Activation of conventional dendritic cells, plasmacytoid dendritic cells and macrophages by *Salmonella* Enteritidis, *Cryptococcus neoformans* and parapoxvirus ovis

Der Fakultät für Biowissenschaften, Pharmazie und Psychologie

der Universität Leipzig

eingereichte

D I S S E R T A T I O N

zur Erlangung des akademischen Grades

Doctor rerum naturalium

Dr. rer. nat.

vorgelegt

von Diplom-Biochemikerin Sabine Siegemund

geboren am 16. 05. 1978 in Radolfzell am Bodensee

Leipzig, den 26. 10. 2007

„Investment in knowledge pays the best reward.“

Benjamin Franklin

To those who teach well

and thus motivate

Table of contents

1 Introduction

Every day we are surrounded by infectious and possibly disease-causing microorganisms such as bacteria, fungi, and viruses. Nevertheless we get sick very rarely. This is due to a fine tuned defence system elaborated during evolution, whose task is to keep our body free from foreign agents not belonging to our body and therefore was termed immune system (Latin: immunis = free, untouched, clean).

1.1 Dendritic cells and macrophages orchestrate immune responses by the release of cytokines

Immune responses are stimulated upon threats against health. Defence against infectious agents including rapidly growing bacteria, viruses and fungi requires an immediate host response in order to limit microbial growth and dissemination, and then stimulation of a prolonged specific response to clear the infection and to prevent re-infection. The immune system meets these dual needs of an immediate response to danger and an initiation of long-term protection by the elaboration of immunoregulatory cytokines.

Dendritic cells (DC) and macrophages (MΦ) are among the frontline sentinel cells that release cytokines in response to pathogenic stimuli such as bacteria, fungi and viruses. Cytokines including interleukins (IL), interferons (IFN), and tumor necrosis factors (TNF) are glycoproteins that regulate immune responses by signalling between cells in an autocrine, paracrine and endocrine manner (1). Cytokines have no intrinsic activity, they mediate their effects by binding to their corresponding receptor on the cellular target. In addition to secreted cytokines, membrane-bound cytokines, whose effect is dependent on the interaction of cells, exist (2). Cytokines are pleiotropic acting on a large number of different target cells and mediating different effects on different cell types. However, many different cytokines may act in the same way on a single cellular target (redundancy). Some cytokines mediate stronger effects in association with other cytokines, a process termed synergy. However, cytokines are also able to antagonize the effects of other cytokines. Thus, by the release of cytokines DC and MΦ are able to orchestrate immune responses (3;4).

1.2 Dendritic cells and macrophages induce immediate and prolonged immune responses

MΦ and immature DC reside within non-lymphoid tissues, where they actively capture and process antigens (5;6). Upon contact with microbial products DC and MΦ are activated and produce chemokines that recruit innate immune cells to the site of infection (7) including further macrophages and immature DC, neutrophils able to phagocytose the invading microorganism, and natural killer (NK) cells that induce apoptosis of infected cells. Furthermore, DC and MΦ are able to release soluble effector molecules and type I interferons mediating immediate anti-microbial effects.

An important soluble effector molecule is nitric oxide (NO). NO itself is not highly toxic to microorganisms, but it can react with superoxide anion to produce very toxic derivates such as peroxynitrite and nitrogen dioxide radical. Moreover, NO can attack the iron centers (8) in various key proteins such as nitrogenase (9) and ribonucleotide reductase (10) and also induces modification of thiol-containing proteins (11). Thus, the production of NO contributes to killing of the invading pathogen and restricts further pathogen spreading. In addition, NO is able to modulate cytokine production by immune cells (12).

Type I including IFN-α. interferons are produced in response to viral infection and are absolutely critical for anti-viral resistance. A major function of type I interferons is to enhance the expression of proteins mediating anti-viral effects such as kinases phosphorylating the α-subunit translation initiation factor 2 (eIF2α) leading to down-regulation of mRNA translation (13;14) and the 2'-5' oligoadenylate synthetase which activates RNase L promoting RNA degradation (15). Moreover, expression of adenosine deaminase acting on RNA (ADAR)-1, which deaminates viral RNA replication intermediates (16) leading to mutations in the viral genome, is up-regulated by type I interferons.

Furthermore, type I interferons enhance the expression of intrinsic immunity proteins. Intrinsic immunity proteins include tripartite motif protein (TRIM) 5α (17) which aims at capsid proteins of invading retroviruses, as well as Mx proteins, large GTPases targeting nucleoproteins of bunya- and orthomyxoviruses (18). Besides, apolipoprotein B mRNA-editing enzyme catalytic polypeptide like editing complex (APOBEC) 3G deaminates retroviral genomes causing mutations that impair subsequent virus replication (17).

Additionally, type I interferons sensitize cells to apoptosis upon subsequent viral infection (19), thus preventing further virus spreading, and they contribute to NK cell and cytotoxic T lymphocyte immune responses (20;21). Moreover, type I interferons facilitate cross-presentation by DC for presentation of viral antigens to $CD8^+$ T cells (22). Thus, by the

recruitment of innate immune cells and the release of anti-microbial effector molecules DC and MΦ mediate an immediate immune response to limit uncontrolled pathogen growth.

DC then migrate to the draining lymph nodes and concomitantly undergo maturation. DC maturation is characterized by increased antigen-processing capacity and enhanced expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules including CD86 and CD80 (23;24), which are necessary for successful activation of prolonged immune responses mediated by T lymphocytes (25). In the lymph nodes DC stay in T lymphocyte containing areas to present antigens that derive from phagocytosed microbes and are displayed on the cell surface as a stable peptide-MHC complex. Additionally, DC produce stimulatory cytokines promoting lymphocyte proliferation and differentiation (26).

Also MΦ have been reported to express co-stimulatory molecules (27;28) and to be able to activate T lymphocytes (29). Similar to DC, MΦ and B cells also belonging to the antigen-presenting cell types are able to trigger the activation of memory T cells (30;31). However, when DC and MΦ were more intensively studied, it became clear that DC are the principle stimulators of mixed leukocyte reactions (32). While both, DC and MΦ are able to induce proliferation of naïve allogeneic T cells, only DC can induce naïve syngeneic T cells to proliferate (33). Clustering with DC has been shown to precede and to be essential for T cell expansion (34), whereas clustering with MΦ did not result in T cell proliferation (35). Therefore, the ability to induce proliferation of naïve T cells is a unique feature of DC. By the activation of naïve T lymphocytes, DC initiate pathogen-specific immunes responses and the establishment of an immunological memory to prevent re-infection.

1.3 Dendritic cells and macrophages induce cellular immune responses essential for protection against intracellular pathogens

DC and MΦ promote cell-mediated and humoral immune responses upon contact to intracellular and extracellular pathogens. Intracellular residence is believed to confer significant advantages to microbes by insulating them from soluble components of the immune systems such as neutralizing antibodies (36). Initiation of cell-mediated immune responses therefore is essential for protection against intracellular pathogens (37), since soluble antibodies are unable to reach the pathogen in the cell and thus are unable to mediate beneficial effects against intracellular microorganisms. Additionally, cell-mediated immune responses support anti-viral immune responses by the eradication of virus-infected cells and thus prevent further spreading of the virus.

Cell-mediated immune responses are based on effector mechanisms mediated by T lymphocytes. In general, mature T lymphocytes express either CD8 or CD4 molecules allowing for the identification of cytotoxic $CD8^+$ T cells (T_{cyt}) and $CD4^+$ T helper (Th) cells. While cytotoxic $CD8^+$ T cells are able to recognize and destroy infected host cells (38), $CD4^+$ Th cells support other immune cells in their microbicidal function. Already 20 years ago two different types of Th cells termed Th1 and Th2 cells were identified (39). The latter are important for supporting the humoral immune response (40) and mediate their effects by Th2-related cytokines including IL-4 (41). Th1 cells are able to activate anti-microbial defence mechanisms such as the production of reactive oxygen and/or nitrogen intermediates (42;43) and are essential for mounting an effective cell-mediated immune response (44) by releasing IFN-γ. Recently a new set of T helper cells, characterized by the production of interleukin (IL)-17 has been identified (45). By IL-17 release these cells (now termed Th17 cells) are able to complement antimicrobial resistance mediating recruitment of neutrophilic granulocytes to the site of infection (46;47).

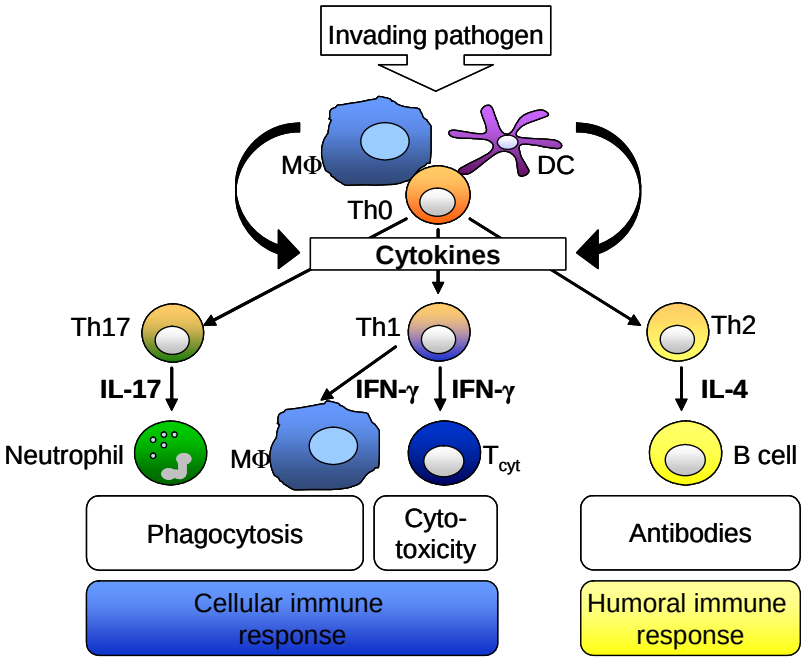

Fig. 1 Schematic representation of the immune responses initiated by DC and MΦ upon antigen encounter.

By the release of cytokines, DC and MΦ orchestrate the differentiation of naïve T helper cells into different T helper subsets, which then mediate either cellular immune responses for protection against intracellular microorganisms or humoral immune responses for protection against extracellular microorganisms. Only the main Th-mediated immune responses are shown. DC = dendritic cell; MΦ = macrophage; T_{cyt} = cytotoxic T cell; Th = T helper cell

It has been shown that for mounting a cellular Th1-driven immune response IL-12 produced by antigen-presenting cells including DC and MΦ is important (48;49). IL-12 is a heterodimeric cytokine consisting of a IL-12/23p40 and a IL-12p35 subunit (50). The search for new cytokine family members led to the detection of the structurally related cytokine IL-23, consisting of covalently linked IL-12/23p40 and IL-23p19 subunits (51). IL-23 was shown to promote the proliferation of IL-17 producing T cells (45;52), involved in autoimmunity (53). Moreover, IL-23 is able to provide antimicrobial resistance by recruiting neutrophils via induction of IL-17 (54). The latest member of the IL-12 cytokine family is IL-27 consisting of the non-covalently linked subunits Epstein Barr virus induced gene-3 (EBI-3) that is structurally similar to IL-12/23p40 and a IL-27p28 chain structurally related to IL-12p35 and IL-23p19 (55). IL-27 was shown to account for the early induction of the transcription factor T-bet and subsequently for the expression of IL-12Rβ2 subunit, which is a

prerequisite for mounting an IL-12-dependent Th1-driven immune response (56). When IL-27 was first described, it was shown to induce naïve $CD4^+$ T cell proliferation (55). Recent studies demonstrated that IL-27 inhibits the proliferation of IL-17 producing T cells (57;58). In contrast to IL-12p35 and IL-23p19, which are released only as heterodimers, murine IL-27p28 is also secreted as monomer additionally to heterodimeric IL-27 (55). Monomeric IL-27p28 is not able to induce the proliferation of naive $CD4^+$ T cells (55) but can inhibit the proliferation of IL-17 producing T cells, albeit to a lower degree than heterodimeric IL-27 (57).

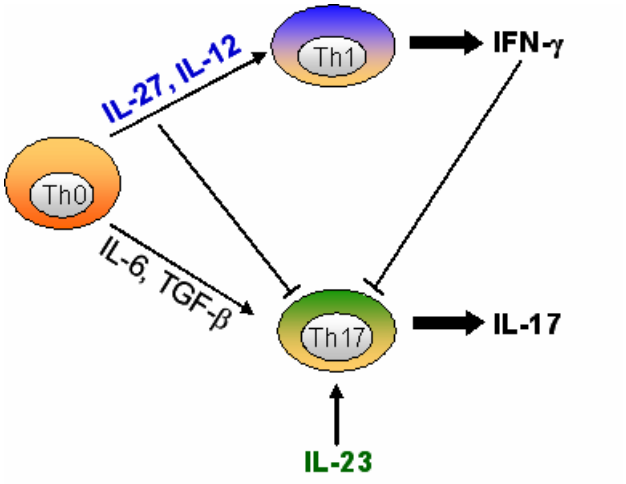

Fig. 2 IL-12 family members orchestrate T cell differentiation.
Under the influence of IL-12 and IL-27, naïve T cells differentiate into IFN-γ secreting Th1 cells. In the presence of IL-6 and TGF-β naïve T cells differentiate into IL-17 producing T cells (Th17 cells). IL-23 mediates proliferation of Th17 cells. Generation of Th17 cells is antagonized by IL-27 and by IFN-γ.

1.4 Dendritic cells and macrophages sense microorganisms with innate pattern recognition receptors

DC and MΦ detect microorganisms by germ line-encoded pattern-recognition receptors (PRR) that are able to bind highly conserved pathogen-associated molecular patterns (PAMP). PRR comprise any PAMP receptor capable of triggering antimicrobial function in leukocytes. These include C-type lectins, which are calcium-dependent carbohydrate-binding proteins such as the mannose receptor and dectin-1 (59), scavenger receptors, a family of cell surface glycoproteins binding modified lipoproteins (60), and the cytosolic nucleotide oligomerization domain-like (NLR) receptor family sensing bacterial products including peptidoglycans, flagellin, bacterial RNA and bacterial toxins (61).
Upon ligand binding some PRR are able to induce the expression of innate response genes, including those encoding co-stimulatory molecules, cytokines or chemokines. Induction of

those genes is critical not only for immediate protection against pathogen dissemination, as exemplified by the importance of interferons in resistance to viral infection (62), but also for coupling innate with adaptive immunity (63;64). So far, only few PRR families have been shown to be able to induce gene expression including virus-sensing retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) helicases and toll-like receptors (TLR).

TLR are a family of at least 12 members characterized by the extracellular domains containing varying numbers of leucin-rich repeat motifs and a cytoplasmic signalling domain homologous to that of the IL-1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain. Upon TLR engagement the TIR domain initiates a network of intracellular signalling cascades invariably involving the intracellular adaptor proteins myeloid differentiation primary response gene 88 (MyD88) and/or TIR-domain containing adaptor protein inducing interferon (TRIF). MyD88 is involved in signalling by various TLR (65;66), as shown by crucial evidence obtained from $MyD88^{-/-}$ mice (67;68). These mice were shown to be profoundly unresponsive to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9. However, $MyD88^{-/-}$ DC but not $TLR4^{-/-}$ DC were found to be responsive to lipopolysaccharid (LPS) stimulation (69;70) leading to the detection of TRIF, which is now known to mediate the TLR4-induced MyD88-independent pathway, and also to be the exclusive adaptor protein used by TLR3 (71). The intracellular signalling cascades lead to the activation of nuclear factor-kappa B (NF-κB) and interferon regulatory factors (IRF) involved in the transcription of proinflammatory cytokines and type I interferons, respectively. Most TLR family members (TLR1, TLR2, TLR4, TLR5, TLR6, TLR11) are expressed on the surface of DC and MΦ, whereas TLR3, TLR7, and TLR9 are expressed in the membranes of endosomal compartments (Fig. 3). TLR either directly or in collaboration with other molecules recognize PAMP including lipid, carbohydrate, peptide and nucleic-acid structures that are broadly expressed by bacteria, fungi and viruses (72).

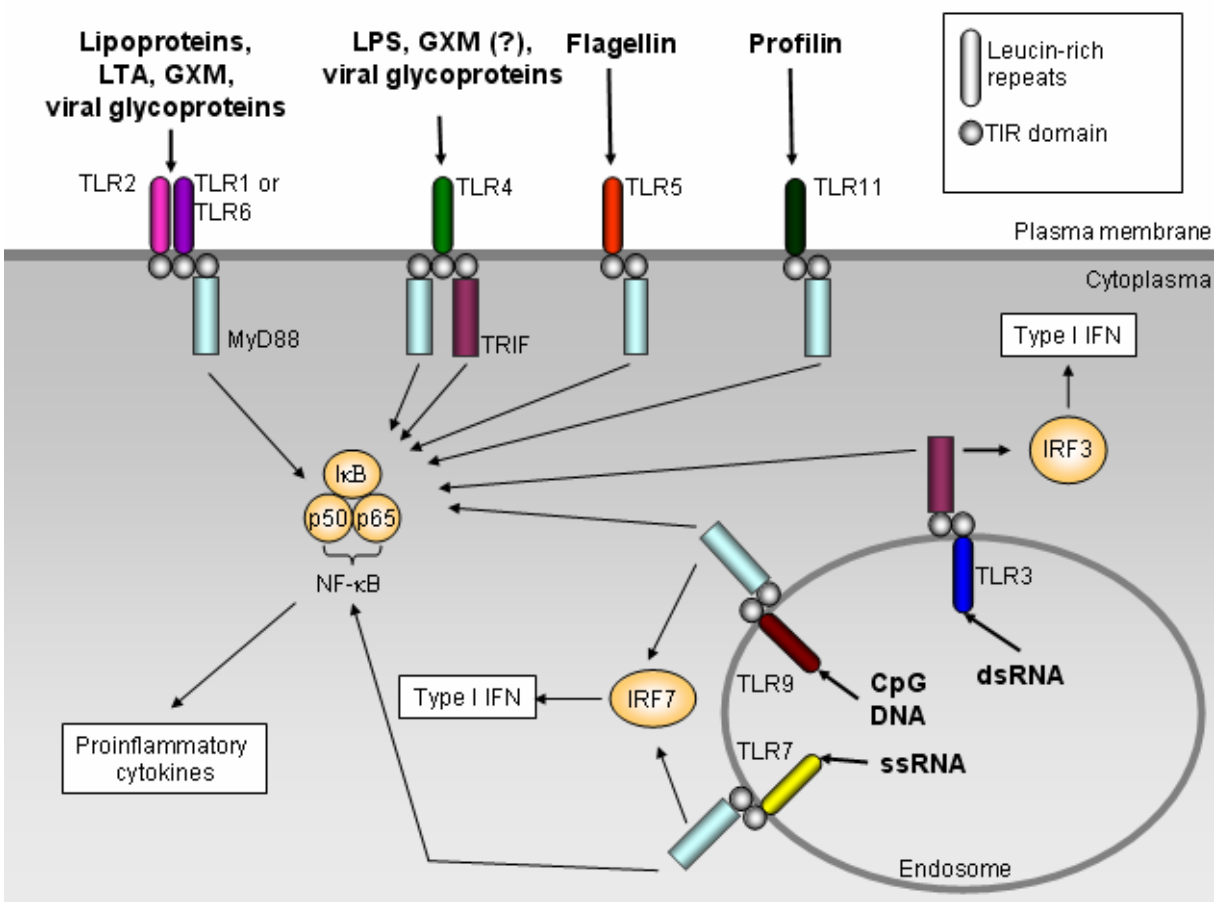

Fig. 3 Schematic representation of the structure and main signalling pathways of murine TLR family members in response to selected ligands.

Only the main adaptor molecules and signalling pathways used by the different TLR with known ligands are shown. In reality, the pathways that are triggered by the different receptors are diverse. For example, Toll-like receptor (TLR) signalling includes not only nuclear factor-kappa B (NF-κB) activation, but also mitogen-activated protein kinases, phosphatidylinositol-3-kinase and several other pathways that remarkably influence the immune response to the engagement of TLR. TLR8 is inactive in mice, TLR10 is not found in mice but in humans. The ligands for murine TLR12 and TLR13 (not shown) are currently unknown. ds = double-stranded; GXM = glucuronoxylomannan; IFN = interferon; IκB = inhibitor of NF-κB; IRF = IFN regulatory factor; LPS = lipopolysaccharide; LTA = lipoteichoic acid; MyD88 = myeloid differentiation primary response gene 88; ss = single-stranded; TLR = Toll-like receptor; TRIF = TIR-domain containing adaptor protein inducing IFN. The picture was adapted from (72).

1.4.1 Recognition of bacterial components

LPS, the major component of the cell wall of Gram-negative bacteria, is recognized by TLR4. TLR2 is involved in the recognition of a variety of microbial components including lipoteichoic acid expressed by Gram-positive bacteria, and lipoproteins expressed by Gram-negative and Gram-positive bacteria, and lipoarabinomannan, a cell wall component of mycobacteria. TLR2 interacts physically and functionally with TLR1 or TLR6, which appear to be involved in the discrimination of changes in the lipid portion of lipoproteins. Flagellin, the major component of flagella of mobile bacteria is detected by TLR5. Bacterial genomic DNA is also an immunostimulant and is recognized by TLR9. Its stimulatory effects is due to the presence of unmethylated CpG dinucleotides in a particular base context designated CpG-DNA. These CpG motifs are abundant in bacterial DNA, whereas in mammalian genomes its frequency is restrained and it is highly methylated, preventing activation of TLR9 by the genomic DNA of the host (73).

1.4.2 Recognition of fungal components

In addition to sensing bacterial PAMP, TLR2 and possibly TLR4 are involved in the recognition of fungal PAMP including glucuronoxylomannan (GXM), the major cell wall component of *Cryptococcus neoformans* (*C. neoformans*). TLR2 can collaborate with Dectin-1, a C-type lectin receptor binding β-glucan, in response to yeast and elicit a strong proinflammatory immune response (73). Dectin-2 is associated with recognition of hyphal (rather than yeast/conidial) components of *Candida albicans*, *Microsporum audouinii*, and *Trichophyton rubrum* (74). In addition further receptors such as the type 3 complement receptor, the mannose receptor and DC-SIGN are implicated in the recognition of fungi (73).

1.4.3 Recognition of viral components

Viruses contain genetic material composed of either DNA or RNA that encodes viral structural components and enzymes involved in replication. DC and MΦ sense various viral components including viral DNA, that is rich in CpG motifs, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and surface glycoproteins. Among the TLR family members, cell-surface expressed TLR2 and TLR4 are involved in the recognition of viral glycoproteins, whereas TLR3, TLR7, and TLR9 expressed in the endosome membranes sense dsRNA, ssRNA and CpG-DNA, respectively (62;73). In addition to the nucleic acid sensing TLR family members, which are only able to recognize nucleic acids that are in the endosome, cytosolic RNA-helicases including RIG-I and MDA5 are involved in recognizing viral

dsRNA (62;75). Besides, very recently the existence of a novel cytoplasmic DNA receptor termed DNA-dependent activator of IFN-regulatory factors (DAI) has been reported (76), which could be involved in the recognition of viral DNA.

To study responses of TLR, highly purified TLR ligands including the synthetic TLR3 ligand polyinosinic-polycytidylic acid (poly I:C), LPS binding to TLR4, synthetic anti-viral imidazoquinoline components such as the TLR7 ligand R-848, and synthetic stimulatory CpG oligodesoxynucleotides (CpG-ODN) binding to TLR9 can be used (62).

1.5 DC subtypes: Conventional DC versus plasmacytoid DC

Since the first description of DC in 1973 (77), it has become increasingly clear that they are heterogeneous comprising two phenotypically and functionally distinct populations. Conventional DC have a dendritic form and exhibit typical DC functions such as antigen uptake, processing, and presentation to T cells. By flow cytometry murine conventional DC are characterized as $CD11c^+$ $CD11b^+$ $B220^-$ (78). Furthermore, they do not express $mPDCA\text{-}1^-$ (personal observation). Following antigen uptake in the peripheral tissues conventional DC migrate through the lymphatic vessels into the lymph nodes and activate T cell dependent immune responses as described in 1.2.

Plasmacytoid DC, also known as natural interferon producing cells, are major producers of type I interferons in response to stimulation with viruses (79). They are a rare cell population found in the peripheral blood, lymphoid organs, bone marrow, thymus, liver and lung. Murine plasmacytoid DC are defined by flow cytometry as $CD11c^{low}$ $CD11b^-$ $B220^+$ $m\text{-}PDCA\text{-}1^+$ (80-82). Murine splenic plasmacytoid DC express high levels of TLR7 and TLR9 but only low levels of TLR3 compared to conventional DC, making plasmacytoid DC likely to more sensitively recognize dsDNA and ssRNA (83).

1.6 Objectives

The goal of the present Ph.D. thesis was to define the mechanisms of activation of conventional and plasmacytoid DC subsets and MΦ upon stimulation/infection with (i) bacterial, (ii) fungal and (iii) viral pathogens. Therefore the activation of DC and MΦ by (i) the facultative intracellular bacterium *Salmonella (S.)* Enteritidis, (ii) the facultative intracellular yeast *Cryptococcus (C.) neoformans*, and (iii) parapoxvirus ovis was characterized. Furthermore, the molecular mechanisms of recognition leading to DC and MΦ activation were investigated.

S. Enteritidis

For protection against facultative intracellular pathogens such as *S*. Enteritidis Th1-mediated immune responses orchestrated by IL-12 family members are essential (37;84;85). However, when this Ph.D. thesis was started, the exact cellular sources of IL-12 family production were unclear. Therefore, this Ph.D. thesis aimed to investigate the contribution of DC and MΦ to the production of IL-12 family members in response to *S*. Enteritidis *in vitro* and *in vivo*. Moreover, the role of TLR2 and TLR4 in the induction of IL-12 family members by *S*. Enteritidis was defined.

C. neoformans

Similar to *S*. Enteritidis, immunity to *C. neoformans* is dependent on IL-12 and is complemented by IL-23 (86;87). However, the IL-12 family member producing cell types during cryptococcosis were elusive when this Ph.D. thesis was started. Thus, production of IL-12 family members by bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMMΦ) in response to *C. neoformans* was studied *in vitro*. The involvement of TLR2, TLR4 and MyD88 in *C. neoformans*-induced IL-12 family member production was investigated.

C. neoformans has been reported to inhibit activation of antigen-presenting cells (88). However, a side-by-side analysis of conventional DC and MΦ had never been performed. Moreover, the role of plasmacytoid DC, a recently discovered DC subset, in response to infection with *C. neoformans* had not been studied. Thus, another objective of the present Ph. D. thesis was to compare the activation of conventional bone marrow-derived DC (BMDC), plasmacytoid BMDC and bone marrow-derived MΦ (BMMΦ) as assessed by the expression of MHC-II, CD86 and CD80 upon *in vitro* stimulation with *C. neoformans*.

Parapoxvirus ovis

Chemically inactivated parapoxvirus ovis (iPPVO) mediates immunostimulatory effects enhancing antimicrobial resistance (89;90). Current available data point to innate immune cells which are activated by iPPVO. However, the exact cellular targets of iPPVO and the mechanism(s) of recognition remained elusive. Therefore, another goal of this Ph. D. thesis was to characterize the activation of conventional BMDC, plasmacytoid BMDC and BMMΦ as assessed by production of IFN-α and other cytokines, as well as by the expression of MHC-II, CD86, and CD80. This Ph.D. thesis further aimed to identify the BMDC-activating constituent of iPPVO and to define the role of MyD88 and TLR9 in the activation of BMDC by iPPVO.

2 Animals and Material

2.1 Mice

C57BL/10ScSn (wild-type) mice, TLR4^{def} (C57BL/10ScN) and TLR2^{def} (C57BL/10ScSn-TLR2$^{-/-}$) mice were bred at the animal facility of the Max-Planck-Institute of Immunobiology (Freiburg, Germany) and kept under specific-pathogen-free (SPF) conditions and in accordance with the guidelines approved by the Animal Care Usage Committee of the Regierungspräsidium Leipzig, Germany at the Institute of Immunology (College of Veterinary Medicine (Leipzig, Germany) and the Max-Planck-Institute of evolutionary Anthropology (Leipzig, Germany). C57BL/6J wild-type mice, IL-12p35$^{-/-}$ (49) and IL-23p19$^{-/-}$ (91) were bred at the Institute of Immunology (College of Veterinary Medicine (Leipzig, Germany) and the Max-Planck-Institute of evolutionary Anthropology (Leipzig, Germany). Female mice were used at the age of 8 to 16 weeks for all experiments. Food and water were given *ad libitum*.

2.2 Reagents

Agarose	Invitrogen, Karlsruhe, Germany
β-Mercaptoethanol	Sigma-Aldrich, Taufkirchen, Germany
Bromine phenol blue	Sigma-Aldrich, Taufkirchen, Germany
CpG-ODN 2216[1]	TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany
DMSO	Sigma-Aldrich, Taufkirchen, Germany
dNTP´s (25 mM)	Diagonal, Münster, Germany
EDTA (sodium salt)	Merck, Darmstadt, Germany
Ethanol	Carl Roth GmbH, Karlsruhe, Germany
Ethidium bromide	Sigma-Aldrich, Taufkirchen
FCS	PAA Laboratories GmbH, Cölbe, Germany
Formaldehyde (37 Vol %)	Carl Roth GmbH, Karlsruhe, Germany
Gelatine (Pig)	Merck, Darmstadt, Germany
Glycerol	Sigma-Aldrich, Taufkirchen, Germany
Glucose	Sigma-Aldrich, Taufkirchen, Germany
L-Glutamin (200 mM)	Sigma-Aldrich, Taufkirchen, Germany

[1] CpG-ODN 2216 had the sequence 5'-GsGsGGGACGATCGTCsGsGsGsGsG-3' (s = phosphothioate)

GolgiPlug®	Miltenyi Biotec, Bergisch-Gladbach, Germany
HEPES solution, pH 7,4	PAA Laboratories, Cölbe, Germany
KCl	Merck, Darmstadt, Germany
KH_2PO_4	Fluka-Chemie Ag, Buchs, Germany
K_2HPO_4	Carl Roth GmbH, Karlsruhe, Germany
LD-Columns	Miltenyi Biotec, Bergisch-Gladbach, Germany
LPS (from *Salmonella* Abortus equi, S-Form)	Alexis, Grünberg, Germany
$MgCl_2$	Promega, Mannheim, Germany
MOPS	Merck, Darmstadt, Germany
NaCl	Carl Roth GmbH, Karlsruhe, Germany
Na_2CO_3	Carl Roth GmbH, Karlsruhe, Germany
$NaHCO_3$	Sigma-Aldrich, Taufkirchen, Germany
NaOH	Carl Roth GmbH, Karlsruhe, Germany
Na_2HPO_4	Carl Roth GmbH, Karlsruhe, Germany
Naphtylethylendiamide-Dihydrochloride	Sigma-Aldrich, Taufkirchen, Germany
Penicillin/Streptomycin (10^4 U/ml, 10^4 µg/ml)	Sigma-Aldrich, Taufkirchen, Germany
Phenol red	Sigma-Aldrich, Taufkirchen, Germany
Polyinosinic–polycytidylic acid potassium salt (poly I:C)	Sigma-Aldrich, Taufkirchen, Germany
R-848 (resiquimod)	Alexis, Grünberg, Germany
Saponin	SERVA FEINBIOCHEMIKA GmbH & Co KG, Heidelberg, Germany
SDS	Sigma-Aldrich, Taufkirchen, Germany
Sodium pyruvate (1 mM)	Sigma-Aldrich, Taufkirchen, Germany
Sulphanilamide	Sigma-Aldrich, Taufkirchen, Germany
Tris Base	AppliChem GmbH, Darmstadt, Germany
Trypan blue	Sigma-Aldrich, Taufkirchen, Germany
Tryptone	Carl Roth GmbH, Karlsruhe, Germany
Türk's Solution	Merck, Darmstadt, Germany

2.3 Pathogens and biologicals

2.3.1 *Salmonella enterica* serovar Enteritidis

The auxotrophic (ade^-, his^-) vaccine strain *Salmonella enterica* serovar Enteritidis SalmoVac® (kindly provided by the Impfstoffwerke Dessau-Tornau, Rosslau), originally developed from the parental strain *Salmonella enterica* serovar Enteritidis 64/03 (ade^+/his^+) (92;93), was used in all experiments. It was kept as frozen stock in FCS/10% DMSO at -80°C.

2.3.2 *Cryptococcus neoformans* var. *neoformans*

The encapsulated highly virulent *C. neoformans* strain 1841, serotype D, was originally obtained from F. Hoffmann-La Roche Ltd, Basel, Switzerland (86;94;95). The *C. neoformans* serotype D strain CAP67, an acapsular mutant strain was kindly provided by Dr. Bettina Fries, Albert-Einstein College of Medicine, Bronx, NY. The acapsular *C. neoformans* serotype D strain C566 was kindly provided by Prof. Dr. Kwon-Chung, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Diseases, NIH, Bethesda, MD. All strains were kept as frozen stocks in 10% skim milk at -20°C. Prior to *in vitro* stimulations, *C. neoformans* strains were cultured under constant shaking in Sabouraud medium at 30°C for 16 h. Before stimulation, *C. neoformans* was washed twice in PBS.

2.3.3 Parapoxvirus ovis

Chemically inactivated parapoxvirus ovis was kindly provided by Dr. Rüdiger Raue (Pfizer® Ltd., Sandwich, UK). Heat inactivated PPVO (hiPPVO) was obtained by incubating iPPVO for 60 min at 60°C in a water bath.

2.4 Cell lines

L929 cells naturally producing M-CSF were kindly provided by Prof. Dr. T. Jungi (University of Bern, Switzerland). Ag8653 cells, transfected with a gene for murine GM-CSF (96), were a kind gift from Prof. Dr. B. Stockinger (National Institute for Medical Research, London, GB). All cells were stored in liquid N_2 and regularly tested for *Mycoplasma spp.*.

2.5 Enzymes

NADPH oxidase	Biomaster, Köln
SA-HRP	Southern Biotech, Birmingham, AL, USA
Trypsin/ EDTA	Sigma-Aldrich, Taufkirchen

2.6 Antibodies

All used antibodies are listed in the appendix. In addition, the following commercial Kits were purchased for ELISA measurements:

TNF-α (Duoset®)	R&D-Systems, Wiesbaden
IL-10 (Duoset®)	R&D-Systems, Wiesbaden
IL-17 (Duoset®)[2]	R&D-Systems, Wiesbaden
IL-27p28 Quantikine®	R&D-Systems, Wiesbaden
TGF-β (Duoset®)	R&D-Systems, Wiesbaden

2.7 Protein standards

Recombinant murine IL-23 was expressed and purified at Schering-Plough Corporation, Palo Alto, CA; USA (51) and provided as a kind gift by Dr. Robert A. Kastelein; recombinant murine IL-12 and homodimeric p40 (IL-12/23p40) were expressed and purified at Hoffmann-La Roche (Roche), Nutley, NJ (97) and provided as a kind gift by Dr. Maurice Gately. Recombinant murine IFN-γ, and recombinant murine IL-6 were obtained from Hoffmann-La Roche Ltd, Nutley, NJ, USA. Recombinant murine IFN-α was purchased from Lee Biomolecular Research Laboratories, Inc., San Diego, CA, USA, and is measured in international reference units (IRU)/ml as provided by the National Institutes of Health (98;99).

2.8 Growth factors

Human Fms-like tyrosine kinase 3 ligand (Flt3L) was kindly provided by Amgen, Seattle, USA. GM-CSF as M-CSF-containing supernatants were obtained as described in 3.1.2 and 3.1.1, respectively.

2.9 Media, Buffer, Solutions

2.9.1 Buffers and solutions for cell isolation

1x PBS:

0.80 % (w/v) NaCl
0.02 % (w/v) KCl
0.23 % (w/v) Na_2HPO_4 x 2 H_2O
0.02 % (w/v) KH_2PO_4 pH = 7.4

[2] This ELISA quantifies IL-17A

PBS / EDTA:

50.5 mM EDTA in 10 % 10x PBS pH = 7.4; sterile filtered

Gey´s-Solution (for lysis of erythrocytes):

Sol.A:	0.65 M	NH_4Cl
	24.80 mM	KCl
	4.20 mM	Na_2HPO_4
	0.81 mM	KH_2PO_4
	25.23 mM	Glucose
	0.14 mM	Phenolred
Sol.B:	0.42 % (w/v)	$MgCl_2$ x 6 H_2O
	0.07 % (w/v)	$MgSO_4$
Sol.C:	2.25 % (w/v)	$NaHCO_3$

Working solution: 20 % (v/v) sol. A, 5 % (v/v) sol. B and 5 % (v/v) sol. C

Trypan blue:

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

MACS buffer (for cell depletion):

5 % FCS
2 mM EDTA in 1x PBS, sterile filtered; pH7.4

2.9.2 Buffers and solutions for flowcytometry

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

Permeabilization buffer:

0.55% (w/v)	Saponin
5% (v/v)	FCS
2mM	HEPES, pH 7.4 in 1x PBS

2.9.3 Buffers and Solutions for ELISA

10x PBS:

0.35 % (w/v)	KH_2PO4_4
1.58 % (w/v)	Na_2HPO_4 (anhydrous)
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 (Pig)	
0.5 % (w/v)	BSA	in 1x PBS

Serum diluent:

0.10 % (w/v)	Gelatine (Pig)	
0.05 % (v/v)	Tween 20	in 1x PBS

Washing buffer:

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

Substrate-buffer:

TMB-Peroxidase-Substrate	KPL, Medac, Wedel, Germany

2.9.4 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 H3PO4
Working solution: 1:1 dilution of solution A and B (stable for 12 h)

2.9.5 Media and solutions for murine, bacterial and fungal cell culture

If not indicated otherwise, all cell culture reagents were purchased PAA-Laboratories, Cölbe, Germany.

DMEM culture medium for the cultivation of L929 cells and BMMΦ:

Dulbecco´s modified Eagle medium (DMEM), high glucose

10 % (v/v)	FKS
1 % (v/v)	L-Glutamin (200 mM)
1 % (v/v)	Penicillin/Streptomycin (10^4 U/ml, 10^4 µg/ml)

IMDM culture medium for the *ex vivo* cultivation of PEC and DLN cells:

Iscove's Modified Dulbecco's Medium (IMDM) Medium

10 % (v/v)	FKS
1 % (v/v)	Penicillin/Streptomycin (10^4 U/ml, 10^4 µg/ml)

Pluznik´s Medium in DMEM for the generation of BMMΦ:

30 % (v/v)	M-CSF-containing supernatant	
10 % (v/v)	FCS	
5 % (v/v)	Horse serum	
1 mM	Sodium pyruvate	
1 % (v/v)	L-Glutamine (200 mM)	
1 % (v/v)	Penicillin/Streptomycin (10^4 U/ml, 10^4 µg/ml)	
50 µM	β-Mercaptoethanol (0,175 %ig)	Sigma-Aldrich, Taufkirchen

Fms-like tyrosin-kinase 3 ligand (Flt3L) culture medium in RPMI for the generation of plasmacytoid and conventional BMDC:

RMPI	Medium	
10 % (v/v)	FCS	
1 % (v/v)	Penicillin/Streptomycin (10^4 U/ml, 10^4 µg/ml)	
300 ng/ml	huFlt3L	kindly provided by Amgen, Seattle, USA

RPMI culture medium in for the generation of GM-CSF-containing supernatant:

RMPI	Medium
10 % (v/v)	FCS
1 % (v/v)	Penicillin/Streptomycin (10^4 U/ml, 10^4 µg/ml)

Cryomedium for cell lines:

10 % (v/v)	DMSO in FCS

Solid medium for growth of *S.* Enteritidis:

XLD-Agar	SIFIN, Berlin, Germany

Cryomedium for *S. Enteritidis* stock:

10 % (v/v)	DMSO in FCS

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

2% glucose (w/v)
1% peptone (w/v) in ddH2O

Cryomedium for *C. neoformans* stock

10 % (w/v) skim milk in PBS

2.10 Kits

Venor® *GeM* Mycoplasma detection kit	Minerva Biolabs GmbH, Berlin, Germany

2.11 DNA standards

100 bp DNA Ladder	Invitrogen, Karlsruhe, Germany

2.12 Equipment

ELISA equipment:

ELISA-Reader: Spectra-max 340	Molecular Devices, Munich, Germany
ELISA-Washer (Ultrawash PLUS)	DYNEX Technologies Inc., Chantilly, VA, USA
Immunoplates Maxisorb	NUNC, Wiesbaden, Germany

Flow cytometry equipment:

FACS Calibur	Becton Dickinson, Heidelberg, Germany

Cell culture equipment:

CO_2-incubator for cell culture	Heraeus, Osterode, Germany
Incubator for fungal growth	Memmert, Schwabach, Germany
Incubator for bacterial growth	Heraeus, Osterode, Germany
Cell culture plates	TPP®, Trasadingen, Switzerland
Cell Strainer	Becton Dickinson, Franklin Lakes, NJ, USA
Cell counter	Beckman Coulter Vi-cell™ cell viability analyser
Benchtop incubator GFL 3031	Gesellschaft für Labortechnik mbH, Burgwedel, Germany
Cell Scraper, sterile	Costar®, Cambridge, MA, USA
Laminar flow hoods: HERA-safe	Heraeus, Osterode, Germany
pulse welding apparatus	Rische & Herfurth Ltd., Germany
Polystar 100 GE	Maschinen- und Apparatebau, Hamburg, Germany

Centrifuges:

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

Gel electrophoresis equipment:

DNA-Sub Cell™ electrophoreses chamber	Bio-Rad Laboratories, Hercules, CA, USA
Power supply model 200/2.0	Bio-Rad Laboratories, Hercules, CA, USA
UV-Transilluminator	MWG-Biotech, Ebersberg

Scales:

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

Microscopes:

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

Miscellaneous:

iCycler	Biorad, München, Germany
pH-Meter: inoLab® Level2	WTW Ltd, Weilheim, Germany
Ultra-Turrax homogenizer	Ika-Werke GmbH, Staufen, Germany
Waterbath: Isotemp 205	Scientific Support, Hayward, CA, USA

Consumables:

Test tubes, syringes	Becton Dickinson, Franklin Lakes, NJ, USA
Needles	Neolis®, Terumo ®, Leuven, Belgium
Pipette tips	Eppendorf, Hamburg, Germany

2.13 Software

BD CellQuest™ pro	Becton Dickinson, Heidelberg; Acquisition and analysis of flow cytometry data

Graph Pad Prism™ 4	GraphPad Sofware™, San Diego, CA, USA; Statistics and data visualization
Reference Manager Professional 10/11	Thomson Research Soft, Carlsbad, CA, USA; Management of bibliographies
Softmax Pro 3.1.2 /5.0.0	Molecular Devices, Sunnyvale, CA, USA Acquisition and analysis of ELISA-Reader data
Microsoft® Office 2002/2003	Microsoft, Redmond, WA, USA Spreadsheet analysis, data presentation

3 Methods

3.1 *In vitro* procedures

3.1.1 Preparation of M-CSF-containing supernatant

To obtain M-CSF-containing supernatant, L929 cells were expanded in DMEM culture medium at 37°C in an humidified atmosphere containing 8% CO_2. Since for the production of M-CSF-containing supernatant the adherence of L929 cells is advantageous, 1×10^5 cells/ml were seeded into teflon bags with the hydrophile side introverted and the hydrophobe side outwards. After a 7 day incubation period, cell free supernatants were harvested, apportioned and stored at – 80°C for up to two years.

3.1.2 Preparation of GM-CSF-containing supernatant

To obtain GM-CSF-containing supernatant, Ag8653 cells transfected with the murine gene for GM-SCF (96) were cultured in RPMI culture medium with a starting concentration of 5×10^5 cells/ml at 37°C in a humidified atmosphere containing 5% CO_2. Cells were split every 3 - 4 days by resuspending 20 ml cell suspension in 80 ml of fresh culture medium. Cell free supernatants were harvested, filtered sterile, apportioned and stored at -80°C for up to 9 months.

3.1.3 Generation of bone marrow-derived cell cultures

Conventional BMDC were cultured as described elsewhere (100). In brief, femurs of C57BL/10 wt, $TLR2^{def}$ or $TLR4^{def}$ mice were removed and the bone marrow was flushed out with PBS containing 5% FCS using a syringe with a 27 gauge diameter needle. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1% penicillin/streptomycin, 50 µM β-mercaptoethanol and 10% supernatant from a cell line (routinely tested for the absence of *Mycoplasma spp*.) transfected with the murine GM-CSF gene (96). At day 10 the nonadherent cells were harvested. For further experimental procedures cells were adjusted to 5×10^5 cells/ml in culture medium supplemented with 5% of the GM-CSF-containing supernatant, plated in 24-well plates, and stimulated after 24 h incubation.

For generation of a mixed culture of plasmacytoid and conventional DC, bone marrow cells from C57BL/6 mice were cultivated for 7 d at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 supplemented with 10 % heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol (Sigma, Taufkirchen,

Germany), 0.2% vitamins, essential and non-essential amino acids, and 300 ng/ml Flt3L (kindly provided by Amgen Inc., Seattle, USA). For stimulation, cells were seeded into 24-well plates in the culture medium and stimulated after 1-2 h incubation.

For differentiation of bone marrow-derived macrophages (BMMΦ) bone marrow cells (1×10^5 cells/ml) were cultivated for 10 days at 37°C un Pluznik's medium containing M-CSF-containing supernatant derived from the cell line L929 (101). After a 10-day differentiation period at 37°C in humidified atmosphere with 8% CO_2, BMMΦ were washed twice with a serum-free, high-glucose formulation of DMEM and incubated overnight (5×10^5 cells/ml) in a 24-well plate until stimulation.

3.1.4 *In vitro* stimulation

The capsulated *C. neoformans* strain 1841 and the acapsular mutants *C. neoformans* C566 and *C. neoformans* Cap67 were cultured under constant shaking in Sabouraud dextrose medium at 30°C for 16 h. Before stimulation, *C. neoformans* was washed twice in PBS. A final concentration of 1×10^7 cfu/ml (MOI = 20) was found to optimal for cytokine responses following *in vitro* stimulation after titration experiments. *S.* Enteritidis was thawed and washed twice in PBS. For stimulation, *S.* Enteritidis was used at a MOI = 10. Chemically inactivated parapoxvirus ovis was thawed and used, if not indicated otherwise, at a MOI = 5. For control, cells were stimulated with poly I:C (100 µg/ml), LPS (5 µg/ml), R-848 (1 µg/ml) or CpG-ODN (1 µM). All used stimuli were resuspended in the appropriate cell culture medium prior to cell stimulation.

3.2 Infection and Dissection of mice

3.2.1 Infection of mice

For infections, one vial of the frozen *S.* Enteritidis stock (SALMOVAC) was thawed and washed twice with sterile PBS. Inocula were resuspended in 500 µl sterile PBS, and injected intraperitoneally (i.p.). 129 Sv/Ev mice were infected with 5×10^6 cfu/ml, for C57BL/6J mice an infection dose of 2.5×10^6 was found to be optimal. The accuracy of the infection dose was confirmed by plating serial dilutions of the infection dose on XLD agar plates. The control group was infected with 500 µl sterile PBS.

3.2.2 Time points of dissection

The present study focussed on the contribution of DC and MΦ to the production of IL-12 family members during innate and adaptive immune responses. For innate mechanisms, 1 day

post infection (dpi) was chosen for analysis. Since it was reported, that after i.p. infection with *S.* Enteritidis organ burdens of the spleen and liver rise until 14 dpi and decline thereafter (102) day 14 was additionally chosen for analysis to investigate the production of IL-12 family members by peritoneal DC and MΦ at the time point of the T-cell dependent adaptive immune response.

3.2.3 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 suberic boards. After superficial disinfection the ventral skin was opened by a median caudocranial cut from the pelvis to the neck, and blood was collected via cardiac puncture. Following peritoneal lavage (PEL), if applicable, spleen, liver and parathymic lymph nodes were removed.

3.2.4 Peritoneal lavage

Ice-cold PBS/EDTA (2 ml) was 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 bacterial burden determination as well as cytokine and NO assessment. A second lavage with a volume of 5 ml PBS/EDTA was conducted as described above. Cells of both lavages were pooled for flow cytometry analysis analyses and counted on a hemacytometer. To obtain enough cells for *ex vivo* culture, PEC of all animals belonging to the same group were pooled.

3.2.5 Organ removal

The peritoneal cavity was opened in the median by an incision from the pelvis to the neck. The thorax was opened, and parathymic lymph nodes, liver and spleen were removed. Organs were weighed and stored in sterile tubes at 4°C for further processing.

3.3 Specimen processing and evaluation

3.3.1 Determination of bacterial burden in organs and peritoneal lavage

At the indicated time points control samples of weighed organs were homogenized in 1 ml of sterile PBS using an Ultra-Turrax homogenizer. Serial dilutions of the homogenates and fluids of the first PEL (see 3.2.4) were plated on XLD agar plates. Colonies of *S.* Enteritidis were counted after 16-24 h of incubation at 30°C.

3.3.2 *Ex vivo* cell culture

For *ex vivo* culture, PEC were adjusted to 5×10^6 PEC/ml in IMDM containing additional 10% FCS and 1% penicillin/streptomycin and cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

3.3.3 Peritoneal DC and MΦ depletion

PEC were depleted by high-gradient cell separation (MACS; Miltenyi Biotec, Bergisch–Gladbach, Germany). $CD11c^+$ DC or $F4/80^+$ MΦ were removed with PE-labelled anti-mouse-CD11c or anti-mouse-F4/80, respectively, followed by incubation with an anti-PE-antibody coupled to magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions using LD MACS columns and the VarioMACS system (Miltenyi Biotec). Success of depletion was confirmed prior to *ex vivo* culture by FACS analysis. PEC depleted either of DC or of MΦ were adjusted to $3 – 5 \times 10^6$ cells/ml in IMDM supplemented with 10% FCS, 1% penicillin/streptomycin and 1 mM L-glutamine and plated in 24-well plates. Following overnight incubation, PEC were cultured for another 24 h in the absence or presence of *S.* Enteritidis.

3.3.4 Flow cytometric analyses

All FACS staining incubations were conducted at 4 - 8°C. For each stained sample an unspecific Ig isotype control was added. FACS analyses were conducted with a FACScalibur flow cytometer. At least 1×10^5 cells were used per stain.

Cells were transferred to Eppendorf or Falcon reaction tubes and spun down at 300 × g for 8 min at 4°C. The supernatant was removed and the pellet was adjusted to 2×10^8 cells/ml in FACS buffer containing 25 µg/ml anti-mouse CD16/CD32 monoclonal antibody. The cell suspension was seeded into V-bottom plates at a volume of 5 µl/well and incubated for 10 min. Then another 5µl containing fluorophore-conjugated monoclonal antibodies (for concentrations see Chapter 0) was added. Incubation for 25 minutes was followed by washing the cells twice (300 × g, 4°C, 2 min) with FACS buffer. The pellet was finally resuspended in fixation buffer and stored at 4°C protected from light until measurement.

To enumerate absolute cell numbers of peritoneal and DLN cell populations, total cells of single animals were counted using the Vi-CELL™ cell viability analyser from Beckman Coulter. Absolute numbers of the different populations were calculated by multiplication of

the percentages of each population acquired by FACS analysis with the total number of PEC or DLN cells.

3.3.5 Intracellular cytokine staining of BMDC

3.3.5.1 IL-12/23p40

For intracellular IL-12/23p40 staining, BMDC were stimulated with heat-inactivated or viable *S.* Enteritidis with a MOI of 10 or iPPVO with a MOI of 5 for the indicated time in the presence of GolgiPlug (according to BD PharMingen's Cytofix/Cytoperm Plus kit instructions) for the final 3-5 h. This was followed by surface staining in the presence of 25 µg/ml anti CD16/CD32 with anti-CD11c (FITC or APC-labelled) and, if plasmacytoid BMDC were stained, anti-B220 (FITC-labelled), permeabilization for 30 min at 4°C with permeabilization buffer, and intracellular IL-12/23p40 staining using anti-IL-12/23p40 (PE-conjugated).

3.3.5.2 IFN-α

For intracellular IFN-α staining, a mixed culture of conventional and plasmacytoid BMDC were stimulated with iPPVO with a MOI of 5 in the presence of GolgiPlug for the final 2-3 h as described above. In addition to the murine DC marker CD11c, plasmacytoid DC express B220, in contrast to conventional DC which are $CD11c^+$ $B220^-$. Thus, these two markers allow the identification and separate analysis of both subsets in a mixed culture of conventional and plasmacytoid DC such as Flt3L-generated BMDC. The B220 antibody (FITC conjugated) required for the identification of plasmacytoid BMDC derives from rats as does the unconjugated anti-IFN-α antibody. Thus, the secondary PE-labelled anti-rat antibody used for detection of the rat-anti-IFN-α antibody is also able to bind the anti-B220 antibody, which leads to abrogation of the B220-signal necessary for the identification of plasmacytoid BMDC. To avoid the latter BMDC were first stained for intracellular IFN-α with the primary and secondary antibody followed by staining of the surface markers with directly fluorochrome-labelled antibodies. At least 1×10^6 cells were incubated in the presence of 25 µg/ml anti CD16/CD32 for 10 min, washed once in FACS buffer and fixed in fixation buffer for 20 min. This was followed by permeabilization of the cells in permeabilization buffer for 20 min, and intracellular IFN-α staining using a purified monoclonal rat anti-IFN-α antibody and a polyclonal rabbit anti-IFN-α antibody. After 20 min of incubation, cells were washed twice with permeabilization buffer, resuspended in permeabilization buffer and incubated another 20 min with secondary anti-rat-IgG and anti-rabbit-IgG antibodies (PE-labelled). Then, cells were washed once with permeabilization buffer and twice in FACS buffer

followed by surface staining with anti-CD11c (APC labelled) and anti-B220 (FITC-labelled). After incubation for 25 minutes cells were washed twice with FACS buffer. The pellet was finally resuspended in FACS buffer and measured within 24 h.

3.3.6 Quantification of nitric oxide

NO in oxygen-containing solutions is chemically unstable and undergoes rapid oxidation to NO_2. In the presence of various biological tissue components this oxidation is catalyzed and further oxidation of NO_2 to NO_3 is promoted. Therefore, it is necessary to measure both NO_2 and NO_3 to accurately analyse the level of total NO. Thus, NO_3 in lavage fluids 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). Oxidation of NO_2 to NO_3 does not occur in supernatants of cell cultures. Thus, NO_2 in cell culture supernatants of *in vitro* and *ex vivo* cultured cells could be measured without prior handling. The concentrations of NO_2 can be determined by Griess reagent reaction (103). 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).

3.3.7 Quantification of cytokines

Cytokines were quantified by sandwich ELISA. For that, 96 well U-bottom plates were incubated over night with 50 µl of cytokine-specific capture antibody diluted in carbonate buffer (IL-12p40, IL-23, IFN-γ) or PBS (all other capture antibodies). On the next day, the plate was washed once and unspecific binding was blocked by incubating the plate with blocking buffer for 2 h at room temperature (RT). Following another washing step, samples and the cytokine standard were plated at a volume of 50µl and incubated for 2 h at RT. Of note, when IFN-α was measured, samples and standards were incubated at least 4 h at RT. Then, the plate was washed three times, and the biotinylated detection antibody was applied and incubated for 2 h at RT. For IFN-α ELISA, unbiotinylated detection antibody was used. This was followed by four washing steps, incubation with streptavidin or secondary antibody (for IFN-α) conjugated to horse radish peroxidase. After another 30-60 min incubation, plates were washed 5-times. For the final colorimetric reaction the TMB Microwell Peroxidase System (KPL, Gaithersburg, USA) was used as substrate. ELISA plates were measured at 650 nm and 480 nm with a Spectra-max 340 ELISA reader.

All washing steps were preformed with washing buffer. Detection antibodies and standards were dissolved in serum diluent prior to application.

3.3.8 Statistical analysis

Kruskal-Wallis statistics followed by Dunns post test was performed using GraphPad PRISM® software (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 Characterization of bone marrow-derived conventional and plasmacytoid dendritic cell and macrophage cultures

For *in vitro* studies, bone marrow cells were differentiated into (i) conventional bone marrow-derived dendritic cells (BMDC) under the influence of GM-CSF-containing supernatant generated by cells that are transfected with the murine gene for GM-CSF (96), (ii) a mixed culture of plasmacytoid and conventional BMDC under the influence of recombinant human fms-like tyrosin-kinase 3 ligand (Flt3L) (82), or (iii) bone marrow-derived macrophages (BMMΦ) under the influence of M-CSF-containing supernatant of L929-cells (101). GM-CSF-generated BMDC display phenotypical and functional features similar to the conventional or myeloid DC which are present in the secondary lymphoid organs such as lymph nodes or spleen (80;100). Conventional BMDC express the same TLR, produce similar cytokine responses and induce similar T cell proliferation as splenic DC (104-106). BMDC generated in the presence of Flt3L comprise plasmacytoid and conventional DC closely resembling steady-state murine splenic plasmacytoid DC, $CD8^+$ conventional DC and $CD8^-$ conventional DC. The common hallmarks of Flt3L-generated BMDC and their splenic counterparts include the expression of surface markers, transcription factors, TLR receptors, chemokine receptors, and TLR-mediated chemokine and cytokine responses (82;107-109). M-CSF generated BMMΦ share characteristic activities with macrophages freshly isolated from animals. These include adhesion properties, phagocytic activity, and production and secretion of endogenous mediators such as inflammatory cytokines upon stimulation with LPS and other microbial ligands (110-112). Thus, GM-CSF-generated BMDC, Flt3L-generated BMDC and M-CSF-generated BMMΦ are adequate tools to investigate immunological mechanisms *in vitro*.

Murine DC express CD11c on their surface, whereas MΦ are mainly characterized by the expression of F4/80 (113;114), thus allowing DC and MΦ identification. We found the cells differentiated with GM-CSF to consist of mainly $CD11c^+$ $F4/80^{low}$ BMDC. In addition, a small population of IgM^+ B cells (4,5-9,5 %) and a few $Gr\text{-}1^+$ granulocytes (1-5%) develop, as described for cells cultured under these conditions (100). No $F4/80^{high}$ $CD11c^-$ macrophages are detectable after cultivating bone marrow cells in the presence of GM-CSF-containing supernatant (Fig. 4A).

Since one of the goals of this study was to compare the production of the IL-12 cytokine family of conventional BMDC and BMMΦ in response to *S. Enteritidis*, it was necessary to test whether any of the $CD11c^-$ IgM^+ or $CD11c^-$ $Gr\text{-}1^+$ cells in the heterogeneous cell culture gained with GM-CSF-containing supernatant could be a possible source of the IL-12 cytokine family. Therefore, the cells were stimulated with *S*. Enteritidis *in vitro* for 24 h in the presence of a protein transport inhibitor during the last 3-5 h of stimulation, thus preventing the release of synthesized IL-12/23p40. Then, the different cell populations were analysed for IL-12/23p40 synthesis by FACS. The ability to produce IL-12/23p40 after 24 h stimulation with heat-inactivated *S. Enteritidis* was restricted to the population of $CD11c^+$ BMDC and did not occur in the $CD11c^-$ IgM^+ or $CD11c^-$ $Gr\text{-}1^+$ cell populations (Fig. 4B). This demonstrates that DC are the only IL-12/23p40-producing cell type in this culture upon stimulation with *S*. Enteritidis.

During stimulation some of the cells differentiated with GM-CSF-containing supernatant became adherent. Since it was reported that some cells of GM-CSF-derived cell cultures are able to differentiate into adherent BMMΦ (115), we compared this adherent cell fraction with the non-adherent cell fraction for their expression of the DC marker CD11c and the MΦ marker F4/80 after stimulation with heat-inactivated *S.* Enteritidis for 48 h. Both, the adherent and the non-adherent cell fraction expressed CD11c on their surface, the adherent cells even more than the non-adherent cells. The expression of F4/80 remained low or was even abolished after this time of stimulation on both populations (Fig. 4C). The same data were obtained by cultivating cells in medium for 48 h without heat-inactivated *S.* (data not shown). Therefore, we conclude that both, the adherent and the non-adherent cells in our experimental system are BMDC.

In the presence of Flt3L, bone marrow cells differentiate into a mixed culture of conventional and plasmacytoid DC (82). In addition to the DC marker CD11c, plasmacytoid DC express the marker B220 and m-PDCA-1, whereas conventional DC are $CD11c^+$ $B220^-$ $mPDCA\text{-}1^-$ (116;117). Cells cultured in the presence of Flt3L were found to comprise of 68-82 % conventional BMDC positive for CD11c and negative for B220 and/or mPDCA-1, and of 12-25 % plasmacytoid BMDC positive for CD11c and B220 and/or mPDCA-1 in the different experiments (Fig. 5A).

Cells differentiated in the presence of M-CSF-containing supernatant were found to be 95% positive for F4/80, and <5% positive for CD11c (Fig. 5B).

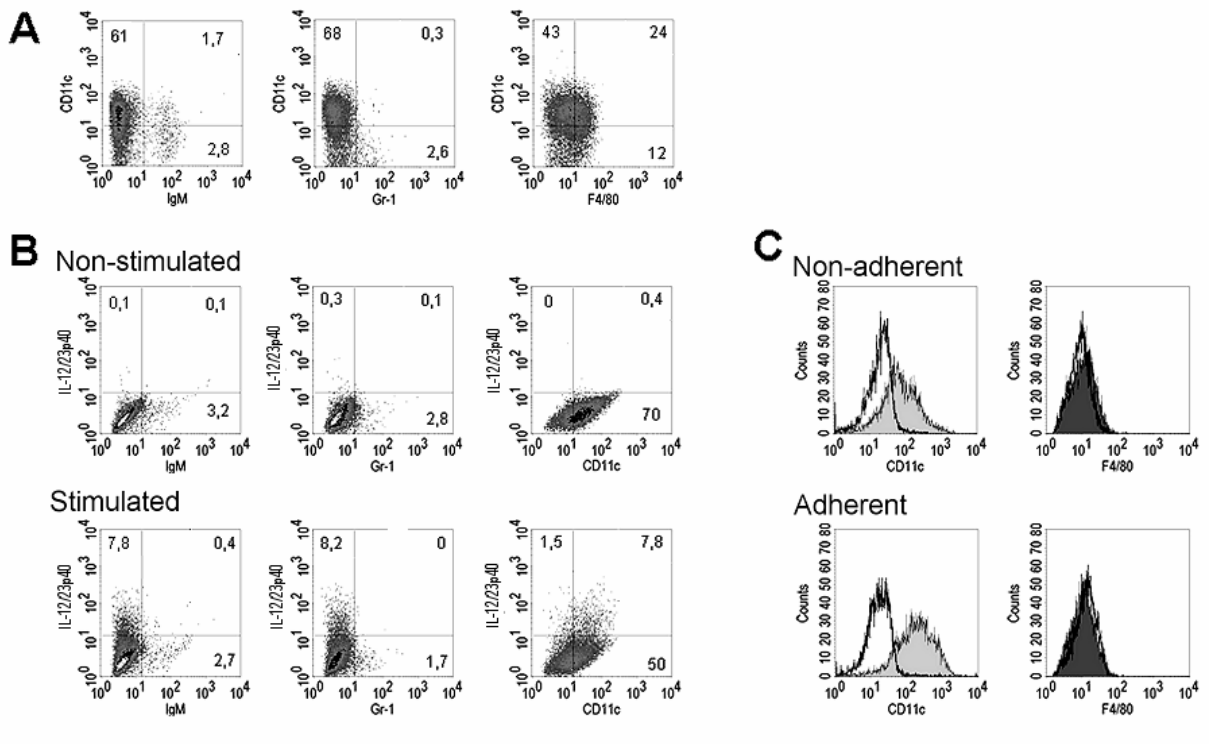

Fig. 4 CD11c$^+$ BMDC are the only producers of IL-12/23p40 within a GM-CSF-generated bone marrow-derived cell culture in response to *S. Enteritidis*.

(A) Bone marrow cells cultured for 10 d with GM-CSF-containing supernatant comprise mainly CD11c$^+$ cells but also IgM$^+$ and Gr-1$^+$ cells. (B) The cell culture was incubated for 24 h with medium (*upper panel*) or heat-inactivated *S.* Enteritidis (5×10^6 cells/ml; *lower panel*). The CD11c$^+$, IgM$^+$ and Gr-1$^+$ cells were analysed for intracellular IL-12/23p40 by FACS staining. (C) During incubation with heat-inactivated *S.* Enteritidis some cells become adherent. The non-adherent (*upper panel*) and adherent (*lower panel*) cells incubated for 48 h with heat-inactivated *S.* Enteritidis (5×10^6 cells/ml) or medium were harvested separately and analysed for the DC marker CD11c and the MΦ marker F4/80. Shown is one representative of three independent experiments.

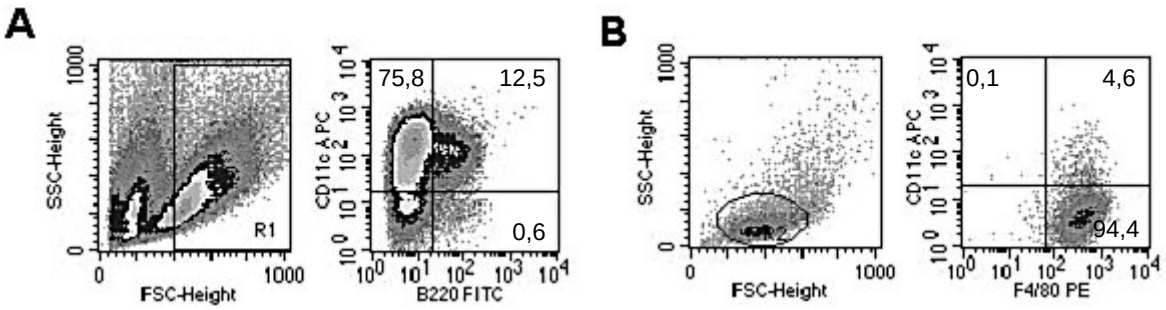

Fig. 5 Characterization of BMDC generated in the presence of Flt3L and of BMMΦ generated in the presence of M-CSF-containing supernatant

(A) Bone marrow cells cultured for 7 d with Flt3L comprise $CD11c^+$ $B220^-$ conventional BMDC and $CD11c^+$ $B220^+$ plasmacytoid BMDC. (B) The cell culture differentiated in the presence of M-CSF-containing supernatant consists of $F4/80^+$ BMMΦ and 0-5% $CD11c^+$ BMDC. Shown is one representative of at least three independent experiments.

4.2 Production of the IL-12 family members by DC and MΦ activated by the facultative intracellular bacterium *Salmonella* Enteritidis

4.2.1 Background: *Salmonella*

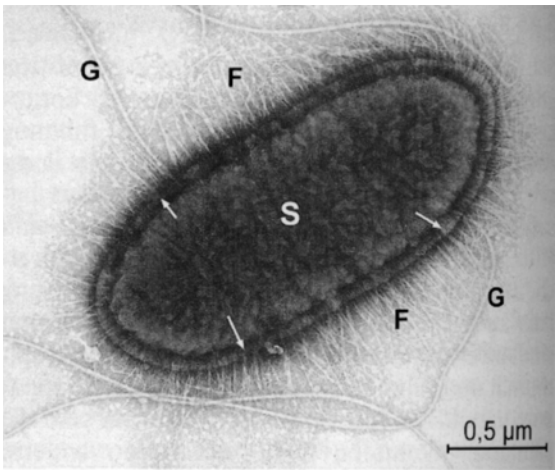

Fig. 6 Morphology of *Salmonella enterica spp.*.
Salmonella (S) are 0.7 – 1.5 × 2.0 – 5.0 µm big rods with flagella (G) and fimbria (F) originating at the plasma membrane (arrows). The picture was adapted from (118).

Salmonella enterica spp. enterica serovar Enteritidis (*S.* Enteritidis) is a Gram-negative bacterium that is transmitted by ingestion of contaminated food or water and causes food-borne salmonellosis pandemic in humans. *Salmonella* infections increased continuously beginning in the mid-1970ies (119). In 2006 there were 3616 infections with *Salmonella spp*. reported in Germany, with *S.* Enteritidis being the most prevalent cause of disease accounting for 43,94% of all reported *Salmonella* infections (Robert-Koch-Institut; Epidemiologisches Bulletin/ 19.01.2007/Nr.3).

Salmonella is facultative intracellular bacterium that has developed several mechanisms to avoid its elimination within the cell (120). In particular, it has evolved mechanisms that allow its survival in the harsh intracellular milieu of phagosomes. It can thus survive in phagocytes that should otherwise kill ingested bacteria (121-123). Therefore, phagocytes such as DC and MΦ can mediate systemic spreading of *Salmonella* (124-126). Several studies in humans as well as in murine models demonstrated that protective immunity against the Gram-negative facultative intracellular bacteria *S.* Enteritidis, *S*. Typhimurium or *S.* Dublin critically depends on IL-12 and might be complemented by IL-23 (85;127-130). Thus, it is of great interest to identify the essential cellular sources which need to be activated by *Salmonella* for production

of the IL-12 family members. After infection of MΦ, *S.* Typhimurium were reported to replicate in splenic MΦ *in vivo* (131) and induce cytokines of the IL-12 cytokine family in a TLR4-dependent manner (112;132-134). LPS/TLR4 signalling was shown to be essential for protection against experimental infection of mice with *S.* Typhimurium (135;136), and MΦ had been found to be important effector cells upon challenge with *S.* Typhimurium of immunized mice. However, MΦ were dispensable for induction of protective immunity by vaccination with attenuated *S.* Typhimurium (137).

Besides MΦ in recent years DC came into focus as important cells mediating immune responses against *Salmonella*. DC located below the intestinal epithelium layer were reported to sample *S.* Typhimurium from the intestinal lumen (138). Also, Peyer´s patch DC were found to internalize and harbour an attenuated *S.* Typhimurium strain used for immunizing mice (139). Similar to MΦ, *Salmonella* is able to induce antigen presentation and production of IL-12 in DC after infection (140-142). Presently the significance of DC vs. MΦ as cellular sources of the members of the IL-12 family is not clear. In this study cytokine responses of DC and MΦ upon activation with *S. Enteritidis* were compared *in vitro* and *in vivo*.

4.2.2 Results

4.2.2.1 Conventional BMDC produce more IL-12/23p40, IL-12, IL-23 and IL-27p28 than BMMΦ in response to LPS or *S.* Enteritidis

Comparing the ability of BMDC and BMMΦ to produce IL-12, IL-23, IL-27p28, and IL-12/23p40 in response to LPS or *S.* Enteritidis revealed that both types of APC, conventional BMDC and BMMΦ, produced constitutively the IL-12/23p40-subunit at low levels. Upon stimulation with LPS (derived from *Salmonella*) or *S.* Enteritidis, the levels of IL-12/23p40 in the culture supernatants of conventional BMDC and BMMΦ increased and were about 20-fold higher in culture supernatants of conventional BMDC compared to those of BMMΦ (Fig. 7A). Moreover, LPS and *S.* Enteritidis induced the release of IL-12 and IL-23 by conventional BMDC but not by BMMΦ. Both APCs produced IL-27p28 following stimulation with LPS or *S.* Enteritidis, with about 20-fold higher levels detectable in the culture supernatants of conventional BMDC than those of BMMΦ, an observation similar to that seen for the IL-12/23p40-subunit. These data suggest that DC are more potent producer of IL-12 family members in response to *S.* Enteritidis than MΦ.

In addition the levels of the pro-inflammatory cytokines IL-6 and TNF-α, cytokines that have been reported to be important for DC maturation (143;144) and MΦ activation and

differentiation (145-148), were analyzed in the culture supernatants of conventional BMDC and BMMΦ. Culture supernatants of BMDC contained about 10-fold more TNF-α than those of BMMΦ but similar amounts of IL-6 in response to 12 h stimulation with *S.* Enteritidis (Fig. 7B).

A recent study by our group using BMMΦ from IL-$10^{-/-}$ and wt mice demonstrated that the lack of IL-12 and IL-23 release by wt BMMΦ is due to the presence of endogenous IL-10 in the culture supernatant (133). Therefore, it was interesting to compare the IL-10 levels released by conventional BMDC to those released by BMMΦ. Both, conventional BMDC and BMMΦ, produced similar levels of IL-10 in response to LPS or *S.* Enteritidis (Fig. 7B), indicating that the observed differences of IL-12 family member production by BMDC and BMMΦ were not due to the presence of endogenous IL-10. To characterize the effect of exogenous IL-10 on the release of IL-12/23p40, IL-12 and IL-23 by BMDC, varying concentrations of recombinant IL-10 (0.01 – 10.0 ng/ml) were added to *S.* Enteritidis-stimulated BMDC. As a consequence production of IL-12/23p40, IL-12 and IL-23 by *S.* Enteritidis-stimulated BMDC was markedly inhibited. Even the lowest concentrations of IL-10 (0.01 ng/ml) reduced considerably the release of the IL-12/23p40-subunit. The output of IL-12 and IL-23 by BMDC was down-regulated in a dose-dependent manner by exogenous recombinant IL-10 (Fig. 7C). Thus, similar to BMMΦ (133), exogenous IL-10 has inhibitory effects on the IL-12 release by conventional BMDC.

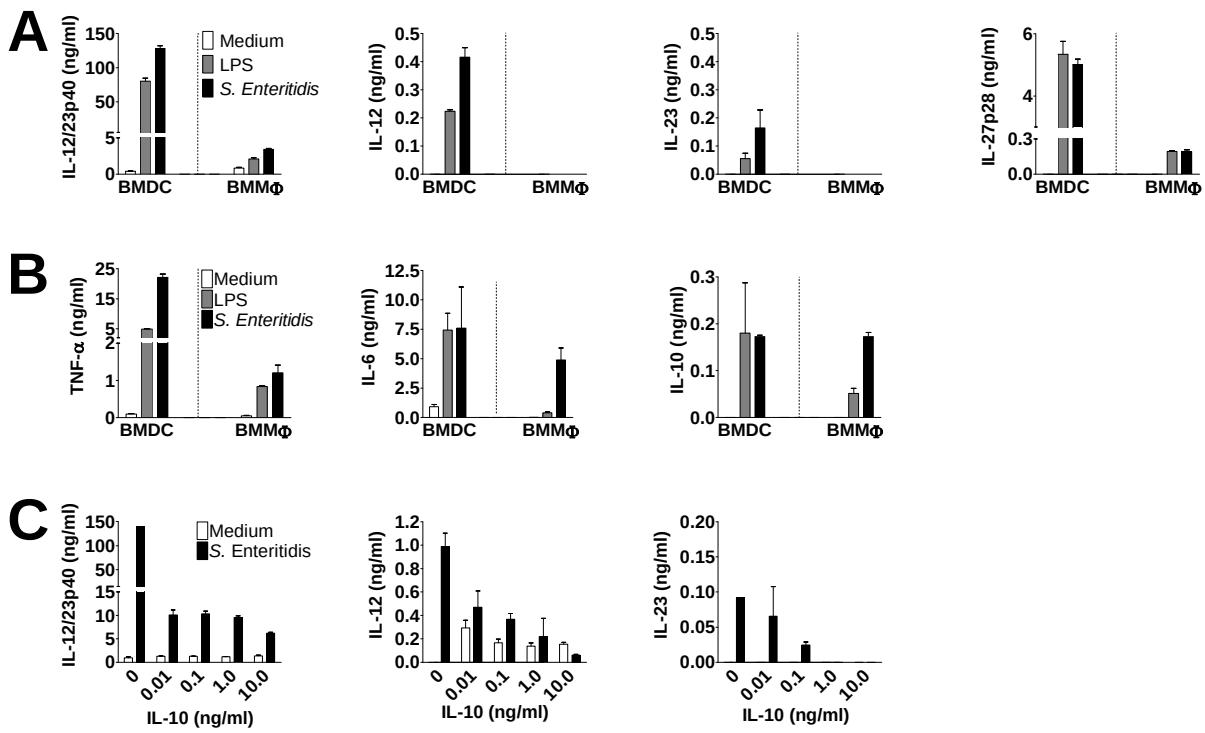

Fig. 7 Conventional BMDC are more potent producers of the IL-12 cytokine family members in response to *S.* Enteritidis or LPS than BMMΦ.

(A) Conventional BMDC and BMMΦ were stimulated for 12 h with medium, LPS (5 µg/ml) or *S.* Enteritidis (5×10^6 cfu/ml). Cell-free supernatants were prepared and analysed by ELISA for IL-12/23p40, IL-12, IL-23, IL-27p28. (B) Contents of TNF-α, IL-6 and IL-10 in culture supernatants of conventional BMDC and BMMΦ following stimulation as described in (A). (C) Exogenous IL-10 inhibits the release of IL-12/23p40, IL-12 and IL-23 by conventional BMDC in response to *Salmonella*. BMDC were stimulated for 12 h with *S.* Enteritidis (5×10^6 cfu/ml) in the presence of the indicated concentrations of recombinant IL-10. Cell free supernatants were analyzed by ELISA for IL-12/23p40, IL-12 and IL-23. Mean ± SEM (triplicate samples) of one of three independent experiments are shown.

4.2.2.2 The secretion of IL-12/23p40, IL-12, IL-23 and IL-27p28 in response to *S.* Enteritidis by conventional BMDC follows differential kinetics and is mediated by LPS/TLR4 interaction

It was shown previously by us that mRNA transcripts production of the IL-12 family members by BMMΦ follows differential kinetics (133). To characterize the time course of IL-12/23p40, IL-12 and IL-23 secretion by conventional BMDC after *in vitro* activation with LPS or *S. Enteritidis*, the levels of these cytokines were monitored for 48 h. As shown in Fig. 8A, the IL-12/23p40 concentration in the culture supernatants increased until 12-24 h reaching a plateau thereafter. The level of IL-12 reached its maximum 12 h after stimulation and was still elevated 48 h post stimulation. IL-23 increased to a peak at 6 h after stimulation decreasing continuously thereafter. Also the kinetics of the IL-10 and TNF-α release was investigated. The level of IL-10 reached its maximum 24 h after stimulation and was still elevated 48 h post stimulation. The amount of TNF-α reached its maximum concentration already 6 h after stimulation and decreased continuously thereafter, an observation similar to that seen for IL-23 (Fig. 8B).

Next, the question was addressed by which TLR *S. Enteritidis* induces the secretion of IL-12/23p40, IL-12, IL-23 and IL-27p28. Since *Salmonella* is a Gram-negative bacterium expressing LPS in its cell wall, the LPS receptor TLR4 (149) is a prime candidate for signal uptake and transduction. In addition, the potential contribution to the IL-12 inducing activity of *S. Enteritidis* by TLR2 ligands such as lipopeptides was assessed (111;150). By stimulating conventional BMDC of wt, $TLR2^{def}$ and $TLR4^{def}$ mice we show that LPS triggers the secretion of IL-12/23p40, IL-12, IL-23, and IL-27p28 in a TLR4-dependent and TLR2-independent manner. Surprisingly, the *S. Enteritidis*-induced secretion of IL-12, IL-23 and IL-27p28 is triggered exclusively by TLR4 similar to LPS, whereas low-level production of IL-12/23p40 is induced also by other PRR than TLR4 when stimulated with *S. Enteritidis* (Fig. 8C). Similar to the release of IL-12 and IL-23, the induction of IL-10 in BMDC with LPS or *S. Enteritidis* is TLR4-dependent. TNF-α, being released by BMDC of all three genotypes constitutively at low levels, is induced by LPS, as expected, in a TLR4-dependent manner. Its induction by *S. Enteritidis* is also triggered by other PRR than TLR4. Similar as for IL-12/23p40 the TLR4-independent induction of TNF-α is considerably weaker than the induction by TLR4 (Fig. 8D). These results indicate that *S. Enteritidis* induces IL-12 cytokine family secretion mainly by LPS/TLR4 interaction with a minor role for other PRR.

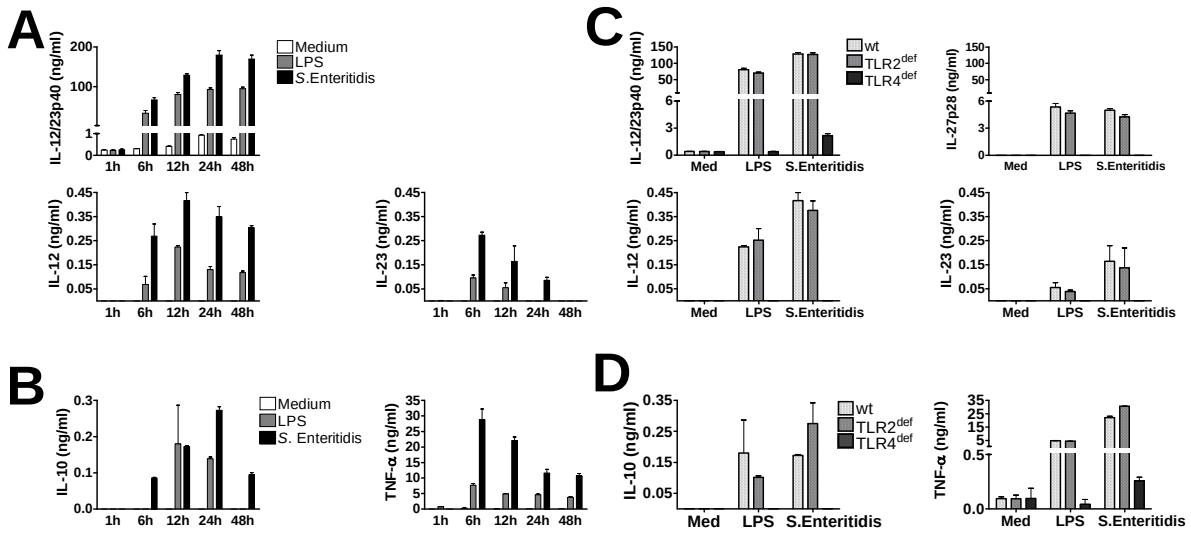

Fig. 8 The release of IL-12/23p40, IL-12, IL-23, IL-10 and TNF-α by BMDC in response to *S. Enteritidis* or LPS follows differential kinetics and is mainly triggered by TLR4.

(A) BMDC were stimulated with medium, LPS (5 µg/ml) or *S.* Enteritidis (5×10^6 cfu/ml) for the indicated time points. Cell free supernatants were analysed by ELISA for IL-12/23p40, IL-12 and IL-23. Similar data was obtained by stimulation with heat-inactivated *S.* Enteritidis (5×10^6 hk*S*E/ml; data not shown). (B) The release of IL-10 and TNF-α by BMDC following stimulation as described in (A). (C) BMDC originating from wt, $TLR2^{def}$ and $TLR4^{def}$ mice were stimulated for 12 h as described in (A), and the release of IL-12/23p40, IL-12, IL-23 and IL-27p28 was measured by ELISA. (D) The release of IL-10 and TNF-α following stimulation as described in (C). Mean ± SEM (triplicate samples) of one of three independent experiments are shown.

4.2.2.3 Investigations with primary DC and MΦ following i.p. *Salmonella* infection

To analyse whether the *in vitro* results resembled *in vivo* responses to *S.* Enteritidis, mice were infected intraperitoneally (i.p.) with *S.* Enteritidis. After i.p. infection the peritoneal cavity represents the site of infection, which is drained primarily by the parathymic lymph node (DLN) representing the site of immune response induction. It has been shown previously by our group, that after i.p. infection *S. Enteritidis* spreads systemically and the organ burden of spleen and liver increases continuously until 14 days after infection dropping rapidly thereafter (151). This observation probably indicates that after 14 days the T-cell dependent

immune response is able to clear the infection. Previous data point to the involvement of both $CD4^+$ and $CD8^+$ T cells in control of *S. Enteritidis* (152). To investigate the production of IL-12 family members by peritoneal DC and MΦ at the time point of the T-cell dependent adaptive immune response, day 14 was chosen for analysis. In addition, to study the contribution of peritoneal DC and MΦ to early innate immune mechanisms, day 1 after infection was analysed.

4.2.2.4 DC and MΦ expansion in response to i.p. *Salmonella* infection *in vivo*

We wished to investigate whether DC and MΦ would expand in response to i.p. *S.* Enteritidis infection. To determine the kinetic of the $CD11c^+$ DC and $F4/80^+$ MΦ expansion to i.p. *S. Enteritidis* infection at the peritoneal cavity and the DLN, peritoneal exudate cells (PEC) and DLN cells were isolated, and quantified for total cell numbers as well as DC and MΦ numbers at 1 dpi and at 14 dpi. After i.p. infection *S.* Enteritidis was rapidly cleared from the peritoneal cavity. However, the mice were still infected, as apparent by the increasing organ burdens of spleen and liver due to spreading of the bacterium (Fig. 9A). Clearance of *S.* Enteritidis from the peritoneal cavity had begun already 1 dpi and correlated with a significant influx of cells in the peritoneal cavity (Fig. 9B). Until 14 dpi the number of PEC decreased again but still remained significantly elevated compared to naïve mice. The expansion of $CD11c^+$ DC in the peritoneal lavage fluid followed a similar kinetic trend: DC numbers of naïve mice were about 1.2×10^5 (median number). During the first 24 h of infection the greatest expansion of DC occurred, the numbers of DC in the peritoneal cavity increased almost 15-fold slightly declining thereafter until 14 dpi. In contrast, MΦ numbers did not change in the peritoneal cavity upon *S. Enteritidis* infection (Fig. 9B).

Upon activation, DC do not stay at the site of infection but migrate to the DLN (134;153). DLN of naïve mice comprised 2.0×10^6 cells. Until 1 dpi the number of DLN cells had started to increase and continued to rise until 14 dpi (Fig. 9C). The increasing cell numbers were associated with macroscopically visibly enlarged DLN. The DLN of uninfected mice contained about $4{,}4 \times 10^4$ (median) DC. During the first 24 h of infection, DC numbers hardly changed in the DLN. Then, the DC expanded to almost 10-fold higher numbers until 14 dpi. In contrast, the number of MΦ tended to increase slightly in the DLN until 14 dpi, however, the increase did not become statistically significant (Fig. 9C). Thus, during *S.* Enteritidis infection DC expand in the peritoneal cavity and, with a little delay, in the DLN, whereas MΦ do not significantly change in either compartment.

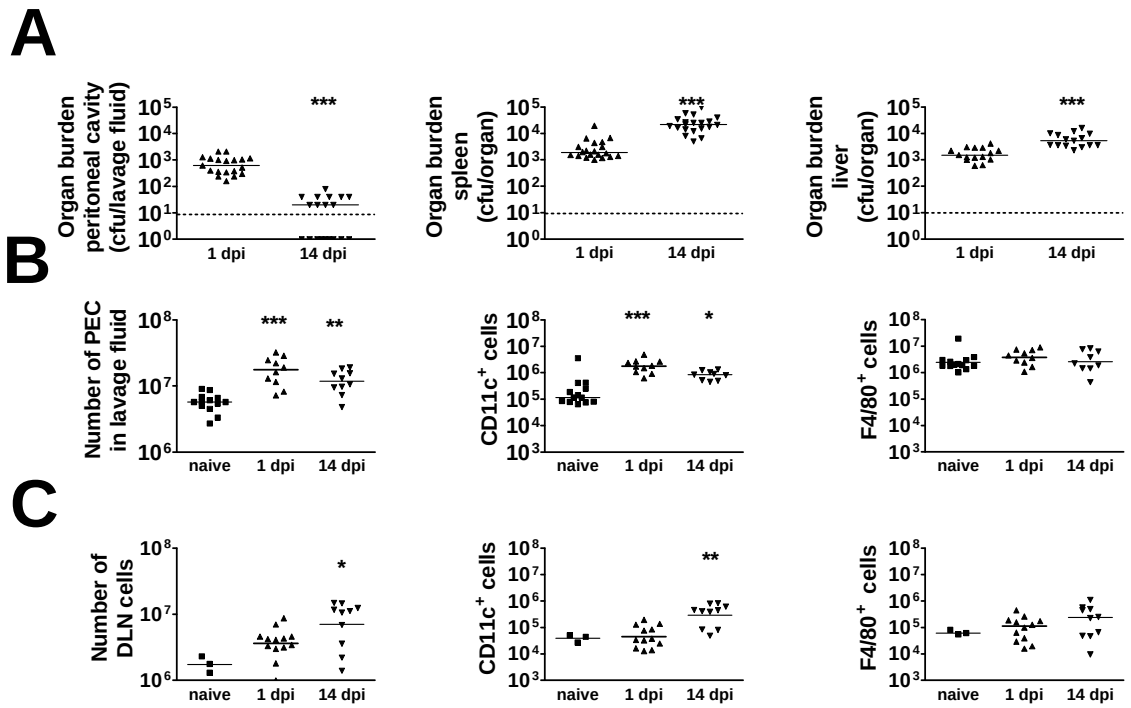

Fig. 9 Evaluation of the organ burden and cell numbers of DC and MΦ in the lavage fluid (peritoneal cavity) and the DLN in response to *S.* Enteritidis.

(A) Organ burden of the peritoneal cavity, spleen and liver of mice infected i.p. with 5×10^6 *S.* Enteritidis 1 dpi and 14 dpi. Pooled data of two (liver) resp. three (lavage fluid, spleen) independent experiments are displayed with median. Statistics was calculated using the Mann-Whitney U test (* p = 0,05; ** p = 0,01; *** p = 0,001). The *dashed line* displays the detection limit of 10 cfu/organ. (B) Total numbers of all PEC as DC and MΦ in the lavage fluid of naive versus infected mice. PEC were analysed for $CD11c^+$ and $F4/80^+$ cells by FACS. Absolute numbers were calculated by multiplication of the absolute numbers of PEC with the percentage rates of $CD11c^+$ or $F4/80^+$ cells. Pooled data of two independent experiments are displayed (n = 10). Statistics was calculated by Kruskal-Wallis Test and Dunn's Post test (* p = 0,05; ** p = 0,01; *** p = 0,001 vs. naïve animals). (C) Total numbers of DLN cells as DC and MΦ in the DLN. DLN cell suspensions were prepared and analysed as for PEC cells described in (B).

To investigate, which DC subsets increased upon *S.* Enteritidis infection, the increasing $CD11c^+$ DC subpopulations were quantified more closely by the additional surface markers

CD11b characteristic for conventional DC, and mPDCA-1, which is expressed on plasmacytoid DC. PEC of naïve animals contained 2.45×10^5 (median number) conventional DC. Conventional DC expanded significantly during the first 24 h up to a peak of 2.10×10^6 cells (median) decreasing again thereafter. The numbers of peritoneal plasmacytoid DC of naïve mice were modest compared to conventional DC (median 2.70×10^3). Upon infection plasmacytoid DC expanded rapidly during the first 24 h and further increased during the course of infection up to almost 3-fold higher levels (Fig. 10A top panels). This demonstrates that both, conventional DC and plasmacytoid DC increase during *Salmonella* infection, indicating that plasmacytoid DC which are in general associated with protection against viral infections, in addition to conventional DC, are involved in the immune response to *Salmonella* and maybe to other bacterial infections at the site of infection. To analyze whether peritoneal DC were activated by *S*. Enteritidis, DC of the peritoneal cavity expressing MHC-II, CD86 and CD80, necessary for antigen presentation and T cell activation, were quantified. At 1 dpi PEC contained more than 2,5-fold more MHC-II$^+$ DC than naïve animals. Until 14 dpi the numbers of MHC-II$^+$ DC slightly declined again. The amount of CD86$^+$ DC increased significantly already during the first 24 h to 5-fold higher levels and continued to expand up to 24-fold higher levels on day 14 (Fig. 10A bottom panels). In contrast, the quantity of peritoneal CD80$^+$ DC did not critically change during the course of infection (data not shown).

The DLN of naïve animals comprised almost twice as many conventional DC than plasmacytoid DC. Conventional DC expanded significantly already during the first 24 h and continued to increase up to more than 12-fold higher levels than naïve animals during the course of infection. In contrast, numbers of plasmacytoid DC of the DLN did not critically change in response to *S*. Enteritidis infection. Whereas MHC-II$^+$ DC almost doubled during the first 24 h after infection and continued to increase up to 4-fold higher amounts until 14 dpi, the number of CD86$^+$ DC and CD80$^+$ DC did not change in the DLN (Fig. 10B and data not shown). Thus, while both, conventional and plasmacytoid DC expand in the peritoneal cavity following i.p. *S*. Enteritidis infection, only conventional DC accumulate in the DLN. These data point to conventional DC as important antigen-presenting cells inducing adaptive immune responses at the DLN in *Salmonella* infection.

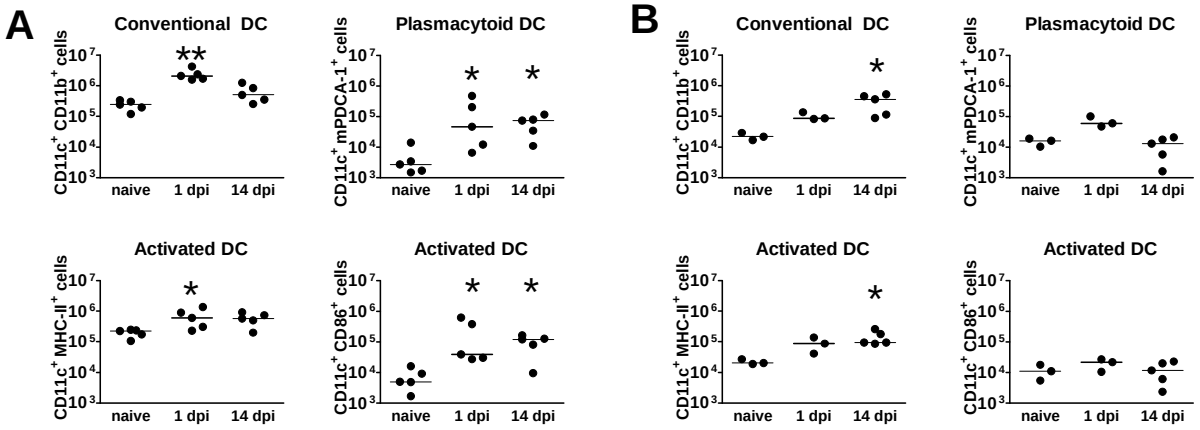

Fig. 10 Characterization of the different DC subsets increasing upon *S.* Enteritidis infection.

PEC (A) and DLN cells (B) of naïve and infected mice were analysed for the indicated DC subsets by FACS. Absolute numbers were calculated by multiplication of the absolute numbers of PEC (A) resp. DLN cells (B) with the relative percentage rates of the indicated DC subsets. Statistics was calculated by Kruskal-Wallis and Dunns post test (* p = 0,05; ** p = 0,01; *** p = 0,001 vs. naïve animals).

4.2.2.5 Production of IL-12/23p40 and NO occurs at the site of infection rather than at the secondary lymph node

Upon antigen encounter including *Salmonella* DC and MΦ are among the front line sentinel cells producing pro-inflammatory cytokines and anti-microbial effectors molecules such as IL-12 family members and NO (43;154). Following *Salmonella* uptake and processing, DC migrate to the DLN (134) to induce T cell dependent immune responses. The T cell differentiation from naïve to effector T cells is critically orchestrated by IL-12 family members (37;155). Thus, is it of great interest to characterize the production of IL-12 family members at the peritoneal cavity and the DLN in response to *S. Enteritidis* infection. Therefore, PEC and DLN cells were isolated of naïve and infected animals at 1 dpi and 14 dpi, cultured *ex vivo* for another 24 h, and the levels of IL-12/23p40, IL-12, IL-23 and NO of the culture supernatants and in the lavage fluid of the peritoneal cavity were measured. Additionally, the production of NO at both compartments was analysed. In the peritoneal lavage fluid of naïve animals the levels of IL-12/23p40 and NO were close to or even below

the detection limits. Upon infection, IL-12/23p40 and NO levels started to increase continuously until significantly elevated levels at 14 dpi (Fig. 11A). IL-12 and IL-23 could be detected in the peritoneal lavage fluids only of single animals, likely because of high dilution of the cytokines due to the lavage procedure (data not shown).

In agreement with cytokine and NO responses observed in the lavage fluid representing *in vivo* immune responses, *ex vivo* cultured PEC of naïve animals released neither IL-12/23p40 nor NO, whereas PEC of infected mice released high amounts of IL-12/23p40 and NO at 1 dpi as well as at 14 dpi (Fig. 11B). In contrast, *ex vivo* cultured DLN cells of 1 day-infected secreted neither IL-12/23p40 nor NO. However, low levels of IL-12/23p40 and NO compared to PEC could be detected in the supernatants of *ex vivo* cultured DLN cells of 14 days-infected mice. No production of IL-12/23p40 or NO could be observed by *ex vivo* cultured DLN cells of uninfected animals (Fig. 11C). Thus, upon *S.* Enteritidis infection IL-12/23p40 and NO are produced at the site of infection rather than at the site of immune response induction. The observed preferential production of IL-12/23p40 at the site of infection vs. the site of immune response induction is surprising considering the essential role of the IL-12 family for the orchestration of T cell differentiation, and point to an additional immunoregulatory role of IL-12 family members at the site of infection.

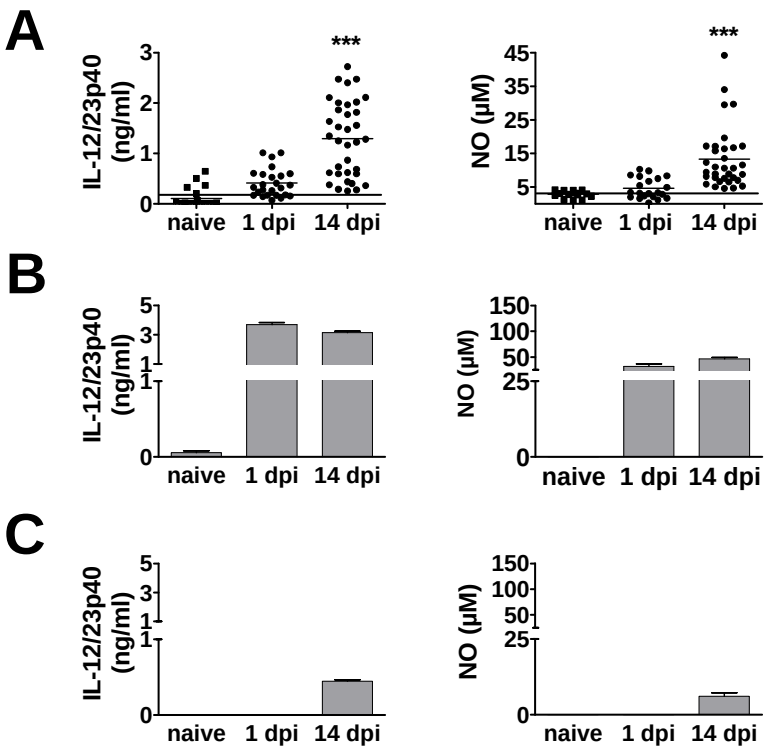

Fig. 11 Levels of IL-12/23p40 and NO in lavage fluids, *ex vivo* cultured PEC and DLN cells.

(A) The peritoneal cavities of naïve and infected mice were flushed with 2 ml of PBS/EDTA and the levels of IL-12/23p40 and NO in the lavage fluid determined by sandwich ELISA and Griess reagent, respectively. Shown are pooled data of at least three independent experiments. Statistical analysis was done by Kruskal-Wallis and Dunns post test (* p = 0,05; ** p = 0,01; *** p = 0,001 vs. naïve animals). (B) Levels of IL-12/23p40 an NO in culture supernatants of 24 h *ex vivo* cultured PEC. Levels of IL-12/23p40 of the cell culture supernatants were analysed by ELISA and Griess reagent, respectively. Mean ± SEM (n = 3) of one of at least two independent experiments are shown. (C) Levels of IL-12/23p40 and NO in culture supernatants of parathymic DLN cell suspensions cultured *ex vivo* as described in (B).

4.2.2.6 IL-12/23p40 vs. NO production: task sharing between peritoneal DC and MΦ

One aim of the present Ph. D. thesis was to study the contribution of peritoneal DC and MΦ to the production of IL-12/23p40 and NO in the peritoneal cavity upon *S*. Enteritidis infection. Therefore, PEC of infected mice were isolated at the indicated time points and depleted either of $CD11c^+$ DC or $F4/80^+$ MΦ prior to *ex vivo* culture. The degree of depletion was verified by FACS analysis, and all fractions used contained less than 2% contaminating $CD11c^+$ DC or $F4/80^+$ MΦ (data not shown). The depletion of DC of PEC from 1 day-infected animals completely abrogated IL-12/23p40 release by PEC during the following *ex vivo* culture. When PEC of 14 days-infected mice were depleted of DC, levels of IL-12/23p40 in the culture supernatants were considerably lower compared to unseparated PEC, whereas lack of MΦ had essentially no effect on the IL-12/23p40 levels (Fig. 12A).

Production of NO by PEC of 1 day and 14 days-infected animals was not critically changed by depletion of DC. In contrast, loss of MΦ after depletion dramatically decreased NO levels in the culture supernatants of PEC isolated at both time points (Fig. 12B). Thus, in response to i.p. infection with *S. Enteritidis*, peritoneal DC are the main IL-12/IL-23p40 producing cell type, whereas peritoneal MΦ are the main NO producing cell type. These data suggest a distinct task sharing between peritoneal DC and MΦ at the site of infection and support the *in vitro* results described in chapter 4.2.2.1.

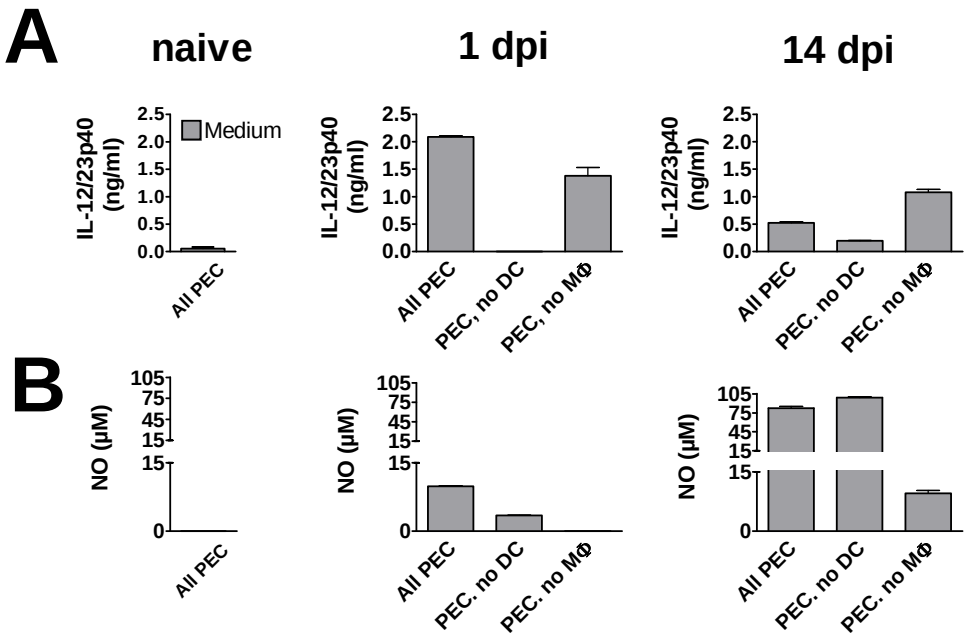

Fig. 12 Levels of IL-12/23p40 and NO in supernatants of *ex vivo* cultured PEC depleted of DC or MΦ.

PEC of infected mice were isolated at the indicated time points and depleted of either $CD11c^+$ DC or $F4/80^+$ MΦ with Miltenyi anti-PE-Microbeads according to the manufacturer's protocol. Following *ex vivo* culture for another 24 h, levels of IL-12/23p40 and NO in the PEC culture supernatants were analysed by ELISA and Griess reagent, respectively. Mean ± SEM (n = 3) of one of at least two independent experiments are shown.

4.2.2.7 Production of IL-17 and IFN-γ in response to *S.* Enteritidis

By the production of IL-12 family members DC and/or MΦ are able to orchestrate the differentiation of naïve T cells into different T cell subsets (Fig. 2). Thus, IL-12 family members influence the outcome of the subsequent T cell-derived cytokine response. It is widely accepted that IL-23 and IL-12 are able to induce production of IL-17 and IFN-γ, respectively, by T cells (156). It has been shown by our group earlier, that culture supernatants of *Borrelia burgdorferi*-stimulated BMDC induce the production of IL-17 in T cells. This induction of IL-17 was found to depend on IL-23 (157). To investigate the induction of IL-17 and IFN-γ following i.p. *S.* Enteritidis infection more closely, PEC and DLN cells from 1 day- and 14 days-infected mice were isolated and cultured *ex vivo* for 24 h.

Then the levels of IL-17 and IFN-γ in the culture supernatants and in the lavage fluid recovered from the peritoneal cavity were determined. Whereas no IL-17 was detected in the lavage fluid of naïve mice, IL-17 amounts increased upon infection reaching statistically elevated levels after 14 days of infection. Consistent with the observed IL-17 production upon infection *in vivo*, e*x vivo* cultured PEC of 1 day-infected mice released high levels of IL-17. However, PEC of 14 days-infected animals were poor producers of IL-17 (Fig. 13A top panels). Taken together these results indicate that IL-17 is synthesized early in response to *Salmonella* and accumulates at the site of infection.

Similar to production of IL-17, IFN-γ production in the peritoneal cavity increased in response to *S*. Enteritidis infection. The IFN-γ levels in the lavage fluid reached significantly elevated levels already at 1 dpi and these high IFN-γ levels remained until 14 dpi. Surprisingly, *ex vivo* cultured PEC released, only low levels of IFN-γ (Fig. 13A bottom panel).

In the supernatants of *ex vivo* cultured DLN cells of 1 day-infected mice no measurable levels of IL-17 or IFN-γ were produced, whereas after two weeks of infection DLN cells secreted some IL-17 and high amounts of IFN-γ (Fig. 13B). These data are consistent with IL-12 and/or IL-23-induced differentiation of naive T cells in the DLN later than 1 day after infection but prior to day 14.

The high production of IL-17 by PEC isolated 1 day after infection was striking. Thus it was of interest to address the question, whether the high levels of IL-17 produced from PEC of 1 day-infected mice were dependent on IL-23. Therefore, PEC from wild-type (wt) mice, IL-23p19$^{-/-}$ mice unable to produce IL-23, and of IL-12p35$^{-/-}$ lacking the ability to produce IL-12, were isolated 1 day after infection and cultured *ex vivo* in the presence or absence of *S*. Enteritidis. PEC derived from IL-12p35$^{-/-}$ mice released higher levels of IL-17 than wt mice (Fig. 13C). This effect is due to the lack of the IL-12/IFN-γ axis, that is known to counteract the IL-23/IL-17 axis (158). The effect was even more profound when PEC were stimulated in the presence of *S*. Enteritidis, nevertheless, similar observations were made during *ex vivo* culture in the absence of *S*. Enteritidis. In contrast, PEC deriving from IL-23p19$^{-/-}$ mice lacking endogenous IL-23 failed to produce measurable amounts of IL-17 when cultured in the absence of *S*. Enteritidis. However, in the presence of *Salmonella*, IL-23p19$^{-/-}$ PEC were able to release IL-17, albeit at considerable lower levels than those of wt PEC (Fig. 13C top panel). Therefore, the production of IL-17 of *ex vivo* cultured PEC 1 day after infection is partially dependent of the presence of endogenous IL-23.

IFN-γ was produced exclusively when PEC of all three genotypes were cultured in the presence of *S*. Enteritidis but not in the absence of *S*. Enteritidis as observed before (Fig. 13C bottom panel; for comparison see Fig. 13A). Following *ex vivo S*. Enteritidis stimulation, the IFN-γ output of IL-23p19$^{-/-}$ PEC were similar to those of wt PEC, whereas IL-12p35$^{-/-}$ PEC barely released any IFN-γ. These data demonstrate that the induction of IFN-γ by *S*. Enteritidis is dependent of IL-12 consistent with the known role of IL-12 in immunity to *Salmonella* infection (84;127;130).

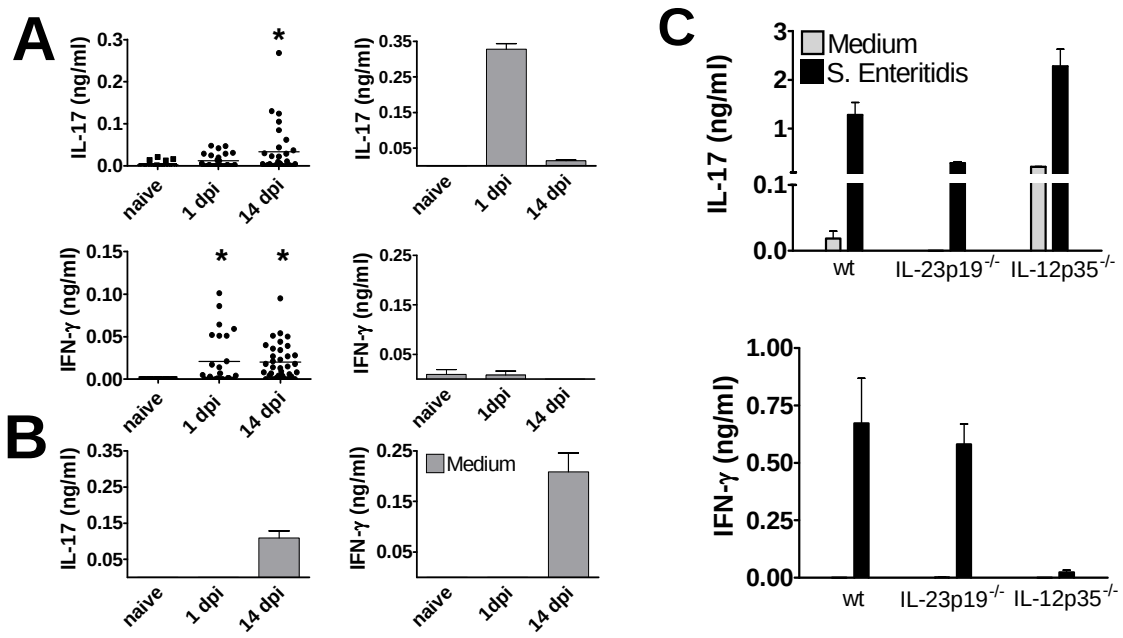

Fig. 13 Levels of IL-17 and IFN-γ produced *in vivo* in the peritoneal cavity and *ex vivo* by cultured PEC and DLN cells.

(A) The peritoneal cavity of naïve and infected mice was flushed with 2 ml of PBS/EDTA and the levels of IL-17 and IFN-γ in the lavage fluid determined by sandwich ELISA. Shown are pooled data of at least three independent experiments. Statistics were calculated by Kruskal-Wallis and Dunns post test (* p = 0,05; ** p = 0,01; *** p = 0,001 vs. naïve animals). Each column *(right panel)* represents the mean ± SEM of at least duplicate *ex vivo* PEC cultures. One of at least two independent experiments is shown. (B) Levels of IL-17 and IFN-γ in culture supernatants of parathymic DLN cells isolated and cultured *ex vivo* as described in (A). (C) PEC of three to five mice of the indicated genotypes were isolated 1 day after infection, pooled and *ex vivo* cultured in the presence or absence of *S*. Enteritidis. After 24 h the levels of IL-17 and IFN-γ were assayed by sandwich ELISA. Shown are pooled data of two independent experiments. Each column represents the mean ± SEM of at least triplicate cultures.

4.2.3 Summarizing Remarks

The *in vitro* data presented show that conventional BMDC rather than BMMΦ are potent producers of IL-12 family members in response to *S.* Enteritidis. Moreover, the present data demonstrate that despite the expression of many different TLR ligands on the surface *S.* Enteritidis mediates the induction of IL-12 family members primarily by LPS/TLR4 interaction. These *in vitro* observations are in line with the results obtained with primary cells that demonstrate that production of IL-12 family members upon i.p. infection with *S.* Enteritidis IL-12 family members at the site of infection primarily occurs by peritoneal DC rather than MΦ. However, MΦ were found to be the major NO producing cell type, indicating an important role of MΦ for the mediation of killing of *S.* Enteritidis. This suggests a distinct task sharing between DC and MΦ during *Salmonella* infection. Interestingly, similar as for NO, IL-12 family members are rather produced at the site of infection than at the DLN, pointing to an additional important immunoregulatory role of IL-12 family members at the site of infection.

Furthermore, the obtained results indicate that following i.p. infection *S.* Enteritidis induces early production of IL-17 at the site of infection, which is partially dependent on the presence of IL-23. Further studies will be necessary, however, to identify the cellular sources of IL-23-dependent and -independent IL-17 production. Taken together these data point to DC as major APC producing IL-12 and other members of the IL-12 family in *Salmonella* infection.

4.3 Activation of IL-12 family members by conventional bone marrow-derived dendritic cells, plasmacytoid bone marrow-derived dendritic cells and bone marrow-derived macrophages by *Cryptococcus neoformans*

4.3.1 Background: *Cryptococcus neoformans*

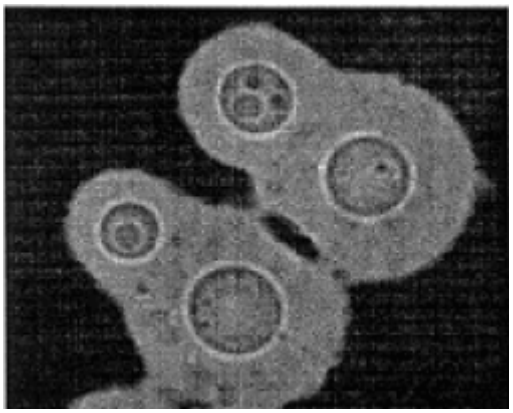

Fig. 14 Morphology of *Cryptococcus neoformans spp.*

C. neoformans cells are round to oval. The typical vegetative form of *C. neoformans* is the yeast form with a cell diameter of 2.5 µm to 10 µm (159). The cells are encountered by a polysaccharide capsule. The figure was adapted from (159).

Cryptococcus neoformans (*C. neoformans*) is an ubiquitous encapsulated pathogenic yeast which can cause life-threatening meningoencephalitis in immunosuppressed patients, especially those with HIV infection (160). Especially *C. neoformans* variants of the serotypes A (*C. neoformans* var. *grubii*) and D (*C. neoformans* var. *neoformans*), which are distributed worldwide (161), are responsible for most cases of cryptococcosis in immunocompromised patients such as AIDS patients (162). *C. neoformans* infection is believed to be acquired by inhalation of infectious particles (163). In the immunocompetent host, the infection is often limited to the lung. However, in patients with impaired immunity, extrapulmonary dissemination can occur (164;165). When *C. neoformans* invades the brain, it can cause severe meningoencephalitis (166), which is the most common clinical presentation of cryptococcosis (167).

The host defence against *C. neoformans* is critically regulated by cell-mediated immunity and both, $CD4^+$ T and $CD8^+$ cells play a central role in eradication of this infection (168-171). The

balance between Th1 cytokines and Th2 cytokines strongly influences the course of the infection: predominant production of Th1 cytokines was shown to provide protection, whereas infection is aggravated by Th2 cytokines (172). IL-12, essential for the differentiation of naïve Th cells into Th1 cells, was found to be a critical factor for protection against cryptococcal infection (87;94). Early studies comparing IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice pointed to additional IL-12/23p40-dependent effects other than IL-12 involved in the immune response to *C. neoformans* (94). These data were corroborated in 2006 when IL-23, another member of the IL-12 cytokine family, was found to complement protective immunity to *C. neoformans* (86). However, while there are many studies focussing on the mechanisms of action of IL-12 in cryptococcal infection (86;87;173-178), the major cellular sources of IL-12 and IL-23 in response to *C. neoformans* are unknown.

Potent producers of the cytokines of the IL-12 family are dendritic cells (DC) and macrophages (MΦ) (37). However, the contribution of DC and MΦ to the production of IL-12 family members in the course of cryptococcal infection is poorly defined. MΦ exist in great numbers in tissues, making alveolar MΦ most likely to be among the first cells confronted with *C. neoformans* cells after inhalation. Uptake of *C. neoformans* has been shown for rat alveolar MΦ (179), human microglia (180), and murine MΦ *in vitro* (181-183) and *in vivo* (184). Nevertheless, it was also demonstrated *in vitro*, that the capsule of *C. neoformans* has inhibitory effects on the release of IL-12 by human PBMC and murine MΦ (185;186), raising the question whether other cells such as DC might account for IL-12 production in cryptococcal infection. Recently, the uptake of *C. neoformans* by DC *in vivo* has been reported (187). In mice immunized with a protective *C. neoformans*-derived antigen, DC accumulated in the draining lymph node, in contrast to mice immunized with non-protective cryptococcal antigen. Accumulation of DC was associated with increased numbers of activated CD4^{+} T cells (188), making it tempting to speculate that DC are potent producers of IL-12 family members and the major antigen-presenting cells in response to *C. neoformans.*

Little is known of the receptors through which DC and MΦ sense *C. neoformans*. Toll-like receptors (TLRs) are innate immune-pattern recognition receptors that recognize a wide range of microbes including fungi (189). It was demonstrated *in vitro* that glucuronoxylomannan, the major component of the capsule of *C. neoformans*, binds to Chinese hamster ovary cells transfected with TLR2, TLR4 and/or CD14 (190). Using mice deficient for TLR2, TLR4 CD14 and the intracellular adaptor molecule MyD88, which is used for intracellular signalling by all known TLR but TLR3, Biondo *et al.* showed that MyD88$^{-/-}$ mice have significantly reduced survival compared to wild-type mice after intravenous and pulmonary

infection with a serotype A strain of *C. neoformans*. In the same study, $TLR2^{-/-}$ mice died significantly sooner following pulmonary infection but nor intranvenous infection, whereas $CD14^{-/-}$ mice tended to a reduced survival follwing intravenous infection (191). The role of TLR2, TLR4 and MyD88 for the induction of IL-12 family members in response to *Cryptococcus*, however, remains elusive. In the present study we investigated whether TLR2, TLR4 and MyD88 are involved in the induction of IL-12 family members upon stimulation by *C. neoformans*.

Upon activation by *C. neoformans*, conventional DC and MΦ are able to modulate the expression of MHC-II and co-stimulatory molecules such as CD86 and CD80 (192-195) critical for the successful induction of T cell responses (196). Studies on the role of plasmacytoid DC in cryptococcal infection have not yet been performed. The present study for the first time provides a side-by-side comparison of the activation of conventional bone marrow-derived DC (BMDC), plasmacytoid BMDC and bone marrow-derived macrophages (BMMΦ) by *C. neoformans* in terms of production of IL-12/IL-23p40 and the expression of MHC-II and the co-stimulatory molecules CD86 and CD80.

4.3.2 Results

4.3.2.1 *C. neoformans* induces IL-12/23p40 in conventional BMDC but not in BMMΦ

Upon stimulation with the capsulated *C. neoformans* 1841 strain conventional BMDC responded with increased production of IL-12/23p40. Similarly, the uncapsulated mutant strain *C. neoformans* CAP67 induced augmented IL-12/23p40 release. Levels of IL-12/23p40 tended to be higher in response to stimulation with the uncapsulated strain CAP67, indicating that the capsule of *C. neoformans* 1841 inhibits further induction of IL-12/23p40, which has been shown previously (185). Unlike conventional BMDC, BMMΦ did not secrete IL-12/23p40 upon stimulation with *C. neoformans* 1841. Even the acapsular strain *C. neoformans* CAP67 failed to induce release of BMMΦ-derived IL-12/23p40. However, when stimulated with *S*. Enteritidis for control, BMMΦ as well as conventional BMDC increased IL-12/23p40 production demonstrating that both cell types are able to produce IL-12 family members (Fig. 15A). Interestingly, conventional BMDC additionally produced IL-12 and IL-23 in response to *S*. Enteritidis which was not detectable following stimulation with *C. neoformans* (data not shown). Taken together our data point to conventional DC rather than MΦ as potent producers of IL-12 family members in response to *C. neoformans*.

It was reported that together with IL-12, TNF-α is important for the development of a protective immune response against *C. neoformans* (197-199). To investigate the role of conventional DC and MΦ as a source of TNF-α in response to *C. neoformans*, the production of TNF-α of conventional BMDC and BMMΦ was compared. Upon stimulation with *C. neoformans* 1841 conventional BMDC responded with an augmented release of TNF-α. As seen for IL-12/23p40, stimulation with the acapsular strain *C. neoformans* CAP67 led to higher levels of TNF-α in the supernatants of conventional BMDC. In BMMΦ, however, the capsulated strain *C. neoformans* 1841 and the acapsular strain *C. neoformans* CAP67 failed to induce TNF-α. The capsulated strain *C. neoformans* 1841 even markedly down-regulated constitutive production of TNF-α by BMMΦ (Fig. 15B). These data suggest that conventional BMDC rather than BMMΦ are a source of TNF-α in response to *C. neoformans*. Furthermore, the capsule of *C. neoformans* 1841 seems to inhibit stronger induction TNF-α, an observation similarly seen for IL-12/23p40.

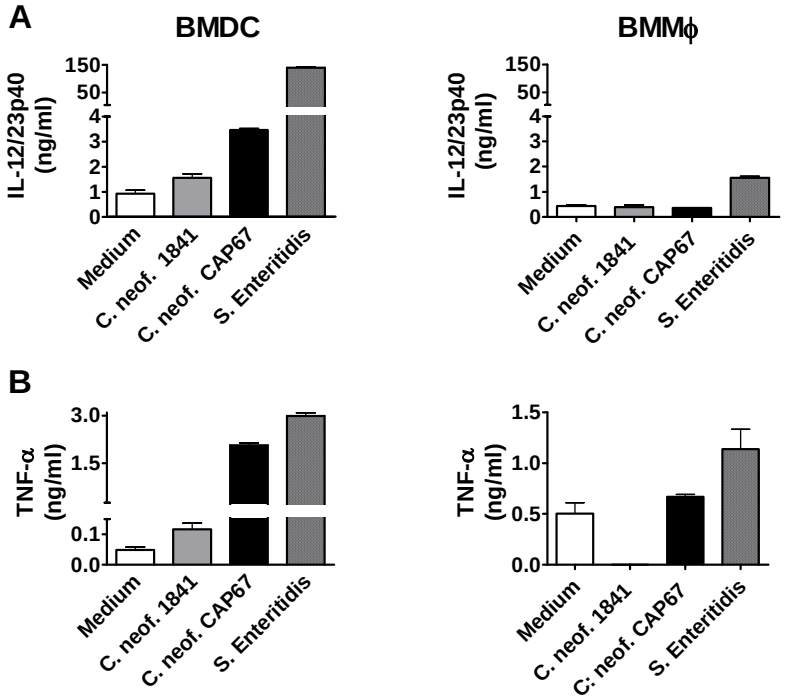

Fig. 15 *C. neoformans* induces the release of IL-12/23p40 in conventional BMDC but not in BMMΦ.

Conventional BMDC and BMMΦ were stimulated with *C. neoformans* 1841 at a MOI = 20 for 48 h, and the amount of IL-12/23p40 (A) and TNF-α (B) was analysed by sandwich ELISA. For control, antigen-presenting cells were stimulated with the acapsular strain *C. neoformans* CAP67 (MOI = 20), CpG-ODN (1 μM), or *S. Enteritidis* (5×10^6 cfu/ml). Shown is one representative of at least two independent experiments (mean ± SEM, duplicate samples).

4.3.2.2 The induction of IL-12/IL-23p40 by conventional BMDC upon stimulation with *C. neoformans* is independent of TLR2 or TLR4 but clearly depends on MyD88

The contribution of TLR2 and TLR4 for protective immunity against *C. neoformans* is controversially discussed (190;191;200;201). To determine, whether TLR2 or TLR4 play a role in the induction of IL-12/23p40, conventional BMDC originating from TLR2^{def} or TLR4^{def} mice were stimulated with *C. neoformans* 1841 and the levels of IL-12/23p40 in the culture supernatants were analysed. Following stimulation with the TLR4 ligand LPS, wild-type and TLR2^{def} conventional BMDC released high levels of IL-12/23p40, whereas almost no IL-12/23p40 could be detected in the supernatants of TLR4^{def} conventional BMDC. When stimulated either with *C. neoformans* 1841 or the acapsular strain *C. neoformans* C566, IL-12/23p40 levels detected in the culture supernatants of BMDC derived from TLR2^{def} and TLR4^{def} mice were similar compared to those of BMDC derived from wild-type mice (Fig. 16), indicating that other pattern recognition receptors than TLR4 and TLR2 are essential for the induction of IL-12/23p40 by *C. neoformans*.

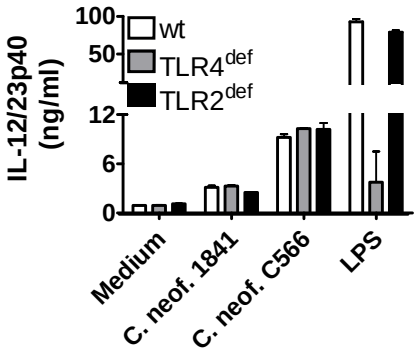

Fig. 16 The induction of IL-12/23p40 by *C. neoformans* is independent of TLR2 and TLR4.

Bone marrow cells originating from wild-type (wt), TLR2 deficient (TLR2^{def}) and TLR4 deficient (TLR4^{def}) mice were cultured for 10 days in the presence of GM-CSF-containing supernatant. Conventional BMDC were stimulated with *C. neoformans* 1841 (MOI = 20) or with the uncapsulated mutant *C. neoformans* C566 (MOI = 20), medium, or LPS (5 µg/ml) as control. After 24 h cell culture supernatants were harvested and analysed for IL-12/23p40 by ELISA. Shown is one representative of two independent experiments (triplicate samples; mean ± SEM).

Production of IL-12/23p40 in response to *C. neoformans* is, however, dependent on MyD88, as demonstrated by reduced IL-12/23p40 levels in culture supernatants of MyD88$^{-/-}$ BMDC upon *C. neoformans* stimulation. As expected, CpG-ODN induced IL-12/23p40 dependent on MyD88, whereas IL-12/23p40 upon LPS stimulation occurred MyD88-independent (Fig. 17), which is consistent with the known fact that TLR4 is able to signal independent of MyD88 by the intracellular adaptor molecule TRIF (202).

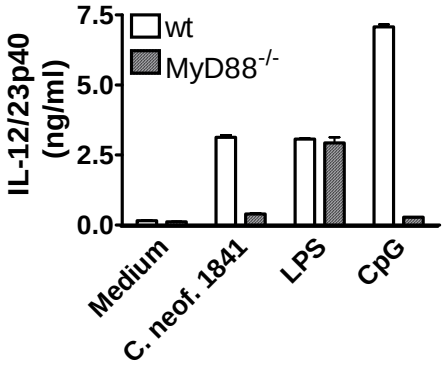

Fig. 17 The induction of IL-12/23p40 by *C. neoformans* is dependent of MyD88.
Bone marrow cells originating from wild-type (wt) and MyD88 deficient (MyD88$^{-/-}$) mice were cultured for 7 days in the presence of Flt3L. Plasmacytoid and conventional BMDC were incubated in the presence of *C. neoformans* 1841 (MOI = 20), medium, LPS (5 μg/ml) or CpG-ODN (1 μM). After 48 h cell culture supernatants were harvested and analysed for IL-12/23p40 by ELISA. (duplicate samples; mean ± SEM).

4.3.2.3 Distinct regulation of the expression of MHC-II and CD86 on conventional BMDC, plasmacytoid BMDC and BMMΦ in response to *C. neoformans*

Besides production of IL-12/23p40 we wished to study the antigen-presenting function of DC vs. MΦ by assessing the expression of MHC-II and co-stimulatory molecules such as CD86 and CD80 following stimulation with *C. neoformans*. In addition, we aimed to study the precise role of the plasmacytoid and conventional DC subsets in response to *C. neoformans*. Therefore, bone marrow cells were cultured in the presence of Flt3L instead of GM-CSF, to induce a mixed culture consisting of plasmacytoid and conventional BMDC (82). To confirm that Flt3L-generated BMDC responded similarly to *C. neoformans* as GM-CSF-generated BMDC, we assessed the IL-12/23p40 response upon stimulation. CpG-ODN were used as control stimulus for plasmacytoid BMDC. BMDC generated in the presence of Flt3L were

found to produce similar amounts of IL-12/23p40 as GM-CSF-generated BMDC in response to *C. neoformans* 1841. As expected, upon CpG-ODN stimulation high amounts of IL-12/23p40 were produced (Fig. 18A; for comparison see Fig. 16A).

In addition to the murine DC marker CD11c, plasmacytoid DC express B220, in contrast to conventional DC which are $CD11c^{+}B220^{-}$. Thus, these two markers allow the identification and separate FACS analysis of each cell-subset in a mixed culture of conventional and plasmacytoid DC as obtained by cultivating bone marrow cells in the presence of Flt3L. On conventional BMDC the stimulation with *C. neoformans* 1841 led to an increase of the expression of MHC-II and CD86 (Fig. 18B, top panels). The expression of CD86 and MHC-II remained, however, almost unchanged on plasmacytoid BMDC (Fig. 18B, middle panels). BMMΦ even down-regulated CD86 and MHC-II in response to *C. neoformans* 1841 (Fig. 18B, bottom panels). Interestingly, on all three cell types the expression of CD80 barely changed in the presence of *C. neoformans*. CpG-ODN used as control stimulus induced the up-regulation of MHC-II, CD86 and CD80 on both subsets of BMDC, but, in agreement with reports on M-CSF-attenuated CpG-ODN responsiveness (203), not on BMMΦ (Fig. 18B). Taken together these data provide evidence that conventional BMDC rather than plasmacytoid BMDC or BMMΦ are activated by *C. neoformans* and point to conventional DC as important antigen-presenting cells in the immune response to *C. neoformans* infection.

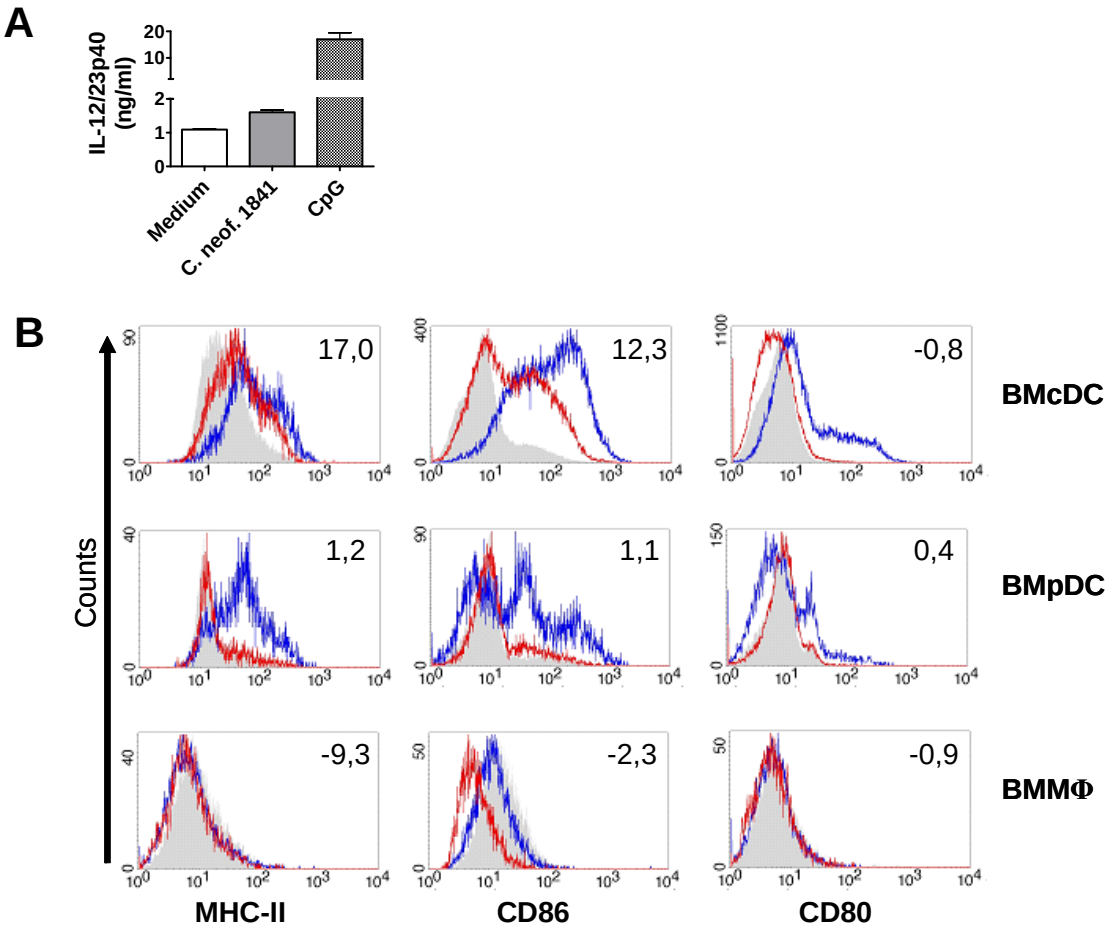

Fig. 18 Differential regulation of MHC-II, CD86 and CD80 on conventional BMDC, plasmacytoid BMDC and BMMΦ in response to *C. neoformans*.

(A) Flt3L-generated BMDC were stimulated with the indicated stimuli for 48 h, and IL-12/23p40 levels were assessed by sandwich ELISA. (B) Conventional BMDC (BMcDC; *top panels*), plasmacytoid BMDC (BMpDC; *middle panels*) and BMMΦ (*bottom panels*) were stained for MHC-II, CD86 and CD80 and analysed by FACS measurement following cultivation for 48 h in the presence of *C. neoformans* (MOI = 20; *red line*), CpG-ODN (1 µM; *blue line*) or medium (*grey curve*) as control. Numbers in the top right corners describe the difference of the fluorescence intensity medians of the indicated surface marker displayed by *C. neoformans*-stimulated and medium-cultured cells. Positive values represent up-regulation of the expression, whereas negative values display down-regulation of the indicated marker expression. Shown is one representative of at least two independent experiments.

4.3.2.4 The up-regulation of surface molecules on conventional BMDC and plasmacytoid BMDC in response to *C. neoformans* depends on MyD88

Since the production of IL-12/23p40 clearly depended on the intracellular adaptor molecule MyD88, we wished to study whether the up-regulation of surface molecules necessary for the activation of T cells was also dependent on MyD88. Therefore, FLt3L-generated BMDC derived from wild-type and MyD88$^{-/-}$ bone marrow cells were incubated with *C. neoformans*, and the expression of MHC-II, CD86, CD80 and CD40 was analysed on conventional BMDC and plasmacytoid BMDC. Again, conventional BMDC and plasmacytoid BMDC were distinguished by the expression of CD11c and B220. For control, conventional and plasmacytoid BMDC were incubated with CpG-ODN and R-848, which are known to induce MyD88-dependent signalling (204;205), and poly I:C and LPS, which are known to induce MyD88-independent signalling cascades (206).

The increase of the expression of MHC-II, CD86, CD80 and CD40 on conventional BMDC in response to *C. neoformans* clearly required the presence of MyD88 (Fig. 19 top panels). On plasmacytoid BMDC the up-regulation of MHC-II was poor compared to conventional BMDC, but also clearly depended on MyD88. Again, the expression of CD86 and CD80 hardly changed on plasmacytoid BMDC upon stimulation with *C. neoformans*. Similarly, CD40 was not remarkably up-regulated on plasmacytoid BMDC in response to *C. neoformans* (Fig. 19 bottom panels). As expected, surface molecule expression was enhanced on MyD88$^{-/-}$ conventional and plasmacytoid BMDC by poly I:C and LPS but not by CpG and R-848 (Fig. 19).

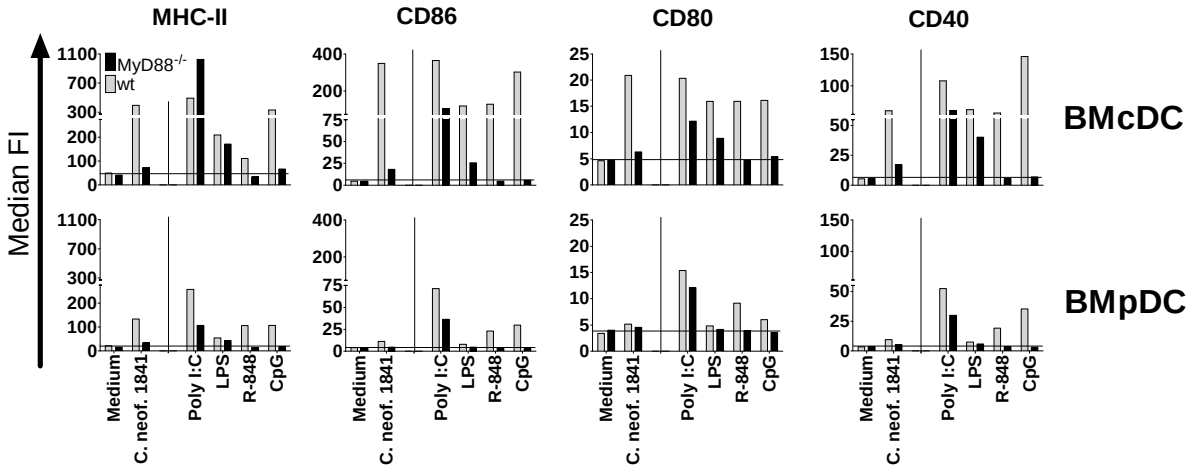

Fig. 19 *C. neoformans*-induced up-regulation of MHC-II, CD86, CD80 and CD40 on conventional BMDC derived from MyD88$^{-/-}$ and wt mice.

BMDC were stimulated with *C. neoformans* (MOI = 20) or poly I:C (100 µg/ml), LPS (5 µg/ml), R-848 (1 µg/ml) and CpG-ODN (1 µM; CpG) for control. Following 48 h incubation, BMDC were harvested and conventional BMDC (BMcDC) and plasmacytoid BMDC (BMpDC) were analysed separately for the expression of MHC-II, CD86, CD80 and CD40 by FACS analysis. The median fluorescence intensities (FI) of the indicated molecules are shown. The *horizontal line* displays the expression level of the indicated surface molecule displayed by medium cultured cells.

4.3.3 Summarizing Remarks

The obtained data show that *C. neoformans* induces the release of IL-12/23p40 by BMDC but not by BMMΦ. The induction of IL-12/23p40 in BMDC is independent of TLR2 and TLR4, whereas the intracellular signalling molecule MyD88 is essential. These data indicate that *C. neoformans* induces IL-12/23p40 in BMDC by other MyD88-signalling PRR than TLR2 and TLR4. Moreover this study demonstrates that *C. neoformans* activates conventional BMDC rather than plasmacytoid BMDC to increase the expression of MHC-II and CD86, whereas on BMMΦ the expression of MHC-II and CD86 even declines in response to *C. neoformans*. The up-regulation of surface molecules on conventional BMDC clearly depends on MyD88. Taken together these results point to conventional DC as important APC in immunity to *C. neoformans*.

4.4 Immunostimulatory effects of parapoxvirus ovis on conventional bone marrow-derived dendritic cells, plasmacytoid bone marrow-derived dendritic cells and bone marrow-derived macrophages

4.4.1 Background: parapoxvirus ovis

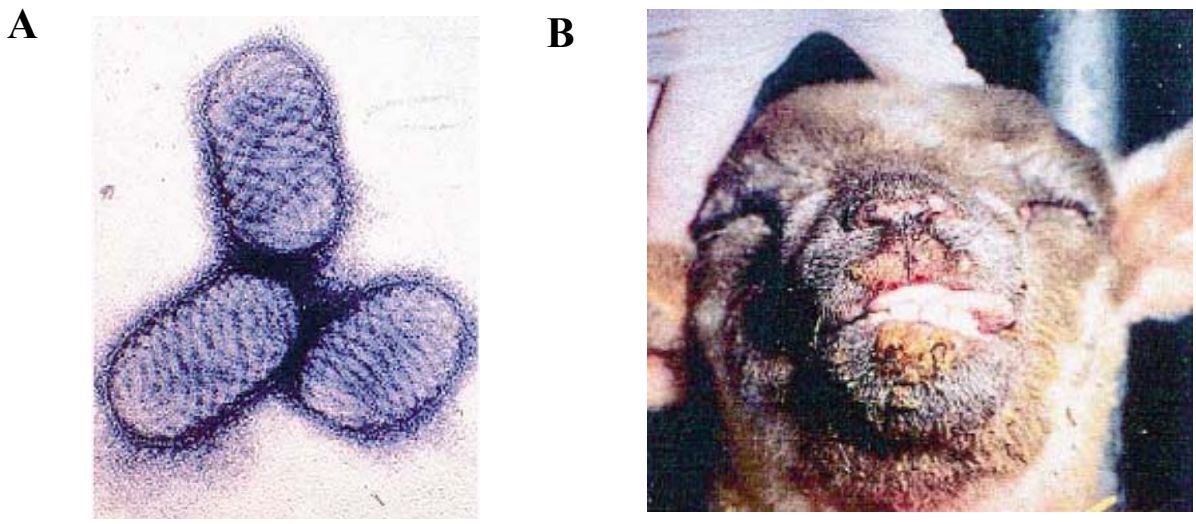

Fig. 20 Morphology of parapoxvirus ovis, that can cause contagious pustular dermatitis. (A) Parapoxvirus ovis virions are oval and display a characteristic basket weave pattern. The particle size is about 200 nm. (B) Infections are localised to the skin or buccal cavity. The pictures were adapted from (207).

Parapoxvirus ovis[3], a member of the *Parapoxvirus* genus of the *Poxviridae* family and the *Chordopoxvirinae* subfamily, is an ovoid-shaped epitheliotrophic dsDNA virus with a genome of approx. 140 kb and a particle size of about 200 nm (Fig. 20A). It infects damaged or scarified skin causing ecthyma contagiosum (contagious pustular dermatitis known as orf) in sheep (Fig. 20B) and goats worldwide, and occasionally is able to infect man (208). Parapoxvirus ovis has been shown to stimulate the immune system for release of inflammatory cytokines (209;210). Thus, parapoxvirus ovis infection might be able to modulate the outcome of concomitant infections by other pathogens. In fact, treatment with

[3] Virus nomenclature: According to the guidelines of the International Committee on Taxonomy of Viruses (ICTV) the first letters of the virus family and subfamily are capitalized and printed in italics in formal taxonomic usage. When referring to the virus being studied rather than to the taxonomic group the virus belongs to, the virus name is written in lower case Roman script without capitals.

chemically inactivated parapoxvirus ovis (iPPVO) was demonstrated to efficiently reduce susceptibility to hepatitis B virus and herpes simplex virus infection (90). Due to its immunostimulatory capacities the registered biological Baypamune® (which now is termed Zylexis®) comprising iPPVO as active component has been used in Germany as adjuvant for the prophylaxis and metaphylaxis of infections of farm animals, small animals and horses since 1990.

Among the avant-garde sentinel cells that initiate a response to immunomodulators such as iPPVO are DC and MΦ. DC are heterogeneous comprising several subsets expressing different morphological shapes and functions. In addition to conventional DC, which have a dendritic shape and exhibit DC functions such as antigen uptake, processing and presentation, plasmacytoid DC, non-dendritic shaped round cells with the ability to produce large amounts of type I interferons in response to viral pathogens, recently came into focus for protective immune responses to virus infections and immunostimulatory effects upon iPPVO application (210;211). However, the exact cellular targets and potentially cell-specific mechanisms of activation induced by iPPVO are poorly understood.

Until today two groups of innate pattern recognition receptors have been linked to virus detection and the induction of immune responses. The first group, the membrane associated toll-like receptors (TLR), is primarily expressed by DC and MΦ and comprises at least 12 TLR (72). Ligands for TLR3, TLR7 and TLR9 which are expressed at the membranes of endosomal compartments, are dsRNA, ssRNA and dsDNA with unmethylated CpG motifs (Fig. 3), respectively (62). Among the TLR expressed on the cell surface, TLR1, TLR2 and TLR6 are involved in the recognition of hepatitis C virus core protein (212). Besides, TLR2 binds herpes simplex virus (210;213), human cytomegalovirus (214) and hemagglutinin protein of measles virus (215) whereas TLR4 recognizes the fusion protein of respiratory syncytial virus (210;216) and mouse mammary tumor virus (210;214).

Ligand binding to a TLR triggers complex signalling cascades which are dependent on the intracellular adaptor molecules MyD88 and/or TRIF. Activation of MyD88 and/or TRIF triggers further downstream signalling cascades leading to the transcription of proinflammatory cytokines. All known TLR use either MyD88 or TRIF, only TLR 4 is known to signal via both adaptor molecules (62). Of note, proinflammatory cytokines and IFN are critically regulated by either MyD88 or TRIF alone but not by pathways using both adaptor molecules (217).

The second group of innate pattern recognition receptors for viruses belongs to the family of RNA helicases such as RIG-1 and MDA-5 involved in the detection of cytosolic dsRNA.

MDA-5 recognizes poly I:C and measles virus (218;219), while RIG-I sensors influenza virus and Epstein-Barr virus (220-222) leading to the expression of type I interferons. Lately the existence of novel cytosolic DNA sensor activating innate immune responses was reported (76) and provides evidence that additional antimicrobial recognition mechanisms exist.

The purpose of the third part of the present Ph. D. thesis was (i) to characterize the immunostimulatory effects of PPVO on the activation and maturation of conventional vs. plasmacytoid bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMMΦ), and (ii) to define pattern recognition receptors involved in the recognition of iPPVO.

4.4.2 Results

4.4.2.1 Inactivated parapoxvirus ovis induces IFN-α, IL-12/23p40 and TNF-α, in BMDC rather than BMMΦ

Type I interferons including IFN-α induce important anti-viral effector mechanisms such as the enhanced expression of anti-viral proteins, sensitization of bystander cells to apoptosis upon subsequent viral infection, and stimulation of anti-viral effector cells such as NK cells and cytotoxic T lymphoctes (for a detailed description see 1.2). To investigate whether the reported immunostimulatory effects of iPPVO were associated with the induction of IFN-α in DC and/or MΦ, Flt3L-generated BMDC comprising conventional and plasmacytoid BMDC (82) and BMMΦ were compared in terms of IFN-α production upon iPPVO stimulation. Additionally, the release of proinflammatory cytokines such as IL-12/23p40, and TNF-α and of the anti-microbial effector molecule NO was investigated. CpG-ODN, characterized as potent IFN-α inducer (223) and LPS, known as strong stimulator of BMDC and BMMΦ (102;224) were used for control.

Upon iPPVO stimulation BMDC were potent producers of IFN-α compared to BMMΦ, which released only poor amounts of IFN-α. Moreover, BMDC augmented IL-12/23p40 and TNF-α production in response to iPPVO, whereas BMMΦ did not enhance IL-12/23p40 release and hardly augmented TNF-α production upon iPPVO stimulation. In contrast, iPPVO-induced NO production was exclusively observed for BMMΦ pointing to a role of BMMΦ for antimicrobial effector functions rather than for an immunoregulatory role. Similar to iPPVO, CpG-ODN induced the release of IFN-α, IL-12/23p40, and TNF-α in BMDC but, in agreement with reports on M-CSF-attenuated CpG-ODN responsiveness (203), not in BMMΦ. An increase of BMMΦ-derived IL-12/23p40, TNF-α and NO levels, however, was observed upon LPS stimulation (Fig. 21A) indicating that BMMΦ had no intrinsic defect in

their responsiveness. The induction of cytokine responses by BMDC was dose-dependent (Fig. 21B). Taken together these data demonstrate that BMDC are potent producers of IL-12/23p40, IFN-α and TNF-α whereas BMMΦ play an important role for NO synthesis in response to iPPVO. This suggests that iPPVO is able to stimulate both, DC and MΦ, whereas the responses by DC and MΦ are different.

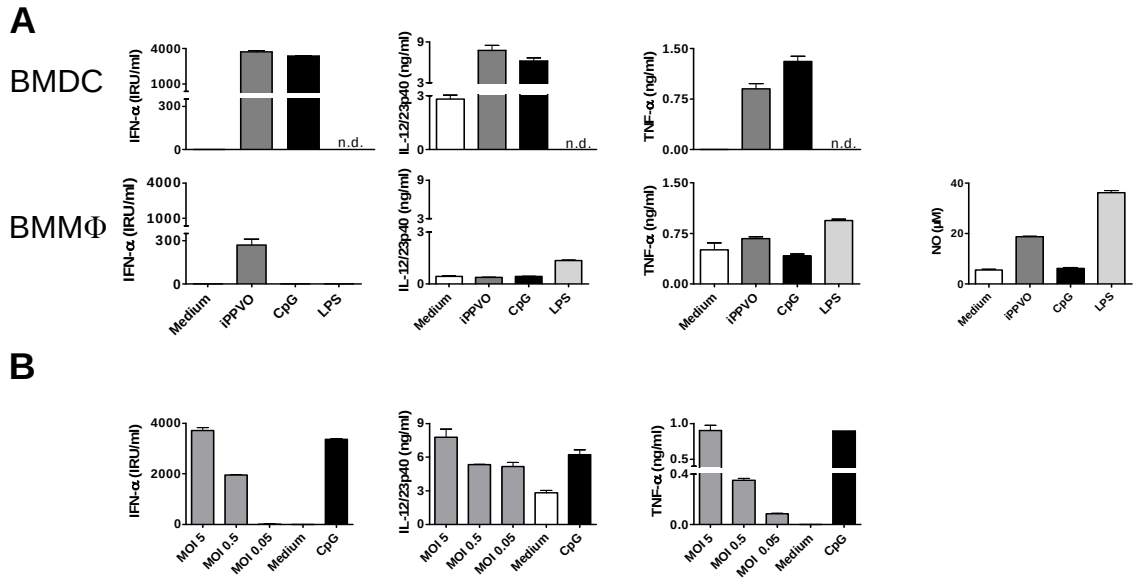

Fig. 21 Production of IFN-α, IL-12/23p40, and TNF-α by BMDC and BMMΦ in response to parapoxvirus ovis.

(A) BMDC and BMMΦ were stimulated with iPPVO (MOI = 5), CpG-ODN (1 μM) and LPS (5 μg/ml). After 48 h of incubation, concentrations of IL-12/23p40, TNF-α, IFN-α and NO in the culture supernatants were measured by sandwich ELISA and the Griess reaction, respectively. (B) Cytokine responses of BMDC following stimulation with the indicated concentrations of iPPVO and analysis as described in (A). Each column represents the mean ± SEM of triplicate cultures. BMDC were found to potently respond to LPS stimulation in other experiments (data not shown). Shown is one representative of at least three independent experiments. *n.d. = not done*

4.4.2.2 IFN-α and IL-12/23p40 production is biased towards plasmacytoid and conventional BMDC, respectively

Flt3L-generated BMDC represent a mixed culture of plasmacytoid and conventional BMDC (82). Both DC subsets express the murine DC marker CD11c on their surface (80), whereas plasmacytoid BMDC additionally express B220 (82;211). Thus, these two markers allow identification and separate analysis of these two BMDC subsets in a mixed culture of conventional and plasmacytoid DC as obtained by incubating bone marrow cells in the presence of Flt3L. To address the question, which DC subset was major producers of IFN-α and IL-12/23p40 in response to iPPVO, Flt3L-generated BMDC were stimulated with iPPVO in the presence of a protein transport inhibitor, thus inhibiting the release of iPPVO-induced, freshly synthesized IFN-α and IL-12/23p40. Subsequently, the plasmacytoid and the conventional BMDC subset were analysed for the presence of intracellular IFN-α or IL-12/23p40 by FACS measurement. CpG-ODN were used as control stimulus.

Upon stimulation with iPPVO, plasmacytoid BMDC were preferential producers of IFN-α, whereas IL-12/23p40 production was biased to conventional BMDC (Fig. 22A). Interestingly, production of IFN-α and IL-12/23p40 occurred with different kinetics. While IFN-α^+ cells reached a peak at 12 h declining thereafter upon iPPVO or CpG-ODN stimulation, the amounts of IL-12/23p40$^+$ cells continuously increased throughout the experiment (Fig. 22B). In consistence with these data, a strong increase of IFN-α levels was observed in the supernatants of iPPVO-stimulated BMDC between 6-12 h, whereas IL-12/23p40 levels were found to rise continuously (Fig. 22C). The observation of different kinetics and cell type-dependent characteristics for iPPVO-stimulated production of IFN-α vs. IL-12/23p40 indicates that differential cell-specific modes of production of these cytokines are operative.

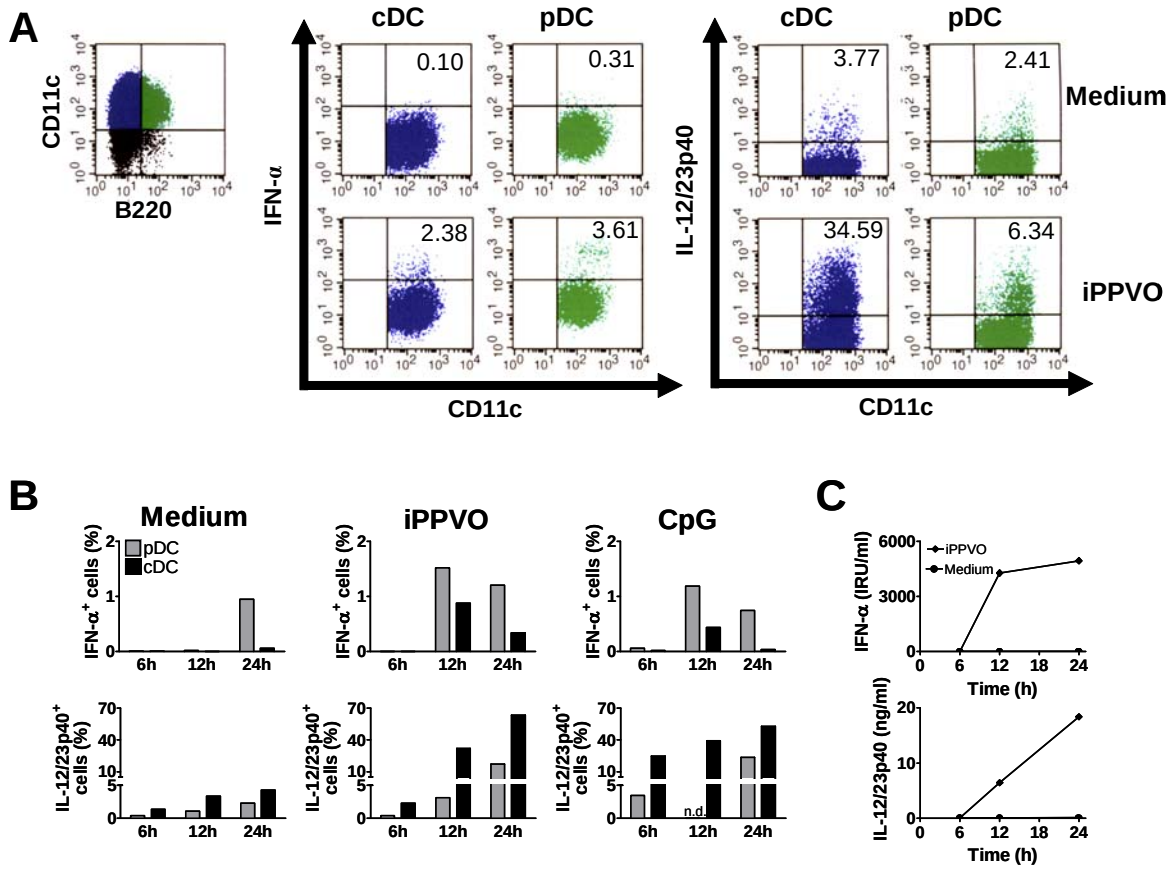

Fig. 22 iPPVO-induced IFN-α and IL-12/23p40 production is biased towards plasmacytoid and conventional BMDC, respectively and follows distinct kinetics.

(A) Flt3L-generated conventional BMDC (cDC; *blue*) and plasmacytoid BMDC (pDC; *green*) were stimulated for 12 h with medium or iPPVO (MOI = 5) and analysed for intracellular IL-12/23p40 and IFN-α synthesis as described in *Methods*. Numbers in the upper right corner describe the number of IFN-α^+ resp. IL-12/23p40$^+$ BMDC. Quadrants were set according to the isotype control staining, all background staining signals were below 0,2% (data not shown). (B) Kinetics of the intracellular production of IFN-α and IL-12/23p40 by Flt3L-generated conventional and plasmacytoid BMDC in stimulated for the indicated time points and analysed as in (A). *n.d. = not done.* (C) Time course of the secretion of IFN-α and IL-12/23p40 by Flt3L-generated plasmacytoid and conventional BMDC. BMDC were stimulated with iPPVO (MOI = 5). Supernatants were harvested at the indicated time points, and levels of IFN-α and IL-12/23p40 were analysed by sandwich ELISA. Shown is one representative of two independent experiments.

4.4.2.3 Parapoxvirus ovis induces the up-regulation of MHC-II, CD86 and CD80 in conventional rather than plasmacytoid BMDC or BMMΦ

The expression of co-stimulatory molecules on antigen-presenting cells directly correlates with their ability to present antigen to T cells and to activate naïve T cells. Therefore, it was interesting to characterize and compare the expression of MHC-II and the co-stimulatory molecules CD86 and CD80 on plasmacytoid vs. conventional BMDC vs. BMMΦ by FACS. Again, conventional and plasmacytoid BMDC were distinguished by the expression of B220 and CD11c. On plasmacytoid BMDC, MHC-II and CD86 were found to be up-regulated upon stimulation with iPPVO (Fig. 23A top panels). However, up-regulation of MHC-II and CD86 was much stronger in conventional BMDC (Fig. 23A middle panels), whereas the expression of CD80 was increased only weakly on both, plasmacytoid and conventional BMDC in response to iPPVO. BMMΦ hardly augmented the expression of CD86 in response to iPPVO and even down-regulated MHC-II and CD80 (Fig. 23A bottom panels). Upon CpG-ODN stimulation plasmacytoid and conventional BMDC expressed similar levels of MHC-II, CD86 and CD80 as upon iPPVO-stimulation. The x-fold up-regulation of CD86 upon CpG-ODN stimulation was identical in conventional and plasmacytoid BMDC. BMMΦ only weakly up-regulated CD86, which is consistent with reports on attenuated CpG-ODN responsiveness in the presence of M-CSF (203) (Fig. 23A). The up-regulation of MHC-II, CD86 and CD80 on BMDC was dose-dependent (Fig. 23B). These data suggest conventional BMDC rather than plasmacytoid BMDC or BMMΦ to be potent antigen presenting cells in response to iPPVO.

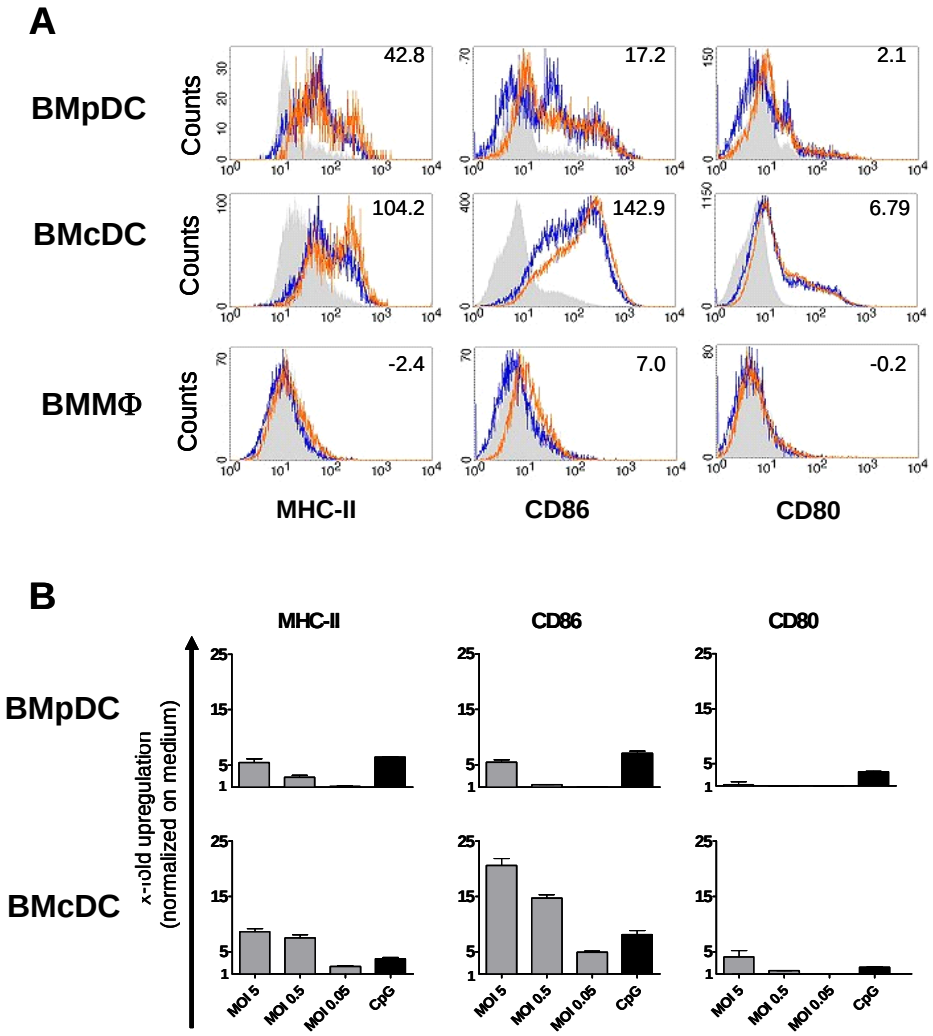

Fig. 23 iPPVO-induces up-regulation of MHC-II, CD86 and CD80 on conventional BMDC rather than plasmacytoid BMDC or BMMΦ.

(A) Following cultivation for 48 h in the presence of iPPVO (MOI = 5*; orange line*), medium (*grey curve*) and CpG-ODN (1 µM; *blue line*) for control, plasmacytoid BMDC (BMpDC) and conventional BMDC (BMcDC) as BMMΦ were stained for MHC-II, CD86 and CD80 and analysed separately by FACS. The numbers in the top right corners display the difference of the fluorescence intensity medians of the indicated molecule of iPPVO- vs. medium-cultured cells. Negative values indicate a down-regulation of the indicated molecule by iPPVO. (B) The x-fold up-regulation of MHC-II, CD86 and CD80 on plasmacytoid BMDC (BMpDC) and conventional BMDC (BMcDC) following stimulation as in (A) was calculated by division of the fluorescence intensity medians of the indicated surface molecule after cultivation in the presence of the indicated MOIs of iPPVO by those of medium cultured cells. The up-regulation of the indicated surface molecules of medium-cultured cells was normalized to 1. Each column represent mean ± SEM of triplicate cultures. Shown is one representative of at least two independent experiments.

4.4.2.4 The BMDC-activating constituent of iPPVO is heat-stable

iPPVO bears several pathogen-associated molecular patterns, which could account for the immunostimulatory effects of iPPVO on BMDC and BMMΦ including virus-associated proteins and viral dsDNA. To define the role of iPPVO-DNA for the activation of BMDC, iPPVO was incubated for 60 min at 60°C prior to BMDC stimulation. At this temperature proteins are gently denaturated, whereas DNA is heat-stable. Additionally, purified viral DNA was analysed for immunostimulatory capacities on BMDC and compared to iPPVO-induced BMDC activation, as assessed by measurement of IFN-α, TNF-α and IL-12/23p40 release and the up-regulation of MHC-II, CD86 and CD80. Again, CpG-ODN were used as control stimulus.

BMDC stimulated with heat-incubated iPPVO produced similar levels of IFN-α and TNF-α as iPPVO-stimulated BMDC. Moreover, heat-incubated iPPVO induced IL-12/23p40 production. These data indicate that the observed cytokine induction is triggered by a heat-stable component such as heat-stable protein domains or possibly viral DNA. In fact, IL-12/23p40 release was triggered even upon stimulation with purified viral DNA. Moreover, initial results of our group show that even iPPVO that has been boiled at 95°C induces the release of IL-12/23p40 (data not shown). However, neither IFN-α nor TNF-α was secreted after stimulation with viral DNA or boiled iPPVO, suggesting that viral DNA plays a pivotal role for the induction of IL-12 family members including IL-12/23p40. Stimulation with the control stimulus CpG-ODN induced high amounts of IFN-α, TNF-α and IL-12/23p40 (Fig. 24).

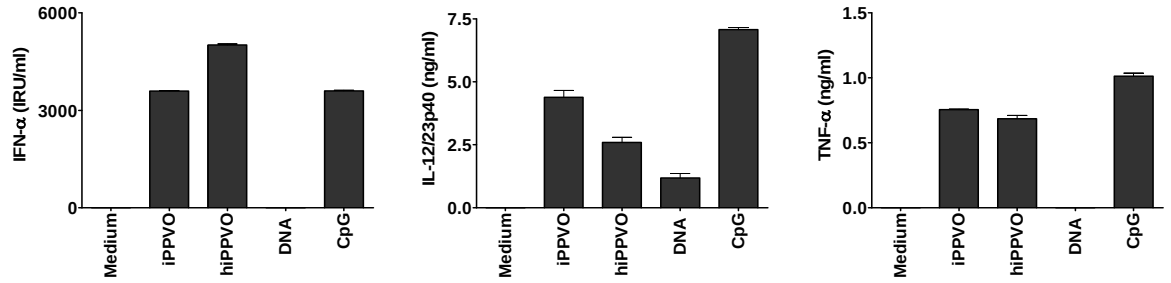

Fig. 24 Levels of BMDC-derived IFN-α, IL-12/23p40 and TNF-α following stimulation with iPPVO, heat-incubated iPPVO (hiPPVO) and viral DNA.

BMDC were cultured with iPPVO (MOI = 5), hiPPVO (MOI = 5) and viral DNA (2 µg/ml) for 48 h, and supernatants were subsequently analysed for IFN-α, TNF-α and IL-12/23p40 by sandwich ELISA. Each column represent mean ± SEM of duplicate cultures.

In addition to cytokine responses, the expression of MHC-II, CD86 and CD80 on plasmacytoid and conventional DC upon stimulation with heat-incubated iPPVO were analyzed. Expression of MHC-II and CD80 was increased to the same degree by heat-incubated iPPVO and non-heat-incubated iPPVO on plasmacytoid BMDC, whereas the expression of CD86 tended to be reduced (Fig. 25 top panel). Similar results were observed for the expression of MHC-II, CD86 and CD80 on conventional BMDC, which was not markedly affected by heat-incubation of iPPVO. CpG-ODN, as expected, activated plasmacytoid and conventional BMDC to up-regulate MHC-II, CD86 and CD80. However, purified iPPVO DNA barely changed the expression levels of MHC-II, CD86 or CD80 (Fig. 25). These observations suggest that pure DNA at the used concentration of 2 µg/ml is not able to induce surface molecule expression possibly due to a poor uptake of pure DNA into the BMDC. Nevertheless, activation of plasmacytoid and conventional BMDC by heat-incubated iPPVO point to a heat-stable component as supposedly heat-stable protein domains or viral DNA as important inducer of plasmacytoid and conventional DC activation, when viral DNA is delivered into the cell. This conclusion is supported by the fact, that boiled iPPVO is able to induce up-regulation of MHC-II, CD86 and CD80 (F. Herden, personal communication; data not shown).

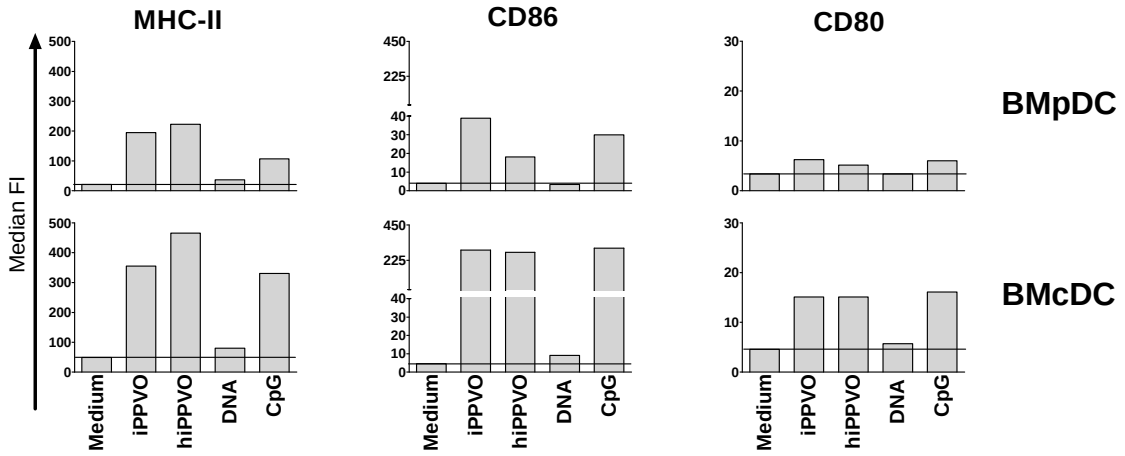

Fig. 25 Expression of MHC-II, CD86, and CD80 by plasmacytoid BMDC and conventional BMDC after stimulation with iPPVO, heat-incubated iPPVO, and viral DNA.

Conventional BMDC (BMcDC) and plasmacytoid BMDC (BMpDC) were cultured in the presence of the iPPVO (MOI = 5), heat-incubated iPPVO (hiPPVO; MOI = 5), viral DNA (2 µg/ml) and CpG-ODN (CpG; 1 µM). Following an 48 h incubation, BMDC were harvested, and electronically gated conventional and plasmacytoid BMDC subsets were analysed for the expression of the indicated surface molecules. Columns represent median fluorescence intensities (FI). The *horizontal line* indicates expression levels of medium-cultured cells.

4.4.2.5 Parapoxvirus ovis induces IFN-α, IL-12/23p40 and TNF-α partially MyD88-dependent

The immediate steps leading to iPPVO-induced activation of BMDC have not yet been defined. PPVO belongs to the poxviruses which contain dsDNA (225). TLR9 is known to recognize double-stranded bacterial and in some instances also viral DNA (205;226). Similar to other currently known TLR (except TLR3 and TLR4), TLR9 mediates intracellular signalling exclusively by the adaptor molecule MyD88 (72). Therefore, investigations on the role of MyD88 in iPPVO-induced BMDC activation allows to draw conclusions on the TLR involved in iPPVO recognition. To this end, BMDC originating from wt and MyD88$^{-/-}$ mice were stimulated with iPPVO. Poly I:C, LPS, R-848 and CpG-ODN, known ligands recognized by TLR3, TLR4, TLR7 and TLR9, respectively, were used as control stimuli.

Upon iPPVO stimulation IFN-α levels were the same for wt and MyD88$^{-/-}$ BMDC, pointing to other signalling pathways for the transcription and secretion of IFN-α than those using

MyD88. As expected, CpG-ODN was the only control stimulus triggering IFN-α release, and the induction was MyD88-dependent. The release of IL-12/23p40 and TNF-α by BMDC following iPPVO stimulation was markedly reduced in MyD88$^{-/-}$ BMDC as compared to wt BMDC. However, low-level-production of IL-12/23p40 and marginal levels of TNF-α were also induced in MyD88$^{-/-}$ BMDC, indicating that the induction of IL-12/23p40 and TNF-α is partially but not exclusively mediated by MyD88. As expected, poly I:C and LPS, which can activate TRIF-dependent signalling pathways, induced IL-12/23p40 independently of MyD88, whereas CpG-ODN and R-848, which both depend on MyD88 exclusively, did not. Interestingly, TNF-α release, however, was triggered by MyD88-dependent signalling pathways by all used control stimuli (Fig. 26).

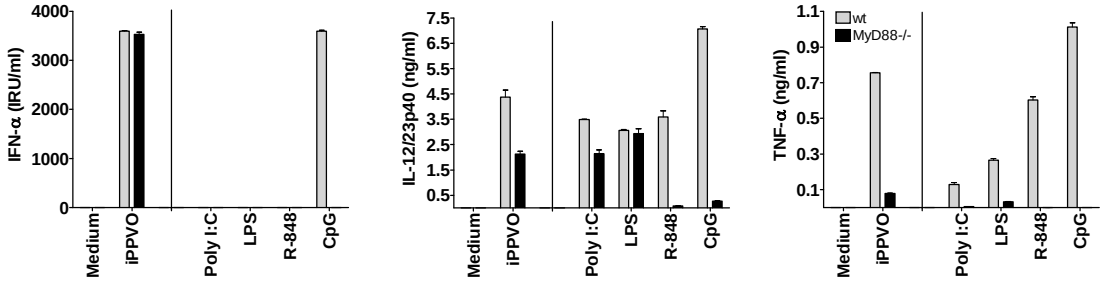

Fig. 26 The release of IFN-α, IL-12/23p40 and TNF-α by a mixed culture of plasmacytoid and conventional BMDC derived from wt and MyD88$^{-/-}$ bone marrow following iPPVO stimulation.

BMDC were cultured in the presence of iPPVO (MOI = 5) for 48 h. For control BMDC were stimulated with poly I:C (100 µg/ml; TLR3 ligand), LPS (5 µg/ml; TLR4 ligand), R-848 (1 µg/ml; TLR7 ligand) and CpG-ODN (1 µM; TLR9 ligand; CpG). BMDC culture supernatants were harvested and cytokine amounts measured by sandwich ELISA. Each column represents mean ± SEM of duplicate cultures.

4.4.2.6 Parapoxvirus ovis enhances the expression of MHC-II, CD86 and CD80 on DC partially MyD88-dependent

Furthermore, the MyD88-dependance of the activation of plasmacytoid and conventional BMDC was assessed by the expression of MHC-II, CD86 and CD80. Consistent with the results for cytokine induction by iPPVO, MyD88$^{-/-}$ plasmacytoid and conventional BMDC

displayed reduced levels of MHC-II and CD86 compared to wt plasmacytoid BMDC upon iPPVO stimulation. The same observations were made for CD80 on conventional BMDC. As expected, TLR3 ligand poly I:C and TLR4 ligand LPS, both ligands known to be able to signal by the intracellular adaptor molecule TRIF (206), induced the up-regulation of MHC-II, CD86, and CD80 in MyD88$^{-/-}$ BMDC, whereas R-848 and CpG-ODN, both ligands using MyD88-dependent signalling (204;205), did not. This indicates that BMDC recognize iPPVO by a MyD88-dependent TLR (e.g. TLR9 possibly sensing iPPVO-DNA) and this recognition mechanism contributes to the observed stimulation of enhanced expression of co-stimulatory molecules. Besides, other mechanisms must exist which contribute to the observed stimulatory effect (Fig. 27).

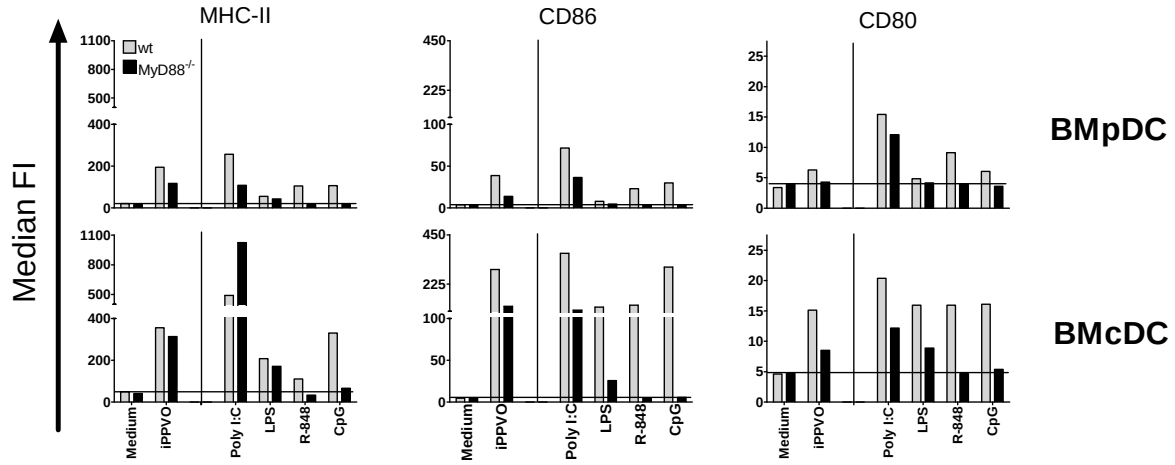

Fig. 27 The expression of MHC-II, CD86 and CD80 on wt and MyD88$^{-/-}$ plasmacytoid and conventional BMDC in response to iPPVO.

BMDC were stimulated with iPPVO (MOI = 5) or poly I:C (100 µg/ml; TLR3 ligand), LPS (5 µg/ml; TLR4 ligand), R-848 (1 µg/ml; TLR8 ligand) and CpG-ODN (1 µM; TLR9 ligand; CpG) for control. Following 48 h incubation, BMDC were harvested and plasmacytoid BMDC (pDC) and conventional BMDC (cDC) were analysed separately for the expression of MHC-II, CD86 and CD80 by FACS analysis. The median fluorescence intensities (FI) of the indicated molecules are shown. The *horizontal line* displays the expression level of the indicated surface molecule displayed by medium cultured cells.

Taken together these data for the first time demonstrate that PPVO-induced activation of conventional and plasmacytoid BMDC is partially dependent on MyD88, providing substantial evidence that iPPVO activates additional MyD88-independent signalling pathways especially for the induction for type I interferons. The involvement of MyD88 in the recognition of iPPVO points to the recognition of iPPVO by TLRs inducing MyD88-

dependent signalling pathways. However, the MyD88-independent signalling mechanisms indicate recognition mechanisms by additional yet undefined receptors. The role of TLR9 and the MyD88$^{-/-}$-independent mechanisms in the recognition of iPPVO merit further investigation.

4.4.3 Summarizing Remarks

The present study shows for the first time that iPPVO activates conventional and plasmacytoid BMDC rather than BMMΦ partially by MyD88, leading to the release proinflammatory cytokines such as IFN-α, IL-12/23p40, and TNF-α, and the enhanced expression of surface molecules required for T cell activation. Therefore, these results point to DC rather than MΦ as mediators of immunostimulatory effects induced by iPPVO. On the other hand BMMΦ but not BMDC were found to produce NO in response to iPPVO. This argues for a significant role of MΦ in antimicrobial effector functions. Moreover, the present data demonstrate for the first time that iPPVO activates MyD88-dependent and -independent signalling pathways, making it tempting to speculate that iPPVO is recognized by more than a single receptor. Therefore, although additional studies are necessary to further define the molecular interactions between iPPVO and innate immune receptors, this study substantially contributes to the understanding of the immunostimulatory effects by iPPVO.

5 Discussion

By the production of IL-12 family members antigen-presenting cells such as DC and MΦ orchestrate $CD4^+$ Th1 cell-mediated immune responses essential for the protection against intracellular pathogens including *Salmonella* and *Cryptococcus* (37;155). DC, comprising conventional and plasmacytoid DC subsets, and MΦ recognize pathogens by pattern recognition receptors. Among PRR, the TLR family is known to recognize many microbial components such as LPS, CpG oligonucleotides, lipopeptides, and viral glycoproteins (72). Upon engagement TLR trigger a complex network of intracellular signalling cascades invariably involving the intracellular signalling proteins MyD88 and/or TRIF (72). Intracellular signalling results in cytokine release including IL-12 family members and expression of MHC-II and co-stimulatory molecules such as CD80 and CD86 necessary for successful induction of T cell responses. Additionally, plasmacytoid DC have the unique ability to quickly produce high amounts of IFN-α upon TLR engagement, a type I interferon interfering with virus replication and protein synthesis.

To gain a better understanding of the activation of conventional and plasmacytoid DC subsets and MΦ and their contribution to the induction of cell-mediated immune responses to (i) bacterial, (ii) fungal, and (iii) viral pathogens, in the present study conventional DC, plasmacytoid DC, and MΦ were compared in terms of cytokine production (mainly IL-12 family members) and the expression of MHC-II, CD86 and CD80 in response to (i) the facultative intracellular bacterium *S*. Enteritidis, (ii) the facultative intracellular fungus *C. neoformans*, or (iii) parapoxvirus ovis.

5.1 Production of the IL-12 family members by DC and MΦ activated by the facultative intracellular bacterium *Salmonella* Enteritidis

5.1.1 Production of IL-12 family members and NO in response to *S*. Enteritidis: task sharing of DC and MΦ

Observations from human patients as well as data generated with murine infection models have shown that the members of the IL-12 cytokine family are essential for immunity to *Salmonella* (85;127-130). Several studies have suggested that macrophages are producers of the IL-12 cytokine family in response to *Salmonella* infection (133;227). Data in more recent years have pointed to the dendritic cell as important target cells for *Salmonella* and as a

source for IL-12 (24;140;142;228). However, the major cellular sources for IL-12 in immunity to *Salmonella* infection remained elusive (229;230).

To investigate IL-12 family production in response to *S*. Enteritidis *in vitro*, conventional bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMMΦ), that share characteristic biological activities with primary conventional DC and macrophages (100;110;231), were used. This study shows that both, BMDC and BMMΦ, are able to produce IL-12/23p40 and IL-27p28 in response to *Salmonella* with BMDC producing about 20-fold more IL-12/23p40 and IL-27p28 than BMMΦ. It is demonstrated that only BMDC but not BMMΦ release IL-12 and IL-23 upon stimulation with *S.* Enteritidis. The results obtained *in vitro* are corroborated by subsequent investigations with primary DC and MΦ studied *ex vivo*, which disclose that following i.p. infection with *S*. Enteritidis, peritoneal production of IL-12/23p40 is dominated by DC.

For the successful induction of T cell responses, antigen-presenting cells such as DC and MΦ have to express antigenic peptides as MHC:peptide complex on the cell surface together with co-stimulatory molecules in addition to the production of cytokines. Whereas both antigen-presenting cell types, BMDC and BMMΦ, are able to up-regulate the expression of co-stimulatory molecules and production of pro-inflammatory cytokines after infection with *S*. Typhimurium, only BMDC have been shown to migrate to the draining lymph nodes and to induce a *Salmonella*-specific T cell response following injection into the footpad (134). Depletion of MΦ during vaccination with attenuated *S.* Typhimurium did not result in loss of protection against challenge with virulent *S.* Typhimurium (137), indicating that MΦ are neglible for the induction of adaptive immune responses. In agreement with these results, monocytes recruited to the site of infection, which later give rise to tissue-resident MΦ, were unable to process bacteria and present *Salmonella*-derived antigen to T cells (229) after oral *S*. Typhimurium infection. DC, however, were reported to be critical for initiating adaptive immune responses *in vivo* to orally acquired *S*. Typhimurium (232). In this context, IL-12 has been shown to be important for the induction of Th1-mediated immune responses (154;233) while IL-23 has been reported to be important for the maintenance of IL-17 producing T-cells (234;235). Moreover, in a study published in 2006, IL-27p28 was demonstrated to inhibit the development of IL-17-producing T cells (57). Preferential production of the T-cell differentiating cytokines of the IL-12 family by conventional DC as compared to MΦ is consistent with the known function of DC as critical inducers of adaptive T cell mediated immune responses. Although in the present study DC were not directly tracked for their migration patterns, it was striking that conventional DC but not plasmacytoid DC and MΦ

significantly expanded in the parathymic lymph node (LN) draining the peritoneal cavity in response to i.p. *S.* Enteritidis infection. Elevated numbers of DC in the parathymic LN could be the consequence of DC migration from the site of infection (peritoneal cavity) to the DLN. The fact, that MHC-II$^+$ DC expanded in the DLN upon *S*. Enteritidis infection point to conventional DC as major antigen-presenting cells in response to *Salmonella*. Peritoneal MΦ, however, were main producers of NO as assessed by depletion experiments in this study. This observation supports and extends results from an oral *Salmonella* infection model, where inflammatory monocytes recruited to the intestinal tissue were the major producers of iNOS, whereas DC made little if any contribution to host iNOS during infection (229). Moreover, MΦ have been found to be effector cells during salmonellosis which may even exert an immunopathological function in response to infection with virulent *S*. Typhimurium strains (137).

Our results are in line with data reported by other *in vitro* and *ex vivo* studies also pointing to distinct cytokine and chemokine production by DC vs. MΦ in response to *Salmonella* (142;227;236;237). It is noteworthy that murine MΦ have mostly been found to produce IL-12/23p40 but no or only low amounts of IL-12p70 (132;133;227). The importance of DC as major IL-12-producing cell population was also demonstrated in one study conducted *in vivo* using a *Toxoplasma* infection model (238).

5.1.2 Regulation and kinetics of the *S.* Enteritidis-induced release of IL-12 family members

It has been reported that the secretion of IL-12 is limited by endogenous IL-10 (239). In a previous study by our group using *S.* Enteritidis-stimulated BMMΦ derived from IL-10$^{-/-}$ mice, a powerful suppression of both, IL-12 and IL-23 by endogenous as well as exogenous IL-10 was demonstrated (133). Similar to BMMΦ the secretion of IL-12 and IL-23 was down-regulated in BMDC by the addition of exogenous IL-10 when stimulated with *S.* Enteritidis. However, comparison of the secretion of endogenous IL-10 in our experimental system revealed that BMDC and BMMΦ released comparable amounts of IL-10. Therefore, although IL-10 is able to down-regulate the release of IL-12 and IL-23, other factors and/or mechanisms may contribute to the regulation of the release of the IL-12 cytokine family members.

We characterized the time course of the secretion of IL-12, IL-23 and IL-12/23p40 by BMDC. The fact that the production of each cytokine follows its own kinetics indicates that

the synthesis of the members of the IL-12 cytokine family is regulated separately for each subunit as described earlier (240). In a previous study looking at cytokine mRNA levels our group observed that the synthesis of IL-12 family members follows differential kinetics in BMMΦ (133). Interestingly, the kinetics of the IL-12 family member release by BMDC is very similar to that of IL-12 family member mRNA-production in BMMΦ (see Fig. 3). Since in BMDC the same differential production kinetics were obtained using either heat-inactivated *S.* Enteritidis (data not shown) or viable *S.* Enteritidis, we conclude that replication of *Salmonella* is not critical for the induction of IL-12/23p40, IL-12 and IL-23.

IL-27p28 was released in higher amounts compared to IL-12 and IL-23 by BMDC and BMMΦ. Higher levels of IL-27p28 mRNA compared to those of IL-12p35 and IL-23p19 were observed by our group earlier in BMMΦ (133). Smits et *al.* also reported the up-regulation of IL-27p28 and IL-23p19 mRNA in response to non-pathogenic Gram-negative bacteria in human monocyte-derived DC yet without quantifying absolute levels of transcribed IL-27p28 and IL-23p19 (241). The present study for the first time demonstrates that IL-27p28 protein is released in larger amounts than IL-12 and IL-23 by antigen-presenting cells *in vitro*. Unfortunately, measurement of the complete heterodimeric IL-27 by ELISA is not possible yet. When IL-27 was first described, the release of murine monomeric IL-27p28 in addition to the output of heterodimeric IL-27 from transfected cell lines was already reported (55). The release of IL-27p28 was also demonstrated in a septic peritonitis model (242). The fact that IL-27p28 can be secreted as monomer is a feature distinct from the related cytokine subunits IL-12p35 and IL-23p19 which only can be secreted as heterodimers bound to the IL-12/23p40-subunit. (51;55;243). The biological function of IL-27 is still discussed controversially. While Th1-inducing effects have been demonstrated in initial studies (55;244;245), more recent studies suggest a role in limiting the intensity and duration of Th1 responses (246-248). Investigations of the biological function of IL-27p28 revealed that in contrast to heterodimeric IL-27, monomeric IL-27p28 is unable to induce T cell proliferation (55). Very recently others demonstrated that IL-27p28 was able to inhibit the development of IL-17-producing T-cells, albeit to a lower degree than heterodimeric IL-27 (57). Further studies will be necessary to elucidate the biological functions of IL-27 and IL-27p28.

5.1.3 *S.* Enteritidis induces IL-12 family members chiefly by LPS/TLR4 interaction

The present data demonstrate that the *Salmonella*-induced release of IL-12, IL-23 and IL-27p28 by BMDC is induced by LPS/TLR4 interaction, whereas the secretion of

IL-12/23p40 is triggered also by other PRR than TLR4. Considering how many TLR ligands are expressed by *Salmonella*, the prime role of LPS in induction of the IL-12 cytokine family is surprising. Many TLR ligands such as CpG DNA, lipoteichoic acid, lipopeptides, flagellin, and poly I:C have been shown to induce the transcription or release of IL-12 (249;250), IL-23 (251;252) and IL-27 (251;253). Moreover, the ligation of TLR4 by LPS was reported to be a critical but not an exclusive event leading to DC and MΦ responses to Gram-negative bacteria such as *Salmonella* (254;255). Nevertheless, our data corroborate other studies also reporting the importance of TLR4 for the induction of the IL-12 cytokine family by *Salmonella* (111;133) and for protective immunity to *Salmonella* (135;256-259). In contrast to IL-12, IL-23 and IL-27p28, production of IL-12/23p40 was also triggered by other PRR than TLR4. However, the signals resulting from such activation were insufficient for high-level production of secreted IL-12/23p40. We also observed TLR4-independent low-level TNF-α production. These findings are consistent with data reported from Li *et al.* describing a TLR4-independent low-level production of TNF-α induced by *Salmonella* in BMMΦ (111;260). Taken together, the present data show that although *S.* Enteritidis bears several ligands for PRR on its surface, LPS has a prime role in inducing IL-12, IL-23 and IL-27p28. Therefore, BMDC and BMMΦ share the mechanism of recognition of *Salmonella* but differ in their cytokine response to *Salmonella*.

5.1.4 Preferential production of IL-12/23p40 at the site of infection *in vivo*

Production of IL-12/23p40 preferentially occurred by peritoneal exudat cells obtained from the peritoneal cavity compared to IL-12/23p40 production observed by DLN cells. Since the IL-12 family in general is associated with the orchestration of T cell differentiation, the preferential production of IL-12/23p40 at the site of infection sheds new light on a current immunological concept of T cell induction and local effector responses. IL-12 has been shown to be essential for the control of the growth of intracellular bacteria in MΦ such as *Salmonella* and *Listeria* (84;261). IL-23, another member of the IL-12 family, is able to induce IL-17 (45;262;263), a cytokine which mediates the recruitment of neutrophilic granulocytes (264). The present data demonstrates a rapid production of IL-17 in response to *Salmonella*. Interestingly, IL-23-induced IL-17 was also reported to be important for optimal Th1-dependent IFN-γ production, necessary to mediate protective immune responses to *mycobacteria* (263;265). IL-12/23p40 was reported to play a role for the migration of DC to the DLN in response to *mycobacteria*, a prerequisite for the induction of naïve T cell

differentiation (266). Thus, production of IL-12 family members at the site of infection seems conceivable additionally orchestrating bactericidal immune mechanisms.

Our data support reports from others who demonstrated that in response to *Aspergillus fumigatus* the majority of DC were activated and displayed a mature phenotype as assessed by the release of TNF-α and the enhanced expression of co-stimulatory molecules. However, in the same study only DC, which had internalized the fungus, up-regulated CCR7 and thus may have acquired the capacity to migrate into secondary lymph nodes (267). Indirect maturation of DC without direct pathogen association has also been shown for *Salmonella* (144). Therefore, it is conceivable that in response to *S*. Enteritidis the majority of DC present at the site of infection is activated, releases cytokines including IL-12 family members and enhances the expression of co-stimulatory molecules, whereas only DC with internalized *salmonellae* migrate to the DLN to induce T cell differentiation. In fact, it is currently unclear whether migrating skin DC, recruited blood-borne DC, or lymph node resident DC are involved in T cell activation to local microbial infection. It was demonstrated that after pulmonary infection with *C. neoformans* the polarization of $CD4^+$ T cells to IFN-γ producing Th1 cells occurred at the site of infection (268). Revindran *et al.* demonstrated that after intradermal infection with *Salmonella* T cell activation required CCR6-dependet cell migration. Interestingly, DC populations in the DLN did not alter in CCR6-deficient mice after infection, although T cell activation was abrogated if CCR6 was missing. By transfer of blood phagocytes into CCR6-deficient mice, T cell activation in response to *Salmonella* infection could be restored, but antigen presentation by blood phagocytes was not required to re-establish the activation of T cells. Therefore, Revindran *et al.* propose that following *Salmonella* infection blood phagocytes are recruited to the site of infection in a CCR6-dependent manner, engulf the bacteria and migrate to the lymph nodes where antigen is transferred to resident DC that can then mediate T cell activation (269). A role for blood-borne DC has also been shown in a very recent study demonstrating that upon dermal infection with *Leishmania* (*L*.) *major* monocytes are recruited from the blood to the site of infection and there differentiate into DC. These monocyte-derived DC were the only DC capable of capturing and presenting *L. major* resulting in a protective Th1-mediated immune response, whereas dermal DC were not involved in antigen presentation (270).

Interestingly, the present study for the first time demonstrates that not only conventional DC but also plasmacytoid DC, actually best characterized for their ability to respond to viral infections (271), increased at the site of infection but not at the secondary lymph nodes in response to *S*. Enteritidis. This is intriguing in light of a recent study demonstrating that the

TLR5 ligand flagellin is able to recruit plasmacytoid DC whereas LPS is not (272). In fact, little is known about the role of plasmacytoid DC during bacterial infection. Expansion of plasmacytoid DC was described in response to infection with *Listeria monocytogenes* (273). In response to CpG oligonucleotides, which can be found in viral and bacterial DNA, interaction of plasmacytoid DC with conventional DC was essential to facilitate the induction of IL-12 production by conventional DC (274). Additional *in vivo* studies are required illuminate the role of plasmacytoid DC and other cell types such as blood phagocytes, skin DC and/or resident lymph node DC in bacterial infections.

5.1.5 Early production of IL-17 in response to *S*. Enteritidis infection

S. Enteritidis induced the release of IFN-γ, as reported by others earlier (275;276). The production of IFN-γ was dependent on IL-12 as assessed by *S*. Enteritidis infection of IL-12p35$^{-/-}$ mice lacking the ability to produce IL-12. IFN-γ production of IL-12p35$^{-/-}$ PEC was almost completely abrogated, demonstrating the induction of IL-12 in response to *S*. Enteritidis *in vivo*. The IL-12/IFN-γ axis has been shown to be essential for protection against *Salmonella* in mice and men (84;277). Lately, IL-23 was reported to promote IL-17 production by T cells (45). While in this very first study memory cells were suggested to be the IL-17 producing cell type, later studies reported antigen- or polyclonally activated naïve T cells to be potent producers of IL-17 in response to IL-23 in mice and men (157;278). Since these IL-17-producing T cells expressed CD4 characteristic for T helper (Th) cells, they were nominated Th17 cells (53). Currently available data suggest that the induction of Th17 cells is mediated by TGF-β and IL-6, whereas IL-23 is essential for proliferation and survival of Th17 cells (279).

The IL-23/IL-17 axis was found to complement IL-12-induced cell-mediated immune responses (52;86). IL-17 induces the expression of proinflammatory cytokines (such as IL-6 and TNF), chemokines and matrix metalloprotease mediating tissue infiltration and tissue destruction (46). Moreover, IL-17 is also involved in the chemotaxis of neutrophils (264;280;281) and it co-stimulates T cells and enhances the maturation of dendritic cells (46).

The present study demonstrates that *S*. Enteritidis induced high production of IL-17 in PEC cultured *ex vivo* at 1 day after infection. Early induction of IL-17 was also reported in response to *Mycobacterium tuberculosis* (265;282) and *E. coli* infection (283). As source of IL-17 γδ-T cells were identified, and production of IL-17 depended on IL-23 (265;283). Interestingly, the present data demonstrate that in response to *S*. Enteritidis the induction of

IL-17 was partially but not exclusively dependent on IL-23, suggesting a role for other still undefined mechanisms involved in the induction of IL-17. The characterization of the cellular sources of IL-17 and the mechanisms leading to IL-23-independent production of IL-17 in response to *S*. Enteritidis merit further investigations.

In summary, the present study demonstrates that despite of a similar mechanism of recognition BMDC are more potent producers of IL-12/23p40, IL-12, IL-23, and IL-27p28 than BMMΦ in response to *Salmonella*. Consistent with these *in vitro* results, the *ex vivo* production of IL-12/23p40 by peritoneal exudat cells is dominated by peritoneal DC. Interestingly, IL-12/23p40 production preferentially occurs at the peritoneal cavity rather than at the DLN, suggesting an important role for IL-12 family members at the site of infection in addition to the well known function for the orchestration of T cell differentiation in the secondary lymph nodes. Moreover, the present data show that both, conventional and plasmacytoid DC but not MΦ expand at the site of infection, whereas only conventional DC also expand in the DLN. These data point to conventional DC as major antigen-presenting cell producing IL-12 and other members of the IL-12 family.

5.2 Production of IL-12 family members by conventional BMDC, plasmacytoid BMDC and BMMΦ activated by *Cryptococcus neoformans*

Data generated with murine infection models have shown that IL-12 and IL-23 are essential for cell-mediated immunity to intracellular fungal pathogens such as *C. neoformans* (86;87;94;175). However, it is still unclear which innate immune cells are activated by *C. neoformans* to produce IL-12 family members and to induce T cell responses. This *in vitro* study for the first time includes plasmacytoid DC in the analysis of innate immune cell types which may be responsive to *C. neoformans* and shows that *C. neoformans* preferentially activates conventional BMDC rather than plasmacytoid BMDC and down-regulates BMMΦ. We demonstrate that *C. neoformans* induces the release of IL-12/23p40 by BMDC generated either in the presence of GM-CSF-containing supernatant or Flt3L but not by BMMΦ. The induction of IL-12/23p40 occurred independently of TLR2 or TLR4 in conventional BMDC. Finally, conventional but not plasmacytoid BMDC or BMMΦ acquire surface molecules required for antigen presentation in response to stimulation with *C. neoformans*. The present results provide evidence for MyD88-dependent mechanisms of both, IL-12/23p40 production

and up-regulation of MHC-II and CD86 expression by *C. neoformans*-activated conventional DC.

Both, DC and MΦ were shown to be able to respond to *C. neoformans* by internalization (187;195;284). Subsequently their responses apparently differ greatly. It was demonstrated for murine MΦ and human PBMC *in vitro* that the capsule of *C. neoformans* has inhibitory effects on the release of IL-12 (185;186). On the other hand in a study investigating DC phenotypes lower numbers of $CD11c^{+}MHC\text{-}II^{+}$ DC in the lung-associated lymph nodes were accompanied by significantly lower levels of IL-12 in the lymph nodes when mice were treated with an anti-TNF-α monoclonal antibody during pulmonary infection with *C. neoformans*, (285). Consistent with a major role of DC for production of IL-12 it was also reported that accumulation of DC in the draining lymph nodes after immunization with protective cryptococcal immunogen (cryptococcal culture filtrate antigen together with complete Freund's adjuvant) was associated with higher numbers of activated $CD4^{+}$ T cells in the draining lymph node (188). This suggests that DC induce protective T cell-mediated immunity against *C. neoformans*. Thus, preferential production of IL-12 family members and the remarkable T-cell stimulatory capacity by DC compared to MΦ is consistent with the known immunoregulatory function of DC in the lymph node. In agreement with our data derived from activation with the serotype D strain 1841 others also observed production of IL-12/23p40 by conventional BMDC using a serotype A strain of *C. neoformans* (200). This corroborates the responsiveness of conventional DC to *C. neoformans*. MΦ, however, have been implicated to be important effector cells in cryptococcosis by producing NO mediating stasis or killing of *C. neoformans* (286-290). However, in this study BMMΦ did not release NO in response to *C. neoformans* stimulation (data not shown), indicating that additional signals are necessary for the induction of NO by MΦ *in vivo*. In fact, IL-12-induced production of IFN-γ by NK cells has been shown to be essential for the production of MΦ-derived NO (291). The lack of additional signals might also account for the absence of measurable amounts of IL-12 and IL-23 in our *in vitro* system. This is in agreement with other reports where induction of IL-12 by GXM- or encapsulated *C. neoformans*-stimulated antigen-presenting cells could not be observed (186;292).

In addition to the lack of IL-12/23p40 production by BMMΦ, we observed that MHC-II and CD86 is down-regulated on BMMΦ. This is in line with data from Monari *et al.* who reported down-regulation of MHC-II on MΦ by GXM (293). Up-regulation of MHC-II on MΦ has been shown, however, *in vivo* (192;193), and T-cell derived IFN-γ was found to contribute to the up-regulation *in vivo* (193). Thus, a possible explanation for the lack of MHC-II up-

regulation on BMMΦ *in vitro* could be the lack of IFN-γ, since as expected BMMΦ did not release IFN-γ in response to *C. neoformans* (data not shown). On the other hand this suggests that conventional DC (which up-regulated MHC-II and CD86 in the absence of IFN-γ) do not depend on T-cell derived IFN-γ for their potent role in antigen presentation during cryptococcosis as opposed to MΦ.

Our study shows for the first time that *C. neoformans* induces the up-regulation of MHC-II and CD86 on conventional BMDC rather than plasmacytoid BMDC. *In vitro* the activation of DC by cryptococcal mannoproteins has been described (194), whereas the polysaccharide capsule of *C. neoformans* was found to inhibit DC maturation of human monocyte-derived DC (88). This study using not only *C. neoformans*-derived molecules but the whole fungus, however, corroborates results from a pulmonary cryptococcosis infection model describing the up-regulation of MHC-II, CD80 and CD86 by lung DC 7 days after inoculation (195). Previously, it has been reported that *in vivo* there is an association of the ratio of "lymphoid" to "myeloid" DC in draining lymph nodes with the type and protective nature of the immune response following immunization with cryptococcal antigen (188). Thus, "lymphoid" DC as opposed to "myeloid" DC were found to negatively regulate protective cell-mediated immunity to *C. neoformans* (188). However, with the availability of more recent DC lineage data the terms "myeloid" and "lymphoid" for the subclassification of DC subsets have been modified (80). Currently DC subsets are defined as conventional DC comprising both the former "myeloid" and "lymphoid" DC and, in addition, a distinct subset has been described termed plasmacytoid DC also known as natural interferon-producing cells (80). It remains to be shown *in vivo* which subtype of conventional DC is associated with protective cell-mediated immunity to *C. neoformans* infection and whether plasmacytoid DC play a role.

In summary this study demonstrates that *C. neoformans* preferentially activates conventional BMDC rather than plasmacytoid BMDC and BMMΦ leading to the production of IL-12 family members. Moreover, our study provides novel evidence that conventional BMDC rather than plasmacytoid BMDC up-regulate surface molecules needed for antigen presentation and T cell stimulation in response to *C. neoformans*.

5.3 Immunostimulatory effects of parapoxvirus ovis on conventional BMDC, plasmacytoid BMDC and BMMΦ

Inactivated parapoxvirus ovis (iPPVO) was reported to mediate immunostimulatory effects (294), enhancing anti-viral activity against Hepatitis B virus infection and herpes simplex

virus type 1 (90). Studies on the basic mechanisms for the immunostimulatory capacities of iPPVO suggest that cells of the innate immune system are activated by iPPVO. Although monocytes or other antigen presenting cells were believed to be involved in the initiation of the protective mechanisms, up to date a detailed analysis of the cellular sources that are activated by iPPVO and the molecular mechanisms leading to the activation of innate immune cells had not been performed.

The present study demonstrates that bone marrow-derived dendritic cells (BMDC) rather than bone marrow-derived macrophages (BMMΦ) produce IFN-α, IL-12/23p40, and TNF-α in response to iPPVO. Type I interferons including IFN-α were shown to be essential for the induction of cytotoxic T lymphocytes in response to certain viruses (295). Furthermore biological activities of type I interferons such as IFN-α enhance the expression of intracellular anti-viral proteins subsequently interfering with viral replication and protein synthesis (296). Furthermore type I interferons increase antibody production by DC stimulation (297), enhance NK cell activity (298), trigger up-regulation of MHC-I expression (299), and induce/activate proapoptotic genes and proteins together with concomitant repression of antiapoptotic genes (299). IL-12 family members were shown to orchestrate T cell differentiation (37) leading to the production of IFN-γ essential for the activation of cellular immune responses. TNF-α plays an important role in immunoregulation mediating proinflammatory and immunoregulatory effects (300). Therefore, DC-derived IFN-α, IL-12/23p40, and TNF-α are likely to be involved in enhanced resistance observed following iPPVO application (90).

iPPVO-induced production of IL-12/23p40 was dominated by conventional BMDC, whereas both, conventional and plasmacytoid BMDC contributed to the production of IFN-α. These data point to conventional DC as critical cells for the induction of T cell responses upon iPPVO stimulation. This conclusion is supported by the observation that conventional BMDC were preferential producers of IL-12 family members necessary for the induction of Th1-mediated cellular immune responses. Production of IFN-α, however, was biased towards plasmacytoid BMDC, indicating an important role for plasmacytoid DC for the mediation of anti-viral effects. Interestingly, the interaction of plasmacytoid and conventional DC has been shown to be essential for the production of conventional DC-derived IL-12 in response to *L. monocytogenes* (301). Whether interaction of plasmacytoid and conventional DC also adds to the immunostimulatory effects of iPPVO merits further investigation.

In consistence with the dominant role of conventional DC in the IL-12 cytokine family response to iPPVO, the expression of maturation markers including MHC-II, CD86 and

CD80, was up-regulated to higher levels on conventional BMDC than on plasmacytoid BMDC, and it was poor on BMMΦ suggesting a primary role of conventional DC for the induction of T cells responses upon iPPVO application. Interestingly, *Canarypox virus*-induced maturation of dendritic cells was mediated by apoptotic cell death and TNF-α secretion rather than directly by the virus (302). Induction of apoptosis of monocytes by iPPVO has been reported (303), and the present data demonstrates that iPPVO induces TNF-α in BMDC. However, the fact that also BMMΦ up-regulated MHC-II and CD86 despite the lack of iPPVO-induced TNF-α release points to the ability of iPPVO to directly induce cell activation. The induction of TNF-α might nevertheless additionally promote DC maturation in response to iPPVO.

BMMΦ were the only cell type producing NO in response to iPPVO. NO has antimicrobial activity against bacteria, parasites, and fungi (12). Anti-viral effects of NO are also known for some types of virus, most typically DNA viruses such as murine poxvirus (ectromelia virus, EV), murine cytomegalovirus and herpes viruses including HSV, and Epstein-Barr virus (EBV), and some RNA viruses such as coxsackievirus and lymphocytic choriomeningitis virus (12;304). Therefore, our data suggest that by the production of NO MΦ contribute to the iPPVO-mediated enhanced resistance to infections. Taken together the present data demonstrate the activation of both, DC and MΦ by iPPVO, and point to DC as critical cells for mediating immunoregulatory mechanisms and MΦ mediating antimicrobial effector function upon iPPVO application.

The present study for the first time shows that DC activation by iPPVO is partially dependent on MyD88. MyD88 is used by all known TLR but TLR3 to initiate intracellular signalling cascades leading to the release of cytokines and the up-regulation of surface molecules necessary to activate naïve T cells. Therefore, it is likely that a receptor belonging to the TLR family is involved in recognition of iPPVO. The genetic material of iPPVO is dsDNA (225) with a base content of about 63 % cytosine and guanosine (294). Therefore TLR9, sensing double-stranded CpG DNA motifs as occurring in bacterial and viral DNA, is a prime candidate for the recognition of iPPVO. Moreover, due to chemical treatment iPPVO is not able to replicate. Therefore no transcription of the viral genome takes place, and no mRNA transcripts are generated which could possibly be recognized by TLR3 and TLR7 or cytosolic receptors such as RIG-1 or MDA-5 known to bind viral RNA (305). Gentle heat incubation by at 60°C did not result in loss of iPPVO-induced activation. Initial results of our group indicate that even boiling of iPPVO at 95°C does abrogate the activation of BMDC. Thus, heat-stable protein domains or a non-proteinous ligand such as DNA would explain the heat

stability of iPPVO-induced BMDC activation. Indeed, purified PPVO-DNA was able to induce IL-12/23p40 release by BMDC, pointing to a role for iPPVO-derived DNA in the induction of BMDC activation.

Intriguingly, iPPVO induces BMDC activation by additional MyD88-independent pathways, indicating the involvement of more than one receptor in the recognition of iPPVO. Recently adenovirus, a double-stranded DNA virus, has been reported to stimulate interferon and proinflammatory cytokine responses by TLR-independent mechanisms (306). This points to the existence of a yet unknown receptor(s) sensing viral double-stranded DNA. The involvement of cytosolic RNA receptors including RIG-I and MDA-5 in recognition of measles virus and influenza virus A has been shown (218;222). Very recently, the discovery of a cytosolic DNA receptor activating innate immune responses has been reported (76). Therefore, although further studies are required to define the MyD88-independent iPPVO-induced mechanisms leading to DC activation, binding of iPPVO to cytosolic DNA receptors which then mediate signalling cascades leading to BMDC activation seems conceivable. In summary this study for the very first time shows that iPPVO induces activation of BMDC rather than BMMΦ in a MyD88-dependent as well as a MyD88-independent manner leading to cyokine production and up-regulation of surface molecules required for the induction of T cell responses.

6 Future Perspectives

The results of the present study provide a detailed characterization of the activation of conventional DC, plasmacytoid DC and MΦ by bacterial, fungal and viral pathogens. The study broadens our knowledge of how microbial stimuli are recognized by conventional and plasmacytoid DC subsets and MΦ and initiate IL-12 and IFN-α synthesis required for cellular immune responses essential for the protection against intracellular pathogens and anti-viral effects. The better understanding of (i) which cells are preferentially activated and (ii) which PRR mediate activation in response to microbial stimuli contributes to facilitating the development of better vaccination and therapeutic strategies tailored to different infections in the future.

7 Summary

Within the present Ph. D. thesis the activation of both, conventional and plasmacytoid dendritic cells (DC) and of macrophages (MΦ) by the facultative intracellular bacterium *Salmonella enterica* serovar Enteritidis (*S*. Enteritidis), the facultative intracellular fungus *Cryptococcus neoformans* (*C. neoformans*) and parapoxvirus ovis, a virus belonging to the family of the *Poxviridae*, was investigated. Conventional DC, plasmacytoid DC and MΦ are among the first line sentinel cells that sense invading pathogens and activate immediate anti-microbial effector mechanisms such as the release of type I interferons interfering with virus replication and NO mediating microbicidal effects. Furthermore, DC and MΦ induce adaptive immune responses to clear the infection and mediate long-term protection of the host. For the orchestration of T-helper cell 1 (Th1)-driven cellular immune responses required for protection against intracellular pathogens such as *S*. Enteritidis and *C. neoformans* the production of interleukin (IL)-12 family members is essential. The IL-12 family is a new cytokine family comprising the prototypic IL-12, a heterodimeric cytokine consisting of the subunits IL-12/23p40 and IL-12p35, and the structurally related cytokines IL-23 (heterodimer of IL-12/23p40 and IL-23p19), IL-27 (heterodimer of IL-27EBI-3 and IL-27p28) and the shared p40-subunit of IL-12 and IL-23 termed IL-12/23p40.

In 2003 (when this Ph.D. thesis was started), the contribution of DC and MΦ to IL-12 family member production during salmonellosis was poorly defined. The present Ph.D. thesis demonstrates that the *in vitro* production of IL-12 family members in response to *S*. Enteritidis is dominated by conventional bone marrow-derived DC (BMDC) as compared with bone marrow-derived macrophages (BMMΦ). The production of IL-12 family members by BMDC is down-regulated by the addition of exogenous IL-10. Surprisingly, BMDC and BMMΦ released similar amounts of endogenous IL-10 in response to *S*. Enteritidis, pointing to additional regulatory mechanisms responsible for the preferential IL-12 family member production by BMDC vs. BMMΦ.

DC and MΦ sense microorganisms by the recognition of conserved pathogen-associated molecular patterns with pattern recognition receptors such as toll-like receptors (TLR). The data obtained show that despite the expression of many TLR ligands on *S*. Enteritidis, the production of IL-12, IL-23, IL-12/23p40 and of the counter-regulatory cytokine IL-10 surprisingly is chiefly mediated by LPS/TLR4 interaction.

The preferential production of IL-12 family members by DC vs. MΦ was corroborated in subsequent experiments conducted with primary DC and MΦ studied *ex vivo*. After

intraperitoneal (i.p.) infection of mice with *S*. Enteritidis peritoneal DC were identified as the main producers of IL-12/23p40, whereas peritoneal production of NO was dominated by MΦ. This was associated with expansion of conventional DC at the peritoneal cavity and the parathymic lymph node draining the peritoneal cavity (DLN), whereas MΦ numbers did not increase in response to *S*. Enteritidis infection. This points to conventional DC as important antigen-presenting cell in salmonellosis. Surprisingly, the production of IL-12/23p40 in response to *S*. Enteritidis preferentially occurred at the site of infection (peritoneal cavity) rather than at the DLN, pointing to an additional important immunoregulatory role of IL-12 family members at the site of infection. Moreover, plasmacytoid DC usually associated with protection against viral infections, expanded in the peritoneal cavity but not in the DLN upon i.p. infection with *S*. Enteritidis, suggesting a novel role for plasmacytoid DC in salmonellosis and possibly other bacterial infections. Moreover, the present data demonstrate that *S*. Enteritidis-induced rapid production of IL-17 partially depends on IL-23, whereas IFN-γ production is completely IL-12-dependent.

Immunity to *C. neoformans* also critically depends on IL-12 and is complemented by IL-23. The present Ph.D. thesis provides the first side-by-side study of the activation of conventional BMDC, plasmacytoid BMDC, and BMMΦ in response to *C. neoformans* stimulation. Activation of these antigen-presenting cells was assessed by analyzing the production of IL-12/23p40 and TNF-α and the expression of MHC-II and co-stimulatory molecules such as CD86 and CD80 which are required for T cell activation. The results demonstrate for the first time that *C. neoformans* activates conventional BMDC rather than plasmacytoid BMDC, whereas BMMΦ are even down-regulated by *C. neoformans*. These data point to conventional DC as important antigen-presenting cells in the immune response to *C. neoformans* infection. The *C. neoformans*-induced production of IL-12/23p40 was found to be independent of TLR2 and TLR4 but clearly dependent of the TLR adaptor molecule myeloid differentiation primary response gene 88 (MyD88). The up-regulation of MHC-II, CD86 and CD80 also required MyD88, pointing to TLR involved in the activation of DC by *C. neoformans*.

Parapoxvirus ovis is a double-stranded (ds) DNA virus which causes ecthyma contagiosum in sheep and goats and occasional infections of humans. Chemically inactivated parapoxvirus ovis (iPPVO) has immunostimulatory capacities enhancing anti-microbial resistance. However, the exact cellular targets and mechanisms of immune activation have not been defined. The data of the present Ph.D. study show that iPPVO induces the release of IFN-α, IL-12/23p40 and TNF-α preferentially by BMDC, whereas BMMΦ exclusively produced NO upon stimulation with iPPVO. The production of IFN-α and IL-12/23p40 was biased towards

plasmacytoid BMDC and conventional BMDC, respectively, and followed distinct kinetics. Upon iPPVO stimulation conventional BMDC up-regulated the expression of MHC-II, CD86 and C80 to higher levels than plasmacytoid BMDC, whereas expression of MHC-II, CD86 and C80 barely changed on BMMΦ. These data point to conventional DC as important antigen-presenting cells upon iPPVO application.

Furthermore, the achieved data revealed that a heat-stable viral constituent such as heat-stable protein domains or possibly viral DNA is responsible for the activation of BMDC. The latter conclusion is strengthened by demonstrating activation of BMDC by isolated viral DNA The genetic material of iPPVO is dsDNA with a base content of about 63 % cytosine and guanosine. Therefore, endosomal TLR9, that is associated with the recognition of CpG-containing dsDNA, might be responsible for DNA-mediated recognition of internalized iPPVO. Upon ligand binding TLR9 (similar to all other known TLR but TLR3) induces signalling cascades involving the adaptor molecule MyD88. Using BMDC derived from bone marrow of MyD88$^{-/-}$ mice it could be shown for the first time that iPPVO induces its immunostimulatory effects in a partially MyD88-dependent manner. This suggests a role for TLR9 in the recognition of iPPVO. In addition, the very recently discovered cytosolic DNA receptor DNA-dependent activator of IFN-regulatory factors (DAI) and/or other yet unknown possibly cytosolic receptors for DNA may contribute to recognition of iPPVO.

The results of the present study provide a detailed characterization of the mechanism responsible for the recognition of a selected bacterial, fungal and viral pathogen and the subsequent activation of the conventional and plasmacytoid DC subsets and MΦ. The better understanding of (i) which cells are preferentially activated and (ii) which receptors mediate recognition and activation in response to microbial stimuli will profoundly foster the development of better vaccination and therapeutic strategies adapted to different infections in the future.

References

1 Banyer, J. L., Hamilton, N. H., Ramshaw, I. A., and Ramsay, A. J. 2000. Cytokines in innate and adaptive immunity. *Rev.Immunogenet.* 2:359-373.

2 Sasaki, K., Ikeda, K., Ogami, K., Takahara, J., and Irino, S. 1995. Cell-to-cell interaction of cytokine-dependent myeloblastic line constitutively expressing membrane-bound stem cell factor abrogates cytokine dependency partially through granulocyte-macrophage colony-stimulating factor production. *Blood* 85:1220-1228.

3 Yi, A. K., Yoon, J. G., Yeo, S. J., Hong, S. C., English, B. K., and Krieg, A. M. 2002. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. *J.Immunol.* 168:4711-4720.

4 Rodriguez-Galan, M. C., Bream, J. H., Farr, A., and Young, H. A. 2005. Synergistic effect of IL-2, IL-12, and IL-18 on thymocyte apoptosis and Th1/Th2 cytokine expression. *J.Immunol.* 174:2796-2804.

5 Rosenberger, C. M. and Finlay, B. B. 2003. Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. *Nat.Rev.Mol.Cell Biol* 4:385-396.

6 Austyn, J. M. 1996. New insights into the mobilization and phagocytic activity of dendritic cells. *J Exp.Med.* 183:1287-1292.

7 Caux, C., Vanbervliet, B., Massacrier, C., Ait-Yahia, S., Vaure, C., Chemin, K., Dieu-Nosjean And MC, and Vicari, A. 2002. Regulation of dendritic cell recruitment by chemokines. *Transplantation* 73:S7-11.

8 Lancaster, J. R., Jr. and Hibbs, J. B., Jr. 1990. EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc.Natl.Acad.Sci.U.S.A* 87:1223-1227.

9 Michalski, W. P. and Nicholas, D. J. 1987. Inhibition of bacteriochlorophyll synthesis in Rhodobacter sphaeroides subsp. denitrificans grown in light under denitrifying conditions. *J Bacteriol.* 169:4651-4659.

10 Lepoivre, M., Flaman, J. M., and Henry, Y. 1992. Early loss of the tyrosyl radical in ribonucleotide reductase of adenocarcinoma cells producing nitric oxide. *J Biol Chem.* 267:22994-23000.

11 Hausladen, A., Privalle, C. T., Keng, T., DeAngelo, J., and Stamler, J. S. 1996. Nitrosative stress: activation of the transcription factor OxyR. *Cell* 86:719-729.

12 Bogdan, C. 2001. Nitric oxide and the immune response. *Nat.Immunol.* 2:907-916.

13 Williams, B. R. 2001. Signal integration via PKR. *Sci.STKE.* 2001:RE2.

14 Berlanga, J. J., Ventoso, I., Harding, H. P., Deng, J., Ron, D., Sonenberg, N., Carrasco, L., and de Haro, C. 2006. Antiviral effect of the mammalian translation initiation factor 2alpha kinase GCN2 against RNA viruses. *EMBO J* 25:1730-1740.

15 Player, M. R. and Torrence, P. F. 1998. The 2-5A system: modulation of viral and cellular processes through acceleration of RNA degradation. *Pharmacol.Ther.* 78:55-113.

16 Samuel, C. E. 2001. Antiviral actions of interferons. *Clin.Microbiol.Rev.* 14:778-809, table.

17 Bieniasz, P. D. 2004. Intrinsic immunity: a front-line defense against viral attack. *Nat.Immunol* 5:1109-1115.

18 Haller, O., Kochs, G., and Weber, F. 2007. Interferon, Mx, and viral countermeasures. *Cytokine Growth Factor Rev.* 18:425-433.

19 Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. 1998. How cells respond to interferons. *Annu.Rev.Biochem.* 67:227-264.

20 Le Bon, A., Durand, V., Kamphuis, E., Thompson, C., Bulfone-Paus, S., Rossmann, C., Kalinke, U., and Tough, D. F. 2006. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. *J Immunol* 176:4682-4689.

21 Stetson, D. B. and Medzhitov, R. 2006. Type I interferons in host defense. *Immunity* 25:373-381.

22 Le Bon, A., Etchart, N., Rossmann, C., Ashton, M., Hou, S., Gewert, D., Borrow, P., and Tough, D. F. 2003. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat.Immunol* 4:1009-1015.

23 Lateef, Z., Fleming, S., Halliday, G., Faulkner, L., Mercer, A., and Baird, M. 2003. Orf virus-encoded interleukin-10 inhibits maturation, antigen presentation and migration of murine dendritic cells. *J.Gen.Virol.* 84:1101-1109.

24 Svensson, M., Johansson, C., and Wick, M. J. 2000. *Salmonella enterica* Serovar Typhimurium-Induced Maturation of Bone Marrow-Derived Dendritic Cells. *Infect.Immun.* 68:6311-6320.

25 Liwski, R. S., Chase, J. C., Baldridge, W. H., Sadek, I., Rowden, G., and West, K. A. 2006. Prolonged costimulation is required for naive T cell activation. *Immunol Lett.* 106:135-143.

26 Quah, B. J. and O'Neill, H. C. 2005. Maturation of function in dendritic cells for tolerance and immunity. *J Cell Mol.Med.* 9:643-654.

27 Hein, R. M. and Woods, M. L. 2007. TIM-1 regulates macrophage cytokine production and B7 family member expression. *Immunol Lett.* 108:103-108.

28 Rugtveit, J., Bakka, A., and Brandtzaeg, P. 1997. Differential distribution of B7.1 (CD80) and B7.2 (CD86) costimulatory molecules on mucosal macrophage subsets in human inflammatory bowel disease (IBD). *Clin.Exp.Immunol* 110:104-113.

29 Rosenwasser, L. J. and Rosenthal, A. S. 1978. Adherent cell function in murine T lymphocyte antigen recognition. II. Definition of genetically restricted and nonrestricted macrophage functions in T cell proliferation. *J Immunol* 121:2497-2501.

30 Tzehoval, E., De Baetselier, P., Ron, Y., Tartakovsky, B., Feldman, M., and Segal, S. 1983. Splenic B cells function as immunogenic antigen-presenting cells for the induction of effector T cells. *Eur J Immunol* 13:89-94.

31 Ott, P. A., Tary-Lehmann, M., and Lehmann, P. V. 2007. The secretory IFN-gamma response of single CD4 memory cells after activation on different antigen presenting cell types. *Clin.Immunol* 124:267-276.

32 Steinman, R. M., Gutchinov, B., Witmer, M. D., and Nussenzweig, M. C. 1983. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J Exp.Med.* 157:613-627.

33 Guidos, C., Wong, M., and Lee, K. C. 1984. A comparison of the stimulatory activities of lymphoid dendritic cells and macrophages in T proliferative responses to various antigens. *J Immunol* 133:1179-1184.

34 Austyn, J. M., Weinstein, D. E., and Steinman, R. M. 1988. Clustering with dendritic cells precedes and is essential for T-cell proliferation in a mitogenesis model. *Immunology* 63:691-696.

35 Austyn, J. M. and Morris, P. J. 1988. T-cell activation by dendritic cells: CD18-dependent clustering is not sufficient for mitogenesis. *Immunology* 63:537-543.

36 Sibley, L. D. 2004. Intracellular parasite invasion strategies. *Science* 304:248-253.

37 Brombacher, F., Kastelein, R. A., and Alber, G. 2003. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol.* 24:207-212.

38 Andersen, M. H., Schrama, D., Thor, S. P., and Becker, J. C. 2006. Cytotoxic T cells. *J Invest Dermatol.* 126:32-41.

39 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.

40 Abbas, A. K., Murphy, K. M., and Sher, A. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787-793.

41 Guy, R. and Hodes, R. J. 1989. Antigen-specific, MHC-restricted B cell activation by cell-free Th2 cell products. Synergy between antigen-specific helper factors and IL-4. *J Immunol* 143:1433-1440.

42 Alam, M. S., Akaike, T., Okamoto, S., Kubota, T., Yoshitake, J., Sawa, T., Miyamoto, Y., Tamura, F., and Maeda, H. 2002. Role of nitric oxide in host defense in murine salmonellosis as a function of its antibacterial and antiapoptotic activities. *Infect.Immun.* 70:3130-3142.

43 Eriksson, S., Chambers, B. J., and Rhen, M. 2003. Nitric oxide produced by murine dendritic cells is cytotoxic for intracellular *Salmonella* enterica sv. Typhimurium. *Scand.J Immunol* 58:493-502.

44 Mosmann, T. R. and Coffman, R. L. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu.Rev.Immunol* 7:145-173.

45 Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J., and Gurney, A. L. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J.Biol.Chem.* 278:1910-1914.

46 Kolls, J. K. and Linden, A. 2004. Interleukin-17 family members and inflammation. *Immunity* 21:467-476.

47 Witowski, J., Pawlaczyk, K., Breborowicz, A., Scheuren, A., Kuzlan-Pawlaczyk, M., Wisniewska, J., Polubinska, A., Friess, H., Gahl, G. M., Frei, U., and Jorres, A. 2000. IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO alpha chemokine from mesothelial cells. *J Immunol* 165:5814-5821.

48 Magram, J., Connaughton, S. E., Warrier, R. R., Carvajal, D. M., Wu, C. Y., Ferrante, J., Stewart, C., Sarmiento, U., Faherty, D. A., and Gately, M. K. 1996. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity.* 4:471-481.

49 Mattner, F., Magram, J., Ferrante, J., Launois, P., Di, P. K., Behin, R., Gately, M. K., Louis, J. A., and Alber, G. 1996. Genetically resistant mice lacking interleukin-12 are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. *Eur.J.Immunol.* 26:1553-1559.

50 Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chan, S., Loudon, R., Sherman, F., Perussia, B., and Trinchieri, G. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp.Med.* 170:827-845.

51 Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F., and Kastelein, R. A. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* 13:715-725.

52 Khader, S. A., Pearl, J. E., Sakamoto, K., Gilmartin, L., Bell, G. K., Jelley-Gibbs, D. M., Ghilardi, N., deSauvage, F., and Cooper, A. M. 2005. IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-gamma responses if IL-12p70 is available. *J.Immunol.* 175:788-795.

53 Harrington, L. E., Mangan, P. R., and Weaver, C. T. 2006. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr.Opin.Immunol.* 18:349-356.

54 Happel, K. I., Dubin, P. J., Zheng, M., Ghilardi, N., Lockhart, C., Quinton, L. J., Odden, A. R., Shellito, J. E., Bagby, G. J., Nelson, S., and Kolls, J. K. 2005. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. *J.Exp.Med.* 202:761-769.

55 Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W. M., Mattson, J. D., Wagner, J. L., To, W., Zurawski, S., McClanahan, T. K., Gorman, D. M., Bazan, J. F., de Waal, M. R., Rennick, D., and Kastelein, R. A. 2002. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity.* 16:779-790.

56 Lucas, S., Ghilardi, N., Li, J., and de Sauvage, F. J. 2003. IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. *Proc.Natl.Acad.Sci.U.S.A* 100:15047-15052.

57 Stumhofer, J. S., Laurence, A., Wilson, E. H., Huang, E., Tato, C. M., Johnson, L. M., Villarino, A. V., Huang, Q., Yoshimura, A., Sehy, D., Saris, C. J., O'Shea, J. J., Hennighausen, L., Ernst, M., and Hunter, C. A. 2006. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. *Nat.Immunol.* 7:937-945.

58 Batten, M., Li, J., Yi, S., Kljavin, N. M., Danilenko, D. M., Lucas, S., Lee, J., de Sauvage, F. J., and Ghilardi, N. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. *Nat.Immunol.* 7:929-936.

59 Robinson, M. J., Sancho, D., Slack, E. C., LeibundGut-Landmann, S., and Reis e Sousa 2006. Myeloid C-type lectins in innate immunity. *Nat.Immunol* 7:1258-1265.

60 Peiser, L., Mukhopadhyay, S., and Gordon, S. 2002. Scavenger receptors in innate immunity. *Curr.Opin.Immunol* 14:123-128.

61 Franchi, L., McDonald, C., Kanneganti, T. D., Amer, A., and Nunez, G. 2006. Nucleotide-binding oligomerization domain-like receptors: intracellular pattern recognition molecules for pathogen detection and host defense. *J Immunol* 177:3507-3513.

62 Kawai, T. and Akira, S. 2006. Innate immune recognition of viral infection. *Nat.Immunol.* 7:131-137.

63 Janeway, C. A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb.Symp.Quant.Biol* 54 Pt 1:1-13.

64 Medzhitov, R. and Janeway, C. A., Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91:295-298.

65 Muzio, M., Natoli, G., Saccani, S., Levrero, M., and Mantovani, A. 1998. The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J Exp.Med.* 187:2097-2101.

66 Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A., Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol.Cell* 2:253-258.

67 Takeuchi, O., Takeda, K., Hoshino, K., Adachi, O., Ogawa, T., and Akira, S. 2000. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *Int.Immunol* 12:113-117.

68 Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11:115-122.

69 Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P. F., Sato, S., Hoshino, K., and Akira, S. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* 167:5887-5894.

70 Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K., and Akira, S. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol* 166:5688-5694.

71 O'Neill, L. A. and Bowie, A. G. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat.Rev.Immunol* 7:353-364.

72 Trinchieri, G. and Sher, A. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat.Rev.Immunol* 7:179-190.

73 Akira, S., Uematsu, S., and Takeuchi, O. 2006. Pathogen recognition and innate immunity. *Cell* 124:783-801.

74 Sato, K., Yang, X. L., Yudate, T., Chung, J. S., Wu, J., Luby-Phelps, K., Kimberly, R. P., Underhill, D., Cruz, P. D., Jr., and Ariizumi, K. 2006. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J Biol Chem.* 281:38854-38866.

75 Bowie, A. G. and Fitzgerald, K. A. 2007. RIG-I: tri-ing to discriminate between self and non-self RNA. *Trends Immunol* 28:147-150.

76 Takaoka, A., Wang, Z., Choi, M. K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., Ohba, Y., and Taniguchi, T. 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 448:501-505.

77 Steinman, R. M. and Cohn, Z. A. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp.Med.* 137:1142-1162.

78 Wick, M. J. 2007. Monocyte and dendritic cell recruitment and activation during oral Salmonella infection. *Immunol Lett.*

79 Cao, W. and Liu, Y. J. 2007. Innate immune functions of plasmacytoid dendritic cells. *Curr.Opin.Immunol* 19:24-30.

80 Shortman, K. and Naik, S. H. 2007. Steady-state and inflammatory dendritic-cell development. *Nat.Rev.Immunol* 7:19-30.

81 Fallarino, F., Orabona, C., Vacca, C., Bianchi, R., Gizzi, S., Asselin-Paturel, C., Fioretti, M. C., Trinchieri, G., Grohmann, U., and Puccetti, P. 2005. Ligand and

cytokine dependence of the immunosuppressive pathway of tryptophan catabolism in plasmacytoid dendritic cells. *Int.Immunol* 17:1429-1438.

82 Brawand, P., Fitzpatrick, D. R., Greenfield, B. W., Brasel, K., Maliszewski, C. R., and De Smedt, T. 2002. Murine Plasmacytoid Pre-Dendritic Cells Generated from Flt3 Ligand-Supplemented Bone Marrow Cultures Are Immature APCs. *J Immunol* 169:6711-6719.

83 Reis e Sousa, C. 2004. Toll-like receptors and dendritic cells: for whom the bug tolls. *Seminars in Immunology* 16:27-34.

84 Jouanguy, E., Doffinger, R., Dupuis, S., Pallier, A., Altare, F., and Casanova, J. L. 1999. IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. *Curr.Opin.Immunol* 11:346-351.

85 Lehmann, J., Bellmann, S., Werner, C., Schroder, R., Schutze, N., and Alber, G. 2001. IL-12p40-dependent agonistic effects on the development of protective innate and adaptive immunity against Salmonella enteritidis. *J.Immunol.* 167:5304-5315.

86 Kleinschek, M. A., Muller, U., Brodie, S. J., Stenzel, W., Kohler, G., Blumenschein, W. M., Straubinger, R. K., McClanahan, T., Kastelein, R. A., and Alber, G. 2006. IL-23 enhances the inflammatory cell response in *Cryptococcus neoformans* infection and induces a cytokine pattern distinct from IL-12. *J.Immunol.* 176:1098-1106.

87 Kawakami, K., Tohyama, M., Xie, Q., and Saito, A. 1996. IL-12 protects mice against pulmonary and disseminated infection caused by Cryptococcus neoformans. *Clin.Exp.Immunol* 104:208-214.

88 Vecchiarelli, A., Pietrella, D., Lupo, P., Bistoni, F., McFadden, D. C., and Casadevall, A. 2003. The polysaccharide capsule of *Cryptococcus neoformans* interferes with human dendritic cell maturation and activation. *J.Leukoc.Biol.* 74:370-378.

89 Fachinger, V., Schlapp, T., Strube, W., Schmeer, N., and Saalmuller, A. 2000. Poxvirus-induced immunostimulating effects on porcine leukocytes. *J.Virol.* 74:7943-7951.

90 Weber, O., Siegling, A., Friebe, A., Limmer, A., Schlapp, T., Knolle, P., Mercer, A., Schaller, H., and Volk, H. D. 2003. Inactivated parapoxvirus ovis (Orf virus) has antiviral activity against hepatitis B virus and herpes simplex virus. *J Gen.Virol.* 84:1843-1852.

91 Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. A., Gorman, D., Kastelein, R. A., and Sedgwick, J. D. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.

92 Martin, G., Hanel, I., Helmuth, R., Schroeter, A., Erler, W., and Meyer, H. 1996. [Immunization with potential Salmonella enteritidis mutants--1. Production and in vitro characterization]. *Berl Munch.Tierarztl.Wochenschr.* 109:325-329.

93 Springer, S., Lehmann, J., Lindner, T., Thielebein, J., Alber, G., and Selbitz, H. J. 2000. [A new live Salmonella enteritidis vaccine for chickens--experimental evidence of its safety and efficacy]. *Berl Munch.Tierarztl.Wochenschr.* 113:246-252.

94 Decken, K., Kohler, G., Palmer-Lehmann, K., Wunderlin, A., Mattner, F., Magram, J., Gately, M. K., and Alber, G. 1998. Interleukin-12 is essential for a protective Th1 response in mice infected with Cryptococcus neoformans. *Infect.Immun.* 66:4994-5000.

95 Mueller, U., Stenzel, W., Koehler, G., Werner, C., Polte, T., Hansen, G., Schuetze, N., Straubinger, R. K., Blessing, M., McKenzie, A. N., Brombacher, F., and Alber, G. IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with *Cryptococcus neoformans*. J.Immunol . 2007.
Ref Type: In Press

96 Zal, T., Volkmann, A., and Stockinger, B. 1994. Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J Exp.Med.* 180:2089-2099.

97 Gately, M. K., Carvajal, D. M., Connaughton, S. E., Gillessen, S., Warrier, R. R., Kolinsky, K. D., Wilkinson, V. L., Dwyer, C. M., Higgins, G. F., Jr., Podlaski, F. J., Faherty, D. A., Familletti, P. C., Stern, A. S., and Presky, D. H. 1996. Interleukin-12 antagonist activity of mouse interleukin-12 p40 homodimer in vitro and in vivo. *Ann.N.Y.Acad.Sci.* 795:1-12.

98 Brennan, G. L. and Kronenberg, L. H. 1983. *Biotechniques* 1:78.

99 Finter, N. B. 1969. *J.Gen.Virol.* 5:419.

100 Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rossner, S., Koch, F., Romani, N., and Schuler, G. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223:77-92.

101 Burgess, A. W., Metcalf, D., Kozka, I. J., Simpson, R. J., Vairo, G., Hamilton, J. A., and Nice, E. C. 1985. Purification of two forms of colony-stimulating factor from mouse L-cell-conditioned medium. *J.Biol.Chem.* 260:16004-16011.

102 Schuetze, N. Untersuchungen zur Expression der Interleukin-12-Zytokinefamilieund ihrer Rezeptoren im murinen *Salmonella* Enteritidis Modell. 2006.
Ref Type: Serial (Book,Monograph)

103 Stone, W. L., Yang, H., and Qui, M. 2006. Assays for nitric oxide expression. *Methods Mol.Biol* 315:245-256.

104 Boonstra, A., Asselin-Paturel, C., Gilliet, M., Crain, C., Trinchieri, G., Liu, Y. J., and O'Garra, A. 2003. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *J Exp.Med.* 197:101-109.

105 Hochrein, H. and Wagner, H. 2006. Toll-like Receptors. In Lutz, M. B., Romani, N., and Steinkasserer, A., eds., pp. 119-127.

106 Morelli, A. E., Zahorchak, A. F., Larregina, A. T., Colvin, B. L., Logar, A. J., Takayama, T., Falo, L. D., and Thomson, A. W. 2001. Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood* 98:1512-1523.

107 Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F., and Trinchieri, G. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat.Immunol* 2:1144-1150.

108 Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. H., O'Keeffe, M., Shao, Q. X., Chen, W. F., Villadangos, J. A., Shortman, K., and Wu, L. 2005. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* 174:6592-6597.

109 Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., and Colonna, M. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat.Med.* 5:919-923.

110 Freudenberg, M. A., Keppler, D., and Galanos, C. 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. *Infect.Immun.* 51:891-895.

111 Lembo, A., Kalis, C., Kirschning, C. J., Mitolo, V., Jirillo, E., Wagner, H., Galanos, C., and Freudenberg, M. A. 2003. Differential contribution of Toll-like receptors 4 and 2 to the cytokine response to *Salmonella enterica* serovar Typhimurium and Staphylococcus aureus in mice. *Infect.Immun.* 71:6058-6062.

112 Sing, A., Merlin, T., Knopf, H. P., Nielsen, P. J., Loppnow, H., Galanos, C., and Freudenberg, M. A. 2000. Bacterial induction of beta interferon in mice is a function of the lipopolysaccharide component. *Infect.Immun.* 68:1600-1607.

113 Daro, E., Pulendran, B., Brasel, K., Teepe, M., Pettit, D., Lynch, D. H., Vremec, D., Robb, L., Shortman, K., McKenna, H. J., Maliszewski, C. R., and Maraskovsky, E. 2000. Polyethylene glycol-modified GM-CSF expands CD11b(high)CD11c(high) but notCD11b(low)CD11c(high) murine dendritic cells in vivo: a comparative analysis with Flt3 ligand. *J Immunol* 165:49-58.

114 Morris, L., Graham, C. F., and Gordon, S. 1991. Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. *Development* 112:517-526.

115 Lutz, M. B. 2004. IL-3 in dendritic cell development and function: a comparison with GM-CSF and IL-4. *Immunobiology* 209:79-87.

116 Barchet, W., Cella, M., and Colonna, M. 2005. Plasmacytoid dendritic cells--virus experts of innate immunity. *Semin.Immunol* 17:253-261.

117 Ohbayashi, M., Manzouri, B., Flynn, T., Toda, M., Ikeda, Y., Nakamura, T., and Ono, S. J. 2007. Dynamic changes in conjunctival dendritic cell numbers, anatomical position and phenotype during experimental allergic conjunctivitis. *Exp.Mol.Pathol.* 83:216-223.

118 Michael Rolle and Anton Mayr 2002. *Medizinische Mikrobiologie, Infektions- ud Seuchenlehre*, 7 edn. Enke Verlag, Stuttgart.

119 Guard-Petter, J. 2001. The chicken, the egg and Salmonella enteritidis. *Environ.Microbiol.* 3:421-430.

120 Jones, B. D. and Falkow, S. 1996. Salmonellosis: host immune responses and bacterial virulence determinants. *Annu.Rev.Immunol.* 14:533-561.

121 Fields, P. I., Swanson, R. V., Haidaris, C. G., and Heffron, F. 1986. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. *Proc.Natl.Acad.Sci.U.S.A* 83:5189-5193.

122 Jantsch, J., Cheminay, C., Chakravortty, D., Lindig, T., Hein, J., and Hensel, M. 2003. Intracellular activities of *Salmonella enterica* in murine dendritic cells. *Cell Microbiol.* 5:933-945.

123 Miller, S. I. and Mekalanos, J. J. 1990. Constitutive expression of the phoP regulon attenuates Salmonella virulence and survival within macrophages. *J Bacteriol.* 172:2485-2490.

124 Holden, D. W. 2002. Trafficking of the Salmonella vacuole in macrophages. *Traffic.* 3:161-169.

125 Rescigno, M., Rotta, G., Valzasina, B., and Ricciardi-Castagnoli, P. 2001. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology* 204:572-581.

126 De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y., and Fang, F. C. 1997. Periplasmic superoxide dismutase protects Salmonella from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc.Natl.Acad.Sci.U.S.A* 94:13997-14001.

127 Altare, F., Lammas, D., Revy, P., Jouanguy, E., Doffinger, R., Lamhamedi, S., Drysdale, P., Scheel-Toellner, D., Girdlestone, J., Darbyshire, P., Wadhwa, M., Dockrell, H., Salmon, M., Fischer, A., Durandy, A., Casanova, J. L., and Kumararatne, D. S. 1998. Inherited interleukin 12 deficiency in a child with bacille Calmette-Guerin and *Salmonella* enteritidis disseminated infection. *J Clin.Invest* 102:2035-2040.

128 Kincy-Cain, T., Clements, J. D., and Bost, K. L. 1996. Endogenous and exogenous interleukin-12 augment the protective immune response in mice orally challenged with Salmonella dublin. *Infect.Immun.* 64:1437-1440.

129 Maclennan, C., Fieschi, C., Lammas, D. A., Picard, C., Dorman, S. E., Sanal, O., Maclennan, J. M., Holland, S. M., Ottenhoff, T. H., Casanova, J. L., and Kumararatne, D. S. 2004. Interleukin (IL)-12 and IL-23 Are Key Cytokines for Immunity against *Salmonella* in Humans. *J Infect.Dis.* 190:1755-1757.

130 Mastroeni, P., Harrison, J. A., Chabalgoity, J. A., and Hormaeche, C. E. 1996. Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. *Infect.Immun.* 64:189-196.

131 Salcedo, S. P., Noursadeghi, M., Cohen, J., and Holden, D. W. 2001. Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo. *Cell Microbiol.* 3:587-597.

132 Chong, C., Bost, K. L., and Clements, J. D. 1996. Differential production of interleukin-12 mRNA by murine macrophages in response to viable or killed *Salmonella* spp. *Infect.Immun.* 64:1154-1160.

133 Schuetze, N., Schoeneberger, S., Mueller, U., Freudenberg, M. A., Alber, G., and Straubinger, R. K. 2005. IL-12 family members: differential kinetics of their TLR4-mediated induction by *Salmonella* Enteritidis and the impact of IL-10 in bone marrow-derived macrophages. *Int.Immunol* 17:649-659.

134 Zhao, C., Wood, M. W., Galyov, E. E., Hopken, U. E., Lipp, M., Bodmer, H. C., Tough, D. F., and Carter, R. W. 2006. *Salmonella* typhimurium infection triggers dendritic cells and macrophages to adopt distinct migration patterns *in vivo*. *Eur.J.Immunol.*

135 Vazquez-Torres, A., Vallance, B. A., Bergman, M. A., Finlay, B. B., Cookson, B. T., Jones-Carson, J., and Fang, F. C. 2004. Toll-like receptor 4 dependence of innate and adaptive immunity to *Salmonella*: importance of the Kupffer cell network. *J.Immunol.* 172:6202-6208.

136 von Jeney N., Gunther, E., and Jann, K. 1977. Mitogenic stimulation of murine spleen cells: relation to susceptibility to Salmonella infection. *Infect.Immun.* 15:26-33.

137 Wijburg, O. L., Simmons, C. P., van, R. N., and Strugnell, R. A. 2000. Dual role for macrophages in vivo in pathogenesis and control of murine Salmonella enterica var. Typhimurium infections. *Eur.J.Immunol.* 30:944-953.

138 Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J. P., and Ricciardi-Castagnoli, P. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat.Immunol* 2:361-367.

139 Hopkins, S. A., Niedergang, F., Corthesy-Theulaz, I. E., and Kraehenbuhl, J. P. 2000. A recombinant *Salmonella* typhimurium vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. *Cell Microbiol.* 2:59-68.

140 Johansson, C. and Wick, M. J. 2004. Liver dendritic cells present bacterial antigens and produce cytokines upon *Salmonella* encounter. *J.Immunol.* 172:2496-2503.

141 Kirby, A. C., Yrlid, U., Svensson, M., and Wick, M. J. 2001. Differential involvement of dendritic cell subsets during acute *Salmonella* infection. *J.Immunol.* 166:6802-6811.

142 Marriott, I., Hammond, T., Thomas, E., and Bost, K. 1999. *Salmonella* efficiently enter and survive within cultured CD11c+ dendritic cells initiating cytokine expression. *Eur J Immunol.* 29:1107-1115.

143 Park, S. J., Nakagawa, T., Kitamura, H., Atsumi, T., Kamon, H., Sawa, S., Kamimura, D., Ueda, N., Iwakura, Y., Ishihara, K., Murakami, M., and Hirano, T. 2004. IL-6

regulates in vivo dendritic cell differentiation through STAT3 activation. *J.Immunol.* 173:3844-3854.

144 Sundquist, M. and Wick, M. J. 2005. TNF-alpha-dependent and -independent maturation of dendritic cells and recruited CD11c(int)CD11b+ Cells during oral *Salmonella* infection. *J.Immunol.* 175:3287-3298.

145 Clemons-Miller, A. R., Cox, G. W., Suttles, J., and Stout, R. D. 2000. LPS stimulation of TNF-receptor deficient macrophages: a differential role for TNF-alpha autocrine signaling in the induction of cytokine and nitric oxide production. *Immunobiology* 202:477-492.

146 Kawashima, T., Murata, K., Akira, S., Tonozuka, Y., Minoshima, Y., Feng, S., Kumagai, H., Tsuruga, H., Ikeda, Y., Asano, S., Nosaka, T., and Kitamura, T. 2001. STAT5 induces macrophage differentiation of M1 leukemia cells through activation of IL-6 production mediated by NF-kappaB p65. *J.Immunol.* 167:3652-3660.

147 Silva, J. S., Vespa, G. N., Cardoso, M. A., Aliberti, J. C., and Cunha, F. Q. 1995. Tumor necrosis factor alpha mediates resistance to Trypanosoma cruzi infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. *Infect.Immun.* 63:4862-4867.

148 Yu, R. Y., Wang, X., Pixley, F. J., Yu, J. J., Dent, A. L., Broxmeyer, H. E., Stanley, E. R., and Ye, B. H. 2005. BCL-6 negatively regulates macrophage proliferation by suppressing autocrine IL-6 production. *Blood* 105:1777-1784.

149 Beutler, B., Du, X., and Poltorak, A. 2001. Identification of Toll-like receptor 4 (Tlr4) as the sole conduit for LPS signal transduction: genetic and evolutionary studies. *J Endotoxin.Res.* 7:277-280.

150 Galanos, C., Gumenscheimer, M., Muhlradt, P., Jirillo, E., and Freudenberg, M. 2000. MALP-2, a Mycoplasma lipopeptide with classical endotoxic properties: end of an era of LPS monopoly? *J.Endotoxin.Res.* 6:471-476.

151 N.Schuetze 2005. *Untersuchungen zur Expression der Interleukin-12-Zytokinfamilie und ihrer Rezeptoren im murinen Salmonella Enteritidis-Modell*.

152 Lehmann, J., Bellmann, S., Werner, C., Schroder, R., Schutze, N., and Alber, G. 2001. IL-12p40-dependent agonistic effects on the development of protective innate and adaptive immunity against Salmonella enteritidis. *J Immunol* 167:5304-5315.

153 Khader, S. A., Partida-Sanchez, S., Bell, G., Jelley-Gibbs, D. M., Swain, S., Pearl, J. E., Ghilardi, N., Desauvage, F. J., Lund, F. E., and Cooper, A. M. 2006. Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. *J.Exp.Med* 203:1805-1815.

154 Alber, G., Al-Robaiy, S., Kleinschek, M., Knauer, J., Krumbholz, P., Richter, J., Schoeneberger, S., Schuetze, N., Schulz, S., Toepfer, K., Voigtlaender, R., Lehmann, J., and Mueller, U. 2006. Induction of immunity and inflammation by interleukin-12 family members. *Ernst.Schering.Res.Found.Workshop*107-127.

155 Hunter, C. A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat.Rev.Immunol* 5:521-531.

156 Langrish, C. L., McKenzie, B. S., Wilson, N. J., de Waal, M. R., Kastelein, R. A., and Cua, D. J. 2004. IL-12 and IL-23: master regulators of innate and adaptive immunity. *Immunol Rev.* 202:96-105.

157 Knauer, J., Siegemund, S., Muller, U., Al-Robaiy, S., Kastelein, R. A., Alber, G., and Straubinger, R. K. 2007. Borrelia burgdorferi potently activates bone marrow-derived conventional dendritic cells for production of IL-23 required for IL-17 release by T cells. *FEMS Immunol Med Microbiol.* 49:353-363.

158 Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., and Dong, C. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat.Immunol* 6:1133-1141.

159 Buchanan, K. L. and Murphy, J. W. 1998. What makes Cryptococcus neoformans a pathogen? *Emerg.Infect.Dis.* 4:71-83.

160 Levitz, S. M. 1991. The ecology of Cryptococcus neoformans and the epidemiology of cryptococcosis. *Rev.Infect.Dis.* 13:1163-1169.

161 Bennett, J. E., Kwon-Chung, K. J., and Howard, D. H. 1977. Epidemiologic differences among serotypes of Cryptococcus neoformans. *Am.J Epidemiol.* 105:582-586.

162 2002. European Confederation of Medical Mycology (ECMM) prospective survey of cryptococcosis: report from Italy. *Med.Mycol.* 40:507-517.

163 Mitchell, T. G. and Perfect, J. R. 1995. Cryptococcosis in the era of AIDS--100 years after the discovery of Cryptococcus neoformans. *Clin.Microbiol.Rev.* 8:515-548.

164 McAdams, H. P., Rosado-de-Christenson, M. L., Templeton, P. A., Lesar, M., and Moran, C. A. 1995. Thoracic mycoses from opportunistic fungi: radiologic-pathologic correlation. *Radiographics* 15:271-286.

165 Miller, K. D., Mican, J. A., and Davey, R. T. 1996. Asymptomatic solitary pulmonary nodules due to Cryptococcus neoformans in patients infected with human immunodeficiency virus. *Clin.Infect.Dis.* 23:810-812.

166 Walsh, T. J. and Groll, A. H. 1999. Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. *Transpl.Infect.Dis.* 1:247-261.

167 Zuger, A., Louie, E., Holzman, R. S., Simberkoff, M. S., and Rahal, J. J. 1986. Cryptococcal disease in patients with the acquired immunodeficiency syndrome. Diagnostic features and outcome of treatment. *Ann.Intern.Med.* 104:234-240.

168 Hill, J. O. and Harmsen, A. G. 1991. Intrapulmonary growth and dissemination of an avirulent strain of *Cryptococcus neoformans* in mice depleted of CD4+ or CD8+ T cells. *J Exp.Med.* 173:755-758.

169 Huffnagle, G. B., Yates, J. L., and Lipscomb, M. F. 1991. Immunity to a pulmonary Cryptococcus neoformans infection requires both CD4+ and CD8+ T cells. *J Exp.Med.* 173:793-800.

170 Mody, C. H., Chen, G. H., Jackson, C., Curtis, J. L., and Toews, G. B. 1994. In vivo depletion of murine CD8 positive T cells impairs survival during infection with a highly virulent strain of Cryptococcus neoformans. *Mycopathologia* 125:7-17.

171 Mody, C. H., Lipscomb, M. F., Street, N. E., and Toews, G. B. 1990. Depletion of CD4+ (L3T4+) lymphocytes in vivo impairs murine host defense to Cryptococcus neoformans. *J Immunol* 144:1472-1477.

172 Koguchi, Y. and Kawakami, K. 2002. Cryptococcal infection and Th1-Th2 cytokine balance. *Int.Rev.Immunol* 21:423-438.

173 Edwards, L., Williams, A. E., Krieg, A. M., Rae, A. J., Snelgrove, R. J., and Hussell, T. 2005. Stimulation via Toll-like receptor 9 reduces *Cryptococcus neoformans*-induced pulmonary inflammation in an IL-12-dependent manner. *Eur J Immunol* 35:273-281.

174 Harrison, T. S. and Levitz, S. M. 1996. Role of IL-12 in peripheral blood mononuclear cell responses to fungi in persons with and without HIV infection. *J Immunol* 156:4492-4497.

175 Hoag, K. A., Lipscomb, M. F., Izzo, A. A., and Street, N. E. 1997. IL-12 and IFN-gamma are required for initiating the protective Th1 response to pulmonary cryptococcosis in resistant C.B-17 mice. *Am.J Respir.Cell Mol.Biol* 17:733-739.

176 Kawakami, K., Qureshi, M. H., Zhang, T., Koguchi, Y., Shibuya, K., Naoe, S., and Saito, A. 1999. Interferon-gamma (IFN-gamma)-dependent protection and synthesis of chemoattractants for mononuclear leucocytes caused by IL-12 in the lungs of mice infected with Cryptococcus neoformans. *Clin.Exp.Immunol* 117:113-122.

177 Qureshi, M. H., Zhang, T., Koguchi, Y., Nakashima, K., Okamura, H., Kurimoto, M., and Kawakami, K. 1999. Combined effects of IL-12 and IL-18 on the clinical course and local cytokine production in murine pulmonary infection with Cryptococcus neoformans. *Eur J Immunol* 29:643-649.

178 Zhang, T., Kawakami, K., Qureshi, M. H., Okamura, H., Kurimoto, M., and Saito, A. 1997. Interleukin-12 (IL-12) and IL-18 synergistically induce the fungicidal activity of murine peritoneal exudate cells against Cryptococcus neoformans through production of gamma interferon by natural killer cells. *Infect.Immun.* 65:3594-3599.

179 Bolanos, B. and Mitchell, T. G. 1989. Phagocytosis of Cryptococcus neoformans by rat alveolar macrophages. *J Med.Vet.Mycol.* 27:203-217.

180 Lee, S. C., Kress, Y., Zhao, M. L., Dickson, D. W., and Casadevall, A. 1995. Cryptococcus neoformans survive and replicate in human microglia. *Lab Invest* 73:871-879.

181 Levitz, S. M. and DiBenedetto, D. J. 1989. Paradoxical role of capsule in murine bronchoalveolar macrophage-mediated killing of Cryptococcus neoformans. *J Immunol* 142:659-665.

182 Tucker, S. C. and Casadevall, A. 2002. Replication of Cryptococcus neoformans in macrophages is accompanied by phagosomal permeabilization and accumulation of

vesicles containing polysaccharide in the cytoplasm. *Proc.Natl.Acad.Sci.U.S.A* 99:3165-3170.

183 Kitz, D. J., Johnson, C. R., Kobayashi, G. S., Medoff, G., and Little, J. R. 1984. Growth inhibition of Cryptococcus neoformans by cloned cultured murine macrophages. *Cell Immunol* 88:489-500.

184 Feldmesser, M., Kress, Y., Novikoff, P., and Casadevall, A. 2000. Cryptococcus neoformans is a facultative intracellular pathogen in murine pulmonary infection. *Infect.Immun.* 68:4225-4237.

185 Kawakami, K., Qureshi, M. H., Koguchi, Y., Nakajima, K., and Saito, A. 1999. Differential effect of *Cryptococcus neoformans* on the production of IL-12p40 and IL-10 by murine macrophages stimulated with lipopolysaccharide and gamma interferon. *FEMS Microbiol.Lett.* 175:87-94.

186 Retini, C., Kozel, T. R., Pietrella, D., Monari, C., Bistoni, F., and Vecchiarelli, A. 2001. Interdependency of interleukin-10 and interleukin-12 in regulation of T-cell differentiation and effector function of monocytes in response to stimulation with *Cryptococcus neoformans*. *Infect.Immun.* 69:6064-6073.

187 Syme, R. M., Spurrell, J. C., Amankwah, E. K., Green, F. H., and Mody, C. H. 2002. Primary dendritic cells phagocytose Cryptococcus neoformans via mannose receptors and Fcgamma receptor II for presentation to T lymphocytes. *Infect.Immun.* 70:5972-5981.

188 Bauman, S. K., Nichols, K. L., and Murphy, J. W. 2000. Dendritic cells in the induction of protective and nonprotective anticryptococcal cell-mediated immune responses. *J Immunol* 165:158-167.

189 Levitz, S. M. 2004. Interactions of Toll-like receptors with fungi. *Microbes.Infect.* 6:1351-1355.

190 Shoham, S., Huang, C., Chen, J. M., Golenbock, D. T., and Levitz, S. M. 2001. Toll-like receptor 4 mediates intracellular signaling without TNF-alpha release in response to *Cryptococcus neoformans* polysaccharide capsule. *J Immunol* 166:4620-4626.

191 Biondo, C., Midiri, A., Messina, L., Tomasello, F., Garufi, G., Catania, M. R., Bombaci, M., Beninati, C., Teti, G., and Mancuso, G. 2005. MyD88 and TLR2, but not TLR4, are required for host defense against *Cryptococcus neoformans*. *Eur J Immunol* 35:870-878.

192 Kawakami, K., Kohno, S., Morikawa, N., Kadota, J., Saito, A., and Hara, K. 1994. Activation of macrophages and expansion of specific T lymphocytes in the lungs of mice intratracheally inoculated with Cryptococcus neoformans. *Clin.Exp.Immunol* 96:230-237.

193 Kawakami, K., Kohno, S., Kadota, J., Tohyama, M., Teruya, K., Kudeken, N., Saito, A., and Hara, K. 1995. T cell-dependent activation of macrophages and enhancement of their phagocytic activity in the lungs of mice inoculated with heat-killed Cryptococcus neoformans: involvement of IFN-gamma and its protective effect against cryptococcal infection. *Microbiol.Immunol* 39:135-143.

194 Pietrella, D., Corbucci, C., Perito, S., Bistoni, G., and Vecchiarelli, A. 2005. Mannoproteins from *Cryptococcus neoformans* promote dendritic cell maturation and activation. *Infect.Immun.* 73:820-827.

195 Wozniak, K. L., Vyas, J. M., and Levitz, S. M. 2006. In vivo role of dendritic cells in a murine model of pulmonary cryptococcosis. *Infect.Immun.* 74:3817-3824.

196 Coyle, A. J. and Gutierrez-Ramos, J. C. 2001. The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. *Nat.Immunol* 2:203-209.

197 Huffnagle, G. B., Toews, G. B., Burdick, M. D., Boyd, M. B., McAllister, K. S., McDonald, R. A., Kunkel, S. L., and Strieter, R. M. 1996. Afferent phase production of TNF-alpha is required for the development of protective T cell immunity to Cryptococcus neoformans. *J Immunol* 157:4529-4536.

198 Kawakami, K., Qureshi, M. H., Koguchi, Y., Zhang, T., Okamura, H., Kurimoto, M., and Saito, A. 1999. Role of TNF-alpha in the induction of fungicidal activity of mouse peritoneal exudate cells against Cryptococcus neoformans by IL-12 and IL-18. *Cell Immunol* 193:9-16.

199 Kawakami, K., Qifeng, X., Tohyama, M., Qureshi, M. H., and Saito, A. 1996. Contribution of tumour necrosis factor-alpha (TNF-alpha) in host defence mechanism against Cryptococcus neoformans. *Clin.Exp.Immunol* 106:468-474.

200 Nakamura, K., Miyagi, K., Koguchi, Y., Kinjo, Y., Uezu, K., Kinjo, T., Akamine, M., Fujita, J., Kawamura, I., Mitsuyama, M., Adachi, Y., Ohno, N., Takeda, K., Akira, S., Miyazato, A., Kaku, M., and Kawakami, K. 2006. Limited contribution of Toll-like receptor 2 and 4 to the host response to a fungal infectious pathogen, *Cryptococcus neoformans*. *FEMS Immunol.Med.Microbiol.* 47:148-154.

201 Yauch, L. E., Mansour, M. K., Shoham, S., Rottman, J. B., and Levitz, S. M. 2004. Involvement of CD14, toll-like receptors 2 and 4, and MyD88 in the host response to the fungal pathogen *Cryptococcus neoformans in vivo*. *Infect.Immun.* 72:5373-5382.

202 Weighardt, H., Jusek, G., Mages, J., Lang, R., Hoebe, K., Beutler, B., and Holzmann, B. 2004. Identification of a TLR4- and TRIF-dependent activation program of dendritic cells. *Eur J Immunol* 34:558-564.

203 Sweet, M. J., Campbell, C. C., Sester, D. P., Xu, D., McDonald, R. C., Stacey, K. J., Hume, D. A., and Liew, F. Y. 2002. Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages. *J Immunol* 168:392-399.

204 Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat.Immunol* 3:196-200.

205 Barton, G. M., Kagan, J. C., and Medzhitov, R. 2006. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat.Immunol* 7:49-56.

206 Hoebe, K. and Beutler, B. 2004. LPS, dsRNA and the interferon bridge to adaptive immune responses: Trif, Tram, and other TIR adaptor proteins. *J Endotoxin.Res.* 10:130-136.

207 Haig, D. M. and McInnes, C. J. 2002. Immunity and counter-immunity during infection with the parapoxvirus orf virus. *Virus Res.* 88:3-16.

208 Smith, K. J., Skelton, H. G., III, James, W. D., and Lupton, G. P. 1991. Parapoxvirus infections acquired after exposure to wildlife. *Arch.Dermatol.* 127:79-82.

209 Buttner, M., Czerny, C. P., Lehner, K. H., and Wertz, K. 1995. Interferon induction in peripheral blood mononuclear leukocytes of man and farm animals by poxvirus vector candidates and some poxvirus constructs. *Vet.Immunol Immunopathol.* 46:237-250.

210 Friebe, A., Siegling, A., Friederichs, S., Volk, H. D., and Weber, O. 2004. Immunomodulatory effects of inactivated parapoxvirus ovis (ORF virus) on human peripheral immune cells: induction of cytokine secretion in monocytes and Th1-like cells. *J.Virol.* 78:9400-9411.

211 Barchet, W., Cella, M., and Colonna, M. 2005. Plasmacytoid dendritic cells--virus experts of innate immunity. *Semin.Immunol.* 17:253-261.

212 Chang, S., Dolganiuc, A., and Szabo, G. 2007. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. *J Leukoc Biol*.

213 Aravalli, R. N., Hu, S., Rowen, T. N., Palmquist, J. M., and Lokensgard, J. R. 2005. Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus. *J Immunol* 175:4189-4193.

214 Burzyn, D., Rassa, J. C., Kim, D., Nepomnaschy, I., Ross, S. R., and Piazzon, I. 2004. Toll-like receptor 4-dependent activation of dendritic cells by a retrovirus. *J Virol.* 78:576-584.

215 Bieback, K., Lien, E., Klagge, I. M., Avota, E., Schneider-Schaulies, J., Duprex, W. P., Wagner, H., Kirschning, C. J., Ter, M., V, and Schneider-Schaulies, S. 2002. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. *J Virol.* 76:8729-8736.

216 Kurt-Jones, E. A., Popova, L., Kwinn, L., Haynes, L. M., Jones, L. P., Tripp, R. A., Walsh, E. E., Freeman, M. W., Golenbock, D. T., Anderson, L. J., and Finberg, R. W. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat.Immunol* 1:398-401.

217 Hirotani, T., Yamamoto, M., Kumagai, Y., Uematsu, S., Kawase, I., Takeuchi, O., and Akira, S. 2005. Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta. *Biochem.Biophys.Res.Commun.* 328:383-392.

218 Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R. A., Diamond, M. S., and Colonna, M. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc.Natl.Acad.Sci.U.S.A* 103:8459-8464.

219 Berghall, H., Siren, J., Sarkar, D., Julkunen, I., Fisher, P. B., Vainionpaa, R., and Matikainen, S. 2006. The interferon-inducible RNA helicase, mda-5, is involved in measles virus-induced expression of antiviral cytokines. *Microbes.Infect.* 8:2138-2144.

220 Samanta, M., Iwakiri, D., Kanda, T., Imaizumi, T., and Takada, K. 2006. EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. *EMBO J* 25:4207-4214.

221 Opitz, B., Rejaibi, A., Dauber, B., Eckhard, J., Vinzing, M., Schmeck, B., Hippenstiel, S., Suttorp, N., and Wolff, T. 2007. IFNbeta induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. *Cell Microbiol.* 9:930-938.

222 Le Goffic, R., Pothlichet, J., Vitour, D., Fujita, T., Meurs, E., Chignard, M., and Si-Tahar, M. 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *J Immunol* 178:3368-3372.

223 Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z. K., Endres, S., Krieg, A. M., and Hartmann, G. 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol* 31:2154-2163.

224 Siegemund, S., Schuetze, N., Freudenberg, M., Lutz, M. B., Straubinger, R. K., and Alber, G. Production of I-12, IL-23 and IL-27p28 by bone marrow-derived conventional dendritic cells rather than macrophages after LPS/TLR4-dependent induction by *Salmonella* Enteritidis. Immunobiology . 2007.
Ref Type: In Press

225 Cottone, R., Buttner, M., Bauer, B., Henkel, M., Hettich, E., and Rziha, H. J. 1998. Analysis of genomic rearrangement and subsequent gene deletion of the attenuated Orf virus strain D1701. *Virus Res.* 56:53-67.

226 Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.

227 Bost, K. L. and Clements, J. D. 1997. Intracellular *Salmonella* dublin induces substantial secretion of the 40-kilodalton subunit of interleukin-12 (IL-12) but minimal secretion of IL-12 as a 70-kilodalton protein in murine macrophages. *Infect.Immun.* 65:3186-3192.

228 Yrlid, U., Svensson, M., Johansson, C., and Wick, M. J. 2000. *Salmonella* infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response. *FEMS Immunology and Medical Microbiology* 27:313-320.

229 Rydstrom, A. and Wick, M. J. 2007. Monocyte recruitment, activation, and function in the gut-associated lymphoid tissue during oral Salmonella infection. *J Immunol* 178:5789-5801.

230 Kirby, A. C., Yrlid, U., and Wick, M. J. 2002. The innate immune response differs in primary and secondary *Salmonella* infection. ***J.Immunol.*** 169:4450-4459.

231 Shortman, K. and Liu, Y. J. 2002. Mouse and human dendritic cell subtypes. ***Nat.Rev.Immunol.*** 2:151-161.

232 Salazar-Gonzalez, R. M., Niess, J. H., Zammit, D. J., Ravindran, R., Srinivasan, A., Maxwell, J. R., Stoklasek, T., Yadav, R., Williams, I. R., Gu, X., McCormick, B. A., Pazos, M. A., Vella, A. T., Lefrancois, L., Reinecker, H. C., and McSorley, S. J. 2006. CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. *Immunity* 24:623-632.

233 Trinchieri, G., Pflanz, S., and Kastelein, R. A. 2003. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 19:641-644.

234 Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R., and Weaver, C. T. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441:231-234.

235 Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., and Stockinger, B. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. ***Immunity.*** 24:179-189.

236 Norimatsu, M., Harris, J., Chance, V., Dougan, G., Howard, C. J., and Villarreal-Ramos, B. 2003. Differential response of bovine monocyte-derived macrophages and dendritic cells to infection with *Salmonella* typhimurium in a low-dose model *in vitro*. *Immunology* 108:55-61.

237 Pietil, T. E., Veckman, V., Kyllonen, P., Lahteenmaki, K., Korhonen, T. K., and Julkunen, I. 2005. Activation, cytokine production, and intracellular survival of bacteria in *Salmonella*-infected human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol*jlb.

238 Liu, C. H., Fan, Y. T., Dias, A., Esper, L., Corn, R. A., Bafica, A., Machado, F. S., and Aliberti, J. 2006. Cutting edge: dendritic cells are essential for *in vivo* IL-12 production and development of resistance against *Toxoplasma gondii* infection in mice. *J Immunol* 177:31-35.

239 D'Andrea, A., ste-Amezaga, M., Valiante, N. M., Ma, X., Kubin, M., and Trinchieri, G. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. ***J.Exp.Med.*** 178:1041-1048.

240 Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat.Rev.Immunol* 3:133-146.

241 Smits, H. H., Van Beelen, A. J., Hessle, C., Westland, R., De Jong, E., Soeteman, E., Wold, A., Wierenga, E. A., and Kapsenberg, M. L. 2004. Commensal Gram-negative bacteria prime human dendritic cells for enhanced IL-23 and IL-27 expression and enhanced Th1 development. *Eur J Immunol* 34:1371-1380.

242 Wirtz, S., Tubbe, I., Galle, P. R., Schild, H. J., Birkenbach, M., Blumberg, R. S., and Neurath, M. F. 2006. Protection from lethal septic peritonitis by neutralizing the biological function of interleukin 27. *J.Exp.Med.* 203:1875-1881.

243 D'Andrea, A., Rengaraju, M., Valiante, N. M., Chehimi, J., Kubin, M., Aste, M., Chan, S. H., Kobayashi, M., Young, D., Nickbarg, E., and . 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J.Exp.Med.* 176:1387-1398.

244 Yoshida, H., Hamano, S., Senaldi, G., Covey, T., Faggioni, R., Mu, S., Xia, M., Wakeham, A. C., Nishina, H., Potter, J., Saris, C. J., and Mak, T. W. 2001. WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection. *Immunity.* 15:569-578.

245 Chen, Q., Ghilardi, N., Wang, H., Baker, T., Xie, M. H., Gurney, A., Grewal, I. S., and de Sauvage, F. J. 2000. Development of Th1-type immune responses requires the type I cytokine receptor TCCR. *Nature* 407:916-920.

246 Yamanaka, A., Hamano, S., Miyazaki, Y., Ishii, K., Takeda, A., Mak, T. W., Himeno, K., Yoshimura, A., and Yoshida, H. 2004. Hyperproduction of proinflammatory cytokines by WSX-1-deficient NKT cells in concanavalin A-induced hepatitis. *J.Immunol.* 172:3590-3596.

247 Hamano, S., Himeno, K., Miyazaki, Y., Ishii, K., Yamanaka, A., Takeda, A., Zhang, M., Hisaeda, H., Mak, T. W., Yoshimura, A., and Yoshida, H. 2003. WSX-1 is required for resistance to Trypanosoma cruzi infection by regulation of proinflammatory cytokine production. *Immunity.* 19:657-667.

248 Villarino, A., Hibbert, L., Lieberman, L., Wilson, E., Mak, T., Yoshida, H., Kastelein, R. A., Saris, C., and Hunter, C. A. 2003. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. *Immunity.* 19:645-655.

249 Salazar-Gonzalez, R. M. and McSorley, S. J. 2005. *Salmonella* flagellin, a microbial target of the innate and adaptive immune system. *Immunol Lett.* 101:117-122.

250 Albrecht, I., Tapmeier, T., Zimmermann, S., Frey, M., Heeg, K., and Dalpke, A. 2004. Toll-like receptors differentially induce nucleosome remodelling at the IL-12p40 promoter. *EMBO Rep.* 5:172-177.

251 Wirtz, S., Becker, C., Fantini, M. C., Nieuwenhuis, E. E., Tubbe, I., Galle, P. R., Schild, H. J., Birkenbach, M., Blumberg, R. S., and Neurath, M. F. 2005. EBV-induced gene 3 transcription is induced by TLR signaling in primary dendritic cells via NF-kappa B activation. *J.Immunol.* 174:2814-2824.

252 Vanden, E. S., Goriely, S., De, W. D., Goldman, M., and Willems, F. 2006. Preferential production of the IL-12(p40)/IL-23(p19) heterodimer by dendritic cells from human newborns. *Eur.J.Immunol.* 36:21-26.

253 Schnurr, M., Toy, T., Shin, A., Wagner, M., Cebon, J., and Maraskovsky, E. 2004. Extracellular nucleotide signalling via P2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the cAMP pathway. *Blood*.

254 Rescigno, M., Urbano, M., Rimoldi, M., Valzasina, B., Rotta, G., Granucci, F., and Ricciardi-Castagnoli, P. 2002. Toll-like receptor 4 is not required for the full maturation of dendritic cells or for the degradation of Gram-negative bacteria. *Eur J Immunol* 32:2800-2806.

255 Royle, M. C., Totemeyer, S., Alldridge, L. C., Maskell, D. J., and Bryant, C. E. 2003. Stimulation of Toll-like receptor 4 by lipopolysaccharide during cellular invasion by live *Salmonella* typhimurium is a critical but not exclusive event leading to macrophage responses. *J Immunol* 170:5445-5454.

256 Bernheiden, M., Heinrich, J. M., Minigo, G., Schutt, C., Stelter, F., Freeman, M., Golenbock, D., and Jack, R. S. 2001. LBP, CD14, TLR4 and the murine innate immune response to a peritoneal *Salmonella* infection. *J.Endotoxin.Res.* 7:447-450.

257 Bihl, F., Salez, L., Beaubier, M., Torres, D., Lariviere, L., Laroche, L., Benedetto, A., Martel, D., Lapointe, J. M., Ryffel, B., and Malo, D. 2003. Overexpression of Toll-like receptor 4 amplifies the host response to lipopolysaccharide and provides a survival advantage in transgenic mice. *J.Immunol.* 170:6141-6150.

258 MacVittie, T. J., O'Brien, A. D., Walker, R. I., and Weinberg, S. R. 1982. Inflammatory response of LPS-hyporesponsive and LPS-responsive mice to challenge with Gram-negative bacteria *Salmonella* typhimurium and *Klebsiella pneumoniae*. *Adv.Exp.Med.Biol.* 155:325-334.

259 Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van, H. C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282:2085-2088.

260 Li, Q. and Cherayil, B. J. 2003. Role of Toll-like receptor 4 in macrophage activation and tolerance during *Salmonella enterica* serovar Typhimurium infection. *Infect.Immun.* 71:4873-4882.

261 Tripp, C. S., Gately, M. K., Hakimi, J., Ling, P., and Unanue, E. R. 1994. Neutralization of IL-12 decreases resistance to Listeria in SCID and C.B-17 mice. Reversal by IFN-gamma. *J Immunol* 152:1883-1887.

262 Happel, K. I., Zheng, M., Young, E., Quinton, L. J., Lockhart, E., Ramsay, A. J., Shellito, J. E., Schurr, J. R., Bagby, G. J., Nelson, S., and Kolls, J. K. 2003. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to Klebsiella pneumoniae infection. *J Immunol* 170:4432-4436.

263 Khader, S. A., Bell, G. K., Pearl, J. E., Fountain, J. J., Rangel-Moreno, J., Cilley, G. E., Shen, F., Eaton, S. M., Gaffen, S. L., Swain, S. L., Locksley, R. M., Haynes, L., Randall, T. D., and Cooper, A. M. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat.Immunol* 8:369-377.

264 Wu, Q., Martin, R. J., Rino, J. G., Breed, R., Torres, R. M., and Chu, H. W. 2007. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory Mycoplasma pneumoniae infection. *Microbes.Infect.* 9:78-86.

265 Umemura, M., Yahagi, A., Hamada, S., Begum, M. D., Watanabe, H., Kawakami, K., Suda, T., Sudo, K., Nakae, S., Iwakura, Y., and Matsuzaki, G. 2007. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. *J Immunol* 178:3786-3796.

266 Khader, S. A., Partida-Sanchez, S., Bell, G., Jelley-Gibbs, D. M., Swain, S., Pearl, J. E., Ghilardi, N., Desauvage, F. J., Lund, F. E., and Cooper, A. M. 2006. Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. *J Exp.Med.* 203:1805-1815.

267 Gafa, V., Lande, R., Gagliardi, M. C., Severa, M., Giacomini, E., Remoli, M. E., Nisini, R., Ramoni, C., Di Francesco, P., Aldebert, D., Grillot, R., and Coccia, E. M. 2006. Human dendritic cells following Aspergillus fumigatus infection express the CCR7 receptor and a differential pattern of interleukin-12 (IL-12), IL-23, and IL-27 cytokines, which lead to a Th1 response. *Infect.Immun.* 74:1480-1489.

268 Lindell, D. M., Moore, T. A., McDonald, R. A., Toews, G. B., and Huffnagle, G. B. 2006. Distinct compartmentalization of CD4+ T-cell effector function versus proliferative capacity during pulmonary cryptococcosis. *Am.J Pathol.* 168:847-855.

269 Ravindran, R., Rusch, L., Itano, A., Jenkins, M. K., and McSorley, S. J. 2007. CCR6-dependent recruitment of blood phagocytes is necessary for rapid CD4 T cell responses to local bacterial infection. *Proc.Natl.Acad.Sci.U.S.A* 104:12075-12080.

270 Leon, B., Lopez-Bravo, M., and Ardavin, C. 2007. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity* 26:519-531.

271 Colonna, M., Trinchieri, G., and Liu, Y. J. 2004. Plasmacytoid dendritic cells in immunity. *Nat.Immunol* 5:1219-1226.

272 Tsujimoto, H., Uchida, T., Efron, P. A., Scumpia, P. O., Verma, A., Matsumoto, T., Tschoeke, S. K., Ungaro, R. F., Ono, S., Seki, S., Clare-Salzler, M. J., Baker, H. V., Mochizuki, H., Ramphal, R., and Moldawer, L. L. 2005. Flagellin enhances NK cell proliferation and activation directly and through dendritic cell-NK cell interactions. *J Leukoc Biol* 78:888-897.

273 Tam, M. A. and Wick, M. J. 2006. Differential expansion, activation and effector functions of conventional and plasmacytoid dendritic cells in mouse tissues transiently infected with Listeria monocytogenes. *Cell Microbiol.* 8:1172-1187.

274 Kuwajima, S., Sato, T., Ishida, K., Tada, H., Tezuka, H., and Ohteki, T. 2006. Interleukin 15-dependent crosstalk between conventional and plasmacytoid dendritic cells is essential for CpG-induced immune activation. *Nat.Immunol* 7:740-746.

275 Ramarathinam, L., Niesel, D. W., and Klimpel, G. R. 1993. Salmonella typhimurium induces IFN-gamma production in murine splenocytes. Role of natural killer cells and macrophages. *J Immunol* 150:3973-3981.

276 Thatte, J., Rath, S., and Bal, V. 1993. Immunization with live versus killed Salmonella typhimurium leads to the generation of an IFN-gamma-dominant versus an IL-4-dominant immune response. *Int.Immunol* 5:1431-1436.

277 Foster, N., Hulme, S. D., and Barrow, P. A. 2003. Induction of antimicrobial pathways during early-phase immune response to Salmonella spp. in murine macrophages: gamma interferon (IFN-gamma) and upregulation of IFN-gamma receptor alpha expression are required for NADPH phagocytic oxidase gp91-stimulated oxidative burst and control of virulent Salmonella spp. *Infect.Immun.* 71:4733-4741.

278 Vanden, E. S., Goriely, S., De, W. D., Willems, F., and Goldman, M. 2005. IL-23 up-regulates IL-10 and induces IL-17 synthesis by polyclonally activated naive T cells in human. *Eur.J.Immunol.* 35:469-475.

279 Bettelli, E., Oukka, M., and Kuchroo, V. K. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat.Immunol* 8:345-350.

280 Laan, M., Cui, Z. H., Hoshino, H., Lotvall, J., Sjostrand, M., Gruenert, D. C., Skoogh, B. E., and Linden, A. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* 162:2347-2352.

281 Witowski, J., Pawlaczyk, K., Breborowicz, A., Scheuren, A., Kuzlan-Pawlaczyk, M., Wisniewska, J., Polubinska, A., Friess, H., Gahl, G. M., Frei, U., and Jorres, A. 2000. IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO alpha chemokine from mesothelial cells. *J Immunol* 165:5814-5821.

282 Lockhart, E., Green, A. M., and Flynn, J. L. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol* 177:4662-4669.

283 Shibata, K., Yamada, H., Hara, H., Kishihara, K., and Yoshikai, Y. 2007. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *J Immunol* 178:4466-4472.

284 Cross, C. E., Collins, H. L., and Bancroft, G. J. 1997. CR3-dependent phagocytosis by murine macrophages: different cytokines regulate ingestion of a defined CR3 ligand and complement-opsonized Cryptococcus neoformans. *Immunology* 91:289-296.

285 Herring, A. C., Falkowski, N. R., Chen, G. H., McDonald, R. A., Toews, G. B., and Huffnagle, G. B. 2005. Transient neutralization of tumor necrosis factor alpha can produce a chronic fungal infection in an immunocompetent host: potential role of immature dendritic cells. *Infect.Immun.* 73:39-49.

286 Alspaugh, J. A. and Granger, D. L. 1991. Inhibition of Cryptococcus neoformans replication by nitrogen oxides supports the role of these molecules as effectors of macrophage-mediated cytostasis. *Infect.Immun.* 59:2291-2296.

287 Goldman, D., Cho, Y., Zhao, M., Casadevall, A., and Lee, S. C. 1996. Expression of inducible nitric oxide synthase in rat pulmonary Cryptococcus neoformans granulomas. *Am.J Pathol.* 148:1275-1282.

288 Lee, S. C., Dickson, D. W., Brosnan, C. F., and Casadevall, A. 1994. Human astrocytes inhibit Cryptococcus neoformans growth by a nitric oxide-mediated mechanism. *J Exp.Med.* 180:365-369.

289 Lovchik, J. A., Lyons, C. R., and Lipscomb, M. F. 1995. A role for gamma interferon-induced nitric oxide in pulmonary clearance of Cryptococcus neoformans. *Am.J Respir.Cell Mol.Biol* 13:116-124.

290 Tohyama, M., Kawakami, K., Futenma, M., and Saito, A. 1996. Enhancing effect of oxygen radical scavengers on murine macrophage anticryptococcal activity through production of nitric oxide. *Clin.Exp.Immunol* 103:436-441.

291 Kawakami, K., Koguchi, Y., Qureshi, M. H., Yara, S., Kinjo, Y., Uezu, K., and Saito, A. 2000. NK cells eliminate Cryptococcus neoformans by potentiating the fungicidal activity of macrophages rather than by directly killing them upon stimulation with IL-12 and IL-18. *Microbiol.Immunol* 44:1043-1050.

292 Blackstock, R., McElwee, N., Neller, E., and Shaddix-White, J. 2000. Regulation of cytokine expression in mice immunized with cryptococcal polysaccharide, a glucuronoxylomannan (GXM), associated with peritoneal antigen-presenting cells (APC): requirements for GXM, APC activation, and interleukin-12. *Infect.Immun.* 68:5146-5153.

293 Monari, C., Bistoni, F., Casadevall, A., Pericolini, E., Pietrella, D., Kozel, T. R., and Vecchiarelli, A. 2005. Glucuronoxylomannan, a microbial compound, regulates expression of costimulatory molecules and production of cytokines in macrophages. *J Infect.Dis.* 191:127-137.

294 Haig, D. M. 2006. Orf virus infection and host immunity. *Curr.Opin.Infect.Dis.* 19:127-131.

295 Fonteneau, J. F., Larsson, M., and Bhardwaj, N. 2002. Interactions between dead cells and dendritic cells in the induction of antiviral CTL responses. *Curr.Opin.Immunol* 14:471-477.

296 Pestka, S., Langer, J. A., Zoon, K. C., and Samuel, C. E. 1987. Interferons and their actions. *Annu.Rev.Biochem.* 56:727-777.

297 Le Bon, A., Schiavoni, G., D'Agostino, G., Gresser, I., Belardelli, F., and Tough, D. F. 2001. Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* 14:461-470.

298 Ortaldo, J. R., Mason, A., Rehberg, E., Moschera, J., Kelder, B., Pestka, S., and Herberman, R. B. 1983. Effects of recombinant and hybrid recombinant human leukocyte interferons on cytotoxic activity of natural killer cells. *J Biol Chem.* 258:15011-15015.

299 Pestka, S. 2007. The interferons: 50 years after their discovery, there is much more to learn. *J Biol Chem.* 282:20047-20051.

300 Chatzantoni, K. and Mouzaki, A. 2006. Anti-TNF-alpha antibody therapies in autoimmune diseases. *Curr.Top.Med.Chem.* 6:1707-1714.

301 Tam, M. A. and Wick, M. J. 2006. Differential expansion, activation and effector functions of conventional and plasmacytoid dendritic cells in mouse tissues transiently infected with Listeria monocytogenes. *Cell Microbiol.* 8:1172-1187.

302 Ignatius, R., Marovich, M., Mehlhop, E., Villamide, L., Mahnke, K., Cox, W. I., Isdell, F., Frankel, S. S., Mascola, J. R., Steinman, R. M., and Pope, M. 2000. Canarypox virus-induced maturation of dendritic cells is mediated by apoptotic cell death and tumor necrosis factor alpha secretion. *J Virol.* 74:11329-11338.

303 Kruse, N. and Weber, O. 2001. Selective induction of apoptosis in antigen-presenting cells in mice by Parapoxvirus ovis. *J Virol.* 75:4699-4704.

304 Akaike, T. and Maeda, H. 2000. Nitric oxide and virus infection. *Immunology* 101:300-308.

305 Kawai, T. and Akira, S. 2005. Pathogen recognition with Toll-like receptors. *Curr.Opin.Immunol.* 17:338-344.

306 Nociari, M., Ocheretina, O., Schoggins, J. W., and Falck-Pedersen, E. 2007. Sensing infection by adenovirus: Toll-like receptor-independent viral DNA recognition signals activation of the interferon regulatory factor 3 master regulator. *J Virol.* 81:4145-4157.

Appendix

Abbreviations

2'-5' OAS	2'-5' oligoadenylate synthetase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
eIF2a	α subunit translation initiation factor 2
ADAR-1	adenosine deaminase acting on RNA-1
APC	allophycoerythrin
APOBEC	apolipoprotein B mRNA-editing enzyme catalytic polypeptide like editing complex
BMDC	bone marrow-derived dendritic cell(s)
BMMΦ	bone marrow-derived macrophages
i	chemically inactivated
CpG-ODN	CpG oligodesoxynucleotides
C	*Cryptococcus*
Tcyt	cytotoxic T cell(s)
dpi	day post infection
def.	deficient
DC	Dendritic cells
dNTP	desoxynucleotidetriphosphate
DNA	desoxyribonucleic acid
DMSO	dimethylsulfoxide
DAI	DNA-dependent activator of IFN-regulatory factors
ds	double-stranded
DLN	draining lymph node
ELISA	enzyme linked immunosorbent assay
EDTA	ethylene diaminetetraacetic acid
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FACS	fluorescent-activated cell sorting
Flt3L	fms-like tyrosin kinase 3 ligand
GM-CSF	granulocyte macrophage-colony stimulating factor
hi	heat-incubated and chemically incativated

HRP	horse radish peroxidase
h	hour(s)
Ig	immunglobulin
IRF	interferon regulatory factor(s)
IFN	interferon(s)
IL	interleukin(s)
i.p.	intraperitoneal(ly)
LPS	lipoplysaccharid
LN	lymph node
M-CSF	macrophage-colony stimulating factor
MΦ	macrophages
MHC	major histocompatibility complex
MDA5	melanoma-differentiation-associated gene 5
MOI	multiplicity of infection
MyD88	myeloid differentiation primary response gene 88
NK cell(s)	natural killer cell(s)
NO	nitric oxide
n.d.	not done
NF-κB	nuclear factor-kappa B
NLR	nucleotide oligomerization domain-like
PPVO	parapoxvirus ovis
PAMP	pathogen-associated molecular patterns
PRR	pattern-recognition receptors
PEC	peritoneal exudate cells
PBS	phosphate-buffered saline
PE	phycoerythrin
poly I:C	polyinosinic-polycytidylic acid
RIG-I	retinoic-acid-inducible gene I
RNA	ribonucleic acid
RT	room temperature
S	*Salmonella*
ss	single-stranded
SDS	sodium dodecyl sulfate
Sol	solution

SEM	standard error of the mean
SA	streptavidin
spp	subspecies
Th cell	T helper cell
TMB	tetramethylbenzidine
TRIF	TIR-domain containing adaptor protein inducing IFN
TIR	Toll/IL-1R homology
TLR	toll-like receptor(s)
TRIM 5α	tripartite motif protein 5α
TGF-β	Tumor growth factor-β
TNF	tumor necrosis factor(s)
wt	wild-type
XLD	xylose-lysine-desoxycholat

Antibodies

Tab. A 1 Antibodies for cell depletion (MACS)

antibody (isotype)	clone	labelling	final concentration	source
Anti-PE	unknown	magnetic bead		Miltenyi Biotec, Bergisch-Gladbach
anti-ms CD11c (ham IgG1)	HL3	PE	12.5 µg/ml	BD Pharmingen™, Heidelberg
anti-ms F4/80 (rat IgG2b)	CI:A3-1	PE	2.5 µg/ml	Caltag Laboratories, Burlingame, USA

Tab. A 2 Capture antibodies for cytokine sandwich ELISA

antibody	clone	final concentration	source
anti-ms IFN-α	RMMA-1	2 µg/ml	R&D-Systems, Wiesbaden
anti-msIFN-γ	AN-18	5 µg/ml	Hoffmann La-Roche Ltd., Basel, Switzerland
anti-msIL-6	MP5-20F3	2,5 µg/ml	BD Pharmingen™, Heidelberg
anti-msIL-12p40	5C3	25 µg/ml	Hoffmann La-Roche Ltd., Basel, Switzerland
anti-msIL-12p70	48110	2 µg/ml	R&D-Systems, Wiesbaden
anti-msIL-23p19	PAB 565	2 µg/ml	Schering-Plough Biopharma, Palo Alto, USA

Tab. A 3 Detection antibodies for cytokine sandwich ELISA

antibody	clone	labelling	final concentration	source
polyclonal rabbit anti-msIFNα	-	purified	1:4000 diluted	R&D-Systems, Wiesbaden
anti-msIFN-γ	XMG1.2	HRP	1:1000 diluted	ATCC, Manassas, VA, USA
anti-msIL-6	MP5-32C11	Biotin	1:400	BD Pharmingen™, Heidelberg
anti-msIL-12/23p40	goat anti mouse serum	Biotin	1:3000	Hoffmann La-Roche Ltd., Basel, Switzerland

Tab. A 4 Secondary antibodies for cytokine sandwich ELISA

antibody	clone	labelling	final concentration	source
donkey anti rabbit IgG F(ab')$_2$	unknown	HRP	1:2500	dianova, Hamburg

Tab. A 5 Antibodies for flow cyotmetric analyses

antibody (isotype)	clone	labelling	final concen-tration	source
anti-ms CD16/CD32 (rat IgG)	2.4G2	purified	25.0 µg/ml	BD Pharmingen™, Heidelberg
anti-ms CD11b (rat IgG2b)	M1/70.15	FITC, PE	2.5 µg/ml	Caltag Laboratories, Burlingame, USA
anti-ms CD11c (ham IgG1)	HL3	FITC, PE, APC	12.5 µg/ml	BD Pharmingen™, Heidelberg
anti-ms CD80 (rat IgG2a)	RMMP-2	PE	2.5 µg/ml	Caltag Laboratories, Burlingame, USA
anti-ms CD86 (rat IgG2a)	RMMP-1	PE	2.5 µg/ml	Caltag Laboratories, Burlingame, USA
anti-ms CD45R/B220 (rat IgG2a)	RA3-6B2	FITC, PE	2.5 µg/ml	Caltag Laboratories, Burlingame, USA
anti-ms F4/80 (rat IgG2b)	CI:A3-1	FITC, PE	2.5 µg/ml	Caltag Laboratories, Burlingame, USA
anti-ms Ly-6G and Ly-6C (rat IgG2b)	RB6-8C5	FITC	12.5 µg/ml	BD Pharmingen™, Heidelberg
anti-ms I-A^b (ms IgG2a)	AF6-120.1	FITC	12.5 µg/ml	BD Pharmingen™, Heidelberg
anti-ms IFN-α (rat IgG1)	RMMA-1	purified	2 µg/ml	R&D-Systems, Wiesbaden
polyclonal rabbit anti ms-IFNα (rabbit serum)	n.a.	purified	1:4000 diluted	R&D-Systems, Wiesbaden
donkey anti-rat IgG F(ab')$_2$	n.a.	PE	2.5 µg/ml	dianova, Hamburg
donkey anti-rabbit IgG F(ab')$_2$	n.a.	PE	2.5 µg/ml	dianova, Hamburg
polyclonal anti-ms IgM	n.a.	FITC	1:40 diluted	Southern Biotech, Birmingham, AL, USA
anti-ms-IL-12/23p40	C15.6	PE	5.0 µg/ml	BD Pharmingen™, Heidelberg
anti-ms mPDCA-1 (rat IgG2b)	JF05-1C2.4.1	FITC, PE	1:40 diluted	Miltenyi Biotec, Bergisch-Gladbach

Index of Figures

Acknowledgements

I would like to thank my supervisor Professor Dr. Gottfried Alber. It was a great opportunity to work under his supervision, and I am grateful that I was able to learn how to successfully plan, organize, conduct and publish scientific research projects from him. He always took the time for critical but supportive discussions which beside arousing fascination and enthusiasm for the scientific questions substantially contributed to the fact that I very much loved my work and enjoyed my time at the Institute of Immunology.

Moreover I am grateful to Professor Dr. Annette Beck-Sickinger. Whenever I needed advice from somebody outside of the Institute of Immunology, she was the one lending me her ear. Especially her encouraging comments on how to arrange a scientific career as a woman were very helpful to me. By her good teaching during my degree studies of biochemistry she very much contributed to my fascination for life sciences.

Together Professor Dr. Gottfried Alber and Professor Dr. Annette Beck-Sickinger substantially contributed to the success of my fellowship applications.

I am also very thankful to Dr. Nicole Schütze, who taught me most of the immunological techniques necessary for the successful conduction of my project, and to Dr. Uwe Müller who introduced me to handling *cryptococci*. Both of them share their profound knowledge on a lot of immunological topics in many helpful discussions until now. I very much appreciate their editorial advice on my manuscripts before submission as well as their great team work and technical help that both of them are always willing to provide.

My thanks also go to PD Dr. Reinhard Straubinger for his supervision during Professor Alber's sabbatical as well as his valuable comments on my first manuscript draft. His opinion very often provided a yet unseen point of view which very often caused me to reconsider things from a different angle. Working together with PD Dr. Straubinger was a very valuable experience I would not want to miss.

Furthermore I would like to thank the remaining former and present team of the Institute of Immunology, by name Juliane Richter, Dipl.-Biol. Silke Schulz, Dr. Jens Knauer, veterinarian Inke Krupka, Dipl.-Biol. Martin Koethe, Dr. Katharina Töpfer, Christina Wiegand and Dr. Samiya Al-Robaiy. The working atmosphere together with them was great and extraordinary, and I would not have been successful without their great team work and technical help they were always willing to provide. I am sure it will be hard to find such a great team again. In this context I would also like to thank Professor Dr. Manfred Blessing and his team of the Center of Biotechnology and Biomedicine for always providing help, technical advice and resources when I asked for it.

For many helpful comments and discussions on macrophages and DC I would like to thank Professor Dr. Marina Freudenberg from the Max Plank Institute of Immunology in Freiburg and Professor Dr. Manfred Lutz from the Institute of Virology and Immunology of the University of Würzburg. Their helpful comments substantially improved my understanding of the topic and many manuscript drafts. Moreover, TLR2^{def} and TLR4^{def} mice were kindly provided by Professor Dr. Marina Freudenberg, and I am grateful to Professor Dr. Manfred Lutz for providing GM-CSF-transfected cells. L929-cells were a kind gift of Professor Dr. Thomas Jungi from the University of Bern. For their helpful discussions and technical help on work with plasmacytoid DC I would also like to thank Dr. Sabine Vollstedt, Dr. Meredith O'Keefe and her technician Ben Fancke. Moreover, I am grateful to Dr. Rüdiger Raue and Pfizer® Ltd. for providing iPPVO, Professor Mathias Büttner for providing DNA derived from PPVO, and Dipl.-Biol. Andrea Hartl and Dr. Tim Sparwasser for providing bone marrow from MyD88$^{-/-}$ and TLR9$^{-/-}$ mice. Besides I thank Dipl.-Biochem. Doreen Thor for critically reading parts of previous drafts of this manuscript.

For the breeding and maintenance of the mice for my studies and the excellent collaboration I am grateful to the animal facility staff at the Institute of Immunology, especially Dr. Uwe Müller, Rowina Voigtländer, Eva Marquardt and Norman Kirchhoff.

I am also grateful to Dr. Andreas Grahnert and Professor Dr. Mike Gurish. Due to their good supervision I learnt a lot during my first experiences in a laboratory. Their endless patience and friendliness caused me to never hesitate asking also possibly stupid questions. The same accounts for Professor Dr. Alber. I hope I will be able to teach, support and motivate young scientists at some point in the future as well as they do.

For his extraordinary teaching in Biology back in school I am grateful to Mr. Kobschätzki. His enthusiastic teaching sparked my fascination for life sciences. In this context I am also very grateful to my parents, who never query my way and support me wherever they can. Without their support I would not be where I am today.

The present study was financially supported by the Hanns Seidel Foundation, the German Research Foundation (grant Al 371/3-3), Pfizer® Ltd, and the HWP of the state of Saxony.

Last but most important I would like to thank my beloved husband Thomas. I always wonder how I could possibly deserve such abundant love and support I receive from him. I am deeply grateful that I met him and that I am so unbelievably lucky he shares his life with me.

Publikationsliste

Publikationen:

1. Schuetze, N., Schoeneberger, S., Mueller, U., Freudenberg, M. A., Alber, G., and Straubinger, R. K. 2005. IL-12 family members: differential kinetics of their TLR4-mediated induction by *Salmonella* Enteritidis and the impact of IL-10 in bone marrow-derived macrophages, *Int. Immunol*. 17: 649-659

2. Knauer, J., Siegemund, S., Muller, U., Al-Robaiy, S., Kastelein, R. A., Alber, G., and Straubinger, R. K. 2007. *Borrelia burgdorferi* potently activates bone marrow-derived conventional dendritic cells for production of IL-23 required for IL-17 release by T cells. *FEMS Immunol Med Microbiol.* 49:353-363.

3. Siegemund, S., Schuetze, N., Freudenberg, M. A., Lutz, M. B., Straubinger, R. K., and Alber, G. 2007. Production of IL-12, IL-23 and IL-27p28 by bone marrow-derived conventional dendritic cells rather than macrophages after LPS/TLR4-dependent induction by *Salmonella* Enteritidis. *Immunobiol*. (in press)

4. Siegemund, S., Alber, G. *Cryptococcus neoformans* activates conventional dendritic cells rather than plasmacytoid dendritic cells and down-regulates macrophages" *FEMS Immunol Med. Microbiol.* (submitted)

5. Siegemund, S., Schuetze, N., Schulz, S., Straubinger, R. K., and Alber, G. 2008. Task Sharing between dendritic cells and macrophages: Initiation of the IL-12/IFN-γ and the IL-23/IL-17 axis versus NO production in response to *Salmonella.* (manuscript in preparation)

6. Siegemund, S., Hartl, A., Herden, F., Kirschnig, K., Büttner, M., Sparwasser, T., and Alber,G. 2008. Role of TLR9 in the activation of conventional and plasmacytoid bone marrow-derived dendritic cells by *parapoxvirus ovis.* (manuscript in preparation)

Buchkapitel:

1. Alber, G., Al-Robaiy, S., Kleinschek, M., Knauer, J., Krumbholz, P., Richter, J., Schoeneberger, S., Schuetze, S., Schulz, S., Toepfer, K., Voigtlaender, R., Lehmann, J., and Mueller, U. 2006. Induction of Immunity and Inflammation by Interleukin-12 Family Members. In: R. Numerof, Dinarello C.A., and K. Asadullah (eds.) Cytokines as Potential Therapeutic Targets for Inflammatory Skin Diseases, Springer-Verlag, Berlin Heidelberg, 107-127.

Erklärung über die eigenständige Abfassung der Arbeit

Hiermit versichere ich, dass die vorliegende Dissertation ohne unzulässige Hilfe und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt wurde und dass die aus fremden Quellen direkt oder indirekt übernommenen Gedanken in der Dissertation als solche kenntlich gemacht worden sind.
Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht.

Leipzig, den 26. Oktober 2007

Sabine Siegemund