatgcgc
1861 aggtgacccc ggagggcggc gcgggaaagg gaagaactcc gcgaggccgc gcggtgggga
1921 agcgggagaa ↓gccgctcttc ctattccact cgcagtctgc gtgtggggga aacgagtgcc
1981 cggcgtatga aacgcctaac ttcgcgaaat aaagagagac gtataaaagt tcaagaattc
2041 tgtccagact caagggccct ttctcattta ggggcaaccc tgtcactaca tcataaactt
2101 ttaaatccgt gatccccacg ttacaaaagc agaagtccct tttagacttt tagcgaaa ↓ac
2161 tgaactttgc cggtgtccca cacggaggga gggaggacgg gaggccacgc cagggctgcg
2221 gggctgcagg gcgtggacgc atcctggccg gggcgtccac tgtcgacgtc tccacatccc
2281 aggagggtcg agacggccgc gggaagcagt ctgcgagccg tgcggtccca ttccaaggac
2341 cccgccagtg tgagtcactg acagcttcg ↓c gaatcaacgg tgcccagagg aaaaaacttc
2401 tcatttggac ttctaggccc ggcagtggcc ggcggtcagg gccccccagt agggcagggc
2461 ggggcggggc ggggcgcaca gagccagagg ggcttgcgag cggcggctga gggaccgcgg
2521 ggaggggggcg ccgagcggct ccagcgcaga gactctcact gcacgccgga ggccccttcc
2581 tcgctcgcgc ccgcgcgacc gcgcgcccca gtcccgcccc gccccgctaa ccgccccaga
2641 cacagcgctc gccgagggtc gcttggaccc tgatcttacc cgtgggcacc ctgcgctctg
2701 cctgccgcga agaccggctc cccgacccgc agaagtcagg agagagggtg aagcggagca
2761 gcccgaggcg gggcagcctc ccggagcagc gccgcgcaga gcccgggaca atggggccgc
                                                          ----------
                                                        M  G  P  R
2821 ggcggctgct gctggtggcc gcctgcttca gtctgtgcgg cccgctgttg tctgcccgca
     ---------------------------------------------------------------------------
        R  L  L   L  V  A   A  C  F  S  L  C  G  P  L  L  S  A  R  T
2881 cccgggcccg caggc
     -----------------------
        R  A  R  R
```

Figure 10: Nucleotide sequence of the PAR1 promoter gene (Gene Bank accession number U35634). The sequence shown in red corresponds to the promoter size used in this work. Arrows are designating the 5' ends of each deletion.

The strategy we used for cloning of promoter deletion fragments was as follows: the fragments were amplified by PCR, using genomic DNA as a template, and treated with the restriction endonucleases. At the same time, the vector was also treated with the restriction enzymes. The vector and the promoter fragment were ligated using the DNA ligase. After the ligation, the bacteria were transformed with the construct, and grown on selective medium. The DNA was isolated from bacteria.

DNA of promoter deletion fragments was generated by PCR amplification, using the following primers:

Construct	5' primer	3' primer
PAR1-1106 (1,2kb)	ATGCGAGCTCCCAGTGGCAAAGCAACTT	ATTACTCGAGCCTCCGGCGTGCAGTGAGAGTCTCTGC
PAR1-863 (957bp)	ATGCGAGCTCTTCTGTGGTCCCAGGTAATCCG	ATTACTCGAGCCTCCGGCGTGCAGTGAGAGTCTCTGC
PAR1-526 (620bp)	AGCTGAGCTCGCCGCTCTTCCTATTCCACTCGCA	ATTACTCGAGCCTCCGGCGTGCAGTGAGAGTCTCTGC
PAR1-298 (392bp)	GATCGAGCTCACTGAACTTTGCCGGTGTCCCA	ATTACTCGAGCCTCCGGCGTGCAGTGAGAGTCTCTGC
PAR1-87 (181bp)	ACTAGAGCTCCGAATCAACGGTGCCCAGAGGA	ATTACTCGAGCCTCCGGCGTGCAGTGAGAGTCTCTGC

PCR conditions: 35 cycles, 94^{o}C 30 s, 64^{o}C 30 s, 72^{o}C 90 s. PCR reactions were run on a GeneAmp PCR System 2400 PE and a GeneAmp PCR System 9700.

For PCR amplification the Advantage GC Genomic PCR kit was used. This kit contains GC melt, necessary for breaking strong guanine-cytosine bonds in the genomic DNA. Magnesium acetate is present in the reaction mixture because Mg^{2+} ions are cofactors for polymerase activity. The following

reaction mixture was made: 10 µl 5x GC Genomic buffer (200 mM Tris, pH 9.3), 10 µl GC melt (5 M), 2.2 µl magnesium acetate (25 mM), 1 µl dNTP mix (10 mM each), 2 µl template DNA (0.1 µg/µl), 1 µl polymerase mix, 2 µl primer mix (5 µM), 21.8 µl Milli Q water.

After the PCR amplification, the PAR1 promoter deletions and the vector (pGL3 enhancer) were treated with restriction endonucleases (New England Biolabs), to create the sticky ends, necessary for the ligation. The reaction contained: 2 µl 10x buffer, 2 µl PAR1 plasmid (1.21 µg/ml), 0.5 µl BSA (10 mg/ml), 1 µl of each enzyme SacI, XhoI (100000 U/ml), 13.5 µl Milli Q water.
The digestion mixture was incubated 1 h at 37^{o}C.
The vector was additionally incubated with 20 U alkaline phosphatase for 1 hour at 37^{o}C, to prevent its religation. Phosphatase was inactivated for 10 minutes at 80^{o}C. The fragments obtained after digestion were separated by horizontal 1% agarose gel electrophoresis in 0.5x TBE buffer, containing ethidium bromide (10 µg/ml). The gels were run for 20 minutes at 180 V. After separation, the fragments were purified from the gel using QIAquick Gel Extraction Kit (as described in the 2.2.3. section).

Ligation

The vector and the inserts were ligated using Rapid DNA ligation kit, containing DNA ligase, which anneals the sticky ends of the vector and the DNA fragment. The composition of the reaction mixture was as follows: 3 µl insert, 3 µl vector, 2 µl DNA dilution buffer, 10 µl 2x DNA ligation buffer, 1 µl ligase, 2 µl Milli Q water.
The mixture was incubated 10 min at room temperature, and then used for the transformation of bacteria.

Transformation

In order to select a cloned plasmid-construct, the bacteria were transformed.
5 µl of ligation mixture was incubated with 70 µl of DH5α, chemically competent bacteria (*E. coli*), for 30 min on ice. Bacteria were then heat-shocked for 30 seconds at 42^{o}C, and then again placed on ice. This bacterial

suspension was then plated on LB agar with selection antibiotic ampicillin (100 µg/ml), and incubated overnight at 37°C. Only bacteria transformed with pGL3 construct can grow on this agar, because the plasmid contains the ampicillin resistance gene.

Amplification of the positive clones

Single colonies were inoculated into LB medium with ampicillin (100 µg/ml) and incubated overnight at 37 °C. Plasmids were isolated using QIAprep Spin Miniprep Kit (as explained in the 2.2.3. section). Isolated DNA was again digested with SacI and XhoI enzymes, and the products were separated on horizontal agarose gel electrophoresis, to prove whether the fragments have the right size.

2.2.3. DNA plasmid purification

The QIAGEN Plasmid Purification Kit was used for purifying the cloned constructs. This method is based on an alkaline lysis of bacteria, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. RNA, proteins, dyes and low-molecular weight impurities were removed by a medium-salt wash. The plasmid DNA is eluted in a high-salt buffer, then concentrated, and desalted by iso-propanol precipitation.

Maxiprep

A single colony was picked from the selective plate and inoculated in a starter culture of 2-5 ml LB medium containing Ampicillin (100 µg/ml) and incubated for ~8 h at 37°C with vigorous shaking (~ 300 rpm). 200 µl of starter culture was diluted into 100 ml of selective LB medium and grown for 12-16 h at 37°C with vigorous shaking (300 rpm). The bacterial cells were harvested by centrifugation at 6000 g at 4°C for 15 min. The pellet was resuspended in 10 ml of Buffer P1 (with RNAse) and vortexed to resuspend the bacteria. 10 ml of Buffer P2 was added, gently but thoroughly mixed by inverting 4-6 times, and incubated at room temperature for 5 min. After adding 10 ml of buffer P3, the samples were mixed gently by inverting 4-6 times and incubated 20 min on

ice. Samples were centrifuged for 30 min at 20,000 g at 4^{o}C and the DNA plasmid containing supernatant was removed promptly. In the meantime, the column QIAGEN-tip 500, was equilibrated with equilibrating (QBT) buffer. The supernatant was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The column was washed 2 times with washing (QC) buffer and DNA was eluted with elution (QF) buffer. DNA was precipitated, by addition of isopropanol. The pellet was collected by centrifuging 30 min at 15,000 g at 4^{o}C, and the supernatant was carefully decanted. The DNA pellet was washed with ethanol and centrifuged at 15,000 g for 10 min. The supernatant was carefully decanted without disturbing the pellet. The pellet was air-dried (5-10 min) and redissolved in a suitable volume of buffer (TE, pH 8.0) or autoclaved Milli Q water. Absorbance at 260 nm was measured using an UV spectrophotometer, and the DNA concentration was calculated using the formula:

DNA (µg/ml) = A_{260} x 50 x dilution

For the DNA purity check, the ratio of A_{260}/A_{280} should be over 1.8.

Miniprep

After inoculating a single colony from the selective plate, in a starter culture of 2-5 ml LB medium containing Ampicillin (100 µg/ml), and after incubation for ~ 8 h at 37^{o}C with vigorous shaking (~ 300 rpm), 2 ml of this culture was taken and centrifuged for 5 minutes at 400 g. The bacterial pellet was resuspended in 250 µl Buffer P1, then 250 µl Buffer P2 was added and the tubes were mixed 4-6 times gently by inverting. 350 µl Buffer N3 was added and the tubes were immediately gently mixed and centrifuged for 10 min at 10,000 g. The supernatants were applied to the column, centrifuged, and the flow-through was discarded; then they were washed by adding 750 µl Buffer PE and centrifuged again. To elute the DNA, 50 µl of water was pipetted to the centre of each QIAprep column, which was allowed to stand for 1 minute and then centrifuged for 1 minute as above.

PCR product purification

The QIAquick PCR Purification Kit was used to purify DNA fragments from PCR and enzymatic digestions, by the same principal of DNA plasmid binding to QIAGEN Anion-Exchange Resin.

Five volumes of Buffer PB were added to 1 volume of PCR sample, mixed, applied to the QIAquick column and centrifuged for 30 seconds at 10,000 g. The flow-through was discarded, the columns were washed with 750 µl Buffer PE and again centrifuged for 30 seconds at 10,000 g. To elute DNA, 50 µl water was applied to the centre of the QIAquick membrane, the column was allowed to stand for 1 minute and was then centrifuged as described above.

DNA purification from the gel

The main advantage of this kit is that after separation by electrophoresis, the DNA could be extracted from the gel and purified.

Three volumes of Buffer QG were added to one volume of the gel (gel volume was calculated by weight). The mixture was incubated at 50^{o}C for 10 min and mixed by vortexing every 2-3 minutes during incubation. After the gel slice was completely dissolved, the colour of the mixture turns yellow. The adsorption of DNA to the QIAquick resin is efficient only at pH<7.5. Buffer QG contains a pH indicator, which turns yellow at pH<7.5 and orange or violet at higher pH. This allows easy determination of the optimal pH for DNA binding. One gel volume of isopropanol was added, mixed, and applied to the QIAquick column. Sample was centrifuged for 1 min at 10,000 g, washed with 750 µl of Buffer PE, and the flow-through was discarded. To elute the DNA, 50 µl of water was applied to the centre of the QIAquick membrane, the column was allowed to stand for 1 minute and was then centrifuged as given above.

2.2.4. Transient transfection of HEK 293 cells

In all transient transfection experiments SuperFect transfection reagent was used. SuperFect has a defined spherical architecture, with branches radiating from a central core and terminates with charged amino groups. This reagent assembles DNA into compact structures, optimising the entry of DNA into the

cell. SuperFect/DNA complexes possess a net positive charge, which allows them to bind to negatively charged receptors (e.g. sialylated glycoproteins) on the surface of eukaryotic cells. Once inside, SuperFect Reagent buffers the lysosome after it has fused with the endosome, leading to pH mediated inhibition of lysosomal nucleases, which ensures stability of the SuperFect/DNA complexes.

HEK293 cells cultivated in the flask were trypsinized at more then 80% confluency, then they were resuspended in DMEM (with 10% FCS), and seeded on the dishes in concentrations indicated in the table below. The cells were transfected using the SuperFect Transfection Reagent and the plasmid DNA in the following ratios:

	plasmid DNA	SuperFect	DMEM-serum free
24 well plate (500 000 cells/well)	0.6 µg	5 µl	50 µl
6 well plate (2x 10^6 cells/well)	2 µg	10 µl	90 µl
10 cm Petri dish (8x 10^6 cells/well)	10 µg	70 µl	300 µl

The mix of plasmid DNA, serum-free medium and SuperFect was vortexed for 10 seconds and then incubated for 10 min at room temperature prior to adding to the cells. In order to have the same DNA concentration in all samples, in co-transfection experiments the plasmids of empty vectors were used for adjusting the DNA concentration (in the samples with less DNA). After 4 h, the cells were washed with PBS, fresh medium was added, and the cells were cultivated for 48 h.

2.2.5. Luciferase gene-reporter assay

The firefly luciferase reporter gene was used for the analysis of the PAR1 promoter activity. Firefly luciferase catalyses luciferin oxidation using ATP and Mg^{2+} ions as a co-substrate and generates a flash of light (Figure 7). The activity of the promoter was proportional to the amount of luciferase activity,

measured as a light emission in the presence of substrate.

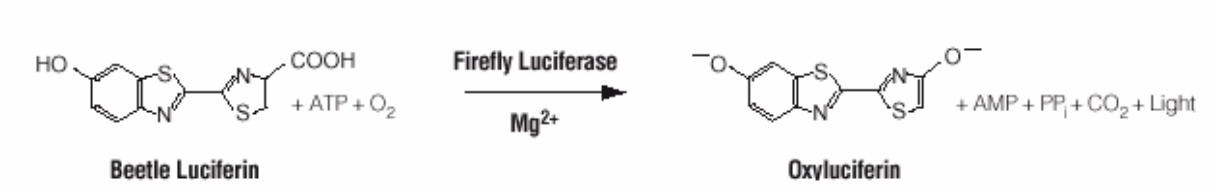


Figure 11: Bioluminiscence reaction catalysed by the firefly luciferase (Promega, Manheim, Germany).

All PAR1 promoter constructs were cloned into pGL3 enhancer vector in the multiple cloning region between SacI and XhoI restriction sites, and this plasmid was used for the transfection of HEK293 cells. pGL3 enhancer vector (Figure 10) contains the ampicillin resistance gene (important for the positive clone selection) and the luciferase gene.

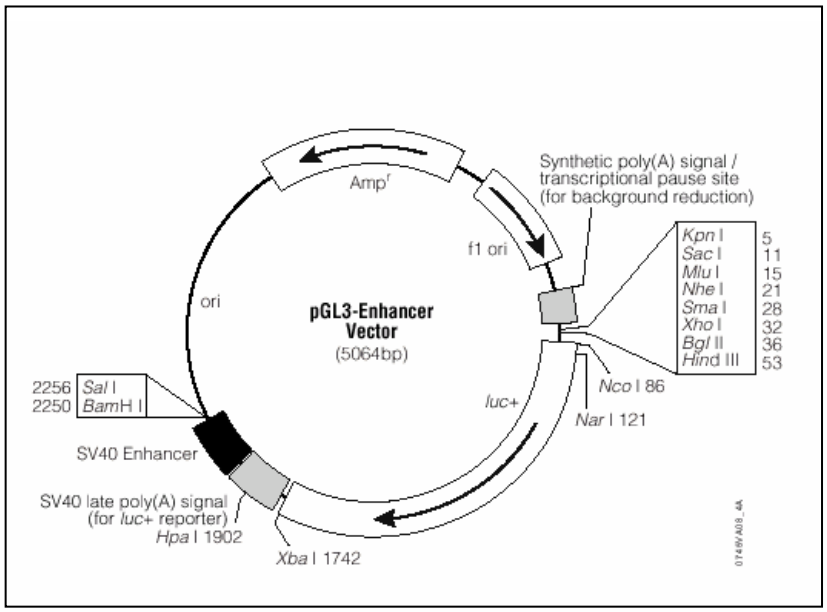


Figure 12: The pGL3 – Enhancer vector circle map. *Luc*+ is the cDNA encoding the firefly luciferase; Amp^r gene conferring ampicillin resistance in *E. coli*; ori, origin of replication in E. coli (Promega, Manheim, Germany).

HEK 293 cells were transiently transfected in 24 well plates, using 0.5x 10^6 cells/well. 48 h after transfection, the medium was removed and the cells were washed with PBS and lysed with 100 µl of Passive lysis buffer (Promega). After 15 min shaking the lysate at room temperature, and one freeze/thaw cycle, lysates were centrifuged for 5 min at 4000 g. 20 µl of the lysates were

transferred into 96 well plate and luciferase activity was measured in a plate luminometer. 50 µl of 0.2 mM luciferin solution (in Luciferase assay buffer) was added to each well. The samples were measured for 2 seconds.
Since the transfection efficiency was not always the same, it was necessary to normalise the results, i.e. RLU (relative luciferase units) were normalised to total protein concentration. Protein concentration was measured using Micro BSA Assay Kit (Pierce), by microplate reader Dynatech MR7000. This method utilizes bicinchoninic acid (BCA) as a detection reagent for Cu^{1+}, which is formed when Cu^{2+} is reduced by protein in an alkaline environment. A purple-coloured reaction product is formed by the chelation of two molecules of BCA with one Cu^{1+} ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations. The luciferase assay results were presented as the RLU/total protein concentration.

2.2.6. Western blots

Western blot analysis was used to investigate the protein expression. Proteins migrate in an electric field through the gel, and become separated based upon size and charge. Separated proteins can be transferred to a solid membrane, and the membrane can be immunostained with the antibody specific for the protein.

Electrophoresis

Cells were harvested by scraping, the medium was removed and 1x Sample Lysis Buffer was added. The cell lysates were then sonicated using 3 times 5 second intervals, and incubated for 5 min at 95^{o}C. The denatured proteins were separated on SDS-PAGE in Tris-Tricine buffer system, and detected with specific antibodies. 8% polyacrylamide running gel and 4% stacking gel were made as indicated in the following table:

	Running gel	Stacking gel
3x GB (Gel buffer)	3.3 ml	1.66 ml
AB 30% (48% acrylamide, 1.5% bis-	2.66 ml	0.66 ml

acrylamide)		
50% glycerol	2.12 ml	-
Water	1.8 ml	2.63 ml
0.1% APS (Ammonium persulfate)	100 µl	50 µl
TEMED(N,N,N',N'-Tetramethylethylenediamine)	10 µl	5 µl

Samples were run 10 min at 75 V and then for 45 min at 120 V, using the Mini Protean 3 Electrophoresis Cell (BioRad). Molecular weight Marker RPN 800 (Amersham Biosciences) was used as a standard for protein size from 10-250 kDa.

Electroblotting

The proteins were transferred from gel to a blotting membrane by semi-dry method. Prior to blotting, the nitrocellulose membrane (0.45 µm pore size, Schleicher & Schuell Bioscience) was equilibrated in blotting buffer. Gels were washed in blotting buffer for 45 minutes, to remove SDS, and then blotting sandwich was assembled avoiding bubbles: blotting paper, membrane, gel, blotting paper. The semidry apparatus was run at 180 mA (for 1 gel – 75 cm^2) and 360 mA (for 2 gels – 150 cm^2) for 60 min.

Detection of proteins by specific antibodies

The membranes were incubated on the shaker in TBS-T+ 5% milk for 1 h at room temperature, to block the non-specific binding sites, and then incubated with primary antibody diluted in TBS-T+ 5% milk powder (in a concentration recommended by the manufacturer) for 1 h at room temperature or overnight at 4°C. The membranes were then washed three times for 10 min in TBS-T to wash away the unbound primary antibody, and incubated with HRP-conjugated secondary antibody diluted in TBS-T for 1 h at room temperature, and then again washed 3 times with TBS-T for 10 min. The proteins were visualized by enhanced chemiluminescence (Super Signal West Pico

Chemiluminiscent Substrate, Pierce) for 5 min and exposed to Hyperfilm ECL (Amersham).

Stripping

In the case when the membrane had to be reprobed with another antibody, the previously used antibody was removed by stripping.

The membranes were incubated in the stripping solution first for 15 min at 50^{o}C, then 15 min at room temperature on the shaker. Afterwards, the membranes were washed 3 times with TBS and then again blocked in 5% milk and used for immunostaining.

2.2.7. Electrophoretic mobility shift assay (EMSA)

EMSA is used for determining protein-DNA interactions. This assay is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments. The gel shift assay is carried out by first incubating proteins (such as nuclear extract) with a ^{32}P end-labelled DNA fragment containing the putative protein binding site. The reaction products are then analyzed on a non denaturing polyacrylamide gel. The specificity of DNA-binding protein for the putative binding site is established by competition experiments using non-labelled DNA fragments or oligonucleotides containing a binding site for the protein of interest.

Nuclear protein extraction

We investigated the effect of PMA and TNF-α stimulation on the PAR1 promoter. This stimulation leads to activation of regulatory proteins (transcription factors) and their translocation to the nucleus. The nuclear extracts of stimulated and non-stimulated cells were prepared.

10x 10^6 HEK 293 cells per sample were stimulated with 50 ng/ml PMA and 50 ng/ml TNF-α, for different time points. Since PMA was dissolved in DMSO, the controls treated with DMSO were included.

The protocol for nuclear extract preparation was as follows[131].

Buffer A: 10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF.

Buffer B: 50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% glycerol.

After stimulation, the cells were washed with cold PBS, scraped and centrifuged. Then they were resuspended in the 3 fold volume of of the cell pellet of Buffer A, and incubated on ice for 5 min. After centrifugation, 5 min at 4000 g, the pellet was suspended in approximately 0.5 fold the pellet volume of Buffer B, and incubated on ice for 30 min. The suspension was centrifuged for 15 min 15,000 g, and the nuclear extract was collected. The 280 nm and 260 nm absorbance of the samples was measured with an UV spectrophotometer. The protein concentration was calculated from the formula:

Protein (mg/ml) = 1.55 x A_{280}– 0.76 x A_{260}

Nuclear extracts were snap frozen in liquid nitrogen and stored at -80°C.

Labelling of the probe

For labelling, we used a probe corresponding to the NF-κB motif of the mouse-immunoglobulin light chain enhancer (5' AGC TTG GGG ACT TTC CAC TAG TACG 3')[132] labelled with ^{32}P.

End labelling was done with T4 polynucleotide kinase, which incorporates the radioactively labelled phosphate group from the (γ-^{32}P) ATP to the 5' end of the DNA oligonucleotide. The reaction mixture contained: 2 µl double-stranded oligonucleotide (1.75 pmol/µl), 1 µl T4 polynucleotide kinase 10x buffer (70 mM Tris-HCl, pH 7.6), 1 µl (γ-^{32}P) ATP (10 µCi/ µl), 1 µl T4 polynucleotide kinase (10000 U/ml), 5 µl nuclease-free water.

Reaction mixture was incubated at 37^{o}C for 10 min, stopped by adding 10 µl 0.5 M EDTA and 89 µl of TE buffer. To purify the probe from the non-incorporated ATP, we used the Micro spin G25 (Pharmacia) column. The specific activity of the probe was measured in a Scintillation counter, taking 2 ml of scintillation liquid (Ultima Gold XR) and 1 µl of probe. The radioactivity of the probe was adjusted to 50,000 cpm/µl with water, and subsequently stored at 4^{o}C for a maximum one week.

Analysis of protein-DNA binding

5 µg of protein extracts were incubated with ^{32}P labelled double stranded NF-κB consensus oligonucleotide. The binding assay contained: nuclear extract (5 µg), 0.5 mM DTT, 1 µg poly (I:C) in 30 µl binding buffer (10 mM Tris- HCl pH 7.5, 1 mM $MgCl_2$, 0.5 mM EDTA, 4% glycerol) and 50000 cpm of the labelled probe. To prove that the radioactively labelled DNA-protein complex was specific, we added non-labelled DNA in a 100-fold excess, as a competitor. The samples were incubated for 15 min at room temperature before adding the probe and then 30 min after adding the radioactively labelled DNA. The samples were separated on 5% non-denaturing polyacrylamide gel for 2 h at 200 V in 0.5x TBE buffer, pH 8.0. Gels were dried at 80^{o}C on a Gel dryer, autoradiographed, scanned using a FLA-3000 phosphoimager, and quantified by video densitometry using Aida image analyser v.4.00 software.

Super shift assay

For supershift analysis, antibody against RelB (10 µg/ml) was added to the mixture. After 30 min on ice, the labelled probe was added and the assay was performed as described.

2.2.8. *In vitro* DNase I footprint analysis

DNase I Footprinting assay is a method of studying DNA-protein interaction and identifying the DNA sequence to which a protein binds. First, a target DNA fragment about 100-300 bp in length is radioactively labelled (at only one end) and incubated with proteins (nuclear extract), followed by controlled digestion with DNase I, which cuts the probe randomly, but only once. The digested DNA is recovered from the reaction and resolved on a polyacrylamide gel along with G+A chemical sequencing reaction, which uses the same probe as template, and cleaves the DNA after every guanine and adenine base. The regions bound by proteins will be protected from DNase I digestion and will be shown as a blank area on the gel, while the exact

protein-bound sequence can be read out by comparing the location of the blank with the sequencing reaction.

Labelling of the probe

The PAR1 promoter fragment (taken from the 5' end of the promoter, position -1105 to -863 bp), was cut from the pGL3 enhancer vector using SacI and EcoRI enzymes, and purified using the QIAquick PCR Purification Kit. For the dephosphorylation of the fragment, CIP-Calf intestinal phosphatase (New England Biolabs) was used as follows: 10 µl DNA fragment (~ 1 µg), 2 µl Buffer (NEBuffer 3) (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9), 1 µl CIP (10000 U/ml), 7 µl Milli Q water.

Digestion was performed for 1 h at 37°C. After digestion, the DNA fragment was additionally purified using the QIAquick PCR Purification Kit. For labelling, T4 polynucleotide kinase, which transfers γ-radioactive phosphate from ^{32}P-ATP to the DNA fragment, was used. The composition of the reaction mixture was as follows: 30 µl DNA (6 pmol), 10 µl Buffer for T4 enzyme (70 mM Tris, pH 7.6), 5 µl (γ^{32}P) ATP (10 µCi/µl), 2.5 µl T4 enzyme (10000 U/ml), 52.5 µl Milli Q water.

The reaction was incubated for 1 h at 37°C, and the enzyme was inactivated by heating at 65°C for 5 minutes. This way, the two-end labelled probe was created. In order to obtain only a 5' labelling, the probe was digested once more with EarI enzyme that cuts DNA near the 3' end.

SacI

↓

* 5'cccagtggcaa agcaacttaa gctgcaggct cctcctgccc acccccagcc gcgacgcccc tgggggccct tagcagactg ccaagcgagt gtccccgccc tgtgccttga atttagccaa actcctttaa tatcaggcct ggcgcggtga ttcacgcctg taatccccgc actttaggag gctgaggcag gaggatcgct tgaggccagc agtgaaatta gccggacatg gtggcgcgcg cct*ttctgtg gtcccaggta atccggaggc tggggcgaaa gggtggcctg agccagggag atcgaggctg taagtgagcc gtgttggcgc cactgcactc cagcctgggc gacagggcaa gaccctgcct caaaaacaaa acaaactgta1 atatcgttca agaacgtgtg aaggacactt ggaaagttac caggccattt ctctcctggg cgcccgcggc cctagaacgc cggcctcacc ggcagacgcg cgccctcctc ccagatgcgc aggtgacccc ggagggcggc gcgggaaagg gaagaactcc gcgaggccgc gcggtgggga agcgggagaa gccgctcttc c↓tattccact cgcagtctgc gtgtggggga aacgagtgcc cggcgtatga aacgcctaac ttcgcgaaat aaagagagac gtataaaagt tcaag 3'

EarI ↑

EcoRI

Figure 13: Digestion strategy for the PAR1 promoter 5' fragment. Asterisk (*****) represent the radioactive labelling site, and the sequence written in red, represents the EarI cleavage site.

1 µl of enzyme was added to reaction mixture containing radioactively labelled fragment, and incubated 30 minutes at 37^{o}C. Small, 5' labelled ends were removed with Sephadex Micro Spin G-25 columns. The activity of the probe was 15,000 – 70,000 cpm/µl; it was stored at 4^{o}C, for the maximum of one week.

In vitro footprinting

In this step, the radioactively labelled probe is incubated with the proteins, and then digested with DNase I.

The reaction mixture containing the probe, poly (I:C), T3x buffer (75 mM HEPES, 15 mM $MgCl_2$, 12 mM $CaCl_2$) and nuclear extract, was incubated on ice for 10 min. Then, DNase I was added, and the reaction was incubated for 2 min on ice, and stopped by adding 50 µl of DNase I Stop Solution (0.1% SDS, 100 mg/ml tRNA, 200 mg/ml proteinase K, 1 mM DTT, 0.5 mM PMSF). The samples were incubated at 50^{o}C for 45 min to allow protein degradation by proteinase K. The DNA was extracted first with 80 µl phenol (equilibrated with TE buffer) and then with 80 µl chloroform. The aqueous phase was recovered and the DNA was precipitated with 300 µl cold 100% ethanol. After 15 min on ice the DNA was pelleted for 15 min at 15,000 g and 4^{o}C. The pellet was resuspended in 300 µl 70% ethanol and centrifuged at 4^{o}C for 5 min at 15,000 g. The pellet was air-dried, resuspended in 5 µl loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol), and denaturated for 5 min at 95^{o}C before loading onto the gel.

Chemical cleavage of DNA - A/G ladder

For chemical cleavage, 10 ng of DNA resuspended in 20 µl water was used. 25 µl of pure formic acid was added and the mixture was incubated for 3 min at 20^{o}C, placed on ice, and 200 µl of Stop solution (0.3 M Sodium-acetate, 1 mM EDTA) was added.

The DNA was precipitated with 750 µl 100% ethanol and incubated at –20°C for 20 min. After centrifugation for 10 min at 15,000 g, the supernatant was removed carefully, and the pellet dissolved in 250 µl 0.3 M sodium-acetate pH 5.5. DNA was re-precipitated with 750 µl 100% ethanol for 15 min at 4°C and centrifuged for 10 min. The pellet was resuspended with 70% ethanol, incubated for 15 min at 4°C, centrifuged for 5 min and air-dried. 100 µl of 1 M piperidine solution was used to dissolve the DNA. It was incubated for 30 min at 95°C, then cooled at room temperature, centrifuged, and piperidine was removed using a speedvac for approximately 1 h. The dried pellet was dissolved in 5 µl of loading buffer and was ready for loading on to the gel.

Sequencing gel

In order to separate the DNA fragments obtained by DNase I treatment, we used a 6% polyacrilamide gel. The gel contained: 21 g urea, 7.5 ml acrylamide/bis-acrylamide(30%), 5 ml 10x TBE, 350 µl 10% APS, 12 µl TEMED, up to 50 ml Milli Q water was added.

The gel was poured between the glass plates: 20 cm x 40 cm x 0.5 cm with spacers 1.5 cm x 40 cm x 0.4 mm. After polymerization, the gel was pre-run at 1800 V for 1 h. The samples were loaded, and the gel was run at 2000 V, 40 W and 25 mA for ~ 2 h. After transfer of the gel onto Whatman 3MM filter paper, the gel was dried for 1 h at 70°C (Biometra gel dryer), then incubated on the phosphoimager screen for autoradiography overnight. The results were scanned with FLA-3000 phosphoimager and analysed using Image Quant software.

2.2.9. RNA isolation

For isolation of RNA, two different methods have been used: TRIZOL reagent and Magnetic Dynal beads.

1) Total RNA isolation using TRIzol Reagent

During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, but it disrupts cells and dissolves cell components. Addition of chloroform and centrifugation separates the solution into an

aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase.

1 ml of TRIzol was used to lyse approximately $2x10^6$ cells. The cells were homogenised by pipeting and after adding 200 µl chloroform, and the samples were centrifuged at 12,000 g for 15 min at 4°C. Following centrifugation, the mixture separated into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol Reagent used for homogenisation. The aqueous phase was transferred to a fresh tube. The RNA was precipitated by isopropanol, and recovered by centrifugation at 12,000 g for 10 min at 4°C. The RNA precipitate, often visible after centrifugation, forms a pellet at the bottom of the tube. The pellet was washed with 75% ethanol, and air-dried for 5 to 10 min. The RNA was dissolved in 25 µl water and the absorbance at 260 nm was measured using Gene Quant pro RNA/ DNA calculator. RNA concentration was calculated using the formula:

RNA (µg/ml) = A_{260} x 40 x dilution

<u>Reverse transcriptase (RT) reaction</u>

For the synthesis of first-strand cDNA, the M-MLV RT (Invitrogen) was used: ~ 5 µg total RNA, 1 µl oligo $(dT)_{12-18}$ (500 µg/ml), 1 µl dNTP (10 mM each), up to 12 µl Milli Q water.

This mixture was heated to 65°C for 5 min and quickly chilled on ice, in order to allow oligo(dT) and mRNA annealing. The content of the tube was collected by brief centrifugation and the following components were added: 4 µl 5x First-Strand Buffer (250 mM Tris-HCl, 375 mM KCl, pH 8.3), 2 µl 0.1M DTT, 1 µl RNase OUT Recombinant Ribonuclease Inhibitor (40 U/µl).

The samples were gently mixed and incubated at 37°C for 2 min. 1 µl (200 U/ml) of M-MLV RT was added and mixed by pipeting gently up and down. The reaction mixture was incubated at 37°C for 50 min, and then inactivated by heating at 70°C for 15 min.

2) mRNA isolation using Dynabeads mRNA Direct Kit

This kit is based on the Dynbeads bio magnetic separation technology. Oligo $(dT)_{25}$ is bound to the bead surface and is used to capture mRNA and as a primer for the reverse synthesis of the first strand cDNA.

The cells were lysed using Lysis/Binding Buffer, 1 ml per sample of $4x10^6$ cells, and pipetted repeatedly through a syringe (BD Microlance 3 needle) in order to reduce the viscosity as a result of DNA release during lysis.

50 µl of prewashed magnetic beads were added and incubated at 4^oC 10 min on the shaker. The lysates were washed three times with washing buffer and three times with 1^{st} strand buffer (200 µl). Washing with the first strand buffer was done in order to prepare the samples for the RT reaction (the same buffer was used for RT). To collect the beads, the reaction tubes were kept on the magnetic stander and the supernatant was easily removed. The beads without any supernatant were used in a reverse transcription reaction, by adding the rest of the reaction components directly in to the tube with the beads.

Reverse transcriptase (RT) Reaction

For the first Strand cDNA synthesis we used SuperScript II RT, and the mixture for reverse transcriptase reaction contained: 5 µl 5x RT buffer, 2.5 µl 0.1 M DTT, 2.5 µl dNTP (10 mM each), 1 µl SuperScript II RT(200 U/ml), up to 25 µl Milli Q water.

After incubation for 60 min at 42^oC (with vortexing every 10 min), RT reaction was completed. 50 µl of 1x First strand buffer was added and heated to 95^oC for 3 min. This step leads to denaturation and separation of the mRNA template from the newly synthesized cDNA, which is bound to the beads. The beads were washed in 50 µl of TE buffer once and stored in 50 µl of TE buffer at 4^oC for 1 month.

PCR reaction

For the PCR amplification reaction, Taq polymerase and the following components were used: 3 µl 10x PCR buffer without Mg (200 mM Tris-HCl, 500 mM KCl, pH 8.4), 1 µl 10 mM dNTP mixture, 0.9 µl 50 mM $MgCl_2$, 4 µl primer mix (5 µM each), 1-20 µl (~ 5 µg) template DNA, 0.5 µl Taq DNA Polymerase (5 U/ml), the volume was filled up to 30 µl with Milli Q water.

The primers used in the PCR amplification reactions are listed below:

Primer	sense	antisense
PAR1	CGCCTGCTTCAGTCTGTGC	GCCAGACAAGTGAAGGAAGCC
PAR2	TGGATGAGTTTTCTGCATCTGTCC	CGTGATGTTCAGGGCAGGAATG
PAR3	TCCTGCTTCTGTTGCCCACTT	TACCACAACCATCTATGATCGTATGC
PAR4	AACCTCTATGGTGCCTACGTGC	CCAAGCCCAGCTAATTTTTG
GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC
IL-12	TAATCGTCCAAAAGTCTTA	TCCTGTTCTGCTGTGTCTTG
IL-8	ATGACTTCCAAGCTGGCCGTGCT	TCTCAGCCCTCTTCAAAAACTTCTC
IL-6	TACATCCTCGACGGCATCTCA	AGTTGTCATGTCCTGCAGCCA
TNF-α	CAGAGGGAAGAGTTCCCCAG	CCTTGGTCTGGTAGGAGACG
MIP-3α	TGCAACCAGTTCTCTGCATC	ACAGGGGAACTCTCAGAGCA
CCL22	ACTGCACTCCTGGTTGTCCTC	CACGGTCATCAGTAGGCTC
CCL17	ATGGCCCCACTGAAGATGCTGG	TCAAGACCTCTCAAGGCTTTGC
MCP-1	CATGAAAGTCTCTGCCGCCCTTCT	AGTTAGCTGCAGATTCTTGGGTTG

PAR1 specific primers were used as in Colognato et al[133]. Primers for PAR2, PAR3 and PAR4 were ordered according to Kahn et al[134]. RT-PCR for MCP-1 was performed according to Seino et al[135]. IL-12, IL-6 and IL-8 primers were taken from the literature[136-138], CCL17 and CCL22 from the group of Caligaris-Cappio[139].

PCR conditions:

Product (size)	No of cycles	Denaturation	Annealing	Elongation
PAR1 (650 bp)	33	94^{o}C 15 s	55 ^{o}C 30 s	72 ^{o}C 1 min
PAR2 (599 bp)	36	94^{o}C 30 s	65 ^{o}C 30 s	72 ^{o}C 1 min
PAR3 (850 bp)	35	94^{o}C 15 s	65 ^{o}C 30 s	72 ^{o}C 1 min

PAR4 (541 bp)	35	94°C 30 s	55 °C 30 s	72 °C 1 min
GAPDH (650 bp)	21	94°C 30 s	55 °C 30 s	72 °C 1 min
IL- 12 (754 bp)	36	94°C 30 s	50 °C 30 s	72 °C 1 min
IL-8 (289 bp)	35	94°C 30 s	52 °C 30 s	72 °C 30 s
IL-6 (399 bp)	35	94°C 30 s	52 °C 30 s	72 °C 30 s
TNF-α (325 bp)	33	94°C 30 s	65 °C 30 s	72 °C 30 s
MIP-3α (300 bp)	28	94°C 30 s	60 °C 30 s	72 °C 45 s
CCL-22 (289 bp)	23	94°C 45 s	55 °C 30 s	72 °C 45 s
CCL-17 (284 bp)	28	94°C 45 s	60 °C 30 s	72 °C 45 s
MCP-1 (337 bp)	28	94°C 15 s	55 °C 30 s	72 °C 50 s

The RT-PCR products were separated on 1% agarose gel (containing 10 µg/ml ethidium-bromide), at 100 V in 1x TBE buffer. The PCR products were visualised using a Herolab Transilluminator and semiquantitatively analysed by Easy Win 32 software. The intensity of the signal was normalised to GAPDH as a house-keeping gene.

2.2.10. DNA Sequencing

The identity of the PCR products was checked by sequencing with a ABI PRISM 310 Genetic Analyzer.

To improve the template quality, the DNA was cleaned from primers, dNTPs, residual salts, using the QIAGEN PCR Purification Kit (as described in section 2.2.3.). After purification, the samples had to be fluorescence labelled. Big Dye Terminator Cycle Sequencing Kit contains four different fluorescent dyes, for labelling of each nucleic acid base. The following reaction mixture was made: 10-100 ng DNA sample, 1-10 pmol 3' (or 5') primer, 4 µl Big Dye Terminator Cycle Sequencing Kit, the volume was filled up to 20 µl with Milli Q water.

PCR conditions: 25 cycles, 96°C 10 s, *°C 5 s, 60°C 4 min, * depending on the primers used.

Purification of the samples after PCR

In this purification step it is necessary to precipitate the DNA, and to redissolve it in sequencing reagent.

DNA was precipitated with ethanol (end concentration was 70%). The DNA was recovered by centrifugation at 15,000 g, washed once with 70% ethanol. The pellet was resuspended in 25 µl Template Suppression Reagent (TSR), incubated for 20 min at 90°C, then cooled on ice and transferred into the tube for sequencing. The sequences were analysed by BLAST software.

2.2.11. Measurement of the cytosolic calcium

Dendritic cells were loaded with Fura-2 acetoxymethyl ester. This dye is nonpolar and can easily penetrate the cell membrane. Once the dye is inside the cell, the ester becomes hydrolysed, yielding Fura-2. The polarity of the acid prevents diffusion of the dye outside of the cell.

Fura-2 fluorescence was measured at two wavelengths, with excitation at 340 and 380 nm and emission at 510 nm. The ratio of the fluorescence at the two wavelengths is proportional to the calcium concentration in nM:

$$[Ca^{2+}] = Kd \times ((R-R_{min}) / (R_{max}-R)) \times (S_{f2} / S_{b2})$$

R_{max} and R_{min} are the fluorescence ratio values under saturating and Ca^{2+} free conditions, respectively, and Sf2 / S_{b2} is the ratio of fluorescence values for Ca^{2+} bound / Ca^{2+} free indicator measured at the wavelength 380 nm used to monitor the Ca^{2+} free indicator. The Kd of Fura-2 is 244nM[140].

The cells were washed and resuspended in HEPES buffered salt solution (in mM: 25 HEPES, 3.5 HCl, 125 NaCl, 1.2 $MgCl_2$, 5 $NaHCO_3$, 5 glucose, 5 Na-pyruvat, pH 7.4) containing 0.1% bovine serum albumin. The dendritic cells were incubated with 5 µM Fura-2 acetoxymethyl ester for 30 minutes at 37°C. The cells were washed 3 times and resuspended in a buffer salt solution supplemented with 1.2 mM $CaCl_2$. One million cells were transferred into a quartz cuvette and allowed to equilibrate at 37°C for 3 minutes prior to

measurement in a PTI Fluorescence Detection System. The cells were challenged with thrombin and peptide WKYMV, which was used as a positive control[141].

2.2.12. Flow cytometry (FACS)

Fluorescent-activated cell sorting is a method for sorting a suspension of cells, one cell at a time, based upon specific light scattering and fluorescence characteristics of each cell. This method allows analysis of the cell surface fluorescence in order to check the expression of antigens, without breaking the cell membrane.

The cells were incubated in FACS buffer (PBS with 0.5% BSA, 0.1% NaN_3 and 2 mM EDTA). Primary antibody was added so that the final concentration was 8 µg/ml, and incubated for 1 h on ice. For washing, 1 ml of FACS buffer was added and the cells were centrifuged at 800 g for 5 min at 4^{o}C. Secondary antibody, conjugated with phycoerythrin (PE), was added in 1:100 dilution and incubated for 1 h in the dark at 4^{o}C. The cells were washed again and fixed with paraformaldehyde dissolved in PBS, so that the final concentration of paraformaldehyde was 2%. When the fluorescence was measured on the same day, cells were not fixed. Samples were analysed for antigen expression using a FACScan (BD Biosciences, San Jose, USA).

2.2.13. Mixed leukocyte reaction (MLR)

This experiment reveals the ability of dendritic cells to provoke a T cell response *in vitro*. Initially, it was used as a model for graft rejection. Leukocytes from one individual, the potential transplant donor, were mixed with T cells from the responder or graft recipient. If donor and recipient are mismatched at the Major Histocompatibility Complex (MHC), the T cells begin to proliferate, release cytokines and become cytotoxic T lymphocytes (CTLs).

Isolation of naïve $CD4^{\pm}$ T cells

For T cell isolation, we used the naïve $CD4^{+}$ T cell isolation Kit (Miltenyi Biotech). The principal of this method is an indirect magnetic labelling for the

isolation of naïve $CD4^+$ helper T cells from human peripheral blood mononuclear cells (PBMC). Memory T helper cells and non-T helper cells ($CD45RO^+CD4^+$T helper cells), $CD8^+$ Tcells, γ/δ T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes and erythroid cells, are indirectly magnetically labelled using a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD36, CD45RO, CD56, Cd123, TCR γ/δ and Glycophorin A, and Anti-Biotin MicroBeads. Isolation of highly pure naïve $CD4^+$ T cells is achieved by depletion of the magnetically labelled cells.

PBMC were isolated from human whole blood. 25 ml of blood was mixed with 1.625 ml of 77 mM EDTA. After centrifugation for 20 min at 307 g at room temperature, plasma was removed, and the rest, i.e., erythrocytes and PBMC were diluted with 0.9% NaCl up to 25 ml. The gradient, containing 55% Percol in NaCl (0.9%), was overlaid with blood and centrifuged for 23 min at 465 g on room temperature. After centrifugation, the white band, containing the PBMCs, on the top of the Percoll gradient, was collected and washed once with 0.9% NaCl.

The cells were counted and resuspended in washing buffer (PBS, pH 7.2, 0.5% BSA and 2 mM EDTA) – 40 µl of buffer for 10^7 cells. Biotin antibody cocktail was added (10 µl per 10^7 cells), mixed and incubated 10 min on ice. Then, 30 µl of Washing buffer and 20 µl of Anti-Biotin MicroBeads per 10^7 cells was added, mixed, and incubated for 15 min on ice. The cells were washed by adding 1-2 ml buffer per 10^7 cells, and centrifuged at 300 g for 10 min. Finally, the cells were resuspended – up to 10^8 cells in 500 µl of buffer.

The LS column (Miltenil Biotech), was placed in the magnetic field of a MACS Separator, and prepared by rinsing with 3 ml Washing buffer. The cell suspension was applied onto the column; the cells were allowed to pass through and collected. These unlabeled cells, represented the pure naïve $CD4^+$ T helper cell fraction. For the determination of purity of isolation, the cells were characterised by FACS staining with anti-CD4 antibody.

Co-culture of dendritic cells with T cells

In order to test the ability of dendritic cells to stimulate T cells proliferation *in vitro*, we co-cultivated these two cell types.

Allogeneic $CD4^+$ T helper cells were co-cultured in 96-well round bottomed culture plates (10^5 T cells/well) with graded number of dendritic cells: 1000, 2000, 10 000 cells/well. The cells were cultured in RPMI 1640 medium with 10% FCS, in a final volume of 200 µl per well at 37°C and 5% CO_2. After 3 days, the cells were pulsed with [^{3}H]-thymidine. Radioactively labelled thymidine was incorporated in to the DNA of proliferating cells, meaning that the measured radioactivity is proportional to the increase in cell number. 0.5 µCi of [^{3}H]-thymidine was added per well for 16 h, after which the cells were harvested and analysed in a scintillation counter.

2.2.14. Chemotaxis assay

Chemotaxis was determined by measuring the number of cells migrating through a polycarbonate filter (5 µM pore size) in a 24-well Transwell chamber system. The upper chamber contained $2x10^5$ cells diluted in 100 µl of RPMI 1640 serum-free medium with 0.1% BSA, and the lower chamber contained 220 µl of the same medium with or without chemokines. Dendritic cells were allowed to migrate for 2 h at 37°C in a humidified atmosphere with 5% CO_2. After the migration, the filters with the attached cells were fixed in 800 µl of 4% paraformaldehyde for 30 minutes. After washing in deionized water, the polycarbonate filters were dehydrated by incubation in 70%, 90% and 100% ethanol for 5 minutes each. After washing in deionized water, the filters were stained with hematoxyllin for 3 minutes. The mixture of 75% ethanol and 25% HCl was used for destaining of the cell cytoplasm, leaving the nuclei stained. The filters were washed again, but this time with tap water, and incubated for 5 minutes in each of the following solutions: 70%, 90% and 100% ethanol. Migrating cells on the outer surface of the membrane were counted using a microscope. Oil immersion objective was used with 100x magnification, and 24 different fields per membrane were counted. Data are expressed as a chemotactic index, which is the ratio between the number of migrating cells in the presence of chemoattractants and the spontaneously migrated cells (in the absence of chemoattractants).

2.2.15. Flow cytometric analysis of actin polymerization

In order to reduce the serum-mediated effects, the cells were serum starved for 12 h prior to experiment.

After 12 h of serum starvation, $5x10^5$ cells/ml were incubated at 37°C for 5 minutes prior to the addition of the chemoattractants. Thrombin and WKYMV (positive control) were added in a 10 µl volume so that the final concentration was: 1 U/ml for thrombin, and 10 nM for WKYMV. At defined time points, the reactions were stopped by fixation in 300 µl 4% paraformaldehyde for 15 minutes. After washing, the cells were permeabilised in cold 0.1% Triton X-100. Then, the dendritic cells were stained with TexasRed-Phalloidin (diluted 1:100 in PBS) in the dark for 30 minutes, and analyzed by flow cytometry. For each time point, the mean fluorescence intensity was normalized to control, i.e. non-stimulated cells.

2.2.16. *In vitro* Rho-kinase (ROCK) assay

To analyze the activity of ROCK in thrombin-stimulated dendritic cells, we immunoprecipitated the kinase from dendritic cell lysates, and performed the assay *in vitro* using MYPT as a substrate. MYPT is a recombinant protein and represents a part of MLCP (myosin light chain phosphatase), which becomes phosphorylated by ROCK.

The dendritic cells were serum starved for 12 h, washed and stimulated with 1 U/ml of thrombin for 5 minutes. The cells were lysed in lysis buffer (in mM: 50 Tris pH 7.4, 100 NaCl, 10 $MgCl_2$, 10 NaF, 1 Na_3VO_4, 1 PMSF, 0.5% Triton X-100, 10% glycerol, protease inhibitor cocktail 1:100 diluted) for 10 minutes at 4°C. Cell lysates were precleared by incubation with the protein A/G Plus agarose. After discarding the beads, Rho-kinase (ROCK) was immunoprecipitated by incubating the cell lysates for 2 h at 4°C with anti-ROCK2 antibody, followed by 1 h incubation with protein A/G-Plus agarose. The beads were washed three times with lysis buffer, and resuspended in 30 µl kinase buffer (in mM: 50 Tris pH 7.4, 100 NaCl, 2 $MgCl_2$, 2 $MnCl_2$, 10 NaF, 1 Na_3VO_4, 1 PMSF, 0.05% Triton X-100, 10% glycerol, protease inhibitor cocktail 1:100 diluted) with 100 µM ATP and 0.5 µg MYPT and incubated for

30 minutes at 30 ^{o}C. Proteins were separated on 8% SDS-PAGE gels, and immunoblotted with antibody against phosphorylated MYPT. Staining of the membrane with anti-ROCK2 antibody was used as a loading control.

2.2.17. Statistical analysis

Statistical significance was calculated with the Newman-Keuls test. Data shown represent the mean ± SEM. P values lower then 0.5 were considered significant.

3. Results

3.1. Analysis of the PAR1 and PAR3 promoters

Molecular characterisation of the protease activated receptor (PAR) 1 gene revealed that its promoter is TATA-less, and gene regulation include a Sp1 and AP-2 like binding sites[142]. Our aim was to investigate consensus-binding sequences for different regulatory proteins that potentially modulate the expression of the human PAR1. Functional analysis of the PAR1 and PAR3 promoters was performed using gene reporter assay. For this, HEK 293 cells were transfected with PAR1 or PAR3 promoters, then the cells were stimulated with proinflammatory agents, and the luciferase activity was measured. The level of luciferase activity corresponds to the promoter activation.

The 1.2 kb protease-activated receptor (PAR) 1 promoter, was cloned into the pGL3 enhancer vector, and used for HEK 293 transfection. In order to investigate, how this promoter can be activated, we treated the HEK 293 cells with different proinflammatory agents: 1 µg/ml poly (I:C) (mimics viral infection), 0.5 µg/ml LPS (mimics bacterial infection), 50 ng/ml PMA (imitates DAG and primarly activates PKC), 50 ng/ml TNF-α (activates NF-κB and AP-1),and 5 U/ml thrombin (activates PAR1 and PAR3), for the last 24 h of cultivation. 48 h after transfection, the cells were lysed and the firefly luciferase activity was measured.

3.1.1. PAR1 and PAR3 promoters are activated by PMA/TNF-α

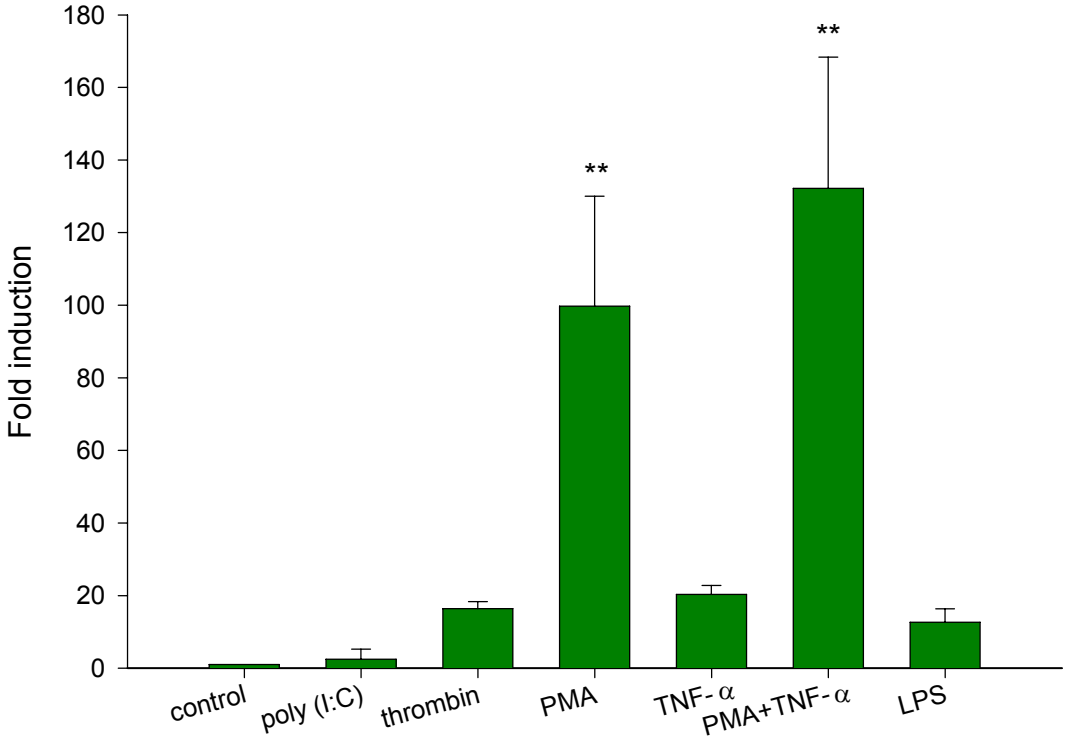


Figure 14: Proinflammatory stimuli activate the 1.2 kb PAR1 promoter. HEK 293 cells were transfected with the PAR1 promoter. 24 h later, the cells were stimulated with one of the following agents: poly (I:C) (1 µg/ml), thrombin (5 U/ml), PMA (50 ng/ml), TNF-α (50 ng/ml), the combination of PMA (50 ng/ml) and TNF-α (50 ng/ml), or LPS (0.5 µg/ml). Bars represent mean (± SEM) of three independent experiments, performed in triplicates. The data were normalised to the protein concentration. **p<0.01 vs. control.

The luciferase activity in the control, non stimulated cells was very low. All stimuli gave an increase in PAR1 promoter activity, compared to the control. The luciferase activity was highly increased in PMA treated cells. Treatment with TNF-α or thrombin resulted in similar activation (~ 25 fold). The PMA+TNF-α combination, had an additive effect, compared to the stimuli used alone, indicating that different pathways activated by PMA and TNF-α, contributed to the activation of PAR1 promoter.

The analogous experiment was done with the 2 kb long PAR3 promoter, cloned into the pGL3 enhancer vector.

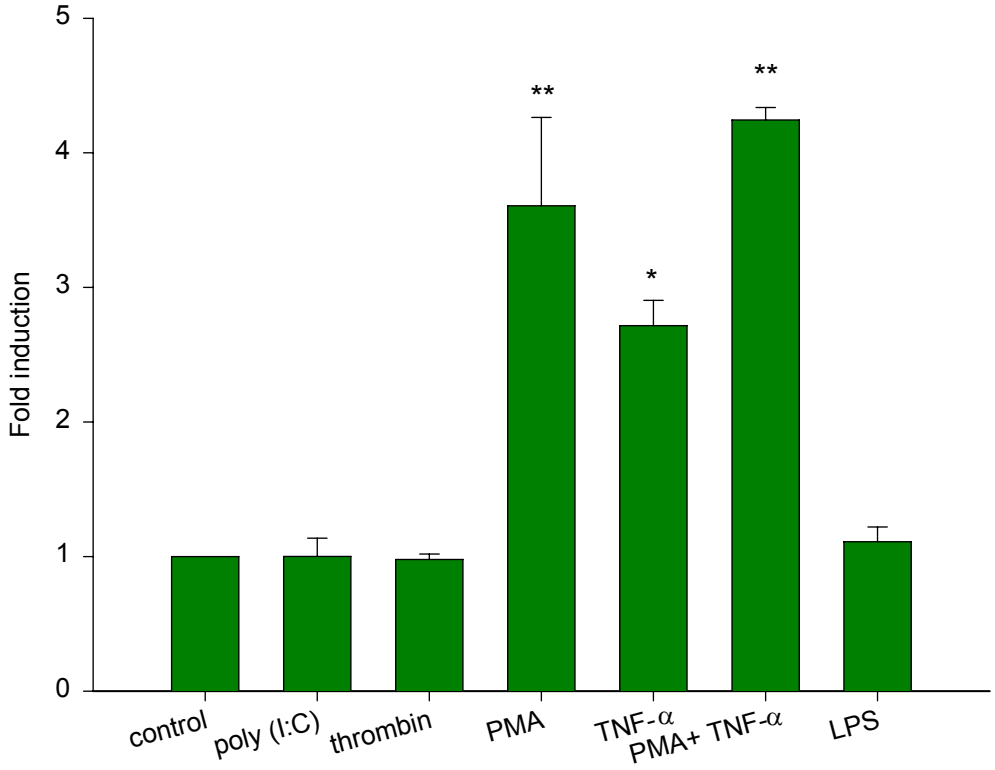


Figure 15: Proinflammatory stimuli activate the 2 kb PAR3 promoter. HEK 293 cells were transfected with the PAR3 promoter. 24 h later, the cells were stimulated with one of the following agents: poly (I:C) (1 µg/ml), thrombin (5 U/ml), PMA (50 ng/ml), TNF-α (50 ng/ml), the combination of PMA (50 ng/ml) and TNF-α (50 ng/ml), or LPS (0.5 µg/ml). Bars represent mean (± SEM) of three independent experiments, performed in triplicates. The data were normalised to the protein concentration. * $p<0.05$, ** $p<0.01$ vs. control.

The PAR3 promoter had a much lower activation level, compared to the PAR1 promoter. Activation of the PAR3 promoter with either poly (I:C) or thrombin, showed the same luciferase activity as the control cells. LPS stimulation gave no effect, while PMA and PMA+TNF-α, similar to PAR1 promoter, induced the highest promoter activation. However, no additive effect of PMA and TNF-α was observed with the PAR3 promoter.

3.1.2. The PAR1 and PAR3 promoters are activated by NF-κB proteins

The previous experiments have shown that PAR promoters could be activated by PMA and TNF-α. These stimuli can lead to NF-κB activation[143,144], and AP-1 activation[145,146]. Therefore, we further tested the co-transfection of expression plasmids, encoding the different NF-κB subunits: p50, p52, p65 cRel and RelB (cloned in to the pcDNA expression vector), together with the PAR promoters, in HEK 293 cells. Different combinations of NF-κB proteins were tested in order to find the synergistic effect on the promoter activation. The cells were lysed 48 h after transfection, and the luciferase activity was measured as described before.

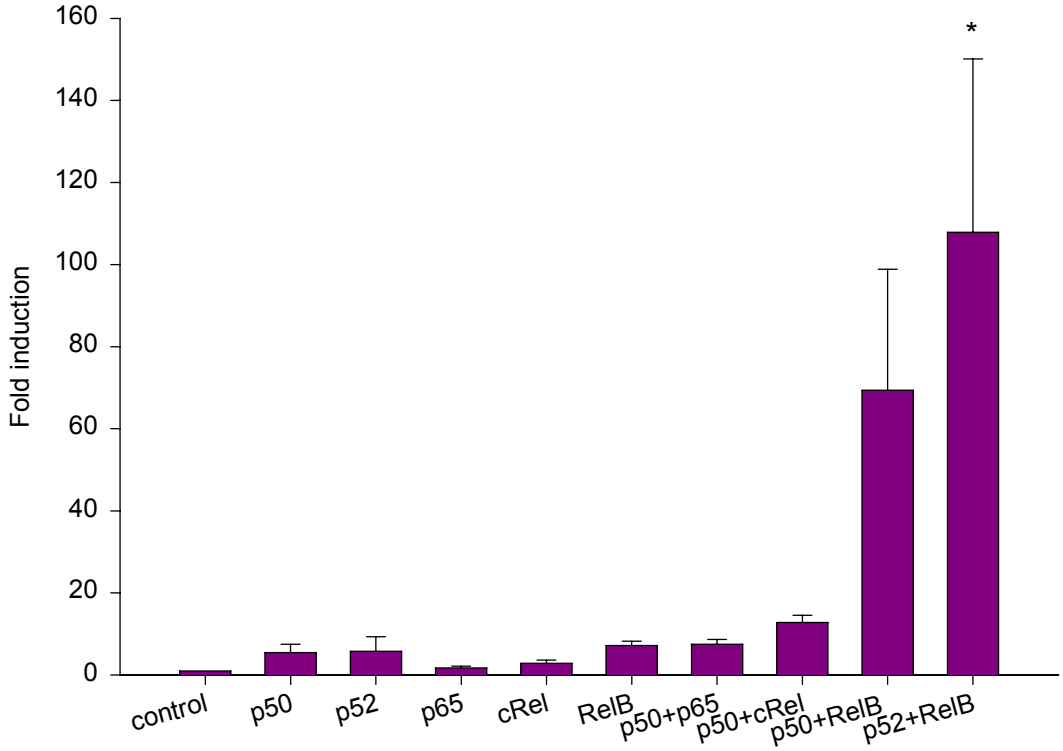


Figure 16: NF-κB proteins activate the PAR1 promoter in HEK 293 cells. HEK 293 cells were co-transfected with the PAR1 promoter and different NF-κB subunits for 48 h. The cells were lysed, the luciferase activity was measured and normalised to the total protein concentration. Bars represent mean (± SEM) of three independent experiments, performed in triplicates. * $p<0.05$ vs. control.

Control cells, that were transfected only with the PAR1 promoter and empty vector pcDNA showed a low level of luciferase activity. All NF-κB subunits, except p65, stimulated the promoter. p65 had minimal effect on PAR1 promoter activation, when used alone and with p50. c-Rel activated promoter, but in combination with p50, no additional activation was observed, suggesting a lack of synergy. These data showed only minor activation when p50 and RelB were used alone, yet a striking approximately ~ 70 and ~110 fold activation when the combinations p50/RelB and p52/RelB were used, respectively. NF-κB dimer, p50/p65 showed almost the same level of promoter activation as p65 alone.

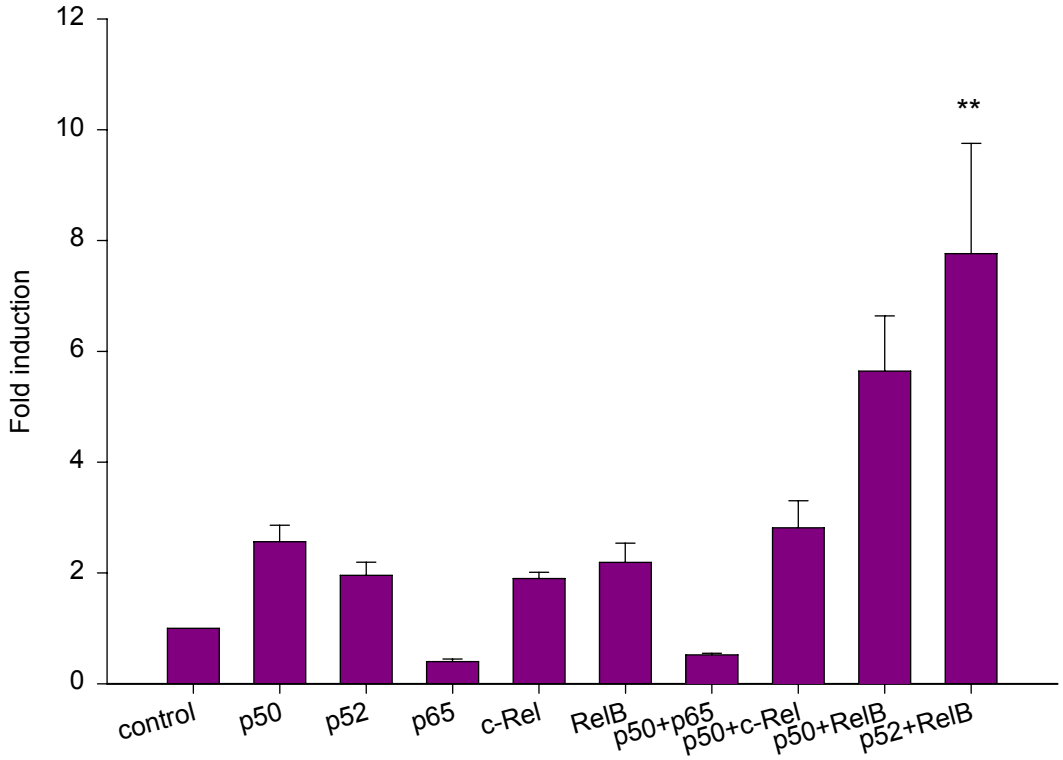


Figure 17: Effect of the NF-κB proteins on the activity of the PAR3 promoter in HEK 293 cells. HEK 293 cells were co-transfected with the PAR3 promoter and different NF-κB subunits, for 48 h. The cells were lysed, the luciferase activity was measured and normalised to the total protein concentration. Bars represent mean (± SEM) of three independent experiments, performed in triplicates. ** $p<0.01$ vs. control.

P50, p52, c-Rel and RelB gave two times fold activation, when used alone. The p50/RelB and p52/RelB combinations gave the highest luciferase activity.

3.1.3. The VCAM1 promoter is activated by p65

From all NF-κB subunits, PAR1 and PAR3 promoters did not respond to p65 alone. Therefore, we took the VCAM1 promoter (cloned in to the pcDNA expression vector) as a positive control to test if p65 is expressed in HEK 293 cells and is able to activate a NF-κB dependent gene expression.

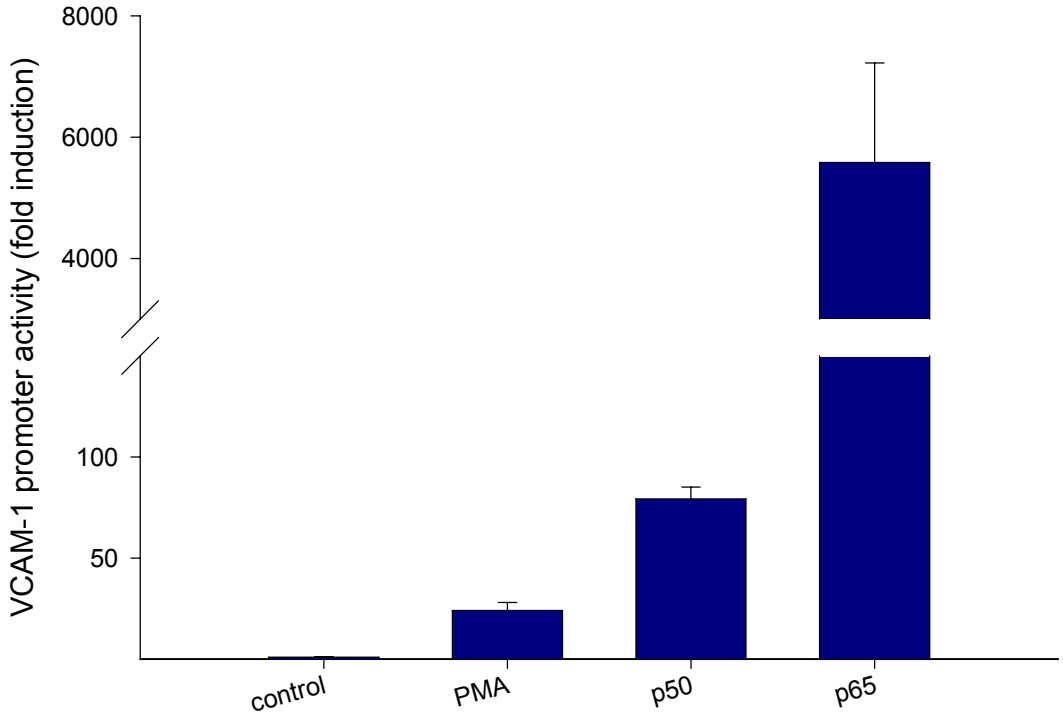


Figure 18: The VCAM1 promoter is activated by PMA (50 ng/ml), p50 and p65 in HEK 293 cells. HEK 293 cells were transfected with the VCAM1 plasmid and were 24 h later stimulated with PMA (50 ng/ml) or transfected with p50 and p65. After 48 h of transfection, the cells were lysed, the luciferase activity measured and normalised to total protein concentration. Bars represent mean (± SEM) of two independent experiments, performed in triplicates.

These data clearly show the ability of p65 to activate the VCAM1 promoter approximately 200 times more than PMA, which was used as a positive control. This data are in agreement with the literature stating that p65 homodimers can activate the VCAM1 promoter [147].

Together these data showed that the PAR1 and PAR3 promoters are activated by pro-inflammatory stimuli, which could lead to the NF-κB

activation. The NF-κB subunits were over-expressed, and as a result, the PAR1 and PAR3 promoters were activated. This indicates that both promoters might contain an NF-κB binding regulatory element.

3.1.4. 5' Deletion mutations of the PAR1 promoter

We next tried to identify binding site for the regulatory proteins in the PAR1 promoter. In order to do so, we cloned four different 5' deletion mutants into pGL3 enhancer vector and tested their activity in the luciferase assay.

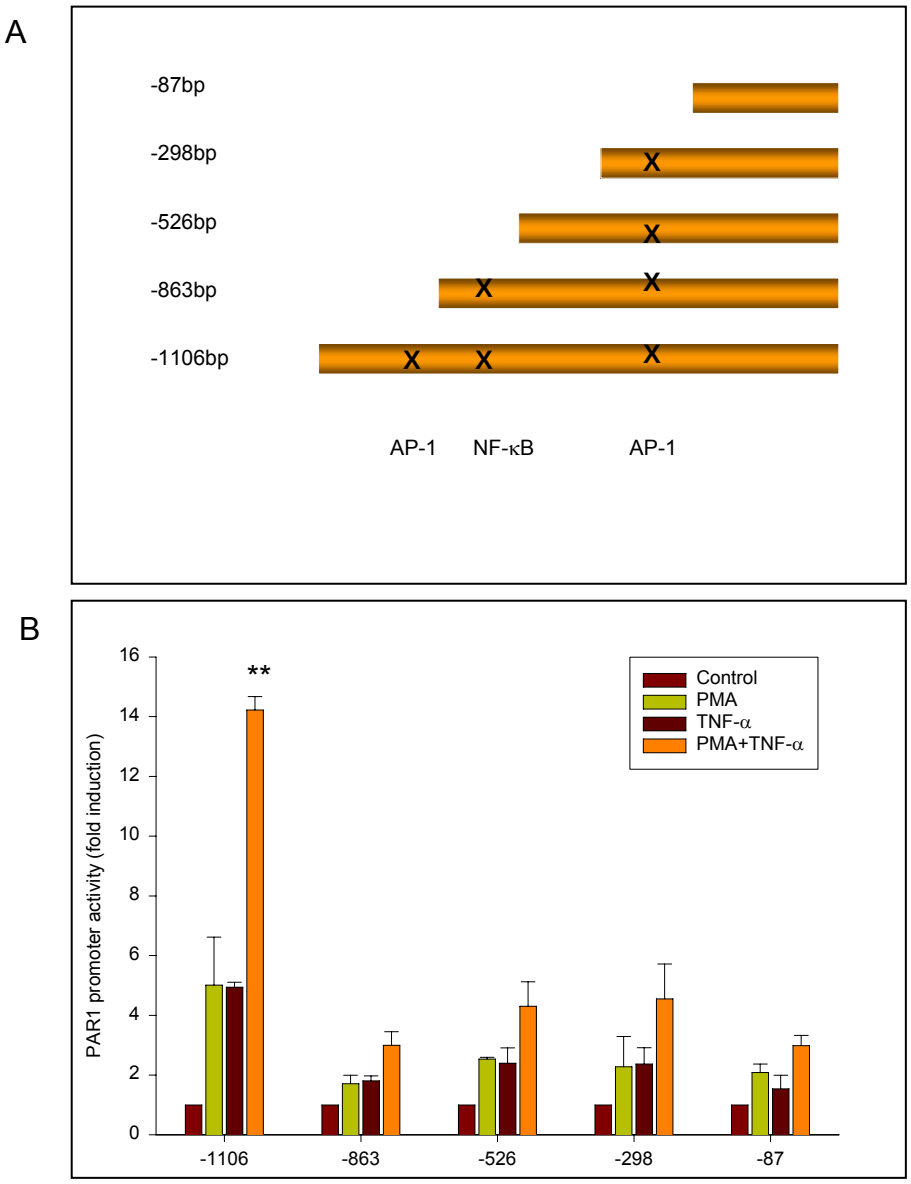


Figure 19: Delineation of the PMA/TNF-α response element by 5' deletions of the PAR1 promoter. A Scheme of the PAR1 5' deletion constructs. The numbers represent the base pairs upstream from the major transcription start site (according to Schmidt et al[148]), and X

represent the potential binding sites for NF-κB and AP-1. **B** Promoter activity of the different 5' deletion mutations. HEK 293 cells were transfected with the promoter deletion constructs cloned into the pGL3 enhancer vector. The cells were stimulated with PMA (50 ng/ml), TNF-α (50 ng/ml) or the combination of PMA (50 ng/ml) and TNF-α (50 ng/ml) for 24 h. 48 h after transfection, the cells were lysed and the luciferase activity was measured as described before. Data are presented as a mean (± SEM) of two independent experiments, performed in triplicates. **p<0.01 vs. control.

The potential binding sites for NF-κB and AP-1 were analysed using Genomatix software for transcriptional factor analysis-Matinspector. It utilizes a library of matrix descriptions to locate transcription factor binding sites in the sequence.

The PAR1 promoter is TATA-less, meaning that it has more than one transcription start sites. For labelling of the 5' deletion mutants, we used the transcription start site according to Schmidt et al[148].

Luciferase activity of the PAR1 5' deletion mutants was compared to the activity of the whole promoter (designated as –1106), which showed the highest response.

Our results show that after the first ~ 250 bp deletion, the promoter activation after PMA/TNF-α stimulation is diminished, and remains more or less at the same level, after introducing the further deletions. This indicates that the NF-κB binding region might be located between -1106 and -863 bp.

We also observed with PMA+TNF-α stimulation, that the level of the promoter activity is higher then when the cells are stimulated with individual stimuli. This effect was not simply additive but synergistic.

3.1.5. Analysis of PAR1 promoter regulatory elements

To investigate the potential regulatory binding site, we conducted the DNA footprinting assay using the nuclear extracts from non-treated HEK 293 cells and nuclear extracts from HEK 293 cells stimulated with PMA (50 ng/ml) and TNF-α (50 ng/ml). Before conducting the DNase I footprinting, two preliminary experiments were done, in order to determine the proper HEK 293 stimulation time and to control the quality of the nuclear extracts.

3.1.5.1. RelB expression in HEK 293 cells

Analysis of the PAR1 and PAR3 promoter activity in the gene reporter assays has shown an increased promoter activity when co-transfected with p50/RelB and p52/RelB. For this reason, we monitored RelB expression. The optimal stimulation time for the HEK 293 cells, i.e. the time when the RelB production was the highest, was defined by Western blot, of the whole cell lysates, on a time scale from 1 to 12 h.



Figure 20: Upregulation of RelB expression in HEK 293 cells, stimulated with PMA and TNF-α. HEK 293 cells were stimulated with PMA (50 ng/ml) and TNF-α (50 ng/ml) for indicated time periods, and the whole cell lysates were analysed by immunobloting using RelB antibody.

The RelB expression reached a maximum after 8 h. 12 h stimulation gave almost the same level of RelB expression as 8 h. Therefore, it was decided to stimulate the HEK 293 cells with PMA/TNF-α for 8 h and use these cells for preparation of nuclear extracts for the footprinting experiment.

3.1.5.2. Electromobility shift assay (EMSA) analysis of the nuclear extract quality

The quality of nuclear extracts isolated from non-treated HEK 293 cells and from 8 h PMA (50 ng/ml) +TNF-α (50 ng/ml) stimulated HEK 293 cells was tested by EMSA, using the NF-κB consensus sequence (5'-AGCTTGGGGACTTTCCACTAGTACG - 3') as a labelled probe[149].

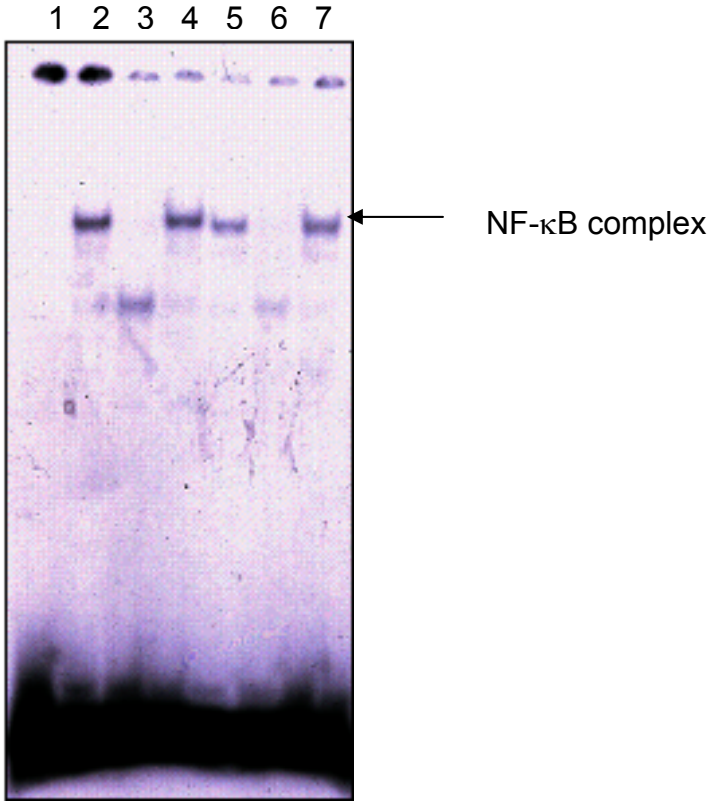


Figure 21: Nuclear extracts from untreated or cells treated with PMA and TNF-α from nucleoprotein complex with NF-κB consensus oligonucleotide. The treated nuclear extracts were obtained by stimulation of HEK 293 cells with 50 ng/ml PMA and 50 ng/ml TNF-α for 8 h. The arrow indicates the NF-κB complex. 1- NF-κB probe alone. 2 – probe + control nuclear extract (i.e. nuclear extract from non-stimulated HEK 293 cells). 3 – probe + control nuclear extract + cold NF-κB probe (100 fold excess). 4 - probe + control nuclear extract + RelB antibody (10 µg/ml final concentration). 5 - probe + treated nuclear extract. 6 – probe + treated nuclear extract + cold NF-κB probe (100 fold excess). 7 - probe + treated nuclear extract + RelB antibody (10 µg/ml).

EMSA revealed the presence of activated NF-κB proteins in non-stimulated and stimulated nuclear extracts. The nonlabelled NF-κB probe was used as a competitor for the protein binding in a 100-fold excess. The absence of the band on the gel proves the specificity of the nucleoprotein complex.

However, the NF-κB complexes did not have the same migration in non-stimulated and in stimulated cells. It appears that in stimulated HEK 293 nuclear extract, the NF-κB complex had a lower size, which could point to the difference in the composition of the NF-κB dimers.

Surprisingly, the supershift with the RelB antibody was not detected, even though the Western blot showed high RelB expression. We suspect this was due to the antibody used. The antibody used recognised RelB in denatured form in Western blot, and probably doesn't recognise native RelB.
Thus, the EMSA analysis showed that non-treated and treated nuclear extracts can form NF-κB complex, but of a different size.

3.1.5.3. Localizing the regulatory protein binding site

Nuclear extracts tested by EMSA, were further used in DNA footprinting experiments. We have shown that after deletion of 250 bp from the 5' promoter region, the luciferase activity was significantly decreased. For this reason, a DNA sequence located between -1106 and –863 bp was used in the footprint experiment as a probe. Three different samples were used: "naked" DNA (no proteins were added), DNA incubated with nuclear extracts from non-treated HEK 293 cells, and DNA incubated with nuclear extracts from the cells stimulated with PMA and TNF-α. A "naked" DNA was used for comparison to the DNA incubated with nuclear extracts. All samples were digested with DNase I, and the fragments obtained by digestion were separated by electrophoresis.
As a ladder, the sample was chemically cleaved, with formic acid and piperidine (Maxam-Gilbert method[150]), braking the DNA after every adenine and guanine base. The ladder was used to determine the sequence of the probe, and thus to locate the footprints. DNA, incubated with the nuclear extracts, was digested with two different concentrations of DNase I, in order to obtain the longer and shorter fragments (higher DNase I concentration resulted in formation of shorter fragments, localised on the bottom of the gel, and *vice versa*, lower DNase I concentration resulted in formation of longer fragments). The regions of the DNA sequence to which the regulatory proteins are bound, are protected from the DNase I digestion, and they appear on the gel as the regions where the bands disappear, compared to the ladder. These regions are called footprints.

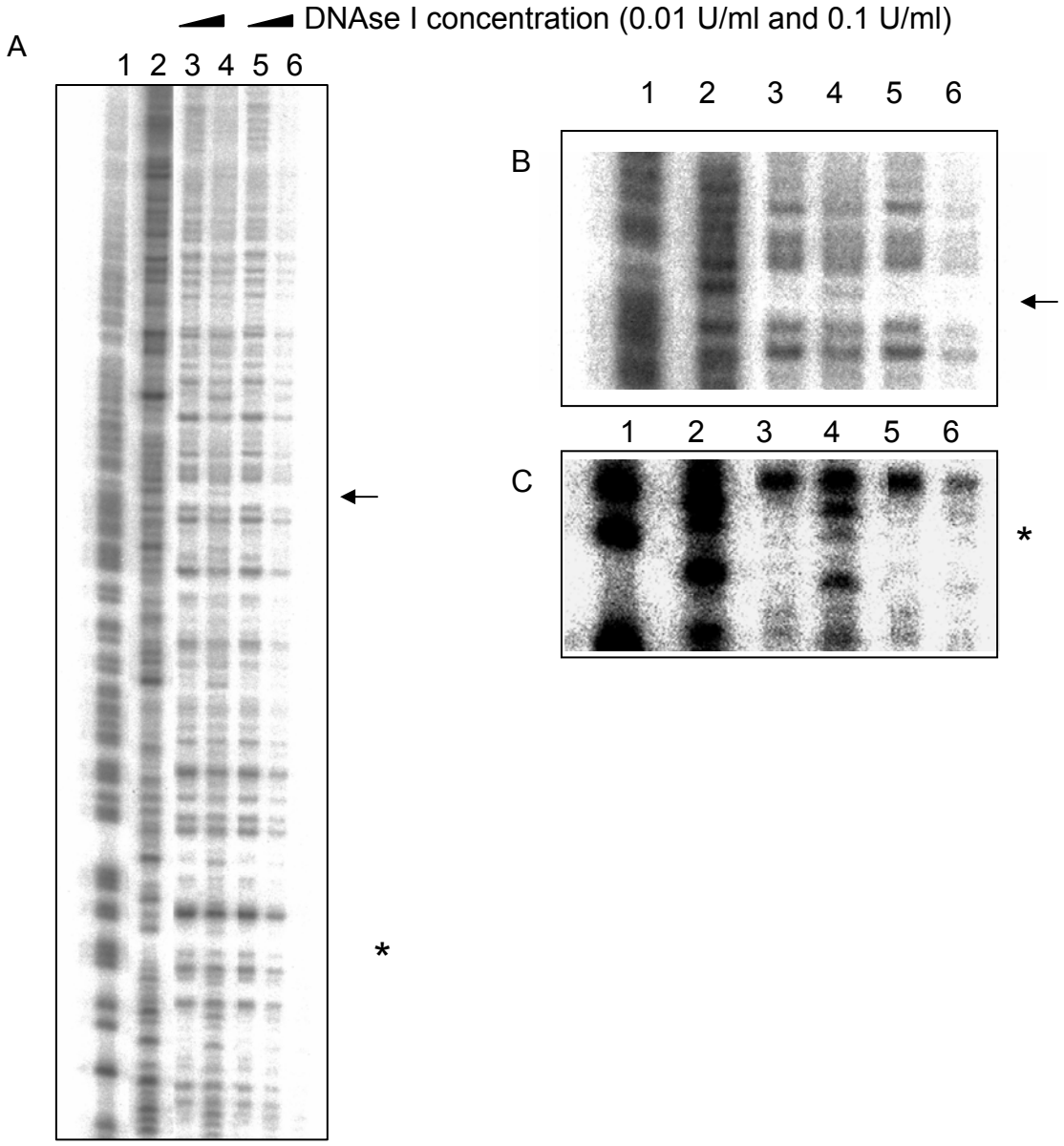


Figure 22: Control and PMA/TNF-α stimulated nuclear extracts from HEK 293 cells, incubated with the PAR1 promoter 5' region (position -1106bp to –863 bp), have different DNase I digestion patterns. A. Arrow and asterisk indicate the potential binding site of the regulatory element. 1 – A/G ladder. 2 – DNA without proteins (treated with 0.1U/ml DNase I). 3, 4 – DNA incubated with nuclear extract from non-treated HEK 293 cells, and digested with 0.01 and 0.1 U/ml of DNase I, respectively. 5, 6 – DNA incubated with nuclear extract from the HEK 293 cells stimulated with PMA and TNF-α, and digested with 0.01 and 0.1 U/ml of DNase I, respectively. B. Enlarged picture of the footprint. C. Enlarged picture of the false footprint.

Our results show that the "naked" DNA can be completely digested by DNase I, which gives many closely positioned bands on the gel. DNA incubated with

the nuclear extract shows some regions where the bands are very faint or even completely missing, compared to the “naked” DNA. These regions are called footprints and they are the result of the protein binding to DNA. On the other hand, some bands in the samples incubated with the nuclear extracts appear to be of higher intensity compared to the bands in the “naked” DNA. These are so called hypersensitive sites, not so exposed to DNase I cleavage, but the protein binding to DNA leads to change in DNA conformation, which enhances the cleavage.

Samples of DNA incubated with the control nuclear extracts showed clearly some binding regions. Then we looked for the differences between the footprints in the probes incubated with non-treated and treated nuclear extracts; located between the position -938 and -931, we detected a footprint present only in samples with treated nuclear extract.

In the control nuclear extract digested with 0.01 U/ml DNase I (Figure 16, lane 3), we were able to detect the footprint in this region. With the higher DNase I concentration, this effect was abolished and the band reappeared (lane 4). In the samples containing the nuclear extracts from PMA+TNF-α treated HEK293 cells, the footprint was detected with both DNase I concentrations (lane 5, 6). These data suggest that in the nuclear extract from pretreated cells, the protein complex has a higher binding affinity to this part of the DNA sequence, or that the PMA+TNF-α stimulation induces the binding of a complex with different composition. In some other parts of the sequence, we were able to detect the footprint elements (marked with an asterisk in figure 22). However, more detailed analysis, and the repeating of the experiment, showed the same DNase I digestion pattern in all samples.

Thus, the *in vitro* DNase I footprinting assay revealed one potential regulatory site, localised between -938 and -931 position in the PAR1 promoter.

3.1.5.4. EMSA analysis of the potential regulatory site

In order to define the composition of the protein complex binding to the PAR1 promoter upon PMA+TNF-α stimulation, we used EMSA analysis. Non-

stimulated and stimulated nuclear extracts were incubated with different oligonucleotides.

A 20 base pair oligonucleotide from the footprint region was designed, labelled at the 5' end with ^{32}P and used as a probe for EMSA. The sequence of the oligo was following:

5' TAA TCC CCG CAC TTT AGG AGG CTG AGG CAG 3'

The red letters denote the core sequence for AP-1 transcription factor, according to MatInspector software analysis, and the underlined sequence represents the footprinting region.

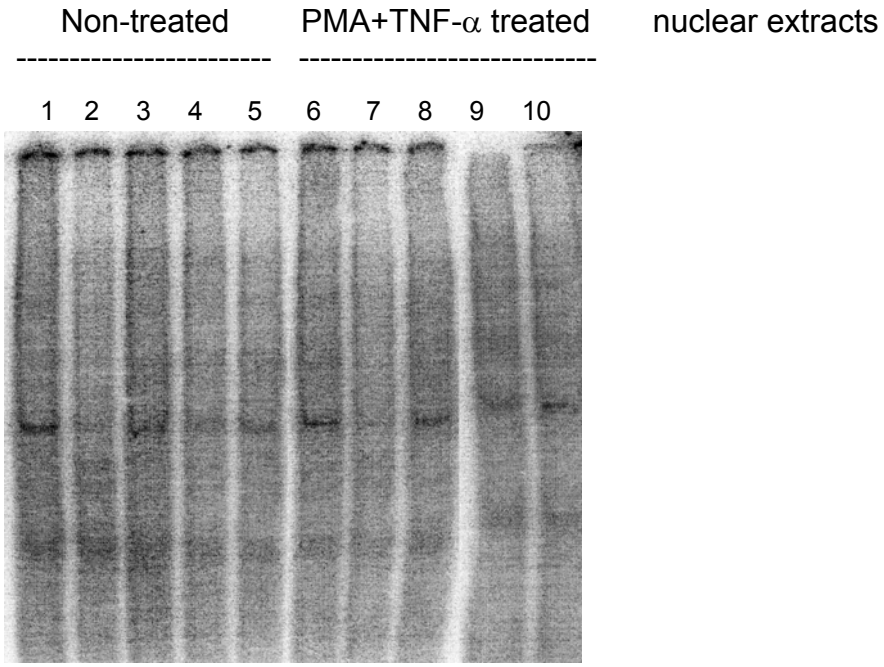


Figure 23: EMSA analysis of the NF-κB potential binding site. 1 – Probe + control nuclear extract (from the non-stimulated HEK 293 cells). 2 – Probe + control nuclear extract + nonlabelled probe. 3 – Probe + control nuclear extract + NF-κB oligo. 4 – Probe + control nuclear extract + AP-1 oligo. 5 – Probe + control nuclear extract + Sp1 oligo. 6 - Probe + treated nuclear extract (from the HEK293 cells stimulated with PMA (50 ng/ml) and TNF-α (50 ng/ml)). 7 – Probe + treated nuclear extract + nonlabelled probe. 8 – Probe + treated nuclear extract + NF-κB oligo. 9 – Probe + treated nuclear extract + AP-1 oligo. 10 – Probe + treated nuclear extract + Sp1 oligo.

The radioactively labelled probe was incubated with the nuclear extracts of non-treated HEK 293 cells, and of HEK 293 cells stimulated with PMA (50 ng/ml) and TNF-α (50 ng/ml). Nonabelled NF-κB, AP-1 and Sp1 oligos were

used as competitors for the DNA/protein complex formation, and were added in 100-fold excess.

Using the non-radioactively labelled probe as a competitor resulted in a complex disappearing (lanes 2 and 7), which proves the specificity of the protein binding to the oligonucleotide.

EMSA revealed that the protein binding in the footprinting region observed, could not be antagonised by NF-κB or by Sp1 oligo (lanes 3, 5 and 8, 10), neither in control nor in treated cells. When AP-1 was used as a competitor, the specific band (lanes 4 and 9) was reduced in the control and treated nuclear extract samples. In the nuclear extracts from pretreated cells, the nucleo-protein complex was of a different size. This implies that the composition of the AP-1 complex in control nuclear extracts is different than that in nuclear extracts from pretreated cells.

Our results demonstrated the presence of AP-1 complex in non-stimulated and stimulated nuclear extracts. However, the exact composition of these complexes was not resolved by EMSA and remains elusive since it is possible that the different members of the AP-1 family are forming the regulatory protein dimers.

Gene reporter analysis, of the PAR1 promoter, demonstrated an up-regulation of the promoter activity due to NF-κB proteins, implying that promoter activation is NF-κB dependent. Footprinting analysis did not show any NF-κB binding site on the PAR1 promoter. Since we have found a potential AP-1 binding site, it is possible that NF-κB activates the PAR1 promoter indirect via induction of AP-1.

3.2. Expression and function of PARs in dendritic cells

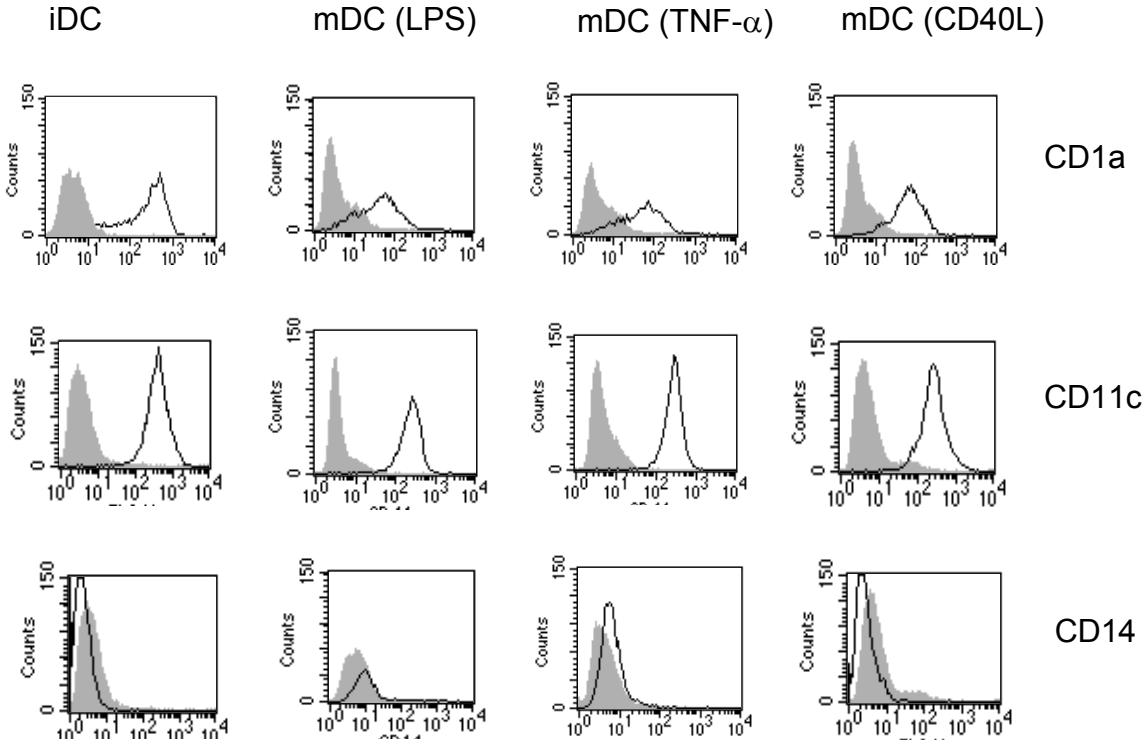
Dendritic cells are present in almost all tissues as immature dendritic cells. To fulfil their “mission” as antigen presenting cells, they need to encounter an antigen and to receive a stimulus for maturation.

The aim of this work was to investigate the expression and function of the protease-activated receptors in dendritic cells. It has been shown that immature dendritic cells have very low expression of PAR1 and PAR3 on mRNA level [133]. Our task was to analyse if the PAR expression changes during the process of dendritic cell maturation, and if changes in the expression are dependent on the maturation stimulus used.

We have used three different maturation stimuli, LPS (mimics bacterial infection), TNF-α (an inflammatory cytokine), and CD40L (involved in the dendritic cell-T cell cross talk).

3.2.1. Phenotypic characterisation of dendritic cells

The expression profile of surface markers of was analysed in all tree types of mature dendritic cells, in comparison to that of immature dendritic cells.



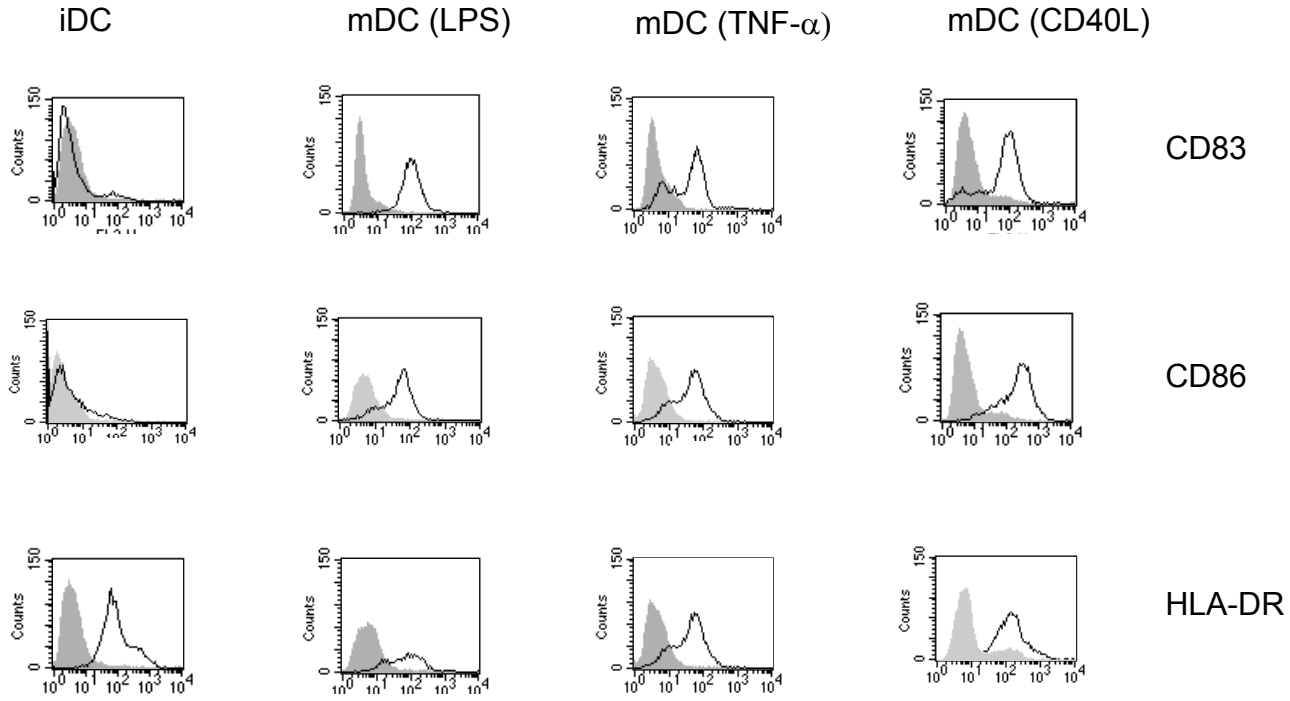


Figure 24: Phenotypic characterisation of immature and mature dendritic cells. Immature dendritic cells were cultivated for 6 days in the presence of GM-CSF (100 ng/ml) and IL-4 (50 ng/ml). Mature dendritic cells were generated by additional treatment for 48 h with LPS (0.5 µg/ml), TNF-α (50 ng/ml) and CD40L (0.5 µg/ml). The dendritic cells were incubated for 1 h with primary antibodies (anti-CD1a, anti-CD11c, anti-CD14, anti-CD83, anti-CD86 and anti-HLA-DR), washed, incubated for 1 h with secondary anti-mouse phycoerythrin-labelled antibody, washed again, and the fluorescence was measured by FACScan. Grey peaks represent isotype controls.

CD1a is a marker specific for thymocytes and dendritic cells[1]; it is similar to MHC class I, and has the role of presenting non-peptide antigens to some T cells. Our results show that immature and mature dendritic cells are CD1a positive.

CD11c is expressed on monocytes and all monocyte derived cells, granulocytes and NK cells; it has the function to bind fibrinogen and represents a part of the complement receptor type 4. FACS analysis shows that CD11c is expressed on the surface of immature and mature dendritic cells.

CD14 is a marker specific for monocytes, and functions as LPS receptor. Negative staining for this molecule verifies that the differentiation process from monocytes to dendritic cells is complete.

CD83 is a mature dendritic cells marker. Activated T and B cells also express CD83. It belongs to the immunoglobulin superfamilly and its function is still unknown. CD83 staining shows clear distinction between immature dendritic cells ($CD83^{low}$) and mature dendritic cells ($CD83^{high}$).

CD86, also called B7-2, is a costimulator for T cell activation. T cells express CD28 and CTL-4 - both of them bind to CD86. With dendritic cell maturation, the expression of this marker increases.

HLA-DR antibody reacts with the human class II antigen of the major histocompatibility complex (MHC). This molecule plays a major role in the cellular interactions during antigen presentation. HLA-DR is expressed primarily on B cells, monocytes, macrophages, thymic epithelial cells, dendritic cells and activated T lymphocytes. Our data show approximately the same expression in immature and mature dendritic cells, indicating that all dendritic cell types express MHC class II on their surface. In the literature, we found data demonstrating no differences in MHC class II protein expression between immature and mature dendritic cells, but suggesting that only the half-life of MHC class II differs between immature and mature dendritic cells[151]. Immature dendrtitc cells have a faster turnower rate of MHC clas II than mature dendritic cells.

Thus, these results indicate, that monocyte-derived immature dendritic cells are, $CD1a^{+}$, $CD11c^{+}$, $CD14^{-}$, $CD83^{-}$, $CD86^{-}$ and $HLA\text{-}DR^{+}$. During maturation, an upregulation in CD83 and CD86 is observed, so that dendritic cells become $CD83^{+}$ and $CD86^{+}$. The phenotypic profile of immature and mature dendritic cells is in the agreement with the literature[9,24,31].

3.2.2. Expression of PARs at the mRNA level

In order to analyse the expression of PARs, we isolated RNA from dendritic cells, and analyzed the RNA quality by agarose gel electrophoresis.

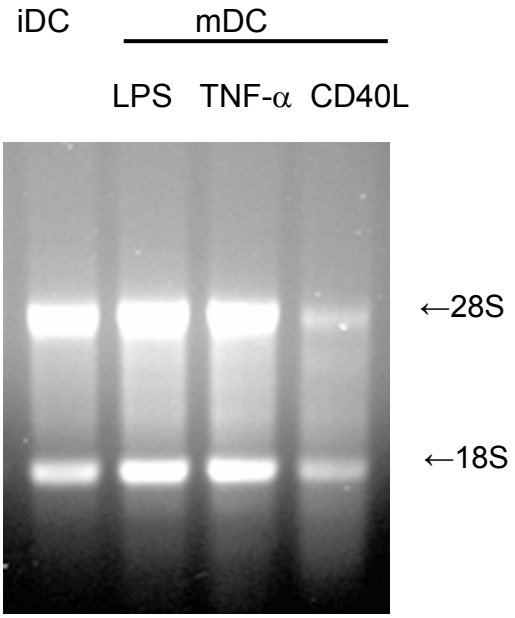


Figure 25: The total RNA isolated from dendritic cells is intact. Immature dendritic cells were cultivated for 6 days in the presence of GM-CSF (100 ng/ml) and IL-4 (50 ng/ml). For maturation dendritic cells were treated additionally for 48 h with LPS (0.5 µg/ml), TNF-α (50 ng/ml) and CD40L (0.5 µg/ml). 2x 10^6 cells/sample were lysed using TRIsol and total RNA was isolated as described in Methods.

Agarose gel electrophoresis shows clearly two bands, corresponding to 28S and 18S rRNA. The intensity of the 28S rRNA band is approximately two times higher than the intensity of the 18S rRNA band. This 2:1 ratio (28S: 18S) is a good indication that the RNA is completely intact.

3.2.2.1. Analysis of the PAR1 and PAR3 expression in dendritic cells

FACS analysis proved that we obtained phenotypically fully mature dendritic cells by using all three maturation stimuli. Further, we analysed expression of the thrombin receptors, i.e. PAR1 PAR3 and PAR4 in dendritic cells.

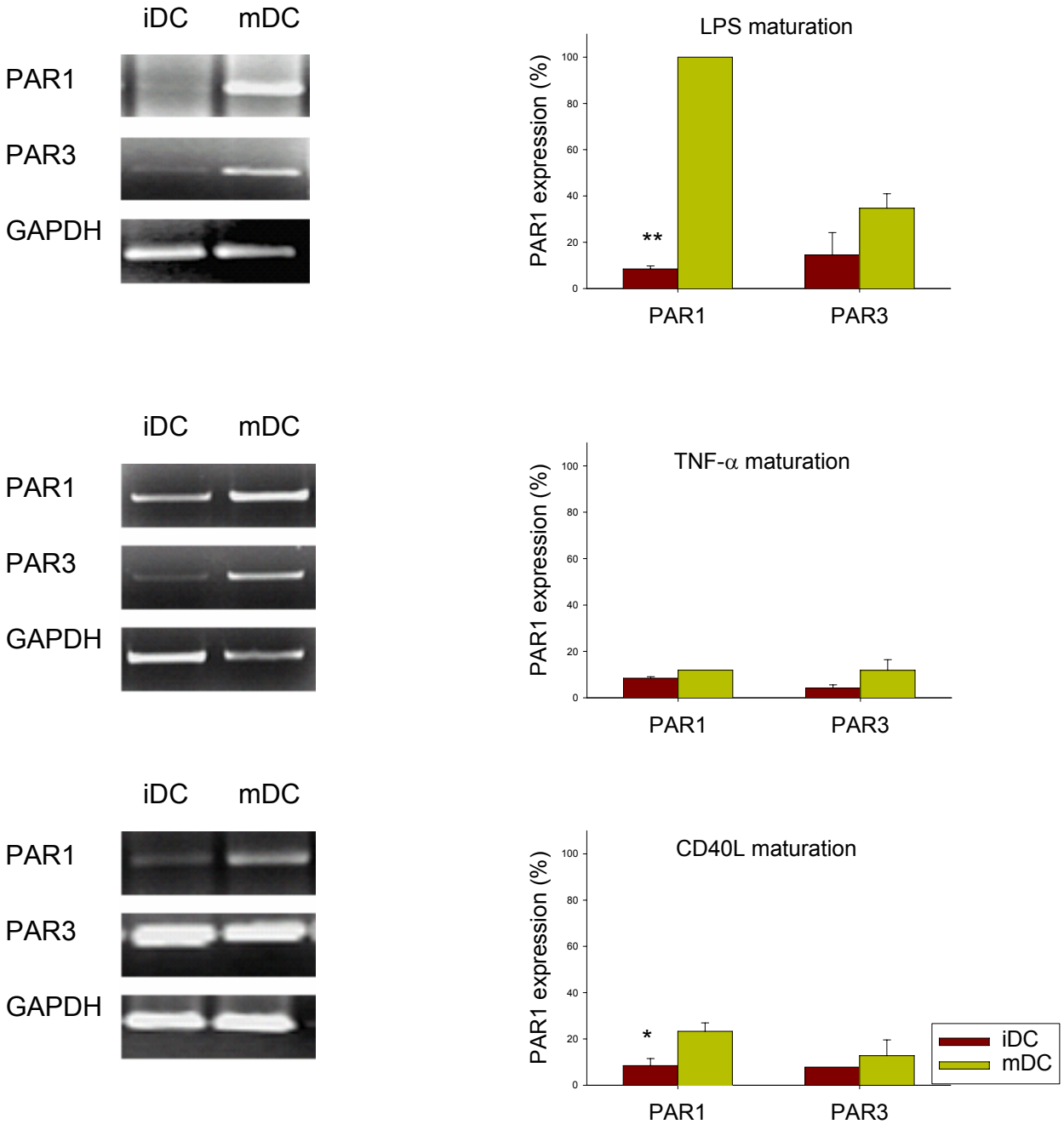


Figure 26: Semiquantitative analysis of the PAR1 and PAR3 mRNA expression in immature, LPS-, TNF-α– and CD40L-matured dendritic cells. mRNA was isolated using TRIzol reagent. The cDNA produced in a RT reaction was used as a template for the PCR

with primers specific for PAR1 and PAR3. The PAR1 and PAR3 expression was normalised to GAPDH. The PAR1 expression in LPS-matured dendritic cells was taken as 100%. The expression of all the other PARs in all three maturation types were calculated relative to PAR1 expression in LPS-matured dendritic cells. Bars represent mean (± SEM) of three independent experiments, * $p<0.05$, ** $p<0.01$ vs. immature dendritic cells. The pictures on the left are showing one representative experiment out of three.

Analysis of the PAR1 and PAR3 mRNA expression revealed that immature dendritic cells have a very low level of expression. A significant increase of the PAR1 expression in LPS-matured dendritic cells was detected compared to immature dendritic cells. No changes in the PAR1 expression is observed in TNF-α-matured dendritic cells and an approximately two fold increase in the PAR1 expression upon CD40L maturation. The PAR3 mRNA level was approximately 50% higher in LPS-matured dendritic cells compared to immature dendritic cells. We detected a slight change in the PAR3 expression with TNF-α-matured-dendritic cells, and no changes in PAR3 in CD40L-matured dendritic cells. If the three different maturation stimuli are compared, LPS induces the highest PAR1 and PAR3 expression.

3.2.2.2. Analysis of PAR4 expression in dendritic cells

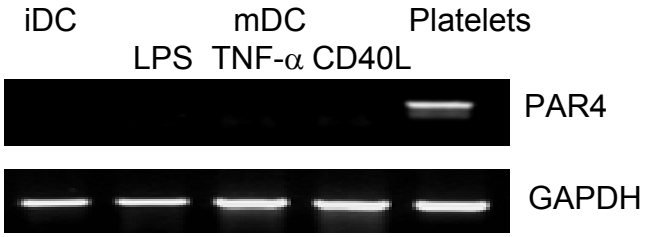


Figure 27: Expression of PAR4 mRNA in dendritic cells. Platelets mRNA served as a positive control and GAPDH was used for normalisation. Platelets were isolated from the blood and TRIzol reagent was used for the mRNA was isolation. The picture is showing one experiment out of three.

PAR4 mRNA was not detected in mature dendritic cells, but it was present in mRNA isolated from platelets, used as a positive control.

Thus, the mRNA expression analysis showed that only PAR1 and PAR3 mRNA are present in dendritic cells. The third thrombin receptor, PAR4, was absent in dendritic cells. For this reason, in further experiments, we analysed the expression and function of only PAR1 and PAR3.

3.2.3. Expression of PAR1 and PAR3 at the protein level

To analyse the expression of PARs at the protein level in dendritic cells, we prepared whole cell lysates and analysed them by immunoblotting using antibodies against PAR1 and PAR3.

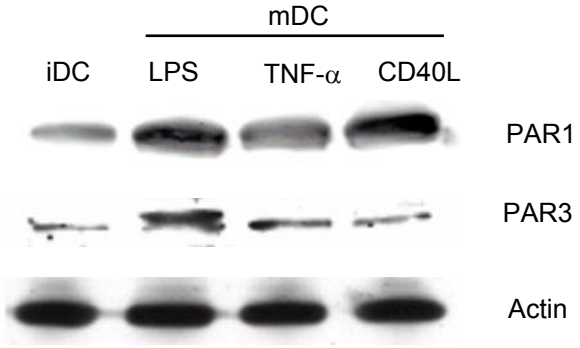


Figure 28: Protein expression of PAR1 and PAR3 in immature and mature dendritic cells. Immature dendritic cells were cultivated for 6 days in the presence of GM-CSF (100 ng/ml) and IL-4 (50 ng/ml). For maturation dendritic cells were treated additionally for 48 h with LPS (0.5 µg/ml), TNF-α (50 ng/ml) and CD40L (0.5 µg/ml). The Western blot was made from the whole cell lysate of dendritic cells. Actin was used as a loading control. The picture shows one representative experiment out of three.

Western blot analysis of dendritic cell lysates, demonstrated low level of PAR1 and PAR3 expression in immature dendritic cells. PAR1 protein expression was increased when LPS and CD40L were used as maturation stimuli. A slight upregulation of PAR3 protein expression is observed only with LPS stimulation.

3.2.4. Surface expression of PAR1 and PAR3

RT-PCR and Western blot results revealed the expression of PAR1 and PAR3 in dendritic cells. However, only the receptors expressed on the surface of the cells can be activated by thrombin cleavage. Therefore, we stained the surface of dendritic cells with PAR1 and PAR3 antibodies, and measured the expression of these receptors by flow cytometric analysis.

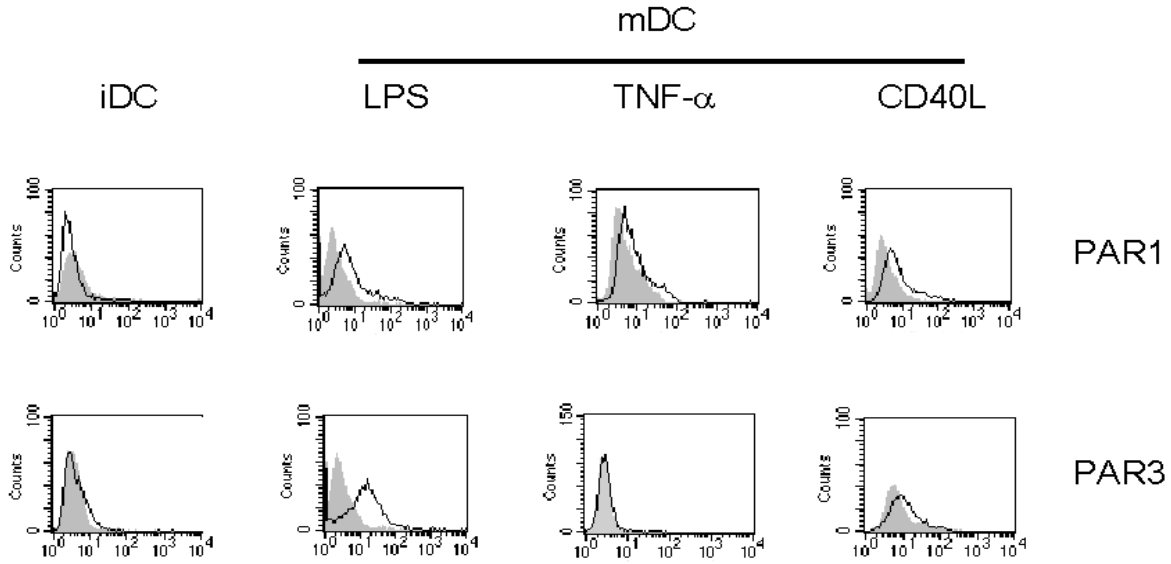


Figure 29: FACS analysis of PAR1 and PAR3 expression on the surface of dendritic cells. Immature dendritic cells were cultivated for 6 days in the presence of GM-CSF (100 ng /ml) and IL-4 (50 ng/ml). For maturation dendritic cells were treated additionally for 48 h with LPS (0.5 µg/ml), TNF-α (50 ng/ml) and CD40L (0.5 µg/ml). The dendritic cells were immunostained either with anti-PAR1 or anti-PAR3 antibodies for 1 h, washed and incubated with the secondary phycoerythrin conjugated antibody, washed again, and the fluorescence was measured using a FACScan. Grey peaks represent an isotype control, and the white peaks PAR1 and PAR3 antibody staining. Picture is showing one representative experiment out of three.

The FACS data imply that immature dendritic cells express neither PAR1 nor PAR3 on their surface or that the level of expression is too low to be detected. However, upon maturation only in LPS-matured dendritic cells a significant shift in PAR1 and PAR3 fluorescence was observed. With TNF-α and CD40L maturation only slight changes of fluorescence intensity were detected.

3.2.5. Cytosolic calcium release as a response to thrombin stimulation

The presence of thrombin receptors on the surface of dendritic cells has been analysed, and the next step was to prove their functionality. Therefore, we stimulated LPS-matured dendritic cells with thrombin, and measured the cytosolic calcium response, as a secondary messenger known to be released after thrombin stimulation in monocytes, macrophages and U937 cells[98,152].

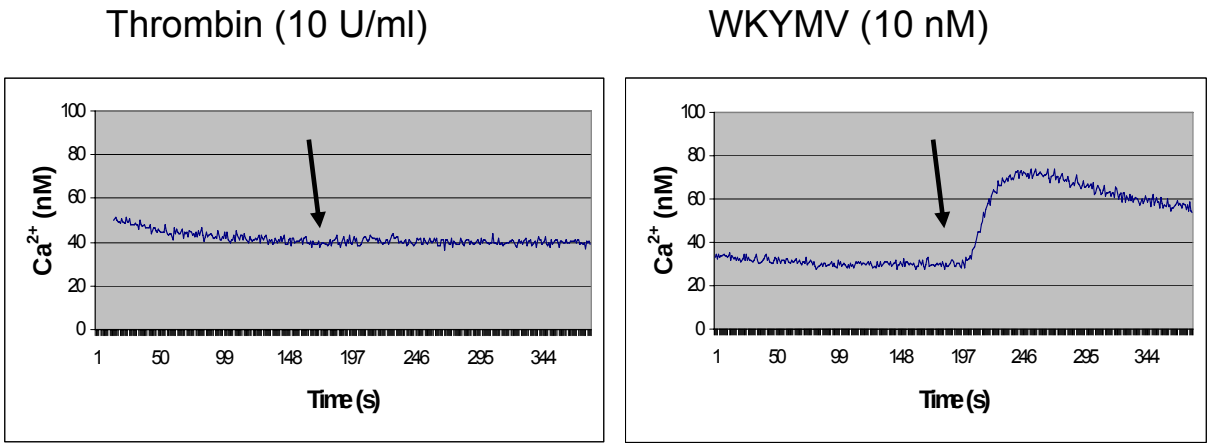


Figure 30: Thrombin stimulation does not increase cytosolic Ca $^{2+}$ levels in LPS-matured dendritic cells. (A) After 12 h of serum starvation, LPS-matured dendritic cells, were loaded with 5 µM Fura-2 acetoxymethyl ester for 30 minutes, and then stimulated with 10 U/ml thrombin. 10 nM WKYMV was used as a positive control. The fura-2 fluorescence was measured at two wavelengths, with excitation at 340 and 380 nm and emission at 510 nm. The ratio of the fluorescence at the two wavelengths is proportional to the calcium concentration in nM. One representative experiment out of three is shown.

Surprisingly, thrombin stimulation of mature dendritic cells did not result in any changes of the cytosolic Ca^{2+} level when different concentrations of thrombin were used (0.1 U/ml, 1 U/ml and 10 U/ml). WKYMV activates the formyl peptide receptor and is able to induce a Ca^{2+} flux in monocytes, immature and mature dendritic cells[141].

Thus, thrombin stimulation of LPS-matured dendritic cells does not induce an cytosolic calcium increase.

3.2.6. Thrombin stimulation slightly upregulates pERK

Thrombin signalling provides a pathway to mitogen-activated kinases (MAPK) [153]. In HUVEC cells thrombin activates p38 MAPK[154] and in Jurkat T cells p38 as well as ERK are activated by thrombin[155].
After thrombin signaling in dendritic cells,we monitored the activation of p38 and ERK.

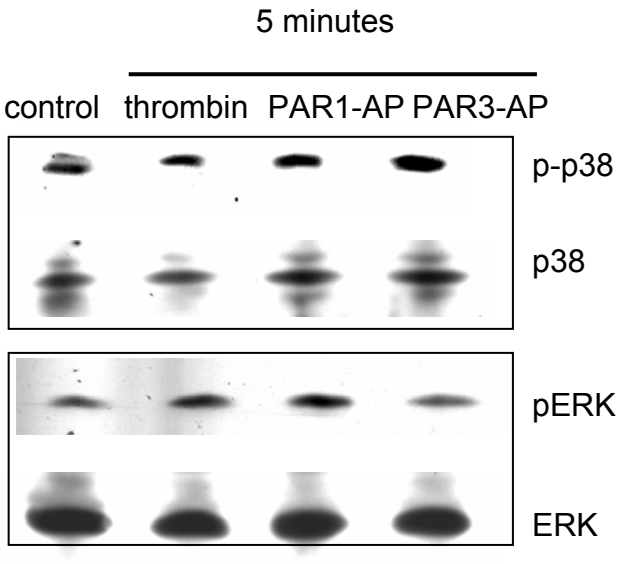


Figure 31: Activation of PAR1 and PAR3 with thrombin, PAR1- or PAR3-AP slightly induce ERK phosphorylation. LPS-matured dendritic cells were serum starved for 12 h, and stimulated with thrombin (1 U/ml), PAR1-AP (100 µM) or PAR3-AP (100 µM). Western blots were made from whole cell lysates. p38 and ERK were used as loading controls for the corresponding phosphorylated forms. One representative experiment out of three is shown.

Dendritic cells were stimulated with thrombin, PAR1- or PAR3-AP. These peptides have the same sequence as the N-tethered ligand, created after the thrombin cleavage of the receptor. Using PAR-activating peptides as stimulators, we could verify that the thrombin effect on dendritic cells is the result of PAR1 and PAR3 activation.
Thrombin and PAR1/3-AP stimulation of dendritic cells did not induce phosphorylation of p38.
Phosphorylation of ERK was somewhat up-regulated when thrombin and PAR1-AP were used as stimuli, but not with PAR3-AP.

Western blot analysis showed only minor changes in the phosphorylation of ERK. Therefore, we concluded that thrombin signaling does not induce significant changes in p38 and ERK.

3.2.7. Thrombin induces expression of inflammatory cytokines

Cytokine production is an important feature of dendrtic cells. It enables their communication with the interacting cells. To define the function of thrombin receptors in dendritic cells, we analysed the cytokine expression in dendritic cells after thrombin stimulation.

As readout, we have chosen eight cytokines: IL-12, IL-8, IL-6, MCP-1, CCL17, CCL22, MIP-3α and TNF-α.

IL-12 is a dominant cytokine produced by dendritic cells, important for inducing Th1 type of T cells[156]. Dendritic cells are expressing the chemokines IL-8, MCP-1, and their receptors[157]. IL-8 is a chemoatractant for neutrophils and T lymphocytes and may function as a key mediator in diverse inflammatory disorders[158]. MCP-1 is a monocyte, eosinophil, and lymphocyte chemoattractant, and plays an important role in chronic inflammatory and allergic diseases. CCL17 (TARC) and CCL22 (MDC) play an important role in the secondary lymphoid organs by recruiting naive T cells that express the receptors for these cytokines[159,160]. TNF-α is produced after maturation, and has an important role in the induction of the immune response[161]. MIP-3α is expressed predominantly in inflamed and mucosal tissues and is chemotactic for $CD34^+$ derived dendritic cells and T cells[162]. The cytokine expression was analysed at the mRNA level.

3.2.7.1. Optimisation of the PCR conditions

The primers were taken from the literature, yet it was necessary to optimise the PCR conditions concerning the number of cycles to be used. The number of cycles versus the amount of the PCR product ratio has to be in the linear range. If the number of cycles is too low or too high, the differences in the mRNA expression upon different stimulation may not be seen.

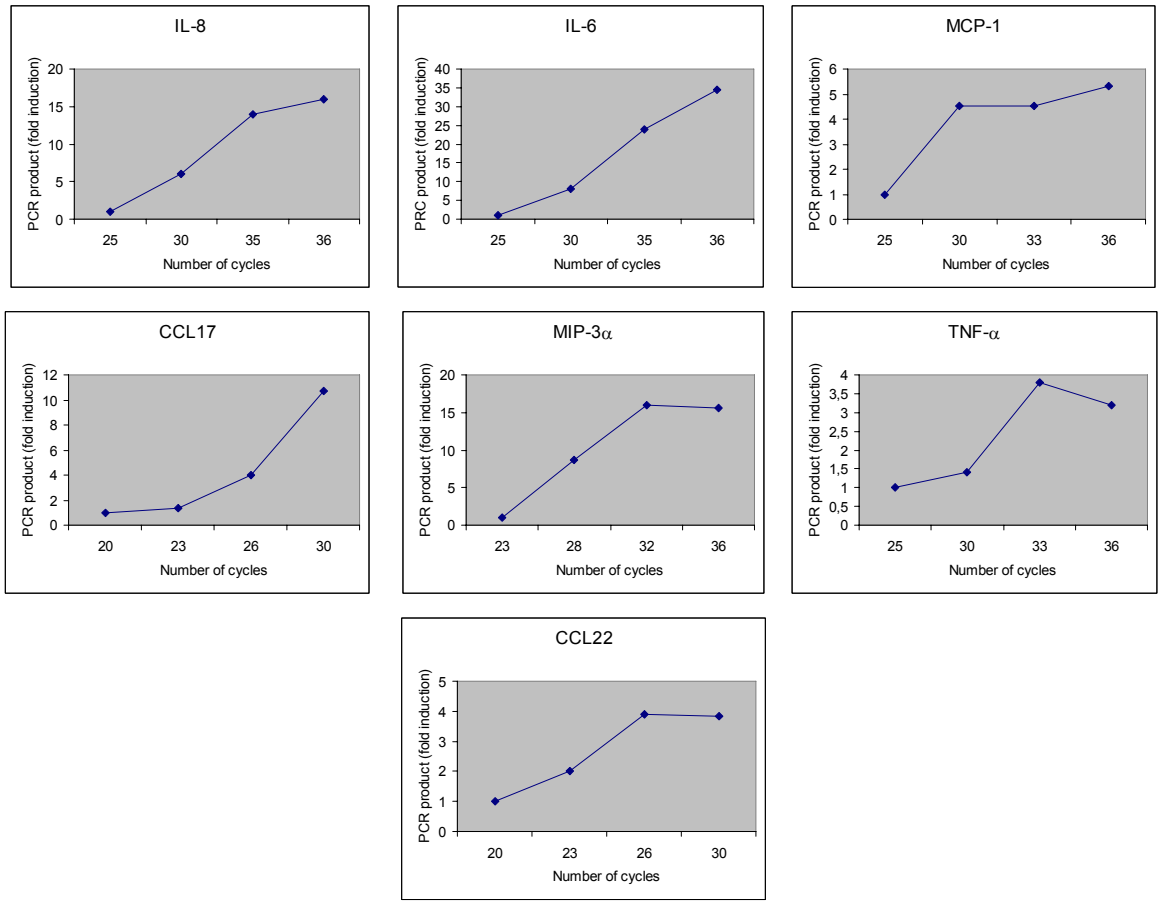


Figure 32: The amount of PCR product versus the number of cycles. LPS-matured dendritic cells were incubated in serum free medium for 12 h and then stimulated with thrombin and activation peptides for 8 h. Their mRNA was isolated, and cDNA was synthesized by RT reaction. The same PCR reaction mixtures were amplified for different numbers of cycles. The amount of the PCR product was quantified by densitometric analysis and normalised to the PCR product obtained with the lowest number of cycles (fold induction).

The optimum number of cycles for the tested cytokines was as follows: IL-8 -33 cycles, IL-6 -35 cycles, MCP-1 -28 cycles, CCL17 -28 cycles, MIP-3α -28 cycles, TNF-α -32 cycles and CCL22 -23 cycles.

3.2.7.2. The cytokine expression profile of thrombin-stimulated mature dendritic cells

Dendritic cells were incubated in serum-free medium for 12 h and then stimulated with thrombin (10 U/ml) for 8 h.

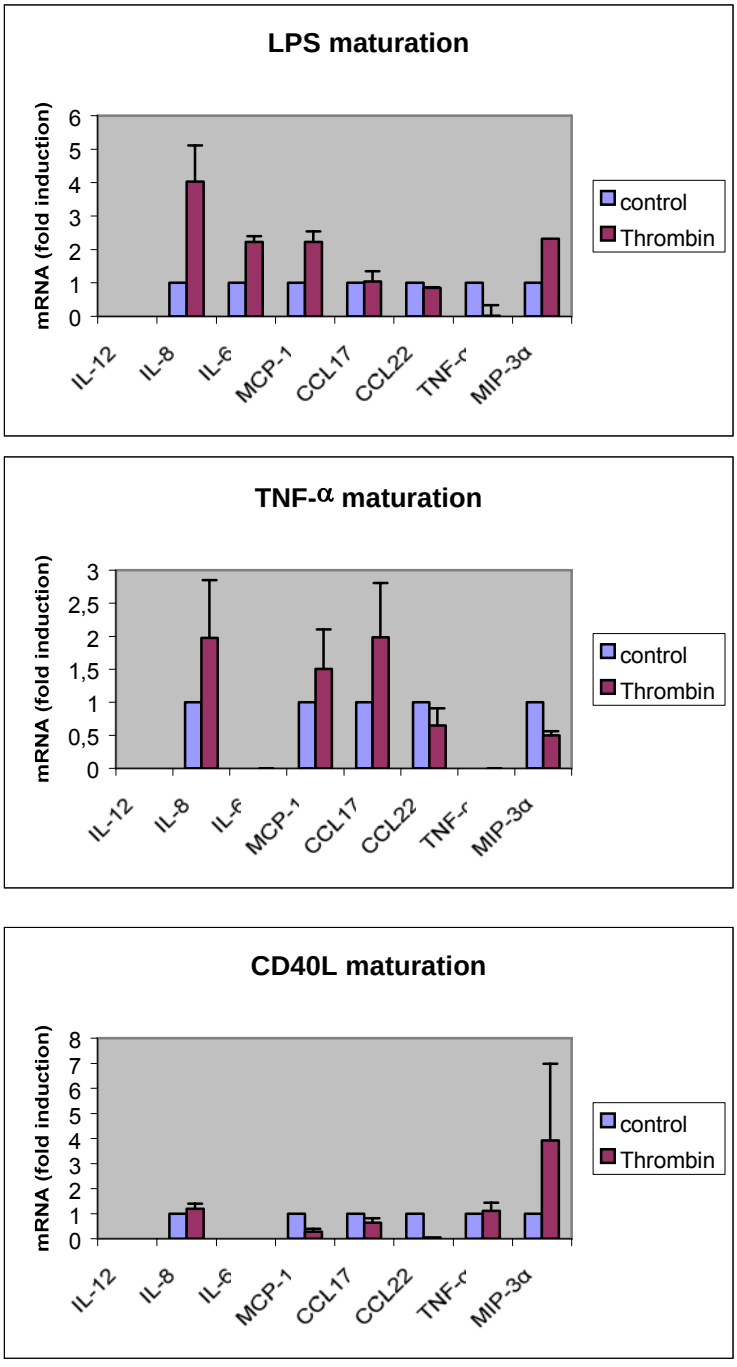


Figure 33: Thrombin regulates expression of various cytokines in LPS, TNF-α and CD40L matured dendritic cells. The dendritic cells were treated with thrombin (10 U/ml) for 8 h. The cells were lysed, mRNA isolated and after the RT reaction, the cytokine expression

was analysed by PCR. Data are represented as mean (± SEM) of four independent experiments. The level of cytokine expression was quantified by densitometric analysis, normalised to GAPDH, and then normalised to the control (non-treated cells).

Comparison the cytokine expression profiles of LPS-matured dendritic cells, with the two other maturation stimuli, implicated that LPS-matured dendritic cells have the broadest cytokine repertoire. They express all tested cytokines except IL-12. The TNF-α–matured dendritic cells do not express IL-12, IL-6 and TNF-α. The CD40L-matured dendritic cells do not express IL-12 and IL-6. Upon thrombin stimulation, of LPS-matured dendritic cells, we observed IL-8 mRNA expression increase, and a minor, only two fold increase of IL-6 and MIP-3α. The TNF-α expression was downregulated.

Analysis of the cytokine expression in TNF-α and CD40L-matured dendritic cells revealed no significant changes upon thrombin stimulation.

Thus, when we compare the three different types of mature dendritic cells, we can say that only LPS-matured dendritic cells can respond to thrombin stimulation with the synthesis of IL-8 and MIP-3α.

3.2.7.3. Upregulation of the IL-8 and MIP-3α expression after stimulation with PAR1- and PAR3- activating peptides

Dendritic cells were stimulated with either thrombin, PAR1- or PAR3-AP. As mentioned earlier, PAR-activating peptides are thrombin agonists.

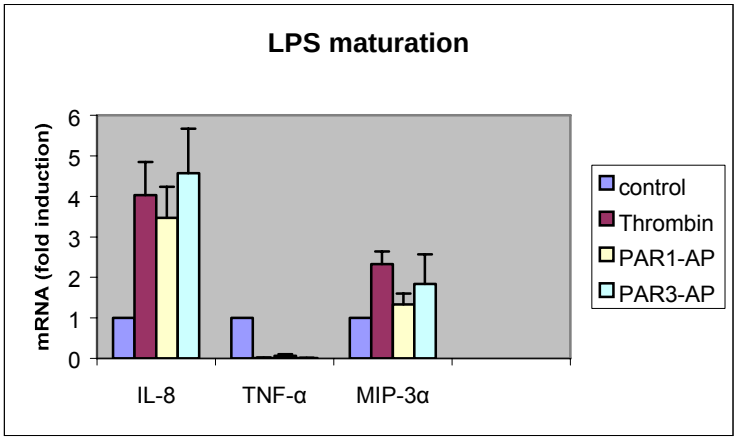


Figure 34: In LPS-matured dendritic cells, PAR1 and PAR3 activation, leads to mRNA expression of IL-8 and MIP-3α, whereas TNF-α is downregulated. Dendritic cells were serum starved for 12 h, and treated with thrombin (10 U/ml), PAR1-AP (100 μM), PAR3-AP

(100 μM) for 8 h. The cells were lysed, mRNA was isolated, and the cytokine expression was analysed by RT-PCR. Data are represented as mean (± SEM) of four independent experiments. The level of cytokine expression, normalised to GAPDH, and then normalised to the control (non-treated cells), was quantified by densitometric analysis.

In LPS-matured dendritic cells, we detected an increase in IL-8 and MIP-3α mRNA expression upon thrombin stimulation.

This increase was also observed when either PAR1- or PAR3-AP was used, suggesting hat the upregulation of the cytokine production was a result of the thrombin signalling through PARs.

Upregulation of the IL-6 expression after thrombin stimulation was not followed by an upregulation after PAR1 or PAR3-AP stimulation. These data suggest that the IL-6 mRNA increase was a result of thrombin signalling trough receptors other then PARs.

The effect of trombin on TNF-α downregulation was repeated with synthetic peptides, which lead to the conclusion that it is PAR activation dependent.

To summarise, PAR1 and PAR3 activation leads to IL-8 and MIP-3α upregulation and TNF-α downregulation. This effect is observed only in LPS-matured dendritic cells. No significant changes in the mRNA expression after thrombin stimulation were detected in TNF-α or in CD40L matured dendritic cells.

3.2.7.4. IL-12 production by dendritic cells

IL-12 is one of the most important cytokines produced by dendritic cells, because it induces T cell differentiation towards Th1 type[163]. However, we were unable to amplify it, therefore we used different templates, to detect IL-12 mRNA in other cell types.

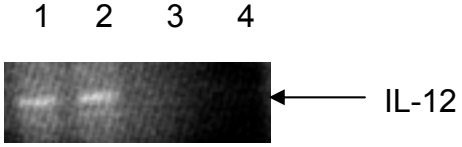


Figure 35: IL-12 mRNA is expressed in monocytes and macrophages, but not in dendritic cells. mRNA was isolated using TRIzol from: monocytes (1), macrophages –

differentiated from monocytes for 7 days in the presence of M-CSF (15 ng/ml) (2), immature dendritic cells – cultivated for 6 days in the presence of GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) (3), mature dendritic cells – cultured the same way as the immature dendritic cells and additionally treated for 48 h with LPS (0.5 µg/ml) (4). The cells were lysed, RNA isolated, and 1µg of RNA from each cell type was used for the RT-PCR. The PCR products were run on the 1% agarose gel. The picture is showing one representative experiment out of two.

These data demonstrate that we detected IL-12 mRNA in monocytes and macrophages, but not in dendritic cells. For this experiment, 36 cycles of PCR amplification was used. These results suggest that after 48 h maturation with LPS dendritic cells do not express IL-12.

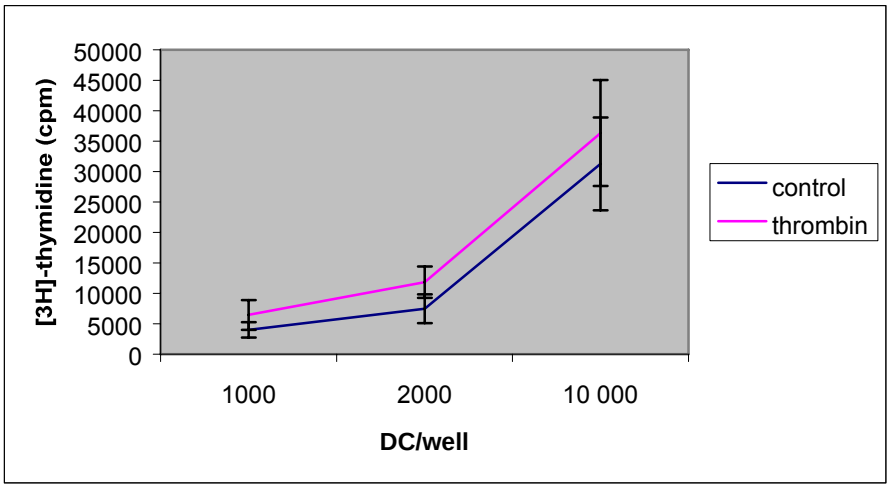
3.2.8. Thrombin-stimulated dendritic cells induce T cell proliferation

Dendritic cells have the potency to initiate T cell-dependent immune responses.
Becouse we found that only LPS-matured dendritic cells can respond to thrombin, we tested their capacity to stimulate allogeneic naïve T cells, before and after the thrombin stimulation.

3.2.8.1. Mitomycin C treatment of dendritic cells

Before co-cultivation, dendritic cells are often irradiated or treated with mitomycin C, in order to prevent their proliferation. If the pre-treatment is not used, a mixed leukocyte reaction could represent two-way reactions, in other words, dendritic cells can stimulate T cell proliferation and T cells in a feedback, can stimulate dendritic cells. However, in these experiments the small number of dendritic cells used as stimulators contributed negligible to proliferation compared with the large population of responder T cells. Non-proliferating dendritic cells in the MLR were also used by other groups[164-166].

A



B

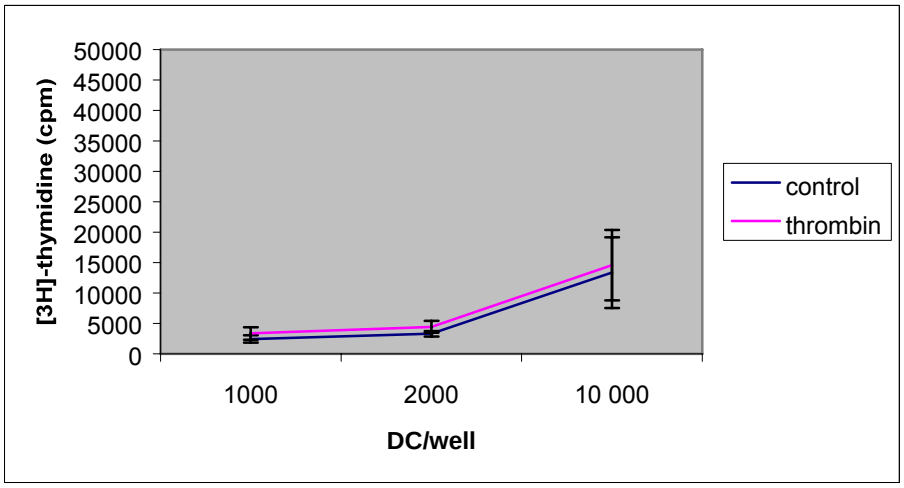


Figure 36: The influence of mitomycin C on the dendritic cell proliferation. LPS-matured dendritic cells were incubated for 1 h without (A) or with 50 µg/ml mitomycin C (B), washed and then treated with thrombin (10 U/ml), for 1 h. The cells were washed and co-cultivated with naïve T lymphocytes (10^5 cells/well) for 3 days. The last 16 h of co-culturing, 0.5 µCi of [^{3}H]-thymine per well was added. The cells were harvested, and the incorporated radioactivity was measured in the scintillation counter. The results represent the mean counts per minute (cpm) ± SEM of three independent experiments each performed in quadruplicate.

Co-culture experiments indicate, that when mitomycin was used, the dendritic cells were not able to induce the T-lymphocyte proliferation. We were not able to see any difference between control and thrombin stimulated dendritic cells. Therefore, for further experiments we used dendritic cells non-treated with mitomycin C.

3.2.8.2. PAR1- and PAR3-activated dendritic cells induce T cell proliferation

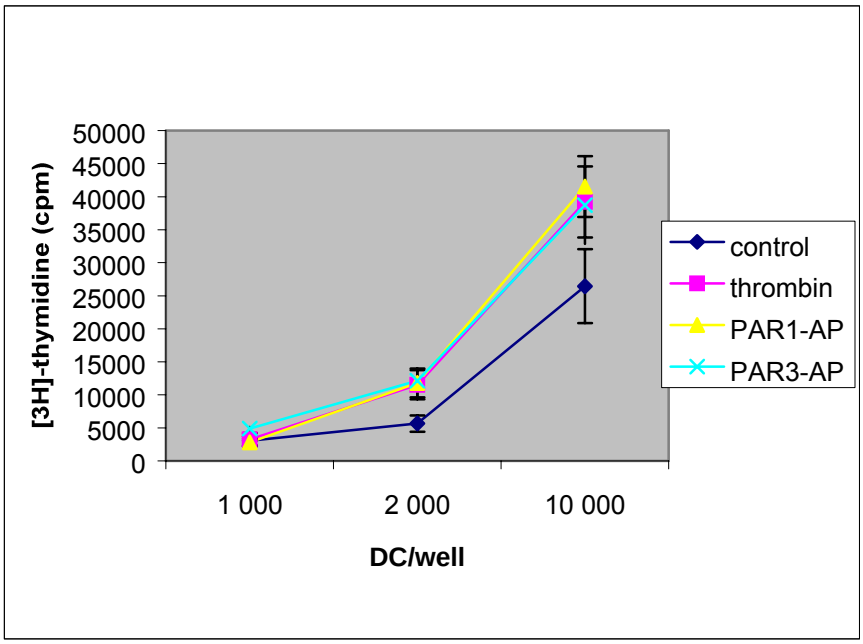


Figure 37: Allogeneic T cell stimulatory capacity of LPS-matured dendritic cells. LPS matured dendritic cells were serum starved for 12 h treated with thrombin (10 U/ml), or PAR1/3-AP (100 µM each) for 1 h, washed, and co-cultivated with naïve T cells (10^5 cells/well) for 3 days. The last 16 h of co-culturing, 0.5 µCi of [^{3}H]-thymine per well was added. The cells were harvested and the incorporated radioactivity was measured in a scintillation counter. The results represent the mean counts per minute (cpm) ± SEM of three independent experiments, each performed in quadruplicate.

The allostimulatory capacity of dendritic cells on T cells was higher when dendritic cells were stimulated with thrombin or with PAR1 and PAR3-AP, compared to control (non-treated cells). We can observe that with the increased number of dendritic cells, the T lymphocyte proliferative response was also increased. Minimal autologous lymphocyte proliferation resulted when 2000 dendritic cells per well were used. Significant differences between control and thrombin stimulated cells were observed only when 10000 dendritic cells/well were used.

These results imply that only mitomycin C un-treated dendritic cells, cultured in a ratio 1:10 with T cells, can induce proliferation when stimulated with thrombin, PAR1- or PAR3-AP.

3.2.9. Thrombin stimulation induces chemotaxis

The ability of dendritic cells to migrate is a very important aspect of their function[141,195,219]. Here we investigated dendritic cells migration towards thrombin.

3.2.9.1. Concentration-response curves of dendritc cells migration

First we monitored a concentration-response effect for thrombin, PAR1- and PAR3-AP on the chemotaxis of LPS-matured dendritic cells.

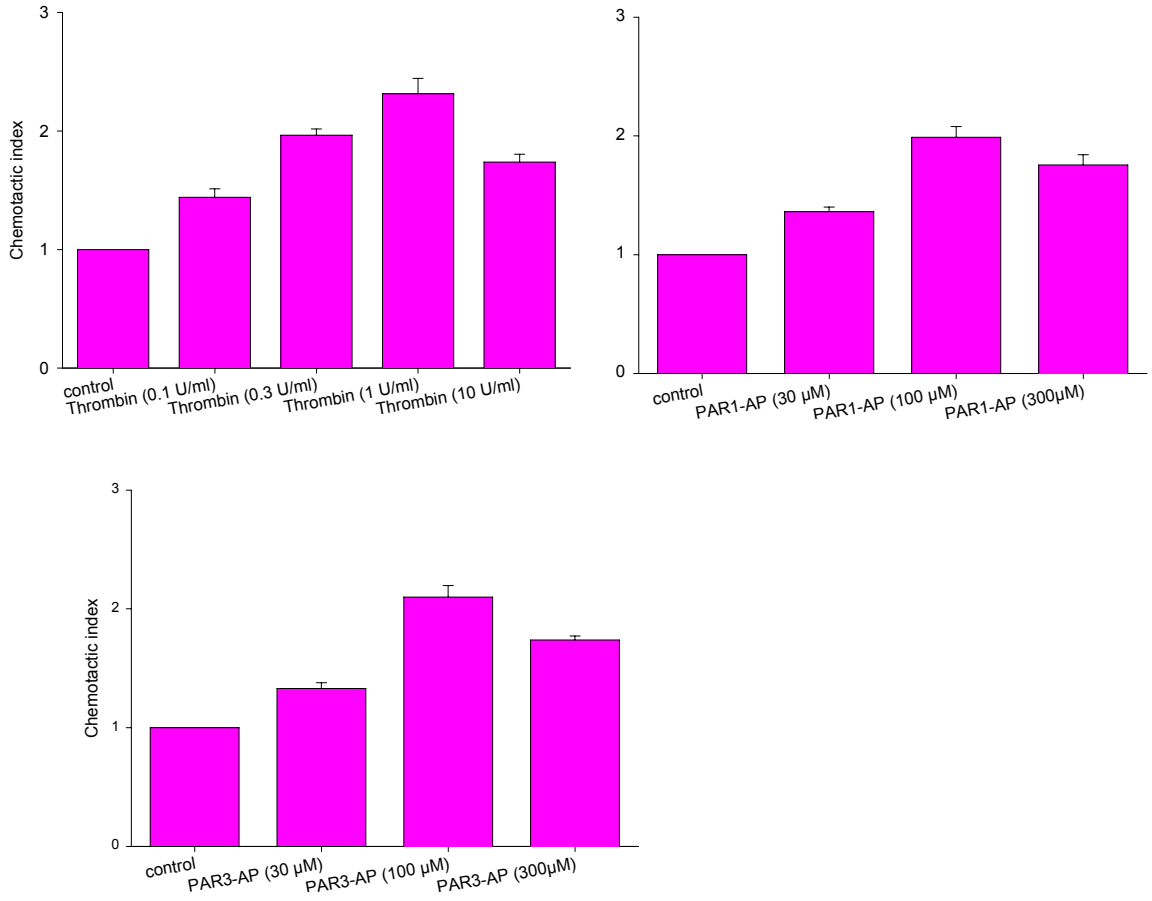


Figure 38: Thrombin, PAR1-AP and PAR3-AP induce migration of LPS-matured dendritic cells. The experiments were performed in Transwell 24-well chemotaxis chambers, with 5 µm pore size. 0.2x 10^5 cells were seeded in the upper chamber and incubated for 2 h at 37°C and 5% CO_2. After migration, the cells on the membranes were fixed, stained, and those that migrated through the membrane were counted. Results are given as means ± SEM

of three independent experiments. The chemotactic index is the ratio of the migration in the presence of chemotactic stimulus and the spontaneous migration (medium only).

A thrombin concentration of 0.1 U/ml was not enough to induce dendritic cells migration. With 0.3 U/ml, a rapid increase of the chemotactic index was observed, and the peak of stimulation was reached with 1 U/ml of thrombin.

PAR1- and PAR3-AP were not as strong stimuli as thrombin, yet 100 µM concentrations resulted in an almost two fold induction.

In further experiments we used the following concentrations of stimulators 1 U/ml thrombin, 100 µM PAR1-AP, and 100 µM PAR3-AP.

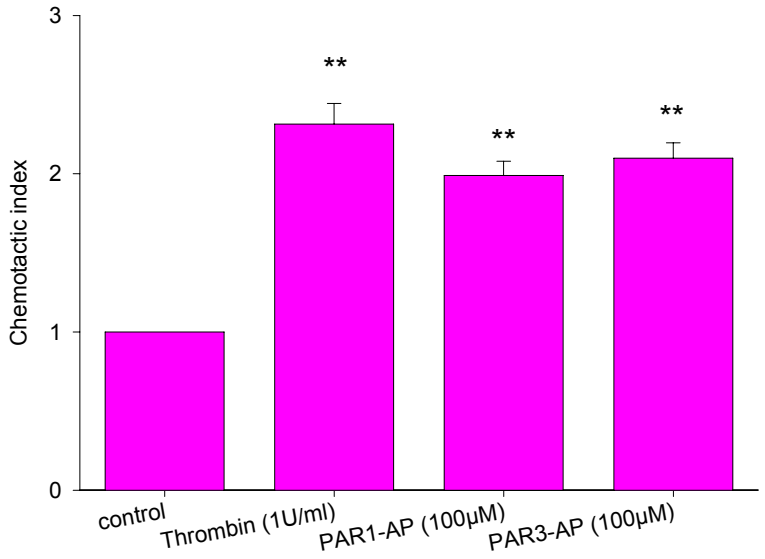


Figure 39: Comparison of the thrombin, PAR1-AP and PAR3-AP-induced chemotaxis of LPS-matured dendritic cells. The experiments were performed in Transwell 24-well chemotaxis chambers, with 5 µm pore size. 0.2x 10^5 cells were seeded in the upper chamber and 1 U/ml of thrombin, 100 µM PAR1-AP and 100 µM PAR3-AP were used as chemoattractants in the lower chamber. The cells were incubated for 2 h at 37°C and 5% CO_2. After migration, the cells on the membranes were fixed, stained, and those migrated through the membrane were counted. Results are given as means ± SEM of three independent experiments. The chemotactic index is the ratio of the migration in the presence of chemotactic stimulus and the spontaneous migration (medium only). **p<0.01 vs. control.

Thrombin as chemotattractant agent elicited the strongest response. PAR1 and PAR3-AP stimulation resulted in approximately the same response, which was slightly lower then that of thrombin. The fact that PAR1-AP and PAR3-AP

also elicited a chemotactic response, comparable to that of thrombin indicated the thrombin might exert its effect via PAR1 and PAR3.

3.2.9.2. Proteolytic blockage of PAR1 and PAR3 using antibodies

In order to confirm that thrombin signals through PAR1 and PAR3, specific antibodies were used to block the receptor activation by thrombin.

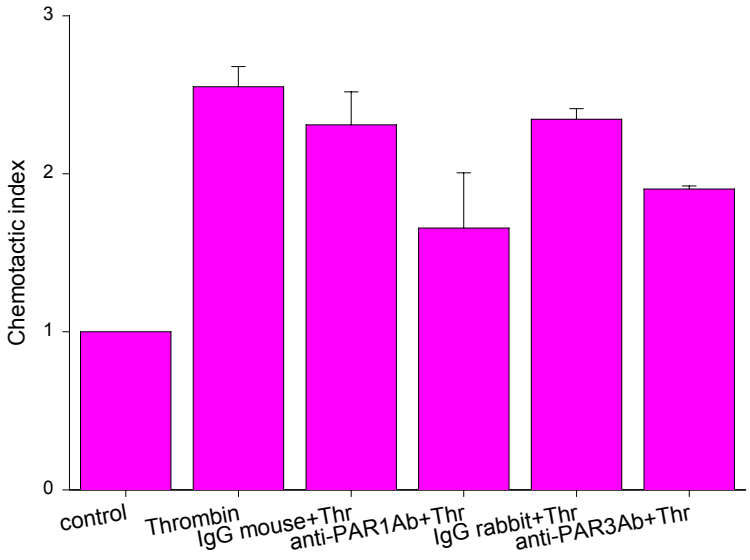


Figure 40: Effects of PAR1 and PAR3 antibodies on the thrombin-induced migration of LPS-matured dendritic cell migration. Dendritic cells were incubated with PAR1 antibody (50 µg/ml) or PAR3 antibody (10 µg/ml) for 30 min and then chemotaxis towards thrombin (1 U/ml) was monitored. The experiments were performed in Transwell 24-well chemotaxis chambers, with 5 µm pore size. 0.2x 10^5 cells were seeded in the upper chamber and incubated for 2 h at 37°C and 5% CO_2. After migration, the cells on the membranes were fixed, stained, and those migrated through the membrane were counted. Results are given as means ± SEM of three independent experiments. The chemotactic index is the ratio of the migration in the presence of chemotactic stimulus and the spontaneous migration (medium only).

Mouse IgG and rabbit IgG were used as an isotypic controls for PAR1 and PAR3 antibodies, respectively. The migration of the dendritic cells in the presence of IgGs was almost unchanged. Preincubation of the dendritic cells with PAR1 and PAR3 antibodies diminished the chemotactic response. When PAR1 or PAR3 antibodies were used, the chemotactic response was not

completely abolished suggesting that blockage of PAR1 receptor still allowed migration by signaling through PAR3 and *vice versa*.

3.2.9.3. Immature dendritic cells do not respond to thrombin

A good evidence that the thrombin indeed initiated a chemotactic response via PAR1 and PAR3 was obtained from the experiment with immature dendritic cells.

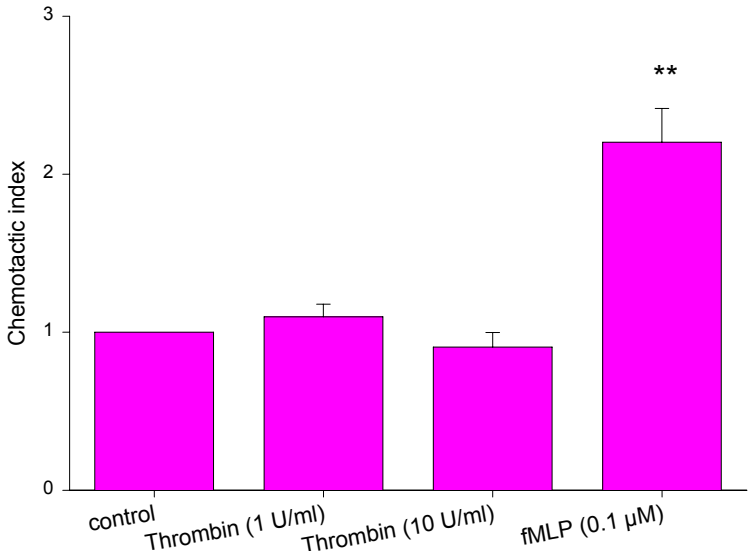


Figure 41: Immature dendritic cells do not respond to thrombin. Experiments were performed in Transwell 24-well chemotaxis chambers, with 5 µm pore size. 0.2x 10^5 cells were seeded in the upper chamber and in the lower chamber, 1 U/ml, 10 U/ml and 0.1 µM fMLP were used as stimuli. The cells were incubated for 2 h at 37°C and 5% CO_2. After migration, the cells on the membranes were fixed, stained, and those migrated through the membrane were counted. Results are given as means ± SEM of three independent experiments. The chemotactic index is the ratio of the migration in the presence of chemotactic stimulus, and the spontaneous migration (medium only). **p<0.01 vs. control.

Thrombin stimulation of immature dendritic cells did not result in a chemotactic response. These data are consistent with our finding that immature dendritic cells express no PARs, therefore, could not be activated by thrombin.

FMLP was used as a positive control, because it is known that immature dendritic cells express formyl receptors, which could be activated by fMPL[141]. To conclude, we can say that immature dendritic cells do not respond to thrombin in a chemotactic manner, whereas mature dendritic cells do. This response is mediated through PAR1 and PAR3, because the specific agonists of these receptors provoke the same response as thrombin.

3.2.10. Cytoskeleton rearrangement in chemotaxis

Directional cell migration is accompanied by polarization of the cell body. The main cytoskeleton rearrangement during cell migration involves actin polymerization at the leading edge and myosin activation at the rear of the cell enabling cell contraction and detachment [167].

3.2.10.1. Thrombin induces actin polymerization

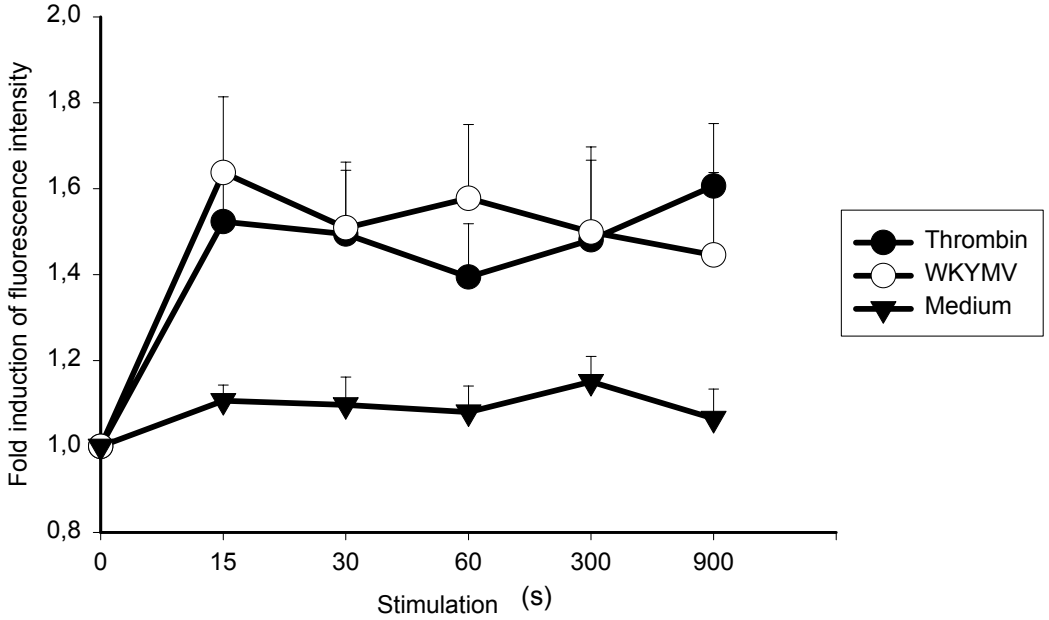


Figure 42: Thrombin stimulation of LPS-matured dendritic cells induces actin polymerization. Dendritic cells at a concentration $5x10^6$ cells/ml were stimulated with 1 U/ml of thrombin for the indicated time periods. The cells were fixed, permeablised and stained with Texas Red Phalloidin. The fluorescence intensity was measured using a FACScan, and the mean fluorescence intensity was determined by the Cell Quest program. Peptide WKYMV

(10 nM) was used as the positive control. Results are presented as mean ± SEM of three independent experiments. *p<0.05 vs. control for 0 time point.

Within 15 s of thrombin stimulation, a rapid 1.5-fold actin polymerization was detected. It remained on the same level, between 1.4 and 1.6 fold induction for the observation period of 15 minutes. WKYMV stimulation resulted in a slightly stronger, but equally persistent response. Medium was used as a negative control.

3.2.10.2. Myosin phosphorylation as a result of PAR signaling

In migrating cells, phosphorylation of the motor protein myosin II has an important role in regulating actomyosin contractility. Myosin consists of two heavy and two light chains[168]. Myosin II activation is mainly controlled by its light chain (MLC) phosphorylation.

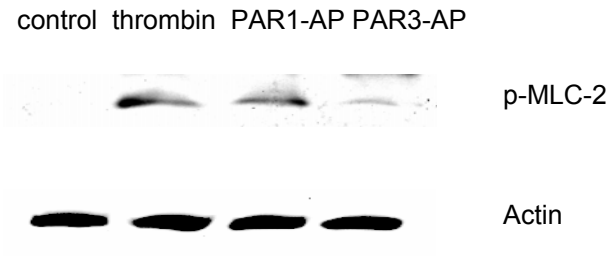


Figure 43: Thrombin stimulation of LPS-matured dendritic cells leads to phosphorylation of the myosin light chain (MLC). Dendritic cells were stimulated with 1 U/ml of thrombin, 100 µM PAR1- or 100 µM PAR3-AP. 5 minutes and 30 minutes after stimulation, total cell lysates were prepared and immunoblotted using anti phospho-MLC2 antibody. Actin was used as loading control. The picture is showing one representative experiment out of three.

The Western blot results clearly demonstrated myosin light chain phosphorylation after thrombin stimulation. The signal coming from stimulation with PAR3 – activating peptide is weak, but still significant compared to control.

3.2.11. Rho-GTPases are involved in dendritic cell migration to thrombin

In migrating cells, Rho regulates the formation of the stress fibers, focal adhesion complexes, and contractile actin-myosin filaments. Rho-GTPases cycle between an active, GTP-bound, and inactive, GDP-bound conformation[169]. In an active state, Rho can activate its kinase - ROCK. ROCK is a serine/threonine kinase which activates MLC in two different ways, either by direct phosphorylation of MLC[170], or by phosphorylation and inactivation of MLC phosphatase (MLCP), which leads to a decreased rate of MLC dephosphorylation. We further investigated the role of ROCK in dendritic cell migration to thrombin.

3.2.11.1. Upregulation of ROCK

To test the ROCK activation, we used MYPT, a recombinant protein representing a part of the MLCP catalytic subunit, as a substrate in an *in vitro* kinase assay.

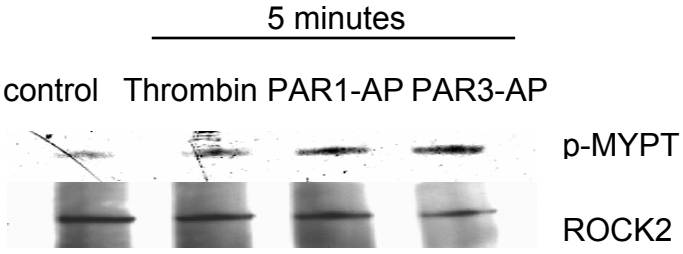


Figure 44: Thrombin stimulation of LPS-matured dendritic cells activates ROCK-2. Dendritic cells were stimulated with 1 U/ml of thrombin for 5 minutes. The cells were lysed and the ROCK-2 kinase was immunoprecipitated using ROCK-2 antibody and protein A/G sepharose beads. The kinase assay was performed at 30^{o}C for 30 min with 0.1 mM ATP and 0.5 µg of substrate - MYPT. Samples were run on 8% SDS-PAGE gels and immunoblotted using phospho-MYPT antibody. One representative experiment out of three is shown.

The activity of ROCK-2 in non-stimulated cells was very low. Thrombin, PAR1- and PAR3-AP induced an increased substrate phosphorylation, indicating the stimulated higher kinase activity.

3.2.11.2. The ROCK inhibitor, Y27632, abolished MLC phosphorylation

Using the ROCK specific inhibitor, Y27632, to block the thrombin signaling pathway, we validated ROCK as a pathway member. The cells were pretreated with Y27632[171] and allowed to migrate using thrombin as a stimulus. 3 µM inhibitor was used to block the kinase activity and tested if that would prevent myosin light chain 2 (MLC2) phosphorylation. MLC2 is a downstream member of the thrombin signaling pathway.

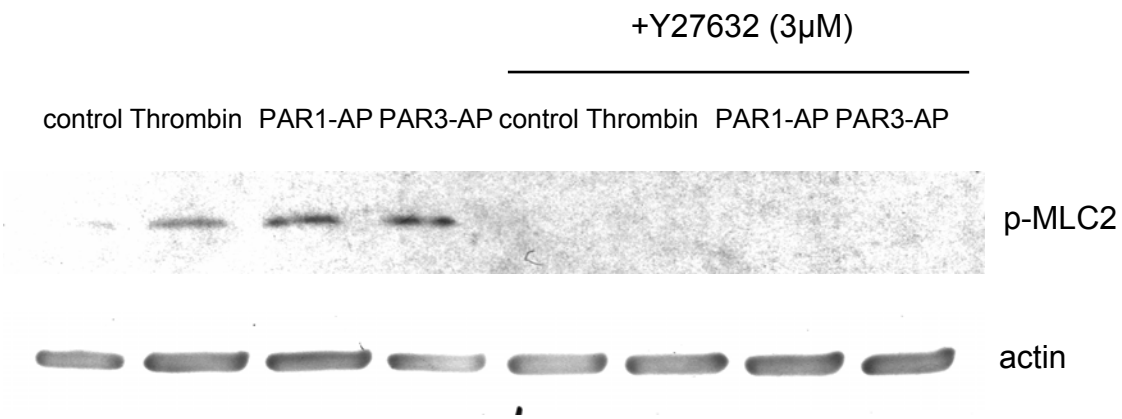


Figure 45: The ROCK inhibitor Y27632 blocks the thrombin-induced MLC2 phosphorylation. LPS-matured dendritic cells were preincubated with Y27632 (3 µM) for 30 minutes, washed and stimulated with 1 U/ml of thrombin, 100 µM PAR1- or 100µM PAR3-AP for 5 minutes, lysed and immunoblotted as described above. The picture is showing one representative experiment out of three.

Two batches of cells were used, control dendritic cells, and dendritic cells pre-treated with the inhibitor. Both groups were stimulated with thrombin and PAR1 and PAR3-AP for 5 minutes. The ROCK inhibitor completely abolished the myosin light chain phosphorylation.
This indicates that thrombin signaling in dendritic cells activates the ROCK pathway and that ROCK activity can be efficiently inhibited by Y27632.

3.2.11.3. The ROCK inhibitor, Y27632, prevents dendritic cell chemotaxis to thrombin

After verifying that inhibition of ROCK abolished the MLC phosphorylation, we preformed the chemotaxis experiment with inhibitor-pretreated cells.

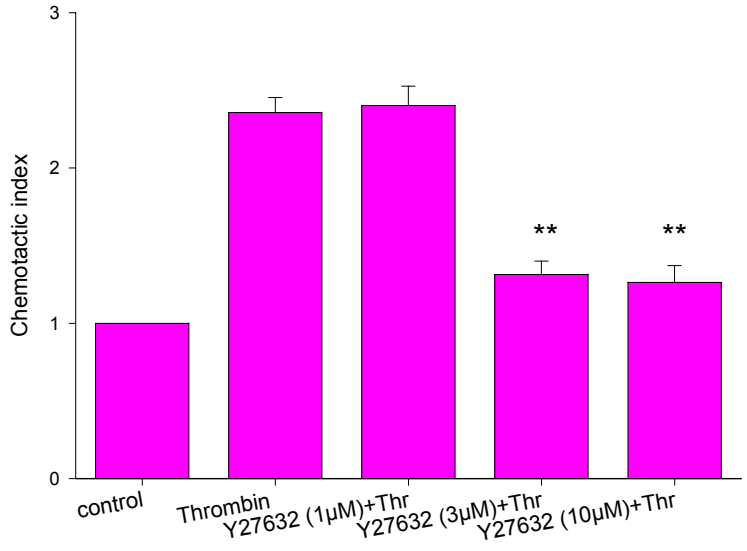


Figure 46: The ROCK-specific inhibitor abolishes the thrombin-induced migration of dendritic cells. 0.2x 10^5 cells were incubated with the Y27632 for 30 minutes and then added to the upper wells of the chambers. 1 U/ml of thrombin was added to the lower well. The cells were incubated for 2 h at 37^{o}C and 5% CO_2. After migration, the cells on the membranes were fixed, stained, and those migrated through the membrane were counted. Results are given as means ± SEM of three independent experiments. The chemotactic index is the ratio of the migration in the presence of chemotactic stimulus, and the spontaneous migration (medium only). **p<0.01 vs. inhibitor non-treated cells.

Different concentrations of the ROCK inhibitor showed that 1 μM was not enough to prevent the dendritic cells migration towards thrombin. 3 μM and 10 μM gave the same response, i.e. significantly diminished the chemotactic response to thrombin.

The fact that we prevented dendritic cell migration by using ROCK specific inhibitor proves that ROCK is an important member of the thrombin signaling pathway.

4. Discussion

4.1. PAR1 and PAR3 promoter analysis

The thrombin receptor PAR1 is the first identified member of the protease-activated receptor family. The activation of PAR1 results in different types of mitogenic and proinflammatory signals[172]. However, little is known about the regulation of PAR1 gene expression. Although PAR1 has been cloned[148] and the potential regulatory sequences in the PAR1 promoter region have been identified[173], determination of the transcription factors involved in PAR1 activation by different proinflammatory signals, is still not quite clear.
A potential AP-2/Sp1 potential binding sites have been described in PAR1 promoter[174]. AP-2 has an important role as a tumor suppressor in melanoma cells. The loss of AP-2 correlates with the overexpression of PAR1 which contributes to malignant phenotype of human melanoma[122]. Sp1 is a transcription factor responsible for the transcription of housekeeping genes.
The regulation of the PAR3 gene has still not been elucidated. Potential binding sites for the GATA transcription factor have been localized, as well as octamer sequences, which represent a binding sites for Oct transcription factors[124]. We can say that the analysis of regulation of both PAR1 and PAR3 promoters is still at the very begining.

In this work, the effect of different inflammatory agents on the PAR1 and PAR3 promoter activity was tested. Our results show that both PAR1 and PAR3 promoters are activated by PMA, as well as the combination of PMA/TNF-α. PAR1 and PAR3 responded similarly to PMA and TNF-α, suggesting that the same regulatory elements could be involved in activation of both promoters. Literature data imply that the downstream effect of PMA and TNF-α stimulation could be the activation of NF-κB and/ or AP-1 transcription factors[145,146,175]. Therefore, we investigated the role of NF-κB proteins in PAR1 and PAR3 promoter activation, by co-transfection of different NF-κB subunits with promoters.

The gene reporter assays demonstrate that NF-κB proteins activated PAR1 and PAR3 promoter. Specifically heterodimers of p50/RelB and p52/RelB, induced a rapid increase in PAR1 promoter activation. These dimmers had ten times stronger activation potency than p50, p52 or RelB alone, suggesting a synergistic effect. Activation of the PAR3 promoter was not as pronounced as PAR1; nevertheless, p50/RelB and p52/RelB also activated this promoter in a synergistic manner. The NF-κB activation proceeds in two different pathways: classical (canonical) and non-canonical[176]. The classical pathway is driven by the activation of a multi-subunit IκB kinase (IKK) complex, which contains two catalytic subunits, IKK1/IKKα and IKK2/IKKβ, and a regulatory subunit, NEMO/IKKγ[177]. The IKK complex leads to the inducible phosphorylation of IκB proteins their ubiquitination and subsequent proteasome-mediated degradation, thereby releasing NF-κB from their inhibitory influence Once released, NF-κB in the form of p50/p65 heterodimers, is free to migrate to the nucleus and to bind to the promoter of specific genes possessing its binding site. In addition to the above classical pathway, an alternative (or non-canonical) pathway of activation involves processing of p100/NF-κB2 into p52 subunit and formation of p52/RelB heterodimers. Unlike the classical pathway, which involves IKKβ and NEMO, activation of the alternative NF-κB pathway is critically dependent on NIK and IKKα[178].

In our model system, p50/p65 heterodimers had no effect on the PAR1 or PAR3 promoter activity. Therefore, we can exclude the classical NF-κB activation pathway and assume that the upregulation of the PAR1 and PAR3 promoters is a result of the alternative NF-κB pathway activation. P52/RelB are important for lymphoid organ development[179,180]. During maturation of dendritic cells, p50/RelB dimmers are exchanged by p52/RelB, suggesting an importance of this complex in the gene regulation during dendritic cells maturation.

The gene reporter data imply that the activity of the PAR1 and PAR3 promoters is upregulated by inflammatory stimuli and NF-κB proteins in a similar manner; both promoters are activated by PMA/TNF-α, and by

p52/RelB. So far, there is no evidence in the literature that PAR1 and PAR3 are regulated by NF-κB.

In order to localise the regulatory region we introduced 5' deletion mutations in PAR1 promoter, which is responsible for the PMA/TNF-α activation. When a 250 base pairs long region (positioned between -1106 and -863 bp upstream from the transcription start site) was deleted, the PAR1 promoter response to the proinflammatory agents was abolished. Introduction of further deletions had no effect on the PAR1 promoter activity. We assumed that the regulatory region is located between -1106 and -863 bp.

Surprisingly, when we compared the sequences of the PAR1 and PAR3 promoters, using the Basic Local Alignment Search Tool (BLAST), we found the homology of PAR1 and PAR3 sequences in exactly this, 250 bp region, on the PAR1 promoter 5' site, indicating that this homology region could reveal the regulatory site.

In order to find the NF-κB binding element in the PAR1 promoter, we performed footprinting experiments. DNase I digestion pattern revealed one footprint, and further analysis of this footprint region by EMSA demonstrated AP-1 binding.

Even though the gene reporter assay showed that NF-κB proteins activate the PAR1 promoter, no NF-κB binding site was detected. This includes the possibility that activated NF-κB dimer does not bind directly to the DNA but activates AP-1, which then occupies the AP-1 binding site on the promoter. In multiple myeloma, NF-κB activates the IL-6 gene, but the NF-κB binding to IL-6 promoter is not required. In stead, this factor cooperates with c-Jun, which occupies the AP-1 binding site on the IL-6 promoter. Such indirect effect of NF-κB on AP-1 activation has been described as a novel transcriptional mechanism for the activation of NF-κB-driven genes[181].

4.2. Function of PARs on dendritic cells

The dendritic cells used in *in vitro* studies have been derived from peripheral blood monocytes, cultured in the presence of the GM-CSF and IL-4. These cells are immature, having a high endocytic capacity and expressing low levels of MHC class II, CD83 and other co-stimulatory molecules[182]. Upon maturation with TNF-α, CD40 ligand, or LPS, dendritic cells down-regulate the mechanisms of antigen capture, including endocytic activity and the expression of Fc receptors and increase the expression of co-stimulatory and adhesion molecules[183]. These phenotypic changes parallel the functional transition of dendritic cells from antigen capturing to antigen presenting cells.
Similar changes indicating maturation have also been reported using PMA[184], poly (I:C) – a synthetic double stranded RNA[185], conditioned medium of adherent autologous monocytes in culture, named monocyte-conditioned medium (MCM) [186], and calcium ionophore[187].
We have chosen three different dendritic cell maturation stimuli, LPS as a model of bacterial infection, TNF-α as an inflammatory stimulus, and CD40 ligand, which mimics the T cell-dendritic cell cross talk.
FACS analysis of CD1a, CD11c, CD14, CD83, CD86, and HLA-DR (MHC class II) expression was corresponding to the literature data concerning the ability of the three above-mentioned stimuli to induce dendritic cells maturation[156,188,189]. All cells were CD1a positive, since they are developed from the myeloid progenitor[190], CD14 negative, meaning that the differentiation process from monocytes is completed, and they expressed CD11c, a marker for monocyte-derived cells. All three types of mature dendritic cells had a high expression of co-stimulatory molecules CD83 and CD86 compared to immature dendritic cells. MHC class II was expressed on all cells, providing no distinction between immature and mature dendritic cells. However, the level of MHC expression is not a parameter of maturation status, but the half-life of this molecule on the cell surface[27]. Immature dendritic cells have a significantly lower MHC class II half-life then mature dendritic cells.

However, this phenotypic characterisation of the expression of maturation markers could not provide any quantitative data concerning the possible level of maturation. Gad et al[191] have designated an additional stage between immature and mature dendritic cells – so called semi-mature, which can not be distinguished from mature dendritic cells by their surface markers. These semi-mature dendritic cells do not produce proinflammatory cytokines and therefore cannot activate T cells.

Expression of the protease-activated receptors in immature dendritic cells has been investigated by Colognato et al[133] and only very low levels of PAR1 and PAR3 mRNA were detected. The authors suggest that the expression of PARs is down regulated on transcriptional level in immature dendritic cells, compared to their precursors monocytes.

In this work, the expression of thrombin receptors PAR1, PAR3 and PAR4, in immature and mature dendritic cells was analysed. PAR2, being a trypsin receptor was not investigated here. The role of PAR2 in dendritic cells was studied by Fields and colleagues[92]. Activation of PAR2 on immature dendritic cells, by trypsin and trypsin agonists stimulates dendritic cell development, i.e. maturation. No evidence of thrombin effect on dendritic cells was found in the literature.

By RT-PCR analysis, only PAR1 and PAR3 mRNA were detected. PAR4 mRNA, could not be amplified using the primers according to Kahn et al[192]. When LPS was used for maturation, the most striking, a 10-fold PAR1 induction, at mRNA level, was detected. PAR3 mRNA expression was also increased in LPS-matured dendritic cells. CD40L-matured dendritic cells had an increase in PAR1 expression only. TNF-α maturation gave no significant changes in the mRNA expression of PARs in mature compared to immature dendritic cells.

Western blot analysis from whole cell lysates, detected an up-regulation of the PAR1 and PAR3 protein expression only in LPS-matured dendritic cells. FACS analysis revealed no presence of these receptors on the TNF-α and CD40L-matured dendritic cell surface.

Our results demonstrate that different maturation stimuli do not have the same effect on the PAR expression. The functional state of the dendritic cells, induced by different stimuli, may be relevant for the immune response outcome. The comparison of LPS and TNF-α stimulation by oligonucleotide microarrays[193], shows that only LPS-matured dendritic cells express genes required for the activation and control of the immune response. For instance, the production of cytokines IL-1β, IL-6 and IL-12 is upregulated in LPS, but not in TNF-α-matured dendritic cells. TNF-α appears to be a mild stimulus unable to drive dendritic cells to terminal differentiation. CD40L used alone as a maturation stimulus may not be sufficient. The combination of CD40L and LPS induces full maturation with strong cytokine expression[194].

An important feature of dendritic cell function is the cytokine production. They are synthesised in the dendritic cell environment, and by dendritic cells, regulating their migration and recruitment of other cells involved in the immune response. Dendritic cells migration is dependent on the expression of particular chemokine receptors on their surface, which enable them to move along the chemotactic gradient, driving the maturating dendritic cells toward the lymphatics and generating an immune response[157]. So far, human immature dendritic cells have been shown to express high levels of receptors for IL-8, MIP (macrophage inflammatory protein)-1α, MIP-1β, MCP (monocyte chemoattractant protein)1 and MCP-4 and RANTES, whereas mature dendritic cells express receptors for ELC (EBI-1 ligand), SDF-1 (stromal cell derived factor), SLC (secondary lymphoid tissue chemokine), MIP-3β and TARC (thymus and activation regulated chemokine)[195-198]. This differential sensitivity to cytokines is important for two reasons; first, it is necessary to enhance the recruitment of immature dendritic cells to the inflamed site, and second, with maturation dendritic cells respond to different cytokines, allowing them to leave the inflamed tissues and preparing them for trafficking to lymph nodes[196].

Not only that dendritic cells respond to cytokines in their environment, but they also produce a vide variety of cytokines themselves. IL-12 is released when dendritic cells are activated by microbial stimuli[199], and plays a major role in

cellular immunity by inducing the differentiation of naive T cells to Th1 cells[163]. IL-8 is produced after LPS stimulation[200], and is a chemokine for T cells and granulocytes[201]. MIP-3α is responsible for the recruitment of immature dendritic cells to the sites of injury[202], and is chemotactic for T cells[203]. IL-6 stimulates proliferation of B cells and their differentiation into antibody-secreting plasma cells[204], TNF-α is produced after maturation[161], as well as CCL17 (TARC) and CCL22 (MDC), which facilitate the attraction of T lymphocytes[205,206].

The maturation signals delivered through TNF-α, LPS or CD40L differ remarkably in their quality to stimulate the cytokine production by dendritic cells[193,207].

Analysis of the cytokine expression profile in these three maturation types as a result of thrombin stimulation revealed the up-regulation of the cytokine expression only in LPS-matured dendritic cells. We were not able to detect any IL-12 mRNA in thrombin stimulated dendritic cells.

The cytokine production profile of TNF-α and CD40L-matured dendritic cells showed no significant changes in the expression after thrombin stimulation. This implies again, that TNF-α and CD40L, when used alone, are weak stimuli for maturation of dendritic cells, which are able to respond to thrombin. Our data demonstrate that thrombin stimulation of LPS-matured dendritic cells, results in IL-8 and MIP-3α upregulation. This suggests that thrombin activates dendritic cells to produce the cytokines chemotactic for T cells, and therefore accelerates the formation of immunological synapse.

The final outcome of the dendritic cells maturation is interaction with the other cells of the immune system, and induction of the immune response. Dendritic cells activate T lymphocytes by inducing their differentiation and proliferation. Differentiation is a process that converts naïve T lymphocytes to effector cells, induced by cytokines and accessory molecules. Concerning differentiation, there are two possible outcomes, dendritic cells are inducing Th1 or Th2 differentiation of naïve T cells[208].

Proliferation is a response to antigen recognition. The *in vitro* model that we used to examine the proliferation of T cells was the co-culture of dendritic cells

with naïve $CD4^+$ T cells. Prior to co-culture, dendritic cells were stimulated with thrombin or PAR1- or PAR3- activating peptides for one hour.

The results suggest that thrombin stimulated dendritic cells have a higher ability to induce T cell proliferation, compared to non-stimulated dendritic cells. This effect was also observed with PAR1- and PAR3- activating peptide stimulation. The number of stimulator (dendritic cells) and responding cells (T cells) was varieted in order to find the optimal ratio. Dendritic cells vs. T cells ratio of 1:100 resulted in no proliferation effect, suggesting that not enough dendritic cells were present in the co-culture. Minimal T lymphocyte proliferation was observed when dendritic cells/T cell ratio was 1:50, and optimal with 1:10. Co-culture experiments indicate that thrombin stimulation activates dendritic cells toward more efficient T cell stimulator.

However, this experiment does not provide the information concerning the outcome of this interaction. We are not able to say whether thrombin-stimulated dendritic cells induced Th1 or Th2 subtype of T cells. Monocyte derived dendritic cells generated with GM-CSF and IL-4, and activated with LPS or MCM generate predominantly Th1 effectors[209,210]. In experiments where IL-12 production was inhibited by siRNA, dendritic cells were producing IL-10 and stimulating the production of Th2 cytokines by T cells in vitro[211]. Myeloid dendritic cells, have the ability to polarise T cells towards Th2 by downregulating IL-12[212]. If we look at the cytokine profile of LPS-matured dendritic cells, we find no IL-12 mRNA, even after thrombin stimulation. Therefore, we can assume that thrombin-stimulated dendritic cells induce T cell differentiation towards the Th2 type. Precise characterisation of these cells could be accomplished by further experiments of measuring the cytokine release (for example IL-4, IL-5) of co-cultivated T cells.

Thrombin is effective in stimulating monocyte and lymphocyte migration by signalling through PAR1 and induction of an cytosolic calcium increase in these cells[72]. Chemotaxis of eosinophils is also induced by thrombin in a dose-dependent manner[73], as well as chemotaxis of macrophage-like continuous cell lines, and neutrophil chemotaxis and aggregation[213-215].

One of the major findings in this work is that mature dendritic cells could also migrate towards thrombin. Peptide agonists of PAR1 and PAR3 clearly

produced thrombin-like effects. However, the chemotactic index of agonist stimulation was approximately the same as the thrombin response. This implies redundancy of the PAR1 and PAR3 signalling in the presence of thrombin, which activates both receptors. PAR1 and PAR3 antibodies were effective in blocking the thrombin response. When PAR1 antibody was used, thrombin signalling is mediated only through PAR3 and as a result, the chemotactic index was approximately 50% lower, compared to the cells with active PAR1 and PAR3. The results were the same under the opposite conditions – when PAR3 was blocked with specific antibody, and only PAR1 could respond to thrombin.

Further, we analysed thrombin signalling pathway in dendritic cells. Thrombin induces Ca^{2+} mobilisation in U937, monocytes and macrophages[98,216]. Thrombin stimulation releases calcium as a result of signalling through PARs. PARs are G-protein-coupled receptors, whose activation leads to a conformational change, and to activation of the G protein, which exchanges bound GDP with GTP. The α subunit of the G protein dissociates from $\beta\gamma$ subunits and activates PLC (phospholipase C). PLC hydrolyses phosphatidyl-inositol-1,2-bisphosphate to diacilglycerol (DAG) and inositol-3-phospahte (IP_3). IP_3 leads to the opening of the calcium channels and the release of Ca^{2+} ions from the intracellular pools. Diacylglycerol activates protein kinase C, which phosphorylates downstream proteins[217].

In dendritic cells the calcium release can be elicited by a variety of stimuli such as cell adhesion, platelet activating factor (PAF), interaction with T cells[218], formyl peptides[141], chemokines: RANTES, MIP-1α, MCP-3[219], SDF-1 (stromal cell- derived factor 1)[195,220], SLC (secondary lymphoid tissue chemokine)[220].

Surprisingly, Ca^{2+} as a secondary messenger was not involved in the thrombin signalling of dendritic cells. As will be discussed later, by Rho-kinase activation, thrombin signalling pathway in dendritic cells is calcium-independent.

Migration of dendritic cells is central for the induction of immune response. This process necessitates cytoskeleton rearrangement, i.e. actin polymerisation on the front of migration and depolymerization at the rear side of the cell[221]. Configuration of dendritic cell cytoskeleton requires Cdc 42, Rac and Rho[222]. Cdc 42 is a GTPase involved in cell polarisation, lamelipodia extension, dendritic cells endocytosis, and since mature dendritic cells have no endocytic ability, Cdc 42 is downregulated[223]. Rac leads to accumulation of the actin network at the cell periphery producing membrane ruffling and lamelipodia[223], important for dendritic cells movement. Rac1 is required for the dendritic cell maturation, the phagocytosis, the antigen uptake, and the cross-presentation to T cells. Rho is important for the organisation of the actin cytoskeleton in dendritic cells and in macrophages[224].

Thrombin stimulation of dendritic cells resulted in a rapid actin polymerisation, necessary for the cell movement toward stimulus, i.e. thrombin.

Since actin polymerisation can be regulated by Rho GTP-ases[225], we analysed the activity of the Rho kinase (ROCK). The ROCK assay proved that the higher activity of this kinase is a result of thrombin stimulation.

The contraction of the cell body can be regulated by Rho. Rho acts via ROCK to affect MLC (myosin light chain) phosphorylation, both by inhibiting MLC phosphatase and by directly phosphorylating MLC[226,227]. MLC phosphorylation is also regulated by MLC kinase (MLCK), which is activated by calcium, and stimulated by ERK and MAPKs[228]. As mentioned previously, we detected no calcium response. Western blot analysis showed a slight change in ERK activation and no change in p38 (member of the MAPK family) activation. Therefore, the activity of MLCK was not further investigated.

There is now considerable evidence suggesting that thrombin signalling involves Rho/ROCK activation, which results in MLC phosphorylation[34, 35]. We detected an increase in phosphorylated MLC, after thrombin stimulation. Dendritic cell treatment with the specific pharmacological inhibitor of ROCK (Y-27632) completely abolished MLC phosphorylation suggesting that ROCK is involved in MLC phosphorylation. These results verify that thrombin induces chemotaxis of dendritic cells through Rho/ROCK pathway.

This work provides evidence that dendritic cells are activated by thrombin. In physiological conditions, there are two possible situations where dendritic cells could come in contact with thrombin.

One finding is that, after LPS activation and antigen capturing at the inflammation/infection site, dendritic cells are migrating to the lymphoid tissues. Kudo et al[229] detected the presence of some coagulation factors in human Payer patches. Histological staining revealed traces of thrombin and a significant amount of pro-thrombin. Our results indicate that mature dendritic cells could be additionally activated in the lymphoid tissues, by the thrombin present there, and that this activation enhances T cells differentiation.

The other possibility for dendritic cells to come into contact with thrombin is in the atherosclerotic changes on blood vessels. The presence of dendritic cells was described in atherosclerotic plaques[60]. Other immune cells were also localized in the atherosclerotic plaques, a significant amount of T lymphocytes[214] and macrophages, which are migrating to the site of plaque formation, ingesting lipids and turning into foam cells[215]. In atherosclerotic plaques, the number of dendritic cells markedly increases with more than 90% accumulating in the plaque shoulders[230,231]. The formation of clusters of dendritic cells with T cells and NK cells is associated with plaque destabilisation[232]. The fact that thrombin is present in the human atherosclerotic plaque[233] indicates a possible role of thrombin as activator of the dendritic cells localised in the plaque. A model that we used, LPS-matured dendritic cells display functional PAR1 and PAR3 on their surface. We can hypothesize that monocytes circulating in the blood are accumulating at the site of plaque formation, differentiate into immature dendritic cells, because the cytokines necessary for their differentiation are provided by the endothelial cells[234], and then these cells could mature locally, in the presence of LPS. The TLR4 (LPS receptors) and their role in the initiation and progression of atherosclerosis was investigated by Pasterkamp et al[235]. As a matter of fact, LPS promotes atherosclerosis by its effect on endothelial cells to release cytokines[236].

In conclusion, this work provides evidence for a role of thrombin in dendritic cell activation. Thrombin affects dendritic cell migration via PAR1 and PAR3.

The chemotactic effect of thrombin is transduced through the RhoA/ROCK pathway. Thrombin-activated dendritic cells have the ability to strongly stimulate T lymphocytes and evoke the immune response. If we are focusing on the role of dendritic cells in atherosclerosis, and their contribution to the atherosclerotic plaque destabilisation[60], we can assume that targeting dendritic cells in the vascular endothelium, in a way to downregulate their activity, could prevent a plaque destabilisation and become a novel therapeutic strategy for the treatment of atherosclerosis.

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Publications:

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