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Beyond Th1 and Th2:
A non-classical immune pathway induced by Interleukin (IL)-23 complements IL-12 in immunity to *Cryptococcus neoformans* infection

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to Gregor

for his continuous support and encouragement

$(p40)_2$	homodimeric p40
°C	degrees Celsius
µ	micro (10^{-6})
µg	microgramm
µl	microliter
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ABTS	azino-bis(3-ethylbenz-thiazolin-6-sulfate)
AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
BCA	bicinconinic acid
C. neoformans	*Crytptococcus neoformans*
CD	cluster of differentiation
$CD4^+$ T cell	T cell expressing CD4 surface molecule
$CD8^+$ T cell	T cell expressing CD8 surface molecule
cDNA	complementary DNA
CFU	colony forming units
CHO	Chinese hamster ovary cell line
CIA	collagen-induced arthritis
CLMF	cytotoxic lymphocyte maturation factor
CMI	dell-mediated immunity
ConA	Concavalin A
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	desoxyribonucleinacid
DTH	delayed type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EBI3	Eppstein Barr virus-induced gene 3
EBV	Eppstein Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraaceticacid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
Fig.	figure
FITC	fluoresceine isothiocyanate
FIV	feline immunodeficiency virus
G-CSF	granulocyte-colony stimulating factor
GXM	glucuronoxylomannan
H&E	haemotoxilin and eosin
HIV	human immunodeficiency virus
hkCn	heat-killed C. neoformans
HRP	horse radish peroxidase
IEC	ion-exchange chromatography

IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-12R	interleukin-12 receptor
IL-23R	interleukin-23 receptor
iNOS	inducible nitric oxide synthetase
kDa	kilodalton
L	liter
LPS	lipopolysaccharide
M	molar
M	milli (10^{-3})
M. bovis BCG	*Mycobacterium bovis* Bacillus Calmette Guerin
M. tuberculosis	*Mycobacterium tuberculosis*
mBSA	methylated bovine serum albumine
MCP-1	macrophage chemoattractant protein-1
MHC	major histocompatibility complex
Ml	milliliter
mM	millimolar
NADPH	nicotinamide adenine dinucleotide hydrophosphated
NK cell	natural killer cell
NKSF	natural killer cell stimulatory factor (synonyme of IL-12)
NO	nitric oxide
p.i.	post infection
p19	p19 subunit of IL-23
p35	p35 subunit of IL-12
p35/40	p35 and p40 subunit of IL-12
p40	p40 subunit of IL-12
PAMP	pathogen associated molecular pattern
PBS	phosphate-buffered saline
PEC	peritoneal exudated cells
PEL	peritoneal lavage
PRR	pattern recognition receptor
rm	recombinant murine
SCID	severe combined immunodeficiency
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
STAT	signal transducer and activators of transcription
T. gondii	*Toxoplasma gondii*
TBS	tris buffered saline
TBS-T	tris-buffered saline with Tween20
TCR	T cell receptor
TEMED	N, N, N', N'-tetramethylethylendiamine

Th	T helper
TNF	tumor necroses factor
TNP	trinitrophenole
V	volt
vCn	viable *C. neoformans*
WT	wild-type

FIGURES

TABLES

1 INTRODUCTION

Every second we are subject to attack by various pathogenic microbes trying to invade our bodies and infect organs and tissues. Though at constant risk, the occurrence of an established infection is rare - proof of an efficient protective system. In addition to physical barriers such as skin and mucosa, a highly specialized network of cells and soluble factors, called the immune system, is the key feature of host defense. In higher organisms such as vertebrates (especially mammals and birds) the unspecific innate immune system is complemented by an antigen-specific adaptive compartment providing a more versatile means of defense. If protection fails, pathogens establish a focus of infection from which they can spread and sometimes cause fatal disease. This could be either because of the pathogen using strategies to elude or overcome the immune mechanisms or because of inborn (primary) or acquired (secondary) immunodeficiency.

Edward Jenner's breakthrough smallpox vaccination in 1796 and Robert Koch's postulates on the criteria characterizing infectious disease in the late 19th century, paved the way for extensive research on the nature of infection and host defense, bringing to light valuable insights in immune system processes (SILVERSTEIN 1979a). In 1884 Elie Metchnikoff identified phagocytic cells as the key players in unspecific immunity (METCHNIKOFF 1884). These cells, which he termed *macrophages* and *microphages* (neutrophils), were able to engulf and digest microbes. At this time the community of immunologists was split in two schools, the *cellularists*, headed by Metchnikoff and Louis Pasteur, and the *humoralists*, who hypothesized that immunity is mediated solely by soluble factors (SILVERSTEIN 1979b). The latter of theories was supported by Emil von Behring and Shibasaburo Kitasato who found evidence for the existence of antibodies and were the first to use antiserum to treat active disease. We now know that both, cellular and humoral components attribute to host defense. At the time of Behring's description of antibodies, Paul Ehrlich published his "side-chain-theory". Therein he described cells that bear antigen-specific chains on their outer surface which can be shed on occasion and are able to bind pathogens, thereby providing the earliest description of what we now call B cells (EHRLICH 1897). Although at the beginning of the 20th century James B. Murphy established a role for small white blood cells termed *lymphocytes* in tissue and tumor graft rejection (MURPHY 1926), it was not before 1959 that James Gowan could focus the immunologists' attention to these cells (SILVERSTEIN 2001). Soon thereafter B (bursal-derived) and T (thymus-derived) lymphocytes became the recognized elements in humoral and cellular immunity, respectively. A further breakthrough

in the understanding of adaptive immune responses was achieved by Robert L. Coffman and Timothy R. Mosmann in 1986 when they published the basis of our classical understanding of the dichotomy of T helper cell responses (MOSMANN *et al.* 1986). They described that the two branches of adaptive immunity, antibody-mediated and cell-mediated immunity, are governed by two different subsets of T lymphocytes, T helper type 1 (Th1) and T helper type 2 (Th2) cells, which differ in their cytokine profile

Cytokines form a group of proteins that act as intercellular communication signals. Produced by one cell, they affect the behavior of the cells bearing the appropriate receptor (JANEWAY *et al.* 2001). Their important role in coordinating the fine-tuned processes of immune defense put them in the center of immunological research since the 1980s and led to the development of novel treatment strategies. These strategies aim to enhance or suppress specific immune reactions by either administering or neutralizing cytokines. Understanding the role of cytokines will lead to understanding of the coordination of immune responses in host defense, and how failure of such protective mechanisms become manifest in autoimmune diseases and cancer.

1.1 AIM OF STUDY

Recently, a novel cytokine, interleukin (IL)-23, was discovered (OPPMANN *et al.* 2000) which is closely related to IL-12, a cytokine essential for protection in various models of intracellular infection (TRINCHIERI 2003). Meanwhile, an essential contribution of IL-23 in various models of autoimmune disease could be demonstrated (ALBER *et al.* 2006; MCKENZIE *et al.* 2006). This key role for IL-23 in chronic inflammation raised the question for its physiological role in host defense (HUNTER 2005; LANGRISH *et al.* 2004). Although there is evidence for a protective function of IL-23 in acute infections (MCKENZIE *et al.* 2006), its role in chronic infection could not yet be clearly defined.

Due to its structural relationship to IL-12, first studies on the biological function of IL-23 focused on the shared biological functions of both cytokines. The present work was performed in the light of the recently defined implication of IL-23 in a non-classical cellular immune response. The aim of the here presented study was (i) to define the role of this IL-23-mediated immune pathway in a chronic infection model, murine cryptococcosis, and (ii) to unveil a possible mode of action for IL-23 in this context.

2 REVIEW OF LITERATURE

2.1 CELLULAR IMMUNITY TO INFECTION

2.1.1 Innate immunity: first line of defense

Invading microorganisms are confronted immediately with a network of phagocytic cells, such as dendritic cells (DCs) and macrophages (mononuclear phagocytes) in tissues as well as polymorphonuclear phagocytes, such as neutrophils, and monocytes in the blood-stream.

Macrophages are most important in initiating the local inflammatory response (JANEWAY *et al.* 2001). They mature continuously from circulating monocytes and reside in tissues. Conserved structures displayed on the pathogen surface, so-called pathogen-associated molecular patterns (PAMPs), enable phagocytes to recognize and ingest the pathogens by specific pattern recognition receptors (PRRs) (KAISHO and AKIRA 2000). Not only internalization in a membrane-bounded vesicle known as phagosome with subsequent digestion of the pathogen is mediated by binding to PRRs, moreover, upon such stimulation macrophages and DCs release cytokines and chemoattractant proteins (chemokines) leading to local inflammation (JANEWAY *et al.* 2001). Important inflammatory cytokines produced by macrophages are IL-1β, IL-6 and tumor necrosis factor (TNF)-α. IL-1β and TNF-α induce activation of the vascular endothelium and increase vascular permeability; processes crucial for the infiltration of further leukocytes as well as increased drainage to lymph nodes. TNF-α is most important in the containment of infection; its neutralization leads to a systemic spread of otherwise local infections in mouse models. In addition, TNF-α stimulates DC migration to lymph nodes thus triggering the initiation of adaptive immunity (see below). IL-6 strongly induces the expression of hepatic acute-phase proteins. All three cytokines synergize in activating lymphocytes and, systemically, in inducing fever (JANEWAY *et al.* 2001).

Chemokines produced by macrophages upon activation cause the influx of further inflammatory cells. Important such mediators are CXCL8 and monocyte chemoattractant protein 1 (MCP-1) with overlapping, but also distinct effects on cell recruitment (MUKAIDA *et al.* 1998). CXCL8 (or KC in mice) recruits a second major group of phagocytic cells from the blood to the site of infection, the neutrophils. They are short-lived cells that like macrophages internalize and destroy pathogens or produce microbicidal oxygen-derived products such as H_2O_2 in a process termed respiratory burst or toxic nitrogen oxides (NO). The production of MCP-1 leads to the influx of further macrophages as well as lymphoid cells, such as natural killer (NK) cells, another cell type of innate immunity.

NK cells bear invariant receptors; upon stimulation they release their cytotoxic granules onto the surface of the bound target. NK cells are activated in response to macrophage-derived cytokines such as IL-12, IL-18 and TNF-α. They are important in the early phase of infection with intracellular pathogens by means of cytotoxicity and interferon (IFN)–γ production (HAMERMAN *et al.* 2005). IFN-γ is a pleiotropic cytokine that plays an essential role in innate and adaptive immunity (SHTRICHMAN and SAMUEL 2001). Mutations of components in the IFN-γ signaling pathway are associated with strongly elevated susceptibility to infection mainly with intracellular pathogens (DORMAN and HOLLAND 1998; JOUANGUY *et al.* 1996). Therefore, activated NK cells serve to contain infections with intracellular pathogens while adaptive immunity (also triggered by NK cell-derived IFN-γ) generates more specific means to clear the infection (see next section).

The means of innate immunity described so far are efficient in repelling a broad range of microorganisms. Pathogens, however, have developed strategies to overcome such mechanisms. In these circumstances innate immunity paves the way for adaptive immune responses. DCs are most important in bridging innate and adaptive immunity as they, after antigen-uptake, migrate from tissues to the local lymph nodes where they initiate the specific immune response by priming naïve T cells which then become effector cells (JANEWAY *et al.* 2001). DCs, together with macrophages and B cells, function as antigen-presenting cells (APCs). They process phagocytized antigen and load the resulting peptides on major histocompatibility complex class 2 (MHC-II) molecules expressed on the surface thereby presenting it to T cells (RAMACHANDRA *et al.* 1999). Activation of APCs by microbial products up-regulates the expression of MHC molecules, thus enhancing their antigen-presenting capacities. DC-primed T cells that migrate from lymph nodes to tissues are retained at the site of infection basically by MHC-presented antigen. In addition, APCs upon activation up-regulate co-stimulatory molecules that enhance the MHC-induced signal in T cells (COYLE and GUTIERREZ-RAMOS 2001). Macrophages and DCs are further the source of cytokines important for the induction and maintenance of adaptive cellular immunity. In this context the members of the IL-12 family of cytokines such as IL-12, IL-27 and IL-23 (see chapter 2.2.1) as well as IL-18 play an important role (BROMBACHER *et al.* 2003).

Therefore, the two major tasks of innate immunity are the containment of infection or even rapid elimination of pathogens by macrophages, neutrophils and NK cells, as well as the initiation of adaptive immune response by macrophages and DCs.

2.1.2 Adaptive immunity: specificity and memory as strategies

The central cells of adaptive immunity are T and B lymphocytes. Both, T and B lymphocytes respond to antigen presentation by specific recognition through their antigen receptors, the T cell and B cell receptor.

T cells recognize peptide antigen only when it is presented on MHC-molecules. There are two classes of MHC molecules that are corresponding to two different T cell subsets. MHC class 1 (MHC-I) is expressed on almost every cell type and is recognized by cytotoxic T cells (CTL), expressing the cluster of differentiation (CD) antigen CD8 (co-receptor) on their surface. CTL are programmed to destroy their target cells. Upon recognition of altered or foreign antigen presented on MHC-I they release the cytotoxic granules onto the bound cell and induce cell death. While $CD8^+$ T cells also express cytokines such as IFN-γ the most important producers of cytokines and thereby regulators of cellular immune responses are T helper (Th) cells, characterized by expression of co-stimulatory receptor CD4. Th cells recognize antigen presented on MHC-II molecules by their T cell receptor (TCR), hence they are only activated by APCs.

TCR signaling is enhanced by co-stimulatory signals provided by APCs. The best characterized co-stimulatory molecules are the glycoproteins of the B7 complex, CD80 and CD86. Binding of APC-expressed B7 molecules to their common receptor CD28 on the T cell surface leads to the production of IL-2, most important for proliferation and clonal expansion of T cells and also activates NK cells. Conversely, inhibition of B7 molecule binding inhibits T cell responses (JANEWAY *et al.* 2001) demonstrating the importance of co-stimulatory signals provided by APCs.

Upon TCR/CD28 signaling depending on the surrounding cytokine milieu naïve Th cells can commit to different lineages either promoting cell-mediated immunity (Th1) or enhancing B cell induced antibody-mediated immunity (Th2).

The processes involved in Th1 commitment are still subject to extensive research. While IL-12 is shown to be essential for the Th1 maintenance, recently discovered cytokines such as IL-27 and IL-23 add to our understanding of the regulation of early and late-stage Th1 responses. NK cell-derived early IFN-γ and APC-derived IL-27 initiate the Th1 program in naïve T ells in the context of TCR/CD28 activation characterized by expression of both IL-12R chains and consecutive production of IFN-γ (BROMBACHER *et al.* 2003). By activation via APC-derived IL-12 IFN-γ production is enhanced and the receptor for IL-18 is expressed on the cell surface. Signaling through functional IL-12 and IL-18 receptors on effector Th1 cells induces high-level IFN-γ production, the hallmark cytokine of the Th1

response (BROMBACHER *et al.* 2003). IFN-γ can activate microbicidal activity as well as cytokine production in macrophages; amongst others enhancing TNF-α-induced NO production (JANEWAY *et al.* 2001). IL-12 enhances their antigen presenting capacities (TRINCHIERI 2003). Moreover, IL-12 activates NK cells and induces IFN-γ production in such cells.

A valuable tool in assessing Th1 responses is the delayed-type hypersensitivity (DTH) reaction, a local Th1 cell-mediated inflammatory response. The prototypic DTH response is an artifact of modern medicine - the tuberculin test. The mechanisms involved in the generation of a DTH response resemble the events of a regular effector Th1 response: Th1 cells are retained at the site of antigen injection by MHC-II encountered antigen and produce cytokines such as TNF-α and IFN-γ as well as chemokines thereby inducing influx of macrophages and local inflammation.

Collectively, Th1 responses lead to an enforcement of the cellular effector mechanisms of innate immunity thus making it a strong weapon in the struggle against intracellular pathogens.

Th2 commitment in contrast is mediated by IL-4. Th2 cells are characterized by the production of IL-4 as well as IL-13, IL-5 and IL-10. Th2 responses are focused on the activation of B cells and the inhibition of macrophage activation. B cells bind and ingest antigen via their B cell receptor (membrane-bound immunoglobulin (Ig)) which leads to activation and the production of antigen-specific IgM antibodies. While this process is independent of T cells, the switch of Ig classes and the establishment of B cell memory are only possible when Th cells recognize MHC-II-bound antigen presented by B cells and in turn stimulate those B cells via CD40L/CD40 ligation. In case of Th2 cell/B cell interaction, subsequent IL-4 production leads to an Ig class switch to IgE, a hallmark Ig for Th2 responses. Th2 responses are directed against extracellular pathogens that are accessible for antibodies. Conversely, antibody-mediated immunity is little effective against intracellular pathogens providing a rationale for the fact that Th2 responses are inhibited by the Th1 pathway and vice versa.

A most important feature of adaptive immunity is the establishment of immunological memory, the basic concept of vaccination. T memory cells can rapidly recall the Th1 or Th2 cytokine pattern that was induced during primary stimulation. High-level cytokine secretion occurs within hours of secondary stimulation, TCR activation alone is sufficient to induce this robust recall response.

The outcome of inborn or acquired deficiencies of the adaptive immune system proves its importance. In horses, mainly Arabian horses, a mutant of the gene encoding the DNA-dependent protein kinase leads to the absence of T and B cells in homozygous carriers. Those foals develop a severe combined immunodeficiency (SCID) and die of infections with opportunistic pathogens. In people the most frequent severe acquired immunodeficiency is AIDS caused by infection with the human immunodeficiency virus (HIV). The loss of $CD4^+$ T cells in the devastating course of infection is hallmarked by secondary infections with opportunistic pathogens, such as *Mycobacterium* ssp., *Toxoplasma gondii* or *Cryptococcus neoformans*.

2.2 INTERLEUKIN-23

2.2.1 Interleukin-23, a member of the IL-12 family of cytokines

IL-23 is a recently discovered cytokine that possesses strong proinflammatory potential. Because of its close relationship to the prototypical Th1 cytokine IL-12 it is assigned to the IL-12 family cytokines (Fig. 1). The members of this cytokine family, including IL-23, are produced by activated APCs (D'ANDREA *et al.* 1992; OPPMANN *et al.* 2000; PFLANZ *et al.* 2002), whereas their receptors are expressed on broad range of myeloid and lymphoid cells (PARHAM *et al.* 2002; PRESKY *et al.* 1996). Microbial products are important stimuli for the expression of IL-12 family members by APCs which indicates their important role in immunity to infection (SCHUETZE *et al.* 2005; FEDELE *et al.* 2005; SMITS *et al.* 2004). IL-12 family cytokines are considered some of the most important soluble signals for induction of cellular immunity, acting at the interface of innate and adaptive immunity (BROMBACHER *et al.* 2003; LANGRISH *et al.* 2004; TRINCHIERI *et al.* 2003). The accurate interplay between these cytokines is an essential process in the orchestration of T cell responses. This implies that, to gain a better understanding of this crucial step in host defense, new findings concerning one cytokine of this family necessarily have to be put in context to the action of the others. Therefore, in the following, a brief introduction of the IL-12 family members will be given. Since the present work focused on exploring the role of IL-23 in host defense in a chronic infection model in which IL-12 has been shown to be an important player previously (DECKEN *et al.* 1998; KAWAKAMI *et al.* 1996), mainly the relationship between IL-23 and IL-12 will be highlighted.

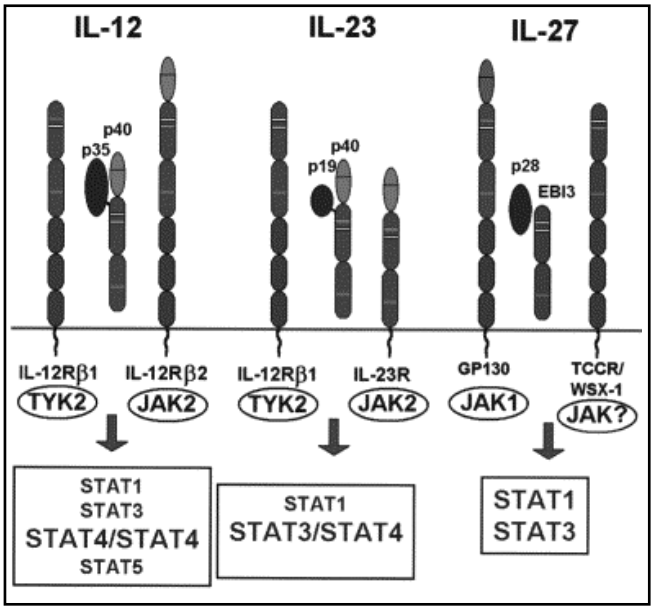


Fig. 1. IL-12 family of cytokines. Shown are the heterodimeric ligands IL-23, IL-12 and IL-27 as well as their two-chained receptors. Downstream signaling involves characteristic phosphorylation of signal transducer and activator of transcription (STAT) molecules. In addition to the depicted cytokines, the p40 subunit of IL-23 and IL-12 is physiologically expressed in its monomeric as well as homodimeric form. Figure adapted from TRINCHIERI *et al.* (2003).

2.2.1.1 IL-12 – an important player in the maintenance of Th1 responses

In 1989 two different groups of scientists discovered IL-12 simultaneously and named it based on the observed biological activity NKSF (natural killer cell stimulatory factor) (KOBAYASHI *et al.* 1989) and CLMF (cytotoxic lymphocyte maturation factor) (STERN *et al.* 1990). Its first revealed immunological function was the induction of IFN-γ production in NK cells. Further research mainly in IL-12 deficient mice demonstrated an essential role for IL-12 in the induction (MAGRAM *et al.* 1996; MATTNER *et al.* 1996) and maintenance (PARK *et al.* 2000; YAP *et al.* 2000) of Th1 responses, which are especially required for protection against intracellular pathogens. IL-12 was the first cytokine to be found with a heterodimeric structure. It is composed of a 40 kDa subunit named p40 covalently linked to a 35 kDa subunit p35 (KOBAYASHI *et al.* 1989; TRINCHIERI 2003). While the p35 subunit shares homology with other class I cytokines such as IL-6 and granulocyte-colony stimulating factor (G-CSF) (MERBERG *et al.* 1992) the p40 subunit, surprisingly, is homologous to the extracellular domain of the IL-6 receptor α chain (IL-6Rα) (GEARING and COSMAN 1991).

2.2.1.2 $(p40)_2$ – an antagonist of IL-12 with also proinflammatory potential

Besides the production of IL-12 by activated macrophages and DCs a from 10 to 1000-fold excess production of the p40 subunit, but not the p35 subunit, was found (D'ANDREA *et al.* 1992). Soluble p40 was shown to antagonize IL-12 bioactivity *in vitro* (MATTNER *et al.* 1993) by binding to the IL-12 receptor (GILLESSEN *et al.* 1995), whereby the homodimeric form ($(p40)_2$) showed higher bioactivity than the monomeric form of p40. *In vivo*,

pretreatment with $(p40)_2$ reduced LPS-induced circulating IFN-γ levels and protected mice from death (MATTNER *et al.* 1997). Moreover, mice genetically engineered to overexpress p40 in the liver developed impaired Th1 responses (YOSHIMOTO *et al.* 1998). In contrast, an agonistic function of $(p40)_2$ is discussed but not yet defined in molecular terms. It could be shown that addition of $(p40)_2$ to a mixed lymphocyte culture enhanced the induction of $CD8^+$ T cells (PICCOTTI *et al.* 1997). Moreover, in *Mycobacterium bovis* BCG-infected mice double-deficient for IL-12p35 and p40 the administration of $(p40)_2$ was able to restore the observed defect in antigen-specific DTH response (HOLSCHER *et al.* 2001).

2.2.1.3 IL-23 – a proinflammatory cytokine structurally similar to IL-12

In the year 2000 the novel cytokine IL-23 was identified by a computational database search for IL-6 related cytokines. IL-23 not only has a heterodimeric structure like IL-12, it even shares the p40 subunit with IL-12, which is in case of IL-23 covalently linked to a novel 19 kDa subunit p19 (OPPMANN *et al.* 2000). Similar to the structural relationship of IL-23 and IL-12, also their heterodimeric receptors are composed of a common chain termed IL-12 receptor β1 (IL-12Rβ1) complemented by IL-23 receptor IL-23R for IL-23 (PARHAM *et al.* 2002) and IL-12 receptor β2 for IL-12 (PRESKY *et al.* 1996) (Fig. 1). IL-23R as well as IL-12Rβ2 are crucial for transmembrane signaling by providing binding sites for signal transducer and activator of transcription (STAT) molecules. While IL-23 mainly activates STAT3 and STAT4 heterodimers, IL-12 signals via STAT4 (PARHAM *et al.* 2002). This difference, amongst others, accounts for the unexpected distinct effects of IL-23 as compared to IL-12 which will be discussed in the following sections.

2.2.1.4 IL-27 – an initiator but also regulator of Th1 responses

The IL-12 family member identified latest is termed IL-27, a heterodimer composed of Epstein-Barr Virus (EBV)-induced gene 3 (EBI3) and a novel 28 kDa polypeptide p28 (Fig 1) (PFLANZ *et al.* 2002). While EBI3 shares homology with IL-12/IL-23p40, p28 is closely related to IL-12p35 and IL-23p19. Besides the ability of IL-27 to promote IFN-γ production in naïve but not memory T cells *in vitro* (PFLANZ *et al.* 2002) there is evidence that the specific IL-27 receptor chain WSX-1 (named after an amino acid motif in the intracellular domain) that associates with gp130 to form the functional IL-27R (PFLANZ *et al.* 2004) is essential for early initiation of Th1 responses *in vivo* (CHEN *et al.* 2000; YOSHIDA *et al.* 2001). In contrast, EBI3-deficient mice were resistant to Th2-mediated immunopathology associated with oxazolone-induced colitis (NIEUWENHUIS *et al.* 2002).

Recent studies point to an immunoregulatory function of IL-27 since WSX-1 deficient mice developed an immunopathological Th1 response in the course of *Toxoplasma gondii* (VILLARINO *et al.* 2003) and *Trypanosoma cruzi* (HAMANO *et al.* 2003) infection. Further studies in p28-deficinet mice will provide a clearer understanding of the biological function of IL-27 and its interplay with the other IL-12 family members.

2.2.2 Immunological functions of IL-23

2.2.2.1 IL-23 in adaptive immunity

The IL-12 family of cytokines is commonly associated with Th1 promoting activities. Therefore, after discovery of IL-23 its effects on T cells were investigated first. In contrast to IL-12, IL-23 was able to induce IFN-γ production in memory cells but had no effect on naïve T cells (OPPMANN *et al.* 2000). Since IFN-γ is the hallmark cytokine of a Th1 response, this finding caused confusion about the contributions of IL-12 and IL-23 to Th1 commitment. However, mice specifically lacking IL-23 (IL-23p19$^{-/-}$ mice) displayed normal Th1 development and IFN-γ production (MCKENZIE *et al.* 2006). In addition, the magnitude of DTH response in IL-23p19$^{-/-}$ mice immunized with methylated bovine serum albumin (mBSA) was comparable to WT mice at 18 hours post induction (GHILARDI *et al.* 2004). Interestingly, however, at later time points the DTH reaction was greatly diminished indicating a clear contribution of IL-23 in T cell functions (GHILARDI *et al.* 2004).

Indeed, more recently, a more exclusive function could be contributed to IL-23: the promotion of IL-17 production in a certain T cell subset (AGGARWAL *et al.* 2003; LANGRISH *et al.* 2005). IL-17 is a potent inflammatory cytokine with effects on mononuclear as well as polymorphonuclear leukocytes (AGGARWAL and GURNEY 2002). It was identified about a decade ago and could be shown to be mainly produced by CD4^{+} T cells. IL-17 promotes inflammatory responses in primary and stable cell cultures and is elevated in rheumatoid arthritis, asthma, multiple sclerosis, psoriasis, and transplant rejection (KOLLS and LINDEN 2004). Mice specifically lacking IL-23 or IL-17 show an impaired antigen-specific cellular immune response (GHILARDI *et al.* 2004; NAKAE *et al.* 2002).

More recently, IL-17 producing T cells were described as a T cell subset distinct from Th1 and Th2 and termed Th17 (HARRINGTON *et al.* 2005; LANGRISH *et al.* 2005). Development of such cells was driven by IL-23 *in vitro*. Th17 cells besides IL-17 produced significant amounts of TNF-α and IL-6 but only little IFN-γ (LANGRISH *et al.* 2005). Moreover, IL-12 and/or IFN-γ appear to antagonize IL-23-induced IL-17 production *in vitro* (HARRINGTON *et al.* 2005; LANGRISH *et al.* 2005). Accordingly, in mice lacking IL-12

(IL-12p35$^{-/-}$ mice) IL-17 production is elevated as compared to WT mice (MURPHY *et al.* 2003, unpublished observation). While a role for Th17 cells in promoting autoimmunity was shown (LANGRISH *et al.* 2005, see chapter 2.2.3) further work is required to characterize their physiological role.

Little is known about the direct effects of IL-12 family cytokines on B cells. In IL-23p19$^{-/-}$ mice B cells seem to develop normally (GHILARDI *et al.* 2004). Consistently, IL-23p19$^{-/-}$ mice display normal levels of serum Igs and develop a normal T-cell-independent IgM response to trinitrophenol (TNP)-LPS or TNP-Ficoll (GHILARDI *et al.* 2004). T cell-dependent antigens such as ovalbumin and type II collagen, however, induce impaired antibody responses (GHILARDI *et al.* 2004; MURPHY *et al.* 2003) suggesting a secondary effect of IL-23 on antibody-mediated immunity via CD4^{+} T cell help.

2.2.2.2 IL-23 in innate immunity

Though having important roles in bridging innate and adaptive immunity, IL-23 and IL-12 also directly affect the cells of the innate immune system. IL-12 was shown to induce IFN-γ production in NK cells (TRINCHIERI 2003). In addition to the receptor for IL-12 human NK cells bear the IL-23R while a functional relevance has not been reported yet (PARHAM *et al.* 2002.)

The murine IL-23R is also expressed on cells that are the source of IL-23, macrophages and DCs, suggesting an autocrine or paracrine function for this cytokine (PARHAM *et al.* 2002). Indeed, exogenous IL-23 enhanced IL-12 production in DCs. Moreover, IL-23-pretreated peptide-pulsed DCs induced a stronger DTH response than mock-pretreated cells by a mechanism independent from IL-12 (BELLADONNA *et al.* 2002). Other indications for a role of IL-23 in innate immunity come from *in vivo* studies. Parallel to the multiorgan inflammation associated with elevated levels of serum TNF-α and IL-1β observed in mice genetically engineered to overexpress the p19 subunit of IL-23 (WIEKOWSKI *et al.* 2001), peritoneal macrophages produce TNF-α and IL-1β upon intraperitoneal injection of IL-23 (CUA *et al.* 2003). These findings, however, do not necessarily reflect direct interaction of IL-23 with myeloid cells as it is possible that the expression of inflammatory cytokines is mediated by other IL-23-induced factors, such as IL-17.

IL-23 effects on neutrophils are not sufficiently investigated yet. Neutrophils produced IL-17 in a model of LPS-induced airway neutrophilia (FERRETTI *et al.* 2003). While the authors pointed out IL-15 as a possible trigger, one might speculate about a role for IL-23. In a very recent report the IL-23/IL-17 axis was shown to be important for regulation of

granulopoiesis (STARK *et al.* 2005). Normal neutrophils migrate to tissues, where they become apoptotic and are phagocytized by macrophages and DCs. The authors show that this leads to phagocyte secretion of IL-23, which is controlling IL-17 production by T cells. IL-17 then induced granulopoiesis via a G-CSF dependent mechanism. Antibody blockade of the p40 subunit of IL-23 could reduce neutrophil numbers in wild-type mice. Together these findings identified a role for IL-23 in homeostasis of neutrophil production *in vivo*.

2.2.3 Biological and clinical relevance of IL-23

2.2.3.1 IL-23 in host defense

Despite its strong proinflammatory potential, a role for IL-23 in host defense has not yet been clearly defined. In acute *Klebsiella pneumoniae* infection IL-23-induced IL-17 is crucial for neutrophil recruitment to the infected airways and subsequent clearance of pathogen (HAPPEL *et al.* 2005a; YE *et al.* 2001a). In an acute model of *Toxoplasma gondii* infection IL-17R$^{-/-}$ mice show a higher mortality associated with impaired neutrophil recruitment to the infected sites (KELLY *et al.* 2005), whereas in another model of acute toxoplasmosis IL-23p19$^{-/-}$ mice were as resistant as wild-type mice (LIEBERMAN *et al.* 2004).

Attempts to address the role of IL-23 in host defense during chronic infection have raised many questions. In a very recent report on *M. tuberculosis* infection, IL-23 was dispensable for protection, although it was crucial for the promotion of antigen specific IL-17 (KHADER *et al.* 2005). However, the authors showed that in the absence of IL-12, IL-23 provides a moderate level of protection. This observation parallels previous reports which showed, that mice lacking the p40 subunit of IL-12/IL-23 are more susceptible to chronic infection with intracellular pathogens than mice lacking the p35 subunit of IL-12 (COOPER *et al.* 2002; DECKEN *et al.* 1998; ELKINS *et al.* 2002; HOLSCHER *et al.* 2001; LEHMANN *et al.* 2001; LIEBERMAN *et al.* 2004). In the murine model of infection with *C. neoformans* it was shown that IL-12p35$^{-/-}$ and p40-deficient mice both develop a Th2 response associated with elevated susceptibility (DECKEN *et al.* 1998). This pointed to the essential role of IL-12 in immunity to *C. neoformans*. Nevertheless, IL-12p35$^{-/-}$ mice still were able to form granulomata when infected with *C. neoformans*, whereas granuloma formation in mice lacking the p40 subunit was greatly impaired (DECKEN *et al.* 1998). From these data the authors concluded that another p40-dependent factor may contribute to the protective cellular immunity to *C. neoformans* infection. Today's knowledge of IL-23 and the availability of IL-23p19$^{-/-}$ mice allow for assessing the particular role of IL-23 in chronic granulomatous inflammation induced by *C. neoformans* infection, as herein presented.

2.2.3.2 IL-23 in cancer

A physiological role of IL-23 in preventing transformation of cells has not yet been investigated. Documented studies so far focused on assessing antitumor activity of IL-23 in tumor cells engineered to express this cytokine, either by viral transduction or gene-gun therapy. In models of colon carcinoma and melanoma cells, retroviral transduction of tumor cells revealed potent antitumor activities for IL-23 that were mediated by CTL (LO *et al.* 2003). In a further study, combined gene-gun therapy with IL-23 and IL-18 resulted in a significant suppression of implanted melanomas similar to combined IL-12/IL-18 therapy (WANG *et al.* 2004). While IL-12/IL-18 therapy strongly induced IFN-γ, IL-23/IL-18 treatment only moderately enhanced IFN-γ production in stimulated splenocytes. Interestingly, both treatment regimens led to comparable CTL-mediated killing activity (WANG *et al.* 2004). Taken together, IL-23 gene-therapy-mediated antitumor activity seems to rely on an IFN-γ-independent enhancement of cell-mediated killing.

2.2.3.3 IL-23 in autoimmunity

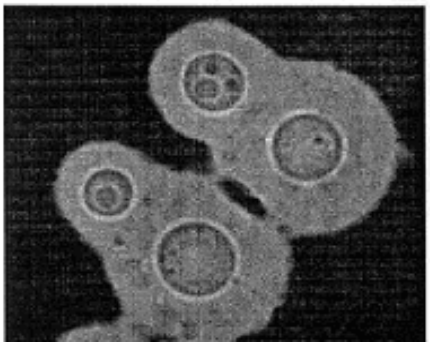
In the past many studies investigating the mechanisms of autoimmune inflammation focused on describing a role for the IL-12/IFN-γ axis and were performed either in p40-deficient mice or with p40-neutralizing antibodies. In both cases IL-12 as well as IL-23 functions were inhibited. Meanwhile, in two models of chronic T cell-mediated autoimmune inflammation, experimental autoimmune encephalomyelitis (EAE) (CUA *et al.* 2003) and collagen-induced arthritis (CIA) (MURPHY *et al.* 2003), IL-23 rather than IL-12 was shown to be the critical cytokine in the development of chronic inflammation. EAE serves as a model for human multiple sclerosis, whereas CIA is an accepted model for rheumatoid arthritis. In both studies mice specifically lacking IL-23 (IL-23p19$^{-/-}$ mice (OPPMANN *et al.* 2000)) were completely resistant to disease. Conversely, in EAE, transfer of *in vitro* generated antigen- and IL-23-driven IL-17-producing T cells was decisive for disease onset in naïve mice (LANGRISH *et al.* 2005). There are indications in the recent literature that IL-23 might also be involved in the pathogenesis of other T cell-mediated autoimmune diseases such as psoriasis vulgaris, inflammatory bowel disease and type I diabetes (MCKENZIE *et al.* 2006). Collectively, IL-23 seems to be a key player in organ-specific autoimmune chronic inflammation.

2.3 CRYPTOCOCCOSIS

2.3.1 *Cryptococcus neoformans* – causative agent of cryptococcosis

Cryptococcus neoformans is an encapsulated yeast (Fig. 2) and was first isolated from a tibia lesion by the German physician Otto Busse in 1894 (KNOKE and SCHWESINGER 1994).

Cryptococcus spp.



Kingdom:	Fungi
Phylum:	Basidiomycota
Subphylum:	Hymenomycetes
Class:	Heterobasidiomycetes
Subclass:	Tremellomycetidae
Order:	Filobasidiales
Genus:	*Cryptococcus*

Fig. 2: Morphology and taxonomical classification of *Cryptococcus neoformans spp*.
C. neoformans cells are round to oval; budding yeasts measure 4-6 µm. The cells are encountered by a polysaccharide capsule with a diameter of 1 to >30 µm (CASADEVALL and PERFECT 1998). Figure adapted from BUCHANAN and MURPHY (1998).

The genus *Cryptococcus* (for taxonomic classification see Fig. 2) includes around 37 species. Among these, *C. neoformans* is the only species that is facultative pathogenic. *C. neoformans* was generally accepted to include three varieties, var. *gattii*, var. *grubii* and var. *neoformans* (LEVITZ 1991; FRANZOT *et al.* 1999). Differences between the three varieties with regard to pathogenicity and geographical distribution have been described. *C. neoformans* var. *neoformans* and var. *grubii* are responsible for most cases of cryptococcosis in immunocompromised patients, and *C. neoformans* var. *gattii* has been associated with infections in subjects with a normal immunologic status (ELLIS 1987; SPEED and DUNT 1995). *C. neoformans* var. *neoformans* and var. *grubii* have a worldwide distribution (BENNETT *et al.* 1977), whereas *C. neoformans* var. *gattii* has been reported to be mainly restricted to tropical and subtropical areas (BARO *et al.* 1999).

2.3.2 Pathogenesis of *C. neoformans* infection and host defense

The infection process by *C. neoformans* commonly starts following inhalation of the yeasts into the lung (MITCHELL and PERFECT 1995). Infecting fungal cells are initially confronted by alveolar macrophages as well as DCs, and subsequently by T cells, macrophages, granulocytes, and natural killer cells. If the host is immunocompromised, *C. neoformans* yeasts escape from host defense using various pathogenic mechanisms. The

polysaccharide capsule and phenol oxidase enzyme of *Cryptococcus neoformans*, as well as its ability to grow at 37°C, are its major virulence factors (MITCHELL and PERFECT 1995). Phenol oxidase enzyme functions in production of melanin. The melanizing enzyme presumably prevents formation of toxic hydroxy radicals and thus protects the fungal cell from oxidative stress as well as the immune defense mechanisms of the host (CASADEVALL *et al.* 2000; JACOBSON 2000). *C. neoformans* can then disseminate in other tissues especially the brain, causing lethal meningoencephalitis (MITCHELL and PERFECT 1995). The importance of the capsule shows in experiments with acapsular mutant strains, which are non-virulent in mouse models of cryptococcosis (FROMTLING *et al.* 1982). Glucuronoxylomannan (GXM), the major capsular polysaccharide of *C. neoformans* is antiphagocytic, blocks both recruitment of inflammatory cells and the increase in costimulatory molecules (BREEN *et al.* 1982; KOZEL and MASTROIANNI 1976). GXM was found to suppress DTH and may reduce antibody production in response to fungal infection (BLACKSTOCK and CASADEVALL 1997; BREEN *et al.* 1982). The mentioned characteristics of *C. neoformans* are crucial for the yeasts survival in the host, yet they are complemented by other virulence factors that will not be further discussed here.

Although *C. neoformans* was originally recognized as an extracellular pathogen, *in vitro* and *in vivo* studies accumulated evidence for its intracellular parasitism within macrophages (FELDMESSER *et al.* 2001). This facultative intracellular parasitism of *C. neoformans* accounts to the fact, that the host's antibody response is not very effective against this pathogen (MITCHELL and PERFECT 1995). In contrast, cell-mediated immunity is essential for eradicating *C. neoformans* as shown by the severe exacerbation of disease in mice lacking essential components of the Th1 pathway, such as $CD4^+$ T cells, IFN-γ or IL-12 (CHEN *et al.* 2005; DECKEN *et al.* 1998; HILL and HARMSEN 1991).

2.3.3 Clinical manifestation in humans and domestic mammals

Given the neurotropic nature of the fungus, the most common clinical form of cryptococcosis in humans and small companion animals is meningoencephalitis (MITCHELL and PERFECT 1995). The course of the infection is usually subacute or chronic.

The most commonly encountered predisposing factor for development of cryptococcosis in humans is AIDS (MITCHELL and PERFECT 1995). Cryptococcosis presents the most common fungal infection of the brain in AIDS patients in the USA and is the fourth leading cause of death. Less commonly, organ transplant recipients or cancer patients receiving

chemotherapeutics or long-term corticosteroid treatment may develop cryptococcosis (KORFEL *et al.* 1998; URBINI *et al.* 2000).

Although cryptococcosis is dramatically potentiated in humans by concomitant infection with HIV, disseminated cryptococcosis is not strongly associated with cats that are infected with FIV. Still, *C. neoformans* infection presents one of the most common systemic fungal diseases in cats and dogs and is associated with respiratory and central nervous symptoms (HORMANSDORFER and BAUER 2000). In horses cryptococcosis presents as a rhinitis or sinusitis whereas in cattle mastitis caused by *C. neoformans* is seen on occasion. Pigeons and other birds are resistant, most likely because of their high body temperature and are considered the most important reservoir of *C. neoformans*.

2.3.5 Experimental *C. neoformans* infection

Most strains of mice are highly susceptible to experimental infection with a small number of yeasts (MITCHELL and PERFECT 1995). The availability of diverse inbred mouse strains and the variety of mice with targeted gene disruptions thereby pave the way for extensive studies on immunity against *C. neoformans* infection to provide more effective treatment options for cryptococcosis itself. On the other hand, however, experimental cryptococcosis displays a valuable tool in investigating the orchestration of cell-mediated immunity in general since this branch of the immune system has been proven to be essential for host defense in cryptococcosis.

2.4 EXPERIMENTAL APPROACH OF PRESENT STUDY

The importance of cell-mediated immunity in *C. neoformans* infection is the cause for the elevated susceptibility to infection observed in mice lacking the p35 or the p40 subunit of IL-12 (DECKEN *et al.* 1998). Although both genotypes develop a sustained Th2 response, p40-deficient mice are more susceptible to infection than IL-12p35$^{-/-}$, pointing to a contribution of a p40-dependent but Th1-independent host defense mechanism (DECKEN *et al.* 1998). The present study seeks to identify the means of this non-classical immune pathway and define the role of IL-23 in its regulation.

In a first step of the present work the question, if IL-23 accounts for the observed difference between the two IL-12 deficient mouse strains was addressed by administration of IL-23 to p40-deficient mice.

In a second series of *in vivo* studies the particular role of IL-23 in protection to *C. neoformans* infection was investigated by infection of IL-23p19$^{-/-}$ mice with a main focus on the cellular immune response and the IL-23-induced cytokine profile.

The third set of experiments focused on further defining the means of IL-23 actions. By administration of recombinant IL-23 to wild-type (WT) mice as well as mice deficient for genes encoding for factors important for adaptive immunity, the effects of IL-23 on the adaptive and innate immune system were assessed. The main focus herein was the cellular composition of inflammatory infiltrates to the site of infection and the local cytokine expression.

The last part of the present work describes a protein purification process of homodimeric p40 from insect cell culture supernatants. This work was performed as a basis for further investigations of the interplay between IL-23 and other IL-12 family members in the course of infection.

3 ANIMALS, MATERIALS AND METHODS

3.1 MICE

Wild-type, IL-12p35$^{-/-}$ and p35/40$^{-/-}$ (MAGRAM *et al.* 1996; MATTNER *et al.* 1996) mice on a C57BL/6 background were bred and maintained in individually ventilated caging units (Ehret, Berlin, Germany) under specific pathogen-free conditions within the animal facility at the Institute of Immunology, Faculty of Veterinary Medicine, University of Leipzig and in accordance with the guidelines approved by the Animal Care and Usage Committee of the Regierungspräsidium Leipzig, Germany (record numbers 24-916811-5/01 and 24-9168.11-36/04). WT, RAG2$^{-/-}$, IL-12p35$^{-/-}$, p40$^{-/-}$ (MAGRAM *et al.* 1996; MATTNER *et al.* 1996) and IL-23p19$^{-/-}$ (CUA *et al.* 2003) mice on a C57BL/6 background were also maintained at DNAX Research Institute (Palo Alto, CA, USA). RAG1$^{-/-}$ mice on a C57BL/6 background were obtained from the Jackson Laboratory, Bar Harbor, Maine, USA. All animal procedures were approved by the DNAX Institutional Animal Care and Use Committee, in accordance with AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) guidelines. Female mice were used at 8-12 weeks of age. As p35/40$^{-/-}$ and p40$^{-/-}$ mice showed no differences in the parameters of infection and immunity that were investigated in the present study, they are referred to as p40-deficient mice hereafter.

3.2 MATERIALS

3.2.1 Equipment

ELISA and multiplex array equipment:

ELISA-Reader: Spectra-max 340	Molecular Devices, Munich, Germany
ELISA-Washer (Ultrawash PLUS)	DYNEX Technologies, Chantilly, VA, USA
Immunoplates Maxisorb	NUNC, Wiesbaden, Germany
LUMINEX 100™	LUMINEX corp., Austin, TX, USA

Flow cytometry equipment:

FACS Calibur	Becton Dickinson, Franklin Lakes, NJ, USA

Microscopes:

Axiovert 25	Carl-Zeiss, Jena, Germany
Axioskop 2 plus	Carl-Zeiss, Jena, Germany

Cell culture equipment:

CO_2-incubator for cell culture	Heraeus, Osterode, Germany
Incubator for fungal growth	Memmert, Schwabach, Germany
Cell culture plates	Becton Dickinson, Franklin Lakes, NJ, USA
Cell strainer	Becton Dickinson, Franklin Lakes, NJ, USA

Centrifuges:

Megafuge 2.0 R	Heraeus, Osterode, Germany
Multifuge3 S-R	Heraeus, Osterode, Germany
Centrifuge 5417C	Eppendorf, Hamburg, Germany

Gel electrophoresis and Western Blotting equipment:

Electrophoreses chamber	Bio-Rad Laboratories, Hercules, CA, USA
Power supply model 200/2.0	Bio-Rad Laboratories, Hercules, CA, USA
Scopix LR 5200 imager	AGFA, Morstel, Belgium

Chromatography system:

AKTAPrime	Amersham Biosciences, Buckinghamshire, UK
HiPrep CM Sepharose FF column	Amersham Biosciences, Buckinghamshire, UK

Scales:

Mettler PM 4000	Mettler Toledo GmbH, Giessen, Germany
Mettler AB184-S-A	Mettler Toledo GmbH, Giessen, Germany

Miscellaneous:

pH meter inoLab® Level2	WTW GmbH, Weilheim, Germany
Benchtop incubator GFL 3031	Ges. f. Labortechnik mbH, Burgwedel, Germany
Laminar flow hoods: HERA-safe	Heraeus, Osterode, Germany
Ultra-Turrax homogenizer	Ika-Werke GmbH, Staufen, Germany
Waterbath: Isotemp 205	Scientific Support, Hayward, CA, USA
Pipettes and pipettors	Eppendorf, Hamburg, Germany
Mouse restrainer	Braintree Scientific, Braintree, MA, USA
Vacuum manifold	Millipore, Billerica, MA, USA

Consumables:

Test tubes, syringes, needles	Becton Dickinson, Franklin Lakes, NJ, USA
Pipette tips	Eppendorf, Hamburg, Germany
Serum separator	Becton Dickinson, Franklin Lakes, NJ, USA

3.2.2 Software

LUMINEX software	LUMINEX corp., Austin, TX, USA Acquisition of multiplexing bead array data by LUMINEX^{100} instrument
Masterplex QT	MiraiBio, Alameda, CA, USA Analysis of LUMINEX data

Softmax Pro 3.1.2	Molecular Devices, Sunnyvale, CA, USA Acquisition and analysis of ELISA-Reader data
BD CellQuest™ pro	Becton Dickinson, Franklin Lakes, NJ, USA Acquisition and analysis of flow cytometry data
GraphPad Prism™ 4	GraphPad Software, San Diego, CA, USA Statistics and data visualization
Reference Manager Pro 11	Thomson Research Soft, Carlsbad, CA, USA Management of bibliographies
Adobe Photoshop CS	Adobe Systems inc., San Jose, CA, USA Digital imaging
Excel® XP	Microsoft, Redmond, WA, USA Spreadsheet analysis
Word® XP	Microsoft, Redmond, WA, USA Word-processing

3.2.3 Reagents

3.2.3.1 Natural products

Fetal calf serum (FCS)	PAA Laboratories, Coelbe, Germany
Gelatine (porcine)	Merck, Darmstadt, Germany
Glucose	Sigma-Aldrich, Taufkirchen, Germany
Penicillin/Streptomycin (10^4 U/ml, 10 mg/ml)	Sigma-Aldrich, Taufkirchen, Germany
Tryptone	Roth GmbH, Karlsruhe, Germany
LPS (from *Salmonella abortus equi*, S-Form)	Alexis, Grünberg, Germany
Nitratereductase	Roche, Nutley, NJ, USA
NADPH (Nicotinamide adenine dinucleotide hydrophosphated)	Roche, Nutley, NJ, USA

3.2.3.2 Chemical reagents

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)	Sigma-Aldrich, Taufkirchen, Germany
Acrylamide	Roth GmbH, Karlsruhe, Germany
Agar-Agar	Roth GmbH, Karlsruhe, Germany
Ammonium peroxodisulfate (APS)	Merck, Darmstadt, Germany
Bromphenol blue	Sigma-Aldrich, Taufkirchen, Germany
EDTA (sodium salt)	Merck, Darmstadt, Germany
Ethanol (analytical grade)	Appli Chem, Darmstadt, Germany
Formaldehyde (37 vol %)	Roth GmbH, Karlsruhe, Germany
Isoflurane	Baxter, Munich, Germany
Isopentane	Fluka, Neu-Ulm, Germay

Naphtylethylendiamide-dihydrochloride	Sigma-Aldrich, Taufkirchen, Germany
N, N, N', N'-tetramethylethylendiamine (TEMED)	Sigma-Aldrich, Taufkirchen, Germany
Polyoxyethylene sorbitan-monolaurate (Tween 20)	Roth GmbH, Karlsruhe, Germany
Sodium dodecylsulfate (SDS)	Sigma-Aldrich, Taufkirchen, Germany
Sulfanylamide	Sigma-Aldrich, Taufkirchen, Germany
Trypan blue	Sigma-Aldrich, Taufkirchen, Germany

All other chemicals were analytical grade and obtained from Roth, Sigma-Aldrich or Merck. Only high purity solvents were used for ÄKTAPrime gelfiltration runs.

3.2.4 Biological material

3.2.4.1 Cryptococcus neoformans

The encapsulated highly virulent *C. neoformans* strain 1841, serotype D, was obtained from F. Hoffmann-La Roche Ltd, Basel, Switzerland (originally isolated from an AIDS patient) and kept as frozen stock in 10% skim milk at -20°C (DECKEN *et al.* 1998). The acapsular *C. neoformans* serotype D strain CAP67 was kindly provided by Dr. Bettina Fries, Albert-Einstein College of Medicine, Bronx, NY, and maintained in the same conditions as strain 1841.

3.2.4.2 Cytokines and antibodies

3.2.4.2.1 Antibodies and protein standard for ELISA

Analyte	Capture Ab	Detection Ab	Protein std.	Source
IgE	R35-72	R35-118 (biot.)	rmIgE C38-2*	BD Pharmingen
murine p40	5C3	Polyclonal goat anti mouse p40 (biot.)	CHO derived murine $(p40)_2$	provided by M. Gately, F. Hoffmann-La Roche Ltd, Nutley, NJ, USA
IFN-γ	AN-18	XMG (peroxidase-labeled)	rmIFN-γ*	F. Hoffmann-La Roche Ltd, Basel, Switzerland

Table 1: Antibodies and protein standards for ELISA. *, rm = recombinant murine.

3.2.4.2.2 Antibodies for flow cytometry

Cell type	Surface antigen	Conjugate	Antibody isotype
T cells	TCR-β CD4	Allophycocyanin FITC	hamster IgG1 rat IgG2a
NK cells	NK1.1	PE	mouse IgG2a
macrophages	F4/80 CD86	PE FITC	rat IgG2b rat IgG2a
dendritic cells	CD11c	Allophycocyanin	hamster IgG1
granulocytes	Gr1	PE	rat IgG2b

Table 2. Antibodies for flow cytometry. Unspecific binding was prevented by blocking Fcγ III/II receptors with purified anti-mouse CD16/CD32. For intracellular cytokine staining IFN-γ-Allophycocyanin (rat IgG1) was used. All antibodies and isotype controls were purchased from BD Pharmingen, Franklin Lakes, NJ, USA.

3.2.4.2.3 Cytokines for in vivo *treatment*

rmIL-23 was expressed and purified at DNAX Research, Palo Alto, CA; USA (OPPMANN *et al.* 2000) and provided as a kind gift by Dr. Robert A. Kastelein; **rmIL-12** and **homodimeric p40 ($(p40)_2$)** were expressed and purified at Hoffmann-La Roche (Roche), Nutley, NJ (GATELY *et al.* 1996) and provided as a kind gift by Dr. Maurice Gately. For *in vivo* treatment, cytokines were diluted in PBS containing 1% genotype-specific mouse normal serum.

3.2.5 Media for murine and fungal cell culture

RPMI medium (for splenocyte cultures)

RPMI 1640	1x medium containing glutamine (Mediatech, Herndon, VA, USA)
10 % (v/v)	fetal calf serum (FCS)
1 % (v/v)	Penicillin/Streptomycin (10^4 U/ml, 10 mg/ml)

Sabouraud medium (for growth of *C. neoformans*)

2% glucose (w/v)	
1% peptone	in ddH_2O

Sabouraud solid medium (for growth of *C. neoformans*)

1% Agar Agar	in Sabouraud medium

Cryomedium for *C. neoformans* stock

10 % (w/v) skim milk	in PBS

3.2.6 Buffers and solutions

3.2.6.1 Buffers and solutions for cell culture

PBS

0.80 % (w/v) NaCl
0.02 % (w/v) KCl
0.23 % (w/v) $Na_2HPO_4 \times 2\ H_2O$
0.02 % (w/v) KH_2PO_4 pH = 7.4 in double distilled water (ddH_2O)

PBS/FCS

3% FCS (v/v) in PBS

Trypan blue

0.9 % (w/v) NaCl
0.4 % (w/v) trypan blue

3.2.6.3 Buffers and solutions for ELISA

10x PBS

0.35 % (w/v) KH_2PO4_4
1.58 % (w/v) Na_2HPO_4
8.50 % (w/v) NaCl

Carbonate-buffer

1.73 % (w/v) $NaHCO_3$
0.86 % (w/v) Na_2CO_3 pH = 9.5

Blocking buffer

0.1 % (w/v) gelatine (porcine)
0.5 % (w/v) BSA in 1x PBS

Serum diluent

0.10 % (w/v) gelatine (porcine)
0.05 % (v/v) Tween 20 in 1x PBS

Washing buffer

0.05 % (v/v) Tween 20 in 1x PBS

Substrate-buffer

10 ml McILVAIN´s buffer (96.3 mM Na_2HPO_4, 53.2 mM citric acid)
10 mg ABTS
10 µl H_2O_2 to 10 ml McILVAIN´s buffer

3.2.6.4 Buffers and solutions for nitric oxide determination

Griess reagent

Sol. A: 0.1 % (w/v) naphtyethylendiamide-dihydrochloride
Sol. B: 1.0 % (w/v) sulphanilamide in 5 % concentrated H_3PO_4
Working solution: 1:1 dilution of solution A and B (stable for 12 hrs)

3.2.6.5 Buffers and solutions for SDS-PAGE

Separating gel (10 ml):

3.34 ml 30% acrylamide-bisacrylamide (37:1)
2.5 ml 1.5 M Tris/HCl (pH 8.8)
4 ml ddH_2O
100 µl 10% (w/v) SDS
5 µl TEMED (N, N, N', N'-tetramethylethylendiamine)
50 µl 10% (w/v) APS (ammonium peroxodisulfate)

Stacking gel (5 ml):

1.5 ml 30% acrylamide-bisacrylamide (37:1)
2.5 ml 0.5 M Tris/HCl pH 6.8
5.5 ml ddH_2O
100 µl 10% (w/v) SDS
30 µl TEMED
30 µl 10% (w/v) APS

Running buffer:

50 mM Tris
192 mM glycine
0.1 % (w/v) SDS in ddH_2O

Sample buffer (2x)

60 % glycerol
0.5 M Tris/HCl pH 6.8
10% (w/v) SDS
0.05% (w/v) bromphenol blue

3.2.6.6 Buffers and solutions for Western blot assays

Blotting buffer (pH 9.2)

48 mM Tris/HCl pH 9.2
39 mM glycine
1.3 mM SDS
20% (v/v) Methanol

TBS buffer (pH 7.5)

20 mM Tris/HCl pH 7.6
137 mM sodium chloride

TBS-T buffer (pH 7.5)

TBS Buffer (pH 7.5)
0.1 % Tween 20

Blocking solution

TBS-T buffer containing 3% (w/v) gelatine

3.2.6.7 Buffers and solutions for liquid chromatography

Phosphate buffer 20mM pH 7.2 (with and without NaCl)

0.2 M monobasic stock
13.9 g sodium phosphate monobasic
500 ml dH20

0.2 M dibasic stock
53.65 g sodium phosphate dibasic heptahydrate
1 L dH_20

0.1 M buffer pH 7.2
600 ml dH_2O
84 ml monobasic stock
216 ml dibasic stock

20 mM sodium phosphate buffer pH 7.2
dilute 0.1 M buffer 1:5 in dH_2O

20 mM sodium phosphate buffer pH 7.2 0.5 M NaCl
add 0.5 mol NaCl to 1 L of 20 mM sodium phosphate buffer pH 7.2 for salt gradient

3.2.6.2 Buffers and solutions for flow cytometry

FACS buffer (washing and dilution buffer)

3.0 % (v/v)	FCS	
0.1 % (w/v)	NaN_3	in 1x PBS

Fixation buffer

1.48 % (v/v) formaldehyde in 1x PBS

3.2.7 Kits and ready-made solutions

Cell culture:	
0.02% EDTA solution in DPBS	Sigma-Aldrich, Taufkirchen, Germany
RBC lysis buffer	Sigma-Aldrich, Taufkirchen, Germany
Protein purification:	
SilverQuest	Invitrogen, Carlsbad, CA, USA
BCA Protein Assay Kit	Pierce Biotechnology, Rockford, IL, USA
LUMINEX assay reagents:	
Beadlyte Mouse Multi-Cytokine Detection System 2	Upstate Biotech., Lake Placid, NY, USA
Additional IL-17 and MCP-1 bead sets	Upstate Biotech., Lake Placid, NY, USA
Histology:	
ornithine carbamyl transferase (O.C.T.)	

embedding medium	Miles Scientific, Naperville, IL, USA
Vectastain® Elite® ABC-Kit	Vector Laboratories, Burlingame, CA, USA
mRNA analysis:	
RNA STAT-60™	Iso-Tex Diagn., Friendswood, TX, USA
RNeasy®	Qiagen, Valencia, CA, USA

3.3 METHODS

3.3.1 *In vivo* procedures

3.3.1.1 Infection of mice and monitoring of survival

C. neoformans strain 1841 was cultured in Sabouraud dextrose medium (2% glucose, 1% peptone) in cultures grown overnight at 30°C. Cells were washed twice with sterile PBS and counted in a hematocytometer. Inocula were resuspended in sterile phosphate-buffered saline. Since it was reported previously that injection of 10,000 CFU of *C. neoformans* strain 1841 results in a chronic, eventually lethal infection in C57BL/6 mice (DECKEN *et al.* 1998), this inoculum was used in the present study to assess long-term T cell functions. Therefore, mice were infected with 10,000 CFU of *C. neoformans* strain 1841 resuspended in either 500 µl PBS for intraperitoneal (i.p.) injection or in 100 µl PBS for intravenous (i.v.) application. Both, i.p. and i.v. infection resulted in the development of chronic infection with a similar course of infection (survival period, organ burden).

Infected mice were monitored daily for morbidity and mortality. In accordance to animal welfare guidelines mice were euthanized when moribund.

3.3.1.2 Cytokine injections

Recombinant murine IL-23 (rmIL-23) was expressed and purified at DNAX Research, Palo Alto, CA, USA (OPPMANN *et al.* 2000) and provided as a kind gift by Dr. Robert A. Kastelein. For reconstitution of p40-deficient mice rmIL-23 (2 µg per injection) or PBS containing 1% of genotype specific mouse normal serum was administered i.p. beginning two days before infection, continued daily until day 20 post infection (p.i.) and twice a week thereafter. WT mice were treated with either rmIL-23 (2 µg per injection) or rmIL-12 (kindly provided by Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ) (0.1 µg per injection) or PBS containing 1% of genotype specific mouse normal serum in the same manner. In experiments with an end point at day 8 p.i., p40-deficient, $RAG1^{-/-}$ and $RAG2^{-/-}$ mice were treated with rmIL-23 (2 µg per injection) or PBS intraperitoneally starting two days before infection and continued daily until day 7 p.i. All cytokine injection solutions were prepared with sterile PBS containing 1% mouse normal serum; PBS for control group injections also contained 1%

mouse normal serum. Homologous mouse normal serum was obtained from male mice of the appropriate genotype.

3.3.1.3 Tail-bleeding

At days 21, 35, and 49 p.i. tail vein blood was obtained from WT and IL-23p19$^{-/-}$ mice to assess the immunological status in the serum. Therefore, mice were put in a mouse restrainer and the lateral tail vein was incised with a razor blade. The dripping blood was collected in serum separator tubes, centrifuged at 7,000 g for 5 min and stored at -80°C until further analysis.

3.3.3 Dissection of mice

3.3.3.1 Time points of dissection

Since the present study focused mainly on the cellular immune response, in a series of preparative experiments and time course analyses the optimal time points to assess organ specific inflammation in this model of *C. neoformans* infection were determined (data not shown). Four time points of interest were identified: The inflammatory response in the liver peaked at day 21 p.i.; at day 35 and 49 p.i. a pronounced inflammatory response in the brain was observed. The processes at the site of infection were assessed at day 8 p.i. as cytokine treatment showed effective at this time point. The specific days of section are indicated in the results section.

3.3.3.2 Experimental procedure

Dissections were performed under sterile conditions in a laminar flow hood. Mice were anesthetized with CO_2 according to animal welfare guidelines and fixed on styrofoam-boards. After superficial disinfection the ventral skin was opened in the median by a caudocranial incision from the pelvis to the neck. Following cardiac puncture and peritoneal lavage (PEL), if applicable, organs were removed. In experiments where brain samples were taken for immunohistochemical analysis mice were perfused intracardially through the left ventricle with 0.9 % saline under deep isoflurane anesthesia before organ removal.

3.3.3.2.1 Cardiac puncture

Following CO_2 anesthesia blood was collected via cardiac puncture. With a 23 gauge needle attached to a 1 ml syringe the right ventricle was punctured by inserting the needle cranial of the xiphoid cartilage into the unopened thorax. The blood was transferred into serum separator tubes, centrifuged at 7,000 g for 5 min and stored at -80°C until further analysis.

3.3.3.2.2 Peritoneal lavage

2 ml of ice-cold PBS/EDTA were instilled into the abdomen through a 23 gauge needle inserted in the inguinal region. Peritoneal lavage fluid was recovered by insertion of the needle through the cranial peritoneum. This first PEL fluid was taken for fungal burden determination as well as cytokine and chemokine assessment. A second lavage with a volume of 5 ml PBS/EDTA was performed as described above and pooled with the cells of the first lavage to obtain enough cells for flow cytometry analysis.

3.3.3.2.3 Organ removal

The peritoneal cavity was opened by a median cut from the pelvis to the xiphoid cartilage. Liver and spleen were removed. Then, the skin was opened by a median incision at the back of head and neck. Through the foramen magnum scissors were inserted into the cranial cavity, and after removal of the skullcap the brain was taken out. Organs were weighed and dissected in sterile petri dishes for further processing.

3.3.4 Specimen processing and evaluation

3.3.4.1 Determination of fungal burden in organs and peritoneal lavage

At time points indicated weighed sections of organs were homogenized in 1 ml of sterile PBS using an Ultra-Turrax homogenizer. Serial dilutions of the homogenates and PEL fluids (recovery of first lavage, see above) were plated on Sabouraud dextrose agar plates. Colonies of *C. neoformans* are soft, glistening to dull, smooth, usually mucoid, and creamy to slightly pink or yellowish brown in color and were counted after 48-72 hours of incubation at 30°C. Microscopical analyses showed that on Sabouraud agar, *C. neoformans* produces round, budding yeast cells. No true hyphae were visible.

3.3.4.2 Histopathological analysis of livers

Sections of livers were fixed in 4% buffered formalin. Further processing of samples was performed by Sharon Osborn, DNAX Research, Palo Alto, CA, USA. After embedment in paraffin wax, sections (5 µm) were stained with hematoxylin and eosin (H&E). Mucicarmine staining was performed for detection of cryptococci using muci-10 concentrate (American Master Tech Scientific, Inc, Lodi, CA, USA). Stained sections were evaluated microscopically by Dr. Scott E. Brodie, Schering Plough Corporation, Lafayette, NJ to determine the distribution, character and extent of granulomatous inflammation.

3.3.4.3 Immunohistochemistry of brain sections

Brains were mounted on thick filter paper with Tissue Tek O.T.C. embedding media (Miles Scientific, Naperville, IL, USA), snap-frozen in isopentane (Sigma-Aldrich, Taufkirchen, Germany) precooled on dry ice and stored at -80^{o} C according to a previous report (STENZEL *et al.* 2005). Specimen processing and immunohistochemical analysis were performed by Dr. Werner Stenzel, Institute of Neuropathology, University of Cologne, Germany. 10-µm thick frozen sections were prepared. The following rat anti-mouse monoclonal antibodies were derived from hybridomas obtained from the ATCC (Manassas, VA, USA): CD4 (clone GK.1.5), CD8 (clone 2.43), MHC class II (I-A^{b,d,q} haplotypes, clone M5.114.15.2), and Ly6-G (clone RB6-8C5). Immunohistochemistry was performed using the Vectastain® Elite® ABC-Kit (Vector Laboratories, Burlingame, CA, USA) with biotinylated secondary antibodies. The peroxidase reaction product was visualized using 3,3'-diaminobenzidine (Sigma-Aldrich, Taufkirchen, Germany) as chromogen and H_2O_2 as co-substrate. Consecutive histological sections were stained and evaluated microscopically.

3.3.4.4 Quantitative RT-PCR

Sections of the brain were snap-frozen in liquid nitrogen, transferred to dry ice and stored at -80°C. Sample processing and quantitative PCR was performed by Terrill McClanahan and Wendy M. Blumenschein, DNAX Research, Palo Alto, CA, USA. Total RNA was prepared from frozen brain tissue using RNA STAT-60^{TM} (Iso-Tex Diagnostics, Friendswood, TX, USA) which includes phenol and guanidinium thiocyanate in a mono phase solution. Brain samples were homogenized in the RNA STAT-60^{TM}. Upon addition of chloroform, the homogenate separates into two phases: aqueous phase and organic phase. The total RNA remains exclusively in the aqueous phase while DNA and proteins are extracted into an organic phase and interphase. After isopropanol precipitation, total RNA was reextracted with phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, Taufkirchen, Germany) via phase-lock light tubes (Eppendorf, Hamburg, Germany). For mRNA analysis of PEC the RNeasy kit (Qiagen, Valencia, CA, USA) was used according to the manufacturer's protocol with at least 5 × 10^{6} PEC per sample.

Total RNA (5 µg) was subjected to treatment with DNase (Ambion, Austin, TX, USA) to eliminate genomic DNA and then reverse-transcribed using the reverse transcriptase Superscript II (Invitrogen, Carlsbad, CA, USA) with oligo $(dT)_{15}$ primers (Roche, Nutley, NJ, USA) and random hexamers (Promega, Madison, WI, USA). Cytokine-, chemokine- and cell marker-specific mRNA was measured using the designated primer pairs (Table 3) by real-

time quantitative PCR (Taqman ABI 5700) with SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA). Gene expression levels were normalized to expression of the housekeeping gene ubiquitin for each sample; relative expression levels of infected mice were further normalized to naïve controls and compared between groups as x-fold upregulation.

Gene	Forward primer	Reverse primer
CD3	CCTCCTAGCTGTTGGCACTTG	CCAGGTGCTTATCATGCTTCTG
F4/80	GAGACGTTTGCCCTGAACATG	AGGATCTGAAAAGTTGGCAAAGA
IFN-γ	GGATATCTGGAGGAACTGGCAA	TGATGGCCTGATTGTCTTTCAA
IL-1β	TCGCAGCAGCACATCAACA	ACAGCTTCTCCACAGCCACAAT
IL-5	CTCACCGAGCTCTGTTGACAAG	CCAATGCATAGCTGGTGATTTTTAT
IL-17	ACCGCAATGAAGACCCTGAT	CAGGATCTCTTGCTGGATGAGA
MCP-1	GCTGGAGCATCCACGTGTT	AGCCAGATGCAGTTAACGCCCCACT
ubiquitin	TGGCTATTAATTATTCGGTCTGCAT	GCAAGTGGCTAGAGTGCAGAGTAA

Table 3. Primer pairs used for Taqman RT-PCR analysis.

3.3.4.5 Splenocyte cultures and ex vivo *stimulation*

The characterization of the cytokine/chemokine pattern in the spleen in the course of systemic *C. neoformans* infection provides valuable insights into the immunological status of the host. Two approaches were used herein: (i) T cell functions were assessed by polyclonal stimulation as well as antigen-specific restimulation to characterize the type of Th response; (ii) In mice lacking T cells the activation state of APCs was assessed by stimulation via PRRs either with LPS or cryptococcal antigen.

Therefore, spleens were passed through a 100-µm mesh. The retrieved splenocyte single cell suspension was cleared from red blood cells using RBC lysis buffer according to the manufacturer's instructions, washed with RPMI 1640 cell culture medium, adjusted to 5×10^6 cells per ml, plated in 24-well plates and stimulated after 2 hrs incubation at 37°C in a humidified CO_2 enriched [5%] atmosphere. For polyclonal stimulation, wells were precoated with anti-CD3 (10 µg/ml) and washed before cells were added or Concavalin A (ConA, 5 µg/ml) was added to splenocyte cultures. For antigen-specific restimulation, the weakly virulent acapsular *C. neoformans* strain CAP67, serotype D, was used as it was found to have better restimulatory capacities than the encapsulated strain 1841 (unpublished observation, BIONDO *et al.* 2005). Splenocytes were either stimulated with viable (10^6 cells per ml) or heat-killed (10^7 cells per ml) *C. neoformans* organisms. After 48 hrs incubation at 37°C in a humidified CO_2 enriched atmosphere, supernatants were collected and stored at -20°C until assayed for cytokines and chemokines.

3.3.4.6 Flow cytometry

3.3.4.6.1 Surface stain of PEC

1×10^6 PEC per stained cell marker were transferred to Eppendorf reaction tubes and spun down at 300 × g for 10 min at 4°C. The supernatant was removed and the pellet then resuspended in FACS buffer (3% FCS, 0.1% NaN_3 in PBS) containing 2 µg anti-mouse CD16/CD32 monoclonal antibody (Fc Block™, BD, Franklin Lakes, NJ, USA) in 10 µl buffer per 10^6 cells. After 1 hr incubation at 4°C, 10 µl of cell suspension (containing 10^6 cells) was incubated with fluorophore-conjugated monoclonal antibodies each at 500 ng per 1×10^6 cells (in FACS buffer) in V-bottom plates (BD, Franklin Lakes, NJ, USA). Incubation at 4°C for 15 minutes was followed by washing of cells three times with FACS buffer. The pellet was finally resuspended in fixation buffer.

For each sample an unstained and an unspecific Ig isotype control was added. Single stained samples for each conjugated dye were generated on pooled cells of each experimental group for setting up the flow cytometer.

3.3.4.6.2 Intracellular cytokine staining of splenocytes

For intracellular cytokine staining, splenocytes were prepared and plated as described above and stimulated with plate-bound anti-CD3 plus soluble anti-CD28 (1 µg/ml) in the presence of Golgi-plug (according to BD PharMingen's Cytofix/Cytoperm Plus kit instructions) for the final 4 hrs. This was followed by surface staining with anti-CD4 (FITC-labeled), permeabilization with Cytofix/Cytoperm buffer, and intracellular cytokine staining using anti-IFN-γ (allophycocyanin-conjugated). Cells were analyzed with a FACScalibur flow cytometer. All antibodies and staining buffers were purchased from BD Biosciences, Franklin Lakes, NJ, USA.

3.3.4.7 Cytokines and chemokines in peritoneal lavage fluids, splenocyte culture supernatants and sera

Supernatants of splenocyte cultures, PEL fluids and brain homogenates as well as sera were assayed using LUMINEX xMAP® bead-based multiplexing technology. This technology has developed from the principles of a conventional sandwich ELISA but in contrast presents a possibility to detect multiple parameters simultaneously in one reaction (Fig. 3).

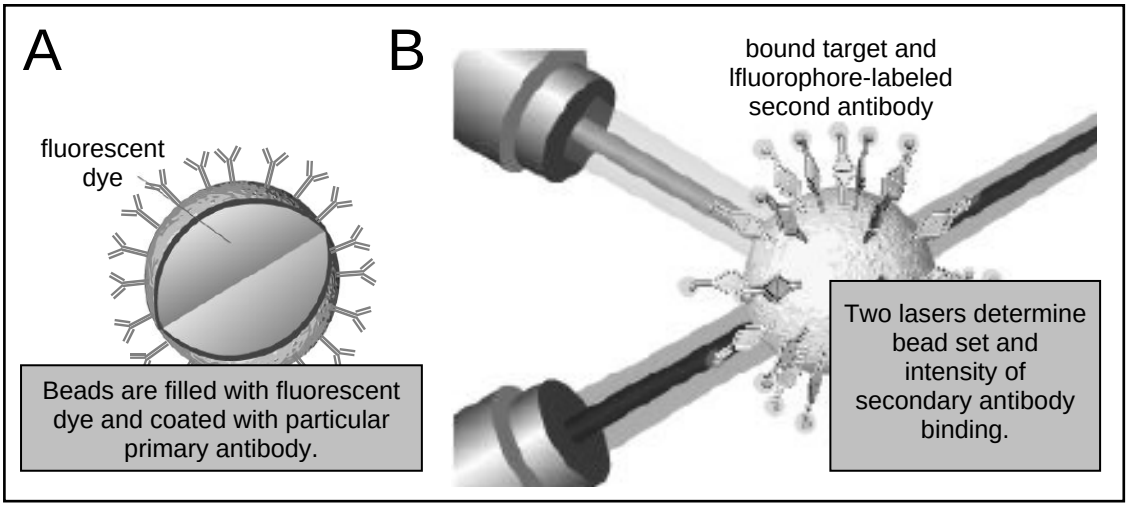


Fig. 3. Principle of LUMINEX xMAP technology. A, primary antibody coated beads are labeled with a fluorescent dye. Each bead-specificity is associated with a certain intensity of the dye and enables characterization of up to 100 different bead sets using a specific flow cytometer, the LUMINEX^{100} instrument. B, fluorescence-based determination of particular bead set and intensity of secondary antibody binding using LUMINEX^{100} instrument.

As a direct result of this ability to multiplex the reactions, LUMINEX xMAP® technology confers a number of advantages over conventional ELISA-based procedures. These include high throughput screening and reduced reagent consumption and sample usage. The latter factor is of importance particularly when working with murine specimens as naturally only small sample amounts can be obtained from one animal. The use of a 12-plex reaction in the present study led to an 18-fold reduction of the amount of sample needed when compared to conventional ELISA thereby opening the possibility to screen for a broad range of target analytes in critical samples like sera or brain homogenate supernatants.

In the present study the Beadlyte Mouse Multi-Cytokine Detection System 2 was used for detection of IL-1β, IL-2, IL-4, IL-5, IL-6, TNF-α, IFN-γ and customized with additional bead sets for IL-17 and MCP-1 detection. Following the manufacturer's recommendations (Upstate Biotechnology, Lake Placid, NY, USA), the protocol was modified for the use of the high biotin-containing RPMI 1640 medium for analyses of splenocyte culture supernatants. When serum samples or supernatants of organ homogenates were assessed, the protocol was modified for the analysis of sera according to the manufacturer's recommendations. In detail, 50 µl of the diluted standards or samples as well as culture media or serum diluent as a negative control were added to each well with the addition of the cytokine capture antibody beads. After two hours incubation, plates were subjected to a vacuum manifold to control for high biotin in the medium. Reporter solution was added and incubated for 1.5 hrs. Diluted streptavidin-phycoerythrin solution was added to each well, and plates were incubated for 30

min. After stopping the reaction, plates were subjected to vacuum and after addition of assay buffer, read on the Luminex^{100} machine, which was programmed with specific bead-signature numbers representing the cytokines assayed. The instrument was set to read a minimum of 50 events per bead set, and the median value was obtained for each reaction to minimize the effect of any outliers.

3.3.4.8 Determination of IgE in sera

The IgE concentration was determined by sandwich ELISA following a standard protocol using the capture antibody R32-72 (BD Pharmingen) and a biotinylated anti-mouse-IgE antibody (BD Pharmingen; R35-118) for detection after incubation with peroxidase-labeled streptavidin (Southern Biotech Assoc., Birmingham, AL, USA) and ABTS as substrate.

3.3.4.9 Quantification of nitric oxide in sera

NO in oxygen-containing solutions is chemically unstable and undergoes rapid oxidation to nitrite (NO_2^-). The presence of various biological tissue components catalyzes this oxidation and promotes further oxidation of NO_2^- to nitrate (NO_3^-). Therefore, it is necessary to measure both NO_2^- and NO_3^- to accurately determine the level of total NO. NO_3^- in sera was first reduced to NO_2^- by incubation the samples for 30 min with nitrate reductase (0.25 units/ml, Roche, Nutley, NJ, USA) in the presence of 100 µM NADPH (Roche, Nutley, NJ, USA). The concentrations of NO_2^- can be determined by Griess reagent reaction as described by STONE *et al.* (STONE *et al.* 2006). Therefore, the samples were incubated with a freshly prepared 1:1 working solution of Griess solution A and B for 10 minutes in the dark at room temperature. The plate was then read at 570 nm wavelength on a plate reader (Molecular Devices, Sunnyvale, CA, USA).

3.3.5 Protein purification of p40-homodimer

In collaboration with Dr. Samiya Al-Robaiy, BBZ, Institute of Immunology, University of Leipzig, and Karin vanderHeijden-Liefkens, Intervet, Boxmeer, Netherlands, murine p40 was expressed in Sf9 cells by infection with baculovirus carrying a p40-encoding plasmid. Sf9 is an insect cell line derived from *Spodoptera frugiperda* ovarian cells and is a natural host for baculovirus infection. This eukaryotic cell expression system was superior to prokaryotic systems (e.g. *Escherichia coli* expression) since eukaryotic expression allows for the glycolsylation of proteins which in the case of p40 is a prerequisite for its biological activity. Physiologically murine p40 is expressed in homodimeric (disulfide-bonded) as well monomeric form. Therefore, in the present work, homodimeric p40 had to be purified from

non-specific insect cell-derived proteins as well as monomeric p40 contamination using methods described below.

3.3.5.1 SDS-PAGE

By sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) proteins from a complex protein mixture are resolved by their molecular weight and can be visualized with different staining techniques (see below). Non-reducing SDS-PAGE was performed with 10% polyacrylamide mini gels (11 cm × 7 cm × 0.1 cm) according to Laemmli (LAEMMLI 1970). Protein samples were mixed with sample buffer in a 1:2 dilution and heated for 10 min at 85°C. The mini gel runs were performed at 127 V for the stacking gel and 200 V for the separating gel. Subsequently either silver staining was performed to depict the total proteome or Western blotting to visualize p40-specific proteins.

3.3.5.2 Assessment of total protein content

3.3.5.2.1 Quantification of total protein content

Quantification of total protein content was achieved by using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's microwell plate protocol (detection limit: 5 µg/ml). Absorbance at 560 nm was measured on a plate reader (Molecular Devices, Sunnyvale, CA, USA).

3.3.5.2.2 Qualitative analysis of total protein content by silver stain

Silver staining is a very sensitive method for the in-gel detection of proteins. With the herein used SilverQuest staining kit (Invitrogen, Carlsbad, CA, USA) less than 10 ng of protein can be detected, whereas for other common stains like Coomassie staining the detection limit is about 50 times higher. Silver staining of SDS-gels was performed according to the manufacturer's recommendations. Briefly, the SDS-gel was incubated in fixing solution for 60 min. Then the gel was washed with 30% ethanol for 10 min and treated with the sensitizing solution provided in the kit for another 10 min. After washing with ultra-pure water, the staining occurred for 15 minutes in the silver-nitrate-containing staining-solution. Gels were washed for 45 seconds with ultra-pure water and incubated in the developing solution. When bands appeared in the desired intensity the procedure was stopped by addition of the stopper solution. After briefly washing with ultra-pure water, gels were scanned on a flat bed scanner (HP, Palo Alto, CA, USA).

3.3.5.3 Detection of murine p40

3.3.5.3.1 Quantification of murine p40

Quantitative determination of murine p40 in Sf9 cell culture supernatants and further preparations was performed by sandwich ELISA according to a standard protocol using a matching pair of mAb consisting of the mAb 5C3 (25 µg/ml) as capture and biotinylated polyclonal goat anti-mouse IL-12 (8 µg/ml) as detection Ab (both Abs were provided by Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ, USA) combined with streptavidin-horseradish-peroxidase (1:3000; Southern Biotechnology Associates), with recombinant mouse IL-12 and $(p40)_2$ (provided by Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ, USA) as a standard (detection limit: 80 pg/ml).

3.3.5.3.2 Qualitative analysis of p40-proteins

Distinguishing monomeric and homodimeric murine p40 was achieved by p40-specific Western blot. After running the samples over non-reducing 10% polyacrylamide gels proteins were transferred onto nitrocellulose membranes (MembraPure, Bodenheim, Germany). Therefore, the membrane and two sheets of Whatman paper were incubated in blotting buffer for 10 min. Thereafter, all components were assembled as follows:

Cathode −

Whatman paper
SDS gel
Nitrocellulose membrane
Whatman paper

Anode +

The gel was blotted onto the nitrocellulose membrane with 100V (60 min). Subsequently, membranes were briefly washed with TBS-T pH 10 and blocked for 1 hr with 3% gelatine in TBS-T pH 7.5. After another washing with TBS-T pH 10 membranes were incubated in biotinylated goat anti-mouse IL-12 mAb (4 µg/ml TBS-T pH 7.5 containing 1% gelatine) for 1 hr. After three washes in TBS-T pH 10 membranes were incubated for 1 hr in horseradish peroxidase-conjugated streptavidin (diluted 1:100,000 in TBS-T buffer). Washing was repeated six times. Development was performed using the SuperSignal® WestPico enhanced chemiluminescence (ECL) kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's recommendations. Membranes were incubated for 1 min on Hyperfilm™-ECL (Amersham Biosciences, Buckinghamshire, UK) or CL-Xposure film (Pierce Biotechnology, Rockford, IL, USA) in an x-ray cartridge. After exposure films were

developed using the wet laser imager (Scopix LR 5200, AGFA, Morstel, Belgium) of the radiology division in the Department of Small Animal Medicine, Faculty of Veterinary Medicine, University of Leipzig and scanned on a flat bed scanner (HP, Palo Alto, CA, USA).

3.3.5.1 Liquid chromatography

By liquid chromatography the murine p40-containing Sf9 cell culture supernatant was run through a solid phase and the eluent, murine p40, was retarded by the matrix. From the several liquid chromatography techniques applicable, in the present study ion-exchange chromatography (IEC) was performed since this method was reported previously for the purification of murine $(p40)_2$ expressed in stably transfected CHO cells (GATELY *et al.* 1996). By IEC proteins are separated by their surface charge which depends on the pH of the surrounding medium. The basic principle of IEC is illustrated in Fig. 4. In the present work a cation exchange chromatography was performed at pH 7.2 since murine p40 is positively charged at such pH. A linear salt gradient was used to displace bound protein from the column matrix dependent on the strength of binding.

A 1-ml column with a CM Sepharose FF matrix (Amersham Biosciences, Buckinghamshire, UK) was used on the ÄKTAprime liquid chromatography system (Amersham Biosciences, Buckinghamshire, UK). 15 ml of Sf9 supernatant containing a total of 90 µg/ml mouse p40 as determined by ELISA was loaded on the column. After washing with 5 volumes of 20 mM sodium phosphate buffer pH 7.2 the column was eluted with a 40 ml linear salt gradient from 0 mM to 500 mM NaCl in 20 mM sodium phosphate buffer pH 7.2. The eluate was collected in fractions of 1 ml and analyzed by SDS-PAGE followed by silver staining and Western blot analysis.

3.3.5.4 Biological activity

Since homodimeric p40 has been shown to be an antagonist for IL-12 at the IL-12 receptor (GILLESSEN *et al.* 1995; MATTNER *et al.* 1993), biological activity of the purified $(p40)_2$ was assessed *in vitro* by measuring its inhibitory effect on IL-12-induced production of IFN-γ. Splenocytes from WT mice (5×10^6 cells/ml) were incubated with $(p40)_2$ in different concentrations ranging from 0.1 ng/ml to 100 ng/ml for 12 hrs and then stimulated with IL-12 (1 ng/ml) for another 48 hrs. The cell supernatants were analyzed for IFN-γ by ELISA.

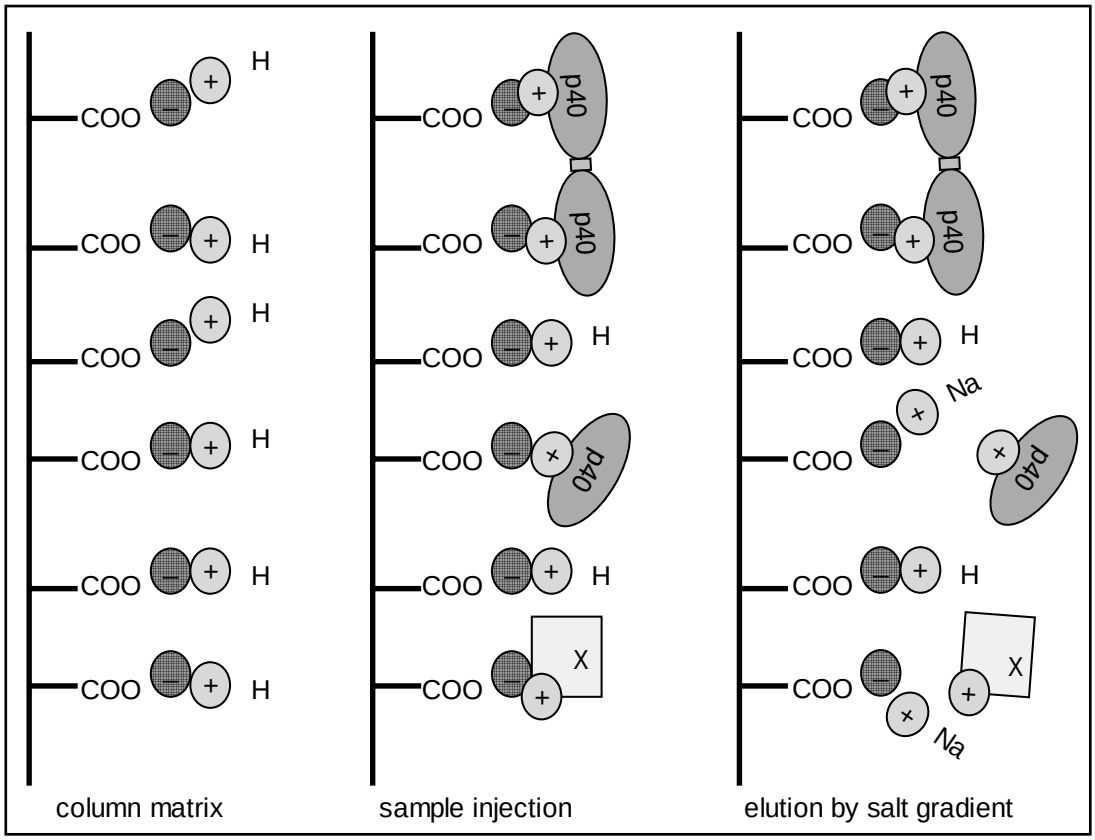


Fig. 4. Principle of ion exchange chromatography (IEC). Depicted is the principle of a cation exchange column as used for the purification of p40 in the present work. The column matrix has a negative charge thereby binding positively charged proteins such as homodimeric and monomeric p40 as well as irrelevant positively charged proteins (X) in the injected sample. The amount of positive charges determines the strength of binding to the matrix. By usage of a linear salt gradient, proteins are eluted in a certain time course depending on the strength of their binding.

3.3.6 Statistical analysis

Mann-Whitney-Rank sum test was used for comparisons of two groups. When comparing three or more groups, Kruskal-Wallis statistics followed by Dunns post test was performed. Survival proportions were displayed using the Kaplan-Meier method; statistical differences were assessed by the Log Rank test. Median survival times were compared using a paired Student's *t*-test. In either case, GraphPad PRISM software was used (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined to be based on a *P* value less than 0.05.

4 RESULTS

4.1. CONTINUATION OF PREVIOUS STUDIES

In a previous report it was shown that p40-deficient mice (which lack all p40-dependent IL-12 family members: IL-12, IL-23, p40, $(p40)_2$) show a higher susceptibility to infection with *C. neoformans* than IL-12p35$^{-/-}$ mice (which lack only IL-12) (DECKEN *et al.* 1998). At this time point the existence of IL-23 was not known so that the authors suggested monomeric or homodimeric p40 to be the responsible cytokine for the observed difference. Reconstitution of p40-deficient mice with either factor, however, could not restore resistance to *C. neoformans* infection. This led the authors to hypothesize the existence of another p40-dependent cytokine (DECKEN *et al.* 1998). The discovery of IL-23 in the year 2000 suggested a candidate molecule to test this hypothesis (OPPMANN *et al.* 2000).

4.1.1 Reconstitution of *C. neoformans*-infected p40-deficient mice with recombinant murine IL-23

To address the question if the elevated susceptibility in p40-deficient mice to *C. neoformans* infection reported by DECKEN *et. al*. was due to the lack of IL-23, WT, IL-12p35$^{-/-}$ and p40-deficient mice were infected with the *C. neoformans* serotype D strain 1841 as used in the previous study (DECKEN *et al.* 1998). The lack of IL-12 (IL-12p35$^{-/-}$ mice) was associated with a significantly reduced survival time (Fig. 5) consistent with a previous report (DECKEN *et al.* 1998). In addition, the combined lack of IL-12 and IL-23 (p40-deficient mice) resulted in an even shorter survival time (Fig. 5). Recombinant murine IL-23 (rmIL-23) or PBS was administered to p40-deficient mice beginning two days before infection, continued daily until day 20 post infection and twice a week thereafter. The administration of rmIL-23 resulted in a significantly prolonged survival period of p40-deficient mice as compared to their PBS-treated littermates (Fig. 5; $P<0.001$). IL-23 treated p40-deficient mice reached survival periods comparable to those of IL-12p35$^{-/-}$ mice (Fig. 5). Besides these effects on survival, rmIL-23 treatment resulted in a slightly reduced fungal load in liver and brain of p40-deficient mice at day 8 p.i. with *C. neoformans* (data not shown).

These findings demonstrate a protective effect of recombinant IL-23 on the outcome of infection in IL-12/IL-23-deficient mice, strongly suggesting that IL-23 is the responsible molecule for the higher resistance of IL-12p35$^{-/-}$ mice compared to p40-deficient mice.

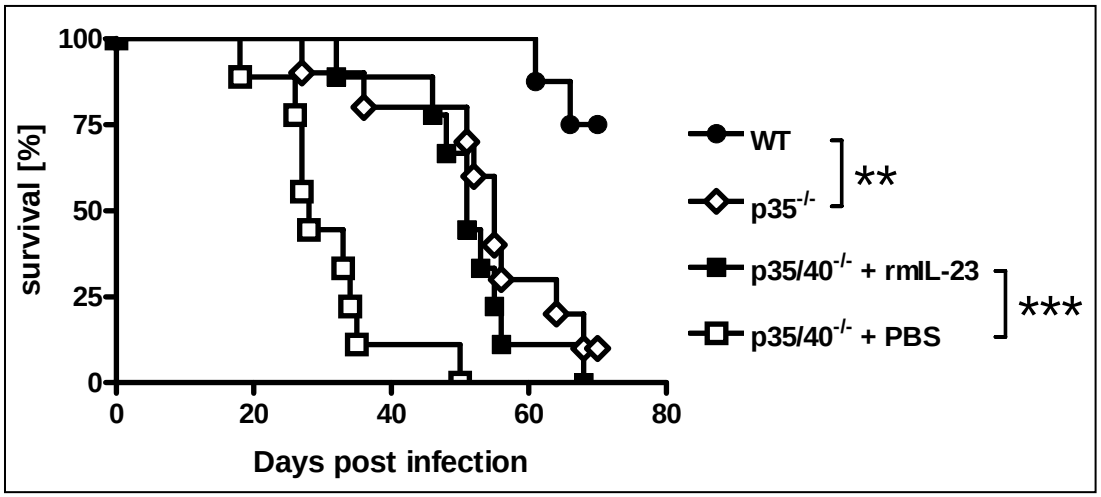


Fig. 5. IL-23 administration prolongs survival after *C. neoformans* infection in the absence of IL-12. The p40-deficient mice were treated with either rmIL-23 or PBS throughout the observation period of the experiment beginning two days before infection with 10,000 CFU of *C. neoformans*. Survival of WT, IL-12p35$^{-/-}$ and reconstituted as well as mock-treated p40-deficient mice (n = 8-10 mice) was monitored. Kaplan-Meier projections of survival curves were compared by log rank test. ***, $P<0.001$. The experiment shown is representative of two independently performed experiments. Another IL-23 reconstitution experiment using p40$^{-/-}$ mice showed similar results.

4.2 THE ROLE OF ENDOGENOUS IL-23 IN EXPERIMENTAL CRYPTOCOCCOSIS

The findings reported in the previous section indicate, that in mice lacking IL-12 the additional lack of IL-23 leads to reduced resistance to *C. neoformans* infection pointing to a role for IL-23 in immunity to *C. neoformans*. Further investigations focused on defining the particular contribution of endogenous IL-23 to immunity in cryptococcosis. Therefore, a series of *in vivo* experiments was performed in IL-23p19$^{-/-}$ mice, which specifically lack IL-23 but are still able to produce other p40-dependent cytokines, such as IL-12. The parameters which were assessed focused on (i) changes in resistance to *C. neoformans* infection, (ii) the type of immune response established in the course of *C. neoformans* infection, and (iii) the local inflammatory response at infected sites in mice lacking endogenous IL-23.

4.2.1 Infection of IL-23p19$^{-/-}$ mice with *C. neoformans*: mortality and fungal burden

To address the question if the specific lack of IL-23 but an unchanged ability to produce IL-12 renders mice more susceptible to *C. neoformans* infection, IL-23p19$^{-/-}$ were infected with *C. neoformans*. A major parameter to assess changes in resistance to infection of a specific mouse strain is the survival time. Therefore, in five independently performed

experiments survival was monitored. Comparing median survival periods of IL-23p19$^{-/-}$ and WT mice obtained in all five experiments, IL-23p19$^{-/-}$ mice showed a significantly reduced median survival time (P<0.05, Fig. 6 A). Statistics performed on each of the five experiments individually revealed a significantly reduced survival time of IL-23p19$^{-/-}$ mice in two experiments (P<0.05), and a trend for reduced survival times in IL-23p19$^{-/-}$ mice in three experiments, including the one shown (Fig. 6 B).

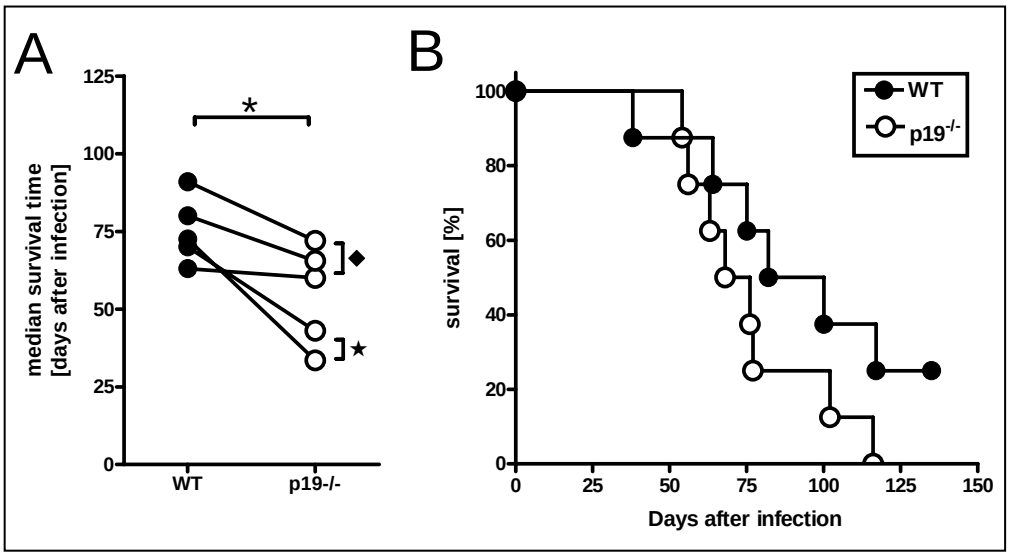


Fig. 6. Reduced survival of IL-23p19$^{-/-}$ mice following *C. neoformans* infection. IL-23p19$^{-/-}$ mice and WT controls were infected with *C. neoformans*. Survival was monitored through the course of infection. (A) Median survival times of WT and IL-23p19$^{-/-}$ mice in 5 independent experiments. Lines connect associated data points for either experiment. Statistics performed individually on each of the five experiments show a significantly reduced survival time of IL-23p19$^{-/-}$ in two experiments (★, P<0.05), the other three experiments (◆) including the depicted one (B) showed a trend for reduced survival times in IL-23p19$^{-/-}$ mice. *, P<0.05, n = 8-10 mice for each experiment. Median survival times were compared using paired t-test, survival curves were compared by Log Rank test as described in *Materials and Methods*.

The reduction of the survival period observed in IL-23p19$^{-/-}$ mice was accompanied by a significant difference in cryptococcal clearance. At day 21 after infection, both IL-23p19$^{-/-}$ and WT mice had a comparable fungal burden in liver. However, on day 35 post infection, only WT mice showed a reduction of liver burden, whereas the fungal load in livers of IL-23p19$^{-/-}$ mice was similar to organ burden observed at day 21 p.i. (P<0.05, Fig. 7 A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of *C. neoformans* organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 7

B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown).

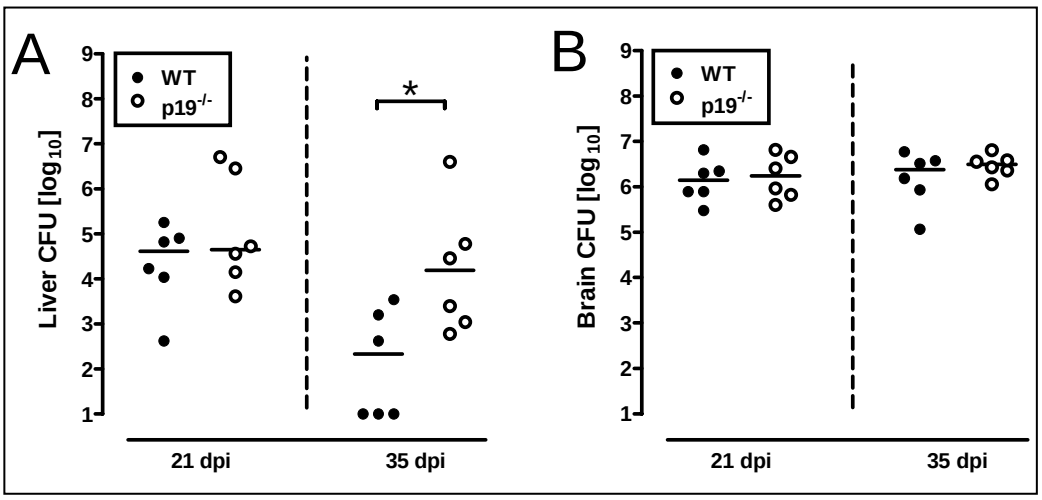


Fig. 7. Delayed cryptococcal clearance in the liver of IL-23p19$^{-/-}$ mice. WT and IL-23p19$^{-/-}$ mice were infected with *C. neoformans* and sacrificed at day 21 and day 35 p.i. Colony forming units (CFU) in liver (A) and brain (B) were determined at both time points (bars indicate median). Mann-Whitney rank sum test was used for comparison of the two groups at either time point. *, $P<0.05$. Each data point represents one individual mouse. The data shown is pooled data from two independent experiments (n = 3 mice/experiment).

These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to *C. neoformans*, particularly at later time points.

4.2.2 Cytokine pattern in sera and restimulated splenocytes of infected IL-23p19$^{-/-}$ mice

Subsequent experiments aimed on exploring the basis for the increased susceptibility of IL-23p19$^{-/-}$ mice to *C. neoformans* infection. Protection in this infection model is shown to be strongly dependent on cellular immune mechanisms, underlining the importance of Th1 promoting IL-12 effects (CASADEVALL and PERFECT 1998; NICHOLS *et al.* 1991). With IFN-γ as a marker cytokine for a Th1 response and IgE, the IL-4 dependent immunoglobulin class, as a marker for a Th2 response the Th1/Th2 balance was assessed in infected mice. Following infection with *C. neoformans*, IL-23p19$^{-/-}$ mice showed similar serum levels of IgE compared with WT controls at day 21 and 49 p.i. (Fig. 8 A). Furthermore, both IL-23p19$^{-/-}$ and WT mice had comparable amounts of circulating IFN-γ (Fig. 8 B). In contrast, p40-deficient mice that additionally lack IL-12 show a clear Th2 shift by elevated serum IgE levels ($P<0.05$) and no detectable circulating IFN-γ (Fig. 8 A and B).

In addition, when T cell functions were assessed at day 21 p.i. by restimulation of splenocytes of *C. neoformans*-infected mice with either a polyclonal stimulus (anti-CD3) or

antigen-specific stimuli such as viable (v*Cn*) and heat-killed (hk*Cn*) *C. neoformans* organisms, WT and IL-23p19$^{-/-}$ responded similarly with pronounced IFN-γ production. In contrast, IFN-γ expression in p40-deficient mice was significantly reduced after antigen-specific stimulation (Fig. 8 C). In agreement with these findings, IL-4 levels were significantly higher in infected p40-deficient mice than in WT and IL-23p19$^{-/-}$ which showed similar IL-4 expression after polyclonal stimulation (Fig. 8 C).

To specifically analyze Th cells in infected WT, IL-23p19$^{-/-}$ and p40-deficient mice, intracellular FACS staining of CD4^{+} T cells was performed for IFN-γ after anti-CD3/anti-CD28 activation of splenocytes. The percentage of IFN-γ^{+} CD4^{+} T cells was comparable between WT and IL-23p19$^{-/-}$ mice, whereas p40-deficient mice showed a reduced frequency of CD4^{+} IFN-γ producers (Fig. 8 D). As IL-23 was shown to promote a distinct IL-17 producing T cell subset (AGGARWAL *et al.* 2003; LANGRISH *et al.* 2005), the ability of splenocytes of infected WT, IL-23p19$^{-/-}$ and p40-deficient mice to produce IL-17 in response to polyclonal and antigen-specific stimulation was assessed. WT mice showed a pronounced IL-17 response after either stimulus. IL-17 production in IL-23p19$^{-/-}$ and p40- deficient splenocytes, however, was impaired when stimulated polyclonally and not detectable after antigen-specific restimulation with both viable *C. neoformans* (v*Cn*) and heat-killed *C. neoformans (*hk*Cn)* at day 21 (Fig. 8 C).

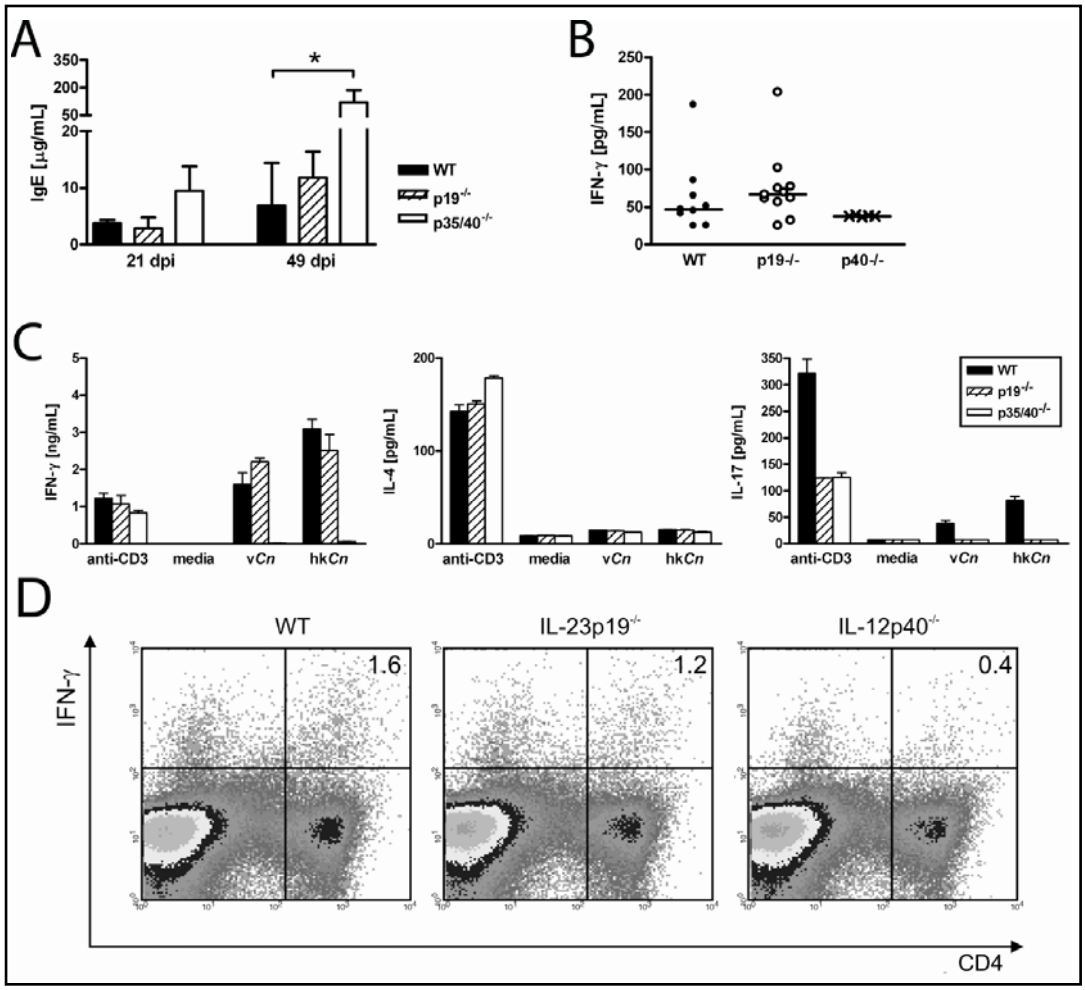


Fig. 8. Unaltered Th1/Th2 balance but impaired production of IL-17 in IL-23p19$^{-/-}$. WT, IL-23p19$^{-/-}$ and p40-deficient mice were infected with *C. neoformans* and sacrificed or tail-bled at indicated time points. (A and B) Serum analysis for IgE (n=3-4 mice) were performed at days 21 and 49 p.i. (A) and for IFN-γ at day 35 p.i. Each data point represents one animal. (B). *C*, At day 21 p.i. pooled splenocytes of either genotype were plated at 5×10^6 and stimulated in triplicates with anti CD3 (wells precoated with 5 μg/ml), 10^6 viable (v*Cn*) or 10^7 heat-killed (hk*Cn*) *C. neoformans* organisms for 48 hrs. Culture supernatants were assessed for IFN-γ, IL-4 and IL-17 by LUMINEX multiplexing protein assay. *D*, At day 21 p.i. splenocytes of WT, IL-23p19$^{-/-}$ and p40-deficient mice were co-stained for surface CD4 and intracellular IFN-γ after anti-CD3/anti-CD28 stimulation. Each dot represents the acquired signal of one single cell. Numbers in FACS blots indicate percentage of IFN-γ producers of all CD4$^+$ lymphocytes. ANOVA was performed using Kruskal-Wallis statistics followed by Dunn's post test. *, P<0.05. Experiments shown are representative of at least two independently performed experiments.

At day 35 p.i. antigen specific induction of IL-17 was even more pronounced in WT mice, but still absent in mice lacking IL-23 (IL-23p19$^{-/-}$ and p40-deficient mice) (data not shown). The data demonstrate that the lack of IL-23 does not alter the Th1/Th2 balance in *C. neoformans* infection but leads to a significantly impaired IL-17 production.

4.2.3 Granuloma formation in the livers of infected IL-23p19$^{-/-}$ mice

Protective immunity in *C. neoformans* infection is characterized by mononuclear cell recruitment to the site of infection leading to the formation of granulomata in most tissues (CASADEVALL and PERFECT 1998; MITCHELL and PERFECT 1995). Previously an impaired ability of p40-deficient mice to form granulomata when compared with IL-12p35$^{-/-}$ mice was reported (DECKEN *et al.* 1998). Since both genotypes of mice used in those studies lack IL-12, the following analyses focused on the individual contribution of IL-23 to the granulomatous response in cryptococcal infection in the presence of IL-12. Therefore, granuloma formation in the liver was compared between infected IL-23p19$^{-/-}$ and WT mice by light microscopy.

As described above, liver burdens differed between the genotypes at day 35 p.i. but not at day 21 p.i. (see Fig. 7). For this reason the number of granulomata was normalized to the liver fungal burden by calculating the ratio between the mean number of granulomata per 100x field and the colony forming units (CFU) in the liver for each animal. On day 21 after infection with *C. neoformans* no difference between WT and IL-23p19$^{-/-}$ mice could be found, whereas at day 35 p.i. WT mice had a significantly elevated normalized number of liver granulomata as compared with IL-23p19$^{-/-}$ mice (Fig 9). No significant differences were seen in terms of granuloma size or cellular composition in livers of infected WT and IL-23p19$^{-/-}$ mice (data not shown).

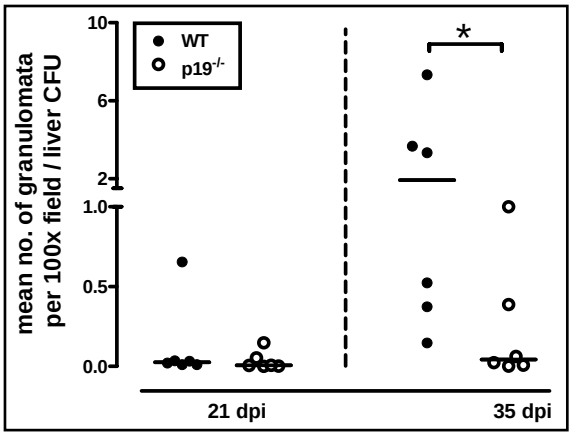


Fig. 9. Defective granuloma formation in the absence of IL-23. WT and IL-23p19$^{-/-}$ mice were infected with *C. neoformans* and the granulomatous response was quantified microscopically. Mean number of granulomata per 100× field was determined. To compensate for differences in organ burden the ratio of the number of granulomata and the organ burden in the liver was calculated for each animal as represented by depicted data points. *, $P<0.05$. Data shown is pooled data of two independently performed experiments (n = 3 mice per experiment).

These findings provide evidence for a role of IL-23 in granuloma formation even in the presence of IL-12.

4.2.4 Inflammatory response in brains of infected IL-23p19$^{-/-}$ mice

C. neoformans shows a strong tropism for the CNS, causing a sustained meningoencephalitis which subsequently leads to death (CASADEVALL and PERFECT 1998; MITCHELL and PERFECT 1995). Therefore, it was of particular interest to investigate the inflammatory processes in the brain. Histopathological examination of brain sections at day 49 p.i. revealed a pronounced infiltration of the brain by *C. neoformans* organisms in both WT and IL-23p19$^{-/-}$ mice. Cryptococcal foci were associated with leukocyte infiltrations in both genotypes (Fig. 10 A and B). Interestingly, H&E stains of brain sections revealed a diminished inflammatory response in the IL-23p19$^{-/-}$ mice in four individually performed experiments. To verify this observation, the quality and composition of inflammatory cells was characterized by immunohistochemistry and the immunostained cell populations were quantified. Immunostaining for the activation induced cell surface molecule MHC-II revealed that in WT mice *C. neoformans* foci were surrounded by MHC-II-positive macrophages and abundantly activated MHC-II-positive microglia cells, whereas in the IL-23p19$^{-/-}$ mice macrophages were significantly less frequent and activation of microglial cells was markedly reduced (Fig. 10 C, D and E). Differences in T cell recruitment were also obvious: IL-23p19$^{-/-}$ showed a reduction in CD4^{+} (Th cells) and CD8^{+} (CTL) T cells compared with WT mice (Fig. 10 F-K). Granulocytic leukocyte infiltration of the brain was greater in infected WT versus IL-23p19$^{-/-}$ mice when sections were evaluated using Gr1 staining (Fig. 10 L-N).

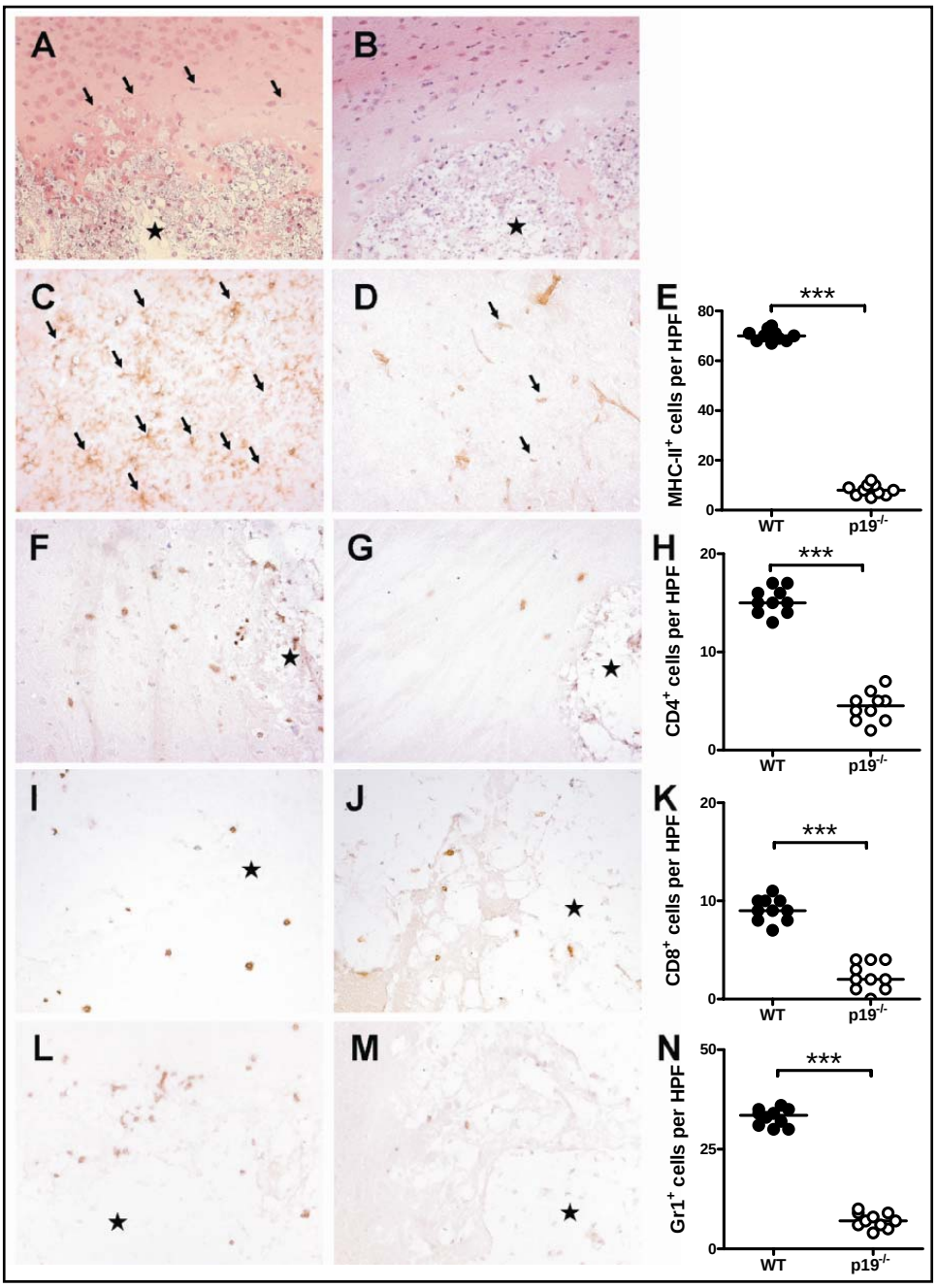


Fig. 10. Impaired activation of microglia and reduced cellular infiltration in brain tissue of infected IL-23p19$^{-/-}$ mice. Representative brain sections of either WT (A, C, F, I, L) or IL-23p19$^{-/-}$ (B, D, G, J, M) mice from day 49 p.i. with *C. neoformans* are shown to illustrate the granulomatous response. Stars indicate the location of cryptococcal foci. (A and B) H&E staining. In the brain parenchyma cryptococcal foci infiltrated by inflammatory cells were found in both WT (A) and IL-23p19$^{-/-}$ mice (B). Surrounding microglia cells were only visible in WT animals (arrows). (C and D) Anti-MHC-II immunostaining. Abundant MHC-II^{+} macrophages/microglia were found in the surroundings of cryptococcal foci in WT (C) and to a much lesser extend in IL-23p19$^{-/-}$ (D) mice (arrows indicate microglial cells). (F and G) Anti-CD4 immunostaining. In WT mice abundant CD4^{+} T cells were detected in the center and vicinity of cryptococcal foci (F) whereas in IL-23p19$^{-/-}$ mice only few CD4^{+} T cells were found (G). (I and J) Anti-CD8 immunostaining. CD8^{+} T cells were found in the

center and at the border of the cryptococcal foci in larger numbers in infected WT (I) than in IL-23p19$^{-/-}$ mice (J). (L and M) Anti-Gr1 immunostaining. In WT mice many Gr1^{+} granulocytes were found in the center and at the border of cryptococcal foci (L) whereas in IL-23p19$^{-/-}$ mice very few Gr1^{+} granulocytes were detected (M). (E, H, K and N), Diagrams show cell counts and statistical analysis referring to 10 evaluated high power fields (HPF) (0.16 mm^2) in sections of the respective genotype. Sections shown are representative of four to six mice per group from one experiment. In four more individually performed experiments, H&E stained brain sections revealed a diminished inflammatory response. Original magnification, ×50.

The results from the immunohistochemical analyses were supported by cell marker mRNA analyses of the brains. The levels of transcripts for the mononuclear cell markers F4/80 (mainly found on macrophages) and CD3 (part of the T cell receptor) were reduced approximately 5-fold in brains from IL-23p19$^{-/-}$ mice as compared to brains from WT mice at day 21 p.i. (Fig. 11).

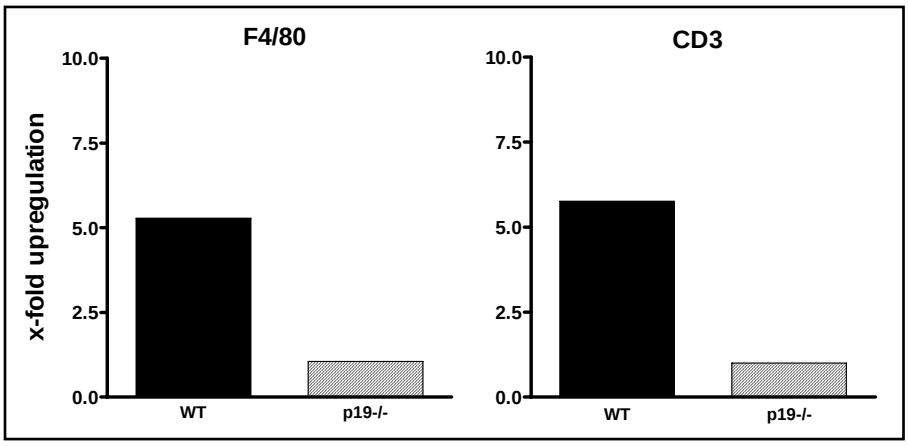


Fig. 11. Reduced expression of mRNA for mononuclear cell markers in brain tissue of infected IL-23p19$^{-/-}$ mice. WT and IL-23p19$^{-/-}$ mice were infected with *C. neoformans* and sacrificed at day 21 p.i. Expression of brain mRNA for F4/80 and CD3 was assessed by quantitative RT-PCR and normalized to ubiquitin expression. X-fold up-regulation is shown as a ratio between infected and naïve mice (pooled samples of 5 individual mice) of either genotype. Experiment shown is representative of two independently performed experiments.

To characterize the functional differences in the brain inflammatory response of both genotypes, the mRNA expression profile of cytokines and chemokines in the brain of infected mice was analyzed.

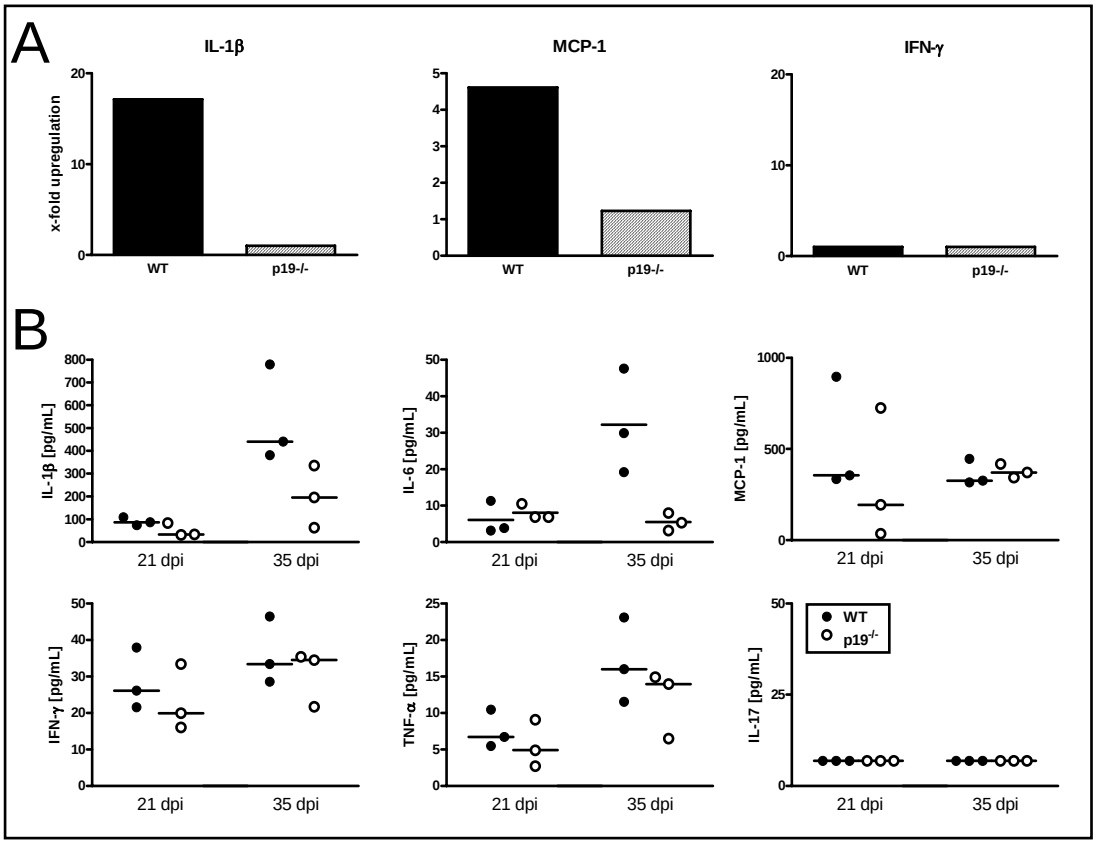


Fig. 12. Reduced expression of proinflammatory cytokines and chemokines in brain tissue of infected IL-23p19$^{-/-}$ mice. WT and IL-23p19$^{-/-}$ mice were infected with *C. neoformans*. (A) Total mRNA of the brain was assessed for cytokine and chemokine expression at day 21 p.i. by quantitative RT-PCR. X-fold up-regulation normalized to ubiquitin is shown as a ratio between infected and naïve mice of either genotype (pooled samples of three individual mice). (B) Supernatants from brain homogenates were assessed for protein levels of IL-1β, IL-6, TNF-α, MCP-1, IFN-γ and IL-17 by LUMINEX multiplexing protein assay. Each data point represents one individual mouse. Experiments shown are representative of two independently performed experiments (n = 3 mice per experiment).

At day 21 p.i., WT, but not IL-23p19$^{-/-}$ mice, showed increased expression of mRNA for the proinflammatory cytokine IL-1β and the mononuclear cell chemoattractant MCP-1. At this time point, however, the proinflammatory cytokines TNF-α, IL-6 and IFN-γ as well as the nitric oxide synthesizing enzyme iNOS were not expressed at appreciable levels (Fig. 12 A and data not shown). Similarly, elevated levels of MCP-1 protein were seen in brain homogenates of WT mice at day 21 p.i. Moreover, elevated levels of IL-1β and IL-6 protein were present in brain homogenates of WT, but not of IL-23p19$^{-/-}$ mice (Fig. 12 B).

Remarkably such changes were more prominent at day 35 p.i. than on day 21 (Fig. 12 B). Although in models of autoimmunity IL-23p19$^{-/-}$ mice showed a markedly impaired production of TNF-α (CUA *et al.* 2003; MURPHY *et al.* 2003), in the brain homogenates of *C. neoformans*-infected mice this defect was not that obvious. There was no difference in IFN-γ levels when comparing the two genotypes and IL-17 was not detectable (Fig. 12 B).

The data depicted in Fig. 12 are representative of a trend seen in 2 independent experiments but does not reach statistical significance. Nevertheless these data suggest an important mechanism for the significantly reduced cellular infiltration in brains of infected IL-23p19$^{-/-}$ mice (described in Fig. 10) and points to a role for MCP-1, IL-1β, and IL-6 in IL-23-dependent inflammation.

Taken together, these findings demonstrate that the absence of IL-23 is associated with an impaired inflammatory response in the brains of mice infected with *C. neoformans*, mainly characterized by reduced recruitment and activation of mononuclear cells.

4.3 EFFECTS OF EXOGENOUS IL-23 IN EXPERIMENTAL CRYPTOCOCCOSIS

Infection studies in IL-23p19$^{-/-}$ mice described in the previous section revealed a contribution of endogenous IL-23 to resistance in *C. neoformans* infection by enhancing the cellular immune response and inducing a distinct cytokine pattern. In this section the above described proinflammatory activities of IL-23 are further explored by a distinct approach: Recombinant murine IL-23 was administered to *C. neoformans*-infected mice. This approach not only presents a possibility to further investigate the IL-23-induced mechanisms, but also to define a potential therapeutic activity of IL-23 treatment in chronic fungal infection.

For all experiments in this section mice were infected intraperitoneally. The intraperitoneal infection led to a disease course similar to the disease course observed following intravenous infection (as stated in section 3.3.1.1), but opens the possibility for mechanistical analysis at the site of infection, the peritoneal cavity. In a first set of experiments the effect of exogenous IL-23 on the outcome of infection in WT mice was characterized. The main questions addressed were: (i) Does exogenous IL-23 improve resistance as assessed by monitoring survival period and determining fungal burden at infected sites? (ii) Which type of Th response is associated with effects mediated by IL-23 treatment? Subsequently, analyses were performed in p40-deficient mice to address the IL-23 effects specifically, and independent of the IL-12 pathway, and in T and B cell-deficient mice to investigate the effect of IL-23 on innate immunity. Parameters of interest in either immunodeficient mouse strain were (i)

fungal burden at the site of infection, (ii) cytokine and chemokine profile induced by *C. neoformans* and (iii) composition of inflammatory cell infiltrations at the site of infection.

4.3.1 Administration of IL-23 to *C. neoformans*-infected WT mice

To address the question if exogenous IL-23 mediates protective effects in cryptococcosis, WT mice were infected with *C. neoformans* and treated with IL-23, IL-12 (positive control) or PBS (negative control) starting two days prior to infection, continued daily until day 20 p.i. and twice a week thereafter.

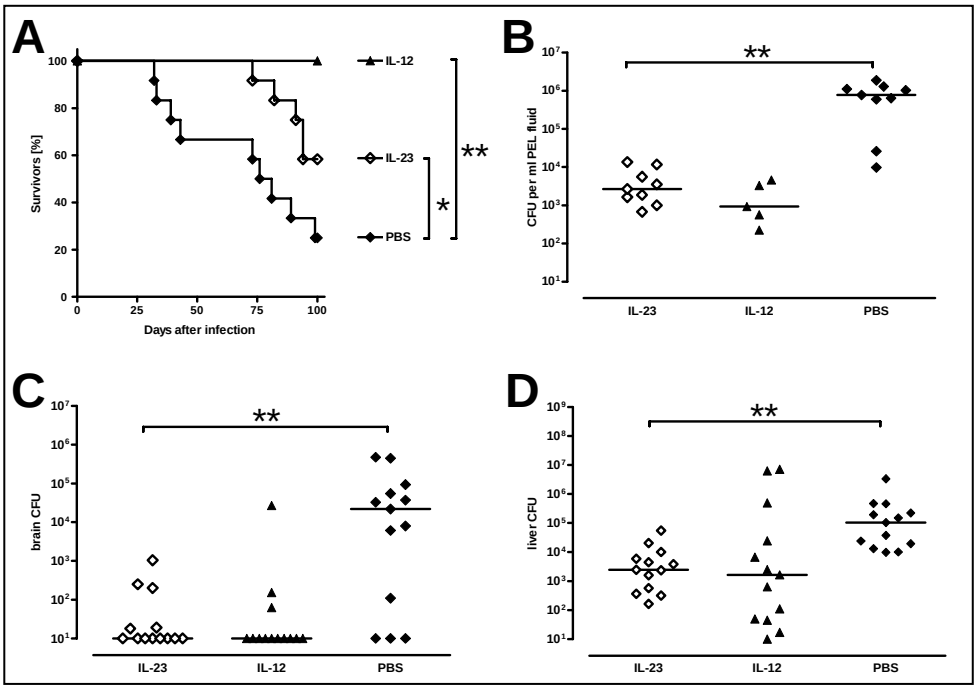


Fig. 13. Exogenous IL-23 enhances resistance to *C. neoformans*. *C. neoformans*-infected WT mice were treated with IL-23, IL-12 or PBS. (A) Mice were monitored for morbidity and mortality. (B) At day 8 p.i. the fungal burden in the peritoneal cavity was assessed. (C, D) At day 21 p.i. mice were sacrificed. Fungal burden of brain (C) and liver (D) were determined. Each data point represents one mouse. Data shown are representative (A, B) or cumulative data (C, D) of two individually performed experiments (n = 5-8 mice per experiment). Bars in scatter plots indicate median. **, $P<0.01$; *, $P<0.05$.

At day 100 p.i. 78% of mock-treated mice had died whereas, consistent with a previous report (DECKEN *et al.* 1998), IL-12 treatment resulted in 100% survival during the complete observation period (Fig. 13 A). The administration of IL-23 led to significantly prolonged survival periods compared to the PBS treatment; however 40% of IL-23-treated WT mice died up to day 100 p.i. (Fig. 13 A). Both, IL-23 and IL-12 treatment resulted in reduced fungal burden at the site of infection, the peritoneal cavity, at day 8 p.i. (Fig. 13 B), whereas

fungal burden in livers and brains was comparable between all three treatment groups at this time point (data not shown). At day 21 p.i. however, cryptococcal burden in brain and liver was significantly reduced in IL-23-treated WT mice compared to the mock-treated controls (Fig 13 C and D).

Analysis of sera at day 21 p.i. revealed a pronounced production of the Th1 associated cytokine IFN-γ as well as TNF-α and the effector molecule nitric oxide (NO) following IL-12 treatment, whereas serum levels of these factors were below detection limit following either IL-23 or PBS treatment (Fig. 14 A-C). In contrast, a significant production of the Th2-associated immunoglobulin class IgE was found in the IL-23- and the PBS-treated group at comparable levels but not in the IL-12-treated group (Fig. 14 D). This Th2 shift after IL-23 or PBS treatment, as well as the Th1 cytokine expression following IL-12 treatment was evident already at day 8 p.i. (data not shown). IL-17 was not detectable in sera at either time point (data not shown).

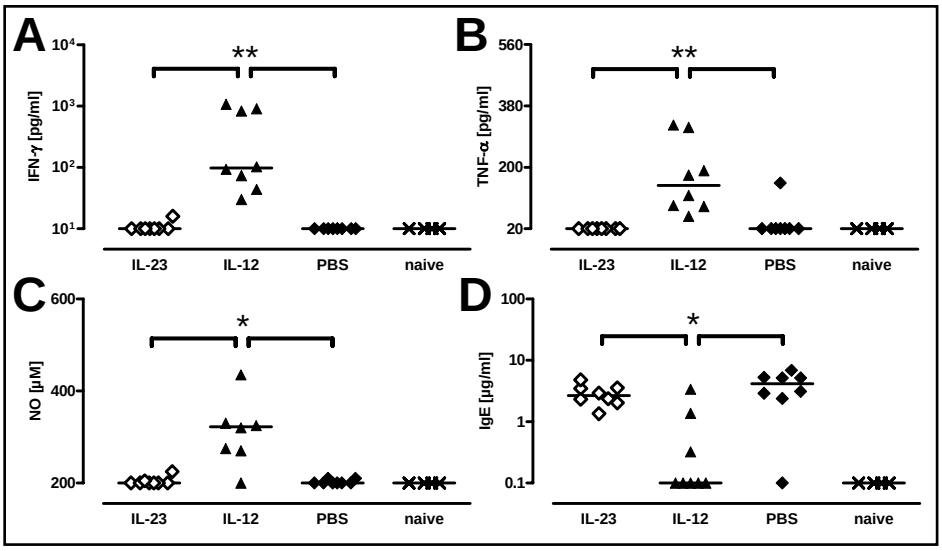


Fig. 14. Exogenous IL-23 does not alter the Th1/Th2 balance in *C. neoformans* infection. *C. neoformans*-infected WT mice were treated with IL-23, IL-12 or PBS. At day 21 p.i. sera were assessed for cytokines, nitric oxide and IgE. Each data point represents one animal. Experiment shown is representative of two individually performed experiments (n = 5-8 mice). Bars in scatter plots indicate median. **, $P<0.01$; *, $P<0.05$.

Taken together, administration of either IL-12 or IL-23 was associated with enhanced resistance to *C. neoformans* infection as determined by prolonged survival rates and reduced fungal burden early at the site of infection (day 8 p.i.) as well as later (day 21 p.i.) in infected organs. The importance of IL-12 in immunity to intracellular infection and its association with the establishment and maintenance of a Th1 response clearly showed. In contrast, IL-23-

treated mice despite their elevated resistance failed to mount a protective Th1 response but show a default Th2 pattern.

4.3.2 Administration of IL-23 to *C. neoformans*-infected p40-deficient mice

4.3.2.1 Fungal burden at site of infection and cytokine profile

To investigate the mechanisms by which exogenous IL-23 mediates its protective effects subsequent experiments explored the processes that led to the observed IL-23- induced reduction of fungal burden in the peritoneal cavity. To see whether the protective action of administered IL-23 depends on IL-12, studies were performed in p40-deficient mice that lack all p40-dependent IL-12 family members including IL-23 and IL-12.

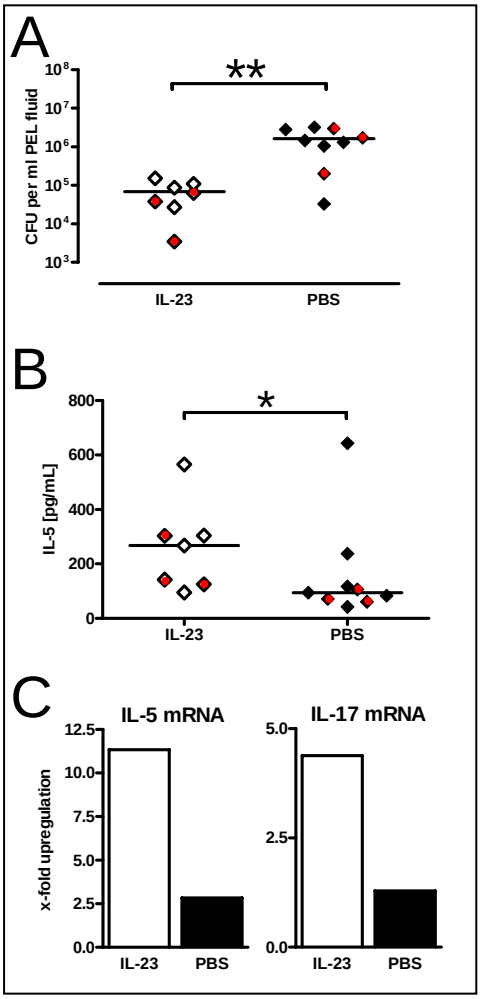


Fig. 15. Exogenous IL-23 leads to reduced fungal burden in *C. neoformans*-infected p40-deficient mice and enhanced expression of IL-5 and IL-17 at the site of infection. p40-deficient mice were infected intraperitoneally with *C. neoformans* and treated with either IL-23- or PBS. (A) Fungal load in the PEL fluid was determined at day 8 p.i. (B) Supernatants of PEL fluids from IL-23- or mock-treated p40-deficient mice were analyzed for cytokines at day 8 p.i. IL-5 was detected by LUMINEX multiplexing protein assay. (C) PEC of three mice per treatment group (red-labeled data points in panel A and B) were pooled and total mRNA was analyzed for cytokine expression by quantitative RT-PCR at day 8 p.i. X-fold upregulation normalized to ubiquitin is shown. Data shown are cumulative data of two individually performed experiments for either genotype (n = 3-5). Bars in scatter plots indicate median. **; $P<0.01$; *$P<0.05$.

Mice were intraperitoneally infected with *C. neoformans* and treated with either IL-23 or PBS. At day 8 p.i. IL-23-treated p40-deficient mice showed a reduction of fungal burden at the site of infection, the peritoneal cavity (Fig. 15 A). Surprisingly, IL-23 treatment resulted in elevated protein levels of the commonly Th2-associated cytokine IL-5 in PEL fluids (Fig. 15 B) as well as up-regulation of mRNA for IL-5 and IL-17 in PEC (Fig. 15 C). IL-17

protein was not detectable in the PEL fluids (data not shown).Protein levels of IFN-γ, TNF-α, IL-6, IL-1β, IL-2, IL-4 and MCP-1 in PEL fluids and mRNA levels of those factors in PEC did not differ between IL-23 and PBS treatment groups (data not shown).

According to the elevated mRNA levels, the cryptococcal antigen specific recall response in splenocyte cultures was characterized by an elevated protein expression of IL-17 and IL-5 in the IL-23 treated group (Fig. 16). Moreover, elevated levels of the proinflammatory cytokines IL-1β, TNF-α, IL-6 and IL-2 showed in the IL-23-treated group after stimulation with either viable (v*Cn*) or heat-killed cryptococci (hk*Cn*). In case of IL-17 and IL-5 the difference in production level between treatment groups was even more pronounced when cells were stimulated with a polyclonal stimulus (ConA) (Fig. 16).

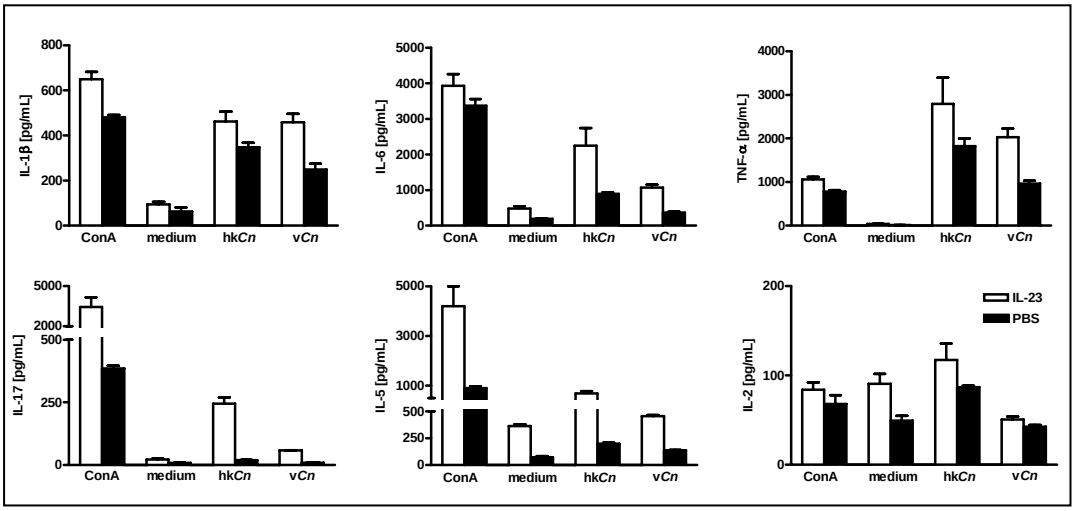


Fig. 16. Enhanced production of proinflammatory cytokines and IL-5 in restimulated splenocytes of IL-23-treated p40-deficient mice infected with *C. neoformans*. At day 8 p.i. pooled splenocytes from IL-23- or mock-treated p40-deficient mice (n = 5 mice) were plated and stimulated in triplicates with ConA (5 µg/ml), 10^6 viable (v*Cn*) or 10^7 heat-killed (hk*Cn*) *C. neoformans* organisms per ml. Culture supernatants were assessed for IL-1β, IL-6, TNF-α, IL-17, IL-5 and IL-2 by LUMINEX multiplexing protein assay and results are shown as mean and SEM. Experiment shown is representative of two individually performed experiments.

The data indicate that the IL-23 treatment can lead to a reduction of fungal burden at the site of infection independent of IL-12. This effect was associated with an elevated local production of IL-17 and IL-5. A pronounced expression of both cytokines was also seen in stimulated splenocyte cultures. In addition, splenocytes of IL-23-treated mice showed a pronounced expression of other proinflammatory cytokines and chemokines.

4.3.2.2 Cellular immune response at the site of infection

The magnitude and composition of the cellular immune response determine the degree of protection in *C. neoformans* infection (CASADEVALL and PERFECT 1998; MITCHELL and PERFECT 1995; NICHOLS *et al.* 2002). In the previous section mice lacking endogenous IL-23 were shown to develop an impaired cellular response to *C. neoformans* infection. To assess the effect of exogenous IL-23 on cellular composition of the inflammatory cell infiltrations at infected sites, PEC were taken from IL-23- and mock-treated p40-deficient mice 8 days after intraperitoneal infection with *C. neoformans*. Total cell counts were comparable between both treatment groups (Fig. 17 A). Cellular composition of PEC was analyzed by flowcytometry. A comparison of PEC from both treatment groups by size and granularity did not reveal apparent differences (Fig. 17 B). When gating on lymphocytes (red gate in Fig. 17) it turned out that IL-23 treatment resulted in a significantly higher frequency of TCR-β^+ cells. Interestingly, the same trend was seen for NK1.1$^+$ cells (Fig. 17 C). The percentage of CD11c$^+$ cells (mainly DCs) of all viable PEC (blue gate in Fig. 17) was significantly higher in the IL-23-treated group (Fig. 17 D) whereas F4/80$^+$ (mainly macrophages) and Gr1$^+$ (mainly granulocytes) cells did not differ in frequency (data not shown).

These findings underline the implication of endogenous IL-23 in cellular immunity by demonstrating an IL-23-induced enhancement of T cell and DC frequency at the site of infection. These effects are mediated independently of IL-12 or other p40 cytokines.

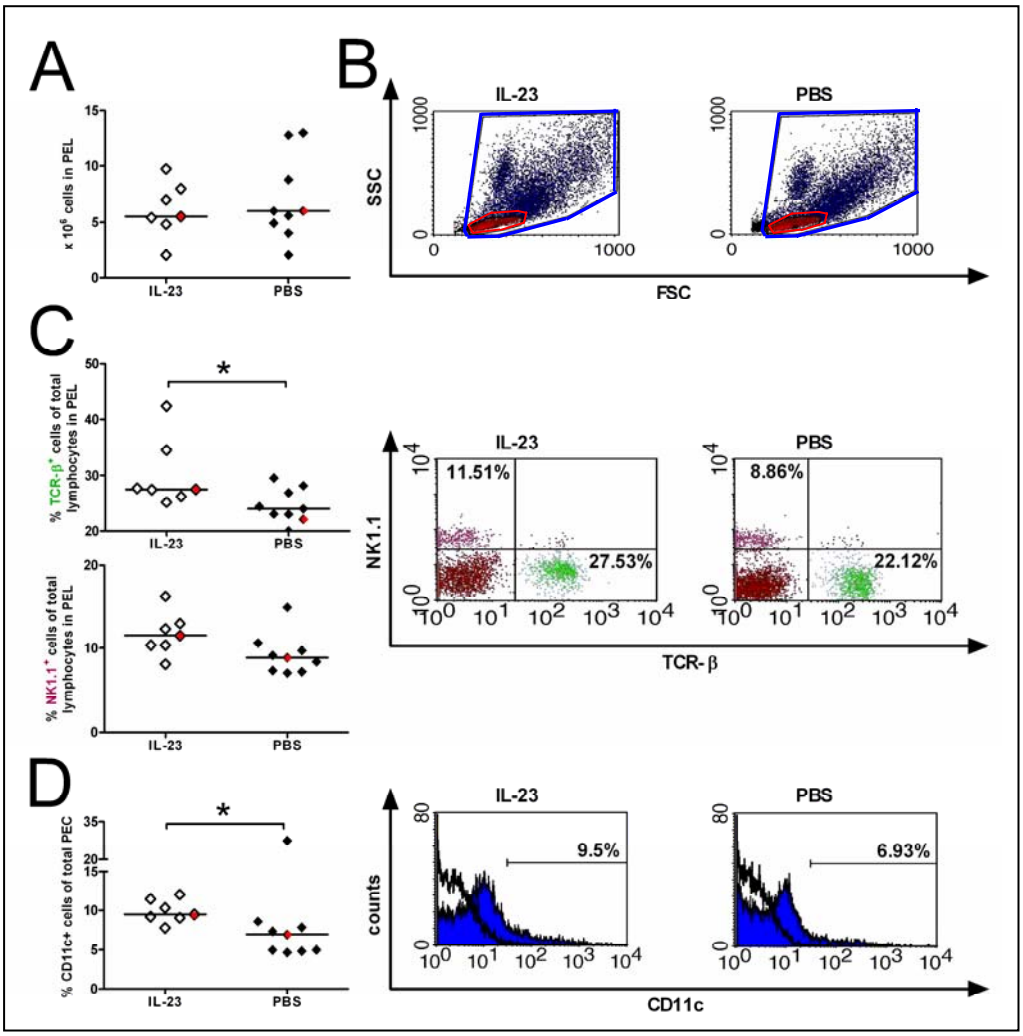


Fig. 17. Exogenous IL-23 augments infiltration of T cells and DCs to the site of infection in p40-deficient mice infected with *C. neoformans*. PEL fluids were obtained from IL-23- or mock-treated p40-deficient mice at day 8 after intraperitoneal infection with *C. neoformans*. (A) Total number of cells in PEL fluid was assessed microscopically. (B-F) Cellular composition of PEL fluids was determined by flow cytometry (FACS). (B) Representative FACS plots showing PEC of p40-deficient mice after IL-23 or mock treatment by size (forward scatter, FSC) and granularity (side scatter, SSC), each dot represents the signal of one cell. (C) Frequency of TCR-β^+ and NK1.1$^+$ cells in PEC is shown as percentage of all lymphoid cells (red gate in panel B). Representative FACS dot plots show percentage of NK1.1$^+$ (upper left quadrant) and TCR-β^+ (lower right quadrant) cells. (D) Frequency of CD11c$^+$ cells is shown as percentage of all viable PEC (blue gate in panel B). Representative FACS histogram plots show isotype control (open curve) and cells stained for CD11c (filled curve). Bars indicate analysis region for CD11c$^+$ cells. Scatter plots show cumulative data of two individually performed experiments (n = 3-5 mice in each experiment). Representative FACS plots show representative data of one mouse per treatment group and refer to the data points labeled red in the scatter plots. *, $P<0.05$.

4.3.3 Administration of rmIL-23 to *C. neoformans*-infected mice lacking T and B cells

4.3.3.1 Fungal burden at site of infection and cytokine/chemokine profile

The innate immune system is the first line of defense against invading pathogens. In cryptococcosis innate immunity plays an important role in containing the infection (MITCHELL and PERFECT 1995). Previous experiments showed that IL-23 enhances the inflammatory response independent of the major Th1 cytokine IL-12, but associated with a pronounced antigen specific production of IL-17. To address the question if IL-23 actions completely rely on such T cell dependent mechanisms, it was of interest to investigate also the effects of IL-23 on innate immunity. Therefore, RAG-deficient mice ($RAG1^{-/-}$ or $RAG2^{-/-}$) that lack T and B cells were infected with *C. neoformans* intraperitoneally. At day 8 p.i. IL-23-treated RAG-deficient mice had a significantly reduced fungal burden at the site of infection (Fig. 18).

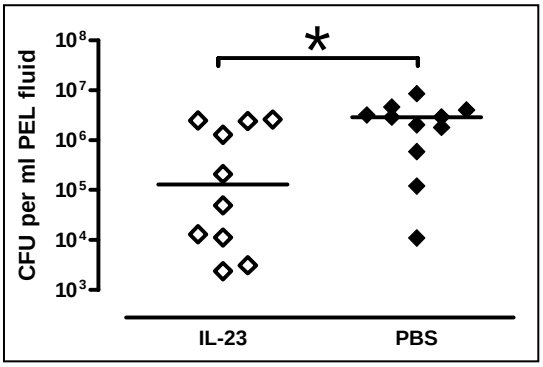


Fig. 18. Exogenous IL-23 leads to reduced fungal burden at the site of infection in T and B cell deficient mice infected with *C. neoformans*. RAG-deficient mice were infected intraperitoneally with *C. neoformans*, treated with either IL-23 or PBS and sacrificed at day 8 p.i. Fungal load in PEL fluids was determined. Data shown are cumulative data of two individually performed experiments. Bars indicate median. n = 5-6 mice per experiment. *, $P<0.05$.

Moreover, expression of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α as well as IL-5 and the chemokine MCP-1 were strongly enhanced after IL-23 treatment, in contrast to IFN-γ which was not elevated in either group (Fig. 19). Paralleling these findings, an *ex vivo* analysis of the activation of innate immune cells in the spleen revealed a pronounced expression of IL-1β, IL-6 and TNF-α, IL-5, MCP-1 and IL-17 in IL-23-treated RAG-deficient mice (Fig. 20).

These data indicates that the reduction of fungal burden at the site of infection by IL-23 treatment is independent of T and B cells. In addition, the elevated cytokine and chemokine expression at the site of infection as well as in stimulated splenocyte cultures does not rely on the means of adaptive immunity.

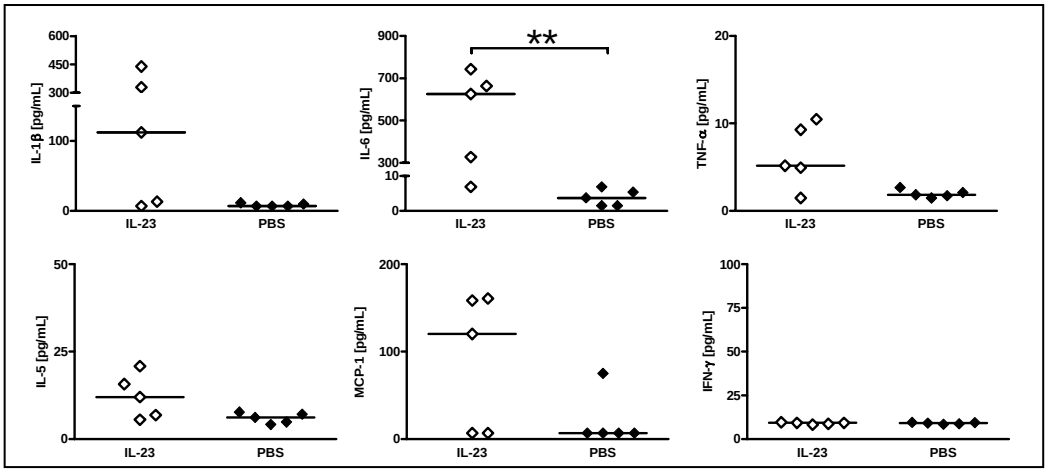


Fig. 19. Exogenous IL-23 enhances cytokine and chemokine production at the site of infection in T and B cell deficient mice infected with *C. neoformans*. RAG2$^{-/-}$ mice were infected intraperitoneally with *C. neoformans*, treated with either IL-23 or PBS and sacrificed at day 8 p.i. Supernatants of PEL fluids were assessed for IL-1β, IL-6, TNF-α, IL-5, MCP-1 and IFN-γ by LUMINEX multiplexing protein assay. Experiment shown is representative of two individually performed experiments. n = 5-6 mice. Bars indicate median. **, P<0.01.

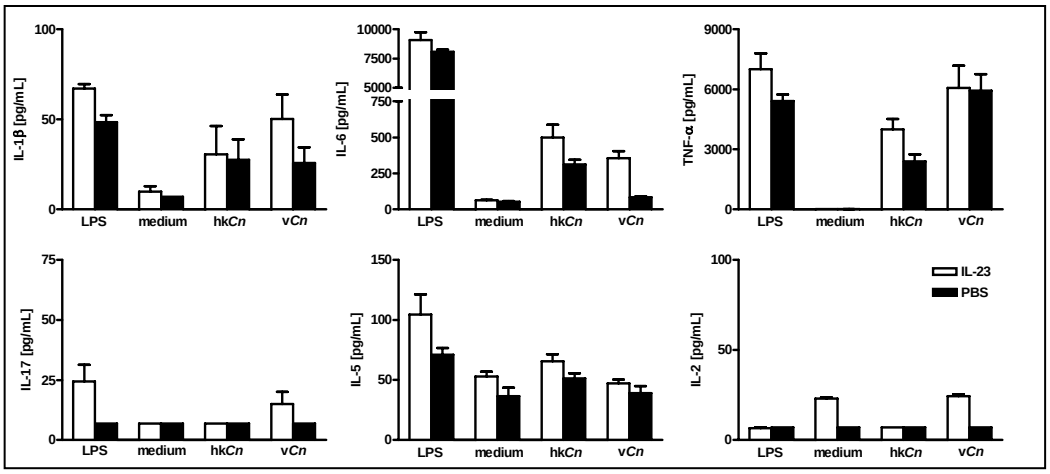


Fig. 20. Exogenous IL-23 enhances cytokine production in restimulated splenocytes from *C. neoformans*-infected mice lacking T and B cells. At day 8 p.i. splenocytes from IL-23- or mock-treated RAG1$^{-/-}$ mice (n = 5) were pooled, plated and stimulated in triplicates for 48 hr with LPS (5 µg/ml), 10^6 viable (v*Cn*) or 10^7 heat-killed (hk*Cn*) *C. neoformans* organisms per ml. Culture supernatants were assessed for IL-1β, IL-6, TNF-α, IL-17, IL-5 and IL-2 by LUMINEX multiplexing protein assay and results are shown as mean and SEM. Experiment shown is representative of two individually performed experiments.

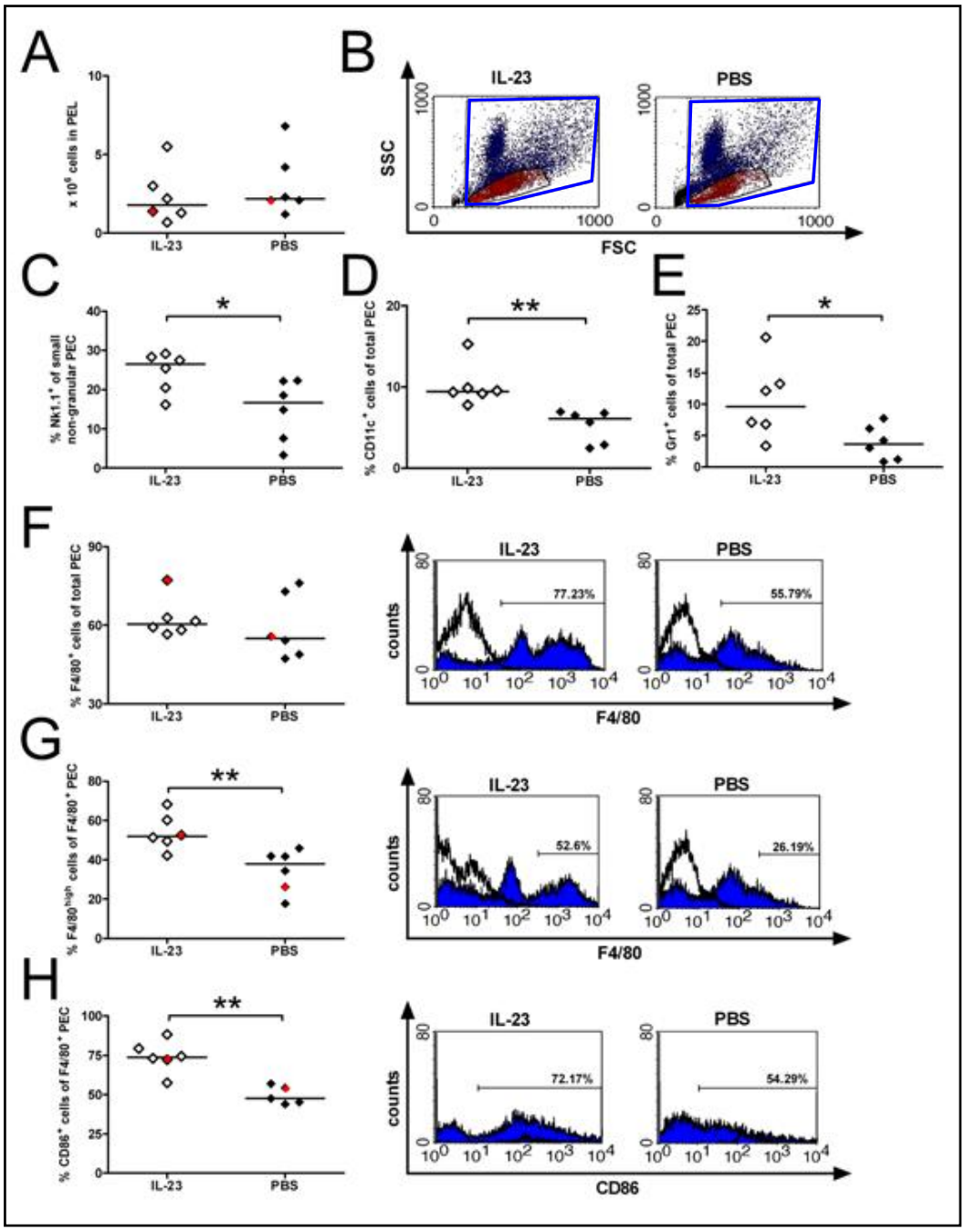


Fig. 21. Exogenous IL-23 augments inflammatory cells infiltration to site of infection and enhances activation of F4/80$^+$ PEC in mice lacking T and B cells. PEL fluids were obtained from *C. neoformans*-infected IL-23- or mock-treated RAG2$^{-/-}$ mice at day 8 p.i. (A) Total number of cells in PEL fluid was assessed microscopically. (B-H) Cellular composition of PEC was determined by flow cytometry. (B) Representative FACS plots of PEC by size (FSC) and granularity (SSC). Frequency of NK1.1$^+$ (C) is shown as percentage of small sized non-granular cells (red gate in panel B). Although RAG-deficient mice are lacking mature T and B lymphocytes, besides NK cells this gate contains leukocyte precursors, *C. neoformans* organisms and less defined leukocyte subpopulations. Moreover, the gate overlaps with DC, granulocyte and macrophage containing regions. CD11c$^+$ (D), Gr1$^+$ (E) and F4/80$^+$ (F) cells is shown as percentage of all viable PEC (blue gate in panel B). (G) Rate of F4/80^{high} cells is shown as percentage of total F4/80$^+$ PEC. (H) F4/80$^+$ cells were gated (indicated regions in

histogram plots in panel F) and frequency of $CD86^+$ $F4/80^+$ cells was determined. Representative FACS plots refer to the data points labeled red in scatter plots. Histogram plots show isotype control (open curve) and cells stained for indicated cell surface antigens (filled curves); regions of positively stained cells are indicated by bars. Experiment shown is representative of two individually performed experiments (n = 6 mice). **, $P<0.01$; *, $P<0.05$.

4.3.3.2 Cellular immune response at the site of infection

In a next step the cellular basis for the local IL-23-dependent antifungal effect and the associated enhanced cytokine and chemokine expression was explored. Therefore, PEC of IL-23- or mock-treated RAG-deficient mice were analyzed at day 8 p.i. Total cell counts were no different between both treatment groups (Fig. 21 A). Moreover, analysis for size and granularity of PEC in both treatment groups showed no difference (Fig. 21 B). Staining for a number of cell type specific surface antigens, however, revealed a higher percentage of $NK1.1^+$ (mainly NK cells, Fig. 21 C), $CD11c^+$ (mainly DCs, Fig. 21 D) and $Gr1^+$ (mainly granulocytes, Fig. 21 E) cells when gated on viable PEC. The frequency of $F4/80^+$ cells (mainly macrophages) was comparable in both treatment groups (Fig. 21 F), however, further analysis of these cells revealed morphological differences: A higher frequency of $F4/80^+$ cells expressed F4/80 at a high level (Fig. 22 G) and, in addition, the expression of the co-stimulatory molecule CD86 by $F4/80^+$ cells was significantly higher following IL-23 treatment (Fig. 21 H).

These findings confirm the effect of IL-23 on DC recruitment observed in p40-deficient mice. Additionally, they reveal its independence of T and B cells. In such immunodeficient conditions, the effects of exogenous IL-23 on inflammatory cell recruitment are even more prominent, as the frequency of NK cells and granulocytes was elevated.

4.4 PROTEIN PURIFICATION OF P40-HOMODIMER

Cytokines of the IL-12 family are considered the most important group of APC-produced cytokines orchestrating cellular immune responses (ALBER *et al.* 2006; BROMBACHER *et al.* 2003; LANGRISH *et al.* 2004; TRINCHIERI *et al.* 2003). In the current understanding of the interplay between IL-12 family cytokines, however, many questions remain unsolved. Homodimeric p40 has been shown to antagonize IL-12 effects *in vitro* (GILLESSEN *et al.* 1995; MATTNER *et al.* 1993) and *in vivo* (MATTNER *et al.* 1997), but was also associated with proinflammatory agonistic effects (HOLSCHER *et al.* 2001). In addition, in a very recent report homodimeric p40 could be shown to impair IL-23-induced IL-17 production (SHIMOZATO *et al.* 2006). The herein described protein purification of homodimeric p40

from transfected insect cell culture supernatants forms the basis for a future series of *in vivo* and *in vitro* studies that aim to provide a deeper insight in the networking of all p40-dependent cytokines. The insect cell line Sf9 was transfected with a murine p40-encoding plasmid by a baculovirus vector in collaboration between the Institute of Immunology and Intervet, Boxmeer, The Netherlands. Supernatants of transfected cell cultures were provided for the herein described purification.

4.4.1 Expression level of $(p40)_2$ in Sf9 cell culture supernatants

In a first step the protein profile and the expression of murine p40 in Sf9 cell culture supernatants was characterized. The total protein content was 2 mg/ml. The proteome of Sf9 culture supernatants was visualized by SDS PAGE and subsequent silver staining (Fig. 22 A).

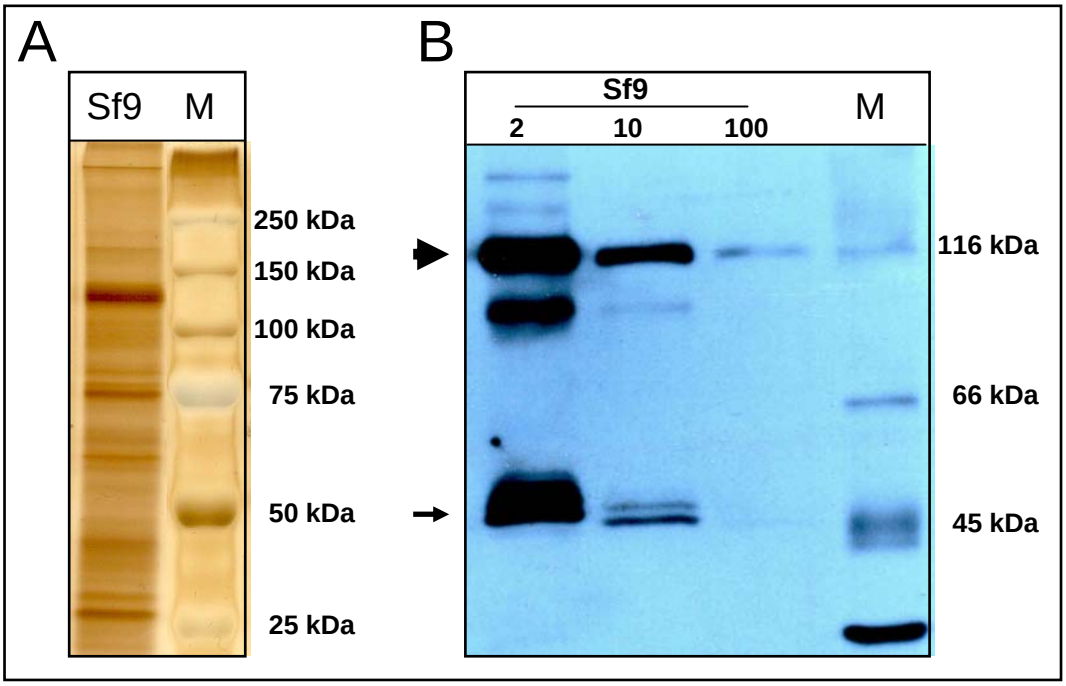


Fig. 22. Analysis of p40 content in Sf9 cell culture supernatants. A non-reducing SDS-PAGE was performed with cell culture supernatant from p40-transfected Sf9 cells. (A) By silver-staining the proteome of the supernatants was visualized. (B) p40-specific Western blot developed by chemiluminescence and visualized on x-ray film. Sf9, Sf9 cell culture supernatants; Numbers, dilution factor; M, molecular weight marker; bold arrow, $(p40)_2$ at 116 kDa; thin arrow, p40 at approximately 45 kDa.

As determined by ELISA the concentration of murine p40 in Sf9 culture supernatants was 6 µg/ml. The expression of p40 was further characterized by p40-specific Western blot (Fig. 22 B). Besides a strong band at 116 kDa consisting of p40-homodimer another intense band showed at 40 kDa referring to monomeric p40. Interestingly, at approximately 97 kDa a further band showed that in size does not match yet reported p40-cytokines (Fig. 22 B). The

ratio between homodimeric and monomeric p40 was estimated as 2:1 by comparing the intensity of the bands that showed in the p40-specific Western blot.

Taken together, transfection of Sf9 cells by baculovirus containing a p40-encoding plasmid resulted in a strong expression of homodimeric as well as monomeric p40.

4.4.2 Ion exchange chromatography of Sf9 cell culture supernatants

In a previous report, GATELEY *et al.* achieved purification of murine p40 expressed by stably transfected CHO cells by ion exchange chromatography. As murine p40 has a positive charge in a neutral pH it can be bound to a negatively charged matrix. Therefore, a cation exchange column packed with a matrix bearing free carboxylgroups was used in combination with a liquid chromatography system. A total amount of 90 µg of murine p40 was loaded on the column. By washing with five volumes of sodium phosphate buffer most of the negatively charged and therefore unattached protein was eliminated from the column (data not shown). Subsequently, the column was eluted with a 40 ml linear salt gradient from 0 mM to 500 mM NaCl in sodium phosphate buffer.

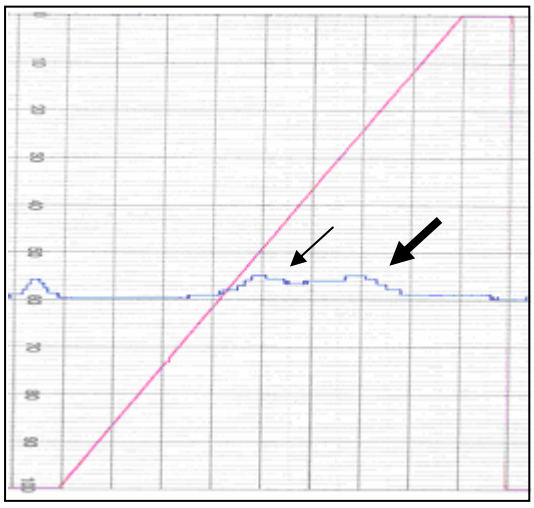


Fig. 23. Ion-exchange chromatography of Sf9 cell culture supernatants containing $(p40)_2$. Purification of murine p40-homodimer from transfected Sf9 cells was achieved by ion exchange chromatography using a negatively charged matrix. Sf9 culture supernatant containing 90 µg total murine p40 was loaded on the column. After washing off non-bound protein the column was eluted with a linear salt gradient ranging from 0 mM to 500 mM. The red line in the chromatogram displays the salt concentration, the blue line the absorption at 260 nm wavelength measured in the eluate. Arrows indicate protein peaks.

The positively charged Na^+ ions compete with the proteins of the Sf9 cell cultures for the binding sites on the column matrix. With rising salt concentration even strongly attached protein is removed from the column. The resulting chromatogram showed two protein peaks during the elution process (Fig. 23).

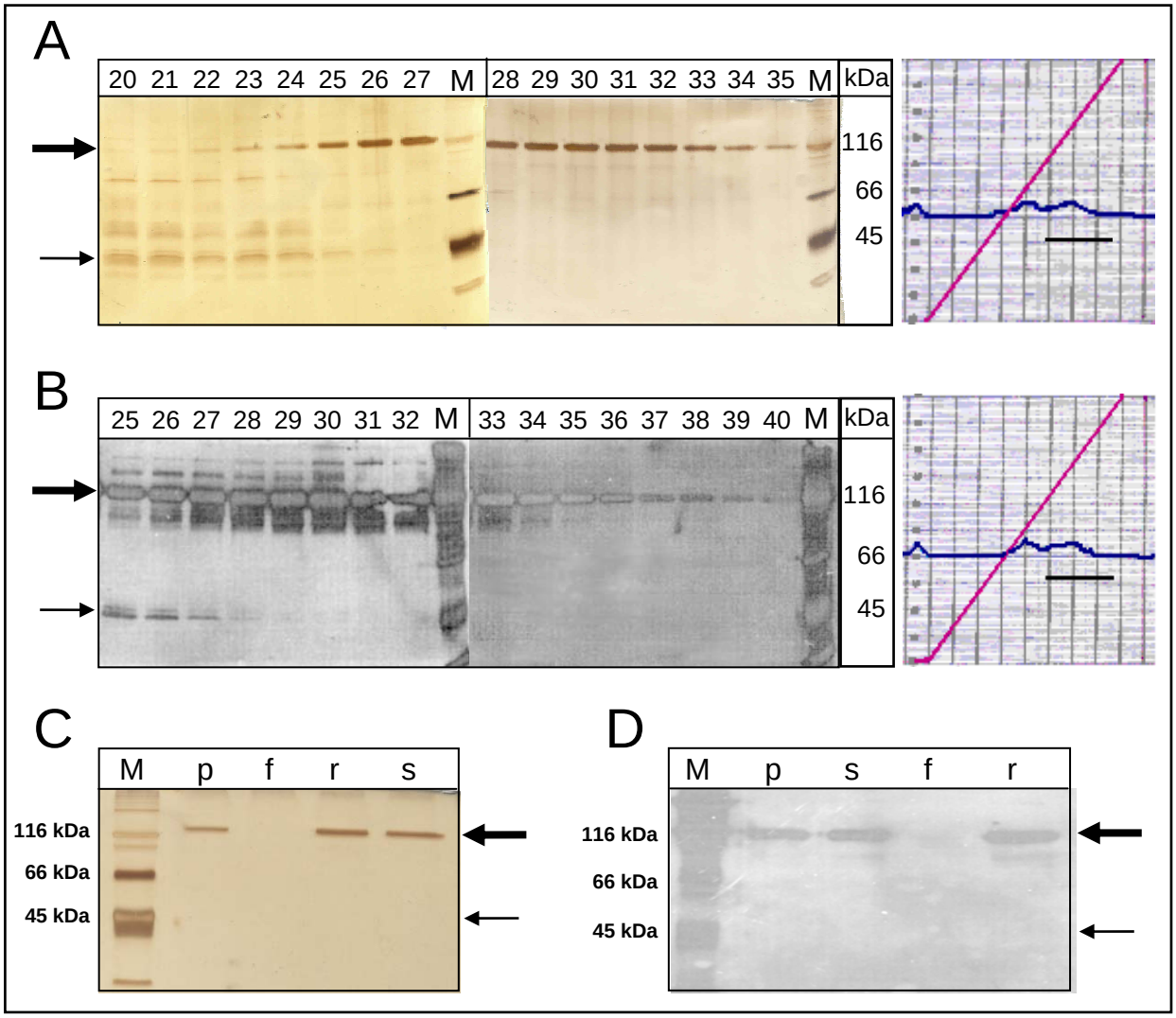


Fig. 24. Ion-exchange chromatography of p40-containing Sf9 cell culture supernatants. The eluate obtained by cation exchange chromatography was collected in fractions of 1 ml volume. Total protein and p40 content in selected fractions was analyzed by non-reducing SDS-PAGE followed by silver staining (A) or p40-specific Western blot (B). The fractions analyzed are depicted in the chromatogram on the right side of both panel A and B. Non-reducing SDS-PAGE and subsequent silver-staining (C) and p40-specific Western blot (D) of pooled fractions #31-40 (p), after 10-fold concentration (filtrate = f; retentate = r) and subsequent sterile filtration (s). Numbers, number of fraction; M, molecular weight marker; bold arrow, $(p40)_2$; thin arrow, monomeric p40.

The eluate was collected in fractions of 1 ml which were analyzed by SDS-PAGE followed by silver staining (Fig. 24 A) and p40-specific Western blot analysis (Fig. 24 B). This analysis revealed that the first peak recorded in the chromatogram (Fig. 23) referred to monomeric p40 and other cationic proteins, whereas the second peak reflected homodimeric p40. Homodimeric p40 containing fractions without detectable contamination of monomeric p40 (fractions #31-40) were pooled, concentrated 10-fold and subsequently sterile-filtered.

Protein purity of the final preparation was assessed by non-reducing SDS-PAGE followed by silver-staining (Fig. 24 C) or p40-specific Western blot (Fig. 24 D). Since no monomeric p40 was detected in the final preparation the p40-concentation of 5 µg/ml as determined by ELISA referred in total to homodimeric p40. This was further confirmed by assessing the amount of total protein in the final preparation (5 µg/ml).

In summary, homodimeric p40 could be purified from Sf9 culture supernatants and distinguished from monomeric p40 and other p40-independent proteins by ion exchange chromatography.

4.4.3 Biological activity of purified $(p40)_2$

An important step at the end of the purification process is the determination of the biological activity of the purified protein. Since homodimeric p40 has been shown to be an antagonist for IL-12 at the IL-12 receptor (MATTNER *et al*., 1993) biological activity of the purified $(p40)_2$ was assessed *in vitro* by measuring its inhibitory effect on IL-12-induced production of IFN-γ.

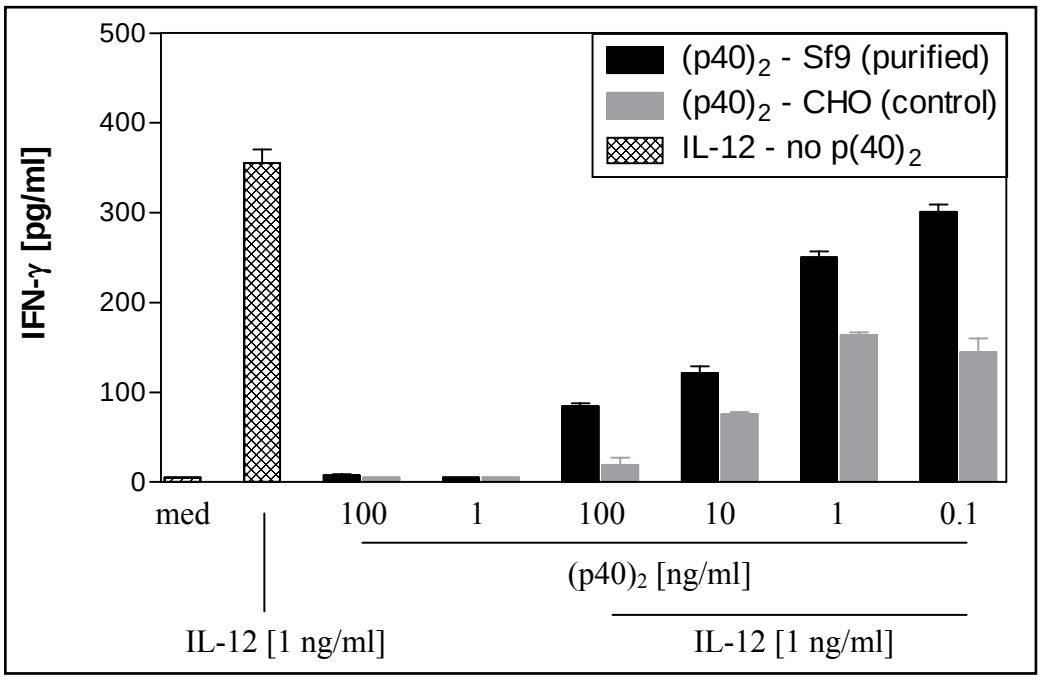


Fig. 25. Determination of biological activity of purified Sf9 cell culture supernatant $(p40)_2$. Biological activity of purified $(p40)_2$ was measured *in vitro* by determination of its inhibitory effect on IL-12 induced IFN-γ production. Cells were incubated with different concentration of p40-homodimer. After 12 hrs IL-12 (1 ng/ml) was added. IFN-γ was assessed in culture supernatants after 48 hrs. As controls splenocytes were stimulated with medium only as well as either cytokine individually. Black bars, purified Sf9 cell-derived $(p40)_2$; grey bars, CHO cell-derived $(p40)_2$ as a control.

Therefore, primary splenocytes from WT mice were incubated with different concentrations of the purified Sf9-cell derived homodimeric p40 as well as CHO cell-derived homodimeric p40 as a control. Cells were incubated for 12 hrs with homodimeric p40 before addition of IL-12 to ensure sufficient binding of homodimeric p40 to the IL-12 receptor. After 48 hrs IFN-γ was measured in the culture supernatants. This assay showed that purified Sf9 cell-derived $(p40)_2$ blocked IL-12 activity in a dose-dependent manner and to a similar extent as seen for the CHO-derived $(p40)_2$-control (Fig. 25). This data demonstrates that bioactive p40-homodimer could be purified from Sf9 cell culture supernatants.

4.4.4 Comments on p40 protein purification yields

The above purification procedure resulted in highly purified and bioactive murine $(p40)_2$. Assuming that the amount of $(p40)_2$ in the Sf9 cell supernatants is half the amount of total p40 (which is 6 µg/ml), approximately $^1/_9$ of the $(p40)_2$ loaded on the column could be recovered. To purify an appropriate quantity of $(p40)_2$ for *in vivo* and *in vitro* use, e.g. 10 mg, the rate of recovery would have to be improved. Since the resolution of the ion-exchange chromatography can barely be enhanced, it is recommended to take the eluate achieved by ion-exchange chromatography for a further purification step. The difference in molecular weight between monomeric and homodimeric p40 is large enough to perform a gel filtration over Sephacryl S-200 HR (Amersham) utilizing the same liquid chromatography system as described above. Thereby those fractions containing homodimeric p40 contaminated with monomeric p40 could be finally purified.

5 DISCUSSION

The IL-12 family of cytokines plays a key role in the orchestration of cellular immune responses. Years of basic and clinical research on the prototypical Th1 cytokine IL-12 revealed its importance in immunity to intracellular nonviral infections, cancer and autoimmune diseases (ALBER *et al.* 2006; TRINCHIERI 2003). After the discovery of IL-23 and its structural relationship to IL-12, re-analysis of IL-12 studies revealed, that IL-23, rather than IL-12, is the key player in autoimmune disease models (CUA *et al.* 2003; MURPHY *et al.* 2003), inducing a non-classical T helper cell population that is mainly characterized by the production of IL-17. Knowing about this strong proinflammatory potential of IL-23 it is intriguing to investigate its role in infectious diseases. In a series of *in vivo* experiments the present work aimed to explore the contribution of the novel IL-23-induced immune pathway to host defense in chronic fungal infection. A protective function of IL-23 in the absence of IL-12 could be identified by reconstitution of p40-deficient mice with recombinant IL-23. In addition, the presented results describe a role for endogenous IL-23 in protective immunity even in the presence of IL-12. Corroborating theses findings, administration of recombinant IL-23 enhanced the inflammatory response to *C. neoformans* infection and improved elimination of fungal cells even in the absence of T and B cells.

5.1 IL-23 IS PROTECTIVE IN THE ABSENCE OF IL-12

It has been shown previously that p40-deficient mice are more susceptible to infection with the opportunistic pathogen *C. neoformans* than IL-12p35$^{-/-}$ mice (DECKEN *et al.* 1998). Survival times and organ burden of p40-deficient mice could not be restored by treatment with either monomeric or homodimeric p40 (DECKEN *et al.* 1998), which are produced physiologically in a large excess relative to IL-12 or IL-23 (D'ANDREA *et al.* 1992). In the present study, administration of recombinant IL-23 greatly improved the survival of infected p40-deficient mice, restoring the survival period of treated mice to the level of IL-12p35$^{-/-}$ mice. Collectively, these data indicate that IL-23 has a role in resistance to *C. neoformans* infection, although IL-23 is not sufficient for complete protection in the absence of IL-12. Recently, a similar effect was attributed to IL-23 reconstitution in *Toxoplasma gondii* (*T. gondii*)-infected p40-deficient mice (LIEBERMAN *et al.* 2004). These data demonstrate that IL-23 is able to protect against *C. neoformans* and *T. gondii* independently of IL-12.

5.2 A ROLE FOR ENDOGENOUS IL-23 IN IMMUNITY TO *C. NEOFORMANS* INFECTION

The use of genetically modified mice provides an essential and broadly used tool for the understanding of the function(s) of a particular gene product. To define the contribution of endogenous IL-23 to immunity in cryptococcosis in the present work, parameters of infection were assessed in mice deficient for IL-23p19. These mice were generated by targeted gene disruption in 129/SvEv stem cells (OPPMANN *et al.* 2000). Thus bred mice were backcrossed onto a C57BL/6 background. C57BL/6 mice are broadly accepted to be a Th1 biased strain. To infection with the herein used *C. neoformans* strain 1841 C57BL/6 mice were shown to be relatively resistant (DECKEN *et al.*). For the present study the infectious dose was optimized for the use of this mouse strain and chronic lethal infection was induced.

5.2.1 IL-23 contributes to immunity to *C. neoformans* infection in the presence of IL-12

The shorter survival time and higher organ burden observed in *C. neoformans*-infected IL-23p19$^{-/-}$ compared to WT C57BL/6 mice provide evidence for a role of endogenous IL-23 in host resistance. Interestingly, IL-23p19$^{-/-}$ mice infected with *T. gondii* did not show an altered resistance after infection (LIEBERMAN *et al.* 2004). This may be related to the different nature of *T. gondii* versus *C. neoformans* infection and, in addition, only acute stages of infection were investigated in this study (LIEBERMAN *et al.* 2004). In the present studies of *C. neoformans* infection both early and late time points were examined. It was found that in IL-23p19$^{-/-}$ mice clearance of *C. neoformans* organisms is delayed late in infection (i.e. day 35 p.i.), presumably after initial control of the infection has been established. Consistent with this observation, impaired clearance has been documented in p40-deficient mice when compared with IL-12p35$^{-/-}$ mice following *Francisella tularensis* infection (ELKINS *et al.* 2002). In a very recent report on chronic *Mycobacterium tuberculosis* infection, IL-23p19$^{-/-}$ mice show an elevated bacterial burden in the spleen only at a late time point (KHADER *et al.* 2005).

Notably, the differences observed in WT versus IL-23p19$^{-/-}$ mice following *C. neoformans* infection are not as pronounced as those seen comparing IL-12p35$^{-/-}$ and p40-deficient mice (DECKEN *et al.* 1998). Therefore, the protective capacity of IL-23 is more apparent in the absence of IL-12, as is the case for *T. gondii* and *M. tuberculosis* infection (LIEBERMAN *et al.* 2004; KHADER *et al.* 2005). It was shown that IL-12 inhibits IL-23 induced T cell production of IL-17 (AGGARWAL *et al.* 2003). Moreover, two reports demonstrate an enhancement of IL-23 effects in IL-12-deficient mice (IL-12p35$^{-/-}$ mice) in autoimmune

chronic inflammation (CUA *et al.* 2003; MURPHY *et al.* 2003). The subtle effects of IL-23 in the presence of IL-12 in the model of *C. neoformans* infection could therefore be due to suppression of IL-23 functions by endogenous IL-12/IFN-γ by a yet unknown mechanism.

5.2.2 Endogenous IL-23 contributes to maintenance of the granulomatous reaction

In the chronic phase of infection, a granulomatous inflammatory response predominantly characterized by mononuclear cells is an important feature of resistance to *C. neoformans* infection (CASADEVALL and PERFECT 1998). As previously reported, p40-deficient mice show a defect in granuloma formation that was not observed in IL-12p35$^{-/-}$ mice (DECKEN *et al.* 1998). These findings are consistent with other reports on p40-dependent, IL-12-independent defects in granuloma formation during infection with *Salmonella* Enteritidis or *M. bovis* BCG (HOLSCHER *et al.* 2001; LEHMANN *et al.* 2001). Of note, in all of these studies p40-dependent granuloma formation was explored in mice lacking IL-12. In the present study it showed that the lack of IL-23 leads to impaired granuloma formation even in the presence of IL-12. These findings are supported by a recent study using a murine model of tuberculosis. Reconstitution of *M. tuberculosis*-infected p40-deficient mice with recombinant IL-12 only partially restored their ability to form granulomata (FENG *et al.* 2005). Granulomata were fewer in IL-12-reconstituted p40-deficient mice than in WT controls suggesting that IL-12 contributes in parts to granuloma formation and that other factors such as IL-23 are likely required for a sustained granulomatous inflammatory response (FENG *et al.* 2005). Indeed, the higher susceptibility to disease observed in p40-deficient mice when compared to IL-12p35$^{-/-}$ mice was associated with an impaired granuloma formation in the former genotype (HOLSCHER *et al.* 2001). In mice competent for IL-12 production, however, the specific lack of IL-23 did not affect granuloma formation in *M. tuberculosis* infection pointing to a more crucial role of IL-12 in this infection model (KHADER *et al.* 2005). IL-23 was shown to be responsible for the higher resistance to murine tuberculosis observed in IL-12p35$^{-/-}$ mice compared to p40-deficient mice and was demonstrated to induce IFN-γ in the absence of IL-12 in *M. tuberculosis* infection (KHADER *et al.* 2005). In *C. neoformans* infection, however, IL-12p35$^{-/-}$ similarly as p40-deficient mice fail to produce IFN-γ and mount a Th2 response (DECKEN *et al.* 1998). Although neither in the presence nor in the absence of IL-12 IL-23 was shown to induce IFN-γ in *C. neoformans* infection, in both cases granuloma formation is impaired by IL-23 deficiency. Collectively these data indicate that dependent on the nature of the infectious agent IL-23 can contribute to granuloma formation by means distinct from IL-12 and IFN-γ.

After initially being able to form hepatic granulomata with a frequency comparable to WT mice, at a later time point the granulomatous reaction was significantly impaired in IL-23p19$^{-/-}$ mice. Consistent with this data, in EAE, a mouse model of multiple sclerosis, susceptible WT and resistant IL-23p19$^{-/-}$ mice initially show comparable leukocyte infiltration into the brain, whereas sustained inflammation of the CNS is only seen in WT mice (CUA *et al.* 2003). Similarly, the magnitude of DTH response in IL-23p19$^{-/-}$ mice immunized with methylated BSA was comparable to WT mice at 18 hrs post induction but greatly diminished thereafter (GHILARDI *et al.* 2004). In summary, such findings point to a crucial role for IL-23 in the maintenance of inflammatory processes.

5.2.3 IL-23 enhances the inflammatory process by induction of chemokines

C. neoformans shows a strong tropism for the CNS where it causes a sustained meningoencephalitis with subsequent death if untreated (CASADEVALL and PERFECT 1998). Therefore, the immune response in the brain is of particular interest. Aggregates of *C. neoformans* accumulate in the brain of WT and IL-23p19$^{-/-}$ mice, eliciting an inflammatory response in both genotypes. In IL-23p19$^{-/-}$ mice, however, the density of inflammatory infiltrates was reduced as compared to WT mice. Numbers of T cells, macrophages and neutrophils were significantly reduced. Interestingly, MCP-1 mRNA and protein concentrations were markedly lower in IL-23p19$^{-/-}$ mice. MCP-1 is a CC type chemokine that mainly attracts mononuclear leukocytes (LU *et al.* 1998). In pulmonary cryptococcosis, MCP-1 has been shown to be crucial for recruitment of CD4^{+} T cells and macrophages, production of TNF-α and IL-6, and clearance of organisms (HUFFNAGLE *et al.* 1995). Also, it has been described to be present at elevated levels in brains of mice protectively immunized against *C. neoformans* (UICKER *et al.* 2005). The role of IL-23 in the maintenance of inflammatory processes might therefore partially rely on cell recruitment via induction of MCP-1.

5.2.4 IL-23 augments antigen presentation thereby maintaining the cellular response

It was shown in this study that MHC-II expression in microglial cells was impaired in IL-23p19$^{-/-}$ mice when compared to WT controls. Also, 5-fold more MHC-II mRNA was induced in brains of WT than in IL-23p19$^{-/-}$ mice (data not shown). Similar observations have been described for EAE (CUA *et al.* 2003). Moreover, a role for IL-23 in enhancing antigen presentation has previously been shown for DCs (KOPP *et al.* 2003; BELLADONNA *et al.* 2002). Since primed effector T cells are retained at the site of infection if antigen is

encountered in proper MHC context, the above findings describe another IL-23-dependent mechanism leading to the maintenance of localized immune responses.

Although infected IL-23p19$^{-/-}$ mice show an impaired inflammatory response in the brain, the organ burdens do not significantly differ from WT controls. Previously it was reported that IL-12 deficiency leads to severely reduced survival times after *C. neoformans* infection, but organ burdens at day 21 p.i. in multiple organs including the brain did not differ between IL-12p35$^{-/-}$ mice and resistant WT controls (DECKEN *et al.* 1998). Therefore, in this model of cryptococcosis the fungal organ burden found relatively early in the course of cryptococcal infection is not necessarily predictive for the level of resistance as determined by survival time.

5.2.5 The IL-23 induced cytokine pattern is hallmarked by IL-17, IL-6 and IL-1β

In the present work it is shown, that the lack of IL-23 does not affect the Th1/Th2 balance. These data are consistent with findings in other studies looking at IL-23p19$^{-/-}$ mice in autoimmune disease or infection models (CUA *et al.* 2003; LIEBERMAN *et al.* 2004; MURPHY *et al.* 2003; KHADER *et al.* 2005) and resemble the previously reported finding that IL-12p35$^{-/-}$ and p40-deficient mice do not differ in IFN-γ production following *C. neoformans* infection (DECKEN *et al.* 1998). Interestingly, however, pronounced IL-17 expression in WT but none in IL-23p19$^{-/-}$ mice could be observed. It is intriguing that antigen-specific production of IL-17 seems to be completely dependent on IL-23, whereas polyclonal stimulation of *C. neoformans*-infected splenocytes of IL-23-deficient mice led to the production of IL-23-independent IL-17, albeit to a lower level than seen in WT splenocytes after polyclonal stimulation.

Because IL-23p19$^{-/-}$ mice produce less IL-17 it is conceivable that IL-17 acts as a mediator of IL-23. Herein reported is the observation of impaired expression of myeloid cell-derived proinflammatory cytokines such as IL-6 and IL-1β during cryptococcal infection of IL-23p19$^{-/-}$ mice. Of note, increased expression of IL-1β and TNF-α in the brains of infected WT mice is associated with protective immunity in cryptococcosis (UICKER *et al.* 2005). Indeed IL-17 was shown to stimulate production of TNF-α and IL-1β in human macrophages (JOVANOVIC *et al.* 1998). In addition, it promotes production of MCP-1 in various adherent cells (AGGARWAL and GURNEY 2002). A protective role for the IL-23/IL-17 axis has been defined in acute *Klebsiella pneumoniae* infection. IL-23-induced IL-17 was shown to be crucial for neutrophil recruitment to the infected airways and subsequent clearance of pathogen (HAPPEL *et al.* 2003; YE *et al.* 2001b). In a model of chronic *M. tuberculosis*

infection, IL-23p19$^{-/-}$ mice showed, consistent with the herein reported findings, a severe defect in antigen-specific IL-17 production that was, however, not associated with altered resistance to infection in these mice (KHADER *et al.* 2005). In the light of the above described predominant contribution of IFN-γ to cellular responses in tuberculosis, IL-17 effects might be completely obliterated by the IL-12/IFN-γ axis. In *C. neoformans* infection IL-23 induced expression of IL-1β, IL-6 and MCP-1 in infected brains but no detectable IL-17. Therefore, direct effects of IL-23 on macrophages could be considered. Indeed, macrophages have been shown to bear the IL-23R (PARHAM *et al.* 2002). Studies in IL-17$^{-/-}$ mice have to be performed to provide a deeper insight in the regulation of cellular responses by IL-23/IL-17 in *C. neoformans* infection.

5.3 A PROTECTIVE ROLE FOR EXOGENOUS IL-23 IN *C. NEOFORMANS* INFECTION

The polysaccharide capsule of *C. neoformans* biases the immune response towards a suitable environment for survival of the fungus by down-regulating the expression of proinflammatory mediators (BUCHANAN and MURPHY 1998). Due to such immunosuppressive capacities, in the herein employed lethal model of cryptococcosis, observations in mice deficient for proinflammatory cytokines such as IL-23 might reveal only a faint signature of the possible protective effects of such factors. Therefore, the administration of IL-23 as performed in the present work was likely to give a better insight in the mechanism of action employed by IL-23. In cryptococcosis, this methodical approach was used in the past for a broad range of cytokines such as IFN-γ, IL-12, G-CSF and IL-18 (BAVA *et al.* 1995; DECKEN *et al.* 1998; KAWAKAMI *et al.* 1996; KAWAKAMI *et al.* 1997; UCHIDA *et al.* 1992; ZHANG *et al.* 1997).

5.3.1 IL-23 improves resistance to *C. neoformans* infection without inducing Th1

As discussed in section 5.1, recombinant IL-23 enhanced the resistance to *C. neoformans* infection in the absence of IL-12. Further experiments showed that exogenous IL-23 is also capable of protecting mice that are competent in IL-12 production. The enhanced resistance of IL-23-treated mice to *C. neoformans* infection adds to the finding of reduced resistance in IL-23 deficiency.

However, the observed effect by IL-23 is weaker than the protection observed after IL-12 treatment. IL-12 is a potent mediator of resistance in *C. neoformans* infection. Treatment of infected mice with IL-12 completely protects those mice from death. The strong enhancement of Th1 cytokine production following such treatment shows the importance of this pathway

in immunity to *C. neoformans*. Similar protective effects could be demonstrated following IFN-γ (BAVA *et al.* 1995) or also IL-18 (KAWAKAMI *et al.* 1997) treatment of *C. neoformans* infected mice. IL-23 treatment, however, though protective, is not associated with an enhancement of Th1. Moreover, the IL-23 treated mice develop a Th2 response similar to mock treated control mice.

In contrast to treatment with IL-23 or IL-12, administration of homodimeric p40 to wild-type mice had a detrimental effect on disease outcome in WT mice (DECKEN *et al.* 1998). While it is was known that homodimeric p40 blocks IL-12-induced IFN-γ production on the receptor level (GILLESSEN *et al.* 1995), a recent study shows that also IL-23 effects are antagonized by homodimeric p40 (SHIMOZATO *et al.* 2006). In the light of such findings together with today's knowledge about the novel IL-12 family members more defined studies on the controversially discussed pro- or anti-inflammatory role of homodimeric p40 should be performed. As a preparative step for such studies, a p40 protein purification was performed and described in the present work. A surprising result was the finding of a 90 kDa protein band which occurs in association with the Sf9 cell $(p40)_2$ band. This band is detectable in a p40-specific Western blot (Figs. 22 and 24). Analysis of current literature on p40 showed, that this second band, which has not been explicitly described so far, also occurs in supernatant of p40-transfected CHO cells (HOLSCHER *et al.* 2001: Fig. 3). Since this band did not gain intensity during a time course experiment (data not shown), it is unlikely to be a degradation product, but rather a glycosylation variant of homodimeric p40. Follow-up experiments should focus on assessing the biological importance of this finding and might add to our understanding of the conflicting data on the physiological role of homodimeric p40 in the interplay of IL-12 family cytokines.

5.3.2 Exogenous IL-23 elicits antimicrobial effects

The prolonged survival was accompanied by lower organ burden at the site of infection at an early time point as well as in infected organs later in the course of infection. Two other studies so far described an antimicrobial effect of endogenous IL-23 at the site of infection: In a model of pulmonary tuberculosis IL-23 administration into infected lungs by an adenoviral vector could significantly reduce the mycobacterial burden in this organ (HAPPEL *et al.* 2005b). Secondly, recombinant IL-23 reduced the parasite burden in the peritoneal cavity of p40 deficient mice infected with *T. gondii* (LIEBERMAN *et al.* 2004). These findings complement the observation of increased fungal burden in the IL-23p19$^{-/-}$ mice. Moreover, the IL-23 mediated reduction of fungal burden at day 8 post infection

provided a fruitful model for further investigations on the mechanisms underlying such antimicrobial effects.

5.3.2 IL-23 produces a distinct cytokine pattern hallmarked by proinflammatory cytokines as well as IL-5

Treatment with IL-23 led to the induction of proinflammatory cytokines. Interestingly, not a Th1 pattern was induced since up-regulation or enhanced production of IFN-γ could not be observed. In contrast, IL-17 production was markedly enhanced in the IL-23 treated group. Recently, the role for IL-23 in promoting a IL-17 producing T cell subset (AGGARWAL *et al.* 2003; CUA *et al.* 2003) which is meanwhile considered a Th cell lineage distinct from Th1 and Th2 (AGGARWAL *et al.* 2003; HARRINGTON *et al.* 2005) could be defined. Besides IL-17, this T cell subset (Th17) was shown to produce IL-6 and TNF-α, both of which were found in elevated levels in the study presented here. In addition, mice genetically engineered to overexpress p19 develop severe systemic inflammation accompanied by high serum levels of TNF-α and IL-1β, but not IFN-γ (WIEKOWSKI *et al.* 2001). A more recent study investigated the outcome of local overexpression of IL-23 in a pulmonary tuberculosis model. Paralleling the here presented findings, the reduced bacterial burden following IL-23 transfer was associated with the enhanced expression of IL-17 (HAPPEL *et al.* 2005b).

Interestingly, IL-23 treatment showed a clear association with elevated IL-5 expression. IL-5 is commonly known as a Th2 cytokine, leading to the maturation and recruitment of eosinophils (WELTMAN and KARIM 2000). Although in the present study consistently elevated levels of IL-5 were found, at the site of infection as well as in restimulated spleen cell cultures, no increase of eosinophil counts could be observed. HUFFNAGLE *et al.* (1998) showed by neutralization of IL-5 of mice with pulmonary *C. neoformans* infection that besides its crucial effects on eosinophil counts in infected lungs, IL-5 also played an important role in the recruitment of macrophages, T lymphocytes, and B lymphocytes to the infected site. Interestingly, neutralization of IL-5 did not affect neutrophil recruitment (HUFFNAGLE *et al.* 1998). Thus, IL-5 might play a more distinct role in cryptococcosis.

Above findings underline the herein reported observations in IL-23 deficient mice and clearly demonstrate that IL-23 induces a cytokine pattern that is distinct from Th1 or Th2. Besides IL-17 as the major hallmarking cytokine, the IL-23 induced immune pathway is characterized by the expression of IL-1β, IL-6, TNF-α, IL-5 and MCP-1.

5.3.3 IL-23 enhances recruitment of inflammatory cells to the site of infection

Independently of IL-12, the administration of IL-23 led to enhanced recruitment of inflammatory cells such as T cells, NK cells and DCs to the site of infection. The elevated numbers of T cells observed after treatment with recombinant IL-23 resemble findings in a recent study on pulmonary tuberculosis. The delivery of IL-23 into infected lungs by an adenoviral vector led to higher counts of activated CD4 positive cells in this organ (HAPPEL *et al.* 2005b). Transgenic overexpression of p19 led to multiorgan inflammation with an inflammatory infiltrate mainly consisting of lymphocytes, macrophages and neutrophils (WIEKOWSKI *et al.* 2001), paralleling the herein reported findings. Moreover, administration of IL-23 to *C. neoformans*-infected mice also increased the recruitment of DCs to the site of infection. DCs are the most effective APC for inducing CMI responses (BANCHEREAU *et al.* 2000). In cryptococcosis DCs were demonstrated to be involved in the induction of protective immunity (BAUMAN *et al.* 2000). More recently, their specific involvement in bridging innate and adaptive immunity during *C. neoformans* infection was even more defined by their non-redundant capability of inducing T cell responses to mannoproteins, major antigens of *C. neoformans* (MANSOUR *et al.* 2006). Effects of IL-23 on DC recruitment were also found in mice engineered to coexpress p40 and IL-23p19 in keratinocytes. Expression of the transgene led to increased numbers of Langerhans cells in the skins of such mice (KOPP *et al.* 2003). Consistent with the herein reported finding of elevated CD86 expression in inflammatory macrophages, those cells showed a marked up-regulation of costimulatory molecules.

The effect on inflammatory cell recruitment observed after IL-23 treatment closely resembles the findings associated with endogenous IL-23 reported in section 4.2. Together these data clearly demonstrate a role for IL-23 in inflammatory cell recruitment / granuloma formation during *C. neoformans* infection.

5.3.4 IL-23 treatment is effective even in the absence of T cells

The importance of cells of the adaptive immune system in immunity against *C. neoformans* infection was convincingly demonstrated in SCID mice which lack T and B cells and are highly susceptible to cryptococcosis (HUFFNAGLE *et al.* 1991). SCID mice were first phenotypically described in the late 1980s and then found to have a genetic mutation at chromosome 16 leading to a defect of the ubiquitous DNA double-strand break repair machinery (HENDRICKSON 1993; JORGENSEN *et al.* 1995). During lymphocyte development DNA break repair systems are crucial for successful V(D)J recombination

defining the specificity of the antigen receptor. However, DNA breaks occur also in other cell types, so that the outcome of such a general mutation is hard to predict. The finding of the lymphocyte specific RAG proteins involved in V(D)J recombination and the availability of mice deficient for these DNA recombinases generated by targeted disruption of *RAG1* or *RAG2* provided a more specific tool to investigate the role of adaptive versus innate immunity. Mice deficient for RAG genes were used in this study and showed, as expected, a higher susceptibility to *C. neoformans* infection than WT mice (data not shown). However, treatment of infected RAG-deficient mice with IL-23 clearly elicited proinflammatory effects that also led to increased resistance. RAG-deficient mice, although lacking adaptive immunity, have a functional innate immune system. In terms of resistance to cryptococcal infection the most important of such cell types are DCs, macrophages and NK cells, while controversial data exists on the contribution of neutrophils to resistance or susceptibility in this infection (CASADEVALL and PERFECT 1998).

Macrophages have been shown to be significant effector cells in immunity to *C. neoformans* (LEVITZ 1994). They are most efficiently activated by IFN-γ and TNF-α, major cytokines expressed during Th1 responses. Addition of TNF-α to *C. neoformans* infected macrophage cultures enhanced their phagocyitc activity drastically (COLLINS and BANCROFT 1992). In addition to such direct activation, NK cells have been shown to activate macrophages even in the absence of T cells (KAWAKAMI *et al.* 2000). Addition of IL-12 or IL-18 increased this bystander activity of NK cells *in vitro* (KAWAKAMI *et al.* 2000).

Collectively, these data point to two possible mechanisms of action for exogenous IL-23 in RAG-deficient mice: (i) IL-23 directly activates macrophages, and (ii) IL-23 activates cells, such as NK cells, which then produce factors activating macrophages. Both hypotheses find support in the literature. Parham *et al.* showed that message for the IL-23R is expressed not only on T cells but also on macrophages and NK cells (PARHAM *et al.* 2002). Granulocytes have been shown to produce IL-17 (FERRETTI *et al.* 2003), whereas yet it is not known if those cells also express the IL-23 receptor. Moreover, we still lack clear evidence for a direct effect of IL-23 on cells of the innate immune system. *In vitro*, stimulation of murine bone-marrow-derived macrophages did not lead to detectable cytokine production or regulation of cell surface proteins (unpublished data). *In vivo*, CUA *et al.* showed an effect on peritoneal macrophages by injecting rIL-23 in the peritoneal cavity of mice. Peritoneal macrophages obtained by peritoneal lavage were shown to express higher mRNA levels for IL-1β and TNF-α. Although strongly resembling the results obtained following intraperitoneal

application of IL-23 into RAG-deficient mice, those experiments were performed in T cell competent mice, still leaving open the possibility of an indirect effect on the peritoneal macrophages by T cell expressed factors, e.g. IL-17.

Thus, although the induction of Th17 cells is clearly an important and exclusive function of IL-23 and is observed during *C. neoformans* infection, yet undefined T cell-independent effects of IL-23 can elicit an antifungal inflammatory environment.

5.4 CONCLUSIONS

Taken together, IL-23 complements the more dominant role of IL-12 in protection against *C. neoformans* by mechanisms distinct from IL-12. The IL-23 pathway is characterized by the promotion of IL-17, as well as IL-1β, IL-6, TNF-α and MCP-1, all of which affect cell recruitment and maintenance of inflammatory responses (Fig. 26).

However, while in models of organ-specific autoimmunity the proinflammatory effects of IL-23 are crucial for disease progression, the contribution of endogenous IL-23 to the protective inflammatory response in this chronic fungal infection is limited. In contrast, in models of acute *Klebsiella pneumoniae* and *Toxoplasma gondii* infection, as well as in a murine model of sepsis IL-23 and/or IL-17 were shown to play a crucial role by inducing a protective neutrophil response (HAPPEL *et al.* 2005a; KELLY *et al.* 2005; RICE *et al.* 2005). This gives a clue to a physiological protective role for IL-23 and could be the basis for its evolutionary preservation. However, the in the present study reported findings clearly show a proinflammatory effect of IL-23 involving cells others than neutrophils, pointing to a more versatile role for IL-23 that still needs to be further defined..

Treatment with IL-23 shows a protective effect even in severely immunocompromised mice infected with *C. neoformans*. The lack of T cells as in RAG-deficient mice or that of Th1 responses as in p40-deficient mice resemble the situation of an AIDS patient; a high risk scenario for a cryptococcal infection. Current treatment with amphotericine B reduces mortality drastically; however, it leads to severe liver damage in the course of the inevitable long-term treatment regimen. Although in this context a supportive immunotherapy would be truly beneficial, for long-term IL-23 treatment the risk of developing autoimmune disorders cannot be ruled out.

A more fruitful approach might be the development of IL-23 antagonists. IL-23 advanced to a most promising target for the treatment of organ-specific autoimmune diseases, such as psoriasis or rheumatoid arthritis. Currently, TNF-α antagonists are used for the treatment of rheumatoid arthritis; antibodies against p40 were found to be beneficial in psoriasis

treatment. Both such therapies, though highly efficacious, are associated with an elevated risk for infections as seen in AIDS patients. Particularly opportunistic chronic infections such as tuberculosis, histoplasmosis or cryptococcosis are reported (HAGE *et al.* 2003; RYCHLY and DIPIRO 2005; TRUE *et al.* 2002). Moreover, cases of neoplasias were observed in association with anti-p40 treatment of psoriasis or Crohn's Disease (KAUFFMAN *et al.* 2004; MANNON *et al.* 2004). Novel drugs have to be more specific in their inhibition of autoimmune inflammation without affecting immunity to infections and cancer; drugs that antagonize IL-23 might lead to this goal.

The herein reported findings not only present an important contribution to the understanding of the biological function of IL-23, but also give important input for potential safety considerations in the development of drugs aiming to enhance or to block IL-23 effects.

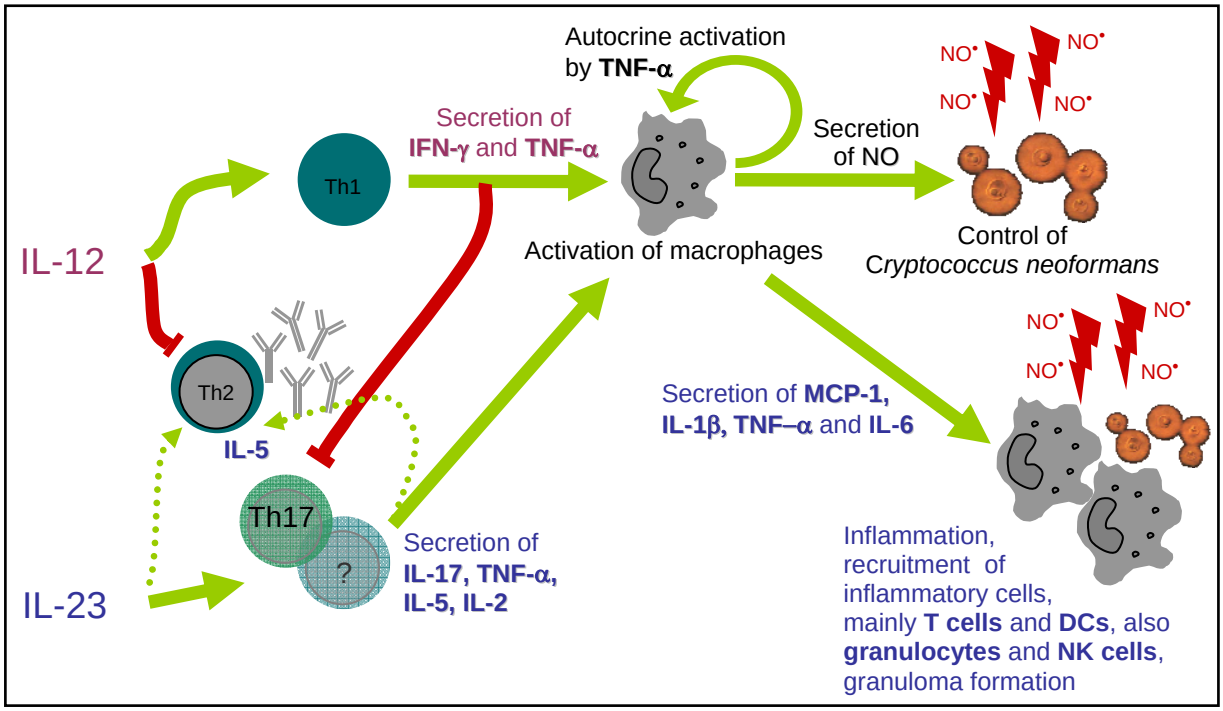


Fig. 26. Mechanism of action for IL-23 versus IL-12 in immunity to *C. neoformans* infection. IL-23 and IL-12 employ distinct mechanisms which result in activation of macrophages and subsequent elimination of *C. neoformans* organisms.

6 SUMMARY

Beyond Th1 and Th2: A non-classical immune pathway induced by Interleukin (IL)-23 complements IL-12 in immunity to *Cryptococcus neoformans* infection

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The interleukin (IL)-12 family of cytokines plays a key role in the orchestration of cellular immune responses, bridging innate and adaptive immunity. The founding member, IL-12, was discovered in the late 1980s as the first heterodimeric cytokine, composed of a 40 kDa (p40) and 35 kDa (p35) subunit. Years of basic and clinical research on this prototypical T helper type (Th)1 cytokine revealed its importance in immunity to intracellular non-viral infections, as well as in cancer and autoimmune diseases. Since the discovery of IL-23 as another cytokine composed of the p40 subunit of IL-12 in the year 2000, IL-23, rather than IL-12, could be shown to be the key player in rodent models of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. With accumulating evidence revealing IL-23 as the crucial regulator of a non-classical pathway of cellular immunity which is hallmarked by IL-17 producing T cells it is intriguing to gain understanding of the importance of such findings in immunity to infections. The present work describes a series of *in vivo* studies investigating the role of endogenous as well as exogenous IL-23 in a murine model of chronic fungal infection, cryptococcosis.

To address the role of endogenous IL-23, wild-type (WT), IL-12- (IL-12p35$^{-/-}$), IL-23- (IL-23p19$^{-/-}$) deficient, as well as IL-12- and IL-23- double deficient (p40-deficient) mice on a C57BL/6 background were infected with *Cryptococcus neoformans (C. neoformans)*. Following infection, p40-deficient mice demonstrated higher mortality than IL-12p35$^{-/-}$ mice. Reconstitution of p40-deficient mice with recombinant murine IL-23 prolonged their survival to levels similar to IL-12p35$^{-/-}$ mice. IL-23p19$^{-/-}$ mice showed a moderately reduced survival time and delayed fungal clearance in the liver. While interferon (IFN)-γ production was similar in WT and IL-23p19$^{-/-}$ mice, production of IL-17 was strongly impaired in the latter. IL-23p19$^{-/-}$ mice produced fewer hepatic granulomata relative to organ burden and showed defective recruitment of mononuclear cells to the brain. Moreover, activation of microglia cells and expression of IL-1β, IL-6, and MCP-1 in the brain was impaired.

The second part of the present work explores the mechanisms underlying the IL-23 effects by characterizing the role of exogenous IL-23. *C. neoformans*-infected C57BL/6 WT mice treated with recombinant murine IL-23 showed significantly prolonged survival time as compared to mock-treated control mice. However, complete survival throughout the observation period (100 days) was only achieved following IL-12 treatment. At day 21 post infection (p.i.) the IL-23-treated mice as well as the IL-12 group had a significantly lower fungal burden in the brain than the control mice. However, while IL-12 treatment was associated with elevated serum levels of the proinflammatory mediators IFN-γ, tumor necrosis factor (TNF)-α and nitric oxide, IL-23-treated animals, although more resistant, developed a Th2 response similar to the control group as measured by serum IgE levels. Further experiments to assess the mechanism of action were based on the finding of reduced fungal burden at the site of infection, the peritoneal cavity, at day 8 p.i. following IL-23 treatment. This microbicidal effect was also seen in p40-deficient as well as in T and B cell deficient (RAG-deficient) mice. Administration of IL-23 led to enhanced recruitment of inflammatory cells, not only of T cells but also cells of the innate immune system such as DCs, natural killer cells and granulocytes to the infected site. Although numbers of macrophages were not altered following IL-23 treatment, co-stimulatory molecules were markedly up-regulated on such cells. The chemokine/cytokine pattern induced by IL-23 treatment was hallmarked by proinflammatory mediators such as MCP-1, IL-1β, IL-6, TNF-α and IL-17, but also the Th2 associated cytokine IL-5.

From these results it can be concluded that a non-classical immune pathway induced by IL-23 complements the more dominant role of IL-12 in protection against *C. neoformans.* This novel immune response is characterized by an enhancement of the inflammatory cell response and the production of a proinflammatory cytokine pattern hallmarked by IL-1β, IL-6, TNF-α and IL-17.

7 ZUSAMMENFASSUNG

Jenseits von Th1 und Th2: Eine nicht-klassische, durch Interleukin (IL)-23 induzierte Immunantwort komplementiert die IL-12-vermittelte Immunität gegen *Cryptococcus neoformans*

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In der zellulären Immunantwort kommt den Zytokinen der Interleukin (IL)-12 Familie eine Schlüsselrolle als Bindeglied zwischen angeborener und adaptiver Immunität zu. IL-12 wurde erstmals in den späten 1980er Jahren als proinflammatorisches Zytokin beschrieben, das aus einer 40 kDa (p40) und einer 35 kDa (p35) Proteinuntereinheit zusammengesetzt ist. Mittlerweile konnte seine Rolle als prototypisches Zytokin der Typ-I Immunantwort und somit seine wichtige Funktion in der Immunabwehr gegen nicht-virale intrazelluläre Infektionen sowie bei Krebs und Autoimmunität definiert werde. Seit der Entdeckung von IL-23 als weiterem heterodimeren Zytokin, welches aus der p40 Untereinheit von IL-12 und der IL-23-spezifischen Untereinheit IL-23p19 besteht, konnte gezeigt werden, dass eher IL-23 als IL-12 eine essentielle Rolle in Nager-Modellen für Autoimmunerkrankungen wie Multipler Sklerose und Rheumatoider Arthritis zukommt. Mit wachsender Evidenz für einen durch IL-23 induzierten nicht-klassischen Arm der zellulären Immunantwort, der gekennzeichnet ist durch IL-17, stellt sich die Frage nach seiner Relevanz in der Wirtsabwehr. In der vorliegenden Arbeit wurde in einer Reihe von *in vivo* Experimenten die Rolle von endogenem sowie exogenem IL-23 am Mausmodell einer chronischen Pilzinfektion, der Kryptokokkose, untersucht.

Zur Untersuchung von endogenem IL-23 wurden Wildtyp (WT), IL-23- (IL-23p19$^{-/-}$) und IL-12- (IL-12p35$^{-/-}$) defiziente sowie IL-12- und IL-23- doppelt-defiziente (p40-defiziente) C57BL/6 Mäuse mit *Cryptococcus neoformans* (*C. neoformans*) infiziert. Nach Infektion zeigten p40-defiziente Mäuse eine erhöhte Mortalität verglichen mit IL-12p35$^{-/-}$ Mäusen. Rekonstitution der p40-defizienten Tiere mit rekombinantem IL-23 führte zu einem verlängerten Überleben vergleichbar mit IL-12p35$^{-/-}$ Tieren. IL-23p19$^{-/-}$ Mäuse zeigten mäßig reduzierte Überlebenszeiten sowie eine verzögerte Erreger-Eliminierung in der Leber. Während der Interferon (IFN)-γ-Spiegel in WT und IL-23p19$^{-/-}$ Mäusen vergleichbar war, war die Produktion von IL-17 in IL-23p19$^{-/-}$ Mäusen deutlich vermindert. Relativ zur

Keimlast bildeten IL-23p19$^{-/-}$ weniger Granulome als WT Mäuse. Des Weiteren war die Rekrutierung von Entzündungszellen ins Gehirn sowie die Aktivierung von Microgliazellen in der Abwesenheit von IL-23 vermindert. Zudem war in diesen Tieren die Expression von IL-1β, IL-6 und MCP-1 verringert.

Im zweiten Teil der vorliegenden Arbeit wurde die Rolle von exogenem IL-23 in der Kryptokokkose untersucht und somit die Mechanismen, die den IL-23-Effekten zugrundeliegen naeher untersucht. *C. neoformans*-infizierte C57BL/6 WT Mäuse, die mit rekombinantem IL-23 behandelt wurden, zeigten gegenüber den Kontrolltieren signifikant verlängerte Überlebenszeiten. 100%iges Überleben während des gesamten Beobachtungszeitraumes (100 Tage) konnte jedoch nur mit einer IL-12-Behandlung erzielt werden. An Tag 21 *post infectionem* (p.i.) zeigte sich eine Reduktion der Keimlast im Gehirn in der IL-12- sowie in der IL-23-behandelten Gruppe. Während die IL-12-Behandlung mit erhöhten IFN-γ, Tumor Nekrose Faktor (TNF)-α und Stickstoffmonoxid (NO) Serumspiegeln einherging, entwickelten IL-23-behandelte Tiere trotz der erhöhten Resistenz eine Th2-Antwort (gemessen am IgE Serumspiegel) vergleichbar mit den Kontroll-behandelten Tieren. IL-23-Behandlung von WT Mäusen führt zu einer Reduktion der Keimlast am Ort der Infektion, der Peritonealhölhle, an Tag 8 p.i. Dieser Effekt zeigte sich unabhängig von IL-12 in p40-defizienten Mäusen sowie in T- und B-Zell-defizienten (RAG-defizienten) Mäusen. Gabe von IL-23 führte zur verstärkten Rekrutierung von T-Zellen sowie Zellen der angeborenen Immunität wie z. B. dendritischen Zellen, natürlichen Killerzellen und Granulozyten zum Ort der Infektion. Obwohl sich kein Unterschied in der Anzahl von Makrophagen zeigte, führte IL-23 doch zu einer vermehrten Expression von ko-stimulatorischen Molekülen auf diesen Zellen. Das durch IL-23 induzierte Zytokinmuster zeigt die charakteristische erhöhte Produktion von proinflammatorischen Mediatoren wie MCP-1, IL-1β, IL-6, TNF-α und IL-17, aber auch des Th2-Zytokins IL-5.

Aus diesen Ergebnissen läßt sich schlußfolgern, daß eine nicht-klassische Immunantwort induziert durch IL-23 die dominantere Rolle von IL-12 in der Immunität gegen *C. neoformans* komplementiert. Charaktaristisch für diese Immunantwort ist eine verstärkte Entzündungszellrekrutierung sowie ein proinflammatorisches Zytokinmuster welches hauptsächlich durch IL-1β, IL-6, TNF-α und IL-17 geprägt ist.

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