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Mechanismen der Signalübertragung von PI3Kg in T-Zellen

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Dedicated to my family

Contents

Summary

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases involved in the regulation of diverse important cellular functions. The members of PI3K class I exhibit dual enzymatic specificity both as lipid kinases and protein serine kinases, and they are known to be indispensable for the development and proper functions of T lymphocytes. The involvement of PI3Ks in the activation of T cells is clearly demonstrated and so far mostly related to the PI3K class I_A enzymes (α, β and δ isoforms), but it was found that PI3Kγ, the single member of class I_B PI3K, is also involved in the regulation of activation-induced proliferation and cytokine production of T cells. How PI3Kγ participates in this mechanism is largely elusive.

Earlier reports proved that PI3Kγ KO mice have a reduced number and an impaired differentiation of thymocytes, as well as a reduced number of CD4 T cells in spleen. We confirmed this earlier findings regarding disrupted differentiation of PI3K$\gamma^{-/-}$ thymocytes, but in addition results presented here showed that this gene disruption had an even stronger impact on the mouse phenotype, significantly reducing the number of T cells in spleen and lymph nodes and effecting both major subpopulations of mature T cells (CD4 and CD8 cells).

In published studies PI3K$\gamma^{-/-}$ T cells showed reduced proliferation upon T cell receptor (TCR) stimulation. Costimulation with specific anti-CD28 antibody or activation with Ionomycin and phorbol ester is able to rescue this proliferation, in contrast to subsequent interleukin-2 (IL-2) and interferon-γ production. In our experiments IL-2 costimulation of TCR-activated PI3K$\gamma^{-/-}$ T cells was also sufficient to rescue this impaired proliferation, which clearly implicated that the lack of cytokines may be the major cause of their functional defect.

In order to elucidate how PI3Kγ couples the activation of TCR and IL-2 production in Jurkat cells, we used the PI3Kγ specific inhibitor AS041164. In our experimental settings

AS041164 did not exhibit any detectable cytotoxicity within the applied concentration range. Furthermore, this substance had no significant influence on overall PI3K activity in Jurkat cells proving its selectivity and specificity in our model.

The results obtained by applying AS041164 on Jurkat cells implicated that PI3Kγ may not be involved in the signalling events in the proximity of activated TCR, and that PI3Kγ is dispensable for certain aspects of T cell activation, such as CD69 expression. Nevertheless, treatment with AS041164 clearly and reproducibly reduced IL-2 production of activated Jurkat cells in dose-dependent manner. Jurkat cells have constant high level of PIP3, the major product of PI3K lipid-kinase activity; therefore it is most likely that this effect was caused by a lack of PI3Kγ protein kinase-activity.

PI3Kγ specifically interactions with several isoforms of protein kinase C (PKC) in Jurkat cells were also proved in this thesis. These interactions seem to be important for the proper function of interacting PKC isoforms upon T cell activation and for the subsequent IL-2 production. Finally, our results showed that PI3Kγ may be involved in the control of IL-2 production on several levels from transcription to secretion, and one of these levels is apparently PKC-related.

In conclusion, results from this thesis clearly demonstrate that PI3Kγ is an important modulator of thymocyte proliferation and differentiation. Moreover, PI3Kγ is necessary for the TCR-induced IL-2 production, a process known to be crucial for the normal proliferation of T cells after antigen receptor activation. PI3Kγ interacts with several PKC isoforms and modulate their activity upon TCR engagement, which is necessary for the IL-2 production. Described interactions depend on overall PI3K lipid-kinase activity as well as PI3Kg protein-kinase activity. This various modes of protein-protein interactions and kinase activity clearly show the PI3Kγ is a multifunctional enzyme important for proper development and function of lymphocytes.

Zusammenfassung

Phosphorinositol-3- Kinasen (PI3Ks) gehören zu einer Lipidkinasenfamilie, die an der Regulation verschiedener zellulärer Funktionen beteiligt ist. Die Enzyme der PI3Ks Klasse I besitzen eine doppelte Spezifität, sowohl als Lipidkinasen, als auch als Proteinserinkinasen. Sie sind dafür bekannt bei der Entwicklung und Funktion von T- Lymphozyten unverzichtbar zu sein. Die Beteiligung der PI3Ks an der Aktivierung von T-Zellen wurde hauptsächlich den PI3K I_A Klasseenzymen (a, ß, d Isoformen) zugeordnet. Man entdeckte jedoch, dass PI3K?, ein einzelnes Mitglied der PI3K I_B Klasse, auch bei der Regulation der durch Aktivierung induzierten Proliferation und Zytokinproduktion der T- Zellen eine Rolle spielt. In welcher Weise PI3K? an diesem Mechanismus beteiligt ist, ist in großen Teilen noch nicht bekannt.

Aus früheren Experimenten mit PI3K? Knockout Mäuse ist bekannt, dass diese sowohl eine geringere Anzahl und eine eingeschränkte Differenzierung der Thymozyten, als auch eine verringerte Zahl CD4+ T-Zellen in der Milz aufweisen. Wir konnten diese frühere Endeckungen, die die gestörte Differenzierung in PI3K?$^{-/-}$ Thymozyten betreffen, bestätigen, jedoch belegen unsere hier dargestellten Ergebnisse auch, dass diese Gendisruption sogar einen stärkeren Einfluss auf den Mausphänotyp hat. So kommt es zu einer signifikanten Verringerung der T- Zellen in der Milz und den Lymphknoten und zu einer Beeinflussung beider Hauptpopulationen reifer T- Zellen ($CD4^+$ und $CD8^+$ Zellen).

In publizierten Studien zeigten PI3K?$^{-/-}$ T- Zellen eine reduzierte Proliferation bezüglich der T-Zellrezeptor (TCR) Stimulation. Eine CD28 Kostimulation oder eine Aktivierung mit Ionomycin und Phorbolester ist in der Lage diese gestörte Proliferation, im Gegensatz zu anschließenden Interleukin-2 (IL-2) und Interferon-? Produktion, aufheben. In unseren Experimenten war auch eine IL-2 Kostimulation der TCR- aktivierten PI3K?$^{-/-}$ T-Zellen fähig, die gestörte Proliferation wiederherzustellen, was deutlich zeigt, dass ein Fehlen der Zytokine die Hauptursache ihres funktionellen Defektes sein könnte.

Um aufzuklären, auf welche Weise PI3K? die Aktivierung des T- Zellrezeptors mit der IL-2 Produktion in Jurkat Zellen verbindet, wurde der spezifische PI3K? Inhibitor AS041164 verwendet. In unserem experimentellen Rahmen wies AS041164 in der applizierten Konzentrationsbreite keine detektierbare Zytotoxizität auf. Die Substanz hat keinen signifikanten Einfluss auf die PI3K Gesamtaktivität in Jurkatzellen, was ihre Selektivität in unserem Modell beweist.

Die mit dem spezifischen Inhibitor AS041164 erzielten Ergebnisse implizieren, dass PI3K? nicht in Signalwege in der Nähe des aktivierten TCRs verwickelt ist, und dass das Enzym für bestimmte Aspekte der T-Zellaktivierung, wie die CD69 Expression, verzichtbar ist. Dennoch verringerte die Behandlung mit AS041164 deutlich und reproduzierbar die IL-2 Produktion in aktivierten Jurkat Zellen in einer dosisabhängigen Weise. Jurkat Zellen besitzen einen konstant hohen Spiegel an PIP3, dem Hauptprodukt der PI3K Lipidkinase Aktivität, daher ist es am wahrscheinlichsten, dass dieser Effekt durch ein Fehlen der Proteinkinase Aktivität der PI3K? verursacht wird.

Im Rahmen der Arbeit wurde klar gezeigt, dass PI3K? spezifisch mit verschiedenen Isoformen der Proteinkinase C (PKC) in Jurkatzellen interagiert. Es hat den Anschein als seien diese Interaktionen für die richtige Funktion der interagierenden PKC Isoformen bei der T- Zellaktivierung und für die IL-2 Produktion wichtig. Abschließend zeigen unsere Ergebnisse, dass PI3K? in die Kontrolle der IL-2 Produktion auf verschiedenen Ebenen von der Transkription bis zur Sekretion verwickelt ist und dass eine dieser Ebenen anscheinend mit der PKC zusammenhängt.

Zusammengefasst zeigt diese Studie klar, dass PI3K? ein wichtiger Modulator der Thymozytenproliferation und- differenzierung ist. Darüber hinaus ist PI3K? für die TCR-induzierte IL-2 Produktion wichtig, einem Prozess, der entscheidend für die normale T-Zellproliferation nach der Antigen- Rezeptor Aktivierung ist. PI3K? interagiert mit verschiedenen PKC Isoformen und moduliert ihre Aktivität bezüglich der TCR Bindung, was

für die IL-2 Produktion nötig ist. Die beschriebenen Interaktionen hängen sowohl von der gesamten PI3K Lipidkinase Aktivität, als auch von der PI3K? Proteinkinase Aktivität ab. Diese verschiedenen Arten von Protein- Protein Interaktion und Kinase Aktivität zeigen klar, dass PI3K? ein multifunktionales Enzym ist, das für eine ordnungsgemäße Entwicklung und Funktion von Lymphozyten wichtig ist.

1. Introduction

1.1. Cell signalling

All eukaryotic cells have elaborate system of proteins that are capable to convert an extracellular signal into an intracellular biochemical event or process, and this signal transformation is commonly known as signal transduction. These refined signalling mechanisms enable intercellular communication and coordination of many physiological processes. Cellular signalling systems include different kinds of proteins: cell-surface and intracellular receptor proteins, protein kinases, protein phosphatases, adaptor proteins, GTP-binding proteins and many other which interact with above mentioned signal transducers. All the proteins involved in signal transduction, regardless whether they catalyse particular biochemical reactions or facilitate interactions of other proteins, exhibit high selectivity for their targets (Alberts *et al*, 1994).

Numerous types of cell receptors can recognise a huge variety of specific external stimuli, activate appropriate intracellular signalling pathways and transmit a signal to different sites within the cell. The results of the signalling processes are manifolds, including changes in cell metabolism, changes of the cytoskeleton and of cell motility or, as drastic as cellular suicide (programmed cell death). Many signalling pathways transmit their signals to the nucleus where trough activation or inhibition of different transcription factors fundamental changes in gene expression can occur.

1.2. T cells, lymphocytes and other cells of immune system

During evolution all higher organisms developed a complex system for the protection of their organism from foreign pathogens. This system, known as immune system, is entirely based on the function of highly specialised cells called leukocytes, commonly known as white blood cells. The first line of an organism's defence is innate immunity, a part of the immune system that provides an immediate response to a wide range of pathogens. The innate

immunity mostly involves macrophages and all granulocytes (neutrophils, eosinophils and basophils). T and B lymphocytes are cellular components of adaptive immunity, a system that provides a highly specific immune response enabled by the presence of specific antigen receptors on the cell surface of lymphocytes. The adaptive immune response, unlike the innate immune response, requires prior exposure to pathogens and, in many cases, provides lifelong resistance to the same pathogen.

Leukocytes originate in the bone marrow along with other blood cells, and they all derive from the same progenitors – pluripotent haematopoietic stem cells. These pluripotent cells produce two more specialised types of stem cells: a common lymphoid progenitor that gives rise to T and B lymphocytes, and a common myeloid progenitor that gives rise to erythrocytes, megakaryocytes and different other types of leukocytes. A brief overview of the blood cell differentiation is given in Figure 1.1. B cells mature in the bone marrow, while T cells complete their differentiation in the thymus. Immature T cells in the thymus or thymocytes undergo a complex process of differentiation, and distinct phases of this process are marked with changes in their cell surface molecules. Components of the T cell receptor (TCR) along with CD4 and CD8 co-receptor proteins are most important for distinguishing different stages of thymocyte differentiation. The cells in the first stage of development are called ‘double-negative’, because they do not express CD4 and CD8. The majority of thymocytes from this phase proceeds into the next step by becoming ‘double positive’ cells which express both CD4 and CD8. At this stage thymocytes start to express pre-T-cell receptor and they enlarge and divide. Later they become small, resting double-positive cells expressing low levels of TCR. Most of them fail positive selection and undergo apoptosis. Those who survive lose expression of either CD4 or CD8, become ‘single-positive’ thymocytes, and, after maturation, exported from the thymus.

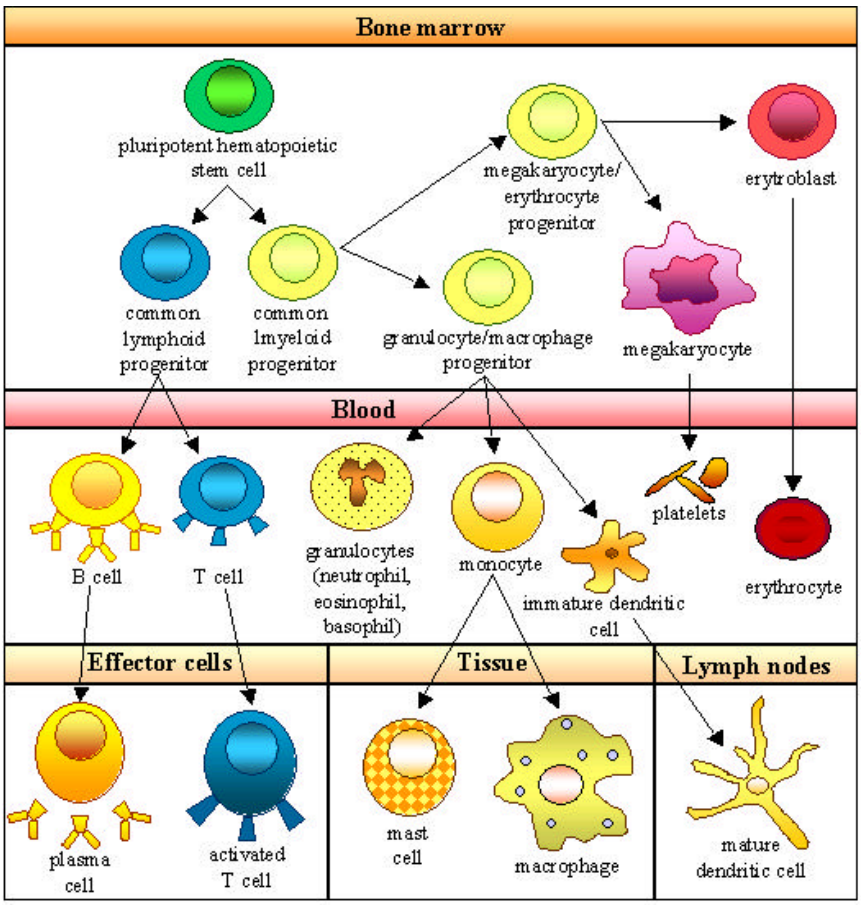


Figure 1.1. Differentiation of blood cells. All blood cells, including lymphocytes, arise from haematopoietic stem cells in the bone marrow. The first step in the differentiation of these pluripotent cells are two more specialised types of the stem cell, the common lymphoid progenitor that gives rise to the B cells, T cells and natural killer cells, and the common myeloid progenitor that gives rise to different types of other leukocytes, erythrocytes and megakaryocytes. Natural killer cells, unlike other lymphocytes, lack the antigen-specificity and they are a part of the innate immune system. Other leukocytes that belong to the myeloid linage are monocytes which give rise to mast cells and macrophages, dendritic cells and three types of granulocytes: neutrophils, basophils and eosinophils. Macrophages and dendritic cells are known as professional antigen presenting cells. They engulf pathogens and antigens, expose them on the cell surface and by contact activate antigen specific T cells (modified after Janeway *et al*., Immunobiology, 5th ed., 2001).

There are three major subsets of mature T lymphocytes: CD8 T cells or cytotoxic T cells, and two types of CD4 T cells or helper T cells – T_H1 cells activate macrophages (and B cells) while T_H2 cells stimulate B cells to differentiate. Mature T and B lymphocytes circulate between the blood and the lymphatic system and guard peripheral tissues. After encounter

with an antigen, lymphocytes become activated, start to proliferate (a process known as clonal expansion), and finally differentiate into effector cells capable to fight with the infection. B cells become antibody-secreting plasma cells, whereas T cells differentiate into effector T cells whose function depends on the sub-type of each activated T cell.

1.3. Activation of T cells

The T cell receptor (TCR) is a rare example of a receptor complex which determines many aspects of the cell function during the whole life span of a single T lymphocyte. From development and differentiation of a T cell to its activation, clonal expansion, effector function and apoptosis or survival, TCR is capable to deliver various signals that can result in completely different cell fate decisions (Werlen *et al*., 2003). This capability is enabled by the huge complexity of the TCR signalling process that involves numerous signalling proteins.

T cell activation by contact with an antigen induces antigen specific T cell clonal expansion and differentiation. This response is regulated by signal transduction pathways initiated by the antigen receptor (TCR) and costimulatory molecules. An antigen is by default a foreign peptide bound to major histocompatibility complex (MHC) molecules presented on the surface of professional antigen presenting cells, such as dendritic cells. TCR is comprised of an α/β subunit that recognises the antigen-MHC complex and signal transduction subunits γ, δ, ε (CD3 complex) and ζ chains.

Binding of antigen-MHC to TCR initiates signalling processes by recruiting and activating protein tyrosine kinases (PTK) of Src, Syk and Tec families (Lin & Weiss, 2001; Cantrell, 2002). TCR does not have any intrinsic kinase activity, but conformational changes induced by the activation of TCR expose immunoreceptor tyrosine-based activation motifs (ITAM) of the signal transducing subunit (CD3 and ζ chain) allowing their phosphorylation by Lck, a member of Src kinase family. The ζ–chain associated protein kinase of 70 kDa (ZAP-70), a member of the Syk family, binds to phosphorylated ITAMs via its tandem Src-homology 2 (SH2) domains. This sets off phosphorylation and activation of ZAP-70, which

leads to the subsequent phosphorylation of its substrates, such as linker of activated T cells (LAT) and Src homology 2-domain-containing leukocyte protein of 76 kDa (SLP-76). A major negative regulator of these initial TCR activation steps is the transmembrane tyrosine phosphatase CD45 that is also responsible for maintaining the basal level of Lck activation (Cantrell, 2002).

LAT and SLP-76 are just two among a growing number of adapter proteins involved in TCR signal transduction. Their major role is to couple tyrosine kinases to the downstream effectors by forming scaffolds to assemble signalling protein complexes in the correct intracellular location (Simeoni *et al*., 2005a). At this point signals emerging from the triggered TCR disseminate from the inner side of the plasma membrane to different signalling pathways that end in the nucleus. This links TCR to the signalling pathways indispensable for T cell activation. One of such pathways is the activation of Ras- and Rho-family guanine nucleotide binding proteins (GTPases). These GTPases are essential during T cell development, while in mature T cells they appear to be essential for the activation of the mitogen activated protein kinase (MAPK) pathway, which is necessary for the induction of proliferation, actin skeleton rearrangement and cytokine production upon activation.

Early events in TCR signalling include activation of enzymes which are involved in metabolism of inositol phospholipids. Phospholipase C gamma 1 (PLCγ1) is recruited to the plasmamembrane and activated by tyrosine kinases (Lck, ZAP70, Itk and Rlk). Activated PLCγ1 hydrolyses phosphatidilinositol(4,5)biphosphate (PtdIns(4,5)P_2) and produces diacylglycerol (DAG) and inositol(1,4,5)triphosphate (Ins(1,4,5)P_3). DAG binds to the specific domains in a number of signalling proteins, while Ins(1,4,5)P_3 initiates an increase in intracellular calcium. These two events set off the activation of protein kinase C (PKC) and protein kinase D (PKD) isoforms among other enzymes, and they seem to be the key steps of TCR receptor triggering. The increased intracellular Ca^{2+} also activates calcium-calmodulin-

dependent phosphatase calcineurin, resulting in nuclear translocation of the transcription factor known as nuclear factor of activated T cells (NFAT).

TCR activation also stimulates the activity of phosphatidilinositide 3-kinases (PI3Ks). The product of their kinase activity is phosphatydilinosytol(3,4,5)triphosphate (PtdIns(3,4,5)P_3), an important second messenger. It is known that PI3K activation occurs within seconds of TCR activation, and PI3Ks remain active for nine hours or more (Costello *et al*., 2002). The molecular mechanism by which TCR is coupled to the PI3Ks remains unclear, but it is known that these enzymes are essential for T cell activation and proliferation (Okkenhaug & Vanhaesebroeck, 2003b).

The above described main signalling pathways emerging from activated TCR, along with other signalling events, culminate in the activation of transcription factors in the nucleus, including nuclear factor kappa B (NFκB), nuclear factor of activated T cells (NFAT) and activating protein-1 (AP-1). These factors initiate transcription of genes required for differentiation, proliferation as well as effector actions of activated T cells.

Proliferation and differentiation of activated T cells are driven by the cytokine interleukin 2 (IL-2), which is produced by the activated T cells itselves. In addition, IL-2 is known to stimulate B cells, natural-killer (NK) cells, monocytes, macrophages and oligodendrocytes. Human IL-2 is a protein with globular structure consisting of 133 amino acid residues and predicted molecular mass of 15.4 KDa. Its synthesis is one of the earliest effects of T cell activation and it coincides with the synthesis of the α chain of IL-2 receptor (also known as CD25). The expression of this receptor chain on the cell surface is considered as an early marker of the activation. The IL-2 receptor (IL2R) has three chains: α, β and γ. Resting T cells express the βγ heterodimer which has moderate affinity for IL-2, while the αβγ heterotrimer of activated cells has a high affinity, allowing the cells to respond to very low doses of IL-2. In primary cells costimulatory signals (like CD28 co-receptor activation) are necessary for the production of IL-2: it requires the coordinated action of several

signalling pathways that integrate on the level of multiple transcription factors, including aforementioned NFAT, AP-1, NK-κB and Oct-1 (Jain, *et al*., 1995; Eder *et al*., 1998).

1.4. Phosphoinositide 3-kinases

Phosphoinositide 3-kinases (PI3Ks) are a family of evolutionarily conserved lipid kinases involved in regulation of diverse biological functions, including cell growth, differentiation, survival, proliferation, migration and metabolism. All enzyme members of this family phosphorylate the 3-position of the inositol ring of their substrates (Figure 1.2). These enzymes, isolated from a wide range of species, share a high sequence homology within their kinase domain. Based on their substrate, specificity and utilisation, their structure and regulatory mechanisms, PI3Ks are divided into four distinct classes, referred to I_A, I_B, II and III (Domin&Waterfield, 1997).

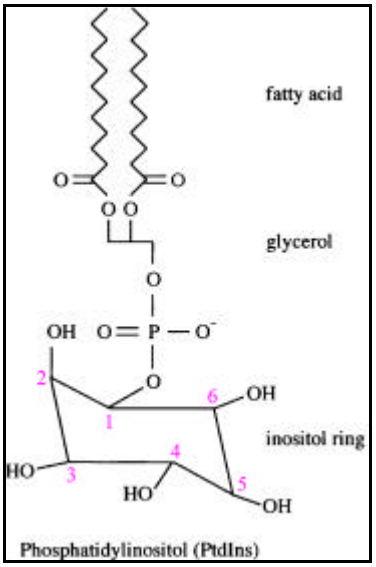


Figure 1.2. Phosphatidylinositol (PtdIns). Inositol-containing lipids are a class of phospholipids consisting of phosphatidic acid to which an inositol ring is attached via its 1' -OH group. In the scheme phosphatidylinositol is presented without any phosphate group on the inositol ring. In living cells all free -OH groups except for the 2' and 6' groups can be phosphorylated in different combinations. These phosphorylated forms of phosphatidylinositol are cold phosphoinositides (PIs) and each of them has a specific function inside the cell. Enzymes members of the PI3K family phosphorylate position 3' of the inositol ring.

The class II PI3K family consists of three members: PI3K-C2α and PI3K-C2β are expressed ubiquitously, whereas PI3K-C2γ is expressed primarily in hepatocytes. Their size is approximately 170 KDa. All enzymes of this class phosphorylate PtdIns and PtdIns(4)P and PtdIns(4,5)P *in vitro* to generate PtdIns(3), PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively, with a strong preference for PtdIns over PtdIns(4)P or PtdIns(4,5)P_2. Nevertheless, their activity and their function *in vivo* are poorly described. It is known that epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, integrin ligation, and the chemokine known as monocyte chemoattractant protein (MCP-1) can induce increased lipid kinase activity in class II PI3K. Class II PI3Ks have two distinct domains at their C termini. The first, pox homology (PX) domain can bind PtdIns(3)P and PtdIns(3,4)P_2 and in this way recruits class II PI3Ks to the plasmamembrane. The function of their second domain, a C2 domain, is not clear, but it is confirmed that it can bind to phospholipids in a Ca^{2+}-independent manner *in vitro*. Unlike the members of class I PI3Ks, adaptor molecules for class II enzymes are not described so far. The cellular localisation of class II PI3K is also not clearly defined – conflicting reports suggest different compartments, but it seems that class II PI3Ks enzymes are predominantly associated with the membrane fraction of cells (Koyasu, 2003; Vanhaesebroek *et al.*, 2001).

Class III PI3Ks are homologues of the yeast Vps34p involved in vesicular protein sorting. A single class III PI3K catalytic subunit has been identified in all eukaryotic species. Both in yeast and mammals, this catalytic subunit exist in a complex with a Ser/Thr protein kinase named Vps15p in yeast and p150 in mammals. All eukaryotes appear to have vps34p and vps15p analogous. They are highly related enzymes and both display N-terminal myristoylation. This lipid modification of Vps15p recruits Vps34p to the membrane in yeast. Apparently p150a has a similar role in mammals. In yeast Vps34p/Vps15p heterodimers regulate vesicle trafficking trough proteins containing FYVE finger domains that can bind PtdIns(3)P. The mammalian ortholog is involved in movement of proteins trough the

lysosome. Class III PI3Ks can use only PtdIns as a substrate *in vitro*, and they are probably responsible for the generation of most PtdIns(3)P in cells. Class III PI3Ks are constitutively active *in vitro*, but the regulation their activity *in vivo* is largely unknown (Vanhansebroek *et al*, 2001; Koyasu, 2003).

1.5. PI3Ks class I – structure, function and regulation

All enzymes of class I PI3Ks can utilize PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 as a substrate and produce PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 *in vitro*, respectively. Considering that the predominant substrate in cells is PtdIns(4,5)P_2, the major product of their enzymatic activity *in vivo* is PtdIns(3,4,5)P_3. It is an important lipid second messenger that controls a wide range of cellular responses (Figure 1.3.). Resting mammalian cells contain significant and constant levels of PtdIns(3)P. While the basal levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are relatively low, their concentration can rise sharply upon different kinds of cellular stimulation. In addition, all lipid substrates and products of PI3Ks are restricted to the membrane compartments, and their accessibility appears to be a critical factor for their metabolism and function (Vanhaesebroeck *et al.*, 2001).

PI3K class I_A are heterodimers comprised of a catalytic subunit designated as p110 and a regulatory subunit. There are three different enzymatic subunits of this class named p110α, p110β and p110δ, all encoded by different genes. p110α and p110β are ubiquitously expressed while the expression of p110δ is largely restricted to the cells of the immune system. Class I_A enzymes have five different regulatory subunits: p85α, p55α and p50α as two splicing variants of the first one, and p85β and p85γ encoded by separate genes. p85α is most abundantly expressed (Fruman *et al.*, 1999).

PI3K class I_B is represented by a single heterodimer named PI3Kγ. The enzymes catalytic subunit known as p110γ is predominantly expressed in leukocytes. p110γ interacts

with a specific type of regulatory subunit, a unique adaptor molecule named p101. This regulatory subunit does not have any structural or functional homology to known proteins.

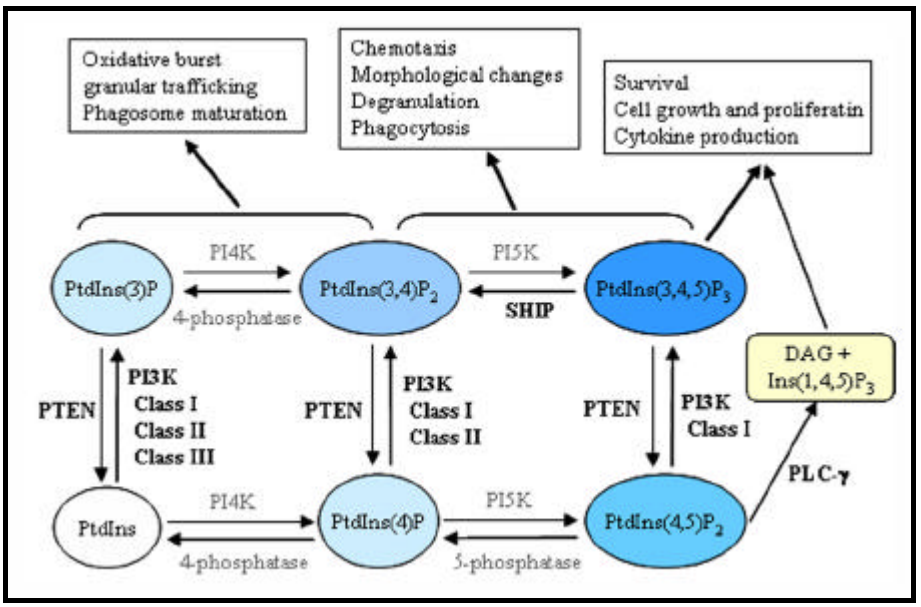


Figure 1.3. Metabolism of phosphoinositides and their functions in cellular processes of immune system. Different classes of PI3K phosphorylate 3-position of the inositol ring and can utilize different substrates. The amount of PtdIns(3,4,5)P_3 is regulated by two phosphoinositide phosphatases, SH2 domain-containing 5 inositol phosphatase (SHIP) and the tumor suppressor gene product phosphatase and tensin homolog (PTEN). PtdIns(4,5)P_2 is cleaved by phospholipase C-gamma (PLC-γ) providing two distinct second messengers: inositol-1,4,5-trisphophate (Ins(1,4,5)P_3), which triggers intracellular calcium flux, and diacylglycerol (DAG), which acts on PKC enzymes. The phosphorylation on the 4- and 5-position of the inositol ring is regulated by distinct phosphoinositide kinases and phosphatases. All enzymes that are not discussed in the text are shown in grey letters (after Koyasu, 2003).

All four enzymatic subunits of class I_A and I_B (p110) shares the following homologous regions: conserved C-terminus catalytic domain, PIK domain, C2 domain and Ras-binding domain (Ras-BD). The catalytic domain of PI3Ks is structurally organized into two lobes. The N-terminal lobe contains the nucleotide-binding loop and a conserved lysine residue responsible for ATP biding. In many regards this lobe is similar to the corresponding region of other protein kinases. The C-terminal lobe, apart from contributing to ATP binding,

contains the activation loop that determines the substrate specificity of PI3Ks (reviewed by Vanhaesebroeck *et al.*, 2001). Ras-BD is responsible for the interaction of p110 with an activated form of the proto-oncogene Ras. The affinity of this interaction is considerably lower then the interaction of p110 subunits with their regulatory subunits. The PIK domain is a helical domain typical for lipid kinases and it acts as a structural spine around which the other domains are anchored. The C2 domain is a protein module with phospholipid binding properties and it is involved in the recruitment of the enzymes to the membrane. Class I_A catalytic subunits contain a N-terminal region responsible for constitutive interaction with Class I_A regulatory subunits, while the corresponding region of PI3Kγ facilitates interaction with p101.

Isoforms of class I_A subunit p110 do not seem to have binding specificity for distinct regulatory subunits (Vanhaesebroeck *et al.*, 1995). For the regulatory subunits of class I_A distinct domains are known, two Src homology domain 2 (SH2) domains separated by 'inter SH2 domain' which is responsible for constitutive interaction with the catalytic subunits of class I_A, and it was proved that regulatory subunits stabilize the unstable p110 catalytic subunits (Yu *et al.*, 1998; Vanhaesebroeck *et al.*, 2005). The two SH2 domains can mediate interaction with tyrosine-phosphorylated proteins, which appears to be critical for recruitment of p110 subunits to the inner surface of the plasmamembrane, the location of their lipid substrate. This translocation also brings the catalytic subunit in the vicinity of phosphotyrosine residues in receptors, adaptor proteins and other molecules.

Besides translocation, the activation of catalytic subunits of class I_A enzymes is closely connected to activation of tyrosine-kinase-associated receptors. How this activation is achieved is still not fully elucidated (Okkenhaug and Vanhaesebroeck, 2003a). Activation of PI3K class I_A by Ras is well documented. This interaction elevates lipid-kinase activity of PI3K class I_A above the level induced by phosphotyrosin engagement of their regulatory subunits (Rodrigez-Vinciana *et al.*, 1994, 1996). There are reports that GPCR ligands can also

stimulate protein members of PI3K Class I_A. Such kind of up-regulation was observed in cell lines that do not express detectable levels of PI3Kγ and it seemed to be related mostly to the β-isoform of PI3K (reviewed by Vanhaesebroeck and Waterfield, 1999).

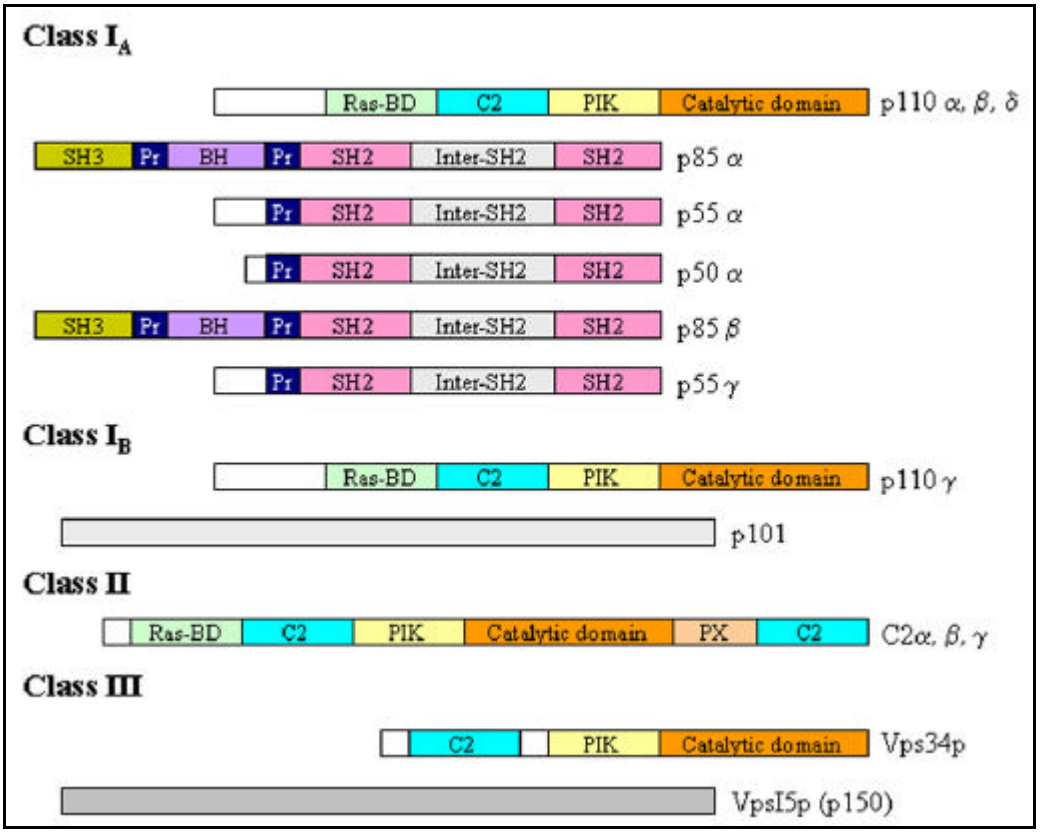


Figure 1.4. Schematic presentation of functional domains found in different PI3K family members. All enzymatic subunits of PI3Ks contain the highly conserved catalytic domain and the C2 domain, which is involved in recruiting PI3K to membrane. These two domains are connected by a helical domain (PIK). In addition, both class I and II enzymes, contain a Ras-binding domain (Ras-BD). All regulatory subunits of class I_A contain dual SH2 domains connected with an inter-SH2 domain. The two SH2 domains bind to tyrosine-phosphorylated peptide motifs, leading to activation of p110 subunits. The inter-SH2 domain constitutively interacts with the N-terminal domain of p110 α, β, δ in order to maintain the stability of p110 in the cell. p85α and p85β have on their N-terminus an Src homology domain 3 (SH3), a Brc homology domain (BH) and an additional proline–rich region (Pr), the functions of which are not fully understood. p101, the regulatory subunit of class I_B, specifically interacts with the N-terminal domain of p110γ. The Phox homology domain (PX), found only in the class II PI3K family, binds PI(3)P and PI(3,4)P_2 and may recruit class II PI3Ks to the membrane. Vps34 forms a heterodimer with the serine/threonine kinase Vps15p and regulates vesicle trafficking (after Koyasu, 2003).

The enzymatic activity of PI3Kγ is primarily regulated by G protein-coupled receptors (GPCR) (Stoyanova *et al*., 1995). The Gβγ subunit of the G-protein mediates activation of PI3Kγ and directly activates PI3Kγ *in vitro*. Both subunits of PI3Kγ have been shown to bind Gβγ *in vitro*, but p101 does so with a five-fold higher affinity (Stephens *et al*., 1997; Voigt et *al*., 2005). p101 binds to Gβγ released from heterotrimeric GPCR and recruits the p110γ to the plasmamembrane, which is a prerequisite for the GPCR-induced PI3Kγ activation *in vivo* (Brock *et al*., 2003). An N-terminal domain of p101 mediates heterodimerisation with p110γ, whereas the C-terminal domain of p101 is responsible for the interaction with Gβγ (Voigt *et al*., 2005). p101 appears to be essential for Gβγ-mediated activation of PI3Kγ in living cells, but surprisingly, the lipid-kinase activity of PI3Kγ can be stimulated by Gβγ in the absence of p101 (Leopoldt *et al*., 1998). PI3Kγ, like the PI3K class I_A, also interacts with Ras in a GTP-dependent manner. (Rodriguez-Viciana et al., 1994; Rubio et al., 1997; Vanhaesebroeck et al., 1997). Ras forms a transient complex with PI3Kγ and activates it *in vivo* and *in vitro*. Moreover, conformational changes of PI3Kγ induced by Ras binding implicate that allosteric mechanism, in addition to membrane recruitment, may be important for the activation of PI3Ks (Suire *et al*., 2002; Pacold *et al*., 2000).

The major downstream targets of lipid-kinase activity of PI3Ks are the proteins with Plekstrin Homology (PH) domain. PH domains are globular protein domains of about 120 amino acids that can bind phospholipids, although some PH domains can mediate protein-protein interactions (Vanhaesebroeck and Waterfield, 1999). Many, if not most PH domains, bind PtdIns(3,4,5)P_3 with high affinity. PH domains have been identified in all eukaryotes in about 200 different proteins including kinases, phospholipases, structural proteins, nucleotide-exchange factors and adaptor proteins (Vanhaesebroeck & Waterfield, 1999; Sasaki *et al*., 2002). In most of the cases it is proved that phospholipid binding affects localisation, conformation and/or activity of these proteins (Sasaki *et al*., 2002). The most important known targets of PI3Ks enzymatic activity are two serin/threonin kinases: Phosphoinositide-

dependent kinase 1 (PDK1) and Protein Kinase B (PKB) also known as Akt. PDK1 is important and/or essential for activation of many signalling proteins such as Protein Kinase A (PKA) (Cheng *et al*., 1998), Protein Kinase B (reviewed by Vanhaesebroeck and Waterfield, 1999), Protein Kinase C (PKC) isoforms (Le Good *et al*., 1998; Chou *et al*., 1998), serum- and glucocorticoid-iduced protein kinase (SGK) (Park *et al*., 1999; Kobayashi and Cohen, 1999) and $p70^{S6}$ and $p90^{S6}$ kinases (Pullen *et al*., 1998). After translocation to the membrane PKB/Akt is phosphorylated by PDK1 on Thr308 and subsequently within its hydrophobic motif on Ser473 by kinases dubbed 'PDK2' which leads PKB/Akt to full activation. Although several proteins have been proposed to act as PDK2, the enzyme with this function has not been identified so far. The PI3K-PDK1-PKB/Akt signalling cascade is in the focus of many authors as the one of the most relevant anti-apoptotic pathways. PKB/Akt–mediated regulation of forkhead transcription factors (FOXO) and cyclin D1 allows cell cycle entry, while phosphorylation of IκB kinase (IκBK) and of proteins of the cell-death machinery such as BAD and caspase-9 inhibits their activity and promotes cell survival (Wymman & Marone, 2005). In addition, PKB/Akt plays an important role in the insulin signalling and activation of endothelial nitric oxide synthetase (eNOS) (reviewed by Vanhaesebroeck & Waterfield, 1999).

The aforementioned members of the protein kinase C (PKC) family also require phosphorylation within their activation loop by PDK1 (reviewed by Sasaki *et al*., 2002). This phosphorylation is only a priming step in the autophosphorylation required to generate a catalytically competent form of PKC that needs diacylglycerol binding and/or relief from autoinhibition for full activation (Parekh *et al*., 2000a). Their binding to PDK1 also suggests a potential role of PDK1 in regulation of PKCs subcellular localisation (Ward & Catrell, 2001).

The other targets of PI3Ks lipid kinase activity are tyrosine kinases of Tec family, Bruton tyrosine kinase (BTK) and Inducible T cell kinase (Itk), enzymes indispensable for normal development and function of B and T lymphocytes, respectively. Presence of PH

domains and PtdIns(3,4,5)P_3-dependent activity are also confirmed for some GTP/GDP exchange factors (GEFs) and GTPase-activating proteins (GAPs), e.g. GTPases of the Rho, Ras and Arf families. Examples for this are GEFs specific for the Rho family of GTPases (Rho, Rac and cdc42). It has been proved that Rac is activated during chemotaxis stimulated by tyrosine kinases in a PI3K-dependent manner (Han *et al.*, 1998).

Different PI3K catalytic subunits of class I_A and I_B exhibit dual specificity both as lipid kinases and protein serine kinases. All PI3K class I enzymes have one single catalytic centre responsible for both kinase activities. Their protein kinase activity is directed towards auto-phosphorylation and/or phosphorylation of their regulatory subunits. This kind of enzymatic activity has been demonstrated *in vitro*, but no other proteins were identified as a substrate of these enzymes *in vivo* so far. Hence, the protein kinase activity of p110α is directed toward phosphorylation of p85 adaptor protein, whereas p110δ is selective for an autophosphorylation site (Ward and Cantrell, 2001). It has been shown that PI3Kγ protein kinase activity can activate a mitogen activated protein kinase (MAPK) in a transient transfection system. In this study authors used a PI3Kγ mutant without lipid kinase activity but with retained protein kinase activity (Bondeva *et al*., 1998). It is possible that different protein kinase activities may give unique cellular functions to different PI3K isoforms.

Two phophosphoinositide phosphatases are major negative regulators of PI3Ks lipid kinase activity – phosphatase and tensin homologue (PTEN) and haematopoietic-specific SH2-domain containing inositol 5-phosphatases (SHIP). PTEN cleaves the phosphate from the 3-position of PtdIns(3,4,5)P_3 generating PtdIns(4,5)P_2 and in this way counteracts all PI3Ks. PTEN is a tumour suppressor that is often inactivated in different types of cancer, leading to the constitutive activation of the class I PI3K-signaling pathways. On the other hand, SHIP removes phosphate group from the 5-position of PtdIns(3,4,5)P_3 and converts it to PtdIns(3,4)P_2. Members of the SHIP phosphatases have inhibitory role in the immune system and, probably, in insulin signalling. SHIP mediates an important negative feedback

mechanism in lymphocytes. Loss of SHIP results in an unbalanced immune response (reviewed by Ward & Cantrell, 2001). Furthermore, phospholipase Cγ (PLCγ) cleaves PtdIns(4,5)P_2 creating two important second messengers: inositol-1,4,5-phosphate and diacylglycerole. PLCγ does not directly counteract the activity of PI3K, but it reduces the amount of the major PI3K substrate.

Two unrelated pharmacological inhibitors of PI3Ks, Wortmannin and LY294002, were highly useful research tools for proving the involvement of PI3Ks in many signalling events. Both substances are cell-permeable, low-molecular-weight compounds, which are, at relatively low doses, specific inhibitors of most PI3Ks. Unlike LY294002, Wortmannin binds irreversibly to the PI3K ATP binding pocket of the catalytic subunit, a reaction that has been competed by ATP and PtdIns(4,5)P_2 but not PtdIns (Wymann *et al*., 1996). LY294002 is a competitive inhibitor of the ATP binding site. Wortmannin is usually applied at final concentrations of 20-100 nM while concentrations of LY294002 is in the range of 5-30 µM. It must be mentioned that Wortmannin, in contrast to LY294002, is rather unstable in aqueous solutions and also has a tendency to react with serum proteins, which makes it inappropriate for treatment of the cell culture for periods longer then few hours (Vanhaesebroeck & Waterfield, 1999). All mammalian class I, II and III PI3K members show a similar *in vitro* sensitivity to Wortmannin and LY294002 (Ward *et al*., 1996, Vanhaesebroeck & Waterfield, 1999). Moreover, both compounds have serious inhibitory activity on numerous cellular functions resulting in their high cytotoxicity. For example, pharmacological inhibitors of PI3Ks block or severely compromise development and functions of the cells of the immune system. The first studies *in vivo* conducted by Gunther *et al*., in 1989 (reviewed by Okkenhaug and Vanhaesebroeck, 2003) already indicated a high potential of Wortmannin as an immune suppressor but also its high toxicity. Wortmannin and LY294002 were therefore never considered as potential therapeutic substances.

1.6. Phosphoinositide 3-kinases in the immune system

Activation of tyrosine-kinase-related receptors in the cells of the immune system is known to activate class IA PI3Ks. For example, cytokines such as interleukin 2 (IL-2), IL-3, IL-6, IL-7, IL-15, granulocyte colony-stimulating factor, erythropoietin, oncostatin M and interferons activate class IA PI3Ks in many types of immune cells. In addition, cytokine receptors with intrinsic tyrosine-kinase activity (macrophage colony-stimulating factor receptor and c-Kit) can also activate class IA PI3Ks. Finally, these enzymes are known to be activated upon antigen recognition by B cell receptor (BCR) and TCR (Koyasu, 2003).

On the other hand, several lines of evidence indicate that PI3Kγ, the only member of class I_B PI3K, is involved in chemokine-induced cell migration of different immune cells. For example, binding of interleukin 8 (IL-8 or CXCL1) and stromal-cell-derived factor 1 (SDF1 or CXCL12) to their specific receptors, which are GPCR, induce wortmannin-sensitive chemotaxis of neutrophils and T cells, respectively (reviewed by Curnock *et al*., 2002).

The established pharmacological inhibitors of PI3Ks are highly toxic, and do not discriminate between different isoforms of PI3Ks. Therefore, alternative approaches were applied in order to investigate the involvement of PI3Ks in the regulation of immune functions. Different experimental designs, many of them including overexpression of mutant PI3K subunits in different cell models, gave rise to conflicting, and sometimes opposite results. The examples of such controversies are the recruitment/activation of PI3Ks after CD28 ligation in T cells or the impact of wortmannin and LY294002 on T cell activation. Pharmacological inhibitors of PI3Ks clearly inhibit activation of primary T cells, but in many regards they are considered equally ineffective on certain transformed T cell lines, especially on Jurkat cells (reviewed by Kane & Weiss, 2003). A similar situation emerged around the involvement of PI3K in IL-2 production upon T cell activation. The first clear evidence of PI3K involvement in IL-2 expression in activated T cells came from Eder et al. (1998). The authors came to the conclusion that class I_A PI3K has an essential role in T cell receptor-

mediated IL-2 gene expression in *normal* T cells, as the authors state in the title of their manuscript.

The abovementioned controversies required the application of a new approach to investigate PI3Ks involvement in the regulation of immune functions *in vivo*, so gene targeting seemed to be the next reasonable step.

1.6.1. Targeting the p85α subunit of class I_A

p110α and p110β knockout mice ware generated and both KO died *in utero* between embryonic days 9,5 and 10,5 or shortly after embryo implantation, respectively. Double heterozygous $P110\alpha^{+/-}p110\beta^{+/-}$ mice grew normally, which suggests that both isoforms have distinct roles in foetal development (Bi *et al*., 1999, 2002). Thus, the effect of these mutations on immune cells could not be determined. The alternative approach was to target the regulatory subunit of Class I_A PI3Ks.

Two groups independently created p85α KO mice using different approaches. Suzuki *et al*. (1999) deleted the first exon of the gene, so the p55α and p50α splicing isophormes were retained; these animals were viable. On the other side, Fruman *et al*. (1999a) disrupted the expression of all three variants. The mice generated in such manner died shortly after birth. Fruman *et al*. (1999a) therefore used a RAG (recombination-activating gene) complementary approach to investigate function of lymphocytes in their model. The results of those two groups are mostly consistent. Both groups could not find any remarkable defect in T cell development or functions. In contrast to that, B cell development and functions were seriously compromised: transition from pro-B to B cell was partly blocked resulting in 50-80% reduction in the number of mature B cells, and reduction or absence of peritoneal CD5+ B-1 cells. Proliferation induced by activating B cell receptor (BCR) or anti-CD40 activation was impaired. However, Fruman *et al*. (1999a) reported that the proliferative response to anti-CD40 activation can be rescued with interleukin-4 (IL-4) costimulation. T cell-dependent, but not T cell-independent antibody production was also impaired. Finally, further investigation

by both groups described reduced expression of all catalytic subunits of class I_A and increased expression of p85β. Mast cells of those mice exhibited reduced SCF- or IL-3-induced proliferation, moderately reduced number of tissue mast cells and complete absence of gastrointestinal mast cells, while dendritic cells showed increased IL-12 production and reduced sensitivity to *Leishmania major* infection (reviewed by Vanhaesebroeck *et al*., 2005). Conflicting data about alterations in some specific signalling pathways imply that these p85 KO mice cannot be considered as PI3K knockouts (Okkenhaug & Vanhaesebroeck, 2003).

P85β knockout mice are viable and do not have any apparent defect in the immune system (Ueki *et al*., 2003). Targeting of the gene encoding p55γ has not been reported so far.

1.6.2. Targeting the p110 subunits of class I_A

p110δ knock-out mice have been generated and they are viable (Clayton *et al*., 2002). Nevertheless, the most valuable data about the role of p110δ in immune system are coming from the group of Vanhaesebroeck who applied a more elegant strategy: instead of deleting the whole gene they introduced a point mutation creating a knock-in (KI) mouse line that expresses a kinase-dead enzyme. This method avoids disturbing the molecular balance of different PI3K isoforms (Okkenhaug *et al*., 2002). The phenotype of this mouse named $p110\beta^{D910A/D910A}$ is reviewed in detail by Okkenhaug and Vanhaesebroeck (2003). In p110δ KO mice expression of the regulatory class I_A subunits was reduced in B cells, whereas, in p110δ KI cells p85, p55 and p50 levels were not altered. The phenotypes of p110δ KO and KI mice were similar with respect to signalling and functional defects in lymphocytes, although they were not directly compared. Briefly, those phenotypes in many regards resemble the phenotype of p85α KO mice, at least regarding the B cells: both p110δ KO and KI exhibited a nearly 50% reduction in the number of B cells in the spleen, anti-IgM-induced Akt/PKB activation was almost completely lost and calcium influx was attenuated. Further, BCR induced activation and proliferation was impaired, just like anti-CD40 and IL4 induced

proliferation of B cells. In addition, both T cell-independent, as well as T cell-dependent antibody production was impaired as a consequence of their impaired T cell functions.

The phenotype of T cells also revealed interesting facts: Akt/PKB activation upon anti-CD3 activation of the p110$\delta^{D910A/D910A}$ T cells was almost ablated, calcium flux was attenuated, the number of T cells in the spleen reduced, and mature T cells had a more 'naive' phenotype. However, the size and cellularity of the thymus was comparable to those in the WT mice. Moreover, it was possible to rescue reduced anti-CD3-induced proliferation with anti-CD28 costimulation, while cytokine production remained normal. Mutation of p110δ also induced minor functional defects in mast cells, and possibly in neutrophils (Vanhaesebroeck *et al*., 2005). An important finding in p110δ KI mouse was the differential sensitivity of different receptor systems to p110δ inactivation. This indicated that p110δ selectively couples to specific receptor systems (Vanhaesebroeck *et al*., 2005). Further supported with some additional results, above-mentioned authors believe that the p110δ isoform is the major provider of PI3K activity downstream of BCR and TCR.

1.6.3. PI3Kg and immune system

Three groups at approximately same time generated PI3Kγ knockout mice by using different strategies (Li *et al*., 2000; Sasaki *et al*., 2000; Hirsch *et al*., 2000). All mice were viable, fertile and displayed normal life span in standard conditions. The focus of those preliminary investigations was mainly on the haematopoietic cells. There was no apparent effect of this deletion on B cell development and function, except that the production of antibodies containing the lambda light chains in response to T cell-independent antigens were altered (Li *et al*., 2000). However, the main phenotype of PI3Kγ KO mice is mostly related to the cells of the innate immune system. Although the total number of neutrophils of PI3Kγ KO mice increased, they exhibited reduced chemotaxis both *in vivo* and *in vitro*. Accumulation of neutrophils in the peritoneal cavities was significantly reduced in both casein- and *Listeria*

monocytogenes-treated KO mice in comparison to control animals. *In vitro* chemotaxis induced by GPCR agonists (N-formil-Met-Leu-Phe (fMLP) and C5a) of PI3Kγ$^{-/-}$ neutrophils was reduced by 70%. The results indicated that observed reduction in chemotaxis was caused by impaired motility and not by altered adhesion of the cells. In addition, the GPCR-induced respiratory burst was reduced in freshly isolated bone marrow neutrophils from PI3Kγ KO mice (Sasaki *et al*. 2000; Li *et al.*, 2000; Hirsch *et al*., 2000). Macrophages of PI3Kγ KO mice had chemotaxis reduced by 52-85%; it was induced by different GPCR agonists such as: RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), macrophage inflammatory protein-5 (MIP-5), macrophage derived chemokine (MDC), stromal cell derived factor-1 (SDF-1) and C5a *in vitro*. Interestingly, PI3Kγ$^{-/-}$ peritoneal macrophages also exhibited reduction of chemotaxis by 85% toward vascular endothelial growth factor (VEGF), an agonist known to bind to the receptor tyrosine-kinase. Moreover, PI3Kγ KO mice had impaired inflammatory response to septic peritonitis: 12 h after bacteria injection the number of peritoneal PI3Kγ$^{-/-}$ macrophages was reduced by 90% in comparison to the WT. 48 h after intraperitoneal injection of *Staphylococcus aureus*, there were ten-fold more bacteria in PI3Kγ KO mice peritoneum, in spite of the fact that PI3Kγ$^{-/-}$ macrophages did normally phagocytose bacteria (Hirsch *et al*., 2000). PI3Kγ was also proved to positively regulate SDF1-induced T cell migration both in a cell line model (Curnock *et al*., 2002) and in *in vitro* assays with mouse T cells (Reif *at al*., 2004). PI3Kγ$^{-/-}$ bone marrow derived mast cells were less sensitive to antigen-IgE stimulation than WT mast cells. The release of the histamine-containing granules and the rise of the intracellular calcium concentration were also diminished in PI3Kγ$^{-/-}$ mast cells (Sasaki *et al*., 2000; Laffargue *et al*., 2002). *In vivo* and *ex vivo* migration of dendritic cells was also reported to be reduced in PI3Kγ KO mice (Del Prete *et al*., 2004).

One of the most intriguing features of PI3Kγ KO mice seems to be the phenotype of their T cells. PI3Kγ KO mice had reduced thymic cellularity and a reduced size of the thymus

itself. Thymocytes exhibited enhanced apoptosis when stimulated with anti-CD3ε antibody *in vivo*, or in combination with adenosine analogues. The KO mice thymocytes also showed a defect in the proliferative expansion that accompanies development from the double negative (DN) to the double positive (DP) stage (Sasaki *et al*., 2000). Later findings confirmed that PI3Kγ modulates T cell differentiation at several stages. PI3Kγ deficiency moderately impairs pre-TCR-induced DN thymocytes differentiation - PI3Kγ KO mice showed an increase in DN cell numbers. The lack of PI3Kγ affected CD4/CD8 lineage commitment during positive selection and augmented CD8 cell differentiation. In addition to this effects, it was confirmed that the lack of PI3Kγ reduced the migration of mature thymocytes to the periphery (Rodriguez-Bolardo *et al*., 2003).

Sasaki *et al*. (2000) also reported that PI3Kγ KO mice had a reduced number of CD4 T cells, but no CD8 T cells in spleen, and a normal ratio of different T cell sub-types in other peripheral lymphoid organs. The response of the mature PI3K$\gamma^{-/-}$ T cells to activation by phorbol ester and Ca^{2+} ionophore (TPA/Ionomycin) was normal, while proliferation induced by anti-CD3 antibody and concanavalin A (Con A) was impaired. A functional defect in interleukin 2 (IL-2) and interferon gamma (INF-γ) production upon activation was more pronounced after TCR-induced activation. It was possible to rescue impaired TCR-induced proliferation with anti-CD28 costimulation, unlike activation-induced cytokine production. $CD8^{+}$ T cell-dependent antiviral response and functional T helper cell-dependent response to hapten antigens *in vivo* were impaired as well (Sasaki *et al*., 2000).

PI3Kγ knock-in mice which express full length kinase-dead enzyme have been generated and compared to the PI3Kγ KO mice (Patruccio *et al*., 2004). Although these mice had a similar defect in immune cells, they displayed important differences in their cardiac phenotype. PI3Kγ seems to be the part of the protein complex?which is indispensable for the proper function of the phosphodiesterase 3B (PDE3B). This enzyme degrades cAMP, and the loss or deregulation of PDE3B activity in cardiac cells upon PI3Kγ KO resulted in increased

cAMP levels, leading to an activation of protein kinase A and an increased heart contractility. This effect was absent in PI3Kγ KI mice, which indicates that this particular role of PI3Kγ is not related to its kinase activity but to the proposed scaffolding function of this protein. This is an example of how different gene-silencing approaches can lead to different functional outcomes with important implications for understanding of enzyme functions and also for their potential use as pharmacological targets (Vanhaesebroeck *et al*., 2005).

Ever since the first experiments with PI3Kγ KO mice it became obvious that this enzyme, along with the PI3Kδ isoform, may become a very promising drug target (Wetzker and Romel, 2004). First, both KOs are viable and fertile and without proved physiological disturbances as long as their immune systems are not challenged. Second, both isoforms are predominantly expressed in the cells of the immune system and both enzymes play important roles in functions of those cells. Moreover, PI3Kγ is biochemically distinct from the class I_A PI3K (Vanhaesebroeck *et al*., 2005) and its crystal structure is determined (Walker *et al*., 2000; Pacold *et al*., 2000). Finally, PI3Kγ is doubted to be one of the key modulators in inflammation and allergy (Wymann *et al*., 2003).

Indeed, in the last couple of years Serono Pharmaceutical Research institute (Serono International, Geneva, Switzerland) in cooperation with several academic research groups designed and developed a series of chemical substances which are potent and selective PI3Kγ inhibitors (Camps *et al*., 2005). All designed substances are small-molecule ATP analogues with high cellular permeability. Some of those substances exhibit a high inhibitory potency toward PI3Kγ, while maintaining a favourable selectivity profile against other PI3K isoforms, as well as against a broad range of tyrosine and serine-threonine protein kinases. The compound named AS-605240 was found to be the most superior PI3Kγ inhibitor with improved biopharmaceutical profile, and it was able to mimic the effect of PI3Kγ deficiency in the whole panel of *in vivo* and *in vitro* assays. The same substance was also successfully applied *per os* to the experimental animals, and this treatment suppressed the

progression of joint inflammation and damage in two distinct mouse models of rheumatoid arthritis (Camps *et al*., 2005). The oral application of AS-605240 was also successful in the case of MRL-*Ipr* mice, a mouse model of human systemic lupus erythematosus (SLE), a chronic inflammation caused by deregulation of T cell-mediated B cell activation resulting in glomerulonephritis and renal failure. The treatment reduced the glomerulonephritis and prolonged the lifespan of the animals, proving again that PI3Kγ may be a useful target in the treatment of chronic inflammations (Barber *et al*., 2005).

1.7. Aim of this work

PI3Ks are important signal mediators involved in the regulation of diverse biological functions, including cell growth, differentiation, survival, proliferation, migration and metabolism. PI3Ks are indispensable for the development and proper functions of T lymphocytes and other cells of the immune system. The involvement of PI3Ks in the activation and proliferation of T cells was mostly related to the PI3K class I_A enzymes, but the mechanism of this involvement is still elusive. Ever since PI3Kγ KO mice have bin generated (Li *et al*., 2000; Sasaki *et al*., 2000; Hirsch *et al*., 2000), many convincing evidences emerged about the involvement of the PI3Kγ, the single member of class I_B PI3K, in T cell activation, proliferation and cytokine production.

It was proved that PI3K$\gamma^{-/-}$ mice had disrupted tymic development, reduced number of thymocytes and of CD4 T cells in spleen (Sasaki *et al*., 2000; Rodriguez-Bolardo *et al.* 2003). PI3K$\gamma^{-/-}$ T lymphocytes exhibited impaired TCR-induced proliferation and subsequent cytokine production *in vitro*. It was possible to rescue impaired proliferation of PI3K$\gamma^{-/-}$ T cells by the activation with Ionomycin and phorbol ester, but not the cytokine production. Moreover, CD8 T cell-dependent antiviral response as well as functional T helper cell-dependent response were impaired *in vivo* (Sasaki *et al*., 2000).

In work presented here we analysed the profile of T cells of PI3K$\gamma^{-/-}$ mice in more details and tried to elucidate if their impaired TCR-induced IL-2 production is one of the major causes of reduced proliferation of PI3K$\gamma^{-/-}$ T cells.

In order to reveal new details about involvement of PI3Kγ in TCR-induced activation and subsequent IL-2 production of the T cells, we used AS041164, a specific pharmacological inhibitor of PI3Kγ. In our experiments we used the Jurkat cell line, the most commonly used model of human T cells. Specific features of this cell line in respect to the regulation of PI3Ks

activity made it possible to elucidate whether lipid-kinase or protein-kinase activity of PI3Kγ is important for the analysed processes.

In order to find out more about the TCR signalling mechanism which involves PI3Kγ, we tried to identify interacting partners of PI3Kγ in Jurkat cells. The focus of our attention were proteins whose interaction with PI3Kγ is regulated by T cell receptor activation, and which are relevant for the cellular functions of the T cells at the same time.

2. Results

2.1. Thymocytes and T cells of PI3Kg KO mice

The PI3Kγ knock-out (KO) mice, used as a model in our study, were generated as described by Hirsch *et al*. (2000). It was proved that PI3Kγ$^{-/-}$ T cells have altered thymic development, impaired TCR-induced proliferation and diminished subsequent cytokine production (Rodriguez-Bolardo *et al*., 2003; Sasaki *et al*., 2000). In order to analyse in more details the role of PI3Kγ in T cells, we thoroughly investigated the total number of PI3Kγ$^{-/-}$ T cells and thymocytes in lymphoid organs, as well as the ratio of different cellular subpopulations.

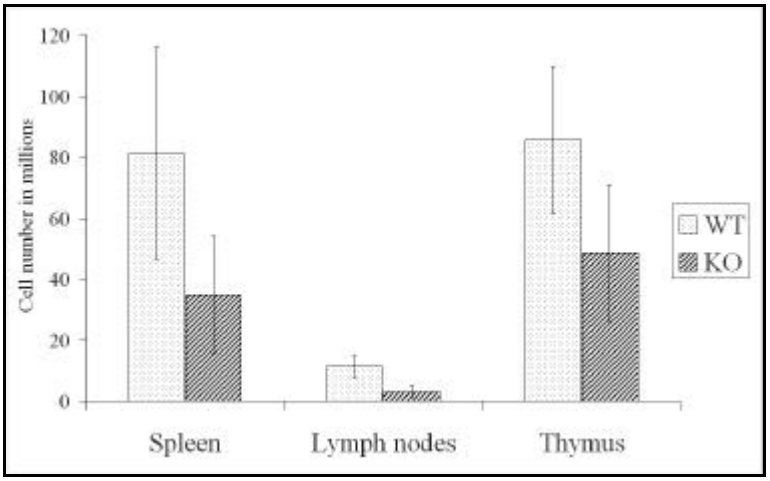
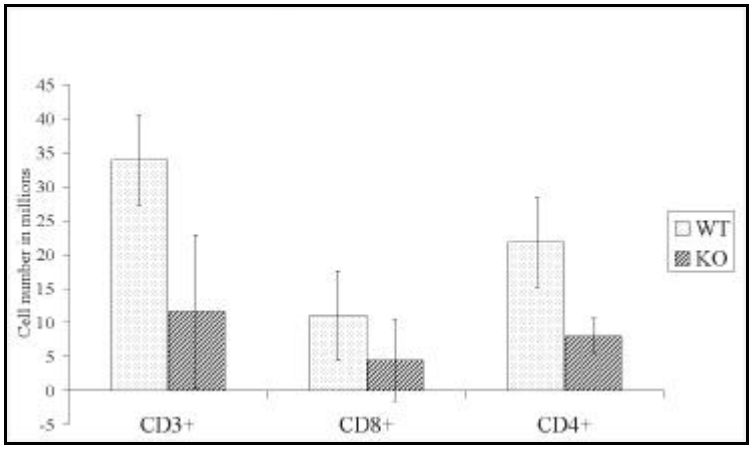


Figure 2.1. The total number of cells in spleen, lymph nodes and thymus. The organs were homogenised to a single cell suspension, and the cells were counted using the Neubauer counting chamber. Tripan-blue exclusion was used to confirm cell viability and only viable cells were taken into account. No significant differences in percentage of viable cells between different samples were observed.

The cell analyses were performed on two occasions with seven KO and seven wild-type (WT) animals in total, all between two and five months of age. All the animals were fit, healthy and the KO animals were without any apparent morphological and morphometrical deviation. The total cell number of thymus, spleen and lymph nodes was determined after the

examined organs had been homogenized to a single cell suspension. The percentage and number of T cells in total and different T cell subpopulation, as well as different thymocytes subpopulations was examined by flow cytometry. The results revealed that in all examined peripheral lymphoid organs of PI3Kγ KO mice the total number of T cells was reduced by about 50% or more (Figure 2.1.). The size of thymus and spleen in KO mice was reduced approximately by 30-50% in comparison to the corresponding organs of WT mice exhibiting much softer consistence. Lymph nodes of PI3Kγ KO mice were significantly reduced in size as well, and difficult to visualize in the majority of cases.



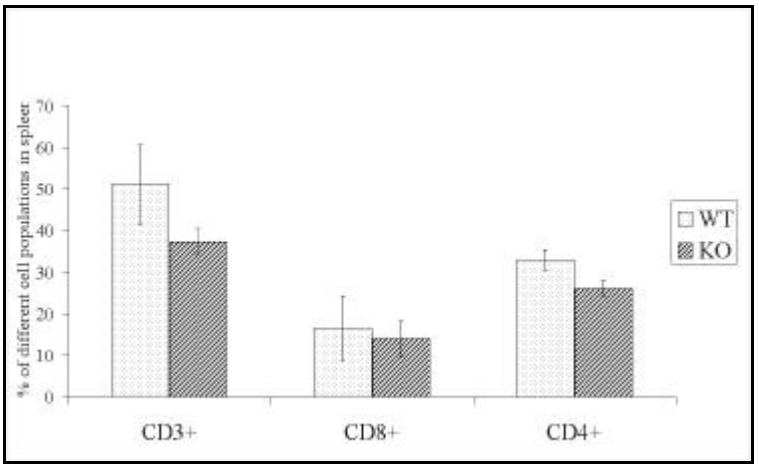


Figure 2.2. A) The total number of all T cell (CD3+), CD8+ and CD4+ T cells in spleen, cell number in millions. **B) The ratio of T cell, CD8+ and CD4+ T cells in spleen**, results given as percentage of all spleenocytes. Analysis performed by flow cytometry upon staining with specific antibodies; subpopulations of the T cells were identified as double positive cells (CD4+/βTCR or CD8+/βTCR).

Number of T cells in spleen of PI3Kγ KO mice was reduced to almost one third in comparison to the WT (Figure 2.2). Cell number of both subpopulations of mature T cells (CD4+ and CD8+) contributed to this difference. Comparison of percentages of different T cell populations in the KO and WT spleen shows slightly reduced percentage of both major subpopulations (CD4+ and CD8+) in PI3Kγ KO as well. These results are in slight discrepancy with previous finding by Sasaki *et al*. (2000), who observed only reduction of CD4+ T cells in the spleen.

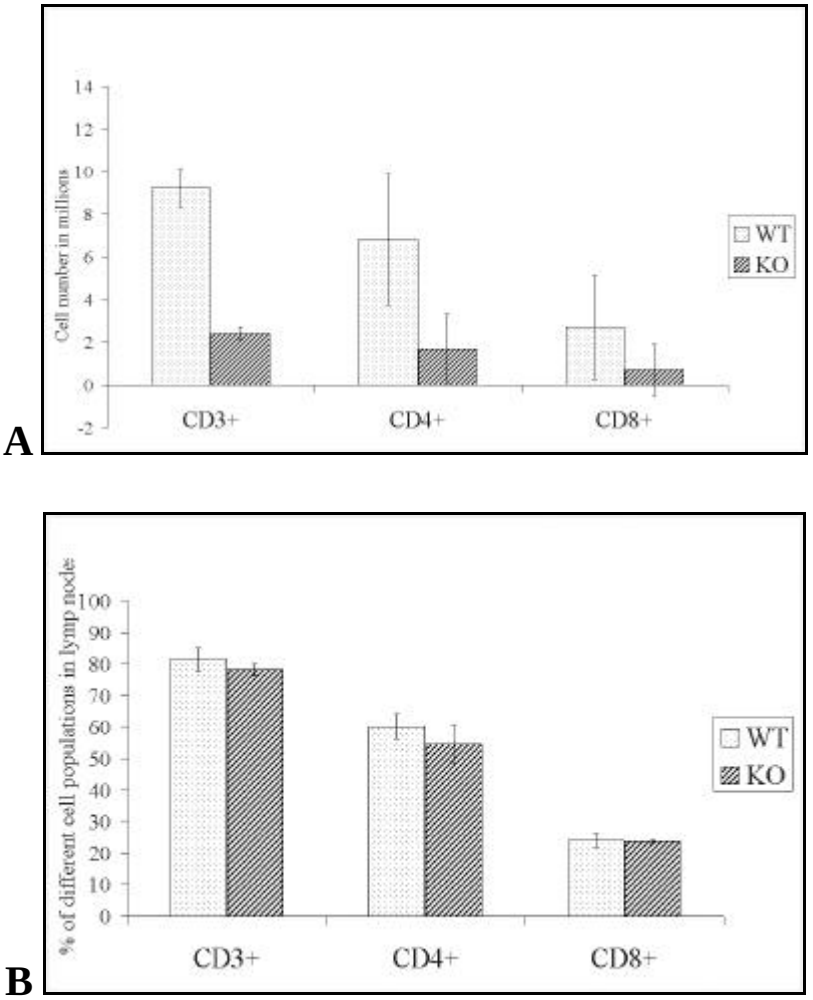


Figure 2.3. A) The total number of all T cell (CD3+), CD8+ and CD4+ T cells in lymph nodes, cell number in millions. **B) The ratio of all T cell (CD3+), CD8+ and CD4+ T cells in lymph nodes**, results given as percentage of all cells from the lymph nodes. Analysis performed by flow cytometry upon staining with specific antibodies; subpopulations of the T cells were positively identified as cells positive for double staining (CD4+/βTCR or CD8+/βTCR).

The cell number reduction in the PI3Kγ KO lymph nodes was severe – WT animals had nearly four-fold more T cells (Figure 2.3). The ratio between of CD4+ and CD8+ T cells in the lymph nodes of PI3Kγ KO mice showed nearly no differences to the WT. Again, such effect was not described by Sasaki *et al*. (2000).

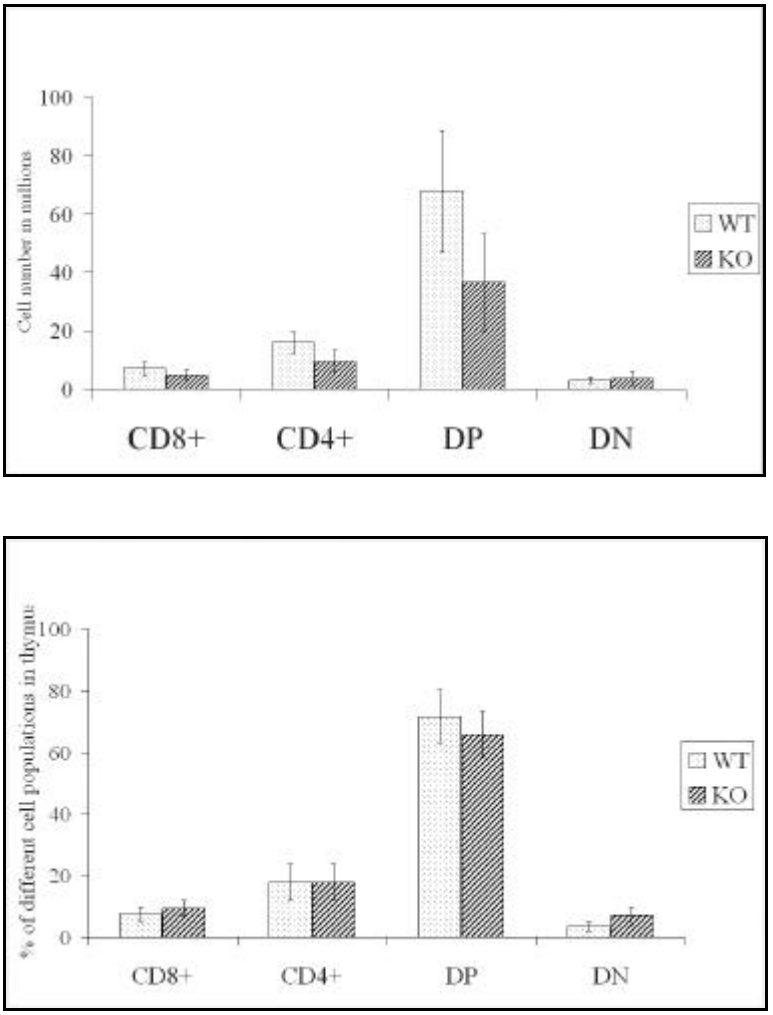


Figure 2.4. A) The total number of single positive (CD8+ and CD4+), double positive (DP, CD4+/CD8+) and double negative (DN, CD4-/CD8-) thymocytes, cell numbers in million. **B) The ratio of single positive, double positive and double negative thymocytes**, results given as percentage of all thymocytes. Analysis performed by flow cytometry upon staining with specific antibodies.

The total number of thymocytes in KO was nearly half of the number of WT thymocytes and this difference was mostly caused by reduction of double-positive cells (Figure 2.4) which, by default, represent the major subpopulations of thymocytes. Small

reduction in number of single-positive cells could also be observed. There were no striking differences in the ratio of major thymocyte subpopulations (CD4+, CD8+, double positive – DP and double negative – DN) in WT and KO. A relatively small decrease in percentage of DP and a small increase in percentage of DN and CD8+ thymocytes were observed.

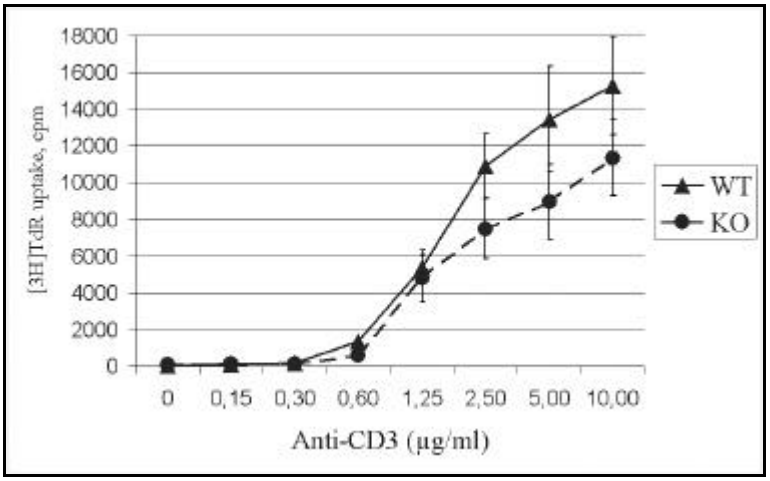
Our results about the number of PI3Kγ$^{-/-}$ thymocytes proved involvement of PI3Kγ in positive regulation of thymic growth and T cell differentiation in thymus and in many regards confirmed earlier findings by Sasaki *et al*. (2000) and Rodrigues-Bolardo *et al*. (2003). In contrast to these earlier findings, we observed stronger reduction of mature T cells number in all peripheral lymphoid organs of PI3Kγ KO mice, and both major subpopulations of mature T cells were affected. This appears to be a consequence of impaired development and differentiation of PI3Kγ$^{-/-}$ thymocytes.

Additional flow cytometry analyses included number/ratio of cells positive for γδTCR, CD44, CD45RB, CD62L and CD25 (data not shown). With exception of the reduced total number in peripheral lymphoid organs, ratio of different subpopulations of resting mature PI3Kγ$^{-/-}$ T cells did not exhibit any significant difference in comparison to the WT T cells.

2.2. Activation and proliferation of PI3Kg$^{-/-}$ T cells

Further more, we investigated proliferation of PI3Kγ$^{-/-}$ T cells purified from the spleen by negative depletion. At first, in order to examine the ability of PI3Kγ$^{-/-}$ T cell to proliferate, the cells were activated by different mitogens. The proliferation was measured as a [^{3}H]thymidine uptake 72 h upon the activation. A smaller number of samples (from two KO and two WT mice) were activated with phytohemagglutinin (PHA), lipopolysaccharides (LPS), Concavalin A (Con A) and Ionomycin + phorbol ester (IONO+TPA). These substances are commonly used as positive controls for the activation of lymphocytes because of their wide-range none-specific activating effect. These experiments did not show any significant differences in proliferation between PI3Kγ$^{-/-}$ and WT T cells (data not shown).

TCR-induced (anti-CD3-induced) proliferation was also performed. PI3K$\gamma^{-/-}$ T cells exhibited approximately 30% reduced anti-CD3-induced proliferation (Figure 2.5.A), which represents a weaker reduction than observed by Sasaki *et al*. (2000). We also confirmed previous findings by the same authors that impaired proliferation of PI3K$\gamma^{-/-}$ T cells can be rescued by anti-CD28 costimulation (data not shown).



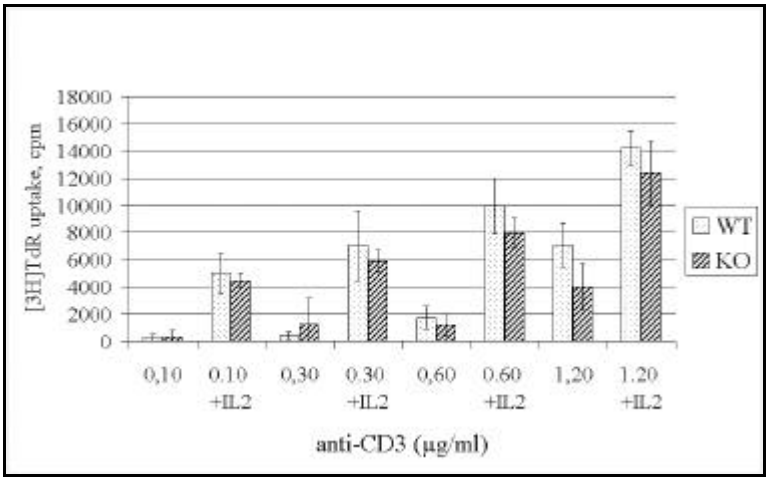


Figure 2.5. A) TCR-induced proliferation of T cells purified from spleen. B) TCR-induced proliferation of T-cells purified from spleen, +/- IL-2 costimulation (5 ng/ml of human recombinant IL-2 where indicated). The activation of T cells was done with anti-CD3 antibody immobilised on the solid surface using indicated concentrations. Proliferation was measured by [^{3}H]-thymidine uptake and results are given as counts per minute (cpm). The results represent readouts from one of the two independent experiments which show the same trends. In both types of experiments samples from at least 3 WT and 3 KO animals were used.

In order to elucidate if the reduced cytokine production upon activation can be the cause of impaired anti-CD3-induced proliferation of PI3Kγ$^{-/-}$ T cells, we activated T cells from the KO and WT mice with and without IL-2 costimulation. Indeed, recombinant IL-2 enhanced anti-CD3-induced proliferation both in KO and WT T cells and rescued proliferation of PI3Kγ$^{-/-}$ T cells (Figure 2.5B).

Proliferation of PI3Kγ$^{-/-}$ T cells activated with different mitogens was comparable with proliferation of WT T lymphocytes, while PI3Kγ$^{-/-}$ T cells activated trough TCR exhibited reduced proliferation. This clearly indicated involvement of PI3Kγ in some of the signalling pathways emerging from activated TCR. Moreover, IL-2 costimulation was able to rescue impaired proliferation of PI3Kγ$^{-/-}$ T cells. Considering that reduced cytokine production of KO T lymphocytes upon TCR-induced activation is caused by the absence of PI3Kγ (Sasaki *et al*., 2000), our results implicate that the lack of cytokines, especially IL-2, may be the major cause of PI3Kγ$^{-/-}$ T cell functional defect.

2.3. Inhibitory potential and selectivity of AS041164, a specific inhibitor of PI3Kg

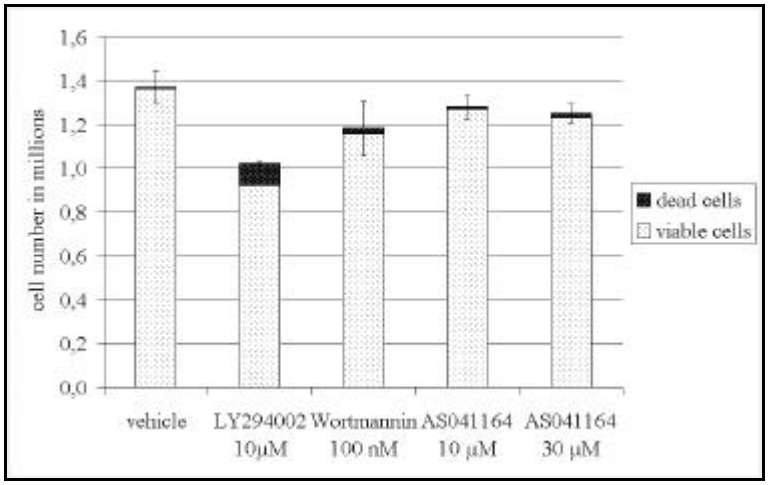
In order to elucidate how PI3Kγ couples the activation of TCR and IL-2 production we took the opportunity to use the PI3Kγ specific inhibitor AS041164. This substance is one from the series of complex compounds developed by SERONO (Geneva, Switzerland) and it was kindly provided by this company. The AS041164 binds non-covalently and reversibly to PI3Kγ ATP-binding site and blocks its kinase activity. The substance is a derivate of hiadolizine-2,4-dione with molecular weight of 249,24. The AS041164, as well as other substances from the same series, does not show notable activity against a wide panel of protein kinases at low µM concentration. The AS041164 does not exhibit high selectivity: *in vitro* it shows nearly four times higher affinity to bind to PI3Kγ than PI3Kα (unpublished

data, SERONO Company). In our experiments the AS041164 was applied in the range of concentration between 3 µM and 30 µM according to the manufacturer's recommendations.

Possible cytotoxicity of AS041164 was tested in our system. We used Jurkat cell line as the most commonly used model of human T cells. The cells were grown in the standard conditions and the impact of AS041164 on the cell proliferation and viability was examined after 24 and 48 hours of treatment. Standard concentrations of Wortmannin (100 nM) and LY294002 (10 µM) were used as control treatments. Given the fact that the chemical stability of AS041164 under these conditions was unknown, 24 hours after starting the test the same amount of all inhibitors and vehicle was added to the probes for 48 h treatment. To avoid maximum cell density before the measurement, the initial number of cells in samples for 48 h treatment was reduced by 50%. The cells were counted with Beckman-Coulter Z2 particle counter and the cell samples were stained with propidium-iodide (PI) and fluorescein diacetate (FDA) prior to flow cytometry analysis. We made an assumption that the ratio of metabolically active cells (FDA positive) could not significantly differ from the percentage of viable cells under this conditions. Indeed, there was less then 1% of the cells that were double negative or double positive for PI and FDA staining.

Results in Figure 2.6. show that 24 h treatment of Jurkat cells with 10 µM AS041164 insignificantly reduces the cell number and has practically no impact on the cell viability. 30 µM AS041164 after 24 h showed slightly stronger reduction of the cell number but the margins of error were still close to those of mock-treated cells. 48 h treatment with both concentrations of AS041164 showed insignificant difference to the mock-treated cells. The relatively small cytotoxicity of Wortmannin, especially after 24 h treatment, can be explained by its documented chemical instability. Presented data confirmed preliminary results obtained with Neubauer counting chamber and tripan-blue exclusion (data not shown). The results pointed to the two conclusions: that AS041164 showed very low, practically insignificant cytotoxicity under described conditions, and also that AS041164 was differing from

Wortmannin and LY294002 under given conditions regarding the impact on the proliferation and viability of Jurkat cells.



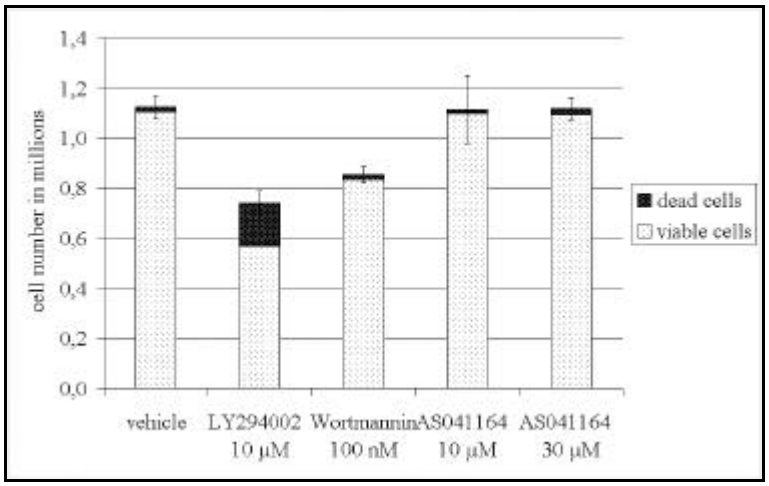


Figure 2.6. The number and viability of Jurkat cells after 24 h (A) and 48 h (B) of treatment with different PI3K inhibitors; used concentrations are indicated. 24 h after the commencement of the test, the same amount of all inhibitors and vehicle was added to the probes for 48 h treatment as the chemical stability of AS041164 under this conditions was unknown. Cells were counted by Beckman-Coulter Z2 particle counter, and the analysis was performed by flow cytometry; PI positive cells were accounted as dead and FDA positive cells were considered as viable.

In order to elucidate the inhibitory potential and selectivity of AS041164, several simple inhibitory analyses were performed. The major aim of this experiment was to compare possible inhibitory effects of AS041164 with the effects of wortmannin and LY294002 in Jurkat cells. We made an assumption that the level of activation/phosphorylation of Akt/PKB

could be considered as a reflection of intracellular PtdIns(3,4,5)P_3 level, or indirectly as a reflection of overall PI3K activity. Jurkat cells are exceptional in this regard because they do not express (or at least do not express functional) PTEN and SHIP, and their basal level of PtdIns(3,4,5)P_3 is constantly high, but can be further enhanced by various kinds of stimuli. We therefore examined the efficiency of wortmannin- and LY294002-induced inhibition of Akt/PKB phosphorylation in Jurkat cells under different conditions.

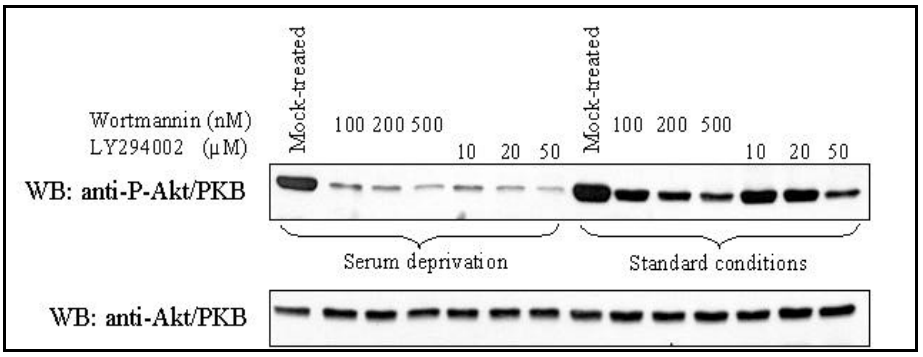


Figure 2.7. Inhibition of Akt/PKB activation in Jurkat cells by wortmannin and LY294002. Serum deprived and Jurkat cells maintained in standard conditions were incubated with indicated concentration of PI3K inhibitors for 30 minutes. The cells were lysed, and protein lysat was resolved on 10% SDS-PAGE followed by immuno-blotting with antibody specific for activated Akt/PKB. Western blot for Akt/PKB obtained from the same membrane after the stripping represents loading control.

Standard concentrations, two-fold and five-fold higher concentration of both inhibitors were applied to the serum-deprived cells and to the cells in the standard conditions. Both substances show clear, dose-dependent and rapid inhibition of PI3Ks in Jurkat cells. This effect is significantly enhanced and/or accelerated by serum-deprivation (Figure 2.7.).

The next step was to compare the effect of AS041164 and wortmannin in a short time-course treatment with serum-deprivation and in the standard conditions (Figure 2.8.). AS041164 was apparently a weak inhibitor of Akt/PKB activation and it seemed to have a small impact on overall PI3K kinase activity, especially in the standard conditions.

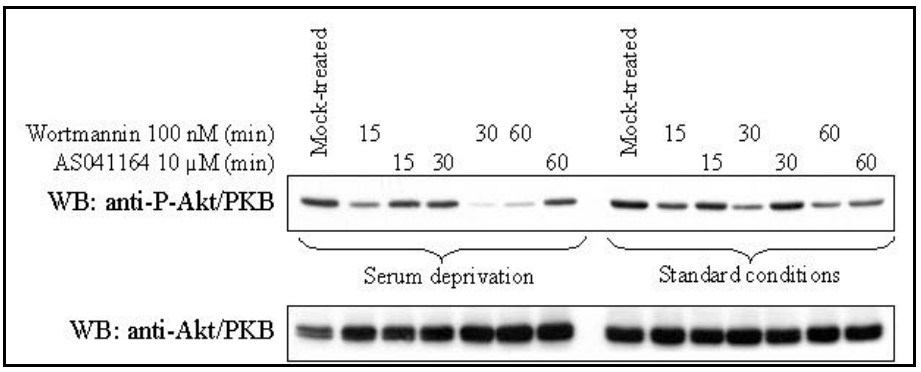


Figure 2.8. Inhibition of Akt/PKB activation in Jurkat cells by wortmannin and AS041164. Serum deprived and Jurkat cells maintained in the standard conditions were incubated with the indicated concentration of inhibitors during a time-course. The cells were lysed at indicated time points, and protein lysat was resolved on 10% SDS-PAGE followed by immunoblotting with antibody specific for activated Akt/PKB. Western blot for Akt/PKB obtained from the same membrane after the stripping represents loading control.

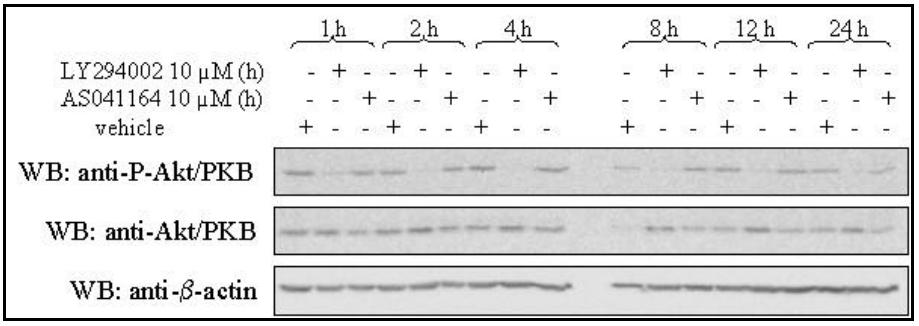


Figure 2.9. Inhibition of Akt/PKB activation in Jurkat cells by LY294002 and AS041164 during 24 hors time-course. Jurkat cells maintained in the standard conditions were incubated with indicated concentration of inhibitors during a 24 h time-course. The cell samples were lysed at indicated time points, the protein lysat was resolved on 10% SDS-PAGE followed by immuno-blotting with antibody specific for activated Akt/PKB. Western blots for Akt/PKB and β-actin, obtained from the same membrane after the stripping, represent loading controls.

The inhibitory effect of AS041164 on Akt/PKB activation was also compared to the effect of LY294002 during a 24 h time-course treatment of Jurkat cells in the standard conditions (Figure 2.9.). These results again showed that PI3Kγ specific inhibitor was

differing from LY294002 and that it had minor effect on overall PI3K enzymatic activity under given conditions.

It was proved that activation of G-protein coupled receptors (GPCR) by lisophosphatidic acid (LPA) also induces activation of PI3Kγ and bifurcation of its lipid- and protein-kinase activity which leads to activation of Akt/PKB and MAPK, respectively (Bondeva *et al*., 1998). Thus, we decided to test the ability of AS041164 to inhibit Akt/PKB and Erk 1/2 activation with and without LPA stimulation in Jurkat cells. As shown in Figure 2.10., AS041164 is a week inhibitor of Akt/PKB activation in comparison to wortmannin, both before and after LPA stimulation. On the other side, AS041164 appears to be a relatively good inhibitor of LPA-induced activation of Erk under the same conditions.

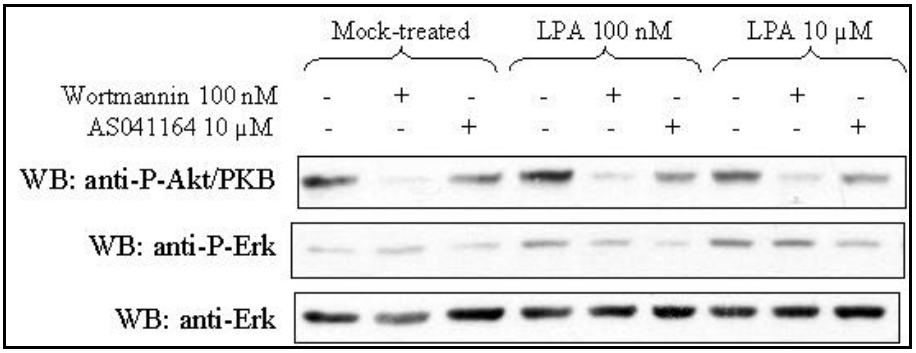


Figure 2.10. Inhibition of Akt/PKB and Erk activation in Jurkat cells by wortmannin and AS041164 +/- LPA stimulation. Serum-deprived cells were treated with indicated concentrations of inhibitors 30 minutes prior to stimulation, stimulated with indicated concentrations of LPA for 10 minutes and lysed. Protein lysat was resolved on 10% SDS-PAGE. Immuno-blotting was done with specific antibodies for activated Akt/PKB and activated Erk 1/2. Western blots against Erk 1/2 obtained from the same membrane after the stripping represent loading control.

Our results confirmed that wortmannin and LY294002 perform strong, rapid and dose-dependent inhibition of PI3Ks in Jurkat cells, which was enhanced by serum deprivation. On the other side, AS041164, unlike wortmannin and LY294002, performed a week inhibition of Akt/PKB activation in serum-deprived Jurkat cells. The same substance had undetectable influence on overall PI3K enzymatic activity of Jurkat cells kept in the standard conditions

and no significant cytotoxicity within the applied range of concentrations. This data, along with the information provided by the manufacturer, confirmed that AS041164 could be considered and used as PI3Kγ specific inhibitor in our model system.

2.4. PI3Kg specific inhibitor AS041164 reduces IL-2 production upon anti-CD3-iducet activation of Jurkat cells

The next aim was to examine whether the application of AS041164 on anti-CD3-activated Jurkat cells can reduce IL-2 production and in this way reproduce some of the features of PI3K$\gamma^{-/-}$ T cells. Mouse monoclonal antibody against human CD3 complex (clone OKT3) coupled to the solid surface via goat anti-mouse IgG (GAMI) was used for the cell activation. Treatment with Ionomycin (IONO) and phorbol ester (TPA) was used as a positive control for the activation, and the treatment of activated cells with 10 µM LY294002 was taken as a confirmative control of PI3Ks inhibition. The cells were kept in the standard conditions during the whole experiment. The inhibitors were applied 15-20 minutes prior to the activation, and the treatment took 24 hours. IL-2 concentration in cell culture supernatant was measured by ELISA. As shown in Figure 2.11., AS041164 reduced IL-2 production of activated Jurkat cells in a concentration dependent manner, unlike 10 µM LY294002, which completely blocked anti-CD3-induced IL-2 production. We could not exclude the possibility that the activated Jurkat cells were more susceptible to a possible cytotoxic effect of AS041164, so reduced IL-2 production may have been caused by a reduced number and/or viability of the activated cells. The additional viability tests with PI and FDA staining were therefore performed with the same samples (Figure 2.12.).

Our results showed that AS041164 reduced IL-2 production in anti-CD3-activated Jurkat cells in a dose-dependent manner. The reduction could not be justified by reduced cell viability as a consequence of treatment with the inhibitor. Diminished cell activation or disrupted signalling downstream from TCR caused by AS041164 treatment is more likely to

be the reason for this reduction. Moreover, the kinase activity and not only the presence of PI3Kγ appeared to be important for TCR-induced IL-2 production in Jurkat cells.

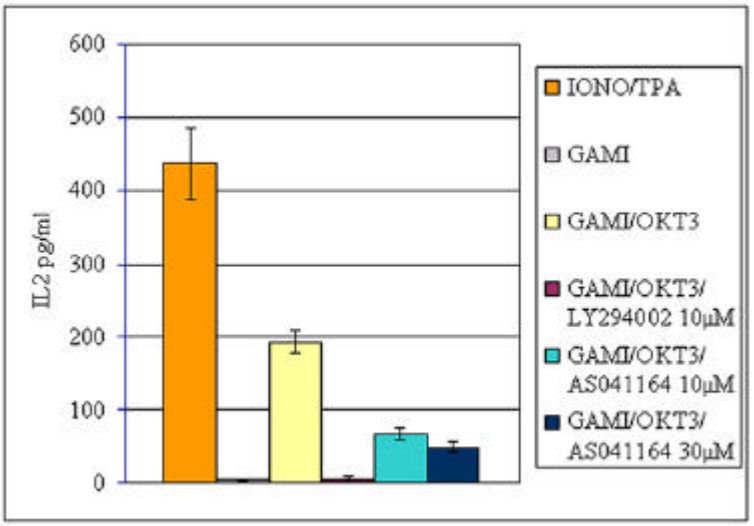


Figure 2.11. **IL-2 production upon activation of Jurkat cells**. Cells were activated by mouse monoclonal anti-CD3 antibody (clone OKT3) immobilised to the solid surface via goat anti-mouse IgG (GAMI) or by IONO/TPA as positive control. Cells were treated with indicated concentrations of inhibitors. Samples were taken 24 h after the activation, and IL-2 concentration in the cell culture supernatant was measured by ELISA.

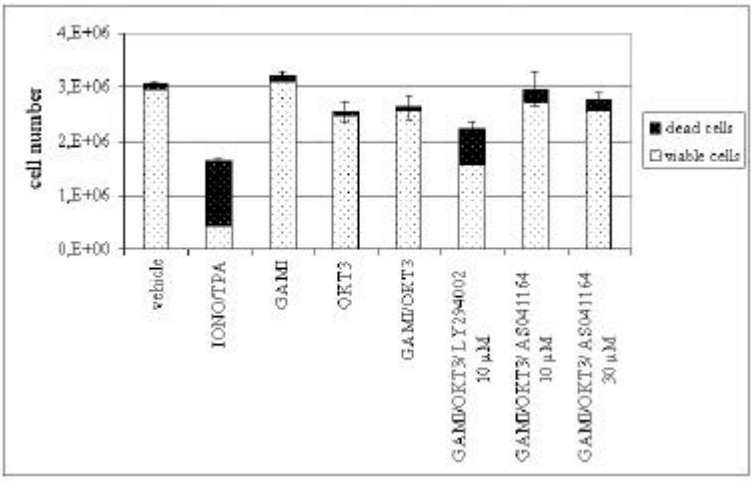


Figure 2.12. Cell number and the viability of Jurkat cells 24 h after the activation. Analysis performed by flow cytometry after PI and FDA staining.

2.5. The impact of PI3Kγ specific inhibitor AS041164 on some signalling pathways related to the activation of TCR in Jurkat cells

Presented data along with previously known facts, clearly implicated that PI3Kγ is involved in activation-induced IL-2 production in T cells. We therefore investigated the possible impact of AS041164 on some of TCR-induced signalling pathways in Jurkat cells. TCR-induced activation of PI3Ks is well documented, but the mechanism of this activation is not completely elucidated, and several proposed models are exclusively related to PI3K class I_A enzymes (reviewed by Okkenhaug and Vanhaesebroeck, 2003b). Wortmannin and LY294002 generally inhibit activation of primary T cell but they are considered to be relatively ineffective in Jurkat cells (reviewed by Kane and Weiss, 2003). These discrepancies regarding the inhibitory impact of those two substances on Jurkat cells activation are mostly related to CD28 co-stimulation (Crooks *at al*., 1995). However, in our experiments we did not use any type of costimulation parallel with CD3 stimulation. Our earlier results proved that wortmannin and LY294002 perform rather strong and consistent PI3K inhibition under the conditions we used for the following experiments (serum deprived Jurkat cells, the inhibitor was applied 30 minutes prior to the stimulation). Mouse monoclonal antibody against TCR, clone C305 (an antibody which reacted with idiotypic-like determinants expressed on Jurkat cells; Weiss *et al*., 1984) was used for the activation. Soluble C305 antibody binds and activates TCR of Jurkat cells and induces signalling processes downstream and in the proximity of TCR, but it does not cross-link the receptors and does not induce full-scale activation of the cells. Nonetheless, it is a useful and commonly used tool for investigation of the initial steps of TCR-induced T cell activation.

Activation of several relevant signalling proteins was investigated in parallel: Akt/PKB activation as one of the major target of PI3K enzymatic activity, and activation of three parallel MAPK pathways (represented by Erk 1/2, p38 and SAPK/JNK) upon TCR-induced activation.

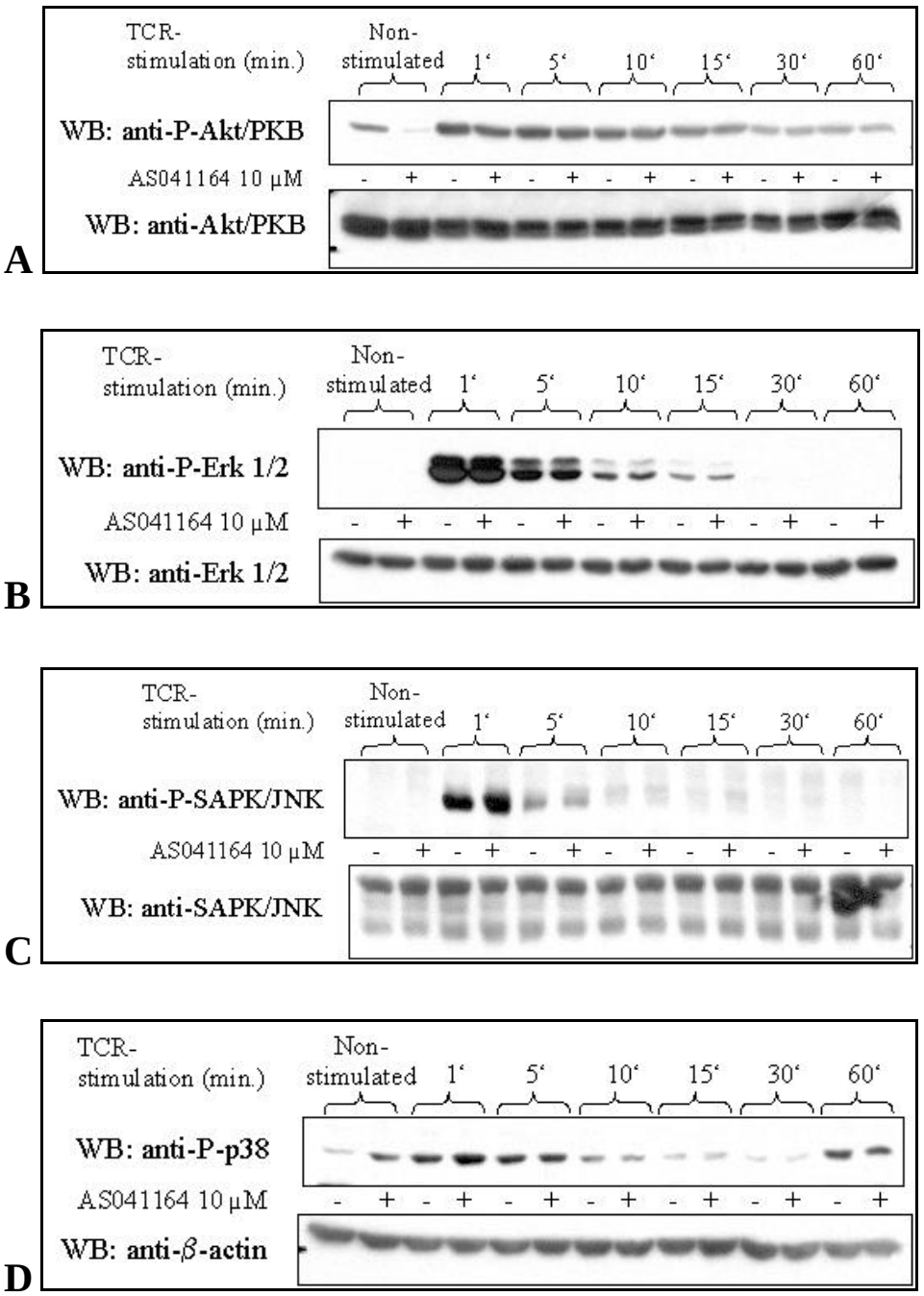


Figure 2.13. The effect of 10 µM AS41164 on A) Akt/PKB activation, B) Erk 1/2 activation, C) SAPK/JNK activation and D) p38 activation upon TCR stimulation of Jurkat cells with C-305 monoclonal antibody. The stimulation was stopped at indicated time-points, cells were lysed and total cell lysat was resolved on 10% SDS-PAGE followed by immunoblotting with specific antibodies. Second western blots are obtained from the same membranes after the stripping and represent corresponding loading controls.

Results presented in Figure 2.13. were obtained with 10 µM AS041164. This concentration was approximately at the middle of recommended range. In all our experiments

where this concentration induced clear alterations of read-outs we could not detect any unspecific inhibition. 10 µM AS041164 reduced Akt/PKB activation in serum-deprived none-stimulated Jurkat cells (Figure 2.13.A), but did not have any detectable impact on Akt/PKB activation upon TCR activation. Moreover, there was not any detectable impact of the same substance on Erk 1/2 (Figure 2.13.B) and SAPK/JNK (Figure 2.13.C) phosphorylation. Interestingly, the treatment of Jurkat cells with 10 µM AS041164 slightly enhanced p38 activation, both in none-stimulated cells and at least at the beginning of the TCR-stimulation (Figure 2.13.D). The significance of this result remains unclear. The pattern of tyrosine phosphorylation of proteins typical for TCR activation was examined as well (data not shown). Again, there were no detectable changes after the treatment of activated Jurkat cells with the inhibitor.

In conclusion, AS041164 did not exhibit any significant changes in the activation of Akt/PKB and MAPK in activated Jurkat cells, implicating that PI3Kγ may not be directly involved in the regulation of these pathways upon T cell activation, or at least, PI3Kγ may not be involved in the initial, acute phase of TCR-induced activation of Jurkat cells.

2.6. PI3Kg interacts with different PKC isoforms in Jurkat cells

Our next goal was to try to identify interacting partners of PI3Kγ in Jurkat cells, especially the proteins whose interaction with PI3Kγ appears to be regulated by TCR signalling. Jurkat cells were stimulated with anti-TCR mouse monoclonal antibody C305 for 10 minutes. Anti-PI3Kγ immunoprecipitation was performed, the samples with corresponding controls were resolved on SDS-PAGE and the gels were silver-stained (Figure 2.14.). Particular bands, which seemed specific and altered by the stimulation were isolated, digested by tripsin and the peptide fragment samples were analysed and identified by mass-spectrometry (MS). The most important finding of those analyses was identification of several

isoforms of protein kinase C (PKC). The results were confirmed by several independent immunoblotting experiments.

We confirmed that a PI3Kγ in Jurkat cell constitutively interacts with four isoforms of PKC. The amount of those coprecipitated proteins, and possibly the strength of interaction declines in a range PKCα>PKCβ1>PKCβ2>PKCζ. The signal coming from coprecipitated PKCζ was much weaker then signals from other isoforms, slightly above the level of background and it should be therefore carefully evaluated. Any similar interactions between PI3Kγ and PKCε, PKCδ, PKCθ and PKCη isoforms were not detectable. This was an additional proof that registered signals were not artefacts coming from unspecific binding of PKCs to PI3Kγ, to the antibody or to the G-protein sepharose beads.

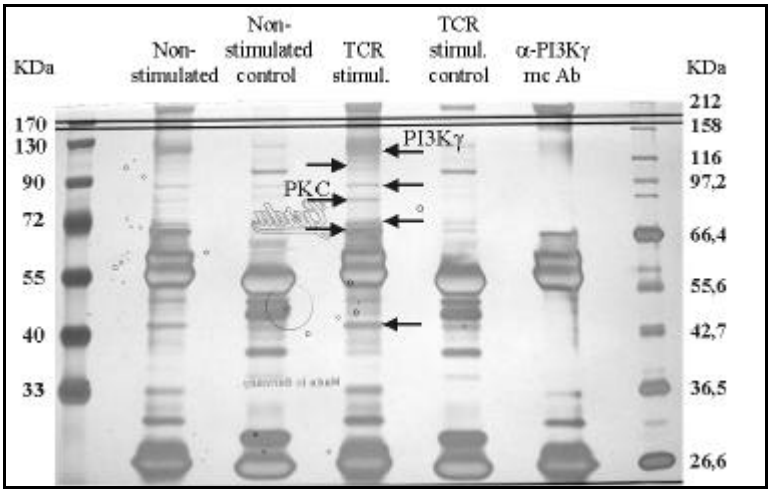


Figure 2.14. Non-stimulated Jurkat cells or cells stimulated with anti-TCR antibody C305 for 10 minutes were lysed and the anti-PI3Kγ immunoprecipitation was performed with specific mouse monoclonal antibody, clone 641. The samples and corresponding controls were resolved on 10% SDS-PAGE and the gel was stained with silver. Bends representing potential specific interacting partners of PI3Kγ were extracted from the gel and analysed by MS. The arrows point to some of the analysed samples. The samples identified as PKC and PI3Kγ (as positive control) are indicated. The image presents one of four such gels from independent experiments.

We tried to elucidate the nature of this interaction by performing an inhibitory analysis. Four different inhibitors have been used: LY294002 as strong, unspecific inhibitor

of all PI3Ks, AS041164 as specific PI3Kγ inhibitor and Bisindolylmaleimide I and Gö 6976 as two different, but well documented inhibitors of PKCs (Figure 2.15). Non-stimulated Jurkat cells were treated with the inhibitors and anti-PI3Kγ immunoprecipitation was performed. Immunoblotting was made with antibodies specific for eight aforementioned PKC isoforms .

LY294002 almost completely abolished interaction between PI3Kγ and four interacting PKC isoforms indicating that these interactions were highly correlated with Akt/PKB (and probably PDK1) activation and seemed likely to be PtdIns(3,4,5)P_3-dependent. AS041164 also reduced these interactions but to a lesser extent in comparison to LY294002 (Figure 2.15.). That was an indication that these interactions may not be fully dependent on kinase activity of PI3Kγ; it seemed more likely that PI3Kγ kinase activity enhanced these already existing, constitutive interactions in Jurkat cells. Surprisingly, the inhibition of all four PKC isoforms by both PKC inhibitors enhanced the amount of coprecipitated PKC isoforms, meaning that PKC inhibition actually increased the interactions between PI3Kγ and PKCs.

The attempts to coprecipitate any PKC isoforms by PI3Kγ immunoprecipitation from resting human CD4 T cells isolated from peripheral blood gave no results (data not shown). It was already mentioned that Jurkat cells have extremely high level of PtdIns(3,4,5)P_3 due to the fact that PTEN and SHIP in these cells are mutated and dysfunctional. Hence, the results obtained from human CD4 T cells support an assumption that those interactions are indeed PtdIns(3,4,5)P_3-dependent. Consistent with this is the fact that anti-CD3 activation of Jurkat cells further elevated the level of PtdIns(3,4,5)P_3 and enhanced the Akt/PKB activation, but also increased the interaction of PI3Kγ and PKCβ1 and PKCβ1 (Figure 2.15.D).

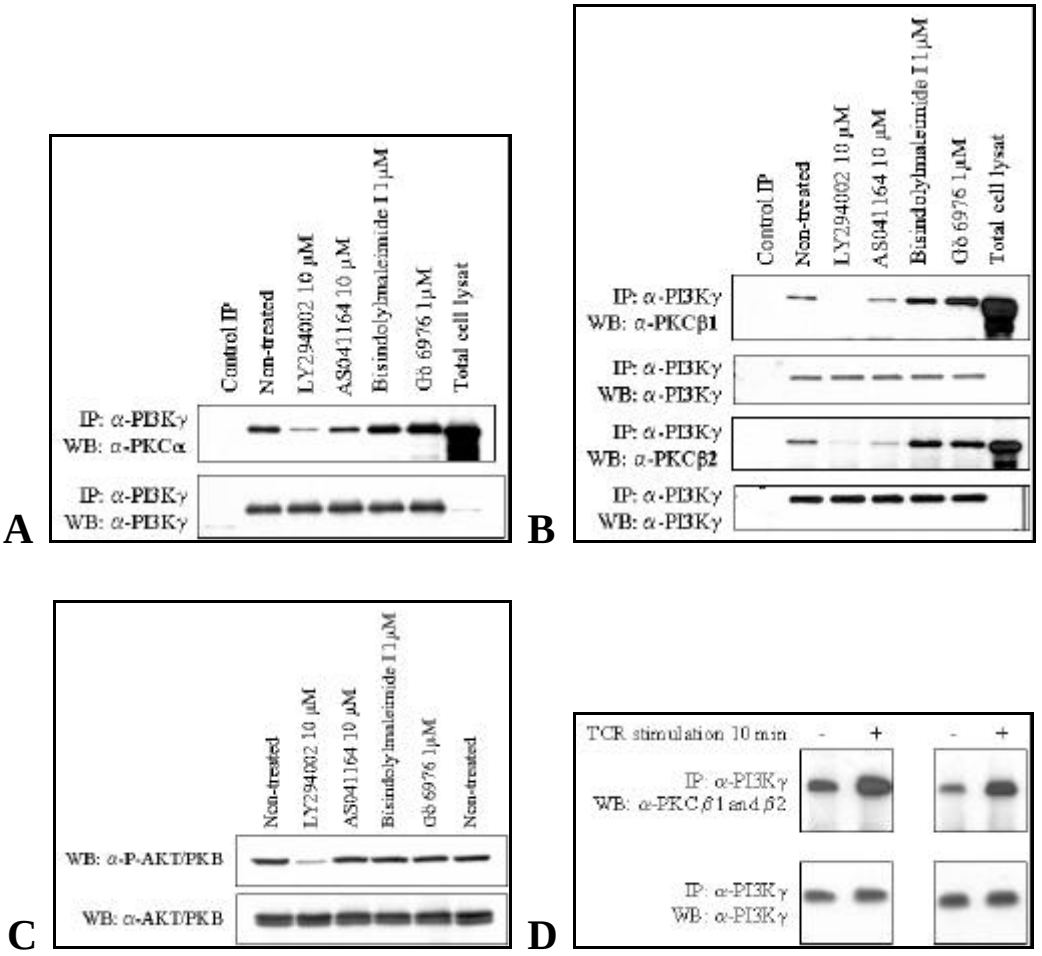


Figure 2.15. A) Co-immunoprecipitation of PKCa. B) Co-immunoprecipitation of PKCb1 and PKCb2. Jurkat cells were kept in standard conditions, treated with indicated concentrations of inhibitors for 30 minutes, lysed and anti-PI3Kγ immunoprecipitation was performed. **C) The impact of the same inhibitors on phosphorylation of Akt/PKB. D) Co-immunoprecipitation of PKCb1 and PKCb2 after the stimulation of Jurkat cells TCR**. Cells were serum-deprived, stimulated with C305 antibody for 10 minutes, lysed and α-PI3Kγ immunoprecipitation was performed. Corresponding loading controls were obtained with the same membranes after the stripping.

On the western blot images presented in Figure 2.15A and -B, it can be visualised that the signals for PKCs coming from the total cell lysat were much stronger then the signals coming from the immunoprecipitation samples. The amount of the total cell lysat loaded on the gel was roughly 10% of the lysat used for the immunoprecipitation. Concerning the fact that PKCα, PKCβ1 and PKCβ2 are abundant cytosolik molecules, this may suggests several

explanations which do not necessarily exclude each other: first, a relatively small portion of total PKCs interacts with PI3Kγ under given conditions; second, these interactions may be rapid and transient; third, the interactions may be mediated *via* one or more other molecules; and fourth, stechiometry of this interaction may be in favour of PI3Kγ.

The newly discovered interaction between two signalling proteins, especially between two kinases, always brings the question if this is an enzyme-substrate interaction. Several attempts with different conditions and stimulations, followed by immunoprecipitations and *in vitro* protein-kinase assays, did not confirm this assumption - no transphosphorylation between PI3Kγ and any of PKC isoforms was detected (data not shown). It should be taken into consideration that similar interactions have been registered in a different model, where certain results implicated that PI3Kγ can be the substrate for of PKCs (personal communication to Prof. Matthias Wymann, University of Basel, unpublished data).

Taken all together, PI3Kγ is in constitutive interactions with several PKC isoforms (PKCα, PKCβ1, PKCβ2 and probably PKCζ) in Jurkat cells. These interactions seem to be PtdIns(3,4,5)P_3-dependent and their constitutive nature is apparently specific for Jurkat cells. Described protein-protein interactions were enhanced by TCR activation, and surprisingly, by PKC inhibition. Moreover, these interactions were facilitated by the kinase activity of PI3Kγ, but without evidence that interactions between PI3Kγ and PKC isoforms were kinase-substrate interactions.

2.7. PKC inhibitor and PI3Kg specific inhibitor reduce TCR activation-induced IL-2 production of Jurkat cell in a similar manner

The activation of T cells with Ionomycin (IONO) and phorbol-ester (TPA) is commonly used *in vitro* as a positive control for lymphocytes activation and cytokine production. Treatment with Ionomycin and TPA completely by-passes all cell-surface receptors including TCR, boosts intracellular calcium influx and activates the members of

protein kinase C family beside other signalling proteins, respectively. It is known that TCR mediated Ca^{2+} flux of PI3K$\gamma^{-/-}$ T lymphocytes is comparable with the WT T cells, ergo observed differences in T cell activation and cytokine production cannot be explained by differences in Ca^{2+} flux (Sasaki *et al.*, 2000). We therefore examined if there may be any correlation in inhibition of PI3Kγ and PKCs related to the reduced IL-2 production.

The additional samples in our cell activation and IL-2 production assay were included: IONO/TPA activation inhibited with 30 µM AS041164 and 1 µM Bisindolylmaleimide I, and also, anti-CD3 activation inhibited with three different concentrations of Bisindolylmaleimide I and AS041164 (Figure 2.16.). It is known that the PKCs inhibitors diminish both TCR-induced and IONO/TPA-induced activation and cytokine production. Although 1 µM Bisindolylmaleimide I represents relative excess of the inhibitor, the aim for using this concentration was to obtain full PKC inhibition and clear-cut differences. The cells were activated with immobilised OKT3 antibody as described for the previous experiments.

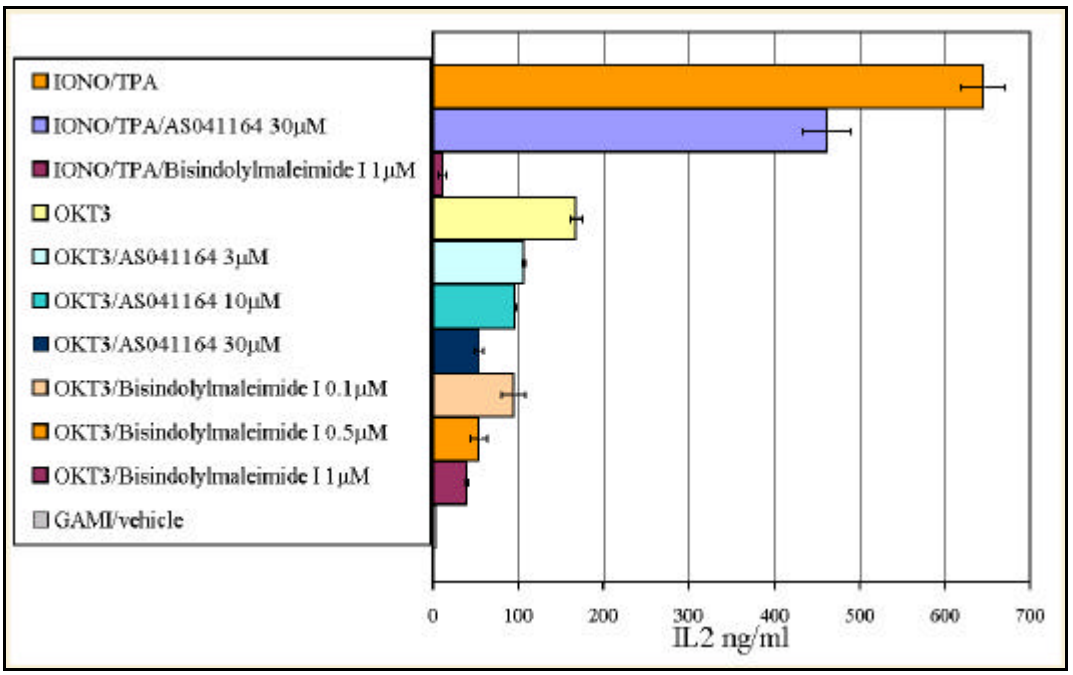


Figure 2.16. The inhibition of PI3Kg and PKCs reduces the IL-2 production of activated Jurkat cells. The cells were treated with indicated concentrations of inhibitors during 24 hours of activation in standard conditions. The IL-2 concentration in the cell supernatant was measured by ELISA.

1 µM Bisindolylmaleimide I blocked IONO/TPA-induced IL-2 production while 30 µM AS041164 reduced it for about 30%. Interestingly, the reduction of anti-CD3-induced IL-2 production in a concentration-dependent manner showed similar pattern for both (AS041164 and Bisindolylmaleimide I) inhibitors. In this experiment, as well as in all other performed experiments, AS041164 showed a consistently stronger inhibition of CD3-induced IL-2 production than IONO/TPA-induced IL-2 production. On the other side, Bisindolylmaleimide I showed the opposite trend in the inhibition efficiency regarding the two different types of the cell activation.

2.8. PI3Kg regulates IL-2 expression of activated Jurkat cells on different levels

The results presented above indicated that PI3Kγ involvement in the control of IL-2 expression upon the activation of Jurkat cells was related to the role of different PKC isoforms, and this involvement probably might take place on different levels, e.g. transcriptional, post-translational or secretion levels. We therefore decided to perform additional experiments and IL-2 assay in order to focus on the two different but closely related issues: the expression of early activation markers on the cell surface as one of first results of altered transcription, and the level of IL-2 transcription and secretion as one of the ultimate outcomes of T cell activation. First of all, extra samples were included and new IL-2 assay was performed after 24 hour treatment (see Figure 2.17.). The cells from the samples were used for the analysis of the activation by flow cytometry.

CD69 is a type II integral membrane protein and it is a phosphorylated, disulfide linked 27/33 kDa homodimer composed of differentially glycosylated subunits with an extracellular C-type lectin domain. The CD69 is considered as a very early marker of lymphocyte activation and it is commonly used as a parameter of T cell activation. We measured the percentage of activated Jurkat cells as ratio of viable (PI negative) and CD69+ cells, 24 h after the stimulation (Figure 2.17.). 1 µM Bisindolylmaleimide diminished

IONO/TPA-induced activation of Jurkat cell. This is to be expected if one of the major targets of activation and the only target of inhibition are PKCs. The inhibitory effect of 10 µM LY294002 was much weaker in this case, while 10 µM and 30 µM AS041164 did not show any reduction of CD69+ cells. The CD3-induced CD69 expression was reduced to a similar extent both by 1 µM Bisindolylmaleimide and 10 µM LY294002, while 10µM AS041164 again did not show any influence on CD69 expression. Considering that all observed effects of AS041164 were clearly dose-dependent and highly reproducible, it is very unlikely that 3µM AS041164 would have any influence on the percentage of CD69+ cells in these experiments. On the other side, in experiments with CD3-iduced activation 30 µM AS041164 performed inhibition comparable to the effects of Bisindolylmaleimide I and LY294002, raising the question about the selectivity of the inhibitor at this particular concentration under given conditions (Figure 2.17.).

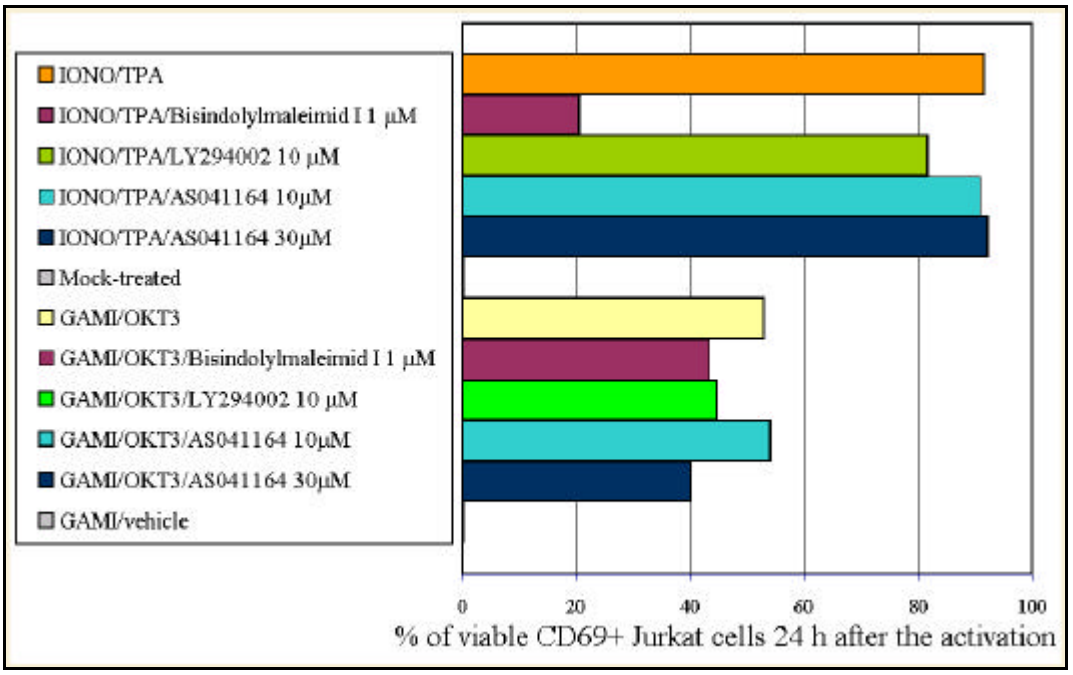


Figure 2.17. Percentage of viable CD69+ Jurkat cells 24 hours after the activation with IONO/TPA or CD3-induced activation. Only viable cells (PI negative) were taken into account.

Measurements of IL-2 production from the same experiments (Figure 2.18.) proved again that the inhibition of PI3Kγ kinase activity reduces IL-2 production both in Jurkat cells activated with IONO/TPA and those activated via TCR. This inhibition was always concentration-dependent and stronger in TCR-activated samples. AS041164-induced inhibition of IL-2 production was considerably weaker than inhibition caused by 10 µM LY294002, although both PI3Kγ specific inhibitor and LY294002 act in a similar way and both substances were applied within the same range of concentration. Again, if there were any doubts about the selectivity of 30 µM AS041164, the effects of 3 µM and 10 µM AS041164 on IL-2 production were clear and highly reproducible, while the selectivity of these lower concentrations of the inhibitor appeared to be beyond any doubts.

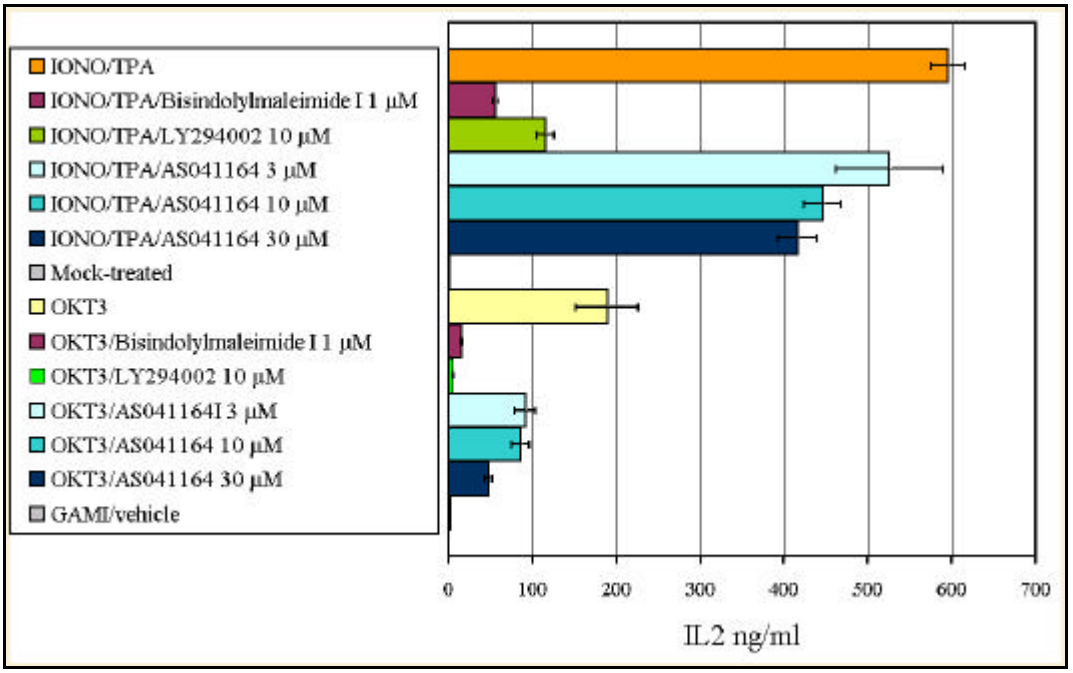


Figure 2.18. The inhibition of PI3Kg reduced IL-2 production of activated Jurkat cells. The cells were activated with IONO/TPA or with anti-CD3 (OKT3) antibody and treated with indicated concentrations of inhibitors during the 24 hours of activation in standard conditions. IL-2 concentration in the cell supernatant was measured by ELISA.

Additional experiments with the same pattern of activation and inhibition were performed in order to correlate the amount of secreted IL-2 and its transcription (Figure 2.19.). RNA was isolated at the same time point as for the other analyses, 24 hours upon activation. This time

point was taken as a compromise in order to obtain comprehensive analysis of all read-outs obtained from this type of experiments.

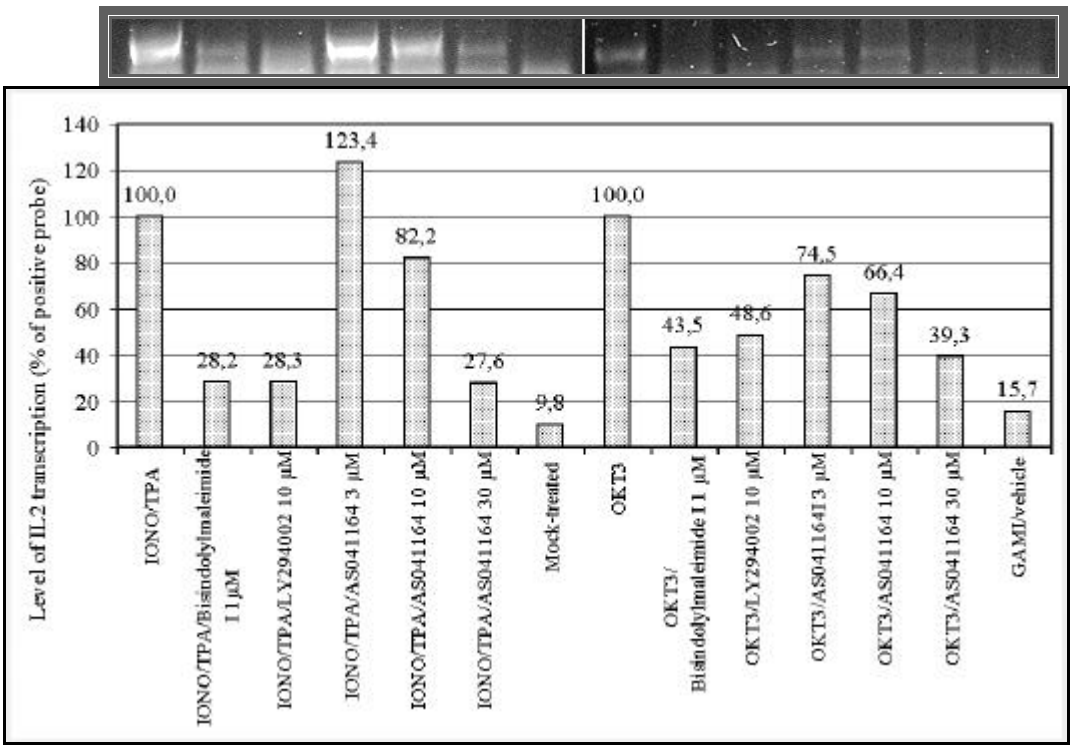


Figure 2.19. The inhibition of IL-2 transcription in activated Jurkat cells by different concentrations of AS041164. Jurkat cells were activated with IONO/TPA or with anti-CD3 (OKT3) antibody and treated with indicated concentrations of inhibitors during the 24 hours of activation in the standard conditions. The intensity of RT-PCR signal for IL-2 was normalised according to the intensity of the signals for β-actin obtained from the identical samples. The values are recalculated as the percentage of the positive probe for the given type of activation. The results represent one of the five independent experiments.

Both Bisindolylmaleimide I and LY294002 were strong inhibitors of IL-2 transcription in IONO/TPA-activated Jurkat cells, while different concentrations of AS041164 exhibited drastic differences in their performance: treatment with 3 µM AS041164 slightly increased IL-2 transcription, most probably due to the delayed peak of activation. 10 µM AS041164 showed week repression and 30 µM AS041164 inhibited this transcription to the approximately same extent as Bisindolylmaleimide I and LY294002. TCR(CD3)-induced IL-2 transcription was much weaker and its suppression by different inhibitors was slightly different: while Bisindolylmaleimide I and LY294002 reduced the IL-2 transcription to less

than half, AS041164 inhibited the transcription in a concentration-dependent manner – from moderate to strong inhibition like non-selective PKC and PI3K inhibitors.

Our results showed that specific inhibitor of PI3Kγ effected the expression of CD69 in Jurkat cells activated via TCR only when it was applied as 30 µM, the concentration with questionable selectivity under described conditions. In all other cases of activation/inhibition AS041164 had no effect on CD69 expression. This implicates that PI3Kγ was dispensable for certain aspects of cell activation and subsequent transcriptional regulation. On the other side, inhibition of the PI3Kγ effected IL-2 production in a concentration-dependent manner both in the cells activated with IONO/TPA and in the cells activated via TCR, in which case AS041164 exhibited a considerably stronger inhibitory effect. At the same time, the reduction of IL-2 transcription induced by the treatment with different concentrations of AS041164, could not fully justify the reduction in secreted IL-2. Discrepancies between experimental results from IL-2 RT-PCR, level of CD69 expression and measurements of secreted IL-2 indicate once more that used inhibitors, and especially AS041164, might have impact on IL-2 expression in activated Jurkat cells on different levels, from transcription to secretion.

3. Discussion

3.1. PI3Ks and T cell activation

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases involved in regulation of diverse biological functions, including cell growth, differentiation, survival, proliferation, migration and metabolism. PI3Ks are indispensable for the differentiation and functions of the T cells. This family of enzymes is a key component of the TCR signalling pathway. The major product of their lipid-kinase activity, phosphatidyinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3), is an important second messenger that regulates the activity of numerous TCR-related proteins, such as PDK1, Akt/PKB, Vav-1, Itk, PLCγ1, Ras and many others. To our knowledge, in previously published works, the TCR-induced activation of PI3Ks was almost exclusively related to the class I_A PI3K (α, β and δ isoforms). Several models of this PI3K activation were proposed, but none of them was so far fully accepted. All of them include few important prerequisites for the full activation of PI3K: translocation of PI3K to the plasmamembrane, indirect association with ITAMs on the cytoplasmatic tail of TCR via regulatory subunits and involvement of protein tyrosine kinases and/or different transmembrane adaptor proteins. PI3K activation occurs within seconds of TCR activation, and PI3Ks remain active for nine hours or more (Costello *et al*., 2002). The first clear evidences of PI3Kγ involvement in the T cell activation-induced proliferation and cytokine production came with the generation of PI3Kγ KO mice (Sasaki *et al*., 2000). In our study we tried to elucidate the nature of PI3Kγ involvement in T cell functions and the mechanism behind this.

3.2. The features of PI3K$g^{-/-}$ T cells

PI3Kγ KO mice show reduced number of thymocytes and mature T cells, and the functions of p110$\gamma^{-/-}$ T cells are impaired. All previous results clearly indicate that PI3Kγ is

not indispensable for T cell homeostasis and function, but rather an important modulator of development, activation and activation-induced cytokine production in T cells. Our data showed reduction of the number of thymocytes and mature T cells in peripheral lymphoid organs and partly confirm earlier findings (Sasaki *et al*., 2000; Rodriguez-Bolardo *et al.***,** 2003). We described that disruption of p110γ had an even stronger impact on the mouse phenotype, especially regarding the number of T cells in spleen and lymph nodes, effecting both major subpopulations of mature T cells (CD4+ and CD8+ cells). There could be a few reasons for this discrepancy. First, slightly different gene-targeting strategies were applied on the mouse strain with a different genetical background. Sasaki *et al*. (2000) used C57BL/6 (commonly known as 'Black 6') mice while the mice we used for our study belonged to 129/sv inbred line (provided by the group of E. Hirsch). Second, the animals we used were in average several weeks older, many of them being close or at the beginning of their sexual maturity.

Our results concerning the reduced number and altered ratio of major thymocytes subpopulations also clearly indicated severely impaired thymic growth and cellularity, as well as disrupted T cell development and differentiation. Rodriguez-Bolardo *et al***.** (2003) described this effect in more details by proving that PI3Kγ is involved in thymocyte development on at least two levels. First, PI3K$\gamma^{-/-}$ thymocytes have impaired DN-to-DP transition at pre-TCR stage (designated as DN stage III) and diminished proliferative expansion that accompanies this stage. Furthermore, PI3K$\gamma^{-/-}$ thymocytes exhibit altered CD4/CD8 lineage commitment during positive selection. Nonetheless, it seems more likely that such seriously disrupted thymic growth and development should also have serious consequences on the number of peripheral mature T cells, instead of having minor effects as described by Sasaki *et al*. (2000). In contrast to the reduced number, the ratio of different subpopulations of resting mature PI3K$\gamma^{-/-}$ T cells (who passed the bottle-neck in their thymic development), including the ratio of $CD4^+$ and $CD8^+$, was comparable with the ratio of WT.

However, our results about proliferation of mouse PI3K$\gamma^{-/-}$ T lymphocytes confirmed an earlier observation from Sasaki *et al*. (2000). PI3K$\gamma^{-/-}$ T cells do have reduced proliferation in response to activation by an anti-CD3 antibody and CD28 costimulation was able to rescue this impaired proliferation. We found out that the impaired proliferation of the same T cells could also be rescued by IL-2 costimulation. Given the fact that reduced cytokine production of TCR-activated PI3K$\gamma^{-/-}$ T cells cannot be rescued by CD28 co-stimulation, as well as with Ionomycin- and phorbol ester-induced activation, this clearly indicated that the lack of IL-2 expression (and probably some other cytokines as INFγ) may be the major reason for this malfunction. The same fact may explain impaired functions of PI3K$\gamma^{-/-}$ T cells *in vivo* (Sasaki *et al*., 2000), where costimulation (like CD28 activation) of T cells is present by default during TCR-induced activation.

3.3. Jurkat cells are good model to investigate the function of PI3Kg in the T cells

For already more than twenty years Jurkat cells have been the most commonly used model for investigating human T lymphocytes. The majority of the facts known nowadays about TCR structure, its function and related signalling pathways, have emerged from studies performed with this cell line (Abraham and Weiss, 2004). The core aspects of these processes that have been defined in Jurkat cells are relevant in physiological settings. Nevertheless, Jurkat cells, like any other model, show some limitations and disadvantages. Shan *et al*. (2000) proved that this particular cell line does not express a detectable level of PTEN, while other authors (Astoul *et al*., 2001) proved the same for SHIP. The absence of these two phosphatases in Jurkat cells results in an abnormally high level of PtdIns(3,4,5)P_3 and slower termination of PI3K signalling pathways upon treatment with PI3K inhibitors. According to Astoul *et al*. (2001) Jurkat cells have a five-fold higher basal level of the PtdIns(3,4,5)P_3 than basal level of PtdIns(4,5)P_2, and further, a two-fold increase of PtdIns(3,4,5)P_3 level can be detected upon TCR activation. The same authors used A20 cells for comparison, and in

contrast to Jurkat cells, A20 cells had a very low basal level of PtdIns(3,4,5)P_3, lower than the basal level of PtdIns(4,5)P_2. Triggering the B cell receptor of A20 cells induced a 10- to 12-fold increase in the PtdIns(3,4,5)P_3 level. Consequences of the abnormalities in Jurkat cells are constitutive membrane localisation of Itk (Shan *et al*., 2000,) and Akt/PKB, enhanced activity of PDK1 (Baier, 2003), as well as constantly high phosphorylation of glycogen synthase kinase 3 (GSK3), an endogenous substrate for activated Akt/PKB, and abnormal GTPase-controlled actin regulation (Astoul *et al*., 2001) (Figure. 3.1.).

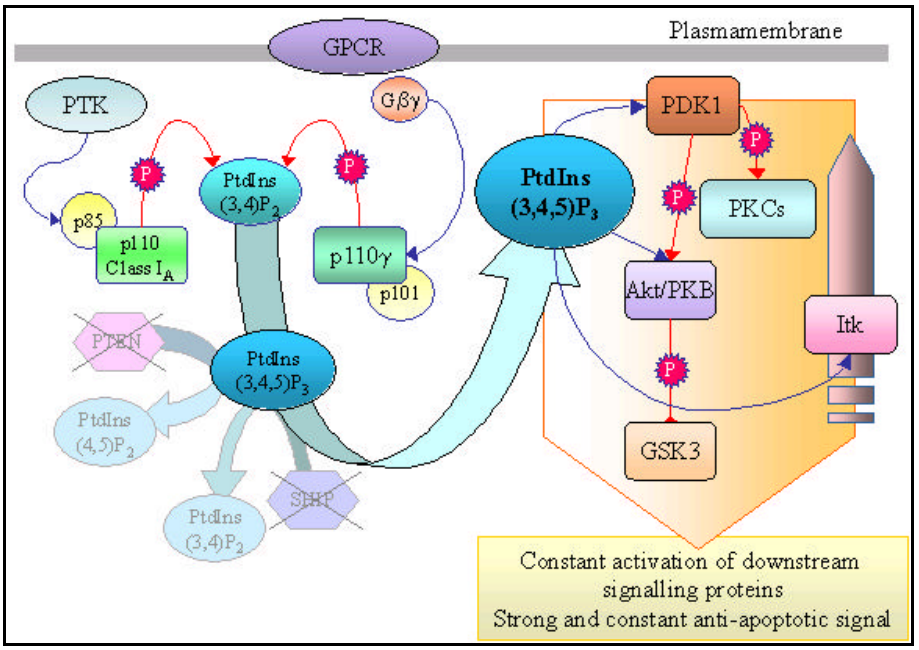


Figure 3.1. Specific features of Jurkat cells. Like in all other cells lines the activation of PI3K class I is connected to the protein tyrosine kinases (PTK), while PI3Kγ is activated via G-protein coupeled receptors (GPCR). Two lipid phosphatases PTEN and SHIP, the negative regulators of the PtdIns(3,4,5)P_3 level, are absent in Jurkat cells. Contribution of the PI3Kγ to the overall PI3Ks lipid-kinase activity in Jurkat cells is relatively small. The basal level of PtdIns(3,4,5)P_3 is constantly high resulting in the recruitment of the proteins containing PH domains (e.g. Itk, PDK1, Akt/PKB) to the plasmamebrane. In addition, this translocation enhances the activity of PDK1, which in return activates Akt/PKB and PKCs. Activated Akt/PKB phosphorylates and blocks GSK3, BAD and caspase-9, and in this way induces a strong anti-apoptotic signal.

Does all this make Jurkat cells an inappropriate model for investigating the role of PI3K in T cells? *In vivo* T cells follow a chemokine gradient that recruits them into the relevant secondary lymphoid organ where they can encounter the antigen presenting cells. This migration requires activation of both chemokine and integrin signalling pathways which are both linked to the activation of PI3Ks. Moreover, memory T cells respond to the cytokine interleukin 15 (IL-15), which also activates PI3Ks. Hence, *in vivo* TCR triggering will normally occur in the cells that have activated PI3Ks already. Jurkat cells are therefore a good model to study TCR signalling in chemokine-activated normal cells or IL15-stimulated memory cells, as long as the data obtained from these cells are interpreted bearing in mind that PI3K signalling is abnormal (Astoul *at al*., 2001).

Our results indicated that the contribution of PI3Kγ to the overall PI3Ks lipid-kinase activity in Jurkat cells is relatively small, practically insignificant in standard conditions when the huge excess of PtdIns(3,4,5)P_3 is constantly present within the cell. Nevertheless, the inhibition of PI3Kγ in activated Jurkat cells induced a specific, clear-cut effect, rather similar to the effect of gene disruption in KO mice. It was therefore justified to use Jurkat cells in our experiments as a model to investigate the role of p110γ by using the specific inhibitor AS041164.

3.4. AS041164 is a selective inhibitor of PI3Kg in Jurkat cells

It is well known that both Wortmannin and LY294002 are cytotoxic for primary cells. However, Jurkat cells appear to tolerate relatively low concentrations of both substances because of their abnormally high level of PtdIns(3,4,5)P_3. In our experiments 100 nM Wortmannin, and especially 10 µM LY294002, significantly reduced the rate of cell proliferation and viability of the Jurkat cells. On the other side, 10µM and 30 µM AS041164 did not have any significant impact on cell viability and cell-cycle progression of Jurkat cells in the standard conditions. Different kinds of lymphocyte activation *in vitro* always induce

apoptosis, meaning that the number and viability of the proliferating cells are reduced to a different extent. Activation with Ionomycin and TPA dramatically reduced viability of the activated Jurkat cells in contrast to the CD3-induced activation. 10µM and 30 µM AS041164 did not reduce the viability of Jurkat cells activated in both ways. Moreover, the inhibitor appeared to rescue the activated cells from apoptosis, unlike 10 µM LY294002, which reduced both total cell number and the percentage of activated Jurkat cells. This was another proof that the inhibitor kept desirable selectivity in our experimental setting. Hence, any outcome of the AS041164 application on Jurkat cells in the low micro-molar range could not be caused by the cytotoxic effect of this inhibitor.

Akt/PKB is one of the major targets of PI3Ks lipid-kinase activity. Actually, Akt/PKB binds PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 via its PH domain, and translocates to the plasmamembrane where PDK1 phosphorylates threonin 308 in the kinase activating loop of Akt/PKB (Komander *et al*., 2004). Further phosphorylation of serine 473 in the C-terminal regulatory domain is associated with full activation of AKT/PKB and it is performed by one still unknown kinase designated as PDK2 (Seminario and Wange, 2003). PDK1 is also recruited to the plasmamembrane after interaction of its PH domain with PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2. Binding of PDK1 to these lipids does not appear to change the intrinsic kinase activity of the enzyme; it seems that this interaction enhances PDK1 kinase activity by bringing the enzyme and its substrates in close contact. There are few data that indicate how PDK1 activity is regulated, but PDK1 is considered to be constitutively active (Seminario & Wange, 2003). The constitutively high level of PtdIns(3,4,5)P_3 in Jurkat cells enhances kinase activity of PDK1 and the activation of Akt/PKB. Indeed, the Jurkat cells we used exhibited relatively high level of Akt/PKB activation even after some hours of serum-deprivation. The level of full Akt/PKB activation, that is Akt/PKB phosphorylation on serine 473, can therefore be considered as a read-out of the intracellular PtdIns(3,4,5)P_3 level and reflection of overall PI3K activity.

In order to clear-out aforementioned controversies about the efficiency of the PI3K inhibition by Wortmannin and LY294002 in Jurkat cells (reviewed by Kane and Weiss, 2003, and Seminario and Wange, 2003) we performed a set of simple inhibitory analyses. We observed that inhibition of the Akt/PKB phosphorylation by Wortmannin and LY294002 was faster and/or more efficient in serum-deprived Jurkat cells than in the cells maintained in the standard conditions, but in both cases the inhibition was clear and concentration-dependent. For that reason, in order to avoid any possible side-effects caused by large surplus of the inhibitors, for further experiments we decided to use the lowest concentrations of these inhibitors applied in our initial tests.

The next step was to compare possible influence of the AS041164 on Akt/PKB phosporylation in Jurkat cells with the impact of wortmannin and LY294002. Our data showed that 10 µM AS041164 was a week inhibitor of Akt/PKB phosphorylation in serum-deprived Jurkat cells and it did not show a detectable inhibitory effect in Jurkat cells maintained in the standard conditions, both during the short-term (1 h) and long-term (24 h) treatment. Again, AS041164 exhibited significant differences to general PI3K inhibitors. If we postulate that AS041164 displays acceptable selectivity under the described conditions, we can also assume that the contribution of the PI3Kγ to the overall PI3K lipid-kinase activity in Jurkat cells (without any specific stimuli and under the standard conditions) is relatively small.

LPA stimulation of Jurkat cells induced PI3Kγ-dependent activation of both Akt/PKB and Erk 1/2. This activation was Wortmannin sensitive, that is PI3K-dependent, and the only PI3K isoform clearly proved to be activated by GPCR agonists is PI3Kγ (Lopez-Ilasaca *et al.*, 1997). 100 nM Wortmanin practically blocked Akt/PKB activation, both in serum-deprived non-activated cells and in LPA-stimulated Jurkat cells. The reduction of Erk 1/2 activation by 100 nM Wortmanin under the same condition appeared to be less effective, especially with the higher concentration of the LPA. On the other side, 10 µM AS041164 exhibited different

pattern of the inhibition: it was a week inhibitor of Akt/PKB activation, and a potent inhibitor of LPA-induced Erk 1/2 activation. One would expect that under given conditions both inhibitors should perform nearly the same pattern of the inhibition, but these inhibitors are apparently two substances with different features and different modes of action.

Direct involvement of PI3Kγ in the signalling pathways connected to the TCR and related tyrosine kinases was never proved, but our results, along with earlier findings, clearly indicated that inhibition PI3Kγ kinase activity disrupted at least some of the signalling events and/or processes initiated by the TCR activation. We therefore decided to examine the impact of the PI3Kγ specific inhibitor on the activation of Akt/PKB and MAPK upon TCR activation. The investigated pathways are known to be activated by TCR in a PI3K-dependent manner, and they are linked to the activation of transcription factors responsible for TCR-induced IL-2 production. Serum deprived Jurkat cells were activated by a soluble clonotypic C305 antibody which induces a strong, rapid and transient activation of Akt/PKB, Erk 1/2, p38 and SAPK/JNK, as well as typical pattern of tyrosine phosphorylation of many proteins involved in TCR signalling. Possible effects of the inhibition were monitored during a 60 minutes time-course. Above mentioned results, with exception of the p38 activation, did not show any changes in activated Jurkat cells pre-treated with the PI3Kγ specific inhibitor. Surprisingly, the phosphorylation of p38 was slightly enhanced, but we were not able to elucidate the significance of this observation. Taken all together, these results indicated that, whatever the nature of PI3Kγ involvement in the TCR-induced activation of the Jurkat cells may be, it does not seem to be in the proximity to the TCR in signal transduction and it does not seem to regulate the initial steps of this process. Nevertheless, no matter how insignificant may look the reduction of Akt/PKB activation in serum-deprived non-stimulated samples, it should not be overlooked. Retained expression of PTEN in Jurkat cells had minor effects on early PI3K-dependent TCR signalling events, in contrast to distal signalling events, including IL-2 production, CD69 up-regulation and activation of NFAT/AP-1, which were all inhibited

by PTEN expression (Seminario *et al.*, 2003). PTEN ineffectively opposed the acute changes in PtdIns(3,4,5)P_3 level induced by TCR activation, but the phosphatase clearly downregulated the basal level of inositide.

3.5. Selective inhibitor of PI3Kγ reduces IL-2 production in activated Jurkat cells

The PI3Kγ specific inhibitor AS041164 was a weak inhibitor of Akt/PKB activation in serum-deprived Jurkat cells and showed practically no impact on Akt/PKB activation in the cells maintained in standard conditions, as well as in activated Jurkat cells. AS041164 seemed to influence neither level of PtdIns(3,4,5)P_3 nor the activation of PDK1, and it certainly did not reduce Akt/PKB activation under standard conditions. In contrast, AS041164-induced reduction of IL-2 production in activated Jurkat cells was clear, dose-dependent and highly reproducible. These results, along with the data obtained from PI3Kγ KO mice, point to the conclusion that cytokine production of activated p110$\gamma^{-/-}$ T cells was most probably not impaired due to the absence of p110γ or a lack of its lipid-kinase activity, because the excess of PtdIns(3,4,5)P_3 is constantly present in Jurkat cells. It seems more likely that this effect was caused by the lack of p110γ protein-kinase activity itself or by other properties of the enzyme (e.g. subcellular localisation or certain protein-protein interactions) which depend on the protein-kinase activity.

3.6. p110γ interact with different PKC isoforms in Jurkat cells

Our results proved that PI3Kγ interacts with four different PKC isoforms in Jurkat cells. For more than a decade the protein members of the PKC family have been known to play a key role in the T cell activation (Barry and Nishizuka, 1990; Dreikhausen *et al.*, 2003). It is well known that pleiotropic PKC inhibitors block T cell activation (Wilkinson *et al.*, 1998), but T lymphocytes contain up to eight different species of PKC isoforms, making it difficult to determine the specific cellular function of these individual enzymes (Baier, 2003).

Moreover, the PKC signalling system appears to be highly versatile, cell-type- and differentiation status-dependent. Regulation of PKC expression and activity operate at the transcriptional, translational and post-translational levels, including the active mechanisms for PKC degradation (Baier, 2003). It is therefore not surprising that the reports on involvement of particular PKC isoforms in the processes like T cell activation and IL-2 production often gave conflicting results.

PKCα was considered to be the major PKC isoform involved in TCR/CD28 signalling pathways and responsible for the generation of the transcription factors AP-1 and NFAT (Genot *et al*., 1995; Dreikhausen *et al*., 2003). Dreikhausen et al. (2003) proved overlapping biological activities of PKCα and PKCβ in activation of the MAPK pathway and NF-κB, but claimed that PKCβ1 is the major regulator of TCR-CD28-induced IL-2 gene transcription and secretion in Jurkat cells. Another model and another approach led to a rather different result: Long *et al*. (2001) proved that PKCβ is specifically involved in the secretion but not in the transcription of IL-2 in activated HUT 78 cells. Many studies in different T cell lines also indicated that PKCθ, a lymphocyte specific PKC isoform, plays a significant role in T cell activation (Ghaffari-Tabrizi *et al*., 1999; Kempiak *et al*., 1999; Altman *et al*., 2000), which was confirmed with the generation of PKCθ KO mice (Pfeifhofer *et al*., 2003). Peripheral T cells lacking PKCθ failed to activate NF-κB and AP-1, and failed to express IL-2 upon TCR-induced activation. However, stimulation of these cells with Ionomycin and phorbol ester restored IL-2 transcription and proliferation. Trushin *et al*. (2003) proved that both PKCα and PKCθ are required for the activation of T cells and subsequent expression of IL-2R and IL-2 production. Their results clearly placed PKCα upstream from PKCθ in these signalling events, implicating that PKCα is responsible for the activation of transcription factors NF-κB and NF-AT, but not AP1, upon CD3/CD28-induced activation. The same authors used both Jurkat cells and human peripheral blood T cells in their study, combining genetic analysis, pharmacological inhibitors and RNA interference.

Interaction of p110γ with PKCα, PKCβ1, PKCβ2 and PKCζ in Jurkat cells is constitutive and LY294002 sensitive. Treatment of the cells with this PI3Ks inhibitor drastically diminished those interactions, which highly correlated with a reduced level of PtdIns(3,4,5)P_3 and a reduction of Akt/PKB activation. The enzyme members of the protein kinase C family are multi-domain proteins with a complex multi-level regulation of their activation. The following pattern of activation refers to the group of conventional PKCs (α, βI, βII and γ isoforms), but other PKC isoforms are activated in a similar manner. First, PKCs are allosterically regulated by their N-terminal pseudosubstrate – the release of the pseudosubstrate from the kinase core is an initial step of the activation. Besides, binding of diacylglycerol (*in vivo*) or phorbol ester (*in vitro*) to the C1 domain of PKCs strongly increases affinity of the enzyme for membranes. Binding of Ca^{2+} ions to the C2 domain also facilitates recruitment of PKCs to the membrane. The functions of both C1 and C2 domains appear to be synergistic, but independent. Three stages of phosphorylation are required for the full activation of PKCs. The initial and rate-limiting step in this process is phosphorylation of the target threonin residue in activation loop by PDK1. This is followed by phosphorylation of the threonin residue in turn motif, which locks PKC in a catalytically competent, thermally stable, and phosphatases resistant conformation (Edwards *et al*., 1997; Newton, 2001). Finally, phosphorylation of (predominantly) a serine residue in the C-terminal hydrophobic motif affects the sub-cellular localisation and stability of PKCs. It is known that all PKC isoform subclasses can form complexes with PDK1 and that PKC phosphorylation in the activation loop by PDK1 is blocked by LY294002 (reviewed by Parekh *et al*., 2000). Taking in consideration abnormally high levels of PtdIns(3,4,5)P_3, and therefore the same abnormally high PDK1 kinase activity in Jurkat cells (Baier, 2003), as well as the proved pattern of PKC activation, it can be assumed that the level of PKC kinase activity in Jurkat cells must also be elevated, but still be Wortmannin and LY294002 sensitive.

On the other side, inhibition by AS041164 reduced the interaction between p110γ and PKC isoforms which implicates that p110γ kinase-activity might not be essential, but important to enhance the interactions of p110γ and PKC isoforms in Jurkat cells. Very surprisingly, two PKC inhibitors (Bisindolylmaleimide I and Gö 6976) also enhanced the interactions between p110γ and PKC isoforms. This may be explained by results of Hu and Exton (2004) who investigated interaction between PKCα and Phospholipase D1. They came to some unexpected results: first, inhibition of PKCα by Bisindolylmaleimide I and Ro-31-8220 induced phosphorylation of threonin residue(s) of the enzyme *in vivo*, and then, the same inhibitors induced translocation of PKCα to the plasmamembrane. Bisindolylmaleimide I and Gö 6976 also blocked PMA-induced translocation of PKCα to the perinuclear region, but not PMA-induced translocation of PKCα to the plasmamembrane. This suggests the conclusion that predominantly or only active and not inhibited p110γ and translocated PKC isoforms interact in the vicinity of the plasmamembrane. This could explain why it was not possible to detect these interactions in non-stimulated human peripheral blood CD4+ T cells, and why those interactions are enhanced in Jurkat cells upon TCR stimulation. The answer could be that interactions between p110γ and activated PKC isoforms in primary human T lymphocytes take place only after the antigen receptor activation, and that this interaction is probably rapid and transient.

3.7. The interaction of 110g and PKC isoforms is important for IL-2 production of activated Jurkat T cells

The specific p110γ inhibitor AS041164 and PKCs inhibitor Bisindolylmaleimide I reduced anti CD3-induced production of IL-2 in a similar, dose-dependent way. When IL-2 production was induced by Ionomycin and phorbol-ester bypassing TCR and directly activating PKCs among other targets, the impact of AS041164 on IL-2 production was much weaker, unlike the effect of Bisindolylmaleimid I, which completely blocked IL-2 production.

This implicates that p110γ kinase-activity is required for the proper functioning of the aforementioned PKC isoforms upon TCR activation in order to induce cytokine production. One possibility is that all this enzymes are part of a larger signalling complex, and it is likely that p110γ facilitates the recruitment of PKCα, -β1, and -β2 (and –ζ) to the plasmamembrane, into the vicinity of PDK1, the major physiological activator of PKCs (Figure 3.2.). Ionomycin and TPA activation of Jurkat cells can obviously overcome the problem of PKCs translocation to the plasmamembrane when PI3Kγ is inhibited. This may be the reason why AS041164 is less effective inhibitor of IL-2 production when the cells are activated in this way. Bearing in mind that PDK1 activation is also dependent on the PtdIns(3,4,5)P_3 level, this may explain why p110γ-PKCs interactions are more sensitive to Wortmannin and LY294002 than to AS041164.

The involvement of PI3Kγ in IL-2 expression upon Jurkat cell activation may be located on several levels from the regulation of transcription to the export of IL-2 from the cells. It is known that the induction of transcription from one of the IL-2 promoter's sites by NFAT requires both calcium- and PKC/Ras-dependent pathways (Rao *et al*., 1997; Kane and Weiss, 2003). PI3K$\gamma^{-/-}$ cells produce reduced amounts of IL-2 and interferon-γ (INF-γ) in response to treatment with anti-CD3ε and anti-CD28ε or ConA. This functional defect was also present in PI3K$\gamma^{-/-}$ cells activated with IONO/TPA, a stimulus that bypasses the initial TCR-activation signalling events. Moreover, TCR-mediated Ca^{2+} flux, tyrosine phosphorylation, and activation of tyrosine kinases were comparable among PI3K$\gamma^{+/-}$ and PI3K$\gamma^{-/-}$ T cells (Sasaki *et al*., 2000). It therefore seems that the cause of reduced IL-2 production of activated PI3K$\gamma^{-/-}$ T cells on the transcriptional level may be disruption of a PKC/Ras-dependent but not a calcium-dependent pathway.

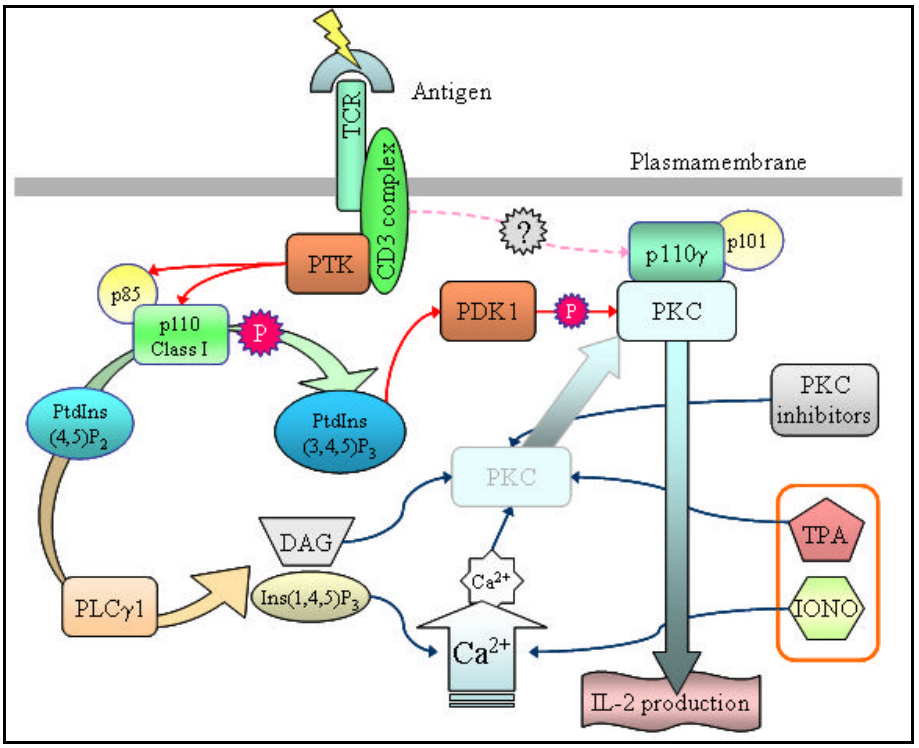


Figure 3.2. The interaction of PI3Kg with PKCa, -b1, -b2 and –z in activated T cells. Antigen binding to the TCR induces signalling cascade that activates PI3Ks class I_A, while the direct influence of TCR activation on the PI3Kγ remains elusive. The activated enzymes rapidly increase the level of PtdIns(3,4,5)P_3 which enhances the kinase activity of PDK1 among other enzymes. Activated PLCγ1 creates DAG which binds to the C1 domain of PKCs, and Ins(1,4,5)P_3 which induces Ca^{2+} influx. Calcium ions bind to the C2 domain of PKCs and together with DAG induce translocation of PKCs to the plasmamebrane. *In vitro* activation with Ionomycin and TPA makes the same effect on PKCs, like some PKC inhibitors which also facilitate the translocation of PKCs. PI3Kγ facilitates the recruitment of PKC isoforms in the vicinity of the plasmamembrane, where PKCs become phosphorylated by PDK1. Proper activation of different PKCs is necessary for the IL-2 expression.

The expression of IL-2 is regulated in very complex way, which is quiet common for many cytokines. The complexity of regulation enables tight control of cytokine production and release. Antigen recognition by the TCR and activation of the T lymphocytes induces specific transcription factors. Several studies demonstrated a requirement for cooperative binding of the three transcription factors, AP-1, NF-AT, and NF-κB for maximal IL-2 production (Chen and Rothenberg, 1994; Garrity *et al.*, 1994; Novak *et al.*, 1990). NFAT

binds to the promotor region of the IL-2 gene and activates its transcription. Induction of transcription does not lead by default to the production of IL-2: in primary T cells CD28 costimulation is required for the stabilisation of IL-2 mRNA. Cytokine promotors have constant, low basal activity, but their mRNAs are short-lived because of the 'instability' of their 3' untranslated region. Stabilisation of the IL-2 mRNA increases IL-2 synthesis by 20- to 30-fold. Activation of transcriptional factors AP-1 and NF-κB leads to a further 3-fold increase in IL2 production, so these two effects together increase IL-2 production by as much as 100-fold (Janeway *et al*., 2001). The peak of IL-2 transcription in Jurkat cells activated by phorbol ester and ionophore can be registered 6-7 hours after the stimulation, while TCR-induced IL-2 transcription reaches its peak several hours later (Weiss *et al*., 1987; Wiskocil *et al*., 1985).

RT-PCR results for IL-2 clearly showed that AS041164 inhibited IL-2 transcription in a dose-dependent manner. Ionomycin/phorbol ester-induced IL-2 transcription was strongly inhibited both by Bisindolylmaleimide I and LY294002 confirming the importance of both PKCs and PI3Ks in this process. Two different kinds of cell activation gave a slightly different pattern for the inhibition by AS041164. In ionomycin/phorbol ester-activated cells treatment with the lowest concentration of AS041164 sligtly increased transcription of IL-2, whereas higher concentrations of the inhibitor reduced IL-2 transcription in a dose-dependent manner. In contrast secretion of IL-2 was downregulated with all concentrations of the inhibitor. A sligtly different picture emerged upon CD3-activation of Jurkat cells. In this case secretion and transcription of IL-2 were reduced by all concentrations of the PI3Kγ-specific inhibitor AS041164 in a dose-dependent manner. This shows that PI3Kγ may be involved in the control of IL-2 production on several levels from transcription to secretion, and that at least one of these levels is connected to PKCs. Although the level of reduction may be slightly different the significant differences on the various regulatory levels point to the conclusion that various modes of protein activity of PI3Kγ allow distinct interactions with

different signalling pathways, suggesting that PI3Kγ is a multifunctional enzyme important for the regulation of lymphocytes at different cellular levels.

4. Materials and Methods

4.1. Materials

4.1.1. Cells

Jurkat cells were purchased from German Collection of Microorganisms and Cell Culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig), DSMZ No ACC 282, LOT 7.

Antibodies

4.1.2. Primary antibodies used for western blotting

Mouse monoclonal anti-β-actin antibody, clone AC-15, Sigma

Rabbit polyclonal antibody to Human PKC α (C-20), (cs-209), Santa Crus Biotechnology

Rabbit polyclonal antibody to Human PKC βI (C-16), (cs-209), Santa Crus Biotechnology

Rabbit polyclonal antibody to Human PKC βII, (cs-210), Santa Crus Biotechnology

Rabbit polyclonal antibody to Human PKC θ (C-18), (cs-212), Santa Crus Biotechnology

Rabbit polyclonal antibody to Human PKC δ (C-17), (cs-213), Santa Crus Biotechnology

Rabbit polyclonal antibody to Human PKC ε (C-15), (cs-214), Santa Crus Biotechnology

Rabbit polyclonal antibody to Human PKC ζ (C-20), (cs-216), Santa Crus Biotechnology

Rabbit polyclonal antibody to Human PKC η (C-15), (cs-215), Santa Crus Biotechnology

Rabbit polyclonal antibody to Phospho-SAPK/JNK (Thr183/Tyr185), #9251, Cell Signaling Technology, NEB

Rabbit polyclonal antibody to SAPK/JNK, #9252, Cell Signaling Technology, NEB

Rabbit polyclonal antibody to Phospho-p38 MAP Kinase (Thr180/Tyr182), #9211, Cell Signaling Technology, NEB

Mouse monoclonal antibody to Phospho-p42/44 MAPK (P-Erk 1/2) (Thr202/Tyr204), #9106, Cell Signaling Technology, NEB

Mouse monoclonal antibody to p42/44 MAPK (Erk 1/2) (L34F12), #4696, Cell Signaling Technology, NEB

Rabbit polyclonal antibody to Phospho-Akt (P-Akt) (Ser473), #9271, Cell Signaling Technology, NEB

Rabbit polyclonal antibody to Akt, #9272, Cell Signaling Technology, NEB

4.1.3. Secondary antibodies used for western blotting

Goat anti-mouse IgG (H+L), Peroxidase-Labeled (KPL, Gaithersburg, MD, USA)

Goat anti-rabbit IgG (H+L), Peroxidase-Labeled (KPL, Gaithersburg, MD, USA)

4.1.4. Antibody used for immunoprecipitation

Anti-PI3Kγ mouse monoclonal antibody, clone 641, hybridoma culture supernatant (hybridoma cells are the property of 'AG Molekulare Zellbiologie, Klinikum der FSU Jena')

Mouse IgG2A isotype control (Sigma)

4.1.5. Antibodies and conjugates used for purification of mouse T cells

FITC-anti-B220 (Caltag, Burlingame, CA, USA)

FITC-anti-CD11b (Caltag, Burlingame, CA, USA)

FITC-anti DX5 (BD Pharmingen)

BioMag® Sheep anti-fluorescein particles (Polysciences Inc., Warrington, PA, USA)

4.1.6. Antibodies used for activation of mouse T lymphocytes and Jurkat cells

Hamster monoclonal Anti-Mouse CD3ε antibody, clone 145-2C11, cat. Nr. 553057 (BD Pharmingen)

Goat Anti-Mouse IgG + IgM (H+L) (GAMI) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA)

Mouse Monoclonal Anti-Human CD3, clone OKT3 (eBioscience, San Diego, CA, USA)

C305 mouse monoclonal antibody which reacted with idiotypic-like determinants expressed on Jurkat cells (Weiss *et al*., 1984), hybridoma culture supernatant (a kind gift from Professor B. Schraven, Institute of Immunology, University of Magdeburg)

4.1.7. Antibodies used for the flow cytometry analysis

FITC-Conjugated Mouse Anti-Human CD69 Monoclonal antibody, clone FN50, Catalogue Number: 555530 (BD Pharmingen)

FITC-Conjugated Anti-Mouse CD3 Molecular Complex Monoclonal Antibody, clone 17A2, Catalogue Number: 555274 (BD Pharmingen)

R-Phycoeritrin (R-PE) conjugated anti-mouse CD4 (L3T4) monoclonal antibody, clone H129.19, Catalogue Number: 553652 (BD Pharmingen)

PER-CP-conjugated anti-mouse CD8 monoclonal antibody, clone 53-6.7, Catalogue Number: 553036 (BD Pharmingen)

FITC-conjugated anti-mouse TCR β-chain monoclonal antibody, clone H57-597, Catalogue Number: 553171 (BD Pharmingen)

4.1.8. Buffers

Dulbeccos Phosphate Buffer Saline, D-PBS (Gibco)

FACS buffer (D-PBS, 1% BSA; Gibco)

Lysis buffer for immunoblotting (20 mM HEPES, 10 mM EGTA, 40 mM β-glycerophosphate, 2,5 mM $MgCl_2$, 1% NP-40 alternative, 10 mM NaF, 2 mM Na_3VO_4, 100 µM PMSF and 1 µg/ml Aprotinin)

5x gel loading buffer, GLB (10% SDS, 25% glycerol, 25% β-mercaptoethanol)

Anode buffer for semi-dry transfer (0,3 M Tris, 20% methanol, pH 10,4)

Cathode buffer for semi-dry transfer (25 mM Tris, 20% methanol, 40 mM 6 amino-n-caproic acid, pH 9,4)

Tris buffer saline/Tween 20 - TBST (50 mM Tris, 150 mM NaCl, 2,7 mM KCl, 0,1% Tween 20, pH 7,5)

Lysis buffer for immunoprecipitation (100 mM TRIS/HCl pH 7.5, 150 mM NaCl, 1 mM MgCl2 and 0.25% NP-40 substitution)

Wash buffer for immunoprecipitation (100 mM TRIS/HCl pH 7.5, 100 mM NaCl, 1mM $MgCl_2$ and 0.05% Tween-20)

*4.1.9. **Primers** (MWG-Biotech, Germany)*

IL-2 sense: AACCTCAACTCCTGCCACAATG

IL-2 anti-sense: CAAGTTAGTGTTGAGATGATGC

β-actin sense: TACATGGCTGGGGTGTTGAA

β-actin anti-sense: AAGAGAGGCATCCTCACCCT

4.1.10. Other reagents and chemicals

$(dT)_{18}$ primer (MWG-Biotech, Germany)

[*methyl*-^{3}H]thymidine (Amersham)

10 mM dNTP's (MWG-Biotech, Germany)

3MM Whatman filter papers (Biometra, Göttingen, Germany)

AMV Reverse Transcriptase (Promega)

AS04116 (Serono Company, Geneva)

Biomax® film (Kodak)

Bisindolylmaleimide I (Calbiochem)

Biomag Sheep anti-fluorescein particles (Polysciences Inc.),

Bradford Reagent (Sigma)

BSA, cell culture tested (Sigma)

Cell culture flasks (Grainer)

Cell strainers, pore size 70 µm (BD Falcon)

DNase I, RNase free (Roche)

Enhanced Chemiluminescence reagent - ECL (Western Lightning®; Pierce)

Ethidium bromide (Sigma)

Fluorescein diacetate (FDA; ICN Biomedicals)

Foetal calf serum (Gibco)

Glass Fibre Filters, Filtermat A (Wallac Oy, Turku, Finland)

Gö 6976 (Calbiochem)

G-protein Sepharose beads (GammaBind®; Amersham)

Imobilion-P Transfer Membrane (PVDF; Millipore)

Ionomycin, Free Acid, from *Streptomyces conglobatus* (Calbiochem)

LPA (L-a-lysophospatidic acid, Oleoyl, Sodium; Sigma)

LY-294002 (Alexis Biochemicals)

Non fett milk powder (Roth, Karlsruhe, Germany)

p(dN_6) random primer (Roche)

Penicillin/streptomycin for the cell culture(Gibco)

PMA (TPA or Phorbol 12-myristate 13-acetate; Alexxis Biochemicals)

Ponceau S (AppliChem, Germany)

Propidium iodide (PI; Sigma)

Quantykine Human IL2 ELISA (R&D System, Minneapolis, USA)

Recombinant Human Interleukin 2 (IL-2; BD Pharmingen)

Rneasy Mini Kit (Qiagen)

RPMI 1640 medium with GlutaMax® (Gibco)

Sequencing grade methylated trypsin (Sigma)

Tag Polymerase (Qiagen)

Tripan Blue (Sigma)

U-bottomed 96-well plate (Costar)

Wortmannin (Alexis Biochemicals)

β-mercaptoethanol, 2-mercaptoethanol (Sigma)

4.2. Methods

4.2.1. Cell cultivation

Jurkat cells were maintained in the standard conditions for the activation of the cells with immobilised OKT3 antibody, both before and during the assay, as well as for the inhibitory analysis where indicated. Cells were maintained at 37^0C with 5% CO_2 in 250 ml plastic cell culture flasks, RPMI 1640 medium with supplemented with 10% foetal calf serum. The medium was exchanged every 2-3 days, depending on the cell density, before they reached density of $1.5x10^6$/ml.

Serum deprivation of Jurkat cells was performed over night (approximately 16 hours) in RPMI 1640 medium supplemented with 0,25% of cell culture tested BSA, incubation at 37^0C with 5% CO_2.

Mouse T lymphocytes purified from the spleen and used for the functional assays were maintained under standard conditions: incubation at 37^0C with 5% CO_2 in plastic cell culture flasks, RPMI 1640 medium supplemented with 10% foetal calf serum, 100U/ml penicillin, 100 µg/ml streptomycin and 3,5 µl/l of β-mercapto-ethanol.

4.2.2. Isolation of the mouse lymphocytes

The experimental animals were sacrificed with pressurised CO_2. The whole intact lymphoid organs (thymus, spleen, lymph nodes) were extracted under aseptic conditions and transferred into the petri dishes with ice-could PBS, washed and the blood and residues of the

surrounding tissue were removed (Figure 2.1.). The organs were transferred into separate cell strainers with pore size of 70 µm. Each cell strainer was submersed in the petri dish filled with 10 ml ice-could medium. The organs were homogenised inside of the cell strainer by using 1 ml syringe plunger with rubber top. Cell suspension was collected from the petri dish, transferred to the 15 ml polypropylene conical centrifuge tubes and centrifuged for 10 minutes at 1200 rpm, 4^0C. The cell pellet was resuspended in 10 ml of ice-could medium and the washing step was repeated three times in total. The cells were counted with Neubauer counting chamber using tripan-blue exclusion.

4.2.3. Purification of the mouse T cells

Mouse T lymphocytes used for the functional assays were purified from spleen by negative selection. Isolated lymphocytes samples were resuspended in 200 µl PBS/1% BSA. Following antibodies ware added in the cell suspension: 10 µl of FITC-anti-B220, 10 µl of FITC-anti-CD11b and 5 µl of FITC-anti DX5. The cells were incubated with the antibodies for 30 minutes at 4^0C and afterwards washed three times with 1 ml of PBS/1% BSA. After the final washing step cell pellet was resuspended in 500 µl of PBS/1% BSA. 1,5 ml anti-FITC magnetic beads suspension (Biomag Sheep anti-fluorescein particles), washed three times with 500 µl of PBS/1% BSA, were retained with the magnet, resuspended in 500 µl of PBS with 1% BSA and mixed with 500 µl of the cell suspension. The mixture was gently rotated for 30 minutes at 4^0C. The beads were separated with a magnet twice, each time retaining the supernatant with T cells. Purified T cells were resuspended in the medium and prepared for the further use. The cell purity was confirmed by flow cytometry analysis (FACScalibur, BD) after the staining with FITC-anti-CD3 antibody. The obtained yield was 91 - 94%.

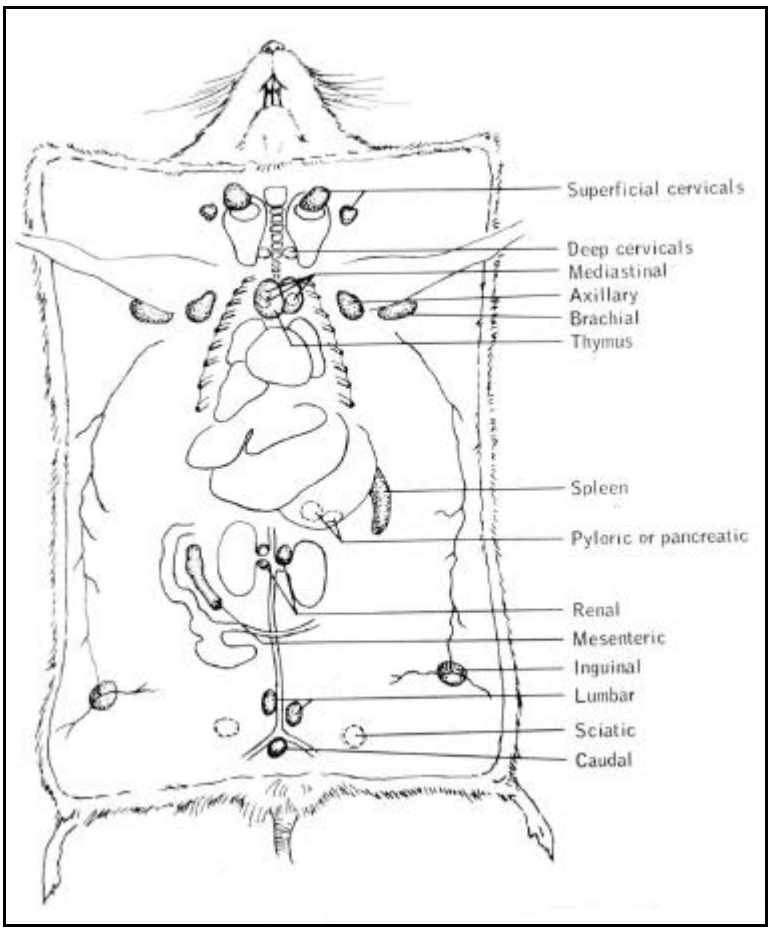


Figure 4.1. Mouse lymphoid organs. The whole intact thymus and spleen were extracted for the analyses; caudal, inguinal, brachial, axillary, deep and superficial cervical lymph nodes were extracted and used for the analysis of the T cells in lymph nodes. T cells purified from the spleen were used for functional assays.

4.2.4. Staining of the mouse T cells with specific antibodies for the flow cytometry analysis

For each sample, 1 x 10^6 cells were stained with antibodies and analysed on a FACSCalibur® using the CellQuest® software (Becton Dickinson). All the antibodies were purchased from BD Biosciences and the cell staining was performed according to the manufacturer's recommendation. Briefly: the 100-fold dilution of antibody mixture was prepared in cold FACS buffer. The samples were distributed in U-bottomed 96-well plate, the plate was centrifuged (1000 rpm, 4^0C) and the cell pellet was resuspended in 100 µl of

antibody mix solution. After the incubation at 4^0C for 30 minutes, the cells were washed three times with FACS buffer, transferred to the FACS tubes and resuspended in final volume of 500 µl FACS buffer.

4.2.5. Activation of the mouse T cells and proliferation assay ([^{3}H]-thymidine uptake)

Purified mouse T cells (2.5 x 10^4 cells/well) were cultured under standard conditions in a U-bottomed 96-well plate in the presence of plate-bound anti-mouse CD3 antibody (Simeoni *et al*., 2005b). The plate was pre-incubated for 2 h at 37^0C with 100 µl per well of the indicated antibody concentrations. The cells were transferred in the plate, cultured for 72 h and labelled with 0.5 µCi/well of [3H]thymidine during the last 6 h. The incubation was terminated by filtration through Glass Fibre Filters by using TOMTEC Mach III cell harvester (Orange, CT, USA), and the filter was dried and frozen at -20^0C. The read-out was obtained 24 hour later on Wallac 1450 (Wizard automatic Microbeta counter, Wallac Oy, Turku, Finland).

4.2.6. Flow cytometry analysis of the Jurkat cells viability (PI and FDA staining) and Jurkat cell activation (CD69 staining)

For viability test 2,5x10^5 Jurkat cells per sample were stained with fluorescein diacetate (FDA) in concentration of 0.04 µg per 100 µl of cell suspension and propidium iodide (PI) in concentration of 25 µg per 100 µl of cell suspension in FACS buffer (Ross *et al*., 1989). Samples were incubated for 15 min at room temperature in dark, washed and resuspended in FACS buffer.

For the analysis of the activation 2,5x10^5 Jurkat cells per sample were stained with FITC-conjugated mouse anti-human CD69 monoclonal antibody. The staining was performed according to the manufacturer's protocol: the cells were suspended in 100 µl of FACS buffer,

incubated with 5 µl of antibody for 30 min in dark at 4^0C. The cells were washed and resuspended in FACS buffer.

Stained Jurkat cells were analysed using a FACS®Calibur (Becton Dickinson) and CellQuest Pro (BD) analysis software. Excitation and emission settings were 488 nm and 525–550 nm (FL1 filter) for FDA and FITC-anti-CD69 and 488 nm and 564–606 nm (FL2 filter) for PI staining, respectively.

4.2.7. Immunoblotting

The samples of the Jurkat cells were washed with ice-could PBS, resuspended by vortexing in 100 µl per 1 million cells of ice-could lysis buffer and left on ice for 10 minutes. The samples were centrifuged for 20 minutes at 15000 rpm, 4^0C, and the supernatant containing proteins was transferred into the new 1,5 ml micro test tubes. The protein concentration was measured with Bradford reagent (Sigma) according to the manufacturer's protocol (absorbance at 595 nm). Total cell protein lysate was kept at -20^0C until further use. The aliquots of the lysate containing 25-50 µg of proteins were mixed with appropriate volume of the 5xGLB, boiled for 5 minutes at 95^0C and resolved on 10% SDS-PAGE (Sambrook and Russell, 2001). The resolved proteins were transferred on the PVDF membrane using semi-dry system according to the manufacturer's protocol with slight modifications. Briefly, in semi-dry blotting device the gel and the membrane were sandwiched between three sheets of 3MM Whatman filter papers soaked with anode and cathode buffer. Constant current of 2.5 mA/cm^2 was applied for one hour. The efficiency of the transfer was controlled with Ponceau S staining of the membrane. The membranes were blocked either in 5% non fat milk or 5% BSA dissolved in TBST for one hour at room temperature. The detection of the proteins was performed by using respective mouse monoclonal or rabbit polyclonal antibodies diluted according to the manufacturers' recommendation, followed by incubation with appropriate secondary antibody conjugated to

the HRP. Both after the incubation with primary and the secondary antibody the membranes were washed five times for five minutes in TBST buffer. The proteins were visualised by applying ECL solution on the membrane for one minute, the membrane was wrapped in a transparent foil and finally, Biomax® film (Kodak) was exposed to the membrane.

4.2.8. The stimulation of Jurkat cells with C305 antibody

The samples of Jurkat cells in serum-free medium were aliquoted in 1,5 ml micro test tubes (10^6 cells in 400 µl per tube) and kept during stimulation in the Thermomixer at 37^0C, 1000 rpm. 100 µl of hybridoma culture supernatant containing C305 mouse monoclonal antibody (a kind gift from Professor B. Schraven, Institute of Immunology, University of Magdeburg) was added to the samples, and the activation was terminated at indicated time-points by adding 1 ml of ice-could PBS. The cells were centrifuged for 2 minutes at 4^0C, 1000 rpm. The cell pellet was washed with 1 ml of ice-could PBS and resuspended in 100 µl of lysis buffer for immunoblotting.

4.2.9. Anti-CD3-induced activation of Jurkat cells

24-well plates were incubated with 250 µl per well of 2 µg/ml Goat-anti-mouse IgG (GAMI) for 2 h at 37^0C. Plates were washed with D-PBS and afterwards incubated with 250 µl per well of 2 µg/ml anti-CD3 monoclonal antibody OKT3 for 2 h at 37^0C. Plates were washed with D-PBS. Jurkat cells were collected, counted, centrifuged and resuspended in standard medium at cell density of 10^6 cells/ml. The aliquots of the cell suspension were treated for 15 minutes prior to the activation with following amounts of inhibitors where indicated: 10 µM LY294002, 3, 10 or 30 µM AS041164 (as indicated in the text), 1 µM Bisinolylmaleimide I. 500 µl aliquot from each cell suspension was distributed to one well. Each treatment was made in triplicate. Treatment with 1 µg/ml of Ionomycin, (IONO) and 10 µM Phorbol 12-myristate 13-acetate (PMA or TPA) was used as a positive control. Negative

controls were mock-treated cells and cells treated only with GAMI (2 µg/ml). Homogeneous cell suspension from each sample was collected 24 h later, samples were centrifuged at 1000 rpm for 2 minutes, and 100 ml of supernatant was used for IL-2 measurement. Remaining cell pellet was used for RNA extraction.

4.2.10. Measurement of IL-2 production in activated Jurkat cells

Concentration of IL-2 in supernatant was determined with Quantykine Human IL2 ELISA according to the manufacturer's instructions. Briefly, a monoclonal antibody specific for IL-2 has been pre-coated onto a microplate. 100 µl of prepared standards and samples were pipetted into the wells along with 100 µl of assay buffer and any IL-2 present was bound by the immobilized antibody during 2 hours of incubation at RT. After three washing steps, an HRP-coupled polyclonal antibody specific for IL-2 was added to the wells for another 2 hours of incubation. Following three more washing steps, a substrate solution was added to the wells. The colour development was stopped with stop-solution and the intensity of the colour was measured with TECAN Ultra plate reader (Tecan Group, Mannedorf/Zurich, Switzerland), readout at 450 nm wave length, and referent absorbance at 620 nm.

4.2.11. Immunoprecipitation of PI3Kg from Jurkat cells

10^7 Jurkat cells were lysed in 500µl of lysis buffer for immunoprecipitation. The lysate from each sample was incubated with 100 µl of culture supernatant from 641 hybridoma (mouse monoclonal anti-PI3Kγ antibody, Lot 121204) for 2 h at 4^0C with rotation. G-protein Sepharose beads (GammaBind®) were aliquoted into separate micro test tubes, 15 µl of slurry that approximately corresponds to 10 µl of the solid beads, washed and equilibrated with lysis buffer, and the total cell lysate was transferred into the micro test tubes with the Sepharose beads. Samples were incubated for another 2 h at 4^0C with rotation. The beads were washed three times with wash buffer, and 100 µl of 1xGLB was added to each sample. Samples were boiled at 95^0C for 5 minutes and resolved in 10% SDS-PAGE.

4.2.12. Silver staining and distaining of SDS-PAGE

SDS-PAGEs with the samples after PI3Kγ immunoprecipitation were silver-stained according to Schevchenko *et al*. (1996). Briefly, the proteins were fixed in the gel with 50% methanol and 5% acetic acid for 20 minutes, then in 50% methanol for 10 minutes, followed by 10 minutes washing in the water. Sensitisation was made with 0,02% of Sodium-thiosulphate for 1 minute, followed by two 1 min washing steps with water. The staining was performed with 0,1% of silver-nitrate for 20 minutes at the 4^0C in the dark, followed by two 1 min washing steps with the water. The staining was developed with 0,04% formaldehyde and 2% Sodium-carbonate. The developing was terminated with 5% acetic acid. The gels were stored in 1% acetic acid at 4^0C until further procedure.

Distaining of the protein samples excised from the gel was performed according to the Gharahdaghi *et al*. (1999) in order to improve in-gel digestion and enhance the sensitivity of mass spectrometry identification. Briefly, distaining solution was made by mixing equal volumes of 30 mM potassium hexacyanoferrate (III) and 100 mM sodium-thiosulphate. The peaces of the polyacrylamide gel containing the protein samples were incubated in 1,5 ml micro test tubes with 200 µl of distaining solution for 8 minutes in Thermomixer (Eppendorf) at the room temperature, 1000 rpm. The samples were washed four times with the water, one minute each time with vortexing.

4.2.13. In-gel tripsin digestion of proteins

The peaces of the PAA gel after the distaining procedure were digested in-gel according to Mortz *et al*. (1994) with small modifications. The samples were soaked in the mixture of acetonitrile/water (2:3) and incubated for 20 minutes in Thermomixer (Eppendorf), 1000 rpm, at room temperature. The liquid was removed and the peaces of gel were dried in the vacuum centrifuge for 20 minutes. The samples were rehydrated/alkalized with 50 µl of 50 mM ammonium-hydrogencarbonate for 15 minute also in Thermomixer, 1000 rpm at room

temperature. Acetonitril/drying/rehydrating steps were repeated three times in total, except that the very last rehydrating step was replaced with the following one: 50 µl of the tripsin solution, concentration 60 µg/ml (sequencing grade methylated trypsin), was added to the each sample and incubated on ice for 20-30 minutes. The residue of the tripsin solution was removed and the peaces of the gel were soaked in 50 mM ammonium-hydrogencarbonate. The tripsin digestion was performed at 30^0C for 15 min followed by 37^0C incubation over night. Afterwards, the incubation solution was removed and stored in the separate tubes, while new 100 µl of the acetonitrile/water (3:2) solution was added to the samples and incubated on the Thermomixer for another 3 hours, 1000 rpm at room temperature. The removed incubation solution and the very last extraction solution were combined and the samples were lyophilised. The samples were stored at -80^0C until the further analysis.

4.2.14. Mass spectrometry of peptides

Mass spectrometry of peptides was done in the collaboration with Institute for General Botany and Plant Physiology, FSU-Jena, Germany. Lyophilised samples were resuspended in 5 µl of water/acetonitrile/formic acid (95:5:0,1) prior to LC-MS analysis. Peptide analyses, analyte sampling, chromatography and acquisition of data were performed on a LC (Famous-Ultimate; LC-Packings, Amsterdam, Netherlands) coupled with an LCQ Deca XP ITMS (Thermoelektron, San Jose, CA) according to the manufacturer's instructions. The measured MS-MS spectra were matched with the amino acid sequences of tryptic peptides from the *H.sapiens* database in FASTA format. Raw MS-MS data were analysed by the Finnigan Sequest/Turbo Sequest software (revision 3.0; ThermoQuest, San Jose, CA). To identify corresponding loci, identified protein sequences were subjected to search with IPI data base (EMBL-EBI; http://srs.ebi.ac.uk).

4.2.15. RNA extraction

RNeasy mini kit (Qiagen) was used for the extraction of total RNA from the Jurkat cells according to the manufacturer's protocol. Briefly, $2x10^6$ Jurkat cells per sample were collected 24 h after the activation along with corresponding control samples. The cells were lysed with 350 µl of RTL buffer per sample, and the lysate was homogenised with a QIAshredder spin column. Equal volume of 70% ethanol was added to the homogenised lysate and the samples were applied on the RNeasy spin columns. The columns were centrifuged for 15 second at 13 000 rpm. On-column DNase digestion was performed and the columns were first washed with RW1 buffer followed with two washing with RPE buffer. Finally, RNA was eluted from each column with 30 µl of RNase-free water and stored at -20^0C.

4.2.16. Semi-quantitative RT-PCR

Reverse transcription was performed using AMV Reverse Transcriptase (Promega). 10 mg of total RNA was mixed with 100 pmol (dT)18 and 100 pmol random hexamer primer and heated at 70^0C for 5 min. Master mix, containing 5 µl AMV RT 5x buffer, 2.5 µl 10 mM dNTP's and 2.5 µl AMV Reverse Transcriptase (300 u/ml), was added, volume was adjusted with RNAse free water to 25 µl and probes were incubated at 42^0C for 1.5 h. Semi-quantitative RT-PCR protocol was performed as described by Halford *et al*. (1999) and Spadoni *et al*. (2003). Briefly, 5-fold or 10-fold serial dilutions of cDNA, prepared from total RNA, were used as RT-PCR templates. One twentieth volume of the resulting dilutions was subjected to PCR amplification using polymerase buffer (Qiagen) at 1x concentration containing 1.5 mM magnesium chloride, supplemented with 0.2 mM dNTP's, 50 pmol PCR of each primer and 2.5 units Tag polymerase (Qiagen) in total volume of 50 µl. The PCR cycling was performed using an Eppendorf Mastercycler. The cDNA mixture was denatured at 95^0C for 2 min, followed by 30 (β-actin) or 40 cycles (IL-2) of 30 sec. denaturation at

95^0C, 30 sec. annealing at the temperature specific for each primer set (61^0C for β-actin, 57^0C for IL-2), and 60 s extension at 72^0C. PCR products were separated by electrophoresis in 1,5% agarose gels containing EtBr (0.5 mg/ml) and visualised by UV transillumination. As an internal control of cDNA, PCR was performed on the same cDNA using primers for β-actin. The quantification of the signals' intensity was made with AIDA Image Analizer Software version 3.22 (Raytest, Germany).

5. References

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6. Abbreviations

AP-1 - activating protein-1

ATP - Adenosine 5'-Triphosphate

BAD – BCL2 antagonist of cell death

BCR - B cell receptor

BTK - Bruton tyrosin kinase

cAMP – cyclic adenosine monophosphate

Con A - Concavalin A

cpm- counts per minute

DAG – diacylglycero

DN - double negative thymocytes (CD4-/CD8-)

DP - double positive thymocytes (CD4+/CD8+)

EGF - epidermal growth factor

ELISA - Enzyme Linked Immunoabsorbent Assay

eNOS - endothelial nitric oxide synthetase

ERK - Extracellular Signal-Regulated Kinase

EtBr – ethidium bromide

FACS - Fluorescent Activated Cell Sorter

FDA - fluorescein diacetate

fMLP - N-formil-Met-Leu-Phe

FOXO - forkhead transcription factors

FYVE domain - highly conserved zinc-binding domain named after four proteins first identified to contain it: Fab1p, YOTB, Vac1p, and EEA1

GAPs - GTP-ase-activating proteins

GEFs - TP/GDP exchange factors

GLB – gel loading buffer

GPCR – G-protein coupled receptor

GTP - Guanosine 5'-Triphosphate

IL-2 – interleukin 2

IL-2R – interleukin 2 receptor

INF-γ - interferon-γ

Ins(1,4,5)P_3 - inositol(1,4,5)triphosphate

IONO – Ionomycin

ITAM - immunoreceptor tyrosine-based activation motifs

Itk - inducible T cell kinase

IκBK - IκB kinase

KI – knock-in

KO – knock-out

LAT - linker of activated T cells

Lck- lymphocyte-specific protein-tyrosin kinase

LPA - L-a-lysophospatidic acid

LPS – lipopolysaccharides

MAPK - mitogen activated protein kinase

MCP-1 - monocyte chemoattractant protein 1

MDC - macrophage derived chemokine

MHC - major histocompatibility complex

MIP-5 - macrophage inflammatory protein-5

MS - mass-spectrometry

NFAT - nuclear factor of activated T cells

NFκB - nuclear factor kappa B

NK cells – natural killer cells

p38 – MAPK protein of 38 KDa

PBS – Phosphate Buffer Saline

PCR – polymerase chain reaction

PDE3B - phosphodiesterase 3B

PDGF - platelet-derived growth factor

PDK1 – phosphoinosytide-dependent protein kinase 1

PH domain – plekstrin homology domain

PHA – phytohemagglutinin

PI - propidium-iodide

PI3K - phosphatidilinositide 3-kinase

PIK domain - phosphatidilinositide kinase domain

PKA - protein kinase A

PKB/Akt - protein kinase B, also known as Akt

PKC - protein kinase C

PKD - protein kinase D

PLCγ1 - Phospholipase C gamma 1

PtdIns – phosphatidylinositol

PtdIns(1,4,5)P_3 - phosphatidilinositol(1,4,5)triphosphate

PtdIns(3,4,5)P_3 - phosphatidilinositol(3,4,5)triphosphate

PtdIns(4,5)P_2 - phosphatidilinositol(4,5)biphosphate

PtdIns3P - phosphatidilinositol(3)phosphate

PTEN - phosphatase and tensin homologue

PTK - protein tyrosin kinase

PVDF - polyvinylidene difluoride

PX domain - pox homology domain

RANTES - Regulated on Activation, Normal T cell Expressed and Secreted

Ras-BD - Ras-binding domain

Rlk – resting lymphocyte kinase

SAPK/ JNK - Stress-Activated Protein Kinase/Jun N-terminal Kinase

SDF1 - stromal-cell-derived factor 1

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SGK - serum- and glucokorticoid-iduced protein kinase

SH2 - Src-homology domain 2

SHIP - SH2 domain-containing 5 inositol phosphatase

SLE - systemic lupus erythematosus

SLP-76 - Src homology 2-domain-containing leukocyte protein of 76 kDa

TCR - T cell receptor

TPA or PMA - phorbol 12-myristate 13-acetate

VEGF - vascular endothelial growth factor

Vps34p – vacuolar protein sorting protein of 34 KDa

WT – wild-type

ZAP-70 - ζ–chain associated protein kinase of 70 kDa

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