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“Differential regulation of IL-12p70 and IL-23 in murine dendritic cells”

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1 SUMMARY

Dendritic cells are the sentinels between the innate and the adaptive immunity. They are professionals that capture invading pathogens, recognize specific microbial structures and induce naïve T lymphocytes to polarize into a specific T cell subset. To initiate the T cell polarization DCs secrete cytokines which are induced upon Toll-like receptor activation by microbial structures. The recognition of these structures and the discrimination between non-self and self structures by TLRs is fine tuned, but under defined circumstances deregulation of immune responses appears. Consequently, this can result in immune disorders such as autoimmunity, chronic inflammatory diseases or cancer. In this thesis the investigations are focused on the regulation of the IL-12 family members IL-12p70 and IL-23 in DCs. The objective was to investigate three different endogenous and exogenous factors that regulate IL-12p70 or IL-23.

In the first part Selenium, an essential trace element and important factor in several metabolic pathways including the cellular redox status and reactive oxygen species (ROS) dependent signaling was applied as supplement in immature Langerhans cell culture. Because Selenium also plays a role in the immune system the TLR-induced IL-23 production of the DCs upon Selenium treatment was analyzed. In the immature Langerhans cell line XS-52 the strongest inducer of IL-23 was TLR4 ligand LPS. Furthermore increased levels of TLR4-induced IL-23 in cells treated with Selenium were detected in a concentration dependent manner. Whereas the IL-23 subunit p40 was upregulated upon Selenium treatment the second subunit p19 was completely unaffected. This effect was detected on mRNA and protein level. In addition, as expected, IFN-γ inhibited the TLR4-induced IL-23 secretion of both, Selenium treated and untreated cells.

In the second part of this thesis $p47^{phox}$, an organizing protein of the NADPH oxidase was analyzed regarding its potential to regulate IL-12p70 and/or IL-23 secreted by different DC subtypes. Since it was demonstrated that $p47^{phox}$ deficiency is associated with enhanced autoimmunity and chronic inflammation we wanted to prove whether it has a function in addition to that within the NADPH oxidase. We found some hints that $p47^{phox}$ may be interact with proteins of the TLR signaling pathway and thus we hypothesized that $p47^{phox}$ may have a function for the regulation of TLR-mediated cytokine production in DCs. In several

experiments with DCs from the spleen of different p47^{phox} deficient mice we detected an increased production of TLR9-induced IL-12p70 compared to wild type cells. In contrast TLR4 stimulation with LPS displayed no significant differences between p47^{phox} deficient and wild type cells. In spleen cells IL-23 was not detected. Confirming the results of this new negative feedback by p47^{phox} on IL-12p70 rats, with a single nucleotide polymorphism in the p47^{phox} gene, were investigated. Interestingly this polymorphism is located in the phosphorylation site of IRAK4, an important kinase in the TLR pathway. In rats with a methionine residue at this position in the p47^{phox} protein enhanced IL-12p70 level were found, compared to the rats with threonine, which can be phosphorylated by IRAK4. All analyzed mice and rats have defects in the NADPH oxidase function due to a non functional p47^{phox} protein which results in a defective ROS production. To determine whether the observed negative feedback mechanism is connected to the lack of ROS production experiments with gp91^{phox} deficient mice, which also have a defective NADPH oxidase function, were performed. In several experiments the enhanced IL-12p70 production in cells from p47^{phox} deficient mice could be confirmed, but no differences between gp91^{phox} deficient and wild type mice have been observed. In further studies was found that the inhibition of the NADPH oxidase function did not alter the negative feedback on TLR9-induced IL-12p70 secretion by p47^{phox}. Interestingly upon treatment with the inhibitor a feedback mechanism in wild type cells also after TLR4 stimulation was observed. Hence, blocking a ROS-dependent TLR4 pathway by the inhibitor uncovered the LPS induced ROS-independent pathway of the TLR4 signaling. These findings strongly approve a NADPH oxidase/ROS-independent function of p47^{phox} in DCs. Because splenic DCs do not secrete IL-23, *in vitro* differentiated DCs from the bone marrow were investigated regarding the negative feedback mechanism. In DCs from p47^{phox} deficient mice, differentiated with GM-CSF, the upregulation of IL-12p70 was confirmed, whereas Flt3-L cultured DCs did not display the negative feedback. In contrast to IL-12p70 no difference for the IL-23 production between wild type and p47^{phox} deficient cells has been detected. Thus, we concluded that IL-23 production is not regulated by p47^{phox}. IL-12p70 is the major cytokine in the Th1 polarization whereas IL-23 is important for the maintenance and survival of Th17 cells. To prove whether the regulation of IL-12p70 influences the T cell response immunization experiments closely resembling the classical DTH-like

protocols were performed. Groups of $p47^{phox}$ deficient and wild type mice received either PBS, OVA alone or mixed with TLR9 ligand CpG2216 in IFA s.c. to activate and polarize naïve T cells towards Th1 or Th17 cells. After ten days isolated lymph node cells were incubated in an ELISA spot assay with or without OVA and the frequency of IFN-γ and IL-17 producing T cells was quantified. *In vitro* recall of OVA immunization of wild type and $p47^{phox}$ deficient mice resulted in an increased IFN-γ and IL-17 frequency in the $p47^{phox}$ deficient cells. The combination with CpG2216 as adjuvant and inducer of the 3rd signal enhanced the frequency of IFN-γ and IL-17 producing T cells in wild type mice significantly. However, in $p47^{phox}$ deficient cells the IFN-γ and IL-17 response, being already detectable without *in vitro* OVA re-stimulation, was strongly augmented upon OVA re-stimulation. These findings confirmed our *in vitro* data for IL-12p70. Hence, the data supports our hypothesis that the $p47^{phox}$ dependent regulation of IL-12p70 and the consequences for the T cell response is an important mechanism to prevent uncontrolled immune responses.

In the last part of this thesis the immunomodulatory property of vitamin D_3 on the IL-12p70 production of DCs was examined. Since it was shown that VD_3 influences the differentiation and maturation of monocytes and DCs, splenic DCs from C57BL/6 and BALB/c mice were investigated regarding their IL-12p70 production after VD_3 treatment. Spleen cells, stimulated with LPS or CpG2216, exhibited a decreased IL-12p70 production when treated with VD_3 before stimulation phase. In contrast treatment with VD_3 only during TLR stimulation had no influence on the IL-12p70 production. Since it was demonstrated that VD_3 stimulates the expression of $p47^{phox}$ mRNA cells from $p47^{phox}$ deficient mice were also treated with VD_3. In initial experiments only a slight inhibition of IL-12p70 has been detected in $p47^{phox}$ deficient cells compared to the wild type.

In summary the thesis displays three different possibilities to influence the TLR-induced cytokine secretion of DCs, although with different intensities and specificities.

2 INTRODUCTION

2.1 Innate and adaptive immunity

2.1.1 Innate immunity – antigen presenting cells

The innate immunity is a universal and ancient system of host defense against invading pathogens. Components of the innate immunity are physical and chemical barriers, blood proteins including the complement system and phagocytic cells such as neutrophils, macrophages and dendritic cells (DCs). Microorganisms penetrating the epithelial surface are sensed immediately by phagocytic cells that engulf the microbes upon binding to their cell surface (Janeway, 6th edition). In parallel proteins of the complement system get activated and initiate proteolytic reactions against the invading pathogens. Specific pattern recognition receptors (PRRs), which are germline encoded and located on the cell surface or in intracellular compartments of phagocytic cells such as DCs recognize common structures of microorganisms and lead to the induction and secretion of cytokines such as interleukins, interferons and chemokines (Janeway, 1989; Medzhitov, 2002; Janeway, 2002; Akira, 2006). Release of those proteins by phagocytic cells initiates the process of inflammation which is characterized by heat, pain, redness and swelling triggered by vasodilation and an increasing permeability of the blood vessels (Janeway, 6th edition). In the initial phase primarily neutrophils are recruited to the site of inflammation where they engulf and destroy the pathogens. Shortly after monocytes arrive and differentiate into macrophages which supporting the reaction. During these activities DCs loaded with pathogens migrate from the peripheral tissue via afferent lymphatics to the regional lymph nodes. The mature DCs present pathogenic antigens to naïve T lymphocytes via their major histocompatibility complex (MHC). Because DCs discriminate between self and non-self antigens an activation of T lymphocyte response depends on co-stimulatory molecules such as B7.1 (CD80) or B7.2 (CD86) expressed by DCs upon PRR stimulation. Those molecules interact with the T lymphocyte receptor CD28. In parallel DCs secrete different cytokines upon PRR stimulation that are necessary for the differentiation of naïve T lymphocytes into specific T helper (Th) cells. Thus, DCs producing the cytokine interleukin (IL) -12 initiate the

development of Th1 cells whereas IL-23 results in the proliferation of Th17 cells (Mosmann, 1989; Aggarwal, 2003, Langrish, 2005).

2.1.2 Adaptive immunity – T lymphocytes

In contrast to the innate immune recognition which is characterized by a spectrum of germline-encoded receptors the adaptive immunity consists of B and T Lymphocytes that respond to specific antigens presented by antigen presenting cells (APCs) such as DCs. The main differences between the innate and the adaptive immunity are listed in the table below (**Table 1**).

Table 1: Innate and adaptive immunity (Janeway et al., 2002)

Property	Innate immune system	Adaptive immune system
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments Rearrangement necessary
Distribution	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Recognition	Conserved molecular patterns (LPS, LTA, mannans, glycans)	Details of molecular structure (proteins, peptides, carbohydrates)
Self-Nonself discrimination	Perfect: selected over evolutionary time	Imperfect: selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation of effectors
Response	Co-stimulatory molecules Cytokines (IL-1β, IL-6) Chemokines (IL-8)	Clonal expansion or anergy IL-2 Effector cytokines: (IL-4, IFNγ)

Naïve T lymphocytes circulate continuously between the blood and the lymphoid organs via high endothelial venules. Upon contact with antigen loaded APCs in lymphoid organs naïve T lymphocytes differentiate and proliferate to effector cells. After several days proliferated effector T lymphocytes migrate to the site of infection. Thus, effector T lymphocytes reenter the blood stream and are recruited to the infected tissue by expression of selectins (P- and E-selectin), adhesion molecules and chemokines on the vascular endothelium (Dustin, 1991, Springer, 1995). T lymphocytes can be divided into two main subtypes: T helper (Th) lymphocytes characterized by the expression of CD4 and cytotoxic T lymphocytes (CTL) that express CD8 on their surface. Whereas Th lymphocytes were activated via MHC class II molecules CTLs recognize MHC class I on the surface of DCs. In contrast to MHC class II molecules expressed by antigen presenting cells MHC

class I molecules are expressed on the surface of many cells in order that intracellular viruses and other pathogens can be recognized by immune cells. CTLs are effector cells that directly respond to infected cells by releasing of cytotoxic proteins such as Perforin, Granzymes and Granulysin from lytic granules (Barry, 2002). Consequently it is clear that CTLs require a stronger co-stimulation either directly by antigen presenting cells via B7 molecules that bind CD28 on CTLs or by the presence of Th cells. Unlike CTLs different subtypes of Th cells are described, some are discovered quiet recently. The classical subtypes Th1 and Th2 are described in the late eighties by Mosmann et al. (Mosmann, 1989). Th1 cells are defined by their clearance of intracellular pathogens and the production of interferon (IFN) -γ. In contrast Th2 cells control specific parasitic infections through the production of interleukin 4 (IL-4), IL-5 and IL-13. Deregulation or uncontrolled activation of Th1 and Th2 cells, respectively are associated with immune pathogenesis. Whereas Th1 cells have been linked to autoimmune reactions Th2 cells are associated with atopy and asthma. Recently a distinct subtype called Th17 was described (Wynn, 2005; Langrish, 2005 JEM). Characteristic for those cells is the production of IL-17 and their impact on autoimmune diseases and recently described also tumorigenesis (Cua, 2003; Murphy, 2003; Langrish 2003; Langowski, 2006; Kortylewski, 2009).

2.2 Autoimmunity

In normal healthy state self-tolerance mechanisms exist. These mechanisms include the discrimination between self and non-self antigens. Already in the early stage of lymphocyte development selection of nonself-reactive from self-reactive lymphocytes is performed. Impaired function of this system often results in autoimmune diseases which can be divided in systemic or organ-specific autoimmune diseases. Prominent examples for systemic autoimmune reaction are systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). They are defined by characteristic auto-antibodies such as antibody against chromatin in SLE or the rheumatoid factor (RF) against the fragment crystallizable (Fc) region of the immunoglobulin G (IgG) in RA. Because systemic autoimmune diseases affect multiple organs and auto-antibodies can not be cleared from the body they often become chronic. In addition to the systemic autoimmune diseases organ-

specific autoimmune diseases are limited to one or a few organs such as inflammatory bowel diseases (IBD) or type I insulin-dependent diabetes mellitus (T1D) or multiple sclerosis (MS). In T1D the immune system attacks the insulin producing pancreatic β cells which leads to the complete loss of insulin production (Green, 1999). MS is an autoimmune disease characterized by the destruction of myelin sheath of the central nervous system that at least influences the signal conductivity of the neurons (Steinman, 1996). Autoimmune diseases were often connected to genetic and environmental risk factors, respectively. Several studies have shown that sun light exposure and thus the Vitamin D_3 (VD_3; 1,25-dihydroxycholecalciferol; 1,25-$(OH)_2D_3$) level is one environmental factor connected to MS or T1D (Cantorna, 2000; The EURODIAB substudy 2 study group, 1999). In addition genetic risk factors as trigger for autoimmune diseases are increasingly described. Genetic loci associated with autoimmune disorders often encode for genes important during immune response such as intracellular signaling molecules and PRRs, transcription factors, cytokines and cytokine receptors, membrane receptors and co-stimulatory molecules (**Table 2**). Interferon regulatory factor (IRF) 5 has been associated with SLE and the IBD ulcerative colitis (Sigurdsson, 2005; Sigurdsson, 2008; Graham, 2005; Graham, 2007; Harley, 2008; Hom, 2008). Furthermore signal transducer and activator of transcription (STAT) 4, a key molecule in the IL-12p70 signaling pathway, was identified as a risk gene for autoimmune diseases (Remmers, 2007). In patients treated with anti-rheumatic drugs the high expression of STAT4 in DCs in the synovium disappears (Walker, 2006). In addition to the transcription factors also cytokines and its receptors are associated with autoimmune diseases such as the IL-23 receptor (Duerr, 2006; Barret, 2008). Because there is also an association of IL-12p40 with the IBD Crohn's disease and psoriasis the balance between both cytokines IL-12p70 and IL-23 seems to be important for the pathogenesis of these diseases (Cargill, 2007; Nair, 2007; Barret, 2008).

Table 2: Genetic loci associated with autoimmune diseases (Gregersen et al., 2009)

Gene	Location	Function	Diseases[a]
Intracellular signaling molecules and receptors			
PTPN22	1p13.3	TCR and BCR signaling and other?	RA, SLE, AITD, T1D
BANK1	4q22	B cell activation/BCR signaling	SLE
TNFAIP3	6q23	Ubiquitin editing enzyme; inhibitor of TNFR signaling/NF-κB pathway	RA, SLE, CD
BLK	8p23	B cell activation	SLE
PTPN2	8p11.3	Negative regulator of T cell activation	CD, T1D
TRAF1	9q33	Regulates TNFR signaling/NF-κB pathway	RA
Intracellular pattern-recognition receptors			
IFIH1	2q24	Receptor for viral dsRNA	T1D, GD
NOD2/ CARD15	16q12	Intracellular receptor for bacteria, signals via NF-κB	CD
Transcription factors			
REL	2p13	Member of NF-κB	RA
STAT4	2q32.2	Regulates IFN-γ pathway	RA, SLE
IRF5	7q32	Regulates type 1 IFN pathway	SLE
NKX2-3	10q24.2	Regulates development of intestinal and secondary lymphoid organs and B and T cell homing	CD
Cytokines and cytokine receptors			
IL2/Il21	4q26	T cell regulation	T1D, RA, Celiac disease
IL23R	1p31.1	Th17 homeostasis	PSA, PSO, CD, AS
IL7RA	5p13	Memory T cell homeostasis	MS
IL2RA	10p15.1	T cell/Treg homeostasis	MS, T1D, GD
IL12B	15q31.1	Development of T cell subsets, Th1 and Th17	PSO, CD
Membrane receptors and costimulatory molecules			
CTLA4	2q33	T cell costimulation inhibitory	T1D, RA
ITGAM	16p11.2	Immune complex clearance/leukocyte adhesion	SLE
CD40	20q12	B/T cell costimulation	RA
		Production of IgM, TNF-α, IL-2 via NF-κB pathway	
Autophagy related			
ATG16L1	2q37.1	Autophagy	CD
IRGM	5q33.1	Autophagy	CD
Enzymes			
ARTS1	5q15	Peptide trimming for MHC I	AS
PADI4	1p36.13	Enzymatic peptide citrullination	RA
Autoantigens			
INS	11p15.5	Target autoantigen	T1D
TSHR	14q31	Target autoantigen	AITD

[a]Abbreviations: AITD, autoimmune thyroid disease; AS, ankylosing spondylitis; CD, Crohn's disease; GD, Graves' disease; MS, multiple sclerosis; PSA, psoriatic arthritis; PSO; psoriasis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T1D, type 1 diabetes

2.3 Dendritic cells

DCs are sentinels and widely distributed through the body. They are specialized to capture, process and present antigens to naïve T cells and induce different T cell responses or tolerance (Banchereau and Steinman, 1998). Several subtypes of DCs differ in their location, migratory pathways and immunological function (Shortman and Liu, 2002). In an immature state DCs screen the peripheral tissue for foreign antigens and capture them. Loaded with the pathogenic structure they migrate to the lymph node where they achieve a mature phenotype. DCs have a hematopoietic origin and invade as precursor cells (pre-DCs) lymphoid and non-lymphoid tissues were they rest. Pre-DCs are plasmacytoid DCs (pDCs), precursor of conventional DCs (cDCs) and monocytes (Shortman and Naik, 2007, Onai 2007). They share a common progenitor in the bone marrow (BM) known as macrophage and DC precursor (MDP) that is characterized by the expression of Fms-like tyrosine kinase 3 (Flt3) (Liu, 2009). Consequently it is clear why differentiation from pre-DCs into cDCs and pDCs requires Flt3-(ligand)L. Although granulocyte macrophage-colony stimulating factor (GM-CSF) is also effective for the generation of DCs from BM progenitors it is not essential. In mice lacking GM-CSF numbers of steady state DCs in lymphoid tissues was not or only slightly altered (Vremec, 1997). In contrast monocytes from the BM differentiate into inflammatory DCs upon cultivation with GM-CSF (Sallusto, 1994). Typical monocytes-derived cells are immature Langerhans cells in the skin (Ginhoux, 2006).

2.3.1 Conventional dendritic cells

Conventional DCs in steady state appear in lymphoid and non-lymphoid tissues and in the circulation (Wu, 2007). Lymphoid tissues containing resident cDCs are thymus, spleen and lymph nodes. Subtypes of cDC are localized in different tissues such as in the skin (Langerhans cells and dermal DCs), the liver, the mucosa and the lung. In addition to the resident cDCs which are immature and active for antigen uptake and processing migratory cDCs exist (Wilson, 2003). They have a mature phenotype and shutdown their antigen uptake function. Furthermore cDCs differ in the expression of their surface cluster of differentiation

(CD) marker and their cytokine production upon activation by microbial stimuli. Immature cDCs in spleen and lymph nodes can be divided in a $CD8^+$ or $CD8^-$ phenotype. The subtype of $CD8^+$ cDCs that is also $CD4^-$, $CD205^+$ and $CD11b^-$ was described as the main producer of IL-12p70 (Reis e Sousa, 1997, 1999; Hochrein, 2001). In contrast $CD8^-$ and $CD4^-$ cDCs produce high amounts of IFN-γ in response to IL-18 but no IL-12p70 or IFN-α (Hochrein, 2001). cDCs differentiated from pre-DCs of the BM are highly $CD11c^+$, $CD11b^+$, $CD45RA^-$ and $B220^-$ and thereby differ clearly from pDCs described below (Diao, 2004).

2.3.2 Plasmacytoid dendritic cells

Although cDCs and pDCs share a common precursor, the morphology and the phenotype of pDCs differ from cDCs. They are round without dendrites, circulate, relatively long-lived and known as the natural type I IFN-producing cell (Liu, 2005). They express specific PRRs recognizing microbial and viral RNA and DNA. Upon activation by these stimuli pDCs mature towards a cDC-like phenotype and produce large amounts of type I IFN (Asselin-Paturel, 2001). Although in the mature state they exhibit a cDC-like population they are poor antigen presenting cells and consequently have a weak capacity to stimulate T cells (Asselin-Paturel, 2001; Krug, 2003). Depending on the maturation state of pDCs they are characterized by their expression of B220, CD45RA, CD11c, Ly6C and GR-1 (Asselin-Paturel, 2001; O'Keeffe, 2002). In contrast to cDCs they do not express CD11b and CD205 such as cDCs and furthermore do not respond to bacterial products (Asselin-Paturel, 2001; O'Keeffe, 2002). pDCs are also described as regulators of the immune response because they activate naïve T lymphocytes to produce IL-10 (Liu, 2005). Recent publications describe the T lymphocyte homing chemokine receptor CCR9 as a marker for the tolerogenic phenotype of pDCs (Hadeiba, 2008; Wendland, 2007). CCR9 is exclusively expressed on immature pDCs which are efficient to induce regulatory T lymphocytes (Hadeiba, 2008).

2.4 Toll-like receptors

Toll-like receptors (TLRs) are PRRs that recognize pathogen-associated molecular patterns (PAMPs). The entire family of TLRs discovered consists of 13 subtypes,

which recognizes a variety of PAMPs (**Table 3**; Akira, 2006). TLRs are type I transmembrane proteins composed of three domains. First the ectodomain which consists of leucine-rich repeats (LRRs) mediate the recognition of the respective PAMPs. Second the transmembrane domain and third an intracellular domain that is known as the Toll/IL-1 receptor (TIR) domain. This domain is homologous to that of the IL-1R and is required for the initiation of downstream signaling pathways (Bowie and O'Neill, 2000). TLRs differ in their localization in the cell. Some TLRs are expressed on the cell surface and others are localized intracellular in different compartments such as endosome, lysosome or the endoplasmatic reticulum (ER). TLRs located on the cell surface include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11. Typical intracellular TLRs are TLR3, TLR7, TLR8 and TLR9. Apart from a cell membrane and intracellular location of TLRs additionally their downstream signaling components differ regarding adaptor molecules and subsequent kinase cascades. Two main adaptor molecules mediate the signaling upon TLR activation are described (Akira, 2004). Myeloid differentiation primary response gene 88 (MyD88) is the universal adaptor for all TLRs except TLR3. Signals via MyD88 activate inflammatory pathways. The association of TLRs and MyD88 results in the recruitment of IL-1R-associated kinase (IRAK) family members such as IRAK1, IRAK2, IRAK4 and IRAK-M. Phosphorylation of IRAKs leads to the dissociation from MyD88 and that result in the interaction with the tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6. This interaction promotes the activation of the transforming growth factor (TGF) -activated kinase 1 (TAK1) that is combined with TAK1-binding protein (TAB) 1, TAB2 and TAB3. Subsequently TAK1 activates the inhibitor of nuclear factor-κB (IκB)-kinase (IKK) complex composed of the catalytic subunits IKKα and IKKβ and the regulatory subunits IKKγ/nuclear factor-κB (NF-κB) essential modulator (NEMO) which then supports the phosphorylation of the IκB proteins. Degradation of the IκB proteins results in the NF-κB translocation to the nucleus. Simultaneously TAK1 activates the mitogen-activated protein kinase (MAPK) pathway which mediates the activation of the transcription factor AP-1. In addition IRF5 is recruited to the MyD88-IRAK4-TRAF6 complex, phosphorylated and translocated to the nucleus. NF-κB, adaptorprotein-1 (AP-1) and IRF5 induce the transcription of inflammatory cytokine genes. TIR-containing adaptor inducing IFN-β (TRIF) is the second important adaptor molecule of the TLR signaling pathway. Whereas TLR4

activation resulted in the recruitment of both, MyD88 and TRIF TLR3 exclusively mediates its signal via the adaptor TRIF. The signaling cascade downstream the adaptor TRIF is also via TRAF6 and TAK1 but additionally TRAF3 is recruited which subsequently activates the TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK1)/IKKi complex that phosphorylates IRF3. In contrast to NF-κB and AP-1, which induce inflammatory cytokines, IRF3 regulates the expression of type I interferons in the TRIF-dependent pathway.

Table 3: Recognition of PAMPs by the different TLRs (Akira et al., 2006)

Microbial Components	Species	TLR Usage
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B *Streptococcus*	TLR6/TLR2
PG	Gram-positive bacteria	TLR2
Porins	*Neisseria*	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites		
tGPI-mutin	*Trypanosoma*	TLR2
Glycoinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma gondii*	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
ND	HCMV, HSV1	TLR2
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4

2.4.1 Toll-like receptor 4

TLR4 recognizes lipopolysaccharides (LPS), a major membrane compound of Gram-negative bacteria. For a robust induction of cytokines TLR4 requires a tight association with another LRR protein, MD-2 which functions as a LPS-binding component on the cell surface. Furthermore LPS-binding protein (LBP) and CD14 localized in the cell membrane or cytoplasm are involved in the response to LPS (Miyake, 2007). TLR4 activates both signaling pathways the MyD88-dependent and alternatively the TRIF-dependent pathway. Whereas other TLRs such as TLR2 and TLR5 induce only proinflammatory cytokines TLR4 can induce both, a proinflammatory and an IFN response (Kawai and Akira, 2006). These differences can be explained by further adaptor proteins that determine which pathway downstream of TLR4 is activated. For example the combination of TIR domain-containing adaptor protein (TIRAP) and MyD88 activates the MAPK and NF-κB pathway resulting in a pro-inflammatory response, whereas in contrast TRAM and TRIF activate TBK1 and IκB that then induce a type I IFN response (Barton, 2009). Although it is generally believed that the two pathways are induced simultaneously, in a recent publication Kagan et al. demonstrated that they are activated sequentially. In the first step LPS-activated TLR4 in combination with the adaptor complex MyD88-TIRAP is endocytosed. During the early endosome formation the MyD88-TIRAP complex is released from the membrane and the TRAM-TRIF complex engage the TIR domain of the TLR4 and induce the second step leading to the gene expression of type I IFN (Kagan, 2008). In addition to LPS TLR4 recognizes envelope proteins of viruses such as respiratory syncytial virus or mouse mammary tumor virus (Akira, 2006). Furthermore TLR4 and another cell surface receptor, TLR2 are also implicated in the recognition of endogenous molecules such as heat shock proteins, fibrinogen, hyaluronic acid, fatty acids, high-mobility group box 1 (HMGB1) and β-defensin 2 which are released during inflammation. Thus, these TLRs not only recognize exogenous structures of microbes but may be sensors for endogenous danger signals (Miyake, 2007).

2.4.2 Toll-like receptor 9

In contrast to TLR4 which is localized on the cell membrane, TLR9 is an intracellular receptor and localized in the endosome, lysosome or ER. TLR9 is

primarily expressed on pDCs and B cells (Hemmi, 2000; Takeshita, 2001; Hornung, 2002) and recognizes unmethylated CpG motifs in DNA. In pDCs TLR9 act as a sensor of viral infections (Lund, 2003; Krug, 2004; Krug, 2004). Prior stimulation TLR9 is resident in the ER but upon stimulation TLR9 rapidly translocates to the endolysosomes (Latz, 2004). During this process the TLR9 is cleaved by proteases, passes through the Golgi and sorted to the endolysosomal system (Ewald, 2008). Although in different DCs TLR9 stimulation induces the expression of proinflammatory cytokines only in pDCs TLR9 stimulation results in a strong IFN-α production. These differences in response to TLR9 ligands can possibly be explained by the expression of IRF7 exclusively in pDCs (Honda, 2005). In this context IRF7 forms a complex with MyD88, IRAK1, IRAK4 and TRAF6 and translocates to the nucleus (Kawai, 2004; Honda, 2004). Synthetic CpG-containing oligodeoxynucleotides (ODNs) are potent ligands for TLR9. Type A ODNs such as CpG2216 (human TLR9 agonist) are characterized by mixed phosphodiester-phosphorothioate backbones and a single hexameric purine-pyrimidine-CG-purine-pyrimidine motif flanked by self-complementary bases that form a stem loop with a poly-G tail at the 3' end (Verthelyi, 2001). In contrast type B ODNs such as CpG1668 (mouse TLR9 agonist) have phosphorothioate backbones that contain thymine cytosine guanine (TCG) motifs. Although both types (A and B) can induce IFN-α in pDCs, type A ODNs fail to stimulate B cells (Verthelyi, 2001; Krug, 2001). When both ODNs are applied simultaneously, type B ODN suppress the type A ODN induced secretion of IFN-α (Gursel, 2002).

2.5 Cytokines

Cytokines are immunomodulating products of immune cells such as interleukins, chemokines, interferons and tumor necrosis factors that mediate many functions of these cells. In parallel to inflammatory and regulatory cytokines produced during an immune response growth and differentiation factors exist that are primarily necessary for the development of cells. In response to microbial stimuli phagocytes and DCs secrete cytokines such as IL-12p70, IL-23 or IFN-α. The release of these cytokines activates in turn cells of the innate and adaptive immunity such as natural killer (NK) cells and T cells, respectively to produce

TNFα, IFN-γ or IL-17. In the following sections cytokines investigated in the context of this thesis are described in detail.

2.5.1 Interleukin-12

Kobayashi et al. identified a 70 kD glycoprotein produced by an Epstein-Barr-Virus-transformed B cell line that activates and enhances the cytotoxicity and IFN-γ secretion of NK cells (Kobayashi, 1989). This protein, better known as IL-12p70 is a heterodimeric cytokine composed of the subunits p35 and p40 and induces Th1 cell response (Hsieh, 1993; Manetti, 1993). IL-12p70 further promotes the expansion and activity of CTLs both directly and indirectly by Th1 cells (Colombo and Trinchieri, 2002). In addition to the virus-transformed B cell lines, where IL-12p70 was discovered, DCs and monocytes are known as the main IL-12p70 producers in response to microbial stimuli (Mosmann, 1989; D'Andrea, 1992; Macatonia, 1995). Although the gene expression of both subunits is regulated independently they have to be expressed in one cell. Whereas the expression of the subunit p35 is tightly regulated and therefore the rate limiting step for the formation of the bioactive heterodimer, p40 is expressed at quantities higher than required (Snijders, 1996). Thus, it is clear that p40 also forms homodimers that have been never found for p35 (Wolf, 1991). Both p40 mono- and homodimers have been proposed as natural inhibitors for bioactive IL-12p70 (Gillessen, 1995). IL-12p70 is initially induced rapidly and independent of T cell signals such as IFN-γ (Scharton-Kersten, 1996). The pro-inflammatory cytokine IL-12p70 needs to be strictly controlled by negative and positive regulatory mechanisms. Microbial products including bacterial wall components, structures of fungi and parasites, bacterial and viral RNA and DNA that are recognized by TLRs are strong inducers of IL-12p70 (Ma, 2001). Thereby the amount of IL-12p70 depends on the expression pattern of TLRs in the different subsets of DCs and phagocytes. During the inflammatory response IL-12p70 is enhanced by IFN-γ produced by Th1 or NK cells via a positive feedback mechanism (Ma, 1996; Hayes, 1998). Furthermore stimulation of CD40 expressed by DCs through CD40 ligand expressed by T cells increases the production of IL-12p70 (Bianchi, 1999; Schulz, 2000). Interestingly also Th2 derived cytokines IL-4 and IL-13 enhances IL-12p70 by induction of gene

expression of both subunits p35 and p40 (D'Andrea, 1995; Marshall, 1997). All of these positive enhancers of IL-12p70 production serve as regulators for an adequate immune response upon microbial invasion. In contrast inhibitory mechanisms for IL-12p70 are needed to avoid uncontrolled, dysregulated immune responses. Maintaining the fine balance between immune reaction against pathogens and detrimental systemic inflammation potent inhibitors of IL-12p70 such as IL-10 are expressed. IL-10 suppresses the transcription of both subunits p35 and p40 (Aste-Amezaga, 1998; Demangel, 2002; Xia, 2003). In addition transforming growth factor β influences the p40 mRNA stability (Du, 1998). Furthermore type I interferons inhibit the IL-12p70 production in mature DCs (Heystek, 2003). Surprisingly the pro-inflammatory cytokine TNFα is also able to suppress IL-12p70 (Ma, 2001). Cells influenced by IL-12p70 such as T cells, NK cells and also DCs and B cell lines express the IL-12p70 receptor (IL-12R). The IL-12R is composed of the IL-12Rβ1 and IL-12Rβ2 chain that activates the janus kinase (JAK)/STAT pathway mainly via STAT4 (Thierfelder, 1996; Kaplan, 1996). During immune response activation of T cells through the TCR induces the expression of the IL-12R chains. Furthermore transcription is enhanced by IL-12p70 itself, IFN-α, TNF and through co-stimulation of CD28. Several years ago it was discussed whether under defined circumstances the deregulation of IL-12p70/Th1 pathway are the main cause for the development of autoimmune reactions. Since the discovery of other IL-12 family members such as IL-23 and IL-27 it was shown that IL-12p70 and the resulting Th1 response are not the main players in autoimmune diseases (Becher, 2002; Gran, 2002; Cua, 2003; Murphy, 2003; Hunter, 2005). All known IL-12 family members and their receptors are represented in the figure below (**Fig.1**).

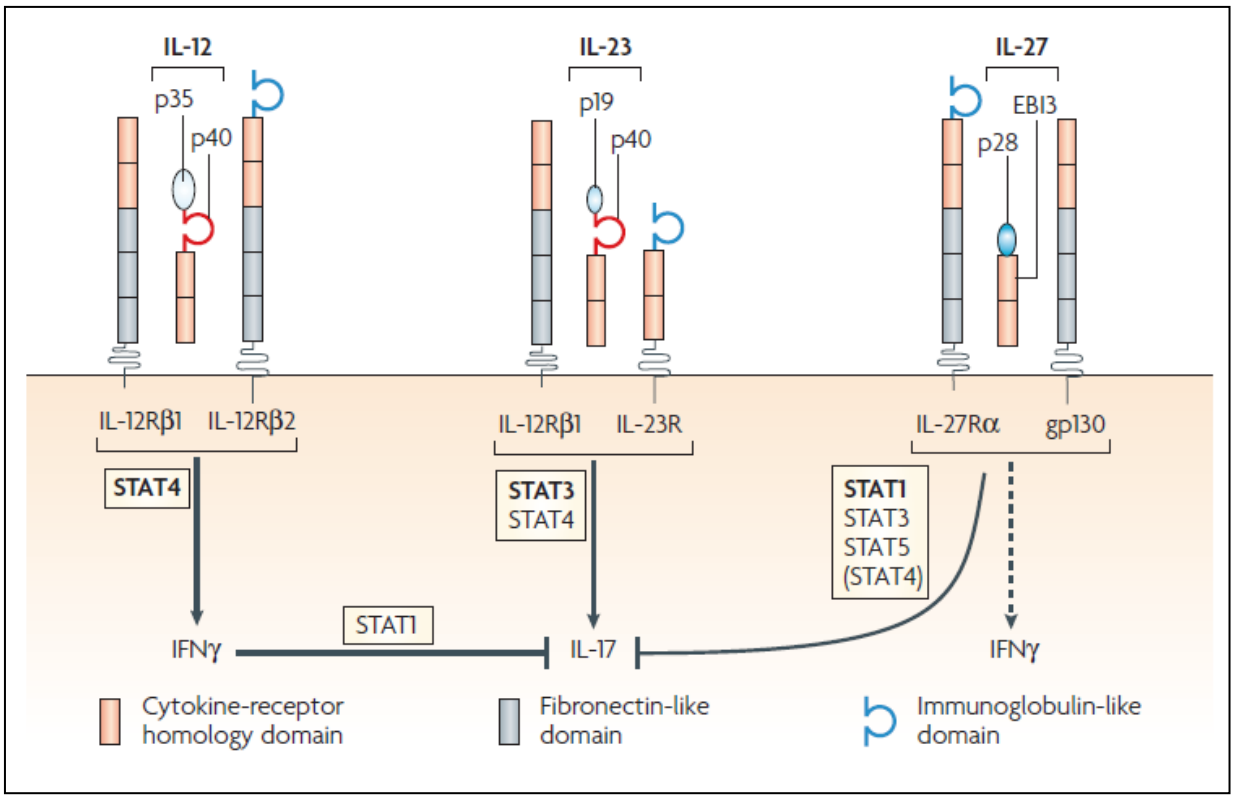

Fig. 1: IL-12 family and their respective receptors.
IL-12p70 is composed of the subunits p35 and p40. IL-23 shares with IL-12p70 the subunit p40 and additional consist of the subunit p19. The third member is IL-27 that is composed of the subunits p28 and EBI3. (Goriely et al., Nature, 2008)

Whereas the role of IL-12p70 in autoimmune diseases is controversial, its anti-tumor potential has been described in several publications (Gerosa, 2002; Kaplan, 1998; Shankaran, 2001; Colombo, 2002; Trinchieri, 2003; Airoldi, 2009). Herewith a clear opposite function between the two IL-12 family members, IL-12p70 and IL-23, became evident (IL-23 is described in the next section).

2.5.2 Interleukin-23

With the discovery of IL-23 in the year 2000 a new member of the IL-12 family was found (Oppmann, 2000). IL-23 consists of p40, the common subunit with IL-12p70, and the unique subunit p19. IL-23 is produced by DCs upon TLR activation through microbial stimuli. In comparison to IL-12p70 that induces a Th1 response IL-23 promotes the expansion and survival of Th17 cells, a new T cell subset characterized by the production of IL-17 (Langrish, 2005). The receptor for IL-23 is expressed on T cells and NK cells and is composed of the recently cloned IL-23R and IL-12Rβ1, of which the latter in turn is also a moiety of the IL-12R (Parham,

2002). Although IL-23 and IL-12p70 are both secreted by DCs and have comparable functions for the immune response, IL-23 is causative for several autoimmune diseases as shown by different groups recently. In a transgenic mouse model overexpression of p19 led to multi-organ inflammation, runting, infertility and premature death (Wiekowski, 2001). IL-23 is also responsible for the establishment and maintenance of different autoimmune diseases as shown in animal models for collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE) or IBD, whereas p19-deficient mice are resistant to these diseases (Cua, 2003; Murphy, 2003; Ghilardi, 2004; Langrish, 2005; Yen, JCI, 2006).

Furthermore an *in vivo* study using a monoclonal antibody against p19 demonstrates reduced EAE in mice (Chen, 2006). Although IL-23 drives autoimmune diseases like IL-12p70, at the same time it has an opposite role to IL-12p70 in tumorigenesis. IL-23 reduces $CD8^+$ T cells in tumor and promotes tumor angiogenesis (Langowski, 2006, 2007). Recent findings for the transcription factor STAT3 that is overexpressed in tumors may clarify the different functions of IL-12p70 and IL-23 regarding tumorigenesis. Thereby STAT3 signaling in the tumor microenvironment shifts an anti-tumor IL-12p70 program to a pro-tumor IL-23 program through the transcriptional activation of the IL-23 subunit p19 and simultaneously augments the transcriptional suppression of the IL-12p70-specific p35 gene. This transcriptional regulation in tumors was observed by co-activation of the p19 promoter with NF-kB/p65 and on the other hand a suppression of c-Rel binding to the p35 promoter (Kortylewski, 2009).

2.5.3 Interferon-α

Type I IFN in general was discovered as a protein secreted by cells infected with an inactivated influenza virus. This protein was able to interfere with the viral replication and the viral cell entry which established the name “interferon” (Isaacs and Lindenmann, 1987). Since the discovery of IFNs two main subtypes of the IFN family are known, type I IFNs which include IFN-α and IFN-β and type II IFNs with their most prominent protein IFN-γ (described below). IFN-α is mainly produced by pDCs upon viral infections or stimulation with bacterial CpG-containing DNA. In addition to its direct antiviral activity IFN-α also has immunomodulatory functions.

During viral infection IFN-α enhances the production of IL-12p70 by cDCs. Interestingly several studies showed that high levels of IFN-α applied exogenously decrease the expression of the p40 subunit (Cousens, 1997; Dalod, 2002). Inhibition of IFN-α signaling or depletion of pDCs recovers the IL-12p70 production (Dalod, 2002). In contrast low amounts, which are comparable to the endogenous levels of IFN-α during an infection, increase the efficiency of the p35 transcription (Gautier, 2005). Furthermore IFN-α increases the cytotoxic properties of NK cells and macrophages (Ortaldo, 1983). As described above in addition to the IL-12p70 production IFN-α supports the maturation of cDCs and induces the proliferation of regulatory T cell (Ito, 2001). IFN-α enhances the expression of MHC-I molecules which results in an increased presentation of viral but also self antigens (Biron, 1999; Biron, 2001). Because IFN-α exhibits such a complex and important role in the immune response several studies investigating the potential of recombinant IFN-α in clinical applications have been performed both to use its antiviral as well as anti-tumor potential (US NIH, clinicaltrials.gov).

2.5.4 Interferon-γ

IFN-γ was first detected 40 years ago as an antiviral IFN (Wheelock, 1965). T lymphocytes and NK cells are the main sources of IFN-γ, but also B cells, macrophages, and DC produce IFN-γ (Yoshimoto, 1997; Puddu, 1997; Ohteki, 1999). Induction of IFN-γ is initiated by the IL-12p70 production of antigen-presenting cells such as DCs. In turn IFN-γ increases the IL-12p70 production in a positive feedback loop (described in 2.5.1). IFN-γ represents the characterizing cytokine for a Th1 response. During inflammatory responses IFN-γ suppresses the production of regulatory IL-10 (Flores, 2007). The IFN-γ receptor consists of two chains, the IFN-γR1 and IFN-γR2. Whereas the IFN-γR1 binds the ligand IFN-γ the second chain IFN-γR2 is necessary for the functional receptor complex and the signal transduction. The signals upon receptor activation are transferred via the Jak/STAT1 pathway, whereas the different JAKs are interchangeable (Kotenko, 1996). Release of IFN-γ, stimulated by activated DCs correlates with a potent tumor-specific cytolytic activity (Furomoto, 2004; Miconnet, 2002; Vicari, 2002).

2.5.5 Interleukin-17

IL-17 is a proinflammatory cytokine produced by activated T lymphocytes (Th17) and was originally described as CTL-associated antigen 8 and later identified as IL-17 (Rouvier, 1993; Yao, 1995). IL-17 stimulates epithelial, endothelial and fibroblastic cells to express different proinflammatory cytokines such as IL-6 and IL-8 (Kolls, 2004). Five family members of IL-17 have been discovered (IL-17A-F) whereas only IL-17A, IL-17E and IL-17F are characterized in considerable detail (Moseley, 2003). IL-17A and IL-17F equally produced by Th17 cells can form heterodimers. In the differentiation of Th17 cells two major transcription factors are involved, STAT3 and retinoid-related orphan receptor (ROR)-γt. Furthermore, for a differentiation of IL-17 producing T cells from naïve lymphocytes IL-6 and TGFβ are required (Bettelli, 2006; Veldhoen, 2006; Mangan, 2006). Although for their initial differentiation IL-23 only plays a minor role, the expansion and survival of Th17 cells depends on IL-23, produced by DCs (Veldhoen, 2006). Although Th17 cells lack the ability of self-amplification such as Th1 and Th2 cells, they produce the cytokine IL-21 which induces the expression of the IL-23 receptor on Th17 cells and thus supports the expansion of these cells (Zhou, 2007). IL-23 also induces its own receptor on T lymphocytes (Langrish, 2005; Chen, 2007). Several groups showed that IL-17 production is strongly connected to autoimmune diseases and chronic inflammation. IL-17 is increased in the synovium of RA patients and may be involved in the development of MS, as exemplified in EAE models (Kotake, 1999; Lock, 2002). Furthermore IL-17 is elevated in patients with an active IBD and psoriasis (Fujino, 2003; Zaba, 2007).

2.6 Selenium

Selenium, an essential trace element, is an important factor in several metabolic pathways including the cellular redox status and ROS dependent signaling, the thyroid hormone metabolism and also immune functions (Beckett and Arthur, 2005; Cunningham-Rundles, 2005; Ryan-Harshman and Aldoori, 2005). In the inorganic form Selenium is incorporated as selenocysteine at the active site of many selenoproteins. These selenoproteins are mostly members of the glutathione peroxidase (GPx) and thioredoxin reductase (TR) families (Allan, 1999; Schrauzer, 2000; McKenzie, 2002). Because selenoproteins are involved in many

inflammatory cytokine signaling pathways, e.g. IL-1, Selenium has important functions in the immune system. In 2003 Matsue et al. reported that ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one or PZ51), an organic Selenium-containing compound with different antioxidant activities, efficiently inhibits the LPS-induced cytokine production of immature Langerhans cell line (iLCs, XS-52), the DC dependent proliferation of Th1 and Th2 cells and the ROS production of BM DCs and iLCs (Matsue, 2003). In contrast HIV-positive individuals have diminished Selenium levels that were associated with decreased Th1 cytokine production (Baum, 2000). In mice the supplementation with Selenium results in decreased skin damage and tumor formation upon UVB light exposure (Overvad 1985; Pence, 1994). In humans subnormal levels of Selenium have been associated with an increased risk to develop skin cancer (Clark, 1984). In a study in 2005 it was demonstrated that Selenium levels can be associated with tumorigenesis in a chemical induced tumor model in rats (Björkhem-Bergman, 2005). On the contrary there are investigations in humans regarding the prevention of tumorigenesis by Selenium (Clark, 1996; Lippman, 2009). Analyzing these contrasting studies it appears likely that divergent roles of Selenium could possibly be explained by the different concentrations applied. Hence, it was shown that very low (<10 nM) and very high doses (>10 µM) resulted in cell death. While on the one hand moderate levels of Selenium exhibit antioxidant properties increasing concentrations may reverse its protective potential into a strong oxidative reagent (reviewed in Selenius, 2009).

2.7 Different roles of $p47^{phox}$

2.7.1 $p47^{phox}$ as component of the NADPH oxidase

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a multi subunit complex enzyme converting molecular oxygen into superoxide anion (O_2^-) (Babior, 2004; Cross, 2004). This enzyme is mainly expressed by neutrophils, eosinophils, monocytes and macrophages. During microbial infections the activation of the NADPH oxidase is an important innate function to kill invading pathogens (Segal, 2005; Nathan, 2006). In further enzymatic processes O_2^- is used for production of reactive oxygen species (ROS) supporting phagocytic reactions to defend pathogenic components. The NADPH oxidase complex

consists of membrane (Cybb/gp91/NOX2, Cyba and small G-protein Rap1A) and cytosolic ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and small G-proteins Rac2 and Cdc42) components (**Fig. 2**; Sheppard, 2005). The cytosolic subunit $p47^{phox}$ is necessary for the activation of the NADPH complex, to which end it is phosphorylated by serine and threonine kinases (Ago, 2003; Dang, 2006; Johnson, 1998). Phosphorylation of $p47^{phox}$ unmasks a PX-domain and allows the transport of $p67^{phox}$ and the interaction with the membrane located cytochrome b_{558}/Cybb (Sheppard, 2005). Only this complete NADPH complex is active and functional to produce ROS.

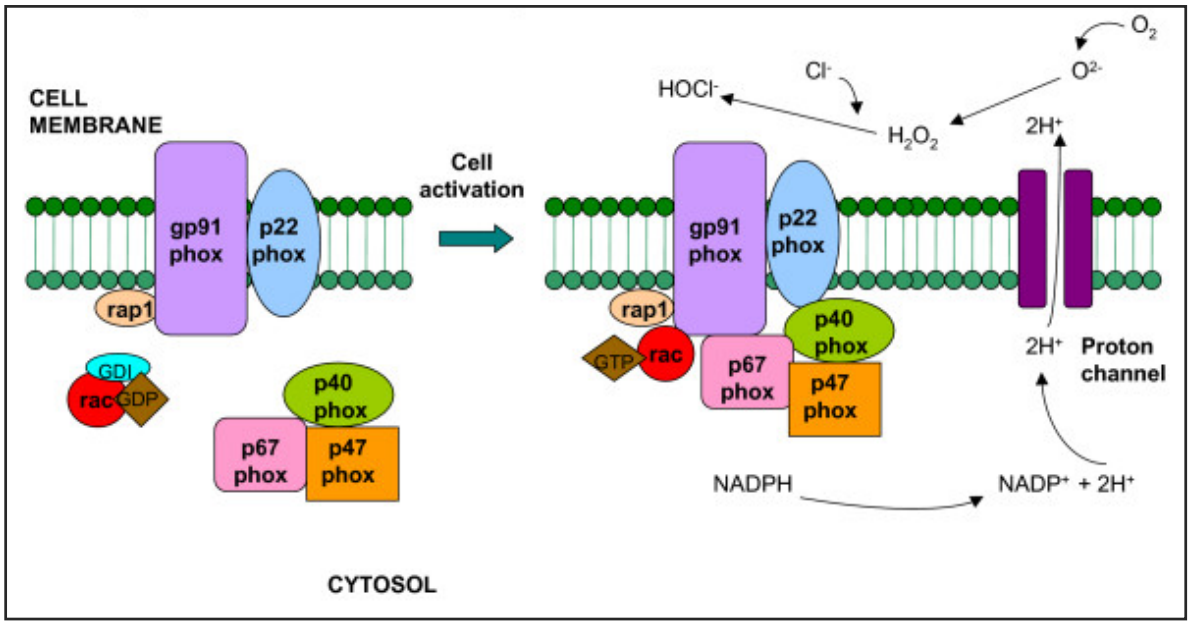

Fig. 2: Activation of the NADPH oxidase. (Assari et al., 2006)

Because the phagocytic NADPH oxidase and ROS production plays an important role in host defense against microbial pathogens, it is clear that mutations in genes of this enzyme lead to dramatic effects. One such genetic disorder is the chronic granulomatous disease (CGD), which is associated with life-threatening bacterial and fungal infections and is characterized by an absence of ROS production due to a deficiency in one of the components of the NADPH oxidase (Meischl and Roos, 1998; Kannengiesser, 2008). Almost 30% of CGD patients suffer from a genetic defect in the $p47^{phox}$ gene *Ncf1*. In addition to $p47^{phox}$ the homologue NOX organizing protein 1 (NOXO1), which is an analogue of $p47^{phox}$, was discovered by different groups (Banfi, 2003; Takeya, 2003; Geiszt, 2003).

2.7.2 p47^{phox} in autoimmune diseases

A connection between a lack of ROS production by NADPH oxidase and increased autoimmunity and severity of RA in mice and rats was proposed (Olofsson, 2003; Gelderman, 2007). A similar effect on RA was demonstrated in a mouse strain with a mutation affecting splicing of the p47^{phox} gene *Ncf1* leading to a defect in the oxidative burst response mediated by the NADPH oxidase complex (Hultqvist, 2004). In some CGD patients along with the progression of the disease inflammation of the gastrointestinal tract (gut) develops. Especially in CGD patients with a p47^{phox} defect these gut inflammatory processes are characterized as chronic IBD such as Crohn's disease or colitis (Roos, 2006). Because the symptoms of CGD are typical for inflammatory processes in patients with an established IBD sometimes a diagnosis of CGD is difficult and confirmed only late (Huang, 2004).

2.7.3 p47^{phox} in TLR signaling

Although the evidence for a connection of p47^{phox} with the TLR signaling pathway was only circumstantial at the beginning of this study, there are some hints for a potential role of p47^{phox} along TLR signaling. Recently it was shown that IRAK4 is able to phosphorylate p47^{phox}. Interestingly, unlike protein kinase C (PKC) IRAK4 not only phosphorylates serine residues but also threonine residues of the p47^{phox} protein (Pacquelet, 2007). Since IRAK4 is strongly involved in the signal cascades of TLRs we wondered whether alternative phosphorylation of p47^{phox}, different from PKC, may point to an important role of p47^{phox} (Kim, 2007). This could have an effect not only for NADPH oxidase activation, but also on the modulation of TLR downstream signaling. Another hint towards a link between p47^{phox} and the TLR pathways was suggested by Takeshita et al. as they discovered an interaction between TRAF4 and the TLR signaling molecules TRAF6, TRIF and IRAK1 (Takeshita, 2005). Results from yeast two-hybrid screening indicated that TRAF4 was able to physically form a complex with TRAF6 and p47^{phox}.

2.8 Vitamin D_3

VD_3 is a steroid hormone that regulates several cellular and physiological responses. Furthermore VD_3 is known to possess a variety of immunomodulatory properties including effects on both myeloid and lymphoid cells (Manolagas, 1990). The influence of VD_3 on T lymphocytes is studied extensively. Th1 proliferation and cytokine production is inhibited by VD_3 (Bhalla, 1984; Lemire, 1992; Mattner, 2000; Boonstra, 2001). In contrast to Th1 response the effect of VD_3 on Th2 response is controversial. Whereas it is proposed that VD_3 upregulates the Th2 cytokines IL-4 and IL-5 (van Etten, 2005) in earlier studies other groups showed an inhibition of the Th2 response (Cantorna, 1998; Staeva-Vieira, 2002). Furthermore VD_3 inhibits IL-6, an important cytokine for the differentiation of Th17 cells (Xue, 2002). In addition to the T lymphocytes also the differentiation and maturation of monocytes and DCs are influenced by VD_3 which interfere with the activation of T lymphocytes (Abe, 1981; Kreutz, 1990; Schwende, 1996; Berer, 2000; Penna, 2000; Griffin, 2001). Although in macrophages phagocytosis and bacterial killing is stimulated by VD_3 treatment the antigen-presenting capacity is suppressed i.e. by the inhibition of MHC-II expression (Lemire, 1992; Griffin, 2000). During differentiation of monocytes into macrophages VD_3 stimulates the phagocytosis by upregulation of NADPH oxidase activity which is mediated by an increased $p47^{phox}$ expression and its translocation to the membrane (Fuhrman, 2004). This correlates with an enhanced antimycobacterial activity of monocytes treated with VD_3 (Sly, 2001). The discovery of the VD receptor (VDR) led to a better understanding of these immunomodulatory effects of VD_3. VDR is a member of the nuclear hormone receptor superfamily and apart from its role for calcium and phosphate homeostasis it has been identified in mononuclear cells, DCs, APCs and activated T and B lymphocytes (Arnson, 2007). Additionally, in primary lymphoid organs such as BM and thymus VDR expression has been approved (Deluca, 2001; Langub, 2000).

2.9 Objectives

In many immune disorders such as autoimmunity, chronic inflammatory diseases and cancer cytokines play an important role. Although it is well accepted that most of these disorders are strongly affected by the adaptive immunity including T and B cells, in the last years the role of DC more and more shifted into the focus of research.

In this thesis differences in the regulation of the IL-12 family members IL-12p70 and IL-23 through exogenous and endogenous factors should be investigated. A variety of DC subtypes exist and their potential to produce cytokines differs between them. In the experiments planned DCs from different immune organs have to be investigated. Some DCs produce only one of the both selected cytokines. DCs from the skin, iLCs, only produce IL-23 whereas in contrast splenic cDCs are characterized by their exclusive IL-12p70 secretion. As a model for DCs capable to secrete both cytokines BM cells, expanded and differentiated into DCs, will be used in several experiments.

In the following the influence of three different factors on the expression and secretion of either IL-12p70 or IL-23 will be investigated. Therefore this thesis is divided into three major chapters:

1. Regulation of IL-23 by Selenium

To investigate the role of inorganic Selenium on the IL-23 secretion of the iLC line XS-52 in different experiments cells will be cultured with and without Selenium, incubated with different stimuli to induce the cytokine production and finally analyzed. As an iLC-specific read-out levels of IL-23 are determined. In addition to the heterodimeric protein IL-23 that is secreted into the supernatant, also measurements of its subunits p19 and p40 on mRNA and protein level will be presented.

2. Regulation of IL-12p70 and IL-23 by $p47^{phox}$

Based on the finding that $p47^{phox}$ deficiency is associated with autoimmune diseases in mice and rats we asked the question, whether $p47^{phox}$ may also have a function in addition to that within the NADPH oxidase complex? To address this

question published data were collected that raised evidence towards a role of $p47^{phox}$ in the TLR signaling pathway. Although rare, some interesting hints in these investigations convinced us to analyze the influence of $p47^{phox}$ for the TLR-dependent IL-12p70 and IL-23 production. Therefore different mouse and rat models with different $p47^{phox}$ mutations were planned to use for *in vitro* and *in vivo* investigations. For *in vitro* studies DCs from different immune organs should be isolated and the cytokine production upon TLR stimulation has to be measured. Furthermore the ROS level of $p47^{phox}$ deficient and wild type (WT) mice have to be cosidered. Based on these *in vitro* results indicating the innate immune response of DCs *in vivo* experiments investigating the effects on the adaptive immunity have been planned to follow.

3. Role of Vitamin D_3 on IL-12p70 expression

Because VD_3 exhibits immunomodulatory properties such as the inhibition of T cell differentiation or antigen-presenting capacity of DCs in this study the direct influence of VD_3 on the cytokine production of DCs should be determined. In several experiments with different mouse strains the VD_3 influence on IL-12p70 secretion should be detected. Finally a possible connection between VD_3 and $p47^{phox}$ should be tested. Therefore experiments with $p47^{phox}$ deficient and WT mice have to be performed.

3 MATERIAL AND METHODS

3.1 Mouse and rat strains

3.1.1 C57BL/6 strain

The C57BL/6 strain is one of the most common inbred mouse strains. Because the C57BL/6 strain is very robust and relatively easy to breed they are widely used as genetic background for genetically modified mice which were developed as models of human diseases. It is well known that the C57BL/6 strain is biased towards Th1 response and furthermore characterized by the activation of type-I macrophages (Mills, 2000). C57BL/6 strains are i.e. resistant to the intracellular pathogen *Leishmania major*, whereas the BALB/c strain is susceptible for the infection (Mills, 2000). For our experiments C57BL/6 mice were obtained from Harlan Winkelmann GmbH (Borchen, Germany).

3.1.2 BALB/c strain

In contrast to the C57BL/6 strain is the BALB/c mouse characterized by a typical Th2 response including the activation of type-II macrophages. They were mainly used for cancer and immunology research. Since 1920 the original colony was systematically inbred by several laboratories and new BALB/c substrains has been developed. The most substrains reach over 200 generations to date. BALB/c mice for our studies were purchased from Harlan Winkelmann GmbH (Borchen, Germany).

3.1.3 $p47^{phox-/-}$ mice

CGD is a rare genetic disease in which phagocytes do not produce oxidative metabolites such as hydroxyl radical, superoxide, hydrogen peroxide and hypochlorous acid which are important for the host defence. Mutations in the different components of the NADPH oxidase are known as causes for the disease. The most common form is the X-linked form of CGD characterized by mutations in the membrane-bound $gp91^{phox}$ (60% of cases), followed by autosomal recessive forms caused by mutations in $p47^{phox}$ (30% of cases), $p67^{phox}$ (5% of cases) and $p22^{phox}$ (5% of cases). CGD patients suffer from severe bacterial and fungal

infections combined with aberrant inflammatory responses and tissue granuloma formation (Meischl and Roos, 1998; Kannengiesser, 2008).

The first mouse model for CGD with a mutated $p47^{phox}$ was generated by Steven M. Holland (Jackson Laboratory, Bar Harbor, Maine, USA). The $p47^{phox}$ gene was disrupted by the insertion of a neomycin cassette into exon 7 (Jackson, 1995). Those $p47^{phox-/-}$ mice were backcrossed to the C57BL/6 background and bred at the local animal facility under specific pathogen-free conditions.

For our *in vivo* experiments we also used $p47^{phox-/-}$ mice with a neomycin cassette in exon 3, produced by Jürgen Roes (Harbord, 2002). Those mice, backcrossed to C57BL/6 for ten generations, were kindly provided by A.M. Shah (Li, 2003).

3.1.2 Natural mutated $p47^{phox}$ mice

Additional to the generated $p47^{phox-/-}$ mouse models we used an earlier identified mouse strain with a natural mutation in the $p47^{phox}$ gene (Huang, 2000). This mutation, located at a splicing site of exon 8 of the $p47^{phox}$ gene, results in different splice transcripts. Consequently those truncated proteins influence the NADPH oxidase function, resulting in the loss of ROS production. Mice were backcrossed to B10.Q strain by Rikard Holmdahl and signed as $Ncf1^{*/*}$ (Karolinska Institutet, Stockholm, Sweden).

3.1.3 $gp91^{phox-/-}$ mice

As a model for the X-linked form of CGD $gp91^{phox}$ knockout mice were generated by insertion of a neomycin cassette in exon 3 (Pollock, 1995). Comparable to the $p47^{phox-/-}$ mice $gp91^{phox}$ knockouts are characterized by an absent ROS production. For our experiments we obtained the $gp91^{phox-/-}$ mice from the Jackson Laboratory (Bar Harbor, Maine, USA).

3.1.4 $TRIF^{-/-}$ mice

$TRIF^{-/-}$ mice were generated by initiation of a program for random germline mutagenesis, resulted in a single base-pair deletion in the TRIF gene (Hoebe, 2003). Consequently 24 amino acids have been removed from the carboxy terminus. Due to this mutation the TRIF-dependent TLR signaling is impaired. The

TRIF$^{-/-}$ mice were kindly provided by U. Kalinke and Z. Waibler (PEI, Langen) with the consent of B. Beutler (Scripps Research Institute, La Jolla, USA).

3.1.5 MyD88$^{-/-}$ mice

MyD88 is the adaptor protein for both pathways, TLR4 and TLR9. In 1998 a MyD88 knockout mouse was created. The knockout of the MyD88 gene was generated by the replacement of 2 exons with a neomycin cassette (Adachi, 1998). Those mice show an impaired IL-1β and IL-18 signaling. We also obtained the MyD88$^{-/-}$ mice from the PEI with the consent of S. Akira (Osaka University, Osaka, Japan).

3.1.6 p47^{phox} mutated rats

Investigating the human rheumatoid arthritis different models for mice and rats were established. Pristane induced arthritis (PIA) sudden and strong developed in the highly susceptible DA rats, but not in the E3 rats. Polymorphisms in arthritis-linked gene loci were investigated in both strains and two polymorphisms resulting in amino acid substitutions were identified (Olofsson, 2003). Only one polymorphism of both, at position 153 resulted in complete protection to RA in E3 rats (153Thr) and high susceptibility in DA rats (153Met), respectively. This fragment from the E3 allele was transferred onto the DA background to obtain a congenic strain. Excluding interference from other E3 loci this congenic strain was backcrossed with DA rats for 12 generations. The backcrossed strain DA.*Ncf1*.^{E3} is completely protected to RA whereas the “wildtype” DA.*Ncf1*.^{DA} are highly susceptible to RA.

3.2 Methods of cell biology

3.2.1 Cultivation and stimulation of the immature Langerhans cell line XS-52

XS-52 medium:	Iscove's medium
	2 mM L-glutamine
	100 IU/ml Penicillin
	100 µg/ml Streptomycin
	1 mM sodium pyruvate
	100 µM non-essential amino acids
	50 µM β-ME

The immature Langerhans cell line XS-52 was established by Xu et al. (Xu, 1995). Therefore cells were isolated from epidermis of newborn BALB/c mice and cultivated *in vitro* for several month in RPMI medium supplemented with 10% of keratinocyte supernatant and 5 ng/ml GM-CSF.

In our lab iLCs were plated at density of $2.5x10^6$ in 10ml medium supplemented with 5% FCS. Additionally cells were cultivated with GM-CSF (5ng/ml) and passaged once a week. In all experiments with iLCs cells were plated $2.5x10^6$ in 10ml medium supplemented with 5% FCS and cultivated for 3 days. For the Selenium experiments iLCs were cultivated in medium containing Na_2SeO_3 in different concentrations (defined in the respective experiments). Afterwards cells were centrifuged and cultivated in serumfree medium for 24 hrs. Next day cells were stimulated with different TLR ligands and/or IFN-γ for 24 hrs.

3.2.2 Isolation of primary cells from spleen and lymph nodes

ACK buffer:	0.15 M NH_4Cl
	10 mM $KHC0_3$
	0.1 mM Na_2EDTA

Spleen cell medium:	Iscove's medium
	2 mM L-glutamine
	100 IU/ml Penicillin
	100 µg/ml Streptomycin
	1 mM sodium pyruvate
	100 µM non-essential amino acids
	50 µM β-ME
Lymph node cell medium:	RPMI 1640 with Glutamax
	100 IU/ml Penicillin
	100 µg/ml Streptomycin
	100 µM non-essential amino acids

Whole spleens or lymph nodes were dissected from mice and stored in PBS or medium without FCS until further processing. Spleens were gently homogenized with a glass tissue homogenizer. In contrast lymph nodes were homogenized using a cell strainer and the back of a plastic syringe. After rinsing the homogenizer or cell strainer thoroughly cell suspensions were centrifuged (1500rpm) for 10 min at room temperature. For removal of red blood cells pellets were suspended in ACK buffer and incubated for 3 min at 37°C. Blocking the reaction 2 volumes of medium with 5% FCS was added. Finally cell suspension was washed twice with serumfree medium and cells were counted.

3.2.3 Purification of $CD11c^+$ dendritic cells from the spleen

Collagenase D buffer:	100 mg Collagenase D
	10 mM HEPES
	0.15 M NaCl
	5 mM KCl
	1 mM MgCl2
	1.8 mM CaCl
	ad 50 ml H_2O (molecular grade)
	sterile filtered trough a 0.22 µm filter unit

MACS buffer:	PBS
	0.5% BSA
	2 mM EDTA

Spleen cells were prepared by enzymatic disaggregation using Collagenase D buffer (Roche Diagnostics, Mannheim). Spleens were cut into small pieces and disrupted using a cell strainer. Cells were suspended in MACS buffer. Subsequently spleen cells were magnetically labeled with CD11c MicroBeads (Miltenyi, Bergisch Gladbach), incubated and separated using a magnetic field according to the manufacture's protocol. Cells from positive and negative fraction were count for further processing. Purity of the CD11c positive fraction was determined by FACS analysis.

3.2.4 Isolation and differentiation of BM-derived cells

BM cell medium:	RPMI 1640 with Glutamax
	100 IU/ml Penicillin
	100 µg/ml Streptomycin
	1 mM sodium pyruvate
	1/100 HEPES
	50 µM β-ME

In the murine system BM cells were isolated from Femur and Tibia. Therefore mice were sacrificed and complete bones were dissected. Femur and Tibia were separated and both ends of each bone were cut. With a syringe filled with PBS the BM was flushed out into a dish until bones got white and clear. The BM was suspended with PBS and centrifuged (1100rpm) for 6 min at RT. Cell pellet was suspended with ACK buffer for red blood cell lyses and incubated at RT for 1 min. Reaction was blocked with medium with 10% FCS. Cell suspension was centrifuged as described above. The cell pellet was suspended with fresh medium containing 10% FCS and cell number was determined.
For the differentiation of BM cells into DCs two well established protocols are generally used (Waibler, 2007). pDCs were generated with 100 ng/ml murine Flt3-

L whereas mDCs require 100 ng/ml murine GM-CSF for their differentiation. Freshly isolated BM cells were seeded $2x10^6$/ ml in the presence of Flt3-L to differentiate into pDCs. In contrast 10^6 BM cells per ml were incubated with GM-CSF for 7 days to generate myeloid DCs. After 4 days two-thirds of medium was exchanged and Flt3-L and GM-CSF, respectively were added.

3.2.5 Cultivation and stimulation of primary and BM-derived, differentiated cells

Primary spleen cells were seeded at $3x10^6$ cells/ ml in T25 cell culture flasks with medium supplemented with 5% FCS and cultivated over night in a humidified incubator (5% CO_2 and 37°C). Next day medium was changed to serumfree medium and cells were stimulated with TLR ligands for 20 hrs. TLR4 was stimulated with 1 µg/ml LPS (Sigma Aldrich, Seelze, Germany) and TLR9 with 2.5 µM CpG ODN2216, CpG ODN2088 and GpC ODN2216 (InvivoGen, Toulouse, France), respectively. Other stimulations were defined in the respective experiments.

Purified $CD11c^+$ cells were seeded in 6-well plates (10^6 cells/ ml) in medium with 5% FCS and incubated over night. Next day cells were stimulated in serumfree medium as described above.

BM-derived, *in vitro* differentiated DCs were seeded in serumfree medium (10^6 cells/ ml) and directly stimulated with TLR ligands. After 20 hrs supernatant was collected and cytosolic protein or mRNA were isolated.

3.2.6 FACS analyses

FACS buffer:	PBS 1% FCS 1% NaN_3

Original spleen cells, magnetically separated CD11c positive and negative cells were stained for CD11c surface expression with anti-CD11c antibody. Dendritic cells differentiated from BM cells were measured for their typical CD markers (**Table 2**). Fluorescence labeled antibodies were used for the detection are listed

in the table below. Isotype controls were used as controls. Subsequently cells were washed twice with FACS buffer and fixed with 1% paraformaldehyde. Expression of CD11c was detected with FACSCalibur Flow Cytometer (BD) and evaluated with CellFlow software.

Table 4: Antibodies and Isotyp controls for FACS analysis

antigen	fluorescence dye	company
CD11c	Allophycocyanin (APC)	Miltenyi, Bergisch Gladbach
Hamster IgG	Allophycocyanin (APC)	BD Bioscience, Heidelberg
B220	Fluorescein isothiocyanate (FITC)	Miltenyi, Bergisch Gladbach
Rat IgG2A	Fluorescein isothiocyanate (FITC)	AbD serotec, Oxford, UK

3.2.7 Concentration of the supernatant

Following incubation with different stimuli cells were scraped and centrifuged (1500rpm) for 10 min at 4°C. Cell-free supernatants were concentrated six-fold with 10 kD Centriprep columns by centrifugation (3000rpm) for 45 min at 4°C (Millipore, Schwalbach, Germany). Centripreps were equilibrated with H_2O (Gibco) and centrifuged (3000rpm) for 8 min before use.

3.2.8 Protein isolation from whole cells

During all isolation steps samples were kept at 4°C. Cell pellets were suspended in PBS containing phosphatase inhibitor cocktail. Subsequent cell suspension was sonified on ice for cells lyses. After sonification the detergent Nonidet P-40 (1:16) and 10mM HEPES were added and suspension was kept on ice for 15 min. Following suspension was centrifuged (3000rpm) for 10 min. The pellet contains the nuclear fraction whereas the supernatant contains membrane and cytosolic proteins.

3.2.9 Protein assays

Protein concentrations of the supernatant and the cytosol/membrane fraction were quantitatively determined with BCA assay (Pierce, Rockford, IL). Samples were diluted in H_2O and incubated with BCA reagent in a 96-well plate at 37°C. With a

more sensitive protocol, micro BCA, very low protein concentrations were detectable. Standard curves were created with BSA.

3.3.10 Detection of Reactive Oxygen Species (ROS)

Reaction buffer:	10 mM HEPES
	Tyrode's salt
	adjusted to pH 7.4 at 37°C

In spleen cells ROS production of cells was stimulated with 100nM PMA (Alexis Biochemicals) and inhibited using 5 µM DPI (diphenylene iodonium chloride) from Sigma Aldrich. H_2O_2 release of spleen cells was measured fluorometrically with Amplex Red reagent purchased from Molecular Probes (Paisley, UK). The Amplex Red reagent in combination with horseradish peroxidase (Sigma Aldrich, Seelze, Germany) reacts with H_2O_2 in a 1:1 stochiometrically to produce resorufin (oxidation product). In the experimental setting spleen cells were isolated and stimulated with LPS, CpG2216 or left untreated for a 20 h period as described before. Subsequently $5x10^5$ spleen cells were seeded in 96-well plates in 200 µl/well reaction buffer containing Amplex Red and horseradish peroxidase (HRP). ROS release of seeded cells was measured continuously from the start and, after the addition of PMA at 35 min, performed for a total period of 75 min.

3.3 Immunological methods

3.3.1 SDS PAGE

Resolving Gel (12.5%)	3.4 ml H_2O
	2.5 ml TRIS (1.5M, pH 8.8)
	4.1 ml Polyacrylamide
	0.1 ml SDS (10%)
	0.1 ml APS (10%)
	4 µl TEMED

Stacking Gel (5%)	1.4 ml H_2O
	0.25 ml TRIS (1.5M, pH 6.8)
	0.33 ml Polyacrylamide
	20 µl SDS (10%)
	20 µl APS (10%)
	2 µl TEMED

Proteins of whole cell lysates were separated by SDS PAGE. SDS polyacrylamid gels were prepared in two steps. First the resolving gel was prepared, cast and covered with isopropanol. After polymerization isopropanol was decanted and the gel was rinsed twice with H_2O and dried with paper. The stacking gel was prepared, cast over the resolving gel and combs were adopted. Protein samples were mixed with loading dye containing β-mercaptoethanol to generate reducing conditions. Afterwards samples were boiled for 5 min and loaded on the gel. Finally Gel was into SDS buffer and electric current was applied (0.04-0.08 A). After 1-1.5 hrs depending on the size of the favored proteins and the current applied gel was blotted (3.3.2).

3.3.2 Western Blot

Blotting buffer A:	0.3 M Tris
	20% Methanol
	Adjusted to pH 10.4
Blotting buffer B:	25 mM Tris
	20% Methanol
	Adjusted to pH 10.4
Blotting buffer C:	25 mM Tris
	0.04 M ε-amino-n-capronacid
	20% Methanol
	Adjusted to pH 9.4

Wash buffer 1 (PBST):	PBS
	0.05% Tween20
	Adjusted to pH 7.5
Wash buffer 2 (TBST):	100mM Tris
	0.15 M NaCl
	0.05% Tween20
	Adjusted to pH 7.5

Table 5: Antibodies for Western Blot

protein	primary antibody	secondary antibody
p19	goat anti-p19 (R&D)	rabbit anti-goat HRP (Dako)
p40	BAF499 (R&D)	Streptavidin-HRP (R&D)

Gels from SDS-PAGE were blotted on a PVDF membrane. Therefore filter papers were soaked in buffer A, B and C, respectively. In defined order filter papers, gel and PVDF membrane were stacked into the blotting apparatus (**Fig. 2**). It is important to avoid air between the several layers. Blotting apparatus was closed and electric current (0.08-0.12 A) was applied for 45-60 min, depending on the number of gels. Afterwards blots were washed in wash buffer 1 and 2, respectively for 15 min. For detection of p19 protein blots were blocked for 1 hr with 3% non-fat dried milk in PBST, for p40 protein with 1% BSA in PBST. Blots for $p47^{phox}$ detection were blocked with 5% non-fat dried milk in PBST. Blots were washed again with wash buffer. Afterwards blots were incubated with the respective antibodies over night. Next day blots were washed and stained with their corresponding secondary antibodies for 1-1.5 hrs. After a further wash step blots were incubated 1 min with ECL reagent and developed with hyperfilms (Amersham). Confirm the constant loading of gels with proteins blots were stripped with stripping buffer for 20 min at 37°C and stained for β-actin with the respective antibody.

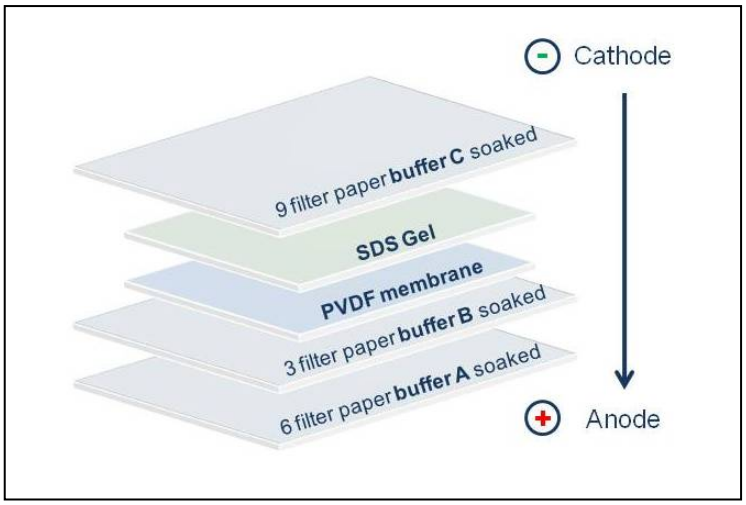

Fig. 3: Assembly of the blotting apparatus

3.3.3 Enzyme-linked immunosorbent assay

ELISA wash buffer:	PBS 0.05% Tween20

Cytokines of interest were measured in the supernatant. Therefore mouse specific ELISA assays for detection of IL-12p70 (eBiosciences), IL-10 (R&D Systems) and IFN-α (PBL Biomedical Laboratories) were used. Except for the IFN-α ELISA 96well plates were pre-coated with the capture antibody and incubated over night at 4°C. In the IFN-α ELISA kit plates are already pre-coated with antibody. Next day plates were blocked with blocking buffer, washed 3 times and samples and standard dilutions respectively were pipette into the wells. After 1.5-2 hrs plates were washed again and detection antibodies were incubated for 2 hrs. Finally antibody-coupled enzymes were used and substrates were pipette. Enzyme-substrate reaction was stopped with H_2SO_4 and plates were measured at 450nm. Results were standardized with standard curves and normalized to whole cell protein measured by BCA protein assay.

3.3.4 ELISPOT assay

ELISPOT assays were performed as described (Radeke, 2002): in brief, ELISPOT plates (Millipore, Schwalbach, Germany) were coated with capture antibody for murine IFN-γ (BD, Heidelberg, Germany) overnight at 4°C. Plates were then washed and blocked with 1% cell culture-grade BSA/PBS for 1 hr at room temperature. After washing freshly isolated lymph node cells were seeded at $3x10^5$/ well or spleen cells at $5x10^5$/well in RPMI medium (Gibco, Invitrogen, Karlsruhe, Germany), supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 µM non-essential amino acids and 10% fetal calf serum. Cells were stimulated with or without 13.5 µg/ml ovalbumin and cultured for 24 hrs at 37°C. Thereafter cells were removed and plates were washed with PBS and PBS/Tween. Detection antibody for mIFN-γ (BD, Heidelberg, Germany) was added in 1%BSA/PBST and incubated overnight. Plates were washed with PBST and PBS before developing the colorimetric assay by addition of 3-amino-9-ethylencarbazole/DMF in 0.1 M sodium acetate buffer (1:30). Colorimetric reaction was stopped with distilled water and air dried. ELISPOT plates were subjected to the computerized ELISPOT reader system A.EL.VIS (ELI.Scan) and enumerated with the ELI.Analyse Software V5.0 (A.EL.VIS GmbH, Hannover, Germany).

3.4 Molecular biological methods

3.4.1 Isolation of mRNA

Cellular mRNA was isolated with the RNeasy Mini Kit (Qiagen). For cell lyses pellets were suspended in RLT buffer containing β-mercaptoethanol. Cell lysates were homogenized using QiaShredder columns (Qiagen). Homogenates were mixed with 1 volume EtOH and loaded onto RNeasy columns and centrifuged (12000rpm) for 2 min. Columns with bound mRNA were washed with RW1 and RPE buffer and centrifuged in between. Finally columns were dried by centrifugation and mRNA was eluated with H_2O (molecular grade). Concentration of RNA was determined photometrically by using an UV Spectrometer (260 nm). Purity was measured by the ratio RNA to protein (260/280 nm).

3.4.2 cDNA synthesis

Primer Mix (10µl):	2 µg mRNA
	1 µl Oligo$(dT)_n$
	1 µl Random Hexamers
	8 µl H_2O (molecular grade)
Mastermix (10µl):	4 µl 5x cDNA Synthase buffer
	1 µl 0.1 M DTT
	1 µl H_2O (molecular grade)
	2 µl 10 mM dNTP Mix
	1 µl RNAse Out
	1 µl RT

First strand cDNA synthesis was performed using the ThermoScript RT-PCR system (Invitrogen). Primer Mix and 2 µg of mRNA sample were mixed and denatured for 5 min at 65°C. Afterwards 10 µl of Mastermix were pipette to the samples and the following program applied.

Table 6: RT-PCR program

Temperature	Time
25°C	10 min
50°C	45 min
85°C	5 min

Finally samples were incubated with 1 µl RNAse H for 20 min at 37°C.

3.4.4 Standard PCR

PCR Mastermix:	5 µl 10x PCR buffer
	1.5 µl $MgCl_2$
	1 µl dNTPs
	38 µl H_2O (molecular grade)
	1 µl forward primer (10 µM)
	1 µl reverse primer (10 µM)
	0.5 µl Taq-polymerase

For the PCR 48 µl mastermix and 2 µl cDNA sample were mixed in a PCR tube and centrifuged to avoid air bubbles in the sample. For cDNA product amplification of p19 and p40, subunits of IL-23, specific forward and reverse primers were used. As a control for the same amount of cDNA in the reaction the house keeping cDNA of GAPDH was also analyzed.

Table 7: Primer sequences (MWG Biotech)

Primer	Sequence
p19 forward	CAGCAGCTCTCTCGGAAT
p19 reverse	ACAACCATCTTCACACTGGATACG
p40 forward	TCTTTGTTCGAATCCAGCG
p40 reverse	GAAAACTGGAAAAAGCCAACC
GAPDH forward	ACCACAGTCCATGCCATCAC
GAPDH reverse	TCCACCACCCTGTTGCTGTA

Afterwards PCR was performed with the following standard program, whereas the annealing temperature depends on the melting temperature of the primer.

Table 8: Standard PCR program

Temperature	Time	Step	
94°C	2 min	activation of the Taq polymerase	
94°C	1 min	Denaturation	30-36 cycles
60°C	1 min	Annealing	
72°C	2 min	Elongation	
72°C	10 min	Final elongation	

3.4.3 Real time PCR

Table 9: Primer sequences (MWG Biotech)

Primer	Sequence
p19 forward	5’ TGCTGGATTGCAGAGCAGTAA
p19 reverse	5’ GCATGCAGAGATTCCGAGAGA
p19 probe	5’ TATGGCTGTTGCCCTGGGTCACTCA
p35 forward	5’ CCACCCTTGCCCTCCTAAAC
p35 reverse	5’ GTTTTTCTCTGGCCGTCTTCA
p35 probe	5’ CCTCAGTTTGGCCAGGGTCATTCCA
p40 forward	5’ GGAAGCACGGCAGCAGAATA
p40 reverse	5’ AACTTGAGGGAGAAGTAGGAATGG
p40 probe	5’ CATCATCAAACCAGACCCGCCCAA
18s forward	5’ GAAACGGCTACCACATCCAAG
18s reverse	5’ CGGGTCGGGAGTGGGT
18s probe	5’ AAGGCAGCAGGCGCGCAAA

Primers (1 µM) and fluorogenic probes (1 µM; labeled with 5’ FAM and 3’ TAMRA) for murine p19, p35, p40 and 18s were purchased from Thermo Fisher Scientific (Ulm, Germany). Real Time PCRs were performed in duplicates with 2.5 µl of cDNA and 0.5 µl forward primer, 0.5 µl reverse primer, 0.5 µl probe, 8.5 µl distilled water and 12.5 µl Platinum Quantitative PCR SuperMix-UDG (Invitrogen) with the following cycling program (45 cycles).

Table 10: Real Time PCR program

Temperature	Time	Step	
50°C	2 min	activation of the Taq polymerase	
95°C	2 min		
95°C	15 sec	amplification	45 cycles
60°C	45 sec		

3.4.4 Polyacrylamidgel-electrophoresis

Polyacryamidgel:	0.2 ml 50x TAE buffer
	4.0 ml Polyacrylamid
	5.8 ml H_2O
	0.1 ml APS (10%)
	4 µl TEMED
50x TAE buffer:	2 M Tris acetate
	50 mM EDTA, pH 8.0

Gel was prepared, poured between 2 glass plates and a comb was inserted. After polymerization of the gel the comb was removed and 10 µl PCR sample mixed with 2 µl 6x loading dye was loaded onto the gel. Subsequently 1x TAE buffer was filled into the tray containing the loaded gel and was run at 100-120 V for 1 hr. To enables the analysis of DNA fragment size in the sample a molecular-weight marker was included.

3.5 *In vivo* immunization

Referring to classical protocols described to elicit delayed-type hypersensitivity three groups of five mice were injected s.c. at the base of the tail at 7-9 week of age. OVA (Sigma Aldrich, Seelze, Germany) alone or with CpG2216 (InvivoGen, Toulouse, France) and PBS alone as a control, respectively, were mixed with Incomplete Freund's Adjuvant (IFA; Sigma Aldrich, Seelze, Germany) at a 1:1 ratio and emulsified. All mice received injections of 200 µl emulsion each either with 50 µg OVA /mouse, with a combination of 50 µg OVA and 50 µg ODN2216 /mouse, or with PBS in IFA, and were sacrificed after 10 days. Inguinal and axillary lymph nodes were isolated and single lymphocyte suspensions used for ELISPOT analysis.

4 RESULTS

4.1 Regulation of IL-23 by Selenium

In this study we investigated the effect of Selenium (sodium selenite, Na_2SeO_3) on the expression of IL-23 subunits (mRNA and protein level) and secretion of the bioactive heterodimer in iLCs that following stimulation exclusively produced IL-23 but no IL-12p70.

4.1.1 Regulation of IL-23 by different TLR ligands in combination with Selenium

In the initial experiments of this study iLCs were cultivated in medium supplemented with different concentrations of Selenium (50, 100, 250 nM) or left untreated for 1-2 weeks. Following iLCs were stimulated with different TLR ligands for 24 hrs. Finally IL-23 secretion was measured in the supernatant. We detected an increased IL-23 secretion upon TLR4 stimulation with LPS and a further up-regulation with Selenium treatment dose dependently. TLR9 and TLR 7/8 stimulation with CpG2216 and R848, respectively resulted in IL-23 secretion, but was not further influenced by Selenium treatment. Cells stimulated with TLR5 ligand Flagellin did not secrete IL-23 (**Fig. 4**).

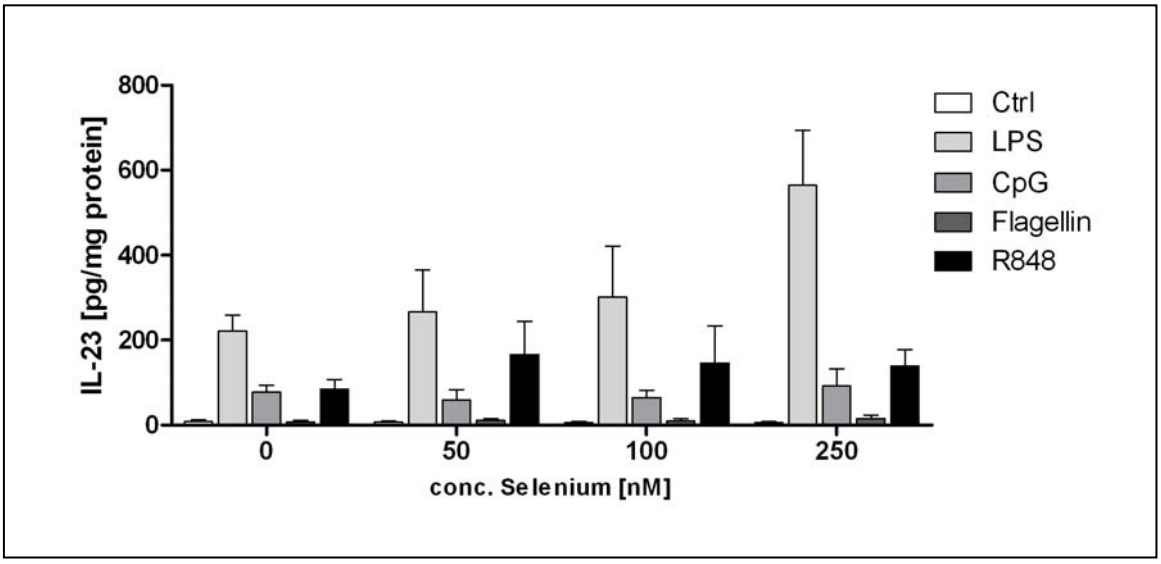

Fig. 4: IL-23 secretion upon TLR stimulation and Selenium treatment.
Selenium treated iLCs were further stimulated with TLR ligands. TLR4 stimulation with LPS [1 µg/ml] resulted in enhanced IL-23 secretion that was additional dose dependently increased by Selenium. Stimulation with CpG2216 [2.5 µM] and R848 [5 µM] increased IL-23 secretion, but no further up-regulation was detectable when cells were treated with Selenium. TLR5 ligand Flagellin [50 ng/ml] did not induce IL-23 production.

4.1.2 Regulation of IL-23 subunits p19 and p40 by Selenium

Since we have measured an up-regulation of IL-23 by Selenium supplementation we wanted to know whether both subunits of the heterodimer IL-23, p19 and p40 are directly influenced. First we cultured iLCs 1-2 weeks with medium containing Selenium (250 nM) and pure medium, respectively. Following iLCs were stimulated with TLR4 ligand LPS or IFN-γ or a combination of both and incubated for 24 hrs. Finally mRNA was isolated, measured and cDNA was synthesized with RT-PCR. Whereas p19 mRNA was constitutively expressed, p40 mRNA was induced by LPS and furthermore enhanced by treatment with Selenium (**Fig. 5**).

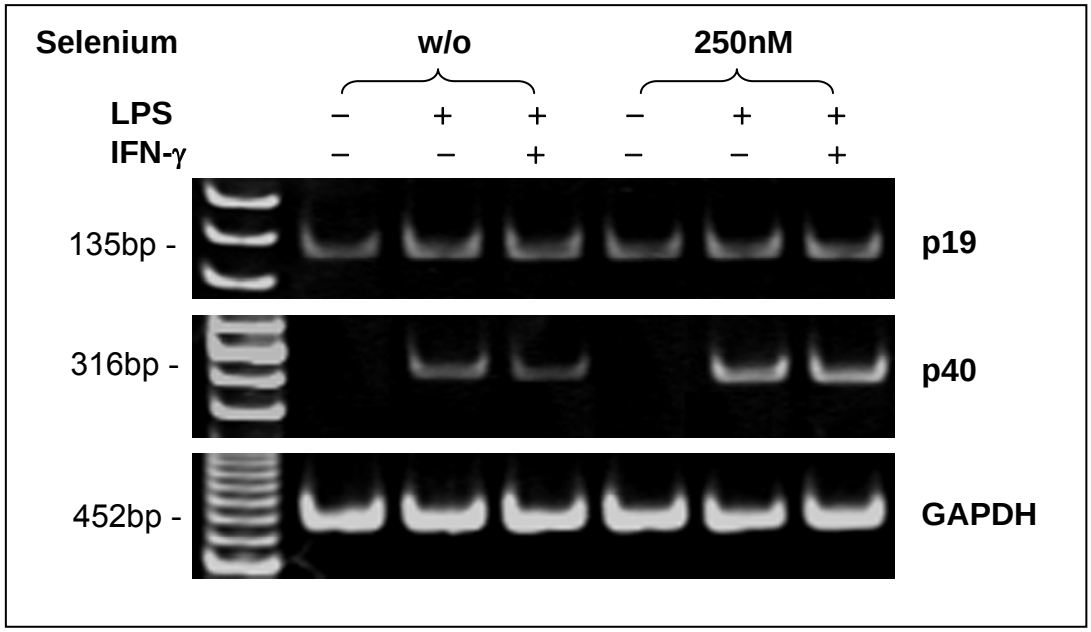

Fig. 5: mRNA expression of p19 and p40.
iLCs, treated with Selenium were stimulated with LPS [1 µg/ml] and IFN-γ [10 ng/ml] and mRNA expression of both subunits p19 and p40 was measured by standard PCR. p19 mRNA was constitutively expressed and not up-regulated by LPS or Selenium treatment. In contrast p40 mRNA was induced by LPS and further up-regulated by Selenium.

From mRNA results we were further interested in the protein levels of both IL-23 subunits. Therefore LPS and IFN-γ stimulated and Selenium treated iLCs were centrifuged and supernatant was concentrated 6-fold. With Western Blot analysis and specific antibodies we detected secreted monomers p19 and p40 in the supernatant. Whereas p19 mRNA was constitutively expressed the protein was initially translated upon LPS stimulation (**Fig. 6a**). Additional stimulation with IFN-γ decreases p19 protein, whereas treatment with Selenium had no influence on p19 protein (**Fig. 6a**). In contrast p40 was not decreased after IFN-γ stimulation, but in the presence of Selenium p40 protein was down-regulated (**Fig. 6b**).

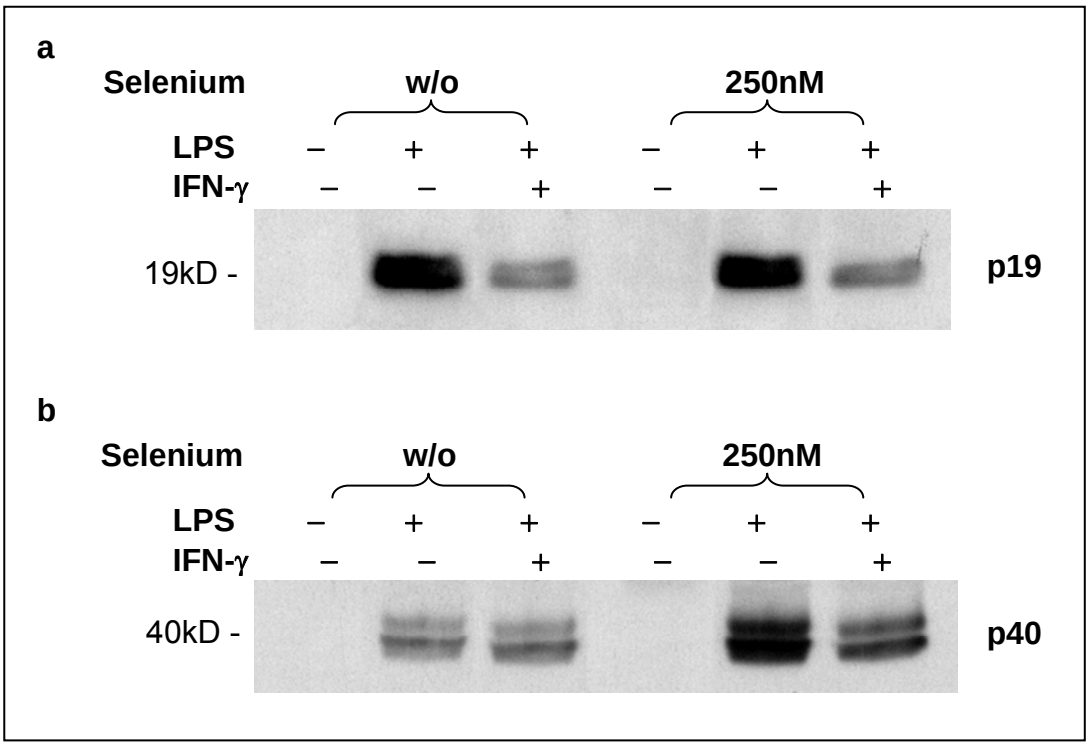

Fig. 6: Western blot analysis of IL-23 subunits p19 and p40.
Protein induction of p19 and p40 was measured in the supernatant from LPS and IFN-γ stimulated iLCs that where cultured in medium containing Selenium or left untreated. Whereas p19 mRNA was constitutively expressed protein was initial induced by LPS and inhibited by IFN-γ addition (a). In contrast p40 protein was also induced by LPS, but IFN-γ had no influence (b). Selenium treatment did not change the p19 protein induction (a), but p40 protein was increased in iLCs cultivated in Selenium-containing medium (b).

From the mRNA data and the protein results of both IL-23 subunits we finally measured the IL-23 heterodimer secretion in the supernatant of those iLCs, treated with Selenium and stimulated with LPS and IFN-γ. As expected and known from the initial experiment LPS did induce IL-23 secretion (**Fig. 7, gray bars**). Furthermore this was enhanced by Selenium treatment (250nM). Additional stimulation with IFN-γ resulted in an inhibition of the LPS induced IL-23 secretion in both, Selenium-treated and untreated iLCs (**Fig. 7, black bars**).

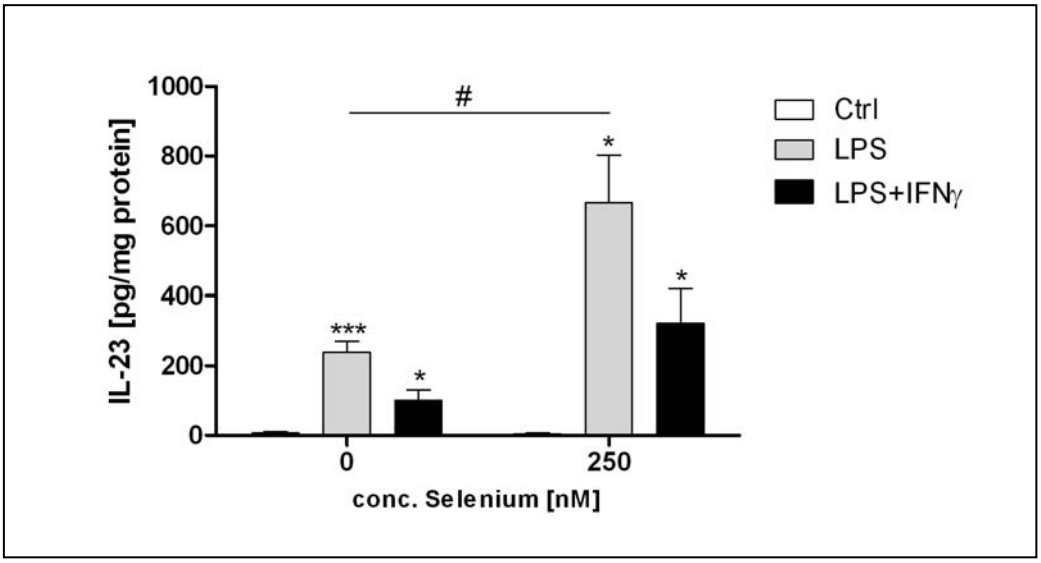

Fig. 7: IL-23 secretion of iLCs measured by mouse specific ELISA.
LPS stimulated iLCs secrete IL-23 which was further enhanced by Selenium supplementation (gray bars). Stimulation with IFN-γ resulted in an inhibition of LPS induced IL-23 secretion in Selenium treated and untreated cells (black bars).

In summary we found a Selenium-dependent increase of IL-23 protein in dendritic cells (immature Langerhans cell line). Interestingly only IL-23 subunit p40 was regulated by Selenium on both mRNA and protein level, p19 was unaffected. Stimulation with IFN-γ resulted in the reduction of LPS-induced IL-23 whereas only p19 protein was downregulated, not p40. In combination with Selenium treatment p40 protein was also diminished.

4.2 Regulation of IL-12p70 and IL-23 by p47^{phox}

Supplementation with Selenium resulted in increased IL-23 production of iLCs. Since it was shown that Selenium influences the redox status of the cell and consequently the antioxidant activity it would be interesting whether a subunit of the ROS producing enzyme NADPH oxidase, p47^{phox}, influences the cytokine signaling in DCs. Based on the findings that deficiency of p47^{phox} resulted in an increased susceptibility to RA and EAE in animal models, with the following part of this thesis the question should be answered whether p47^{phox} deficiency is associated with an increased immune response of DCs.

4.2.1 IL-12p70 regulation upon TLR stimulation in primary spleen cells

4.2.1.1 Regulation of IL-12p70 in p47^{phox} knockout mice

Our initial investigations were targeted at the regulation of IL-12p70 in spleen cells from p47^{phox} knockout and wild type (WT) C57BL/6 mice. Spleen cells were isolated and stimulated for 20 hrs with LPS and CpG2216. We found in p47$^{phox-/-}$ spleen cells an enhanced mRNA expression of IL-12p35 compared to WT mice (**Fig. 8a**). In addition, a difference between TLR4 and TLR9 signaling was discernible, because in WT cells CpG2216 stimulation resulted in a lower expression of p35 compared to LPS (TLR4) stimulation (**Fig. 8a**). IL-12p40 mRNA expression was neither different significantly between WT and p47$^{phox-/-}$ nor between TLR4 and TLR9 stimulatory conditions (**Fig. 8b**).

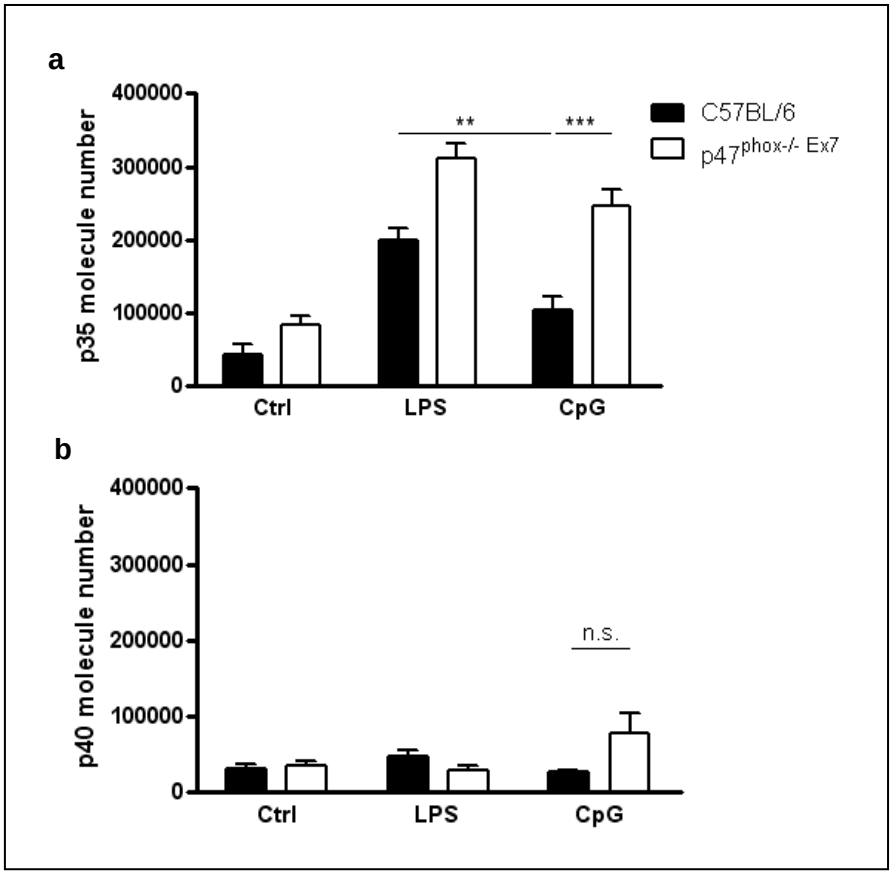

Fig. 8: Analysis of p35 and p40 mRNA.
With Real Time PCR analysis the expression of IL-12p70 subunits p35 and p40 was determined. Enhanced mRNA expression of IL-12p70 subunits p35 (a) was established after TLR9 stimulation with CpG (2.5 µM) in p47$^{phox-/-}$ cells in comparison to wild type cells. p40 mRNA was not significantly altered (b). Statistical significance is indicated (n=3, ***P<0.005, **P<0.01).

In parallel to the detection of mRNA regulation we were interested in the IL-12p70 protein level. With a mouse specific IL-12p70 ELISA we measured the IL-12p70 protein in the supernatant from LPS and CpG2216 stimulated spleen cells. Corresponding to mRNA protein data revealed an enhanced TLR9-induced IL-12p70 protein secretion in p47$^{phox-/-}$ cells compared to WT cells (**Fig. 9a**). We could not detect any difference between p47$^{phox-/-}$ and WT upon TLR4 stimulation with LPS (**Fig. 9a**). To prove the specificity of the agonistic TLR9 ligand CpG2216 with respect to the negative feedback regulation we performed additional experiments with antagonistic and control TLR9 ligands. Antagonistic CpG2088 and control CpG alone did not stimulate IL-12p70. Although as expected increasing concentrations of CpG2088 added to CpG2216 resulted in reduced IL-12p70 secretion, we observed no substantial change in the TLR9-induced negative feedback of IL-12p70 in WT cells (**Fig. 9b**).

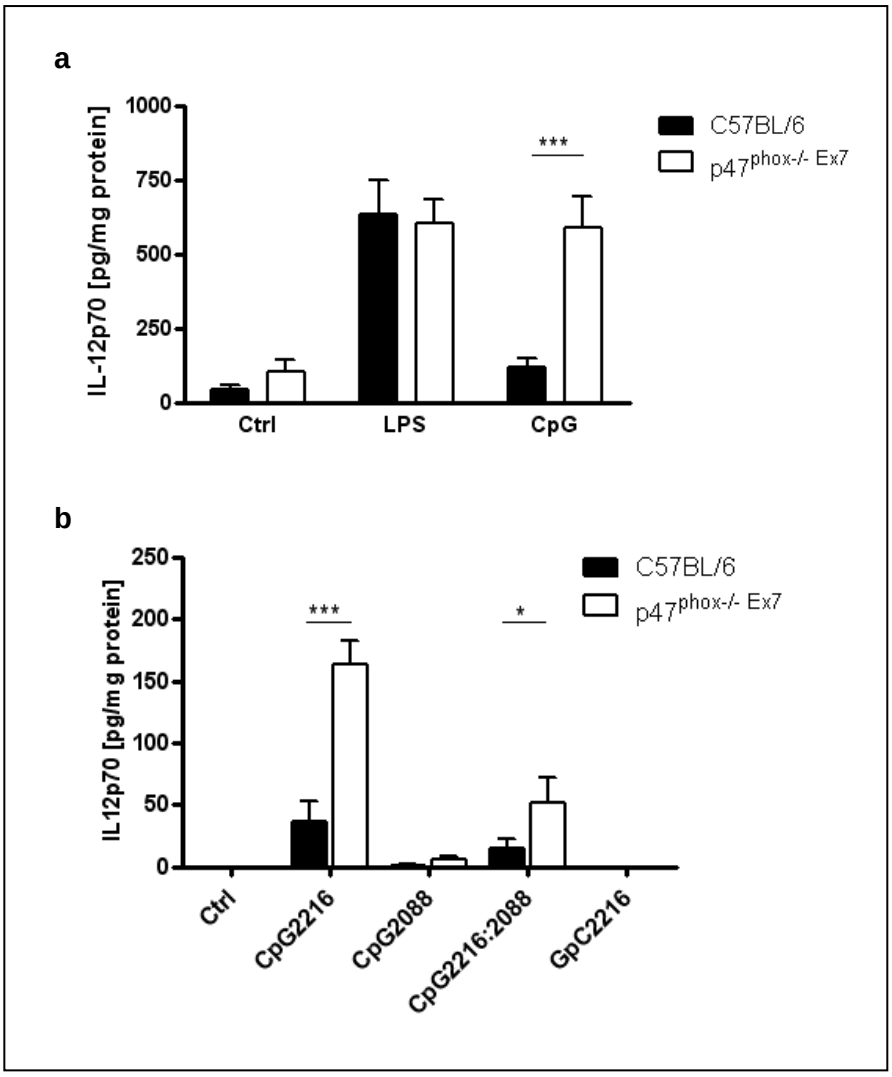

Fig. 9: IL-12p70 protein secretion in spleen cells upon TLR stimulation.
Secretion of IL-12p70 protein, measured by specific ELISA, was also increased in CpG2216 stimulated p47$^{phox-/-}$ cells (a). TLR4 stimulation with LPS (1 µg/ml) showed no significant differences between p47$^{phox-/-}$ and wt cells (a). Co-stimulation of CpG2216 and CpG2088 resulted in diminished IL-12p70 secretion, but CpG2088 did not influence the negative feedback in wt (b). Statistical significance is indicated (n=3, ***P<0.005, *P<0.05).

4.2.1.2 Regulation of IL-12p70 in p47^{phox} mutated mice

A problem with the p47^{phox} knockout mouse is that it does not only differ by the defective p47^{phox} gene in comparison with WT C57BL/6 but also with a large and unknown linked chromosomal fragment derived from the 129 strain. To exclude the possibility of such linked genetic polymorphisms, as well as differences in the remaining background of the poorly defined BL/6 strains, we used a genetically better controlled strain with a mutation in the p47^{phox} gene, leading to ROS deficiency, on the B10.Q background (Ncf1*/* mice). To confirm our hypothesis we

investigated spleen cells of Ncf1*/* and B10.Q WT mice regarding TLR4- and TLR9-induced IL-12p70. Indeed we observed a negative feedback regulation in TLR9-induced IL-12p70 in WT cells, while in Ncf1*/* cells IL-12p70 was elevated (**Fig. 10**).

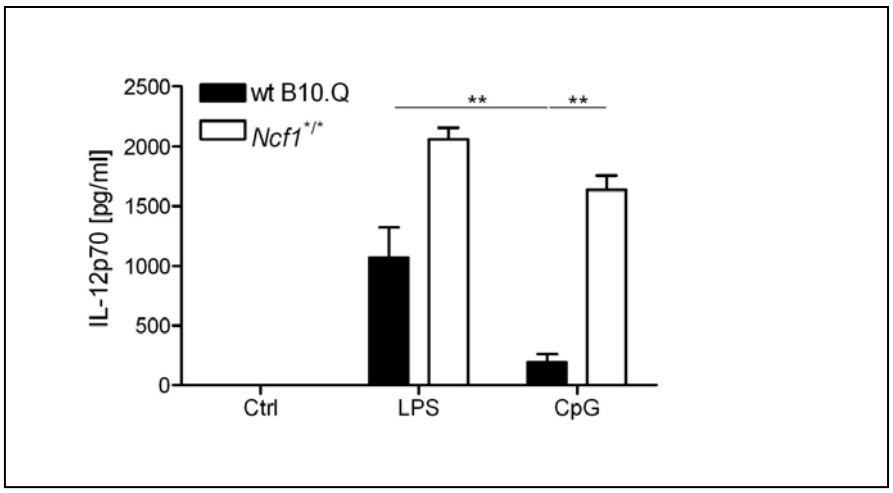

Fig. 10: IL-12p70 secretion in Ncf1*/* spleen cells upon TLR stimulation.
Secretion of IL-12p70 protein, measured by specific ELISA, was increased in CpG2216 stimulated Ncf1*/* cells compared to wt cells. After TLR4 stimulation with LPS (1 µg/ml) no significant difference between Ncf1*/* and wt cells was observed. Statistical significance is indicated (n=5, **P<0.01).

4.2.1.3 Regulation of IL-12p70 in p47^{phox} mutated rats

In addition to mouse models with a p47^{phox} defect we were interested in the rats with a SNP at position 153 in the p47^{phox} protein. The most important point is that this SNP is located at a phosphorylation position in p47^{phox}. Threonin at this position can be phosphorylated by IRAK4, but not by PKC which is one of the common enzymes activating p47^{phox}. IRAK4 as a kinase in the TLR signaling pathway is related to p47^{phox} which fits in our hypothesis. As described before rats with threonin (DA.*Ncf1*.^{E3}) are completely protected to RA and EAE whereas rats with methionin (DA.*Ncf1*.^{DA}) showed fast onsets and severe progressions of both diseases. In our experiments spleen cells from both rat strains were stimulated with LPS and CpG2216 and IL-12p70 protein was measured in the supernatant. Upon LPS stimulation in both rats no IL-12p70 was detectable (**Fig. 11**), whereas stimulation with CpG2216 resulted in an increased IL-12p70 response in the DA.*Ncf1*.^{DA} rats compared to DA.*Ncf1*.^{E3} (**Fig. 11**).

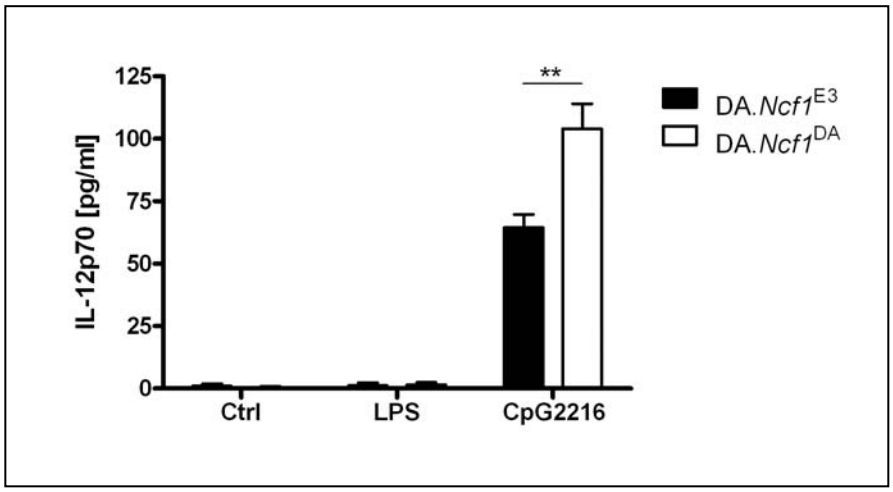

Fig. 11: IL-12p70 regulation in rat spleen cells upon TLR stimulation.
Rat spleen cells were stimulated with LPS [1 µg/ml] and CpG2216 [2.5 µM]. LPS did not induce IL-12p70 in both rat strains. In contrast stimulation with TLR9 ligand CpG2216 resulted in the induction of IL-12p70 with a significant enhanced secretion in DA.*Ncf1*.^{DA} cells compared to DA.*Ncf1*.^{E3}. Statistical significance is indicated (n=4, **P<0.01).

4.2.2 IL-23 production upon TLR stimulation in primary spleen cells

Since IL-23 is also produced by dendritic cells and play an important role in chronic inflammation we investigated the IL-23 response to TLR4 and TLR9 ligands in spleen cells of p47$^{phox-/-}$ and WT mice. We measured mRNA of the IL-23 subunit p19 with Real time PCR and the whole protein IL-23 in the supernatant. Because IL-23 shares with IL-12p70 the subunit p40 which was investigated before we focused in this part on the IL-23 subunit p19. With Real-time PCR analyses we measured p19 subunit in TLR stimulated and control spleen cells. We could not detect any regulation of p19 neither upon TLR4 nor upon TLR9 stimulation compared to the control (**Fig. 12**).

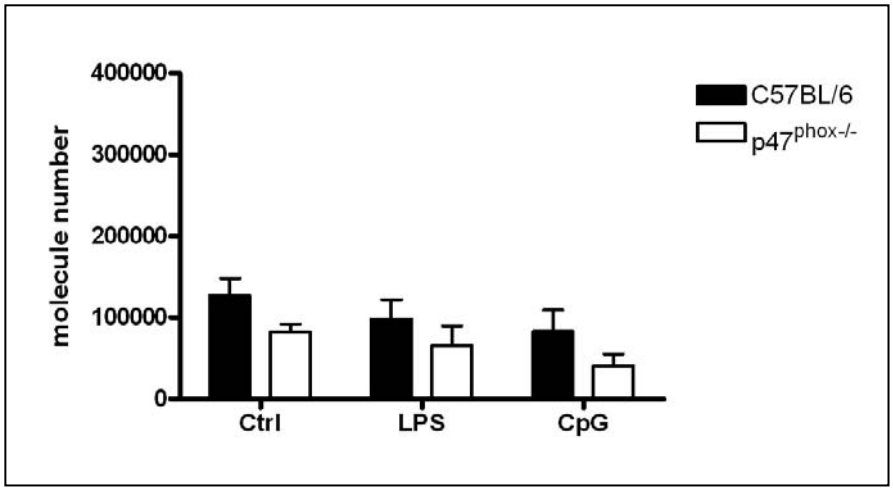

Fig. 12: Analysis of p19 mRNA expression.
With Real Time PCR expression of p19 subunit was measured. Molecule number of p19 mRNA was not significantly altered upon TLR stimulation with LPS [1 µg/ml] or CpG2216 [2.5 µM] in WT and $p47^{phox-/-}$ cells.

With mouse specific IL-23 ELISA we measured IL-23 in the supernatant of TLR4 and TLR9 stimulated spleen cells. We detected neither upon LPS nor upon CpG2216 stimulation IL-23 in the concentrated supernatant (**Fig. 13**). The detection limit of this ELISA is 15.6 pg/ml. Because TLR4 stimulation with LPS should induce IL-23 production we conclude from our results that IL-23 producing dendritic cells are absent in the spleen.

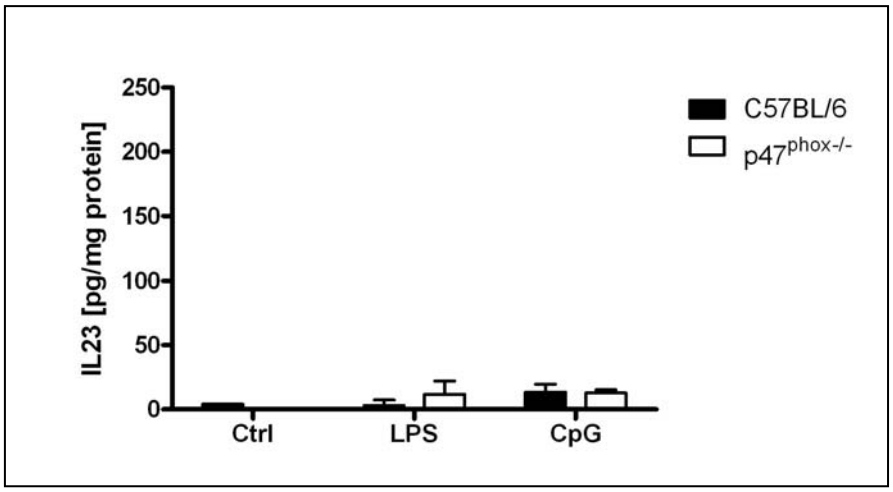

Fig. 13: IL-23 ELISA of TLR stimulated spleen cells.
In TLR4 [1 µg/ml LPS] and TLR9 [2.5 µM CpG2216] stimulated spleen cells from WT and $p47^{phox-/-}$ no IL-23 secretion was detectable. One representative experiment out of 4 is shown.

4.2.3 Regulation of other cytokines in primary spleen cells

4.2.3.1 Regulation of IFN-α in p47^{phox} knockout mice

In order to confirm the differences between TLR4 and LR9 signaling our interest focused also in IFN-α as an important factor in anti-viral immune response. For this purpose we stimulated spleen cells of p47$^{phox-/-}$ and WT mice with LPS and CpG2216 and measured IFN-α secretion in the supernatant. We found that IFN-α was exclusively induced after stimulation with TLR9 ligand CpG2216, but not after LPS, in both groups of mice (**Fig. 14**). Additionally we observed significant higher levels of IFN-α secretion in spleen cells of p47$^{phox-/-}$ mice compared to WT (**Fig. 14**).

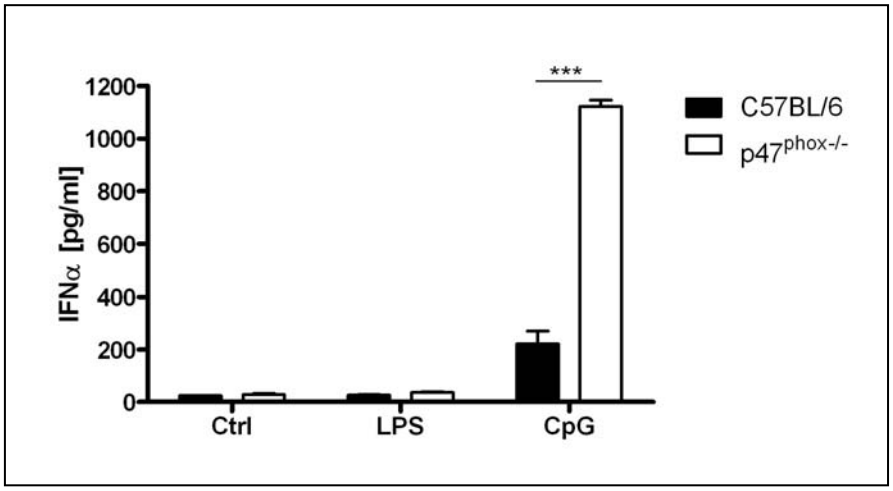

Fig. 14: IFN-α secretion in TLR stimulated spleen cells.
In concentrates of supernatants IFN-α was measured by mouse specific ELISA. Exclusively TLR9 stimulation with CpG2216 (2.5 µM) resulted in secretion of IFN-α. In p47$^{phox-/-}$ cells IFN-α is significantly increased in comparison to wt cells. Statistical significance is indicated (n=4, ***P<0.005).

4.2.3.2 Regulation of IL-10 in p47^{phox} knockout and mutated mice

To follow up our hypothesis regarding p47^{phox} regulation in TLR signaling we extended our investigations towards additional immune regulatory cytokines. To this end we stimulated spleen cells of p47$^{phox-/-}$ and WT mice with LPS and CpG2216 and measured IL-10 secretion after a 20 hrs incubation period. IL-10 protein levels were significantly elevated in p47$^{phox-/-}$ cells after TLR9 stimulation compared to WT conditions (**Fig. 15a**). In contrast, a TLR4 mediated response

through stimulation with LPS led to IL-10 secretion with no difference between p47$^{phox-/-}$ and WT cells (**Fig. 15a**). In parallel we measured also IL-10 secretion in Ncf1$^{*/*}$ mice and detected significantly lower levels after TLR9 stimulation in WT compared to p47^{phox} mutants (**Fig. 15b**). In contrast to the experiments with the p47^{phox} knockout mice we also measured decreased IL-10 secretion in WT cells upon TLR4 stimulation with LPS (**Fig. 15b**).

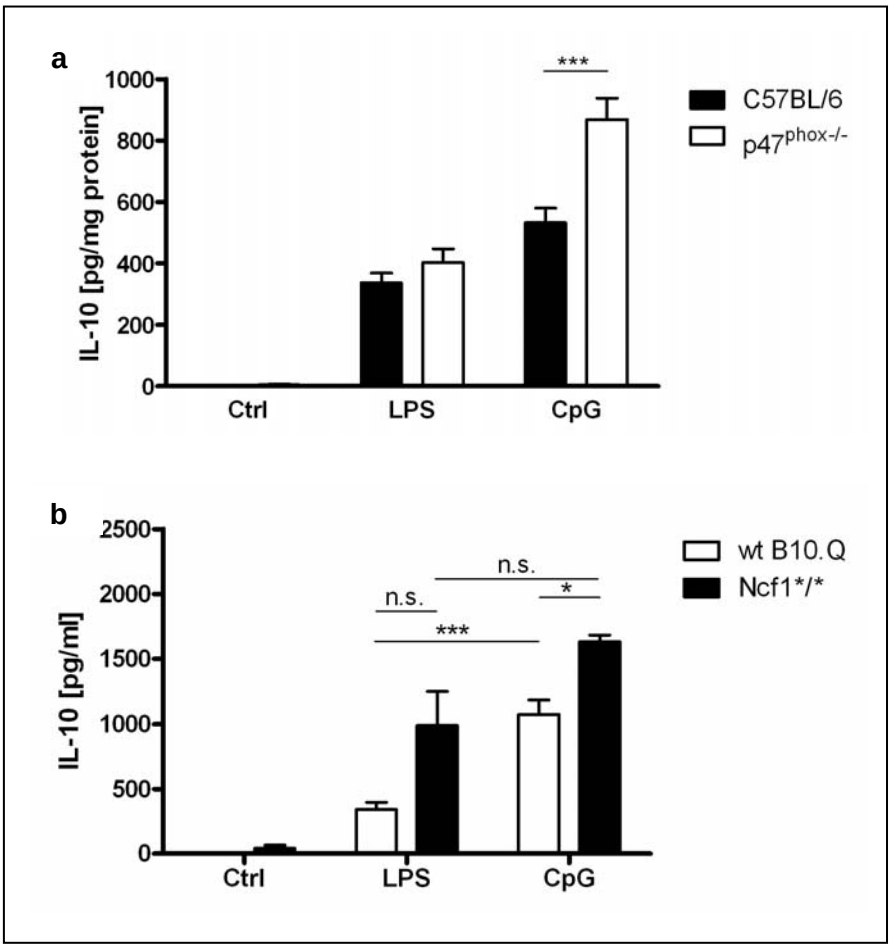

Fig. 15: IL-10 secretion of TLR stimulated spleen cells.
IL-10 secretion in TLR9-stimulated p47$^{phox-/-}$ cells was significantly increased compared to wt cells (a). Upon TLR4 stimulation there is no significant difference in IL-10 secretion detectable (a). In Ncf1$^{*/*}$ spleen cells IL-10 is increased upon TLR9 and TLR4 stimulation compared to the WT cells. Statistical significance is indicated (n=4, ***P<0.005, *P<0.05).

4.2.4 ROS independent feedback regulation of IL-12p70

4.2.4.1 Comparison of p47^{phox} and gp91^{phox} knockout spleen cells

To investigate whether the observed unrestrained TLR9 mediated IL-12p70 response in cells lacking p47^{phox} is independent of the lack of ROS production we

performed control experiments with spleen cells from gp91$^{phox-/-}$ mice. Like p47$^{phox-/-}$ cells functionally these are also unable to assemble an active NADPH oxidase. Stimulation of spleen cells from gp91$^{phox-/-}$ mice with the TLR9 ligand resulted in a decreased IL-12p70 secretion as shown in WT cells before, whereas again p47$^{phox-/-}$ mice secrete significantly more IL-12p70 after CpG2216 stimulation (**Fig. 16**).

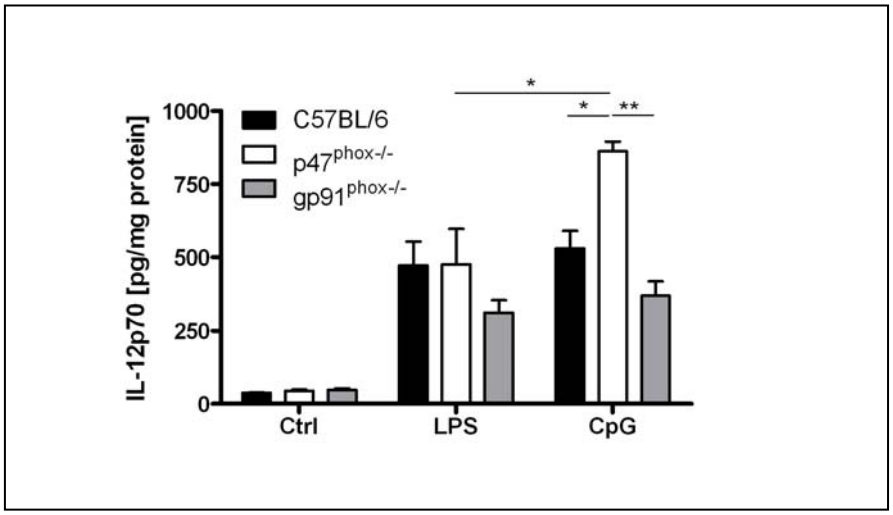

Fig. 16: Comparison of p47$^{phox-/-}$ and gp91$^{phox-/-}$ spleen cells regarding IL-12p70.
IL-12p70 was measured in the supernatants of spleen cells upon incubation with LPS [1 µg/ml] and CpG ODN2216 [2.5 µM] for 20 hrs. In both, WT and gp91$^{phox-/-}$ spleen cells TLR9-induced IL-12p70 secretion was comparable. In contrast IL-12p70 in p47$^{phox-/-}$ cells was significantly increased after TLR9 stimulation. Statistical significance is indicated (n=3, *P<0.05, **P<0.01).

4.2.4.2 Inhibition of ROS with DPI

Further experiments to confirm a ROS-independent role of p47^{phox} were performed with an inhibitor of ROS production, DPI. Spleen cells were investigated regarding their inducible ROS production by pre-stimulation with LPS or CpG2216. Additional samples were stimulated with PMA to induce ROS production and measured at different time points. In the process we detected higher levels of ROS in WT cells pre-treated with LPS and CpG2216, whereas as expected ROS production in p47$^{phox-/-}$ cells was not detectable (**Fig. 17a**). Parallel to ROS measurement IL-12p70 secretion was determined in p47$^{phox-/-}$ and WT cells. Although ROS formation was undetectable after DPI treatment (**Fig. 17b**) no impact on feedback regulation by p47$^{phox-/-}$ in TLR9 signaling was observed (**Fig.**

17c). Surprisingly, after blockade of ROS production the feedback regulation is now clearly observable also in TLR4 signaling (**Fig. 17d**).

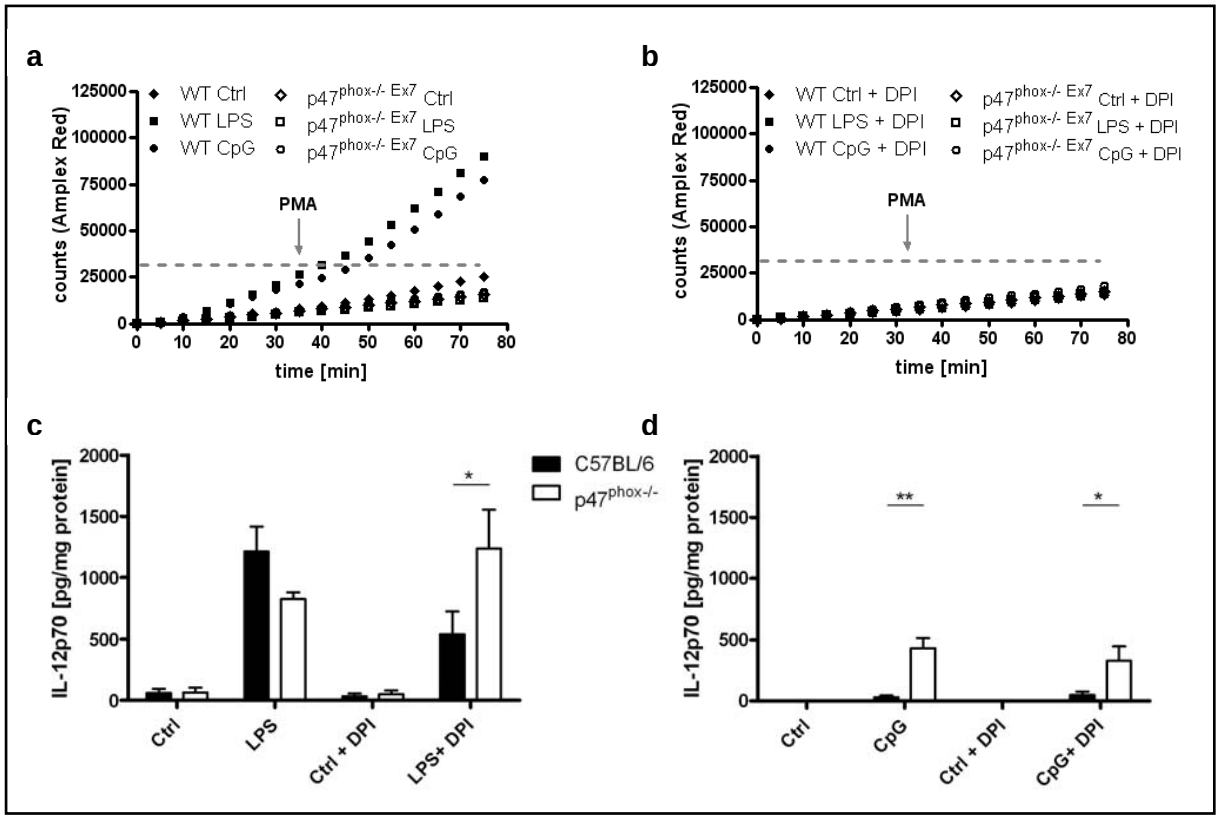

Fig. 17: Measurement of ROS and ROS-independent regulation of IL-12p70.
Measurement of ROS production via Amplex Red detects after PMA treatment [100 nM] increased ROS only in WT cells pre-stimulated with LPS [1 µg/ml] or CpG2216 [2.5 µM], whereas ROS production in $p47^{phox-/-}$ cells was absent (a). After inhibition of NADPH oxidase by DPI [5 µM] ROS production was completely abolished (b). The grey line marked the basal production of ROS. One representative experiment out of four is shown. IL-12p70 secretion was measured in the supernatants of spleen cells after incubation with LPS [1 µg/ml] and CpG2216 [2.5 µM] or in combination with DPI [5 µM] after 20 hrs. Secretion of IL-12p70 protein was increased in $p47^{phox-/-}$ cells after TLR9 stimulation alone as well as in combination with Inhibitor DPI compared to wt cells (c). DPI treatment resulted also in decreased levels of IL-12p70 in TLR4 stimulated wt cells. This effect was not observable in wt cells treated with LPS [1 µg/ml] alone (D). Statistical significance is indicated (n=3, *P<0.05, **P<0.01).

4.2.5 MyD88 dependent regulation of IL-12p70

In order to evaluate the relative contribution of different TLR downstream signaling adaptor modules we performed further experiments with $MyD88^{-/-}$ and $TRIF^{-/-}$ mice. In $MyD88^{-/-}$ and $TRIF^{-/-}$ spleen cells, stimulated with the TLR4 ligand LPS, IL-12p70 secretion was markedly reduced due to lack of either the MyD88- or TRIF-dependent signaling pathway. When cells were stimulated with TLR9 agonist

CpG2216 IL-12p70 secretion was completely abolished in both MyD88$^{-/-}$ and TRIF$^{-/-}$ cells (**Fig. 18**).

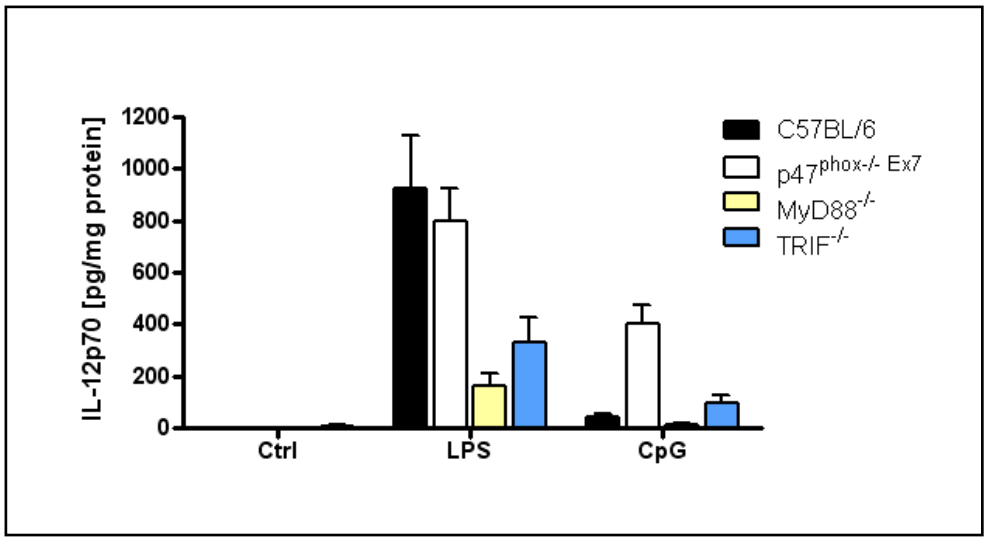

Fig. 18: IL-12p70 secretion in spleen cells of MyD88$^{-/-}$ and TRIF$^{-/-}$ mice.
After LPS stimulation decreased IL-12p70 was observed in both, MyD88$^{-/-}$ and TRIF$^{-/-}$ compared to wt and p47$^{phox-/-}$ cells. After TLR9 stimulation with CpG2216 exclusively in p47$^{phox-/-}$ cells IL-12p70 was detected. One representative experiment out of two is shown.

4.2.6 IL-12p70 regulation in CD11c^{+} enriched spleen cells

Since all experiments so far had been performed in whole spleen cells we reasoned which specific cell type might be responsible for the negative feedback regulation of IL-12p70. In the spleen, dendritic cells are one of the major IL-12p70 producers. To investigate the role of dendritic cells we isolated CD11c positive cells from the spleen as described before and stimulated CD11c^{+} and CD11c^{-} cells with TLR4 and TLR9 ligands. IL-12p70 was enhanced after TLR4 stimulation in both, p47$^{phox-/-}$ and wild type CD11c^{+} cells (**Fig. 19a**). Stimulation with TLR9 ligand CpG2216 only resulted in elevated IL-12p70 secretion in p47$^{phox-/-}$ CD11c^{+} cells compared to wild type CD11c^{+} cells (**Fig. 19a**). In comparison to CD11c^{+} spleen cells CD11c^{-} cells clearly showed a lower IL-12p70 secretion after TLR4 stimulation and hardly detectable IL-12p70 after TLR9 stimulation (**Fig. 19b**).

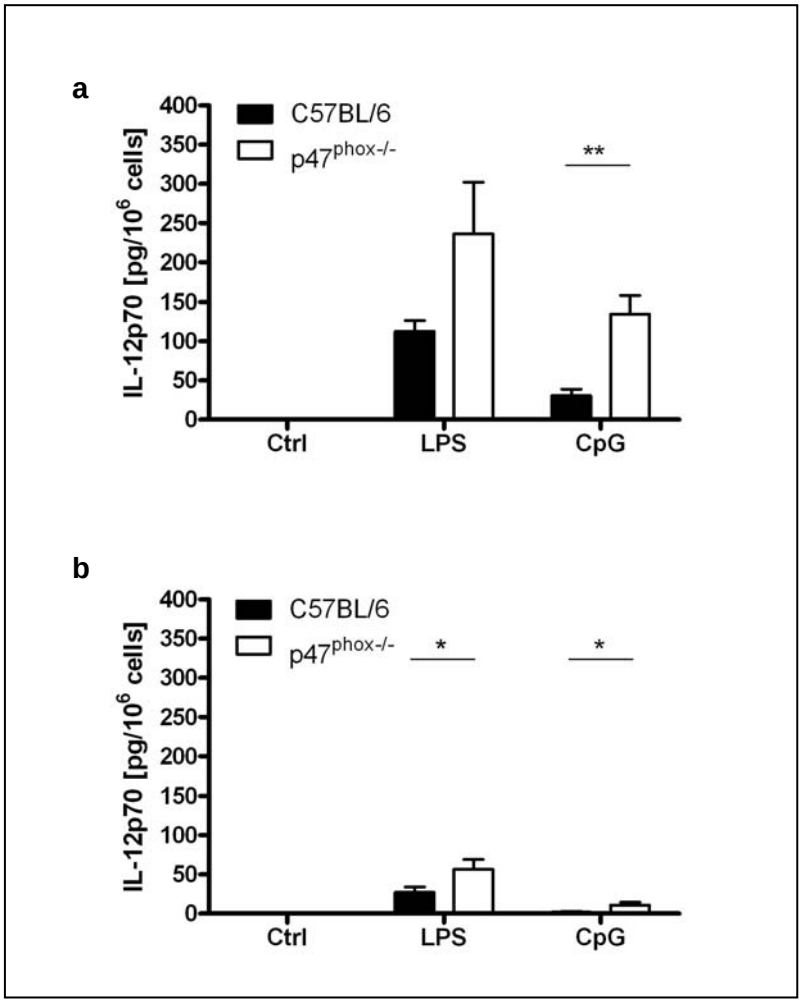

Fig. 19: IL-12p70 secretion of CD11c$^+$ dendritic cells.
Purified CD11c$^+$ spleen cells from p47$^{phox-/-}$ mice stimulated with CpG2216 [2.5 µM] exhibited an increased IL-12p70 secretion compared to WT cells (a). Upon TLR4 stimulation with LPS [1 µg/ml] showed no significant difference between WT and p47$^{phox-/-}$ cells (a). In contrast CD11c$^-$ cells secreted no IL-12p70 upon TLR9 stimulation with CpG2216 and hardly IL-12p70 upon LPS stimulation (b). Statistical significance is indicated (n=3, *P<0.05, **P<0.01).

In parallel as a control we monitored the isolation and purification of spleen cells by FACS. Therefore cells from all three fractions; original, CD11c$^+$ and CD11c$^-$ were incubated with CD11c FACS antibodies for 15 min. Finally cells were measured and counted by FACS. Results show that we were able to enrich CD11c$^+$ cells from 3.5% in whole spleen cell suspension to about 78% of CD11c$^+$ positive cells (**Fig. 20**).

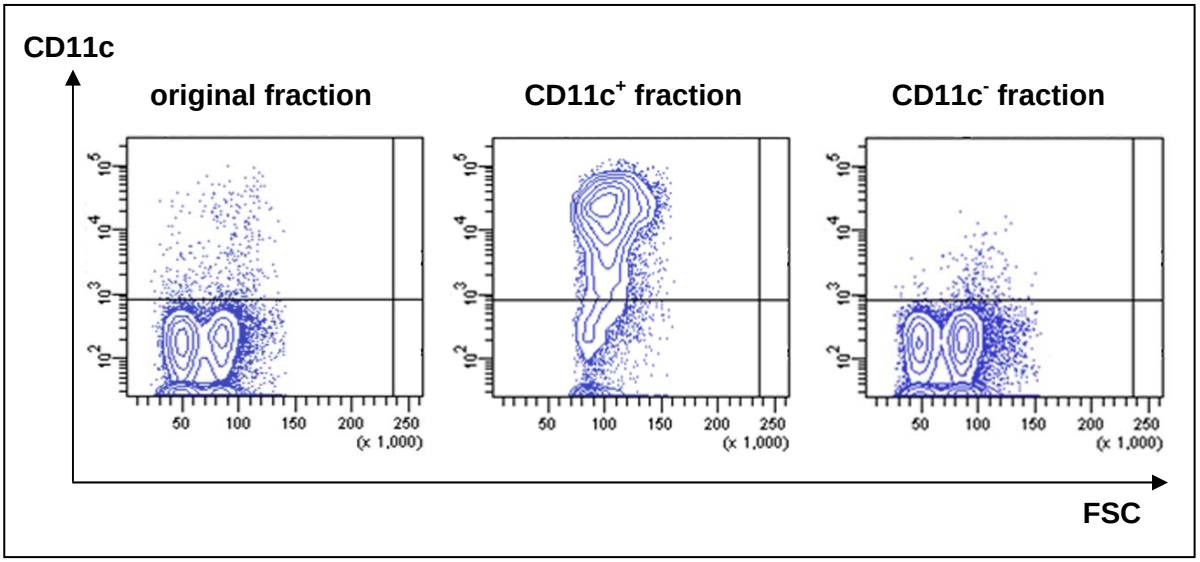

Fig. 20: FACS analysis of CD11c purified spleen cells.
Original, CD11c$^+$ and CD11c$^-$ spleen cells were measured by FACS. From 3.5% CD11c$^+$ cells in the original spleen cell fraction 78.1% CD11c$^+$ cells were enriched. Data are representative for 3 different experiments.

4.2.7 IL-12p70 and IL-23 regulation in BM-derived DCs

4.2.7.1 IL-12p70 and IL-23 regulation in BM-derived mDCs

Because in the spleen we determined only DCs producing IL-12p70 we decided to investigate other DC sources such as the BM. The advantage over the purification of splenic DCs is that from BM precursor cells much higher numbers of specific DCs can be differentiated with certain protocols. We used two different protocols to obtain mDCs and pDCs, respectively (described in 3.2.5). In the following experiments we used cells from WT C57BL/6 and p47$^{phox-/-}$ mice.

Upon stimulation of differentiated mDCs with TLR4 (1 µg/ml LPS) and TLR9 (2.5 µM CpG2216) ligands we detected IL-12p70 secretion. Whereas upon LPS stimulation in p47$^{phox-/-}$ cells IL-12p70 was comparable to WT cells upon TLR9 stimulation IL-12p70 was enhanced in p47$^{phox-/-}$ (**Fig. 21a**). Additionally to IL-12p70 we investigated also the IL-23 response of those cells and observed in contrast to the variations of the IL-12p70 secretion upon TLR9 stimulation no significant difference of IL-23 between WT and p47$^{phox-/-}$ under the same conditions (**Fig. 21b**).

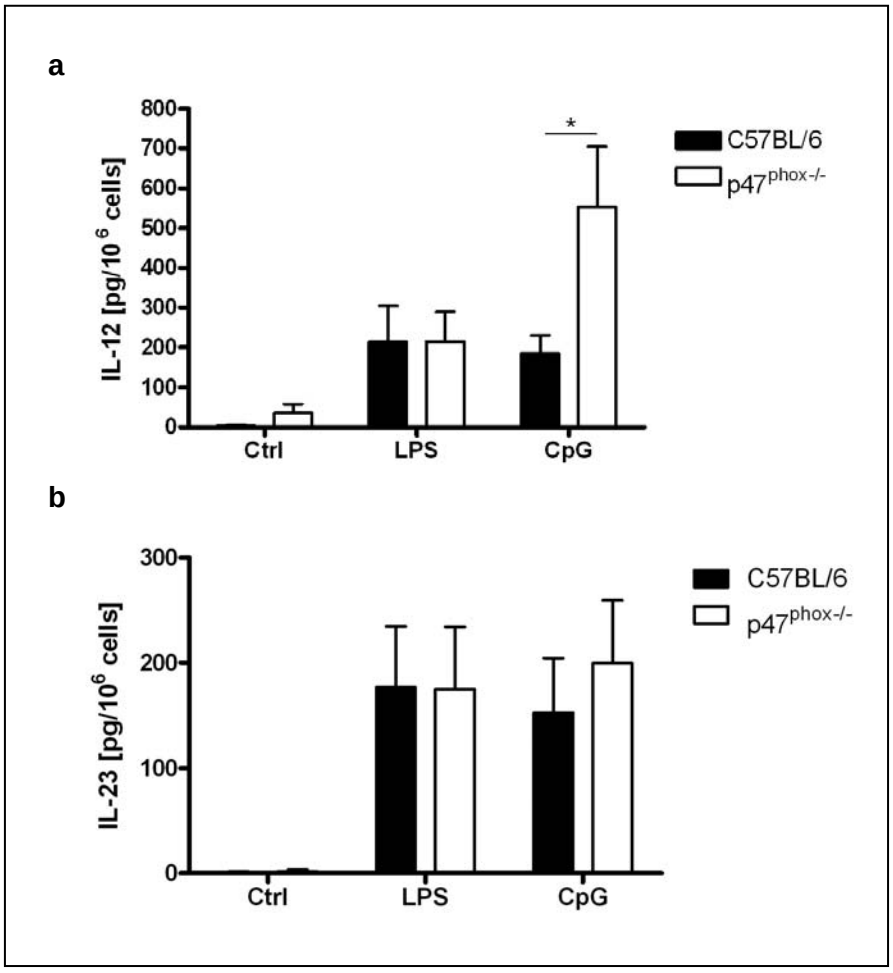

Fig. 21: IL-12p70 and IL-23 regulation in BM-derived mDCs.
IL-12p70 secretion was increased in TLR9 stimulated mDCs from p47$^{phox-/-}$ mice compared to WT (a). Upon LPS stimulation no difference was observed (a). In contrast to IL-12p70 secretion we detected no difference of IL-23 between p47$^{phox-/-}$ and WT cells upon TLR4 and TLR9 stimulation (b). Statistical significance is indicated (n=4, *P<0.05).

4.2.7.3 IL-12p70 and IL-23 regulation in BM-derived pDCs

We showed that BM-derived differentiated mDCs produce both cytokines, IL-12p70 and IL-23. Furthermore we observed differences in TLR9 stimulated IL-12p70 secretion between WT and p47$^{phox-/-}$ mDCs. In parallel to the mDCs we were also interested in the secretion of both cytokines by pDCs. We stimulated BM-derived pDCs from WT and p47$^{phox-/-}$ mice with TLR4 and TLR9 ligands and measured IL-12p70 and IL-23. Upon TLR4 stimulation pDCs produced no detectable amounts of IL-12p70, whereas upon TLR9 stimulation IL-12p70 was increased in both, p47$^{phox-/-}$ and WT cells (**Fig. 22a**). On the other hand IL-23

secretion was completely absent in TLR4 stimulated pDCs and hardly detectable upon TLR9 stimulation in p47$^{phox-/-}$ and WT cells (**Fig. 22b**).

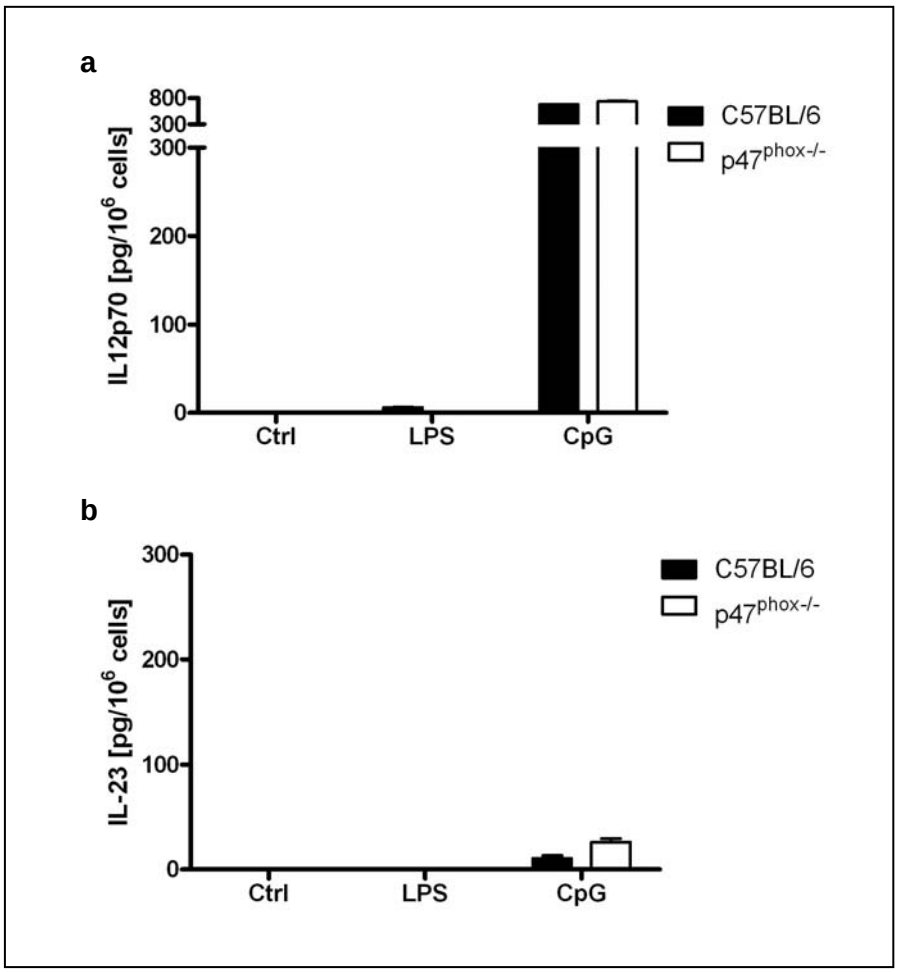

Fig. 22: IL-12p70 and IL-23 regulation in bone-marrow derived pDCs.
IL-12p70 secretion was increased in TLR9 stimulated pDCs from both, p47$^{phox-/-}$ and WT cells (a). Upon LPS stimulation no IL-12p70 was detectable (a). In contrast to IL-12p70 secretion we measured less IL-23 upon TLR9 stimulation in p47$^{phox-/-}$ and WT cells, but no IL-23 upon TLR4 (b). One representative experiment out of 3 is shown.

4.2.7.3 Characterization of BM-derived mDC and pDC by CD antigens

BM-derived cells, differentiated with GM-CSF or Flt3-L, can be characterized by specific CD antigens. Although there are different CD antigens for the characterization of DCs in this experiment only two typical marker, CD11c and B220, were applied. GM-CSF differentiated BM cells contained two main populations. First, one fraction is CD11c$^+$ and B220$^-$ (48.76%), which is the typical DC fraction (**Fig.23a**). The other fraction is negative for both markers (49.5%, **Fig.23a**). In contrast Flt3-L differentiated cells are divided into three main populations. First there is a CD11c$^+$ and B220$^-$ population (25.57%), second a

CD11c$^+$ and B220$^+$ fraction (13.78%) and third a population which is negative for both markers (48.25%, **Fig.23b**).

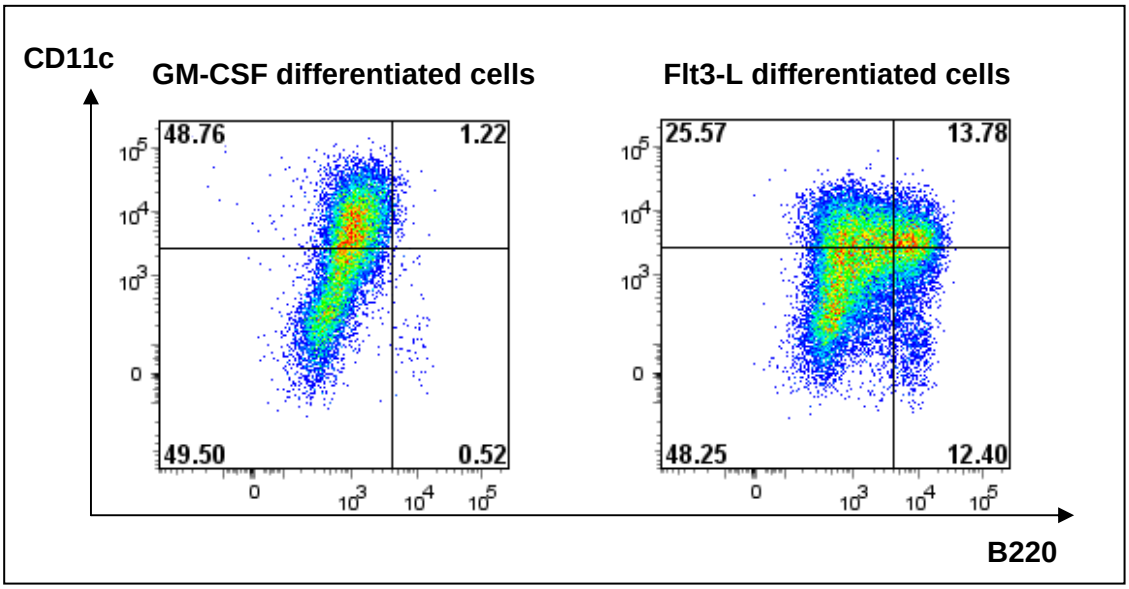

Fig. 23: FACS analysis of BM cells differentiated with GM-CSF or Flt3-L
GM-CSF differentiated cells include a fraction of cells positive for CD11c and negative for B220 (48.76%). In contrast Flt3-L differentiation results in two different populations, a CD11c$^+$ and B220$^-$ fraction (25.57%) but additionally a population which is CD11c$^+$ and B220$^+$ (13.78%). With both differentiation protocols a population completely negative for CD11c and B220 (approx. 50%) arises. One representative analysis out of 3 is displayed.

4.2.8 Adjuvant-guided T cell response *in vivo*

4.2.8.1 Th1 response of OVA re-stimulated LN cells and spleen cells

Reasoning that the induction of the 3rd signal, i.e. IL-12p70 elicited by the TLR9 ligand CpG2216 in dendritic cells, should strongly enhance a Th1-polarized antigen-specific response in p47$^{phox-/-}$ mice *in vivo*, too, we performed immunization experiments closely resembling the classical DTH-like protocols. Mice received either PBS, OVA alone or mixed with CpG2216 in IFA s.c. to activate and polarize naïve T-cells towards IFN-γ producing Th1 cells. After ten days isolated LN cells from immunized p47$^{phox-/-}$ and WT mice were incubated in an ELISA spot assay with or without OVA and the frequency of IFN-γ producing T cells was quantified. LN cells from both mouse strains treated with PBS *in vivo* exhibited no IFN-γ production upon re-stimulation with OVA (**Fig. 24a**). *In vitro* recall of OVA immunization of WT and p47$^{phox-/-}$ mice resulted in an increased IFN-γ frequency in the p47$^{phox-/-}$ LN cells (**Fig. 24b**). Providing a polarization signal, in our case IL-12p70 induced by TLR9 activation with CpG2216, enhanced the

frequency of IFN-γ-producing Th1 in WT mice significantly (**Fig. 24c**). However, in p47$^{phox-/-}$ LN cells the IFN-γ response, being already detectable without *in vitro* OVA re-stimulation, was strongly augmented upon OVA re-stimulation (**Fig. 24c**).

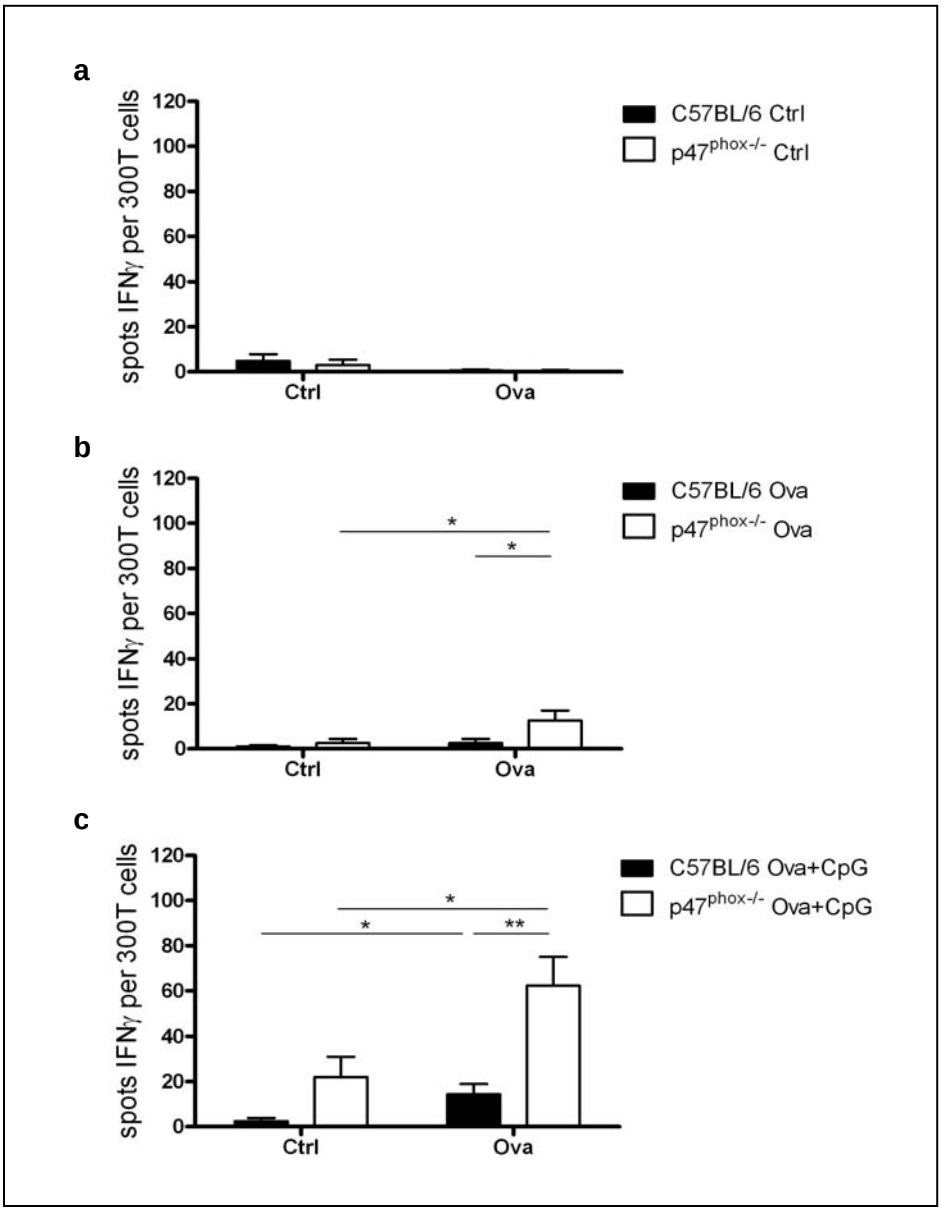

Fig. 24: Th1 response of lymph node cells from immunized mice.
Lymph node cells from *in vivo* immunized mice were *in vitro* stimulated with OVA and IFN-γ frequency was measured by ELISPOT. LN cells from control groups (PBS) produced no IFN-γ upon re-stimulation with OVA (a). OVA immunization resulted in IFN-γ response upon OVA re-stimulation only in p47$^{phox-/-}$ LN cells (b). Combined injection of OVA and CpG2216 increased the IFN-γ response in both, p47$^{phox-/-}$ and WT cells upon re-stimulation with OVA (c). Additionally p47$^{phox-/-}$ cells already produce IFN-γ without OVA re-stimulation which is further enhanced upon OVA treatment (c). Statistical significance is indicated (n=5, *P<0.05, **P<0.01).

In parallel to LN cells we investigated also spleen cells from immunized mice regarding their Th1 response. Therefore freshly isolated spleen cells were seeded in ELISPOT plates, re-stimulated with OVA or left untreated and IFN-γ frequency was measured. In comparison to the LN cells we detected no significant increase of IFN-γ in spleen cells from control and OVA immunized mice (**Fig. 25a, b**). Combined treatment of OVA and CpG2216 resulted in enhanced IFN-γ response in p47$^{phox-/-}$ and WT cells upon OVA re-stimulation with a higher extent in p47$^{phox-/-}$ spleen cells (**Fig. 25c**).

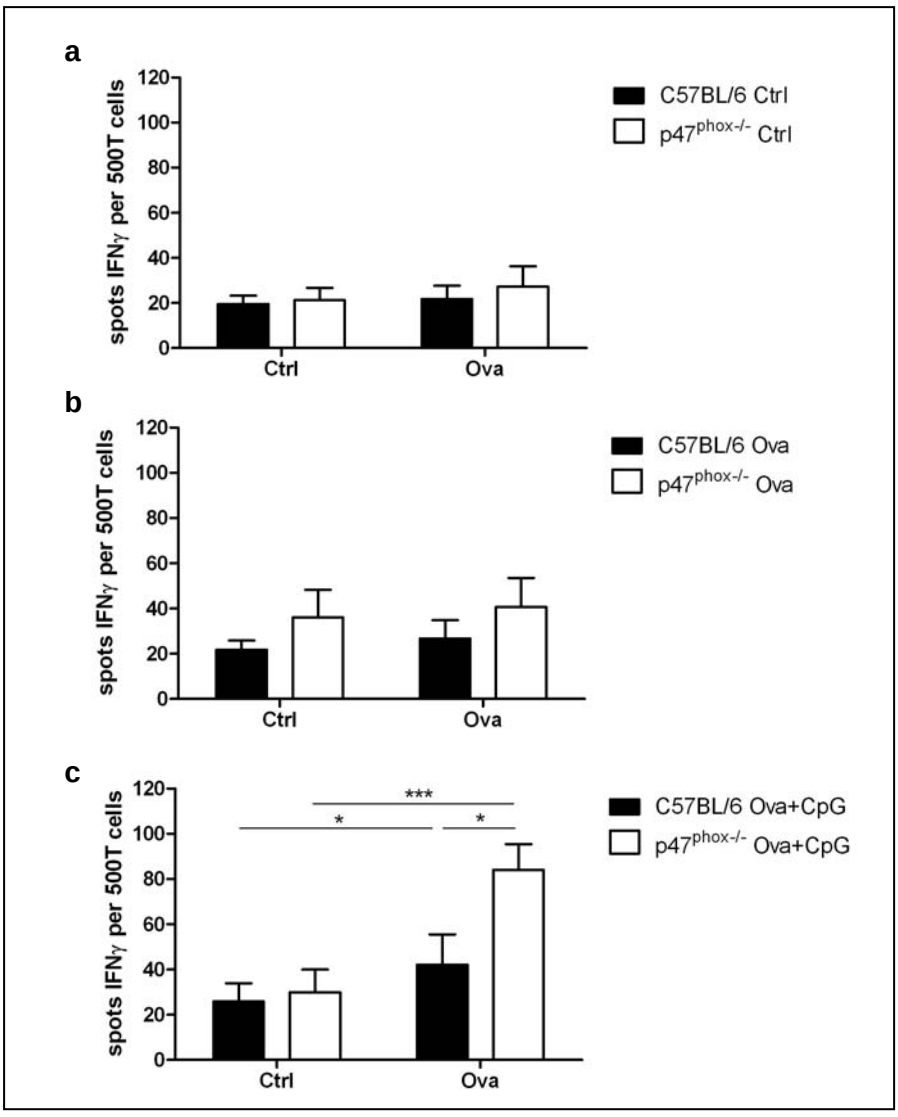

Fig. 25: Th1 response of spleen cells from immunized mice.
IFN-γ frequency of control, OVA and OVA/CpG immunized mice was detected by ELISPOT. Spleen cells of control and OVA immunized mice showed no enhanced IFN-γ response upon OVA re-stimulation (a, b). Both, p47$^{phox-/-}$ and WT spleen cells treated with OVA and CpG exhibited increased IFN-γ frequency upon OVA stimulation *in vitro* (c). Furthermore spleen cells from p47$^{phox-/-}$ mice, immunized with OVA and CpG produced higher amounts of IFN-γ upon OVA re-stimulation compared to WT cells (c). Statistical significance is indicated (n=5, *P<0.05, ***P<0.001)

4.2.8.2 IL-12p70 response in LN cells from immunized mice *in vitro*

Because the polarization of naïve T cells into T helper cells requires a 3rd signal produced by antigen presenting cells like dendritic cells. For the Th1 polarization thie main signal is IL-12p70 which is secreted by dendritic cells upon uptake of antigens and simultaneous stimulation of pathogen recognition receptors, i.e. TLRs. To investigate whether the Th1 response is induced by IL-12p70 we stimulated LN cells from immunized p47$^{phox-/-}$ and WT mice with TLR4 ligand LPS (1 µg/ml) and TLR9 ligand CpG2216 (2.5 µM) *in vitro* and determined the IL-12p70 response by mouse specific ELISA. LN cells of control WT mice exhibited low amounts of IL-12p70 in response to LPS and CpG2216, whereas in p47$^{phox-/-}$ LN cells IL-12p70 was increased upon CpG2216 stimulation (**Fig. 26a**). Mice immunized with OVA showed roughly the same response as the PBS treated mice (**Fig. 26b**). In contrast to WT mice LN cells from p47$^{phox-/-}$ mice treated with OVA and CpG2216 *in vivo* are characterized by an enhanced IL-12p70 response upon TLR4 and TLR9 stimulation (**Fig. 26c**).

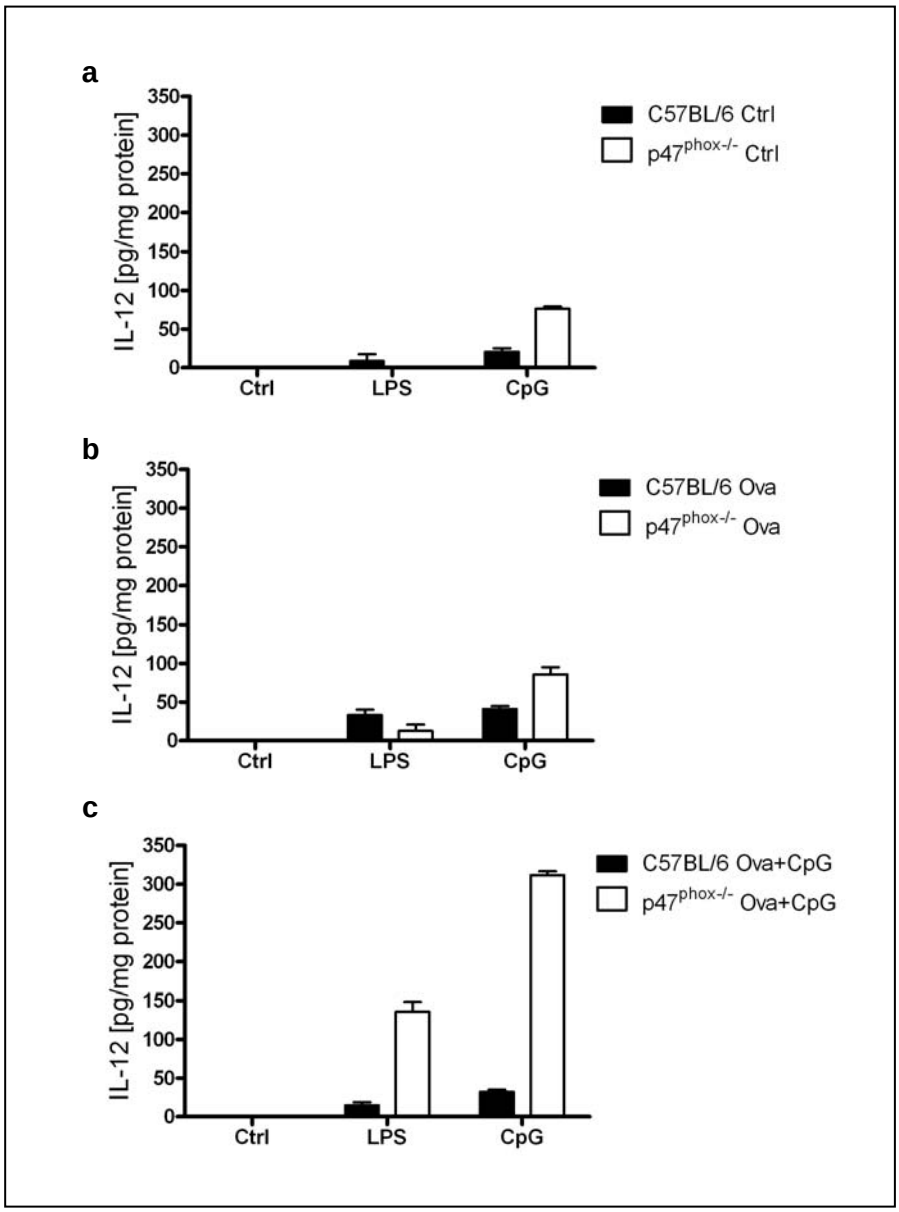

Fig. 26: IL-12p70 response of LN cells measured by mouse specific ELISA.
LN cells from control mice (PBS treated) stimulated with TLR4 ligand LPS showed no IL-12p70 response (a). TLR9 stimulation with CpG2216 resulted only in $p47^{phox-/-}$ control cells in enhanced IL-12p70 (a). OVA immunized LN cells exhibited only low amounts of IL-12p70 upon TLR4 and TLR9 stimulation with a slight increase in $p47^{phox-/-}$ cells upon TLR9 stimulation (b). Immunization with OVA combined with CpG2216 resulted only in $p47^{phox-/-}$ LN cells to an upregulation of IL-12p70 upon TLR4 and TLR9 stimulation, respectively (c). One representative experiment out of 2 is shown.

4.2.8.3 Th17 response of OVA re-stimulated LN cells and spleen cells

In chronic inflammation not only Th1 response but also Th17 cells play an important role. Th17 cells are characterized by the production of IL-17. In contrast to the Th1 development that involves IL-12p70 Th17 cells were stabilized and survived by the availability of IL-23. In our experiments $p47^{phox-/-}$ and WT mice were immunized with OVA, a combination of OVA and CpG2216 or with PBS as control for 10 days. Subsequently LN and spleen cells were isolated, seeded into ELISPOT plates and directly re-stimulated with OVA or left untreated. After 24 hrs incubation time, plates were washed, specific IL-17 detection antibodies were used and finally IL-17 production was detected as spots on the plates. In control mice no IL-17 response has been detected in LN cells re-stimulated with OVA *in vitro* (**Fig. 27a**). LN cells from OVA immunized mice exhibited also no detectable IL-17 response except LN cells from $p47^{phox-/-}$ upon re-stimulation with OVA (**Fig. 27b**). In contrast immunization of mice with OVA in combination with CpG2216 resulted in increased Th17 response upon OVA re-stimulation with a higher extent in $p47^{phox-/-}$ cells (**Fig. 27c**). Additionally OVA and CpG2216 immunized $p47^{phox-/-}$ mice already showed enhanced IL-17 spots in *in vitro* untreated LN cells (**Fig. 27c**).

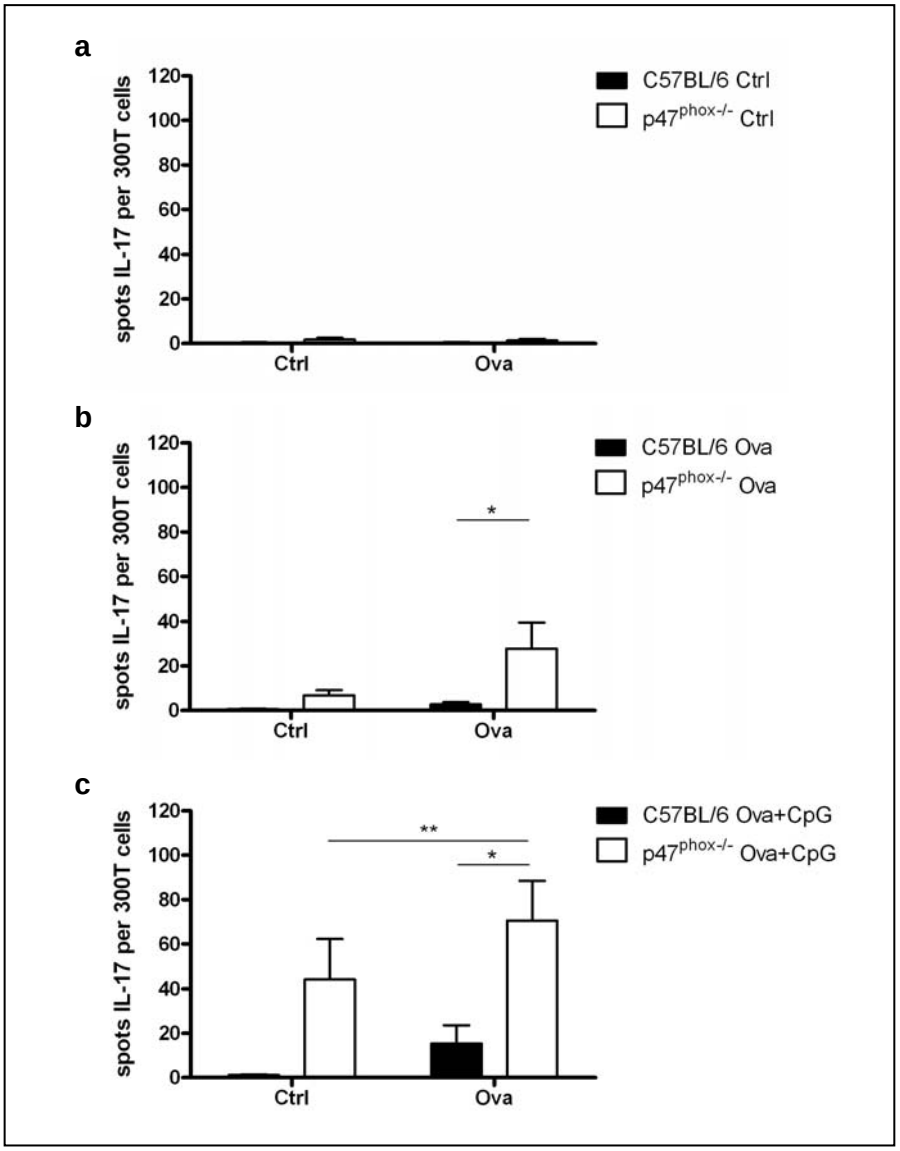

Fig. 27: Th17 response of LN cells from immunized mice.
IL-17 response was measured with ELISPOT using specific IL-17 antibodies. LN cells from control mice exhibited no IL-17 response (c). In OVA immunized group only LN cells from p47$^{phox-/-}$ mice showed increased IL-17 spots upon OVA re-stimulation *in vitro* (b). In contrast both, p47$^{phox-/-}$ and WT cells from OVA and CpG2216 immunized mice produce IL-17 upon re-stimulation with OVA, whereas p47$^{phox-/-}$ cells already secrete IL-17 without OVA re-stimulation *in vitro* (c). Statistical significance is indicated (n=5, *P<0.05, **P<0.01)

In addition to the LN cells from immunized mice we further determined the Th17 response of spleen cells from those mice. Therefore freshly isolated spleen cells were in an IL-17 specific ELISPOT investigated. As described before spleen cells were *in vitro* re-stimulated with OVA or left untreated. We measured in spleen cells from the control group low amounts of IL-17 with a slight increase in p47$^{phox-/-}$ treated with OVA *in vitro* (**Fig. 28a**). Mice immunized with OVA alone showed an

enhanced Th17 response upon OVA re-stimulation, whereas p47$^{phox-/-}$ mice exhibited a clear increase of IL-17 of both, untreated and OVA re-stimulated cells (**Fig. 28b**). When mice were immunized with a combination of OVA and CpG2216 IL-17 response in spleen cells of both, p47$^{phox-/-}$ and WT was significantly increased upon re-stimulation with OVA with a higher extent in p47$^{phox-/-}$ cells (**Fig. 28c**).

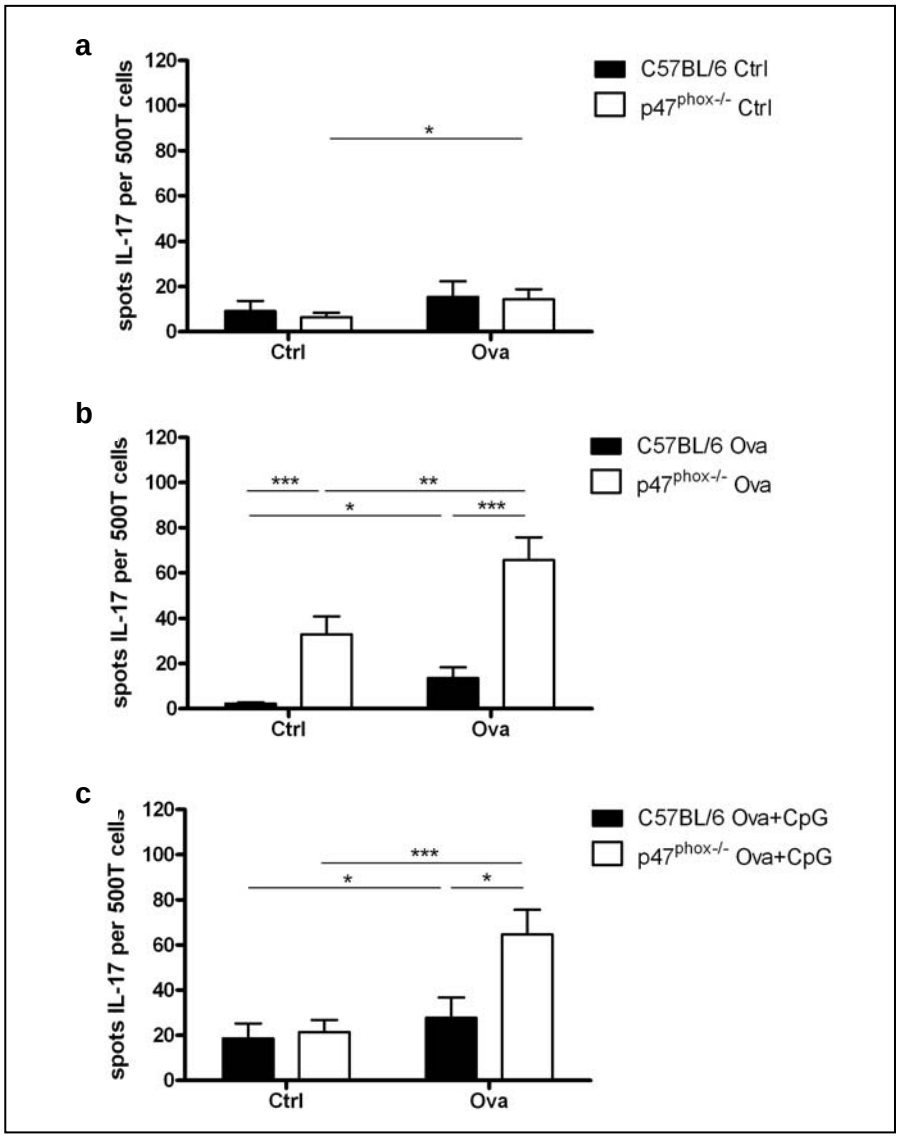

Fig. 28: Th17 response of spleen cells from immunized mice.
With IL-17 ELISPOT Th17 response of spleen cells from immunized mice was detected. Spleen cells of control mice exhibited a slight increase of IL-17 when treated with OVA *in vitro*, but only significantly in p47$^{phox-/-}$ cells (a). Immunization with OVA resulted in enhanced IL-17 response upon OVA re-stimulation in both mice, whereas IL-17 spots of p47$^{phox-/-}$ cells are already enhanced without OVA (b). Spleen cells from mice treated with OVA and CpG2216 showed increased IL-17 response upon re-stimulation with OVA *in vitro* (c). Statistical significance is indicated (n=5, *P<0.05, **P<0.01, ***P<0.001)

4.2.8.4 IL-23 response LN cells from immunized mice *in vitro*

Based on the fact that IL-23 plays an important role for the development and survival of Th17 cells we wanted to know whether IL-23 secretion is influenced by $p47^{phox}$ as shown before for IL-12p70. In this context we stimulated LN cells from immunized $p47^{phox-/-}$ and WT mice with TLR4 and TLR9 ligands. Therefore LN cells were thawed and seeded 10-15x10^6/ml in T25 culture flasks. Cells were incubated for 6-8 hrs in medium containing FCS. Afterwards LN cells were stimulated in serum-free medium with LPS and CpG2216, respectively and after 20 hrs IL-23 was measured in the supernatant by ELISA. We neither detected in $p47^{phox-/-}$ nor WT cells any secretion of IL-23 after TLR stimulation (**Fig. 29a-c**). In this respect it was irrelevant whether LN cells were derived from control or immunized mice (**Fig. 29a-c**).

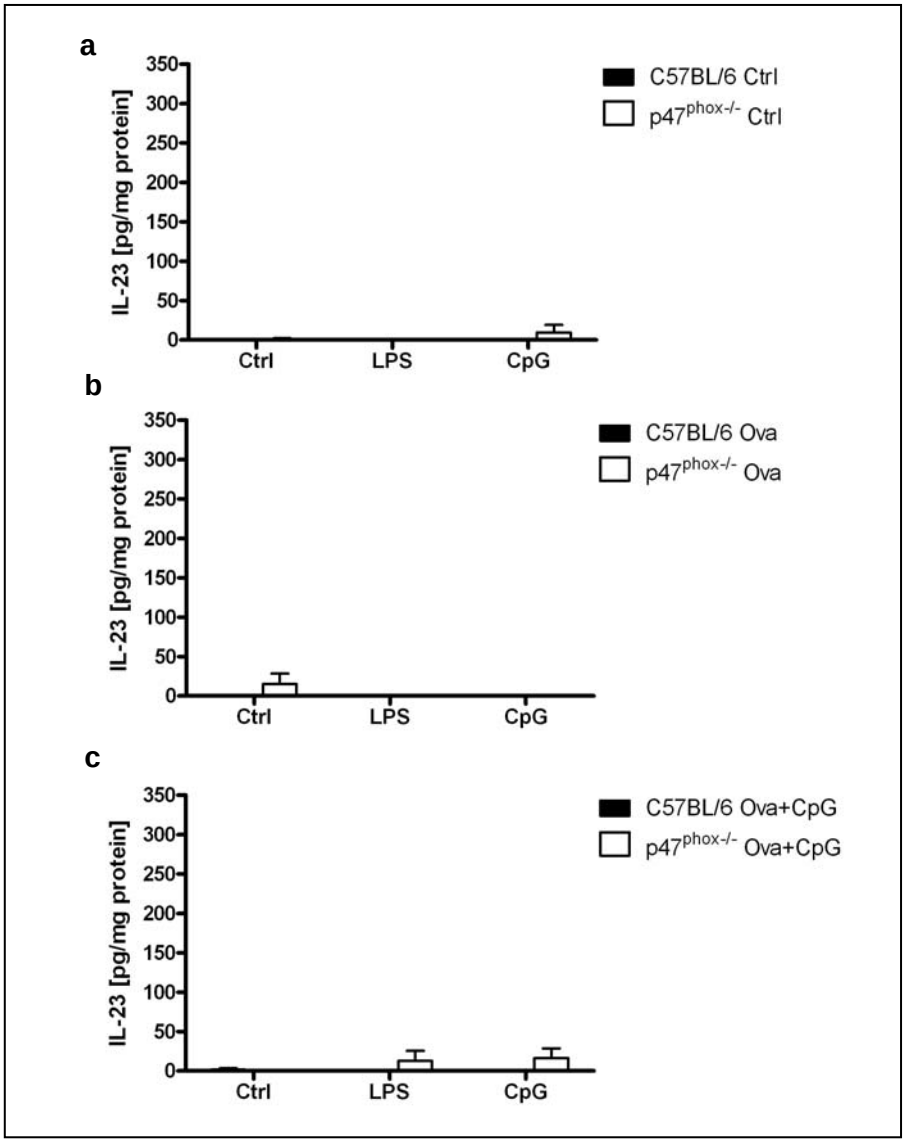

Fig. 29: IL-23 response of LN cells measured by mouse specific ELISA.
LN cells from control and immunized p47$^{phox-/-}$ and WT cells were stimulated with LPS and CpG2216, respectively. There was no IL-23 secretion detectable in LN cells from p47$^{phox-/-}$ and WT mice of all three groups (a-c).

4.3 Role of Vitamin D_3 on IL-12p70 expression

It is known that VD_3 has immunomodulatory functions. Since it was shown that VD_3 stimulates the phagocytosis and the bacterial killing capacity but suppresses the antigen-presentation in the following experiments the influence of VD_3 treatment on IL-12p70 secretion by DCs has to be investigated. The enhanced phagocytic activity upon VD_3 treatment was shown to be mediated via an upregulation of $p47^{phox}$. Based on the previous results that IL-12p70 is increased when $p47^{phox}$ is absent in the following experiments also the association between VD_3 and $p47^{phox}$ should be noticed.

4.3.1 IL-12p70 regulation by VD_3 in different mouse strains

4.3.1.1 IL-12p70 regulation in C57BL/6 spleen cells

In an initial experiment we wanted to know whether short term treatment with VD_3 influences the ability of spleen DCs to secrete IL-12p70 upon stimulation with TLR ligands. Freshly isolated spleen cells were cultivated in medium containing VD_3 (10^{-7}M) before TLR stimulation (0-24 hrs), before and during TLR stimulation (0-48 hrs), only during TLR stimulation (24-48 hrs) or left untreated (w/o). Cells were stimulated with LPS (1 µg/ml) or CpG2216 (2.5 µM) for 24 hrs and IL-12p70 was measured in the supernatant. LPS induced IL-12p70 in untreated cells. VD_3 treatment inhibited LPS-induced IL-12p70 when cells were treated before stimulation (**Fig. 30a, 0-24 hrs**). Spleen cells incubated with VD_3 before and during stimulation exhibited comparable inhibition of IL-12p70 (**Fig. 30a, 0-48 hrs**), whereas treatment with VD_3 only during LPS stimulation had no influence (**Fig. 30a, 24-48 hrs**). In contrast to high secretion upon LPS spleen cells stimulated with CpG2216 produced low levels of IL-12p70, as observed in spleen cell experiments before. However, VD_3 treatment before stimulation (0-24 hrs) as well as over the whole period (0-48 hrs) resulted in decreased IL-12p70 (**Fig. 30b**). Incubation with VD_3 during CpG2216 stimulation did not significantly inhibit IL-12p70 (**Fig. 30b, 24-48 hrs**).

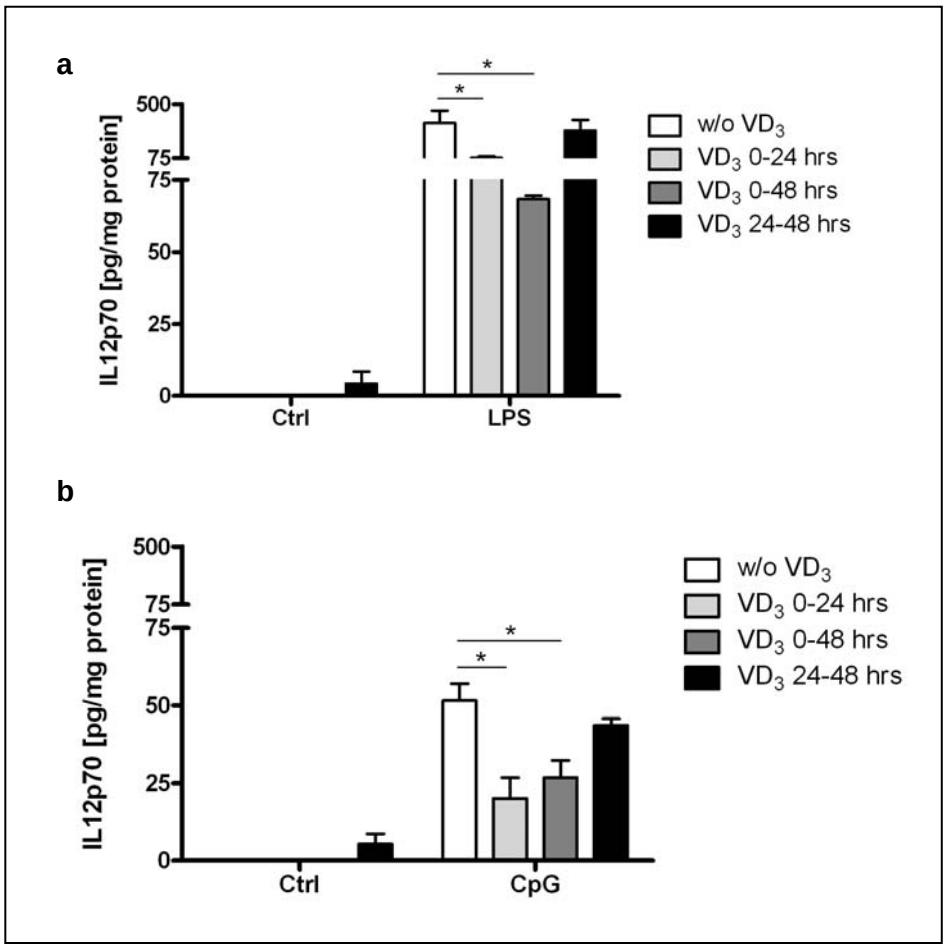

Fig. 30: IL-12p70 regulation by VD_3 in C57BL/6 spleen cells.
VD_3 treatment before stimulation with LPS [1 µg/ml] or during the whole period resulted in decreased IL-12p70 (a, 0-24 hrs, 0-48 hrs). In contrast treatment with VD_3 only during stimulation had no effect on IL-12p70 secretion (a, 24-48 hrs). Although upon CpG2216 [2.5 µM] stimulation IL-12p70 response was much lower than upon LPS the IL-12p70 pattern upon VD_3 treatment was comparable (b). Statistical significance is indicated (n=5, *P<0.05, **P<0.01, ***P<0.001)

4.3.1.2 IL-12p70 regulation in BALB/c spleen cells

From the experiments with C57BL/6 mice we know that IL-12p70 is regulated by VD_3. Since it was shown that TNBS-induced colitis in BALB/c mice was improved by *in vivo* VD_3 application (Daniel, 2008) we wanted to clarify whether spleen cells from those mice also have an altered IL-12p70 response due to a VD_3 treatment *in vitro*. Therefore spleen cells from 10-12 weeks old BALB/c mice were isolated and cultivated in medium containing VD_3 (10^{-7} M) before TLR stimulation (0-24 hrs), before and during TLR stimulation (0-48 hrs), only during TLR stimulation (24-48

hrs) or left untreated (w/o). TLR stimulation with LPS and CpG2216 was performed as described above. In comparison to the C57BL/6 mice spleen cells from BALB/c mice exhibited almost the same IL-12p70 pattern. VD_3 treatment resulted in decreased IL-12p70 response when applied before TLR stimulation with LPS (**Fig. 31a**) or CpG2216 (**Fig. 31b**). In contrast to the C57BL/6 experiment VD_3 influences the IL-12p70 response in BALB/c cells upon LPS stimulation also when applied only during TLR stimulation (**Fig. 31a, black bar**). Upon TLR9 stimulation with CpG2216 this significant altered IL-12p70 response was not observable (**Fig. 31b, black bar**).

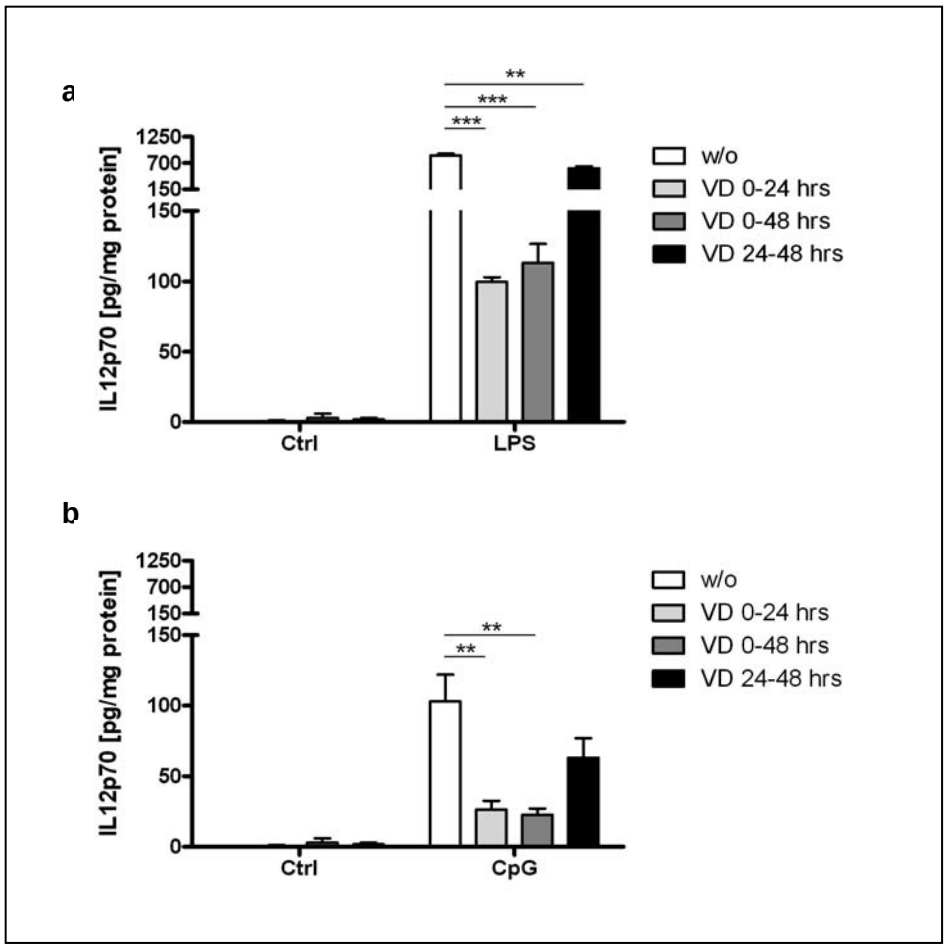

Fig. 31: IL-12p70 regulation by VD_3 in BALB/c spleen cells.
IL-12p70 response upon LPS or CpG2216 stimulation was decreased by VD_3 treatment before (0-24 hrs) or before and during (0-48 hrs) TLR stimulation (a, b, gray bars). Cultivation of spleen cells exclusively during TLR stimulation resulted only upon LPS stimulation in significantly decreased IL-12p70 response (a, black bar). Statistical significance is indicated (n=5, **P<0.01, ***P<0.001)

4.3.2 IL-12p70 regulation by VD_3 in $CD11c^+$ cells

In summary so far we figured out that VD_3 regulates the IL-12p70 response in spleen cells from C57BL/6 and BALB/c mice. To confirm that these effects arise from the DCs in the spleen we additionally purified CD11c positive DCs. We performed an experiment where we cultured $CD11c^+$ cells in medium containing VD_3 (10^{-7} M) or left untreated and stimulated those cells again with TLR4 and TLR9 ligands. As expected from the previous experiments we detected a decreased IL-12p70 secretion from cells treated before (0-24 hrs) and before/during (0-48 hrs) TLR stimulation, respectively (**Fig. 32a, b**).

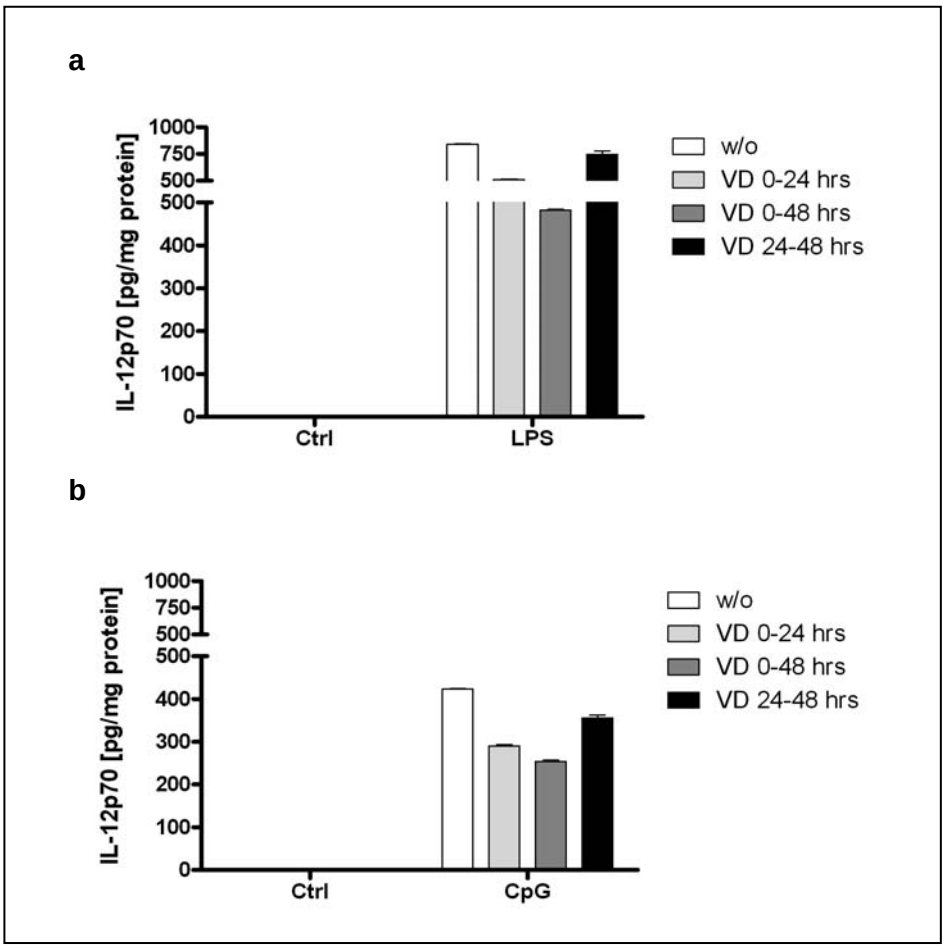

Fig. 32: Regulation of VD_3 in $CD11c^+$ spleen cells.
IL-12p70 secretion was diminished in $CD11c^+$ cells treated with VD_3 before and before/during TLR4 and TLR9 stimulation (a, b, gray bars). This experiment only was performed 2 times.

4.3.3 IL-12p70 regulation by VD_3 in $p47^{phox-/-}$ and WT spleen cells

Since it was described that VD_3 influences the mRNA expression of $p47^{phox}$ (Fuhrman, 2004) we wanted to know whether those finding could be confirmed in our cells regarding the impact on the IL-12p70 response. Therefore spleen cells from $p47^{phox-/-}$ and WT mice were isolated and directly treated with VD_3 or left untreated. After 24 hrs cells were stimulated with TLR4 ligand LPS and TLR9 ligand CpG2216. In comparison to the untreated cells (100%) VD_3 treatment before stimulation resulted in reduced IL-12p70 upon TLR4 stimulation in WT and $p47^{phox-/-}$ cells by 45-55% (**Fig. 33a**). In contrast to LPS stimulation VD_3 treatment diminished TLR9 induced IL-12p70 only by 20% in $p47^{phox-/-}$ cells whereas in WT cells IL-12p70 was again reduced by 40% (**Fig. 33b**)

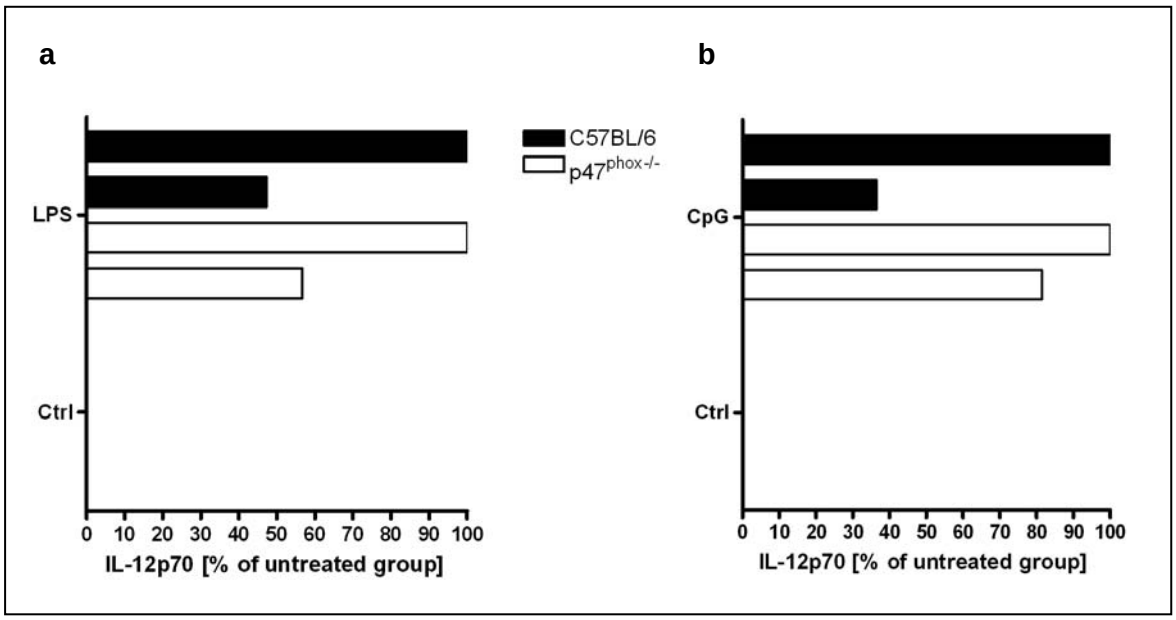

Fig. 33: Percentage of IL-12p70 reduction by VD_3 in $p47^{phox-/-}$ and WT cells.
TLR4 induced IL-12p70 secretion was reduced by 45-55% upon VD_3 treatment in WT and $p47^{phox-/-}$ spleen cells (a). VD_3 treated WT cells stimulated with TLR9 ligand demonstrated also 40% reduction of IL-12p70, whereas only 20% reduction was observed in $p47^{phox-/-}$ cells. One of 2 independent experiments is shown.

With the previous investigations it was demonstrated that short term treatment with VD_3 resulted in diminished IL-12p70 production of DCs. Whether $p47^{phox}$ is associated with this VD_3 effect on the IL-12p70 expression has to be determined in more detail.

5 Discussion

DCs as the major producer of IL-23 and/or IL-12p70 play an important role in the regulation of immune responses. They do not only induce the T cell response by presenting antigens, they also determine the polarization of T cells by supporting the 3rd signal - their specific cytokines, such as IL-12p70 or IL-23. This thesis focused on the regulation of TLR-mediated cytokine secretion of different DC populations. Because these two cytokines are associated with several immune disorders, such as autoimmunity or cancer, our investigations of three different molecules, regulating these cytokines, may be demonstrate some new and promising approaches. Although these three molecules have distinct functions, they are all associated with autoimmune disorders and, demonstrated in this thesis, represent regulatory potential on the TLR-mediated cytokine secretion of DCs.

5.1 Regulation of IL-23 by Selenium

Investigating the role of Selenium on the expression and secretion of IL-23 the main focus was on DCs from the skin, the cell line XS-52 as a model for iLCs. It has been reported that Selenium deficiency correlates with decreased T cell numbers in HIV patients and enhanced susceptibility to skin tumor formation in human and mice (Clark, 1984). Supplementation with inorganic Selenium which rapidly incorporates into selenoproteins increases the number of skin Langerhans cells and thus protects mice from skin tumor formation upon UVB irradiation (Rafferty, 2003). The findings of the experiments in this thesis clearly demonstrated a stimulatory effect of Selenium on the IL-23 secretion by iLCs. In the experiments concentrations between 10 and 250 nM exhibited enhanced IL-23 production. It has been shown that Ebselen in relatively high concentrations (10-20 µM) inhibits the IL-12p40 production of iLC (Matsue, 2003). These findings are the opposite to the results of this thesis whereas the Selenium concentration was much lower than in the cited publication.

In colon cancer cells treatment with Selenium resulted in an enhanced oxidative stress response and increasing ROS levels (Huang, 2009). The induction of free radicals by Selenium treatment is further associated with the induction of apoptosis (Nilsonne, 2006) which should explain the anti-tumor effects of

Selenium. From the obtained data it is clear that LPS induces IL-23 and this is further enhanced by Selenium. Whether the increased ROS production influences the LPS-induced IL-23 secretion was not determined. Thus, for the better understanding of the results it will be necessary to investigate this point more detail. As demonstrated for mRNA and protein level IL-23 subunit p19 is completely unaffected by Selenium treatment whereas p40 is upregulated. In the literature there is no evidence that IL-23 or specifically its subunit p40 is upregulated by Selenium. As discussed above the opposite function of Selenium was demonstrated. To strengthen the findings of this study further investigation regarding ROS induction and activation of selenoproteins such as Trx reductase should be performed.

5.2 Regulation of IL-12p70 and IL-23 by $p47^{phox}$

In this part of the thesis the hypothesis has been addressed that $p47^{phox}$, independently of its role for NADPH oxidase activation, is involved in the regulation of TLR signaling. Maintaining the balance of the immune response is essential to avoid autoimmune reactions or chronic inflammatory processes. Since it was shown that the absence of $p47^{phox}$ is associated with enhanced autoimmunity (Olofsson, 2003; Hultqvist, 2004) the question had to be answered whether $p47^{phox}$ directly interact with the TLR signaling to influence the IL-12p70 and IL-23 production of DCs. In the first set of experiments spleen cells of $p47^{phox}$ deficient mice exhibited an increased IL-12p70 secretion after stimulation of the intracellular TLR9 with CpG2216 compared to WT cells. Consequently the reduced secretion of IL-12p70 observed after TLR9 stimulation in WT cells seems to be controlled by a negative feedback mechanism. This observation may important to prevent excessive proinflammatory immune responses against foreign molecular structures derived from invading pathogens. That TLR4 induced IL-12p70 secretion was not different between $p47^{phox-/-}$ and WT cells is explainable, because TLR4 signaling pathways are mediated via two different adaptor molecules. First via the adaptor MyD88, this is equally to the TLR9 signaling and second via TRIF, which is not involved in the TLR9 pathway. It has been found that type A CpGs, including the applied CpG2216, do not induce IL-12p70 in human PBMCs (Ghosh, 2007), whereas type B CpGs can also activate B cells in

humans and induce the secretion of IL-12p70 in GM-CSF-differentiated BM cells derived from C57BL/6 mice (Theiner, 2008). Furthermore it has been described for pDCs that type A CpGs are weak inducers of TLR9-dependent NF-κB signaling, resulting in IL-12p70 expression, but strong inducers of IFN-α (Rothenfusser, 2002; Verthelyi, 2003; Krieg, 2007). Thus, in this study it should be demonstrated, that $p47^{phox}$ beeing involved in the down-regulation of TLR9-mediated IL-12p70 secretion in WT cells, is the main factor for this effect. In addition to the genetically engineered $p47^{phox}$ deficient mice a strain with a spontaneous mutation in the $p47^{phox}$ gene *Ncf1*, resulting in less or no functional splice variants of $p47^{phox}$ protein, have been investigated. In these mice it has been shown earlier that *Ncf1* polymorphism is associated with RA and that the deficiency of $p47^{phox}$ led to more severe inflammation (Hultqvist, 2004). With regard to the regulation of IL-12p70 it has been demonstrated that also in this natural mutated mice the lack of $p47^{phox}$ results in increased IL-12p70 production by splenocytes. Because little is known about the mechanism how $p47^{phox}$ could influence the IL-12p70 expression by interaction with the TLR signaling pathway in a further experiment rats with a SNP in the $p47^{phox}$ were investigated regarding the IL-12p70 secretion. Although in the rats just one single amino acid is different compared to the normal $p47^{phox}$ protein, the IL-12p70 secretion of splenocytes was significantly increased compared to WT rats. In disease models those rats were shown to be susceptible to RA and EAE when a SNP at amino acid position 153 resulted in methionine instead of threonine (Olofsson, 2003). Interestingly this amino acid position is one of the most important phosphorylation sites for the IRAK4, one of the key kinases in the TLR pathway (Pacquelet, 2007). Possibly with these findings and the fact that IRAK4 phosphorylates $p47^{phox}$ a further piece of evidence is collected to uncover a connection between $p47^{phox}$ and the TLR signaling cascade.

To test whether only proinflammatory IL-12p70 is affected by $p47^{phox}$ activity also two other important cytokines were investigated. In addition to the antiviral response IFN-α the expression of the more regulatory IL-10 after stimulation with LPS and CpG2216 was determined. Thereby significantly lower IFN-α and IL-10 levels upon TLR9 stimulation were detected in WT cells whereas in $p47^{phox-/-}$ splenocytes both cytokines are upregulated. After TLR4 stimulation no difference between WT and $p47^{phox}$ deficient cells was discernible for IL-10 and furthermore IFN-α response was not detected. The mechanism of IL-10 regulation via different

TLR pathways and the role of $p47^{phox}$ is unclear, and published data regarding a connection between IL-10 and IL-12p70 regulation and the respective cellular sources of either cytokine are manifold and partly contradictory (Flores, 2007; Boonstra, 2006; Bohnenkamp, 2007). Because the spleen cell system consists of a variety of immune cells, the cells producing IL-12p70 may differ from the IL-10 producing cells. The high amounts of secreted IFN-α determined in splenocytes from $p47^{phox-/-}$ cells and moderate levels in WT cells points to an involvement of IFN-producing DCs such as pDCs. Thus, pDCs, which are the major producers of IFN-α, may be specifically sensitive to $p47^{phox}$ feedback. In addition, the type of CpG being agonistic and resulting in a strong IFN-α response support a pDC involvement (Asselin-Paturel and Trinchieri, 2005; Krieg, 2002). To prove whether pDCs are also sensitive to the negative feedback regarding IL-12p70 further experiments, including DCs prepared from other compartments, were performed. In the experiments with BM-derived cells this part was investigated in more detail and will be discussed later.

One of the main questions in this study was, whether the lack of ROS production due to a nonfunctional NADPH oxidase has any influence on the observed feedback mechanism. Therefore splenocytes of $gp91^{phox}$ deficient mice were compared with $p47^{phox}$ deficient cells with respect to the negative feedback. The results demonstrated that the negative effect on TLR9-mediated IL-12p70 secretion was dependent on $p47^{phox}$ rather than on a functional NADPH oxidase complex. In subsequent inhibition experiments with the flavoprotein inhibitor DPI it was clearly demonstrated that the negative feedback regulation of TLR9-mediated IL-12p70 secretion is not influenced by $gp91^{phox}$ (Cross, 1986; Radeke, 1991), which confirms a ROS-independent mechanism. Surprisingly, in this experimental series a negative feedback regarding IL-12p70 secretion also after stimulation of LPS in combination with DPI was discovered. These data suggest that the TRIF and MyD88 pathways downstream of TLR4 exhibit a differential sensitivity towards ROS inhibition. Hence, blocking a ROS-dependent TRIF signaling by DPI uncovered the LPS induced MyD88 pathway of the TLR4 signaling, which again is regulated by $p47^{phox}$. Based on the fact that the spleen consists of diverse cell types, not only DCs but also macrophages and neutrophils could be involved in ROS production. However there is a major difference between these phagocytic cells and DCs, especially with regard to the time course and level of ROS

production and the acidification in the phagosomal compartments. Whereas DCs, with their main function being to present processed peptides as antigens to naïve T lymphocytes, are characterized by a continuous and sustained production of ROS, macrophages and neutrophils produce very rapidly high amounts of ROS, called “burst” (Savina, 2006). In addition, the function of ROS during antigen presentation by DCs may be limited by the secretion of catalases (Thoren, 2007). The obtained data demonstrating ROS production following TLR stimulation alone suggest that ROS in DCs might have a special function independent of phagocytosis. It has been shown that in DCs the NADPH oxidase is cryptic, which means that oxidase activation and ROS production, respectively, are only recovered upon TLR activation (Elsen, 2004).

According to our hypothesis that $p47^{phox}$ interacts with the TLR signaling cascade and based on the previous findings that the negative feedback regulation by $p47^{phox}$ is apparent only in a TLR/MyD88 dependent pathway, mice deficient for the adaptor TRIF and MyD88, respectively were investigated. These experiments showed that the negative feedback regulation was also detectable in spleen cells of $TRIF^{-/-}$ mice which support the idea that the feedback is restricted to the TLR-MyD88 pathway. With respect to the confirmed ROS-independent results regarding IL-12p70 regulation by $p47^{phox}$ it can be concluded that the signaling involved is only TLR-MyD88 dependent. It has been published that IL-12p70 production in DCs is abolished when MyD88 is absent (Kawai, 1999; Hemmi, 2003). In a more recent publication it has been described that only IL-12p70 was absent, whereas intracellular IL-12p40 was still detectable (Theiner, 2008). This finding reinforces the idea that $p47^{phox}$ influences primarily IL-12p35 expression via the TLR-MyD88 pathway and, to a lesser extent, IL-12p40 expression. It is not yet clear, whether only the heterodimeric IL-12p70 or if, which of its subunits p35 and p40 are more important regulators in autoimmune diseases (Harbord, 2002; Becher, 2002; Gutcher and Becher, 2007). However, from the data of this study it could be supposed that the regulation of IL-12p35 and IL-12p70, respectively, by $p47^{phox}$ particularly in DCs via the TLR/MyD88 pathway influences the development of autoimmune diseases. Mice lacking TLR9 or its adaptor molecule MyD88 are protected from experimental autoimmune encephalitis (Prinz, 2006). This fact is clearly supportive for our suggestion that the regulation and fine tuning

of the TLR9/MyD88 pathway resulting in IL-12p70 production plays a major role in autoimmune diseases.

Although all of the data so far point towards DCs as carriers of the observed $p47^{phox}$ dependent feedback mechanism of TLR9-induced IL12p70, the continuation of our investigations includes the purification of DCs from the spleen with a typical DC marker, CD11c. As expected the negative feedback mechanism on IL-12p70 was detectable in the CD11c-positive fraction of splenocytes. Thus, by further refining the negative feedback by $p47^{phox}$ to the $CD11c^{+}$ DC fraction it stresses its crucial role for the regulation of chronic inflammation and autoimmune diseases. Focusing more stringently on the point whether pDCs are the main carriers of the negative feedback, in following experiments DCs were generated from the BM of $p47^{phox}$ deficient and WT mice. The data clearly demonstrated that the feedback sensitive to $p47^{phox}$ only arises in GM-CSF differentiated BM cells. Although Flt3-L differentiated cells exhibited a strong IL-12p70 response the $p47^{phox}$ effect was not discernible in those cells. Differentiation with Flt3-L resulted in a mixture of DCs including cDCs characterized by CD11c expression but no expression of B220 and pDCs which are alternate in their CD11c expression but are described as B220 positive cells. Thus, the feedback mechanism is maybe masked by the different cell types. The fact that GM-CSF differentiated cells also including inflammatory DCs derived from monocytes could explain the feedback mechanism in this population. Furthermore these cells produce IL-23 after TLR4 and TLR9 stimulation but interestingly $p47^{phox}$ deficiency has no influence on this cytokine. In contrast Flt3-L differentiated cells do not secrete IL-23 upon TLR4 or TLR9 stimulation. Although several hints are found within this study that IL-12p70 producing, TLR9-positive and $CD11c^{+}$ DCs exhibit the negative feedback mechanism by $p47^{phox}$ it is not completely clarified which specific subtype is the important one. Based on recent findings, that changes the classical view of DC development, one promising subpopulation carrying the feedback mechanism could be the monocyte-derived, GM-CSF-dependent, TLR9-positive inflammatory DC (Naik, 2007; Onai, 2007; Liu, 2009). Of course, to proof this hypothesis further investigations are needed.

In mice and rats with different mutations of $p47^{phox}$ it was demonstrated that this lack of $p47^{phox}$ is associated with enhanced autoimmunity (Olofsson, 2003; Hultqvist, 2004). The $p47^{phox}$ mediated mechanism for this effect, however, is not

clarified, and currently several different possibilities have been suggested. First, release of ROS into endosomes may affect the processing of antigens and thereby change the priming capacity of DCs (Savina, 2006); second, $p47^{phox}$ has been proposed to be critical for tryptophan metabolism in DC and thereby makes them less proinflammatory (Romani, 2008); and third, macrophages may be proinflammatory and more efficient antigen presenting cells, if they are deficient in ROS production (Gelderman, 2007). These mechanisms are all dependent on the function of $p47^{phox}$ as a part of a NADPH oxidase complex. In contrast, the present investigation defines an additional, somewhat more appealing, mechanism that is independent of the oxidative burst: $p47^{phox}$ may negatively regulate TLR9-mediated IL-12p70 secretion of DCs by acting through an adaptor on the TLR/TRAF6 signaling cascade.

After assembling all of this data regarding the regulatory function of $p47^{phox}$ in DCs *in vitro*, the next interesting point was to investigate if the Th1 response mediated by IL-12p70 is influenced. Although our *in vivo* experiments with a delayed-type hypersensitivity-like immunization protocol would point toward an increased Th1 response in $p47^{phox}$ deficient mice, the consequence of this type of regulation is not completely clear. As detected in our previous results that type I IFNs are influenced by $p47^{phox}$ and activated together with an increased IL12p70 secretion, it might operate to both drive and protect against chronic inflammation (Baccala, 2007; Treschow, 2005). However, the results of our initial short-term *in vivo* experiments clearly point toward a prominent role of a missing negative feedback regulation by $p47^{phox}$ for the development of an IL-12p70-dependent, Th1-polarized response. As described before immunization with antigens in the absence of a polarizing adjuvant, i.e., in IFA only, usually leads to a Th2-type response (Yip, 1999). Classically, immunization with “complete Freund’s adjuvant” (IFA containing *Mycobacterium tuberculosis*) or IFA with inflammatory type A CpGs and antigen facilitate a Th1 response (Chu, 1997; Yip, 1999). In addition to the Th1 response the IL-23 driven Th17 axis has been investigated. Interestingly Th17 memory cells were detected and also a feedback mechanism comparable to the Th1 response was observed while no IL-23 expression was measurable in the LN cells ten days after immunization. From the data of the BM-derived DCs we knew already that IL-23 is not regulated by $p47^{phox}$. Of course it is known that other cytokines are involved in the differentiation and proliferation of Th17 cells but IL-23

is the cytokine responsible for the survival of Th17 cells (Veldhoen, 2006). Though it may be possible that factors like IL-6 and TGFß may be involved in the initial skewing of naïve T cells towards Th17, and thus make IL-23 suspendible, the observed effects on Th17 axis are not completely understood and further studies have to be done into this specific finding.

Concluding the data of the *in vitro* and *in vivo* experiments with $p47^{phox-/-}$, herein for the first time a new and strong regulator of the proinflammatory IL-12p70/Th1 response has been identified, i.e. $p47^{phox}$. These findings offer the possibility to exploit the role of $p47^{phox}$ for both the guidance of an anti-inflammatory therapy in autoimmunity or vice versa to elicit a strong anti-cancerogenic response.

5.3 Role of Vitamin D_3 on IL-12p70 expression

In the third part of this thesis the immunomodulatory function of VD_3 before and during stimulation of DCs with TLR ligands was investigated. In two different mouse strains splenocytes and $CD11c^+$ purified cells treated with VD_3 diminished IL-12p70 secretion upon TLR stimulation was detected. Thereby the strongest effect was observed in cells which were incubated with VD_3 also before TLR stimulation. These findings confirm the published results that VD_3 inhibits the differentiation and maturation of DCs and furthermore the production of IL-12p70 and specifically its subunit p35 in DCs (Lyakh, 2005). Already fifteen years ago the down-regulation of c-rel, an important NF-κB protein, which regulates the expression of p35 was demonstrated (Yu, 1995). Furthermore other NF-κB proteins such as rel-B are regulated by VD_3 (Dong, 2003). VD_3 was shown to inhibit factors essential for the differentiation of DCs such as IRF4 (Gauzi, 2005). In mice deficient for IRF4 $CD4^+$ DCs are completely absent (Tailor, 2006). Thus, it is obvious that the immunomodulatory function of VD_3 is widely distributed on different factors controlling the immune response.

Since it was shown that VD_3 regulates the redox status by elevating the expression of $p47^{phox}$ the association of VD_3 with $p47^{phox}$ regarding the IL-12p70 secretion have been investigated (Deutsch, 1995; Sly, 2001). Herein, in initial experiments with WT and $p47^{phox}$ deficient mice I could demonstrate that VD_3 may have a stimulatory effect on $p47^{phox}$ which consequently resulted in decreased IL-12p70 upon TLR9 stimulation. Although also in $p47^{phox}$ deficient mice TLR9 induced IL-12p70 is down-regulated by VD_3 treatment the effect on WT cells is

about 40% stronger than in $p47^{phox-/-}$ cells. Because in the cell VD_3 has many different effects the influence on $p47^{phox}$ may be only one part and thus may is not completely translated into the amount of IL-12p70 secretion. To investigate this influence in more detail further experiments are needed including the $p47^{phox}$ expression and activation upon VD_3 treatment.

In conclusion the results of this thesis present three factors, Selenium, $p47^{phox}$ and VD_3, which at different levels of efficiency regulate the TLR mediated immune response of DCs. In the last years DCs became more important due to their specific regulation of immune responses either for prevention of autoimmunity or in cancer therapy. One potential regulatory mechanism for these approaches could be the IL-12p70 regulation by $p47^{phox}$, because with respect to this modulator the results obtained in this thesis are very promising.

6 OUTLOOK

In this thesis the regulation of IL-12p70 and IL-23 in DCs was investigated. Although in this thesis different possibilities to regulate one of the both cytokines have been presented, future prospective research will definitely and preferentially focus on the role of $p47^{phox}$. Exploitation of its function or IL-12p70 induction upon TLR9 stimulation holds the greatest promise.

A direct interaction of $p47^{phox}$ with a protein of the TLR pathway resulting in the upregulation of IL-12p70 is not completely clarified. In future experiments different candidates, seems to be interesting as interaction partner, should be investigated in more detail (**Fig.34**, colored proteins).

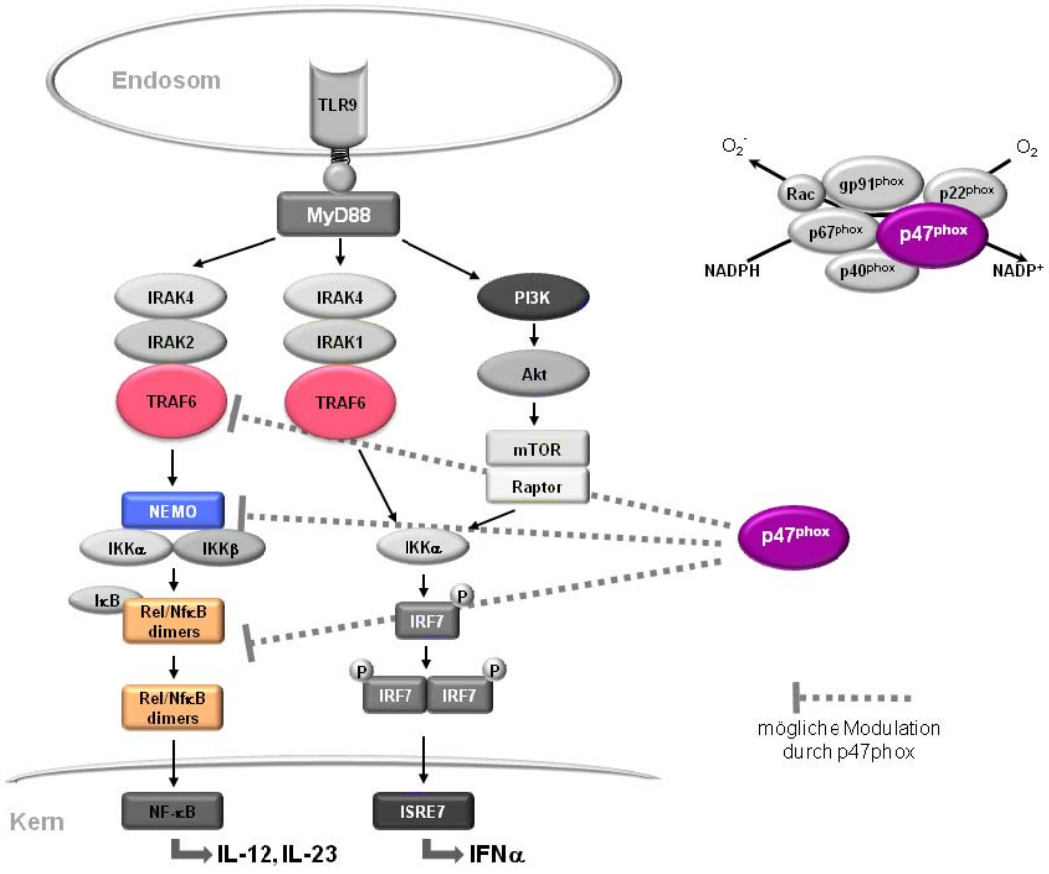

Fig. 34: Possibilities of $p47^{phox}$ to influence proteins of the TLR signaling pathway

Although the DC subpopulation comprises the $p47^{phox}$ feedback seems to be TLR9-positive, $CD11c^{+}$, IFN-α and IL-12p70 producer, further studies have to be performed within this field.

7 LITERATURE

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8 APPENDIX

8.1 Abbreviations

A	Adenine
APC	Antigen presenting cell
APS	Ammonium peroxo-disulfate
bp	Basepairs
BCA	Bicinchoninic acid
BM	Bone marrow
BSA	Bovine serum albumin
C	Cytosine
CD	Cluster of differentiation
cDC	Conventional DCs
cDNA	Copy DNA, complementary DNA
CGD	Chronic granulomatous disease
CIA	Collagen-induced arthritis
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
DNA	Desoxy ribonucleic acid
dNTP	Desoxynucleoside triphosphate
DTT	Dithiotreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme linked Immunosorbent assay
ELISPOT	Enzyme linked immuno spot
ER	Endoplasmatic reticulum
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
Fc	Fragment crystallizable
FCS	Fetal calf serum
Flt3	FMS-like tyrosine kinase 3
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor

HEPES	N-(2-Hydroxyethyl)piperazine-N'-ethanesulfonic acid
HMGB1	High-mobility group box 1
HRP	Horseradish peroxidase
IBD	Inflammatory bowel diseases
IFA	Incomplete Freund’s adjuvant
IFN	Interferon
IgG	Immunoglobulin G
IKK	Inhibitor of nuclear factor-κB (IκB)-kinase
IL	Interleukin
iLC	Immature Langerhans cells
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
JAK	Janus kinase
kb	Kilobase
kD	Kilodalton
LN	Lymph node
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRRs	Leucin rich repeats
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene (88)
NADPH	Nicotinamide adenine dinucleotide phosphate
NEA	Non-essential amino acids
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor κB
NK	Natural killer
NP-40	Nonidet P-40
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAA	Polyacrylamide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

pDC	Plasmacytoid DCs
phox	Phagocyte oxidase
PIA	Pristane induced arthritis
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
rpm	Rounds per minute
RT-PCR	Reverse transcribed-polymerase chain reaction
s.c.	subcutan
SD	Standard deviation
SDS	Sodiumdodecyl sulfate
Se	Selenium
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
T	Thymine
T1D	Type I insulin-dependent diabetes mellitus
TAE	Tris Acetate EDTA
TAB	TAK1-binding protein
TAK1	transforming growth factor (TGF) -activated kinase 1
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK binding kinase 1
TEMED	N,N,N',N' - tetramethylethylene diamine
Th	T helper
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
U	Units
UV	Ultraviolet (light)
VD_3	Vitamin D_3
VDR	VD receptor
WT	Wild type

8.2 Deutsche Zusammenfassung

Dendritische Zellen gelten als „Wächter“ zwischen der angeborenen und der erworbenen Immunität. Sie sind professionelle Antigen-präsentierende Zellen, die eindringende Pathogen finden, über spezielle Rezeptoren, wie Toll-like Rezeptoren, mikrobielle Strukturen erkennen und daraufhin eine gezielte T-Zell Antwort induzieren. Für die Ausrichtung der naiven T-Zellen sind TLR-induzierte Zytokine verantwortlich. Die Erkennung und Unterscheidung mikrobieller, fremder Muster und körpereigener Strukturen über TLRs ist fein abgestimmt, aber dennoch kann es unter bestimmten Umständen zu einer Dysregulation der Immunantwort kommen. Die Konsequenz kann eine unkontrollierte Immunantwort sein, welche sich in Autoimmunität, chronisch-entzündlichen Erkrankungen oder Krebs wiederspiegelt. In dieser Dissertation lag der Fokus auf zwei Zytokinen der IL-12 Familie, IL-12p70 und IL-23, produziert von DCs. Das Ziel der Arbeit war die Untersuchung von drei endogenen bzw. exogenen Faktoren, welche IL-12p70 oder IL-23 regulieren könnten.

Im ersten Teil der Arbeit wurde Selen, ein essenzielles Spurenelement und ein wichtiger Faktor in vielen Stoffwechselwegen inklusive Sauerstoffradikal-/Redox - Status, als Zusatz in einer DC Zellkultur verwendet. Da Selen auch eine wichtige Rolle im Immunsystem zugesprochen wird, wurde die TLR-induzierte IL-23 Produktion der DCs nach Selenbehandlung analysiert. In der unreifen Langerhanszelllinie XS-52 war der stärkste Stimulus für die IL-23 Produktion der TLR4 Ligand LPS. Desweiteren konnte eine erhöhte Menge TLR4-induziertes IL-23 in den Selen-behandelten Zellen, konzentrationsabhängig, beobachtet werden. Während die IL-23 Untereinheit p40 durch Selen herauf reguliert wurde, war die zweite Untereinheit p19 unbeeinflusst durch Selen. Diesen Effekt konnte sowohl auf mRNA als auch auf Protein Ebene detektiert werden. Zusätzlich, wie auch zu erwarten, hat die gleichzeitige Stimulation mit IFN-γ die IL-23 Produktion in nicht behandelten und Selen-behandelten inhibiert.

Im zweiten Abschnitt der Dissertation sollte $p47^{phox}$, ein Organisationsprotein der NADPH oxidase bezüglich seines regulatorischen Potenzials für die IL-12p70 und IL-23 Produktion in verschiedenen DC Typen analysiert werden. Seit gezeigt wurde, dass $p47^{phox}$ Defizienz mit erhöhter Autoimmunität und chronischer Entzündung assoziiert ist, sollte geprüft werden, ob $p47^{phox}$ eine zusätzliche Funktion neben seiner Rolle innerhalb der NADPH oxidase besitzt. Es gibt einige

Hinweise, dass $p47^{phox}$ mit bestimmten Proteinen des TLR Signalweges interagieren kann. Daraus ableitend wurde für diese Arbeit die Hypothese aufgestellt, dass $p47^{phox}$ eine regulatorische Funktion für die TLR-induzierte Zytokinproduktion in DCs haben könnte. In mehreren Experimenten in Milzzellen $p47^{phox}$ defizienter Mäuse konnte eine erhöhte IL-12p70 Antwort nach TLR9 Stimulation im Vergleich zu Wildtyp Zellen detektiert werden. Im Gegensatz zu TLR9 war die TLR4-induzierte IL-12p70 Produktion nicht unterschiedlich. In Milzzellen konnt generell kein IL-23 gemessen werden. Um die Ergebnisse bezüglich dieser neu beschriebenen negativen Rückkopplung auf IL-12p70 durch $p47^{phox}$ zu bestätigen wurden Ratten mit einem Einzelnukleotid-Polymorphismus im $p47^{phox}$ Gen untersucht. Interessanterweise befindet sich dieser Polymorphismus an einer Phosphorylierungsstelle der IRAK4, einer wichtigen Kinase im TLR Signalweg. In Ratten, welche für Methionin an dieser Stelle kodieren, war die TLR-induzierte IL-12p70 Antwort im Vergleich zu den Ratten mit einem Threonin an dieser Position deutlich erhöht. Threonin kann im Gegensatz zu Methionin durch IRAK4 phosphoryliert werden. Alle untersuchten Mäuse und Ratten sind durch einen Defekt in der NADPH Oxidase gekennzeichnet, welcher durch ein nicht funktionelles $p47^{phox}$ Protein entsteht und sind somit nicht in der Lage Sauerstoffradikale, ROS, zu produzieren. Um zu zeigen, dass die fehlende negative Rückkopplung in $p47^{phox}$ defizienten und mutierten Zellen nicht durch das Fehlen von Sauerstoffradikalen zustande kommt, wurden Kontrollexperimente mit $gp91^{phox}$ Mäusen durchgeführt. Diese Tiere sind ebenfalls durch eine defekte NADPH Oxidase Funktion charakterisiert. In mehreren Experimenten konnte gezeigt werden, dass die $gp91^{phox}$ defizienten Zellen keine Unterschiede bezüglich der negativen Rückkopplung auf IL-12p70 zu Wildtyp Zellen aufweisen. In weiteren Untersuchungen mit einem Inhibitor der NADPH Oxidase wurden ebenfalls keine Effekte auf die negative Rückkopplung gemessen. Interessanterweise wurde aber mit Hilfe des Inhibitors auch nach TLR4 Stimulation ein negativer Rückkopplungseffekt auf IL-12p70 in Wildtyp Zellen detektiert. Somit konnte nachgewiesen werden, dass die Blockade des ROS-abhängigen TLR4 Weges den ROS-unabhängigen Teil des TLR4 Signalweges aufdeckt, welcher zuvor nicht sichtbar war. Diese Befunde zeigen deutlich, dass $p47^{phox}$ eine NADPH Oxidase-unabhängige Funktion besitzt. Da Milzzellen kein IL-23 produzieren wurden DCs, welche zuvor aus dem Knochenmark differenziert

wurden, hinsichtlich der negativen Rückkopplung untersucht. In DCs aus $p47^{phox}$ defizienten Mäusen, differenziert mit GM-CSF, konnte eine erhöhte IL-12p70 Produktion nachbewiesen werden. Im Gegensatz dazu zeigten Flt3-L differenzierte Knochenmarkzellen aus $p47^{phox}$ defizienten und Wildtyp Mäusen keine Unterschiede in der IL-12p70 Menge. Für IL-23 konnte weder in GM-CSF noch in Flt3-L differenzierten Zellen eine negative Rückkopplung detektiert werden. Somit konnte gezeigt werden, dass IL-23 nicht durch $p47^{phox}$ reguliert wird. IL-12p70 ist das wichtigste Zytokin für die Ausrichtung naiver T Zellen in Th1 Zellen, während IL-23 für die Erhaltung und das Überleben von Th17 Zellen essenziell ist. Um zu überprüfen, ob die Regulation von IL-12p70 durch $p47^{phox}$ auch die T-Zell Antwort beeinflusst, wurden Immunisierungsexperimente, vergleichbar zu den klassischen DTH Protokollen, durchgeführt. Verschieden Gruppen von $p47^{phox}$ defizienten und Wildtyp Mäusen erhielten s.c. entweder PBS, OVA oder OVA in Kombination mit dem TLR9 Liganden CpG2216 in IFA. Dies sollte die Aktivierung und Polarisierung naiver T-Zellen in Th1 oder Th17 Zellen fördern. Nach 10 Tagen wurden die regionalen Lymphknoten entnommen, die Zellen in einem ELISA spot assay mit oder ohne OVA re-stimuliert und die Frequenz an IFN-γ bzw. IL-17 produzierenden T-Zellen gemessen. Die erneute Stimulation mit OVA *in vitro* verstärkte die IFN-γ und IL-17 Antwort in $p47^{phox}$ defizienten Zellen. Die Kombination mit CpG2216 als Adjuvans und Auslöser des 3. Signals erhöhte die Frequenz von IFN-γ bzw. IL-17 in Wildtyp Zellen deutlich. Darüberhinaus war die Antwort in $p47^{phox}$ defizienten Zellen, welche bereits ohne erneute OVA Stimulation verstärkt war, gegenüber dem Wildtyp signifikant erhöht. Somit bestärken diese Befunde auch die *in vitro* Daten bezüglich IL-12p70 Regulation und darüberhinaus die Wichtigkeit der $p47^{phox}$ Regulation als zusätzlicher Mechanismus zur Kontrolle der Immunantwort.

Im letzten Teil der Arbeit wurde die immunregulatorische Funktion von Vitamin D_3 untersucht. Nachdem gezeigt wurde, dass VD_3 die Differenzierung und Reifung von Monozyten und DCs beeinflusst, wurden DCs aus Milzzellen von C57BL/6 und BALB/c Mäusen bezüglich IL-12p70 Produktion nach VD_3 Behandlung untersucht. Milzzellen welche mit LPS oder CpG2218 stimuliert wurden zeigten eine verminderte IL-12p70 Produktion wenn sie mit VD_3 vor der Stimulation behandelt wurden. Dagegen hatte die Behandlung mit VD_3 ausschließlich während der Stimulation keinen Einfluss. Es ist bekannt, dass VD_3 die mRNA von

$p47^{phox}$ herauf regulieren kann. Somit wurden in ersten Experimenten auch $p47^{phox}$ defiziente Zellen mit VD_3 behandelt und es konnte, verglichen zum Wildtyp, nur eine leichte Inhibition der IL-12p70 Menge detektiert werden.

Zusammenfassend zeigen die Daten dieser Dissertation drei verschiedene Möglichkeiten die TLR-induzierte Zytokinproduktion von DCs zu regulieren, wenn auch mit unterschiedlicher Intensität und Spezifität.

8.3 Figures and Tables

8.4 Journal publications and congress contributions

C. Richter, M. Herrero San Juan, J. Will, R.P. Brandes, U. Kalinke, S. Akira, J.M. Pfeilschifter, M. Hultqvist, R. Holmdahl and H. H. Radeke. **p47^{phox} provides a ROS-independent negative feedback regulation of TLR9-induced IL-12p70 in murine dendritic cells.** 2009, 182(7):4183-91.*J. Immunology*

16th European Congress of Immunology, Paris, 2006
Differential regulation of interleukin-23 subunits in dendritic cells by Selenium and gamma-interferon. C. Richter, M. Herrero San Juan, J. Will and H.H. Radeke

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p47^{phox} provides a negative feedback regulation for TLR9 induced IL-12p70 in interferon-alpha producing dendritic cells independent of its NADPH oxidase activating function. C. Richter, Jutta Will, M. Herrero San Juan, C. Daniel, J.M. Pfeilschifter and H.H. Radeke

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C. Richter, J. Will, M. Herrero San Juan, K. Palfi, R. Brandes, E. Ramos-Lopez, J.M. Pfeilschifter and H. H. Radeke

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Regulatory potential of the NADPH oxidase subunit p47^{phox} in TLR signaling – crucial point for defective immune responses? C. Richter, J. Will, M. Herrero San Juan, R. Brandes, E. Ramos-Lopez, J.M. Pfeilschifter, H.H. Radeke

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ROS-independent regulation of TLR9-induced IL-12p70 by p47^{phox} in murine dendritic cells. C. Richter, J. Will, M. Herrero San Juan, C. Daniel, J.M. Pfeilschifter, and H.H. Radeke

Frühjahrstagung der DGPT, Mainz, 2009
p47^{phox} as an important modulator of IL-12p70 in dendritic cells and the consequences for the Th1 response. C. Richter, M. Herrero San Juan, J.M. Pfeilschifter, R. Holmdahl, R.P. Brandes and H.H. Radeke

World Immune Regulation Meeting III, Davos, 2009
p47^{phox} as an important modulator of IL-12p70 in dendritic cells and the consequences for the Th1 response. C.Richter, M. Herrero San Juan, J.M. Pfeilschifter, R. Holmdahl, R.P. Brandes and H.H. Radeke

IL-23 regulation by p47^{phox} in bone-marrow-derived dendritic cells and their impact on the Th17 response. K. Immig, C. Richter, J.M. Pfeilschifter, R. Holmdahl, R.P. Brandes and H.H. Radeke

9 DANKSAGUNG

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10 CURRICULUM VITAE

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- Nachwuchsforscherstipendium der Uniklinik Frankfurt, 2009

Preise

- Posterpreis des „World Immune Regulation Meeting III“, 2009, Davos
- Preis für den Vortrag während des „World Immune Regulation Meeting III“, 2009, Davos

11 EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel:

"Differential regulation of IL-12p70 and IL-23 in murine dendritic cells"

in dem Institut für Allgemeine Pharmakologie und Toxikologie unter Betreuung und Anleitung von Prof. Dr. Heinfried H. Radeke mit Unterstützung durch PD Dr. Ulrike Köhl und Prof. Dr. Heiko Mühl ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Die vorliegende Arbeit wurde in Teilen in folgendem Publikationsorgan veröffentlicht:

Richter C, Herrero San Juan M, Will J, Brandes RP, Hultqvist M, Holmdahl R, Kalinke U, Akira S, and Radeke HH. *Ncf1* provides a reactive oxygen species-independent negative feedback regulation of TLR9-induced IL-12p70 in murine dendritic cells. 2009. *Journal of Immunolgy* 182: 4183–4191

Frankfurt am Main, 05.10.2009 Cornelia Richter