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Mgr. Ondřej Štěpánek

Regulation of signal transduction by leukocyte surface proteins

Mechanizmy regulace signální transdukce povrchovými proteiny leukocytů

Doktorská dizertační práce

Školitel:
Mgr. Tomáš Brdička, PhD.
Ústav molekulární genetiky, AV ČR

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Prohlášení:

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Podpis

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1. Contents

2. Abstract

The core of the doctoral thesis "Regulation of signal transduction by leukocyte surface proteins" consists of three publications in international peer-reviewed journals dealing with leukocyte signaling both at the level of individual signaling pathways and in the context of a multicellular organism.

Most attention is paid to signaling via the T cell receptor (TCR), which plays a central role in the development and function of T cells and represents a key signaling pathway for proper function of the adaptive component of the immune system. Transmembrane protein tyrosine phosphatase CD148 was considered a negative regulator of TCR signaling through dephosphorylation of LAT and PLCγ1 proteins. This study brings evidence that CD148 is able to modulate signaling also at the level of Lck, both positively and negatively. The net effect of CD148 activity on the TCR signaling is determined by the intracellular biochemical context, notably, the presence of another tyrosine phosphatase CD45. The second project dealt with the characterization of a transmembrane adaptor protein PRR7. This adapter inhibits TCR signaling via down-regulation of the intracellular Lck and cell surface TCR levels.

The research concerning the signaling in the environment of a multicellular organism is represented by the analysis of CD148 expression at different stages of T cell development. Not only CD148 expression changes during the T-cell life cycle but significant differences between mouse and human were identified as well. These differences possibly contribute to different TCR signaling thresholds between the two species and among different developmental stages of T cells and their thymic precursors. To this category also belongs the last project of this thesis which was focused on the analysis of interaction of secreted glycoprotein vitronectin with apoptotic and necrotic cells. Our data combined with previously published studies suggest that vitronectin binds unremoved dead cells in stressed tissues and organs with a high frequency of apoptosis (spleen, thymus). The dead cell-bound vitronectin probably activates integrin and urokinase receptor signaling pathways in leukocytes and other cells, thus contributing to the tissue repair and remodeling. Visualization of the interaction of vitronectin with necrotic/apoptotic cells could be employed to distinguish different stages of cell death.

3. Abstract (Czech version)

Jádro dizertační práce „Mechanizmy regulace signální transdukce povrchovými proteiny leukocytů“ tvoří tři publikace v zahraničních impaktovaných časopisech, které se zabývají leukocytární signalizací jak na úrovni jednotlivých signálních drah tak v kontextu mnohobuněčného organizmu.

Největší pozornost je věnována signalizaci přes T buněčný receptor, která hraje ústřední roli ve vývoji a funkci T buněk a je tedy zásadní signální drahou pro správnou funkci specifické složky imunitního systému. Transmembránová proteinová tyrozin fosfatáza CD148 byla považována za negativní regulátor T receptorové signalizace prostřednictvím defosforylace proteinů LAT a PLCγ1. Součástí této práce je studie ukazující, že CD148 je schopna ovlivňovat signalizaci i na úrovni Lck, a to jak pozitivně tak negativně. O tom, který směr regulace T receptorové signalizace převáží, pravděpodobně rozhoduje biochemická situace v buňce např. aktivita jiné fosfatázy CD45. Druhý projekt, který je součástí dizertační práce, se týká charakterizace transmembránového adaptorového proteinu PRR7. Tento adaptor inhibuje signalizaci T buněčného receptoru tím, že negativně reguluje množství Lck v buňce a T buněčného receptoru na buněčném povrchu.

Studium signalizace z hlediska mnohobuněčného organizmu je reprezentováno expresní analýzou CD148 v různých stadiích T buněčného vývoje. Nejen, že exprese CD148 se mění v průběhu životního cyklu T buňky, ale byly identifikovány i zásadní rozdíly mezi myší a člověkem. Tyto odlišnosti mohou vést k rozdílně nastaveným prahům citlivosti T receptorové signalizace mezi druhy i mezi jednotlivými stadii T buněk a jejich prekurzorů v brzlíku. Další projekt se zabýval analýzou interakcí sekretovaného glykoproteinu vitronectinu s apoptotickými a nekrotickými buňkami. Naše data v kombinaci s dříve publikovanými studiemi naznačují, že vitronectin váže neodstraněné mrtvé buňky v poškozených tkáních a v orgánech s vysokou frekvencí apoptózy (slezina, brzlík). Takto vázaný vitronectin spouští signalizaci přes integriny a urokinázový receptor v leukocytech i dalších buňkách, čímž přispívá k regeneraci a přestavbě poškozených tkání. Sledování interakce vitronectinu s mrtvými buňkami může být využito pro odlišení jednotlivých fází buněčné smrti.

4. Preface

During my 4-year graduate appointment at the Laboratory of Molecular Immunology at the Institute of Molecular Genetics, Czech Academy of Sciences, Prague I was concerned with signaling in cells of the immune system. As the leukocyte signaling represents a very broad research field involving a lot of individual signal transduction pathways with hundreds of participating proteins, I had to focus on particular issues. I was predominantly interested in the early steps in signal transduction. As the majority of signaling pathways starts at the plasma membrane, I focused on plasma membrane associated proteins. My PhD thesis consists of three projects dealing with three proteins playing roles in triggering and regulation of signal transduction by leukocyte surface receptors in mammals. Each project has been finished by a publication in an international peer-reviewed journal.

My primary interest was receptor-like protein tyrosine phosphatase CD148 and its role in TCR signaling. Second project involved identification and characterization of a novel transmembrane adaptor protein PRR7 which is a part of a long term research topic in our laboratory. I mainly contributed to the functional characterization of this adaptor protein in T cells. The last project focused on an extracellular ligand for several immunologically important receptors, vitronectin. I characterized the interaction of vitronectin with apoptotic and necrotic cells.

My dissertation thesis includes following sections: introduction summarizing current knowledge of TCR signal transduction with emphasis on the early events, aims of my research projects, methods, three sections dedicated to description and discussion of the particular projects and resulting publications, conclusions, and reprints of manuscripts that were either published or accepted for publication.

5. Introduction

5.1. Cell signaling

Multicellular organisms consist of highly orchestrated cells that have to subordinate their behavior to the interests of the whole organism. The most typical example of failure of this principal rule is cancer. An indispensable role in the coordination of cellular processes in the organism is played by intercellular communication based on generation and reception of molecular signals. Not only is it essential for the development and homeostasis of the organism but it also enables reactions to various exogenous stimuli including infection.

Immune system protects the organism from invading pathogens. This function is provided mainly by consortium of leukocytes, highly specialized bone marrow-derived cells. Leukocytes are responsible for the recognition as well as the eradication of pathogens. The immune reaction is a complex process involving several leukocyte subtypes (most importantly granulocytes, monocytes/macrophages, dendritic cells, mast cells, T cells, B cells and/or NK cells). The actions of individual leukocytes must be tightly coordinated to achieve complete elimination of infection on one hand and only minimal harm to the host organism on the other. If not working properly, the immune system can be a cause of a wide range of pathologies. When the immune system over-reacts to harmless stimuli, allergy or autoimmunity can occur. On the contrary, immunodeficiency is a condition when the ability of the immune system to suppress infections is compromised.

Cell signaling plays a crucial role in the orchestration of immune response. The signals, that trigger specific cellular programs of individual leukocytes, can be produced either by pathogens or by other host cells. Hundreds of ligands and receptors working on a key-lock based principle constitute for a highly complex dynamic environment governing proper development and function of leukocytes. Importantly, defects in a particular signaling pathway could lead to severe malfunctions of the whole immune system.

The T-cell receptor signaling cascade represents one of the most intensively studied pathways because of its central role in the adaptive immune response. Moreover, it has become a model receptor for studying the signal transduction from the extracellular environment into the cell interior. This also is one of the reasons why I have decided to describe it in more detail. The second and more important reason is that two of the three projects described in this dissertation concern the TCR signaling directly.

5.2. T-cell receptor ligands

The TCR is one of two main receptors of the adaptive immunity (the other one is B-cell receptor). Very important feature of TCR is a random rearrangement of the encoding genes during T-cell development resulting in clonal variability. Each T-cell clone expresses a unique TCR that is able to bind a unique set of potential ligands. Ligands for canonical αβTCR heterodimer are peptides in complexes with Major histocompatibility complex (MHC) I or II (pMHC) on the surface of other (presenting) cells. Due to the phenomenon of positive selection, only those αβT cells expressing TCR with a potential to bind any pMHC complex are capable of accomplishing their development.

In cytotoxic (CD8+) T cells, αβTCR binds MHCI loaded with a peptide produced in the presenting cell. Clones recognizing MHCI loaded with an endogenous peptide are eliminated during negative selection in thymus, thus the TCR on mature T cells should be triggered only when a peptide originating from an intracellular pathogen (virus) is presented. TCR triggering results in the activation of the cytotoxic T cell that subsequently makes the infected presenting cell die. As MHCI is expressed on the majority of cells in the body, cytotoxic cells represent a potent barrier for reproduction and spreading of intracellular pathogens throughout the organism.

In helper (CD4+) T cells, TCR recognizes pMHCII. MHCII is loaded with peptide fragments from particles taken up by specialized leukocytes (macrophages, dendritic cells, and B cells). Again, autoreactive clones are eliminated during negative selection and helper T cells get activated only after a contact with antigen presenting cell that engulfed an exogenous particle (e.g. bacteria). Subsequently, helper T cells produce signals supporting the activity of the presenting cell and other leukocyte subsets.

5.3. TCR triggering

The intracellular signaling platform of αβTCR is represented by associated CD3 and ζ chains that together contain 10 immunoreceptor tyrosine-based activation motifs (ITAMs) (1). It is well documented that the ITAMs are phosphorylated by kinases from Src family after TCR triggering which is crucial for activation of more distal parts of the TCR signaling pathway (1-3). Surprisingly, the mechanism governing inducible ITAM phosphorylation after TCR-pMHC engagement has not been completely elucidated yet. There are three major models explaining the signal transduction after TCR triggering: segregation model, conformational change model, and aggregation model (4). As these models are not mutually exclusive, it is probable that several mechanisms act in synergy to perform phosphorylation of the ITAMs in the TCR/CD3 complex, as suggested for aggregation and conformational change model (5).

The segregation model hypothesizes that the equilibrium between protein tyrosine phosphatases and kinases changes after TCR engagement. This shift was suggested to be caused by two events: (1) the recruitment of Src family kinase (SFK) Lck to the TCR/CD3 complex by CD8 or CD4 co-receptors that bind MHCI and MHCII, respectively (2,6,7) and (2) exclusion of large receptor-like phosphatases from the narrow space between T cell and antigen presenting cell where TCR signaling is initiated (8-10). Although supported by several pieces of evidence, this model poorly explains the situation when TCR signaling is

induced without engagement of CD4/CD8 co-receptors and forming of the immunological synapse (e.g. via anti-TCR antibody).

According to the conformational change model, TCR engagement invokes a conformational change in TCR/CD3 resulting in exposure of ITAMs that are inaccessible for SFKs under resting conditions (11-13). This simple model nicely explains both physiological and non-physiological (e.g. via anti-TCR antibody) manners of TCR triggering. However, little evidence supporting this model is available so far (4,14).

The aggregation model is based on observation that TCR/CD3 cross-linking via specific antibody or soluble multimeric pMHC induces TCR multimerization followed by phosphorylation and down-stream signaling. However, the specific pMHC recognized by the TCR is present in very low concentrations on the surface of the presenting cell (4). Thus, it seems unlikely that many TCR molecules can be co-aggregated by simultaneous binding of specific pMHC. Indeed, just one agonist pMHC complex can be recognized and activate TCR signaling cascade (15). The recent modification of the aggregation model suggests that pseudodimers containing one TCR-agonist pMHC (strong interaction) and one TCR-self pMHC (weak interaction) pair are formed (16).

5.4. Role of Src family kinases in TCR signal transduction

Src family kinases (SFK) represent a group of 8 structurally similar proteins (Fgr, Lyn, Src, Yes, Lck, Hck, Blk, and Fyn). The SFKs are associated with plasma membrane via N-terminal myristoyl and some family members also palmitoyl anchors (17). From N- to C-terminus, SFKs possess SH3, SH2, and catalytic domains followed by a C-terminal tail (18). Two key tyrosine residues are crucial for the regulation of SFK activity. When phosphorylated, the tyrosine in the C-terminal tail (Y527 in chicken Src) participates in an intramolecular interaction with the SH2 domain, promoting closed inactive conformation of the SFK (18). The auto-inhibited conformation is further stabilized via SH3/polyproline type II helix intramolecular interaction (18). The phosphorylation of this inhibitory tyrosine is mediated by C-terminal Src kinase (Csk) and in part by Csk-homologous kinase (Chk) (19). The dephosphorylation of the C-terminal tyrosine leads to the release of the closed conformation. The priming of the kinase could be mediated by multiple tyrosine phosphatases, CD45 and CD148 being predominant in leukocytes (20,21).

In contrast to the C-teminal tyrosine residue, the tyrosine in the catalytic domain (Y416 in chicken Src) plays a positive role and its phosphorylation is required for the full activity of the SFK (18). The activation tyrosine represents an auto-phosphorylation site. CD45 and

Lyp/PEP are believed to be the main phosphatases dephosphorylating the activation tyrosine in T cells (22-24). In contrast to some older models, Lck does not get activated after TCR triggering. Instead, a substantial fraction of Lck is constitutively active and TCR phosphorylation by this kinase is regulated by other means, which may include some of the mechanisms mentioned above (25). Thus, Csk, CD45, and other Lck regulators are important for setting the signaling threshold rather than for driving the intracellular signaling after TCR engagement.

Two members of Src family play a prominent role in TCR signaling: Lck and Fyn. Lck is a major SFK in T-cell signaling cascade, while Fyn probable plays a more subtle role (26). As thymic T-cell development is dependent on the TCR signaling, genetically engineered mouse strains missing essential components of TCR signaling pathway exhibit severe T cell developmental defects. This was the case of $Lck^{-/-}$ mouse, where partial development block was observed, while $Fyn^{-/-}$ thymocytes seemed to be unaffected (7). However, $Lck^{-/-}$ /$Fyn^{-/-}$ double knock-out animals showed almost complete block of thymocyte development, indicating that there is no other SFK able to compensate for their loss (27). Importance of Lck and Fyn in TCR signal transduction was also demonstrated directly by the analysis of Lck deficient cell lines and murine T-cells (26,28-30).

After TCR engagement, Lck and Fyn phosphorylate ITAMs in the TCR/CD3 complex, which represents one of the earliest events in the TCR signaling cascade (7). Phosphorylated ITAMs function as docking sites for 70 kDa zeta-associated protein (ZAP-70), a kinase from Syk family (31,32). Binding to phosphorylated ITAMs both recruits ZAP-70 to the proximity of its substrates and induces release of auto-inhibited conformation of the kinase. For the full activation of ZAP-70, phosphorylation of a crucial tyrosine residue in the kinase domain is necessary (33). This phosphorylation event is probably catalyzed by Lck and/or trans-auto-phosphorylation (33,34). Active ZAP-70 phosphorylates Linker for activation of T cells (LAT) and SH2 domain-containing leukocyte protein of 76 kDa (Slp-76), two adaptors that subsequently constitute for a core of a signalosome complex recruiting a number of other signaling molecules (34-38). LAT is a member of transmembrane adaptor protein family and it is responsible for anchoring of the signalosome complex to the lipid raft membrane microdomains. Although LAT itself does not possess any enzymatic activity, its role in TCR signal transduction is crucial as revealed by impaired TCR signaling in LAT deficient T-cell lines and mice (39,40).

5.5. Down-stream events in TCR signaling cascade

After the LAT/Slp-76 signalosome is formed, the signaling pathway branches. One arm starts at phospholipase-Cγ (PLCγ) that interacts with both phosphorylated LAT and Slp-76 adaptors (41,42). Recruited PLCγ is activated by phosphorylation mostly mediated by IL-2-inducible T-cell kinase (Itk) (43,44). Active PLCγ generates membrane bound diacylglycerol and soluble inositol-1,4,5-trisphosphate (45). The latter second messenger induces intracellular calcium influx by opening calcium channels in the membrane of the endoplasmic reticulum (subsequently calcium channels in the plasma membrane open as well) (38). Increased intracellular calcium activates phosphatase calcineurin that dephosphorylates Nuclear factor of activated T-cells (NF-AT) resulting in its nuclear translocation and turning on the genes governing T-cell activation and differentiation (e.g. IL-2) (46).

Diacylglycerol induces activation of Protein kinase-Cθ (PKCθ) that stimulates phosphorylation of members of the Inhibitor of NF-κB (IκB) family by IκB kinase (IKK) complex. Phosphorylation of IκB leads to its degradation and release of active NF-κB transcription factors (47). NF-κB signaling pathway is involved in T-cell maturation, differentiation, proliferation, survival, and effector functions by enhancement of transcription of a subset of TCR-response genes, including IL-2 (47,48). Additionally, diacylglycerol activates Ras guanyl-releasing protein (RasGRP) both directly and indirectly via PKCθ activity (38). RasGRP functions as a guanyl nucleotide exchange factor activating a small GTPase Ras (49).

Ras can also be activated by a guanyl nucleotide exchange factor Son of sevenless homolog 1 (SOS1) that represents another arm of the signaling cascade emanating from LAT/Slp-76 signalosome (35,50). The recruitment of SOS1 to the phosphorylated LAT is mediated by Growth factor receptor-bound protein 2 (Grb2) adaptor protein (51). Activated Ras stimulates a mitogen-activated kinase (MAPK) cascade leading to activation of Extracellular signal-regulated kinases (Erk) 1 and 2 important for T-cell development, proliferation, differentiation, and effector functions (52-54). Via up-regulation of Activator protein-1 (AP-1) transcription factor`s subunit Fos, Erk also contributes to IL-2 production (38).

Third arm of the LAT/Slp-76 dependent TCR signal transduction pathways starts at Vav, a guanyl nucleotide exchange factor for Rho/Rac family of small GTPases (38). Vav binds

phosphorylated Slp-76 and gets activated by tyrosine phosphorylation (55,56). Subsequently, Vav induces cytoskeletal reorganization supporting the formation of the immunological synapse (57). Moreover, Vav potentiates the other branches of the TCR signal transduction pathway (58).

An important role in TCR signaling in naïve and memory T cells is played by co-stimulating receptors, the most prominent example being CD28 (38). CD28 binds CD80 and CD86 ligands on the surface of mature dendritic cells. CD28 engagement stimulates activity of Phosphatidylinositol 3-kinase (PI3K) that produces phosphatidylinositol (3,4,5) trisphosphate (PIP3) in the inner leaflet of the plasme membrane (59). PIP3 serves as a docking site for several proteins involved in TCR signal transduction, including PLCγ1 activator Itk (38). PIP3 also attracts 3-phosphoinositide-dependent protein kinase 1 (PDK1) and its substrate, another kinase Akt/PKB. Activated Akt/PKB prevents apoptosis, stimulates glucose metabolism, and enhances the NF-κB signaling pathway by phosphorylation of a handful of substrates (59,60).

5.6. Regulation of TCR signaling

The TCR signal transduction pathway is regulated in terms of the signaling threshold, duration, and magnitude to set the proper T cell response. As already mentioned, the first phosphorylation step of TCR signal transduction is catalyzed by Lck and Fyn kinases. The activity of the kinases is regulated by phosphorylation of the C-terminal tyrosine by Csk and opposed by CD45 that functions also as Lck inhibitor by dephosphorylating the catalytic loop tyrosine (19,21). The amount of active Lck at the moment of TCR engagement probably contributes to the regulation of the TCR signaling threshold and magnitude (25,61,62).

Other protein tyrosine phosphatases typically regulate TCR in a negative manner. SH2 domain-containing phosphatase 1 (SHP-1) dephosphorylates many proteins involved in the TCR signaling cascade, including Lck and ZAP-70, and probably regulates the magnitude as well as shut-down of the TCR signaling (63-65). Recruitment of SHP-1 to the signalosome is mediated by the interactions with kinases Lck and Zap-70 and phosphorylated immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (63,65). ITIMs are inhibitory signaling platforms found in the intracellular part of various inhibitory receptors (CTLA-4, PD-1, CEACAM1) negatively regulating the TCR signaling (38). Loss of SHP-1 decreases the signaling thresholds for both positive and negative selection during T-cell development (66).

An unexpected role in the attenuation of the TCR signaling is played by LAT. After TCR activation, a SHIP-1/Dok/LAT/Grb2 complex is formed (67). Src homology 2 domain-containing inositol-5-phosphatase-1 (SHIP-1) inhibits TCR signaling by counteracting the activity of PI3K and dephosphorylating the substrate of PLCγ1 (68). Adaptors from Downstream of tyrosine kinases (Dok) family inhibit Ras signaling and are suggested to compete for ITAM binding with ZAP-70 (69). The inhibitory function of LAT was first demonstrated in LAT Y136A knock-in mice where Tyrosine 136 (PLCγ1 binding site) of LAT is mutated to Alanine. Besides impaired T-cell development these mice exhibited hyper-responsiveness in the periphery (70).

During the termination of the TCR signaling, internalization and degradation of the TCR/CD3 complex and other signaling molecules occur. E3 ubiquitin-ligases from Casitas B-lineage lymphoma proto-oncogene (Cbl) family bind to the TCR-induced signalosome and mark the TCR/CD3 complex and other signaling molecules for degradation (38,71-74). Loss of this negative regulation in Cbl-b$^{-/-}$ mice leads to the full T-cell activation even in the absence of co-stimulation, resulting in the induction of the immune response, and subsequent development of autoimmunity (72,75).

TCR signaling is also regulated at the organismal level. Under some circumstances, the once TCR-activated T cells are eliminated via apoptosis to prevent autoimmunity and hyper-responsiveness. This rule applies during the negative selection in thymus and during the termination of the immune response via activation-induced cell death and activated cell autonomous death of terminally differentiated T-cells at the periphery (76,77). The exceptions for the latter principle are long-lived memory T cells, bearers of the adaptive immunological memory (78).

6. Aims of the study

The study was focused at three proteins (CD148, PRR7, and vitronectin) involved in early steps of leukocyte signal transduction. Interestingly, the initialization of the three projects was completely different. CD148 had been an intensively studied enzyme (22) and we concentrated on its role in T cells that had not been completely understood at that time. We were especially interested in the role of CD148 in regulation of SFKs involved in TCR signaling. Thus, we investigated a novel role of a well-known enzyme.

Contrastingly, the other two projects were directed at identification and characterization of proteins based on their unique properties. PRR7 was discovered in a genome-wide screen employed to identify poorly described proteins with structural features of transmembrane adaptors. Subsequently, we focused on functional characterization of PRR7 in terms of T-cell physiology. We concentrated mostly on the role of PRR7 in TCR signaling and apoptosis of T-cells.

The process of vitronectin identification was principally different. We had an antibody (called 2E12) to an unknown structure present in apoptotic cells. As numerous structures appearing upon apoptosis commitment are involved in apoptotic cell recognition, it was likely that our structure could act as a ligand triggering cell signaling pathways. Indeed, we identified the structure as extracellular protein vitronectin that is recognized by various leukocyte receptors (integrins and uPAR). Subsequently, we focused on the analysis of the character of vitronectin binding to dead cells which eventually resulted in the development of a modified approach for characterization of apoptotic and necrotic cells both *in vitro* and *in vivo*.

6.1. Specific aims

1. Expression analysis of CD148 in murine and human T-cells and their precursors.

2. Functional analysis of the role of CD148 in regulation of SFKs involved in TCR signaling.

3. Testing a *phospho-tyrosine displacement model* of SFK/phosphatase interaction using CD148 as a member of R3 subtype of receptor-like tyrosine phosphatases.

4. Characterization of PRR7 effects on TCR signaling.

5. Identification of the structure recognized by 2E12 antibody and its further characterization.

6. Development of thr method to monitor the progression of apoptosis and necrosis.

7. Materials and methods

Two main types of models were employed in the research reported in my dissertation thesis. The first type is represented by human cancer cell lines, especially Jurkat T-cell line and its derivates. These cells were genetically modified to perform reverse genetic studies. The gene manipulations included ectopic expression using transduction with retroviral

vectors, inducible gene expression system, and RNA interference. The other model was represented by primary cells isolated from human and mouse blood and immune tissues. These cells were used mainly for expression analysis.

Data were collected using a wide range of individual protocols. The four key approaches were (1) flow cytometry including measurement of calcium influx in real time, (2) western blotting including quantitative detection using an Odyssey Infrared Imaging System and a set of phospho-specific antibodies, (3) immunoprecipitation including the use of a phosphatase substrate-trapping approach, and (4) confocal microscopy on Leica SP5 system.

One method has been developed as an outcome of my graduate research. It is an improved flow cytometry-based protocol for detection and characterization of apoptotic and necrotic cells *ex vivo* and *in vitro*. The method includes Annexin-V/propidium iodide/vitronectin triple staining.

8. Role of protein tyrosine phosphatase CD148 in T-cell receptor signaling

8.1. Protein tyrosine phosphatases

In eukaryotic cells, protein tyrosine phosphorylation is much rarer post-translational modification than phosphorylation at serine or threonine residues. Nevertheless, the protein tyrosine phosphorylation plays an essential role in the early steps of many signaling pathways. Tyrosine phosphorylation is mediated by tyrosine kinases and counteracted by protein tyrosine phosphatases (PTP). Human genome encodes 107 PTPs that could be divided into two main groups: non-transmembrane and receptor-like (79,80). The specificity of phosphatase domains towards various phospho-tyrosine substrates *in vitro* is rather low, suggesting that the *in vivo* specificity could be determined rather by access to the substrate than by specificity of the catalytic domain *per se*. Indeed, soluble non-transmembrane phosphatases often contain structural domains mediating specific protein-protein interactions (80). A nice example is SHP-1. Besides the phosphatase domain, it contains tandem SH2 domains that interact with immunoreceptor tyrosin-based inhibitory motifs and recruit the phosphatase to the submembrane compartment where it dephosphorylates its substrates (66).

Receptor-like PTPs contain a single transmembrane segment and usually possess a large extracellular ectodomain that can be heavily glycosylated (80). In analogy to receptor kinases, it has been speculated that the ectodomains of transmembrane phosphatases could serve for ligand binding accompanied with a change in the enzymatic activity. So far, very few such ligands have been described, indicating that the general function of the extracellular domains is probably different (81). It could mediate exclusion from cell-cell contact sites, modulation of cell-cell contacts via trans-dimerization, or serve as a steric barrier for self-inhibitory homodimerization (9,10,82,83). It is also possible that specific functions for ectodomains of particular receptor-like phosphatases evolved independently and no general rule applies. There is surprisingly little evidence concerning the relevance of the extracellular domain for the physiological function of the receptor-like phosphatases. CD45 and CD148 phosphatases can be found among the exceptions as it has been shown that shortening of their extracellular domain modulates their biological activity (9,84).

8.2. Two structurally distinct receptor-like phosphatases CD45 and CD148

CD148 and CD45 represent two members of the receptor-like PTP superfamily. Their phosphatase domains are structurally very similar as they both belong to the class I Cys-based protein tyrosine phosphatases (79). However, besides the catalytic domains, these phosphatases are very different: (1) CD45 but not CD148 contains additional pseudo-phosphatase domain, (2) the ectodomains do not exhibit high level of similarity, and (3) CD148 contains a unique intracellular C-terminal tail (80,85).

CD45 is a leukocyte specific phosphatase dephosphorylating C-terminal inhibitory tyrosine in SFKs (21,22). CD45 deficient cell lines, mice, and humans exhibit severe defects in T-cell signaling and development (82,86-89). Interestingly, other leukocyte subtypes seem much less affected by CD45 deficiency in the knock-out mice (22). In addition to the positive role in TCR signal transduction, CD45 acts as a negative signaling regulator by dephosphorylating the activation tyrosine of SFKs and possibly also other substrates (23,90).

Until recently, CD148 was considered an inhibitory phosphatase in various cell types including T-cells, where it dephosphorylated signal transducers LAT and PLCγ (9,91-93). However, increasing pieces of evidence are showing that CD148 is able to activate various SFKs through dephosphorylation of the inhibitory tyrosine (20,94-96). Most importantly, $CD45^{-/-}CD148^{-/-}$ doubly deficient mice exhibited much stronger signaling defect in B cells

and macrophages than CD45$^{-/-}$ or CD148$^{-/-}$ singly deficient mice, indicating that CD148 is able to compensate for the loss of CD45 in these cells (20).

In contrast, CD148 apparently does not rescue CD45 deficiency in T-cell signaling and development (20,22). Additionally, CD148 was shown to inhibit TCR signaling after its ectopic expression in Jurkat cell line (9,91). One possibility explaining differential impact of CD148 on T-cell and B-cell signaling and development was that thymocytes did not express CD148 during the key phases of their development. Alternatively, CD148 might be unable to dephosphorylate Lck, a specific T-cell SFK playing a key role in TCR signal transduction. Major task of my PhD project was to find answers to the open questions concerning regulation of SFKs by CD148 in TCR-mediated signaling.

8.3. Expression of CD148 T cells

Analysis of published data on expression of CD148 suggested that its expression in T cells and thymocytes might differ between mice and humans (97-101). However, this contrast seemed to remain unnoticed and no comparative analysis had been carried out. Moreover, the information about CD148 expression during thymocyte development was very limited. The situation was further complicated by the fact that the only available antibody to murine CD148 (clone 8A-1) did not give very good results upon staining for flow cytometry.

The first aim was to develop satisfactory procedure for CD148 staining. In the end, I used quite simple three-step protocol, where cells were first stained with hamster anti-CD148 antibody (8A-1), followed by staining with two secondary antibodies: goat anti-hamster antibody conjugated with Dy-light 549 fluorophore and subsequent donkey anti-goat antibody conjugated again with Dy-light 549. In addition, quantitative real time PCR on sorted murine thymocyte populations was employed to confirm the data from flow cytometry. Staining of human CD148 was performed using one step protocol with mouse anti-CD148 PE-conjugated antibody.

Comparative analysis revealed that the expression of CD148 is completely different between mice and humans. CD148 is expressed in the early phases of development of murine thymocytes but its production is down-regulated during maturation. Contrastingly, human thymocytes express very low level of CD148 until they reach the very last stage of T-cell maturation when CD148 is heavily up-regulated. Interestingly, both human and murine thymocytes express very low amount of CD148 during the CD4+CD8+ double positive stage when the TCR dependent positive selection takes place and when the developmental block in CD45 deficient mouse occurs.

These observations allowed us to make two major conclusions: 1. CD148 is expressed in low amounts in the critical double positive thymocyte stage both in mice and humans. Thus, it seems probable that CD148 is unable to compensate for CD45 deficiency in thymocyte development because of its low expression during the positive selection. 2. CD148 is expressed in human mature thymocytes and T cells where it might play some role in TCR signaling. Unfortunately, the mouse model is not relevant here, as the CD148 is not expressed in the corresponding cells.

8.4. Redundant roles of CD45 and CD148 in T-cell receptor signaling

To experimentally assess whether CD148 is able to prime Lck in T-cells, we used CD45 deficient leukemic T-cell lines. Majority of experiments were done using Jurkat derived JS-7 cells (102) but two additional CD45 T-cell lines were employed for the key experiments, giving essentially the same results. These cell lines did not express endogenous CD148 which can be associated with their pre-mature phenotype and/or tumor suppressing activity of CD148 described in other tissues. We expressed CD148, catalytically dead CD148 C1239S mutant, and CD45 in these cells and observed their effects on Lck and Fyn phosphorylation and TCR signaling. Not only Lck and Fyn co-precipitated with CD148 but the phosphorylation of their inhibitory C-terminal tyrosines was decreased in CD148 expressing cells.

Substrates of protein tyrosine phosphatases can be identified using a substrate trapping approach. It is based on the substrate trapping mutant of the phosphatase (D1205A in the case of CD148) that binds a substrate but is unable to perform the dephosphorylation step (103). The stabilized substrate-phosphatase intermediate can be immunoprecipitated and disrupted by sodium dodecyl sulfate, enabling the identification of the substrate by western blotting or another method. We used this approach to reveal that Lck is a direct substrate of CD148. Interestingly, data obtained in this experiment suggested that the activation tyrosine is a substrate for CD148 as well.

CD45 deficient T-cell lines exhibited defective TCR signaling measured as calcium influx, CD69 up-regulation, Erk phosphorylation, and an increase in global tyrosine phosphorylation. Ectopic expression of CD148 rescued TCR signaling in these cells in a manner comparable to CD45. Additionally, we confirmed that expression of CD148 in CD45 sufficient Jurkat cells leads to inhibition of TCR signaling. Apparently, CD148 influences TCR signaling both positively and negatively. The net effects of CD148 activity are dependent on the intracellular environment, in this case presence or absence of CD45.

Analysis of the phosphorylation status of Lck using phospho-specific antibodies revealed that expression of CD148 influences the phosphorylation of the Lck activation tyrosine positively in CD45 deficient T-cell lines but negatively in CD45 sufficient cells. Thus, CD148-induced changes of the Lck activation tyrosine phosphorylation nicely correlated with the changes in TCR signaling intensity. According to the recently published study, the phosphorylation status of the Lck activation tyrosine is the decisive factor determining the activity of the kinase, which is in agreement with our observations (25).

We hypothesize that CD148 influences the phosphorylation of the Lck activation tyrosine both directly (negative effect) and indirectly by dephosphorylation of the inhibitory tyrosine (positive effect). Here, the similarity to CD45 is striking, as CD45 also dephosphorylates both critical tyrosines in Lck (22). Moreover, change in the expression of CD45 produces varying effects depending on the specific amount of CD45 in the cells. When the expression of CD45 is very low, its increase enhances TCR signaling. Interestingly, maximum TCR activity is observed at the levels of CD45 which are still sub-physiological and further addition of CD45 partially inhibits TCR signaling (61,62).

In the context of the up-to-date knowledge of the function of CD45, our data indicate that CD148 behaves very similarly to CD45 in terms of TCR signaling. It should be also noted here that we cannot exclude the possibility that CD45 and CD148 differ in the ability to dephosphorylate some other substrates.

8.5. Roles of CD45 and CD148 in setting a TCR signaling threshold

We propose that CD45 and CD148 constitute for an aggregate pool of phosphatase activity modulating the threshold for TCR signaling by setting the amount of active Lck molecules. Although it seems that the negative effects of CD148 on TCR signaling are dominant in wild type T cells, it is important to take into account that CD148 has the potential to regulate TCR signaling in a positive manner. T-cell signaling occurs during various physiological events and under variable circumstances with respect to the developmental phase (thymocyte stages, naïve T cells, memory T cells), lineage (CD4+ vs. CD8+, Th1 vs. Th2 vs. Treg etc.), ligand dependency (tonic signaling or pMHC induced), and environment (e.g. level of oxidative stress). Thus, it is plausible that the net effects of CD148 on TCR signaling differ in various physiological conditions. Likewise, it has been shown recently that CD45 differentially regulates basal and inducible TCR signaling in thymocytes (61).

Expression of both CD45 and CD148 is strictly regulated during human T cell development. CD45 switches from CD45RO to CD45RA splice isoforms after thymocyte maturation and back to CD45RO after T-cell activation at the periphery (104). The biological role of the alternative splicing remains to be explained (105). However, some studies suggested different impacts of individual isoforms on TCR signaling (106,107). The regulation of expression of CD148 during thymocyte development has been described in this thesis. Additionally, CD148 is up-regulated after peripheral T-cell activation both in mice and humans (97,101).

Expression variability of both CD45 and CD148 during the T-cell life cycle suggests changes in the pool of Lck-directed phosphatase activity, possibly leading to differentially set thresholds in particular T-cell subtypes. This assumption is supported by the principles of positive selection in thymus and self-tolerance in the periphery. Thymocytes respond to self-pMHC ligands during the positive selection, whereas naïve peripheral T cells cannot get activated by the same self-pMHC molecules to avoid autoimmune reactions (108).

Moreover, we describe dissimilar expression of CD148 between mice and humans, implying that either the signaling threshold is differently set in murine versus human mature naïve T cells or the effects of this discrepancy are compensated by additional interspecies variability.

Our findings concerning the role of CD148 in TCR signaling together with previously published data are shown in Figure 1. We propose that CD148 helps to set up the proper threshold of TCR signaling in T cells and thus contribute to balance the T-cell activation potential between hyper-sensitivity on one hand and hypo-responsiveness on the other.

Further research in this field could involve identification of additional substrates of CD45 and CD148, investigation of potential functional differences between CD45 and CD148 based on their structural differences, and analysis of the role of CD148 in human primary T-cell subsets using RNA interference.

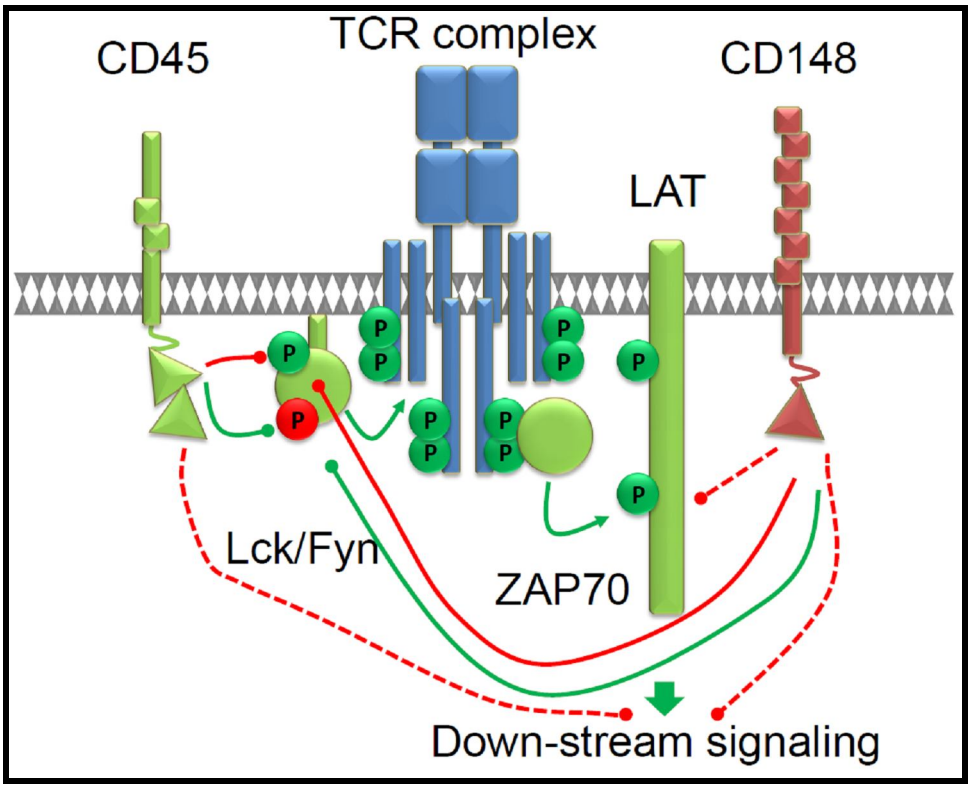

Figure 1 Role of CD148 in TCR signal transduction. The ability of CD148 to dephosphorylate both activation and inhibitory tyrosines in SFKs involved in TCR signaling was identified in my PhD project. The other interactions were published previously (see the text for details and citations).

8.6. Phospho-tyrosine displacement model and R3 receptor-like tyrosine phosphatases

The ability to dephosphorylate the SFKs' C-terminal inhibitory tyrosines is shared by several members of the phosphatase superfamily. However, it still remains to be elucidated, how the phosphatases access the phosphate as the C-terminal phospho-tyrosine is masked by the intramolecular interaction with the SH2 domain. According to a phospho-tyrosine displacement model, the phosphatase itself is phosphorylated at a C-terminal tyrosine that interacts with the SH2 domain of the SFK and displaces the phospho-tyrosine/SH2 intramolecular interaction in the SFK. As a result, the SFK opens, enabling the phosphatase to access and dephosphorylate the SFK's inhibitory tyrosine (Figure 2). This model was suggested and experimentally verified for RPTPα, a receptor-like phosphatase of R4 subtype (109).

CD45 does not have any tyrosine close to its C-terminus but CD148 possesses three tyrosines at its C-teminal tail, one of which (Y1320) is conserved not only among different vertebrate species but also among members of R3 subtype of receptor-like PTPs (110). Although flanked by different sequence than the critical tyrosine in RPTPα, the C-terminal

tyrosines corresponding to CD148 Y1320 in three R3 subtype members were reported to bind Fyn after pervanadate-induced phosphorylation (85).

As we had a nice functional read-out, we decided to test the tyrosine displacement model using CD148 as a member of R3 subtype receptor-like tyrosine phosphatase in JS-7 cells. We mutated all three C-terminal tyrosines of CD148 to alanines. The triple-tyrosine mutant was still able to complement CD45 deficiency implying that the phosphotyrosine displacement model does not apply to CD148. Due to the sequence similarity of all R3 subtype receptor-like tyrosine phosphatases, it is likely that the C-terminal tyrosines of other members are not required for the dephosphorylation of the inhibitory tyrosines of SFKs as well.

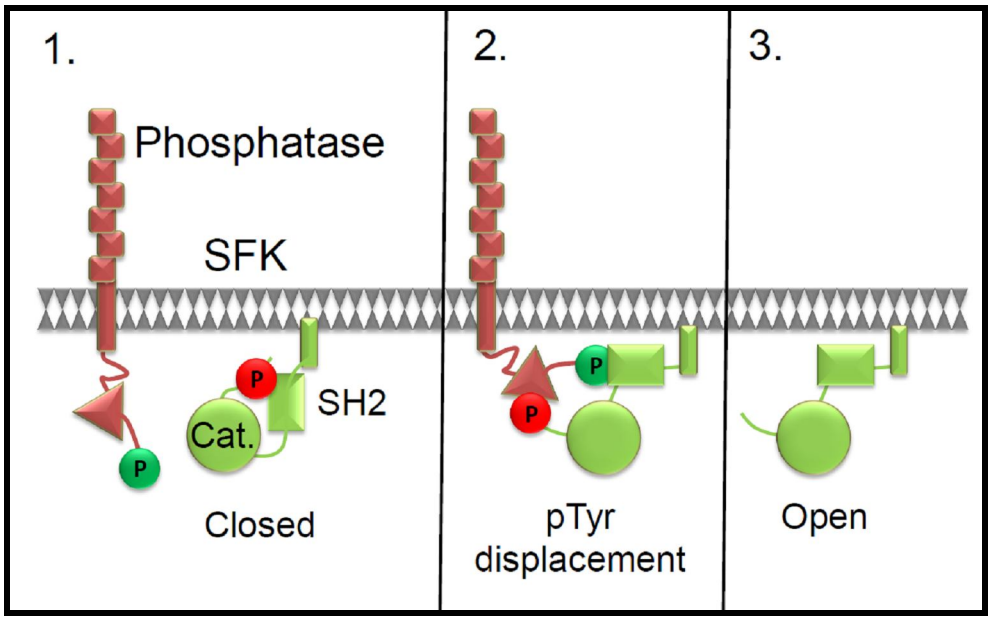

Figure 2 A phosphotyrosine displacement model. See the text for details and citations.

9. Role of transmembrane adaptor protein PRR7 in T cells

9.1. Transmembrane adaptor proteins

Adaptor proteins are common constituents of cell signaling pathways. Unlike kinases, phosphatases, lipases and other typical signaling modulators, they do not exhibit any enzymatic activity. Instead, they bind other components of the signal transduction cascade and bring them together. Thus, adaptor proteins usually play a critical role in the signalosome assembly. From the stochastic point of view, adaptor proteins increase the probability of specific protein-protein interactions resulting in efficient signal transduction between the particular components of the signaling cascade.

Transmembrane adaptor proteins (TRAPs) represent a specific subgroup of adaptor proteins. Typically, they exhibit very short extracellular domain, transmembrane segment, and intracellular domain including protein-protein interaction motifs (111). Membrane anchoring of TRAPs results in the recruitment of TRAP interaction partners to submembrane compartment. Moreover, several TRAPs residing in lipid rafts are believed to drive immunoreceptor signal transduction by attraction of the signaling components to these membrane microdomains (112).

Some transmembrane adaptor proteins are tightly associated with receptors and serve as their intracellular signaling platforms. For instance, ITAM containing CD3 and ζ chains constitute one signaling unit with TCR (1). Other TRAPs do not seem to be physically connected to any receptor and are involved in later steps of signaling cascades. The most important example is LAT that is crucial for TCR-induced signalosome formation and downstream signaling (35).

Identification of novel TRAPs is a long term research interest of our laboratory. The effort has resulted in the discovery of Cbp/PAG, NTAL, and LIME (113-115). To identify further potential TRAPs, genome-wide screen was performed for proteins fulfilling the following criteria: short N-terminal extracellular domain, palmitoylation CxC or CxxC motif between aa 20-60, tyrosine based phosphorylation motifs (YxxI/L/V) and/or Group I PDZ binding motifs (S/TxL/V). Among the predicted novel TRAPs, protein called proline rich 7 (PRR7) was found.

9.2. PRR7

PRR7 was initially described as a component of post-synaptic density associated with NMDA receptor in a rat forebrain (116). However, neither expression nor functional analysis of PRR7 was published until we started to work on this topic. The protein contains short N-terminal extracellular segment, transmembrane domain, and long intracellular sequence containing several putative interaction sites including six predicted phospho-tyrosine motifs.

Expression analysis revealed that PRR7 is strongly expressed in brain and weakly in numerous other tissues. Interestingly, TCR-induced activation of primary human peripheral T-cells resulted in up-regulation of PRR7.

9.3. Effects of PRR7 in T cells

We studied the functional effect of PRR7 after over-expression in Jurkat T-cell line, using inducible expression system for key experiments. It was observed that expression of PRR7 led to massive induction of apoptosis in Jurkat T-cells but not in cell lines derived from solid tissues. The sensitivity of T cells to PRR7 induced apoptosis could be linked to the fact that the programmed cell death is one of possible physiological outcomes of TCR signaling in thymocytes (negative selection) and peripheral T cells (activation induced cell death) (77).

Additionally, PRR7 was identified as a potent inhibitor of TCR signaling. Use of apoptosis inhibitor Z-VAD revealed that the inhibition of TCR signal transduction by PRR7 is at least partially independent of the pro-apoptotic effect. We performed thorough analysis of the whole TCR signaling cascade to identify the particular site affected by PRR7. It showed that the very first step of TCR signal transduction, phosphorylation of the TCR/CD3 complex, is impaired in PRR7 over-expressing cells. We found that cellular levels of Lck and surface TCR are diminished after the induction of PRR7 expression, most likely explaining the observed phenotype. Mechanism of down-regulation of these key molecules by PRR7 remains unsolved and should be a topic of further studies.

Collectively, these results indicated that PRR7 might act as a negative regulator of TCR signaling and inductor of apoptosis in T cells after activation. One of physiological roles of PRR7 might be participation in the elimination of effector T cells during shut-down of the immune reaction and prevention of harmful hyper-responsiveness of activated T-cells.

10. Integrin ligand vitronectin interacts with apoptotic and necrotic cells

10.1. Apoptosis and the tools for its monitoring

Apoptosis, a most common type of programmed cell death, can be induced by both endogenous and exogenous signals. The decision to enter apoptosis is made when the pro-survival signaling pathways are over-balanced by pro-apoptotic signals. Apoptosis plays an important role in the development of multicellular organisms and tissue regeneration. Moreover, apoptosis is essential for the proper function of the immune system as it serves for removal of aged neutrophils, auto-reactive clones in lymphocyte development, effector lymphocytes at the end of the immune response, and infected cells from the body.

In contrast to uncontrolled cell death (necrosis), apoptosis does not lead to the immediate disruption of the plasma membrane. Instead, the potentially harmful cell content is retained in the apoptotic cells/bodies prior to their removal by phagocytes. Engulfment of apoptotic cells by scavenger cells does not invoke an inflammatory response at this stage (117). If the apoptotic cell is not removed by phagocytes timely, the permeability of its membrane increases and the cell progresses to the phase called late apoptosis or secondary necrosis. In this phase, cellular content leaks to the body and inflammation can be induced (118). The transition to the phase of late apoptosis is usually viewed as a potentially harmful process, because defective apoptotic cell clearance is associated with autoimmunity (119).

Several techniques for the detection of early apoptotic, late apoptotic, and necrotic cells have been developed including those focused on DNA fragmentation, protease activity, morphological changes, changes of plasma membrane etc. (120). The latter method belongs to the most popular ones because it can be routinely performed on a single cell level by flow cytometry. A very common protocol employs a fluorophore conjugated Annexin-V together with propidium iodide (PI) staining. Annexin-V binds to phosphatidylserine exposed on the surface of apoptotic and necrotic but not viable cells. PI is a cell non-permeable nucleic acid-binding dye that is excluded by early apoptotic and viable cells. Thus, Annexin-V-/PI- cells are identified as viable cells, Annexin-V+/PI- marks early apoptotic cells, and necrotic and late apoptotic cells stains both with Annexin-V and propidium iodide (121).

However, the permeability of the cell membrane for small charged dyes like PI, does not necessarily means that the membrane is leaky also for macromolecules (proteins, nucleic acids) that could be a potent source of auto-antigens upon defective apoptotic cell clearance. Indeed, it seems that the inclusion of PI precedes the membrane permeability for proteins (122). The disability of the membrane of late apoptotic and necrotic cells to retain macromolecules is usually measured as a leakage of some well detectable protein (e.g. lactate dehydrogenase) to the cultivation media. However, this approach is not applicable for analysis on a single cell basis neither for monitoring the progression of apoptosis *in vivo*.

10.2. Identification of vitronectin as a marker of "very late apoptosis"

Newly described antigens specific for apoptotic cells might become useful tools for detection of apoptotic cells and could help us to improve our understanding of the biology of apoptosis, apoptotic cell clearance, and other processes accompanying cell death in

tissues. Numerous extracellular proteins were reported to bind apoptotic and necrotic cells to facilitate their recognition by phagocytes and mediate their engulfment (117,118). From the signaling point of view, these proteins serve as ligands triggering pro-phagocytic signaling pathways in the scavenger cells.

Mouse monoclonal antibody 2E12 was described to bind apoptotic cells from *in vitro* cultures but the structure recognized by the antibody remained elusive (123,124). We found out that the 2E12 antibody recognizes bovine glycoprotein vitronectin from fetal bovine serum that was present in the cultivation medium. The interaction of vitronectin (a component of plasma and extracellular matrix) with dead cells could be inhibited by heparin and was not mediated by integrins, surface vitronectin receptors.

Further characterization revealed that the plasma protein vitronectin interacted with a so far unidentified structure inside the necrotic cells and cells at the latest stage of apoptosis, occurring about 24 hours after the progress to the PI-positive phase commonly referred to as late apoptosis. We developed Annexin-V/PI/vitronectin triple staining procedure for flow cytometry that can be used to distinguish three stages of apoptosis in respect to the plasma membrane integrity: early apoptosis (exclusion of PI and vitronectin), late apoptosis (staining by PI but exclusion of vitronectin), and "very late apoptosis" (staining by both PI and vitronectin). Monitoring of apoptosis and necrosis revealed that progress from PI+/vitronectin- to the PI+/vitronectin+ stages is substantially slower in apoptosis than in necrosis (Figure 3). This indicates that even after the cell energy metabolism probably stops working during the late apoptosis, there might be mechanisms delaying the complete loss of membrane integrity. We also detected vitronectin positive cells in the immunologically important thymus and spleen in mice, where the programmed cell death commonly occurs. This finding implied that the interaction of vitronectin with the "very late apoptotic" and/or necrotic cells takes place *in vivo* and is not an artifact of the *in vitro* cultivation.

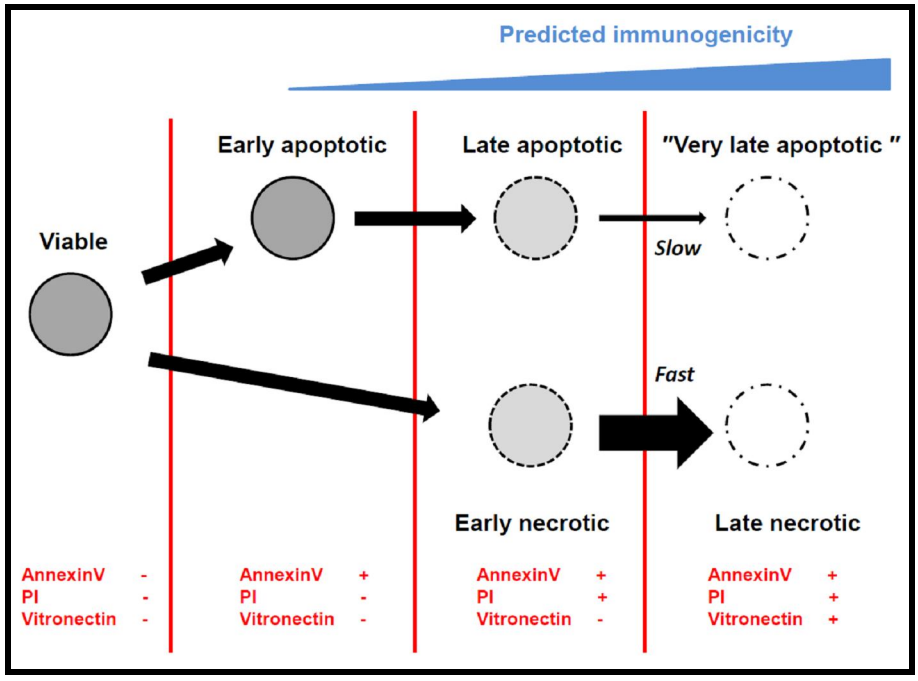

Figure 3 Phases of apoptosis and necrosis progression as revealed by AnnexinV/PI/Vitronectin triple staining. See the text for details.

10.3. Role of vitronectin in injured and inflamed tissues

The identification and characterization of vitronectin interaction with cells in late phases of cell death enabled us to develop a method for more precise monitoring of stages of apoptosis and necrosis. It can be used to distinguish between two late stages of the cell death that differ both from the morphological and immunological points of view. However, the fact, that vitronectin directly binds late apoptotic and necrotic cells, is interesting *per se*.

It has been well documented that vitronectin accumulates at sites of inflamed, injured, necrotic, and cancer tissues including cirrhotic liver, atherosclerotic plaques, injured skin, Alzheimer plaques, myocardial infarction, and colorectal carcinoma (125-133). The mechanism leading to the vitronectin enrichment in the stressed tissues is not fully understood. Two main hypotheses suggest specific transport of vitronectin to the sites of injury and its up-regulation in the stressed tissues, respectivelly. Our data indicate that anchoring of vitronectin to necrotic and "very late apoptotic" cells might contribute to the vitronectin incorporation into the sites of injury, inflammation, and disease.

Vitronectin is a ligand for important surface receptors ($\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_1$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, and $\alpha IIb\beta_3$ and the urokinase receptors). It is well documented that vitronectin can modulate biological processes at the sites of injury, stress, or disease by induction of signaling in leukocytes and surrounding cells. Vitronectin is involved in tissue healing and remodeling,

cell survival, induction of inflammation, and attraction of leukocytes to the site of injury or disease on one hand but is also belived to stimulate tumor invasiveness and development of chronic injuries on the other (126,129,134-139). Our observation of vitronectin binding to the necrotic and/or late apoptotic cells both *in vitro* and *in vivo* contributes to the understading of the behaviour and biological function of this multireceptor ligand.

Further aims should be directed at the identification of the intracellular structure binding vitronectin after the membrane disruption and mapping the parts of vitronectin mediating this interaction.

11.Conclusion

In my dissertation thesis, I discussed three projects dealing with signaling of leukocytes by surface receptors. Two projects concerned characterization of novel regulation mechanisms of TCR signal transduction pathway. These projects were focused on two transmembrane proteins, protein tyrosine phosphatase CD148 and adaptor PRR7, and their involvement in the early steps in the TCR signaling cascade. CD148 regulates TCR signaling by modulating of Lck activity both, in negative and positive manner, depending on the cellular context. In this respect, CD148 resembles another protein tyrosine phosphatase CD45. Expression of PRR7 results in down-regulation of both Lck and surface TCR and inhibition of the TCR signaling.

Second arm of my research concerned characterization of interaction of plasma protein vitronectin with late apoptotic and necrotic cells *in vitro* and *in vivo*. The interaction of vitronectin with dead cells most probably contributes to the accumulation of vitronectin at sites of injury. Vitronectin is a well characterized ligand for various surface receptors (integrins and urokinase receptor) of leukocytes and non-immune cells, triggering pathways important for healing and remodeling of injured tissues. The finding that vitronectin binds necrotic and apoptotic cells at the terminal stage of cell death indicates that vitronectin-dependent signaling pathways are triggered by dead cells at sites of injury and inflammation. Moreover, a newly developed method for analysis of stages of apoptosis and necrosis based on the vitronectin binding can be used for detection of potentially immunogenic cells in organisms as well as for monitoring of physiological outcome of pro-apoptotic signaling pathways (including TCR in some conditions).

Results shown and discussed in my dissertation are integrated into three research publications in peer-reviewed journals (two first author and one third author papers).

11.1. Summary of major findings

1. Expression of CD148 is strongly regulated during T-cell development and substantially differs between mouse and human T-cells.

2. CD148 complements CD45 deficiency in cell lines by Lck and Fyn priming. Moreover, we showed that CD148 regulates Lck both positively and negatively.

3. The *tyrosine displacement model* of SFK dephosphorylation does not apply for CD148 as a member of R3 subtype receptor-like PTPs.

4. PRR7 inhibits TCR signaling by down-regulating Lck and surface TCR.

5. Multireceptor ligand vitronectin binds inside cells at the final stage of apoptosis and necrosis *in vitro* and *in vivo*, possibly explaining the vitronectin enrichment in injured tissues.

6. Annexin-V/PI/vitronectin triple staining for flow cytometry can be used for distinguishing of three stages of apoptosis on a single cell level.

12. Publications

The texts of the publications can be found at the end of this thesis.

12.1. List of publications

1. Stepanek O, Kalina T, Draber P, Skopcova T, Svojgr K, Angelisova P, Horejsi V, Weiss A, Brdicka T. **Regulation of SRC-family kinases involved in T-cell receptor signaling by protein tyrosine phosphatase CD148.** J Biol Chem. 2011 May 4. [Epub ahead of print] (IF_{2009} = 5.33)

2. Hrdinka M, Dráber P, Stepánek O, Ormsby T, Otahál P, Angelisová P, Brdicka T, Paces J, Horejsi V, Drbal K. **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. 2011 Apr 1. [Epub ahead of print] (IF_{2009} = 5.33)

3. Stepanek O, Brdicka T, Angelisova P, Horvath O, Spicka J, Stockbauer P, Man P, Horejsi V. **Interaction of late apoptotic and necrotic cells with vitronectin.** PLoS One. 2011 May 4;6(5):e19243. (IF_{2009} = 4.35)

12.2. Contribution

Ad 1. I performed design (Figure 1, Figure 2A,C,E, Figure 4-8 and partially Figure 2B,D and Figure 3), data collection (Figure 1, Figure 2A,C,E, Figure 4B-E, Figure5-8), and data analysis (Figure 1-2,4-8 and partially Figure 3) for majority of experiments and wrote the manuscript under the supervision of Tomáš Brdička.

Ad 2. I joined the project in its final phase and therefore I was not involved in the initial characterization of PRR7. I performed and analyzed experiments concerning the impact of PRR7 on TCR signaling (Figure 6C-D and Figure 8A,C-D).

Ad 3. I joined the project after vitronectin had been already identified as an antigen recognized by 2E12 antibody. Nevertheless, I contributed to further development of the project (Figures 3-7). Additionally, I remade several previous experiments in a new setup to meet standards required for a scientific publication. Briefly, I designed (Figure 1C,D, Figure 3-7, Figure S2,S3, and partially Figure 1E-F), performed and analyzed (all figures except for Figure 2 and Figure S1) majority of experiments and wrote the manuscript (with corrections by Tomáš Brdička and Václav Hořejší).

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14. Reprints of publications

A. Regulation of SFKs involved in TCR signaling by protein tyrosine phosphatase CD148

B. PRR7 is a transmembrane adaptor protein expressed in activated T cells involved in regulation of TCR signaling and apoptosis

C. Interaction of late apoptotic and necrotic cells with vitronectin