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Studies on immunoreceptor signaling molecules

Studium signalizačních molekul imunoreceptorů

Disertační práce

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Laboratoř molekulární imunologie

Ústav molekulární genetiky AV ČR

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Prohlášení:

Prohlašuji, že jsem disertační práci vypracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 21. července 2011

Tereza Ormsby

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1 Summary

A delicate balance in the number, specific type and function of leukocytes is required for proper functionality of the mammalian immune system. Innate immunity, which quickly recognizes pathogens, represents the first line of defense. Later, a more specific response is generated via adaptive immunity. Deregulation of the immune system is manifested by the inability to control infection (see 3.2.2), development of allergic, autoimmune disorders or even cancer (see 3.3), and ultimately can lead to death. To fulfill their functions, cells develop an intricate network of intra- as well as extra-cellular molecules organized into signaling cascades, which allows them to communicate between each other. Better understanding of the molecular mechanisms of signaling pathways in leukocytes is critical for design of efficient therapies.

In this thesis, leukocyte signaling was studied in several aspects. First, the role of adhesion molecules in pathogenesis of cervical cancer and the regulation of their expression was investigated.[1] The second publication describes a new transmembrane adaptor protein (TRAP), called prolin rich 7 (PRR7), as a potentially interesting regulator of signaling and apoptosis in activated T cells.[2] The final publication characterized the role of the Btk kinase downstream of the triggering receptor expressed on myeloid cells 1 (TREM-1), which was shown to be involved in inflammatory processes; suppression of its activity had beneficial effects in the treatment of septic shock.[3]

This thesis is based on three publications:

1. Textor S., Accardi R., **Havlová T.**, Hussain I., Sylla B.S., Gissmann L., Cerwenka A. 2010. NF-κ B-dependent upregulation of ICAM-1 by HPV16-E6/E7 facilitates NK cell/target cell interaction, *Int J Cancer* 128: 1104-1113

2. Hrdinka M., Dráber P., Štepánek O., **Ormsby T.**, Otáhal P., Angelisová P., Brdička T., Pačes J., Hořejší V., Drbal K. 2011. PRR7 is a transmembrane adaptor protein expressed in activated T cells involved in regulation of T cell receptor (TCR) signaling and apoptosis, *J Biol Chem* 286: 19617-29

3. **Ormsby T.**, Schlecker E., Ferdin J., Tessarz A.S., Angelisová P., Köprülü A.D., Borte M., Warnatz K., Schulze I, Ellmeier W., Hořejší V., Cerwenka A. 2011. Btk is a positive regulator in the TREM-1/DAP12 signaling pathway, Blood [ahead of print, doi:10.1182/blood-2010-11-317016]

2 Shrnutí

Správná funkce imunitního systému savců je zajišťována křehkou rovnováhou mezi množstvím a nejrůznějšími typy leukocytů, které vykonávají velmi specifické funkce. Přirozená imunita, která rychle reaguje na přítomnost patogenů představuje první obrannou linii organismu. Teprve později se zahajuje specifičtější odpověď tím, jak se aktivuje adaptivní imunita reprezentovaná T a B lymfocyty a produkcí protilátek. Deregulace imunitního systému se projevuje neschopností potlačit infekci, rozvojem alergických a autoimunitních onemocnění nebo dokonce rakovinného bujení, takže v konečném důsledku může vést až k smrti. K tomu, aby leukocyty mohly vykonávat své funkce, vyvinuly spletitou síť intra- i extracelulárních molekul uspořádaných do signálních kaskád, které jim umožňují vzájemnou komunikaci. Pochopení molekulárních mechanismů signálních drah leukocytů je zásadním předpokladem pro vyvinutí účinných terapií.

V této disertační práci byla leukocytární signalizace studována v několika aspektech. Nejprve bylo zjišťováno, jakou roli hraje zvýšená exprese adhezivních molekul v patogenezi rakoviny děložního čípku a jak je tato exprese regulována na molekulární úrovni.[1] Druhá publikace představuje nový transmembránový adaptorový protein, nazvaný PRR7 (prolin rich 7), jako potenciálně zajímavý regulátor signalizace a apoptózy aktivovaných T buněk.[2] Finální práce se zabývá funkcí kinázy Btk v signalizaci receptoru TREM-1 (triggering receptor expressed on myeloid cells 1), který se ukazuje být velmi důležitý v zánětlivých procesech a potlačení jeho funkce má příznivý vliv při léčbě septického šoku.[3]

Tato disertační práce je založena na třech publikacích:

1. Textor S., Accardi R., **Havlová T.**, Hussain I., Sylla B.S., Gissmann L., Cerwenka A. 2010. NF-κ B-dependent upregulation of ICAM-1 by HPV16-E6/E7 facilitates NK cell/target cell interaction, *Int J Cancer* 128: 1104-1113

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3 Introduction

3.1 Cells of the immune system[4]

Different functions of the immune system are mediated by a wide variety of cells of hematopoietic origin and plasma proteins. Leukocytes make up the cellular component of immunity. They originate from hematopoietic stem cells in the bone marrow and give rise to either myeloid or lymphoid common progenitors. The myeloid progenitors further differentiate into monocytes, granulocytes, mast cells, dendritic cells (DCs) and megakaryocytes, whereas T cells, B cells and natural killer (NK) cells evolve from the lymphoid progenitor.

The first line of defense is mediated by cells of the innate immunity. Innate immune cells exert rapid effector functions through a variety of pattern recognition receptors (PRRs; see 3.4.1.4) with broad specificity against common molecular features of different pathogens. Innate immunity is mediated by two main categories – myeloid cells and NK cells.

Granulocytes represent the most abundant group of white blood cells (70%) and are characterized by the presence of cytoplasmic granules and a uniquely shaped nucleus. Three types of granulocytes –neutrophils, basophils and eosinophils– can be distinguished according to different staining properties of their cytoplasmic granules. Neutrophils, the most numerous group of granulocytes, play an important role in antibacterial and antifungal defense mechanisms. They migrate from the blood into inflamed tissues early in an acute inflammatory response. They efficiently kill pathogens either intracellularly following phagocytosis or extracelularly by releasing toxic mediators stored in their cytoplasmic granules. Basophils and eosinophils play a role in immune responses against parasites. They are also involved in the development of allergic reactions.

Monocytes are blood-circulating cells, which, upon migrating into tissues, differentiate into macrophages. They engulf pathogens or dead cells by phagocytosis, contributing to the clearance of infection. They also function as antigen-presenting cells (APCs) and produce a wide variety of cytokines.

Mast cells protect the internal surfaces of the body against pathogens and are involved in the response to parasitic worms. They are important in wound healing and play a prominent role in the development of allergic responses and anaphylactic shock. Their cytoplasm contains granules rich in inflammatory mediators such as histamine and heparin, which are released upon activation.

Although they are of lymphoid origin, NK cells belong to the innate part of the immune system. They have the ability to recognize cells with a low expression of major histocompatibility complex (MHC) class I glycoproteins, i.e. exhibiting so called “missing self”. NK cells play an important role in destroying virus-infected or transformed cells. Upon activation, they release cytoplasmic granules

onto the surface of the bound target cell, and the effector proteins contained in the granules penetrate the cell membrane and induce cell death. NK cell killing depends on the balance between activating and inhibitory receptors. The activating receptors recognize several common cell surface ligands and signal the NK cell to kill the bound cell. On the other hand, the inhibitory receptors associate with MHC class I molecules and inhibit the signaling of activating receptors. If the inhibitory signal is lost, the cell is killed.

The link between innate and adaptive immunity is demonstrated by DCs in their roles as professional APCs. They both phagocytose particulate materials and continually ingest large amounts of the extracellular fluid and its contents by macropinocytosis. Ingested pathogens are degraded and presented to T cells in the form of a peptide bound to MHC class II molecules. In addition, DCs are also potent producers of different cytokines, and therefore, they can modulate functions of other cell populations.

Adaptive immunity is mediated by a wide diversity of clonally specific antigen receptors produced by somatic recombination in lymphocytes. Each lymphocyte matures bearing a unique version of the prototypic antigen receptor. Upon activation, a lymphocyte proliferates and gives rise to huge number of cells highly specific to a given antigen. There are two types of antigen-specific lymphocytes: B cells and T cells. The antigen receptor of B cells (BCR) is a membrane-bound immunoglobulin (Ig). Upon activation, B cells proliferate and differentiate into plasma cells, which produce secreted antibodies of the same specificity as the original receptor. T cells recognize antigen-derived peptides bound to MHC molecules. Cytotoxic T cells kill cells infected with intracellular pathogens, using similar mechanisms as NK cells. They recognize antigens as peptide-MHC class I glycoprotein complexes. On the other hand, helper T cells provide essential, additional signals that cause antigen-stimulated B cells to differentiate and produce antibodies and cause macrophages to become more efficient in killing engulfed pathogens. Helper T cells recognize peptide-MHC class II glycoprotein complexes. Special type of T cells are regulatory T cells which play an important role in suppressing the activity of other cells, thereby providing a control mechanism in the immune system.

3.2 Inflammation and sepsis[4]

Inflammation is a physiological reaction of the immune system, intended to protect the host from various hazards, including invading pathogens. The purpose is to restore and maintain normal tissue homeostasis after an injury or an infection, and ultimately, to repair damaged tissue and efficiently get rid of pathogens. Inflammation is essentially beneficial; however, prolonged or excessive inflammation can cause serious harm, such as septic shock (see 3.2.2), or lead to the

development of immunopathological diseases. Inflamed tissue is characterized by pain (dolor), redness (rubor), heat (calor) and swelling (tumor).

Depending on the duration of the immune response, inflammation can be divided into acute and chronic. Acute inflammation is the immediate response of the body to harmful stimuli, which lasts for minutes to days, and results in clearing of the infection and repair of the injured tissue. The impairment of negative regulatory mechanisms or the ineffective elimination of pathogenic microbes can lead to chronic inflammation. Chronic inflammation lasts for longer periods of time, and it is accompanied by a change in the type of cells present at the site of inflammation and by parallel damage and healing of the tissue from the inflammatory responses.

3.2.1 Mechanisms of inflammation

Inflammation begins when tissue-resident cells, mainly resident macrophages, DCs and mast cells, recognize highly conserved structures of pathogens called pathogen-associated molecular patterns (PAMPs), which are bound by PRRs. PRRs represent a large group of extra- and intracellular receptors with different structural features and specificities (see 3.4.1.4). Activation of the PRRs leads to the release of various pro-inflammatory mediators and the onset of inflammation.

Mast cells produce a number of biologically active substances including histamine and leukotriens, which cause contraction of endothelial cells of blood vessels. The resultant increased space between the endothelial cells affords higher capillary permeability, which enables recruitment of leukocytes to the site of inflammation. Macrophages secrete pro-inflammatory cytokines (such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1)) and chemokines (such as IL-8) and engulf the pathogens and dead tissue cells by phagocytosis. Cytokines activate various adhesion molecules, e.g. E-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1; see 3.4.1.3.2), on the surface of the endothelial cells of blood vessels. Corresponding molecules on the surface of leukocytes, called integrins (VLA-1, LFA-1; see 3.4.1.3.1), attach to these adhesion molecules, allowing the leukocytes to slow down, roll, flatten and squeeze through the space between the endothelial cells in the process of diapedesis or extravasation.

Secreted chemokines diffuse into the blood vessels, forming a concentration gradient and attracting other immune cells into the inflamed tissues. Among the first cells migrating into the site of inflammation are neutrophils, followed by monocytes, which differentiate into macrophages. Neutrophils phagocytose and kill microorganisms by releasing the contents of their cytoplasmic granules. They also secrete pro-inflammatory cytokines and chemokines, attracting other immune cells and intensifying the ongoing inflammation.

Furthermore, DCs are activated by PRRs and differentiate into mature APCs. They express antigens of phagocytosed microorganisms as a complex of an MHC molecule and a peptide. Mature

dendritic cells migrate into the draining lymph node where they present antigens to T cells and thus activate the adaptive arm of the immune system. In addition, blood coagulation mechanisms are activated, which helps to repair the physical barriers of the host body.

According to the specific nature of the infection or injury and the current state of the immune system, acute inflammation can result in: (1) complete clearance of infection with no or little tissue destruction; (2) fibrosis or scaring due to incomplete tissue regeneration; (3) progression into chronic inflammation if the acute inflammatory response could not be attenuated; (4) septic shock (see 3.2.2) when pathogens massively enter the blood stream.

3.2.2 Sepsis

Sepsis is a complicated clinical syndrome, which develops when the adequate immune response to an infection becomes deregulated, leading to the overactivation of the immune system. The average mortality rate is estimated between 25% and 50%, ca 40% in the elderly population and up to 50% in immunocompromised individuals.[5]

Sepsis commences with the large-scale release of damage-associated molecular patterns (DAMPs) from compromised tissue or invading pathogens, resulting in the overstimulation of PRRs. Signaling of PRRs (see 3.4.1.4) leads to massive secretion of various pro-inflammatory mediators including cytokines, such as TNF-α and IL-1, free radicals and enzymes. These mediators are advantageous in moderate amounts, but at excessively high concentrations, they can be quite harmful.

An imbalance in homeostasis at various levels, affecting not only the immune system, is the main hallmark of septic shock. The autonomic nervous system acts through catecholamines, which bind to adrenergic receptors on the surface of macrophages, neutrophils and DCs and activate the transcription factor NFκB. Catecholamines display pleiotropic effects - altered lymphocyte trafficking, vascular perfusion, cell proliferation and apoptosis. A high concentration of catecholamines in the early phases of sepsis has been shown to increase the inflammatory response. Later, apoptosis of adrenal medullary cells causes a decrease of catecholamines` concentration, leading to the dysfunctional modulation of heart and blood vessels and contributing to cardio-vascular failure.[6]

The presence of microorganisms in blood results in excessive activation of complement system. Anaphylatoxin C5a, which is produced by the C5 convertase of the complement cascade, plays a crucial role in the immunopathogenesis of sepsis. In the early stages of septic shock, C5a deregulates the coagulation cascade and promotes secretion of pro-inflammatory cytokines, including migrating-inhibitory factor (MIF), and high mobility group box 1 (HMGB1). In the later stages, a high concentration of C5a leads to increased apoptosis of lymphocytes and adrenal medullary cells and to neutrophil dysfunction, called immune paralysis and characterized by the termination of intracellular signaling. This accounts for the high vulnerability to secondary infection as well as septic

cardiomyopathy (heart failure) and multi-organ failure. The complement cascade is tightly connected to coagulation and the fibrinolysis system.

Sepsis disrupts normal equilibrium between procoagulant and anticoagulant factors. Increased expression of the tissue factor leads to enhanced generation of thrombin, which cleaves fibrinogen into fibrin. On the other hand, expression of anticoagulant proteins, such as antithrombin, protein C and the tissue factor pathway inhibitor, is down-regulated. Mobilization of the coagulation system and suppression of fibrinolysis at the same time represent a starting point for the development of disseminated intravascular coagulation (DIC). This pathological process is characterized by an acute risk of fatal thrombosis together with global haemorrhage. Finally, prolonged tissue hypoxia and hypoperfusion cause multiple organ failure and death.

3.3 Cancer and the immune system[4,7,8]

Cancer is a multifactorial disease caused by uncontrolled proliferation of a single transformed cell which invades the organism and causes destruction of adjacent tissue. Treatment of cancer is usually based on surgical removal of the majority of tumor mass followed by the destruction of the remaining malignant cells by chemotherapy and/or radiation therapy, which is accompanied by severe side effects. A more elegant way of curing cancer would be to directly stimulate the immune responses precisely against the tumor cells.

Evasion of malignant cells from detection of the immune system is described by the immunoediting model, which divides this complex process into three “E” phases – elimination, equilibrium and escape. At the beginning, malignant cells are readily recognized and immediately destroyed by immune effector cells. The crucial role in this phase is played by activated CD8 T cells and NK cells. However, immune cells contribute to the selection of cells resistant to the action of the immune system, which first leads to the establishment of a balance between continuous tumor growth and tumor destruction. In addition, tumor cells promote tissue remodeling and angiogenesis, leading to the evolution of tumor cell variants with low immunonogenicity. Finally, as the tumor progresses, it starts to actively suppress attack by the immune system and completely escapes its control. The destiny of a tumor is already decided during the equilibrium phase, in which the tumor is infiltrated by different subsets of effector, helper and regulatory T cells, NK cells and myeloid cells, such as myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). These cells help to create a state of immunological tolerance; they shape the tumor microenvironment, support angiogenesis and generally suppress various components of immunity.

The rate of tumor growth is determined by the magnitude of immunological tolerance, which is established within the tumor, between the tumor cells and the surrounding microenvironment

including immune cells. In general, tumors have low immunogenicity because they do not express abnormal peptides, or they decrease the expression of MHC class I or co-stimulatory molecules. Moreover, the tumor or surrounding parenchyma expresses increased amounts of T cell inhibitory molecules including PD-L1, B7-H3, B7x, HLA-G and HLA-E. For some tumors, like epithelial tumors, these expression levels correlate with the outcome of tumor growth. The tumor secretes various factors (IL-10, transforming growth factor β (TGFβ), including vascular endothelial growth factor (VEGF)), which dampen the development of inflammatory responses, support angiogenesis, and recruit suppressive populations of cells including regulatory T cells, immature DCs, MDSCs and M2 macrophages. Finally, tumor cells can secrete molecules such as collagen that form a physical barrier around the tumor, preventing access by lymphocytes.

3.3.1 Cancers caused by human papillomaviruses (HPVs)

In 2008, Harald zur Hausen received the Nobel Prize for Medicine for his research regarding human papillomaviruses (HPVs). He discovered that cervical cancer is not caused by the herpes simplex virus, as previously believed, but rather by HPVs. He found out that HPVs are a heterogeneous group of small dsDNA viruses and infection of the cervix by subtypes 16 and 18 may lead to cancer development.[9] Thanks to improvement in diagnosis, the incidence of cervical cancer dramatically decreased in industrialized nations; however, in less developed countries, cervical cancer is still the second most common type of cancer in women, and over 99% is caused by HPVs.[10]

HPVs are small, non-enveloped dsDNA viruses that infect keratinocytes in the basal layer of stratified squamous epithelia. These cells maintain their replicating potential and may get exposed due to micro-abrasions. There are two main HPV types that infect the genital mucosa. Alpha papillomaviruses, referred to as high-risk (HR), are represented by HPV16 and 18 and are found in about 70% of malignant cervical cancers.[11] By contrast, beta papillomaviruses, also called low-risk (LR) HPVs, include HPV5, 8 and 38 and cause only cutaneous infection without major clinical syndroms in healthy individuals.[12]

Importantly, infection by HR HPV types is necessary but not sufficient for progression to malignant tumor, since only a small percentage of women infected by HR HPVs in the end develop cervical cancer. Therefore, additional environmental or endogenous factors are involved in triggering of the malignant progression.[13]

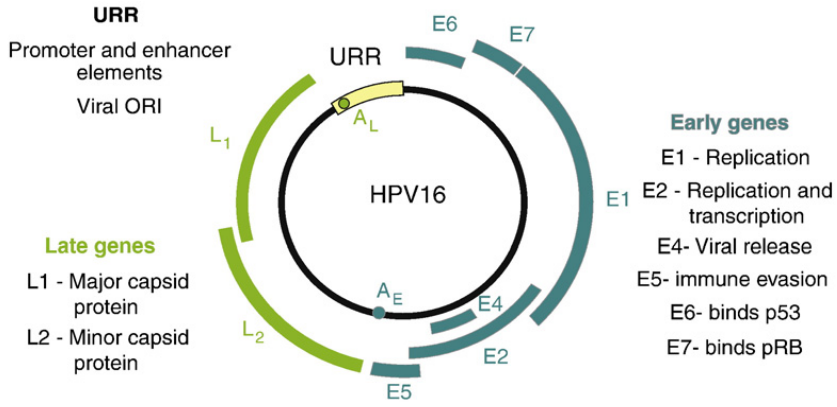


Figure 1: The HPV genome. HPV, human papillomavirus; ORI, origin; pRb, retinoblastoma protein; URR, upstream regulatory region. The picture is adapted from Stanley.[9]

The genome of HPVs is circular dsDNA, which can be divided into three parts: (1) a noncoding upstream regulatory region (URR); (2) a region encoding early genes, E6, E7, E1, E2, E4 and E5; and (3) a sequence coding two late proteins of the capsid, L1 and L2 (Figure 1). The genome is found in the nucleus as low copy extrachromosomal DNA units called episomes, which replicate autonomously from host chromosomal DNA. The genome does not encode polymerases or other proteins necessary for viral replication, thus it completely relies on the cellular DNA synthesis machinery. First, the virus invades the basal layer of cervical epithelia. Shortly afterwards, the virus initiates its own replication independently of the cell cycle and generates around 50-100 copies of itself per cell. The infected cell starts proliferating. At this stage, the viral transcriptional rate is minimal and expression of E6/E7 transcripts is hardly detectable, which promotes evasion of immune control. Later, the infected keratinocytes leave the basal layer and move into the differentiating section, which begins enormous up-regulation of expression of viral proteins. At this point, the viral copy number is at least 1000 copies per cell. Capsid proteins, L1 and L2, are expressed, and virions are assembled, exiting the cell without lysis or necrosis, which further eases the spread of infection because it prevents an onset of the inflammatory response (Figure 2).[9]

Interestingly, in precancerous lesions, most HPV genomes are maintained as episomes, whereas in many high-grade lesions, viral DNA is found integrated into the host DNA. Places with genomic instability are susceptible to integration of foreign DNA. It is speculated that integration plays a role in malignant progression of cervical cancer and involves impairment in expression of the E2 protein, which inhibits the expression of early viral genes including E6 and E7.[14]

HPVs exert an interesting strategy to escape the recognition by the host immune system. Postponing expression of high amounts of viral antigens in areas that are not easily accessed by immune cells allows the HPVs to stay hidden, but it also brings one obstruction. The HPVs do not

bear their own DNA replication enzymes and, in this respect, fully depend on the host. As keratinocytes differentiate, they stop proliferation and down-regulate the expression of factors driving the cell cycle. To overcome this problem, the virus has to maintain the differentiated cell in the active cell cycle. The primary viral proteins with immortalization activity are E6 and E7. Both E6 and E7 are small proteins localized in the nucleus which lack intrinsic enzymatic or DNA-binding capacity; instead they bind different factors important for regulation of the cell cycle. E7 binds the retinoblastoma (Rb) family of proteins and targets them for degradation. In normal cells, the Rb family blocks the activity of the E2F transcription factors, which interact with promoter regions of many genes implicated in cell cycle progression, and the cells enters S phase. Consequently, abrogated levels of Rb lead to increased stabilization of the tumor suppressor, p53. However, the E6 protein directs p53 toward degradation and promotes the state of chromosomal instability by preventing DNA repair. In addition, HR E6 enhances telomerase expression, a reverse transcriptase needed for elongation of the 3' end of chromosomal DNA.[10,14]

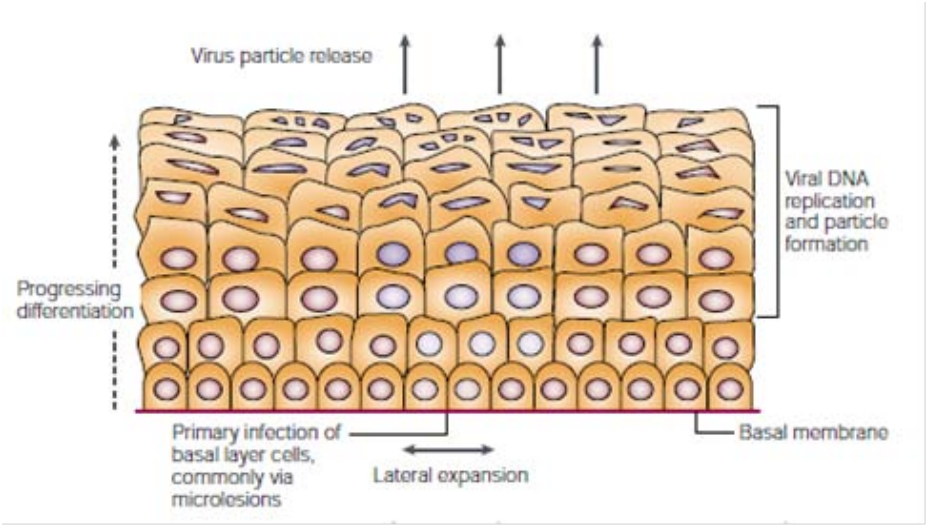


Figure 2: Infectious cycle of high-risk HPVs. First, HPVs infect the proliferating layer of basal epithelial cells and replicate to a level of 50-100 copies/cell. When the cell starts to differentiate, the HPVs rapidly proliferate into ca 1000 copies/cell, which are subsequently released by budding. Viral proteins E6 and E7 are critical for oncogenesis, since they induce mitosis in terminally differentiated cells. The picture is adapted from zur Hausen.[15]

3.4 Signaling mechanisms in cells of the immune system

Signal transduction is the process of transfer of information from the cellular environment to the inside of the cell. Signaling begins when a receptor binds its specific ligand. Receptors are expressed either on the cell surface or intracelullarly. A specific series of events, called a signaling cascade, is activated downstream of the receptor and includes activation or inhibition of protein kinases and release of intracellular second messengers. Signaling ultimately changes the transcriptional activity of a cell and, dependent on the precise context of events, results in proliferation, apoptosis, growth, migration, adhesion, secretion of various cytokines, hormones or other mediators or the change in the expression of proteins on the cellular surface. Signaling represents the foundation of the molecular mechanisms of immune processes, an understanding of which is necessary for the development of new therapies.[16]

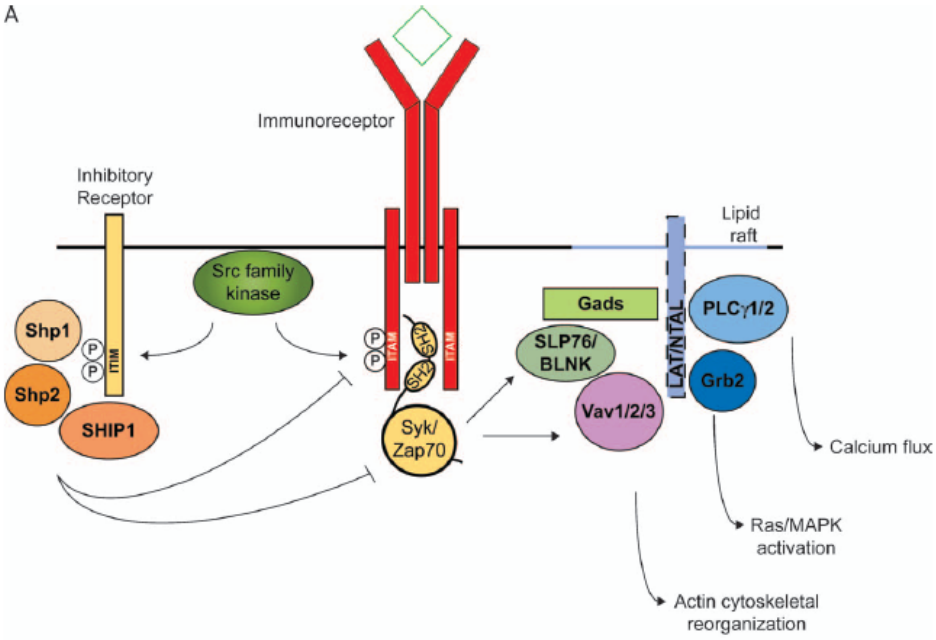
3.4.1 Receptors of immune cells

3.4.1.1 Classical immunoreceptors[4,17,18]

Classical immunoreceptors are comprised of a T-cell receptor (TCR), a B-cell receptor (BCR), Fc-receptors (FcRs), and activating receptors of NK cells. Although these receptors bind different ligands, their intracellular signaling pathways are more or less similar. These immunoreceptors consist of a recognition module, which binds its respective ligand, and a signaling module, which is responsible for transfer of the signal into the cell through a complex network of signaling cascades. The signaling module is represented by a non-covalently associated transmembrane adaptor protein, which bears one or more immunoreceptor tyrosine-based activation motifs (ITAMs). For example, TCR uses the CD3ζ chain, BCR signals via the immunoglobulin α (Igα) and Igβ chains (CD79a,b), Fc receptors couple to the common γ-chain (FcRγ) and the activating receptors of NK cells couple to DAP12. The ITAM consists of two tyrosine residues in the sequence, YxxL/I, which are six to twelve amino acids apart, so the consensus sequence is YxxL/I-x_{6-12}-YxxI/L. Receptor engagement leads to activation of different members of the Src kinase family (SFK) (see 3.4.2.1), which includes Src, Lck, Fyn, Lyn, Yes, Fgr, Hck, and Blk, depending on the cell type (Figure 3). SFKs then phosphorylate ITAMs, and phosphorylated ITAMs serve as docking sites for the tandem SH2 domains of Syk kinases (see 3.4.2.2), ζ-associated protein of 70 kDa (ZAP-70) or spleen tyrosine kinase (Syk), which trigger kinase activation and downstream signaling.

Upon activation of Syk kinases, receptor-proximal signaling complexes are formed. They consist of the transmembrane adaptor proteins, linker of activation of T cells (LAT) and/or non-T cell

A



B

Immunoreceptor	ITAM adapter	Src family kinase	Syk/Zap70	Inhibitory receptors
T-cell receptor	CD3ζ	Lck	Zap70	CTLA4, PD1
B-cell receptor	Igα and β	Lyn	Syk	FcγRIIB, CD22
Fc receptors	FcRγ chain	Hck/Fgr/Lyn	Syk	PIR-B, SIRPα, FcγRIIB
NK-activating receptors	DAP12	Lck	Zap70/Syk	KIRs
TREM1/2	DAP12	Hck/Fgr/Lyn	Syk	PIR-B, SIRPα, FcγRIIB

Figure 3: Signaling pathway downstream of immunoreceptors. (A) Upon ligand binding, SFKs phosphorylate ITAMs, which in turn leads to relocalization and activation of Syk kinases. Syk kinases phosphorylate a number of downstream signaling proteins such as SLP76, LAT and/or NTAL, and members of the Vav family. In addition, Src kinases also phopshorylate ITIM motifs in a variety of cell surface receptors. Phoshorylated ITIMs recruit phosphatases, which downmodulate the activation pathway. **(B)** The table lists immunoreceptors and the coupled ITAM-containing adaptors, as well as SFKs and Syk kinases involved in the initiation of downstream signaling. ITIM-containing inhibitory receptors are also listed. The figure was adapted from Abram *et al.*[17]

activation linker (NTAL), as well as the cytoplasmic adaptor proteins, SH2 domain-containing leukocyte protein of 76 kDa (SLP76) and SH2 domain-containing leukocyte protein of 65 kDa (SLP65, also known as BLNK). Then members of the Tec kinase family (see 3.4.2.3) phosphorylate phospholipase Cγ (PLCγ). Activated PLCγ generates second messengers such as inositol 1,4,5-trisphisphate (IP_3), which triggers Ca^{2+} mobilization, and diacylglycerol (DAG), which in turn activates protein kinase C (PKC). In addition, various Vav isoforms mediate cytoskeleton rearrangements. Growth factor receptor-bound protein 2 (Gbr2) binds to phosphorylated tyrosine motifs of LAT/NTAL, and thus, the Ras/MAPK pathway is activated. Finally, transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), the activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT) are activated, and new transcripts are synthesized.

There are also receptors whose main function is to down-regulate activating pathways. They often contain the immunoreceptor tyrosine-based inhibition motif (ITIM), which upon phosphorylation, associates with a phosphatase such as the SH2 domain-containing phosphatases 1 and 2 (SHP1 and SHP2). These phosphatases then dephosphorylate different tyrosine residues in the proteins mentioned above, leading to downmodulation of signaling.

3.4.1.2 Triggering receptor expressed on myeloid cells 1 (TREM-1)

TREM-1 belongs to the Ig-like superfamily of receptors and plays an important role in inflammatory responses.[19,20] TREM-1 cDNA was first identified by a bioinformatic search for sequences with regions homologous to the activating receptor of NK cells, NKp44.[19] The gene encoding TREM-1 is mapped to human chromosome 6p21.1 and mouse chromosome 17C.[21] Besides TREM-1, this region contains genes for several related receptors, including TREM-2 and the TREM-like transcripts (TLT) 1-5. Some of these genes are considered to be pseudogenes.[22] Orthologs of TREM proteins were found in chickens, cattle, and dogs as well as in skates and teleost fish, indicating that these receptors are highly evolutionarily conserved molecules.[23-27]

TREM-1 is a 30 kDa glycoprotein which possesses a single extracellular V-type Ig-like domain, a transmembrane segment with a positively charged residue, and a short intracellular part that lacks intrinsic signaling motifs.[28,29] Independent studies were performed to solve the structure of TREM-1. While the first report proposed existence of head-to-tail dimers of the TREM-1 molecule,[30] other reports used X-ray crystallography as well as analytical centrifugation and nuclear magnetic resonance spectroscopy to show that TREM-1 exists in solution as a monomer.[28,29]

The existence of an alternatively spliced mRNA of TREM-1, svTREM-1, which would encode a protein of molecular weight 17.5 kDa, lacking the N-glycosylation and the transmembrane region, was suggested.[31] Furthermore, a soluble TREM-1, sTREM-1, was detected in serum of septic patients by western blotting, as a band with an apparent molecular mass of 27 kDa.[32] The appearance of sTREM-1 coincided with a decrease of TREM-1 expression at the cell surface. Metalloproteinase inhibitors increased the stability of TREM-1 at the cell surface while reducing the levels of sTREM-1 in LPS-treated monocytic cultures.[33] These data and the fact that svTREM-1 has not been detected so far, support the idea of proteolytic shedding of TREM-1 from the cell surface. sTREM-1 might negatively regulate the functions of TREM-1 through neutralization of the putative ligand(s) and might be useful as a therapeutic or diagnostic tool for septic shock.[32]

TREM-1 expression is associated with a mature stage of myeloid development.[31] In humans, TREM-1 is expressed on blood neutrophils and $CD14^{high}$ monocytes.[19] Monocytes and macrophages isolated from secondary lymphoid organs also express functional TREM-1 on the cell surface. In

healthy individuals, a vast majority of resident intestinal macrophages completely lack TREM-1.[34] However, inflamed lesions of patients with inflammatory bowel diseases contained increased amounts of TREM-1$^+$ macrophages, which positively correlated with enhanced disease activity.[35] In mice, TREM-1 is expressed by subsets of blood monocytes, blood granulocytes and by BMDCs.[36] Engagement of Nod-like receptors (NLRs)[37] or Toll-like receptors (TLRs)[38] up-regulates TREM-1 expression. Depending on the specific signal and receptor engaged, TREM-1 expression is regulated in a MyD88-dependent (for LPS and TLR4) or in a TRIF-dependent (lipoteichoic acid and TLR2) manner.[39] At a transcriptional level, NFκB acts as a positive regulator of *TREM-1* gene expression, whereas PU.1 is a negative regulator.[40]

Binding of TREM-1 to endogenous ligand(s) expressed on granulocytes and platelets and present in the sera of septic patients, as well as to exogenous ligands on Marburg and Ebola viruses, has been described.[41-44] The exact nature of the TREM-1 ligand(s), however, remains elusive. Therefore, agonistic monoclonal antibodies (mAbs) are currently used to trigger TREM-1 signaling pathways.

By this approach it was discovered that TREM-1 engagement results in respiratory burst, degranulation, phagocytosis, the secretion of pro-inflammatory cytokines (such as TNF-α), the secretion of the chemokines, such as IL-8 and monocyte chemotactic protein-1 (MCP-1), and in the up-regulation of cell surface expressed differentiation/activation markers.[19] In animal models of LPS-induced septic shock and microbial sepsis caused by live *Escherichia coli*, application of a soluble TREM-1-Ig fusion protein greatly increased survival of experimental animals, indicating the importance of TREM-1 in the amplification of inflammation.[20] Gibot *et al*[45] showed that a moderate dose of TREM-1 siRNA improved survival in the mouse model of bacterial peritonitis, whereas a high dose of siRNA compromised the neutrophil respiratory burst and increased mortalility. Moreover, TREM-1 is up-regulated on myeloid cells in patients or animals with acute pancreatis,[46] inflammatory bowel disease[35,47] or rheumatoid arthritis.[48-50] High TREM-1 expression often correlates with disease severity. On the other hand, patients with cystic fibrosis show low levels of TREM-1 expression on lung-resident macrophages and circulating monocytes. Those monocytes express high amounts of PU.1, which was revealed to negatively regulate TREM-1 expression. It is speculated that low TREM-1 expression on those cells contributes to a failure to mount appropriate inflammatory responses.[51] Furthermore, increased TREM-1 expression was observed on TAMs in malignant effusion and tumor tissue of non-small cell lung cancer, which correlated with poor prognosis.[52] Thus, TREM-1 seems to function as a mediator of acute as well as chronic inflammatory processes. It helps to clear infection; however, excessive stimulation of TREM-1 can have detrimental effects for the host, resulting in the development of septic shock or long-lasting chronic inflammatory complications.

TREM-1 possesses a short intracellular part that lacks intrinsic signaling motifs. Instead, it is coupled to the ITAM-containing adaptor protein, DAP12. DAP12 contains an aspartic acid residue within its transmembrane region to non-covalently pair with receptors harboring complementary,

positively charged amino acid residues in their transmembrane domain.[53] TREM-1 engagement in primary human monocytes leads to Ca^{2+} mobilization and phosphorylation of several proteins including DAP12, extracellular-signal regulated kinase (Erk1/2) and PLCγ (Figure 4).[19] Moreover, phosphorylation of the adaptor protein NTAL was shown.[54] NTAL then binds Grb2, son of sevenless (Sos) and the ubiquitin ligase casitas B-lineage lymphoma (c-Cbl). Using siRNA mediated knock-down in a myelomonocytic cell line, it was demonstrated that NTAL negatively regulates TREM-1-induced Erk1/2 phosphorylation and TNF-α and IL-8 production. In addition, the absence of NTAL led to delayed and decreased Ca^{2+} flux.[54,55] At a transcriptional level, enhanced amounts of NFκB subunits p50 and p65/RelA were observed upon TREM-1 stimulation in primary human monocytes.[32] Recently, it was reported that the adaptor protein caspase recruitment domain 9 (CARD9) binds B cell lymphoma/leukemia 10 (Bcl-10) after TREM-1 stimulation. This complex is essential for TREM-1-induced secretion of TNF-α, IL-2 and IL-12p40 by mouse bone marrow-derived dendritic cells (BMDCs) probably due to its involvement in the activation of NFκB.[56]

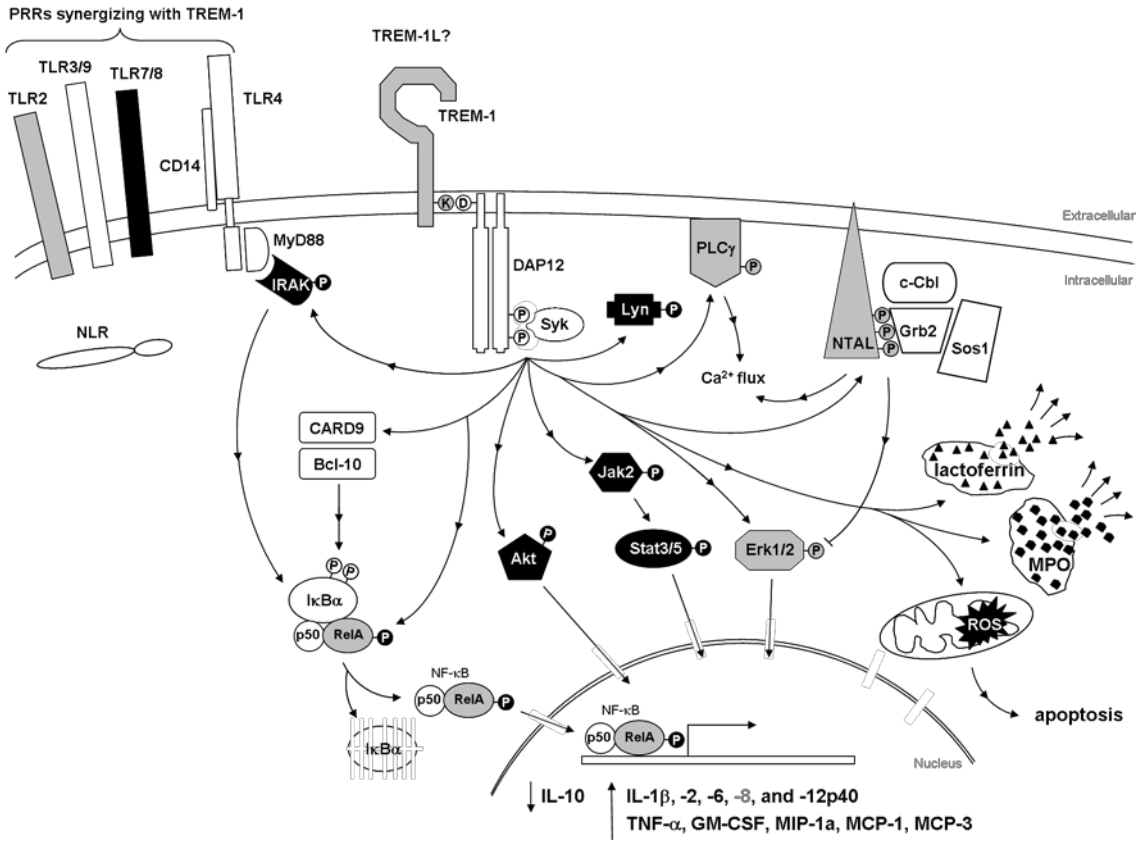


Figure 4: TREM-1/DAP12 signaling in myeloid cells. Signaling proteins experimentally demonstrated to be involved in TREM-1 signaling in monocytes (white), in neutrophils (black), or both (grey). The picture was adapted from Tessarz *et al.*[55]

The TREM-1/DAP12 signaling pathway was also studied in neutrophils (Figure 4). According to Fortin *et al*, TREM-1 is relocalized to the lipid rafts after engagement in neutrophils.[57] Several proteins are phosphorylated following TREM-1 ligation, including Src kinase Lyn, Janus kinase 2 (Jak2), PLCγ, protein kinase B (PKB/Akt), Erk1/2, p38MAPK, NTAL and, interestingly, IL-1R-associated kinase 1 (IRAK-1), which was previously only implicated downstream of TLRs.[54,57,58] In addition, TREM-1 stimulation in neutrophils leads to activation of transcription factors such as signal transducers and activators of transcription (STAT)5, STAT3 and p65/RelA.[57] The final result of TREM-1 signaling in neutrophils is secretion of IL-8, myeloperoxidase (MPO), and lactoferrin,[57] production of reactive oxygen species (ROS), [57-59] degranulation of cytosolic granules and phagocytosis.[59]

3.4.1.3 Cell adhesion molecules (CAMs)

Cell adhesion is mediated by specialized proteins, called CAMs, which bind to extracellular matrix or other cells. CAMs are transmembrane proteins whose intracellular parts associate with the cytoskeleton through a set of adaptor proteins while the extracellular part mediates ligand binding. CAMs function not only as a simple "glue" between cells; they also transmit important information from the cellular environment, and mediate migration, cell growth and proliferation. They play an important role in tumor invasion and metastasis formation. CAMs can be divided into four main families: the Ig superfamily (Ig-CAMs), integrins, selectins and cadherins.[60]

3.4.1.3.1 Integrins

Integrins are a large family of heterodimer molecules, composed of two chains, α and β. There are 19 types of α subunit and 8 types of β subunit, and 25 distinct αβ-receptors with a wide range of specificities are known.[17] Integrin molecules are formed when a single β chain associates with any one of the α chains.[60] Leukocytes express β_2 integrins including leukocyte-function-associated antigen 1 (LFA1) (CD11a/CD18; $\alpha_L\beta_2$), macrophage-adhesion-ligand 1 (Mac1) (CD11b/CD18; $\alpha_M\beta_2$), and β_1 integrins, such as very late antigen 4 (VLA4) ($\alpha_4\beta_1$). Among the typical ligands of integrins are members of Ig-CAMs, like intercellular adhesion molecule 1-5 (ICAM-1-5), extracellular matrix proteins (fibrinogen) and complement proteins (iC3b).[17]

Integrin signaling consists of two stages, called inside-out and outside-in. In resting cells, integrins exist in a bent or folded conformation with low affinity to ligand binding. Cell activation triggers a sequence of events inside the cell, which results in a segregation of the cytoplasmic part of the integrin heterodimers. Subsequent unfolding of the extracellular domains dramatically increases

the affinity for ligand binding and facilitates clustering of the high-affinity receptors on the cell surface. This process is called inside-out signaling.[17]

Once the high-affinity receptor binds its ligand, intracellular signaling cascades are triggered in a process termed outside-in signaling. Outside-in signaling leads to firm adhesion, spreading, ROS production and secretion of cytokines. The outside-in signal resembles signaling through classical immunoreceptors. Activation of SFKs and Syk and increase in total level of tyrosine phosphorylation was observed.[17] In addition, SLP76,[61] Vav,[62] PLCγ,[63] Pyk2, Akt,[17] and PKC isoforms[64] are important for integrin function. Recently it was shown that ITAM-containing adaptor proteins DAP12 and FcRγ are indispensable for integrin outside-in signaling. *DAP12*$^{-/-}$ or *FcRγ*$^{-/-}$ mice exhibit partial impairment in integrin-mediated ROS production, degranulation, and firm adhesion. Double knock-out mice have a complete block of integrin function.[65]

3.4.1.3.2 Ig superfamily (Ig-CAMs) with focus on ICAM-1

Ig-CAMs are cell-surface glycoproteins which contain several extracellular Ig-like loops. In contrast to the other families of CAMs, ligand binding is Ca^{2+}-independent. Most Ig-CAMs are integral transmembrane proteins, but some are linked to a glycophopshatidyl inositol (GPI) anchor.[66]

ICAM-1 (CD54) is a type I transmembrane glycoprotein with a molecular weight between 80-110 kDa. It is expressed on the endothelial cells of blood vessels and on hematopoietic cells as well. Binding of LFA1 and Mac1 on leukocytes by ICAM-1 causes firm adhesion of leukocytes on the blood vessel and allows their trans-migration into the tissues. In addition, ICAM-1 is important for interactions between APCs and T cells during the formation of the immunological synapse.[67] Its expression is greatly induced by pro-inflammatory cytokines (TNF-α, IL-1β). The ICAM-1 promotor contains binding sites for transcription factors NFκB, AP-1, AP-2, and AP-3. It was shown that TNFαR signaling leads to increased ICAM-1 expression via PI3K- and atypical PKCζ-mediated activation of NFκB.[68]

Existence of a soluble form of ICAM-1 (sICAM-1) has been observed in plasma. Elevated amounts of sICAM-1 were detected in the serum of patients with cardiovascular disease and autoimmune disorders, as well as cancer. Moreover, serum levels of sICAM-1 correlated with the severity of these diseases.[67]

ICAM-1 is able to initiate outside-in signaling.[69] Its cytoplasmic domain lacks any typical signaling motifs but it contains a tyrosine residue (Y512) and many positively charged amino acids, which may be important for signaling.[70] ICAM-1 ligation leads to phosphorylation of various molecules, including SFKs, PLCγ, PKC and MAPKs and also those involved in cytoskeleton rearrangement, such as the GTPases of the Rho family.[67]

3.4.1.4 Pattern recognition receptors (PRRs)

PRRs are very important for host defense against bacterial and virus infection because they sense the presence of microorganisms by binding to evolutionarily conserved structures, called PAMPs, on the surface of pathogens. In addition, some of them are able to recognize endogenous molecules, known as DAMPs, which are released from damaged tissue. Four different classes of PRRs have been described so far: transmembrane TLRs and C-type lectin receptors (CLRs), and cytoplasmic retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NLRs. Their respective ligands and cellular localization are summarized in Table 1. Generally, signaling of PRRs up-regulates transcription of genes involved in inflammatory responses such as pro-inflammatory cytokines, chemokines, type I interferons (IFNs), antimicrobial proteins and proteins implicated in modulation of PRRs signaling.[71]

Table 1: Pattern recognition receptors, their localization and ligands. The table was adapted from Takeuchi *et al.*[71]

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

3.4.1.4.1 Toll-like receptors (TLRs)

TLRs are type I integral membrane proteins characterized by the presence of extracellular leucine-rich repeats, responsible for ligand recognition and a cytoplasmic Toll/IL-1R homology (TIR) domain for induction of the intracellular signaling cascade.[72] Ligand binding leads to dimerization followed by a conformational change and allows association with downstream signaling molecules.[73] Particular TLRs slightly differ in their cytoplasmic signaling cascades according to which

TIR-domain-containing adaptor proteins are preferentially used. There are five of those adaptor proteins including myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain-containing adaptor protein (TIRAP/Mal), TRIF-related adaptor molecule (TRAM) and Sterile-α and Armadillo motif-containing protein (SARM). The downstream signaling can be roughly divided into two distinct pathways, MyD88-dependent and TRIF-dependent.[71]

The MyD88-dependent pathway is initiated by the relocalisation of MyD88 by TIRAP to the cytoplasmic TIR domain of TLRs. MyD88 then recruits IRAK1 and IRAK4. Next, IRAK4 phosphorylates IRAK1, allowing association with TNF receptor-associated factor 6 (TRAF6). Phosphorylated IRAK1 and TRAF6 dissociate from the receptor and form a complex with TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1) and TAB2. IRAK1 is degraded, and the complex translocates to the cytoplasm where it interacts with ubiquitin ligases. Ubiquitinylation of TRAF6 induces activation of TAK1. TAK1 in turn activates the IκB kinase (IKK) complex and MAP kinase kinase 6. The IKK complex phosphorylates the inhibitor of NFκB (IκBa), marking it for ubiquitinylation and subsequent degradation by proteasome. Free NFκB can translocate to the nucleus and activate transcription of pro-inflammatory cytokine genes. Activation of the MAPK pathway accounts for the formation of the transcription factor, AP-1, which drives expression of additional pro-inflammatory genes.[71-73]

The TRIF-dependent pathway starts with the recruitment of TRIF to the TIR domain through adaptor protein TRAM. TRIF mediates formation of a complex consisting of TRAF3, TRAF6 and receptor-interacting protein 1 (RIP1). RIP1 is implicated in the activation of NFκB, while TRAF3 is important for interferon (IFN)-regulatory factor 3 (IRF3) activation. TRAF3 activates TBK1/IKK-*i*, which phosphorylates IRF3 and IRF7. The dimer of IRF3 and IRF7 then translocates to the nucleus, resulting in induction of type I IFNs. In addition, IKK-*i* phosphorylates STAT1, further contributing to the transcription of IFN-inducible genes.[71-73]

TLR1, TLR2, TLR5, TLR6, TLR7 and TLR9 use the MyD88-dependent pathway. TLR3 is the only one that signals exclusively via the TRIF-dependent pathway; TLR4 can utilize both pathways.

3.4.2 Kinases and phosphatases

In general, kinases are a group of enzymes called phosphotransferases. They catalyze the transfer of phosphate groups from high-energy donor molecules, such as ATP, to specific substrates. Obversely, phosphatases are enzymes that remove phosphate groups.

Protein kinases specifically add a phosphate group to the tyrosine, serine or threonine residues of proteins. Over 500 protein kinases exist in the human/mouse genomes. According to their structural

features and specific functional characteristics, protein kinases can be grouped into various subfamilies.[74] Stimulation of kinases is the hallmark of signal transduction. Similarly, dephosphorylation of proteins is catalyzed by protein phosphatases, which are therefore important regulators of physiological responses. Kinases and phosphatases modulate all aspects of cellular biology, including development and cell cycle regulation. Deregulation of their function can lead to various diseases, even cancer.[16]

3.4.2.1 Src kinases (SFKs)

The Src family of non-receptor tyrosine kinases consists of eight members: Src, Fyn, Yes, Fgr, Lck, Hck, and Blk, which all display similar domain organisation and modes of regulation. In addition, these kinases can be subdivided into two subfamilies: Lyn, Hck, Lck and Blk may all be referred to as Lyn-related SFKs and Src, Yes, Fyn and Fgr are Src-related. Furthermore, there are three distantly related SFK-like kinases: Brk, Frk and Srm.[74]

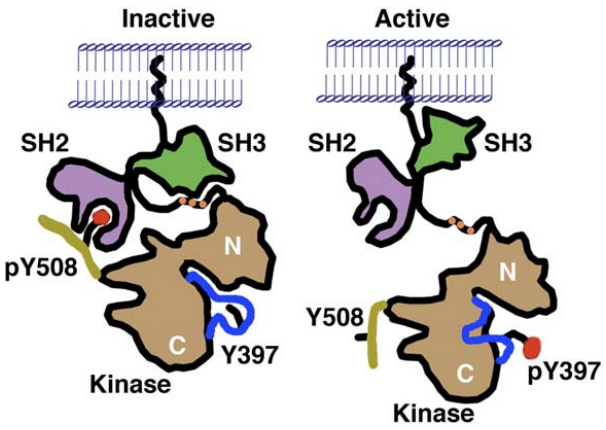


Figure 5: Schematic of SFK in its active and inactive conformation. In the resting state, the kinase exhibits a closed conformation held together by the intra-molecular association between the SH2 domain and the phosphosphorylated C-terminal tyrosine, and stabilized by interactions between the SH3 domain and a proline sequence at the beginning of the kinase domain. Upon activation, the C-terminal tyrosine is dephosphorylated by phosphatases, like CD45, which disrupts the interaction with the SH2 domain, and the kinase assumes open conformation. The picture is adapted from Ingley.[74]

All members share common structural motifs that are important for their function and regulation (Figure 5). The N-terminal sequence (60 aa) contains a myristoylation and/or a palmitoylation motif, allowing membrane localization of the kinase. Acylation permits not only localization proximal to receptors, but also targeting to specific parts of the plasma membrane called lipid rafts.[75] Furthermore, the N-terminal sequence of Lck is responsible for interaction with coreceptors, CD4 and CD8;[76] in Fyn it may mediate association with TCR.[77] The N-terminal sequence is followed by a Src homology 3 (SH3) domain (40-70 aa; see 3.4.3), which mediates intra-molecular association with a conserved proline motif in the catalytic domain, and thus negatively regulates kinase activity. The SH2 domain (approx. 100 aa; see 3.4.3) binds to specific phosphotyrosine motifs, for instance, pYEEI in the case of SFKs. The SH2 domain associates with a pY motif at the C-terminus of the molecule, locking the kinase domain in a close conformation Therefore, it is critical

for negative regulation of kinase activity. In the active state of the kinase, both the SH2 and SH3 domains contribute to protein-protein interactions with substrates.[78] The catalytic domain consists of ca 250 C-terminal aa and possesses the classical kinase activation loop (A-loop) with a tyrosine residue, which is phosphorylated in the fully active kinase.[79] At the very end of the kinase polypeptide chain, there is the C-terminal tyrosine-based regulatory sequence that mediates intra-molecular interaction with the SH2 domain. C-terminal Src kinase (Csk) and the related Csk homologous kinase (Chk) are responsible for phosphorylation of this tyrosine; dephosphorylation is accomplished by transmembrane phosphatase CD45.

The best-studied function of SFKs is their role in initiation of the signaling downstream of immunoreceptors. Following receptor ligation, SFKs are activated via mechanisms, which are thus far imprecisely understood. Activated SFK phosphorylates tyrosine residues in the ITAM sequences of TCR, BCR or FcRs. This allows recruitment of Syk kinases to the receptor and their subsequent activation by SFKs.

Because of the functional redundancy between SFKs, knock-out mice deficient in a single SFKs usually exhibit rather mild phenotypes. Double or triple knock-out mice are often embryonically lethal or have seriously compromised immune systems.[77,80]

3.4.2.2 Syk kinases

The Syk family of non-receptor tyrosine kinases consists of two members in mammals: Syk (72 kDa), expressed by B cells, myeloid cells and NK cells, and ZAP-70 (70 kDa), expressed only by T cells and NK cells. Syk-related kinases are also found in invertebrates, suggesting an ancient evolutionary origin.[18] *Syk*$^{-/-}$ mice are perinatally lethal. The major phenotype of a *Syk*$^{-/-}$ embryo is embryonic hemorrhage caused by a defect in the separation of lymphatic vessels from the blood circulation network and a lack of mature B cells.[81]

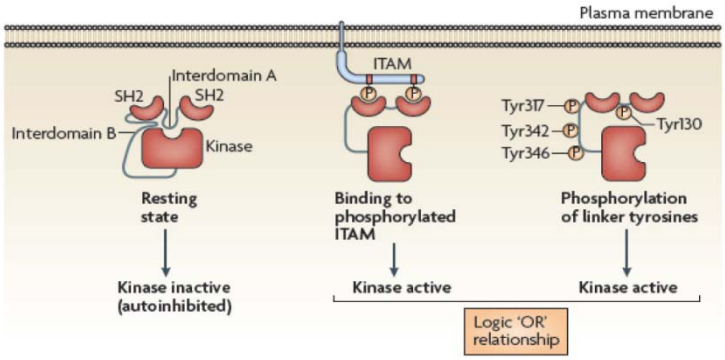


Figure 6: Structural basis of Syk activation. There are three different modes of Syk activation. In the resting state, Syk is autoinhibited by the binding of interdomains A and B to the kinase domain. Binding of the two tandem SH2 domains to ITAMs or phosphorylation of tyrosine residues in the linker regions allows employment of the active conformation. The picture is adapted from Mocsai *et al.*[18]

Syk kinases play an important role downstream of classical immunoreceptors (TCR, BCR and various FcRs) as well as in integrin[65] and C-type lectin signaling,[82-84] bone resorption,[85] and vascular development.[81,86,87] Inhibitors of Syk, fostamatinib and R112, are used in the treatment of some allergic and autoimmune disorders such as allergic rhinitis, autoimmune thrombocytopenia and rheumatoid arthritis.[88-91] Syk is also involved in the development of B cell lineage leukemias and lymphomas.[92,93]

Syk kinases consist of two tandem Src homology 2 (SH2) domains and a C-terminal kinase domain. These domains are connected by two linker regions: the region between the SH2 domains is called interdomain A, and the sequence which lies between the C-terminal SH2 domain and the kinase domain is termed interdomain B. Syk and ZAP-70 share a 56% overall sequence conservation with 60% similarity in the kinase domain.[94] The kinase domain of Syk is inactive in the resting state. To become activated, either the SH2 domains both have to bind to dually phosphorylated ITAM sequences, or tyrosine residues in the linker regions need to be phosphorylated to open the catalytic center into the active conformation (Figure 6).[95] Many of these features are also conserved in its homolog, ZAP-70.[96]

3.4.2.3 Tec kinases

Tec kinases are a group of non-receptor tyrosine kinases which consist of five members: tyrosine kinase expressed in hepatocellular carcinoma (Tec),[97] bone marrow kinase on the X chromosome (Bmx/Etk),[98,99] Bruton`s tyrosine kinase (Btk),[100-102] IL-2-inducible tyrosine kinase (Itk/Emt/Tsk),[103,104] and resting lymphocyte kinase (Rlk/Txk).[105,106] The importance of Tec kinases is exhibited in people with a mutation in the *BTK* gene, who suffer from a rare hereditary disease called X-linked agamaglobulinemia (XLA).[101,102] (Figure 7) is primarily restricted to the hematopoietic system, although Bmx has been detected in endothelial cells and the liver. Low levels of Rlk have been reported in testis.[107]

All members share common structural features (Figure 8). With the exception of Rlk, Tec kinases have a pleckstrin homology domain (PH; see 3.4.3) at the N-terminus, responsible for recruitment to the plasma membrane. The PH domain has an autoregulatory function and is followed by a Tec homology (TH) domain, which is formed by a Btk homology (BH) motif and by one or two proline-rich regions.[108] Interestingly, the BH motif is a highly conserved zinc finger motif, which binds Zn^{2+}. Mutations affecting Zn^{2+} binding lead to an extremely unstable protein.[109] The SH3 domain (see 3.4.3) binding proline-rich sequences has been implicated in autoregulation as well as in protein-protein interactions. The SH2 domain (see 3.4.3) associates with pY motifs and mediates

protein-protein interactions. Finally, at the C-terminus, there is a kinase domain.[110] Rlk is atypical because it contains a palmitoylated cysteine-rich sequence (as opposed to a PH domain and a BH motif), which is responsible for membrane targeting.[111]

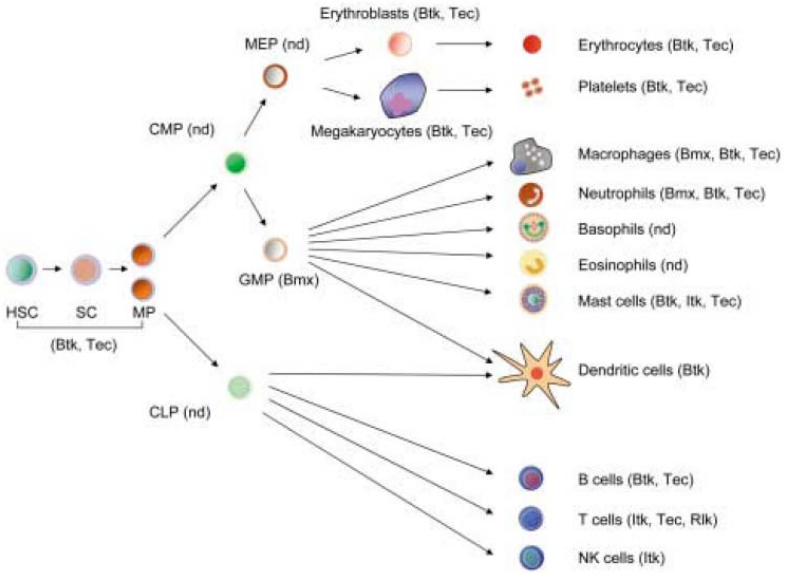


Figure 7: Expression pattern of Tec family kinases in the hematopoietic system. The picture is adapted from Schmidt *et al.*[107]

Activation of Tec kinases proceeds in several steps. First, upon receptor engagement, phosphotidylinositol (3,4,5)-trisphosphate (PIP_3) is generated by PI3-kinase (PI3K) and interacts with the PH domain of a Tec kinase. In the second step, a tyrosine residue in the activation loop of the kinase domain is phosphorylated by SFKs. Subsequently, autophosphorylation of a tyrosine residue

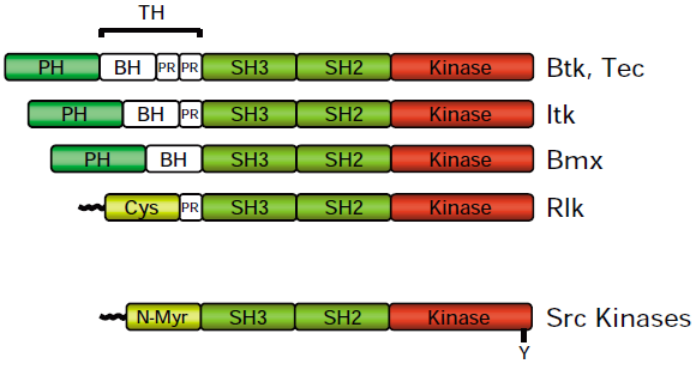


Figure 8: Structural comparison of Tec family and Src family kinases. The picture is adapted from Schmidt *et al.*[107]

within the SH3 domain results in full activation of the Tec kinase. Binding to adaptor proteins such as SLP76 and SLP65 allows incorporation into BCR and TCR signaling cascades and represents another

level of regulation.[112] Tec kinases are regulated by intramolecular interactions between the TH and SH3 domains, preventing binding to potential ligands.[108] In addition, Rlk is directed to the membrane by a PI3K-independent mechanism due to palmitoylation, suggesting a different method of regulation.[111]

3.4.2.3.1 Bruton`s tyrosine kinase (Btk)

Btk is involved in signaling via a variety of receptors including the BCR, FcRs,[113] TLRs,[114,115] G protein-linked receptors,[116,117] the death receptor[118] and cytokine receptors.[119-121] Btk is critical for B cell development, differentiation and signaling. Loss of Btk function causes XLA, a rare primary immunodeficiency disease, which is manifested by an almost complete absence of peripheral B cells and serum Igs of all classes due to a blockage of transition between the pro- and pre-B cell stages.[101,102] T lymphocyte numbers are normal or slightly enhanced. Secondary lymphoid organs such as lymph nodes or tonsils are often smaller.[122] Affected individuals suffer from recurrent bacterial and enteroviral infections which often begin within the first year of life when the maternal Igs have been catabolized. Patients receive prophylactic antibiotics regularly and intravenous Igs every three to four weeks.[122,123] The mouse model of the disease is called *Xid*. *Xid* is characterized by the spontaneous mutation of a CpG site in the *Btk* gene, changing residue 28 in the PH domain from arginine to cysteine.[100,124] *Xid* mice maintain about half the number of splenic B cells because Tec is able to partially replace Btk in mice.[125] The phenotype of *Btk*$^{-/-}$ knock-out mice is similar to *Xid*.[126]

The best-studied role of Btk in signaling is its role in triggering Ca^{2+} flux. It was shown that Btk phosphorylates PLCγ at several tyrosine residues, including Y783, which is necessary for its maximal lipid hydrolase activity.[127] Nevertheless, Btk is a multidomain protein bringing together a broad spectrum of signaling proteins into a single platform and playing a role in many cellular processes.[123,128] Plasma membrane signaling of Btk is predominantly located in lipid rafts and/or caveolae.[129-131] A low concentration of Btk can be detected in the nucleus, suggesting that it is a nucleo-cytoplasmic shuttling protein. The role of Btk in the nucleus is not clear.[132] Btk interacts with F-actin through the PH domain, indicating a role in cytoskeletal rearrangement.[133] Btk has pro- as well as anti-apoptotic functions depending on the specific context.[134] *Btk*$^{-/-}$ DT40 cells do not undergo radiation-induced apoptosis due to the increased activity of STAT3.[135] On the other hand, Btk associates with the Fas receptor and abolishes its interaction with Fas-associated protein with death domain (FADD); thereby, Btk inhibits apoptosis.[118] Btk is implicated in tumor development. Mutations inactivating Btk were found in lymphoid tumors and in colorectal cancer. In addition, *Xid* mice are resistant to certain experimentally induced tumors, while activating mutations cause a transformed phenotype.[128]

Btk is directly involved in modulating the transcriptional status of cells. It interacts with several transcription factors and phosphorylates some of them, thereby inducing their activity. For example, NFκB activity is profoundly impaired in *Btk*$^{-/-}$ cells.[136,137] In addition, Btk plays a role in activation of NFAT[138] as well as BAP-135/TFII-I.[139]

Btk is encoded by a single gene on the X chromosome. No alternatively spliced variants of Btk have been detected so far. Homologs of human Btk have been identified in chimpanzees, fruit flies, zebra fish, clearnose skates, chickens, and the marine sponge, *Suberites domuncula*. Although the Btk sequence is highly conserved among species, its functional role may differ dramatically throughout evolutionary history. In the fruit fly, Btk is necessary for the development of male reproductive organs, while in higher organisms, Btk plays a role in the normal development of the immune system.[123]

Mutations causing XLA have been described in coding as well as in non-coding sequences of the *BTK* gene. To date, more than 1100 different mutations in 970 unrelated families, of which over 600 of them are unique, have been discovered. Thus, most of the mutations are reported in a single family.[140] All types of mutations, including missense, nonsense, mutation at the site of splicing, deletion or insertion, have been detected, all of them either generating functionally compromised Btk or missing it all together.[123] Approximately one third of the mutations are missense.[140] Usually, functionally significant, conserved residues and CpG nucleotides are affected. Interestingly, no patient with the replacement of the Y551 has been reported so far.[141] Typically mutations affect the PH, the TH or the kinase domain. Most of the Btk PH domain mutations are concentrated in the binding site region where they disturb interactions with membrane lipids. About half of all the mutations are located in the kinase domain. They are mainly on one face of the kinase domain which is responsible for ATP, Mg^{2+} and substrate binding.[122]

Various mutants of Btk were used to study Btk function. The E41K mutation of Btk was shown to be hyperphosphorylated[142] and to exhibit a stronger association with membrane phospholipids,[143] resulting in higher amounts of Btk at the plasma membrane.[142] Increased Ca^{2+} mobilization was observed after BCR engagement in *Btk*$^{-/-}$ DT40 cells reconstituted with Btk E41K.[144] However, transgenic mice expressing Btk E41K suffer from similar but more severe B cell defects relative to *Xid* mice. A possible explanation is that the E41K mutation not only blocks the development of follicular recirculating B cells but also causes an enhanced blast formation of splenic B cells *in vitro*.[145] The R28C mutation alters the positive charge on the surface of the PH domain and abolishes the interaction with membrane-resident lipid ligands.[146,147] Thus, Btk R28C cannot relocate from the cytosol to close proximity of the plasma membrane, where activation of PLCγ takes place.[148] Interestingly, according to the analysis of the structure of the PH domain, the E41K mutant is able to bind two molecules of PIP_3 as compared to one molecule in WT.[147] The Btk K430E mutation targets the kinase domain of Btk, rendering it inactive. In contrast, in *Btk*$^{-/-}$ DT40 cells reconstituted with Btk K430E, Ca^{2+} mobilization is fully restored.[149] In this study, it was proposed that in addition to its

kinase activity, Btk might also function as an adaptor protein that facilitates Ca^{2+} flux. Similarly, Btk's tumor suppressor activity is fully restored by the kinase inactive form of Btk.[150] Of note, Btk R525Q, another kinase inactive mutant, did not reconstitute BCR-induced Ca^{2+} flux.[151]

In the promoter of the *BTK* gene, two active NFκB-binding sites, which bind both of the NFκB subunits p50 and p65, have been identified. Moreover, transcription of Btk was suppressed following inhibition of the NFκB signaling pathway.[152] *Xid* and *Btk*$^{-/-}$ mice display decreased functionality of NFκB pathway.[153] Thus, Btk can positively regulate its own transcription via NFκB signaling and Btk and NFκB create an autoregulatory feedback loop.

A low resolution model of Btk's structure revealed that Btk displays an extended conformation with no, or few, inter-domain interactions.[154] Prolonged activation of Btk is prevented by other proteins, negatively regulating its activity. In this respect it was shown that PKCβ phosphorylates a key serine in the TH domain (S180), which interferes with membrane targeting and subsequent activation of Btk.[144] In contrast, other isoforms of PKC such as PKCθ have a rather positive regulatory role on the kinase activity of Btk.[155] Recently, an inhibitor of Btk (IBtk) was identified by a yeast-two-hybrid screen using Btk as the bait. IBtk physically associates with Btk and down-regulates its kinase activity.[156] The mechanism is not completely clear; however, it is dependent on interaction with the PH-TH region of Btk.[157] In addition, Yamadori *et al*[158] showed that the protein Sab, which binds the SH3 domain, negatively regulates the auto- and transphosphorylation activity of Btk. Ubiquitin ligase c-Cbl also associates with the SH3 domain and may possibly target Btk for degradation. Upon BCR activation, Btk is transported into lipid rafts and caveolae where it is either reactivated, transported to the cytosol and reused or simply degraded. Interaction of caveolin-1 with Btk results in dramatic down-regulation of the kinase activity of Btk.[131,159] Peptidyl-prolyl cis-trans isomerase, Pin1, catalyzes conformational changes of protein substrates and influences their function and stability. Pin1 does not influence the kinase activity of Btk but rather it modulates its expression levels in a cell cycle-dependent manner.[160]

Btk is a promising target for immunotherapy, therefore the design of efficient inhibitors of its function is under constant investigation. First, using an *in vitro* assay to block interaction between Btk and PKC, Kawakami *et al*[161] developed a quinine epoxide, called terreic acid, which seemed to efficiently inhibit Btk kinase activity. However, its high toxicity and low specificity makes it unsuitable for clinical applications. Mahajan *et al*[162] used a modeling approach to design leflunomide metabolite analogs with a high likelihood of binding favorably to the catalytic site within the kinase domain. However, LFM-A13 was also shown to inhibit other members of the Tec PTKs as well as other kinases.[163,164] Recently, PCI-32765 was shown to bind irreversibly into the active site of Btk and block Btk activity at IC_{50} = 11 nM. This inhibitor has thus far been used successfully in a clinical study using spontaneous B-cell non-Hodgkin lymphoma in dogs.[165]

Btk`s function in innate immunity

In innate immune cells, the role of Btk is less clear. Myeloid cells usually express more than one member of the Tec kinase family, and they often display redundant functions in signaling. Btk has been implicated in several pathways in myeloid cells,[112] and some of them are described in the following pages.

Monocytes/macrophages

Recently, it was shown that Tec family kinases are involved in macrophage survival via M-CSF signaling. Stimulation of M-CSF resulted in phosphorylation of Btk. Using a suboptimal amount of M-CSF, *Btk*$^{-/-}$*Tec*$^{-/-}$ bone marrow-derived macrophages (BMDMs) displayed increased cell death, correlating with a severe drop in macrophage numbers as compared to WT. In addition, Tec and Btk are required for expression of GM-CSFRα in BMDMs but not in BMDCs, suggesting that Tec kinases contribute to the lineage-specific regulation of GM-CSFRα expression.[166] FcγR-mediated, CR1- and CR3 (CD11b/CD18)-dependent phagocytosis and LPS-induced chemotaxis of monocytes isolated from XLA patients were reduced.[167] Moreover, in mouse peritoneal macrophages, Btk is involved in Fcγ receptor-mediated phagocytosis.[168]

Using a yeast two-hybrid system and subsequent overexpression studies in HEK293T cells, Jefferies *et al*[114] showed that Btk can associate with the TIR domain of TLR4, 6, 8 and 9, with adaptor proteins MyD88 and MyD88 adaptor-like (Mal), and with IRAK-1. In human monocytes and macrophages, Btk is activated downstream of TLR2 after Pam3Cys treatment and TLR4 after LPS stimulation.[169,170] Treatment of the mouse macrophage cell line RAW 264.7 with the Btk inhibitor LFM-A13 abolished LTA-induced activation (acts through TLR2) of Btk and subsequent expression of TNF-α and MIP-2.[115] *Xid* macrophages exhibit impaired phosphorylation and transactivation of the p65 subunit of NFκB upon LPS,[171] CpG (ligand of TLR9) or R848 (ligand of TLR7 or 8)[172] treatment. Macrophages from *Xid* mice produce significantly lower amounts of the pro-inflammatory cytokines, TNF-α and IL-1β, in response to LPS challenge, although the induction of MHC and costimulatory molecules was not affected.[173] Furthermore, IL-10 production in response to LPS was reduced in *Xid* macrophages, while IL-6 production was enhanced.[174] In addition, *Xid* macrophages show impaired burst of reactive oxygen species.[175]

Contradictory results were obtained from studies of TLR stimulation of monocytes of XLA patients. In one report, relative to healthy controls, monocytes of XLA patients secreted reduced amounts of TNF-α upon LPS stimulation.[169] On the other hand, Perez *et al*[176] reported that monocytes of XLA patients showed similar TNF-α expression upon TLR4 triggering, compared to healthy individuals. Interestingly, incubation of XLA monocytes with M-CSF leads to an increase in the

expression of Tec and restores the ability to produce TNF-α upon LPS stimulation, suggesting a compensatory role for Tec, which has been previously reported for murine B cells.[169]

Xid mice seem to be less susceptible to the development of various inflammatory and autoimmune diseases in experimental models.[175,177,178] The induction of experimental autoimmune encephalomyelitis is slower and less severe in *Xid* mice relative to WT. Moreover, Mangla *et al*[175] observed less severe disease progression in models of a dextran sodium sulphate-induced colitis and of a Carrageenan-induced acute edema.

Btk is also crucial for suppressing inflammatory responses. Btk can phosphorylate Mal.[179] Phosphorylated Mal then interacts with suppressor of cytokine signaling 1 (SOCS1) which results in ubiquitinylation of Mal and its proteasomal degradation.[180]

Neutrophils

Human neutrophils express Bmx, Btk and Tec (Figure 7). The Btk inhibitor, LFM-A13, suppresses tyrosine phosphorylation, production of superoxide anions, adhesion, chemotaxis and phospholipase D activity after treatment with the bacterially derived chemotactic factor fMLP. LMF-A13-pretreated cells display decreased accumulation of PIP_3 in the plasma membrane accompanied with reduced amounts of translocated Rac-2, RhoA, ADP ribosylation factor-1, Tec, Bmx and Btk after fMLP stimulation.[181] LFM-A13 has also been reported to inhibit degranulation and Ca^{2+} mobilization of neutrophils upon CD16b cross-linking.[163] Recently, Btk was implicated as an important regulator of neutrophilic granulocyte maturation and function. Fiedler *et al*[182] reported that GM-CSF- and TLRs-induced differention is impaired in *Btk*$^{-/-}$ neutrophils. They found that Btk is critical for expression of lineage-determining transcription factors C/EBPα, C/EBPβ and PU.1. Moreover, expression of MPO, gelatinase, elastase and other granule proteins was dependent upon Btk activity.

XLA patients show an increased neutropenia, typically coinciding with severe infection.[183,184] However, neutropenia is not a characteristic of XLA patients on sufficient Igs therapy. Moreover, no defect in respiratory burst, MAP kinase signaling or shedding of surface CD62L was observed in XLA neutrophils upon TLR4 or TLR7/8 triggering.[185]

Dendritic cells

In dendritic cells, the impact of Btk in signaling is not completely clear. Gagliardi *et al*,[186] found that monocyte-derived DCs from XLA patients and healthy individuals exhibit no difference in DC differentiation, antigen presentation, or LPS-induced maturation. On the other hand, the work of Taneichi *et al*[187] revealed that responses to ligation of TLR2, 3, 4, 7/8 are compromised in monocyte-derived DCs of XLA patients, leading to impaired phenotypic maturation based on CD83

expression accompanied with lower production of TNF-α. In addition, Sochorova *et al*[188] reported that XLA patients have normal numbers of circulating DCs which display no apparent defects in response to stimulation of TLR1/2, 2/6, 3, 4 and 5, but they do exhibit a profound impairment of IL-6 and TNF-α in response to stimulation by TLR8 agonist. This observation is further strengthened by the fact that XLA patients suffer from recurrent enteroviral infections.

In the mouse system, *Btk*$^{-/-}$ BMDCs appear to be more mature and exihibit an increased *in vitro* and *in vivo* T cell stimulatory function compared to WT BMDCs. The role of Btk in DCs seems to be mediated by IL-10, which activates STAT3. *In vivo*, *Btk*$^{-/-}$ mice show enhanced inflammatory responses in Th2-driven asthma and Th1-mediated contact sensitivity.[189]

Osteoclasts

The role of Tec kinases in the development of osteoclasts, cells necessary for bone resorbtion, has been proposed.[190,191] *Tec*$^{-/-}$*Btk*$^{-/-}$ mice show an osteopetrotic phenotype due to a defect in bone resorption. *In vitro* osteoclast differentiation of BMMs in the presence of the receptor activator of NFκB ligand (RANKL) and M-CSF was severely impaired in the absence of Btk and almost completely abrogated in the absence of Tec and Btk.[191] Shinohara *et al*[191] suggested a mechanism of this defect. Upon RANKL activation, a complex between phosphorylated Btk and adaptor molecules such as SLP65 is formed. The complex formation requires ITAM-mediated signaling since it is abolished in *DAP12*$^{-/-}$*FcγR*$^{-/-}$ cells. Btk signaling leads to the phosphorylation of PLCγ1 and PLCγ2 and subsequent activation of the critical transcription factor, NFATc1, for osteoclast differentiation. Indeed, NFATc1 activation, as well as Ca^{2+} flux and phosphorylation of PLCγ1 and PLCγ2, was seriously impaired upon RANKL stimulation in *Tec*$^{-/-}$*Btk*$^{-/-}$ mice. In addition, transduction of Btk rescued NFATc1 activation.[190,191] Together these data suggest that Tec kinase provides a link between the RANKL and ITAM-mediated signaling pathways.

Osteoclasts from XLA patients showed defective resorption activity *in vitro* but bone density and bone turnover markers were not altered in XLA patients. In serum of XLA patients, increased levels of inflammatory cytokines were detected. Addition of this serum restored the activity of XLA osteoclasts and led to the normalization of bone density *in vitro*.[192]

3.4.3 Adaptor proteins

Adaptor proteins lack intrinsic enzymatic activity and instead they mediate protein-protein and/or protein-lipid interactions. Shaw *et al*[193] specify the functions of adaptor proteins in four ways: (1) they operate as a scaffold onto which signaling molecules assemble; (2) they distribute signaling components to specific places in a cell; (3) they organize positive and negative feedback signals necessary to alter the signaling pathways; and (4) they protect activated signaling molecules from

inactivation (e.g. by phosphatases). Therefore, adaptor proteins enhance specificity and efficiency of signal propagation by concentrating signaling molecules at a specific site at the right time.[193] In their sequence, they contain various structural domains, which enable specific binding.

The *SH2 domain* was first identified in the Src kinase. The SH2 domain is an evolutionarily highly conserved structural module containing ~100 aa organized into two α helices and seven β strands. The SH2 domain plays an important role in signal transduction because it associates with a phosphorylated tyrosine (pY) residue. Selectivity for a particular, phosphorylated site is defined by residues located three to five amino acids C-terminally to pY.[194]

Similarly to the SH2 domain, the *phosphotyrosine-binding (PTB) domain* binds to a pY residue. However, the prototypical recognition sequence was determined to be NPx(pY/Y/F) (x represents any aa), where residues located N-terminally of the tyrosine are important. Moreover, the tyrosine residue does not have to be phosphorylated at all. Interestingly, PTB domains are structurally very similar to PH domains and can also interact with phospholipids. Thus, PTB domains exhibit much broader binding specificity than SH2 domains.[195]

The *SH3 domain* predominantly prefers peptides that contain a central motif, PxxP (x represents any aa). Residues surrounding this core motif determine the exact binding preferences of a particular SH3 domain. There are 13 different subtypes of SH3 domains; some of them do not even recognize the typical PxxP motif. It seems that most of them specifically interact with the structural module called PPII (polyproline type II), a left-handed helix with three residues per turn. In addition, positively charged residues such as arginine and lysine have been known to play an important part in binding an SH3 domain.[196]

The *WW domains* are relatively small structural units containing around 40 amino acids. The name is derived from two conserved tryptophan residues. Their structure is formed by a triple stranded β-sheet. WW domain-containing proteins are localized inracellularly or in the cell nucleus. Several types of WW domains with different consensus binding sequences are defined. The biggest group recognizes the motif, PPxY; the second group interacts with the PPLP-containing peptides. Group III of the WW domains binds poly-P motifs flanked by R or K, while Group IV of the WW domains associates with short sequences with phospho-S or phospho-T followed by P, in a phosphorylation-dependent manner.[197]

PDZ domains consist of 80-90 aa comprising six β strands and two α helices arranged in a globular structure. Proteins often consist of multiple copies of PDZ domains, and they are almost always cytoplasmic. They bind the carboxyl-terminal sequences of proteins, usually transmembrane receptors and channels. Various C-terminal hydrophobic residues are important for binding of the PDZ domain.[198,199]

The *Plecstrin homology (PH) domain* was first identified in the protein, pleckstrin, from which it received its name. The PH domain generally favors phosphoinositides and is usually responsible for anchoring proteins to the plasma membrane. Similarly to the other structural domains, the PH domains

also display heterogenous binding specifities and are involved not solely in protein-lipid interactions but also in protein-protein interactions. PH domains fold into a 7-stranded β-sandwich structure. The β1–β2 loop between the first two β-strands marks a deep binding pocket with a basic motif, $Kx_n(K/R)xR$, which is primarily needed for interaction with the phosphate group.[195,200]

The name of the *C1 domains* is derived from 'conserved region-1' of PKC. They contain a zinc-finger motif with a standard sequence $Hx_{12}Cx_2Cx_{13–14}Cx_2Cx_4Hx_2Cx_7C$ (in which C is cysteine, H is histidine and x is any residue). Typical C1 domains bind diacylglycerol, and the functions of atypical C1 domains remain unknown.[200]

C2 domains are named after another homology region in PKC. They associate with a wider range of lipids including phosphatidylserine. Conventional C2 domains bind both Ca^{2+} and membrane phospholipids (phosphatidylserine). However, not all of the C2 domains fulfill these criteria. They are characterized by an 8-stranded, antiparallel β-sandwich of ~130 aa, with three critical inter-strand loops that are required for binding both Ca^{2+} (when relevant) and membranes. Compared to PH domains, they lack the basic binding pocket responsible for association with membrane lipids. The interaction with negatively charged lipids is mediated by Ca^{2+}.[200]

According to the cellular distribution, adaptor proteins (SLP76, SLP65, and MyD88) can be localized intracellularly, while others (LAT, NTAL, PAG, SIT and TRIM) are transmembrane (TRAPs). All TRAPs display similar structural features which somewhat resemble ITAM-containing adaptor proteins associated with the classical immunoreceptors DAP12 and FcRγ. They all possess only a very short N-terminal extracellular sequence, followed by a transmembrane region. A C-terminal cytosolic part of the molecule contains a number of structural features which allows binding and recruitment of cytoplasmic proteins. Tyrosine-based motifs, which do not resemble ITAM motifs, are especially common. These tyrosines are phosphorylated/dephopshorylated after a receptor triggering. In addition, some of the TRAPs contain a cysteine-based palmitoylation motif in a juxtamembrane position. These TRAPs then reside in a special compartment of plasma membrane called lipid rafts.[201]

4 Results and discussion

Publications summarized below are to be found as an attachment at the end of this Thesis.

4.1 NF-κ B-dependent upregulation of ICAM-1 by HPV16-E6/E7 facilitates NK cell/target cell interaction[1]

Cervical cancer is one of the most common female cancers in the world. The causative agents of cervical cancer are high-risk HPVs (HPV16 and HPV18), small dsDNA viruses which infect the epithelial layer of the cervix.[9]

This publication is based on the observation that simultaneous transduction of primary human keratinocytes with viral proteins E6 and E7 of HPV16 directly induces strong ICAM-1 expression at mRNA as well as at protein levels. In the next step, we investigated the molecular mechanisms underlying this observation. It was shown that HPV-E6E7 induce expression of NFκB- and AP-1-responsive genes. Moreover, NFκB, AP-1, GAS and C/EBP are implicated in the transcriptional regulation of ICAM-1.[202] Indeed, we found that HPV-E6E7 cells contain decreased amounts of IκBα, increased phosphorylation of p65 at Ser536 and enhanced levels of p65/p50 in the cell nuclei. Transduction of HPV-E6E7-expressing keratinocytes with an NFκB superrepressor (ΔN-IκBα), a deletion mutant of IκBα lacking the phosphorylation sites Ser32 and Ser36, led to reduction of ICAM-1 expression, indicating that ICAM-1 up-regulation is at least partially mediated via the NFκB pathway.

Finally, contribution of various types of HPVs to the up-regulation of ICAM-1 was investigated. Low-risk mucosal Alpha PV type, HPV6, and cutaneous Beta PV type, HPV38, did not enhance ICAM-1 levels. The other cutaneous Beta PV types, HPV5 and HPV8, induced an increased expression of ICAM-1 but in an NFκB-independent manner, suggesting the involvement of other signaling pathways. HPV38, although belonging to the same group as HPV5 and HPV8, is not considered a high-risk type. In addition, high-risk mucosal Alpha PV type, HPV18, induced an intermediate up-regulation of ICAM-1 and the highest expression was induced by HPV16, indicating that the amounts of ICAM-1 expression correlate with oncogenicity of the respective HPV type.

The enhanced ICAM-1 expression led to increased conjugate formation with the NK cell line, NKL. Furthermore, NKL cell-mediated killing of the HPV^+ cervical carcinoma cell line, CaSki, was greatly reduced by blocking of LFA-1, the ligand of ICAM-1. The role of ICAM-1 in tumor progression is not clear. Up-regulation of ICAM-1 may lead to better retention of leukocytes inside the tumor; attraction of NK cells and CD8 T cells causes increased killing of tumor cells. On the other hand, increased ICAM-1 levels result in a higher risk of metastasis development. For example,

increased amounts of ICAM-1 on breast cancer cells correlated with a better prognosis and survival rate of patients,[203] but in melanoma and liver cancer patients, it correlated with increased risk of metastasis.[204-206] Thus, ICAM-1 up-regulation may account for different prognoses, depending upon the type of tumor. So far, there are no reports describing the impact of ICAM-1 expression in patients with cervical cancer. Therefore, we cannot draw a conclusion as to whether up-regulation of ICAM-1 supports tumor growth or enhances recognition of tumor cells by leukocytes. Nevertheless, our study suggests that cervical carcinoma should be well recognized by NK cells. In addition, activating NK cell receptor ligands such as CD155 and MICA are often up-regulated, and inhibitory NK cell receptor ligands like MHC class I are frequently down-regulated in cervical cancer *in situ*.[207] However, NK cell activity is often suppressed by the tumor microenvironment and by other immune cells such as regulatory T cells.[208] Efficient activation of NK cells might be a promising strategy for successful treatment of cervical cancer.

I contributed to this work by performing retroviral transductions of primary keratinocytes for revision of the manuscript.

4.2 PRR7 is a transmembrane adaptor protein expressed in activated T cells involved in regulation of T cell receptor (TCR) signaling and apoptosis[2]

Signaling is mediated by the activity of many different proteins. Among them, several transmembrane adaptor proteins play regulatory roles when they, for instrance, bring kinases/phosphatases and their substrates into close proximity. Transmembrane adaptor protein (TRAP), PRR7, was first described as a component potentially involved in modulation of neural activities.[209] This work investigated its role in immune cells. PRR7 is a 274-aa highly evolutionarily conserved (94% among placental mammals) protein with structural features typical for TRAPs. It has a short N-terminal, extracellular sequence, a single transmembrane segment and a cytoplasmic region containing several conserved binding motifs. These motifs include multiple SH2 domain binding and/or endocytic tyrosine-based motifs (YxxI/L/V/A), multiple prolin-rich SH3 binding motifs (PxxP), group I of WW domain binding motifs (PPxY), and a C-terminal PDZ domain binding motif (TTAV). In addition, the potential palmitoylation motif (CCxC) is localized in the submembrane region.

Expression of PRR7 at the mRNA level was detected in various tissues including the brain, esophagus, lungs, ovaries, thymus and lymph nodes. Rapid up-regulation of PRR7 was observed in PHA-stimulated PBLs. At protein level, PRR7 was expressed in PBLs activated with anti-CD3 and anti-CD28 mAbs, PHA, or PMA and ionomycin as detected by immunoprecipitation. Furthermore, lymphoid cell lines Jurkat, Ramos and MOLT-4 expressed PRR7. PRR7 was palmitoylated and localized into large but non-buoyant detergent-resistant complexes.

Because overexpression of PRR7 led to rapid apoptosis, a PRR7 inducible expression system was used for generating stable PRR7-expressing clones in the Jurkat cell line, called J-iPRR7. Using this system, we found that a region (aa151 - aa171) surrounding Tyr166 is critical for induction of apoptosis. Full-length PRR7 was localized at the plasma membrane as well as intracellularly, in large vesicular, perinuclear structures. The same sequence (aa151 - aa171) responsible for induction of apoptosis was required for localization into the perinuclear structures. Interestingly, tyrosines were rather dispensable for this effect. PRR7 was phosphorylated after TCR engagement most probably by the kinase Src which also associated with PRR7. J-iPRR7 cells treated with the SFKs inhibitor, PP2, displayed reduced phosphorylation of PRR7. However, PP2 treatment had no effect on apoptosis induction.

Besides induction of apoptosis, the typical phenotypic feature of J-iPRR7 cells was a partially activated state characterized by spontaneous up-regulation of the surface activation marker CD69 and enhanced secretion of IL-2 after PMA and ionomycin treatment. In contrast, attenuated Ca^{2+} flux and reduced phosphorylation of signaling molecules, including ZAP70, LAT, PLCγ1, Erk1/2 and Jnk, were observed simultaneously after TCR stimulation, suggesting a negative regulatory role of PRR7 in the proximal steps of TCR signaling. Moreover, phosphorylation of TCRζ both in basal state and after stimulation was decreased. These effects might be explained by the observation that PRR7 induction also leads to decreased expression and phosphorylation of the Src kinase, Lck. So PRR7 might, by an unknown mechanism, down-regulate the activity of Lck, which is, in turn, manifested by impaired proximal signaling. Interestingly, expression as well as phosphorylation of transcription factor c-Jun was greatly increased. It is speculated that enhanced activity of c-Jun accounts for the partially primed state of J-iPRR7 cells. In this context, it seems that c-Jun acts independently of Lck, because treatment with the SFKs kinase inhibitor, PP2, only minimally affected c-Jun expression. In this respect, it was shown that c-Jun is regulated by ubiquitin-mediated degradation.[210] Furthermore, potential binding sites for E3 ubiquitin ligases were found in the sequence of PRR7. However, no interaction was detected by immunoprecipitation.

Although PRR7 induction has dramatic effects on TCR signaling, it remains to be determined whether it is also important under normal physiological conditions. Expression of PRR7 seems to be very tightly regulated in resting T cells, while in COS-7 or HEK293FT cells, it is well tolerated. Using a conditional *PRR7* gene knock-out may provide more answers.

I helped with this project at the very beginning by carrying out the initial cloning of PRR7 and preparing the recombinant protein for immunization and subsequent testing of the monoclonal antibodies.

4.3 Btk is a positive regulator in the TREM-1/DAP12 signaling pathway[3]

Innate immune responses are orchestrated via multiple cell surface receptors, including G-protein linked receptors, complement receptors, cytokine receptors, TLRs, FcRs and other receptors of the Ig-like superfamily. TREM-1, which belongs to the Ig-like superfamily, was recently implicated in the production of pro-inflammatory cytokines and chemokines during bacterial infection and sepsis. TREM-1 is coupled to an ITAM-containing transmembrane adaptor protein DAP12.[19] Our aim was to identify novel regulators in the TREM-1/DAP12 pathway.

First, we focused on proteins known to be involved in Ca^{2+} flux in other ITAM-based signaling pathways, such as BCR-mediated signaling in B cells. In this publication, we demonstrate that Btk, a member of the Tec family of kinases, becomes phosphorylated on Y551 (which lies in the activation loop of the kinase) upon TREM-1/DAP12 triggering in the human myelomonocytic cell line U937 and primary human PBMCs containing 14% $CD14^+$ cells. This phosphorylation was dependent on the activity of Src and Syk kinases.

To investigate the role of Btk in TREM-1/DAP12 signaling, we generated U937 cells in which expression of Btk was diminished by shRNA-mediated knockdown. In these cells, Erk1/2 and PLCγ phosphorylation, Ca^{2+} mobilization, up-regulation of activation/differentiation markers and production of pro-inflammatory cytokines, TNF-α and IL-8, were all reduced after TREM-1 triggering. These data were further confirmed by introducing a Btk construct insensitive to the shRNA.

Next, the molecular mechanisms underlying Btk activity in the TREM-1 pathway were investigated. Cell lines bearing various Btk mutants were used. The E41K mutation in the PH domain of Btk was shown to be hyperphosphorylated[142] and to exhibit stronger association with membrane phospholipids.[143] On the other hand, the R28C mutation prevents Btk from binding to PIP_3 in the plasma membrane.[148] Thus, Btk R28C cannot relocalize from the cytosol to the close proximity of the plasma membrane, where activation of PLCγ takes place. Finally, the Btk K430E mutation targets the kinase domain of Btk, rendering it inactive. Our data revealed that the E41K mutant acted as a gain-of-function mutant, leading to increased Erk1/2 phosphorylation, Ca^{2+} flux, up-regulation of CD11c and CD86, and cytokine secretion upon TREM-1 triggering. On the contrary, R28C and K430E mutants showed the opposite effects. These data suggest that intact membrane localization and kinase activity of Btk were required for function of Btk in TREM-1 signaling.

To investigate the role of Btk in primary cells, BMDCs from $Btk^{-/-}$, $Tec^{-/-}$, and $Btk^{-/-}Tec^{-/-}$ knock-out mice were used. TNFα production was significantly reduced in $Btk^{-/-}$ and almost completely abolished in $Btk^{-/-}Tec^{-/-}$ cells. It might be that Tec partially compensates for the lack of Btk in $Btk^{-/-}$ BMDCs.

Finally, blood from patients suffering with XLA, a rare immunodeficiency caused by nonfunctional or missing Btk, was collected. PBMCs from the majority of tested XLA patients displayed reduced TNF-α production upon TREM-1 triggering. Since the exact nature of the TREM-1

ligand(s) remains elusive, the consequences of nonfunctional TREM-1 signaling in the clinical manifestation of XLA are not clear. Elevated levels of inflammatory cytokines, including TNF-α, were detected in the serum of XLA patients.[192] Our data suggest that these high levels of TNF-α arise from pathways unrelated to TREM-1. The stimulation of monocytes of XLA patients with LPS resulted in controversial findings. One study reported defects in TNF-α production by monocytes of XLA patients upon LPS stimulation,[169] whereas another study observed similar levels of TNF-α expression.[176] Thus, the contribution of TLR4 signaling to the high TNF-α serum levels in XLA patients requires further investigation.

Collectively, these results indicate that Btk is a positive regulator in the TREM-1/DAP12 pathway. Therefore, it is tempting to speculate that inhibition of Btk with specific small molecules might be a promising strategy in the treatment of inflammation and sepsis.

In this project, I designed and performed the vast majority of experiments needed, analyzed the data and wrote the manuscript.

5 Concluding remarks

The aim of this thesis was to contribute to the understanding of selected physiologically relevant signaling pathways in leukocytes. The main results can be summarized as follows.

1. We found that the adhesion molecule, ICAM-1, is up-regulated upon transduction of E6 and E7 of HPV16. Increased expression of ICAM-1 was mediated by the NFκB signaling pathway. The level of ICAM-1 expression correlated with the oncogenicity of the respective HPV type. ICAM-1 up-regulation led to enhanced conjugate formation with NK cells. Moreover, NK cell-mediated killing of the cervical carcinoma cell line, CaSki, was reduced by blocking of the ligand of ICAM-1, LFA-1. Therefore, strategies to improve recognition of cervical carcinoma cells by NK cells might prove useful for treatment.

2. We identified a new transmembrane adaptor protein, PRR7. Expression of PRR7 seems to be very tightly regulated in resting T cells. Induction of PRR7 leads to apoptosis and reduced proximal signaling events after TCR stimulation. On the other hand, expression of the transcription factor, c-Jun, was up-regulated. A remarkable effect of PRR7 overexpression in TCR signaling is decreased expression and phosphorylation of Lck. In conclusion, these data provide evidence that PRR7 is a potential regulator of signaling and apoptosis in activated T cells.

3. We discovered that Btk, a member of Tec protein tyrosine kinases, is phosphorylated after TREM-1 stimulation in myeloid cells. Btk positively regulates TREM-1-induced Erk1/2 phosphorylation, Ca^{2+} flux, up-regulation of activation/differentiation and production of pro-inflammatory cytokines in the TREM-1 signaling pathway. Intact membrane localization and a functional kinase domain are required for Btk activity in TREM-1-mediated signaling. Interestingly, PBMCs derived from patients lacking a functional *BTK* gene displayed reduced TNF-α secretion after TREM-1 stimulation. These data suggest that Btk plays an important role in inflammatory processes, and that manipulating its activity may be beneficial during inflammation.

6 Abbreviations

aa	amino acid
AP-1	activator protein 1
APC	antigen-presenting cell
Bcl-10	B cell lymphoma/leukemia
BCR	B cell receptor
BH	Btk homology
BMDCs	bone marrow-derived dendritic cells
BMDMs	bone marrow-derived macrophages
Bmx	bone marrow kinase on the X chromosome
Btk	Bruton's tyrosine kinase
CAMs	cell adhesion molecules
CARD9	caspase-recruiting domain, member 9
c-Cbl	Casitas B-lineage lymphoma
CD	cluster of differentiation
CLRs	C-type lectin receptors
Csk	C-terminal Src kinase
DAG	diacylglycerol
DAMPs	damage-associated molecular patterns
DC	dendritic cells
DIC	disseminated intravascular coagulation
Erk1/2	extracellular-signal regulated kinase
FADD	Fas-associated protein with death domain
fMLP	N-formyl-methionine-leucine-phenylalanine
GM-CSF	granulocyte macrophage colony-stimulating factor
Grb2	growth factor receptor-bound protein
ICAM-1	intracellular adhesion molecule, CD54
IFNs	type I interferons
IP_3	inositol 1,4,5-trisphosphate
IRF	IFN-regulatory factor
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
IRAK	IL-1R-associated kinase
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
Itk	IL-2-inducible tyrosine kinase
HPV	human papillomavirus
HMGB1	high mobility group box 1
LFA1	leukocyte-function-associated antigen 1
LPS	lipopolysacharide
(m)Ab	(monoclonal) antibody
Mac1	macrophage-adhesion-ligand 1
Mal	MyD88 adaptor-like
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MCP-1	monocyte chemotactic protein-1
MDSCs	myeloid derived suppressor cells
MHC	major histocompatibility complex
MIF	migration inhibitory factor
MPO	myeloperoxidase
MyD88	myeloid differentiation primary response gene 88
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLRs	Nod-like receptors
NTAL	non-T cell activation linker

PAMPs	pathogen-associated molecular patterns
PBLs	peripheral blood lymphocytes
PBMCs	peripheral blood mononuclear cells
PH	pleckstrin homology
PHA	phytohemagglutinin
PLCγ	phospholipase Cγ
PMA	phorbol 12-myristate 13-acetate
PRR7	proline rich 7
PRRs	pattern recognition receptors
pY	phosphorylated tyrosine
RANKL	receptor activator of NFκB ligand
Rb	retinoblastoma
RIP	receptor-interacting protein
Rlk	resting lymphocyte kinase
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
SFK	Src family kinase
SH	Src homology
SHP	SH2 domain-conatining phosphatase
SLP76/65	SH2 domain-containing leukocyte protein of 76/65 kDa
SOCS	suppressor of cytokine signaling
Sos	Son of sevenless
STAT	signal transducers and activators of transcription
Syk	Spleen tyrosine kinase
ROS	reactive oxygen species
TAB	TAK1 binding protein
TAK1	TGF-β-activated kinase
TAMs	tumor-associated macrophages
TCR	T cell receptor
Tec	tyrosine kinase expressed in hepatocellular carcinoma
TGFβ	transforming growth factor β
TH	Tec homology
TIR	Toll/IL-1R homology
TIRAP/Mal	TIR domain-containing adaptor protein
TLRs	toll-like receptors
TLT	TREM-like transcript
TNFα	tumor necrosis factor α
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRAPs	transmembrane adaptor proteins
TREM-1	triggering receptor expressed on myeloid cells 1
TRIF	TIR domain-containing adaptor inducing IFN-β
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA	very late antigen
WT	wild type
XLA	X-linked agammaglobulinemia
ZAP70	ζ-associated protein of 70 kDa

7 References

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8 Attachments