Streptococcus pneumoniae capsular polysaccharide-mediated CD4$^+$ T cell-dependent abscess formation requires IL-6 and TLR-4

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To my parents

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1. Introduction

1.1 General introduction

The immune system is a fascinating and precious defence mechanism. It is essential for maintaining the integrity of the human organism. Host defence mechanisms consist of several defence lines such as natural barriers and non-specific (innate) as well as specific (adaptive) immune responses. Natural barriers, also called physical and chemical barriers, are the first line of defence. Physical barriers include the skin, which is the toughest one to overcome for a pathogen unless it is breached for instance by injury or catheters. Chemical barriers come into action wherever the skin protection is not consistent (e.g. eye). Mucous membranes are protected by secreted anti-microbial immunoglobulins or lysozyme which can be found in tears or saliva. Each region of the body has its own strategy to contributing to host defence against infections. Mucus and cilia located in nose and throat prevent pathogens from entering these open cavities by provoking coughing or sneezing. The acidic pH in the gastro-intestinal tract is an inhospitable environment for most pathogens (Janeway et al., 2005). The immune system is a highly differentiated and adaptable defence mechanism consisting of two components, the innate and the adaptive immune system. Recognition and differentiation between self- and foreign antigens and defending the body from diverse infectious invaders are the major tasks of the immune system.

The second line of defence is the innate immune system, which combats antigens that penetrated the first line of defence. The innate immune mechanism is non-specific and acts immediately on the clearance of pathogens but does not generate lasting protective immunity (Litman et al. 2005). Microorganisms, which we encounter in daily life, are detected and destroyed within minutes or hours by the various mechanisms of the innate immunity and only occasionally cause perceptible disease in a normal, healthy individual (Janeway et al., 2005). Phagocytes play a crucial role in the innate immune system as they have the capacity to effectively eliminate pathogens. Professional phagocytes are macrophages, mast cells, most dendritic cells (DCs) and granulocytes including neutrophils. These early team-players of the immune system eliminate antigens by breaking them down with e. g. digestive enzymes in their lysosomes. Macrophages are, like neutrophils,

professional phagocytes that have the capacity to attract other cells such as T cells from the blood to the site of infection by the release of chemoattractive cytokines and thus are part of the adaptive immunity. The complement system with its classic, lectin, and alternative pathways is an early warning mechanism for pathogen detection and lysis. Complement can attach to the bacterial surface, which promotes phagocytosis and is a phenomenon called opsonization. Only if an infectious organism can breach these early lines of defence will an adaptive immune response ensue (Janeway et al., 2005).

The third line of defence is the adaptive immune system. The cells of the adaptive immune system are the antigen-specific B-cell and T-cell lymphocytes. Adaptive immunity is triggered in vertebrates when a pathogen evades the innate immune system and generates a threshold level of antigen (Janeway et al., 2005). As soon as the innate immune system signals the presence of danger to the T cells of the adaptive immune system, T cells get involved with the B cells that have the ability to rearrange genes of the immunoglobulin family, permitting the creation of a large diversity of antigen specific clones and immunological memory (Gourley et al., 2004). The adaptive immune system then activates antigen-specific lymphocytes to proliferate and to differentiate into effector and memory cells that eliminate pathogens. Memory cells can prevent recurrence of disease caused by the same microorganism. Yet this highly sophisticated and potent adaptive immune system, which is a well-organized orchestra, needs to be conducted, instructed and regulated by antigen presenting cells (APCs).

1.2 Antigen-presenting cells

Phagocytes include professional APCs, which are dendritic cells (DCs) as well as macrophages and B cells. Bone-marrow (BM)-derived APCs are distributed in different compartments such as peripheral blood, lymphatic tissues, and solid organs to guard the host (Janeway et al., 2005). A main function of DCs is not only to engulf and degrade antigens intracellularly, but also to carry pathogen antigens to the secondary lymphatic organs, the lymph nodes and the spleen.

TABLE 1.1: Main characteristics of immature and mature DCs. Immature DCs have tremendous phagocytic activity to ingest antigens. Mature DCs have a phenotype that is associated with high levels of MHCI and MHCII proteins, and co-stimulatory molecules.

	Immature DCs (iDCs)	**Mature DCs (mDCs)**
Phagocytosis	↑↑	↓
MHC molecules	↓	↑↑
Co-stimulatory molecules	↓	↑

DCs exist in different stages of maturation which are associated with specific functional traits. The respective characteristics are given in Tab 1.1. Immature DCs have a tremendous phagocytic activity to ingest antigens either via pinocytosis or receptor-mediated endocytosis. Upon significant stimulation, an iDC matures to a mDC to activate the adaptive immune response, a process which is associated with loss of phagocytic activity and a high surface display of B7 molecules (CD80 and CD86) which is essential for T cell activation.

Upon antigen-mediated activation, DCs secrete cytokines that influence both the innate and the adaptive immune responses, which make these cells to essential gatekeepers that determine whether and how the immune system responds to the presence of infectious agents (Janeway et al., 2005). Activated DCs mature into highly effective APCs, a process that is associated with their loss of phagocytic activity. Mature DCs are generally considered to be terminally-differentiated cells, which have a short life span and no proliferation capacity (Shortman and Wu, 2004).

1.3 Antigen processing and MHC-dependent antigen presentation in APCs

Activation of the adaptive immune system by DCs is mediated by cytokine secretion as well as by direct presentation of antigens to T cells (Fig. 1.1). T cells circulate between blood and peripheral lymphoid tissue searching for their specific antigen. Naïve T cells are mature recirculating T cells that have not yet encountered their antigen. T cells consist of helper, cytotoxic and regulatory T cells. The $CD4^+$ helper T cells depend on the major histocompatibility complex (MHC) class II:peptide-contact

to initiate and modulate receptor- and cytokine-mediated functions of the immune system (Kaufmann and Schaible, 2005). MHC is a membrane glycoprotein encoded in a cluster of genes and a molecule that displays peptide antigen to T cells. $CD8^+$ cytotoxic T cells recognize endogenous antigens presented by MHC class I and mediate the cytotoxic immune response, as for instance the elimination of virus-infected cells (Pamer et al., 1998). Regulatory T helper (Treg) cells suppress the immune response and are vital to protect the organism from the aggressive effector mechanisms of the immune system.

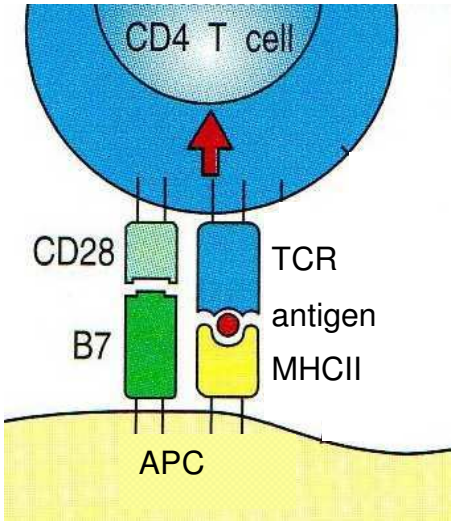


FIGURE 1.1: APC:T cell interaction. The MHCII molecule on the APC presents the antigen to the T cell receptor (TCR). Together with the B7:CD28 binding, APCs can activate T cells and induce the clonal expansion of T cells (modified from Janeway et al., 2005).

Extracellular pathogens and proteins internalized by APCs are either recycled to the plasma membrane or transported to the lysosome for fragmentation and then presented via MHC class II (MHCII) to $CD4^+$ T cells to induce an immune response (Janeway et al. 2005; Watts, 2004). Proteins are enclosed by endosomes, which become increasingly acidic as they progress from early to late endosome while travelling into the interior of the cell and eventually fuse with the lysosome. Proteases as well as glycosidases and lipases are present in endolysosomes and are activated by low pH yielding protein degradation into peptides. Cells can also generate an oxidative burst, including the production of nitric oxide (NO). NO and potentially other oxidants process the antigen to low molecular weight (MW) peptides. In order to

present the antigen to a $CD4^+$ T cell, the peptide needs to be loaded onto the MHCII molecule (Smith et al., 1998; Jardetzky et al., 1990). On the contrary to MHCI, the MHCII molecule is only present on professional APCs. The MHCII molecule consists of a noncovalently bound complex of two transmembrane glycoprotein chains, the α and β chains. The MHCII molecule possesses - on the contrary to the MHCI molecule - open ends on the peptide-binding groove. An uncontrolled early binding is prevented by the invariant chain which fills and blocks the peptide binding groove of MHCII as well as targets it to an acidic endosomal compartment. The MHCII:invariant chain complex enters the acidic endosomal pathway where activated proteases cleave the invariant chain leaving a short fragment of it, called CLIP (class II-associated invariant-chain peptide). Intracellular antigen loading of MHCII is dependent on HLA-DM, a MHCII-like molecule in humans (H-2M in mice), which is found predominantly in the MHCII compartment (MIIC) (Denzin and Cresswell, 1995). It first binds to the MHCII molecule, catalyzes the release of the CLIP fragment from the MHCII binding groove, stabilizes the MHCII molecule to prevent aggregation and aids in rapid and tight binding of peptides to the empty MHCII molecule. The MHCII:peptide complex is then transported from the lysosome to the cell surface and the peptide presented to $CD4^+$ T cells (Abbas et al., 2000).

Upon recognition, the $CD4^+$ T cells are specialized to activate other effector cells of the immune system, e.g. macrophages, which are activated to kill the intravesicular pathogens they harbour or B cells to secrete immunoglobulins against foreign molecules. The B-cell antigen receptors are a membrane-bound form of the antibodies, which will be released upon activation, plasma cell differentiation and proliferation of the B cell with a specific antigen. The MHCII:peptide complex must be stable at the cell surface. Otherwise, the pathogen in the infected cell could escape detection or be picked up by MHC molecules of an uninfected cell and be wrongfully destructed by cytotoxic T cells (Janeway et al., 2005).

T cells bear about 30,000 antigen-receptor molecules on their surface (Janeway et al., 2005). These receptors are termed T-cell receptor (TCR). The TCR receptor is a heterodimer and consists of two different polypeptide chains, the TCRα and the TCRβ, which are linked by a disulfide bond. The TCR on their cell surface recognizes processed and presented peptide antigens. These α:β heterodimers have a similar structure to the Fab fragment of an immunoglobulin molecule. They account

for antigen recognition by most T cells (Janeway et al., 2005). About 5 % of the T cells though possess a TCR consisting of γ and δ chains.

1.4 DCs stimulate T cell activation

The mature DCs interact with T cells by forming an immunological synapse made up of MHCII:peptide complexes on the DC and the TCR on the T cells. Other ligand interaction within the synapse are CD40 and CD40 ligand (CD40L), CD80 (B7.1) or CD86 (B7.2) and CD28, DC-SIGN and intercellular adhesion molecule 3 (ICAM-3), ICAM-1 or ICAM-2 to LFA-1, and LFA-3 to CD2, each on the DC and the T cell surface (Steinman, 2000). This specific interaction activates the T cell by intracellular signalling pathways, and induces their differentiation and proliferation. Naïve T cells transiently bind to APCs through low-affinity LFA-1:ICAM-1 interactions for screening the MHC molecules on the APC surface for the presence of specific peptide (Janeway et al., 2005). Once a naïve T cell recognizes its MHC:peptide ligand, signalling through the TCR induces a conformational change in LFA-1, which greatly increases its affinity for ICAM-1 and ICAM-2. The conformational change in LFA-1 stabilizes the T cell:APC association and prolongs cell-cell contact which can persist for several days. During that time, the naïve T cell proliferates and its progeny, which also adhere to the APC, differentiate into armed effector T cells (Janeway et al., 2005). By secreting specific cytokines, DCs can further direct T cell development towards either Th1 or a Th2 phenotype (de Jong et al., 2005). Interaction of immature or tolerogenic DCs with T cells can result in T cell deletion, anergy, or the generation of Treg cells (Roncarolo et al., 2001).

1.5 Superantigens and T cell-independent antigens

In contrast to conventional processed antigens, superantigens do not require internalization for their biological activity. Superantigens are for example, the toxic shock syndrome toxin-1 (TSST1) or staphylococcus aureus enterotoxins. Superantigens can bind to the α chain of MHCII on the APC surface and a specific β-chain of the αβTCR on a $CD4^+$ T cell. Immunoglobulins and TCR are highly variable molecules and possess a variable (V) region that binds to antigens. Bacterial

superantigen toxins hyperstimulate the immune system through the direct activation of APCs and T cells, which possess TCR-specific variable β-chain (Vβ) elements (Marrack and Kappler, 1990). Fragmentation of the superantigens, as in e.g. antigen processing, would only lead to loss of their biological activity. Their biological activity depends on binding as an intact protein to the outside surface of an MHC class II molecule which has already bound peptide (Janeway et al., 2005).

MHCII-mediated $CD4^+$ T cell activation has been considered to be strictly limited to protein antigens (Watts and Powis, 1999). When presented to T cells by MHCII, peptide antigens generally elicit a T cell-dependent immune response typified by the production of Th1 or Th2 cytokines as well as immunoglobulin G (IgG) and the induction of immunologic memory.

In contrast, the immunologic response to polysaccharide antigens is considered T cell-independent and characterized by a primary humoral immune response of IgM production, rare IgG class switching, and no immunologic memory (Lesinski and Westerink, 2001; Abbas et al., 2000). Vaccine research with carbohydrate-protein conjugates has demonstrated that attachment of some polysaccharides or oligosaccharides to immunogenic carrier proteins like tetanus toxoid (TT) can generate an antigen capable of inducing significant IgG production (Kasper et al., 1996), T cell activation, and immune memory specifically directed toward the carbohydrate (Baker et al., 1999).

1.6 Zwitterionic polysaccharides as T cell-dependent antigens

Until recently, MHCII was only known to present protein peptides or superantigens. Indeed, most bacterial polysaccharides usually find their end station in lysosomes of antigen-presenting cells (APCs) without further processing or presenting on the APC surface to T cells (Watts, 2004). Thus, the discovery of the MHCII pathway also being responsible for carbohydrate antigen presentation and ultimately T cell recognition alters the fundamental model of MHCII-antigen binding. Recently, *in vivo* and *in vitro* studies showed that some polysaccharides of certain commensal bacteria are transported from lysosomes to the cell surface where they are presented by MHCII to activate $CD4^+$ T cells *in vitro* and induce $CD4^+$ T cell-dependent abscesses *in vivo* (Velez et al., 2009; Cobb and Kasper, 2008; Stephen et al., 2007; Cobb et al., 2004;

Kalka-Moll et al., 2002). The $CD4^+$ T cell activation by bacterial polysaccharides has been shown to depend on retrograde transport via MHCII tubules of the bacterial polysaccharides. The functional role of HLA-DM helps the bacterial polysaccharide to compete with peptides for MHCII binding (Cobb and Kasper, 2008; Stephen et al., 2007).

Few naturally occurring polysaccharides such as PSA1 and PSA2, Sp1, and CP5 from the commensal bacteria *Bacteroides fragilis*, *Streptococcus pneumoniae* serotype 1 and *Staphylococcus aureus*, respectively, are characterized by opposite charge motifs on each repeating unit and are therefore called zwitterionic polysaccharides (ZPS) (Choi et al., 2002; Wang et al., 2000; Lindberg et al., 1980). This zwitterionic motif is critical for antigenic function of ZPSs. Removal of either a positive or negative group has been demonstrated to result in an immunologically inert carbohydrate unable to stimulate $CD4^+$ T cells (Tzianabos et al., 1993).

1.7 Peritonitis and abscess formation as a T cell-dependent immune response

Secondary peritonitis, which is by far the most common form of peritonitis, results from loss of integrity in the gastrointestinal tract, leading to contamination of the peritoneal space by commensal intestinal bacteria (Farthmann and Schoffel, 1998). Intraperitoneal abscesses develop as a classic host response to bacterial infection that follows colonic leakage of commensal intestinal bacteria. It might serve to localize the pathogens within the peritoneal cavity. Intraperitoneal abscesses can be seen in patients with inflammatory bowel diseases (IBD) such as Crohn's disease or ulcerus colitis. Despite improved diagnostic modalities, potent antibiotics, modern intensive care, and aggressive surgical treatment, up to one third of patients still die from generalized peritonitis (Marshall and Innes, 2003). The cornerstones of successful surgical treatment for generalized peritonitis are thorough peritoneal lavage, drainage of localised abscesses, repairing, which may require removal of the contaminating source, and effective drainage of the peritoneal cavity. However, even with optimal therapy including the administration of antibiotics, residual abscesses form in many patients, resulting in substantial morbidity and mortality (Nichols, 1985).

Abscess formation is known to be a $CD4^+$ T cell-dependent immune response induced by ZPS (Tzianabos et al., 1993). The zwitterionic charge promotes $CD4^+$ T

cell-dependent abscess induction in experimental murine and rat models (Tzianabos et al., 2000b). Chemical abrogation of the zwitterionic charge demonstrated that the zwitterionic nature of ZPS is critical to its ability to induce abscess formation. Elimination of the charged groups on each repeating unit does not induce $CD4^+$ T cell-dependent immune responses *in vitro* and *in vivo* (Tzianabos et al., 1993). Vice versa, uncharged polysaccharides induce T cell-dependent immune responses after they are modified to possess a zwitterionic charge (Tzianabos et al., 2000a, 1994, and 1993). In this study, experiments are performed with the ZPS model antigen Sp1 from *Streptococcus pneumoniae* serotype 1.

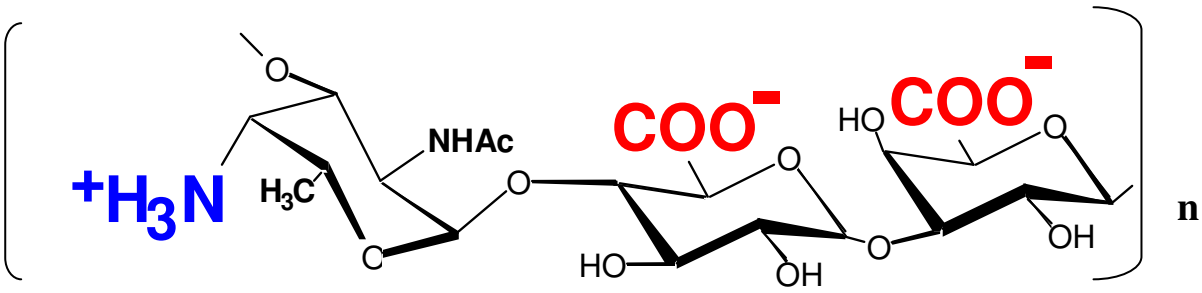
1.8 *Streptococcus pneumoniae*

S. pneumoniae is a spherical sometimes ovoid gram-positive bacterium, which is an oxidase- and catalase- negative, non-sporing, non-motile, alpha-hemolytic, and aerotolerant anaerobe diplococcus (Cowan and Steel, 1993). It is a significant human pathogen as it is a major cause of pneumoniae. Other infections caused by *S. pneumoniae* are meningitis, otitis media, bacteraemia, endocarditis but also brain abscesses as well as peritonitis. In humans, *S. pneumoniae* serotype 1 is predominantly detected in specimens from abscesses located in lungs, muscles and soft tissue (personal communication with R. R. Reinert, National Reference Laboratories for *Pneumococci*, Aachen). The capsule is the main virulence factor which permits also a discrimination of upto 91 different known serotypes till date. Sp1, the zwitterionic polysaccharide used in this study, is an integral part of the capsule of *S. pneumoniae* serotype 1.

Two vaccines are available today which confer immunization to the capsular components of *S. pneumoniae*. One is called Pneumovax 23® which is a 23-valent pneumococcal-free vaccine composed of purified polysaccharides derived from the surface capsule of the bacterium (de Roux et al., 2008). These polysaccharide antigens generally elicit T cell-independent immune responses and are recommended for over 2 year-olds (Pebody et al., 2005). The other one is called Prevenar® and is a conjugate vaccine which is recommended for infants and toddlers upto 2 years of age. In this vaccine the capsular polysaccharide is conjugated with a protein to induce a T cell-dependent response against seven serotypes (Bernatoniene and Finn, 2005, Reinert, 2004).

1.9 ZPS and Sp1 structure

Sp1 is a right handed helix with eight residues per turn and a pitch of 20 Å (Choi et al., 2002). Sp1 is a linear polymer of an average molecular size of 70 kDa corresponding to 130 trisaccharide repeating units. Each tri-saccharide unit possesses two negatively charged carboxyl groups and one positively charged amino group which identifies Sp1 as ZPS (Lindberg et al., 1980) (Fig. 1.2).



-3)-α-*D*-AAT-(1-4)-α-*D*-GalA-(1-3)-α-D-GalA-(1-

FIGURE 1.2: Structure of Sp1. The molecular size of one Sp1 trisaccharide unit is 537 Da. Each Sp1 repeating unit contains of one positively charged galactose (2-acetamido-4-amino-2,4,6-trideoxygalactose-AAT) and two negatively charged galacturonic acids (GalA) and illustrate the zwitterionic nature of the polysaccharide (Choi et al., 2002; Stroop et al., 2002; Tzianabos et al., 1993).

1.10 ZPS-mediated $CD4^+$ T cell activation

Sp1 is endocytosed, processed and loaded onto newly synthesized MHCII molecules by means of HLA-DM (Cobb et al., 2004). The MHCII:Sp1 complex is transported via tubuli in DCs from the lysosomal compartment to the cell surface where the Sp1 is presented to T cells to induce a $CD4^+$ T cell-dependent immune response (Stephen et al., 2007) (Fig. 1.3).

Direct T cell-interaction with the MHCII of APCs is essential for a ZPS-mediated $CD4^+$ T cell proliferation (Kalka-Moll et al., 2002). The high density of charge on Sp1 molecules facilitates electrostatic interactions between APCs and T cells (Choi et al., 2002). ZPS promote $CD4^+$ T cell proliferation via the first signal which is mediated by the MHCII molecule on the APC and initiated by the TCR and the CD3 molecule on the T cell. Essential is also the second signal that is provided by the co-stimulatory molecules B7.2 (CD86) and CD40 of the same activated APCs to CD28 and CD40L

of the same T cell (Stephen et al., 2005). ZPS requires these co-stimulatory molecules to promote intra-abdominal abscess formation through $CD4^+$ T cell activation (Tzianabos et al. 2000b; Stephen et al., 2005). Abscess formation in response to ZPS failed in animals lacking $\alpha\beta CD4^+$ T cells (Chung et al., 2003). The CD40L interaction on T cells with the CD40 on APC is critical to elevate expression of co-stimulatory molecules and induces the expression of a variety of cytokines.

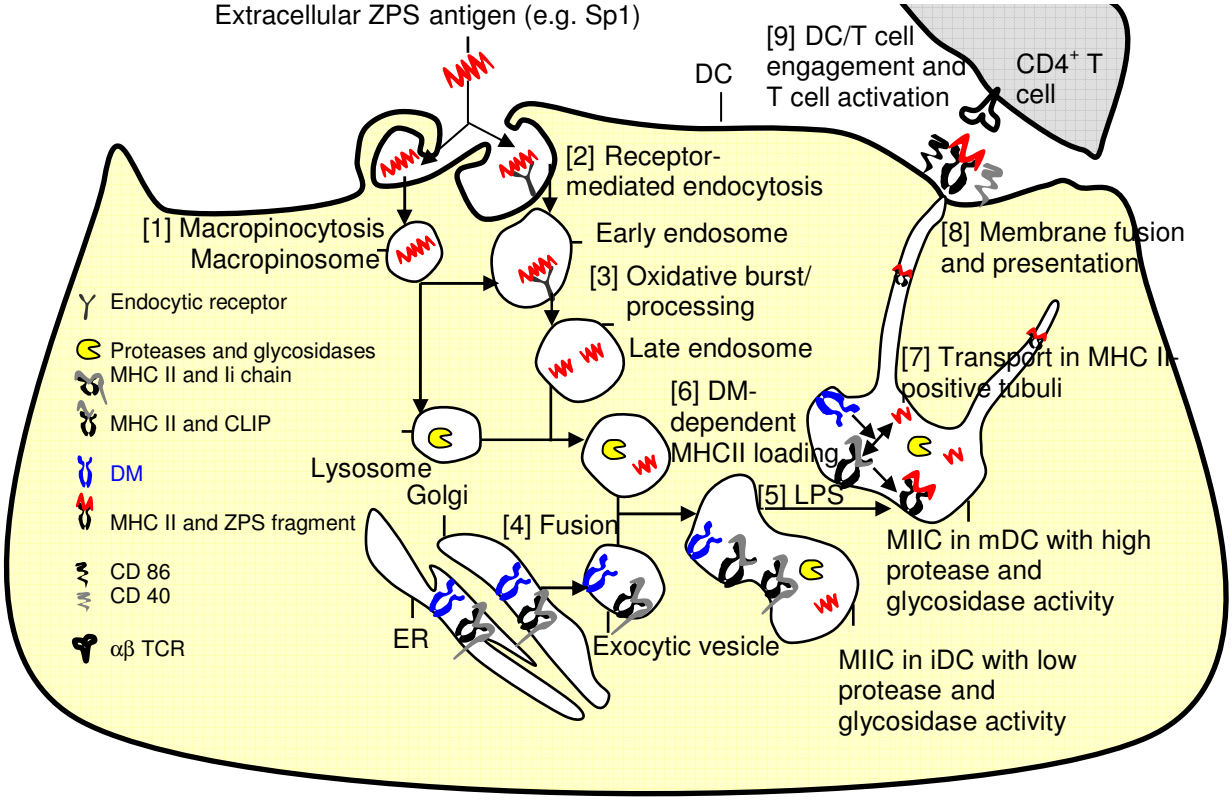


FIGURE 1.3: Model of Sp1 internalization, processing and presentation to $CD4^+$ T cells. [1], [2] ZPS is internalized by DCs either by macropinocytosis or receptor-mediated endocytosis. [3] ZPS-internalization induces an oxidative burst in the DC that is required for the processing of ZPS antigens to fragments. [4] The late endosome containing the ZPS fragments fuses with the MHCII- and DM-containing exocytic vesicle. [5] DC maturation is induced by for example LPS and leads to high protease and glycosidase activity in the MIIC. [6] DM catalyses the exchange of CLIP for the ZPS fragment antigen contained within the MIIC. [7] The MHCII:ZPS fragment complex is transported via tubuli from the endolysosomal compartment to the cell surface. [8] After the membrane fusion of the tubuli, the antigen is presented to the T cell where [9] then the DC/T cell engagment and T cell activation occur (Cobb et al., 2004, Stephen et al., 2007, Cobb and Kasper, 2008).

1.11 Cytokines involved in T cell-dependent immune responses

Cytokines gain increasing importance in $CD4^+$ T cell-induced abscess formation. It has been suggested that cytokines may be responsible for triggering the migration of immune cells into the peritoneal cavity following contamination with *B. fragilis* (Gibson et al., 1996). Cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, IL-12, IL-15, and IL-18, are involved in T cell activation and proliferation (Banchereau et al., 2000). Gibson et al. (1998) demonstrated that ZPS PSA1 induces the production of TNF-α and IL-1α on peritoneal cells which act as significant immune mediators for abscess formation. If TNF-α is blocked, expression of the ICAM-1 is significantly reduced so that accumulation of polymorphonuclear leukocytes (PMNLs) within the abdominal cavity, the hallmark of peritoneal sepsis, is inhibited. Furthermore, PSA1 stimulates the secretion of chemokines, such as IL-8, from T cells (Tzianabos et al., 2002). In mice challenged with whole *B. fragilis*, $CD4^+$ T cells mediate abscess formation by an IL-17-dependent mechanism (Chung et al. 2003). Treatment of mice with an IL-17-neutralizing antibody significantly inhibited abscess formation.

During sepsis and peritonitis, along with TNF and IL-1, IL-6 is also an important mediator of immunologic alterations in the host (Ertel et al., 1991; Shalaby et al., 1989). High titres of IL-6 result from complex amplification mechanisms involving IL-1 and TNF (Xu et al., 2007; Brouckaert et al., 1993). Evidence suggests that IL-6 is the most potent of the proinflammatory cytokines because it stimulates the expression of the broadest spectrum of acute phase proteins in inflammation and infection (Gomez et al, 2006; Helle et al., 1988). IL-6, a pleiotropic inflammatory cytokine, is produced by a variety of cells and acts on a wide range of tissues (Hirano, 1998). It stimulates the activation, survival, proliferation of $CD4^+$ T cells and acts on T cells as an anti-apoptotic factor (Takeda et al., 1998; Teague et al., 1997; Van Snick, 1990; Lotz, et al. 1988). IL-6 prevents apoptosis of normal and resting T cells in the absence of additional cytokines. IL-6 has also been identified as a migration factor for human primary T cells (Weissenbach et al., 2004). *In vitro* experiments showed chemotactic activity of IL-6 on peripheral blood lymphocytes, lymphokine-activated killer cells, $CD4^+$ and $CD8^+$ T lymphocytes (Cao et al., 1998; Delens et al., 1994; Bacon et al., 1990). Additionally, IL-6 has been linked to T cell-dependent immune diseases and

autoimmune diseases such as the inflammatory bowel diseases, ulcerative colitis and Crohn's disease (Mitsuyama et al., 2006; Van Kemseke et al., 2000).

1.12 Toll-like receptor signalling and adapter molecules

The innate immune system provides an immediate but non-specific defence against pathogens by recognizing specific pathogen-associated molecular patterns (PAMPs) (Kawai and Akira, 2007; Janeway, 1989). The innate immune system recognizes PAMPs by means of pattern-recognition receptors (PRR) (Sanjuan et al., 2009; Ishii et al., 2008). Such PRRs include the Toll-like receptors (TLR), homologues of the *Drosophila* Toll gene (Pasare and Medzhitov, 2004).

Until now, 13 murine TLRs and 10 human TLRs have been identified of which each recognizes several different PAMPs (Kumagai et al., 2008; Pasare and Medzhitov, 2004). TLRs that reside extracellularly on the cell membrane or phagosomes are e.g. TLR1/2 or 2/6, TLR4, TLR5 and TLR11, which recognize proteoglycans or lipopeptides of gram-positive bacteria, lipopolysaccharides (LPS) from gram-negative bacteria, flagellin and propellin, respectively. TLR3, TLR7/8 and TLR9 are expressed exclusively intracellularly in endosomes and recognize nucleic acids: dsRNA, ssRNA and ssDNA (containing CpG motifs), respectively (Kumagai et al., 2008; Uehara and Takada, 2007). An overview for the common TLRs and their ligands and adapter molecules are given in Fig. 1.4.

All TLRs elicit conserved inflammatory pathways. The proximal events of TLR-mediated intracellular signalling are initiated by Toll/interleukin-1 receptor (TIR)-domain-dependent heterophilic interactions with TIR-domain-containing adapters. These cytoplasmic adapters, which regulate TLR-mediated signalling pathways by providing specificity for individual TLR downstream signalling cascades are myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adapter protein (TIRAP)/Mal, TIR domain-containing adapter inducing IFNβ (Trif) and Trif-related adapter molecule (TRAM) (Takeda and Akira, 2005; Akira and Takeda, 2004; Martin and Wesche, 2002). TLR signalling can occur either in a MyD88-dependent (TLR1, 2, 4, 5, 6, 7, 8, 9, 11) or in a MyD88-independent (TLR3, 4) manner. TLR4 can activate NF-κB via the MyD88-dependent pathway as well as the TRIF-dependent (MyD88-independent) signalling pathway. TLR2 and TLR4 recruit TIRAP

and MyD88 for the MyD88-dependent pathway and TLR4 recruits TRAM and Trif in the MyD88-independent pathway.

The triggering of TLRs (e.g. TLR4) on DCs leads to the induction of co-stimulatory signals (e.g. MHCII, CD80 and CD86) and to the production of cytokines (e.g. IL-6) that orchestrate T cell recruitment, activation, differentiation and survival and are essential for the induction of adaptive immune responses (Ulevitch and Tobias, 1995; Yamamoto et al., 2002; Pasare and Medzhitov, 2003, Kubo et al., 2004; Liang et al., 2004; Yang et al., 2004).

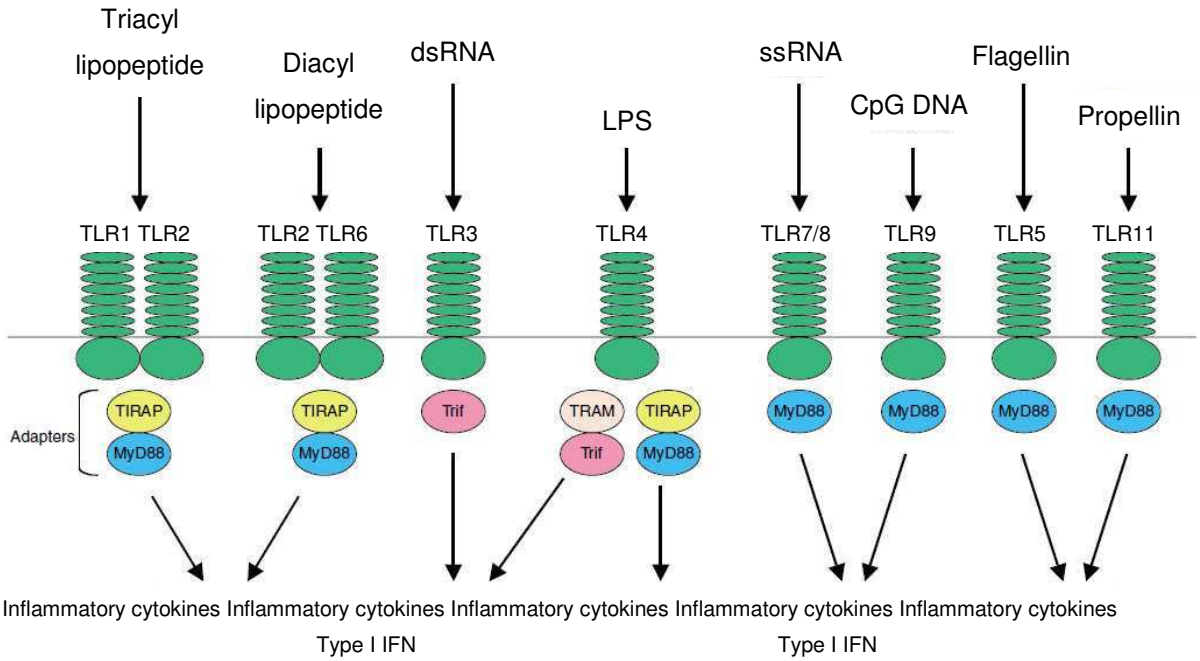


FIGURE 1.4: Toll-like receptors. Extracellularly located TLRs are TLR1/2 or TLR2/6, TLR4, TLR5, and TLR11 and recognize lipopeptides, LPS, flagellin and propellin, respectively. Intracellularly located TLRs are TLR3, TLR7/8 and TLR9 and recognize dsRNA, ssRNA and CpG DNA, respectively. All TLRs, except TLR3 mediate MyD88-dependent signalling (modified from Kawai and Akira, 2006).

1.13 NF-κB activation and translocation

Upon TLR stimulation with ligands, MyD88 death domain interacts with the death domain of IL-1 receptor-associated kinase (IRAK) (Kawai and Akira, 2007). IRAK is phosphorylated and associates with TNF receptor-associated factor 6 (TRAF-6), which activates transforming growth factor-β (TGFβ)-activated proteinkinase 1

(TAK1) in an ubiquitin-dependent manner (Chen, 2005). TLR-signalling leads mainly to the activation of the transcription factor “nuclear factor of ‘kappa-light-chain-gene-enhancer’ of activated B-cells” (NF-κB) which induces specific gene transcription (Kawai and Akira, 2006, Moynagh, 2005).

The NF-κB family comprises out of five members: RelA (p65), RelB, c-Rel, p105 (NF-κB; a precursor of p50) and p100 (NF-κB2; a precursor of p52) (Karin and Greten, 2005). NF-κB proteins form homo- or hetero-dimers. Under non-stimulated conditions, NF-κB is inactive in the cytoplasm and bound to family members of inhibitory proteins known as IκB proteins (Kawai and Akira, 2007). The IκB kinase (IKK) complex consists of IKKα and IKKβ protein kinases and a regulatory molecule, IKKγ/Nemo. TAK1 activates the IKK complex that leads to NF-κB activation (Fig. 1.4). Upon stimulation with various TLR ligands, IKK complex phosphorylate e.g. IκB-α, at serine residues. Phosphorylated IκB-α is then degraded via the proteosomal pathway, releasing the NF-κB dimers as for e.g. p65 to translocate from the cytoplasm to the cell nucleus (Kawai and Akira, 2006). A TRAF-6-mediated activation of NF-κB is required for the transcription of inflammatory cytokines (Fig. 1.5).

Though many TLR ligands have been identified so far, which contain carbohydrate moieties such as LPS, so far the noncarbohydrate portion of the molecules seemed critical for TLR ligation and activation (Alexander and Rietschel, 2001). Only recently, pure carbohydrates have been suggested as potential TLR ligands and to activate TLR signalling. Polysaccharides from cell culture of *Acanthopanax senticosus* in particular leads to APC activation via TLR (Han et al., 2003). More interestingly, the *B. fragilis* ZPS PSA1 has been demonstrated to stimulate APCs through a TLR2-dependent mechanism. TLR2 was revealed to be the receptor for PSA to activate NF-κB and hence lead to co-stimulatory molecule expression as well as cytokine production (Wang et al., 2006). This study investigates further TLRs for ZPS-mediated immune responses.

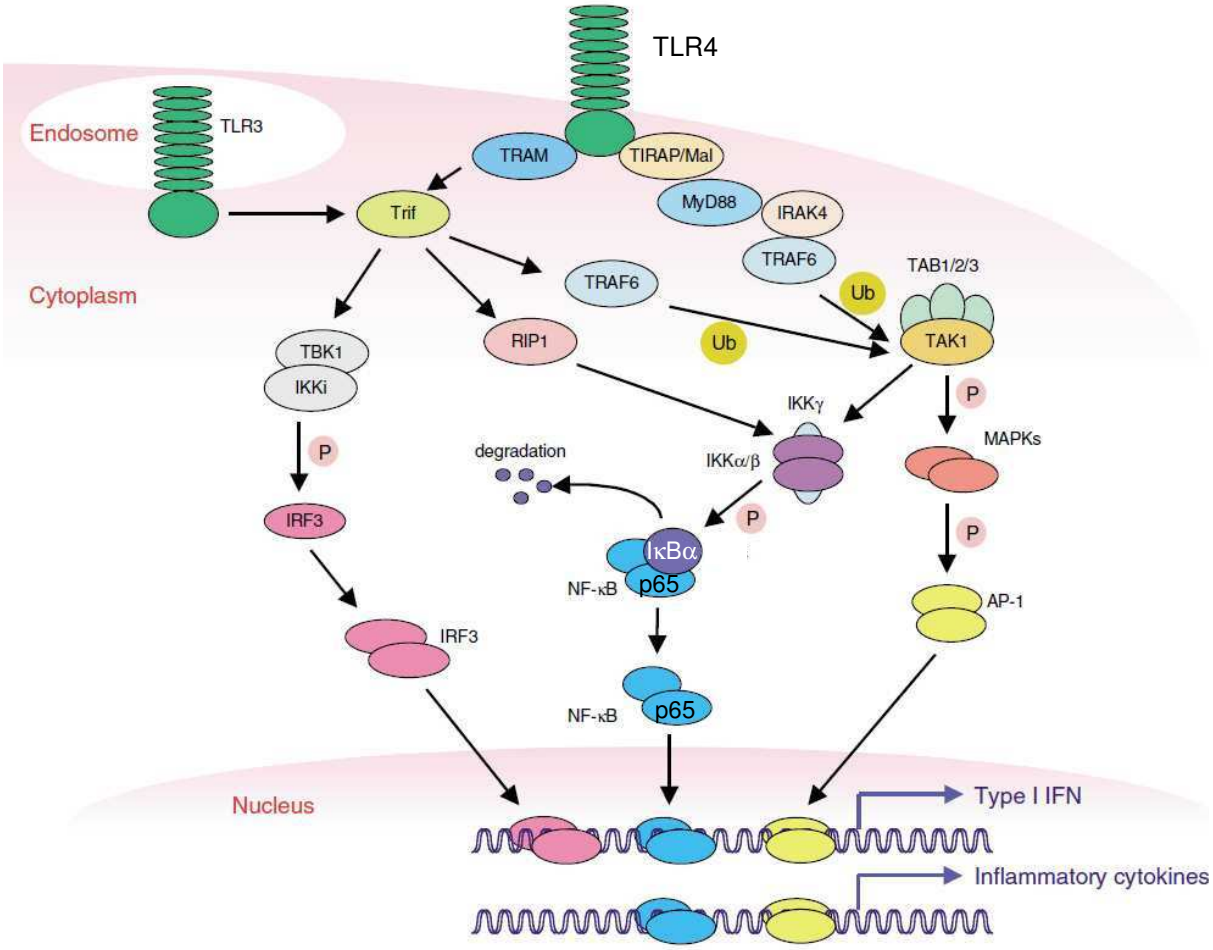


FIGURE 1.5: NF-κB activation and translocation. TLR4 activates the MyD88-dependent or the Trif-dependent pathway. In the Myd88-dependent pathway, TRAF-6 activates TAK1, which then activates the IKK complexes or the mitogen-activated protein (MAP) kinases. IKK complex leads to ubiquitination and phosphorylation of IκB-α, which releases NF-κB subunits, e. g. p65 to translocate to the nucleus. MAP kinases activate first JNK, p38 and ERK to then activate AP-1. In the Trif-dependent pathway, TLR4 recruits TRAM, which interacts with TBK1 which together with IKKi mediates phosphorylation of IRF3. Activation of IRF3, NF-κB and AP-1 is required for induction of type I IFN, particularly IFNβ. TLR3, which resides in endosomal vesicles, utilizes Trif but not MyD88, TIRAP or TRAM for its signaling (modified from Kawai and Akira, 2006).

1.14 Aim of the present study

Microbial glycobiology is still a vast field of many unanswered questions, especially when it comes to ZPS which have the potential to induce $CD4^+$ T cell-dependent immune responses *in vitro*. ZPS are also capable of inducing abscess formation *in vivo* in a $CD4^+$ T cell-dependent manner (Stephen et al., 2007; Cobb et al., 2004; Kalka-Moll et al., 2002; Tzianabos et al., 1993).

To date, very little is known about the role of IL-6 in Sp1-mediated abscess formation *in vivo* and $CD4^+$ T cell responses *in vitro*. The purpose of this study is to shed light on the IL-6 secretion following Sp1 challenge and its involvement in abscess formation. Professional APCs will be tested for their ability to internalize Sp1 and to secrete IL-6 upon Sp1 uptake. The effect of IL-6 on Sp1-induced T cell migration and T cell apoptosis will be explored. Further, Sp1 will be tested as a TLR ligand to activate NF-κB and IL-6 expression.

2. Materials and Methods

2.1 Materials

2.1.1 Consumables

Item	Company, Place
15 mL polypropylene test tubes, Cellstar	Greiner Bio-One, Frickenhausen
50 mL polypropylene test tubes, Cellstar	Greiner Bio-One, Frickenhausen
Cannula (sterile)	
26 G	Terumo Neolus, Belgium
20 G, 27 G	BD Microlance3, Spain
Cell strainer (sterile), 40 µm, 70 µm, 100 µm	BD Falcon, Heidelberg
Cell scraper (25 cm)	Sarstedt, Nümbrecht
Combitips plus	Eppendorf, Hamburg
Cover slips (glass) ∅12 mm, ∅ 18 mm	Menzel, Braunschweig
Cover glasses for Haemocytometer	Superior Marienfeld Laboratory Glassware, Lauda-Königshofen
Cryo vials (sterile) 1.8 mL, Cellstar	Greiner Bio-one, Frickenhausen
Eppendorf tubes, 0.5 –2.0 mL	Sarstedt, Nümbrecht
FACS tubes	BD Falcon, Heidelberg
Filters (sterile), 0.45 µm, 0.22 µm	Nunc, Langenselbold
Lab-Tek Slide Culture Chambers	Nunc, Langenselbold
Lab-Tek®II Chamber #1.5 German Coverglass System	Nunc, Langenselbold
MatTek Poly-D-lysine-pre-coated 1.5 coverslips glass bottom plates	MatTek, USA
Microscope slides	Engelbrecht, Edermünde
Microtitre plates, 96 wells, non-sterile	Nunc, Langenselbold
Neubauer chamber/Haemocytometer	Brand, Wertheim
Parafilm M	Brand, Wertheim
Pasteur pipette (230 mm)	Volac, Wertheim
Petri Dish, 90 mm	Nunc, Langenselbold

Consumable	Company, Place
Plastic cuvettes	Sarstedt, Nümbrecht
Transfer pipettes (sterile)	Sarstedt, Nümbrecht
5 mL, 10 mL, 25 mL, and 50 mL	Sarstedt, Nümbrecht
Sealing tape	Dynex, Frankfurt
Syringes (sterile), 1 mL, 5 mL, 10 mL	BD Falcon, Heidelberg
Syringes, Micro-fine (sterile), 1 mL, 29 G, U-100	BD Falcon, Heidelberg
Steriflip, 0.2 µm pore size	Millipore, Schwalbach
Tissue culture plates	
6 wells, 12 wells, 24 wells, 96 wells	Nunc, Langenselbold
Tissue culture dishes	Nunc, Langenselbold
Tissue culture flasks	Nunc, Denmark

2.1.2 Instruments

Item	Company, Place
Autoclave	Fritz Gössner GmbH, Hamburg
Blotting chamber Criterion, Electroporation unit	Biorad, Munich
Centrifuge	
Multifuge 3 S-R	Heraeus, Hanau
Multifuge 4KR	Heraeus, Hanau
Biofuge fresco, Table top	Heraeus Instruments, Hanau
Centrifuge-rotors, max. 4600 rpm	Heraeus, Hanau
Conductivity measurement device, Cond 340i	WTW, Weilheim
Electrophoresis power supply, PowerPac-300	Biorad, Munich
Fluorescent Activated Cell Sorter	
(FACS System) Calibur	BD, Heidelberg
Fast Protein Liquid Chromatography ÄKTA(FPLC)	Amersham Biosciences, Munich
Fraction collector, Frac-950	Amersham Biosciences, Munich
Freezer (-20°C) Liebherr Premium	Liebherr, Ochsenhausen
Freezer (-80°C), Hera Freeze, Kendro	Heraeus, Hanau
Fridge	Liebherr, Ochsenhausen

Instrument	Company, Place
Gel-documentation unit, Gel Doc 1000	Biorad, Munich
Ice machine	Ziegra, Isernhagen
Incubators	
CO2 incubators, Thermo Scientific Forma	Thermo, Langenselbold
Incubator with shaker, Innova 4200	New Brunswick Scientific, USA
IR lamp, Clatronic	LGA, Nürnberg
Laminar flow, LaminAir Holten, Thermo Scientific	Thermo, Langenselbold
Liquid nitrogen tank, Arpege 170	AirLiquide, Langenfeld
Lyophiliser, Lyovac GR2	SRK Systemtechnik, Riedstadt
Magnetic stirrer, IKA, Combimag, RCH	Janke & Kunkel AG, Staufen
Magnet for cell isolation	
MidiMacs	MiltenyiBiotec, Bergisch-Gladbach
EasySep	StemCell, Cologne
Microscope	
Light microscope (cell count), Axiovert-25	Zeiss, Cologne
Fluorescent microscope Olympus AH2 & IX81	Olympus, Hamburg
Confocal laser scanning microscope FV1000	Olympus, Hamburg
Micropipette	
0.2 - 2 µL, 2 - 20 µL, 20 - 200 µL, 100 - 1000 µL	Thermo LabSystems
Multipipette plus	Eppendorf, Hamburg
NANOpure Diamond Reinstwassersysteme	Werner, Barnstedt
NMR DRX500	Bruker Biospin, Switzerland
Orbital Shaker, MR3000	Heidolph, Schwabach
pH-meter 766 Calimatic	Knick, Zweibrücken
Pipette boy, Accujet	Brand, Wertheim
Refractive Index (RI) Detector Varian 356LC	Varian, Darmstadt
Rotator, Heidolph Reax-2	Heidolph, Schwabach
Scales, Sartorius Laboratories	Sartorius, Heidelberg
Special accuracy weighing machine, Genius	Sartorius, Heidelberg
Spectral photometer, Genesys 20	Thermo, Langenselbold
Stirred Ultrafiltration Cell (Amicon)	Millipore, Schwalbach
Thermo mixer comfort, heating block	Eppendorf, Hamburg
Vacuum pump, Model RV3	Edwards, Kruetzwertheim

Instrument	Company, Place
Vortex, Genie 2	Scientific Industries, USA
Water bath, GFL 1086	GFL, Burgwedel

2.1.3 Buffers, media and solutions

All buffers and solutions were prepared or diluted with either Nanopure water, sterile aqua ad iniectibilia or PBS.

Item	Company, Place
Aqua ad iniectabilia	Delta Select, Pfullingen
Phophate buffered saline-Dulbecco (PBS)	Biochrom AG, Berlin
VLE (very low endotoxin)-RPMI 1640 Medium	Biochrom AG, Berlin

2.1.4 Chemicals and reagents

Item	Company, Place
Complete protease inhibitor cocktail tablet	Roche, Switzerland
DTT(1,4 Dithio-DL-threitol)	Fluka, Sigma, Steinheim
EDTA(Ethylenediaminetetraacetic acid)	Sigma, Steinheim
EGTA (Ethyleneglycol bis (2-aminoethyl) tetraacetic acid	Roth, Karlsruhe
Formaldehyde	Roth, Karlsruhe
Glycerol	Merck, Darmstadt
Hepes	Gibco, Karlsruhe
$MgCl_2$	Sigma, Steinheim
NaCl	Roth, Karlsruhe
NaF	Merck, Darmstadt
$NaVo_3$	Roche, Switzerland
Nonidet P40	Fluka, Steinheim
PMSF (Phenylmethylsulfonyl fluoride)	Roche, Mannheim
Tween®20 (Polyoxyethylenesorbitanmonolaurate)	Sigma, Steinhein

2.1.5 Purification of *Streptococcus pneumoniae* type 1 capsular polysaccharide (Sp1)

Item	Company, Place / Composition
Software:	
BCA assay	Revelation Version 4.06
FPLC	UNICORN™ 3.2
2 M NaOH	
2 M Acetic acid	
Phenol (90%)	Fluka, Steinheim
H_2SO_4 (96%)	Merck, Darmstadt
10,000 Da MW cut-off membrane (Amicon)	Millipore, USA
BCA test kit	Pierce, Rockfort, USA
BCA plate reader, MRX Tc Revelation	Dynex Technologies, Berlin
Blood agar plate, Heipha Dr. Müller GmbH	Heipha, Eppelheim
Chromic sulphuric acid	Riedel-de-Haen, Seelze
D_2O (Heavy H_2O for NMR)	Deutero GmbH, Kastellaun
Dialysis buffer, pH 7.3:	0.05 M PBS with 0.05% NaN_3
LAL test	Charles River, Charlston, USA
Lyophilisate of *S. pneumoniae* serotype 1, capsular polysaccharide	ATCC®, Mansas, USA
Nitrogen flask	Linde, Vulkan, Pullach
Sodium azide (NaN_3) (0.05%)	Fluka, Steinheim
Sephacryl® S-400	Pharmacia Biotech, Goettingen
Ultrafiltration cell	Amicon

2.1.6 Labelling and modification of Sp1

Item	Company, Place
Acetic anhydride	Sigma-Aldrich, Steinheim
Alexa Fluor® 488-Hydrazide	Molecular Probes, Leipzig
Alexa Fluor® 594-Hydrazide	Molecular Probes, Liepzig
Ethylene glycol	Sigma, Steinheim

Item	Company, Place
PD10 column (desalting)	GE Healthcare, Munich
0.1 M sodium acetate buffer (pH 5.5)	
Sodium bicarbonate ($NaHCO_3$)	Biochrom AG, Berlin
Sodium-m-periodate	Sigma, Steinheim
Sodium phosphate buffer (0.1 M, pH 7.0)	

2.1.7 Animals and abscess induction

Item	Company, Place
Anti-mIL-6 purified rat monoclonal IgG_1 (MP520F3)	R&D Systems, Wiesbaden
Ketavet®, API: Ketaminhydrochloride	Pfizer, Berlin
Rompun, 2%, API: Xylazinhydrochloride	Bayer, Leverkusen
SCCA (Sterile caecal content adjuvant)	caecal contents of C57BL/6 mice
Mice:	
C57BL/6 wildtype mouse	Charles River, France
IL-6-deficient mouse	Bluethmann, Switzerland
TLR2-deficient mouse	Kirschning, Munich
TLR4-deficient mouse	Kirschning, Munich
MyD88-deficient mouse	MPI, Freiburg

2.1.8 Intracellular cytokine staining, flow cytometry and fluorescence microscopy

Item	Company, Place / Composition
Software:	
FACS	CELLQuest™Pro, BD
Fluorescence and light microscopy	CellP Soft Imaging System
Confocal microscopy	FluoView1000, FV10
Binding buffer (Apoptosis assay)	0.1 M Hepes/NaOH (pH 7.4)
	1.4 M NaCl
	25 mM CaCl
Cytofix/Cytoperm™ (ICS)	BD Biosciences, Heidelberg

Item	Company, Place / Composition
FACS buffer	PBS with 10% FBS
FACS fix (to fix cells, if delay in FACS analysis):	PBS 1% FBS 2.5% Formaldehyde
FACSRinse, FACSFlow, FACSClean	BD Biosciences, Belgium
Perm/Wash™ Buffer (ICS)	BD Biosciences, Heidelberg
Staining Buffer (ICS)	PBS (100 mL) 1% FBS (1 mL) 0.09% Sodium Azide (NaN_3)
Sodium hypochlorite solution	Roth, Karlsruhe
Starvation buffer (Apoptosis assay)	RPMI-1640 supplemented with 0.1% FBS
Apoptosis Detection Kit	BD Pharmingen, Heidelberg
Propidium iodide	
Annexin V FITC conjugated	
Antibodies:	
CD4-APC anti-mouse (clone: RM4-5)	BD Pharmingen, Heidelberg
CD8-APC anti-mouse (clone: 53-6.7)	BD Pharmingen, Heidelberg
CD11b-PE anti-mouse (clone: M1/70)	BD Pharmingen, Heidelberg
CD11b-FITC anti-mouse (clone: M1/70)	BD Pharmingen, Heidelberg
CD11c-PE anti-mouse (clone: HL3)	BD Pharmingen, Heidelberg
CD11c-FITC anti-mouse (clone: HL3)	BD Pharmingen, Heidelberg
CD16/CD32 Fc block anti-mouse (clone: 2.4G2)	BD Pharmingen, Heidelberg
CD45R/B220 PerCP anti-mouse (clone: RA3-6B2)	BD Pharmingen, Heidelberg
IgG1α anti-rat (clone: G28-5)	BD Pharmingen, Heidelberg
IL-6 FITC anti-mouse (clone: MP520F3)	R&D, Wiesbaden-Nordenstadt

2.1.9 Immunohistochemistry

Item	Company, Place / Composition
Avidin Biotin Blocking Kit	Vector Laboratories, Eching
Cryostat CM3050S	Leica Microsystems, Wetzlar
DAB substrate	Vector Laboratorie, Eching
Fast red	Dako, Hamburg
Glass cuvettes	Glassware, Wertheim
Isopentane (2-Methylbutane)	Roth, Karlsruhe
Peroxidase blocking	0.3% H_2O_2 in Methanol
Phosphatase	Dako, Hamburg
Antibodies :	
IL-6, rat anti-mouse (clone : MP5-20F3)	Biosource, Hamburg
F4/80, rat anti-mouse (clone: A3-1)	Serotec, Duesseldorf
CD11c, hamster anti-mouse (clone: HL3)	BD Pharmingen, Heidelberg
Goat anti-hamster IgG:Biotin	Serotec, Duesseldorf

2.1.10 Cell culture and cell counting

Item	Company, Place
2-Mercaptoethanol, 50 mM	Gibco, UK
CD11c (N418) micro beads, mouse	MiltenyiBiotec, Bergisch-Gladbach
DMSO	Sigma, Steinheim
Easy Sep, Mouse biotin selection kit	Stem Cell, Cologne
Erythrocyte lysis	NaCl (0.2%, 1.6%)
Fetal Bovine Serum (FBS) LPS-free Heat inactivated before use, 30 min at 56°C	Gibco, Karlsruhe
Heparin (5000 U)	dilute 1:1000 in PBS
Hepes, 1 M	Gibco, Karlsruhe
Histopaque-1083	Sigma-Aldrich, Steinheim
L-Glutamine, 200 mM (Low Endotoxin, LE)	Biochrom AG, Berlin
LPS, *Escherichia coli* 026:B6	Sigma, Steinheim
Penicillin/Streptomycin (10,000 U)	Biochrom AG, Berlin

Item	Company, Place
Sodium Pyruvate, 100 mM	Gibco, Karlsruhe
Trypan blue	Sigma, Steinheim
Antibodies:	
CD4 anti-mouse APC (clone: RM4-5)	BD Pharmingen, Heidelberg
CD8 anti-mouse APC (clone: 53-6.7)	BD Pharmingen, Heidelberg
CD34 biotin	eBioscience, Cologne
Cells:	
Bone marrow-derived dendritic cells	
Bone marrow-derived macrophages	
Lavage cells	
Splenocytes	
$CD4^+$ T cells	
Peritoneal macrophages	

2.1.11 ELISA and nuclear extraction

Item	Company, Place / Composition
Software:	
Flow Cytomix Pro 1.0 Software BMSFFS/1.0	Bender MedSystems, Vienna
ELISA:	
mIL-6 ELISA	Diaclone, Cologne
Multiplex Bead Assay	Bender MedSystems, Vienna
Trans AM NFκB p65 Activation Assay ELISA	Active Motif, Rixensart
ELISA plate reader, Dyna Tech MR5000	Heidolph GPM, Tangstedt
Methanol (CH_3OH)	J. T. Baker, Netherlands
Modified Sp1	N-acetylated purified Sp1
Nuclear extraction buffers	
Buffer A:	10 mM Hepes pH 7.9
	10 mM KCl
	0.1 mM EDTA
	0.1mM EGTA

Item	Company, Place / Composition
	add fresh:
	1 mM DTT
	1 mM PMSF
Buffer C:	20 mM Hepes pH 7.9
	0.4 M NaCl
	1 mM EDTA
	1 mM EGTA
	add fresh:
	1 mM DTT
TLR ligands:	
Flagellin (TLR5 ligand)	Invivogen, France
Gardiquimod (TLR7 ligand)	Invivogen, France
LPS, Ultrapure *E. coli* 0111:B4 (TLR4 ligand)	Invivogen, France
ODN (TLR9 ligand)	Invivogen, France
Pam3CSK4 (TLR2 ligand)	Invivogen, France
Poly I:C (TLR3 ligand)	Invivogen, France

2.1.12 Migration Assay

Item	Company, Place / Composition
Software:	Wallac 1420 Manager
BSA (Albumin Bovine Fraction)	Serva, Heidelberg
Buffer	PBS with 0.1% BSA
Calcein AM (0.5 mg/mL DMSO)	Invitrogen, Spain
$CD4^+$ T cell Isolation kit, mouse	MiltenyiBiotec, Bergisch-Gladbach
Chemotaxis Plates (5 µm)	Neuro Probe, USA
Cotton swabs (Q-Tips)	
MACS Buffer:	PBS pH7.2
	0.5% BSA
	2 mM EDTA
MACS Multistand	MiltenyiBiotec, Bergisch-Gladbach
MACS Separation Columns	MiltenyiBiotec, Bergisch-Gladbach

Item	Company, Place / Composition
rmIL-6 cytokine	R&D Systems, Wiesbaden
SLC (Secondary Lymphoid Chemokine)	Peprotech, Hamburg
Wallac Victor$^2_{TM}$ 1420 MultiLabel Counter	Wallac, Finland

2.1.13 Genotyping

Item	Company, Place / Composition
Software:	Biorad Quantity one-4.4.4 Basic
50 X TAE buffer pH 8.5	242 g Tris-Base ($C_4H_{11}NO_3$)
	57 mL Acetic Acid
	37 g Na_2EDTA x2H_2O (TitriplexIII)
Agarose UltraPure^{TM}	Invitrogen, Spain
GeneRuler^{TM} 100bp DNA Ladder	Fermentas, St. Leon-Rot
DNeasy Blood & Tissue kit	Qiagen, Hilden
Ethidium bromide	Roth, Karlsruhe
Gel chamber	Biorad, Munich
Geldoc2000	Biorad, Munich
Illustra^{TM} PuReTaq^{TM} Ready-To-Go^{TM} PCR beads	GE Healthcare, Munich
PCR Thermocycler T3 & Tgradient	Biometra, Goettingen
Power Pac3000 Power Supply	Biorad, Munich
Primers	Sigma Genosys

TABLE 2.1: IL-6 primer used for genotyping.

Primer	Sequence	Expected product size
IL-6c-pr1	5'TTCCATCCAGTTGCCTTCTTGG3'	
IL-6c-pr2	5'TTCTCATTTCCACGATTTCCCAG3'	1314 bp (IL-6-/-), 174 bp (WT)
IL-6-neo	5'CCGGAGAACCTGCGTGCAATCC3'	380 bp (IL-6-/-)

2.1.14 Surface expression and T cell proliferation

Item	Company, Place / Composition
γ-Radiator, Biobeam 8000/2000	Gamma-Service Medical GmbH
Antibodies:	
Anti-CD14	
Anti-TLR2	
Anti-TLR4	
Anti-rat IgG FITC (2nd antibody)	Sigma, Steinheim
CD80	BD Pharmingen, Heidelberg
CD86	BD Pharmingen, Heidelberg
HLA-DR	BD Pharmingen, Heidelberg
Mouse IgG1 (clone: P3)	eBioscience, Cologne
Rat IgG2a (clone: R35-95)	BD Pharmingen, Heidelberg
TLR2 anti-mouse, purified (clone: T2.5)	ebioscience, Cologne
TLR4 anti-mouse, purified (clone: MTS510)	BD Pharmingen, Heidelberg

2.1.15 Western blot and whole cell extraction

Item	Company, Place / Composition
ECL Plus Western blotting detection reagents	
Detection Reagent 1 and 2	Amersham Biosciences, UK
Hyperfilm	Amersham Biosciences, UK
Film Developer Curix60	Agfa, Cologne
Fixer and Developer solutions	Agfa, Cologne
Immobilon-pSQ Transfer Membrane (0.2 µm)	Millipore, USA
Loading dye	5 mL Tris (pH 6.8)
	10 mL Glycerol
	20 mL SDS (10%)
	0.1 g Bromophenol blue
LPS *Salmonella abortus equi* (ATCC 9842)	Sigma, Steinheim
Prestained protein marker	Cell Signal, Frankfurt
Semi-dry transfer cell	Biorad, Munich

Item	Company, Place / Composition
SDS PAGE:	
10% Resolving gel	30% acrylamide mix (26.6 mL)
	1.5 M Tris pH 8.8 (20 mL)
	10% SDS (0.8 µL)
	10% APS (0.8 µL)
	TEMED (0.032 µL)
	Water (31.8 mL)
5% Stacking gel	30% acrylamide mix (5.1 mL)
	1 M Tris pH 6.8 (3.675 mL)
	10% SDS (0.3 µL)
	10% APS (0.3 µL)
	TEMED (0.03 µL)
	Water (20.4 mL)
Buffers:	
Antibody and blocking buffer	5% Milk powder
	in 1 x TBS-0.01% Tween 20
RIPA buffer	1 M Hepes (20 mM final conc.)
	5 M NaCl (350 mM final conc.)
	Glycerol (20% final)
	1 M $MgCl_2$ (1 mM)
	500 mM EDTA (0.5 mM)
	0.1 M EGTA (0.1 mM)
	Upto 50 mL with water
	add fresh:
	$NaVo_3$
	NaF
	NP-40
	Protein inhibitor
Transfer buffer	5 x Tris/Glycin (1 L)
	Methanol (1L)
	10% SDS (5 mL)

Item	Company, Place / Composition
	Water upto 5 L
10 x SDS buffer	Glycine (288 g)
	Tris base (60 g)
	SDS (20 g)
	Water upto 2 L
Wash buffer	1 x PBS with 0.1% Tween 20
1° Antibodies: (diluted in antibody buffer)	
Phospho-IκBα (mouse mAb) (1:1000)	Cell Signal, Frankfurt
Total-IκBα (C-21, Rabbit) (1:400)	Santa Cruz, Heidelberg
α-Tubulin (mouse) (1:4000)	Sigma, Steinheim
2° Antibodies: (diluted in antibody buffer)	
Sheep-anti-mouse IgG, HRP linked (1:3000)	GE Healthcare, Munich
Donkey-anti-rabbit IgG, HRP linked (1:3000)	GE Healthcare, Munich
Sheep-anti-mouse IgG, HRP linked (1:3000)	GE Healthcare, Munich

2.2 Methods

2.2.1 Purification of *Streptococcus pneumoniae* type 1 capsular polysaccharide

All instruments and devices used in the antigen purification process were deproteinated by treatment with sulphuric and chromic acid. The material was depyrogenated by heat inactivation for 4 h at 240°C or by treatment with 2 M NaOH. Heat-sensitive material such as plastic tubings of the FPLC and the ultrafiltration stirred cell (Amicon) were cleansed with 2 M NaOH-solution and pyrogen free water and subsequently sterilized in 4% formaldehyde. Buffer and solutions were sterile filtered through 0.22 µm-filter units.

Pneumococcal polysaccharide powder which contains the purified capsular components of *S. pneumoniae* type 1 is an intermediate product in the production of the 23-valent polysaccharide-vaccine against pneumococci from Merck & Co. (Whitehouse Station, New Jersey USA) and is marketed by the ATCC. The aim of the purification was to obtain the zwitterionic polysaccharide Sp1 from the whole capsular polysaccharide complex of *S. pneumoniae* obtained from ATCC and free it from C-substance, proteins and nucleic acid.

2.2.1.1 Base treatment to separate the C-substance

Base treatment with 2 M NaOH cleaved the phospho-diester bonds of the C-substance so that the low molecular weight fragments of the C-substance can be separated from Sp1 by the subsequent gel filtration chromatography. *S. pneumoniae* capsule lyophilisate (200 mg) was dissolved in 2 M NaOH (10 mg/mL) under vortexing, aliquoted into 2 mL Eppendorf tubes and incubated at 80°C for 1 h. Aliquots were then pooled and neutralized (pH 7) with 2 M acetic acid. The solution was ultrafiltrated in Amicon® stirrer cells fitted with a 10,000 Da cut-off membrane. The units were kept at 4°C and under 1.5 bar nitrogen pressure. Molecules larger than 10,000 to 30,000 Da were retained while smaller molecules were removed. By ultrafiltration, the solution was concentrated to a smaller volume. Simultaneously the ultrafiltration aided the filtrate to reach the same conductivity as the 0.05 M PBS-NaN_3 buffer, which was also used for equilibrating the FPLC. The concentrated supernatant was collected and filtered through a 0.22 µm sterile filter prior to loading onto the FPLC.

2.2.1.2 Gel filtration chromatography to separate Sp1 from C-substance and other contaminants

Gel filtration chromatography was performed using Sephacryl S-400 high resolution columns. Sephacryl High Resolution is a composite medium prepared by covalently cross-linking ally dextran with N, N'-methylene bisacrylamide to form a hydrophilic matrix of high mechanical strength and broader fractionation range. The principle of gel filtration chromatography is that smaller molecules such as the C-substance will be retained longer in the matrix of the chromatography column as they require longer to diffuse in and out of the interspaces. Larger molecules such as Sp1 with an average size of 70 kDa, however, pass faster through the gel bed. Molecules can thereby be separated according to the molecular weight and size.

The S-400 HR column was connected to the semi automated ÄKTA FPLC system run by the UNICORN^{TM} software. Sterility of the system was tested by incubating the run-through buffer on blood agar plates. Conductivity and refraction index were recorded throughout the run by the RI detector to detect polysaccharide contents in the solution. The polysaccharide solution with a volume of 50 mL was loaded and eluted with 500 mL buffer at a flow rate of 1.5 mL/min and collected in 4 mL fractions.

2.2.2 Analysis of purified Sp1

2.2.2.1 Phenol/sulphuric acid assay for determination of carbohydrate content

Fractions were additionally analysed for polysaccharide content by the phenol-sulphuric acid method. Reaction of phenol and sulphuric acid with polysaccharide yield a yellow-brownish colour development. For this test, 200 µL of polysaccharide sample were mixed with 500 µL sulphuric acid and 5 µL phenol and swayed after 10 min to check for a colour development. Positive control was sucrose and water was used a negative control. Fractions showing yellow-brownish colour development and were detected positive by the RI measurements were pooled together. The polysaccharide solution was dialysed and concentrated against water by Amicon® stirrer cells until the conductivity was equal to that of water (around 1 µSiemens). Carbohydrate-containing fractions were filtered through 0.22 µM sterile-flipped, lyophilized, and stored at 1 mg/mL (in PBS). Samples were checked for the presence of protein, LPS and Sp1.

2.2.2.2 Bicinchoninic acid test for determination of protein contamination

The principle of the bicinchoninic acid (BCA) test is that peptide bonds in proteins reduce Cu^{2+} from the cupric sulphate to Cu^{1+} whereby colour change reactions from green to purple are proportionally stronger to increasing protein concentrations (Levin and Brauer, 1951). BSA was used as a positive and water as a negative control. BSA was prepared as a 1:2 dilution series from a fresh 2 mg BSA/mL stock solution for standardization and quantification of protein measurements. The assay was performed according to the manufacturer's instructions.

2.2.2.3 Limulus amoebocyte lysate test for determination of endotoxin contamination

The Limulus amoebocyte lysate (LAL) is a sensitive method to measure endotoxin. It is based on the principle that endotoxin produces gelation in LAL that is readily recognized macroscopically (Hochstein and Seligmann, 1979). The test was performed in 96-well microtitre plates with serial dilutions of the standard ranging from 1 EU to 0.0075 EU and a Sp1 concentration of 5 mg/mL; 25 µL of either standard or Sp1 were pipetted per well and after an addition of 25 µL LAL to all wells, the plate was incubated for 1 h at 37°C. Endotoxin-free water was used as negative control and the serially diluted control standard endotoxin (CSE) from *E. coli* strain 055:B5 as positive control and for quantification. Gelation in wells was observed by eye. As 3 EU correspond to 1 ng endotoxin, equal gelation levels for standard and sample can be used to calculate EU as well as endotoxin levels in 100 µg Sp1.

2.2.2.4 Proton NMR Spectroscopy

NMR spectra were obtained from a sample of 700 µg of lyophilized purified Sp1- or modified Sp1-containing samples, which were reconstituted in 700 µL of D_2O. NMR experiments were performed on a Bruker DRX500 instrument with a proton resonance frequency of 500.13 MHz. The ^1H spectra were recorded at 80°C using pre-saturation to suppress the water signal. Chemical shifts were referenced in relation to $^1H^2HO$ resonance at 4.36 ppm.

2.2.3 Oxidation, labelling and modification of Sp1

2.2.3.1 Oxidation of Sp1

Sp1 is a linear polymer of an average molecular size of 70 kDa corresponding to 130 trisaccharide repeating units with a respective molecular size of 537 Da. Each repeating unit of Sp1 contains one positive and two negative charge groups on galacturonic acid residues [GalA, residues a and c] and 2-acetamido-4-amino-2,4-6-trideoxygalactose [Aat, residue b] with a sequence of 3)-a-D-GalA (a)-(1 3)-a-D-Aat (b)-(1 4)-a-D-GalA (c)-(1 (Tzianabos et al., 1993, Choi et al., 2002, Lindberg et al. 1980) (Fig. 1.1). Sp1 was reconstituted in water. For fluorescence labelling of Sp1, the adjacent hydroxyl groups on residue c (MW 175 g/mol) were oxidized by 0.01 M sodium-m-periodate for 90 min in the dark to create highly reactive aldehyde functional groups. Reaction was stopped by addition of ethylene glycol and oxidized Sp1 was desalted using PD-10 columns, which are pre-packed, disposable gel filtration columns. They contain Sephadex^{TM} G-25 medium for group separation of high (M_r > 5000) from low (M_r < 1000) molecular weight substances and were equilibrated with 25 mL water prior to loading. Oxidized Sp1 was loaded to the column in a total volume of 2.5 mL and eluted with 3.5 mL water. Subsequently, the column was rinsed with 10 mL water and 15 mL NaN_3, locking the final 5 mL NaN_3 in the column till the next use; each column was used 2 to 3 times. Sp1 was lyophilized and stored in sterile pyrogen-free glass vials under vacuum till usage or used immediately for the labelling experiments.

2.2.3.2 Labelling of oxidized Sp1 by Alexa Fluor® Hydrazide

The desalted and freeze-dried oxidized Sp1 was weighed and reconstituted in 0.1 M sodium acetate buffer (pH 5.5) (22.5 µL sodium acetate buffer/mg Sp1). Lyophilized Alexa Fluor® Hydrazide sodium salt was reconstituted in 50 mM potassium phosphate solvent buffer (pH 7) to yield a 50 mM Alexa Fluor® dye stock solution. Alexa Fluor® Hydrazide was added to Sp1 (2.5 µL Alexa/mg Sp1) and incubated for two h at RT in the dark, shaking every 10 min. After incubation, the total volume was increased to 2 mL with 0.1 M sodium acetate buffer (pH 5.5) and neutralized with 3 M NaOH. The unbound Alexa was separated from the Sp1 solution by passing through PD 10 columns twice in the same manner as described above under 2.2.3.1 for desalting. Desalted and labelled Sp1 was sterile-filtered, freeze-dried and later

weighed, aliquoted and stored as above till usage. The complete labelling protocol was performed in the dark. Biological activity of labelled Sp1 was proven by abscess induction studies previously.

2.2.3.3 Modification of Sp1

Modification of Sp1 was conducted by N-acetylation of the free positive amino groups on Sp1. Sp1 was dissolved in 1 mg to 10 mg/mL sodium phosphate buffer (0.1 M) and kept on ice under constant stirring which is vital to ensure a good yield of acetylation. Acetic anhydride with 5% $NaHCO_3$ was prepared and added at a 10-fold molar excess of the amount of amines present in Sp1 to the cold, stirring Sp1 solution. The addition of the acetic anhydride occurred slowly and in several aliquots over a time course of at least 1 h to ensure a good yield of acetylation. Subsequently, the actylated Sp1 was purified over a PD10 gel filtration as described in 2.2.3.1, sterile-filtered, freeze-dried, aliquoted and used immediately for experiments.

2.2.4 Animals

2.2.4.1 Mouse strains

C57BL/6 mice were obtained from Charles River Laboratories or the Institute for Physiology of the University of Cologne and held in the animal facilities of the Institute for Medical Microbiology, Immunology and Hygiene. IL-6-deficient mice of C57BL/6 background were kindly provided by H. Bluethmann (Hoffmann-LaRoche Basel, Switzerland) (Kopf et al., 1994) and held under specific pathogen-free conditions at the Institute of Pathology of the University of Cologne. Homozygosity was confirmed by PCR. TLR2- and TLR4-deficient mice and MyD88-deficient mice, all from C57BL/6 background, were kindly provided by C. Kirschning (TU Munich) and the Max-Planck-Institute of Immunobiology in Freiburg, respectively. These animals were held at the Institute for Medical Microbiology, Immunology and Hygiene. All animals were maintained in an environmentally controlled facility at the Medical Microbiology Institute for at least 1 week before experimental abscess induction.

2.2.4.2 Genotyping of IL-6-deficient mice

Total DNA was purified (DNeasy Blood & Tissue Kit) from tail biopsies of IL-6-/- mice to genotype and ensure their IL-6-deficiency. As control, a tail biopsy of a C57BL/6 wildtype mouse was used. PCR is a method to amplify specific DNA sequences. The DNA or purified water was added along with 10 pmol/µL of the appropriate primers (Table 1 in 2.1.12) (Kopf et al. 1994) and purified water to PCR beads (total volume 25 µL). Each DNA sample was added to two different primer reaction set-ups. Reaction 1 contained the forward (IL-6c-pr1) and reverse primer 1 (IL-6c-pr2). Reaction 2 contained the forward primer 1 and the reverse primer neo (IL-6-neo). The Genotyping PCR protocol was adopted from Jackson Laboratories and the thermocycler was programmed as in Tab. 2.2.

TABLE 2.2: IL-6 genotyping PCR protocol

Step	Temperature	Time
1. Initial denaturation	94°C	3 min
2. Denaturation	94°C	30 sec
3. Annealing	61°C	1 min
4. Elongation	72°C	1 min
5. Final elongation	72°C	2 min

Steps two to four were repeated 35 times. The final hold was at 10°C.
An amount of 10 µL of PCR product was electrophoresed on a 1.5% agarose gel (+ethidium bromide). The combination of IL-6c-pr1 and IL-6c-pr2 gave a band of 1314 bp for the IL-6 mutated allele (IL-6-/+ and IL-6-/- mice) and of 174 bp for the wt allele. The combination of IL-6c-pr1 and IL-6-neo gave a band of 380 bp for the IL-6-/- mutated allele.

2.2.5 Abscess induction studies and evaluation of the cellular peritoneal influx

In abscess induction studies, mice were injected intraperitoneally with Sp1 (100 µg of Sp1 in PBS mixed with sterile cecal content adjuvant [SCCA]; 1:1 v/v, 0.2 mL total volume) (Stephen et al., 2007). SCCA was prepared by mashing the cecal content of C57BL/6 mice intestine through a 100 µm sieve with PBS. After autoclaving, the

suspension was centrifuged and the supernatant aliquoted and stored at –20°C till use. SCCA itself does not induce peritoneal abscesses but mimics peritoneal leakage.

In order to block IL-6 intraperitoneally, C57BL/6 wildtype mice were injected with 250 µg anti-mIL-6 intraperitoneally at the time of challenge and 6 h thereafter. Six days after challenge, mice were killed and macroscopically examined for the presence of abscesses within the peritoneal cavity by two double-blinded examiners. Abscesses were isolated and their diameter was measured before snap-freezing with iso-pentane in liquid nitrogen for immunohistochemistry analysis. Only abscesses larger than 2 mm were counted as significant.

At different time points following intraperitoneal challenge with mod. Sp1, Sp1, or Alexa-594-labelled Sp1 (100 µg), WT and IL-6-/- mice underwent peritoneal lavage with 2 mL to 4 mL of ice-cold RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin to assess secreted cytokines and the cellular influx into the peritoneal cavity. A total cell count was performed by trypan blue staining and a haemocytometer. Each sample was then analyzed by flow cytometry or fluorescent microscopy. In each experiment, three to six mice per group were tested. The experiment was performed three times in an independent manner. The studies described here were performed in accordance with the guidelines established by the German animal protection legislation (legislation number K05/07 for spleen isolation and K16.5/06 for iv, sc, ip injections).

2.2.6 Isolation of bone marrow-derived DCs and macrophages

BM-derived dendritic cells (BMDCs) were generated from mouse bone marrow by adapting a previously described method (Inaba et al., 1992). In brief, bone marrow cells from mice were cultured in VLE RPMI 1640 supplemented with 5% FBS, 500 U recombinant mouse granulocyte/macrophage-colony stimulating factor (GM-CSF), 20 µg/mL gentamycin, and 50 µM 2-mercaptoethanol. DC medium was exchanged in two-day intervals. DCs were isolated by magnetic cell sorting with a CD11c-specific monoclonal antibody (mAb).

Bone-marrow (BM)-derived macrophages were generated from C57BL/6 (WT) mice as previously described (Inaba et al., 1992). Erythrocytes were lysed with 0.2%

and 1.6% saline. After washing, cells were cultured in VLE-RPMI 1640 medium supplemented with 10% FBS, 15% L929 supplement, 100 µg/mL penicillin/streptomycin, 2 mM glutamine, 2.383 g/L Hepes, and 0.11 mg/mL sodium pyruvate. An equal amount of fresh macrophage medium was added to the medium on day 6. Cells were used for experiments on days 10 or 11.

2.2.7 Cytokine detection by Multiplex bead assay and IL-6 ELISA

The multiplex fluorescent bead immunoassay (Bender MedSystems) was performed to quantify proinflammatory cytokines in the peritoneal fluid at different time points following Sp1 challenge or SCCA alone (control). Cells were removed from the lavage fluid by centrifugation. The standard, bead mixture, biotin-conjugate mixture and streptavidin-PE solution were prepared according to the manufacturer's manual. Wells were pre-wetted with the provided assay buffer, before standard dilutions and samples were added. The protocol was used as suggested by the manufacturer and the samples were analysed by flow cytometry and the Flow Cytomix Software.

BMDCs were stimulated with different Sp1 concentrations (20 µg to 100 µg) at different time intervals. Cells were harvested, centrifuged and the supernatant collected. The murine IL-6 ELISA was performed as instructed by the manufacturer (Diaclone) and absorption was read in the Dyna Tech MR5000 ELISA plate reader at 450 nm (reference wavelength 620 nm).

2.2.8 Flow cytometry

For surface marker staining, lavage cells were stained with specific antibodies for 30 min on ice, washed, and then analyzed by flow cytometry. For intracellular cytokine staining (ICS), the lavage cells were incubated with Fc block for 15 min at 4°C and ICS performed according to the manufacturer's protocol. In brief, cells were stained for surface markers for 30 min on ice with specific antibodies, washed, fixed with Cytofix/Cytoperm for 20 min on ice. Subsequently, cells were washed and permeabilized with Perm/Wash Solution (1:9) and stained with interleukin-specific antibodies for 30 min at 4°C.

Cells prepared for flow cytometry were analyzed – after gating for viable cells by forward and sideward scatter - by FACScan™ using CELLQuest™ software. The results were expressed as percentage (%) of fluorescence-labelled cells in a population. Experiments were performed a minimum of three times in an independent manner.

2.2.9 Fluorescence microscopy

To investigate the Sp1-uptake and IL-6 production within Sp1-positive APCs, fluorescence and confocal microscopy were performed. After the surface marker staining and ICS, cells were transferred onto poly-D-lysine-pre-coated 1.5 cover slips attached to 35 mm dishes and then fixed in Cytofix solution (BD). Cells were kept on ice until further observation under the confocal microscope.

2.2.10 Immunohistochemistry

Snap-frozen abscesses were cryo-sectioned (5-6 µm), fixed in 4% buffered formalin for 1 min and then used for immunohistochemical analyses. To determine the IL-6 secretion of macrophages present in the abscess capsule, a co-staining for IL-6 and macrophages was performed, consecutively. After blocking of endogenous peroxidase by 0.3% H_2O_2 in methanol and endogenous biotin by the avidin-biotin blocking kit each for 30 min, sections were treated with normal goat serum, and then incubated with anti-IL-6 antibody solution (1:50) overnight. Next, an incubation with goat AP-conjugated anti-rat antibodies (1 h at RT) and development with Fast Red for 30 min followed. Subsequently, macrophages were stained by incubation with biotin-linked anti-F4/80 rat anti-mouse antibodies (1:100) for 1 h and a further treatment (1 h) with streptavidin-conjugated HRP that was then envisioned with DAB substrate. Sections were counterstained with hemalaun. DCs were visualized with purified anti-mouse CD11c hamster IgG and goat anti-hamster IgG:Biotin.

2.2.11 Apoptosis assay

To test the effect of Sp1 on apoptosis of $CD4^+$ T cells, spleen cells from C57BL/6 WT and IL-6-deficient mice (Kopf et al., 1994) were isolated by grinding the tissues through a 70 µm mesh. Erythrocytes were lysed (0.2% saline for 30 sec), cells were washed and incubated at 1 x 10^6 cells/mL in starvation buffer or non-starvation buffer (RPMI-1640 supplemented with 5% FCS) for 12 h in the presence of chemically modified Sp1 (100 µg/mL), Sp1 (100 µg/mL), or in medium alone. The cells were then stained with an APC-conjugated anti-CD4 mAb, or its appropriate isotype control, for 20 min on ice. After washing, cells were dissolved in binding buffer and stained with FITC-conjugated Annexin V (5 µl/1x10^6 cells) for 15 min on ice. Cells were washed, and 5 µl propidium iodide (PI) was added to the cells for 2 min. The PI staining was stopped by the addition of 5 mL of ice-cold PBS. Cells were washed and prepared for flow cytometry.

2.2.12 Quantification of $CD4^+$ T cells in blood, spleen, and peritoneal lavage

Quantification of the $CD4^+$ T cells in blood, spleen, and peritoneal lavage of C57BL/6 WT and IL-6-deficient mice was performed 24 h following Sp1 challenge. Lavage cells were isolated as described above (2.2.5). Spleen cells were isolated by grinding the tissues through a 70 µm mesh. Blood was collected in 50 µL Heparin (1:1000 in PBS) to prevent clotting. Erythrocytes in blood and spleen samples were lysed (0.2% NaCl for 30 sec, 1.6% NaCl), the cells washed, stained with trypan blue, and counted with a haemocytometer. The cells were then stained with an anti-CD4 or anti-CD8 mAb and its appropriate isotype control, and analysed by flow cytometry.

2.2.13 Migration assay

$CD4^+$ T cells were purified with the MACS-kit and incubated overnight in RPMI medium containing 5% FBS and 1% penicillin/streptomycin. The next day, cells were harvested, washed in PBS with 0.1% FBS (buffer) and taken up in 2 mL volume. Cells were incubated with the fluorescent dye calcein (6 µL calcein/mL cells) for 20

min at 37°C and washed twice thereafter with buffer. After cell counting, cells were adjusted to 5 x 10^6 cells/mL in degassed buffer. Chemoattractant dilutions of different IL-6 concentrations (50 ng to 200 ng/mL) were prepared in buffer and then carefully, pipetted onto the bottom plate (29 µL/well). The filter was gently stacked onto the bottom plate before 25 µL of cell suspension (1.3 x 10^5 $CD4^+$ T cells/well) were pipetted onto the filter. SLC (secondary lymphoid-tissue chemokine; 500 ng/mL final conc.) was used as positive and buffer as negative control.

Cell migration through the 5 µm filter to the bottom chamber at 37°C was assessed 4 h later. The filter was washed and dried gently with Q tipps. Migration of the fluorescent $CD4^+$ T cell from filter to bottom plate was quantified in the Wallac Victor by reading plate as well as filter with the Wallac Manager software. Each concentration was pipetted in sextets and the migration assay was performed at least three times.

2.2.14 Nuclear extraction and NFκB ELISA

BMDCs and BM-derived macrophages were treated with Sp1, modified Sp1, and with the known TLR ligands LPS (1 µg/mL), Pam3CSK4 (0.5 µg/mL), Poly I:C (1 µg/mL), Flagellin (0.05 µg/mL), Gardiquimod (0.5 µg/mL) and ODN (2 µM/mL) for different time intervals. Cells were washed with PBS, scraped, pooled, washed and cell counted, centrifuged and taken up in 1 mL PBS. For the preparation of nuclear extracts, the cells were resuspended in 400 µL low salt buffer A and incubated 15 min on ice to allow the nuclei to swell. An amount of 50 µL 10% NP-40 was added under shaking to gently lyse the cell leaving the nuclei intact. After sedimenting the nuclei, 70 µL of the high salt buffer C was added slowly. The nucleoplasm was extracted into the buffer while the nuclear envelop stayed intact and retained the genomic DNA. The extract was separated from the nuclear envelop/DNA by centrifugation at 13,000 rpm for at least 8 min. A BCA assay (see 2.2.2.2) was performed to determine the protein concentration. NFκB ELISA was performed according to manufacturer's instructions. An amount of around 16 µg protein were used. Each experiment was repeated at least three times.

2.2.15 Whole cell extraction, SDS-PAGE and Western blot

Peritoneal macrophages were starved for 4 h in FBS-free culture medium and then treated with Sp1 (100 µg/mL), LPS (1 µg/mL) or Sp1 plus LPS for different time intervals. Cells were washed with PBS, scraped, and spun down. Pellets were lysed for whole cell extraction in RIPA buffer plus inhibitors and incubated on ice for 30 min. Cells were centrifuged, the supernatant was collected and used for protein determination (see 2.2.2.2) prior to protein sample separation on a SDS-PAGE.
Protein samples were mixed with loading dye and degraded at 95°C for 5 min. After a quick spin, at least 10 µg/mL of protein were transferred to the SDS gel consisting of a 10% resolving gel and a 5% stacking gel. The proteins were electrophoresed in a blot chamber for 60 min to 90 min at 100 V.

An Immobilon-pSQ transfer membrane was pre-wetted in methanol for 5 sec before equilibration in transfer buffer for at least 10 min. The proteins were transferred from the SDS gel onto the membrane using a semi-dry transfer cell at 20 V for 45 min.

After protein transfer, the membrane was washed in washing buffer for 5 min at RT and then treated in blocking solution for 1 h. The membrane was rinsed once after blocking with washing buffer and incubated with a Phospho/IκB-α specific antibody overnight at 4°C. After four washing steps with washing buffer, 10 min each, the secondary HRP-linked antibody was added for 45 min at RT. Three washes for 10 min each followed. Detection was performed by incubation and rotation with the chemiluminescence solution for 5 min. The membrane was developed by exposure to a X-ray film for 5 min, 10 min and 15 min.

For reprobing the membrane with total-IκB-α and α-tubulin specific antibodies, the membrane was stripped. For stripping, the membrane was washed 5 min with water, incubated for 5 min at RT with 0.2 M NaOH and washed twice with water for 5 min each. Membranes were treated again for 20 min with blocking buffer and then incubated with total-IκB-α specific antibody overnight at 4°C or α-tubulin specific antibody for 2 h at RT. The second antibody incubation and washes were performed as described.

2.2.16 TLR surface expression and blocking experiments

Human monocytes or BMDCs (4 x 10^6 cells/mL in 24-well plate) were incubated in culture medium alone, or in culture medium containing Sp1 (50 µg/mL) for 8 h and prepared for flow cytometry analysis.

For the T cell proliferation assay, T cells were isolated by centrifugation in Ficoll-Hypaque gradients and purified with nylon wool and immunomagnetic beads (Finberg et al. 1992). The purity of the $CD3^+$ and $CD4^+CD8^-$ cell populations was confirmed by flow cytometry (≥95%). The proliferation assays were performed in 24-well plate transwells and inserts with a 0.1-µm pore size. The lower compartment contained 10^6 gamma-irradiated mononuclear cells as APCs and the upper compartment 5 x 10^5 $CD4^+$ T cells. The cells were incubated in culture medium alone, in culture medium containing Sp1 (50 µg/ml, or in culture medium containing Sp1 and a mouse IgG2a isotype control antibody, and azide- and endotoxin-free blocking antibodies to TLR2, and TLR4 (each at 10 µg/ml) at 37°C, 5% CO_2. The antibodies were added 30 min before the antigens. T cell proliferation was quantitated by [^{3}H]thymidine incorporation (1 µCi/well) for 6 h. Assays were performed at least three times at independent time points. The results were expressed as counts per minute (cpm) or stimulation index.

2.2.17 Statistical analysis

Results of peritoneal cytokine and cellular influx assays in the various groups were compared by Student's t-test. Comparison of groups with regard to abscess formation was made by chi-square analysis.

3. Results

3.1 Purification of Sp1

Experiments of this study were performed with the zwitterionic polysaccharide (ZPS) Sp1, isolated from the capsule of the gram-positive bacterium *Streptococcus pneumoniae* serotype 1. Sp1 was separated from C-substance, a contaminating cell wall polysaccharide, and other contaminating components such as proteins, lipids and endotoxins by purification via base treatment, ultrafiltration, and gel filtration chromatography (Stephen et al., 2005; Kalka-Moll et al., 2002). Polysaccharide containing fractions were detected by appraisal of the refraction index peak. Sp1 purity was ensured by ruling out protein contamination by the BCA method, nucleic acid contamination by measurement of the UV absorbance at 260 nm and LPS contamination by the LAL test. In addition, Sp1 was subjected to high-resolution proton NMR spectroscopy at 500 MHz (Kalka-Moll et al., 2000). Sp1 was found to contain no detectable protein and no detectable nucleic acid. Endotoxin was not detectable according to the LAL test with a sensitivity of <8 pg of LPS/mg of Sp1 (<0.4 pg of LPS/mL of culture medium containing 50 µg of Sp1) which corresponds to <0.028 EU/mg of Sp1 (<0.0012 EU/mL of culture medium containing 50 µg of Sp1).

3.2 Sp1 modification

As negative controls, medium, PBS, or modified Sp1 (mSp1) were used in biological assays. The positive amino groups as well as the negative carboxyl groups contribute together to the zwitterionic charge of Sp1 and its ability to induce a T cell-dependent abscess formation (Tzianabos et al., 1993). The removal of the positive charge of the amino group altered the zwitterionic charge and yielded a polysaccharide with a net negative charge. Modification of Sp1 was attained by N-acetylation of the free amino group with acetic anhydride. This treatment resulted in the loss of the free amino group on the 2-acetamido-4-amino-2,4,6-trideoxygalactose (see Fig 1.1). mSp1 was evaluated by ^{1}H NMR spectroscopy which revealed the successful modification of Sp1 (Fig. 3.1).

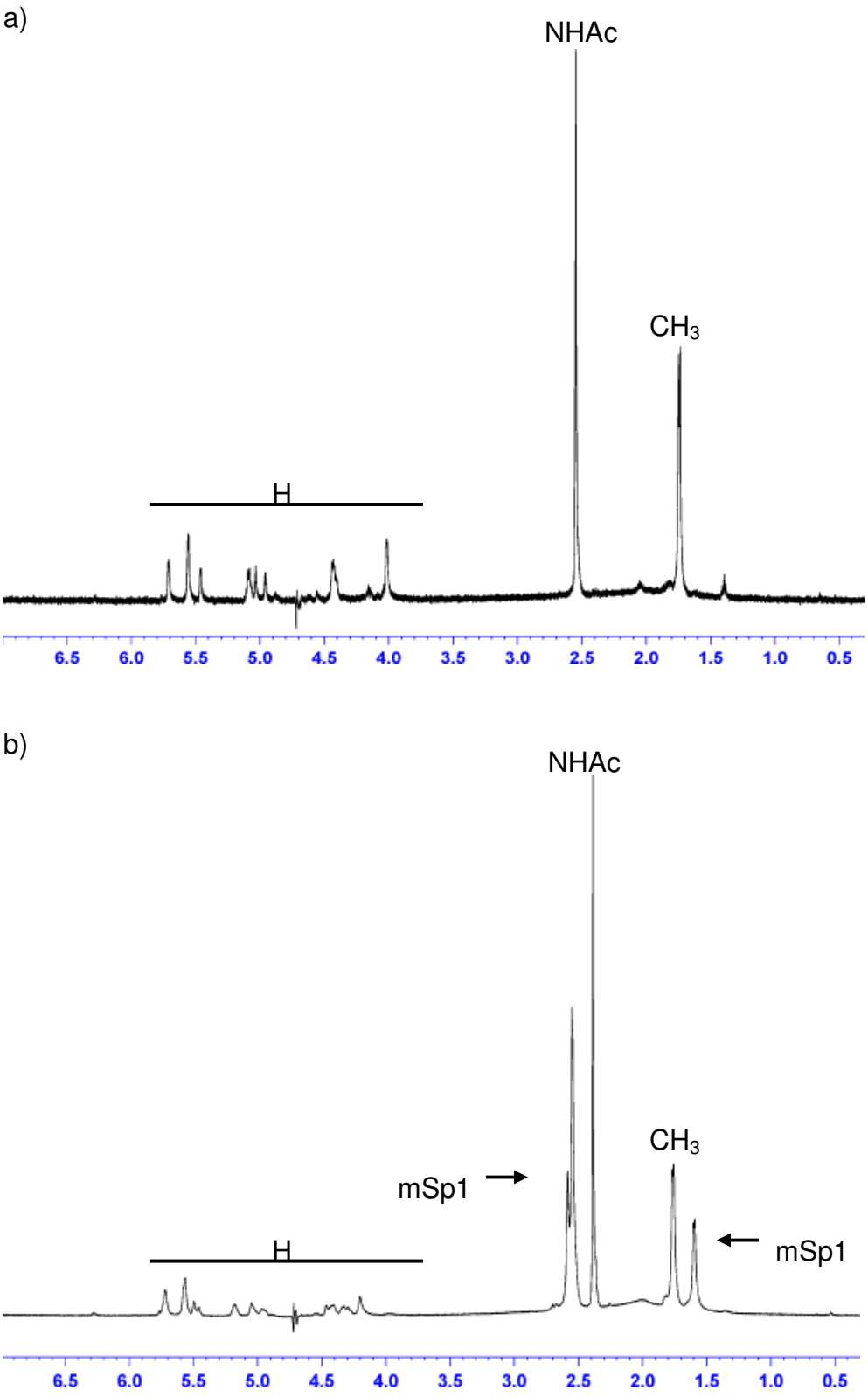


FIGURE 3.1: Sp1 is chemically modified by acetic anhydride. ^{1}H NMR spectra were recorded at 80 °C of a) native Sp1 and b) mSp1. The chemical shift for each proton is shown as parts per million (ppm, x-axis). (H, hydroxyl groups of Sp1 including carbohydrate rings)

The black bar (__H__) from about 4 ppm to 5.7 ppm indicated the region of hydroxyl group signals of the Sp1. A high water signal was suppressed (negative peak at 4.75 ppm) as it was common practice to yield more pronounced peaks and a plain base line. The successful modification of Sp1 was confirmed with the presence of new peaks at 1.6 ppm and 2.6 ppm. Those indicated new CH_3 groups, which composed the mSp1.

3.3 Bioactivity of Sp1 and mSp1

ZPS-mediated abscess formation is a T cell-dependent immune response (Stephen et al., 2007; Cobb et al., 2004; Kalka-Moll et al., 2002). Figure 3.2 a) shows a typical abscess on day 6 after intraperitoneal Sp1 challenge. Modified ZPS had been shown to abrogate the ability of abscess formation and $CD4^+$ T cell activation (Tzianabos, 1993). Bioactivity of Sp1 as well as of mSp1 was tested by intraperitoneal challenge with sterile cecal content adjuvant (SCCA) alone or plus Sp1 or mSp1. Six days after challenge, mice were opened and examined for abscesses. SCCA that mimics the leakage of intestinal flora into the peritoneal cavity did not result in abscess formation (Fig. 3.2 b)). SCCA plus mSp1-challenged mice did not form any abscesses whereas SCCA plus Sp1-challenged C57BL/6 mice showed a significant increase in abscess formation ($p<0.05$). The ability of purified Sp1 to induce abscess formation confirmed its bioactivity. The abrogated bioactivity of mSp1 proved it to be a negative control in further experiments.

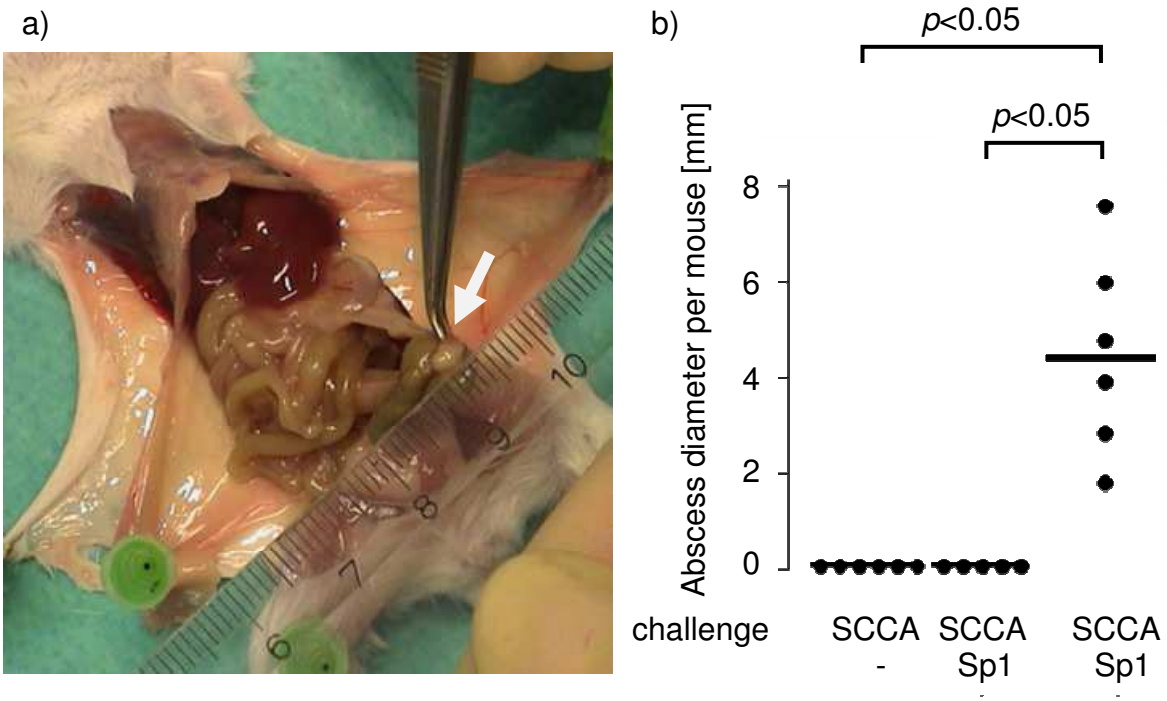


FIGURE 3.2: Sp1-mediated T cell-dependent abscess formation. a) Abscess induction confirmed the bioactivity of Sp1. Six days after intraperitoneal challenge with Sp1, mice were killed and checked for peritoneal abscess formation. One abscess is held with the forceps (indicated by the white arrow). b) C57BL/6 were challenged intraperitoneally with sterile SCCA alone or SCCA plus mSp1 or Sp1. After 6 days, intraperitoneal abscess formation was examined at autopsy. One dot represents the total abscess diameter per mouse; bars indicate the median abscess size per group. This figure shows a representative result out of at least three experiments performed independently.

3.4 Genotyping of IL-6KO mice

To test the importance of IL-6 in ZPS-mediated abscess formation, IL-6 knock-out (KO) mice were used. IL-6KO mice were kindly provided by Prof. Bluethmann and homozygosity was assured by genotyping. The combination of primers IL-6-Pr1 and IL-6-Pr2 yielded a band of 1314 bp for the IL-6 mutated allele (-/- mice) and a band of 174 bp for the wt allele (+/+) (PC, positive control). The combination of the primers IL-6-Pr1 and IL-6-neo was to detect an inserted neomycin selection cassette in the IL-6KO mice. Homozygosity was confirmed when reaction 1 yielded a 1314 bp band and reaction 2 a band of 380 bp for the IL-6 mutated allele (-/-). All tested IL-6-deficient mice were homozygous. The combination of the bands 174 bp and 380 bp

would have indicated the heterozygosity (+/-) of the IL-6KO mice which was not the case for the mice used in this study (Fig. 3.3).

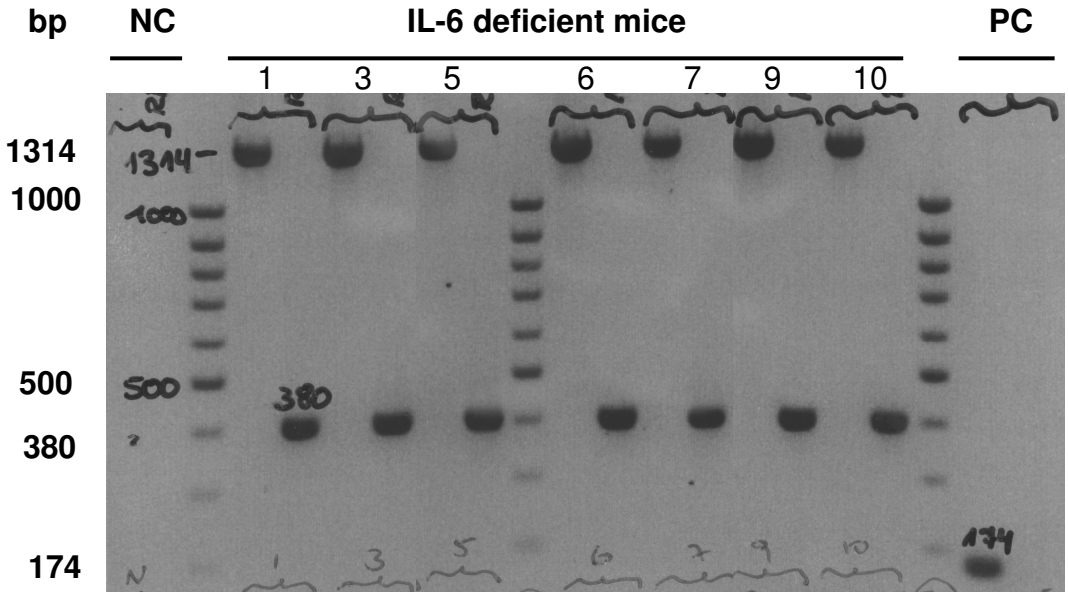


FIGURE 3.3: Tail biopsy genotyping of IL-6KO mice confirmed homozygosity. The total DNA was isolated, amplified by PCR and analysed by gel electrophoresis. Each of the IL-6KO mice 1, 3, 5, 6, 7, 9 and 10 has two lanes in the above figure, one for each reaction. The IL-6KO mice were shown to be homozygous KO mice with a 1314 bp band in reaction 1 (lane 1) and a 380 bp band in reaction 2 (lane 2); negative control (NC): water, positive control (PC): WT mice.

3.5 Intraperitoneal Sp1-induced cytokines in abscess formation

Cytokines known to be involved in T cell activation and proliferation include TNF-α, IL-1 and IL-6 among other (Banchereau et al., 2000). IL-1 and TNF-α have been demonstrated to play a pivotal role in *B. fragilis*- and PSA1-induced abscess formation in rats (Gibson et al., 1996; Gibson et al., 1998).

However, the cytokines involved in Sp1-induced peritoneal abscess formation and their further role in this immune response were barely investigated so far. In this study, the known pre-dominant pro-inflammatory cytokines IL-1, TNF-α and IL-6 were tested in a murine model for their involvement in ZPS-induced intraperitoneal abscess formation with Sp1 as the model antigen. IL-6 was demonstrated as the predominant cytokine (Fig. 3.4). Six h after Sp1 plus adjuvant challenge, IL-6 secretion was significantly higher than after SCCA alone challenge ($p<0.05$).

Interestingly, IL-1 and TNF-α were detected at lower levels after Sp1 challenge than IL-6. Besides, there was no significant increase in IL-1 or TNF-α secretion after Sp1 plus adjuvant-challenge compared to adjuvant challenge alone.

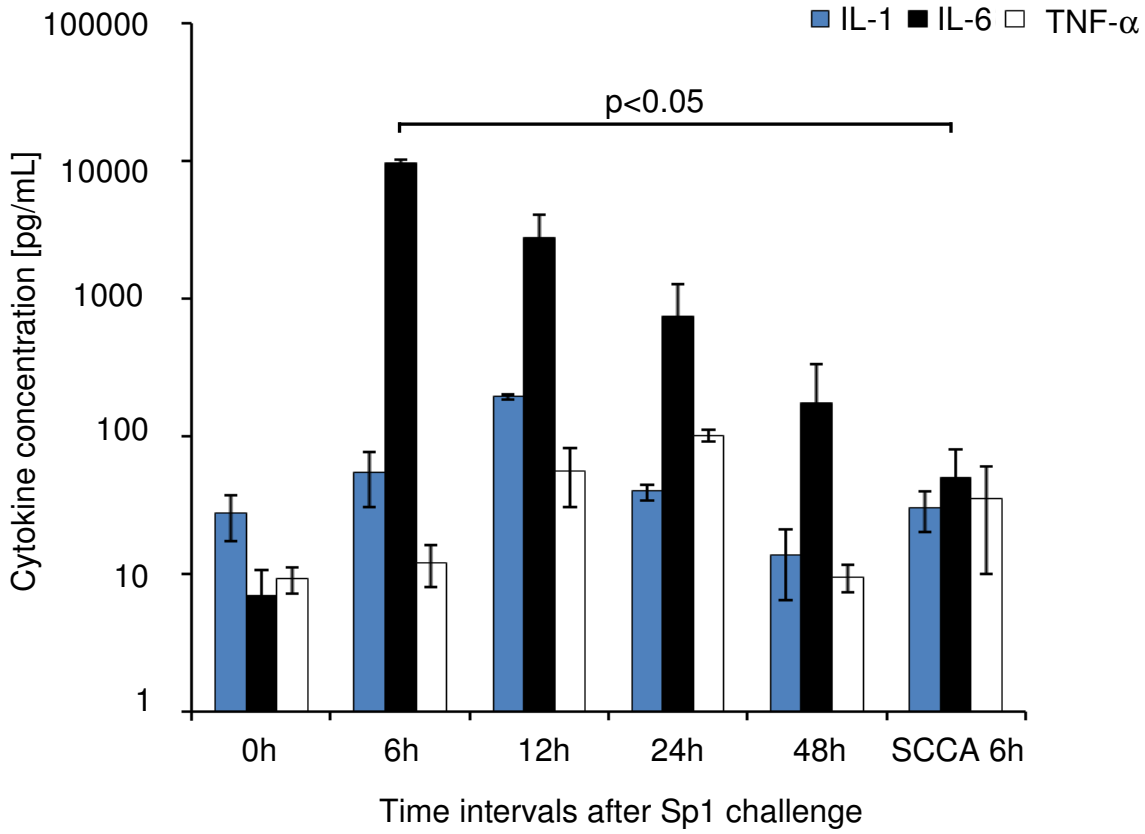


FIGURE 3.4: Intraperitoneal administration of Sp1 induced peritoneal IL-6. At different time points after intraperitoneal Sp1 challenge, peritoneal lavage fluid was analyzed for IL-1, IL-6, and TNF-α by a multiplex bead assay. As a control, peritoneal fluid of mice challenged with SCCA alone was taken at different time points, pooled, and analyzed for cytokines. The experiments were performed three times independently, each with four mice per group.

3.6 Sp1-induced IL-6 secretion in peritoneal and BM-derived macrophages

The absolute number of F4/80$^+$ macrophages has been reported to be 20–50% of the peritoneal cell population in normal mice (Yang et al., 2002). Experimental ZPS-induced abscess walls consist of macrophages, CD4$^+$ T cells, and DCs (Stephen et al., 2007; Tzianabos and Kasper, 2002) whereas dead and dying neutrophils and

other granulocytes make up the major component of the pus in the abscess center (Janeway et al., 2005). Macrophages constitute - as in a septic abscess - a main part of the wall of an experimental abscess (Tzianabos and Kasper, 2002). IL-6 is produced in large quantities by peritoneal host cells in response to Sp1. To identify a cellular source of IL-6 secretion and pivotal IL-6-mediated functions in the process of abscess formation, the effect of intraperitoneal Sp1 challenge on macrophages was addressed. CD11b-positive macrophages migrated into the peritoneal cavity upon Sp1 challenge (Fig. 3.5). Macrophages represent 50% of incoming cells six h after Sp1 challenge. SCCA alone (negative control) attracted significantly less macrophages and total cells into the peritoneum (*, $p<0.05$).

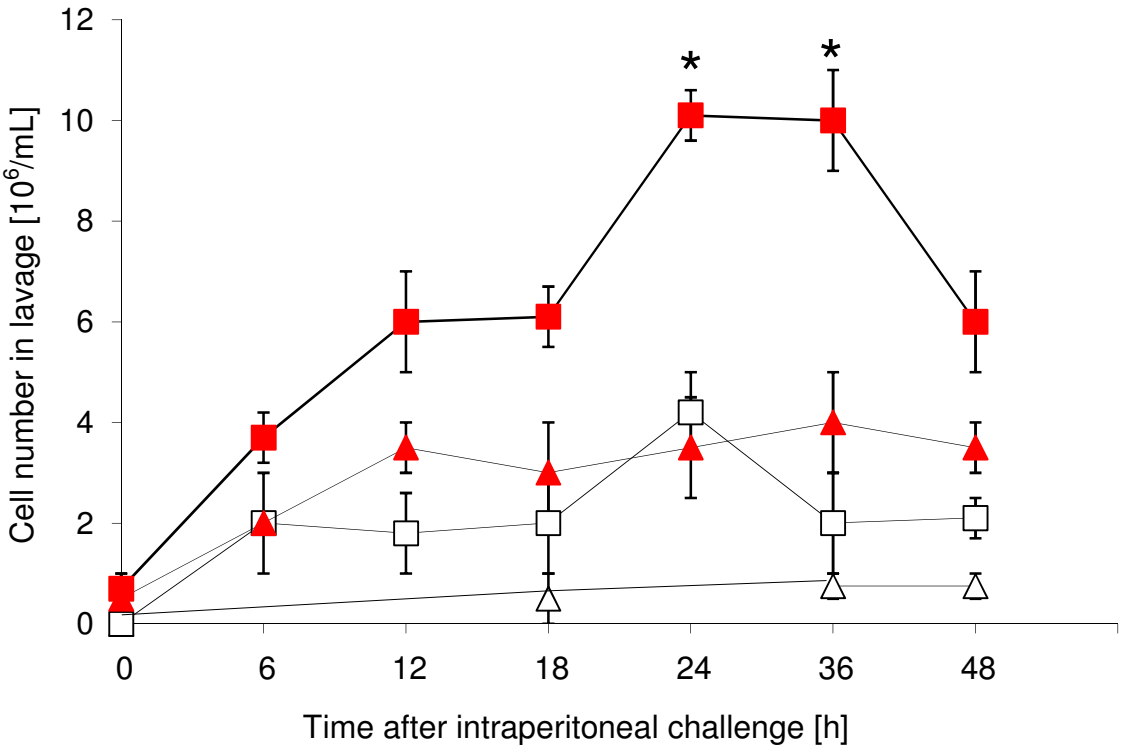


FIGURE 3.5: Influx of total cells and macrophages into the peritoneum upon Sp1 challenge. Mice were i.p. challenged with either Sp1 (red symbols) or SCCA controls (white symbols). Influx of total cells (square) and CD11b-positive macrophages (triangles) into the peritoneum was measured in WT mice (six per group). The peritoneal lavage was performed at different time intervals (x-axis) after challenge. Cell numbers were determined by cell counting and flow cytometry. Bars indicate standard errors within a group tested ($p<0.05$).

Twenty-four h after intraperitoneal Sp1 challenge, about 28% of cells attracted into the peritoneal cavity were CD11b macrophages (Fig. 3.6).

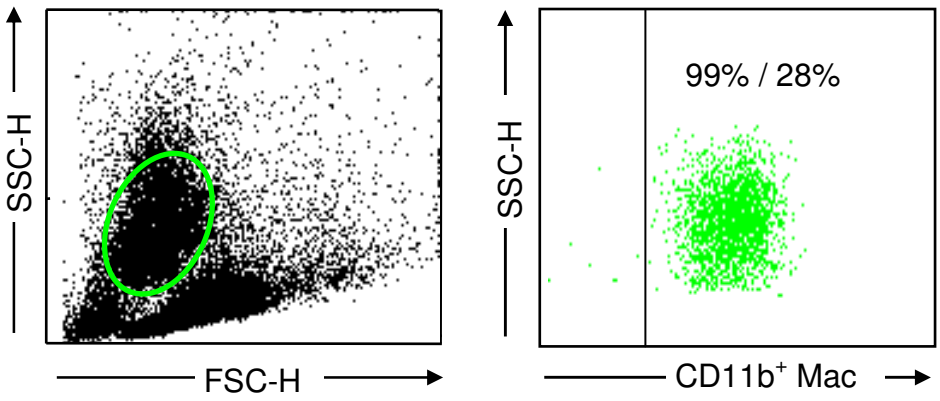


FIGURE 3.6: Sp1-induced macrophage influx into the peritoneum. Lavage cells were isolated 24 h after intraperitoneal Sp1 challenge. Macrophages (Mac) of the peritoneal influx were stained with PE-conjugated anti-CD11b mAb and analyzed by FACS. Numbers in the right dot plot represent the percentage of positive macrophages, gated as indicated in the left dot plot and non-gated cells, respectively.

Macrophages are known to internalize extracellular antigens by pinocytosis, phagocytosis or endocytosis. Therefore, the next step was to examine whether the peritoneal macrophages internalize Sp1. Fluorescence microscopy revealed that fluorescence-labelled Sp1 was internalized by about 90% of peritoneal macrophages (Fig. 3.7).

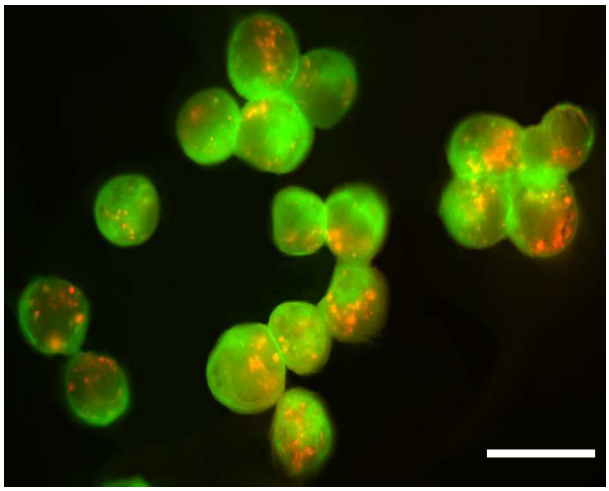


FIGURE 3.7: Peritoneal macrophages internalize Sp1. Mice were challenged intraperitoneally with Sp1-Alexa 594 (100 µg). Twenty-four h after Sp1 challenge, lavage cells were isolated and stained with CD11b-FITC (green) and analyzed by fluorescent microscopy. Scale bar: 10 µm.

BM-derived macrophages were also treated with Sp1-Alexa594. Confocal microscopy of 100 $CD11b^+$ macrophages revealed that 92% of in vitro macrophages internalized Sp1 (Fig. 3.8).

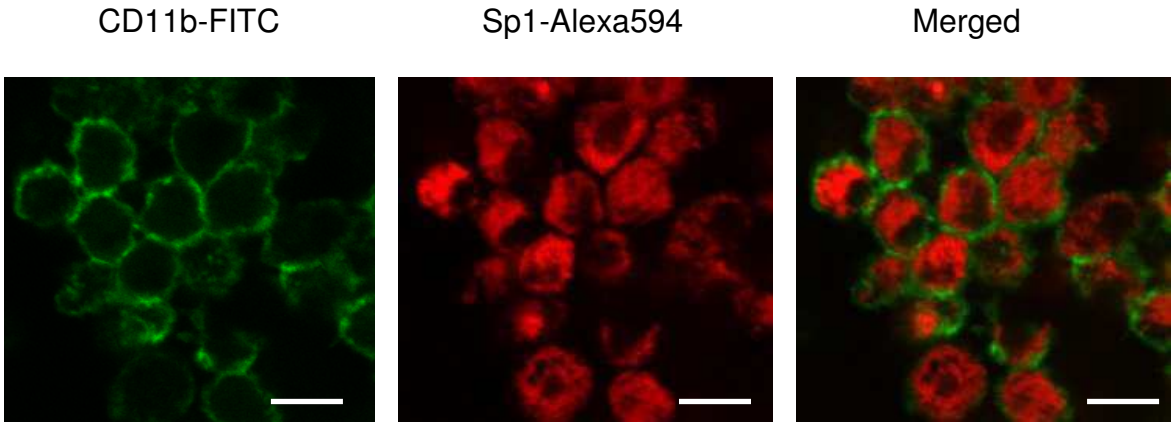


FIGURE 3.8: BM-derived macrophages internalize Sp1. BM-derived macrophages were treated with Sp1-Alexa594 (red) for 24 h, stained with the surface marker CD11b-FITC (green) and then analyzed under the confocal microscope. The merged image visualized the Sp1-Alexa594 uptake in $CD11b^+$ macrophages. The experiment was performed four times in an independent manner. Scale bar: 10 µm.

The next step was to investigate whether macrophages, which internalize Sp1 also produce IL-6. Quantitative analysis of the confocal microscopy analysis of the cytokine staining of the macrophages revealed that 95% of cells positive for intracellular Sp1-Alexa594 synthesized IL-6 (Fig. 3.9).

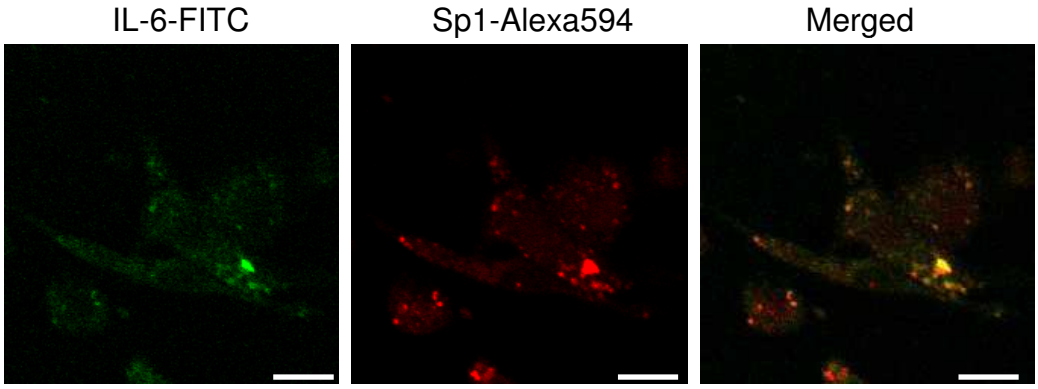


FIGURE 3.9: IL-6 accumulation in Sp1-positive macrophages. BM-derived macrophages were treated for 33 h with Sp1. Sp1-positive macrophages (Alexa594, red) were analysed after ICS for IL-6 (FITC, green) production with confocal microscopy. The merged image visualized IL-6 production in Sp1-positive macrophages. The experiment was performed three times independently. Scale bar: 10 µm.

Further it was investigated whether Sp1 internalization by macrophages lead to IL-6 secretion. Intracellular cytokine staining of peritoneal macrophages 6 h after Sp1 challenge demonstrated that almost 90% of the Sp1-induced peritoneal macrophages showed cytoplasmic expression of IL-6 (Fig. 3.10).

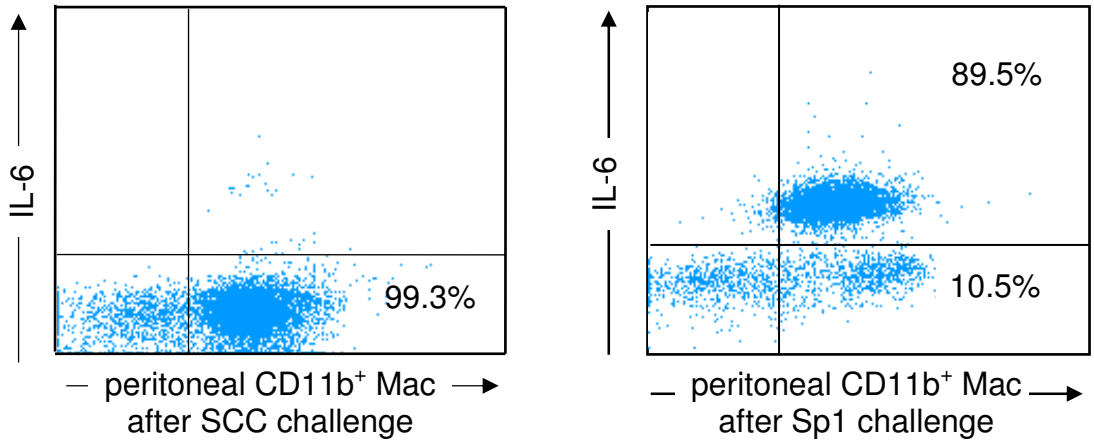


FIGURE 3.10: IL-6 secretion by Sp1-positive macrophages. Six h after intraperitoneal SCC or Sp1 challenge, macrophages (Mac) of the peritoneal influx were surface-stained with PE-conjugated anti-CD11b mAb, and intracellularly with FITC-conjugated anti-IL-6, and then analyzed by flow cytometry. Numbers in the quadrants of the dot blot represent the percentage of positive macrophages.

Macrophages represent the principal constituent of the abscess wall (Tzianabos and Kasper, 2002). Intraperitoneal abscesses were isolated six days after Sp1 challenge and analysed immunohistochemically. Immunohistochemical analyses of abscesses demonstrated incorporation of IL-6-positive macrophages in the wall (Fig. 3.11). Taken together, this result demonstrated that IL-6 secreted by macrophages was a predominant cytokine in Sp1-mediated abscess formation.

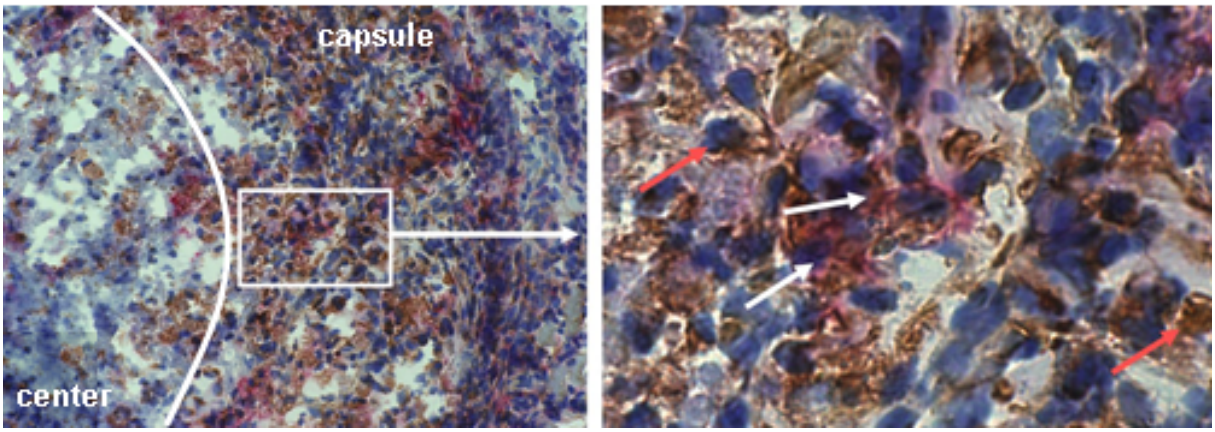


FIGURE 3.11: IL-6 secreting macrophages localize within abscess capsule. Six days after SCCA plus Sp1 challenge, intraperitoneal abscesses were isolated from C57BL/6 mice and snap frozen in iso-pentane. Cryo-sectioned abscesses were stained with anti-F4/80 to stain macrophages (brown, peroxidase and DAB) and anti-IL-6 (red, phosphatase and fast red), and analyzed by light microscopy (20x). The left panel shows an overview of a partial abscess. The right panel demonstrates the abscess capsule highlightened in the rectangular box in the left panel. White arrows indicate double-stained cells, red arrows indicate F4/80-positive IL-6-negative cells.

3.7 Sp1-mediated IL-6 secretion in BM-derived DCs

DCs are known to be the most potent APCs. Besides macrophages, CD11c-positive DCs, which are also capable of migrating into the peritoneal cavity upon Sp1 challenge, play an important role in an experimental model of abscess formation. About 7% of the total cells attracted into the peritoneal cavity by Sp1 application are CD11c-positive DCs (Stephen et al., 2007). BM-derived DCs were shown via fluorescence microscopy to internalize Sp1 (Fig. 3.12).

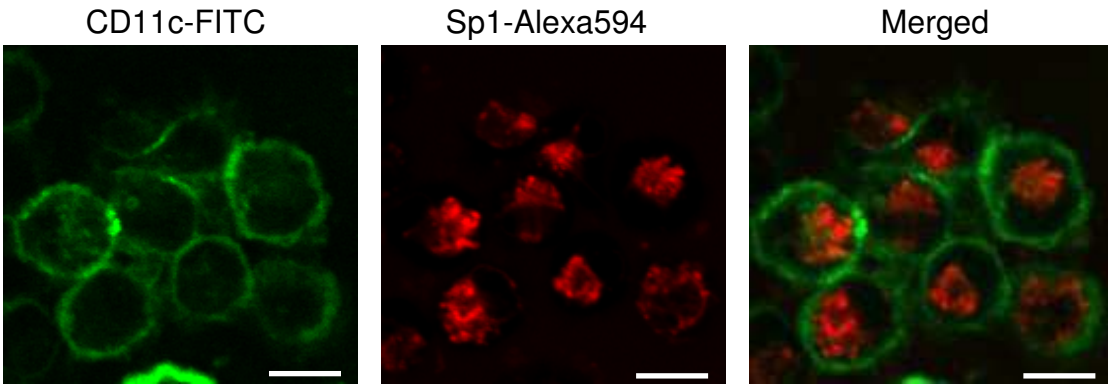


FIGURE 3.12: BM-derived DCs internalize Sp1. BM-derived DCs were stained with CD11c-FITC (green) after 24 h treatment with Sp1-Alexa594 (red) and analyzed under the confocal microscope. The merged image visualized the Sp1 uptake in CD11c-positive DCs. The experiment was performed at least five times in an independent manner. Scale bar: 10 µm.

To investigate whether Sp1-internalized BM-derived DCs secrete IL-6, an ICS was performed. Hundred DCs positive for intracellular Sp1 were counted. ICS of Sp1-Alexa594-treated DCs demonstrated that about 98% Sp1-containing DCs accumulated IL-6 (minimum 97%, maximum 98% in three experiments performed independently). IL-6 production inside Sp1-positive BM-derived DCs was revealed by confocal microscopy (Fig. 3.13).

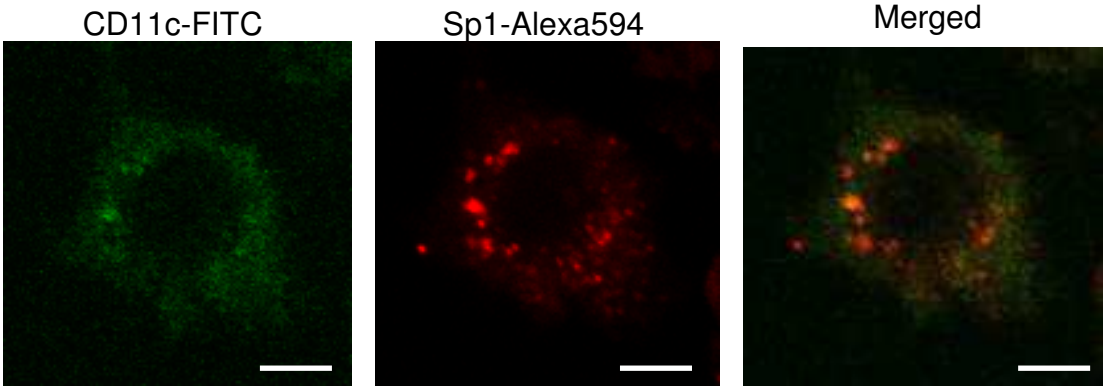


FIGURE 3.13: IL-6 production in Sp1-internalized DCs. BM-derived DCs were treated for 33 h with Sp1-Alexa 594 (red). Sp1-positive BM-derived DCs were analysed for intracellular IL-6 accumulation (FITC, green) by confocal microscopy. The merge image visualized Sp1-positive DCs to produce IL-6. Scale bar: 10 µm.

DCs were shown here to migrate into the peritoneum upon Sp1 stimulation and to be able to internalize Sp1. To gain information about the specificity of the Sp1-induced IL-6 synthesis in APCs, the IL-6 secretion by DCs in vitro was investigated.
BM-derived DCs treated for 33 h with different concentrations of Sp1 in vitro secreted IL-6 in a dose-dependent manner (Fig. 3.14). Besides medium alone, mSp1 was used as a negative control. Medium alone and chemically modified Sp1 failed to induce dose-dependent IL-6 secretion by DCs. This result demonstrated that IL-6 secretion depended on the zwitterionic charge motif of Sp1 and its concentration.

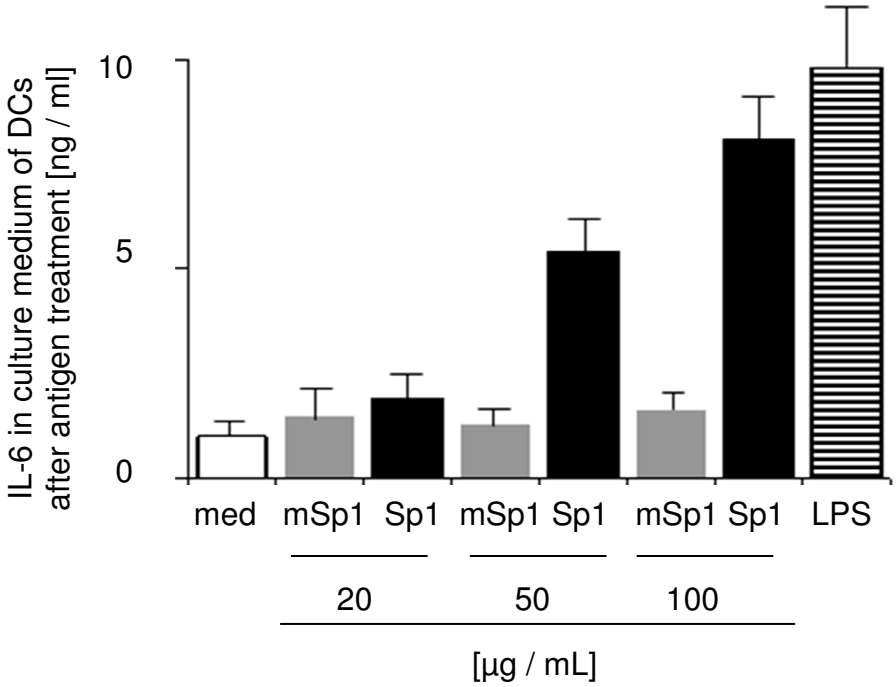


FIGURE 3.14: Sp1-dose-dependent IL-6 secretion in BM-derived DCs. BM-derived DCs were treated for 33 h in medium (NC, negative control, white bar), mSp1 (grey bar) or Sp1 (black bar) at different concentrations, or LPS (PC, positive control, striped) (x-axis). Supernatants were analyzed for IL-6 by ELISA. Mean and standard deviations were calculated from three individual experiments.

Sp1-induced intraperitoneal abscesses were analyzed for IL-6-positive DCs being incorporated into the abscess wall. Immunohistochemistry of snap-frozen abscesses demonstrated that IL-6 secreting DCs were detained in the abscess wall (Fig. 3.15).

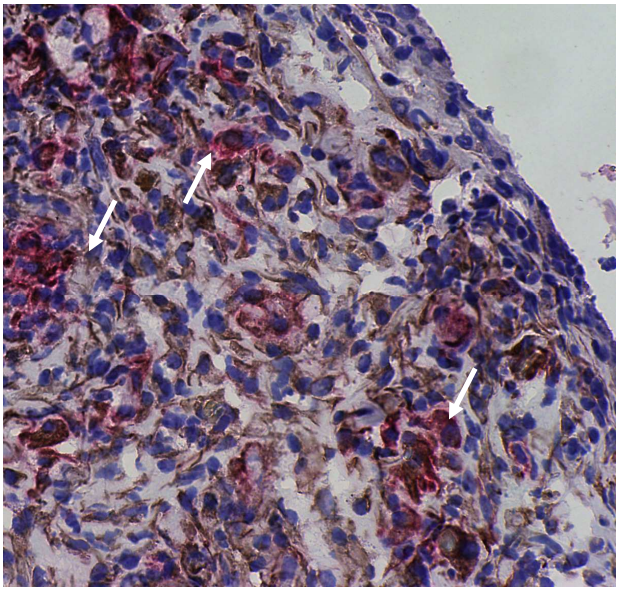


FIGURE 3.15: IL-6 secreting DCs localize within abscess capsule. Six days after Sp1 challenge, cryo-sectioned intraperitoneal abscesses were stained with a CD11c-specific antibody to stain DCs (brown) and anti-IL-6 (red), and analyzed by light microscopy (20x). The white arrows indicate the CD11c-positive and IL-6-positive cells.

3.8 IL-6 dose-dependent $CD4^+$ T cell migration

IL-6 was reported to be a direct mediator of T cell migration. The IL-6-dependent migration was demonstrated for primary human T cells and human T cell lines (Weissenbach et al., 2004). To test the hypothesis of IL-6-dependent migration for murine T cells, a migration assay was performed. Within 4 h, $CD4^+$ T cells isolated from spleens of WT mice migrated through a transwell with a 5 µm pore size towards IL-6 in a dose-dependent manner (Fig. 3.16).

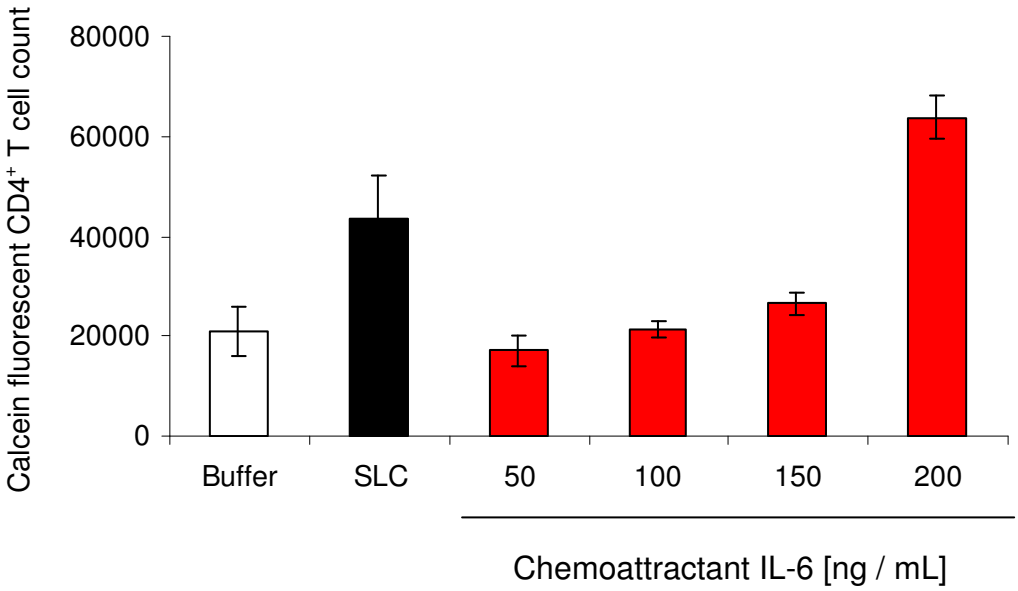


FIGURE 3.16: IL-6 dose-dependent CD4⁺ T cell migration. Purified CD4⁺ T cells (1.3 x 10^5 cells in 25 µL / well) from murine splenocytes migrated in an IL-6-dose-dependent manner through 5 µm filters. IL-6 concentrations (red bars) were prepared in buffer (PBS + 0.1% BSA). SLC (secondary lymphoid-tissue chemokine, 500 ng/mL, black bar) was used as positive control and buffer (white bar) as negative control. The experiments were performed three times in an independent manner and each concentration was pipetted in sextets per experiment.

3.9 Sp1-induced peritoneal CD4⁺ T cell influx depends on IL-6

Abscess formation depends on CD4⁺ T cell migrating into the peritoneal cavity (Tzianabos et al., 2000b). IL-6 has been described as a migration factor for CD4⁺ T cells in different tissues. Therefore, the CD4⁺ T cell, macrophage and DC cell counts in the peritoneal lavage following Sp1 challenge of WT mice were measured, IL-6-deficient mice, and IL-6-deficient mice treated intraperitoneally with IL-6. As controls, cell counts were made from peripheral blood and spleens of WT and IL-6-deficient mice. The CD4⁺ T cell counts in the spleen and peripheral blood of WT and IL-6-deficient mice were similar (Tab. 3.1). However, compared to WT mice, the peritoneal lavage of IL-6-deficient mice showed a 73% decrease in the CD4⁺ T cell count. The peritoneal macrophage cell count was similar in WT and IL-6-deficient mice. The number of DCs was reduced in IL-6-deficient mice when compared to WT mice. Exogenous IL-6 cytokine treatment of IL-6 KO mice restored the cell count of macrophages, DCs, and CD4⁺ T cells in the peritoneal cavity to levels of WT mice.

The results suggest a functional role of IL-6 as a migration factor for $CD4^+$ T cells into the peritoneum.

TABLE 3.1: T cell counts in C57BL/6 WT and IL-6-deficient mice. C57BL/6 WT and IL-6KO mice were challenged intraperitoneally with Sp1 (100 µg) plus SCCA (5 mice per group). One group of IL-6KO mice were treated intraperitoneally with mouse IL-6 (100 µg/kg) at the time of challenge, and at 6 h and 12 h following Sp1 challenge. Twenty-four h after challenge, cells from C57BL/6 WT and IL-6KO mice were isolated from the peritoneum by lavage, and from blood and spleen, stained with anti-CD4, and analyzed by flow cytometry. In addition, peritoneal lavage cells were stained with anti-CD11b and anti-CD11c antibodies to stain macrophages and DCs, respectively. Numbers represent the percentage of cells of the total cells or the absolute number.

	Blood	**Spleen**	**Peritoneum**			
	[%]	**[%]**	**[%]**	**[%]**	**[%]**	**[cells x10^3/ml]**
	CD4	**CD4**	**Mac**	**DC**	**CD4**	**CD4**
WT	33.6±2.0	21.2±5.8	26.5±13.9	7.3±0.7	4.1±0.4	39.3±3.5
IL-6KO	35.2± 3.0	18.8±5.9	22.6±5.3	1.7±1.1	1.1±0.1	9.5±0.7
IL-6KO (+IL-6)	n.d.	n.d.	28.1±10.1	6.1±2.4	4.3±0.1	41.2±4.0

3.10 Anti-apoptotic effect of Sp1-mediated IL-6 secretion on $CD4^+$ T cells

Sp1 induced $CD4^+$ T cell activation in vitro in a MHC class II- and co-stimulation-dependent manner (Kalka-Moll et al., 2002, Stephen et al., 2005). IL-6 stimulated the activation, survival, and proliferation of $CD4^+$ T cells, acting on T cells as an anti-apoptotic factor (Takeda et al. 1998; Teague et al., 1997; Van Snick 1990; Lotz et al., 1988). Whether Sp1 was also capable of protecting $CD4^+$ T cells from apoptosis and whether that effect required IL-6 were further questions to investigate. Spleen cells of C57BL/6 WT mice were starved to induce apoptosis. $CD4^+$ T cells were purified and examined for apoptosis in the presence and absence of Sp1 and chemically modified Sp1. As a control, $CD4^+$ T cells were incubated in non-starving medium. Fluorescence staining of $CD4^+$ cells with the apoptosis marker annexin V, revealed

that in contrast to medium and modified Sp1, native Sp1 decreases apoptosis (16% apoptotic cells versus 39% and 42%) (Fig. 3.17). At the same time, in the presence of Sp1, 15% of $CD4^+$ T cells stained positively with propidium iodide (PI), a marker for necrosis and cell death. Thirty-seven per cent and 42% were stained with PI in the absence of Sp1 and in the presence of chemically modified Sp1, respectively. This observation shows an anti-apoptotic effect of Sp1 on $CD4^+$ T cells. The analysis was applied to spleen cells of IL-6-deficient mice of the C57BL/6 background. IL-6-deficient cells treated with Sp1 did not inhibit apoptosis of $CD4^+$ T cells. Sixty-five per cent of CD4+ T cells stained positively with annexin V. In contrast, 36% and 42% of cells left untreated, or treated with modified Sp1 as controls were positive for annexin V, respectively (Fig. 3.17). The result correlated with the findings of the PI staining, revealing that in the absence of IL-6 the inhibitory effect of Sp1 on cell death was abrogated. Sixty-two percent of $CD4^+$ T cells were dead in the presence of Sp1. Thirty-one per cent and 43% of $CD4^+$ T cells stained positively for PI when left untreated or treated with modified Sp1, respectively. In summary, the anti-apoptotic effect of Sp1 depends on IL-6.

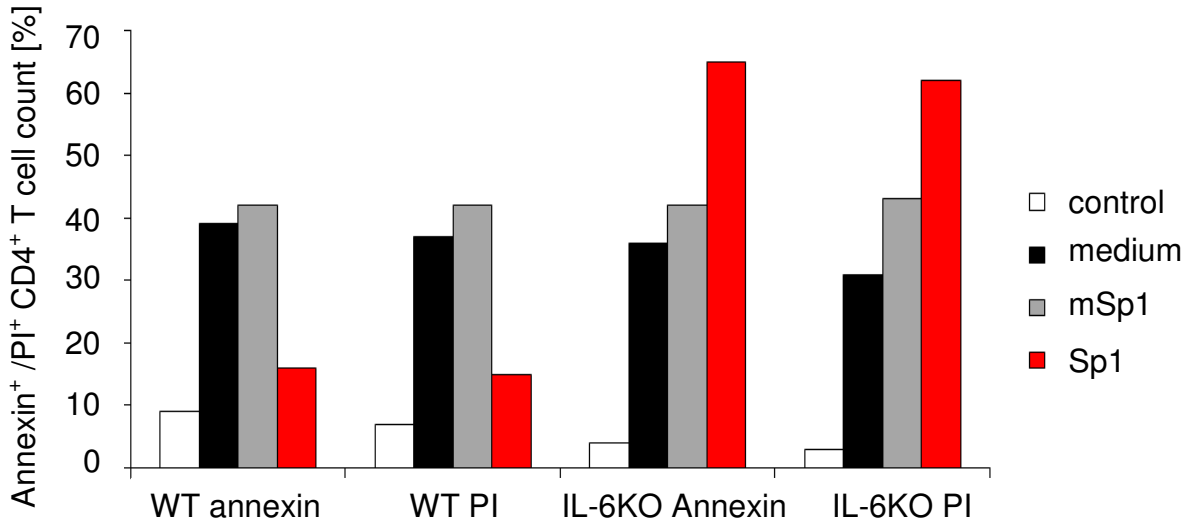


FIGURE 3.17: Sp1 inhibits apoptosis through IL-6. $CD4^+$ T cells from WT and IL-6KO mice were treated with starvation medium alone (black), plus mSp1 (100 µg/mL; grey) or plus Sp1 (100 µg/mL; red) for 8 h. As a control, cells were incubated in non-starvation medium alone (white). $CD4^+$ T cells were stained for apoptosis with annexin V and for cell death with PI and analyzed by FACS.

3.11 Sp1-mediated abscess formation depends on IL-6

Now, that Sp1 was shown to induce IL-6 in APCs which protected $CD4^+$ T cells from apoptosis and lead to T cell migration into the peritoneal cavity, the role of IL-6 in Sp1-induced abscess formation was investigated. Peritoneal administration of Sp1 to WT mice induced abscesses whereas in IL-6KO mice abscess formation by Sp1 was significantly reduced ($p<0.05$). SCCA alone or challenge with mSp1 (negative controls) did not induce abscesses ($p<0.05$). Furthermore, peritoneal administration of an anti-IL-6 neutralizing mAb with the intraperitoneal Sp1 challenge and 6 h thereafter resulted in 50% reduction of abscess formation in WT mice (Fig. 3.18).

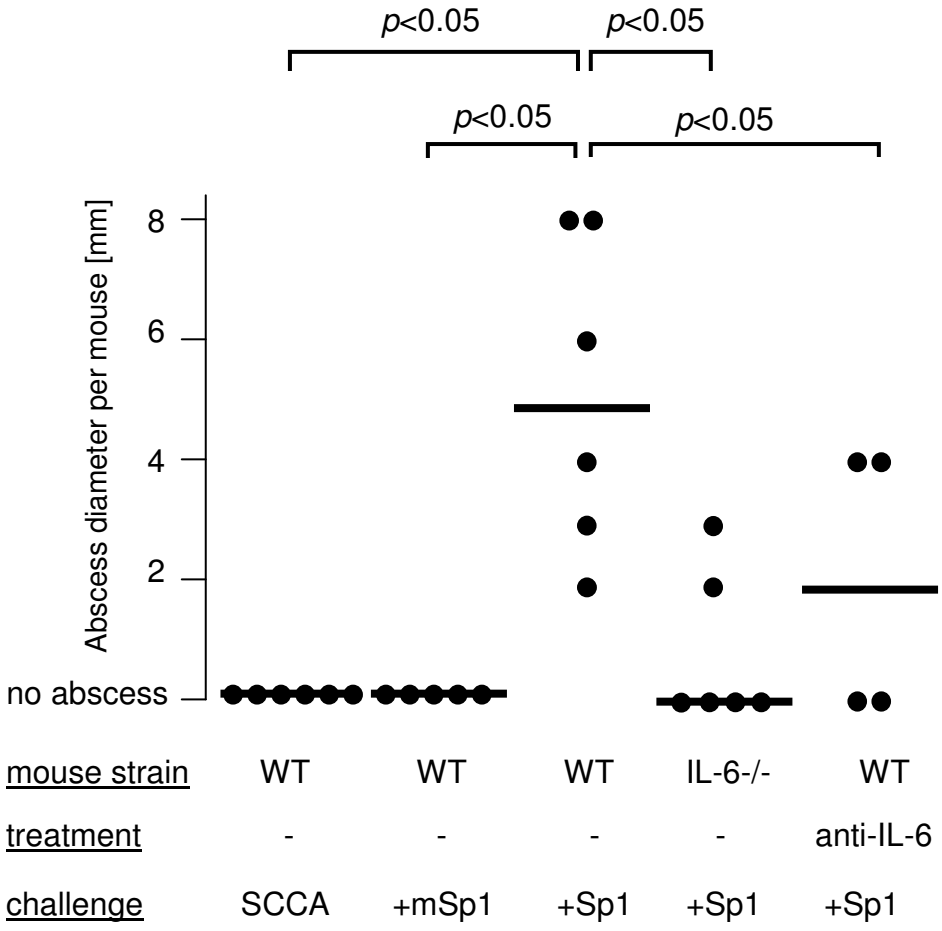


FIGURE 3.18: IL-6-dependency of Sp1-mediated T cell-dependent abscess formation. C57BL/6 or IL-6KO mice were challenged intraperitoneally with SCCA alone or plus mSp1 as controls, or plus Sp1 in the presence or absence of anti-IL-6 antibody. After 6 days, intraperitoneal abscess formation was examined at autopsy. One dot represented the total abscess diameter per mouse; bars indicated the median abscess size per group. This figure showed a representative result out of at least three experiments performed independently.

Altogether, the data so far demonstrated that IL-6 seemed to be required for Sp1-induced abscess formation. This study showed firstly, that Sp1 challenge lead to macrophage and DC influx into the peritoneum. Secondly, these APCs internalized Sp1. Thirdly, IL-6 was produced and secreted by Sp1-positive APCs and led fourthly, to IL-6-mediated T cell migration. Fifthly, T cell apoptosis was reduced by Sp1-induced IL-6, and finally Sp1-induced IL-6 secretion lead to a T cell-dependent abscess formation in which IL-6-secreting APCs were retrieved in the abscess capsule. Next was to identify the pathway of how Sp1 induced IL-6 secretion.

3.12 Sp1-mediated NF-κB activation in murine macrophages is impaired

BM-derived macrophages were treated with Sp1 to test for NF-κB activation. Cells were either left untreated or treated with Sp1, LPS, or both for 5 min, 30 min and 60 min. The whole protein extracts were collected for SDS-PAGE electrophoresis and subjected to Western blot using antibodies that recognize IκB-α phosphorylation. LPS-treated macrophages showed increased phosphorylated IκB-α levels after 5 min whereas extracts of Sp1-treated macrophages showed no increased IκB phosphorylation for the different time points tested and remained at similar low levels as the non-stimulated cells (Fig. 3.19 a)). A decrease in total IκB-α was observed for LPS-treated macrophages after 30 min. Sp1-treated macrophages also showed decreased levels of total IκB-α levels after 30 min. After 60 min of LPS or Sp1 – treatment, total IκB-α levels remained stable or increased, respectively. Macrophages treated with both, LPS plus Sp1, decreased constantly in IκB-α levels and increased in IκB-α phosphorylation levels with increasing time.

In addition, Sp1-induced NF-κB activation in macrophages was tested by detecting the translocation of the NF-κB subunit p65 to the nucleus by ELISA. Nuclear extracts of BM-derived macrophages were collected after 15 min of either mSp1, Sp1 or LPS treatment. NF-κB ELISA did not detect p65-translocation into the nucleus of the Sp1-treated WT macrophages and had similar low levels as untreated cells or mSp1-treated cells, which were used as controls. In contrast, LPS-treated macrophages showed high levels of p65 in the nuclear extracts (Fig. 3.19 b)).

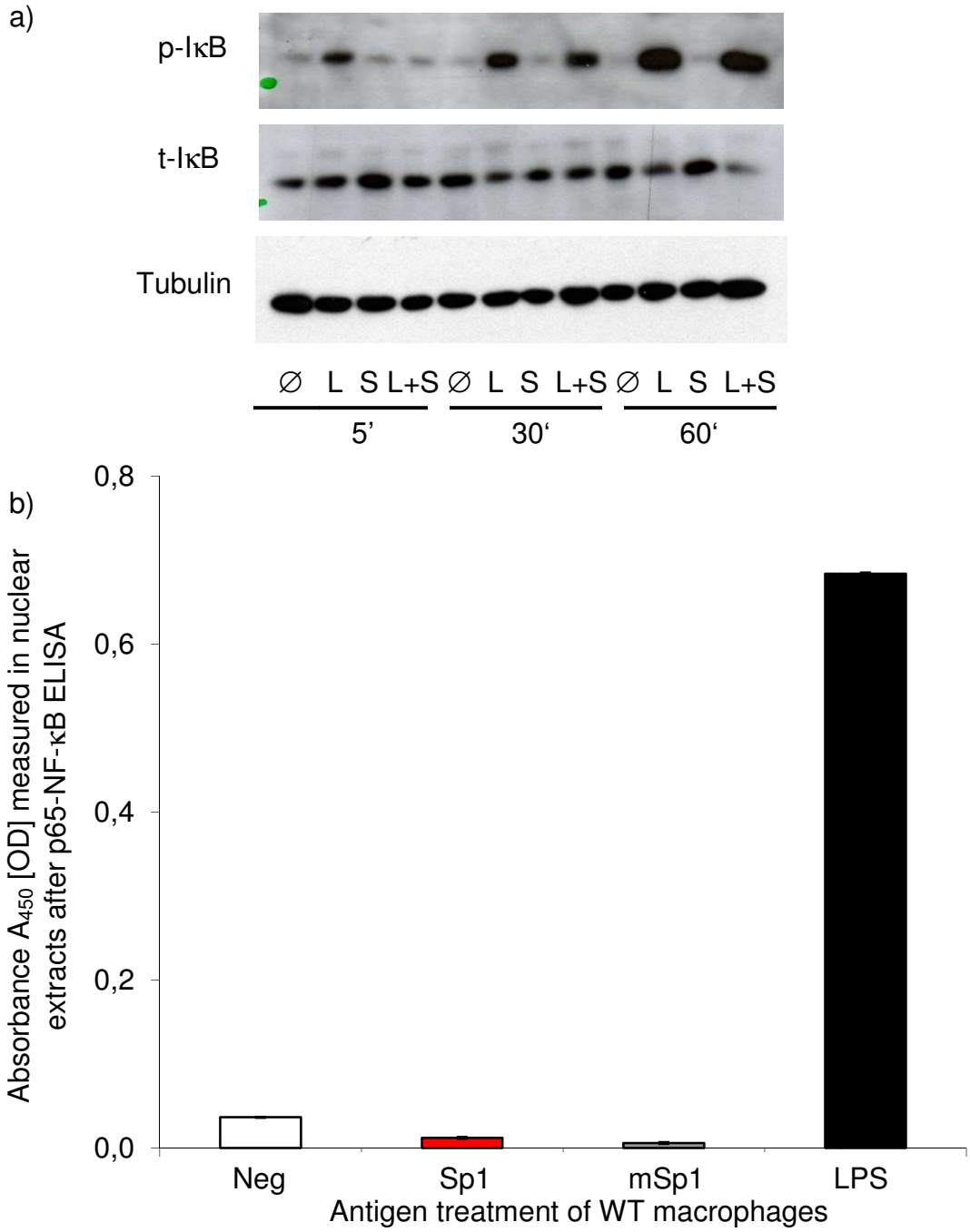


FIGURE 3.19: BM-derived macrophages show no Sp1-induced NF-κB activation. a) BM-derived macrophages were treated with LPS (L), Sp1 (S) or LPS and Sp1 (L+S) for 5, 30 and 60 min. Then, the whole extracts were collected and tested for total and phosphorylated IκB by Western blot (IκB MW: 40 kDa). b) BM-derived macrophages were treated with medium alone, mSp1, Sp1 or LPS for 15 min. Nuclear extracts of macrophages were used for NF-κB ELISA measurements. The experiments were repeated two times in an independent manner in triplicates.

3.13 Sp1-mediated NF-κB activation in murine BM-derived DCs

BM-derived DCs were incubated with various TLR ligands alone or in the presence of Sp1 to test for competition to determine possible receptors for Sp1. BM-derived DCs treated with Sp1 alone were capable of activating the NF-κB subunit p65 in contrast to untreated cells. The TLR2, TLR3, TLR5, TLR7 and TLR9 ligands Pam3, Poly I:C, Flagellin, Gardiquimod and ODN, respectively, showed either slight or even no difference between the incubation with the ligand alone and co-incubation of ligand and Sp1. Interestingly, LPS-treated DCs showed an increased level in p65-translocation to the nucleus in comparison to LPS plus Sp1-treated cells. The co-incubation of LPS and Sp1 lead to a 50% decrease in NF-κB activation (Fig. 3.20). TLR4 was hence investigated in further detail as potential receptors for Sp1. TLR2 has been demonstrated to be the receptor for PSA, which upon stimulation activates NF-κB (Wang et al., 2006). Therefore, it was of interest to further investigate whether TLR2-signalling was induced by other ZPS, like Sp1, too.

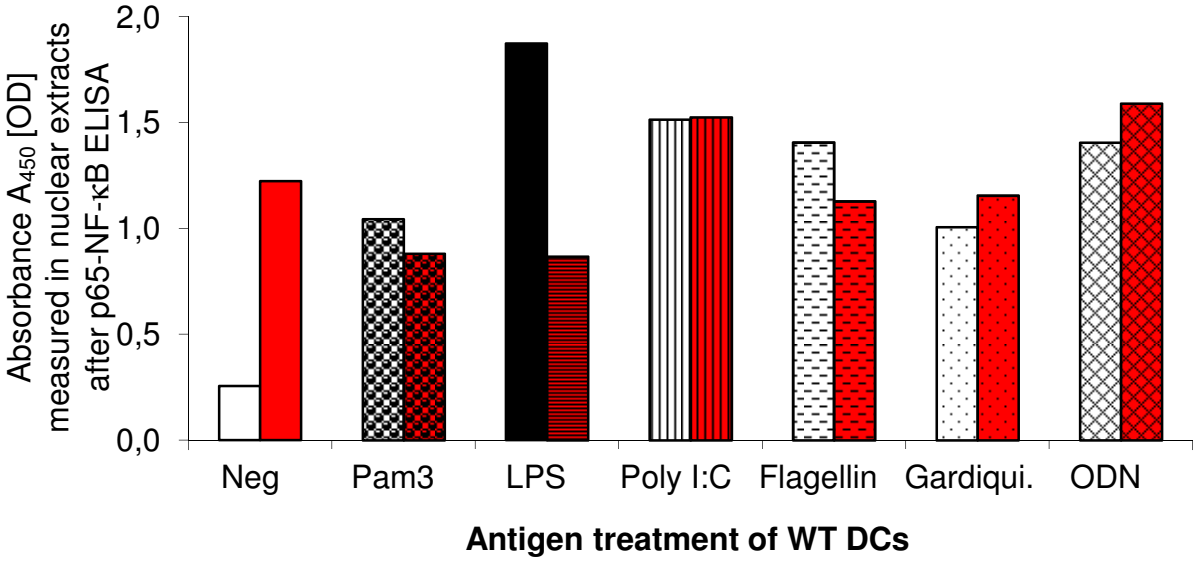


FIGURE 3.20: LPS and Sp1 compete for TLR4. BM-derived DCs were incubated for 15 min in the presence (red bars) or absence (white bars) of Sp1 with medium (Neg) or various TLR ligands: Pam3 (TLR2 ligand), Poly I:C (TLR3 ligand), LPS (black bar; TLR4 ligand), Flagellin (TLR5 ligand), Gardiquimod (=Gardiqui., TLR7 ligand) and ODN (TLR9 ligand). Cells were washed, nuclei extracted and analysed for p65-translocation (NF-κB activation) by NF-κB ELISA.

3.14 Sp1 induces TLR2 and TLR4 expression in human monocytes

As TLR2 and particularly TLR4 seemed to be potential Sp1 receptors, the surface expression of these two receptors was checked upon Sp1 treatment. In contrast to TLR4 surface expression in untreated human monocytes (1.8%), Sp1-treated human monocytes showed a TLR4 expression of 26.1%. TLR2 expression increased from 7.9% in untreated cells to 17.3% in Sp1-treated cells (Fig. 3.21).

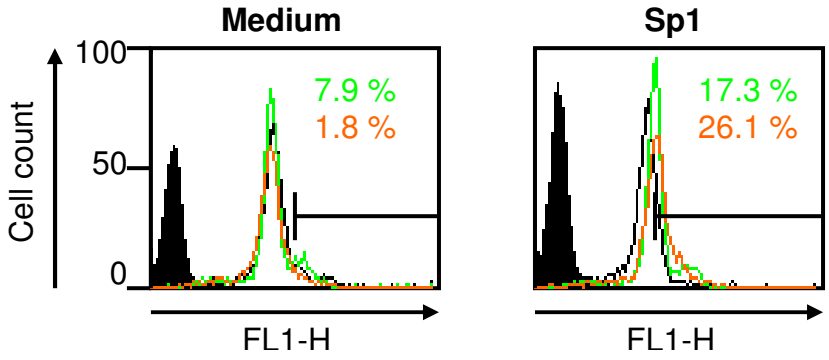


FIGURE 3.21: Sp1 increase surface expression of TLR2 and TLR4 in human monocytes. Human monocytes were treated with Sp1 or medium alone for 8 h and analysed by FACS. The results indicate non-stained cells (filled black histogram), cells stained with the isotype control IgG2a (black line), with the antibody to TLR2 (green line), and the antibody to TLR4 (orange line). Percentage of positively stained cells are indicated above the marker, for TLR2 (green), for TLR4 (orange).

3.15 Sp1-internalization by TLR2KO and TLR4KO DCs

To test whether DCs of TLR2KO mice and TLR4KO mice were capable of internalizing Sp1, BM-derived DCs were treated with fluorescence-labelled Sp1 and observed for Sp1 internalization. TLR4KO as well as TLR2KO DCs efficiently internalized Sp1, demonstrating that Sp1 uptake was independent from TLR2 or TLR4 recognition (Fig. 3.22) and internalized Sp1 like WT DCs as shown in Fig. 3.12.

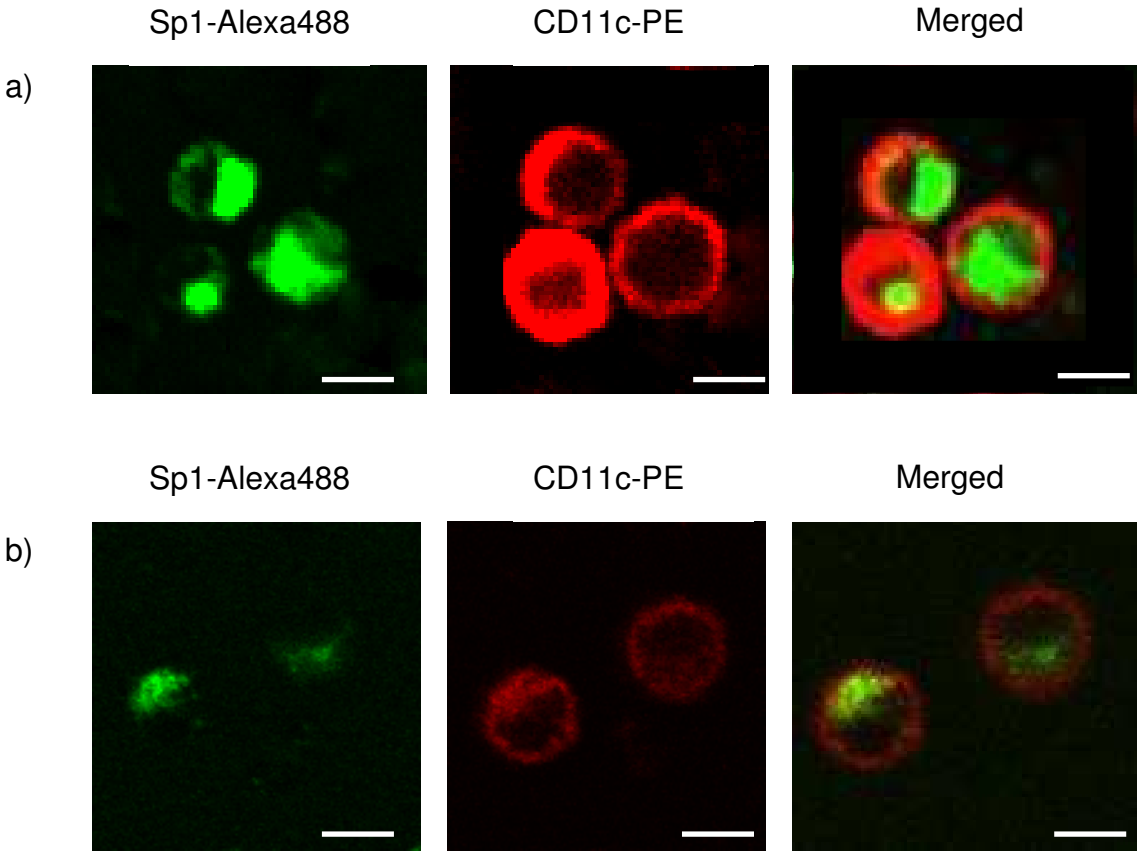


FIGURE 3.22: Sp1 is endocytosed by TLR2KO and TLR4KO DCs. BM-derived DCs from a) TLR2KO mice and b) TLR4KO mice were incubated for 24 h with Sp1-Alexa488 (green) and then stained with an anti-CD11c-PE (red). Scale bar: 10 µm.

3.16 Sp1 induces TLR2 and TLR4 expression in WT and KO mice DCs

As BM-derived DCs of TLR2KO and TLR4KO mice were capable of internalizing Sp1, DCs of WT as well as TLR2KO, TLR4KO and MyD88KO mice were investigated for TLR2 and TLR4 expression upon Sp1 treatment. MyD88KO DCs were used to determine whether Sp1 utilized a MyD88-dependent or MyD88-independent pathway to activate NF-κB. Upon Sp1 treatment WT DCs showed a high TLR2 as well as TLR4 expression of 12% and 11% respectively. TLR2KO DCs showed upon Sp1 treatment a low TLR2 expression of 1% and a TLR4 expression of 8%. TLR4KODCs upon Sp1 treatment expressed highly TLR2 (8%) and low TLR4 (2%). Interestingly, TLR2 as well as TLR4 expression were also impaired in MyD88KO DCs with 1% and 4% respectively after Sp1 treatment (Fig. 3.23).

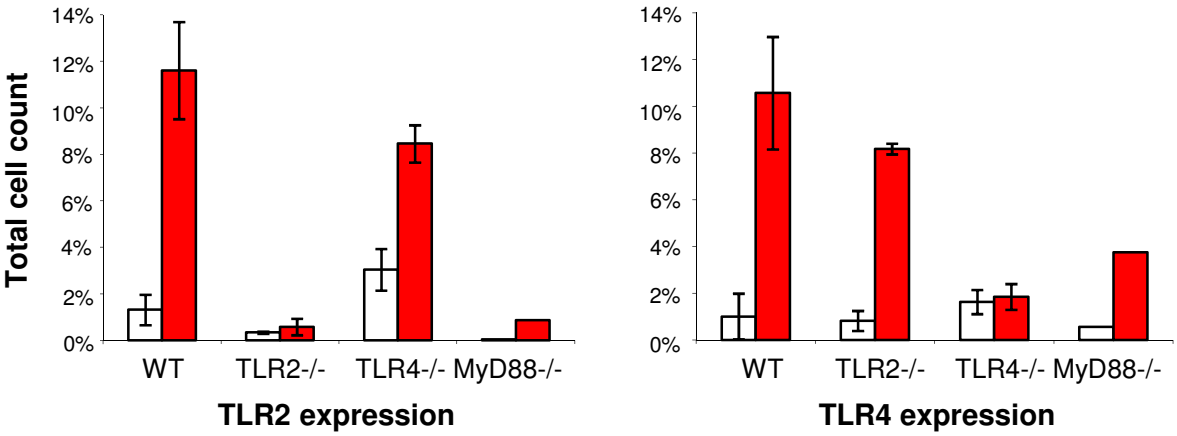


FIGURE 3.23: Sp1 increase surface expression of TLR2 and TLR4 in murine BM-derived DCs. Murine DCs were treated with medium alone (white bars) or Sp1 (red bars) for 8 h, stained with TLR2 or TLR4 antibodies and analysed by FACS. Each experiment was repeated at least three times, except for MyD88KO, which was performed once due to limited numbers of mice.

So far it was shown that TLR2KO DCs as well as TLR4KO DCs internalize Sp1. It was also demonstrated that upon Sp1 uptake, WT DCs expressed TLR2 as well as TLR4. Sp1 might induce TLR4 expression in BM-derived DCs in a MyD88-independent manner.

3.17 Sp1-induced cell maturation is mediated by TLR4

Cell maturation as well as cytokine production can occur in response to TLR activation. Maturation of DCs is characterized by the up-regulation of MHC class II and co-stimulatory molecules such as CD80 and CD86. Sp1-induced IL-6 production and secretion by WT DCs had been demonstrated in this study. To test whether Sp1 leads to cell surface maturation in APCs, human monocytes were incubated for 8 h with medium alone, Sp1 alone, or Sp1 with blocking antibodies. In comparison to untreated cells or isotype-treated cells, Sp1-treated human monocytes showed high HLA-DR and co-stimulatory surface expression for CD80 and CD86. Treating the cells with Sp1 plus isoltype control, anti-CD14 or anti-TLR2 did not lead to mentionable differences. However, monocytes treated with Sp1 plus anti-TLR4 showed a strong decrease for all three tested surface maturation markers (Fig. 3.24).

This result demonstrated clearly that TLR4 is required for Sp1-induced cell maturation as well as that anti-TLR4 antibodies lead to a decreased expression of the co-stimulatory molecules.

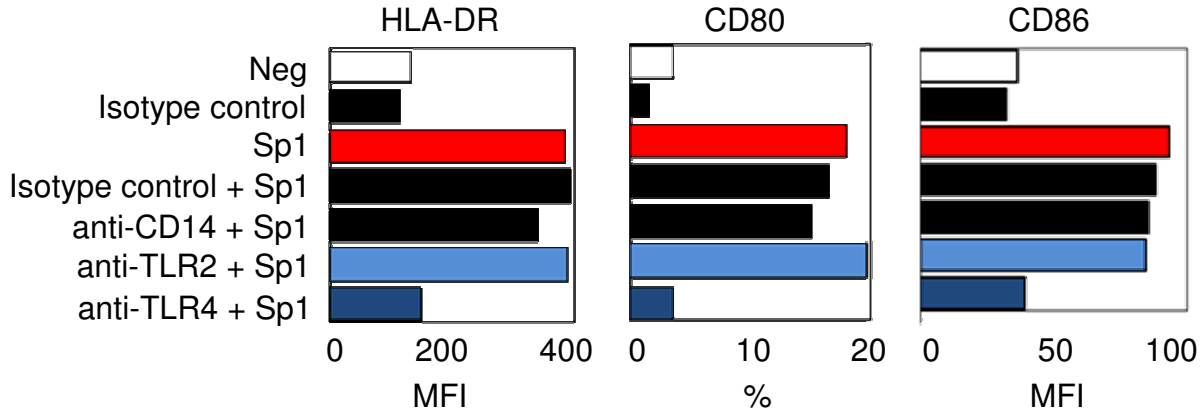


FIGURE 3.24: Anti-TLR4 antibody inhibits Sp1-mediated APC maturation. Human monocytes were incubated in medium alone, in Sp1 (50 µg/mL) alone, or in Sp1 with blocking antibodies to either CD14, TLR2, or TLR4 (10 µg/mL each). Surface expression of HLA-DR, CD80, and CD86 was detected after 8 h of incubation by FACS; MFI (Mean Fluorescence Intensity), % (Total cells).

3.18 Sp1-induced T cell proliferation is impaired by TLR4 blocking antibody

Direct interaction of T cells with APCs had been shown to be essential for ZPS-mediated T cell activation (Kalka-Moll et al., 2002). Polysaccharide-mediated $CD4^+$ T cell activation can also be measured *in vitro* by monitoring T cell proliferation (Tzianabos et al., 2000b).

Therefore APCs and T cells were incubated either in presence or absence of Sp1 and with or without blocking antibodies. Cells were then tested for Sp1-induced $CD4^+$ T cell proliferation. This experiment disclosed an almost 9-fold increase of T cell proliferation after Sp1 treatment compared to untreated cells or untreated cells plus isotype control. Cells treated with Sp1 plus anti-TLR2 did not affect the increase in T cell proliferation upon Sp1 treatment. Blocking antibodies against TLR4, however, reduced the Sp1-induced T cell proliferation to about levels of untreated cells (Fig. 3.25). This confirmed that TLR4 was definitely required for Sp1-mediated T cell activation. Next, Sp1-induced NF-κB activation in TLR2KO mice, TLR4KO and MyD88KO DCs was investigated.

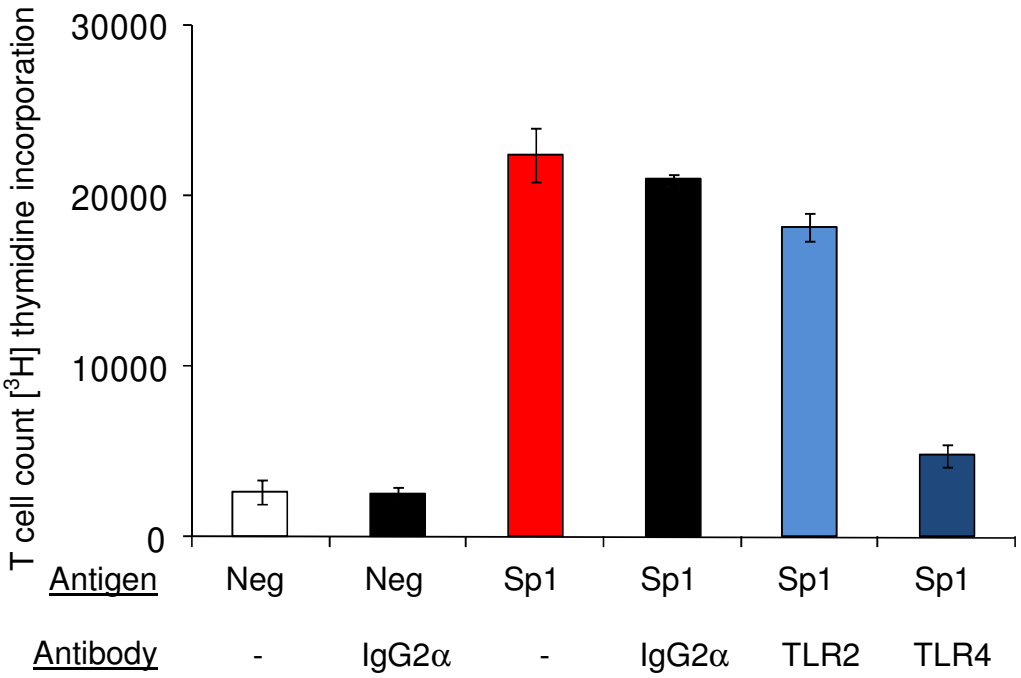


FIGURE 3.25: Anti-TLR4 antibody inhibits Sp1-induced CD4$^+$ T cell proliferation. The cells were treated for 6 h with medium alone, Sp1, or Sp1 with monoclonal blocking antibodies against TLR2 or TLR4 or the isotype control IgG2α. T cell proliferation was quantitated by [^{3}H]thymidine incorporation (1 µCi/well). Mean and standard deviation were calculated from three individual experiments.

3.19 Sp1-induced NF-κB activation is impaired in TLR4KO DCs

To explore the appropriate TLR receptor for Sp1 that mediates NF-κB-activation, DCs of WT as well as TLR2KO and TLR4KO mice were investigated for NF-κB activation after Sp1 treatment. BM-derived DCs were incubated with medium alone (untreated) or treated with LPS or Pam3 in the absence and presence of Sp1 and investigated for the nuclear translocation of the p65 subunit. MyD88KO DCs were treated with medium alone, mSp1, Sp1, LPS, Sp1 and LPS.

Sp1-treated WTDCs had an increased p65-translocation level compared to untreated WTDCs or mSp1-treated WTDCs, which were used as control. The untreated DCs and mSp1-treated DCs of WT as well as KO mice were all at similarly low levels. Sp1-induced p65-translocation in TLR2KO DCs was clearly increased and had similar levels of p65-translocation as in WTDCs after Sp1 treatment (Fig. 3.26). In TLR2KO DCs, NF-κB activation was decreased after treatment with Pam3 alone. In contrast, Pam3 plus Sp1-treated DCs were able to activate NF-κB in an almost

equal level as after treatments with Sp1 alone or LPS plus Sp1 in TLR2KO DCs. Interestingly, Sp1-induced p65-translocation level was 60 % decreased in TLR4KO DCs compared to in Sp1-treated WT DCs. Levels of p65-translocation in TLR4KO DCs was also decreased after treatment with LPS in the absence and presence of Sp1 and at similar low levels as after treatment with Sp1 alone. The levels of NF-κB activation after Sp1, LPS and Sp1 plus LPS treatment was either only slightly increased or at similar levels of p65-translocation in untreated TLR4KO DCs or mSp1-treated MyD88KO DCs, respectively. Treatment with the TLR2 ligand Pam3 in absence or presence of Sp1 showed similarly high increased p65-translocation in WT DCs as well as TLR4KO DCs. This experiment showed TLR4 as the potential receptor for Sp1 in order to activate NF-κB. As Sp1-induced NF-κB activation was shown to be impaired in TLR4KO DCs but not in TLR2KO DCs, in the next step, Sp1-induced IL-6 production was compared between WT and KO mice.

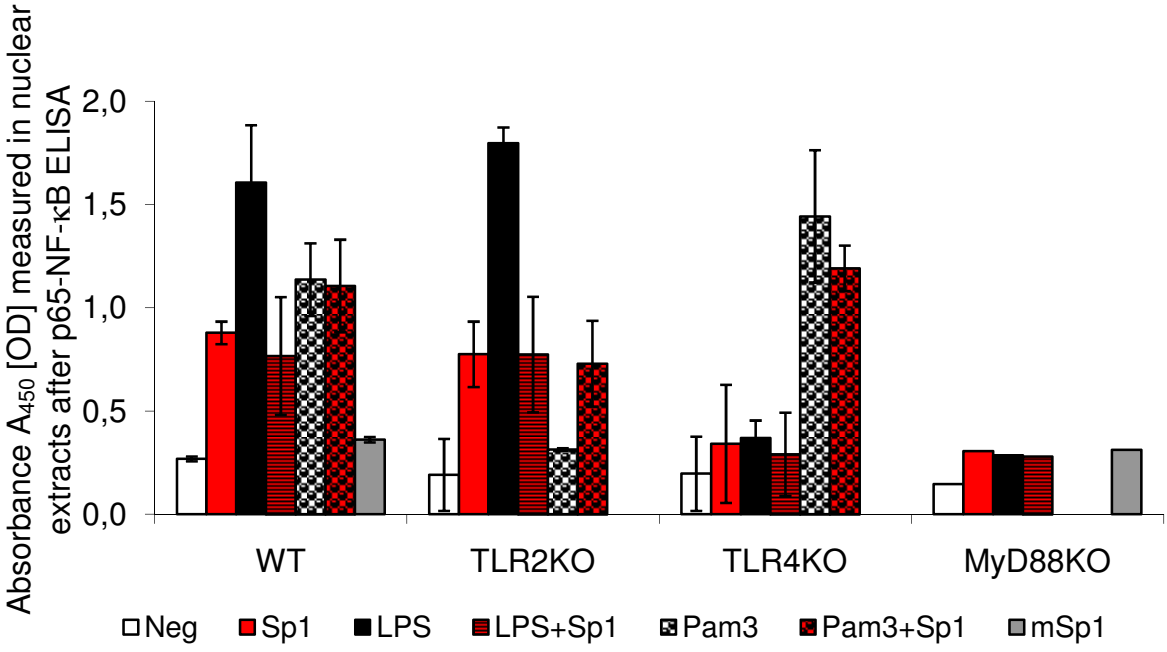


FIGURE 3.26: NF-κB activation in WT and KO DCs Sp1 treatment. BM-derived DCs from WT, TLR2KO, TLR4KO and MyD88KO mice were treated for 15 min with LPS, Pam3, mSp1, or medium alone in the presence of absence of Sp1. Cells were washed, nuclei extracted and the p65-translocation was measured with a p65-NF-κB ELISA. Mean and standard deviation were calculated from at least three independent experiments.

3.20 Decreased IL-6 production in Sp1-positive TLR4KO DCs

DCs of WT, TLR2KO and TLR4KO mice as well as control IL-6KODCs, were treated with Sp1 and checked then via ICS for IL-6 production. Sp1-induced IL-6 secretion was increased in WTDCs compared to untreated DCs. Sp1-induced IL-6 production in IL-6KO DCs was impaired and similar to untreated cells. TLR2KO DCs had an Sp1-induced IL-6 production almost as high as in WT DCs (Fig. 3.27). Interestingly, IL-6 accumulation was reduced in TLR4KO DCs and showed a similarly low level of about 2.7% IL-6-production in CD11c-positive DCs as in IL-6KO DCs. The data so far supported TLR4 as an essential Sp1 receptor and therefore TLR4 remained in focus for the further experiments. Sp1 could induce NF-κB activation as well as IL-6 production in TLR2KO DCs and so far showed no impact in Sp1-mediated immune responses and was therefore omitted in the next experiments.

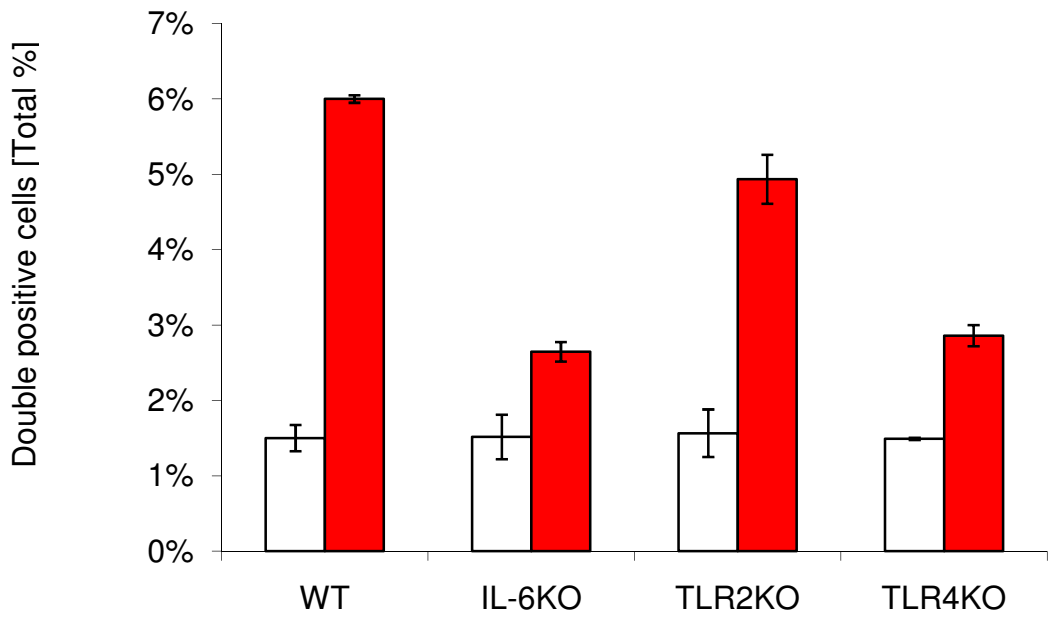
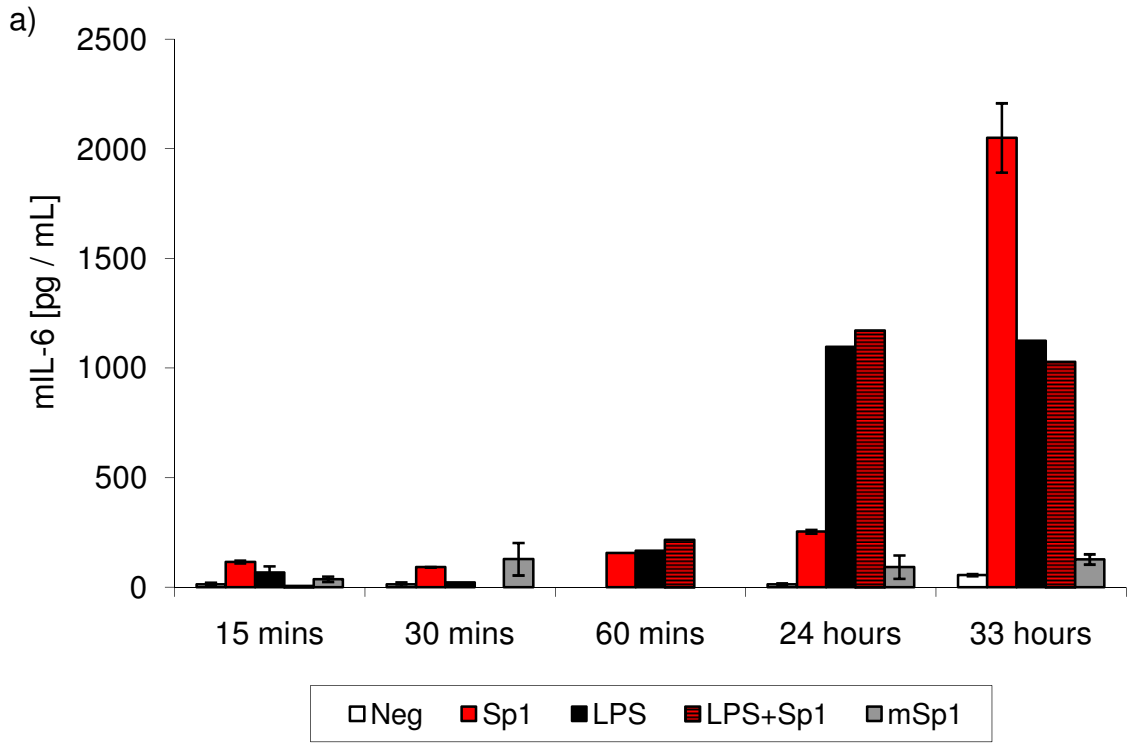


FIGURE 3.27: IL-6 production is impaired in TLR4KO DCs upon Sp1 treatment. DCs from WT as well as KO mice were treated with medium alone (white bars) or with Sp1 (100 µg / mL, red bars) for 33 h. An ICS was performed with an anti-IL-6-FITC murine antibody and DCs were stained with CD11c-(PE)-specific murine antibody. DCs were then analysed by FACS for double positive cells. Mean and standard deviation were calculated from three individual experiments.

3.21 Impaired IL-6 secretion in Sp1-treated TLR4KO DCs

IL-6 secretion upon Sp1 treatment was tested by IL-6 ELISA. BM-derived WT DCs were treated for different time points with Sp1, LPS, and Sp1 plus LPS and the culture supernatants were checked for IL-6 secretion after 15 min, 30 min, 60 min, 24 h and 33 h. The highest IL-6 secretion in WT DCs was detected in this kinetic study 33 h after Sp1 treatment (Fig. 3.28 a)). The Sp1-induced IL-6 secretion was even higher than the LPS-induced IL-6 secretion which was added as control.

Then, BM-derived DCs of WT, TLR4KO, IL-6KO and MyD88KO mice were treated with Sp1, LPS or both for 33 h and supernatants were measured for IL-6 secretion by IL-6 ELISA. IL-6KO DCs were taken as control and they showed almost no measurable values for IL-6 secretion. Similar results were also obtained for TLR4KO DCs treated with Sp1, LPS, both or mSp1 (Fig. 3.28 b)). In WT DCs treated with LPS in absence and presence of Sp1, 1125 and 1028 pg/mL IL-6 were secreted, respectively. In MyD88KO DCs these values were lower. In MyD88KO DCs, 92 pg/mL IL-6 were measured after Sp1 treatment compared to 2050 pg/mL IL-6 in Sp1-treated WT DCs. LPS alone lead to an IL-6 secretion of 98 pg/mL and LPS plus Sp1 treatment of MyD88KO DCs to 203 pg/mL IL-6. Next to be investigated, was the requirement of TLR4 in Sp1-mediated abscess formation.



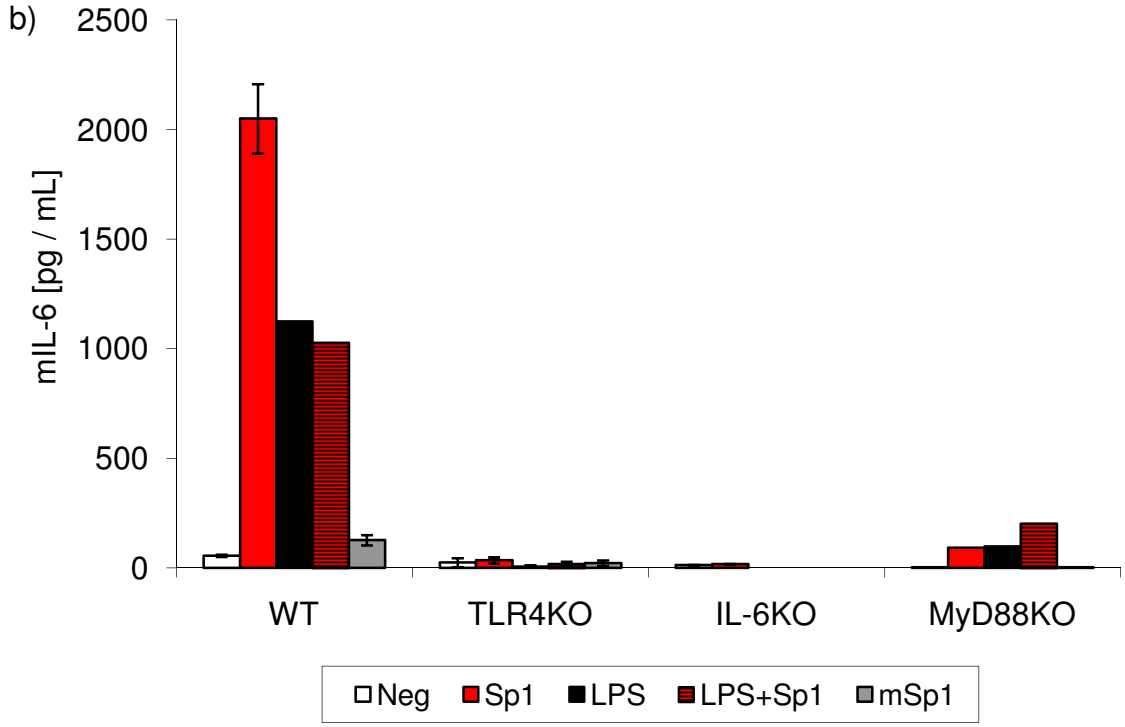


FIGURE 3.28: IL-6 secretion 33 h after Sp1 treatment in WT DCs. a) Kinetics of IL-6 secretion of WT DCs treated with medium, Sp1, LPS, LPS plus Sp1 or mSp1 after 15 min, 30 min, 60 min, 24 h and 33h. IL-6 secretion was measured with an IL-6 ELISA. b) After 33 h, supernatants of WT, TLR4KO, IL-6KO and MyD88KO DCs treated with medium, Sp1, LPS, Sp1 and LPS, or mSp1 were collected and analysed by IL-6 ELISA for IL-6 secretion. The experiments were repeated three times in an independent manner, except for MyD88KO DCs due to mice shortage.

3.22 Sp1-mediated abscess formation is impaired in TLR4KO mice

Intraperitoneal abscess formation is a complex host response to bacterial infection that required several cell types. Activation of $CD4^+$ T cells by ZPS antigens is shown to be one essential factor in that process (Chung et al., 2003). In this experiment, it was investigated whether the role of $CD4^+$ T cell-dependent process required TLR4.

WT as well as TLR4KO mice were challenged with SCCA alone as negative control or Sp1 plus SCCA. WT mice injected with SCCA alone showed a significantly reduced abscess formation compared to WT mice injected with Sp1 plus SCCA ($p<0.05$). All Sp1-challenged WT mice formed abscesses. In contrast to WT mice, TLR4KO mice exhibited a significantly reduced ability to form abscesses upon Sp1

challenge ($p<0.05$) (Fig. 3.29). Therefore TLR4 was shown to be essential in forming Sp1-induced abscesses.

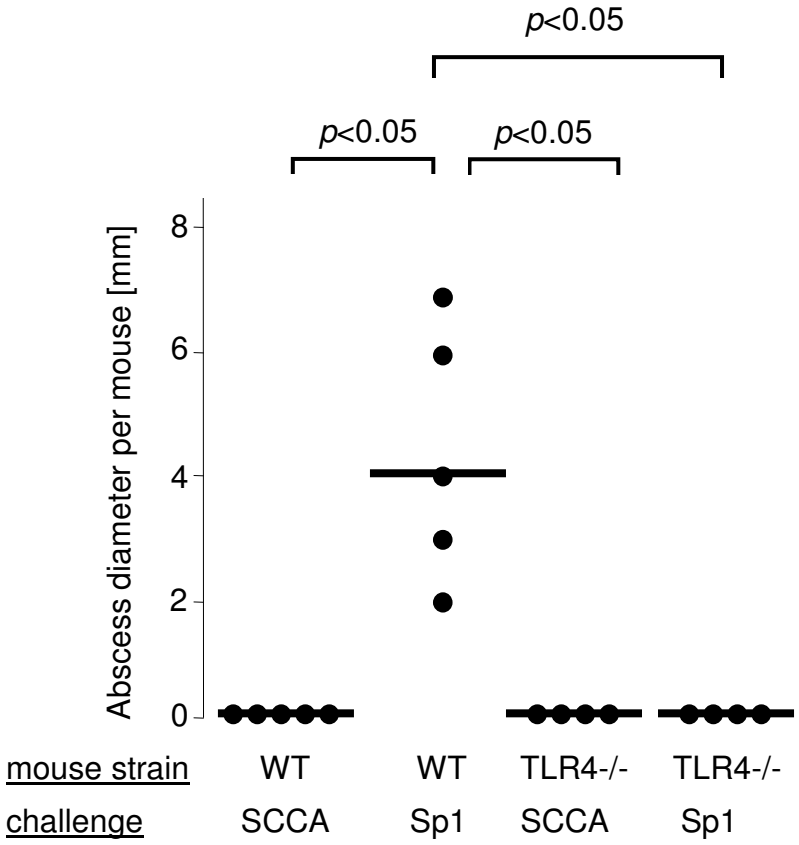


FIGURE 3.29: TLR4 is required for abscess formation in vivo. WT and TLR4KO mice were challenged intraperitoneally with sterile SCCA alone as negative control or Sp1 plus SCCA. Six days later, the mice were examined at autopsy for the presence of intraabdominal abscess formation. Each dot represents the total abscess size per mouse. Bars indicated the median abscess size per group. This figure showed a representative result out of at least three experiments performed independently.

The obtained results lead to the assumption that Sp1-activated NF-κB-mediated IL-6 production required TLR4. Sp1 treatment led to impaired NF-κB activation and IL-6 secretion in TLR4KO and MyD88KO DCs in comparison to WT DCs. Furthermore, co-stimulatory maturation molecule expression and $CD4^+$ T cell activation were decreased in Sp1-treated cells upon TLR4 blockade. Finally, no abscess formation was seen in TLR4KO mice upon Sp1 treatment.

4. Discussion

For long polysaccharides were believed to be T cell-independent antigens. Only recently, polysaccharides have been shown to elicit $CD4^+$ T cell-dependent immune responses due to their zwitterionic charge. ZPS-induced peritoneal abscess formation is a $CD4^+$ T cell-dependent immune response and can lead to peritonitis. Peritonitis is a life-threatening situation. Hence, it is important to understand the underlying mechanisms of ZPS-mediated abscess formation in order to develop effective methods of treatment. This study focused on the role of IL-6 in the ZPS-mediated abscess formation and used Sp1 as a model antigen.

4.1 Sp1 is an efficient model antigen for ZPS-induced abscess formation

For studies on ZPS, various capsular polysaccharides of *B. fragilis*, *S. aureus* and *S. pneumoniae* are available (Kalka-Moll et al., 2001, Tzianabos et al., 2001). Advantage of using Sp1 is that the capsule-lyophilisate of *S. pneumoniae* serotype 1 is commercially available. Besides, if required bacteria can be cultivated in the own laboratory under aerobe conditions. Furthermore, purification of ZPS of *B. fragilis* and *S. aureus* demand complex methods (Moreau et al., 1990). Also, the outer membrane of the capsule of *B. fragilis*, being a gram-negative bacterium, consists of LPS. Sp1 is isolated following a modified standardized method (Choi et al., 2002; Stephen et al., 2005). The purification procedure excludes a contamination with biological active substances by using pyrogen free and sterile materials. Sp1 was confirmed to be free of contamination by BCA test, LAL test and UV absorbance as well as NMR. The bioactivity of the Sp1 used in the experiments was ensured by abscess formation.

The removal of the positive charge of the amino group yielded an overall net negative charge Sp1. In this study, mSp1 had proven as reliable negative control due to its loss of biological activity to induce a $CD4^+$ T cell-dependent abscess formation.

4.2 IL-6 is the predominant intraperitoneal Sp1-induced cytokine in abscess formation

Crohn's disease is frequently associated with intraperitoneal sepsis and abscess formation and interestingly, in contrast to ulcerative colitis, shows higher IL-6 serum

concentrations (Van Kemseke et al., 2000; Niederau et al., 1997). The activation of the mucosal immune system is characterized by production of proinflammatory cytokines (Strober et al., 2002; Blumberg et al., 1999; Fuss et al., 1996). In particular, IL-6 and IL-6 signalling appear to play a pivotal role in inflammatory bowel diseases (IBDs). IL-6 is produced by APCs and T cells in IBD patients.

In this study, we demonstrate the need of IL-6 for the formation of polysaccharide-mediated intraperitoneal abscesses. In contrast to experimental abscess models in rats using whole *B. fragilis* and PSA1 as causative agents, Sp1 challenge in mice induces significantly higher levels of IL-6 than IL-1 and TNF–α (Gibson et al., 1996; Gibson et al., 1998). The peak response to the Sp1 challenge of IL-6 was at 6 h, of IL-1 at 12 h and of TNF-α at 24 h. In this case, IL-6 was the first of the three cytokines to be expressed. Please note that Fig. 3.4 (p. 50) shows a logarithmic scale as y-axis and otherwise even the peak values obtained in the kinetics for IL-1 and TNF-α would be barely visible compared to the IL-6 values.

Several methods and experiments were performed to test other cytokines but IL-6. In brief, we have performed TNF-α ELISAs and multiplex bead assays to screen for IL-1a, IL-2, IL-5, IL-10, IFN-γ, TNF-α, GM-CSF, IL-4, and IL-17 besides IL-6. These experiments did not show a notable increase of the other cytokines. We think that the presentation of IL-1, TNF-α, and IL-6 is -although restricted- preferable because they are the most important cytokines described so far for secondary peritonitis and abscess formation. IL-2 and IL-17 are also cytokines involved in abscess formation (Chung et al., 2003, Tzianabos et al., 1999) but as these are secreted by the $CD4^+$ T cells, they were not included here because this study clearly focuses on the cytokine expression by APCs and not T cells. ZPS such as PSA1, PSA2, Sp1 and CP8 have in common the zwitterionic charge motif that contains at least one positive and one negative charge per repeating unit (Tzianabos et al., 2001; 1993). However, for example, PSA1 has exactly one positive charge and one negative charge and Sp1 has two negative charges and one positive charge per repeating unit. Also, the saccharides and their number per repeating unit differ because Sp1 is a trisaccharide whereas PSA is a pentasaccharide. The other studies in regard to ZPS-mediated cytokine secretion were performed partly in rats with whole ZPS-positive bacteria or ZPS. Consequently, we believe that there are slight differences in the mechanism of abscess formation induced by Sp1 and other ZPS.

Besides the animal species, the differences include the structural differences, the production of cytokines and the signalling pathways (Wang et al., 2006).

IL-6 has been recognized as a major participant in elevating the inflammatory response in IBD such as Crohn's disease and ulcerative colitis. Both are severe intestinal inflammatory conditions which show increased expression of proinflammatory cytokines in inflammed biopsy (Rescigno, 2008; Leon et al., 2006; MacDonald and Monteleone, 2005). However, there is evidence suggesting that the unaffected tissue areas of IBD undergo an abnormal immune activation as shown by the expression of increased levels of the proinflammatory cytokines IL-6, TNF-α (Reimund et al., 1996), and IL-18, in intestinal biopsies from Crohn's disease patients (Monteleone et al., 1999). Cytokines are immunomodulators that regulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and differentiation of various cells (Fu, S. Y. et al., 2007).

4.3 IL-6 secretion by peritoneal and BM-derived macrophages and BM-derived DCs depends on the dose and ZPS charge of Sp1

Peritoneal as well as BM-derived macrophages and BM-derived DCs were shown in this study to internalize Sp1. This is a novelty compared to previous studies which showed Sp1 uptake only with human Raji or RJ2.2.5 B cells (Velez et al., 2008).

Peritoneal macrophages and DCs, both participating in the formation of the abscess wall, secrete IL-6 upon Sp1 treatment. Macrophages are an important cellular element, since they reside in the peritoneal cavity and help initiate immune responses through phagocytosis and cytokine secretion (Valle et al., 1995). DCs that represent a minor population of APCs migrating into the peritoneal cavity upon Sp1 challenge, are also incorporated into the abscess wall (Stephen et al., 2007). For unknown reasons, detection of IL-6 by DCs *in vivo* is rare. Yet, BM-derived DCs secrete IL-6 when treated with Sp1 *in vitro*. We show in BM-derived DCs *in vitro,* that the induction of IL-6 in DCs clearly depends on the zwitterionic charge motif because Sp1 lacking the free amino group and thus resembling a common negatively-charged polysaccharide induces a significantly lower IL-6 level. Besides, IL-6 secretion in BM-derived DCs is Sp1-dose dependent.

4.4 Sp1-mediated IL-6 secretion has migratory and anti-apoptotic effects on $CD4^+$ T cells

In comparison to WT mice, in IL-6-deficient mice the total number of macrophages and DCs is decreased by 28% (33.8% of total cells in WT mice and 24.3% of total cells in IL-6-deficient mice). However, the number of influx $CD4^+$ T cells is reduced by 73% in the peritoneal cavity in IL-6-deficient mice. Table 3.1 (p. 61) shows the numbers of peritoneal macrophages and DCs in IL-6KO and WT mice. Exogenous IL-6 cytokine treatment of IL-6KO mice restored the cell count of macrophages, DCs, and $CD4^+$ T cells to levels of WT mice. Besides suggesting a pleiotropic effect of IL-6 on APCs, the result also identifies IL-6 as a potential $CD4^+$ T cell migration factor in the peritoneum. We showed that murine $CD4^+$ T cells migrate into the peritoneum in an IL-6-dependent manner. $CD4^+$ T cells migration was increased almost 4 times from 50 ng/mL to 200 ng/mL IL-6.

Sp1 induces T cell activation and proliferation of mammalian $CD4^+$ T cells *in vitro* and *in vivo* (Kalka-Moll et al., 2002; Stephen et al., 2007; Velez et al., 2008). Activation and proliferation of $CD4^+$ T cells is sustained by IL-2 secretion. Prevention of apoptosis through IL-6 has been shown in T cells at different stages of the cell cycle (Teague et al., 1997). Testing the effect of Sp1 on apoptosis of $CD4^+$ T cells, we observe that Sp1 inhibits their apoptosis. This effect is abrogated when the ZPS is converted into a non-ZPS and in the absence of IL-6. This result implies that IL-6 is an essential cofactor for the protection from cell death and might indirectly support IL-2-mediated cellular activation and proliferation in response to ZPS (Tzianabos et al., 1999; Tzianabos et al. 2000a).

4.5 Anti-IL-6 antibodies protect WT mice from Sp1-mediated abscess formation

The crucial question is whether abrogation of the pleiotropic functions of IL-6 blocks abscess formation. Indeed, abscess formation is significantly inhibited in IL-6-deficient mice. Various options for the prevention and treatment of abscesses are still being investigated. For example, in a peritonitis model in rats, treatment with tissue-type plasminogen and urokinase during the first 24 h after leakage of intestinal contents into the peritoneal cavity was very effective in preventing intra-abdominal abscess formation (Buyne et al., 2008). Besides the prophylaxis and treatment of pathologies with anti-IL-6 monoclonal antibodies that has been shown to be effective

against lethal *Escherichia coli* infection, lethal TNF-α challenge in mice, and Crohn's disease, we see that treatment of mice with a neutralizing IL-6-specific antibody reduces abscess formation by 50% (Adachi et al., 2008; Mitsuyama et al., 2006; Starnes et al. 1990). As a consequence, early application of an IL-6-specific antibody, or targeting of IL-6 signalling, in secondary peritonitis or abdominal surgery involving intestinal incisions may offer a prophylactic strategy for the prevention of intra-peritoneal abscess formation (Mitsuyama et al., 2006).

4.6 Sp1-induced NF-κB activation

BM-derived macrophages were tested for NF-κB activation after Sp1 treatment. NF-κB activation was not detected by using total and phosphorylated IκB antibodies and subjecting the SDS PAGE to Western blot. In addition, the nuclear translocation of NF-κB subunit p65 by NF-κB ELISA did also not show a significant increase after Sp1 treatment of macrophages. Sp1-mediated NF-κB activation seemed to utilize different mechanisms in BM-derived macrophages than in BM-derived DCs. BM-derived DCs were used for further investigating Sp1-mediated IL-6 secretion. IL-6 possess an NF-κB binding site in their promoter regions (Gruss et al., 1992). Therefore, in this study it was suspected that the Sp1-induced IL-6 secretion in murine DCs may involve TLR-mediated NF-κB activation. DCs treated with various TLR ligands in absence and presence of Sp1, showed LPS and Sp1 competing for TLR4-mediated NF-κB activation. NF-κB activation was increased after Sp1 treatment in WT DCs and TLR2KO DCs of this study. TLR4KO DCs and MyD88KO DCs, in contrast, showed rather impaired NF-κB activation and IL-6 secretion after Sp1 treatment. T cell activation requires TLR-mediated recognition of PAMPs, induction of co-stimulation and cell maturation in DCs and production of IL-6 by DCs in response to TLR ligation during infection (Pasare and Medzhitov, 2003). The special focus was on Sp1-induced NF-κB activation mediated by TLR4.

4.7 TLR4-blocking antibodies inhibit Sp1-induced APC maturation and $CD4^+$ T cell proliferation

In APCs cytokines are mainly regulated via TLR signalling (Underhill et al., 1999). In recent years, TLRs have emerged as critical to recognise a variety of PAMPs of

infectious agents (Akira et al., 2006). In mammalian tissues, TLRs are highly expressed by resident immune cells, including DCs and tissue macrophages. Upon binding ligands, all known TLRs except for TLR3 can activate downstream signalling cascades through the adaptor protein MyD88 to induce the production of inflammatory cytokines. These cytokines are interferon-α (IFN-α), IFN-β, IL-12, TNF-α, IL-6, and IL-1. Especially TLR-activation in DCs induce NF-κB-dependent IL-6 and IL-1 production (Kubo et al., 2004). Trif-dependent (MyD88-independent) pathways induce NF-κB or IRF-mediated type I IFN production (Tamura et al., 2008; Kawai and Akira, 2006). IRF4 has been shown to play a key regulatory role for IL-6 production and consecutive activation of T lymphocytes in experimental colitis in mice (Mudter et al., 2008).

The CD40 ligand interaction on T cells with the CD40 on APCs is critical to elevate expression of other co-stimulatory molecules and the secretion of a variety of cytokines including IL-6 (Banchereau et al., 2000). It is feasible that the previously published requirement of co-stimulatory interactions via B7-CD28 and CD40-CD40L for ZPS-dependent $CD4^+$ T cell activation and abscess formation contribute significantly to the up-regulation of IL-6 synthesis (Tzianabos et al., 2000b; Stephen et al., 2005).

In contrast to PSA1 from *B. fragilis* that causes macrophages and DCs to secrete IL-12 and TNF-α in a TLR2-dependent pathway, macrophages and DCs do not secrete TNF-α or IL-12 in response to Sp1 (unpublished data and Wang et al., 2006). Thus, the immune response to the ZPS Sp1 with predominant IL-6 secretion seemed to be conferred by another TLR than TLR2. Stimulation of murine macrophages with the TLR4 ligand LPS has been shown to induce enhanced cell surface antigen expression such as MHCII, CD80 and CD86 as well as IL-6 production (Ulevitch and Tobias, 1995; Yamamoto et al., 2002). Sp1-treatment also induce increased HLA-DR, CD80 as well as CD86 surface display in human monocytes (Stephen et al., 2005). Previous experiments revealed Sp1-induced cell maturation by increased MHCII, CD80 and CD86 surface display also in murine DCs (data not shown). In this study, the TLR responsible for Sp1-mediated APC maturation was identified. Human monocytes were treated with Sp1 in absence and presence of TLR-blocking antibodies. We showed TLR4 blocking antibodies to inhibit Sp1-induced co-stimulatory expression on APCs. Sp1 plus TLR2 blocking antibodies showed increased APC maturation as in cells treated with Sp1 alone.

Sp1 is shown to induce T cell-proliferation (Kalka-Moll et al., 2002; Stephen et al., 2007; Velez et al., 2008). In this study, Sp1-induced T cell-proliferation was inhibited upon the TLR4 blocking antibody application. Blocking antibodies to TLR2 had no inhibitory effect on APC maturation or T cell proliferation and behaved like the cells treated with Sp1 alone affirming the importance of TLR4 in Sp1-mediated $CD4^+$ T cell immune responses.

4.8 Sp1-induced abscess formation is TLR4-dependent

We found the Sp1-internalization capabilities of TLR2KO DCs and TLR4KO DCs to be similar to the Sp1-internalization by WT DCs. This indicated that the internalization of Sp1 was not necessarily receptor-specific. A lack of TLR4 did not inhibit the Sp1 uptake. Interesting was the TLR4 surface display after Sp1 treatment in MyD88KO DCs. The low TLR2 surface display and the increase –though comparably slight- of TLR4 surface display in these MyD88KO DCs after Sp1 treatment hinted towards a possible involvement of a MyD88-independent pathway. Sp1 is an extracellular model antigen. Therefore, an extracellular TLR is required which not only recognizes Sp1 but also, based on this result, activates the MyD88-independent pathway. TLR4 being an extracellular TLR as well as being capable to activate a MyD88-dependent as well as MyD88-independent pathway, supports the assumption that TLR4 is a possible receptor for Sp1. MyD88-deficient mice lose their ability to produce proinflammatory cytokines, such as TNF-α, IL-1, IL-6 and IL-12p40, in response to LPS. Albeit with delayed kinetics compared with wild-type cells, DCs of MyD88-deficient mice can still trigger activation of NF-κB as well as retain their capacity to produce IFN-inducible genes and co-stimulatory molecules and to mature in response to LPS (Kawai et al., 1999; 2001 and Kaisho et al. 2001). Most other TLRs compared to TLR4 are completely ineffective at activating NF-κB in MyD88KO mice (Kawai et al., 1999). This indicated that Sp1 might utilize the TLR4 pathway to activate NF-κB and produce IL-6.

We showed that Sp1 treatment lead to impaired IL-6 production in TLR4KO DCs, whereas TLR2KO DCs produced IL-6 at similar levels as WTDCs. In addition, this study showed by means of p65-NF-κB-ELISA that the Sp1-induced IL-6 secretion in TLR4KO DCs is impaired and comparable to the levels of IL-6 secretion of IL-6KO DCs. With the abscess formation model, finally, we proved TLR4 to be

essential for Sp1-mediated abscess formation. TLR4KO mice showed significantly decreased abscess formation in comparison to WT DCs after Sp1 challenge.

4.9 Other possible receptors for Sp1-mediated IL-6 secretion

The immune system is complex and therefore TLR4 might not be the only receptor to detect Sp1 to mediate Sp1-induced immune responses.

Many studies have shown that NOD1 and NOD2 signalling, like TLR signalling, are capable of activating NF-κB as well as IL-6 production (Uehara and Takada, 2007; Akira et al., 2006; Ogura et al., 2001). NOD1 and NOD2 are intracellular receptors. *Ex vivo* studies showed that *S. pneumoniae* whole bacteria was sensed by NOD2 and might mediate NF-κB-dependent cell activation (Opitz et al., 2004). WT DCs treated with NOD1 or NOD2 ligands in absence or presence of Sp1 showed, however, no difference in the NF-κB activation (data not shown). Sp1 has been shown to be transported retrogradually from endosome to lysosomes and then via tubuli to the cell surface (Stephen et al., 2007). Therefore, most likely, Sp1 does not entertain a direct contact with cytoplasm and NOD1 or NOD2 receptors.

Another possible receptor might be DC-SIGN. This C-type lectin receptor is present on DCs and has been shown to recognize specifically *Streptococcus pneumoniae* serotypes 3 and 14 (Koppel et al., 2005). Besides functioning as an adhesion molecule, a recent study has also shown that DC-SIGN can initiate innate immunity by modulating toll-like receptors (den Dunnen et al., 2009). But the detailed mechanism is not yet known and an involvement in IL-6 secretion has not been demonstrated yet.

4.10 Conclusion and future perspective

Based on these results we conclude that in peritoneal abscess formation induced by the ZPS Sp1, macrophages and DCs recognize ZPS and secrete IL-6. This stimulates the IL-6 dose-dependent migration of $CD4^+$ T cells to the peritoneum, and inhibits their apoptosis. Peritoneal macrophages, BM-derived macrophages and BM-derived DCs are capable of internalizing Sp1and thereafter to produce IL-6. Inhibition of abscess formation in the absence of IL-6 confirms the biologic importance of this innate immune mediator for the cellular adaptive host response. TLR4 is essential for Sp1-mediated APC maturation and T cell proliferation. Sp1-mediated NF-κB required TLR4. TLR4 has been shown to be essential in Sp1-mediated IL-6 secretion and T cell-dependent abscess formation. Therefore, this study shows that Sp1 can induce NF-κB-mediated IL-6 secretion and abscess formation via TLR4 signalling.

Despite many remaining questions, it is clear that TLR regulation of the various transcription factors is crucially important in regulating the expression of genes that mediate innate and adaptive immunity. This area offers much potential in terms of therapeutic exploitation. Recently, TLR9-induced type I IFN has been shown to be protective from colitis (Katakura et al., 2005). Such studies and the results obtained with the anti-IL-6 antibody treatment to prevent abscess formation and the observation that TLR4KO mice do not form abscesses upon Sp1-challenge open up further opportunities for therapeutical research.

Summary

Abscess formation associated with secondary peritonitis causes severe morbidity and can be fatal. Formation of abscesses requires the presence of $CD4^+$ T cells. Zwitterionic polysaccharides (ZPS) represent a novel class of immunomodulatory bacterial antigens that stimulate $CD4^+$ T cells in a MHC class II-dependent manner. The capsular polysaccharide Sp1 of *Streptococcus pneumoniae* serotype 1 possesses a zwitterionic charge with free amino groups and promotes T cell-dependent abscess formation in an experimental mouse model. So far, nothing is known about the function of IL-6 in a Sp1-mediated intraperitoneal abscess formation. ZPS are only recently been investigated to activate a Toll-like-receptor-mediated cytokine secretion. To date, a receptor for Sp1 has not yet been investigated, which is capable of mediating a Sp1-induced cytokine production.

In this study, we demonstrated that macrophages and DCs, the most prevalent professional antigen-presenting cells (APCs) involved in the formation of abscesses, internalize Sp1, secrete IL-6 and are incorporated in the abscess capsule upon Sp1 challenge *in vivo*. We found IL-6 secretion to occur in BM-derived DCs in a Sp1 dose-dependent manner. The Sp1-induced migration of $CD4^+$ T cells into the peritoneal cavity requires IL-6 and Sp1 inhibits apoptosis of $CD4^+$ T cells in an IL-6-dependent manner. Abrogation of the Sp1-induced pleiotropic effects of IL-6 in IL-6-deficient mice and wildtype mice treated with an IL-6-specific neutralizing antibody results in significant inhibition of abscess formation.

We show that Sp1-induced APC maturation and T cell-proliferation are inhibited in presence of TLR4 blocking antibodies. Sp1-induced NF-κB activation and IL-6 secretion are impaired in *in vitro* experiments of TLR4-deficient DCs. Sp1-mediated abscess formation is abrogated in TLR4-deficient mice. Altogether, the data shows TLR4 as a possible receptor for Sp1 and delineates the essential role of IL6 in the linkage of innate and adaptive immunity in Sp1-mediated abscess formation.

Zusammenfassung

Eine Abszessbildung, die mit einer sekundären Peritonitis einhergeht, kann schwerwiegenden Krankheitssymptome hervorrufen und fatale Folgen mit sich bringen. Damit sich Abszesse bilden können, müssen $CD4^+$ T Zellen vorhanden sein. Außerdem sind Makrophagen und DCs (Dendritische Zellen) die am häufigsten in der Entstehung von Abszessen vorkommenden professionellen Antigen-präsentierenden Zellen (APZ).

Bislang besagte das immunologische Dogma der Antigenpräsentation, dass Polysaccharide T Zell-unabhängige Immunreaktionen auslösen und nach der Internalisierung in den Lysosomen verbleiben. Zwitterionische Polysaccharide (ZPS) stellen jedoch eine neuartige Klasse von immunmodulatorischen bakteriellen Antigenen dar, welche $CD4^+$ T Zellen in einer MHC Klasse II-abhängigen Art stimulieren. Das kapsuläre Polysaccharid Sp1 von *Streptococcus pneumoniae* Serotyp 1 besitzt eine zwitterionische Ladung und kann so die T Zell-abhängige Abszessbildung in einem experimentellen Mausmodel begünstigen. Bislang ist über die Funktion von IL-6 in der Sp1-induzierten intraperitonealen Abszessbildung nichts bekannt. ZPS wurden erst in den letzten Jahren bezüglich einer TLR (Toll-like-receptor) vermittelten Zytokinausschüttung erforscht. Hier wurden nun der Zusammenhang von Sp1-induzierten Abszessen in Abhängigkeit von IL-6 als auch ein geeigneter Rezeptor für Sp1 untersucht.

In dieser Arbeit wird gezeigt, dass APZ in WT (Wildtyp) Mäusen nach Sp1 Gabe das Sp1 internalisieren, IL-6 ausschütten und Bestandteile der Abszesskapsel bilden. Für die durch Sp1-induzierte Migration von CD4 T^+ Zellen in die Peritonealhöhle ist IL-6 erforderlich. Zusätzlich konnte nachgewiesen werden, dass die Inhibition der $CD4^+$ T Zellapoptose durch Sp1 von IL-6 abhängig ist. Die Unterdrückung der Sp1-induzierten pleiotropischen Effekte von IL-6 in IL-6 defizienten Mäusen sowie WT Mäusen, die mit IL-6-spezifisch neutralisierenden Antikörpern behandelt wurden, resultieren in einer signifikanten Inhibition der Abszessbildung.

Außerdem konnte belegt werden, dass die Sp1-induzierte Reifung von APZ und die Proliferation von T Zellen durch TLR4-blockierende Antikörper eingeschränkt wurde. Die NF-κB-Aktivierung und IL-6 Ausschüttung durch Sp1 sind in TLR4-defizienten DCs

stark eingeschränkt. Überdies konnte in TLR4-defizienten Mäusen keine Sp1-vermittelte Abzessbildung nachgewiesen werden.

Diese Daten beschreiben die essentielle Rolle von IL-6 in der Verknüpfung der angeborenen und erworbenen Immunität in der Polysaccharid-vermittelten Abszessbildung. Folglich ermutigen die Daten die Erforschung weiterer Therapieansätze mittels IL-6 und TLR4 Antikörpern zur Behandlung von ZPS verursachter Peritonitis.

List of abbreviations

Å	Ångström
Ab	Antibody
AP-1	Adapter-protein-1
APC	Antigen-presenting cell
APC	Allophycocyanine
APS	Ammonium persulphate
ATCC	American Type Culture Collection
BCA	Bicin choninic acid
BM	Bone-marrow
bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLIP	Class II-associated invariant chain peptide
CP	*Staphylococcus aureus* type capsular polysaccharide
CpG	Cytosine-phosphate-guanine
cpm	Counts per minute
CSE	Control standard endotoxin
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-Grabbing Non-integrin
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds	Double stranded
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
e. g.	exempli gratia
EGTA	Ethylene glycol bis2-aminoethyl-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

EU	endotoxin unit
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FPLC	Fast performance liquid chromatography
FSC	Forward scatter
GMCSF	Granulocyte-macrophage colony stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HRP	Horseradish peroxide
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
ICS	Intracellular cytokine staining
iDC	Immature DC
IFN	Interferon
Ig	Immunoglobulin
IH(C)	Immunohistochemistry
IκB	Inhibitory κB
IKK	IκB kinase
IL	Interleukin
i.p.	Intraperitoneal
IRAK	IL-1 receptor-associated kinase
IRF	IFN regulatory factor
i.v.	Intravenous
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
KO	Knock-out
LAL	Limulus amoebocyte lysate
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
MFI	Mean fluorescence intensity

MHC	Major histocompatibility complex
Mod	Modified
mDC	Mature DC
mSp1	Modified Sp1
MyD88	Myeloid differentiation primary response protein 88
NaCl	Sodium chloride
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa B
NLR	NOD-like receptor
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
OD	Optical density
PAMP	pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phospate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PMNL	Polymorphonuclear leukocytes
PRR	Pattern-recognition receptors
PSA	Polysaccharide A from *Bacteroides fragilis*
RNA	Ribonucleic acids
s.c.	Subcutaneous
SCCA	Sterile cecal content adjuvant
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
Sp1	Capsular polysaccharide from *Streptococcus pneumoniae* type 1
ss	Single stranded
SSC	Sideward scatter
TAE	Tris-acetate-EDTA-buffer
TAK1	TGFβ activated proteinkinase 1
TCR	T cell receptor
TEMED	Tetramethylethylenediamine

TGFβ	Transforming growth factor-β
Th	T helper cell
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	Trif-related adapter molecule
TriDAP	Tripeptide L-Ala-D-Glu-mDAP
Trif	TIR domain-containing adapter inducing IFNβ
TSST1	Toxic shock syndrome toxin-1
TT	Tetanus toxoid
UC	Ulcerative colitis
VLE	Very low endotoxin
WB	Western blot
WT	Wildtype
ZPS	Zwitterionic polysaccharide

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Interleukin-6 is essential for zwitterionic polysaccharide-mediated abscess formation

Running title: IL-6 in polysaccharide-mediated abscess formation

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ABSTRACT

Abscess formation associated with secondary peritonitis causes severe morbidity and can be fatal. Formation of abscesses requires the presence of $CD4^+$ T cells. Zwitterionic polysaccharides (ZPS) represent a novel class of immunomodulatory bacterial antigens that stimulate $CD4^+$ T cells in a MHC class II-dependent manner. The capsular polysaccharide Sp1 of *Streptococcus pneumoniae* serotype 1 possesses a zwitterionic charge with free amino groups and promotes T cell-dependent abscess formation in an experimental mouse model. So far, nothing is known about the function of IL-6 in intraperitoneal abscess formation. Here, we demonstrate that macrophages and DCs, the most prevalent professional antigen-presenting cells involved in the formation of abscesses, secrete IL-6 and are incorporated in the abscess capsule. Sp1 inhibits apoptosis of $CD4^+$ T cells and causes IL-17 expression by $CD4^+$ T cells in an IL-6-dependent manner. Abrogation of the Sp1-induced pleiotropic effects of IL-6 in IL-6-deficient mice and mice treated with an IL-6-specific neutralizing antibody results in significant inhibition of abscess formation. The data delineate the essential role of IL-6 in the linkage of innate and adaptive immunity in polysaccharide-mediated abscess formation.

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INTRODUCTION

Secondary peritonitis, which is by far the most common form of peritonitis, results from loss of integrity in the gastrointestinal tract, leading to contamination of the peritoneal space by commensal intestinal bacteria.[1] Despite improved diagnostic modalities, potent antibiotics, modern intensive care, and aggressive surgical treatment, up to one third of patients still die from generalized peritonitis.[2] The cornerstones of successful surgical treatment for generalized peritonitis are thorough peritoneal lavage, drainage of localised abscesses, repairing, which may require removal of the contaminating source, and effective drainage of the peritoneal cavity. However, even with optimal therapy including the administration of antibiotics, residual abscesses form in many patients, resulting in substantial morbidity and mortality.[3]

Abscess formation is known to be a T cell-dependent immune response induced by the capsular polysaccharide of commensal bacteria.[4,5] So far, only protein antigens were believed to be capable of inducing direct T cell responses, whereas polysaccharide antigens were said to be T cell-independent.[6] Indeed, most bacterial polysaccharides usually find their end station in lysosomes of antigen-presenting cells (APCs) without further processing or presenting on the APC surface to T cells.[7] However, *in vivo* and *in vitro* studies showed that polysaccharides of certain commensal bacteria are transported from lysosomes to the cell surface where they are presented by MHC class II to activate $CD4^+$ T cells *in vitro* and induce $CD4^+$ T cell-dependent abscesses *in vivo*.[8-10] These polysaccharides such as PSA1 and PSA2, Sp1, and CP5 from the bacteria *Bacteroides fragilis*, *Streptococcus pneumoniae* serotype 1 and *Staphylococcus aureus*, respectively, are characterized by opposite charge motifs on each repeating unit and are therefore called zwitterionic polysaccharides (ZPS).[11-16] Elimination of their charged groups abrogates T cell-dependent immune responses *in vitro* and *in vivo*.[5] Zwitterionic polysaccharides promote intra-abdominal abscess formation and $CD4^+$ T cell

activation via the co-stimulatory molecules B7-2 and CD40 on activated APCs.[17,18] In intraperitoneal abscess formation, not only cell activation and co-stimulation but also cytokines and adhesion molecules are important. Gibson et al. demonstrated that ZPS PS A1 induces the production of TNF-α and IL-1α on peritoneal cells which act as significant immune mediators for abscess formation.[19,20] If TNF-α is blocked, expression of the intercellular adhesion molecule 1 (ICAM-1) is significantly reduced so that accumulation of polymorphonuclear leukocytes within the abdominal cavity, the hallmark of peritoneal sepsis, is inhibited. Furthermore, PS A1 stimulates the secretion of chemokines, such as IL-8, from T cells.[21] In mice challenged with whole *B. fragilis*, $CD4^+$ T cells mediate abscess formation by an IL-17-dependent mechanism.[22] Treatment of mice with an IL-17-neutralizing antibody significantly inhibited abscess formation.

During sepsis and peritonitis, besides the release of cytokines such as TNF-α and IL-1, IL-6 is also an important mediator of immunologic alterations in the host.[23,24] High titres of IL-6 result from complex amplification mechanisms involving IL-1 and TNF-α.[25-28] Evidence suggests that IL-6 is a very potent proinflammatory cytokine because it stimulates the expression of the broadest spectrum of acute phase proteins in inflammation and infection.[29,30]. IL-6 a pleiotropic inflammatory cytokine, is produced by a variety of cells and acts on a wide range of tissues.[31] It stimulates the activation, survival, proliferation of $CD4^+$ T cells and acts on T cells as an anti-apoptotic factor.[32-35] It prevents apoptosis of normal and resting T cells in the absence of additional cytokines. IL-6 has also been identified as a migration factor. *In vitro* experiments showed chemotactic activity of IL-6 on peripheral blood lymphocytes, lymphokine-activated killer cells, $CD4^+$, and $CD8^+$ T lymphocytes.[36-38] IL-6 has also been identified as a migration factor for breast cancer cells, corneal epithelial cells and keratinocytes, and T cells *in vitro.*[39-44] Additionally, it has been linked to T cell-

dependent immune diseases and autoimmune diseases such as inflammatory bowel disease.[45, 46]

IL-6 and the transforming growth factor-β (TGF-β) are required for the *in vivo* and *in vitro* differentiation and function of a distinct subset of proinflammatory $CD4^+$ T cells that produce IL-17 (TH-17 cells).[47] TH-17 cells are thought necessary for the control of a variety of bacterial and fungal infections at mucosal surfaces and have been shown to have critical functions in human diseases, including Crohn's disease.[48-51]

So far, nothing is known about the role of IL-6 in ZPS-mediated abscess formation and CD4+ T cell responses. Here, we demonstrate that IL-6 plays a critical role in abscess formation. Macrophages and DCs secrete IL-6 in response to the ZPS Sp1. Sp1-induced migration of $CD4^+$ T cells into the peritoneal cavity, inhibition of $CD4^+$ T cells apoptosis, and IL-17-expression of $CD4^+$ T cells require IL-6 secretion. Together, this data illustrates the requirement of IL-6 secretion by cells of the innate immune system for $CD4^+$ T cell immune responses to zwitterionic polysaccharides.

MATERIALS AND METHODS

Antigens

S. pneumoniae type 1 capsular polysaccharide complex was obtained from the American Type Culture Collection (ATCC) and further purified to obtain homogeneity as previously described.[10, 18] *S. pneumoniae* type 1 capsular polysaccharide complex was treated with 2 M NaOH for 1 h at 80°C to remove C substance (a contaminating cell wall polysaccharide). After purification by gel-permeation chromatography with Sephracyl S-400 HR (Amersham Pharmacia Biotech), the Sp1 was concentrated by ultrafiltration and lyophilization and stored at a concentration of 1 mg/ml in 0.15 M phosphate-buffered saline. Chemical modification of Sp1 by neutralization of the free amino group on the 2-acetamido-4-amino-2,4,6-trideoxygalactose by *N*-acetylation that creates a polysaccharide with a net negative charge was performed as previously described.[5]

The polysaccharide antigen was purified aseptically with sterile water. The instruments and devices used in the antigen purification process were deproteinated by treatment with sulfuric and chromic acid and depyrogenated by heat inactivation for 4 h at 240°C or by treatment with 1 M to 2 M sodium hydroxide buffer. The antigens were analyzed for protein by the bicinchoninic acid method (Perbio) and by UV absorbance at 280 nm, for nucleic acid by UV absorbance at 260 nm, and for endotoxin (lipopolysaccharide [LPS]) by the limulus amebocyte lysate test (Charles River Endosafe). In the limulus test, the antigens were evaluated alone and in the presence of LPS; LPS alone served as a positive control. Sp1 was found to contain no detectable protein and no detectable nucleic acid. Endotoxin was not detectable in the above preparations according to the limulus test with a sensitivity of <8 pg of LPS/mg of Sp1 (<0.4 pg of LPS/ml of culture medium containing 50 μg of Sp1) which corresponds to <0.028 endotoxin units [EU/mg of Sp1 (<0.0012 EU/ml of culture medium

containing 50 µg of Sp1). In addition, the polysaccharide antigen was subjected to high-resolution (500 MHz) proton nuclear magnetic resonance spectroscopy [15, 52] Fluorescent labelling of Sp1 with Alexa-Fluor® 594 (Invitrogen) (Sp1-Alexa 594) was performed as previously described.[10] LPS (026:B6) was obtained from Sigma.

Abscess induction studies and evaluation of the cellular peritoneal influx

Animal experiments were performed in accordance with the German animal protection legislation guidelines (license number K07/05 and K16.5/06). Homozygous IL-6-deficient (IL-$6^{-/-}$) mice were generously provided by Prof. H. Bluethmann (Basel, Switzerland).[53] Homozygosis was confirmed by PCR. Wildtype mice C57BL/6 were obtained from Charles River Laboratories.

In abscess induction studies, 6-8 week-old C57BL/6 and IL-$6^{-/-}$ mice were injected intraperitoneally with Sp1 or modified Sp1 (100 µg in PBS mixed with sterile cecal content adjuvant [SCCA]; 1:1 v/v, 0.2 ml total volume) or SCCA alone (as control).[10] In order to block IL-6 intraperitoneally, C57BL/6 wildtype mice were injected intraperitoneally with 250 µg of anti-mIL-6 (clone MP520F3, R&D) at the time of challenge and 6 h later.

At different time points following Sp1 challenge, mice underwent peritoneal lavage with 4 ml of ice-cold RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin to assess secreted cytokines and the cellular influx into the peritoneal cavity. The cellular influx was assessed at different time points following intraperitoneal challenge of WT and IL-$6^{-/-}$ mice with modified Sp1, Sp1, or Alexa-594-labelled Sp1 (100 µg). A total cell count was performed by trypan blue staining and a haemocytometer. Each sample was then analyzed by flow cytometry or fluorescent microscopy. In each experiment, three to six mice per group were tested. The experiment was performed three times in an independent manner.

Six d after challenge, mice were macroscopically examined for the presence of abscesses within the peritoneal cavity by two double-blinded examiners. Abscesses were isolated and their diameter was measured. In each experiment, four to six mice per group were tested. The experiments were performed three times in an independent manner.

Isolation of bone marrow-derived DCs and macrophages

DCs were generated from mouse bone marrow by adapting a previously described method.[54] In brief, bone marrow cells from C57BL/6 mice were cultured in RPMI 1640 supplemented with 5% FBS, 500 U recombinant mouse granulocyte/macrophage-colony stimulating factor (GM-CSF), 20 µg/ml gentamicin, and 50 µM 2-mercaptoethanol. DC medium was exchanged in two-day intervals. DCs were isolated by magnetic cell sorting with a CD11c-specific monoclonal antibody (mAb) (Miltenyi Biotec).

Bone-marrow (BM)-derived macrophages were generated from C57BL/6 (WT) mice as previously described.[54] Erythrocytes were lysed with 0.2% and 1.6% saline. After washing, cells were cultured in RPMI medium supplemented with 10% FBS, 15% L929 supplement, 1% penicillin/streptomycin, 1% glutamine, 1% Hepes, and 1% sodium pyruvate. An equal amount of fresh macrophage medium was added to the medium on day 6. Cells were used for experiments on d 10 or 11.

Cytokine detection

The multiplex fluorescent bead immunoassay (Bender MedSystems) was performed to quantify proinflammatory cytokines in the peritoneal fluid 6 h following Sp1 challenge or SCCA alone (control). Cells were removed from the lavage fluid by centrifugation. The

protocol used was as suggested by the manufacturer and the samples were analysed by flow cytometry and the Flow Cytomix Pro 1.0 Software BMSFFS/1.0 (Bender MedSystems).

BM-derived dendrite cells (DCs) were stimulated with different Sp1 concentrations (20 µg to 100 µg). Cells were harvested, centrifuged and the supernatant collected to measure the IL-6 secretion via ELISA. The murine IL-6 ELISA was performed as instructed by the manufacturer (Diaclone) and absorption was read in the UV/Vis spectrometer at 450 nm (Perkin Elmer Lambda 40).

Flow cytometry and intracellular cytokine staining

For surface marker staining, lavage cells were stained with specific antibodies for 30 min on ice, washed, and then analyzed by flow cytometry. For intracellular cytokine staining (ICS), lavage cells were stimulated with anti-CD3 (5 ng/ml) and CD28 (500 ng/ml) for 6 h at 37°C and 5% CO_2. After the first hour of incubation, Golgi Stop (BD Harlingen) was added. Cells were stained for surface markers for 30 min on ice with specific antibodies, washed, fixed with Cytofix/Cytoperm (BD Pharmingen) for 20 min on ice, and then, permeabilized with Perm/Wash Solution (BD Pharmingen). For interleukin staining, cells were treated with interleukin-specific antibodies for 30 min at 4°C.

CD4 (clone RM4-5, BD), CD11b (clone M1/70, BD), CD11c (clone HL3, BD), and the respective isotype controls were used for surface marker staining. For ICS, anti-mouse mIL-6 (MP5-20F3, R&D), IL-17 (TC11-18H10, BD), and the respective isotype controls were used. Cells prepared for flow cytometry were analyzed – after gating for viable cells by forward and side scatter - by FACScan™ (Becton Dickinson) using CELLQuest™ software (Becton Dickinson). The results were expressed as percentage (%) of fluorescent-labelled cells in a

population. Experiments were performed a minimum of three times in an independent manner.

Fluorescent microscopy

To investigate the Sp1-uptake and IL-6 production within Sp1-positive APCs, fluorescent microscopy was performed. After the surface marker and intracellular cytokine staining, cells were transferred on to poly-D-lysine-pre-coated number 1.5 cover slips attached to 35 mm dishes (MatTek, USA) and fixed in Cytofix solution (BD). Cells were observed immediately under the microscope (Olympus FluoView FV1000 Confocal).

Immunohistochemistry

Snap-frozen abscesses were cryo-sectioned (5-6 µm), fixed in 4% buffered formalin for 1 min and then used for immunohistochemical analyses. To determine the IL-6 secretion of macrophages and DCs present in the abscess capsule, a co-staining for IL-6 and cells was performed, consecutively. After blocking endogenous peroxidase by 0.3% H_2O_2 in methanol and endogenous biotin by a avidin-biotin blocking kit (Vector Laboratories, Eching, GER) for 30 min each, sections were treated with normal goat serum, and then incubated with an anti-IL-6 antibody (1:50; rat anti-mouse clone MP5-20F3, Biosource) for 18h. Next, incubation with a goat alkaline phosphotase-conjugated anti-rat antibody (1 h at 20-22°C) and development with Fast Red (Dako, Hamburg) for 30 min were performed. Subsequently, macrophages were stained with a biotin-linked anti-F4/80 rat anti-mouse antibody (clone A3-1; 1:100; Serotec) and a further treatment with streptavidin-conjugated HRP for 1 h. DCs were stained with a purified hamster anti-mouse CD11c (clone HL3, BD), biotinylated goat anti-hamster IgG (STAR79B, Serotec), and further treatment with streptavidin-conjugated

HRP for 1 h. The staining was envisioned with DAB substrate (Vector Laboratories). Sections were counterstained with hemalaun.

Apoptosis assay

To test the effect of Sp1 on apoptosis of $CD4^+$ T cells, spleen cells from C57BL/6 WT (Charles River Laboratories) and IL-6-deficient mice were isolated by grinding the tissues through a 70 µm mesh. Erythrocytes were lysed (0.2% saline for 30 sec), cells were washed and incubated at 1 x 10^6 cells/ml in starvation buffer (RPMI-1640 supplemented with 0.1% FCS) or non-starvation buffer (RPMI-1640 supplemented with 5% FCS) for 12 h in the presence of chemically modified Sp1 (100 µg/ml), Sp1 (100 µg/ml), or in medium alone. The cells were then stained with an APC-conjugated anti-CD4 mAb (clone RM4-5, BD), or its appropriate isotype control, for 20 min on ice. After washing, cells were dissolved in binding buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl (BD)) and stained with FITC-conjugated Annexin V (5µl/1x10^6 cells; BD Apoptosis Detection Kit) for 15 min on ice. Cells were washed, and 5µl propiumiodide (PI) (BD) was added to the cells for 2 min. The PI staining was stopped by the addition of 5 ml of ice-cold PBS. Cells were washed and prepared for flow cytometry.

Quantifying CD4+ T cells in blood, spleen, and peritoneal lavage

Quantifying the $CD4^+$ T cells in blood, spleen, and peritoneal lavage of C57BL/6 WT and IL-6-deficient mice was performed 24 h following Sp1 challenge. Spleen cells were isolated by grinding the tissues through a 70 µm mesh. Erythrocytes in blood and spleen samples were lysed (0.2% saline for 30 sec), the cells washed, stained with trypan blue, and counted with a haemocytometer. The cells were then stained with an anti-CD4 mAb (clone RM4-5, BD) and its appropriate isotype control, and analysed by flow cytometry.

Statistical analysis

Results of peritoneal cytokine and cellular influx assays in the various groups were compared by Student's t-test. Comparison of groups with regard to abscess formation was made by chi-square analysis.

RESULTS

Peritoneal Sp1-induced cytokines in abscess formation

IL-1 and TNF-α have been described as essential mediators of *B. fragilis*- and PS A1-induced abscess formation in rats.[19, 20] Here, we tested the cytokines IL-1, TNF-α, and IL-6 for their involvement in ZPS-induced intraperitoneal abscess formation with Sp1 as the model antigen in a murine model. We demonstrate IL-6 as the predominant cytokine (Fig. 1). In contrast to the sterile cecal content adjuvant (SCCA) alone, which is used as a control to mimic the leakage of intestinal flora into the peritoneal cavity and does not result in abscess formation [10], Sp1 plus adjuvant induces significantly higher secretion of IL-6 that peaks at 6 h following Sp1 challenge ($p<0.05$). Interestingly, IL-1 and TNF-α are detected at significantly lower levels after Sp1 challenge than IL-6 ($p<0.05$).

Sp1 induces IL-6 secretion by peritoneal macrophages

IL-6 is produced in large quantities by peritoneal host cells in response to Sp1. To identify a cellular source of IL-6 secretion and pivotal IL-6-mediated functions in the process of abscess formation, we first addressed the effect of intraperitoneal Sp1 challenge on macrophages that constitute – as in a septic abscess – a main part of the wall of an experimental abscess.[21] CD11b-positive macrophages migrate into the peritoneal cavity upon Sp1 challenge (Fig. 2a). Within 6 h after Sp1 challenge they represent more than 50% of the incoming cells. Administration of SCCA alone, as a control, attracts significantly less macrophages and total cells into the peritoneum. At 24 h after Sp1 challenge, about 28% of cells attracted into the peritoneal cavity are CD11b-positive macrophages (Fig. 2b). Macrophages are known be capable of internalizing extracellular antigens by various mechanism, such as, pinocytosis, phagocytosis, and endocytosis. Using fluorescent microscopy we studied whether the

peritoneal macrophages internalize Sp1. Indeed, administration of fluorescent-labelled Sp1 results in Sp1 internalization by about 90% of peritoneal macrophages (Fig. 2c). We investigated whether Sp1 internalization by macrophages leads to IL-6 secretion. Intracellular cytokine staining of peritoneal macrophages 6 h after Sp1 challenge demonstrates that almost 90% of the Sp1-induced peritoneal macrophages show cytoplasmic expression of IL-6 (Fig. 2d). Macrophages represent the principal constituent of the abscess wall. Immunohistochemical analyses of abscesses examined 6 d following Sp1 challenge, demonstrate incorporation of IL-6 positive macrophages in the wall (Fig. 2e). Altogether, this result demonstrates that IL-6 secreted by macrophages is a predominant cytokine in Sp1-mediated abscess formation.

Sp1-mediated IL-6 secretion by DCs in vitro

In an experimental model of abscess formation, besides macrophages, CD11c-positive DCs play an important role. They also migrate into the peritoneal cavity upon Sp1 challenge and are retrieved from the abscess capsule.[10] About 7% of the total cells attracted into the peritoneal cavity by Sp1 application are CD11c-positive DCs.[10] IL-6-positive DCs are incorporated into the abscess wall secrete (Fig. 3a). To gain information about the specificity of the Sp1-induced IL-6 synthesis in APCs, we decided to examine the IL-6 secretion by DCs *in vitro*. Bone marrow-derived DCs treated for 33 h with different concentrations of Sp1 *in vitro* secrete IL-6 in a dose-dependent manner (Fig. 3b). As negative controls, medium and chemically modified Sp1 were used. Chemical modification by neutralization of the free amino group on the 2-acetamido-4-amino-2,4,6-trideoxygalactose by *N*-acetylation creates a polysaccharide with a net negative charge. Medium alone and chemically modified Sp1 failed to induce dose-dependent IL-6 secretion by DCs. This result demonstrates that IL-6 secretion depends on the zwitterionic charge motif of Sp1.

Sp1-induced peritoneal CD4$^+$ T cell influx depends on IL-6

Abscess formation depends on CD4$^+$ T migrating into the peritoneal cavity.[17] IL-6 has been described as a migration factor for CD4$^+$ T cells in different tissues. We measured the CD4$^+$ T cell, macrophage and DC cell counts in the peritoneal lavage following Sp1 challenge of wildtype mice, IL-6-deficient mice, and IL-6-deficient mice treated intraperitoneally with IL-6. As controls, cell counts were made from peripheral blood and spleens of wildtype and IL-6-deficient mice. The CD4$^+$ T cell counts in the spleen and peripheral blood of wildtype and IL-6-deficient mice were similar (Table 1). However, compared to wildtype mice, the peritoneal lavage of IL-6-deficient mice showed a 73% decrease in the CD4$^+$ T cell count. The peritoneal macrophage cell count was similar in WT and IL-6-deficient mice. The number of DCs was reduced in IL-6-deficient mice when compared to WT mice. Exogenous IL-6 cytokine treatment of IL-6 KO mice restored the cell count of macrophages, DCs, and CD4$^+$ T cells in the peritoneal cavity to levels of WT mice. The results suggest a functional role of IL-6 as a migration factor for CD4$^+$ T cells into the peritoneum.

Anti-apoptotic effect of Sp1-mediated IL-6 secretion on CD4$^+$ T cells

Sp1 induces MHC class II- and co-stimulation-dependent CD4$^+$ T cell activation *in vitro*.[8, 18] IL-6 stimulates the activation, survival, and proliferation of CD4$^+$ T cells, acting on T cells as an anti-apoptotic factor.[32-35] We therefore tested whether Sp1 supports the survival and inhibits apoptosis of CD4$^+$ T cells; and whether the effect requires IL-6. Spleen cells of C57/Bl6 wildtype mice were starved and CD4$^+$ T cells were examined for apoptosis in the presence and absence of Sp1 and chemically modified Sp1. As a control, CD4$^+$ T cells were incubated in non-starving medium. Fluorescence staining of CD4$^+$ cells with the apoptosis marker annexin V, revealed that in contrast to medium and modified Sp1, native Sp1

decreases apoptosis (16% apoptotic cells versus 39% and 42%) (Fig. 4). At the same time, in the presence of Sp1, 15% of $CD4^+$ T cells stained positively with PI, a marker for cell death. Thirty-seven % and 42% were stained with PI in the absence of Sp1 and in the presence of chemically modified Sp1, respectively. This observation shows an anti-apoptotic effect of Sp1 on $CD4^+$ T cells. The analysis was applied to spleen cells of IL-6-deficient mice of the C57/Bl6 background. IL-6-deficient cells treated with Sp1 did not inhibit apoptosis of $CD4^+$ T cells. Sixty-five % of CD4+ T cells stained positively with annexin V. In contrast, 36% and 42% of cells left untreated, or treated with modified Sp1 as controls were positive for annexin V, respectively (Fig. 4). The result correlated with the findings of the PI staining, revealing that in the absence of IL-6 the inhibitory effect of Sp1 on cell death was abrogated. Sixty-two % of $CD4^+$ T cells were dead in the presence of Sp1. Thirty-one % and 43% of $CD4^+$ T cells stained positively for PI when left untreated or treated with modified Sp1, respectively. In summary, the anti-apoptotic effect of Sp1 depends on IL-6.

Sp1-induced IL-6 activates IL-17 expression in $CD4^+$ T cells

In an experimental abscess model with the ZPS PS A1, the induction of abscesses was shown to be dependent on IL-17 secreting $CD4^+$ T cells. We first addressed whether the ZPS Sp1 induced cytoplasmic expression of IL-17 in $CD4^+$ T cells and then analyzed the contribution of IL-6 on the IL-17 cytokine expression. Eighteen h after Sp1 challenge, lavage $CD4^+$ T cells of wildtype and IL-6-deficient mice were studied by intracellular IL-17 staining. Control mice were challenged with SCCA alone. In Figure 5, the results are summarized in a histogram overlay. In wildtype mice, 59% of peritoneal $CD4^+$ T cells express cytoplasmic IL-17 upon Sp1 challenge. In contrast, in IL-6-deficient mice challenged with Sp1, 6% of peritoneal $CD4^+$ T cells express IL-17. Altogether, cytoplasmic expression of IL-17 by $CD4^+$ T cells upon Sp1 challenge requires IL-6.

The requirement of IL-6 in Sp1-induced abscess formation

We then tested whether IL-6 is essential for the formation of Sp1-induced abscesses. Peritoneal administration of Sp1 to wild type mice produced abscesses (Fig. 6). In contrast, abscess formation by Sp1 in IL-6 deficient mice was significantly reduced ($p<0.05$). SCCA alone and challenge with chemically modified Sp1 used as negative controls did not induce abscesses ($p<0.05$). Furthermore, peritoneal administration of an anti-IL-6 neutralizing mAb with the intraperitoneal Sp1 challenge and again 6 h later resulted in a 50% reduction of abscess formation.

DISCUSSION

In this study, we demonstrate the need of IL-6 for the formation of polysaccharide-mediated intraperitoneal abscesses. In contrast to experimental abscess models in rats using whole *B. fragilis* and PS A1 as causative agents, Sp1 challenge in mice induces significantly higher levels of IL-6 than IL-1 and TNF-α.[19,20] ZPS have in common the zwitterionic charge motif that contains at least one positive and one negative charge per repeating unit.[5] However, for example, PS A1 has exactly one positive charge and one negative charge and Sp1 has two negative charges and one positive charge per repeating unit. Also, the saccharides and their number per repeating unit differ. Besides the animal species, structural differences might explain distinction of the cytokine production. IL-6 has been recognized as a major participant in elevating the inflammatory response in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis. Interestingly, in contrast to ulcerative colitis, Crohn's disease which is frequently associated with intraperitoneal sepsis and abscess formation, higher IL-6 serum concentrations are found.[46, 55]

Peritoneal macrophages and DCs, both participating in the organization of the abscess wall, secrete IL-6 upon Sp1 treatment. Macrophages are an important cellular element, since they are resident in the peritoneal cavity and help initiate immune responses through phagocytosis and cytokine secretion.[56] DCs that represent a minor population of APCs migrating into the peritoneal cavity upon Sp1 challenge, are also incorporated into the abscess wall.[10] We show in bone marrow-derived DCs *in vitro*, that the induction of IL-6 clearly depends on the zwitterionic charge motif because Sp1 lacking the free amino group and thus resembling a common negatively-charged polysaccharide induces a significantly lower IL-6 level.

In recent years, Toll-like receptors (TLRs), which recognise a variety of pathogen-associated molecular patterns of infectious agents, have emerged as critical for this recognition.[57] In mammalian tissues, TLRs are highly expressed by resident immune cells, including above all

DCs and tissue macrophages. Upon binding ligands, all known TLRs except for TLR3 can activate downstream signalling cascades through the adaptor protein MyD88 to induce the production of inflammatory cytokines. These cytokines are interferon-α (IFN-α), IFN-β, IL-12, TNF-α, IL-1, and IL-6. In contrast to PS A1 from *B. fragilis* that causes macrophages and DCs to secrete IL-12 and TNF-α in a TLR2-dependent pathway, these APCs neither secrete TNF-α nor IL-12 in response to Sp1 (unpublished data).[58] Thus, the immune response to the ZPS Sp1 with predominant IL-6 secretion might be conferred by another TLR than TLR2. The CD40 ligand interaction on T cells with the CD40 on APCs is critical to elevate expression of other co-stimulatory molecules and the secretion of a variety of cytokines including IL-6.[59] It is feasible that the previously published requirement of co-stimulatory interactions via B7-CD28 and CD40-CD40L for ZPS-dependent $CD4^+$ T cell activation and abscess formation contribute significantly to the up-regulation of IL-6 synthesis.[17,18].

In comparison to WT mice, in IL-6-deficient mice the total number of macrophages and DCs is decreased by 28% (33.8% of total cells in WT mice and 24.3% of total cells in IL-6-deficient mice). However, the number of influx $CD4^+$ T cells is reduced by 73% in the peritoneal cavity in IL-6-deficient mice. Besides suggestion a pleiotropic effect of IL-6 on antigen presenting cells, the result also identifies IL-6 as a potential $CD4^+$ T cell migration factor in the peritoneum. Sp1 induces T cell activation and proliferation of mammalian $CD4^+$ T cells *in vitro* and *in vivo*.[8,10,60] Activation and proliferation of $CD4^+$ T cells is sustained by IL-2 secretion. Prevention of apoptosis through IL-6 has been shown in T cells at different stages of the cell cycle.[35] Testing the effect of Sp1 on apoptosis of $CD4^+$ T cells, we observe that Sp1 inhibits their apoptosis. This effect is abrogated when the ZPS is converted into a non-ZPS and in the absence of IL-6. This result implies that IL-6 is an essential cofactor for the protection from cell death and might indirectly support IL-2-mediated cellular activation and proliferation in response to ZPS.[61,62]

Here, we observe intracellular IL-17 expression by peritoneal $CD4^+$ T cells. This observation confirms the reported result by Chung et al. who demonstrated IL-17 expression by $CD4^+$ T cells in response to whole *B. fragilis*.[22] Interestingly, we did not detect IL-17 in significant quantities in the lavage fluid (data not shown) but by intracellular staining. This finding might be explained by the low quantity of $CD4^+$ T cells accumulating in the peritoneal cavity 24 h after Sp1 challenge which only represent about 1% of the total cells.[10] We show here that IL-17 expression of peritoneal $CD4^+$ T cells in response to the model ZPS Sp1 is IL-6-dependent. IL-6 and TGF-β have been identified as differentiation factors for TH17 cells.[63,64,65] Presently, it is unclear what the physiological conditions are that allow TH17 differentiation despite the presence of interference by pre-existing TH1 and TH2 cells. Presumably, APCs ubiquitously produce low amounts of TGF-β in response to an activation stimulus, but the local TGF-β production at the site of interaction of APCs with naïve T cells seems to be crucial for the TH17 development.[66,67] Future studies will address the role of TGF-β in ZPS-induced TH17 cells.

The crucial question is whether abrogation of the pleiotropic functions of IL-6 blocks abscess formation. Indeed, abscess formation is significantly inhibited in IL-6-deficient mice. Various options for the prevention and treatment of abscesses are still being investigated. For example, in a peritonitis model in rats, treatment with tissue-type plasminogen and urokinase during the first 24 h after leakage of intestinal contents into the peritoneal cavity was very effective in preventing intra-abdominal abscess formation.[68] Besides the prophylaxis and treatment of pathologies with anti-IL-6 monoclonal antibodies that has been shown to be effective against lethal *Escherichia coli* infection, lethal TNF-α challenge in mice, and Crohn's disease, we see that treatment of mice with a neutralizing IL-6-specific antibody reduces abscess formation by 50%.[45,69,70] As a consequence, early application of an IL-6-specific antibody, or targeting of IL-6 signalling or trans-signalling, in secondary peritonitis or abdominal surgery involving

intestinal incisions may offer a prophylactic strategy for the prevention of intra-peritoneal abscess formation.[45]

Based on these results we conclude that in peritoneal abscess formation induced by the ZPS Sp1, macrophages and DCs recognize the ZPS and secrete IL-6 which stimulates the IL-17-expression of $CD4^+$ T cells and inhibits their apoptosis. Inhibition of abscess formation in the absence of IL-6 confirms the biologic importance of this innate immune mediator for the cellular adaptive host response.

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LEGENDS

Fig. 1: Intraperitoneal administration of Sp1 induces peritoneal IL-6. At different time points after intraperitoneal Sp1 challenge, peritoneal lavage fluid was analyzed for IL-1, IL-6, and TNF-α by a multiplex bead assay. As a control, peritoneal lavage fluid of mice challenged with SCC adjuvant alone was taken at different time points, pooled, and analyzed for cytokines. The figure shows the result of 3 experiments performed independently each with 4 mice per group.

Fig. 2: Sp1 induces IL-6 secretion by peritoneal macrophages. **a**, Influx into the peritoneum, of total cells (squares) and CD11b-positive macrophages (triangles) was measured in C57Bl/6 mice (six per group) challenged with Sp1 (filled symbols) and SCCA controls (open symbols). The peritoneal lavage was performed at different time intervals (x-axis) after challenge. Cell numbers were determined by cell counting and flow cytometry. Bars indicate standard errors within a group tested. **b**, Twenty-four h after intraperitoneal Sp1 challenge, macrophages (Mac) of the peritoneal influx were stained with PE-conjugated anti-CD11b mAb and analyzed by flow cytometry. Numbers in the right dot plot represent the percentage of positive macrophages, gated as indicated in the left dot plot and non-gated cells, respectively. **c**, Peritoneal macrophages internalize Sp1. Mice were challenged intraperitoneally with Sp1-Alexa 594 (100 µg). Twenty-four h after Sp1 challenge, cells of the peritoneal influx were stained with FITC-conjugated anti-CD11b and analyzed by fluorescent microscopy. Scale bar, 10 µm. **d**, Six h after intraperitoneal challenge with Sp1 plus SCC or SCC alone, macrophages (Mac) of the peritoneal influx were surface-stained with PE-conjugated anti-CD11b mAb, and intracellularly with FITC-conjugated anti-IL-6, then analyzed by flow cytometry. Numbers in the quadrants of the dot blot represent the percentage of positive macrophages. **e**, Six d after Sp1 challenge, cryo-sectioned

intraperitoneal abscesses were stained with anti-F4/80 to stain macrophages (brown) and anti-IL-6 (red), and analyzed by light microscopy (20 x). The left panel shows an overview of a partial abscess. The right panel demonstrates the abscess capsule highlightened in the rectangular box in the left panel. White arrows indicate double-stained cells, red arrows indicate F4/80-positive IL-6-negative cells.

Fig. 3: Effect of Sp1 on IL-6 secretion by DCs. **a**, Six d after Sp1 challenge, cryo-sectioned intraperitoneal abscesses were stained with an CD11c-specific antibody to stain DCs (brown) and anti-IL-6 (red), and analyzed by light microscopy (20 x). **b**, Bone marrow-derived DCs were treated in medium alone (empty bar), with chemically modified Sp1 (gray bars) and Sp1 (black bars), at different concentrations, or LPS as positive control (100 ng/ml) (striped bar) for 33 h (x-axis). Supernatants were analyzed for IL-6 by ELISA.

Fig. 4: Sp1 inhibits apoptosis through IL-6. Spleen cells from wildtype mice and IL-6-deficient mice were treated in starvation medium alone, with chemically modified Sp1 (100 µg/ml), and Sp1 (100 µg/ml) for 12 h. As a control, cells were incubated in non-starvation medium alone. Apoptosis of $CD4^+$ T cells was evaluated by Annexin V staining, cell death by PI staining. $CD4^+$ T cells were analyzed by flow cytometry. Numbers above the marker represent the percentage of annexin V- or PI-positive $CD4^+$ T cells of total $CD4^+$ T cells. Dotted black line, non-starvation medium alone (control); gray line, starvation medium alone; dashed gray line, modified Sp1-treated cells; black line, Sp1-treated cells.

Fig. 5: Effect of Sp1-induced Il-6 on IL-17 expression by $CD4^+$ T cells. Twenty-four h after intraperitoneal Sp1 challenge (100 µg) or SCCA challenge as a control, the peritoneal lavage $CD4^+$ T cells of challenged WT and IL-6-deficient mice were analyzed for intracellular IL-17

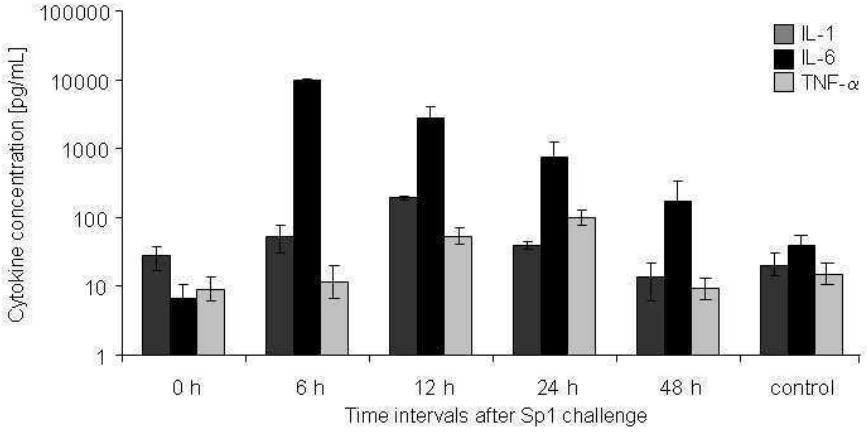
(x-axis) by flow cytometry. Filled black line, isotype control of $CD4^+$ T cells isolated from SCCA-challenged mice; white line, IL-17 of $CD4^+$ T cells isolated from SCCA-challenged mice; gray line, isotype control of $CD4^+$ T cells isolated from Sp1-challenged mice; black line, IL-17 of $CD4^+$ T cells from Sp1-challenged mice. The figure shows the representative result out of 4 experiments performed independently with 3 mice in each group.

Fig. 6: IL-6-dependency of Sp1-mediated T cell-dependent abscess formation. **a**, C57BL/6 or IL-6 $^{-/-}$ mice were challenged intraperitoneally with sterile cecal adjuvant alone (SCCA) and modified Sp1 as control, or Sp1 in the presence or absence of anti-IL-6 antibody simultaneously applied into the peritoneum. After 6 d intraperitoneal abscess formation was examined at autopsy. One dot represents the total abscess diameter per mouse; bars indicate the median abscess size per group.

Table 1: T cell counts in C57BL/6 wildtype and IL-6-deficient mice. C57BL/6 WT and IL6$^{-/-}$ mice were challenged intraperitoneally with Sp1 (100 µg) plus SCC (5 mice per group). One group of IL-6$^{-/-}$ mice were treated intraperitoneally with mouse IL-6 (100 µg/kg) at the time of challenge, and at 6 h and 12 h following Sp1 challenge. Twenty-four h after challenge, cells from C57BL/6 WT and IL6$^{-/-}$ mice were isolated from the peritoneum by lavage, and from blood and spleen, stained with anti-CD4, and analyzed by flow cytometry. In addition, peritoneal lavage cells were stained with anti-CD11b and anti-CD11c antibodies to stain macrophages and DCs, respectively. Numbers represent the percentage of cells of the total cells or the absolute number.

	Blood	Spleen	Peritoneum			
	[%]	[%]	[%]	[%]	[%]	[cells x10^3/ml]
	CD4	CD4	Mac	DC	CD4	CD4
Wildtype	33.6±2.0	21.2±5.8	26.5±13.9	7.3±0.7	4.1±0.4	39.3±3.5
IL6$^{-/-}$	35.2± 3.0	18.8±5.9	22.6±5.3	1.7±1.1	1.1±0.1	9.5±0.7
IL6$^{-/-}$ (+IL6)	n.d.	n.d.	28.1±10.1	6.1±2.4	4.3±0.1	41.2±4.0

Figure 1, Meemboor et al.



Intraperitoneal administration of Sp1 induces peritoneal IL-6.

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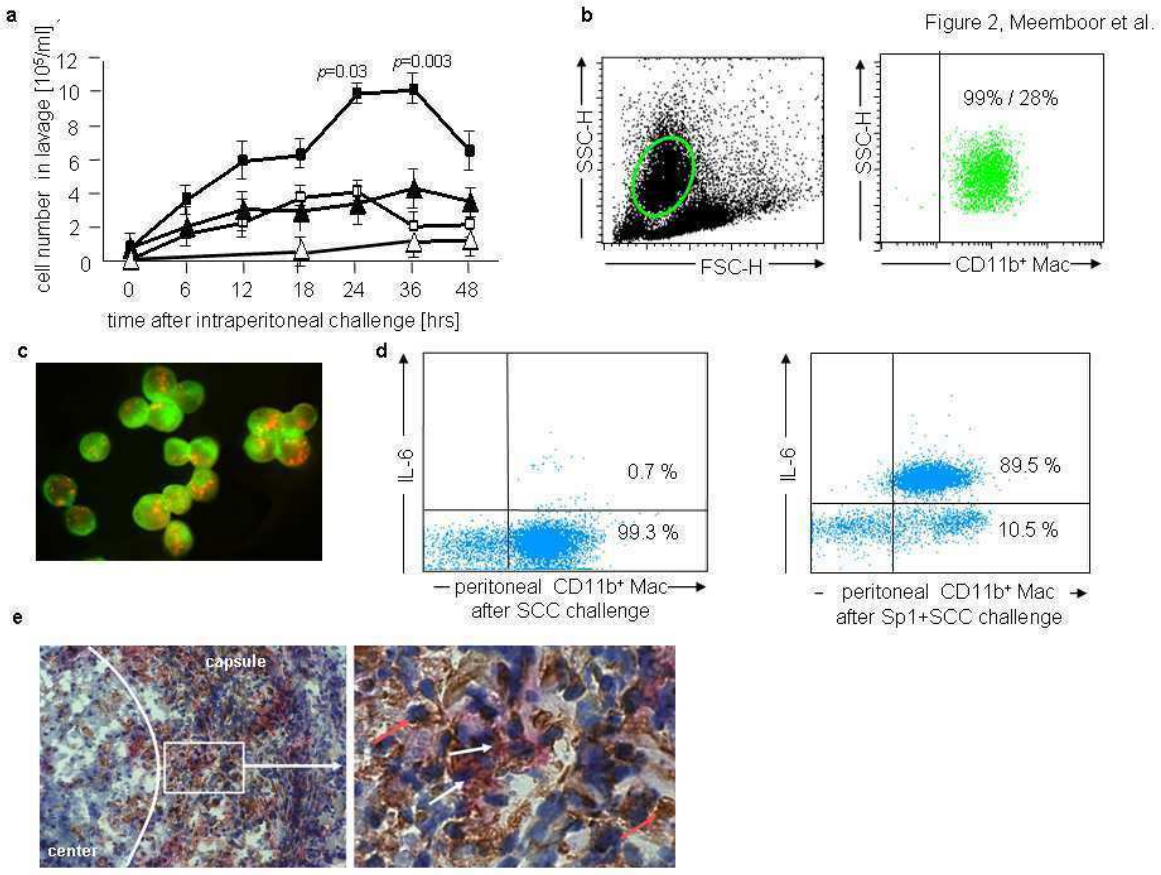

Sp1 induces IL-6 secretion by peritoneal macrophages.

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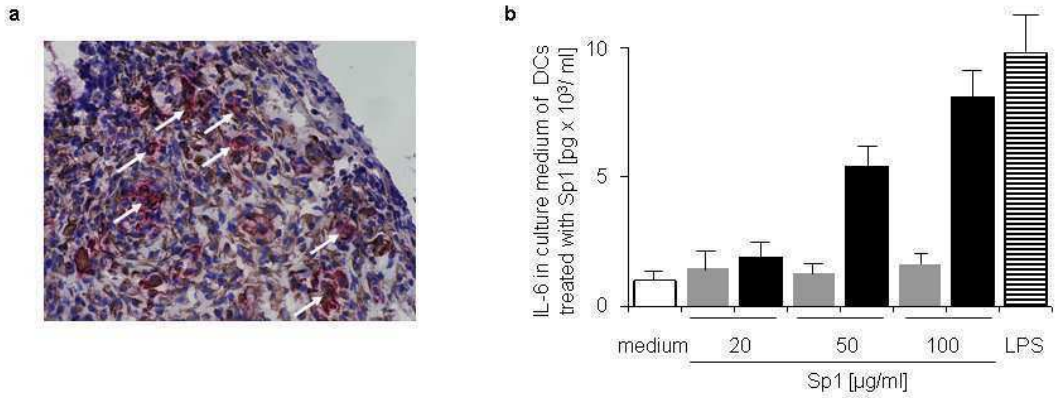
Figure 3, Meemboor et al.



Effect of Sp1 on IL-6 secretion by DCs.

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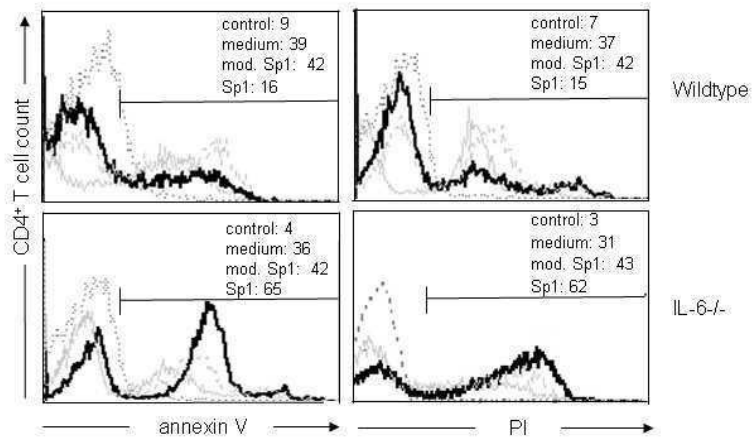
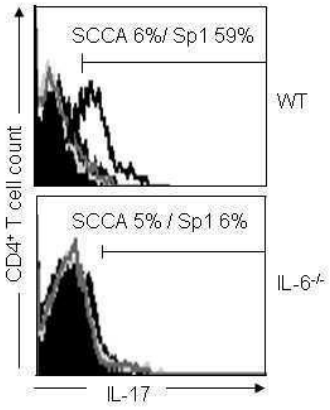
Figure 4, Meemboor et al.



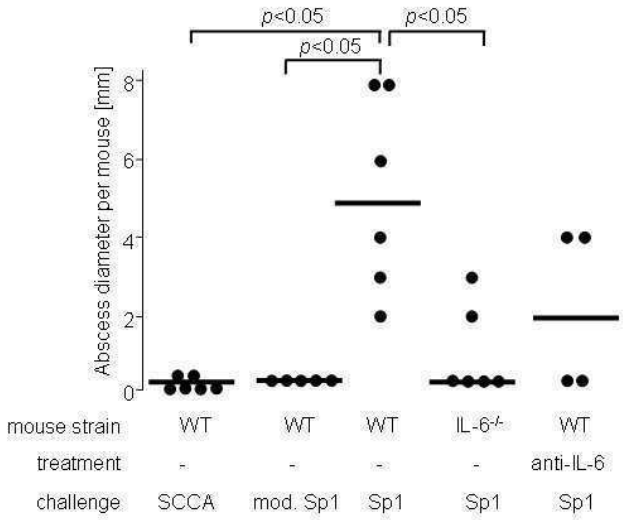
Sp1 inhibits apoptosis through IL-6.

Figure 5, Meemboor et al.



Effect of Sp1-induced IL-6 secretion on TH17 polarization.

Figure 6, Meemboor et al.



IL-6-dependency of Sp1-mediated T cell-dependent abscess formation.

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