More than a Danger Signal – The Versatility of the Heat Shock Protein Gp96 in Innate and Adaptive Immunity

Mehr als ein Alarmsignal -
Die vielfältigen Rollen des Hitzeschockproteins Gp96 in der angeborenen und erworbenen 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)
ACAMP	Apoptotic cell-associated molecular patterns
APC	Antigen presenting cell
BMDC	Bone marrow-derived DC
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
CLIP	Class II-associated invariant chain peptide
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
DMSO	Dimethylsulfoxid
dsRNA	Double-stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	fluorescence-activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage colony stimulating factor
Gp96	96 kDa glucose-regulated protein
HAU	haemagglutinating units
HLA	Human leukocyte antigen
HSP	Heat shock protein
Ig	Immunglobulin
IL	Interleukin
***Imd* gene**	Immune deficiency gene
IMDM	Iscove's Modified Dulbecco's Medium
IRAK	IL-1receptor-associated protein kinase
ITAM	Immunoreceptor tyrosine-based activation motif
Jnk	c-Jun N-terminal kinase
LBP	LPS-binding protein
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	mitogen-activated protein kinase
MBL	Mannan-binding lectin
MDC	Monodansylcadaverine
MSR	Macrophage scavenger receptor
MyD88	Myeloid differentiation factor 88
OAS	2'-5'-oligoadenylate synthase (OAS)
OVA	Ovalbumin
Pam_3Cys	(S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteine
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline

PDI	Protein disulfide-isomerase
PE	Phycoerythrin
Poly(I:C)	Polyinosine-polycytidylic acid
PRP	Platelet rich plasma
PRR	Pattern recognition receptor
SAPK	Stress-activated protein kinases
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TIR domain	Toll/IL-1 receptor domain
TIRAP	TIR domain containing adaptor protein
TNF-α	Tumor necrosis factor-α
TRAF	TNF receptor activated factor
TRIF	TIR domain-containing adapter inducing IFN-β

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

Summary

Immunization with tumor lysates induces tumor protection against the original tumor in mouse models. Surprisingly, all tumor antigens purified from the lysates belong to the heat shock protein (HSP) family and are not mutated compared to the proteins in normal tissue. Instead, their immunogenicity is based on tumor-specific peptides bound to the HSPs. The investigation of the mechanisms that allow the induction of efficient immune responses with low doses of HSP:peptide complexes was the focus of this work, which was performed using mainly the ER-resident HSP Gp96.

We were able to show (chapter 3) that HSP-mediated immunity depends on its receptor-mediated uptake by professional antigen presenting cells (APCs). This is a prerequisite for the efficient shuttling of the associated peptides to the MHC molecules of the APC. Moreover, specific immune responses require the unspecific activation of APCs by danger signals. These lead to the upregulation of costimulatory molecules and the secretion of pro-inflammatory cytokines. Danger signals can be pathogen-derived as well as endogenous substances. Here we provide evidence that Gp96 itself is a danger signal (chapter 4). Gp96 triggers APC activation via the Toll-like receptors (TLRs) 4 and 2 pathways which are also used by pathogen-derived danger signals (chapter 6). TLR-mediated activation of the immune system is of importance for the specific immune response (chapter 7). Priming of cytotoxic T-lymphocytes (CTLs) after a viral challenge depends on either $CD4^+$ T cells or TLR-mediated signals. If both are missing, CTL priming is impaired. For minor H specific CTL responses, TLR signaling is strictly required. A similar importance of TLR-mediated signals can be expected for Gp96-induced immune responses.

The strong inflammatory signals triggered by Gp96 have to be controlled *in vivo*. In chapter 5, it is shown that human platelets are able to control the immunostimulatory capacities of Gp96. Platelets bind high amounts of Gp96, especially after their activation by thrombin. Thereby, free Gp96 is withdrawn resulting in a lower APC activation. This mechanism might have a physiological role during wound healing to prevent the adverse effects of chronic inflammation.

HSPs are more than endogenous danger signals released from dying cells. They also deliver detailed information about the events that caused the death. They combine everything in a single protein that is required for the induction of an efficient and specific immune response.

Zusammenfassung

Die Injektion von Tumorlysaten bewirkt im Mausmodell eine protektive Immunität gegen den verwendeten Tumor. Alle in den Arbeiten von P. Srivastava identifizierten Tumorantigene gehören jedoch zur Familie der Hitzeschockproteine (HSPs) und sind nicht gegenüber dem Protein im Ursprungsgewebe verändert. Die Immunogenität rührt vielmehr von an das HSP gebundenen, tumorspezifischen Peptiden. Die Aufklärung der Mechanismen, die die Induktion von Immunantworten mit geringsten Dosen von HSP-Peptid Komplexen ermöglichen, stand im Mittelpunkt dieser Arbeit, die vorwiegend mit dem ER-ständigen HSP Gp96 durchgeführt wurde.

Es konnte gezeigt werden, dass eine Voraussetzung HSP-vermittelter Immunität dessen Rezeptor-vermittelte Endozytose ist (Kapitel 3). Diese ist grundlegend für die effektive Repräsentierung der assoziierten Proteine in den MHC-Molekülen der APCs. Eine spezifische Immunantwort bedarf darüber hinaus auch unspezifischer Alarmsignale, die unter anderem die verstärkte Expression von costimulatorischen Molekülen auf professionellen Antigen-präsentierenden Zellen (APCs) bewirken. Diese Alarmsignale können Pathogen-assoziierte Signaturen oder endogene Substanzen sein. Wir konnten zeigen, dass Gp96 selbst ein solches Alarmsignal ist (Kapitel 4). Dabei vermitteln die Toll-like Rezeptoren (TLR) 4 und 2 die Gp96-induzierte Aktivierung (Kapitel 6), die auch Rezeptoren für Pathogen-assoziierte Alarmsignale sind. TLR-vermittelte Aktivierung des Immunsystems ist in anderen Systemen von Bedeutung für die spezifische Immunantwort (Kapitel 7): Für die Aktivierung cytotoxischer T-Zellen (CTLs) nach einer Virusinfektion sind entweder $CD4^+$ T-Helferzellen oder TLR-Signalwege nötig. In Abwesenheit beider Signalwege findet kein derartiges „Priming“ von CTLs statt. Für das CTL-Priming gegen Minorhistokompatibilitätsantigene sind TLR-Signale sogar unabdingbar. Eine ähnliche Bedeutung der TLR-Signalwege kann für Gp96 erwartet werden.

Die von Gp96 ausgelöste Entzündungreaktion muss *in vivo* begrenzt werden. In Kapitel 5 wird ein solcher Kontrollmechanismus durch Thrombozyten beschrieben, die besonders nach ihrer Aktivierung Gp96 sehr gut binden und die Aktivierung von APCs unterbinden.

HSPs sind mehr als nur Alarmsignale, die aus sterbenden Zellen freigesetzt das Immunsystem unspezifisch aktivieren. Sie liefern zusätzlich Informationen über den Grund, der zum Tod ihrer Ursprungszelle führte, und ermöglichen so eine effiziente Immunantwort.

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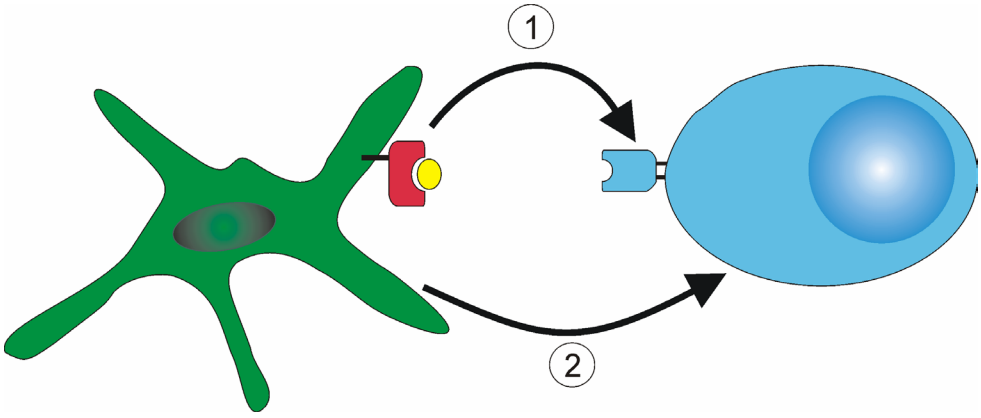
GENERAL INTRODUCTION



Multicellular organisms are first class residences for microorganisms. The supply with nutrition is overwhelming and, especially true for endothermic animals, the environmental conditions are comfortably constant. Therefore, it is not surprising that some bacteria and fungi have chosen a parasitic life style to exploit these advantages. Moreover, viruses got rid of an exhausting metabolism on their own and use the “you-know-what-you-get”-conditions inside higher life forms to fulfil the sole aim in nature, self-replication. Multicellular organisms had to evolve strategies to fight against colonization by microorganisms otherwise they would be easily outgrown by the rapidly dividing invaders – which by the way would be no real advantage for both. These strategies are summarized under the term immune system which is made up of two arms, innate and adaptive immunity. The former is specific for infectious nonself and recognizes infection through several germ line encoded receptors that recognize conserved microbial patterns. Adaptive immunity is based on a diverse and numerous receptor repertoire which is clonally distributed on T and B cells and specific for virtually all nonself antigens. This repertoire is not germ line encoded but emerges from the somatic recombination of a few inherited gene segments. Recognition of pathogens by the innate immune system results in immediate activation of effector mechanisms against the invaders. These early processes play also a central role in the induction of the adaptive response which is therefore to some extend under control of innate immunity. Although innate effector mechanisms are not able to defeat pathogenic microorganisms, they limit expansion of the pathogen until the slower, but more precise adaptive immune response is ready to do the job.

Adaptive immunity

Specificity for virtual every nonself antigen and memory are the striking advantages of adaptive immunity. Its flexibility is achieved by distinct antigen receptors on every individual B and T lymphocyte. This diversity is not germ line encoded but is created by the random recombination of variable gene segments during cell development. The randomized process of receptor generation implies that also lymphocytes specific for self antigens are generated. But self-reactive lymphocytes are eliminated in early phases of cell development, a prerequisite for the prevention of autoimmunity. The number of lymphocytes specific for a single antigen is relatively

small. After recognition of its specific antigen, however, B and T cells rapidly divide and develop into effector cells that eliminate the infectious agent. A subset of these clonally expanded cells, which have proven to be useful during the first infection, differentiates into memory cells that can be rapidly reactivated if the pathogen is encountered again.

Lymphocyte antigen recognition receptors

The immunoglobulins (Ig) were the first proteins involved in adaptive immunity that have been characterized. They are produced by B cells where they occur as membrane-bound antigen recognition receptors. Igs with the same specificity are secreted as antibodies by plasma cells being the terminally differentiated type of B cells. This differentiation of naïve B cells into plasma cells is triggered after recognition of the specific antigen by the membrane-bound Ig. Immunoglobulins are composed of two 50 kDa heavy chains, associated via disulfide bridges, and two 25 kDa light chain (kappa or lambda), each linked to one heavy chain. Heavy and light chains consist of four or two domains with typical immunoglobulin fold, respectively. In these densely packed moieties of about 110 amino acids, two β sheets are packed together and linked by a disulfide bond. The aminoterminal domains (V domains) of one heavy and one light chain build together the variable antigen binding side. The other, constant domains (C domains) do not vary between the Igs of different B cells and are responsible for the triggering of effector functions after antigen binding. The two antibody arms (F(ab) portions) defined by the light chain together with the associated first two Ig domains of the heavy chain are connected to the Fc portion through a flexible hinge region. This flexibility allows binding of two antigenic epitopes by both arms of an antibody with little steric constraints. Several different gene segments encode for the C domains of the heavy chain that can associate sequentially with the same variable region by DNA recombination, defining the five antibody isotypes IgM, IgD, IgG, IgE and IgA that differ in structure, localization, serum concentration and function.

The T cell receptor (TCR) resembles very much a membrane-bound F(ab) fragment of an antibody with an aminoterminal variable and a constant region. It is a glycosylated heterodimer of two polypeptide chains with two Ig domains each, termed α and β chains. Both subunits are membrane-anchored possessing an

unusual positively charged transmembrane region. A minor subset of T cells expresses an alternative, but structurally very similar γδ TCR.

Generation of B and T cell receptor diversity

The amazing point about lymphocyte antigen receptors is the enormous diversity that is created out of a limited number of inherited gene segments. The variable antigen-recognizing domains are encoded by separate gene segments that are brought together by somatic recombination in distinct stages of lymphocyte development. The light chains of immunoglobulins and the α chains of the TCR are composed of V (variable) and J (joining) gene segments, the heavy chains of Igs and the β chain of the TCR are further diversified by the contribution of D (diversity) elements. There are up to 100 different copies for each gene segment in the genome. Moreover, the Ig light chains are encoded in two loci (κ and λ). The random combinatorial diversity of Igs – taking into account that both light and heavy chain V domains build the antigen recognition site together – gives rise to approximately $3.5x10^6$ different antibodies in theory. Moreover, the joining of the gene segments is imprecise raising further the diversity to approximately 10^{14} possible antibodies and 10^{18} TCRs. For antibodies, somatic hypermutation provides an additional source of diversification which occurs after a B cell has encountered its specific antigen. During the initiated proliferative cycles, point mutations occur at a very high rate in the V regions of the rearranged heavy and light chain genes. If a clone with improved antigen affinity is created, it is preferentially selected for proliferation and differentiation into plasma cells (affinity maturation). Therefore, several mechanisms contribute to an enormous diversity of lymphocyte antigen receptors, enabling the immune system to recognize all possible antigens.

The random generation of TCR and Ig diversity implies the disadvantage that also antigen receptors against self-structures are created. These self-reactive lymphocytes have to be deleted or rendered unfunctional to prevent autoimmunity. The processes accounting for the self-tolerance of the lymphocyte repertoire take place during cell development in the bone-marrow or thymus for B or T cells, respectively. The fate of immature B cells as soon as they express IgM on their surface is determined by the signal they receive via their B cell receptor. Strong autoantigens that crosslink the IgM receptors lead to the elimination of the B cells by

apoptosis (Chen et al., 1995) or to the replacement of the receptor by “receptor editing”. During this process, light-chain gene rearrangement is re-entered until a self-tolerant Ig has been created (Tiegs et al., 1993). Autoantigens that are not able to crosslink the B cell receptor render the developing cell non-functional with downregulated receptor and blocked signal transduction (Goodnow et al., 1988), a state termed anergy. For T cells, the shaping of the immature cell repertoire is even more complex. On the one hand, autoreactivity has to be prevented (negative selection), on the other hand mature T cells have to interact functionally with self MHC molecules (positive selection, Zinkernagel et al., 1978). Only thymocytes that bind moderately but not excessively to self antigens on thymic cortical epithelial cells survive and continue their maturation. Strong interaction of the TCR with self antigens leads to the deletion of the autoreactive T cell in the thymus by apoptosis. Bone-marrow derived macrophages and dendritic cells in the thymus are believed to mediate this process of negative selection. However, for both subsets of lymphocytes some autoreactive cells mature and make their way to the periphery, mainly because some autoantigens are not expressed in bone-marrow or thymus. But also in these cases, control mechanisms exist to prevent autoimmune reactions. If a B cell recognizes a ubiquitous autoantigen in the periphery, it becomes anergic due to the lack of T cell help. The same holds true for T cells if the antigen is encountered on peripheral tissue without costimulatory signals that usually can only be given by professional antigen presenting cells (APCs).

B cells - antigen recognition by immunoglobulins

The effector function of B cells is the secretion of antibodies after they encountered their specific antigen. These antibodies bind to structures on the pathogen’s surface resulting in the neutralization and destruction of the invaders by cells of the innate immune system or by complement activation.

On mature B cells, membrane-bound Igs (IgM or IgD) are associated with a heterodimer of Igα and Igβ that is responsible for signal transduction into the cell after ligation of the B cell receptor. Igα and Igβ contain conserved tyrosine containing motifs that can be phosphorylated by Src family kinases. These sequences are called immunoreceptor tyrosine-based activation motifs (ITAMs). Syk, another protein tyrosine kinase, binds to fully phosphorylated ITAM motifs, gets itself phosphorylated

and thereby activated, kicking of the downstream propagation of the signal via several pathways including the MAP kinase and NF-κB pathways. This signal is 1.000- to 10.000-fold enhanced by the contribution of the B cell co-receptor that contains CD19, CD21 and CD81. Moreover, the membrane-spanning tyrosine protein phosphatase CD45 is required for B cell signaling. CD45 associates with the B cell receptor and removes an inhibitory phosphate group from Src family kinases. In the nucleus, several transcription factors trigger proliferation and cell maturation resulting in the development of clonally expanded plasma cells.

On the interaction of T cells with MHC molecules

In contrast to secreted antibodies that are directed against extracellular pathogenic structures, T cells can detect cells infected with intracellular pathogenes such as viruses and some bacteria. Those cells present peptide fragments from pathogen-derived proteins on their surface (Townsend et al., 1985). The peptides are generated inside the cell as a by-product of protein turnover and are delivered to the cell surface bound to specialized polymorphic glycoproteins, the major histocompatibility complex (MHC) molecules. These proteins are encoded in the MHC region which was initially identified as the gene locus that is linked with the rejection or acceptance of tissue transplants.

Two classes of MHC molecules can be distinguished. MHC class I proteins consist of a membrane-bound 43 kDa α chain associated with the invariant β_2 microglobulin (12 kDa). The two N-terminal domains (α_1 and α_2) fold into a structure in that two α helices lay on top of a basal β sheet. This composition builds a long peptide binding groove. MHC class II molecules consist of two membrane bound subunits (α and β chain). The N-terminal domains (α_1 and β_1) form together the peptide binding cleft that structurally resembles that of class I molecules although it is more open at both ends.

The ligand for the variable TCR is not peptide alone, but the peptide bound to self-MHC molecules (Zinkernagel and Doherty, 1974). This MHC restriction is based on the nature of the interaction between the TCR and its ligand and on the polymorphism and polygenicity of the MHC molecules. The TCR makes intense contacts with both, the MHC molecule and the peptide (Garboczi et al., 1996). Therefore, the MHC portion influences the binding significantly. However, many MHC

residues at the TCR interface are conserved and the MHC's allelic variability is restricted to the residues that form the peptide binding cleft. Different MHC alleles therefore differ in their requirements for the peptides to be bound, and MHC restriction of T cells is to a big portion due to the fact that different MHC alleles present distinct sets of peptides. The single binding site of a MHC molecule is able to bind a variety of different peptides with high affinity while other peptides do not bind at all. The crystal structure of MHC:peptide complexes gives insights into this binding characteristic (Bjorkman et al., 1987). The peptide is anchored in the MHC groove via two or three residues that point into the direction of the MHC. Most peptides that bind to a given MHC allele possess the same or chemically related amino acids at these anchor residues while the sequence at other positions of the peptide is more or less unrestricted. Therefore, a peptide binding motif can be assigned for every MHC variant (Falk et al., 1991). Moreover, MHC class I molecules establish stable interactions with the N- and C-termini of the peptide. Therefore, MHC binding peptides are restricted in length to 8-10 amino acids. MHC class II peptides in contrast are variable in length. Due to the open conformation of their binding cleft the class-II peptides protrude at both ends of the MHC molecule. However, due to the moderate sequence restriction by two or three anchor positions a bundle of different peptides generated in a cell binds to the MHC molecules of a cell. In addition, the polygenicity and polymorphism of both classes of MHC molecules ensure that several different MHC variants are expressed on every cell of an individual, each bearing a different peptide repertoire Therefore, it can be assumed that the peptide fragments presented on the surface reflect a very detailed picture of the protein content inside the cell. On the other hand, the MHC restriction of T cells does not only reflect differences between MHC alleles in residues that directly establish contact between MHC and TCR, but more importantly differences in the bound peptide repertoire between MHC variants.

T cell effector functions

Beside the trimeric MHC:peptide:TCR contact, activation of naïve T cells requires the interaction of one of the T cell co-receptors CD4 or CD8 with conserved structures on MHC molecules. The two subsets of T cells can be distinguished by their expression of these co-receptors. $CD8^+$ T cells interact with MHC class I molecules that are expressed on almost all tissues. They scan peripheral cells for the presence of

foreign peptide fragments in their MHC class I molecules as a sign of infection. If the cognate antigen is recognized, $CD8^+$ T cells possess profound weapons to eliminate infected cells. $CD8^+$ T cells are therefore called cytotoxic T lymphocytes (CTLs). $CD4^+$ T cells (helper T cells) are MHC class II restricted and can be differentiated into two subtypes, T_H1 and T_H2 cells. MHC class II molecules are expressed on professional antigen presenting cells (APCs) only and on normal tissue after IFN-γ stimulation. The main task of the IFN-γ secreting T_H1 cells is the activation of infected macrophages that enhances the microbicidal functions of the latter. T_H2 cells support the differentiation of naïve B cells. The activation of B cells, macrophages and dendritic cells (DCs) by $CD4^+$ T cells is mediated by the interaction of CD40 ligand (CD40L) on the T cell with CD40 on the APC (Schoenberger et al., 1998a).

The signal provided by the TCR and the T cell coreceptor alone is not sufficient for the activation of naïve T cells. A second, costimulatory signal is required which usually can only be provided by APCs after their activation. The best characterized costimulatory molecules are the homodimeric glycoproteins CD80 and CD86 (reviewed in Carreno and Collins, 2002). Binding to their receptor on the T cell side (CD28) is necessary for the synthesis of IL-2. IL-2 in turn induces T cell proliferation in an autocrine and paracrine fashion. Another pair of costimulatory molecules with also backward directed APC activating capacities are 4-1BB on the T cell and 4-1BB ligand on the APC (DeBenedette et al., 1995). The upregulation of costimulation on APCs requires their maturation. This process can be initiated on the one hand in an antigen-specific manner by $CD4^+$ cells via CD40:CD40L interaction and on the other hand via innate immune recognition mechanisms, as will be discussed later. However, the requirement for a second signal ensures that an immune response is not triggered preliminarily – e.g. against autoantigens expressed in the periphery.

The three paths of antigen presentation

MHC class I processing pathways

The peptides presented in MHC molecules are generated by distinct antigen processing pathways (Morrison et al., 1986). Most of the peptides bound to MHC class I molecules are generated in the cytosol (Stoltze et al., 2000a). There, proteins to be degraded are cut into small peptide fragments by the proteasome irrespective of their origin (viral, bacterial or self proteins). Beside this normal protein turnover, a

significant source of peptides are defective ribosomal products (DRiPs), which consist of prematurely terminated and misfolded polypeptides (Yewdell et al., 1996; Khan et al., 2001). The generated fragments are further N-terminally trimmed by cytosolic and ER-resident aminopeptidases (Stoltze et al., 2000b; Serwold et al., 2002; Saric et al., 2002). A small subset of these peptides escapes breakdown to the amino acid level and is transported into the ER by the "Transporter associated with Antigen Processing" (TAP) in an ATP-dependent manner. In the ER, the pre-built, chaperone-stabilized MHC class I molecule is physically linked to the TAP transporter by tapasin, ready to bind freshly imported peptides. After successful peptide loading the MHC molecule is released from the associated chaperones and is transported to the cell surface.

MHC class II processing

MHC class II molecules are loaded with peptides that are generated in low-pH endocytic vesicles through various cathepsins as the predominant endosomal proteases. Exogenous proteins, which are uptaken from the environment by endocytosis or which derive from intravesicular pathogens, or self proteins, residing in or traveling through endosomal compartments, are substrates for theses enzymes and therefore possible sources of class II presented peptides.

MHC class II molecules are – as all non-mitochondrial transmembrane proteins – synthesized into the ER membrane. However, not the ER, but a specialized endosomal compartment is the place of MHC class II peptide loading (MIIC; MHC class II compartment). Newly synthesized MHC class II molecules associate with the invariant chain (Ii) protein. A polypeptide stretch of the Ii occupies the peptide binding groove, thereby preventing premature binding of peptides. Moreover, the Ii allows the release of the MHC molecule from the ER and directs its transport to the MIIC. There, the Ii is cleaved by acidic proteases leaving a short peptide, CLIP (class II-associated invariant chain peptide), bound to the MHC peptide cleft. CLIP is replaced by other peptides with the help of the MHC class II-like molecule HLA-DM (H2-M in mice) that stabilizes class II molecules and catalyzes peptide exchange (Morris et al., 1994; Fling et al., 1994; Kropshofer et al., 1997). The prolonged facilitation of peptide exchange by HLA-DM also selects for high affinity peptides, a process called *peptide editing*. The physiological role of HLA-DO, another class II-like molecule (H2-O in mice) is unclear as it acts as a negative regulator of HLA-DM in B

cells and thymic epithelial cells (Denzin et al., 1997). Finally, stable MHC class II peptide complexes leave the MIIC and are transported to the cell surface.

Cross-presentation

The separation of the two antigen processing pathways makes perfect sense with regard to the distinct functions performed by the two classes of MHC molecules. Virus-infected cells display viral epitopes in their MHC class I molecules and thereby can be killed by specific CTLs. MHC class II-bearing APCs on the other hand require activating signals by T helper cells after the uptake of exogenous antigens to perform their several function. However, the priming of e.g. naïve $CD8^+$ T cells depends on the presence of costimulatory molecules and antigenic peptide bound to MHC class I molecules on the same cell. For viral infections, this implies that APCs should be susceptible for infection by all kinds of viruses. Indeed, dendritic cells as the "most professional" APCs can be infected by almost all viral pathogens. But some viruses, such as the human papillomavirus, infect exclusively peripheral tissue. Despite this, a potent immune response is initiated against this pathogen. Moreover, MHC class I-negative tumors are targets of CTL responses. These obeservations suggest that exogenous antigen has access to the MHC class I presentation pathway.

The existence of such a pathway was first noted when mice primed with cells expressing different minor H antigens and MHC molecules generated a self-MHC restricted CTL response against the foreign minor H antigens (Bevan, 1976a; Bevan, 1976b), implying a transfer of antigen from the donor cells to the MHC molecules of the host APCs. This phenomenon has been termed *cross-presentation* and the resulting T cell priming is referred to as *cross-priming* (reviewed in Heath and Carbone, 2001). In particular, these definitions are used to describe the shuttling of exogenous antigens into the MHC class I presentation pathway of the APC. Dendritic cells, macrophages and even B cells have been shown to be able to cross-present exogenous antigen *in vitro*. In mice, a subset of DCs – the $CD8a^+$ lymphoid population – cross-presents antigens constitutively and most efficiently *in vivo* (den Haan et al., 2000; Pooley et al., 2001), although $CD8a^-$ myeloid DCs seem to have the same capacities after activation e.g. by FcγR ligation (den Haan and Bevan, 2002). Due to their activation-independent cross-presentation $CD8a^+$ DCs are believed to play an essential role in the induction of tolerance to peripheral antigen through *cross-tolerization*, when no DC activating stimuli are present.

The efficiency of cross-presentation is enhanced if the antigen is delivered as apoptotic material (Albert et al., 1998b), associated with heat-shock proteins (Srivastava et al., 1994), in immuncomplexes (Rodriguez et al., 1999) or exosomes (Wolfers et al., 2001). The latter are a population of small membrane vesicles secreted by cells and enriched with distinct proteins. The preference for antigen in these forms seems to be a direct result of the efficient uptake via receptor-mediated mechanisms (Albert et al., 1998a; Singh-Jasuja et al., 2000c). The cross-presentation pathway towards the MHC class I molecule, once the antigen has entered the APC, is not yet fully understood. There are several reports suggesting that for processing of exogenous antigens proteasomal cutting and TAP are required (Regnault et al., 1999; Norbury et al., 1995; Kovacsovics-Bankowski and Rock, 1995), especially if the C-terminus of a MHC epitope has to be trimmed (Castellino et al., 2000). The mechanism of antigen delivery to the cytosol is still enigmatic. Alternatively, exogenous antigen is degraded in endosomal compartments, followed by “regurgitation” of generated peptides and their surface loading on MHC class I molecules (Pfeifer et al., 1993). However, the rules that define whether an antigen is processed via either one or both of these routes still have to be determined.

Pattern recognition – the common theme in innate immunity

To respond or not to respond – during a possible infection this decision for the immune system is believed to be done by innate immune recognition of pathogen associated molecular patterns (PAMPs) (Janeway, Jr. and Medzhitov, 2002). This implies that the recognized motifs cannot be mutated by the pathogens without life-threatening loss of function. As a matter of fact, many known PAMPs are typical nucleic acids or conserved components of cell wall structure from microorganisms. PAMPs are detected by pattern recognition receptors (PRRs) which include membrane-bound receptors and soluble proteins in extracellular fluids and intracellular compartments. PRRs in contrast to the variable antigen receptors of lymphocytes are fixed in the genome and each cell of a given subtype bears the same limited set of receptors. Due to selection mechanisms during evolution the self-foreign discrimination by PRRs is as perfect as possible, while the self-nonself decision by lymphocyte antigen receptors is error-prone – owing to their generation

by random gene rearrangement and despite all control mechanisms that evolved to avoid self-reactivity.

Toll-like receptors

Toll-like receptors (TLRs) comprise a growing list of pattern recognition receptors with different ligand specificities (Table 1). Their recent discovery beginning in 1997 with the cloning and characterization of TLR4 by Medzhitov et al. (Medzhitov et al., 1997) gave new impulses to the field of innate immunity. TLRs are type I transmembrane proteins with an extracellular leucine-rich repeat domain and a conserved intracellular domain. The latter is homologous with the cytosolic domain of the IL-1 receptor and therefore called Toll/IL-1 receptor (TIR) domain. The TIR domain is found in several transmembrane and cytosolic proteins in animals and plants. The majority of them plays a role in host defense.

Table 1. Pathogen-associated molecular patterns (PAMPs) from various pathogens and the corresponding Toll-like receptors (TLRs).

PAMP	Pathogen	TLR
lipoarabinomannan	mycobacteria	TLR2
zymosan	yeast	TLR2
lipoproteins	bacteria, *Borrelia*	TLR2
peptidoglycans	gram-positive bacteria	TLR2
glycophosphatidyl anchors	*Trypanosoma cruzi*	TLR2
hemagglutinin	measles virus	TLR2
Hsp60	*Chlamydia*, human	TLR4, TLR2
Gp96	mouse	TLR4, TLR2
Hsp70	human	TLR4, TLR2
LPS	gram-negative bacteria	TLR4, CD14, MD-2, LBP
RSV fusion protein	respiratory syncytial virus	TLR4, CD14
unmethylated CpG	bacteria	TLR9
double-stranded RNA	viruses	TLR3
flagellin	flagellated bacteria	TLR5
imidazoquinolines	(antiviral drugs)	TLR7, TLR8

Drosophila's Toll and insect immunity

The Toll gene of *Drosophila melanogaster* was discovered in 1985 as the first member of the Toll family (Anderson et al., 1985). During embryogenesis it is an essential component of a pathway that controls dorsoventral axis formation. However, Toll is also an important part of a very effective innate immune system relying on pattern recognition (reviewed in Hoffmann and Reichhart, 2002). *Drosophila* Toll mutants are unable to withstand fungal infections due to the failure to produce the antifungal peptide Drosomycin (Lemaitre et al., 1996) which is synthesized and secreted into the hemolymph by the insect's fat body cells – a functional liver equivalent. Activation of the Toll signalling pathway requires cleavage of the extracellular polypeptide spaetzle by a serine protease. Ligation of Toll by trimmed spaetzle leads via the adaptor protein Tube and the protein kinase Pelle to activation of the NF-κB family member Dif (Drosophila immunity factor) inside the cell. The upstream events that couple pathogen recognition to cleavage of spaetzle are not yet fully understood. *semmelweis (seml)*, an induced mutation in a gene encoding for a peptidoglycan-recognition protein, abolishes Toll-dependent response to infection with Gram-positive bacteria while antifungal defense is not affected (Michel et al., 2001). This suggests the existence of two different pathways leading to spaetzle cleavage depending on the type of pathogen. Recently, mutations in the *persephone* gene encoding a serine protease have been described showing impaired Toll-mediated response to fungal infections (Ligoxygakis et al., 2002). Whether the *seml* and *persephone* gene products are directly involved in spaetzle cleavage and whether they are part of a protease cascade is still unclear.

Recognition of Gram-negative bacteria is not Toll-mediated in *Drosophila* (Lemaitre et al., 1995). The presence of these pathogens triggers the activation of the Imd pathway which is similar to mammalian tumor necrosis factor-α (TNF-α) signaling. The *immune deficiency (imd)* gene encodes a cytosolic protein containing a death domain. This pathway results in the activation and nuclear translocation of the transcription factor Relish, another member of the Rel / NF-κB protein family. Relish promotes the transcription of several genes encoding antibacterial peptides (e.g. Drosocin). However, the receptor that links Gram-negative infection to the Imd pathway is unknown.

Mammalian Toll-like receptors in pathogen defense

Today ten different mammalian members of the TLR family have been identified. TLRs are expressed differentially in cells of the immune system and respond to different stimuli.

TLR4

LPS from the outer membrane of Gram-negative bacteria induces a severe and generalized inflammation in mammals leading to a life-threatening condition called endotoxin shock. The LPS-binding protein (LBP) in the serum concentrates LPS and recruits it to the principal LPS receptor on mononuclear cells, CD14. However, cells from CD14-deficient mice respond to LPS, although only at markedly higher concentrations. Moreover, CD14 lacks a cytoplasmic domain and soluble CD14 can substitute for the membrane bound form. These findings suggested that other co-receptors are involved in LPS signaling that transmit the information across the cell membrane. In 1998, the mutation of the C3H/HeJ mouse strain was analyzed, which is hyporesponsive to LPS. This strain carries a missense point mutation within the region of the *Tlr4* gene that encodes for the cytoplasmic tail of the receptor. This mutation leads to the exchange of a highly conserved prolin to a histidine residue and abrogates LPS-mediated cell activation (Poltorak et al., 1998). Transfection of TLR4 alone does not confer LPS responsiveness to cells, suggesting that at least one other molecule is involved in the TLR4 receptor complex. Subsequently, the missing factor was identified as the secreted molecule MD-2 which binds to the extracellular domain of TLR4 (Shimazu et al., 1999). Moreover, TLR4 is not able to reach the plasma membrane in MD-2 deficient cells (Nagai et al., 2002). TLR4 is not only a receptor for LPS, but has been shown to be essential for the cell activating signals induced by the heat-shock proteins Hsp60, Hsp70 and Gp96 (Asea et al., 2002; Vabulas et al., 2001; Vabulas et al., 2002; Asea et al., 2002). Moreover, defensins have been shown to trigger DC maturation via TLR4 (Biragyn et al., 2002).

TLR2

Several reports suggested that TLR2 is also involved in LPS signaling (Yang et al., 1998; Kirschning et al., 1998). However, cells carrying a null allele for TLR2 show a normal response to LPS (Heine et al., 1999). Therefore, it was suggested that

expression of TLR2 may render cells sensitive to minor contaminations in LPS preparations. Highly pure LPS does not signal via TLR2 (Hirschfeld et al., 2000). Thereafter, lipoproteins 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 carry a lipid at the N-terminal cysteines and are produced by a variety of bacteria. Moreover, TLR2 recognizes cell wall components from different pathogens including zymosan of yeast (Underhill et al., 1999), mycobacteria (Means et al., 1999a; Means et al., 1999b), peptidoglycans (PGNs) from Gram-positive bacteria (Schwandner et al., 1999), and glycophosphatidylinositol anchors from *Trypanosoma cruzi* (Campos et al., 2001). Homodimers of TLR2 are not able to induce cytokine production (Ozinsky et al., 2000), functionality and some ligand specificity is provided by heterdimerization with other TLRs, such as TLR1 and TLR6: The diacetylated mycoplasmal macrophage-activating lipopetide 2 (MALP-2) is not recognized by cells defective in TLR 2 or TLR6, while triacetylated lipoproteins of bacterial origin trigger cell activation in the absence of TLR6 (Takeuchi et al., 2001).

TLR9

Another conserved molecular pattern of bacteria is hidden in their DNA structure. In vertebrate DNA the sequence motif CpG occurs at about one fifth of the randomly expected frequency only and, if present, it is more likely to be methylated at the cytosine than bacterial CpG sequences. Unmethylated bacterial CpG DNA has a stimulatory effect on mammalian immune cells including, B cells, macrophages and dendritic cells (Krieg et al., 1995; Stacey et al., 1996; Sparwasser et al., 1998) resulting in secretion of T_H1-like cytokines and in expression of costimulatory molecules. In 2000, TLR9 was identified as the receptor mediating CpG signals and was therefore the third TLR with known ligand specificity (Hemmi et al., 2000). TLR9 is not localized at the cell surface but resides in endosomes (Ahmad-Nejad et al., 2002). In line with this, CpG- but not LPS-induced cell activation requires endocytosis and endosomal maturation. Interestingly, the DNA-dependent protein kinase (DNA-PK), an enzyme activated by DNA and involved in DNA repair of double strand breakes, is also essential for CpG-dependent cell activation (Chu et al., 2000). However, the relationship between TLR9 and DNA-PK in the CpG response remains unclear.

TLR5

Flagellin is a major component of the bacterial flagella and is recognized by the innate immune defense of organisms as diverse as plants, flies and mammals. Recently, the signal transducing receptor has been identified as TLR5 (Hayashi et al., 2001). Expression of flagellin in non-flagellated *Escherichia coli* conferred the ability to activate cells via TLR5. Therefore, diverse conserved patterns of bacteria - comprising DNA structure, cell wall compounds and flagella - are recognized by a set of different TLRs. This strategy makes it difficult for bacteria to circumvent the innate immune response simply by mutation.

TLRs in antiviral defense

Fewer implications of TLRs in antiviral immunity are known. One reason for this might be that viruses comprise a highly diverse and variable group of pathogens with not much more in common as being some kind of packaged nucleic acid that bears the information for self replication. PAMPs are therefore not likely to be shared between different classes of viruses. Viruses lack an own metabolism and rely completely on the intracellular environment of their host. This mainly intracellular appearance of viruses is an additional argument against the existence of extracellularly acting PAMPs. However, there are some hints for an involvement of TLRs in antiviral immune responses. Double-stranded RNA (dsRNA) is recognized by TLR3 (Alexopoulou et al., 2001) and is associated with viral infection. dsRNA occurs at some point during the replication cycle of most viruses and is absent in non-infected cells. Moreover, murine and human TLR7 and human TLR8 have been shown to mediate the response to imidazoquinolines, low molecular weight compounds used in the treatment of external genital warts caused by papillomavirus infection (Hemmi et al., 2002; Jurk et al., 2002). These molecules are structurally similar to purine bases. This argues for the existence of until now unknown viral PAMPs as natural ligands of TLR7 and TLR8 originating from nucleic acid. But also structural proteins of the outer viral coat are recognized by TLRs: The fusion protein of respiratory syncytial virus, an important respiratory pathogen of humans, activates cells in a TLR4- and CD14-dependent matter. It has been shown that the virus persists longer in the lungs of infected TLR4-deficient mice compared to wildtype mice (Kurt-Jones et al., 2000). Similarly, the coat protein hemagglutinin of measles

virus activates the innate immune system via TLR2 (Bieback et al., 2002). Vaccinia virus evolved a mechanism to interfere with TLR-mediated signaling suggesting an important role of this pathway in antiviral immunity: two ORFs of the viral genome share amino acid sequence homology with the TIR domain. Expression of the corresponding protein products in mammalian cells inhibits IL-1, IL-18 and TLR4 signaling but not MyD88-independent pathways (Bowie et al., 2000). However, up to now our understanding of TLR involvement in antiviral responses is incomplete.

TLR signaling pathways

The TLR and IL-1 receptor signaling pathways share most of their participating molecules which is not surprising with regard to their homologous intracellular domains (figure 1). Binding of PAMPs to TLRs leads to the recruitment of the adaptor protein myeloid differentiation factor (MyD) 88 to the receptor via homophilic interaction of the TIR domains (Muzio et al., 1997; Wesche et al., 1997; Medzhitov et al., 1998). The TIR domain of MyD88 is connected to a death domain. MyD88 transduces the TLR signal via this module to IL-1R-associated protein kinase (IRAK), a serine kinase related to Pelle in *Drosophila* that contains another death domain. Phosphorylated IRAK dissociates from the receptor complex and associates with tumor necrosis factor (TNF) receptor-activated factor 6 (TRAF6). Via further unknown mechanisms this signal leads to activation of the transcription factors c-Jun N-terminal kinase (Jnk), p38 mitogen-activated protein kinase (MAPK) and NF-κB. In MyD88-deficient mice ($MyD88^{-/-}$), cell activation in response to most TLR-mediated stimuli is completely abolished (Kawai et al., 1999). However, retarded activation of NF-κB and p38 after LPS stimulation of macrophages can be observed, while cytokine secretion is severely impaired. Moreover, CD80 and CD86 are upregulated in $MyD88^{-/-}$ but not $TLR4^{-/-}$ derived BMDCs after LPS treatment. These observations suggested the existence of a TLR4-dependent but MyD88-independent signaling pathway. Recently, the additional adaptor proteins TIRAP (TIR domain containing adaptor protein; also called MAL) (Horng et al., 2001; Fitzgerald et al., 2001) and TRIF (TIR domain-containing adapter inducing IFN-β) (Yamamoto et al., 2002b; Oshiumi et al., 2003) have been described. TIRAP is involved in signaling of TLR4, 2, 6 and 1 and is required for cytokine secretion by mouse DCs after stimulation via these TLRs even in the presence of MyD88 (Horng et al., 2002; Yamamoto et al.,

2002a). Due to these results TIRAP is not involved in the MyD88-independent signaling pathway described above. TRIF mediates most probably MyD88-independent signaling via TLR3. However, MyD88 is the most universal adaptor protein, because it transduces signals from all known TLRs and from the IL-1 and IL-18 receptors. Bone-marrow derived dendritic cells from MyD88-deficient mice reveal a markedly reduced cytokine secretion in response to most PAMPs or non at all (Kawai et al., 1999), underlining the importance of MyD88 in TLR signaling. Whether Tollip, a recently discovered component of the IL-1 receptor signaling pathway, plays a role in TLR signaling still has to be determined. Tollip is associated with IRAK before IL-1 receptor stimulation and occurs transiently in the activated IL-1 receptor complex (Burns et al., 2000). Therefore, it was suggested that Tollip helps in IRAK recruitment to the activated IL-1 receptor.

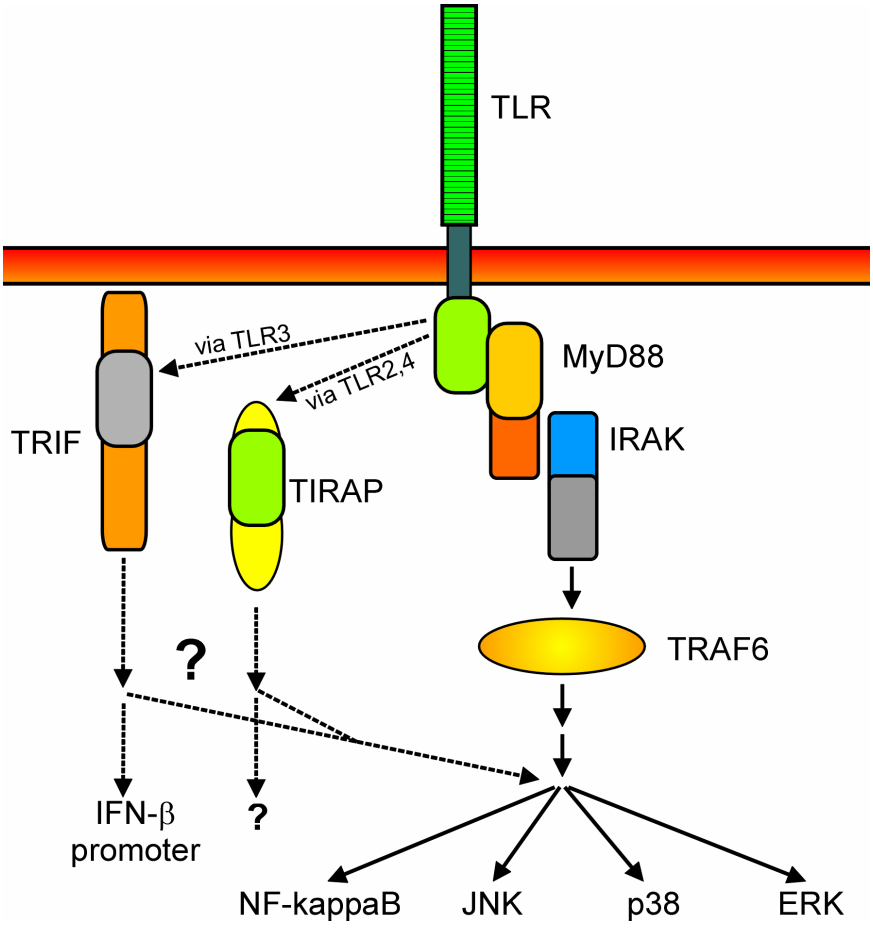


Figure 1. TLR signaling pathways. Ligation of TLRs leads to the recruitment of an adaptor protein to the intracellular TIR domain of the receptor. The adaptor MyD88 interacts with all TLRs and associates with IRAK that gets autophosphorylated. Phosphorylated IRAK associates with TRAF6. This leads to the activation of several transcription factors. Signaling via TLR2 and 4 depends on the presence of the additional adaptor TIRAP. The third known adaptor TRIF is important for the IFN-β production after TLR3 stimulation. For TLR4 another MyD88- and TIRAP-independent signaling pathway has been described that is able to mediate upregulation of costimulation, although with delayed kinetics.

Other pattern recognition receptors

TLRs are important, but not the only pattern recognition receptors used by the innate immune system. There are several other proteins on and in the cell involved in PAMP recognition. Mannan-binding lectin (MBL), C-reactive protein and serum amyloid protein are liver-produced soluble PRRs. They belong to the acute phase proteins, which are secreted into the blood stream in early stages of infection. These receptors recognize cell wall compounds of microorganisms, such as terminal mannose residues in the case of MBL. Inside the cell, several PRRs are known to be involved in antiviral responses. Viruses gain access to the cytosol where they start their reproductive machinery. The double-stranded RNA-dependent protein kinase PKR is activated by dsRNA which occurs during replication of RNA and some DNA viruses (reviewed in Clemens and Elia, 1997). Activated PKR inhibits the translation initiation factor eIF2α by phosphorylation and thereby blocks all cellular protein synthesis. Moreover, PKR induces via NF-κB and MAP kinase signaling pathways the production of Type-I interferons. These exert antiviral effects by the reduction of protein synthesis in neighboring cells. Another intracellular PRR for dsRNA is 2'-5'-oligoadenylate synthase (OAS). OAS ligated by dsRNA synthesizes the formation of 2'-5'-oligoadenylate. This second messenger activates RNAseL which destroys all cellular RNA, resulting in complete block of protein synthesis and as a consequence thereof in apoptosis.

The human NOD1 and NOD2 proteins, that are structurally related to apoptosis factor Apaf-1/Ced-4 and to a class of plant resistance genes, have been shown to confer responsiveness to bacterial lipopolysaccharides (Inohara et al., 2001). Interestingly, NOD proteins contain like TLRs a leucine-rich repeat domain.

On the cell surface, macrophage mannose receptor and dectin-1 are additional PRRs (Brown et al., 2002), that recognize bacterial and yeast surface carbohydrates. Macrophage scavenger receptors (MSRs) have a broad binding specificity for polyanions including dsRNA, LPS and lipoteichonic acid. MSR deficient mice show an increased susceptibility to bacterial and viral infections (Suzuki et al., 1997) suggesting a role for MSRs in host defense against pathogens.

The list of mentioned PRRs is by no means complete and certainly more PRRs will be discovered after the field of innate immune recognition has been brought into focus again with the discovery of the Toll-like receptors.

Effector functions of the innate immune response

Once a pathogen is recognized by the innate immune system several effector mechanisms are initiated to fight the invaders and to limit their spread until the adaptive immune system has lined up. Activated macrophages phagocytose pathogens and digest them in their phagolysosomes by lytic enzymes. Low pH, bactericidal peptides and reactive oxygen species produced in a so called respiratory burst contribute to the inhospitableness of these compartments. Moreover, cytokines and chemokines are released that induce inflammation and recruitment of neutrophils, macrophages and finally also cells of the adaptive immune system to the site of infection. IL-6 induces the production of acute phase proteins in the liver that contribute to host defense. Inflammation leads to an increased permeability of the vascular epithelia. By this, a group of serum proteins belonging to the complement system gains access to the site of infection. The mannan-binding lectin (MBL) recognizes pathogen surfaces and triggers a proteolytic cascade. Finally, the complement system forms holes in the pathogen's membrane by a "membrane-attack complex", the pathogen is opsonisized for phagocytosis and inflammatory mediators are released. By these means, the immune system is able to defeat the majority of invading microorganisms and all invertebrates are perfectly happy with only these defense mechanisms at hand.

Links between innate and adaptive immunity

The immune system functions by discriminating nonself from self. In the original Self-Nonself (SNS) model proposed by Burnet and supported by experiments from Medawar this decision is exclusively made by cells of adaptive immunity (B and T cells). Lymphocytes are taught the definitions of self early in their development. However, in 1975 Lafferty and Cunningham performed experiments that suggested the requirement of a second signal for T cell activation (Lafferty and Cunningham, 1975). They named this signal "costimulation", which T cells receive from stimulator cells (today known as APCs). Costimulation was later assigned to molecules on the APC site (such as CD80 and CD86) that specifically interact with receptors on the T cell site (e.g. CD28). This interaction leads to IL-2 responsiveness in the T cell and therefore allows its proliferation and maturation. It has been reported very early that the costimulatory capacities of APCs are activation dependent – B cells that had

been stimulated for 3 days with LPS and dextran sulfate gained T cell stimulatory capacities in a mixed lymphocyte reaction while unstimulated B cells were poor stimulators (Krieger et al., 1986). The concept of a second signal implies that the decision to initiate an immune response is dependent on cells without diversified antigen-specific receptors. Therefore, the SNS model had to be refined in order to account for the importance of costimulation. In 1989, Janeway suggested a distinct sense of self-nonself discrimination (Janeway, Jr., 1989). He proposed that APCs are quite poor T cell stimulators (due to lack of costimulation) until they are activated by PAMPs recognized via a few pattern recognition receptors. According to this the decision made by APCs is between "infectious-nonself" (INS) and "noninfectious-self". Due to the ultimate importance of the activation status of the antigen presenting cell deciding between activation and anergy of antigen specific T cells, the role of innate immunity had to be re-evaluated. The ancient innate immune system makes the decisions; the sophisticated adaptive immune system just fulfils the jobs. In 1994, the model was even refined by Polly Matzinger who introduced the "danger model" (reviewed in Matzinger, 2002). Due to this, the immune system does not care about self and nonself or infectious and harmless, but is activated on endogenous danger signals from injured cells, such as those exposed to pathogens, toxins or mechanical damage. Several danger signals have since been described, e.g. mammalian DNA, RNA or heat shock proteins released from necrotic cells, type-I interferons secreted by virus-infected cells, or CD40L on activated platelets or hyaluron after vessel damage (Gallucci et al., 1999). The PAMPs from Janeway's theory are included into the danger model as exogenous danger signals.

Recently, the importance of another control mechanism for adaptive immunity besides costimulation was rediscovered. The concept of regulatory T cells which has already been postulated in the early 1970s (originally termed suppressor T cells) is now well established (Maloy and Powrie, 2001). $CD4^+$ $CD25^+$ regulatory T cells are able to inhibit harmful immunopathological responses against self or foreign antigens. Their suppressive function on effector T cell proliferation is most probably mediated by the anti-inflammatory cytokines IL-10 and TGF-β and by cell-cell contact dependent mechanisms leaving the T cells unresponsive to IL-2. However, recently it has been reported that APCs that have been activated via TLR ligands, are able to block the suppressive function of regulatory T cells, thereby enabling an immune response (Pasare and Medzhitov, 2003). This block of suppression depends on IL-6

and at least one other soluble mediator secreted by the activated APC. On the other hand, regulatory T cells itself express TLR4, 5, 7 and 8 and can be activated by LPS rendering them even more suppressive on $CD4^+$ T cell mediated responses *in vitro* (Caramalho et al., 2003).

These newly discovered links between TLR-mediated activation and T cell responses and possibly other observations to follow confirm the ideas of Matzinger and Janeway that adaptive immunity is ruled by innate immune recognition.

2

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THE HEAT-SHOCK PROTEIN GP96 LINKS INNATE AND SPECIFIC IMMUNITY

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Among other heat shock proteins (HSPs) the ER-resident chaperone Gp96 has been described as a potent tumour vaccine in animal models. A growing list of data underlines that Gp96 triggers both arms of pathogen defence - innate and specific immunity - in a synergistic and most efficient way: It enables specific immune responses by transferring immunogenic peptides that have been acquired in the ER to the MHC class I pathway of antigen presenting cells (APCs). For this, two important features of Gp96 are required. First, its ability to bind immunogenic peptides. Second, its acquisition by specialised antigen presenting cells capable of inducing cellular immune responses. Due to specific receptors on the surface of APCs, this uptake from the extracellular space occurs very efficiently and rapidly. Serving the innate branch of immunity, Gp96 unspecifically activates APCs, which then provide a pro-inflammatory cytokine milieu and co-stimulation to cytotoxic T cells. Thus, Gp96 uses all resources of the immune system to trigger cytotoxic T cell responses against associated peptides.

This chapter was written by the author of this thesis together with Harpreet Singh-Jasuja.

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Chaperones – essential stress tolerance for the cell

Proteins are essential for all biological processes and their structural integrity is crucial for every cell. A variety of stress situations like high temperature, anoxia, heavy metals or glucose starvation promotes the denaturation of proteins and therefore directly threatens life. To ensure survival, all organisms respond to these conditions with the expression of a group of highly conserved proteins called heat shock proteins (HSPs) which confer elevated stress tolerance to the cells they are expressed in (Lindquist, 1986). HSPs bind to hydrophobic stretches of denatured proteins thereby preventing their aggregation. Moreover, HSPs initiate the refolding of their bound substrate or proteolytic degradation if the unfolding was irreversible. However, most HSPs are constitutively expressed in high copy numbers and fulfil their chaperone function – assistance in protein folding, subunit assembly and transport between cellular compartments - also under non-stressed conditions (Bukau et al., 2000).

HSPs are divided into 6 subfamilies according to their molecular weight: small HSP, HSP40, HSP60, HSP70, HSP90 and HSP100. Due to the limitation of this review we will focus on the HSP families with shown immunological relevance (table 2). The most intensely studied family of 70 kDa heat shock proteins is present in all major cellular compartments of eukaryotic cells. Hsp70 (Hsp72) and Hsc70 (Hsp73), the stress-inducible and constitutively expressed forms, respectively, are found in the cytosol. BIP (Grp78) represents the ER-resident form of HSP70, Grp75 the mitochondrial form. Hsp60, a large multimeric complex of 2 stacked heptameric rings, resides in the mitochondrion.

The HSP families differ in the way they contribute to proper protein folding, subunit assembly and cellular integrity. While the members of the HSP70 family bind as monomers to hydrophobic regions of nascent polypeptide chains or denatured proteins and keep them in a folding competent state, Hsp60 assists partially folded proteins in an ATP-dependent manner to reach their native state inside its large central channel (Bukau and Horwich, 1998).

Table. 2. Structural and functional properties of immunological relevant chaperones and their protein families

chaperone family	structural features	family member with immunizing properties	localization	function
HSP60	two stacked heptameric rings	Hsp60 (60 kDa chaperonin)	mitochondrium	ATP-dependent assistance in folding of proteins to the native state, binds hydrophobic surfaces in collapsed conformation
HSP70	monomeric	Hsc70 (Hsp73, constitutive form)	cytoplasmic	ATP-dependent stabilization of hydrophobic regions in *de novo* synthezied and denatured proteins, bind polypeptide in extended conformation
		Hsp70 (Hsp72, inducible form)		
HSP110 subfamily	multimeric complex together with Hsc70 and Hsp25	Hsp110 (Hsp105)	cytoplasmic	chaperone function together with Hsc70 proposed: holds unfolded proteins in folding competent state, but has no refolding activity by itself (ATP dependent?)
GRP170 subfamily	interacts with Gp96 and Grp78	Grp170 (Orp150)	ER lumen	chaperone function proposed
HSP90	non-covalent homodimers	Hsp90 α/β (Hsp86 / Hsp84)	cytoplasmic	ATP-dependent role in refolding of misfoded and denatured proteins and interaction with proteins involved in cell signaling (e.g. steroid hormone receptors)
		Gp96 (Grp94, Endoplasmin)	ER lumen	ATP-independent chaperone function at late stages of folding proposed
Calreticulin family	monomeric, binds Ca^{2+} ions	Calreticulin (Calregulin, Erp60)	ER lumen	lectin-like chaperone with glycosylated protein substrates, Ca^{2+} homoeostasis

The Heat Shock Protein Gp96

The ER-resident 96 kDa glucose-regulated protein (Gp96, Grp94, Endoplasmin) belongs to the 90 kDa HSP family (HSP90 family) and shares 47 % sequence identity with its cytosolic homologues Hsp90 α and β (Gupta, 1995). Although an ER homologue is absent in yeast, it can already be found in protozoa (Larreta et al., 2000). The high conservation between all Gp96 proteins from different species indicates a very early gene duplication event in the evolution of eukaryotic organisms leading to the separation of the ER and cytosolic homologues.

Gp96 possesses in addition to Hsp90 an amino-terminal signal peptide and a C-terminal KDEL retention signal guiding the nascent polypeptide chain to the ER. Most of our knowledge about the structure of HSP90 proteins derives from intense studies on cytosolic Hsp90. However, due to the high sequence homology many conclusions can be transferred to Gp96. Both proteins consist of two conserved domains which are connected by a highly charged and flexible linker region. At present, there is a crystal structure only for the N-terminal domain of Hsp90. An antiparallel β sheet forms the bottom of a 15 Å deep pocket while the edges consist of α helices (Stebbins et al., 1997; Prodromou et al., 1997). HSP90 chaperones assemble into elongated homodimers via their C-terminal dimerization domains (Wearsch and Nicchitta, 1996; Nemoto et al., 1995). Above 50 °C, higher order oligomerization has been observed, which is most likely due to a conformational change leading to the appearance of a new hydrophobic oligomerization site (Chadli et al., 1999; Wearsch et al., 1998; Wearsch et al., 1998).

The HSP families differ in the way they contribute to proper protein folding, subunit assembly and cellular integrity. While the members of the HSP70 and HSP60 families take part in the refolding process of denatured proteins in an ATP-dependent manner, the 90 kDa heat shock proteins are supposed to bind late folding intermediates until folding and subunit assembly have been terminated (Jakob et al., 1995). In addition, the functionality of a growing list of proteins - mostly steroid hormone receptors and non-receptor tyrosine kinases - is dependent on interaction with cytosolic Hsp90. In the case of hormone receptors, Hsp90 is believed to stabilize the client protein in a metastable conformation keeping it competent for hormone binding. Similarly, Gp96 has been shown to interact among others with unassembled immunoglobulins (Chadli et al., 1999), Major Histocompatibility Complex (MHC) class

II polypeptide chains (Schaiff et al., 1992) and her2/neu protein (Chavany et al., 1996). During immunoglobulin assembly, Gp96 interacts with late, fully oxidized intermediates sequentially after the Hsp70 homologue BiP (Melnick et al., 1994). Moreover, Gp96 is essential for the proper and functional folding of Toll-like receptors while the functionality of the majority of proteins in the secretory pathway is not dependent on the presence of Gp96 (Randow and Seed, 2001). In line with this, the viability and even the stress resistance of at least certain cell lines is not altered without Gp96, although knock-out mice die in early stages of embryonic development (Stoilova et al., 2000).

The nucleotide binding pocket of HSP90 chaperones

For the 70 kDa heat shock proteins it is known now for several years that ATP is an important regulator of substrate binding. ATP, bound to the conserved N-terminal domain of Hsp70, induces a conformational change in the molecule which allows rapid binding and release of substrate. The substrate can be trapped by hydrolysis of ATP to ADP and its release is again dependent on ADP/ATP exchange (Pierpaoli et al., 1997). Our understanding of the role of nucleotides in the mechanism of substrate binding to HSP90 chaperones received a major impulse from X-ray crystallographic studies of the N-terminal Hsp90 domain in association with ATP or the antitumour drug geldanamycin, revealing an unusual nucleotide binding pocket which is very similar to that of DNA gyrase B (Prodromou et al., 1997). In this pocket ATP does not bind in the usual extended form, but in a kinked conformation strongly resembling the structure of the anti-tumour drug geldanamycin. Interestingly, Hsp90 and Gp96 are the only cellular proteins that bind this drug (Whitesell et al., 1994; Chavany et al., 1996; Chavany et al., 1996).

Although ATP binds with very low affinity to Hsp90, ATP binding and hydrolysis has been shown to be crucial for Hsp90 function *in vivo* (Obermann et al., 1998). Recently, it has been observed that ATP binding induces a transient association of the two N-terminal domains resulting in the formation of a 'molecular clamp' (Prodromou et al., 2000), but the functional role of this conformational change remains enigmatic. Interestingly, ATP and geldanamycin have been shown to reduce the heat induced oligomerization of Hsp90 (Chadli et al., 1999). Similar, but not identical observations have been made for the ER-resident Gp96. After incubation at 50 °C or induced by the fluorophore bis-ANS (1,1’-bis(4-anilino-5-

naphthalenesulfonic acid)), a tertiary conformational change can be observed that goes along with oligomerization, increased peptide binding and chaperone activity (Wearsch et al., 1998; Wassenberg et al., 2000; Blachere et al., 1997). Radicicol, like geldanamycin an established inhibitor for the nucleotide binding pocket, blocked this bis-ANS or heat shock induced Gp96 activation (Wassenberg et al., 2000). Although both Gp96 and Hsp90 bind geldanamycin, the properties of the nucleotide binding pockets differ. The adenosine derivative 5'-(N-ethylcarboxamido)adenosine (NECA) associates exclusively with Gp96 with a stoichiometry of 1 mol NECA per 1 mol of Gp96 dimer (Rosser and Nicchitta, 2000). Weak binding of other adenosine derivatives including ATP and ADP is also reported in this work, but in contrast to Hsp90 no ATPase activity was observed. Based on these results it has been proposed that Gp96 substrate binding is allosterically regulated by ATP and ADP as negative regulators and possibly another yet unidentified ligand involved (Wassenberg et al., 2000).

HSPs – versatile tools in immunology

Peptides as HSP binding substrates

By definition, chaperones interact with non-native proteins. They do so by binding to hydrophobic stretches which are normally buried and not exposed on the surface of the protein. The HSP70 family members use their hydrophobic binding pocket to bind to small hydrophobic segments in extended conformation (Zhu et al., 1996). As the affinity for the bound peptide segment seems not to be altered by distant parts of the substrate proteins, small peptides bind to HSP70s equally strong as peptides in the context of partially denatured proteins. The same might be true for Gp96 although no X-ray structures of Gp96 substrate complexes are available. Hsp90 and Gp96 have been shown to bind peptides and in some cases direct isolation and sequencing of the antigenic peptide from purified heat shock proteins was successful (Breloer et al., 1998; Ishii et al., 1999; Nieland et al., 1996). Gp96 preferentially interacts with peptides enriched in hydrophobic and aromatic residues (Spee and Neefjes, 1997). Recently, the peptide binding site of Gp96 has been mapped in the C-terminal domain near the dimerization site by cross-linking experiments (Linderoth et al., 2000). This finding is surprising with regard to the two observed binding sites on the

highly homologous Hsp90 (Scheibel et al., 1998): the N-terminal domain of the cytosolic chaperone seems to have a high affinity for unfolded proteins and peptides longer than 10 amino acids while the C–terminal domain, corresponding to the dimerization domain of Gp96, binds preferentially partially folded proteins.

Despite the fact that there are still many open questions concerning the way in which peptides meet HSPs, there is no doubt that peptides can associate with Gp96 *in vivo*. Most peptides inside the cell are generated by the proteasome. They are produced as intermediates of protein turnover and further degraded by cytosolic peptidases. However, a fraction of these peptides in mammals escapes further degradation and is translocated into the ER by TAP in an ATP-dependent manner. In this compartment, a small subset of peptides is loaded onto MHC class I molecules. These MHC-peptide complexes are finally transported to the cell surface and presented to CD8-positive cytotoxic T cells.

Chaperones are involved in different steps along the way of MHC-peptide complex formation. The specialized chaperone tapasin, which establishes the physical interaction between some MHC class I allelic products and TAP, influences the peptide loading process (Garbi et al., 2000). Gp96 together with Grp170 and Calreticulin is one of the major peptide binding proteins in the ER and it has been shown to bind peptides of immunological relevance in a TAP-dependent and TAP-independent way (Arnold et al., 1997; Lammert et al., 1997a; Spee and Neefjes, 1997; Spee et al., 1999). It has been proposed that peptides are transferred directly from one HSP to the other without ever occurring as free peptides - neither in the cytosol nor in the ER - until they finally reach the MHC class I molecule. However, so far there is only little evidence supporting this intriguing ‘relay line hypothesis’ (Srivastava et al., 1994), although Gp96, which has also been shown to interact with unassembled MHC class II molecules (Schaiff et al., 1992), would be the ideal candidate to fulfil this job in the ER. Concerning the cytosolic chaperones Hsp70 and Hsp90, specific inhibition by the immunosuppresive drug deoxyspergualin abrogated the ability of cells to present antigenic peptides through MHC class I molecules and this inhibition could be overcome by additional Hsp70 (Binder et al., 2001a). These results prove an involvement of cytosolic chaperones in antigen presentation, but a direct evidence that peptide binding to Hsp70 and Hsp90 in the cytosol is essential for the presentation of these peptides is still missing.

However, due to Gp96's location at the heart of the MHC class I presentation pathway it can be assumed that the peptides associated with Gp96 represent the entire protein content of a cell in a similar fashion as the peptides on MHC class I molecules do on the cell surface. The pool of available peptides may be even more diverse due to the less stringent binding specificities of heat shock proteins. Interestingly, another feature links Gp96 to the MHC class I presentation pathway. Recently, it was demonstrated convincingly that Gp96 possesses an intrinsic aminopeptidase activity. However, because of the very low activity it is still unclear whether this plays a role in the N-terminal trimming of peptides translocated into the ER (Menoret et al., 2001).

Heat shock protein - peptide complexes are immunogenic

The immunogenic potential of Gp96-peptide complexes was first demonstrated by Srivastava and co-workers (reviewed in Srivastava et al., 1998). Gp96 purified from tumour cells was able to protect mice against a subsequent challenge of the same tumour. Moreover, treatment of mice with HSPs could also be used for therapy against the pre-existing primary tumour and its metastases (Tamura et al., 1997). In the latter case only Gp96 from tumour but not from liver cells was able to elicit an immune response against the cancer cells due to tumour-specific peptides bound to the heat shock protein. The requirement for peptide binding has been validated by findings that HSPs deprived of peptides do not induce immunity (Udono and Srivastava, 1993) and that loading of 'empty' HSPs with peptides results in reconstitution of HSP immunogenicity (Blachere et al., 1997).

Although Gp96 is the best described HSP concerning its role in the induction of immune responses, other HSPs have been shown since to perform similar functions: the constitutive and inducible form of the HSP70 protein (Udono and Srivastava, 1993), HSP90 (Udono and Srivastava, 1994), Calreticulin (Basu and Srivastava, 1999; Nair et al., 1999), Hsp110 and Grp170 (Wang et al., 2001). All these proteins have the ability in common to bind intracellular peptides forming potent immunogenic HSP-peptide complexes which can be used as vaccines against the cells from which they are purified. As the immunity induced by these complexes relies on associated peptides and the HSP acts primarily as an antigen carrier molecule, heat shock

proteins have also been called adjuvants of mammalian origin (Srivastava et al., 1998).

Gp96 targets antigen-presenting cells

How does the HSP-peptide complex induce an immune response? To answer this question we will look shortly at basic mechanisms of antigen processing and presentation. The adaptive immune system relies on the presentation of immunogenic peptides at the cell surface embedded in the extremely polymorphic structure of MHC molecules. Complexes of MHC molecules and peptides are recognized specifically by the T cell receptor (TCR) leading to activation (or in some cases silencing) of the T cell. There are two occasions when the interaction of the MHC-peptide complex and the TCR are required in the immune response, e.g. against a virus: first, during the initial activation of naive T cells by professional antigen presenting cells (APCs) that display viral antigens on their MHC molecules, a process referred to as T cell *priming*. Second, during the *elimination* of virus infected cells by now activated cytotoxic T cells (CTLs). Members of the APC family are macrophages, dendritic cells (DCs) and B cells. The dendritic cell has been suggested to be the most sophisticated professional APC being able to prime T cells as well as to induce tolerance by carefully regulating its arsenal of co-stimulatory surface molecules (Banchereau et al., 2000).The efficient induction of immune responses elicited by HSP-peptide complexes led to the hypothesis that HSP-induced immunity uses existing pathways of antigen presentation. In consequence, this means that HSP-peptide complexes are taken up by specialized antigen-presenting cells and peptide is transferred from HSPs to MHC molecules inside these APCs for recognition by the T cell (Srivastava et al., 1994). Meanwhile, substantial evidence has accumulated to underline this hypothesis. Macrophages and dendritic cells that have been pulsed with Gp96-peptide complexes re-present the associated peptides on their MHC class I molecules for activation of CTLs *in vitro* (Suto and Srivastava, 1995; Singh-Jasuja et al., 2000c; Castellino et al., 2000). The depletion of phagocytic cells leads to the abrogation of the HSP-mediated immune response *in vivo* (Udono and Srivastava, 1994). DCs that have been pulsed with Gp96 are able to induce protective immunity against tumours *in vivo* (Nicchitta, 1998).

Another important feature of HSP-mediated immunity is the independence of the induced protection from the MHC haplotype of the cell from which the HSPs were isolated. Peptide associated to HSPs purified from cells with one MHC haplotype can be re-presented by antigen-presenting cells of another haplotype (Arnold et al., 1995). This phenomenon – the independence of priming from cellular MHC haplotype – has been coined *cross-priming* (Bevan, 1976b) and has recently been shown to be required if the antigen is not expressed by the professional APC itself (Sigal et al., 1999). *In vivo*, cross-priming would be essential in inducing immune responses against non-APCs that have been infected by viruses or other pathogens or have turned into tumour cells. The viral or tumour antigens would then be transferred to APCs where they are cross-presented for eliciting a T cell response.

Receptor-mediated uptake of heat shock proteins

The ER-resident chaperone Protein disulfide-isomerase (PDI) has been shown to bind peptides that have been translocated into the ER by TAP (Lammert et al., 1997b; Spee and Neefjes, 1997). Its peptide-binding abilities even exceed those of Gp96 making PDI the dominant peptide acceptor in the ER. However, all experiments utilising PDI-peptide complexes to induce immune responses against the associated peptides have failed so far (Lammert et al., unpublished observations; P. Srivastava, personal communication). Indeed, in contrast to Gp96 and other HSPs, PDI is not found to bind to cell surface receptors (Singh-Jasuja et al., 2000a). This shows that other features of HSPs besides their ability to build stable complexes with peptides are required. Therefore and in line with the extraordinary efficiency of immunizations with Gp96, it has been proposed early that APCs possess specific receptors for HSPs on their surfaces enabling these cells to clear Gp96 efficiently from the extracellular fluid (Srivastava et al., 1994). Lately, evidence for this hypothesis has been provided. The first indication of a receptor-mediated uptake of HSPs by APCs was reported in EM binding studies using labelled Hsc70 and Gp96 and macrophages (Arnold-Schild et al., 1999). This observation was confirmed in confocal microscopy studies (Wassenberg et al., 2000). Evidence for binding to specific receptors was finally provided by flow cytometry studies using fluorescein isothiocyanate (FITC)-labelled Hsc70 (Castellino et al., 2000) or Gp96 (Singh-Jasuja et al., 2000c). In the latter studies saturation and competition by non-labelled Gp96

was shown demonstrating the specificity of the HSP-receptor interaction. It was also revealed that only APCs like macrophages, DCs and B cells but not T cells bind Gp96 in a receptor-mediated fashion. Both studies also show cross-presentation of HSP-associated peptide on MHC class I molecules for activation of CTL lines. Interestingly, for both molecules – Gp96 and Hsc70 – receptor-mediated uptake of the HSP is essential for re-presentation of HSP-associated peptides on MHC class I molecules. Non-specific endocytosis and macropinocytosis was not able to do so. Consequently, HSP receptors do not only facilitate efficient uptake of HSP-peptide complexes but also seem to be responsible for shuttling HSP-associated antigen into the right processing pathway. For Hsc70 it has also been demonstrated that this pathway is dependent on the nature of the antigen and the requirement for further processing. If C-terminal processing of the peptide for transfer onto MHC molecules is required, re-presentation is dependent on the activity of the proteasome and TAP. If C-terminal processing is not required, transfer of peptide from HSP to MHC can occur inside the endosome as confirmed by confocal microscopy (Castellino et al., 2000).

The search for the identity of one or more HSP receptors is still going on. The first receptor for Gp96 has been identified as CD91 by cross-linking Gp96 chemically to plasma membrane fractions of macrophages and sequencing the bound protein (Binder et al., 2000b). Meanwhile CD91 has also been identified as a receptor for the heat shock proteins Hsp70, Hsp90 and Calreticulin (Basu et al., 2001). CD91, also known as α_2-macroglobulin receptor or low density lipoprotein receptor related protein, consists of a 420 kDa α- and a 85 kDa β-unit. Its functions are believed to be the clearance of extracellular plasma products as well as regulation of lipid metabolism (reviewed by Strickland et al., 1995). The question whether CD91 is the only receptor for Gp96 or whether CD91 is also a receptor for other HSPs, still needs to be resolved. Data from our lab hint into the direction that other receptors are likely to be involved in the binding and uptake of extracellular Gp96 (Singh-Jasuja et al., unpublished observation).

Interestingly, the classical ligand of CD91, the plasma protein α_2-macroglobulin, also has the ability to bind peptides and the resulting α_2-macroglobulin-peptide complexes similar to Gp96-peptide complexes are able to prime CD8 T cell responses *in vivo* (Binder et al., 2001b).

Gp96 activates dendritic cells

To efficiently prime naive T cells at least two signals have to be provided by the APC: The first is the recognition of MHC-peptide complexes by the TCR and its co-receptor. To fulfil this requirement, APCs and especially DCs possess unique pathways to shuttle exogenous antigen effectively into the antigen processing pathway leading to the formation of MHC-peptide complexes (Rodriguez et al., 1999). The second signal depends on the interaction of co-stimulatory molecules with e.g. CD28 on the T cell side. The absence of co-stimulatory activity rather leads to tolerance and not priming. The ability to carefully regulate its co-stimulatory molecules makes the dendritic cell exceptional among the APCs. Co-stimuli can be provided by several surface markers including CD80, CD86, 4-1BB ligand and others (reviewed in Watts and DeBenedette, 1999) which are upregulated during DC maturation.

There are numerous sources responsible for the maturation of DCs: bacterial products like lipopolysaccharid (LPS), pro-inflammatory cytokines like TNF-α or the engagement of CD40 expressed on APCs by CD40 ligand (CD40L) on T helper cells. It has also been shown, that the content of cells undergoing necrosis (but not apoptosis) is able to activate macrophages and DCs (Sauter et al., 2000). However, the substances that are released during necrosis and that are responsible for APC-stimulation, still have to be determined. The question whether HSPs - one of the most abundant classes of cellular proteins - can induce the activation of macrophages and DCs has been clarified in the past year. Hsp60 was the first heat shock protein to be identified as an activator of macrophages in a CD14- (Kol et al., 2000) and Toll-like receptor 4-dependent (Ohashi et al., 2000) manner. However, Hsp60 is unlikely to be involved in cross-priming as it does not possess peptide-binding abilities. Gp96 has been demonstrated to activate human and mouse dendritic cells by up-regulating co-stimulatory molecules and subsequently enhance stimulation of T cells. Furthermore, upon activation by Gp96 DCs release pro-inflammatory cytokines resulting in the induction of an inflammatory response by the innate part immune system (Singh-Jasuja et al., 2000b). Heat-denatured Gp96 was not able to activate DCs, while the same heat-treatment did not diminish the activity of LPS, demonstrating that contaminating LPS in the protein preparations was not the cause of the described effects. Maturation of DCs by Gp96 is dependent on a signal transduction pathway involving the transcription factor NF-κB (Basu et al.,

2000). Recently, we were able to show that Toll-like receptors (TLRs) are involved in Gp96-induced DC maturation (Vabulas et al., 2002). Bone-marrow derived dendritic cells from TLR4-deficient mice showed no activation in response to Gp96. TLR4 was first identified as the receptor mediating the response of cells to the bacterial cell wall component LPS. In transfection studies also TLR2 was able to mediate Gp96 induced signals, but knock-out mice did not show an altered response. Most probably, TLR4 can compensate for TLR2 *in vivo*, while TLR4 signalling is absolutely required. Interestingly, blocking of receptor-mediated endocytosis by the transglutaminase inhibitor monodansylcadaverine abrogates Gp96-induced DC maturation while LPS activation is not affected (Vabulas et al., 2002). A similar effect has been observed for Hsp60 (Vabulas et al., 2001). One interpretation is that Gp96 might be recruited to endosomes by its endocytosis receptor CD91 where it meets its activation receptor TLR4. Once activation has been triggered via TLRs several signalling pathways get involved like stress-activated protein kinases, JNK and p38 finally leading to the activation of NF-κB (Vabulas et al., 2002). In this respect, it is very important to notice that APCs use the same receptors and signalling cascades to trigger their activation by heat-shock proteins as they use for pathogen-associated molecular patterns (PAMPs) like LPS, bacterial DNA or double stranded RNA. Therefore, TLRs are not only involved in the innate immune response to pathogens, but have to be considered as danger signal receptors in general also recognizing occurring cell death indicated by the presence of HSPs in the extracellular space.

Scepticism about the *in vivo* relevance of HSP-mediated cross-presentation and APC activation has been raised because of the rather high concentrations of HSP used for DC activation, ranging from 10 to 100 µg/ml *in vitro*. However, the highly immunostimulating potential of HSPs has been demonstrated *in vivo* by showing maturation of dendritic cells in mice by exogenously applied HSP. The injection of 1 µg of Gp96 led to the massive infiltration of CD11c-positive DCs into the draining lymph nodes from the point of injection. These DCs were of the mature phenotype and were highly efficient in the stimulation of T cells (Binder et al., 2000a).

Taken together recent reports show conclusively that HSPs alone can act as highly stimulating agents to antigen presenting cells, especially dendritic cells. The activation of APCs enables DCs to communicate efficiently with T cells resulting in subsequent stimulation of a strong immune response.

HSPs as necrosis messengers, HSP receptors as sensors for necrosis

Experiments identifying CD91 as a receptor for Gp96 showed that the re-presentation of Gp96-associated peptides after receptor-mediated uptake can be blocked by antibodies against CD91 or by α_2-macroglobulin (Binder et al., 2000b). The authors have incorporated the latter observation into an intriguing model for the general role of HSPs in the regulation of APC function. In the blood uptake of Gp96 (and possibly other HSPs) is inhibited by α_2-macroglobulin which is present in the serum in large amounts. In tissues, however, extracellular Gp96 can access CD91 and is taken up via receptor-mediated endocytosis leading to the subsequent presentation of its associated peptides on MHC molecules. Another regulator of extracellular Gp96 activity is also present in the blood. Platelets have been described to possess receptors for Gp96 and to interfere with Gp96-induced DC activation. Therefore, they might be involved in the neutralization of Gp96 and possibly other HSPs in the blood and in wounds (Hilf et al., 2002).

As Gp96 is known to be localized to the ER lumen the question regarding the source of extracellular Gp96 and other HSPs arises. Indeed, early experiments identified Gp96 as a cell surface protein on fibroblasts (Pouyssegur and Yamada, 1978). However, there is no basis so far for regulated secretion of HSPs. It seems much more attractive that HSPs are released in large amounts as result of necrotic cell death. Indeed, it has been demonstrated that necrotic cells induced by repeated freeze-thaw cycles or by virus-infection release Gp96, Hsp90, Hsp70 and Calreticulin while apoptotic cells generated by UV radiation do not (Basu et al., 2000; Berwin et al., 2001). Both, necrosis and apoptosis, although finally leading to the death of a cell, have very different starting points. Apoptosis, also known as programmed or ‘silent’ cell death, occurs all the time, especially during development of the organism and is a primary ‘tool’ in the education of the immune system inside the thymus. On the other hand, necrosis is the result of severe stress or trauma, either caused by injury or by infection. Upon necrosis, cellular proteins including Gp96 are released into the surrounding extracellular fluid. APCs in the neighbourhood might acquire Gp96 and other HSPs and shuttle the associated peptides into the correct processing pathway for presentation on MHC molecules. Simultaneously, Gp96 could activate APCs inducing the release of pro-inflammatory cytokines and the up-regulation of co-stimulatory molecules (Singh-Jasuja et al., 2000b; Basu et al., 2000). Presentation of immunogenic peptide on MHC molecules and providing co-stimuli would result in a

T cell response against the source of the HSP. This immune response would be directed against infected cells in case of viral infection or would simply elicit a preventive danger response in case of injury. In the case of apoptosis, apoptotic cells or their remnants, so called apoptotic bodies, expose phosphatidylserin on the outer leaflets of their membranes. Phosphatidylserin is sensed by the phosphatidylserin receptor present on some macrophages leading to an anti-inflammatory cytokine response including IL-10 and TGF-β release (Fadok et al., 2000). Although apoptotic cells are able to transport antigen to APCs leading to the cross-presentation of these antigens (Albert et al., 1998a), co-stimulatory activity is not induced (Sauter et al., 2000). Antigen presentation without co-stimulus results in tolerance and not priming. Other receptors such as CD14 or CD36 recognize other apoptotic cell-associated ligands (ACAMPs) and can transmit similar tolerogenic signals to the APC (Devitt et al., 1998). Taken together this model proposes that apoptosis creates a tolerogenic milieu, necrosis on the other hand an immunogenic milieu. HSPs may well play a vital role in these processes: heat shock proteins as necrosis messengers, HSP receptors as necrosis sensors.

To the biochemists heat shock proteins and chaperones are known as multifunctional proteins. They facilitate folding and unfolding of proteins, they participate in vesicular transport processes, they prevent protein aggregation in the densely packed cytosol, they are involved in signalling processes etc. This multifunctionality of HSPs and especially Gp96 - the most intensely studied HSP from the immunological point of view - has now been extended even further: Gp96 carries peptides that represent cellular proteins, transfers these peptides to MHC molecules after binding to receptors on DCs and other APCs and additionally it behaves as danger signal by activating DCs to express co-stimuli and pro-inflammatory cytokines and it regulates the surface expression of its own receptors (Singh-Jasuja et al., 2000b). These combined features make Gp96 a powerful weapon. It activates both, the innate and the adaptive immune system. Because of having so many functions, Gp96 has also been called ‘the Swiss Army Knife of the Immune System’ (Schild and Rammensee, 2000). Several clinical trials are ongoing which use tumour derived Gp96 as cancer vaccine to exploit the immunological properties of this HSP. The striking advantage of this strategy is obvious: Gp96 isolated from autologous tumour material provides an individualized vaccine against multiple tumour antigens without the need to characterize single antigenic peptides. Large clinical vaccination studies with

optimized immunization protocols have to prove whether the promising pre-clinical results with Gp96 will lead to the development of a powerful tumour vaccination therapy.

OUTLINE OF THIS THESIS

When this work was started in 1999 very little was known about the mechanisms by which vaccination with HSP:peptide complexes confers protective immunity against the tissue the protein had been purified on. Our main area of interest was therefore the characterization of the receptors involved in the uptake of the heat shock protein Gp96 and the elucidation of the internalization process in more detail leading to the results shown in chapter 3. However, the strikingly low amount of Gp96:peptide complex required to confer specific immunity without the need of further adjuvants prompted us to analyze possible peptide-independent immunostimulatory capacities of Gp96 itself (chapter 4). This work provides a link between innate immune recognition and specific immunity condensed in one protein, Gp96. Further search for interactions between HSPs and cells of the innate immune system resulted in discoveries regarding receptor-mediated binding of Gp96 to human platelets and functional consequences thereof, as described in chapter 5. Moreover, we analyzed the signaling events occurring in APCs after Gp96-mediated activation (chapter 6). The striking finding that the TLR-mediated pathways used during HSP-mediated cell activation are the same as those required for the recognition of pathogen-associated patterns, such as LPS, revealed the importance of innate immune recognition for adaptive immune responses in general. Therefore, we set out to analyze the contribution of TLR-mediated immune recognition to CTL responses. The promising results obtained so far are discussed in chapter 7.

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CROSS-PRESENTATION OF GP96-ASSOCIATED ANTIGENS ON MHC CLASS I MOLECULES REQUIRES RECEPTOR-MEDIATED ENDOCYTOSIS

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Heat-shock proteins (HSPs) like Gp96 (grp94) are able to induce specific cytotoxic T-cell (CTL) responses against cells from which they originate. Here, we demonstrate that for CTL activation by Gp96-chaperoned peptides specific, receptor-mediated uptake of Gp96 by antigen presenting cells (APC) is required. Moreover, we show that both in humans and mice only professional APCs like dendritic cells (DCs), macrophages and B cells, but not T cells are able to bind Gp96. The binding is saturable and can be inhibited using unlabelled Gp96 molecules. Receptor-binding by APCs leads to a rapid internalization of Gp96 which co-localizes with endocytosed MHC class I and class II molecules in endosomal compartments. Incubation of Gp96 molecules isolated from cells expressing an Adenovirus type 5 (Ad5) E1B epitope with the dendritic cell line D1 results in the activation of E1B-specific CTLs. This CTL activation can be specifically inhibited by the addition of irrelevant Gp96 molecules not associated with E1B peptides. Our results demonstrate that only receptor-mediated endocytosis of Gp96 molecules leads to MHC class I-restricted re-presentation of Gp96-associated peptides and CTL activation; non-receptor-mediated, nonspecific endocytosis is not able to do so. Thus, we provide evidence on the mechanisms by which Gp96 is participating in the cross-presentation of antigens from cellular origin.

The author of this thesis contributed substantially to figures 1, 2 and 3 of this chapter together with Harpreet Singh-Jasuja.

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Introduction

Activation of cytotoxic T lymphocytes (CTLs) with exogenous cell-associated antigens requires efficient uptake and presentation of these antigens by bone marrow-derived antigen presenting cells (APCs). This phenomenon was first observed by Bevan (Bevan, 1976b; Bevan, 1976a) for the induction of CTLs against minor H antigens. Because the antigens were expressed in foreign donor cells with different MHC molecules, this process was termed ‚cross-priming'. Since then it has been shown that soluble protein antigens (Staerz et al., 1987; Rock et al., 1990), antigens expressed in MHC matched cells (Huang et al., 1994; Toes et al., 1996; Kurts et al., 1996) or antigens encoded by naked DNA (Corr et al., 1996) also require uptake and re-presentation by MHC molecules expressed on the surface of professional APCs. Therefore, the term 'cross-presentation' was introduced to describe the general re-presentation of exogenous cell-associated antigens by MHC class I (Kurts et al., 1996) and MHC class II molecules (Adler et al., 1998). In addition to CTL activation, cross-presentation can also lead to the induction of CTL tolerance (Kurts et al., 1997a; Kurts et al., 1997b; Pfeifer et al., 1993).

The nature of the APCs that are able to take up and re-present cell-associated antigens on MHC class I molecules remains elusive *in vivo*. However, *in vitro* studies suggest that dendritic cells (DCs) (Kurts et al., 1997a; Kurts et al., 1997b; Albert et al., 1998b; Pfeifer et al., 1993; Golenbock and Fenton, 2001), macrophages (Debrick et al., 1991; Kovacsovics-Bankowski et al., 1993; Norbury et al., 1995; Srivastava, 1991; Zheng et al., 2001) or B cells (Ke and Kapp, 1996) might be involved.

Several pathways for antigen uptake have been described, ranging from non-specific mechanisms such as phagocytosis, pinocytosis or macropinocytosis (Heath and Carbone, 1999; Jondal et al., 1996; Rock, 1996; Sallusto et al., 1995; Reis e Sousa et al., 1993; Steinman, 1991; Chen et al., 1999; Kaisho and Akira, 2001; Aderem and Ulevitch, 2000; Schnare et al., 2001) to specific, receptor-operated mechanisms that include mannose and scavenger-type receptors (Jondal et al., 1996). Depending on the nature of the antigens, and consequently on the mode of uptake, antigens might be targeted to different processing compartments and be able to gain access to different antigen presentation pathways. CTL activation can be mediated by macropinocytosis or phagocytosis of exogenous, soluble antigens (Bachmann et al., 1996; Kovacsovics-Bankowski and Rock, 1994; Norbury et al.,

1997; Cella et al., 1996). However, these pathways require high antigen concentrations and might therefore be of limited relevance in providing a mechanism for cross-presentation *in vivo* (Heath and Carbone, 1999).

More recently, apoptotic bodies were shown to be phagocytosed by immature dendritic cells resulting in the activation of MHC class I-restricted T cells (Albert et al., 1998b; Rovere et al., 1998; Fantuzzi and Dinarello, 1999). This uptake involves CD36 and the integrin receptor $\alpha_v\beta_5$ (Albert et al., 1998a), which explains the high efficiency.

An additional pathway with potential relevance for cross-presentation became evident when the induction of tumor immunity and CTL activation through the injection of heat-shock proteins, such as Gp96, HSP70 and HSP90, was discovered (reviewed in Srivastava et al., 1998). The specificities of the CTL response were directed against the cells from which the HSPs were isolated. This can be explained by the association of the HSPs with peptides of cellular origin. Immune responses against several cellular antigens including minor H, tumor and viral antigens were induced (reviewed in Schild et al., 1999) by using as little as 1-2 ng HSP/peptide complex in one particular case (Blachere et al., 1997). It was postulated that the extremely efficient MHC presentation of HSP-associated peptides is accomplished by the receptor-mediated uptake of HSPs by professional APCs (Srivastava et al., 1994). Recently, binding of Hsp70 and Gp96 to a macrophage- and dendritic-like cell line was observed (Arnold-Schild et al., 1999). This observation provides a possible explanation for the high immunogenic potential of HSPs in situations in which they are injected into mice or released from dying cells in that they shuttle antigenic peptides to APCs (Srivastava et al., 1994). Receptor-mediated endocytosis of HSPs by professional APCs will lead to the accumulation of these peptide chaperones in cells crucially involved in the activation of CTLs.

We therefore decided to characterize the cell populations involved in receptor-mediated endocytosis of HSPs in detail, to follow the fate of endocytosed HSPs and to test whether or not receptor-mediated endocytosis of HSPs indeed results in the re-presentation of HSP-associated peptides and subsequent activation of CTLs. The latter issue in particular is of crucial importance for the understanding of HSP-mediated cross-presentation since antigen uptake by APCs does not necessarily correlate with the ability to cross-present antigens. Despite the fact that macrophages

and DCs phagocytose apoptotic cells, only immature DC are able to cross-present antigens and to activate CTLs (Albert et al., 1998a).

We now describe that members of the family of professional APCs, such as macrophages, DC and B cells, are able to bind the ER-resident HSP Gp96 specifically. The binding was saturable and could be competed for with unlabelled Gp96 molecules. The uptake of Gp96 isolated from cells expressing the Adenovirus type 5 (Ad5) E1B epitope by the dendritic cell line D1 resulted in the activation of E1B-specific CTLs. More importantly, activation of Ad5 E1B-specific CTLs could be inhibited by competition with Gp96 not associated with E1b peptide. This result clearly demonstrates that CTL activation is the consequence of receptor-mediated endocytosis of Gp96 molecules followed by the class I-restricted re-presentation of associated peptides and supports the participation of HSPs in cross-presentation of cell-associated antigens.

Materials and Methods

Mice, cells, antibodies and proteins

The DEC205-knockout mice were kindly provided by Michel Nussenzweig and Ralph Steinman. BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). MHC class II-deficient mice ABBN5 (Grusby et al., 1991) and littermate ABBN6 were obtained from Taconic (Germantown, NY). P388D1, RMA and RMA-S mouse cell lines (ATCC, Manassas, VA) were cultured in Minimal Essential Medium (α-MEM). The cell line D2SC/1, representing an early progenitor of mouse splenic DC, and D1, a non-transformed, growth-factor dependent long-term DC culture (Winzler et al., 1997), were cultured in Iscove's modified Dulbeccos's Medium (IMDM). All tissue culture media were supplemented with 10% FCS, 0.3 mg/ml L-glutamin, 100 U/ml penicillin / streptomycin and 50 µM β-mercaptoethanol. To grow D1 cells, medium was additionally supplemented with 30% conditioned medium from the fibroblast cell line R1. Antibody to Gp96 (SPA-850) was obtained from StressGen Biotechnologies (Victoria, BC, Canada). Following labelled antibodies to mouse and human antigens were obtained from Pharmingen (San Diego, CA): H2-K^b-Biotin, H2-A^b-Biotin, CD8-FITC, Interferon-γ-PE, CD16/CD32 (Fc block), CD45R/B220-PE, CD19-PE, CD14-PE, CD90.2 (Thy1.2)-PE, CD86 (B7.2)-PE, CD11c-PE, Mac3-PE, CD1a-PE, CD83-PE, IgG1-PE and IgG2a-PE isotype

controls. Goat-anti-rabbit-Alexa™ 546 and Streptavidin-Alexa™ 546 (Molecular Probes, Leiden, Netherlands) were used as secondary reagents. Bovine Serum Albumin (BSA), biotinylated BSA, Ovalbumin (OVA) and Fluorescinisothiocyanate (FITC) were obtained from Sigma-Aldrich (St. Louis, Missouri). Streptavidin-PE was purchased from Jackson Laboratories, West Grove, PA. BSA and OVA were labeled with FITC or Biotin according to standard protocols. Free FITC molecules were removed by reaction with Tris and gelfiltration through a Sephadex G-25 (Sigma-Aldrich) column. Gp96 and Gp96-FITC from the mouse cell line IGELa2 were obtained from Immatics (Tübingen, Germany). All animal studies were performed according to our institutional guidelines and approved by our Institutional Review Board.

Purification of Gp96

The TAP-deficient RMA-S SigE1B cell line has been generated by transfection of RMA-S with the adenovirus early region 1 H2-D^b-restricted E1b epitope (VNIRNCCYI) targeted to the endoplasmic reticulum in a TAP-independent fashion (Toes et al., 1995). Gp96 was purified from RMA, RMA-S and RMA-S SigE1B cell lines as described (Arnold et al., 1995). The approximate concentrations were determined by measuring the OD at 280 nm using an extinction coefficient of 1.0.

Cytometry (FACS) binding assay

100,000 cells were incubated for 30 min on ice in 100 µl IMDM, 10% FCS containing 30 µg/ml Gp96-FITC or Ovalbumin-FITC and washed three times and fixed in 1% paraformaldehyde. For competition experiments, a given excess of unlabelled Gp96 was added together with 50 µg/ml Gp96-FITC simultaneously. For staining of mouse spleen cells (including erythrocytes) and human peripheral blood lymphocytes (PBL), PE-conjugated antibodies were added as markers for different cell types. Immature DCs were prepared from bone-marrow of C57BL/6 mice (Inaba et al., 1992) and human blood monocytes (Bender et al., 1996) as described. Cytometry measurements were performed on a FACSCalibur® (Becton Dickinson).

Internalization studies in confocal microscopy

Bone-marrow derived, immature DCs (BMDCs) were prepared from C57BL/6 mice. On day 6 of their preparation the BMDCs were tested for CD11c, CD86 and MHC class II expression and seeded on cover slips, pre-cooled and incubated for 30 min on ice with IMDM containing 10% FCS and 50 µg/ml Gp96-FITC („pulse“). The coverslips were washed twice and incubated in IMDM medium for 15 min or longer at 37°C („chase“), washed and fixed in 3.7% Paraformaldehyde in PBS. For the co-localization experiments cells were pre-incubated with Fc-Block (α-CD16/CD32) followed by biotinylated antibodies to H2-K^b or H2-A^b and 50 µg/ml Gp96-FITC together with Streptavidin-Alexa™ 546. For staining of lysosomes cells were fixed with methanol/aceton (1:1, -20°C) and incubated with α-Lamp-1 (kindly provided by M. Fukuda) and goat-anti-rabbit-Alexa™ 546. For microscopy a Zeiss LSM 510 laser scanning microscope was used. „Bleeding“ of emission into other detection channels was excluded usingh the multitracking modus of the LSM 510. Thickness of the optical plane was adjusted by the pinhole to be less than 1 µm.

Immunization of mice with Gp96

C57BL/6 mice were immunized intraperitoneally with 30 µg Gp96 purified from RMA-S SigE1B cells. After 10 days mice were killed and the spleen cells restimulated with E1B-expressing XC3 cells or Ad5 E1B peptide (50 ng/ml). Specific lysis of RMA-S SigE1B cells by CTLs contained in the spleen culture was determined by a standard chromium release assay 5 days after restimulation and after a second restimulation with XC3 cells or Ad5 E1B peptide (50 ng/ml).

CTL cross-presentation assay

The cytotoxic T-lymphocyte (CTL) clones 100B6, 0.1C2 and LN5 were described previously (Kast et al., 1989; Toes et al., 1995). CTL clones were restimulated on a weekly basis by incubation with the Ad5 E1B/E1A-expressing tumor cell line XC3. The E1B peptide was synthesized on a ABI 432 A peptide synthesizer (Applied Biosystems) applying Fmoc strategy.

Activation of CTL clones was assessed by measurement of intracellular Interferon-γ production. 25,000 D1 cells were incubated with 20 µg/ml Gp96 purified from RMA-S SigE1B, RMA or RMA-S cells for 2 hours at 37°C, for competition experiments an

excess of Gp96 from RMA or RMA-S was added, washed four times and incubated with 250,000 CTLs for 12 hours at 37°C. 10 µg/ml Brefeldin A were added for additional 5 hours at 37°C. Cells were washed, fixed and perforated with Saponin. The fixed cells were stained with PE-labeled anti-IFN-γ or isotype control and FITC-labeled anti-CD8 antibodies and measured in flow cytometry.

Results

Gp96 binds specifically to antigen presenting cell lines

Recent experiments demonstrated that HSPs are able to interact specifically with a macrophage- and a DC-like cell line (Arnold-Schild et al., 1999). We therefore further characterized the cell types able to interact with Gp96 in a specific manner. For this purpose, we incubated several cell lines with FITC-labelled Gp96, always at 4°C to exclude endocytosis. We only observed a specific interaction of Gp96 with APC lines, like P388D1, D2SC/1 and D1, but not with the lymphoma cell lines RMA, EG.7 and T1 (Fig. 1A, B). Increasing the total concentration of Gp96-FITC the binding displayed saturation at a total concentration of 30 µg/ml (Fig. 1C) and could only be competed for by unlabelled Gp96, but not by ovalbumin (Fig. 1A) or BSA (not shown). A 1-fold excess of unlabelled Gp96 resulted in a 50% reduction, a 5-fold excess in a approx. 75% reduction of Gp96-FITC binding at saturation point, which could be inhibited completely using an excess of up to a 100-fold (Fig. 1D). These data correspond to the theoretical values of 50% and 83% (1:1 and 1:5 dilution of Gp96-FITC with unlabelled Gp96), demonstrating that the FITC-labelling of Gp96 did not affect the binding characteristics to its putative receptor significantly. No inhibition was observed using an excess of up to 400-fold of ovalbumin or BSA (data not shown). These data demonstrate the presence of a specific Gp96 receptor that is expressed on APCs but not on other cell lines (Fig. 1A, B, C, D).

Gp96 interacts specifically with primary antigen presenting cells in mice and humans

More importantly, Gp96 (Fig. 1E) but not BSA (Fig. 1F) bound efficiently to immature bone-marrow-derived primary DCs prepared (as described in Inaba et al., 1992) from C57BL/6 mice, and could be competed for by increasing amounts of unlabelled Gp96 (Fig. 1G) but not by BSA (Fig. 1H). Specific binding was also observed when mouse spleen cells from BALB/c mice were incubated with Gp96-FITC (Fig. 2). Gp96 interacted specifically with cells that stained positive for MHC class II and CD45 (B220) but not with cells positive for CD90 (Thy-1) molecules. Setting the forward and sideward scatter gate on the bigger cells, including cells of the myeloid lineage, CD11c and Mac-3 positive cells were also positive for Gp96-FITC, indicating that the expression of the Gp96 receptor is restricted to professional APCs. No staining was observed using OVA-FITC (all panels on the left) or BSA-FITC (data not shown). The identical outcome was observed using spleen cells from C57BL/6 mice (not shown). A similar Gp96-FITC staining pattern was obtained for human PBL. HLA-DR, CD86, CD19 and CD14, but not CD2 or CD3 positive cells interacted specifically with Gp96-FITC (Fig. 3A). Again, no staining was observed using OVA-FITC. Gp96 binding to monocytes was slightly better than to B cells in human PBL. As expected, DCs expressing CD1a and CD83 were not detected. To determine Gp96-FITC binding to this cell type, we differentiated DCs from human PBL by the application of GM-CSF and IL-4. The whole DC population generated stained positive with Gp96 but not BSA (Fig. 3B).

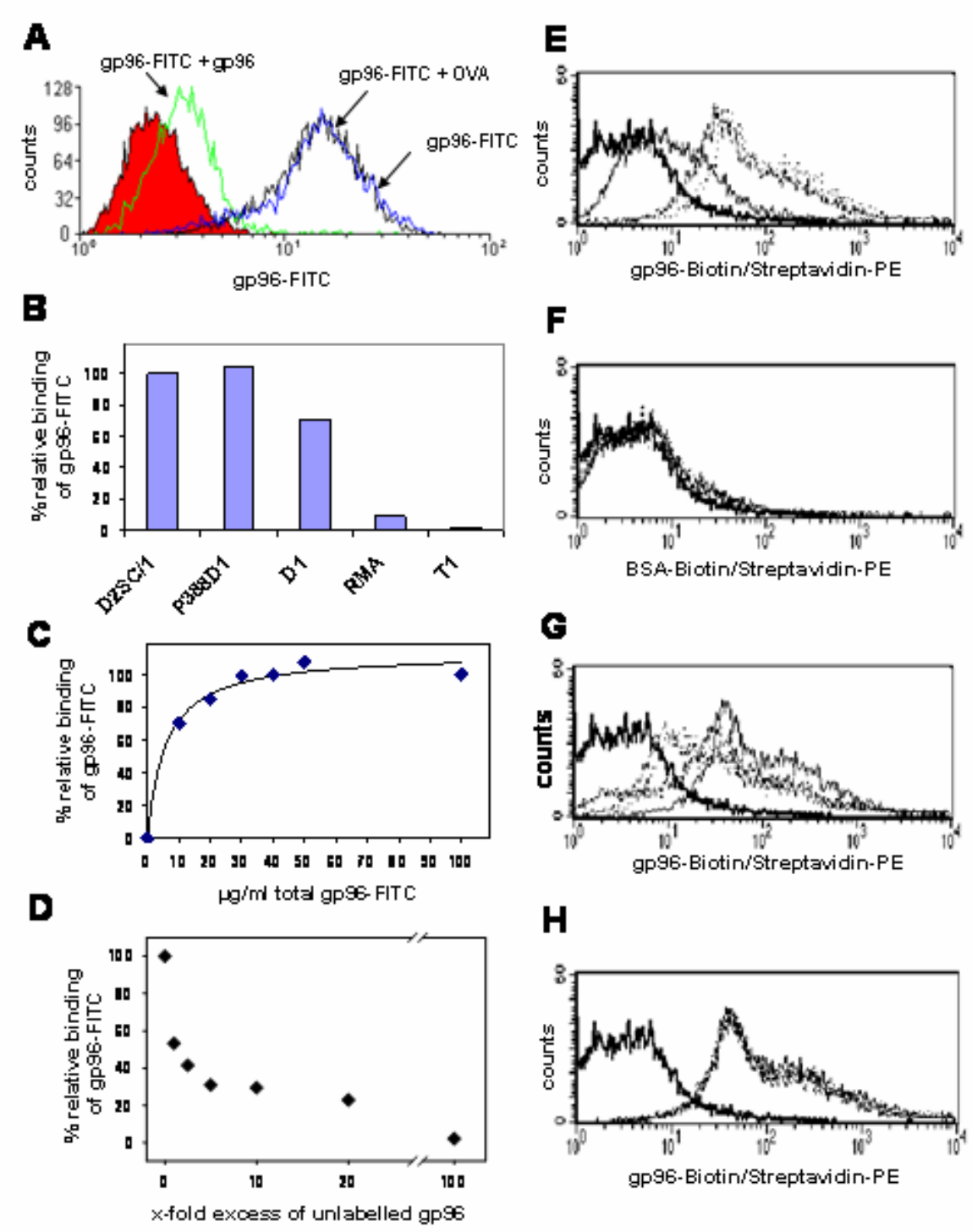

Figure 1. Specific binding of Gp96-FITC to APC cell lines and BMDC. Binding of 3 µg Gp96-FITC to 100,000 D2SC/1 cells was performed always at 4°C in 100 µl IMDM containing 10% FCS. This binding could be competed by a 10-fold excess of unlabelled Gp96 but not Ovalbumin (**A**). Specific binding of Gp96-FITC was observed on D2SC/1 (DC progenitor), P388D1 (macrophage) and D1 (DC) but not on RMA and T1 cells (**B**). Binding could be saturated at approx. 30 µg/ml for 100,000 D2SC/1 cells (**C**) and competed almost completely by an 100-fold excess of unlabelled Gp96 (**D**). Binding is given as relative values where 100% represents maximum binding of Gp96-FITC. The concentration values shown give total concentration of Gp96-FITC added to the cells. (**E**) Binding of 1 µg (—), 5 µg (---) and 10 µg (**…**) Gp96-Biotin/Streptavidin-PE to immature bone-marrow derived primary DCs (BMDCs) from C57BL/6 mice, no binding was observed for BSA-Biotin/Streptavidin-PE (**F**); binding of 10 µg Gp96-Biotin (—) to BMDC is competed in a similar fashion to **D** by unlabelled Gp96 (**G**), but not by unlabelled BSA (**H**).

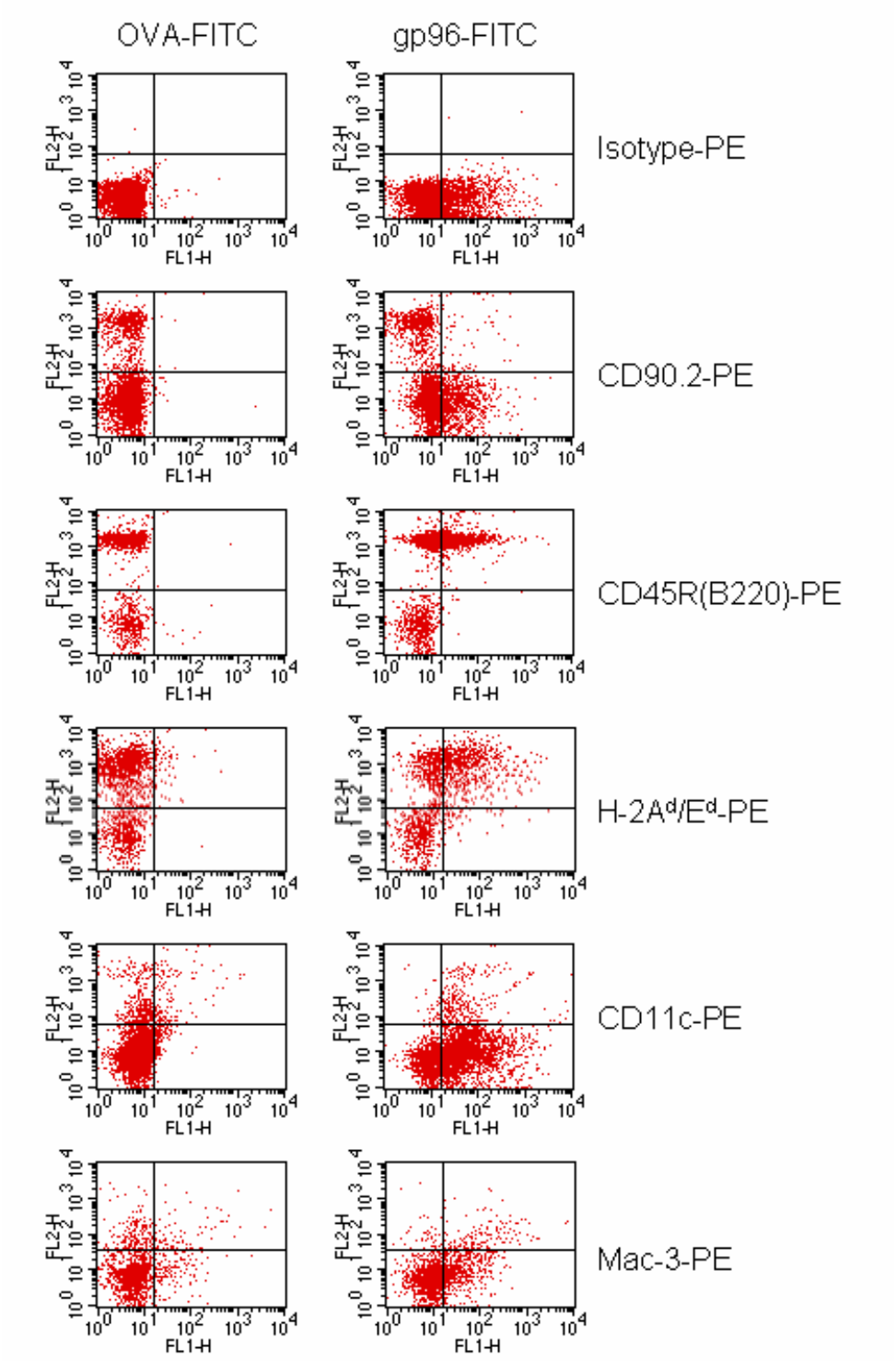

Figure 2. Specific binding of Gp96-FITC to B cells, macrophages and dendritic cells, but not to T cells of a spleen cell culture. 100,000 fresh Balb/c spleen cells were stained with 5 µg Ovalbumin-FITC (left panels) or Gp96-FITC (right panels) and different PE-labelled cell type marker antibodies to: CD90.2 (Thy-1.2, T cells), CD45R/B220 (B cells), I-A^d/E^d, CD11c (dendritic cells) and Mac-3 (monocytes and macrophages). Macrophages and DCs were counted in a different gate than lymphocytes with a higher forward scatter value.

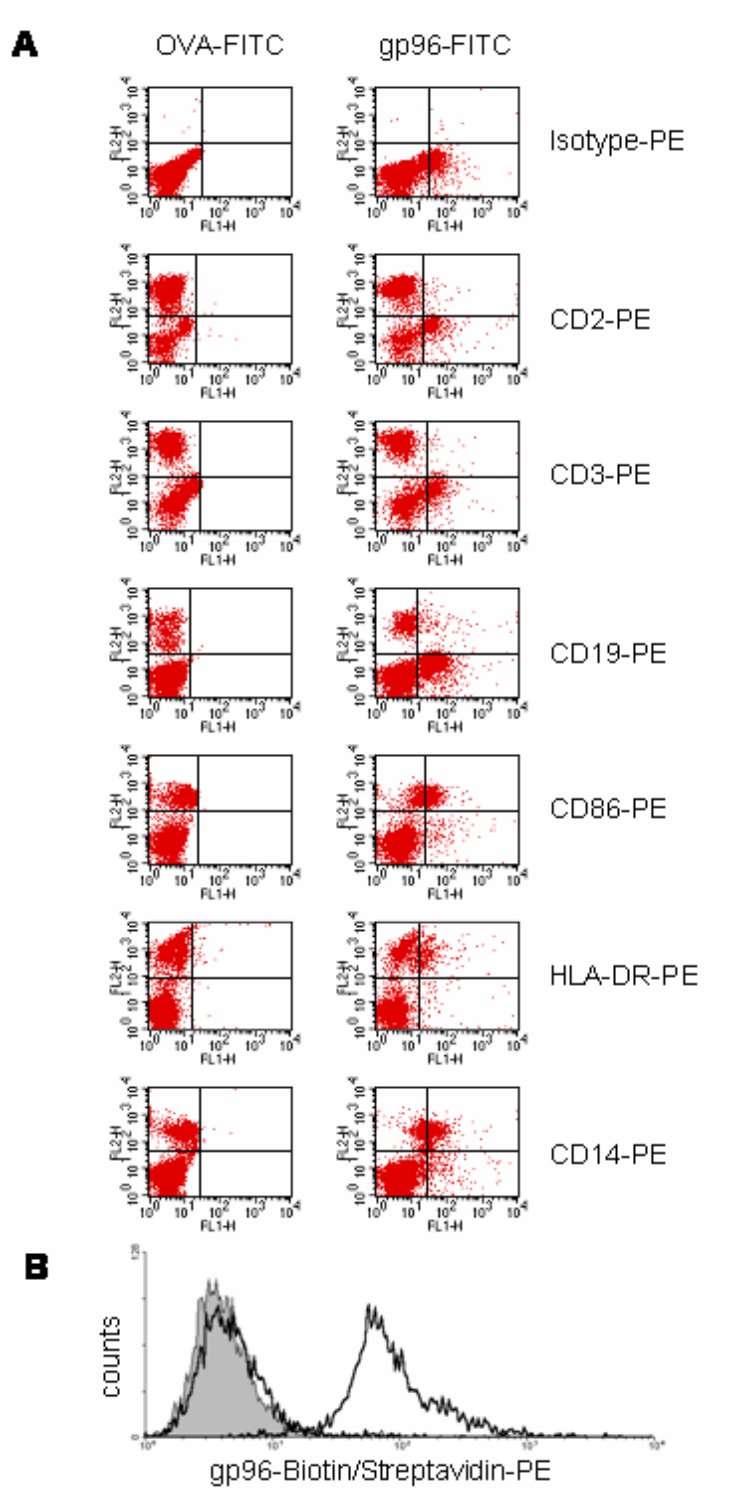

Figure 3. Gp96-FITC binds to APCs in human PBL culture but not to T cells. (**A**) 100,000 fresh human PBL were stained with 5 µg Ovalbumin-FITC (left panels) or Gp96-FITC (right panels) together with PE-labelled antibodies to following cell surface antigens: CD2 (T and NK cells), CD3 (T cells), CD19 (B cells), CD86, HLA-DR and CD14 (monocytes). The gate was set on all living cells. Therefore monocytes appear as a population with a slightly higher autofluorescence than lymphocytes in both fluorescence channels. Comparing the shifts of each population monocytes showed slightly better binding of Gp96 than B cells. (**B**) Binding of 0 µg (filled grey) and 5 µg (—) Gp96-Biotin to immature DCs prepared from human PBL. 10 µg of BSA-Biotin (—) did not display binding.

DEC-205 and MHC class II molecules are not required for Gp96 binding

Since Gp96 molecules contain a single, high mannose oligosaccharide (Wearsch and Nicchitta, 1996; Welch et al., 1983; Gallucci and Matzinger, 2001), we addressed the question whether this might allow the uptake by the DEC-205 receptor. DEC-205 is expressed on DCs and thymic epithelial cells and is capable of directing captured soluble, exogenous antigens to a specialized antigen processing compartment (Swiggard et al., 1995). DCs were prepared from bone marrow of wild-type and DEC-205$^{-/-}$ mice (kindly provided by Michel Nussenzweig and Ralph Steinman) and incubated with increasing amounts of Gp96-FITC. FACS analysis revealed identical staining (Fig. 4A), suggesting that the DEC-205 receptor is not involved in the binding of Gp96 molecules by DCs.

We further speculated whether MHC class II might function as a receptor for Gp96 because Gp96 showed binding to all MHC class II-positive mouse spleen cells and human PBL. Binding together with marker antibodies to spleen cells from MHC class II-knockout mice and their littermates did not reveal any difference (shown for MHC class II antibody in Fig. 4B, other markers not shown), indicating that MHC class II molecules do not function as Gp96 receptors.

Gp96 is endocytosed efficiently and co-localizes with recycled MHC class I and class II molecules

Recently it has been suggested that Gp96-FITC bound to peritoneal macrophages is endocytosed into early endosomes but does not reach the stage of lysosomes (Wassenberg et al., 1999). We also attempted to determine the faith of receptor-bound Gp96 at the cell surface of APCs by confocal microscopy (Fig. 5) using authentic dendritic cells (bone-marrow derived DCs from C57BL/6 mice). Initial binding of FITC-labelled Gp96 to the cell surface, at 4 °C to prevent endocytosis, revealed a patched pattern. Further incubation at 37 °C led to efficient endocytosis of Gp96. Co-localization with lysosomes, labelled with Lamp-1 antibody (Carlsson et al., 1988), was not observed after 15, 30, 45, 60 and 90 min of endocytosis (shown for 60 min in Fig. 5).

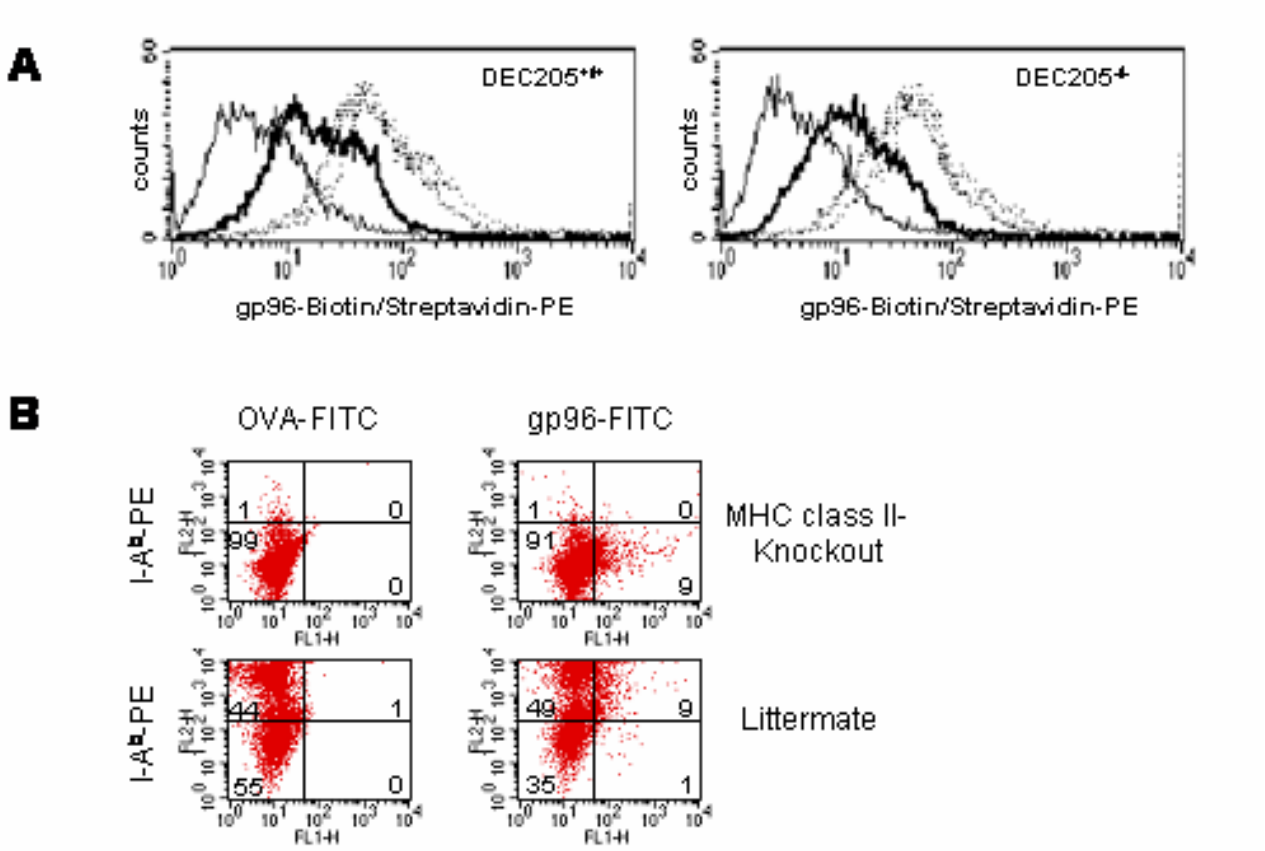

Figure 4. DEC-205 and MHC class II do not function as receptors for Gp96. Binding of Gp96-Biotin to bone-marrow derived DCs from wildtype and DEC205$^{-/-}$ mice **(A)** as well as binding of Gp96-FITC to spleen cells from MHC class II$^{-/-}$ mice and their littermate **(B)** showed identical staining. For staining of spleen cells in (B), different cell surface markers were used (see Fig. 2), only antibody to MHC class II is shown.

Recently, it has been reported that internalized cell surface MHC class I, like class II molecules, are able to bind their antigen in endosomal compartments suggesting these vesicles to be putative MHC class I and class II loading compartments for exogenously derived antigen (Gromme et al., 1999). We therefore attempted to determine whether Gp96 taken up by receptor-mediated endocytosis can be found in compartments containing recycled MHC class I and class II molecules. Indeed, after 15 min of endocytosis nearly all of the endocytosed H2-K^b and H2-A^b molecules co-localized with Gp96. Similar results were obtained using Gp96-FITC bound to the cell surface of the D2SC/1 cell line, where after 15 min of endocytosis Gp96 co-localized with Transferrin-Texas Red (as marker for early endosomes) and endocytosed H2-K^d molecules but was excluded from lysosomes after 30 min (data not shown).

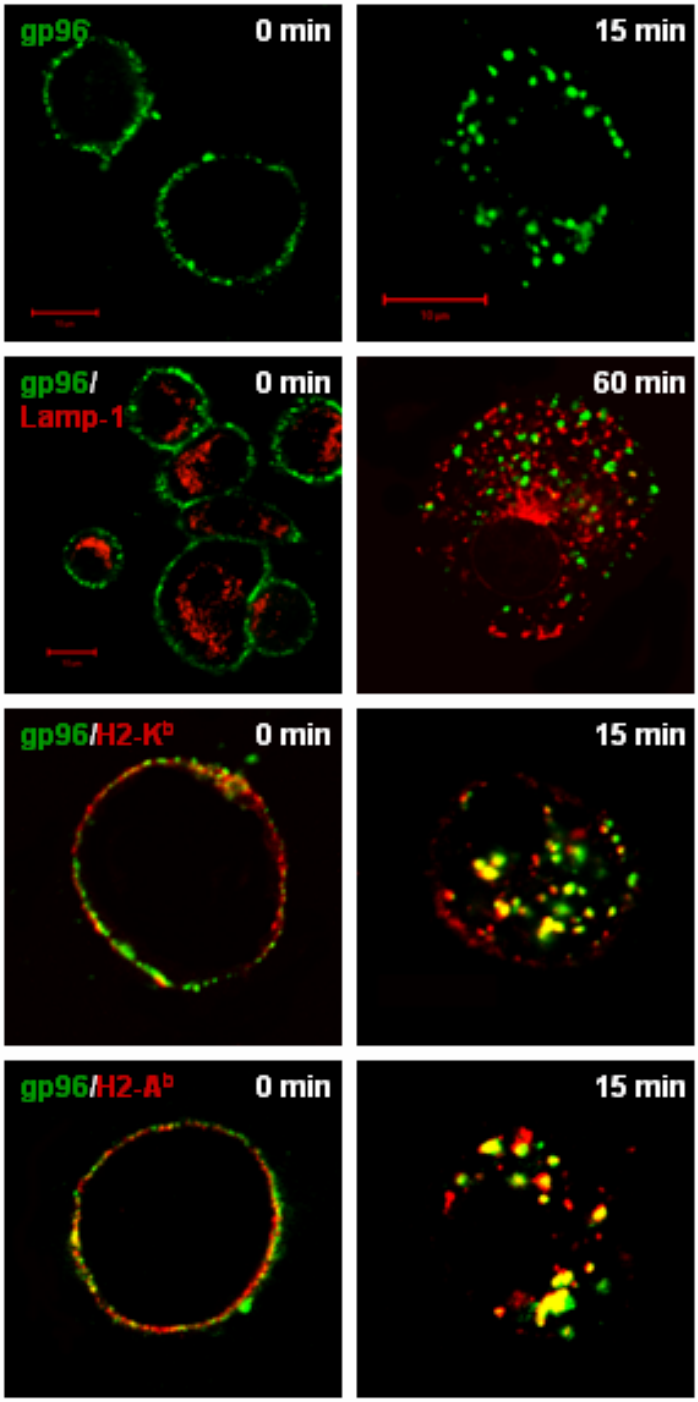

Figure 5. Gp96-FITC is endocytosed by bone-marrow derived DCs efficiently and co-localizes with endocytosed MHC class I and class II molecules but does not target to lysosomes. Internalization of Gp96-FITC was followed with confocal microscopy, representative sections are displayed. Coverslip-grown BMDCs were incubated with 50 µg/ml Gp96-FITC (shown in false colour green) on ice, washed, chased for 15 min or longer at 37 °C and fixed in paraformaldehyde. To follow the fate of Gp96-FITC after 15, 30, 45, 60 and 90 min (only 60 min is shown) of endocytosis cells were fixed, permeabilized with methanol/aceton and stained with antibody to Lamp-1 and secondary Alexa™ 546-coupled antibody to visualize lysosomes (shown in false colour red). No co-localization of Gp96 and Lamp-1 was observed. Furthermore, cells were stained with biotinylated antibodies to MHC class I (H2-K^b) and class II (H2-A^b) and secondary Streptavidin-Alexa™ 546-coupled anitbodies (both shown in red) as well as Gp96-FITC (green) on ice, washed and chased at 37°C for 15 min. After 15 min of endocytosis nearly all vesicles containing endocytosed Gp96 and MHC class I and class II molecules co-localize (shown in yellow as result of overlapping green and red).

Gp96-associated peptides are loaded onto MHC class I molecules as a result of receptor-mediated endocytosis

Gp96 molecules have been observed to enter APCs by receptor-mediated endocytosis as well as by non-receptor-mediated, non-specific endocytosis or macropinocytosis (Arnold-Schild et al., 1999; Wassenberg et al., 1999). The latter non-specific pathways have been described many times before to introduce exogenous proteins into the MHC class I-restricted antigen pathway, but unlike receptor-mediated endocytosis require high concentrations of antigens (reviewed in Jondal et al., 1996).

To investigate whether receptor-mediated endocytosis can lead to cross-presentation of Gp96-associated antigens, we have isolated Gp96 from RMA-S SigE1b cells that stably express the H2-D^b-restricted E1B epitope of Adenovirus type 5, fused with an ER-targeting signal sequence. C57BL/6 mice immunized with these Gp96 molecules generated CTLs that recognized RMA-S SigE1B and RMA cells pulsed with the Ad5-E1B peptide efficiently but not RMA cells, demonstrating the presence of the Ad5-E1B epitope on Gp96 molecules. Immunization with control Gp96 molecules from RMA-S cells did not induce Ad5-E1B specific CTL responses (Fig. 6).

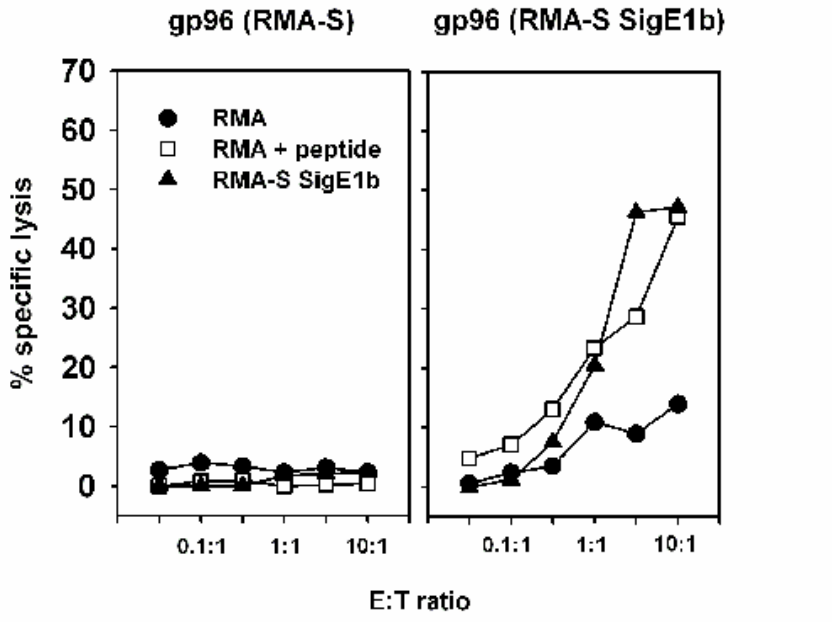

Figure 6. Gp96-E1B complexes generate an CTL response *in vivo*. Gp96 was purified from RMA-S SigE1B and RMA-S cells. 30 µg of Gp96 from either cell type were injected into C57BL/6 mice intraperitoneally. The specificity of the generated CTLs was assayed by ^{51}Cr-release of RMA-S SigE1B cells (▲), RMA cells incubated with 100 ng/ml Ad5 E1B peptide (□) or RMA cells (●). The figure shows one representative of three independent experiments.

To test whether the E1b epitope attached to Gp96 was re-presented to CTLs after uptake by APCs, Gp96 isolated from RMA-S SigE1B (or control Gp96 from RMA-S cells) was incubated with the DC cell line D1 for 2 h at 37°C. The D1 cells were further incubated overnight with the Ad5-E1B-specific CTL clones 100B6, 0.1C2 or control CTL clone LN5, specific for the Ad5-E1A epitope. Intracellular IFN-γ production was measured to determine CTL activation via the re-presentation of the Ad5 E1B peptide. As shown in Fig. 7A, incubation of 0.1C2 CTLs with D1 cells pulsed with RMA-S SigE1B Gp96 resulted in the activation of T cells. This activation was not observed if control Gp96 isolated from RMA-S cells was used or if Gp96 isolated from RMA-S SigE1B cells was incubated with the CTLs in the absence of D1 cells. The latter experiment clearly demonstrates that D1 cells, which efficiently bind Gp96 molecules (see Fig. 1B), are required for the re-presentation. The T cells themselves are not able to bind Gp96 (see Fig. 2 and 3) and consequently do not stimulate each other.

Most importanly, however, the activation of Ad5 E1B-specific CTLs by Gp96 from RMA-S SigE1B cells could be inhibited by the addition of a 2-fold excess of irrelevant Gp96 molecules from RMA-S and RMA cells. This excess of Gp96 was able to reduce the binding of Gp96-FITC by 60% (Fig. 1D) and eliminated the activation of 0.1C2 CTLs by Gp96 molecules from RMA-S SigE1B cells almost completely. The identical scenario was observed for a different Ad5-E1B specific CTL clone, 100B6 but not for LN5 CTLs, which are specific for the control Ad5-E1A CTL epitope (Fig. 7B). No competition was observed by using a 2-fold excess of BSA as control (data not shown).

Discussion

HSPs have been shown previously to induce specific immune responses against tumor, minor H and viral antigens (reviewed in Schild et al., 1999; Srivastava et al., 1998). This feature is based on peptides that are associated with HSPs and on the fact that by an unknown mechanism, HSPs can interact very efficiently with APCs to result in the re-presentation of HSP-associated peptides and subsequent activation of T cells (Blachere et al., 1997; Suto and Srivastava, 1995). We have now shown in this study that indeed specific binding of low amounts of Gp96 to a receptor present

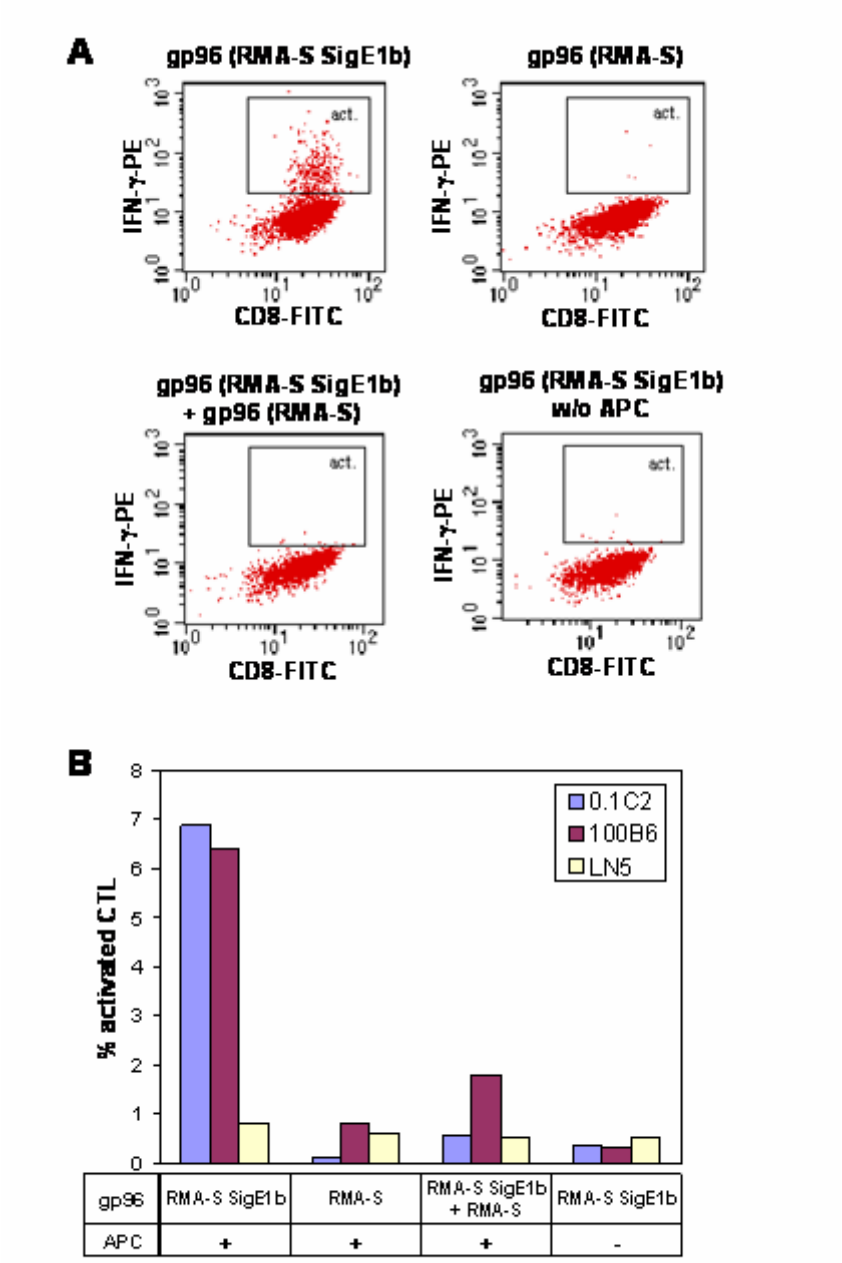

Figure 7. Specific activation of CTLs by dendritic cell-mediated cross-presentation of Gp96-associated antigen requires receptor-mediated endocytosis of Gp96-antigen complexes.
(**A**) Activation of Ad5 E1B specific CTL clones 0.1C2 was assayed by intracellular Interferon-γ staining in flow cytometry. D1 dendritic cells as APCs could activate the CTLs after prior incubation of D1 with Gp96-E1B complexes purified from RMA-S SigE1B (upper left panel), but not with irrelevant Gp96 isolated from RMA-S (upper right panel) or RMA cells (data not shown) or in the absence of D1 cells (lower right panel). Moreover, activation by Gp96-E1B complexes could be competed with a 2-fold excess of Gp96 from RMA-S (lower left panel) or RMA (not shown) but not with the same excess of BSA (not shown) indicating the presence of a receptor-mediated pathway responsible for processing of Gp96 by D1 cells. PE-labelled isotype control antibody was always negative (data not shown). Results are representative for at least three experiments. (**B**) Summary of the activation of Ad5 E1B specific CTL clones 0.1C2 and 100B6 as well as control CTL clone LN5 specific for Ad5 E1A. Graph shows the percentage of activated CTLs present in the gate shown in **A**. Addition of Ad5 E1B peptide to D1 cells resulted in the activation of around 25% of CTL clones 0.1C2 and 100B6 (data not shown).

on mouse and human professional APCs is required for the MHC class I-restricted re-presentation of Gp96-associated peptides. The nature of the Gp96 receptor still remains unclear. We reported earlier that Gp96 binding to the macrophage line P388D1 cannot be inhibited by mannan, thus arguing against the participation of the mannose receptor. Using DCs from DEC-205$^{-/-}$ mice, we show here that this receptor as well, which displays strong homology to the mannose receptor present on macrophages (Swiggard et al., 1995), is unlikely to be involved, because Gp96-FITC binding is indistinguishable from that observed for DCs of wild-type mice (Fig. 4A). Because DnaK and HSP73 molecules have been reported to bind to certain allelic products of MHC class II (Auger and Roudier, 1997; Rich et al., 1998), they could represent another potential receptor for HSPs on the surface of APCs. The observation that Gp96 binds to all MHC class II positive cells could indicate that Gp96, too, uses MHC class II molecules as a receptor. However, anti-MHC class II antibodies were not able to inhibit the binding of Gp96-FITC molecules (data not shown) and cells from MHC class II$^{-/-}$ mice showed identical Gp96 binding compared to wildtype mice, thus arguing against MHC class II molecules being the receptor for Gp96 (Fig. 4B).

We further demonstrate in this study that the specific interaction of Gp96 molecules with DCs results in re-presentation of associated peptides and specific activation of CTLs. Gp96 molecules isolated from RMA-S SigE1B cells that carry the Ad5 E1B CTL epitope (Fig. 6) are able to activate Ad5 E1B-specific CTLs after incubation with the DC line D1, as visualized by intracellular IFN-γ staining. The control CTL line LN5 is not activated by any of the Gp96 preparations tested (Fig. 7). More importantly, we are able to show here for the first time that receptor-mediated endocytosis of Gp96 is indeed required for the re-presentation and subsequent activation of CTLs. By inhibiting the specific binding of RMA-S SigE1B-derived Gp96 with a 2-fold excess of unrelated Gp96 molecules that have been shown to reduce Gp96-FITC binding by 60 % (Fig. 1D), we completely abolish the activation of Ad5 E1B specific CTLs (Fig. 5). This low excess of unrelated Gp96 was chosen on purpose to exclude potential toxic effects of a high Gp96 concentration. Using synthetic E1b peptide approx. 25 % of CTLs could be activated (data not shown) compared to 6-7% activated CTLs as shown in Fig. 7. Therefore, the amount of RMA-S SigE1b Gp96 was not able to activate all possible CTLs, most likely because of limiting amounts of peptide. As the activation of CTLs requires the activating signal to be above a certain

threshold the amount of antigen presented by MHC class I molecules in the presence of competitor could easily be below this threshold explaining the lack of a CTL response with a 2-fold excess of irrelevant Gp96 not associated with E1b peptide.

Because only receptor-mediated endocytosis of labelled Gp96, but not unspecific, nonreceptor-mediated uptake such as pinocytosis or macropinocytosis, can be inhibited by an excess of unlabelled Gp96 (Arnold-Schild et al., 1999), our results clearly demonstrate that receptor-mediated endocytosis of Gp96 molecules is the cellular pathway responsible for re-presentation of Gp96-associated peptides by MHC class I molecules. Therefore, our results provide evidence for the hypothesis that professional APCs possess receptors that are able to interact specifically with HSPs (Srivastava et al., 1994) and direct HSP-associated peptides into the MHC class I-restricted antigen presentation pathway. This now explains why very small amounts of Gp96/peptide complexes can activate T cells.

The exact intracellular pathway for the re-presentation of Gp96-associated peptides requires further clarification. Confocal microscopy data point into the direction that Gp96 heads for early endosomes but does not enter lysosomes. We could show that Gp96 after receptor-mediated uptake enters compartments containing MHC class I and class II molecules. It can be speculated that these compartments function as putative loading compartments where antigen could be transferred to MHC class I and class II molecules (Gromme et al., 1999) but it cannot be excluded that Gp96-antigen-complexes enter the cytosol specifically, as recently suggested for immunoglobulin-antigen complexes after endocytosis by Fc receptors in dendritic cells (Rodriguez et al., 1999).

Further identification of the pathway responsible for the re-presentation of Gp96-associated peptides will also contribute to the understanding of the phenomenon termed cross-presentation. Until now, cross-presentation of MHC class I-restricted antigens has been shown to be induced by receptor-mediated phagocytosis of apoptotic bodies (Albert et al., 1998a; Rovere et al., 1998; Fantuzzi and Dinarello, 1999), exosomes (Zitvogel et al., 1998), bacteria (Rescigno et al., 1998) and proteins, either denatured or immobilized (Bachmann et al., 1996), by phagocytic or nonphagocytic mechanisms (Jondal et al., 1996). Unlike the latter two pathways, which require in most cases high concentrations of the antigens, receptor-mediated endocytosis of HSPs operates efficiently at antigen concentrations around 1-2 ng per mouse (Blachere et al., 1997) and might be as efficient as receptor-mediated

phagocytosis of apoptotic cells or receptor-mediated endocytosis of proteins by surface-immunoglobulins on B cells. One can envisage that HSPs, released from dying cells, bind to HSP receptors of professional APCs and are endocytosed before the associated peptides are re-presented by MHC class I molecules.

The antigen carriers in apoptotic cells or exosomes are unknown but one interesting possibility is that HSPs chaperone the antigenic peptides, thus protecting them from further degradation and directing them to the correct intracellular loading compartment. In line with this speculation is the observation that HSP70 is one of the proteins found in close association with the transferrin receptor in exosomes derived from reticulocytes (Mathew et al., 1995). Whether or not the induction of apoptosis leads to a general increase of HSPs is still controversial and might depend on factors that are still to be determined. For tumor cells, it was reported that apoptotic death was associated with low HSP expression levels (Melcher et al., 1998), whereas for polymorphonuclear leukocytes, increased apoptosis coincided with induction of Hsp72 (Hennigan et al., 1999). Nevertheless, an increase of HSP expression levels generally seems to correlate with increased immunogenicity (Menoret et al., 1995; Melcher et al., 1998), supporting the above mentioned hypothesis.

The finding that TAP-deficient cells are still able to cross-prime as efficiently as wild-type cells (Schoenberger et al., 1998b) does not contradict the involvement of HSPs in cross-presentation. It shows that the ER-resident HSP Gp96 alone is not essential for cross-priming, but it also does not exclude the participation of other HSPs such as HSP70 or HSP90 that might compensate for the absence of immunogenic Gp96/peptide complexes. Another argument formulated against the participation of HSPs in cross-presentation of cellular antigens is based on an experiment performed by Bevan and Carbone (Carbone and Bevan, 1990), in which splenocytes were incubated with ovalbumin or β-galactosidase, washed and injected into mice. Because of the non-specific coating of cells with the soluble proteins, an association with HSPs might be difficult to imagine. The incubation conditions (37°C, 10 mg/ml protein, 10 min.), however, do not exclude the pinocytic or phagocytic uptake of the proteins by splenocytes, which process and release antigens, possibly even associated with HSPs. In addition, several different pathways for cross-presentation, including apoptotic cells, exosomes and receptor-mediated endocytosis of HSPs, might exist in parallel, each one able to induce the cross-presentation of different types of antigens.

More detailed knowledge about the Gp96 receptor, its intracellular transport and the regulation of expression in different cell types will deepen our understanding of the role of Gp96 and possibly HSPs in general in cross-presentation and could greatly improve the application of Gp96 for the induction of specific immune responses *in vivo*.

4

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THE HEAT SHOCK PROTEIN GP96 INDUCES MATURATION OF DENDRITIC CELLS AND DOWN-REGULATION OF ITS RECEPTOR

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Peptides associated with the heat shock protein Gp96 induce a specific T cell response against cells from which Gp96 is isolated. Recently, we have shown that Gp96 binds to a yet unknown receptor present on dendritic cells (DCs) and that receptor-mediated uptake is required for cross-presentation of Gp96-associated peptides by DCs. We now describe that Gp96 mediates maturation of DCs as determined by up-regulation of MHC class II and CD86 molecules, secretion of the cytokines IL-12 and TNF-α and enhanced T-cell stimulatory capacity. Heated, and therefore denatured Gp96 is not able to induce DC maturation and cytokine secretion. Furthermore, we show that mature DCs are no longer able to bind Gp96 molecules. Hence, the Gp96 receptor is down-regulated on mature DCs suggesting that this receptor behaves similar to other receptors involved in antigen uptake like the scavenger receptor CD36, the mannose receptor or the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Alltogether, our findings provide an additional explanation for the remarkable immunogenicity of Gp96 as a cross-priming antigen carrier and direct activator of dendritic cells.

The author of this thesis contributed substantially to figures 1 and 3 of this chapter togehter with Harpreet Singh-Jasuja, and to figure 2 together with Harpreet Singh-Jasuja and Hans-Ulrich Scherer.

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Introduction

Dendritic cells are very effective activators of T cells. Immature DCs are experts in antigen acquisition, whereas mature DCs are specialized in T cell activation most likely caused by increased expression of MHC and co-stimulatory molecules, like CD86. DC maturation can be induced by multiple stimuli including LPS, bacteria and viruses, CpG oligonucleotides and signaling molecules, like CD40L (Bender et al., 1998; Caux et al., 1994; MacPherson et al., 1995; Rescigno et al., 1998; Sparwasser et al., 1998). Especially the ability to present exogenous antigens through a process called 'cross-presentation' is a key feature of DCs. These exogenous antigens include proteins, bacteria and apoptotic cells (Heath and Carbone, 1999). In addition, we have shown recently that also peptides chaperoned by heat shock proteins (HSPs), like Gp96 can be represented by APCs in the context of MHC class I molecules to CTLs. The representation requires the uptake of Gp96 via a so far unidentified receptor expressed by DCs (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000c).

Heat shock proteins, like HSP60 and HSP70, also induce the activation of monocytes and the secretion of the pro-inflammatory cytokines TNF-α and IL-12 via interaction with CD14 molecules (Asea et al., 2000; Chen et al., 1999; Kol et al., 2000; Corr et al., 1996). Thus, HSPs may not only serve as a vehicle for antigenic peptides recognized by T cells but also as a danger signal to the innate immune system when released from stressed cells, as postulated (Matzinger, 1994). In line with this is the finding that increased HSP70 expression in tumor cells, induced by non-apoptotic cell death or transfection, resulted in enhanced tumor immunogenicity (Melcher et al., 1998) via a T-cell mediated pathway (Todryk et al., 1999).

Among all HSPs analyzed, the ER-resident heat shock protein Gp96 has the best documented history with respect to the induction of specific CTL responses and tumor protection (Schild et al., 1999; Srivastava et al., 1998). These features were attributed to the fact that Gp96 is associated with peptides derived from intracellular proteins which are efficiently represented on MHC class I molecules on DCs after receptor-mediated endocytosis of Gp96 (Singh-Jasuja et al., 2000c; Srivastava et al., 1998). Nonspecific phagocytosis did not result in CTL activation (Singh-Jasuja et al., 2000c). However, recently it has been demonstrated that for activation of CTLs from their naive precursors immunogenic peptides need to be presented by DCs that

display their full potential of co-stimulatory molecules. Given these insights into the requirements for T-cell priming we tested the effect of Gp96 on DC maturation and T cell activation. We found that Gp96-treated immature DCs secrete TNF-α and IL-12 and convert to the mature phenotype, expressing increased levels of CD86, MHC class II and CD83 molecules in the case of human DCs. This change in phenotype has functional consequences which are visualized by the increase in activation of allogenic T cells. Interestingly, after maturation DCs loose their capacity to bind exogenous Gp96. This observation is well in line with the reduced abilities of mature DCs to acquire antigen (Sallusto and Lanzavecchia, 1994).

Materials and Methods

Generation of dendritic cells

The medium used throughout was Iscove's Modified Dulbecco's Medium (IMDM, BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine (GibcoBRL Life Technologies, Paisley, UK), 100 IU/ml Penicillin/Streptomycin (Gibco), 10% FCS (PAA, Linz, Austria) and cytokines as indicated below. Mouse immature DCs were generated from bone-marrow of C57BL/6 or BALB/c mice according to Inaba et al., 1992. Briefly, bone marrow cells were incubated with 150 U/ml GM-CSF (PeproTech, London, UK) for 6-8 days renewing medium every two days. Approx. 90-100 % of all cells in the FACS® gate used for monocytes were DCs as determined by flow cytometry with antibodies obtained from Pharmingen, San Diego, CA: they were CD11c-, CD86- and MHC class II-positive and CD14-negative. Human immature dendritic cells were prepared from PBMCs according to Bender et al., 1996. Briefly, $CD14^+$ monocytes were purified by 1 h adherence to culture dishes and extensive washing; monocytes were incubated with 1000 IU/ml IL-4 and 800 IU/ml GM-CSF for 6-8 days renewing cytokines every three days. The cells generated in this way showed a large number of dendrites up to day 12 and were only lightly adherent. They expressed CD1a, low CD14, CD86, HLA-DR and very low CD83 on their surface as determined by antibodies (Pharmingen).

Stimulation of dendritic cells

Mouse and human DCs were stimulated by addition of 30 to 100 µg/ml Gp96 or 2 µg/ml LPS (from *Salmonella typhimurium*, Sigma Chemicals, St. Louis, MO) for 24 h. In some cases Gp96 or LPS were pre-treated at 95°C for at least 20 min. Gp96 (kindly provided by Immatics, Tübingen) was purified from mycoplasma-free IGELa2 mouse cell line as described (Arnold et al., 1995). FPLC fractions preceeding and following fractions not containing Gp96 according to Western blot are referred to as 'flanking fractions' (provided by Immatics, Tübingen). Endotoxin that might be present in Gp96 preparations was tested by a Limulus Amebocyte Lysate Kit (QCL-1000, BioWhittaker) according to the guidelines published by the US Food and Drug Administration (FDA). The endotoxin content determined in all cases was at or below 0.05 EU/µg Gp96. For detection of possible mycoplasma contaminations of the IGELa2 cell line and Gp96 FPLC fractions the Mycoplasma Plus™ Kit by Stratagene, La Jolla, CA was used.

Cytokine detection

Mouse IL-12 (p40) and TNF-α were measured in culture supernatants using standard sandwich ELISA protocols. Antibodies and recombinant standards of both cytokines were obtained from Pharmingen. The capture antibody was bound to the ELISA plate (MaxiSorb™, Nunc, Roskilde, Denmark), the biotinylated detection antibody was recognized by streptavidin-conjugated horse-radish peroxidase and detected by 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (Sigma) emitting at 415 nm.

Stimulation of alloreactive T cells

Human or BALB/c DCs were stimulated in a 96-well plate as described above, washed extensively and incubated with PBL from a different donor or C57BL/6 spleen cells, respectively, for 4 days at different responder/stimulator ratios. On day 4, 1 µCi of ^{3}H-Thymidin was added per well, cells were harvested after additional 16 h and incorporated ^{3}H-Thymidin was detected using a Wallac 1450 MicroBetacounter.

FACS® analysis

Cell surface staining was performed using antibodies as mentioned above, Ovalbumin-FITC or Gp96-FITC (kindly provided by Immunosome, Tübingen) which were incubated with cells for 30 min on ice in IMDM containing 10% FCS. Dead cells were excluded by PI staining. All FACS® analysis was performed on a FACSCalibur® (Becton Dickinson, Mountain View, CA) using Cell Quest Software.

Results

Gp96 induces maturation of human DCs

To study the effect of Gp96 on phenotypical changes of DCs, immature DCs were generated through incubation of human PBMCs with GM-CSF and IL-4 for 7 days. For additional 24 h Gp96 or LPS as a positive control were added to the cultures. As shown in Figure 1, Gp96 as well as LPS induced maturation of DCs, now displaying increased levels of CD83 and CD86 on their surface. Denaturation of Gp96 by heat destroyed its ability to activate DCs, whereas LPS was not affected by this treatment. These latter observations strongly argue for Gp96-mediated DC activation not being a consequence of endotoxin contamination but the result of binding of native Gp96 to its receptor (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000c; Kurts et al., 1996). This was further supported by the finding that the Gp96-flanking fractions from the FPLC purification (lacking Gp96) did not induce DC maturation and that normal medium and Gp96 did not differ in their endotoxin content as measured by the *Limulus* amebocyte assay kit (data not shown). In addition, Gp96 was purified from a cell line not infected with mycoplasma. This is important to note because it has been shown recently that supernatant from mycoplasma-infected cells is able to induce DC maturation (Salio et al., 2000). BSA and Concanavalin A added as control proteins in similar amounts as Gp96 did not activate DCs (data not shown).

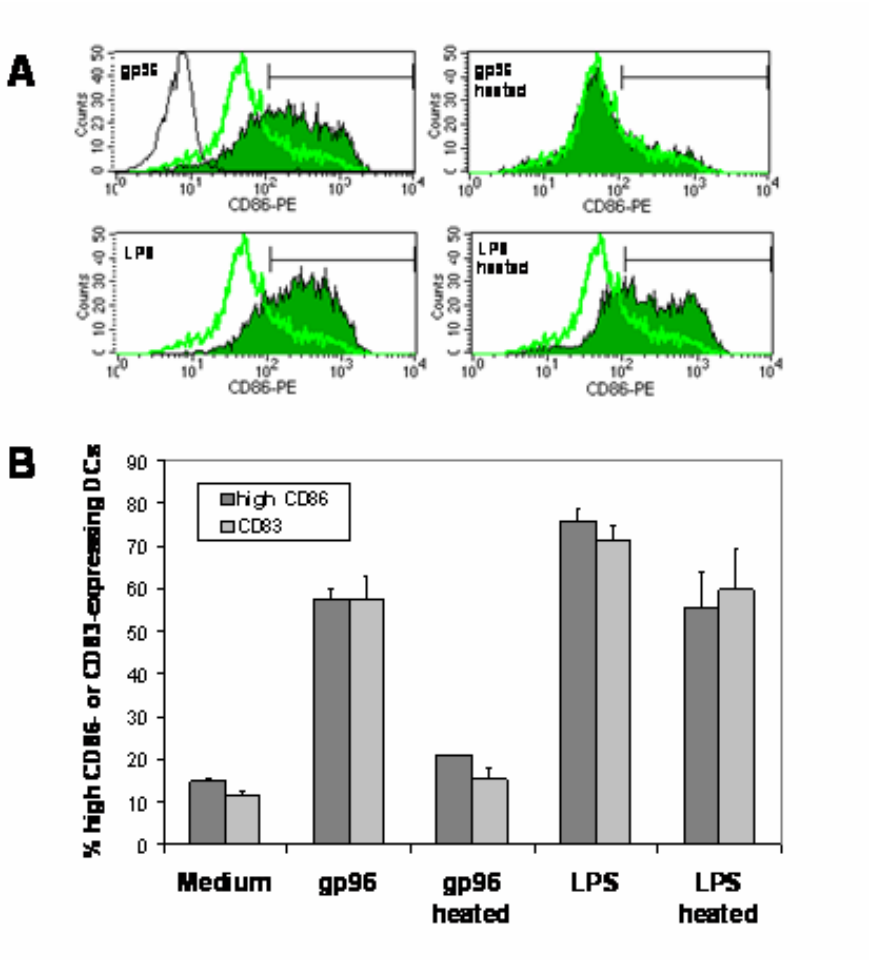

Figure 1. Gp96 activates human dendritic cells. Human dendritic cells were prepared *in vitro* from $CD14^+$ PBMCs with GM-CSF and IL-4 after 7 days and incubated with Gp96, heat-treated Gp96, LPS or heat-treated LPS for 24 h. **(A)** CD86 expression levels of DCs treated with Gp96/LPS (filled histogram) or non-treated DCs (in grey; black line represents isotype control antibody which showed same fluorescence intensity for all treatments). **B** shows the percentage of high CD86 (as indicated by marker bar in A) and activation marker CD83 expressing DCs after treatment with the different effector molecules. Error bars give standard deviation. The results are representative of three independent experiments.

Gp96 induces maturation of mouse DCs

A comparable Gp96-mediated activation was also obtained using mouse bone-marrow derived DCs. Incubation of immature DCs with Gp96 at different concentrations induced a heat-labile maturation of DCs, as visualized by increased expression of CD86 molecules (Figure 2A) and MHC class II molecules (data not shown). In addition to cell surface expression of maturation markers, Gp96 also induces the secretion of pro-inflammatory cytokines IL-12 and TNF-α (Figure 2B). Again, the effect is heat-sensitive and not due to endotoxin contaminations possibly present in our Gp96 preparations.

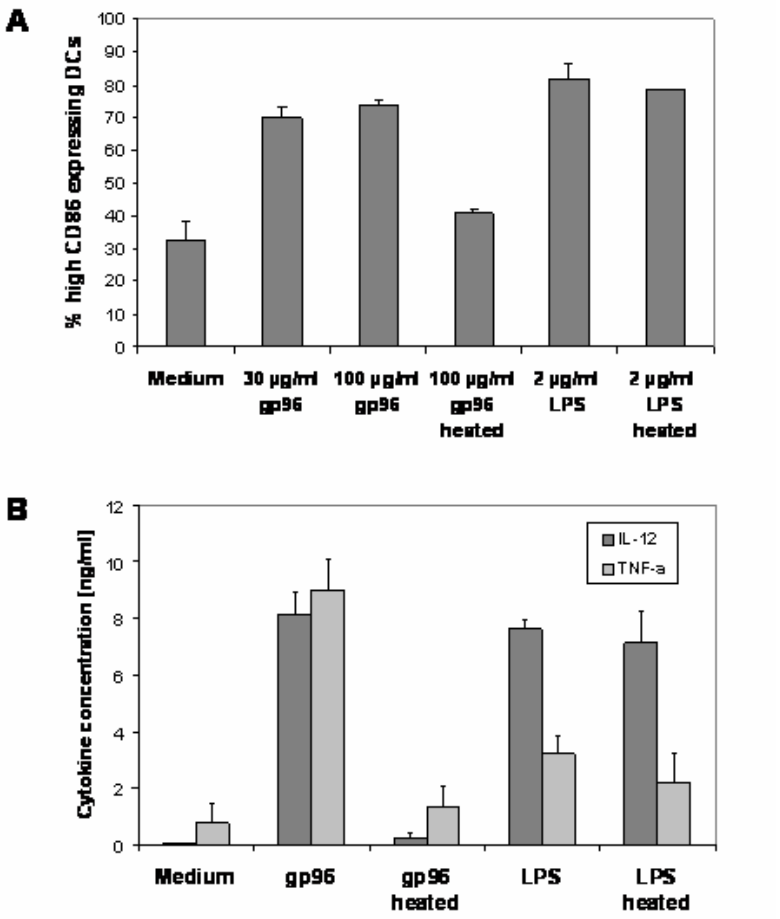

Figure 2. Gp96 activates mouse dendritic cells. Mouse dendritic cells were prepared from bone marrow of C57BL/6 or BALB/c mice with GM-CSF after 7 days. **(A)** Treatment with 30 and 100 µg/ml Gp96 and 2 µg/ml LPS and heat-treated LPS after 24 h led to an up-regulation of CD86 (as measured by FACS double staining with CD11c and CD86 antibodies) while heat-treated Gp96 did not activate DCs. **(B)** Supernatants of the experiment above were analysed by ELISA for contents of the cytokines IL-12 and TNF-α showing similar results as in A. Error bars show standard deviation of triplicates. The results are representative of at least three independent experiments.

Gp96-activated DCs induce strong T cell proliferation

To investigate whether the Gp96-mediated DC maturation has functional consequences, DCs matured by Gp96 or LPS were incubated with allogenic PBMCs for 4 days and cell proliferation was determined by incubation with ^{3}H-thymidin. As shown in Figure 3, DCs displaying a mature phenotype either after LPS or Gp96 activation for 24 h, induced 3-fold better T cell proliferation than immature DCs incubated with medium only. As observed before, the Gp96-mediated effect is heat-sensitive because DCs incubated with heated Gp96 did not display an enhanced T cell stimulatory capacity. Comparable T cell proliferation was observed using mouse DCs and allogenic splenocytes (data not shown).

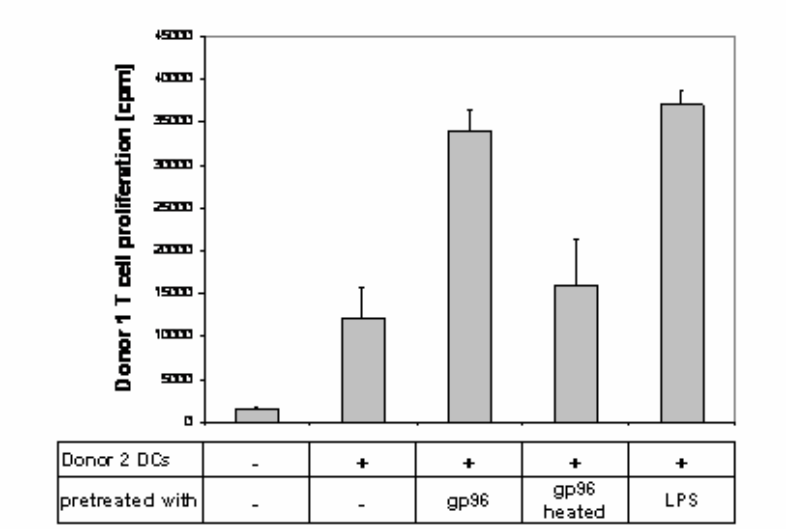

Figure 3. Human and mouse dendritic cells activated by Gp96 are able to induce strong proliferation of alloreactive T cells. Human DCs were prepared and treated with 30 µg/ml Gp96, heat-treated Gp96 or 2 µg/ml LPS for 24 h as described above. After extensive washing, these pre-treated DCs were incubated with 10^5 PBMCs of a different donor for 4 days in different stimulator/responder ratios (shown is ratio 1:30). Proliferation of T cells was assayed by addition of 1 µCi ^{3}H-Thymidin for 16 h. Error bars give standard deviation of triplicates. Results are representative of two independent experiments. Similar results were obtained for mouse BALB/c DCs inducing proliferation of C57BL/6 T cells (data not shown).

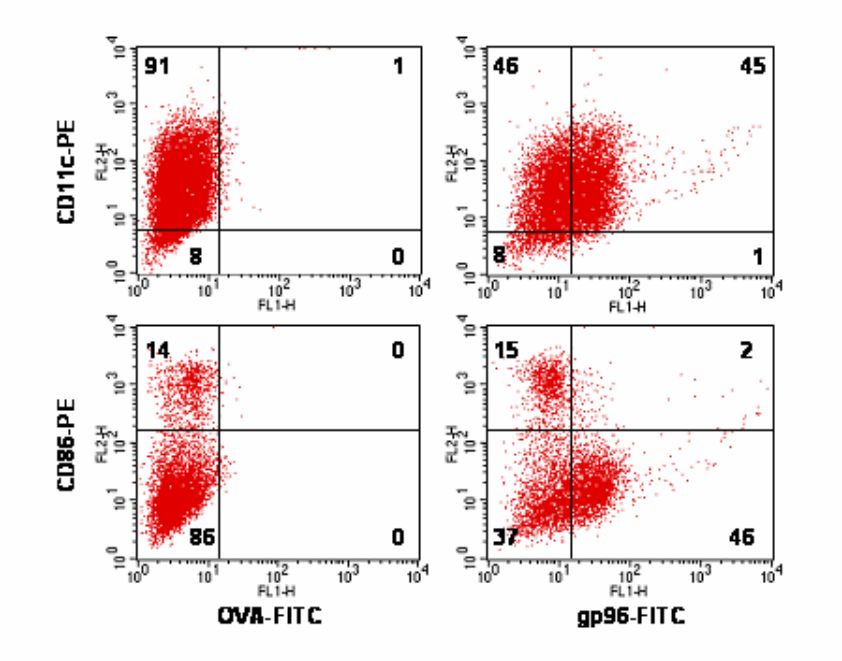

Figure 4. Activated DCs down-regulate the Gp96 receptor. Bone-marrow derived DCs from C57BL/6 mice show a heterogenous population of activated (high CD86 positive on FL-2) and non-activated (low CD86 positive) CD11c-positive DCs. OVA-FITC as control protein did not bind at all, while Gp96-FITC bound only to non-activated DCs (lower panels). The upper panels show CD11c expression of the DC preparation and binding of Gp96-FITC. The values give percentage of total cells in the specified quadrant. Results are representative of three different experiments.

Mature DCs express reduced levels of the Gp96 receptor

Maturation of DCs induces upregulation of MHC class II, CD83 and CD86 molecules, resulting in increased T cell proliferation. In addition, once activated, the DCs are unable to further receive Gp96-mediated stimuli. As shown in Figure 4, all CD11c positive mouse DCs bind Gp96 but not ovalbumin. However, only immature DCs, expressing low levels of CD86, are able to bind Gp96. As Gp96 is complexed with peptides from the cell it has been isolated from (Schild et al., 1999; Srivastava et al., 1998) and DCs are able to cross-present these peptides on MHC class I molecules (Singh-Jasuja et al., 2000c), mature DCs can be expected to no longer be able to present Gp96-associated peptides to T cells.

Concluding remarks

Our experiments show that the ER-resident heat shock protein Gp96 is able to induce maturation of mouse and human DCs (Huang et al., 1994; Corr et al., 1996). This observation is especially remarkable in the light of previous findings where Gp96 has been shown to specifically bind to DCs resulting in MHC class I-restricted cross-presentation of Gp96-associated peptides (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2000c). The finding of down-regulation of the Gp96 receptor on the surface of mature DCs also allows the first speculations on the nature of the Gp96 receptor expressed on DCs. Possible candidates are endocytic receptors like the scavenger receptor CD36 or the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, all of which have been shown to be down-regulated upon DC maturation (Albert et al., 1998a).

We provide here the first evidence that Gp96 is not only a peptide carrier directing the associated peptides to professional APCs but also a direct activator of DCs, inducing a conversion to the mature phenotype highly efficient in T cell activation. Gp96 might therefore act as a danger signal when released from necrotic or stressed cells delivering both unspecific and specific stimuli to the immune system.

Once activated, the DCs loose the capacity to acquire new Gp96-associated peptides and gain the ability to communicate efficiently with T cells specific for the presented MHC/peptide complexes. This situation resembles closely what has been observed initially for the presentation of soluble antigens on MHC class II molecules (Sallusto et al., 1995), where it has been described that DCs are ‘locked’ in a state of antigen presentation and highly efficient in the activation of T cells.

This new feature of Gp96 provides an additional, so far unknown, explanation for its high immunogenicity and will allow to improve its application in the induction of specific immune responses *in vivo*.

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HUMAN PLATELETS EXPRESS HEAT SHOCK PROTEIN RECEPTORS AND REGULATE DENDRITIC CELL MATURATION

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Immunizations using the ER-resident heat shock protein Gp96 induce specific immune responses. Specificity is based on the MHC class I restricted cross-presentation of Gp96-associated peptides derived from endogenous proteins. Initiation of the immune response depends on the ability of Gp96 to induce the production of pro-inflammatory cytokines by macrophages and dendritic cells (DCs) and their maturation in a fashion presumably independent of associated peptide. Both events are mediated by Gp96 receptors on antigen presenting cells. It is known that Gp96 is released from cells upon necrosis induced e.g. by virus infection. While this event will support the efficient induction of immune responses on one hand, it might on the other hand interfere with processes that are susceptible to chronic inflammation, like wound healing after tissue damage. Therefore, Gp96-mediated stimulation of the immune system requires tight regulation. Here, we describe that human thrombocytes specifically interact with Gp96 and that binding of Gp96 to platelets is more than 10-fold enhanced upon activation by thrombin. Gp96 does neither interfere with thrombin-induced platelet activation nor with platelet aggregation. However, the presence of platelets during Gp96-mediated DC activation reduces secretion of pro-inflammatory cytokines and activation of DCs. This effect is independent of soluble platelet factors and cell-to-cell contact between DCs and thrombocytes. Thus, we provide evidence for a regulatory mechanism that neutralizes Gp96 molecules systemically, especially in the blood. This effect might be of significance in wounds where chronic inflammation and immune responses against autoantigens have to be prevented.

The major part of the work and ideas reported in this chapter was performed by the author of this thesis with minor contributions of the co-authors.

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Introduction

The endoplasmic reticulum (ER)-resident heat shock protein (HSP) Gp96 plays multiple roles in mammalian organisms. As a chaperone it assists protein folding and prevents aggregation of partially unfolded proteins in the ER (Melnick et al., 1994). In this key compartment of the major histocompatibility (MHC) class I presentation pathway, Gp96 is also one of the major peptide binding proteins and associates with a peptide pool representative for the protein content of the cell (Lammert et al., 1997a; Spee and Neefjes, 1997). Gp96 is released from cells after tissue damage caused by severe injury and as a consequence of necrotic cell death induced by freeze-thaw cycles (Basu et al., 2000) or virus infection (Berwin et al., 2001). Its presence in the extracellular space reveals surprising immunostimulatory properties: immunization with Gp96 preparations from tumor cells has been shown to elicit protective and therapeutic immune responses against the tumor the HSP had been purified from (Udono and Srivastava, 1994; Tamura et al., 1997). The specificity of this immune response is due to tumor-derived peptides associated with Gp96 (Udono and Srivastava, 1994). After receptor-mediated endocytosis of Gp96 by professional antigen presenting cells (APCs) these peptides are presented on MHC class I molecules (Singh-Jasuja et al., 2000c). This process is usually referred to as 'cross-presentation' (Carbone et al., 1998; Bevan, 1976b) and is one of the key events during priming of naive T cells (Sigal et al., 1999). In addition, Gp96-mediated APC activation results in the upregulation of co-stimulatory molecules and in the release of the pro-inflammatory and T_H1 promoting cytokines tumor necrosis factor alpha (TNF-α) and interleukin-12 (IL-12) (Singh-Jasuja et al., 2000b). The release of the anti-inflammatory and T_H2 promoting cytokine IL-10 is only slightly stimulated. The resulting cytokine milieu is an important prerequisite for the triggering of a cytotoxic T cell response. So far, receptor-mediated interactions for Gp96 have only been described for professional APCs comprising DCs, macrophages and B cells. The alpha-2 macroglobulin receptor (CD91) has been identified as one receptor on a macrophage cell line (Binder et al., 2000b). Binding of Gp96 to the scavenger receptor CD36 has been shown by Panjwani et al. (Panjwani et al., 2000). More recently, physical interaction between Gp96 and Toll-like receptors (TLR) 1, 2 and 4 in the ER has been described (Randow and Seed, 2001), and we were able to show that Gp96 signals via the TLR4 pathway, at least in bone-marrow derived mouse

DCs (Vabulas et al., 2002). However, the potent ability of Gp96 to induce a pro-inflammatory milieu might be a mixed blessing for the organism in some situations. After injuries, transient inflammation during the first phase of wound healing is helpful, but chronic inflammation prevents proper tissue formation (Singer and Clark, 1999). In this scenario, the pro-inflammatory potential of Gp96 has to be regulated, otherwise wound healing might be impaired and the uncontrolled maturation of DCs would favor the development of autoimmunity. A major difference between for example virus-induced cell lysis and cell death caused by injury is the presence of platelets. These small, non-nucleated cells play a key role in hemostasis by forming a plug which physically stops blood loss. In addition, activated platelets release several mediators from their granules that contribute to proper wound healing. Platelet aggregation and activation is triggered by many different stimuli, of which the most prominent are thrombin, collagen and ADP. The latter two substances are normally not visible to platelets unless blood vessels are disrupted. From this point of view, collagen and ADP might be considered as messengers for cell death and injury with platelets as appropriate sensors. Although platelets carry MHC class I molecules on their surface, they do not themselves stimulate primary T cell responses (Gouttefangeas et al., 2000). However, thrombocytes possess several immunomodulary properties: Activated platelets have been shown to induce an inflammatory reaction on vascular endothelial cells via CD40 ligand (CD40L) which was originally identified on activated $CD4^+$ T cells (Henn et al., 1998). In addition, the maturation of DCs by fixed, activated platelet preparations was demonstrated (Gatti et al., 2000). On the other hand, upon activation thrombocytes secrete pro-inflammatory cytokines and chemokines (e.g. platelet factor 4, RANTES) as well as anti-inflammatory mediators (e.g. TGF-β) (Tykocinski et al., 1996; Power et al., 1995; Kameyoshi et al., 1992; Brindley et al., 1983; Assoian and Sporn, 1986).

In this work, we show for the first time that human platelets express receptors for the ER-resident heat shock protein Gp96. We investigated in detail the binding of Gp96 to the surface of human platelets and consequences therefrom concerning platelet function. Furthermore, we analyzed the influence of human platelets on the Gp96-mediated activation of DCs.

Materials and methods

Materials

Purified mouse Gp96 and fluorescein isothiocyanate (FITC)-labeled Gp96 were provided by Immatics Biotechnologies (Tübingen, Germany). Gp96 lots were tested endotoxin-free with a limulus amebocyte lysate assay (QCL-1000, Biowhittaker, Verviers, Belgium). Recombinant ovalbumin and FITC-labeled bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Ovalbumin was labeled with FITC according to standard protocols and purified by gel filtration on a Sephadex G-25 column (Amersham Pharmacia Biotech, Freiburg, Germany).

Platelet isolation

Nine volumes of freshly taken blood from healthy donors were mixed with one volume of 110 mM sodium citrate as anticoagulant. The citrated blood was centrifuged at 100 g for 15 min at room temperature to obtain platelet rich plasma (PRP) in the upper phase. For activation, flow cytometry and co-culture experiments with DCs, platelets were separated from plasma according to the following procedure. The PRP was overlaid on top of a 2 ml 34 % (w/v) BSA cushion and centrifuged at 550 g for 10 min. Platelets were collected from the interphase and washed twice with serum-free cell culture medium. For activation, platelet suspensions were incubated for 3 min with 0.2 U/ml thrombin at 37 °C if not stated otherwise. Thereafter, cells were fixed for 2 min on ice by the addition of paraformaldehyde (PFA) to a final concentration of 1 % (w/v). Residual PFA was removed by two additional washing steps. Non-fixed platelets were used within three hours after preparation. Contamination by other blood cells (mainly erythrocytes) in thrombocyte preparations used for DC co-cultures was always lower than 0.5 %.

Dendritic cell preparation

Human DCs were prepared from freshly drawn blood from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient (Lymphoprep; Nycomed, Oslo, Norway). The obtained cells were washed twice with phosphate-buffered saline (PBS) and resuspended in X-Vivo 15 medium (Biowhittaker) supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml

streptomycin. PBMCs were plated at a density of 6 x 10^6 cells / ml. After 2 h at 37 °C non-adherent cells were removed by washing with PBS. 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 interleukin-4 (IL-4, R&D Systems, Minneapolis, USA) and 20 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. Only immature DCs being $CD14^-$, $CD83^-$, $CD86^{low}$ were used for activation experiments. The amount of CD1a on DCs varied between different DC preparations between low expression and complete absence. This is in accordance with earlier findings (Duperrier et al., 2000). The fraction of activated DCs, as analyzed by CD83 expression was always <5 %.

Antibodies and staining for flow cytometry

The following antibodies were used for fluorescence-activated cell sorter (FACS) analysis: FITC-labeled monoclonal antibody (moAb) against CD41 and phycoerythrin(PE)-labeled CD40L-moAb (both Coulter Immunotech, Marseille, France), PE-labeled moAb specific for the α subunit of CD91 (Research Diagnostics Inc., Flanders, USA), PE-CD1a-moAb, PE-CD14-moAb, PE-CD36-moAb, PE-CD83-moAb, PE-CD86-moAb (all BD Biosciences, Heidelberg, Germany). For FACS analysis, aliquots of 1 x 10^7 platelets were incubated with labeled antibodies or proteins in FACS buffer (PBS, 1 % (w/v) BSA, 0.02 % (w/v) sodium azide) for 30 min on ice. Staining with Gp96-FITC was performed in cell culture medium supplemented with 10 % (v/v) FCS. Platelets were washed three times with FACS buffer and fixed in 1 % (w/v) PFA prior to analysis on a FACScalibur cytometer (BD Biosciences, Heidelberg, Germany). Appropriate mouse isotype controls were included to evaluate background staining. If indicated, platelets were preincubated with competitors for 30 min on ice. For competition experiments the anti-CD36 antibody clones CB38 (BD Biosciences) and SM0 (Sigma-Aldrich) and a moAb against the 85 kDa subunit of CD91 (clone 5A6, Research Diagnostics Inc., Flanders, USA) were used.

Analysis of platelet function

Freshly isolated platelets from different donors were pre-incubated for 15 min at 37 °C with different effectors. 2.5 µM ADP, being a weak inducer of platelet activation at this concentration, was used as positive control. Thereafter, thrombin was added in different concentrations (0, 1, 5 or 100 mU/ml). After 5 min of incubation at 37 °C, platelets were fixed by addition of PFA to a final concentration of 1 % (w/v). Residual PFA was removed by two washing steps with FACS buffer. Platelets were stained with a PE-labeled antibody specific for the platelet activation marker CD40L. CD40L expression levels after activation with 500 mU/ml thrombin was set as 100 %. For aggregation assays freshly prepared PRP from healthy donors who had not taken Aspirin™ for 10 days was used. Platelet concentration was adjusted to 2.5 x 10^5 / µl with platelet poor plasma which had been obtained by centrifugation (2500 g, 15 min, RT) of the remaining blood after PRP preparation. Aggregation was analyzed using a APACT 4 aggregometer (Labor GmbH, Ahrensburg, Germany). 300 µl of stirred PRP was incubated at 37 °C with 50 µg/ml Gp96, 50 µg/ml ovalbumin or buffer alone for 3 min. Thereafter, ADP, collagen or adrenaline were added and aggregation was measured for additional 5 min.

Platelet – dendritic cell co-culture

2 x 10^4 platelets / µl in 200 µl cytokine-free medium were pre-incubated for 45 min with 20 µg/ml Gp96 or 20 ng/ml LPS in a 96-well plate. Thereafter, 2 x 10^5 immature DCs were added. After 24 and 48 hours 100 µl of the cell culture supernatant were assayed for IL-10, TNF-α and IL-12 by sandwich ELISA with antibodies obtained from BD Biosciences. In addition, the maturation state of DCs was measured by determining the amount of $CD83^+$ and $CD86^{high}$ cells by flow cytometry after 48 h. To exclude endotoxin contaminations as the reason for DC activation in the Gp96 lots used, boiled Gp96 was included, as Gp96 is heat-sensitive while LPS is not (Singh-Jasuja et al., 2000b). In some experiments direct cell-to-cell contact was prevented with transwell inserts for 96-well plates (NUNC, Roskilde, Denmark). In these experiments platelets filled into the chamber of the insert were separated from DCs by a membrane (0.2 µm pore diameter) allowing only the exchange of soluble factors. Total culture volume in transwell experiments was 230 µl.

Results

Gp96-FITC binds specifically to human platelets

We first investigated whether Gp96 molecules interact with human platelets. Freshly purified platelets were fixed directly or stimulated by 0.2 U/ml thrombin for 3 min at 37 °C before fixation. To control the homogeneity of the cells used, thrombocytes were stained with an antibody against the platelet-specific marker CD41 (Figure 1 A). This marker identified a homogenous platelet preparation with little variations in fluorescence intensity between activated (filled gray) and non-activated (black line) cells. In contrast, only activated thrombocytes show staining with an antibody against CD40L. This is in line with previous findings reporting CD40L expression exclusively

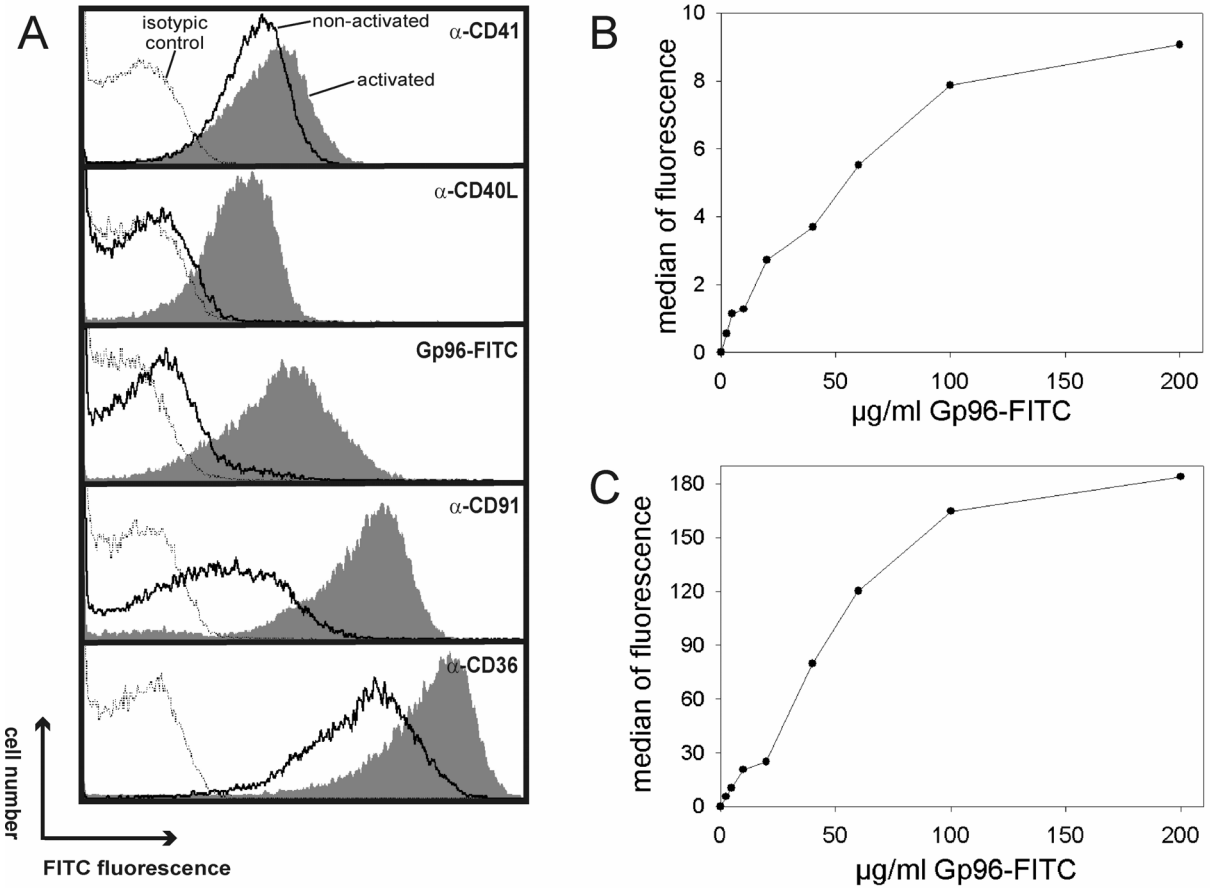

Figure 1. Gp96 binds to non-activated and thrombin-activated human platelets. Freshly prepared platelets from human blood were incubated for 3 min at 37 °C with 0.2 U/ml thrombin or without effector and fixed with paraformaldehyde. **(A)** After extensive washing platelets were stained with antibodies specific for CD41, CD40L, CD91 and CD36 or with 50 µg/ml FITC-labeled Gp96 and analyzed by flow cytometry. Isotypic controls are shown as dotted lines, stainings of non-activated platelets as solid lines and of thrombin-activated platelets in filled gray. Non-activated **(B)** or thrombin-activated **(C)** platelets were stained with different concentrations of Gp96-FITC for 30 min at 4 °C and analyzed by flow cytometry. The data shown are representative for at least four independent experiments.

on activated platelets (Henn et al., 1998). Gp96-FITC binds to non-activated and to thrombin-activated platelets (Figure 1 A, third panel). Comparing fluorescence intensities, binding of Gp96 is approximately tenfold enhanced on activated platelets. FITC-labeled ovalbumin as a control showed no binding on thrombocytes (data not shown). Figure 1 A (fourth panel) shows that CD91, which has been already identified as a Gp96 receptor, is also expressed on human platelets and strongly up-regulated after thrombin-induced activation correlating with the binding characteristics of Gp96-FITC. The scavenger receptor CD36, which has been suggested as an additional receptor for Gp96 recently (Panjwani et al., 2000), is already present at high levels on non-stimulated platelets and is only slightly up-regulated after activation (Figure 1 A, last panel). To analyze the specificity of the observed binding of Gp96 to platelets we tested whether saturation could be achieved. Non-stimulated and thrombin-activated thrombocytes showed typical saturation curves when incubated with different concentrations of Gp96-FITC (Figure 1 B,C). Maximal binding is again more than tenfold higher on activated than on non-activated platelets. In both cases, half-maximal binding is achieved approximately at 40 µg/ml Gp96-FITC which is comparable to the binding characteristics on monocytes and DCs (Singh-Jasuja et al., 2000c). Another feature of specific binding of a labeled ligand to its receptor at saturating concentrations is that it can be specifically competed by the same unlabeled ligand. Figure 2 A shows the binding of 50 µg/ml Gp96-FITC to activated platelets in the presence of different amounts of unlabeled Gp96. Increasing concentrations of competitor reduce the Gp96-FITC binding as expected for specific ligand-receptor interaction. Ovalbumin and BSA as control proteins were not able to compete Gp96-FITC binding (Figure 2 A,B). The same competition pattern was observed for non-stimulated platelets (Figure 2 B). We tried to compete Gp96-FITC binding with monoclonal antibodies against CD36 and CD91, to investigate whether these two receptors are also involved in Gp96 binding to platelets. While competition with appropriate isotypic controls did not result in reduced Gp96-FITC staining, two anti-CD36 clones and an antibody against the 85 kDa subunit of CD91 competed with Gp96 binding (Figure 2 C). An antibody against the platelet marker CD41 did not alter Gp96-FITC staining (data not shown), supporting the specificity of the observed competition. This suggests that both, CD36 and CD91, are involved in Gp96 binding to platelets.

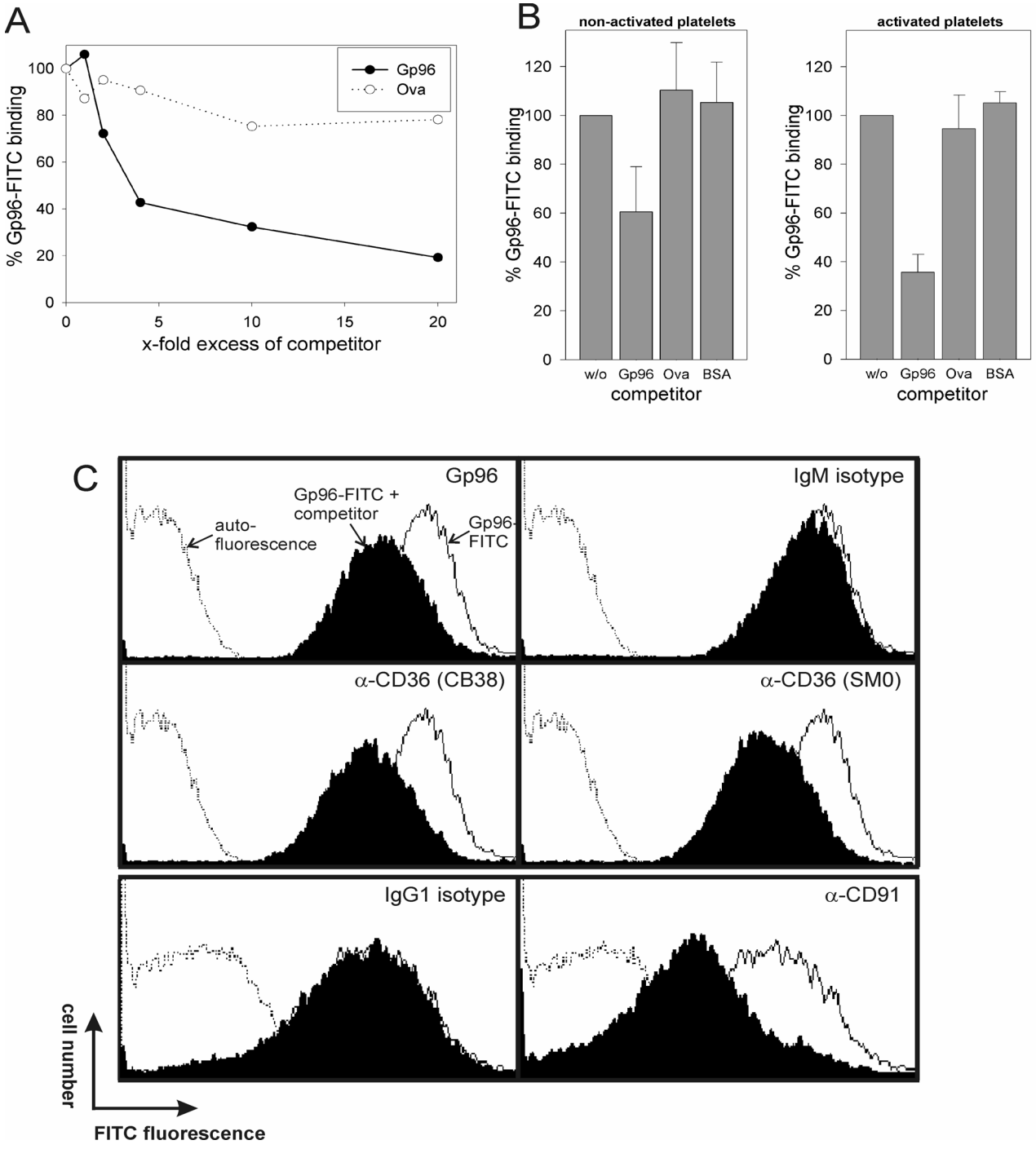

Figure 2. Gp96-FITC binding to human platelets can be specifically competed by unlabeled Gp96 and by antibodies against CD36 and CD91. Platelets were fixed and incubated with 50 µg/ml Gp96-FITC after pre-incubation with competitor for 30 min on ice. Fluorescence of stained cells was analyzed by flow cytometry. **(A)** Different amounts of unlabeled Gp96 or ovalbumin were used for competition on thrombin-activated cells. Data shown are representative for three independent experiments. **(B)** Non-stimulated and thrombin-activated platelets were stained using a 10-fold excess of the indicated competitors. Fluorescence intensity without competitor was set as 100 %. Means of triplicates are shown and error bars represent SEM. **(C)** 500 µg/ml unlabeled Gp96, isotypic controls, monoclonal antibodies against CD36 (CB38, SM0; both IgM isotype) or against the 85 kDa subunit of CD91 (IgG1 isotype) were used for competition prior to staining of thrombin-activated platelets with 50 µg/ml Gp96-FITC (concentration of competing antibodies: 50 µg/ml). Autofluorescence is shown as dotted line, uncompeted and competed staining as solid line and filled histogram, respectively. The experiments were performed in triplicates.

Gp96 does not influence platelet activation and aggregation

We tested whether Gp96 was able to interfere with thrombin induced platelet activation. Freshly isolated platelets were pre-incubated with 100 µg/ml Gp96 or ovalbumin as control for 15 min at 37 °C or were left untreated. 2.5 µM ADP, a weak inducer of platelet activation at this concentration, was included as positive control. Thereafter, thrombin was added in different concentrations. After 5 min incubation with thrombin, cells were fixed and expression of the platelet activation marker CD40L was measured by flow cytometry. Due to variations between different donors in their response to thrombin, data for individual donors are shown (Figure 3). Without thrombin only ADP-treated platelets show a slight increase in CD40L expression. Therefore, Gp96 does not trigger thrombocyte activation. Moreover, compared to control samples, Gp96 had no influence on platelet activation induced by saturating or lower concentrations of thrombin. It cannot be excluded that a weak activating effect of Gp96 was not visible in our experiments due to pre-activation during cell preparation. However, this seems very unlikely because no significant staining with anti-CD40L antibody compared to the isotypic control was observed. Thus, the influence of platelet pre-activation was negligible. The second important component of platelet function is their aggregation after injury in order to stop bleeding physically. To analyze the effect of Gp96 on thrombocyte aggregation freshly prepared PRP was incubated at 37 °C with 50 µg/ml Gp96 under continuous stirring in an aggregometer. No formation of aggregates could be observed (Figure 3B, horizontal parts of the curves before addition of other stimuli). Even after 15 min of incubation, there was no induction of aggregation by Gp96 alone (data not shown). After 3 min platelet aggregation was induced either by 2.5 or 10 µM ADP, 10 µg/ml collagen or 50 µM adrenaline. Independent of the effector used no differences in the kinetics or final degree of aggregation could be observed between Gp96-pretreated and control samples.

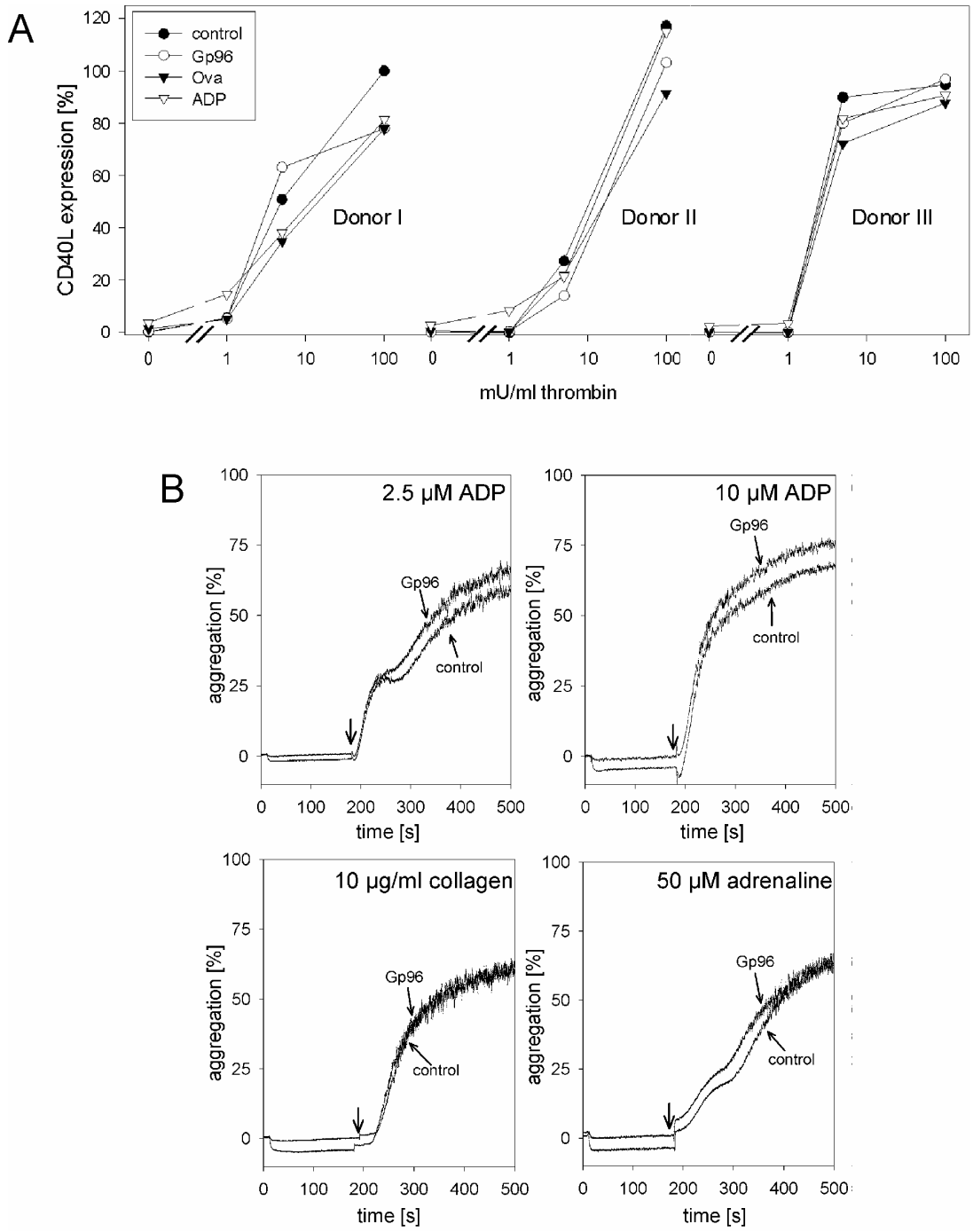

Figure 3. Gp96 does not interfere with platelet function. (A) Freshly isolated platelets from three different healthy donors were pre-incubated with 2.5 µM ADP, 100 µg/ml Gp96 or ovalbumin at 37 °C for 15 min. Thereafter, thrombin at different concentrations was added. Following an additional 5 min incubation at 37 °C platelets were fixed and activation was analyzed by flow cytometry after staining with PE-labeled anti-CD40L antibody. Due to varying levels of CD40L expression between different donors, the obtained fluorescence intensities were converted into relative activation values. The median of fluorescence after maximal stimulation with 500 mU/ml thrombin was set as 100 % activation (donor 1: 23.71; donor 2: 28.13; donor 3: 18.11). The value for non-stimulated platelets was set as 0 % (2.94 ± 0.24). **(B)** The influence of Gp96 on the aggregation of platelet rich plasma (2.5 x 10^5 cells/µl) was measured in an aggregometer. 10 s after start of measurement Gp96 was added to a final concentration of 50 µg/ml. The control samples were treated with buffer only. After 3 min 2.5µM or 10 µM ADP, 10 µg/ml collagen or 50 µM adrenaline were added and aggregation was followed for additional 320 s. Experiments were done in triplicates and repeated for three different donors.

Platelets inhibit Gp96 induced DC maturation

While no interference of Gp96 with platelet function was apparent in our experiments, we now addressed the question whether the binding of Gp96 to platelets might interfere with the immunostimulatory effect of Gp96. We concentrated on dendritic cells, as the key cell type in the initiation of an immune response. Immature DCs were cultured with Gp96 for 2 days in the presence or absence of 2 x 10^4 thrombin-activated autologous platelets per µl (concentration in blood: 1.5 x 10^5 – 4 x 10^5 per µl). After 24 h a reduced concentration of the pro-inflammatory cytokines TNF-α and IL-12 in the supernatant could be observed when platelets were present in the cell culture (Figure 4 A). No difference was visible in the secretion of the anti-inflammatory and T_H2 promoting cytokine IL-10. A similar cytokine pattern was

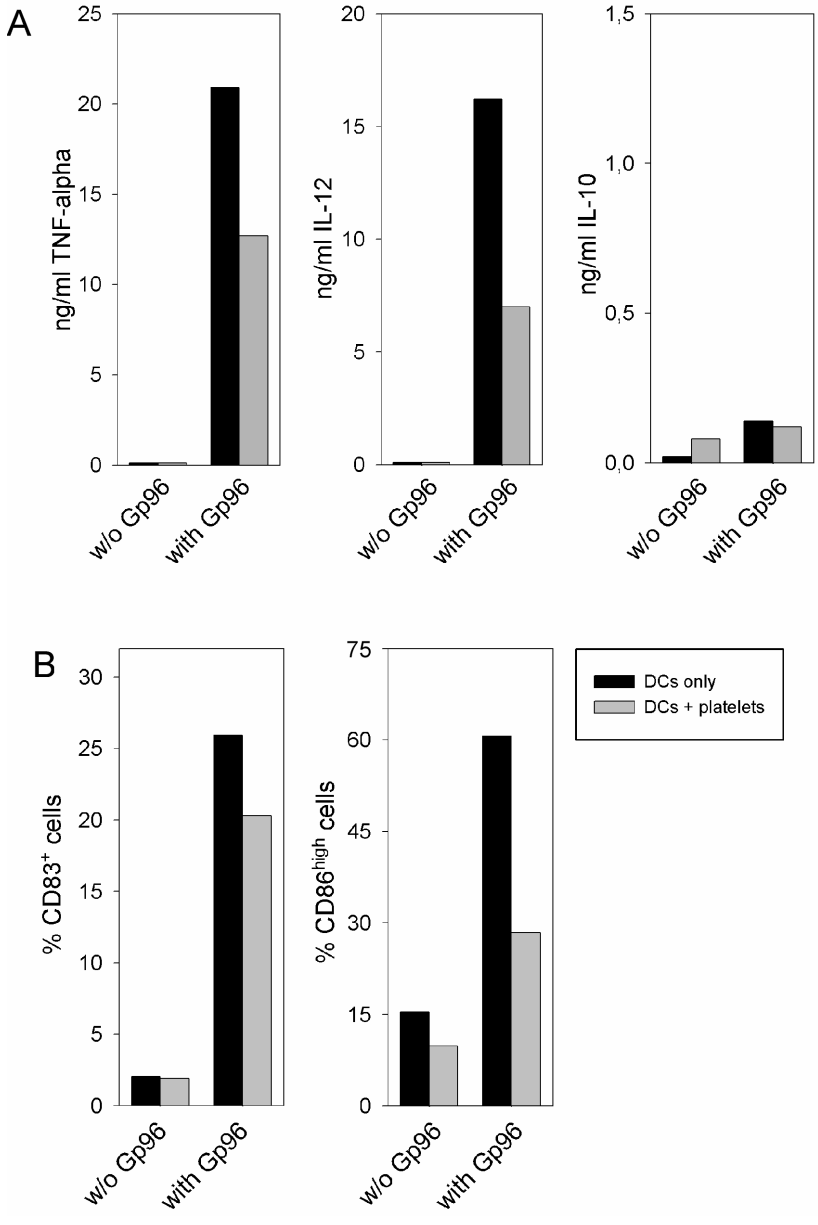

Figure 4. Autologous platelets interfere with Gp96 induced DC maturation. Human monocytes were cultured with GM-CSF and IL-4 to obtain immature DCs. After 6 days 4 x 10^6 thrombin-activated platelets from the same donor were pre-incubated with 20 µg/ml Gp96 in a 96-well plate for 45 min followed by addition of 2 x 10^5 immature DCs per well.
(A) Cytokine concentrations of IL-12, TNF-α and IL-10 in the cell culture supernatant were determined by ELISA after 24 h.
(B) The maturation of DCs was measured by determining the number of $CD83^+$ and $CD86^{high}$ cells by flow cytometry. Values for DCs without platelets are shown as black bars, DC-platelet co-cultures as light bars. Data shown are representative for three independent experiments with cells from different donors.

observed after 48 h culture, and to a lower extent when non-stimulated platelets were used (data not shown). Additionally, DCs showed reduced upregulation of the activation markers CD83 and CD86 after 48 h activation with Gp96 in the presence of platelets (Figure 4 B). While CD83 expression was only reduced by approximately 20 % in all experiments performed, the reduction in CD86 expression was greater than 50 %. The DC activating capacities of Gp96 were not caused by low amounts of endotoxin in the Gp96 preparations, since boiled Gp96 showed no stimulating effect on DCs (data not shown). In contrast, LPS-induced activation is heat-resistant as we have shown previously (Singh-Jasuja et al., 2000b). When PBMCs from the same donor were used instead of platelets, Gp96-mediated DC activation was not influenced (data not shown). Thus, the anti-inflammatory effect of platelet preparations was not due to contamination by other blood cells. Therefore, we conclude that platelets themselves reduce Gp96-induced DC activation resulting in lower secretion of pro-inflammatory cytokines and in a less pronounced differentiation to mature DCs. To analyze further the mechanism of this effect we performed transwell experiments to avoid cell-to-cell contacts between DCs and platelets. This method also enabled us to increase the platelet: DC ratio without affecting DC viability (1×10^5 platelets / µl, which is closer to the physiological thrombocyte concentration in blood). As shown in Figure 5, freshly prepared platelets were able to reduce Gp96-induced TNF-α production and PFA-fixed platelets, which had been activated with thrombin prior to fixation, completely abrogated TNF-α production without direct cell-to-cell contact (Figure 5 A). The same was observed for the upregulation of the maturation markers CD83 and CD86 (Figure 5 B). In contrast to this, LPS-induced DC maturation was not affected by the presence of platelets (Figure 5 B). The finding that fixed platelets act immunosuppressive strongly suggests that no platelet derived soluble factor can be involved in the observed effect. This favors a more passive mechanism where platelets simply compete with DCs for Gp96 binding. In line with this, the inhibitory effect of platelets was more pronounced when thrombin-activated, fixed platelets were used (Figure 5 A), while fixed non-stimulated platelets caused a lower reduction of DC activation (data not shown).

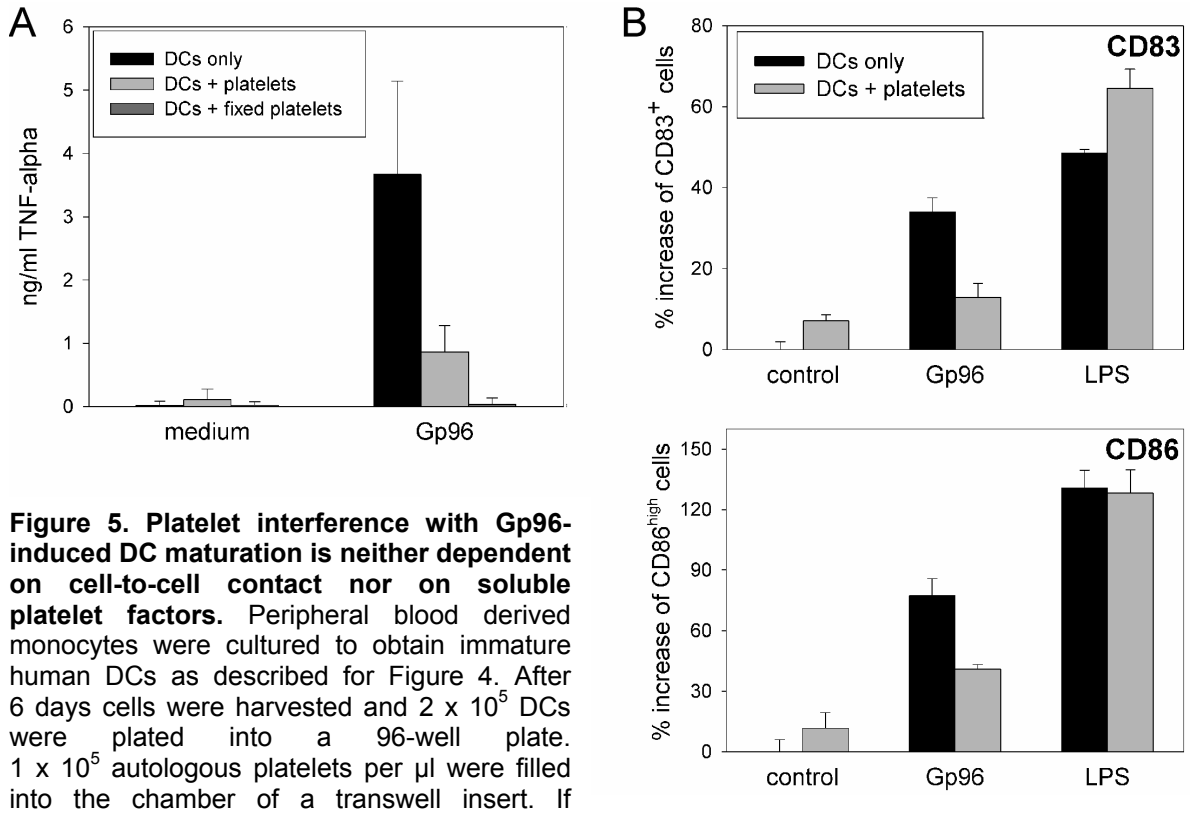

Figure 5. Platelet interference with Gp96-induced DC maturation is neither dependent on cell-to-cell contact nor on soluble platelet factors. Peripheral blood derived monocytes were cultured to obtain immature human DCs as described for Figure 4. After 6 days cells were harvested and 2 x 10^5 DCs were plated into a 96-well plate. 1 x 10^5 autologous platelets per µl were filled into the chamber of a transwell insert. If indicated, 25 µg/ml Gp96 or 1 µg/ml LPS were added to the platelets. **(A)** Freshly prepared platelets or thrombin-activated platelets which were fixed with PFA after activation were used. After 20 h transwell culture TNF-α concentration in the supernatant was determined. Mean values of duplicates are shown. The experiment was repeated twice. **(B)** DCs were left untreated or stimulated with Gp96 and LPS in the absence or presence of fresh platelets. After 48 h CD86 and CD83 expression was analyzed by flow cytometry. Percent increase of activated cell numbers compared to non-stimulated DCs in the absence of platelets is shown. The percentage of activated cells in control samples for CD83 and CD86 was 33% and 26 %, respectively. Mean values of triplicates are shown with error bars representing SEM.

Discussion

The ER-resident HSP Gp96 is released during necrotic cell death and activates dendritic cells. This feature, in combination with its ability to transfer intracellular peptides for their MHC class I restricted presentation allows Gp96, together with other HSPs like HSP70 and HSP90, to be a very efficient messaging system alerting the organism of bacterial or viral infections and possibly injury. Since HSPs are also released during mechanical tissue damage, control mechanisms have to exist that limit the HSP-mediated DC activation locally and prevent the release of pro-inflammatory cytokines in healing wounds. One mechanism has been postulated when CD91 was identified as one of the receptor molecules for Gp96. Interaction of Gp96 with CD91 in the blood stream is inhibited by the presence of α_2-macroglobulin

which binds to CD91 as well (Binder et al., 2000b). Our data reported here indicate a second, more general mechanism: The immunostimulatory capacity of Gp96 is neutralized in the blood stream by the binding of Gp96 to thrombocytes. This interaction is specific as the binding of Gp96-FITC can be inhibited by unlabeled Gp96 molecules (Figures 1 and 2). By competition experiments with monoclonal antibodies the two known receptor candidates CD91 and CD36 could be identified as Gp96 receptors on platelets. Competition experiments with both antibodies in combination have been performed (data not shown), but did not show significant additive competition. However, further investigations with more sensitive techniques are needed to reveal whether CD36 and CD91 act together in a receptor complex or whether they form two independent receptors. More interestingly, Gp96-FITC binding is approximately tenfold enhanced after the activation of thrombocytes (Figure 1). This implies an even greater neutralizing effect of platelets on the immunostimulatory capacity of Gp96, when activated during tissue damage accompanied by blood vessel disruption (Figure 6). The presence of physiological amounts of platelets reduces the Gp96-induced secretion of the pro-inflammatory cytokines TNF-α and IL-12 (Figure 4 A) and maturation of DCs as analyzed by the diminished upregulation of the activation markers CD83 and CD86 (Figure 4 B). Since the activation status of DCs correlates with their ability to stimulate T cells, the induction of immune responses will be impaired. Moreover, the immature DC phenotype and the reduced

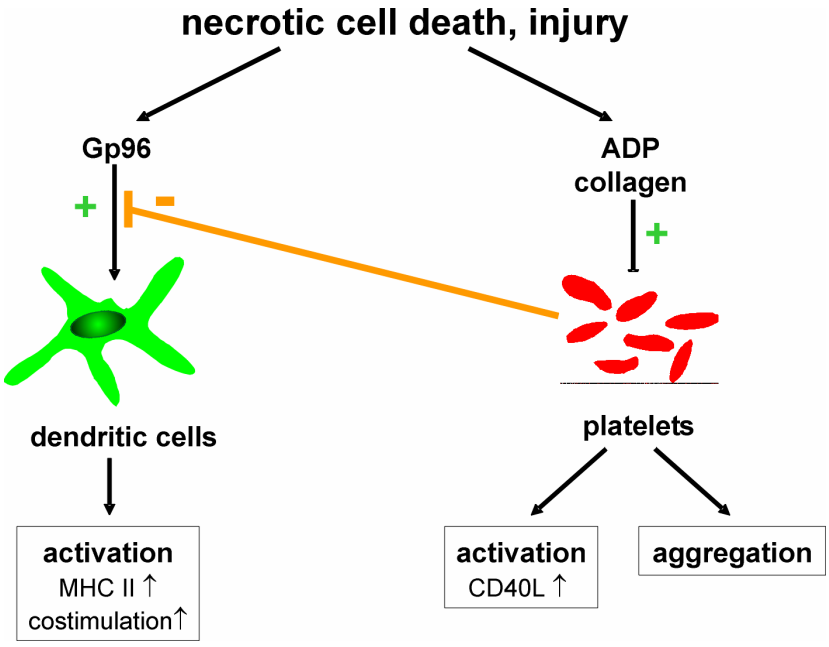

Figure 6. Interaction of two danger signal pathways. After necrosis or injury cytosolic compounds are released. Among these, HSPs such as Gp96 have been shown to activate dendritic cells. ADP and collagen trigger platelet activation and aggregation. However, these two pathways are not additive. Platelets and even stronger activated platelets interfere with Gp96-induced DC activation by competing for HSP binding.

levels of pro-inflammatory cytokines in the presence of platelets might even favor the induction of tolerance towards self antigens released during mechanical tissue damage and possibly presented by immature DCs (Dhodapkar et al., 2001; Huang et al., 2000; Sauter et al., 2000; Steinman et al., 2000). It is important to note that the binding of Gp96 to platelets does not interfere with their function. This is demonstrated by unchanged platelet activation and aggregation in the presence of Gp96 (Figure 3 A and B). The functional insensitivity of platelets towards Gp96 binding makes perfect sense because it ensures that while Gp96 is neutralized wound healing still can take place properly. The binding of Gp96 on platelets has an impact on activation of DCs by HSPs when both are present in culture. This inhibition might be the result of competition: the high number of Gp96 binding sites on platelets is likely to reduce the concentration of free heat shock protein available for binding to APCs. Since 80 % of the surface of platelets is invaginated building an open canalicular system, a great portion of bound Gp96 is not accessible at all for other cells but hidden in these invaginations. It cannot be excluded, however, that other mechanisms enhance or diminish the observed immunosuppressive effects of platelets. Thrombocytes themselves may respond to Gp96 binding in a yet unidentified way leading to an altered DC activation. But at least in our system secreted platelet factors are not involved in immunosuppression, otherwise fixed platelets would not have been able to reduce DC activation (Figure 5 A). Another possible mechanism could be that activated platelets adhere to DCs and modify their response to external stimuli through direct interaction, as has been shown for other cell types: Isolated monocytes produce various chemokines in the presence of activated platelets (Gawaz et al., 1998; Neumann et al., 1997; Weyrich et al., 1996). Autologous platelets enhance the IL-1 and TNF-α response of PBMCs after LPS stimulation (Aiura et al., 1997). For the latter effect a direct platelet adhesion to monocytic cells via P-selectin on the platelet surface has proven to be essential, although the activation signal itself is given by other, probably soluble, mediators (Weyrich et al., 1996). P-selectin also mediates the specific interaction between neutrophils and activated thrombocytes (Hamburger and McEver, 1990), but the implications on inflammatory events are controversially discussed (Gamble et al., 1990; Nagata et al., 1993). Our results suggest that, unlike monocytes, DCs are not activated by platelets themselves, at the indicated concentrations (Figure 4 B). In contrast, the inflammatory response to Gp96 is diminished. This effect is not

dependent on cell-to-cell contact as shown by transwell experiments (Figure 5). Nevertheless, a direct contact between platelets and DCs might contribute to the observed immunosuppressing effect of thrombocytes or might alter the activation state of DCs. Recently, it has been reported that Langerhans cells, a special DC subtype, are activated if co-cultured with fixed activated platelets from a heterologous source (Gatti et al., 2000). The authors attribute the observed maturation of Langerhans cells to the stimulating capacities of CD40L molecules on activated platelets (Henn et al., 1998): In our experiments using only low concentration of autologous thrombocytes, no DC activation or pro-inflammatory cytokine production could be observed without addition of Gp96 (Figure 4). Taken together, platelets might influence DC activation on at least three different levels: First, neutralization of DC activating substances (e.g. Gp96); second, release of cytokines; third, direct interaction via membrane associated molecules (e.g. CD40L/CD40). Another fact supports the relevance of the Gp96 neutralizing effect of platelets *in vivo*. The platelet:DC ratio used in our experiments is far lower than that in peripheral blood. With 0.6 % DCs among all PBMCs and up to 2 x 10^6 PBMCs per µl of blood, a maximal DC concentration of 12 cells per µl can be calculated, while the platelet concentration varies between 1.5 x 10^5 and 4 x 10^5 per µl. Thus, the platelet : DC ratio in human blood is around 10^4 : 1. In our experiments we used ratios of 20 : 1 (co-culture) or 115 : 1 (transwell experiment) and observed a significant influence on Gp96-induced DC maturation. There are no reliable data on the number of DCs and platelets in a scenario of tissue injury with blood vessel disruption, but it seems very unlikely that the platelet : DC ratio might be lower than 20 : 1. However, in wounds the relevance of the observed DC platelet phenomenon can only be finally judged when data on the ratio of this cell types in damaged tissue become available. Recently, other HSPs have been shown to interact with the Gp96 receptor CD91 (Basu et al., 2001; Binder et al., 2000c), which is also expressed on thrombocytes. It has to be evaluated whether these proteins bind to platelets in a similar way as Gp96 does. Nevertheless, the interaction of Gp96 and possibly other HSPs with thrombocytes can be expected to have important implications in the prevention of systemic inflammation and in reducing the secretion of pro-inflammatory cytokines in healing wounds.

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THE ER-RESIDENT HEAT SHOCK PROTEIN GP96 ACTIVATES DENDRITIC CELLS VIA THE TLR2/4 PATHWAY

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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 (BMDC) from mice lacking functional TLR2 and/or TLR4 molecules. We find that the Gp96-TLR2/4 interaction results in the activation of NF-κB-driven reporter genes, mitogen- and stress-activated protein kinases and induces IκBα degradation. BMDCs 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 the experiments resulting in figure 2, and contributed substantially to figures 3, 4, 6 and 7 and to the concept of this work together with Sibylla Braedel.

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Introduction

The immunogenic potential of heat shock proteins (HSPs) is a well established phenomenon, first observed by Srivastava and co-workers 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 CTL lines specific for a variety of antigens from mice immunized with HSP molecules (Blachere et al., 1993; Suto and Srivastava, 1995; Arnold et al., 1995). The HSPs that mediated this effect include the cytosolic Hsp70 and Hsp90 and the ER-resident chaperones calreticulin and Gp96 (Udono and Srivastava, 1994; Basu et al., 2001). Furthermore, Gp96 molecules have been shown to induce CTL 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 MHC expression (Suto and Srivastava, 1995; Arnold et al., 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 to understand the mechanisms contributing to the efficient induction of immune responses against the HSP-associated peptides.

A receptor responsible for the uptake of HSP-peptide complexes has been identified as the α_2-macroglobulin (α_2m)-receptor CD91, expressed on professional antigen presenting cells (APCs) (Basu et al., 2001; Binder et al., 2000b). Only receptor-mediated endocytosis has been shown to result in the representation of HSP-associated peptides, thus explaining the high efficiency of this process (Singh-Jasuja et al., 2000c). 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 MHC class II and costimulatory molecules as well as in TNF-α and IL-12 secretion (Asea et al., 2000; Basu et al., 2000; Singh-Jasuja et al., 2000b). 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 so 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 (TLR) (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 (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). The TLR signaling pathway shares most components with the IL-1 receptor (IL-1R) signaling pathway responsible for the 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 non responder 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 as well as 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 found much earlier in phylogeny, as is the TLR and IL-1R family. Therefore, it remains unclear if the HSP/TLR-pathway is solely used by the innate immune system to fight pathogens by unspecific mechanisms or if HSPs with peptide-binding ability, like Gp96, also use this mechanism to link nonspecific immunostimulatory capacities with their specific, peptide-based features and the

activation of the adaptive immune system. In this study, we analyzed the functional consequences of the interaction of Gp96 with members of the TLR family.

Methods

Reagents, antibodies and plasmids

Gp96 and FITC-labelled Gp96 were provided by Immatics Biotechnologies, Tübingen, Germany. LPS from *Salmonella minnesota* RE 595, monodansylcadaverine (MDC) and anisomycin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Phosphothioate stabilized CpG deoxyoligonucleotide 1668 (TCC-ATC-ACG-TTC-CTG-ATG-C) was purchased from TIB MOLBIOL (Berlin, Germany).

Antibodies to ERK1/2 were obtained from Upstate Biotechnology (Lake Placid, NY), other antibodies used in cell signaling studies were from New Engalnd Biolabs (Frankfurt a. M., Germany) including: anti-phospho-JNK1/2 (Thr183/Tyr185), anti JNK1/2, anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-IκB-α, anti-phospho-ERK1/2 (THR202/Tyr204), anti-ERK1/2, anti-phospho-STAT1 (Tyr701) and anti-STAT1. Antibodies for FACS analysis were purchased from BD Biosciences (Heidelberg, Germany).

The expression vectors for the N-terminus of human flag-tagged TLR2 and TLR4 were gifts from Tularik, Inc. (South San Francisco, USA). The human MD2 expression vector was kindly provided by K. Miyake (Sage Medical School, Nabeshima, Japan). The luciferase reporter driven by a synthetic enhancer harbouring 6 NF-κB binding consensus sites was a gift from P. Baeuerle (München, Germany).

Analysis of signaling pathways in RAW264.7

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

deoxyoligonucleotide 1668. Cells were then lysed in lysis buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DGTA, 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 (PMSF). Lysates were boiled in SDS-sample buffer, sonicated and centrifuged at 10,000 g for 10 min. Electrophoresis of the lysates was carried out on a 10% SDS-PAGE and Western blotting was performed using Protran nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany). The membranes were blocked in 5% skim milk solution, probed with the indicated antibodies and visualized using Renaissance Chemiluminescence Reagent (NEN, Köln, Germany). When indicated 100 µM MDC was added 20 minutes before stimulation. All following steps were performed as described above.

Luciferase-reporter assay in 293T fibroblasts

Human embryonic kidney fibroblasts 293T were cultured in DMEM (Biochrom KG) supplemented with 100 IU/ml penicillin/streptomycin (Biochrom KG) and 10% FCS (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 per electroporation by addition of empty expression vector. Cells were electroporated in a final volume of 400 µl (RPMI-25% FCS) at 200 V and 960 µF (Gene Pulser, Bio-Rad-Laboratories, Munich, Germany). Following 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 instruction.

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), C3H/HeJ / TLR2$^{-/-}$. All other mice were obtained from Charles River (Sulzfeld, Germany). For the generation of mouse bone-

marrow derived DCs, IMDM (BioWhittaker, Verviers, Belgium) was used supplemented with 2 mM L-glutamine (GibcoBRL Life Technologies, Paisley, GB), 100 IU/ml penicillin/streptomycin (GibcoBRL), 10% FCS (PAA, Linz, Austria) and 200 U/ml GM-CSF. Bone marrow cells were incubated in GM-CSF containing medium for 6-8 days and fresh medium with GM-CSF was replaced 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 mononuclear blood cells (PBMCs) were isolated using a Ficoll density gradient (Lymphoprep; Nycomed, Oslo, Norway). The obtained cells were washed twice with PBS and resuspended in X-Vivo 15 medium (Walkersville, USA) supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. PBMCs were plated at a density of $20x10^6$ cells / well. After 2 h at 37 °C non-adherent cells were removed by washing with PBS. 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 interleukin-4 (IL-4, R&D Systems, Minneapolis, USA) and 20 ng/ml GM-CSF (Leukomax, Novartis Pharma GmbH, Nürnberg, Germany). The differentiation state of DCs was examined by flow cytometry. Only immature DCs being $CD1a^+$, $CD14^-$, $CD83^-$ 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 addition of 1 µM Gp96, 1 µM heat pre-treated Gp96 or 2 µg/ml LPS. After 24 h, IL-12 (p40) IL-10 concentrations in the supernatants were measured using standard sandwich ELISA protocols. Antibodies and recombinant standards of both cytokines were obtained from Becton Dickinson Biosciences (Heidelberg, Germany). The capture antibody was bound to the ELISA plate (MaxiSorb™, Nunc, Roskilde, Denmark), the biotinylated detection antibody was revealed by streptavidin-conjugated horseradish peroxidase and ABTS 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™, Becton Dickinson). 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 the presence or absence of 250 µM MDC and 0.5% DMSO in both cases for 16 h. Supernatants were assayed for TNF-α and IL-12 by sandwich ELISA as described above.

Confocal microscopy

Human monocyte-derived dendritic cells (day 7) were seeded on cover slips. The DCs were pre-cooled and incubated for 30 min on ice with IMDM containing 10% FCS and 100 µg/ml Gp96 -FITC („pulse“) in the presence and absence of 250 µM monodansylcadaverine (MDC) and in the presence of 0.5% DMSO in both cases. The coverslips were washed twice and were fixed immediately or incubated in IMDM 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 less than 1 µm.

Results

Gp96 interacts with TLR2 and TLR4

Among the members of the TLR family, the ligands for TLR2, TLR4 and TLR9 are 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 (Basu et al., 2000; Singh-Jasuja et al., 2000b). For this purpose we incubated the human embryonic kidney fibroblast cell line 293T 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).

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$^{-/-}$/4-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.

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

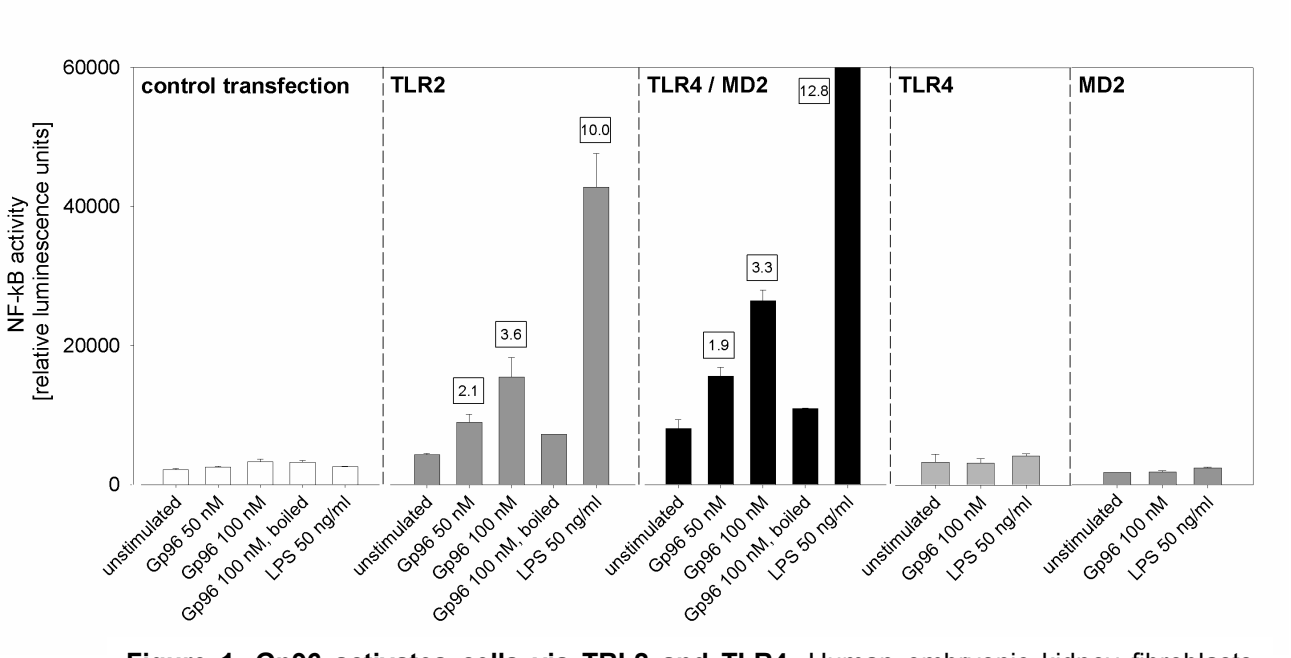

Figure 1. Gp96 activates cells via TRL2 and TLR4. Human embryonic kidney fibroblasts 293T were transiently transfected with the indicated Toll-like receptors. At the same time they were co-transfected with luciferase reporter driven by a synthetic enhancer harbouring NF-κB binding consensus sites. Luciferase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

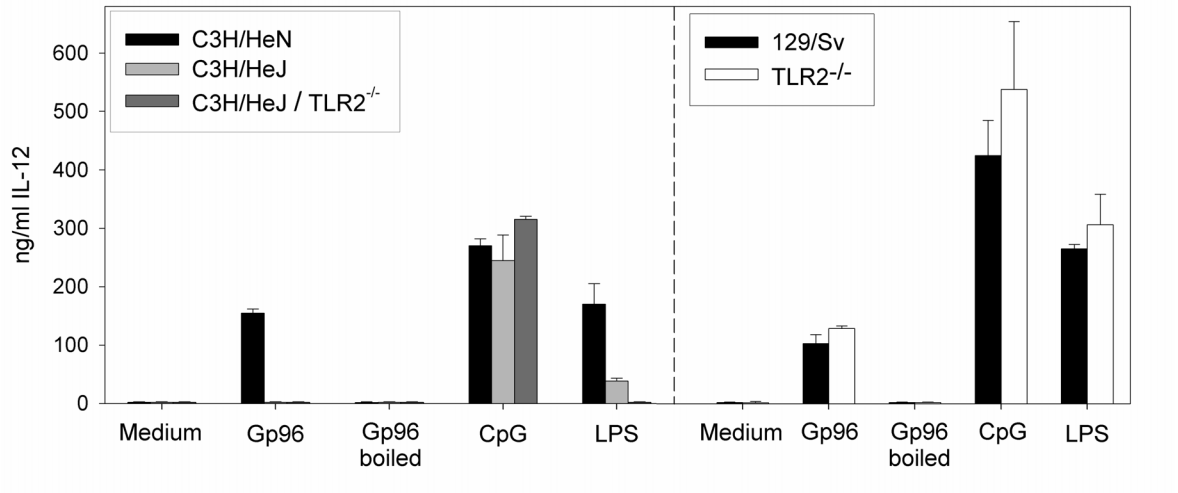

Figure 2. Gp96 -induced IL-12 production by dendritic cells is TLR4 dependent. Bone marrow-derived dendritic cells from the indicated mice strains were cultured in the presence of Gp96 (1 µM), boiled Gp96, CpG (deoxyoligonucleotide 1668, 2 µM) or LPS (2 µg/ml). After 20 h IL-12 concentration in cell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

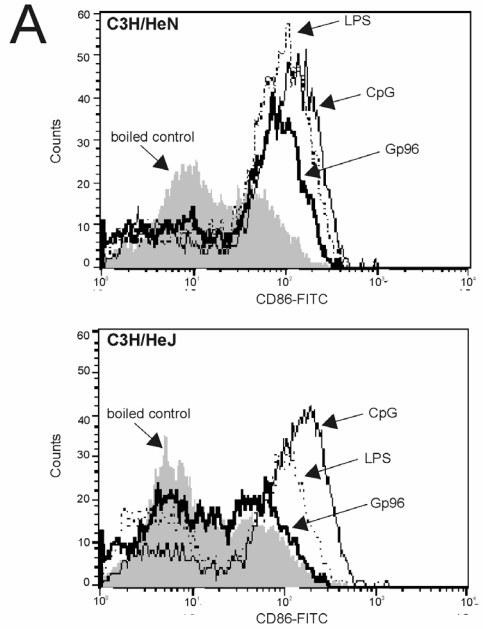

Figure 3. Gp96 mediates DC activation via TLR4 and TLR2. Bone marrow-derived dendritic cells were stimulated with Gp96, CpG or LPS (same concentrations as described in Fig. 2). After two days surface expression of CD86 was evaluated. Appropriate isotype control stainings were included. **(A)** CD86 surface expression by BMDCs from C3H/HeJ and C3H/HeN control mice is shown. C3H/HeJ derived dendritic cells do not upregulate CD86 in response to Gp96, while CpG activation is not and LPS activation 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 mean. Gp96-induced DC maturation is impaired in C3H/HeJ mice and 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.

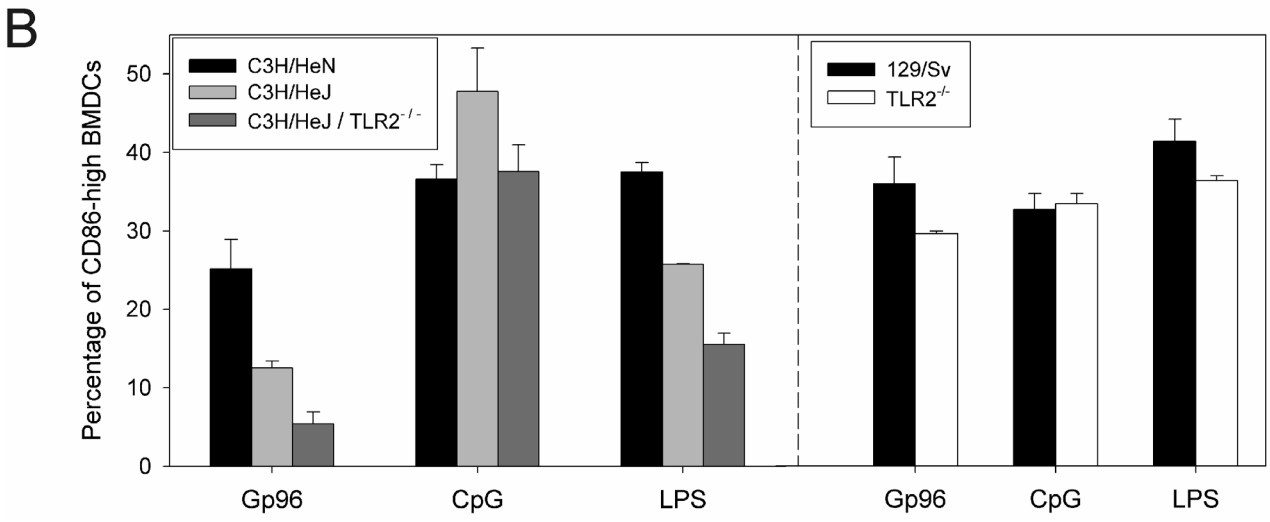

Fig. 3A shows CD86 upregulation for one C3H/HeN and one C3H/HeJ mouse, Fig. 3B represents mean values of CD86 upregulation of three individual mice. This effect was again heat-sensitive. CpG-DNA induced CD86 upregulation on all BMDCs, whereas LPS was impaired in BMDCs from C3H/HeJ and TLR2/4$^{-/-}$ mice. A minimal upregulation of CD86 molecules in C3H/HeJ compared to 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.

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 the kinases c-Jun N-terminal kinase 1/2 (JNK1/2), p38, 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 MHC 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 (Singh-Jasuja et al., 2000c; Arnold-Schild et al., 1999), 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

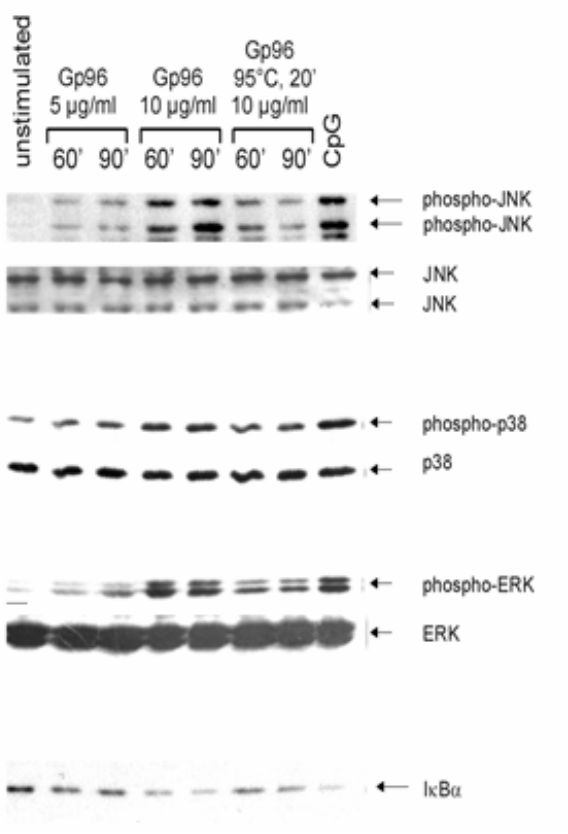

Figure 4. Gp96 triggers classical signaling cascades. RAW267.4 macrophages were incubated with different concentrations of Gp96 for 60 or 90 min and with 2 µg/ml CpG-DNA as a positive control for 30 min. Cells were lysed and the amount of phospho-JNK, phospho-p38, phospho-ERK and was determined by western 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.

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.

Indeed, MDC was found to inhibit the endocytosis of FITC-labeled Gp96 molecules by DCs (Fig.5). The presence of 0.5% DMSO alone used to dissolve MDC did not influence Gp96 uptake compared to medium control lacking DMSO (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 TNF-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 they were also affected as exemplified by the strongly reduced 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.

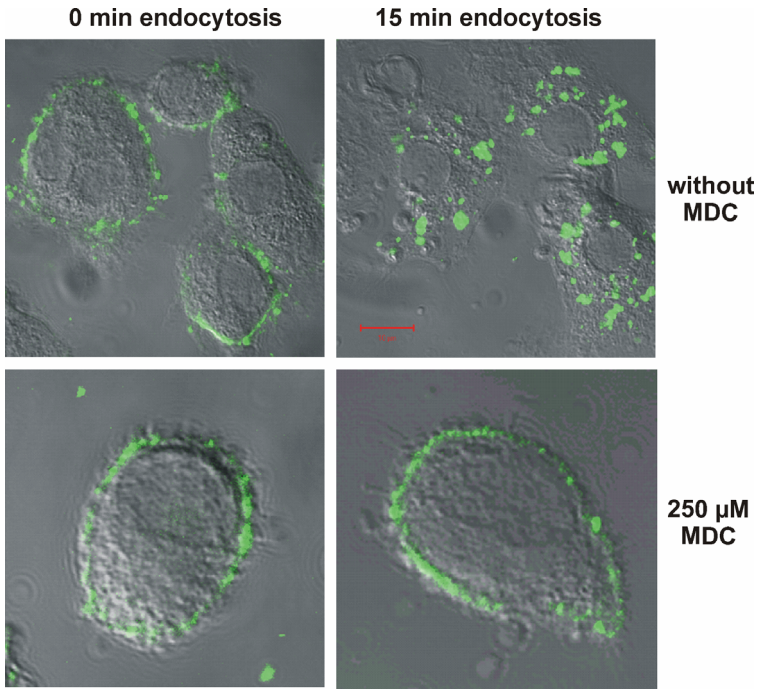

Figure 5. Monodansylcadaverine inhibits receptor-mediated endocytosis of Gp96. Human monocyte-derived, immature dendritic cells were incubated with Gp96-FITC at 4°C, washed and fixed (no endocytosis) or treated at 37°C for further 15 min (endocytosis) and fixed with methanol/acetone. The confocal micrographs 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 trypanblue staining) in 0.5% DMSO and 250 µM MDC was approximately 95% and 90%, respectively. Representative sections are shown. Identical results were obtained in at least four independent experiments.

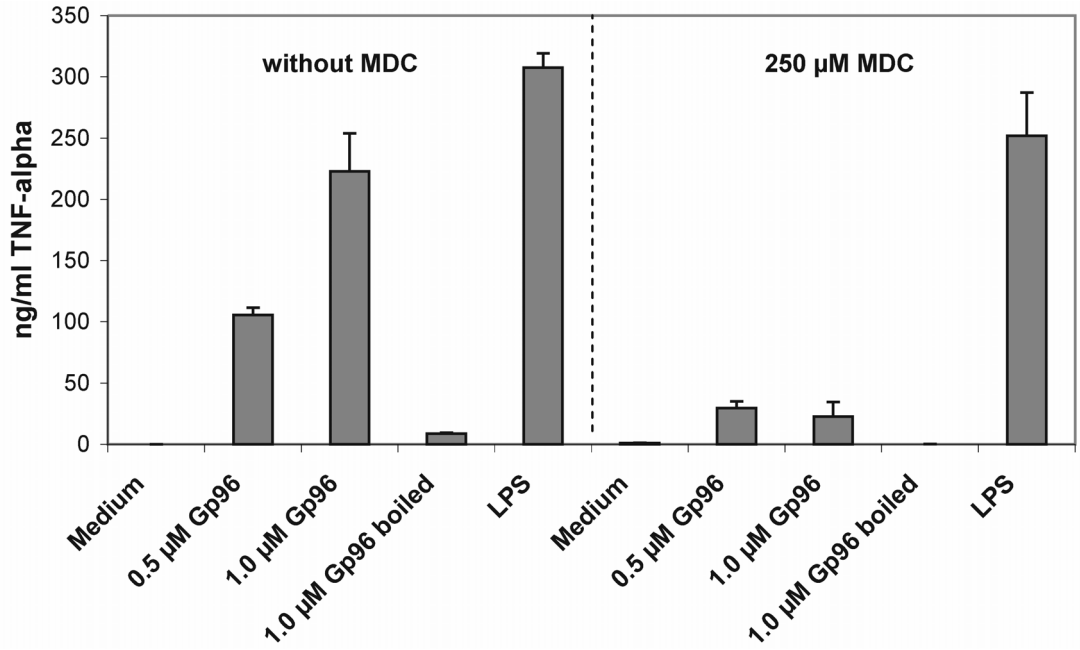

Figure 6. Receptor-mediated endocytosis is required for Gp96-mediated TNF-α secretion by DC. Human monocyte-derived, immature dendritic cells were activated by Gp96 and LPS in the presence and absence of 250 µM MDC. Boiled Gp96 was used as a control to exclude the possibility of endotoxin contaminations in Gp96 preparation. MDC specifically inhibited Gp96-mediated DC activation while 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 SEM.

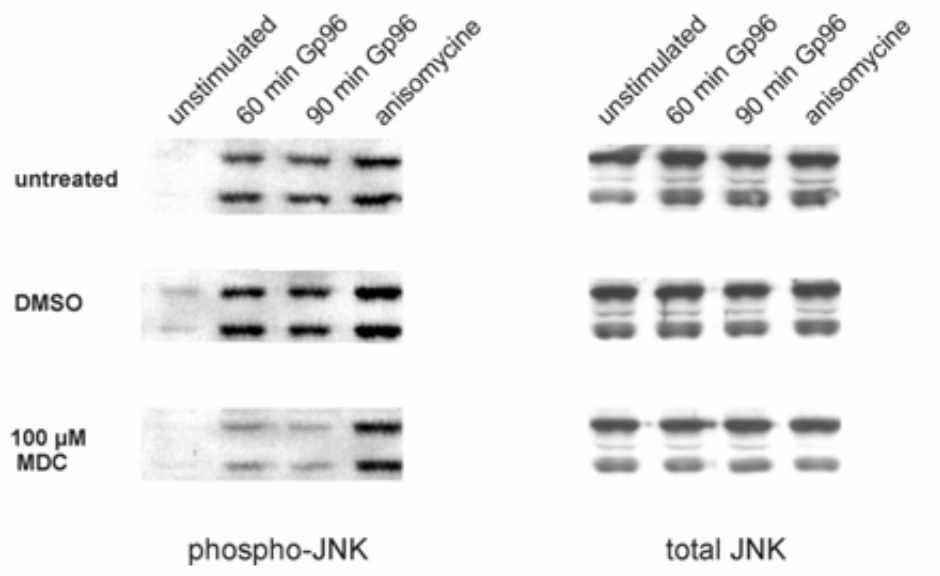

Figure 7. Endocytosis of Gp96 is required for activation of JNK. RAW267.4 macrophages were stimulated with different concentrations of Gp96 for 60 or 90 min or with 0.2 µg anisomycine for 10 min. Cells were incubated either in standard medium, in medium containing 100 µM MDC or in medium containing DMSO matching the amount used for solvation of MDC. The assay of JNK was performed as described for 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 ER-resident chaperone Gp96. We find that TLR2 and TLR4 mediate NF-κB driven luciferase induction (Fig. 1) and mainly TLR4, but to small extent also TLR2, are responsible for the upregulation of CD86 and the secretion of IL-12 and TNF-α (Fig. 2 and Fig. 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 (Basu et al., 2000; Singh-Jasuja et al., 2000b). 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 activation of the NF-κB pathway and activation of the stress-activated protein kinases (SAPK) 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 (Fig. 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 PAMP of HSPs that allows their specific interaction with TLR2 and TLR4 is unknown. We are currently investigating the possibility if 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). We observe here the same effect for the new TLR2 and TLR4 ligand, Gp96. 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., 2000c) which 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 LBP on the cell surface (reviewed in Golenbock and Fenton, 2001), Gp96 -mediated DC activation might depend on the presence of CD91 molecules which 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. While 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$^{-/-}$/4 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 only induce 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 ER 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 PAMPs 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-reacts with TLRs (Vabulas et al., 2001). Our results now demonstrate that the exclusive association of TLRs with PAMPs 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 example of how the innate and adaptive immune system can be stimulated simultaneously by the same molecule which 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., 2000c). The unique combination of both features now allows the MHC-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.

7

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RELEVANCE OF TOLL-LIKE RECEPTOR SIGNALING FOR PRIMING OF CYTOTOXIC T-LYMPHOCYTES *IN VIVO*

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Manuscript submitted.

The efficient priming of cytotoxic T-lymphocytes (CTLs) requires the interaction with antigen-presenting cells (APCs). APCs themselves have to undergo maturation processes to express sufficient levels of co-stimulatory molecules and to 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. For CTL priming, the redundant and complementary roles of these two activation pathways have not yet been investigated. Using mice deficient in the TLR-associated adaptor protein myeloid differentiation factor 88 (MyD88), and $CD4^+$ T cell-depleted mice, we now show that the two signaling pathways can compensate each other during the priming of virus-specific CTLs. In the case of minor H-specific CTL induction, however, both pathways are necessary. Thus, the requirement for MyD88- or $CD4^+$ T cell-mediated activation signals for CTL priming depends on the nature of the antigen.

The author of this thesis performed all experiments shown in this chapter with the exception of figure 1 C,D, figure 3 B and figure 5 A,B. Figures 1 C,D and 5 A,B resulted from the work of Sibylla Braedel and figure 3 B from the work of Daniele Arnold-Schild. Sibylla Braedel contributed also to the concept of this work and to figure 5 C.

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Introduction

Cytotoxic T cells play a major part in the adaptive immune response against pathogens and tumor cells. Their activation requires the interaction with professional APCs, such as dendritic cells (DCs). For efficient priming of CTL responses, DCs have to develop from an immature to a mature state that is characterized by upregulation of costimulatory molecules and production of pro-inflammatory cytokines (Banchereau and Steinman, 1998). This maturation process is initiated by various stimuli, including cellular interactions with $CD4^+$ T cells via CD40/CD40L (Cella et al., 1996) or small pathogen-derived molecules recognized as danger signals by members of the TLR family (Bendelac and Medzhitov, 2002). In mammals there are at least ten different TLRs, each recognizing a distinct subset of conserved microbial products called PAMPs (pathogen associated molecular patterns) (Medzhitov, 2001). Ligation of TLRs leads to the recruitment of MyD88, a cytosolic adaptor protein, and triggers the activation of Jun, Fos and NF-κB transcription factors via a signal kinase cascade (Medzhitov, 2001; Medzhitov et al., 1998; Wesche et al., 1997). MyD88 is an essential component of the signaling pathways of TLRs (Kawai et al., 1999) and also of the receptors for IL-1 and IL-18 (Adachi et al., 1998), although additional adaptor proteins have been described. TIRAP (Toll-IL-1 receptor domain-containing adaptor protein) is involved in TLR4 and TLR2 signaling (Horng et al., 2001; Fitzgerald et al., 2001; Yamamoto et al., 2002a; Horng et al., 2002). The recently discovered adapter TRIF (TIR domain-containing adapter inducing IFN-β) mediates most probably MyD88-independent signaling via TLR3 (Yamamoto et al., 2002b). However, bone-marrow derived dendritic cells (BMDCs) from MyD88-deficient mice reveal a greatly reduced cytokine secretion in response to most PAMPs or non at all (Kawai et al., 1999), underlining the importance of MyD88 in TLR signaling.

The priming of CTLs against viruses or 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 the maturation of dendritic cells. Numerous reports describing DC maturation by the interaction of PAMPs with TLRs, and the recent finding that type 1 T helper (T_H1) cell 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 recently that double-stranded (ds) RNA is able to trigger DC activation via TLR3 (Alexopoulou et al., 2001). This might provide an explanation for the helper T cell independence of CTL activation against RNA viruses like influenza.

However, the involvement of MyD88-mediated signals in CTL priming has not yet been directly assessed. Therefore, we investigated the induction of cytotoxic T cell responses using MyD88$^{-/-}$ mice. Our results indicate that TLR- and CD4^{+} T cell-mediated signals compensate and cooperate in the induction of CTL responses in an antigen-dependent manner. For strong antigens, such as viruses, either MyD88- or CD4^{+} T cell-dependent signals are required and each can compensate for the lack of the other. Weak antigens on the other hand, such as minor H antigens, demand the contribution of both signaling pathways. Therefore, minor H responses, which provide a model for tumor rejection, depend on activation of the innate immune system by danger signals. This might provide an explanation for the absence of an immune response against many tumors.

Material 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). Lipopolysaccharide (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 synthezised 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).

Immunizations and ^{51}Cr release assay

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 assays were 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.

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 ABTS 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 oligonucleotides 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.

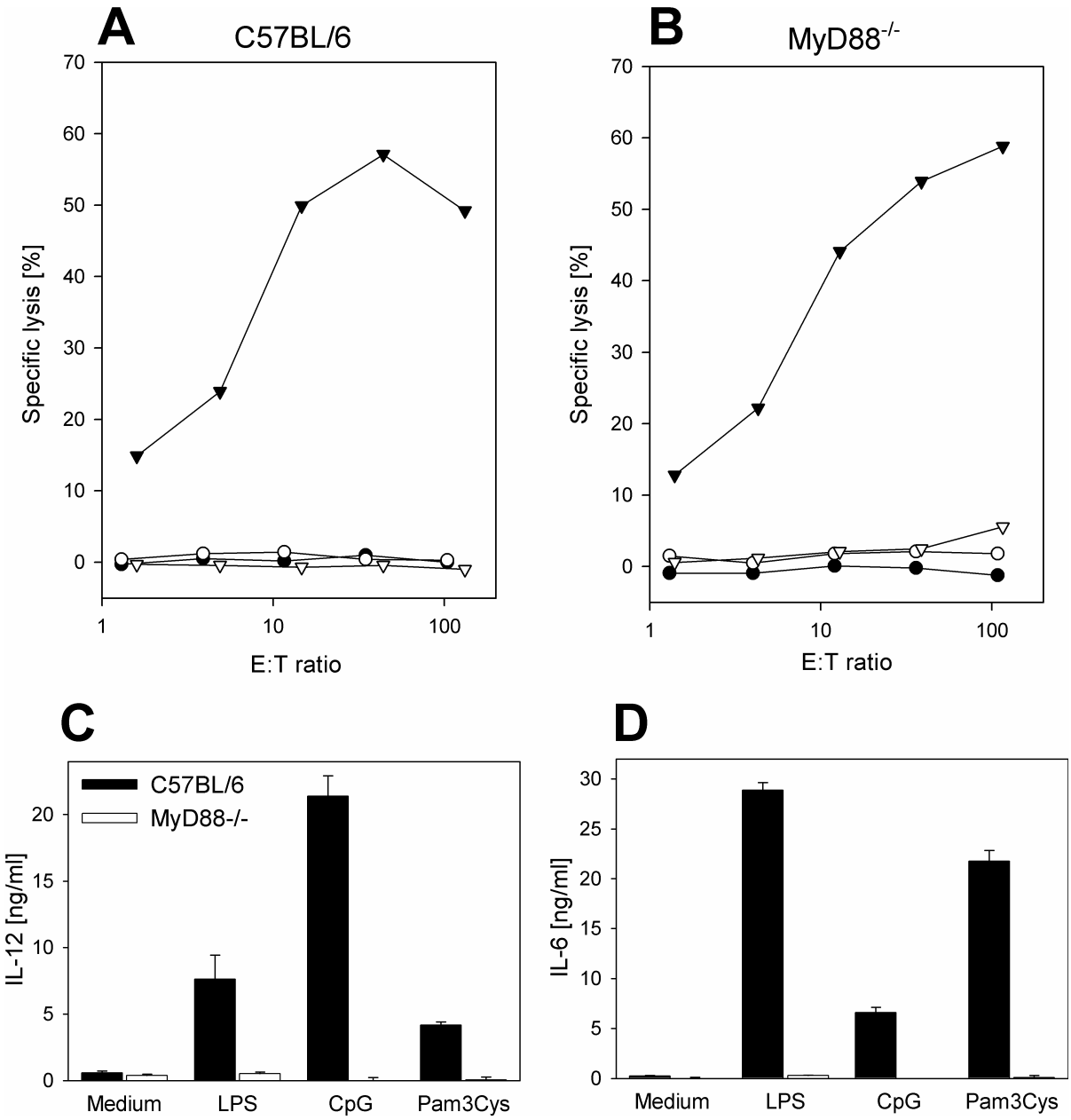

Figure 1. CTL activity against human influenza virus is not affected in MyD88$^{-/-}$ mice. **(A,B)** C57BL/6 (A) or MyD88$^{-/-}$ mice (B) were injected i.p. with 500 HAU of human influenza virus (▼). Control mice were left untreated (●). After 9 days splenocytes were stimulated *in vitro* with the 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, IL12 (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.

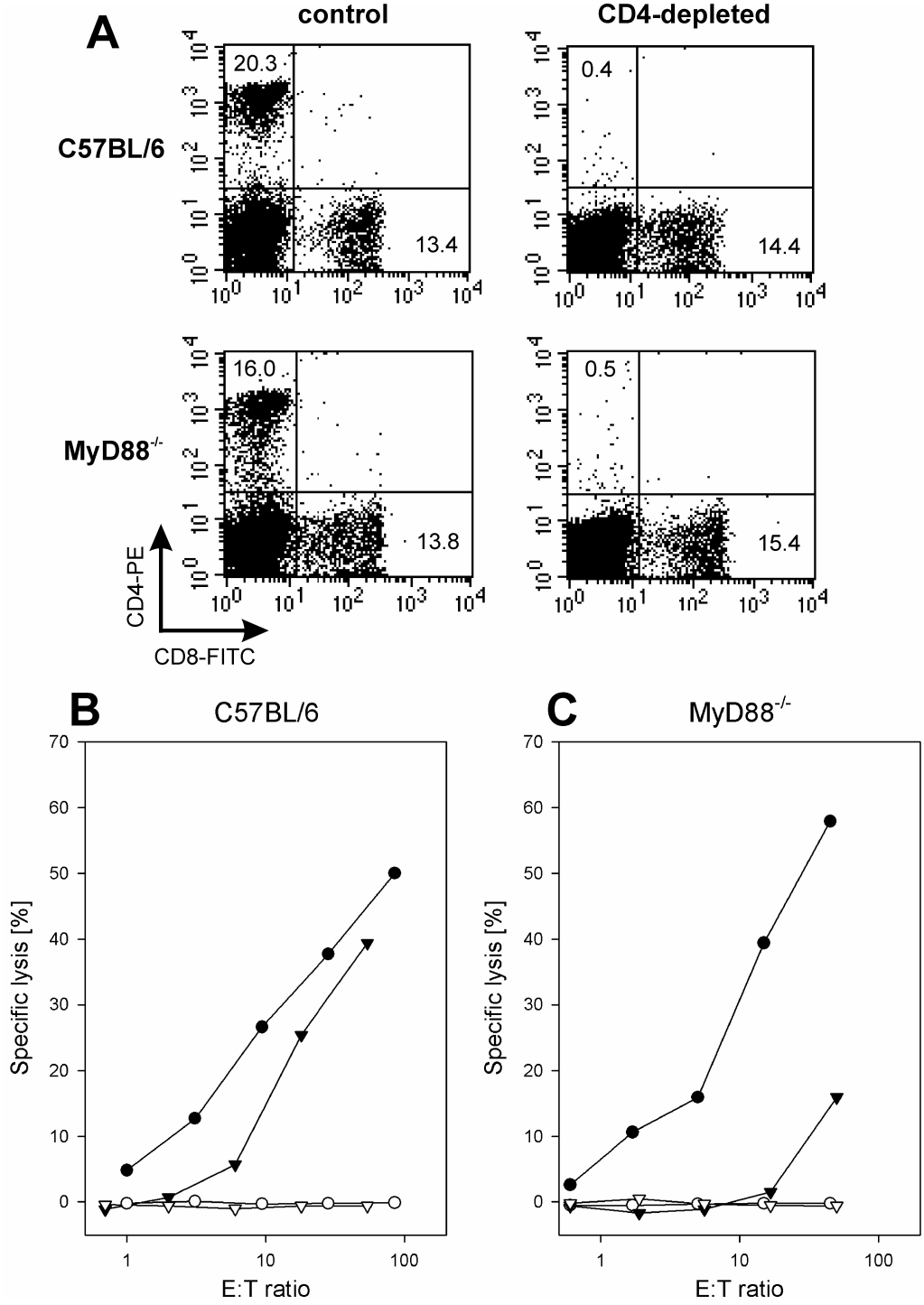

Figure 2. CTL response against human influenza virus in MyD88$^{-/-}$ mice is dependent on CD4$^+$ T helper cells. C57BL/6 and MyD88$^{-/-}$ mice were immunized with human influenza virus and splenocytes were stimulated with peptide as described for Fig. 1. CD4$^+$ T cells had been depleted in half of 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; Tripp et al., 1995; Rahemtulla et al., 1991). 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.

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

To investigate the role of MyD88-mediated stimuli in a system of weaker antigenicity, we studied the induction of minor H-specific CTLs in $MyD88^{-/-}$ mice. $CD4^+$ T cells have been shown to be necessary for the generation of minor H-specific CTLs (Schild et al., 1987). As shown in Fig. 3a,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. 3a,c) mount comparable minor H CTL responses. This could be because in this situation, CTL priming is independent of host APCs and mediated directly by the interaction of naïve T cells with donor APCs not defective in the MyD88 pathway. To investigate this possibility, we injected cells from C57BL/6 or $MyD88^{-/-}$ mice in 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. 4a), we found that the immunization using spleen cells from $MyD88^{-/-}$ mice was unable to prime CTLs (Fig. 4b). However, cells from $MyD88^{-/-}$ as well as C57BL/6 mice were able to induce the activation of alloreactive CTLs (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 that present the antigen.

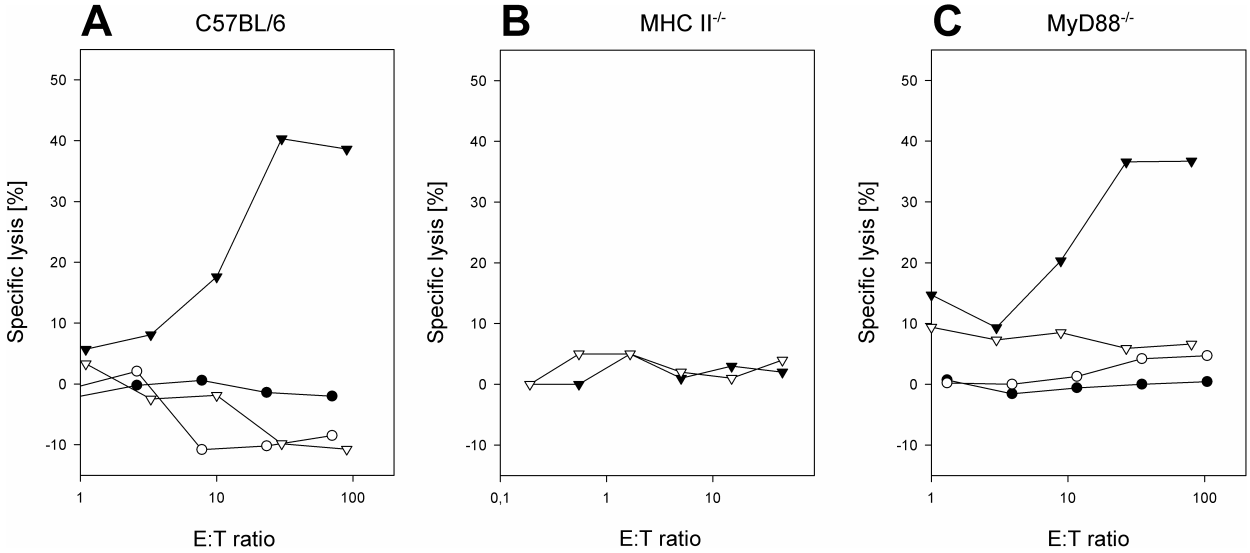

Figure 3. CTL responses against BALB minor H antigens require CD4$^+$ T helper cells, but not MyD88-dependent signals in host APCs. 1x10^7 irradiated BALB.B splenocytes were injected i.p. into C57BL/6 **(A)**, MHC class II-deficient **(B)** or MyD88$^{-/-}$ mice **(C)**. 9 days later, mice were sacrificed 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.

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 might 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. 5a) and IL-12 (data not shown) and by the upregulation of CD86 molecules (Fig. 5b), 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). When the poly(I:C) activated BMDCs from MyD88$^{-/-}$ mice analyzed in Fig. 5a and 5b were injected 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. 5c).

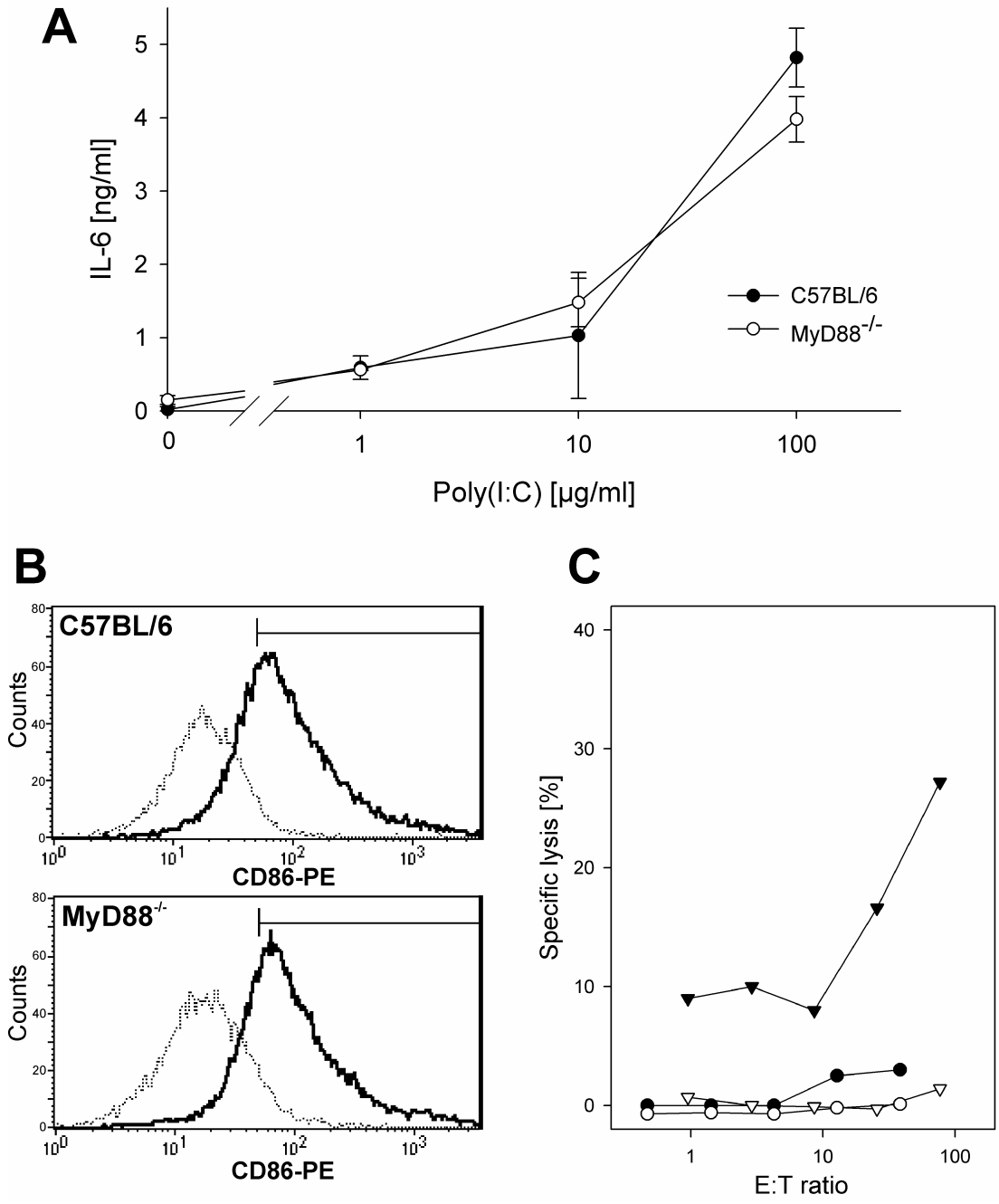

Figure 5. MyD88-dependent signals during CTL priming against minor H antigens can be compensated by MyD88-independent activation of donor APCs. (A,B) Immature BMDCs from C57BL/6 (●) and MyD88-deficient (○) mice were stimulated with the indicated concentrations of poly(I:C). **(A)** After 20 h IL-6 concentrations 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 is CTL priming severely impaired but either one of the two is nevertheless able to maintain CTL activation. This suggests that during a viral challenge sufficient activation of APCs can be achieved along two distinct pathways: either by the engagement of CD40 by CD40L on activated helper T cells or via the MyD88 pathway, probably triggered by PAMP - TLR interaction.

A different situation is observed for the induction of CTLs against minor H antigens. Here, both $CD4^+$ T cells and MyD88-mediated signals in the antigen-bearing donor cells are required (Fig. 3 and Fig. 4). 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. 5c). The reason for this is most likely the TLR3-dependent but MyD88-independent upregulation of CD86 molecules by poly(I:C) (Fig. 5b), as reported previously (Alexopoulou et al., 2001). However, in contrast to earlier reports, we also observed the secretion of pro-inflammatory cytokines by poly(I:C) stimulated BMDCs from $MyD88^{-/-}$ mice to levels comparable with that of wildtype BMDCs (IL-6, Fig. 5a; IL-12, data not shown), whereas other known MyD88-dependent stimuli, such as Pam_3Cys or CpG oligonucleotides, failed to induce the activation of BMDCs from $MyD88^{-/-}$ mice both in earlier experiments (Fig. 1) and in the same analysis (data not shown). This discrepancy still demands an explanation, but LPS contamination of the used poly(I:C) preparation, which was tested endotoxin-free by limulus amoebocyte lysate assay, can be ruled out. Moreover, LPS evokes a strongly reduced cytokine response in $MyD88^{-/-}$ derived cells compared to wildtype cells (Kawai et al., 1999).

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 (Schoenberger et al., 1998a; Ridge et al., 1998; Bennett et al., 1998). In addition, CD40-independent DC sensitization and direct lymphokine-dependent $CD4^+$ - $CD8^+$ T cell communication might contribute to the priming of CTLs (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., 2001; Kadowaki et al., 2001). Other virus-derived PAMPs might also contribute to TLR-mediated APC activation. 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).

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 can only be speculated on. One possible candidate that is able to connect both pathways might be 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 (McArthur and Raulet, 1993; Lichtman et al., 1988). 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). But for T_H1 responses at least, IL-1β and IL-18 are not generally required because mice deficient in caspase-1 which cleaves the IL-1β and IL-18 precursors into their biologically active forms (Fantuzzi and Dinarello, 1999), show a normal antigen-specific T_H1 response (Schnare et al., 2001). Alternatively, endogenous danger signals that are not pathogen-derived might activate the immune system in a TLR- and therefore MyD88-dependent manner during minor H specific CTL priming. Heat-shock proteins released during cell death are possible candidates for this. 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., 2002). The contribution of the recently discovered additional adaptor proteins TIRAP and TRIF (Horng et al., 2001; Fitzgerald et al., 2001; Yamamoto et al., 2002b) to the activation signals required for the development of CTLs is probably of minor importance because it is

not able to compensate for the MyD88-dependent signals in MyD88$^{-/-}$ APCs during priming of minor H specific CTLs in BALB.B mice (Fig. 3c) or influenza-specific CTLs in MyD88$^{-/-}$ mice after CD4^{+} T cell depletion (Fig. 2c).

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. 3C): During the induction of minor H specific CTLs in an H2-matched combination (C57BL/6 or MyD88$^{-/-}$ cells injected into BALB.B mice or vice versa), transfer of antigens from donor cells to host APCs (cross presentation) does not take place. Instead, minor H antigens are recognized exclusively on donor cells. 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 would be that 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.

T_H1 immune responses have been reported to be compromised in MyD88-deficient mice (Schnare et al., 2001; Kaisho et al., 2002). Here, we observe a similar situation for the induction of minor H-specific CTL responses. Apparently, the induction of CTL responses against weak antigens, such as minor H, requires the contribution of both CD4^{+} T cell- and MyD88-mediated stimuli. A similar scenario can be expected for the priming of CTLs against other cell-associated antigens, such as tumor antigens. The absence of PAMPs and danger signals in many tumors might contribute to the lack of an efficient antitumor response. However, in the case of CTL priming against strong antigens, as shown here for the influenza virus, MyD88-dependent stimuli are not required as long as CD4^{+} T cells are present. Thus, our experiments provide evidence for an antigen-dependent requirement of MyD88 signaling for the induction of primary CTL responses.

DISCUSSION AND OUTLOOK

The vaccination with HSP:peptide complexes is very efficient in terms of the amount of antigen required (Udono and Srivastava, 1994). However, it took several years before major insights were gained into the immunobiology of HSPs (Figure 1). The low concentration of antigens sufficient for the induction of an immune response can be explained by the receptor-mediated uptake of the HSP:peptide complexes (chapter 3). This process is quite well understood and CD91 has been identified as the receptor that mediates the uptake of several HSPs, such as Hsp70, Gp96, Hsp90 and calreticulin (Basu et al., 2001; Binder et al., 2000b). But other receptors may also be involved. There is some evidence that the scavenger receptor CD36 interacts with Gp96 (Panjwani et al., 2000, chapter 5).

The second prerequisite for the successful vaccination with HSP-associated peptides is the adjuvants function of HSPs (chapter 4). Gp96 (Basu et al., 2000; Singh-Jasuja et al., 2000c), Hsp70 (Asea et al., 2000) and Hsp60 (Kol et al., 1998; Chen et al., 1999) have been shown to activate APCs. The maturation signal given is mediated

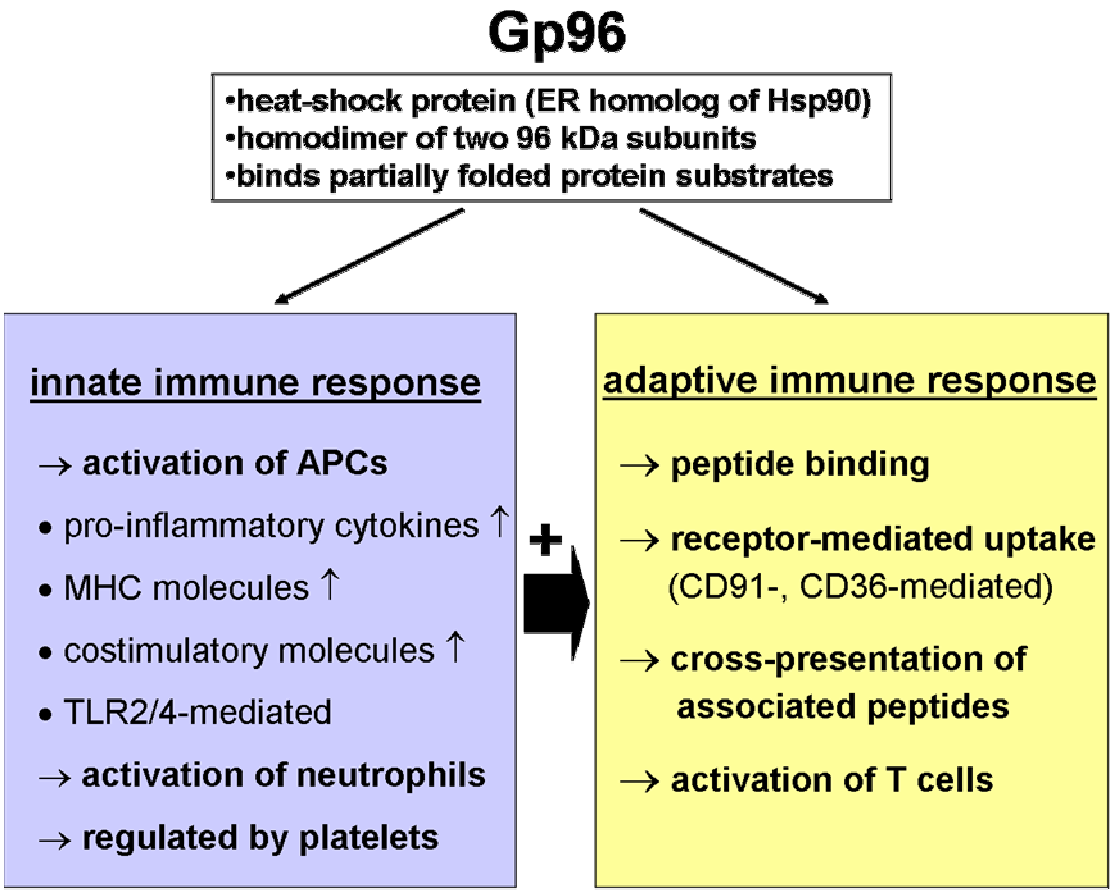

Figure 1. Main biological functions of Gp96. White box: General features. Blue box: Interactions with the innate immune system. Yellow box: Involvment in the induction of specific immune responses.

by TLR4 and TLR2 (chapter 6). Activated APCs secret pro-inflammatory cytokines and express elevated levels of costimulatory molecules. This second signal is required to trigger the development of naïve T cells into effector T cells. Without costimulation, TCR engagement leads to anergy of the T cell. Therefore, delivery of antigen by HSPs is not sufficient and their adjuvanticity is crucial for the induction of immunity. The timing of these two functions is perfect: The activating signal is not given until the HSP:peptide complex is internalized (chapter 6), and at this point the antigen has already entered the APC. Too early maturation of e.g. Langerhans cells in the skin would trigger their migration towards lymph nodes before antigen uptake is terminated. How the receptors responsible for endocytosis (CD91, CD36) contribute to HSP-induced maturation signals via TLRs is still unknown.

The activating stimuli provided by HSPs have been controversely discussed. It is a severe concern that these observations are due to minor endotoxin contaminations in the protein preparations. But there is growing evidence, that for Gp96 at least the protein itself is responsible for APC maturation: First, Gp96-induced, but not endotoxin-induced DC maturation is heat-sensitive and polymyxin-insensitive (Braedel et al., unpublished observations). Second, cells transfected with membrane-bound Gp96 (Zheng et al., 2001; Hilf et al., unpublished obervations) and cells that secrete Gp96 (Baker-LePain et al., 2002) activate APCs in the absence of bacterial contaminations. Third, the threshhold for DC activation is independent of the observed endotoxin content of the protein preparations and achieves saturation at around 50 µg/ml Gp96 (Hilf et al., unpublished observations). Interestingly, this amount equals the concentration that favours complete dimerization of the cytosolic equivalent Hsp90 (Richter et al., 2001). Therefore, the activating capacities of Gp96 might depend on the protein dimer.

Of course, the fact that endotoxins and HSPs use the same receptors to mediate their maturation signal tempts speculations about endotoxin contaminations and it is still possible that Gp96 potentiates the effect of very low LPS concentrations by binding and delivering it via receptor-mediated uptake to cells. But on the other hand, several different exogenous PAMPs from very different pathogens have been shown to bind to e.g. TLR2 (see chapter 1, table 1). Therefore and in line with Polly Matzinger's danger hypothesis (Matzinger, 2002), it is not surprising that several and - in the case of HSPs - also endogenous danger signals bind to one single TLR. The

message given by LPS and released HSPs is the same: Something is wrong and adaptive and innate immunity have to be alert.

It is a matter of speculation whether the *in vivo* concentrations of Gp96 and other endogenous heat shock proteins after necrotic cell death are high enough to elicit APC activation. However, measurable concentrations of Gp96 have been detected in the supernatant of cells infected with a lytic virus (Berwin et al., 2001) and in the wound fluid of operated patients (S. Herter, unpublished observations). Moreover, Gp96 is the most abundant ER protein. Release of the ER content after necrosis might therefore lead to a very high local concentration of this HSP. Taking into account that all endogenous danger signals released after necrotic cell death may act synergistically it is very likely that sufficient concentrations for APC activation can occur *in vivo*.

If cell death leads to APC activating concentrations of danger signals, this signal has to be controlled in situations in which sustained inflammation and potential autoimmune responses have to be prevented. Systemic release of danger signals e.g. in the blood might lead to excessive secretion of pro-inflammatory cytokines and therefore to a life-threatening condition similar to the endotoxin shock after systemic infection with bacteria. The effects of Gp96 in the serum are neutralized by excessive amounts of the CD91 ligand, α_2 macroglobulin, in the blood (Binder et al., 2000b) and by the huge number of Gp96-binding platelets (chapter 5). The control of Gp96 action by platelets might be even more effective after injury. In this scenario, activated platelets express 10-fold higher amounts of Gp96 receptors and are able to interfere with Gp96-induced DC activation.

Platelets were the first non-APCs shown to interact with HSPs. Meanwhile, neutrophils and monocytes have also been identified as targets of Gp96 (Radsak et al., 2002). Especially neutrophils comprise the major fraction of blood leukocytes and are an important part of the innate immune response. The binding of Gp96 to them triggers IL-8 release and enhances phagocytic activity. Thus, another link between Gp96 and innate immunity has been identified that is not mediated by APCs. All cell types that have been shown to interact with HSPs are shown in figure 2.

What is still missing in our understanding of HSP-mediated immunity? Although we have now a very precise knowledge about the path of Gp96 to the cell and the receptors involved in its uptake, we still do not understand the processes inside the

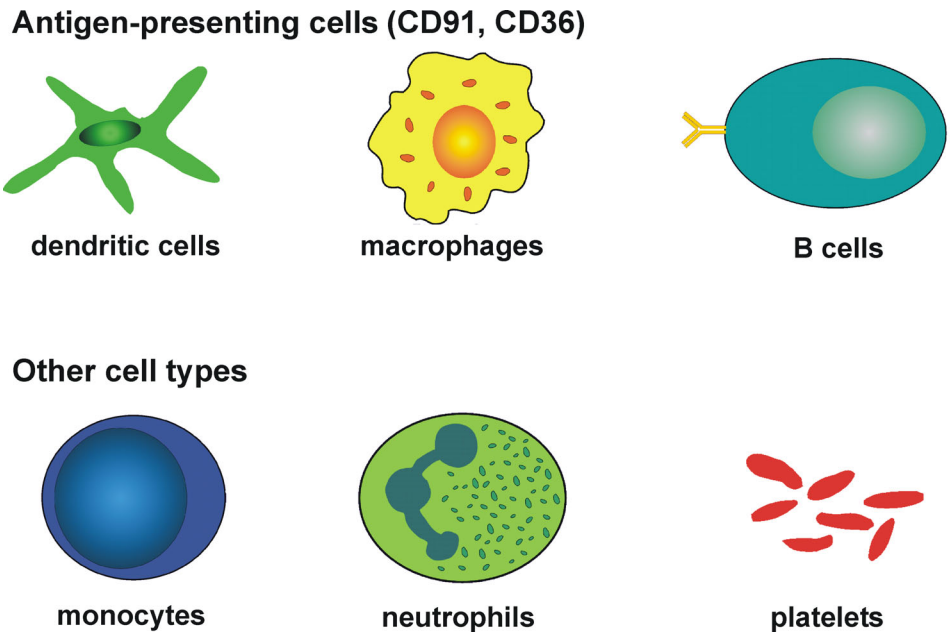

Figure 2. Cell types with HSP-binding capacities.

APC. How does the peptide travel from Gp96 into the binding groove of the MHC molecules? Two general pathways for this "cross-presentation" of antigens have been suggested (Heath and Carbone, 2001). On the one hand, a path requiring antigen transfer into the cytosol has been described. This pathway is proteasome- and TAP-dependent. On the other hand, the antigen can be processed in endosomal compartments and the generated peptides are loaded onto MHC molecules after "regurgitation" on the cell surface or in specialized vesicular compartments. For Hsp70-associated peptides, it has been demonstrated that C-terminal processing requires the participation of the proteasome and therefore the cytosolic pathway (Castellino et al., 2000). Peptides with correct C-terminus for presentation in MHC class I molecules can be represented via the regurgitation pathway. Similar results may be expected for Gp96. However, the participating cellular proteins and the mechanism that allows peptide transfer into the cytosol are not known.

The representation machinery works very efficiently as small Gp96:peptide amounts are sufficient for the induction of specific immunity. Until now, the roles of Gp96 in adaptive and innate immunity have only been looked at separately. It is still an open question whether the capacity of Gp96 to activate APCs is really required for successful induction of specific immunity by HSP:peptide complexes. The ideal method to answer this question is to perform immunization experiments in mice that do not respond to Gp96-mediated activation signals – in other words MyD88- or

TLR4/TLR2-deficient mice. For virus infections, we were able to demonstrate that normal CTL responses are induced in the absence of MyD88- and TLR-mediated signals (chapter 7). However, TLR signaling is required to compensate for an induced lack of $CD4^+$ T cell help. This shows that unspecific innate immune recognition via TLRs is able to achieve APC maturation levels sufficient for T cell priming *in vivo* even without antigen-specific $CD4^+$ T cell help (Figure 3). Moreover, in a system of weaker immunogenicity of the antigen (minor H antigens) TLR signaling is generally required for priming of CTLs. Of course, the danger signal that is involved in APC activation during minor H specific T cell priming has still to be determined, but the experiments performed provide further evidence for the importance of innate immune recognition for adaptive immunity. Similar results can be expected for immunizations with Gp96:peptide complexes that therefore might not work in mice deficient in TLR4/2 signaling.

A recent publication from C. Nicchitta's lab suggests that the tumor protective effect of Gp96 might be independent of bound peptide (Baker-LePain et al., 2002). In these experiments, immunization with transfected cells that secrete soluble Gp96 confers some tumor protection (slower tumor growth) irrespective of the cell type chosen for

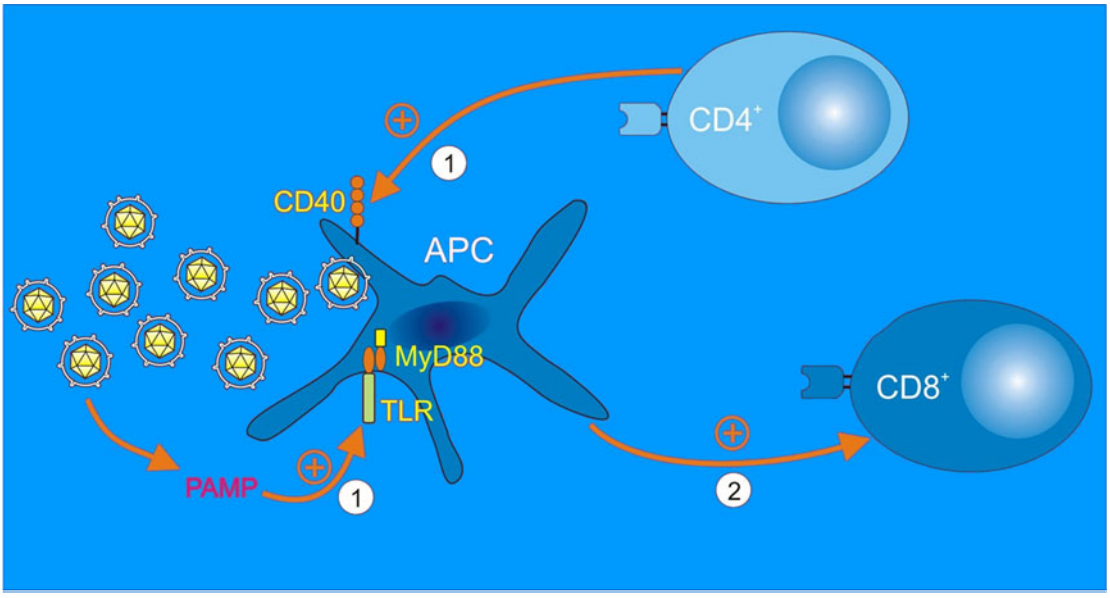

Figure 3. APC activating signals during CTL priming against viral antigens. During a viral challenge APCs are activated at least via two pathways that are each sufficient for CTL priming. One signal is provided by specifc $CD4^+$ T cells that recognize MHC class-II restricted viral eptiopes. The APCs 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.

immunization. Moreover, Gp96 was able to retard tumor growth even in the absence of its C-terminal domain which has been demonstrated to possess a peptide binding site (Linderoth et al., 2000). These results obviously contradict the early observations of Pramod Srivastava and of others that tumor protection is restricted to the tumor the Gp96 had been purified from. However, with the tumor system chosen by Nicchitta and coworkes no specific immune response could be induced, even with tumor lysate – the tumor line was simply not immunogenic. In the experiments published by P. Shrivastava the tumor lysate did induce tumor protection and the success of Gp96 vaccination was limited to the tumor the protein was purified from (Srivastava et al., 1998). But even in this work a slight tissue-independent retardation of tumor growth was also observed that is likely to be mediated by the general immunostimulatory capacities of Gp96.

Gp96 and other HSPs are certainly potent, endogenous danger signals. But they are even more. Due to their ability to carry antigenic material to APCs in a form that is predestined for uptake and representation, they deliver everything to the APC that is required to trigger an effective immune response - specificity and activation signals. In the case of pathogen-induced necrotic cell death, Gp96 not only calls the police, it provides also a detailed photograph of the murderer. These functions demonstrate the tight link between innate and adaptive immunity, condensed to one protein.

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Meiner lieben Frau Tanja, die mich auffing und unterstützte, auch wenn das Labor einmal wieder nur Frustrationen und Fehlschläge zu bieten hatte. Dieser Dank kann jedoch niemals ausdrücken, welche Bedeutung Tanja für alle Bereiche meines Lebens hat.

Curriculum vitae

Name:	Norbert Hilf
Date of birth:	15.09.1972
Place of birth:	Dortmund, Germany

1980-1983	Elementary School in Dortmund-Wichlinghofen, Germany
1983-1992	Goethe-Gymnasium, Dortmund (Grammar School)
June 1992	Abitur
08/1992 - 09/1993	Civil Service as an ambulance assistant at the Arbeiter-Samariter-Bund, Witten, Germany
10/1993 – 08/1999	Biochemistry studies at the Eberhard-Karls-University in Tübingen
10/1995	Vordiplom in Biochemistry
04/1997	Chief Editor of the “Leitfaden Biochemie” (first edition)
04/1997 – 03/1998	Practical training at the Max-Planck-Institute for Biochemistry in Martinsried
12/1998 - 08/1999	Diploma thesis at the Max-Planck-Institute for Developmental Biology, Department of Biochemistry (Tübingen) supervised by PD Dr. Hoch and Prof. Dr. U. Schwarz, title: "Analysis of the Agrin Receptor – Characterization of Oligomeric Protein Complexes of the Muscle-specific Kinase"
10.06.1999	Marriage to Tanja Gabriele Hilf, née Hipp
08/1999	Diploma in Biochemistry (graduation)
16.08.1999	Birth of Lena Sophia Hilf
12/1999-02/2003	PhD thesis at the University of Tübingen, Dept. of Immunology (Prof. Rammensee) supervised by PD Dr. Schild, Title: “More than a danger signal – the versatility of the heat shock protein Gp96 in innate and adaptive immunity”
10/2000	Poster prize at the II. International Conference on Heat-Shock Proteins in Immune Response“, Farmington, Conneticut, USA
Since 04/2002	Supported by the doctoral programme “Cellular mechanismns of immune-associated processes“ financed by the Deutschen Forschungsgemeinschaft

Akademische Lehrer

Akademischen Lehrer an der Universtität Tübingen und (*) während eines einjährigen Aufenthaltes am Max-Planck-Institut für Biochemie in Martinsried waren (in alphabetischer Reihenfolge):

Prof. Albert, Prof. Bisswanger, Prof. Bohley, Prof. Braun, Prof. Eisele, Prof. Frommherz*, Prof. Gauglitz, Prof. Gönnenwein, PD Dr. Günzl, Prof. Hagenmaier, Prof. Hamprecht, Prof. Hanack, Prof. Hofschneider*, Dr. T. Holak*, Prof. G. Jung, PD Dr. Kapurniotu, Prof. Kreutzberg*, Prof. Lindner, Prof. E. Maier, Prof. Dr. W.-E. Mayer, Prof. Mecke, Dr. J. Meier, Prof. C. Müller, Prof. Nakel, Prof. Ninnemann, Prof. Oberhammer, Prof. Oelkrug, Prof. Oesterheld*, Prof. Overath, Prof. Pfeiffer, PD Dr. Pommer, Prof. Probst, PD Dr. G. Raivich*, Prof. Rammensee, Prof. Reuter, Dr. Reinecke, PD Dr. Schild, Prof. Schmid, PD Dr. Stefanovic, PD Dr. Stoeva, Prof. Strähle, Dr. R. Timpl*, Prof. Voelter, Prof. Wegmann, Prof. Weser, PD Dr. Wiesinger, PD Dr. Wiesmüller, Prof. Wohlleben

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Geburtsdatum:	15.09.1972
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1980-1983	Grundschule in Dortmund-Wichlinghofen
1983-1992	Goethe-Gymnasium, Dortmund
Juni 1992	Abitur
08/1992 - 09/1993	Zivildienst beim Arbeiter-Samariter-Bund, Witten als Rettungssanitäter
10/1993 – 08/1999	Biochemie-Studium an der Eberhard-Karls-Universität, Tübingen
10/1995	Vordiplom
04/1997	Editor der Erstausgabe des „Leitfaden Biochemie”
04/1997 – 03/1998	Aufenthalt am Max-Planck-Institut für Biochemie, Martinsried
12/1998 - 08/1999	Diplomarbeit am Max-Planck-Institut für Entwicklungsbiologie, Abteilung Biochemie (Tübingen) unter der Anleitung von PD Dr. Hoch und Prof. Dr. U. Schwarz; Titel: „Untersuchungen zum Agrinrezeptor – Charakterisierung oligomerer Proteinkomplexe der muskelspezifischen Kinase"
10.06.1999	Heirat mit Tanja Gabriele Hilf, geborene Hipp
08/1999	Diplom in Biochemie
16.08.1999	Geburt der Tochter Lena Sophia
12/1999 - 02/2003	Doktorarbeit an der Universität Tübingen, Institut für Zellbiologie, Abteilung Immunologie (PD Dr. Schild, Prof. Dr. Rammensee); Titel: “Mehr als ein Alarmsignal – die vielfältigen Rollen des Hitzeschockproteins Gp96 in der angeborenen und erworbenen Immunität”
10/2000	Posterpreis der „II. International Conference on Heat-Shock Proteins in Immune Response“, Farmington, Conneticut, USA
seit 04/2002	Gefördert durch das Graduiertenkolleg “Zellbiologische Mechanismen Immunassoziierter Prozesse“ der Deutschen Forschungsgemeinschaft