universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

The PI3K/PTEN pathway in innate immune responses with particular focus on atherosclerosis and rheumatoid arthritis

angestrebter akademischer Grad

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CONTENT

ABSTRACT

Conversion of PtdIns-4,5-biphosphate (PIP2) to PtdIns-3, 4,5-triphosphate (PIP3) by Phosphoinositide 3-kinases (PI3K) is crucial in various diverse cellular processes such as metabolism, growth, survival and migration. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is the most famous opponent of PI3K activity. Knockout of PTEN and thus hyperactive PI3K signaling was shown to limit immune responses and excessive host tissue damage. We hypothesized that myeloid specific deletion of PTEN modulates inflammatory responses in murine models of chronic inflammation.

In atherosclerosis, myeloid specific PTEN knockout led to increased foam cell formation *in vitro* independent of scavenger receptor expression. Atherosclerotic plaque formation was not significantly altered in mice aged 16 weeks, but an effect is expected to be seen in older mice.

In rheumatoid arthritis, PTEN deficiency in myeloid cells was demonstrated to be beneficial for mice suffering from Collagen-induced arthritis (CIA). In this model of non-infectious exacerbated immune response, mice with sustained PI3K activation had a significantly lower or no response at all. Histology revealed less inflammation and bone erosion in synovial joints of PTEN -/- mice and additionally, plasma cytokine release was diminished.

PTEN deficient bone-marrow derived dendritic cells also demonstrated anti-inflammatory properties, such as reduced IL-6 production and release. Important to mention in this context are the diminished IL-12 and IL-23 levels, which are important in T helper cell differentiation.

To sum up, PI3K signaling was shown to act anti-inflammatory and inhibition of PTEN might be a potent target for treatment of various autoimmune diseases such as rheumatoid arthritis and possibly also multiple sclerosis.

ZUSAMMENFASSUNG

Umwandlung von PtdIns-4,5-biphosphat (PIP2) zu PtdIns-3, 4,5-triphosphat (PIP3) durch Phosphoinositid 3-kinasen (PI3K) ist essentiell in verschiedenen zellulären Prozessen wie z. B. Metabolismus, Wachstum, Überleben und Chemotaxis. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) ist der bekannteste Gegenspieler zur PI3K Aktivität. Durch Knockout von PTEN und dadurch Verstärkung des PI3K Signalweges wurde bereits gezeigt, dass die Immunantwort und auch übermäßiger Gewebsschaden vermindert war. Daraufhin stellten wir die Hypothese auf, dass Defizienz von PTEN einen bedeutenden Einfluss auf die Immunantwort in Mausmodellen für chronische Entzündung hat.

Im Modell für Atherosklerose führte PTEN Knockout in myeloiden Zellen zu verstärkter Bildung von Schaumzellen in einem Prozess unabhängig von der Expression von Scavenger Receptors. Die Bildung von atherosklerotischen Plaques war allerdings nicht signifikant verändert in 16 Wochen alten Mäusen. Wir erwarten aber, dass ältere PTEN Knockout Mäuse sehr wohl unterschiedlich reagieren.

Diese Diplomarbeit zeigt, dass PTEN Defizienz in myeloiden Zellen sich günstig auswirkt auf KOllagen-induzierte Arthritis. In diesem Mausmodell für rheumatoide Arthritis zeigten Mäuse mit einer verstärkten PI3K Aktivierung signifikant reduzierte oder sogar keine Krankheitszeichen. In histologischen Gelenksschnitten von PTEN -/- Mäusen wurde verminderte Entzündung und Knochenerosion beobachtet. Außerdem war die Freisetzung von Zytokinen ins Plasma vermindert.

In vitro wurden PTEN defiziente dendritische Zellen aus Knochenmark gezüchtet, welche auch die oben beschriebenen anti-inflammatorischen Eigenschaften, wie verminderte IL-6 Produktion und Freisetzung zeigten. Wichtig dabei ist auch die Verringerung der IL-12 und IL-23 Level, die wichtig in der T Helfer Zell Differenzierung wichtig sind.

Zusammenfassend wurde gezeigt, dass der PI3K Signalweg anti-inflammatorisch wirkt und dass PTEN ein potentielles Zielgen zur Behandlung von Autoimmunkrankheiten wie rheumatoider Arthritis darstellen kann.

1. INTRODUCTION

1.1 Signaling via the PI3K/PTEN pathway

1.1.1 The Phosphoinositide 3-kinase family

Phosphoinositide 3-kinases, abbreviated PI3K, are signal-transducing lipid kinases that are defined as catalysing the phosphoylation of phosphoinositide PtdIns-4,5-biphosphate (PIP2) on the D3 carbon of the inositol ring to produce PtdIns-3,4,5-triphosphate (PIP3). (Figure 1.1)

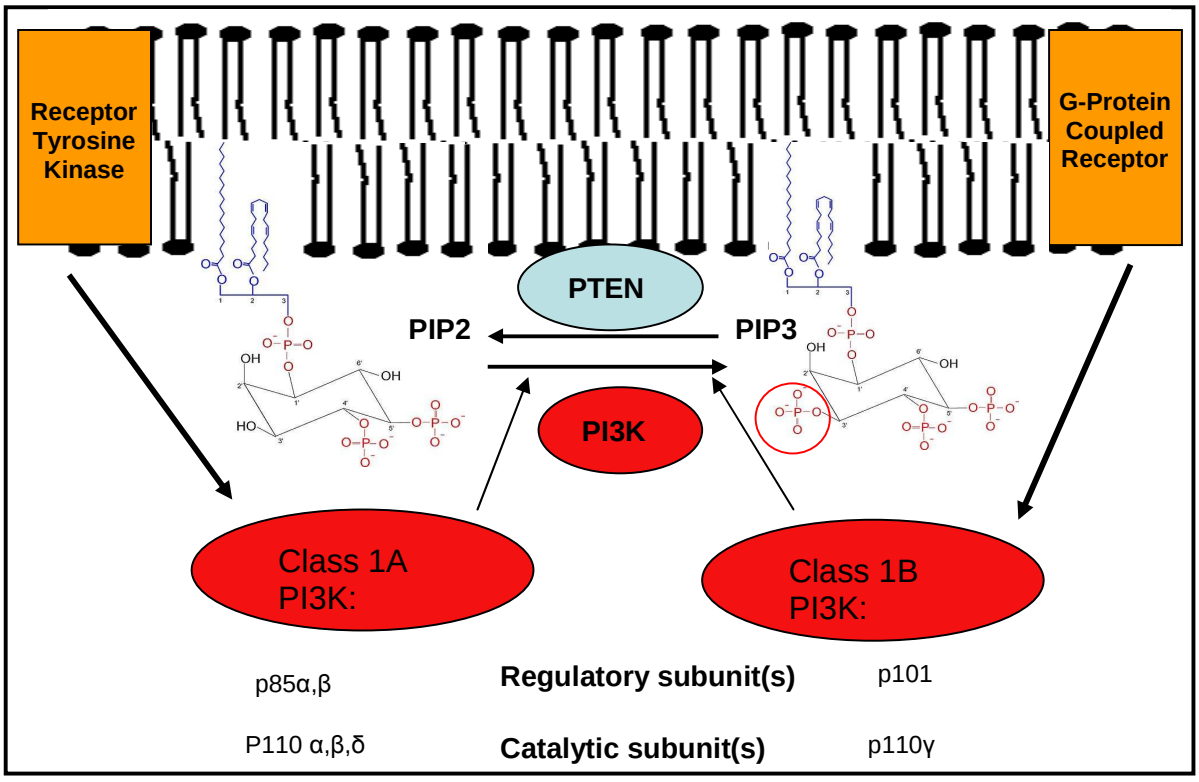


Figure 1.1: Schematic representation of the activation of different class 1 PI3-kinases. Class 1 A PI3K is activated by RTKs and consists of catalytic subunit p110α, p110β or p110δ associated with regulatory subunit p85α or p85β. Class 1 B PI3K is activated by GPCRs and consists of catalytic p110γ and regulatory p101. Both subclasses generate PI(3,4,5)P3 out of PI(4,5)P2. PTEN as a PI3K antagonist dephosphoylates PIP3 at position 3. (modified from (Gunzl and Schabbauer, 2008))

PI3Ks have been quite early correlated with viral oncoproteins (Whitman et al., 1985) and are since then seen as important players in human cancer. (Marone et al., 2008) Only recently, PI3K has shown to be implicated in the pathogenesis of inflammatory diseases, with effects being more or less independent form originally found PI3K functions such as cell growth, proliferation and survival.(Fruman et al., 1999)
According to Domin & Waterfield, 3 main classes of PI3K were defined based on structure and regulation. (Domin and Waterfield, 1997) (Figure 1.2) The probably most important one and thus the best understood is Class I. It can be subdivided into class IA and class IB.

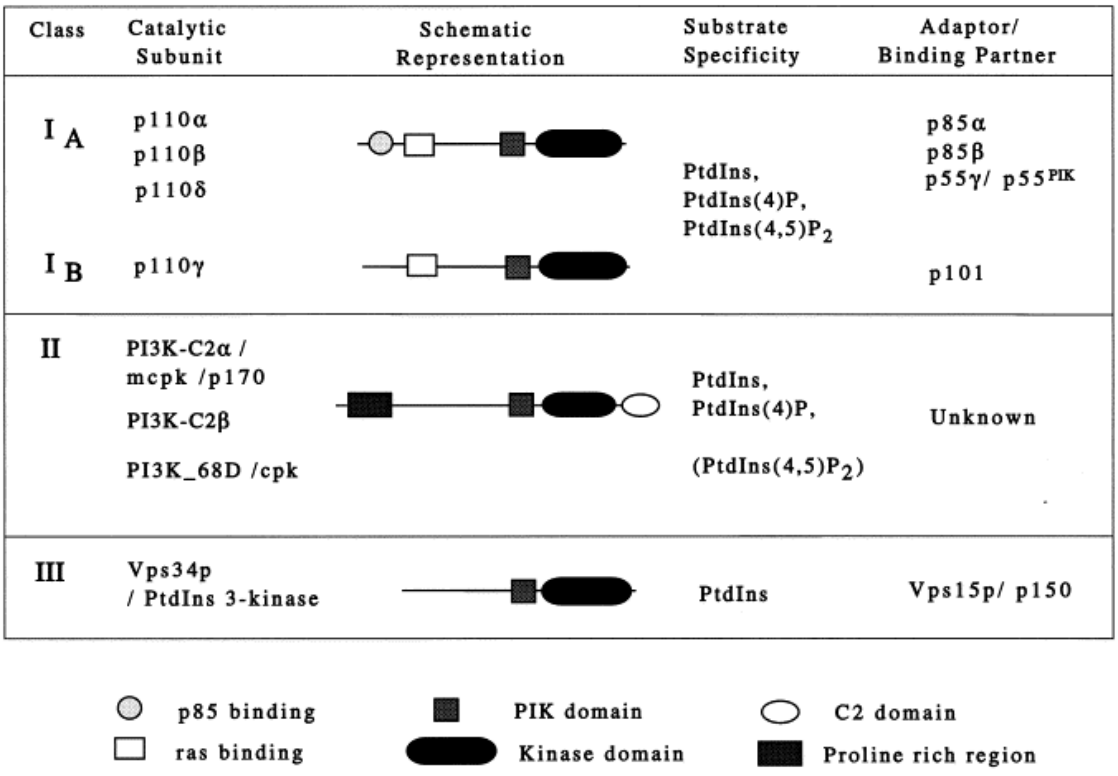


Figure 1.2: The 3 different classes of PI3-Kinases as defined by Domin & Waterfield. ((Domin and Waterfield, 1997))

Class 1A enzymes consist of a catalytic domain (p110α, p110β or p110δ) associated with regulatory subunits (p85α, p85β, p55α, p50α or p55γ).(Fruman et al., 2000)

Class 1B consists of p110γ as a catalytic subunit and p101 as regulatory domain.(Carpenter et al., 1990)

1.1.2 Activation of PI3 kinases

PI3Kinases can be activated upon a plethora of stimuli including growth factors, cytokines, hormones and neurotransmitters. In terms of activation, there are some major differences between class 1A and class 1B PI3K. Whereas the first becomes mainly activated by receptor tyrosine kinases and ligands such as insulin or growth factors, the latter is stimulated by G-protein coupled receptors, which are influenced mainly by chemokines.(Figures 1.1 and 1.3)

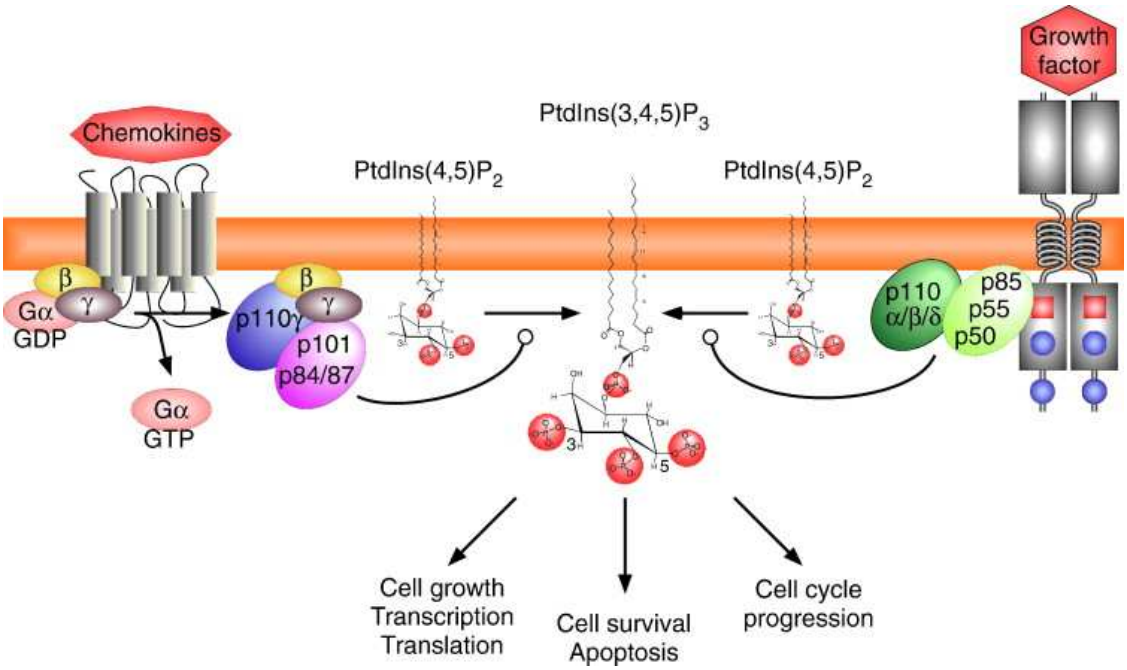


Figure 1.3: Simplified scheme for activation and downstream effects of PI3K.(Marone et al., 2008)

1.1.3 Cellular processes upon PI3K activation

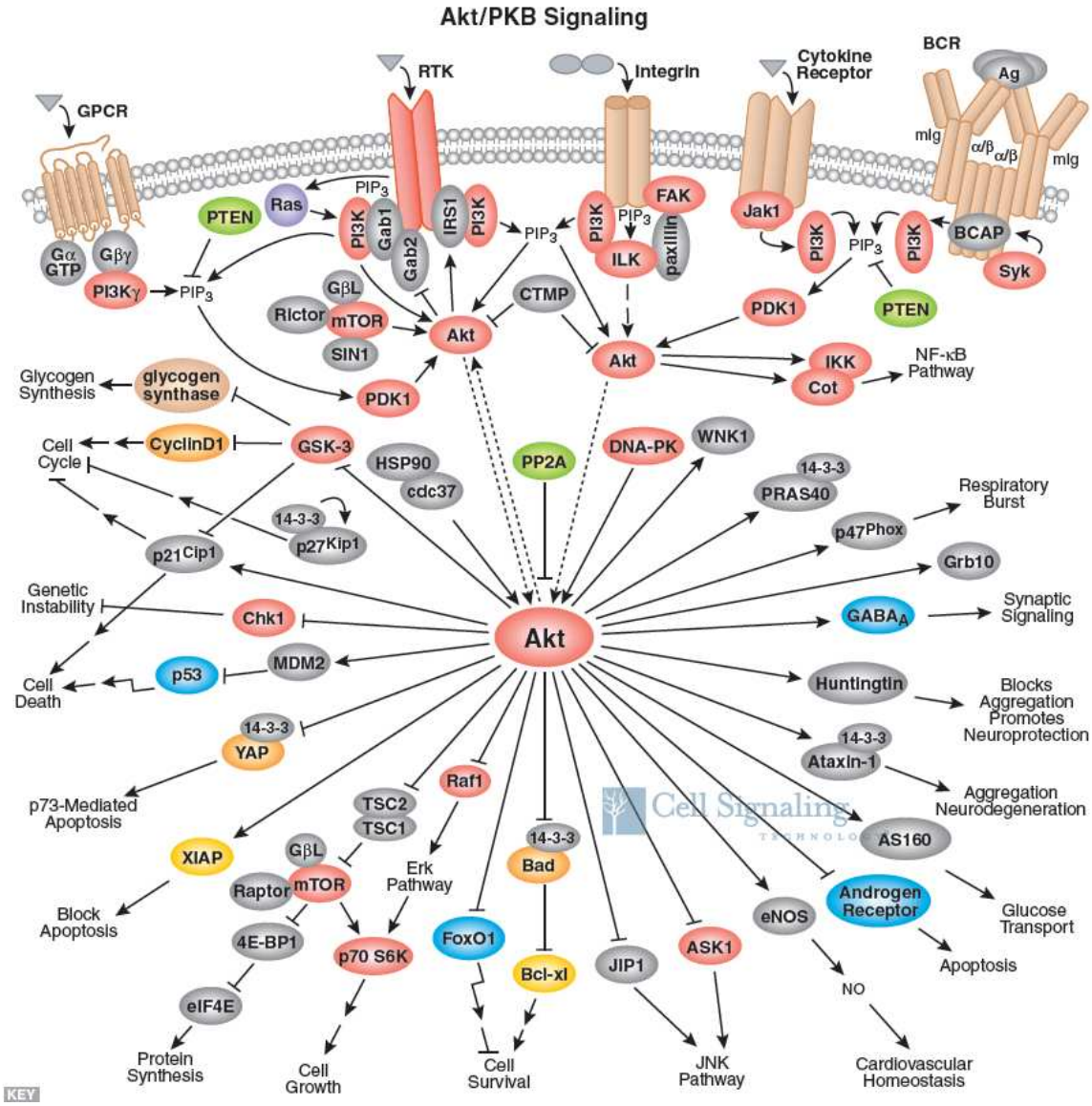


Figure 1.4: Summary of the PI3K-Akt signalling pathway (by Cell Signaling technologies)

The formation of PIP3 leads to the activation of downstream targets containing a pleckstring homology (PH) domain. The most important in this context is Akt, also known as protein kinase B (PKB), which is a serine/threonine protein kinase

discovered in 1995.(Franke et al., 1995) Burgering and Coffer first suggested a role for Akt/PKB in PI3K signal transduction by inhibiting PI3K with Wortmannin and PI3K silencing. (Burgering and Coffer, 1995)
Akt has now been reported to be involved in many cellular processes, notably cell proliferation and survival. Today there are 3 isoforms of Akt described: AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ, which are all highly homologous in terms of peptide sequence. (Manning and Cantley, 2007) Through binding to PIP3, Akt is phosphoylated at Thr308 and Ser473, mainly by PDK1 (phosphoinositide dependent kinase 1) and mTORC2 (mammalian target of rapamycin complex 2).

Phosphoylated Akt then influences a variety of downstream genes depicted in figure 1.4, for example inhibition of GSK-3 and Bad, which are responsible for the anti-apoptotic effects of PI3K. Akt is considered as a classical oncogene, as many regulated genes influence cell growth, proliferation and survival.

Negative regulation of Akt signaling can happen directly via dephosphoylation by PHLPP or indirectly through inhibition of PI3K signaling.

1.1.4 PTEN and negative regulation of PI3K signaling

A negative regulator of the PI3K pathway is the phosphatase and tensin homologue deleted on chromosome 10, abbreviated PTEN. PTEN antagonizes PI3K by removing a phosphate group from PI(3,4,5)P3 to generate PI(4,5)P2. It has originally been found as an important tumour suppressor gene, as it stops PI3K-mediated proliferation and survival, but is now also seen as important in inflammatory context. (Li et al., 1997; Suzuki et al., 2001) It is a dual-specificity phosphatase and hydrolyzes ester bonds on thyrosine and serine/threonine residues of lipid substrates. (Myers et al., 1997)

Regulation of PTEN takes place at multiple levels, the most prominent one being probably phosphoylation and thus inactivation by casein kinase II. (Gericke et al., 2006)
Another inositol phosphatase family is SHIP (Src homology 2-containing-inositol 5'-phosphatase).(Kalesnikoff et al., 2003)

1.1.5 PI3K/PTEN in innate immune reactions

It is still a controversially debated issue in research whether the PI3K/PTEN pathway acts predominantly pro- or anti-inflammatory. Using pharmacologic inhibition of PI3K signalling by the fungal metabolite Wortmannin or by the synthetic inhibitor LY294002, different studies found proofs for reduced as well as enhanced pro-inflammatory gene expression. These contradictory effects might be explained by non-specific inhibition of targets other than PI3K. (Gunzl and Schabbauer, 2008)
Using genetically modified mice, a clearer picture of the involvement of PI3K in innate immune responses can be drawn. In p85 alpha deficient cells, IL-12 expression upon TLR stimulation has been shown to be elevated. (Fukao et al., 2002) Yu et al. demonstrated that flagellin induced activation of PI3K via TLR5 and that chemical inhibition of PI3K as well as PI3K knockout in mice induced MAPK activation followed by enhanced expression of proinflammatory genes such as IL-6 and KC. (Yu et al., 2006) In complete PI3K knockout mice generated by L. Cantley and in PTEN deficient cells it was shown that the PI3K-Akt pathway inhibits MAPK activation upon LPS stimulation, but that the NF-kappaB pathway was only marginally affected. (Luyendyk et al., 2008) Contrary to this are findings made by Artin et al. who claim that AKT and GSK3b have a direct effect on NF-kappaB activity. (Martin et al., 2005)
In terms of inflammatory modulators, reduction in PI3K signalling leads to elevated TNF-alpha and IL-6 levels. (Luyendyk et al., 2008) In animal models of sepsis it has been shown that inhibition of PI3K by Wortmannin leads to increased cytokine production and reduced survival time in endotoxemia or cecal ligation and puncture-induced polymicrobial sepsis. (Schabbauer et al., 2004; Williams et al., 2004)

PTEN has also recently become of interest in migration of neutrophils, where it functions as a discriminator that prioritizes responses to different chemoattractant signals. (Heit et al., 2008) This migratory defect has also been observed in our studies using flPTEN LysMcre mice in pneumonia. (Matt et al., manuscript in preparation)

Recent data obtained in our laboratory suggest that the anti-inflammatory properties of the PI3K pathway are at least in part mediated by IL10/DUSP regulation. (Günzl et al., manuscript in preparation) Figure 1.5 depicts our current working hypothesis.

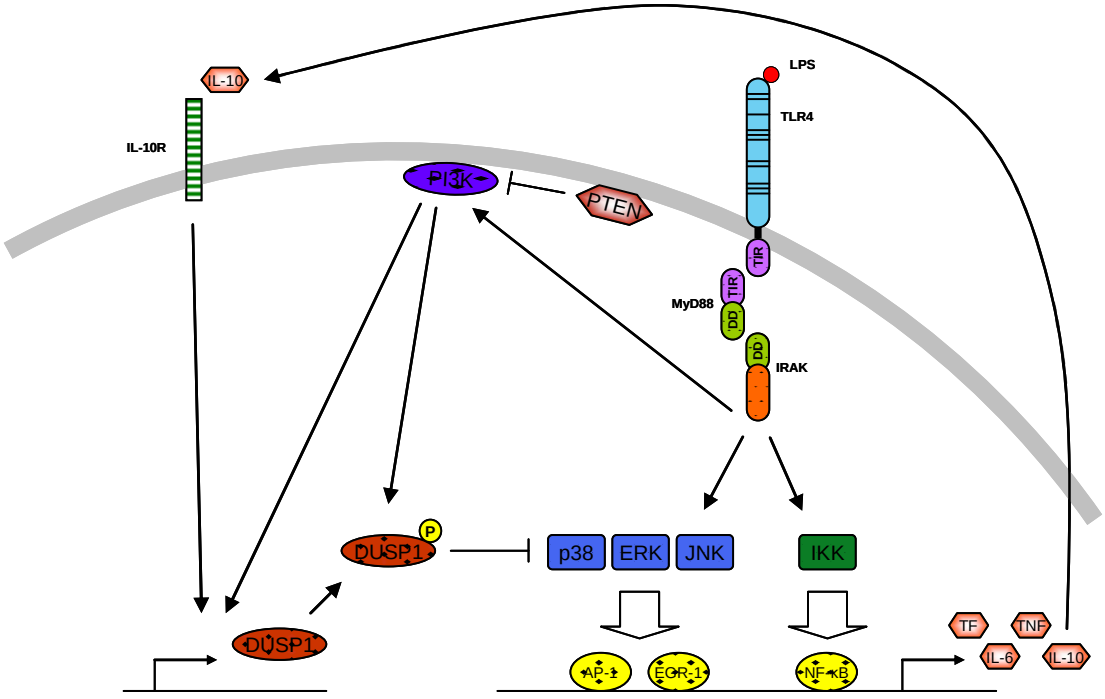


Figure 1.5: PI3K negatively regulates TLR signalling by dampening the MAP-Kinase response through regulation of DUSP1 on mRNA and protein level. (Günzl et al., manuscript in preparation)

1.1.6 Knockout mice available to study the PI3K/PTEN pathway

In this diploma thesis, different types of knockout mice were used. To study PTEN function, a complete knockout mouse has been designed by Cristofano et al. (Di Cristofano et al., 1998) As it shows embryonic lethality at approximately day 7.5,

conditional PTEN knockout mice using the cre-loxP system have been developed. To study the function of myeloid cells in immune function, we used PTEN fl/fl LysMcre mice in which the PTEN gene is excised after induction of the Lysozyme M promoter. (Clausen et al., 1999)

Another mouse strain using cre recombinase is the ap2cre flPTEN mouse, where PTEN is specifically excised in adipose tissue. (Kurlawalla-Martinez et al., 2005) Recent results obtained using conditional PTEN knockout mice have been reviewed by Suzuki et al. (Suzuki et al., 2008)

For PI3K itself, many different strains are available. Of particular interest for our research purposes was the PI3K class 1A subunit p85α, for which two different knockout strains have been developed in the past. The first, published by L. Cantley (Fruman et al., 2000), lacks all isoforms (p85 alpha, p55 alpha and p50 alpha) and shows perinatal lethality with only a fraction of animals surviving the first weeks postnataly. One of the most obvious phenotypes of this mouse is excessive liver necrosis. The second available p85α-deficient mouse strain, developed by S. KOyasu, still expresses the smaller variants of p85α, namely p50α and p55α, leading to a quite normal phenotype. It can thus be concluded that these isoforms can at least partially compensate for the deletion of the full gene product. (Terauchi et al., 1999)

1.2 The innate immune system

1.2.1 Inflammation

Inflammation (Latin: inflammo – to set on fire) is the reaction of the body to noxious stimuli and conditions such as infections and tissue injury. (Medzhitov, 2008) Its primary goal is to remove potentially dangerous substances and allow tissue regeneration.

Inflammation can be subdivided into acute and chronic forms. Acute inflammation represents the immediate answer of the body to invading pathogens or tissue injury

and is usually cleared within a period of a few days. If however the inflammatory stimulus, e.g. allergens, bacteria or chemicals, persists, the inflammation cannot be resolved. This situation is then called chronic inflammation. Another example of a pathological situation is dysregulated inflammation as it is the case in septic patients.

1.2.2 The human immune system

The human immune system with its two arms, innate and adaptive, has to fulfil the 3 basal principles to help its host survive: (Beutler, 2004)

- Recognition of pathogens (as many as possible)
- Kill pathogens upon recognition
- Establish self-tolerance

In this system, mechanisms and cells of innate immunity provide initial defense and act within hours upon infection. It consists mainly of physical barriers, phagocytes, complement, NK cells and its respective receptors and will be discussed in greater detail later on. As a contrast, adaptive immune responses are more specific and act later on in the time course of infection. The respective cells are T and B cells, but also antibodies as the humoral part. (Medzhitov and Janeway, 1998) As our research mainly focused on innate innate immune responses and T helper cell activation, the other components of the adaptive immune system will not further be described.

1.2.3 The innate immune system - receptors

1.2.3.1 Pattern recognition receptors

Pattern recognition receptors (PRRs) are receptors of the innate immune system that recognize pathogen-associated molecular patterns (PAMPs) and trigger antimicrobial functions of leukocytes. (Medzhitov, 2001) These receptors can either be membrane-bound, cytoplasmatic or secreted components. Examples for such receptors are toll-like receptors (TLRs), scavenger receptors and NOD-like receptors (NLRs). (Medzhitov, 2008)

1.2.3.2 Toll-like receptors

TLRs have been first described by Medzhitov et al. in 1997 as human homologues of the Drosophila melanogaster protein toll and were shown to activate the NF-kappaB pathway and subsequently adaptive immune system. (Medzhitov et al., 1997) Probably the most prominent example for a PRR-PAMP interaction is the recognition of the bacterial endotoxin lipopolysaccharide (LPS) by TLR-4, first described by B. Beutler's group in 1998. (Poltorak et al., 1998) For correct recognition of LPS, accessory molecules like LBP (LPS binding protein), CD14 and MD-2 are necessary. (Medzhitov, 2001) TLR-2 recognizes a wide variety of microbial surface products as summarized in figure 1.6. This is possible due to interaction with TLR1 and TLR6.

TLR-3 is predominantly expressed in dendritic cells and has been shown to recognize dsRNA. (Alexopoulou et al., 2001) TLR-5 is a sensor for bacterial flagellin which in turn activates the NF-kappaB pathway and thus TNF-alpha. (Hayashi et al., 2001)

Another innate immune receptor is TLR-9 which recognizes unmethylated CpG DNA. (Hemmi et al., 2000)

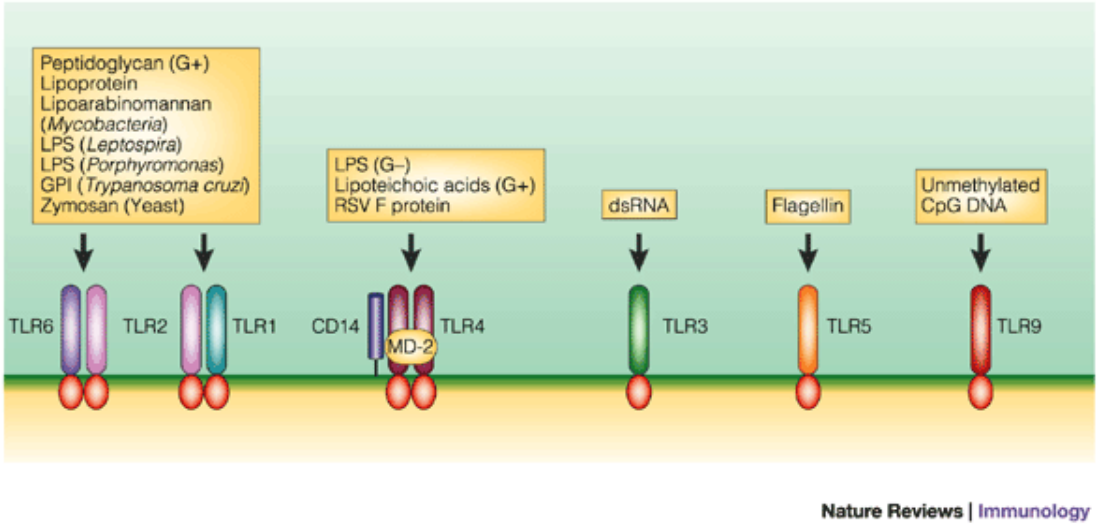


Figure 1.6: Toll-like receptors recognize a variety of pathogens, for example microbial surface proteins such as LPS, Flagellin or PGN. (Medzhitov, 2001)

Signalling downstream of TLRs is often shared and requires MyD88, TOLLIP (Toll-interacting protein), IRAK (IL-1R associated kinase) and TRAF-6 (TNF-receptor associated factor 6). Toll-like receptors are also seen as important activators of adaptive immunity as summarized in figure 1.7.

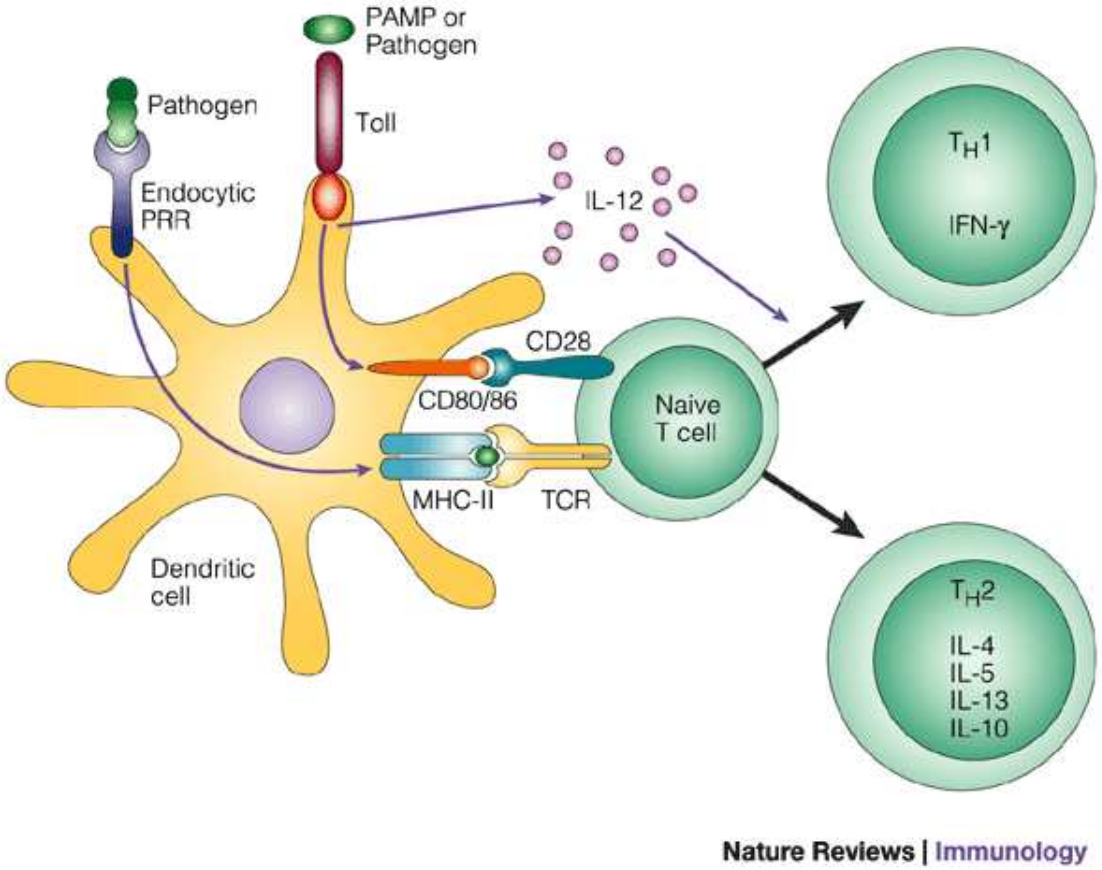


Figure 1.7: TLRs expressed on dendritic cells (DCs) are important stimulators of the adaptive immune system. Pathogens bind to Pathogen Recognition Receptors (PRRs)/TLRs on DCs and stimulate naïve T cells through direct interaction or release of cytokines. As a consequence, T cells differentiate into different subtypes such as Th1 or Th2. (Medzhitov, 2001)

1.2.3.3 Scavenger receptors

Scavenger receptors (SRs) are a six subgroups comprising family of glycoproteins present on the cell surface that were originally defined by binding modified low density lipoproteins (mLDL). SRs play important roles in atherogenesis but also more

general in innate immune regulation by macrophages. (Peiser and Gordon, 2001) The principal receptors responsible for uptake of modified LDL into macrophages and subsequent foam cell formation are SR-A and CD36. (Kunjathoor et al., 2002) According to Peiser and Gordon, SRs not only play a role in endocytosis of mLDL, but also in adhesion, phagocytosis and pattern recognition. (Peiser and Gordon, 2001) Oxidized phospholipids, especially four oxidized 1-palmitoyl-2-arachidonyl-phosphatidylcholine (oxPAPC) species, have been shown to serve as ligands for SR CD36 and are enriched in atherosclerotic lesions. (Podrez et al., 2002a; Podrez et al., 2002b)

1.2.4 The innate immune system – cells of the (innate) immune system

1.2.4.1 Macrophages

Macrophages are mononuclear phagocytes derived from blood monocytes that belong to the myeloid lineage of leukocytes. They are present all over the body and appear as diverse species in different organs, for example brain microglial cells or Kupffer cells present in the liver. (Beutler, 2004) Also for research purposes, different macrophage lineages have to be considered, like alveolar, bone-marrow-derived, thioglycollate-elicited, or osteoclasts.

Macrophages as well as dendritic cells play an essential role in the initial phase of inflammation as they recruit polymorphonuclear leukocytes (neutrophils, basophils, eosinophils) to the site of infection. (Beutler, 2004) Macrophages are also able to engulf and kill cells, but the more specialized cells to do this task are neutrophils, which are short-lived cells present in high numbers in conditions of severe infection.

In terms of activation of the adaptive immune response, myeloid cells of the innate immune system play a vital role as antigen presenting cells (APCs) and in producing pro-inflammatory cytokines such as IL-12, CD40L, IL-1, type I interferons, and TNF. (Beutler, 2004)

1.2.4.2 Dendritic cells

Dendritic cells are cells of myeloid or lymphoid origin that were first described in 1973 as cells similar to macrophages present in small numbers lymphoid organs. (Steinman and Cohn, 1973) Dendritic cells (DC) are now known to influence many different classes of lymphocytes (T, B, NK cells) and many types of T cell responses (Th1/Th2/Th17, regulatory T cells, peripheral T cell deletion). (Lebre and Tak, 2008) In their function as professional antigen presenting cells, they migrate to the T cell zones in lymphoid organs and activate T cells to respond to antigen by secreting cytokines and by direct cell-cell contact via B7 receptor (on DC) – CD28 (on lymphocyte) interaction. (Guermonprez et al., 2002)

1.2.4.3 T helper cells

T cells are divided into CD8 positive cytotoxic and CD4 positive T helper cells. It has long been established, that T helper cells can further be separated into Th1 and Th2 subsets. In this respect, IL-12 is an important cytokine that drives CD4 positive T cells into Th1 helper cell differentiation, which then produce IFN-gamma. The Th1 driven immune response is cell-mediated, B-cells mainly produce opsonising IgG antibodies, pathogens are immediately destroyed with the side effect of massive tissue destruction. (Szabo et al., 2003) There is no evidence for a Th2-inducing cytokine in dendritic cells that induce Th2 differentiation, but it is assumed, that indirect effects such as inhibition of IL-12 production by IL-10 favour a Th2 response. (Moser and Murphy, 2000) Th2 cells are important in humoral immunity and are thought to play a role in allergy pathogenesis.

Only recently, a new subtype of Th cells has been described. Its main characteristic is that it produces IL-17 and is thus called Th17. Originally described in 2005, development and function of Th17 cells now provide a fast-evolving area of research. (Harrington et al., 2005) The main stimulus for Th17 cell development seems to be IL-6, but also mediated at least in part by TGF-beta and IL-1. IL-23 is thought to be essential in final steps of Th17 differentiation. (Dong, 2008)

The process of differentiation is summed up in figure 1.8.

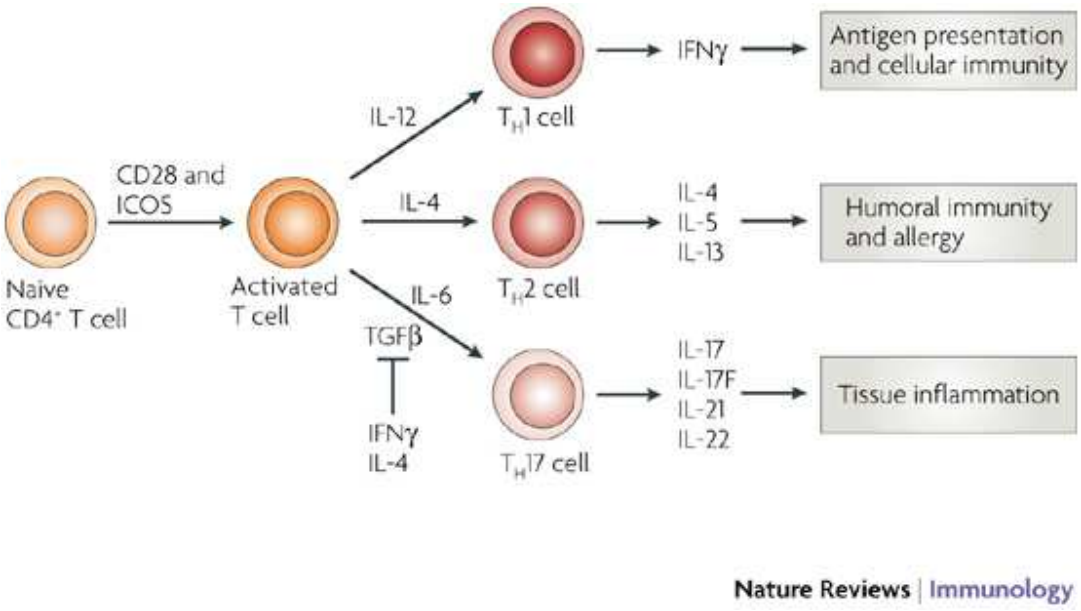


Figure 1.8: Differentiation of naïve CD4 positive T cells to T helper cell subsets. (Dong, 2008)

1.3 Atherosclerosis

1.3.1 Atherosclerosis - epidemiology

Cardiovascular disease (CVD) is still the single most common cause of death in Western countries. (Heald et al., 2006) As an example, 38% of all deaths in the United States in 2001 were due to CVD. (Murray et al., 2006) Among these, coronary heart disease and stroke account for the major part. Ischaemic heart disease and cerebrovascular disease are the leading causes of death in low- as well as high-income countries. (Figure 1.9)

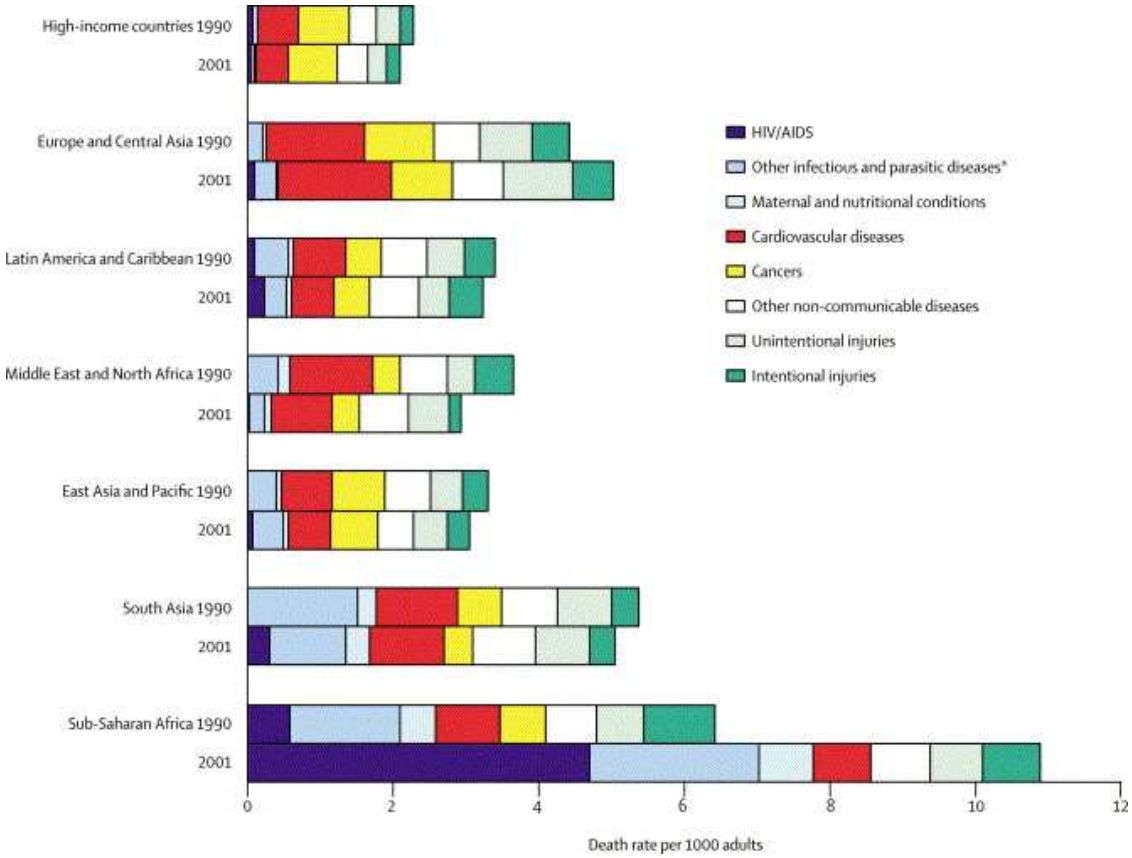


Figure 1.9: Death rates by disease group and region in 1990 and 2001 for adults aged 15–59 years. The red areas represent cardiovascular diseases, which are the most common cause of death in Europe and Central Asia. (Lopez et al., 2006)

Most important risk factors for these diseases are high arterial blood pressure, elevated cholesterol and glucose levels and cigarette smoking. (Devereux and Alderman, 1993) As the number of risk factors increases, so does the severity of atherosclerosis, even in young people. (Berenson et al., 1998)

Atherosclerosis as a disease affecting the blood vessels is seen as the main physiological contributor to CVD. The relationship between hypercholesteraemia and atheroma formation has been established for a long time. A more recent approach is the association of inflammatory processes and atherogenesis. (Libby, 2002)

1.3.2 Vessel wall anatomy

Atherosclerosis is a disease caused by accumulation of lipids and fibrous elements in the large arteries. (Libby, 2002) To further describe formation of these lesions, known as atherosclerotic plaques, basic microscopic anatomy of the arterial wall is necessary.

Directly surrounding the arterial lumen is the intima, which consists of a single epithelial layer and a basement membrane. The middle layer, called media, contains mainly smooth muscle cells embedded in extracellular matrix. The outermost layer is the tunica externa, also known as adventitia, composed of connective tissue. The layers are separated by internal and external elastic laminae. (Figure 1.10)

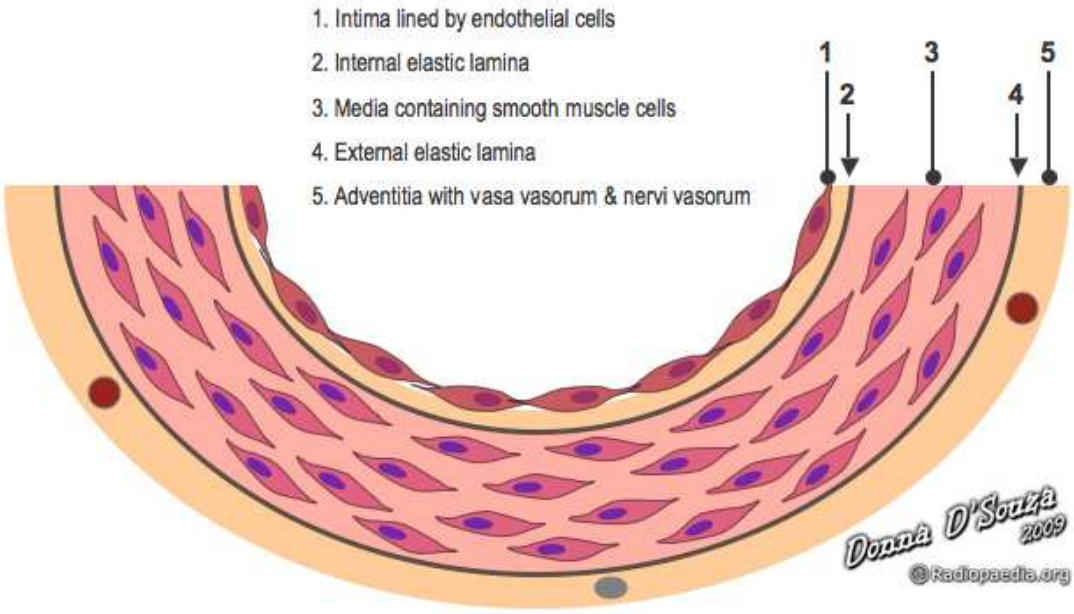


Figure 1.10: Anatomy of a healthy vessel wall: Surrounding the vessel lumen are Intima, Media and Adventitia, each separated by elastic laminae. (Picture from http://radiopaedia.org/articles/histology-of-blood-vessels)

In pathological situations, different inflammatory cells, dead cells and cholesterol accumulate in the arterial intima and thus form an atherosclerotic plaque. A schematic view of an advanced atherosclerotic plaque is depicted in figure 1.11.

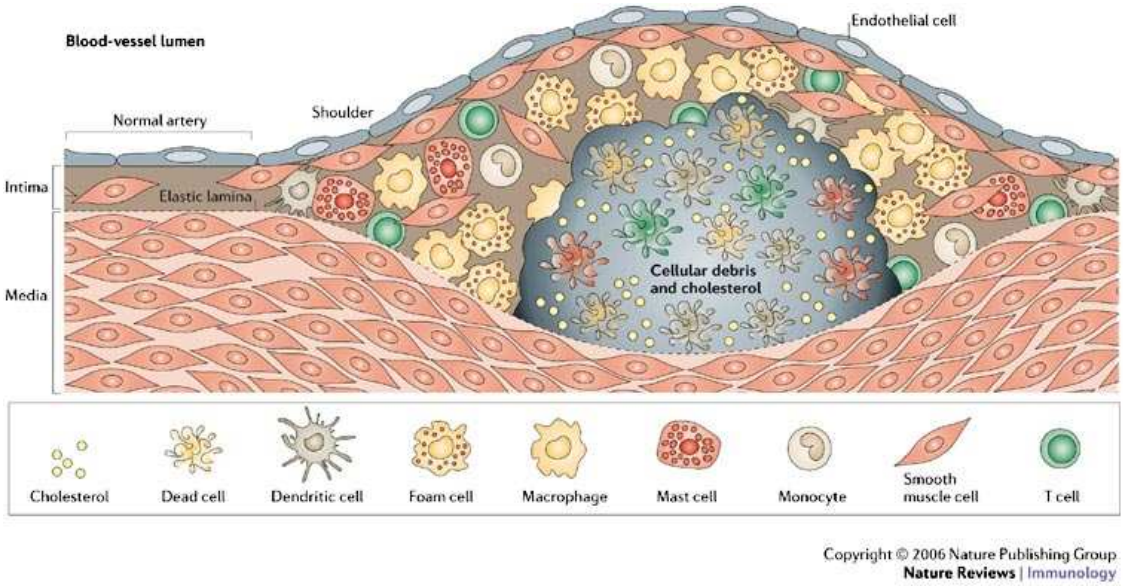


Figure 1.11: Content of an atherosclerotic plaque. Accumulation of lipid loaden foam cells and macrophages in the arterial intima is the main characteristic of an atherosclerotic plaque. In late lesions, cellular debris and cholesterol accumulate in the necrotic core. (Hansson and Libby, 2006)

1.3.3 Atherogenesis

Atherosclerotic plaque formation starts with expression of vascular cell-adhesion molecule 1 (VCAM-1) on endothelial cells, caused by intimal cholesterol accumulation. (Cybulsky et al., 2001) VCAM-1 is the main contributor to attract monocytes and later also T lymphocytes to the endothelium. After adhering to the endothelium, monocytes start entering the intima. Responsible for this process is mainly the chemokine macrophage chemotactic protein-1 (MCP-1). (Gu et al., 1998)
Macrophage colony-stimulating factor (M-CSF) then allows intimal monocytes to differentiate into mature macrophages. This is accompanied by enhanced expression of scavenger receptor A (SRA) and CD36. Through these cell surface receptors, macrophages are able to take up lipids from the periphery and form so-called foam cells, named after their characteristic lipid-laden cytoplasmatic droplets. In advanced lesions, death of foam cells and resulting cell debris leads to further inflammation which worsens the outcome.
Figure 1.12 nicely summarises the role of mononuclear phagocytes in atherogenesis.

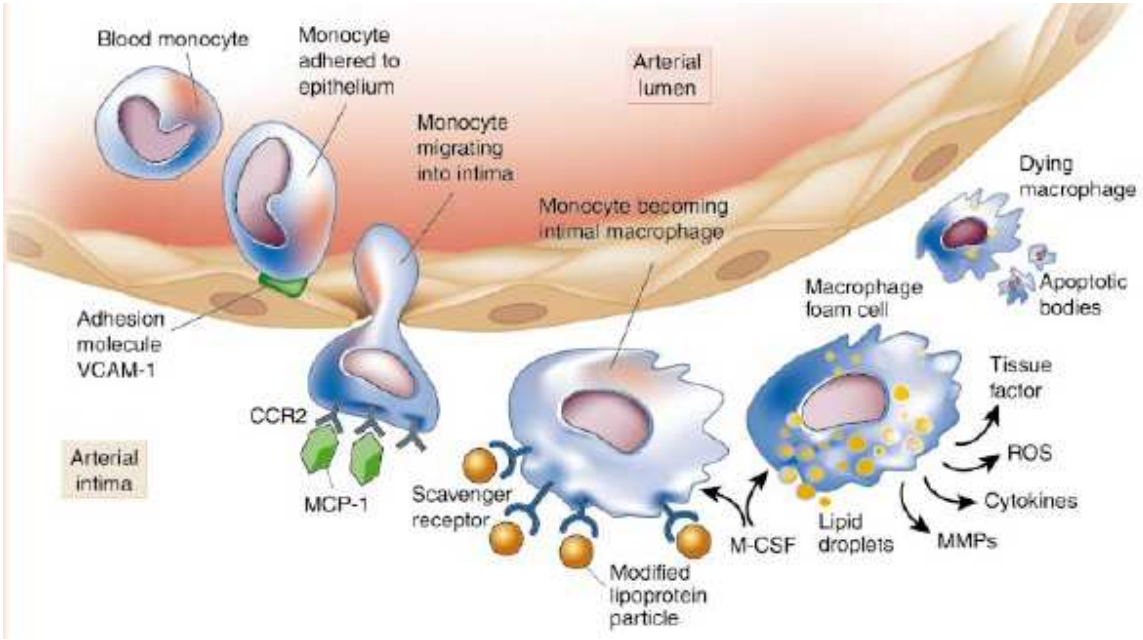


Figure 1.12: The role of macrophages in atherosclerotic plaque development: Blood monocytes are recruited to the arterial intima by VCAM-1. MCP-1 then drives migration of monocytes into the intima. Monocytes then differentiate into macrophages and take up modified lipoprotein particles via scavenger receptors, forming foam cells. (Libby, 2002)

Histologically, different stages of plaque formation can be distinguished: The initial lesion with only isolated foam cells is present in almost every individual. Also the slightly advanced fatty streaks are no major problem in terms of CVD risk. It starts getting dangerous in the conversion of early to advanced atheromas, which is mainly characterized by thinning of the fibrous cap. Thin fibrous caps are prone to rupture, resulting in thrombosis and its possible clinical complications, mainly myocardial infarction. (Libby, 2002)
In all these processes, immune responses, innate as well as adaptive mechanisms, are involved. (Libby et al., 2002)

1.3.4 Experimental systems to study atherosclerosis in mice

Up to now, two different animals have proven to be particularly useful to mimic atherosclerotic processes in mice. Knockout of the gene for apolipoprotein E (ApoE),

a key component of the cholesterol metabolism in mice leads to development of spontaneous hypercholesteraemia and atherosclerotic disease patterns. (Plump et al., 1992) Feeding mice an atherogenic, Western-type diet further enhances this process. Another model is deficiency for the low-density-lipoprotein receptor (LDLR) which is usually intensified by feeding of a high fat diet to develop sufficient hypercholesteraemia and atherosclerotic plaques. (Hansson and Libby, 2006) In this context it is important to mention that, contrary to humans, cardiovascular events are not observed in dyslipidemic mice because mice usually keep their thick fibrous cap.
To assess the effect of possible atherosclerosis-related genes, cross-breeding with ApoE -/- mice or bone marrow transplantation in LDLR -/- mice can offer important insights.

1.3.5 A potential role for PI3K/PTEN in atherosclerosis

Biwa et al. showed that stimulation of macrophages with oxidized LDL leads to activation of the PI3K signalling axis and that this further induces proliferation of macrophages using the pharmacologic PI3K inhibitor Wortmannin. (Biwa et al., 2000) Also Joe Witztum showed activation of PI3K and downstream AKT signaling by minimally oxidized LDL. (Miller et al., 2005; Miller et al., 2003)
Also clinically efficient lipid lowering drugs, the HMG-CoA reductase inhibitors, known as statins were shown to activate PI3K signalling. (Dimmeler et al., 2001)
In terms of *in vivo* studies, L. Cantley's group demonstrated that deletion of the gene for PI3K subunit p110 gamma attenuates murine atherosclerosis. (Chang et al., 2007)
In terms of the PI3K signalling regulator, no effect on atheroma development in ApoE -/- PTEN haplodeficient mice could be seen. (Andres et al., 2006) But as PTEN heterozygosity does not always show an effect, full PTEN knockout mice have to be studied.

1.4 Arthritis

1.4.1 Rheumatoid arthritis, an overview

Arthritis (Greek for joint inflammation) is one of the leading causes of disability in people aged over 55 years. Different forms of this disease are described, subdivided by the main cause of joint destruction. The most common type is osteoarthritis, which is usually caused by trauma of the joint, joint infection or age. Examples for other primary forms of arthritis are rheumatoid arthritis (RA), gout and septic arthritis.

Parts of this diploma thesis focus on rheumatoid arthritis, which is described as a chronic autoimmune disorder and affects about 1% of the Western population. (Lebre and Tak, 2008) Clinical symptoms of rheumatoid arthritis are pain, swelling, stiffness and loss of function of the joints. Areas that are most often impaired by RA are higher extremities, especially joints of the hands and fingers, but as the disease progresses, also other joints of the body can be inflamed. Epidemiologically, women are 3 times more affected than men and in contrast to osteoarthritis, also younger individuals are subjects to RA.

Current therapy focuses to alleviate symptoms, reduce joint destruction and prevent disease onset. Disease modifying antirheumatic drugs (DMARDs) are defined as useful drugs to slow down rheumatoid arthritis disease progression. The most important ones currently in use are methotrexate and different TNF inhibitors.

To study physiological processes in RA, the animal model of collagen induced arthritis (CIA) is probably most useful to provide new insights into disease progression. In this model, collagen II is injected into mice aged 4-12 weeks together with complete Freund's adjuvant (CFA) and Mycobacterium tuberculosis. (Luross and Williams, 2001) Using this model, effects of gene knockout can be studied in greater detail. It is also clinically relevant as collagen type II antibodies are present in patient samples and also reflect inflammatory activity measured by TNF-alpha and IL-6 levels in these individuals. (Cook et al., 1996; Kim et al., 2000)

1.4.2 Potential role for the PTEN/PI3K pathway in rheumatoid arthritis

L-12, which is composed of p40 and p35 subunits seems to have an important role in the development and establishment of CIA and thus RA. (Joosten et al., 1997) IL12 is a cytokine that classically drives the Th1 type of inflammatory response, which is characterized by excessive IFN-gamma production. A consequence of this predominant Th1 response is massive tissue destruction as it is present in RA.

IL-12 production has been shown to be suppressed by PI3K. Furthermore, production of Th2 cytokines, namely IL-4 and IL-5 has been shown to be reduced in PI3K knockout mice. (Fukao et al., 2002) Fukao et al. also state, that differentially regulated p38 in PI3K knockout cells might account for the different IL-12 levels. (Fukao et al., 2002) In PTEN deficient cells, the opposite was observed, namely they produce less IL-12 and TNF, but more IL-10. (Kuroda et al., 2008)

A Swiss group demonstrated that PI3K p110 gamma knockout mice are protected in animal models of rheumatoid arthritis. Also, a specific PI3K gamma inhibitor suppresses disease progression. They claim that the effect is caused by decreased neutrophil influx in the -/- mice. (Camps et al., 2005)

Somewhat contradictory to their findings are the obervations made in the neutrophil specific (Ela2cre) PTEN knockout mouse which was found to be protected in a serum-transfer model of arthritis. They also demonstrate reduced neutrophil influx due to impaired chemotaxis in PTEN KO animals. (Heit et al., 2008)

Another group investigated adenovirus-mediated PTEN transfer into rats and found amelioration of CIA symptoms. (Wang et al., 2008)

2. OBJECTIVES

The aim of this thesis was to further elucidate the beneficial role of the PI3K-PTEN pathway in different experimental settings. In order to ensure relevance of this issue *in vivo* and not only in the cell culture dish, different animal models have been applied.

PTEN has already been studied in various cancer-related contexts, but has only recently attracted the attention of immunologists. (Suzuki et al., 2001) It was shown to be important in neutrophil chemotaxis,(Heit et al., 2008) but also in limiting the cytokine release upon endotoxin challenge. (Luyendyk et al., 2008)

Inflammation plays an important role in the pathology of atherosclerosis. (Glass and Witztum, 2001; Libby, 2002) An important process in atherogenesis is the influx of macrophages in the arterial intima and subsequent foam cell formation. As our PTEN deficient macrophages showed decreased inflammatory response in various experimental settings, we speculated that PTEN deficiency might also influence the outcome in an animal model of atherosclerosis. We thus used *in vivo* as well as *in vitro* methods to determine the outcome of macrophage specific PTEN knockout on atherosclerotic processes.

Another animal model where an overwhelming inflammatory response is detrimental is the collagen-induced arthritis model for autoimmunity. In this context, Fukao et al. nicely demonstrated that disruption of the PI3K signalling pathway leads to enhanced IL-12 production and, as a consequence, a dominant Th1 driven immune response. (Fukao et al., 2002) As knockout of PTEN enhances PI3K dependent signalling, we speculated that in our animal model of rheumatoid arthritis, PTEN might play a beneficial role by limiting pro-inflammatory T helper cell responses. We thus used myeloid specific PTEN knockout mice to study the role of this gene in the interplay between innate and adaptive immunity.

3. MATERIALS AND METHODS

3.1 Materials

Lysis buffer (ready to use) for genotyping of mice contains:

Tris-Cl 100mM (pH 8.0)	12.11g/100ml
EDTA 5mM (pH 8.0)	18.61g/100ml
SDS 0.2%	20g/100ml
NaCl 200mM	11.69g/100ml

TAE (50X) (Tris, Acetic Acid, EDTA)

900ml dH2O
242g Tris Base
57,1ml Glacial Acetic Acid
18,6 g EDTA
dH2O up to 1 liter

PBS (10x) (Phosphate Buffered Saline)

800ml dH2O
80g NaCl
2.0g KCl
14,4g Na2HPO4
2,0g KH2PO4
adjust pH to 7,4 (with NaOH)
dH2O up to 1 liter

PBST (Phosphate Buffered Saline Tween)

100ml PBS (10x)
5g Tween-20

dH2O up to 1 liter

RPMI-1640 full medium contains:

RPMI-1640 (Invitrogen, Karlsruhe, D)

10% FBS (Invitrogen, Karlsruhe, D)

1% PSF (100U/ml penicillin, 100g/ml streptomycin, 0.25g/ml amphotericin) (Invitrogen, Karlsruhe, D)

1% L-Glutamine (Invitrogen, Karlsruhe, D)

3.2 Mice experiments

3.2.1 Mice handling

PTEN fl/fl mice were obtained by T.W. Mak (Suzuki et al., 2001), LysM cre transgenic mice were kindly provided by R. Johnson. (Peyssonnaux et al., 2005) ApoE transgenic mice were a kind gift of C.J. Binder.

Mice were bred in the basement facility of our institute in compliance with Austrian laboratory animal law and institutional guidelines. The health status of our mice was regularly checked.

A convenient surrounding for mice was provided by continuous ventilation, constant temperature (20-22℃) and a 12h day/night light-cyc le. Breeding cages usually consisted of 1 male and 2-3 females. Pups were weaned at 3-4 weeks of age according to sex and genotype. At approximately 5 weeks of age, mice were genotyped.

Some mice were kept on a high fat diet (Western Diet; SNIFF, Soest, Germany) to enhance lesion formation in atherosclerotic mice.

Experiments were always performed with age-matched littermate controls. All mice were backcrossed onto a C57BL/6 background for at least 6 generations.

3.2.2 Anaesthesia of mice

For short time sedation, the inhalational anesthetic Forane® was used. Its huge advantage is that it does not interfere with inflammatory pathways and that it acts immediately and thus it is perfect for short procedures such as drawing blood.

A mixture of Ketamine as a dissociative anesthetic and Xylazine as a powerful sedative/analgesic was used to anesthesize mice for longer periods of time. Usually we took 10% Ketaminol® and 5% Xylasol® diluted in sterile Ringer's Solution and injected 10 times the bodyweight of a mouse (in µl) for up to two hours of anesthesia.

3.2.3 Induction of collagen induced arthritis in mice

According to materials and methods by Kai et al., 200µg of chicken type II collagen in 0.05 M acetic acid together with an equal volume of complete Freund's adjuvant (CFA) and heat-killed Mycobacterium tuberculosis were injected intradermally at the tail base of mice. (Kai et al., 2006) To ensure that effects are not caused by the adjuvant, a CFA-only control was used. A second injection (boost) was performed 3 weeks later, and this day was designated as day 0.
Mice were expected to develop severe hind paw arthritis 5 to 10 weeks after the second boost. Clinical scores of mice affected by arthritis were done on a regular basis. Mice were scored as described previously by assessing joint swelling and grip strength. (Redlich et al., 2002)

Swelling was judged in all four paws by using a semi quantitative score:

0 no swelling
1 mild swelling of toes and ankle
2 moderate swelling of toes and ankle
3 severe swelling of toes and ankle

Grip was similarly assessed:

0	normal grip strength
–1	mildly reduced grip strength
–2	severely reduced grip strength
–3	no grip at all

Both scores were then combined and are indicated as arthritis score. All assessments were performed in blinded fashion.

Animals were killed 2 to 5 weeks after the boost. Histology of hind paws was performed by Birgit Niederreiter to visualize signs of arthritis such as cellular infiltrations, hyperplasia of synovial tissue and bone erosions. (Redlich et al., 2002) Histological TRAP stainings were then analysed by OsteoMeasure Analysis System (OsteoMetrics).

Autoantibody levels were also kindly determined by members of the Division of Rheumatology, MUW. (Redlich et al., 2002)

3.2.4 Genotyping mice

3.2.4.1 Tissue preparation

Mice were earmarked using an ear-tag punch (Fisher Scientific) according to the following scheme (Figure 3.2). Punched tissue or pieces of the tail were put into a 1.5 Eppendorf tube.

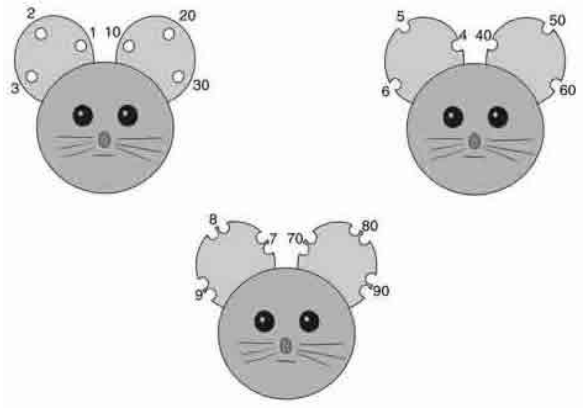


Figure 3.1: Scheme for earmarking of mice

To lyse cells, 95µL lysis buffer (Tris-Cl 100mM, EDTA, SDS 0.2%, NaCl 200mM) and 5 µL Proteinase K 10mg/mL, recombinant, PCR Grade (Roche Diagnostics, Mannheim, D) were added to each tissue sample. After at least 3 hours incubation (55℃, 650rpm), Proteinase K was diluted using 1 mL ddH2O. In a centrifugation step (5 min, RT, 13 krpm) unlysed tissue was pelleted. Proteinase K was then finally inactivated through heat (10 min, 99℃, 650 rpm). Samples were spinned down and cooled.

Polymerase chain reaction

For amplification of the isolated mouse DNA, Polymerase chain reaction (PCR) was performed using the following mastermix:

5 µL	5x buffer (containing 1.5 mM MgCl2)
2.5 µL	dNTPs (2 mM)
1µL	Primer (10 pm/µL each)
1 µL	DNA lysate
0.125 µL	GoTaq™ DNA Polymerase (Promega, Madison, WI, USA, 5u/µL)
15.375 µL	ddH2O

Primers:

ApoE		
ApoE forward	1:10	5'-GCC TAG CCG AGG GAG AGC CG-3'
ApoE revers	1:20	5'-TGT GAC TTG GGA GCT CTG CAG C-3'
ApoE revers mut	1:20	5'-GCC GCC CCG ACT GCA TCT-3'
PTEN		
PTEN forward	1:10	5'-CTC CTC TAC TCC ATT CTT CCC-3'
PTEN revers	1:10	5'-ACT CCC ACC AAT GAA CAA AC-3'
Cre		
Cre forward	1:10	5'-TCG CGA TTA TCT TCT ATA TCT TCA G-3'
Cre revers	1:10	5'-GCT CGA CCA GTT TAG TTA CCC-3'

PCR programs:

PTEN / Cre

94°	3:00	
94°	0:45	35x
60°	0:45	
72°	1:00	
72°	5:00	
15°	4ever	

Expected bands: WT (PTEN) band: 228bp
KO (floxed) band: 335bp
Cre band: 500bp

ApoE

94°	3:00	
94°	0:20	30x
68°	0:40	
72°	2:00	
72°	5:00	
15°	4ever	

Expected bands: WT band: 155bp
KO band: 245bp

PCR samples were applied on a 2% Agarose gel (5g Agarose in 250 mL 1x TAE-buffer) containing EtBr (1:10000).

3.2.5 Collection of blood

Blood was either collected from the inferior vena cava or from the retroorbital sinus of anesthetized mice. Blood was mixed with sodium citrate (3.2% wt/vol) at a ratio of

10:1. To obtain plasma samples, whole blood was centrifuged (10min, 8 krpm) and the supernatant in different dilutions could be used for ELISA analysis.

3.3 In vitro experiments

3.3.1 Isolation of peritoneal macrophages

2mL of Thioglycollate (4% in dH2O) were injected peritoneally to elicit the recruitment of macrophages into the peritoneum. After 3 days, mice were anesthetized with approximately 10x bodyweight (in µL) of a solution containing Ketamine and Xylazine. A peritoneal lavage was performed with 5 mL of RPMI -1640 media to obtain thioglycollate elicited macrophages. The cells were then centrifuged (5', 1500rpm) and diluted in RPMI containing 10% Fetal Calf Serum, glutamine and antibiotics for a final concentration of 10^6 cells per mL.

3.3.2 Isolation of bone marrow and differentiation of dendritic cells

Mice were sacrificed either on dry ice or through cervical dislocation. Skin and muscles were removed from the long bones of the hind paws. Femur and tibia were flushed with pure RPMI-1640 medium to obtain murine bone marrow which then could be used to differentiate BM-derived dendritic cells.
Dendritic cells were generated using RPMI full medium containing 10 to 15 µg/mL GM-CSF. After 7-9 days, cells in the supernatant were harvested and plated to use in experiments.
BM-derived DCs were then analyzed by Stefan Blüml using standard FACS procedures to ensure proper differentiation.

3.3.3 Stimulation of macrophages

Cells with a concentration of 106 /mL were plated on 12 well dishes. After approximately 2 hours incubation, the incubation medium was changed to remove non-adhering cells. On the following day, stimuli were added in the following final concentrations:

LPS (Invitrogen Corp., Carlsbad, CA, USA): 1µg/mL
OxLDL (see protocol for oxidation of LDL): 50µg/mL
CpG: (Invivogen Corp., Carlsbad, CA, USA): 1-5 µM
oxPAPC (see protocol for OxPL use): 20µg/mL

3.3.4 Staining of foam cells using Oil Red O

Medium was removed from the cells and fixative (IHC zinc fixative, BD) was added to the cells for at least 15 minutes at room temperature. After removing the fixative, the wells were washed once with 60% isopropanol. Then Oil Red O working solution (see instructions) was added to the dry wells. After 10 minutes, Oil Red O was removed and the wells were washed 5 times under running tap water.
In order to elute Oil Red O, cells were washed with 30% isopropanol and then the stain was eluted with 100% isopropanol. Flourescence could be measured in a Bio-Tek EL808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) at 500nm. To counterstain the nuclei, cells were incubated with hematoxylin for 10 seconds and washed again with water.

3.3.5 Oxidation of LDL

After determination of protein concentration using Bradford assay, LDL (kindly provided by V. Botchkov) was dialyzed against 3 litres of PBS at 4℃. After 6 to 8 hours, the buffer was changed and dialysis continued overnight. On the next day, possible volume changes were noted and a small aliquot was taken as unoxidized

control. This aliquot was mixed with 0,01% BHT (butylated hydroxytoluene) and 200 µM EDTA to avoid further oxidation. Remaining dialysed LDL was oxidized overnight at 37℃ by adding 10mM CuSO4 to a final concentration of 10 µM. As a control for the oxidation process, conjugated dienes of oxidized and native LDL were measured by determining the optical density of a 20 µg/ml dilution at 235 nm.
200 µM EDTA was then added to the oxidized LDL and further dialysis at 4℃ overnight against 3 litres of PBS including 200 µM EDTA was done to stop the oxidation process. To remove precipitated proteins for long time storage, oxLDL was then centrifuged at 500 rpm for 20 min at 4℃. To assure purity, oxLDL was then sterile filtered (0,2 µm filter). To determine the exact protein concentration, a common used Bradford was performed.
This oxidation was performed according to (Wang et al., 2008) and (Uda et al., 2006).

3.3.6 Preparation of oxidized phospholipids for use in cell culture

Phospholipids dissolved in chloroform (10 mg/mL) and stored at -80℃ were kindly provided by Olga Oskolkova. Phospholipid suspensions were prepared freshly for each experiment. Phospholipids were transferred into Falcon tubes and chloroform was evaporated under a stream of argon. Dried phospholipids were resuspended in prewarmed RPMI-1640 full (37℃) by vortexing at maximal speed for 30 seconds, incubating for 5 min at 37℃ and again vortexing for 1 min.

3.4 Isolation and staining of mouse aorta

3.4.1 Isolation

Mice were sacrificed on dry ice to prevent vascular damage of larger vessels. Blood was drawn from vena cava (heparinised 1mL syringe, 27G needle). Then, the vena cava was cut to allow perfusion with 10-20mL of 1x PBS to remove blood. To fix

tissue and plaques, the aorta was then perfused with 4% PFA in PBS. Then, organs, except for heart and kidney could be removed. Finally the aorta was dissected. Fat and the adventitia were removed to allow better staining. Then the aorta was opened longitudinally. During this procedure, the aorta and the heart / kidneys were still associated. After incubating the aorta in fixative for 3 days, they it could be pinned onto a black wax dissection pan.

3.4.2 Staining of aorta

Pinned aortas were incubated in 0.5% Sudan IV solution in 70% ethanol/acetone (1:1) for 15 minutes. Sudan IV was removed and 80% ethanol was added to the aortas 2-5 minutes until normal aortic regions containing no plaques appeared white. Ethanol was removed by washing the aortas 15 minutes under running tap water. Pictures were taken to allow quantification by Image J.

3.5 Analysis methods

3.5.2 ELISA enzyme-linked immunosorbent assay - Measurement of Cytokines/Chemokines

Blood samples (0.1 mL) were collected from the retro-orbital sinus. Plasma concentrations of TNF-alpha, IL-6, IL-10, E-selectin were measured using commercial DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA).

3.6.2 Real-Time Polymerase Chain Reaction

Total RNA was isolated from lysed cells using 0.8 mL of Trizol (Invitrogen). Samples were then used to synthesize cDNA using the SuperScript First-Strand Synthesis kit

(Invitrogen). To quantitate the levels of the desired mRNA, PCR primers were used at a final concentration of 900 nM and the probe at a final concentration of 900 ng per reaction under standard thermocycling conditions using a Fast SYBR Green Mastermix (Applied Biosystems, Foster City, CA, USA) and StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The melting curve was analysed after all SYBR runs to ensure that only a single PCR product is formed. Samples were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Sequences of used primers are summed up in table 3.1

Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
GAPDH	GGTCGTATTGGGCGCCTGGTCACC	CACACCCATGACGAACATGGGGGC
IL-6	TGCAAGTGCATCATCGTTGTTC	CCACGGCCTTCCCTACTTCA
IL-10	TGGCCCAGAAATCAAGGAGC	CAGCAGACTCAATACACACT
PTEN	ACACCGCCAAATTTAACTGC	TACACCAGTCCGTCCCTTTC
IL12p40	ACAGCACCAGCTTC-TTCATCAG	CCTCACCCTCGGCATCCAGCAGC
IL12p35	CCAAGGTCAGCGTTCCAACA	AGAGGAGGTAGCGTGATTGACA
IL23p19	TGGCTGTGCCTAGGAGTAGCA	TTCATCCTCTTCTTCTCTTAGTAGATTCATA
CD36	GGAGCAACTGGTGGATGGTT	TTGAGACTCTGAAAGGATCAGCA
SR-A3	GCTTCAGAAGATGCTGCTAGCC	TGACTGCTTCTGGTGGAGAGC
SR-B1	ACGGCCAGAAGCCAGTAGTC	CGGTGTCGTTGTCATTGAAG
SR-B2	GGTGTTGAACATCAGCATCTGC	AACGAACTTCTCGTCGGCTTG
CD36	GGAGCAACTGGTGGATGGTT	TTGAGACTCTGAAAGGATCAGCA
CD68	TGGCGCAGAATTCATCTCTTC	GGTCAAGGTGAACAGCTGGAG
MARCO	TGATGCGACTGTCTTCTGTCG	CATTGTCCAGCCAGATGTTCC
SR-A3	GCTTCAGAAGATGCTGCTAGCC	TGACTGCTTCTGGTGGAGAGC
SR-B1	ACGGCCAGAAGCCAGTAGTC	CGGTGTCGTTGTCATTGAAG
SR-B2	GGTGTTGAACATCAGCATCTGC	AACGAACTTCTCGTCGGCTTG

Table 3.1: Oligonucleotides for cDNAs analysis by semi quantitative real-time PCR

3.6.3 Western Blotting

For visualization of different proteins on Western blot, stimulated macrophages were harvested and lysed in Laemmli buffer, denatured (96℃, 10min) and separated by

SDS-PAGE (10%). Proteins were then blotted to a polyvinylidene fluoride membrane (Immobilion PVDF Transfer Membrane; Millipore, Bedford, MA, USA), blocked in 5% milk in PBST for at least 20 min, probed overnight with primary antibody, then washed 3 times with PBST and incubated with horseradish peroxidase-coupled secondary antibody (GE Healthcare UK Limited, Buckinhamshire, GB) for at least 2 hours. Membranes were developed using the chemiluminescence reagent assay SuperSignal® West Femto (Pierce Biotechnology, Rockford, IL, USA) and exposed in chemiluminescence-imager FluorChem HD2.
For normalization, membranes were incubated in stripping buffer for 30 min at 37℃, washed 3 times with PBST and then reprobing was performed as described earlier.

Primary antibodies used (all Cell Signaling Technology, Beverly, MA)

- PTEN
- phospho-ERK
- ERK
- p38
- phospho p38
- Actin
- pGSK3 beta (Ser9)
- phospho-Akt (Thr308)

Statistical analysis

Data are given as mean ± standard deviation. Values were compared by using the unpaired two-tailed Student's t test. Significance is indicated by ✶ ($p<0.05$). Statistical analysis was performed using Microsoft Excel and GraphPad Prism.

4. RESULTS

4.1 PTEN in Atherosclerosis

4.1.1 Knockout of PTEN in LysMcre macrophages was shown on protein as well as mRNA level

Mice were routinely checked for their genotypes using earmarks or pieces of the tail. Standard PCR was performed on the cre and on the floxed allele. A representative genotyping picture is depicted in figure 4.1.A

We also wanted to investigate whether the floxed allele is actually excised in macrophages. Thus thioglycollate-elicited macrophages were harvested from LysMcre positive or negative fl/flPTEN mice and 27 cycles of the same PCR protocol as used above revealed that the floxed band disappeared in LysMcre positive samples while the cre-band was still present. (Figure 4.1.B)

Also real-time PCR performed on the same samples showed highly significant downregulation of PTEN mRNA. (Figure 4.1.C)

On protein level, determined by Western blot analysis, it was even more obvious that PTEN is almost completely absent. (Figure 4.1.D)

To allow atherosclerosis research, mice were crossed into an ApoE -/- background. A representative picture showing +/-, +/+ and -/- is shown in figure 4.1.E. For correct atherosclerosis studies, only ApoE -/- animals were taken into consideration.

Fig. 4.1

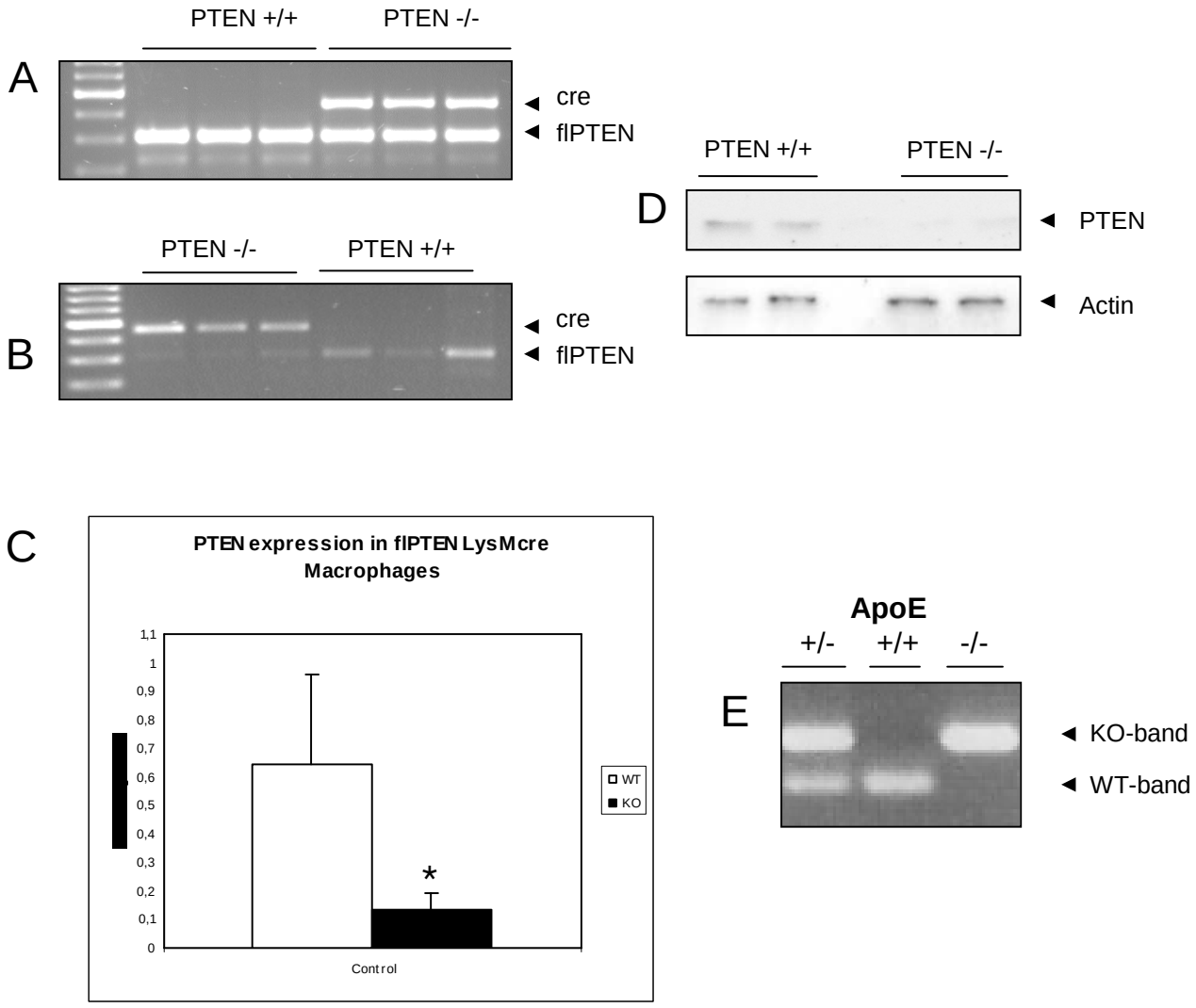


Figure 4.1: PTEN is efficiently deleted in LysMcre PTEN fl/fl on DNA, mRNA and Protein level. **(A)** Pieces of mouse tissue were subjected to genotypic analysis. **(B)** PTEN alleles are successfully excised in PTEN -/-macrophages which are positive for LysMcre recombinase. **(C)** PTEN mRNA expression normalized to GAPDH is significantly reduced in LysMcre PTEN fl/fl thioglycollate-elicited macrophages. **(D)** PTEN protein is absent in Western blots of PTEN -/- peritoneal macrophages. **(E)** Representative picture of routinely performed ApoE genotyping. Only ApoE -/- animals were used for *in vivo* atherosclerosis studies.

4.1.2 PTEN deficiency in macrophages results in enhanced intracellular levels of modified lipoprotein particles and enhanced foam cell formation

Thioglycollate-elicited macrophages from LysMcre flPTEN ApoE double deficient mice were isolated and plated. After fixation, they were stained with Oil Red O and Hematoxylin as a counterstain. A microscopic picture of stained foam cells was taken (Figure 4.2.A) and the number of positive stained cells per field was counted (Figure 4.2.B, right graph). A tendency to more positively stained foam cells in PTEN knockouts was visible.

Additionally, an essay for *in vitro* foam cell formation was used. Thioglycollate-elicited macrophages from LysMcre fl/fl PTEN mice were incubated with or without oxLDL o/n. Lipid-loaden foam cells were stained with the method previously described. Figure 4.2.C confirms the data obtained with *in vivo* generated foam cells by showing more staining in PTEN -/- wells. Counterstain was performed using hematoxylin to ensure that equal cell numbers were used. (Data not shown)

Figure 4.2.B (left graph) shows the optical density of Oil Red O eluted by Isopropanol and measured in an ELISA plate reader in *in vitro* generated foam cells.

Fig. 4.2

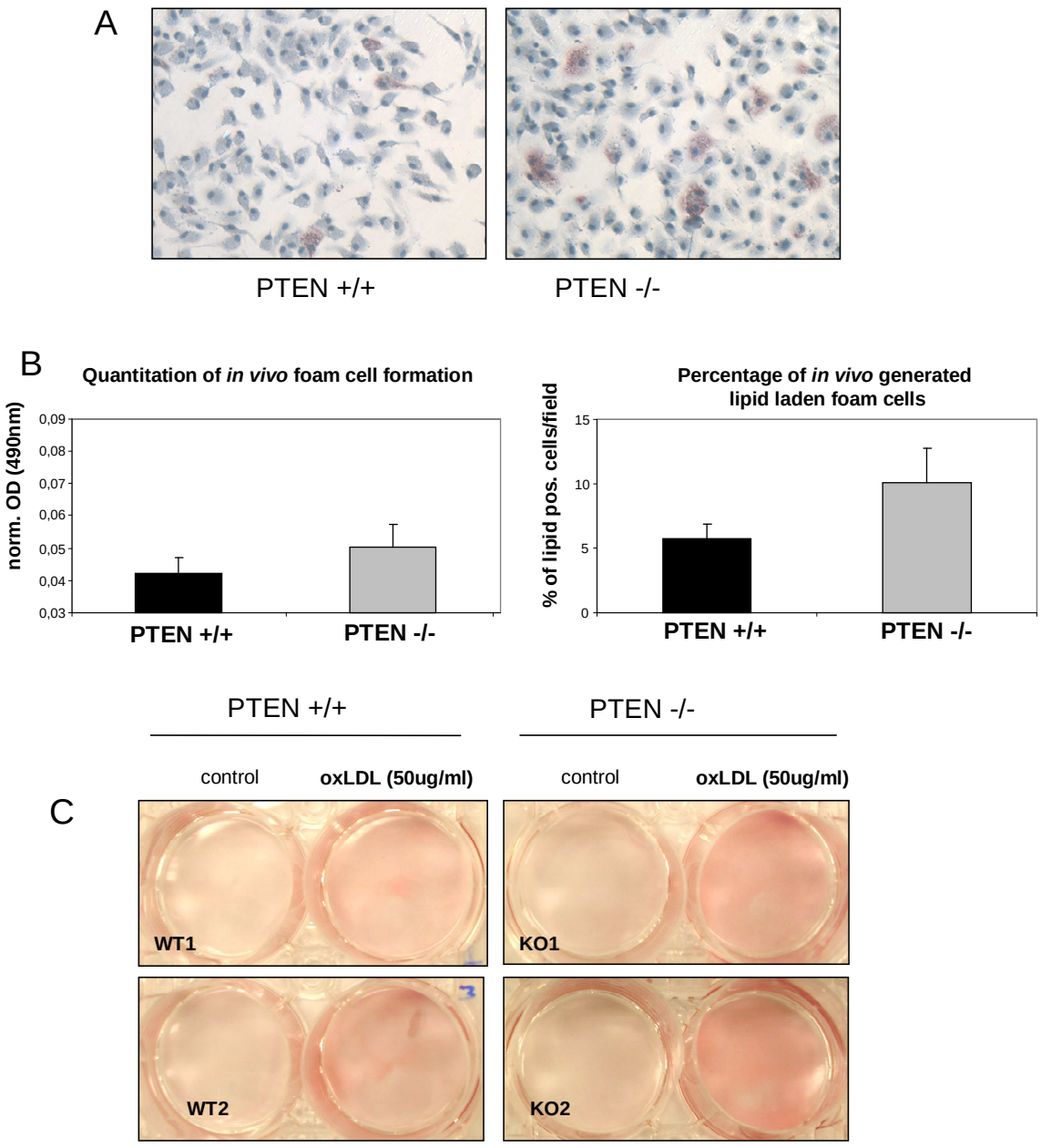


Figure 4.2: Foam cell formation in LysMcre PTEN fl/fl mice **(A)** Microscopic picture of lipid laden foam cells, isolated from ApoE -/- PTEN KO and WT mice. **(B)** Quantification of *in vitro* generated foam cells by Oil Red O elution (left) and percentage of positively stained *in vivo* generated foam cells per microscopic field. (n = 3 mice per group). **(C)** Macroscopic picture of wells with *in vitro* generated foam cells staine with Oil Red O.

4.1.3 Effects of PTEN knockout in macrophages in atherosclerosis associated signalling

Thioglycollate-elicited macrophages of PTEN -/- and +/+ mice were stimulated with oxidized low-density-lipoprotein (oxLDL) or Oxidized-1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine (oxPAPC) over night. Cells were harvested in SDS-PAGE sample buffer and subjected to Western blot analysis. Incubation with phospho AKT antibody in comparison to total AKT revealed that PI3K dependent phosphoylation of AKT is induced by oxLDL and oxPAPC. (Figure 4.3.A)

One of the hypotheses we wanted to test was, whether expression of scavenger expression is different in PTEN knockout macrophages. We thus took thioglycollate-elicited macrophages from LysMcre PTEN fl/fl mice and WT control mice and isolated RNA. Real time PCR nicely demonstrated that expression of scavenger receptors SR-AIII, CD36, SR-BI, CD68, SR-BII in PTEN deficient macrophages does not differ from wildtype controls. (Figure 4.3.B) Therefore we concluded that differential SR expression is not responsible for enhanced foam cell formation.

Fig. 4.3

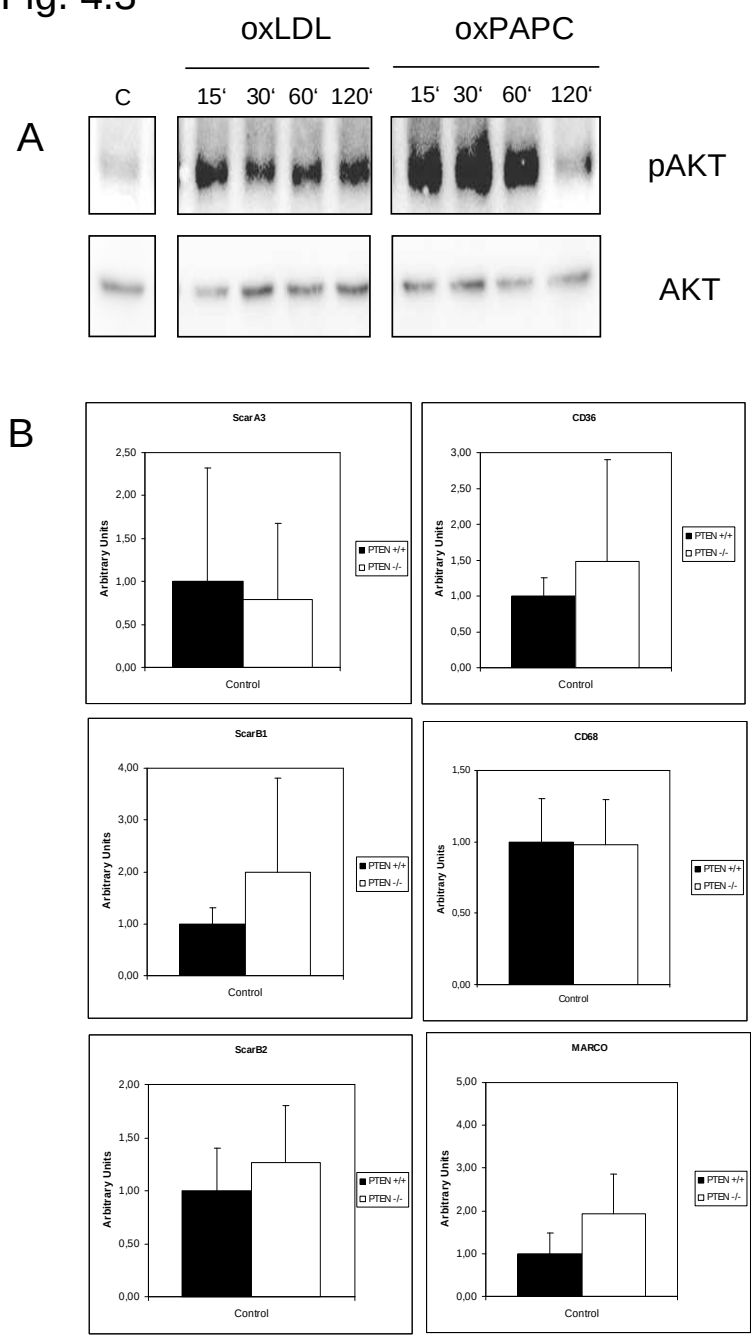


Figure 4.3: Activation of PI3K signaling by oxLDL and oxPAPC and comparison of scavenger receptor expression in unstimulated PTEN deficient peritoneal macrophages **(A)** Enhanced levels of phospho-AKT compared to total AKT levels upon stimulation of WT macrophages with oxLDL or oxPAPC o/n. **(B)** Enhanced foam cell formation of PTEN deficient peritoneal macrophages is not caused by differential expression of scavenger receptors SR-AIII, CD36, SR-BI, CD68, SR-BII and MARCO (n=4 mice per group). Target genes were normalized to GAPDH, results are not efficiency corrected.

4.1.4 Analysis of atherosclerotic plaques in en face preparations of aortas of LysMcre flPTEN mice

LysMcre fl/fl PTEN mice were kept on a normal diet for 16 weeks after birth and then sacrificed on dry ice. Weight was determined and aortae of mice were harvested and stained with Sudan IV. A representative picture of stained aortae pinned on a black wax dissection pan is shown in figure 4.4.A. Red stained lesion areas were quantified by ImageJ and are given as percentage of total aortic area. Group 1 (Figure 4.4.B) gives the result of 12 mice, shown separately for males and females. Only in males a difference between WT and KO could be shown. Fitting to the data on foam cell formation, lesion area in PTEN -/- mice was enhanced.

To allow better statistics, a second group (10 mice) was analysed. To our surprise, this time WT mice had more stained areas than compared to KO. (Figure 4.4.C)

Overall, the analysis of aortic plaques did not give us statistically significant results. The main reason might be that the mice were still too young and should have been kept on an additional high fat diet to see more effects. The conclusion was to analyse mice aged 24 weeks or older because there the lesion area will be at least above 1% and we also expect genotype differences to be enhanced.

Fig. 4.4

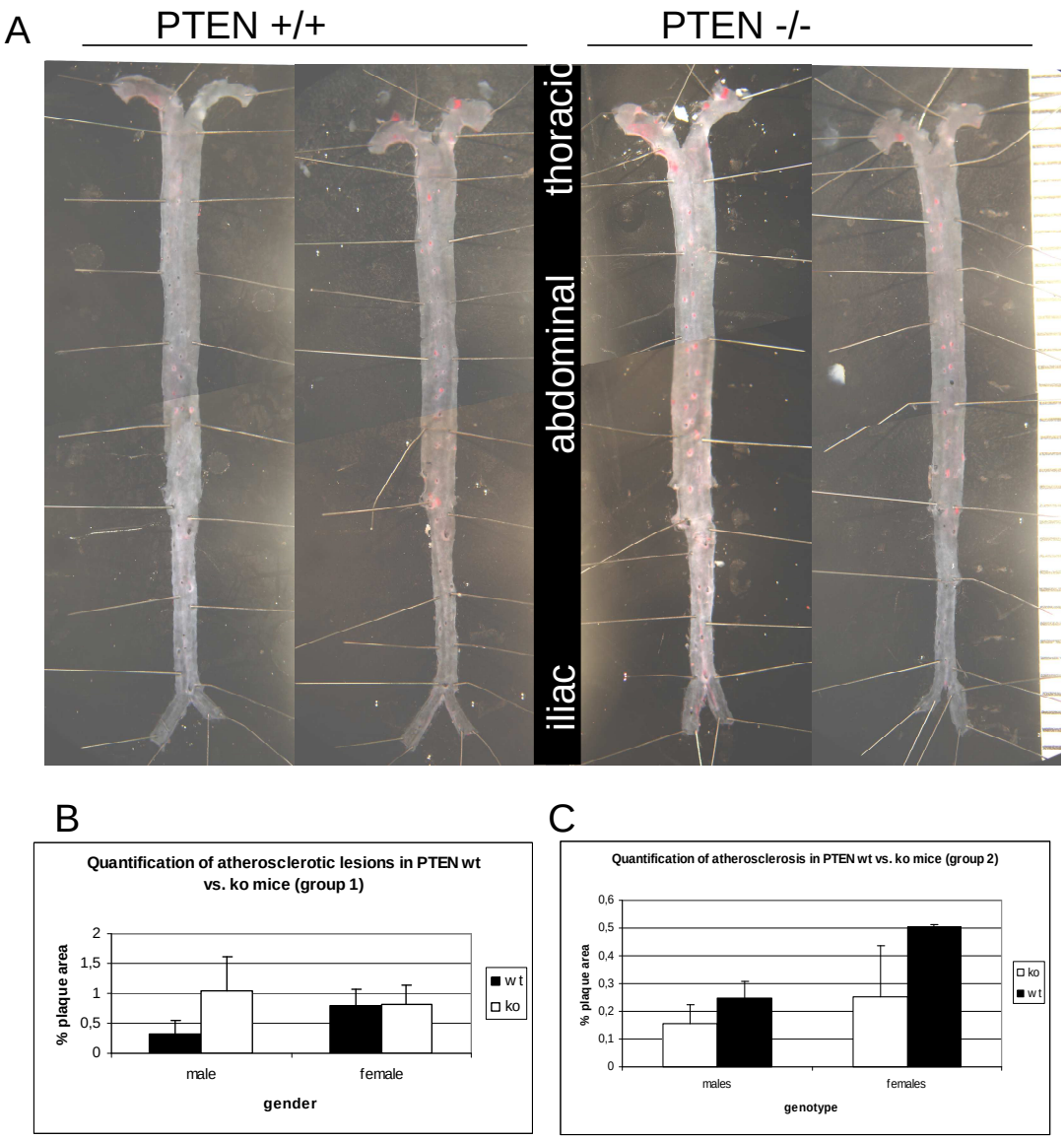


Figure 4.4: Analysis of atherosclerotic plaques in ApoE -/- LysMcre PTEN fl/fl mice. **(A)** Representative picture of en face preparations of ApoE -/- PTEN WT and KO murine aortae. In these animals, which were part of the first group analysed, the difference is mainly in the abdominal section. Quantification of lesion area of group 1 (n=12) **(B)**, and group 2 (n=10) **(C)** with ImageJ analysis software, separated by gender and genotype.

4.2 PTEN in Rheumatoid Arthritis

4.2.1 Mice deficient for PTEN in myeloid cells show significantly decreased symptoms for arthritis than wildtype control mice

CIA was induced in LysMcre PTEN fl/fl (PTEN KO) mice using type II collagen and CFA containing M. tuberculosis as described in the material and methods section.
Clinical score was assessed weekly and 2 weeks after the second boost, first symptoms were visible in the wildtype control mice. At this time point, no significant joint arthritis could be detected in PTEN KO mice, whereas WT mice were already affected. (Figure 4.5.A and C) In a time course until 6 weeks after boost, this effect persisted. KO mice only developed mild arthritis in comparison to CFA-control, but in wildtype mice disease severity as indicated by arthritis score increased. (Figure 4.5.A)
Figure 4.5.B shows the phenotype of PTEN WT and KO hind paws, which are mostly affected in this model of CIA because they are closest to the tail base where collagen is injected.
Histology was then performed by Birgit Niederreiter on synovial joints of KO and WT control mice to distinguish inflamed pathological tissue from the respective normal one. (Figure 4.5.D) Using a TRAP stain and the analysis software OsteoMeasure, detailed comparison of different parameters of histological sections was possible.
What we found is that joint inflammation and bone erosion in KO tissue samples was significantly reduced in comparison to wild-type mice and almost resembled the CFA-control. (Figure 4.8.D)

Fig. 4.5

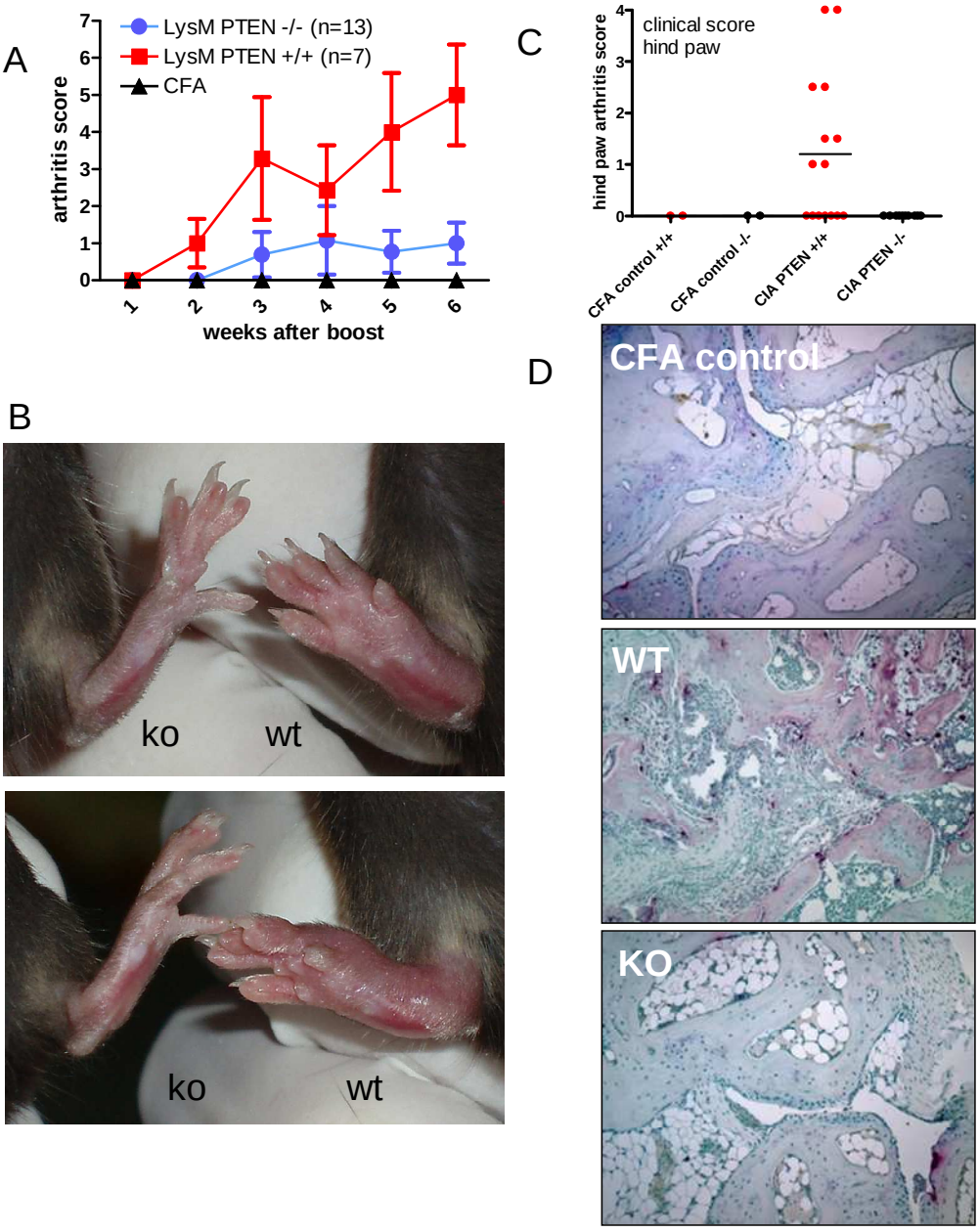


Figure 4.5: Symptoms for arthritis are reduced in LysMcre PTEN fl/fl mice. **(A)** Significant lower arthritis score in PTEN -/- mice. **(B)** Phenotype of arthritis affected hind paws of 2 representative PTEN WT and KO pairs. **(C)** Arthritis score dot plot of CIA-mice hind paws, 2 weeks after the second boost. **(D)** Histology of synovial joints of CIA-mice hind paws. (OsteoMeasure analysis depicted in Fig. 4.7.D) Arthritis score includes swelling and grip strength.

4.2.2 Reduction of inflammatory markers in plasmas of PTEN KO CIA mice but no effect on collagen autoantibodies level

Blood samples of PTEN WT and KO mice at day 0 and 3, 4 and 5 weeks after CIA boost were collected. (See scheme in figure 4.6) Plasma levels of IL-6, KC and soluble E-selectin were measured by ELISA. Consistent with previous data obtained by our group and others, levels of different proinflammatory cytokines were reduced in PTEN KO mice. Only 5 weeks upon challenge, protein levels of IL-6 and KC were similar, so one can assume that the effect of PTEN is already present in early arthritis. (Figure 4.7.A, B and C)

Autoantibody levels to type II collagen in plasma samples were also measured to check if different antibody titers might correspond with the effect seen *in vivo*. Surprisingly only 3 weeks after the second boost, antibody levels were slightly decreased in PTEN knockout mice subjected to CIA, but at all the other timepoints, no significant difference could be detected. (Figure 4.7.D)

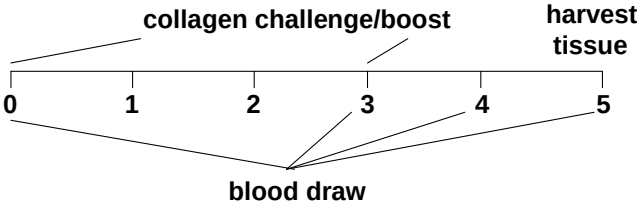


Figure 4.6: Scheme for Collagen-induced arthritis: Blood was collected 0, 3, 4 and 5 weeks after the first collagen challenge. 5 weeks after CIA induction, histology samples were collected.

Fig. 4.7

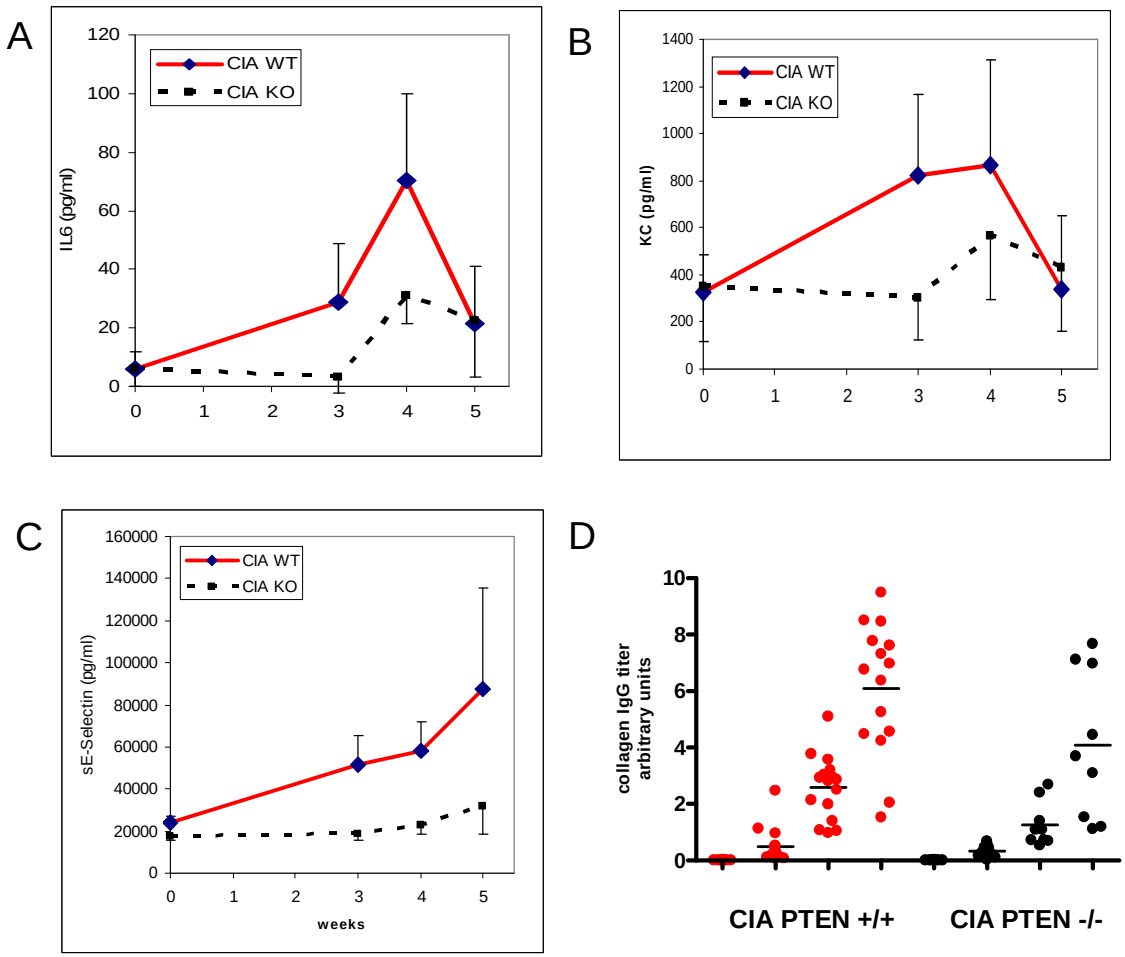


Figure 4.7: Reduced proinflammatory cytokines, but similar autoantibody levels in PTEN -/- CIA mice. **(A, B and C)** Levels of IL-6, KC and soluble E-selectin in plasmas of arthritic mice, determined by ELISA. **(D)** Anti-Chicken IgG in arthritic mice, ELISA.

4.2.3 Generation of bone-marrow derived dendritic cells which showed the expected PTEN knockout phenotype

To test the hypothesis whether the Lysozyme M specific knockout of PTEN also affects other cells of the myeloid lineage different from macrophages, bone marrow (BM) of LysMcre fl/fl PTEN mice was isolated and differentiated with GM-CSF to obtain *in vitro* generated BM-derived dendritic cells (DCs). These cells are usually non-adherent and were thus isolated from the supernatant. In order to see a potential PTEN knockout in DCs, samples for RNA and protein analysis were taken. Another aliquot of the cells was taken to check for expression of DC-specific surface markers in FACS analysis.

Real-time PCR results nicely demonstrated that PTEN mRNA levels are significantly reduced in LysMcre + fl/flPTEN dendritic cells. (Figure 4.8.A) The remaining 10% of PTEN mRNA might be due to contamination by other, non-myeloid cells such as fibroblasts or B-cells.

DCs harvested in Laemmli sample buffer were applied on a SDS-polyacrylamid-gel. Western blots were developed for total AKT, phospho-AKT (Thr 308) and phospho-GSK3beta (Ser9). As expected, levels of phospho-AKT, which is the most important downstream signalling component of the PI3K/PTEN pathway, were enhanced in PTEN -/- cells whereas total AKT levels are equal in WT and KO samples. Additionally, pGSK3beta as the probably most important downstream target of AKT/PKB is also enhanced in PTEN -/- cells. (Figure 4.8.B)

Analysis of BM-derived DCs in flow cytometry revealed equal expression of surface markers in WT and KO cells. Furthermore, correct differentiation into DCs was confirmed by measuring mean fluorescence intensity of CD80, CD86 and CD40. (Figure 4.8.C)

Also in LPS-stimulated DCs 2, 4 and 6 hours after stimulation, the knockout effect on PTEN was present. (Figure 4.8.D)

Fig. 4.8

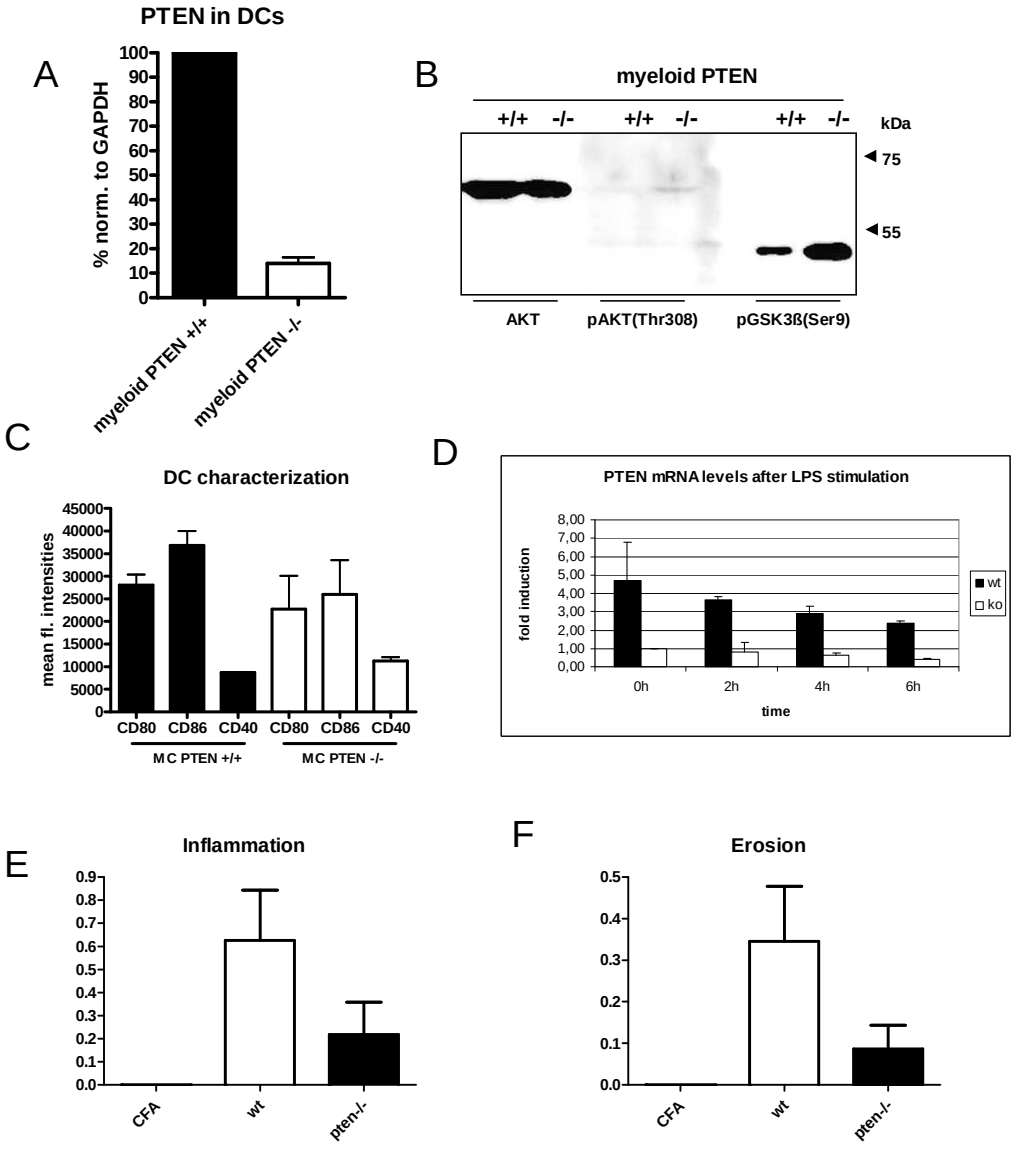


Figure 4.8: Generation of DC containing the expected PTEN KO phenotype was confirmed. **(A)** PTEN mRNA measured in real-time PCR is markedly reduced in LysMcre PTEN fl/fl mice. **(B)** Levels of phospho-AKT and phospho-GSK3beta are enhanced due to hyperactive PI3K signalling. **(C)** Flow cytometry data on PTEN WT and KO dendritic cells. **(D)** Also in LPS stimulated cells, PTEN mRNA is reduced in KO dendritic cells. **(E and F)** Quantification of joint inflammation and bone erosion for histologic pictures as depicted in figure 4.5.D.

4.2.4 Inflammatory cytokines are reduced in in vitro generated BM-derived PTEN -/- dendritic cells

Dendritic cells were differentiated out of bone marrow as described previously. Thereafter, they were stimulated with different PAMPs o/n and samples for RNA and ELISA analysis were prepared.

Figure 4.9.A shows effects of LPS, PGN and the Triacyl-lipopeptide Pam3Cys on induction of IL-6 and IL-10 mRNA induction. Pam3Cys, which stimulates TLR-2 shows the smallest effects on mRNA induction. The effects of peptidoglycan and lipopolysaccharide are more pronounced. In these samples a clear difference between WT and KO can be seen. As already demonstrated in macrophages, KO of PTEN in DCs also leads to downregulation of proinflammatory IL-6 mRNA.

Contrary to this is the finding that IL-10, which is assumed to be anti-inflammatory, is reduced in KO samples, but it was shown in several independent experiments. Interestingly, macrophages show the opposite effect, namely upregulation of IL-10 in the PTEN -/- cells.

Additionally, protein levels in supernatants of LPS, PGN, Pam3Cys and CpG DNA stimulated PTEN +/+ and -/- cells were measured by ELISA. These effects further supported the data obtained by real-time PCR. IL-6 as well as IL-10 protein levels were always profoundly reduced in KO in comparison to WT.

Figure 4.9.B depicts a timecourse of IL-6 and IL-10 mRNA induction upon LPS challenge. Not only after o/n stimulation, but also 2, 4 and 6 hours after stimulation the above mentioned effect was present. IL-6 and IL-10 levels were always reduced in KO samples, even though the reduction was not always statistically significant.

Fig. 4.9

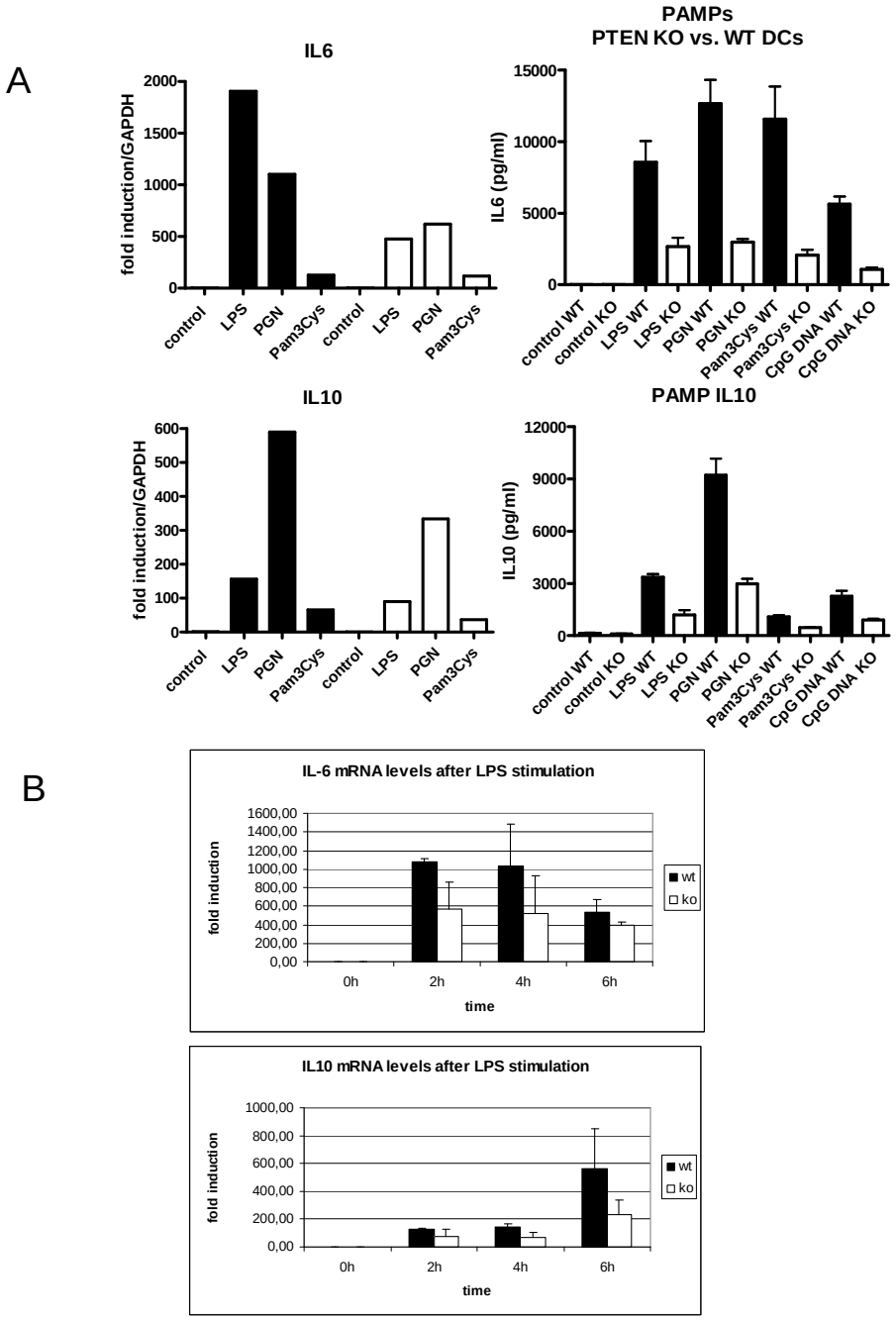


Figure 4.9: Reduced inflammatory cytokine production in PTEN -/- BM-derived DCs. **(A)** IL-6 and IL-10 mRNA measured in real-time PCR is reduced in LysMcre PTEN fl/fl mice, stimulated with LPS, PGN and Pam3Cys (left panels). Also, IL-6 and IL-10 protein levels go in line with the data obtained on mRNA level (right panels) **(B)** IL-6 and IL-10 mRNA levels 2, 4 and 6 hours after LPS stimulation are reduced in PTEN KO BM-derived DCs.

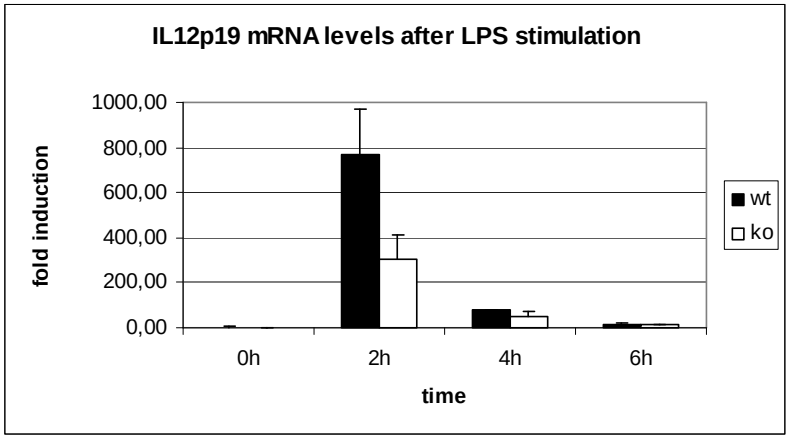
4.2.5 Reduced levels of all subunits of IL-12 may account for the anti-inflammatory effect of the PTEN knockout

In vitro generated BM-derived dendritic cells were stimulated with LPS for 2, 4 and 6 hours. RNA was isolated of the respective samples and real-time PCR was performed. Figure 4.10 depicts the outcome on mRNA induction of IL-12 subunits. It could be demonstrated, that all IL-12 subunits were reduced in PTEN -/- cells in comparison to wt. The p19 subunit, which forms active IL-12 together with p40, shows the most prominent reduction. (Figure 4.10.A) Subunit p35, a component of IL-23, is also markedly reduced in PTEN KO. (Figure 4.10.B) Surprisingly, also subunit p40, which was previously assumed not to be affected by PTEN, was slightly downregulated. (Figure 4.10.C)

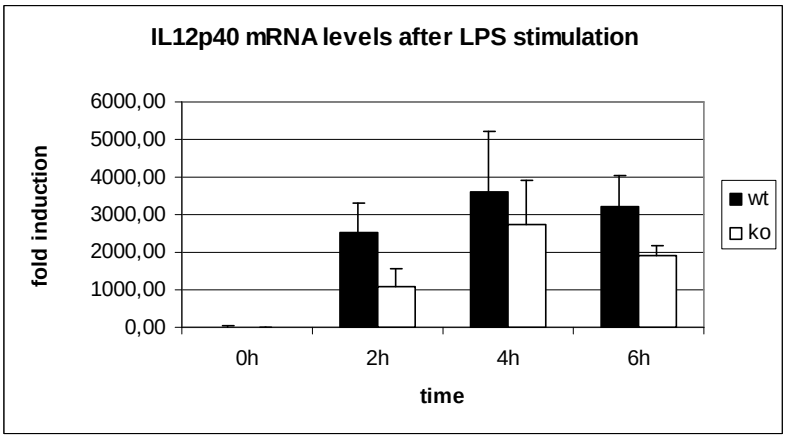
These results perfectly fit to the data obtained by Fukao et al. which showed the opposite effect in PI3K knockout cells. (Fukao et al., 2002)

Fig. 4.10

A



B



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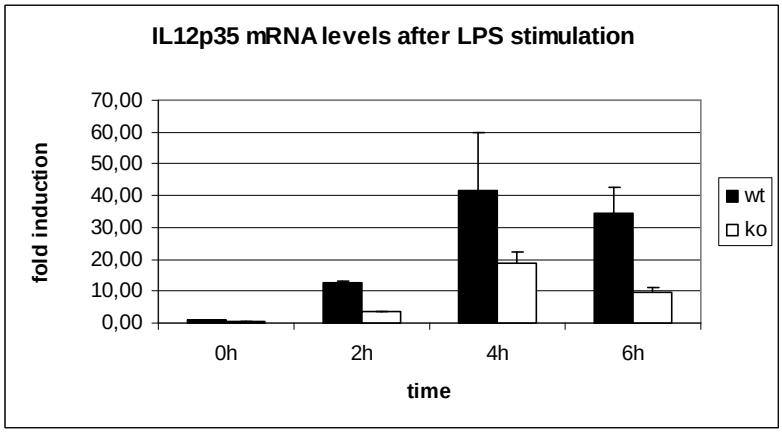

Figure 4.10: Subunits of IL-12 and IL-23 less abundant in PTEN KO BM-derived DCs. **(A)** IL-23 subunit p19, **(B)** IL-12 and IL-23 common subunit p40 and **(C)** IL-12 subunit p35 are reduced on mRNA level 2, 4 and 6 hours after LPS stimulation.

4.2.6 Differential regulation of MAPK signalling might account for the downregulatory effects on cytokine levels by enhanced PI3K signalling

MAPK signalling plays an essential role in the regulation of cytokine production in inflammatory conditions. We wanted to test the hypothesis whether enhanced levels of PIP3 as present in our PTEN KO DCs influence phosphoylation of MAPK and thus account for the reduced cytokine production.

Thus *in vitro* differentiated BM-derived DCs were stimulated with LPS for 10, 20 and 30 minutes. Samples were harvested in Laemmli buffer and then subjected to Western blot analysis. Development with phospho-ERK1/2 antibody revealed that levels of phosphoylated protein are reduced in PTEN -/- DCs whereas total ERK1/2 levels did not differ. (Figure 4.11.A) Normalization to Actin confirmed loading of equal amounts of protein.

As expected, levels of phospho-GSK3beta were enhanced in PTEN KO due to excessive PI3K signalling. Only slight changes in GSK3beta phosphoylation upon LPS stimulation can be seen (Figure 4.11.A)

Even more important, reduced p38 MAPK phosphoylation was found in BM-derived PTEN deficient DCs upon stimulation with CpG DNA or LPS. (Figure 4.11.B)

Fig. 4.11

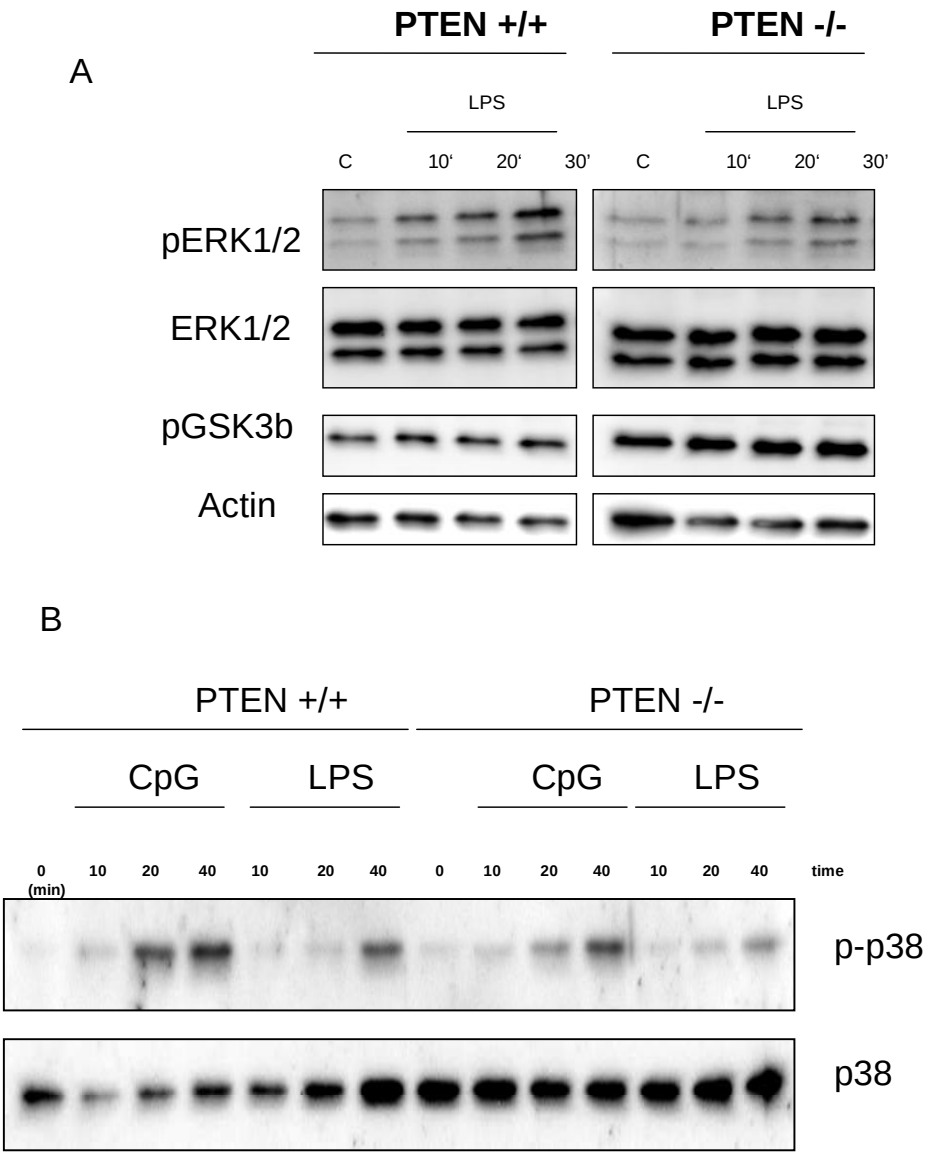

Figure 4.11: Reduced phosphoylation of MAPK p38 and ERK1/2 in PTEN -/- DCs. **(A)** Western blot of BM-derived DCs stimulated with LPS for 10, 20 and 30 minutes with phospho ERK1/2, total ERK, phospho GSK3beta and Actin. **(B)** Phospho p38 and total p38 Western blot of PTEN WT and KO BM-DCs stimulated with LPS and CpG.

4.3 Manuscripts in preparation

4.3.1 Myeloid PTEN enhances inflammation but impairs bactericidal activities during murine pneumococcal pneumonia

Gernot Schabbauer, Ulrich Matt, Philipp Günzl, Tanja Furtner, Eva Hainzl, Ildiko Mesteri, Bianca Doninger, Bernd R. Binder, Sylvia Knapp

Manuscript submitted for publication in Journal of Immunology in May 2009

4.3.2 Anti-inflammatory properties of the PI3K pathway are mediated by IL10/DUSP regulation

Philipp Günzl, Eva Hainzl, Ulrich Matt, Barbara Dillinger, Benedikt Mahr, Sylvia Knapp, Bernd R. Binder, Gernot Schabbauer

Manuscript in preparation, will be submitted to European Journal of Immunology in May 2009

5. DISCUSSION

In this diploma thesis, genetically modified mice were used to dissect the PI3K/PTEN pathway in different animal models of human diseases. Our group focuses mainly on anti-inflammatory properties PI3K as described already by Luyendyk and others. (Luyendyk et al., 2008) We therefore utilised two different animal models of chronic inflammation. In rheumatoid arthritis, which is mainly an autoimmune disease, excessive immune activation is detrimental. Peter Libby has recently reviewed the common link between RA and atherosclerosis, which is expression of proinflammatory cytokines such as IL-1, IL-6 and TNF-alpha. (Libby, 2008)

5.1 Atherosclerosis

Formation of atherosclerotic plaques in mice as well as in humans is nowadays seen as a process of chronic inflammation. By knocking out genes for important inflammatory mediators such as MCP-1 or VCAM-1, mice are protected from atherosclerosis. In this thesis, I evaluated a possible effect of the PTEN gene, which has already been described as a regulator of inflammatory processes, in the formation of foam cells and atherosclerotic plaques.

It could be shown *in vitro* as well as *in vivo*, that in PTEN fl/fl LysMcre+ mice, the PTEN gene expression shown on mRNA level is markedly reduced. Proper excision of the PTEN fl allele was observed in semi-quantitative PCR. On protein level, PTEN cannot be detected in significant amounts in LysMcre+ cells.

To assess the effects of PTEN on foam cell formation, the first step was to get a clue whether PTEN KO enhances or reduces accumulation of intracellular lipids in comparison to wildtype control. I found increased lipid accumulation on microscopic as well as macroscopic scale in *in vivo* as well as *in vitro* generated foam cells stained by Oil Red O.

We then hypothesized, that this effect might be due to differential expression of scavenger receptors, which are described as being important in lipid uptake by

macrophages. To our surprise, SR expression was not significantly different in PTEN KO cells, so this factor can be excluded.
Indicating a role for the PI3K/PTEN pathway in atherosclerotic processes is the fact, that treatment of cells with oxLDL and oxPAPC increases AKT phosphoylation, which is the most important mediator downstream of PI3K. Additionally supporting our view that PI3K signalling plays a role in atherogenesis is the finding, that minimally modified LDL activated PI3K as well as Akt and led to phosphoylation of extracellular signal-regulated kinase ½ (ERK1/2).(Miller et al., 2005)

A parallel approach was the analysis of *in vivo* effects on atherosclerotic plaque formation. For this purpose, PTEN fl/fl LysMcre+ were backcrossed onto an ApoE deficient background and analyzed for aortic lesion areas. So far, no precise conclusion can be drawn on basis of the data presented in this thesis. In the first group analysed, a tendency to more lesion formation was present, whereas in the second group, the opposite was the case. We assumed, that the reason for the large variation in the actual numbers was that the lesion area of mice was generally less than 1%, which is far too less to allow adequate analysis.
The future plan is to isolate aortas of mice aged 24 weeks or older which are expected to develop more advanced lesions. Additionally, further data on mRNA induction upon oxLDL stimulation has to be collected to evaluate possible changes in gene expression in PTEN knockout mice that are relevant in atherosclerotic processes.
Another approach to study PTEN function in atherosclerosis is the use of inducible conditional cre recombinase systems. As we failed to induce sufficient knockout in the tamoxifen-inducible estrogen-receptor cre mouse (Zhang et al., 1996), we are currently crossing PTEN fl/fl mice to a Mx-cre mouse, which is inducible upon polyI-polyC injection. (Kuhn et al., 1995) Using this system, we try to address other cells important in atherosclerosis, such as endothelial cells.

5.2 Arthritis

Autoimmune diseases such as rheumatoid arthritis are largely caused by excessive immune activation and reduced anti-inflammatory signals. RA is characterized by an infiltration of the synovia of multiple joints by inflammatory cells such as monocytes/macrophages, neutrophils and B-cells. Heit et al. already demonstrated a role for neutrophil specific (Ela2cre) knockout of PTEN in a K/BxN serum-transfer model of arthritis. (Heit et al., 2008) In this model, joint inflammation and degree of hindpaw swelling in Ela2cre PTEN fl/fl mice were significantly lower than in littermate control mice. They argue that differential neutrophil chemotaxis is primarily responsible for this effect. Nevertheless, it was demonstrated by Camps et al. that mice deficient in the class 1B PI3K subunit p110 gamma, which is responsible for transduction of G-protein coupled receptors, neutrophil infiltrates are reduced compared to wildtype. (Camps et al., 2005)
As a starting point for studying RA in PTENfl/flLysMcre+ mice, we hypothesised that the anti-inflammatory properties of a hyperactive PI3K signalling axis, which were already described by our group and others in various projects, (Luyendyk et al., 2008; Yu et al., 2006) might also play a role in the pathogenesis of the disease. Cytokines are important in coordinating immune responses to resolve pathogenic infections, but play a detrimental role if their production is dysregulated. Thus, we focussed on differences in cytokine production and subsequent physiological consequences in CIA mice.

In first *in vivo* studies, a clear protective effect in knockout mice of the PTEN gene in comparison to wt littermate control mice subjected to CIA could be observed. PTEN -/- mice showed significantly decreased paw swelling and grip strength scores, but similar anti-collagen II antibody titers. These data indicate that reduced anti-collagen response through impaired dendritic cell migration may not play an important role, (Del Prete et al., 2004) because activation of antigen-specific B-cells takes place.
We then decided to do histology on synovial joints of arthritic mice to get a clearer picture of the actual disease region. Evaluation of TRAP stains revealed significant

decrease in joint erosion and inflammation in PTEN -/- synovial joints which goes in line with the above described arthritic score.

To evaluate whether the signs of this local inflammation are also present on a systemic level, pro-inflammatory cytokine production was measured in murine blood after CIA induction. IL-6, KC and E-selectin were found to be profoundly reduced in PTEN KO plasmas. To confirm this *in vivo* effect on a cell-based *in vitro* system, bone marrow-derived dendritic cells were stimulated with PAMPs and again, similar differences in cytokine levels were detected. This goes in line with previous data obtained on macrophages that were shown to produce less IL-6 and TNF-alpha after stimulation with the TLR ligands LPS, CPG and PGN. (Kuroda et al., 2008) Additionally, our group gained similar results, *in vitro* as well as *in vivo* in thioglycollate-elicited peritoneal macrophages (unpublished data).

What was surprising is that IL-10 production also seemed to be reduced in LysMcre fl/fl PTEN dendritic cells, which is contrary to the findings in macrophages made by A. Suzuki and us (unpublished data) in macrophages. (Kuroda et al., 2008)

Of particular interest is the expression of IL-12 cytokine family members, namely IL-12 and IL-23, which are key players in the regulation of T cell responses. (Gee et al., 2009) These cytokines are mainly produced by monocytes, macrophages and dendritic cells as a response to infection. IL-12 and IL-23 share the common p40 subunit, but differ in their small components, p35 and p19, respectively. Although they are quite similar in terms of receptor and subsequent JAK/STAT signalling activation, they fulfil highly specific roles in immune regulation. Whereas Il-12 is important in production of IFN-gamma and subsequent Th1 cell differentiation, IL-23 has a key role in Th17 cell development. (Gee et al., 2009) Concerning the role of the PI3K/PTEN pathway in T helper cell development, it has already been demonstrated that p85 PI3K knockout mice are resistant to Leishmania infection due to DC overproduction of IL-12 and thus excessive Th1 polarization. (Fukao et al., 2002) This finding has also been confirmed in the opposite direction as LysMcre PTEN fl/fl mice show increased susceptibility and delayed healing as response to Leishmania infection.(Kuroda et al., 2008)

Due to their important role in T helper cell activation and thus also in autoimmunity, a neutralising antibody to IL-12 subunit p40 has been developed for use in multiple sclerosis, an autoimmune disease similar to rheumatoid arthritis in terms of inducing agents. (Longbrake and Racke, 2009) Subunit p40 antibodies proved to prevent autoimmune encephalomyelitis in murine models,(Leonard et al., 1995) but so far, Phase II clinical trials failed, probably because only very early disease stages can be addressed by the antibody. (Longbrake and Racke, 2009)
In our myeloid-cell specific PTEN knockout system, mice were also protected from RA and *in vitro* cytokine levels for IL-12 and IL-23 were found to be reduced in BM-derived DCs. Thus it can be speculated, that there is an effect on T helper cell differentiation in PTEN -/- mice.
As the reduction in IL-12 levels was found to support the view of Fukao et al., who show increased IL-12 levels in PI3K p85 alpha knockout mice, we also tried to find differences in MAPK signalling which are supposed to be responsible for regulation of IL-12 expression. (Fukao et al., 2002) We found reduced levels of phospho p38 as well as phospho ERK1/2 upon LPS as well as CpG stimulation. These effects demonstrate that hyperactive PI3K signalling leads to decreased levels of mitogen-activated protein kinases and thus reduced cytokine mRNA induction. (Lu et al., 1999)

We now conclude that myeloid cell specific PTEN knockout reduces phosphoylation of MAPK and as a consequence also reduces proinflammatory cytokine production in antigen presenting cells. This presumably results in markedly reduced numbers of auto-inflammatory Th1/Th17 cells and thus a better clinical outcome in collagen-induced arthritis and possibly other immune diseases such as experimental autoimmune encephalomyelitis (EAE).

Further projects will now aim to investigate the T helper cell response in PTEN knockout animals. On the one hand, if Th17 cells are less abundant, related cytokines such as IL-17, IL-21 and IL-22 should also be present in lower amounts in draining lymph nodes. On the other hand, Th2 or Treg cytokine levels such as IL-4 and IL-5

might be elevated. Figure 5.1 demonstrates our current view how hyperactive PI3K signalling in PTEN deficient myeloid cells regulates adaptive immune responses.

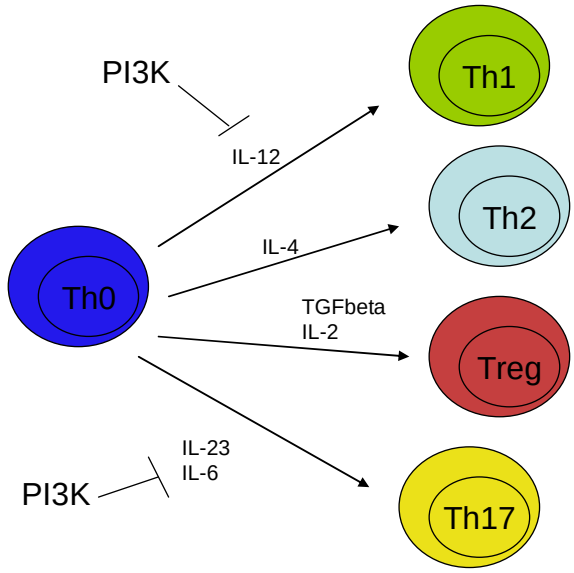

Figure 5.1: Differentiation of CD4 positive naïve T cells to Th1 and autoinflammatory Th17 cells is reduced in PTEN deficient mice whereas Th2 and probably also regulatory T cells are more abundant. This happens due to reduced MAPK signalling and subsequent diminished release of proinflammatory IL-6, IL-23 and IL-12. (modified from (La Cava, 2009))

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Submission to Journal of Immunology in April 2009

Anti-inflammatory properties of the PI3K pathway are mediated by IL10/DUSP regulation

Philipp Günzl, Eva Hainzl, Ulrich Matt, Barbara Dillinger, Benedikt Mahr, Sylvia Knapp, Bernd Binder, Gernot Schabbauer

Manuscript in preparation, will be submitted to European Journal of Immunology in April/May 2009

PTEN as a link between innate and adaptive immunity in an animal model of rheumatoid arthritis

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- Inhibition of foam cell formation by oxidized phospholipids (together with V. Bochkov)
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