Achieving Protective Immunity Against Intracellular Bacterial Pathogens: A Study on the Efficiency of Gp96 as a Vaccine Carrier

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Abstract

Effective vaccination using *in vitro* peptide-loaded heat shock proteins, tumor-derived heat shock proteins, and heat shock-fusion proteins has been shown in viral, parasite, and tumor model systems. We demonstrate protective DNA-vaccination using gp96-peptide fusion proteins against the intracellular bacterial pathogen *Listeria monocytogenes* in a mouse model. In contrast to previous studies using pathogen-derived heat shock proteins as vaccine vehicles, we used recombinant endogenous (*mus musculus*) gp96 (grp94) as a carrier for immunodominant listerial peptides. Analyses of the cellular immune response revealed profound epitope-specific IFN-γ and cytotoxic T-cell responses. Our findings suggest a predominantly MHC-I restricted T-cell response to DNA vaccination with gp96-fusion proteins in the model employed. Most importantly, DNA vaccination induced protection against an otherwise lethal dose of *L.monocytogenes*.

Introduction

The symptoms of an infection result from a complex interaction of microbial factors and host responses: in order to produce an infection, a pathogenic microorganism must be able to survive in the environment, be transmitted, and establish itself in its host. The immune system plays an essential role in host defense (Janeway et al., 2001a, 2001b; Kaufmann, 2003). Microorganisms must evade the immune system long enough to ensure their replication and spread. Understanding the strengths and weaknesses of the immune system in effecting a protective response against infectious agents is paramount to the development of new vaccine strategies (Del Giudice et al., 1998). In this manuscript, we report the development of a vaccine against an intracellular bacterial pathogen. We discuss: i) the essential components of the immune system, ii) the pathogenesis of listerial infections, iii) development and evaluation of a DNA-based vaccine against this bacterial pathogen.

Innate and Adaptive Immunity

Following its introduction into a host a pathogen faces what can be artificially divided into two types of immune responses: an innate and an adaptive response. These two responses differ in their kinetics, their effectors, and the receptors involved (review in Medzhitov et Janeway, 2000).

Innate Immune Response

The innate immune response is present before the introduction of a microbe (Medzhitov et Janeway, 2000; Beutler, 2004). All cells of an organism possess innate immune mechanisms. Contrary to the adaptive arm of immunity, the receptors recognize conserved structures of the microbe: cell wall components, DNA, or RNA. Those structures are therefore referred to as pathogen associated molecular patterns (PAMP), and the receptors involved in their recognition pattern are referred to as pattern recognition receptors (PRR) (Medzhitov et Janeway, 2000; Beutler, 2004). The interactions of receptors of the Toll receptor family (TLR) with PAMPs and their subsequent initiation of signaling cascades, illustrates well this type of innate immune mechanism. The TLR were first discovered on account of their role in Drosophila immunity, an insect that lacks circulating immune cells (Lemaitre et al., 1996; Hoffmann, 2003). In mammals the TLR are a family of germline encoded receptors with the ability to recognize various microbial structures. Because these receptors are already present at the time of infection, they constitute a first line of defense and recognize rapidly dividing pathogens (Kopp et Medzhitov, 2003).

The innate immune response involves different mechanisms that help contain the infection in its early stage. One of the major mechanisms is the induction of an inflammatory response (O'Riordan et al., 2002). On the cellular level, transcription factors of the NF-κB family are important inducers (review in Karin et Lin, 2002). A number of extracellular signals, such as stress, cytokines, and bacterial infection can induce their activation. Once activated, these transcription factors upregulate the production of multiple cytokines, soluble factors, that activate more specific components of the immune system. Many other innate immune mechanisms assist in the containment of infection (O'Riordan et al., 2002; Beutler, 2004). The process of phagocytosis is central, as it enables cells to internalize and degrade foreign structures. It can be performed by polymorphonuclear cells (PMN), or other cells that have

specialized mechanisms for microbial killing, such as proteases, reactive oxygen, or reactive nitrogen species (Shiloh et al., 1999).

Adaptive Immune Response

Contrary to the innate immune system, the adaptive immune response exists only in multi-cellular organisms and relies on specialized immune cells, the lymphocytes (Medzhitov et Janeway, 2000). Following the recognition of a microbe, the adaptive immune system requires a longer time to mount a response. It functions with the following sequence of events: antigen presentation, clonal expansion, and differentiation into effector cells, either B or T lymphocytes. Antigen receptors, immunoglobulin (Ig) and T-cell receptor (TCR), respectively, are produced during a somatic process of recombination (Bassing et al., 2002). In the lymphocytes, the random juxtaposition of genetic elements produces a repertoire of receptors, of which those that correspond to specific antigens are selected. Both Ig and TCR are large multi-chain molecules: Ig are formed by light and heavy chains, while TCR are composed of either α and β polypeptide chains, or, less often, of γ and δ polypeptide chains (Bassing et al., 2002).

The adaptive immune response consists of two arms. The humoral arm consists of soluble Ig or antibody, which is produced by B lymphocytes. The cellular arm is mediated by cytotoxic T lymphocytes (CTL), so called because they are able to lyse cells presenting foreign antigen on their cell surface. An important subset of T lymphocytes orchestrates the immune response and is also part of the cellular response: T helper (T_h) cells are required for activation of CTL and B cells. Depending on the patterns of cytokines they produce, they activate separately the humoral and the cellular arms of the adaptive immune system (Romagnani et al., 2000). T_h1 cells

produce IFN-γ, IL-2, and TNF-α, and support macrophage activation and CTL responses. T_h2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, all of which assist in B cell activation and antibody production (Romagnani et al., 2000). The humoral immune response is especially adapted to extracellular pathogens, while the CTL response is particularly important during intracellular infections (Wong et Pamer, 2003). CTL killing operates via cell lysis, or by inducing the target cells to undergo apoptosis. When a CTL binds to a target cell, perforins are released. These polymerize in the target cell membrane to form trans-membrane channels. Serine proteases and other proteins are released and enter the cell through the trans-membrane channels, thus inducing cell lysis. In addition, non-secretory surface receptor mediated triggering of apoptosis may occur through cross-linking of apoptosis-inducing cell surface molecules on the target, such as Fas and the tumor necrosis factor (TNF) receptor.

T cells are further classified according to their cell surface expression of CD4 and CD8. T helper cells express CD4, while CTL express CD8 on their surface. As we will see later, CD4 - and CD8 proteins act as co-receptors for MHC class II and MHC class I molecules, respectively. CTL are usually CD8 +, and are stimulated by endogenous antigens, or antigens derived from intracellular pathogens, in association with MHC class I molecules (Kloetzel et Ossendorp, 2004; Lehner et Cresswell, 2004). T_h are usually CD4 + and are stimulated by exogenous antigens in association with MHC class II molecules (Bryant et Ploegh, 2004; Al-Daccak et al, 2004).

The regulation of the magnitude and duration of the immune response is critical when considering pathology and disease development. Contrary to the innate immune response, the adaptive immunity is characterized by the existence of memory (Sprent et Surh, 2002). The re-introduction of an antigen long after its clearance results in a rapid expansion of antigen specific memory cells. The development of

memory T cells is of primary interest when designing effective vaccines (Sprent et Surh, 2002).

A Link Between Adaptive and Innate Immune Systems - The Danger Theory

Innate and adaptive immunity work in a concerted fashion. We already mentioned that innate immunity is involved in the initial stages of infection, but it also plays a role in the initial tuning of the adaptive immune response. The participation of the innate immune response may be better understood when one concedes that the immune system is responsive to the situations of danger (Matzinger, 2002): Polly Matzinger first introduced the theory that the activation of the immune system is induced by alarm signals from injured cells or tissues, rather than by the recognition of non-self, or foreign, structures. Cells distressed on account of infection by pathogens, exposure to toxins, or mechanical damage, are perceived to release molecules and cell components that are recognized by surface receptors on surrounding cells and induce the activation of signaling cascades associated with the immune response (Matzinger, 2002).

The adaptive immune system is extremely sensitive to the context in which it is stimulated. In addition to antigen recognition, T cells require an additional co-stimulation in order to be activated (Appleman et Boussiotis, 2003). This co-stimulation is provided by cell surface molecules as well as by cytokines produced mainly by antigen presenting cells (APC). Recent discoveries show these co-stimulatory molecules are regulated in a danger dependent manner (Matzinger, 2002). After the entry of a pathogen into an organism, its cells encounter a stress situation that solicits components of the innate system, such as the transcription factor NF-κB. This situation is perceived at the level of the APC, by macrophages or dendritic cells

(DC). In these cells, the danger signal induces a double process of maturation and of activation. In addition, co-stimulatory molecules are induced at the cell surface of mature, activated DC and macrophages (Matzinger, 2002). Consequently, a vigorous adaptive immune response is initiated.

The danger theory is supported by the recent observation that many receptors of the innate immune system recognize intracellular components in addition to the previously described PAMPs (Matzinger, 2002). These receptors include not only the TLR, but also another newly discovered family of intracellular proteins, called nucleotide-binding oligomerization domain (NOD). Intracellular components only become apparent to the innate receptors in conditions of cell disruption or distress. As we will see later, a family of proteins known as heat shock proteins play an important role as danger signals (Lehner et al., 2000; Banerjee et al., 2002; Wallin et al., 2002; Pockley, 2003).

Antigen Presentation and Crosspriming

T lymphocytes recognize antigenic fragments in association with major histocompatibility complex (MHC) molecules from APC (Roy , 2003; Lehner et Cresswell, 2004; Kloetzel et Ossendorp, 2004). MHC class I molecules can be found in all nucleated cells, while MHC class II molecules are expressed only by specific APC, such as B cells, macrophages, or dendritic cells (van den Elsen et al, 2004). Depending on the cellular compartment in which the pathogen or the antigen is localized, there are two distinct pathways (Figure I): i.) cytosolic antigens are derived from intracellular pathogens, or those with the ability to egress from the phagosome. They are efficiently degraded into small peptides by the proteasome, a large cellular proteolytic complex, and are transported to the lumen of the endoplasmic reticulum

(ER) via either transporter associated with peptide transport (TAP) -dependent or TAP-independent mechanisms (Kloetzel et Ossendorp, 2004). In the ER these peptides are loaded onto newly synthesized MHC class I molecules, and transported to the cell surface. ii.) extracellular pathogens are engulfed by APC and degraded in a membranous vacuole, termed phagosome. In the phagosome, antigenic peptide fragments associate with MHC class II molecules (Bryant et Ploegh, 2004). MHC class II peptide complexes are then recycled to the cell surface.

In discrepancy to this apparent dichotomy, phemonena of crosspriming have been described (Figure II). Crosspriming refers to the ability of exogenous peptides to access the MHC class I presentation pathway. Both the mechanisms at work in crosspriming and its relevance to the *in vivo* situation are a matter of intense discussion (Pfeifer et al., 1993; Kovacsovics-Bankowski et Rock, 1995; Delneste et al., 2002). Recently it has been proposed that the phagosome compartment may be competent for the antigen processing ending in crosspresentation via the MHC class I pathway (Houde et al., 2003; Guermonprez et al., 2003). A good example of crosspriming involves the phagocytosis of apoptotic blebs containing antigen by DC (Yrlid et Wick, 2000; Delneste et al., 2002; Schaible et al., 2003). DC then may crosspresent this exogenous antigen, thus allowing its association with MHC class I molecules (Figure II). Crosspriming is an important factor in the design of non-live vaccines against intracellular pathogens, as protection requires that the antigen applied extracellularly is presented both by MHC class II and MHC class I molecules.

Listeria monocytogenes

Genus and Basic Microbiology/Disease Manifestations

Listeria monocytogenes (*L.monocytogenes*) is a gram-positive, facultative anaerobic, rod (review in Vazquez-Boland et al, 2001). It is a motile bacterium without a capsule, and does not produce spores. Listeria grows at 4°C and is most frequently contracted by ingestion of contaminated food (Vazquez-Boland et al, 2001). *L. monocytogenes* is a facultative intracellular bacterium, and is known to cause severe pathology in humans, especially immuno-compromised individuals (Vazquez-Boland et al, 2001): in pregnant women, a listeria infection can cause abortion and severe malformation. In adults, listeria infection causes meningitis and bacteremia, both with a serious pathology and high mortality rates.

Following ingestion, *L.monocytogenes* crosses the intestinal barrier. Mechanisms employed at this step are not completely clear, but could involve the M cells, a specialized cell type of the intestinal epithelium covering lymphoid follicles (Roy, 2003). From there, *L.monocytogenes* is rapidly carried to mesenteric lymph nodes, spleen, and liver. *L.monocytogenes* is an intracellular pathogen, which replicates essentially in hepatocytes (Cossart et al., 2003). The intracellular infectious cycle is well described (Figure III). Efficient internalization is a main virulence determinant of *L.monocytogenes*. Two proteins, internalin A and B, are responsible for the receptor-mediated uptake of *L.monocytogenes* in epithelial cells (Gaillard et al., 1991; Cossart et al., 2003). Internalin A interacts specifically with E-cadherin, while internalin B activates the tyrosine kinase receptor Met, in addition to interacting with other ubiquitous receptors and proteoglycans (Cossart et al, 2003). In contrast to *Salmonella* and *Shigella*, which induce membrane ruffling, *L.monocytogenes* internalization is achieved by a zipper-like mechanism: the bacteria is internalized without any membrane protrusion (Cossart et al., 2003). Following invasion, *L.monocytogenes* is contained in a membraneous vacuole. Virulent bacteria are able to break the vacuole at an early step of its maturation. This prevents access of lysosomal

hydrolases to the bacterium. Internalized bacteria escape the vacuole on account of their ability to secrete a pore-forming toxin known as listeriolysin (LLO). This toxin is a member of a family of cholesterol-dependent pore-forming toxins. LLO requires the acidic pH in the endosome to become able to interact with the cholesterol of membranes (Glomski et al., 2002). It then forms oligomers, and disrupts the membrane, thus forming a pore, which allows *L.monocytogenes* to egress into the cytoplasm, where it replicates. In the cytosol, an efficient proteasomal degradation restricts the membrane-disruptive ability of LLO (Decatur et Portnoy, 2000; Lety et al:, 2002). As a result, the pore-forming ability of LLO is restricted to the endosome. The proteasomal degradation of LLO is dependent on an amino-terminal PEST-sequence (Decatur et Portnoy, 2000; Lety et al:, 2002). In order to achieve a more effective transmission, *L.monocytogenes* express a cell-surface molecule called ActA, which induces a polar polymerization of cytosolic actin monomers (Loisel et al., 1999). This allows cell-to-cell transmission of bacteria, without exposing progeny to the extracellular environment (Figure III). Therefore *L.monocytogenes* exists largely hidden from antibodies and other soluble effector molecules, frequently inside macrophages (O'Riordan et al., 2003). These virulence strategies highlight the importance of a cell-mediated immune response in *L.monocytogenes* infection.

Immune Response Against Listeria

Though the innate immune response effected by PMN and macrophages probably plays a role in the limitation of initial stages of infection, it is insufficient for sterile clearance. Two well established mechanisms of anti-microbial defense in macrophages are the production of reactive oxygen intermediates by phagocyte oxidase, and of reactive nitrogen intermediates by inducible nitric oxide synthase.

Macrophages genetically deficient in these two systems are unable to kill virulent *L.monocytogenes* (Shiloh et al., 1999).

Because bacterial replication occurs in the host cell cytosol, listerial antigens are processed via the MHC class I pathway, and are recognized by CD8 T cells. There are two groups of CD8 T cells (Wong et Pamer, 2003): The first group recognizes short, hydrophobic, bacterial peptides that contain N-formyl-methionine at the amino-terminus (Princiotta et al., 1998). These N-formyl leader sequences are found only in bacteria and in mitochondria, which are of bacterial origin. N-formyl-methionine-peptides are presented via MHC class Ib molecules, which are present and invariant in most laboratory mouse strains (H-2M3 allele) (Princiotta et al., 1998). Responses to N-formyl-methionine-peptides are strong in C56/Bl6 mice, but virtually absent in Balb/C mice. CD8 T cell responses to these peptides are different from CD8 T cell responses to peptides presented by MHC class Ia molecules. MHC class Ib restricted responses are more rapid and larger than those mediated by MHC Ia. In contrast, the memory responses to peptides presented by MHC Ia are more robust (Wong et Pamer, 2003). The second group of CD8 T cells recognizes peptides generated by cytosolic degradation of secreted bacterial proteins. These peptides are presented by classical MHC class I a molecules (Wong et Pamer, 2003).

The dominant listerial CD8 T cell epitopes, presented by MHC class Ia molecules, have been defined in Balb/C mice. The peptides LLO-91-99 (GYKDGNEYI), and p60-217-225 (KYGVSVQDI) derived from the proteins LLO and p60, respectively, were found to be immunodominant (Pamer et al, 1991). The contribution of the p60 protein in virulence is unknown, but it could assist listerial entry into host cells (Figure III) (Kuhn et Goebel, 1989; Hess et al., 1996b). It is currently unclear, which characteristic is responsible for the immuno-dominance of the LLO-91-99 and p60-217-225 peptides. Their immuno-dominance could relate to

efficient cytosolic degradation, MHC-peptide stability, the T cell repertoire, or competition between T cells (Wong et Pamer, 2003).

While CD8 T cells confer protection against *L.monocytogenes*, the mechanisms of bacterial clearance remain undefined. Peptide-specific IFN-γ expression is dispensable for protection, as is cytolytic function. (Wong et Pamer, 2003). The production of chemokines, such as MIP 1-α (macrophage inhibitory factor) could be important for protection, but further studies are required to clarify their role (Wong et Pamer, 2003). Though the CD8 + T cell response is dominant in adaptive immunity to this pathogen, not all *L.monocytogenes* invading the host cell are able to lyse the phago-lysosome. Some bacterial proteins are processed in the phago-lysosomal compartment and presented in association with MHC class II molecules. Thus, CD4 T cells are also activated in response to *L.monocytogenes* infection (Ladel et al., 1994).

Because experimental murine listeriosis reproduces many features of human infection, this model is particularly well suited to the study of intracellular pathogens and the cellular immune response. In addition, it provides an opportunity for the analysis of mechanisms of immune evasion and manipulation of the host response. An important technical consideration here is the comparatively short duration of infection (5-7 days). In this study both LLO and p60 CD8 T cell epitopes were used as model antigens in Balb/C mice.

Gp96/Grp94

Heat Shock Proteins - Definition

Heat shock proteins are a family of proteins that are produced in response to various types of cellular stress: heat shock, osmotic shock, exposure to free radicals, nutrient deprivation, infection, inflammation, and malignancy (Hartl et Hayer-Hartl,

2002). Because of their role as scaffolding proteins during protein biosynthesis in the cell, HSP are also known as molecular chaperones (Meunier et al., 2002). HSP bind to, and stabilize, an otherwise unstable conformation of an unrelated protein. Controlled binding and release of the substrate protein ensures its correct fate *in vivo*. This can be folding, oligomeric assembly, transport to another cellular compartment, or controlled switching between active and inactive conformations (Hartl et Hayer-Hartl, 2002). HSP bind a wide range of proteins. Their primary function is the chaperoning of intracellular proteins during protein folding at the ribosome. This involves HSP binding to exposed, hydrophobic surfaces of the protein/peptide substrate (Meunier et al., 2002). During cellular stress, rapid up-regulation of HSP expression helps prevent extensive protein degradation and to reinstate cellular homeostasis. HSP are highly conserved, have remarkable cross-species homology, and are present in all organisms, prokaryotes and eukaryotes (Robert et al., 2001). HSP are residents of numerous cellular compartments, including the cytosol, mitochondria, and ER (Hartl et Hayer-Hartl, 2002).

Gp96-Structure and Function

Gp96 is an ER-resident protein chaperone (Lee, 2001). Gp96 is expressed constitutively, but is upregulated during cellular stress. Like Bip/Grp78, gp96 belongs to the family of Glucose-regulated-proteins (Grp), and is therefore also referred to as Grp94. This family of Grp is transcriptionally regulated in a manner distinctly different from other HSP. The transcription factors ATF6 (activating transcription factor 6) and CREB (cAMP-response element-binding protein) are key regulators of Grp synthesis (Lee, 2001). Transcriptional regulation of those HSP not belonging to the Grp family involves HSF 1(heat shock factor), HSF2 and HSF3 (Morimoto, 1998).

The murine gp96 is produced as a pre-protein of 802 aminoacids (AA). The first 21 AA constitute a signal peptide that targets gp96 to the ER. Following the removal of this signal sequence, the mature gp96 protein consists of 781 AA. Gp96 is eventually N-glycosylated. The amino-terminal domain of gp96 accounts for its ability to chaperone proteins in the ER and is able to bind ATP or ADP with low affinity (Soldano et al., 2003). The carboxy-terminus of gp96 contains a KDEL motif that mediates ER residency. This short carboxy-terminal motif determines the retrieval of the proteins that carry it from post-ER compartments by allowing retrograde transport mechanisms to the ER (Stornaiuolo et al., 2003). In some instances, gp96 can be found associated with the cell surface (Altmeyer et al., 1996; Robert et al., 1999). The mechanisms involved in gp96 secretion are unclear, but could be of pathological relevance, and have been shown to constitute a danger signal (Altmeyer et al., 1996; Robert et al., 1999).

Gp96 binds a variety of peptides *in vitro* (Ishii et al., 1999). Association of gp96 with peptides occurs also *in vivo* and is probably not an artifact of cell disruption (Menoret et al, 1999). No sequence specificity has yet been demonstrated. Gp96 can bind peptides of up to 20 or more AA, but prefers peptides with a length between 7 and 15 AA. A protocol that would allow an unbiased estimation of the diversity of peptides associated with gp96 has not yet been established (Liu et al, 2004). The repertoire of peptides comprises both some which require TAP for their loading on gp96, as well as some which do not (Arnold et al., 1997). Although no crystal structure of gp96 peptide complexes are presently available, computational analysis of its possible structure, and mutagenesis studies have defined a peptide binding pocket between AA 624 and 630, in the C-terminal region of gp96 (Linderoth et al, 2000 and 2001). ATP /ADP have no effect on peptide binding activity (Wearsch et Nicchitta, 1997). In the ER gp96 is an obligatory homo-dimer, and exists also in higher order

oligomers (Wearsch et al., 1998; Linderoth et al., 2001). The location of the dimer assembly domain may influence the conformation of the peptide-binding pocket (Wearsch et al., 1998).

Gp96 as a Vehicle for Peptide Immunization

The ability of gp96 to bind peptides is important for its immune function. Gp96 stimulates the efficient presentation of these peptides by MHC class I molecules, leading to the subsequent induction of a cellular immune response (Udono et al., 1994; Suto et Srivastava, 1995). This ability was first documented in the context of cancer immunotherapy (Udono et al., 1994; Suto et Srivastava, 1995): Immunization of mice and rats with gp96 preparations isolated from syngeneic cancers cells was shown to elicit a strong tumor-specific immune response. This immune response was directed at the peptides and not at gp96 *per se* (Blachere et al., 1997). These initial results identified gp96 as a good vehicle to stimulate presentation of peptides via MHC I molecules.

The ability of gp96 to present peptides to MHC class I molecules probably has its origin in the cellular uptake and processing mechanisms of gp96-peptide complexes. In order to be re-presented by professional APC, the gp96-peptides complexes need to be endocytosed (Singh-Jasuja et al., 2000a). Several candidate receptors for the gp96-peptide complexes have been described, and they are presented in Table I. The only receptor for whom genetic evidence has been provided, is the Scavenger-receptor-A (SRA): macrophages that are genetically deficient in SRA show reduced binding and internalization of gp96 complexes (Berwin et al., 2003). The later steps for processing are less clear. Following receptor-mediated uptake, two pathways have been suggested: i.) A first pathway postulates the existence of a retrograde

transport step between the early endosome and the ER. In the ER a TAP-mediated transport of gp96 associated peptides into the cytosol would render them accessible to the proteasome. Consequently, small peptides derived from proteasomal digestion could be loaded on nascent ER-MHC class I molecules. The existence of this first pathway is demonstrated by the use of TAP knock-out (k/o) mice, as well as the use of proteasome-inhibitory drugs, such as lactacystin. (Basu et al., 2001) ii.) However, in some cases, HSP-peptide complex re-presentation occurs without any involvement of the TAP or the proteasome (Castellino et al., 2000). A direct loading of gp96 associated peptides onto recycling MHC class I molecules in an Fc-γ–receptor positive, endosomal compartment, could account for this second pathway (Berwin et al., 2002b and 2003).

Recent studies demonstrated that the HSP of the Grp-family are endowed with an immuno-stimulatory function independent of their ability to bind peptides (MacAry et al., 2004). Gp96 proteins are able to stimulate the expression of MHC molecules and of co-stimulatory signals on the surface of professional APCs, especially DCs (Cho et al., 2000b; Singh-Jasuja et al., 2000b; Binder et al., 2000b). These immuno-modulatory properties of gp96 might account for its function as a danger signal, and its function as an adjuvant in vaccine formulations. Grp, and thus gp96, is released by compromised cells either by secretion or as a consequence of necrotic cell death (Basu et al., 2000; Berwin et al., 2001). This then leads to activation of pro-inflammatory signaling cascades such as NF-κB, and the induction of cytokine release. The TLR family might be implicated in the immuno-stimulatory signaling induced by extracellular gp96, since interaction of HSP complexes with TLR2 and TLR4 have been demonstrated to induce NF-κB activation (Asea et al., 2002; Vabulas et al., 2002a and 2002b).

Project Objective:

Rationale for Using Chaperones in an Anti-listerial Vaccine, i.e. a Vaccine against Intracellular Pathogens

This study focuses on further understanding of host interactions with intracellular bacterial pathogens and exploitation of novel or improved therapeutic strategies. Experimental murine listeriosis was used as a model system for the investigation of the immunological function of gp96 and its potential as a vaccine vehicle. For this purpose we designed a DNA-vaccine encoding gp96 peptide-antigen fusion-proteins, using the immunodominant listerial antigens LLO and p60. We aimed to establish the efficacy of gp96 as a vaccine vehicle and stimulator of protective immunity against an intracellular bacterial pathogen. The induction of a strong cellular, i.e. cytotoxic, immune response is of particular importance in a potent vaccine against intracellular pathogens. We therefore investigated the ability of gp96 peptide fusion vaccines to induce a protective, CD8 T cell dependent, peptide-specific immune response in Balb/C mice.

Material and Methods

Material

Cell Lines and Tissue Culture Media

- J774 Macrophage cell line (H-2Kd)
- P815 Macrophage cell line (H-2Kd)

DMEM Medium:

Dulbeccos MEM 0435 (Life Technologies) 3,7g/l $NaHCO_3$.

We added 10% Fetal Calf Serum, 1% Glutamin, 10 U/ml Penicillin, 100 U/ml Streptomycin, and 1% Sodiumpyruvate.

Bacterial Culture Media and Agar

Agar Plates: 1,5 % Bacto-Agar was added to Culture Media

Culture Media:

LB Medium	10g Trypton
	5g Yeastextract
	10g NaCl

Listerial Peptides (H-2Kd)

Peptide design was based on the original protein sequence. Synthesis of peptides : Jerini Biotools

LLO-91-99: GYKDGNEYI

LLO-elongated: VTNVPPRK **GYKDGNEYI** VVEKK

p60 217-225: KYGVSVQDI

p60 elongated: WALSV **KYGVSVQDI** MSWNN

Elongation of peptides was based on an estimation of those AA after which proteasomal cleavage is most likely to occur. Nonamer peptide sequences were elongated just enough to assure that potential proteasomal cleavage would not affect the immunodominant nonamer sequence. The AA sequence for the elongated peptides is based on the respective protein sequence.

Bacteria

- E.coli B21D3 — Stratagene
- L.monocytogenes EGD — American Type Culture Collection, 15313, wildtype Listeria

Methods

general methods were performed according to standard protocols,

Current Protocols in Molecular Biology, Vol 1-4 (2002), Wiley and Sons Inc., New York

Tissue Culture

All cell lines were cultivated in DMEM Medium at 37°C and 7% CO2

Transformation of E.coli

DNA was introduced by heat shock of B21D3 E.coli according to standard procedures.

Thaw bacteria, and add 1µl DNA, keep on ice for 30 minutes. Heat shock for 1 minute in 42°C water bath, return immediately to ice, and add LB medium without antibiotics. Shake at 37°C for 60 minutes, and then plate on LB Agar plates (with antibiotic). Incubate overnight in 37°C incubator. Pick single colonies next day and culture in 2-5 ml LB with antibiotics overnight.

DNA Preparation:

Small volumes of plasmid DNA (‚Miniprep‘) was extracted from E.coli cultures using Quiagen Spin Columns standard protocol.

Restriction Digests

1 µg DNA was incubated with restriction enzymes in a total volume of 20 µl for 3 hours. Buffers and temperatures as indicated. Samples were then applied to an 1% agarose gel. 0,5 µl/ml Ethidiumbromide was added to 100ml of 1% agarose in TAE buffer before agarose gels were poured. 1KD Marker was used (Stratagene) as a standard. Fragments were separated by electrophoresis for 1 hour at 90 V.

SDS PAGE

Stacking gel 4%	Separative gel 10%
1,3ml Acrylamid	2,5ml Acrylamid
2,5ml Tris(1,5M), pH8,8	2,5ml Tris(1,5M), pH8,8
0,1ml SDS 10%	0,1ml SDS 10%
6,1ml H_2O	4,8ml H_2O

5 µl TEMED	5 µl TEMED
50 µl APS 10%	50 µl APS 10%

Electrophoresis was performed at 150mA for approximately 40 minutes or until Bromphemol-blue marker reached the end of the gel. Broad-Range Rainbow Marker (BioRad) was applied as a standard.

Following electrophoresis polyacrylamide gels were stained for 10 minutes in Coomassie Blue. Excess Coomassie Blue was removed by washing for 1 hour.

WESTERN Blot

Blotting Buffer I: (all Buffers ad 1,5 l) 18,15g TRIS (0,3M), 10% Methanol, pH 10

Blotting Buffer II: 1,5g TRIS (0,025M), 10% Methanol, pH 10

Blotting Buffer III: 1,5g TRIS (0,025M), 10% Methanol, 0,01 % SDS, 0,04 M Glycin (1,5g), pH 9,4

Washing Buffer: (ad 1 l) 30ml 5M NaCl (8,8g), 20ml 1M TRIS, pH7,5, aqua dest.

Tween-20 (Sigma)

Skim Milk Powder (Fluka)

Antibody to gp96 (SPA 850) 1µg/ml was purchased from Stressgen

ECL-Hyperfilm, ECL Western Blotting detection Reagents (Amersham Lifescience)

Generation of formyl-35S-MGWII and Binding Assay

Resin bound IGWII peptide was synthesized by Jerini Biotools and coupled to Fmoc protected 35S-Methionine (Amersham, U.K.) by the HBTU method (Knorr et al.1989). Specific radioactivity of methionine was 1Ci/mmol. The resin bound peptide was deprotected by treatment with 20% piperidine and formylated with an excess of formic acid in the

presence of dicyclohexyl carbodiimide. The formylated peptide was cleaved from the resin by treatment with 95% TFA. Purity and identity of the peptide was confirmed by thin layer chromatography and mass spectrometry. Peptide binding assays were performed using PAGE on 8% polyacridamide gels. 100pmol protein was incubated with 1nmol F-35S-MIGWII (1µCi) in 50 µl 20mM Tris-HCl pH 7,5 1mM MgCl2. The sample was mixed with 50 µl 0.2% SDS, 10 % glycerol, without heating and applied to the gel. Following electrophoresis the gel was dried and the radioactive band visualized and quantified with a Phosphoimager (Ray-test).

<u>Purification of Recombinant Gp96</u>

(Wearsch et Nicchitta, 1996; Srivastava, 1997; von Bonin et al., 2003)

<u>Lysis of Bacteria</u>

20mM Tris HCl pH 8

25% Sucrose

1mM EDTA

50mM NaCl

0,1% Triton X 100

10mM Imidazol

The bacterial pellet was suspended in 20ml Lysis buffer and vortexed.

15	µl	Leupeptin/Pepstatin
50	µl	DNAse
300	µl	Lysozym
300	µl	Pefablock

The above cocktail was added to the sample before ultrasound treatment, followed by two cycles of freeze-thaw (1hour at 4°C)

Finally 200 µl 1M $MgCl_2$ was added and the sample was left at room temperature for 30 minutes.

The sample was then applied to POROS MC 20 Nickel column. (Ni^{2+}-NTA; 2ml).

The column was equilibrated with 20mM Tris pH 8, 500mM NaCl, and 10mM Imidazol. Recombinant protein was eluted with a gradient of 10-250mM Imidazol in the above buffer in 20ml. Flow rate was set at 2ml/min and detection at 280nm. Fraction size was 1ml.

Gp96 Denaturation

8M Urea was added to a sample of gp96 protein and incubated at room temperature for 10 minutes. The sample was then applied to a high resolution Superdex column (HR 10/30, Pharmacia). The column was previously equilibrated in PBS and standards had been measured for BSA (70 kD), Cytochrome C (12 kD), Ovalbumin (45 kD), Catalase (230 kD). Chromatography was performed at 0.1ml/min and detection was set at 280nm.

Removal of Excess Peptide After Fluorescence Binding Assay by HPLC

Anion Exchange column AX 300, 3 micrometer, 0.5ml (Applied Biosystems)

The column was equilibrated in PBS. Elution was performed with a linear gradient until a max of 1M NaCl. Flow rate was set at 2ml/min and 500 µl fractions were collected. Detection was set at 200-600 nm.

Desalting columns Sephadex G10

Sephadex G25

Sephadex G50

Sephadex G75

All columns were equilibrated with PBS. Flow rate, detection, and elution were performed as above. The total volume of all samples applied was 50µl. Chromatography using desalting columns was followed by an evaluation of fluorescence in the fractions collected. Quantification and analysis revealed less than 96 % separation of unbound peptide.

Immunization Experiments

Mice

All experiments were performed using 8 to 12 week old BALB/c female mice. Mice were obtained from our breeding facilities at the 'Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin' (Berlin, Germany). Naive mice and mice vaccinated with empty vector DNA were used as negative controls. Mice vaccinated with 1 x 10^3 viable *L.monocytogenes* EGD were used as positive control. An additional group was immunized with gp96 DNA devoid of antigenic peptide. For survival studies 10 mice were used per group, for all other assays 3 mice were used per group and time-point.

Construction of Vectors Expressing Fusion Protein

Gp96-fusion proteins were constructed by site specific mutagenesis (Stratagene). Each antigenic peptide was fused directly to the C-terminus of the gp96 protein. Fusion proteins were then cloned into the pCI expression vector (Invitrogen). All 5 constructs were sequenced, and DNA for vaccination was purified from overnight bacterial cultures using Endofree GigaPrep Kits (Qiagen).

Naked DNA Vaccination

DNA vaccination was performed 3 times at 3-week intervals. Mice were anaesthetized with 200-300µl i.p. Avertin (2-methyl-2-butan, 2,2,2-tribrom-methanol), and the hind legs of mice

were shaved. DNA (50µg) was applied intramuscularly into each left and right hind leg of BALB/c female mice.

Depletion of CD4+ and CD8+ T Cells

CD4+ and / or CD8+ T cells were depleted 3 times in 7 day intervals, beginning 2 days prior to the 2nd boost. Antibodies (anti-CD8α Clone # 169; anti-CD4 Clone # 191) were administered at a dose of 200µg in a volume of 200µl by intraperitoneal injection.

Determination of CD8+TCell Response

Elispot

Spleens from vaccinated mice were prepared to a single cell suspension and were restimulated *in vitro* with 10^{-6}M peptide for 2 days. P815 cells were used as APC and were pulsed with 10^{-6} M peptide for 2 h prior to application in the assay. All tissue cultures were done in RPMI medium (Biochrom, Berlin, Germany), supplemented with 10% FCS, 1mM L-Glutamine, 100µg/µl Penicillin, and 100 U/µl Streptomycin (all Biochrom, Berlin, Germany). APC were added at $1x10^5$ per well to 96-well Elispot plates (Millipore, Molsheim, France) together with $1x10^5$ – $1x10^6$ spleen cells. Plates were incubated at 37°C and 7% CO_2 for 22-24 h. We used the IFN-γ mAb R4-6A2 (rat anti-mouse), and XMG-1.2 (biotin rat anti-mouse), and SIGMA FAST ™ substrate tablets (Sigma, Steinheim, Germany) for detection of IFN-γ specific spots. Each value presents the mean of triplicate values.

CTL Assay

Spleens from 3 mice were prepared to a single cell suspension and were cultured at $4x10^6$spleen cells /ml together with $3x10^6$/ml (3000 rad) syngeneic stimulator cells. The stimulator cells had been pulsed at 37°C with 10^{-6}M peptide for 1 h. The cells were incubated

in a 25-cm^2 flask (Falcon, Heidelberg, Germany) at 37°C and 7%CO_2 for 5 days. P815 cells were used as targets and were restimulated with 10^{-6}M peptide and 100µCi ^{51}Cr for 2 hours prior to the assay. Targets were washed twice and added at 1x10^4 targets per well to a round-bottom 96-well tissue culture plate (Falcon) together with responder cells at different E:T ratios. After 3-4 h of incubation, 25µl of supernatant was collected and ^{51}Cr activity was determined. The percentage of peptide specific lysis was calculated as: (experimental value - spontaneous release)/(maximum release – spontaneous release) x100. Each value presents the mean of triplicate values.

Protection Assay

Mice were challenged with 5 x 10^4 *L. monocytogenes* EGD (5 x LD 50) 3 weeks after the 2nd boost (63 days after the first vaccination). Survival was monitored for 10 days. 10 mice were used per group.

Statistical Analysis

Statistical analysis for IFN-γ Elispots was performed using an unpaired Student's t test, with $p<0.05$ indicating significant differences (GraphPad Prism 3.0). Statistical analyses for protection assays were performed using the Log Rank Test, with $p<0.05$ indicating significant differences (GraphPad Prism 3.0).

Results

Biological Activity of Recombinant Protein

A binding assay using a ^{35}S-Methionine labeled bacterial peptide known to bind endogenous gp96 (i.e. isolated from murine tissues, or J774 macrophage cell line) was performed to verify the biological activity of the recombinant protein. The binding assay was performed according to the protocol described. Efficiency of binding was compared at two temperatures, 0° C and 40°C (figure 1).

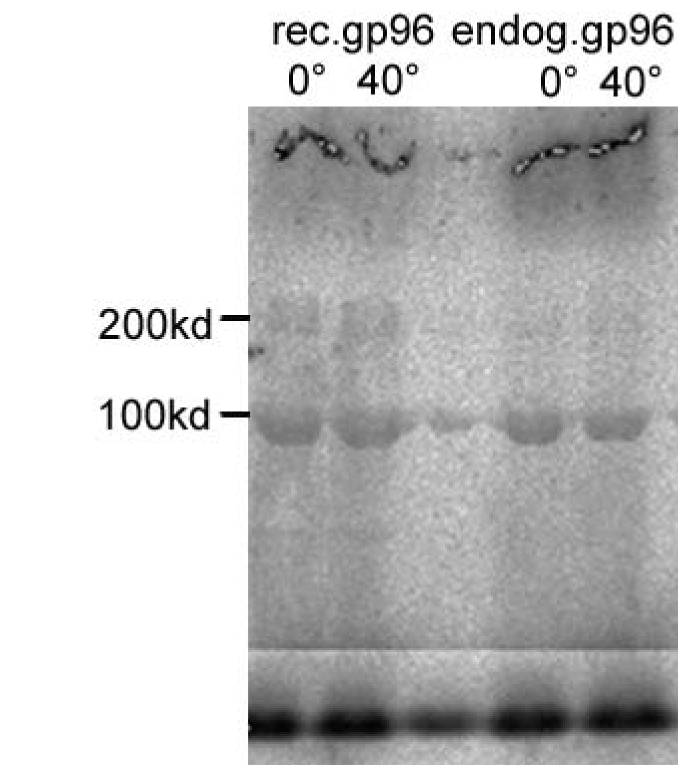


Fig.1. Comparison of peptide binding at 0° C and 40° C.

Recombinant gp96 and gp96 purified from J774 macrophages were incubated with radioactively labeled peptide at the indicated temperatures. The samples were mixed with Laemmli-buffer without heating and applied to SDS-PAGE. 200kd bands represent dimeric protein

Radioactively labeled fMIGWII peptide had been shown previously to bind to endogenous gp96. F-MIGWII is an N-formylated peptide derived from proteins of the

intracellular bacterium *L. monocytogenes* and has been shown to generate a protective CD8 T cell response in C57BL/6 mice. The binding assay was performed according to the protocol described. Subsequent binding assays were performed (data not shown) in which samples were incubated for either 15 or 30 minutes at 0°C, 37°C, and 60°C. Competition studies were performed using a 100fold excess of unlabeled p60 nonamer peptide. The p60 217-225 epitope is an immunodominant epitope derived from the p60 protein of *L. monocytogenes.*

The resulting temperature kinetics did not reveal significant differences in binding efficiency. Note, however, that at least two distinct bands developed (figure 1). The appearance of the 200 kDa band was more pronounced for the recombinant than for the tissue-culture derived protein. This phenomenon was further analyzed using size exclusion gel chromatography (as shown in figures 2 and 3). Recombinant as well as organ-derived gp96 preparations revealed two populations at molecular masses of approximately 300 and > 400 kDa. Determination of molecular sizes was based on standards used to calibrate the column. Denaturation of recombinant as well as organ derived gp96 with urea led to a significant shift of the 300 kDa peak towards 200kDa. This suggests that protein eluting at 300 kDa is at least a dimeric form.

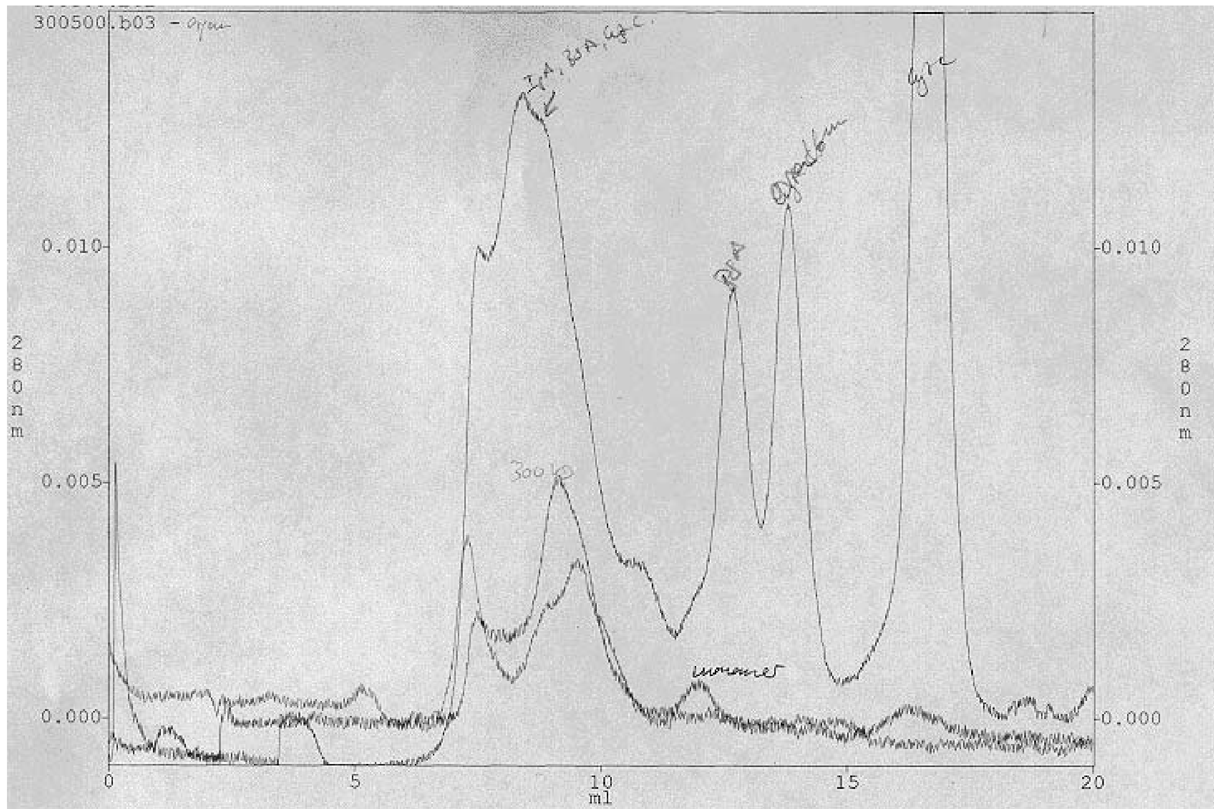


Fig.2. Chromatographic fractionation according to molecular mass, using organ-derived and recombinant gp96 after purification.

blue: standard, red: organ derived gp96, green: rec. gp96; the first peak at 7 ml represents the >400 kDa form, the second peak at 10 ml, represents the 300 kDa form.

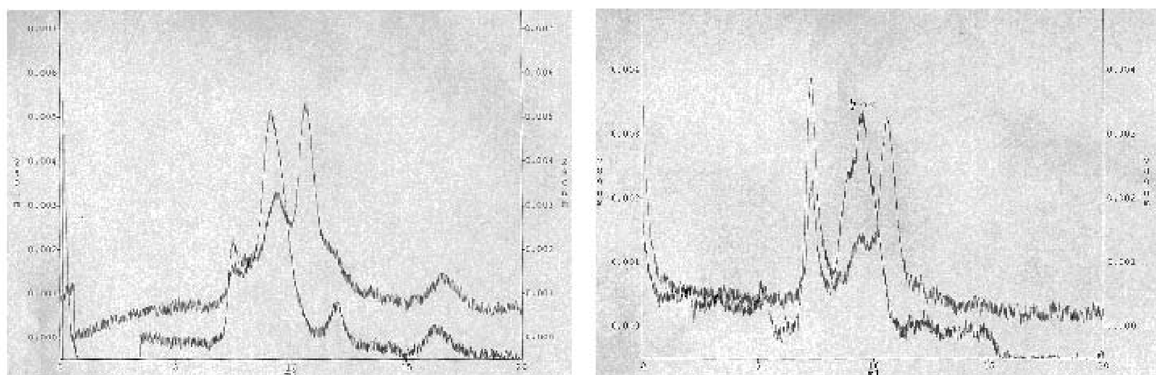


Fig.3. Chromatographic fractionation of denatured gp96.

3a. organ-derived gp96, 3b. rec. gp96, in both figures red represents the native protein, and green represents the urea-denatured protein; the shift between the green and red peaks represents a shift from 300-400 kDa to 200kDa forms of gp96.

Removal of Excess Peptide After Binding Assay

Because all binding assays sofar were performed using a 100 fold excess of peptide, it was necessary to identify a method for efficient removal of excess unbound peptide before further analysis of samples was performed using either fluorescence spectrometry or macrophage assays. This proved to be difficult, because the total volume of the binding assay was < 50 µl containing minute amounts of protein. Initial efforts using centricon spin columns failed on account of the distribution of the protein complexes on the membrane and the difficulty of eluting sufficient proportions off the membrane after washing of peptide. In a second approach, Nickel beads were applied to the sample after the binding assay. His –tagged recombinant gp96-peptide complex bound to the beads and free peptide was removed by several washes. Efficient elution of the protein-peptide complex from the beads proved to be complicated as most conventional methods threatened to disrupt peptide binding. Finally, our efforts centered on purification using various chromatography columns. Nickel columns as well as several Sephadex gel filtration columns were tested as described. Chromatography revealed only insufficient separation of unbound peptide as this was assumed to form aggregates. Various anion exchange columns were tested. These proved to provide the most effective and reliable separation of unbound peptide from protein –peptide complexes as well as ensuring almost quantitative recovery of the protein applied. All subsequent binding assays were followed by application over anion exchange HPLC. Quantitative analysis was then performed using a fluorescence spectrometer. Standard curves correlating amount of peptide and measured fluorescence were set up and used as reference to estimate the binding efficiency.

The gp96 protein has been shown previously to be unstable. Frequent freeze-thaw of the Cy3 labeled sample resulted in sudden protein degradation. It was considered likely that

the bisfunctional fluorescent label increased the instability or led to crosslinking of the protein. Bradford analysis revealed that unlabeled protein aliquots treated in the same manner did not degrade equally. A new aliquot of recombinant protein was labeled, but this time a monofunctional Cy5 dye was used. The Cy5 dye was preferred to the Cy3 dye on account of the better distinction between emission spectra produced in combination with Fluoro labeled peptide.

Subsequent studies were performed using Cy 5 labeled protein and Fluoro labeled elongated LLO peptide. This peptide is a 25 mer constructed such that the dominant epitope is flanked by 3-5 amino acids. Final analysis of the binding assay was done using HPLC. This allowed simultaneous washing of excess peptide and fluorescence analysis of protein-peptide complexes as revealed by chromatography. Protein and peptide were incubated for 15 minutes at 0°C, 37°C, or 60°C (figure 4, 5, 6, and 7).

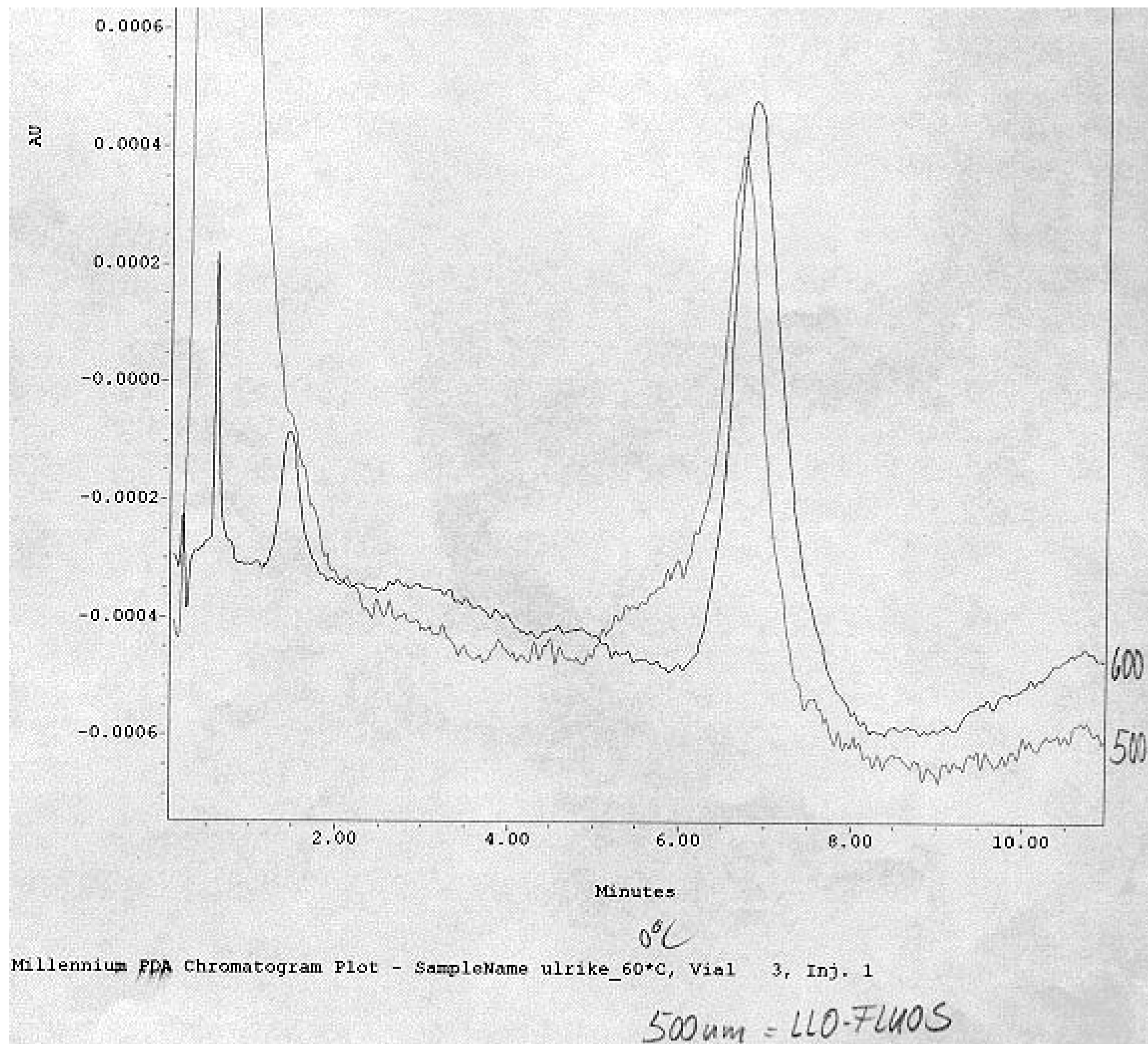


Fig.4. Chromatographic fractionation of gp96-peptide complexes after *in vitro* binding assay at 0°C.

black: 600nm, gp96-Cy5; red: 500nm, LLO-Fluos; Cy 5 labeled gp96 and Fluos labeled LLO peptide were incubated for 15 min. at 0°C. The free/excess peptide peak is just before the gp96-peptide complex peak. This makes it very difficult to collect a fraction of gp96-peptide complex cleared of excess peptide.

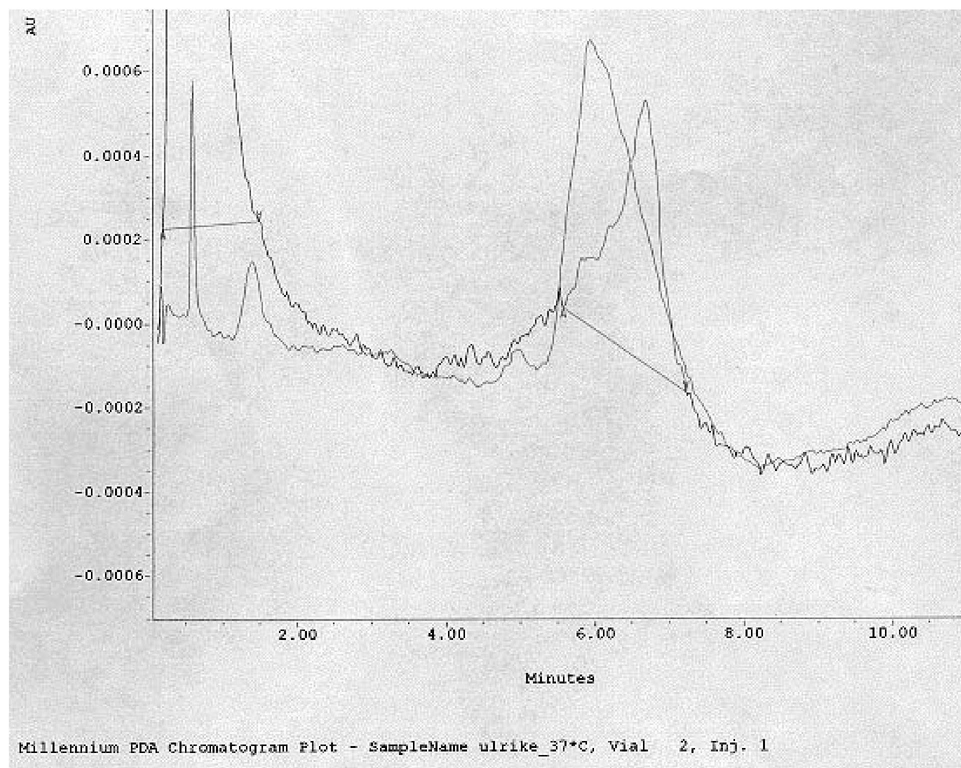


Fig.5. Chromatographic fractionation of gp96-peptide complexes after *in vitro* binding assay at 37°C.

black/green:600nm, gp96-Cy5; red:500nm, LLO-Fluos; gp96-Cy5 and LLO-Fluos were incubated for 15 min at 37°C. The free/excess peptide peak is just before the gp96-peptide complex peak. This makes it very difficult to collect a fraction of gp96-peptide complex cleared of excess peptide. The green line indicates the fraction overlap.

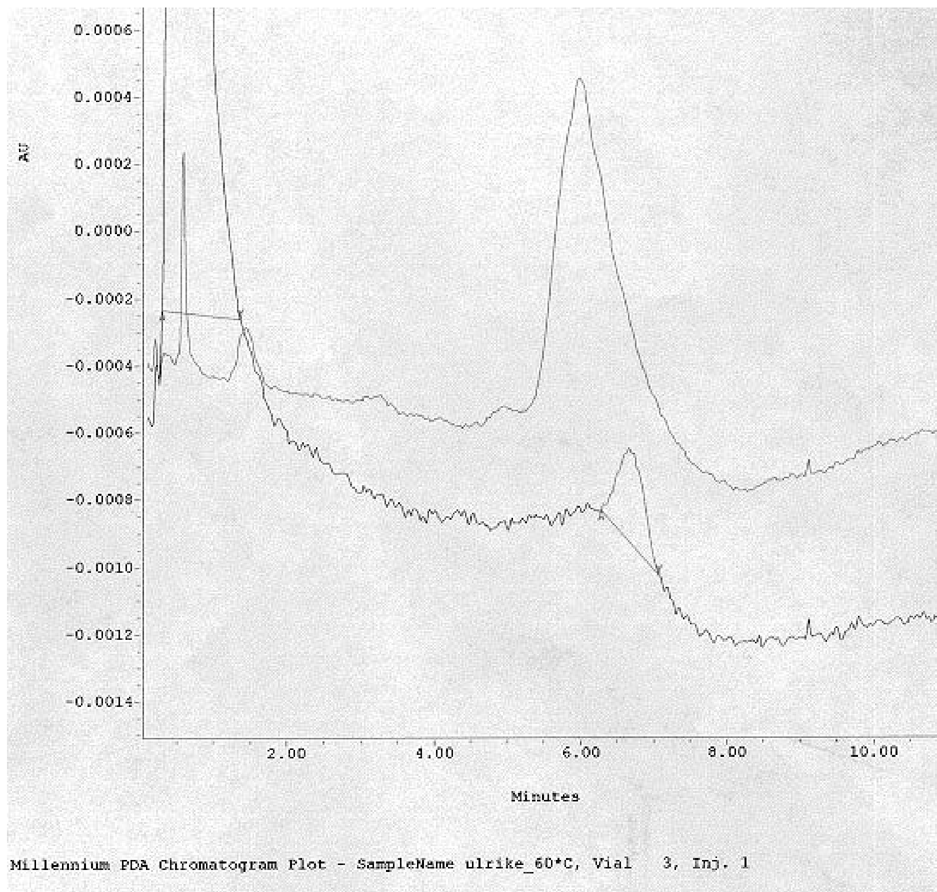


Fig.6. Chromatographic fractionation of gp96-peptide complexes after *in vitro* binding assay at 60°C.

black:600nm, gp96-Cy5; red:500nm, LLO-Fluos; gp96-Cy5 and LLO-Fluos were incubated for 15 min. at 60°C. The free/excess peptide peak is just before the gp96-peptide complex peak. This makes it very difficult to collect a fraction of gp96-peptide complex cleared of excess peptide. The green line indicates the fraction overlap.

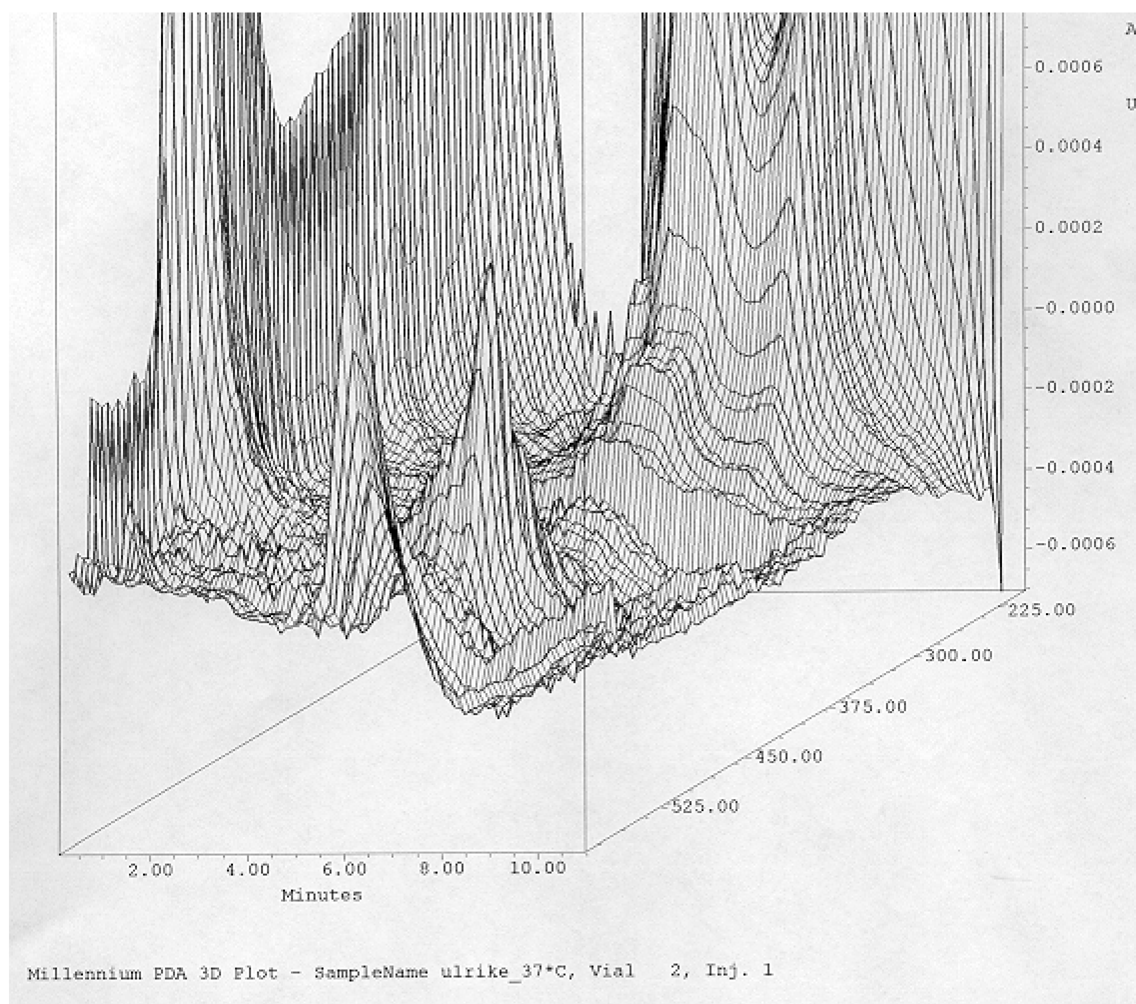


Fig.7. **3D plot of chromatography results after incubation for 15min at 37°C.**

The peaks represent unbound peptide, and protein peptide complex. The first peak with the coordinates 500nm and 6 minutes (unbound peptide) is just in advance and overlaps with the beginning of the second peak with the coordinates 600nm and 7 minutes (protein-peptide complex).

Interim Summary:

The basic question when regarding the efficacy of gp96 complexes as vaccine vehicle is: can a gp96-antigen-complex induce a protective, CD4+ T-cell independent immune response in the *L. monocytogenes* model? Previous work involved the isolation and purification of gp96 from spleens and livers of infected and noninfected mice and *L.monocytogenes* infected J774 macrophages. Thus far we described initial work based on the *in vitro* loading of antigenic peptides onto recombinant gp96-HIS. Preliminary results of in vitro HSP peptide binding assays suggested a low binding affinity of the selected peptides to

gp96. In view of the basic question posed in this project, we decided that a vaccination study required the structure of the vaccine vehicle and the ratio of HSP-peptide to be stable.

Construction and Administration of Gp96/peptide-antigen Complexes

Consistent with studies focusing on *in vitro* loading of antigenic peptides onto various HSP we found an average stable peptide binding efficiency <10% (data not shown). In order to optimize the construction of HSP/peptide complexes with a predictable 1:1 stoichiometry of HSP: fusion-partner we constructed DNA encoding HSP fusion proteins. These DNA constructs encode gp96 fusion proteins with the antigenic peptide fused directly to the C-terminus of recombinant *mus musculus* gp96 (GRP94) by site specific mutagenesis. We then cloned the gp96 fusion protein into the eukaryotic expression vector pCI. We tested 4 DNA constructs expressing different gp96-fusion proteins, using 2 immuno-dominant *L.monocytogenes* epitopes, LLO, and p60, as fusion partners: LLO 91-99 (referred to as LLO-99 in the following), LLO 82-104 (referred to as LLOe), p60 217-225 (referred to as p60-217), and p60 212-230 (referred to as p60e). Since the cellular processing of HSP complexes has not been firmly elucidated, we constructed elongated versions of the originally described nonamer epitopes LLO 91-99 and p60 217-225, such that proteasome processing would be less likely to destroy the original peptide epitope sequence. Naked DNA vaccine constructs encoding gp96-fusion proteins were applied intramuscularly 3 times in total, with an interval of 3 weeks between each vaccination.

Immunization Using Gp96-peptide Fusion Proteins:

Application of Gp96-peptide Fusion Proteins:

Two routes of application were envisaged for the administration of fusion proteins. First, DNA immunization and second, protein immunization. For the DNA immunzation, DNA was purified from *E.coli* using endofree plasmid purification protocols from Qiagen. DNA vaccines were administered intramuscularly in the left and right hind leg of mice at a dose of 50µg per leg. DNA was applied three times in three-week-intervals. Mice were challenged for survival with 5×10^{4} *L. monocytogenes* (5xLD50), intravenously, 2-3 weeks after the 2nd boost. In addition, peptide specific T cell activation was measured by IFN-γ production using Elispot analysis. ^{51}Cr release assays were performed to assay peptide specific lysis and the production of CTL. The work described here will focus only on the DNA vaccination data, as this was the focus of this thesis project.

DNA-immunization Schedule:

prime	1st boost	2nd boost	challenge	read-out
day -61	day -41	day -21	day 0	days 7 through 28

Results of DNA-immunization:

Peptide Specific IFN-γ Response Induced by DNA Vaccination with Gp96 Fusion Proteins

We first analyzed peptide specific IFN-γ responses in vaccinated mice using a standard Elispot assay between day 7 and 28 after the 2nd boost (figure 8). Spleen cells were re-stimulated with peptide for 2 days and used as effector cells. P815 and J774 cells were pulsed with peptide for 2 hours before the assay and used as APC. Naive control, pCI vector control, and gp96 alone induced negligible IFN-γ responses. In contrast, on day 14 after the 2nd boost, all gp96-fusion proteins induced significant IFN-γ responses when restimulated either with HKL or homologous peptide antigen, as compared to negative controls ($p<0.05$). Gp96-p60e and gp96-p60-217 induced equal IFN-γ responses when restimulated with HKL or homologous antigen, which were both significantly higher than negative controls ($p<0.05$). In the case of gp96-LLOe and gp96-LLO-99 both constructs induced IFN-γ responses which were significantly higher than negative controls when restimulated with either HKL or homologous antigen ($p<0.05$). However, gp96-LLOe induced a significantly higher response than gp96-LLO-99 when restimulated with homologous antigen ($p<0.05$). The IFN-γ response on day 21 after the 2nd boost was markedly lower than on day 14 (data not shown).

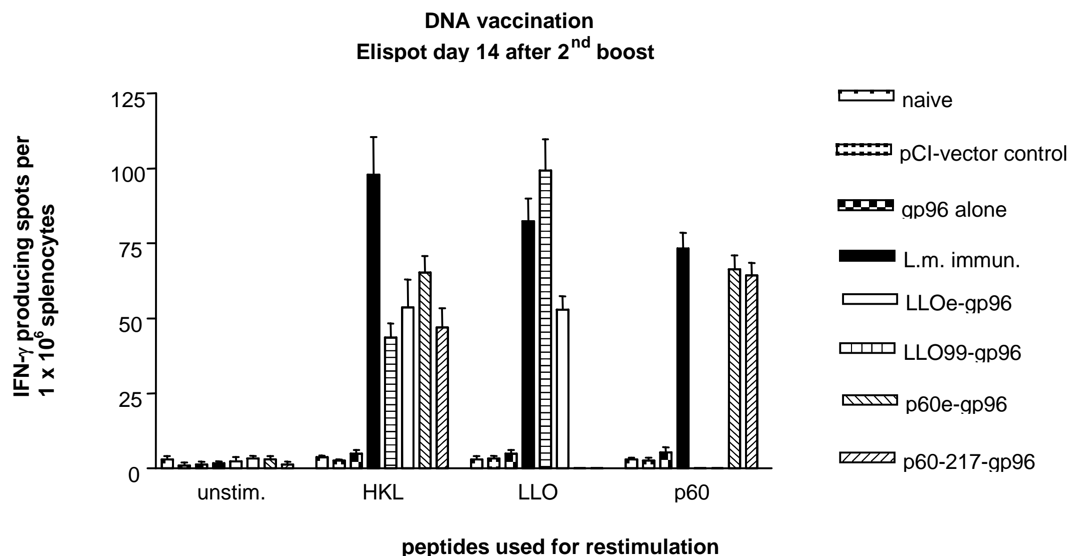


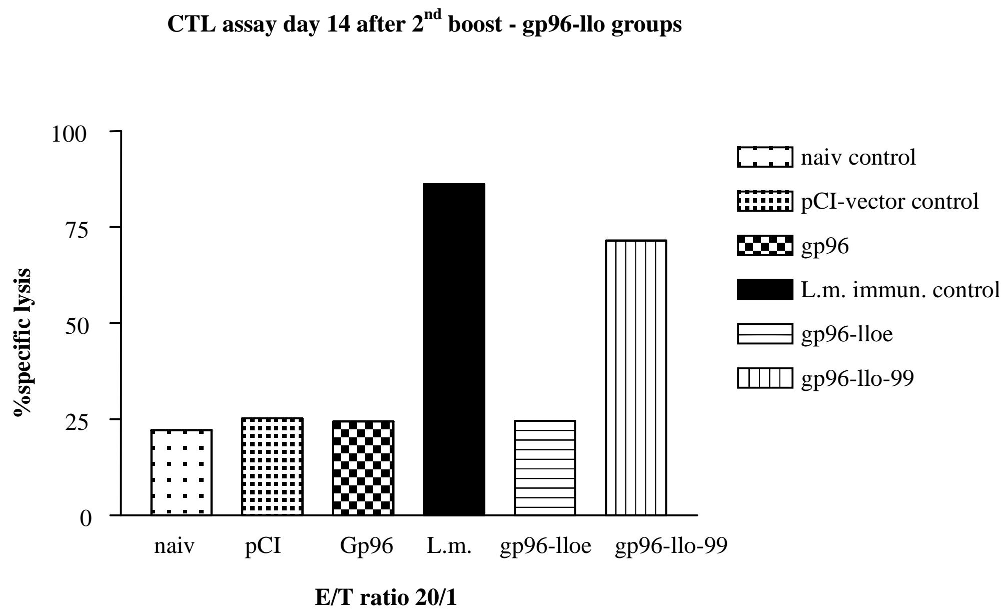
Fig. 8. Peptide specific IFN-γ responses in DNA vaccinated mice

IFN-γ responses were determined by standard Elispot assays after the 2nd boost. (a) IFN-γ response 14d after 2nd boost (LLOe/p60e:gp96 fused to elongated peptides, LLO-99/p60-217: gp96 fused to nonamer peptides). Spleen cells were restimulated with either Heat killed Listeria (HKL), LLO-91-99 peptides (LLO), or p60-217-225 (p60) peptides, in addition to unstimulated controls (unstim.). Naive control, pCI vector control, and gp96 alone induced negligible IFN-γ responses. All gp96-antigen fusion constructs induced significant IFN-γ responses when restimulated either with HKL or homologous peptide, as compared to negative controls ($p<0.05$). Gp96-p60e and gp96-p60-217 caused equal IFN-γ release when restimulated with HKL or homologous peptide, which were both significantly higher than controls ($p<0.05$), and did not differ from each other significantly. Also gp96-LLOe and gp96-LLO-99 induced IFN-γ responses which were significantly higher than negative controls when restimulated with either HKL or homologous antigen ($p<0.05$). However, gp96-LLOe induced a significantly higher response than gp96-LLO-99 when restimulated with homologous antigen ($p<0.05$). Representative results from 1 of 2 experiments with similar results. Shown are mean +/- standard deviations (X+/- SD) of 3 mice.

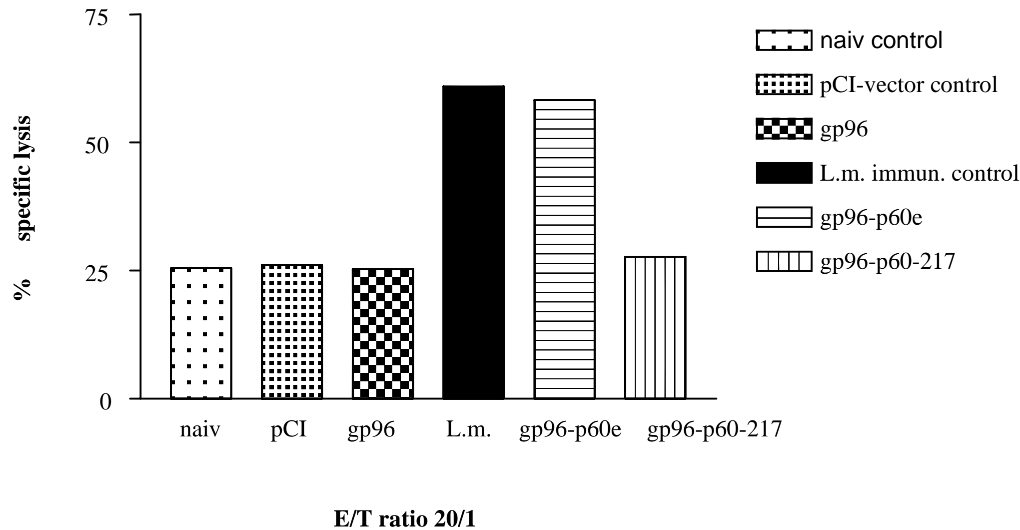
Peptide Specific CTL Response Induced by DNA Vaccination with Gp96 Fusion Proteins

Second, we analyzed the induction of a peptide specific CTL response between day 7 and day 28 after the 2nd boost. Spleen cells were re-stimulated with peptide in the presence of irradiated, naive spleen cells as APC for 5 days and used as effector cells. Peptide specific CTL activity was measured in a standard ^{51}Cr release assay using peptide pulsed P815 cells as targets (figure 9a, and 9b). Spleen cells from mice vaccinated with gp96 alone did not induce a measurable CTL response. The CTL response induced by the gp96-p60-e and gp96-LLO-99 constructs on day 14 after the 2nd boost was profound and comparable to the CTL response

induced by *L. monocytogenes* infection. In contrast, the gp96-p60-217 and the gp96-LLO-e constructs induced only marginal peptide specific lysis not exceeding the lysis observed for the control groups. The CTL responses measured on day 21 after the 2nd boost did not exceed the lysis observed for the control groups (data not shown).



a.



b.

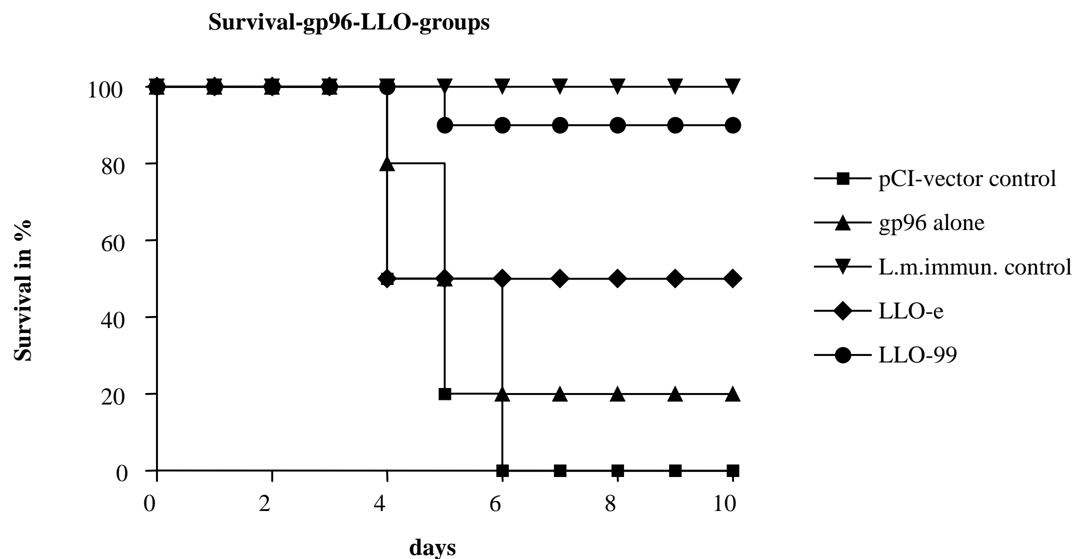
Fig.9. a-b **Induction of a peptide specific CTL response in DNA vaccinated mice**

CTL response 14d after 2nd boost. (a) P815 restimulated with LLO peptides (b) P815 restimulated with p60 peptides (abbreviations as described in fig. 1). For reasons of comprehensibility, the figures show specific target cell lysis only at E/T ratio 20:1, although killing was measured over a range of ratios from 60:1 to 2:1 at 3-fold dilutions. Representative results from 1 of 2 experiments with similar results.

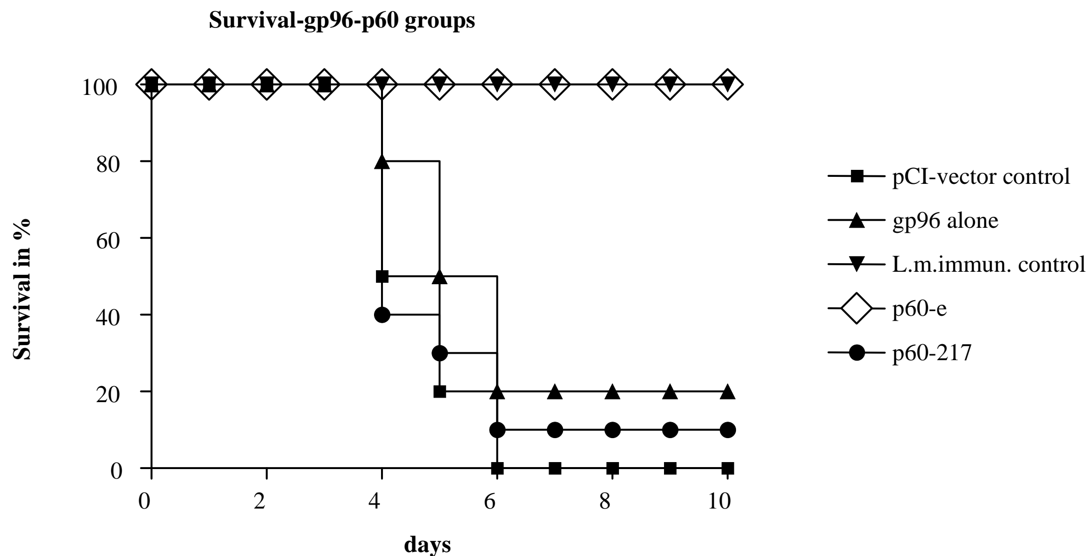
Protection Against Lethal *L.monocytogenes* by DNA Vaccination with Gp96-fusion Proteins

We next determined whether HSP-based DNA vaccination was able to confer protective immunity against an otherwise lethal dose of *L.monocytogenes* (figure 10a, and 10b). All 4 gp96-fusion protein constructs were tested. As expected, the gp96 alone and the pCI-vector control did not protect against lethal challenge with *L.monocytogenes*. The gp96-p60-e construct induced complete and the gp96-LLO-99 construct induced almost complete protection against *L.monocytogenes*, and in both cases protection was significant ($p<0.05$). The protection induced by gp96-LLO-99 was significantly higher than the negative control ($p<0.05$), and there was no significant difference between the gp96-LLO-99 construct and the

positive control (p>0.05). Though the gp96-LLOe construct afforded 50% protection, this was not significantly higher than the protection induced by the negative control (p>0.05). The gp96-p60e construct induced equal protection as compared to the positive control, and significantly higher protection than the negative control (p<0.05). However, there was no significant difference between the protection induced by the gp96-p60-217 construct and the negative control (p>0.05). Interestingly, the same 2 HSP-fusion protein constructs for which we observed the most efficient induction of peptide specific CTL (see figure 2) were able to induce statistically significant protection against a normally lethal dose of *L.monocytogenes*.



a.



b.

Fig.10. a-b Protection against L.monocytogenes. by DNA vaccination

Mice were challenged with 5 x10^4 *L.monocytogenes* i.v. 3 weeks after the 2nd boost (abbreviations as described in fig. 1). Survival was monitored for 10 days. The pCI vector control and the gp96 alone induced significantly lower protection, as compared to the positive control (vaccination with viable *L.monocytogenes*) ($p<0.05$). (a) gp96-LLO constructs. The protection induced by gp96-LLO-99 was significantly higher than negative controls ($p<0.05$), and there was no significant difference between the gp96-LLO-99 construct and the positive control ($p>0.05$). Though the gp96-LLOe construct afforded 50% protection, this was not significantly higher as compared to the negative control ($p>0.05$). (b) gp96-p60 constructs. The gp96-p60e construct induced equal protection as compared to the positive control, and significantly higher protection than the negative control ($p<0.05$). However, there was no significant difference between the protection induced by the gp96-p60-217 construct and the negative control ($p>0.05$). Ten mice were used per group. Representative results from 1 of 2 experiments with similar results.

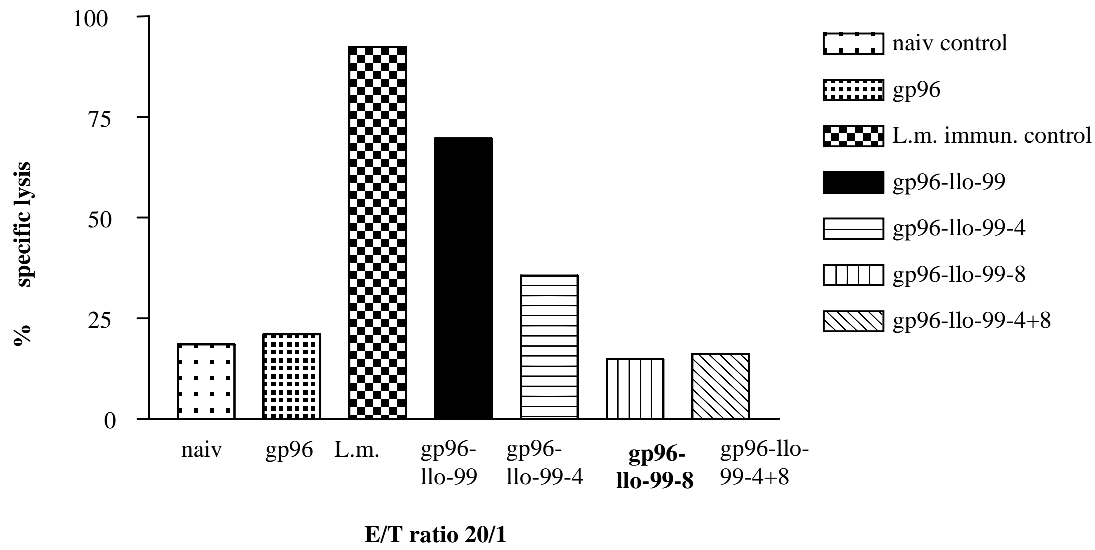
Which T-cell Population is Responsible for the Observed Cellular Response and the Protective Immunity?

Finally, we wanted to define the T-cell population critical for the cellular response to vaccination. To this end, we depleted CD4+, CD8+, or both CD4+ and CD8+ T-cells using the appropriate mAb before the 2nd boost. Results from CTL assays performed between day 7 and day 28 after the second boost, as well as survival studies, revealed that the CTL response and protective immunity were mediated primarily by CD8+ T-cells, and largely independent of CD4+ T-cells (see figure 11a, which shows day 14 results).

In the protection assay (figure 11b, and 11c), as expected, there was no significant difference between the protection afforded by the *L.monocytogenes* vaccinated positive control and the gp96-LLO-99 construct in non-depleted mice ($p>0.05$). Also, there was no significant difference between mice vaccinated with gp96-LLO-99 and depleted of CD4+ T cells, and non-depleted mice ($p>0.05$), and both groups showed a significantly higher survival than the negative control ($p<0.05$). However, the protection induced in mice immunized with gp96-LLO-99 and depleted of either CD8+ T cells, or CD4+ plus CD8+ T cells was significantly reduced as compared to the survival observed for the non-depleted mice ($p<0.05$). Vaccination with gp96 alone did not induce significant protection as compared to the negative control ($p>0.05$) (figure 11b).

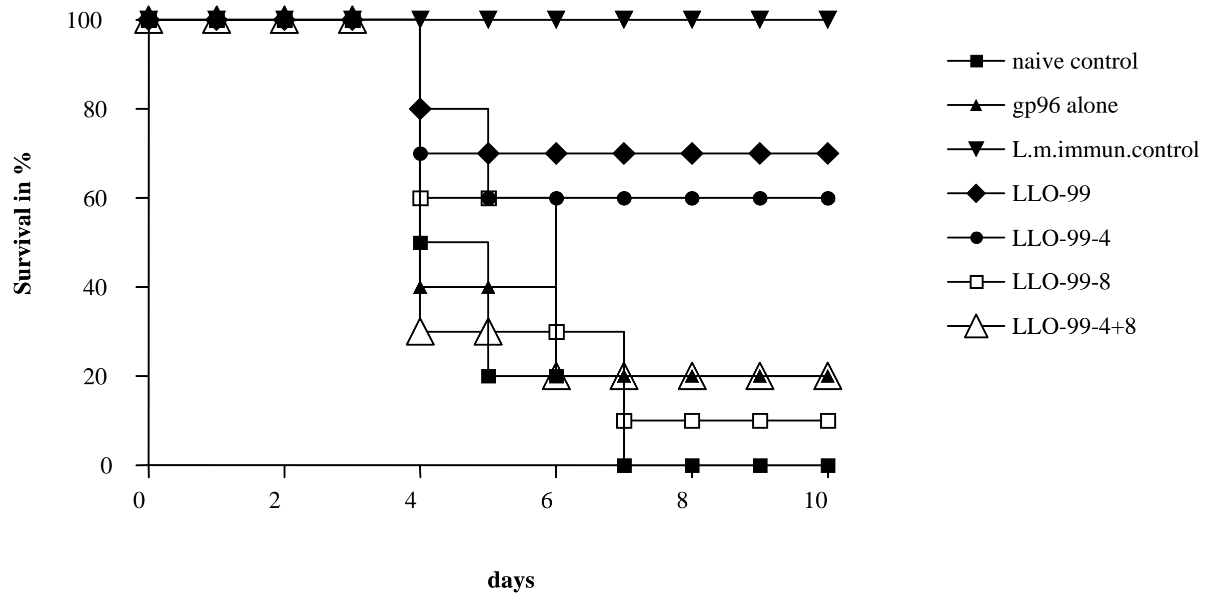
With regard to the protection afforded by the gp96-p60-e construct (figure 11c), there was no significant difference between the protection afforded by the *L.monocytogenes* vaccinated positive control and the gp96-p60-e construct in non-depleted mice ($p>0.05$). Protection in mice vaccinated with gp96-p60-e and depleted of CD4+ T cells, and non-depleted mice did not differ ($p>0.05$), and both groups showed a significantly higher survival than the negative control ($p<0.05$). However, the protection induced in mice vaccinated with gp96-p60-e and depleted of either CD8+ T cells, or CD4+ plus CD8+ T cells was significantly reduced as compared to the non-depleted mice ($p<0.05$). Thus, both for gp96-LLO and for gp96-p60 vaccination, depletion of CD8+ T cells abrogated protection, while depletion of CD4+ T cells did not do so. We conclude that DNA constructs encoding gp96/peptide-fusion proteins are capable of inducing potent CD8+ T cell responses, which protect against an intracellular bacterial pathogen.

CTL assay day 14 after 2nd boost - CD4/CD8 Tcell depleted groups

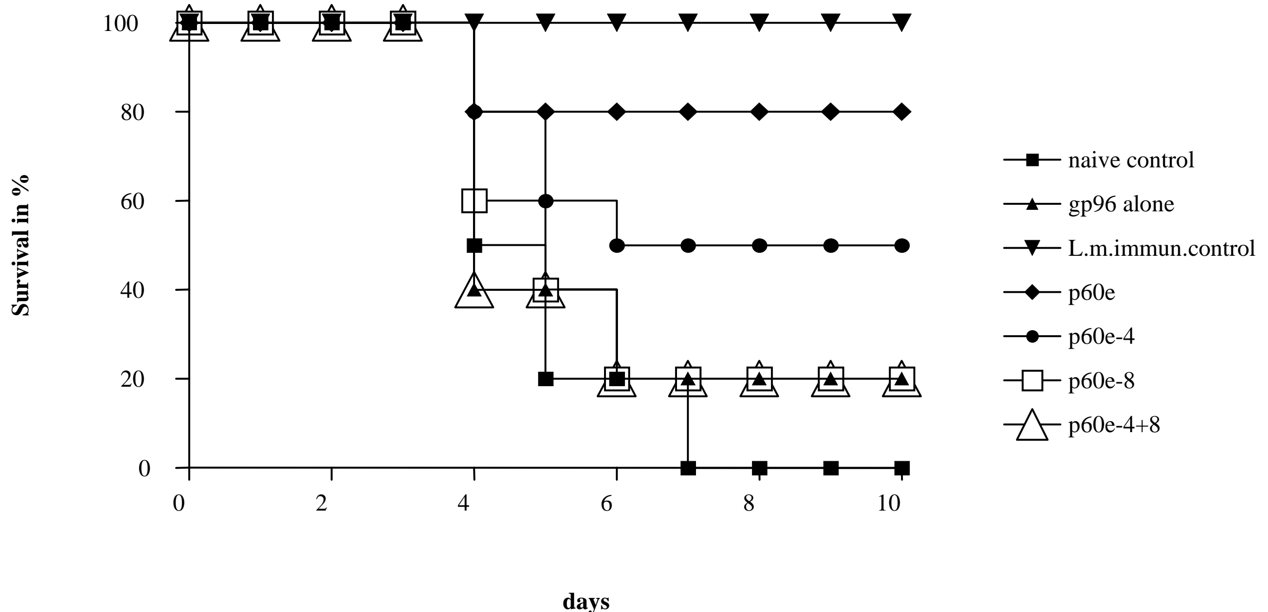


a.

Survival after CD4+/CD8+ T cell depletion- gp96-LLO-99 groups



b.



c.

Fig.11 a-c Phenotype of T-cell population critical for DNA vaccine induced immunity

CD4+, CD8+, or both CD4+ and CD8+ T-cells were depleted using the appropriate mAb before the 2nd boost.
(a) CTL assay day 14 after 2nd boost, using P815 restimulated with LLO peptide. For reasons of comprehensibility, the figures show specific target cell lysis only at E/T ratio 20:1, although killing was measured over a range of ratios from 60:1 to 2:1 at 3-fold dilutions. Representative results from 1 of 2 experiments with similar results.
(b) Protection assay with gp96-LLO constructs. There was no significant difference between the protection afforded by the positive control and the gp96-LLO-99 construct in non-depleted mice ($p>0.05$). Similarly, protection in mice vaccinated with gp96-LLO-99 and depleted of CD4+ T cells, did not significantly differ from non-depleted mice ($p>0.05$), and both groups showed a significantly higher survival than the negative control ($p<0.05$). However, the protection induced in mice vaccinated with gp96-LLO-99 and depleted of either CD8+ T cells, or CD4+ plus CD8+ T cells was significantly lower than the survival observed for non-depleted mice ($p<0.05$). Protection in CD8+ T cell, or the CD4+ plus CD8+ T cell depleted mice did not differ from the negative control ($p>0.05$). Vaccination with gp96 alone also failed to afford significant protection as compared to the negative control ($p>0.05$).
(c) Protection assay with gp96-p60 constructs. Protection in the positive control and the gp96-p60-e vaccinated non-depleted mice was equally high ($p>0.05$). Protection in mice vaccinated with gp96-p60-e and depleted of CD4+ T cells, and that in non-depleted mice did not differ significantly ($p>0.05$), and both groups showed a significantly higher survival than the negative control ($p<0.05$). However, the protection in mice vaccinated with gp96-p60-e and depleted of either CD8+ T cells, or CD4+ plus CD8+ T cells was significantly reduced as compared to non-depleted mice ($p<0.05$). Neither the CD8+ T cell, nor the CD4+ plus CD8+ T cell depleted mice differed from the negative control ($p>0.05$). Protective immune responses were determined as described in legends to figure 10 (LLO-99:no depletion, LLO-99-4:depletion of CD4+, LLO-99-8:depletion of CD8+, LLO-99-4+8:depletion of both CD4+ plus CD8+ T-cells, abbreviations are the same for gp96-p60e construct). For the CTL assay, 3 mice were used per group, for the protection assay, 10 mice per group were used (see also Material and Methods). Representative results from 1 of 2 experiments with similar results.

Discussion

DNA-Vaccination with Gp96 Peptide Fusion Proteins

We describe here efficacious antibacterial protection afforded by a naked DNA vaccine encoding gp96-peptide fusion proteins. Previous studies in virus and parasite model systems have focused on the use of pathogen-derived HSP as vaccine carriers, most frequently cytosolic bacterial HSP60 or HSP70 (Liu et al., 2000; Harmala et al., 2002; Anthony et al., 1999). *In vitro* loaded HSP/peptide complexes or HSP-fusion proteins were applied subcutaneously or intradermally as purified recombinant proteins. Alternatively, recombinant HSP-fusion proteins were applied intramuscularly as naked DNA (Chen et al., 2000; Lima et al., 2003). The basic rationale behind the use of HSP as vaccine vehicles is based primarily on two observations: First, the ability of HSP to cross-prime and to induce dominant activation of the MHC I restricted CD8+ T-cells (Udono et al., 1994; Suto et Srivastava, 1995; Blanchere et al., 1997; Robert et al., 2002), and second, the strong general immuno-stimulatory quality of pathogen-derived HSP (Harmala et al., 2002; Van Eden et al., 2003).

The application of endogenous HSP was avoided initially because of the anticipated risk of inducing unwanted autoimmune responses to the HSP. However, studies have shown that probably due to the high sequence homology between endogenous and pathogen-derived HSP, the induced cellular response is similar (Paul et al., 2000; Van Eden et al., 2003). We chose endogenous gp96, because we aimed to use the HSP not as an antigen, but solely as a carrier for the fused listerial antigen. The ability of HSP to cross prime and to activate the MHC I antigen presentation pathway reserved usually for cytosolic, as opposed to endosomal antigens, makes them particularly interesting vaccine vehicles to combat intracellular

pathogens, where the induction of a potent CD8+ T-cell response is essential (Kaufmann, 2003). Because HSP shuttle antigens towards the MHC I antigen presentation pathway, we expected that the use of HSP as a vaccine vehicle would promote CD8 T cell responses against the dominant listerial epitopes.

In our study we observed induction of a profound peptide specific IFN-γ response and peptide specific CTL activity for 2 gp96-fusion protein constructs: gp96-LLO 91-99, and gp96-p60-212-230. The 2 other constructs tested were able to induce a peptide specific IFN-γ response, but negligible peptide specific CTL lysis. Interestingly, those 2 constructs, which induced a significant CTL response, also induced efficient protection against challenge with an otherwise lethal dose of *L.monocytogenes.* Our findings argue against antigen-specific IFN-γ production as a robust correlate of protection in experimental listeriosis, and point to CTL responses as the more reliable surrogate.

We can not explain at this point what caused the superior efficacy of the gp96-LLO-91-99 and gp96-p60-212-230 constructs. However, we assume that the reason lies in the processing of the gp96 constructs in the cell and the integrity of the resulting immuno-dominant peptide epitope. Proteasome cleavage site prediction programs and analysis of immunoproteasome cleavage motifs by MALDI and mass spectrometry may prove helpful in understanding the intracellular processing and efficacy of individual HSP fusion proteins (Toes et al., 2001). Analyses of the T-cell populations responsible for the observed IFN-γ and CTL responses are consistent with the assumption that protective immunity induced by HSP-based DNA vaccination is mediated largely by CD8+ T-cells. This is in agreement with previously published data by others in parasite and viral model systems (Blanchere et al., 1997; Anthony et al., 1999; Robert et al., 2002). We can not formally exclude, however, a contribution of CD4+ T cells to the immune response we observed. Also, we did not analyze the contribution of CD4+ T cells during the priming phase (Cho et al., 2000a; Hamilton et al., 2001; Shedlock et al., 2003a and 2003b; Kaech et Ahmed, 2003). However, the use of

dominant listerial MHC I epitopes, and the lack of listerial MHC II epitopes in our system make a major contribution of listeria specific CD4+ T cells unlikely.

We observed no measurable IFN-γ and CTL response to immunization with gp96 DNA without peptide. This indicates a low immuno-stimulatory potential of endogenous gp96 at the dose applied, and implies the lack of autoimmune reactivity to the vaccine carrier employed. We conclude that endogenous gp96 served solely as a carrier molecule for the protein or peptide associated with it, and was responsible, albeit by a mechanism not yet fully understood, for shuttling the associated peptides into the MHC Ia presentation pathway.

Our results confirm that gp96 can be used as an efficient and safe vaccine carrier. They give credit to the observations that were obtained previously, mostly by the use of purified gp96. These studies used purified HSP prepared from bacterial extracts, and so it remains to be verified in how far the observed effects can be ascribed to contaminating endotoxin, CpG or ConA. Our study was based on DNA purified using a stringent endotoxin removal protocol. We do not assume eventual endotoxin contamination to be responsible for the observed cellular immune response for two reasons: First, the fusion proteins are generated *de novo* in the cell after transfection with naked DNA. Second, eventual endotoxin contamination should have induced a more significant unspecific immune response to immunization with all constructs equally, and the gp96-minus peptide construct in particular. Much attention has been focused on how to enhance antibody and T cell responses to the antigen encoded in DNA vaccines. A prerequisite for an effective adaptive immune response, however, is the activation of an innate immune response, particularly in immuno-compromised patients. As mentioned earlier, gp96 appears to function as a danger signal when released from affected cells. The gp96 vaccine vehicle can therefore activate an innate immune response, while the antigen fused to gp96 induces a specific adaptive T cell response.

Comparison With Other Effective Vaccines Against Intracellular Pathogens

Several other efficient vaccine systems have been established, using i.) subunit vaccines, or preparations based on killed microorganisms, and ii.) live vaccines based on attenuated and / or recombinant bacterial strains (Kochi et al., 2003; Moron et al., 2004). Two important questions must be addressed in the evaluation of an efficient vaccine candidate: i) the strength and specificity of the cellular response induced, and ii) the generation of a sustained memory response. The successful vaccination of CD4 T cell deficient immuno-compromised individuals (such as in HIV) poses an additional challenge, as it requires safety, and CD4 T cell independence, without limiting establishment of a robust memory T cell population.

Vaccines consisting of killed microorganisms or purified microbial components appear to provide a safe mechanism for immunization. However, their efficiency is generally low. Functional memory T cell development is dependent on effector cell induction. The main defect in response to killed microorganisms is inefficient induction of clonal expansion. This failure is due to a contracted, but co-stimulation dependent activation phase, resulting in a rapid, but abortive T cell growth (Janeway et al., 2001; Moron et al., 2004).

There are three live bacterial strains currently licensed for application in humans: *Mycobacterium bovis Bacille Calmette Guerin* (BCG), *Salmonella typhi*, *Ty21*, and *Vibrio Cholerae, CVD103 HgR* (Mollenkopf et al., 2001; Gentschev et al., 2001; Moron et al., 2004). The response to live microorganisms is generally characterized by a protracted early T cell sequestration in lymphoid tissues. Because live vaccines provide stronger effector induction and a longer initial response, they also provide a better memory (Janeway et al., 2001; Moron et al., 2004). Attenuated live vaccines are usually designed such that they do not cause serious infection, but they are considered potentially dangerous for an immuno-compromised individual. Efficient experimental live attenuated vaccines have been designed using mainly

Listeria, and *Salmonella* (Hess et al., 1996a; Gentschev et al., 2002; Spreng et al., 2003). They have also been used as vectors to deliver plasmid DNA vaccines (Catic et al, 1999; Gentschev et al., 2000). W. Goebel, S.H.E. Kaufmann and others have developed a series of vaccines in which they use attenuated strains of Listeria and Salmonella as carriers (Gentschev et al., 1996a and 1996b; Gentschev et al., 2001; Darji et al., 2003). Their primary purpose was to develop a vaccine model against intracellular pathogens, which allows efficient transport of a variable antigen to the phagosome or cytosol. They used two different approaches to achieve this: i.) expression of antigens in the bacteria and secretion of these antigens into the phagosome, via the α-hemolysin secretion system of *E.coli*, or the cytosol, via the listeriolysin of *L.monocytogenes* (Gentschev et al., 1996b; Dietrich et al., 2001; Spreng et al., 2003). ii.) transport of plasmid DNA encoding the antigen into the cytosol by attenuated bacteria (Gentschev et al., 2000). In addition, they investigated the efficiency of attenuated bacteria expressing both cytolysin and plasmid DNA (Gentschev et al., 2001). Vaccination using both approaches has proved successful in the murine listeria model, and in all cases those bacterial strains expressing listeriolysin were significantly more efficient at shuttling the antigen to the cytosol and inducing a cellular immune response (Gentschev et al., 2001). Those systems which lead to a sustained presence of the antigen in the phagosome as well as efficient shuttling of the antigen to the cytosol lead to a strong presentation via both the MHC class I and class II pathways. These models may be particularly useful when the induction of strong CD4 and CD8 T cell responses are required for clearance of and protection against a pathogen. In the light of current discussion regarding the possible importance of CD4 T cell help in the establishment of a memory response at least in the priming phase, further study of the type and magnitude of memory responses achieved by these vaccine models is of great interest.

Rationale for an Efficient Cellular Vaccination – Perspectives for Improvement of HSP (gp96) Vaccines

We describe here efficacious antibacterial protection afforded by a naked DNA vaccine encoding gp96-peptide fusion proteins. Protection was accompanied by profound antigen specific IFN-γ secretion and CTL activation. CD8+ T-cells were shown to be crucial for all 3 activities: protection, IFN-γ, and CTL. Still, many questions regarding the mechanism of action of this vaccination model remain unanswered. Which cell type ensures a successful vaccination by expressing gp96-complexes? Different scenarios are possible: Should APC or muscle cells be transfected? An intradermal or subcutaneous application may allow for transfection of Langerhans cells or dendritic cells. Is the gp96 complex expressed in transfected cells secreted? How is the gp96 complex presented to surrounding APC?

The establishment of a strong immunological memory is an essential requirement for a successful vaccination. Memory has been defined as the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously, and reflects the pre-existence of a clonally expanded population of antigen-specific lymphocytes (Janeway et al., 2001). Memory responses also differ qualitatively from primary responses. Though much remains unknown about the mechanisms regulating T cell memory, there have been some recent advances (Kaech et al., 2002; Schluns et Lefrancois, 2003; Geginat et al., 2003): Memory T cells have been further differentiated into central and effector memory T cells (Sallusto et al., 1999). The initiation of a primary immune response leads first to the induction of T effector cells and later to the induction of effector memory T cells in the periphery. Effector memory T cells may then develop further into central memory T cells, which play an important role in immune surveillance and in the memory response initiated after secondary infection. Central memory T cells (T_{CM}) reside in lymphoid organs and display L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7) on their cell surface. These

surface molecules play an essential role in lymph node homing, as they interact with surface molecules of endothelial cells mediating attachment. Effector memory T cells (T_{EM}) reside in peripheral tissues and have downregulated CCR7 expression, but upregulated CCR2 and CCR5 expression (Sallusto et al., 1999; Schluns et Lefrancois, 2003). CCR2, CCR5, and CCR7 are surface proteins that are involved in cell adhesion and chemotaxis, thus promoting migration to either the peripheral tissues or the lymph nodes (Kaech et al., 2002; Moser et al., 2004).

In experimental models with dominant antigen, clonal expansion leads to a 1000 fold increase in precursor frequency of antigen specific T cells. Differentiation of naive into memory T cells involves a reprogramming of gene-expression profiles by changes in the profiles of active transcription factors. As a result, naive and memory T cells exhibit a different pattern of surface molecules (Schluns et Lefrancois, 2003; Kaech et al, 2002). In contrast to naive T cells, IFN-γ and cytotoxic molecules, such as perforin, are constitutively expressed in T_{EM} and T_{CM}. How are these memory T cell populations regulated? IFN-γ appears to play a role in the initial stimulation, as well as in the downregulation of the effector T cell population after infection (Sallusto et al., 1999; Schluns et Lefrancois, 2003). This implies an important function in the control of infection and in the regulation of effector T cell numbers. Cytokines that regulate CD4 T memory cells have not yet been well defined (Geginat et al., 2001), but IL-2 and IL-15 have been suggested to influence the expression of CCR7 and CD62L on their cell surface, which may influence their migration pattern (Seder et Ahmed, 2003; Foulds et al., 2002). Recent studies show that IL-2, IL-15 and IL-7 may make an important contribution to the regulation of CD8 memory T cell proliferation (Seder et Ahmed, 2003; Geginat et al., 2003; Foulds et al., 2002). Their precise role, however, remains to be elucidated.

We have described previously the different roles of CD4 and CD8 T cells in the response to listerial infection. What are the differences in CD4 and CD8 T memory cell

populations, and what determines their size? The precise mechanisms that control the rate and extent of CD4 and CD8 T memory cell proliferation are unknown. We know however, that the magnitude of proliferation is dependent on the abundance of antigen: the greater the antigen load, the larger the number of effector T cells (Foulds et al., 2002). The threshold for CD8 T cell activation appears to be much lower than for CD4 T cell activation. This may be due to extrinsic factors causing a faster accumulation of TCR signals, thus reducing the requirement for co-stimulation (Seder et Ahmed, 2003; Foulds et al., 2002; Lanzavecchia et Sallusto, 2002). When the activation threshold of CD8 T cells is overcome, they initiate a programmed proliferative response. CD8 T cells have a higher proliferative potential than CD4 T cells, as the rise in number of CD8 T cells is substantially higher. CD4 T cells have a slower rate of division and proliferation peaks approximately 1-2 days after CD8 T cells (Foulds et al., 2002). CD8 T memory cell populations are maintained for a long time due to homeostatic cell proliferation. In contrast, CD4 T memory cell populations decrease slowly over time. IL-15 has been implicated in the induction of CD8 T memory cell proliferation, but does not affect the maintenance of CD4 T memory cells (Seder et Ahmed, 2003; Foulds et al., 2002; Lanzavecchia et Sallusto, 2002).

What is known about T memory cell lineage? The lineage of T memory cell development is not fully understood. There are currently two basic models: i.) T memory cells are direct descendants of T effector cells. ii.) T memory cells arise from a second independent lineage (Wherry et al., 2003; Badovinac et Harty, 2003; Kaech et al., 2002). The use of CRE/LOXP and adoptive transfer systems have provided data in support of the first model (Kaech et al, 2002). Both studies indicate a maintenance of T effector cells in the T memory cell pool after the contraction phase. Thus the T memory cells appear to be formed directly from the T effector cells (Wherry et al., 2003; Badovinac et Harty, 2003; Kaech et al., 2002; Hu et al., 2001). The size of the CD8 T memory cell pool has been shown to correlate directly with the size of the T effector cell population. However, recent observations suggest that

some T memory cells may develop without passing through an effector stage. This would imply that T memory cell development is nonlinear and results in two different subsets (Kaech et al, 2002). Many aspects of the T_{CM} - T_{EM} model require further examination: Most importantly, their developmental relationship requires more precise classification. What is the role of these subpopulations in the secondary immune response? Can antigen stimulation induce T_{CM} to develop into T_{EM}? Do T_{CM} and T_{EM} provide different levels of protection?

What are the implications for vaccine development? Because T cell memory is determined by magnitude of expansion and the extent of effector cell death, strategies that interfere with cell death may enhance T cell memory in response to vaccination. Another concern is the condition under which T cell activation occurs: High levels of IL-4, IL-7, and IL-15 can increase the numbers of antigen specific CD4 and CD8 T cells, and IL-15 can enhance protective immunity (Kaech at al., 2003; Schluns et Lefrancois, 2003). It remains to be investigated how long-lived the effects of these cytokines are on T memory cells (Messi et al., 2002; Barber et al., 2003). The number of T memory cells formed is largely dependent on burst size. Therefore it is important for a vaccine to induce as large an effector T cell population as possible. Killed or subunit vaccines frequently provide a lower level of antigen, that is short-lived. In contrast, DNA-vaccines are more stable and may provide a higher level of antigen. Still, the precise duration and magnitude of antigen expression by a DNA-vaccine, as well as the types of effector and memory T cells induced remain to be elucidated. Further analysis of the current T memory cell models is required to delineate exactly those aspects of their regulation that could be integrated into the design of optimized vaccines.

It has become possible to enumerate CD8 T cells by staining them with tetrameric MHC-peptide complexes (Busch et al., 1998; Appay et al., 2002; Ravkov et al., 2003). Cell surface markers specific for memory T cells are also the focus of recent investigation (Ravkov et al., 2003). Is memory dependent on the persistence of antigen? Memory T cells are thought to persist either: i.) because antigen has programmed them for a longer lifespan, ii.) because a

low level of residual antigen preserves them by repetitive sub-threshold signaling, or iii.) because the process that allows naive T cells to survive in the periphery acts more effectively on memory T cells (Janeway et al., 2001). Compared to other formulations, DNA vaccines may allow a prolonged persistence of antigen in the organism.

Are memory responses in *L.monocytogenes* dependent on CD8+ or CD4+ T-cells, or both? This question has been approached in a series of recent studies. Most investigations revealed CD4+ T-cells to be essential during the priming, but not during the recall response to infection (Hamilton et al., 2001; Shedlock et al., 2003a and 2003b; Badovinac et al., 2003; Kaech et Ahmed, 2003; Sun et Bevan, 2003). In our study, we depleted CD4+ T-cells or CD8+ T-cells directly before the 2nd booster immunization. Hence CD4+ T cells could have participated in the priming of CD8+ T cells. Yet CD4+ T-cells were not essential for recall and protective responses in our system. These insights may make it possible to design a vaccine uniquely suited for the vaccination of CD4 T cell deficient, immuno-compromised patients. However, it may be beneficial for a vaccine to induce CD4 T cell activation even if the clearance and protective immunity are largely CD8 T cell mediated. Dendritic cells for instance, require CD4 T cell help in order to stimulate CD8 T cells, even when using highly immunogenic antigens.

How could DNA vaccines be improved? The use of DNA in a vaccine leads to some safety concern, because of its possible integration into the genome of the host cell. The application of suicidal DNA-vaccines offer a promising perspective, as these vaccines would induce host cell apoptosis, reducing the risk of genome integration. Also, cell death can provide additional immuno-stimulation on account of the release of danger signals (Racanelli et al., 2004). CD8 + DC selectively phagocytose dying cells. If the phagocytosed antigen derived from apoptotic host cells can be cross-presented for cross-priming (Yrlid et Wick, 2000; Delneste et al., 2002; Schaible et al., 2003), the result would be a strong CD8 effector cell priming. It may be beneficial if DNA vaccines could be targeted directly to specialized

APC such as DC. Unfortunately, no efficient protocol for the direct transfection of DC has currently been established. DNA vaccination offers some other advantages over other strategies: An important benefit is the fact that one has the ability to deliver antigens that would normally be unstable or difficult to purify. A successful vaccine needs to trigger both innate and adaptive immune responses. For DNA vaccines, the crucial role of TLR in the induction of an immune response has recently been recognized (Moron et al, 2004). TLR9 is of particular interest, because it recognizes unmethylated CpG, and TLR3, because it binds double-stranded (ds) RNA. The recognition of ds RNA by intracellular PRR may be a reason for their high immunogenicity, as the cell signaling following PRR activation results in recruitment of lymphocytes. A strong innate immune response may also lower the threshold of activation for lymphocytes and thus assist in enabling rapid activation of an effective adaptive response.

Concluding Remarks

The immune response to infectious organisms depends on various features of the infectious process and varies between pathogens, their route of infection, and the specific host environment misused by the microbe. The resulting infection may be acute, chronic, or latent. In the case of intracellular pathogens, specifically *L.monocytogenes*, but also mycobacteria, therapeutic intervention focuses on the prevention of acute or chronic infection. The primary goal is the achievement of long-lasting immunological memory with a minimal vaccine dose and few side effects. Recent advances in molecular biology and cellular immunology have opened the door to novel strategies for vaccine development. Amongst these are the applications of antigenic peptide alone, or together with an adjuvant, application of naked DNA as well as various controlled-release preparations. Another approach suggests immunization with antibodies to cellular receptors mimicking the cell surface receptor the

pathogen uses for entry (reviewed in Sanz et al., 2004). This prevents subsequent invasion of the pathogen, as the antibody binds to the anti-receptor site on the pathogen. The construction of live attenuated organisms, as well as the design of vector systems for antigen delivery, has established itself amongst the more reliable and low risk methods available. Bacterial toxins and cytolysins delivery antigens directly to the cytosol, where they can access the classical MHC class I presentation pathway (Moron et al., 2004). There are two basic questions that arise when establishing HSP chaperones, and specifically gp96, as a candidate for effective vaccination. The first is directed at the specificity of the immune response induced, while the second focuses on advantages to other vaccination strategies. Since effective vaccination using gp96 peptide complexes is achieved, there are a number of interesting variations of the system worth consideration. These are based on the concept of using gp96 as a vector to introduce a designed set of antigens, or pharmaceutical agents. Another approach might suggest a complex capable of inducing endogenous upregulation of gp96 expression. Further elucidation of the mechanism of processing and presentation of gp96 antigen complexes, their cross-presentation, and the stimuli required for memory induction are required. These will allow for an optimization of this vaccine model.

There have been some clinical trials using HSP based vaccines (Chu et al., 2000; Janetzki et al., 2000). HSP vaccines were applied successfully in tumor therapy as recombinant protein vaccines (recombinant bacterial HSP60 or HSP 70), or as endogenous protein vaccines (tumor biopsy derived gp96), and have been so successful as to warrant commercial exploitation (Stressgen/Antigenics). In addition, HSP-based vaccines against Herpes Virus are currently being established (Stressgen). Preliminary results from tumor vaccination studies look promising and encourage a continued evaluation of the application of HSP vaccines with equal success in infection control.

Summary

Our results demonstrate that DNA vaccination with gp96-fusion proteins induces a potent listerial antigen specific CD8+ T-cell response, and confers protection against an otherwise lethal challenge with *L.monocytogenes.* Though many vaccines based on naked DNA, or bacterial carriers have been tested in the listeria model, they have not provided the same degree of protective immunity against lethal challenge (Hess et al., 1996a; Szalay et al., 1995; Fensterle et al., 1999; Lauvau et al., 2001). These observations suggest DNA vaccination with gp96-fusion proteins provides a promising strategy for achieving protective immunity against intracellular pathogens. More importantly, the fact that the efficacy of HSP-based vaccines is at least partially independent of CD4+ T-cells implies an intriguing potential for application in patients with CD4+ T-cell immune deficiencies. To further analyze the degree of independence of CD4+ T-cells in HSP vaccination, their role during priming with HSP-antigen constructs should be determined in the future. Further studies directed at elucidating the precise physiological and patho-physiological role of HSP and the regulatory and signaling circuits involved in the cellular response to HSP based vaccination, will benefit greatly the production of optimized HSP vaccines against intracellular pathogens.

Zusammenfassung:

Unsere Untersuchungen ergeben, daß DNA-Immunisierung mit gp96-Fusionsproteinen in der Lage ist, eine starke, antigen-spezifische CD8 T-Zell Antwort, sowie Schutz gegen eine normalerweise lethale Dosis *L.monocytogenes*, zu induzieren. Trotzdem viele auf nackter DNA, oder auf bakteriellen Tägersystemen basierende Vakzine gegen *L.monocytogenes* experimentell erprobt wurden, haben diese bisher keinen vergleichbaren Immunschutz gegen eine lethale Dosis *L.monocytogenes* geboten (Hess et al., 1996a; Szalay et al., 1995; Fensterle et al., 1999; Lauvau et al., 2001). DNA-Immunisierung mit gp96-Fusionsproteinen könnte daher eine vielversprechende Strategie zur Induktion eines Immunschutzes gegen intrazelluläre Pathogene darstellen. Als besonders interessant erweist sich die überwiegende CD4+ T-Zell - Unabhängigkeit des beobachteten Immunschutzes, bei der Immunisierung von CD4+ T-Zell immundefizienten Patienten. Es bedarf weiterführender Untersuchungen, um über die Rolle der CD4+ T-Zellen bei der primären Immunantwort auf gp96-Fusionsproteine Klarheit zu schaffen. Eine bessere Kenntnis der physiologischen und pathophysiologischen Funktion von HSP, wie auch der Signaltransduktionswege, welche nach Immunisierung mit HSP-Fusionsproteinen aktiviert werden, fördert sicher die Optimierung und Weiterentwicklung von HSP Vakzinen gegen intrazelluläre Pathogene.

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Declaration:

I herewith declare that the work described in this thesis was completed entirely on my own, and only with the assistance cited herein. No part of this dissertation was submitted previously for examination purposes.

Erklärung:

Ich erkläre hiermit, dass ich die vorliegende Dissertation selbständig, und nur unter Zuhilfenahme der genannten Literatur und der erwähnten Hilfsmittel, angefertigt habe. Diese Dissertation lag in keiner Form je einem anderen Prüfungsverfahren vor.

Unterschrift Datum

Ligand	Chaperone	Evidence provided
CD91	Gp96, Hsp90 Calreticulin	Binding of fluorescence-labeled Hsps correlate with expression of CD91 α2 macroglobulin, a physiological ligand of CD91, inhibits the presentation of peptides carried by Gp96, Hsp70 and calreticulin (Binder et al., 2000; Basu et al., 2001) These conclusions have been later contradicted by Berwin et al. (2002).
TLR2 / 4	Hsp70, Gp96	Following TLR2 / 4 transfection into 293 cells, purified Hsp70 becomes able to activate NFkB in a MyD88-dependent fashion (Asea et al., 2002; Vabulas et al, 2002a) Bone marrow-derived DCs lacking functional TLR2 / 4 exhibit reduced activation parameters: stress kinases, NFkB, cell surface expression of the costimulatory molecule CD86 (Vabulas et al., 2002b)
CD40	Hsp70	Transfection-induced increased expression of CD40 augments the binding of Hsp70-peptides complexes to the cell surface of a macrophage cell line, and their subsequent internalization (Becker al., 2002)
LOX1	Hsp70	CHO transfected with the scavenger receptor LOX1 become able to bind Hsp70-peptide complexes. A monoclonal antibody recognizing LOX1 interferes with the ability of DC to present the peptides carried by Hsp70 (Delneste et al., 2002).
Scavenger Receptor A (SRA)	Gp96	293 cells transfected with a cDNA encoding SRA become able to bind and internalize gp96-peptides complexes. Macrophages derived from SRA-/- mice are impaired in Gp96 binding and uptake (Berwin et al., 2003)

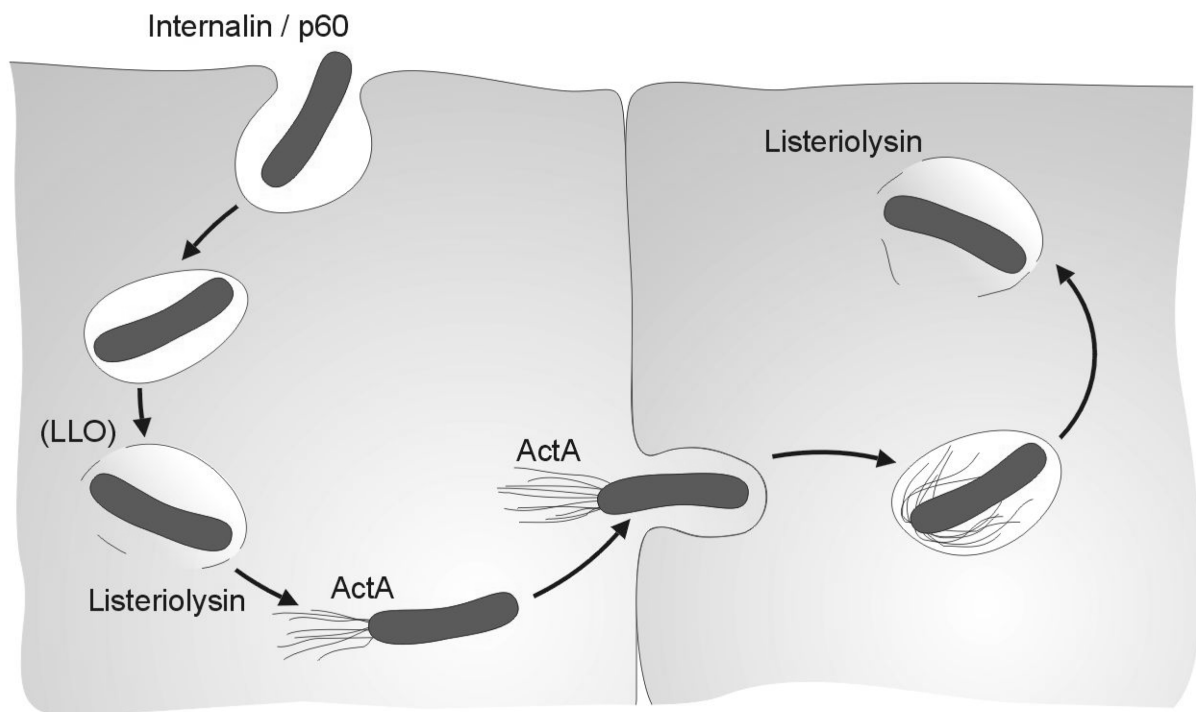

Figure III. Listeria Virulence.

Listeria virulence is determined by three components: i.) Two proteins, internalin A and B, are responsible for the receptor-mediated uptake of Listeria in epithelial cells. Though its exact role is not yet clearly defined, p60 may also promote cell entry. ii.) Listeriolysin (LLO), a pore forming toxin, which allows Listeria to egress from the phagosome into the cytosol, where it replicates. iii.) ActA, a cell surface molecule of Listeria, which induces a polar polymerization of host cell cytosolic actin monomers. This allows cell-to-cell transmission of bacteria, without exposing progeny to the extracellular environment.

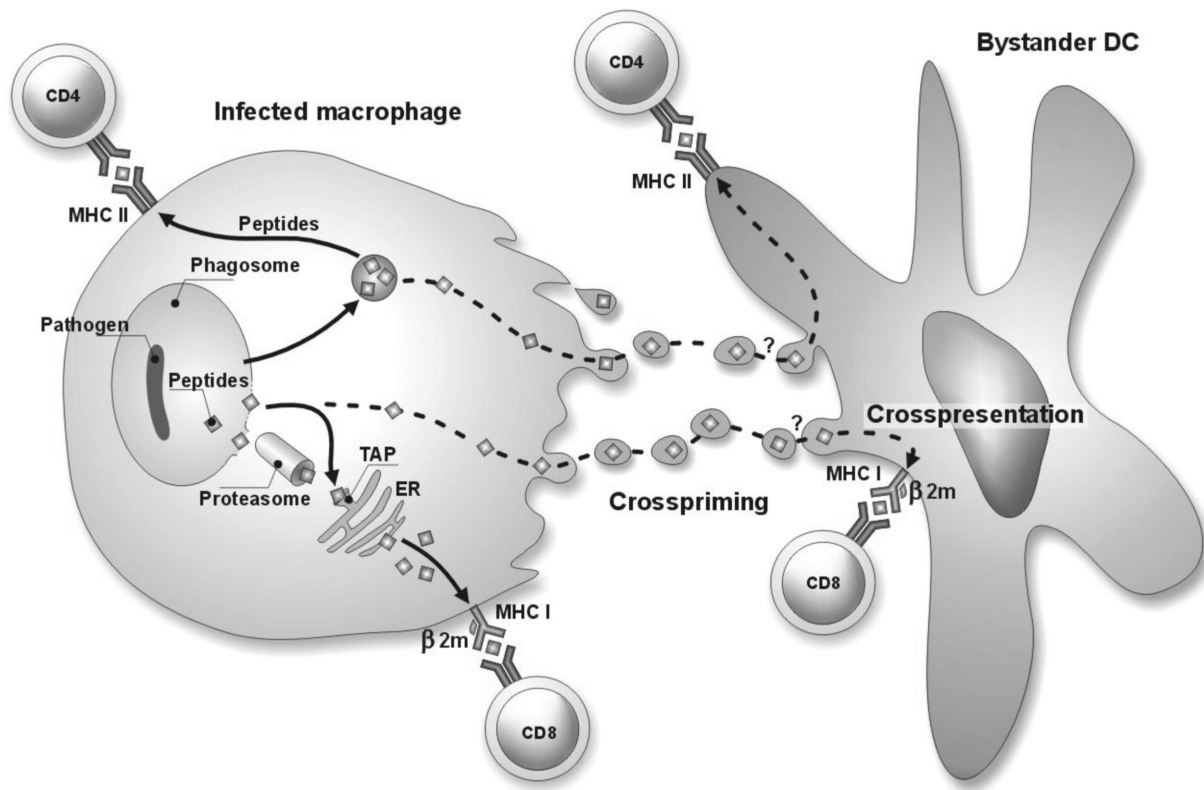

Figure II. Crosspriming and Crosspresentation.

Crosspriming and crosspresentation refer to the ability of exogenous peptides to access the MHC class I presentation pathway. Crosspriming: A good example of crosspriming involves the phagocytosis of apoptotic blebs containing antigen by dendritic cells. Dendritic cells may then present these antigens in association with MHC class II molecules, or they may allow their association with MHC class I molecules. Recently it has been proposed that the phagosome compartment may be competent for the antigen processing ending in crosspresentation via the MHC class I pathway. This has been referred to as crosspresentation. (Figure courtesy of SHE Kaufmann)

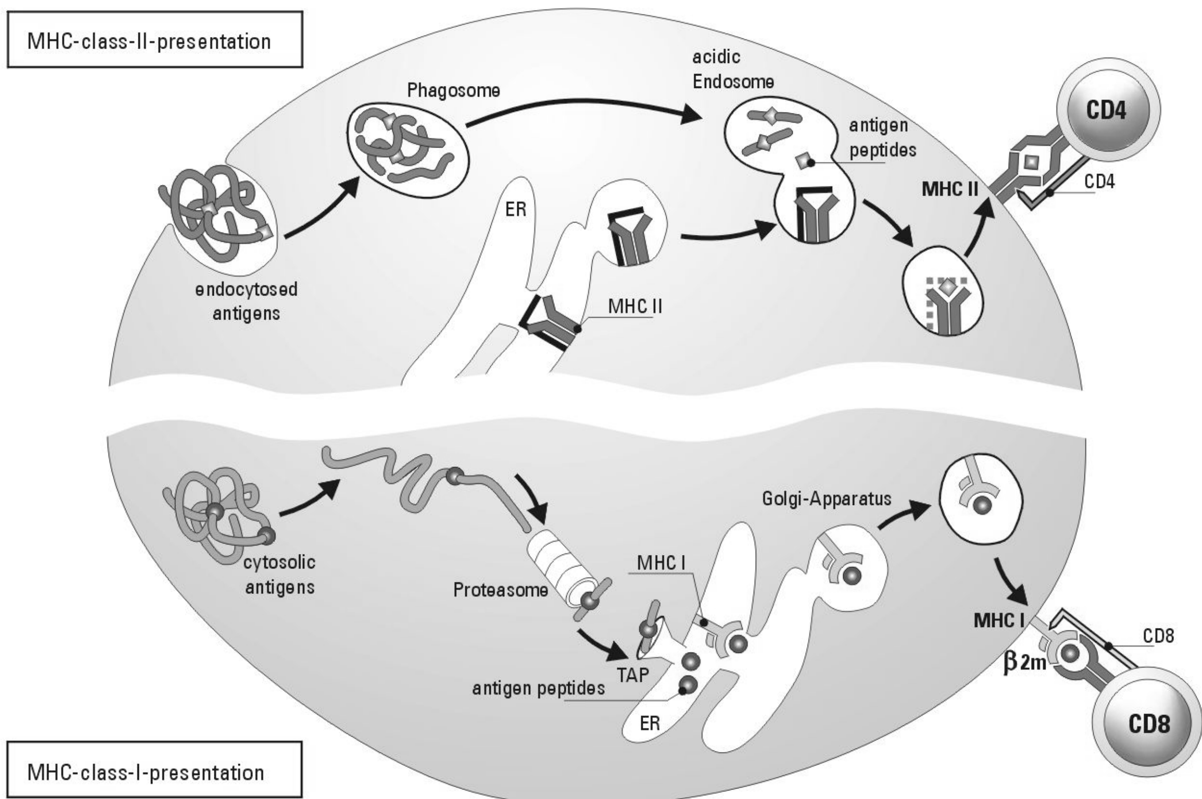

Figure I. Antigen presentation via the MHC class I and MHC class II pathways.

The mode of antigen entry into cells and the site of antigen processing determine whether antigenic peptides associate with MHC class I in the ER, or with MHC class II molecules in the endocytic compartments. MHC class I presentation: endogenous antigens are degraded in the cytoplasm by the proteasome complex. Peptides are then transported accross the ER membrane by an ATP-binding transporter called TAP. In the ER the physical association of TAP with an MHC class I molecule promotes the capture of antigenic peptides by the MHC class I molecule even before they are exposed to the ER lumen. The MHC I-peptide complex is then transported via the Golgi complex to the plasma membrane, where it can interact with CD8+ T cells. MHC class II presentation: exogenous antigens are endocytosed and processed first in the phagosome and then in the endosome. In the endosome antigenic peptides can associate with MHC class II molecules. The endocytic vesicle containing the MHC II-peptide complex then fuses with the plasma membrane. This allows the MHC II-peptide complex to be presented on the cell surface, where it can interact with CD4+ T cells. (Figure courtesy of SHE Kaufmann)

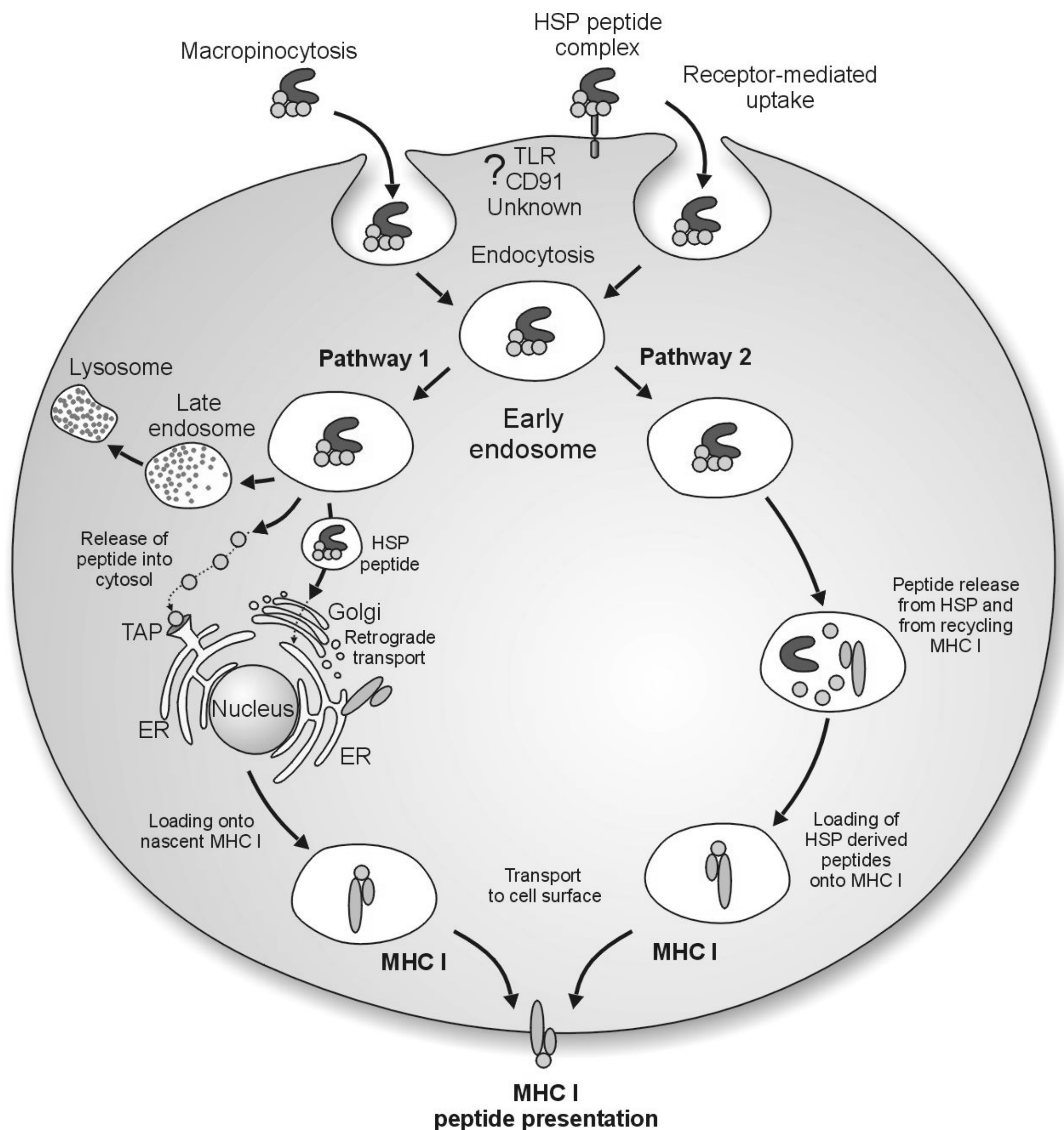

Figure IV. Cellular Uptake and Processing of GR-HSP/Peptide Complexes

Cellular uptake of GR-HSP/peptide complexes by antigen presenting cells may involve the CD91 scavenger receptor, a member of the TLR family, or an as yet unknown receptor. Two cellular processing pathways have been suggested. Pathway I involves endosomal/lysosomal processing of the GR-HSP/peptide complex. This initiates transport of peptide to the ER, where it is loaded onto MHC I molecules and translocated to the cell surface for re-presentation. The peptide may enter the ER via the TAP transporter, or via retrograde transport from the Golgi System. Pathway II involves dissociation of the GR-HSP/peptide complex in the early endosome, transfer of peptide onto recycling MHC I molecules in this same compartment, and translocation of this MHC I/peptide complex to the cell surface for re-presentation. (Figure courtesy of Rapp and Kaufmann, Glucose-regulated Stress Proteins and Antibacterial Immunity (2003) Trends in Microbiology, 11: 519-526)