Toll-like Receptors –
Link between Innate and Adaptive Immunity

Toll-ähnliche Rezeptoren -
Mittler zwischen angeborener und erworbener Immunität

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Abbreviations

For peptide sequences the one- or three-letter amino acid code was used. SI units and standard abbrevations are not explained in the table.

ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
AIRE	Autoimmune regulator
AP-1	Activator protein 1
APC	Antigen presenting cell
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CD	Cluster of differentiation
CD154	Also called CD40 ligand
CD80	Also called B7.1
CD86	Also called B7.2
CFSE	Carboxyfluorescein diacetate succinimide ester
CLIP	Class II-associated invariant chain peptide
CMV	Cytomegalovirus
CpG DNA motif	Cytosine-phosphate-guanine DNA motif
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
CTLA-4	Also called CD152
DC	Dendritic cell
DD	Death domain
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DRiPs	Defective ribosomal products
dsRNA	Double-stranded RNA
ECSIT	Evolutionarily conserved signalling intermediate in Toll pathways
ER	Endoplasmic reticulum
ERAAP	Endoplasmic reticulum aminopeptidase associated with antigen processing
ERK	Extracellular-regulated kinase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating facto
Gp96	96-kDa glycose-regulated protein
HAU	Haemagglutinating units
HCV	Hepatitis C virus
Hib	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Hsp	Heat-shock protein
HSV-1	Herpes simplex virus 1
Ig	Immunoglobulins
Ii protein	Invariant chain protein
IKKs	IκB kinases
IL	Interleucin

INF	Interferon
IRAK4, IRAK1	IL-1R-associated kinase 4, IL-1R-associated kinase 1
ISRE	Interferon-stimulated response elements
ITAM	Immunoreceptor tyrosine-based activation motif
IκB	Inhibitor of NF-κB
JNK	JUN N-terminal kinase
kDa	Kilo Dalton
LBP	Lipopolysaccharide binding protein
LCMV	Lymphocytic choriomeningitis virus
LDL	Low density lipoprotein
LPS	Lipopolysaccharide, also called endotoxin
LRR motif	Leucin-rich repeat motif
LTA	Lipoteichonic acid
MAL	MyD88-adaptor-like protein
MALP-2	Macrophage-activating lipopeptide-2 kDa
MALT	Mucosa-associated lymphoid type
MAP	Mitogen-associated protein
MARCO	Macrophage receptor with collagenous structure
MBL	Mannan-binding lectin
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MIIC	MHC class II compartments
MLA	Monophosphoryl lipid A
MM-LDL	Minimally modified-LDL
MSR	Macrophage scavenger receptors
MTB	*Mycobacterium tuberculosis*
MyD88	myeloid differentiation factor 88, myeloid differentiation primary-response protein 88
NF- κB	Nuclear factor-κB
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
PA28	Proteasome-activator
Pam_3Cys	(S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteine
PAMP	Pathogen associated molecular pattern
PE	Phycoerythrin
pH	Potentia Hydrogenii
PI3K	Phosphatidylinositol 3-kinase
PKR	IFN-inducible dsRNA-dependent protein kinase
Poly(I:C)	Polyinosine-polycytidylic acid (dsRNA analogue)
PPR	Pattern recognition receptor
RNA	Ribonucleic acid
RP105	CD180, radioprotecitve 105
RSV	Respiratory syncytial virus
SAP	Serum amyloid protein
SAPK	Stress-activated protein kinases
SAP-kinase	Stress-associated protein kinase
SIGIRR	Single immunoglobulin IL-1R-related molecule
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signalling
ssRNA	Single-stranded RNA
TAB1/TAB2/TAB3	TAK1-binding proteins

TAK1	Transforming growth factor-β(TGF-β)-activated kinase
TAP	Transporter associated with antigen processing
TAPA-1	Also called CD81
TCR	T cell receptor
T_H	T helper cell
TICAM1	TIR-domain-containing molecule 1, also known as TRIF
TICAM2	TIR-domain-containing molecule 2, also known as TRAM
TIR domain	Toll/IL-1 receptor domain
TIRAP	TIR domain containing adaptor protein, also known as MAL
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOLLIP	Toll-interacting protein
TRAF	Tumor-necrosis factor (TNF)-receptor-associated factor
TRAM	TRIF-related adaptor molecule, also known as TICAM2
TRIF	TIR domain-containing adapter inducing IFN-β, also known as TICAM1
VV	Vaccinia virus

Figures are numbered for each chapter separately. If not otherwise stated, the mentioned figure numbers refer to the figures in the same chapter.

Summary

Toll-like receptors (TLR) function as pattern recognition receptors (PRR) and recognize highly conserved pathogen-associated molecular patterns. They not only activate an immediate innate immune defense but are additionally able to induce an adaptive immune response. The questions, which substances are recognized via TLRs, which specific TLRs are involved by a given danger signal and which mechanisms finally lead to the activation of the adaptive immune system, were the focus of this thesis.

Investigation of the bacteria *Legionella pneumophila*, the causative agent of Legionnaires' disease, revealed that lipopolysaccharide (LPS) purified from the cell wall as well as the whole bacteria activate antigen-presenting cells (APC) via TLR2-dependent signal transduction. Thus, LPS was identified as the main structure for the recognition of *L. pneumophila* by the innate immune system (**Chapter 2**). Furthermore, TLRs play a central role in the activation of the innate immune system by antigens of the fungus *A. fumigatus*, which causes invasive aspergillosis. Thereby, the involvement of TLR2 and TLR4 results in the release of different pro-inflammatory cytokines by APCs (**Chapter 3**). TLRs, however, do not only recognize exogenous pathogen-derived molecules, but also endogenous alarm structures. This could be demonstrated for the ER-resident heat shock protein Gp96, which, as carrier of tumor-specific peptides, is able to elicit protective immunity against tumors. The Gp96-induced activation of dendritic cells via TLR4 and TLR2 (**Chapter 4**) finally leads to the expansion of antigen-specific CD8-positive T cells *in vivo* and *in vitro* (**Chapter 5**). The relevance of TLR-mediated activation of the immune system for the induction of adaptive immune responses could also be demonstrated in a more general system: "priming" of cytotoxic T cells (CLTs) during a virus infection requires either $CD4^+$ T helper cells or TLR-mediated signals. In the absence of both, CTL priming is impaired. In a system of weaker immunogenic antigens, TLR signals are even strictly required (**Chapter 6**).

Toll-like receptors are more than recognition receptors of the innate immune system – they link innate and adaptive immunity.

Zusammenfassung

Toll-ähnliche Rezeptoren (TLR) erkennen als Pattern-Recognition- Rezeptoren (PRR) stark konservierte Pathogen-assoziierte molekulare Strukturen. Sie aktivieren nicht nur eine sofort einsetzende angeborene Immunabwehr, sondern sind zudem in der Lage, eine adaptive Immunantwort auszulösen. Die Fragen, welche Substanzen als Gefahrensignale über TLRs erkannt werden, welche speziellen TLRs diese Aufgabe für individuelle Gefahrenmoleküle übernehmen und welche Mechanismen schließlich zur Aktivierung des erworbenen Immunsystems führen, standen im Mittelpunkt dieser Arbeit.

Untersuchungen des Bakteriums *Legionella pneumophila*, des Erregers der Legionärskrankheit, zeigten, dass sowohl das Bakterium als Ganzes als auch aus der Zellwand isoliertes Lipopolysaccharid (LPS) Antigen-präsentierende Zellen (APC) über TLR2 aktivieren. LPS wurde somit als Hauptstruktur zur Erkennung von *L. pneumophila* durch das angeborene Immunsystem identifiziert. (**Kapitel 2**). Auch Antigene des Pilzes *Aspergillus fumigatus*, der die invasive Aspergillose hervorruft, wurden als TLR-vermittelte Gefahrensignale identifiziert. Dabei führt die Involvierung von TLR2 und TLR4 zur Freisetzung unterschiedlicher pro-inflammatorischer Zytokine durch APCs (**Kapitel 3**). TLRs erkennen aber auch endogene Gefahrenstrukturen. Dies konnte für das ER-ständige Hitzeschockprotein Gp96, das als Trägermolekül von tumorspezifischen Peptiden eine protektive Immunität gegen Tumoren hervorruft, gezeigt werden. Die Gp96-induzierte Aktivierung dendritischer Zellen über TLR4 und TLR2 (**Kapitel 4)** mündet schließlich in eine Expansion von antigen-spezifischen $CD8^+$ T Zellen (**Kapitel 5**). Die Bedeutung der TLR-vermittelten Aktivierung des Immunsystems für die Entwicklung einer adaptive Immunantwort konnte auch in einem verallgemeinerten Modell verdeutlicht werden: Für das „Priming“ cytotoxischer T-Zellen während einer Virusinfektion sind entweder $CD4^+$ T-Helferzellen oder aber TLR-vermittelte Signalwege nötig. Sind beide Signalwege blockiert, findet kein Priming statt. In einem System mit schwächer immunogenen Antigenen sind TLR-Signale sogar absolut notwendig (**Kapitel 6**).

TLRs sind mehr als nur Erkennungsrezeptoren des angeborenen Immunsystems – sie verknüpfen angeborene und erworbene Immunität.

1

GENERAL INTRODUCTION

No living being can exist on its own. During its life, it never ceases to interact with other life forms. These interactions can be of mutual avail, as is the case for symbiotic relationships. In most cases, however, such interactions are harmful or even lethal for at least one of the protagonists. To protect themselves against affection by parasitizing or even live-threatening microorganisms, multicellular organisms have developed a whole plethora of defense strategies, which are subsumed under the term immune system. In vertebrates, its present form consists of two pillars: the innate and adaptive immunity.

The evolutionarily ancient innate immune system is specific for pathogenic nonself and senses infections through a limited number of germ line encoded receptors that recognize conserved pathogen-associated key molecules. The adaptive immune system is specific for virtually all nonself antigens and gains this ability via a random and highly diverse receptor repertoire. This is not germ line encoded but is generated out of a set of inherited gene segments through somatic recombination and other intricate mechanisms. The price to be paid for this high specificity is a delayed adaptive immune response. This gap is bridged by the innate immune system, which, upon recognition of an infectious agent, reacts immediately in order to keep the pathogen in check until the more sophisticated and versatile adaptive immune system is ready for battle. But the innate immunity is not just the advance guard, on the contrary, the innate effector mechanisms induce and instruct the development of adaptive immune responses. Thus, the adaptive immunity is to a certain extent under the control of the innate immunity.

Adaptive immunity

Specificity and memory are the hallmarks of adaptive immunity: these unique features enable the adaptive immune system to recognize virtually every potential antigen specifically and to retain this ability throughout the lifetime. This memory provides enhanced protection against re-infection and is the basic principle of active immunization.

The nearly unlimited possible antigenic structures are recognized by distinct antigen receptors unique to every single B and T lymphocyte. This vast receptor diversity is not germline encoded per se but is generated by random rearrangement of a limited group of inherited receptor gene subunits during lymphocyte development (Tonegawa, 1983; Tonegawa, 1993). However, this randomized process bears the risk of generating antigen receptors that are specific for self antigens. Normally, these self-reactive lymphocytes are eliminated early in cell development in order to prevent autoimmunity.

Prior to infection, the number of lymphocytes specific for any given antigen is relatively small. But upon recognition of their specific antigen, B and T lymphocytes rapidly divide in a process called clonal expansion and differentiate into effector cells able to fight against the infectious agent. This is why adaptive immune responses occur only after a delay of several days. The thus generated effector cells have only a limited life-span but a subset of the clonally expanded cells, which have proven to be useful during the first infection, differentiates into memory cells. These in turn can rapidly be reactivated upon subsequent encounter with the same pathogen thereby providing lasting protective immunity (Immunobiology, Janeway, 6th edition).

Lymphocyte antigen recognition receptors

B cell receptor (BCR)

Immunoglobulins (Ig) are the antigen-recognition molecules of B lymphocytes and exist in two forms: the membrane bound form functions as antigen receptor and is known as B-cell receptor (BCR). Upon antigen recognition by virtue of their membrane-bound Ig, naïve B cells differentiate into plasma cells, which are able to secrete Ig of the same antigen specificity as soluble antibody. Both Ig forms, the BCR and the antibody, differ only in a small C-terminal region which contains a hydrophobic sequence for anchoring the BCR in the membrane or a hydrophilic sequence allowing the secretion of the antibody.

The large Ig molecules (150 kDa) are composed of two types of protein chains (Figure 1), heavy chains (50 kDa) and light chains (25 kDa). Two identical heavy (H) chains and two identical light (L) chains (either lambda or kappa) are joined by disulfide bonds in such a way that each H chain is linked to a L chain and the two H chains are linked together forming a Y-shaped structure with three equal-sized portions. The two identical antigen-binding sites are at the tips of the arms, which are tethered to the trunk of the Y by a flexible hinge region allowing simultaneous binding of two identical antigenic structures by both arms of the antibody.

Decisive for the three dimensional structure of the Ig molecule are the so-called Ig domains that comprise about 110 amino acids. In this distinctive immunogloblulin fold, strands of polypeptides chains are linked together by a disulfide bridge to form a roughly barrel-shaped structure. The H chain contains four of these Ig domains, the L chain two.

Only the first amino-terminal Ig domain of the H and L chains are variable in their sequence and are therefore called variable (V) domains. The V domains of one heavy and one light chain together make up the variable antigen binding site. The remaining domains are constant (C) domains and build up the C region. Several different genes encode for the C domains of the heavy chain and can be linked sequentially with the same variable region by DNA recombination, a process known as isotype switching. The resulting five major classes or isotypes of immunoglobulin, namely IgM, IgD, IgG, IgA and IgE, differ in structure, effector function, localization and serum concentration. As mentioned, IgM is the first Ig to appear on the B cell surfaces and the first to be secreted, whereas IgG is the most abundant class of immunoglobulins found in the plasma. IgD is expressed as surface immunoglobulin on mature naïve B cells but its function is more or less unknown. IgA isotype is secreted mainly by mucosal lymphoid tissues. IgE is involved in allergic reactions. Again, Igs of all isotypes can be produced either as a membrane-bound B-cell receptor or as a secreted antibody by alternative RNA splicing.

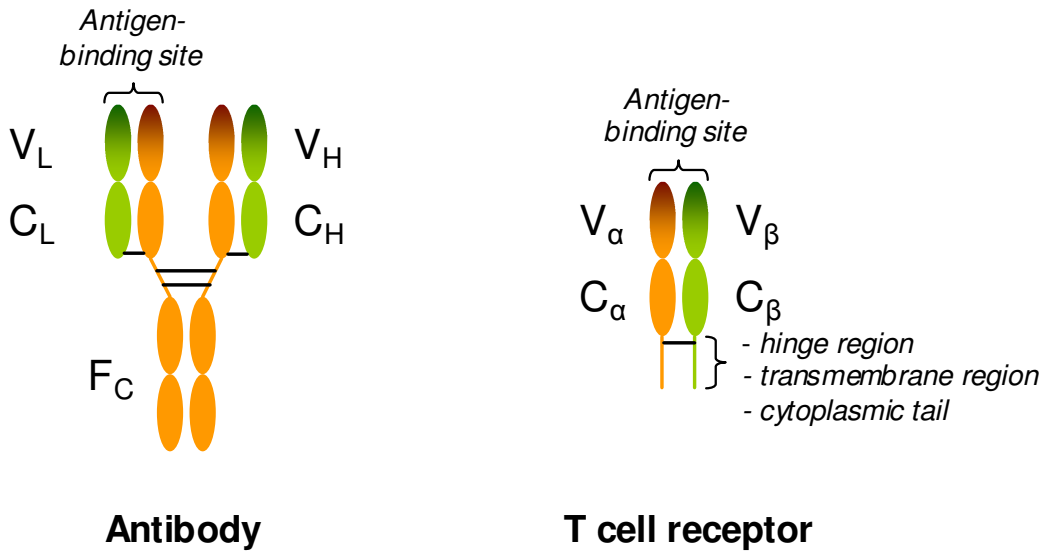

Figure 1. Schematic representation of antibody and T cell receptor. Description in main text.

T cell receptor (TCR)

The heterodimeric T cell receptor (TCR) is composed of two glycosylated polypeptide chains (Figure 1), TCRα and TCRβ, and structurally and functionally resembles the N-terminal part of an immunoglobulin molecule: each TCR chain consists of an aminoterminal variable region and an adjacent constant region, and again, two Ig domains form these characteristic structures. TCRα and TCRβ are connected to each other by a disulfide bond within a short hinge region. Both subunits are membrane-anchored by an unusual positively charged transmembrane segment, which is followed by a short cytoplasmic tail. A minor subset of T cells bears an alternative, but structurally similar γδ TCR.

In contrast to the B cell immunoglobulin, the TCR bears only one antigen-binding site and exists only in the membrane-bound but not in the secreted form.

Generation of B and T cell receptor diversity

Nature has evolved an elegant mechanism to create a tremendous repertoire of diverse lymphocyte receptors out of a limited number of inherited genes. The variable antigen-recognizing domains of the lymphocyte receptors are not encoded by just one single gene. Instead, separate shorter gene segments - with each segment present in multiple copies in the germline genome - are combined in a process of gene rearrangement called somatic recombination to yield a variable-region gene. This occurs in distinct stages during lymphocyte development. The light chain of the immunoglobulin molecule and the α chain of the TCR are made up of two different types of gene segments , V (variable) and J (joining), whereas the diversity of the heavy chain of the immunoglobulin and the β chain of the TCR is further augmented by a third gene segment type, D (diversity). The number of multiple copies of each gene segment type in the human genome ranges from 2 up to about 80. Additionally, the immunoglobulin light chain is encoded in two loci (κ and λ). Since the selection of gene segments occurs at random and since heavy- and light chain and α- and β chain, respectively, pair to form together the antigen-binding site, these two sources of combinatorial diversity give theoretically rise to approximately 1.9×10^6 different immunoglobulins and 5.8×10^6 different TCRs. Furthermore, the joining of the gene segments is imprecise and thus, junctional diversity further increases the repertoire of possible different immunoglobulins and TCRs to approximately 5×10^{13} and 1×10^{18}, respectively. An additional source of diversification in case of B cells takes place, once a naïve B cell is activated by its specific antigen in the periphery along with signals from activated T cells: in activated B cells, point mutations are introduced at a very high rate in the V regions of rearranged heavy- and light chains. If by chance, such a somatic hypermutation confers improved antigen affinity on a particular clone (a process called affinity maturation), it is subsequently selected for proliferation and differentiation into plasma cells. It is this ingenious interplay of several strategies which creates that enormous diversity of lymphocyte antigen receptors, which theoretically allows the immune system to recognize nearly all possible antigenic structures.

Establishment of primary lymphocyte pools: positive and negative selection

However, these sophisticated mechanisms bear their own risks: the creation of antigen receptors that are directed against self-components. To solve this problem, clonal selection of lymphocytes has to select potentially foreign-reactive lymphocytes while autoreactive lymphocytes have to be deleted or inactivated in order to prevent autoimmunity (Burnet, 1957; Klinman, 1996; Rajewsky, 1996). In order to ensure self-tolerance of the antigen receptor repertoire, the developing lymphocytes must undergo major control steps, called positive and negative selection. As soon as a developing lymphocyte expresses an antigen receptor on the cell surface, the receptor is tested for its antigen-recognition properties, which comprises specificity and affinity. As test ligands serve molecules present in the immediate environment that is the bone marrow and fetal liver for B-cells and the thymus for T cells. In general, a developing lymphocyte whose receptor interacts weakly with self antigen is selected to survive (positive selection), whereas another whose receptor binds strongly to self antigen receives death signals and is removed from the repertoire (negative selection). For example, the adult mouse bone marrow produces about 15 million immature B cells per day, but only about 10% exit the marrow, and fewer than half of these new émigrés survive to join the mature peripheral B cell pool (Allman et al., 1993; Rolink et al., 1998).

The fate of an immature B cell that successfully rearranged its immunoglobulin genes to express IgM on the cell surface is guided by the signals it receives through its B cell receptor. Strong autoantigens that crosslink the IgM receptor lead to the elimination (negative selection) of theses autoreactive B cells mediated either through cell death (Nossal and Pike, 1975; Nemazee and Buerki, 1989) by apoptosis (Chen et al., 1995; Norvell et al., 1995) or through the replacement of the receptor by receptor editing. In the latter process, the B cell continues light chain gene rearrangement opening the possibility to be rescued by creating a self-tolerant receptor (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993). Thus, receptor editing is an important salvage mechanism for immature B cells that failed positive selection (Diamant et al., 2005). If the developing B cell encounters an only weakly cross-linking autoantigen of low valence, such as a small soluble protein, it becomes permanently unresponsive. This so-called

anergy is accompanied by downregulation of surface IgM and defective signal transduction (Goodnow et al., 1988), and the anergic cell dies relatively quickly due to the failure of survival signals from T cells. Another potential fate of a self-reactive B cell is ignorance. The binding affinity to its autoantigen is so low that only little, if any, intracellular signals are generated. Last but not least, some autoreactive B cells may not encounter their antigen during development as it is not present in the compartments of lymphopoisis. Positive selection of B lymphocytes was controversially discussed in the past, but there is accumulating evidence that positive selection events play a quite important role in mediating differentiation, lineage commitment, and longevity of B cells (reviewed in Cancro and Kearney, 2004). Apart from BCR signal strength, both the ultimate positive and negative selective fate are further determined by the composite influence of clonotypic composition, the developing cohort, cellular microenvironment (Cyster et al., 1994; Cyster, 1997), and selecting ligands. Although self-epitopes serve as selecting ligands for the establishment of the primary B cell repertoire, they are rather useless to induce survival signals for memory B cells, since those have possibly lost their sufficient self-affinity upon affinity maturation. Instead, it has been found that besides noncognate T cell help constitutive expression of Toll-like receptors (which are to be discussed later) can maintain B cell memory (Bernasconi et al., 2002).

For T lymphocytes, the generation of the immature T cell pool is even more complicated, since T cells recognize antigen as peptides bound to self-MHC (major histocompatibility complex) molecules. That means that on the one hand, negative selection must prevent auto-reactivity, but on the other hand, positive selection must ensure that mature T cells are able to interact functionally with the self-structure of MHC molecules (Zinkernagel et al., 1978). T lymphocytes, whose TCRs bind strongly to ubiquitous self antigens presented by dendritic cells or macrophages, are eliminated in the thymus by apoptosis. Only T cells binding moderately to self-antigens on the thymic cortical epithelial cells are positively selected to survive. However, there are many tissue-specific self-antigens that would not be expected to be expressed in the thymus. Interestingly, some stromal cells present in the thymic medulla have been found to express such otherwise extrathymic proteins in a process controlled by a gene

called the autoimmune regulator (AIRE) (Anderson et al., 2002; Anderson et al., 2005). Nevertheless, self-antigens restricted to the periphery are the main source of potential auto-reactivity for both, B and T lymphocytes. Therefore, the immune system has evolved a complex system of checks and balances. Without help from a T cell, a mature B cell recognizing a self-antigen in the periphery becomes anergic and dies. Without co-stimulatory signals provided by a professional antigen presenting cell, a mature T cells encountering autoantigen on peripheral tissues is rendered unfunctional.

B cell effector function

The effector function of B cells makes up the humoral immune response. It is mediated by antibodies secreted by plasma cells after they have encountered their specific antigen in extracellular spaces. These antibodies protect the host from infection by binding to structures on the surface of pathogens. Thus, neutralization by antibodies prevents attachment to and entrance of the invaders into the host cells but can also clear destructive agents such as bacterial toxins. A second antibody effect called opsonization is achieved by coating the pathogen with antibodies thereby promoting phagocytosis and subsequent elimination of the pathogen. As a third possibility, antibodies can mediate the activation of the complement system, which strongly enhances opsonization but can also directly kill certain bacterial cells by forming pores in their membranes. The type of effector mechanism engaged in a particular response depends in part on the isotype or on the class of antibodies produced by the effector B cell.

In order to carry out its effector function, the B cell has to transmit the antigen recognition signal into the cells' interior. The BCR itself is not able to generate this signal, but needs the help of an associated heterodimer composed of antigen-nonspecific invariant signaling molecules: Igα and Igβ. Each of them contains a conserved cytosolic amino acid sequence called immunoreceptor tyrosine-based activation motif (ITAM). Upon antigen binding, the tyrosine residues within these ITAMs become phosphorylated by receptor-associated Src-family tyrosine kinases. The ITAMs are then able to recruit a second family of protein tyrosine kinases, called

Syk, thus initiating further signaling pathways including the MAP kinases and NF-κB pathways. BCR signaling is further enhanced up to 10,000-fold by aggregation with the so-called B-cell co-receptor complex, which is expressed on mature B cells and comprises the cell-surface molecules CD21, CD19 and CD81 (also called TAPA-1). Activation of CD21 by binding of the complement fragment C3dg promotes co-ligation of the complex with the BCR. This induces phosphorylation of tyrosine residues in the cytoplasmic domain of CD19 by BCR-associated tyrosine kinases thereby recruiting Src-family kinases. CD19 signaling also plays an important role in regulation of positive selection and maturation of B cells, and lack of CD19 imposes developmental arrest of immature B cells and consequential stimulation of receptor editing (Diamant et al., 2005). Finally, these antigen signaling pathways lead to the activation of several transcription factors that trigger proliferation and lymphocyte maturation resulting in clonally expanded plasma cells.

Passive immunization impressively demonstrates the action of humoral immune responses. Already in the 1890s, von Behring discovered the specific antitoxic activity of serum derived from animals immune to diphtheria or tetanus and could thereby treat patients infected shortly before. The injection of antibody or immune serum into a naïve recipient confers short-lived immunity.

B cell activation by armed helper T cells

Besides performing antibody responses, B cells function as professional antigen presenting cells. The BCR delivers the bound antigen to intracellular compartments for degradation and presentation on the B-cell surface as peptides bound to MHC molecules. This in turn is a prerequisite for the maturation of the B cell itself. A general mechanism of adaptive immunity to avoid autoimmune responses implies that naïve antigen-specific lymphocytes are difficult to activate by antigen alone. Instead, naïve B cells require accessory signals that can be provided by an armed helper T cell or, in rare cases, directly from microbial constituents ensuring a rapid response to many important pathogens. However, only the interaction of antigen-stimulated B cells

with helper T cells can induce somatic hypermutation and isotype switching leading to more variable and more functionally versatile antibodies.

T cell antigen recognition in the context of MHC molecules

In contrast to B cells, that defense extracellular spaces by secreting antibodies capable of binding to pathogenic structures, T cells evolved the ability to sense and destroy cells infected with intracellular pathogens. These cells display protein fragments (peptides) derived from invaded virus or bacteria on their cell surface (Townsend et al., 1985). These peptides are delivered to the cell surface by specialized host-cell glycoproteins, the major histocompatibility complex (MHC) molecules. The genes encoding the MHC proteins are located within the MHC region on chromosome 6 for human and chromosome 17 for mice, and are called human leukocyte antigen (HLA) genes or histocompatibility antigen 2 (H-2), respectively. The term MHC was chosen as their most prominent protein product, the MHC-molecules (first named transplantation antigen by Peter Gorer in the 1930s), are predominantly responsible of histocompatibility, that means the acceptance or rejection of tissue- and organ transplants. They present self and foreign peptides derived from endocytosed extracellular or from cytosolic proteins on the cell surface for recognition by T lymphocytes.

Two classes of MHC molecules can be distinguished according to their function and structure as well as their expression pattern on different cell types. MHC class I molecules are expressed on nearly all nucleated cells, although the level of constitutive expression varies with the cell type. They consist of a MHC-encoded, membrane-bound α- or heavy chain (43 kDa), which comprised three extracellular immunoglobulin-like domains (α_{1-3}), a transmembrane region, and a short cytoplasmic stretch. The extracellular part of the heavy chain is non-covalently associated with the non-MHC encoded β-chain, called β_2-microglobulin (12 kDa).

MHC class II molecules are normally expressed only by specialized antigen-presenting cells (APCs) such as dendritic cells, macrophages, B lymphocytes, and thymic stromal cells. They are formed by two transmembrane heavy chains, α (34 kDa) and β

(29 kDa). Each chain consists of two extracellular immunoglobulin-like domains ($\alpha_{1\text{-}2}$, $\beta_{1\text{-}2}$), a transmembrane region, and a short cytoplasmic part. The extracellular parts of the α- and β-chain are non-covalently linked to each other.

The structural basis for the presentation of peptides by MHC-molecules is the formation of the peptide binding groove. For MHC-class I molecules, the two N-terminal α_1 and α_2 domains of the heavy chain fold into a basal β-sheet and two α-helices thereby forming a binding cleft that is relatively closed at both ends. This implies that the bound peptides are restricted in their length to 8 to 10 amino acid residues. In a similar way, the α_1- and β_1-domains of MHC-class II molecules together create a binding groove, which, however, is more open at both ends, thus allowing the binding of peptides of variable lengths (between 9 and 25 amino acids).

The diverse T cell receptor does not recognize the presented peptide alone, but makes intense contacts with both, the MHC molecules and the bound antigenic peptide (Garboczi et al., 1996). MHC restriction, (Zinkernagel and Doherty, 1974) therefore affects the antigen specificity of T cells and is based on the great variation in MHC molecules enabling them to present a broad range of peptides. The MHC locus is characterized by polymorphism and polygenicity, meaning that it contains several different MHC class I and MHC class II genes and that, within the population as a whole, there are multiple alleles of each gene.

Although the MHC portion in the MHC:peptide:TCR complex influences the binding properties significantly, many MHC residues at the TCR interface are conserved, and the highly polymorphic regions are favorably found in residues within the peptide binding cleft. The specific binding groove of the MHC molecule is capable of binding a variety of very different peptides with significant affinity while other peptides cannot be bound at all. Besides the length of the peptide, two or three so-called anchor amino acid residues are responsible for the binding characteristic between the peptide and the MHC molecule. Thus, most peptides that are able to bind to and to stabilize a given MHC allele display the same or chemically related amino acids at these anchor positions. Hence, it is possible to describe a peptide binding motif for every MHC variant (Rotzschke et al., 1990; Falk et al., 1991; Stevanovic and Jung, 1993). For MHC class I molecules, additional interaction occurs between the free N- and C-termini of the peptide with invariant regions of the MHC groove (Bouvier and Wiley,

1994). The crystal structure of MHC molecules gives impressive insights into these binding features (Bjorkman et al., 1987; Saper et al., 1991).

Altogether, the peptide sequence restriction to only two or three anchor positions ensures the presentation of a large number of different peptide fragments, generated in a cell, by a given MHC molecule. And, as a result of polygenicity and polymorphism of both classes of MHC molecules and the codominant expression of several MHC genes, each cell of an individual expresses several different MHC variants, each presenting a different peptide repertoire. Thus, each cell is able to display its entire protein content in a very detailed and exhaustive manner to ensure best surveillance by the immune system. To give an idea, a normal cell presents around 10^4 different peptides on its 10^5 to 10^6 MHC class I molecules. Last but not least, the great variation among MHC molecules within the population contributes to the ability of the immune system to respond to the multitude of different and rapidly evolving pathogens and reflects the selective pressure imposed by these nearly unlimited possible infectious agents.

T cell effector function

T cells are grouped into two major classes that differ in their effector functions. The two classes can be distinguished by the class of MHC molecules they recognize and by the expression of the co-receptors CD4 and CD8. Activation of naïve T cells not only requires the formation of the trimeric MHC:antigen:TCR complex but also the interaction of the co-receptor with invariant sites on the MHC molecule. $CD8^+$ T cells recognize antigen in the context of MHC class I molecules which are expressed by nearly all cell types. They untiringly recirculate through the body on the search of foreign or unexpected peptides presented by MHC class I molecules as a sign of infection or illness. Their effector function is the elimination of these dangerous infected or mutated cells, and they are therefore named cytotoxic T lymphocytes (CTL) or killer cells. They destroy their target cells by releasing cytotoxins such as perforin, granzymes or granulysin or through the action of their Fas-ligand (CD178). Perforin polymerizes in the target-cell membrane to form transmembrane pores,

whereas granzymes, granulysin, and binding of the Fas-ligand to Fas (CD95) on the target cell (Suda et al., 1993; Fisher et al., 1995) induce death by apoptosis with granulysin having additionally antimicrobial functions (Shiver et al., 1992; Lieberman, 2003).

$CD4^+$ T cells recognize peptides presented by MHC class II molecules, which are generally solely expressed by professional antigen presenting cells, but whose expression can be induced in normal tissue cells upon IFN-γ stimulation. $CD4^+$ T cells are conferred to as helper T cells and comprise two functional subtypes: T_H1 and T_H2. T_H1 cells secrete as main cytokine IFN-γ, which activates macrophages that are infected by or have ingested pathogens. In addition, they produce lymphotoxin (also called TL-α or TNF-β), which activates macrophages, concomitantly inhibits B cells and is directly cytotoxic for some cells. Thus, T_H1 cells function mainly as inflammatory immune cells. T_H2 cells, however, act predominantly as regulators in humoral immune responses as they secrete the B-cell growth factors IL-4, IL-5, IL-9, and IL-13, and thereby provide the help required for activation of naïve B cells and their differentiation into plasma cells and memory B cells. On the other hand, they can inhibit macrophage activation by secreting IL-10. Furthermore, $CD4^+$ T cells mediate activation of B cells, macrophages and dendritic cells by ligation of CD40 ligand (CD40L) on the T cell with CD40 on the APC resulting in increased antigen-presentation and co-stimulatory capacity (Schoenberger et al., 1998; Seder and Ahmed, 2003).

Although binding of the MHC:antigen complex by the TCR and the co-receptor is sufficient to trigger armed effector T cells into action, this specific signal is not able on its own to prime naïve T cells. Instead, clonal expansion of naïve T cells and their differentiation into armed effector T cells requires the antigen specific signal plus a second signal, the co-stimulatory signal, simultaneously delivered by the same antigen-presenting cell. The requirement for one APC to deliver these two independent signals is crucial for preventing immune responses to self antigens. Antigen recognition by a naïve T cell in the absence of co-stimulation leads to anergy and thus induces tolerance in the peripheral T cell population. Co-stimulatory molecules are

exclusively expressed by APCs upon activation and subsequent maturation. The best-characterized co-stimulatory molecules are the glycoproteins CD80 (B7.1) and CD86 (B7.2), whose interaction with receptor CD28 on the T cell surface induces the activated T cell to synthesize the T cell growth factor IL-2 and its high affinity receptor. IL-2 then induces in an autocrine and paracrine way T cell proliferation and clonal expansion and also promotes differentiation into armed effector T cells.

As mentioned before, APCs themselves require activation and maturation in order to upregulate co-stimulatory molecules. Thereby, T cell activation and APC activation go hand in hand in a 'T-cell:APC dialogue', which ensure a rapid immune response. Upon activation, naïve T cell express CD40 ligand (CD154), whose binding to CD40 on APCs transmits activating signals to both cell types. Another receptor-ligand pair of co-stimulatory molecules with such a bidirectional stimulatory effect is the 4-1BB (CD137) on T cells and 4-1BBL on APCs (DeBenedette et al., 1995). To avoid an endless circle of activation, the interaction between CD28 and CD80/CD86 must be disturbed thereby limiting IL-2 production. To this end, activated T cells are programmed to express CTLA-4 (CD152) that binds CD80 or CD86 about 20 times more avidly than does CD28 and delivers an inhibitory signal to the T cell. Apart from this T-cell:APC dialogue, upregulation of co-stimulatory molecules on APCs can also be initiated independent on T cell interaction via innate immune recognition mechanisms, as will be discussed later.

The three paths of antigen presentation

Classical MHC class I processing pathway

The peptides presented by MHC molecules derive from different exogenic and endogenic protein sources and, therefore, their generation involves distinct antigen processing pathways (Morrison et al., 1986). Most of the peptides presented on MHC class I molecules are processed in the cytosol (Stoltze et al., 2000a). They derive from self protein or from viruses or certain bacteria that replicate in the cytosol or in the contiguous nuclear compartment. Not only aged and unemployed but otherwise flawless proteins removed by the normal protein turnover constitute the peptide

source, but rather the major contribution to the peptide pool comes from defective ribosomal products (DRiPs) (Yewdell et al., 1996; Reits et al., 2000; Schubert et al., 2000). These are faulty or misfolded polypeptide chains that result from a premature termination during the error prone translation process of RNA into proteins by ribosomes.
The immune system benefits from this apparently rather wasteful production process, since the immediate processing of the DRiPs and subsequent peptide presentation give the T cells a 'head start' (Schild and Rammensee, 2000b) in recognizing infected cells.

The MHC class I processing pathway begins with the degradation of cytosolic proteins by the proteasome, a large multicatalytic protease complex. Stimulation with IFN-γ replaces the constitutive proteasome, found in all cells, by the immunoproteasome (Tanaka and Kasahara, 1998) and induces the production of the PA28 proteasome-activator molecules (Dick et al., 1996; Groettrup et al., 1996). Together, the formation of the immunoproteasome due to replacement of three catalytic subunits by their inducible counterparts and the induction of the PA28 activator changes the cleavage specificity of the proteasome. As a result, the immunoproteasome preferably produces peptide fragments of 8 to 11 amino acid residues in length (Wenzel et al., 1994; Ehring et al., 1996; Kisselev et al., 1998) and with C-terminal residues that are preferred anchor residues for binding to most MHC class I molecules.
Downstream of the proteasome, the generated peptide fragments are shortened at their N-terminus by cytosolic (Stoltze et al., 1998; Beninga et al., 1998; Geier et al., 1999; Stoltze et al., 2000b) proteases. Only a small number of these peptides escapes further degradation to the amino acid level and is translocated into the ER lumen by the 'transporter associated with antigen processing' (TAP). Here, the imported peptides can be further trimmed by the INF-γ inducible aminopeptidase ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing (Saric et al., 2002; Serwold et al., 2002; York et al., 2002) to generate the correct epitope. In the ER, the newly synthesized MHC class I molecule is associated with the TAP via tapasin, and chaperones such as Calnexin, Erp57 and calreticulin stabilize the MHC molecule, until loading with a peptide completes the folding of the MHC molecule. The successfully

assembled peptide:MHC complex is then released from the TAP and finally transported through the Golgi apparatus to the cell surface.

Classical MHC class II processing pathway

The peptides presented by MHC class II molecules are processed and loaded in acidified endocytotic vesicles. Besides self proteins that reside in or pass through endosomal compartments, the peptide antigen source are extracellular foreign antigens, such as bacteria or bacterial products that have been taken up by unspecific or receptor-mediated endocytosis, as well as invaded parasites or bacteria that replicate in intracellular vesicles. In the endosomes, the progressively decreasing pH activates acid proteases such as Cathepsin B, C, S und L, which degrade the engulfed proteins (Nakagawa et al., 1998; Nakagawa et al., 1999). A complex biosynthetic process has evolved for the synthesis of the MHC class II molecules in the ER, their loading in uncomfortably acidic environment and their subsequent transport to the cell surface. The newly synthesized MHC class II α- and β-chains associate with the invariant chain (Ii) protein to form first a trimeric complex and finally a nonameric $(\alpha/\beta\text{-Ii})_3$ complex (Cresswell, 1996; Pieters, 1997). Thereby, a part of Ii occupies the MHC binding cleft which prevents premature binding of peptides to the MHC molecule.

Additionally, Ii targets the MHC complex for transport to specialized late endosomal compartments (MIIC, MHC class II compartments), the place where MHC class II peptide loading occurs. During this transport, Ii is selectively truncated by acid proteases, leaving only a short peptide, CLIP (class II-associated invariant chain peptide) in the MHC binding groove. Subsequently, an MHC class II-like molecule, HLA-DM, catalyses the release of the CLIP fragment and allows the binding of peptide to the binding groove (Fling et al., 1994; Morris et al., 1994; Kropshofer et al., 1997). In a process of 'peptide editing' it stabilizes the empty MHC molecule (Denzin and Cresswell, 1995) and keeps removing unstably bound peptides thus selecting for high affinity peptides. In thymic epithelial cells and B cells, a second atypical MHC class II molecule, HLA-DO, act as potential negative regulator of the HLA-DM dependent peptide loading onto different allo-specific class II molecules. It raises the selection pressure in antigen loading and, thus influences the peptide reservoir of the

MHC class II molecules. Once successfully loaded with antigenic peptide, the MHC molecule is released from the associated chaperons, continues its travel to the cell surface and now waits for the checking by passing lymphocytes.

Alternative pathway: Cross-presentation

The previously described differentiation between the classical MHC class I and II antigen processing pathways is based on the distinction between endogenous and exogenous sources of proteins to be processed. Exogenous proteins are uptaken by APC cells and processed in the MHC class II pathway for recognition by $CD4^+$ T helper cells. Endogenous cytosolic proteins, however, are presented on MHC class I molecules for recognition by $CD8^+$ T cells, which is the basis for elimination of virus-infected cells via $CD8^+$ killer cells. Thus, a viral infection leads to the presentation of endogenous viral proteins by MHC class I molecules, which targets the virus-infected cells for elimination by specific $CD8^+$ killer cells. However, the priming of naïve $CD8^+$ T cells implies that the same antigen presenting cell provides both activation signals, the antigenic signal in form of the antigenic peptide presented on MHC class I molecules and the co-stimulatory signal. That means that APC, the only cells to provide co-stimulatory signals, must be susceptible to all kinds of virus infections. But some viruses do not infect professional APC, such as the human papillomavirus, which infects exclusively peripheral tissues, or some viruses, such as the herpes simplex virus, downregulate co-stimulatory molecules thereby subverting the ability of DCs to prime T cell in the classical way (Mueller et al., 2002; Bosnjak et al., 2005).

Nevertheless, these pathogens elicit potent adaptive immune responses characterized by virus-specific $CD8^+$ T cells. This paradox was solved, when it was found that MHC class I-restricted CTL responses can be induced against peptides derived from exogenous or extracellular protein sources (Bevan, 1976b; Bevan, 1976a). This phenomenon of transferring exogenous antigen into the MHC class I antigen processing machinery has been termed *cross-presentation*. And the physiological outcome may either be priming of $CD8^+$ T cells referred to as *cross-priming* or induction of tolerance (*cross-tolerance*).

These mechanisms are of great physiological relevance, not only for viral antigens, but also for self antigens or tumor antigens that cannot efficiently access the classical

MHC class I processing pathway of DCs. For naturally occurring CTL responses against tumor cells, cross-presentation is elementary, since most tumor cells neither express MHC class II molecules nor co-stimulatory molecules, and – of course - do not infect DCs. Nevertheless, necrotic tumor material can be uptaken by surrounding stroma cells, which are able to present the tumor derived proteins on MHC class I molecules. However, cross-presentation of tumor-derived antigens requires sufficient APC activation (van Mierlo et al., 2002).

Although cross-presentation has also been observed in some macrophages (Kovacsovics-Bankowski et al., 1993; Rock et al., 1993), B cells (Ke and Kapp, 1996; Heit et al., 2004) and even in liver endothelial cells (Limmer et al., 2000) *in vitro*, the major cell type known for its capacity *in vivo* to cross-present antigens are DCs. Thus, transient DC depletion of murine bone-marrow derived $CD11c^+$ DCs abrogated priming of CTLs to exogenous cell-associated antigens, which displays the crucial role of DC in CTL response induction (Jung et al., 2002). In another setting, a subset of murine spleenic DCs, the $CD8a^+$ population, was shown to shuttle endogenous antigens constitutively and most efficiently in the cross-presentation pathway (den Haan et al., 2000; Pooley et al., 2001). In contrast, $CD8a^-$ DCs gain these abilities only after activation e.g. by ligation of their Fcγ-Receptor (den Haan and Bevan, 2002). Because of their activation-independent cross-presentation ability, $CD8^+$ DCs are thought to ensure tolerance to peripheral antigen through the mechanisms of cross-tolerization, when no DC activating stimuli are present.

Protein antigens are most efficiently uptaken and cross-presented if they are delivered in apoptotic bodies (Albert et al., 1998), immune complexes (Rodriguez et al., 1999; Regnault et al., 1999) or exosomes, which are small membrane vesicles secreted by living tumor cells and many other cell types (Zitvogel et al., 1998; Wolfers et al., 2001; Stoorvogel et al., 2002), or shuttled by heat-shock proteins (Srivastava et al., 1994; reviewed in Srivastava, 2002), which play a special role in chaperoning non-cell-associated free antigens to APCs (Zheng et al., 2001). The cross-priming efficiency of antigens in these forms is achieved through specific receptor-mediated

uptake of these structures by immature DCs (Albert et al., 1998; Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000b).

The precise mechanisms for cross-presentation, however, are not yet fully understood. In the peptide exchange model, internalized exogenous proteins are degraded in the endocytic pathway and the thus newly generated peptides replace previous loaded peptides on MHC class I molecules (Chefalo and Harding, 2001; Pfeifer et al., 1993) that could have been endocytosed from the cell surface (reviewed in Ackerman and Cresswell, 2004). Although partial proteolysis may occur in the endocytic pathway, exogenous proteins must be further processed in the cytosol for effective cross-presentation (Rock, 1996). This model involves classical MHC class I processing mechanisms, such as proteasomal proteolysis, TAP-mediated translocation to the ER (Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1997) and C-terminal trimming of the MHC epitope in the ER (Castellino et al., 2000). Unraveling the exact molecular mechanisms of cross-presentation will ultimately define the still controversially discussed (Freigang et al., 2003) physiological relevance of cross-presentation and emphasize its critical role in the initiation of immune responses.

Innate immunity: Pattern recognition

During a possible infection, the immune system has to take the decision to respond or not to respond. However, reliable detection of pathogens is a difficult task due to their molecular heterogeneity and rapid evolution. One strategy of sensing infection is based on innate immune recognition of a limited number of microbial ‘molecular signatures’, the pathogen associated molecular patterns (PAMPs) (Janeway, Jr. and Medzhitov, 2002). This implies that PAMPs are produced only by pathogens and not by host cells allowing the distinction between ‘self’ and ‘microbial nonself’. Furthermore, the recognized motifs are essential for the pathogen’s survival and cannot be mutated for escape strategies by the pathogen without life-threatening loss of function and in most cases, they are expressed constitutively. Indeed, many known PAMPs are typical nucleic acids or conserved components of cell wall structure from microorganisms. Moreover, the highly conserved PAMPs are invariant between microorganisms of a given class. This allows the detection of PAMPs by a limited number of pattern

recognition receptors (PRRs), which include membrane-bound receptors in the cell surface or in intracellular compartments, or soluble proteins secreted into the blood stream and tissue fluids. In contrast to the variable lymphocyte antigen receptors of the adaptive immune system, the PRRs of the innate immune system are defined in the genome, and each cell of a given subtype is able to produce the same limited set of receptors. The ancient innate immune system developed a self-foreign discrimination by defined PRRs as perfect as possible, whereas the self-nonself recognition by the variable lymphocyte antigen receptors of the adaptive immune system is error-prone due to the aim to generate a diversity as high as possible by mechanisms such as random gene rearrangement and despite all the control mechanisms to avoid self-reactivity.

Toll-like receptors

Toll-like receptors (TLRs) form a group of pattern recognition receptors with a great variety of different ligand specificities (Table 1). Their discovery in 1997 by cloning and characterization of TLR4 (Medzhitov et al., 1997) gave new impulses in the field of innate immunity and in the understanding how innate and adaptive immunity are tightly interwoven. TLRs are type I transmembrane glycoproteins characterized by an extracellular leucine-rich repeat domain and a conserved intracellular domain, which is homologous to the cytosolic domain of the IL-1 receptor and therefore named Toll/IL-1 receptor (TIR) domain. The TIR domain is found in numerous transmembrane and cytosolic proteins in animals and plants, and most of them play a role in host defense.

***Drosophila* Toll: selectivity in insect immune defense**

The *Toll* gene of the fruit fly *Drosophila melanogaster* was the first member of the Toll family identified. It was discovered due to its essential function for the dorso-ventral axis formation during embryogenesis (Anderson et al., 1985). However, the transmembrane receptor Toll also plays an important role in a very effective innate immune system in insects that is based on pattern recognition (in (Hoffmann and Reichhart, 2002). In contrast to vertebrates, insects do not have any kind of adaptive

immune system and immunological memory. Nevertheless, they are able to fight immediately and very effective against invading microbes by producing specific anti-microbial peptides. This immune defense is even selective: dependent on the type of infection, anti-bacterial or anti-fungal peptides are expressed. Thereby, the selective activation of the Toll pathway plays a key role. *Drosophila* Toll mutants do not survive fungal infections due to the failure to produce the antifungal peptide Drosomycin (Lemaitre et al., 1996). Fungal infection triggers cleavage of the extracellular polypeptide Spaetzle by a serine protease generating the endogenous ligand for Toll. The physiological active Spaetzle then induces ligand-dependent Toll receptor dimerization (Levashina et al., 1999). But the upstream events leading to Toll activation are not yet fully understood. An induced mutant, *semmelweis (seml)*, which involves a gene encoding for a peptidoglycan-recognition protein, blocks Toll activation by Gram-positive bacteria but does not affect Toll activation by fungal infection (Michel et al., 2001). This finding suggests that two different pathways are triggered by infection with Gram-positive bacteria and fungi, respectively, leading to spaetzle cleavage.

In contrast, Gram-negative bacteria are not recognized by *Drosophila* Toll (Lemaitre et al., 1995). Instead, responses to these pathogens are abolished in *immune deficiency (imd)* mutants, a recessive mutation in a gene encoding a cytosolic protein containing a death domain. The Imd pathway triggered by Gram-negative bacteria has similarities to the mammalian tumor necrosis factor-α signaling pathway. However, it is unknown which receptor links Gram-negative infection to the Imd pathway.

The involvement of *Drosophila* Toll in insect immune responses to fungal infections had a pioneering effect for the identification of Toll-like receptors in mammals.

Mammalian Toll-like receptors in immune defense

Today, thirteen different mammalian TLRs have been identified, with their genes dispersed throughout the genome. Individual TLRs are differentially expressed among immune cells and various tissues, they respond to different stimuli and differ in the signal transduction pathways they activate.

Receptor	PAMP	PAMP origin	Synthetic analogues [Fully synthetic small molecules]
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria	Triacyl lipopeptides
	Soluble factors	Neisseria meningitidis	
TLR2	Lipoproteins/lipopeptides	Various pathogens	Di- and triacyl lipopeptides
	Peptidoglycan	Gram-positive bacteria	
	Lipoteichoic acid	Gram-positive bacteria	
	Lipoarabinomannan	Mycobacteria	
	Phenol-soluble modulin	Staphylococcus epidermidis	
	Glycoinositolphospholipids	Trypanosoma cruzi	
	Glycolipids	Treponema maltophilum	
	Porins	Neisseria	
	Atypical LPS	Leptospira interrogans	
	Atypical LPS	Porphyromonas gingivalis	
	Zymosan	Fungi	
	Hsp 70*	Host	
	Gp96*	Mouse	
TLR3	Double-stranded RNA	Viruses	Poly I:C
TLR4	LPS	Gram-negative bacteria	LPS/lipid A mimetics, such as monophosphoyl lipid A (MPL) [Synthetic lipd A, E5564]
	Taxol	Plants	
	Fusion protein	Respiratory syncytial virus	
	Envelope protein	Mouse mammary-tumor virus	
	Hsp60*	Chlamydia pneumoniae	
	Hsp70*	Host	
	Gp96*	Mouse	

Receptor	PAMP	PAMP origin	Synthetic analogues [Fully synthetic small molecules]
TLR4 continued	Type III repeat extra domain a of fibronectin*	Host	
	Oligosaccarides of hyaluronic acid*	Host	
	Polysaccaride fragments of hepara sulphate*	Host	
	Fibrinogen*	Host	
TLR5	Flagellin	Bacteria	Discontinuous 13-amino-acid peptide
TLR6	Diacyl lipopeptides	Mycoplasma	Diacyl lipopeptides
	Lipoteichoic acid	Gram-positive bacteria	Oligonucleotides [Imidazole quinolines (imiquimod, resiquimod) Guanosine nucleotides (loxoribine)]
	Zymosan	Fungi	
TLR7	Imidazoquinoline	Synthetic compounds	
	Loxoribine	Synthetic compounds	
	Bropirimine	Synthetic compounds	
	Single-stranded RNA	Viruses	
TLR8	Imidazoquinoline	Synthetic compounds	[Imidazol quinolines (imiquimod)]
	Single-stranded RNA	Viruses	
TLR9	CpG-containing DNA	Bacteria and viruses	CpG oligodeoxy-nucleotides
TLR10	N.D	N.D.	
TLR11	N.D	Uropathogenic bacteria	
	profilin-like molecule	Toxoplasma gondii	
TLR12	N.D.	N.D.	
TLR13	N.D.	N.D.	

Table 1. Toll-like receptors (TLRs) and the corresponding pathogen-associated molecular patterns (PAMPs) from various pathogens. Table content is based on (Akira and Takeda, 2004). *These ligand preparations might be contaminated with lipopolysaccaride and/or other potent microbial components, so more-precise analysis is required to conclude that TLRs recognize these endogenous ligands. N.D.: not determined.

TLR2 (TLR1 and TRL6)

Early reports on TLR ligands suggested that, in a transfection model, TLR2 is involved in LPS (lipopolysaccharide) signaling (Kirschning et al., 1998; Yang et al., 1998). However, cells carrying a null allele for TLR2 display a normal response to LPS (Heine et al., 1999). Almost simultaneously, TLR4 was shown to be required for LPS signaling by using TLR4-deficient and TLR4-knockout mice (Sultzer, 1968; Poltorak et al., 1998a; Qureshi et al., 1999). Subsequently, it could be demonstrated, that highly pure LPS does not signal via TLR2. It is rather the overexpression of TLR2 in transfection models which renders cells sensitive to minor protein-contaminations in LPS preparations thus pretending a contribution of TLR2 to LPS signaling (Hirschfeld et al., 2000). Thereafter, it became a paradigm that LPS-signaling requires the four molecules LBP, CD14, MD-2, and TLR4 (as described below for TLR4). In the mean time, however, TLR2 was found to function as a receptor for atypical LPS produced by *Leptospira interrogans* (Werts et al., 2001), *Porphyromans gingivalis* (Hirschfeld et al., 2001), Rhizobium species (Girard et al., 2003), and *Legionella pneumophila* (Girard et al., 2003; Braedel-Ruoff et al., 2005) which are structurally different from the prototypic enterobacterial LPS.

Additionally, lipoproteins and lipopeptides from different organisms have been identified as TLR2 ligands (Aliprantis et al., 1999; Brightbill et al., 1999; Hirschfeld et al., 1999; Takeuchi et al., 2000). Lipoproteins are proteins carrying lipid covalently linked to N-terminal cysteines and are produced by a variety of organisms, including Gram-negative and Gram-positive bacteria and mycoplasmas. Moreover, TLR2 recognizes cell wall components from different pathogens including peptidoglycan (PGNS) from Gram-positive bacteria (Schwandner et al., 1999; Takeuchi et al., 1999), zymosan of yeast (Underhill et al., 1999), mycobacterial lipoarabinomannan (Means et al., 1999; Underhill et al., 1999), modulin produced by *Staphylococcus epidermidis* (Hajjar et al., 2001), glycosylphosphatidylinositol anchors from *Trypanosoma Cruzi* (Campos et al., 2001), as well as Glycolipid and lipoteichoic acid (LTA) from Treponema (Opitz et al., 2001; Schwandner et al., 1999).

This unusual broad range of ligands recognized by TLR2 is – to some extent – explained by cooperation of TLR2 with other TLRs. It was found that homodimerization of TLR2 does not induce cytokine production in macrophages upon receptor stimulation (Ozinsky et al., 2000), whereas heterodimerization of TLR2 with other TLRs, such as TLR1 or TLR6, conferres functionality as well as some ligand specificity. Thus, bacterial lipoproteins, which are mostly triacylated at the N-terminal cysteines, trigger immune responses via TLR2 upon heterodimerization with TLR1.

In contrast, mycoplasmal macrophage-activating lipopeptide-2 kDa (MALP-2), which is only diacylated, requires in addition to TLR2 the presence of TLR6: in a mouse model, $TLR6^{-/-}$ cells are unresponsive to the diacylated MALP-2, but respond normally to triacylated lipopeptides from other bacteria. However, $TLR2^{-/-}$-cells fail to recognize either type of lipopeptide. Reconstitution experiments in $TLR2^{-/-}$/$TLR6^{-/-}$-cells reveal that co-expression of both, TLR2 and TLR6, is absolutely required for MALP-2 responsiveness. So, it seems that TLR6 is responsible for discriminating between the N-terminal lipoylated structures of MALP-2 and lipopeptides derived from other bacteria (Takeuchi et al., 2001). Subsequently, it was observed for human cells, that TLR6 colocalizes with TLR2 at the plasma membrane and that TLR2-TRL6 signaling does not require endosomal maturation (Nakao et al., 2005). Blockage of TLR6 using specific antibodies inhibits recognition of synthetic MALP2 or peptidoglycan, which indicates that TLR6 recognizes its ligands at the cell surface. But TLR6 is not sufficient for MALP2 signaling: in the absence of functional TLR2, MALP2 failed to induces cell stimulation even in the presence of TLR6 (Nakao et al., 2005).

However, the model proposing that triacylated lipopeptides signal through TRL2-TLR1 heteromers, whereas diacylated lipopeptides induce signaling through TLR2-TRL6 heteromers has been reanalyzed using new synthetic lipopeptide derivates. As result, not only triacylated, but also diacylated lipopeptides like Pam2CSK4 and MALP2-SK4 induced proliferation of B lymphocytes independently of TLR6. These results redefine the role of TLR2 heteromers: both the lipid and the N-terminal peptides of lipoproteins seem to contribute to the specificity of recognition by TLR2 heteromers (Buwitt-Beckmann et al., 2005).

In addition, TLR2 is also involved in viral defense: it recognizes the outer coat protein hemagglutinin (H) of wild-type measles virus. In attenuated strains, however, a single amino acid mutation in the H protein abolishes the ability to activate the innate immune system via TLR2 (Bieback et al., 2002). Furthermore, TLR2 and CD14 control the inflammatory cytokine response to human cytomegalovirus (CMV) virions during the prereplication phase of the virus. However, inflammation induced by CMV may not lead to viral clearance but may rather facilitate its replication and dissemination (Compton et al., 2003). For Herpes simplex virus 1 (HSV-1), TLR2-mediated induction of inflammatory cytokines is not protective, but associated with lethal viral encephalitis upon HSV-1 infection (Kurt-Jones et al., 2004). The *mycobacterium tuberculosis* (MTB) evades host immune surveillance and persists inside macrophages through the action of a 19-kDa lipoprotein that inhibits MHC class II expression and antigen processing in a TLR2-dependent manner (Noss et al., 2001). Thus, TLR activation is a double-edged sword, protective or detrimental, depending on the pathogen and the location of the infection.

TLR3

Double-stranded RNA (dsRNA) is a molecular pattern associated with viral infection, since it is produced by most viruses at some point during their replication cycle (and is absent in non-infected cells). Its immunostimulatory activity is known since long, partly because it is able to activate the dsRNA-dependent protein kinase PKR (Williams, 1999). However, cells derived from PKR-deficient mice are still capable to respond to dsRNA and to polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue, suggesting the existence of another receptor. This receptor is thought to be TLR3, as TLR3-deficient mice showed reduced responsiveness to poly(I:C), as well as to viral dsRNA (Alexopoulou et al., 2001).

TLR3 was also demonstrated to directly contribute to the immune response of respiratory epithelial cells to influenza A virus, a highly contagious single-stranded RNA virus responsible for the “flu” syndrome, and hereby, the viral replicative intermediate dsRNA is a prerequisite (Guillot et al., 2005) and critical for the outcome of the infection (reviewed in Jacobs and Langland, 1996; Majde, 2000).

However, recently, the challenge of TLR3-deficient mice with different viruses (lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), murine cytomegalovirus (MCMV), and retroviruses) hints that TLR3 is not universally required for induction of antiviral responses, because the absence of TLR3 does not alter viral pathogenesis or impair induction of adaptive antiviral responses to these viruses (Edelmann et al., 2004).

A possible explanation for the controversial role of TLR3 in antiviral responses is offered by a recent publication showing that TLR3 plays a role in cross-priming *in vivo* against virus-infected cells. DC activation by dsRNA present in virally infected cells but absent from uninfected cells requires signaling through TLR3. Thus, TLR3 permits cross-priming of CTLs against viruses that do not directly infect antigen-presenting cells (Schulz et al., 2005). TLR3 is not expressed on the cell surface but is intracellularly located, as shown for various cell types (Matsumoto et al., 2003), human dendritic cells; (Guillot et al., 2005), epithelial cells) and therein coincides with TLR7 and TLR9, all of which are involved in the recognition of nucleic-acid-like structures. Thus, the importance of TLR3 in antiviral immunity remains to be further investigated.

Apart from this, some viral proteins have been found to subvert the host TLR3 defense system for the virus' own benefit. For example, the A52R protein of the vaccinia virus (VV), the poxvirus used to vaccinate against smallpox, blocks the activation of the transcription factor NF-kB by TLR3 and other TLRs by associating with two key proteins important for TLR signal transduction (Harte et al., 2003). The hepatitis C virus (HCV) expresses a serine protease, NS3/4A, which is able to disrupt the poly(I:C)-activated TLR3 pathway by cleavage of the adaptor protein TRIF (Li et al., 2005).

TLR4

Lipopolysaccharide (LPS or endotoxin) constitutes the major surface component of the outer membrane of Gram-negative bacteria (Rietschel and Wesphal, 1999). In

mammals, LPS induces a severe and generalized inflammation leading to a life-threatening condition called endotoxin or septic shock. It is defined as a collapse of the circulatory and respiratory systems, which is caused by the overwhelming secretion of cytokines as a result of an uncontrolled systemic bacterial infection. LPS is recognized by TLR4 in a complex manner that requires several accessory molecules.

The first identified molecule involved in LPS signaling was the LPS-binding protein (LBP), a 60 kDa acute-phase protein produced by the liver (Tobias et al., 1986; Tobias et al., 1988; Ramadori et al., 1990; Grube et al., 1994) and secreted into the plasma. Although constitutively expressed, LBP is produced in greater amounts after LPS challenge (reviewed in Beutler and Rietschel, 2003). The LBP in the serum concentrates LPS and recruits it to an additional, concomitantly identified receptor, CD14 (Wright et al., 1989; Schumann et al., 1990). This high-affinity LPS receptor CD14 is either expressed as a glycophosphoinositol (GPI)-linked protein on the surface of cells from the myeloid-lineage (mCD14) (Wright et al., 1990), or exits as soluble protein (sCD14) in the serum. The latter one is either directly secreted by the CD14-expressing cells or derived from protease-dependent shedding of the membrane-bound molecules (Bazil and Strominger, 1991; Bufler et al., 1995). sCD14 competes with mCD14 for LPS binding and is able to neutralize LPS-induced responses *in vitro* and *in vivo* (Schutt et al., 1992; Haziot et al., 1994; Haziot et al., 1995). Additionally, sCD14 mediates the LPS-induced activation of non-CD14-expressing cells (Haziot et al., 1993; Pugin et al., 1993). However, cells derived from CD14-deficient mice respond to LPS as well as to live Gram-negative bacteria, although only at very high concentrations of LPS or bacteria (Haziot et al., 1996). These findings and the fact that mCD14 lacks a cytoplasmic domain for triggering activation signals inside the cell, suggested the existence of other receptors involved in LPS signaling that are able to transmit the information across the cell membrane.

Already in the late 1970's, the mouse strain C3H/HeJ was known to be insensitive to endotoxin and the responsible gene was conferred as *Lps* gene (O'Brien et al., 1980). It took until 1998 to discover that TLR4 is the *Lps* gene product. The unresponsiveness to LPS in this mouse strain is due to a missense point mutation in the *Tlr4* gene,

leading to the exchange of a stringently conserved prolin to a histidine residue within the TIR (Toll/interleukin-1-receptor) domain of the cytoplasmic tail of the receptor, thereby abrogating downstream signaling (Poltorak et al., 1998b; Poltorak et al., 1998a; Qureshi et al., 1999). Studies using *Tlr4* knockout mice (C57BL/10ScCr mice lack TLR4 entirely) confirmed that TLR4 functions as the signal-transduction receptor for LPS (Hoshino et al., 1999). However, transfection of TLR4 alone does not confer LPS responsiveness on a recipient cell line, suggesting the requirement for at least one other factor for LPS signaling (Wright, 1999). The missing molecule was identified as MD-2, a small glycoprotein that lacks a transmembrane region and is either secreted as a soluble, active protein (Visintin et al., 2001a) or expressed on the cell surface in physical association with the extracellular domain of TLR4 (Shimazu et al., 1999; Schromm et al., 2001). Moreover, formation of an active TLR4:MD-2 complex in the Golgi apparatus is essential for TLR4 distribution and TLR4 is not able to reach the plasma membrane in MD-2 deficient cells (Nagai et al., 2002a). Thus, activation of the TLR4 signaling complex requires the coordinated interplay of LBP, CD14, MD-2, and TLR4.

For quite a while, yet another protein was thought to cooperate with TLR4 in LPS recognition, at least in B cells: RP105 is a B-cell surface molecule (CD180) and was the first mammalian toll-like protein described. It was found to be involved in immune responses to LPS, since B cells lacking RP105 show hyporesponsiveness to LPS (Ogata et al., 2000). Structurally similar to the TLR4:MD-2-complex, RP105 associates with a soluble extracellular molecule, MD-1. MD-1 is important for RP105 with respect to B-cell surface expression and LPS recognition and signaling (Miyake et al., 1998; Nagai et al., 2002b).
However, very recently, the role of RP105 was redefined as negative regulator of TLR4 signaling (Divanovic et al., 2005b). Although the extracellular portion of RP105 resembles that of other TLRs, it does not have a cytoplasmic TIR domain required for TLR signaling. RP105 mostly resembles TLR4, not only in the requirement of an accessory molecule, but also in the amino-acid sequence. Moreover, Divanovic et al. demonstrated that RP105 is expressed by the same subset of human peripheral blood mononuclear cells and possesses the same expression pattern as TLR4. However,

RP105 is not able to trigger LPS signaling. Instead, expression of RP105:MD1 in a transfection model specifically inhibited the TLR4:MD2-mediated response to LPS. The inhibition does not involve RP105 signaling but interaction between RP105 and TLR4 mediated through MD1-MD2 interaction. In RP105 deficient mice, stimulation with LPS but not with other TLR ligands resulted in significantly increased immune responses, and these mice suffered a more severe endotoxicity after LPS challenge than did wild-type mice.

TLR4 is not only a pattern-recognition receptor for LPS, but has also been shown to be required for the cell activating signals induced by the heat-shock proteins Hsp60, Hsp70 and Gp96 (Vabulas et al., 2001; Asea et al., 2000; Asea et al., 2002; Vabulas et al., 2002b). Furthermore, TLR4 was demonstrated to be involved in the recognition of lipoteichoic acids (LTA) from Gram-positive bacteria (Takeuchi et al., 1999). Moreover, defensins, small antimicrobial peptides produced by the innate immune system upon microbial infection, have been shown to trigger DC maturation via TLR4 (Biragyn et al., 2002).

Also structural proteins of the outer viral coat are recognized by TLR4: the fusion protein of respiratory syncytial virus (RSV), an important respiratory pathogen of humans, activates cells in a TLR4- and CD14-dependent manner. The virus persists longer in the lungs of infected TLR4-deficient mice compared to wild-type mice (Kurt-Jones et al., 2000). It is not yet clear whether the F protein of RSV represents an example of a viral PAMP, in that some conserved feature of the F protein is shared with fusion proteins of other viruses. Although TLR4 was demonstrated in TLR4-deficient mouse models to act in viral clearance (Haynes et al., 2001), an alternative possibility is that RSV developed the ability to stimulate TLR4 for its own benefit (Monick et al., 2003). Vaccinia virus evolved a mechanism to interfere with TLR signaling suggesting an important role of TLR-mediated antiviral immunity. Two ORFs (open reading frames) of the vaccinia virus genome share amino acid sequence homology with the TIR domain of TLRs. Expression of the corresponding cytoplasmic proteins in infected mammalian cells interferes with IL-1, IL18 and TLR4 signaling but has no effect on MyD88-independent pathways (Bowie et al., 2000). However, as

mentioned for TLR2, the current understanding of the role of TLRs in responses to viruses is still incomplete.

TLR5

Flagellin is the main component of the bacterial flagella and is recognized by the innate immune defense of organisms as diverse as plants, flies and mammals (Hayashi et al., 2001). Recently, mammalian TLR5 was identified as PPR for bacterial flagellin from both Gram-positive and Gram-negative bacteria. Expression of flagellin in non-flagellated bacteria conferred the ability to activate cells via TLR5, whereas deletion of the flagellin genes from flagellated bacteria strains abrogated the TLR5-stimulating activity (Hayashi et al., 2001).

Flagellin is an unusual PAMP in that it is a protein, and proteins are notoriously easy to mutate in response to selection, and it does not undergo any posttranslational modification that would distinguish it from host cellular proteins. However, the C- and the N-terminus as well as the three-dimensional structure of flagellin are extremely conserved (Samatey et al., 2001). Additionally, flagellin as motor of mobility is vital for the bacteria. These aspects render flagellin interesting as a PAMP. Actually, TLR5 recognizes the same motif in flagellin that is also necessary for propulsion, which is required for successful propagation of the bacteria (Reichhart, 2003), thereby precluding its mutation in response to evolutionary pressure by the host immune system. The recognition site comprises a cluster of 13 amino acid residues and is buried in the flagellar filament. Thus, monomeric flagellin, but not the filamentous molecules, activates TLR5 (Smith et al., 2003). Insufficient TLR5 signaling in response to flagellin has been linked to diseases such as Legionnaires' disease. A common stop codon polymorphism in the extracellular domain abolishes TLR5-mediated flagellin signaling and is associated with susceptibility to pneumonia caused by *Legionella pneumophila* (Hawn et al., 2003). *Helicobacter pylori*, which colonizes the human gastric mucosa and is associated with gastritis, peptic ulcers, gastric adenocarcinomas and gastric mucosa-associated lymphoid type (MALT) B-cell lymphomas, seems to evade detection by TLR5 thereby escaping immune clearance. Unlike other Gram-negative microbes, it does not release flagellin upon invading the

host, and recombinant *H. pylori* flagellin is significantly less potent in activating TLR5-mediated immune responses than flagellin of other species (Gewirtz et al., 2004).

TLR7 and TLR8

Genomic structure and sequence similarities suggest that with TLR7 and TLR8 form a subfamily with TLR9. Natural ligands for TLR7 and TLR8 were not known until recently, but synthetic products with potent anti-viral and anti-tumor properties, such as the imidazoquinoline compounds Imiquimod and R-848, stimulate humoral and cellular immune responses via these receptors (Hemmi et al., 2002; Jurk et al., 2002; Lee et al., 2003a; Heil et al., 2003). These compounds are structurally similar to nucleic acids, and subsequently, it was proposed that TLR7 and TLR8 mediate the immunostimulating capacities of stabilized RNA (Scheel et al., 2004). Moreover, it was demonstrated lately that uracil-rich, single stranded RNAs (ssRNAs) found in many viruses are physiological ligands for TLR7 and TLR8. A sequence from the U5 region of HIV-1 RNA, which is rich in guanosine and uracil, stimulates dendritic cells to produce antiviral and proinflammatory cytokines. Cells from TLR7-deficient mice are unresponsive to the viral ssRNA, but mice lacking TLR8 responded normally. In contrast, analysis of human TLR8 suggests that this TLR may sense viral ssRNA, pointing to species differences among the TLRs (Heil et al., 2004): while the human TLR8 is a functional receptor for R-848, mouse TLR8 might be nonfunctional (Hemmi et al., 2002; Jurk et al., 2002). Concomitantly, it was shown, that mice lacking TLR7 fail to activate dendritic cells in response to genomic ssRNA from influenza virus and synthetic polyU RNA. Recognition of these PAMPs by TLR7 depends on endocytosis and endosomal maturation. It is proposed that during infection, phagocytes and dendritic cells take up virus-infected apoptotic cells, and subsequently, viral particles are degraded by endosomal proteases in phagosomes exposing the viral RNAs (Diebold et al., 2004). Accordingly, TLR7 and TLR3, both of which are receptors for viral RNAs, are localized in intracellular acidic compartments and are delivered to phagosomes after particle uptake (Matsumoto et al., 2003; Lee et al., 2003a). The intracellular distribution of TLR7 is mediated by a targeting sequence

within its transmembrane domain, whereas targeting of TLR3 to the same intracellular compartments is achieved by a short 23 amino acid sequence present in the linker region between the transmembrane domain and the TIR domain (Funami et al., 2004; Nishiya et al., 2005). TLR8 as well localizes intracellularly, but with a small fraction on the cell surface (Nishiya and DeFranco, 2004). Nevertheless, mainly TLR8 and also TLR7 detect RNA of the human parechovirus 1, a ssRNA virus, in endosomal compartments (Triantafilou et al., 2005). Although, TLR7 and TLR8 are structurally and functionally closely related, TLR agonist studies revealed that TLR7 and TLR8 agonists differ in their target cell selectivity and cytokine induction profile (Gorden et al., 2005). TLR ligands mimicking PAMPs are intensively studied for their therapeutic use against different diseases as well as vaccine adjuvants. These include the above mentioned synthetic TLR7 and TLR8 ligands Imiquimod and R-848, as well as the TLR9-stimulating CpG oligonucelotides (as described in more detail below for TLR9) (Weeratna et al., 2005). Recently, it was shown that using imiquimod as adjuvant for immunization of mice with a CTL epitope is highly effective in activating T cells (Rechtsteiner et al., 2005). This strategy provides a basis for vaccine development against cancer or virus-associated diseases.

TLR9

Another PAMP of bacteria is hidden in their DNA structure as unmethylated double-stranded cytosine-phosphate-guanine (CpG) motif. In vertebrate DNA, the CpG sequence motifs are highly suppressed and occur only at about one fifth of the randomly expected frequency. And, if present, vertebrate CpG sequences are most likely to be methylated at the cytosine residues, whereas bacterial CpG sequences lack equivalent cytosine methylation.

Unmethylated CpG motifs in bacterial, plasmid or viral DNA (Sato et al., 1996; Roman et al., 1997) and synthetic oligodeoxynucleotides (CpG ODN) (Krieg et al., 1995) are potent stimulants for immune cells including B cells (Krieg et al., 1995), macrophages (Stacey et al., 1996) and dendritic cells (Sparwasser et al., 1998) to support expression of co-stimulatory molecules and production of T_H1-type and pro-inflammatory cytokines (Jakob et al., 1998; Hartmann et al., 1999; Hacker et al.,

1999). In 2000, TLR9 was identified as receptor recognizing CpG motifs by using *Tlr9*-knockout mice (Hemmi et al., 2000). In humans, functional TLR9 expression is predominantly seen in B cells and plasmacytoid DCs (pDCs) (Krug et al., 2001; Hornung et al., 2002). Unlike the TLR4/MD2 complex, TLR9 is not localized at the cell surface but is expressed in the endoplasmic reticulum (ER). In line with this, CpG-DNA- but not LPS-induced cell activation requires endocytosis and endosomal maturation (Ahmad-Nejad et al., 2002). Fluorescently tagged CpG-DNA was observed to traffic to early endosomes and subsequently moves to a tubular lysosomal compartment. Concurrently, TLR9 redistributes from the ER to the CpG-DNA-containing structures in the endosome (which also accumulate MyD88), where TLR9 binds to CpG-DNA and initiates signaling (Leifer et al., 2004; Latz et al., 2004b).

Distinct classes of synthetic CpG ODNs have been described that are capable of stimulating cells that express human TLR9 (Verthelyi et al., 2001). TLR9 seems to recognize CpG DNA directly, since it can distinguish between different immunostimulatory CpG motifs: human TLR9 and mouse TLR9 are optimally stimulated by different CpG motifs, which vary only slightly in the flanking sequences of the CpG dinucleotides (Bauer et al., 2001). These species differences probably reflect sequence differences in TLR9. However, it was shown recently that this sequence specificity is restricted to phosphorothioate (PS)-modified ODN and is not observed when a natural phosphodiester (PO) backbone is used (Roberts et al., 2005). Lately, ligand-binding studies revealed that (PO)-CpG DNA directly binds to TLR9. Interestingly, this binding is CG sequence-independent and stimulatory as well as nonstimulatory CpG DNA sequences are able to bind to TLR9, indicating that each construct might be able to modulate inflammatory responses. Indeed, it was shown, that nonstimulatory CpG DNA potently inhibits TLR9 signaling (Latz et al., 2004b).

Interestingly, CpG-DNA provides a survival signal to DCs by activating two different pathways, the NF-kappaB and PI3K/Akt pathways. This CpG-driven cell survival might be one of the mechanisms by which bacterial DNA stimulates and maintains the innate immune responses (Park et al., 2002; Yi and Krieg, 1998). Additionally, the DNA-dependent protein kinase (DNA-PK), an enzyme activated by DNA and

involved in DNA repair of double strand breaks, is also necessary for cell activation by CpG-DNA (Chu et al., 2000). Quite recently, DNA-PK was found to trigger CpG-DNA responses by activating the nuclear factor Akt for translocation into the nucleus, whereas TLR9 is not involved in this pathway (Dragoi et al., 2005). This raises the question about the relationship between TRL9 and DNA-PK in regard to CpG signaling.

TLR10

Human TLR10 was first described in 2001. It contains 811 amino acid residues and is most closely related to TLR1 and TLR6 in regard to the overall amino acid identity (Chuang and Ulevitch, 2001). Furthermore, the TLR10 gene lies in a locus that also contains TLR1 and TLR6, both of which function as co-receptors for TLR2. But until now, no specific ligand for TLR10 has been identified. However, TLR10 was demonstrated to directly associate with MyD88, the common TLR adapter protein (Hasan et al., 2005). The murine TLR10 gene is nonfunctional and disrupted by a retroviral insertion (Hasan et al., 2005).

TLR11

Murine TLR11 was identified during the search for sequence homologies to the TIR domain of TLR4. Further analysis revealed that TLR11 is particularly abundant in the mouse kidney and bladder. When infected with uropathogenic baceria, mice lacking TLR11 harbored 10,000 times as many bacteria in their kidneys as normal mice (Zhang et al., 2004). Humans only have a truncated form of TLR11, which is probably not functional. This might be why humans are predisposed to urinary tract infections.
For mice, TLR11 was shown, very recently, to play a role in innate responses to parasite infections. A first hint for TLR engagement was the requirement of the TLR adaptor molecule MyD88 for the host resistance to *Toxoplama gondii* (Scanga et al., 2002; Del Rio et al., 2004). Subsequently, the protein profilin and TLR11 were identified as new ligand:TLR pair. The profilin-TLR11 interaction confers resistance to infection with *T. gondii* and moreover, TLR11-deficient mice survived acute

infection with this parasite (Yarovinsky et al., 2005). It is assumable that profilin homologues from other related parasites are also recognized by TLR11 thereby inducing protective responses.

TLR12 and TLR13

Mouse TLR12 and TLR13 were identified through database search based on TIR domain homology, but up to now, no specific ligands have been found. Humans only have a *tlr12* pseudogene in the orthologous position and also lack a TLR13 ortholog (Tabeta et al., 2004).

In summary, a set of different TLRs recognize quite a number of diverse conserved patterns from all kinds of pathogens – ranging from DNA structures to cell membrane or cell wall components, with a growing list of newly identified TLR ligands and receptors interacting with each other. This quite simple strategy enables the innate immune system to effectively fight against invading pathogens. Moreover, TLRs are able to tailor the innate responses to pathogens by engaging different signaling pathways involving specific usage of adaptor and regulative molecules by the different TLRs.

TLR signaling pathways

TLR signaling cascade

The TLRs belong to a superfamily that includes the interleukin-1 receptors, based on the considerable homology in the cytoplasmic region: TLRs and IL-1Rs have a conserved region of about 200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1 (TIR) domain (Slack et al., 2000). Within the TIR domain, three conserved, highly homologous boxes are crucial for signaling. By contrast, the extracellular regions of the IL-1Rs and the TLRs differ noticeable. The extracellular region of IL-1Rs contains three immunoglobulin-like domains, the extracellular region of TLRs contains 19 to 25 tandem copies of leucin-rich repeat (LRR) motifs, each repeat consisting of 24 to 29 amino acids (Bell et al., 2003).

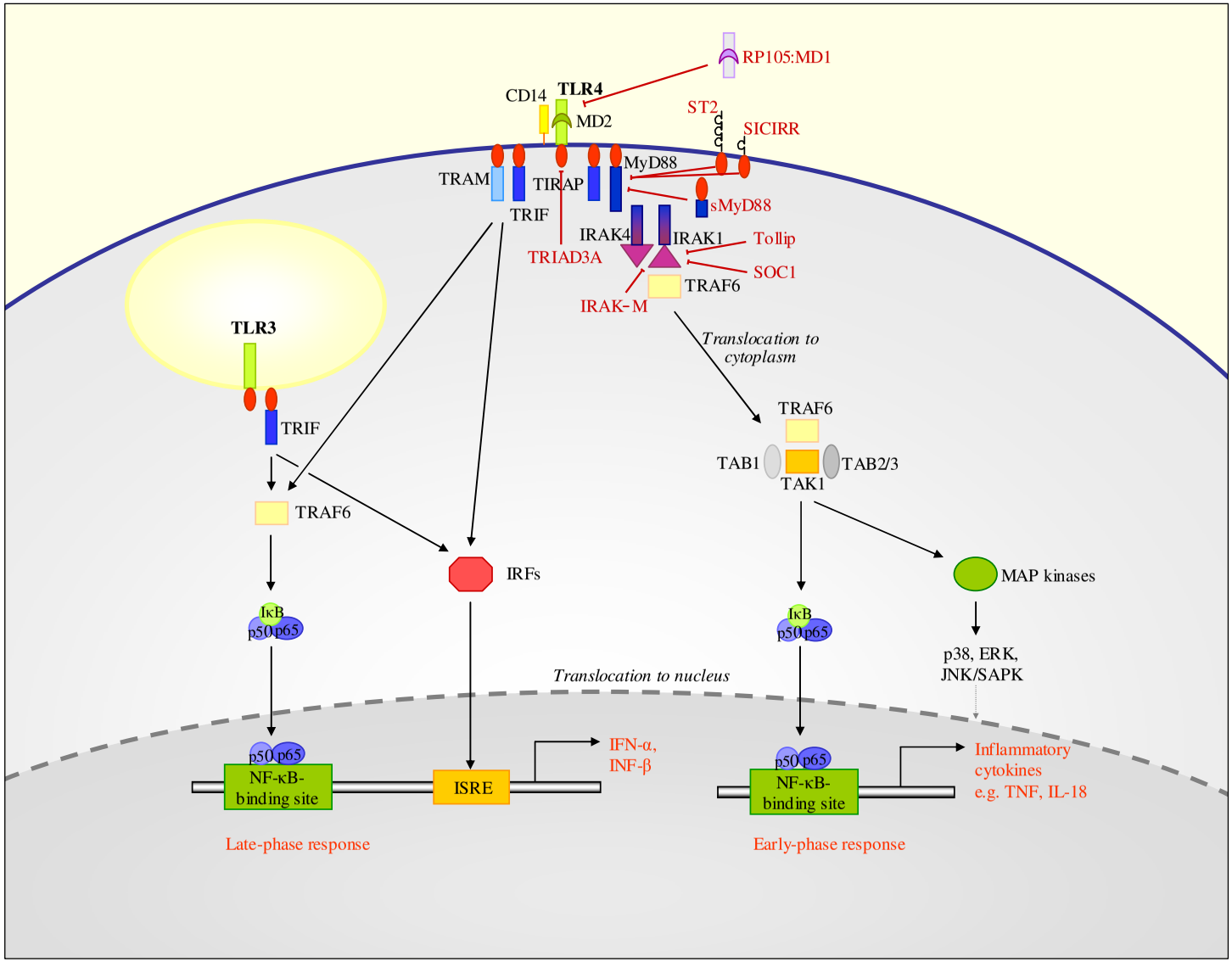

Figure 2. Toll-like receptor signaling. Scheme of Toll-like receptor signaling pathways as discussed in main text. (Based on (O'Neill, 2004) **CD14,** Cluster of differentiation 14; **ERK,** Extracellular-regulated kinase; **IL-18,** Interleucin-18; **INF-α/-β**, Interferon-α/-β; **IRAK-1,** IL-1R-associated kinase 1; **IRAK-M,** IL-1R-associated kinase M; **ISRE,** Interferon-stimulated response elements; **IκB,** Inhibitor of NF-κB; **JNK,** JUN N-terminal kinase; **MAP kinases,** Mitogen-associated protein kinases; **MyD88,** Myeloid differentiation primary-response protein 88; **NF-κB,** Nuclear factor-κB; **p50,** Subunit of NF-κB; p65, Subunit of NF-κB, also known as REL-A); **RP105,** Radioprotecitve 105, also known as CD180; **SAPK,** Stress-activated protein kinases; **SICIRR,** Single immunoglobulin IL-1R-related molecule, also known as TIR8; **sMyD88,** Short MyD88; **SOCS1,** Suppressor of cytokine signalling 1; **TLR,** Toll-like receptor; **TNF,** Tumor necrosis factor; **Tollip,** Toll-interacting protein; **TRAF6,** Tumor-necrosis factor (TNF)-receptor-associated factor 6; **TRAM,** TRIF-related adaptor molecule, also known as TICAM2; **TRIAD3A,** a RING-finger E3 ligase; **TRIF,** TIR domain-containing adapter inducing IFN-β, also known as TICAM1.

Upon ligand binding TLRs/IL-1Rs dimerize, thereby undergoing conformational changes required for the recruitment (Figure 2) of the adaptor molecule **MyD88** (myeloid differentiation primary-response protein 88). MyD88 consists of a C-terminal TIR domain, which interacts with the TIR domain of the receptor, and a N-terminal death domain (DD), which interacts homophilic with the DD of a serin/threonin protein kinase, **IRAK4** (IL-1R-associated kinase 4) (Muzio et al., 1997; Wesche et al., 1997; Burns et al., 1998). Binding to MyD88 enables IRAK4 to phosphorylate the subsequently recruited **IRAK1,** thereby inducing the activation of its kinase activity (Li et al., 2002; Suzuki et al., 2002). Autophosphorylation of IRAK1 then allows **TRAF6** (tumor-necrosis factor (TNF)-receptor-associated factor 6) to bind to this complex (Ye et al., 2002). Subsequently, the IRAK1-TRAF6 complex disengages from the receptor and interacts at the plasma membrane with another preformed complex consisting of **TAK1** (transforming growth factor-β(TGF-β)-activated kinase), **TAB1**, and **TAB2 or TAB3** (TAK1-binding proteins). This interaction induces phosphorylation of TAB2/TAB3 and TAK1, leading to their translocation to the cytoplasm together with TRAF6 and TAB1, whereas IRAK1 is degraded at the plasma membrane. In the cytoplasm, TAK1 is then activated, resulting in the activation of IKKs (IκB kinases), which then phosphorylate the IκBs. This phosphorylation allows ubiquitylation and subsequent degradation of IκB, thereby releasing NF-κB (nuclear factor-κB). NF-κB is consequently free to translocate into the nucleus and induce the expression of its target genes.

Activation of TAK1 also results in the activation of the MAP (mitogen-associated protein)-kinases ERK (extracellular-regulated kinase) and p38 as well as the SAP (stress-associated protein)-kinase JNK/SAPK (JNK for JUN N-terminal kinase), finally leading to the phosphorylation and activation of Jun and Fos, which together form the AP-1 (activator protein 1) transcription factor (reviewed in Akira and Takeda, 2004).

MyD88-dependent and -independent pathways

In MyD88-knockout mice ($MyD88^{-/-}$), cell activation in response to most TLR-mediated stimuli is completely abolished (Kawai et al., 1999). Thus, $MyD88^{-/-}$-mice do not produce TNF or IL-6 when exposed to IL-1 or microbial components which are recognized by TLR2, TRL4, TRL5, TLR7 or TLR9 (Adachi et al., 1998; Takeda et al., 2003). However, LPS stimulation of $MyD88^{-/-}$ macrophages still results in the activation of NF-κB and MAPK, although with delayed kinetics (Kawai et al., 1999). Moreover, co-stimulatory molecules such as CD40, CD80 and CD86 are upregulated, and proliferation of T cells is induced in $MyD88^{-/-}$- but not in $TLR4^{-/-}$-derived BMDCs upon LPS treatment (Kaisho et al., 2001). These observations suggested the existence of a TLR4-dependent but MyD88-independent signaling pathway.

Recently, additional receptor-proximal adaptor proteins, all of which have TIR domains, have been described: TIRAP (TIR-domain containing adaptor protein, also known as MyD88-adaptor-like protein, MAL) (Horng et al., 2001; Fitzgerald et al., 2001), TRIF (TIR-domain-containing adaptor protein inducing IFN-β; also known as TIR-domain-containing molecule 1; TICAM1) (Yamamoto et al., 2002b; Oshiumi et al., 2003a) and TRAM (TRIF-related adaptor molecule, also known as TIR-domain-containing molecule 2; TICAM2) (Bin et al., 2003; Oshiumi et al., 2003b; Yamamoto et al., 2003b; Yamamoto et al., 2003b; Fitzgerald et al., 2003).

TIRAP is involved in signaling of TLR2, TLR4, TLR6 and TLR1 (Horng et al., 2002) and is required for cytokine production upon stimulation via these TLRs but only in the presence of MyD88 (Yamamoto et al., 2002a). These results indicate that TIRAP is essential for MyD88-dependent signaling initiated by TLR2 and TLR4, but does not participate in the MyD88-independent pathway.

TRIF mediates MyD88-independent signaling via TLR3, but is also partially involved in TLR4-signaling (Hoebe et al., 2003; Yamamoto et al., 2003a): TRIF is essential for TLR4-mediated activation of the MyD88-independent pathway leading to the production of type I interferons (IFN-α/β), whereas the TLR4-mediated production of inflammatory cytokines requires both, MyD88-dependent and MyD88-independent/TRIF-dependent signals. In the TRIF-dependent pathway involved in

TLR3 and TLR4-signal transduction, the IRF family of transcription factors plays a role.

Interestingly, TRAM participates specifically in the activation of the MyD88-independent/TRIF-dependent signaling pathway through TLR4 but not TLR3 (Fitzgerald et al., 2003; Oshiumi et al., 2003b), and both, TRAM and TRIF are required for the TLR4-mediated induction of inflammatory cytokines although the precise mechanisms remain to be determined (Yamamoto et al., 2003b). An exiting recent finding demonstrates, that for the MyD88-independent LPS signaling via activation of the TRIF-TRAM pathway CD14 – formerly thought to simply concentrate the LPS signal via TLR4 – is absolutely required (Jiang et al., 2005).

In conclusion, the process of NF-κB activation downstream of TLR4 ligation is rather complex, with the early response involving MyD88 and TIRAP, leading to the production of inflammatory cytokines, and the later response involving TRAM and TRIF, allowing the induction of an additional set of genes, including antiviral genes, such as interferon-α and -β.

Altogether, MyD88 is the most universal adaptor protein, since it triggers signals from all known TLRs and from the IL-1 and IL-18 receptors. Bone-marrow derived dendritic cells from MyD88$^{-/-}$ mice reveal a markedly reduced cytokine secretion in response to most PAMPs (Kawai et al., 1999) which emphasizes the importance of MyD88 in TLR signaling.

Other molecules involved in TLR signaling

Additionally to the described molecules, other intracellular proteins seem to be involved in TLR-signaling pathways. TOLLIP (Toll-interacting protein), for example, is able to bind to the TIR domain of IL-1Rs, TLR2 and TLR4, once the receptors are activated. In the non-activated situation, TOLLIP suppresses the onset of the TLR-signaling cascade: by forming a complex with IRAK1, it blocks the kinase activity of IRAK1 thereby preventing the NF-κB activation. Upon ligand binding to the TLR, the TOLLIP-IRAK1 complex is recruited to the receptor, finally leading to the dissociation of TOLLIP (Burns et al., 2000; Zhang and Ghosh, 2002).

Pellino was first identified in *Drosophila*, were it binds to the IRAK-homologue, Pelle (Grosshans et al., 1999). In mammalians, Pellino forms a complex with IRAK1 after TLR/IL-1R stimulation (Yu et al., 2002). However, Pellino lacks any domain capable of enzymatic activity, and is therefore thought to function as a scaffolding protein facilitating the release of phosphorylated IRAK from the receptor (Akira and Takeda, 2004). Among the other molecules potentially involved in TLR-signaling pathways are phosphatidylinositol 3-kinase (PI3K) (Koyasu, 2003; Li et al., 2003), evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) (Kopp et al., 1999; Xiao et al., 2003), SRC-familiy tyrosine kinases (Jefferies et al., 2003; Meng and Lowell, 1997) and MPKKKs (Dumitru et al., 2000; Huang et al., 2004).

The way, how a given PAMP elicits and modulates the immune response is obviously quite complex. Not only different pathways are involved upon ligation of a specific TLR, but also different TLRs are known to act together to provoke immune responses. The list of the described signaling molecules is by no means complete and it certainly remains exciting which insights in the orchestrating of "specialized proteins" will be discovered.

Negative regulation of TLR signaling

Although inflammatory responses are critical to control the growth of pathogens (Hagberg et al., 1984; Shahin et al., 1987; Eden et al., 1988; Cross et al., 1995), excessive production of inflammatory cytokines as a result of TLR signaling causes live-threatening systemic disorders (Beutler et al., 1985; Danner et al., 1991), such as endotoxic shock, which can be induced by the TLR4 ligand LPS. Organisms therefore evolved strategies to modulate their TLR-mediated responses. Chronically repeated exposure to endotoxin or LPS transiently increases the tolerance against a endotoxin challenge (Beeson and With the Technical Assistance of Elizabeth Roberts, 1947; Greisman et al., 1966; Ziegler-Heitbrock, 1995) and preexposure to LPS *in vivo* and *in vitro* results in a transient state of hyporesponsiveness to subsequent stimulation with LPS (Munoz et al., 1991; Setrakian et al., 1994). This endotoxin (LPS) tolerance is a negative feedback mechanism to protect from endotoxic shock (Henricson et al., 1990; Gustafson et al., 1995; Salkowski et al., 1998). During this process, TLR4 is

downregulated (Nomura et al., 2000) and NF-κB activation is decreased (Goldring et al., 1998; Kastenbauer and Ziegler-Heitbrock, 1999). So far, several cytoplasmic molecules are known to negatively regulate TLR signaling.

An alternative spliced truncated form of MyD88, termed MyD88s, is induced upon LPS treatment and lacks a short intermediate domain separating the DD and TIR domains (Janssens et al., 2002). MyD88s is unable to bind to IRAK4 and to promote IRAK1 phosphorylation leading to the shutdown of LPS-induced NF-κB activation (Burns et al., 2003). Another negative regulator is IRAK-M (Kobayashi et al., 2002) which is expressed in monocytes and macrophages following stimulation with various TLR ligands. IRAK-M, which lacks kinase activity, prevents the dissociation of the IRAK1-IRAK4 complex from MyD88, thereby preventing the formation of the IRAK-TRAF6 complex (reviewed in Janssens and Beyaert, 2003 and Akira and Takeda, 2004). Proteins of the SOCS (suppressor of cytokine signaling) family are induced by cytokines and negatively regulate their signaling pathways (Yasukawa et al., 2000). Although the exact mechanism is unknown, SOCS1 is thought to directly downmodulate TLR-signaling pathways by interacting with IRAK1 (Stoiber et al., 1999; Dalpke et al., 2001; Kinjyo et al., 2002; Nakagawa et al., 2002). Interestingly, also membrane-bound molecules that contain TIR domains have recently been shown to negatively regulate TLR signaling. SIGIRR (single immunoglobulin IL-1R-related molecule) transiently interacts with TLR4, IRAK1 and TRAF6, and SIGIRR-deficient mice are hypersensitive to LPS-induced endotoxic shock (Wald et al., 2003). ST2 associates with MyD88 and TIRAP, and overexpression of ST2 inhibits NF-κB activation. ST2-deficient mice show an increased secretion of inflammatory cytokines upon LPS stimulation and are unable to induce LPS tolerance.

Altogether, LPS tolerance is of great benefit by preventing exaggerated inflammatory responses and shock syndrome. But it can also cause major problems in the treatment of patients that recover from septic shock. Here, hyporesponsiveness to LPS leads to a severely reduced host defence during opportunistic infections leading to high mortality (Docke et al., 1997).

The role of another signaling adaptor molecule, DAP12 (KARAP), containing an immunoreceptor tyrosine-based activation motif (ITAM) is discussed controversially. Hamerman et al. observed enhanced TLR responses in the absence of DAP12. DAP12-deficient macrophages produced increased amounts of inflammatory cytokines in response to a variety of TLR ligands and DAP12-deficient mice were more susceptible to endotoxic shock and showed enhanced resistance to bacterial infections (Hamerman et al., 2005). However, Turnbull et al. found that $DAP12^{-/-}$-mice have improved survival from endotoxemia as well as from septic peritonitis and decreased acute phase responses during sepsis arguing that DAP12 signaling increases the response to microbial products leading to higher mortality rates (Turnbull et al., 2005).

Recently, TRIAD3A, a RING-finger E3 ligase, has been shown to promote ubiquitylation of TLR4 and TLR9, targeting these TLRs for degradation and thereby negatively regulating the intensity and duration of TLR signaling (Chuang and Ulevitch, 2004).

RP105 (radioprotective 105) joins the list of direct negative regulators of TLR signaling, but it is unique in its specificity for TLR4. RP105 is a TLR4 homologue and like TLR4 whose surface expression and signaling depends upon co-expression of the secreted protein MD-2, surface expression of RP105 is dependent upon co-expression of the MD-2 homologue, MD-1. Moreover, the expression pattern of RP105 correlates with that of TLR4. However, RP105 lacks a signaling domain. The RP105:MD-1 complex was shown to directly interact with the TLR4:MD-2 signaling complex, thereby inhibiting the ability of the latter one to bind its ligands. Additionally, RP105 regulated responses to endotoxin *in vivo* emphasizing its role as physiological negative regulator of TLR4 responses (Divanovic et al., 2005b; Divanovic et al., 2005a).

TLRs and cross-presentation

As discussed earlier, cross-presentation enables exogenous antigens to be routed for presentation on MHC class I molecules allowing their recognition by $CD8^+$ T cells and is therefore important for the development of $CD8^+$ CTL responses against tumors and infectious pathogens that do not directly infect APCs. Activation of TLRs on APC induces upregulation of co-stimulatory molecules and cytokine production, thereby shaping an adaptive immune response. For, when present during their priming, TLR ligands increase proliferation and cytotoxicity of CTLs responding to foreign antigens (Schwarz et al., 2003; Reis e Sousa, 2004). Moreover, TLRs seem to play a role in mediating cross-priming by DCs. In an *in vitro* cross-presentation model, TLR3 and TLR9 ligands enhanced cross-presentation by mature DCs (Datta et al., 2003). *In vivo*, immunization experiments of mice with virus-infected cells or cells containing synthetic dsRNA revealed the role of TLR3 in promoting cross-priming of CTLs against viruses (Schulz et al., 2005). Triggering of TLR9 by immunostimulatory CpG DNA is able to induce *in vivo* CTL responses in the absence of $CD4^+$ T cell help (Cho et al., 2000; Horner et al., 2001). The TLR involvement appears to replace the CD40-CD40L interaction between APCs and $CD4^+$ T cells that is necessary for cross-presentation (Bennett et al., 1997; Schoenberger et al., 1998; Albert et al., 2001). But still, the mechanisms underlying TLR-induced cross-presentation are not fully understood. It is independent of endosomal acidification and involves the cytosolic antigen processing machinery, including TAP (transporter associated with antigen processing) (Cho et al., 2002). Covalent linkage of antigen to immunostimulatory CpG DNA reveals that TLR9 has no impact on the observed enhanced antigen-uptake of these conjugates, but is required for APC activation and maturation downstream of endocytosis and subsequent cross-priming of $CD8^+$ T cells. Wildtype but not TLR9-deficient DCs were able to upregulate CD40 and CD80 and to secrete inflammatory cytokines upon uptake of the CpG-antigen conjugates, and wildtype but not TLR9-deficient mice generated specific CTL responses to the conjugates (Heit et al., 2003). Further investigation emphasizes the involvement of TLR ligands in CTL cross-priming by showing that the TLR signaling adaptor molecule MyD88 is required for cross-priming (Palliser et al., 2004). Recently, immature DCs were demonstrated to present processed antigen from captured purified protein in association with class I

MHC molecules in the presence of poly(I:C), but not of LPS. Thus, priming of antigen-specific $CD8^+$ CTLs with purified viral protein *in vivo* is dependent on the use of an appropriate stimulus or involvement of an appropriate TLR, respectively (Fujimoto et al., 2004).

The ability of TLR ligands as non-specific stimuli to mediate cross-presentation by DCs bears the risk of 'innate autoimmunity', whereby non-specific stimuli break cross-tolerance to self-antigens by converting tolerogenic DCs presenting peripheral self antigens into autoimmunogenic DCs (Vella et al., 1995; Vezys and Lefrancois, 2002; Walker and Abbas, 2002; Beutler, 2004); described in TLR and disease section). However, this scenario is discussed very controversially (Hamilton-Williams et al., 2005), for maintenance of self-tolerance is one of the basic concerns of both, the innate and the adaptive immune system.

TLRs and human diseases

Toll-like receptors play a critical role in both innate and adaptive immune responses. It is therefore not surprising that TLR functions also affect human diseases. For example, septic shock is caused by the overwhelming secretion of inflammatory cytokines as a result of LPS triggered TLR4 responses upon uncontrolled systemic bacterial infection. On the other hand, TLR polymorphisms can lead to insufficient TLR functions and increased risk of bacterial infections as observed for altered TLR4 and TLR2 (reviewed in Cook et al., 2004). The same is true for mutations in TLR signaling molecules that result in impaired TLR signaling (Picard et al., 2003).

Surprisingly, TLR4 has been put in the context of atherosclerosis. Abundant TLR4 mRNA and protein were found in atherosclerotic plaques compared to unaffected vessels (Xu et al., 2001). And a role for TLR4 in DC-mediated lesion progression is suggested by the finding that mouse DCs loaded with a heart-specific peptide can induce myocarditis in a TLR-dependent manner (Eriksson et al., 2003). Accelerated atherosclerosis, which is frequently observed in systemic lupus erythematosus (SLE), seems to be mediated through modified endogenous lipoproteins that involve TLR triggering. Minimally modified-LDL (MM-LDL) induces activation of macrophages

and endothelial cells via TLR4 (Miller et al., 2003; Walton et al., 2003), and saturated but not polyunsaturated fatty acids can activate macrophages through TLR4 (Lee et al., 2003b).
Polymorphism of TLR4 is additionally linked to asthma, although the underlying mechanisms are not fully understood and may involve regulatory T cells (Caramalho et al., 2003; Higgins et al., 2003). Paradoxically, exposure to LPS and other TLR ligands in early childhood decreases the incidence of asthma later in life (Braun-Fahrlander et al., 2002; Gehring et al., 2002; Braun-Fahrlander, 2003).

TLRs are also discussed to play a role in breaking cross-tolerance to self-antigens (Figure 3). In such a scenario, lymphocytes with low affinity for self antigen may overcome their state of ignorance in some circumstances upon action of a strong stimulus. This stimulation can occur if self structures are recognized by TLRs during alterations in the overall body composition such as infection or hormonal changes. Triggering by TLRs provides the co-stimulation that is necessary for the activation of autoreactive lymphocytes but missing under normal conditions. One example is the unmethylated CpG motif in DNA recognized by TLR9. Although much more common in bacterial DNA, it can be enriched in activated mammalian cells with substantial DNA demethylation. Extensive cell death coupled with insufficient clearance of apoptotic bodies drives B lymphocytes specific for DNA to internalize the CpG DNA present in these immune complexes via their BCR and to accumulate it in the endosomal compartment. Here, it encounters TLR9 which is then activated by a sufficient concentration of CpG. This activation signal together with the signal provided by the BCR finally activates the previously ignorant B cells to secrete antibodies directed against self (Leadbetter et al., 2002; Viglianti et al., 2003).

Although genetical susceptibility must be considered, this pathway is thought to explain systemic autoimmunity that commonly implies DNA-related autoantigens. An example might be systemic lupus erythematosus (SLE), in which chromatin and DNA enriched in guanine/cytosine content (Sano and Morimoto, 1982) are the major targets of autoreactive lymphocytes in these patients.

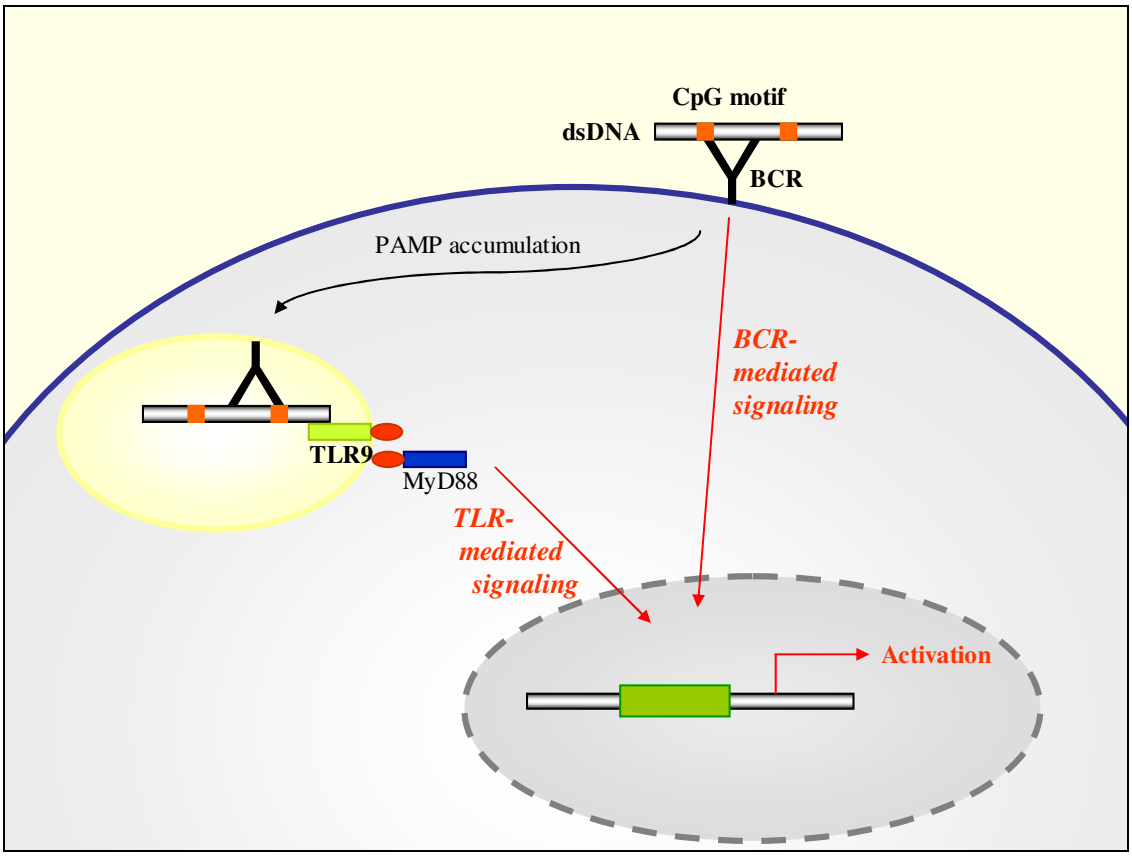

Figure 3. Schematic representation of a possible mechanism of TLRs breaking ignorance to self-antigens. In ignorant B cells, BCR signaling upon encounter of CpG containing dsDNA autoantigen is unsufficient to trigger activation. In a situation of autoimmunity, the BCR is thought to shuttle and accumulate the autoantigen into an intracellular, TLR9 habouring compartment, thus promoting recognition of the CpG motif by TLR9 leading to TLR9 signaling via a MyD88-dependent pathway. Only the interplay of both, TLR9 signaling and BCR signaling, activates the B cell thereby subverting the ignorate state toward autoimmunity.

However, the high risk of 'innate autoimmunity' questions the discussed (Walker and Abbas, 2002; Vezys and Lefrancois, 2002; Beutler, 2004) ability of TLR ligands as non-specific stimuli to convert tolerongenic DCs presenting peripheral self antigens into autoimmunogenic DCs. It would imply that cross-tolerance is regularly converted into autoimmunity by infections with pathogens capable to signal via TLRs or from the use of TLR-ligands as vaccine adjuvants. Instead, self-tolerance is a rather robust state with multiple checkpoints (Arnold et al., 1993; Andre et al., 1996; Heath and Carbone, 2001; Hamilton-Williams et al., 2005). Moreover, it was demonstrated very recently that DC activation by TLR ligands is not sufficient to break peripheral cross-tolerance in the absence of antigen-specific $CD4^+$ T cell help. Without such help,

autoreactive CTLs are deleted despite of persisting self antigen presence. Autoimmunity only occurred by stimulating the early effector phase of autoreactive CTLs when their precursor frequency was unphysiologically high (Hamilton-Williams et al., 2005).

In contrast, the ability of TLRs to directly activate DCs makes them promising candidates for their use in vaccine design and in the development of novel adjuvants for vaccine therapy. Thus, linking the polysaccharide of *Haemophilus influenzae* type b (Hib) to a Hib outer membrane protein complex elicits protective immunity (Donnelly et al., 1990) by the direct engagement of TLR2 (Latz et al., 2004a). In regard to the development of novel adjuvants, the adjuvant effect of LPS could be addressed to its lipid A structure, and equivalent derivatives, such as monophosphoryl lipid A (MLA), are far less toxic but still trigger via TLR4 maintaining their immunomodulatory capacity (reviewed in Persing et al., 2002). Furthermore, CpG DNAs, known to trigger TLR9, are under intensive investigation for their use as adjuvants: in a recent clinical study, CpG DNA was shown to enhance the immunogenicity when added to a hepatitis B vaccine (Cooper et al., 2004). Thus, a detailed understanding of TLR involvement in the development of human diseases enables promising immunotherapeutic strategies for treatment or even prevention of diseases.

Other pattern recognition receptors

Besides the TLR, the innate immune system uses a broad range of pattern recognition receptors. These comprise not only proteins that are expressed on the cell-surface but also cytosolic proteins and even secreted ones. Mannan-binding lectin (MBL), C-reactive protein (CRP) and serum amyloid protein (SAP) are such soluble PRRs. They are produced by the liver and belong to the acute phase proteins, which are released into the blood stream early during infection. These receptors recognize cell wall compounds of pathogens, such as the phosphocholine portion of certain bacterial and fungal cell-wall lipopolysaccharides in the case of CRP or terminal mannose residues on the surface of many bacteria and some viruses, including the human immunodeficiency virus (HIV). Binding of the equivalent PAMP results in its opsonization as well as in the activation of the complement cascade.

Among the secreted PRR are LBP (Lipopolysaccharide binding protein) and CD14, both of which are involved in the recognition of LPS. CD14 not only exists in the soluble form, but is rather anchored in the cell membrane. The class of cell surface PRRs comprises further more the macrophage mannose receptor (Fraser et al., 1998) and dectin-1 (Brown et al., 2002), that recognize bacterial and yeast surface carbohydrates. MARCO (macrophage receptor with collagenous structure) binds to bacterial cell walls. Macrophage scavenger receptors (MSR) show a broad binding specificity for polyanions including LPS, dsRNA, oxidized LDL and lipoteichonic acid. Mice deficient in MSR have an increased susceptibility to bacterial and viral infections (Suzuki et al., 1997) suggesting a role for MSRs in host defense against pathogens.

In the cytosol, several PRRs are known to be involved in antiviral responses. Inside the cell, viruses use the host's cell machinery for their replication. At some point during the replication cycle of most viruses, double stranded RNA is produced, which is recognized by PKR (IFN-inducible dsRNA-dependent protein kinase) (reviewed in Clemens and Elia, 1997). Activated PKR inhibits the eukaryotic translation initiation factor EIF-2 by phosphorylation and thereby blocks further viral replication. Additionally, the induction of type I interferons (IFN-α/β) is partly dependent on PKR (Diebold et al., 2003). These antiviral effector molecules interfere with viral replication, thereby blocking the spread of viruses to uninfected cells.

Intracellular recognition of bacteria involves the cytosolic proteins NOD1 and NOD2 (Nucleotide-binding oligomerization domain proteins), which bind to distinct structures derived from peptidoglycan which are not ligands for TLRs (reviewed in Ulevitch, 2004). NOD proteins initiate cell activation through a multi-protein complex called inflammasome (Martinon and Tschopp, 2004), finally leading to the activation of NF-κB.

The list of above mentioned PPRs is not exhaustive, and even more PPRs are certain to be discovered, now, the field of innate immune recognition gained new interest with the discovery and characterization of the Toll-like receptors.

Links between innate and adaptive immunity

For a long time, the immune system was thought to function by discriminating between self and nonself. Burnet's original self-nonself (SNS) model (Burnet, 1959) suggested that this discrimination is exclusively performed by cells of the adaptive immune system. Lymphocytes recognize foreign structures by their single-specific surface receptor, leading to their activation and to the initiation of an immune response. The finding that nonidentical cattle twins are mutually tolerant of each other's blood cells (Owen, 1945) led to the conclusion that lymphocytes learn the definition of self early in life. This model was supported by Medawar's experiments, in which adult mice accept foreign skin grafts if they had been injected as babies with cells from the donors (Billingham et al., 1953). However, it had to be modified due to the findings that for both B and T lymphocytes, the recognition of their specific antigen is not sufficient for activation. Instead, B lymphocytes die upon antigen recognition (signal one) unless they are rescued by help (signal two) (Bretscher and Cohn, 1970), a trick which eliminates potential autoreactive B cells. This help was later found to be provided by T helper cells. In 1975, Lafferty and Cunningham proposed that T helper cells as well need a "signal two" for activation (Lafferty and Cunningham, 1975). They receive this co-stimulatory signal from stimulator cells, today known as antigen-presenting cells (APC). APCs provide the co-stimulatory signal through the specific interaction of their co-stimulatory surface molecules, such as CD80 or CD86, with the equivalent receptors on the T cells, such as CD28 (Schwartz, 1990; Jenkins et al., 1991; Harding et al., 1992). Upon this interaction, T cells express the IL-2 receptor and produce IL-2, and the autocrine action of this cytokine leads to proliferation and differentiation of the activated T cells into armed effector T cells.

However, APCs themselves need to be activated in order to be able to deliver co-stimulation to naïve T cells (Krieger et al., 1986). They ingest all sorts of self and foreign substances at infection sites and then upregulate co-stimulatory signals on activation through innate immune receptors, the pattern recognition receptors (PRR) that recognize conserved pathogen-associated molecular patterns (PAMPs). Thus, the induction of an adaptive immune response is dependent on innate immune

mechanisms, which do not show diversified antigen-specificity and, consequently, are not able to distinguish between all kinds of self and nonself. In 1989, Janeway therefore proposed in the infectious-nonself (INS) model that APCs discriminate between "infectious-nonself" and "non-infectious-self" (Janeway, Jr., 1989) via the limited set of PRRs. The activation status of the antigen-presenting cell has a key function for the decision whether an antigen specific T cell becomes activated or anergic. The role of adaptive and innate immunity had to be reconceived and the dichotomy separating both had to be overtaken: the ancient innate immune system is a prerequisite for the triggering of the sophisticated adaptive immune system.

In an orchestrated interplay, the PRRs, such as TLRs, link innate and adaptive immune responses: innate recognition of PAMPs through TLRs initiates an inflammatory response leading to the recruitment of cells to the infection sites. Activation of TLRs induces the expression of diffusible chemotactic factors and cell surface adhesion molecules attracting innate immune cells, such as monocytes, neutrophils, basophils, eosinophils and NK cells as well as adaptive immune cells, and facilitating their migration to the inflamed tissue (Huang et al., 2001). Monocytes express a variety of TLRs, and upon activation the infiltrating monocytes can differentiate into either tissue macrophages or DCs that contribute to the generation of adaptive immunity. TLR triggered activation of DCs having captured microbial antigens leads not only to the upregulation of co-stimulatory and MHC molecules but also to a switch in chemokine receptor expression and to the secretion of cytokines and chemokines finally resulting in the generation of effector responses including T helper cell and CTL responses. In this process, different DC populations respond only to the pathogens they have appropriate TLRs for. And even when triggered by the same ligand, the outcome of the TLR stimulation can differ dependent on the DC subtype: stimulation with synthetic TLR7 agonists or with certain synthetic oligonucleotides containing CpG motifs induces pDCs to secrete IFN-α and mDCs to secrete IL-12 (Ito et al., 2002; Hemmi et al., 2003). This impressively shows how DC-mediated control of T cell activation is dependent on innate PRRs (reviewed in Iwasaki and Medzhitov, 2004). The finding that TLRs play a role in cross-presentation by dendritic cells and concomitant cross-priming of cytotoxic T cells further supports the idea that adaptive immune responses

are mediated or shaped by innate PRRs able to discriminate between infectious non-self and non-infectious self.

But in 1994, Matzinger changed the points of view and introduced her danger model: the primary driving force for the immune system is the need to detect and protect against danger (Matzinger, 1994). Danger signals not only arise exogenously by pathogens, but also endogenously by nonforeign alarm signals that derive from cells and tissues which are stressed, virally infected or killed necrotically, but not by healthy cells or those dying apoptotically (Gallucci and Matzinger, 2001). These endogenous danger signals include heat-shock proteins, mammalian DNA (Ishii et al., 2001) or RNA released by necrotic cells, type-I interferons produced by virus-infected cells, CD40L on the surface of activated T cells, reactive oxygen intermediates, neuromediators, hyaluron and other extracellular-matrix breakdown products after vessel damage (Gallucci et al., 1999). In the danger model, the PRRs from the Janeway INS models are considered as sensors for exogenous danger signals. But additionally, the danger model is able to explain situation of dangerous self, caused for example by some mutations, and harmless foreign, including, e.g., fetuses. Moreover, it also tries to explain autoimmunity by suggesting that at least some autoimmune diseases may arise by mutations in genes changing for example the normal clearance processes, or by pathogens or toxins causing cellular stress or death. In these circumstances the immune system is doing its job by responding to alarm signals, but to the harm of the host (Matzinger, 2002).

Although the necessity for co-stimulation largely controls the adaptive immune system, a small subset of T cells powerfully contributes additionally to the regulation of adaptive immune responses. They were originally termed suppressor T cells in the early 1970s as being able to suppress autoimmune responses (Gershon, 1975; Gershon et al., 1972). Today, these $CD4^+$ $CD25^+$ cells are more accurately conferred to as regulatory T cells according to their function in the control of immunopathological responses directed against self or foreign antigens (Maloy and Powrie, 2001). Until now it is not fully understood how regulatory T cells act on and how they affect their target cells to dampen responsiveness. They are able to prevent autoimmune disease *in*

vivo, and can suppress T-cell proliferation in response to a specific antigen or to allogeneic stimulator cells *in vitro*. Suppression seems to be mediated by multiple mechanisms including direct cell-cell contact between regulatory T cells and the target cells as well as indirect action through soluble factors. Regulatory T cells secrete the anti-inflammatory cytokines IL-10 and TGF-β, which inhibit T-cell proliferation and render dendritic cells unable to promote T-cell activation (Janeway, Immunobiology, 6th edition). However, it has been reported recently that TLR signals can influence adaptive response by blocking the suppressive effect of regulatory T cells on effector T cells. This mechanism is independent of co-stimulation and is instead mediated by IL-6 and at least one other soluble factor secreted by the activated APCs in responses to TLR activation. *In vivo*, the blockage of regulatory T cell function requires TLR activation on DCs, and other modes of DC activation such as inflammatory cytokines cannot substitute for this TLR-dependent signal (Pasare and Medzhitov, 2003; Yang et al., 2004). In contrast, Kubo et al. propose that the effect of proinflammatory cytokines produced by TLR-activated mature DCs is to reverse the state of anergy of regulatory T cells rather than to overcome regulatory T cell suppression (Kubo et al., 2004). Conversely, pathogens are known to trigger Toll-like receptor pathways to modulate regulator T cell responses toward their own benefits (Higgins et al., 2003; Netea et al., 2004). On the other hand, regulatory T cells themselves express different Toll-like receptors, such as TLR4, 5, 7, and 8. Without the aid of any APCs, they can be activated by LPS which even increases their suppressive activity on $CD4^+$ T cells *in vitro* (Caramalho et al., 2003).

The ideas of Janeway and Matzinger that innate immune recognition is a prerequisite as well as a control for adaptive immunity have proven true by the exciting discoveries of the nexus of TLR-mediated activation and T cell function. But still, there are many secrets to be discovered!

Outline of this Thesis

In the past years, the knowledge in the exciting field of Toll-like receptors (TLR) and the understanding of how these proteins link innate and adaptive immunity grew dramatically.

When the work on this thesis started in 2001, the controversial discussion about the involvement of TLR2 and TLR4-pathways in response to the bacterial cell wall component lipopolysaccharid (LPS) seemed to be resolved and it was commonly accepted that TLR2 recognizes LPS of Gram-positive bacteria and TLR4 that of Gram-negative bacteria. However, this rule seemed to be proven by exceptions. Therefore, one focus of interest of this thesis was the characterization of the receptor involved in the recognition of LPS species, which are regarded to be structurally and/or functionally atypical. The interesting observation that LPS from the Gram-negative bacteria *Legionella pneumophila* as well as the bacteria as a whole signal via TLR2 and depend on CD14 is presented in Chapter 2.

Another aim of this thesis was to elucidate a possible role of Toll-like receptors in the immune surveillance of invasive aspergillosis. The finding that antigens of the causative agent, *Aspergillus fumigatus*, use different TLRs to differentially induce the secretion of pro-inflammatory cytokines by dendritic cells (Chapter 3) emphasizes the complex role of TLRs in controlling adaptive immune responses.

Other goals of this thesis were the characterization of the molecular basis by which heat shock protein Gp96:peptide complexes confer protective immunity against tumors or pathogens the peptide had derived from. The remarkable finding that Gp96-mediated dendritic cell activation is triggered by TLRs (Chapter 4) and finally results in a CD8-biased T cell response (Chapter 5) revealed the importance of innate immune recognition for adaptive immune responses. To further address the issue of TLRs linking innate and adaptive immunity, we analyzed the contribution of TLR-mediated innate immune recognition to priming of cytotoxic T lymphocytes during the response against viral and minor H antigens. The promising results obtained are presented in Chapter 6.

2

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LEGIONELLA PNEUMOPHILA MEDIATED ACTIVATION OF DENDRITIC CELLS INVOLVES CD14 AND TLR2

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In this study, we analyzed the activation of bone marrow-derived dendritic cells (BMDCs) from mice lacking the cd14*-gene with purified* Legionella pneumophila *lipopolysaccharide and with viable or formalin-killed* L. pneumophila*. We found that low concentrations of LPS and doses of* L. pneumophila *that are relevant to infection are dependent on CD14 to activate BMDCs. Higher concentrations of LPS are able to overcome the lack of CD14 indicating that other receptors are involved. We, therefore, included studies using BMDCs from mice lacking functional TLR2 and/or TLR4 molecules. We found that purified* L. pneumophila *LPS as well as* L. pneumophila *either viable or formalin-killed are able to activate BMDCs from TLR4-deficient C3H/HeJ mice but fail to activate BMDCs from TLR2-knockout mice. Our data show that not only purified LPS from* L. pneumophila *but also the micro-organism itself stimulate BMDCs via TLR2 and that this stimulation is dependent on CD14 in this mouse model.*

The major part of the work and ideas as well as all experiments reported in this chapter were performed by the author of this thesis. Marion Faigle contributed to figure 2 and figure 4 as well as to the concept of this work.

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Introduction

Legionella pneumophila, the causative agent of Legionnaires' disease, is an intracellular pathogen of alveolar macrophages. Pneumonia primarily occurs in immunocompromized hosts suffering from malignancies associated with monocyte dysfunction as well as in patients who receive immunosuppressive drugs. The mechanism by which *L. pneumophila* infection of the lung is controlled is not yet clear. *L. pneumophila* is relatively resistant to innate and humoral immune response. The protective effect of antibody production and polymorphonuclear cells is limited (Ciesielski et al., 1986; Conlan and Ashworth, 1986; Weeratna et al., 1994) and activation of complement does not result in inactivation or killing of the bacteria (Horwitz and Silverstein, 1981). Instead, cell-mediated immunity seems to be important for infection control. Initial investigations have demonstrated that lymphocytes from convalescents undergo proliferation and release cytokine activity into the cell culture supernatant after *in vitro* contact with formalin-inactivated legionellae (Plouffe and Baird, 1981; Plouffe and Baird, 1982; Horwitz, 1983; Friedman et al., 1984a; Friedman et al., 1984b).

The induction of cell-mediated immunity crucially depends on the participation of dendritic cells (DCs) as professional antigen-presenting cells (APCs) which are able to display processed antigens on MHC molecules. This was shown recently also for the presentation of *L. pneumophila* derived antigens (Neild and Roy, 2003). In addition, upon first encounter with the pathogen, DCs are stimulated to mature and thereby to secrete pro-inflammatory cytokines. This is initiated by the recognition of conserved structures of the pathogen, the PAMPs (pathogen associates molecular patterns) by a set of pathogen recognition receptors (PRR) such as Toll-like receptors (TLRs).

In this way, lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria triggers innate effector functions. Several molecules on the surface of innate immune cells are involved in this process. They include the LPS-binding protein (LBP) (Antal-Szalmas, 2000), which interacts with the GPI-anchored cell surface protein CD14 (Wright et al., 1990; Golenbock et al., 1993), the signal transducing molecule TLR4 (Poltorak et al., 1998a) and the accessory molecule MD-2 (Shimazu et al., 1999). CD14 exists in two isoforms, the glycoxylphosphatidylinositol (GPI) anchored

membrane protein and the soluble serum protein (Ulevitch and Tobias, 1994) and both froms of CD14 can be involved in LPS signalling (Antal-Szalmas, 2000).

So far, the requirement for TLR4 is observed for all LPS types derived from bacteria belonging to the *Enterobacteriaceae*. However, recent studies have also shown that the LPS of some Gram-negative bacteria like *Rhizobium* and *Legionella* require TLR2 rather than TLR4 (Girard et al., 2003). In comparison to other Gram-negative bacteria, the lipopolysaccharide (LPS) of *L. pneumophila* exhibits unusual structural features (Zahringer et al., 1995; Helbig et al., 1995) which are predicted to be responsible for the interaction with TLR2 rather than with TLR4 molecules (Horwitz, 1983) and for the strongly reduced interaction with CD14 molecules on human monocytes (Neumeister et al., 1998). The latter study revealed that about 1000-fold higher concentrations of *L. pneumophila* LPS (LPS-Lp) as compared to *Escherichia coli* (LPS-Ec) or *Salmonella enterica* serovar Minnesota-derived LPS (LPS-Sm) had to be used in order to achieve comparable cytokine production. This difference correlated with the inability of LPS-Lp to compete with LPS-Ec for the binding to membrane bound-CD14 and to interact with soluble CD14 molecules. Since competition and binding experiments are limited to a certain range of LPS concentrations that can be used, it remained to be determined whether or not weak and, therefore, so far undetectable interactions with CD14 molecules still take place and contribute to the LPS-Lp-mediated activation of cells of the innate immune system.

We, therefore, examined the contribution of CD14 and TLR molecules to the stimulation of bone marrow-derived dendritic cells (BMDCs) by purified LPS-Lp as well as by viable or formalin-killed *L. pneumophila* using mice lacking CD14 molecules and different TLRs. Since LPS is the major pro-inflammatory constituent of the Gram-negative bacterial cell wall and is recognized by the host as main PAMP, we wondered whether intact bacteria are recognized by the same molecules as the purified LPS-Lp or if different TLRs are involved. We found for both purified LPS-Lp and intact bacteria that signal transduction involves CD14 molecules and strictly depends on TLR2 in our mouse model.

Materials and Methods

Reagents and antibodies

LPS from *Salmonella typhimurium* (LPS-Stm) was purchased from Sigma-Aldrich (Taufkirchen, Germany). LPS from *L. pneumophila* serogroup 1 (LPS-Lp) was extracted according to Moll et al. Phosphothioate-stabilized CpG oligonucleotide 1668 (TCC-ATC-ACG-TTC-CTG-ATG-C) (ODN) was obtained from TIB MOLBIOL (Berlin, Germany). Palmitoyl-3-Cys-Ser-$(Lys)_4$ (Pam_3Cys) was obtained from EMC microcollections GmbH (Tübingen, Germany).

Bacteria

L. pneumophila serogroup 1 strain Pontiac (isolated from a patient with severe *Legionella* pneumonia and passaged less than three times on BCYE α-agar) was kindly provided by Prof. Dr. G Ruckdeschel (Munich, Germany).

Generation of mouse dendritic cells

Mouse immature DCs were generated from bone marrow of C3H/HeN, C3H/HeJ (Tularic Inc., South San Fransisco, CA, USA), BALB/c (Charles River, Sulzfeld, Germany) and C3H/HeJ/TLR2$^{-/-}$, TLR2$^{-/-}$, CD14$^{-/-}$ mice as previously reported (Vabulas et al., 2002b). C3H/HeJ/TLR2$^{-/-}$ and TL2$^{-/-}$ mice were generously provided by H. Wagner/K. Kirschning (Munich, Germany). CD14$^{-/-}$ mice were a kind gift from C. Schütt (Greifswald, Germany). For the generation of mouse bone marrow-derived DCs, Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker, Verviers, Belgium) was used supplemented with 2 mM L-glutamine (GibcoBRL Life Technologies, Paisley, UK), 100 IU/ml penicillin/streptomycin (GibcoBRL), 10% FCS (PAA, Linz, Austria) and 150 U/ml GM-CSF. Bone marrow cells from two mice were pooled and incubated in GM-CSF containing medium for 6-7 days and fresh medium with GM-CSF was replaced every 2 days. The obtained DCs were CD11c positive and CD14 negative.

Pre-treatment of mouse DCs prior stimulation

Immature DCs (6-7days) derived from $CD14^{-/-}$ and B6 mice were washed three times with serum-free medium and were then stimulated either in the presence or absence of serum.

For stimulation with bacteria, bone marrow cells were washed on day 4 with antibiotic-free medium; for all further steps, antibiotic free medium was used.

Stimulation of mouse DCs

Immature DCs were stimulated by addition of *L. pneumophila* LPS, *S.typhimurium* LPS, Pam_3Cys or 1668 ODN in appropriate concentrations. For infection with *L. pneumophila*, the bacteria-to-cell ratio was adjusted to 10:1 and 1:1, respectively, using a 96-well tissue culture plate (Greiner, Frickenhausen, Germany). After 2 h of co-incubation at 37°C and 5% CO_2, non-phagocytosed bacteria were killed by the addition of 75 µg/ml of gentamicin (Invitrogen, Eggenstein, Germany). After an additional incubation time of 20 h, supernatants were removed for detection of secreted cytokines.

Detection of mouse cytokines

Twenty hours after stimulation of mouse DCs, IL-6 and IL-12 (p40) concentrations in the cell culture supernatants were determined using standard sandwich ELISA protocols. Antibodies and recombinant cytokine standards were obtained from Becton Dickinson (Heidelberg, Germany). The capture antibody was bound to the ELISA plate (MaxiSorb™, Nunc, Roskilde, Denmark), the biotinylated detection antibody was visualized by streptavidin-conjugated horseradish peroxidase and TMB substrate (Sigma) and the assay was read at 405 nm.

Results

BMDC activation by *L. pneumophila* LPS requires CD14

To investigate the role of CD14 during *L. pneumophila* LPS-induced activation, BMDCs derived from CD14 knockout mice and from BALB/c wild-type mice were stimulated with purified LPS from *L. pneumophila* in the presence or absence of FCS - which naturally contains soluble CD14 and LBP (Wright et al., 1990) and cell culture supernatants were analyzed for the secretion of IL-6 (Fig. 1) and IL-12 (data not shown).

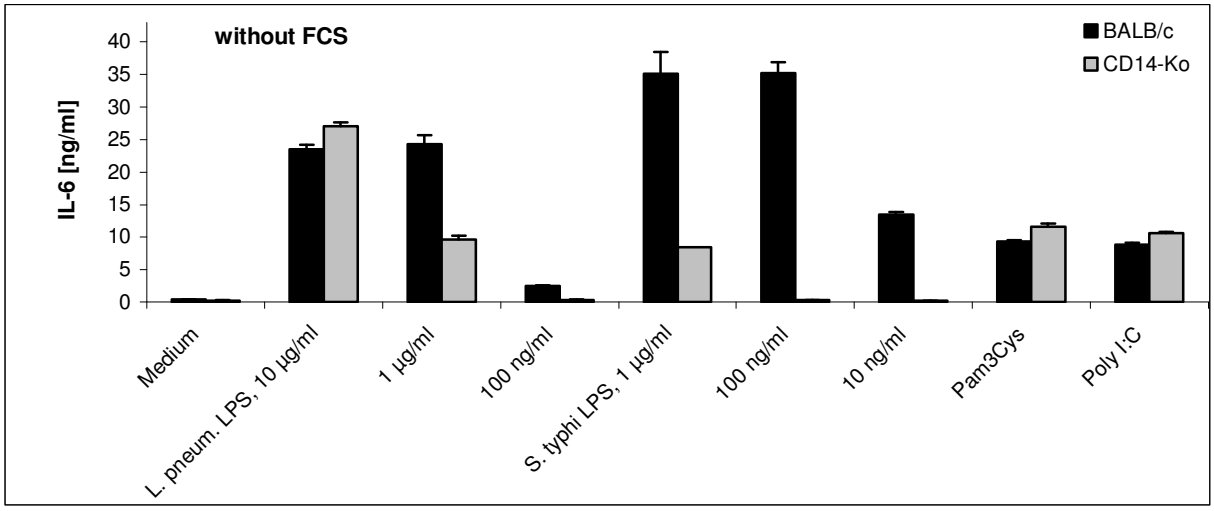
Low concentrations of *L. pneumophila* LPS (e.g. < 1 µg/ml) activated BMDCs in a CD14-dependent manner. Only higher concentrations of *L. pneumophila* LPS (10 µg/ml) activated BMDCs via a CD14-independent way. In the presence of FCS, BMDCs derived from CD14 knockout mice were more susceptible to activation by *L. pneumophila* LPS; however, at 100 ng/ml, BMDCs from CD14 knockout mice produced also reduced amounts of IL-6 (Fig. 1B). As a control for a CD14-dependent stimulation, LPS from *S. typhimurium* was used. As CD14-independent stimuli, Pam_3Cys and poly(I:C) were added to BMDCs.

L. pneumophila interacts with CD14

To analyze the effect of CD14 under more physiological conditions and to elucidate whether additional CD14-independent stimuli play a role in BMDC activation, we used viable and formalin-killed bacteria instead of purified LPS. Both, viable and formalin-killed *Legionella* bacteria were able to stimulate BMDCs derived from wild-type mice to secrete IL-12 (Fig. 2) and IL-6 (data not shown).

This stimulation was clearly CD14-dependent, since under serum-free condition the ability of BMDCs derived from CD14 knockout mice to respond to *L. pneumophila* is drastically reduced. Addition of serum reconstitutes the ability of BMDCs to secrete cytokines partially. The controls were used as describe above.

A



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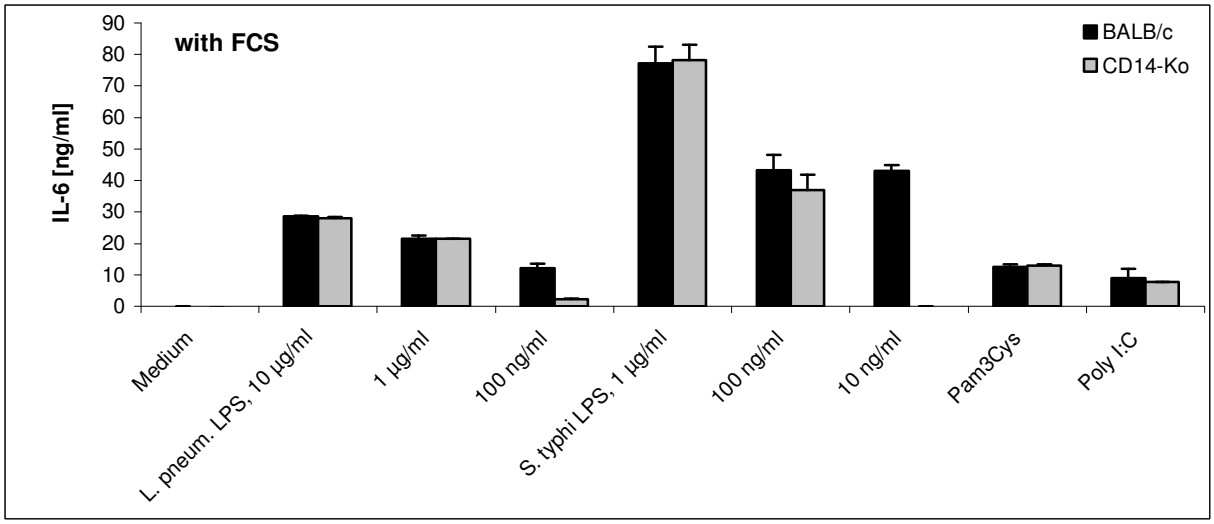
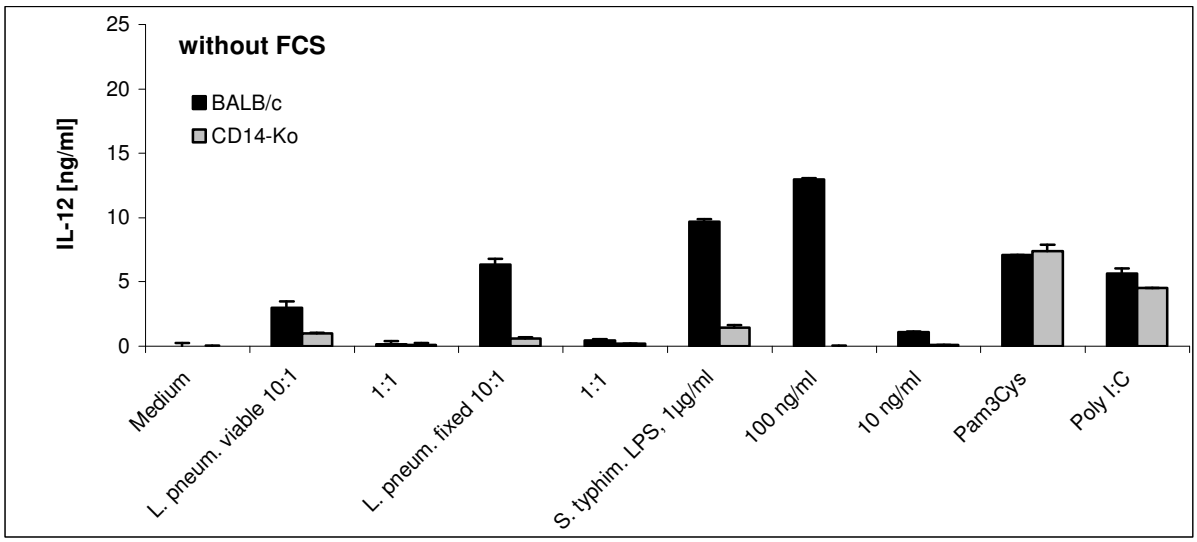


Figure 1. Role of CD14 in *L. pneumophila* LPS-mediated IL-6 production. Bone marrow-derived dendritic cells from the indicated mice strains were cultured with *L.pneumophila* LPS (10 µg/ml, 1 µg/ml or 100 ng/ml) in the presence and the absence of FCS – LPS of *S. typhimurium* (1 µg/ml, 100 ng/ml or 10 ng/ml) was used as CD14-dependent control and Pam_3Cys (2.5 µg/ml) and poly I:C as CD14-independent control. After 20 h, the IL-6 concentration in the cell culture supernatant was measured by ELISA. **(A)** In the absence of FCS, BMDCs derived from CD14 knockout mice produced significantly less IL-6 in response to 100 ng/ml and 1 µg/ml of *L. pneumophila* LPS compared to the wild-type. **(B)** In the presence of FCS, the defect of CD14 is restored at concentrations higher than 1µg/ml. The experiment was performed three times. The error bar represents the deviation of triplicates of one experiment.

A



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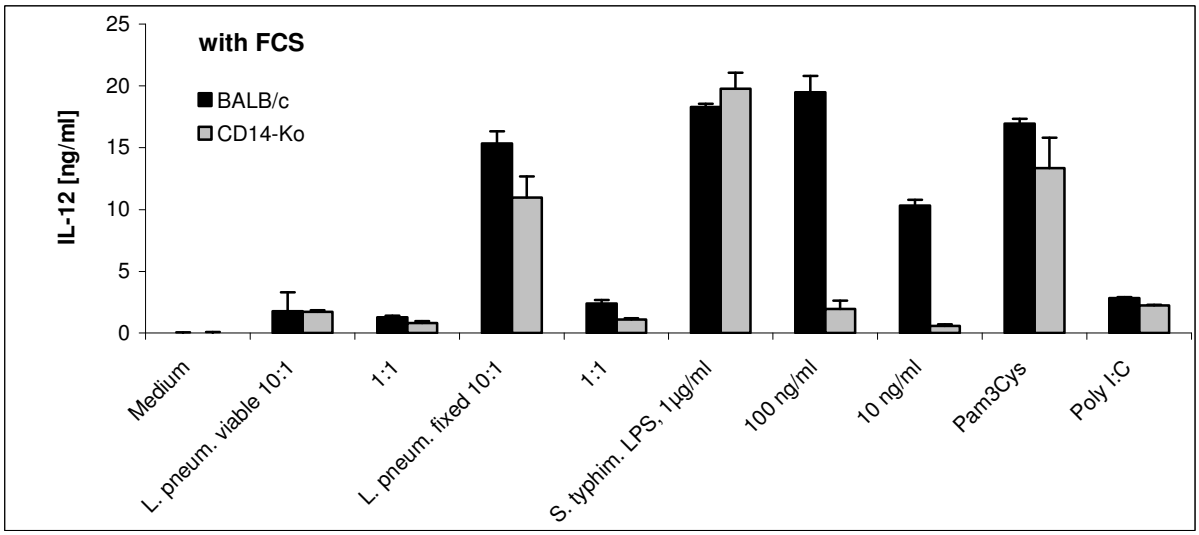

Figure 2. Role of CD14 on IL-12 secretion by BMDCs in response to *L. pneumophila.* In the presence and the absence of FCS, bone marrow-derived dendritic cells from BALB/c and CD14 knockout mice were co-cultured with *L. pneumophila* in bacteria-to-cell ratios of 10:1 and 1:1. Non-phagocytosed bacteria were killed 2 h post-infection by addition of gentamicin. LPS of *S. typhimurium* (1µg/ml, 100 ng/ml or 10 ng/ml) was used as CD14-dependent and Pam_3Cys (2.5 µg/ml) and poly I:C as CD14-independent control. After 20 h, the supernatant was analysed for secretion of IL-12. **(A)** In the absence of FCS, the ability of BMDCs derived from CD14 knockout mice to secrete IL-12 in response to infection by viable or fixed *L. pneumophila* is impaired. **(B)** Addition of FCS to the cell culture restores the ability of BMDCs derived from CD14 knockout mice to secrete IL-12 in response to infection by viable or fixed *L. pneumophila*. The mean value of triplicates is shown together with the standard deviation from one of three independent experiments.

L. pneumophila LPS triggers activation of BMDCs via TLR2 but not via TLR4

Since activation of BMDC by *L. pneumophila* LPS and *Legionella* bacteria requires the presence of CD14, we investigated whether Toll-like receptors (TLRs) are involved. To determine the role of TLRs, *L. pneumophila* LPS-mediated activation of BMDCs from mice lacking functional TLR2 and/or TLR4 molecules was studied. We investigated the secretion of the pro-inflammatory cytokine IL-12 (Fig. 3) and IL-6 (data not shown) in response to *L. pneumophila* LPS, *S. typhimurium* LPS, Pam_3Cys and CpG-ODN. BMDCs from C3H/HeN and C3H/HeJ (a TLR4-deficient mouse) but not from $TLR2^{-/-}$ and C3H/HeJ/$TLR2^{-/-}$ responded to the *L. pneumophila* LPS stimulus by secretion of IL-12. CpG-ODN induced IL-12 secretion in all cultures, Pam_3Cys-mediated activation was abrogated in BMDCs from $TLR2^{-/-}$ mice and *S. typhimurium* LPS was impaired in BMDCs from C3H/HeJ mice, in line with previous reports.

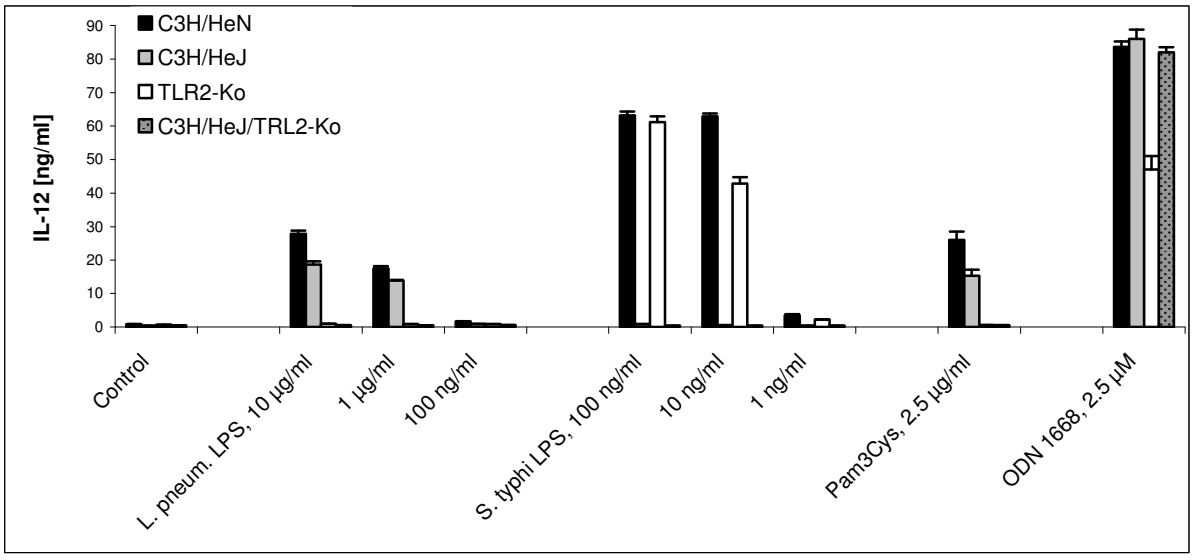

Figure 3. *L. pneumophila* LPS-induced IL-12 production by dendritic cells is TLR2 dependent. Bone marrow-derived dendritic cells from indicated mice strains were stimulated with *L. pneumophila* LPS (10 µg/ml, 1 µg/ml or 100 ng/ml), *S. typhimurium* LPS (100 ng/ml, 10 ng/ml or 1 ng/ml), Pam_3Cys (2.5 µg/ml) and Oligonucleotide 1668 (2.5 µM). After 20 h, the IL-12 concentration in cell culture supernatant was measured by sandwich ELISA. Only BMDCs from C3H/HeN and C3H/HeJ mice secrete IL-12 in response to *L. pneumophila* LPS, BMDCs from $TLR2^{-/-}$ and C3H/HeJ/$TLR2^{-/-}$ mice do not. The mean value of triplicates from one of three indeependnt experiments is shown. Error bars represent standard deviation.

BMDCs derived from TLR2 knockout mice do not respond to *L. pneumophila*

Since activation of BMDCs by *L. pneumophila* LPS was strictely dependent on TLR2 (Fig. 3), we wanted to investigate if the activation of BMDCs by whole bacteria follows the same pathway.

We, therefore, infected the above used BMDCs with viable and formalin-killed *L. pneumophila* and determined the secretion of IL-12. As shown in Figure 4, BMDCs from wild-type mice (C3H/H3N) as well as from TLR4-deficient mice (C3H/HeJ) secreted IL-12 in response to the stimulation with *L. pneumophila*. In contrast, BMDCs from mice lacking TLR2 molecules (TLR2$^{-/-}$ and C3H/HeJ/TLR2$^{-/-}$) did not respond to the stimulation with *L. pneumophila* by secretion of IL-12. Oligonucleotide induced IL-12 secretion in all cultures, *S. typhimurium* LPS triggered activation via TLR4 (impaired activation in BMDCs from C3H/HeJ and C3H/HeJ/TLR2$^{-/-}$ mice) and Pam_3Cys via TLR2 (impaired activation in BMDCs from TLR2$^{-/-}$ and C3H/HeJ/TLR2$^{-/-}$ mice), in line with previous reports. Interestingly, the fixation of *L. pneumophila* by 1% PFA seemed to be required to induce a strong activation of BMDCs.

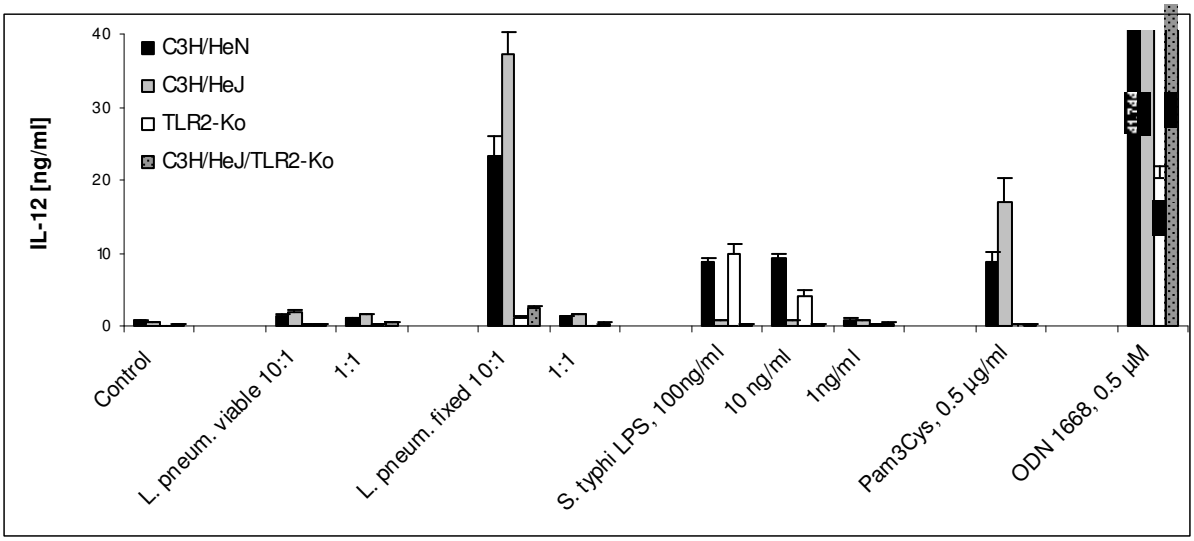

Figure 4. *L. pneumophila* activates dendritic cells to produce IL-12 via TLR2. *L. pneumophila* either viable or fixed by 1% PFA were added to the culture of BMDCs from the indicated mice strains in bacteria-to-cell ratios of 10:1 and 1:1. Two hours later, non-phagocytosed bacteria were killed by gentamicin. LPS from *S. typhimurium* (100 ng/ml), Pam_3Cys (0.5 µg/ml) and oligonucleotide 1668 (0.5 µM) were used as controls. Cell culture supernatant after 20 h was analysed for the secretion of IL-12 by sandwich ELISA. BMDCs from C3H/HeN and C3H/HeJ mice are stimulated by both viable and fixed *L. pneumophila* to secrete IL-12. BMDCs from TLR2$^{-/-}$ and C3H/HeJ/TLR2$^{-/-}$ mice are not able to secrete IL-12 in response to neither viable nor fixed *L. pneumophila*. The mean value of triplicates from one of three independent experiments is shown. Error bars represent standard deviation.

Discussion

It was assumed in the past that the LPS of enterobacteria is restricted to the use of TLR4 as a signal transducing molecule, whereas TLR2 is involved in the recognition of Gram-positive bacteria and *Mycobacteria* (Yoshimura et al., 1999; Flo et al., 2000). However, it was recently demonstrated that even though *Porphyromonas gingivalis* (Hirschfeld et al., 2001), *Leptospira interrogans* (Werts et al., 2001) and *L. pneumphila* (Girard et al., 2003) are Gram-negative bacteria, they activate cells of the immune system via a TLR2-dependent mechanism. Previous studies have also demonstrated that TLR4 was not involved in host defense against pulmonary *L. pneumophila* infection (Lettinga et al., 2002). Whereas TLR4-mediated responses to LPS require the presence of LBP and CD14 molecules, efficient TLR2-mediated signalling can also be observed in the absence of CD14 (Chow et al., 1999; Netea et al., 2002a). We, therefore, analyzed the contribution of TLR2, TLR4 and CD14 to the signaling pathways triggered by intact *L. pneumophila* and by its most abundant cell surface molecule LPS. For this purpose, we used BMDCs from mice lacking CD14 as well as mice with defects in TLR2 and TLR4. We found that both CD14 and TLR2 molecules are involved in the *L. pneumophila* LPS-induced secretion of pro-inflammatory cytokines by mouse BMDCs.

Although it has been recently demonstrated that the LPS of *L. pneumophila* does not interact with CD14 expressed by human monocytes (Neumeister et al., 1998), we obtained first evidence that CD14 might be involved in the activation of antigen-presenting cells by *L. pneumophila* LPS in a functional assay using BMDCs from CD14-knockout mice. Cytokine secretion induced by a low dose of LPS (1 µg/ml) was reduced in BMDCs from CD14-knockout mice, while high concentrations of LPS triggered BMDC activation also in the absence of CD14 (Fig. 1A). The addition of serum containing sCD14 (Bazil et al., 1986) increased the ability of LPS to activate BMDCs even at low concentrations (Fig. 1B).

The involvement of CD14 in the activation of APCs became clearly visible using live and fixed bacteria as a stimulus. At an infectious dose of 10:1 bacteria per cell BMDC, we observed a drastically reduced cytokine secretion in CD14 knockout mice compared to wild-type mice (Fig. 2A). It was not possible to test whether increased

numbers of bacteria would provide a CD14-independent stimulus as observed for *L. pneumophila* LPS because increasing the bacteria to cell ratio killed the BMDCs as observed previously (Husmann and Johnson, 1994).

As observed for purified *L. pneumophila* LPS, addition of serum induced a better cytokine secretion by BMDCs when intact bacteria were used for stimulation (Fig. 2B). In this case, BMDCs from CD14 knockout mice secreted the same amount of cytokines as the BMDCs from wild-type mice. However, in the absence of FCS, the ability of BMDCs from CD14-knockout mice to secrete cytokines is reduced compared to wild-type BMDCs (Fig. 2A).

We next investigated the role of TLRs in the activation of dendritic cells by *L. pneumophila* LPS and intact bacteria. Using BMDCs from C3H/HeJ, C3H/HeJ/ $TLR2^{-/-}$ and $TLR2^{-/-}$ mice, we found that *Legionella LPS* triggered activation of BMDCs exclusively via TLR2 whereas TLR4 was not involved (Fig. 3). This finding was in line with the results obtained by Girard and colleagues who described a TLR2-mediated CD14 expression in bone marrow derived granulocytes as a measurement for activation (Girard et al., 2003) and Kikuchi and colleagues reporting a TLR4-independent signaling of *L. pneumophila* LPS (Kikuchi et al., 2004). Our experiments extended this observation to intact bacteria: only TLR2 appeared to be involved in the *Legionella*-mediated activation of BMDCs without a detectable contribution of TLR4 (Fig. 4). This absolute requirement for TLR2 in BMDC activation by whole bacteria was surprising since a redundant recognition of several different PAMPs (soluble or membrane bound) of a given pathogen was expected. However, our results strongly argue that LPS is the main structure for the recognition of *L. pneumophila* by the innate immune system. We can not finally rule out that other pathogen structures of *L. pneumophila* activate the immune system in a similar TLR2- and CD14-dependent manner although this might be quite unlikely. This finding is in contrast to the stimulation of innate immune responses by other bacteria like the Gram-positive group B streptococci (GBS). Here, a so far unknown secreted GBS factor stimulates macrophages in a CD14/TLR2/TLR6-dependent manner but GBS stimulate cells without the contribution of these molecules (Henneke et al., 2002).

Nevertheless, additional molecules might also influence the interaction of intact *L. pneumophila* with BMDCs. When we compared viable bacteria with bacteria killed by

1% PFA we found that the latter were much more potent in activating BMDCs and inducing the secretion of cytokines (Fig. 4) as reported recently (Kikuchi et al., 2004). This increase in BMDC activation is not due to the fixation procedure since irradiated bacteria behave like fixed bacteria (data not shown). Whether or not soluble factors secreted by live *L. pneumophila* which suppress the activation of cells of the innate immune system are involved remains to be determined.

Conclusions

These results provide strong evidence that *L. pneumophila* LPS is the main PAMP recognized on the surface of *L. pneumophila* and that CD14 contributes to the TLR2 mediated activation.

3

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ASPERGILLUS FUMIGATUS ANTIGENS ACTIVATE INNATE IMMUNE CELLS VIA TOLL-LIKE RECEPTORS 2 AND 4

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Invasive aspergillosis (IA) is a leading cause of mortality in hematological patients. Appropriate activation of the innate immune system is crucial for the successful clearance of IA. Therefore, we studied the Aspergillus fumigatus*-mediated activation of human granulocytes and monocyte-derived immature dendritic cells (DCs), as well as murine bone marrow-derived DCs (BMDCs) from wild-type, toll-like receptor (TLR)4-deficient, TLR2 knockout., and TLR2/TLR4 double-deficient mice.* Aspergillus fumigatus *antigens induced the activation and maturation of immature DCs as characterized by CD83 expression, upregulation of major histocompatibility complex and co-stimulatory molecules. Moreover, fungal antigens enhanced the phagocytosis and production of interleukin (IL)-8 in granulocytes. The release of IL-12 by BMDCs in response to* A. fumigatus *antigens was dependent on the expression of TLR2, whereas the release of IL-6 was dependent on the expression of functional TLR4 molecules. The protein precipitate of* A. fumigatus *supernatant provided strong stimulation of DCs and granulocytes, indicating that a factor secreted by* A. fumigatus *might activate innate immune cells. In conclusion,* A. fumigatus *antigens induced the activation of DCs and granulocytes. Our results indicated that, this activation was mediated via TLR2 and TLR4. Future studies are needed to assess the clinical impact of these findings in patients at high risk for IA.*

The author of this thesis performed the experiments resulting in figures 3 and 4 and contributed to figures 1 and 2 as well as to the concept of this work together with Markus Radsak.

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Introduction

Invasive aspergillosis (IA) has become a major cause of infection-related mortality in patients with haematological malignancies, especially after allogeneic stem cell transplantation (SCT) (Wald et al., 1997). Proven risk factors in humans are defects in phagocyte function (Morgenstern et al., 1997), steroid-induced suppression of macrophage conidiocidal activity (Schaffner et al., 1982; Palmer et al., 1991), and chemotherapy-induced neutropenia (Gerson et al., 1984). Patients with a previous history of IA (Offner et al., 1998) and patients colonized in the lower respiratory tract without signs of tissue-invasive disease (Einsele et al., 1998) have been found to have an increased risk for recurrence of IA during a subsequent episode of neutropenia or immunosuppression (Offner et al., 1998). These observations implicate local cellular defects in the immune effector mechanisms as major predisposing factors of the host to IA (Schaffner et al., 1982; Romani, 1997; Cenci et al., 1998).

In *Drosophila*, toll participates in the defence against fungi by the induction of drosomycin secretion as an early form of an innate immune response (Lemaitre et al., 1996). In mammals, toll-like receptors (TLRs) are involved in the response to pathogens by the recognition of so-called pathogen-associated molecular patterns (PAMPs). These include lipopolysaccharide (LPS), peptidoglycans, lipoproteins and bacterial CpG-DNA, which are recognized by TLR4, TLR2 and TLR9, respectively (Aderem and Ulevitch, 2000; Hemmi et al., 2000).

Toll-like receptors have been demonstrated to induce tumour necrosis factor α (TNF-α) release in murine peritoneal macrophages and transfected human cell lines after stimulation with different biological forms of *Aspergillus fumigatus* (Mambula et al., 2002) and preliminary data indicate a potential role of TLRs in the *A. fumigatus*-induced activation of human monocytes (Wang et al., 2001).

More recently, a critical role of T-helper (T_H) cell responses in the control of IA has been reported in murine models (Clerici and Shearer, 1994; Cenci et al., 1997; Cenci et al., 1998; Cenci et al., 1999; Cenci et al., 2000; Mehrad et al., 1999; Clemons et al., 2000) and in patients with haematological malignancies and allogeneic SCT recipients (Hebart et al., 2002). Dendritic cells (DCs) are recognized as the initiators of specific immune responses to pathogens (Banchereau and Steinman, 1998). In the murine

model, pulmonary DCs were found to internalise *Aspergillus* conidia and hyphae, to undergo functional maturation upon migration to the draining lymph nodes and spleen and to induce T cell priming of $CD4^+$ T lymphocytes (Bozza et al., 2002). *In vitro*, DCs were found to restore *Aspergillus*-specific lymphoproliferation in haematological patients (Grazziutti et al., 2001).

In the current study, we assessed the *A. fumigatus*-induced activation of granulocytes and DCs.

Materials and Methods

Reagents, antibodies and plasmids

Lipopolysaccharide from *Pseudomonas aeruginosa* (kindly provided by T. Schröder, Tübingen) was used or LPS from *Salmonella typhimurium* was purchased from Sigma-Aldrich (Taufkirchen, Germany). Phosphothioate-stabilized CpG oligonucleotide 1668 (TCC-ATC-ACG-TTC-CTG-ATG-C) was purchased from TIB MOLBIOL (Berlin, Germany). Palmitoyl-3-Cys-Ser-$(Lys)_4$ (Pam_3Cys) was obtained from EMC microcollections GmbH (Tübingen, Germany).

Cell staining was performed using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse monoclonal antibodies against CD86, CD40, CD11b (purchased from Pharmingen, Hamburg, Germany); CD80, HLA-DR, CD14 (all purchased from Becton Dickinson, Heidelberg, Germany); CD66b or CD83 (all from Coulter-Immunotech Diagnostics, Hamburg, Germany), and CD1a (OKT6; Ortho Diagnostic Systems, Neckargemund, Germany), and mouse immunoglobulin (Ig)G isotype controls (Becton Dickinson). Samples were analysed on a FACScalibur (Becton Dickinson).

Aspergillus fumigatus antigens

Conidia of *A. fumigatus* (strain CBS 144-89) were inoculated in 150-ml Erlenmeyer flasks containing Sabouraud liquid medium (2% [w/v] glucose, 1% [w/v] mycopeptone). Flasks were shaken for 24 h at 37°C and 200 rpm. Two-liter fermenters (LSL Biolafitte, Saint Germain en Laye, France) containing 1.2 liters of Sabouraud

medium were inoculated with the shaken flask cultures. The 18 h-culture conditions were as follows: inoculum 8% (v/v); temperature, 26°C; aeration, 50 l of air per minute; agitation, 500 rpm. The mycelial mat recovered by filtration was extensively washed with water. Mycelium was disrupted in a glass bead cell homogeniser in 50 mmol/l Tris-HCl buffer pH 7.5 and the water-soluble cellular extracts (EC SAB) were recovered after centrifugation. The ethanol precipitate (PP SAB) of *A. fumigatus* was prepared by precipitating the culture filtrate with 4 volumes of ethanol after 18 h of culture and stored at 4°C. Protein content was measured by the BioRad technique (Bio-Rad, Marne La Coquette, France) according to the manufacturer's instructions and estimated in milligram equivalent BSA per millilitre. EC SAB and PP SAB were tested endotoxin free (< 0.1 endotoxin units/µg protein) by limulus amebocyte lysate assay (BioWhittaker, Verviers, Belgium).

To generate hyphae, conidia (10^6 colony forming units/ml) were harvested after 3 d of culture on Sabouraud glucose agar, filtered through sterile gauze and then incubated in yeast nitrogen base for 18 h at 37°C, followed by a centrifugation for 10 min at 3000 g. Mycelia were washed twice and, for sterilization, incubated in ethanol-posphate-buffered saline (ethanol-PBS; 70%) for 24 h at 4°C.

Preparation and culture of human cells

Polymorphonuclear neutrophils (PMN) were separated from heparinized blood of healthy volunteer donors by dextran sedimentation using Polymorphprep™ (Nycomed, Oslo, Norway) according to the manufacturer's instructions and as described previously (Radsak et al., 2003). Contaminating red blood cells were removed by an in-house lysis buffer [150 mmol/l ammonium chloride, 1 mmol/l potassium bicarbonate, 0.1 mmol/l ethylene diamine tetra acetate (all from Sigma, St Louis, MO, USA) in distilled water, pH 7.3]. The purity of cell preparation was assessed by flow cytometry with CD66b as marker for PMN. Usually, 95-98 % of cells were CD66b positive. For stimulation of PMN, cells were suspended in a concentration of 2 x 10^6 cells/ml in Roswell Park Memorial Institue (RPMI) 1640 (PAN Biotech, Aidenbach, Germany) with 3 % (v/v) heat inactivated (30 min at 56°C) fetal bovine serum (FBS;

PAN Biotech) and cultivated at 37°C with 7.5 % CO_2 in air in the presence of various stimuli diluted in medium as indicated.

Human DCs were generated from heparinized blood of healthy donors as reported previously (Grigoleit et al., 2002). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient (LINARIS, Bettingen am Main, Germany), washed twice in sterile calcium- and magnesium-free Hanks' balanced salt solution (GIBCO BRL, Karlsruhe, Germany) and re-suspended in RPMI 1640 medium supplemented with Glutamax-I, 25 mmol/l HEPES buffer (GIBCO BRL), 200 µg/ml Gentamicinsulfate (Refobacin® 80 mg; Merck, Darmstadt, Germany), and 10% fetal calf serum (FCS; Sigma). PBMCs were plated at a density of 10 x 10^6 cells/well for 2 h at 37 °C and non-adherent cells were removed by washing with PBS. Adherent monocytes were cultured for 6 d in RP10 medium supplemented with 1000 IU/ml interleukin-4 (IL-4; R&D Systems, Minneapolis, MN, USA) and 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Leukomax, Novartis Pharma GmbH, Nürnberg, Germany). The differentiation state of DCs was examined by flow cytometry.

Generation of mouse DCs

Mouse immature DCs were generated from bone marrow of C3H/HeN and C3H/HeJ (Tularic Inc., South San Fransisco, CA, USA). C3H/HeJ / TLR2$^{-/-}$, and TLR2$^{-/-}$ mice were generously provided by H. Wagner/K. Kirschning (Munich, Germany). For the generation of mouse bone marrow-derived DCs (BMDCs) Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Verviers, Belgium) was used supplemented with 2 mmol/l L-glutamine (GibcoBRL Life Technologies, Paisley, GB), 100 IU/ml penicillin/streptomycin (GibcoBRL), 10% FS (PAA, Linz, Austria) and 200 U/ml GM-CSF (Peprotec, Rocky Hill, NJ, USA). Bone marrow cells were incubated in GM-CSF containing medium for 6-8 d and fresh medium with GM-CSF was replaced every 2 d. The obtained DCs were CD11c positive and CD14 negative.

Phagocytosis

For analysis of phagocytic activity, ingestion of PE-labeled polystyrene microspheres (diameter 1 µm, Fluoresbrite Plain Microspheres PCRed; Polysciences, Warrington, PA; USA) was evaluated as described previously (Lehmann et al., 2000; Radsak et al., 2003). Briefly, aliquots of 2 x 10^5 freshly purified PMN were incubated in the presence of stimuli as indicated and 5 x 10^6 microbeads [effector:target (E:T) ratio 1:25] for 60 min at 37 °C, then kept on ice, washed twice in fluorescence-activated cell sorting buffer (FACS) and fixed in 1 % paraformaldehyde in PBS. Analysis was performed by FACS.

Detection of human and mouse cytokines

For analysis for cytokines by enzyme-linked immunosorbent assay (ELISA), supernatants were derived from stimulation with purified human PMN or mouse DCs and frozen at -20 °C until required.

Supernatants of PMN were analysed by ELISA for IL-8 (OptEIA from Pharmingen, Hamburg) according to the manufacturer's instructions.

Supernatants derived from mouse DCs were analysed using commercial IL-6 and IL-12 specific ELISA (BD Pharmingen) as described previously (Vabulas et al., 2002b).

Quantifiable reverse transciption polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNeasy spin columns (Qiagen, Hilden, Germany) followed by cDNA synthesis using the first strand cDNA synthesis kit based on avian myeloblastosis virus (AMV) reverse transcriptase (Roche, Mannheim, Germany). Quantifiable PCR assays were performed by real-time PCR with the LightCycler™ instrument as reported previously (Loeffler et al., 2003). This technique is based on the fluorescence resonance energy transfer (FRET). The samples were quantified by defined external standards, ranging from 10^9 to 10^1 CFU/ml. Primers were specific for TNF-α and IL-12 (5'-). The probes consisted of two parts: one part had been labelled at the 5'-end with the Light Cycler™- Red 640 fluorophore (5'-), the other at the 3'-end with fluorescein (5'-; Tibmolbiol, Berlin, Germany).

Statistics

The statistical significance of the data was analysed using a non-parametric Mann-Witney U-test for comparison between two groups. For multivariate analyses, the data were analysed by two-way analysis of variance (ANOVA). For all analyses, $P < 0.05$ was considered statistically significant.

Results

Aspergillus fumigatus antigens activate human immature monocyte-derived dendritic cells

This study was intended to analyse the effects of *A. fumigatus* antigens on immature DCs. Therefore, monocyte-derived immature DCs were stimulated overnight with different antigen preparations from *A. fumigatus* including hyphae, a cellular extract (EC SAB) and the ethanol precipitate of the culture supernatant of *A. fumigatus* (PP SAB) and analysed for the surface expression of CD83, co-stimulatory and major histocompatibility complex (MHC) molecules as well as for the expression of IL-12p40 and TNF-α by quantifiable RT-PCR. *Aspergillus fumigatus* antigens were found to activate immature DCs as demonstrated by an increased expression of co-stimulatory molecules and HLA-DR, and to induce maturation of immature DCs (Fig. 1). Moreover, stimulation of immature DCs with *A. fumigatus* antigen preparations induced a strong expression of IL-12p40 (up to 1000-fold) and TNF-α RNA (up to 100-fold) (data not shown). PP SAB was found to provide the strongest stimulation, but high input of hyphae yielded comparable result indicating that this effect might result from a higher concentration of TLR ligands in the PP SAB preparation. As PP SAB and EC SAB were found to stimulate immature DCs as effectively as *Aspergillus* hyphae, all further experiments were performed with EC SAB and PP SAB.

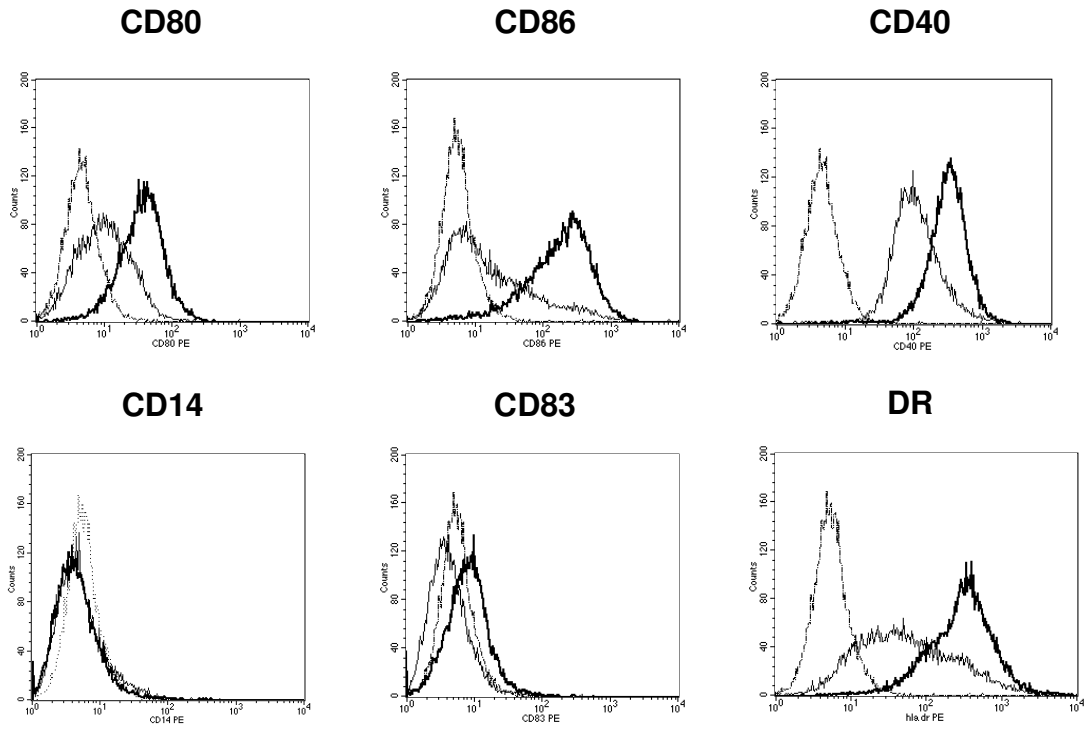

Figure 1: Aspergillus fumigatus hyphae induce activation and maturation of monocyte-derived human immature dendritic cells (DCs). The figure shows the result of one representative experiment. Overnight stimulation with A. fumigatus hyphae (one hyphae/DC) induces activation (upregulation of CD40, CD80, CD86, HLA-DR) and maturation (increased expression of CD83) of immature DCs. CD14 expression was unchanged. Isotype control, dotted line; negative control, thin line; A. fumigatus stimulated DCs, thick line.

Aspergillus antigens stimulate effector functions of human neutrophils

Polymorphonuclear neutrophils play an important part in the first line of defence against microbial pathogens by contributing substantially to the innate host defence with their ability to rapidly extravasate into inflamed tissue and their employment of potent effector mechanisms, i.e. phagocytosis, production of reactive oxygen species, and release of mediators and antimicrobial substances (Van Furth et al., 1973; Lloyd and Oppenheim, 1992; Ben Baruch et al., 1995; Greenberg and Grinstein, 2002).

Therefore, we studied whether PP SAB and EC SAB could also mediate activation of human PMN. In a first set of experiments, we investigated the ingestion of unopsonized fluorochrome-labelled polystyrene beads as measure for unspecific phagocytosis as described previously (Radsak et al., 2003). The phagocytic activity of PMN was markedly enhanced by PP SAB, but not by EC SAB (Table I). The

activation was insensitive to the presence of polymyxin B (not shown) indicating that the observed activation was not due to potential contaminations by bacterial endotoxins.

Table I. *Aspergillus* antigen PP SAB, but not EC SAB, enhances phagocytic activity of PMN.

Stimulus	fold dilution	mean (n=8 experiments)	SD	P-value
Medium		267,0	136.2	
PP SAB	10	935,0	383.8	<0,001
	30	744,3	202.6	<0,001
EC SAB	10	385,5	334.5	ns
	30	256,0	215.0	ns

PMN, polymorphonuclear neutrophil; ns, not significant.
Purified PMN (2 x 10^5) were incubated with the indicated stimuli in the presence of 5 x 10^6 fluorochrome-labelled microspheres as described in *Materials and Methods*. Cells were washed and fixed in 1% paraformaldehyde. Fluorescence intensity was evaluated by FACS. The summarised results of eight independent experiments with different donors are shown as mean with standard deviation (SD). Statistical analysis was performed by Mann-Witney U-test comparing the stimulated cells to the medium control.

In addition, *Aspergillus* antigens stimulated the release of IL-8 in purified PMN, as shown in Fig. 2. In a similar way, *Aspergillus* antigens mediated degranulation in PMN, analysed by upregulation of surface expression of CD66b and Mac-1 (CD11b) indicating mobilization of specific and gelatinase granules (not shown).

These results suggest that these *Aspergillus* antigens present potent stimuli for the activation of innate immune cells.

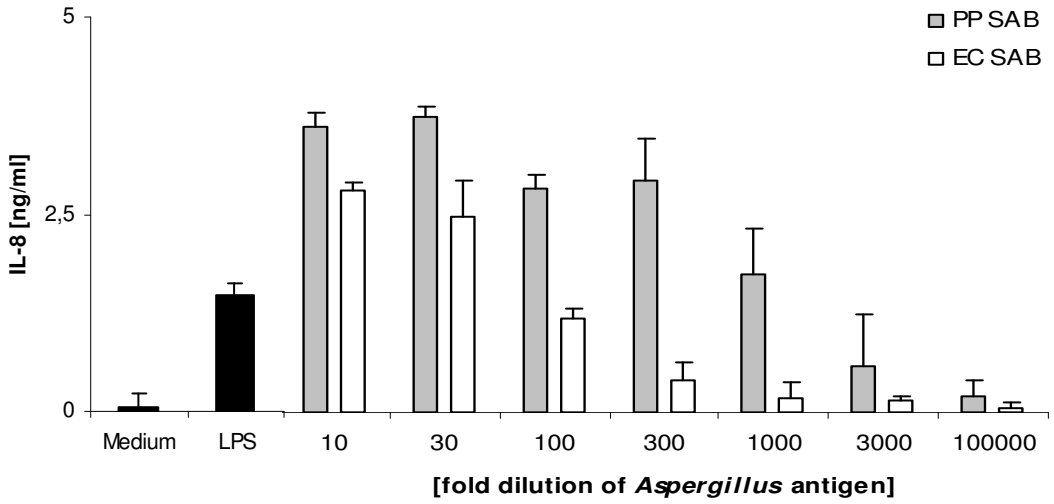

Figure 2. Release of IL-8 by polymorphonuclear neutrophils (PMN) after stimulation with *Aspergillus* antigens. Freshly purified PMN (4 x 10^5) were incubated in presence of the indicated *Aspergillus* antigens PP SAB (grey bars), EC SAB (open bars), or controls (medium alone or Lipopolysaccharide 1 µg/ml; black bars). Culture supernatants were harvested after 6 h and assayed for IL-8 by ELISA. All samples were assayed in triplicates and are depicted as mean and standard deviation. The representative results from one of three independent experiments are shown.

Aspergillus fumigatus mediates DC activation via TLR2 and TLR4

To analyse a potential role of TLR2 and TLR4 in the *A. fumigatus* antigen-mediated activation of innate immune cells, we investigated this interaction in detail in bone marrow-derived DCs (BMDCs) from wild-type mice and mice lacking functional TLR2 or TLR4 molecules. In the first set of experiments, we analysed the secretion of the pro-inflammatory cytokines IL-12 and IL-6 in response to EC SAB and PP SAB or LPS, Pam_3Cys and CpG-DNA as control stimuli. Upon stimulation BMDCs derived from TLR4 deficient mice and wild-type mice produced comparable amounts of IL-12 in response to EC SAB and PP SAB (Fig. 3). In contrast, BMDCs from TLR2 knockout mice were still activated by PP SAB but not by EC SAB, suggesting that activation by EC SAB is strictly dependent on TLR2.

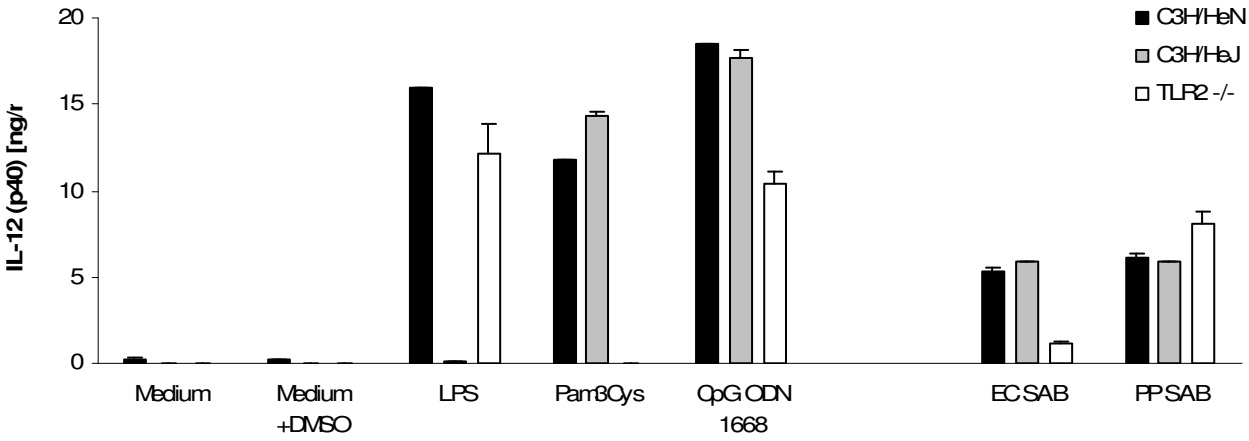

Figure 3. EC SAB-induced IL-12 production by DCs is TLR2 dependent. Bone marrow-derived dendritic cells (BMDCs) from the indicated mice strains were cultured in the presence of EC SAB (200-fold dilution), PP SAB (200-fold dilution), LPS (2.5 µg/ml), CpG ODN 1668 (2 µM) or Pam_3Cys (2 µg/ml). After 20 h IL-12 concentration in cell culture supernatant was measured by sandwich ELISA. BMDCs derived from C3H/HeN mice and C3H/HeJ mice produced comparable amounts of IL-12 in response to EC SAB and PP SAB. In contrast, TLR2 knockout mice-derived DCs responded to PP SAB but not to EC SAB regarding IL-12 production. The mean and standard deviation of triplicate wells is shown. The differences shown are statistically significant ($P < 0.05$ by two-way ANOVA).

In order to look at this more closely, a second set of experiments was performed using also BMDCs from TLR2 and 4 double-deficient mice. As shown in Fig. 4A, BMDCs from wild-type mice (C3H/HeN) and TLR4-deficient mice (C3H/HeJ) again gave a comparable IL-12 response to PP SAB and EC SAB (Fig. 3). However, BMDCs from the TLR2/4 double-deficient mice were did not respond to the PP SAB stimulus. As expected, BMDCs from TLR2/4 double-deficient mice did not respond to the TLR2-dependent IL-12 release induced by EC SAB. Analysing the production of IL-6 only showed a strong response to PP SAB in the wild-type BMDCs, but not to EC SAB (Fig 4B). Interestingly and unlike the production of IL-12, PP SAB-induced secretion of IL-6 was also affected in TLR4-deficient mice.

These results clearly demonstrate that the activation of BMDCs by EC SAB is exclusively dependent on TLR2. The situation is more complex for PP SAB. In the case of IL-12 production induced by PP SAB, TLR2 and TLR4 can fully compensate for each other. However, IL-6 production in TLR4-deficient mice can not be compensated by TLR2.

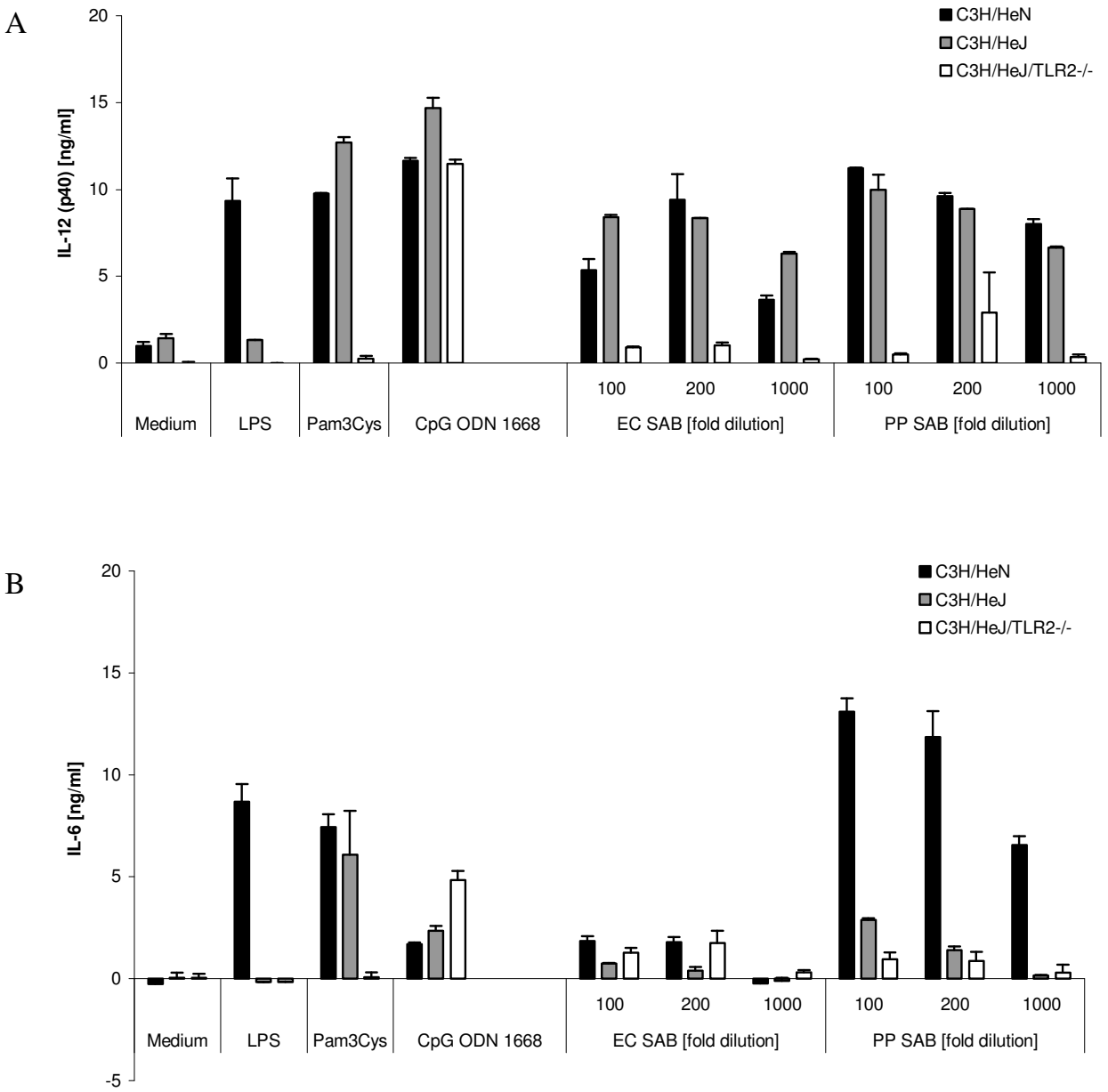

Figure 4. ***Aspergillus fumigatus*** **mediates dendritic cell (DC) activation via TLR2 and TLR4.** Bone marrow-derived dendritic cells (BMDCs) from the indicated mice strains were stimulated with EC SAB, PP SAB (indicated dilution), LPS (10 ng/ml), CpG ODN 1668 (5 µmol/l) or Pam_3Cys (1 µg/ml). Cell culture supernatants were harvested after 20 h and analysed for IL-12 and IL-6 secretion by sandwich ELISA. (A) BMDCs from C3H/HeN mice and C3H/HeJ mice secreted comparable amounts of IL-12 in response to EC SAB and PP SAB. C3H/HeJ/TLR2$^{-/-}$ mice did not respond to either fungal preparation. (B) BMDCs from C3H/HeN produced significant amounts of IL-6 in response to PP SAB, but not to EC SAB. The IL-6 release upon stimulation with PP SAB was more dependent on TLR4 compared with the release of IL-12. The mean and standard deviation of triplicate wells is shown. The differences shown are statistically significant ($P < 0.05$ by two-way ANOVA).

Discussion

In this report, we demonstrated that antigen preparations derived from *A. fumigatus* were potent activators of human immature monocyte-derived DCs, as demonstrated by the upregulation of MHC and co-stimulatory molecules, and an increased TNF-α and IL-12p40 mRNA concentration. The presence of these pro-inflammatory cytokines can be expected to contribute to the development of a T_H1-dominated immune response, which was found to be associated with control of IA. Moreover, CD83 was upregulated, indicating differentiation into mature DCs. In line with these results, the adoptive transfer of DCs pulsed with conidia and DCs transfected with conidial RNA were found to induce T_H1-dependent antifungal resistance in allogeneic bone marrow transplanted mice (Bozza et al., 2003).

The precipitate of the culture supernatant (PP SAB) was found to induce the strongest stimulation of immature DCs in comparison to hyphae and the cellular extract of *A. fumigatus* (EC SAB). However, the high input of hyphae yielded comparable results, indicating that this effect might result from a higher concentration of TLR ligands in the PP SAB preparation. Whereas the cytokine release of DCs from TLR4- and TLR2-deficient mice was only slightly reduced upon stimulation with PP SAB, a complete blockade of the cytokine release was observed in DCs from TLR2 and TLR4 double-deficient mice (Fig. 4). For signals mediated by PP SAB, TLR2 and TLR4 could fully compensate for each other if one was deficient, but deficiency of both, TLR2 and TLR4, could not be compensated for, demonstrating that TLRs are responsible for PP SAB-mediated cell activation. In contrast, signalling induced by the cellular extract of *A. fumigatus* EC SAB was found to be strictly TLR2 dependent, which is in line with data describing a TLR2 dependence of TNF-α release in murine peritoneal macrophages after stimulation with *Aspergillus* conidia and hyphae (Mambula et al., 2002). More recently, *A. fumigatus*-induced activation of murine peritoneal macrophages has been described to be dependent on the expression of TLR2 and TLR4 (Meier et al., 2003).

Interestingly, PP SAB but not EC SAB was found to strongly induce IL-6 release in culture supernatants of murine immature DCs. This finding is of special interest as IL-6 release by DCs has recently been described as essential for blocking the suppressive

effects of regulatory $CD4^+CD25^+$ T cells on the initiation of pathogen-specific adaptive immune responses (Pasare and Medzhitov, 2003). The characterisation of regulatory T cells has opened exciting opportunities to induce tolerance after transplantation and to prevent graft *versus* host disease after allogeneic SCT; however, these cells might induce potentially detrimental effects by down-regulation of immunity to infections and also to tumours (Wood and Sakaguchi, 2003). The characterisation of the PP SAB-derived TLR ligand responsible for IL-6 release in DCs is thus of potential interest for the development of *Aspergillus*-directed immunotherapy protocols.

PP SAB-induced IL-6 release was dependent on the expression of functional TLR4 molecules whereas IL-12 release was TLR2 dependent. This suggests that various pattern recognition receptors might be involved in the complex interaction of microbes and DCs. Recently, the use of different TLRs to differentially induce the release of pro-inflammatory cytokines and chemokines has been described for the interaction of *Candida albicans* and DCs (Netea et al., 2002b). Thus, differences in antigen preparations, cytokines analysed and cell types under study are likely to have a major impact on the results in the study of cell pathogen interactions and to explain differing results, as reported recently for TLR-usage in the *A. fumigatus*-mediated cell activation (Wang et al., 2001; Mambula et al., 2002). Moreover, TLR4-mediated but not TLR2-mediated cytokine signals were found to be lost upon *Aspergillus* germination to hyphae indicating that phenotypic switching during germination might counteract host immune defence mechanisms (Netea et al., 2003).

Our findings obtained with mouse- and human-derived DCs are complemented by the experiments performed with human neutrophils. This cell type has been shown to express high levels of TLR2 and low levels of TLR4 on the surface (Kurt-Jones et al., 2002). In line with the results observed in DCs, PP SAB and EC SAB, although to a lesser extent, were found to augment neutrophil function as assessed by the release of IL-8, phagocytosis and degranulation. Thus, factors stimulated by *A. fumigatus* directly activate anti-*Aspergillus* effector functions of neutrophils and induce the release of IL-8 to attract further effector cells to the site of infection.

In conclusion, our results clearly indicate that *A. fumigatus* antigens induce the activation of immature DCs and granulocytes. According to our results, this activation

is mediated via TLR2 and TLR4. Future studies are needed to assess the clinical impact of these findings in patients at high risk for IA, such as recipients of an allogeneic SCT.

4

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THE ENDOPLASMIC RETICULUM-RESIDENT HEAT SHOCK PROTEIN GP96 ACTIVATES DENDRITIC CELLS VIA THE TOLL-LIKE RECEPTOR 2/4 PATHWAY

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The heat shock protein Gp96 has been shown to induce specific immune responses. On one hand, this phenomenon is based on the specific interaction with CD91 that mediates endocytosis and results in major histocompatibility complex class I-restricted representation of the Gp96-associated peptides. On the other hand, Gp96 induces activation of professional antigen-presenting cells, resulting in the production of pro-inflammatory cytokines and up-regulation of costimulatory molecules by unknown mechanisms. In this study, we have analyzed the consequences of Gp96 interaction with cells expressing different toll-like receptors (TLR) and with bone marrow-derived dendritic cells from mice lacking functional TLR2 and/or TLR4 molecules. We find that the Gp96-TLR2/4 interaction results in activation of nuclear factor κB-driven reporter genes and mitogen- and stress-activated protein kinases and induces IκBα degradation. Bone marrow-derived dendritic cells of C3H/HeJ and more pronounced C3H/HeJ / TLR2$^{-/-}$ mice fail to respond to Gp96. Interestingly, activation of bone marrow-derived dendritic cells depends on endocytosis of Gp96 molecules. Our results provide, for the first time, the molecular basis for understanding the Gp96-mediated activation of antigen-presenting cells by describing the simultaneous stimulation of the innate and adaptive immune system. This feature explains the remarkable ability of Gp96 to induce specific immune responses against tumors and pathogens.

The author of this thesis performed all experiments resulting in figures 1, 2, 3, 4, 6, and 7 and contributed substantially to figure 5 (together with Harpreet Singh and Silvia Herter), and, together, with Norbert Hilf, to the concept of this work.

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Introduction

The immunogenic potential of heat shock proteins (HSPs) is a well-established phenomenon first observed by Srivastava during the immunotherapy of mouse tumors (reviewed in Srivastava, 1991). Specificity of the immune response is based on peptides that associate with HSPs as a consequence of their function as molecular chaperones (Srivastava et al., 1998; Schild et al., 1999). Tumor-specific protection is mediated by $CD8^+$ T cells as shown by *in vivo* cell depletion studies (Udono et al., 1994) and by the ability to generate cytotoxic T lymphocyte cell lines specific for a variety of antigens from mice immunized with HSP molecules (Blachere et al., 1993; Arnold et al., 1995; Suto and Srivastava, 1995). The HSPs that mediated this effect include the cytosolic HSP70 and HSP90 and the endoplasmic reticulum-resident chaperones calreticulin and Gp96 (Basu et al., 2001; Udono and Srivastava, 1994). Furthermore, Gp96 molecules have been shown to induce cytotoxic T lymphocyte cross-priming against viral and minor histocompatibility antigens, supporting the hypothesis that Gp96 molecules are associated with a large repertoire of peptides not influenced by the cellular major histocompatibility complex expression (Arnold et al., 1995; Suto and Srivastava, 1995). HSP molecules can also provide an immunogenic context to synthetic peptides complexed to HSP70 or Gp96 molecules *in vitro* (Blachere et al., 1997; Rock et al., 1990). Because of this, HSP molecules have been called adjuvants of mammalian origin (Srivastava et al., 1998). Recently, progress has been made in understanding the mechanisms that contribute to the efficient induction of immune responses against HSP-associated peptides.

A receptor responsible for the uptake of HSP-peptide complexes has been identified as the α_2-macroglobulin receptor CD91, expressed on professional antigen-presenting cells (APCs) (Binder et al., 2000b; Basu et al., 2001). Only receptor-mediated endocytosis has been shown to result in the representation of HSP-associated peptides by MHC class I molecules, thus explaining the high efficiency of this process (Singh-Jasuja et al., 2000b). However, the ideal adjuvant should not only target the antigen to professional APCs, it also should induce APC activation to provide the proper costimuli required for efficient induction of the immune response. For HSPs, especially HSP70, HSP90 and Gp96, this ability has been demonstrated recently. The

exposure of macrophages or DCs to HSPs resulted in the upregulation of major histocompatibility complex class II and costimulatory molecules as well as in tumor necrosis factor α and IL-12 secretion (Asea et al., 2000; Singh-Jasuja et al., 2000a; Basu et al., 2000). The contribution of this mechanism in situations of physiological relevance is supported by the observation that necrotic but not apoptotic cell death leads to the release of HSPs (Basu et al., 2000; Berwin et al., 2001), thus activating the innate arm of the immune system to attract cells equipped with antigen specific receptors.

The molecular basis for this process, however, has not been understood thus far. Studies investigating the stimulatory effect of HSP60 on epithelial cells and HSP60 and HSP70 on macrophages demonstrated the involvement of CD14 molecules, suggesting the participation of Toll-like receptors (TLRs) (Chen et al., 1999). In *Drosophila*, Toll participates, in addition to the induction (coordination) of dorsal-ventral patterning during embryogenesis, in the defense against fungi by the induction of drosomycin secretion as an early form of innate immune responses against infection (Lemaitre et al., 1996). In mammals, TLRs are involved in the response to pathogens by the recognition of so-called pathogen-associated molecular patterns. These include lipopolysaccharide (LPS), peptidoglycans, and lipoproteins and bacterial CpG-DNA, which are recognized by TLR4, TLR2, and TLR9, respectively (Aderem and Ulevitch, 2000; Hemmi et al., 2000). The TLR signaling pathway shares most components with the IL-1 receptor signaling pathway responsible for activation of the innate immune system (Aderem and Ulevitch, 2000).

Recently, HSPs have been linked to TLRs by the observation that HSP60 failed to activate TLR4 defective macrophages from C3H/HeJ mice (Ohashi et al., 2000). Subsequently, it was shown that genetic complementation of nonresponder cells with TLR4 or TLR2 restores responsiveness (gain of function) to HSP60 while $TLR2^{-/-}$ or TLR4 deficient cells exhibit a “loss of function”. Surprisingly, macrophage activation was equally well induced by bacterial and endogenous mammalian HSP60. Thus, the presence of molecular patterns that interact with members of the TLR family is not limited to pathogen-derived molecules. Because of this, HSP60 has been proposed to serve as a danger signal for the innate immune system (Chen et al., 1999). HSP60 is not associated with antigenic peptides (Arnold-Schild and Schild, unpublished

observation) and is found much earlier in phylogeny, as are the TLR and IL-1 receptor families. Therefore, it remains unclear whether the HSP/TLR pathway is used solely by the innate immune system to fight pathogens by unspecific mechanisms or whether HSPs with peptide binding ability, such as Gp96, also use this mechanism to link nonspecific immunostimulatory capacities with their specific, peptide-based features with the activation of the adaptive immune system.
In this study, we decided to analyze the functional consequences of the interaction of Gp96 with members of the TLR family.

Materials and Methods

Reagents, antibodies, and plasmids

Gp96 and FITC-labeled Gp96 were provided by Immatics Biotechnologies (Tübingen, Germany). LPS from *Salmonella minnesota* RE 595, monodansylcadaverine (MDC), and anisomycin were purchased from Sigma-Aldrich. Phosphothioate stabilized CpG oligonucleotide 1668 (TCCATCACGTTCCTGATGC) was purchased from TIB MOLBIOL (Berlin, Germany).
Antibodies to ERK1/2 were obtained from Upstate Biotechnology (Lake Placid, NY), and other antibodies used in cell signaling studies, including anti-phospho-JNK1/2 (Thr^{183}/Tyr^{185}), anti JNK1/2, anti-phospho-p38 (Thr^{180}/Tyr^{182}), anti-p38, anti-IκB-α, anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-ERK1/2, anti-phospho-STAT1 (Tyr^{701}) and anti-STAT1, were from New Engalnd Biolabs (Frankfurt, Germany). Antibodies for fluorescence-activated cell-sorting analysis were purchased from BD PharMingen.
The expression vectors for the NH_2 terminus of human FLAG-tagged TLR2 and TLR4 were gifts from Tularik, Inc. (South San Francisco, CA). The human MD2 expression vector was kindly provided by K. Miyake (Sage Medical School, Nabeshima, Japan). The luciferase reporter driven by a synthetic enhancer harboring six NF-κB binding consensus sites was a gift from P. Baeuerle (München, Germany).

Analysis of signaling pathways in RAW264.7 cells

The mouse macrophage cell line RAW264.7 (purchased from American Type Culture Collection, Manassas, VA) was grown in VLE-RPMI medium (Biochrom KG, Berlin, Germany) supplemented with 100 IU/ml penicillin/streptomycin (Biochrom KG) and 10% fetal calf serum (Biochrom KG). Before stimulation, cells were incubated with serum-free medium for 2-4 h. Stimulation was performed for the indicated time period by the addition of 10 or 100 nM Gp96, 100 nM Gp96 pretreated at 95°C for 20 min or 2 µM CpG oligonucleotide 1668.

Cells were then lysed in lysis buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Trition X-100, 10 mM pyrophosphate, 20 mM β-glycerophosphate, 1 mM orthovanadate, 10 mM sodium fluoride, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride. Lysates were boiled in SDS sample buffer, sonicated, and centrifuged at 10,000 x g for 10 min. Electrophoresis of the lysates was carried out by 10% SDS-PAGE and Western blotting was performed using Protran nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked in 5% skim milk solution, probed with the indicated antibodies and visualized using Renaissance Chemiluminescence Reagent (PerkinElmer Life Sciences).

When indicated 100 µM MDC was added 20 minutes before stimulation. All following steps were performed as described above.

Luciferase reporter assay in 293T human embryonic kidney fibroblasts

293T human embryonic kidney fibroblasts were cultured in Dulbecco's modified Eagle's medium (Biochrom KG) supplemented with 100 IU/ml penicillin/streptomycin (Biochrom KG) and 10% fetal calf serum (Biochrom KG). For luciferase reporter assays, 5-10 x 10^6 cells were transfected with 1 ng 6x NF-κB luciferase reporter and 10 ng TLR2 or 10 ng TLR4 plus 10 ng MD2 plasmid DNA. The overall amount of plasmid DNA was held constant at 20 µg/electroporation by the addition of empty expression vector. Cells were electroporated in a final volume of 400 µl (RPMI-25% fetal calf serum) at 200 V and 960 microfarads (Gene Pulser, Bio-Rad). After electroporation, cells were washed and cultured in 6-well plates. Cells were subsequently stimulated with 100 nM Gp96, 500 nM Gp96, 500 nM Gp96 pre-treated

at 95°C for 20 min, or 100 ng/ml LPS overnight in serum-free medium. Cell lysis and measurement of luciferase activity in extracts was performed with the Luciferase Assay System Kit from Promega (Mannheim, Germany) according to manufacturer's instructions.

Generation of mouse dendritic cells

Mouse immature DCs were generated from bone marrow of C3H/HeN, C3H/HeJ, C3H/HeJ/TLR2$^{-/-}$, 129Sv/C57BL/6 and TLR2$^{-/-}$ mice. TLR2$^{-/-}$ mice were obtained from Tularic Inc. (South San Fransisco, CA). All other mice were obtained from Charles River (Sulzfeld, Germany). For the generation of mouse bone marrow-derived DCs, we used Iscove's modified Dulbecco's medium (BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine (Invitrogen), 100 IU/ml penicillin/streptomycin (Invitrogen), 10% fetal calf serum (PAA, Linz, Austria) and 200 U/ml granulocyte macrophage colony-stimulating factor. Bone marrow cells were incubated in granulocyte macrophage colony-stimulating factor-containing medium for 6-8 days, and medium was replaced with fresh granulocyte macrophage colony-stimulating factor-containing medium every 2 days. The obtained DCs were CD11c-positive and CD14-negative.

Generation of human dendritic cells

Human DCs were prepared from freshly drawn blood from healthy donors. Peripheral blood mononuclear cells were isolated using a Ficoll density gradient (Lymphoprep; Nycomed, Oslo, Norway). The obtained cells were washed twice with phosphate-buffered saline and resuspended in X-Vivo 15 medium (Bio-Whittaker, Walkersville, MD, USA) supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Peripheral blood mononuclear cells were plated at a density of $20x10^6$ cells/well. After 2 h at 37 °C, nonadherent cells were removed by washing with phosphate-buffered saline. Adherent monocytes were cultured for 6 days in medium supplemented with 1% (v/v) human serum (Peel-Freez, Brown Deer, WI, USA), 1000 U/ml IL-4 (R&D Systems, Minneapolis, USA) and 20 ng/ml granulocyte macrophage

colony-stimulating factor (Leukomax; Novartis Pharma GmbH, Nürnberg, Germany). The differentiation state of DCs was examined by flow cytometry. Only immature DCs that were $CD1a^{+}$, $CD14^{-}$, $CD83^{-}$ and $CD86^{low}$ were used for activation experiments. The fraction of activated DCs analyzed by CD83 expression was always <5 %.

Stimulation of DCs

Mouse DCs were stimulated by the addition of 1 µM Gp96, 1 µM heat pre-treated Gp96, or 2 µg/ml LPS. After 24 h, IL-12 (p40) and IL-10 concentrations in the supernatants were measured using standard sandwich enzyme-linked immunosorbent assay protocols. Antibodies and recombinant standards of both cytokines were obtained from BD PharMingen (Heidelberg, Germany). The capture antibody was bound to the enzyme-linked immunosorbent assay plate (MaxiSorb™; Nunc, Roskilde, Denmark), the biotinylated detection antibody was revealed by streptavidin-conjugated horseradish peroxidase and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma), and the assay read at 405 nm. Furthermore, on day 2 after activation, expression of the costimulatory molecule CD86 was measured by flow cytometry (FACSCalibur™; BD PharMingen). Isotype controls of antibodies were used in all experiments to determine the appropriate background fluorescence.

For experiments in the presence of MDC, human monocyte-derived immature dendritic cells (day 7) were incubated with 0.5 µM or 1.0 µM Gp96, 1.0 µM Gp96 heat-inactivated at 95°C for 20 min, or 2 µg/ml LPS in 0.5% Me_2SO ± 250 µM MDC for 16 h. Supernatants were assayed for tumor necrosis factor α and IL-12 by sandwich enzyme-linked immunosorbent assay as described above.

Confocal microscopy

Human monocyte-derived dendritic cells (day 7) were seeded on cover slips. The DCs were precooled and incubated for 30 min on ice with Iscove's modified Dulbeccos's medium containing 10% fetal calf serum and 100 µg/ml Gp96 -FITC („pulse") in 0.5% Me_2SO ± 250 µM monodansylcadaverine (MDC). The coverslips were washed

twice and fixed immediately or incubated in Iscove's modified Dulbeccos's medium for 15 min at 37°C („chase"). Fixation was done in methanol/acetone (ratio, 1:1) at –20°C. For confocal microscopy, a Zeiss LSM 510 laser scanning microscope was used. Thickness of the optical plane was adjusted by the pinhole to be < 1 µm.

Results

Gp96 interacts with TLR2 and TLR4

Among the members of the TLR family, the ligands for TLR2, TLR4 and TLR9 have been studied in considerable detail. Interestingly, HSP60 has been shown to activate macrophages via TLR4 (Ohashi et al., 2000). Inspired by these observations, we investigated the potential of these TLRs to trigger Gp96-mediated APC activation (Singh-Jasuja et al., 2000b; Basu et al., 2000). For this purpose, we incubated 293T human embryonic kidney fibroblast transiently transfected with different TLRs and the luciferase reporter driven by synthetic enhancer containing NF-κB binding consensus sites with Gp96 or LPS as a control. As shown in Fig. 1, the expression of TLR2 and also TLR4/MD-2 conferred responsiveness to the Gp96 stimulus in a dose-dependent manner. Boiling of Gp96 abolished the induction of luciferase activity, thus demonstrating that possible endotoxin contaminations in the Gp96 preparation were not responsible for the observed effect. Likewise, the presence of polymyxin B (an LPS inhibitor) did not interfere with Gp96-mediated activation (data not shown).

TLR4-mediated Gp96 activation is dependent on the presence of MD2 as transfection with TLR4 or MD2 alone did not result in the induction of luciferase activity (Fig. 1). LPS mediates NF-κB-driven luciferase induction via TLR2 and TLR4. This finding is in line with previous reports showing that in addition to TLR4-mediated activation by LPS, other endotoxin contaminations present in commercially available LPS also mediate APC activation via TLR2 (Hirschfeld et al., 2000). Transfection of TLR3, TLR7, TRL8, and TLR9 did not confer responsiveness to the Gp96 stimulus (stimulation indices between 1.2 and 1.5; data not shown).

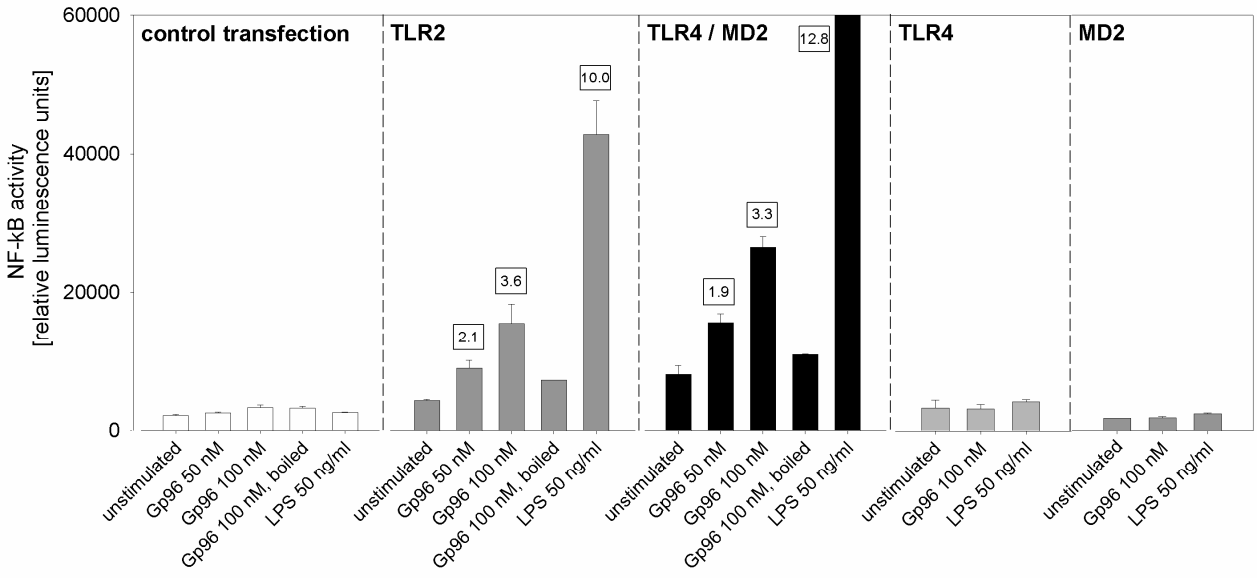

Figure 1. Gp96 activates cells via TRL2 and TLR4. 293T human embryonic kidney fibroblasts were transiently transfected with the indicated Toll-like receptors. At the same time, they were cotransfected with luciferase reporter driven by a synthetic enhancer harboring NF-κB binding consensus sites. Luciferase activity was measured after stimulation by Gp96, boiled Gp96, and LPS. Only cells transfected with TLR2 or TLR4 plus MD2 respond to Gp96 activation; cells without Toll-like receptor transfection did not respond to Gp96 activation. Each error bar represents the deviation of duplicates. The results are representative of three independent experiments. In some cases, the fold induction of luciferase activity relative to the unstimulated cells is indicated.

Gp96 mediates DC activation via TLR4 and TLR2

To analyze the contribution of TLR2 and TLR4 under more physiological situations, we studied the Gp96-mediated activation of bone marrow-derived DCs (BMDCs) from mice lacking functional TLR2 or TLR4 molecules or both. In the first set of experiments, we investigated the secretion of the pro-inflammatory cytokine IL-12 in response to Gp96, LPS, and CpG DNA. As shown in Fig. 2, BMDCs from C3H/HeN, $TLR2^{-/-}$, and TLR2 wild-type but not C3H/HeJ (a TLR4 deficient mouse) and C3H/HeJ/$TLR2^{-/-}$ ($TLR2^{-/-}$/TLR4-deficient) mice responded to the Gp96 stimulus by secretion of IL-12. CpG DNA induced IL-12 secretion in all cultures, and LPS-mediated activation was impaired in BMDCs from C3H/HeJ and C3H/HeJ/$TLR2^{-/-}$ mice, in line with previous reports. Again, boiled Gp96 did not induce any stimulation. The lack of TLR2 did not affect Gp96-mediated IL-12 secretion from BMDCs.

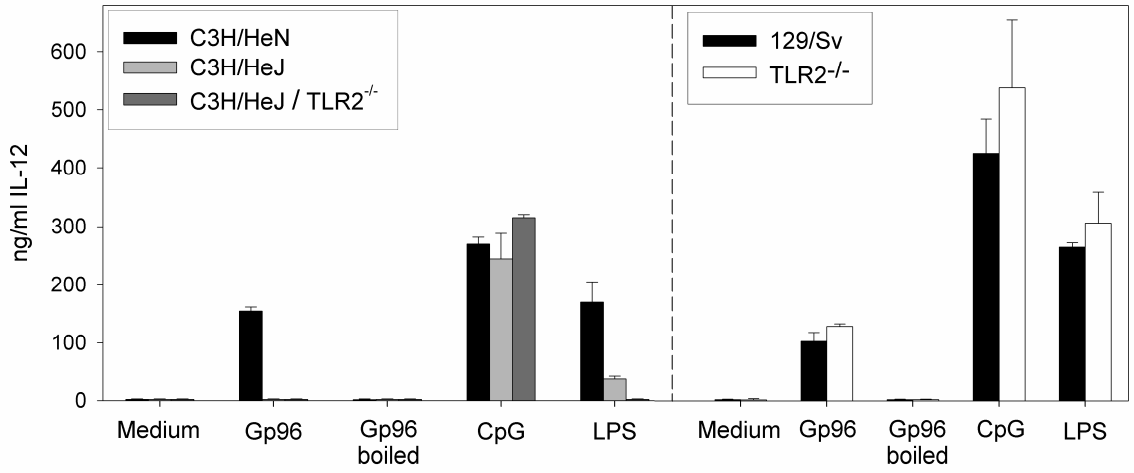

Figure 2. Gp96 -induced IL-12 production by dendritic cells is TLR4-dependent. Bone marrow-derived dendritic cells from the indicated mouse strains were cultured in the presence of Gp96 (1 µM), boiled Gp96, CpG (oligonucleotide 1668, 2 µM), or LPS (2 µg/ml). After 20 h, IL-12 concentration in cell culture supernatant was measured by sandwich enzyme-linked immunosorbent assay. The mean value of triplicates is shown, and error bars represent standard deviation.

During Gp96-mediated DC activation, secretion of IL-12 is accompanied by the up-regulation of the costimulatory molecule CD86 (Singh-Jasuja et al., 2000b; Basu et al., 2000). Therefore, CD86 expression in mice lacking TLR2 and/or TLR4 molecules was investigated. As observed for the secretion of IL-12, BMDCs from C3H/HeN, TLR2$^{-/-}$, and TLR2 wild-type mice but not from C3H/HeJ and C3H/HeJ/TLR2$^{-/-}$ mice up-regulated CD86 molecules after 48 h coculture with Gp96.

Fig. 3A shows CD86 up-regulation for one C3H/HeN mouse and one C3H/HeJ mouse; Fig. 3B represents mean values of CD86 up-regulation of three individual mice. This effect was again heat-sensitive. CpG-DNA induced CD86 up-regulation on all BMDCs, whereas LPS was impaired in BMDCs from C3H/HeJ and TLR2/4$^{-/-}$ mice. A minimal up-regulation of CD86 molecules in C3H/HeJ mice compared with TLR2/4$^{-/-}$ mice was observed in three independent experiments and might be caused by the interaction of Gp96 with TLR2 as observed for the NF-κB driven luciferase induction reported in Fig. 1. In TLR2$^{-/-}$ mice, this effect can obviously be compensated by TLR4.

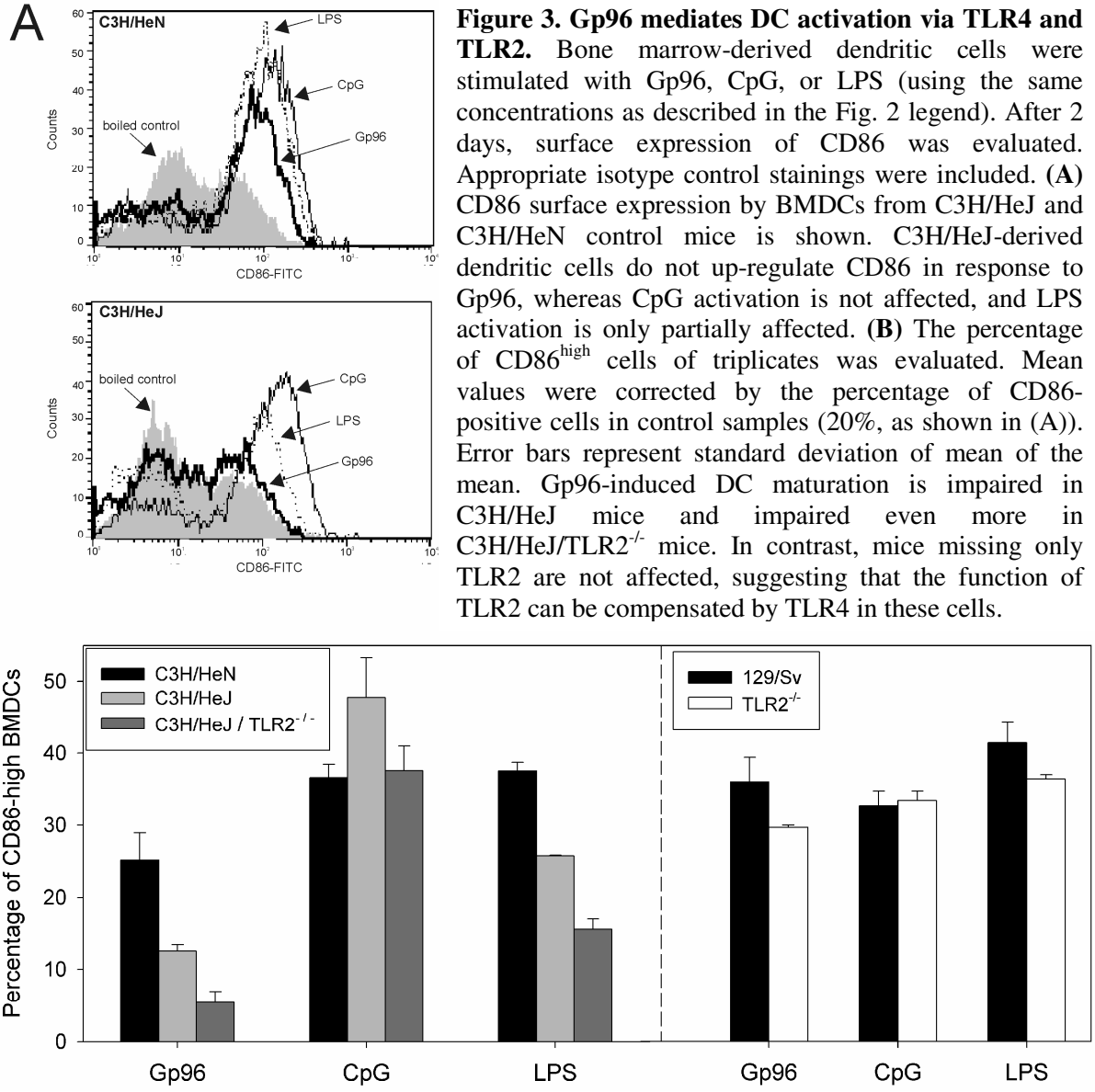

Figure 3. Gp96 mediates DC activation via TLR4 and TLR2. Bone marrow-derived dendritic cells were stimulated with Gp96, CpG, or LPS (using the same concentrations as described in the Fig. 2 legend). After 2 days, surface expression of CD86 was evaluated. Appropriate isotype control stainings were included. **(A)** CD86 surface expression by BMDCs from C3H/HeJ and C3H/HeN control mice is shown. C3H/HeJ-derived dendritic cells do not up-regulate CD86 in response to Gp96, whereas CpG activation is not affected, and LPS activation is only partially affected. **(B)** The percentage of $CD86^{high}$ cells of triplicates was evaluated. Mean values were corrected by the percentage of CD86-positive cells in control samples (20%, as shown in (A)). Error bars represent standard deviation of mean of the mean. Gp96-induced DC maturation is impaired in C3H/HeJ mice and impaired even more in C3H/HeJ/$TLR2^{-/-}$ mice. In contrast, mice missing only TLR2 are not affected, suggesting that the function of TLR2 can be compensated by TLR4 in these cells.

Gp96 activates classical signaling cascades

The interaction of Gp96 with TLR2 and TLR4 suggests that the activation of APCs involves the classical signaling cascades described for other TLR2 and TLR4 ligands (Hemmi et al., 2000; Kaisho and Akira, 2001).To analyze this issue, the macrophage cell line RAW264.7 was incubated with different concentrations of Gp96 and probed for the phosphorylation of JNK1/2, stress-activated protein kinase (SAPK) p38, the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) and the degradation of IκB-α as an indication for the activation of the NF-κB pathway. Fig. 4 shows that all kinases tested were activated. The Gp96 effect was again heat sensitive. CpG-DNA was used as a positive control.

DC activation requires endocytosis of Gp96

Because major histocompatibility complex class I-restricted representation of HSP70- as well as Gp96-associated peptides depends on receptor-mediated, clathrin-dependent endocytosis of these molecules and subsequent transport to multivesicular compartments (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000b), we investigated whether endocytosis of Gp96 might be a prerequisite for DC activation. MDC is an inhibitor of the membrane-bound transglutaminase and interferes with clathrin-mediated receptor trafficking as demonstrated for the α_2-macroglobulin receptor, CD91 (Davies et al., 1980). Interestingly, CD91 has been shown to be responsible for Gp96 uptake and representation of the associated peptides (Basu et al., 2001; Binder et al., 2000b) after receptor-mediated endocytosis (Singh-Jasuja et al., 2000b).

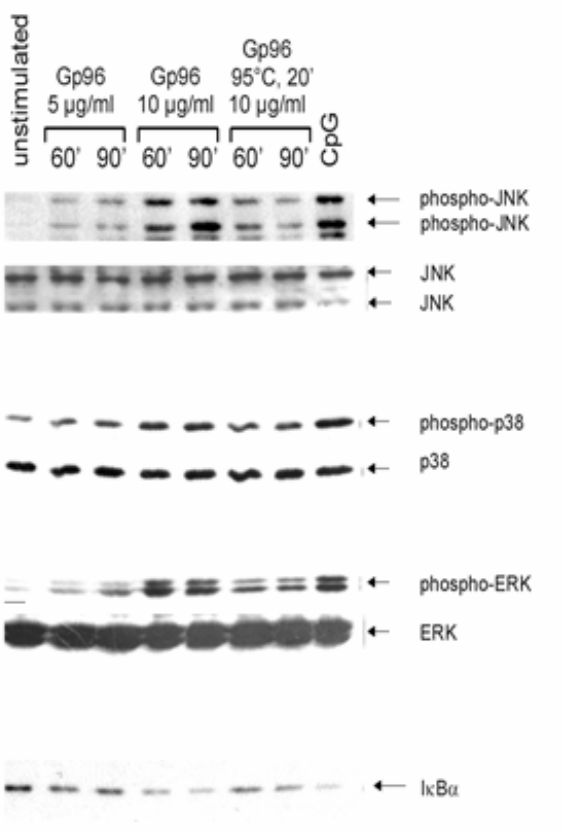

Figure 4. Gp96 triggers classical signaling cascades. RAW267.4 macrophages were incubated with different concentrations of Gp96 for 60 or 90 min and with 2 µg/ml CpG-DNA as a positive control for 30 min. Cells were lysed and the amount of phospho-JNK, phospho-p38, phospho-ERK and was determined by western blot. In addition, the total amount of JNK, p38 and ERK in lysates was determined. JNK1/2, p38 and ERK1/2 were activated upon stimulation with Gp96 in a time and concentration dependent manner. At the same time, the NF-κB pathway was triggered as indicated by the degradation of IκB-α. The stimulatory effect of Gp96 was heat sensitive.

Indeed, MDC was found to inhibit the endocytosis of FITC-labeled Gp96 molecules by DCs (Fig. 5). The presence of 0.5% Me_2SO did not influence Gp96 uptake compared with medium control lacking Me_2SO (data not shown). Therefore, the accumulation of Gp96-FITC at the cell membrane is due to the effect of MDC.

The lack of Gp96 endocytosis in the presence of MDC is accompanied by the lack of tumor necrosis factor α secretion (Fig. 6). As a control, LPS-mediated DC activation is not inhibited by MDC. These results also argue against an endotoxin contamination in the Gp96 preparation being responsible for the observed effect. Similar results were obtained for the Gp96-induced secretion of IL-12 (data not shown).

We next analyzed the effect of MDC on the signal transduction pathways and found that it also interfered with the phosphorylation of JNK1/2 (Fig. 7). The inhibitory effect of MDC was dose-dependent (data not shown) and did not influence the anisomycin-mediated phosphorylation of JNK1/2 (used as control). Thus, Gp96 mediates activation of DCs via TLR2 and TLR4 using the SAPK, MAPK, and NF-kB pathways in a process that requires endocytosis.

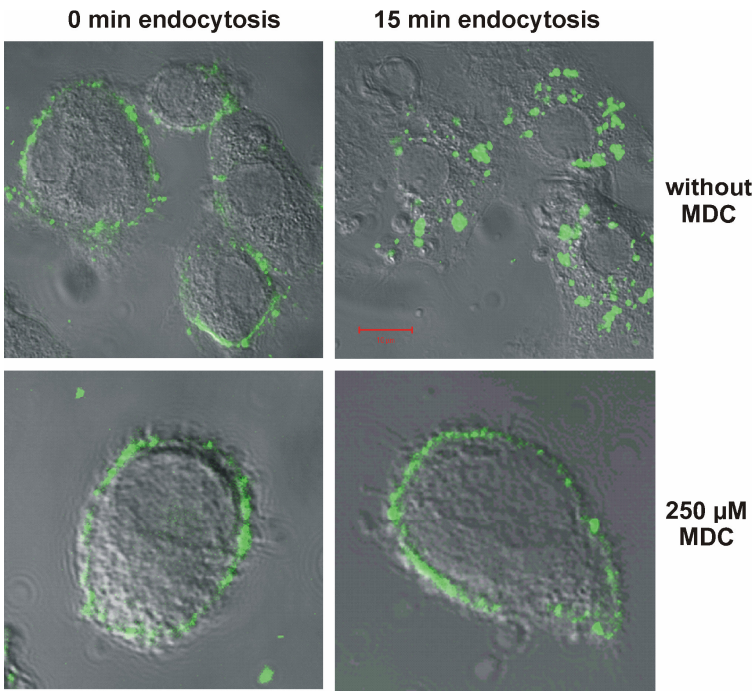

Figure 5. Monodansylcadaverine inhibits receptor-mediated endocytosis of Gp96. Human monocyte-derived immature dendritic cells were incubated with Gp96-FITC at 4°C and washed and fixed (no endocytosis) or treated at 37°C for an additional 15 min (endocytosis) and fixed with methanol/acetone. The confocal micrographs show an overlay of the transmission and fluorescence channels. In the presence of MDC, uptake of Gp96-FITC was substantially inhibited. MDC is an inhibitor of transglutaminase and has been shown to abrogate clathrin-dependent receptor-mediated endocytosis. Viability of cells (tested by trypan blue staining) in 0.5% Me_2SO and 250 µM MDC was approximately 95% and 90%, respectively. Representative sections are shown. Identical results were obtained in at least four independent experiments.

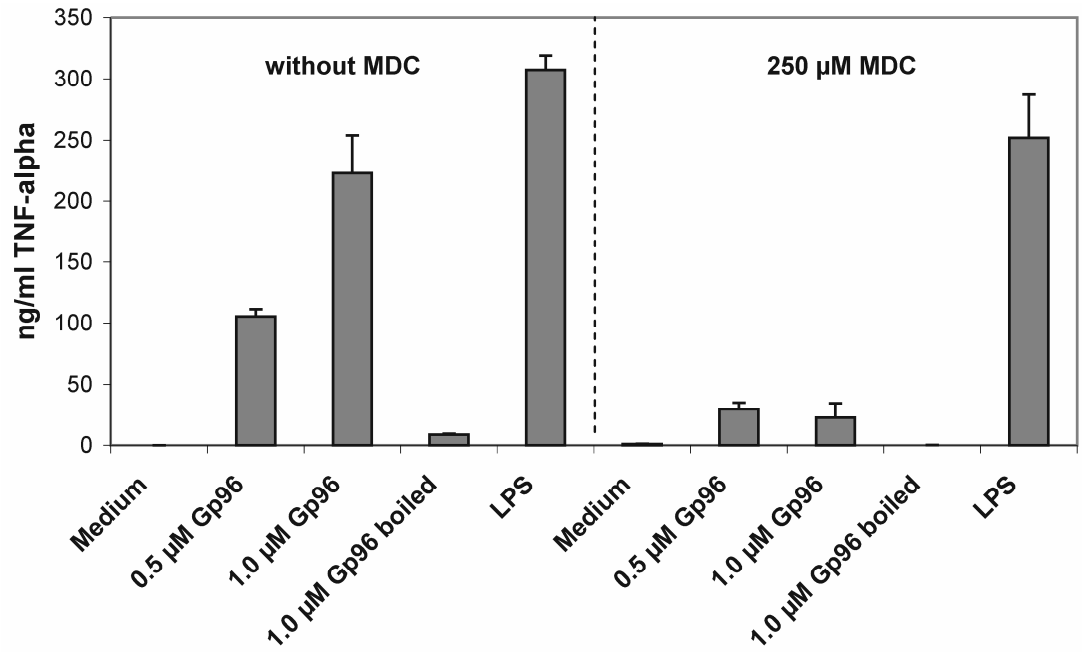

Figure 6. Receptor-mediated endocytosis is required for Gp96-mediated DC activation. Human monocyte-derived immature dendritic cells were activated by Gp96 and LPS in the presence and absence of 250 µM MDC. Boiled Gp96 was used as a control to exclude the possibility of endotoxin contaminations in the Gp96 preparation. MDC specifically inhibited Gp96-mediated DC activation, whereas the activity of LPS was unchanged in the presence of MDC. This demonstrates that receptor-mediated endocytosis is required for Gp96-mediated activation of dendritic cells. Error bars represent S.E.

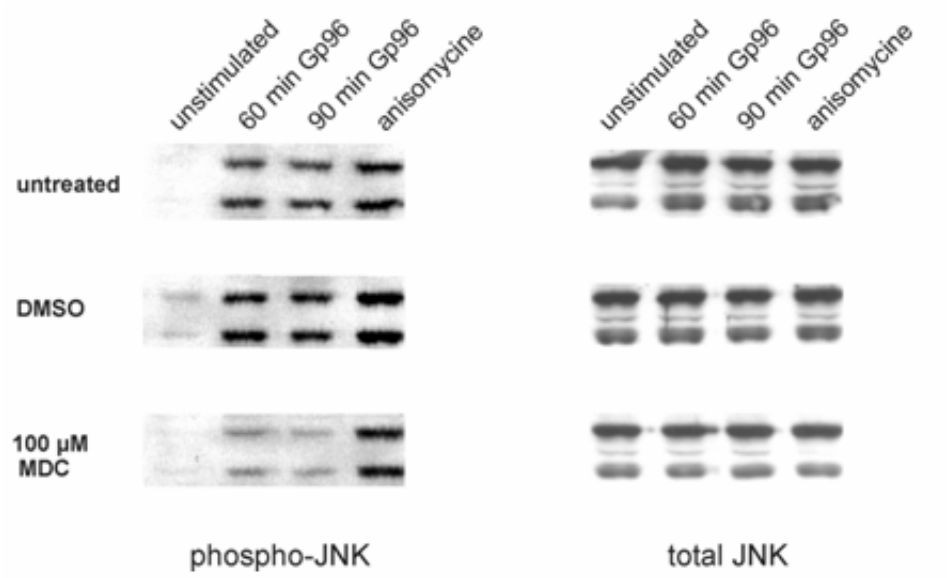

Figure 7. Endocytosis of Gp96 is required for activation of JNK. RAW267.4 macrophages were stimulated with different concentrations of Gp96 for 60 or 90 min or with 0.2 µg anisomycine for 10 min. Cells were incubated in either standard medium, medium containing 100 µM MDC, or medium containing Me_2SO matching the amount used for solvation of MDC. The assay of JNK was performed as described in Fig. 4. The stimulatory capacity of Gp96 is abrogated upon inhibition of endocytosis by MDC. Stimulation by anisomycine was unaffected by MDC treatment. The overall amount of JNK remained the same in all samples.

Discussion

We identified the molecular mechanism of APC activation by the endoplasmic reticulum-resident chaperone Gp96. We find that TLR2 and TLR4 mediate NF-κB-driven luciferase induction (Fig. 1) and that mainly TLR4 and, to a small extent, TLR2 are responsible for the up-regulation of CD86 and the secretion of IL-12 and tumor necrosis factor α (Figs. 2 and 3). IL-10 was not detectable (<1.5 ng/ml; data not shown), which is in line with the previously reported secretion of only pro-inflammatory cytokines (Singh-Jasuja et al., 2000b; Basu et al., 2000). TLR3, TLR7, TLR8, and TLR9 molecules appear not to be involved in Gp96-mediated signaling (data not shown). Expression of CD86 and cytokine secretion is preceded by the NF-κB pathway and activation of the SAPKs JNK1/2 and p38 as well as the mitogen-activated protein kinase ERK1/2 (Fig. 4). DC activation is inhibited by MDC, which interferes with receptor-mediated endocytosis (Figs. 5-7).

Despite the fact that Gp96 and LPS are very different molecules, they both mediate DC activation via TLR4 and TLR2. The nature of the pathogen-associated molecular pattern of HSPs that allows their specific interaction with TLR2 and TLR4 is unknown. We are currently investigating the possibility that Gp96 and microbial components interfere with each others in binding to both TLRs. However, regardless of their shared interaction with TLR2 and TLR4 molecules, several differences in their mode of action are apparent. The most striking observation is that the Gp96-mediated DC activation strictly depends on the endocytosis of Gp96, whereas LPS-mediated DC activation does not require endocytosis and thus functions in the presence of the endocytosis inhibitor MDC. As a consequence, Gp96 - but not LPS-mediated secretion of cytokines is impaired in the presence of MDC (Fig. 6). This, together with the observed sensitivity of Gp96-mediated DC activation to heat denaturation, also demonstrates that an endotoxin contamination in the Gp96 preparation does not account for the observed effects.

This is further supported by the observation that cells expressing Gp96 molecules targeted to their surface induce efficient DC maturation upon cell-to-cell contact (Zheng et al., 2001). Internalization of TLR2 during the activation of macrophages has been observed previously (Underhill et al., 1999). In this series of experiments, TLR2

was found to accumulate in phagosomes of macrophages activated with the yeast cell wall particle zymosan. Whether or not endocytosis was a prerequisite for activation was not investigated. Our results obtained with Gp96 as an agonist for TLR2 and TLR4 suggest this to be the case.

The importance of HSP endocytosis for macrophage activation has very recently been reported for the HSP60/TLR2/4-mediated activation of macrophages (Vabulas et al., 2001). Here, we observe the same effect for the new TLR2 and TLR4 ligand, Gp96, and extent. Our finding parallels the need for Gp96 endocytosis during receptor-mediated uptake and representation of Gp96-associated peptides (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000b) that is mediated through the interaction of Gp96 with CD91, the receptor for α_2-macroglobulin (Basu et al., 2001; Binder et al., 2000b). Interestingly, MDC has been first described as an inhibitor of endocytosis of the α_2-macroglobulin receptor (Davies et al., 1980).

An interesting scenario can be postulated from the above findings: similar to the proposed requirement of LPS-mediated activation for CD14 and LPS-binding protein on the cell surface (reviewed in Golenbock and Fenton, 2001), Gp96-mediated DC activation might depend on the presence of CD91 molecules that endocytose bound Gp96 molecules and subsequently mediate their transport to endocytic vesicles, as described previously (Arnold-Schild et al., 1999). This process will increase the local concentration of Gp96, now able to trigger signaling through TLR2 and TLR4 present in these vesicles (Wagner, 2001) by the recruitment of cytosolic MyD88 to the outer membrane of endocytic vesicles.

The contribution of TLR2 to Gp96-mediated DC activation is not clear. Whereas transfection of TLR2 induces NF-κB-driven luciferase activity (Fig. 1), $TLR2^{-/-}$ mice show normal Gp96-mediated DC activation profiles (Fig. 2 and Fig. 3b). On the other hand, Gp96-mediated DC activation in C3H/HeJ mice is always stronger than in $TLR2^{-/-}$/TLR4-deficient mice, which are not able to respond to a Gp96 stimulus at all. This observation suggests a minor contribution of TLR2. In $TLR2^{-/-}$ mice, the lack of TLR2 signaling can apparently be completely compensated by TLR4, but TLR2 can induce only minimal activation when TLR4 is not functional, as observed for BMDCs

from C3H/HeJ mice. One explanation could be an imbalance in the expression of TLR2 and TLR4 on BMDCs, favoring effects mediated by TLR4.

However, the interaction of both TLR molecules with Gp96 is strongly supported by the recent finding that the interaction of TLR2 and TLR4 with Gp96 inside the endoplasmic reticulum is crucial for the expression of these receptors on the cell surface (Randow and Seed, 2001).

Thus far, TLRs have been described as sensors for pathogen-associated molecular patterns crucial for the initiation of an innate immune response. These mechanisms were developed long before the adaptive immune system. One of the newest additions to the list of TLR ligands identified is HSP60. Interestingly, not only bacterial but also human HSP60 cross-react with TLRs (Vabulas et al., 2001). Our results now demonstrate that the exclusive association of TLRs with pathogen-associated molecular patterns is obsolete. Gp96 is not expressed in bacteria or fungi and provides the first example of a non-pathogen derived ligand of TLRs. More importantly, our results show for the first time how the innate and adaptive immune system can be stimulated simultaneously by the same molecule that is released under physiological situations from necrotic cells (Basu et al., 2000; Berwin et al., 2001). The importance of these TLR-mediated stimuli for the induction of T helper type 1-dominated immune responses has been observed recently using MyD88$^{-/-}$ mice (Schnare et al., 2001).

Gp96 has kept the ability (probably HSP60-derived) to stimulate APCs nonspecifically via TLRs but added a new function: to act as a carrier for antigenic peptides and to promote receptor-mediated uptake by professional APCs (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000b). The unique combination of both features now allows the major histocompatibility complex-restricted presentation of antigenic peptides to cells of the adaptive immune system in an immunostimulatory context and enables DCs to act as coordinators of innate and adaptive immune responses. Being able to understand these mechanisms will make it possible to interfere with the HSP-mediated activation of APCs and to rationally modulate immune responses towards either immunity or tolerance.

5

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GLYCOPROTEIN 96-ACTIVATED DENDRITIC CELLS INDUCE A CD8-BIASED T-CELL RESPONSE

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Heat shock proteins (HSPs) are able to induce protective immune responses against pathogens and tumors after injection into immunocompetent hosts. The activation of components of the adaptive immune system, including cytotoxic T lymphocytes (CTLs) specific for pathogen- or tumor-derived peptides, is crucial for the establishment of immunoprotection. HSPs acquire these peptides during intracellular protein degradation and when released during necrotic cell death, facilitate their uptake and MHC-restricted representation by professional antigen-presenting cells (APCs). In addition, the interaction of HSPs with APCs, including the ER-resident chaperone Gp96, induces the maturation of these cells by Toll-like receptor (TLR)-mediated signalling events. We now provide evidence that in contrast to LPS-mediated DC maturation, the interaction of Gp96 with dendritic cells (DCs) leads to the preferential expansion of antigen-specific CD8-positive T cells in vitro *and* in vivo. *This CD8 preference induced by mouse and human DCs did not correlate with enhanced levels of IL-12 secretion. Thus, despite the fact that both, LPS and Gp96 activate DCs in a TLR4-dependent manner, our experiments clearly demonstrate qualitative differences in the outcome of this maturation process which preferentially favours the expansion of CD8-positive T cells.*

The author of this thesis performed the experiments resulting in figure 1A and D and contributed significantly to the experiments of adaptive transfer reported in this chapter.

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Introduction

Antigenic peptides chaperoned by heat shock proteins have been described as potent tumor vaccines in animal models and are currently studied in clinical trials. Recently, a considerable number of new functions have been uncovered for particularly one heat shock protein, the ER-resident Glycoprotein 96 (Gp96, also known as Glucose-regulated protein 94, Grp94). Gp96 is not only a peptide carrier but also targets surface receptors on antigen-presenting cells, e.g. CD91 for efficient delivery of its peptide cargo into the MHC class I antigen processing pathway. This results in the receptor-mediated cross-presentation of the Gp96-associated peptides on MHC class I molecules and activation of cytotoxic T lymphocytes (Arnold-Schild et al., 1999; Wassenberg et al., 1999; Singh-Jasuja et al., 2000b; Binder et al., 2000b). Furthermore, Gp96 simultaneously activates APCs like dendritic cells *in vitro* (Singh-Jasuja et al., 2000a; Basu et al., 2000) and *in vivo* (Binder et al., 2000a) resulting in increased co-stimulatory activity and release of pro-inflammatory cytokines and nitric oxide (Panjwani et al., 2002). We have also demonstrated that maturation of dendritic cells by Gp96 requires the presence of Toll-like receptor 2 and 4 (Vabulas et al., 2002b). These results support the speculation that heat shock proteins like Gp96 function as local danger signals in response to cellular stress. This has been underlined by several observations: Gp96 is released during necrotic cell death and viral lysis but not after apoptosis (Basu et al., 2000; Berwin et al., 2001). Necrotic lysates from primary tumor tissue able to mature dendritic cells have been shown to be enriched of HSPs and the amount of HSPs in the lysates was critical for the ability of DC maturation (Somersan et al., 2001). At the same time, platelets efficiently bind Gp96, neutralizing its ability to activate DCs, a proposed regulatory mechanism confining the effective area of activating HSPs to the local tissue (Hilf et al., 2002). The APC-activating function of Gp96 is presumably an intrinsic capability independent of the associated peptides (Baker-LePain et al., 2002).

Previously, we have shown that dendritic cells activated by Gp96 exhibit an enhanced T cell stimulatory capacity demonstrated by *in vitro* proliferation assays with allogeneic T cells (Singh-Jasuja et al., 2000a). Investigating this phenomenon in more detail, we now find that human monocyte-derived and mouse bone marrow-derived

dendritic cells matured by Gp96 activate $CD8^+$ cytotoxic T cells rather than $CD4^+$ helper T cells *in vitro* as well as *in vivo*. On the other hand, DC maturation by LPS shows a preference for the expansion of $CD4^+$ T cells. We conclude that the interaction of Gp96 with DCs induces maturation signals that qualitatively differ from those mediated by LPS resulting in the induction of immune responses dominated by $CD8^+$ T cells.

Materials and Methods

Mice

C57BL/6 ($H2^b$, $CD90.2^+$) mice were obtained from Charles River. Congenic C57BL/6J-$Igh^aThy1^aGpi1^a$ ($H2^b$, $CD90.1^+$) mice were obtained from Jackson Laboratories. OT-I (Hogquist et al., 1994) and OT-II (Barnden et al., 1998) mice which have a transgenic T-cell receptor for the H2-K^b-restricted SIINFEKL peptide derived from $Ovalbumin_{257-264}$ or for the H2-A^b-restricted ISQAVHAAHAEINEAGR peptide derived from $Ovalbumin_{323-339}$, respectively, and St42 mice (transgenic T-cell receptor for H2-D^b-restricted Ad5-$E1A_{234-243}$ peptide) were obtained from the animal facility of the Leiden University Medical Center (The Netherlands) (den Boer et al., 2001).

Generation of DC

Mouse immature DCs were generated from bone marrow of C57BL/6 mice according to standard protocols (Inaba et al., 1992) in Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker, Verviers, Belgium) supplemented with 200 mM L-glutamine (GibcoBRL Life Technologies, Paisley, GB), 100 IU/ml penicillin/streptomycin (Gibco), 10% FCS (PAA, Linz, Austria) and cytokines as indicated below. Briefly, bone marrow cells were incubated with 150 U/ml granulocyte-macrophage colony stimulation factor (GM-CSF, PeproTech, London, GB) for 7 days with medium renewed every 2 days. Approximately 90-100% of all cells in the FACS gate used for monocytes were DCs determined by flow cytometry with antibodies (obtained from Pharmingen, San Diego, CA) to be $CD11c^+$, $CD14^-$, $CD86^{low}$ and H2-A^{b+}. Human

immature DCs were prepared from peripheral mononuclear blood cells (PBMC) according to Bender et al. (Bender et al., 1996) in X-Vivo 15 medium (Walkersville, USA) supplemented with 200 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 1% human serum (Peel-Freez, Brown Deer, WI, USA) and cytokines as indicated below. Briefly, monocytes isolated by Ficoll density gradient (Lymphoprep, Nycomed, Oslo, Norway) and plastic adherence were cultured in medium supplemented with 10 µg/ml interleukin-4 (IL-4, R&D Systems, Minneapolis, USA) and 50 µg/ml GM-CSF (Leukomax, Novartis Pharma GmbH, Nürnberg, Germany) for 6-8 days. The cells generated in this way showed a large number of dendrites up to day 12 and were only slightly adherent. They expressed CD1a, low CD14, low CD86, HLA-DR and very low CD83 on their surface as determined by different antibodies (from BD PharMingen) in flow cytometry (data not shown). All FACS analyses were performed on a FACSCalibur® (Becton Dickinson, Mountain View, CA) using Cell Quest Software.

Stimulation of DCs

Mouse BMDCs were stimulated by addition of Gp96 or heat-treated Gp96 (95°C for 20 min) or LPS (from *Salmonella typhimurium*, Sigma Chemicals, St. Louis, MO) for 6 h to 3 days. Gp96 was used at 100 µg/ml and LPS at 100 ng/ml unless indicated otherwise. Gp96 (kindly provided by Immatics Biotechnologies, Tübingen, Germany) was purified from the IGELa2 mouse cell line (tested mycoplasma-free). Endotoxin content in commercial Gp96 preparations was tested using a *Limulus* Amebocyte Lysate Kit (QCL-1000, BioWhittaker) according to the guidelines published by the US Food and Drug Administration. The endotoxin content determined in all cases was below 0.05 EU/µg Gp96.

Supernatants were taken after 18-24 h and mouse IL-12 (p40) was measured using standard sandwich ELISA protocols (antibodies and standards by BD PharMingen), streptavidin-conjugated horseradish peroxidase and 2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) substrate (Sigma). Furthermore, on day 3 after activation, expression of the co-stimulatory molecule CD86 was measured by flow cytometry (data not shown).

Human monocyte-derived, immature dendritic cells (day 7) were stimulated by Gp96, heat-treated Gp96, LPS or heat-treated LPS. Gp96 was used at 100 µg/ml and LPS at 100 ng/ml unless indicated otherwise. Supernatants were assayed for TNF-α or IL-12 by sandwich ELISA as described above (data not shown). On day 3 after activation, expression of co-stimulatory molecules CD83 and CD86 and MICA/B were measured by flow cytometry.

Stimulation of alloreactive T cells

Human DCs were stimulated with different activators in a 96 well plate for 3 days as described above, washed extensively and incubated with PBLs (ratio 1:10) from a different donor for 5 days. CD69 and MHC class II (data not shown) expression, both markers for human T cell activation, was determined on day 1 or 5, respectively, by flow cytometry.

Stimulation of St42 CD8 T cells in vitro

Immature BMDCs were activated as described above for three days, washed extensively and loaded with 1 µg/ml H2-D^b-restricted Ad5-$E1A_{234-243}$ peptide (sequence SGPSNTPPEI) for 1.5 h at 37°C. E1A peptide was synthesized on a ABI 432 A peptide synthesizer (Applied Biosystems) applying Fmoc chemistry. $2x10^4$ loaded BMDCs were washed four times and incubated with $1x10^6$ CFSE (carboxyfluorescein diacetate succinimide ester)-labelled splenocytes from St42 mice. 4 and 5 days later proliferation and intracellular IFN-γ production were measured by flow cytometry. Cells were labelled with CFSE by incubation with 1 µM CFSE in PBS at room temperature for 3 min in the dark and then washed three times with IMDM containing 10% FCS. For flow cytometric analysis intracellular IFN-γ staining was performed using the Cytofix/Cytoperm™ kit (BD Pharmingen, San Diego, CA) according to the instructions of the manufacturer using a phycoerythrin-labelled anti-mouse IFN-γ antibody (BD Pharmingen).

Adoptive Transfer

St42 adoptive transfer: $30x10^6$ CFSE-labelled $CD90.1^+$ heterozygous St42 spleen cells were injected i.v. into $CD90.2^+$ homozygous C57BL/6 on day 0. Transferred CSFE-labelled cells could be detected by flow cytometry in peripheral blood on day 1. On day 2, BMDCs from C57BL/6 mice activated with different stimulators as described above were loaded with 100 µg/ml H2-D^b-restricted Ad5-$E1A_{234-243}$ peptide or 100 µg/ml H2-D^b-restricted Ad5-E1B peptide (sequence VNIRNCCYI) as a negative control peptide for 1 h at 37°C and $4x10^5$ peptide-loaded BMDCs were injected i.p. into the mice. At days 6 and 8 cells from peripheral blood as well as lymph nodes were analyzed by flow cytometry.

OT-I/OT-II adoptive transfer was performed similar to the adoptive transfer of St42 mice except for the following changes: $30x10^6$ CFSE-labelled $CD90.2^+$ homozygous OT-I or OT-II spleen cells were injected i.v. into $CD90.1^+$ homozygous C57BL/6 mice on day 0. BMDCs from C57BL/6 mice were incubated with 100 ng/ml H2-K^b-restricted $Ova_{257-264}$ peptide or 10 µg/ml H2-A^b-restricted $Ova_{323-339}$ peptide for 1 h at 37°C.

Results

Dendritic cells are activated by Gp96

Bone-marrow derived dendritic cells (BMDC) were generated from C57BL/6 mice according to established protocols. Incubation of these immature dendritic cells with Gp96 for 3 days led to up-regulation of the co-stimulatory molecules CD80 and CD86 (data not shown, see Singh-Jasuja et al., 2000a) and release of pro-inflammatory cytokines like IL-12 and IL-6 (Fig. 1A). As lipopolysaccharides (LPS) from gram-negative bacteria are also described as potent TLR4-dependent DC stimulators, the possibility of LPS contaminations in the Gp96 preparation had to be addressed. For this, the following control experiments were performed: A) Gp96 and LPS were both boiled for 20 min prior addition to the cell culture medium. While the activity of Gp96 was completely lost by the heat treatment, the activity of LPS was not affected (Singh-Jasuja et al., 2000a). B) The addition of the endocytosis inhibitor monodansylcadaverine (MDC) abolished the ability of Gp96 but not of LPS to induce

DC activation. Thus, activation of DCs by Gp96 depends upon the endocytosis of Gp96 while activation by LPS is endocytosis independent (Vabulas et al., 2002b). C) Endotoxin levels in the Gp96 preparations by Limulus lysate assay were determined to be below 0.05 E.U./µg. Low amounts of LPS (0.5 ng/ml) which correspond to the level of endotoxin detected in Gp96 preparations were not able to mature dendritic cells (Fig. 1A). D) Fractions obtained from the chromatographic purification of Gp96 which contained no Gp96 but similar levels of endotoxin when compared to the Gp96-containing fraction did not lead to maturation of DCs (Fig. 1A).

Dendritic cells matured by Gp96 show enhanced stimulation of peptide-specific CD8 T-cells *in vitro*

To study the activation of peptide-specific T-cells, BMDCs from C57BL/6 mice were activated by Gp96, boiled Gp96 (both at 100 µg/ml) or LPS at 100 ng/ml. These concentrations were selected because they induce optimal BMDC activation as judged by the expression of CD80, CD86 and the production of the pro-inflammatory cytokines IL-6, IL-12 and TNF-α (data not shown). One day later, the activation status of the BMDCs was measured by determining IL-12 levels in the supernatant (Fig. 1D). 3 days after activation, these DCs were loaded with E1A peptide, washed extensively and co-cultured with CFSE-labelled spleen cells from St42 mice expressing transgenic T cell receptors specific for E1A peptide bound to H2-D^b molecules. The fluorescein-based dye CFSE is split among daughter cells during cell division, thus emission of CFSE-labelled cells is reduced with every round of proliferation (Weston and Parish, 1990). 4 days after T cell stimulation, proliferation of CD8 T cells was measured by determining the level of CFSE (Fig. 1B). On day 5, activation (by determining intracellular IFN-γ production) and proliferation of CD8 T cells in the culture were measured simultaneously (Fig. 1C). Thus, DCs matured by Gp96 induce a significantly stronger proliferation (Fig. 1B) as well as activation (Fig. 1C) of CD8 T cells from St42 mice compared to LPS-matured DCs. This was observed despite the fact that LPS-matured DCs produced higher levels of IL-12 (Fig.1D) or IL-6 and showed no difference in the expression of co-stimulatory molecules (data not shown).

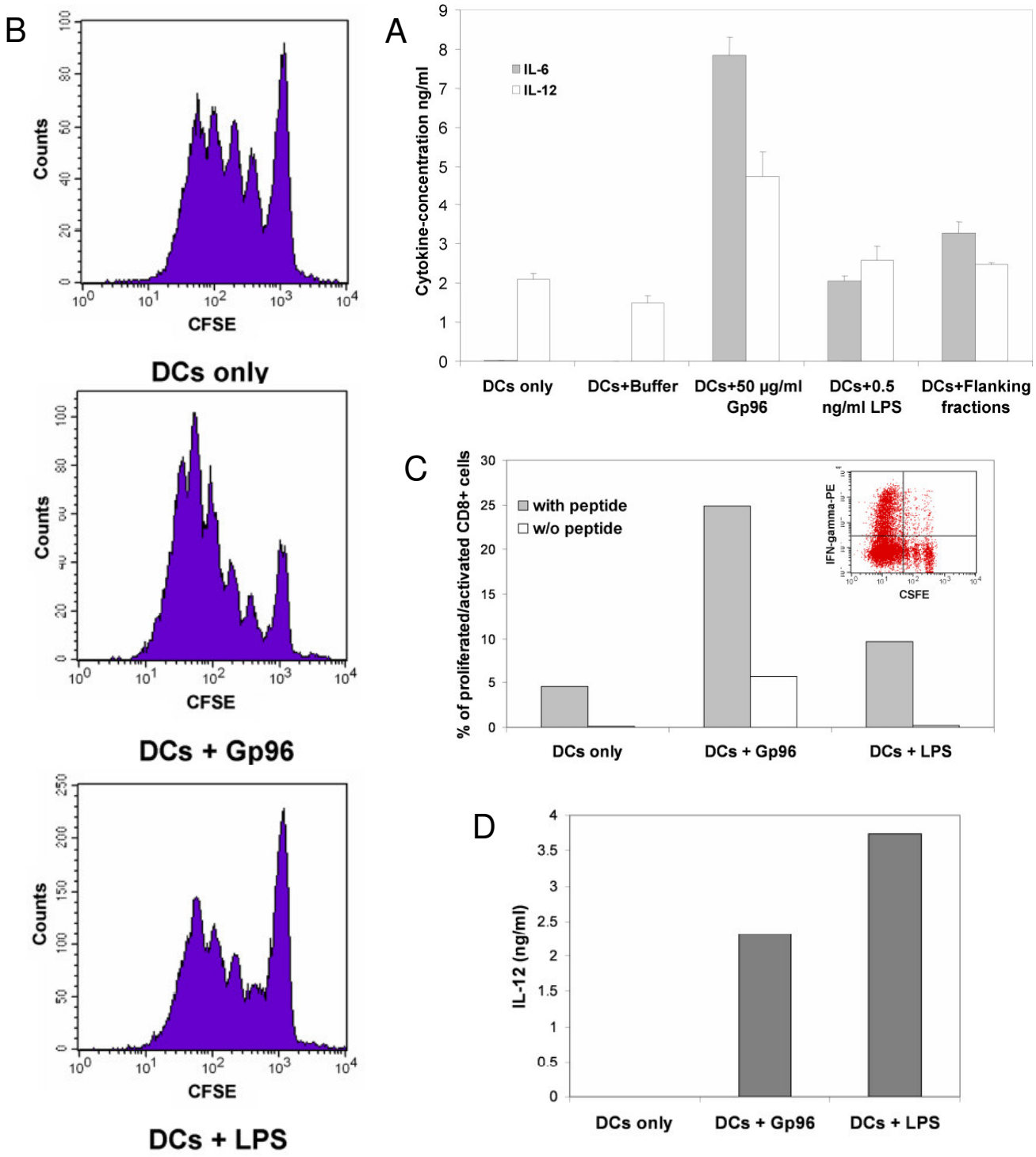

Figure 1. Mouse BMDCs matured by Gp96 induce peptide-specific proliferation and IFN-γ production by CD8 T cells *in vitro*. **(A)** Cytokine production by mouse BMDCs matured by Gp96 or control stimuli for 3 days. **(B)** Proliferation of St42 CD8 T cells *in vitro*. BMDCs from C57BL/6 mice were treated with Gp96 or LPS for 3 days and loaded with Ad5-E1A peptide to be used for co-culture with CFSE-labelled spleen cells from St42 mice. Proliferation of CD8 T cells was assayed by flow cytometric analysis on day 4 after T cell stimulation. **(C)** IFN-γ production of St42 CD8 T cells *in vitro*. The graph shows the percentage of intracellular IFN-γ production of the most proliferated CD8 T cells on day 5 (see insert). **(D)** IL-12 production of the BMDCs used for the *in vitro* activation of St42 T cells. The results are representative of three independent experiments.

Gp96-matured dendritic cells show enhanced stimulation of CD8 T cells *in vivo*

Next, we extended the results obtained for the proliferation of St42 T cells *in vitro* to an *in vivo* model. For this, CFSE-labelled spleen cells from St42 mice ($CD90.1^+$) were injected into the tail vein (i.v.) of $CD90.2^+$ C57BL/6 mice. 24 hours later the injected $CD90.1^+$, CFSE-labelled St42 T cells could be detected in the blood of $CD90.2^+$ mice and represented approximately 1% of all peripheral blood cells (data not shown). One day later, BMDCs matured by Gp96 or LPS for 6 hours or 3 days were loaded with E1A peptide or irrelevant E1B control peptide and injected intraperitoneally (i.p.) into these mice. 8 days after injection of St42 cells peripheral blood and draining lymph nodes were collected to analyze the proliferation of the CFSE-labelled $CD90.1^+$ St42 T cells in response to DC immunization. As shown in Fig. 2 *in vivo* proliferation of St42 T cells in response to BMDCs matured by Gp96 was significantly stronger compared to non-matured or LPS-matured DCs (Fig. 2A, B) although Gp96 and LPS-induced maturation of DCs was comparable as judged on the basis of IL-12 (Fig. 2C) or IL-6 production determined prior to DC injection. No proliferation of St42 T cells was observed without injection of DCs (Fig. 2B) or when DCs loaded with an irrelevant control peptide were injected (data not shown).

Mouse Gp96-matured DCs preferentially activate CD8 T cells vs. CD4 T cells *in vivo*

So far, we demonstrated that although Gp96 and LPS were able to mature BMDCs *in vitro* to a similar extent, Gp96-matured DCs induced a stronger CD8 T cell activation as LPS-matured DCs. To directly compare the effects of DCs matured by Gp96 or LPS on the proliferation of CD8 and CD4 T cells as well, the OT-I and OT-II transgenic mouse systems were selected. T cells from OT-I mice express a transgenic T cell receptor specific for an ovalbumin-derived MHC class I epitope while OT-II transgenic T cells recognize a MHC class II epitope from the same protein. Analogous to the experiments above using T cells from St42 mice, CFSE-labelled OT-I or OT-II spleen cells ($CD90.2^+$) were injected i.v. into C57BL/6 mice ($CD90.1^+$). 48 hours later Gp96- or LPS-matured BMDCs which were loaded with either the MHC class I or class II-restricted peptide from ovalbumin were injected i.p. into these mice. On day 8

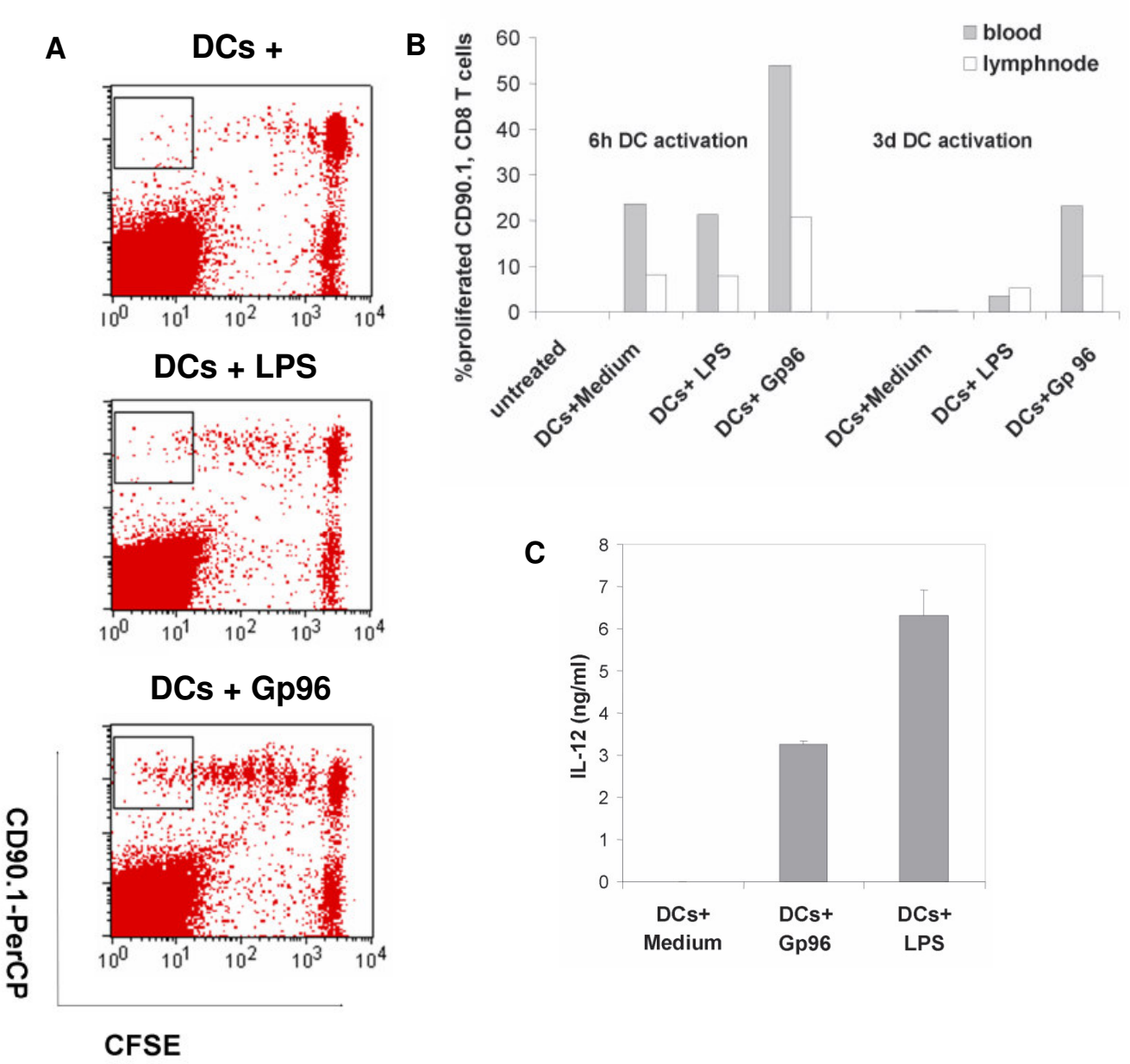

Figure 2. BMDCs matured by Gp96 induce peptide-specific proliferation of CTLs *in vivo*. On day 0 CFSE-labelled St42 spleen cells (CD90.1) were injected i.v. into C57BL/6 mice (CD90.2). BMDCs from C57BL/6 mice were matured by Gp96 or LPS for 6 hours or 3 days and loaded with Ad5-E1A peptide or Ad5-E1B peptide as a negative control. BMDCs were injected i.p. into C57BL/6 mice previously transferred with St42 splenocytes. On day 8 peripheral blood samples and draining lymph nodes were collected. **(A)** Proliferation of St42 CD8 T cells from lymph nodes 6 days after immunization with Ad5-E1A peptide-loaded DCs matured by Gp96 or LPS for 3 days. **(B)** Percentage of proliferated St42 CD8 T cells (gate as shown in (A)) after immunization with peptide-loaded BMDCs stimulated with Gp96 or LPS for 6 hours or 3 days as indicated. Untreated mice received St42 spleen cells only. (C) IL-12 production of BMDCs stimulated with Gp96 or LPS and used for immunization. Error bars give SD of triplicates. The results are representative of two independent experiments.

peripheral blood and lymph nodes were collected to analyze the proliferation of CFSE-labelled CD90.2$^+$ OT-I or OT-II cells. Although DCs were activated to similar levels by Gp96 or LPS as judged by the production of IL-12 *in vitro* (Fig. 3D), proliferation of the CD8$^+$ OT-I T cells was significantly stronger when Gp96-matured DCs were used in comparison to LPS-matured DCs (Fig. 3A, B). On the other hand, proliferation of CD4$^+$ OT-II T cells was more pronounced when LPS-matured DCs were used as stimulator cells (Fig. 3A, C). No proliferation was observed when DCs loaded with a control peptide (Fig. 3A, B, C) or PBS (Fig. 3B, C) were injected.

Human Gp96-matured dendritic cells preferentially stimulate CD8 T cells

Next, the preference for CD8 T cell activation by Gp96-matured DCs was analyzed using human cells. Monocyte-derived human dendritic cells were generated from buffy coats according to established protocols by plastic adherence and culturing of the purified monocytes with GM-CSF and IL-4 for 7 days. Incubation of these immature dendritic cells with Gp96 or LPS for 3 days led to the up-regulation of the co-stimulatory molecule CD86 and the maturation marker CD83 as well as to the release of pro-inflammatory cytokines like IL-12, TNF-α and IL-6 (data not shown). DCs activated by Gp96 or LPS at the concentrations indicated were co-cultured with allogeneic peripheral blood lymphocytes (PBL). Activation of the T cells was determined by the analysis of the activation markers CD69 on day 1 (Fig. 4) and MHC class II molecules on day 5 (data not shown) after DC co-culture. As expected, significantly less T cells were activated by immature dendritic cells ("Medium") compared to dendritic cells matured by Gp96 or LPS (see also (Singh-Jasuja et al., 2000a)). When analysing the expansion of CD8$^+$ versus CD4$^+$ T cells we observed that Gp96-matured DCs preferentially activated CD8$^+$ T cells. This preference was not observed when LPS was used for the maturation of DCs (Fig. 4). Reducing the amount of LPS or Gp96 resulted in a reduced overall T cell activation as evident from the lower numbers of CD69$^+$ T cells but did not influence the ratio of CD8$^+$ vs. CD4$^+$ T cells (Fig. 4 and data not shown).

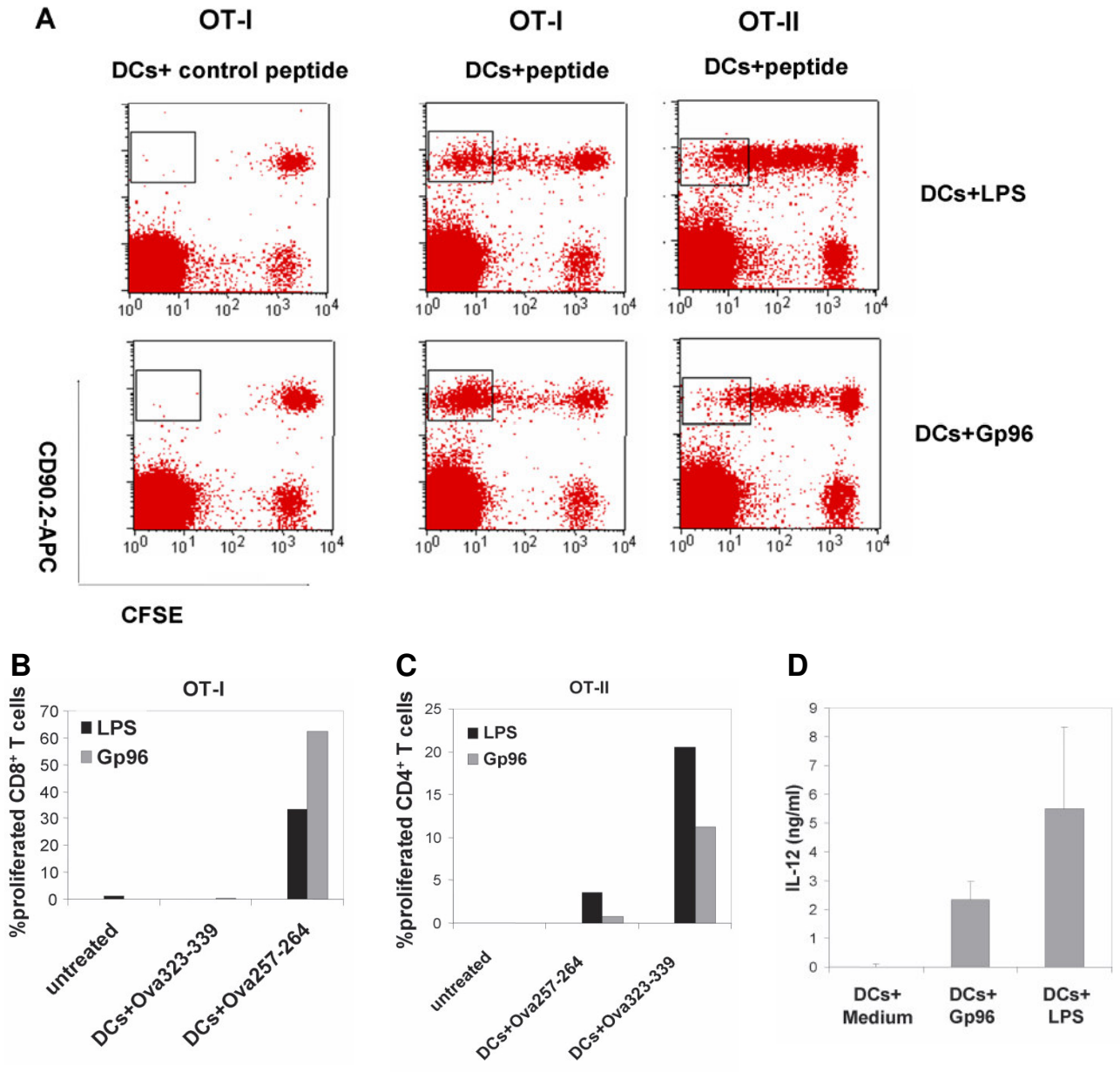

Figure 3. Mouse Gp96-matured DCs preferentially activate CD8 vs. CD4 T cells *in vivo*. On day 0 CFSE labelled OT-I or OT-II spleen cells ($CD90.2^+$) were injected i.v. into C57BL/6 mice ($CD90.1^+$). On day 2, BMDCs from C57BL/6 mice matured by Gp96 or LPS for 6 hours were loaded with H2-K^b-restricted $Ova_{257-264}$ peptide or H2-A^b-restricted $Ova_{323-339}$ peptide and injected i.p. into C57BL/6 mice previously injected with OT-I or OT-II spleen cells. On day 8 peripheral blood samples and draining lymph nodes were collected. **(A)** Proliferation of OT-I or OT-II T cells in lymph nodes. **(B, C)** Summary of the proliferation of OT-I and OT-II T cells *in vivo*. The graphs show the percentage of proliferation present in the gates shown in (A). Untreated mice received OT-I or OT-II spleen cells only. **(D)** IL-12 production of DCs matured by Gp96 and LPS. Error bars give SD of triplicates. The results are representative of two independent experiments.

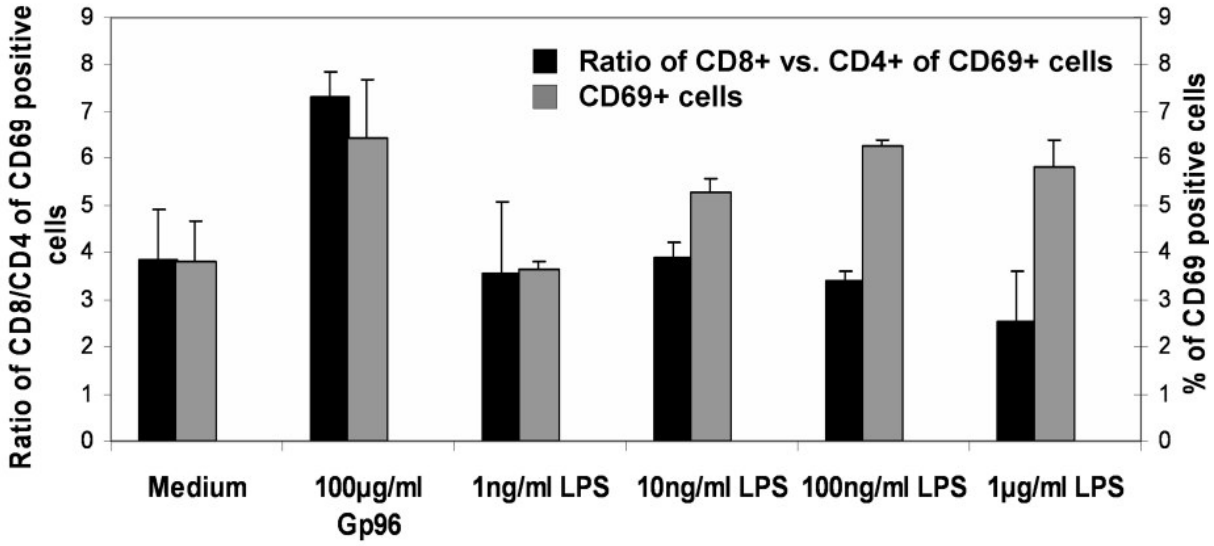

Figure 4. Human DCs matured by Gp96 preferentially induce activation of allogeneic CD8 T cells. Monocyte-derived human dendritic cells were incubated with Gp96 or LPS at the indicated concentrations for 3 days and then co-cultured with allogeneic PBL. The graph shows the percentage of CD69 expressing T cells (gray bars) and the CD8/CD4 ratio of CD69 positive T cells (black bars) 1 day after stimulation with matured DCs. Results are representative of two independent experiments.

Discussion

Heat shock proteins like Gp96 and Hsp70 have been shown to promote the maturation of BMDCs *in vivo* and *in vitro* (Singh-Jasuja et al., 2000a; Basu et al., 2000; Todryk et al., 1999) and to contribute to CTL activation (Srivastava et al., 1998; Schild et al., 1999; Schild and Rammensee, 2000a; Srivastava, 2002). This results in the secretion of pro-inflammatory cytokines (Fig. 1A) and the up-regulation of co-stimulatory molecules in a TLR2/4-dependent fashion (Vabulas et al., 2002a; Vabulas et al., 2002b; Asea et al., 2002).

In this work we analyzed whether BMDC activation by Gp96 induces adaptive immune responses that differ from those induced by LPS-activated BMDCs. The reason to compare these two stimuli is based on the fact that LPS is present during infections with gram-negative bacteria which will be controlled predominantly by antibody-dominated immune responses. Gp96, on the other hand, is released during necrotic cell death, for example as a consequence of viral infections (Berwin et al., 2001); the elimination of many viruses requires the activation of CTLs. Using BMDCs activated with either of the two stimuli we find that Gp96-mediated stimulation

promotes predominantly the activation and expansion of antigen-specific $CD8^+$ T cells *in vitro* (Fig. 1B and 1C) and *in vivo* (Fig. 2 and Fig. 3), whereas LPS-mediated stimulation favours the activation and expansion of antigen-specific $CD4^+$ T cells (Fig. 3). This bias can neither be explained by differences in the production of IL-12 nor IL-6 nor the expression of MHC nor co-stimulatory molecules (Fig. 1D, Fig. 2C, Fig. 3D; data not shown) by Gp96- or LPS-stimulated BMDCs. A similar observation is made for the activation of alloreactive $CD8^+$ T cells by human DCs stimulated by Gp96. Examining the ratio of activated $CD8^+$ versus $CD4^+$ T cells, we find that Gp96-activated human DCs unlike LPS-activated DCs preferentially stimulate the activation of $CD8^+$ T cells. The reduced activation of $CD8^+$ T cells by LPS-activated DCs does not depend on the amount of LPS used for DC activation. Varying the concentration of LPS changes only the number of $CD69^+$, activated T cells but does not affect the ratio of $CD8^+$ vs. $CD4^+$ T cells (Fig. 4).

Our findings suggest qualitative differences in the activation of DCs by Gp96 or LPS. However, the direct or indirect molecular mechanisms or interactions between Gp96-matured DCs and T cells responsible for this preference remain to be determined. We have compared the secretion of several pro-inflammatory cytokines, the up-regulation of various co-stimulatory molecules including CD80, CD86, CD137 (4-1BB ligand), the up-regulation of MHC class I vs. class II molecules and the induction of MIC-A/B molecules which are known to interact with NKG2D molecules present on the surface of $CD8^+$ but not $CD4^+$ T cells (Bauer et al., 1999) (data not shown). All of these molecules are not expressed at significantly different levels between Gp96- and LPS-matured DCs and are therefore unlikely to provide an explanation for our findings.

The previously reported participation of TLR4 in APC activation by heat shock proteins initiated discussions about the contribution of endotoxin contaminations to the observed effects. Several arguments for an endotoxin independent activity of HSPs have been discussed previously. They include that HSP effects were sensitive to heat treatment, the presence of MDC and, in addition, were insensitive to polymyxin B (Singh-Jasuja et al., 2000a; Zheng et al., 2001; Baker-LePain et al., 2002). Furthermore, a study by Reed et al. (Reed et al., 2003) demonstrated that highly purified Gp96 molecules with almost undetectable endotoxin contaminations (< 0.027 pg/µg Gp96) are still able to promote phosphorylation of ERK which is part of the

activation pathway preceding pro-inflammatory cytokine secretion. Nevertheless, experiments using recombinant Hsp70 molecules suggested that endotoxin contaminations are exclusively responsible for the APC-activating capabilities of these HSP (Gao and Tsan, 2003; Bausinger et al., 2002). However, the experiments presented in this study provide additional evidence that endotoxin contaminations present in the HSP preparations are not exclusively responsible for the activation of DCs. One attractive hypothesis that would provide an explanation for this controversy is the possibility that LPS interacts with HSPs as shown recently for Gp96 (Reed et al., 2003) or Hsp60 (Habich et al., 2005) and that this interaction modulates or augments the biological effects of low amounts of LPS present in Gp96 preparations which on their own might be unable to induce DC activation.

In summary, our data show that heat shock proteins such as Gp96 are molecules which are able to participate in the activation of cells of the innate immune system and thereby influence the outcome of adaptive immune responses.

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IMPACTS OF TOLL-LIKE RECEPTOR SIGNALING ON PRIMING OF CYTOTOXIC T-LYMPHOCYTES *IN VIVO*

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Manuscript submitted.

Efficient priming of cytotoxic T-lymphocytes requires their interaction with matured antigen-presenting cells that express sufficient levels of co-stimulatory molecules and secrete pro-inflammatory cytokines. APC maturation is initiated either during the interaction with $CD4^+$ T cells in an antigen-specific manner or directly by the interaction of conserved pathogen-derived components with members of the Toll-like receptor (TLR) family. Using mice deficient in the TLR-associated adaptor protein myeloid differentiation factor 88 (MyD88) we now show that each of these two signaling pathways is sufficient to achieve priming of human influenza virus A-specific CTLs. Only if both pathways are non-functional, is CTL priming impaired. In the case of minor H-specific CTL induction both, MyD88-mediated signals as well as $CD4^+$ T cells are required. This suggests an important role of TLR signaling even in pathogen-free settings, for example during tumor rejection.

The author of this thesis performed figures 1C, 1D, and 6, and, together with Norbert Hilf, contributed to the concept of this work. Figure 4B resulted from the work of Daniele Arnold-Schild and figures 1A, 1B, 2, 3, 4A, 4C, and 5 from the work of Norbert Hilf.

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Introduction

Priming of naive cytotoxic T lymphocytes requires their interaction with professional antigen presenting cells, such as dendritic cells (DCs) which are considered to be the most potent APCs. For efficient CTL priming, DCs have to develop from an immature to a mature state, characterized by upregulation of costimulatory molecules and production of pro-inflammatory cytokines (Pulendran et al., 2001). Lack of costimulation leads to anergy or apoptosis of naive T cells and therefore to tolerance against the presented antigen (Dhodapkar et al., 2001; Steinman et al., 2003). DC maturation can be initiated via several distinct ways: Specific $CD4^+$ helper T cells are able to trigger DC activation via CD40/CD40 ligand interaction (Cella et al., 1996). Apart from this antigen-specific pathway, pathogen-derived molecules can be recognized as danger signals by a limited number of germ-line encoded innate immune recognition receptors triggering APC activation. The Toll-like receptor (TLR) family with at least 11 different members in mammals comprises an important class of this receptor type (Bendelac and Medzhitov, 2002). Each TLR recognizes a distinct subset of conserved microbial products called PAMPs (pathogen associated molecular patterns), such as bacterial LPS or viral double-stranded RNA (Medzhitov, 2001). Ligation of TLRs leads to the recruitment of cytosolic adaptor proteins to the receptor, and triggers the activation of Jun, Fos and NF-κB transcription factors via signal kinase cascades (Wesche et al., 1997; Medzhitov et al., 1998; Medzhitov, 2001).

The adaptor MyD88 is an essential component of the signaling pathways of most TLRs (Kawai et al., 1999) and also for the IL-1 and IL-18 receptors (Adachi et al., 1998). Bone-marrow derived dendritic cells (BMDCs) from MyD88-deficient mice do not reveal any cytokine response to most PAMPs. Nevertheless, MyD88-independent activation has been described for TLR3 and 4, most probably mediated by the alternative adaptor protein TRIF (TIR domain-containing adapter inducing IFN-β) (Yamamoto et al., 2002b; Yamamoto et al., 2003a; Hoebe et al., 2003), but at least for TLR4 signaling DC activation occurs with delayed kinetics and cytokine production is greatly diminished (Kawai et al., 1999; Kaisho et al., 2001). TIRAP (Toll-IL-1 receptor domain-containing adaptor protein), another TLR adaptor, is involved in TLR4 and TLR2 signaling (Fitzgerald et al., 2001; Horng et al., 2001). But TIRAP

does not participate in MyD88-independent signaling pathways (Horng et al., 2002; Yamamoto et al., 2002a), pointing out again the unique role of MyD88 in TLR-mediated signaling.

The role of TLR-mediated stimuli for the induction of CTL responses is still under investigation. Priming of CTLs against certain viruses and bacteria has been shown to be independent from the presence of $CD4^+$ helper T cells and APC activation signals provided by the CD40/CD40L interaction (Larsson et al., 2000; Hamilton et al., 2001). In these cases, it was speculated that other signals can promote sufficient maturation of dendritic cells. Recent findings that type 1 T helper cell (T_H1) responses are impaired in MyD88-deficient mice while T_H2 responses remain unaffected support the assumption that TLR-mediated signals may account for the $CD4^+$ T cell-independent priming of CTLs (Schnare et al., 2001; Kaisho et al., 2002). Moreover, it has been reported that rejection of minor H-antigen mismatched skin grafts requires MyD88 signaling (Goldstein et al., 2003). Protective $CD8^+$ T cell responses after infection with the bacterial pathogen *Listeria monocytogenensis* on the other hand are independent of MyD88 (Way et al., 2003). Apparently, there are differences in the need for TLR/MyD88-mediated signalling during CTL priming that demand further investigation.

As CTL responses play a crucial role in the eradication of virus-infected cells and tumors, we analyzed the contribution of innate immune recognition to CTL priming in appropriate model systems using MyD88-deficient mice. During priming against human influenza virus A either MyD88- or $CD4^+$ T cell-dependent signals are required and each can compensate for the lack of the other. Responses to minor H antigens, which provide a model for the recognition of cellular antigens, demand the contribution of both signaling pathways. Therefore, minor H-specific CTL responses depend on activation of the innate immune system by danger signals. These data might help to understand impaired CTL responses against many tumors.

Materials and Methods

Mice, Reagents and Antibodies

C57BL/6 ($H2^b$) and BALB/c ($H2^d$) mice were obtained from Charles River Wiga GmbH (Sulzfeld, Germany). BALB.B mice ($H2^b$, minor H antigens almost identical to BALB/c) were obtained from Harlan Winkelmann GmbH (Borchen, Germany). MHC class II-deficient mice (C57BL/6Tac-ABB^{tm1}) depleted of mature $CD4^+$ T cells were obtained from Taconic M&B (Ry, Denmark). Generation of $MyD88^{-/-}$-deficient mice (C57BL/6 background, $H2^b$) has been described previously (Adachi et al., 1998). Animals were maintained in the animal facilities of the Department of Immunology, Institute for Cell Biology, University of Tübingen, Germany or the Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Germany.

Phosphothioate-stabilized CpG deoxyoligonucleotide 1668 (TCC ATG ACG TTC CTG ATG CT) was purchased from TIB MOLBIOL (Berlin, Germany). LPS from *Salmonella typhimurium* was obtained from Sigma (Taufkirchen, Germany), poly(I:C) from Amersham (Freiburg, Germany) and (S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteine (Pam_3Cys) from EMC microcollections (Tübingen, Germany). The peptide ASNENMETM derived from influenza nucleoprotein (366-374) was synthesized by the peptide chemistry facilities in the Department of Immunology (Tübingen, Germany).

For flow cytometry, phycoerythrin or fluorescein isothiocyanate-conjugated monoclonal antibodies to mouse CD4 (GK1.5), CD8 (53-6.7), CD11c, CD14, CD86 and appropriate isotype controls were obtained from BD Biosciences (Heidelberg, Germany). Purified GK1.5 antibody for *in vivo* $CD4^+$ T cell depletion was a kind gift of R. M. Toes (Leiden University Medical Center, The Netherlands). Phycoerythrin-conjugated tetramers of H2-D^b/ASNENMETM were obtained using the standard refolding protocol described by Altman et al. (Altman et al., 1996).

Immunizations, ^{51}Cr release assay, *ex vivo* tetramer staining

500 haemagglutinating units (HAU) of human influenza virus (A/PR/8/34) were injected i.p. for the induction of CTL responses against the H2-D^b-restricted immunodominant CTL epitope ASNENMETM. For priming against minor H antigens, 1x10^7 spleen cells were irradiated with 30 Gy and injected subsequently i.p. in a volume of 300 µl PBS. Alternatively, 5x10^6 non-irradiated BMDCs were used. For CD4$^+$ T cell depletion, 100 µg of purified CD4-specific antibody were injected i.p. on days -5, -3, -1, +1 and +6 referring to the time point of immunization. On days 0 and 9, the effectiveness of T helper cell depletion was analyzed by flow cytometry of blood lymphocytes. On day 9 after immunization, mice were sacrificed and splenocytes were stimulated *in vitro* for additional 5 days in Modified Eagle's Medium (alpha modification) containing 10 % fetal calf serum, 2 mM L-glutamine, 50 µM β-mercaptoethanol and antibiotics. If indicated, T cells were restimulated at weekly intervals using medium supplemented with 25 ml supernatant of concanavalin A treated rat splenocytes and 25 mM alpha-methylmannoside. ^{51}Cr release assay was performed as described previously (Arnold et al., 1997). For generation of T cell blasts, mouse spleen cells were cultured for 3 days with 2.5 µg/ml concanavalin A.
For *ex vivo* detection of peptide-specific T cells, 1x10^6 splenocytes were stained with phycoerythrin-conjugated H2-D^b/ASNENMETM tetramer and fluorescein isothiocyanate-conjugated anti-CD8 antibody for 30 min on ice directly after cell isolation. Cell fluorescence was measured by flow cytometry on a FACSCalibur™ (BD Biosciences).

Generation of mouse dendritic cells

Mouse immature DCs were generated from bone marrow of C57BL/6 and MyD88$^{-/-}$ mice using Iscove's Modified Dulbecco's Medium (BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine, antibiotics and 10 % fetal calf serum. Bone marrow cells were incubated in medium containing 150 U/ml granulocyte-macrophage colony-stimulating factor (PeproTech, London, England) for 6-8 days. Medium was replaced every 2 days. The DCs obtained were CD11c$^+$ and CD14$^-$.

Stimulation of BMDCs

Mouse BMDCs were stimulated by the addition of CpG, LPS, poly(I:C) or Pam_3Cys. After 24 h, IL-12 (p40) and IL-6 concentrations in the supernatants were measured using standard sandwich ELISA protocols. Antibodies and recombinant standards of both cytokines were obtained from BD Biosciences. The capture antibody was bound to a MaxiSorb™ ELISA plate (Nunc, Roskilde, Denmark), the biotinylated detection antibody was revealed by streptavidin-conjugated horseradish peroxidase (BD Biosciences) and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid substrate (Sigma). The assay was read at 405 nm. Furthermore, on day 3 after activation, expression of the costimulatory molecule CD86 was measured by flow cytometry on a FACSCalibur™ (BD Biosciences). Isotype controls of antibodies were included in all experiments to determine the appropriate background fluorescence.

Results

MyD88-dependent signals can compensate for missing $CD4^+$ T cell help during priming of influenza-specific CTLs

To investigate the role of MyD88-mediated signals in the generation of virus-specific CTLs, C57BL/6 and $MyD88^{-/-}$ mice were immunized i.p. with 500 HAU human influenza virus (A/PR/8/34). CTL activity was analyzed from spleen cell cultures stimulated for 5 days with the H2-D^b-restricted immunodominant influenza nucleoprotein peptide (366-374) using target cells incubated with peptide. As shown in Fig. 1, $MyD88^{-/-}$ mice (Fig. 1B) produce an influenza-specific CTL response comparable to that of C57BL/6 mice (Fig. 1A), despite the fact that $MyD88^{-/-}$-derived BMDCs display a strongly reduced cytokine response, shown here for IL-12 and IL-6, to various TLR-ligands including LPS, CpG deoxyoligonucleotides and Pam_3Cys (Fig.1C,D), as previously reported (Kawai et al., 1999). These results indicate that MyD88-mediated stimuli are not necessarily required for CTL priming.

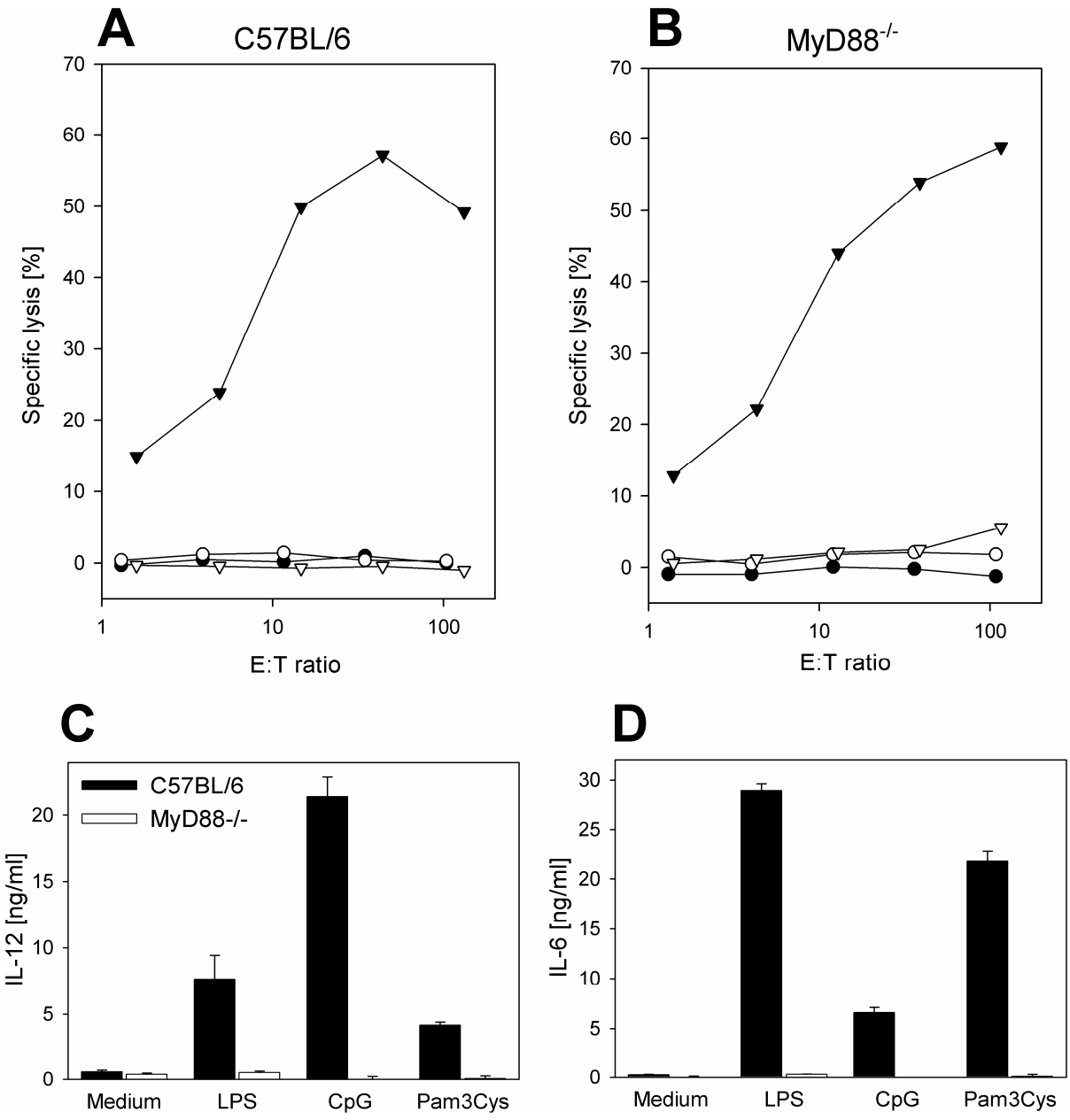

Figure 1. CTL activity against human influenza virus is not affected in MyD88$^{-/-}$ mice. **(A,B)** C57BL/6 (A) or MyD88$^{-/-}$ mice (B) were injected i.p. with 500 HAU of human influenza virus (▼). Control mice were left untreated (●). After 9 days splenocytes were stimulated *in vitro* with the immunodominant influenza CTL epitope. On day 5, CTL activity against EL-4 target cells loaded with peptide (filled symbols) or without (open symbols) was tested in a ^{51}Cr release assay. Spontaneous ^{51}Cr release was < 6 %. Experiments were performed with two mice per group. Results shown are representative of four independent experiments with similar results.
(C,D) Immature BMDCs form the immunized mice used in A,B were stimulated with 1 ng/ml LPS, 2.5 µM CpG or 2.5 µg/ml Pam3Cys. After 20 h, IL-12 (C) and IL-6 (D) concentrations in the culture supernatants were analyzed by ELISA. Mean values of triplicates from C57BL/6 BMDCs are shown with filled bars, from MyD88$^{-/-}$ BMDCs with open bars. Error bars represent SE. Similar results were obtained from more than 5 different experiments.

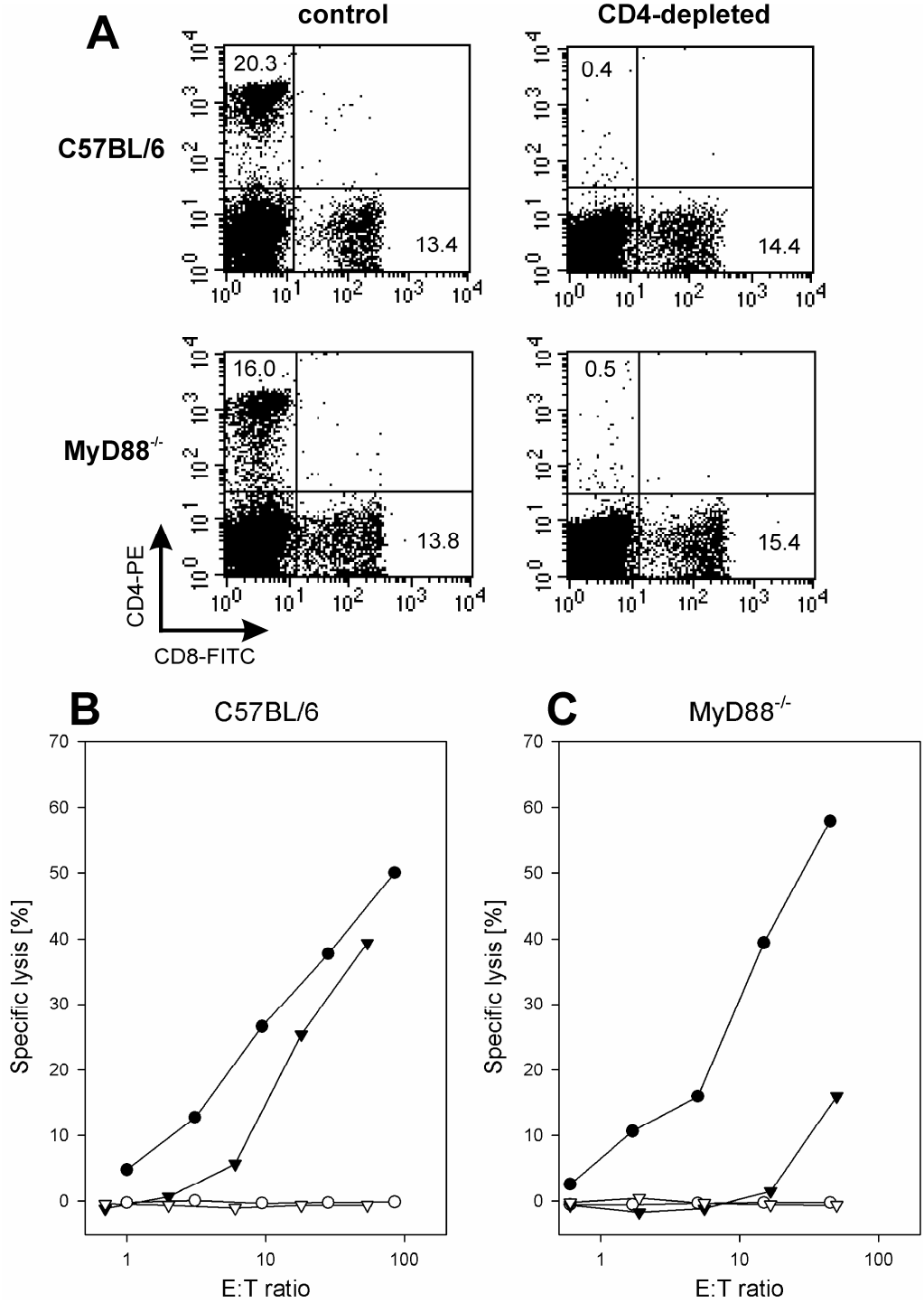

Figure 2. CTL response against human influenza virus in MyD88$^{-/-}$ mice is dependent on CD4$^+$ T helper cells. C57BL/6 and MyD88$^{-/-}$ mice were immunized with human influenza virus and splenocytes were stimulated with peptide as described for Fig. 1. CD4$^+$ T cells had been depleted in half of the mice by repeated i.p. injection of 100 µg GK1.5 antibody on day -5, -3, -1, +1, +6 before and after virus challenge. **(A)** The efficiency of CD4$^+$ T cell-depletion was tested by flow cytometry after staining of blood lymphocytes with fluorochrome-labeled CD8- and CD4-specific monoclonal antibodies at the day of virus injection (day 0). Percentages of CD4$^+$ and CD8$^+$ positive cells among total lymphocytes are indicated for CD4-depleted (right panels) and control mice (left panels) in the upper left and lower right quadrants, respectively. **(B,C)** CTL activity after 5 d *in vitro* stimulation with peptide was assayed in a ^{51}Cr release for lymphocytes from C57BL/6 (B) and MyD88$^{-/-}$ (C) mice (▼ CD4$^+$ T cell-depleted mice; ● undepleted control mice). EL-4 target cells loaded with peptide (filled symbols) and as specificity control without peptide (open symbols) were used. Spontaneous ^{51}Cr release was < 5 %. Results shown are representative of two independent experiments.

The induction of primary CTL responses against many viruses, including influenza, has been shown to take place independently of $CD4^+$ T cells (Buller et al., 1987; Rahemtulla et al., 1991; Tripp et al., 1995). We also observe that the depletion of $CD4^+$ T cells hardly influences the priming of Influenza-specific CTLs in C57BL/6 mice (Fig. 2A, B), but strongly reduces CTL induction in $MyD88^{-/-}$ mice (Fig. 2A,C). Thus, MyD88- or $CD4^+$ T cell-mediated signals can compensate for each other during the priming of influenza virus-specific CTLs. Only if both pathways are missing, CTL priming is impaired.

To determine if the reduced CTL activity in CD4-depleted $MyD88^{-/-}$ mice is due to reduced numbers of influenza-specific CTL we performed *ex vivo* tetramer staining after virus immunization. As shown in Fig. 3, CD4 depletion does not effect the number of influenza nucleoprotein-specific CTL in C57BL/6 mice but strongly reduces the number of T cells reacting with the H2-D^b/ASNENMETM tetramer in $MyD88^{-/-}$ mice.

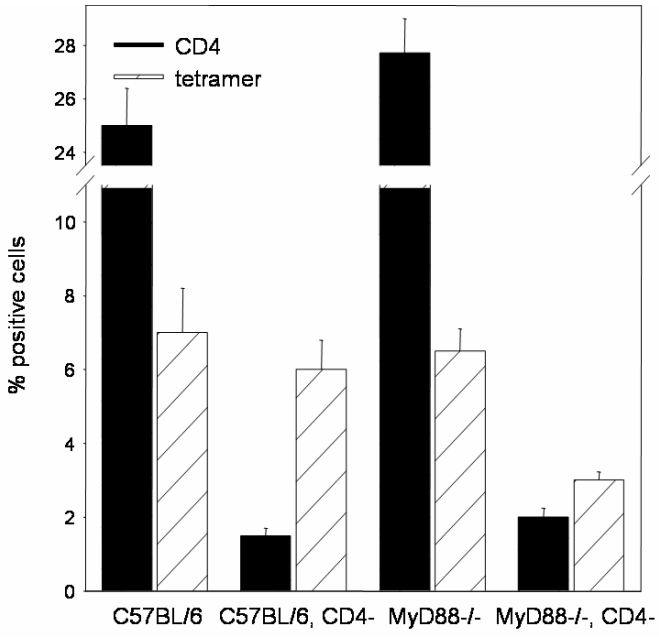

Figure 3. Priming of influenza-specific CTL is reduced in CD4-depleted MyD88-/- mice. C57BL/6 and $MyD88^{-/-}$ mice were immunized with human influenza virus as described in Fig. 1. $CD4^+$ T cells were depleted by repeated i.p. injection of 100 µg GK1.5 antibody on days -5, -3, -1, +1, +6 before and after virus challenge. The efficiency of $CD4^+$ T cell-depletion was tested by flow cytometry after staining of blood lymphocytes with fluorochrome-labeled CD4-specific monoclonal antibodies at the day of virus injection (day 0). Influenza-specific CTL were detected 9 days after infection of influenza virus with phycoerythrin-conjugated H2-D^b/ASNENMETM tetramers and fluorescein isothiocyanate-conjgated anti-CD8 antibody staining. Experiments were performed with three mice per group. Results shown are representative of two independent experiments.

Priming of minor H-specific CTLs requires MyD88- and CD4$^+$ T cell-mediated stimuli

To investigate the role of MyD88-mediated stimuli in a pathogen-free system, we studied the induction of minor H-specific CTLs, which is often used as a model system for tumor rejection. CD4$^+$ T cells have been shown to be necessary for the generation of minor H-specific CTLs (Schild et al., 1987). As shown in Fig. 4A,B, we observe the same CD4$^+$ T cell-dependence for the CTL induction against BALB minor H antigens in C57BL/6 mice (H2^b): After immunization with BALB.B cells (H2^b), no CTL activity is detectable in MHC class II-deficient mice which lack mature CD4$^+$ T cells (strain C57BL/6Tac-Abb^{tm1} N5). In contrast, the presence of a functional MyD88 pathway is not required in host APCs, as MyD88$^{-/-}$ (H2^b) and C57BL/6 mice (Fig. 4A,C) mount comparable minor H CTL responses. Most likely, CTL priming is independent of host APCs in this situation and mediated directly by the interaction of naïve T cells with donor APCs not defective in the MyD88 pathway.

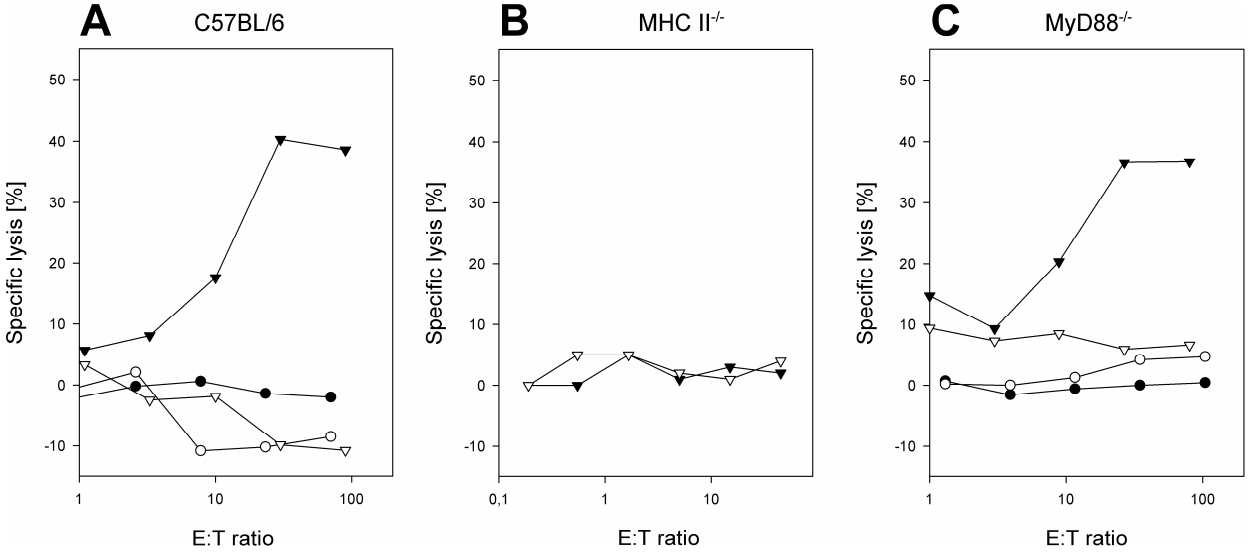

Figure 4. CTL responses against BALB minor H antigens require CD4$^+$ T helper cells, but not MyD88-dependent signals in host APCs. 1x10^7 irradiated BALB.B splenocytes were injected i.p. into C57BL/6 (A), MHC class II-deficient (B) or MyD88$^{-/-}$ mice (C). 9 days later, mice were sacrificed and splenic lymphocytes were stimulated with irradiated spleen cells of BALB.B mice and once restimulated after one week. After additional 5 days, cytolytic activity against BALB.B (filled symbols) and C57BL/6 (open symbols) derived blasts was evaluated in a ^{51}Cr release assay (▼ immunized mice; ● untreated controls, not shown in B). Spontaneous ^{51}Cr release was 19 % for BALB.B and 24 % for C57BL/6 blasts. Experiments were performed with two mice per group. Results shown are representative of two independent experiments.

To investigate this possibility, we injected cells from MyD88$^{-/-}$ mice or C57BL/6 mice as control into BALB.B hosts in order to induce CTLs against C57BL/6 minor H antigens. In contrast to the results obtained by injection of C57BL/6 cells (Fig. 5A), we found that the immunization using spleen cells from MyD88$^{-/-}$ mice was unable to prime CTLs (Fig. 5B). However, cells from MyD88$^{-/-}$ as well as C57BL/6 mice were able to induce the activation of alloreactive CTLs, showing that MyD88$^{-/-}$-derived spleen cells are able to stimulate T cells in general (data not shown). Thus, in contrast to the induction of influenza-specific CTLs, the priming of minor H-specific CTLs requires both the presence of CD4^{+} T cells and a functional MyD88 pathway in the cells used for immunization.

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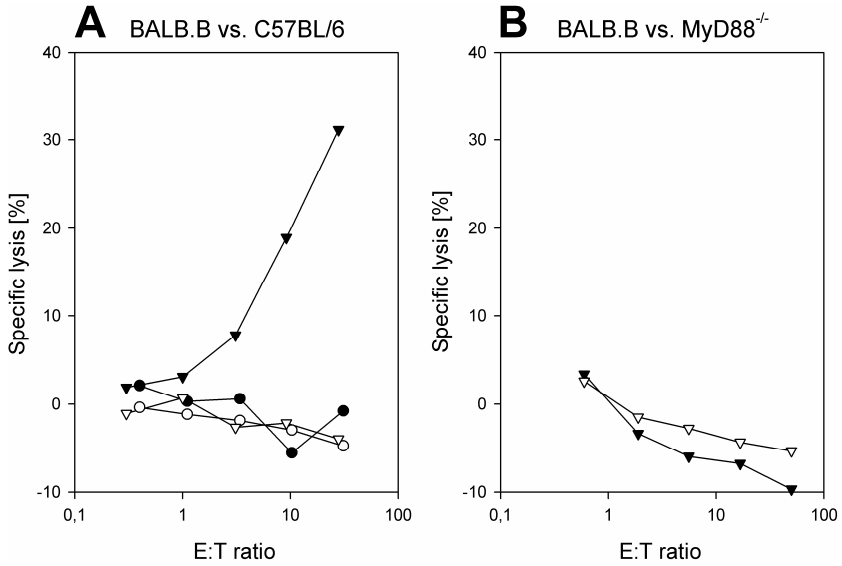


Figure 5. Donor APCs require functional MyD88-dependent signaling pathways to induce minor H-specific CTL responses. 1x10^{7} irradiated splenocytes from C57BL/6 **(A)** or MyD88$^{-/-}$ mice **(B)** were injected i.p. into BALB.B mice. On day 9 after injection, lymphocytes from the injected mice (▼) and from untreated controls (●, not shown in *B*) were stimulated *in vitro* with irradiated C57BL/6-derived splenocytes. Cultures were restimulated twice in weekly intervalls. 5 days after the last restimulation, a ^{51}Cr release assay was performed to analyse CTL activity against C57BL/6 minor H antigens. Filled symbols show CTL activity against C57BL/6 blasts (20 % spontaneous release), open symbols against BALB.B blasts (13 % spontaneous release).

BMDCs from MyD88$^{-/-}$ mice prime minor H-specific CTLs after maturation with poly(I:C)

The inability of MyD88$^{-/-}$ cells to induce minor H-specific CTLs after injection in BALB.B mice might be attributed to their defect in the IL-1R/TLR signaling pathway preventing maturation of professional APCs, such as DCs. If this is the case, activation of MyD88$^{-/-}$-derived BMDCs by MyD88-independent stimuli would overcome this defect. BMDCs from both C57BL/6 and MyD88$^{-/-}$ mice were found to respond to poly(I:C) stimulation by the secretion of IL-6 (Fig. 6A) and IL-12 (data not shown) and by the upregulation of CD86 molecules (Fig. 6B), while the maturation of MyD88$^{-/-}$-derived BMDCs in response to CpG deoxyoligonucleotides and Pam_3Cys was completely abrogated in the same experiment (data not shown). After injection of poly(I:C) activated BMDCs from MyD88$^{-/-}$ mice into BALB.B mice, we observed priming of minor H-specific CTLs whereas immunization using unstimulated BMDCs from MyD88$^{-/-}$ mice was unable to induce minor H-specific CTL activity (Fig. 6C). These results indicate that the lack of MyD88 in donor APCs can be overcome by MyD88-independent maturation of the cells prior to injection.

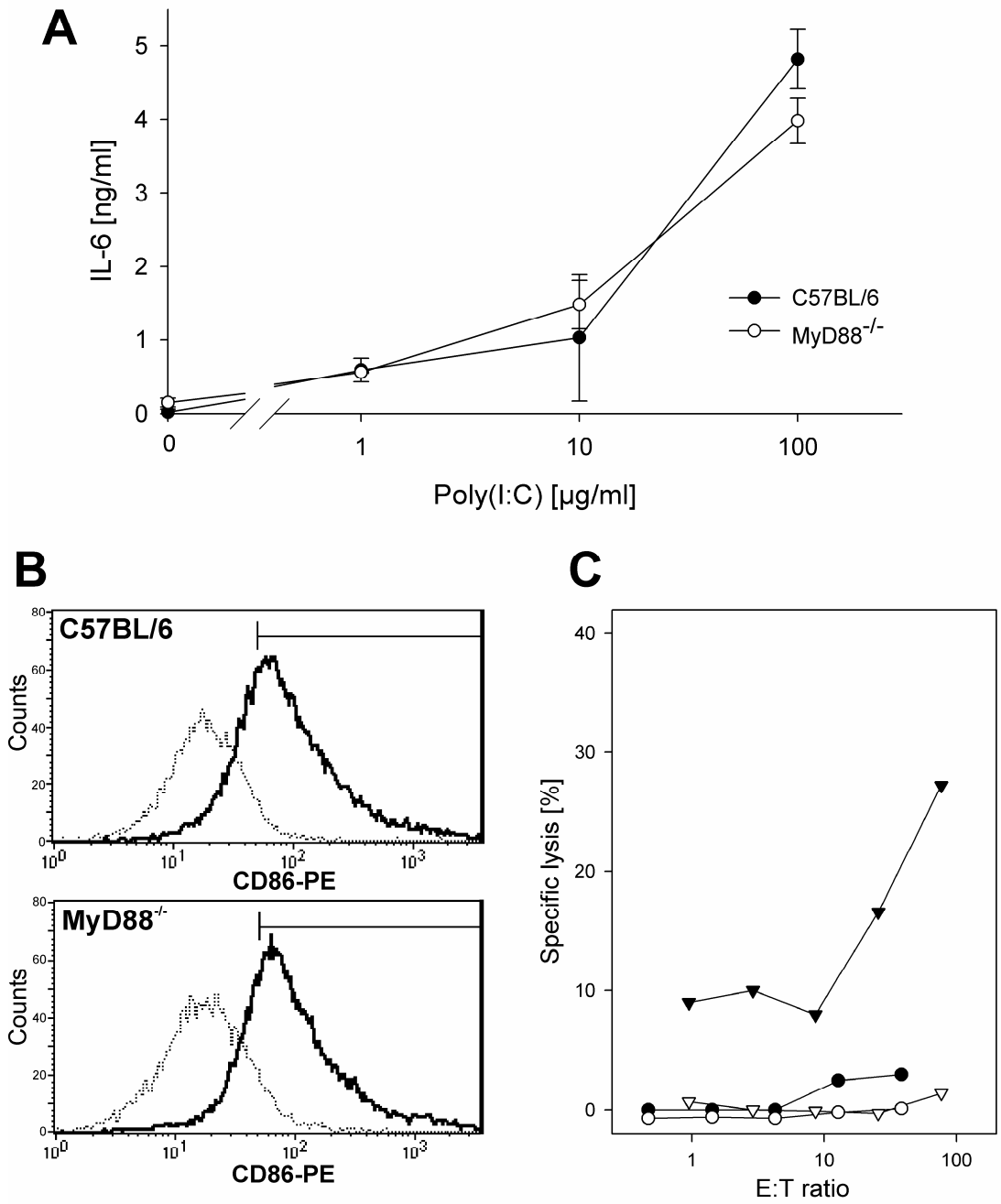


Figure 6. MyD88-dependent signals during CTL priming against minor H antigens can be compensated by MyD88-independent activation of donor APCs. (A and B) Immature BMDCs from C57BL/6 (●) and MyD88-deficient (○) mice were stimulated with the indicated concentrations of poly(I:C). **(A)** After 20 h IL-6 concentrations in the supernatant were analysed by ELISA. Means of triplicates are shown with error bars representing SE. Data shown are representative of three independent experiments. **(B)** After 3 days, maturation status of C57BL/6-derived (upper panel) and MyD88$^{-/-}$-derived (lower panel) BMDCs was measured by flow cytometry after staining with a phycoerythrin-conjugated anti-CD86 antibody (..... unstimulated BMDCs; ▬▬ BMDCs stimulated with 100 µg/ml poly(I:C). Percentages of CD86^{high} cells as defined by the indicated marker were 69.7 ± 1.2 and 69.6 ± 2.3 for C57BL/6 and MyD88$^{-/-}$ mice, respectively (means of triplicates). **(C)** 5x10^5 BMDCs from MyD88$^{-/-}$ (▼ activated by 100 µg/ml poly(I:C); ● unstimulated) were injected i.p. into BALB.B mice. 9 days later, mice were sacrificed and splenocytes were stimulated with irradiated BALB.B cells. After additional 5 days CTL activity against C57BL/6 (filled symbols) and BALB.B blasts (open symbols) was assayed in a ^{51}Cr release assay. Spontaneous ^{51}Cr release was 16 % and 7 % for C57BL/6 and BALB.B blasts, respectively. Results shown are representative of two independent experiments.

Discussion

In this study, we analyzed the contribution of MyD88-mediated signals to the priming of CTLs specific for influenza virus and minor H antigens. We found that the activation of influenza-specific CTLs requires either the presence of $CD4^+$ T cells or a functional MyD88 signaling pathway (Fig. 1 and Fig. 2). Only if both components are missing, CTL priming is severely impaired but either one of the two is nevertheless able to maintain CTL activation. Ex vivo staining of splenocytes of influenza infected mice with H2-D^b/ASNENMETM tetramers reveals that expansion of virus-specific CTL in vivo is strongly reduced in CD4-depleted $MyD88^{-/-}$ but not in C57BL/6 mice (Fig.3). This suggests that during a viral challenge in the absence of $CD4^+$ T cell help sufficient activation of APCs can be achieved via the MyD88 pathway, probably triggered by PAMP - TLR interaction. On the other hand, defects in the MyD88-dependent pathway can be compensated by $CD4^+$ T cells.

The activation signal provided by $CD4^+$ helper T cells is most likely mediated through CD40/CD40L interactions because this help can be replaced by injection of a stimulating anti-CD40 monoclonal antibody (Bennett et al., 1998; Schoenberger et al., 1998). In addition, CD40-independent DC sensitization and direct lymphokine-dependent $CD4^+$ - $CD8^+$ T cell communication might contribute to the priming of CTL (Lu et al., 2000).

One of the signals that stimulates the MyD88 pathway in APCs after the injection of influenza virus is probably induced by the interaction of viral dsRNA with TLR3 (Alexopoulou et al., 2001) which occurs as intermediate during the replication cycle of RNA viruses. The ability of DCs in particular to respond to dsRNA stimuli was reported earlier (Cella et al., 1999) and correlates with the increasing TLR3 expression during the development of monocytes into immature DCs (Visintin et al., 2001b; Kadowaki et al., 2001). However, the contribution of TLR3-mediated signals derived by influenza virus-derived dsRNA remains questionable as they are not able to promote efficient CTL priming in CD4-depleted $MyD88^{-/-}$ mice (Fig.2B). Additional virus-derived PAMPs might contribute to TLR-mediated APC activation as well. For the measles virus, it has recently been reported that the viral hemagglutinin protein activates cells via TLR2 (Bieback et al., 2002). TLR4 has been shown to be involved

in the innate immune response to respiratory syncytial virus but not to influenza virus (Kurt-Jones et al., 2000; Haynes et al., 2001). Especially stimulation of TLR3 and TLR4 induces the expression of interferon regulatory factor 3 (IRF-3), an important transcriptional regulator of the antiviral response (Doyle et al., 2003)). Interestingly, IRF-3 upregulation is mediated MyD88-independently via the TRIF adaptor (Yamamoto et al., 2002b). Therefore, the lack of antiviral response in MyD88$^{-/-}$ mice after CD4^{+} T cell depletion cannot be explained by defective IRF-3 activation. Nevertheless, our results show that TLR- and MyD88-mediated activation cascades are crucially involved during the induction of antiviral CTL responses.

A different situation is observed for the induction of CTLs against minor H antigens. Here, both CD4^{+} T cells and a functional MyD88 signaling pathway are required in the antigen-bearing donor cells (Fig. 4 and Fig. 5). The lack of a functional MyD88 signaling pathway in APCs from MyD88$^{-/-}$ mice can be compensated by poly(I:C)-induced maturation of BMDCs prior to their injection (Fig. 6C). The reason for this is most likely the TLR3-dependent but MyD88-independent upregulation of CD86 molecules by poly(I:C) (Fig. 6B), as reported previously (Alexopoulou et al., 2001). However, in contrast to this report, we also observed the secretion of proinflammatory cytokines by poly(I:C) stimulated BMDCs from MyD88$^{-/-}$ mice to levels comparable with that of wildtype BMDCs (IL-6, Fig. 6A; IL-12, data not shown), whereas other known MyD88-dependent stimuli, such as Pam_3Cys or CpG deoxyoligonucleotides, failed to induce the activation of BMDCs from MyD88$^{-/-}$ mice both in earlier experiments (Fig. 1) and in the same analysis (data not shown). Why TLR3-mediated, MyD88-independent activation of MyD88$^{-/-}$ BMDC rescues the induction of minor H-specific CTL but not the induction of influenza-specific CTL in CD4-depleted MyD88$^{-/-}$ mice is not clear but might by explained by the following reasons. First, the induction of minor H-specific CTL by TLR3-activated BMDC took place in the presence of CD4^{+} T cells whereas these cells were depleted during influenza virus infection in MyD88$^{-/-}$ mice. Second, poly(I:C) treatment induces a stronger activation of BMDCs compared to influenza virus infection (Herter et al., unpublished observation) and only the former might be sufficient for CTL activation.

A comparable MyD88/TLR dependence can be expected for CTL induction against other cell restricted antigens, such as tumor antigens. The absence of PAMPs and

danger signals in many tumors might contribute to the lack of an efficient tumor-directed immune response.

How MyD88-dependent signals contribute to the induction of minor H-specific CTLs and which PAMPs, or in this case, which danger signals interact with which upstream receptors has still to be determined. Heat-shock proteins released during cell death are possible candidates for non-pathogen derived danger signals. For example, Hsp60 and the ER-resident Gp96 have been reported to activate APCs via TLR-mediated pathways (Vabulas et al., 2001; Vabulas et al., 2002b).

Apart from TLRs, another possible candidate is IL-1, which activates target cells via a MyD88-dependent signaling pathway (Adachi et al., 1998). IL-1 is secreted by activated macrophages or T_H2 cells and is required for T_H2 cell proliferation (Lichtman et al., 1988; McArthur and Raulet, 1993). Whether this or the general immunostimulatory capacities of IL-1 play a role in the observed MyD88 dependence of minor H-specific CTL priming still has to be determined. Similarly, IL-18 signaling which has also been shown to be MyD88-dependent (Adachi et al., 1998) has to be considered, because mice deficient in this cytokine show an impaired NK cell activity and T cells from these mice challenged with T_H1 inducing pathogens show impaired IFN-γ production (Takeda et al., 1998). Nevertheless, IL-1β and IL-18 are not generally required for T cell responses, because mice deficient in caspase-1 which cannot produce the biologically active forms of IL-1β and IL-18 (Fantuzzi and Dinarello, 1999) show a normal antigen-specific T_H1 response (Schnare et al., 2001). Moreover, rejection of minor H-mismatched skin allografts is dependent on MyD88, but not on IL-1 or IL-18, suggesting a TLR involvement (Goldstein et al., 2003). However, for prolonged graft survival in the latter study both, donor and recipient mice had to be MyD88 deficient. If the MyD88 pathway was disrupted in only one of them skin grafts were tolerated for about 8 to 10 weeks but eventually rejected. The reason for this obvious difference to our finding where MyD88-deficient cells are not able to induce a CTL response in BALB.B mice might be that the injection of minor-H mismatched cells is less immunogenic compared to skin grafting.

Another interesting aspect emerges from our observation that a functional MyD88-signaling pathway is only required in the cells that are injected, but not in the host cells

(Fig. 4C): During the induction of minor H specific CTLs in an H2-matched combination, only the injection of C57BL/6 but not MyD88$^{-/-}$ cells into BALB.B mice results in the induction of C57BL/6 specific CTL. Obviously, minor H antigens are recognized exclusively on donor cells. One explanation might be that minor H antigens from MyD88-deficient donor cells are insufficiently transferred to host APCs (cross presentation). The efficiency of cross presentation is influenced by the expression levels of the antigen (Kurts et al., 1998). However, this cannot explain the lack of cross presentation in our case because in an H2-mismatched combination, C57BL/6 minor H antigens can be cross presented efficiently by BALB/c mice (Arnold et al., 1997). An alternative explanation could be that minor H antigens are still transferred from donor cells to host APCs but these APCs do not become activated and are therefore unable to induce the priming of minor H specific CTLs. This question, and the role of MyD88-dependent stimuli during the induction of minor H-specific CTLs in an H2-mismatched combination, originally described as cross priming (Bevan, 1976a), are currently under investigation.

In summary, our results show that the TLR / MyD88 pathway does not only play a role in pathogen defense, but also during CTL priming against pathogen-free antigens, such as minor H antigens. However, the immune response to pathogens does not necessarily require MyD88 participation, as shown here and by others (Way et al., 2003). Therefore, the requirement for innate immune recognition via the MyD88 pathway for CTL priming depends on the nature of the antigen.

7

RÉSUMÉ

„Toll“ has first been identified as insect transmembrane protein essential for dorsoventral polarity during embryogenesis. The discovery that Toll is additionally able to protect the fruit fly *drosophila* against microbial infections initialized the search of homologue receptors in mammals. Until now, eleven human and twelve murine Toll-like receptors (TLR) have been identified and characterized. These evolutionarily conserved transmembrane receptors play a crucial role in immunity as pattern recognition receptors (PRR): they enable the innate immune system to recognize pathogen-associated molecular patterns (PAMP) and thus confer a certain degree of specificity (Chapter 1, Table 1). They not only signal the presence of danger and thus activate an immediate innate immune response. Moreover, TLR-mediated signals activate phagocytes and dendritic cells (DCs) to secrete chemokines and cytokines and to up-regulate co-stimulatory molecules in response to these alarm signals, a prerequisite for the induction of the adaptive immune response.
The topic of this thesis is the identification of structures recognized by TLRs and the investigation of the molecular mechanisms by which TLRs link innate and adaptive immunity. Both are important for the development of therapeutics in the fight against pathogens and cancerous tissues.

Although the bacterial cell wall component lipopolysaccharid (LPS) has long been known to be a PAMP recognized by the innate immune system via TLRs, the question, which specific TLR is responsible for recognition, has been the issue of controversial discussion. Finally, it has become the common consensus that TLR2 is involved in the recognition of LPS from Gram-positive bacteria, whereas TLR4 functions as pattern recognition receptor for LPS from Gram-negative bacteria together with the accessory molecule MD-2, the LPS-binding protein (LBP) and CD14. However, doubts have been casted on this paradigm. Therefore, we focused on the characterization of the receptor involved in the recognition of LPS from the Gram-negative bacteria *Legionella pneumophila*, the causative agent of Legionnaires' disease, which was proposed to be structurally and functionally different from those of other Gram-negative bacteria. We observed that purified *L. pneumophila* LPS ranks among the atypical LPS species, since it activates dendritic cells to secrete pro- inflammatory cytokines via a TLR2-dependent mechanism (**Chapter 2**). Although efficient TLR2-

signaling can generally take place in the absence of CD14, we found cell activation by *L. pneumophila* LPS to be dependent on CD14. The physiological significance of these observations is emphasized by the finding that not only the purified LPS but even the micro-organism itself triggers activation signals through TLR2 and CD14. Thus, LPS is the main PAMP for the recognition of *L. pneumophila* by the innate immune system (Figure 1). Comparing viable bacteria with killed bacteria revealed that the latter is more potent in activating immune cells, arguing for soluble factors secreted by live bacteria to be responsible for this suppressive function. Whether these escape mechanisms in turn also involve TLRs is still a matter of speculation.

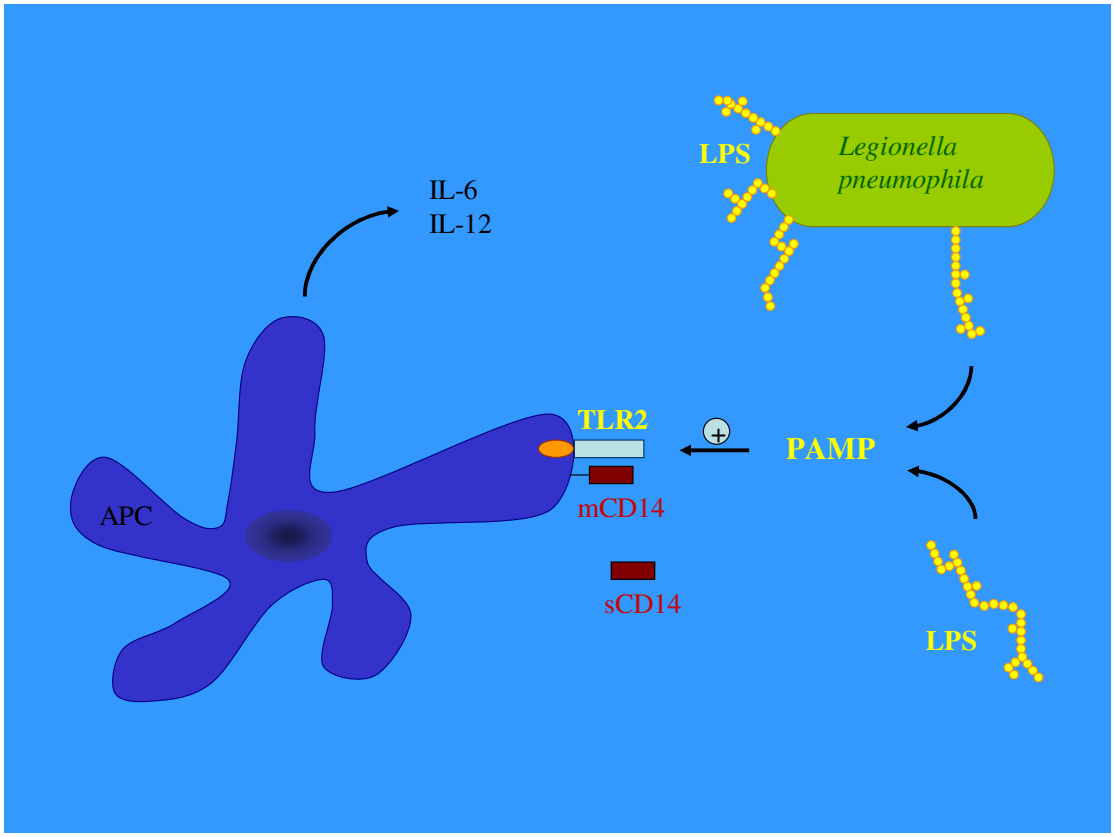
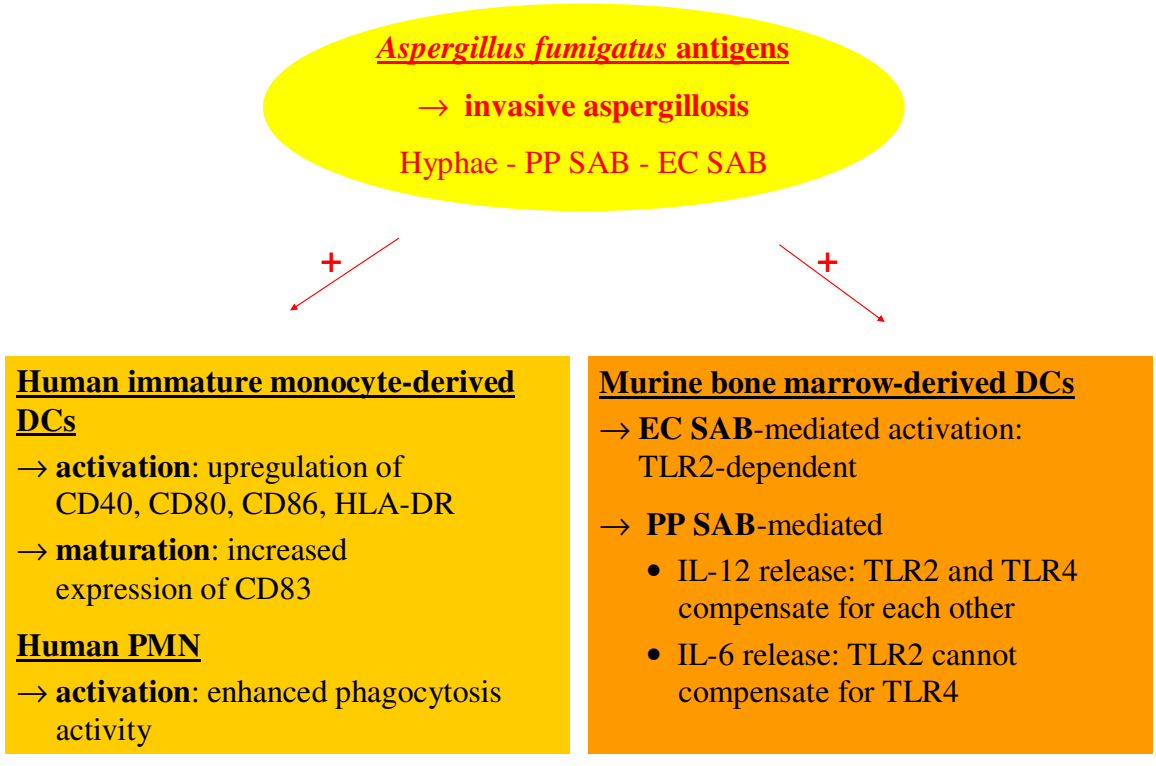


Figure. 1: APC activation by *Legionella pneumophila* LPS and intact bacteria. *L. pneumophila* LPS is an atypical Gram-negative LPS species, since it activates DCs to secrete pro-inflammatory cytokines via TLR2. *L. pneumophila* LPS also engages CD14, either soluble (sCD14) or membrane bound (mCD14). Moreover, the intact bacteria itself triggers activation signals via TLR2 and CD14. Thus, LPS is the main PAMP recogized be the innate immune system.

For antigens of *Aspergillus fumigatus*, a fungus causing invasive aspergillosis (IA), the Toll-like receptor distribution is more complex (Chapter 3). We observed that antigen preparations derived from *A. fumigatus*, i.e. hyphae, PP SAB (ethanol precipitate of A. fumigatus culture supernatant), and EC SAB (cellular extract of A. fumigatus), activate innate immune cells. They stimulate neutrophils for cytokine release (IL-8), phagocytosis and degranulation, and immature DCs for activation and induction of maturation. In this process, the different involvement of TLR2 and TLR4 leads to the secretion of different pro-inflammatory cytokines by DCs: In response to PP SAB, TLR2 and TLR4 can compensate for each other to induce IL-12 release, but TLR2 cannot compensate for TLR4 in case of IL-6 secretion. In contrast, IL-12 production in response to EC SAB is exclusively dependent on TLR2 (Figure 2).

Since appropriate activation of the innate immunity is crucial for the successful clearance of IA, a clear understanding of how TLRs contribute to innate immune recognition of *A. fumigatus* is indispensable.



PP SAB, ethanol precipitate of *A. fumigatus* culture supernatant; EC SAB, cellular extract of *A. fumigatus*; DC, dendritic cell; PMN, polymorphonuclear neutrophils

Figure 2. Activation capacity of *Aspergillus fumigatus* antigens. Yellow ellipse: *A. fumigatus* antigens. Left box: *A. fumigatus* antigens activate human innate immune cells. Right box: Different involvement of TLR2 and TLR4 by *A. fumigatus* antigens differentially induces the secretion of pro-inflammatory cytokines by murine dendritic cells.

In addition to exogenous pathogen-associated molecules, TLRs also recognize endogenous foreign danger signals, such as viral DNA or RNA (Chapter 1) and, as we found, endogenous nonforeign danger signals (Chapter 4).
By analyzing the molecular mechanisms by which the mammalian ER-resident chaperone and heat shock protein Gp96 activates antigen-presenting cells (APC), we demonstrated that TLRs are used to sense this endogenously derived nonforeign alarm signal. Gp96 is of special immunological interest, since vaccination with Gp96:peptide complexes confers protective immunity against the tissue the complex had been purified from. Thereby, Gp96 enables APCs to deliver both signals required to trigger an effective immune response, the antigen-specific signal and the co-stimulatory signal. On the one hand, specificity is obtained by the immunogenic peptide bound to Gp96 as a consequence of its chaperone function. After receptor (CD91)-mediated uptake of the Gp96:peptide complex by APCs, the associated peptide is transferred to the MHC class I antigen-processing pathway for "cross-presentation" to $CD8^+$ T cells. On the other hand, Gp96 unspecifically induces activation and maturation of APCs, which leads to the secretion of pro-inflammatory cytokines and up-regulation of co-stimulatory molecules.

In this thesis, the molecular basis for understanding the Gp96-mediated activation of APCs could be elucidated. We found that Gp96 activates DCs via TLR4 and, to a small extent, via TLR2 signaling pathways and thereby engages classical signaling cascades comprising the NF-κB pathway and activation of the SAPKs JNK1/2 and p38 as well as the mitogen-activated protein kinases ERK1/2. Yet, it turned out that DCs are not activated until Gp96 is endocytosed. Thus, Gp96 has to be internalized for both, the induction of the TLR-mediated maturation signal and the delivery of the specific antigen into the cell for major histocompatibility complex class I-restricted representation ("cross-presentation") ensuring that the same APC delivers the antigen-specific signal as well as the co-stimulatory signal for successful induction of cytotoxic T cell (CTL) responses against the associated antigenic peptide.

However, it is of severe concern that the TLR involvement observed for Gp96 and other HSPs is due to minor endotoxin contaminations in the protein preparations, for, endotoxins and HSPs use the same receptors to trigger their activating signals.

Our group accumulated evidence that at least for Gp96, it is the protein itself that induces APC maturation: First, Gp96-mediated, but not endotoxin-mediated DC maturation, is heat-sensitive and polymyxin-insensitive (Braedel et al., unpublished observations). Second, Gp96-mediated DC activation requires endocytosis of Gp96, while LPS-mediated DC activation is endocytosis independent (Chapter 4). Third, minimal amounts of LPS, which correspond to the endotoxin level detected in Gp96 preparations, are unable to mature DCs, and flanking fractions obtained during the chromatographic purification of Gp96, which contained no protein but similar amounts of endotoxin, do not mature DCs (Chapter 5). Fourth, cells transfected with membrane-bound Gp96 (Zheng et al., 2001, Hilf et al., unpublished observations) activate APCs in the absence of bacterial contaminations. But the most striking argument is provided by the results obtained in Chapter 5: interaction of Gp96 with DCs induces maturation signals that promote predominantly the activation and expansion of antigen-specific $CD8^+$ T cells *in vitro* and *in vivo*, whereas LPS-mediated DC stimulation favors the activation and expansion of antigen-specific $CD4^+$ T cells. Thereby, the ratio of $CD8^+$ versus $CD4^+$ T cells does not depend on the amount of LPS used for DC activation. This bias can neither be explained by differences in the production of pro-inflammatory cytokines nor by the expression level of major histocompatibility complex or co-stimulatory molecules by Gp96- or LPS-stimulated DCs and strongly militates against endotoxin contaminations causing the observed activation of TLR2/4 signaling pathways by Gp96.

To finally rule out speculations about endotoxin contaminations, it remains to investigate, whether Gp96 may function in potentiating the effect LPS traces by binding to and shuttling LPS inside the cells via receptor-mediated uptake.

Altogether, the differences in the qualitative outcome of adaptive immune responses induced by the two TLR4-dependent stimuli, Gp96 and LPS, impressively demonstrate the impact of TLR-mediated activation of innate immune cells for the induction and control of specific immune responses.

Whether or not the minor involvement of TLR2 to the Gp96-triggered cell activation contributes for to the observed differences remains to be investigated. Then, it might be the combination of the nature of given stimuli (which might vary in their binding capacity to the engaged TLR(s)), different expression profiles of the involved TLRs, and their signaling specificity that shape adaptive immunity.

To address the issue of TLR signaling on linking innate and adaptive immunity in more detail, we analyzed the contribution of TLR-mediated innate immune recognition to priming of cytotoxic T lymphocytes (CTL) during the response against viral and minor H antigens (Chapter 6). For infection with human influenza virus A, normal CTL responses are induced in the absence of MyD88- and TLR-mediated signals. However, TLR signaling pathways are required to compensate for an induced lack or $CD4^+$ T cell help (Figure 3). Thus, we could demonstrate that innate immune recognition via TLRs is able to induce APC maturation levels sufficient for CTL priming *in vivo* even in the absence of antigen-specific $CD4^+$ T cell help.

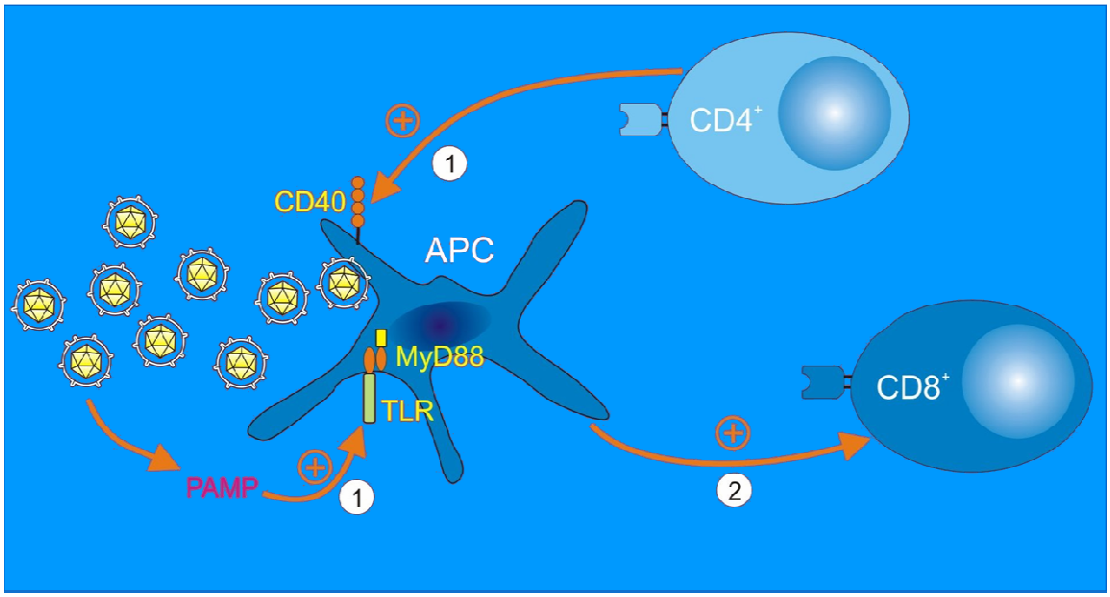


Figure 3. APC activating signals during CTL priming against viral antigens. During a viral challenge APCs are activated at least via two pathways that are each sufficient for CTL priming. One signal is provided by specifc $CD4^+$ T cells that recognize MHC class-II restricted viral eptiopes. The APCs are activated through the engagement of CD40 by CD40L on the T cell. This pathway belongs to the adaptive immunity. The second activation signal is provided in a TLR-mediated fashion by viral PAMPs. This pathway is a part of innate immune recognition. Each of these signals can provide enough activating signals even in the absence of the other to enable priming of $CD8^+$ cytotoxic T cells. From (Hilf, 2003).

For priming against minor H antigens as a model of weaker antigen immunogenicity, TLR signaling is indispensable for CTL responses. Certainly, the PAMP and/or danger signal involved in APC activation during priming against influenza and minor H antigens, respectively, still have to be identified. But the results obtained so far emphasize the importance of innate immune recognition for induction adaptive immune responses and underline the necessity for further studies in this field. A detailed understanding of how innate immunity shapes adaptive immunity provides the basis for new strategies to treat infections as well as for the development of effective vaccines to fight microbes or tumors. A first highlight in this area represent the impressive results of a clinical study conducted by Romero's group (Speiser et al., 2005): a synthetic CpG motif containing oligonucleotide, known to be a potent inducer of human innate immune reponses triggered via TLR9, proved to significantly enhance the immunogenicity of a human tumor vaccine leading to rapid and consistent antigen-specific CTL responses *in vivo*.

This thesis describes the identification of PAMPs and danger signals recognized by TLRs and provides new evidence for the tight **link between innate and adaptive immunity – mediated by TLRs**.

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List of publications

1. Ramirez,S.R., Singh-Jasuja,H., Warger,T., **Braedel-Ruoff,S.**, Hilf,N., Wiemann,K., Rammensee,H.-G., and Schild,H. (2005). Glycoprotein 96-activated dendritic cells induce a CD8-biased T cell response. Cell Stress.Chaperones *10*, 221-229.

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3. **Braedel,S.**, Radsak,M., Einsele,H., Latge,J.P., Michan,A., Loeffler,J., Haddad,Z., Grigoleit,U., Schild,H., and Hebart,H. (2004). Aspergillus fumigatus antigens activate innate immune cells via toll-like receptors 2 and 4. Br.J. Haematol. *125*, 392-399.

4. Scheel,B., **Braedel,S.**, Probst,J., Carralot,J.P., Wagner,H., Schild,H., Jung,G., Rammensee,H.-G., and Pascolo,S. (2004). Immunostimulating capacities of stabilized RNA molecules. Eur.J. Immunol. *34*, 537-547.

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