The Role of Dendritic Cells in the Sepsis-Induced Immunosuppression

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Hemant Agrawal

aus

Mathura, Indien

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1. Gutachter: Prof. Dr. F. U. Schade

2. Gutachter: Prof. Dr. H. Esche

3. Gutachter: Prof. Dr. B. Horsthemke

Vorsitzender des Prüfungsausschusses: Prof. Dr. A. Vortkamp

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Dedicated to

My dearest Mummy and Papa

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Abbreviations

β-ME	Beta-Mercaptoethanol
µg	Microgram
µl	Microliter
µM	Micromolar
7-AAD	7-Aminoactinomycin
°C	Degrees centigrade
Ab	Antibody
APC	Allophycocyanin
APCs	Antigen-presenting cells
BCR	B-cell receptor
BM	Bone marrow
BMCs	Bone marrow cells
BMDCs	Bone marrow derived dendritic cells
BSA	Bovine serum albumin
CARS	Compensatory anti-inflammatory response syndrome
CBA	Cytometric bead array
CLP	Cecal ligation and puncture
CD	Cluster of differentiation
CD40L	CD40 ligand
CFDA-SE	Carboxyfluorescein diacetate Succinimidyl Ester
CM	Culture medium
Con A	Concanavalin A
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DC-LAMP	DC-lysosome-associated membrane protein
DD	Death domain
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ds	Double stranded
EDTA	Ethylenediamine tetraacetatic acid
ELISA	Enzyme-linked immunosorbant assay
FACS	Fluorescence-assisted cell sorting
FADD	Fas-associated death protein
FCS	Fetal calf serum
FcR	Fc receptor
FITC	Fluorescein-isothiocyanate
FL	Flurosence
FSC	Forward scatter
GM-CSF	Granulocyte monocyte-colony stimulating factor
h	*hora* (hour)
HSCs	Hematopoietic pluripotent stem cells
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin

IL	Interleukin
IFN	Interferon
i.v.	Intravenously
KO	Knockout
LBP	LPS-binding protein
LNs	Lymph nodes
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
LT	Leukotriene
LTA	Lipotechoic acid
MACS	Magnetic cell sorting
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major Histocompatibility Complex
min	Minutes
MIP	Macrophage inflammatory protein
ml	Milliliter
mM	Millimolar
NF-kB	Nuclear Factor-kB
ng	Nanogram
NK	Natural killer
nM	Nanomolar
O.D.	Optical density
o/n	Over night
OVA	Ovalbumine
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin-chorophyll-protein complex-cyanine 5.5
pg	Picogram
PGN	Peptidoglycan
pH	Potentia hydrogeni
PI	Propidium iodide
PKC	Protein Kinase C
PL	Peritoneal lavage
PLF	Peritoneal lavage fluid
PMA	Phorbol 12-Myristate 13-Acetate
PMT	Photo multiplier tube
PRRs	Pattern recognition receptors
RANTES	Regulated on activation of normal T cell expressed and secreted
RAIDD	RIP-associated ICH-1/CED-3-homologous protein
RBC	Red blood cells
RIP	Receptor-interacting protein
rm	Recombinant mouse

RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature (20-25°C)
s.c.	Subcutaneously
SD	Standard deviation
SDS	Sodiumdodecylsulfate
SEA	*Shistosoma mansoni* eggs
SEM	Standard error of mean
SIRS	Systemic inflammatory response syndrome
SLC	Secondary lymphoid-tissue chemokine
SN	Supernatant
ss	Single stranded
SSC	Side scatter
TCR	T cell receptor
TGF	Tumor growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Treg	T regulatory cells
TRADD	TNF-receptor associated death domain
TSCs	Total spleen cells
VLE	Very low endotoxin
v/v	Volume per volume
v/w	Volume per weight
WB	Wash buffer
WT	Wildtype

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1. Introduction

1.1. The Immune System

The defense mechanism of vertebrates and, in particular, mammals against pathogens depends on their immune system (Latin: *immunis*: exempt). For example, in response to an attack by infectious agents, the immune system must combat and destroy the "danger" and maintain the homeostatic balance of the body. The entities that define a successful immune defense against an infectious disease are the immunological arms of protection. The immune system can simplistically be divided into two components namely, the innate and the adaptive immune systems (Hoffmann et al., 1999). The successful immune defenses against an infectious agent are the outcome of the close interaction between these two main parts of the immune system.

1.1.1. The Innate System

The first phase of host defense against invading microorganisms relies on the innate arm of the immune system, an evolutionary ancient and universal form of host protection. It has been traditionally considered an immediate but non-adaptive response to infection or tissue injury (Fearon et al., 1996). The innate immune system is composed of numerous and variable components. The skin, mucosal secretions, and stomach and intestinal pH are physical/chemical barriers that form the first line of defense. When an infectious agent evades these barriers and invades the host, cellular innate immune defense come into play that includes natural killer (NK) cells, granulocytes, γδ T lymphocytes, macrophages and dendritic cells (DCs). Although cellular defenses of innate immune responses do not recognize specific epitopes on an antigen in the manner observed with the specific immune responses, they do rely heavily on a limited number of highly conserved cellular receptors termed pathogen-recognition receptors (PRRs) which detect common molecular structures associated with many classes of pathogens; these elements have been termed pathogen-associated molecular patterns (PAMPs). This allows the immune system to discriminate between infectious non-self and non-infectious self (Janeway et al. 2002). Besides

physical/chemical barrier and cellular components, innate responses also comprise a number of soluble factors. These factors include the complement components, natural antibodies, LPS-binding protein (LBP), mannan-binding protein, acute phase proteins, certain chemokines and cytokines, prostaglandins and leukotrienes.

The success of the innate immune system in controlling pathogens is highlighted by the fact that all organisms except for the vertebrates rely exclusively on innate immune responses. Even vertebrates cope with most infections by exclusively using their innate immune system. Under some circumstances, however, the invading pathogen escapes innate immune surveillance. In which case, the adaptive immune response is launched in order to support the innate responses with increased specificity and the ability to generate memory.

1.1.2. The Adaptive System

The adaptive immune response is associated with the activation of B and T lymphocytes which constitute the cellular elements of the adaptive immune system. Unlike cells of innate system, which use a fixed repertoire of inherited receptors, B and T cells undergo a somatic recombination of germ line encoded antigen receptor gene segments to create new and unique antigen receptors (Tonegawa, 1983; Tonegawa, 1988) capable of recognizing virtually any antigen. Cells expressing an antigen-specific receptor are selected upon pathogen binding since receptor triggering induces clonal expansion by proliferation. Furthermore, cell clones selected for a certain antigen are retained, thus constituting antigen specific memory.

Antibodies, encoded by heavy and light immunoglobulin (Ig) genes, are expressed by B cells in a soluble and membrane bound form and serve as the antigen receptors. Secreted antibodies are the main effectors of the humoral response. They recognize antigens in their native three-dimensional structure, thus every chemical structure can serve as an antigenic epitope for B cells. Unlike B cell receptors (secreted or membrane bound antibodies), T cell receptors (TCR) are never secreted and they recognize peptides produced by the proteolytic cleavage of antigens. Thus, T cells recognize the primary structure (amino acid sequence),

and B cells recognize the tertiary structure (three-dimensional) of a protein. Moreover, T cells recognize antigenic peptides only when they are presented by antigen-presenting cells (APCs), which include monocytes, macrophages, endothelial cells, fibroblasts, fibrocytes, DCs (Guermonprez et al., 2002; Knight et al., 1993) on the surface bound to Major Histocompatibility Complex (MHC) class I and II proteins (Davis et al., 1998). B cells can also function as APCs, but not for stimulating naïve T cells in primary immune response (Finkelman et al., 1992). Intracellular antigens (present in cytosol) are processed to a length of 8-10 amino acids by the proteosome and are loaded onto MHC class I molecules which are then delivered to the cell surface, where they are recognized by $CD8^+$ T cells (Flutter et al., 2004). In contrast, extracellular antigens internalized in endosomal compartments are processed in the lysosomes, giving rise to peptides of 12–24 amino acids in length (Chapman, 2006), which are loaded onto MHC class II molecules. These are then delivered to the cell surface for recognition by $CD4^+$ T cells (Abbas et al., 1996).

Together, appropriate innate and adaptive system interactions are needed for highly efficient recognition and clearance of pathogens which could confer optimal protection to host against the invading pathogens.

1.2. Dendritic Cells: The Central Link between Innate and Adaptive Immune System

The signals obtained from the recognition of an invading pathogen by the cellular arm of the innate immune system must be conveyed to the cells of the adaptive immune system in order to mount an effective immune response. This crucial task is efficiently fulfilled by APCs, in particularly by DCs, which provide T cells not only with microbial antigen (once processed) but also with the full information about the nature of the pathogen, thus bridging the essential connection between innate and adaptive immunity (Palucka et al., 1999; Banchereau and Steinman, 1998). DCs are professional antigen presenting cells which play a pivotal role in immunosurveillance against the microbial infections.

1.2.1. Dendritic Cells: Origin and Distribution

In 1970s, Ralph M. Steinman and Zanvil A. Cohn first identified and described the DCs as a novel cell population in the peripheral lymphoid organs of mice (Steinman and Cohn, 1973; Steinman and Cohn, 1974; Steinman et al., 1975). Subsequently, DCs have been described in more and more tissues. Nowadays, it is thought that most organs possess their own DC populations (Hart, 1997). DCs belong to the heterogeneous group of cells that possess varying anatomical location, cell surface markers and function. However, most DCs have several features in common (Table 1).

Table 1: List of common features of dendritic cells

1.) DCs originate from HSCs in bone marrow and migrate to various tissues via bloodstream and reside there as immature cells.
2.) Immature DCs capture antigen or encounter danger signals triggered by infectious agents through receptor and non-receptor-mediated mechanisms and then mature during migration to the secondary lymphoid organs.
3.) Depending on the specific environmental signal, DCs differentiate into different maturation forms.
4.) Mature DCs lose the phagocytic activity and express high density of MHC class I and II and costimulatory molecules to stimulate naïve CD4+ and CD8+ T cells into T helper (Th) cells and cytotoxic T lymphoctes (CTL), respectively.
5.) The particular maturation form of DCs determines the fate of Th cell amplification into Th1, Th2 or Treg lymphocytes.

The development of DCs is considered to occur in distinct stages. Hematopoietic pluripotent stem cells (HSCs), under the influence of yet unknown factors, constantly generate DCs progenitors in the bone marrow, which give rise to circulating precursors in the blood (Wu and Dakic, 2004). These DCs precursors are then seeded via the bloodstream to a large variety of tissues where they reside and give rise to immature DCs which possess high endocytic and phagocytic activity (Banchereau et al., 2000). DCs are a sparsely distributed group of cells constituting about 1-2% of total cells and are present nearly in all organs like, thymus, heart, kidney, lung, intestine, lymph nodes, spleen, skin etc. In the last two decades, with the help of monoclonal antibodies against multiple surface molecules of the diverse DCs subtypes both in human and mouse have been described (Shortman and

Liu, 2002, Wan and Dupasquier, 2005). DCs subsets have been investigated in both lymphoid organs (thymus, bone marrow, spleen, lymph nodes, tonsils etc.) and non-lymphoid organs (blood, skin etc.). In the mouse spleen, conventional DCs express high levels of CD11c (the integrin-α_x chain) and have been divided into three subtypes on the basis of their expression of CD4 and CD8α, namely $CD4^-CD8\alpha^+$, $CD4^+CD8\alpha^-$ and $CD4^-CD8\alpha^-$ (Vremec et al., 2000). These subsets are present in different percentages in the spleen. In the total population of $CD11c^+$ splenic DCs, $CD4^+CD8\alpha^-$ DCs are ~50% while $CD4^-CD8\alpha^+$ and $CD4^-CD8\alpha^-$ DCs are ~25% each (Vremec et al., 2000). $CD4^-CD8\alpha^+$ DCs are mainly concentrated in the T cell areas in the spleen while $CD4^+CD8\alpha^-$ and $CD4^-CD8\alpha^-$ DCs are concentrated in the marginal zones of the spleen, but migrate into the T cell zones on stimulation with microbial products. It has been shown that $CD4^-CD8\alpha^+$ DCs have the ability to produce large amounts of IL-12 as compared to $CD4^+CD8\alpha^-$ and $CD4^-CD8\alpha^-$ DCs (Hochrein et al., 2001). There exist contradictory reports regarding the functional properties of these subsets. Therefore, exact functional differences between these subsets are at present unclear.

1.2.2. Dendritic Cells: Maturation and Migration

When the immature DCs encounter any antigen/pathogen in peripheral tissues, they undergo a change in their phenotype and function which culminate in the complete transition from antigen-capturing cells to antigen-presenting cells. This transition results in the maturation of DCs and their migration from peripheral tissues to the lymphoid organs such as the lymph nodes. As these two events are critical in the life cycle of DCs, they are discussed below together.

1.2.2.1. Dendritic Cells: Activation and Maturation

Numerous factors are involved in DCs activation and transition of immature DCs into mature DCs which includes pathogen-specific PAMPs such as LPS, PGN, bacterial DNA, double stranded RNA (dsRNA) (Guermonprez et al., 2002). Moreover, the balance between pro- and anti-inflammatory cytokines in the local microenvironment, including TNF-α, IL-1β, IL-6, IL-10, TGF-β and prostaglandins (Banchereau et al., 2000; Kalinski et al., 1998)

also induces DCs maturation. Furthermore, certain T cell-derived signals, such as CD40L, play a role in the maturation process (Cella et al., 1996). All these factors can induce immature DCs to mature and migrate to the secondary lymphoid organs where they present captured and processed antigen to naïve T lymphocytes.

Immature DCs are efficient in capturing and processing antigen to form MHC peptide complexes while mature DCs are efficient in presenting the processed antigen and thus prime T cell responses (Banchereau et al., 2000). Besides these properties immature and mature DCs possess numerous other features, figure 1 shows some of them.

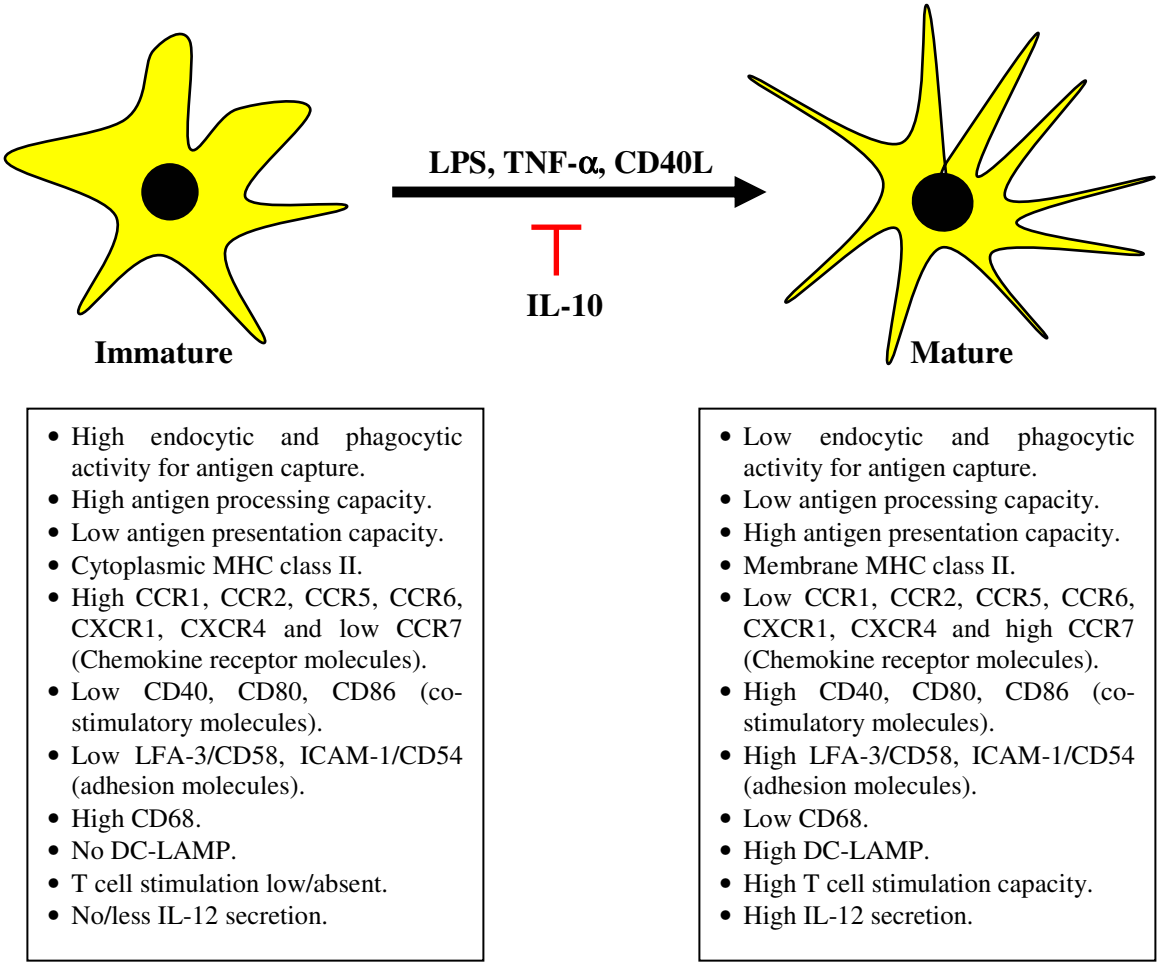


Figure 1: Some features of immature and mature DCs.

1.2.2.2. Dendritic Cells: Migration

Following activation, DCs migrate to the lymphoid organs such as the lymph nodes. There, DCs attract lymphocytes by releasing various chemokines and maintain the viability of recirculating T lymphocytes (Caux et al., 2002; Sozzani, 2005). DCs migration *in vivo* is a tightly regulated function of maturation and is controlled by a variety of cytokines and chemokines (e.g. GM-CSF, TNF-α, IL-1, MIP-1α and β), but also nonchemokine chemotactic agonists, lipid mediators and membrane proteins affect DCs movement and maturation (Sozzani, 2005). During maturation, DCs upregulate CCR7, a receptor for chemokines CCL19 (MIP-3β) and CCL21 (secondary lymphoid tissue chemokines, SLC) (Dieu et al., 1998) and drives DCs migration to the lymphoid vessels and T cells areas of secondary lymphoid organs. As CCR7 also mediates the homing of T cells to secondary lymphoid organs, it plays a key role in helping antigen-bearing DCs to encounter responder T cells (Willimann et al., 1998).

1.2.3. DC-T cell Interactions

Once DCs reach the secondary lymphoid organs such as the spleen and the lymph nodes, they prime T cell responses. DCs present captured antigen to naïve T cells (Thery and Amigorena, 2001), thereby inducing activation and differentiation of naïve CD4+ and CD8+ T cells into T helper (Th) cells and cytotoxic T lymphocytes (CTL), respectively. For T cells to get efficiently activated, they require three signals (Lipscomb and Masten, 2002), namely:

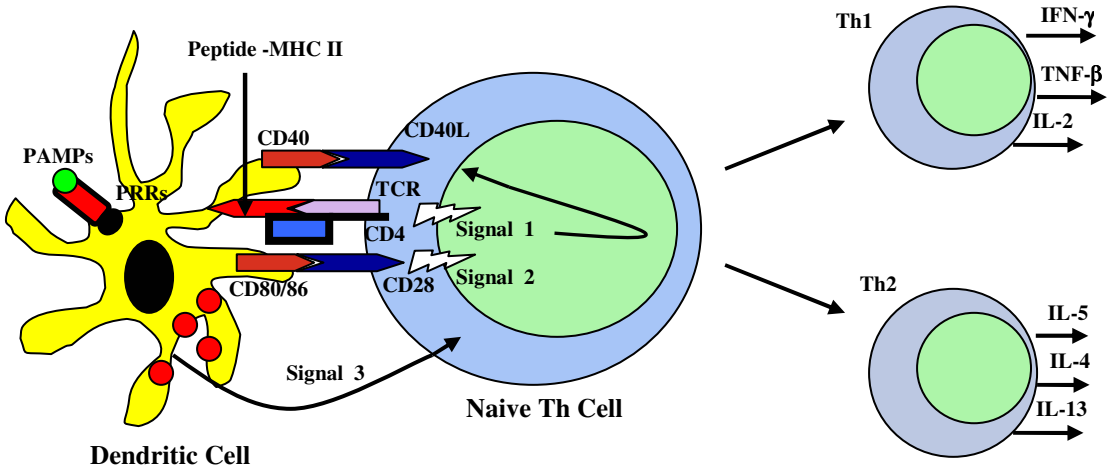
Signal 1: CD4+ and CD8+ T cells need to recognize the displayed antigen on MHC class II & MHC class I molecules, respectively. Each T cell carries a unique T cell receptor & only those T cells that recognize the antigens presented by DCs will become activated (Fig. 2).

Signal 2: T cells need some accessory molecules on DCs known as costimulatory molecules that DCs have upregulated during maturation. This signal makes the T cells to divide and differentiate into effector T cells. In the absence of sufficient costimulation signal, T cells undergo anergy or apoptosis (Fig. 2).

Signal 3: Besides these two signals, a third signal is required to activate T cells. This signal is most probably delivered in the form of soluble molecules such as cytokines and

chemokines, which are produced by DCs themselves and directly affect T cells in a paracrine manner. For example secretion or lack of secretion of IL-12 plays a key role in the final differentiation of naïve Th cells into Th1-type or Th2-type effector cells, respectively (Fig. 2).

Figure 2 illustrates a model for explaining close interaction between DCs and T cells using CD4+ T cells (Th cells) for the development of a protective immune response. This communication between DCs and Th cells is a dialogue rather than a monologue. Th cells respond to DCs and vice versa. The polarization of Th cells towards Th1-type or Th2-type is depending upon the type of signal 3 produced by DCs in response to the interaction of pathogen-specific PAMPs with specialized PRRs on the surface of DCs (Kapsenberg, 2003) (Fig. 2).



	Dendritic cell	**CD4$^+$ T cell**
Signal 1	Peptide-MHC II	TCR and CD4
Signal 2	CD40	CD40L
	CD80	CD28/CTLA-4
	CD86	CD28/CTLA-4
Signal 3	IL-12 production	Th1-type response
	No IL-12 production	Th2-type response
	IL-10 production	Treg-type response
	TGF-β production	Treg-type response

Figure 2: Model for DC-CD4+ T cell interaction.

1.2.4. Dendritic cells: Production of Cytokines and Chemokines

On maturation, DCs secrete a number of soluble factors such as cytokines and chemokines that are critical in determining the nature of the ensuing immune response. Their rate of secretion varies with the maturation state of DCs. DCs produce various cytokines such as IL-12 (Sundquist et al., 2003), IL-10 (Jiang et al., 2002), IL-18, TNF-α, IFN-γ, IL-2, IL-6 etc. in response to bacterial stimuli. Many cytokines act in an autocrine fashion and are involved in the activation or suppression of DCs effector functions, such as TNF-α, IL-1β, IL-6, IL-12 or IL-10. IL-12 is important for the promotion and maintenance of Th1 differentiation and for the initiation of IFN-γ-dependent cell-mediated immunity to many intracellular pathogens (Trinchieri, 1995). The production of IL-10 by DCs inhibits Th1 responses by inhibiting the full maturation of DCs and their capacity to produce IL-12 (Steinbrink et al., 1997) and also promotes the development of regulatory T cells (Wakkach et. al., 2003). IFN-γ is a potent adjuvant of Th1 responses and primes DCs to produce enhanced levels of IL-12 and thus promotes the development of Th1 responses (Snijders et al., 1998). In addition to the production of cytokines, maturation of DCs is also inducing secretion of chemokines that recruit monocytes, DCs (Nakamura et al., 1995), and specific subsets of T cells into the local environment. Mature DCs are known to produce multiple chemokines, including MCP-1/CCL2, CCL3/MIP-1α, CCL5/RANTES, CXCL8 and CXCL10 (Sallusto et al., 1998; Sozzani, 2005). MCP-1 is rapidly produced by DCs upon TLR activation and binds to CCR2, the only known functional receptor. Besides attracting leukocytes, MCP-1 is also known to inhibit Th1 responses and promotes the development of Th2 responses in both infectious (Chensue et. al., 1996; Matsukawa et al., 2000) and allergic disease models (Gonzalo et al., 1998).

In summary, DCs can produce several different cytokines and chemokines and thereby exert important quality control on the immune system.

1.3. Sepsis

Sepsis (in Greek *Σήψις*, putrefaction) is defined as a systemic inflammatory response syndrome (SIRS) initiated by infection that resulted in a disruption of inflammatory homeostatis. Inflammatory dysregulation consequently affects multiple organs via effects on endothelial, epithelial and immune cell types that leads to irreversible damage (Fig. 3).

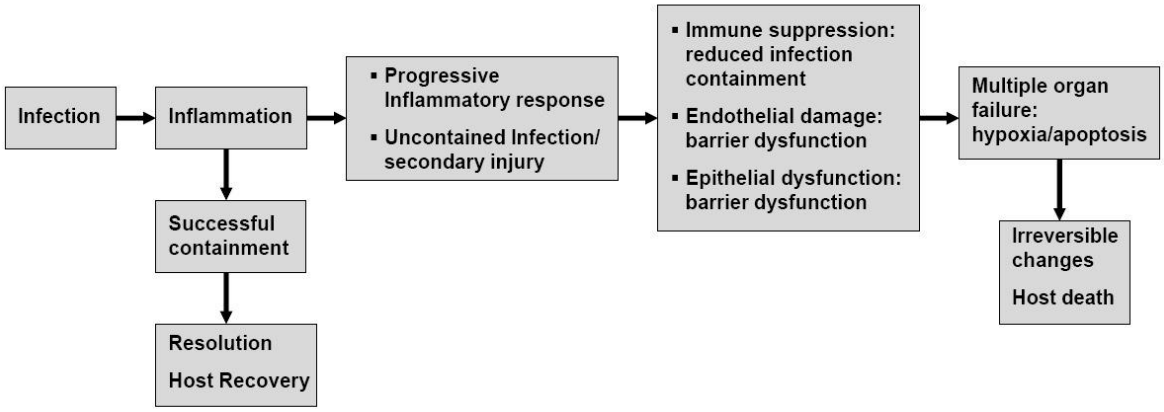


Figure 3: Pathogenesis of sepsis (Adapted from Buras et al., Nat Rev Drug Disc, 4, 854-65).

The poor outcome in sepsis is considered to be a consequence of an overactive systemic inflammatory response elicited by the invading micro-organisms (Ruokonen et al., 1991). The development of SIRS is countered by a mechanism to terminate inflammation following elimination of the infectious agent. This counter regulatory mechanism is known as compensatory anti-inflammatory response (CARS) (Bone et al., 1996). SIRS and CARS are associated with the release of pro- and anti-inflammatory molecules, respectively (Cavaillon et al., 1998). Imbalance and overreaction of either response can result in host damage caused by excessive inflammation or immune dysfunction and thus play a decisive role for the outcome of sepsis (Fig. 4).

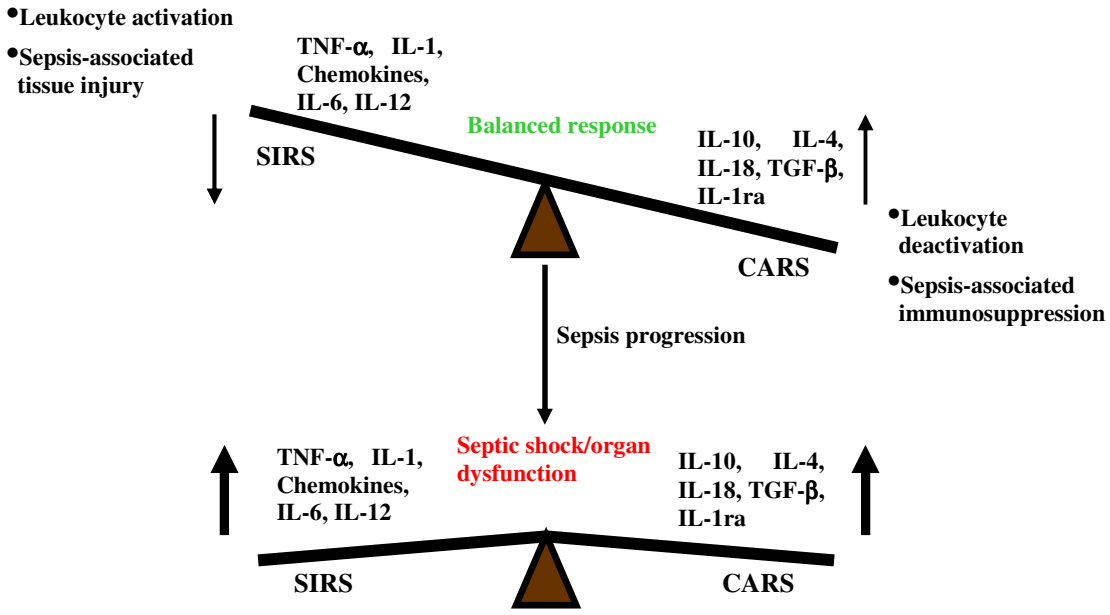


Figure 4: Role of SIRS and CARS in the development of sepsis (Adapted from Buras et al., Nat Rev Drug Disc, 4, 854-65).

Cecal ligation and puncture (CLP) in mice develop symptoms similar to those found in septic patients and serve as a model for polymicrobial sepsis. Pro- and anti-inflammatory cytokines are expressed in large amounts after CLP, and the balance of their expression seems to be critical for the outcome of sepsis (Scott et al., 2002; Torres and De Maio, 2005). IL-12 and its counterpart IL-10 play a major role during sepsis, as modulation of these cytokines influences sepsis-induced mortality after CLP in mice. Application of recombinant IL-10 prior to CLP increases survival (Walley et al., 1996), administration of IL-12 increases sepsis-induced mortality (Echtenacher et al., 2001). However, the time of intervention appears to be decisive for disease development. Absence of IL-10 during the initial phase of sepsis is detrimental (Latifi et al., 2002), whereas neutralization of IL-10 at later time-points is beneficial (Ayala et al., 2001). In addition to the predominance of anti-inflammatory mediators, T as well as B lymphocytes undergo apoptosis in the spleen and other lymphoid organs during sepsis (Hotchkiss et al., 2001). The remaining T cells display reduced responsiveness to mitogens in terms of proliferation and secretion of IL-12 and

IFN-γ (Ayala et al., 1994). This status of dysregulation of a broad range of the innate and adaptive immunity during sepsis has been entitled with terms such as "immunoparalysis".

It has to be emphasized that the changes of the immune system observed during polymicrobial sepsis cannot be simply reduced to effects mediated by bacteria-derived LPS. Mice, which are defective in the LPS signaling molecule Toll-like receptor 4, are susceptible to polymicrobial sepsis but not to LPS-induced shock (Echtenacher et al., 2001). T cell suppression is observed upon polymicrobial sepsis but not upon infusion with LPS (Ayala et al., 1994). These findings demonstrate that multiple factors contribute to polymicrobial sepsis-mediated immunomodulation. Although being a well-described phenomenon, the basic mechanisms leading to sepsis-mediated immunoparalysis have not yet been characterized completely. It has been hypothesized that DCs play a pivotal role in sepsis-mediated immunosuppression. DCs possess high potential for antigen capture, processing and presentation and thus play an important role in orchestrating the body's cell mediated immune response by activating T lymphocytes (as discussed above). Any defects in the cascade of DCs antigen capture, processing and presentation will result in the impairment of the body's ability to clear invading microorganisms such as that seen with sepsis.

1.4. Sepsis and Dendritic cells

Much is known on the role of DCs against the invading microorganisms and this knowledge is expanding by each passing day but little is understood about the effects of polymicrobial sepsis on DCs. Although the potential role played by DCs in the sepsis response is fairly obvious, our understanding of its contribution to sepsis pathogenesis is fairly limited. The limited knowledge in the field of sepsis and DCs may be attributed to the large number of factors involved in the sepsis syndrome. A large number of studies have been conducted to show if DCs are truly the cornerstone in modulating this disease process. Much of the knowledge what we have today about the role of DCs in the inflammatory response comes from studies done in mice models of endotoxemia using bacterial LPS. Although mice endotoxemia model does not recapitulate the sepsis response as seen with mice CLP model (Echtenacher et al., 2001; Remick and Ward, 2005), it does provide a

good model to explore how DCs behave to microbial products and, presumably, microbial invasion. In the last few years several studies were published on the role of DCs during sepsis response in human patients and mice CLP model. In 2002, Hotchkiss et al. reported that the patients who died from sepsis had lost DCs from the spleens (Hotchkiss et al., 2002). The depletion of DCs was further described in spleen (Tinsley et al., 2003; Ding et al., 2004), lymph nodes (Efron et al., 2004), lung (Benjamim et al., 2004) and peritoneum (Ding et al., 2004) in mice CLP model for polymicrobial sepsis. Recently, it was found that the intrapulmonary instillation of bone marrow-derived DCs prevented lethal *Aspergillosis* infection in septic animals (Benjamim et al., 2004). Also, targeted depletion of $CD11c^{hi}MHCII^{hi}$ DCs dramatically increases mortality to sepsis induced by CLP, while adoptive transfer with wildtype bone marrow-derived DCs restores survival in this model of polymicrobial sepsis (Scumpia et al., 2005), suggesting the involvement of DCs during sepsis response.

Besides DCs depletion, their functional properties are also affected by environmental factors such as pro- and anti-inflammatory cytokines seen in sepsis. IL-12 is a pro-inflammatory cytokine, which modulate host immune response. DCs are the major producers of IL-12 (Cella et al., 1996). It was described that splenic DCs from septic mice showed reduced ability for IL-12 production while the peritoneal DCs produced significantly higher amounts of IL-12 than control mice (Ding et al., 2004). Reduced production of IL-12 and TNF-α on the one hand and increased production of IL-10 on the other was seen in isolated lung DCs from septic mice when stimulated with TLR ligands such as LPS, Pam3cys or *Shistosoma mansoni* eggs (SEA) compared with similarly activated DCs obtained from sham mice (Benjamim et al., 2004), suggesting the change in DCs cytokine profile towards the Th2-type. Further, studies were performed in order to investigate the change in expression of DCs surface costimulatory molecules during sepsis, which is a hallmark for DCs activation and maturation. Recently, Ding et al., reported no change in CD40, CD80 and CD86 expression by splenic DCs 24 h after CLP. However, in peritoneum, DCs showed slightly increased expression of CD86 (statistically not significant) while CD40 and CD80 remained the same at 24 h after CLP (Ding et al., 2004).

Similarly, no significant difference was observed in the percentage of mature DCs (MHC II^{hi} and $CD86^{hi}$) in the lymph nodes of CLP versus sham operated mice (Efron et al., 2004). In another study with lung DCs, no marked differences in the expression of CD40 and CD80 were observed between CLP and sham operated animals (Benjamim et al., 2004). The splenic DCs from septic mice with no significant change in their expression of costimulatory molecules was found to have similar antigen presenting capacity as that of DCs from sham mice while peritoneal DCs with slightly increased expression of CD86 showed enhanced T cell proliferation capacity (Ding et al., 2004), suggesting the putative role of DCs maturation in triggering effective T cell response in order to clear the bacterial load.

Although not present in very large number and not really the true effector cell in the body, the DC is an integral part of the immune system. The unique ability of DCs to link innate and adaptive immunities allows the researchers to study its functional properties in more detail and to manipulate DCs to cure many disease processes such as sepsis. Increasing evidences suggested the essential role of DCs in sepsis (Efron and Moldawer; 2003) and has opened the way to utilize DCs in the treatment of sepsis, whether as a vector, a direct effector, or both.

1.5. Aim of the Study

Sepsis is defined as the combination of SIRS and systemic infection. It is associated with immune suppression mediated by a predominance of anti-inflammatory cytokines like IL-10 and profound loss of lymphocytes through apoptosis. It is well known that DCs possess the unique ability to link the innate and adaptive immune systems and play a pivotal role in the defense mechanism against infectious diseases. This is primarily achieved by the ability of DCs to capture and present antigen to T lymphocytes by migrating to the draining lymphoid organs, where they initiate the primary immune response. Numerous scientific investigations have also demonstrated the critical role played by DCs in infectious diseases both *in vitro* and *in vivo*. However, the role of DCs in sepsis-induced immunosuppression remains unclear. Therefore, to address this uncertainty, a set of experiments were designed

to analyze the influence of polymicrobial sepsis on the phenotypic and functional behavior of DCs.

The objective of the present study was to characterize DCs in terms of their number, subset composition, expression of surface markers, cytokine release and T cell stimulatory capacity during sepsis.

Further, depending upon the changes in their phenotypic and functional properties during sepsis, it might be possible to develop a therapy using competent BMDCs that can help to overcome the sepsis-induced immunosuppression.

2. Materials and Methods

2.1. Animals

Naïve female BALB/c (wild type), C57BL/6J (wild type) (Harlan Laboratories, Borchen, Germany), and BALB/c (IL-12p35$^{-/-}$) (kindly provided by Gottfried Alber, Institute of Immunology, University of Leipzig, Leipzig, Germany) (22.0-24.0 g) were used in the present study. All animals were 8-10 weeks old and were allowed to habituate to the animal laboratory conditions for at least one week before starting the experiments. Animals were housed in a group of five in standard plastic laboratory cages with a wire mesh lid. Cages were kept in an air-conditioned, sound-proof room at an ambient temperature of 24.0 ± 0.5°C. The animals had access to standard lab chow and tap water *ad libitum*. A 12 hour light/dark cycle was maintained throughout the experiments. All animal procedures were carried out following institutional guidelines at the Medical Faculty, University of Duisburg-Essen, Germany.

2.2. Animal Model for Polymicrobial Sepsis

Female BALB/c mice were used to induce intra-abdominal polymicrobial sepsis using **C**ecal **L**igation and **P**uncture **(CLP)** model for sepsis with slight modifications. This model of sepsis was developed by the group of Dr. Chaudry (Wichterman et al., 1980) and has been used as a standard model for sepsis for more than three decades. The introduction of CLP model is considered to be of great relevance for sepsis research. The CLP technique comprises of midline laparotomy followed by exposure of cecum, its ligation and puncture. CLP leads to polymicrobial peritonitis; bacteraemia and cytokine responses caused by leakage of faecal material, and mimic the human scenario of ruptured gut. This technique has achieved world wide popularity because of its ease, reproducibility and similarity to human disease progression.

A light anaesthesia was introduced by intra muscular injection of the mixture of Ketamin (0.1 mg/g body weight of the animal) (CEVA, Sante Animale, Duesseldorf, Germany) and

Xylazin (0.01 mg/g body weight of the animal) (CEVA, Sante Animale). Following anaesthesia, a mid-line laparotomy was performed and the cecum was exposed. The exposed cecum was further ligated at half of its length (50% ligation) with a 5-0 suture (Prolene, Norderstedt, Germany) below the ileocecal valve and was punctured once with a 17-gauge needle (KLINIKA, Usingen, Germany) (Fig. 5). The cecum was then gently squeezed to extrude small amount of faeces from the perforation site and after that cecum with the gut content was returned back to the peritoneal cavity and the mice were resuscitated with 1.0 ml of 0.9% of saline intra peritoneal (i.p.).

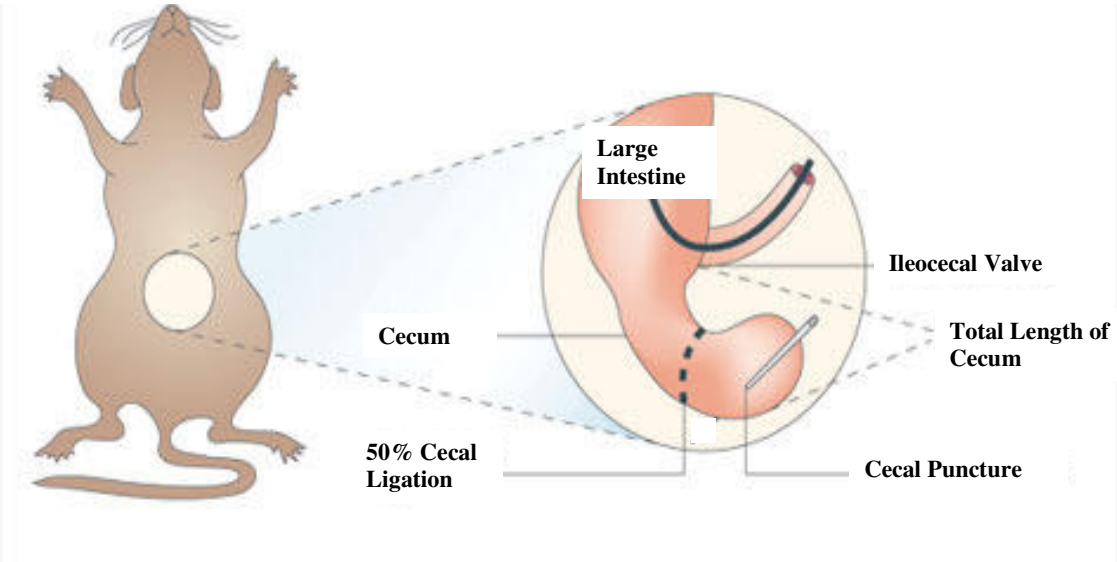


Figure 5: The experimental model for polymicrobial sepsis: cecal ligation and puncture (CLP) (Adapted from Buras et al., Nat Rev Drug Disc, 4, 854-65)

Thereafter, the abdomen was closed in a two layer technique with a 5-0 suture. Sham animals underwent a laparotomy without CLP. Finally, animals were returned to their cages with free access to food and water. This model leads to severe polymicrobial sepsis with a mortality rate of about 60-70% within 36 hours.

2.3. Culture Medium, Chemicals, Reagents, Antibodies and Buffers

All chemicals and reagents used throughout this work were of reasonable purity and purchased from different companies as mentioned below. All solutions and lab ware for cell

cultures were autoclaved or filtered sterile. All reagents were free of detectable lipopolysaccharide (LPS) contaminations as tested using Limulus amoebocyte assay (Bio Whittaker, MD, USA). All numbers given in percent (%) refer to either weight per volume or, in cases of liquids, to volume per volume.

2.3.1. Culture Medium

Very **L**ow **E**ndotoxin medium VLE RPMI 1640 (Biochrom, Berlin, Germany) containing 10% heat-inactivated Fetal Calf Serum (FCS) (Sigma, Deisenhofen, Germany and Biochrom), 10 mM HEPES (BioChrom), 2 mM L-Glutamine (Biochrom), 0.06 mg/ml Penicillin (Sigma), 0.02 mg/ml Gentamicin G (Sigma), and 0.05 mM 2-Mercaptoethanol (Sigma) was used as culture medium (CM).

2.3.2. Chemicals

7-aminoactinomycin D (7-AAD)	Molecular Probes, Karlsruhe, Germany
Ammonium Chloride (NH_4Cl)	Fluka, Deisenhofen, Germany
Buffer, Reference standard, pH 4, 7 and 10	Sigma
Calcium Chloride ($CaCl_2$)	Sigma
Cytofix/Cytoperm™	BD Biosciences, Heidelberg, Germany
di-Sodium EDTA	Promega, Mannheim, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma
FACS Flow	BD Biosciences
GolgiStop™ (monensin)	BD Biosciences
HCl (32%)	Merck, Darmstadt, Germany
Isofluran	DeltaSelect, Munich, Germany
Liberase™ Blendzyme 2	Roche, Grenzach-Wyhlen, Germany
Magnesium Chloride ($MgCl_2$)	Merck
di-Sodiumhydrogen Phosphate	Merck
Sodium Azide	Merck
Sodium Chloride (NaCl)	Merck
Sodium di-Hydrogen Phosphate	Merck

Sodium Hydroxide Pellets	Merck
Perm/Wash™ Buffer	BD Biosciences
Phosphate Buffer Saline Dulbecco's	Gibco, Karlsruhe, Germany
Potassium Hydrogen Carbonate ($KHCO_3$)	Fluka, Deisenhofen, Germany
Potassium Chloride (KCl)	Merck
TBST	Sigma
Vybrant™ CFDA SE Cell Tracer Kit	Molecular Probes

2.3.3. Reagents

Recombinant mouse granulocyte-monocyte colony-stimulating factor (rmGM-CSF), CD40 ligand (CD40L), Interleukin-10 (IL-10) and Interferon-γ (IFN-γ) were purchased from R&D systems, Wiesbaden, Germany. Synthetic phosphorothioated CpG 1668 oligonucleotides (Sparwasser et al., 1997) (sequence **TCCATGACGTTCCTGATGCT**) were purchased from Operon, Cologne, Germany. Bovine Serum Albumin (BSA), Lipopolysaccharide (LPS, *E.coli* 026:B6), Ionomycin, Concanavalin A (Con A), Dimethyl Sulphoxide (DMSO) and Phorbol 12-Myristate 13-Acetate (PMA) were purchased from Sigma.

2.3.4. Antibodies

All antibodies were purchased from BD Biosciences, ebioscience (Frankfurt, Germany), R&D Systems or Immunotools (Friesoythe, Germany).

2.3.4.1. Purified Monoclonal Antibodies

Antibody	Clone	Specificity	Isotype	Company
anti-CD16/CD32	2.4G2	Mouse	Rat IgG_{2b}, κ	BD Pharmingen
anti-IL-10	JES5-16E3	Mouse	Rat IgG_{2b}, κ	BD Pharmingen
anti-IL-10	JES052A5	Mouse	Rat IgG_1	R&D Systems
anti-CD3	17A2	Mouse	Rat IgG_{2b}, κ	BD Pharmingen

2.3.4.2. Fluorescent Dye Labeled Monoclonal Antibodies

Antibody-Labeled	Clone	Isotype	Company
anti-CD3e	FITC145-2C11	Armenian Hamster IgG_1, κ	BD Pharmingen
anti-CD4 FITC	YTS 191.1.2	Rat IgG_{2b}	Immunotools
anti-CD4 FITC	RM4-5	Rat (DA/HA) IgG_{2a}, κ	BD Pharmingen
anti-CD4 PE	RM4-5	Rat (DA) IgG_{2a}, κ	BD Pharmingen
anti-CD8a FITC	53-6.7	Rat (LOU/Ws1/M) IgG_{2a}, κ	BD Pharmingen
anti-CD11b PerCP-CY5.5	M1/70	Rat (DA) IgG_{2a},κ	BD Pharmingen
anti-CD11c APC	N418	Armenian Hamster IgG	eBioscience
anti-CD11c APC	HL3	Armenian Hamster IgG_1, λ	BD Pharmingen
anti-CD11c PE	HL3	Armenian Hamster IgG_1, λ	BD Pharmingen
anti-CD25 APC	PC61	Rat (Outbred OFA) IgG_1, λ	BD Pharmingen
anti-CD40 FITC	HM40-3	Armenian Hamster IgM, κ	eBioscience
anti-CD40 FITC	3/23	Rat (LOU) IgG_{2a}, κ	BD Pharmingen
anti-CD69 PE	H1.2F3	Armenian Hamster IgG_1, λ	BD Pharmingen
anti-CD80 FITC	16-10A1	Armenian Hamster IgG_2, κ	BD Pharmingen
anti-CD86 PE	GL1	Rat (LOU) IgG_{2a}, κ	BD Pharmingen
anti-B220 PE	RA3-6B2	Rat IgG_{2a}, κ	BD Pharmingen
anti-Gr-1 FITC	RB6-8C5	RatIgG_{2b}	Immunotools
anti-H-$2K^d$ PE	SF1-1.1	Mouse(SJL) IgG_{2a}, κ	BD Pharmingen
anti-IA/IE Biotin	2G9	Rat (DA/HA) IgG_{2a}, κ	BD Pharmingen
anti-IA/IE FITC	2G9	Rat (DA/HA) IgG_{2a}, κ	BD Pharmingen
anti-IL-2 PE	JES6-5H4	RatIgG_{2b}	BD Pharmingen
anti-IL-12p40 PE	C15.6	Rat IgG_1	BD Pharmingen
anti-TNF-α PE	MP6-XT22	RatIgG_1	BD Pharmingen
anti-IFN-γ PE	XMG1.2	RatIgG_1	BD Pharmingen

2.3.5. Buffers

2.3.5.1. Erythrocyte or Red Blood Cell (RBC) Lysis Buffer (0.15 M) (100 ml)

Constituent	Weight
NH_4Cl	829.0 mg
$KHCO_3$	100.0 mg
$Na_2EDTA.2H_2O$	3.7 mg

2.3.5.2. Magnetic Cell Sorting (MACS) Buffers

Running Buffer/MACS Buffer (500 ml)

PBS	493.5 ml
FCS (0.5%)	2.5 ml
EDTA (2 mM)	4.0 ml from 250 mM stock solution

Rinsing Solution (500 ml)

PBS	496.0 ml
EDTA (2 mM)	4.0 ml from 250 mM stock solution

Cleaning Solution

70% v/v ethanol

2.3.5.3. Liberase Blendzyme Buffer (10 x) (100ml)

KCl	372.8 mg
$CaCl_2$	266.0 mg
$MgCl_2$	95.2 mg
NaCl	8.8 g
HEPES	10.0 ml from 1 M stock solution

2.4. MACS Kits, Equipment and Accessories

2.4.1. MACS Kits

Basic MicroBeads	Miltenyi Biotec, Moenchengladbach, Germany
CD11c (N418) MicroBeads	Miltenyi Biotec
Pan T cell isolation kit	Miltenyi Biotec

2.4.2. MACS Equipment and Consumables

autoMACS Machine	Miltenyi Biotec
autoMACS separator	Miltenyi Biotec
MACS Pre-Separation Filters	Miltenyi Biotec

2.5. Isolation of Organs and Fluids

2.5.1. Blood

The animals were sacrificed using CO_2 and with the help of sterile forceps and scissors, the chest was cut open. From the opened rib cage, the heart was exposed and cut open. Thereafter, flowing blood was collected into 1.5 ml microcentrifuge tubes (Star Lab, Ahrensburg, Germany) using 1.0 ml pipette tip (shortened at the tip as to increase the area). The blood was kept at room temperature (RT) for 2 h for clotting. After that the blood was centrifuged at 9400 x g for 10 minutes at RT. After centrifugation, serum was collected and supernatant was further analyzed for various cytokines.

2.5.2. Peritoneal lavage Fluid (PLF)

The animals were sacrificed using CO_2 and the peritoneal cavity of individual mouse was cannulated with a 20-gauge needle and 1 ml ice-cold PBS was injected and aspirated repeatedly with a syringe. This procedure was repeated two times. PLF was then centrifuged at 294 x g for 10 minutes at 4˚C. After centrifugation supernatant was collected and used to determine different cytokines by ELISA/CBA (as described in section 2.11.).

2.5.3. Bone Marrow Cells (BMCs) Preparation

The bone marrow cells were prepared from healthy control animals. The method described by Lutz *et al.* (Lutz et al., 1999), with minor modifications (as described in the following lines) was used. Animals were sacrificed using CO_2 and the hind legs were isolated and femurs and tibias were cleanly excised and were freed from excess muscle tissue using sterile forceps and scissors and bones were kept in PBS on ice. After that bones were cleaned two times with 70% alcohol and two times with PBS successively. After that both ends of each femur and tibia were cut off and bone marrow was flushed out with a 27-gauge needle (attached to a syringe with CM). Thereafter, BMCs were resuspended gently to dissolve cell aggregates and the cell suspension was transferred into 15 ml tubes and was centrifuged at 294 x g for 10 minutes at 4˚C. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 2 ml of CM. Cells were then counted and plated in 100 mm Petri dishes (BD Biosciences) at a concentration of 2 x 10^6 Cells/Petri

dish in 10 ml CM supplemented with 20 ng/ml rmGM-CSF (GM-CSF helps in the proliferation and differentiation of hematopoetic cells). On day 3, some Petri dishes with BMCs were used for the analysis of dendritic cell (DC) surface markers like CD11c and MHC II while rests of the Petri dishes were fed with culture medium containing 20 ng/ml rmGM-CSF. On day 7, non-adherent cells were harvested and counted. About 70-75% cells harvested on 7^{th} day were MHC II and CD11c positive as confirmed by staining and FACS analyses (as described below). These cells are termed **B**one **M**arrow derived **D**endritic **C**ells (BMDCs) in the following.

2.5.4. Total Spleen Cells Preparation

The spleen was isolated separately from each animal and any extra tissue attached to it was carefully removed without cutting it. After that spleen was rinsed with 70% alcohol and 500 µl of 0.02 U/ml Liberase Blendzyme 2 (Liberase Blendzyme is a mixture of highly purified collagenases and neutral protease enzymes, formulated for efficient and gentle dissociation of tissue. It acts on the collagen and non-collagen proteins that constitute the intercellular matrix) and was injected into the spleen with the help of a 27-gauge needle attached to a syringe. The spleen was then divided into three equal parts with the help of sterile scissors and kept in 15 ml of Liberase Blendzyme 2 solution at 37°C for 18 min. After treatment with Blendzyme, spleen was transferred to a 70 µm cell strainer (BD Biosciences) kept in the CM containing 5 mM EDTA and meshed gently. After that the cells were resuspended well and incubated for 5 min for dissociation of cell clusters. Thereafter, cells were passed through a 40 µm cell strainer (BD Biosciences) to remove any remaining tissue particle and collected in 50 ml tubes. After that the cells were centrifuged at 294 x g for 10 minutes at 4˚C. After centrifugation, the supernatant was removed and the cell pellet was resuspended in 1 ml erythrocyte lysis buffer. After that the cells were mixed gently and 6 ml CM was added quickly as to stop the lysis process. Thereafter, the cell suspension was underlaid with 1 ml of FCS (all the steps for RBC lysis were done at room temperature) and centrifuged at 294 x g for 10 minutes at 4˚C. After washing, cells were resuspended in 10 ml CM and counted.

2.5.5. Total Lymph Node Cell Preparation

The peripheral lymph nodes, inguinal and popliteal, were isolated and kept in CM on ice. The lymph nodes were transferred into Petri dish and each of them was flushed with CM using a 27-gauge needle. The remaining lymph node tissue was minced through a 40 µm cell strainer. Thereafter, cells were mixed gently and were incubated in CM containing 2 mM EDTA in order to dissociate cell clusters. The cells were then collected in a 15 ml tube and centrifuged at 294 x g for 10 minutes at 4˚C. After washing, the supernatant was discarded and the cells were resuspended in 500 µl of CM and counted.

2.6. Purification of Cells Using MACS Technology

2.6.1. Principle and Method

MACS is an extremely efficient magnetic separation technology using super-paramagnetic MicroBeads with surface antigen recognizing antibodies tagged to it.

Figure 6: The autoMACS™ separator from Miltenyi Biotech is an automated bench-top magnetic cell sorter. It is equipped with strong permanent magnets holding the separation columns and separates the magnetically labeled cells with high speed

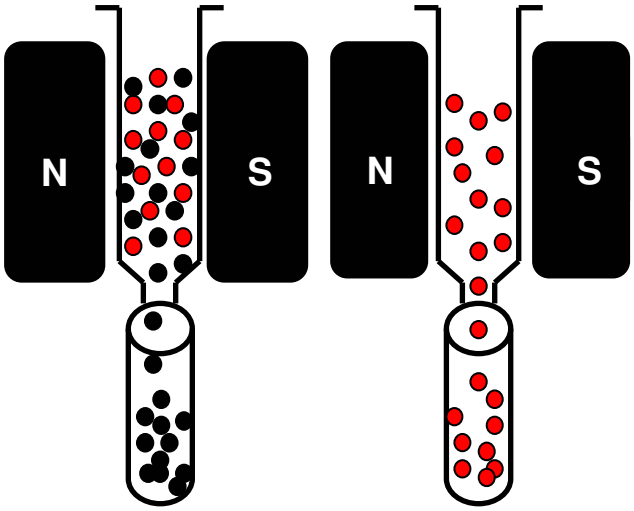


Figure 7: Magnetic cell separation using autoMACS. Labeled cells (shown in red) are retained in the column while unlabeled cells (shown in black) are collected as flow through. On further washing with MACS buffer and under controlled pressure, the retained fraction can be eluted.

Using this technique, even the rarest cells can be isolated. The viability and purity of the separated cells is very high. MicroBeads are very small in size (about 50 nm in size) and

are made up of iron oxide and polysaccharide. They are too small to be detected by optical methods. Therefore, they do not change the scatter properties of the cells during flow cytometer analysis. Moreover, selected cells can be used for culture as MicroBeads decompose during culture and do not change the cell viability and function.

With MACS, cells of interest (positive selection) or cells to be depleted (negative selection) are specifically labeled with MicroBeads. After magnetic labeling, the cells are passed through a separation column which is placed in a strong permanent magnet. The magnetically labeled cells are retained in the column and separated from the unlabeled cells, which pass through. After washing the column with MACS buffer, the retained fraction can be eluted. Both fractions, labeled and unlabeled, are completely recovered.

2.6.2. Usage

2.6.2.1. Isolation of Splenic Dendritic Cells

Splenic dendritic cells (DCs) were purified using CD11c MicroBeads according to the manufacturer's recommendations. Total spleen cells without erythrocytes were magnetically labeled with CD11c MicroBeads and passed through a separation column placed in the magnetic field. The magnetically labeled $CD11c^+$ cells were retained in the column and were collected as positive fraction.

After preparation of TSCs (as described above with erythrocyte lysis step), cells were centrifuged at 294x g for 10 minutes at 4˚C, resuspended in CM and counted. After additional centrifugation, cells were resuspended in 500 µl of binding buffer (PBS + 0.5% FCS) per 10^8 total cells. Then 50 µl of 1:10 diluted Basic MicroBeads per 10^8 cells were added to the cell suspension. The cells were mixed with Basic MicroBeads for a few seconds and were washed with a 10-20 fold volume of binding buffer. After washing, the cells were resuspended in 500 µl of binding buffer per 10^8 cells and were loaded onto the autoMACS column to separate the viable cells without any non-specific material. The Basic MicroBeads treatment depletes material that binds non-specifically to MicroBeads like dead cells and debris. The effluent or unlabeled cells were collected and used further

for DCs isolation. After Basic MicroBeads step, the cells were counted and resuspended in 400 µl of binding buffer per 10^8 total cells. Thereafter, 100 µl of CD11c MicroBeads per 400 µl of cell suspension were added and cells were mixed gently and kept in dark at 4˚C. After 15 minutes, the cells were washed with binding buffer and centrifuged at 294 x g for 10 minutes at 4˚C. After centrifugation, the cells were resuspended in 500 µl of binding buffer per 10^8 cells and were then loaded onto the autoMACS column to separate the CD11c labeled cells from unlabeled cells. The positive fraction (purified DCs) was collected, centrifuged, resuspended in 500 µl of CM and counted. The purity of DCs was generally 85-90% as confirmed by CD11c staining and FACS analysis.

2.6.2.2. Isolation of total splenic T cells

Total splenic T cells were isolated using Pan T cell isolation kit according to the manufacturer's instructions. Using this kit, total mouse T cells were isolated by depletion of non-T cells like B cells, NK cells, dendritic cells, macrophages, granulocytes and erythroid cells which were indirectly labeled with a cocktail of biotin labeled monoclonal antibodies specific for their surface markers, as primary labeling reagent followed by anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. Highly pure T cells were isolated by depletion of magnetically labeled non-T cells.

After preparation of TSCs (as described above but without erythrocyte lysis step), cells were centrifuged at 294 x g for 10 minutes at 4˚C, resuspended in CM and counted. After centrifuging again, the cells were resuspended in 400 µl of binding buffer per 10^8 total cells. To 10^8 total cells, 100 µl of biotin labeled antibody cocktail was added, mixed well and kept for 10 minutes at 4˚C in dark. Thereafter, 300 µl of binding buffer and 200 µl of anti-biotin MicroBeads were added per 10^8 cells. The cells were mixed well and incubated for additional 15 minutes at 4˚C in the dark. The cells were washed and resuspended in 500 µl of binding buffer per 10^8 cells and were then loaded onto the autoMACS column to separate the unlabeled cells from labeled cells. The negative fraction (purified total T cells) was collected, centrifuged, resuspended in 2 ml of CM and counted. The purity of T cells was generally more than 90% as confirmed by CD3 staining and FACS analysis.

2.7. Cell Culture

2.7.1. Total Spleen Cells Culture

The total spleen cells were cultured in CM in 96-well plates with or without 3 μg/ml Con A (Con A is a mitogen for T cells) or 5 ng/ml PMA + 500 ng/ml of Ionomycin (PMA activates Protein Kinase C and Ionomycin increases the concentration of calcium ions inside the cell and their combination brings about activation of T cells) for 2-3 days or in 48-well plates, with or without 5 μg/ml CpG (CpG is a immunostimulatory molecule for antigen presenting cells like DCs) for 18 hours. After incubation, supernatants were collected for protein analysis (as described in section 2.11.) and nonadherent cells were used for FACS analysis.

2.7.2. Splenic Dendritic Cells Culture

The purified splenic DCs were cultured in CM containing 0.3 ng/ml rmGM-CSF in the absence or presence of 100 ng/ml LPS + 2.5 μg/ml CD40L (CD40L binds to CD40 and induces maturation and IL-12 release by DCs) or 5 μg/ml CpG (CpG induces IL-12 release by DCs) in 96-well flat-bottom plates (10^5 cells/well) for 18 h. In some experiments, stimulation of DCs was performed in the presence of rmIFN-γ (10 ng/ml) and in some other experiments neutralizing anti-IL-10 (αIL-10) antibodies (10 μg/ml) or the respective rat IgG1 isotype control (10 μg/ml). For the standardization of the amount of αIL-10 antibodies needed to neutralize 1000 pg/ml IL-10 sufficiently, splenic DCs were purified from healthy control and cultured with or without CpG, in the absence or presence of 10 μg/ml neutralizing αIL-10 antibodies in combination with 1 ng/ml or 5 ng/ml recombinant mouse IL-10 (rmIL-10) or 10 μg/ml of the rat IgG1 isotype control in combination with 1 ng/ml or 5 ng/ml rmIL-10. The combination of αIL-10 and rmIL-10 or rat IgG1 isotype control + rmIL-10 was incubated for 30 minutes on ice prior to the addition to the cells. After addition of the mixture, cells were further incubated for 1 h and then CpG was added. After 18 h, supernatants were collected and were analyzed for IL-12 production. Experimental results have shown that 10 μg/ml αIL-10 antibodies were sufficient to neutralize the activity of 1000 pg/ml IL-10 and therefore, this concentration was used for the experiments.

2.8. Flow Cytometry and Fluorescence Associated Cell analyses (FACS)

2.8.1. Principle and Method

Flow cytometry refers to the technique where measurements of the physical and chemical characteristics of the cells are made when they pass in a fluid stream through a measuring point surrounded by an array of detectors. At the measuring point in a typical flow cytometer the stream of cells intersects a beam of light from a laser (as shown in figure 8). When light interacts with biological particles some of the light is scattered out of the incident beam and this scattered light may be collected over a range of angles by detectors positioned around the measuring point. The light which is scattered in the forward direction, typically up to 20˚ offset from the laser beam's axis, is collected by a detector known as the forward scatter channel (FSC). The FSC intensity roughly equates the particle's size and can also be used to distinguish between cellular debris and living cells. The light which is measured approximately at a 90˚ angle to the excitation line is called side scatter. The side scatter channel (SSC) provides information about the granularity of the particle. Both FSC and SSC are specific for any particle, and the combination of both can be used to differentiate between different cell types in a heterogeneous sample. Additionally, fluorescence measurements taken at different wavelengths can provide quantitative and qualitative data about cell-surface or intracellular molecules which are labelled with different fluorochromes. The separate fluorescence (FL) channels are there in flow cytometers to detect emitted light (FL-1, FL-2 etc) (Fig. 8). The number of these FL channels varies from machine to machine and its manufacturer. In the present study FACScalibur (BD Biosciences) was used which is equipped with four FL channels.

Fluorochromes used in the present study	**FL-channel**
FITC (Fluorescein isothiocyanate)	FL-1
PE (Phycoerythrin)	FL-2
PerCP-Cy5.5 (Peridinin-chorophyll-protein complex-cyanine 5.5)	FL-3
APC (Allophycocyanin)	FL-4

In addition, the light may interact with the natural autofluorescent pigments present within the cell or with fluorescent stains added to the cells prior to analysis. In these cases cell-associated fluorescence is also produced and these photons additionally may be collected by suitable detectors. The most important feature of flow cytometry is that it enables measurements to be made on individual cells at high speeds. This allows one to quantify the heterogeneity of the population of interest rather than merely to obtain average values for a population.

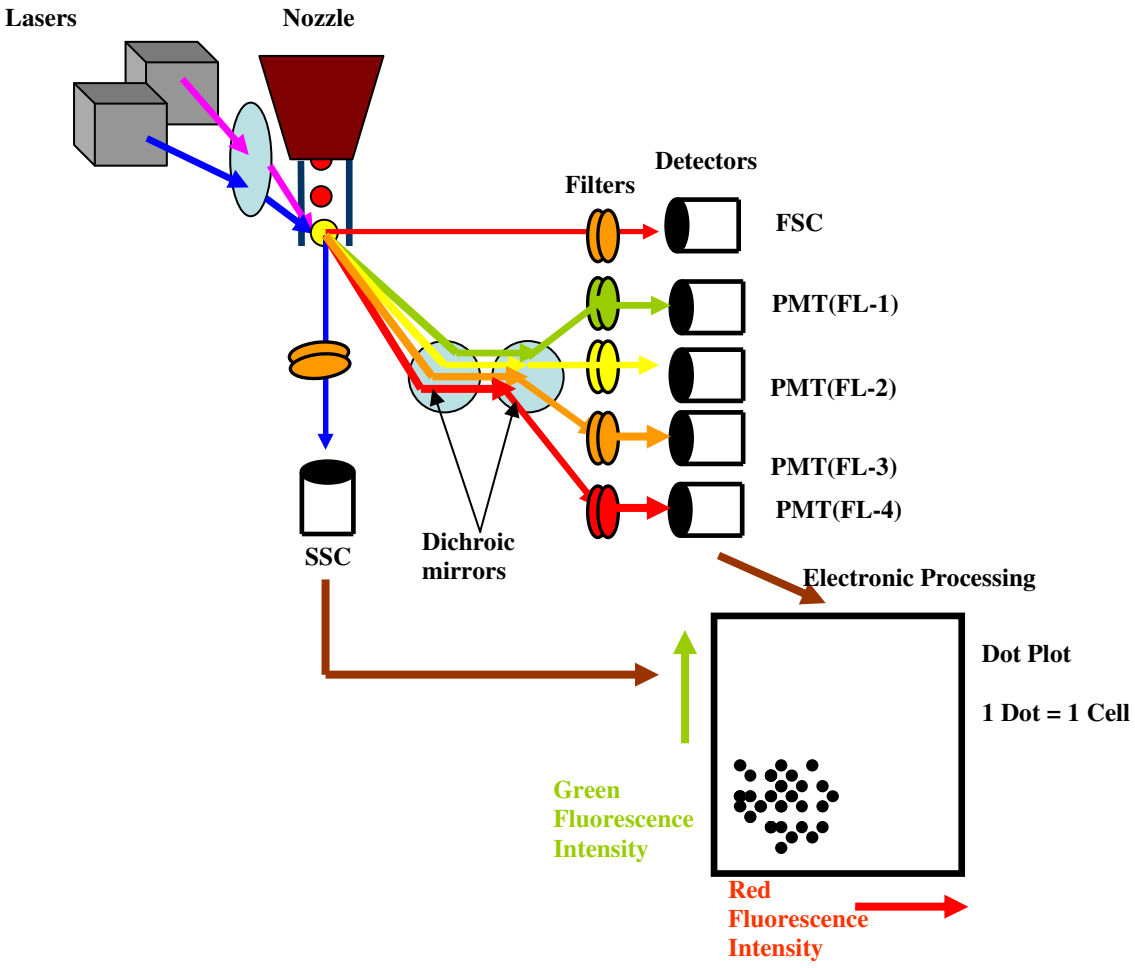


Figure 8: Schematic overview of a typical flow cytometer setup and the principle of flow cytometry.

2.8.2. Usage

2.8.2.1. Surface Stainings

For surface staining, cells (0.5-1x10^6) were washed with Cell Wash (BD). After centrifugation at 460 x g for 6 min at 4˚C, supernatant was discarded and cells were resuspended in 50 µl Cell Wash containing 1 µg Fc Block (BD) for blocking unspecific binding and kept at 4˚C in the dark. After 6 min, for the study of DCs surface and co-stimulatory markers, 50 µl of antibody mixture containing anti-CD11c-APC in combination with anti-CD40-FITC (0.25 µg), anti-CD86-PE (0.005 µg), anti-CD80-FITC (0.5 µg), anti-H2-K^d-PE (0.05 µg) anti-IA^d-FITC (0.03 µg for non-stimulated cells staining and 0.015 µg for stimulated cells staining) , anti-CD8-FITC (0.125 µg), or anti-CD4-PE (0.8 µg) was added and cells were kept for additional 15 min. at 4˚C in the dark. After incubation in antibody mixture, 1 ml of Cell Wash was added and cells were centrifuged at 460 x g for 6 minutes at 4˚C. After centrifugation, supernatant was discarded and cells were resuspended in 250 µl of Cell Wash for FACS analyses.

In case of lymphocyte studies, for the detection of B cells anti-B220-FITC antibody (0.05 µg) was used and for the T cell stimulation study, anti-CD3-FITC antibody (0.25 µg) in combination with anti-CD25-APC (0.05 µg) and anti-CD69-PE (0.1 µg) was used. The same staining process as described for the DCs was followed.

The other cell types like monocytes and granulocytes were studied using anti-CD11b-PerCP-Cy5.5 (0.2 µg) and anti-Gr-1-FITC (titrated amount of 0.5 µl) antibodies respectively. The same staining process as described for the DCs was followed.

2.8.2.2. Intracellular Staining

To measure stimulation-dependent changes in cytokine production on single-cell level, cells were stimulated with CpG (5 µg/ml) and during the last 4-6 h, monensin (GolgiStop 0.66 µl/ml) was added. Monensin is an inhibitor of intracellular protein transport. This results in the accumulation of most cytokines inside the Golgi complex and thus enhances the detectability of cytokine-producing cells. After stimulation, cells were harvested and

centrifuged at 460x g for 6 minutes at 4˚C. After centrifugation, the supernatant was discarded and cells were resuspended in 50 µl Cell Wash containing 1 µg FcBlock (BD Biosciences) for blocking unspecific binding. After 6 min, cells were surface stained using anti-CD11c-APC or anti-CD4-FITC antibodies (as described above). The cells were then fixed and permeabilized using Cytofix/Cytoperm (it is used for simultaneous fixation and permeablization of the cells) for 20 min at RT. Thereafter, intracellular cytokines were stained using anti-IL-12p40-PE (0.4 µg), or anti-IL-2-PE (0.2 µg) antibodies. For analysis of IL-2 synthesis, co-cultures of splenic DCs and T cells were restimulated with 5 ng/ml PMA, 500 ng/ml ionomycin, and monensin (0.66 µl/ml) for 4 h before staining for intracellular IL-2. After washing with permeabilization buffer, cells were resuspended in Cell Wash and analyzed by FACS.

Appropriate isotype controls were used for all stainings. All data were acquired using a FACScalibur (BD Biosciences) and analyzed using CellQuest pro software (BD Biosciences). For all stainings, the FL-3 channel was used to exclude autofluorescent cells. Living cells were selected according to forward and side scatter properties. The mean fluorescence intensity (MFI) (denotes the amount of marker or fluorochrome present on the surface of the cell population) of the respective molecules stained in combination with anti-CD11c antibody was determined on gated, splenic $CD11c^{hi}$ and lymph node $CD11c^{+}$ DCs. Quadrant lines in dot plots were set as indicated in the figure legends. Total DC number per spleen was calculated as % $CD11c^{hi}$ cells x total number of cells per spleen/100.

2.9. Allogenic T cell Assay

2.9.1. Carboxyfluorescein diacetate Succinimidyl Ester (CFDA-SE or CFSE)

CFSE is used for labeling the cells. As the name suggests its chemical structure contains two acetate groups. It enters passively into the cells. It is colorless and nonfluorescent until its acetate groups are cleaved by intracellular esterases to give highly fluorescent, amine reactive carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent complexes which are well retained inside the cell.

CFSE labeled cells induce a signal in the FL-1 channel and can be detected in flowcytometry (Fig. 9).

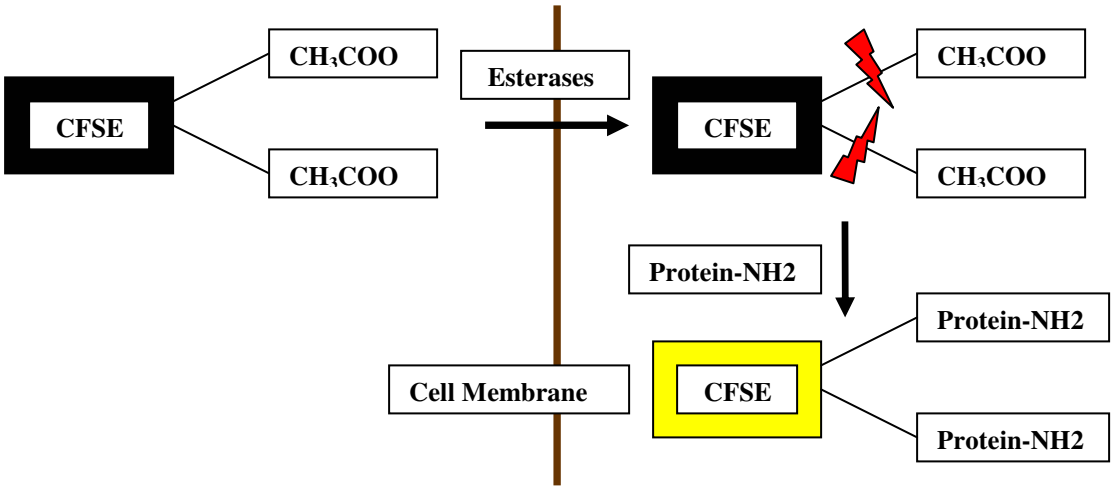


Figure 9: The principle of CFSE staining

2.9.2. CFSE Labeling of T cells

Splenic $CD3^+$ T cells from C57BL/6 or BALB/c mice were purified using Pan T Cell Isolation Kit (as described in section 2.7.2.2.). After isolation, T cells were stained with CFSE. After centrifugation at 294 x g for 10 minutes at 4˚C, the required number of T cells ($4\text{-}8x10^6$ in 500 µl) was resuspended in prewarmed PBS (1.5 µl microcentrifuge tubes were used). To the resuspended cells, 500 nM of CFSE (reconstituted in DMSO) was added and cells were gently resuspended and incubated for 12 minutes at 37˚C in a water bath. After incubation, cells were centrifuged and resuspended in 1 ml of prewarmed medium followed by incubation for 30 minutes at 37˚C in a water bath. After incubation, cells were centrifuged again at 294 x g for 10 minutes at 4˚C and resuspended in prewarmed medium. Cells were then counted and $2x10^5$ T cells were added to the titrated amounts of purified splenic DCs (as described in section 2.9.3.).

2.9.3. Splenic DCs and T cells Co-culture

After labeling of T cells with CFSE, $2x10^5$ T cells were added to the titrated amounts of purified splenic DC per well (96-well, half-area flat bottom plate, Corning, Germany). All

cultures were set up in triplicates. After 3 days, cells were harvested and cell clusters were dissociated through addition of 7.5 mM EDTA for 15 min. After washing with cell wash, T cells were stained using anti-CD3-PE antibody (0.4 µg). Labeled T cells were further mixed with 7-AAD (4 µg/ml) and a constant number of APC CaliBRITE APC beads (BD Biosciences). CaliBRITE APC beads were added in order to allow the acquisition of equal parts per culture. 7-AAD was used to exclude dead cells. For data acquisition, a constant number of CaliBRITE beads were counted. Living T cells ($CD3^+7\text{-}AAD^-$) were gated and the number of divided cells showing less than the maximal CFSE fluorescence intensity was determined.

2.10. Transfer of Mature BMDCs into Septic Animals

BMDCs were prepared as described above. On the 7^{th} day, cells were harvested, centrifuged, resuspended in CM and counted. After counting, $2x10^6$ cells were cultured in a total volume of 2 ml CM (supplemented with 10 ng/ml rmGM-CSF) in presence of 5 µg/ml CpG for 8 h in 6-well plates. After 8 h, non-adherent cells were harvested, washed twice with PBS and counted. The cells were then resuspended in PBS and each mouse was injected with $2x10^6$ cells in 100 µl PBS intravenously (i.v.) or 100 µl PBS.

2.11. Protein Analysis

2.11.1. Enzyme Linked Immuno-Sorbent Assay

2.11.1.1. Principle

The enzyme linked immuno-sorbent assay (ELISA) is a protein assay in which bound antigen is detected by an antibody linked enzyme that converts a colorless substrate into a colored product. A modification of ELISA known as capture or sandwich ELISA is used in the present study to detect secreted products such as cytokines. In sandwich ELISA, cytokine-specific antibodies are bound to the plate, and thus bind cytokine molecules with high affinity even if cytokine molecules are present in very low concentrations. The separate biotinylated antibodies that recognize different epitopes in comparison to the immobilized first antibodies are then added to the bound cytokine molecules. Now, as to detect the sandwiched cytokine molecules, **H**orse **R**adish **P**eroxidase (HRP)-linked

antibodies are added followed by the substrate which gives colored or fluorescent products. The spectroscopic measurement of the color intensity corresponds to the amount of cytokine present.

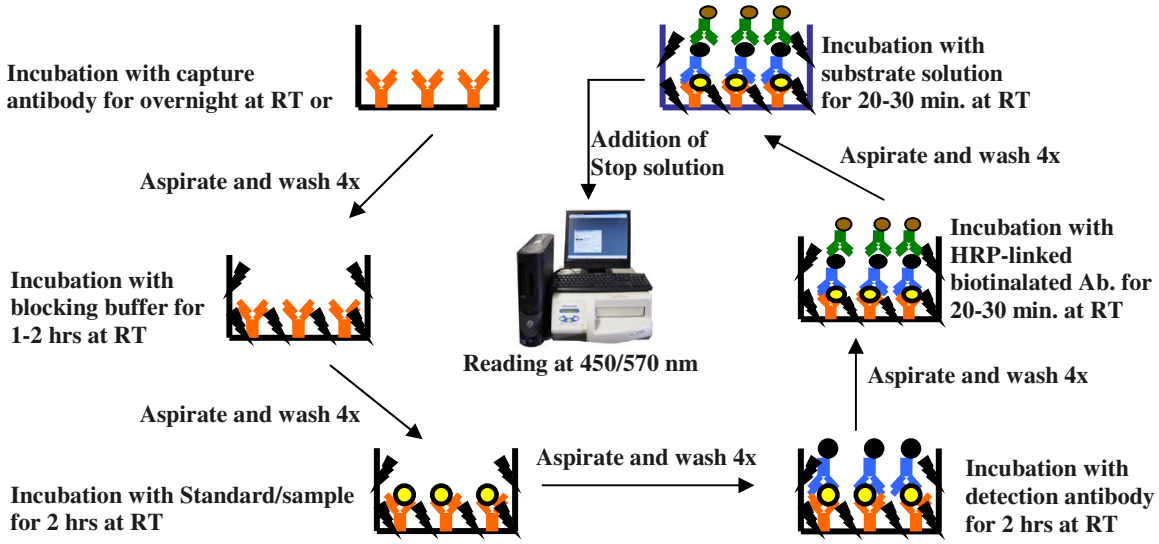


Figure 10: Schematic graphic presentation of ELISA procedure

Flow Diagram for ELISA Procedure

96-well ELISA plate was coated with capture antibody and kept for overnight at RT or at 4˚C

Aspirated and washed 4x

Incubated with blocking buffer for 1-2 hrs at RT

Aspirated and washed 4x

Incubated with Standard/sample for 2 hrs at RT

Aspirated and washed 4x

Incubated with detection antibody for 2 hrs at RT

Aspirated and washed 4x

Incubated with HRP-linked biotinalated antibody for 20-30 min. at RT

Aspirated and washed 4x

Incubated with substrate solution for 20-30 min. at RT

Reading at 450/570 nm

Measurement of the Amount of Cytokine

The amount of cytokine present in the unknown sample was measured by preparing a standard curve. A standard curve was constructed by plotting varying amounts of known standard values and their corresponding optical densities (O.D.). The value of the unknown sample was then measured by comparing with the standard. A standard curve and unknown sample value was generated by using automated microplate reader (ELx 808, Bio-Tek instruments, Wilrijk, Belgium) and Mikro Win Version 3.0 software (Mikrotek, Overath, Germany).

2.11.1.2. Usage in the Detection of Cytokines in Supernatants

To measure stimulation-dependent changes in cytokine secretion, supernatants from stimulated TSCs cultures, purified splenic DCs cultures or from co-culture of splenic DCs and T cells were collected and tested for the production of IL-2, IL-10, IL-12, IFN-γ or TNF-α by ELISA as recommended by manufacturer's (R&D systems, e-bioscience and Biosource, Solingen, Germany).

2.11.2. BD™Cytometric Bead Array (CBA)

2.11.2.1. Principle

The BD™ CBA employs a series of particles (capture beads) with discrete fluorescence intensities to simultaneously detect multiple cytokines from single serum or cell culture supernatant sample. The CBA, combined with flow cytometry, creates a powerful multiple cytokine (multiplex) assay system. The CBA uses the sensitivity of amplified fluorescence detection by flow cytometry to measure cytokines with a particle-based immunoassay. The specific capture beads are mixed with recombinant protein standards or test samples then incubated with phycoerythrin (PE)-conjugated detection antibodies to form sandwich complexes.

Following acquisition of sample data using the flow cytometer (BD FACScalibur), the sample results are generated using the new FCAP Array™ software (BD Biosciences).

Flow Diagram for CBA Procedure

Incubation of capture beads (having distinct fluorescent intensities) with sample/standard for 1 h in dark at RT

↓

Incubation of capture beads plus sample/standard with PE-labeled detection antibodies for addition 1 h in dark at RT

Washed with 1 ml wash buffer

↓

Resuspension of sandwich complexes in 300 µl of buffer

↓

Acquisition and analysis of sample data

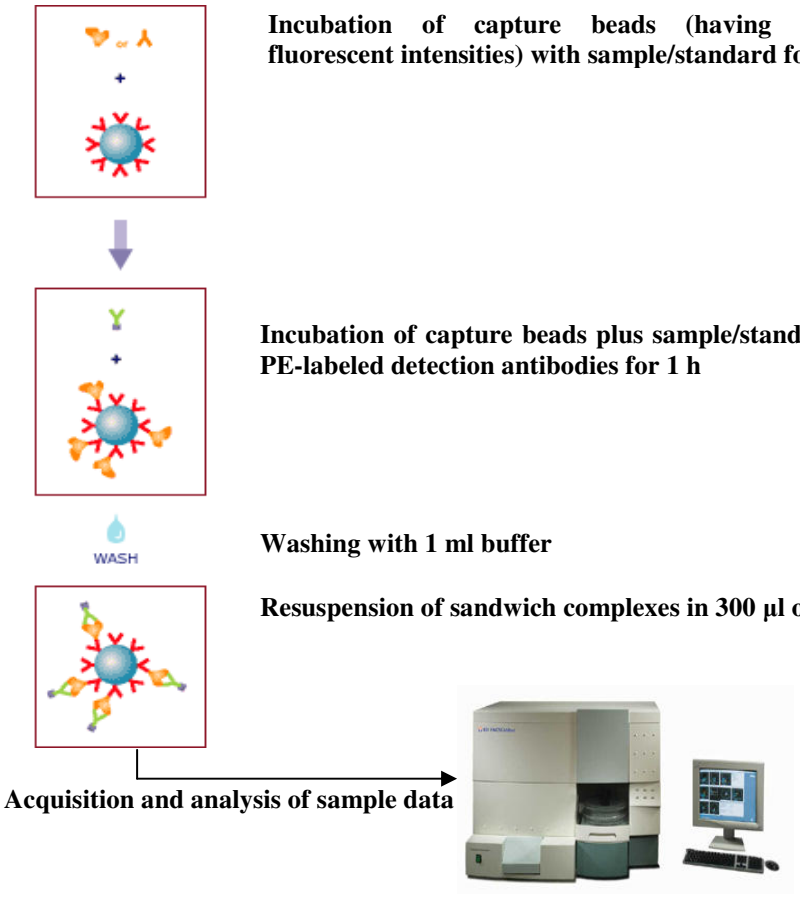


Figure 11: Schematic graphic representation of CBA technique (adapted from BD Biosciences website)

Measurement of the Amount of Cytokine

The amount of various cytokines present in the unknown sample was determined by generating sample results in a graphical and tabular format using FCAP Array software. This software determines cytokine concentrations in the unknown sample based on known concentration values of a set of standards. It measures the MFI of the detector antibody (PE-labeled) for each cytokine. Thereafter, software fits a standard curve to the data from the concentration standards. The standard curve is used to calculate concentration values for each of the measured cytokine in each sample.

2.11.2.2. Usage in the Detection of cytokines in Supernatants

To measure stimulation-dependent changes in cytokine secretion, supernatants from stimulated TSCs cultures and purified splenic DCs cultures were tested for interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1) by using the cytometric bead array CBA Mouse Inflammation Kit. The supernatants from the co-culture of splenic DCs and T cells were tested for interleukin-2 (IL-2), TNF-α and IFN-γ using CBA Mouse Th1/Th2 Kit.

2.12. Statistical Analyses

Results are presented as the mean ± SD or mean ± SEM. Differences between sham and CLP groups were determined by paired Student's *t*-test. The unpaired Student's *t*-test was used to compare differences between replicates of cell cultures within one experiment. One-way analysis of variance (ANOVA) was determined for the differences between sham, CLP and CLP treated with CpG stimulated BMDCs. *Post test* analysis was performed with Tukey's multiple range test. A P value < 0.05 was considered to be significant.

3. Results

3.1. Characterization of Dendritic Cells during Sepsis

3.1.1. Bacteria and Systemic Cytokines

To characterize which bacteria or bacterial species were involved in the development of sepsis in the mouse after CLP, the gut exudate was analyzed using bacteriological methods. The analysis showed that *Escherichia coli* (Gram-negative), *Bacillus species* (mostly Gram-positive) and *Lactobacillus species* (Gram-positive) were present in the gut exudate.

Next, to estimate how fast bacteria spread from the peritoneal cavity to the spleen which represents a systemic compartment, the total spleen cell suspensions were analyzed at different time-points following CLP or sham operation. Spleens were removed at 4, 8, 15 and 36 h after operation and the total spleen cell suspensions were prepared and plated onto blood agar plates. Thereafter, plates were kept for overnight at 37˚C and were analyzed for the presence of bacterial colonies. In control animals for any given time points there were no colonies while bacterial colonies were formed as early as 4 h after CLP in septic animals (Table 2).

[h]	**4**	**8**	**15**	**36**
Control	-	-	-	-
CLP	+	+	+	+

Table 2: The presence of bacteria in spleen during sepsis. Four, 8, 15 and 36 h after CLP or sham operation, the total spleen cell suspensions were prepared and plated onto blood agar plates. After overnight incubation at 37˚C, plates were analyzed for the presence of bacterial colonies. (-) indicates no bacterial colonies and (+) indicates presence of bacterial colonies.

After confirming the systemic presence of bacteria, the presence of different pro- and anti-inflammatory cytokines in the blood was determined. Therefore, at 4, 8, 15 and 36 h after CLP or sham operation, blood was collected and serum was prepared and analyzed for TNF-α and IL-10. For all time points, septic mice showed increased serum levels of TNF-α and IL-10 as compared to control mice. Together, during polymicrobial sepsis the systemic spread of bacteria induces the activation of pro- and ant-inflammatory molecules.

3.1.2. Changes in Cell Number during Sepsis

3.1.2.1. Change in Total Spleen Cells Number

It is known that leukocytes undergo apoptosis during sepsis (Wesche et al., 2005). To investigate how total cell number of the spleen change in our CLP model, total spleen cell suspensions were prepared at different time-points following CLP or sham operation.

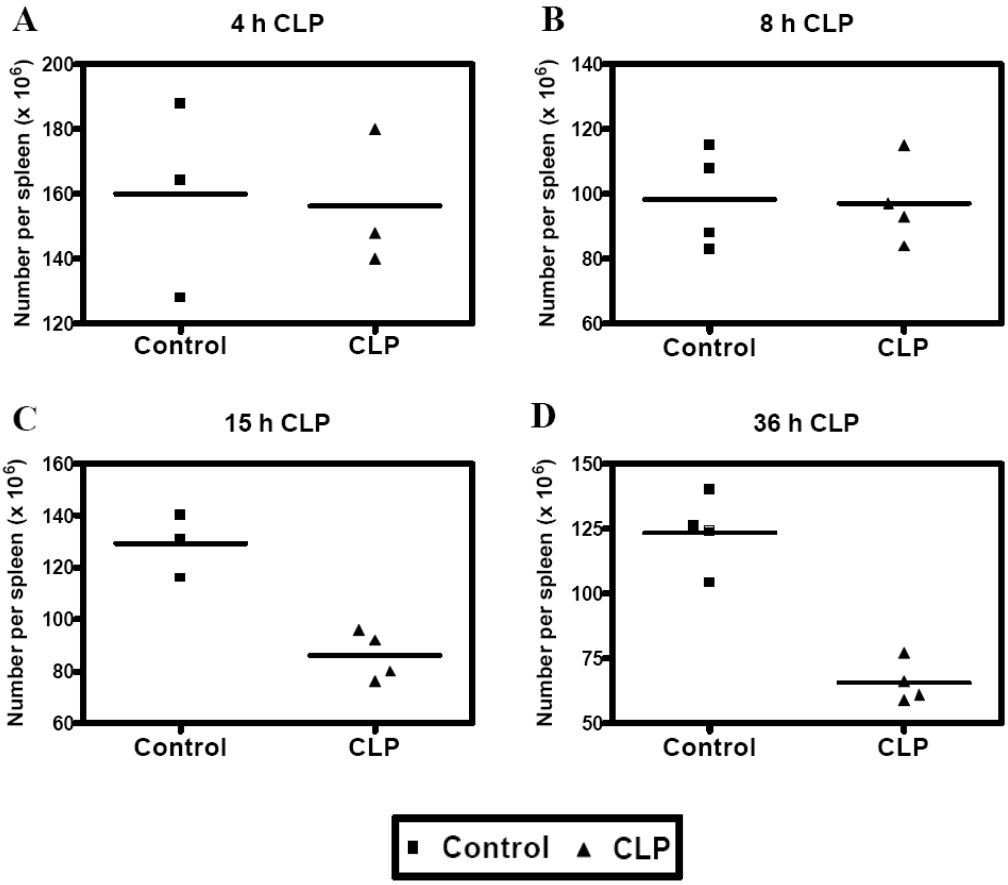


Figure 12: TSCs number during sepsis. Four, 8, 15 and 36 h after CLP or sham operation, TSCs from each animal were prepared and counted using trypan blue to exclude any dead cell. Data show dot plots where each dot show absolute number of total cells/spleen/animal (x 10^6) and are representative of two to six independent experiments per time point after CLP.

Spleens were removed at 4, 15 and 36 h after operation and the total spleen cell suspensions were prepared and counted using trypan blue to exclude any dead cell. There was no change in cell number 4 h (Fig. 12A) and 8 h (Fig. 12B) after CLP while there was marked loss in total cell number 15 h (Fig. 12C) after CLP which gets further reduced 36 h (Fig.

12D) after CLP. Thus, in our sepsis model, the loss of total cell number in the spleen occurs between 8 and 15 h after CLP.

3.1.2.2. Change in Dendritic Cells Number

After looking at the loss in total spleen cell number during sepsis, next question concerning loss in splenic DCs number was addressed. Therefore, total spleen cells were prepared and stained for CD11c. DCs were identified according to their high expression of CD11c (Fig. 13A and 13B). In control animals, 0.94 ± 0.15% of total spleen cells were DCs (Fig. 13C).

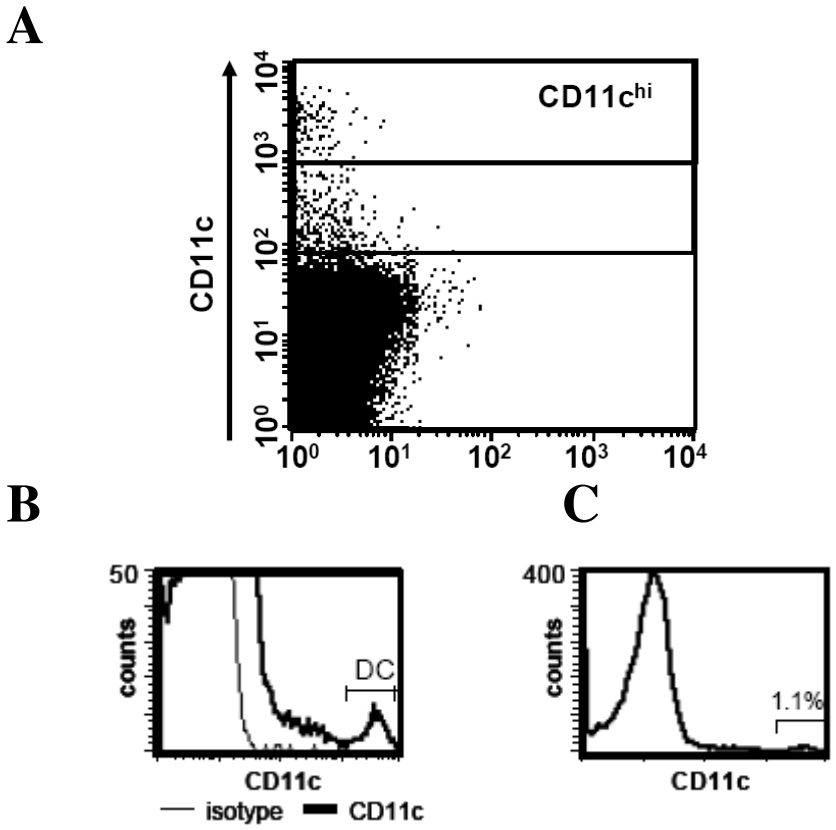


Figure 13: Total DCs number in the spleen. The total spleen cells were stained for CD11c. (A) $CD11c^{hi}$ cells (DCs) were used for gating and analysis. (B) CD11c and isotype control staining of total spleen cells. (C) Same histogram as in (B) but with enlarged scale of the y-axis in order to show the peak of unlabeled cells. Number indicates percentage of $CD11c^{hi}$ cells.

Further, the total number of DCs per spleen in CLP or sham operated mice was calculated. Spleens were removed at 8, 15 and 36 h after CLP or sham operation and stained for CD11c. The absolute number of DCs per spleen from septic and sham mice did not differ until 15 h after CLP. However, 36 h after CLP, the number of DCs in spleens from septic

mice decreased drastically down to 20% of DCs usually found in spleens from control mice (Fig. 14). Thus, polymicrobial sepsis induces profound loss of DCs in the spleen.

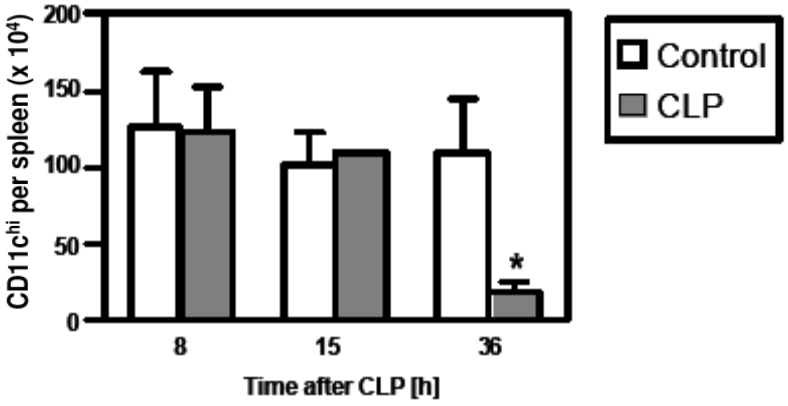


Figure 14: Total DCs number in the spleen during sepsis. Eight, 15, and 36 h after CLP, total spleen cells per group (n=3 to 4) were pooled and stained for CD11c. Sham operation served as control. The total numbers of DCs per spleen of control (open bars) and septic mice (filled bars) were calculated. Data show mean ± SD of 3 to 4 experiments per time point. Asterisk indicates significant difference between CLP and control groups. *, $p<0.05$

3.1.3. Specific Loss of DCs Subpopulations in the Spleen during Sepsis

On the basis of their expression of CD4 and the CD8α chain homodimer (CD8αα), splenic DCs can be further segregated into three subtypes ($CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$). As shown in section 3.1.2.2., 80% of splenic DCs were lost during sepsis. Thus, we addressed the question of whether all DC subpopulations were affected or whether there was a prevalence of a specific DCs subpopulation. Therefore, total spleen cells were prepared 8 and 36 h after CLP or sham operation and the distribution of DC on the three main subpopulations in the spleen was determined. DCs from control mice mainly belonged to the $CD4^+CD8^-$ subpopulation (72%), remaining cells were found in the $CD4^-CD8^+$ (11%) and the $CD4^-CD8^-$ (15%) subsets (Fig. 15A). However, the distribution of DCs from septic mice 36 h after CLP completely differed from this pattern as shown by clearly decreased $CD4^+CD8^-$ (49%) and $CD4^-CD8^+$ (2%) subpopulations but an increased $CD4^-CD8^-$ subpopulation (47%, Fig. 15A). Comparison of the absolute numbers of the three DC subtypes per spleen demonstrated that by 8 h after CLP the size of the three subpopulations did not differ between DCs from control and DCs from septic mice. However, by 36 h after

CLP a profound loss of $CD4^+CD8^-$ and of $CD4^-CD8^+$ DCs was observed. The number of $CD4^-CD8^-$ DCs did not change significantly (Fig. 15B). Thus, the loss of splenic DCs during sepsis can be attributed to a selective depletion of two specific DC subsets.

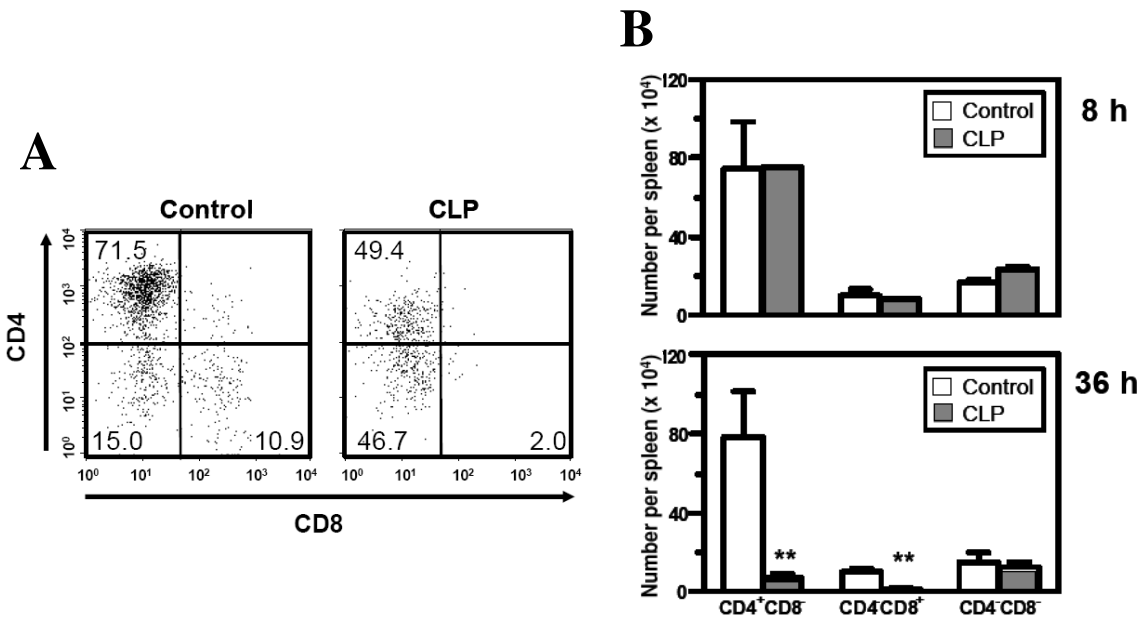


Figure 15: DC subpopulations during sepsis. Eight and 36 h after CLP or sham operation, pooled spleen cells (n=3 to 4 per group) were stained for CD11c, CD4, and CD8. (A) Expression of CD4 and CD8 on $CD11c^{hi}$ DC 36 h after operation. Numbers indicate percentage of cells in the respective quadrant. Data are representative for 4 experiments. (B) Absolute numbers of DCs per spleen belonging to the three subpopulations 8 and 36 h after operation. Data show mean ± SD of 2 (for 8 h) and 4 (for 36 h) experiments. Asterisks indicate differences between CLP (filled bars) and control groups (white bars). **, $p<0.01$.

3.1.4. Maturation of DCs in the Spleen and in the Peripheral Lymph Nodes during Sepsis

3.1.4.1. Rapid Maturation of DCs in the Spleen

To test whether sepsis is associated with a change in the expression of surface markers involved in T cell activation on splenic DCs ($CD11c^{hi}$), total spleen cell suspensions were prepared at different time-points following CLP or sham operation. Spleens were removed at 4, 8, 15 and 36 h after operation and were stained for CD11c in combination with CD40, CD80, CD86, MHC class I and MHC class II.

At 4 h after CLP, DCs from septic mice expressed similar levels of CD40 and slightly enhanced levels of CD86 in comparison with DCs from control mice (Fig. 16). However, at

36 h after CLP, the expression of CD40 and CD86 was strongly enhanced, clearly indicating sepsis-induced maturation (Fig. 16).

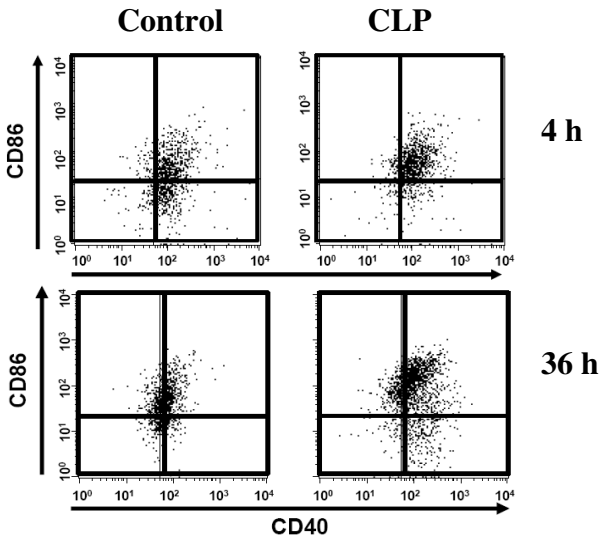


Figure 16: Maturation of splenic DCs during sepsis. Four and 36 h after CLP or sham operation, total spleen cells per group (n=3-4) were pooled and stained for CD11c in combination with CD40 and CD86. Data show CD40 and CD86 expression of DCs 4 and 36 h after CLP or sham operation and are representative of four independent experiments. Quadrant lines were set where isotype controls showed less than 2% false positive cells.

The kinetics showed that CD86 was upregulated further at 8 h after CLP and reached maximal expression at 15 h after CLP (Fig. 17B).

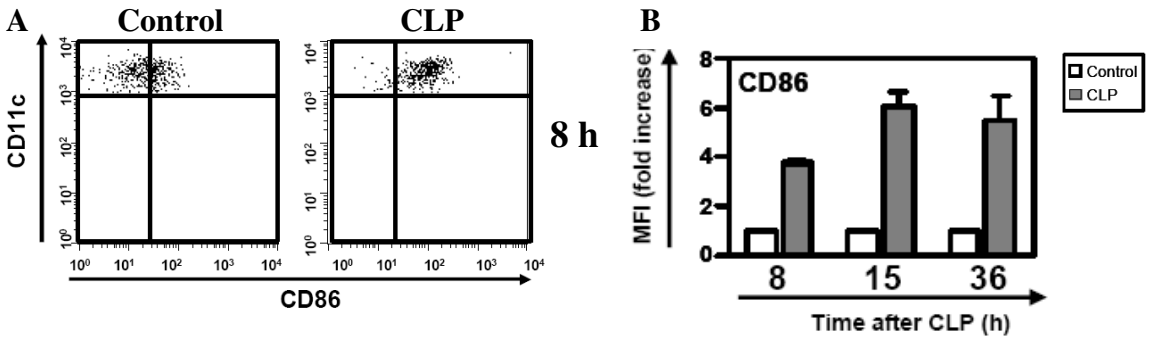


Figure 17: Maturation of splenic DCs during sepsis. Eight, 15 and 36 h after CLP or sham operation, total spleen cells per group (n=3-4) were pooled and stained for CD11c in combination with CD86. (A) Data show CD86 expression of DCs 8 h after CLP or sham operation. These data represent dot plots for any other time points after CLP. Quadrant lines were set where isotype controls showed less than 2% false positive cells. (B) MFI values of the indicated surface markers on DCs (gated on $CD11c^{hi}$, as shown in fig. 13A) were determined. The ratio of MFI values of DCs from septic (filled bars) versus control (open bars) mice was calculated for variations between different experiments. Data show mean ± SD of two (for 8 and 15 h) or four (for 36 h) independent experiments.

Further, increased expression of CD40 was visible at 15 h after CLP, which reached its maximum at 36 h after CLP (Fig. 18B).

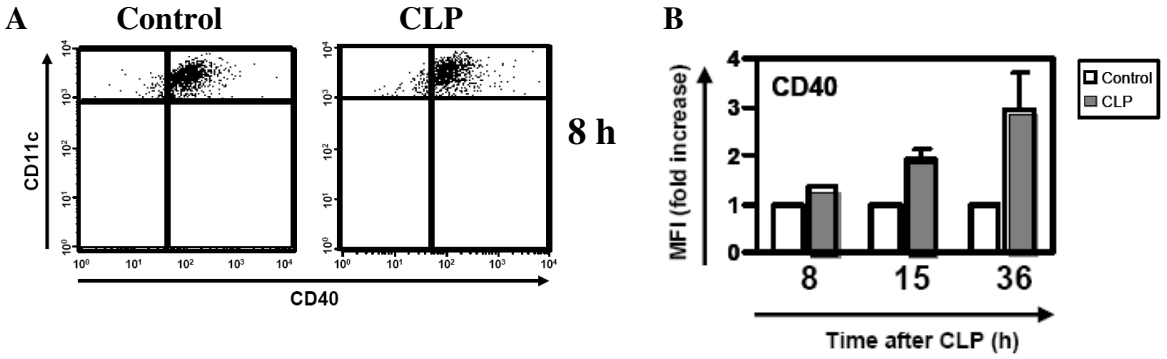


Figure 18: Maturation of splenic DCs during sepsis. Eight, 15 and 36 h after CLP or sham operation, total spleen cells per group (n=3-4) were pooled and stained for CD11c in combination with CD40. (A) Data show CD40 expression of DCs 8 h after CLP or sham operation. These data represent dot plots for any other time points after CLP. Quadrant lines were set where isotype controls showed less than 2% false positive cells. (B) MFI values of the indicated surface markers on DCs (gated on CD11c^{hi}, as shown in fig. 13A) were determined. The ratio of MFI values of DCs from septic (filled bars) versus control (open bars) mice was calculated for variations between different experiments. Data show mean ± SD of two (for 8 and 15 h) or four (for 36 h) independent experiments.

MHC class I expression followed a remarkable kinetics, as it remained unaffected during the first 15 h after CLP but enhanced strongly at 36 h after CLP (Fig. 19B).

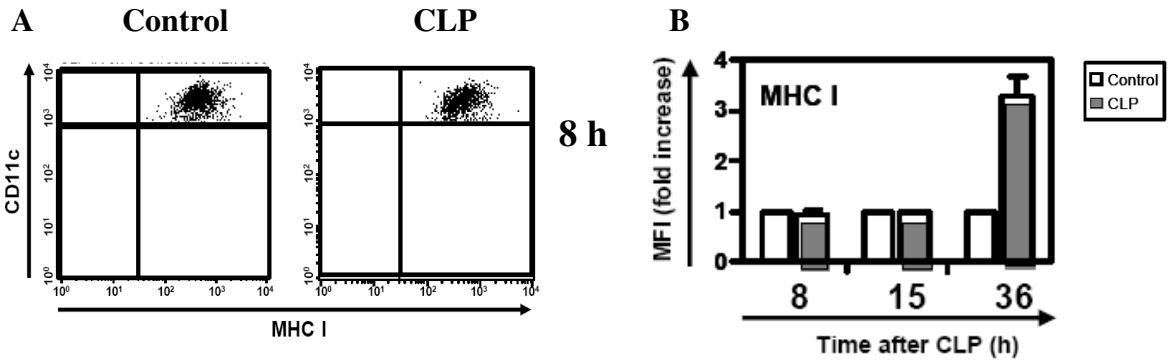


Figure 19: Maturation of splenic DCs during sepsis. Eight, 15 and 36 h after CLP or sham operation, total spleen cells per group (n=3-4) were pooled and stained for CD11c in combination with MHC class I. (A) Data show MHC class I expression of DCs 8 h after CLP or sham operation. These data represent dot plots for any other time points after CLP. Quadrant lines were set where isotype controls showed less than 2% false positive cells. (B) MFI values of the indicated surface markers on DCs (gated on CD11c^{hi}, as shown in fig. 13A) were determined. The ratio of MFI values of DCs from septic (filled bars) versus control (open bars) mice was calculated for variations between different experiments. Data show mean ± SD of two (for 8 and 15 h) or four (for 36 h) independent experiments.

In contrast, sepsis induced moderate or no changes in the expression of CD80 and MHC class II molecules on splenic DCs (Fig. 20B). Thus, polymicrobial sepsis strongly induces maturation of DCs in the spleen.

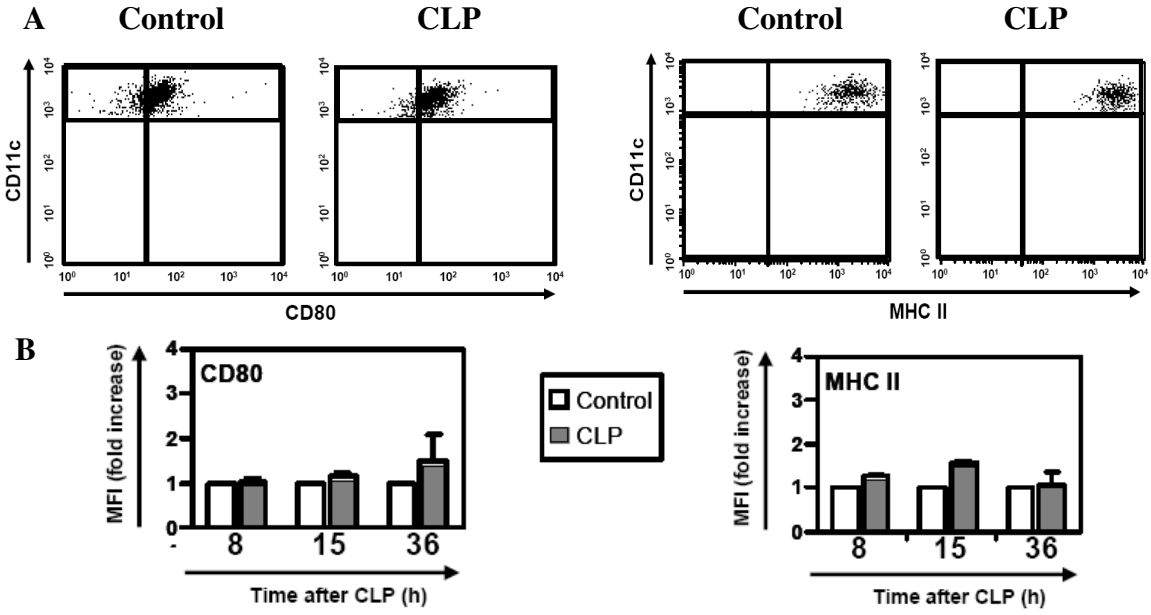


Figure 20: Maturation of splenic DCs during sepsis. Eight, 15 and 36 h after CLP or sham operation, total spleen cells per group (n=3-4) were pooled and stained for CD11c in combination with CD80 and MHC class II. (A) Data show CD80 and MHC class II expression of DCs 8 h after CLP or sham operation. These data represent dot plots for any other time points after CLP. Quadrant lines were set where isotype controls showed less than 2% false positive cells. (B) MFI values of the indicated surface markers on DCs (gated on $CD11c^{hi}$, as shown in fig. 13A) were determined. The ratio of MFI values of DCs from septic (filled bars) versus control (open bars) mice was calculated for variations between different experiments. Data show mean ± SD of two (for 8 and 15 h) or four (for 36 h) independent experiments.

3.1.4.2. Maturation of DCs in the Peripheral Lymph Nodes

Further, to test whether the sepsis-associated maturation was restricted to DCs in the spleen, the expression of surface markers on $CD11c^{+}$ DCs from inguinal and popliteal peripheral lymph nodes, as lymphoid organs distant to the site of infection, was determined at various time points. At 8 h after CLP, CD86, CD40 and MHC class II were expressed at the same levels as found on DCs from controls (Fig. 21). While 15 h after CLP, CD40 and CD86 were increased slightly on DCs from septic mice (Fig. 21). Moreover, 36 h after CLP, lymph node DCs from septic mice upregulated all surface molecules including CD80 and MHC class II, which were only marginally changed on splenic DCs (Fig. 21). MHC class I expression was also enhanced 36 h after CLP as compared to controls (Fig. 21).

Thus, sepsis-induced maturation of DCs in the lymph nodes resembles the maturation of splenic DCs but is characterized by slightly delayed kinetics and followed a different pattern of surface markers upregulation.

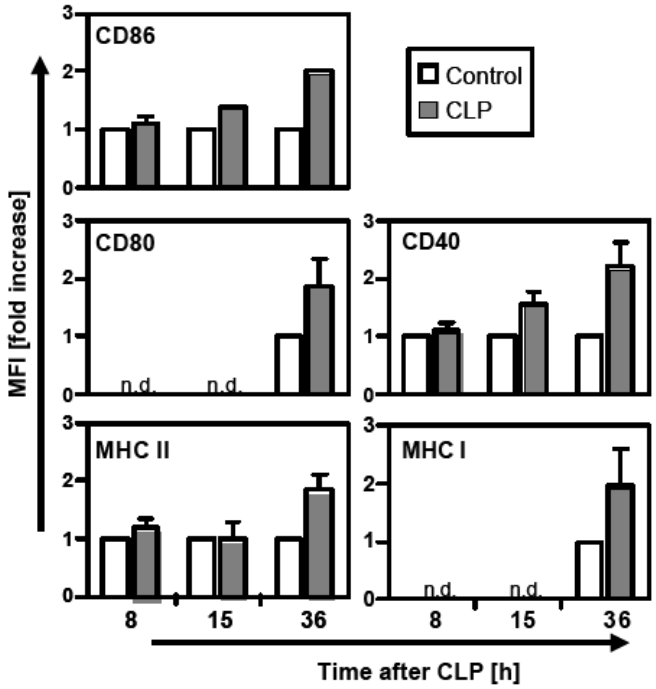


Figure 21: Maturation of peripheral lymph node DC during sepsis. Eight, 15, and 36 h after CLP or sham operation, pooled popliteal and inguinal lymph node cells (n=3 to 4 per group) were stained. $CD11c^+$ DC were gated and MFI values for the indicated surface markers were determined. The ratio of MFI values from DC from septic (hatched bars) versus control (white bars) mice were calculated. Data show mean ± SD of 2 to 4 independent experiments per time point. n.d., not determined

3.1.5. Altered Cytokine Secretion Pattern of DCs during Sepsis

3.1.5.1. Impaired Pro-inflammatory Cytokine Response, but Release of IL-10 by DCs during Sepsis

It is known that containment of an infection induces DCs maturation and release of various pro-inflammatory cytokines like TNF-α, IFN-γ and IL-12 and anti- inflammatory cytokines like IL-10. IL-12 is a key mediator for Th1 cell polarization while its counterpart IL-10 is a major anti-inflammatory cytokine and induces an immunoregulatory response. The effective clearance of bacterial infection requires the development of Th1 cell response. Therefore, the question related to their capacity to produce TNF-α, IFN-γ, IL-12 and IL-10

was addressed. At 8, 15 and 36 h after CLP and sham operation, DCs were purified from the total spleen cells and were cultured with or without CD40 Ligand+LPS (CD40L+L) or immunostimulatory CpG oligonucleotides. After 18 h of culture, supernatants were analyzed for the presence of TNF-α and IFN-γ. The results showed that DCs from control mice secreted substantial amounts of TNF-α and IFN-γ upon stimulation with CpG or CD40L+LPS. However, the secretions of these pro-inflammatory cytokines by DCs from septic mice were reduced by more than 50 % as soon as 8 h after CLP and were further reduced up to 36 h after CLP (Fig. 22).

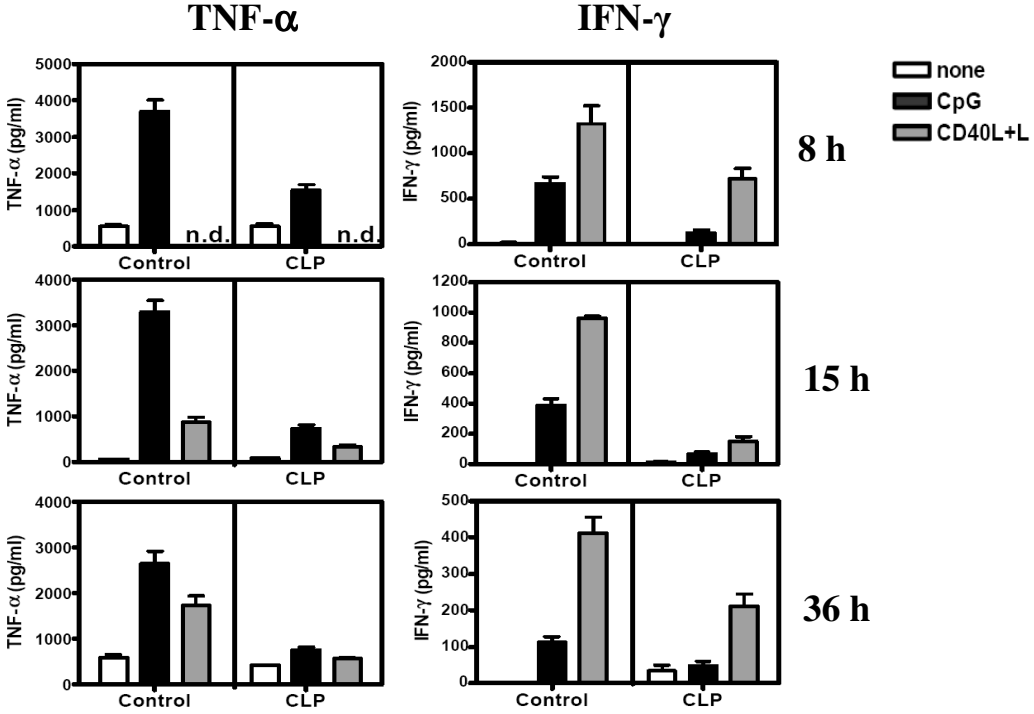


Figure 22: Synthesis of TNF-α and IFN-γ in response to bacterial components. Eight, 15, and 36 h after CLP or sham operation, spleen cells were pooled (n=3 to 4 per group) and DCs were purified and cultured in the presence or absence of 100 ng/ml LPS + 2.5 µg/ml CD40L (CD40L+L) or with 5 µg/ml CpG for 18 h. Supernatants of DCs from septic mice and from control mice were analyzed for TNF-α and IFN-γ. Data show mean ± SD and are representative for 2 experiments per time point. n.d., not determined.

Next, the capacity of DCs to produce IL-12 was analyzed. IL-12 is a heterodimeric pro-inflammatory cytokine comprising of covalently linked p40 and p35 subunit. At 36 h after CLP or sham operation, the total spleen cells were cultured with or without immunostimulatory CpG oligonucleotides. Thereafter, intracellular staining for IL-12p40 was done with total spleen cells in combination with anti-CD11c. Intracellular expression

of IL-12p40 on CD11c^{hi} gated DCs were determined using FACS analysis. The results showed that only very few DCs from control and septic mice produced IL-12p40 in the absence of CpG stimulation while about 33% DCs from control mice produced IL-12p40 as compared to septic DCs in the presence of CpG stimulation (Fig. 23). Only 6% of DCs from septic mice were able to produce IL-12p40 in the presence of CpG stimulation (Fig. 23).

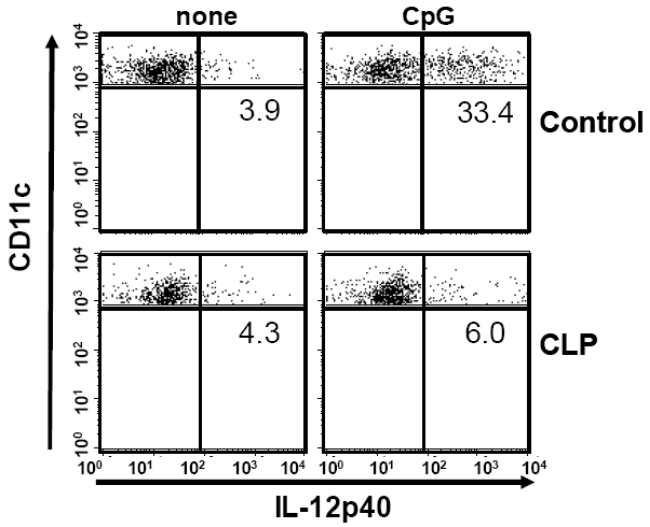


Figure 23: Synthesis of IL-12p40 in response to bacterial components. Thirty-six hours after CLP or sham operation, spleen cells were pooled (n=3 to 4 per group). Thereafter, total spleen cells were cultured with or without 5 µg/ml CpG for 18 h and monensin was added for further 6 h. After surface staining of CD11c, cells were fixed, permeabilized, and stained for intracellular IL-12p40. CD11c^{hi} cells were gated. Quadrant lines were set where isotype control antibodies stained less than 1% of the cells. Numbers indicate percentage of IL-12p40 positive cells. Data are representative of three independent experiments.

In addition, DCs purified from total spleen cells were analyzed for IL-12p70 (bio-active form of IL-12) and IL-10 in the culture supernatants. Therefore, to check their presence, DCs from the total spleen cells were purified at 4, 8 and 36 h after CLP or sham operation and were stimulated with CD40L+LPS or with CpG. After 18 h, supernatants were analyzed for IL-12 and IL-10. At any time-point, supernatants from unstimulated DCs from sham as well as from septic mice showed only low levels of IL-12. DCs from control mice secreted IL-12 in response to CD40L+LPS or CpG but produced only low amounts of IL-10 (Fig. 24). Four hours after CLP, DCs from septic mice showed the same capacity to release IL-12 and IL-10 upon stimulation with CpG as DCs from control mice. However, at 8 and 36 h after CLP, DCs from septic mice secreted only minute amounts of IL-12 upon stimulation with CD40L+LPS or CpG, and the capacity of control DCs to secrete IL-12

remained stable. In contrast, DCs from septic mice released increasing amount of IL-10 even in the absence of any additional stimuli up to 36 h after CLP (Fig. 24). Thus, during polymicrobial sepsis DCs develop an inverse cytokine secretion pattern in response to bacterial stimuli associated with a predominance of IL-10.

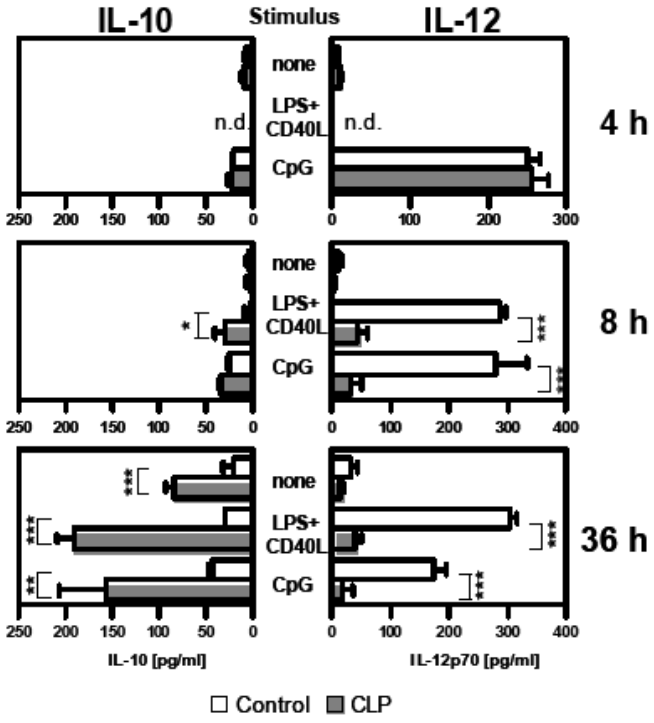


Figure 24: Synthesis of IL-12 and IL-10 in response to bacterial components. Four, 8, and 36 h after CLP or sham operation, spleen cells were pooled (n=3 to 4 per group). Four, 8, and 36 h after operation, DCs were purified from total spleen cells and cultured in the presence or absence of 100 ng/ml LPS + 2.5 µg/ml CD40L or with 5 µg/ml CpG for 18 h. Supernatants of DCs from septic mice (filled bars) and from control mice (white bars) were analyzed for IL-12p70 and IL-10. Data show mean ± SEM of triplicate cultures and are representative for 2 experiments per time point. n.d., not determined. Asterisks indicate significant differences between cultures of sham and CLP groups. *, $p<0.05$; **, $p<0.01$; ***, $p<0.0005$.

3.1.5.2. Endogenous IL-10 is not responsible for Suppressed IL-12 Synthesis by Splenic DCs from Septic Mice

It has been reported that endogenous IL-10 in DCs acts in an autocrine manner and regulates IL-12 synthesis by DCs (Corinti et al., 2001). Thus, the next question regarding putative role of endogenous IL-10 in impaired IL-12 synthesis from DCs during sepsis was asked. The IL-10 neutralizing experiment with the help of anti-IL-10 (αIL-10) antibodies or respective isotype controls was performed. First, the standardization for the amounts of anti-IL-10 antibodies and respective isotype control was made. Second, the effect of IL-10 neutralization on IL-12 release by DCs during sepsis was investigated. Therefore, splenic DCs were purified from healthy control and cultured with or without CpG, in the absence or presence of 10 µg/ml neutralizing αIL-10 antibodies in combination with 1 ng/ml or 5

ng/ml recombinant mouse IL-10 (rmIL-10) or 10 µg/ml of the rat IgG1 isotype control in combination with 1 ng/ml or 5 ng/ml rmIL-10 (Fig. 25). After 18 h, supernatants were analyzed for IL-12 production. The results showed that 10 µg/ml of αIL-10 antibodies were able to neutralize 1000 pg/ml of IL-10 efficiently (Fig. 25).

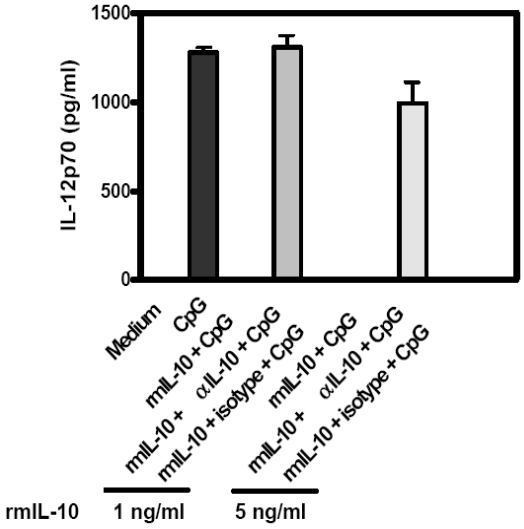


Figure 25: Amount of anti-IL-10 (αIL-10) antibodies needed for sufficient neutralization of 1000 pg/ml of IL-10. Splenic DCs were purified from healthy controls and cultured with or without 5 µg/ml of CpG, in the absence or presence of 10 µg/ml neutralizing αIL-10 antibodies or 10 µg/ml of the rat IgG1 isotype control in combination with 1 ng/ml or 5 ng/ml recombinant mouse IL-10 (rmIL-10) for 18 h. Supernatants of DCs were tested for IL-12. Data show mean ± SD of quadruplicate cultures.

Next, for the second part, splenic DCs were purified 24 h after CLP or sham operation and stimulated with or without CpG in the presence or absence of neutralizing αIL-10 antibodies or the respective isotype control. After 18 h, supernatants were tested for IL-12. In response to CpG, DCs from sham mice released high levels of IL-12 that were further increased in the presence of αIL-10 antibodies but not in the presence of the isotype control antibodies (Fig. 26). However, CpG-stimulated DCs from septic mice did not release IL-12 in the absence or in the presence of anti-IL-10 or isotype control antibodies (Fig. 26).

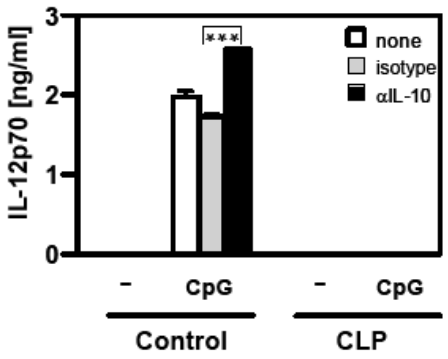


Figure 26: Effect of endogenous IL-10 on IL-12 synthesis. Splenic DCs were purified 24 h after CLP or sham operation and cultured with or without 5 µg/ml CpG each in the absence or presence of 10 µg/ml neutralizing anti-IL-10 (αIL-10) antibodies or 10 µg/ml of the rat IgG1 isotype control for 18 h. Supernatants of DCs from sham mice and septic mice were tested for IL-12. Data show mean ± SEM of triplicate cultures and are representative for 2 experiments. Asterisks indicate significant difference between anti-IL-10 and isotype control-treated cultures. ***, $p<0.0001$

3.1.5.3. Exogenous Treatment with IFN-γ Increases IL-12 Production by Splenic DCs from Septic Mice during Early Sepsis

DCs-derived IL-10 was not responsible for suppressed IL-12 production during sepsis, as neutralization of IL-10 did not restore IL-12 production by splenic DCs from septic mice (section 3.1.5.2.). Thus, the question regarding the role of another cytokine like IFN-γ in IL-12 production by DCs was asked. IFN-γ is known to promote IL-12 release by DCs (Snijders et al., 1998). Therefore, DCs from the total spleen cells were purified at 3, 6 and 24 h after CLP or sham operation and were stimulated with or without CpG in the absence or presence of recombinant mouse IFN-γ (rmIFN-γ) for 18 h. Supernatants were analyzed for IL-12. At any time point, supernatants from unstimulated DCs from sham as well as from septic mice contained only low levels of IL-12. Further, at any time point, in response to CpG, DCs from sham mice released high levels of IL-12, which were increased further in the presence of IFN-γ (Fig. 27). However, CpG-stimulated DCs from septic mice released lower levels of IL-12 as compared to control mice at 3 and 6 h after CLP and this release of IL-12 were further decreased at 24 h after CLP (Fig. 27). Upon IFN-γ treatment, DCs from septic mice released slightly increased levels of IL-12 at 3 h after CLP but this increased release of IL-12 on IFN-γ treatment was absent at 6 and 24 h after CLP (Fig. 27). Taken together, exogenous IFN-γ treatment partially restores IL-12 production by DCs upon CpG stimulation during early hours of sepsis, but it doesn't function in restoring IL-12 synthesis at later times.

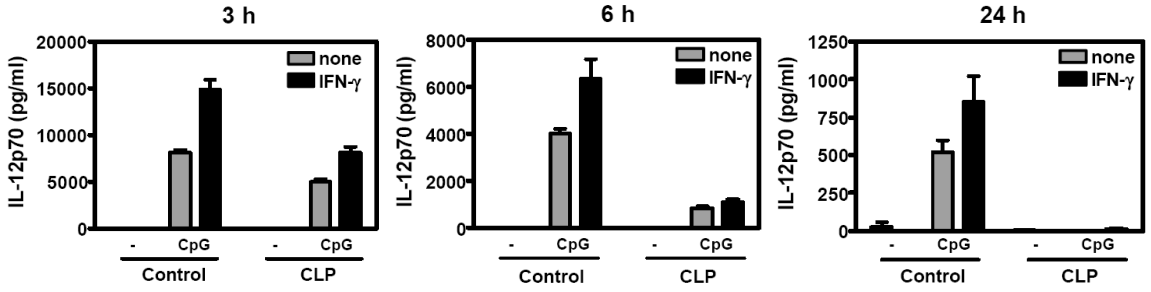


Figure 27: Exogenous treatment with rmIFN-γ increases IL-12 production by DCs from septic mice. Three, 6 and 24 h after CLP or sham operation, splenic DCs were purified and cultured with or without 5 µg/ml CpG and in the absence or presence of 10 ng/ml rmIFN-γ for 18 h. Supernatants of DCs from sham mice and septic mice were tested for IL-12. Data show mean ± SD of triplicate cultures.

3.1.6. Release of the Chemokine MCP-1 by DCs during Late Sepsis

Besides producing various pro- and anti-inflammatory cytokines, DCs also represent an important source of chemokines *in vitro* and *in vivo.* Production of chemokines is strongly increased during maturation of DCs. Mature DCs produce conspicuous amounts of various chemokines like CCL2 (MCP-1), CCL3 (MIP-1α) etc. Therefore, the capacity of DCs to produce MCP-1 during sepsis was addressed. At 8, 15 and 36 h after CLP and sham operation, DCs were purified from the total spleen cells and were cultured with or without CD40 Ligand + LPS (CD40L+L) or immunostimulatory CpG oligonucleotides. After 18 h of culture, supernatants were analyzed for MCP-1. The results showed that DCs from control mice could not be stimulated for MCP-1 release at any time point (Fig. 28). In contrast, 40 h after sepsis, DCs from septic mice showed spontaneous release of MCP-1. This suggested the *in vivo* release of MCP-1 by DCs that was reduced upon further stimulations (Fig. 28).

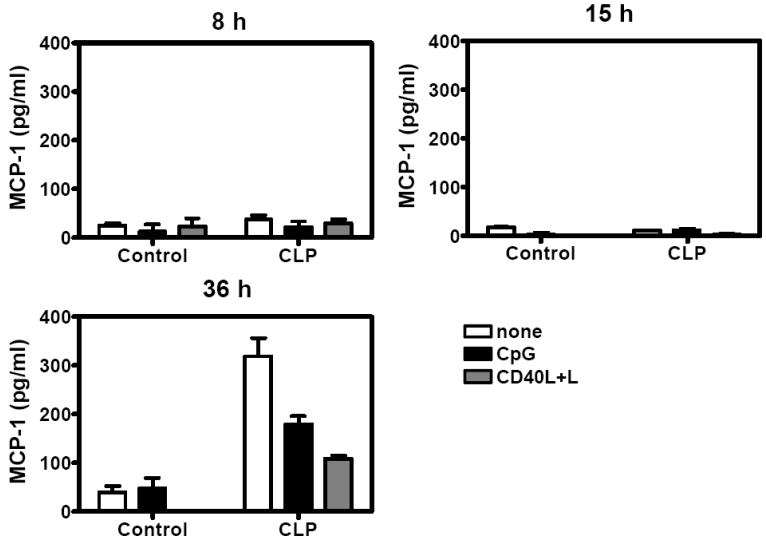


Figure 28: Synthesis of MCP-1 in response to bacterial components. Eight, 15 and 36 h after CLP or sham operation, spleen cells were pooled (n=3 to 4 per group) and DCs were purified and cultured in the presence or absence of 100 ng/ml LPS + 2.5 µg/ml CD40L (CD40L+L) or with 5 µg/ml CpG for 18 h. Supernatants from septic mice and from control mice were analyzed for MCP-1. Data show mean ± SD.

3.1.7. Reduced T Cell-Stimulatory Capacity of DCs during Sepsis

DCs are known potent antigen presenting cells which capture foreign antigen, process and present it to the T cells and in turn activate them. Therefore, the capacity of splenic DCs to

induce T cell activation was addressed. Splenic DCs were purified 36 h after CLP or sham operation and cultured with allogeneic T cells (T cells from C57BL/6J mice). DCs from septic mice were less potent in inducing T cell proliferation than DCs from control mice (Fig. 29). IL-2 is crucial for T cell proliferation and it acts in an autocrine manner. Therefore, the capacity of T cells to produce IL-2 in DC/T cell cocultures was addressed. At 36 h after CLP, $CD4^+$ T cells from mixed lymphocyte cocultures were analyzed for their ability to produce IL-2 upon restimulation with PMA/Ionomycin. The percentage of IL-2-secreting T cells cultured with DCs from septic mice was decreased by more than two-fold in comparison to T cells cultured with DCs from control mice (Fig. 30). Taken together, although expressing high levels of costimulatory molecules, DCs from septic mice were less potent in T cell activation than DCs from control mice.

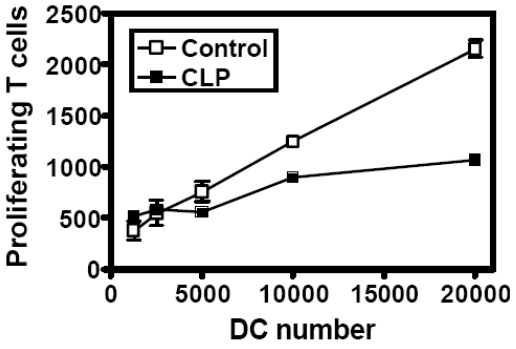


Figure 29: Proliferation test in allogeneic T cell assay. Thirty-Six hours after CLP or sham operation, DCs were purified from pooled spleen cells (n=3 to 4 per group). Titrated numbers of DCs were seeded in 96-well plates and cultured for 18 h. CFSE-labeled allogeneic T cells were added and proliferation was determined after 3 d as described in Material and Methods. Data show mean ± SEM of triplicate cultures with DCs from controls (white squares) and from septic mice (black squares).

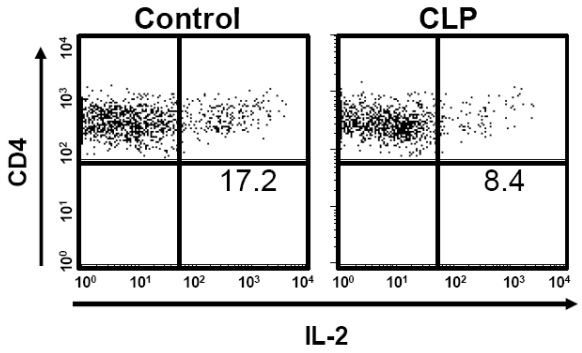


Figure 30: IL-2 synthesis in allogeneic T cell assay. Thirty-six h after CLP or sham operation, DCs were purified from pooled spleen cells (n=3 to 4 per group). Titrated numbers of DCs were seeded in 96-well plates and cultured for 18 h. The unlabeled allogeneic T cells were added to 10^4 DCs. After 4 d, cells were restimulated with PMA/Ionomycin before staining for CD4 and intracellular IL-2. $CD4^+$ cells were gated. Numbers indicate percentage of cells positive for IL-2. Isotype control antibodies stained less than 1%. Data are representative for 2 experiments.

3.2. Restoration of Dendritic Cell Functions during Sepsis

In the present study, the changes in the behavior of DCs was investigated during sepsis and found that there was a significant increase in DC IL-10 release and a significant decrease in DC IL-12 release when compared with controls. This would change the DCs Th polarization properties, yielding a phenotype more likely to promote the development of a Th2 or Treg lymphocyte response. There was also a marked loss in DC number and the remaining DCs showed a reduced ability to drive clonal expansion of T cells in a mixed lymphocyte reaction. From the data collected, it was clear that DCs were affected by environmental factors seen in sepsis. By keeping this information in mind, studies on the development of potential strategy for the restoration of DC functions was aimed.

3.2.1. Strategy Followed for Restoration

DCs play a pivotal role in the establishment of a specific immune response against pathogens and an alteration in DCs function may have accounted for the immunosuppression during sepsis. Treatment strategy which could restore the capacity of DCs for Th1 cell polarization or the normal DCs count seem to represent promising approach to counteract immunoparalysis during sepsis. To address this question, the transfer of *ex vivo* generated Bone Marrow-derived Dendritic Cells (BMDCs) from naïve animals into CLP treated animals was attempted.

3.2.2. Experimental Plan

3.2.2.1. Isolation of Mouse BMDCs

The BMDCs were generated by performing the following steps (details are in materials and methods section) and were further used for transfer into CLP-treated mice.

Figure 31: Schematic presentation for BMDCs generation for injection.

3.2.2.2. Transfer of BMDCs

Six hours after sham and CLP operation, mice were injected with BMDCs or PBS (Fig. 32). The CLP treated mice were divided into three groups. First group was injected with PBS (100 µl/animal) intravenously (i.v.). While second and third groups of CLP treated mice were injected with CpG treated wildtype BMDCs and with CpG treated IL-12p35$^{-/-}$ (IL-12-KO) BMDCs (2 x 10^6 cells/100 µl/animal) i.v., respectively. IL-12KO BMDCs were injected to analyze the role of IL-12, which is required for Th1-type cell response during sepsis. And sham treated mice were injected with PBS (100 µl/animal) i.v. only. The experimental plan followed for injection is shown as below

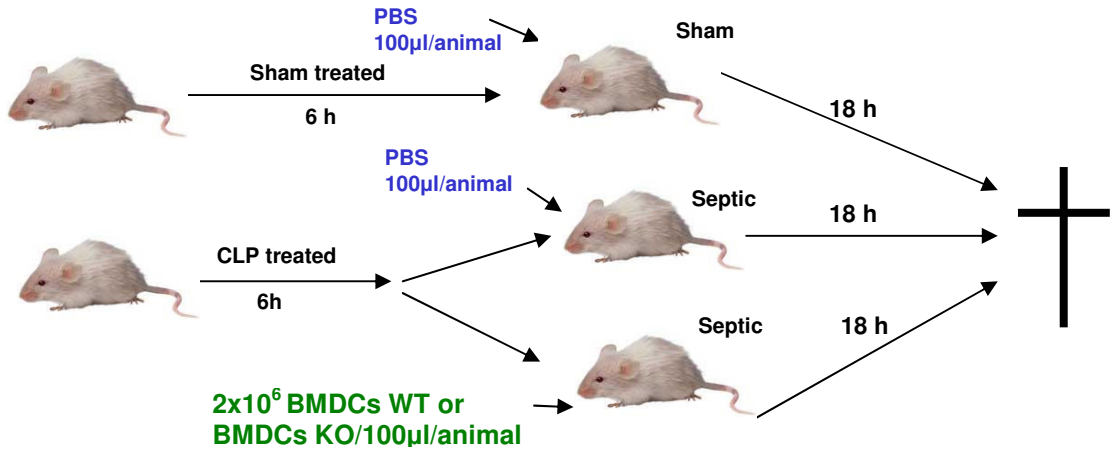


Figure 32: Schematic presentation for BMDCs transfer.

3.2.3. Characterization of BMDCs from Naïve Animal

Prior to transfer experiments, the characterization of BMDCs was done on the basis of various parameters like expression of surface markers involved in T cell activation (MHC class II molecules) and IL-12 release in response to bacterial products like CpG. The cultured bone marrow cells (BMCs) were harvested on 7th day (as described in materials and methods) and stained for CD11c in combination with MHC class II. BMDCs were identified according to their expression of CD11c and MHC class II (Fig. 33B). About 70-75% BMCs harvested on 7th day were CD11c and MHC II positive (Fig. 33B) and were termed BMDCs.

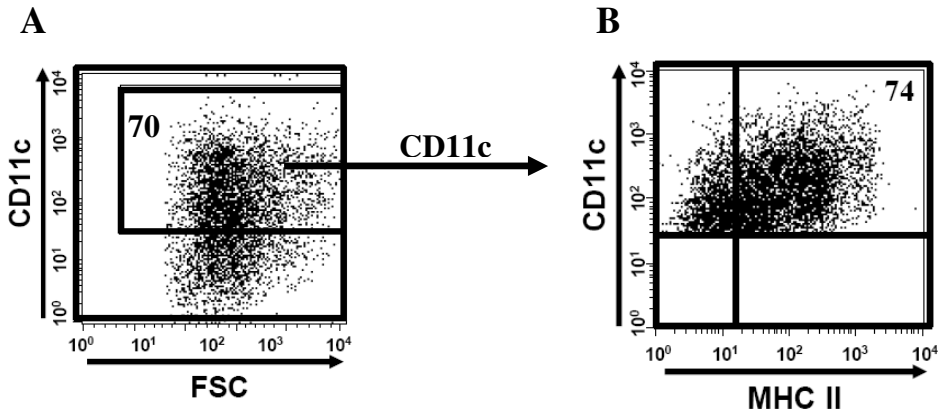


Figure 33: Total number of BMDCs in BMCs preparation on 7th day. BMCs were stained for CD11c and MHC class II (A) Total CD11c cells in BMCs preparation. Number indicates percentage of CD11c cells. (B) Total CD11c cells were gated and analyzed for MHC class II. BMDCs were positive for CD11c and MHC class II. Number indicates percentage of BMDCs.

In addition to surface molecules analysis, BMDCs were analyzed for IL-12 production. On the 7th day, harvested BMDCs were cultured with or without CpG for 18 h. Thereafter, supernatants were tested for IL-12. The results showed that BMDCs in presence of CpG produced high levels of IL-12 (Fig. 34). Together, BMDCs used for therapy expressed high levels of MHC class II molecules and released high amounts of IL-12 upon CpG stimulation.

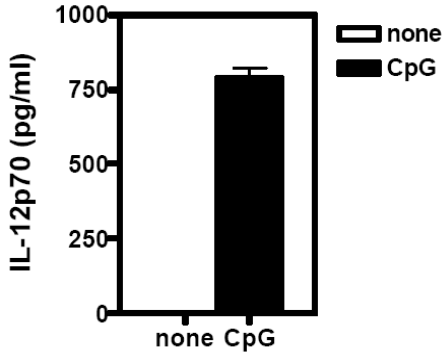


Figure 34: Synthesis of IL-12 in response to CpG. On 7th day BMDCs were cultured with or without 5 µg/ml CpG for 18 h. Supernatants were then analyzed for IL-12p70 production. Data show mean ± SD of triplicate cultures.

3.2.4. BMDCs Transfer Reduces Cell Loss during Sepsis

3.2.4.1. Reduction in Total Spleen Cells Loss

To get insight into the effects of BMDCs transfer during sepsis on the total number of spleen cells (TSCs) in our CLP model, total spleen cell suspensions were prepared at 24 h after CLP or sham operation. The cells were counted and total number of cells per spleen

was calculated. The total number of cells in spleens from septic mice injected with PBS [CLP (P)] declined down to 60 % of total cells usually found in spleens from control mice injected with PBS [Control (P)]. This 40% loss in cell number was partially restored in spleens from septic mice injected with CpG oligonucleotides stimulated wildtype BMDCs [CLP (B+C)] (Fig. 35). Thus, during polymicrobial sepsis transfer of BMDCs in CLP treated mice reduces in part the loss in total cell number in the spleen.

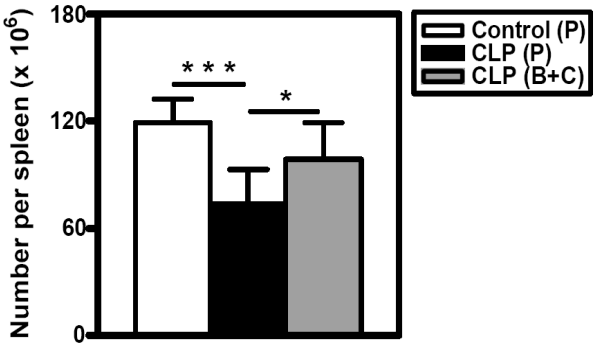


Figure 35: Total cell number in the spleen on BMDCs transfer during sepsis. Twenty-four hours after CLP or sham operation, the total numbers of cell per spleen of control mice injected with PBS (open bar), septic mice injected with PBS (black bar) and septic mice injected with CpG oligonucleotdes treated wildtype BMDCs (gray bar) were calculated. Data show mean ± SD of seven independent experiments. ***, indicates significant difference between sham and CLP groups, $p<0.0005$; and *, indicates significant difference between CLP and BMDCs treated CLP groups, $p<0.05$.

3.2.4.2. Reduction in Total T cells Loss

After looking at the reduction in loss of total spleen cells on BMDCs transfer during sepsis, next question concerning total T cell number in spleen was addressed. According to their expression of CD4 and CD8, two T cell populations can be distinguished in the spleen.

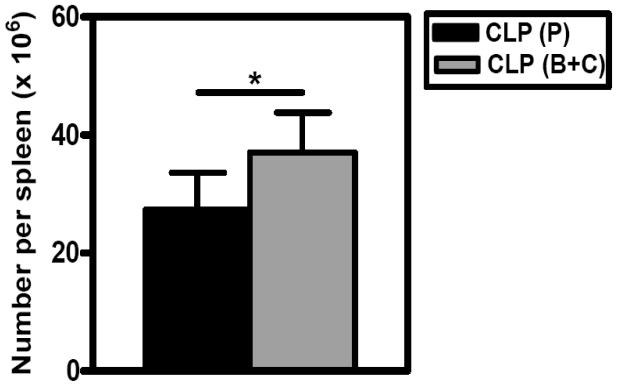


Figure 36: Total T cell number in the spleen on BMDCs transfer during sepsis. Twenty-four hours after CLP or sham operation, the total spleen cells from each animal from each group were stained for CD4 and CD8. These data show the sum of absolute numbers of CD4 and CD8 cells per spleen which represents the total T cell number per spleen of septic mice injected with PBS (black bar) and septic mice injected with CpG oligonucleotides treated wildtype BMDCs (gray bar). Data show mean ± SD of four independent experiments. *, indicates significant difference between CLP and BMDCs treated CLP groups, $p<0.05$.

Total spleen cell suspensions were prepared at 24 h after CLP or sham operation and were stained for CD4 and CD8, counted and the absolute number of CD4 and CD8 T cells per spleen was calculated. Thereafter, to determine the change in the total T cells number, absolute numbers of CD4 and CD8 T cells were summed up. The total number of T cells in spleens from septic mice injected with PBS [CLP (P)] declined down to 60 % of total T cells usually found in spleens from control mice injected with PBS [Control (P)]. This 40% loss in T cell number was partially restored in spleens from septic mice injected with CpG oligonucleotides stimulated wildtype BMDCs [CLP (B+C)] (Fig. 36). Thus, during polymicrobial sepsis transfer of BMDCs in CLP treated mice reduces in part the loss in total T cell number in the spleen.

3.2.5. Altered Cytokine Secretion Pattern of Total Spleen Cells during Sepsis

Next, the capacity of TSCs to produce pro- and anti-inflammatory cytokines was analyzed. Therefore, the release of TNF-α, IFN-γ and IL-10 was measured in TSCs culture supernatants. Cytokine synthesis by untreated TSCs may mirror the activity of TSCs *in situ*. At 24 h after CLP and sham operation, spleens were removed and TSCs were prepared. Thereafter, TSCs were cultured and after 18 h of culture, supernatants were analyzed for the release of TNF-α and IL-10. The results showed that TSCs from control mice secreted no or negligible TNF-α and IL-10. In contrast, TSCs from septic mice released elevated amounts of TNF-α and IL-10 (Fig. 37).

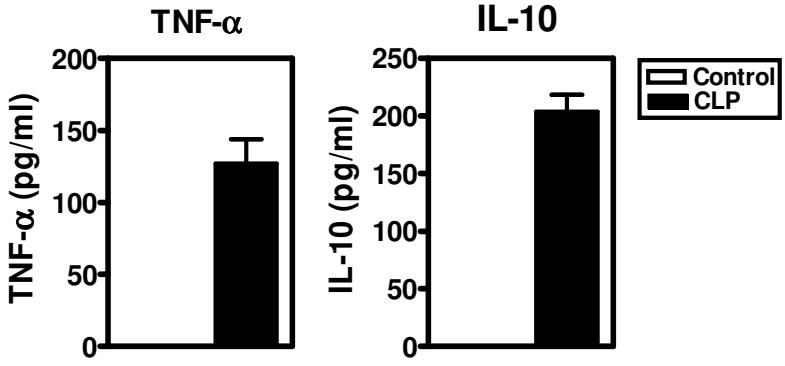


Figure 37: Synthesis of TNF-α and IL-10 during sepsis. Twenty-four hours after CLP or sham operation, spleens were removed and splenocytes were pooled (n=3 to 4 per group) and were cultured for 18 h. Supernatants were analyzed for TNF-α and IL-10. Data show mean ± SD and are representative of 4 to 8 independent experiments.

For checking the IFN- γ release during sepsis, TSCs were prepared and cultured with or without Con A for 48 h. Thereafter, supernatants were analyzed for IFN-γ. There was no or very little IFN-γ production by unstimulated TSCs from control and septic mice (Fig. 38). In contrast, the release of IFN-γ was increased upon stimulation both in control and septic mice but septic mice showed reduced capacity of producing IFN-γ compared to control mice (Fig. 38). Taken together, during polymicrobial sepsis TSCs develop altered cytokine secretion pattern with a predominance of IL-10 release.

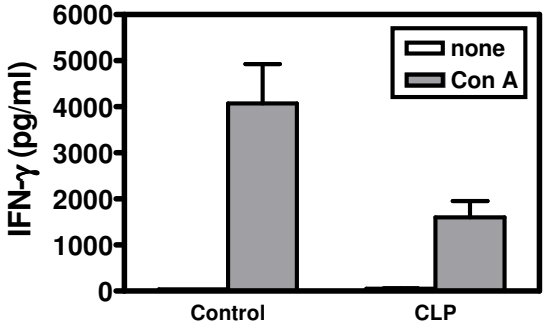


Figure 38: Synthesis of IFN-γ during sepsis. Twenty-four hours after CLP or sham operation, spleens were removed and splenocytes were pooled (n=3 to 4 per group) and were cultured with or without Con A for 48 h. Supernatants were analyzed for IFN-γ. Data show mean ± SD and are representative of seven independent experiments.

3.2.6. BMDCs Transfer Reduces IL-10, TNF-α and IFN-γ Production during Sepsis

It is known that IL-10 production correlates with a Th2 type response and might contribute to immunosuppresion during sepsis. Therefore, the question concerning the modulation of this immunoregulatory cytokine upon BMDCs transfer was addressed. At 6 h after CLP or sham operation, control mice and one group of septic mice were injected with PBS and another group of septic mice were injected with CpG oligonucleotides stimulated wildtype BMDCs. Eighteen hours after injections, spleens were removed and TSCs were prepared. Thereafter, TSCs were cultured without any stimulus for 18 h and supernatants were analyzed for IL-10. TSCs from control mice injected with PBS [Control (P)] released no IL-10. In contrast, TSCs from septic mice injected with PBS [CLP (P)] produced elevated amounts of IL-10. However, this increased IL-10 production was decreased in TSCs from septic mice injected with CpG stimulated wildtype BMDCs [CLP (B+C)] compared to CLP (P) mice (Fig. 39).

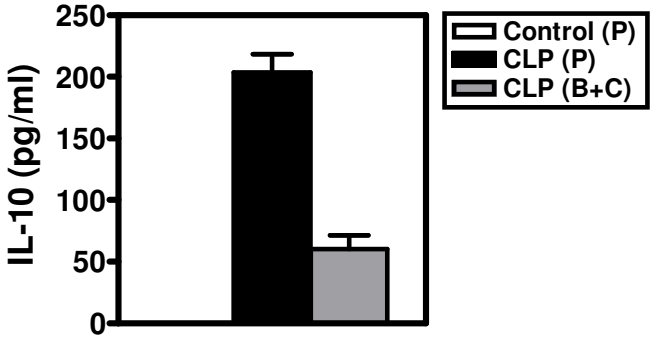


Figure 39: Reduction in IL-10 production on transfer of CpG stimulated wildtype BMDCs during sepsis. Twenty-four hours after CLP or sham operation, spleens were removed and splenocytes were pooled (n=3 to 4 per group) and were cultured for 18 h. Supernatants were analyzed for IL-10. Data show mean ± SD and are representative of six independent experiments.

Further, the effect of BMDCs transfer on the capacity of TSCs to produce TNF-α, which is one of the most potent inducers of inflammation, was analyzed. Therefore, the supernatants generated for IL-10 analysis (as mentioned above) were also analyzed for TNF-α production. TSCs from control (P) mice released no or negligible TNF-α. In contrast, unstimulated TSCs from CLP (P) treated mice produced substantial amounts of TNF-α. However, this increased TNF-α production was decreased in TSCs from CLP (B+C) treated mice compared to CLP (P) treated mice (Fig. 40).

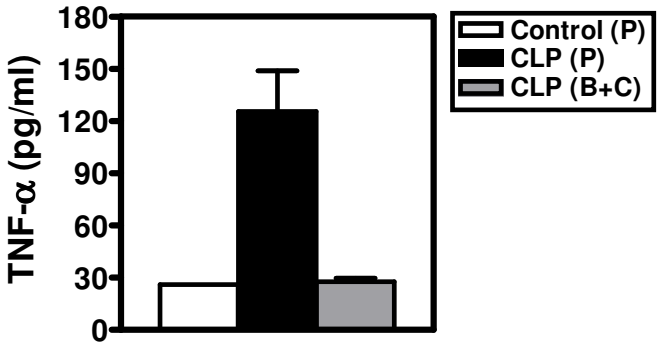


Figure 40: Reduction in TNF-α production on transfer of CpG stimulated wildtype BMDCs during sepsis. Twenty-four hours after CLP or sham operation, spleens were removed and splenocytes were pooled (n=3 to 4 per group) and were cultured for 18 h. Supernatants were analyzed for TNF-α. Data show mean ± SD and are representative of five independent experiments.

Next, the effect of BMDCs transfer on the capacity of TSCs to produce IFN-γ, which is a known Th1 cytokine, was tested. Therefore, TSCs from control (P), CLP (P), and CLP (B+C) were prepared and cultured with Con A for 48 h. Thereafter, supernatants were tested for IFN-γ. In response to Con A, TSCs from control (P) mice released high levels of IFN-γ in comparison with TSCs from CLP (P) treated mice which showed reduced capacity

of producing IFN-γ. Furthermore, this decreased IFN-γ production was decreased in TSCs from CLP (B+C) treated mice compared to CLP (P) treated mice (Fig. 41). Taken together, during sepsis transfer of BMDCs into CLP treated mice reduce IL-10, TNF-α and IFN-γ production by total spleen cells.

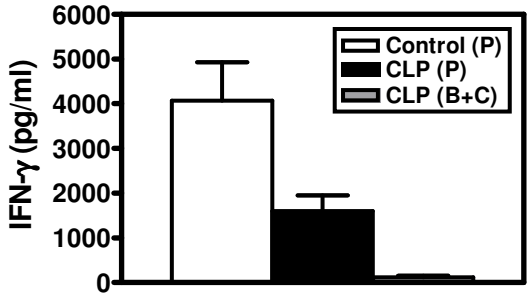


Figure 41: Reduction in IFN-γ production on transfer of CpG stimulated wildtype BMDCs during sepsis. Twenty-four hours after CLP or sham operation, spleens were removed and splenocytes were pooled (n=3 to 4 per group) and were cultured with Con A for 48 h. Supernatants were analyzed for IFN-γ. Data show mean ± SD and are representative of seven independent experiments.

3.2.7. IL-12 is not Responsible for Reduced IL-10 Production on BMDCs Transfer during Sepsis

It has been reported that IL-12 induces the generation of Th1 type cells producing IFN-γ and IL-2 and at the same time, IL-12 inhibits the generation of Th2 cells, which produce IL-10 and IL-4. Thus, the putative role of IL-12 in reduced IL-10 synthesis was investigated. Therefore, BMDCs from IL-12p35$^{-/-}$ (IL-12-KO) mice, which show impaired IL-12 formation, were also generated. At 6 h after CLP or sham operation, CLP treated mice were divided into three groups. The control mice group and one group of septic mice were injected with PBS and the remaining two groups of septic mice were injected with CpG stimulated wildtype BMDCs and CpG stimulated IL-12-KO BMDCs separately. Eighteen hours after injections, spleens were removed and TSCs were prepared. TSCs were then cultured without any stimulus for 18 h and supernatants were analyzed for IL-10. TSCs from control mice injected with PBS [Control (P)] released no IL-10. In contrast, TSCs from septic mice injected with PBS [CLP (P)] produced enhanced levels of IL-10. However, this increased IL-10 production was decreased in both TSCs from septic mice injected with CpG stimulated wildtype BMDCs [CLP (B+C)] and TSCs from septic mice injected with CpG stimulated IL-12-KO BMDCs [CLP (B+C) KO] compared to CLP (P)

mice (Fig. 42). Thus, IL-12 did not attribute to the reduced IL-10 production by total spleen cells on BMDCs transfer into CLP mice.

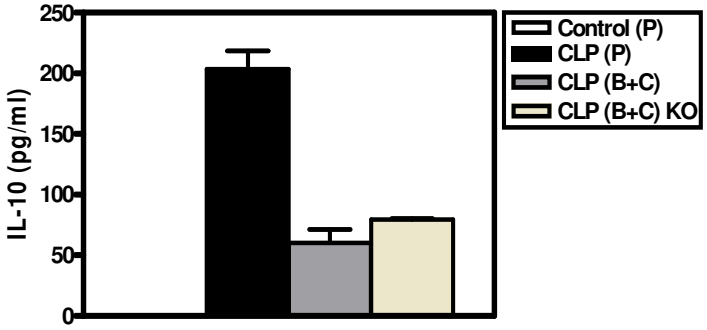


Figure 42: IL-12 did not affect the reduced IL-10 production on transfer of CpG stimulated BMDCs. Twenty-four hours after CLP or sham operation, spleens were removed and splenocytes were pooled (n=3 to 4 per group) and were cultured for 18 h. Supernatants were analyzed for IL-10. Data show mean ± SD and are representative of two independent experiments.

4. Discussion

4.1. Characterization of Dendritic Cells during Sepsis

Immunosuppression following sepsis syndrome remains a poorly understood phenomenon. DCs are known to play a pivotal role in the maintenance and development of cell-mediated immunity against invading pathogens. In the present study, it was hypothesized that the normal behavior and functional properties of DCs get changed during sepsis and might contribute to the immunosuppressed state of the host. It was shown that polymicrobial sepsis induced strong maturation of splenic DCs and a deviated cytokine secretion pattern of these DCs towards IL-10. This maturation process was not restricted to the spleen as a main secondary lymphoid compartment but was also observed in lymph nodes distant to the site of infection. However, maturation of DCs from lymph nodes of septic mice followed a slightly delayed kinetics in comparison to splenic DCs. Additionally, the present study provides evidence that splenic $CD4^+CD8^-$ and $CD4^-CD8^+$ DCs subpopulations were selectively lost during disease development and that remaining DCs from septic mice were less potent in their capacity for T cell activation.

A variety of components released from Gram-negative as well as Gram-positive bacteria, e.g. LPS, PGN etc. have been shown to induce maturation of DCs (De Smedt et al., 1996; Christensen et al., 2002; Reis e Sousa et al., 1999; Riva et al., 1996). Indeed, it has been reported that intraperitoneal bacteria rapidly reach liver and spleen via the blood circulation (Benacerraf et al., 1959). In addition, bacteria were detected in the spleen as early as 4 h after CLP (Table 2). From the peritoneal site of infection direct bacterial spreading via the peritoneal cavity or via the blood circulation may be responsible for the rapid maturation of splenic DCs (Fig. 16, 17, 18 19 and 20). In contrast, DCs from the popliteal or inguinal lymph nodes that do not drain the initial site of infection show a delayed kinetics of maturation (Fig. 21).

Recently, Ding et al. reported that peritoneal and splenic DCs do not enhance the expression of CD86 or CD40 upon CLP-induced sepsis within 24 h (Ding et al., 2004). This observation is in marked contrast to the present findings. However, they also report a lower IL-12 production in splenic DCs from septic mice, which is in alignment with the present findings. Different results in terms of DCs surface marker expression might be explained by different gating strategies during FACS analysis. Another possible explanation for these partially contradictory results is the fact that the inbred strain they used (C3H/HeN) might be less susceptible for sepsis (Watanabe et al., 2004). Efron et al. reported that the total number of DCs in popliteal and inguinal lymph nodes decreases within 24 h after CLP-induced sepsis without any signs for maturation in this time frame. The present data confirm the absence of DCs maturation at least during the early phase of sepsis up to 8 h (Efron et al., 2004). Only marginal effects in terms of DCs maturation was observed by 15 h after CLP, while obvious maturation of lymph node DCs was detected by 36 h after CLP (Fig. 21).

The present study could demonstrate that sepsis did not only induce profound maturation of DCs but also modulated splenic DCs in terms of cytokine secretion. It has been reported that upon bacterial or parasitic infection, DCs secrete a variety of pro-inflammatory cytokines such as IL-1, IL-12, TNF-α (Kadowaski et al., 2000; Langhorne et al., 2004; Pietila et al., 2005) and IFN-γ (Fricke et al., 2006; Ohteki et al., 1999; Pietila et al., 2005; Suzue et al., 2003). These reports are in alignment with the studies in which it was observed that secretion of pro-inflammatory cytokines by DCs and other leukocytes are needed in order to combat the invading pathogen (Tosi, 2005). But in the present study, as early as 8 h after CLP, splenic DCs from septic mice released lower levels of TNF-α and IFN-γ, when stimulated with LPS+CD40L or CpG and the levels of these cytokines decreased further up to 36 h after CLP (Fig. 22). It was reported that monocytes isolated from septic patients when cultured in the presence of LPS showed impaired production of pro-inflammatory cytokines such as IL-1, TNF-α and IL-6 (Munoz et al., 1991). Later on, such altered behavior of monocytes was termed as “monocyte deactivation” which leads to “immunoparalysis” and consequently to the death of the host (Volk et al., 1996; Docke et

al., 1997). Thus, one might speculate that the depressed response of TNF-α and IFN-γ by DCs during sepsis might be the result of their functional reprogramming similar to "monocyte-deactivation". The pathophysiological mechanism leading to "immunoparalysis" is not yet fully understood. Interleukin-10, TGF-β, and PG (prostaglandin) E_2 are known inhibitors of TNF-α and IFN-γ synthesis and might play a role in the development of "immunoparalysis" during sepsis. It has also been reported that the rapid release of IL-10 during endotoxemia that is often used as a model for sepsis in mouse controls TNF-α and IFN-γ production *in vivo* (Marchant et al., 1994). In accord with this study, we can herein suggest that soluble mediators like IL-10, TGF-β or PGE_2 which are released locally in the tissue at high amounts during endotoxic shock (Randow et al., 1995) and sepsis (Cohen, 2002) and therefore, might suppress the capacity of DCs to release pro-inflammatory cytokines like TNF-α, IFN-γ, but also IL-12.

Within 4 to 8 h after CLP, splenic DCs lose their capacity to secrete bioactive IL-12 in response to LPS+CD40L or CpG that both are well known inducers of IL-12 (Fig. 24). DCs secrete IL-12 only transiently for approximately 16 h after stimulation (Langenkamp et al., 2000) before they become unresponsive to further restimulation. This characteristic has been termed "exhaustion" of DCs and might represent a mechanism for protection from infection-induced immunopathology caused by unregulated IL-12 production. A state of impaired IL-12 secretion by splenic DCs upon restimulation with microbial components has been reported for DCs in other models of infectious diseases (Andrews et al., 2001; Reis e Sousa et al., 1999). However, such an immunosuppressive state reported for splenic DCs in the above mentioned models of infection is preceded by a transient phase of IL-12 release and might be explained by DCs exhaustion. The present observations are in contrast to the findings in the above mentioned models of infection since unresponsiveness to restimulation of splenic DCs during sepsis was determined but not a preceding phase of IL-12 secretion. Instead, DCs from septic mice secreted prominent levels of IL-10 *ex vivo* that were further enhanced upon stimulation with otherwise IL-12-inducing agents (Fig. 24). The reason for this aberrant cytokine response of splenic DCs during sepsis remains

unknown. Taken together, during sepsis splenic DCs fail to secrete TNF-α, IFN-γ and IL-12 which are required for the induction of immunity against bacterial infections.

Drug-mediated inhibition of IL-10 several hours after CLP has been associated with significantly increased pro-inflammatory cytokines like IFN-γ and improved bacterial clearance (Kalechman et al., 2002). In another study, *in vivo* neutralization of IL-10 partially reversed the impairment in alveolar macrophage pro-inflammatory cytokine production and phagocytic activity (Reddy et al., 2001). In the present study, DCs-derived IL-10 seemed not to be responsible for suppressed IL-12 production since neutralization of IL-10 did not restore IL-12 production by splenic DC from septic mice (Fig. 26). Therefore, one might speculate that the response of splenic DCs after CLP is influenced by factors other than, or in addition to, IL-10.

Interestingly, sepsis seems not to be associated with a general inhibition of IL-12 production by DCs since it has been reported that DCs in the peritoneal cavity secrete substantial amounts of IL-12 during sepsis (Ding et al., 2004). The underlying mechanisms for this compartment-dependent IL-12 release by DCs remain unclear. We suggest that there exist differences in local micro-environmental factors between spleen and peritoneal cavity during sepsis that might cause different responses of DCs to bacteria. Potential candidates for such factors could be PGE_2, IL-10, and TGF-β that are known to suppress IL-12 production by DCs (Kalinski et al, 1999). IL-10 and PGE_2 have been found in serum and/or spleen early during sepsis (Emmanuilidis et al., 2001; Ertel et al., 1991). PGE_2 inhibits the release of IL-12 by DCs stimulated with bacterial products but does not affect the up-regulation of co-stimulatory molecules (Harizi et al., 2002). In contrast, IL-10 inhibits both IL-12 synthesis and maturation of DCs (De Smedt et al., 1997; Steinbrink et al., 1997). Since splenic DCs from septic mice showed both maturation and suppression of IL-12 synthesis (Fig. 16 and 24), it is unlikely that circulating IL-10 alone mediates the development of the deviated DCs function during sepsis as describe here. Rather, it could be hypothesized that DCs in the peritoneal cavity, representing the primary site of infection, are directly stimulated by gut-derived microbes and are able to respond with adequate IL-

12 production. In contrast, splenic DCs previously exposed to sepsis-induced mediators such as circulating PGE_2 are therefore instructed for an impaired IL-12 secretion when stimulated by spreading bacteria or circulating bacterial products.

Evidence for compartmentalization of the cytokine release during CLP-induced sepsis has also been described for the release of IFN-γ. Mononuclear cells from liver and from the peritoneal cavity but not from the spleen produce IFN-γ (Seki et al., 1998). This compartmentalization of cytokine production might explain why i.v. application of an adenoviral vector coding for IL-10 fails to improve survival after CLP whereas s.c. application protects from CLP-induced mortality (Oberholzer et al., 2002). Splenic DCs seem to be predisposed for IL-10 secretion during sepsis (Fig. 24). Thus, additional IL-10 applied via the i.v. route would further support the predominance of anti-inflammatory mediators in the spleen. In this context it would be of interest whether DCs from peripheral lymph nodes of septic mice are impaired in IL-12 secretion as we observed for splenic DCs. However, the low number of lymph node DCs especially in septic mice was not sufficient for setting up the required cell cultures. Therefore, the behavior of lymph node DCs remains unknown.

It has been shown that IFN-γ can increase the ability of cells to produce IL-12 (Ma et al., 1996). In the present study, IFN-γ treatment of splenic DCs partially restored the decreased IL-12 production in the presence of CpG, only 3 h after CLP but not at 6 and 24 h after CLP while splenic DCs from sham-treated animals showed increased IL-12 production at all time points (Fig. 27). It has been reported that exogenous IFN-γ applied to septic patients restores the LPS-induced TNF-α secretion by monocytes *in vitro* (Docke et al., 1997). In another study *Mycobacterium leprae*-stimulated monocytes produced increased amounts of IL-12 when treated with exogenous IFN-γ (Libraty et al., 1997). These reports contradict the present findings where IFN-γ treatment of splenic DCs only partially restored the IL-12 production. We suggest here that there might be some defect in IFN-γ sensing by splenic DCs during sepsis e.g. through downregulation of the IFN-γ receptor (IFN-γR) which renders them hyporesponsive or largely insensitive when treated with exogenous

IFN-γ. This assumption is in alignment with the report where Kalinski and coworkers have shown that mature DCs express reduced levels of IFN-γR and show decreased production of IL-12 in response to IFN-γ (Kalinski et al., 1999). In another study, it has been reported that when IFN-γR-deficient (IFN-$\gamma R^{-/-}$) mice were infected with *Listeria monocytogenes*, the mutant mice were unable to mount a proper innate immune response against bacterial growth and succumbed to sepsis (Dai et al., 1997). These reports suggest that recognition of IFN-γ is critical for the regulation of cellular responses during infection. Further studies will enhance our understanding of DCs unresponsiveness towards IFN-γ during sepsis.

The present study shows the spontaneous release of MCP-1 by splenic DCs during late sepsis which gets reduced upon stimulation with LPS+CD40L or CpG (Fig. 28). It has been reported that following CLP, the levels of MCP-1 were increased in various tissues (Matasukawa et al., 2000; Benjamim et al., 2003). The elevated levels of MCP-1 have been detected in plasma of patients with sepsis (Bossink et al., 1995), as well as after administration of endotoxin to experimental animals or human volunteers (Jansen et al., 1995; Sylvester et al., 1993). With the understanding of these reports one could say that the cells present in the circulation and tissues become a major source of MCP-1 during sepsis. This release of MCP-1 during CLP-induced sepsis or endotoxic shock has been shown to be associated with the increased survival of the animal (Matsukawa et al., 1999; Zisman et al., 1997). Indeed, in the present study, splenocytes were able to produce MCP-1 36 h after CLP while no/negligible MCP-1 was released at 8 and 15 h after CLP (data not shown). This MCP-1 might be produced by DCs present in total population of spleen cells, as similar pattern for MCP-1 release was observed for splenic DCs (Fig. 28). Following maturation DCs are known to produce MCP-1 (Sallusto et al., 1998; Sozzani, 2005). This is in accord with the present finding where mature splenic DCs released elevated amounts of MCP-1 36 h after CLP. Here, one might argue that splenic DCs showed maturation at 8 and 15 h after CLP also but there was no MCP-1 released at those time points. This can be explained by the observation that at 36 h after CLP, $CD4^{-}CD8^{-}$ became a main subpopulation of DCs and it might represent the main subpopulation of DCs for the production of MCP-1. This assumption is further supported by the recent study where $CD4^{-}$

$CD8^-$ DCs were shown to secrete different amounts of cytokines like TGF-β, IFN-γ and TNF-α secretion as compared to $CD4^+CD8^-$ and $CD4^-CD8^+$ DCs (Zhang et al., 2005). Therefore, it is tempting to speculate that these DCs subpopulations might vary in the release of MCP-1.

It has been shown that MCP-1 can suppress IL-12 production by human monocytes (Braun et al., 2000) and monocyte derived DCs (Omata et al., 2002). Here, one might assume that MCP-1 could have a same effect on mouse splenic DCs as seen with monocytes or monocyte derived DCs. But this assumption is in partial variance with the present findings where splenic DCs showed suppressed IL-12 production capacity *in vitro* even in the absence of MCP-1 during early sepsis (Fig. 24). Therefore, one could imagine that besides MCP-1 there might be other factors which contribute to the suppressed IL-12 production.

In addition, the release of MCP-1 by splenic DCs was coinciding with the release of IL-10 at 36 h after CLP. Here, one might assume that they both influence the release of each other. This assumption is in accord with the present findings where splenic DCs released predominant levels of IL-10 with increase in MCP-1 release at 36 h after CLP. But on the same hand it partly contradicts the findings of IL-10 release, though very little, without any release of MCP-1 by splenic DCs. Therefore, it's difficult to predict that they influence the release of each other. Further studies are needed to elucidate the underlying mechanisms of their release.

The predominance of IL-10 supported by increased release of MCP-1 from splenic DCs during sepsis as shown here might polarize Th cells towards Th2 and in parallel might inhibit the development of a Th1 response that is required for the effective clearance of bacterial infections (Corinti et al., 2001; Omata et al., 2002). The finding that splenocytes from septic mice secrete elevated levels of Th2 cytokines upon mitogenic stimulation (Ayala et al., 1994) support this assumption. The suppressive activity of IL-10 on Th1 cell polarization and proliferation might explain the unexpected finding that DCs from septic mice were inferior to DCs from controls in T cell activation despite the expression of high

levels of co-stimulatory molecules (Fig. 29). Another explanation for the reduced T cell stimulation in response to DCs from septic mice could be the activity of T regulatory (Treg) cells. These Treg cells can inhibit the proliferation and cytokine release of antigen-specific Th1 cells. It has been reported that Treg cells can be induced by "semi-mature" DCs that express MHC class II and CD86 molecules but fail to secrete pro-inflammatory cytokines (Akbari et al., 2001; McGuirk et al., 2002). Therefore, it is tempting to speculate that "semi-mature" DCs develop in the spleen during sepsis and activate Treg cells thereby preventing the induction of a protective Th1 immune response. This hypothesis is supported by the observation that elevated numbers of Treg cells were found in septic patients (Monneret et al., 2003).

One might speculate that the reduced T cell-stimulatory capacity and the impaired IL-12 production by DCs observed 36 h after CLP is a phenomenon characteristic for $CD4^-CD8^-$ DCs. However, several reports argue against divergent properties of the splenic DCs subpopulations with regard to maturation and T cell activation (Maldonado-Lopez et al., 2001; Edwards et al., 2002). Moreover, the finding that splenic DCs failed to secrete IL-12 even during the early phase of sepsis (8 h after CLP, Fig. 24) when all DCs subtypes were present (Fig. 15B) further contradict the assumption of a $CD4^-CD8^-$ DCs restricted phenomenon.

During late sepsis, the number of splenic DCs expressing CD11c was strongly reduced. CD11c is a stable population marker for murine DCs and its expression remains unchanged upon maturation. So far, there is no evidence that DCs downregulate CD11c expression *in vitro* or *in vivo*. Therefore, we assume that the reduced number of splenic $CD11c^+$ cells shown here represents a loss of DCs similar to the loss of $CD11c^+$ DCs that has been observed e.g. during sepsis, endotoxemia, and viral infections (Tinsley et al., 2003; De Smedt et al., 1996; Montoya et al., 2005). It was further shown here that the reduced number of splenic DCs during late sepsis is mediated by a selective loss of the $CD4^+CD8^-$ and $CD4^-CD8^+$ DC subpopulations. In contrast, the total number of $CD4^-CD8^-$ DCs remained unaffected and $CD4^-CD8^-$ DC became a main subpopulation by 36 h after CLP

(Fig. 15). The three DC subpopulations arise from distinct DC lineages and *in vivo* there is no conversion of one subtype into another e.g. through downregulation of CD4 or CD8 expression (Kamath et al., 2002). Likewise, $CD4^+$ or $CD8^+$ DC remain clearly positive for these markers even upon maturation *in vitro* (Vremec et al., 2000). Thus, it is assumed that the $CD4^+CD8^-$ and $CD4^-CD8^+$ DCs are lost during sepsis rather than that these subtypes transform into $CD4^-CD8^-$ DCs.

The disappearance of two DCs subsets during sepsis may occur through migration and/or death. Since apoptosis of DCs has already been reported to be associated with sepsis (both in humans and in mice) (Hotchkiss et al., 2002; Tinsley et al., 2003; Efron et al., 2004), it is supposed that the selective loss of $CD4^+CD8^-$ and $CD4^-CD8^+$ DCs that are shown here is the consequence of apoptosis. It is well known that DCs undergo apoptosis after reaching the final maturation stage e.g. upon administration of LPS (De Smedt et al., 1998). Such a programmed DCs death might represent a tool for regulation of the immune response. However, in this study an enhanced maturation stage of $CD4^+CD8^-$ and $CD4^-CD8^+$ DCs in comparison to $CD4^-CD8^-$ DCs that could result in the predominant loss of these DCs subtypes was never observed (data not shown). Thus, the mechanisms of selective DCs subset depletion remain speculative.

In summary, in the present study a profound maturation of DCs during polymicrobial sepsis was observed. However, DCs acquire a phenotype that might favor the development of Th2 and/or Treg cells and might inhibit effective immunity against the bacterial infection through Th1 cell polarization. Changes in DCs phenotype or functions could therefore contribute to sepsis-mediated immunosuppression. Treatment regimens that restore the capacity of DCs for Th1 cell polarization or the regular DCs number seem to represent promising approaches to counteract immunoparalysis during sepsis. Indeed, the instillation of competent DCs into the lungs of postseptic mice restored an effective antifungal host response in the lung (Benjamim et al., 2005). An attempt to use stimulated DCs as a therapeutic agent during acute sepsis is discussed in the second part of this chapter (section 4.2).

4.2. Restoration of Dendritic Cell Functions during Sepsis

The aim of the second part of this study was to set up a therapy that might prevent the development of sepsis-associated immunosuppression. Based on the previous findings of aberrant DCs function (see section 3.1) during sepsis and on the recent report on the essential role of DCs (Scumpia et al., 2005), a treatment regimen was established. Septic mice were treated with *in vitro* generated competent BMDCs that should overcome the inability of endogenous DCs to mount a Th1-type immune response. Therefore, BMDCs secreting IL-12 and expressing co-stimulatory molecules are required. The BMDCs were treated with CpG for 8 h prior to injection in order to ensure IL-12 secretion and maturation at the time point of application. Moreover, prior induction of maturation of DCs can not be stopped or reversed once the maturation machinery has started. It means that there is a "point of no return" in the life cycle of DCs. Therefore, BMDCs were previously matured before injecting them into the septic animal. These mature BMDCs are not sensitive to "suppressive milieu" present in septic animal that might be responsible for aberrant function of endogenous DCs. This therapy resulted in the diminished loss of TSCs number and in reduced IL-10 production by these cells. The diminished loss of TSCs number was associated with an increased total T cell number in the spleen. In addition to the reduced IL-10 production by TSCs mediated by the treatment with BMDCs, a reduced secretion of TNF-α and IFN-γ was also observed. The present study also provides evidence that reduced IL-10 production by TSCs was not dependent on the IL-12 secreted by injected BMDCs.

It has been documented that $CD11c^+$ DCs are required for survival in murine polymicrobial sepsis (Scumpia et al., 2005). In the present study, we tested the hypothesis that transfer of *ex vivo* generated BMDCs might modulate the immune status of septic mice and might prevent the development of sepsis-induced immunosuppression.

The present study also demonstrates a marked loss in the number of TSCs 24 h after CLP (Fig. 35). The loss of TSCs during sepsis may occur through death. Since apoptosis of leukocyte has already been reported to be associated with sepsis (both in humans and in mice) (Hotchkiss et al., 2003; Wesche et al., 2005), we suppose that the loss of TSCs that

are shown here is the consequence of apoptosis. The loss in number of TSCs during sepsis was associated with the loss in number of total T cells (Results section 4.2.4.2.). This finding is in accordance with the reports of Hotchkiss and coworkers, where they have shown an increase in splenic lymphocyte apoptosis in septic mice, which was associated with an increase in mortality (Hotchkiss et al., 1997; Hiramatsu et al., 1997). Besides lymphocytes many other cell types in the lymphoid organs undergo apoptotic loss during sepsis (Wesche et al., 2005). In the present study, we have observed the loss in number of two DCs subtypes (as discussed earlier) and total T cells. Such a change in the number of specific immune cells might contribute to altered immune response and decreased ability of host to ward off the lethal effects of sepsis.

As discussed in the last paragraph, the loss of TSCs during sepsis is mediated through apoptosis. Since the application of BMDCs was performed at 6 h after CLP when TSCs numbers are unchanged between septic and sham mice, it is more likely that the therapy with BMDCs prevented apoptosis rather than that it induced proliferation. The mechanism how injection of BMDCs might prevent apoptosis-induced cell death in the spleen remains speculative. It has been shown here that treatment with BMDCs results in reduced secretion of TNF-α by TSCs. It is well known that TNF-α triggers the extrinsic pathway of apoptosis via binding to the death receptor TNFR1 (Chen et al., 2002; Cunningham et al., 2002).

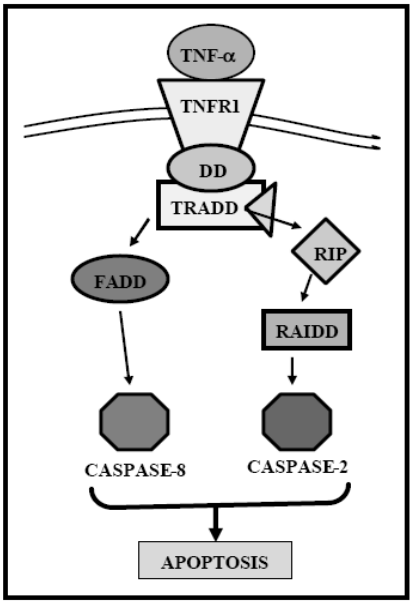


Figure 43: Engagement of TNF-α with its cognate receptor TNFR1, which in turn triggers the apoptotic pathway.

This pathway might be relevant during sepsis development since it was shown that TNF-α mediates apoptosis in the tissue in a TNFR1-dependent manner (Oberholzer et al., 2001). Although these data are conflicting with the work of Ebach et al. who reported that the TNFR1 is irrelevant for the sepsis-associated induction of apoptosis in tissues like spleen and thymus (Ebach et al., 2005). However, given an involvement of the TNFR1 in apoptosis during sepsis, the reduced secretion of TNF-α by TSCs from BMDCs-treated mice in comparison to untreated septic mice might result in decreased TNF-α-induced apoptosis. Certainly, there might exist other mechanisms how the therapy with BMDCs might prevent apoptosis and further studies are needed to clarify them.

In the present study, elevated levels of TNF-α and IL-10 and low levels of IFN-γ were produced by TSCs during sepsis (Fig. 37 and 38). Indeed, it has been reported that key pro-inflammatory and immunomodulatory cytokines such as IL-1, IL-6, IL-10, or TNF-α are induced after activation of TLRs by microbial ligands (Krutzik et al., 2001). But the imbalance in their secretion might lead to poor clearance of invading pathogen as seen in septic patients. In patients with documented sepsis, coexistence of pro- and anti-inflammatory cytokines have been documented in the circulation and presumably within the tissues (Goldie et al., 1995; Vanderpoll et al., 1997; Ertel et al., 1996). It was reported that the expression of TNF mRNA increased in the tissues like spleen, liver and lung following CLP-induced sepsis or intraperitoneal injection of endotoxin (Hadjiminas et al., 1994). This is in alignment with the present findings where elevated levels of TNF-α were produced by TSCs following sepsis. Ayala and coworkers showed that CLP-induced sepsis causes decreased IFN-γ release while increasing IL-10 production by TSCs (Ayala et al., 1994). This is consistent with the present finding where decreased levels of IFN-γ and increased levels of IL-10 were produced by TSCs following CLP-induced sepsis. Thus, present data confirm the dysregulated production of cytokines during the sepsis disease response.

Treatment of septic mice with BMDCs did not only affect the sepsis-associated cell loss in the spleen but additionally modulated the above described aberrant cytokine pattern during

sepsis. Administration of BMDCs resulted in decreased production of IL-10, TNF-α and IFN-γ by TSCs (Fig. 39, 40 and 41). It has been reported that activated DCs following CD40 ligation or TLR ligation produced IL-12p70, which induces IFN-γ production and is important for the generation of Th1 cells (Boonstra et al., 2003). DCs that fail to produce IL-12p70 are assumed to promote the generation of a Th2 response or down-regulate Th1 responses via the action of costimulatory molecules and/or other DCs produced cytokines such as IL-6 or IL-10 (Trembleau et al., 1997; Rincon et al., 1997; Rissoan et al., 1999; Diehl et al., 2002). These reports are consistent with the findings discussed in earlier section that the splenic DCs produced decreased IL-12 and increased IL-10 during sepsis and therefore, failed to initiate protective Th1 immune response against accumulating bacteria. The transfer of IL-12 secreting BMDCs resulted in the decreased production of IL-10 by splenocytes (Fig 39). This can be explained by the reason that the transfer of IL-12 secreting BMDCs during sepsis might effectively prime the Th cells towards Th1 response. The onset of Th1 response might result in the decreased production of IL-10 by TSCs. Also the transfer of IL-12 secreting BMDCs into septic mice leads to decreased production of IFN-γ by TSCs stimulated with Con A (Fig. 41). These findings were unexpected. In a very recent report it has been documented that the cytokine profile of the injected BMDCs plays a key role in the development of Th1 or Th2 response. The BMDCs which were cultured only in GM-CSF were able to secrete IL-12p70 but were unable to induce strong Th1 polarization (Feili-Hariri et al., 2005). This report might explain in part the findings of decreased IFN-γ secretion by TSCs upon transfer of GM-CSF cultured BMDCs. Another reason for decreased IFN-γ production by TSCs upon BMDCs injection could be the presence of a restricted number of IFN-γ secreting T cells in TSCs population. And the number of these cells remains constant even when there was diminished loss in T cell number upon BMDCs transfer. This might resulted in the dilution of IFN-γ concentration produced by restricted number of T cells as seen in the present findings. To elucidate this assumption in more detail, one has to examine the production of IFN-γ per cell by performing intracellular staining. Since, IFN-γ production was not analyzed per cell in the present study, therefore, the mechanism for decreased production of IFN-γ upon BMDCs transfer remains unknown.

The ability of DCs to induce Th1 differentiation has been related to their ability to produce high levels of IL-12p70. This is becoming relevant in clinical settings, where DCs are being used as therapy in the treatment of cancer for example (Schuler et al., 2003). Surprisingly, the treatment of septic mice with IL-12p35KO BMDCs resulted in the decreased production of IL-10 as shown by the transfer of wildtype BMDCs (Fig. 42). These findings suggest that IL-12p70 is not responsible for the decreased IL-10. Here, one might assume that there could be other factor(s) secreted by DCs which can contribute to the decreased IL-10 production by TSCs. The putative factor could be IFN-γ. It has been shown that BMDCs which secrete IFN-γ besides IL-12p70 possess a strong Th1-skewing capability (Feili-Hariri et al., 2005). Therefore, it is tempting to study the effect of the deletion of IFN-γ gene alone besides IL-12 gene or both in the outcome of sepsis response.

Overall, these studies demonstrate that the transfer of DCs into the septic mice resulted in the change of spleen cytokine environment, representing a main secondary lymphoid organ located next to the site of infection. I was able to identify the effects of BMDCs transfer into the septic mice. BMDCs transfer resulted in diminished cell loss and decreased IL-10 production by the spleen cells. These changes might favor the development of Th1 response, which is required for the effective immunity against bacterial infection. Thus, one can say that DCs play a major role in linking the innate and adaptive systems and could serve as a potential tool to intervene in the disease process during sepsis.

5. Summary

The present study comprises of two parts. The first part deals with studies on the role of DCs during polymicrobial sepsis and the second part includes the exploitation of BMDCs as therapeutic agents to prevent the sepsis-induced immunosuppression.

The data obtained from the first part indicated that the sepsis induced altered phenotypic and functional changes in the splenic and the lymph node DCs. There was marked maturation of DCs in the spleen and in the lymph nodes but this process of maturation occurred later in lymph nodes than in the spleen. The splenic DCs developed an altered cytokine secretion pattern, which was associated with the inability of splenic DCs to produce Th1-type cytokines such as IL-12 but released high levels of IL-10. This impairment of IL-12 synthesis was independent from endogenously produced IL-10 as neutralization of IL-10 did not restore the IL-12 secretion by DCs. But IFN-γ treatment restored in part the IL-12 production by splenic DCs only during early sepsis. In addition, the splenic $CD4^+CD8^-$ and $CD4^-CD8^+$ subpopulations were lost during sepsis, and the remaining DCs showed a reduced capacity for allogeneic T cell activation associated with decreased IL-2 synthesis. Thus, during sepsis DCs acquire a phenotype that might favor the development of Th2 and/or Treg cells and might inhibit effective immunity against the bacterial infection through Th1 cell polarization.

The data obtained from the second part highlighted that the administration of competent BMDCs into septic mice changed the cytokine milieu in the spleen, representing a main secondary lymphoid organ situated near to the site of infection. The instillation of competent BMDCs resulted in the diminished loss of splenocytes and in reduced IL-10 production by these cells. The diminished loss of splenocytes was associated with an increased total T cell number in the spleen. In addition to the reduced IL-10 production by splenocytes mediated by the administration of BMDCs, a reduced secretion of TNF-α and IFN-γ was also observed. Moreover, the reduced IL-10 production by splenocytes was not

dependent on the IL-12 secreted by injected BMDCs. These changes in the spleen cytokine environment might favor the development of a Th1 response, which is required for the effective immunity against the bacterial infection.

The present findings reinforce the idea that DCs are affected by sepsis and their replacement has some potentially beneficial contributions to the host's response to infectious challenge. The present data are also in accord with an emerging understanding of the critical role played by DCs in the development of the proper Th1/Th2 cytokine balance during sepsis syndrome in order to ward off the invading bacteria. Thus, these data suggest that DCs are critical cells at the boundary between the innate and adaptive immune response and play an important role in bacterial responses.

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Publications

1. Stefanie B. Flohé, Hemant Agrawal, Daniel Schmitz, Michaela Gertz, Sascha Flohé, and F. Ulrich Schade. "Dendritic cells during polymicrobial sepsis rapidly mature but fail to initiate a protective Th1-type immune response" J Leukoc Biol, March 2006, 79(3), 473-481

2. Agrawal, H., Rani, M., Gertz, M., Schmitz, D., Schade F.U., and Flohé, S.B. "Polymicrobial sepsis induces dysregulated cytokine response in dendritic cells." (Manuscript to be submitted)

3. Stefanie B. Flohé, Jörg M. Bangen, Sascha Flohé, MD, Hemant Agrawal, Katja Bergmann, and F. Ulrich Schade."Origin of immunomodulation after blunt trauma: Involvement of extracellular Heat-Shock Proteins."(Accepted in Shock)

4. Agrawal, H., Rani, M., Gertz, M., Schmitz, D., Schade F.U., and Flohé, S.B. (2005). "Dendritic Cells develop an aberrant cytokine response during polymicrobial sepsis." Abstract in Immunobiology, 210, 6-8, 2005, pp 428.

5. Rani, M., Agrawal, H., Gertz, M., Schmitz, D., Schade F.U., and Flohé, S.B. (2005). "Reprogramming of Dendritic cell progenitors in the bone marrow during polymicrobial sepsis." Abstract in Immunobiology, 210, 6-8, 2005, pp 435-436.

6. Flohé, S.B., Agrawal, H., Gertz, M., Schmitz, D., and Schade F.U. (2005). "Modulation of Dendritic Cells during Polymicrobial Sepsis." Abstract in Shock, 23, Sup 2, 2005, pp 22-23.

7. Agrawal, H., Gertz, M., Schmitz, D., Schade F.U., and Flohé, S.B. (2004). "Alteration in Dendritic Cell Functions during Sepsis." Abstract in Immunobiology, 209, 4-6, 2004, pp 330.

8. Flohé, S.B., Agrawal, H., Gertz, M., Schmitz, D., and Schade F.U. (2004). "Modulation of Dendritic Cells during Polymicrobial Sepsis". Abstract in Conference book of the 8th International Symposium on Dendritic Cells, 17-21 Oct, 2004, Brugge, Belgium, pp 144 (P304).

Posters

1. Agrawal, H., Gertz, M., Schmitz, D., Schade F.U., and Flohé, S.B. "Polymicrobial sepsis induces alteration in dendritic cell functions." The 2nd Summer School of Immunology conducted by German Society of Immunology (DGFI), Ettal, Germany (6-10th March 2006)

2. Agrawal, H., Rani, M., Gertz, M., Schmitz, D., Schade F.U., and Flohé, S.B. "Dendritic cells develop an aberrant cytokine response during polymicrobial sepsis." The 36th Joint Annual Meeting of German Society of Immunology (DGFI) and Scandinavian Society for Immunology (SSI), Kiel, Germany (21-24th Sep 2005)

3. Rani, M., Agrawal, H., Gertz, M., Schmitz, D., Schade F.U., and Flohé, S.B. "Reprogramming of dendritic cell progenitors in the bone marrow during polymicrobial sepsis." The 36th Joint Annual Meeting of German Society of Immunology (DGFI) and Scandinavian Society for Immunology (SSI), Kiel, Germany (21-24th Sep 2005)

4. Agrawal, H., Gertz, M., Schmitz, D., Schade F.U., and Flohé, S.B. "Alteration in dendritic cell functions during sepsis." The Joint Annual Meeting of the Dutch and German Societies for Immunology, Maastricht, The Netherlands (20-23rd Oct 2004)

5. Flohé, S.B., Agrawal, H., Gertz, M., Schmitz, D., and Schade F.U. "Modulation of dendritic cells during polymicrobial sepsis." The 8th International Symposium on Dendritic Cells, 17-21st Oct, 2004, Brugge, Belgium.

CURRICULUM VITAE

PERSONAL DETAILS

Name: Hemant Agrawal
Date of Birth: 14 May 1979
Place of Birth: Mathura, India
Nationality: Indian
Marital Status: Married

EDUCATION

1983 - 1996: Basic Schooling, Mathura, India
1997 - 2000: B.Sc. (Industrial Microbiology), B.S.A. College, Mathura, India
2000 - 2002: M.Sc. (Life Sciences), S.L.S., J.N.U., New Delhi, India
2003 - 2006: Ph.D., Surgical Research, Department of Trauma Surgery, University Hospital, Essen, Germany.

RESEARCH EXPERIENCE

2001 - 2002: M.Sc. dissertation work at the immunology laboratory, S.L.S., J.N.U., New Delhi, India, under project entitled, “Preparation of molecular probes for TLR2 and TLR4 genes and their use in monitoring the expression of these receptors”.
2002 - 2003: Project work at the department of gastroenterology and hepatology, University Hospital, Essen, Germany
2003 – 2006: Ph.D. dissertation work at the department of trauma surgery, University Hospital, Essen, Germany, under the project entitled, “The role of dendritic cells in sepsis-induced immunosuppression."

Essen, 26.06.2006 Hemant Agrawal

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema: "The role of dendritic cells in sepsis-induced immunosuppression" zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Hemant Agrawal befürworte.

Essen, den Aug.2006 Prof. Dr. F. Ulrich Schade

Erklärung:

Hiermit erkläre ich gem. § 6, Abs. 2, Nr. 6 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich die vorliegende Arbeit selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den Aug.2006 Hemant Agrawal

Erklärung:

Hiermit erkläre ich gem. § 6, Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt wurde.

Essen, den Aug.2006 Hemant Agrawal