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Role of CD8- and CD4-LCK interactions in the signaling and development of T cells

Úloha interakce LCK a CD8, CD4 koreceptorů v signalizaci a vývoji T lymfocytů

Doctoral Thesis

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Prohlášení:

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Contents

List of Abbreviations

ADAP	Adhesion and degranulation-promoting adapter protein
AIMT	Antigen-inexperienced memory-like T cells
APCs	Antigen-presenting cells
CD	Cluster of differentiation
CSK	C-Terminal Src kinase
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAG	Diacylglycerol
DN	Double negative
DP	Double positive
FYN	Src-family tyrosine kinase (p59fyn)
IL	Interleukin
IP3	Inositol-1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
LAT	Linker for activation of T cells
LCK	Lymphocyte-specific Src-family tyrosine kinase (p56lck)
MHC	Major histocompatibility complex
OVA	Ovalbumin
PD-1	Programmed cell death protein 1
PKC	Protein kinase C
PLC	Phospholipase C
pMHC	Peptide/MHC complex
RAG	Recombination-activating gene

SH2	Src homology 2
SHP	SH2 domain-containing phosphatase
SLAM	Signaling lymphocytic activation molecule
SLP76	SH2-domain-containing leukocyte protein with size of 76 kDa
STS	Suppressor of TCR signaling
TCR	T-cell Receptor
VDJ	Variable-diversity-joining
ZAP70	Zeta-chain-associated protein kinase with size of 70 kDa

Abstract

Adaptive immune response plays a key role in maintaining homeostasis of the organism. T cells use an immense repertoire of T-cell receptors (TCRs) to discriminate self and foreign antigens with very high sensitivity. Although we have many clues outlining how an ideal TCR repertoire is selected, and a good understanding of the TCR signaling machinery, there are still some key aspects of these processes that remain controversial. The objective of this thesis is to extend our knowledge of the very proximal events of TCR signaling, with special focus on interaction of TCR coreceptors with lymphocyte-specific kinase LCK.

Coreceptor-LCK interaction has been described to regulate several aspects of T-cell development and response. We observed dynamic change of this interaction in course of T-cell development. Interestingly, CD4 and CD8 coreceptors displayed differential dynamics of interaction with LCK. Our data suggest that such disparity in coreceptor-LCK interaction leads to selection of more self-reactive TCR repertoire in $CD8^+$ T cells. Moreover, when the highly self-reactive $CD8^+$ T cells get to the periphery, the homeostatic signals drive their differentiation towards a more tolerogenic memory-like phenotype.

To finally resolve the role of coreceptor-LCK interaction in the T-cell development, we established a murine genetic model with abolished coreceptor-LCK interaction. Our data clearly show that the coreceptor-LCK interaction is essential for proper T-cell development and response, especially to weaker stimuli. Yet again, CD4 and CD8 coreceptors diverge in their function. While both CD4-LCK and CD8-LCK require an enzymatic activity for response to weak stimuli, CD4-LCK seems to have additional role that does not require the LCK kinase activity.

This thesis further includes several collaborative projects. We assisted in uncovering that CD4/CD8 lineage choice precedes any changes in the coreceptor expression, and it is most likely dependent on TCR signal strength. We also helped to identify additional scaffold function of LCK, bridging two other important components of proximal TCR signalosome – kinase ZAP70 and adaptor LAT. Next, our cell line model helped to understand function of phosphatase CD45 as a gatekeeper in TCR signaling, ensuring proper TCR ligand discrimination. Finally, we assisted in identifying a single amino acid site on LAT that evolved for more efficient ligand discrimination.

Abstract (Czech Version)

Adaptivní imunitní odpověď hraje klíčovou roli v udržení rovnováhy organismu. T buňky díky obrovskému repertoáru T-buněčných receptorů (TCR) velice citlivě rozlišují mezi tělu vlastními a cizími antigeny. Ačkoliv již máme celkem dobrou představu o tom, jak T buňky získávají optimální TCR repertoár a jak funguje jejich signální aparát, mnoho aspektů těchto procesů je stále nejasných. Cílem této disertační práce je rozšíření znalostí o fungování T-buněčné signalizace, se zaměřením na úlohu interakce TCR koreceptorů s kinázou LCK, která se specificky vyskytuje v lymfocytech.

Předchozí výzkum ukázal, že interakce koreceptorů s LCK kinázou reguluje mnohé aspekty vývoje a signalizace T buněk. V první části této práce jsme pozorovali dynamickou změnu stechiometrie této interakce v průběhu vývoje T lymfocytů. Tato dynamika se překvapivě výrazně liší u CD4 a CD8 koreceptoru. Naše výsledky naznačují, že odlišná dynamika koreceptor-LCK interakce vyústí v selekci více auto-reaktivního TCR repertoáru u $CD8^+$ T buněk. Když se navíc vysoko auto-reaktivní T buňky dostanou do periferie, v důsledku silných homeostatických signálů diferencují do více tolerogenního fenotypu, podobného paměťovým T buňkám.

Abychom jednoznačně prokázali roli interakce koreceptoru s LCK, vyvinuli jsme myší genetický model s LCK neschopnou vázat koreceptory. Náš model jasně ukazuje nezbytnost koreceptor-LCK interakce pro správný vývoj T buněk a jejich odpověď, obzvláště na slabé antigeny. Znovu však vidíme rozdíly u CD4 a CD8 koreceptorů. Zatímco CD4-LCK i CD8-LCK potřebují enzymatickou aktivitu, aby mohly na slabé antigeny reagovat, CD4-LCK má i dodatečnou funkci nezávislou na enzymatické aktivitě LCK.

Součástí této disertační práce je i několik kolaborativních projektů. Asistovali jsme při odhalení, že rozhodnutí o diferenciaci do $CD4^+$ nebo $CD8^+$ T-buněčné linie předchází jakýmkoliv změnám v expresi koreceptorů, a toto rozhodnutí je pravděpodobně závislé na síle T-buněčné signalizace. Dále jsme pomohli při identifikaci další, adaptorové, role LCK. Její interakce s adaptorem LAT zajišťuje správné skládání T-buněčného signalozomu, a to přemostěním kinázy ZAP70 a jejího substrátu – LAT adaptoru. Náš buněčný model pomohl při objasnění role fosfatázy CD45 jako dozorce nad TCR signalizací, zajišťujícího správné rozlišení TCR ligandů. V neposlední řadě jsme pomohli při identifikaci jedné aminokyseliny v sekvenci LAT adaptoru, která se vyvinula pro lepší diskriminaci mezi TCR ligandy.

Preface

During my graduate studies in the doctoral programme in Immunology at the Faculty of Sciences, Charles University, I worked on my PhD project at the Laboratory of Adaptive Immunity at the Institute of Molecular Genetics of the Czech Academy of Sciences in Prague. The laboratory was newly established, and I was one of the first PhD students supervised by Dr. Ondřej Štěpánek.

The scope of my interest during the entire graduate studies was focused on regulation of T-cell receptor (TCR) signaling and its role in the development of T cells. The main focus of my PhD thesis was the investigation of the interaction of two proximal players in the TCR signalosome, the TCR coreceptors and the lymphocyte specific kinase LCK. We studied how this interaction shapes the development and self-reactivity of the T cells. Eventually, we developed a murine genetic model for studying the role of coreceptor-LCK interaction in T-cell development *in vivo*. Moreover, I contributed to several collaborative projects studying T-cell lineage choice and regulation of TCR signalosome. Finally, Dr. Štěpánek gave me an opportunity to work on a perspective article regarding the role of the coreceptor-LCK interaction in regulation of proper antigen-TCR docking geometry.

My dissertation thesis includes the following chapters: introduction, summarizing current knowledge related to the topic, aims of the research projects, brief description of crucial methods, discussion of individual projects and resulting publications, conclusion, and the reprints of the published manuscripts.

1. Introduction

1.1 Adaptive immune response

Adaptive immune response forms a remarkable network that evolved to ensure homeostasis in organisms. The main distinction from the germ-line encoded innate immunity is the availability of immense anticipatory repertoire of receptors, that are trained to tolerate self, but recognize pathogens or altered-self. There are two main subpopulations bearing such receptors. First population are B cells, originally described in Bursa of Fabricius in birds [1], later also in fetal liver [2] and bone-marrow [3] of vertebrates. Second population are T cells which develop in thymus [4]. Both of these subpopulations differentiate from common lymphoid precursor in the bone-marrow [5]. When differentiation of the progenitor cells continues in the bone-marrow, they become B cells, whereas, if they travel via circulation to the thymus, they give rise to T cells.

B cells play a rather instructive role for innate immune cells. The main outcome of their response is differentiation to plasma cells that produce high yields of antigen-specific antibodies. Antibodies then bind to antigens with very high affinity and specificity, what allows the variety of innate immune cells to recognize the threat and eliminate it with high specificity and effectivity. Still, new aspects of B-cell response are being uncovered, including antibody-independent functions of B cells. Therefore, emergence of new discoveries in B cell response has been reviewed quite recently [6]. On the other hand, T cells differentiate into two main branches. The first branch consists of helper and regulatory T cells that recognize antigen on professional antigen-presenting cells (APCs). These $CD4^+$ T cells then choreograph other immune cells, including B cells [7]. The second branch are the $CD8^+$ T cells that surveil somatic cells. They respond to threats rapidly, with very high specificity and in localized manner [8].

In this project, we study the very proximal events in the signalosome of receptor that defines the T cells: T-cell Receptor (TCR). For this reason, following chapters will focus on outlining current knowledge of conventional αβT cells, their development and response to antigens, with specific emphasis on interaction of TCR coreceptors with the most proximal lymphocyte-specific kinase LCK.

1.2 T-cell receptor signaling

Functional TCR signalosome is crucial for T-cell development and response. Unfortunately, the initial steps of TCR signaling cascade are still not completely

understood. The TCR recognizes ligand loaded on antigen-presentation major histocompatibility complex (MHC) of antigen-presentation cell (APC). Upon TCR recognizing pMHC complex, the lymphocyte specific kinase (LCK), which is loaded on either CD4 or CD8 coreceptor, is recruited to the TCR-pMHC complex. This results in phosphorylation of intracellular components of the TCR complex – namely the immunoreceptor tyrosine-based activation motifs (ITAMs) [9] in the intracellular domains of CD3 chains associated with TCRαβ heterodimer. Zeta-associated protein 70 (ZAP70) is recruited to the phosphorylated ITAMs by its Src homology 2 (SH2) domains [10, 11]. When ZAP70 is activated by LCK phosphorylation on its tyrosine(Y)^{315}, Y^{319} and Y^{493} residues [12], it further phosphorylates two adaptor molecules - Linker for activation of T cells (LAT) [13] and SH2 domain-containing leukocyte protein 76 (SLP76) [14]. These adaptors are recruited to the TCR complex and serve as a core of multimeric protein complex that activates phospholipase Cγ (PLCγ), which then generates second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). Their signaling leads to increased calcium levels in cytoplasm, RAS activation and activation of protein kinase Cθ (PKCθ). Eventually, these signaling pathways lead to T-cell differentiation, proliferation and effector response [15].

1.2.1 Regulation of proximal TCR signalosome

The TCR signalosome is highly dependent on its phosphorylation status. LCK itself is regulated by phosphorylation. LCK activatory tyrosine(Y)^{394} residue site acquires its phosphorylation by LCK autophosphorylation, but it can be dephosphorylated by phosphatases CD45 [16], CD148 [17] and SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1) [18]. Phosphorylation of Y^{505} leads to the inactive LCK conformation [19], where C-terminal negative regulatory loop interacts with SH2 domain of LCK. The inhibitory Y^{505} is dephosphorylated by CD45 [20], but this can be counteracted by C-terminal Src kinase (CSK) facilitated phosphorylation of Y^{505} [21]. As approximately 40% of LCK pool is constitutively active in the resting CD4$^+$ T cells [22], the optimal balance between active and inhibited states of LCK may be already important in priming of the TCR signaling. Since resting T cells retain basal level of CD3ζ phosphorylation [23], the recruitment of ZAP70 to phosphorylated ITAMs occurs even without the TCR stimulation [24]. Only full activation of ZAP70 can lead to subsequent phosphorylation of LAT and SLP76 adaptors. Similarly to LCK, ZAP70 phosphorylation can be also reversed. ZAP70 dephosphorylation is facilitated by two phosphatases of suppressor of TCR signaling family (STS) – STS1 and STS2 [25-27], as well as SHP-1,

which can regulate phosphorylation of both LCK and ZAP70 [18, 28, 29]. The cascade of counteracting kinases and phosphatases suggests existence of various sites for regulation of the TCR signaling strength already within the two most proximal kinases LCK and ZAP70.

To make matters even more complicated, there is a range of costimulatory and coinhibitory receptors that regulate the T-cell signaling [30]. CD28 costimulatory receptor was described as the "second signal" required for triggering of the T-cell response. CD28 regulates T-cell response by stimulation of various signaling pathways, mainly the phosphatidylinositol 3-kinase pathway and the pathway regulating actin remodeling [31]. On the other hand, coinhibitory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) recruit SHP-2 phosphatase for negative regulation of TCR signaling. While PD-1 recruits SHP-2 directly to its immunoglobulin-associated-inhibitory and switch motifs (ITIM, ITSM) [32, 33], it seems CTLA-4 recruits SHP-2 indirectly [34]. Recently, PD-1 inhibitory role was implicated in disruption of CD8-LCK cooperation with TCR complex [35].

1.2.2 Tuning response to weak antigens

Multi-level regulation of TCR signalosome provides many possible targets for regulation of T-cell response. Setting up the optimal TCR signalosome is essential for very sensitive discrimination between unharmful self-antigens and potentially harmful foreign and altered-self antigens. The remarkable discrimination capability of the TCR signalosome can be explained by kinetic proofreading mechanism [36]. Data from murine models bearing transgenic TCR receptors with defined specificity and affinity indeed suggested existence of a ligand affinity threshold required for optimal length of pMHC-TCR interaction that is able to trigger signaling [37-44]. However, such model defining sharp affinity threshold, does not fully explain very strong reactivity of T cells to small number of ligands [45, 46].

Several models have been created to help explain measured TCR-pMHC affinities and their activation thresholds in murine models. Regulation of ligand discrimination based on phosphatase exclusion from TCR signalosome [47, 48], ligand rebinding model [49], influence of physical force exerted by pMHC complex [50] or formation of more stable "catch bonds" [51] has been described. Very recently, improvement of the sensitivity of TCR-pMHC affinity measurements uncovered that TCR ligand discrimination does not have a sharp affinity threshold. Rather imperfect ligand

discrimination with gradual loss of T-cell response upon affinity decrease has been reported [52].

1.3 T-cell development

T cells develop from hematopoietic stem cells that give rise to lymphoid precursors which travel through circulation to thymus. In the thymus, Notch1 signaling initiates final commitment to the T-cell line [53-55] and T cells undergo thymic development. At the initial stage of development, thymocytes are described as double-negative (DN), as they do not express neither CD4, nor CD8 coreceptor. Thymocytes start with the somatic recombination of their *TRB* gene segments, and only when productive recombination event occurs, TCRβ is coupled with surrogate pre-TCRα chain, forming the pre-TCR complex. Successful formation of this complex is then tested in process termed β-selection [56]. Constitutive signaling of pre-TCR complex in DN3 stage provides the signal for further development. However, if TCR signalosome is defective at this stage, thymocyte development is attenuated or even halted, e.g. when the key kinases of TCR signalosome are missing [57]. If the pre-TCR signalosome works properly, rearrangement of the *TRB* gene ceases due to effect of allelic exclusion [58]. Thymocytes subsequently initiate rearrangement of the *TRA* gene segments, and upon productive rearrangement, TCRαβ complex is formed on the surface of developing thymocytes. In this stage, thymocytes express both CD4 and CD8 coreceptors, and are therefore dubbed double-positive (DP). During this stage, thymocytes undergo a process of positive and negative selection (the latter continues also in later developmental stages). Following the positive selection, maturing thymocytes retain only CD4 or CD8 expression, becoming either single-positive thymocytes bearing CD4 coreceptor (SP4) or single-positive thymocytes bearing CD8 coreceptor (SP8). These SP4 and SP8 thymocytes undergo further maturation [59] that results in egress of mature T cells from thymus.

1.3.1 Somatic recombination of genes encoding T-cell receptor

T cells recognize threats to organisms using their T-cell receptor (TCR) with high specificity and sensitivity and elicit a very effective response. The hallmark of T cells is an immense repertoire of receptors, recognizing almost anything they encounter. This repertoire is created by somatic recombination of genes encoding the T-cell receptor (TCR) α and β chains. The variable (V), diversity (D) and joining (J) gene segments are virtually randomly rearranged during T-cell development in thymus [60]. The VDJ-recombination is initiated by recombination activation gene 1 and 2 (RAG1, RAG2)

recombinases [61, 62], resulting in DNA breaks that are repaired upon recombination by non-homologous end joining pathway [63]. This DNA repair strategy allows for further increase in variability of TCR sequence because it can lead to deletion or addition of several nucleotides.

1.3.2 Positive and negative selection in thymus

Although evolution of such a sophisticated system generating vast repertoire of TCRs proved to be very beneficial, there is a very high risk of recognition of endogenous components of the organism. This recognition could possibly result in severe autoimmune reactions. Therefore, development of such repertoire needs to be carefully monitored. The immune cells have to go through a thorough process of selection to obtain the most effective and least harmful repertoire of TCR receptors. T cells undergo two main types of selection during their thymic development – positive and negative selection [64, 65]. Developing T cells need to recognize the antigen-presenting molecules presenting self-peptides (self-pMHC) with at least some strength (positive selection), but they cannot react to the self-peptides too strongly (negative selection). These processes are facilitated by antigen presentation on thymic APCs. There are several subpopulations of thymic APCs, ranging in their localization and type of antigens they present to the developing thymocytes [66].

Optimal TCR repertoire requires the selection window to be defined by the strength of T-cell response. T cells respond to variety of ligands that differ in their affinity to the TCR. Those T cells that do not recognize the self-pMHC do not get a signal to proceed with development and die by neglect. Therefore, the process of positive selection ensures that selected TCRs recognize self-pMHC complex, eliciting at least some level of reactivity that is needed for survival of T cells in periphery [67, 68].

Negative selection starts already in thymic cortex, where positive selection takes place, but continues as the SP thymocytes migrate towards thymic medulla [69]. Negative selection process removes thymocytes with overtly autoreactive TCRs by inducing apoptosis in such thymocytes [64]. Some portion of T cells with highly autoreactive TCRs is however rescued from elimination. Those T cells can become regulatory subpopulation of T cells (T_{reg}s) [70] or specific precursors for intraepithelial CD8αα T cells [71]. In a recent review of thymic selection models, the pool of remaining thymocytes that survive the selection ordeal was estimated to be around 2.5-10% of all thymocytes [72]. These become mature T cells that undertake multiple roles outside the thymus.

1.3.3 Lineage commitment of T cells

In course of their development, T cells retain very different cell fates which are to a large extent defined in the selection process occurring in the thymus. As discussed above, the most important determinant of the selection process is the reactivity of their TCRs to the self-pMHC ligands. Such process ensures development of T-cell pool recognizing ligands exclusively presented on MHC molecules. This so called "MHC-restriction" of TCRs results in development of two main subsets of T cells based on differential expression of CD4 and CD8 coreceptors. The $CD4^+$ T cells recognize antigens presented by MHCII molecules, while $CD8^+$ T cells recognize antigens presented by MHCI molecules.

Though CD4/CD8 lineage commitment has been studied for more than three decades, its mechanism is still unclear. Three main models have been proposed: stochastic, instructive, and kinetic [73]. The stochastic model proposes that the binary lineage commitment is more-or-less random, as after pMHC engagement, one of the coreceptors would be stochastically downregulated and only if the signal endures, the T cell commits to the corresponding lineage. If the signal halts, the T cell is eliminated [74, 75]. The instructive model has two versions differing in type of instruction that is critical for lineage commitment. The instruction is proposed to be either the length or the strength of TCR signaling. These models propose that only weak/short signals resulting from pMHCI-TCR interaction will give rise to $CD8^+$ T cells, and only strong/long signals resulting from pMHCII-TCR interaction will give rise to $CD4^+$ T cells. The third model describes kinetic signaling, where the CD4/CD8 lineage commitment occurs in sequence of signaling events and checkpoints, dependent on combination of signal strength and duration. Several studies have proposed that the double positive thymocytes receiving signal from engaging the self-pMHC complex initially downregulate the CD8 expression to test for continuity of the signaling. If the signaling is interrupted, the thymocytes restore their expression of CD8, while downregulating the CD4 expression. Only then the SP8 thymocytes continue their development. If, however, the signaling upon CD8 downregulation is uninterrupted, the thymocytes commit to their SP4 lineage [76, 77].

1.3.4 Antigen-independent cell fate in periphery

As previously mentioned, there are several T-cell fates determined already by the thymic selection. When T cells get to the periphery, they can adopt various cell fates upon ligand recognition. Naïve T cells can become effector, regulatory or memory cells.

Strikingly, the T cell fate decisions in periphery do not have to be driven by antigen recognition. An intriguing population of antigen-inexperienced memory-like (AIMT) $CD8^+$ T cells has been identified [78]. This population does not seem to emerge neither from thymic selection, as they can differentiate from naïve T cells, nor can they be generated from an antigenic response, as they are found in germ-free and antigen-free mice [78, 79]. In order to survive in the periphery, T cells need to receive homeostatic signal from self-pMHC complexes [67, 68]. Such homeostatic signaling has been suggested to drive the differentiation of AIMT cells, as they have a similar phenotype to memory-like T cells that are known to expand homeostatically in lymphopenic or irradiated mice [78, 80-83]. Although they seem to be able to effectively respond to antigens, the type of the response seems to differ from true memory T cells [84]. Hence, their role in immune response remains to be unveiled.

1.4 Coreceptor-LCK interaction in the T-cell development and signaling

1.4.1 Role of coreceptors in T-cell development

Structurally, CD4 and CD8 coreceptors are members of immunoglobulin superfamily. Both consist of long extracellular segment with immunoglobulin-like domains that are able to interact with antigen-presenting MHC complex. The extracellular domain continues to transmembrane domain and short cytoplasmatic tail, where LCK-interaction site is situated. While CD4 coreceptor is able to form homodimers, CD8 coreceptor generally forms a heterodimer consisting of CD8α an CD8β chains [85]. Interestingly, both MHCI interaction and LCK interaction are facilitated by CD8α [85-88], while CD8β ensures correct membrane domain localization via its palmitoylation [89, 90]. Higher availability of LCK is consistent with the formation of CD8αα homodimers in highly autoreactive T cell subpopulations that differentiate for specialized function as intraepithelial T cells [71].

Coreceptors are indisputably crucial players in the proper T-cell development. Indeed, mice deficient in CD4 or CD8 coreceptors do not develop $CD4^+$ or $CD8^+$ T cells, respectively [91, 92]. Intriguingly, in the CD4-deficient mice, a population of $CD8^+$ cells responding to MHCII-presented antigen was observed [93] and in CD8-deficient mice, MHCI-restricted T cells were also detected [94]. In mice deficient in both CD4 and CD8 coreceptors, substantial population of αβ T cells was detected [95]. These cells were not able to respond to pathogen stimuli, only to alloantigens. Interestingly, mice deficient in CD4 or CD8 did not develop any severe phenotype, but their response to some pathogen

challenges was affected. [96-103]. These mice did not develop severe autoimmunity, suggesting that either the self-reactivity of such mature T cells is very low, or that proper T-cell function can be at least partially preserved even without guided MHC-restriction. For example, to compensate the number of peripheral T cells, homeostatic proliferation driven by self-pMHC recognition can fill the empty T-cell niche [67, 68, 104, 105].

The phenotypes observed in the coreceptor-deficient mice fit with some case reports describing patients lacking CD4 or CD8 expression. These patients did not develop autoimmunity, only partial lympho-deficiency manifested by higher susceptibility to pathogens. While patient lacking CD8 expression suffered recurring bacterial infections [106], the patient lacking CD4 expression suffered with relapsing treatment-resistant warts on both hands and feet [107].

1.4.2 Role of LCK and FYN in the T-cell development

LCK is a lymphocyte specific Src family tyrosine kinase, predominantly expressed in T cells. LCK is tethered to the membrane lipid bilayer via palmitoylation and myristoylation of its N-terminal amino acid residues [108-110]. Two cysteines responsible for interaction with coreceptors are also located in the N-terminal sequence of LCK [111]. Together with two cysteine residues on the coreceptors, they form a zinc-clasp interaction [112]. LCK is further composed of SH3 and SH2 domains important for its scaffolding and interaction with other components of TCR signalosome. Finally, C-terminal domain is responsible for the LCK kinase activity. Mutation of singe lysine residue in this domain leads to complete impairment of its kinase activity [113].

Another Src family tyrosine kinase FYN has two splice variants [114]. The *FynT* variant, preferentially expressed in the T-cells, has a role in T-cell signaling [115, 116]. It has been proposed that although LCK and FYN are both able to propagate TCR response, their membrane localization [117] and some of their targets differ. For example, FYN has been described to preferentially regulate pathways leading to cytokine expression [118]. Activation of family of signaling lymphocytic activation molecules (SLAM) is one of such pathways, as FYN is recruited to the SLAM via SLAM-associated protein (SAP) [119]. Moreover, FYN interacts with adaptor protein ADAP [120] that has been implied to interact with SLP76, leading to activation of NF-κB pathway and IL-2 production [121, 122].

To explore the importance of these SRC kinases in T-cell development, mice deficient in LCK and/or FYN were analyzed. Though mice lacking LCK suffered profound block in the T-cell development [123], only in the absence of both tyrosine kinases expressed in the T-cells, LCK and FYN, the T-cell development was completely abolished [124]. In FYN-deficient mice, thymocyte development appeared normal, though there was less robust response to TCR stimuli [125, 126]. Further study suggests that FYN orchestrates TCR signalosome in regards to commitment of $CD4^+$ T cells to their effector function [127].

1.4.3 Coreceptor-LCK interaction in TCR signaling

Although importance of coreceptor and LCK in T cell development is indisputable, the role of their interaction in TCR signaling is controversial. In light of ever emerging new discoveries, two reviews recently tackled the role of this interaction in T-cell development and signaling [128, 129].

Intuitively, the coreceptor-LCK complex was initially implied to deliver the kinase function of LCK for initiation of TCR signaling [88, 130, 131]. Indeed, improvement of TCR sensitivity to weaker antigens was detected in presence of CD4 and CD8 coreceptors [132, 133]. Correspondingly, coreceptor-LCK interaction has been shown to set up threshold of positive [134] and negative selection [44].

Although CD4 and CD8 coreceptors have been shown to interact with MHCII and MHCI molecules, respectively [130, 135], mathematical model predicted that the main function of coreceptor is bringing LCK to the pMHC-TCR complex, not to stabilize the pMHC-TCR complex [136]. Indeed, at least the CD4-MHCII interaction showed to be much weaker than described previously [137], proposing the CD4-LCK interaction increases the sensitivity to ligand only by 2-20%. The much higher affinity of the CD8 and MHCI compared to CD4 and MHCII [138] suggests possibly differential functions of the two coreceptors.

In contrast to intuitive function of the coreceptor to deliver kinase activity to the TCR signalosome, several studies emerged proposing that the main role of coreceptor-LCK interaction is stabilization of pMHC-TCR complex [139, 140]. Formation of a strong multimolecular complex during interaction of pMHC-TCR with CD8-LCK was implied as the main mechanism of such adaptor function [141]. Here, strong "catch bonds" were formed as consequence of "inside out signaling", as CD8 stabilized the

pMHC-TCR complex from extracellular site, while LCK stabilized the pMHC-TCR complex intracellularly. On top of that, LCK that did not interact with coreceptor was reported to be responsible for triggering the TCR signalosome [142]. Later study even suggested that coreceptor-bound LCK is less active compared to coreceptor-independent LCK [143].

Interestingly, additional function of LCK-CD4 has been identified. LCK has been shown to extend CD4 membrane localization lifetime. LCK was suggested to mask dileucine motif on intracellular tail of CD4, shielding its surrounding serine residues from phosphorylation, therefore effectively preventing clathrin-mediated internalization of CD4 [144, 145]. The CD8 coreceptor does not contain a homologous sequence that would enable similar phenomenon. Thus, CD4- and CD8-LCK interactions seem to have more distinctive function in $CD4^+$ or $CD8^+$ T cells than just correct MHC restriction.

2. Aims of the study

The study was focused on the role of coreceptor-LCK interaction in the development and signaling of T cells. We expanded previous studies by exploring the importance of this interaction in thymic selection process and T-cell reactivity.

In the first part of the study, we aimed to explore dynamics of the coreceptor-LCK interaction throughout the T-cell development. A previous study showed that the stoichiometry of CD4- and CD8-LCK interaction already varies in DP thymocytes [44]. Upon maturation, $CD4^+$ and $CD8^+$ T cells acquire various cell fates, and their signalosome can thus be very different. Hence, we hypothesized, that the dynamics of coreceptor-LCK may vary between $CD4^+$ and $CD8^+$ T cells also upon maturation. As coreceptor-LCK interaction sets up the threshold for the negative selection [44], its differential dynamics could result in distinct characteristics of TCR repertoires in $CD4^+$ and $CD8^+$ T-cell populations. We aimed to employ previously published mathematical model [44] and validate its predictions using *ex vivo* and *in vivo* models.

The coreceptor-LCK interaction is crucial in the proper T-cell development. However, the role of this interaction was mostly studied using cell line models. Therefore, in the second part of the study, we decided to develop the ultimate genetic model lacking the coreceptor-LCK interaction. We aimed to analyze how abolishing coreceptor-LCK interaction affected development of T cells in mice with LCK unable to bind the coreceptors. Moreover, the importance of the kinase-dependent function of coreceptor-

LCK interaction in T-cell response has been challenged. We thus aimed to expand our murine model that to allow uncoupling of enzymatic and adaptor function of this interaction. We aimed to analyze if introduction of kinase-dead LCK, which is able to interact with the coreceptor, into our model could provide the reported adaptor function independent from the LCK kinase activity.

In the third part of the study, we aimed to explore effects of supraphysiological increase in coreceptor-LCK interaction on the T-cell fates. We hypothesized that such increase in homeostatic signaling could lead to more robust differentiation into antigen-inexperienced memory-like T (AIMT) cell phenotype. We therefore set up to explore whether the stronger homeostatic signaling drives the formation of AIMT cells and if so, how do these cells respond to the antigens.

In the fourth part of the study, we further explored the role of TCR signaling strength in CD4/CD8 lineage decision. By manipulating availability of LCK in the TCR signalosome, we aimed to explore whether strength of TCR signaling can affect the lineage choice in developing thymocytes.

In the fifth part of the study, we aimed to develop a simple cell line model that would provide a good platform to study various aspects of TCR signaling. We aimed to develop a Jurkat model with specific TCR recognizing ligands of various affinity. Therefore, we could easily study effects of individual components of TCR signalosome on TCR ligand discrimination.

2.1 Specific aims

1. Analysis of the dynamics of coreceptor-LCK interaction and its role in shaping self-reactivity of developing T cells.
2. Development of murine genetic model with abolished coreceptor-LCK interaction
3. Analysis of T-cell development and response in mice with abolished coreceptor-LCK interaction.
4. Examination of how adaptor function of coreceptor-LCK interaction uncoupled from enzymatic activity affects the T-cell development.
5. Determination of attributes leading to formation of AIMT cells
6. Functional analysis of AIMT-cell response
7. Analysis of the role of signal strength in CD4/CD8 lineage commitment

8. Development of Jurkat cell line model with defined TCR specificity for exploring proximal TCR signaling

3. Materials and methods

In this study, we used two types of models. The first model was based on human cancer T-cell line Jurkat deficient for LCK. These cells were genetically modified to express murine OT-I TCR with known specificity, together with human CD8 coreceptor and LCK. Such xenogeneic model was possible due to similar affinity of murine and human CD8 to the antigen-presentation complex H-2K^b [146]. Using human lymphoblast T2 cell line transfected with murine H-2K^b as antigen-presenting cells, enabled us to study T-cell response to various affinity ligands defined for the OT-I TCR.

The prevalent models in the whole study were murine models. We used mice bearing chimeric CD8.4 coreceptor, CD3ε-deficient mice, RAG2-deficient mice bearing various transgenic OT-I and B3K508 transgenic TCRs. We also developed Lck knock-in mice using Crispr-Cas9 technology. The first LCK knock-in was created by mutation of two N-terminal cysteine residues to alanine. This mutation has been identified to abolish LCK interaction with coreceptors completely [111]. The second LCK knock-in was created by mutation of single lysine residue, responsible for enzymatic function of LCK [113], to arginine.

Data were collected using various experimental protocols. The key approach of the study was execution of *in vivo* or *ex vivo* experiments followed by flow cytometry analysis. The *in vivo* experiments included adoptive transfer, *Listeria monocytogenes* infection response, LCMV response and tumor response. For the *ex vivo* experiments, we analyzed response to variety of ligands presented by antigen-presenting cells, and we also compared ability of T-cells to bind the pMHC complex. Another quite extensive part of the study was phenotypic analysis of murine models by flow cytometry and immunoblotting.

Detailed methods are described in the corresponding publications.

4. Results and discussion

4.1 Coreceptor-LCK interaction shapes T-cell self-reactivity

As mentioned previously, coreceptor-LCK interaction has a very important role in regulation of TCR signaling. There have been several reports, suggesting coreceptor-LCK interaction augments the TCR signaling [42, 132, 147], sets up the threshold for

positive [134] and negative selection [44], ensures MHC-restriction of mature T cells [148] and facilitates productive docking polarity of MHCI restricted T cells [149].

In our project, we explored dynamics of coreceptor-LCK interaction in course of T-cell development (Publication #1). It was previously reported, that the coreceptor-LCK stoichiometry differs between CD4 and CD8α coreceptors in DP thymocytes [44] in favor of CD4. There are two main reasons for higher interaction of LCK with CD4. Firstly, CD4 has slightly higher affinity to LCK compared to CD8α [112]. Secondly, murine thymocytes, but not mature T cells express pool of "tailless" CD8α splice variant, CD8α' unable to bind LCK [150].

We observed that the coreceptor-LCK interaction changes during T-cell maturation. The CD4-LCK interaction increased only 2-fold, while CD8α-LCK interaction increased 13-fold. Based on previously published data [44] and our observations, we employed a previously published mathematical model [44] that predicted $CD8^+$ mature T cells to be more self-reactive and more sensitive to suboptimal antigens compared to mature $CD4^+$ T cells.

We proposed that limited availability of LCK interacting with CD8α in DP thymocytes results in selection of more self-reactive TCR repertoire for $CD8^+$ T cells. This highly self-reactive TCR repertoire compensates lack of available LCK needed to reach the optimal signal strength for positive selection. However, with sudden surge of LCK availability in periphery, the $CD8^+$ T cells become much more self-reactive compared to $CD4^+$ T cells.

We tested our hypothesis *ex vivo* and *in vivo* using murine T cells bearing either MHCI or MHCII restricted transgenic TCR (OT-I and B3K508, respectively). Indeed, the MHCI-restricted, but not MHCII-restricted T cells showed increased reactivity towards antigens with low affinity. Interestingly, the MHCI-restricted T cells responded quite robustly even to the antigen below threshold of negative selection that corresponds to positively selecting self-antigens. This suggests much higher self-reactivity of mature $CD8^+$ T cells compared to $CD4^+$ T cells.

We further tested if the mature $CD8^+$ T cells are more self-reactive by exploring their homeostatic signaling. Basal phosphorylation of CD3ζ chains, ZAP70 and overall tyrosine phosphorylation was indeed higher in $CD8^+$ compared to $CD4^+$ T cells. Moreover, in the absence of regulatory T cells, that are able to remove highly self-reactive

T cells in the periphery [151], the $CD8^+$ T cell population expanded much more compared to $CD4^+$ T cell population.

Previously, $CD4^+$ T cells were considered more self-reactive compared to $CD8^+$ T cells based on higher expression of markers reported to reflect self-reactivity: CD5 and *Nur77*-GFP [152, 153]. We suggest this difference to be a cell-type intrinsic effect not reflecting self-reactivity of the subpopulation. When we mimicked dynamics of coreceptor-LCK interaction from $CD4^+$ T cells in the $CD8^+$ T cells by using CD8.4 chimeric coreceptor, which contains intracellular domain of CD4, we observed lower basal signaling and decrease in *Nur77*-GFP expression on mature $CD8.4^+$ T cells compared to $CD8^+$ T cells.

We observed that by using just two different coreceptors, T-cell development can elegantly and efficiently set up the repertoire of the T cells corresponding to their function. As the $CD4^+$ T cell response leads to very systemic reaction, influencing panoply of immune cells, it is safer to provide very tight control of such response. One step may be providing $CD4^+$ T cells with less autoreactive TCR repertoire, in addition to the need of costimulatory signal from professional antigen-presenting cells to trigger the response. [129]. On the other side, $CD8^+$ T cells provide very rapid but localized response, where such strict control does not have to be so necessary. Thus $CD8^+$ T cells may have much more autoreactive TCR repertoire, to ensure surveillance of transformed cells or pathogens that evolved to disguise by mimicking the host. Moreover, the cells presenting their ligands on MHCI are usually somatic cells which lack the costimulatory molecules. Therefore, much stronger affinity for self-antigens is beneficial for efficient elimination of such threats [129].

4.2 Ultimate genetic model for coreceptor-Lck interaction

Several studies have emerged that contradict the originally described model, where main function of coreceptor-LCK interaction is for coreceptors to bring the LCK to the TCR complex in order to phosphorylate its ITAM motifs. These studies suggest differential function of LCK interacting with coreceptors and "free" Lck. These models suggest that coreceptor-LCK interaction serves as a stabilizing adaptor of pMHC-TCR complex, while "free" LCK is responsible for ITAM phosphorylation [139, 140, 142, 143]. As most of these studies were conducted using cell line models, we decided to resolve this controversy by developing the ultimate genetic model, allowing us to explore the role of coreceptor-LCK interaction in T-cell development *in vivo* (Manuscript #2).

We developed two *Lck* knock-in models. In the first model, two cysteine-alanine mutations were introduced in site responsible for LCK interaction with coreceptors [111], later referred to as Lck^{CA} or binding mutant mouse. As expected, these mice do not have any block in process of β-selection in the DN stage, because thymocytes do not express their coreceptors at this stage. There is however partial block in thymic development during DP stage, which results in lower number of mature SP4 and SP8 thymocytes. Interestingly, number of developing $CD4^+$ T cells in periphery is also reduced, while number of peripheral $CD8^+$ T cells seems to be normal. However, a higher portion of these cells acquires memory-like phenotype. This suggest that $CD8^+$ T cells compensate their numbers by homeostatic proliferation. In a competitive setup of bone marrow chimera, the number of developing T cells from Lck^{CA} mice was 10-times lower compared to T cells from Lck^{WT}mice, confirming defects in their T-cell development. Interestingly, Lck^{CA} mice were able to clear acute LCMV infection with similar efficiency to the Lck^{WT} mice, but their ability to control tumor response was slightly reduced compared to Lck^{WT} mice.

Because the coreceptor-LCK interaction has been suggested to be critical mainly for the response to weaker stimuli, we crossed our mice to models bearing transgenic TCRs with defined specificity. We observed that in mice bearing MHC-I restricted OT-I TCR, the response of LCK^{CA} OT-I T cells to weak ligands was diminished compared to LCK^{WT} OT-I T cells, both *ex vivo* and *in vivo*. Moreover, the LCK^{CA} OT-I T cells had reduced capacity to control growth of OVA-expressing tumors compared to LCK^{WT} OT-I T cells. On the other hand, MHC-II restricted LCK^{CA} B3K508 T cells had already reduced response to the strong, cognate ligands, both *ex vivo* and *in vivo*.

We observed that the MHCII-restricted LCK^{CA} T cells, both from polyclonal and monoclonal mice, had reduced expression of CD4 compared to LCK^{WT} T cells. This effect corresponds to previously reported function of LCK shielding internalization motifs on CD4 [144].

Contrary to previously reported data from transgenic murine model of LCK binding mutant, where absence of coreceptor-LCK interaction led to selection of non-MHC restricted T cells [148], our TCRs clones with defined MHC-specificity were able to retain their MHC-specificity upon maturation.

To explore the kinase-independent adaptor function of coreceptor-LCK interaction, we developed another *Lck* knock-in model, where lysine residue responsible for enzymatic function of LCK [113] was replaced with arginine residue, further referred to as Lck^{KR} or mice bearing kinase-dead LCK. This allowed us to generate mice heterozygous for Lck^{CA} and Lck^{KR} alleles, further referred to as compound heterozygotes. These mice have a partial pool of active LCK unable to interact with coreceptor, complemented with a pool of kinase-dead LCK that can interact with coreceptor. Our data contradicted the possibility of adaptor function for CD8-LCK interaction uncoupled from enzymatic activity, as $CD8^+$ T-cell development in the compound heterozygote mice was not rescued. On the contrary, the presence of kinase-dead LCK had negative effect on the ability of compound heterozygote mice to clear LCMV and regulate tumor growth. Monoclonal $LCK^{CA/KR}$ OT-I T cells had also reduced ability to respond to antigenic stimuli compared to LCK^{CA} OT-I T cells, both *ex vivo* and *in vivo*. Their capacity to regulate growth of OVA-expressing tumors was also reduced.

On the other hand, our data suggest possible adaptor function of CD4-LCK uncoupled from its kinase activity. The availability of kinase-dead LCK in compound heterozygote rescued defect in maturation of MHC-II restricted T cells observed in Lck^{CA} mice. Even the capacity of $CD4^+$ T cells to compete in T-cell development with LCK^{WT} T cells in bone marrow chimeras was partially rescued. Presence of kinase-dead LCK in $LCK^{CA/KR}$ B3K508 T cells lead to rescue in response to strong stimuli compared to Lck^{CA} B3K508 T cells. However, the CD8-LCK adaptor function was not able to rescue defects in response of LCK^{CA} B3K508 T cells to weaker stimuli. The CD4 expression was partially rescued in the compound heterozygote mice compared to binding mutants, confirming that LCK ability to shield CD4 from endocytosis is not dependent on its enzymatic activity. Overall, our data suggest CD4 function independent from LCK kinase activity that can regulate maturation of $CD4^+$ T cells and their response to the strong stimuli.

4.3 Stronger TCR homeostatic signals lead to formation of $CD8^+$ AIMT cells

In the course of thymic development, T cells are selected based on their reactivity to self-antigens. They retain this reactivity upon their maturation, resulting in homeostatic signaling needed for T-cell survival in the periphery. A population of $CD8^+$ antigen-inexperienced memory-like T (AIMT) cells has been suggested to develop based on their homeostatic signaling [78, 154]. In our project (Publication #3), we studied formation of this population and their ability to react to antigenic stimuli.

We observed an increase in AIMT population in our CD8.4 chimeric mice with supraphysiological stoichiometry of CD8-LCK interaction, both in specific-pathogen free and germ-free mice. Increased availability of LCK leads to stronger homeostatic TCR signaling in *CD8.4* $CD8^+$ T cells [155]. Our data thus confirm the suggested role of homeostatic TCR signaling in formation of AIMT cells.

We further observed that the formation of AIMT cells is dependent on the TCR clone the T cells are bearing. While we observed expansion in AIMT cell population in CD8.4 mice bearing highly autoreactive OT-I TCR, we did not detect any AIMT cells in weakly autoreactive F5 TCR [156, 157], regardless of their LCK availability. Although the *CD8.4* T cells bearing F5 TCR elicited stronger response to antigens than *CD8WT* T cells, they do not reach the level of homeostatic signaling needed for differentiation towards AIMT phenotype. To study whether the formation of AIMT cells is indeed clonally dependent, we compared the TCR repertoire used by AIMT and naïve T cells. By cloning several TCRs from these two populations and tracking the development of T cells bearing these TCRs in retrogenic mice, we confirmed that distinct TCR clones drive the T cells towards the corresponding cell fate decision.

Additionally, we tested the ability of AIMT cells to elicit immune response to antigenic stimuli in comparison to naïve and true memory T cells. Even though in some respects, the AIMT cells were able to elicit a slightly stronger response than naïve T cells, they did not reach the full potential of true memory T cells. When we compared the ability of naïve vs AIMT cells bearing the same clone to induce autoimmune diabetes, AIMT cells showed more tolerogenic response. The previously reported reduction in interferon γ production by AIMT cells compared to true memory T cells [84], together with our observations of less efficient upregulation of CD25 and CD49d in AIMT cells compared to naïve T cells in antigenic response, may explain more tolerogenic phenotype of the AIMT cells.

4.4 CD4/CD8 lineage commitment

During the T-cell development, thymic precursors give rise to two major subpopulations, $CD4^+$ T cells and $CD8^+$ T cells. This binary lineage decision is largely believed to be driven by change in TCR signaling upon dynamic change of coreceptor expression [73].

Our collaborators at University College London challenged this perception, looking at more proximal readout of coreceptor expression using single cell RNA sequencing of developing thymocytes (Publication #4). The obtained data suggest that

the CD4/CD8 lineage commitment occurs upon TCR signaling event in the double positive stage, independently of coreceptor expression. Contrary to the coreceptor kinetic model [76], the commitment to SP4 lineage seems to precede commitment to SP8 lineage.

In this project we inspected the role of TCR signaling strength in CD4/CD8 lineage decision. Interestingly, we showed that increase in the TCR signal strength in our CD8.4 chimeric model bearing MHCI-restricted transgenic OT-I TCR, was able to override the lineage commitment and give rise to mature $CD4^+$ T cells.

4.5 Tuning the TCR signaling

Ligand recognition by TCR is essential both for the T-cell development and recognition of pathogens. The engagement of TCR with ligand, however, does not necessarily need to lead to the initiation of T-cell response. In fact, one of the hallmarks of T cells is an efficient discrimination between the self and foreign antigens. Proper regulation of TCR signalosome is therefore essential for ligand discrimination. We developed a simple Jurkat model bearing murine OT-I TCR with defined specificity. This model enables easy testing for the role of individual signaling molecules in TCR signaling, One of the advantages of this model is that it enables studying role of TCR signalosome components in response to weaker stimuli. Moreover, it uses more physiological stimulation of the TCR signaling compared to commonly used cross-linking of signaling molecules.

4.5.1 Scaffold function of LCK in TCR signalosome

Our collaborators at University of California San Francisco (Publication #5), identified an additional role of LCK in coordination of the TCR signaling. In addition to the phosphorylation of ITAMs on TCR/CD3 complex and phosphorylation of ZAP70, LCK scaffold function was described. Interaction of LCK SH3 domain with proline-rich motive PIPRSP on LAT seems to serve as a bridge, gathering LAT and ZAP70 in optimal juxtaposition for efficient TCR signaling. Mutation of the PIPRSP motif leads to inefficient TCR signaling, resulting in partial blocks in murine thymocyte development and inefficient recruitment of LAT upon TCR engagement in our Jurkat model.

4.5.2 CD45 acts as a gatekeeper of TCR signalosome

The balance of phosphorylation in TCR signalosome is tightly regulated by a wide range of kinases and phosphatases. Such signaling cascade allows the mechanisms of kinetic proofreading to properly discriminate between TCR ligands. Our collaborators

examined interplay between CD45 and CSK regulation of TCR signalosome (Publication #6). Increasing inhibition of CSK led to stronger TCR response, but this gradual response was almost completely abrogated in the absence of CD45. Only relatively strong signals were able to elicit some level of response. Increasing levels of CD45 expression in our Jurkat model led to more effective ligand discrimination, underlining the importance of CD45 in the tuning of T-cell response.

4.5.3 Evolutionary tuned ligand discrimination

Our collaborators further unveiled an intriguing mechanism that evolved to improve sensitivity of ligand discrimination (Publication #7). Conservation of uncharged glycine on -1 position of LAT Y^{132} evolved to delay LAT phosphorylation in order to extend the antigen dwell-time requirement for more sensitive kinetic proofreading. As the phosphorylation of LAT Y^{132} is required for PLCγ recruitment, this optimization seems to serve as a checkpoint before production of second messengers with pleiotropic effects. Exchange of the $glycine^{131}$ for positively charged aspartate or glutamate, which are usually conserved at -1 position of other ZAP70 targeted tyrosine residues, resulted in much more robust response of our Jurkat model, even to very weak stimuli. These results confirm the evolutionary conservation of glycine residue as time-limiting step for more sensitive ligand discrimination.

5. Conclusion

In this thesis, I discussed seven projects that shifted our understanding of TCR signaling in T-cell biology.

We unveiled how employment of different coreceptors can set up the proper TCR repertoire that T cells need for their specific function. We showed that coreceptor-LCK interaction stoichiometry is dynamic throughout the process of T-cell maturation, and it varies between CD4 and CD8 coreceptors. Differential regulation of CD4- and CD8-LCK interaction results in formation of more self-reactive repertoire in mature $CD8^+$ T cells, compared to $CD4^+$ T cells. This corresponds to their functions in local vs. systemic response, respectively.

The development of the ultimate murine genetic model where the coreceptor-LCK interaction is abolished helped us crack the controversy around the role of this interaction in TCR signaling. We showed that coreceptor-LCK interaction is essential for proper T-cell development and for the response to weak stimuli. Moreover, we

determined that the availability of enzymatic activity on CD4-LCK and CD8-LCK is required for the response to weak stimuli. Yet again, there is a difference in the role of the two coreceptors. We observed that CD4-LCK, but not CD8-LCK interaction has an additional kinase-independent function in T-cell development and response. We further showed that LCK retains CD4 on the membrane in kinase-independent manner. Our results therefore suggest that CD4 and CD8 coreceptors may define the $CD4^+$ and $CD8^+$ T cells in more ways that just their restriction to different class of MHC molecules.

We further demonstrated that when $CD8^+$ T cells with highly self-reactive TCRs get to the periphery, their strong homeostatic signaling leads to acquisition of memory-like phenotype. We determined that these antigen-inexperienced memory-like T (AIMT) cells display more tolerogenic phenotype. These results suggest that potential autoreactivity of TCR repertoire is not only controlled by thymic selection but can be further regulated by T-cell fate choices in the periphery.

Our collaborators challenged the established models of CD4/CD8 lineage commitment, exposing that MHC discrimination occurs prior to the downregulation of CD4 or CD8 coreceptor expression and the SP4 thymocytes differentiate sooner than SP8 thymocytes. We uncovered that very strong TCR signals can override the CD4/CD8 lineage choice.

We successfully developed a Jurkat cell line model that helped uncover some features of very complex machinery of TCR signalosome. Our model was employed in discovery of additional scaffold role of LCK, bridging together two other proximal signaling molecules ZAP70 kinase and LAT adaptor to the optimal juxtaposition in TCR signalosome. The Jurkat cells bearing TCR with defined specificity also helped elucidate key players that regulate ligand discrimination. We assisted in the demonstration of CD45 phosphatase as crucial gatekeeper of the TCR signalosome, critical for ligand discrimination. Our model further helped in identification of evolutionary conservation of single glycine residue next to the tyrosine phosphorylation site of LAT that enables increase in ligand sensitivity. Elimination of negative charge slows down the phosphorylation of tyrosine residue and therefore enables employment of rate-limiting steps of the kinetic proof-reading.

Overall, our results helped to dissect controversies and challenged the conventional views on the regulation of T-cell development and response. We helped to

unveil some previously unknow steps in the tuning of TCR signalosome. We also produced murine and cell line models that can serve for further detailed inspection of the T-cell response.

The results discussed in my dissertation thesis are integrated in six research publications in peer-reviewed journals together with one manuscript ready for submission. Our experience in studying the role of the coreceptor-LCK interaction in TCR signaling led to an invitation to write a perspective on a publication implicating the role of coreceptor-LCK interaction in proper docking of pMHC on the TCR (Perspective #8).

5.1 Summary of major findings

1. Coreceptor-LCK dynamics changes throughout the process of T-cell maturation, resulting in more self-reactive TCR repertoire of $CD8^+$ T cells

2. Coreceptor-LCK interaction is essential for proper T-cell development

3. CD4-LCK, but not CD8-LCK interaction appears to have additional adaptor function uncoupled from enzymatic activity

4. Homeostatic proliferation of T cells bearing highly self-reactive TCRs leads to differentiation to AIMT-cell phenotype

5. AIMT cells elicit more tolerogenic antigen response

6. MHC-discrimination seems to occur already in DP thymocyte stage and SP4 thymocytes differentiate before SP8 thymocytes

7. Strong TCR signal can over-ride CD4/CD8 lineage decision

8. LCK interacts with LAT adaptor, optimizing its juxtaposition to ZAP70 and proper TCR signaling

9. CD45 phosphatase regulates T-cell ligand discrimination

10. Slow phosphorylation of LAT evolved for more effective ligand discrimination

6. Publications

The full text of the publications can be found in the last section of this thesis.

6.1 List of publications

#1 Dynamics of the Coreceptor-LCK Interactions during T Cell Development Shape the Self-Reactivity of Peripheral CD4 and CD8 T Cells. **Horkova V**, Drobek A, Mueller D, Gubser C, Niederlova V, Wyss L, King CG, Zehn D, Stepanek O. Cell Rep. 2020 Feb 4;30(5):1504-1514.e7. PMID: 32023465 **(IF_{2019} 8.1)**

#2 Unique roles of LCK in CD4 and CD8 T cells. **Horkova V**, Drobek A., Paprckova D., Prasai A., Glatzova D., Krizova K., Kraller M., Platzer R., Weiss A., Huppa J., Stepanek O. Manuscript ready for submission.

#3 Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells. Drobek A, Moudra A, Mueller D, Huranova M, **Horkova V**, Pribikova M, Ivanek R, Oberle S, Zehn D, McCoy KD, Draber P, Stepanek O. EMBO J. 2018 Jul 13;37(14):e98518. PMID: 29752423 **(IF_{2019} 9.9)**

#4 The order and logic of CD4 versus CD8 lineage choice and differentiation in mouse thymus. Karimi MM, Guo Y, Cui X, Pallikonda HA, **Horková V**, Wang YF, Gil SR, Rodriguez-Esteban G, Robles-Rebollo I, Bruno L, Georgieva R, Patel B, Elliott J, Dore MH, Dauphars D, Krangel MS, Lenhard B, Heyn H, Fisher AG, Štěpánek O, Merkenschlager M. Nat Commun. 2021 Jan 4;12(1):99. PMID: 33397934 **(IF_{2019} 12.1)**

#5 Lck promotes Zap70-dependent LAT phosphorylation by bridging Zap70 to LAT. Lo WL, Shah NH, Ahsan N, **Horkova V**, Stepanek O, Salomon AR, Kuriyan J, Weiss A. Nat Immunol. 2018 Jul;19(7):733-741. PMID: 29915297 **(IF_{2019} 20.5)**

#6 CD45 functions as a signaling gatekeeper in T cells. Courtney AH, Shvets AA, Lu W, Griffante G, Mollenauer M, **Horkova V**, Lo WL, Yu S, Stepanek O, Chakraborty AK, Weiss A. Sci Signal. 2019 Oct 22;12(604):eaaw8151. PMID: 31641081 **(IF_{2019} 6.5)**

#7 Slow phosphorylation of a tyrosine residue in LAT optimizes T cell ligand discrimination. Lo WL, Shah NH, Rubin SA, Zhang W, **Horkova V**, Fallahee IR, Stepanek O, Zon LI, Kuriyan J, Weiss A. Nat Immunol. 2019 Nov;20(11):1481-1493. PMID: 31611699 **(IF_{2019} 20.5)**

#8 Perspective: Coreceptor-LCK constrains productive T-cell antigen docking. Stepanek O, Horkova V. Science. 2021 Jun 4;372(6546):1038-1039. PMID: 34083474 **(IF_{2019} 41.8)**

6.2 Contribution

Ad #1 I performed data collection (Fig. 2C-D, Fig. 4A-C, Fig. S1A-D,J-L, Fig. S2B,E-G, Fig. S4A, Fig. S5D,F-H) and data analysis (Fig. 1B-C, Fig. 2A-D, Fig. 4A-C, Fig. 5C-D, Fig. S1B-D,J-L, Fig. S2B-G, Fig. S4A-C, Fig. S5A-D,F-H) for substantial part of experiments and co-wrote the manuscript with my supervisor Dr. Ondřej Štěpánek.

Ad #2 I performed data collection (Fig.1A-D, Fig.2A-C, Fig.3A-C,E, Fig.S2A-H, Fig.S3A-F, Fig.S4A-G,I) and data analysis (Fig.1A-D, Fig.2A-C, Fig.3A-G, Fig.S2A-H, Fig.S3A-F,H, Fig.S4A-I) for majority of the experiments and co-wrote the manuscript with my supervisor Dr. Ondřej Štěpánek.

Ad #3 I joined the project in its final phase. I tested the *ex vivo* response of OT-I T cells (with either wild type or CD8.4 chimeric CD8 coreceptor) to cognate antigen and its altered-ligand peptides. (Figure 5C-D and Figure EV5E). I also contributed by reviewing the manuscript.

Ad #4 I joined the project in its final phase. I contributed with analysis of T-cell development in OT-I Rag2$^{-/-}$ mice bearing wild type or CD8.4 chimeric CD8 coreceptor (Supp. Fig. 9A-B). I also contributed by reviewing the manuscript.

Ad #5 I contributed by development of novel Jurkat cell-line model. I also contributed with characterization of response of these cell lines to antigenic stimuli as well as their interaction with tetramer (Supp.Fig. 8A-B). I also contributed by reviewing the manuscript.

Ad #6 I contributed by development of novel Jurkat cell-line model and review of the manuscript.

Ad #7 I contributed by development of novel Jurkat cell-line model.

Ad#8 I contributed with writing and reviewing the manuscript.

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8. Reprints of publications

1. Dynamics of the Coreceptor-LCK Interactions during T Cell Development Shape the Self-Reactivity of Peripheral CD4 and CD8 T Cells.

2. Unique roles of LCK in CD4 and CD8 T cells.

3. Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells.

4. The order and logic of CD4 versus CD8 lineage choice and differentiation in mouse thymus.

5. Lck promotes Zap70-dependent LAT phosphorylation by bridging Zap70 to LAT.

6. CD45 functions as a signaling gatekeeper in T cells.

7. Slow phosphorylation of a tyrosine residue in LAT optimizes T cell ligand discrimination.

8. Perspective: Coreceptor-LCK constrains productive T-cell antigen docking.