Studies on the role of Coronin 1 and the Actin Cytoskeleton in T cell Signaling and Survival

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Prof. Dr. Eberhard Parlow

The work described in this thesis has been performed from February 2006 to July 2009 at the Biozentrum, Department of Biochemistry, University of Basel in the laboratory and under supervision of Prof. Dr. Jean Pieters.

I want to devote this thesis to my parents who always supported me, provided me with a very good education and made it possible for me to study as well as take this doctoral degree.

The results described in this thesis have been published in:

Mueller P, Quintana A, Griesemer D, Hoth M, Pieters J.

Disruption of the cortical actin cytoskeleton does not affect store operated Ca^{2+} channels in human T cells.

FEBS Letters 2007 Jul 24;581(18):3557-62.

Mueller P, Massner J, Jayachandran R, Combaluzier B, Albrecht I, Gatfield J, Blum C, Ceredig R, Rodewald HR, Rolink AG, Pieters J.

Regulation of T cell survival through coronin-1-mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering.

Nature Immunology 2008 Apr;9(4):424-31.

or are part of the following manuscript:

Philipp Mueller and Jean Pieters

Coronin 1 Maintains the Balance between Survival and Apoptosis in Naive T Cells Independent of F-Actin

Submitted (August 2009)

ABSTRACT

Calcium ions (Ca^{2+}) function as universal second messengers in most if not all eukaryotic cells, including the cells of the immunesystem. Ca^{2+} signals are required for the proper activation of lymphocytes, such as T-lymphocytes, their proliferation, differentiation and effector functions. In lymphocytes store-operated calcium entry (SOCE) through calcium release activated calcium (CRAC) channels in the plasma membrane is the major mechanism to increase cytosolic Ca^{2+} concentrations and is essential for the activation of T and B cells as well as induction of their cytokine gene production. How exactly store operated calcium entry operates in T cells has remained unclear. While one model, based on results obtained using a variety of F-actin-modulating drugs, involves the cortical actin cytoskeleton, the function of F-actin in SOCE has remained controversial during the last decade, since independent studies have found no effects of the same drugs on SOCE in other model systems such as the rat basophile leukemia (RBL) cell line. The first part of this thesis aims at defining the role of the actin cytoskeleton during SOCE in human T cells.

The data presented in this thesis clearly demonstrate that the F-actin cytoskeleton in human T cells has no role in SOCE. These results therefore contribute to our understanding of calcium signaling in cells of the immunesystem.

The second part of this thesis focuses on coronin 1, a member of the conserved WD repeat containing protein family which is encoded in mice (and human) by the gene *coro1a*. Coronin 1, which is specifically expressed in leukocytes, was originally identified as a protein that is maintained around phagosomes containing live mycobacteria, thereby preventing the fusion of the mycobacterial phagosome with lysosomes and mycobacterial destruction.

The aim of this part of the thesis was to define the function of coronin 1 in immune cells by characterizing coronin 1 deficient mice with a special focus on T cell development, T cell receptor signaling, migration and survival as well as the proposed regulatory role of coronin 1 in F-actin dependent processes. We found, in contrast to the long held dogma of coronin 1 being a major regulator of the F-actin cytoskeleton, that coronin 1 regulates cellular signaling rather that F-actin modulation.

Coronin 1 was shown to interact with phospholipase C γ1 (PLC γ1) thereby being an important regulator of inositol-1,4,5-trisphosphate (IP_3) generation from phosphatidylinositol-4,5-bisphosphate (PIP_2). The absence of coronin 1, although not affecting T cell development, resulted in a profound defect in Ca^{2+} mobilization, interleukin-2 (IL-2) production, T cell proliferation and T cell survival in naïve T-cells.

Finally in the last part of this thesis we provide data showing that coronin 1 maintains the balance between survival and apoptosis in naive T cells independent of F-Actin via a calcium/calcineurin dependent pathway.

The data presented in part two and three of this thesis establish the leukocyte specific protein coronin 1 as an essential regulator of T cell receptor signaling as well as naïve T cell homeostasis and survival. This work refutes the proposed role of coronin 1 in the regulation of the F-actin cytoskeleton, instead providing evidence for coronin 1 being a central regulator of Ca^{2+}-dependent signaling in T cells. The work described here further offers new possibilities for the development of compounds for the treatment of T cell mediated disorders of the immunesystem.

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1. INTRODUCTION

1.1. The immune system

The immune system is a network of cells, tissues and organs which work in concert to defend our body against foreign invaders such as viruses, bacteria, fungi and parasites. The human body offers nutrient rich niches with optimal growth conditions for many microbes, and therefore microbial organisms, including many pathogens, continuously try to breach the body's defense system.

In an optimal scenario, physical barriers such as the skin and mucosal membranes, prevent foreign microorganisms from entering our body. However, if this first line of defense fails, the immunesystem needs to seek out the invaders and eliminate them. In doing so, the immunesystem has to ensure that it distinguishes between the body's own cells -"self"- and foreign structures or cells -"nonself"-.

If the immunesystem mistakes self for nonself and launches an attack against the body's own cells this often results in autoimmune diseases such as lupus erythematodes or diabetes. Another example where the immune system fails us are responses against harmless foreign substances such as pollen allergen resulting in allergy. Anything that can induce an immunresponse is termed an antigen. This can be a virus, a microbe, a recombinant protein in a vaccine or tissue from another person (except for genetically identical individuals) in the case of an organ transplantation.

In the following sections different organs, cell populations and signaling mechanisms that are important for the proper functioning of the immunesystem and in particular T lymphocytes will be discussed.

1.1.1. Physical barriers

Our first line of defense against invaders is a physical barrier, mainly comprised of the skin and the mucosal membranes. Skin acts as a mechanical barrier, that in addition is equipped with a low pH of 3-5, retarding microbial entry and growth. The normal flora of the mucous membranes competes with invading microbes for attachment and nutrients. It also entraps foreign microorganisms in the mucus which are then propelled out of the body by the cilia. Normal body temperature already inhibits growth of some bacteria, whereas fever responses block the growth of many invading pathogens. Furthermore, most pathogens that are ingested are killed by the acidic pH of the stomach [1].

1.1.2. The organs of the immunesystem

The organs of the immune system, referred to as lymphoid organs, are positioned throughout the body. There are primary as well as secondary lymphoid organs. The bone marrow, which is a primary lymphoid organ, is the ultimate source of all blood cells, including white blood cell precursors destined to mature into immune cells. The second primary lymphoid organ is the thymus in which lymphocytes known as T lymphocytes or T cells mature.

The secondary lymphoid organs, tonsils, lymph nodes, appendix, peyer's patches and spleen are all locations where cells of the immune system can meet each other as well as get into contact with their cognate antigen. There are only few T and/or B cells, specific for a given antigen (a few thousand). The antigen in addition might not even be accessible for T and/or B cells as they often cannot enter the tissues or organs where the foreign antigens are to be found. The body therefore contains the so called lymphatic system. This system is not under pressure such as the vascular system, bur merely drains the fluid (lymph), which leaks from our blood vessels into the surrounding tissue. The collected lymph is transported mainly by muscle contraction and a series of one-way valves to the upper torso where it reenters the vascular system. On its way back to the blood the lymph passes through the lymph nodes (secondary lymphoid organs). Cells of the immunesystem are retained in the secondary lymphoid organs for a limited amount of time which allows them to get in contact with antigens presented on so called antigen presenting cells (APCs, these will be discussed in

more detail below) or antigens such as viruses and bacteria carried there by the lymph. Immune cells such as T cells, B cells and APCs accumulate in secondary lymphoid organs, where they are brought in close contact with antigens to increase the probability that a particular (nonself) antigen is met by a T and/or B cell carrying its cognate receptor. In addition the stroma network in secondary lymphoid organs which is composed of cells and fibers generates an appropriate microenvironment for cell-cell interactions. The secondary lymphoid organs are thus an important integral part of the adaptive immunesystem and essential for the generation of efficient immunresponses. A schematic outline of the distribution of some lymphoid organs is provided below (Figure 1).

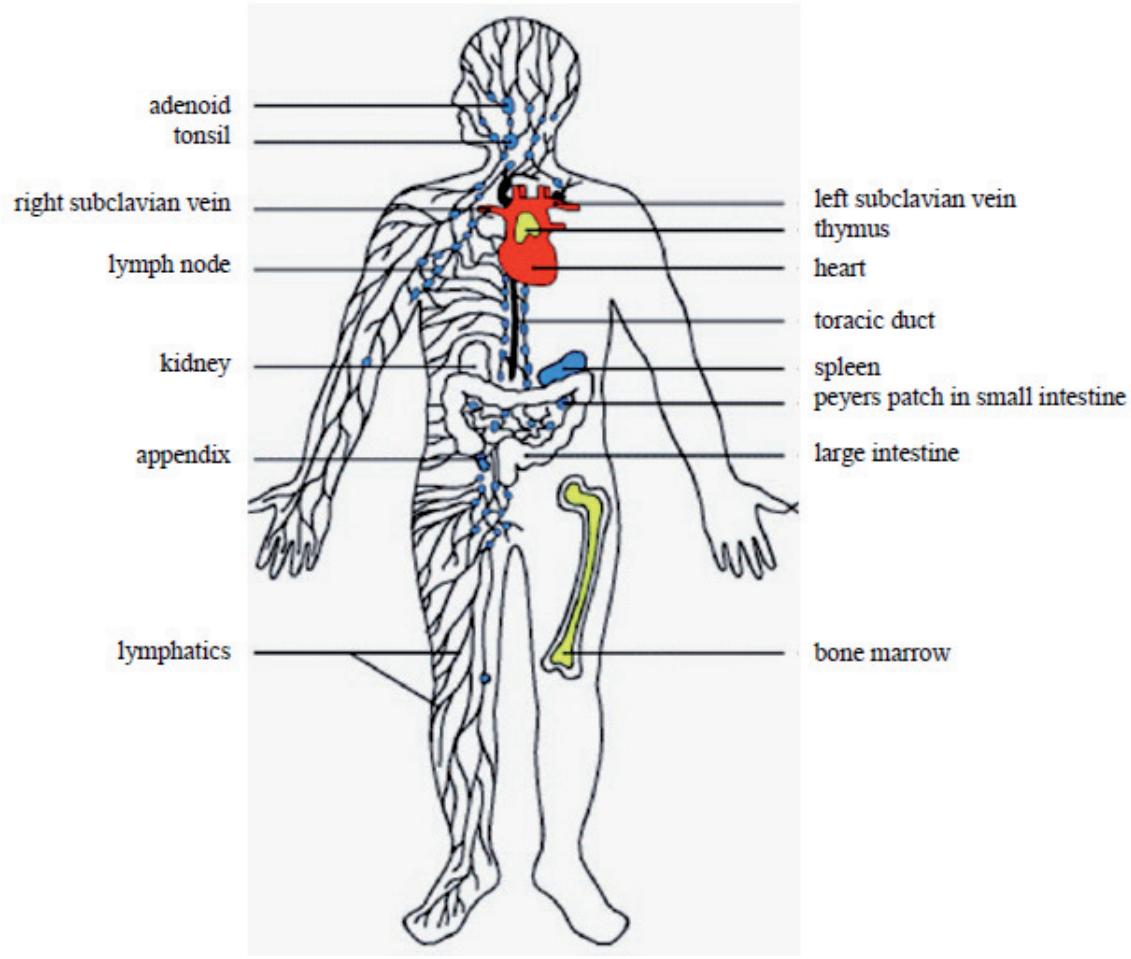


Figure 1:

The distribution of lymphoid tissues in the body (Adapted and modified from [1])

1.1.3. The innate immune system: an overview

As mentioned above, the first line of defense against invaders is comprised of the skin and the mucous membranes of the body. If this defense system is breached the second line of defense, the so called innate immune system, takes over. The innate immune system, besides being based on a number of relatively unspecific components that are effective against a variety of pathogenic organisms, such as the complement system, opsonins and other enzymatic mediators, relies heavily on professional phagocytic cells, namely macrophages and neutrophils. These phagocytic cells can internalize almost any kind of antigen, including soluble as well as particulate forms via receptor mediated endocytosis, phagocytosis and macropinocytosis [2-4]. Expression of specific surface receptors, also known as pattern recognition receptors (PRR), allows them to recognize invading microorganisms. Pattern recognition receptors such as the toll-like receptor (TLR) recognize so called PAMPs (Pathogen-Associated Molecular Patterns). PAMPs are molecular structures, especially of prokaryotes, which are shared by many related pathogens. They are relatively invariable and are not present in the host organism [5, 6]. Examples are the flagellin of bacterial flagella, the peptidoglycan of gram-positive bacteria, the lipopolysaccharide (LPS) of gram-negative bacteria, double-stranded RNAs and unmethylated CpG DNA [7, 8].

PRRs can be subdivided in three groups; (i) Secreted molecules that circulate in blood and lymph. (ii) Surface receptors that bind pathogens, initiating a signal leading to the release of immunostimulatory molecules (cytokines) such as the previously mentioned toll-like receptors. (iii) Finally surface receptors on phagocytic cells that bind pathogens, resulting in their uptake and subsequent degradation in the microbacterial milieu of the lysosomes.

Some pathogens, however, have evolved sophisticated survival strategies allowing them to invade the host, despite the efficient inborn defense mechanisms of the innate immune system. This is probably the main reason why vertebrates do not only have an innate immune system, but during the long process of co-evolution with pathogens, have evolved defense mechanisms that constitute adaptive immunity. The key players of adaptive immunity will be discussed in the following sections.

1.1.4. The adaptive immunesystem: an overview

Vertebrates depend on the innate immune system as a first line of defense, but they can also mount another type of defense, called adaptive immune responses. The adaptive immune responses provide the vertebrate immune system with the ability to recognize, eliminate and remember essentially every pathogen derived antigen and to mount an effective attacks each time the pathogen is encountered. The innate immunesystem and in particular the professional phagocytic cells, which have the ability to present antigens to cells of the adaptive immunesystem and provide co-stimulatory signals, work in concert with the adaptive immune responses to eliminate the pathogens. Unlike the innate immune responses, that only recognizes general PAMPs, the adaptive responses are highly specific for the antigen/pathogen that induced them. Recognition of antigens by the adaptive immune system is based on membrane bound cell-surface receptors of the immunoglobulin super family. A large variety of receptor specificities that in principle are able to recognize almost every structure on foreign antigens, is generated by somatic rearrangements of germ line encoded gene segments that code for the antigen binding regions of those receptors [9]. This already huge diversity becomes even more diverse in B cells and the antibodies they secrete during affinity maturation of effector B cells by a process termed somatic hypermutation [10]. Somatic hypermutations are only generated in the hypervariable regions of the antigen receptors which directly bind the antigen. This whole maturation process is termed clonal selection. During clonal selection, cells which display the highest affinity towards an antigen are selected and proliferate in order to mount an immune response against the pathogen carrying this antigen. This necessity of selection, proliferation and maturation causes a lag phase of several days, only after which the pathogen specific cells of the adaptive immunesystem can participate in the elimination of the pathogen. Until then it is up to the innate immune system to either already eliminate the intruder or at least keep it under control until the adaptive immune cells have multiplied and matured into effector cells. In contrast to the innate immune system, the adaptive immune system has a long term memory in the form of memory cells that can be reactivated if the same pathogen attacks again. In this case the immune response is faster and more efficient than during the initial infection.

Because the responses of the adaptive immunesystem are destructive and can be detrimental for the vertebrates body it is crucial that they be made only in response to antigens that are foreign to the host. The ability to distinguish between foreign and self is a fundamental

feature of the adaptive immunesystem. Occasionally, the system fails to make this distinction and reacts against the host's own antigens, resulting in the potentially fatal condition of autoimmunity. The immunesystem thus has evolved mechanisms to either destroy potentially auto reactive cells of the adaptive immunesystem or render them non responsive, a state which is also referred to as anergy. Such inappropriate responses are normally avoided as the innate immunesystem only calls into action the adaptive immune cells when it recognizes molecules characteristic of invading pathogens, such as PAMPs.

Lymphocytes, the cells of the adaptive immune system, are subdivided into B and T cells. B cells are able to secrete a soluble version of their otherwise membrane bound antigen receptor, which is antigen specific and in its soluble from referred to as antibody. Antibodies are the major effectors of the adaptive, humoral immune responses. They recognize the antigen for which they are specific, in its native, three dimensional structure. Antibodies can either neutralize the antigen as in the case of toxins, opsonized antigen and as such mark it for phagocytosis, activate the complement system or induce ADCC (Antibody-Dependent Cell-Mediated Cytotoxicity) [11, 12].

Antigen recognition by T cells is central to the generation and regulation of effective adaptive immune responses. Unlike B cells or soluble, circulating antibodies, T cells are not able to recognize free antigens. They can only recognize antigenic peptides which are bound and presented by specialized antigen presenting molecules. The most important group of those molecules is represented by the MHC (Major Histocompatibility Complex) class I and II molecules that present polypeptides to different subsets of T cells, depending on the origin of the polypeptide. Other classes of antigen presenting molecules exist, such as the CD1 molecules involved in the presentation of lipids and glycolipid antigens [13, 14].

T cells are subdivided into two functionally distinct populations, the CD8 and the CD4 expressing T cells which recognize antigenic peptides presented on MHC class I respective MHC class II. Antigen recognition on MHC class I induces effector $CD8^+$ T cells to rapidly kill the antigen presenting cell by either secretion of perforin and granzym or surface expression of apoptosis inducing molecules such as the Fas-ligand [15]. In the case of an infection, pathogens residing within in the cytosol are thereby either directly killed alongside with the infected cell or released into the extracellular space, where they can be targeted by the complement system and other humoral defense systems or opsonized by anybodies which render infectious agents non infectious and/or mark them for destruction by phagocytes [16].

$CD4^+$ T cells recognize peptides presented on MHC class II molecules. Those peptides are derived from pathogens taken up and processed via the endocytic system. Activated $CD4^+$ T cells produce cytokines that activate cells such as macrophages, helping these to kill phagocytosed or intracellular pathogens [17]. $CD4^+$ T cells further help B cells to differentiate into plasma cells and produce antigen specific antibodies, and hence are referred to as T helper cells [18]. In the following section the different antigen processing and presentation pathways will be discussed in more detail.

1.1.5. MHC molecules and antigen presentation

T cells can only recognize antigen in form of antigen derived fragments (peptides) that are bound and presented by MHC (Major Histocompatibility Complex) class I and II molecules. As a rule MHC class I molecules display intracellularly derived peptides, whereas MHC class II molecules present peptides originating from extracellularly present antigens. MHC class I molecules are expressed on all nucleated cells, whereas MHC class II molecules are restricted to a set of specialized cells, termed antigen-presenting cells (APCs). APCs, which will be discussed in detail later, comprise a subset of cells such as dendritic cells, macrophages and activated B cells. Depending on the source of the antigen two different antigen processing pathways are employed to load antigenic peptides onto the two classes of MHC molecules. This loading process determines to which T cell subsets an antigen will be presented as well as the type of the immunresponse that will be mounted against the antigen.

1.1.5.1. Class I MHC molecules

The two classes of MHC molecules serve different functions in antigen presentation, binding peptides from different intracellular sources and activating different T cell subsets. These functional differences are reflected by their differential distribution on the body's cells as well as in their structure.

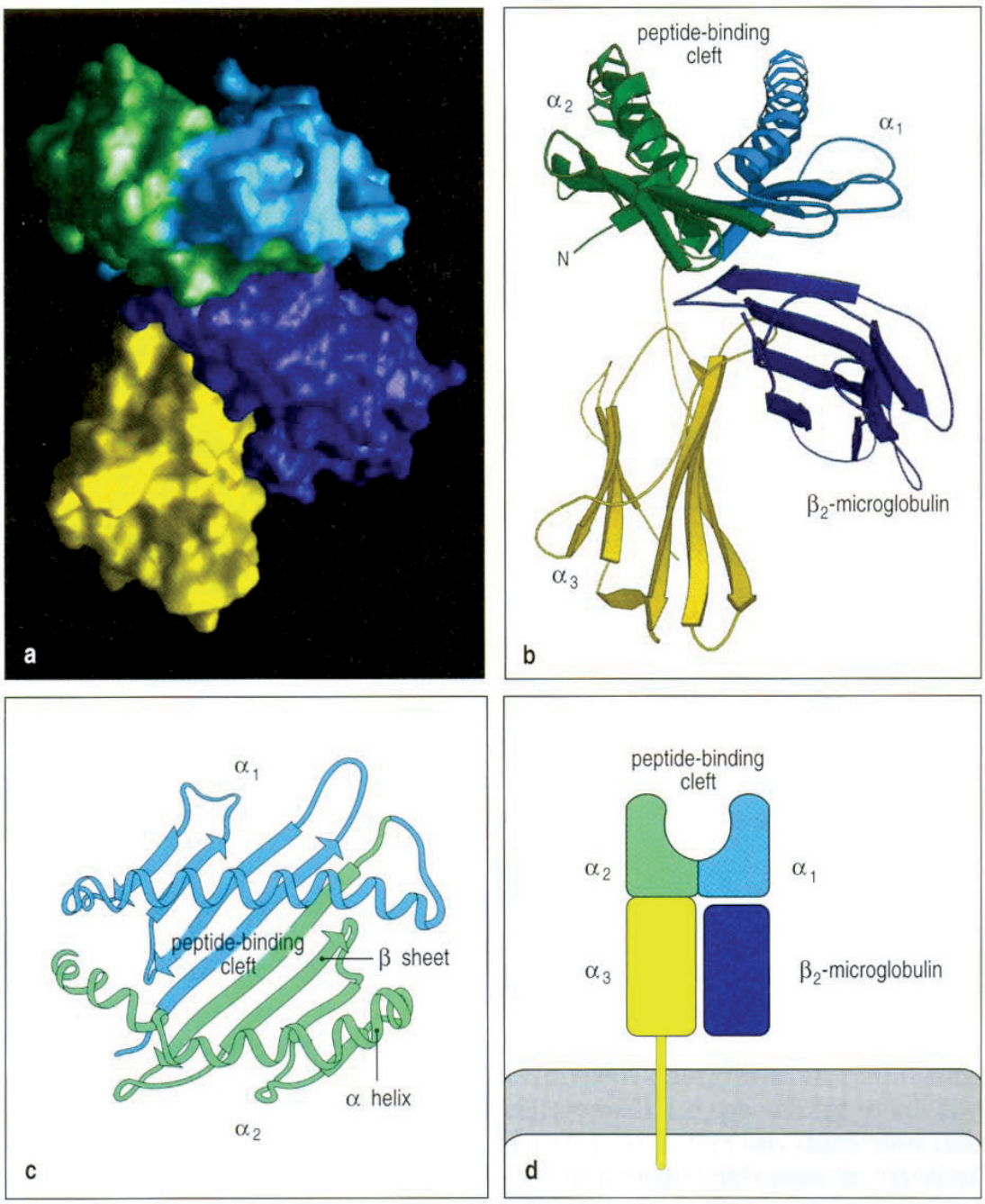


Figure 2

The structure of the MHC class I molecule. A) Computer graphic of the human MHC class I X-ray structure without the transmembrane domain. B) and C) show a ribbon diagram of the structure whereas panel D) shows a schematic outline of the MHC I molecule. The molecule is displayed as a membrane spanning heterodimer with the membrane spanning α chain (43 kDa) and the non covalently bound β_2-microglobulin chain (12 kDa). (Adapted and modified from [1])

The structure of the MHC class I molecule is outlined in Figure 2. The MHC class I molecule is composed of two polypeptide chains. The larger one, the α chain, is encoded by the MHC gene locus, whereas the smaller, non covalently attached β_2-microglobulin chain, which is not polymorphic is encoded in a different locus. The fully assembled molecule consists of 4 domains, three of which are formed by the MHC locus encoded α chain and one made up of the β_2-microglobulin chain. The α_3 domain which harbors the transmembrane domain as well as the β_2-microglobulin chain show amino acid similarities to immunoglobulin C domains and have similar fold structures. The α_1 and α_2 domains form the peptide binding cleft on the surface of the MHC class I molecule [19-21]. They are also sites of polymorphisms which determine T cell antigen recognition.

1.1.5.2. Class II MHC molecules

The MHC class II molecule is a heterodimer consisting of an α and β chain, each of which is made up of two domains, one being a transmembrane domain. Both chains are encoded in the MHC locus. The overall folding of the MHC class II molecules resembles that of the above described MHC I molecule. The major difference lies at the end of the peptide binding cleft made up of the α^1 and β^1 domains in case of the MHC class II molecule (Figure 3) [22-24]. The cleft is more open in the MHC class II molecule compared with the MHC class I molecule. As a consequence, the ends of a peptide presented on MHC class I are mostly buried within the molecule whereas this is not the case for peptides presented on MHC class II. Another consequence is the maximal length of the bound peptide. MHC class I binds short peptides of 8-10 amino acids, whereas peptides which are bound to MHC class II are at least 13 amino acids long, but can be much longer and contain anchoring sites at various distances from the end of the peptide. These differences in peptide binding properties and peptide length reflect the different antigen processing pathway by which peptides are generated and loaded on MHC class I respectively MHC class II molecules. Antigen processing and MHC loading will be discussed in the following section.

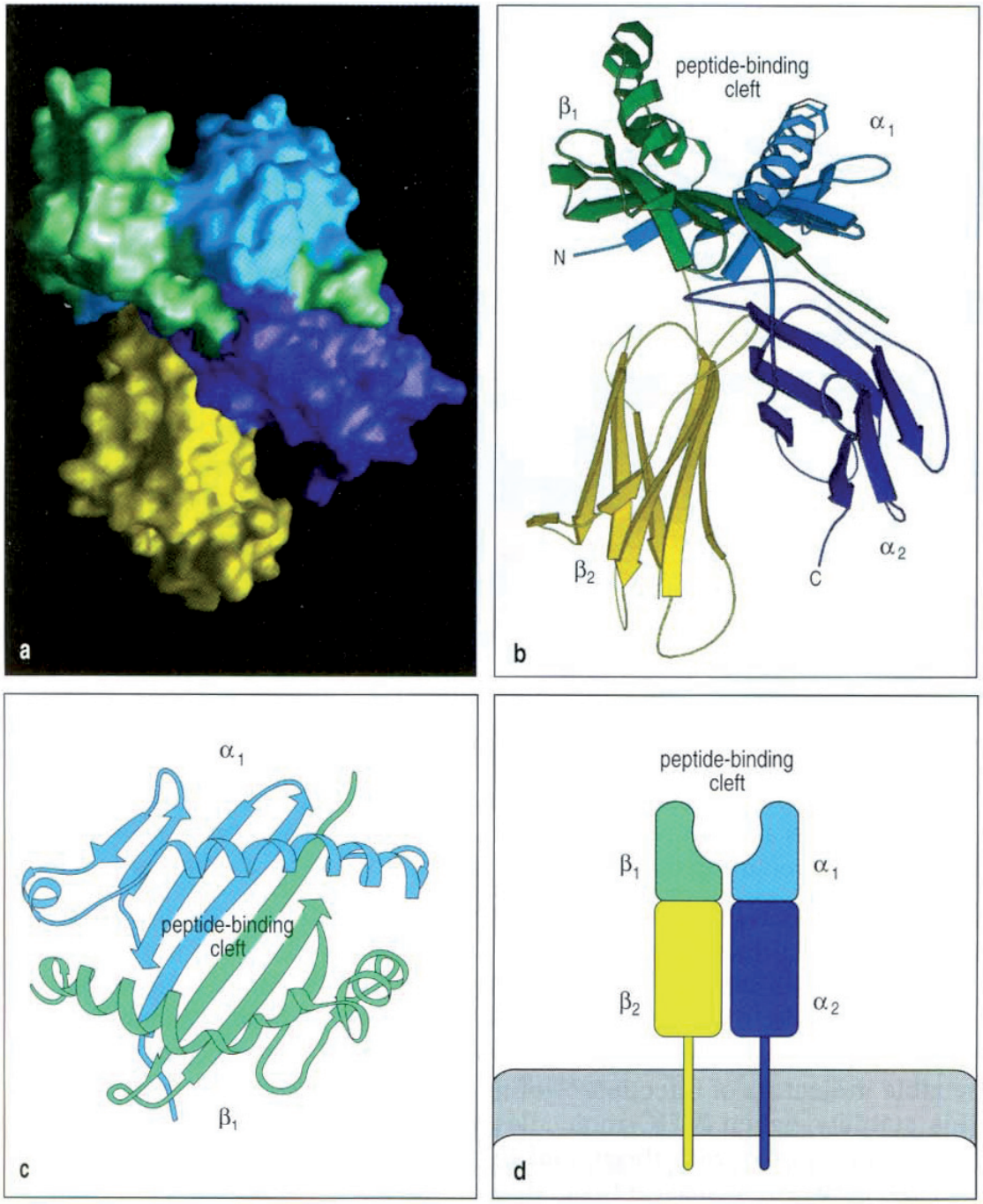


Figure 3:

The structure of the MHC class II molecule. A) Computer graphic of the human MHC class II X-ray structure without the transmembrane domain. B) and C) show a ribbon diagram of the structure whereas panel D) shows a schematic outline of the MHC II molecule. The molecule is compose of two transmembrane glycoprotein chains, α (34 kDa) and β (29 kDa). Each chain has two domains. The two chains together form a compact four domain structure similar to that of the MHC class I molecule. (Adapted and modified from [1])

1.1.5.3. Antigen presentation by MHC class I molecules

MHC class I molecules present peptides that have been generated from antigens processed in the cytoplasm by the proteasome. The proteasome is a large multi subunit protein complex with proteolytic activity. It is responsible for the degradation of proteins that have been marked for destruction by mechanisms, such as ubiquitination [3, 25]. The peptide fragments are transported from the cytosol into the ER (endoplasmic reticulum) where they are loaded onto MHC class I molecules. The sources of proteasome substrates are diverse and include endogenous or viral proteins present in the cytosol as well as incorrectly folded or truncated proteins and proteins retro-translocated from the endoplasmic reticulum to the cytosol [26]. Besides the proteasome, that mainly acts on ubiquitinated protein substrates, other proteases may play a role in antigen processing [27] Following antigen processing by the proteasome, the antigenic peptides are then translocated into the ER lumen by the hetero-dimeric transporter TAP1/TAP2 which is also encoded in the MHC locus and located in the ER membrane. TAP is a member of the large ATP binding cassette (ABC) family of transporters [28]. The partially assembled MHC class I molecules in the ER lumen are associated with chaperones such as calnexin, calreticulin and ERP57, which aid in the assembly of stable MHC class I/peptide complexes. Another MHC encoded protein termed tapasin is believed to bridge TAP and the empty MHC class I molecule waiting to be loaded with a peptide. MHC class I molecules lacking a strong peptide interaction are unstable, ensuring that only functional complexes are found on the cell surface. Stable MHC/peptide complexes are transported to the cell surface via the exocytic pathway [29]. Multiple alleles for MHC proteins exist which comprise different binding properties compensating for the fact that only a minority of the generated peptides can fit into the cleft of a given MHC class I molecule.

The MHC class I/peptide complexes present at the cell surface are constantly checked by $CD8^+$ cytotoxic T cells, which once they recognize a viral, bacterial or otherwise nonself peptide, presented on a MHC class I molecule, kill the nonself peptide bearing target cell. Killing is carried out by secretion of perforin/granzym or surface expression of the Fas ligand, both of which induce apoptosis in the target cell [15]. By destroying the infected cell, the pathogen is either destroyed together with its host cell or is set free to be opsonized by antibodies, recognized by pattern recognition receptors and internalized by phagocytic cells such as macrophages or neutrophils. Antigen processing for presentation on MHC class I, as described above, is schematically depicted below in Figure 4 (left side).

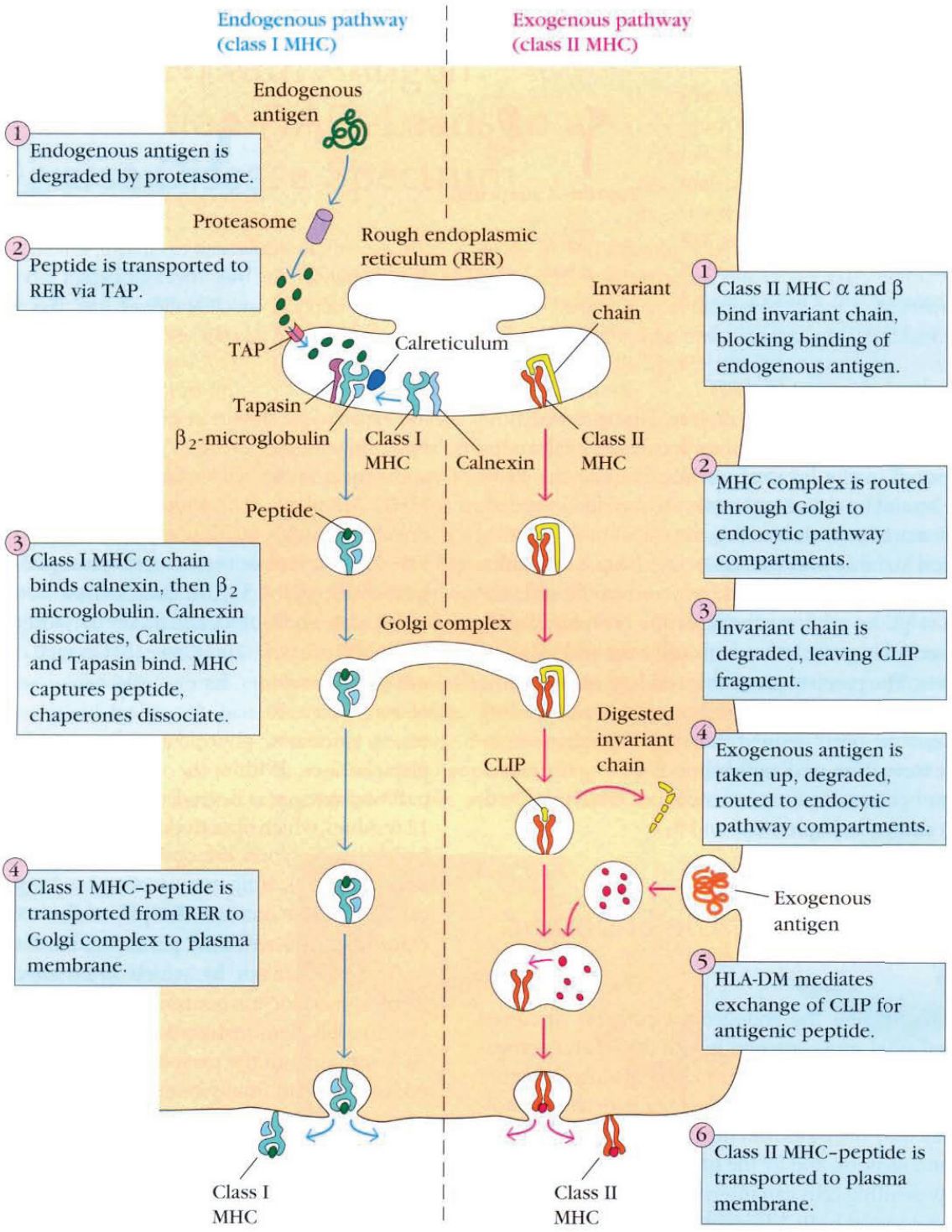


Figure 4:

Separate antigen presenting pathways are utilized for endogenous (green) and exogenous (red) antigens. The mode of antigen entry into cells and the site of antigen processing determine whether antigenic peptides associate with class I MHC molecules in the rough endoplasmatic reticulum or with class II molecules in endocytic compartments. (Adapted and modified from [30])

1.1.5.4. Antigen presentation by MHC class II molecules

MHC class II molecules present peptides that have been generated from antigens engulfed by phagocytic cells such as dendritic cells and macrophages and processed in the endocytic pathway. MHC class II α- and β-chain are bound to a polypeptide termed the invariant chain while in the ER. This complex is transported via the Golgi apparatus to acidic endosomal or lysosomal compartments. These compartments which are also referred to as MHC class II compartment (MIIC) are specialized for the transport and loading of MHC class II molecules with peptide derived from extracellular antigen bearing sources such as bacteria, soluble factors and worms [31-35]. During this transport process the invariant chain is cleaved into smaller peptides, one of which, termed CLIP (class II associated invariant chain peptide), stays bound to the antigen binding pocket of the MHC class II molecule until it is replaced with antigenic peptides destined for presentation on the cell surface [36-38]. Thus, in contrast to MHC I molecules that are loaded with peptide while still in the ER, MHC class II molecules are only loaded after passaging through the Golgi network, within the MHC class II compartment.

Exchange of the CLIP fragment for other peptides is facilitated by a MHC class II-related chaperone molecule called H2-M (HLA-DM) [39-41]. Once the MHC class II has formed a stable complex with a peptide it is transported to the cell surface, where it is recognized by T cell receptors present on $CD4^+$ T cells [42].

MHC class II molecules are mainly expressed on the surface of professional antigen presenting cells, such as macrophages, neutrophiles, dendritic cells and B cell but can be induced by interferon-γ (IFN-γ) stimulation on many cell types [43].

The $CD4^+$ T cell population that recognizes MHC class II/peptide complexes on the cell surface of antigen presenting cells can be further subdivided into subsets such as T_H1- and T_H2-cells. This subdivision if based on the fact that recognition of MHC class II/peptide complexes on the cell surface of phagocytic cells can induce different cytokine secretion patterns. Cytokine production by T cells influences the type of immune response induced by a given antigen. The T_H1 subclass secretes cytokines that aid professional phagocytes in their anti-microbial activity and hyper activates them, whereas T_H2 cells mainly help to initiate and regulate antibody based immune responses [18]. Antigen processing for presentation on MHC class II, as described above, is schematically depicted in Figure 4 (right side).

Besides the thus far described classical mechanisms of MHC class I or II ligand generation, an alternative route for the generation of MHC class I bound peptides exists which is referred to as cross-presentation pathway [44]. Cross-presentation was first described in 1976 [45]. It has two physiological outcomes. It can either lead to induction of tolerance against peripheral antigens [46, 47] or to stimulation of $CD8^+$ T cells. In the latter case, it ensures the generation of anti viral immune response when the professional antigen presenting cells (APCs) are not infected by the virus itself [48, 49] or when the virus interferes with the ability of professional APCs to activate T cells [50-52]. Importantly, cross-presentation has also been implicated in the induction anti-tumor immune responses [53, 54]. Cross-presentation of exogenous antigens can be induced in dendritic cells by inflammatory compounds [55-57], by Fc receptor signaling [58], or by $CD4^+$ T-cells [59], raising the question of whether cross-presentation is regulated similar to the presentation of MHC class II restricted antigens during DC maturation. In these cells, class II molecules are rapidly internalized and recycled for reloading with peptides. [60, 61]. The mechanisms underlying this regulation are not well understood. Enhancement of cross-presentation might be achieved by shifting the proteasomal activity from endogenous to exogenous protein processing [62], by recruitment of MHC class I molecules to "cross-presentation" compartments [63] or "leakiness" of the MHC class II compartments which would allow access of otherwise MHC II restricted peptides to the cytosol and thus to the MHC I processing pathway. Further investigations are necessary to completely understand how cross-presentation is regulated *in vivo*.

1.1.5.5. Antigen presenting cells

Dendritic cells, macrophages and B cells are referred to as professional antigen-presenting cells as they process endogenous and exogenous antigens for the loading on MHC molecules and have the unique ability to stimulate naïve T cells. Stimulation of naïve T cells upon their first encounter with an antigen requires an additional signal delivered by the APC [64]. Professional APCs therefore express high levels of co-stimulatory molecules of the B7 family, which act on T cell receptor associated surface molecules such as CD28 and CTLA-4.

1.1.5.6. Dendritic cells

Dendritic cells (DCs) are regarded as the antigen presenting cells most efficient in the activation of naïve T cells [33, 65]. This has been demonstrated in mice lacking DCs which display severe defects in the initiation of adaptive immune responses [66]. DCs are present to a large extend in tissues such as the skin and are mostly present in an immature state. Similar to macrophages, immature DCs internalize a broad range of antigens via specific and unspecific uptake mechanisms. Stimulation by cytokines or bacterial components causes DCs to migrate from the periphery to the draining lymph nodes, where they come in contact with naïve T cells that are located in the so called T cell zones [67, 68]. During these migratory processes DCs differentiate into professional antigen presenting cell, so called mature DCs. This maturation process involves various phenotypical changes and is accompanied by down regulation of the endocytic capacity, up regulation of co-stimulatory molecules, enhanced antigen processing as well as transport of MHC/peptide complexes to the cell surface [69-72]. Different DC subsets exist *in vivo*, which are generally classified by their tissue distribution and surface marker expression [73, 74].

1.1.5.7. Macrophages

Macrophages such as myeloid dendritic cells and osteoclasts belong to a family of cells termed mononuclear phagocytes. The mononuclear phagocytes share common hematopoietic precursors that are distributed to all tissues of the body via the blood stream as monocytes. Once monocytes have entered the tissues they differentiate into the various cell types mentioned above. Most macrophages become and remain efficient phagocytes throughout their live. Macrophages possess a high endocytic capacity, which accounts for their important role in clearance of invading microorganism during the early phase of a host anti-pathogen response [75]. They can internalize almost any kind of antigen, including soluble as well as particulate forms, both via specific and non-specifically pathways. Both classes of MHC molecules are expressed on macrophages together with co-stimulatory molecules required for the priming of naïve T cells. However these molecules and in particular the MHCs are expressed at significantly lower levels on macrophages when compared to dendritic cells [76]. Even upon activation of macrophages by cytokines or microbial products such as LPS that

results in the up regulation of MHC and co-stimulatory molecules the expression levels do not reach those found on DCs. This may explain why macrophages both *in vivo* as well as *in vitro* were found to be less efficient antigen presenting and T cell activating cells as compared to DCs [65, 77, 78].

1.1.5.8. B cells

Antigen presentation in B cells is linked to their function as antibody secreting plasma cells and surface expression of the antigen specific B cell receptor (BCR). Upon binding of antigens, B cells internalize these together with the BCR and process them via the MHC class II pathway [79].

1.1.6. T cells

T cells belong to a group of white blood cells referred to as lymphocytes. They can be distinguished from other lymphocytes, such as B cells, by the presence of a T cell specific surface receptor called T cell receptor (TCR) (see Figure 6). The abbreviation T, in T cell stands for thymus, since the thymus is the principal organ in which precursors from the bone marrow develop and mature into T cells. TCRs come in two different flavors: αβ and γδ. Over 95 % of the T cells in circulation express αβ TCRs and either CD4 or CD8 co-receptors. The following part will deal only with αβ T cells. In contrast to αβ T cells most γδ T cells do not express CD4 or CD8 on their surface. T cells expressing γδ TCRs are most abundant in areas such as the uterus, the intestine and the tongue which are in contact with the outside world. The repertoire of γδ TCRs is far less diverse than that of αβ TCRs, however much about γδ T cells still remains “mysterious” and controversial. γδ T cells are also found in the thymus but unlike αβ T cells do not seem to require this organ for their development, as mice lacking a functional thymus still have functional γδ T cells. In the following sections the development of αβ T cells as well as signaling via their TCR will be discussed in detail.

1.1.6.1. T cell development

T cells are derived from hematopoietic stem cells in the bone marrow. Early T cell precursors migrate to the thymus where they develop into T cells. The thymus is the primary lymphoid organ where T cells are generated [80]. The thymus can be divided into an outer cortex, where most of the differentiation takes place, and an inner medulla, where newly formed cells undergo final maturation before leaving the thymus and seeding peripheral lymphoid organs [81]. Despite being an integral part of the hematopoietic system, the thymus does not contain self renewing hematopoietic stem cells. That is why the thymus needs to be continuously seeded by small numbers of progenitor cells from the bone marrow, which travel via the blood stream, entering the thymus at the cortex-medulla boundary [82]. T cell development is not driven cell autonomously but relies on signals from non hematopoietic stroma cells such as thymic epithelial cells and mesenchymal fibroblasts as well as antigen presenting cells such as dendritic cells [83]. The thymic stages of T cell development, also referred to as thymocytes, reside in distinct anatomical niches within the thymus that provide the appropriate differentiation signals. During their development thymocytes migrate through these defined thymic sub-regions.

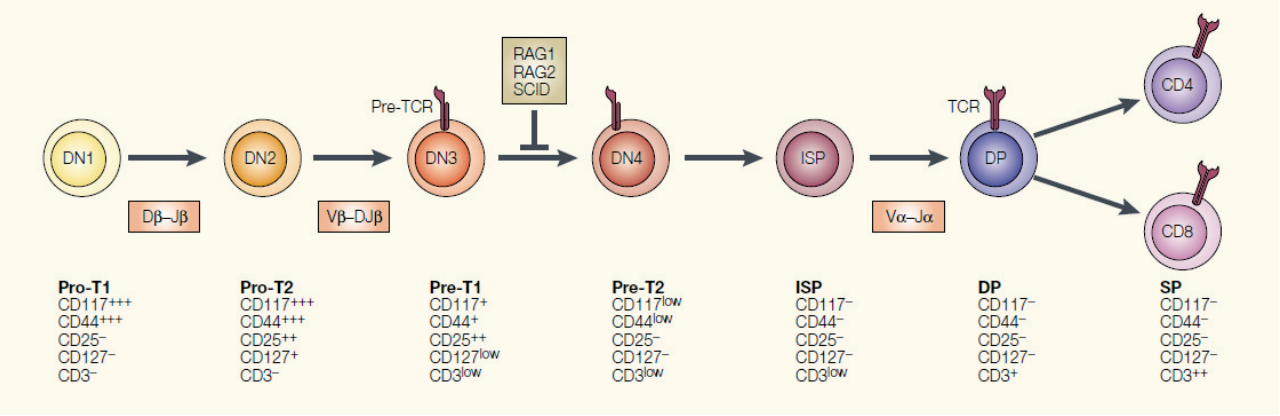


Figure 5:

In this scheme T cell development from less to more mature cells proceeds from left to right. The alternative nomenclature (double negative, DN) is included for comparison. Gene rearrangements are shown boxed in red and the cell surface phenotype of each subpopulation is shown below it. Fluorescence intensity, as measured by flow cytometry on an arbitrary scale from negative (-) through low to very bright (+++) , is indicated for each marker. D, diversity; DP, double positive; ISP, immature single positive; J, joined; RAG, recombination-activation gene; SCID, severe combined immunodeficiency; SP, single positive; TCR, T-cell receptor. (Adapted and modified from [84])

Thymocyte development can be tracked by measuring surface expression of developmental markers as well as co-receptors as described in Figure 5, using flow cytometry. The earliest T cell precursors found within the thymus are negative for both CD4 as well as CD8 and are thus referred to as double negative (DN) thymocytes. Depending on their developmental stage they can be further subdivided into DN1 ($CD3^-$, $CD25^-$, $CD44^{+++}$, $CD117^{+++}$), DN2 ($CD3^-$, $CD25^{++}$, $CD44^{+++}$, $CD117^{+++}$), DN3 ($CD3^{low}$, $CD25^{++}$, $CD44^+$, $CD117^+$) and DN4 ($CD3^{low}$, $CD25^-$, $CD44^{low}$, $CD117^{low}$) [84]. Alongside thymocyte development, rearrangement and expression of the TCR genes occurs. The first crucial check point for efficient rearrangement of TCR genes in αβ T cells is the expression of the so called pre-TCR which is composed of the correctly rearranged β chain and an invariant pre-TCR α chain. If β chain rearrangement has been successful and the pre-TCR is signaling competent, thymocytes can proceed in their development. They will then start the rearrangement of the TCR α chain locus and express the co-receptors CD4 and CD8, developing in CD4/CD8 double positive (DP) thymocytes [85].

As soon as a functional αβ TCR is expressed, DP thymocytes are subjected to a positive and a negative selection process resulting in the elimination of cells which are inefficient in binding MHC/peptide complexes or potentially self reactive [86, 87]. During this selection process a given TCR interacts with peptide loaded MHC molecules present on the surface of thymic epithelial and/or dendritic cells [88]. The resulting signals a DP thymocyte receives via its TCR decide on live or death. If the interaction with the MHC/self-peptide complexes is too weak, meaning that the TCR cannot efficiently interact with the MHC (see below), thymocytes die by "neglect". Conversely if the signal is too strong, thymocytes undergo apoptosis ensuring the removal of potentially self reactive TCRs [89]. The two lineages of T cells, αβ and γδ, differ in their developmental requirements, only αβ T cells require MHC recognition (positive selection). The αβ T cell receptor (TCR), but not its γδ counterpart, contains a conserved motif within the α-chain connecting peptide domain (α-CPM). In transgenic mice expressing an αβ TCR lacking the α-CPM, thymocytes were blocked in positive selection but could undergo negative selection [90, 91]. Thus, the α-CPM seems to participate in the generation of signals required for positive selection, a process that seems to include the cooperation of the TCR with CD4/8 co-receptors. [91-93]. Those cells which survived the selection process continue their development into self restricted CD4 or CD8 single positive (SP) thymocytes. Mature SP thymocytes migrate out of the thymus into the periphery, where they populate the peripheral lymphoid organs waiting to encounter their cognate antigens.

1.1.6.2. The T cell receptor

The T cell receptor is a disulfide linked heterodimer complex of membrane bound polypeptide chains. Similar to the rearrangement and assembly of B cell derived antibody genes the α and δ chains are assembled form V, J and C gene segments, whereas β and γ chains are assembled from V, D, J and C gene segments by means of somatic recombination [9]. Each TCR chain has a highly variable domain which contains three complementarily determining regions (CDRs), responsible for the specificity of TCR binding. The mechanisms responsible for the generation of T cell receptor diversity, such as imprecise end-joining of recombined gene fragments, are essentially the same as the ones which are active for immunoglobulin generation. One exception are somatic point mutations, generated through somatic hypermutation during affinity maturation, which are usually not observed in case of TCRs. Despite the huge diversity observed in the variable regions of the TCR, the constant regions (C) are the same throughout a single species. They are made up of an extracellular domain consisting of an immunoglobulin like domain and a connecting peptide. In case of the α chain the connecting peptide contains the highly conserved α-chain connecting peptide motif (α-CPM), which was previously mentioned in the context of thymic development and positive selection. The TCR chains are anchored in the plasma membrane by a transmembrane region which links the extracellular domains with short cytoplasmatic tails. As these intracellular regions seem to contain no signaling domains, the TCR heterodimer has to tightly associate with accessory proteins such as the CD3 complex, in order to transduce signals from the outside to the inside of a T cell [94, 95]. The CD3 complex is comprised of four different chains (γ, δ, ε and ζ) which form two non-covalently associated heterodimers γ/ε and δ/ε as well as a covalently (disulfide linkage) linked homodimer composed of two ζ chains [96]. All CD3 chains contain at least one so-called immunoreceptors tyrosin-based activation motif (ITAM) (Figure 6). ITAM motifs of the CD3 complex couple TCR α/β chains to intracellular Src tyrosin kinases such as Lck and Fyn [97, 98].

Two other molecules that are also closely associated with the TCR are the co-receptors CD4 and CD8, mentioned before in the context of antigen presentation by MHC molecules. T cells express either CD4 or CD8 which are associated with Lck (or Fyn), Src family tyrosin kinases (SFKs), via their cytoplasmatic domain [98]. In case of an unstimulated TCR, the associated kinase is inactivated by phosphorylation on a C-terminal tyrosin residue (catalyzed by the Csk kinase). Upon contact of a TCR with peptide loaded MHC, the co-receptors bind to

monomorphic determinants on MHC class II respectively MHC class I. Binding of the MHC brings the CD4/8 co-receptor associated Src kinases in close proximity of the TCR and the CD3 complex [99].

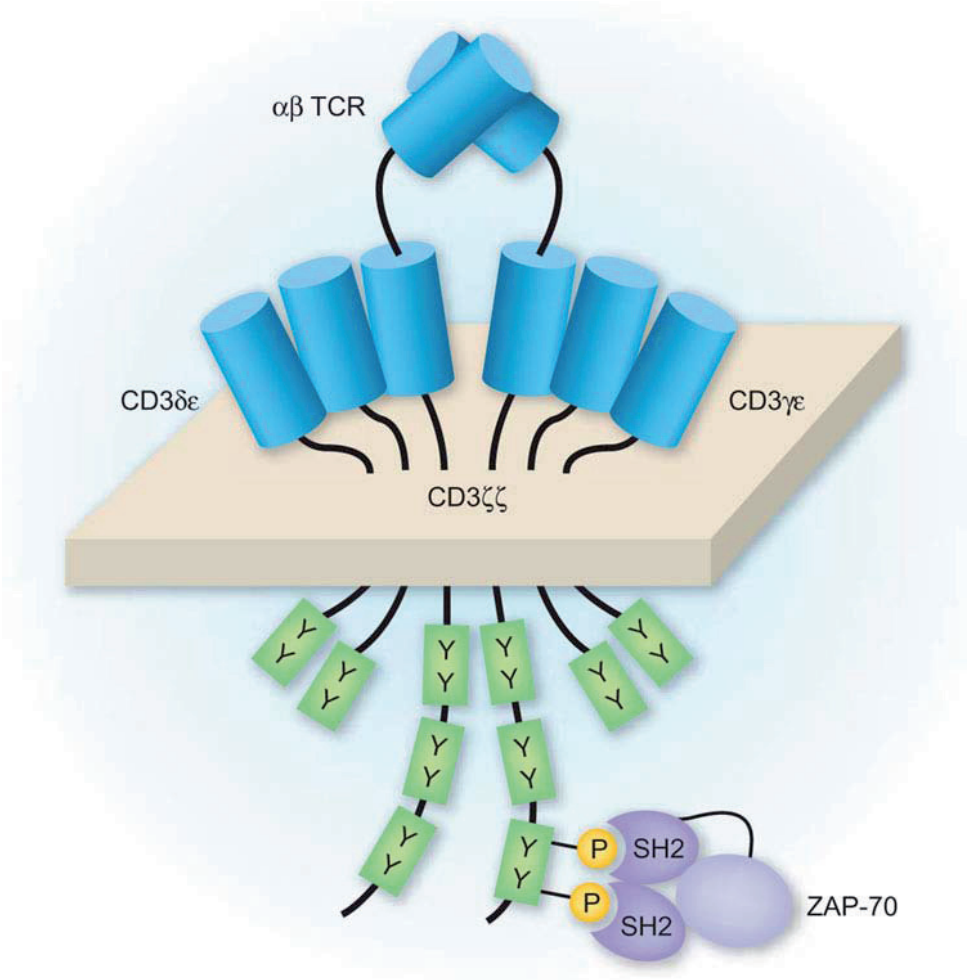


Figure 6:

Schematic representation of the T cell receptor (TCR) complex. The TCR is a covalently linked heterodimer consisting of an α and β chain. The CD3 complex which is associated with the TCR α and β chains comprises four different chains (γ, δ, ε and ζ) which form two non covalently associated heterodimers γ/ε and δ/ε as well as a covalently (disulfide linkage) linked homodimer composed of two ζ chains. ITAMs in the cytoplasmatic tails of CD3 chains provide docking sites for molecules such as ZAP-70 which will be discussed in detail later. (Adapted and modified from [97])

CD45, which is also part of the TCR complex, contains a phosphatase domain in its cytoplasmatic part, which upon receptor stimulation dephosphorylates and activates the Src kinases [100]. The activated Src kinases Leukocyte-specific protein tyrosin kinase (Lck) phosphorylate the ITAMs of the CD3 chains [97]. Phosphorylated ITAMs provide docking sites for the Src Homology 2 (SH2) domains of ζ-chain-associated protein kinase of 70 kDa (ZAP-70) [101], which upon binding is phosphorylated by Lck, allowing propagation of downstream signaling events (Figure 7), discussed in the next chapter.

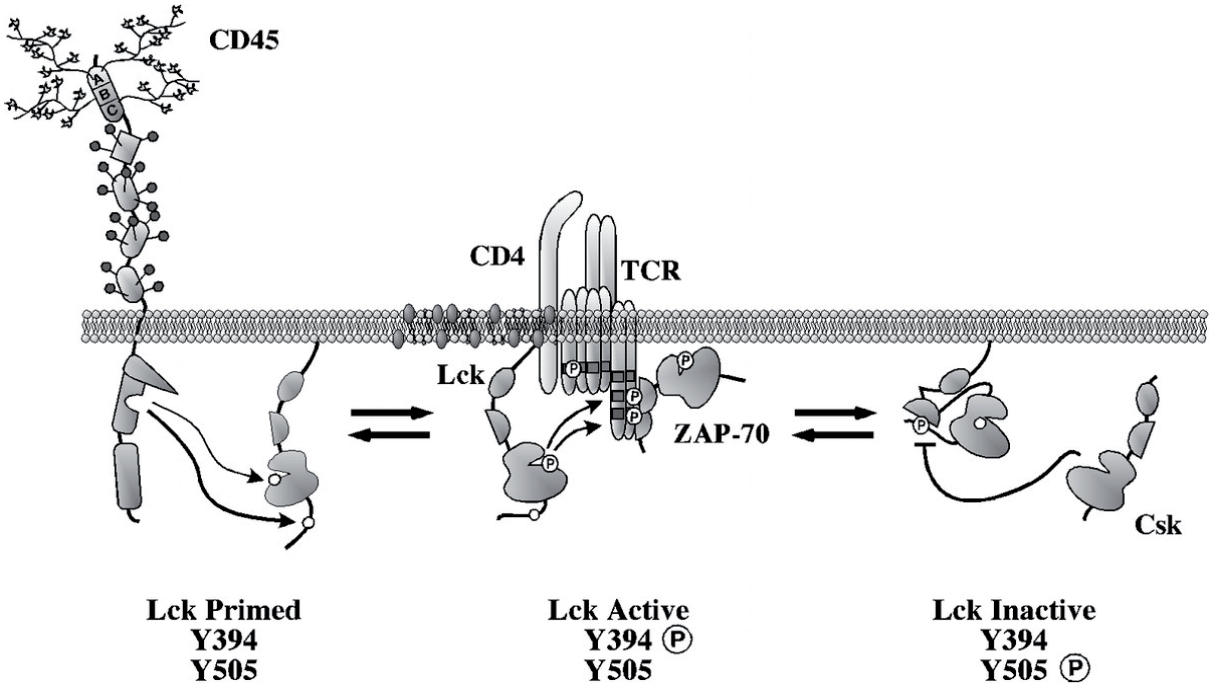


Figure 7

Reciprocal regulation of SFKs by CD45 and Csk. Lck is in a dynamic equilibrium between its inactive and primed conformations due to the reciprocal activity of CD45 and Csk. While CD45 can dephosphorylate both autocatalytic and inhibitory tyrosines of SFKs, the latter is enzymatically favored. This generates a pool of signal-competent SFK. Primed Lck is able to undergo trans-phosphorylation of Y394 generating an active kinase that subsequently phosphorylates the ITAMs of the CD3 chains. The phosphorylated ITAMs provide docking sites for the SH2 domains of ZAP-70, which is then phosphorylated by Lck, allowing propagation of downstream signaling events. SFKs are inactivated by Csk-mediated phosphorylation of the negative regulatory tyrosine. (Adapted and modified from [102]).

1.1.6.3. T cell receptor signaling

Upon T cell receptor engagement with MHC/peptide complexes and tyrosin phosphorylation of ITAMs in the cytoplasmatic regions of the CD3 chains, the phosphorylated ITAMs provide docking sites for the SH2 domains of ZAP-70 (see above), which upon binding is phosphorylated by Lck [103], allowing propagation of downstream signaling events such as the phosphorylation of LAT (linker for activation of T cells) and SLP76 (SRC-homology-2-domain-containing leukocyte protein of 76 kDa) that act as a platform for the recruitment of several key signaling molecules, including phospholipase C-γ1 (PLC-γ1). Lck also phosphorylates the TEC-family kinase ITK (interleukin-2-inducible T-cell kinase) which together with TEC is recruited to the plasma membrane through interaction of their pleckstrin homology (PH) domain with phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 is generated from phosphatidylinositol-4,5-bisphosphate (PIP_2) by the action of phosphatidylinositol-3-kinase (PI3K) [104]. SHIP (SRC homology 2 (SH2)-domain-containing inositol-5-phosphatase and PTEN (phosphatase and tensin homologue) can reduce the levels of PIP_3, thereby decreasing membrane association and activation of certain TEC kinases [105-109]. ITK then forms a complex with several signaling molecules that is nucleated by the adaptors LAT and SLP76 [110, 111]. Activation of the recruited PLC-γ1 by ITK mediated phosphorylation leads to the generation of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$; IP_3), which is required for the induction of Ca^{2+} fluxes within the cell, (see below) and diacylglycerol (DAG), from plasma membrane resident phosphatidylinositol-4,5-bisphosphate (PiP_2). DAD in turn activates members of the protein kinase C (PkC) family and RAS guanyl-releasing protein (RASGRP) [112-114]. This cascade of events results in the downstream activation of mitogen-activated protein kinases such as JNK (JUN amino-terminal kinase), ERK1 (extracellular-signal-regulated kinase 1) and ERK2 as well as calcineurin and other effectors that direct gene transcription via dephosphorylation and nuclear translocation of NFAT and activation of the NF-κB pathway [101, 115]. Signaling intermediates such as Ca^{2+}, IP_3 and DAG are crucial for the production of cytokines and the expression of activation markers by T cells [116]. In addition to activation by DAG, PKC-θ is activated through the guanine nucleotide exchange factor VAV1 and Ras-related C3 botulinum toxin substrate (RAC) mediated pathway [117-119]. The LAT-SLP76 complex also functions as a platform for the accumulation of molecules including VAV1, RAC, the serine/threonine kinase Akt, cell-division cycle 42 (CDC42), Wiskott-Aldrich syndrome protein (WASP), ARP2 (actin-related protein 2 homologue) and ARP3 that regulate the

polymerization of F-actin [120-123]. These molecules, together with other downstream effectors, control TCR-mediated T-cell polarization, adhesion and migration (Figure 8) [124, 125].

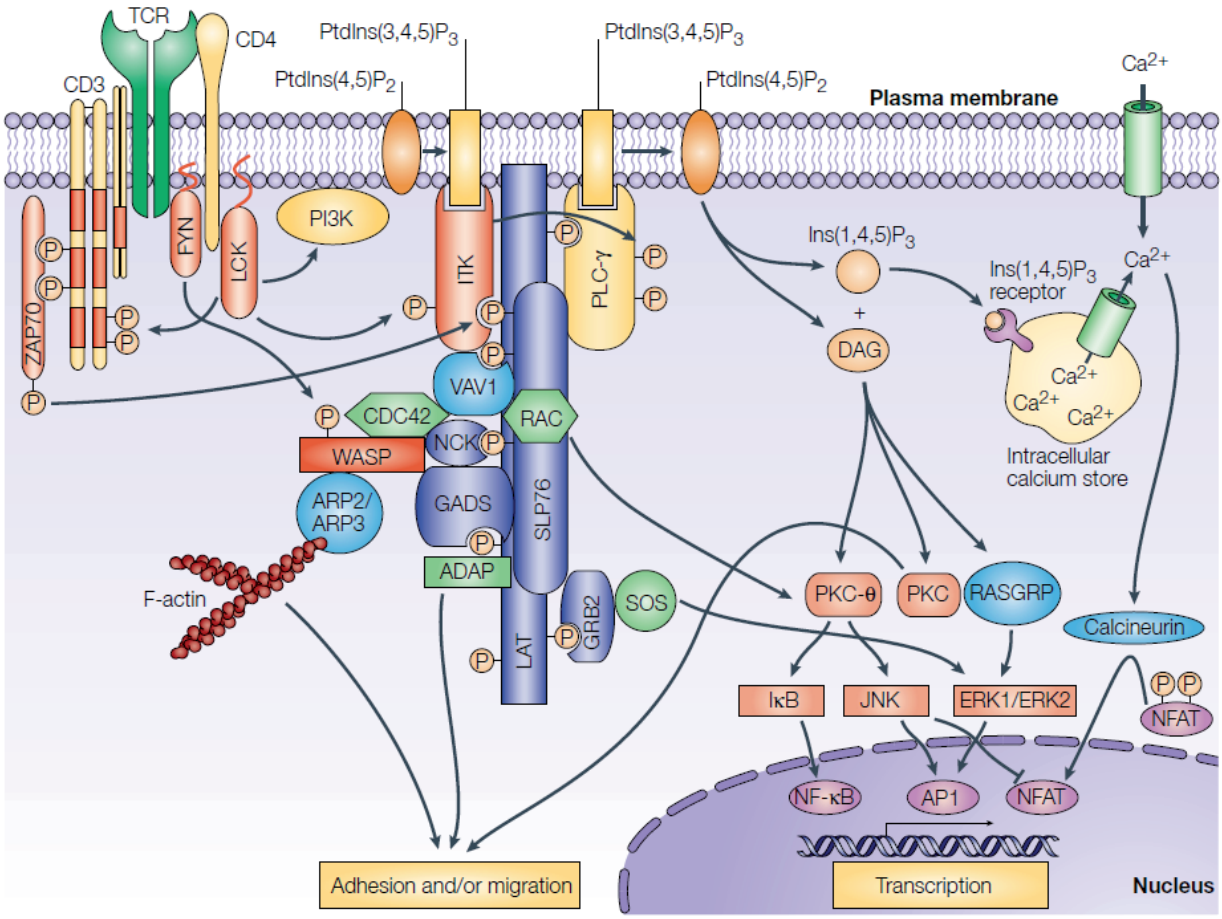


Figure 8:

Signaling via the T cell receptor (TCR) as described in the corresponding text section. Abbreviations: ADAP, adhesion and degranulation promoting adaptor protein; AP1, activator protein 1; GADS, GRB2 related adaptor protein; GRB2, growth-factor-receptor-bound protein 2; IκB, inhibitor of NF-κB; LAT, linker for activation of T cells; NCK, non-catalytic region of tyrosine kinase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; SLP76, SRC-homology-2-domain-containing leukocyte protein of 76 kDa; SOS, son of sevenless homologue; ZAP70, ζ-chain-associated protein kinase of 70 kDa. See also corresponding text section for further abbreviations. (Adapted and modified from [104])

In addition to the CD3 complex and the CD4/8 co-receptors, other co-stimulatory molecules such as CD28 and the cytotoxic T lymphocyte antigen 4 (CTLA-4) are recruited to the TCR upon formation of the immunological synapse (will be described below). Depending on the expression of their ligand on the antigen presenting cell (APC), B7-1 also known as CD80 (for CTLA-4) and B7-2 also known as CD86 (for CD28), subsequent downstream signaling via the PKC θ pathway was shown to be differentially regulated [126, 127].

1.1.6.4. The immunological synapse, T cell activation and homeostasis

Cross-talk between receptor mediated signalling, cytoskeletal reorganization and directed transport of cell surface receptors was first shown in studies utilizing soluble antibodies directed against major components of the TCR complex to cross-link TCRs and other surface molecules for T cell stimulation. Crosslinking results in “capping”, an event characterized by assembly and redistribution of surface receptors, cytoskeletal elements and other molecules such as lipids towards one end of a stimulated cell [128]. Also immunofluorescense analysis of T cell - APC conjugates indicated a marked polarization of the T cell towards the APC. This polarization seemed to particularly involve the microtubule organizing centre (MTOC) which moved to a location underneath the synapse [129]. Using three dimensional visualization techniques scientists around A. Kupfer for the first time gained insight in the spacial organization of T cell - APC complexes. Key molecules such as the TCR and the adhesion integrin leukocyte function associated antigen 1 (LFA1) were found to be polarized at the T cell - APC interface and organized in and restricted to distinct regions within the immunological synapse. These regions were termed supra-molecular activation complexes (SMACs) (Figure 9). The central region of the SMAC (cSMAC) is enriched for the TCR, CD3, CD45 (CD45 enters cSMAC only at a later stage) complex, the co-stimulatory molecules CD28 and CTLA-4 as well as for downstream signaling effectors of the TCR signaling complex such as PKC θ. Cell adhesion and contact formation with the APC seems to be primarily mediated by the peripheral ring structure which surrounds the cSMAC (called the pSMAC). This region is enriched for molecules such as leukocyte function associated antigen 1 (LFA1), CD4/8 and talin. Large and bulky molecules such as CD43, CD44 and most of CD45 (especially at early stages of synapse formation) were confined to an area distal to the synapse, outside the pSMAC, which is known as the dSMAC [130-132]. Recently it has

been proposed that the cSMAC is the site of both TCR signal enhancement as well as TCR degradation and that the balance between these adverse processes is determined by the quality of the antigen, such that the cSMAC can serve as an amplifier for weak agonist signals and integration of co-stimulatory signals (Figure 9) [133, 134].

The precise role of the different SMAC regions remains controversial. It is however now widely accepted that the initiation of TCR signals occurs in peripheral microclusters which are assembled prior to the formation of the immunological synapse (IS). Ligation of TCRs with MHC/peptide complexes initiates the formation of these microclusters which contain the TCR complex and associated signaling molecules (see previous chapter) such as LAT, SLP-76 and ZAP-70. These clusters initiate as well as sustain calcium signals in T cells (Calcium signaling in T cells will be discussed in detail below) [135, 136]. After a short time these clusters converge towards the cSMAC. The formation as well as the translocation of these clusters depends on cytoskeletal dynamics and continues even after the IS has been established. The importance of integrins in T cell activation is emphasized by the key role they play in establishing the IS and maintaining it [137-139]. Sustaining TCR ligation and signaling may help to maintain transcription factors such as NFAT in the nucleus where they initiate specific transcriptional programs [140].

TCR signal induction and propagation are driven by rapid biochemical reactions which either induce or amplify the physical connection and catalytic activity of TCR/IS signaling components. Regulatory mechanisms that oppose these reactions guarantee appropriate responses to external stimuli by controlling or stopping the signals generated in response to these stimuli. Phosphorylation and dephosphorylation are the fastest and most versatile means of regulating signal progression, although other protein modifications such as ubiquitination and de-ubiquitination as well as lipid modifications contribute to the net signal outcome [141].

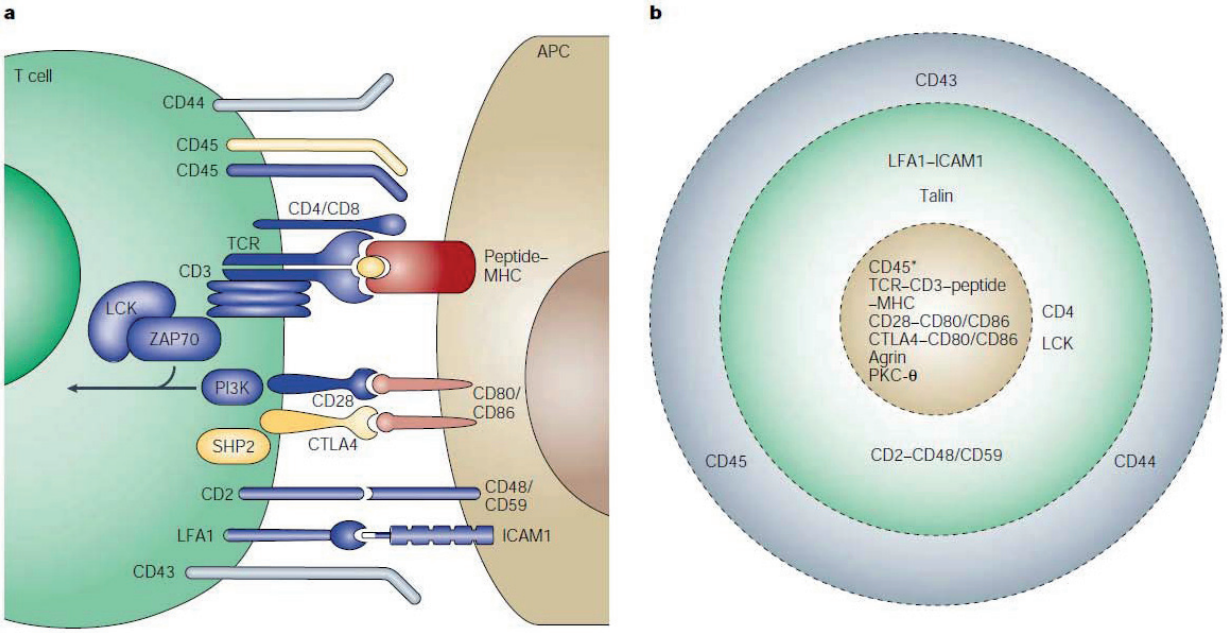


Figure 9:

A) A profile view showing a selection of the key ligand pairs and signaling molecules that are involved in immunological synapse (IS) formation. The stimulatory peptide/MHC complex is shown in red, activating/co-stimulatory molecules are blue, inhibitory molecules are yellow and molecules that are not contributing to signaling are grey. The arrow indicates converging signals that lead to T cell activation. B) The face on view of the synapse with the characteristic 'bulls-eye' zone pattern, including the central region of the supra-molecular activation complex (cSMAC) (yellow), the peripheral ring surrounding the cSMAC (pSMAC, green) and the region distal to the synapse outside the pSMAC (dSMAC, grey) as well the molecules/ligand pairs that are found enriched within. APC, antigen-presenting cell; CTLA4, cytotoxic T lymphocyte antigen 4; ICAM1, intercellular adhesion molecule 1; LFA1, leukocyte function-associated antigen 1; PI3K, phosphatidylinositol 3-kinase; SHP2, SRC homology 2-domain-containing protein tyrosine phosphatase 2; TCR, T-cell receptor; ZAP70, ζ-chain-associated protein 70. *CD45 enters the cSMAC at later stages. (Adapted and modified from [130]).

1.1.6.5 T cell homeostasis and termination of immunresponses

Besides the need for controlling the underlying biochemical reactions, regulation of T cell responses also takes place on the cellular level to appropriately fine-tune immunoreactions. Maintenance of cellular homeostasis in the face of rapid and dynamic alterations in lymphocyte populations upon infections and avoidance of autoimmune responses to self-antigens requires tight and effective mechanisms to prevent and/or terminate lymphocyte reactions. Homeostasis of the immunesystem is ensured by maintaining a continuous balance between positive (activating) and negative (inhibiting) signals as well as the lack of certain

signals. The mechanisms that govern these processes fall into two categories. On the one hand the loss or absence of co-stimulatory signals and stimuli that provide growth and survival signals leads to functional inactivation, failure of initiating an effective immunresponse or programmed cell death. On the other hand lymphocyte activation on its own triggers regulatory systems which control lymphocyte proliferation, differentiation as well as apoptosis. Lymphocyte activation requires two signals, the first one being delivered by the antigen whereas the second one is provided by the APC in the form of a co-stimulatory signal [142]. During an infection the innate immune responses are triggered by molecules and molecular patterns present on the invading pathogens which are recognized by receptors such as the LPS receptor (CD14) and receptors of the Toll-like receptor (TLR) family which leads to the up-regulation of co-stimulatory molecules and enables them to efficiently activate T cells [143]. Self-antigens may be ignored by the immune system largely because the self reactive T cells have been deleted during thymic selection. In addition self-antigens mostly fail to initiate innate immuneresponses that are required for T cell activation. Activated innate immune cells provide the required co-stimulatory signals for T cell activation and effector function. Foreign antigens administered without an adjuvant in case of a vaccination may fail to initiate effective immuneresponses as the adjuvant is needed to efficiently activate innate immuneresponses. Omitting the adjuvant may even induce tolerance instead, a concept which is currently tested in clinical trials, which have the goal of preventing organ transplant rejection and to eliminate auto-immuneresponses. Antigen recognition by lymphocytes without a second signal can lead to a state of functional unresponsiveness, referred to as anergy [144, 145]. Some early studies actually suggest that T cell anergy, at least in some models, may be actively induced rather than being a result of missing co-stimulation. According to these studies anergy is induced because T cells use the inhibitory cytotoxic T lymphocyte antigen-4 (CTLA-4) receptor to engage co-stimulatory B7 molecules on the APC rather than CD28 [146, 147]. CTLA-4 and its importance in the active termination of immuneresponses will be discussed in more detail below.

The frequency of antigen specific T cells is very low (in the order of 1 in 10^6) if an individual has not been exposed to the cognate antigen. However, upon exposure to the antigen under conditions which help to promote efficient and specific immuneresponses this otherwise low frequency can increase to 1/1000 or more within days and return to basal levels within 4-12 weeks [148, 149]. Rapid elimination of lymphocytes by apoptosis is responsible for this fast decline in T cell numbers. Once the antigen respectively its source has been eliminated,

cytokine levels drop, co-stimulatory molecules are no longer expressed at high levels, T cells no longer encounter high numbers of MHC/peptide complexes derived from their cognate antigen and as a consequence die “by neglect” as the T cells become deprived of survival signals and lose expression of anti-apoptotic proteins. Most of these anti-apoptotic proteins belong to the B cell lymphoma (Bcl) family [150-152].

Active termination of T cell immune responses is based on feedback mechanisms that are triggered in lymphocytes during their activation. The inhibitory receptor CTLA-4, for example, is expressed on T cells only following activation. CTLA-4 is induced at a later stage during TCR stimulation and competes with CD28 for B7 (CD80/86) with very high binding affinity [153, 154]. Another molecule that, like CTLA-4, is only expressed on T cells upon activation is the CD28 family protein PD-1 which also binds to B7 family molecules. PD-1 possesses an immunoglobulin domain in the extracellular part and an immunoreceptors tyrosin-based inhibition motif (ITIM) in the cytoplasmic region [155]. PD-1 receptor deficient mice, that develop autoimmune dilated cardiomyopathy, illustrate the critical *in vivo* role of PD-1 [156]. PD-1 functions as a negative regulator of immunresponses and in particular inhibits autoreactive lymphocytes. Judged by the time course of disease onset and progression, the inhibitory mechanism of PD-1 however seems to differ largely from that exerted by CTLA-4. These results suggest that both molecules that are induced upon stimulation in T cells may actually function with different spacial and temporal requirements and restrictions as well as on different cell types and act in a coordinated fashion to keep lymphocyte reactions in check [157].

Activation of T cells also leads to the expression of the death receptor, Fas (CD95/APO-1). The specific interaction of Fas with its cognate ligand, Fas ligand (FasL/CD95L), elicits the activation of a death-inducing caspase (cysteine aspartic acid proteases) cascade, occurring in a transcription-independent manner. Caspase activation executes the apoptosis process by cleaving various intracellular substrates, leading to mitochondrial membrane potential loss, genomic DNA fragmentation and the exposure of signals on the cell surface that attract phagocytic cells and promote uptake as well as degradation of the apoptotic cell(s). The induction of apoptosis in this manner is termed activation induced cell death (AICD). It is likely that Fas mediated T cell death is key to the elimination of cells which repeatedly encounter persistent antigens such as self-antigens. Analysis of Fas or FasL deficient mice support this hypothesis as they do not show prolonged immunresponses towards immunization or viral infections but rather develop autoimmune disease [158-161]. Besides

the molecules mentioned thus far there are many more that participate in the regulation of immunresponses, such as galectin-3 which destabilized the IS, shortens T cell - APC contact time and aids in the down regulation of TCR/CD3 complexes on T cells [162], but to mention them all is beyond the scope of this introduction.

As a last point with respect to the mechanism that fine tune as well as negatively regulate immune responses two sets of cells that have been proposed to play a key role in this process should be mentioned, immature dendritic cells and regulatory T cells (T_{REG}). The paradigm of tolerogenic/immature versus inflammatory/mature dendritic cells has remained controversial regarding the role these APCs play in mediating immune homeostasis and self-tolerance, versus efficient immunresponses mounted against invading pathogens. The discovery of PAMPs and TLRs has provides a molecular basis for understanding the recognition of specific pathogens by DCs and the resulting induction of appropriate innate and adaptive immune responses. During the last years researchers have gained further insight into the mechanism by means of which DCs contribute to establishing and/or maintaining immunological tolerance. The spectrum ranges from non-inflammatory DCs which lack the ability to induce T cell activation to CDs that actively suppress T cell responses and induce regulatory T cell subsets [163, 164]. Murine regulatory T cells are characterized by the expression of CD4, CD25 as well as the T_{REG} specific transcription factor FoxP3 [165]. Disruption of FoxP3 results in the fatal lymphoproliferative disorders of the scurfy mouse. Genetic complementation demonstrated that the protein product of FoxP3, scurfin, is essential for normal immune homeostasis and thus helped to establish FoxP3 as well as the T cell subset that expresses FoxP3 as an important regulatory component of the immunesystem [166, 167]. An early response of $CD4^+$ T cells to an antigen encounter is the production of cytokines, mostly IL-2. Within hours of T cell priming, IL-2 dependent STAT5 phosphorylation occurres primarily in FoxP3 expressing regulatory T cells and only at a later stage in the T cell subset which initially produced the IL-2. As a response to IL-2 signals regulatory T cells proliferate and develop enhanced suppressive activity. This findings indicate that one of the earliest events in a T cell response is the activation of endogenous regulatory cells, probably to prevent autoimmunity [168]. Besides the various mechanism that control T cell responses and help to prevent autoimmunity as well as immune-over-activation, other regulatory mechanisms exist which help to maintain the peripheral pool of mature T cells. The next chapter will address the different requirements of naïve and memory T cells in respect to T cell homeostasis and survival.

1.1.6.6 T cell homeostasis and survival

Positive and negative selection in the thymus allow only a tiny fraction of the initially generated immature thymocytes with a low affinity for MHC/self-peptides to survive and differentiate into mature, naive T cells that can emigrate from the thymus to the periphery. These cells form a long lived population of naive T cells which circulate through the peripheral lymphoid organs in search for their cognate antigen. Activation by antigens expressed on pathogens induces naive T cells to proliferate and acquire the effector functions needed to eliminate the pathogen. As described above, most effector cells die within a few weeks upon clearance of the pathogen and disappearance of their cognate antigen. Only a small fraction of the cells survives for a very long time as memory T cells. Effector and memory states of T cells are separate fates determined by differential TCR signaling [169]. Both naive as well as memory T cells have different requirements for their survival which will be described in this part of the chapter. T cell homeostatic signals are largely derived from contact with MHC/self-peptide complexes and members of the gamma chain family of cytokines, in particular IL-7 and IL-15. Prolonged survival of naive T cells in interphase depends on the combination and availability of contacts with MHC/self-peptide complexes and IL-7 (to a lesser extend IL-15) [170, 171]. Abrogation of TCR expression or of TCR proximal kinases such as Fyn and Lck was shown to reduce the lifespan of naive T cells [172-174]. Similar results were obtained using mice lacking MHC II molecules where T cells are deprived of MHC/self-peptide contacts [175, 176]. The same rules apply if large numbers of naive transgenic T cells with only one fixed TCR compete for few specific MHC/self-peptide contacts but not if these T cells express a diverse repertoire of TCRs, resembling the very low clonal frequency of cells in a polyclonal population [177, 178]. These results again suggest that recognition of specific MHC/self-peptide ligands is required for naive T cell homeostasis. The essential role of IL-7 in mature T cell survival was discovered by the finding that the absence of IL-7 prevented naive T cell survival, whereas IL-7 overexpression or potentiation by use of IL-7/α-IL-7 antibody complexes dramatically increased the pool size of naive T cells [171, 179-181]. During lymphopoiesis IL-7 is produced by stromal and epithelial cells both in the bone marrow as well as in the thymus. For mature T cell homeostasis IL-7 is produced locally within the secondary lymphoid organs where it is most likely retained to a large extend, bound to the extracellular matrix, as described for other members of the gamma chain family of cytokines (Figure 10) [182]. In contrast to naive T cells, longevity as well as

intermittent turnover of long lived memory T cells seems to be largely MHC independent and maintained through contact with a combination of IL-7 as well as IL-15 [183-186].

Finally, this section will deal with some of the key molecules that are involved in the effects exerted by IL-7 and IL-15 in regard to T cell homeostasis and survival (Figure 10). Survival of naive $CD4^+$ and $CD8^+$ T cells under normal physiological conditions requires signals from contact with MHC/self-peptide complexes on DCs and IL-7 on fibroblastic reticular cells (FRC). T cells receive these signals in the T cell zones of secondary lymphoid organs. Naive $CD8^+$ T cells are also partly dependent on signals resulting from contact with IL-15 expressed on DCs. Signaling through IL-7/15 cytokine receptors induces activation of the receptor bound Janus Kinases JAK1 and JAK3, that in turn phosphorylate and activate the STAT5a-b dimer. Suppressor of cytokine signaling-1 (SOCS-1) negatively regulates the responsiveness of naive $CD8^+$ T cells to IL-15 by neutralizing the activity of JAKs. Once phosphorylated, the activated STAT5a-b dimers migrate into the nucleus where, together with signals derived from the TCR, they initiate transcription of genes coding for various proteins. Among these proteins are the anti-apoptotic molecules Bcl-2 (B-cell lymphoma 2) and Mcl-1 (Myeloid cell leukemia sequence 1) that prevent mitochondria-mediated apoptosis by blocking the BH-3-only (Bcl-2-homology domain 3 only) proteins Bim (BCL-2-interacting mediator of cell death) and Bid (BH3 interacting domain death agonist) from activating Bax (Bcl-2–associated X protein) and Bak (Bcl-2 homologous antagonist/killer). Activated Bax and Bak otherwise terminally induce apoptosis by causing loss of cytochome C and other molecules from mitochondria that initiate caspase activation and finally cell death [151, 187-194]. Wojciechowski et al., examined the role of the anti-apoptotic molecule Bcl-2 in combating the pro-apoptotic molecule Bim in the context of naive and memory T cell homeostasis using $Bcl\text{-}2^{(-/-)}$ mice that were additionally deficient in one or both alleles of Bim. Naive T cells were significantly decreased in $Bim^{(+/-)}Bcl\text{-}2^{(-/-)}$ mice, but were largely restored in $Bim^{(-/-)}Bcl\text{-}2^{(-/-)}$ mice, indicating that T cell death is primarily mediated through Bim [193]. Newly initiated protein synthesis via STAT5 and TCR derived signals also leads to repression of IL-7 receptor transcription, thereby reducing the IL-7 receptor expression on the cell surface and rendering the cell to become temporarily starved of IL-7. During such IL-7-deprived state, cell survival appears to depend on intact autophagy [195, 196].

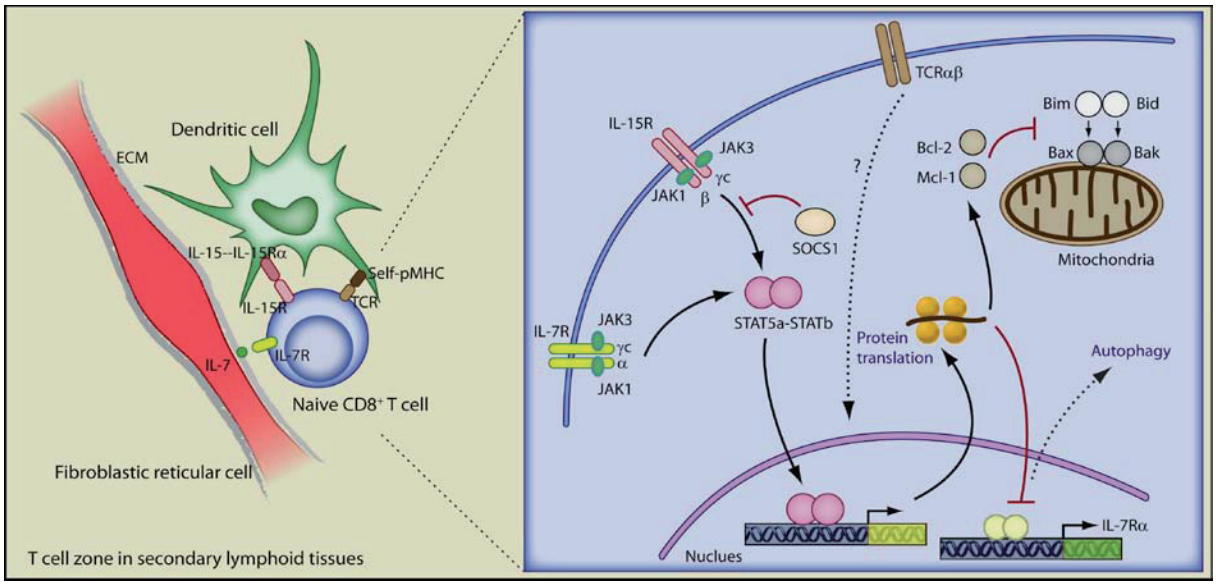


Figure 10:

Survival of naive T cells under normal physiological conditions. Self-peptide MHC (pMHC) ligands on dendritic cells (DCs) together with IL-7 on fibroblastic reticular cells (FRC) (and IL-15 on DCs) stimulate IL-7/15 receptor bound Janus Kinases (JAK). Activated JAKs phosphorylate and activate signal transducer and activator of transcription (STAT). Suppressors of cytokine signaling (SOCS-1) negatively regulates the responsiveness of naive T cells to IL-7/15 by neutralizing the activity of JAKs. Phosphorylated STAT5a-b dimers then migrate into the nucleus, and together with signals transmitted via the TCR, initiates synthesis of various proteins that counteract mitochondria-mediated apoptosis by blocking the BH-3-only proteins. During a IL-7-deprived state, cell survival appears to depend on intact autophagy. For a detailed description of the events depicted in this figure see corresponding text section. (Adapted and modified from [183])

1.1.7. Calcium signaling in T lymphocytes

Calcium signals in cells of the immune system regulate diverse cellular functions, including proliferation, differentiation, gene transcription and effector functions. Engagement of immunoreceptors, such as the T cell receptor (TCR), the B cell receptor (BCR) and Fc receptors result in an increase in intracellular calcium levels. Calcium elevation is essential for activation of signaling molecules such as protein kinase C, calcium dependent adenylate cyclases and the phosphatase calcineurin as well as for the initiation of gene transcription. The major pathways implicated in the induction of an increase in cytosolic calcium concentrations are store-operated calcium entry (SOCE) and calcium-release-activated (CRAC) channels. In this section the signaling pathways leading to calcium influx and the major components of the involved molecular machineries will be discussed.

1.1.7.1. Role of calcium in T lymphocytes

Intracellular calcium elevation occurring downstream of the T cell receptor (TCR) as a result of TCR ligation, is an essential signal for T cell activation by antigens and other stimuli that act upon the TCR. The binding of MHC/antigen complexes to the TCR induces the recruitment of several tyrosine kinases and their substrates to the TCR/CD3 signaling complex. This series of events results in the phosphorylation of phospholipase C γ1 (PLC γ1) [197]. PLC γ1 cleaves the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) in the plasma membrane thereby generating the second messenger inositol-1,4,5-trisphosphate (IP_3). IP_3 binds to IP_3-receptors on the endoplasmatic reticulum (ER) thereby inducing calcium release from this intracellular calcium store, giving rise to transiently elevated cytoplasmatic calcium concentration. This first cytoplasmatic calcium elevation then results in a second wave of calcium influx via calcium permeable channels which span the plasma membrane, thus allowing calcium entry from the extracellular space. The major components of this system will be discussed in detail in the next section. In most cases, calcium influx must be maintained for 1-2 h to efficiently drive the activation events that lead to the expression of factors such as interleukin-2 (IL-2). Once this point has been passed, further T cell activation becomes antigen independent [115]. This requirement for sustained calcium signaling arises mostly from the need to keep specific transcription factors in the

nucleus in a transcriptionally active state. One of the best studied and most important transcription factors not only in T cells, but also in other cells of the immunesystem, is the Nuclear Factor of Activated T cells (NFAT) which upon elevation of the cytoplasmatic calcium concentration becomes dephosphorylated by the phosphatase calcineurin and translocates into the nucleus where it induces the transcription of genes such as IL-2 [198]. In order to efficiently generate the sustained signals in T cells which are needed for cell activation, T cells, once having encountered an APC presenting the right antigen, must switch from an motile to an immobile state. This is essential for maintaining the stability of the immunological synapse formed between the T cell and the APC. *In vitro* experiments have revealed that the interaction of cloned T cells or hybridoma T cells with APCs induces cytosolic calcium elevations within ~ 30 sec. Shortly after this, the T cells round up and stop crawling. It appears that calcium by itself is sufficient to account for the observed changes in motility, as the addition of the ionophore ionomycin as well as the ER calcium store depleting agent thapsigargin induce similar changes independent of the TCR [199, 200].

Today a large number of pathways that participate in the generation of calcium signals in T cells following an antigen encounter are known. Interactions between these different pathways may help to generate the huge complexity of calcium signaling patterns, which transmit different and specific types of information to the cells [201].

1.1.7.2. Mechanisms of calcium signaling in T lymphocytes

The nature of the feedback mechanisms between ER calcium stores and the plasma membrane have remained a mystery for a long time. Early studies revealed that the depletion of the ER calcium stores by themselves, rather than the resulting rise of the cytosolic calcium concentration, is the signal that following inositol-1,4,5-trisphosphate (IP_3, $InsP_3$) induced calcium release from the ER triggers a second wave of calcium influx (SOCE). Calcium influx occurres through calcium channels spanning the plasma membrane [202]. Calcium release activated calcium (CRAC) channels were and still are the best characterized store operated calcium (SOC) channels [203, 204]. However until recently the underlying molecular machinery, which on the one hand senses calcium depletion within the ER and on the other hand couples this depletion with the activation of calcium channels within the plasma membrane, remained ill defined.

The breakthrough came when RNAi screens picked up the STIM 1 proteins as the missing molecular linkers between ER store depletion and SOCE as well as defined the Orai (CRACM) proteins as the pore forming subunits of the CRAC channel [205-209] (see also Figure 11).

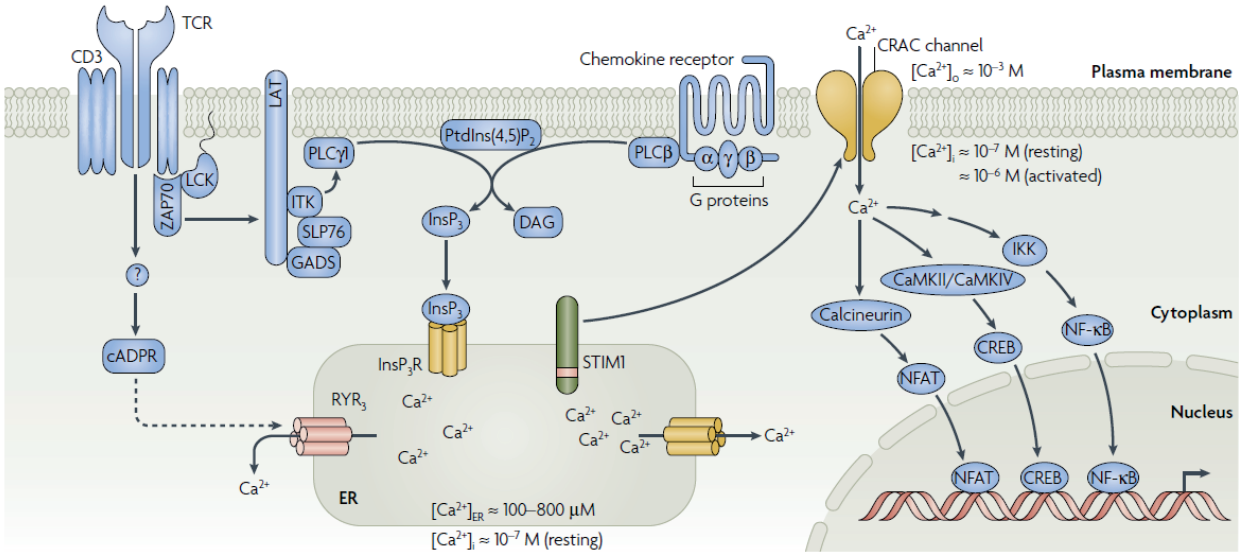


Figure 11:

In resting T cells, a steep gradient in Ca^{2+} concentrations exists between the cytoplasm and the extracellular space, as well as between the cytoplasm and the lumen of the endoplasmic reticulum (ER). The intracellular Ca^{2+} concentrations in T cells is tightly regulated and kept between ~ 100 nM in resting cells and ~ 1 µM following T cell receptor (TCR) stimulation. Antigen recognition through the TCR results in the activation of protein tyrosine kinases, such as LCK and ZAP70 (ζ-chain-associated protein kinase of 70 kDa), which initiate phosphorylation events of adaptor proteins, such as SLP76 (SRC-homology-2-domain-containing leukocyte protein of 76 kDa) and LAT (linker for activation of T cells). This leads to the recruitment and activation of the TEC kinase ITK (interleukin-2-inducible T-cell kinase) and phospholipase Cγ1 (PLCγ1). PLCγ1 catalyses the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PiP_2, $PtdIns(4,5)P_2$) to inositol-1,4,5-trisphosphate (IP_3, $InsP_3$) and diacylglycerol (DAG). $InsP_3$ binds to and opens $InsP_3$ receptors ($InsP_3Rs$) in the membrane of the ER, resulting in the release of Ca^{2+} from intracellular Ca^{2+} stores. A decrease in the Ca^{2+} content of the ER is 'sensed' by stromal interaction molecule 1 (STIM 1), which in turn activates calcium-release-activated calcium (CRAC) channels in the plasma membrane. Ca^{2+} influx though CRAC channels and elevated intracellular Ca^{2+} concentration activate Ca^{2+}-dependent enzymes, such as calcineurin, and thereby transcription factors, such as NFAT (nuclear factor of activated T cells). (Adapted and modified from [210])

In the following part the underlying molecular mechanisms of STIM 1 as well as Orai function will be discussed. STIM 1 molecules are found in a calcium bound state in resting cells in which ER stores are filled and release calcium when ER stores are depleted, thus initiating the activation of CRAC channels. Under normal resting condition, the calcium concentration within the ER is much higher than in the cytoplasm, a state which is maintained by the activity of the ATP dependent SERCA pump. This pump which can be blocked by the drug thapsigargin (induces ER store depletion) permanently pumps calcium from the cytosol back into the ER. In the resting state STIM 1 is found as a dimer which is formed by C-terminal coiled-coil interactions. Upon ER store depletion STIM 1 molecules further oligomerize within seconds, a process mediated by the sterile alpha motif (SAM) domains which are positioned next to the EF hand domains (Figure 12).

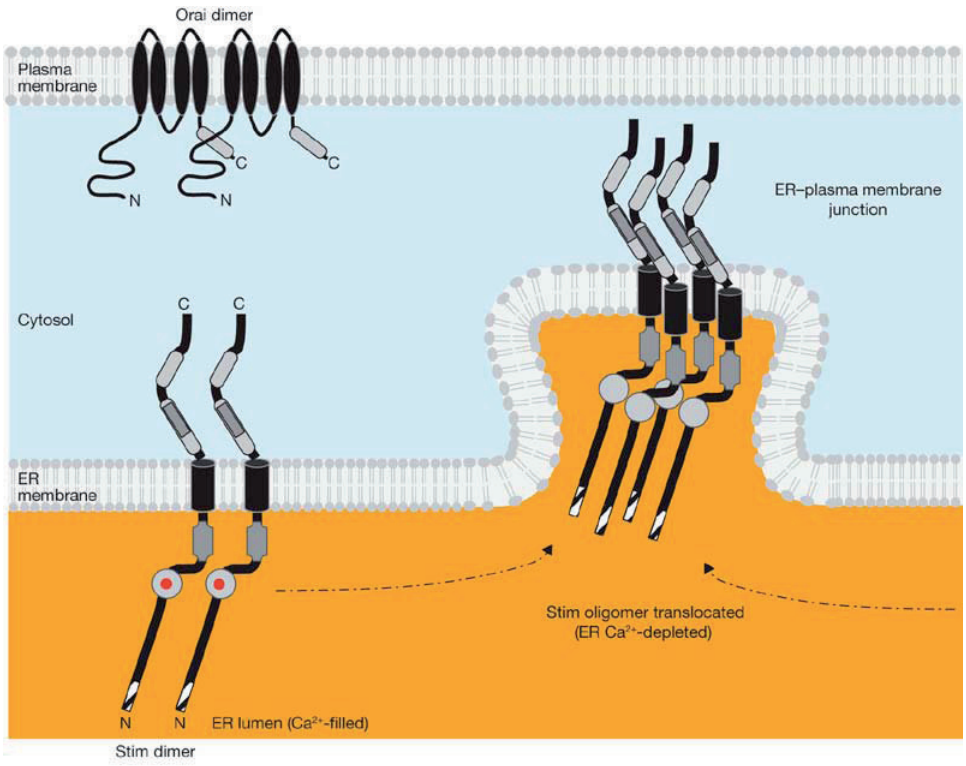


Figure 12:

STIM 1 molecules are shown in the basal state as dimers (left); the Ca^{2+} sensor EF-hand domain has bound Ca^{2+} (red dots) when the ER Ca^{2+} store is filled. Background colours represent basal Ca^{2+} concentrations of ~ 50 nM in the cytosol (blue) and > 400 µM in the ER lumen (brown/yellow). ER Ca^{2+} store depletion causes Ca^{2+} to unbind from the low affinity EF-hand of STIM 1; this is the molecular switch that leads to STIM 1 oligomerization and translocation (dashed arrows) to ER–plasma membrane junctions. Non-conducting Orai channel subunits are shown as dimers. (Adapted and modified from [211])

The function of STIM 1 as an important component of SOCE has been analyzed using high resolution microscopy, RNAi and mutagenesis. It soon became apparent that rendering an EF hand motif, near the N-terminus of STIM 1, which is located in the ER lumen unable to bind calcium by mutagenesis, leads to constitutive calcium influx and CRAC channel activation, independent of ER store depletion [208]. Oligomerization precedes interaction of STIM 1 with and activation of CRAC channels at the plasma membrane [212-215]. Oligomerization of STIM 1, as described above, is necessary and sufficient for its translocation to ER-plasma membrane junctions [215]. STIM 1 molecules were shown to form clusters (puncta) close (10-25 nm) to the plasma membrane shortly after store depletion, whereas they co-localized with ER resident proteins, showing ER distribution if the ER stores were filled [216]. The close proximity of STIM 1 puncta to the plasma membrane allows the direct interaction of STIM 1 with proteins located at or within the plasma membrane. It appears that the protein STIM1 interacts with, Orai, is the subunit from which CRAC channels are build. Work from several laboratories has demonstrated that STIM 1 not only undergoes an interaction with the CRAC channel component Orai and activates CRAC channels but also aids in organizing Orai subunits into plasma membrane clusters, adjacent to the STIM 1 puncta [213, 217, 218] (Figure 13).

Besides the direct activation of calcium influx by STIM 1, additional regulatory mechanisms exist to fine-tune calcium influx and signaling within the cells as well as to guard cells against damage caused by excessive calcium influx. An important calcium buffer within cells are the mitochondria, organelles which have been shown to functionally interact with SOC channels in T lymphocytes [219]. Mitochondria are required to maintain CRAC channel activity by preventing local calcium accumulation around the sites of calcium entry. It has been further shown that mitochondria accumulate at the immunological synapse, a site where strong calcium signals are generated [220]. Consistent with these findings acute blockage of mitochondrial calcium uptake in T cells stimulated via their TCR prevented nuclear translocation of NFAT alongside with CRAC channel activation [219].

Another mechanism of CRAC channel regulation which has been proposed recently is via STIM 2, a molecule similar to STIM 1 that might have arisen by gene duplication alongside STIM 1. STIM 2 is a feedback regulator that stabilizes basal calcium concentrations in the cytoplasm and ER by counteracting STIM 1 [221, 222].

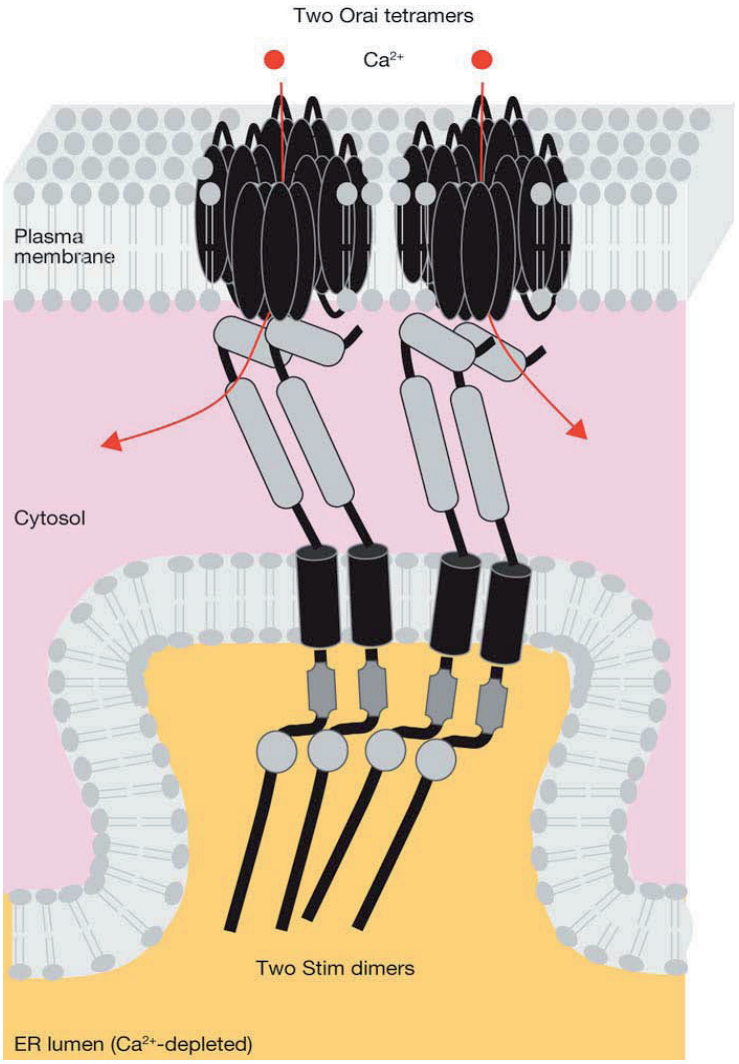


Figure 13:

STIM 1 accumulation induces Orai channels to cluster in the adjacent plasma membrane. The C-terminal effector domain of STIM induces Orai channels to open by direct binding of the distal coiled-coil domain, and Ca^{2+} enters the cell (red arrows). Two STIMs can activate a single CRAC channel consisting of an Orai tetramer; channel activation by STIM may involve a preliminary step of assembling Orai dimers into a functional tetramer. Background colors represent changes in cytosolic Ca^{2+} concentration (from that in the basal state (Ca^{2+} concentrations of ~ 50 nM in the cytosol (blue) and > 400 µM in the ER lumen (brown/yellow) as represented in the previous figure to > 1 µM in the cytosol (now pink) due to Ca^{2+} entry, and ER luminal Ca^{2+} (now lighter yellow) to < 300 µM. (Adapted and modified from [211])

The last molecule implicated in the fast calcium dependent inactivation (CDI) which limits calcium influx into cells via CRAC channels and should be mentioned here is calmodulin. In a recent report, scientists showed that a N-terminal membrane proximal region of Orai interacts with calmodulin. Mutation of the calmodulin interaction site within Orai ablated CDI. Thus a potential explanation for this observation is that upon calcium entry calcium/calmodulin displaces STIM 1 from the N-terminus of Orai and in effect reverses the activation process initiated by STIM 1 [223]. Further work will however be needed to fully understand the mechanisms by which STIM 1, calmodulin, Orai and other molecules act in concert to rapidly induce calcium influx as well as evoke inactivation of the SOC channels.

1.2. The Coronin Family of Proteins

Analysis of actin/myosin complexes isolated from the slime mold *Dictyostelium discoideum* resulted in the identification of a 57 kDa protein. [224]. As antibodies, raised against the newly identified protein stained the plasma membrane surface projections of *Dictyostelium*, which have a crown-shaped appearance, the protein was named "coronin". The deletion of coronin in *Dictyostelium* cells resulted in pleiotropic defects, such as reduced motility and phagocytosis, both of which are actin dependent processes [225-227]. These findings resulted in the classification of coronin as an actin binding protein. Additional experiments, which were carried out using coronin deficient *Dictyostelium* cells as well as *in vitro* actin polymerization assays using purified yeast coronin, prompted scientists to assign coronin as an actin regulating protein. As a consequence of these initial assignations all coronin family members identified thereafter, such as coronins in multicellular invertebrates and mammals have been referred to as actin binding and regulating proteins [228-230].

In the following, our current knowledge in respect to what is known about *Dictyostelium* coronin, *yeast* coronin and coronins from multicellular invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* will be summarized. The remaining part of this chapter will then focus on mammalian coronin 1 (*Coro 1A*), the closest *Dictyostelium* homologue of all known seven mammalian coronin isoforms, which is exclusively expressed in leukocytes.

Recent work, part of which is described in this thesis, based on biochemical, physiological and genetic analysis suggests that coronin 1 has evolved to function, unlike the short *Dictyostelium* coronin, in the regulation of leukocyte specific signaling events, rather than in the regulation of F-actin.

1.2.1. Overview and Molecular Phylogeny:

The coronin gene family comprises seven vertebrate paralogs and at least five unclassified subfamilies in non-vertebrate metazoa, fungi and protozoa. Thus far no representatives in plants or distant protists have been described. All known members exhibit structural conservation in two unique domains of unknown function (DUF1899 and 1900), interspaced by three canonical WD40 domains (plus additional pseudo domains), that form part of a 7-bladed-propeller scaffold and a C-terminal "coiled coil domain" needed for oligomerization.

A

	Species	Protein Name	Synonyme	Gene Name
YEAST Coronin	*Saccharomyces cerevisiae*	Coronin-like protein		CRN1
DICTIOSTELIUM Coronin	*Dictiostelim discoideum*	Coronin	P55	corA
DROSOPHILA Coronin	*Drosophila melanogaster*	LD37992p		coro
Coronin-1	*Mus musculus*	Coronin-1	Coronin-1A Coronin-like protein p57 Coronin like protein A Clipin-A TACO	Coro1a
Coronin-2	*Mus musculus*	Coronin-2	Coronin-1B	Coro1b
Coronin-3	*Mus musculus*	Coronin-3	Coronin-1C	Coro1c
Coronin-4	*Mus musculus*	Coronin-4	Coronin-2A	Coro2a
Coronin-5	*Mus musculus*	Coronin-5	Coronin-2B	Coro2b
Coronin-6	*Mus musculus*	Coronin-6	Coronin-like protein E Clipin-E	Coro6
Coronin-7	*Mus musculus*	Coronin-7	Crn7 70 kDa repeat tumor rejection antigen homologue	Coro7

B

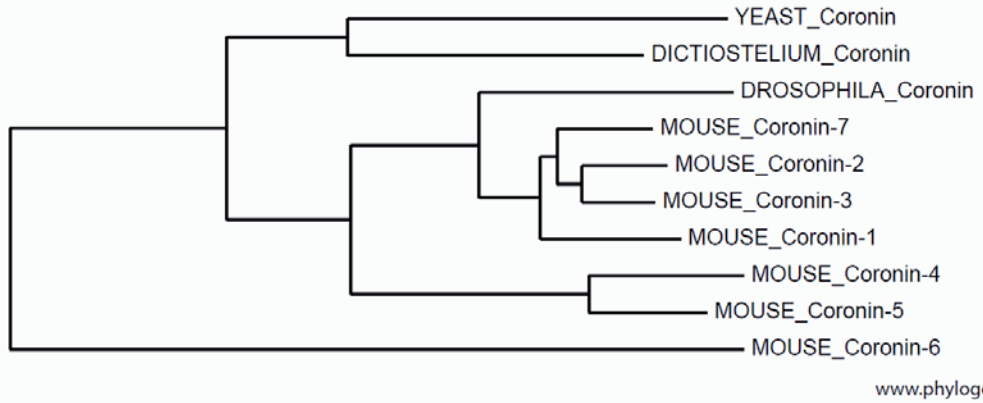


Figure 14:

A) Overview over the seven mouse coronin paralogs as well as the single coronin genes found in *Yeast*, *Dictyostelium* and *Drosophila*. B) Molecular Phylogeny of the coronins listed under (A).

1.2.2. Coronin in Dictyostelium and Yeast

Several years after the identification and characterization of *Dictyostelium* coronin, which by then had been classified as an F-actin binding and regulating protein (see introduction to this chapter), a *yeast* homologue was described [231]. While in *Dictyostelium* two additional coronin like proteins are present besides the short coronin (a coronin 7 homologue and villidin) *yeast* cells only express a single coronin gene.

Despite the *in vitro* observed interactions of *yeast* coronin with the Arp 2/3 complex and inhibitory effects on in vitro F-actin polymerization assays [232, 233], coronin deficient *Yeast* cells, unlike coronin deficient *Dictyostelium* cells (see above), fail to display an obvious phenotype and defects which could be related to F-actin based processes and dynamic [231]. These experimental systems thus do not unequivocally support an *in vivo* function of coronins in F actin regulation. Further work will be needed to define, on a molecular basis, the precise role(s) of coronins in different model organisms.

1.2.3. Coronin in Multicellular Invertebrates

To date only little is known about the function of coronin family members in multicellular invertebrates, such as *Caenorhabditis elegans* and *Drosophila melanogaster*. Both organisms contain a gene (*coro*) with a high homology to *Dictyostelium* coronin. Deletion of the gene in *Drosophila* results in a pleiotropic phenotype which has been attributed to endocytosis / exocytosis events involving F actin-coated vesicles and the establishment of morphogen gradients [234]. As for C. *elegans* coronin, no information is available on its function. Another molecule identified in *C. elegans*, termed Pod-1, shows homology to coronin family members and contains two stretches of WD repeats but unlike other coronins no C-terminal coiled-coil region. It has been reported that Pod-1 may be involved in the embryonic establishment of polarity during the development of *C. elegans* [235]. A Pod-1 homologue in Drosophila has been implicated in neuronal development and axonal growth cone targeting [236].

1.2.4. Mammalian Coronin 1

Mammalian cells can express up to seven coronin isoforms, of which coronin 1 is the closest homologue to *Dictyostelium* coronin (Figure 14) [237-239]. Coronin 1 which will be the major topic during most of this thesis is exclusively expressed in leukocytes. As besides coronin 1 other coronin isoforms are co-expressed in leukocytes but are also found in other cell types, it is reasonable to assume that coronin 1 has evolved to exert a specialized function which is of particular importance in leukocytes.

1.2.5. The Three-Domain structure of Coronin 1

A characteristic feature of all coronins is the central WD (Tryp-Asp) repeat domain linked to a C-terminal coiled-coil region. Coronin 1, the closest mammalian homologue of *Dictyostelium* coronin, is made up of three domains, as outlined in Figure 15 [240, 241]. The first, N-terminal domain, contains the five WD repeats, it is rich in β-sheets and referred to as β-propeller (amino acid 1-355). The second domain, which is referred to as "linker" region has little secondary structure (amino acid 356-429). The third, C-terminal domain is an α-helix rich, coiled-coil containing segment which is needed for oligomerization (amino-acid 430-461).

1.2.5.1. The N-terminal 7-bladed Propeller domain

Biochemical, bioinformatics and structural data obtained for coronin 1 revealed that the WD repeat regions fold into a seven bladed β-propeller with the N- and C-terminal extensions forming the first and the last blade respectively [241, 242]. This structure is reminiscent of the β-propeller fold as it is found in the β-subunits of trimeric G-proteins and the yeast transcription repressor Tup1, both of which are WD repeat containing seven-bladed β-propeller proteins. As this domain mediates protein-protein interactions of G proteins it is possible that it has a similar function in coronin 1 [243-245]. It has been demonstrated recently that coronin 1 interacts with the plasma membrane via its N-terminal domain,

however whether the interaction occurs between coronin 1 and another protein or rather with plasma membrane lipids remains to be determined [241].

1.2.5.2. The C-terminal coiled-coil trimerization motif and the linker domain

Biophysical analysis of the C-terminal coiled-coil domains in coronin 1 revealed that this portion of the molecule folds into a parallel three-stranded coiled coil, mediating trimerization of coronin 1 molecules [241]. Representative electron microscopic pictures, which lead to the current model of coronin 1 oligomerization as well as a cartoon model are shown in Figure 15 and 16. Gatfield et al., were further able to demonstrate that the trimerization of coronin 1 generated a cytoskeleton binding site comprising positively charged residues located within the linker region (amino acids 400-416) [241]. These findings are consistent with previous reports where basic peptides have been shown to be important for the interaction with actin [246, 247]. The assembly of the coiled-coil domain into trimers was further confirmed by X-ray crystallography [248].

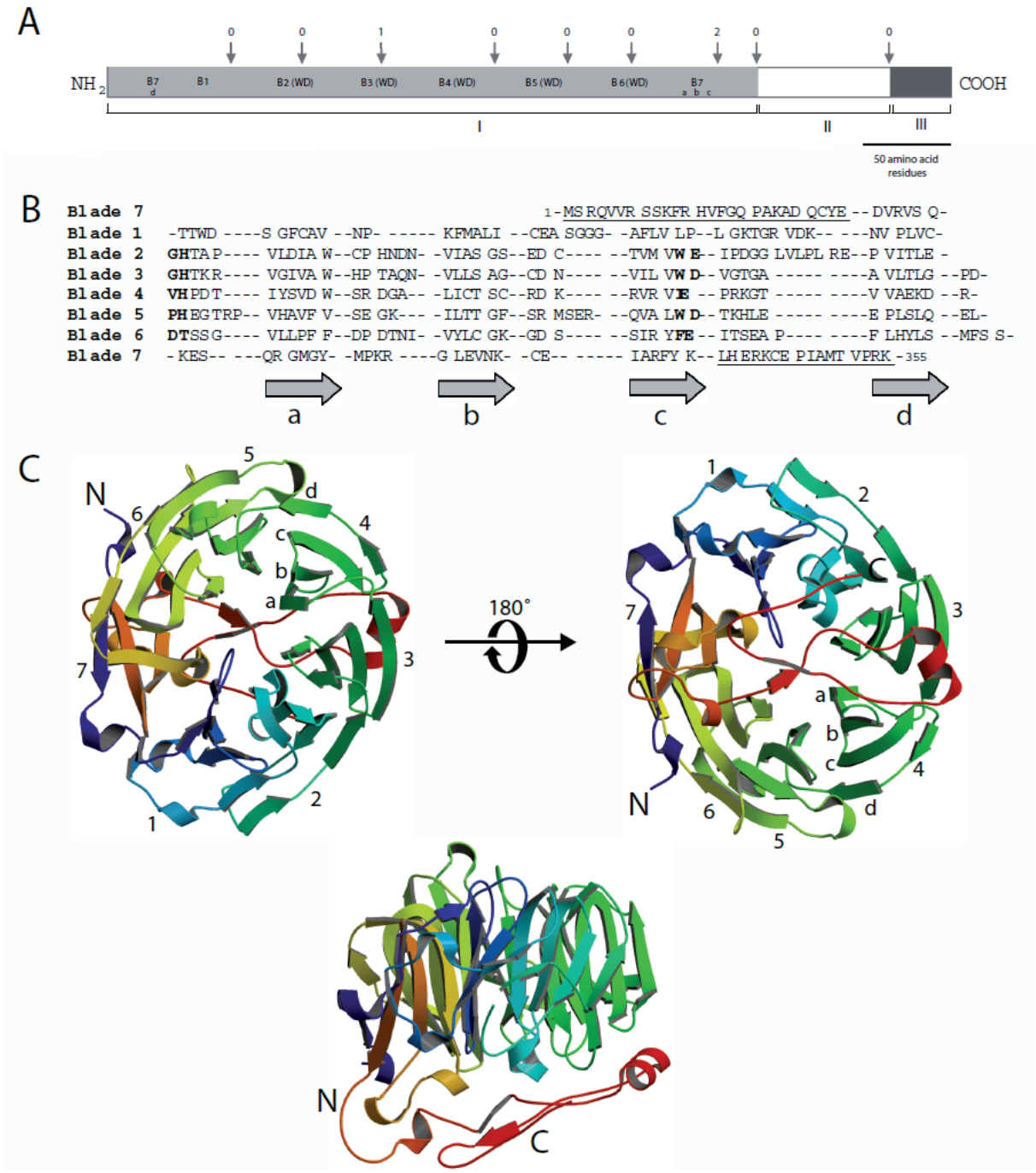


Figure 15:

A) The N-terminal, 7-bladed β-propeller region consists of 5 WD repeats complemented with two stretches of sequence each forming four β-strands, representing two additional blades of the propeller. The propeller region is followed by a linker region. The C-terminal part of coronin 1 is composed of a coiled coil. B) Secondary structure prediction suggests a seven-bladed β-propeller fold reminiscent of the ones of the yeast transcriptional repressor Tup1 and β-subunit of the G protein. The GlyHis and TrpAsp dipeptides of the five WD repeats are highlighted in bold. Predicted blade numbers and corresponding β-strands (gray arrows) are shown on the left and on the bottom of the alignment, respectively. The N-and C-terminal domain extensions are underlined. C) Model of coronin 1 lacking the coiled coil domain based on the X-ray structure [242, 249].

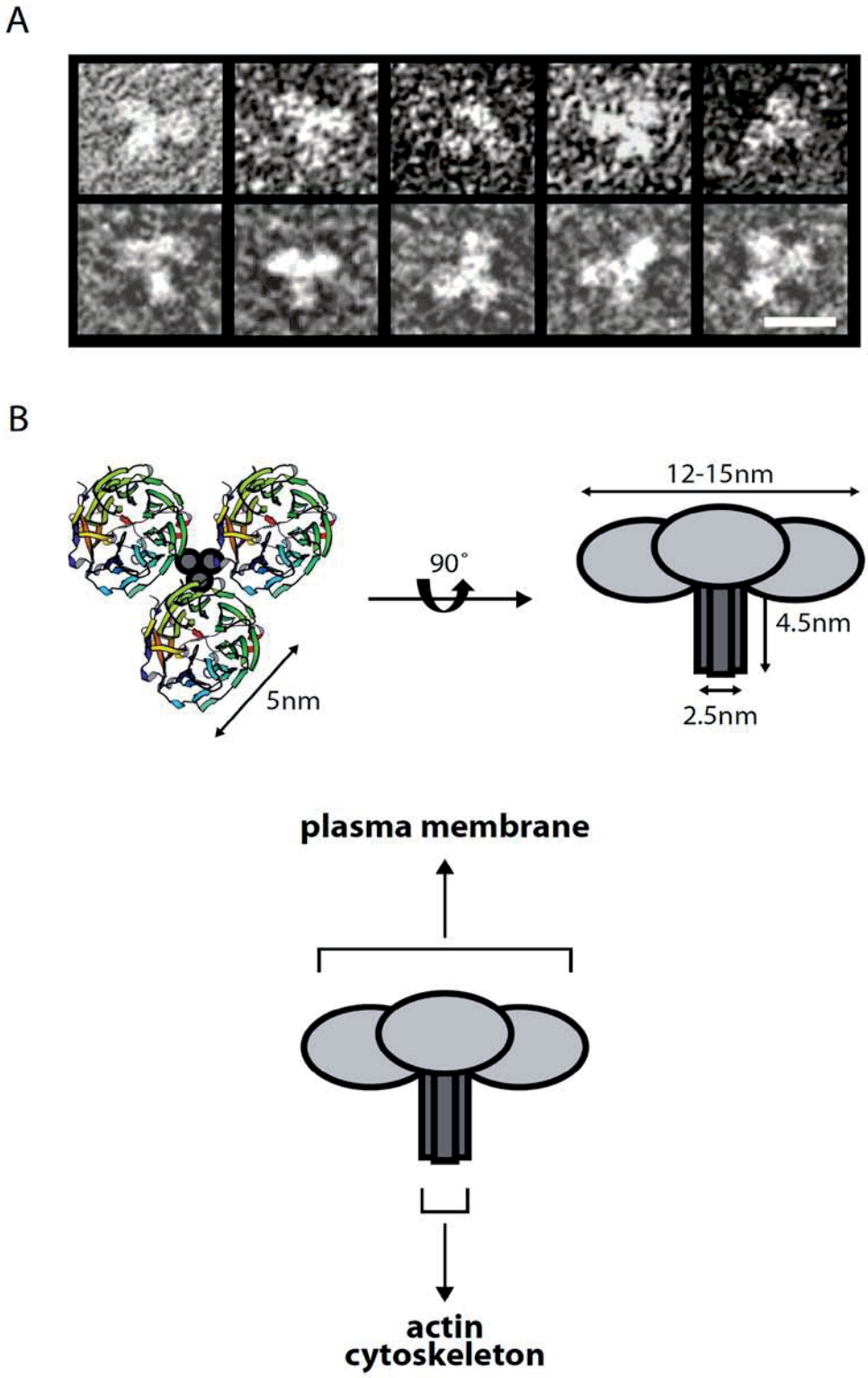


Figure 16:

A) Transmission electron micrographs of affinity-purified and negatively stained coronin 1 complexes isolated from macrophages. The gallery shows multiple examples of the trimeric structure. Scale bar, 10 nm. B) Cartoon presenting the organization of the coronin 1 trimer deduced from the image analysis [249].

1.2.6. Coronin 1 in the innate immune system

A role for coronin 1 in innate immunity was first identified during a screen for host molecules, possibly involved in blocking phagosome-lysosome fusion in macrophages, following an infection with pathogenic mycobacteria. While most bacteria upon internalization into macrophages are rapidly delivered to the bactericidal environment of lysosomes, followed by their destruction, live pathogenic mycobacteria upon internalization, actively block the maturation of their phagosomes to, or fusion with lysosomes. Mycobacteria thereby create their own niche within the otherwise hostile environment of host macrophages.

The screen mentioned above led to the identification of a single detectable protein which was exclusively retained on mycobacterial phagosomes containing live bacteria [237]. The newly identified protein, coronin 1 (at the time known as TACO, for Tryptophane Aspartate containing Coat protein), was shown to distribute equally between cytosol and plasma membrane in resting macrophages. When macrophages were infected with live, pathogenic mycobacteria, coronin 1 was exclusively retained around phagosomes containing live bacteria, whereas in macrophages infected with heat killed mycobacteria, coronin 1 was initially associated with phagosomes but rapidly dissociated after uptake. Based on these results, coronin 1 was suggested to play an essential role in preventing phagosome-lysosome fusion during mycobacterial infection. As at the time when these discoveries were made no coronin 1 deficient mouse models or cell lines were available, the researchers made use of Kupffer cells, the resident macrophages of the liver which were shown to not express coronin 1. Consistent with the above described findings, upon infection of Kupffer cells, mycobacteria were rapidly delivered to lysosomes and degraded [237].

Another pathogen which was shown later to be equally capable of retaining coronin 1 around its phagosomes upon internalization is the human pathogen *Helicobacter pylori* [250]. These findings suggest that the block in phagosome-lysosome fusion mediated by coronin 1 is not only utilized by pathogenic mycobacteria and could be utilized by other, thus far not characterized pathogens.

The mechanisms by which coronin 1, once retained around mycobacterial phagosomes, prevents phagosome-lysosome fusion and mycobacterial survival have remained elusive for a long time. The recent analysis of a coronin 1 deficient mouse model as well as a tissue culture model system of mycobacterial infection (J774 macrophages stably depleted of coronin 1 by

plasmid based expression of coronin 1 specific siRNA) has however shed some light on the molecular activities of coronin 1 in macrophages.

As mentioned earlier, based on the homology to the single coronin isoforms expressed in *Dictyostelium* all thus far identified seven mammalian coronin family members have been classified as F-actin interaction proteins as well as F-actin regulators [228, 229].

However, when coronin 1 deficient macrophages and neutrophils were analyzed, no actin related defects or disturbances of the F-actin cytoskeleton were apparent. All analyzed processes, such as phagocytosis, macropinocytosis, cell spreading, membrane ruffling as well as migration were unaffected by the lack of coronin 1 in macrophages and neutrophils [251, 252]. Instead of regulating F-actin dynamics, coronin 1 was found to be not only retained around mycobacterial phagosomes [237-239, 251] but essential for mycobacteria induced activation of the phosphatase calcineurin. Calcineurin phosphatase activity has been implicated in a large variety of cellular processes covering the range from transcriptional activation to dephosphorylation of proteins involved in endocytic processes and degradation of signalling molecules in order to shut down receptor mediated signalling cascades [253, 254]. The phenotype of coronin 1 deficient macrophages could be mimicked by incubation of wild type cells with the calcineurin blockers cyclosporine A or FK506 which induced lysosomal delivery of the pathogen as observed in the absence of coronin 1. On the other hand, mycobacteria were prevented from being delivered to lysosomes in the absence of coronin 1 by use of the calcium ionophore calcimycin. These results established coronin 1 dependent regulation of calcineurin activation as key event during mycobacterial infections as well as pathogen survival [249, 253]. The above described findings have been summarized in Figure 17 (see below).

A role for coronin 1 in the regulation of mycobacterial trafficking and calcineurin activation fits with a possible role of protein kinase C (PKC) regulated localization of coronin 1 as well as its preferred localization within cholesterol rich membrane regions, which are known to act as signaling platforms [255-261]. Mycobacteria require cholesterol to be efficiently engulfed by macrophages, thus it is reasonable to assume that their uptake in a cholesterol dependent manner has evolved as a mechanism which ensures that once they have been internalized they can reside within coronin 1 coated phagosomes, thus preventing their degradation in lysosomes.

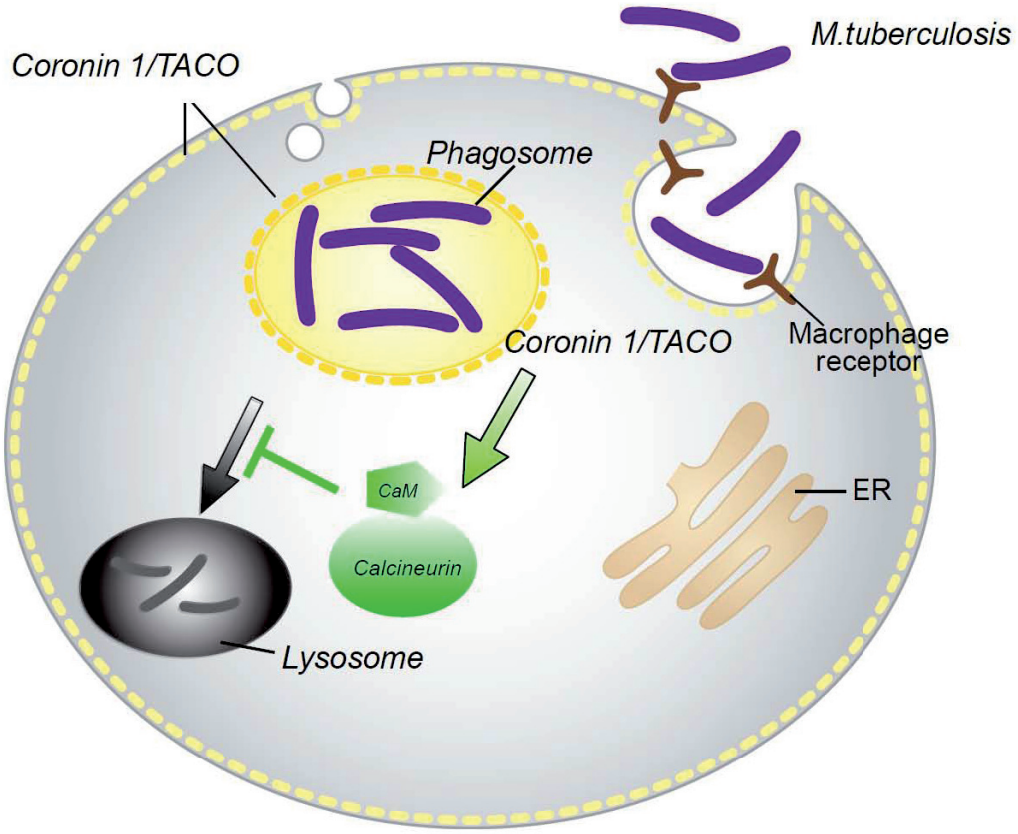


Figure 17:

Model for the activity of coronin 1 in macrophages. In resting macrophages coronin 1 is distributed between the cytoplasm and the cell cortex. Upon the entry of pathogenic mycobacteria, coronin 1 is recruited to and retained at the phagosomal membrane, thereby ensuring the activation of calcineurin. Activation of calcineurin results in a block in the fusion of mycobacterial phagosomes with lysosomes. As a consequence, deletion of coronin 1 or inhibition of calcineurin activity results in the induction of phagosome-lysosome fusion and mycobacterial killing [249].

1.3. Aims of this thesis

1.3.1. Part I

Activation of lymphocytes results in the opening of Ca^{2+} release activated Ca^{2+} (CRAC) channels in the plasma membrane, allowing influx of extracellular Ca^{2+}. CRAC channel activation occurs following depletion of Ca^{2+} stores in the endoplasmic reticulum (ER) [201]. One current model, to explain the coupling of store-emptying to CRAC channel activation, is the secretion-like conformational coupling model [262]. This model proposes that store depletion increases junctions between the endoplasmic reticulum and the plasma membrane, a process which could be regulated by the cortical actin cytoskeleton. It is however unclear to what extend this model, as well as the involvement of the actin cytoskeleton in store operated calcium entry (SOCE), applies to T cells. Thus the aim of the first part of this thesis was to analyzed the involvement of the actin cytoskeleton in CRAC channel activation in Jurkat T cells.

1.3.2. Part II

Coronin 1 is a member of the conserved WD repeat family of coronin proteins that is exclusively expressed in leukocytes. A possible function of coronin 1 in innate immunity was first described following a screen for proteins expressed in macrophages, that are possibly involved in the mycobacterial block of phagosome-lysosome fusion [237]. Macrophages infected with live, pathogenic mycobacteria retained coronin 1 around phagosomes containing live bacteria, whereas in macrophages infected with heat killed mycobacteria, coronin 1 was initially associated with phagosomes but rapidly dissociated from them after uptake. Based on these findings coronin 1 was assigned an essential role in preventing phagosome-lysosome fusion during mycobacterial infection.

However, the normal role of coronin 1 in immune cells has remained unclear. In order to define a molecular role for coronin 1, mice deficient for coronin 1 were generated in the laboratory and backcrossed onto the C57BL/6 background. It was shown that in the coronin 1 deficient animals T cell numbers are significantly reduced.

The aim of the second part of this theses was to build upon these preliminary results and to define a role for coronin 1 in T cell development, proliferation and signaling as well as to elucidate the underlying molecular defect(s) responsible for the observed phenotype.

1.3.3. Part III

The original isolation of the single coronin isoform found in the slime mold *Dictyostelium,* bound to an actin-myosin affinity matrix, has led to the assignment of all coronin protein family members as actin interacting and regulating proteins [228, 229]. Additional i*n vitro* experiments performed with purified yeast coronin suggested that yeast coronin might modulate the formation of F-actin by acting as a regulator of the actin-related protein 2/3 (Arp2/3) complex [233]. However living yeast cells lacking coronin displayed no obvious phenotype [231].

The evidence for F-actin regulation by mammalian coronin isoforms is based to a large extend on *in vitro* polymerization assays which use purified proteins, or on overexpression of coronin domains in a heterologous system. The only *in vivo* evidence linking coronin 1 and modulation of the F-actin cytoskeleton is based on the ~2 fold higher phalloidin fluorescence observed in coronin 1 deficient leukocytes as compared to the wild type control [263]. As a consequence of this observations, the decrease in cell viability as well as migration of naïve T cells derived from coronin 1 deficient mice was attributed to deregulation of the F-actin cytoskeleton.

In contrast to the above mentioned conclusions, the results derived from the research presented in the second part of this thesis suggested that coronin 1 promotes T cell survival by allowing signal transduction downstream of the T cell receptor rather than by modulating the actin cytoskeleton. They further suggest that the migration defect observed in coronin 1 deficient T cells is not a consequence of a non functional F-actin cytoskeleton.

The third and last part of this thesis aims at providing an explanation for this apparent discrepancy and unravel the molecular basis of the observed survival and migration defects, which are specifically observed in coronin 1 deficient naïve T cells.

2. RESULTS

Part I

Mueller P, Quintana A, Griesemer D, Hoth M, Pieters J.

Disruption of the cortical actin cytoskeleton does not affect store operated Ca^{2+} channels in human T cells.

FEBS Letters 2007 Jul 24;581(18):3557-62.

Part II

Mueller P, Massner J, Jayachandran R, Combaluzier B, Albrecht I, Gatfield J, Blum C, Ceredig R, Rodewald HR, Rolink AG, Pieters J.

Regulation of T cell survival through coronin-1-mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering.

Nature Immunology 2008 Apr;9(4):424-31.

Part III

Philipp Mueller and Jean Pieters

Coronin 1 Maintains the Balance between Survival and Apoptosis in Naive T Cells Independent of F-Actin

Submitted (August 2009)

Part I

2.1. Disruption of the cortical actin cytoskeleton does not affect store operated Ca^{2+} channels in human T cells

2.1.1. Abstract

Lymphocyte signaling and activation leads to the influx of extracellular Ca^{2+} via the activation of Ca^{2+} release activated Ca^{2+} (CRAC) channels in the plasma membrane. Activation of CRAC channels occurs following emptying of the endoplasmic reticulum intracellular Ca^{2+} stores. One model to explain the coupling of store-emptying to CRAC activation is the secretion-like conformational coupling model. This model proposes that store depletion increases junctions between the endoplasmic reticulum and the plasma membrane in a manner that could be regulated by the cortical actin cytoskeleton. Here, we show that stabilization or depolymerization of the actin cytoskeleton failed to affect CRAC activation. We therefore conclude that rearrangement of the actin cytoskeleton is dispensable for store-operated Ca^{2+} entry in T-cells.

2.1.2. Introduction

Ca^{2+} is one of the most fundamental signaling messengers in all eukaryotic cells. Cytosolic Ca^{2+} signals are the key to the regulation of a wide variety of physiological events, such as gene transcription, protein folding, apoptosis and exocytosis. Following stimulation of lymphocytes or other non-excitable cells, bi-phasic increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are observed [201, 264] and [265]. The first phase involves activation of phospholipase C by either trimeric G proteins or tyrosine kinase coupled receptors [266]. The active enzyme hydrolyzes phosphatidylinositol bisphosphate, thus generating the second messenger inositol-1,4,5-trisphosphate (IP3), which binds to IP3 receptor molecules within the endoplasmic reticulum (ER) membrane, thereby triggering Ca^{2+} release from ER Ca^{2+} stores [264]. In a second phase, store-emptying triggers a sustained influx of extracellular calcium via the plasma membrane, a process termed store-operated calcium entry (SOC) or capacitive calcium entry [202, 203, 267]. Different models exists as to explain how the emptying of ER stores results in the activation of Ca^{2+} influx via the plasma membrane. A first model postulates a mobile messenger that links store content to plasma membrane channel activation. Such a messenger could be a luminal ER resident protein or a small organic molecule that is released upon ER store depletion, or is generated de novo within the cytosol and/or ER/plasma membrane [268-273]. A second model is based on transport of vesicles to the plasma membrane inserting Ca^{2+} channels into the plasma membrane via fusion following store-emptying [274]. Third, the secretion-like coupling model postulates migration of the endoplasmic reticulum to the plasma membrane in order to locally activate CRAC channels [262, 275]. Coupling of store depletion to the activation of CRAC channels is believed to occur via the stromal interaction molecules (STIM) 1 and 2 by relocalizing within the endoplasmic reticulum to preformed endoplasmic reticulum–plasma membrane contact sites [208, 209, 212, 216, 242, 276].

The secretion-like coupling model is based on results obtained using a variety of F-actin-modulating drugs that suggest the involvement of the cortical actin cytoskeleton in the interaction of ER stores with plasma membrane channels following depletion. For example, studies in a variety of cell types such as smooth muscle cells, platelets, endothelial cells, glioma cells, and pancreatic acinar cells suggest a role for actin in the coupling of store depletion to the activation of store-operated calcium entry (SOC) [262, 277-279]. Induced

polymerization of the cortical actin cytoskeleton by a variety of drugs resulted in the ablation of store-operated calcium entry, suggesting that actin polymerization acts as a physical barrier in preventing the translocation of the endoplasmic reticulum to the plasma membrane [280]. However, in RBL cells, stabilization or disassembly of the actin cytoskeleton fails to affect Ca^{2+} influx via store-operated CRAC channels [281], suggesting that the contribution of the cortical actin cytoskeleton may be cell type dependent. These divergent findings could indicate that coupling to CRAC channels is affected differently by the activity of actin-modulating drugs in different cell types. Alternatively, in some cell types, store-operated calcium entry may predominantly occur through other classes of calcium channels.

A role for the actin cytoskeleton in T-cells has not been described. In this work, we have analyzed the involvement of the actin cytoskeleton in CRAC activation in Jurkat T-cells, and found that modulation of the actin cytoskeleton by either stabilizing or disassembling actin has no significant effect on the activation or function of CRAC channels.

2.1.3. Results

2.1.3.1. Modulation of the T-cell cytoskeleton by actin interfering drugs

To analyze whether in T-cells the cortical actin cytoskeleton can be disassembled by actin-interfering drugs, Jurkat T-cells were left untreated or incubated with Latrunculin B or Cytochalasin D. Whereas both Latrunculin B and Cytochalasin D disrupt F-actin filaments, they do so by different mechanisms. Latrunculin B is a marine toxin isolated from the Red Sea sponge *Latrunculia magnifica*, and inhibits actin polymerization by binding to G-actin [282, 283].

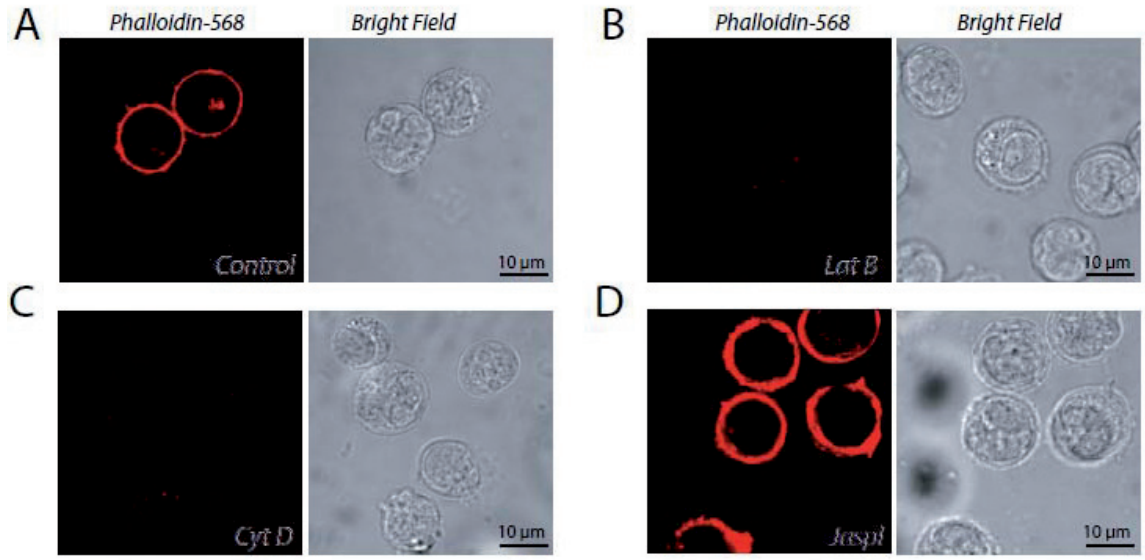


Figure 1: Effect of actin-modulating drugs on the F-actin cytoskeleton.

Jurkat T-cells were left untreated (A) or were treated for 30 min at RT with Latrunculin B (Lat B, 4 µM; B), Cytochalasin D (Cyt D, 10 µM; C) or Jasplakinolide (Jasp, 1 µM; D). Cells were fixed and F-actin was visualized with phalloidin-Alexa Fluor 568. Bar: 10 µM.

Cytochalasin D disrupt the F-actin cytoskeleton by binding to the barbed, fast growing end of actin filaments inhibiting their elongation thereby preventing polymerization [284, 285]. Following treatment, cells were fixed and stained for F-actin using phalloidin coupled to Alexa-568 to visualize F-actin and processed for immunofluorescence microscopy. As shown in Figure 1A–C, while the F-actin cytoskeleton in untreated T-cells was largely cortical, as indicated by the phalloidin staining, following incubation with Latrunculin B and

Cytochalasin D, F-actin was barely detectible. In contrast to the action of Latrunculin B and Cytochalasin D, the fungal peptide Jasplakinolide induces polymerization of F-actin by enhancing the rate of actin filament nucleation [286]. When T-cells were treated for 30 min with Jasplakinolide, followed by fixation and visualization of F-actin by phalloidin, the cortical actin was drastically enhanced compared to non-treated cells (Figure 1A,D).

Induction of F-actin polymerization or depolymerization by these actin-modulating drugs was mirrored using a biochemical assay to separate filamentous actin from G-actin [241, 287]. Cells were left untreated or treated with the drugs indicated in Fig. 2 for 30 min, followed by cell lysis and sedimentation of F-actin. Quantitative recovery of F-actin was achieved by depolymerization using Cytochalasin D followed by SDS–PAGE analysis and immunoblotting. As shown in Figure 2, while in control cells similar amounts of G-versus F-actin were recovered, following Jasplakinolide treatment of the cells virtually all of the actin was recovered from the pelleted fraction, indicating massive polymerization of actin upon Jasplakinolide treatment (Figure 2, *Jasp*). Conversably, when cells were treated with either Latrunculin B or Cytochalasin D, almost all cellular actin was recovered in the soluble fraction, indicating complete depolymerization. Together the morphological and biochemical results suggest that the polymerization and depolymerization of the actin cytoskeleton can be achieved efficiently in human T-cells using these F-actin-modulating drugs.

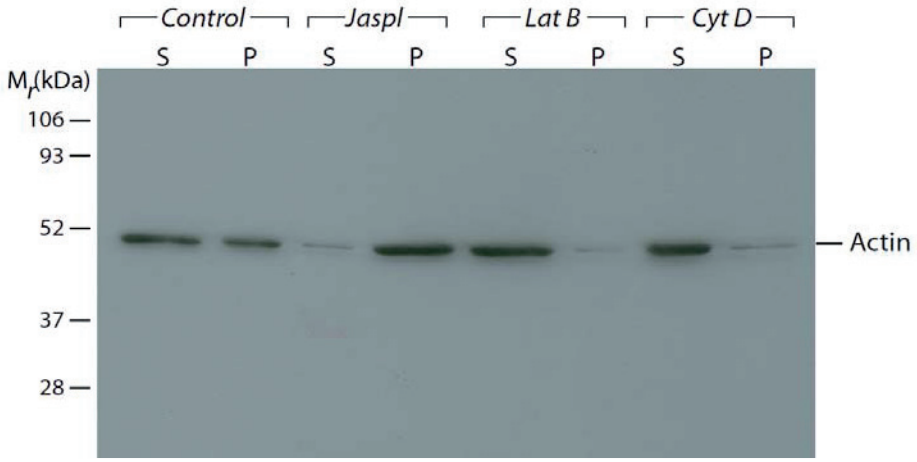


Figure 2: Biochemical analysis of F/G-actin from T-cells treated with actin-modulating drugs.

Jurkat T-cells were left untreated or treated with either Jasplakinolide (1 µM), Latrunculin B (4 µM) or Cytochalasin D (10 µM) for 30 min at RT followed by cell lysis. Proteins present in supernatant (S) and pellet (P) were prepared as described in methods, separated by SDS-PAGE and immunoblotted using anti-actin antibodies. The supernatant resembles the soluble or G-actin whereas the pellet corresponds to the F-actin.

2.1.3.2. Store-operated calcium mobilization following disruption of the actin cytoskeleton

The actin cytoskeleton has been proposed to play an important role in the coupling of the endoplasmic reticulum stores to store-operated Ca^{2+} channels at the plasma membrane. In a variety of cell type, both depolymerization as well as polymerization of F-actin results in the inhibition of store-operated Ca^{2+} influx [262, 279, 280, 288-291].

To analyze whether store-operated Ca^{2+} influx in T-cells is dependent on the actin cytoskeleton, cells were incubated for 30 min. with Jasplakinolide to induce complete F-actin polymerization. Store depletion was initiated by Thapsigargin which induces store depletion by blocking the Ca^{2+} SERCA pumps in the endoplasmic reticulum thereby preventing Ca^{2+} uptake within the ER stores [292]. As shown in Figure 3A, Jasplakinolide incubation did neither alter release of Ca^{2+} from stores by Thapsigargin nor Ca^{2+} influx through CRAC channels as measured by Ca^{2+} re-addition (Figure 3A). The pharmacological modulation of CRAC channels by 2-APB is considered a hallmark of store-operated Ca^{2+} influx through CRAC channels. Whereas 2APB increases CRAC activity at a concentration below 10 μM, it inhibits CRAC activity at higher concentrations [205, 293]. As a control for CRAC channel activity, cells were treated with 3 μM 2-APB after re-addition of Ca^{2+} to elevate Ca^{2+} influx and sequentially with 100 μM 2-APB to completely block Ca^{2+} influx, resulting in the typical bi-phasic behavior of CRAC channel mediated Ca^{2+} entry (Figure 3A).

Similar to the effect of Jasplakinolide, incubation of T-cells with the F-actin depolymerizing drugs Cytochalasin D or Latrunculin B did not significantly modulate Ca^{2+} entry following store depletion (Figure 3B and C). Together these results suggest that polymerization or depolymerization of the actin cytoskeleton do not affect cytosolic Ca^{2+} influx in T-cells.

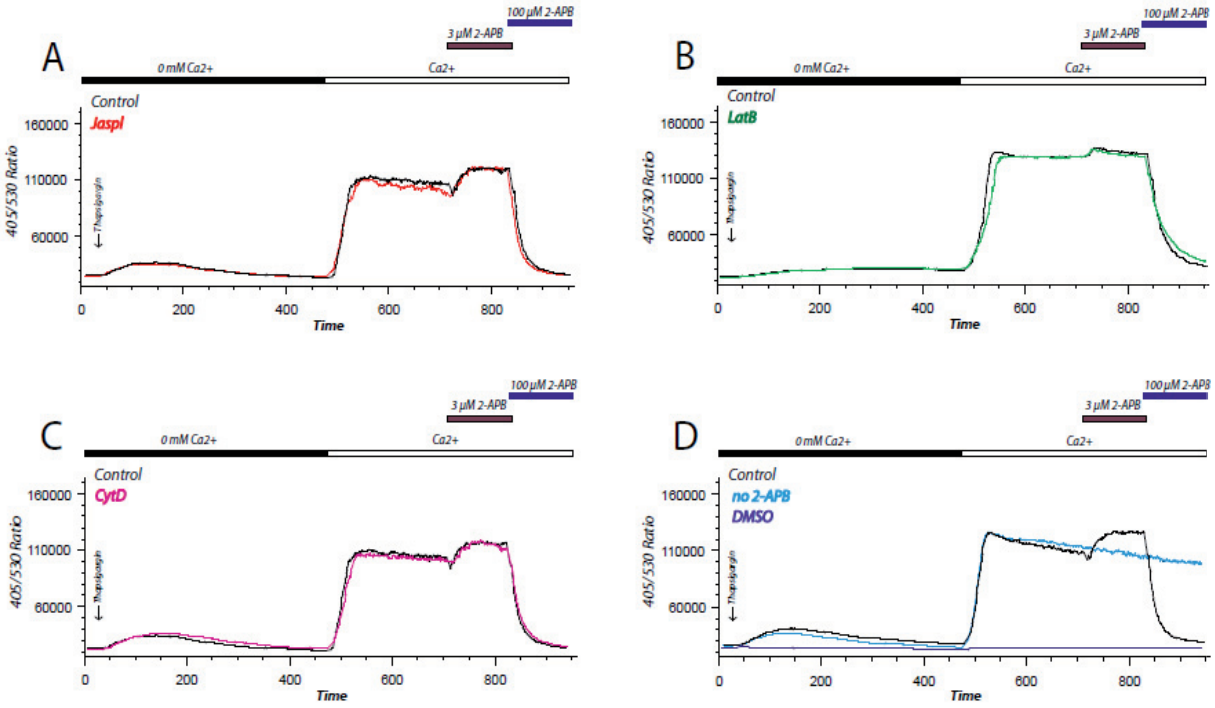


Figure 3: Calcium Fluorimetry in the presence and absence of actin-modulating drugs.

(A–C) Jurkat T-cells were processed as described under methods and calcium influx was analyzed in cells treated for 30 min with the indicated actin-modulating drugs or left untreated as control. Jasplakinolide was used at 1 µM, Latrunculin B at 4 µM and Cytochalasin D at 10 µM. After 8 min calcium was added to the cells, at 12 min 3 µM 2-APB was added to enhance calcium influx and at 14 min 100 µM 2-APB was added to block calcium influx. (D) Additional control samples of untreated cells, as under A–C (Control) or without 2-APB treatment (no 2-APB) or the solvent DMSO used instead of Thapsigargin dissolved in DMSO without 2-APB treatment (DMSO).

2.1.3.3. Patch-clamp analysis in the presence and absence of actin-modulating drugs

To directly analyze the influence of disturbance of the actin cytoskeleton on CRAC channel activity, patch-clamp experiments were performed. As shown in Figure 4, the presence of neither Latrunculin B nor Jasplakinolide significantly influenced the amplitude of CRAC currents in T-cells. We conclude that neither polymerization nor depolymerization of the actin cytoskeleton alter Ca^{2+} influx through CRAC channels in T-cells.

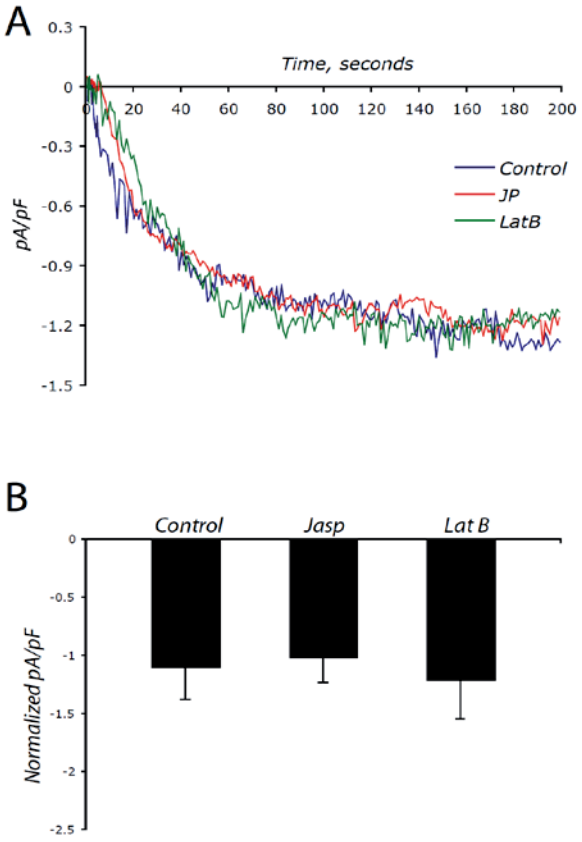


Fig. 4. Effect of actin-modulating drugs on CRAC current.

(A) CRAC current recordings at -80 mV in the presence or absence of Jasplakinolide (1 µM) or Latrunculin B (10 µg/ml) following a 20–30 minutes pre-incubation with the drugs. (B) Average of maximal CRAC currents in control cells ($n = 13$), in latrunculin-treated cells ($n = 8$) or in Jasplakinolide-treated cells ($n = 4$) (Experiments presented in this figure were performed by Quintana A. and Griesemer D.)

2.1.4. Discussion

The processes that regulate the influx of Ca^{2+} following T-cell activation are not fully understood. After T-cell activation, the endoplasmic reticulum Ca^{2+} stores are depleted, which results in the activation of store-operated CRAC/ORAI1 channels at the plasma membrane in a STIM1 dependent manner [202, 205, 208, 217, 276, 294]. While in a variety of cell types, the activation of store-operated Ca^{2+} channels has been suggested to be regulated by the F-actin cytoskeleton, we here describe that in T-cells, as is the case for RBL cells [281], CRAC activation was fully independent of rearrangement of the F-actin cytoskeleton, as analyzed by Ca^{2+} imaging and patch-clamp analysis of cells whose F-actin cytoskeleton was drastically disturbed by actin poisoning reagents.

Evidence for a role of the cortical actin cytoskeleton is supported by experiments in which drugs similar to the ones used here showed a decrease of SOC dependent Ca^{2+} signals in smooth muscle cells, platelets, endothelial cells, glioma cells, and pancreatic acinar cells [280]. How can one reconcile these apparent differences concerning the influence of actin polymerization or depolymerization on SOC channel activity observed between different cells types?

First, it is possible that Ca^{2+} entry channels in the cell types mentioned above are regulated by the actin cytoskeleton; for example, upon growth factor stimulation, members of the transient receptor potential (TRP) calcium influx channels undergo regulated exocytosis to be inserted at the plasma membrane, a step that could well involve the actin cytoskeleton [295, 296]. Also, in platelets and smooth muscle cells, the cortical actin cytoskeleton is tightly linked to the plasma membrane via proteins such as filamin and dystrophin, and polymerization of this cortically located F-actin may prevent any coupling of the ER with plasma membrane Ca^{2+} channels [297, 298]. The precise mode of coupling emptied ER stores to plasma membrane Ca^{2+} channels may thus be distinct in different cell types. For example, it is possible that the subcellular organization of the endoplasmic reticulum in smooth muscle cells and platelets is different from that of RBL and T-cells.

Recent work in T-cells revealed that the majority of contact sites between the ER and the plasma membrane are already preformed in resting cells [216]. Upon Ca^{2+} release, the number of STIM1 junctional contact sites increase significantly, probably since STIM1 proteins within the ER membrane move to those preformed membrane junctions in order to form the

puncta that mediate the activation of CRAC channels. Since formation of STIM puncta beneath plasma membrane mainly depends on STIM recruitment rather than migration of ER membranes towards the plasma membrane, it is plausible that neither depolymerization nor polymerization of cortical actin affects CRAC activity in T-cells, as reported here [217].

An alternative explanation for the observed discrepancies are the methodologies used to analyze Ca^{2+} signaling. While in the study presented here, patch-clamp measurements of CRAC and Ca^{2+} imaging by flow cytometry was used to analyze a potential influence of the actin cytoskeleton on CRAC activity, most of the other studies rely solely on Ca^{2+} imaging. While both techniques have their advantages and disadvantages [299], Ca^{2+} imaging alone does not allow to distinguish effects of the actin cytoskeleton on Ca^{2+} influx channels, membrane potential, Ca^{2+} buffering or Ca^{2+} export mechanisms such as Ca^{2+} ATPases or mitochondrial Ca^{2+} uptake, all of which contribute to Ca^{2+} signals [300]. Interestingly, the conclusion that modulation of the actin cytoskeleton affects store-operated Ca^{2+} entry was solely based on experiments involving Ca^{2+} imaging [262, 277-280, 291]. It therefore can not be exclude that the target of the actin cytoskeleton was not the SOC channel itself but rather for example a K^+ channel, since inhibition of a K^+ channel would depolarize the membrane potential thereby resulting in a reduced Ca^{2+} entry through SOC channels. Harper and Sage have in fact recently shown that in platelets, which have been used as a prominent model system to analyze the influence of the actin cytoskeleton on store-operated Ca^{2+} entry, the Na^+–Ca^{2+} exchanger but not SOC was modulated by the actin cytoskeleton. In T-cells, Na^+–Ca^{2+} exchange appears not to be of functional importance [301, 302], and therefore our conclusion that the cortical actin cytoskeleton does not affect store-operated Ca^{2+} channels in T-cells is consistent with these data.

While our results exclude a role for the F-actin cytoskeleton in CRAC activation in T-cells, the actin cytoskeleton does play a role at other levels of the signaling cascade leading to T-cell activation. In T-cells deficient for the Wiskott–Aldrich syndrome protein (WASp) or the WASP interacting protein WIP, both of which are involved in the regulation of the actin cytoskeleton, T-cell activation is deficient but at a step that lies upstream of store depletion [303-305]. Similarly, T-cells deficient in Vav, a guanine–nucleotide exchange factor for the GTPase Rac which modulates the actin cytoskeleton, cannot properly be activated because of pleiotropic defects related to T-cell receptor clustering [306]. Interestingly, in cells in which expression of the WASP-family verprolin (WAVE)-homologous protein is knocked down,

signaling events immediately downstream of the T-cell receptor are defective [307, 308]; in addition, the absence of WAVE inhibits store-operated Ca^{2+} entry [308]. It should be noted, however, that in natural killer cells the WASp protein regulates the nuclear translocation of transcription factors, which occurs downstream of store-operated Ca^{2+} influx, independently of its effect on the actin cytoskeleton [309].

The process that couples depletion of intracellular stores to plasma membrane Ca^{2+} channels is crucial for the proper functioning of T-cells since a defect in this pathway would render T-cells unresponsive towards immunological stimuli. However, the precise pathways leading to activation of plasma membrane Ca^{2+} channels are far from being defined. Whereas in certain cell types, the cortical actin cytoskeleton may play a role in this activation pathway, our work reported here shows that in T-cells actin rearrangement is dispensable for coupling the intracellular store release to plasma membrane CRAC channels.

2.1.5. Acknowledgement

The research described in this paper was supported by Grants from the Swiss National Science Foundation, the Olga Mayenfisch Stiftung and the Swiss Life Jubileum Fund (to J.P.) as well as the Deutsche Forschungsgemeinschaft (to M.H.).

Part II

2.2. Regulation of T cell survival through coronin-1–mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering

2.2.1. Abstract

T cell homeostasis is essential for the functioning of the vertebrate immune system, but the intracellular signals required for T cell homeostasis are largely unknown. We here report that the WD-repeat protein family member coronin-1, encoded by the gene *Coro1a*, is essential in the mouse for T cell survival through its promotion of Ca^{2+} mobilization from intracellular stores. Upon T cell receptor triggering, coronin-1 was essential for the generation of inositol-1,4,5-trisphosphate from phosphatidylinositol-4,5-bisphosphate. The absence of coronin-1, although it did not affect T cell development, resulted in a profound defect in Ca^{2+} mobilization, interleukin-2 production, T cell proliferation and T cell survival. We conclude that coronin-1, through activation of Ca^{2+} release from intracellular stores, is an essential regulator of peripheral lymphocyte survival.

2.2.2. Introduction

Maintaining T cell homeostasis is central to the ability of vertebrate organisms to mount an effective immune response. Lymphocyte precursors, originating from the bone marrow, home to the thymus, where positive and negative selection result in the production of $CD4^+$ or $CD8^+$ single-positive T lymphocytes [86]. From the thymus, single-positive T lymphocytes seed the peripheral organs, where they circulate for prolonged times between the secondary lymphoid organs and the blood in a naive state [310].

The pool of T cells that are present in the periphery consists of naive T cells as well as effector-memory T cells, which each seem to occupy separate homeostatic niches [170, 311]. The peripheral T cell population is maintained at near-constant numbers; maintenance of naive T cells in the periphery is dependent on the appropriate stimulation of T cells through their T cell receptors (TCRs). Indeed, in hosts that lack peripheral major histocompatibility complex (MHC) class I or class II expression, T cells are unable to survive because of the absence of TCR-mediated T cell stimulation [175, 312]. Also, induction of TCR deletion results in the disappearance of peripheral T cells [172, 173], showing that T cells need input signals through their TCR to support their survival in the periphery [311]. However, the molecular requirements for T cell activation and survival in vivo remain poorly understood.

In mice lacking coronin-1, a leukocyte-specific molecule that is associated with the lymphocyte cortex [241], T cells are deleted from the periphery. This process has been suggested to be linked to prevention of F-actin mediated apoptosis by coronin-1, as judged by the observed increase in phalloidin staining in coronin-1 deficient cells [263]. However, the increased phalloidin signal upon coronin-1 deletion does not reflect altered F-actin abundance (P.M. and J.P., unpublished observations). Accordingly, in macrophages, coronin-1 was found to be fully dispensable for F-actin dynamics [238, 251].

In this work we report that in T cells, instead of regulating F-actin dynamics, coronin-1 interacts with phospholipase C-γ1 (PLC-γ1) and is essential for the generation of inositol-1,4,5-trisphosphate ($InsP_3$) upon TCR stimulation, thereby mediating Ca^{2+} release from intracellular stores that is essential for T cell activation and survival [201, 264]. The function of coronin-1 as a specific survival factor for peripheral T cells may allow a better understanding of T cell homeostasis as well as provide a basis for rational approaches for the development of compounds to treat autoimmune as well as lymphoproliferative disorders.

2.2.3. Results

2.2.3.1. Coronin-1 and F-actin in T cells

In mice lacking the coronin-1 gene *Coro1a* ('coronin-1 deficient mice'), which encodes a leukocyte-specific member of the WD-repeat protein family of coronin molecules, naive T cells are deleted from the periphery ([263] and Supplementary Figs. 1, 2, 3, 4 see below). On the basis of an increase in phalloidin fluorescence in the absence of coronin-1, coronin-1 was suggested to prevent F-actin induced apoptosis [263]; however, other studies showed that in macrophages lacking coronin-1, owing either to siRNA-mediated knockdown of Coro1a or to its deletion [238, 251], all measurable F-actin mediated processes occur normally.

Consistent with the latter results, we found no correlation between the elevated phalloidin staining and F-actin amounts, as analyzed using a sensitive biochemical assay as well as apoptosis as judged by Annexin V staining (P.M. and J.P., unpublished observations). We further evaluated a role for coronin-1 in F-actin distribution and dynamics in T cells by using wild-type ($Coro1a^{+/+}$) or coronin-1 deficient ($Coro1a^{-/-}$) T cells that were seeded onto microscope slides, fixed and stained for coronin-1 and actin, using either an actin monoclonal antibody or phalloidin, followed by confocal laser scanning microscopy. Although in cells lacking coronin-1 the phalloidin fluorescence intensity was increased ~ 2-fold, as anticipated ([263] and P.M. and J.P., unpublished observations), the actin distribution as well as the intensity of cortical actin labeling was identical in wild-type and coronin-1 deficient T cells (Fig. 1a,b). Similarly, analysis of F-actin:G-actin ratio using a biochemical assay [287] showed an identical F:G actin ratio in wild-type and coronin-1 deficient T cells (Fig. 1c). The mean values ± s.d. are listed in the legend to Figure 1.

To further define a possible influence of coronin-1 on actin dynamics, we analyzed TCR-mediated cell spreading on anti-CD3 and anti-CD28 coated microscope slides to define defects in the T cell actin cytoskeleton [308, 313]. The results showed qualitatively identical spreading in wild-type as well as in coronin-1 deficient cells (Fig. 1d). Finally, live cell imaging was used to record the activity of wild-type or coronin-1 deficient T cells seeded onto anti-CD3 plus anti-CD28 coated coverslips; the dynamics of the two types of T cells was similar (Fig. 1e,f and Supplementary Videos 1, 2, 3, 4 online). Together, these data indicated that deletion of the gene encoding coronin-1 did not affect F-actin dynamics in T cells.

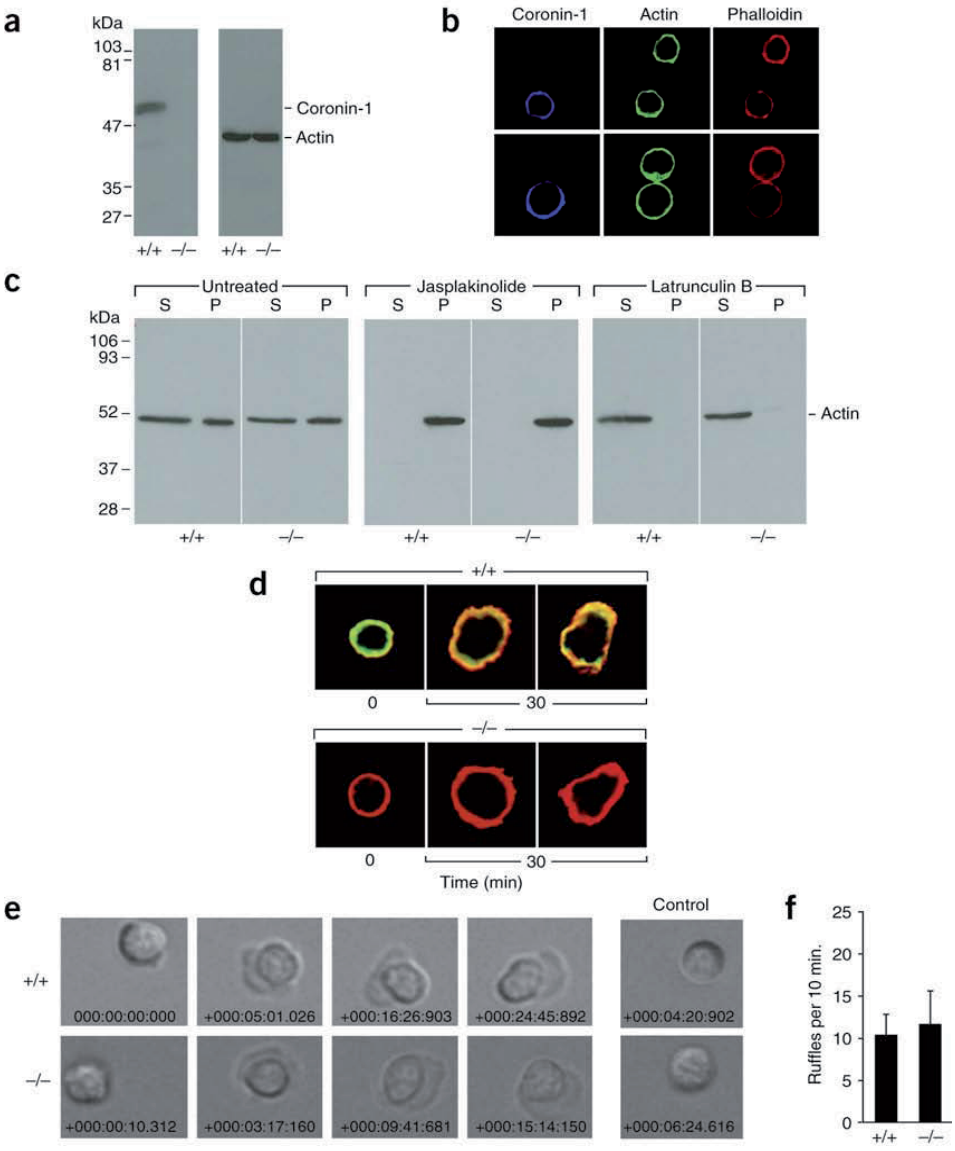


Figure 1: T cell cytoskeleton and F-actin dynamics in the presence and absence of coronin-1.

(a) Immunoblot after separation by SDS-PAGE of total proteins from wild-type (+/+) or coronin-1 deficient (-/-) T cells for coronin-1 (left panel) or actin (right panel). (b) Immunofluorescence microscopy of wild-type (bottom) and coronin-1 deficient (top) T cells on poly-L-lysine-coated slides stained with phalloidin-Alexa Fluor 568 and anti-actin and anti-coronin-1 followed by Alexa Fluor 488-and Alexa Fluor 633-labeled secondary antibodies, respectively. Two representative fields are shown. The ratio of the fluorescence values between the 488 (anti-actin) and 568 (phalloidin) fluorophores was 1.24 for wild-type cells and 0.59 for coronin-1 deficient cells (n = 20 cells). (c) Immunoblot with anti-actin on supernatant (S) and pellet (P) fractions of wild-type or coronin-1 deficient cells left untreated or treated with either jasplakinolide or latrunculin B. Quantitation of F-actin:G-actin ratio: 0.95 ±0.17 in untreated +/+ and 1.00 ±0.18 in untreated -/- cells (n = 4). (d) Immunofluorescence microscopy of T cells left to adhere to anti-CD3 plus anti-CD28 coated or control slides. After fixation, cells were stained with phalloidin Alexa Fluor 568 (red) and antibodies to coronin-1 (green), followed by Alexa Fluor 488 labeled secondary antibodies. (e,f) Video microscopy of wild-type or coronin-1 deficient T cells seeded on either anti-CD3 plus anti-CD28 coated or control slides (e). Quantitation of membrane ruffling in T cells from two independent movies (f). Values were normalized to ruffling over a period of 10 min. Shown are mean values (± s.d.; +/+, n = 17 cells; -/-, n = 23 cells). Data are representative of three independent experiments (Experiments presented in part b, e and f of this figure were performed together with Jayachandran R.).

2.2.3.2. T cell signaling and proliferation in the absence of coronin-1

Because peripheral T cells are known to require a TCR-mediated signal in order to maintain their survival in peripheral lymphoid organs in vivo [173, 314, 315], we analyzed whether coronin-1 performs a role in signaling downstream of the TCR. To analyze TCR stimulation, we examined the capacity of wild-type or coronin-1 deficient cells to respond to allogeneic MHC stimuli. In contrast to wild-type T cells, which proliferated strongly upon allogeneic stimulation, the proliferation of coronin-1 deficient T cells was markedly reduced (Fig. 2a).

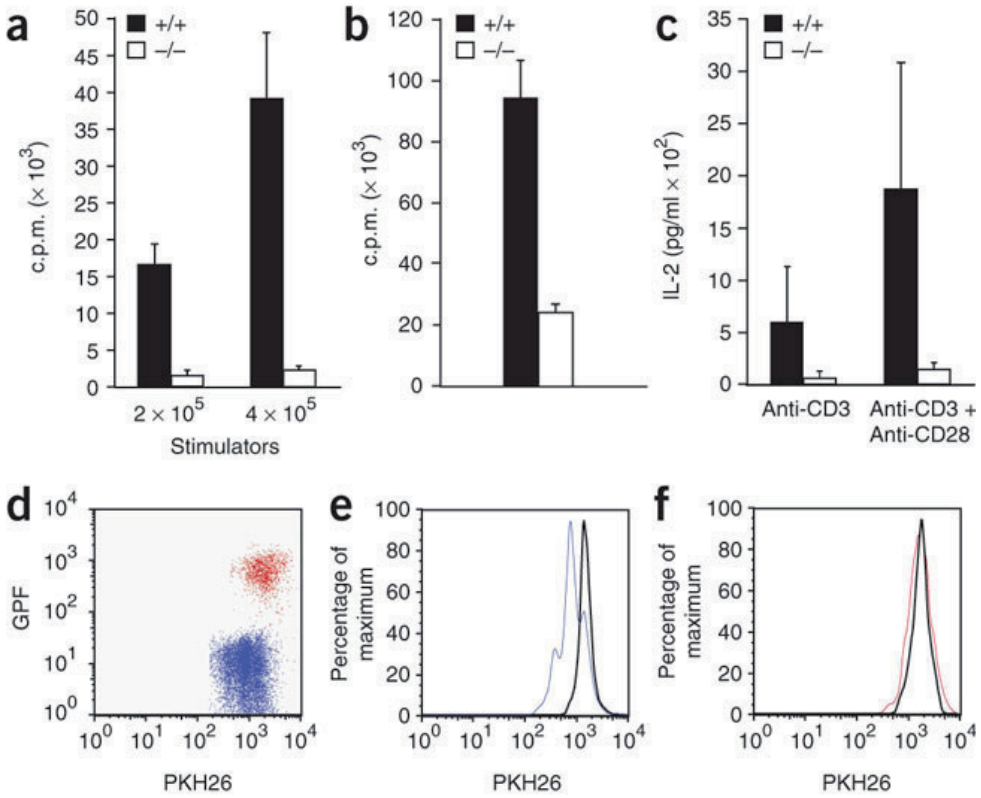


Figure 2: T cell proliferation and cycling in the presence and absence of coronin-1.

(a) Proliferation assay of purified splenic T lymphocytes (1 x105) cultured with the indicated number of mitomycin C treated BALB/c spleen cells for 3 d, followed by measurement of [3H]Thymidine incorporation. (b) Proliferation assay of naive T cells ($CD4^+CD62L^{hi}$, 0.5 x105) from wild-type (+/+) or coronin-1 deficient mice (-/-) stimulated with anti-CD3 (1 µg/ml) on irradiated syngeneic spleen cells (2.5 x105). (c) ELISA for IL-2 production by wild-type or coronin-1 deficient cells after the stimulation indicated below. (d-f) Flow cytometry of total $CD4^+$ T cells (0.5 x105) labeled with the fluorescent cell linker compound PKH26 and stimulated with 0.1 µg/ml of anti-CD3 on 2.5 x105 mitomycin C treated syngeneic spleen cells for 36 h; proliferation of $CD4^+$ T cells was monitored by dilution of the PKH26 dye: dot plot of wild-type cells (blue) and coronin-1 deficient T cells (red) (d); histograms of wild-type (e) or coronin-1 deficient (f) T cells with (blue, red) or without (black) anti-CD3 treatment. Data are representative of three independent experiments. The mean value of triplicate cultures ±s.d. is shown for a-c (Experiments presented in part a of this figure were performed by Massner J.).

Similarly, whereas anti-CD3 stimulation resulted in substantial proliferation and interleukin-2 (IL-2) production, proliferation and release of IL-2 was severely depressed in coronin-1 deficient cells (Fig. 2b,c). Finally, we analyzed TCR-induced cell cycling in T cells; upon incubation of wild-type or coronin-1 deficient naive $CD4^+$ T cells with anti-CD3, wild-type cells readily underwent cell cycle progression (Fig. 2d,e), whereas in the absence of coronin-1, T cells failed to enter the cell cycle (Fig. 2d,f). Together these data suggested that coronin-1 is essential for TCR-mediated proliferation and cell cycle progression of T cells.

2.2.3.3. Coronin-1 and TCR-induced $InsP_3$ generation

To investigate the molecular defect underlying the failure of T cells to proliferate in the absence of coronin-1, we analyzed signaling processes in naive wild-type and coronin-1 deficient T cells (Fig. 3). TCR triggering results in the clustering of TCR molecules, which precedes the activation of adaptor molecules through tyrosine phosphorylation, resulting in the activation of PLC-γ1[316].

We found no difference in clustering of TCRs or tyrosine phosphorylation in wild-type and coronin-1 deficient T cells stimulated with anti-CD3 plus anti-CD28 (Fig. 3a,b). Furthermore, analysis of the phosphorylation as well as localization of the adaptor proteins SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) and LAT (linker for activation of T cells [317]) showed similar phosphorylation and localization upon TCR triggering (Fig. 3c,d,g). TCR triggering of both wild-type and coronin-1 deficient cells resulted in a similar PLC-γ1 phosphorylation and cellular localization (Fig. 3e,g), although in coronin-1 deficient cells PLC-γ1 dephosphorylation occurred slightly faster (Supplementary Fig. 5, see below). Finally, we evaluated recruitment of PKCq the Ca^{2+}-independent protein kinase C family member that integrates TCR-CD28 costimulatory signals, which are essential for productive T-cell activation [118] to the interface between wild-type and coronin-1-deficient T cells stimulated with anti-CD3 and anti-CD28 coated beads; recruitment of PKCq was indistinguishable in both cell types (Fig. 3h,i).

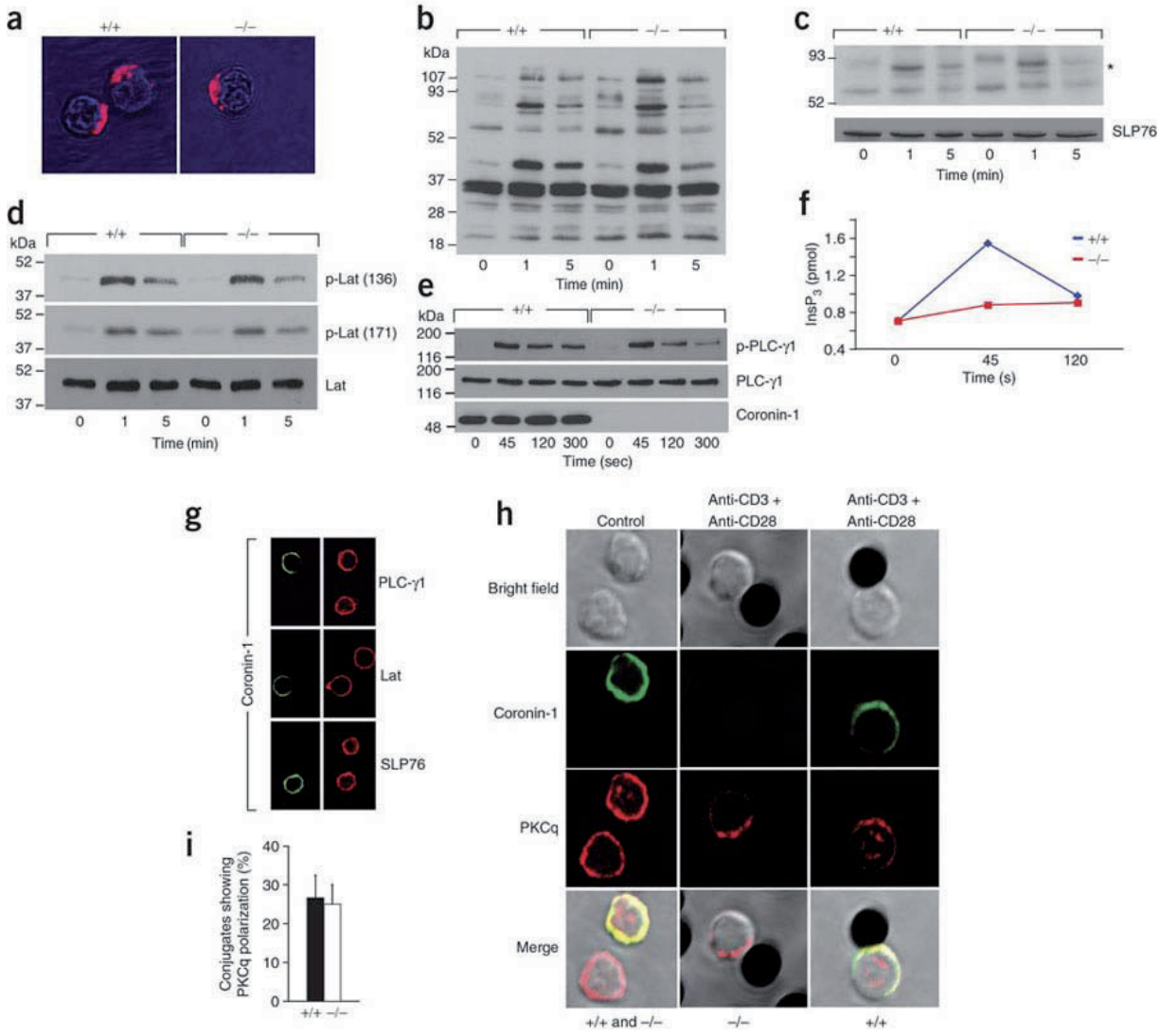


Figure 3: T cell signaling, $InsP_3$ generation and localization of signaling molecules.

(a) Fluorescence microscopy of wild-type (+/+) or coronin-1 deficient (-/-) T cells stimulated with anti-CD3, anti-CD28 and goat anti-hamster Alexa Fluor 568. (b,c) Immunoblot of lysates from wild-type or coronin-1 deficient naive T cells stimulated with anti-CD3, anti-CD28 and mouse anti-hamster for the times indicated. Proteins were separated by SDS-PAGE (12.5%) and immunoblotted with anti-phosphotyrosine (b) or immunoblotted with anti-phosphotyrosine and then reprobed with anti-SLP-76 (asterisk). (d,e) Immunoblots as in b immunoblotted for either phospho- (p)-Lat and total Lat (d) or p-PLC-γ1, total PLC-γ1 and coronin-1 (e). (f) Competitive radio-receptor assay for $InsP_3$ amounts in cells prepared and treated as under b. (g) Fluorescence microscopy of wild-type and coronin-1 deficient naive T cells mixed in a 1:1 ratio and then adhered to anti-CD3 plus anti-CD28 coated slides for 30 min, fixed and stained with either anti-PLC-γ1 (red, top right), anti-SLP-76 (red, bottom right) or anti-Lat (red, middle right) and rat anti-coronin-1 (green, left panels). (h,i) Fluorescence microscopy of wild-type (right column), coronin-1 deficient (middle column) or mixed (left column) splenic T cells incubated with (middle and right columns) or without (left column) beads coated with anti-CD3 plus anti-CD28, and then stained with anti-coronin-1 (green) and anti-PKCq (red). Top, bright field; bottom, merged (red plus green plus bright field). (i) Graph of the percentage of polarized conjugates containing PKCq. (Mean values ±s.d. from three independent experiments are shown.) Data are representative of at least three independent experiments (Experiments presented in part g and i of this figure were performed together with Jayachandran R.).

Activation of PLC-γ1 leads to the generation of $InsP_3$ and diacylglycerol from phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$). $InsP_3$ binds to $InsP_3$ receptors ($InsP_3Rs$) and triggers Ca^{2+} release from intracellular stores [201, 264]. Triggering of the TCRs of coronin-1 deficient T cells resulted in a markedly reduced $InsP_3$ generation compared to that in wild-type T cells (Fig. 3f).

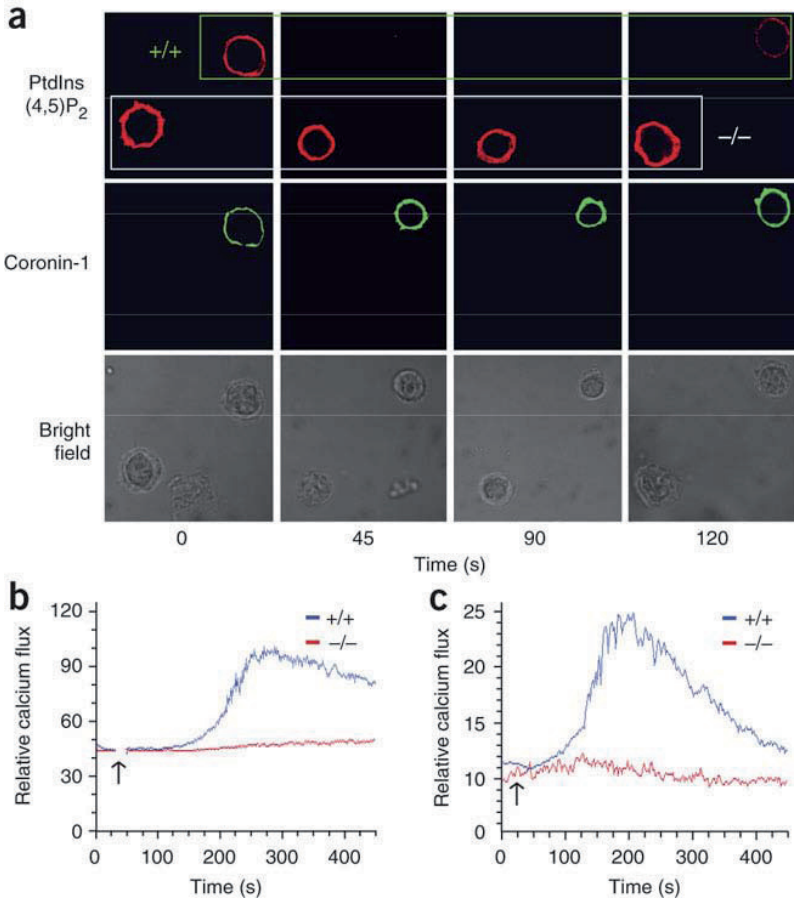


Figure 4: $PtdIns(4,5)P_2$ localization and Ca^{2+} mobilization in wild-type and coronin-1 deficient T cells.

(a) Fluorescence microscopy of wild-type (+/+) or coronin-1 deficient (-/-) naive T cells mixed in a 1:1 ratio and placed on anti-CD3 , anti-CD28 coated slides for the indicated time periods; cells were then incubated with anti-$PtdIns(4,5)P_2$ and rat anti-coronin-1 followed by Alexa Fluor conjugated secondary antibodies (red, $PtdIns(4,5)P_2$; green, coronin-1). (b) Calcium mobilization upon anti-CD3 plus anti-CD28 stimulation of splenic wild-type or coronin-1 deficient T cells loaded with Indo-1. Arrow, time of addition of stimulus. (c) Calcium mobilization upon anti-CD3 plus anti-CD28 stimulation in calcium-free Ringer's solution in splenic wild-type or coronin-1 deficient T cells loaded with Fluo-3. The arrow indicates the time of addition of the stimulus; added after 30 s in the absence of calcium. For details, see Methods. Data are representative of at least three independent experiments (Experiments presented in part a of this figure were performed together with Jayachandran R.).

As visualized using confocal microscopy, in wild-type cells, all PtdIns(4,5)P_2 disappeared within 45 s after TCR triggering (Fig. 4a, top row, green box). Consistent with defective $InsP_3$ generation in the absence of coronin-1, PtdIns(4,5)P_2 amounts remained unaltered when the TCRs of coronin-1 deficient T cells were stimulated (Fig. 4a, top row, white box).

In accordance with defective $InsP_3$ production, no Ca^{2+} flux was generated in coronin-1 deficient T cells after TCR-mediated activation (Fig. 4b), despite normal store content as analyzed using the Ca^{2+} ATPase blocker thapsigargin [292] (data not shown). TCR stimulation in the absence of extracellular Ca^{2+} resulted in normal Ca^{2+} release in wild-type T cells, whereas no Ca^{2+} was released in coronin-1 deficient T cells (Fig. 4c), which is consistent with the lack of $InsP_3$ generation in the absence of coronin-1. In order to assure the viability of the T cells used in this assay, we stained a parallel sample with Annexin V. Less than 5% of the gated cells stained positive (data not shown).

2.2.3.4. Coronin-1 and thymocyte signaling

The observed normal T cell development in the absence of coronin-1 suggested that coronin-1 was crucial for TCR-mediated signaling in peripheral T cells (Figs. 3 and 4) but dispensable for thymocyte development (Supplementary Figs. 2, 3, 4 see below). To understand the relative contributions of coronin-1 to peripheral T cell signaling and thymocyte development, we evaluated coronin-1 expression and TCR-mediated signaling in thymocytes from wild-type and coronin-1 deficient mice. Coronin-1 expression was strongly upregulated in single-positive cells, whereas double-positive cells expressed only small amounts of coronin-1 (Fig. 5a,b and [318]). Double-positive thymocytes showed little Ca^{2+} mobilization after TCR triggering, and this Ca^{2+} mobilization was independent of coronin-1, as no significant differences were observed between wild-type and coronin-1 deficient double-positive thymocytes (Fig. 5c). Single-positive thymocytes from wild-type mice reacted toward TCR triggering with a strong Ca^{2+} mobilization, as expected [319] (Fig. 5d, blue lines). In contrast to that in double-positive thymocytes, calcium mobilization was greatly depressed in coronin-1 deficient single positive thymocytes.

We concluded from these data that, for thymocytes, generation of coronin-1 independent Ca^{2+} fluxes are sufficient to result in proper T cell development; whether or not such Ca^{2+} signaling depends on the expression of other coronin isoforms in the thymocyte subsets, however, remains unknown (Supplementary Fig. 6 and Methods, see below) [320]. Notably, whereas in single-positive thymocytes and naive T cells, coronin-1 was essential for TCR-mediated Ca^{2+} mobilization, in both wild-type as well as coronin-1 deficient memory-effector T cells, TCR triggering did not induce Ca^{2+} fluxes (Supplementary Fig. 7, see below), which is consistent with the normal survival of memory-effector T cells in the absence of coronin-1 [321-324].

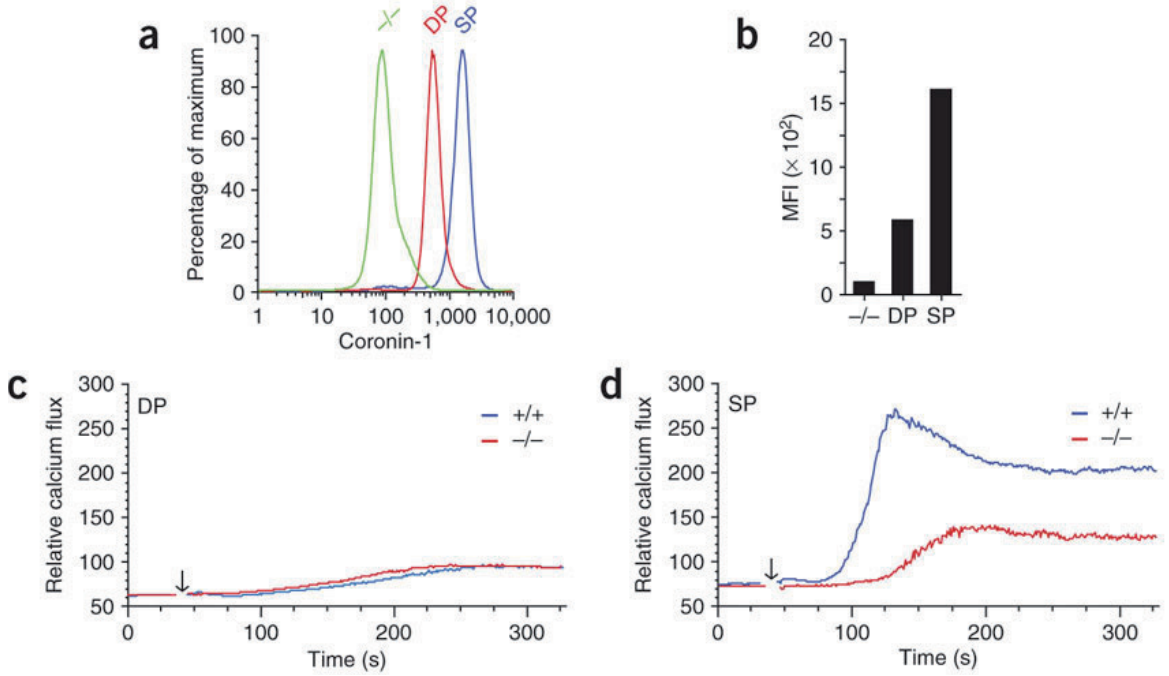


Figure 5: Thymocyte coronin-1 and Ca2+ flux.

(a,b) Flow cytometry of wild-type (+/+) and coronin-1 deficient (-/-) total thymocytes from 5- to 6-week-old mice stained with anti-CD4, anti-CD8 and Alexa Fluor 633 conjugated anti-coronin-1. Histogram of anti-coronin-1 staining for coronin-1 deficient thymocytes and double-positive (DP) and single-positive (SP) wild-type thymocytes (a); corresponding mean fluorescence intensity (MFI) values (b). (c,d) Calcium fluxes upon CD3-induced TCR triggering compared in anti-CD4, anti-CD8 stained, Indo-1 loaded wild-type and coronin-1 deficient thymocytes. Arrow indicates the time of addition of the stimulus. Data are representative of at least three independent experiments.

2.2.3.5. Coronin-1 and PLC-γ1

The data presented thus far suggest that coronin-1 is specifically involved in the activation of Ca^{2+} release through PLC-γ1 mediated formation of $InsP_3$. To analyze the consequences of defective $InsP_3$ production and to investigate whether coronin-1 is specifically involved in PLC-γ1 mediated processes, we evaluated the activation of several signaling molecules downstream of TCR activation. Whereas the activation of the extracellular signal regulated kinases (ERK) 1 and 2 [325, 326], which are PLC-γ1 and Ca^{2+} dependent, was defective in the absence of coronin-1 (Fig. 6a), the phosphorylation pattern of p38 as well as phosphorylation and degradation of IκB-α were unaltered (Fig. 6b). In addition, whereas in the absence of coronin-1 the Ca^{2+}-dependent translocation of NFAT (nuclear factor of activated T cells) to the nucleus was defective upon TCR stimulation, TCR triggering induced nuclear translocation of NF-κB in equal numbers of wild-type and coronin-1 deficient T cells (Supplementary Fig. 8, see below). These results are consistent with a specific role for coronin-1 in $InsP_3$-mediated Ca^{2+} release and furthermore showed that deletion of *Coro1a* does not result in a general defect in TCR-mediated signaling.

To further corroborate a role for coronin-1 in PLC-γ1 mediated $InsP_3$ release, we evaluated T cells after treatment with the PLC activator N-(3-trifluoromethylphenyl)-2,4,6-trimethylbenzenesulfonamide (m-3M3FBS) [327]. Whereas PLC activation in wild-type cells resulted in rapid mobilization of intracellular Ca^{2+}, activation of PLC in the absence of coronin-1 failed to induce Ca^{2+} release (Fig. 6c,d). Similarly, when Jurkat T cells were depleted of coronin-1 by expression of siRNA specific for human coronin-1 (Fig. 6e), activation failed to result in Ca^{2+} mobilization, whereas normal Ca^{2+} mobilization occurred after treatment with siRNA specific for mouse coronin-1 (Fig. 6f).

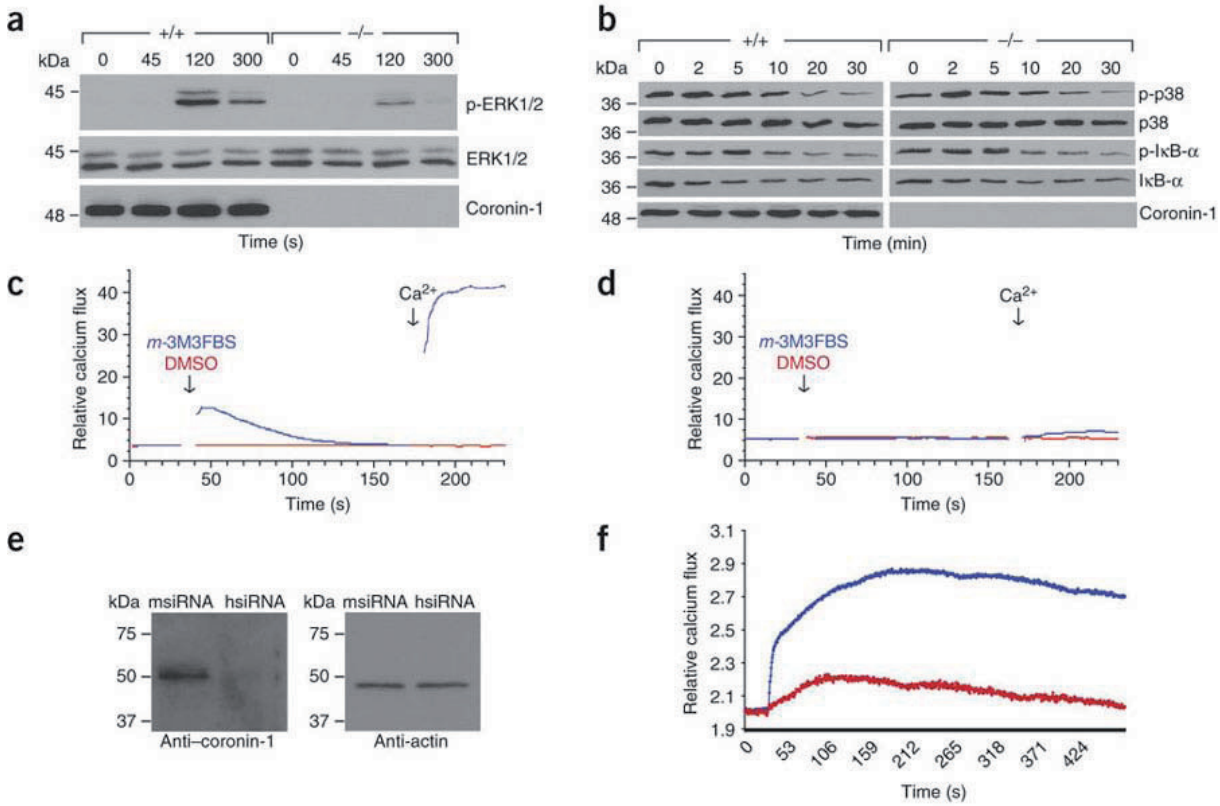


Figure 6: Signaling pathways in wild-type and coronin-1 deficient cells upon TCR and PLC activation.

(a,b) T cells were prepared and treated as described in Figure 3 and immunoblotted using the indicated antibodies. (c,d) T cells from wild-type (c) or coronin-1–deficient (d) mice were loaded with Fluo-3 a.m. and stimulated with m-3M3FBS (blue) or DMSO (red). For details, see Methods. (e) Jurkat T cells transfected with pSUPER::hTACO1 or pSUPER::mTACO1. msiRNA, mouse coronin-1–specific siRNA; hsiRNA, human coronin-1–specific siRNA. Immunoblot of total cell lysates of with coronin-1 or actin-specific antibodies. (f) Measurement of calcium flux by fluorescence in Jurkat T cells transfected as in e (blue, msiRNA; red, hsiRNA) and then loaded with Indo-1 a.m., stimulated with 10 µM m-3M3FBS. Arrow indicates the time of addition of the stimulus. Data are representative of three independent experiments (Experiments presented in part c to f of this figure were performed mainly by Jayachandran R.).

Finally, we analyzed whether coronin-1 directly interacted with PLC-γ1 by performing co-immunoprecipitation from detergent lysates [34] of wild-type T cells. We readily detected coronin-1 by immunoblotting when we immunoprecipitated PLC-γ1 from T cell lysates (Fig. 7a); co-immunoprecipitation of coronin-1 with PLC-γ1 was not due to the association of PLC-γ1 and coronin-1 with the actin cytoskeleton, however, because actin was not detectable in the immune complexes (Fig. 7b-d). Together the above data suggested that coronin-1, by forming a complex with PLC-γ1, acts as an essential cofactor for $InsP_3$ generation and is therefore a key mediator of intracellular Ca^{2+} release.

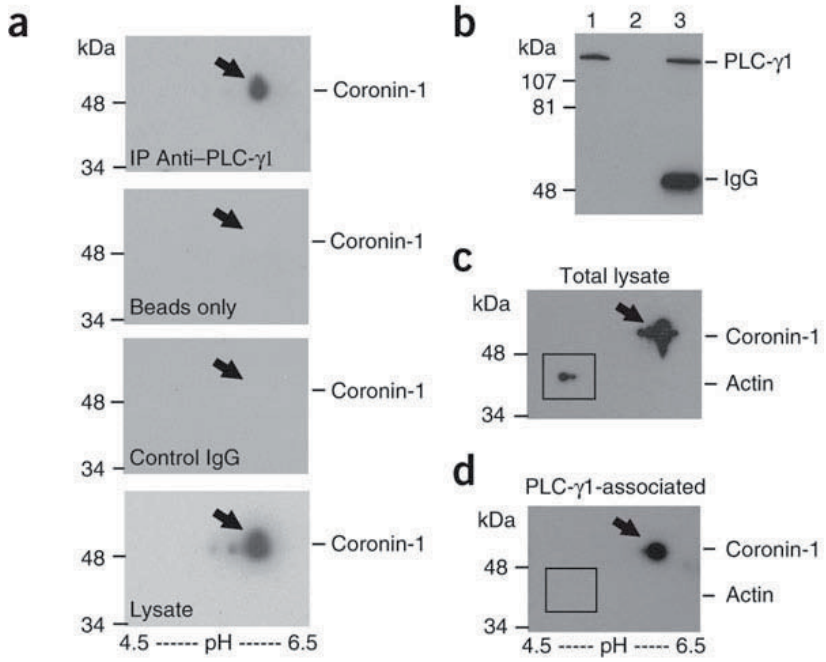


Figure 7: Interaction of Coronin-1 and PLC-γ1.

(a) T cells from wild-type mice were lysed, immunoprecipitated (IP) using PLC-γ1 specific antibodies and separated by two-dimensional IEF SDS-PAGE followed by immunoblotting using coronin-1 specific antibodies (upper panel). As controls, lysates immunoprecipitated with protein A beads only (second panel), control IgG (third panel) or total lysate (lower panel) were similarly separated by two-dimensional IEF SDS-PAGE and immunoblotted for coronin-1 (arrows). (b). Anti-PLC-γ1 immunoblot of 20% of the immunoprecipitated beads from an experiment similar to that in a separated by 7.5% SDS-PAGE to analyze the efficiency of PLC-γ1 immunoprecipitation. Lane 1, total lysate; lane 2, beads only; lane 3, anti-PLC-γ1 immunoprecipitate. (c,d) Blots as in a reprobed with anti-actin. The box indicates the position of actin on the immunoblot. The box indicates the position of actin on the immunoblot. Data are representative of three independent experiments.

2.2.4. Discussion

Coronin-1 is a leukocyte specific molecule that associates with the cell cortex in a cholesterol-dependent manner and has been proposed to be involved in the regulation of leukocyte-specific signal transduction processes [241, 255]. Here, we found that coronin-1 was essential for the survival of naive T cells in the periphery by activating Ca^{2+} release from intracellular stores upon TCR triggering. Coronin-1 associated with PLC-γ1, thereby promoting the generation of the second messenger $InsP_3$, responsible for the release of Ca^{2+} from intracellular stores.

The role defined here for coronin-1 in the generation of $InsP_3$ upon TCR triggering differs markedly from a recent conclusion that coronin-1 is dispensable for TCR-mediated signaling, instead preventing F-actin induced apoptosis [263]. However, in contrast to the results described here, the previous conclusion that coronin-1 is dispensable for TCR-mediated signaling was based on the analysis of mice possessing a fixed T cell repertoire [263]. In such animals, T cells with an activated-memory phenotype develop even in the absence of previous exposure to antigen (ovalbumin); this does not occur in non-transgenic mice [328, 329]. The lack of a signaling defect in the TCR-transgenic T cells used in that study [263] may therefore be because TCR triggering is especially important for the survival of naive T cells, whereas effector-memory cells can survive in the absence of TCR ligation [173, 314, 315]. Consistent with that notion, we found a severe reduction in absolute numbers of naive T cells in coronin-1 deficient mice, although effector-memory cells survive in the absence of coronin-1 [321-323].

Several other lines of evidence support a specific role for coronin-1 in T cell signaling rather than, as reported by a previous study [263], in modulating F-actin dynamics. First, F-actin dynamics were normal in the absence of coronin-1 as analyzed by several independent morphological, biochemical and functional assays, including the analysis of TCR-mediated cell spreading. Second, the conclusion that coronin-1 regulates F-actin dynamics in T cells, thus preventing F-actin induced apoptosis, relies on the observation of increased phalloidin staining in cells lacking coronin-1 [263]. However, enhanced phalloidin staining was detected in all coronin-1 deficient leukocyte subsets, independent of their in vivo depletion, cytoskeletal functioning (P.M. and J.P., unpublished observations) or cortical actin staining (using antibodies to actin). Third, a role for coronin-1 in modulating PLC-γ1 activity rather than in regulating F-actin dynamics is consistent with the capacity of coronin-1 to prevent

lysosomal delivery of pathogenic mycobacteria in macrophages through activation of the Ca^{2+}-dependent phosphatase calcineurin [237, 251, 330], a role which might be mediated by the function of coronin-1 in PLC-γ1 mediated processes described here.

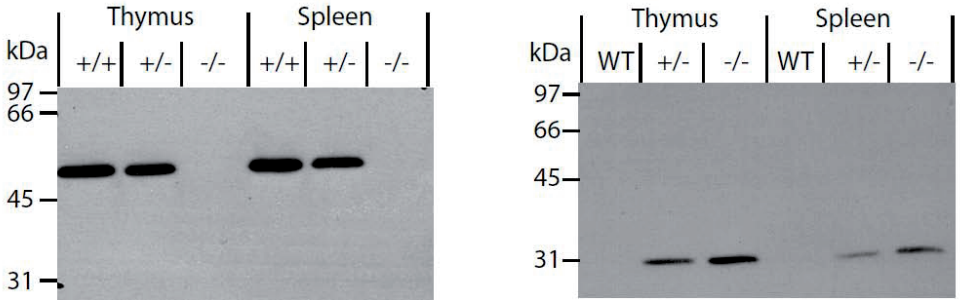
Coronin-1 was initially isolated from mammalian leukocytes co-purifying with a phospholipase activity [240]. The physical interaction between coronin-1 and PLC-γ1 shown here suggests that coronin-1 may function as a docking site for PLC-γ1 required for the generation of $InsP_3$. Alternatively, although the overall localization of PLC-γ1 or its activators does not seem to be altered upon coronin-1 deletion, coronin-1 may be involved in the appropriate positioning of PLC-γ1 at the membrane, which is known to be required for proper generation of $InsP_3$ [241, 331, 332]. In this context, we note that only a fraction of coronin-1 was found to associate with the total PLC-γ1 present in T cells (data not shown), suggesting that either PLC-γ1 interacts with a post-translationally modified form of coronin-1 or, alternatively, that coronin-1 provides a scaffold at the cell cortex to locally modulate the interaction of PLC-γ1 with $PtdIns(4,5)P_2$.

The definition of coronin-1 as an essential signaling component allowing intracellular Ca^{2+} mobilization through $InsP_3$ generation may allow a better understanding of T cell activation and homeostasis as well as provide a basis for rational approaches for the development of compounds to treat autoimmune as well as lymphoproliferative disorders.

2.2.5. Acknowledgments

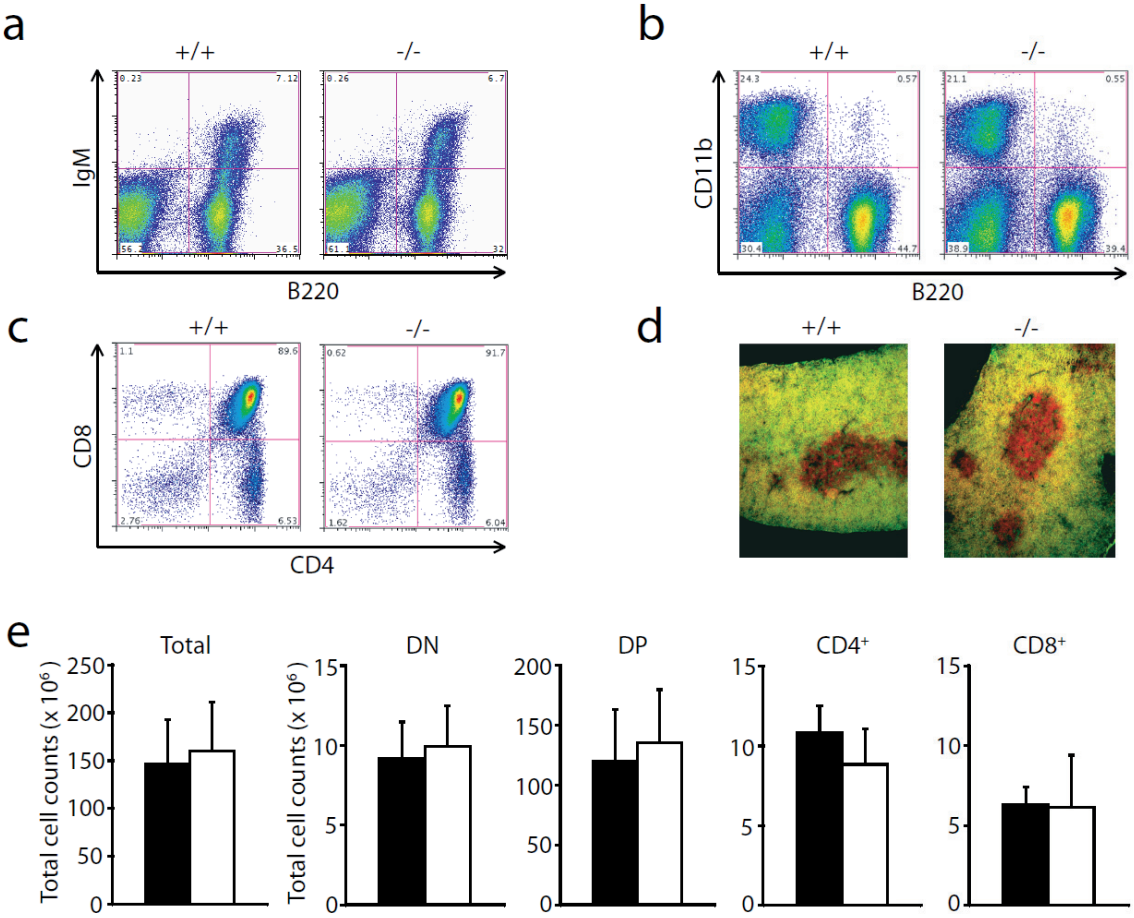
We thank L. Kuhn for assistance with two-dimensional SDS-PAGE and J. Kirberg, K. Huygen, H. Korf, V. Jaeggin, E. Teixeiro, M. Daniels, E. Palmer and P. Demougin for help and discussions. Supported by the German Research Council (SFB 497-B5 to H.-R.R.) and the Kanton Basel-Stadt as well as the Swiss National Science Foundation (to J.P.).

2.2.6. Supplementary Figures and Tables



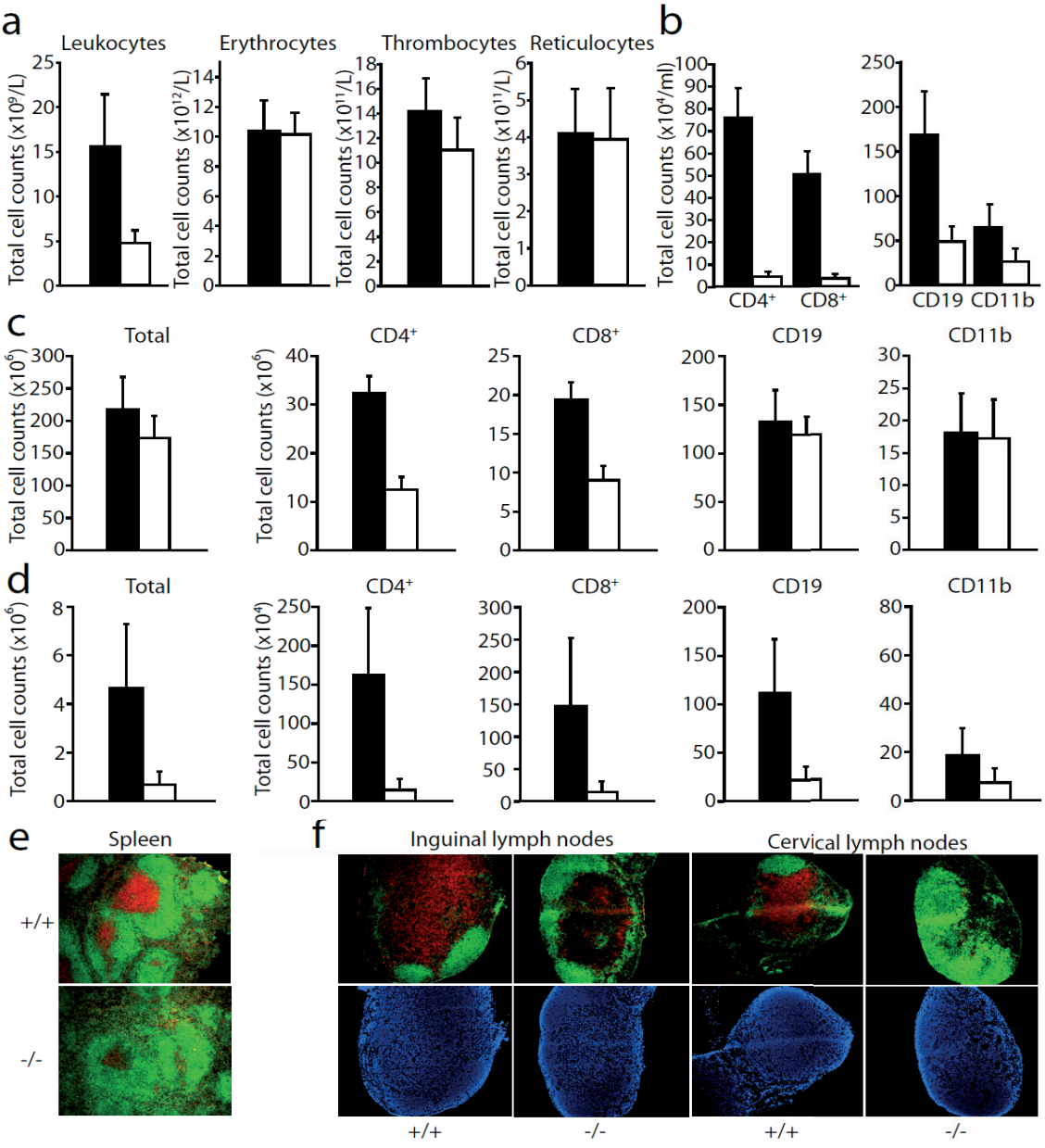
Supplementary Figure 1: Analysis of coronin 1 expression in lymphoid organs from wild type and coronin 1 deficient mice.

Cell lysates were prepared from the indicated organs, and equal protein amounts separated by SDS-PAGE and immunoblotted for the detection of coronin 1 (left panel) and GFP (right panel) (Experiments presented in this figure were performed by Massner J.).



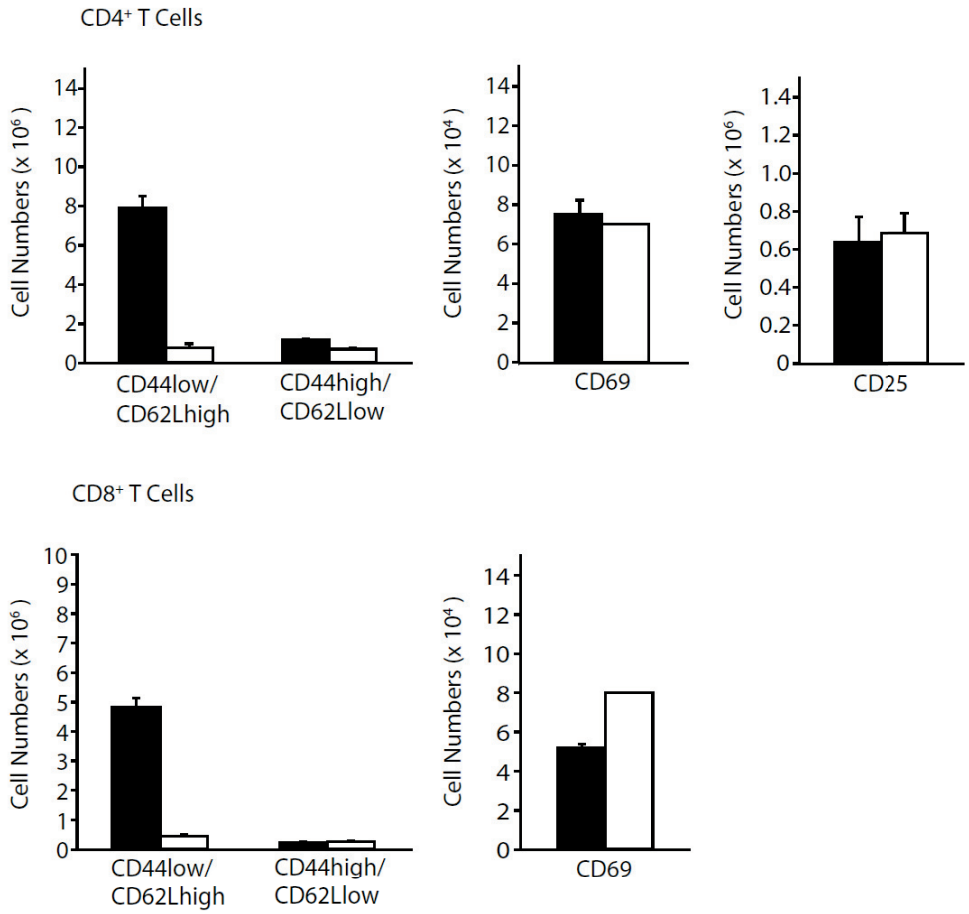
Supplementary Figure 2: Normal lymphocyte development in wild type and coronin 1 deficient mice.

Single cell suspensions of the indicated organs were prepared and stained with antibodies directed against antigens being specifically expressed at the various developmental stages of the lymphoid and myeloid lineage. (a) Bone marrow cells were stained for the B cell lineage marker B220 and IgM. (b) Bone marrow cells of the myeloid lineage were characterized by expression of CD11b and B220. (c) Thymocyte populations were analyzed by CD4 and CD8 staining. (d) Thymi from wild-type (+/+) or coronin 1-deficient (–/–) mice were prepared for histology and stained for $CD4^+$ as well as $CD8^+$ cells using antibodies coupled to APC (CD4) or FITC (CD8). Magnification: 10X. (e) Cell suspensions of the thymus of 6 weeks old homozygous (–/–) or wild-type (+/+) mice were analyzed by flow cytometry using antibodies directed against the indicated thymocyte subsets CD4/CD8 double-negative (DN), double-positive (DP) and single-positive ($CD4^+$ and $CD8^+$) thymocytes). Cells were counted using a Neubauer chamber. Subset specific cell numbers were calculated by referring the percentage of a certain cell type (determined by flow cytometry) to the total cell counts. Filled bars: wild-type, open bars: coronin 1-deficient. Data represent means ± s.d. of 5 animals (n = 5) (Experiments presented in part a, b, c and e of this figure were performed by Massner J. and Ceredig R.; Immunohistochemistry (part d) was performed by Blum C.).



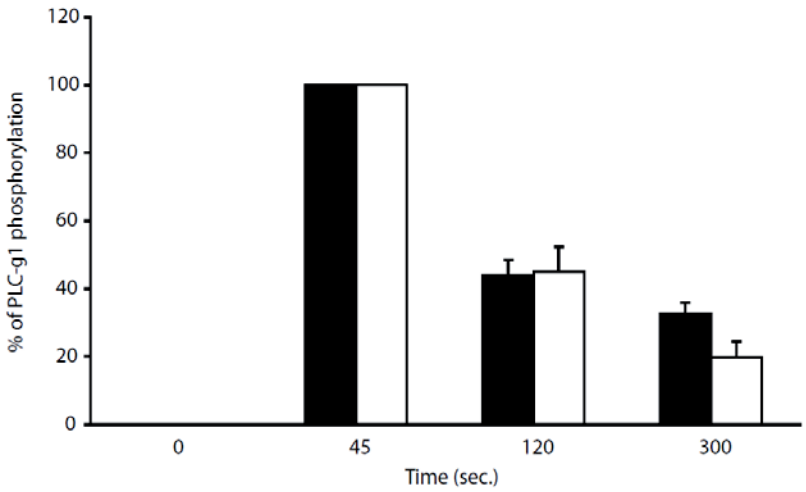
Supplementary Figure 3: Depletion of peripheral T cells in coronin 1-deficient mice.

(a) Tail blood from coronin 1-deficient mice (open bars) and wild-type littermates (filled bars) was hematological analyzed on an ADVIA instrument. Data represent means ± s.d.; +/+, n = 13 animals ; –/–, n = 19 animals). (b). For leukocyte counts, peripheral blood of 6 weeks old mice was analyzed by flow cytometry using antibodies against the indicated leukocyte subsets markers. Cells were counted using a Neubauer chamber and total cell counts were corrected to 1 ml peripheral blood. Subset specific cell numbers were calculated by referring the percentage of a certain cell type (as determined by flow cytometry) to the total cell counts. Data represent means ± s.d. of 5 animals (n = 5). (c, d). Cell suspensions of spleen (c) or inguinal lymph nodes (d) were analyzed by flow cytometry. Lymphocyte specific cell numbers were calculated by referring the percentage of a certain cell type to the total cell counts. Data represent means ± s.d. of 5 animals (n = 5). Spleen (e) or inguinal as well as cervical lymph nodes (f) were prepared for histology and stained for B220 (FITC) and Thy 1.2 (PE) or DAPI (f, lower panel) as described in Supplementary Methods. Magnification: 10X (Experiments presented in part a to d of this figure were performed by Massner J. and Ceredig R.; Immunohistochemistry (part e and f) was performed by Blum C.).



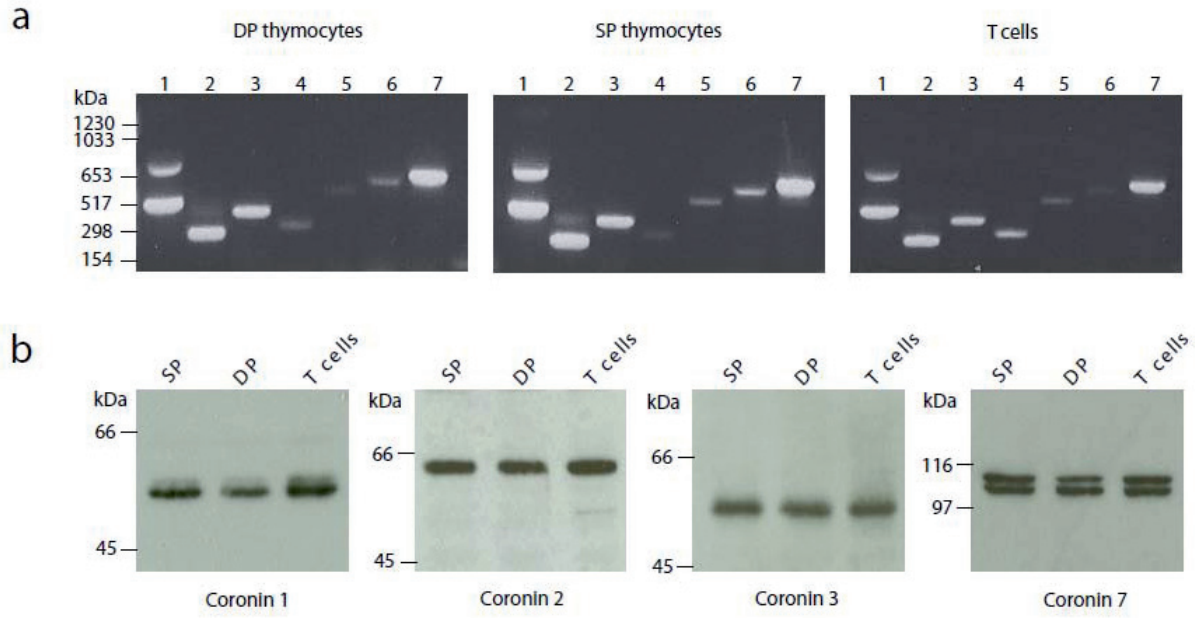
Supplementary Figure 4: T cell populations in the periphery of wild-type and coronin 1-deficient mice.

Erythrocyte depleted splenocytes from wild-type (filled bars) and coronin 1-deficient animals (open bars) were stained with the indicated surface markers and total cell numbers were calculated. Data represent means ± s.d. of 3 animals (n = 3).



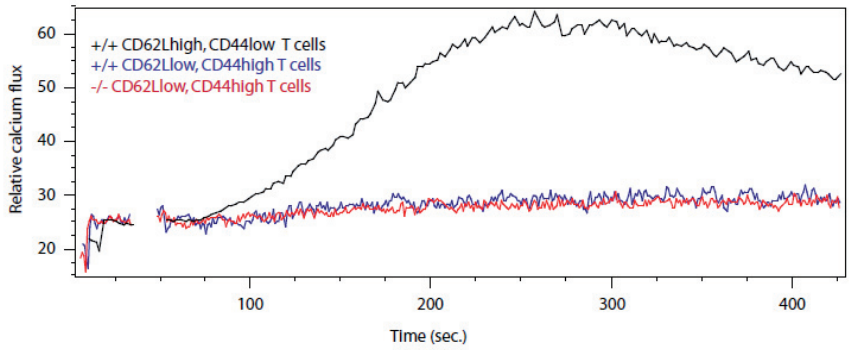
Supplementary Figure 5: Phosphorylation of PLC-γ1 in wild-type and coronin 1-deficient T cells.

PLC-γ1 phosphorylation after TCR cross-linking was quantified in wild-type (filled bars) and coronin 1-deficient naive T cells (open bars) using data sets derived from experiments as shown in Figure 3e. Background subtracted signal intensities were expressed relative to the highest signal intensity at 45 seconds which was set to 100% in all experiments. Data represent means ± s.d. of 4 data sets from 3 independent experiments (n = 4).



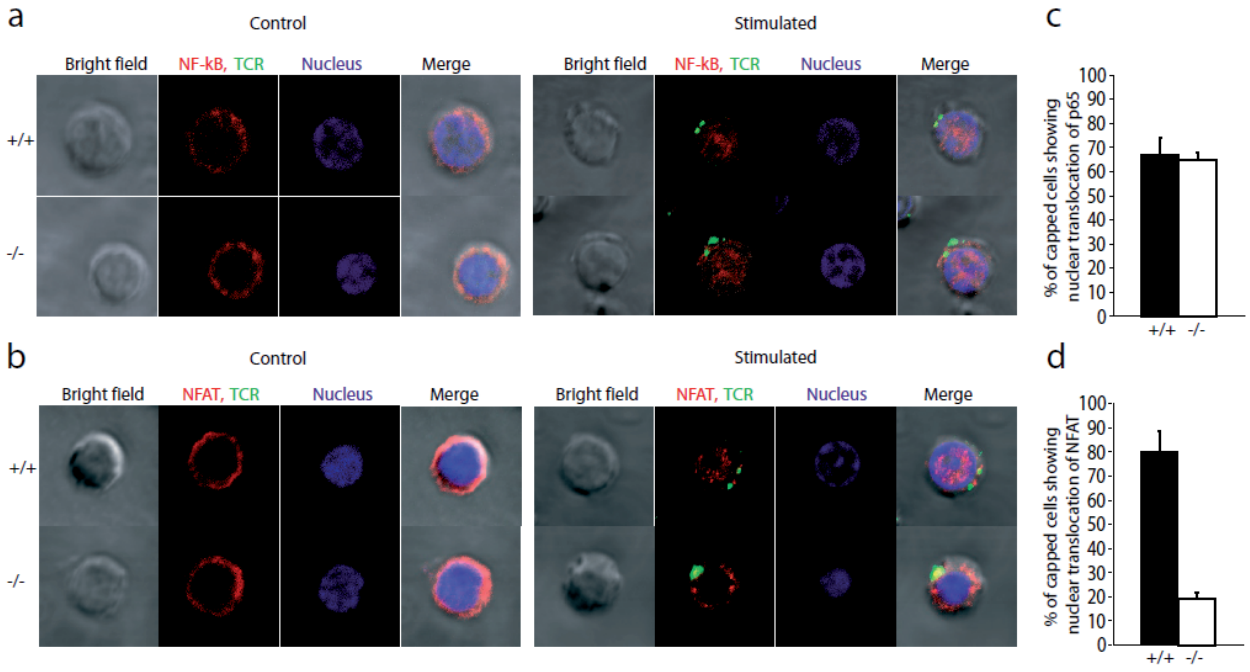
Supplementary Figure 6: Expression of coronin isoforms in thymocyte and T cell subsets.

(a) RT-PCR was carried out using total RNA isolated from single positive thymocytes, double positive thymocytes and splenic T cells, loaded onto a 1.2 % agarose gel and electrophoresed. Coronin 1-7 isoforms are numbered from 1 to 7. (b) Single positive thymocytes, double positive thymocytes and splenic T cells purified from wild-type animals using MACS were lysed and equal protein amounts as determined by BCA protein assay were separated by SDS PAGE (10 %) followed by immunoblotting for the proteins indicated. Data are representative of 3 independent experiment (Experiments presented in this figure were performed together with Jayachandran R. and Combaluzier B.)



Supplementary Figure 7: T cell receptor induced calcium fluxes in memory-effector T cells from wild-type and coronin 1 deficient mice.

Splenic memory-effector T cells from wild-type or coronin 1-deficient mice were stimulated with anti-CD3 + anti-CD28 as described in Supplementary Methods and calcium fluxes were analyzed. Naive wild-type T cells were stimulated and analyzed as described for the memory-effector T cells. Data are representative of 3 independent experiments.



Supplementary Figure 8: Nuclear translocation of NF-kB and NFAT in wild-type and coronin 1-deficient T cells upon T cell receptor triggering.

Wild-type as well as coronin 1-deficient splenic T cells were prepared and stimulated with anti-CD3 + anti-CD28 as described in Supplementary Methods. Cells were stained with anti-p65 (a) or anti-NFAT (b); nuclei were stained using DRAQ5. For quantization of nuclear translocation of p65 (c) or NFAT (d) two times 100 cells were analyzed for both wild-type as well as coronin 1 deficient T cells from two independent experiments.

Part III

2.3. Coronin 1 Maintains the Balance between Survival and Apoptosis in Naive T Cells Independent of F-Actin

2.3.1. Abstract

Coronins are WD repeat containing proteins highly conserved in the eukaryotic kingdom implicated in the regulation of F-actin. Mammalian coronin 1, one of the most conserved isoforms expressed in leukocytes, regulates survival of T cells which has been suggested to be due to its role in preventing F-actin induced apoptosis. We here come to a different conclusion. We show that coronin 1 does not modulate F-actin and that induction of F-actin failed to induce apoptosis. Instead, coronin 1 was required for providing pro-survival signals, in the absence of which T-cells rapidly underwent apoptosis. These results overhaul the long held view that coronin 1 functions through modulation of F-actin and establish coronin 1 as an essential regulator of Ca^{2+}/calcineurin dependent signaling in T-cells.

2.3.2. Introduction

Coronin 1 is a member of the conserved WD repeat family of coronin proteins that is exclusively expressed in leukocytes [237]. In mice lacking coronin 1, naïve T-cells are deleted from the periphery, [239] [263, 333-335], suggesting that coronin 1 has a specific and essential role in the regulation of cellular homeostasis and the survival of these cells.

Coronin 1 is homologous to *Dictyostelium discoideum* coronin, that was originally isolated as an actin/myosin binding protein [224]. Deletion of coronin from these slime molds was subsequently shown to result in pleiotropic defects such as reduced phagocytosis, macropinocytosis, cell locomotion and cytokinesis [225, 226]. In yeast, the single coronin isoform has been suggested to modulate the formation of actin filamentous networks via regulation of the actin-related protein 2/3 (Arp2/3) activity, based on in vitro experiments [233]. However, living yeast cells lacking coronin do not show any obvious phenotype [231].

The original isolation of *Dictyostelium* coronin from an actin-myosin affinity matrix has led to the assignment of all coronin protein family members as actin-interacting and regulating proteins [224, 228, 229]**.** However, the evidence for F-actin regulation by mammalian coronin isoforms is largely based on *in vitro* polymerization assays using purified proteins [336-338], or overexpression of coronin domains in a heterologous system [339, 340]. *In vivo*, the only evidence linking coronin 1 to a direct modulation of the F-actin cytoskeleton is based on the observation that coronin 1 deficient leukocytes display a ~2-fold higher phalloidin fluorescence as compared to wild type cells. Consequently, the decrease in cell viability as well as migration of T-cells lacking coronin 1 as observed in coronin 1 deficient mice was attributed to defective functioning of the F-actin cytoskeleton [263, 333-335]. In contrast to linking a role for coronin 1 in T-cell survival to the modulation of F-actin, recent work suggested that coronin 1 promotes T-cell survival by allowing signal transduction downstream of the T-cell receptor [239].

We here provide an explanation for this apparent discrepancy. We show that phalloidin cannot be used to quantitate F-actin in cells expressing different coronin 1 levels and furthermore that coronin 1 does not directly modulate F-actin. In cells depleted of coronin 1 by either gene deletion or RNA interference, phalloidin levels increased in direct inverse relation to the amount of coronin 1, without affecting the F/G-actin ratio or cell viability.

Conversely, expression of coronin 1 in non-leukocytes resulted in decreased phalloidin fluorescence without affecting F/G actin levels or viability.

Instead of regulating the F-actin cytoskeleton, we describe here that coronin 1 is required for the generation of pro-survival signals upon T-cell receptor ligation, in the absence of which naïve T-cells undergo apoptosis. Importantly, we show that the proposed role for coronin 1 in modulating T-cell migration, is not a cell intrinsic property of coronin 1 deficient T-cells, but is due to imbalanced T-cell receptor signaling. This, in turn, results in the depletion of pro-survival signals and as a result causes defects in cellular survival and migration. The here described results are therefore crucial for a better understanding of the function of coronin 1 as well as the processes that regulate cellular survival and migration.

2.3.3. Results

2.3.3.1. Phalloidin Fluorescence, F-actin and Apoptosis

Coronin 1 has been proposed to prevent apoptosis via the modulation of the F-actin cytoskeleton. This conclusion was based on the observed ~2-fold increase in phalloidin fluorescence intensity in coronin 1 deficient T-cells versus wild type T-cells [263, 333-335]. To analyze the correlation between phalloidin fluorescence and apoptosis, the different leukocyte populations as indicated in Figure 1 and Supplementary Figure 1 (see below) were isolated from wild type or coronin 1 deficient mice, and analyzed for coronin 1 expression, phalloidin fluorescence and Annexin V staining. Staining of all leukocyte populations using Alexa Fluor 568 or 633 -conjugated phalloidin resulted in a ~2-fold increase of fluorescence in coronin 1 deficient cells versus wild type cells (Figure 1A and B).

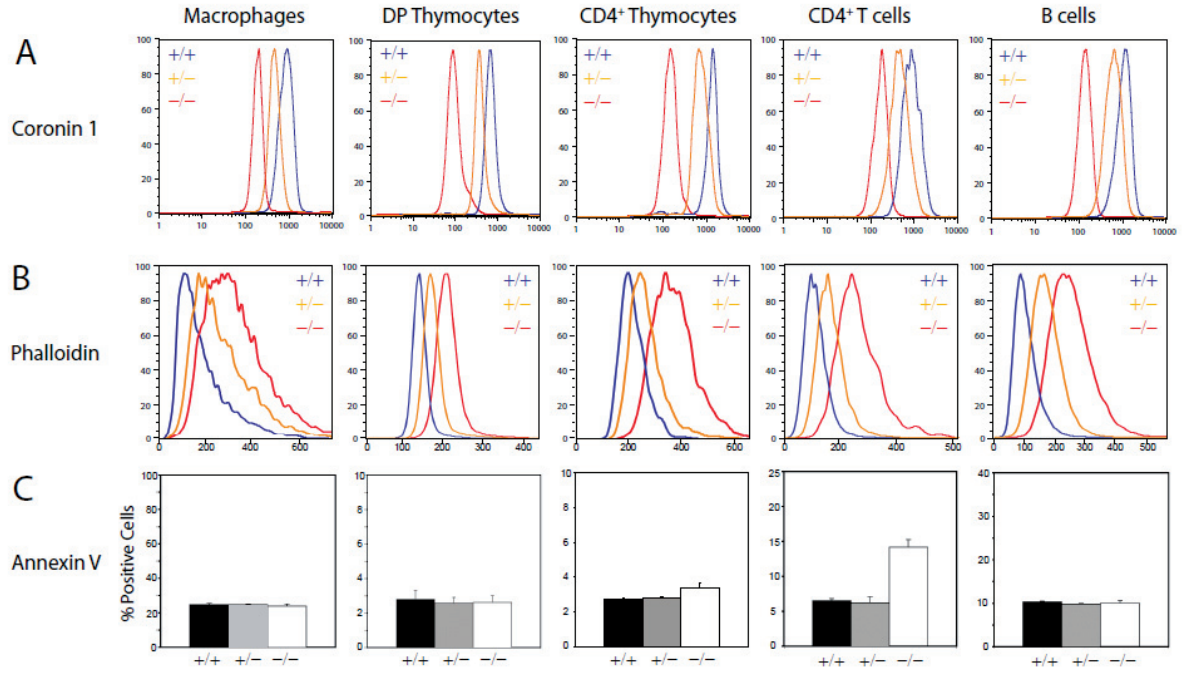


Figure 1: Coronin 1 expression, phalloidin fluorescence and apoptosis in wild type, heterozygous and coronin 1 deficient cells.

The indicated cell populations from wild type, heterozygous and coronin 1 deficient mice were stained for coronin 1 using an AlexaFluor633 conjugated polyclonal anti coronin 1 antibody (A, *upper row*). The same cells were stained using Alexa Fluor 568 or 633 conjugated phalloidin (B, *middle row*) and PE or APC-conjugated Annexin V (C, *lower row*). Depicted are mean +/– SD of three mice in each group. Stainings were analyzed using FACS.

However, with the exception of $CD4^+$ single positive thymocytes as well as T-cells, none of the leukocyte populations lacking coronin 1 showed an increase in apoptosis, as judged by Annexin V labeling (Figure 1C). Notably, the heterozygous $CD4^+$ single positive thymocytes as well as T-cells showed intermediate levels of phalloidin staining but no elevated Annexin V staining.

The above results show that the increased phalloidin fluorescence upon depletion of coronin 1 does not correlate with the induction of apoptosis in the analyzed leukocyte populations. Possibly, the observed increase in phalloidin fluorescence is a result of coronin 1 depletion rather than of increased F-actin. To directly address this possibility, the F/G-actin ratio in wild type or coronin 1 deficient cells was analyzed under conditions where the F-actin cytoskeleton was perturbed by the addition of F-actin interfering drugs. For that purpose, we used either the fungal peptide Jasplakinolide to induce polymerization of F-actin [286] or Latrunculin B to depolymerize F-actin [282, 283]. As expected, the addition of Jasplakinolide resulted in F-actin polymerization while the presence of Latrunculin B induced depolymerization of F-actin in both wild type as well as coronin 1 deficient cells to the same extent (Figure 2A). In order to demonstrate the sensitivity as well as the reproducibility of the biochemical F versus G-actin assay, untreated wild type and coronin 1 deficient T-cells were compared with wild type cells exposed to CCL19, a member of the chemokine compounds that are known to result in the induction of F-actin polymerization [341]. The amount of CCL19 was titrated to yield a similar elevation of phalloidin staining as observed in the untreated coronin 1 deficient T-cells (Figure 2B). Cells treated as described above were subjected to the F versus G-actin assay as in Figure 2A, total proteins were separated by SDS-PAGE, immune-blotted for actin and the F versus G-actin ratio was calculated. As shown in Figure 2, panels C and D, while CCL19 induced the same increase in phalloidin fluorescence as coronin 1 deletion (Figure 2B), only CCL19 triggering resulted in an elevation of the F/G-actin ratio as measured biochemically. Finally, to analyze whether F-actin modulation results in elevated spontaneous apoptosis, as suggested earlier [263], wild type $CD4^+$ single positive thymocytes were incubated with the F-actin modulating reagents as indicated in panel E and the viability of the cells was analyzed by Annexin V and 7-AAD staining. As shown in Figure 2, Panel E, neither depolymerization nor polymerization of the F-actin cytoskeleton in wild type cells resulted in an altered viability while untreated coronin 1 deficient cells showed a severe defect in survival.

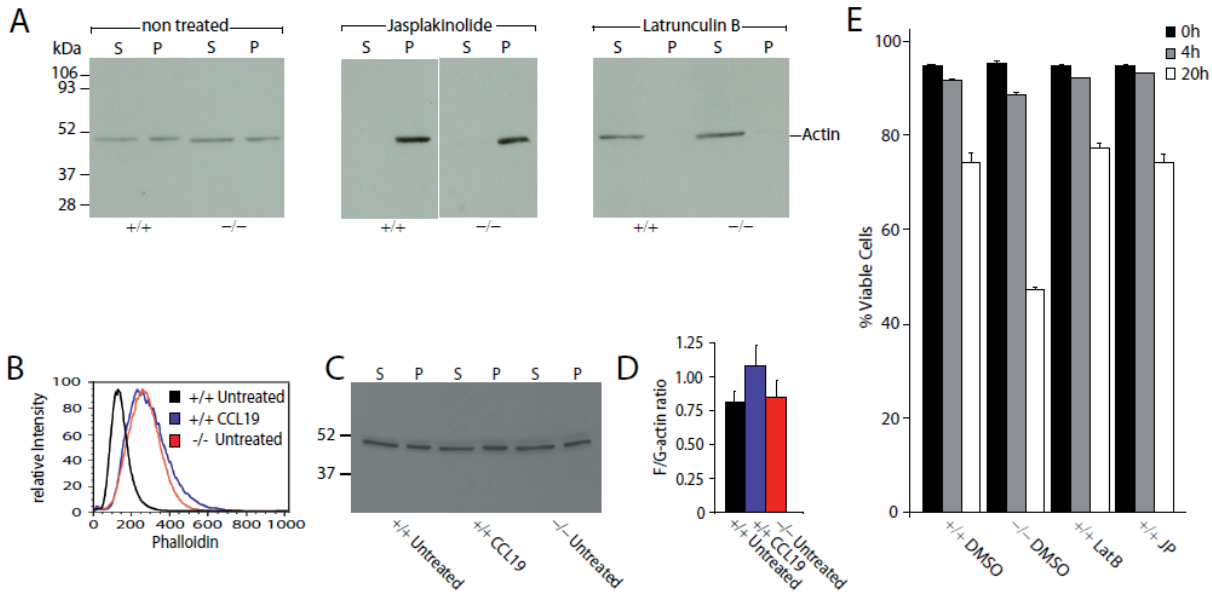


Figure 2: F/G-actin ratio, spontaneous apoptosis and F-actin induced apoptosis in wild type and coronin 1 deficient T-cells.

(A) For the analysis of G- and F-actin content in response to actin modulating drugs, wild type (+/+) or coronin 1 deficient (–/–) splenic T-cells were left untreated or treated with either Jasplakinolide (1 µM) or Latrunculin B (4 µM) for 45 min at room temperature followed by cell lysis. Proteins present in supernatants (S) and pellets (P) prepared as described in methods were separated by SDS-PAGE and immuno-blotted using anti-actin antibodies. (B) Wild type T-cells were treated with CCL19 (250 ng/ml for 60 seconds) to result in the same phalloidin staining as coronin 1 deficient T-cells, shown is a representative histogram of untreated and CCL19 wild type T-cells as well as untreated coronin 1 deficient T-cells stained with phalloidin-633. (C) Cells treated as in B were analyzed for F/G actin as described in methods. (D) Quantitation of a representative experiment out of three as described under B and C. Shown are mean +/– SD of three independent samples. (E) Wild type $CD4^+$ single positive thymocytes were incubated for 4 and 20h with DMSO (carrier), Latrunculin B (LatB, 4 µM) or Jasplakinolide (JP, 1 µM) at 37°C, 5% CO_2 as indicated and stained with Annexin V and 7-AAD at the indicated time points. Coronin 1 deficient cells treated with DMSO were used as an internal control. Cells negative for both Annexin V and 7-AAD were considered as viable cells.

To further analyze the correlation between coronin 1 expression, F-actin accumulation and Annexin V labeling, two additional experiments were performed. First, coronin 1 was depleted in the human T-cell line Jurkat using siRNA specific for mouse (control) or human coronin 1. While coronin 1 expression was effectively knocked down in Jurkat cells expressing the human, but not the mouse siRNA (Figure 3A), the ratio of F/G actin as measured biochemically was similar in wild type and coronin 1 depleted cells (Figure 3B). However, phalloidin fluorescence increased ~2-fold upon coronin 1 depletion (Figure 3C). Analysis of Annexin V labeling revealed no differences (Figure 3D). Also, incubation of Jurkat cells expressing the murine or human specific siRNA with the apoptosis inducing agent tumor necrosis factor-α (TNF-α) revealed no differences in either the time course or degree of

apoptosis induction (Figure 3D, insert). Conversely, while in the non-leukocyte cell line Mel JuSo transfected with cDNA encoding coronin 1 [237], expression of coronin 1 resulted in a decrease of phalloidin fluorescence, as observed in T-cells, no differences were found in the F/G actin ratio or Annexin V labeling of control versus coronin 1 expressing cells (Figure 3E-H). In addition, incubation of control or coronin 1 expressing Mel JuSo cells with the apoptosis inducing agent staurosporin revealed no differences in either the time course or degree of apoptosis induction (Figure 3H, insert). In accordance with these findings, staurosporin induced the same degree of apoptosis in both wild type and coronin 1 deficient B-cells (Supplementary Figure 2, see below). These results therefore corroborate the conclusion that expression or deletion of coronin 1 interferes with phalloidin fluorescence, but does not affect F-actin levels nor has a direct effect on or protects cells from apoptosis.

2.3.3.2. Chemokine induced migration of wild type and coronin 1 deficient T-cells

An important question to address was why, in the absence of an F-actin phenotype, coronin 1 deficient T-cells as opposed to B-cells [334], macrophages [238, 251] and neutrophils [252], show a significantly lower migratory capacity as well as a survival defect when compared to wild type cells. For this purpose wild type and coronin 1 deficient T cell subsets as well as B-cells were analyzed for spontaneous apoptosis as well as the ability to migrate in a transwell migration assay. Naïve $CD4^+$ T-cells (Figure 4A) and B-cells (Figure 4B), isolated from wild type or coronin 1 deficient mice, were left in medium for the indicated time and stained with Annexin V and 7-AAD to assess the number of viable cells. Double negative cells were scored as viable. Cells from the same preparation were analyzed for their ability to migrate in a transwell migration assay towards the indicated concentrations of chemo-attractants. Our results demonstrate that freshly isolated coronin1 deficient naïve T-cells show elevated levels of spontaneous apoptosis and that the number of migration competent naïve T-cells is far lower in case of coronin 1 deficient cell preparations as compared to the wild type control. Our results further show that in B-cells, which in the absence of coronin 1 show elevated levels of phalloidin fluorescence similar to that observed in T-cells (see Figure 1), neither cellular viability nor their capacity to migrate is affected.

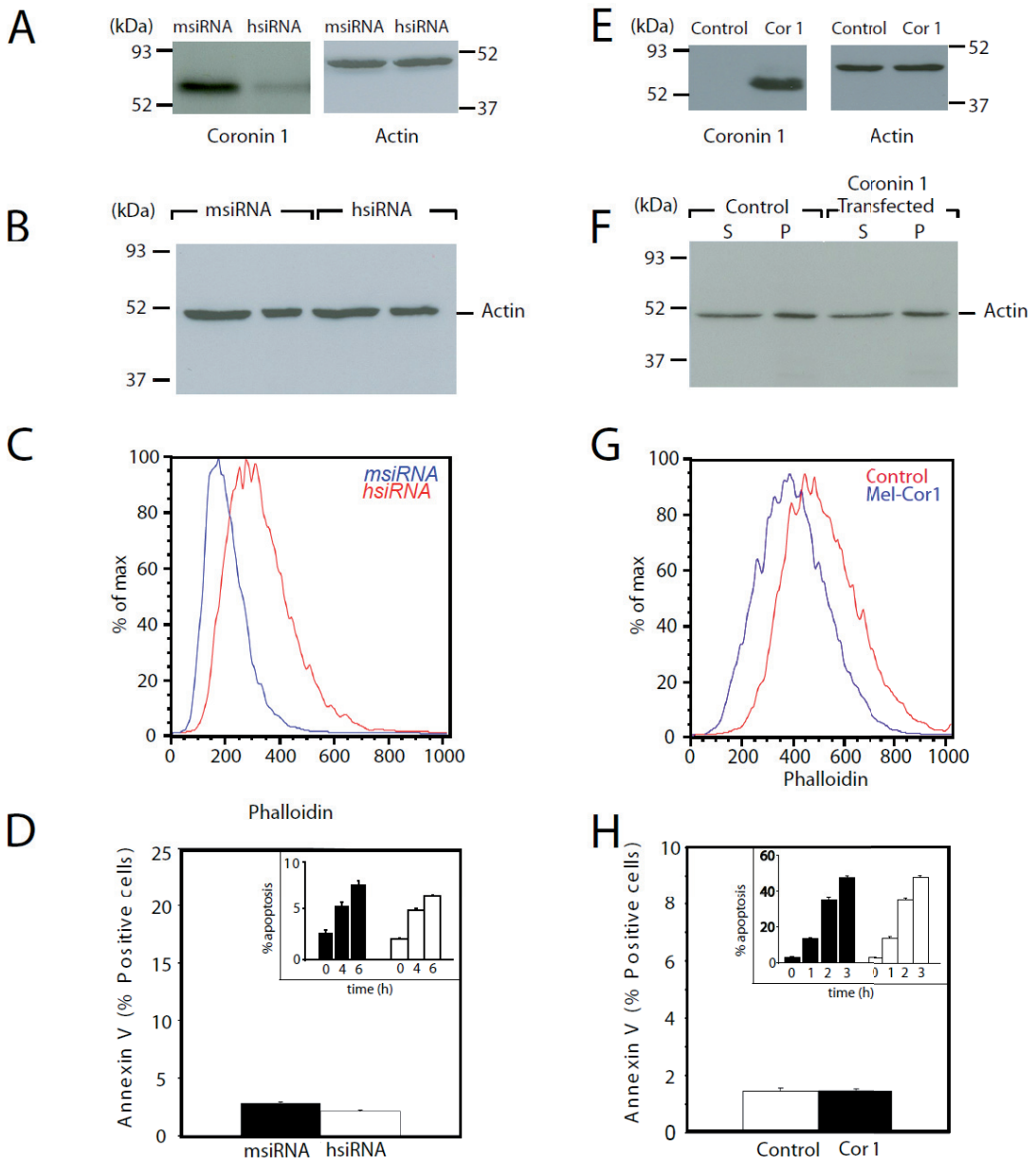


Figure 3: Phalloidin fluorescence, F/G actin ratio and apoptosis in siRNA transfected Jurkat T-cells and coronin 1 expressing Mel JuSo cells.

The human Jurkat T-cell line was transfected for 5 days with human anti-coronin1 siRNA or a control siRNA specific for the murine coronin 1 [238]. (A) Proteins from cell lysates obtained from the same cells as used under B-D were separated by SDS-PAGE and immunoblotted using anti-coronin 1 or anti-actin antibodies. (B) Jurkat T-cells transfected with either the human or control mouse anti-coronin 1 siRNA were analyzed for their G- and F-actin content. Cells were lysed and proteins present in supernatants (S) and pellets (P) prepared as described above were separated by SDS-PAGE and immunoblotted using anti-actin antibodies. (C) The cells used in B were stained using Alexa Fluor 568 phalloidin and analyzed by FACS. (D) Cells as under B and C were stained with PE-conjugated Annexin V and analyzed by FACS. Depicted are means +/- SD of cells from three cell samples analyzed individually. Cells as above were incubated for 0, 4 or 6 hours with 20 ng/ml of human TNFα to induce apoptosis (D, insert). (E-H) Mel JuSo cells stably expressing coronin 1 or coronin 1 negative control cells [237] were analyzed as described for the siRNA treated Jurkat T-cells under A-D with the exception of panel H were staurosporin (1 µM) was used instead of TNF-α (see insert).

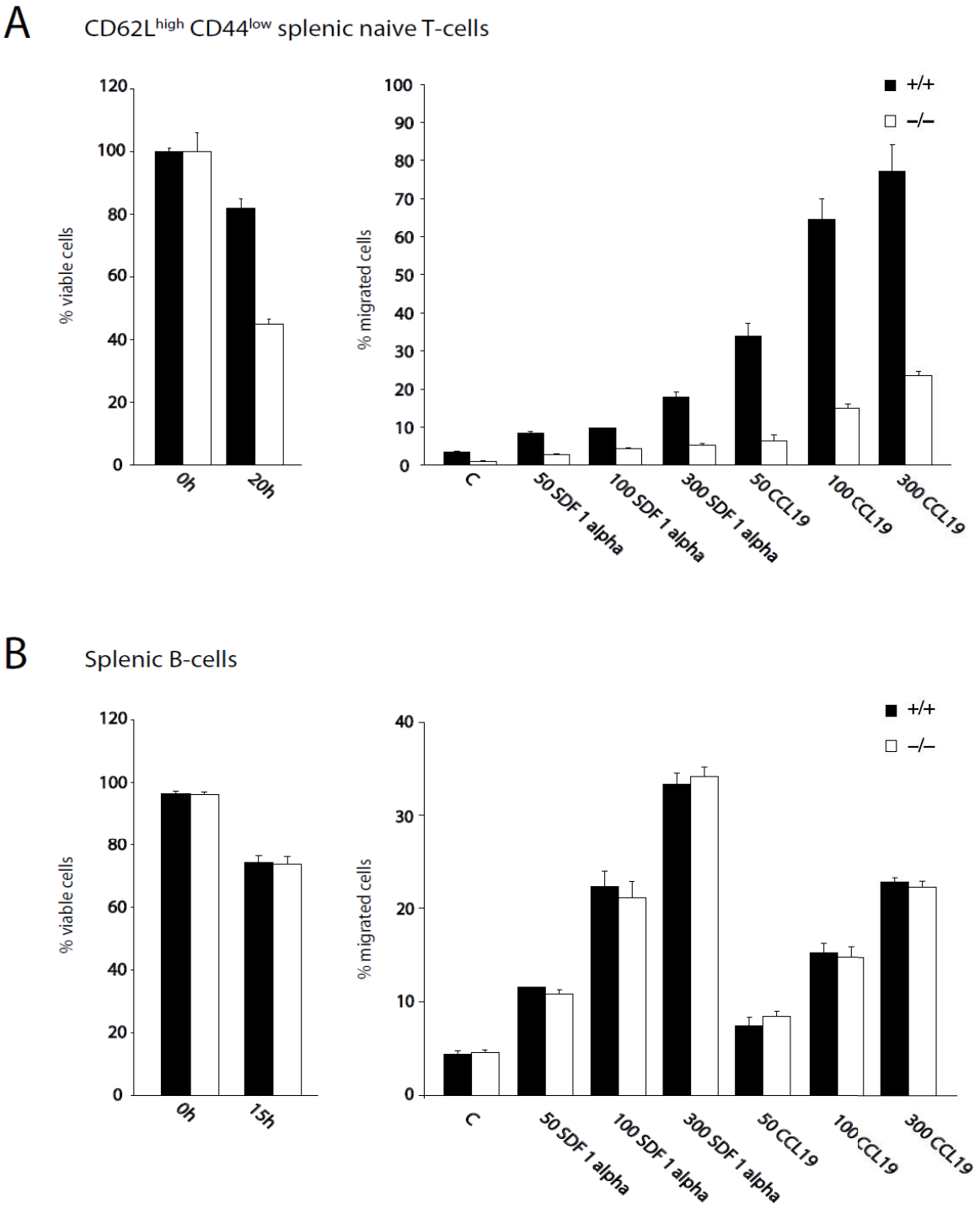


Figure 4: Spontaneous apoptosis and migration of naïve splenic T-cells and B-cells in the presence and absence of coronin 1.

(A) Spontaneous apoptosis and transwell migration of freshly isolated naïve splenic T-cells and (B) B-cells using the indicated chemo-attractants. Depicted are means +/– SD of duplicates. The experiments were repeated at least 3 times.

Using the same experimental setup we also analyzed isolated double positive thymocytes, $CD4^+$ single positive thymocytes and memory/effector T-cells for spontaneous apoptosis as well as their ability to migrate in a transwell migration assay (Supplementary Figure 3, see below). We found that coronin 1 deficient single positive thymocytes are affected to a similar extend as naïve T-cells regarding spontaneous apoptosis as well as transwell migration, but that coronin 1 deficient double positive thymocytes as well as memory/effector T-cells only show a mild increase in apoptosis as well as a minor reduction in transwell migration when compared to the wild type control.

The observation that single positive thymocytes and naïve T-cells are the only leukocytes which show severe defects upon coronin 1 deletion regarding both survival and migration prompted us to hypothesize that the two observed defects are connected and due to the same molecular defect which has to be naïve T-cell specific as well as coronin 1 dependent.

A feature which distinguishes T-cells from other leukocytes is their T-cell receptor. Downstream signaling of the T-cell receptor has been shown to be essential for naïve T-cell survival [183] as well as defective in coronin 1 deficient naïve T-cells [239]. An important question to address was thus whether the observed reduction in viability and migration of freshly isolated coronin 1 deficient naïve T-cells was a primary defect and thus cell intrinsic property of coronin 1 deficient naïve T-cells or rather a secondary defect due to the defect in T-cell receptor signaling.

To analyze whether the migration defect is a cell intrinsic property of coronin 1 deficient cells, or secondary to a T-cell receptor signaling defect, the following experiment was designed (Figure 5 A): Wild type and coronin 1 deficient single positive thymocytes as well as naïve T-cells ($CD62L^{high}$, $CD44^{low}$) were analyzed for phalloidin staining, spontaneous apoptosis as well as their ability to migrate in a transwell migration assay. After this first transwell migration assay, the migrated wild type and coronin 1 deficient cells were washed extensively and further recovered in fresh medium for 1 hour at 37°C. The recovered cells were used to carry out the same analysis as described above for a second time, that is phalloidin staining, spontaneous apoptosis and transwell migration.

If reduced viability and migration were cell intrinsic properties of coronin 1 deficient naïve T cells the previously migrated coronin 1 deficient cells should display the same defects when reanalyzed after recovery using the same assays and conditions.

As can be seen in Figure 5 B and C, freshly isolated coronin 1 deficient $CD4^+$ single positive thymocytes as well as naïve T-cells displayed the above described defects in cellular viability and migration as well as yielded an approximately 2 fold higher phalloidin fluorescence when compared to the wild type control.

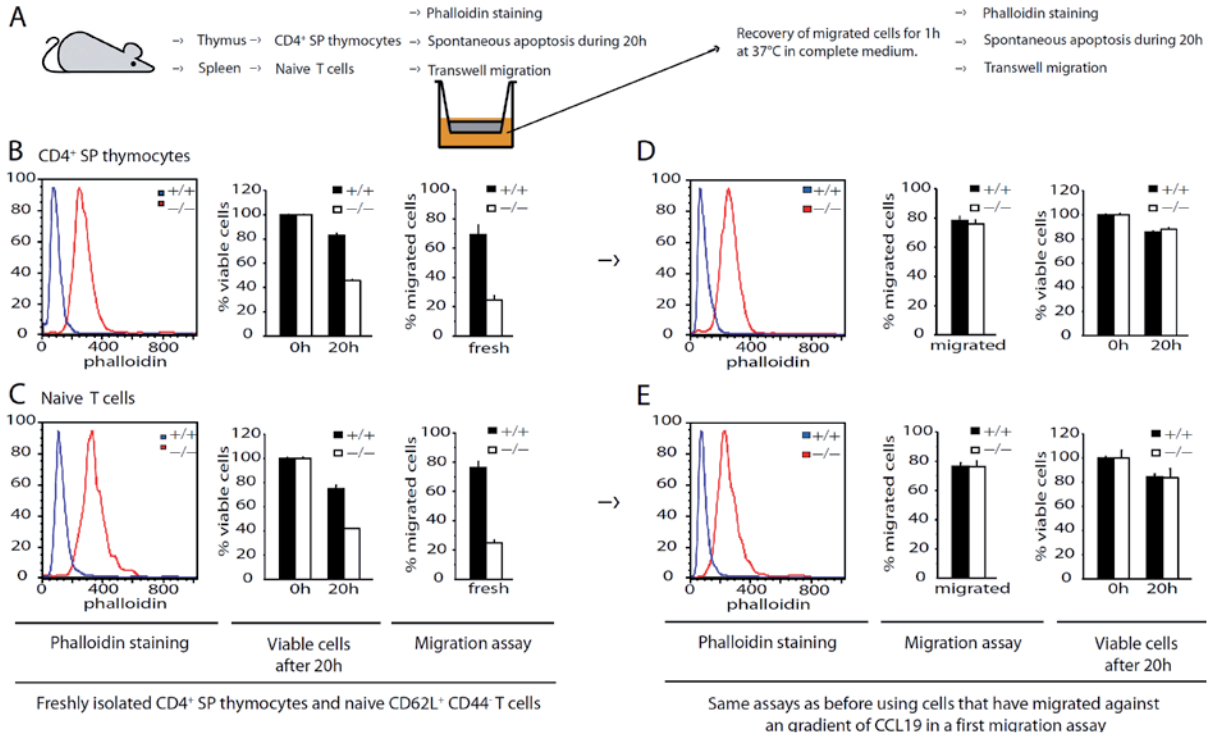


Figure 5: Phalloidin fluorescence, survival and migration of migrated wild type and coronin 1 deficient single positive thymocytes and naive T-cells following recovery.

(A) Schematic outline of the experiment as described under B-E. (B) Freshly isolated $CD4^+$ single positive thymocytes from which all dead and apoptotic cells had been removed (see method) were stained for phalloidin and assayed for spontaneous apoptosis as well as transwell migration against 300 ng/ml of CCL19 in the lower chamber. (C) Assay as described under B performed with naive splenic T-cells. (D and E) Cells as used in B and C were subjected to a transwell migration assay against 300 ng/ml of CCL19 in the lower chamber. Migrated cells were washed to remove all CCL19 and left to recover in complete medium for 1h at 37°C/5% CO_2. After this period, cells were again subjected to phalloidin staining and assayed for spontaneous apoptosis as well as transwell migration as done under B and C. Depicted are means +/– SD of duplicates. The experiments were repeated at least 3 times.

However, when coronin 1 deficient single positive thymocytes as well as naïve T-cells that had migrated in the first assay, were allowed to recover and were subjected to the second round of assays, neither higher levels of spontaneous apoptosis nor a defect in cell migration were observed when compared to the wild type control (Figure 5, panel D and E). In order to exclude that CCL19 by itself was responsible for the observed effects and not selection by migration, the same assay was performed without selection for migrated cells (Supplementary Figure 4). Survival and migration was analyzed for cells that had been left in migration medium for 3h, had been incubated with the same amount of CCL19 used for transwell migration for 3h or had migrated for 3h as described above (Supplementary Figure 4). As can be seen from our data CCL19 by itself had no effect on migration or survival of coronin 1 deficient cells in any of the conditions tested.

Noteworthy is also the fact that migrated and recovered coronin 1 deficient cells still showed the same elevated phalloidin fluorescence as observed for freshly isolated cells. Taken together, these data suggest that the observed migratory defect in coronin 1 deficient T-cells is a secondary defect which is directly linked with T-cell survival and not a result of disturbed F-actin dynamics.

2.3.3.3. T-cell viability and migration.

Our data presented thus far demonstrate a clear correlation between the reduced viability of coronin 1 deficient T-cells and their inability to migrate in a transwell assay. To directly address the importance of cellular viability in T-cell migration, the ability of wild type naïve T-cells in which apoptosis had been induced using the indicated reagents to migrate in a transwell migration assay was analyzed (Supplementary Figure 5). As expected, the manipulated cells were no longer capable of migrating to the same extent as the untreated control cells.

To analyze the result of increasing the viability of coronin 1 deficient naïve T-cells on migration as well as subsequent survival, cells were incubated for 20h with the anti apoptotic cytokine interleukin-7 (IL-7) [179, 187, 342, 343] and the same assays as described above (Figure 5) were performed, namely phalloidin staining, transwell migration and survival with or without added IL-7.

As shown in Supplementary Figure 6, IL-7 restored the viability of coronin 1 deficient naïve T-cells as well as their migratory capacity to wild type levels.

Together these data therefore establish that reduction of T-cell viability severely affects their ability to migrate, and conversively, that increasing the viability of coronin 1 deficient naïve T-cells restores their ability to migrate and survive, again highlighting the causative link between cellular viability and migration.

2.3.3.4. The molecular basis of the observed defects in coronin 1 deficient naïve T-cells

Maintenance of naïve T-cell homeostasis depends on signals that are predominantly delivered via the T-cell receptor [183, 310, 323, 344]. In the absence of coronin 1, naïve T-cells show a specific defect in T-cell receptor dependent signaling and in particular calcium signaling (see Figure 6A and [239]). The specificity of this defect can be demonstrated by using a T-cell receptor specific stimulus versus PMA/Ionomycin to induce T-cell proliferation.

While in the presence of the T-cell receptor specific stimulation proliferation was strongly reduced in coronin 1 deficient naïve T-cells, stimulation with PMA/Ionomycin, which bypasses the T-cell receptor thus directly acting on downstream signaling pathways [101], was comparable between coronin 1 deficient cells and the wild type control (Figure 6B).

A direct consequence of T-cell receptor ligation and subsequent elevation of the cytosolic calcium concentration is activation of the phosphatase calcineurin [115, 345, 346]. Activation of calcineurin results in nuclear translocation of nuclear factor of activated T-cells (NFAT) and subsequent transcriptional activation of target genes like IL-2 [115, 201, 347, 348].

One key molecule in naïve T-cell survival [349, 350], which has been previously reported to depend on coronin 1 for its activation during mycobacterial infection in macrophages is calcineurin [251]. We therefore measured the levels and the activity of calcineurin in freshly isolated wild type or coronin 1 deficient naïve T-cells. While the total levels of calcineurin Aβ, which is the predominantly expressed isoforms in T lymphocytes [350], were identical in wild type and coronin 1 deficient cells (Figure 6C), calcineurin phosphatase activity was severely reduced upon T-cell receptor ligation in the absence of coronin 1 (Figure 6D).

However when coronin 1 deficient as well as wild type naïve T-cells were stimulated with PMA and Ionomycin, no significant differences in calcineurin activation were detected (Figure 6E), consistent with the proliferation data presented above.

Our data therefore demonstrate that the defect in coronin 1 deficient naïve T-cells is T-cell receptor specific as both coronin 1 deficient as well as wild type cells showed similar responses upon stimulation with the T-cell receptor bypassing reagents PMA and Ionomycin, whereas a T-cell receptor specific stimulus resulted in defective calcium signaling, calcineurin activation as well as proliferation in coronin 1 deficient naïve T-cells. We conclude that coronin 1 by regulating calcium/calcineurin signaling upon T-cell receptor stimulation is a key regulator of naïve T-cell survival.

In order to independently show that calcineurin activation is the critical step in the signaling regulated by coronin 1 the following experiment was performed: Migrated wild type and coronin 1 deficient naïve T-cells were incubated with or without the specific calcineurin inhibitor cyclosporine A (CsA) in the presence or absence of either α-CD3/CD28 coated dynal-beads or plate bound α-CD3/CD28 (Figure 7A and B). After 20h, cell viability was determined using Annexin V/7-AAD labeling. The results show that α-CD3/CD28 treatment increases the rate of apoptosis in coronin 1 deficient naïve T-cells as compared to the untreated control. Importantly, the coronin 1 deficient phenotype is phenocopied by incubating wild type cells with CsA. Also, the beneficial effect of IL-7 on cell survival was ablated by α-CD3/CD28 treatment in the absence of coronin 1, indicating that naïve T-cells are susceptible to activation induced cell death upon α-CD3/CD28 stimulation in the absence of coronin 1 or upon inhibition of calcineurin via CsA. One expected consequence of defective calcineurin activation in T-cells is the down-regulation of the anti-apoptotic molecule Bcl-2 [350]. To analyze Bcl-2 downregulation, naïve T-cells were isolated from wild type and coronin 1 deficient mice and the levels of Bcl-2 analyzed in lysates by immunoblotting of SDS-PAGE separated proteins (Figure 7C). Bcl-2 levels were significantly lower in coronin 1 deficient cells as compared to the wild type control. Separately, when wild type cells were treated with the calcineurin inhibitors CsA and FK506, Bcl-2 expression was significantly lower as compared to the control (Figure 7D), again showing that blocking calcineurin in wild type cells phenocopies the coronin 1 deletion.

Furthermore, freshly isolated and migrated naïve T-cells (as described in Figure 5) were analyzed for Bcl-2 expression (Figure 7e). While in freshly isolated cells Bcl-2 level were significantly lower in the absence of coronin 1 migrated coronin 1 deficient cells showed levels similar to the wild type control.

Finally, given the recent report that that inhibition of calcineurin in the presence of T-cell receptor stimulation and co-stimulation via CD28 leads to a super-induction of caspase 3, rendering cells susceptible to apoptosis [351] the levels of caspase 3 in wild type and coronin 1 deficient naïve T cells were analyzed (Figure 7F). While in freshly isolated cells, caspase 3 levels were significantly elevated in the absence of coronin 1, migrated coronin 1 deficient cells showed levels similar to the wild type control.

These findings explain the elevated levels of apoptosis observed in freshly isolated coronin 1 deficient naïve T-cells in molecular terms, namely that the lack of coronin 1 causes a defect in calcineurin activation upon T-cell receptor stimulation which in turn leads to a massive induction of caspase 3 and reduced expression of bcl-2.

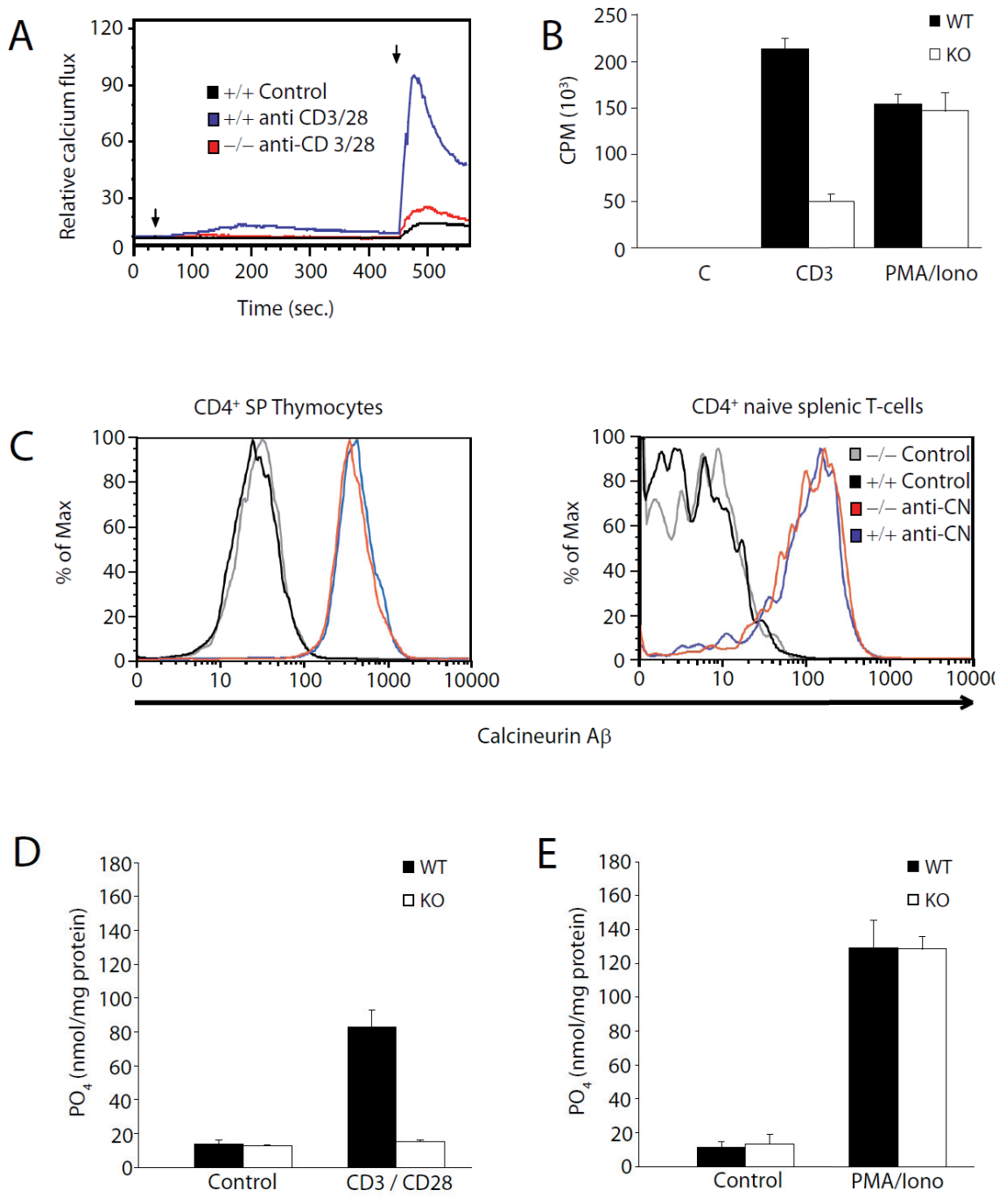


Figure 6: T-cell receptor mediated calcineurin activation in wild type and coronin 1 deficient naive T-cells.

(A) Calcium measurements in Fluo-3 loaded T-cells were performed as described in methods. The first arrow indicates the addition of antibodies in calcium free medium whereas the second arrow indicates the addition of calcium. (B) Proliferation of coronin 1 deficient and wild type naive T-cells using the indicated stimuli as described in methods. Depicted are means +/– SD of triplicate cultures. (C) FACS analysis of Calcineurin Aβ expression in $CD4^+$ single positive thymocytes or naïve splenic T-cells. (D) Calcineurin activity was analyzed in naive coronin 1 deficient and wild type T-cells left untreated or activated with anti CD3/28 and cross linking secondary antibody for 15 minutes, as described in methods. Depicted are means +/– SD of triplicates. (E) Calcineurin activity was analyzed in naive coronin 1 deficient and wild type T-cells left untreated or activated with PMA/Ionomycin for 15 minutes, as described in methods. Depicted are means +/– SD of triplicates.

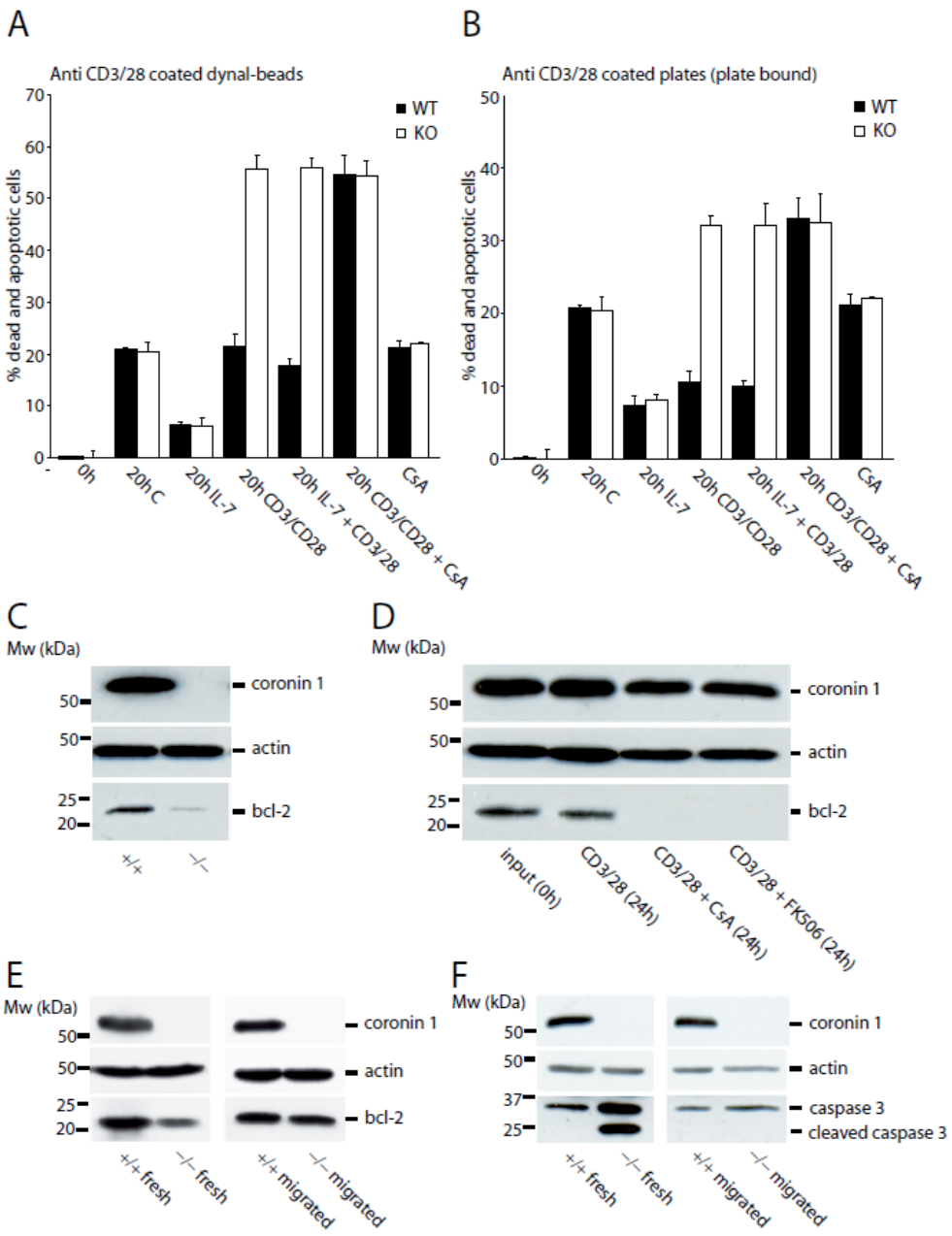


Figure 7: T-cell receptor signaling in wild type and coronin 1 deficient naive T-cells in the presence and absence of IL-7 and calcineurin inhibitors

Migrated wild type and coronin 1 deficient naive T-cells were analyzed for their survival using the indicated reagents and (A) α-CD3/28 coated dynal beads (T-cell expander, Dynal) or (B) plate bound α-CD3/28 (coated with 5/10 µg/ml of the indicated antibodies in PBS at 4°C o/n). Cyclosporine A was used at 5µM and IL-7 at 20ng/ml. (C) Bcl-2 levels were detected in lysates of freshly isolated naive T-cells from wild type or coronin 1 deficient mice using hamster α-mouse Bcl-2 from BD. (D) Bcl-2 levels were detected in lysates of freshly isolated wild type naive T-cells or the same cells incubated o/n on α-CD3/28 plates (coated with 5/10 µg/ml of the indicated antibodies in PBS at 4°C o/n) with or without the addition of the calcineurin inhibitors cyclosporine A or FK506 (5µM and 10µM respectively). (E) Bcl-2 levels were detected in lysates of freshly isolated or migrated naive T-cells from wild type or coronin 1 deficient mice. (F) Caspase-3 levels were detected in lysates of freshly isolated or migrated naive T-cells from wild type or coronin 1 deficient mice.

2.3.4. Discussion

Coronin 1, also known as P57 or TACO (for tryptophan aspartate containing coat protein [237]), is a leukocyte specific molecule that is crucial for the survival and migration of naïve T-cells in the periphery [239, 263, 333-335]. Based on the ~25% homology of coronin 1 with *Dictyostelium* coronin, a molecule that co-purifies with an actin/myosin complex, as well as *in vitro* actin binding assays, it has been widely suggested that coronin 1 allows cellular survival and migration by preventing F-actin formation. Since coronin 1 deletion results in a ~2-fold increase in phalloidin staining, absence of coronin 1 was concluded to lead to excessive F-actin accumulation thereby preventing cell migration and inducing apoptosis [263]. In this paper, we show that coronin 1 does not modulate the F-actin cytoskeleton in leukocytes and non-leukocytes, and furthermore, that F-actin accumulation does not correlate with apoptosis in naïve T-cells as proposed previously [263]. Instead, we find that both survival as well as migration defects occurred as a result of defective signaling and calcineurin activation in coronin 1 deficient naïve T-cells. We further demonstrate that cellular viability and the ability to migrate are linked. When freshly isolated coronin 1 deficient cells are selected for their ability to migrate in a transwell migration assay, the migrated, coronin 1 deficient cells survive and migrate to a similar extend as observed for wild type cells.

We conclude that instead of regulating the F-actin cytoskeleton, coronin 1 functions in balancing pro and anti-apoptotic signals by regulating Ca^{2+} fluxes and calcineurin activation downstream of the T-cell receptor.

2.3.4.1. Coronin 1 and F-actin

The evidence linking coronin 1 to the modulation of the F-actin cytoskeleton is predominantly based on the observed two-fold difference in phalloidin fluorescence between wild type and coronin 1 deficient cells [263, 333, 334]. Several lines of evidence however argue against the differential phalloidin fluorescence being an appropriate measure for altered F-actin dynamics in cells expressing different levels of coronin 1.

First, a sensitive biochemical assay, that accurately measured the influence of chemo-attractant induced F-actin accumulation on the F/G-actin ratio, failed to show any difference in the F/G actin ratio between coronin 1 negative and coronin 1 expressing cells. This is not only the case for T-cells, but also for macrophages deficient for coronin 1 [251] or depleted for coronin 1 using siRNA [238], Jurkat cells depleted for coronin 1 using siRNA and Mel JuSo cells over expressing coronin 1 (this manuscript). Second, depletion of coronin 1 in macrophages, B cells and neutrophils does not affect any of the F-actin dependent functions, such as phagocytosis, macropinocytosis and migration [238, 251, 252, 352]. Third, rather than being an indication of reduced F-actin formation in coronin 1 expressing cells, the reduced phalloidin fluorescence may be a result of a reduction in phalloidin binding to F-actin because of the presence of large amounts of coronin 1 that sterically hinder phalloidin binding to F-actin at the cell cortex. A similar observation has been reported previously, where the authors failed to stain F-actin cables using phalloidin due to accessory F-actin binding factors but were able to label these using anti-actin antibodies [353]. These findings are also consistent with the observation that despite a twofold difference in phalloidin fluorescence we were unable to detect any differences regarding cortical F-actin levels using anti-actin antibodies in coronin 1 deficient versus wild type cells [239].

While *in vitro*, coronin 1 has been shown to co-precipitate with F-actin [240] [354], and modulate F-actin filament dynamics [355-357], a survey of all available literature on coronin 1 reveals that no data exist showing that coronin 1 directly participates in modulating F-actin dynamics *in vivo* (Table S1, see below). Most of the previous studies linking coronin 1 activity to F-actin dynamics rely either on (i) *in vitro* co-sedimentation of coronin 1 fusion proteins with F-actin, (ii) expression of coronin 1 domains in cell lines or (iii) a difference in phalloidin fluorescence (see Table S1, below).

2.3.4.2. F-actin and Apoptosis

One important argument put forward to implicate coronin 1 in preventing F-actin induced apoptosis is based on the observed increase in cell death upon forced F-actin formation in yeast [358] as well as mammalian cell lines [359]. However, when the actin cytoskeleton of primary murine naïve T-cells was polymerized or depolymerized using actin modulating drugs, no differences in apoptosis were apparent (this study). Further, none of the other

leukocyte populations such as B-cells and macrophages, which have been found to be sensitive to F-actin accumulation [360], display a survival defect despite showing elevated phalloidin staining similar to T-cells.

Moreover, induction of apoptosis in a variety of coronin 1 expressing and deficient cell types by TNF-α or staurosporin demonstrated that coronin 1 does not protect cells from apoptosis. It is noteworthy that at least staurosporin induced apoptosis involves F-actin and that proteins which prevent F-actin formation such as human gelsoline, unlike coronin 1, are able to prevent the onset of apoptosis upon staurosporin treatment [361].

We therefore conclude that F-actin modulation cannot account for the elevated levels of apoptosis in coronin 1 deficient cells and that coronin 1 does not protect cells from apoptosis.

2.3.4.3. Coronin 1 and Calcineurin Activation

In coronin 1 deficient cells calcineurin activation following T-cell receptor ligation cannot occur because of defective calcium mobilization, whereas when the T-cell receptor is bypassed using PMA/Ionomycin, calcineurin as such is functional in coronin 1 deficient T-cells. Interestingly, there is a striking resemblance between a recently described calcineurin Aβ deficient mouse model and mice lacking coronin 1 [349] [350]. Both calcineurin Aβ and coronin 1 deficient single positive thymocytes as well as naïve T-cells are specifically depleted *in vivo* and show elevated spontaneous apoptosis *in vitro* while double positive thymocytes as well as memory/effector T-cells and B-cells show none or only mild defects. Experiments with calcineurin Aβ deficient mice have demonstrated that defective activation of the phosphatase leads to susceptibility of T-cells to apoptosis induction due to an imbalance between pro- and anti-apoptotic stimuli, leading to reduced naïve T-cell viability and finally cell death. Consistent with these results, we found that inhibition of calcineurin in naïve wild type T cells phenocopies the coronin 1 deficient T cells, demonstrating that calcineurin activation is the critical step in signaling regulated by coronin 1.

The results presented in this manuscript not only highlight the importance of coronin 1 in calcineurin activation but also provide a rational for the observed migration and survival defects in the absence of coronin 1. As we demonstrate, Bcl-2 levels are reduced and caspase 3 is induced at high level in coronin 1 deficient naïve T-cells as a consequence of defective calcineurin activation. Furthermore, the data described here provide, on the molecular level, an explanation for the diverse phenotypes such as migration and survival defects described for naïve T-cells from coronin 1 deficient mice.

The here presented results highlight the importance of coronin 1 dependent calcium and calcineurin signaling for cellular survival. They also provide a direct link between coronin 1, T-cell receptor mediated calcineurin activation and calcineurin dependent cellular viability. In addition, these data link cellular viability with the migratory capacity of a cell and thus provide an explanation for both the survival as well as the migration defect in coronin 1 deficient naïve T-cells. The results presented here further offer an explanation of why the observed defects are specific for single positive thymocytes as well as naïve T-cells as these cells are heavily dependent on calcineurin Aβ for their survival [38].

The definition of coronin 1 as an essential regulator of calcineurin mediated cellular viability in naïve T-cells and consequently their ability to migrate may allow a better understanding of leukocyte activation and homeostasis.

2.3.5. Acknowledgments

We thank Lotte Kuhn for technical assistance and members of the laboratory for excellent discussions. This work was supported by grants from the Swiss National Science Foundation and the Kanton Basel-Stadt.

2.3.6. Supplementary Figures and tables

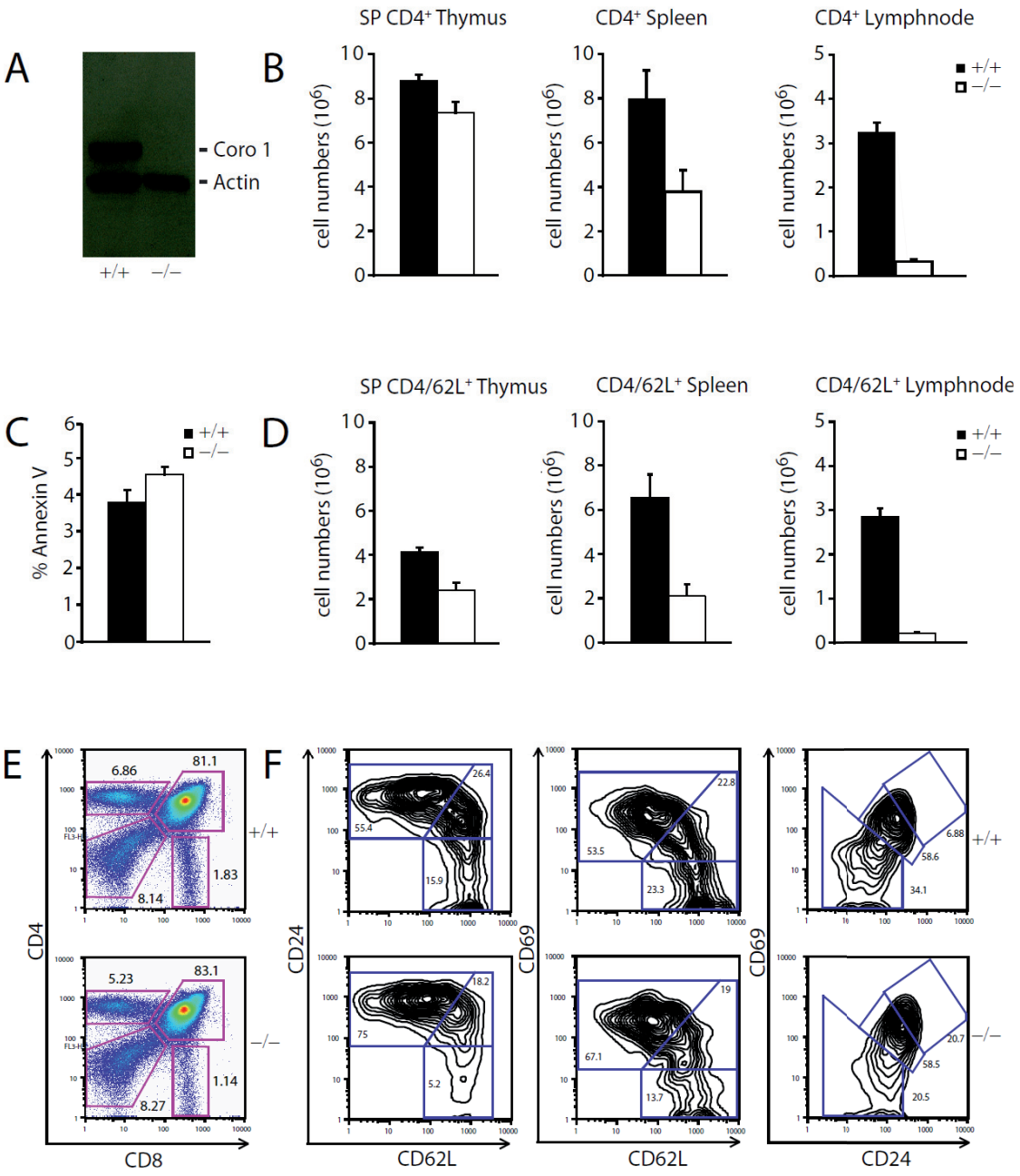


Figure S1: Phenotypic characterization, thymocytes and T-cells in wild type and coronin 1 deficient mice.

(A) T-cells from wild type and coronin1 deficient mice were lysed in SDS sample buffer, separated by SDS page and immune-blotted for coronin 1 and Actin. (B) Single cell suspensions from thymus, spleen and lymph nodes were stained for CD4 positive cells and the indicated cell populations quantitated. (C) Annexin V stainings of $CD4^+$ SP thymocytes from wild type and coronin 1 deficient animals were quantitated. Quantitation was carried out with data obtained from at least 3 animals. Depicted are mean +/- SD. (D) Single cell suspensions from thymus, spleen and lymph nodes were stained for CD4 and CD62L positive cells and the indicated cell populations quantitated. (E) Dot blot of thymus suspensions from representative wild type and coronin 1 deficient animals stained with the indicated antibodies. (F) $CD4^+$ single positive thymocytes from wild type and coronin 1 deficient animals were stained with the indicated antibodies. Shown are representative contour blots.

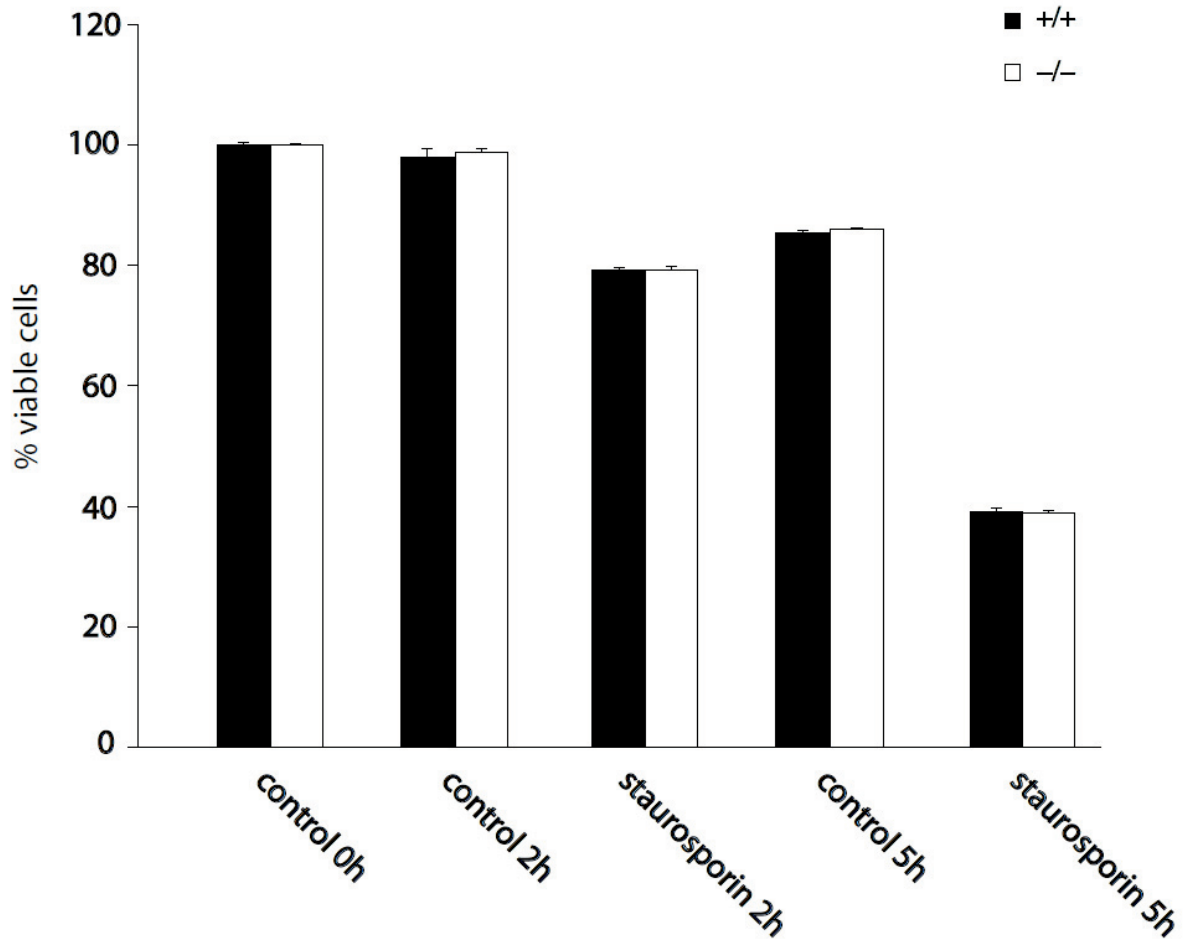


Figure S2: Staurosporin induced apoptosis of wild type and coronin 1 deficient B-cells

Wild type and coronin 1 deficient B-cells were incubated for the indicated time with DMSO (carrier) (C) or staurosporin (STS, 1 µM) and the percentage of viable cells was determined using Annexin V and 7-AAD labeling.

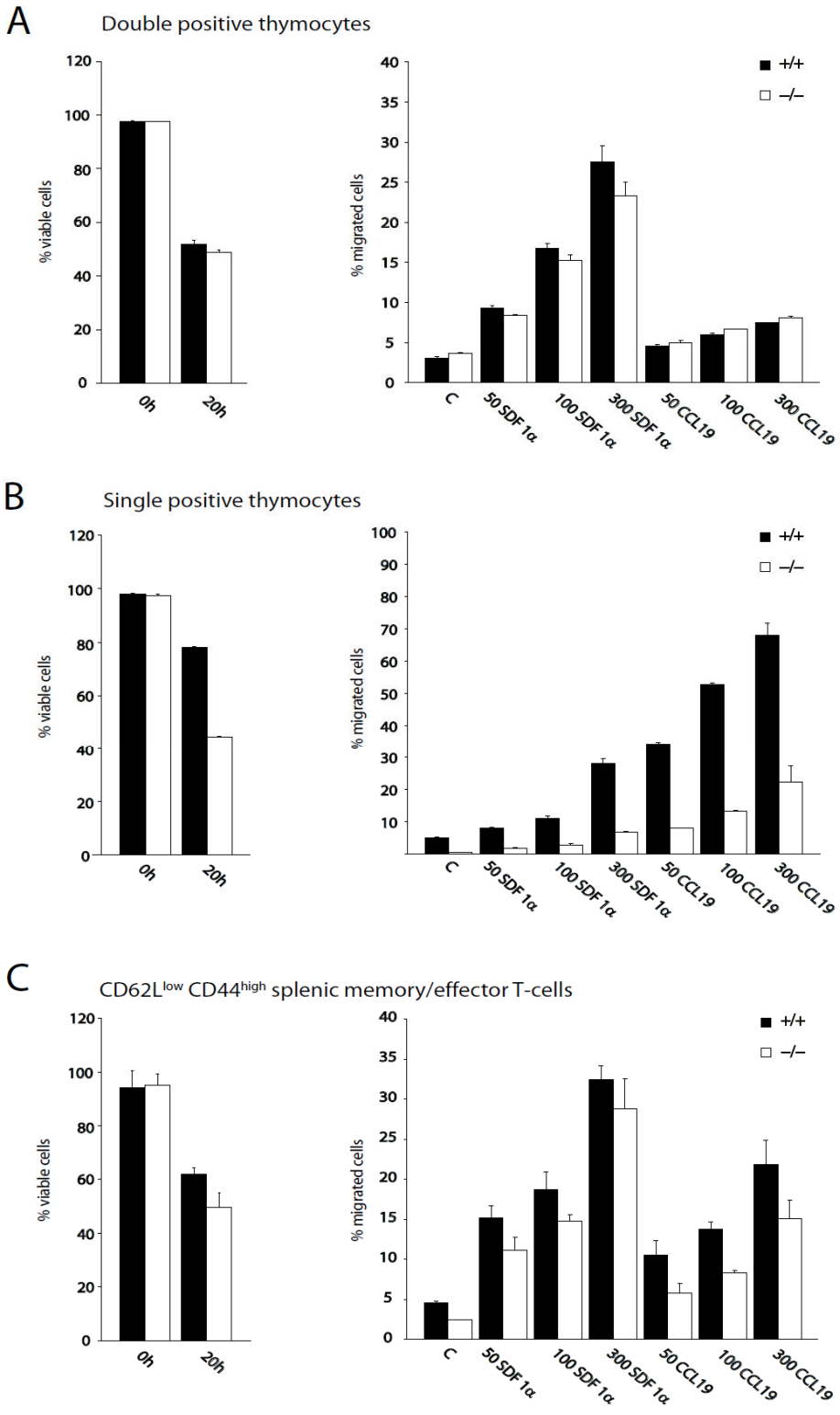


Figure S3: Spontaneous apoptosis and migration of double positive thymocytes, single positive thymocytes and memory/effecter T-cells in the presence and absence of coronin 1.

(A) Spontaneous apoptosis and Transwell migration of double positive thymocytes. (B and C) Same setup as described under A for single positive thymocytes (B) and memory/effector T-cells (C). Depicted are means +/– SD of duplicates. The experiments were repeated at least 3 times.

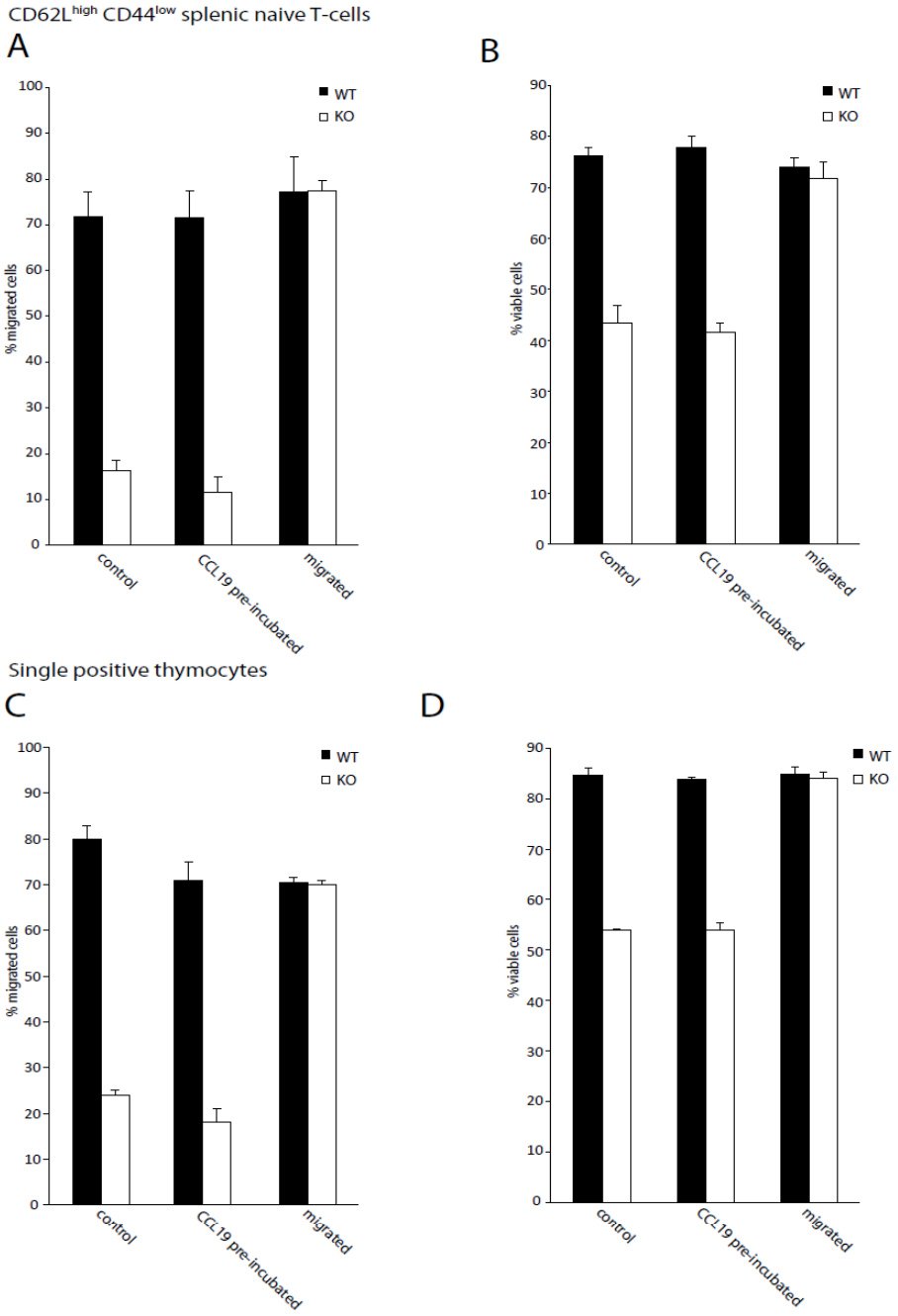


Figure S4: Spontaneous apoptosis and migration of differentially treated naïve splenic Tcells and single positive thymocytes in the presence and absence of coronin 1.

(A/B) Spontaneous apoptosis and transwell migration (3h against 300 ng/ml of CCL19) of naïve splenic T-cells and (C/D) single positive thymocytes analyzed after migration (3h against 300 ng/ml of CCL19), incubation in migration medium (3h) only or migration medium containing 300 ng/ml of CCL19 (3h) as a 9 control. Depicted are means +/– SD of duplicates. The experiments were performed at least 2 times.

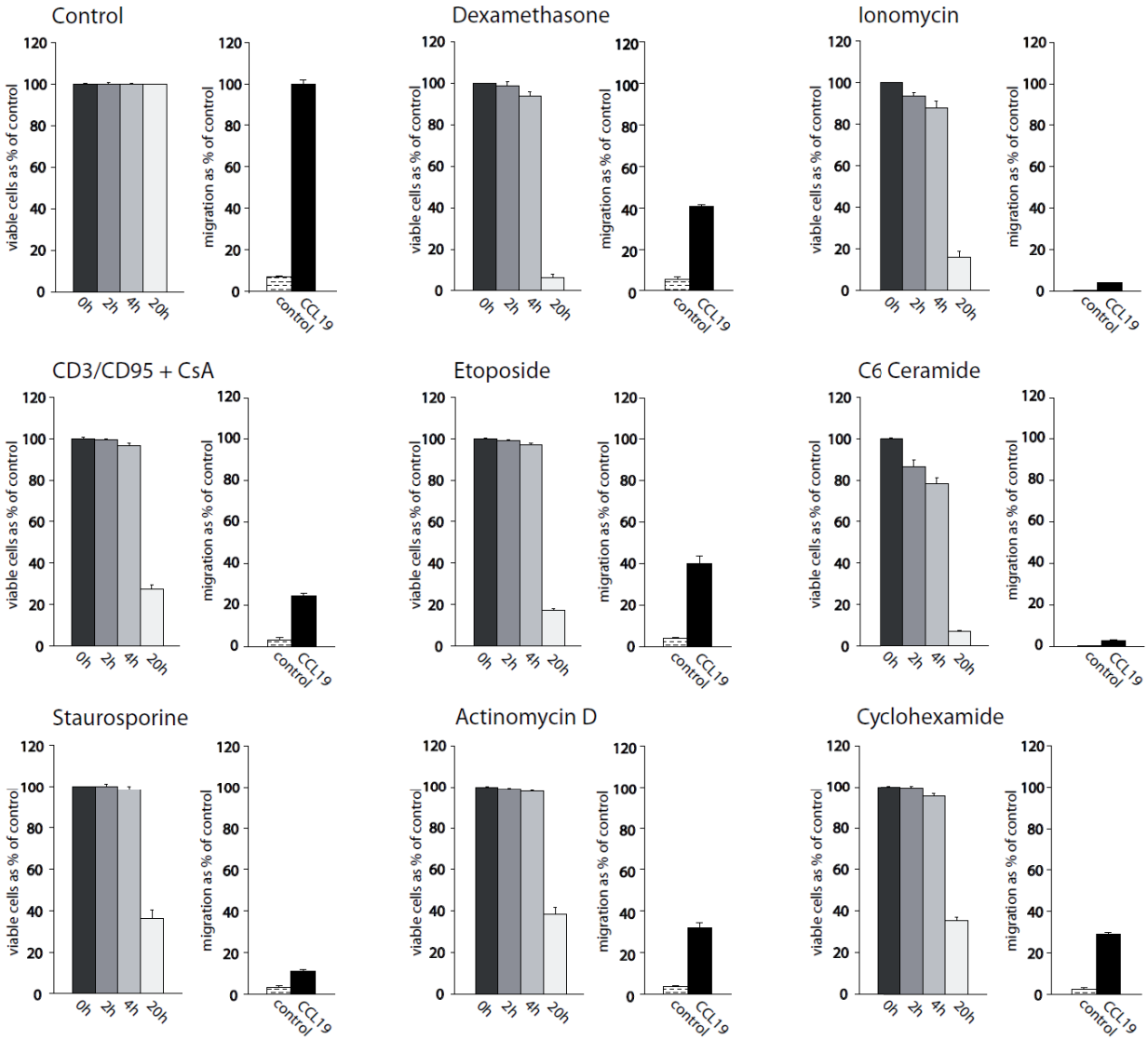


Figure S5: T-cell viability and migration in the presence and absence of coronin 1.

Naïve wild type T-cells were left untreated or exposed to Dexamethasone (0,5 µM), Ionomycin (2 µM), plate bound anti CD3 and anti CD95 (Jo-2) (each 10 µM/ml) supplemented with 10 µM of Cyclosporine A, Etoposide (2 µM), C6 Ceramide (50 µM), Staurosporine (0,1 µM), Actinomycin D (1 µM) or Cyclohexamide (20 µM). At the indicated time points the number of viable cells was determined using Annexin V and 7-AAD staining. The percentage of viable (double negative) cells is expressed as % of the untreated control which was set to 100%. Cells exposed for two hours to the indicated drugs at the indicated concentrations were analyzed for their ability to migrate in a transwell migration assay for two hours against migration medium only as a control or medium supplemented with 300 nM of CCL19, as described in methods. Migrated cells were quantitated on a FACS Calibur. Counts were normalized to the percentage of untreated cells, migrated against CCL19, which was set to 100%. Depicted are means +/– SD of duplicates.

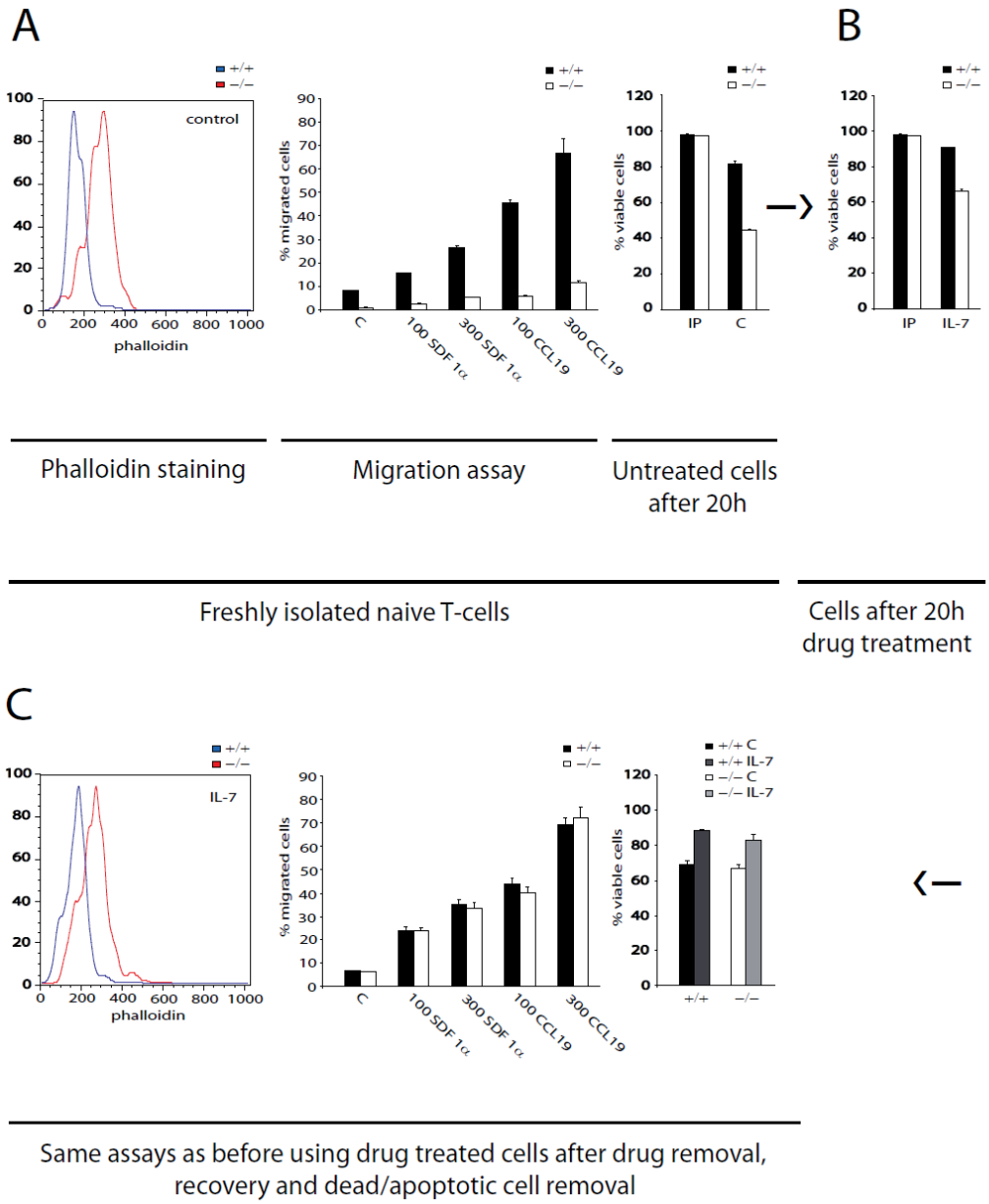


Figure S6: Phalloidin fluorescence, survival and migration of wild type and coronin 1 deficient naive Tcells following IL7 treatment.

(A) Freshly isolated naive splenic T-cells from which all dead and apoptotic cells had been removed (see method) were stained for phalloidin and assayed for spontaneous apoptosis as well as transwell migration against 300 ng/ml of CCL19 in the lower chamber. (B) Cells as used in A were treated with interleukin-7 (IL-7, 20ng/ml, mouse recombinant from R&D). After this period, cells were analyzed using Annexin V/7-AAD staining to determine the number of viable cells. (C) Dead cells were removed from the IL-7 treated population as described in methods, viable cells were subjected to phalloidin staining, assayed for spontaneous apoptosis (with or without the addition of 20ng/ml recombinant IL-7) and transwell migration as done under A. Depicted are means +/– SD of duplicates. The experiments were performed at least 2 times.

Table S1: Mammalian Coronin 1 and F-actin: A Survey of the Existing Coronin 1A Literature

Author	Date of Publication	Coronin1 and F-actin
Suzuki et al.,	1995	- GST-Coronin 1 co-sedimentation with F-actin [240]
Garcia-Higuera et al.,	1996	- No direct F-actin interaction and modulation data [244]
Grogan et al.,	1997	- No direct F-actin interaction and modulation data [362]
David et al.,	1997	- Coronin 1 as part of the *Listeria* actin tail [363]
Machesky et al.,	1997	- Coronin 1 co-elution with Arp2/3 from neutophil lysates [354]
Ferrari et al.,	1999	- No direct F-actin interaction and modulation data [237]
Mishima et al.,	1999	- GST-Coronin 1 co-sedimentation with F-actin - Truncated versions of Coronin were used to define F-actin interacting regions - Truncated versions of Coronin 1 interfere with cell spreading and inhibit lammelipodia extension when over expressed in 3T3 fibroblasts [364]
Reeves et al.,	1999	- No direct F-actin interaction and modulation data [258]
Gatfield et al.,	2000	- No direct F-actin interaction and modulation data [255]
Didichenko et al.,	2000	- No direct F-actin interaction and modulation data [365]
Itoh et al.,	2002	- No direct F-actin interaction and modulation data [257]
Zheng et al.,	2003	- No direct F-actin interaction and modulation data [250]
Oku et al.,	2003	- GST-Coronin 1 co-sedimentation with F-actin - Truncated versions of Coronin were used to define F-actin interacting regions [338]
Nal et al.,	2004	- No direct F-actin interaction and modulation data [318]
Oku et al.,	2005	- No direct F-actin interaction and modulation data [366]
Gatfield.,	2005	- Definition of Coronin 1 domains that are important for trimerization, plasma membrane attachment as well as F-actin interaction [241]
Yan et al.,	2005	- TAT-fusion constructs of Coronin-1 WD domains introduced into RAW 264.7 cells interfere with early steps of phagosome formation - Macrophages depleted of Coronin 1 by siRNA showed reduced phagocytosis [339]

Appleton et al.,	2006	- Crystal structure of murine Coronin 1[242]
Liu et al.,	2006	- GST-Coronin 1 co-sedimentation with F-actin - Truncated versions of Coronin were used to define F-actin interacting regions - Electron microscopy reveals cross linking of F-actin filaments by Coronin 1 [367]
Foeger et al.,	2006	- Phalloidin fluorescence taken as evidence that Coronin 1 deficient naive T-cells accumulate F-actin. Used to explain elevated T-cell death in the absence of Coronin 1 as well as defective T-cell migration - Flag-Coronin 1 WT and S2A but not S2D co-immunoprecipitate with Arp2. Correlation of these results with phalloidin fluorescence of cells over expressing the above mentioned constructs [263]
Brieher et al.,	2006	- In vitro F-actin depolymerization assays of *Listeria* actin comet tails using purified proteins [355]
Yan et al.,	2007	- TAT-fusion constructs of Coronin-1 WD domains introduced into neutrophils interfered with transwell migration, cell adhesion, spreading and phagocytosis - In vitro F-actin polymerization assay using purified GST-Coronin 1 [340]
Jayachandran et al.,	2007	- No defects in F-actin dependent processes such as migration, phagocytosis and cell spreading were detected in Coronin 1 deficient macrophages - F-actin levels were found to be similar in wild type and Coronin 1 deficient cells by biochemical analysis [251]
Jayachandran et al.,	2008	- No defects in actin dependent processes such as migration, phagocytosis and cell spreading were detected in J774 macrophages depleted of Coronin 1 by siRNA - F-actin levels were found to be similar in wild type and Coronin 1 depleted cells by biochemical analysis [238]
Galkin et al.,	2008	- Electron microscopy and 3D reconstitution of Coronin 1 bound to F-actin - Coronin 1 was found to stabilize F-actin [356]
Haraldsson et al.,	2008	- Phalloidin fluorescence taken as evidence that Coronin 1 deficient naive T-cells accumulate F-actin - B-cells were found to be functional and not prone to elevated cell death despite the fact that they displayed elevated phalloidin staining in the absence of Coronin 1 as observed for T-cells [333]

Mueller et al.,	2008	- No defects in actin dependent processes such as cell spreading and membrane ruffling were detected in Coronin 1 deficient naive T-cells [239]
Kueh et al.,	2008	- In vitro F-actin depolymerization assays using purified proteins [357]
Oku et al.,	2008	- Analysis of Coronin 1/F-actin association using EGFP fusion proteins [368]
Shiow et al.,	2008	- Phalloidin fluorescence taken as evidence that Coronin 1 deficient T-cells accumulate F-actin - *In vitro* Coronin 1/F-actin co-sedimentation as well as F-actin polymerization assay [334]
Mugnier et al.,	2008	- Phalloidin fluorescence taken as evidence that Coronin 1 deficient T-cells accumulate F-actin [335]
Shiow et al.,	2008	- No direct F-actin interaction and modulation data [369]
Combaluzier et al.,	2009	- No F-actin defects in coronin 1 deficient B cells [352]
Combaluzier et al.,	2009	- Chemotaxis, phagocytosis as well as cell spreading was found to be unaffected in murine coronin 1 deficient neutrophils when compared to the wild type control [252]

3. MATERIALS AND METHODS

3.1. Mice and tissue culture

Mice lacking coronin-1 were generated as described [251]. Experiments shown used mice that were backcrossed to C57BL/6 for at least four generations, using wild-type littermates as a control. Animal experiments were approved by the Kantonales Veterinäramt Basel-Stadt.

Human Jurkat T-cells were grown in medium consisting of RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Gibco), 10% FBS (Gibco) and penicillin-streptomycin (Mice used in this study were generated by Massner J.).

3.2. Biochemical methods

We performed cell homogenization, lysis and immunoblotting as described [31, 34, 237]. Polyclonal anti-coronin-1 serum has been described[241]. Actin was detected using MAB1501 (Chemicon) at 1:500 dilution. Anti-GFP was from Roche (monoclonal mouse IgG1, clones 7.1 and 13.1). Anti-phosphotyrosine (horseradish peroxidase-conjugated 4G10) was from Upstate. Anti-PtdIns(4,5)P_2 (clone 2C11) was from Santa Cruz. Antibodies to PLC-γ1, phospho–PLC-γ1, ERK1/2, phospho-ERK-1/2, p38, phospho-p38, IκB-α and phospho-IκB-α were from Cell Signaling Technology. Anti-CD3 and anti-CD28 were from BD Biosciences, and the secondary mouse anti-hamster IgG was from R&D (clone MAH1.12). SLP-76 antibodies were from Santa Cruz; Lat and phospho-Lat (171) antibodies were from Cell Signaling Technology; phospho-Lat (136) antibody was from Sigma-Aldrich; and goat anti-hamster Alexa Fluor 568 was from Invitrogen. Antibodies to Calcineurin-Aβ were from Santa Cruz. Anti CD95 (Jo-2). Phalloidin Alexa Fluor-568 and -633 were from Molecular probes. Jasplakinolide and 2-APB were from Calbiochem, Latrunculin B, Cytochalasin D and Thapsigargin were from Sigma, Ionomycin from Calbiochem, Cyclosporine A from Fluka, Etoposide from Sigma, C6 Ceramide from Sigma, Staurosporin from Sigma, Actinomycin D from Sigma, Dexamethasone from Sigma, and Cyclohexamide from Sigma. Annexin V and 7-AAD were from BD Biosciences.

3.3. Analysis of F-actin and G-actin

The relative proportions of G-actin and F-actin in Jurkat T-cells were analyzed by sedimentation of filamentous actin followed by the quantitative determination of F- and G-actin by SDS–PAGE[287]. In brief, cells were left untreated or treated with Jasplakinolide (1 μM), Latrunculin B (4 μM) or Cytochalasin D (10 μM) for 30 min at room temperature followed by lysis (also at RT) in 200 μl F-actin stabilization buffer for 10 min (50 mM PIPES), pH 6.9, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% NP-40, 0.1% Tween 20, 0.1% beta-mercaptoethanol, 1 mM ATP, protease (complete EDTA free cocktail, Roche) and phosphatase inhibitor (HALT™ phosphatase cocktail, Pierce).

In the case of total erythrocyte-depleted splenocytes cells were left untreated or were treated with jasplakinolide (1 μM) or latrunculin B (4 μM) for 45 min at room temperature (23–26 °C) followed by homogenization (also at room temperature) in 200 μl F-actin stabilization buffer for 10 min.

Alternatively cells were incubated with 250 ng/ml CCL19 for 60 seconds at 37 °C in a water bath, followed by lysis (at room temperature) in F-actin stabilization buffer.

Cell lysis was followed by trypan blue exclusion microscopy. Cell lysates were sedimented at 150 000 × *g* for 60 min at 37 °C. The pellets were resuspended in 200 μl ice cold distilled water containing Cytochalasin D (10 μM) and incubated at 4 °C for 60 min to depolymerize the F-actin. The resuspended pellet was gently mixed every 15 min. Ten microliters of supernatant (G-actin) and pellet (F-actin) fractions were subjected to analysis by immunoblotting following SDS–PAGE. Actin was detected using anti-actin antibody (MAB 1501, Chemicon (1:1000)).

3.4. Confocal Laser Scanning microscopy

Cells were adhered on poly-l-lysine treated 10 well Teflon-coated glass slides (Polysciences) for 15 min and left either untreated or treated with Jasplakinolide (1 μM), Latrunculin B (4 μM) or Cytochalasin D (10 μM) for 30 min at room temperature. After fixation (20 min, 4% paraformaldehyde in PBS, 37 C) and permeabilization in 0.2% saponin/3% BSA in PBS, cells were stained overnight with phalloidin-AlexaFluor568 (Molecular Probes) in 0.2% saponin/3% BSA/PBS at 4°C. Overnight labeling was necessary to allow phalloidin-AlexaFluor568 to compete with and displace Jasplakinolide, since Jasplakinolide binds to the same site as phalloidin [286].

In the case of murine T-cells cells were adhered to poly-L-lysine coated, 10-well, Teflon-coated glass slides (Polysciences) for 20 min on ice. We fixed cells with methanol or 4% paraformaldehyde and then blocked and permeabilized them in PBS, 3% BSA, 0.1% TritonX-100. The cells were then stained for 60 min at room temperature with antibodies to actin and/or coronin-1 and subsequently with phalloidin Alexa Fluor 568 (Molecular Probes, 1:25) and/or secondary antibodies (goat anti-mouse Alexa Fluor 546, goat anti-rabbit Alexa Fluor 488; 1:200 each) applied for 45 min at room temperature. We washed slides extensively and mounted them using Fluoroguard antifade mounting medium (Bio-Rad). Slides were analyzed using a LSM 510 Meta confocal laser scanning microscope (Zeiss) and the corresponding software. We analyzed cell spreading as described in the figure legend according to refs. [122, 308].

For quantitation of T cells double-stained with phalloidin Alexa Fluor 568 and anti-actin followed by Alexa Fluor 488–labeled secondary antibodies, we processed the images for the fluorescence intensity in the 488 nm and 568 nm channels after selecting a region of interest for wild-type and coronin-1-deficient cells using the software provided (Zeiss).

3.5. Video microscopy

Wild-type or coronin-1 deficient T cells purified using magnetic cell sorting (Milteny Biotech), resuspended in RPMI, 10 % FBS, L-glutamine, were seeded on eight-well chambered coverglass slides (Nunc) previously coated with anti-CD3 plus anti-CD28 (Pharmingen) (10 µg/ml each) or poly-L-lysine as a control [122], and time-lapse images were acquired every 10 s for a period of 30 min using a Carl Zeiss Axiovert microscope and processed using Open Lab software [241] (Video microscopy was performed by Jayachandran R.).

3.6. Ca^{2+} mobilization measurements

Total spleen cell suspensions were subjected to erythrocyte lysis, washed with RPMI 1640 (Gibco) supplemented with 3% heat inactivated FCS (Gibco) and loaded with Indo-1 or Fluo-3 by incubation in 3 µg/ml of Indo-1 AM or 3 µg/ml of Fluo-3 AM (Molecular Probes) for 45 min at 37 °C in the dark. After loading, cells were washed once or twice with RPMI 1640 containing 3% FCS and stained with anti-CD19 (clone 1D3, BD Pharmingen) conjugated to phycoerythrin (PE) for indo-1 experiments or conjugated to allophycocyanin (APC) for Fluo-3 experiments and with anti-CD11b-PE (Indo-1 experiments) or anti-CD11b-APC (Fluo-3 experiments) (clone M1/70, BD Pharmingen) for 30 min on ice. Cells were subsequently washed twice with RPMI 1640, 3% FCS or with calcium free Ringer's solution. After equilibrating the cells at 37 °C for 5-10 min and establishing a baseline for 30 s, we induced calcium flux by TCR cross-linking using anti-CD3 (7.5-10 µg/ml; clone 145-2C11, BD Pharmingen), anti-CD28 (5 µg/ml) and mouse anti–hamster (5 µg/ml) [370]. Cells were analyzed by flow cytometry on a FACSVantage, FACSCalibur or a LSR II for a further 9-9.5 min, measuring the 405 nm:510 nm ratio (Indo-1) or the 585 nm signal (Fluo-3). Only PE or APC negative cells, respectively, were considered for calcium mobilization measurements. Thymocytes were loaded with Indo-1 as described above, labeled with PE-conjugated anti-CD8 and PE-Cy7-conjugated anti-CD4 for 30 min on ice and triggered with 5-7.5 µg/ml anti-CD3 and 5 µg/ml mouse anti-hamster. For analysis of PLC activation, cells were loaded with Fluo3-AM(2 µM) and labeled with APC-conjugated anti-CD5 antibody (to detect T cells) and PE-Cy7 conjugated anti-CD19 (to exclude B cells). Cells were washed and resuspended in

HBSS without calcium in the presence of 1 mM EGTA. The cells were treated with either PLC activator (m-3M3FBS, 10 μM) or DMSO at the indicated time points, followed by the addition of 2 mM $CaCl_2$. For analysis, only PE-Cy7 negative, $CD5^+$ cells were included. Data were analyzed using FlowJo software. (Experiments were performed together with Jayachandran R.)

For the analysis of Ca^{2+} mobilization in Jurkat T cells, Jurkat cells were transfected with 2 μg each of pSUPER::mTACO1 or pSUPER::hTACO1 as described [238] or left untransfected and were loaded with Indo 1-AM (4 μg/ml) for 45 min in RPMI with 10% FBS at 37 °C. Cells were washed in HBSS without calcium (Invitrogen/GibcoBRL) and seeded at 50,000 cells per well in a 96-well special optics black plates (Costar Corning). Cells were stimulated with 10 μM m-3M3FBS after 30 s and the changes in fluorescence emission at 405 nm and 480 nm measured with excitation at 340 nm using Synergy 2 multi-detection-microplate reader for a period of 8 min. The ratio of 405 nm to 480 nm emission values was calculated and normalized using the software provided. (Experiments were performed together with Jayachandran R.)

Calcium measurements in memory-effector T cells. Splenic memory-effector T cells from wild-type (+/+) or coronin 1-deficient (−/−) mice were purified using the Pan T-cell isolation kit from Miltenyi in a first step to obtain untouched total T cells. In a second step T cells expressing CD62L were depleted using anti CD62L micro beads (Miltenyi). The remaining population of $CD62L^{low}/CD44^{high}$ memory-effector T cells was loaded with Fluo-3 and calcium fluxes following T cell receptor crosslinking (7.5 μg/ml anti- CD3, 5 μg/ml anti-CD28 and 5 μg/ml mouse-anti-hamster) were analyzed on a FACSCalibur. As a control we labeled Fluo-3 loaded erythrocyte depleted wild type splenocytes with PE-Cy7-conjugated antibodies against CD19 and CD11b and APCconjugated antibodies against CD62L on ice. PE-Cy7 negative APC positive cells were considered as naive T cells. Naive T cells were stimulated and analyzed as described for the memory-effector T cells.

In the case of calcium measurements in the context of F-actin and SOC Jurkat T-cells were washed with medium, RPMI 1640 (Gibco)/10% heat inactivated FBS (Gibco)/2 mM L-glutamine (Gibco-BRL) and loaded with 2.0 μg/ml of Indo-1 (Molecular Probes) for 45 min at 37 °C in the dark. After loading, cells were washed two times in medium and left either untreated or were incubated with Jasplakinolide (1 μM), Latrunculin B (4 μM) or Cytochalasin D (10 μM) for 20 min at RT. Cell were washed twice with Ca^{2+} free Ringer

solution in the presence of the drugs (total of 10 min) and taken up in Ca^{2+} free Ringer solution containing 1 mM EGTA (1 ml) and the indicated drugs. After acquiring a baseline for 30 s cells were triggered with 1 µM of Thapsigargin to induce ER Ca^{2+} store release. After a total time of 8 min 500 µl of cell suspension was transferred to a new tube with 500 µL 2 mM calcium containing Ringer. 2-APB, either 3 µM to enhance Ca^{2+} influx or 100 µM to inhibit calcium influx were added as indicated in the corresponding result section. Ca^{2+} measurements were all done on a LSR II (Becton Dickinson). Data were analyzed using FlowJo software.

3.7. Electrophysiology

Patch-clamp experiments were performed at 22-23 °C in the tight-seal whole-cell configuration using fire-polished patch pipettes (2-5 MΩ uncompensated series resistance). Pipette and cell capacitance were electronically cancelled before each voltage ramp with an EPC-9 patch-clamp amplifier controlled by Pulse 8.4 software (Heka elektronik). Membrane currents were filtered at 1.5-2.3 kHz and digitized at a sampling rate of 5-10 kHz. Whole-cell currents were elicited by 200 ms voltage-clamp ramps from -100 mV to +100 mV from a holding potential of 0 mV. To measure leak currents before activation of store-operated currents, 20 voltage ramps were applied within the first 5 s after establishment of the whole-cell configuration, followed by voltage ramps applied every second. All voltages were corrected for a liquid junction potential of -12 mV between internal solutions and the bath solution. The standard pipette solution for whole-cell patch-clamp recordings contained (in mM): 0.05 $InsP_3$, 0.00005 TG, 10 EGTA, 140 Cs-aspartate, 2 $MgCl_2$, 5 Mg-ATP, 0.5 Tris-GTP, 2.5 malic acid, 2.5 Na-pyruvat, 1 NaH_2PO_4 and 10 Hepes (pH 7.2 with CsOH). The extracellular solution contained (in mM): 101.66 NaCl, 3 KCl, 20.66 CaCl2, 1.22 $MgCl_2$, 6.66 d-glucose, and 3.33 Hepes (pH 7.4 with NaOH) (Experiments were performed by Quintana A. and Griesemer D.).

3.8. Mixed lymphocyte reaction and proliferation

We isolated splenic T lymphocytes from coronin-1 deficient mice and wild-type littermates using magnetic beads according to the manufacturers protocol (Pan T-cell isolation kit, Miltenyi). For allogeneic stimulation of T lymphocytes, we isolated spleen cell suspensions from BALB/c mice (RCC) and treated them with 50 µg/ml mitomycin C (Sigma) in PBS for 30 min at 37 °C after erythrocyte lysis. We cultured responding T lymphocytes (1 $x10^5$) together with either 2 $x10^5$ (2:1 ratio) or 4 $x10^5$ (4:1 ratio) stimulating spleen cells in flat-bottom 96-well plates (BD Falcon) using the following medium: RPMI-1640 (Gibco), 1x MEM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 100 units penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 10 µM 2-mercaptoethanol (Gibco), 10% FCS (Gibco). After 3 d incubation in 6% CO2 at 37 °C, and 20 h before harvesting, we added 1 µCi [3H]thymidine (Amersham) to each well. Incorporation of the isotope was measured in a liquid scintillation counter (Packard TopCount) and expressed as the average cpm (± s.d.) per well of triplicate cultures. Proliferation of sorted naive $CD4^+$ T cells (FACs ARIA) and cycling of $CD4^+$ T cells as monitored by dilution of the 4 µM PKH26 dye was performed as described in the figure legend. For IL-2 measurement, erythrocyte-depleted spleen suspensions were stimulated with anti-CD3 (7.5 µg/ml) or with anti-CD3 (7.5 µg/ml) plus anti-CD28 (5 µg/ml) for 24 h. IL-2 production was analyzed using the FlowCytomix assay (Bender MedSystems) according to the manufacturer's protocol.

For PMA/Ionomycin experiments naïve $CD4^+$ splenic T-lymphocytes were isolated from coronin 1 deficient mice and wild-type littermates using magnetic beads according to the manufacturers protocol (MACS, Miltenyi). For stimulation we incubated T-cells with soluble anti-CD3 (0,1 µg/ml) or PMA 20 nM /Ionomycin 100 nM on splenic feeder cells as describedabove. Results were expressed as the average cpm (± s.d.) per well of triplicate cultures (Mixed lymphocyte reactions were performed by Massner J.).

3.9. TCR signaling and IP_3 measurements

Isolated naive splenic T cells were starved for 2 h in medium without FBS at 37 °C in 5% CO2, coated with anti-CD3 (10 µg/ml) or anti-CD3 plus anti-CD28 (10 µg/ml each) for 15 min on ice and washed once in serum-free medium to remove unbound antibody. TCRs were cross-linked with a secondary antibody (mouse anti-hamster) for the times indicated in the figures at 37 °C in a water bath. We terminated reactions by adding 5x SDS sample buffer and boiling for 10 min at 95 °C. Proteins were separated by SDS-PAGE and blotted with antibodies to total phosphotyrosine (horseradish peroxidase conjugated 4G10 antibody from Upstate), phospho-PLC-γ1, total PLC-γ1, ERK1/2, phospho-ERK-1/2, p38, phospho-p38, IκB-α, phospho-IκB-α, Lat, phospho-Lat (171) (all from Cell Signaling Technology), phospho-Lat (136; Sigma-Aldrich) and coronin-1 (1002 rabbit polyclonal antibody [241] or polyclonal rat antibody; where the latter has been used, this is indicated in the figure legend). To measure cellular $InsP_3$, we prepared and treated cells as stated above and extracted $InsP_3$. $InsP_3$ amounts were analyzed using a competitive radio-receptor assay kit according to the manufacturers protocol (Perkin Elmer). For bead binding assays, Dynabeads M450 Epoxy (Invitrogen) were coated with anti-CD3 and anti-CD28 (5 µg/ml each) according to the manufacturer's specifications.

3.10. Immunoprecipitation of PLC γ1

We isolated total T cells from the spleens of WT mice using the Pan T-cell isolation kit (Miltenyi). Cells (8×10^6 in 450 µl lysis buffer per immunoprecipitation reaction) were lysed (50 mM Tris HCl, pH 7.5, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.05% digitonin, protease (complete EDTA-free cocktail, Roche) and phosphatase inhibitor (HALT phosphatase cocktail, Pierce) cocktail) for 20 min on ice with repeated mixing. Nuclei and unbroken cells were sedimented by centrifugation (3,000g, 15 min at 4 °C) and 400 µl of the supernatant transferred to a new tube. Antibody to PLC-γ1, control IgG or buffer (for a control sample) was added at a 1:50 ratio, and the samples were rotated for 1 h at 4 °C. We added 50 µl of a 1:1 slurry of protein A beads in lysis buffer and rotated the samples for another 2 h. The beads were then washed five times with a buffer that had the same composition as the lysis buffer but one-fifth the detergent. After the last wash we removed

20% of the samples and boiled them in SDS sample buffer. These fractions were later separated alongside total lysate corresponding to 1/10 of the input material on a 7.5% SDS-PAGE gel and blotted with antibody to PLC-γ1 to check for efficient immunoprecipitation. The remaining 80% of the samples were taken up in two-dimensional PAGE buffer and separated on 7 cm NL pH 3-10 immobilized pH gradient (IPG) strips (Amersham) according to the manufacturer's protocol. Besides the anti-PLC-γ1 immunoprecipitate, the beads-only control and the control IgG (normal rabbit serum), we also separated total lysate corresponding to 1/10 of the input material on IPG strips of the same pH range. Separation in the second dimension was performed with 10% SDS-PAGE. The two-dimensional gels were blotted to nitrocellulose membrane and probed for coronin-1 and actin.

3.11. Immunoblot for coronin isoforms

Single positive thymocytes, double positive thymocytes and splenic T cells purified from wild-type (+/+) animals using MACS isolation kit (Miltenyi) were lysed in lysis buffer with protease inhibitors and the lysates were analyzed for total protein content by BCA assay. Equal protein amounts were loaded after denaturing with Laemmli buffer onto SDS-PAGE (10 %) and electrophoresed. The gel was transferred onto a nitrocellulose membrane and analyzed by Ponceau staining and incubated with anti-sera raised against the following KLHcrosslinked peptides (coronin 1; amino acid residues 5-20 (raised in rats), coronin 2; amino acid residues 428-439, coronin 3; amino acid residues 419-430 and coronin 7; amino acid residues 910-922, all raised in New Zealand White rabbits). The membrane was incubated with corresponding HRP-tagged secondary antibodies and analyzed using enhanced chemiluminescence. (Experiments were performed together with Jayachandran R. and Combaluzier B.)

3.12. RT-PCR for coronin isoforms

Total RNA was isolated from 5×10^6 single positive thymocytes, double positive thymocytes and splenic T cells purified from wild-type animals using MACS isolation kit (Miltenyi) using Qiagen RNA isolation kit in accordance with the manufacturer's protocol. RNA integrity was checked employing the RNA 6000 Nano Assay Kit (Agilent Technologies). Reverse transcription (RT) reactions were performed according to the manufacturer's protocol using 1 µg total RNA, SuperScript III reverse transcriptase (Invitrogen) and random hexanucleotide primers (Promega). Polymerase chain reactions using coronin-specific primers and cDNA templates from the RT reactions consisted of 30 cycles of 96 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 60 sec. Primer sequences are listed below. (Experiments were performed together with Jayachandran R. and Combaluzier B.)

Coronin isoforms	Forward Primer (FP) Reverse Primer (RP)	Primer Sequence
1	FP	5'-AAACCACTTGGGACAGTGGCT-3'
1	RP	5'-CATCCGGGCCCAGCGTCAGCA-3'
2	FP	5'-GTGAGCGGTCA GGATGCTAATCCAA-3'
2	RP	5'-TTCTCCCTGCTCCTTGACCAG-3'
3	FP	5'-TTTGAGGGGAAGAACGCGGAC-3'
3	RP	5'-AGTGTCTCCCTCTCTGCCCTC-3'
4	FP	5'-CGACTAGGGAT TGTCCTCCA-3'
4	RP	5'-GGTCAGGTGAGGTTTCTCCA-3'
5	FP	5'-GATCCCCATCACCAAGAATG-3'
5	RP	5'-GGCTGC CGTCTGTATTGAAG-3'
6	FP	5'-GTGCTGGACATTGACTGGTG-3'
6	RP	5'-TTGCTTGTGTCCATCTCCTG-3'
7	FP	5'-GAGCTGCCAGTGGAGGTACT-3'
7	RP	5'-GCAACTCATGAC AGCCAGTG-3'

3.13. Flow cytometric analysis and blood counts.

Cell counts were determined for single cell suspensions of the indicated organs using a Neubauer chamber. Flow cytometry was carried out by staining the cells with the relevant monoclonal antibodies at saturating concentrations in PBS + 2 % FCS and analyzing them on a FACS Calibur (Becton- Dickinson). The following monoclonal antibodies and secondary reagents were obtained from BD Pharmingen: PE-labeled α-CD19 (clone 1D3), PE-labeled α-B220 (clone RA3- 6B2), PE-labeled α-CD3 (clone 145-2C11), PE-labeled α-CD11b (clone M1/70), PE- or PECy7-labelled α-CD4 (clone RM4-5), PE-Cy7-labeled-α-CD25 (PL61), Biotinlabeled- α-CD44 (IM7), PE-Cy7-labeled-α-CD69 (H1.2F3), APC-labeled α-CD8a (53-6.7), APC labeled α-CD62L (clone MEL-14), α-CD24 (M1/69) and PECy7-labelled Streptavidin. PElabeled α-CD8a (clone 53-6.7) was obtained from eBioScience. Biotin-labeled antibodies against IgM (clone M41) and CD4 (clone RM4-5) were produced and labeled according to standard techniques. PE or APC labeled Annexin V and 7-AAD (BD Biosciences) was used according to the manufacturer's protocol.

Peripheral blood was obtained by tail bleeding and cell counts as well as the hemoglobin concentration were determined on an ADVIA hematology system (Bayer). For flow cytometry, peripheral blood was subjected to erythrocyte lysis prior to staining and analysis on a FACS Calibur (Experiments were performed together with Massner J. and Ceredig R.).

3.14. Immunohistology

Organs were embedded in OCT medium (Sakura Finetek), snapfrozen and 5-µm sections were cut with a cryostat. Sections were air-dried, acetone-fixed for 8 min and stored at -70 °C. Sections were then rehydrated and blocked in PBS/2 %BSA with 0.1 % NaN_3 and 220 µg/ml mouse IgG (Jackson ImmunoResearch Laboratories). Antibodies, diluted in PBS/2 % BSA, were added directly onto the sections and incubated for 60 min at room temperature in a wet chamber. Antibodies used were: Allophycocyanin (APC)-labeled anti-mouse CD4 (rat IgG2a, clone RM4-5) (Caltag Laboratories), fluorescein isothiocyanate (FITC)-labeled anti-mouse CD8 (rat IgG2a, clone 53-6.7) (BD Biosciences), fluorescein isothiocyanate (FITC)-labeled anti-mouse B220/CD45R (rat IgG2a, clone RA3-6B2) (BD Biosciences), R-Phycoerythrin

(PE)- labeled anti-mouse Thy1.2 / CD90.2 (mouse IgG2b, clone 5a-8) (Caltag Laboratories) and R-Phycoerythrin (PE)-labeled anti-mouse I-Aβ (mouse IgG2a, clone AF6-120.1) (BD Biosciences). Sections were counterstained with DAPI (Serva) for 5 min. After washing the sections were mounted in Fluoromount (Southern Biotechnology Associates). Images were taken on a Zeiss Axioskop with an ORCA ER camera (Hamamatsu) and images were processed by using OPENLAB software (Improvision, Coventry, U.K.) (Immunohistochemistry was performed by Blum C.).

3.15. Nuclear translocation of NF-κB and NFAT.

Wild-type as well as coronin 1-deficient splenic T cells were prepared as described for the signaling experiments. Cells were coated with anti-CD3 (10 µg/ml) and anti-CD28 (5 µg/ml) on ice or were left untreated as a control. After washing, T cell receptors were cross-linked by adding mouse-anti-hamster antibodies to a final concentration of 10 µg/ml. Cells were incubated at 37° C in a water bath for 20 or 30 minutes and reactions were stopped by adding ice cold PBS/2 % FBS/ 0.05 % sodium azide. After washing, cells were incubated with Alexa Fluor-488 goat-anti-mouse on ice for 15 minutes to stain the TCR caps. Washed cells were fixed in 8% PFA for 20 minutes at room temperature and stained with rabbit anti-p65 or NFAT (both Santa Cruz). Nuclei were stained using DRAQ5. For quantization of nuclear translocation of p65 or NFAT two times 100 cells were analyzed for both wild-type as well as coronin 1-deficient T cells from two independent experiments.

3.16. Preparation of $CD4^+$ SP thymocytes, naïve T-cells and B-cells for survival and functional assays

Naïve T-cells were prepared from erythrocyte depleted splenic cell suspensions using the Pan T-cell isolation kit from Miltenyi supplemented with biotinylated antibodies against CD44 at a final concentration of 5µg/ml. Using this procedure we were able to isolate T-cell populations containing ≥ 95% of naïve T-cells. In order to remove dead or apoptotic cells we passed cell suspensions at room temperature for 20 minutes at 800g over a cushion of

HISTOPAQUE-1077 and HISTOPAQUE-1119 (Sigma) mixed at a ratio of 11:3. Using this procedure we were able to recover naïve T-cell populations with ≥ 95% of viable cells (Annexin V, 7-AAD double negative).

For the isolation of $CD4^+$ SP thymocytes we passed thymic single cell suspensions at room temperature for 20 minutes at 800g over a cushion of HISTOPAQUE-1077 and HISTOPAQUE-1119 mixed at a ratio of 11:3, depleted the remaining cell suspensions of CD8 expressing cells using anti-CD8 micro beads from Miltenyi according to the manufacturers protocol. In a next step we positively selected CD8 negative, CD4 positive cells using anti-CD4 micro beads from Miltenyi according to the manufacturers protocol. In order to remove dead or apoptotic cells we passed cell suspensions at room temperature for 20 minutes at 800g over a cushion of HISTOPAQUE-1077 and HISTOPAQUE-1119 mixed at a ratio of 11:3. Using this procedure we were able to recover CD4 single positive thymic cell populations containing approximately 95% of CD4 positive cells which in addition were ≥ 95% viable (Annexin V, 7-AAD double negative).

B-cells were isolated similar to naïve T-cells using anti-CD4, anti-CD8 and anti-CD11b micro beads (Miltenyi) to remove all non B-cells. Using this procedure we were able to recover CD19 positive splenic cells containing ≥ 95% of CD19 positive cells which in addition were ≥ 95% viable (Annexin V, 7-AAD double negative).

3.17. Transwell migration assay

For transwell migration cells were washed extensively with migration buffer: RPMI-1640 supplemented with 0,1% tissue culture grade, lipid free BSA (Sigma) and L-glutamine. Cells were then added to the upper chamber of the migration setup in 100 µl of migration medium. 500 µl of migration buffer containing the indicated stimuli at the indicated concentration were added to the lower wells. Migration was monitored after the indicated time span using a FACS Calibur or Neubauer chamber to quantitate migrated cell numbers. In experiments where wild type as well as coronin 1 deficient cells (express GFP) [251] where tested, we used mixtures of both cell types to minimize experimental variations by having exactly the same condition for both wild type as well as coronin 1 deficient cells. GFP was used to distinguish both cell types.

3.18. Calcineurin activation

To measure calcineurin activity in naïve coronin 1 deficient or wild type T-cells untouched cells were isolated using the Pan T-cell isolation kit from Miltenyi supplemented with biotinylated antibodies against CD44 at a final concentration of 5 µg/ml. Cells were either stimulated for 15 minutes using anti-CD3, anti CD28 as well as secondary antibodies as stated for the calcium measurements or using PMA 50 nM/Ionomycin 200 nM. Upon stimulation cell lysis as well as calcineurin activity measurements were performed using a commercial kit (Calbiochem), according to the manufacturers protocol. Protein concentrations measurements were carried out using a commercial BCA protein assay kit (Pierce) according to the manufacturers protocol.

4. SUMMARY AND OUTLOOK

4.1. Part I

The first part of this thesis addressed the question of whether the F-actin cytoskeleton is important for store operated calcium entry (SOCE). We were able to show, that in Jurkat T cells stabilization or depolymerization of the actin cytoskeleton using actin modulating drugs both failed to affect calcium release activated calcium (CRAC) channel activation and calcium influx. These results lead us to conclude that rearrangement of the actin cytoskeleton is dispensable for SOCE in T cells. Consistent with a previous report, finding no effect of F-actin modulation on SOCE in RBL cells, our data clearly disprove the long held idea that the actin cytoskeleton mediates coupling of intracellular calcium stores to plasma membrane calcium channels in lymphocytes.

These results improve our knowledge of calcium signaling in cells of the immunesystem and allow further refinement of the models we use to explain the molecular events underlying calcium signaling.

4.2. Part II

In the second part of this thesis the *in vivo* role of coronin 1 during T cell development was analyzed, as well as proliferation and T cell receptor (TCR) mediated signaling. We describe that coronin 1 is essential for the proliferation and survival of naive T cells in the periphery. However when investigating T cell receptor downstream events, all analyzed signaling molecules, such as linker for activation of T cells (Lat) and SRC-homology-2-domain-containing leukocyte protein of 76 kDa (SLP-76), down to phospholipase C γ1 (PLC γ1), were expressed to a similar extend as well as phosphorylated equally upon TCR ligation in both wild type and coronin 1 deficient T cells. In depth analysis of PLC γ1 function, substrates and metabolites, revealed that coronin 1 exerts its function in naïve T cells by activating Ca^{2+} release from intracellular endoplasmatic reticulum (ER) calcium stores upon TCR triggering. We were further able to show that coronin 1 associates with PLC γ1, thereby being essential for the generation of the second messenger inositol-1,4,5- trisphosphate (IP_3),

which upon binding to IP_3-receptors at the ER mediates the release of Ca^{2+} from ER calcium stores. The transient increase of cytosolic calcium caused by this release induces a second wave of calcium influx via SOCE channels in the plasma membrane. As a consequence of the elevated calcium concentrations the phosphatase calcineurin is activated and dephosphorylates the transcription factor, nuclear factor of activated T cells (NFAT) in the cytoplasm. Dephosphorylated NFAT translocated into the nucleus where it initiates gene transcription of genes such as the interleukin-2 (IL-2) gene. In line with the finding that in the absence of coronin 1 only weak IP_3 and Ca^{2+} responses are generated both nuclear translocation of NFAT and IL-2 production were markedly decreased in coronin 1 deficient T cells.

The role defined here for coronin 1 in the generation of IP_3 upon TCR triggering differs markedly from a recent report that coronin 1 is dispensable for TCR mediated signaling, instead preventing F actin induced apoptosis. We describe however that in T cells from wild type and coronin 1 deficient mice, F-actin dynamics were normal in the absence of coronin 1 as analyzed by several independent morphological, biochemical and functional assays, including the analysis of TCR-mediated cell spreading.

We therefore conclude that coronin 1, rather than modulating the F-actin cytoskeleton in T cells, is involved in T cell receptor signaling in general and in particular in the regulation of PLC γ1 mediated calcium release from internal stores.

The definition of coronin 1 as a regulator of PLC γ1 and calcium signaling in T cells opens a new and unexpected field of research and possibilities with respect to the development of novel compounds for the treatment of T cell mediated disorders of the immunesystem.

4.3. Part III

In the last part of this thesis we investigated the precise mechanism of T cell death, observed in coronin 1 deficient mice. We found that both naïve T cell survival as well as migration defects observed in coronin 1 deficient naïve T cells occurred as a result of defective PLC γ1/calcium signaling and calcineurin activation. We were further able to demonstrate that cellular viability and the ability of T cells to migrate are intimately linked. When the viable

population of coronin 1 deficient naïve T cells was isolated, these cells neither displayed elevated levels of spontaneous apoptosis nor a migration defect.

As a consequence of defective signaling and calcineurin activation in the absence of coronin 1, Bcl-2 levels were found to be reduced, whereas caspase 3 was induced at high levels. These date are consistent with previous reports that calcineurin deficiency leads to specific defects in naïve T cells and a reduction in Bcl-2 protein levels [350]. Furthermore, the data presented here provide, on a molecular level, an explanation for the diverse phenotypes such as migration and survival defects described for naïve T cells from coronin 1 deficient mice. These data also demonstrate that phalloidin staining, which has been used to quantitate F-actin levels in coronin 1 deficient T cells cannot be used to quantitate F-actin levels if coronin 1 levels differ between cell populations, an effect that might be due to competition of coronin 1 and phalloidin for spatially proximal binding sites. These findings are in agreement with a previous report demonstrating an essential role for coronin 1 in the activation and survival of pathogenic mycobacteria within host macrophages, but not F-actin regulation [251].

The definition of coronin 1 as an essential regulator of calcineurin mediated cellular viability in naïve T cells may allow a better understanding of leukocyte activation and homeostasis, both of which are crucial to the understanding as well as treatment of autoimmune as well as lymphoproliferative diseases. In the future, the precise role of coronin 1 in different T cell subset as well as in different pathological conditions needs to be defined in order to further understand the differential roles of coronin 1 in T cell function as well as activation. Another aspect which might become important during viral and certain bacterial infection is the integrative effect of stimuli received via the TCR as well as other receptors such as the TLRs. Interestingly a recent publication describes that coronin 1 might act as a negative regulator of TLR signaling [371], implying that signaling input via TLRs could be significantly enhanced in the absence of coronin 1 and compensate for missing signals from the TCR as observed for PkCθ deficient T cells [372-374]. Addressing these questions using the coronin 1 deficient mouse model may yield valuable insight into the involvement of coronin 1 in T cell regulation under physiological and pathological conditions.

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6. ABBREVIATIONS

7-AAD	7-Aminoactinomycin
ABC	ATP binding cassette
ADAP	adhesion and degranulation promoting adaptor protein
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
AICD	activation induced cell death
α-CPM	α-chain connecting peptide domain
AP1	activator protein 1
2-APB	2-Aminoethoxydiphenyl Borate
APC	antigen presenting cell
ARP2	actin-related protein 2 homologue
ARP3	actin-related protein 3 homologue
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl	B cell lymphoma
BCR	B cell receptor
BH-3-only	Bcl-2-homology domain 3 only
Bid	BH3 interacting domain death agonist
Bim	BCL-2-interacting mediator of cell death
Ca^{2+}	calcium
caspase	cysteine aspartic acid proteases
CCL	Chemokine (C-C motiv) ligand
CD	cluster designation or cluster of differentiation
CDRs	complementarily determining regions
CDC42	cell-division cycle 42
CDI	calcium dependent inactivation
CLIP	class II associated invariant chain peptide
CaN	calcineurin A
CRAC	calcium release activated
cSMAC	central SMAC
CTLA-4	cytotoxic T lymphocyte antigen 4
CytD	Cytochalasin D
DAG	diacylglycerol

DC	dendritic cell
DMSO	Dimethylsulfoxid
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
dSMAC	distal SMAC
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
FACS	fluorescent activated cell sorter
F-actin	filamentous actin
FasL	Fas ligand
Fc	fragment cristalizable
FoxP3	forkhead box P3
G-actin	globular actin
GADS	GRB2 related adaptor protein
GRB2	growth-factor-receptor-bound protein 2
ICAM1	intercellular adhesion molecule 1
IFN- γ	interferon-γ
IκB	inhibitor of NF-κB
IL-2	interleukin 2
$InsP_3$	nositol-1,4,5- trisphosphate
IP_3	inositol-1,4,5- trisphosphate
IS	immunological synapse
ISP	immature single positive
ITAM	immunoreceptors tyrosin-based activation motif
ITIM	immunoreceptors tyrosin-based inhibition motif
ITK	interleukin-2-inducible T-cell kinase
JAK	Janus Kinase
JNK	JUN amino-terminal kinase
JP	Jasplakinolide
Jaspl	Jasplakinolide
LAT	linker for activation of T cells
LatB	Latrunculin B
Lck	Leukocyte-specific protein tyrosin kinase

LFA1	leukocyte function associated antigen 1
LPS	lipopolysaccharide
MIIC	MHC class II compartment
Mcl-1	Myeloid cell leukemia sequence 1
MHC	Major Histocompatibility Complex
m-3M3FBS	N-(3-trifluromethylphenyl)-2,4,6-trimethylbenzenesulfonamide
NCK	non-catalytic region of tyrosine kinase
NFAT	Nuclear Factor of Activated T cells
NF-κB	nuclear factor-κB
ORAI	In greek mythology “Orai” are the keepers of the gates of heaven
PAMPs	Pathogen-Associated Molecular Patterns
PH	pleckstrin homology
PI3K	phosphatidylinositol-3-kinase
PiP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PkC	Protein kinase C
PLC	phospholipase C
PMA	phorbol-12-myristat-13-acetat
PRR	pattern recognition receptors
pSMAC	peripheral SMAC
PTEN	phosphatase and tensin homologue
RAC	Ras-related C3 botulinum toxin substrate
RAG	recombination-activation gene
RASGRP	RAS guanyl-releasing protein
RBL	rat basophile leukemia
SAM	sterile alpha motif
SCID	severe combined immunodeficiency
SD	standard deviation
SDF	stromal cell derived factor
SDS	sodium dodecyl sulfate
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SFKs	Src family tyrosin kinases
SH2	Src Homology 2
SHIP	SRC homology 2 (SH2)-domain-containing inositol-5-phosphatase

SLP76	SRC-homology-2-domain-containing leukocyte protein of 76 kDa
SMACs	supra-molecular activation complexes
SOC	store operated calcium
SOCE	store operated calcium entry
SOCS-1	Suppressor of cytokine signaling-1
SOS	son of sevenless homologue
SP	single positive
STAT	signal transducer and activator of transcription
STIM 1	stromal interaction molecule 1
TACO	tryptophan aspartate containing coat protein
TCR	T cell receptor
TLR	Toll like receptor
TNF	tumor necrosis factor
TPR	Transient receptor potential
T_{REG}	regulatory T cells
VAV1	vav1 guanine nucleotide exchange factor
WASP	Wiskott-Aldrich syndrome protein
ZAP70	ζ-chain-associated protein kinase of 70 kDa

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LIST OF PUBLICATIONS

Philipp Mueller and Jean Pieters
In Vivo Regulation of Migration and Apoptosis through Coronin 1-mediated Calcineurin Activation
Submitted (August 2009)

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Modulation of macrophage antimicrobial mechanisms by pathogenic mycobacteria.
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LIST OF PATENTS

Patent 1:

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