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**mRNA and protein expression profiling
of CD4+/HLADR-/CD25- Th-cell populations
following CD2 stimulation**

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Abstract

1.1 Deutsch

CD2, auch E rosette receptor, LFA2 (leukocyte function antigen) und T11 genannt, ist ein membranständiges Glykoprotein und gehört zur Immunglobulin Superfamilie. Es ist ein früher T-Zell Marker und wird auch auf NK-Zellen exprimiert. *In vivo* bindet humanes CD2 seinen Liganden CD58 (LFA3) an Antigen Präsentierenden Zellen und ermöglicht dadurch T-Zellen, auf sehr geringe Antigen Konzentrationen zu reagieren. Gleichzeitig werden durch diese Interaktion kostimulatorische Signale in T-Zellen induziert, die an der Regulation der Zytokin-Produktion beteiligt sind, Zytolyse vermitteln, Apoptose von aktivierten peripheren T-Zellen inhibieren und T-Zell Anergie regulieren.

Ziel der Studie war es, die Wirkung von CD2 Stimulation auf T-Zellen, vor allem CD4 positive Helferzellen, auf mRNA und Protein Expression zu untersuchen, da viele Mechanismen in diesem Zusammenhang noch nicht ausreichend geklärt sind.

Aufgereinigte CD4+ /HLADR- /CD25- T-Zellen wurden bereits mit CD2 Antikörpern stimuliert, und mit Hilfe von Amplichip mRNA expressions-Arrays wurden Veränderungen der mRNA Expression im Vergleich zu unstimulierten, sowie zu Anti-CD3 stimulierten CD4+ /HLADR- /CD25- T-Zellen analysiert. Kandidaten-Gene mit deutlich unterschiedlichem mRNA Expressionslevel wurden für weitere Untersuchungen mit Hilfe von TaqMAN Assays ausgewählt.

In dieser Studie wurden einige Gene, deren unterschiedliche mRNA Expression mittels TaqMAN bestätigt wurde und für deren Proteine kommerzielle Antikörper zu Verfügung stehen, genauer untersucht. Die in diesem Zusammenhang untersuchten Gene, beziehungsweise Proteine sind:

CD200 (OX2/MOX2): Teil der Immunglobulin Familie, und ein Zelloberflächen Glykoprotein welches in lymphoiden, neuronalen und Endothelzellen exprimiert wird. Während die mRNA Daten auf eine recht schnell induzierte

Transkription hingewiesen haben, konnten wir in den Western Blot Experimenten jedoch kaum Protein nachweisen.

CD137L: Gehört zur Familie der Tumor Nekrose Faktor Rezeptoren und liefert kostimulierende Signale an T-Lymphozyten. CD137 wird auf aktivierten T-Zellen exprimiert und bindet den Liganden CD137L, den man auf B-Zellen, Makrophagen und Dendritischen Zellen findet. Solche Interaktionen sind in die Antigen Präsentation und die Bildung von Zytotoxischen T-Zellen involviert. Es war uns im Rahmen der Experimente möglich diesen Liganden erstmals auf T-Zellen nachzuweisen, und die gesteigerte mRNA Expression zumindest teilweise zu verifizieren, was eine mögliche neue Einsicht in die Regulation von T-Zellen untereinander zulässt.

α Pak (Pak1 Kinase): Mitglied der PAK Kinase Familie (bestehend aus 6 Isoformen), die unter anderem eine sehr wichtige Rolle in Signalwegen von T-Zellen spielen. Unsere mRNA Daten zeigten eine gesteigerte Transkription von Pak1 nach anti-CD2-Stimulation, die wir auch auf Protein Ebene nachweisen konnten.

NCK 1/2: Ein SH2/3 Adaptor Protein, welches eventuell auch für den CD2 Signalweg relevant sein könnte, da wir zumindest auf mRNA Ebene eine leichte Steigerung nachweisen konnten. Die Proteinkonzentration hingegen wird kaum durch CD2 Stimulation beeinträchtigt.

1.2 English

CD2, also called E rosette receptor, LFA2 (leukocyte function antigen), and T11, is a membrane associated glycoprotein and belongs to the immunoglobulin superfamily. It functions as an early T-cell marker, and is also expressed on NK-cells. *In vivo*, human CD2 binds its ligand CD58 (LFA3), situated on APCs (antigen presenting cells), thus enabling T-cells to respond to very low antigen concentrations. Simultaneously, CD2 induces co-stimulatory signals in T-cells, which are involved in cytokine production, mediate cytotoxicity, inhibit apoptosis of activated peripheral T-cells and regulate T-cell anergy. The goal of this study was to examine the effect of CD2 stimulation on T-cells, particularly CD4 positive helper cells, via mRNA and protein expression patterns, to shed light on mRNA and protein expression levels differentially upregulated upon α CD2 stimulation and to identify signalling or effector molecules so far unknown to be involved in CD2 mediated T-cell activation.

Purified CD4+ /HLADR- /CD25- T-cells were previously stimulated with anti- CD2 antibodies and utilizing Amplichip mRNA expression arrays changes in mRNA expression were compared to both unstimulated and anti-CD3 stimulated CD4+ /HLADR-/CD25- T-cells.

Candidate genes with differential mRNA expression levels were selected for further analysis using TaqMAN assays.

In this study, 4 genes were further investigated for alteration in their respective protein levels following CD2 stimulation:

OX2 (CD200/MOX2): A member of the immunoglobulin super-family, and a membrane associated glycoprotein expressed in lymphoid, neuronal and endothelial tissue. While our mRNA data suggested an increased transcription rate, we were not able to detect proportional amounts of protein during the Western blot experiments to support these findings.

CD137L: A member of the TNF (tumor necrosis factor) receptor family, responsible for delivering co-stimulatory signals in T-lymphocytes. CD137 is expressed on all activated T-cells and binds to its ligand CD137L which is found on B-cells, macrophages and dendritic cells. This interaction is involved in antigen presentation and in the development of cytotoxic T-cells.

During this study, we were able to demonstrate the presence of this ligand on T-cells for the first time, and confirmed the increased mRNA expression rate, at least partly. This might give a new insight into the subject of self regulating T-cell populations.

α PAK (Pak1 kinase): A member of the PAK kinase family (comprising 6 isoforms) which, among other functions, plays an important role in T-cell signalling. Our mRNA data indicated an increase in mRNA transcription upon CD2 stimulation, which we were also able to confirm on the protein level.

NCK 1/2: A member of the SH2/3 adapter protein family, might be part of the CD2 pathway, since we were able to show a slight increase in the mRNA expression. The protein concentration, however, did only change little, if at all, following CD2 stimulation.

Introduction

2.1 No Body is Perfect (The Immune System)

2.1.1 The Innate Immune System

The immune system in higher organisms such as mammals basically consists of two separate compartments. One of them is the innate immune system, which is based on cellular and chemical barriers, inflammation, and the complement system. The skin of the human body for example poses a physical and chemical barrier to microbes due to its keratinized layer of water-impermeable epithelium, as well as an acidic pH level (around 5.5) maintained on its surface, together with a vast amount of enzymes and antibiotic chemical compounds (such as cathelicidin, α -defensins, dermicidin, psoriasin, etc.). Most of these antibiotic substances are produced in eccrine sweat glands, by epithelial cells and keratinocytes, or by an army of specialized cells, which are harbored within the sub-epithelial connective tissue (including mast cells, neutrophils, and macrophages) [1]. Monocytes (e.g. macrophages) are able to recognize potential hazardous materials or microbes using toll like receptors (TLR), and basically devour the intruder.

| Receptor | Ligand |
|----------|--|
| TLR1 | Bacterial lipopeptides |
| TLR2* | Lipopeptides, lipotechoic acid, peptidoglycans |
| TLR3 | Double-stranded RNA, polyinosine:cytosine |
| TLR4 | LPS |
| TLR5 | Flagellin |
| TLR6 | Zymosan, lipopeptides |
| TLR7 | Single-stranded viral RNA, imiquimod |
| TLR8 | Single-stranded viral RNA, imiquimod |
| TLR9 | Unmethylated CpG DNA |
| TLR10 | unknown |

Table 2.1: * TLR-2 cooperates with TLR-1 and TLR-6 to recognize lipoteichoic acid, zymosan, and other ligands. TLR-5, TLR-7, TLR-8, and TLR-9 cooperate to recognize CpG DNA, ssRNA, and flagellin. [2]

Another weapon and major part of the humoral response of the innate immune system, is the complement system. This system consists of more than 30 soluble proteins that are activated in a biochemical cascade triggered by the binding of the glycoprotein C1 to one IgM or several IgG antibodies, which recognize certain microbic surface molecules. The result of this reaction is the recruitment of the membrane attack complex (MAC) to the cell membrane of the pathogen which then basically pokes holes into the microorganism, thus killing it.

Several functions of the innate immune system can be traced back to very simple life forms such as protozoans, single cell organisms which roam the earth for now over 2.5 billion years. This suggests that the innate immune system is the evolutionary older part, since some of its features including phagocytosis can be found in almost all living creatures even the simplest and oldest ones. Even in starfish or sponges specialized phagocytic cells patrol in and around their tissues to protect them from microbic foes. [3] Another conserved and very important aspect of the immune system is the discrimination between „self“ and „non-self“ which, in higher organisms such as mammals, is achieved by specific surface molecules and pattern receptors.

2.1.2 The Adaptive Immune System

The younger and far more specific relative of the innate immune system is the acquired or adaptive immunity, which is branched into a humoral and a cellular compartment. The leading actors of the humoral branch are called B-lymphocytes or B-cells that secrete antibodies, which bind to the surface of foreign „non self“ substances or microbes, triggering an immune response. The „B“ stands for Bursa fabricii, a specialized organ in birds, which is their site of hematopoiesis, hence the name B-cell. In humans the site of B-cell development is the bone marrow and the spleen, yet the „B“ in human B-cells does not stand for bone marrow. [4] B-cell development in the bone marrow starts with so called common lymphocyte precursors (CLP) which then differentiate into the first B-lineage specific pro-B-cells followed by a rearrangement in the immunoglobulin heavy chain characteristic for pre-B-cells (see fig 2.1). These immature (ImmB) cells already express IgM receptors. Upon activation B-cells secrete IgM antibodies (but are able to isotype switch to IgG, IgA and IgE), which are usually the first antibodies to be produced in an immune response and are able to activate the comple-

ment system. Mature B-cells may further develop into plasma cells, which then secrete antigen-specific IgG antibodies, which play a major role in the immunological long term memory. The main task for these immune cells is to produce immunoglobulins with a huge variety of antigen specificities, which then circulate in the blood waiting for a victim in the form of foreign substances or organisms. B-cell TLR signalling also plays an important part in inflammatory responses, hence connecting the cell mediated (BCR) adaptive immune system to the humoral (antibody and TLR) part of the innate immune system. [5]

2.1.3 Attack of the Clones (T-Cells)

The other part of the adaptive immune system is maintained by another cell-population called T-cells. Like B-cells they originate from the bone marrow, but then specialize and mature in the thymus gland, hence the name T-cell. A small fraction of T-cells, however, is present in the fetal liver, which suggests a role of the fetal liver in T-cell development as well. For a vast period of time in the history of medicine, the thymus was thought to be a vestige of evolution, just as the appendix, with no obvious assignment, and even though the name roots in the Greek word for life force „thymos“ suggesting some sort of importance, it took science until 1961 to discover its true purpose. Experiments conducted by Francis Albert Pierre Miller [6] were the first milestones in the research of the thymus and its crucial role in the immune system. After these early discoveries, it soon became clear that the thymus is responsible for the development of one of the main weapons of the adaptive immune system: T-cells. There are two fundamental branches of T-cells: cytotoxic T-cells (Tc or CD8+) and T helper-cells (Th or CD4+). They undergo a sequential maturation, which starts with DN (CD4/CD8 double negative) thymocytes, entering the thymic parenchyma via the vessels around the corticomedullary junction (see fig 2.1). The DN stage is further partitioned into four substages: First the CD44+ CD25- (DN1) stage, followed by CD44+ CD25+ (DN2), the CD44- CD25+ (DN3) and finally CD44- CD25- (DN4) stage. Within the DN3 stage, a selection is made whether the T-cells commit to the T-cell-receptor (TCR) $\alpha\beta$ lineage via pre-TCR (pT) expression, or commit to the TCR $\gamma\delta$ lineage expressing TCR $\gamma\delta$. $\alpha\beta$ lymphocytes are selected positively and negatively via their MHC interaction, while

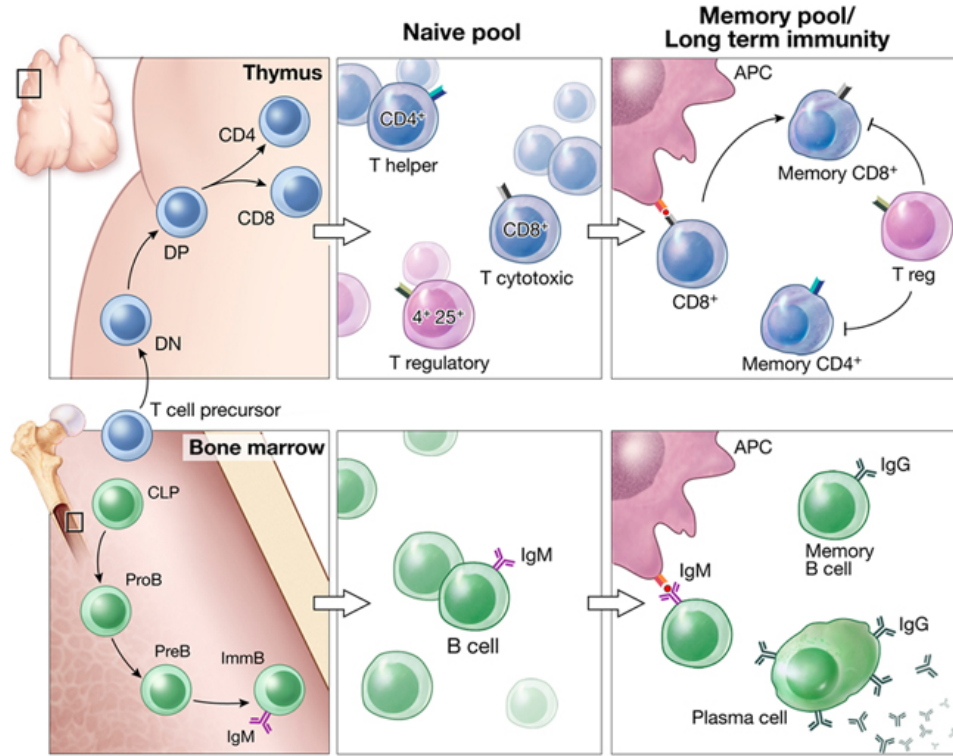


Figure 2.1: T- and B-cell development [7]

the $\gamma\delta$ development and selection process is still under discussion [8]. Most of the TCR $\gamma\delta$ cells have neither CD4 nor CD8 on their surface, which makes sense since the $\gamma\delta$ T-cell receptor does not need a MHC context to bind and recognize foreign substances, instead it binds to organic molecules (often containing phosphorus atoms) in the same manner as antibodies do [9] [10]. The following DP (CD4/CD8 double positive) stage, in which the cells express both CD4 and CD8 on their surface, is characterized by the interaction of DP cells with an MHC receptor of stromal cells, which leads to positive or negative selection, as well as allelic exclusion at the TCR β locus, termination of pT α expression and the initiation of the V(D)J somatic recombination [9]. The V(D)J recombination, standing for **V**ariable, **D**iverse and **J**oining gene, is an important genetic mechanism for a T-cell to randomly assemble their T-cell receptor in order to broaden the responsiveness to possible antigen targets. The V(D)J recombination event is essentially the same for T- and B-cell receptor, as well as for immunoglobulins. In T-cells the recombination starts with the D-to-J recombination in the β chain of the TCR in which ei-

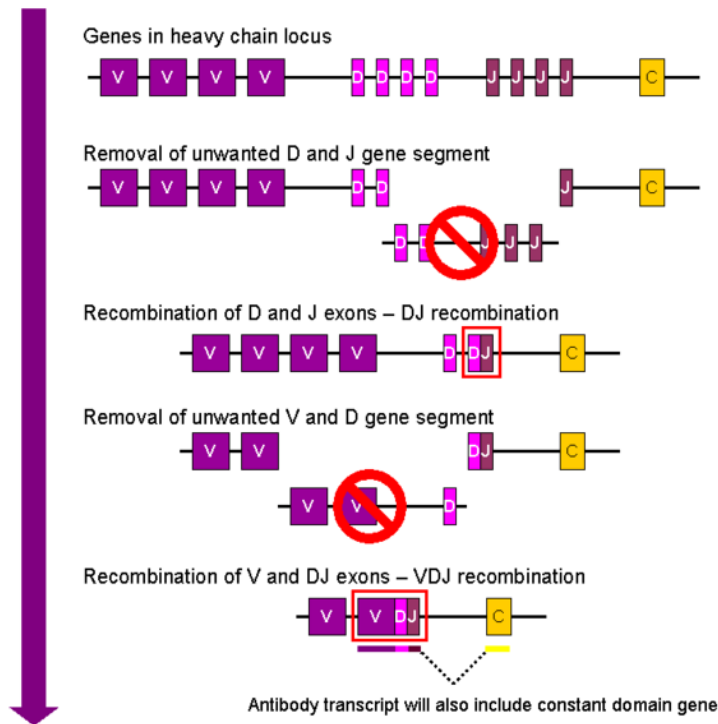


Figure 2.2: basic overview of V(D)J recombination-events of immunoglobulin heavy chains

ther $D_{\beta 1}$ segment joins one of the six possible $J_{\beta 1}$ segments, or alternatively the combination of $D_{\beta 2}$ with one of the seven $J_{\beta 2}$. Next, the V_{β} -to- $D_{\beta}J_{\beta}$ recombination takes place with ultimately leaving a complex of V_{β} - D_{β} - J_{β} with the constant domain gene (V_{β} - D_{β} - J_{β} - C_{β}). Final splicing events delete any remaining intervening sequences and the final construct is translated into the TCR β chain (see Fig 2.2). After the completion of the β chain, the α chain of the TCR is assembled in a similar way with the difference that the α chain lacks the D segment, therefore the first step is the formation of a VJ complex followed by the addition of the constant domain. Finally α and β chains are assembled to the $\alpha\beta$ -TCR which most T-cells carry. Successful (positive selected) cells receive a survival and maturation signal and undergo a change from DP to SP (single-positive), and from there on only bear CD4 or CD8 molecules, restricting CD4+ helper T-cells to MHC class II and cytotoxic CD8+ T-cells to MHC class I [7]. SP cells are then homed to the medulla where they undergo a selection for the deletion of self-reactive T-

cells, in a process called central tolerance. Cells which bear a receptor with high affinity for host or „self“ peptides are sent into apoptosis to prevent potential autoimmune reactions [9]. T-cells which fail to recognize MHC class I or class II receptors, do not receive any survival or maturation signals and die by programmed cell death as well. Compared to the vast number of cells which start their development in the thymus gland, only a few cells endure this harsh yet important selection process, and are then allowed to leave the thymus via venules or lymphatic vessels and finally stand sentinel to defend their host against foreign pathogens.

In this context, crosstalk between the adaptive and innate immunity is crucial for the development of a full immune response to a pathogen (see figure 2.3), but also plays a major role in the progression of autoimmune diseases. For a targeted response to the innumerable bacterial, fungal or viral invaders,

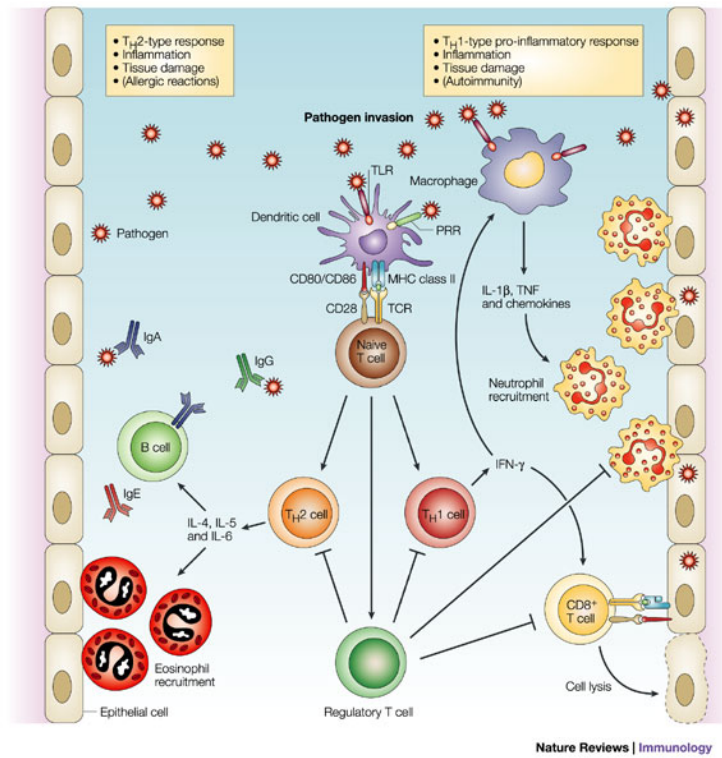


Figure 2.3: simplistic diagram showing the interaction between different immune cells and their crosstalk [11]

T-cells have to rely on contact with other lineages of the immune system, especially antigen-presenting cells (APC). Upon contact with the T-cell in the

lymph nodes or the spleen, the APC then activates and in a broader sense primes the T-cell for its task, the purge of the presented antigen.

This cell-cell contact is conducted via a so called immunological synapse (see chapter 2.4.1) forming around the MHC-TCR receptor interaction. A correct and fitting MHC context has to be established in order to activate the T-cell, which shortly after the first contact with the antigen, shifts into an activated stage, meaning that organelles and the cytoskeleton are rearranged, cell cycle progression is induced and finally clonal expansion starts. The T-cell clones, armed with highly specific receptors for their assigned antigen then drift through blood or lymphatic vessels searching for infiltrators and infected or transformed cells carrying the target antigen in a MHC context. Upon contact with their carefully selected target, another cytoskeletal rearrangement is induced via TCR signalling, allowing the T-cell to direct the secretion of cytolytic substances or cytokines into the newly formed intercellular cleft [12]. With this strategy, single T-cells are able to multi task, meaning that they can simultaneously lyse an infected or transformed cell while forming stimulatory immunological synapses with other cells of the immune system, thanks to a rapid shift of their polarity via rearrangements of their actin fibres.

2.2 Cells with Helper Syndrome (CD4+ Cells)

Contrary to CD8+ cytotoxic T-cells, CD4+ helper cells (Th) are not endowed with any cytolytic potential whatsoever, therefore do not engage the intruder directly, but rather guide, support and/or suppress other cells of the immune system. Therefore, as the name suggests, the main role of CD4+ helper cells is helping to balance the divergent and complex functions of the immune system. Especially in autoimmunity and inflammatory diseases these helper cells play a crucial role, since in these cases they often fail to maintain the composure of an immune system which is frantically attacking a „self“-peptide, or a harmless peptide which is mistaken for a hazardous pathogen [13]. Within the helper cell population there are various subtypes, each with a different task assigned. The first two subpopulations which were identified in the late 1980s were Th1 and Th2 [14] and since then a few new subclasses joined the list of CD4+ T-cells. While the first immune-cells were identified and characterized by their expression of certain CD (cluster

of differentiation) surface molecules, the new subclasses were classified by their cytokine profiles and therefore their function within the meshwork of immunology (see Fig 2.4).

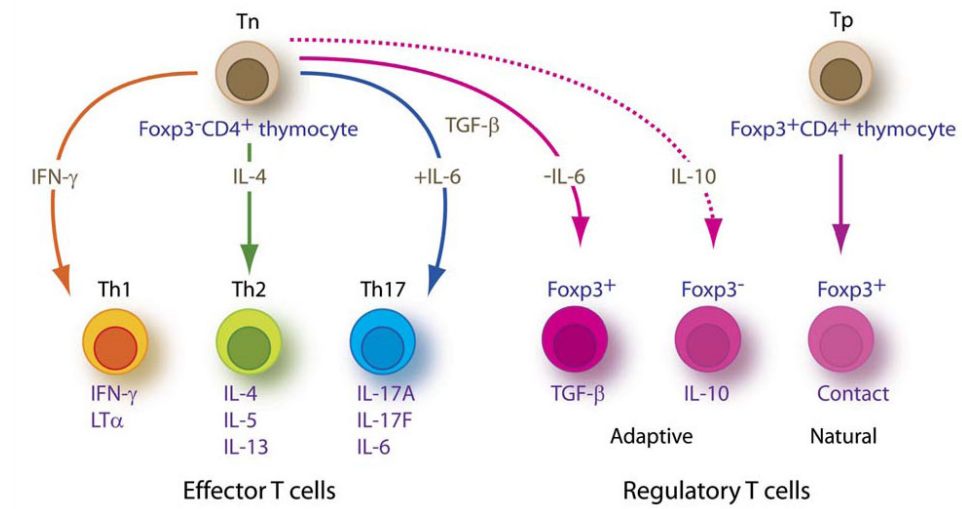


Figure 2.4: Different subsets of CD4⁺ Cells with their distinctive cytokine profile and origin [15]

The classification through cytokine production also helped to develop a better understanding for the communication between cells of the immune system and their lineage commitment. Cytokines secreted from one cell reinforced its own development towards one specific helper-cell population and at the same time inhibited the development into other cell types. These findings of the early 90s from Mosmann et al. [14] as well as similar studies conducted by Coffman in 1986 [16] laid the foundation of our modern understanding of the complex development and maintenance of our immune system. Based on their biological function, there is a basic distinction between regulatory T-cells including nTreg (naturally occurring), iTreg (induced Treg), which are important for the suppression and regulation of the immune system and Th1, Th2, Th17, Tfh and Th9 cells, which play a major role in inflammatory responses [13].

2.3 Commitment Issues (CD4+ Subsets)

2.3.1 Th1

Segmentation of T-cells via cytokine excretion lead to the discovery of Th1 and Th2 helper cells, of which the former are characterized by their production of pro-inflammatory cytokines, namely IFN- γ , TNF- α and TNF- β [14] [13]. Besides their ability to promote IgG2a production in B-cells, their main task is to defend the host against obligate intracellular pathogens such as the protozoan parasite *Leishmania major* [13]. Apart from *Leishmania major*, Th1 response and the resulting IFN- γ production is crucial for the clearance of intracellular bacteria, such as *Mycobacterium avium* [17], *Listeria monocytogenes* [18], *Salmonella typhimurium* [19], as well as fungal parasites such as *Cryptococcus neoformans* [20], and viruses, such as herpes simplex virus (HSV) [21] and influenza A virus [22]. Secondly, Th1 cells seem to be very important in keeping the host free of tumors, which was shown in *in vivo* mouse models by Micallef et al. [23]. It is an intricate balance these cells must achieve between their important role in pathogen clearance, defense against tumor cells, stimulation of the innate and T-cell immune system, and on the other hand refraining from damaging the host by an excessive reaction leading to self reactivity and inflammatory diseases [13]. It is known that Th1 cells play a leading role in the development of inflammatory bowel disease (IBD) [24], diabetes mellitus (type 1 diabetes), graft rejection in patients with bone marrow transplantation [25] as well as autoimmune disorders such as rheumatoid arthritis (RA) [26].

2.3.2 Th2

The role of Th2 helper cells is mainly to produce IL-4, 5, 9, 10 and 13 as well as the rallying of eosinophils and the isotype switching of IgG1 to IgE [14] [13]. In contrast to the tasks Th1-cells are assigned with, the Th2 response focuses more on a humoral response to foreign organisms, especially when dealing with considerable big extracellular hostiles, such as nematodes or helminthes. Within a Th2 response, large amounts of pathogen-specific immunoglobulin are produced in order to deal with the intruder [27]. Another important task for Th2 cells is mucosal immunity in the lung, since an exaggerated Th2 response might lead to chronic inflammation of the airways, atopic asthma, as well as other allergy related pathologies ascribed to exces-

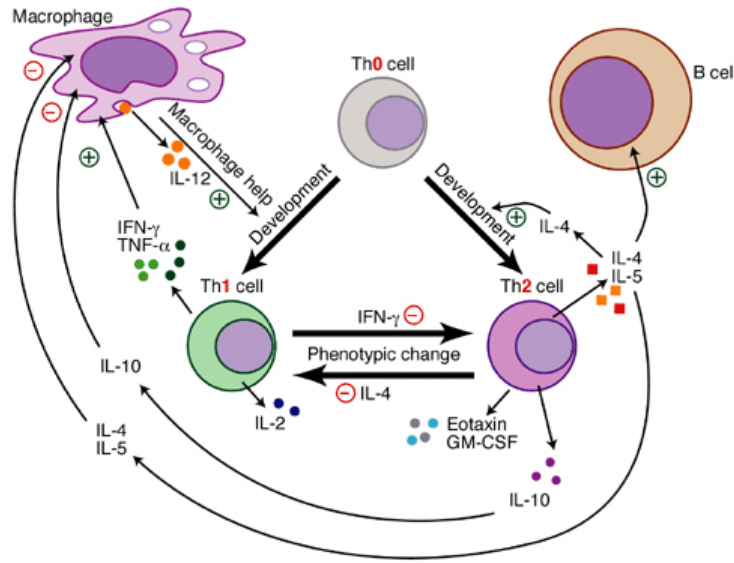


Figure 2.5: A simplified model of the interactions between T helper 1 (Th1) and Th2 responses [Source: <http://journals.cambridge.org/fulltextcontent/ERM/ERM209/S1462399400002143sup007.pdf>]

sive Th2 functions [28]. It is thought that the subset of Th2 cells evolved after their relatives, the Th1 cells, since the targets of the Th2 response seem to be of more recent origin [13] (see Fig 2.5).

2.3.3 Th17

Another important part of the helper-cell population is embodied by a subclass called Th17, which presumably plays an important role in autoimmune tissue injury. As the name suggests, they are defined by their expression of Interleukin 17 (IL-17), namely IL-17A, E and F, as well as IL-21 and 22 [13]. While their status as an independent lineage is widely accepted due to their central role in experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and inflammatory bowel disease (IBD) [29], their common features and differences in the development with the Th1 and Th2 lineage is questioned. Two different models were proposed for Th17 development: The first model suggested that Th17 cells share early stages with the Th1 lineage with both cell lines expressing IL-12 and IL-23 receptors as a common precursor stage, followed by lineage selection via the availability of either IL-12 or IL-23. In contrast, the second model stated that the de-

velopment is distinct from the Th1 lineage, since pertaining to their role in EAE and CIA, T-bet and STAT4 are essential for the development of these diseases, whereas IL-12, IFN- γ and STAT1 are not. Several studies support the second model of Th17 development, since Weaver et al. [15] were able to show that Th1-polarized cells were not able to produce IL-17 after stimulation with IL-23, proposing that Th1 cells are not responsive to Th17 developmental signals. They furthermore showed that IFN- γ actively suppresses Th17 development, indicating that they do not share a common precursor [15]. The importance of Th17 mediated immunity was emphasized in several papers dealing with their role in specific infections such as *Listeria*, *Salmonella*, *Borrelia burgdoferi*, *Mycobacterium tuberculosis*, and several fungal species, indicating that these T-effector-cells are triggered by particular pathogens and are needed for their combating [13].

2.3.4 Treg

Treg cells are considered to be the regulators of the regulators, since their assignment is to modulate the helper cell response posed by Th1 and Th2. They split into nTreg (natural T regulators) and iTreg (inducible T regulators, see Figure: 2.6), they are part of the CD4⁺ subset of lymphocytes, undergo development in the thymus similar to Th cells, but both Treg lines additionally express high levels of CD25 (α -chain of IL-2 receptor), CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), GITR (glucocorticoid-induced tumor necrosis factor receptor family-related gene), LAG-3 (lymphocyte activation gene-3) [30], as well as the transcription factor Foxp3 (forkhead box P3 transcription factor) [31].

iTreg populations further produce membrane-bound forms of TGF- β and IL-10 after stimulation via the TCR [13]. CD25⁺ CD4⁺ Tregs represent only about 5-10% of the peripheral CD4 expressing T-cell population, which is one reason why their function and affiliation is not yet fully defined. Another reason is the fact that surface markers such as CD25 and others listed above are not strictly limited to Treg cells, since they are general markers for activated T-cells, which makes it difficult to isolate them from other T-cell populations. Recent studies, however, indicated their distinction and specific role in the immune system especially for self-tolerance. The lack or dysfunction of a normal Treg population by disruption of the Foxp3 gene for example led to severe autoimmune reactions, such as inflammatory bowel

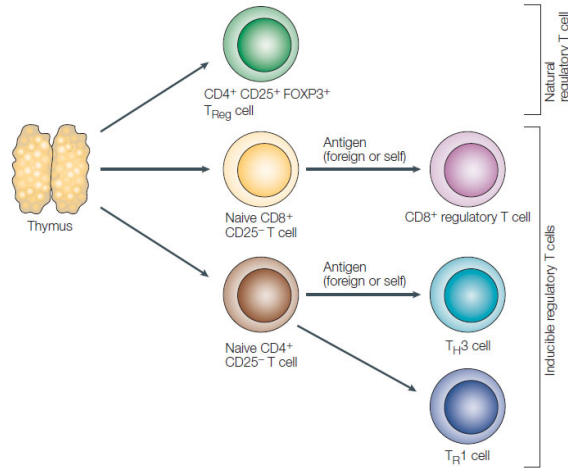


Figure 2.6: Different types of T-cells which all originate in the thymus and then develop into specialized immune-cells such as regulatory T-cells [32]

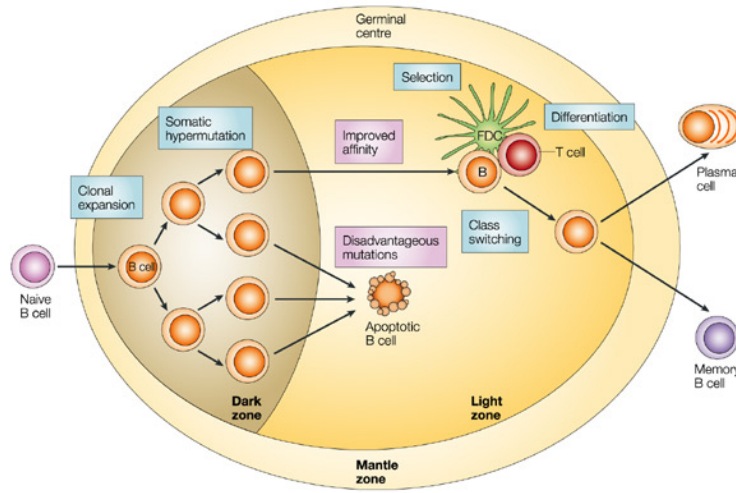
disease, allergy, and type 1 diabetes [31].

2.3.5 Tfh

A small subpopulation of helper cells is stationed in the follicular regions and germinal centers, and the edge of B-cell zones, hence the name follicular helper T-cells (Tfh).

These germinal centers (or GC) are sub-structures within secondary lymphoid tissues such as the spleen or the tonsils and are an important site for B-cell development (see Fig.2.7) [34]. Tfh are characterized by their increased expression of the CXC-motive chemokine receptor 5 (CXCR5), also known as the Burkitt- Lymphoma receptor 1 (BLR-1), which binds to the chemokine CXCL13. This chemokine is highly expressed in B-cell zones, thus homing Tfh cells to these centers [13].

A main task for CD4+ helper cells is the support of B-cell development by promoting processes, important for humoral immunity, such as immunoglobulin isotype-switching and the generation of memory B-cells. For a long time it was thought that this was exclusive Th1/Th2 business, but recent studies have shown that a different subclass of helper-cells is involved in this process as well, i.e. follicular helper-T-cells [35]. Recent studies were able to illuminate the role of this small subset of helper-cells, by pointing out the difference between cytokine expression patterns between Th1, Th2 and Tfh. Tfh cells



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Figure 2.7: development and maturing of B-cells in the germinal centre [33]

produce IL-10 and IL-21 [34], which distinguishes them from Th1 and Th2. Additionally, their independence of TGF- β and retinoid-related orphan receptors for differentiation makes them different from Th17 cells [36], leaving them with an autonomous role as B-cell development helpers in the germinal centers [13].

2.3.6 Th9

It is not clear yet, if Th9 cells actually are a fully differentiated effector cell lineage, since they share a lot of common features with Th2 cells. The main difference seems to be the increased generation of the cytokine IL-9, hence the name Th9, while the secretion of Th2 related cytokines like IL-4, IL-5 and IL-13 seems to be decreased. Additionally, they seem to lack the typical transcription factor for Th2 cells, namely GATA-3, and Treg/Th17 specific factors Foxp3 and ROR γ t [37]. Whether these cells are an autonomous subpopulation or rather specialized Th2 cells has to be further investigated, since there is no confirmed molecular marker ascribed exclusively to these cells yet. Nevertheless, Th9 cells seem to play an important role in the immune response to certain helminth infections [13], a task formally assigned to Th2 cells.

2.4 T-cell Activation

2.4.1 The Immunological Synapse

Activation of T-cells is a complex process, involving cytoskeletal changes and rearrangements of the cell surface. An alteration of surface receptor patterns leads to the formation of a so called „immunological synapse“ which is the main interface for activation and communication between cells of the immune system. The term „synapse“ is usually used for contact junctions of neuronal cells with other neurons, muscle cells, glands, or sensory cells, and has its origin in the Greek words for „syn“ (together) and „haptein“ (grasp or grip). In this context, the term is used for the plane signalling-complexes established between a T-cell and an APC, with the TCR-pMHC (peptide bound MHC) complex forming the core of the synapse, also called central supra-molecular activation cluster (c-SMAC). The TCR complex consists of a TCR α and β chain, together with the CD3 complex composed of one γ , one δ , two ϵ and two ζ chains [38]. Additionally the c-SMAC also harbors other signalling receptors including CD2, CD28, PCK- ϕ , CD4 or in case of cytotoxic T-cells CD8 (see Fig 2.9). In order to stabilize the signalling complexes formed in the c-SMAC domain, the surrounding rings of molecules in the peripheral supra-molecular activation cluster (p-SMAC) contain leukocyte function antigen-1 (LFA-1) and ICAM-1 adhesion proteins [39].

The formation of the synapse relies on TCR-signalling, which, with the aid of co-stimulatory receptors and signals, leads to a reorganization of the cytoskeleton and other receptors, finally resulting in the polarization of the T-cell towards the antigen-presenting-cell (APC) and its peptide bound MHC [40]. In contrast to synapses being formed by neuronal cells, which are stable for years, the immunological synapse is considered to be formed for a very brief period of time only. Lee et al. [42] even stated that constant and tedious stimulation via the TCR it is not essential for T-cell activation, which questions the belief of the central role of the immunologic synapse in T-cell activation. Other studies support that statement by demonstrating that T-cells internalize approximately 90% of their TCRs within thirty to sixty minutes of the first contact with the pMHC [40]. Nevertheless, the importance of the synapse was established in many surveys using biochemical and genetic methods, regardless of its role in actual T-cell activation. One of the first stages in TCR signalling is the phosphorylation of CD3 (see chapter

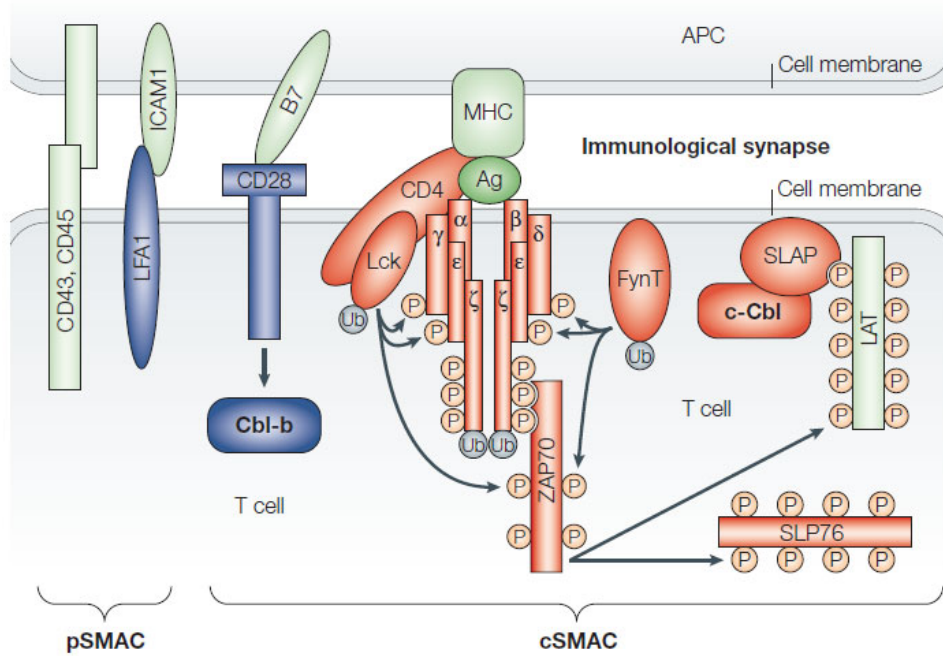


Figure 2.8: The Immunological Synapse [41]

2.4.2), which leads to downstream activation of a variety of other signalling pathways and different genes, resulting in considerable changes in the T-cell protein and mRNA expression patterns and cell cycle progression [40].

2.4.2 CD3 signalling

As far as the TCR signalling complex is concerned, the assignment of the two α and β chains as the binding site for antigens was discovered and described first. Due to its polymorphic nature and relation to the immunoglobulin superfamily it was evident that this was the site for antigen binding [43]. The role of CD3 remained elusive for a long time but was later discovered to be the main signal transducer of the TCR complex. All CD3 chains, in contrast to the two α and β chains, contain long cytosolic tails with at least one immunoreceptor tyrosine-based activation motif (ITAM). On the whole, there are 10 ITAM motifs situated on the cytosolic tails of different CD3-chains, with one sitting on each CD3 γ , δ and ϵ tail, and six on the homodimer CD3 ζ [38]. With their cytosolic tails the CD3 molecules associate with members of the T-cell-specific Src family of protein tyrosine kinases,

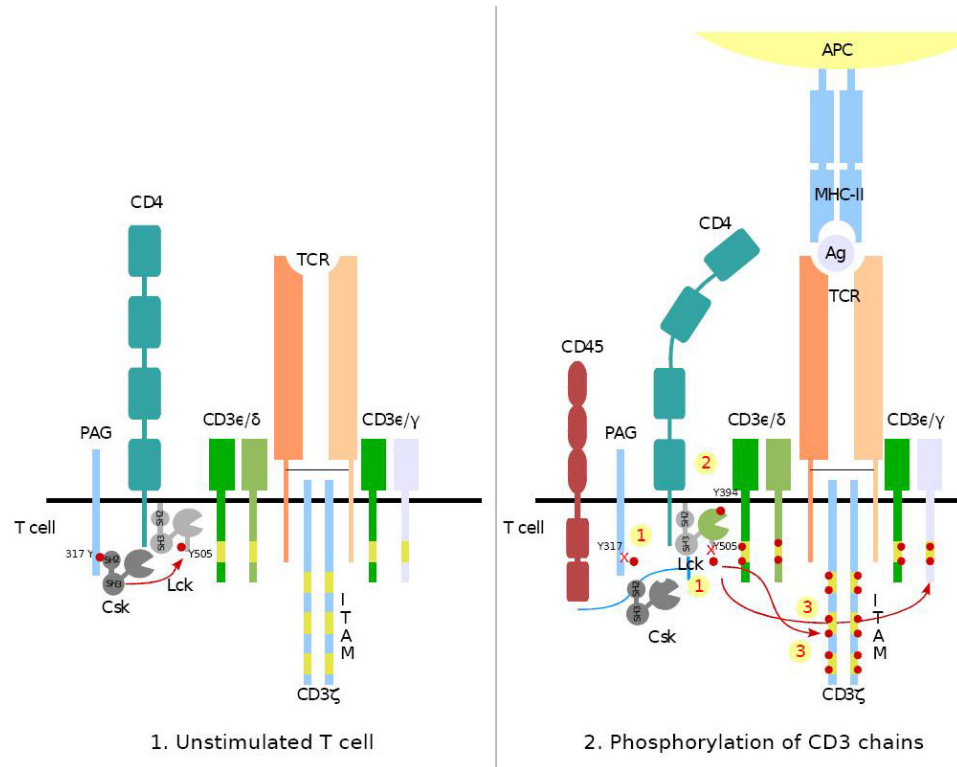


Figure 2.9: CD3 chain phosphorylation upon contact with APC and Antigen [http://www.reactome.org -> TCR signaling]

Fyn and Lck. Other molecules involved in T-cell activation include CD45 and CD4 (or CD8) with the tyrosine kinase Lck bound to their cytoplasmic domain (see figure 2.9). Upon contact with a peptide bound MHC receptor, the kinases Fyn and Lck phosphorylate the CD3-chain ITAM motifs, thus creating docking sites for proteins carrying Src homology 2 (SH2) domains such as the Syk-type kinase ZAP 70 (zeta chain-associated protein of 70 kDa) [12]. Furthermore, it is thought that upon TCR activation, CD3 ϵ undergoes a conformational change, exposing its proline rich region (PRR), which makes it available for binding of SH3 domain carrying proteins such as NCK (see chapter 2.8). Interestingly, this conformational change happens before CD3 ζ and ϵ phosphorylation, which might indicate that the binding of NCK is an important step for the further recruitment of kinases which then accelerate the phosphorylation of the CD3-chains [43] [9]. It is not clear yet, whether the phosphorylation of the ITAMs or the conformational change of the CD3 ζ chain (or both) are equally responsible for the forwarding of

the signal. In addition, the co-receptor, whether it is CD4 or CD8, is also necessary for the correct binding of pMHC thus bringing the bound Lck into proximity of ZAP70 resulting in its phosphorylation and activation. The now activated ZAP70 can bind to and activate a number of intracellular signalling adaptor molecules, such as the linker for the activation of T-cells (LAT) and the SH2 domain containing leukocyte phosphoprotein of 76 kDa (SLP-76) [12]. These two adaptor proteins are crucial for TCR signalling, since studies showed that a loss of function in one of these two genes led to a dysfunctional TCR signalling [43].

2.4.3 The Role of CD2

Regarding T-cell activation and regulation, CD2 (formerly known as T11, LFA-2 and E-rosette receptor) has not enjoyed as much attention and research as CD3, therefore its role as a co-stimulatory molecule is not fully disclosed so far. What is known is that CD2 is an approximately 55kDa transmembrane surface glycoprotein expressed on T-cells and NK cells, important for adhesion in cell-cell contact [44], and it is responsible for the ability of human T-cells to form rosettes with sheep erythrocytes - hence the name E-rosette receptor. There are at least six known epitopes, among which T11.1, T11.2 and T11.3 are the most important ones. The first two epitopes (1 and 2) are expressed on resting T-cells whereas T11.3 is only exposed on activated T-cells [45]. *In vivo*, CD2 (structure shown in Fig 2.10) is able to bind to its ligand CD58 (LFA-3), which is widely expressed on many cell types (including APCs and stromal cells) as well as to CD59, and CD48 (in rodents) [46] [44], contributing to adhesion and T-cell activation in two ways: Firstly, the interaction between CD2 and CD58/CD48 ($K_d \approx 1 \mu M$) leads to cross linking between T-cell (in this case) and target cell surface molecules [47], leading to a process called scanning [48]. „Scanning“ means that the T-cell is able to attach to the APC in a manner, which still allows it to move alongside the APC, frisking its counterpart for antigen bound MHC molecules. Secondly, since CD2 is situated in the c-SMAC domain of the IS in contrast to ICAM1 and LFA1 as mere adhesion molecules in the pSMAC domain, CD2 is suggested that as a (co-) stimulatory molecule, it contributes to the TCR-mediated activation of T-cells as well as other pathways (see Fig 2.11). The loss of CD2 function leads to an immunosuppressive state in both rodents and humans, underlining its important part

in the cellular immune response. In order to distinguish between a mere

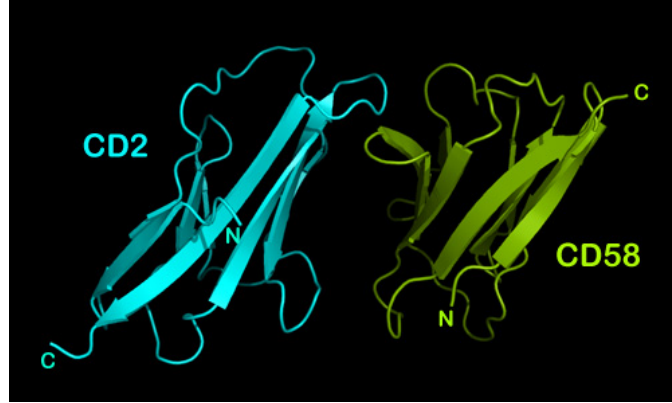


Figure 2.10: Structure of CD2 and CD58

Source: PDB: 1QA9

<http://www.pdb.org/explore/explore.do?structureId=1QA9>

role as an adhesion molecule and actual involvement in T-cell activation, a model has been suggested, which measures the rate of TCR internalization, a process requiring a TCR-pMHC interaction, hence an engaged TCR. In general, it was thought that there are specialized molecules for adhesion (such as ICAM1/LFA1) and a separate set of co-stimulatory molecules intervening with TCR signalling (such as CD28). CD2, albeit not a co-stimulatory molecule such as CD28, has the ability to facilitate both adhesion to APCs and stimulation of T-cells [49]. As a co-stimulatory receptor, it was demonstrated that the binding of CD2 to CD58 leads to the activation of protein tyrosine kinases (PTKs) in the cytoplasm, in particular LCK and FYN as well as the Tec family kinase EMT/ITK/TSK [50]. This CD2 triggered activation is dependent on Lck but independent of CD3 ζ chain, underlining the role of CD2 as a co-stimulatory receptor. In order to understand the role of CD2 in cytoskeletal rearrangements, adhesion or activation, different CD2 cytoplasmic tail interaction proteins were investigated. CD2 cytoplasmic tail-binding protein 1 (CD2BP1) is one of them and is an homolog to the *Schizosaccharomyces pombe* cdc15 which is involved in actin-cytoskeletal regulation [52] [53]. CD2BP1 has the ability to bind to CD2's proline rich sequences (PPLP) with a SH3 domain, but more importantly link CD2 to a protein tyrosine phosphatase (PTP)-PEST. In mice CD2BP1/PSTPIP (the equivalent of PTP-PEST) complexes are able to interact with WASP, which

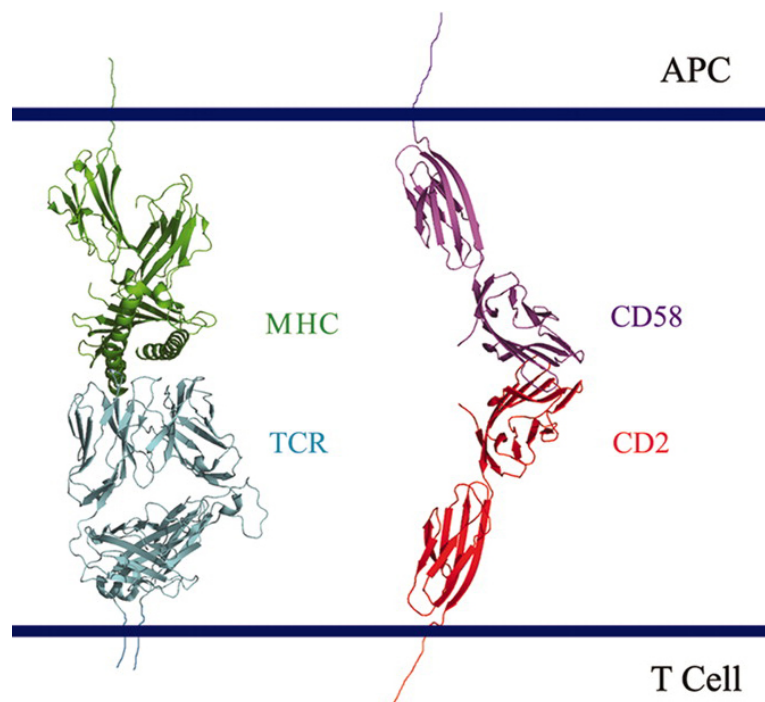


Figure 2.11: Heterophilic interaction of CD2 with CD58. MHC-TCR complex is shown for comparison of molecular dimensions [Source: [51]]

is a key regulator for the actin cytoskeleton. Furthermore, Hutchings et al. were able to show the interaction between the cytoplasmic, proline-rich tail of CD2 and the actin cytoskeleton via the actin associated proteins CMS (human CD2AP), CIN85 and CAPZ, underlining the role of CD2 in the activation of T-cells [54].

A branch of T-cells situated at the mucosal lamina propria (LPTC) show an increased level of IFN- γ expression after CD2 stimulation, due to phosphorylation and enhanced binding of STAT1 and STAT4 to the IFN- γ intronic element. This leads to the conclusion that CD2 stimulation plays a very important role in the regulation of cytokine secretion in the gut, above all IFN- γ which is associated with IBD [55]. Besides IFN- γ production in LPTC, CD2 is also able to induce the production of IL-2 in peripheral T-cell populations, or enhance the production of IL-2 as a co-stimulatory receptor together with the TCR [46].

2.5 CD137L:

CD137L (also called 4-1BBL) is part of the tumor necrosis factor superfamily (TNFSF), thus called TNFSF9, and binds to its receptor CD137 or 4-1BB, which is part of the tumor necrosis factor receptor superfamily (TNFRSF), inducing co-stimulatory signals. This interaction plays a crucial role in processes such as proliferation, apoptosis, and differentiation through the interplay between APCs and other immune cells, such as T and B lymphocytes [56]. Similar to all other members of the TNF superfamily, CD137L is a type II membrane protein with its C-terminus (containing multiple cysteine-rich pseudo-repeats) in the extracellular space, but can also be found in a soluble form in the serum, similar to the TNF receptors, which can either be type I membrane proteins or a soluble form as decoy receptors [57] [58].

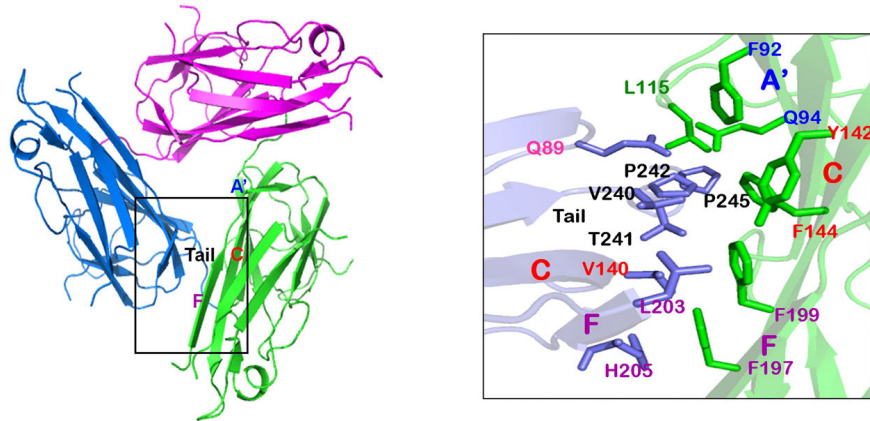


Figure 2.12: CD137L homotrimeric structure and the interaction between the 3 monomers [57]

Interestingly, the signalling between CD137 and its ligand CD137L is bidirectional, meaning that the binding of CD137 to CD137L is able to induce regulatory signals in both participants of this contact, and is not limited to immune cells, such as the monocyte lineage, or B- and T-cells, but can also affect bone marrow cells as well as carcinoma cell lines [56]. Members of the TNF family generally form trimeric structures, as shown in fig. 5.22, but can be further divided into three major groups (regarding their final shape). Group 1 TNFs form bell-shaped trimeric structures, while group 2, due to disulfide bridges, assumes a more globular shape. The third group is basically defined by their lack of sequence homologies with the other members

of the TNF super family. CD137L falls into the last category (Group 3) and shares this group with CD27, CD30L, OX40L and GITRL [57]. The clinical importance of this receptor - ligand duality lies in the fact that T- and B-cell lymphomas in patients with leukemia seem to express both, CD137 and the ligand CD137L, which prolongs their survival and helps to induce proliferation [59]

2.6 CD200 (OX2, MOX1/2):

CD200 (formerly known as OX2) is a type I transmembrane glycoprotein of 45kDa with two extracellular Ig-like domains, a single transmembrane region and a short cytoplasmic tail lacking any known domains or motifs. It is part of the immunoglobulin supergene family, related to a family of co-stimulatory receptors, called B7 [60], and it is found at the 3q13.2 locus on chromosome 3. Many different cell types express CD200 on their surface including activated (but not resting) T-cells, thymocytes, ovarian cells, trophoblasts, dendritic cells, resting B-cells, neurons, endothelial cells and epithelial cells [61]. CD200 presumably has four interaction partners termed CD200R1, -R2, -R3 and -R4, of which CD200R1 is the only one confirmed and seems to be restricted to monocytes, macrophages and dendritic cell lineages, as well as mast cells, basophil cells and certain T-cells. Especially the binding of CD200 to CD200R1 seems to have a grave impact on the modulation of the immune system, since studies show that CD200^{-/-} mice are far more likely to develop autoimmune defects such as spontaneous fetal loss, graft rejection, inflammatory disorders etc. The reason for this is thought to lie in the fact that the cytoplasmic part of CD200R1 is equipped with a NPXY motif, and is therefore able to deliver immunoinhibitory signals [62]. The circumstance that almost only tissues that usually carry CD200 on the surface are affected by this immune frenzy supports that thesis. CD200 - CD200R1 cooperation in general is thought to be one of the future „hot spots“ for the understanding of autoimmune diseases and immune modulation [61].

2.7 α Pak (Pak1):

PAK I (p21-activated kinases) is a family of serin/threonin kinases, which consists of three isoforms (Pak 1-3), all of which are associated with the GTPases Rac and Cdc42. Pak1 (α Pak, p68), Pak2 (β Pak, p65) and Pak3 (γ Pak, p62) are an evolutionary highly conserved group of kinases with dozens of substrates, and are therefore consolidated under the term PAK I, to distinguish them from their less cognate relatives, the PAK II kinases. PAK I kinases share a common structure and are characterized by an N-terminal regulatory domain, harboring a p21 GTPase binding domain (PBD), a CRIB region (for Cdc42 and Rac interactive binding), together with a partially overlapping autoinhibitory domain (AID), followed by proline rich regions, which serve as binding sites for proteins with SH3-domains. Within the PAK I family, the

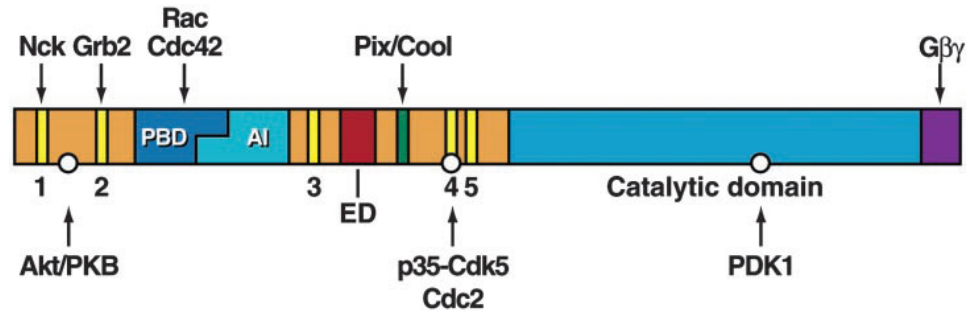


Figure 2.13: Schematic diagram indicating features of PAK1 structure with p21 GTPase binding, autoinhibitory (AI) and catalytic domains. [63]

regulatory domains differ from each other through the number of canonical PXXP SH3-binding motifs (five in Pak1, two in Pak2 and four in Pak3) and a nonclassical (PXP) SH3-binding site [63]. The first binding site in Pak1 is interacting with Nck, an SH2-SH3 adaptor protein, which links the kinase to receptor tyrosine kinases via its SH2 domain [64]. Using Nck, Pak1 can also interact via its PXXP motifs. The C-terminal part hosts the catalytic (kinase) domain, which can be found in different variants, due to alternative splicing of the Pak mRNA. There are two different splice variants of Pak1 known in man (Pak1 and Pak1B), and several splice forms of the Pak3 C-terminus, which are usually highly expressed in neurons. The expression of Pak kinases in leukocytes is restricted to Pak1 and Pak2. In white blood cells (neutrophils, macrophages, mast cells and T-cells) these kinases play an important role in the reorganization of the cytoskeleton, and their ability

to activate the Erk pathway as well as their role in other cellular processes such as cell motility, apoptosis, mitosis, and transcription, underscores their importance in the immune-response [65]. The activation of the NFAT and Erk pathway (see Fig 2.14) via the T-cell receptor relies on Pak1 functionally located downstream of a Nck/Vav/SLP-76 signalling complex [66]. PAKs are usually overexpressed and/or hyperactivated in tumor tissue, which means that their role in cell transformation makes them an attractive target for the development of tumor specific therapies. [67] When not activated via a GT-

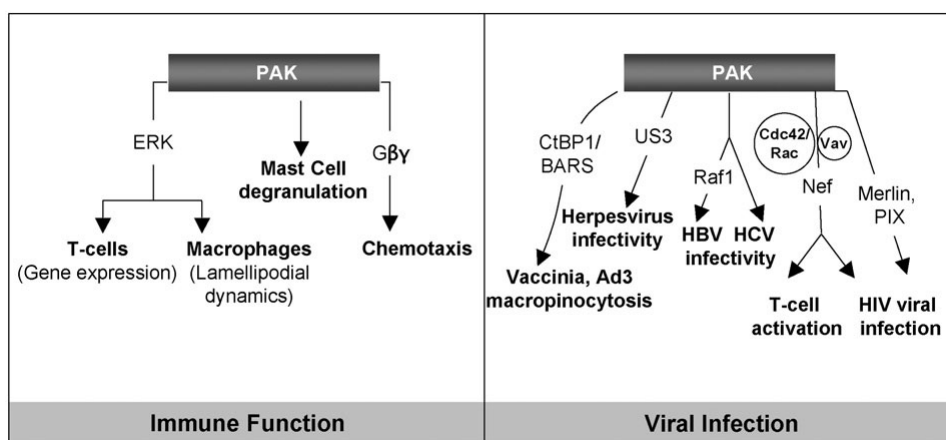


Figure 2.14: The role of PAKs in the activation of immune-relevant pathways [67]

Pase, PAKs form homodimers, which keeps them in their conformationally restricted, inactive state, in which the inhibitory switch domain covers the catalytic domain, thus rendering it catalytically incompetent. Upon activation, a conformational change is induced, due to the binding of an active GTPase to the N-terminal p21 binding domain (PBD). Activation through a GTPase occurs by binding of Rac1, Rac2, Rac3, Cdc42, but also by CHP, TC10 and Wrch-1 to the p21 binding domain, but does not occur when interacting with Rho A-G or other members of the Ras superfamily [63]. This unfolding event allows the autoinhibitory domain to dissociate from the catalytic domain, which leads to autophosphorylation in both the kinase domain, at Thr423 in the activation loop, and at the regulatory domain situated at the N-terminus. The autophosphorylation of Thr423 seems to play an important role in the final activation steps, since it helps to maintain relief from autoihibition and thus promotes further substrate phosphorylation [65].

2.8 Nck:

Nck (non-catalytic region of tyrosine kinase) is a family of cytosolic adapter proteins with a relative molecular mass of 47kDa, which consist of one C-localized SH2 domain, and three SH3 domains connected with linker regions (Figure 2.15). In other words, they could potentially interact with three different downstream or upstream proteins. There are 2 known members of the Nck family in humans, namely Nck1/Nck α and Nck2/Nck β or Grb4, two known members in mice, which are called mNck α and mNck β and one known relative in drosophila (Dock) [68]. In humans the gene coding for Nck1 is localized to the 3q21 locus of chromosome 3, the gene for Nck2 is found on chromosome 2 at the 2q12 locus. These two genes however produce two Nck molecules, which are identical in 68% of their amino acid sequence, with most differences in the linker regions. The similarity between the two Ncks leads to some extent to a functional redundancy, although their expression pattern varies within different tissues. Nevertheless, neither Nck1 nor Nck2 knock-out mice showed any phenotype change, although double knock-outs are fatal *in utero* due to problems with structures derived from mesodermic tissue, indicating that Nck must have a vital part in mammalian development [69].

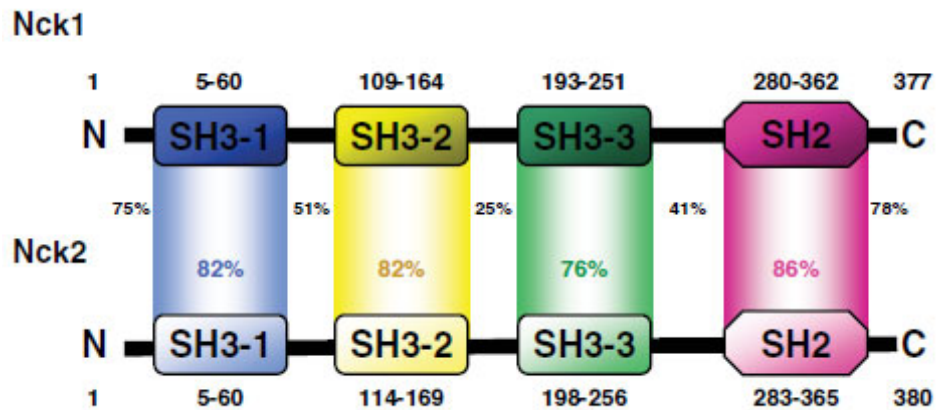


Figure 2.15: Domain-homology between Nck1 and Nck2 in percent [12]

There are, however, studies which have shown that there might be distinct functions for both isoforms, since the regulation of the actin cytoskeleton in fibroblasts and breast carcinomas depends on signalling of platelet-

derived growth factor (PDGF) and epidermal growth factor (EGF) via Nck2 (exclusively) [70]. Furthermore, the control neuritogenesis seems to be related to the function of Nck2 [12]. The importance of Nck proteins is further

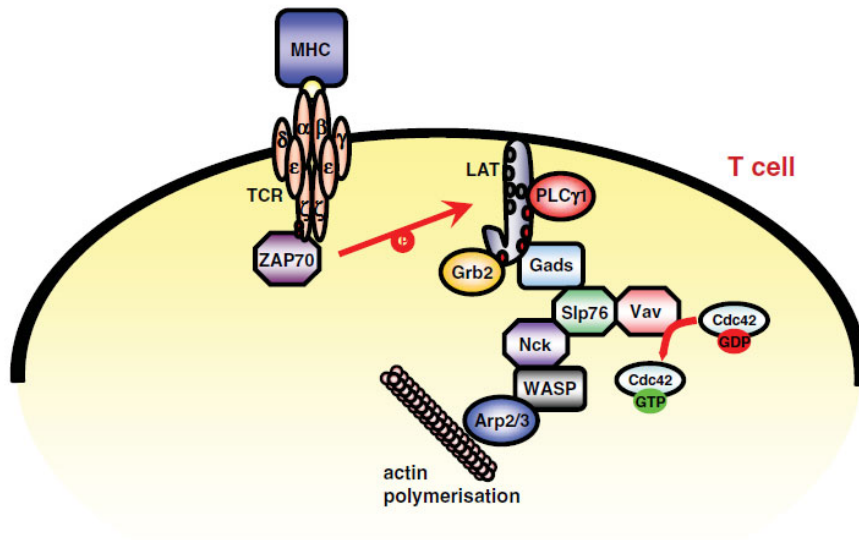


Figure 2.16: Nck in T-cell signalling [12]

illustrated by their role in T-cell signalling, since they link receptor tyrosine kinases (RTKs) with the actin cytoskeleton remodeling machinery, which was demonstrated by co-localization and biochemical studies, indicating that Nck forms a multi protein complex with Vav and SLP-76 (see Figure 2.16) [68]. Additionally, Roy et al showed that Nck knock-out leads to severe T-cell lymphopenia in mice which expressed a transgenic TCR receptor. Thus Nck seems to play a crucial role in the orchestration and modulation of TCR signalling in response to foreign and self antigen stimulation [69].

Another important point is the interaction between Nck and Pak. Recently, new interaction partners were identified: Pix for „Pak interacting exchange factors“, also termed Cool for „cloned out of library“. α Pix (Cool-1) and β Pix (Cool-2) and are both equipped with a SH3 domain, a PH domain and a Dbl-homology (DH) domain [68]. Pak is probably recruited to the plasma membrane by Nck and binds to autophosphorylated receptor tyrosine kinases using its SH2 domain. The N-terminal first proline-rich region of Pak binds to Nck's SH3 domain [12], whereas Pix uses different proline-rich regions

of Pak to bind via its SH3 domain, situated C-terminal of the p21-binding domain (PBD). Studies show that the interaction between Nck and Pak is increased during adhesion or cell-cell contact, and is reduced again after the loss of contact. Since Pak and Pix are recruited to receptor tyrosine kinases via Nck, and Pix can localize Pak to focal complexes, it is likely that the Nck/Pak/Pix complex is responsible for recruiting receptor tyrosine kinases to focal complexes [68].

2.9 Aim of this study:

As mentioned above, the role of CD2 as a co-stimulatory molecule in T-cell activation and anergy is not fully understood yet. We therefore conducted experiments to identify genes and proteins affected by α CD2-stimulation in the context of a CD4+ helper T-cell population. A series of previous TaqMAN assays with the goal to confirm genes showing elevated mRNA expression following CD2 stimulation revealed a handful of genes with significantly increased mRNA levels. We picked four candidate genes to further investigate their protein expression pattern over a time period of 72 hours.

Nck was picked due to its nature as a SH2/SH3 adaptor protein, which has already been shown to interact with the cytosolic tail of CD2 (the proline rich sequences to be exact). Since it is thought to recruit kinases such as α Pak to the cell membrane, Nck is a valuable target to further investigate downstream targets of CD2 signalling.

α Pak seemed to be an interesting target since as a kinase, involved in many cellular processes, it is a main hub for different pathways. The elevated mRNA levels might be an indication for its direct or indirect involvement in CD2 signalling.

As for CD137L, current literature proposes that this ligand is restricted to the myeloid lineage and is only expressed in exceptional cases on members of the lymphoid lineage (T and B-cell lymphomas), it was interesting to find elevated levels of CD137L mRNA in CD2 stimulated T-helper-cells. Therefore we chose to include this protein in our survey to further illuminate this circumstance, since a positive result would bring new light into the regulatory functions of T-effector cells.

OX2 / CD200 also showed increased levels of mRNA in the TaqMAN assays, and was picked for a similar reason as CD137L. It is thought that

CD200 plays a role in T-cell anergy and autoimmune reactions. We therefore suggested that CD2 might be connected to T-cell anergy and self-tolerance amongst others through CD200 and/or CD137L. In this study we tried to investigate a correlation between the results of the TaqMAN assays regarding the mRNA concentration and the actual protein product in CD4⁺/HLADR⁻/CD25⁻ Th-cell populations.

Materials and Methods

3.1 Stock solutions

10% Sodium Dodecyl Sulfate (SDS)

Dissolve 10 g of electrophoresis-grade SDS ($MW = 288.37 \text{ g/mol}$) in 80 ml of distilled H_2O . Add distilled H_2O to make a total volume of 100 ml of solution. Store at room temperature (indefinitely).

1.5 M Tris (pH 8.8)

Dissolve 181.5 g of Tris base ($MW = 121.14 \text{ g/mol}$) in 800 ml of double distilled H_2O . Adjust the pH to 8.8 with concentrated HCl. Adjust the volume to 1 L. Aliquot in convenient volumes. Store at room temperature.

1.0 M Tris (pH 7.3)

Dissolve 12.1 g of Tris base ($MW = 121.14 \text{ g/mol}$) in 80 ml of double distilled H_2O . Adjust the pH to 7.3 with concentrated HCl. Adjust the volume to 100 ml. Store at room temperature.

1.0 M Tris (pH 6.8)

Dissolve 12.1 g of Tris base ($MW = 121.14 \text{ g/mol}$) in 80 ml of double distilled H_2O . Adjust the pH to 6.8 with concentrated HCl. Adjust the volume to 100 ml. Store at room temperature.

5 M NaCl (500 ml)

Dissolve 146.1 g of NaCl ($MW = 58.44 \text{ g/mol}$) in 450 ml of distilled H_2O . Adjust the volume to 500 ml. Store at room temperature.

Phenylmethanesulfonyl Fluoride (PMSF)

Also called α -toluenesulfonyl chloride, PMSF ($MW = 174.19 \text{ g/mol}$) is used as an inhibitor of serine proteases. It is very toxic. Aqueous solutions of PMSF are hydrolyzed very rapidly. Make a 100 mM stock solution (i.e. 174 mg/10 ml) in absolute ethanol or 2-propanol. Aliquot and store in foil

wrapped tubes at -20°C . Add to aqueous solutions immediately before use.

Caution: PMSF is inactivated in aqueous solutions. The rate of inactivation increases with pH and is faster at 25°C than at 4°C . The half-life of a $20\mu\text{M}$ aqueous solution of PMSF is about 35 times shorter at pH 8.0. Thus aqueous solutions of PMSF can be safely discarded at a higher pH than 8.6 and storing for several hours at room temperature. [*Lab Ref. Volume 2*]

10% Ammonium Persulfate (APS)

Dissolve 0.1 g in 1 ml of double distilled H_2O . Mix by vortexing.

Ponceau S

Dissolve 2 g of Ponceau S, 30 g of trichloroacetic acid, and 30 g of sulfosalicylic acid in 100 ml of distilled H_2O .

TBS + 0.25% Tween + 0.25% TritonX (TBS+T)

- 1 M Tris (pH 7.3) 10 ml (10mM)
- 5 M NaCl (MW = 58.44 g/mol) 20 ml (100mM)
- Tween 20 0,5 ml (0.05%)
- Triton X 0,5 ml (0.05%)

Adjust the volume to 1 L with double distilled H_2O . Store at room temperature.

10x Phosphate Buffered Saline (PBS) (ph 7.2-7.4)

- 137 mM NaCl (MW = 58.44 g/mol) 8 g
- 2.7 mM KCl (MW= 74.55 g/mol) 200 mg
- 10 mM Na_2HPO_4 (MW= 141.96 g/mol) 1.44 g
- 2 mM KH_2PO_4 (MW= 136.09 g/mol) 240 mg

Dissolve components in approximately 0.9 liter of double distilled H_2O . Adjust the pH to 7.2 - 7.4 with concentrated HCl and adjust the final volume to 1 L with double distilled H_2O . Store at room temperature.

5x Tris Buffered Saline (TBS) (ph 7.15)

- 1 M Tris-HCl (pH 7.3) 50 ml
- 5 M NaCl (MW = 58.44 g/mol) 150 ml

Dilute components in 1 L of double distilled H₂O.

Lysis buffer II

- Triton X 10%, 10x TBS 1 ml
- PMSF 100mM 100 μ l
- Phosphatase Inhibitor Cocktail (Sigma) 100 μ l
- Protease Inhibitor Cocktail (Sigma) 100 μ l
- double distilled H₂O to make 10 ml

10x Running Buffer (1 L)

- 250 mM Tris base (MW = 121.14 g/mol) 30.3 g
- 2 M glycine (MW= 75.7 g/mol) 150 g
- 1% SDS (MW = 288.38.14 g/mol) 10 g
- double distilled H₂O to make 1 L

10x Western Transfer Buffer (10 L)

- 25 mM Tris base (MW = 121.14 g/mol) 30.3 g
- 0.2 M glycine (MW= 75.7 g/mol) 150 g
- MeOH 20% 2 L
- add distilled H₂O 80% to make 10 L

Sample Buffer II

- 2 M glycerol (MW = 92.10) 2 ml
- 1 M Tris pH 6.8 1.25 ml
- 20% SDS (MW = 288.38.14 g/mol) 1 ml
- bromophenol blue 1,6 mg
- adjust the final volume to 10 ml with double distilled H₂O.
- always add 5% β -mercapto EtOH right before use

Stripping Solution (pH 6.7)

- 100 mM β -mercapto EtOH 142 μ l
- 2% SDS (MW = 288.38.14 g/mol) 4 g
- Tris/HCl (MW = 157.59 g/mol) 1.51 g
- dissolve SDS and Tris/HCl in 180 ml H₂O
- adjust pH to 6.7 with NaOH
- always add β -mercapto EtOH right before use

3.2 Isolation of Mononuclear Cells (MNCs)

Peripheral blood was drawn from healthy volunteers. The study was conducted according to the mandatory ethical regulations posed by the bioethic commission of the Medical University of Vienna.

- Collect blood from a volunteer in vacuette EDTA tubes
- Dilute blood with PBS 1:3
- Dispense 15 ml of Ficoll® into 50 ml Falcon tubes, and carefully overlay with 35 ml of blood-PBS dilution. The blood phase and the Ficoll phase must not get mixed
- Centrifuge the tubes at 1400 rpm for 40 min at room temperature. Set the breaks of the centrifuge to 0.
- Discard the plasma phase and pool the MNC fraction in a new 50 ml Falcon tube (See Fig.: 3.1)
- Adjust the volume of the MNC suspension to 50 ml with sterile PBS
- Centrifuge at 1200 rpm for 10 min at 4°C with breaks enabled
- Discard the supernatant (without disturbing the pellet!), resuspend the pellet in the remaining few μ l and add 25 ml of sterile PBS to wash the cells.
- Centrifuge at 800 rpm for 10 min at 4°C with breaks enabled
- Discard the supernatant (without disturbing the pellet!), resuspend the pellet in the remaining few μ l and add 10 ml of RPMI medium.
- Count Cells.

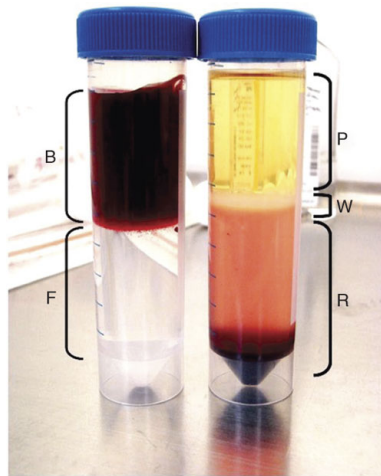


Figure 3.1: On the left: (top down) Blood-PBS (B), Ficoll® (F). On the right: (top down) Plasma (P), MNC layer (W), Ficoll®, red blood cells and granulocytes (R)

3.3 Isolation of CD4⁺ cells

CD4 positive T-helper cells are negatively sorted with *DYNAL® Magnetic Beads* using specific antibodies (table: 3.2) and *DYNAL® Magnetic Beads* coated with sheep anti-mouse IgG antibodies.

- Wash beads to remove preservatives
- Estimate the number of cells to be depleted
- Calculate 4 beads per cell to be depleted and transfer the corresponding volume of beads into a sterile eppendorf 1,5 ml tube
- Place the tube next to the magnet and let it rest for 5 min
- Discard the liquid (carefully!)
- Remove the tube from the magnet
- Add PBS and resuspend the beads
- Put the tube back on the magnet and let it rest again for 5 min
- Repeat the PBS washing step two times
- Resuspend the beads in an adequate volume of PBS or RPMI medium
- Resuspend the MNCs at 2×10^7 cells per ml of RPMI medium
- Add the following antibodies (at the concentration indicated in Table 3.2) for depletion

Table 3.1: Antibodies used for sorting and their dilution (for concentration see: 7.2 References)

| | dilution | target cell |
|-------------------------------------|-----------------|---|
| CD8 | 1:200 | cytotoxic lymphocytes |
| CD13 | 1:200 | myelomonocytic cells |
| CD14 | 1:100 | myelomonocytic cells |
| CD16 | 1:100 | neutrophils, NK-cells, macrophages |
| CD19 | 1:200 | B-cells |
| CD25 | 1:200 | activated T-cells, Treg, B-cells, monocytes |
| CD235a | 1:200 | erythrocytes |
| HLA-DR | 1:200 | activated T-cells, B-cells, monocytes |
| TCR$\gamma\delta$ | 1:200 | subpopulation of T cells |

- Rotate for 30 min at 4°C
- Spin the cells at 1200 rpm, 4°C for 10 min
- Discard the supernatant and resuspend again in RPMI medium
- Add the dynabeads and gently rotate for 30 minutes (maximum 60 min!) at 4°C
- Place the tube next to the magnet and let it rest for 5 min
- Carefully transfer the cell suspension into a new tube (without touching the bead-pellet!)
- Put the new tube with the cells again onto the bead magnet and let it rest for 5 min
- Repeat the last two steps to eliminate all remaining beads from the cell suspension
- Count remaining cells.
- Check the purity of the cell population via fluorescence activated cell sorting (FACS) (see following chapter)

3.4 FACS - Analysis

After the sorting step, check the purity of the resting CD4+ population via FACS using aliquotes of the cell suspension in order to detect contamination with other cell types.

- for each celltype (MNC / T-cell) aliquot 200.000 cells into a FACS tube.
- (MNC only!): add 30 μ l of human IgG (1 mg/ml - Sigma-Aldrich)
- let MNC / IgG incubate on ice for 10 min
- add FITC and PE labelled antibodies (as indicated in Table 3.2) to MNC and T-cells

Table 3.2: FACS-Antibody dilutions

| MNC | Tcell | | FITC | PE | Dilution |
|-----|-------|--|---------|--------------------|----------|
| 1 | 14 | | FITC Co | - | 1:20 |
| 2 | 15 | | - | PE Co | 1:20 |
| 3 | 16 | | CD3 | - | 1:20 |
| 4 | 17 | | - | CD3 | 1:20 |
| 5 | 18 | | CD2 | CD3 | 1:20 |
| 6 | 19 | | CD4 | CD3 | 1:20 |
| 7 | 20 | | CD8 | CD3 | 1:20 |
| 8 | 21 | | CD3 | CD14 | 1:20 |
| 9 | 22 | | CD3 | CD16 | 1:20 |
| 10 | 23 | | CD3 | CD19 | 1:20 |
| 11 | 24 | | CD3 | CD25 | 1:20 |
| 12 | 25 | | CD3 | HLA-DR | 1:20 |
| 13 | 26 | | CD3 | TCR $\gamma\delta$ | 1:20 |

- incubate the cells with the antibodies for 30 min at 4°C in the dark
- add 250 μ l of PBS and spin at 3000 rpm for 10 min at 4°C
- discard the supernatant and resuspend the pellet in 500 μ l PBS (vortex)
- spin at 3000 rpm for 10 min at 4°C
- discard the supernatant and resuspend the pellet in 500 μ l PBS (vortex)
- spin at 3000 rpm for 10 min at 4°C
- discard the supernatant and resuspend the pellet in 500 μ l PBS (vortex)
- FACS Analysis

3.5 Cell Culture

Purified CD4+ cells were distributed into 3x3 wells, at 5×10^6 cells per 3 ml medium per well. Three timepoints (24h, 48h, 72h) with one negative control (no stimulation), CD2 stimulation and CD3 control stimulation each, hence the 3x3 wells. Incubation at 37°C in a humidified atmosphere containing 5% CO₂

10.4

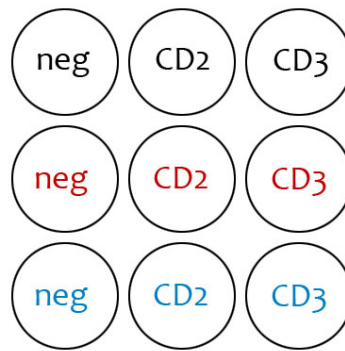


Figure 3.2: Black: 24h / Red: 48h / Blue: 72h

CD2 stimulation:

Two antibodies directed against different epitopes (T11₂ and T11₃) of CD2 were used in order to maximize the stimulation. (See 3.7 for detailed antibody description and concentration)

- aliquot 5×10^6 cells resuspended in 3 ml RPMI medium into each CD2 well
- add 5 µg/ml of Vit13 antibody -> 12 µl
- add 5 µg/ml of 6F10.3 antibody -> 75 µl
- incubate for 24, 48 and 72 hours
- after each timepoint harvest and lyse the cells in lysisbuffer II (see chapter 3.6)

CD3 stimulation:

(See 3.7 for detailed antibody description and concentration)

- dilute 5 µg/ml of OKT3 (CD3) -> 5 µl antibody with 995 µl of 37°C PBS for each CD3 well

- pipet 1 ml of the OKT3 dilution into each CD3 well
- incubate for 30 min at 37°C
- discard the supernatant and wash 3 times with 37°C PBS
- add 5×10^6 cells resuspended in 3 ml medium into each CD3 well
- incubate for 24, 48 and 72 hours and lyse the harvested cells

3.6 Harvesting

For each timepoint:

- transfer the cell suspension from each well into new 15 ml Falcon tubes.
- wash the well 3x with PBS and transfer the suspension into the Falcon tube
- count cells
- branch off 1.600.000 cells for further FACS stainings (see 3.3) into a new Falcon (200.000 for each staining)
- spin the remaining cells at 1200 rpm, 4°C
- discard the supernatant and resuspend the cell pellet in lysis-buffer II (2×10^7 cells per ml lysis buffer)
- lyse 1 h on ice, then spin the lysate at 15000 rpm at 4°C for 10 min and transfer the supernatant into a new eppendorf 1.5 ml tube. Store lysate at -20°C

Check stimulation via FACS using the surface expression of specific activation associated surface molecules (CD69, CD25 and HLA-DR):

add FITC and PE labeled antibodies (as indicated in Tables 3.3 and 3.4) to the T-cell vials

- incubate the cells with the antibodies for 30 min at 4°C in the dark.
- add 250 μ l of PBS and spin at 3000 rpm for 10 min at 4°C
- discard the supernatant and resuspend the pellet in 500 μ l PBS (vortex)
- spin at 3000 rpm for 10 min at 4°C
- discard the supernatant and resuspend the pellet in 500 μ l PBS (vortex)
- spin at 3000 rpm for 10 min at 4°C
- discard the supernatant and resuspend the pellet in 500 μ l PBS (vortex)
- FACS analysis

Table 3.3: FACS scheme for unstimulated and CD2 stimulated T-cells:

| T-cell | | FITC | PE | Dilution |
|--------|--|---------|--------|----------|
| 1 | | FITC Co | - | 1:20 |
| 2 | | - | PE Co | 1:20 |
| 3 | | CD3 | - | 1:20 |
| 4 | | - | CD3 | 1:20 |
| 5 | | CD2 | CD3 | 1:20 |
| 6 | | CD3 | CD69 | 1:20 |
| 7 | | CD3 | CD25 | 1:20 |
| 8 | | CD3 | HLA-DR | 1:20 |

Table 3.4: FACS scheme for CD3 stimulated T-cells:

| T-cell | | FITC | PE | Dilution |
|--------|--|---------|--------|----------|
| 1 | | FITC Co | - | 1:20 |
| 2 | | - | PE Co | 1:20 |
| 3 | | CD3 | - | 1:20 |
| 4 | | - | CD3 | 1:20 |
| 5 | | CD2 | CD3 | 1:20 |
| 6 | | CD2 | CD69 | 1:20 |
| 7 | | CD2 | CD25 | 1:20 |
| 8 | | CD2 | HLA-DR | 1:20 |

3.7 IP / Western Blot

For proteins with a low concentration in the cell lysate, immunoprecipitations were performed to enrich the target molecule.

3.7.1 Immunoprecipitation

- mix 30 μ l of *Protein G Plus-Agarose suspension* (Calbiochem®) with 5 μ l of antibody (final concentration 1:100 with 500 μ l of cell lysate)
- incubate at 4°C for 15-30 min
- add 500 μ l of cell- lysate
- rotate for one hour at 4°C
- spin the samples at 3000 rpm for 5 min
- discard the supernatant
- wash with 500 μ l lysisbuffer II (undiluted)

- spin the samples at 3000 rpm for 5 min
- discard the supernatant
- wash again with 500 μ l lysisbuffer II (undiluted)
- spin the samples at 3000 rpm for 5 min
- discard the supernatant
- wash with 500 μ l lysisbuffer II (diluted 1:10 with TBS)
- spin the samples at 3000 rpm for 5 min
- discard the supernatant
- add 35 μ l of Sample Buffer II

3.7.2 Polyacrylamide Gel elektrophoresis (PAGE)

Hand-casted polyacrylamide gels were used to separate the different proteins in the cell lysate and in the immuno-precipitates according to their molecular weight.

- 9% Resolving Gel (10 ml for 2 gels):
 - * double distilled H₂O 4.3 ml
 - * 30% acrylamide mix 3.0 ml
 - * 1,5 M Tris (pH 8.8) 2.5 ml
 - * 10% SDS 100 μ l
 - * 10% APS 100 μ l
 - * TEMED 6 μ l
 - * overlay with H₂O
 - * let polymerize (aprox. 20 min)
 - * discard the remaining water before continuing
- 5% Stacking Gel (3 ml for 2 Gels)
 - * distilled H₂O 2.1 ml
 - * 30% acrylamide mix 0.5 ml
 - * 1 M Tris (pH 6.8) 380 μ l
 - * 10% SDS 30 μ l
 - * 10% APS 30 μ l
 - * TEMED 3 μ l

- * let polymerize (aprox. 20 minutes)
- add 20 μ l Sample Buffer II to 20 μ l sample (cell lysate or precipitate)
- boil sample at 95°C for 5 min
- load the gel, and let it run for 120 min at 120 V or until the dye front reaches the bottom of the gel

3.7.3 Western blot

After separation via PAGE, the proteins were transferred to a nitrocellulose membrane, which was then incubated with specific antibodies to detect the four target molecules.

- Assemble the sandwich blot (Mini-PROTEAN by Biorad®) in a bowl filled with transfer buffer in the following order:
 - 1) open a blotting cassette (black side at the bottom)
 - 2) soaked sponge
 - 3) 2-3 layers of soaked 3M blotting paper
 - 4) PAGE gel (see 3.7.2)
 - 5) soaked nitrocellulose membrane (Amersham Hybond ECL)
 - 5) 2-3 layers of 3M blotting paperuse a 10 ml pipett to carefully remove any air bubbles that might sit between the gel and the membrane
- 6) soaked sponge
- close and secure the sandwich cassette
- place the cassettes into the Western transfer tank (Mini-PROTEAN by Biorad®)
- fill the tank with Western transfer buffer
- let it run for 90 min at 100 V
- after Western transfer, wash twice with TBST and immerse the membrane in a sufficient amount of Ponceau staining solution and stain for 5 min (do this before blocking!) The total transfered proteins should be visible now.
- after staining, transfer the membrane into water for two washes of 5 minutes each.
- wash with TBST+T (few seconds) until the ponceau stain is fully removed.

- block non-specific binding sites on the membrane with 5% non-fat dried milk in TBST (blocking solution) for 1 h at room temperature on an orbital shaker.
- wash 2x with TBST+T for 15 min
- add primary antibody (diluted according to Table 3.9) in 3 ml TBST+T with 5% non-fat dried milk and incubate over night at 4°C.
- wash 3x with TBST for 20 min
- add secondary (HRP conjugated) antibody (according to Table 3.9 1:4000) and incubate for 1 h
- wash 3x with TBST+T for 20 min

3.7.4 Stripping for reprobing

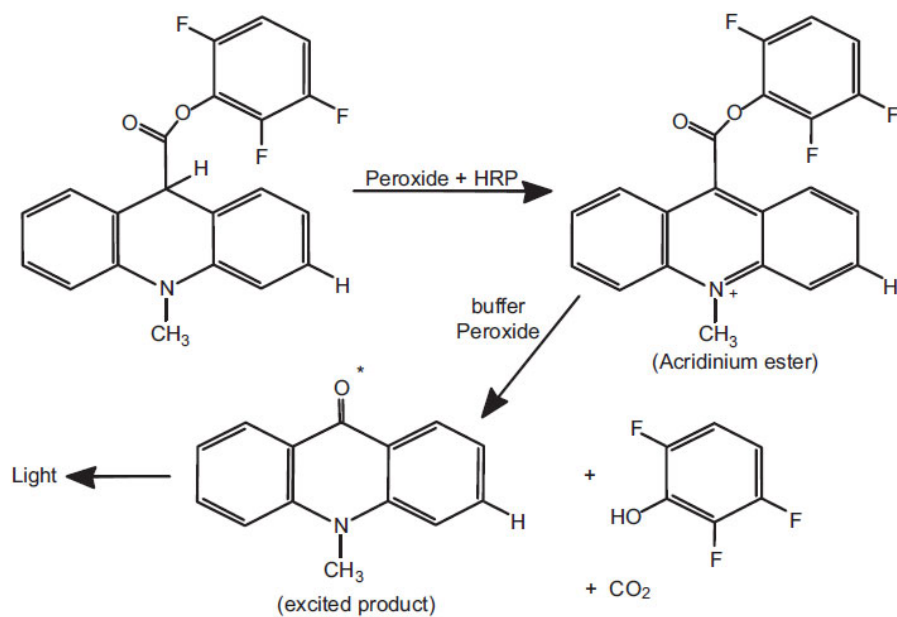
Blots may be stripped for the *de novo* incubation with a different primary antibody.

- place the membrane with the protein side facing up into the stripping solution (+fresh β -Mercapto EtOH)
- incubate at 50°C for 30 min (shake from time to time)
- wash with TBST+T 2x for a few seconds, 3x for 20 minutes
- block the stripped membrane again for 1 h with non fat dry milk (see 3.7.3 for details)
- incubate with new antibody (e.g. α -beta-actin) over night

3.7.5 Detection / ECL

The acronym ECL stands for Enhanced Chemical Luminiscence, and is a non-radioactive method for protein detection on Western blots. It is based on a chemical reaction between the ECL solution and the secondary antibody which is conjugated to horseradish peroxidase (HRP). While most ECL reactions oxidize the cyclic diacylhydrazide luminol, we used an ECL solution that is based on the enzymatic generation of an acridinium ester, which produces a more intense light emission of longer duration (*ECL+* GE Healthcare®). Combined HRP and peroxide catalyzed oxidation of the Lumigen PS-3 Acridan substrate generates thousands of acridinium ester intermediates per minute. These intermediates react with peroxide under slight

alkaline conditions to produce a sustained, high intensity chemiluminescence with maximum emission at a wavelength of 430 nm (see Figure: **3.3**). The resulting light is detected on autoradiography film (Hyperfilm ECL from GE Healthcare®).



3.8 Reagents:

Greiner Bio-One:

VACUETTE EDTA Tubes

Cat. No. 455226

Greiner Bio-One GmbH Bad Haller Straße 32 A-4550 Kremsmünster Österreich

Calbiochem:

Protein G Plus-Agarose Suspension

Cat. No. IP04

EMD Chemicals, Inc. 480 S. Democrat Road 08027 Gibbstown United States

Invitrogen:

Dynabeads® Sheep-anti Mouse IgG

Cat. No. 110-31

Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008.

GE Healthcare:

Ficoll-Paque PLUS

Cat. No. 17-1440-03

Amersham Hybond ECL Membrane

Cat. No. RPN203D

Amersham Hyperfilm ECL

Cat. No. 28-9068-36

ECL Western Blotting Detection Reagents

Cat. No. RPN2109

GE Healthcare Europe GmbH Zweigniederlassung Österreich Euro Plaza,
Building E Wienerbergstrasse 41 A-1120 Wien Austria

PAA:**RPMI 1640 Ready Mix***Cat. No. R15-802***Dulbecco's PBS for Cell Culture***Cat. No. H15-002*

PAA Laboratories GmbH PAA-Strasse 1 4061 Pasching Austria

Sigma-Aldrich:**IgG from human serum***Cat. No. I4506*

GSigma-Aldrich Corporate Offices Sigma-Aldrich 3050 Spruce St. St. Louis,
MO 63103, USA

3.9 Equipment:**Biorad:****PAGE: Mini-PROTEAN Electrophoresis System****Western transfer: Mini Trans-Blot Cell**

Bio-Rad Laboratories, Inc. 1000 Alfred Nobel Drive Hercules, CA 94547
USA

Becton Dickson (BD):**FACS: BD FACS Calibur Flow Cytometer**

Immunocytometry Systems Pharmingen 2350 Qume Drive San Jose, CA,
USA 95131

3.10 Antibodies

Becton Dickson (BD):

Immunocytometry Systems Pharmingen

2350 Qume Drive

San Jose, CA, USA 95131

Santa Cruz Biotechnology:

Santa Cruz Biotechnology, INC.: International Headquarters Santa Cruz

Biotechnology, Inc. 2145 Delaware Avenue Santa Cruz, California 95060

U.S.A.

Beckman Coulter:

Worldwide Headquarters: Beckman Coulter, Inc.: 250 S. Kraemer Boulevard,

P.O. Box 8000 Brea, CA 92822-8000 USA

Antibodies for sorting

| Antibody | Concentration | Company | # | Clone |
|--------------------|----------------------|----------------|----------|--------------|
| CD8 | 0.2 mg/ml | BeckmanCoulter | IM0102 | B9.11 |
| CD13 | 0.2 mg/ml | BeckmanCoulter | IM0713 | SJ1D1 |
| CD14 | 0.2 mg/ml | BeckmanCoulter | IM0643 | RMO52 |
| CD16 | 0.2 mg/ml | BeckmanCoulter | IM0813 | 3G8 |
| CD19 | 0.2 mg/ml | BeckmanCoulter | IM1313 | J3-119 |
| CD25 | 0.2 mg/ml | BeckmanCoulter | 0119 | B1.49.9 |
| CD235a | 0.2 mg/ml | BeckmanCoulter | IM2210 | 11E4B |
| TCR $\gamma\delta$ | 0.1 mg/ml | BeckmanCoulter | IM1313 | IMMU510 |

Table 3.5: FACS Antibodies

| Antibody | FITC/PE | Company | # | Clone |
|--------------------|---------|----------------|--------|----------|
| CD2 | FITC | BD | 555326 | RPA-2.10 |
| CD3 | FITC | BD | 555339 | HIT3a |
| CD4 | PE | BD | 340419 | L120 |
| CD8 | PE | BeckmanCoulter | IM0452 | B9.11 |
| CD14 | PE | BeckmanCoulter | A07764 | RMO52 |
| CD16 | PE | BeckmanCoulter | IM1238 | 3G8 |
| CD19 | PE | BeckmanCoulter | IM1285 | J3-119 |
| CD25 | PE | BeckmanCoulter | IM0479 | B1.49.9 |
| CD69 | PE | BeckmanCoulter | IM1943 | TP1.55.3 |
| HLA-DR | PE | BeckmanCoulter | IM1639 | Immu-357 |
| TCR $\gamma\delta$ | PE | BeckmanCoulter | IM1418 | IMMU510 |

Table 3.6: Antibodies for stimulation

| Antibody | Concentration | Target | Isotype | Clone |
|--------------|---------------|-----------------------|---------|--------|
| CD2 (6F10.3) | 5 μ g/ml | T11.2 epitope | IgG1 | 6F10.3 |
| CD2 (Vit13) | 5 μ g/ml | T11.3 epitope | IgG1 | Vit13 |
| CD3 (OKT3) | 5 μ g/ml | CD3 ϵ/γ | IgG2a | OKT3 |

Table 3.7: 6F10.3:Immunotech (Marseille, France); Vit13: mAb Vit13 was kindly provided by O. Majdic (Institute of Immunology - Medical University of Vienna)

Table 3.8: Primary Antibodies for Western Blot

| Antibody | Manufacturer | Dilution factor | # |
|---------------------|--------------|-----------------|----------|
| α PAK (C-19) | Santa Cruz | 1:400 | sc-881 |
| NCK 1/2 (C-19) | Santa Cruz | 1:400 | sc-290 |
| OX2 (R-17) | Santa Cruz | 1:300 | sc-14388 |
| CD137L (C-20) | Santa Cruz | 1:300 | sc-11817 |

Table 3.9: Secondary Antibodies

| Antibody | Manufacturer | Dilution factor | # |
|----------------------|---------------|-----------------|------------|
| ECL Rabbit IgG (HRP) | GE Healthcare | 1:4000 | NA9310-1ML |
| ECL Mouse IgG (HRP) | GE Healthcare | 1:4000 | NXA931-1ML |
| ECL Goat IgG (HRP) | GE Healthcare | 1:4000 | NA935 |

Preliminary mRNA Data

The mRNA data we used to select our target proteins was acquired in our lab before we started this project. For this, anti-CD2 and anti-CD3 stimulated Cd4+ / CD25- / HLA-DR- cells were lysed, and their gene expression investigated via an Affymetrix© Genotyping chip. Genes that showed an increased mRNA transcription were further examined by reverse transcribing their mRNA into cDNA, which was then measured with real-time quantitative PCR (TaqMAN), to confirm these findings. In the last step, we listed genes with a confirmed upregulated mRNA transcription and picked four proteins which seemed interesting for the context of CD2 signalling, and for which commercial antibodies for Westernblot detection were available. The data below shows the mRNA expression levels [Sunder-Plassmann Lab].

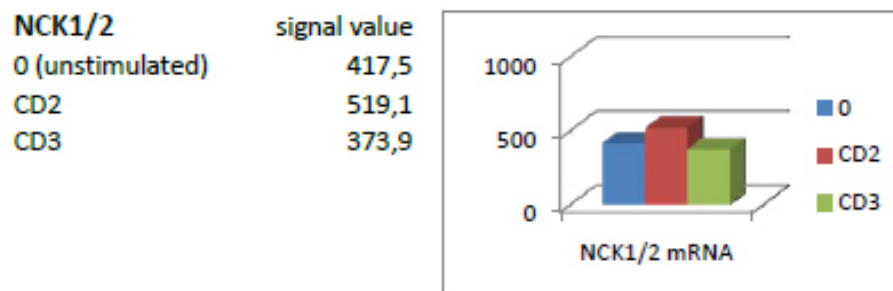


Figure 4.1: **TaqMAN experiments:** NCK mRNA expression of helper-cells after 2 hours of stimulation with anti-CD2 or anti-CD3 mAb (as well as unstimulated cells). The expressed mRNA was transcribed into cDNA and measured with a Real time quantitative PCR (TaqMAN). The „signal value“ represents the measured fluorescence, which was emitted in direct correlation to the initial amount of cDNA, and was normalized to an eukaryotic 18S rRNA endogenous control (Applied Biosystems)

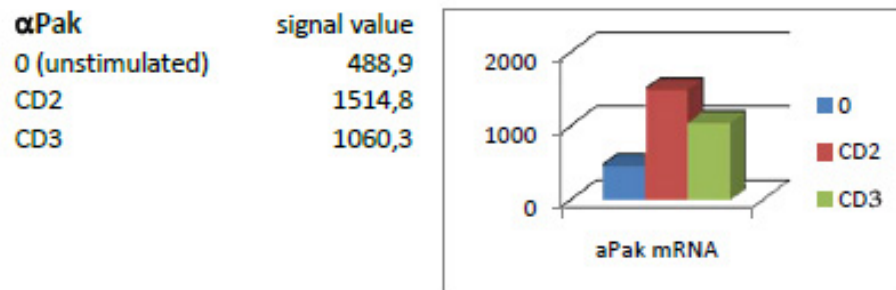


Figure 4.2: **TaqMAN experiments:** α Pak mRNA expression of helper-cells after 2 hours of stimulation with anti-CD2 or anti-CD3 mAb (as well as unstimulated cells). The expressed mRNA was transcribed into cDNA and measured with a Real time quantitative PCR (TaqMAN). The „signal value“ represents the measured fluorescence, which was emitted in direct correlation to the initial amount of cDNA, and was normalized to an eukaryotic 18S rRNA endogenous control (Applied Biosystems)

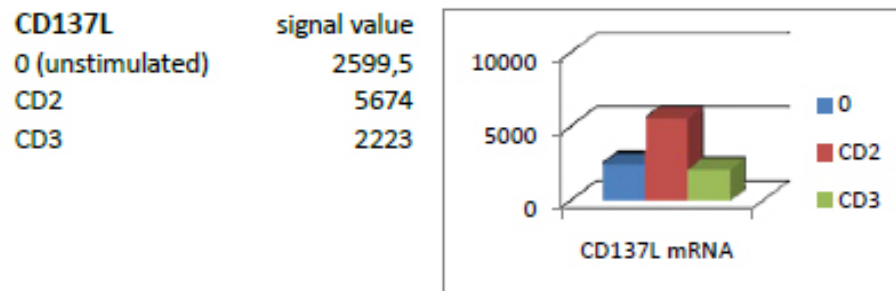


Figure 4.3: **TaqMAN experiments:** CD137L mRNA expression of helper-cells after 2 hours of stimulation with anti-CD2 or anti-CD3 mAb (as well as unstimulated cells). The expressed mRNA was transcribed into cDNA and measured with a Real time quantitative PCR (TaqMAN). The „signal value“ represents the measured fluorescence, which was emitted in direct correlation to the initial amount of cDNA, and was normalized to an eukaryotic 18S rRNA endogenous control (Applied Biosystems)

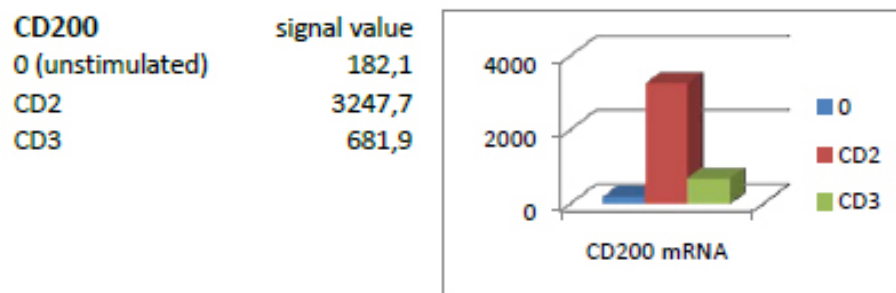


Figure 4.4: **TaqMAN experiments:** CD200 mRNA expression of helper-cells after 2 hours of stimulation with anti-CD2 or anti-CD3 mAb (as well as unstimulated cells). The expressed mRNA was transcribed into cDNA and measured with a Real time quantitative PCR (TaqMAN). The „signal value“ represents the measured fluorescence, which was emitted in direct correlation to the initial amount of cDNA, and was normalized to an eukaryotic 18S rRNA endogenous control (Applied Biosystems)

Results

5.1 The impact of CD2 stimulation on Th-cells

Preliminary raised data from our lab indicated an increased mRNA expression of several genes in purified CD4+ / CD25- / HLA-DR- T-helper-cells after CD2 stimulation. We suggested a direct or indirect impact of CD2 on the transcriptional control of these genes, therefore we decided to pick four genes (NCK, α Pak, CD200, CD137L) which displayed an increased transcription rate, or with an alleged interaction with CD2, and looked at their protein expression pattern after 24, 48 and 72 hours of stimulation with anti-CD2 antibodies. Therefore, we stimulated CD4+ / CD25- / HLA-DR- T-cell populations with α CD2 (6F10.3 + Vit13) to match the mRNA results with actual protein levels detectable in the cell lysate. We were able to confirm a potent stimulation of α Pak production upon α CD2 stimulation, in contrast to CD200 which only showed a very feeble signal on the western blot if at all. The expression pattern of Nck did not change significantly, however we found an increased expression of CD137L, which in this context, has not been described in literature before.

5.2 Purified Th-cells

We aimed to work with a highly pure and strictly CD4+ /CD25 - /HLA-DR- population, to verify the mRNA findings. Therefore, sorting out the right population was vital for the following procedures. We chose to isolate the cells manually instead of FACS assisted sorting, because of the stressing nature of the FACS-procedure and a possible biased result due to the ex ante stress situation. The following FACS results gave us an overview over the composition of the MNC population (cells from multiple lineages) and the sorted population (purified CD4+ / CD25- / HLA-DR- T-helper cells). Situated on the left side of the figures is the MNC population, which is basically a mix of different cell types before sorting, and shown on the right side is the purified T-cell fraction after depletion of unsolicited cells.

The tables on the far right side show the percentage of cells in each sector, according to their staining (**UL** - upper left / **UR** - upper right / **LL** - lower left / **LR** - lower right). Both populations were stained with a FITC (Fluoresceinisothiocyanat) labeled anti human CD3 mAb which served as a T-cell marker. To identify the various cell types of the MNC fraction and to check the purity of the T-cell fraction, both were furthermore stained with PE (Phycoerythrin) labeled mAb directed against cell-type specific surface markers, mentioned below each figure. These figures show the results of one representative experiment.

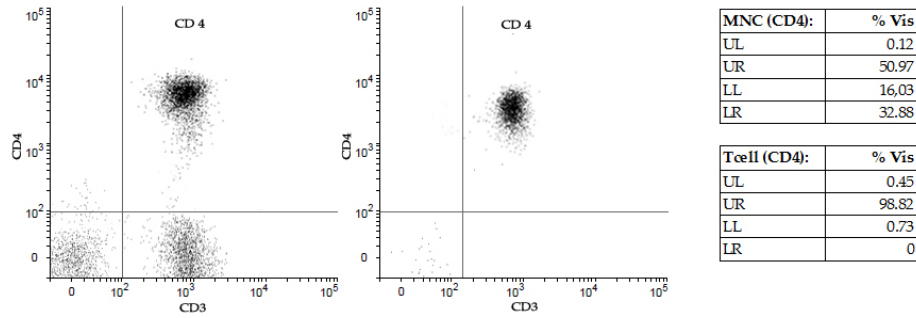


Figure 5.1: CD3 vs CD4 (Th marker)

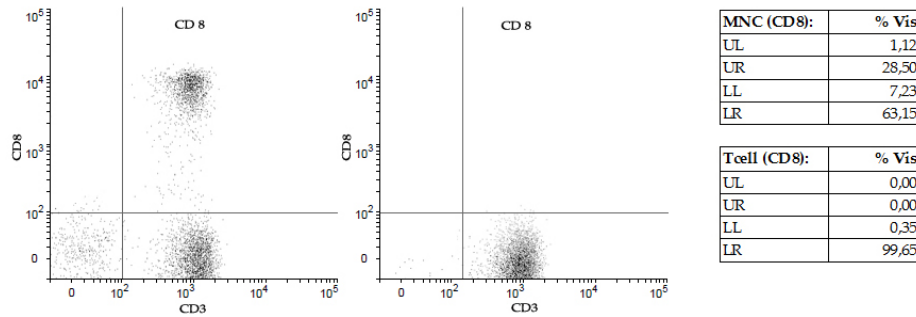


Figure 5.2: CD3 vs CD8 (Tc marker)

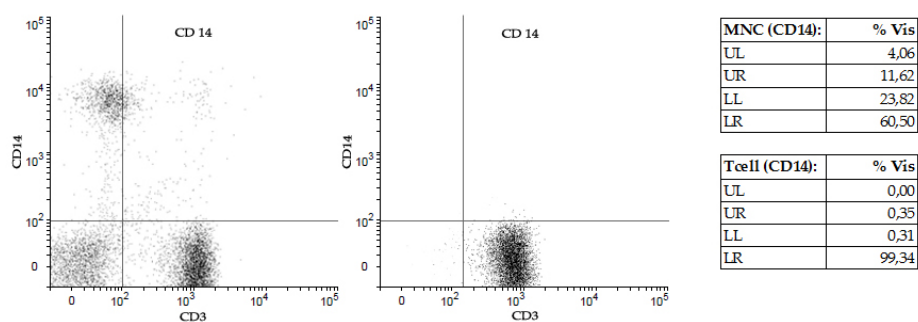


Figure 5.3: CD3 vs CD14 (monocyte marker)

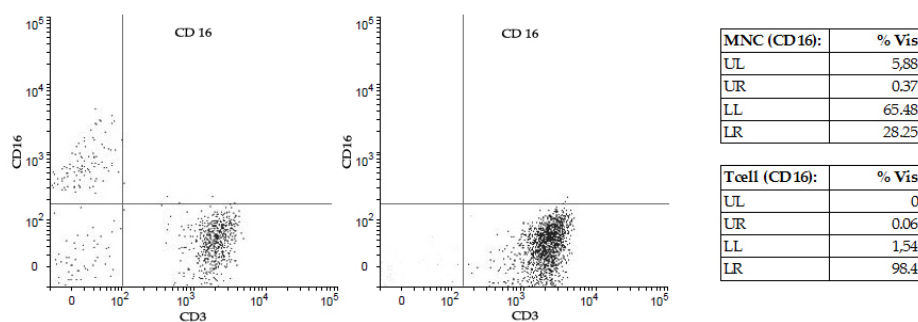


Figure 5.4: CD3 vs CD16 (neutrophils, and NK-cell marker)

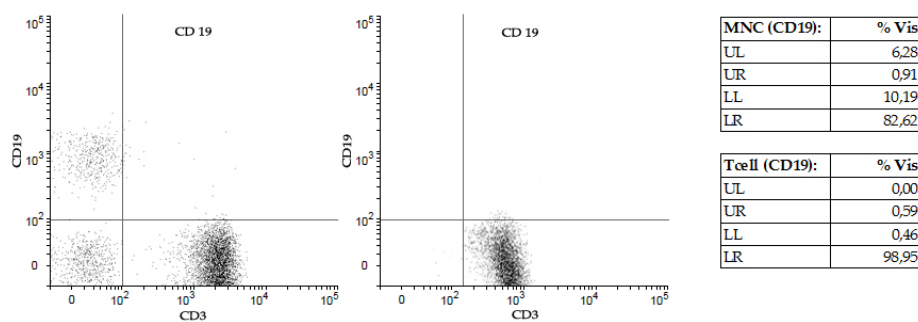


Figure 5.5: CD3 vs CD19 (B-cell marker)

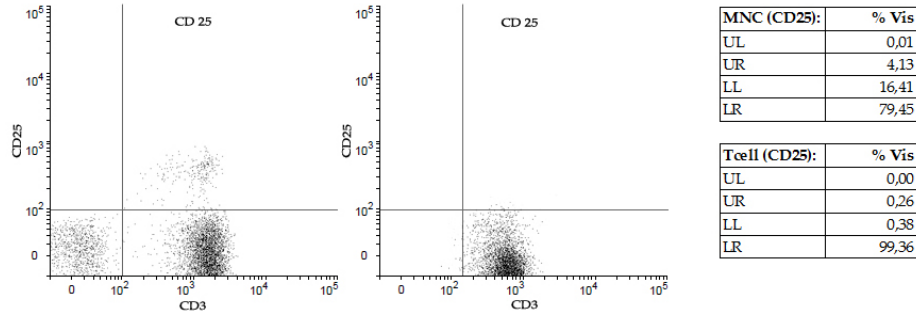


Figure 5.6: CD3 vs CD25 (activated T-cell marker)

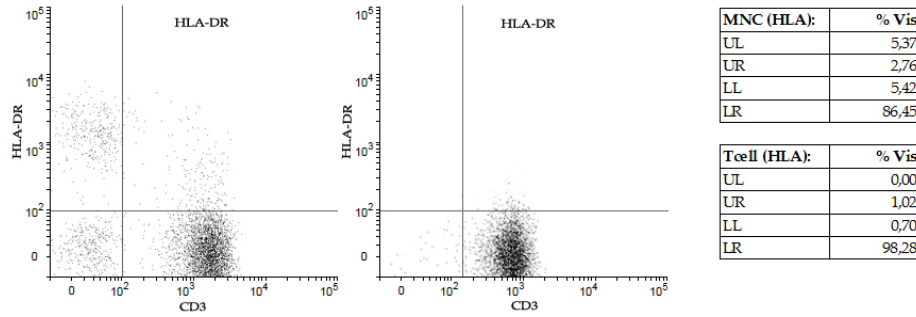
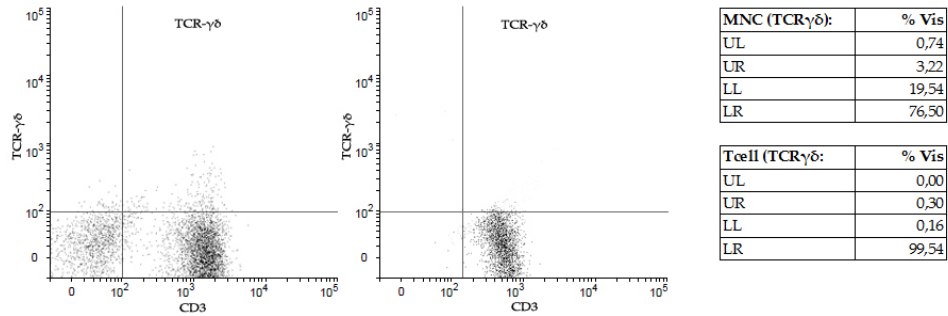


Figure 5.7: CD3 vs HLA-DR (a marker for extended activated T-cell marker)

Figure 5.8: CD3 vs TCR $\gamma\delta$ (marker for TCR $\gamma\delta$ + T-cells)

As figure 5.1 shows, there were 16% of CD3-/CD4- (neutrophils, monocytes, eosinophils, NK-cells), 32% of CD3+/CD4- (cytotoxic T-cells), and about 51% of CD3+/CD4+ (Th) cells in the MNC dotblot (left side), whereas the sorted cells (right side) showed a CD3+/CD4+, or T-helper-cell purity rate of almost 99%. CD8 was used to measure the number of cytotoxic T-cells, and as figure 5.2 indicates, there were 28% cytotoxic T-cells in the

MNC fraction (left side) with 63% of CD3+/CD8- (T-helper cells or double negative cells), and 7.23% of unlabeled cells. The T-cell population was free of Tc cells, since the only CD3+ cells left were CD8-. The next figure (5.3) shows CD14, as a classic monocyte marker, which was used to assess the number of myelomonocytic cells in both populations. The figure shows, that there were around 12% of CD14+/CD3- cells (monocytes) in the MNC fraction (left side), and 0% left in the T-cell population (right side). Figure 5.4 illustrates, that there were 5.8% of CD3-/CD16+/NK cells and neutrophils in the MNC population (left side), which were also successfully removed during sorting, due to the fact that only CD3+/CD16- cells remained (right side). In order to identify B-cell populations (5.5) before, and after sorting, both fractions were incubated with α CD19 antibodies. The figure illustrates that there were 6.3% B-cells (CD3-/CD19+) before (left side) and 0% B-cells (right side) after sorting. Shown in figure 5.6 is CD25, a marker for activated T-cells, which had to be removed prior to our stimulation experiments, since they would corrupt the results. 4.13% of the population was CD3+/CD25+ meaning that they were already activated T-cells dwelling within the MNC population (left side). After sorting, roughly 0.26% remained „CD25+“ (CD25 expression-levels were just above the negative cut-off). HLA-DR+ cells (Fig 5.7) are committed T-cells and were activated for a long time, hence representing a biasing problem for the further stimulation, just as CD25+ cells do. While the MNC fraction (left side) showed a percentage of 5.4 CD3-/HLA-DR+ cells (macrophages, B-cells, dendritic cells) and 2.7% of CD3+/HLA-DR+ (Th-cells), they vanished almost completely in the T-cell population after sorting, leaving only around 1% of very low HLA-DR expressing cells (right side). Finally, TCR $\gamma\delta$ (Fig 5.8) pose a very small subpopulation amongst T-cells. The MNC population (left side) contained around 3.2% of TCR $\gamma\delta$ + T-cells, which were reduced after sorting (right side) to 0.3% which was also just above the negative cut-off.

5.3 T-cell Activation Markers

There are several markers, or surface molecules that are known to be expressed on the surface of activated T-cells. *In vitro* the classical expression of these markers is in a clearly defined sequence: CD69, CD25, HLA-DR and last but not least VLA-1 (not shown). CD69 is usually expressed two to four hours

after stimulation with an antigen, mitogens or pathogen, since the protein is usually already produced and stocked, and just has to be transported to the membrane. CD69 is responsible for the activation of protein tyrosine kinase and calcium flux activity, as well as the transcription of $\text{TNF}\alpha$ and IL-2 [71]. CD25 takes about 12-24 hours to be expressed on activated T-cells, whereas HLA-DR takes around 40-60 hours for upregulation and marks the point after which the immune response moves towards the specific and adaptive phase [72]. These surface molecules indicate the activation state of T-cells, and were used to assess the level of activation following stimulation with anti-CD2 antibodies. After we ensured that the T-cell population was strictly $\text{CD4}^+ / \text{CD25}^- / \text{HLA-DR}^-$, we stimulated the populations *in vitro* in culture wells via diluted antibodies, either directed against the T11(2) and T11(3) epitope of CD2 (CD2 stimulation), or against the CD3 ϵ/γ chain (CD3 stimulation). See 3.7 for detailed antibody description.

5.3.1 Stimulation

The two antibodies (6F10.3 + Vit13) were diluted directly into the culture wells with a concentration of $5\mu\text{g}$ per ml each. Since the expression of CD2 on the surface decreases after stimulation with αCD2 antibodies, we used CD3 (FITC) as a general T-cell marker. In contrast to the CD2 stimulation, the αCD3 antibody (OKT3) was not directly diluted into the RPMI medium, but was crosslinked to the bottom of the culture-dish well prior to the addition of the T-cells. This is important, since soluble OKT3 is a highly potent immunosuppressant and is given to patients after organ transplantations to reduce acute rejection [73]. In contrast, crosslinked anti-CD3 (OKT3), is in fact able to do the opposite, *videlicet* activate resting T-cells [74]. Since stimulation with anti-CD3 antibodies leads to the internalization and therefore decreased detectability of CD3, we used FITC labeled mAb directed against CD2 as a T-cell marker in this case.

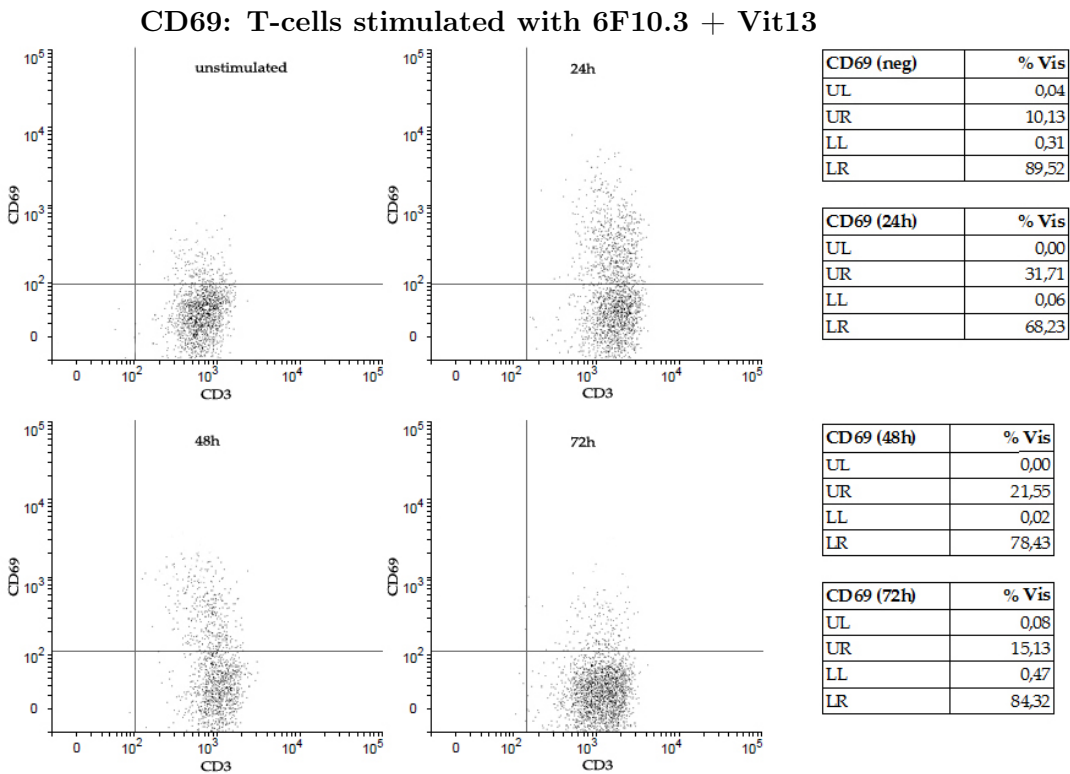
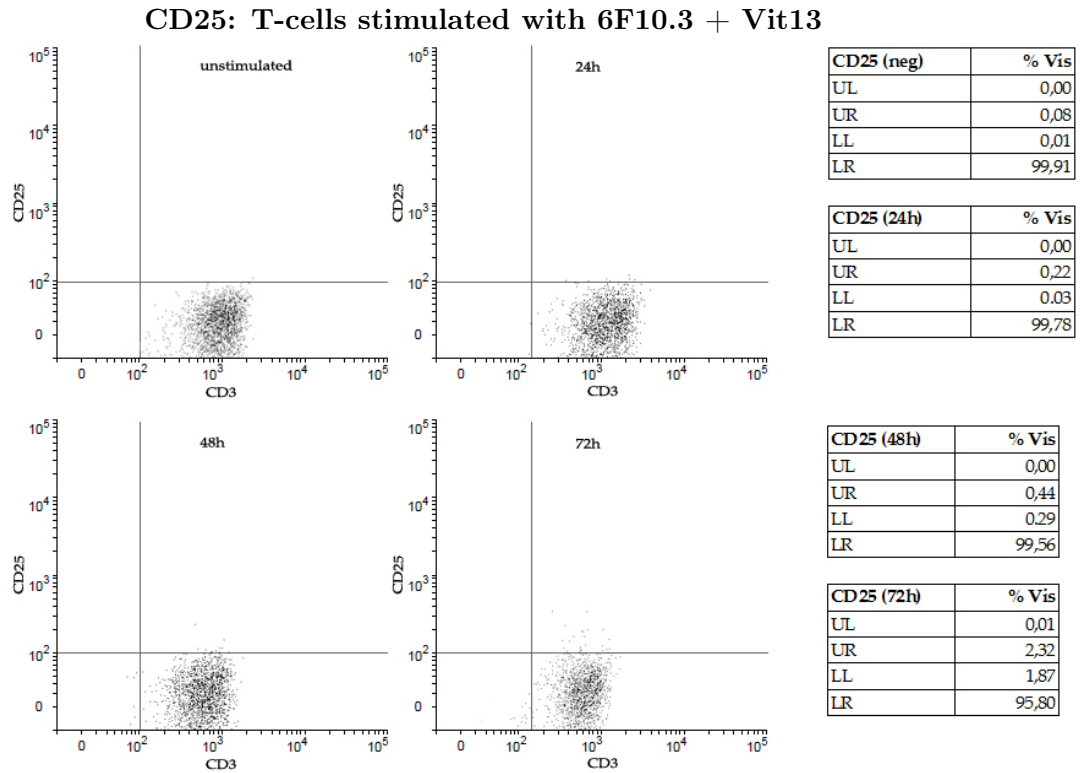
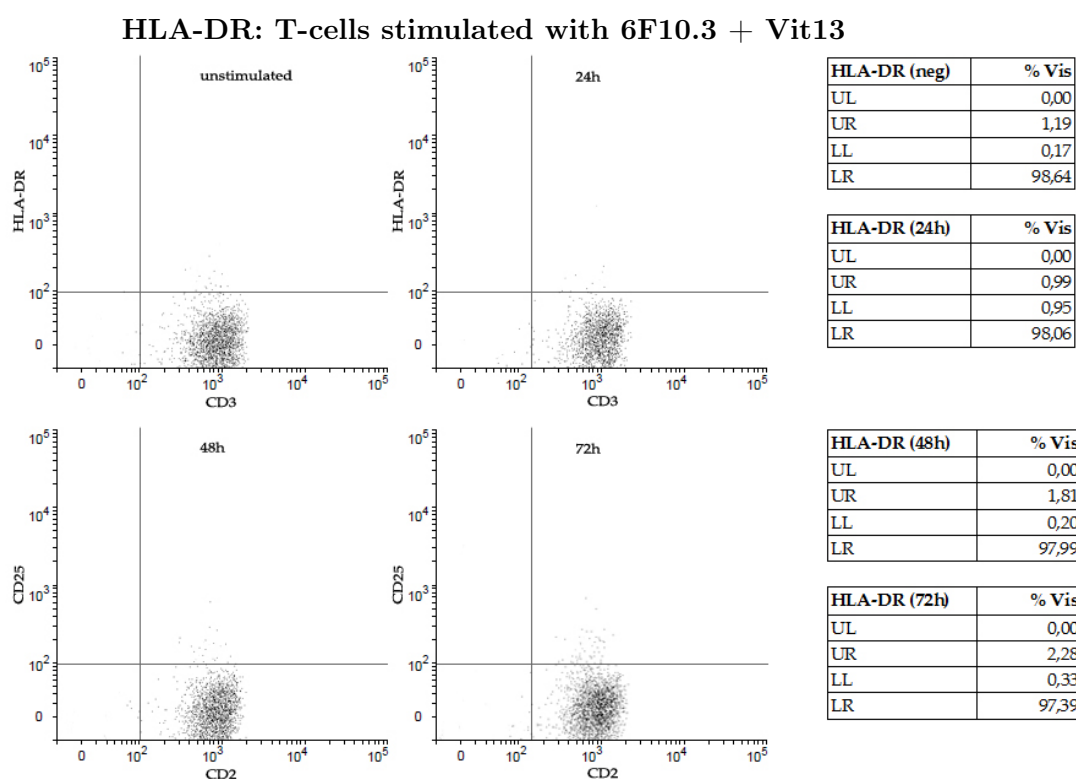


Figure 5.9: **Expression of CD69:**

The figure shows the CD69 expression of the helper-cell population after 24, 48 and 72 hours of stimulation with anti-CD2 (as well as unstimulated cells). CD69 is a C-type lectin protein and is the earliest inducible surface protein expressed lymphoid cells. It therefore serves as an early activation marker for T-cells, since it takes only approximately 1-2 hours from stimulation to presentation of CD69 on the surface of the cell. The figure illustrates the progression of the stimulation. In the unstimulated control, about 10% of the displayed Th-cells carry CD69. After 24 hours of stimulation with anti-CD2 antibodies, it is already a third (31.7%) of all cells expressing this activation marker, which recedes again after 48 hours to 21% and returns to 15% after 72 hours of stimulation.

Figure 5.10: **Expression of CD25:**

The figure shows the CD25 expression of the helper-cell population after 24, 48 and 72 hours of stimulation with anti-CD2 antibodies (as well as unstimulated cells). CD25 expression is associated with recent activated T-cells and is therefore also a marker for the activation status of the T-cell. It furthermore serves as a high affinity receptor for interleukin 2 (IL-2), essential for T-cell proliferation). Within the unstimulated population, there are nearly no (0.08%) CD4⁺/CD25⁺ helper cells which only changes slowly over 24 (0.22%), 48 (0.44%) and 72 (2.32%) hours of stimulation. In contrast to CD69, CD25 takes longer to be expressed on the surface but is not downregulated again as CD69 is.

Figure 5.11: **Expression of HLA-DR:**

The figure shows the HLA-DR expression of the helper-cell population after 24, 48 and 72 hours of stimulation anti-CD2 antibodies (as well as unstimulated cells). HLA-DR is one the human major histocompatibility complexes, a cell surface receptor encoded by the human leukocyte antigen complex on chromosome 6, and in context of Th-cells, only expressed on long term activated T-cells. Unstimulated cells only expressed 1.2% of HLA-DR, which did not alter much after 24 hours (0.99%) of stimulation. A slight increase to 1.8% after 48 hours and a final CD3+/HLA-DR+ percentage of 2.3 after 72 hours were measured.

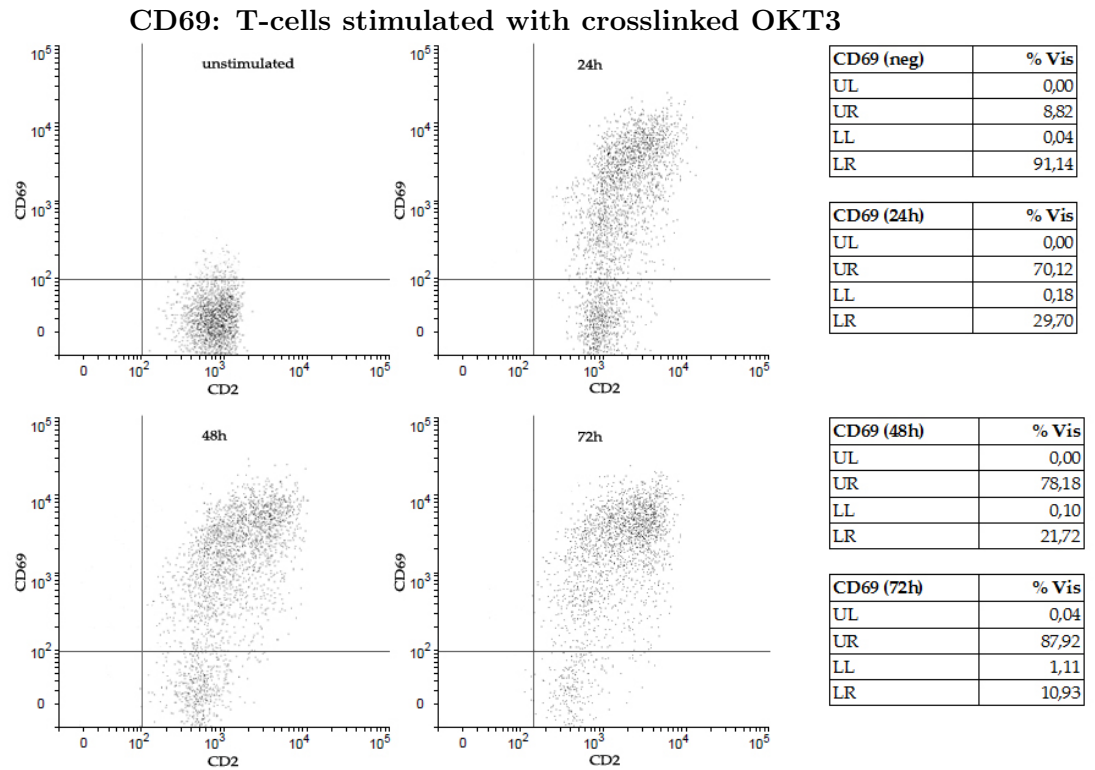
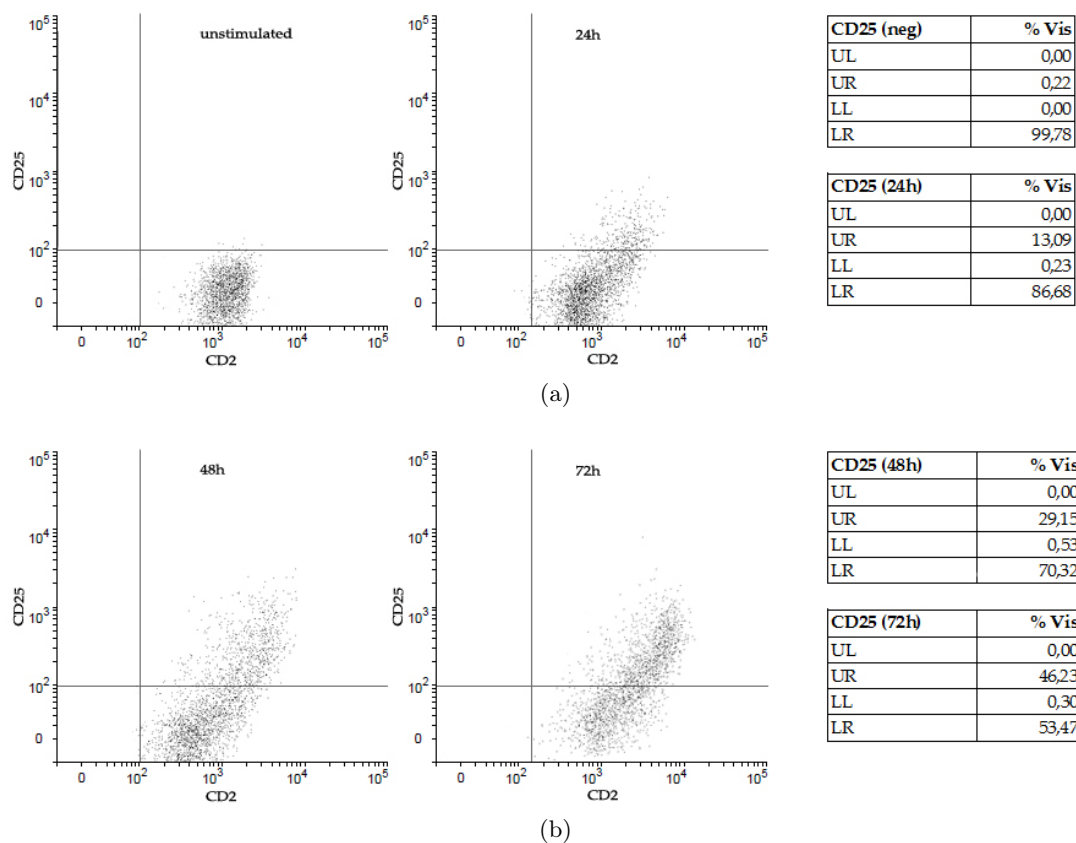


Figure 5.12: **Expression of CD69:**

The figure shows the CD69 expression of the helper-cell population after 24, 48 and 72 hours of stimulation with an anti-CD3 antibody (as well as unstimulated cells). While the unstimulated population already showed 8.82% of CD2+/CD69+ cells, it rapidly increased over 24 hours to 70%, and continued to rise to 78% after 48 hours with a final percentage of 88% CD2+/CD69+ helper-cells after 72 hours.

CD25: T-cells stimulated with crosslinked OKT3**Figure 5.13: Expression of CD25:**

The figure shows the CD25 expression of the helper-cell population after 24, 48 and 72 hours of stimulation with an anti-CD3 antibody (as well as unstimulated cells). About 0,2% of all unstimulated cells were CD2+/CD25+. Upon stimulation the population shifts to 13% of CD2+/CD25+ cells, with an increase to 29% after 24 hours and finally 46.2% after 72 hours.

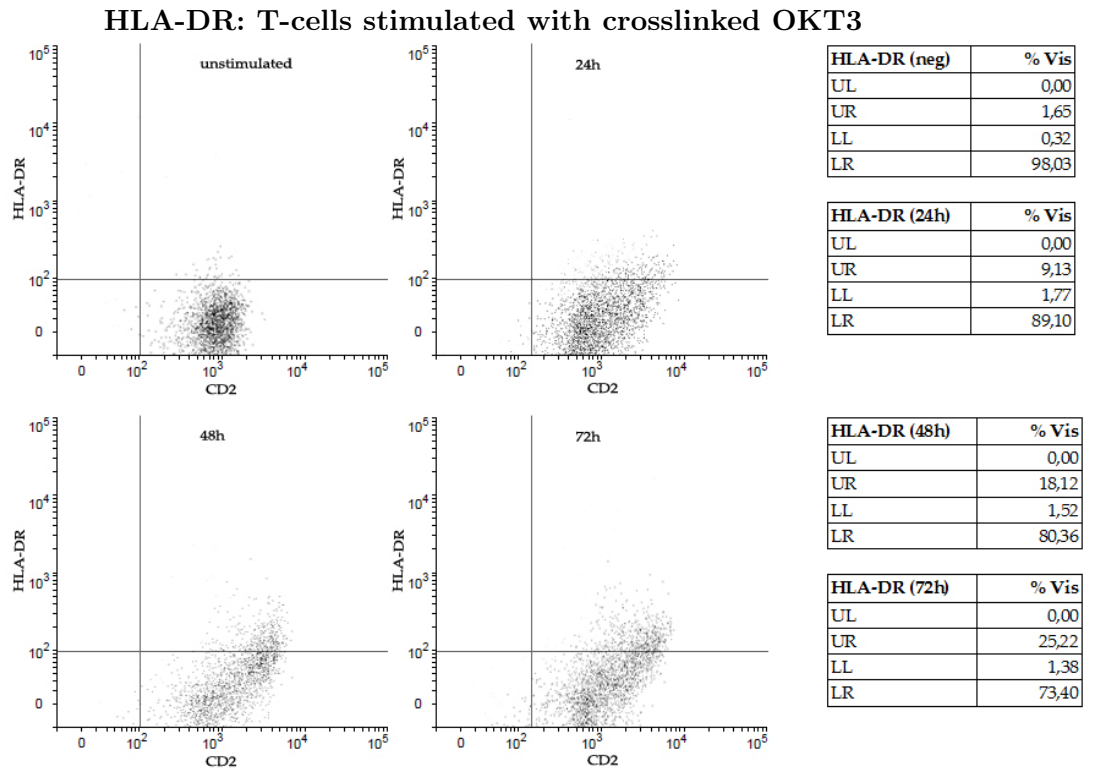


Figure 5.14: **Expression of HLA-DR:**

The figure shows the HLA-DR expression of the helper-cell population after 24, 48 and 72 hours of stimulation with an anti-CD3 antibody (as well as unstimulated cells). 1.65% of the unstimulated population express HLA-DR, which increases to 9.1% upon activation of the T-cells with crosslinked OKT3 after 24 hours. The number further rises to 18% after 48 hours and reaches 25% of CD2+/HLA-DR+ cells after 72 hours.

5.4 Protein Expression Patterns

The following figures show Western blots of one representative experiment (one out of three). The densitometric data shows the mean of all three experiments with the relative signal values, standardized on the respective β -actin signal and compared in relation to each other. The signal value is equal to the relative density and gray scale of the antibody signal on the Western blot.

5.4.1 CD2 and Nck

The densitometric evaluation of the Western blot showed a fairly stable expression of Nck with little or no influence of stimulation with anti-CD2 or anti-CD3 antibodies. There was no significant difference between unstimulated, anti-CD2 stimulated and anti-CD3 stimulated cells.

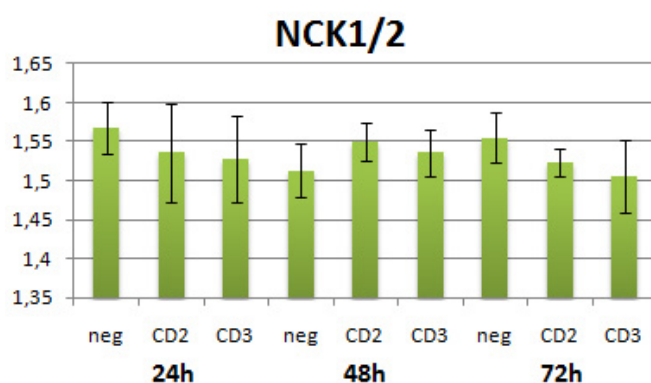


Figure 5.15: NCK1/2 densitometric data

| Time | Stimulation | Signal Mean | SD |
|------|-------------|-------------|--------|
| 24h | neg | 1,5673 | 0,0336 |
| | CD2 | 1,5361 | 0,0628 |
| | CD3 | 1,5281 | 0,0548 |
| 48h | neg | 1,5127 | 0,0341 |
| | CD2 | 1,5501 | 0,0242 |
| | CD3 | 1,5362 | 0,0297 |
| 72h | neg | 1,5553 | 0,0326 |
| | CD2 | 1,5234 | 0,0167 |
| | CD3 | 1,5049 | 0,0469 |

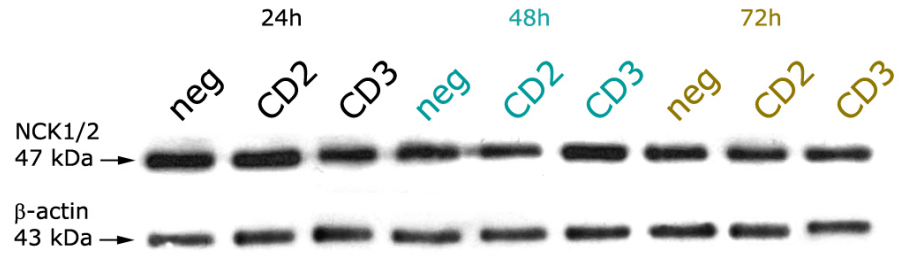


Figure 5.16: Western blot of a whole cell lysate (WCL) with protein levels of NCK (upper lane), and β -actin as a standard (lower lane). The exposure time was 5 minutes in this case, and shows solid, clearly defined NCK bands, as well as a consistent protein concentration, judging by the regular β -actin bands.

5.4.2 CD2 and CD200

The densitometric evaluation of the CD200 Western blots showed a stable but very weak expression of CD200 with little or no influence of stimulation with anti-CD2 or anti-CD3 antibodies. There was no significant difference between unstimulated, anti-CD2 stimulated and anti-CD3 stimulated cells. Each lane was measured in relation to its β -actin signal and compared in relation to each other.

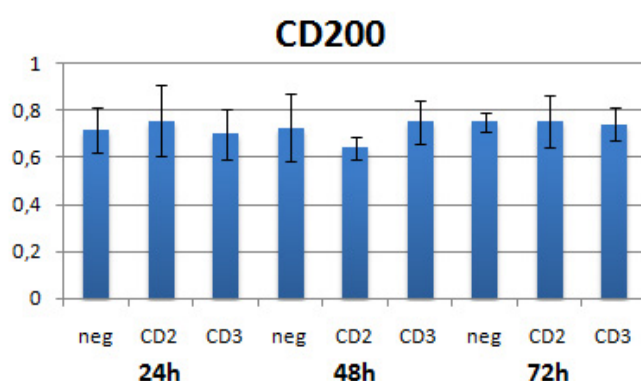


Figure 5.17: CD200 densitometric data

| Time | Stimulation | Signal Mean | SD |
|------|-------------|-------------|--------|
| 24h | neg | 0,7172 | 0,0951 |
| | CD2 | 0,7561 | 0,1525 |
| | CD3 | 0,6998 | 0,1054 |
| 48h | neg | 0,7278 | 0,1417 |
| | CD2 | 0,6412 | 0,0458 |
| | CD3 | 0,7514 | 0,0944 |
| 72h | neg | 0,7524 | 0,0388 |
| | CD2 | 0,7542 | 0,1118 |
| | CD3 | 0,7433 | 0,0704 |

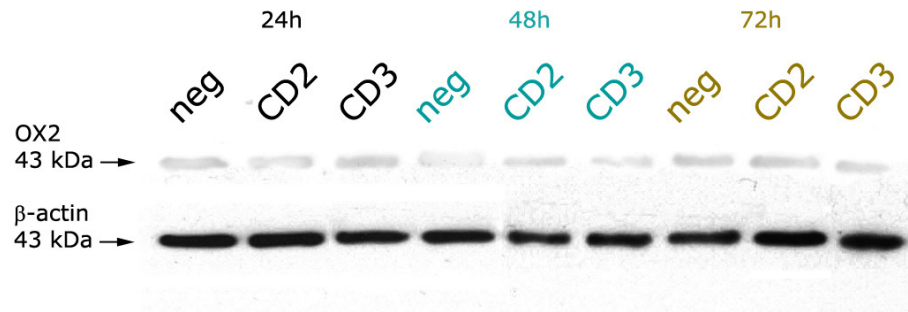


Figure 5.18: Western blot of a whole cell lysate (WCL) with protein levels of CD200 (upper lane), and β -actin as a standard (lower lane).

5.4.3 CD2 and α Pak

The densitometric evaluation of the α Pak Western blot showed a time-dependent, and slight stimulus-dependent (neg/CD2/CD3) expression pattern. Each lane was measured in relation to its β -actin signal and compared in relation to each other.

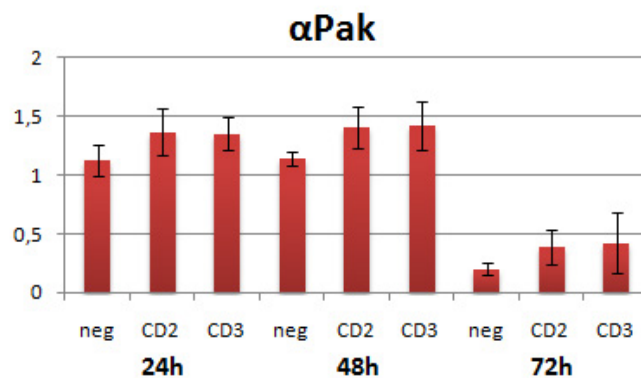


Figure 5.19: α Pak densitometric data

| Time | Stimulation | Signal Mean | SD |
|------|-------------|-------------|--------|
| 24h | neg | 1,0791 | 0,1264 |
| | CD2 | 1,2733 | 0,1980 |
| | CD3 | 1,2771 | 0,1428 |
| 48h | neg | 1,1998 | 0,0606 |
| | CD2 | 1,3016 | 0,1762 |
| | CD3 | 1,3258 | 0,2026 |
| 72h | neg | 0,1619 | 0,0498 |
| | CD2 | 0,5398 | 0,1519 |
| | CD3 | 0,7155 | 0,2614 |

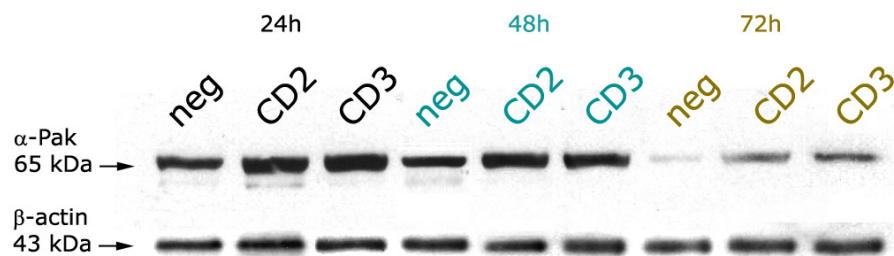


Figure 5.20: Western blot of a whole cell lysate (WCL) with protein levels of α Pak (upper lane), and β -actin as a standard (lower lane).

5.4.4 CD2 and CD137L

The densitometric evaluation of the CD137L Western blot showed a strong time-dependent, and slight stimulus-dependent (neg/CD2/CD3) expression pattern. Each lane was measured in relation to its β -actin signal and compared in relation to each other.

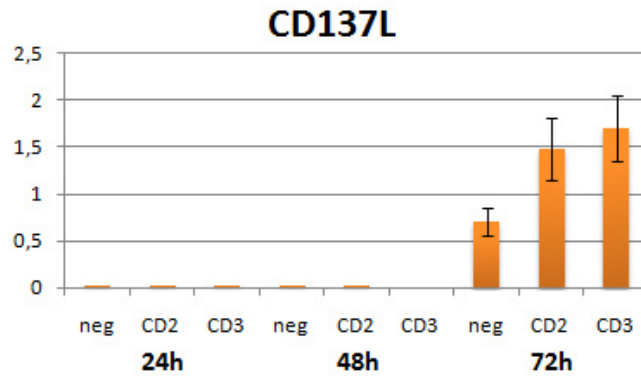


Figure 5.21: CD137L densitometric data

| Time | Stimulation | Signal Mean | SD |
|------|-------------|-------------|--------|
| 72h | neg | 0,7010 | 0,1513 |
| | CD2 | 1,4830 | 0,3385 |
| | CD3 | 1,6995 | 0,3568 |

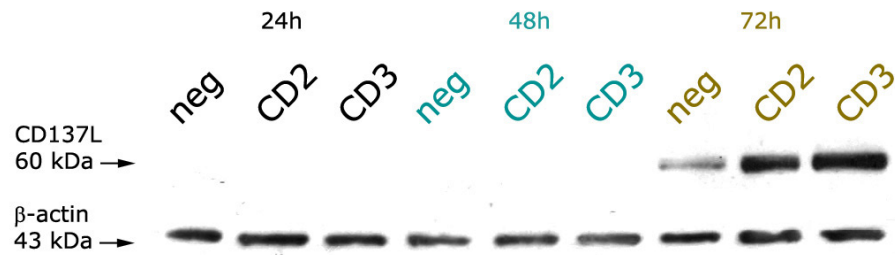


Figure 5.22: Western blot of a whole cell lysate (WCL) with protein levels of CD137L (upper lane), and β -actin as a standard (lower lane).

Discussion and Outlook

The role of CD4+ helper cells in immune and autoimmune processes raised more and more interest over the last twenty years since their first characterization in the late 80s. [14]. It soon became apparent that the immune system is more than just a broad sword which is either raised against intruders or securely sheathed, but rather a versatile applicable scalpel, which *ipso facto* needs a highly regulated hand that leads it. At this point, many fine-regulatory mechanisms are still obscure, especially when it comes to co-stimulation and crosstalk between subpopulations of the immune system. In this work we tried to shed light on the issue of CD2-(co)-stimulation in the context of T-cell signalling and anergy. More precisely we looked at the protein expression patterns of four different alleged targets, namely NCK1/2, CD200, α Pak and CD137L which showed an increased mRNA expression in previous experiments following stimulation with anti-CD2 antibodies. Within the scope of this project we isolated CD4+ / CD25- / HLA-DR- Helper-cells from the blood of volunteers, we then stimulated the cells with anti-CD2 antibodies and observed the change of protein expression over a time period of three days with time points at 24, 48 and finally 72 hours. What we found was an altered expression pattern in two of our target proteins (CD137L and α Pak), a steady and unchanged expression of NCK and finally a surprisingly low level of CD200 in the cell lysates.

6.1 T-cell Anergy and CD2

Anergy is a state of indifference, in which T-cells actively repress TCR signalling as well as the production of IL-2 [75]. This process is vital for immunological tolerance to self and foreign antigens, consequently, the disability for T-cells to properly enter the state of anergy may lead to autoimmune issues. The underlying mechanisms which lead T-cells into anergy are not fully understood yet, but it has been suggested that signalling via TCR (signal 1), in absence of a co-stimulatory signals from CD28 (signal 2) leads to T-cell anergy [76], meaning that TCR-signalling alone triggers a cascade of

inhibitory signals, leading to a metabolic stasis, which can only be suspended by joint signalling of both TCR and CD28. This issue demonstrates the importance of co-stimulatory molecules in the regulation of immune functions, but is also true for other co-stimulatory molecules. The binding of CD28 to either B7-1 or B7-2 for example, can prevent T-cells from entering anergy by inducing IL-2 production and proliferation. In case of CD2, it has been shown that in anergic cells, the CD2R epitope disappears, rendering it unresponsive to signals. Upon exposure to IL-2, the epitope is exposed again and the CD2/LFA-3 signalling is sufficient to shake the cell out of anergy [77].

6.2 CD2 and the Actin Cytoskeleton

Most recent literature suggests CD2's involvement in cytoskeletal rearrangements via interaction with binding proteins, recruited to its cytosolic tail [54] [52] [53]. More specific, the proline rich sequences of the tail are a potential docking site for a vast number of SH3 domain carrying targets. Nck as a SH3 / SH2 adaptor protein falls into that category and raised our attention after the initial finding that the NCKs mRNA was slightly upregulated following anti-CD2 mAb stimulation (see Fig 4.1). Additionally, Buday et al. [68] suggested a connection between Nck and the actin skeleton by showing that its ability to recruit members of the Ser/Thr kinase family (such as α Pak) to the cell membrane via its SH2 domain. As our data shows in figure 5.16, the protein expression levels of NCK, did not seem to alter much after stimulation with anti-CD2 antibodies, whereas the levels of α Pak did. We hypothesize that the slightly increased expression of Nck's mRNA following α CD2 stimulation is important for the cell, in order to have enough transcript to quickly replenish Nck during cell-signalling. Since the interaction between CD2 and CD58 is furthermore important for „scanning“ [48], which involves intense rearrangements of the cytoskeleton (capping, focal adhesions etc.), it would make sense to keep a high level of NCK transcript in the back hand for overcoming NCK shortage. Nevertheless, Nck as a protein is not upregulated after a CD2 stimulus, and the mRNA data shows only a slight increase compared to the unstimulated control. Whether Nck plays a part on the proximal end of the CD2 signalling cascade or not, remains unclear for the time being. Figure 6.1 shows a simplistic scheme of Nck and Pak interaction, showing a RTK as the source of the signal. In case of CD2, Nck

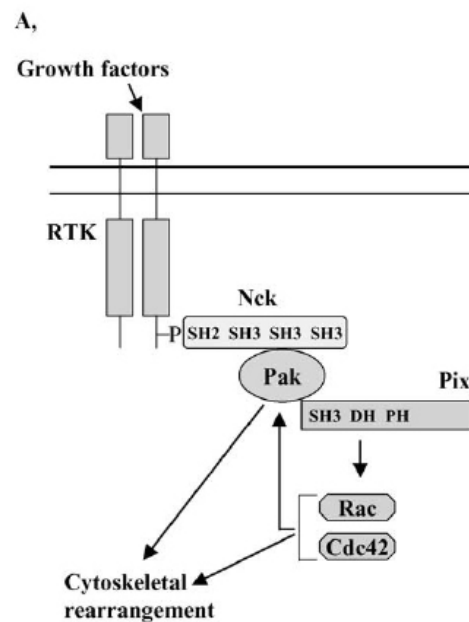


Figure 6.1: Nck recruits Pak to a growth factor receptor. The Pak kinase is then able to either directly or indirectly (via Pix) regulate cytoskeletal rearrangements by activating of multiple signalling pathways [68]

would bind to the cytosolic tail via its SH3 domain instead of the shown SH2 domain. Compared to the mRNA levels of Nck measured, α Pak did show a much higher expression rate (see Fig 4.2) after stimulation via CD2 or CD3. As figure 5.20 illustrates, the protein levels of α Pak are stable over 48 hours upon stimulation and rapidly decrease again after 72 hours. These findings indicate a correlation between T-cell stimulation via CD2/CD3 and regulation of α Pak transcription and translation on some level. Be as it may, this is probably not limited to CD2 stimuli, due to the nature of the Pak kinases as important regulators in several other pathways and immune functions [65] [66]. Interestingly though is the fact that CD2 seems to have a greater impact upon the transcriptional regulation of α Pak than CD3 has. Regarding protein levels, they seem to be almost equally able to stimulate the production of this kinase.

6.3 CD2 as a regulator of T-cell anergy

Despite its role as a co-inducer for T-cell activation, there is vague evidence [78] that CD2 is also involved in regulatory pathways yet to be elucidated. In our preliminary mRNA data, amongst others, two genes showed an increased transcription rate after CD2 stimulation, which immediately caught our attention. One was a glycoprotein, seemingly involved in autoimmune disorders and regulation of immune functions [62], termed CD200 (OX2, MOX2) and the other, was the ligand to a tumor necrosis factor superfamily receptor, termed CD137L. As stated above, the lack of co-stimulatory signals during T-cell activation leads to a state of unresponsiveness and anergy. Besides CD28, the probably best characterized co-stimulatory molecule in this context, there are many other possible co-stimulatory interactions. CD137L amongst other members of the TNF receptor superfamily is able to induce co-stimulatory signals [79] ensuring survival and differentiation of T-cells (both CD4+ and CD8+). While CD200 was known to be expressed on activated T-cells [61], CD137L was thought to be only expressed on APCs and in exceptional cases on T- and B-cell lymphomas [59]. In latter case, the expression of receptor and ligand prolongs their survival and helps to induce proliferation, since studies have shown that the interaction of CD137 and CD137L can enhance the production of IL-2 and IL-4 by CD4+ cells [79], and therefore enhance the overall proliferation. Additionally CD137 signalling is further more important to rescind the suppressive effects, imposed by CD4/CD25+ regulatory T-cells [80], meaning that the additional upregulation of the ligand after 72h might be a countermeasure to evade anergy or extended suppression. Especially in graft rejection this plays an important role, since it has been shown that the jamming of the interaction between CD137 and its ligand CD137L significantly increased the overall allograft survival [81]. It was difficult to initially detect CD137L on our Western blots since it seems to be a protein expressed only after a long time activation of Th-cell populations. Nevertheless the mRNA data (Fig 4.3) shows an increase in transcription after two hours, yet the protein is not detectable until after 72 hours (5.22). In the context of CD2 signalling, this might mean that CD137L is upregulated after approximately 72 hours to evade anergy induced by long term stimulation with anti-CD2 mAb.

CD200 is known to be expressed on activated T-cells [61], and seems to play an important role in the downregulation of excessive immune responses, such as infections with the influenza virus [82]. Additionally it plays a major role as an immunosuppressive molecule which can be found overexpressed in many tumor forms [83], where it modulates the function of T-cells attacking the tumor tissue. Interestingly, in contrast to the mRNA data (Fig 4.4), the Western blots (Fig.5.18) only showed very low concentrations of CD200 detectable in the cell lysate. There are many possible reasons for this outcome, since mRNA does not necessarily have to be translated directly into protein. Therefore large amounts of the transcript might just exist as a backup for a period of time and are then degraded again. Additionally, it is possible that the time points we picked were too late to detect an increase in CD200, since the mRNA detection was conducted two hours after stimulation, whereas the protein expression was examined 24, 48 and 72 hours after stimulation. Nevertheless, since the CD200 - ligand (CD200R1) interaction plays an important part in the general understanding of autoimmune diseases and immune modulation [61], it seems important to confirm the interaction between CD2 and CD200, since this „backdoor“ might help to develop new therapies for autoimmune issues as well as graft rejection and other diseases caused by a T-cell frenzy.

6.4 CD2 as a therapeutic target?

An anti-CD3 mAb is already in use as a therapeutic drug (OKT3), to induce T-cell anergy and prevent skin graft rejection. The potential of CD2 as a therapeutic target is growing ever since its discovery as a co-stimulatory and regulatory molecule. The blocking of CD2-LFA-3 signalling can prolong anergy *in vitro* [77], but not permanently, due to several possible co-stimulatory signals which can be induced by B-cells or APCs or a longer exposure to IL-2. On the other hand, without the blocking of CD2, the anergic effects only last for a maximum of 7 days. One therapeutic CD2 drug (Alefacept) is already in use for the treatment of psoriasis, an inflammatory skin disease. Alefacept is basically an immunoglobulin consisting of an extracellular CD2-binding portion of the human LFA-3 linked to the Fc portion of human IgG1. This design, allows it to effectively bind to cells with upregulated CD2 clusters, while the Fc part binds to the Fc γ RIII receptor on NK cells,

leading to antibody dependent cellular cytotoxicity (ADCC) thus removing cells with an upregulated CD2 expression. We were able to show that stimulation with anti-CD2 antibodies increased the expression of a protein involved in T-cell activation, and anergy, namely CD137L. CD200 which is important to downregulate certain immune functions to prevent autoimmunity, showed an increased mRNA transcription rate, but was not highly expressed on a protein level. These two proteins are already under investigation as immunoregulatory co-stimulators, therefore it would be interesting to reveal the mechanisms between CD2 stimulation and these the transcriptional control of these two proteins, to broaden the possibilities of an therapeutic approach for the treatment of diseases caused by dysfunctional T-cells. However there is still much left to clarify within the issue of CD2 induced direct or indirect (via upregulated co-stimulatory molecules) anergy, therefore only rough speculations are applicable at the moment.

6.5 General Outlook

To further define the task(s) assigned to CD2 in the immunological synapse, it seems necessary to further investigate known and suggested downstream partners, look into additional proteins, which displayed increased mRNA levels, and check their expressions. Concerning our four proteins, it would make sense to identify intermediaries to fully disclose the mechanism behind CD2 induced activation or anergy. Additionally, in case of CD200, it would be important to pick earlier timepoints for lysing the cells, with intervals starting with four hours after CD2 stimulus (the point where mRNA levels were measured) followed by 8 and 12 hour lysing of the cells, which would clarify whether the mRNA is in fact translated into protein or not. For CD137L the opposite would apply, since this protein is only expressed after 72 hours. Therefore we would suggest to shift the time points to 48, 72 and maybe 96 hours. In addition, the FACS dot blots of the α CD2 stimulation (see Fig 5.9) indicate that we were only able to stimulate a relatively small population out of all CD4⁺ / CD25⁻ / HLA-DR⁻ T-cells, since not all CD3 positive cells expressed CD69 following stimulation with anti-CD2 antibodies. This circumstance would be a possible explanation for the poor yield in CD200 protein. Even so, by dealing with such small subpopulations of T-cells it seems difficult to harvest enough cell material for the detailed study

of specific protein expression profiles. In order to do so, more source material (blood) would be necessary, which of course is limited, or the methods for detection have to be refined to detect lower amounts of protein. Possibilities for this or to substantiate interaction partners within the CD2 pathway(s), and further illumine the regulatory mechanisms behind this receptor would be to perform co-immunoprecipitations, yeast-two-hybridassays, or mTRACKing (not published yet) assays.

List of Abbreviations

| | |
|---------------|--|
| ADCC | antibody dependent cellular cytotoxicity |
| AID | autoinhibitory domain |
| APC | antigen presenting cell |
| APs | ammonium persulfate |
| BCR | B-cell receptor |
| BLR-1 | Burkitt-lymphoma receptor 1 |
| CD | cluster of differentiation |
| CD2BP1 | CD2-binding protein 1 |
| CIA | collagen induced arthritis |
| CLP | common lymphocyte precursors |
| CMS | human CD2AP |
| Cool | cloned out of library |
| CRIB | Cdc42 and Rac interactive binding |
| CTLA-4 | cytotoxic T lymphocyte-associated antigen 4 |
| CXCL | CXC chemokine ligand |
| CXCR5 | CXC-motive chemokine recepotor 5 |
| EAE | expermental autoimmune encephalomyelitis |
| ECL | enhanced chemi-luminescence |
| EDTA | ethylene diamine tetraacetic acid |
| EGF | epidermal growth factor |
| FACS | fluorescence activated cell sorting |
| FITC | fluorescein-isothiocyanate |
| Foxp3 | forkhead box P3 transcription factor |
| GC | germinal center |
| GITR | glucocorticoid induced tumor necrosis factor receptor family related gene |

| | |
|----------------|---|
| HLA-DR | human leucocyte antigen-DR |
| HSV | herpes simplex virus |
| IBD | inflammatory bowel disease |
| ICAM | intercellular adhesion molecule |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| ImmB | immature B-cell |
| IS | immunological synapse |
| ITAM | immunoreceptor tyrosine-based activation motif |
| iTreg | inducible T regulator |
| LAG-3 | lymphocyte activation gene-3 |
| LFA | leukocyte function antigen |
| LPTC | lamina propria T-cells |
| mAb | monoclonal antibody |
| MAC | membrane attack complex |
| MHC | major histocompatibility complex |
| MNC | mononuclear cells |
| mRNA | messenger ribonucleic acid |
| Nck | non-catalytic region of tyrosine kinase adaptor protein |
| NFAT | nuclear factor of activated T-cells |
| NK | natural killer cell |
| nTreg | natural T regulator |
| Pix | Pak interacting exchange factors |
| PBD | p21 GTPase binding domain |
| PBS(-T) | phosphate buffered saline (-tween 20) |
| PE | phycoerythrin |
| pMHC | peptide bound MHC |
| PDGF | platelet derived growth factor |
| PRR | pattern recognition receptor |

| | |
|---------------|---|
| PTK | protein tyrosine kinases |
| RA | rheumatoid arthritis |
| PAK | p21 activated kinase |
| PMSF | phenylmethanesulfonyl fluoride |
| RPMI | Roswell Park Memorial Institute |
| SH2 | Src homology 2 |
| SLP-76 | SH2 domain containing leukocyte protein of 76 kDa |
| SMAC | supra molecular activation cluster |
| STAT | signal transducer and activator of transcription |
| Tc | cytotoxic T-cells |
| TCR | T-cell receptor |
| Tfh | follicular helper T-cells |
| TGF | transforming growth factor |
| Th | T-helper-cells |
| TLR | toll-like receptor |
| TNF | tumor necrosis factor |
| WASP | Wiskott-Aldrich syndrome protein |
| V(D)J | variable diverse and joining |

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Appendix

8.1 CDs

CD2 *T11, LFA-2, E-rosette receptor*

expressed on T-cells, thymocytes, NK cells

molecular weight: 45-58 kDa

function: adhesion, costimulation, binds to Lck intracellularly, binds to CD58 (LFA-3).

CD3 *T3*

expressed on T-cells, thymocytes (common T-cell marker)

molecular weight: Three peptides: 20-28

function: associated with the T cell antigen receptor, signal transduction.

CD4 *T4, L3T4*

expressed on thymocytes, T cells, monocytes, macrophages

molecular weight: 55 kDa

function: co-receptor for MHC class II molecules, signal transduction.

CD8a,b *T8, Lyt2,3*

expressed on a thymocyte subpopulation, CTL, Treg cells in the periphery

molecular weight: CD8a: 38 kDa; CD8b: 30kDa

function: Co-receptor for MHC class I molecules.

CD13 *Aminopeptidase N*

expressed on myelomonocytic cells

molecular weight: 150-170 kDa

function: Zink- metalloproteinase, enzyme activity

CD14 *LPS receptor*

expressed on myelomonocytic cells

molecular weight: 53-55 kDa

function: Receptor for lipopolysaccharid- complex and lipopolysaccharid-binding protein (LBP)

CD16 *FcγRIII, FcRIIIA, FcRIIIB*

expressed on neutrophils, NK cells, macrophages

molecular weight: 50-80 kDa

function: deep-affine Fc receptor, mediating phagozytosis and antibody dependend cellular cytotoxicity (ADCC)

CD19

expressed on B cells

molecular weight: 95 kDa

function: in a complex with CD21 (CR2) and CD81 (TAPA-1); co-receptor for B cells

CD25

IL-2Ra, Tac

expressed on activated T cells, regulatory T cells, B cells und monocytes

molecular weight: 55 kDa

function: IL-2 receptor α -chain

CD69

Activation induction molecule (AIM)

expressed on activated T and B cells, activated macrophages and NK cells

molecular weight: 28, 32 Homodimer

function: unknown, early activation marker

Curriculum Vitae

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