Listeria monocytogenes as a vaccine vehicle: generation of attenuated mutants and their immunological characterization

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List of Original Publications

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To my family

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List of Abbreviations

aa	Amino acid(s)
Ad	Adenovirus
AP	Alkaline Phosphatase
APC	Antigen presenting cell
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BHI	Brain heart infusion
BCIP	5-Brom-4-chlor-3-indolylphosphat-*p* toluidine
BSA	Bovine serum albumin
BCG	Bacille Calmette-Guerin
bp	Base pair
°C	Centigrade
CBCs	Cholesterol-binding cytolysins
CFA	Complete Freund's adjuvant
Cfu	Colony forming unit
Co	Company
C-terminal	Carboxy-terminal
CTLs	Cytotoxic T lymphocytes
Cy3	Indocarbocyanin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Desoxynucleoside-5'-triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTH	Delayed Type Hypersensitivity
DTT	Dithiothreitol
EDTA	Ethylene diamine-N, N, N', N'-tetraacetate
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
Fe (II)	Ferrous ion

Fe (III)	Ferric ion
Fig.	Figure
FITC	Fluoresceinisothiocyanate
g	Gravity
h	Hours
HAT	Hypoxanthine; aminopterin; thymidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukine
i.p.	Intraperitoneal
i.v.	Intravenous
Kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani
LLO	Listeriolysin
M	Molar (mol/l)
mAb	Monoclonal antibody
Mcs	Multiple cloning site
MHC I/II	Major histocompatibility complex class I/II
min	Minutes
MOI	Multiplicity of infection
NEA	Non-essential amino acids
Neo	Neomycine
NP	Nucleoprotein
N-terminal	Amino-terminal
OD	Optical density
ori	Origin of replication
OVA	Ovalbumin
P	Promoter
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline

PCD	Programmed cell death
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethylenglycol
PFO	Perfringolysin
PLY	Pneumolysin
PVDF	Polyvinylidendifluoride
rpm	Round per minute
RSV	Respiratory syncytial virus
RT	Room temperature
SCID	Severe Combined Immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Seq.	Sequence
Sp	Signal peptide
TAE	Tris/Acetat/EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline - tween 20
TCA	Trichloroacetic acid
TE	Tris/EDTA
TEMED	N, N, N', N'-Tetramethylethylene diamine
TH	T helper cell
TNF	Tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
UV	Ultraviolet
VGC	Virulence gene cluster
XGal	5-Brom-4-chlorindoxyl-β-D-galactoside
z. B.	zum Beispiel

1. Introduction

1.1. Problems of infectious diseases

The optimism and clinical confidence associated with the development of antimicrobial agents from the 1940s onwards has been tempered by the emergence of new diseases, such as AIDS and infections associated with transplantation and cancer therapy, and by the widespread development of antibiotic resistance. Despite many advances, infectious diseases continue to account for about a quarter of all deaths worldwide (Mandell and Bennett, 2000). Indeed, the dramatic increase in drug-resistant microbes, combined with the lag in development of new antibiotics, the rise of megacities with severe health care deficiencies, environmental degradation, and the growing ease and frequency of cross-border movements of people and produce have greatly facilitated the spread of infectious diseases (Nicoll and Murray, 2002). Regaining the upper hand in the struggle against microbes requires multidisciplinary efforts which include expanding the use of vaccines to prevent infection (Mackay and Rosen, 2001), developing new antimicrobial agents (Byarugaba, 2004), improving surveillance for emerging microbial threats (Nicoll and Murray, 2002), teaching the correct use of antimicrobial therapy (Critchley and Karlowsky, 2004), developing adjunctive immunotherapies (Roilides and Pizzo, 1992), and conducting new basic research on the mechanisms of pathogenesis and drug resistance (Casadevall, 1996).

In the early 20th century, antibody-based (serum) therapy was used to treat a variety of bacterial infections, including those cased by *Corynebacterium diphtheriae*, *Streptococcus pneumoniae, Neisseria meningitides, Haemophilus influenzae*, group A streptococcus, and *Clostridium tetani* (Casadevall and Scharff, 1994; 1995). By the 1930s, serum therapy was a standard treatment for lobar pneumonia. However, when antimicrobial chemotherapy was discovered in the mid-1930s, serum therapy for bacterial infections was rapidly abandoned. Antimicrobial chemotherapy had important advantages over serum therapy: it was more effective and less toxic. The immediate side effects of serum therapy included fevers, chills, and allergic reactions (Feinberg, 1936; Rackemann, 1942). Other disadvantages of serum therapy included the need to establish a precise diagnosis before selecting serum, lot-to-lot variation of serum, and the need for considerable physician production (Casadevall, 1996). In 1975, hybridoma technology provided the means to generate unlimited amounts of monoclonal antibodies (MAbs) (Kohler and Milstein, 1975). In recent years, major advances have been made in the techniques used to generate human antibodies and humanize murine

monoclonal antibodies (Wright *et al*., 1992). The juxtaposition of three recent developments makes the reintroduction of antibody-based therapies an option for serious consideration. First, because of advances in technology, human antibody reagents can be synthesized; thus the toxicities traditionally associated with serum therapy can be avoided. Second, the emergence of new pathogens, the reemergence of old pathogens, and the increased prevalence of drug-resistant microorganisms have caused the effectiveness of existing therapeutic options to decline. Third, the difficulties involved in treating infections in immunocompromised patients suggest the need for adjunctive immunotherapy (Casadevall, 1996). However, both chemotherapy and immunotherapy are only therapeutic but not prophylactic. Indeed, the development of vaccines has been one of the most important contributions of immunology to medicine and public health. Based on stimulation of humoral and cellular immune responses against the corresponding pathogen, the field of vaccinology has experienced success in developing a long term prophylaxes for a variety of infectious disease agents by either historical use of traditional approaches of attenuated or inactivated microorganisms, protein subunits, toxoids or capsular polysaccharides (Hilleman, 2000; Makela, 2000) or recent introduction of gene-based vaccines (Page, 2002).

1.2. Vaccines

More than 70 bacteria, viruses, parasites, and fungi are serious human pathogens (Mackay and Rosen, 2001). Vaccines are available against some of these agents and are being developed against almost all the other bacteria and viruses and about half of the parasites. The first successful human vaccine experiment carried out by Edward Jenner approximately 200 years ago demonstrated that inoculation of a boy with cross-reactive cow-pox virus protected him against two successive infections with small pox virus (Sharma and Khuller, 2001). Since then, the majority of vaccinologists have focused on the development of vaccines. The major breakthrough, occurring a hundred years later, was the preparation by Pasteur, of rabies vaccine, which is based on the intentional attenuation of the pathogen (Allen, 2002).

This paved the way for the development of a big series of viral and bacterial vaccines. These conventional vaccines are based on the entire disease-causing microbial agent and consist of the killed or live attenuated organism that does not lead to infection but is capable of inducing protective immunity. They include also the detoxified toxins of some toxin secreting bacteria, which are effective in preventing the pathology of the bacterial infection (Loosmore *et al*., 1990). The existing conventional vaccines have the ability to eradicate or drastically

diminishe incidence and morbidity of a large number of infectious diseases including major killers such as smallpox, polio and diphtheria (Mackay and Rosen, 2001).

In spite of these tremendous achievements, there are several crucial drawbacks incurred by the current procedures for vaccine preparation. First, microbes that cannot be grown or can be grown only with difficulty *in vitro* pose a special problem to vaccine development. These include several viruses (HBV and HCV), bacteria like *Mycobacterium leprae*, and to a degree *Chlamydia pneumoniae*, and many parasites, of which the *Plasmodia* causing malaria are most important in this context (Makela, 2000). Second, the difficulty of ascertaining adequate killing or attenuation of the vaccine preparation, and the hazard which may be caused by exposure of both the vaccinees and those involved in vaccine production. This consideration is of particular consequences in case of fatal incurable diseases such as AIDS (Arnon and Ben-Yedidia, 2003). Third, the risk of reversion into their original pathogenic forms especially in immunocompromised individuals and infants. Moreover, it is possible that some live vaccine strains can be transmitted from the vaccinee to an unvaccinated individual (Hansson *et al*., 2000).

New approaches are, therefore, being considered for vaccine development, which are not based on the entire organism. These include the use of recombinant DNA technology for the production of relevant microbial protective protein antigens in bacterial, yeast, plant or animal cells for vaccine preparation (Goldfarb *et al*., 1994; Diminsky *et al*., 1999) or production of live vaccines by introducing the relevant gene(s) into the genome of an adequate vector such as vaccinia virus (Oh *et al*., 2003) or Salmonella (Angelakopoulos and Hohmann, 2000). More recently, naked DNA vaccines strategy was applied. It consists of plasmid DNA into which the relevant gene(s) of the microbial agent can be inserted (Ulmer *et al*., 1993; Wang *et al*., 1993). In addition, the utilization of synthetic peptides which constitute the relevant protective epitopes of viruses, bacterial toxins or parasites, was used for eliciting neutralizing immune response towards the disease-causing agent (Tam, 1996; Tourdot *et al*., 1997). A novel means, called synthetic recombinant vaccines, based on synthetic oligonucleotides, which code for the relevant epitope(s) that are inserted into an appropriate vector, for the expression of this external epitope. This approach may allow the inclusion of more than one epitope in the desired vaccine (Klavinskis *et al*., 1989; Newton *et al*., 1989). Although vaccines are traditionally administered to healthy individuals to prevent infection, the rabies vaccine was used therapeutically more than a century ago to avoid deleterious effects on the nervous system after exposure to the virus (Wilde *et al*., 1989; 1996). Recently, attention has been given to the development of vaccines that can be used to stimulate immune defenses in

patient populations after they have been infected with a pathogen or even after they developed a disease (Seder and Hill, 2000; Sela *et al*., 2002; Vandepapeliere, 2002). Such therapeutic vaccines have also been referred to as 'pharmaccines' or 'theraccines' (Vandepapeliere, 2002). Recently, the use of therapeutic vaccines has been extended to cover a variety of chronic, but not acute, diseases such as AIDS, tuberculosis, peptic ulcer, as well as different kinds of cancer diseases and autoimmune diseases where a definite success in developing a drug/vaccine against multiple sclerosis and hopes for myasthenia gravis, lupus and diabetes (Sela *et al*., 2002).

1.2.1. Virus-based vaccine vectors

Vaccines based on live viruses have traditionally been highly effective and relatively easy to produce. For example, the elimination of smallpox was accomplished through mass vaccination with the live vaccinia virus, a mildly pathogenic animal virus related to smallpox. Live attenuated poliovirus developed by Sabin was also responsible for the drastic reduction of the disease all over the world. The live attenuated vaccines are well tolerated and immunogenic and led to effective vaccine against additional infectious diseases, e.g. yellow fever (Co *et al*., 2002), mumps (Cusi *et al*., 2001), and others. These vaccines are usually produced by attenuation of the pathogen by physical means or by selection of naturally occurring mutants that lead to infection with abortive replication of the pathogen, while retaining its immunogenicity (Polo and Dubensky, 2002). Using molecular biology and DNA manipulation methods, it has also been possible to express protective proteins in adequate live vectors and thus design live vaccines against various pathogens. Thus, the development of reverse genetics systems for the recovery of viruses from cDNA has made it possible to rapidly generate recombinant attenuated derivatives of these viruses by either point mutations or by attenuating hazardous sequences that are included in the vaccine (Skiadopoulos *et al*., 2002). Live vaccines can be derived also using genetic engineering techniques since cloning procedures enable the generation of live viruses from plasmid DNA copies containing the whole virus genome. Vaccine candidates can thus be designed by site-directed mutagenesis, gene insertions or deletions and by generation of chimeric viruses (Agabov *et al*., 1998). In addition, engineered viruses may bear a phenotype that facilitates the immune response towards it, for example, expression of cytokines by recombinant RSV (Bukreyev *et al*., 2000). Gene-based delivery of antigens, and in particular application of virus-derived vectors, offers several potential advantages over traditional vaccine technologies. These include, most

notably, high-level production of authentic protein antigens directly within cells of the immunized host, potential adjuvanting effects from the viral delivery system itself and the possibility of efficient delivery of antigen directly to components of the immune system, such as antigen-presenting dendritic cells (DCs) (Polo and Dubensky, 2002; Makela, 2000).

1.2.2. Bacterial-based vaccine vectors

The potential of live, attenuated bacteria as vaccines and vectors has long been recognized and offers a number of advantageous in terms of convinience and immunogenicity compared with vaccines composed of inactivated organisms or antigen subunits. For example, simple modes of inoculation (e.g. oral) may confer protection following a single dose, presumably due to the limited ability of the vector to replicate *in vivo*. During the course of replication, the vector may potentially express many of the immunogens seen during natural infection, including mucosal immune responses that are not normally elicited by systemically administered vaccines. It is estimated that 90% of human infectious diseases are initiated at mucosal surfaces (Kraehenbuhl and Neutra, 1998). Attenuated bacteria may also be genetically engineered to express single or multiple heterologous antigens, providing potential protection against more than one pathogen. Finally, live bacterial vaccines can be inexpensive to manufacture (Trach *et al*., 1997; 2002) and practical for large-scale distribution (Lockman *et al*., 1999).

Advances in the production of live attenuated bacteria expressing heterolougous antigens as vaccines have been greatly facilitated by the development of an increasing number of live vaccine vectors. The empirical development of early bacterial vaccines against typhoid fever (S*almonella typhi* Ty21a) (Germanier and Furer, 1975) and tuberculosis (Mycobacterium *bovis*-BCG) (Guleria *et al*., 1996), for example, resulted in vaccines bearing randomly generated and undefined mutations. The progressive elucidation of microbial biosynthetic pathways hastened the development of second-generation vaccines and vectors that were rationally attenuated by the disruption of genes encoding known metabolic functions. Attenuated bacteria bearing mutations in biosynthetic genes, however, may be affected in their ability to colonize and replicate *in vivo*, diminshing their utility as vectors. To address the issue, highly atenuated strains bearing genes associated with virulence, such as phoP and virG, were created (Sansonetti *et al*., 1991; Homann *et al*., 1996). The effectivnes of virulence-attenuated mutants was based on the notion that such strains would replicate normally *in vivo*, producing superior vaccines and vector candidates.

While a number of attenuated microorganisms show promise as potential vector plateforms, only a relative few have been evaluated in humans. In addition to issues of immunogenicity, a key concern associated with the use of live attenuated bacteria is safety (Trach *et al*., 2002). A vector should be safe for the vaccinated host and the environment as a whole, including unvaccinated contacts exposed to the vaccine vector. Bacterial vaccine vectors candidates that have been conducted for use in preclinical studies include attenuated strains of *Salmonella, Shigella, Listeria, Mycobacterium* and *Vibrio*.

1.2.2.1. *Listeria* vector vaccines

Listeriosis is an invasive infection caused by *L. monocytogenes*. Acquired immunity is multifactoral, involving phagocytes and several T-cell subpopulations (Kaufmann, 1993; Shan *et al*., 1995). Following invasion of the host through the gut, *Listeria* is found in large numbers in the cytosole of splenic APCs, such as macrophages, where the bacteria actively replicate. In the cytosole, *Listeria* antigens are processed by the host's endogenous MHC Class I pathway and presented to CD8+ T-lymphocytes. The ability of *L. monocytogenes* to elicit protective CD8+ T-cell immune response in animals has spurred interest in developing the system as a live bacterial vector for use in humans.

Although outbreaks of listeriosis are relatively uncommon, the organism may nevertheless cause death, particularly in people who are immunocompromised or pregnant. For these reasons, the development of *Listeria* vector candidates for use in humans has proposed with caution. One promising candidate is a *L. monocytogenes* auxotrophic mutant bearing deletions in alanin racemase (*daI*) and D-amino acid aminotransferase (*dat*), two genes required for the biosynthesis of bacterial cell walls (Thompson *et al*., 1998). This approach has the advantage of attenuating a virulent strain and reducing the potential of the mutant to revert to a wild type phenotype. Deletion of *daI* and *dat* resulted in a listerial strain that was highly attenuated in mice. While intravenous or intraperitoneal immunization of mice with a sublethal dose of the *daI dat* double-mutant failed to provide protection against virulent challenge, the addition of D-alanin to the inoculum was sufficient to allow the mutant to survive long enough to elicit protective CTL responses against lethal challenge. More recently, the first human safety study of a virulence attenuated *L. monocytogenes* vaccine (LH1196) was reported. The vaccine candidate was an orally-administered strain of *L. monocytogenes* bearing deletions in *act*A and *plc*B genes necessary for cell-to-cell spreading and escapes from the secondary vacuoles, respectively. Volunteers receiving up to 10^9 cfu shed vaccine organisms for approximately 4

days and showed no serious adverse effects, although two volunteers receiving higher vaccine doses manifested temporary elevation in selected liver enzymes following immunization (Angelakopoulos *et al*., 2002). All volunteers receiving the higher vaccine doses elicited cellular immune responses as shown by the production IFN-γ in ELISPOT assays.

While the uses of attenuated *Listeria* in several applications have been described, the most promising results have been shown in situations where cellular immune responses are required. Recombinant *Listeria* secreting genetic fusions of Hly to lymphocytic choriomeningitis virus (LCMV) NP have been shown to elicit significant MHC Class I-restricted immune responses in mice (Goosens *et al*., 1995). Immunized animals were protected against subsequent challenge with virulent strains of LCMV that establish chronic infection in unimmunized mice. Protection corresponded to the presence of $NP_{118-126}$-specific CD8+ T cells. The effective use of *Listeria* vectors may also be dependent, however, upon the expression of heterologous antigens in appropriate intracellular compartments. This was suggested by results in mice immunized with a virulent strain of *Listeria* expressing either a secreted or a nonsecreted form of the LCMV $NP_{118-126}$ epitope genetically fused to dihydrofolate reductase. Immunised animals elicted $NP_{118-126}$ specific CTL responses of similar magnitude regardless whether the fusion protein was secreted or non-secreted. Furthermore, immunization of mice with either form of the fusion protein was equally efficient at eliciting the production of $NP_{118-126}$-specific memory T- and provides protective immunity against LCMV challenge. In spite of these similarities, however, only mice immunized with *Listeria* expressing the secreted form of the $NP_{118-126}$-DHFR fusion protein were protective against a lethal dose of *Listeria* whereas mice immunized with *Listeria* expressing the non-secreted form of the $NP_{118-126}$-DHFR fusion protein showed no specific resistance to lethal challenge (Shen *et al*., 1996). These results demonstrate that infection with *Listeria* produces discordance between the production of immune CD8+ T cells to the passenger antigen and protection against the vector.

The ability of *L. monocytogenes* to stimulate a vigorous CD8+ T cell response may also have potential as a therapy against some forms of cancer, since experimental evidence suggests that MHC Class I-restricted CD8+ lymphocytes may be a critical mediator of an effective antitumor response (Paterson and Ikonomidis, 1996). A recent study showed that a recombinant strain of *L. monocytogenes* inhibited tumor growth in a murine cancer model. Tumorogenic cells transduced with the influenza virus NP antigen were injected in mice to generate visible, subcutaneous tumors. Immunization of these mice with *L. monocytogenes* expressing NP inhibited tumor growth and ultimately stimulated the regression of

macroscopic tumors (Pan *et al*., 1995). Moreover; this NP-expressing *Listeria* vector was also capable of conferring CD8+ T-cell protection against a lethal challenge of renal carcinoma cells expressing NP.

1.2.2.1.1. *Listeria* delivery of plasmid DNA

The ability of *L. monocytogenes* to enter the host cell cytosole after phagocytosis and deliver plasmid DNA directly to the cytoplasm makes it an attractive DNA delivery candidate to induce cellular immune responses. An early vaccine candidate, *L. monocytogenes* Δ2, was constructed by deleting lecithinase activity, which significantly inhibited cell-to-cell spread by the bacterium (Dietrich *et al*., 1998). In an infected cell, Δ2 was designed to express *in vivo* controlled bacteriophage cytolysin that lysed the *Listeria* cell wall and released the plasmid DNA into the host cell cytosole. Cultured mouse macrophages infected with Δ2 expressing a fluorescent marker was evident in cultures three days post-infection. Bone marrow derived macrophages infected with Δ2 harboring an expression vector encoding a model epitope were able to stimulate an ovalbumin OVA $_{257\text{-}264}$-specific CTL clone to secrete IL-2.
The use of attenuated *Listeria* for genetic immunization has several advantages over the direct injection of naked DNA, including oral administration, a natural tropism for APCs and the presence of recognized immunomodulatory components that may enhance the host's immune response to a vectored antigen (Goosens *et al*., 1995).

1.2.3. Vaccines and T cells

The ultimate goal of a vaccine is to develop long-lived immunological protection, whereby the first encounter with a pathogen is 'remembered', which leads to enhanced memory responses that either completely prevent reinfection or greatly reduce the severity of disease. Specialized cells known as memory T and B cells, and long-lived effector B cells (plasma cells), which constitutively secrete high-affinity 'neutralizing' antibodies, are the basis of immunological memory. The memory T-cell compartment consists of both CD4+ and CD8+ T-cells that can rapidly acquire effector functions to kill infected cells and/or secrete inflammatory cytokines that inhibit replication of the pathogen. Effector CD4+ T cells also help B-cell responses and enhance CD8+ T-cell development, through the activation of antigen presenting cells (APCs) or secretion of cytokines, such as interleukin-2 (IL-2), IL-4 and IL-5. In some situations, protective immunity can be mediated by just one of the branches

of the immune system such as by antibodies or CD8+ T cells but for optimal control of pathogens, both the humoural and cellular immune responses need to be mobilized (Zajac *et al*., 1998).

1.2.3.1. Stages of T-cell responses

The path towards memory T-cell development continues to be delineated, but there are clearly three stages that T cells pass through as they differentiate into memory cells (Ahmed and Gray, 1996). The first stage, the 'expansion' phase, is initiated in the lymphoid tissues, where encounter with antigen induces naive T cells to clonally expand and differentiate into effector T cells, known as T helper (TH) cells or cytotoxic T lymphocytes (CTLs) for CD4+ and CD8+ T cells, respectively. Through the combined abilities of CD4+ and CD8+ effector T cells to secrete inflammatory cytokines and kill infected cells, a typical acute viral or intracellular bacterial infection can be resolved within days. Over the weeks that follow pathogen clearance, the majority (>90%) of effector T cells die, and this second stage is often referred to as the 'death' phase or contraction period which acts as a safeguard to prevent excessive immunopathology by limiting the duration of T cell responses. The surviving T cells enter the third stage, the 'memory' phase, in which the number of memory T cells stabilizes, and these cells are maintained for long periods of time.

1.2.3.2. T-cell differentiation

Recent studies of CD8+ T cells have shown that the link between the commitment to clonal expansion and effector-cell differentiation is remarkably tight; the same duration of antigenic stimulation (2–24 hours) that drove naïve CD8+ T cells to proliferate was sufficient for them to commit to differentiate into effector cells that could secrete IFN-γ, tumour-necrosis factor (TNF) and IL-2, and kill infected cells (Mercato *et al*., 2000; Kaech and Ahmed, 2001; van Stipdonk *et al*., 2001). These data indicate that naïve CD8+ T cells are developmentally programmed to clonally expand and differentiate into CTLs after brief encounter with antigen. Even though CTL effector properties were acquired after as little as 2–24 hours of stimulation, it remains to be determined whether the quality of effector properties is affected by the duration of antigenic stimulation *in vivo*. It seems that T cells that are activated under different conditions, such as with heat-killed bacteria or in the presence of high concentrations of IL-2 or IL-15, might develop suboptimal and/or altered effector CD8+ T-cell functions

(Lauvau *et al*., 2001; Manjunath *et al*., 2001). The programmed development of CD8+ T cells has several advantages. First, it alleviates the need for prolonged confinement of CTLs to the lymphoid organs, which allows their migration to peripheral sites of infection and/or inflammation to remove infected cells. Second, it might also considerably affect the number of memory CD8+ T cells that are generated, because the size of the memory T-cell pool is directly correlated to that of the effector-cell population (Vijh and Pamer, 1997; Busch *et al*., 1998; Murali-Krishna *et al*., 1998). In several models of acute viral and bacterial infection, the number of effector CD8+ T cells peaks 2–3 days after the infectious pathogen is cleared. If each CD8+ T-cell division was regulated strictly by antigen contact, the number of effector CTLs would peak earlier and reach a lower maximum, and consequently, less memory CD8+ T cells would be generated (Kaech *et al*., 2002b).

A similar type of developmental program might also drive the differentiation of activated CD4+ T cells, but the formation of effector CD4+ T cells might be influenced to a greater extent than for CD8+ T cells by extrinsic factors, such as the duration of antigen exposure and the types of cytokines that are present (Kundig *et al*., 1996; Iezzi *et al*., 1998; 1999; Jelley-Gibbs *et al*., 2000; Langenkamp *et al*., 2000). Unlike naive CD8+ T cells, which commit to effector and memory T-cell development within 24 hours of stimulation, naive CD4+ T cells required more than 48 hours of continual antigenic stimulation to commit to the formation of polarized TH1 or TH2 effector phenotypes *in vitro* (that is, the secretion of IFN-γ or IL-4, respectively). Even after 48 hours, a large proportion of the CD4+ T cells did not develop effector properties (that is, they were non-polarized) (Iezzi *et al*., 1999). Nonpolarized CD4+ T cells produced IL-2, but little or no IFN-γ or IL-4 (Langenkamp *et al*., 2000; Iezzi *et al*.,2001). So, it seems that naive CD8+ T cells commit to effector-cell differentiation more readily than CD4+ T cells. As described above, this might result from the apparent differences between CD4+ and CD8+ T cells in their activation requirements or the rates at which their thresholds for activation are reached.

1.2.3.3. T cell migration

As naïve T cells differentiate into effector cells, their migration patterns are altered. Effector T cells have a reduced potential for homing to lymph nodes owing to decreased expression of lymph-node-homing receptors, such as CC-chemokine receptor 7 (CCR7) and L-selectin (CD62L) and a greater capacity to migrate to inflamed tissues owing to increased expression of chemokine receptors such as CCR5 and CCR2. The expression pattern of CD62L on

activated T cells is triphasic and seems to be regulated by the duration of antigenic stimulation. Initially, TCR stimulation induces the rapid shedding of CD62L from the T-cell surface by proteolytic cleavage, but within 24–48 hours, CD62L is re-expressed (Chao et *al*., 1997). However, if TCR stimulation continues, the locus that encodes CD62L becomes transcriptionally silenced and surface expression of CD62L becomes fixed at a low level for an extended period of time (Chao *et al*., 1997; Oehen and Brduscha-Riem, 1998; Teague *et al*., 1999).

After ~24 hours of antigenic stimulation, the levels of CD62L and CCR7 remained high on activated CD4+ T cells, and these cells retained lymph-node-homing properties, whereas migration to peripheral sites, such as the peritoneum and lungs, was inefficient (Iezzi *et al*., 2001). However, if the exposure to antigen was sustained over several days, the T cells lost surface expression of these receptors and trafficking to the lymph nodes was markedly reduced (Langenkamp *et al*., 2000; Sallusto *et al*., 1999; Iezzi *et al*., 2001).

1.2.3.4. Lineage of memory T cells

The precise lineage by which memory cells arise remains unresolved (Murali-Krishna *et al*., 1998; Sprent and Surh, 2002). Conventionally, two models have been proposed for the generation of memory cells. The first is the linear differentiation model, which predicts that memory cells are the progeny of effector CTLs that escape activation induced cell death (AICD) (Jacob and Baltimore, 1999; Opferman *et al*.,1999; Hu *et al*.,2001) as the activated CD8+ T cells seem to be programmed to develop into memory T cells, because CD8+ T cells that were stimulated briefly (~24 hours), proliferated and differentiated into CTLs without further antigenic stimulation but surprisingly, these cells continued to develop into long-lived, protective memory CD8+ T cells (Mercado *et al*., 2000; Kaech and Ahmed, 2001). Therefore, the instructive program that guides effector CD8+ T-cell development is sufficient to guide the formation of memory CD8+ T cells termed “effector memory T cells”.

The other view is that memory cells can directly arise from naive cells without passing through an effector-cell stage (non-linear differentiation), for example when naive T cells receive weak antigenic stimulation towards the end of an immune response and these memory cells are referred to as “central’memory T cells” (Langenkamp *et al*., 2000; Iezzi *et al*., 2001; Lauvau *et al*., 2001; Manjunath *et al*., 2001). If naive cells come under the influence of IL-15 shortly after activation, they differentiate into central memory-like cells. On the other hand, if they encounter sufficiently high doses of IL-2, they differentiate into fully fledged effector

cells. Thus, effector differentiation is not required to generate central memory cells, at least *in vitro*. Moreover, if differentiated effector cells, generated either *in vitro* or *in vivo*, are cultured in IL-15, they too can revert to memory-like cells (Manjunath *et al*., 2001). The difference between memory cells generated with IL-15 from naive cells vs. those generated from effector cells is that while the former express (CD62L) L-selectin and CCR7, the latter have lost these markers indicating that memory cells generated from naive cells resemble central memory cells while those generated from effector cells resemble effector memory cells. Therefore, it is important to consider that memory T-cell development might occur in a non-linear fashion and that it can result in qualitatively different memory T-cell subsets (Langenkamp *et al*., 2000; Campbell *et al*., 2001). Different priming conditions, for example, the duration of antigenic stimulation and the type of cytokines present might affect the formation of these subsets (Kaech *et al*., 2002a).

1.2.3.5. Immunological characters of naive versus memory T cells

Comparisons between naive and memory T cells have begun to reveal the physiological basis for the heightened recall responses of memory T cells. First, as a consequence of clonal expansion during the primary infection, experiments in mice have shown that there can be a substantial increase (~1000-fold) in the precursor frequency of antigen-specific T cells in immune animals compared with naive animals (Busch *et al*., 1998; Murali-Krishna *et al*., 1998; Whitmire *et al*., 1998; Bousso *et al*., 1999). Second, as naive T cells differentiate into memory cells; their gene-expression profile is reprogrammed by changes in chromatin structure and the profile of active transcription factors (Agarwal and Rao, 1998). For example, the genes that encode interferon-γ (IFN-γ) and cytotoxic molecules, such as perforin and granzyme B, are not expressed in naive CD8+ T cells, but are constitutively expressed in effector and memory CD8+ T cells (Yang *et al*., 1998; Bachmann *et al*., 1999; Teague *et al*., 1999; Veiga-Fernandes *et al*., 2000; Grayson *et al*., 2001). Although the synthesis of these proteins occurs in an ‘on–off’ fashion that is regulated by antigen contact, elevated levels of the messenger RNA transcripts endow memory CD8+ T cells with the capacity to produce larger quantities of these proteins more rapidly than naive T cells (Slifka *et al*., 1999; Badovinac *et al*., 2000; Kaech and Ahmed, 2001). Third, memory CD8+ T cells express a different pattern of surface proteins that are involved in cell adhesion and chemotaxis from naive T cells, which allows memory T cells to extravasate into non-lymphoid tissues and mucosal sites (Moser and Loetscher, 2001). This enables memory T cells to survey peripheral

tissues where microbial infections are generally initiated. Fourth, memory T-cell populations are maintained for a long time due to homeostatic cell proliferation, which occurs at a slow, yet steady, pace. Interestingly, the rate of this homeostatic cell division must equal the rate of cell death, because the number of memory CD8+ T cells remains relatively constant over time (Murali-Krishna *et al*., 1998; Homann *et al*., 2001). So, it is the increased number of antigen-specific T cells, and their faster responses, anatomical location (that is, near the sites of microbial entry) and longevity that collectively explain how memory T cells confer long-term protective immunity.

1.3. Interactions of *Listeria monocytogenes* with mammalian host cells and tissues

Listeria monocytogenes is a ubiquitous, rapidly growing, gram-positive bacterium with an unusually broad ecological niche and host range. Infection of humans and animals has been traced to contaminated foods and can lead to serious, often fatal disease. In humans, disease is

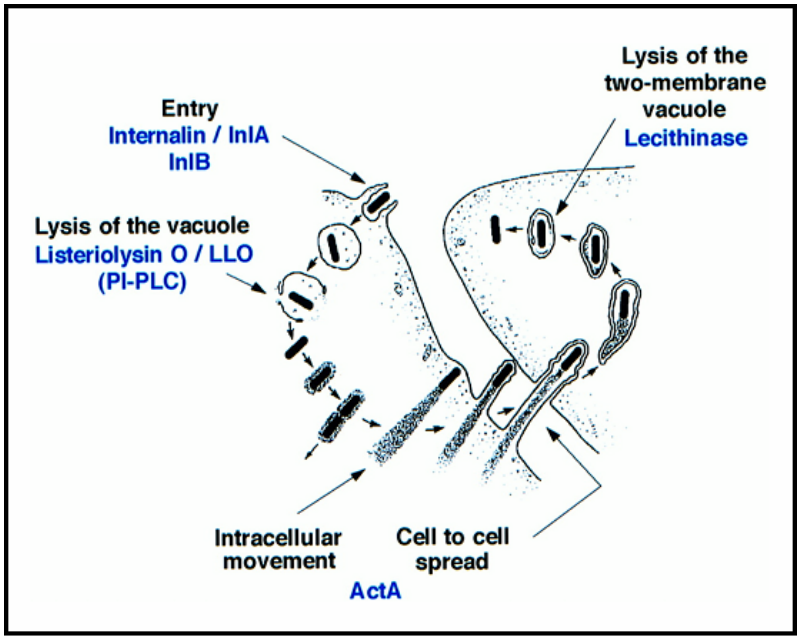

Fig. 1.1. Schematic representation of the cell infectious process by *L. monocytogenes* and *t*he bacterial factors involved. Adapted from Tilney and Portnoy (1989).

most common among pregnant women, newborns, and immunocompromised individuals (Schlech, 2000). These clinical features are due to the unique properties of *L. monocytogenes* to be able to cross three barriers, the intestinal barrier, the fetoplacental barriers and the blood brain barrier (Cossart and Lecuit, 1998). Bacteria cross the intestinal barrier, and via the

lymph and blood proceed to the liver and spleen where the infection is normally stopped by the non-specific defenses of the host, in particular neutrophils. In the immunocompromised host, bacteria multiply in the hepatocytes and finally reach the brain and the placenta. *Listeria* is thus a bacterium able to infect many cell types and tissues where it is always intracellular due to its capacity to trigger its own phagocytosis in cells which are normally nonphagocytic (Cossart, 2002). The different phases of *Listeria* infection in eukaryotic host cells were shown schematically in Fig.1.1. Entry into mammalian cells is mediated by at least two bacterial factors: internalin A (InlA) and B (InlB). Escape from the vacuole requires expression of listeriolysin O (LLO), a pore-forming toxin which in some cells can function synergistically with or be replaced by a phosphatidylinositol-specific phospholipase C (PI-PLC). Intracellular movement requires expression of ActA, and lysis of the two-membrane vacuole is performed by a lecithinase (PLC-B). PI-PLC is synthesized in an active form whereas PC-PLC is produced as an inactive precursor. A bacterial zinc-dependant metalloprotease and a host cell cysteine protease are required to cleave off part of the precursor and activate the phospholipase (Marquis *et al*., 1997).

1.3.1. Entry into cells

Host cell infection begins with the internalisation of the bacteria either by phagocytosis in the case of macrophages or induced phagocytosis (invasion) in the case of normally non-phagocytic cells. Bacterial invasion starts by the interaction with the plasma membrane which progressively enwraps the bacterium. This process usually refers to as the 'zipper' mechanism in contrast to the 'trigger' mechanism used by *Salmonella* or *Shigella* (Finlay and Ruschkowski, 1991; Isberg and Tran Van Nhieu, 1994; Adam *et al.*, 1995; Swanson and Baer, 1995; Mengaud *et al*., 1996). Following internalization, bacteria reside within membrane-bound vacuoles for about 30 min before lysing the membrane. However, significant levels of internalization by non-professional phagocytic cells is often mediated by one or more bacterial surface proteins, collectively named internalins, of which internalin A and B are the best characterized (Braun and Cossart, 2000). Internalin A promotes binding and internalization by E-cadherin, the human receptor for internalin A, whereas internalin B binds to the Met receptor tyrosine kinase and mediates internalization via PI3-kinase activation (Cossart, 2001). As evidenced by the residual level of entry of a Δ*inlAB* mutant, other mechanisms of entry exist. InlA and InlB are two members of the internalin multigene family which contains five other members; InlC, InlC2, InlD, InlE and InlF and it was

anticipated that these genes could also play a role in entry, but this does not seem to be the case (Engelbrecht *et al*., 1996; Domann *et al*., 1997; Dramsi *et al*., 1997). A recent report indicates that ActA, the protein involved in actin polymerization, could also participate in invasion (Alvarez-Dominguez *et al*., 1997).

1.3.2. Escape from a vacuole

Upon phagocytosis by macrophages, there are a number of possible fates awaiting a bacterium (Duclos and Desjardins, 2000). In the case of *L. monocytogenes*, internalized bacteria are either killed or escape into the cytosol. Mutants that fail to escape from a vacuole may survive in tissue culture cell lines, but do not grow (Lety *et al*., 2002). The pore-forming protein listeriolysin O (LLO) is largely responsible for mediating escape from the vacuole, and is consequently an essential determinant of pathogenicity (Vazquez-Boland *et al*., 2001b). Mutants lacking LLO fail to escape from a vacuole in most cells, and synthesis of LLO by other organisms such as *Bacillus subtilis* is sufficient to mediate escape from a vacuole (Bielecki *et al*., 1990). Thus, it is clear that the role of LLO is to mediate vacuolar escape from a phagosome and from a secondary vacuole formed upon cell-to-cell spread (Gedde *et al*., 2000). In addition to LLO, *L. monocytogenes* secretes two phospholipases C (PLCs) that contribute to escape: a phosphatidylinositol- specific PLC (PI-PLC) and a broad-spectrum PLC (PC-PLC) that is synthesized as a proenzyme activated by a secreted *L. monocytogenes* metalloprotease (Vazquez-Boland *et al*., 2001b). Mutants lacking both PLCs show a marked defect in vacuolar escape, and in human epithelial cells such as HeLa cells, PC-PLC and metalloprotease mediate escape from a vacuole in the absence of LLO (Marquis *et al*., 1995). The precise mechanism by which *L. monocytogenes* escapes from a vacuole is not clear but is consistent with the following model: upon phagocytosis, the *L. monocytogenes* containing vacuole acquires markers of a maturing endosome/ phagosome and acidifies to an average pH of 5.9 (Alvarez-Dominguez *et al*., 1997; Beauregard *et al*., 1997). Agents, such as bafilomycin that block acidification, inhibit vacuolar perforation and bacterial escape (Conte *et al*., 1996; Beauregard *et al*., 1997; Glomski *et al*., 2002). It was proposed that LLO insertion into the phagosomal membrane has two functions: one is to dissipate the pH gradient and thereby halt the maturation of the phagosome, and the other is to act as a channel for the passage of proteins from the vacuole (Portnoy *et al*., 2002). The bacterial phospholipases and/or host vacuolar constituents then pass through the channel and act on the vacuole, leading to its dissolution.

1.3.3. Compartmentalization of LLO activity

LLO is one of 23 members of the cholesterol-dependent family of cytolysins (CDCs) secreted by gram-positive bacteria (Billington *et al*., 2000). The best characterized of the CDCs are perfringolysin O (PFO) and streptolysin O (SLO), which are normally secreted by extracellular pathogens and presumably act on cells from outside. Replacement of LLO with PFO in *L. monocytogenes* results in a strain that is able to escape from a vacuole, albeit at a reduced efficiency, but that kills the infected cells from within (Jones and Portnoy, 1994). Thus, LLO is apparently unique in that it acts in a vacuole, but does not kill the host cell upon growth in the cytosol. The properties of LLO as a vacuole-specific lysin can be exploited to deliver macromolecules to the cytosol of macrophages either by incorporation of LLO into acid sensitive liposomes or by expression of recombinant proteins in *Escherichia coli* expressing LLO (Lee *et al*., 1996; Higgins *et al*., 1999). A PEST-like motif has been identified recently close to the N-terminus of mature LLO protein (Rechsteiner and Rogers, 1996; Decatur and Portnoy, 2000; Lety *et al*., 2001). Two different hypotheses were evolved explaining the role of PEST-like sequence in compartmentalization of LLO activity. The first was postulated by Decatur and Portnoy (2000) who found that removal of the PEST-like sequence does not affect LLO activity or vacuolar escape, but results in a strain that is extremely toxic to infected host cells suggesting that this motif may target eukaryotic proteins for phosphorylation and/or degradation by the proteasome, and may generally represent sites of protein-protein interactions (Decatur and Portnoy, 2000; Lety *et al*., 2001). In contrast, Charbit A. and co-workers were recently confirmed that the PEST-like sequence allows efficient disruption of the phagosomal membrane and the ΔPEST mutant remained trapped within phagosomes of bone marrow-derived macrophages (Lety *et al*., 2001). However, both of them have agreed with that the deletion of this motif did not affect the secretion and haemolytic activity of LLO but abolished *in vivo* bacterial virulence. Moreover, a recent study was postulated the absence of correlation between the PEST score in the N-terminus of LLO and the susceptibility of the protein to protease degradation (Lety *et al*., 2002).

LLO is unique among the CDCs in that it has a pronounced acidic pH optimum. Mutation of a single LLO residue (L461T) results in an increase in LLO activity at neutral pH and leads to a 100-fold loss in virulence (Glomski *et al*., 2002). This mutation does not affect escape of *L. monocytogenes* from a vacuole, but causes premature permeabilization of infected host cells after about five bacterial generations. Thus, the acidic pH optimum of LLO as well as the PEST-like sequence restricts LLO activity to a vacuolar compartment. Surprisingly,

transcription and synthesis of LLO continues in the host cytosol (Bubert *et al*., 1999; Moors *et al*., 1999). However, a number of other potential mechanisms may be in place to prevent toxicity to the infected host. For example, PC-PLC secretion is acid dependent and occurs preferentially in host vacuoles (Marquis and Hager, 2000), although this is yet to be documented for LLO.

1.3.4. Growth in the cytosol

Intracellular pathogens can be broadly divided into those that grow within a modified vacuole of the host cell (Duclos and Desjardins, 2000) and those like *L. monocytogenes* that grow in the host cytosol. There is compelling evidence to suggest that the cytosol is a favorable environment for bacterial growth: *Bacillus subtilis* expressing LLO or *E. coli* precoated with LLO can escape from a vacuole and grow in the cytosol of tissue culture cell lines (Bielecki *et al*., 1990; Monack and Theriot, 2001). However, it was recently shown that an *L. monocytogenes* hexose phosphate transporter was virulence-regulated and necessary for growth on glucose-6-phosphate and optimal cytosolic growth (Chico-Calero *et al*., 2002). Thus, although nonpathogens can grow in the cytosol under some circumstances, intracytosolic bacteria have clearly evolved specific mechanisms to enhance intracellular growth. In addition most intracytosolic bacterial pathogens like *L. monocytogenes* have evolved mechanisms of actin-based motility to spread from cell to cell (Goldberg, 2001). When free in the cytosol, *L. monocytogenes* replicates and induces the polymerization of host actin filaments and uses the force generated by actin polymerization to move, first intracellularly and then from cell to cell. These filaments rearrange within 2 h into long comet tails left behind in the cytosol while the bacteria move ahead at a speed of approximately 0.3 mm/second (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Theriot *et al.*, 1992). Remarkably, a single bacterial protein, ActA, is responsible for mediating actin nucleation; actin based motility, and is necessary for pathogenicity. ActA-minus mutants escape normally from vacuoles, but grow in the host cytosol as microcolonies and do not spread from cell to cell or form plaques in tissue culture cell monolayers (Tilney and Portnoy, 1989). The ActA protein provides multiple binding sites for host cytoskeletal components, thereby acting as a scaffold to assemble the cell's actin polymerization machinery (Cameron *et al*., 2000). It was then clear that ActA was sufficient to induce actin polymerisation and movement. However, since bacteria dropped in pure actin do not polymerize actin, it became obvious that ActA was a recruiting factor that allowed actin to polymerize. While a deletion analysis was providing

evidence that the N-terminal part of ActA, called ActA-N (the first 233 amino acids) was sufficient for its activity on the bacterial surface (Lasa *et al*., 1995), the first ActA ligand was identified (Chakraborty *et al*., 1995). It is a protein called VASP (for vasodilator phospho-protein). VASP is a substrate for cGMP-dependent kinases and is found in locations where actin is polymerizing, in particular at the leading edge of locomoting cells. VASP is also a ligand of profilin, an actin-sequestering protein which can also induce actin polymerisation by binding, as an actin-profilin complex to the actin filament barbed ends. It was then shown that VASP binds to the central part of ActA which is dispensable, establishing that VASP together with the central part of ActA stimulates a process that is really generated in the N-terminal part of ActA. ActA-N contains a dimerisation site (Mourrain *et al*., 1997). The key experiment was performed by the group of Mitchison that succeeded to fractionate cell extracts and demonstrate that the critical factor for the polymerisation process is a protein complex which when added to bacteria and actin can induce an "actin cloud" around the bacteria (Welch *et al*., 1997). This Arp2/3 complex contains seven proteins of which two are actin-related proteins which may act as the two first monomers in the actin nucleation process. Recent experiments have shown that purified ActA-N in the presence of purified Arp2/3 complex can stimulate actin polymerisation and also branching of the actin filaments. The interesting discovery that came afterwards is that in mammalian cells, the normal ligands of the Arp2/3 complex are proteins of the Wasp/NWasp/ Scar family (Machesky and Insall, 1998). These proteins upon a signal, such as interaction of a ligand with its receptor, may bind to a GTP-bound small GTPase at the plasma membrane and can then recruit the Arp2/3 complex which itself becomes activated and able to polymerize actin into a branched filament network. Thus ActA and Wasp family proteins are both activators of the actin nucleator Arp2/3 complex. The parallel between ActA and Wasp family proteins also lies at the level of the primary structure. It thus appears that the bacterial ActA mimics mammalian Wasp proteins (Skoble *et al*., 2000; Boujemaa-Paterski *et al*., 2001). An important study was the *in vitro* reconstitution of *Listeria* motility which requires in addition to actin and Arp2/3 complex, cofilin, capping protein, alpha-actinin and also VASP and profilin which accelerate the movement (Loisel *et al*., 1999).

1.3.5. Cell to cell spread

When moving bacteria contact the plasma membrane, they induce the formation of bacterium containing protrusions. Contact between these protrusions and neighbouring cells results in

the internalization of the protrusion. In the newly infected cell, the bacterium is surrounded by two plasma membranes which must be lysed to initiate a new cycle of multiplication and movement. Lysis of the double membrane is mediated by a phospholipase C synthesized by *L. monocytogenes* called phosphatidylcholine-specific phospholipase C (PC-PLC). Thus, once *Listeria* has entered the cytoplasm, it can disseminate directly from cell to cell, circumventing host defences such as circulating antibodies and complement. This ability to disseminate in tissues by cell-to-cell spreading provides an explanation for the early observation that antibody is not protective and that immunity to *Listeria* is T cell-mediated (Cossart and Lecuit, 1998).

1.3.6. The virulence gene cluster of *L. monocytogenes*

Most of the genes coding for the virulence factors which are discussed above are clustered on a 10 kb region of the chromosome. This virulence locus consists of three transcriptional units (Fig. 1.2.). The central position is occupied by the *hly* monocistron , encoding a pore-

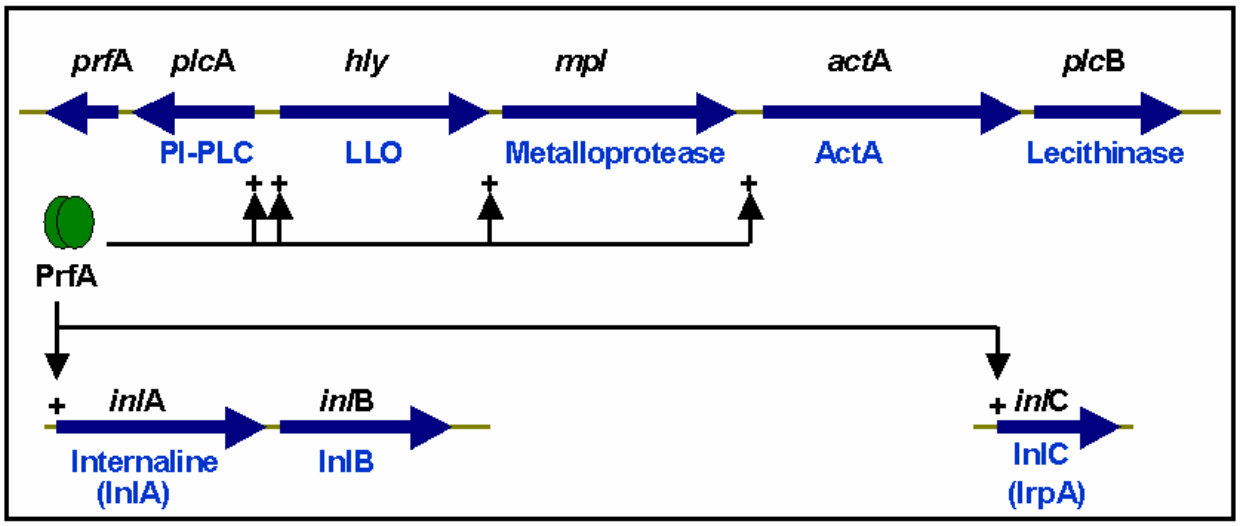

Fig.1.2. Schematic representation of the virulence gene cluster (vgc) of *L. monocytogenes* (*italic*), its corresponding proteins, and its regulation by prfA. The direction of transcription is indicated by arrows.

forming listeriolysin O (Cossart *et al*., 1989; 2001). Downstream from *hly* and transcribed in the same orientation is a 5.7-kb operon comprising three genes: *mpl*, *act*A and *plc*B, encoding a zinc metalloenzyme, a surface protein ActA, a zinc-dependent phospholipase C respectively (Vázquez-Boland *et al*., 1992). Upstream and divergent from *hly* lies the *plc*A-

*prf*A operon that encodes plcA, a phosphatidylinositole-specific phospholipase C. The second gene encodes the prfA protein which is a member of the CAP/FnR family of transcriptional activators and has a critical helix-turn-helix motif similar to that of CAP (Sheehan *et al*., 1996). All known virulence genes, in addition to prfA protein itself, are under the either absolute or partial control of the pleiotropic activator protein PrfA (Mengaud *et al*., 1991; Chakraborty *et al*., 1992). Therefore, *prf*A, like *hly* and *act*A, is absolutely indispensable for *Listeria* pathogenicity. PrfA is the only virulence activator identified to date in *Listeria* and is the main switch of a regulone comprising virulence-associated loci scattered throughout the listerial chromosome, including members of the internaline multigene family (Goebel *et al*., 2000). A number of environmental and growth-phase dependent signals modulate expression of the virulence regulon via PrfA. The activating signals include high temperature (37 °C) (Leimeister-Wächter *et al*., 1992), stress conditions (Sokolovic *et al*., 1990), sequestration of extracellular growth medium components by activated charcoal (Ripio *et al*., 1996), contact with host cells (Renzoni *et al*., 1999) and the eukaryotic cytoplasmic environment (Bubert *et al*., 1999; Freitag and Jacobs, 1999; Moors *et al*., 1999; Renzoni *et al*., 1999). This model predicts a regulatory mechanism involving allosteric activation of PrfA by a putative low molecular weight cofactor, the levels of which would depend on the environmental conditions sensed (Ripio *et al*., 1997; Vega et *al*., 1998). *Prf*A activation leads to the synthesis of more PrfA protein by positive feedback, mediated by a PrfA-dependent promoter, which governs the synthesis of a bicistronic *plc*A-*prf*A mRNA (Vega *et al*., 1998; Mengaud *et al*., 1991).

This 10 kb virulence region is, however, absent from the non-pathogenic species such as *L. innocua*, *L. welshimeri* and *L. gray* but is present, with the same genetic structure and at an identical position, in the chromosome of *L. ivanovii* (Gouin *et al*., 1994; Chakraborty *et al*., 2000). In contrast to well-characterized pathogenicity islands, the *Listeria* virulence gene cluster is rather small and has the same GC content as the rest of the chromosome (Cossart and Lecuit, 1998). The *inlAB* operon is located in another region. *InlAB* was the first identified internalin locus to be identified which was discovered by screening a bank of transposon mutants for defective internalization in epithelial cell monolayers (Gaillard *et al*., 1991). Since then, a number of other internalin loci have been found in *L. monocytogenes* and *L. ivanovii*, and there is evidence that they are also present in nonpathogenic species such as *L. innocua* (Gaillard *et al*., 1991). All these loci form a multigene family exclusive to *Listeria*, encoding proteins with a characteristic domain containing a variable number of leucine-rich repeats (LRRs). There are two subfamilies of internalins. One consists of large proteins (70–80 kDa) which are attached via their C-terminal regions to the bacterial cell wall. This group

is exemplified by the *inlAB*-encoded InlA and InlB polypeptides and includes at least six other members (inlC2, *inlD*, *inlE*, *inlF*, *inlG* and *inlH*), all found in *L. monocytogenes* (Dramsi *et al*., 1997; Raffelsbauer *et al*., 1998). The other group includes proteins generally much smaller in size (25–30 kDa), which lack the C-terminal cell-wall anchor region and are released into the extracellular medium. The prototype is InlC (or IrpA) from *L. monocytogenes* (Engelbrecht *et al*., 1996; Domann *et al*., 1997) but the remaining members of this group (i-InlC, i-InlD, i-InlE, i-InlF and i-InlG) have all been identified in *L. ivanovii* (Engelbrecht *et al*., 1998; González-Zorn *et al*., 2000). The known internalin loci usually comprise from two up to several *inl* genes, forming ´internalin islets´. The only exceptions are the *inlC* and *inlF* genes, which lie alone in the *L. monocytogenes* chromosome (Vazquez-Boland *et al*., 2001a).

1.3.7. Listeriosis model of systemic infections

L. monocytogenes is a food-borne pathogen (Farber and Peterkin, 1991). Although ingestion of these bacteria is likely to be a very frequent event, given their ubiquitous distribution and the high frequency of contamination of raw and industrially processed food, *L. monocytogenes* is an uncommen cause of severe illness in the general human population. Host susceptibility plays a major role in the presentation of clinical disease upon exposure to *L. monocytogenes*. Human infection with *L. monocytogenes* typically occurs in immunocompromised individuals (Schuchat *et al*., 1991), justifying its classification as an opportunistic pathogen. In immunocompetent people, infection with *L. monocytogenes* may be accompanied by flu-like symptoms, and spontaneous resolution is common. Groups at risk are neonates, the elderly, as well as the adults with illness that compromise the immune system like chronic liver disease and AIDS. Pharmacologically immunocompromised adults, such as transplant recipients or patients suffering from autoimmunity, and adults treated with chemotherapy due to cancer are also concerned (Lorber, 1997). In these individuals, *L. monocytogenes* can cause severe invasive infections, which frequently end in a fatal course of meningoencephalitis. An additional frequent form of invasive listeriosis is septicemia (Lorber, 1997). However, recent epidemiological studies have provided compelling evidence that a febrile gastroenteritis syndrome may be the main clinical manifestation of *L. monocytogenes* infection, which is also found in immunocompetent adults upon ingestion of high doses of bacteria (Salamina *et al*., 1996; Dalton *et al*., 1997; Aureli *et al*., 2000).

Another form of clinical presentation is foetomaternal listeriosis (Klatt *et al*., 1986). This results from invasion of the foetus via the placenta and develops as chorioamnionitis. Its consequence is abortion or the birth of a baby with generalized infection, a clinical syndrome known as granulomatosis infantiseptica and characterized by the presence of pyogranulomatous microabscesses disseminated over the body and a high mortality. The infection is usually symptomatic in the mother or may present as a mild flu-like syndrome.

The pathogenesis of listeriosis in humans is still poorly understood. Most of the available information is derived from interpretation of epidemiological, clinical, and histopathological findings or by extrapolation of observations made in experimental infections in animals, particularly in the mouse model which has long been used as a tool to study a rapidly appearing CD8+ T cell-dependent immune response (Mackaness, 1962). As contaminated food is the major source of infection, the gastrointestinal tract is thought to be the primary site of entry of *L. monocytogenes* into the host. The clinical course of infection usually begins about 20 h after the ingestion of heavily contaminated food in cases of gastroenteritis (Dalton *et al*., 1997), whreas the incubation period for the invasive disease is generally much longer, around 20 to 30 days (Linnan *et al*., 1988; Riedo *et al*., 1994).

Before reaching the intestine, the ingested bacteria have to withstand the adverse environment of the stomach. A significant number is probably destroyed by the gastric acidity (Schlech *et al*., 1993). The portal of entry and the mechanism of intestinal translocation used by *L. monocytogenes* are controversial (Marco *et al*., 1997; Jensen *et al*., 1998). However, recent data favours the view that the translocation of *L. monocytogenes* is a passive non-specific process with no preference for enterocytes or M cells (Pron *et al.*, 1998) but with, however, a preferential site of early multiplication in the phagocytic cells underlying the Peyer's patches. This result is in agreement with the early observation that bacteria survive and even replicate in resident phagocytes of the lamina propria (Mackaness, 1962).

Following translocation, bacteria, via the lymph and the blood, reach the spleen and liver. In the latter organ, most of the bacteria are killed by the Kupffer cells, though a fraction reachs the hepatocytes, the principal site of bacterial replication in the liver (Cousens and Wing, 2000), where they induce apoptosis with concomitant release of chemoattractants which leads to an influx of neutrophils (Conlan and North, 1991; Rogers *et al*., 1996). These phagocytic cells ingest bacteria or apoptotic hepatocytes and contribute to the rapid clearing of the infection before a complete sterilization is achieved by the immune response. The step of bacterial multiplication in the liver appears critical for the establishment of a 'successful' infection (Cossart and Lecuit, 1998). 2 to 4 days after infection, influx of neutrophils

gradually changes to infiltrating macrophages and T lymphocytes to form the characteristic granulomas (Mandel and cheers 1980; Heymer *et al*., 1988). These granulomas are the histomorphological correlate of cell-mediated immunity and presumably act as the true physical barriers that confine infectious foci (portnoy, 1992). Between day 5 and day 7 post-infection, bacteria start to disappear from mouse organs until their complete clearance as a result of activated macrophages and a specific immune response primarily involving CTLs and a Th1-biased CD4+ T cell response (Harty *et al*., 1992; Geginat *et al* 1998; Gregory and Liu 2000).

Although it may be hazardous to extrapolate these data to human listeriosis, the scenario of the human infection is believed to begin by ingestion of contaminated food (Schlech *et al*., 1983). Bacteria then reach the gastrointestinal tract and cross the intestinal barrier. In the cases of the immunocompromised host or the pregnant woman, bacteria multiply unrestrictedly in the hepatocytes from which they further disseminate haematogeneously to the brain and placenta (Cossart and Lecuit, 1998).

1.4. Host immune response to *Listeria* infection

1.4.1. The innate response to *Listeria* infection

The innate immune response to *Listeria* involves a coordinated interaction between many cell types and the production of numerous cytokines. These responses have been most clearly studied in SCID (severe combined immunodeficient) mice, devoid of B and T cells, which allow for the dissection of these innate responses. These components are necessary in immunocompetent mice to control infection prior to the development of T cell immunity (Edelson and Unanue, 2000).

Neutrophils play a key role in the early control of *Listeria* growth, appearing at sites of infection within the first 24 hours. The importance of neutrophils was demonstrated by several investigators using monoclonal antibodies to deplete these cells from the host during infection (Rogers and Unanue, 1993; Czuprynski *et al*., 1994a; 1994b). Mice depleted of neutrophils succumbed to an early lethal infection, with large bacteria-laden foci in the liver parenchyma. Rogers *et al*. demonstrated that *Listeria* infection of hepatocytes both *in vitro* and *in vivo* resulted in apoptosis of the hepatocytes and in the release of neutrophil chemoattractants (Rogers *et al*., 1996). Neutrophils migrating into the liver removed apoptotic hepatocytes through release of proteases and destroyed the released bacteria through surface phagocytosis. Indeed, activated neutrophils can produce a variety of cytokines (IL-1b, IL-6

and TNF-α), chemokines [macrophage inflammatory protein 1a (MIP-la), MIP-1b and MIP-2], and other soluble factors (leukotriene B4) that modulate the activities of both resident and immigrating cell populations (Seebach *et al*., 1995).

Recent experiments indicate that the initial elimination of *Listeria* taken up in the liver is not solely a function of immigrating neutrophils; Kupffer cells also play a prominent role. Mice depleted of Kupffer cells by pretreatment with liposome-encapsulated dichloromethylene diphosphonate exhibited an approximate 75% decrease in the number of *Listeria* recovered in the liver 10 min post-infection (Gregory and Wing, 1998). These findings suggest that the majority of *Listeria* initially recovered in the liver is bound to Kupffer cells. Experiments demonstrating reduced blood clearance in mice pretreated i.v. with sugars or neoglycoproteins suggest that clearance in at least some cases is mediated by the interaction of lectins expressed by Kupffer cells and carbohydrate residues on the surface of the bacteria (Ofek and Sharon, 1988). In addition to playing a major role in blood clearance, Kupffer cells are a significant factor in host resistance to both primary and secondary listerial infections of the liver (Pinto *et al*., 1991; Samsom *et al*., 1997). Kupffer cells can inhibit the proliferation of *Listeria* in the liver directly by phagocytosing and killing the organisms, or indirectly by promoting the biological response of other cell populations. Indeed, Kupffer cells can express intercellular adhesion molecule 1 (ICAM-1; CD54) and vascular cell adhesion molecule 1 (VCAM-1; CD106), and produce a variety of soluble factors such as interleukin 1b (IL-1b), IL-6, tumor necrosis factor a (TNF-α), nitric oxide and leukotrienes: all of which can promote the infiltration, localization, and/or antimicrobial activity of neutrophils (Decker, 1990; Essani *et al*., 1995). A similar role in trapping blood-borne pathogens was evaluated in macrophages located in the marginal zone of the spleen (Aichele *et al*., 2003).

Also critical during early infection is the interaction between *Listeria* organisms, macrophages and NK cells (Unanue, 1997a). Infection of macrophages with *Listeria* (or exposure to heat-killed *Listeria*) results in macrophage production of TNF-α and IL-12, two key cytokines of the innate immune system. These two cytokines together synergize to cause NK cell secretion of IFN-γ. In combination with TNF-α, IFN-γ leads to full macrophage activation. Activated macrophages display increased levels of class II MHC molecules and become listericidal through the production of free radicals (Bancroft *et al*., 1991; Beckerman *et al*., 1993). Macrophage activation is clearly necessary for immunity to *Listeria*, as pharmacologic inhibition of nitric oxide production or the gene targeting of inducible nitric oxide synthase and NADPH oxidase results in the inability of mice or macrophages to control infection (Beckerman *et al*., 1993; Shiloh *et al*., 1999). Recently, a report has also

demonstrated that splenic CD8α+ dendritic cells produce IFN-γ in response to *Listeria* infection in the absence of NK cells, potentially serving as an alternative source of IFN-γ (Ohteki *et al*., 1999). It should also be stated that the cytokines IL-1 and IL-6 play a necessary role during the early immune response to *Listeria* infection. Their role has been associated with the neutrophil response (Unanue, 1997a; 1997b). Just recently a novel cytokine, Eta-1 (osteopontin), has been shown to play a role during the very early phase of listeriosis (Ashkar *et al*., 2000). Thus, there exists during the early phase of the infection interplay among IL-1, IL-6, Eta-1, TNF-α and IL-12. Like macrophages, dendritic cells have the ability to phagocytose *L. monocytogenes* at the early stages of infection and produce a variety of proinflammatory cytokines like TNF-α, IL-1, IL-6. and IL-8 as well as IL-12 and IL-18 production (Guzman *et al*., 1995; van Deuren *et al*., 1995; Kolb-Maurer *et al*., 2003) .
Beside its role as a natural killer cell activator, IL-12 is a major Th1-promoting factor (Macatonia *et al*., 1995; Gorak *et al*., 1998). IL-12, in combination with IL-18, induces IFN-γ production by T cells. Both IL-12 and IFN-γ are required for the generation of protective Th1 immune responses against the intracellular bacteria (Yang *et al*., 1997; Sugwara, 2000). A critical role for IL-18 has recently been described in *L. monocytogenes* infected mice (Neighbors *et al*., 2001). Infection was greatly exacerbated by a monoclonal antibody against the IL-18 receptor. Moreover, IL-18 was required for the subsequent release of nitric oxide from the macrophage in response to *L. monocytogenes* infection. IL-18-dependent NO production may be a major mechanism of bacterial clearance.

1.4.2. The adaptive response to *Listeria* infection

1.4.2.1. The T cell response

The adaptive immune response to *Listeria* is dependent on the presence of T lymphocytes. Because the organism is present in both phagosomes and the cytosol of professional antigen-presenting cells during infection, epitopes derived from *Listeria* proteins are presented by MHC class I and class II molecules as well as by the class Ib molecule H2- M3 (Pamer *et al*., 1991; Safley *et al*., 1991; Sanderson *et al*., 1995; Sijts *et al*., 1996; Busch *et al*., 1997; Darji *et al*., 1998). Presentation of *Listeria* antigens (often bacterial secreted proteins) primes both CD4+ and CD8+ T cells. This results in the full clearance of organisms from the host and provides a pool of memory T cells capable of a more rapid secondary immune response to the bacteria. *Listeria*-specific CD4+ and CD8+ T cells can both independently provide resistance to infection although the effector mechanisms used in each case are still not fully elucidated.

It is clear that both CD4+ and CD8+ T cells are activated in an Ag-specific fashion following infection with *L. monocytogenes* (Harty *et al*., 1996). However, a large body of evidence involving specific T cell subset depletion (Mielke *et al*., 1989) and experiments performed in mice deficient in CD4+ and/or CD8+ T cells (Roberts *et al*., 1993; Kaufmann and Ladel, 1994; Ladel *et al*., 1994) indicates that CD8+ T cells are the most effective mediators of antilisterial immunity.

1.4.2.1.1. CD8+ T cell effector mechanisms

Activated CD8+ T cells are capable of elaborating a diverse array of effector functions. Following Ag-specific stimulation, CD8+ T cells produce a broad range of cytokines including IFN-γ and TNF-α (Douglas *et al*., 1998). The ability of activated CD8+ T cells to carry out *in vitro* cytolysis is well documented. Following ligation of the TCR-αβ by the appropriate MHC class I-peptide complex, the CD8+ T cell induces its target to undergo programmed cell death (PCD) (Mielke *et al*., 1989). Two independent pathways account for the majority of *in vitro* target cell lysis by CD8+ T cells (Kagi *et al*., 1994). One is a perforin-dependent pathway, mediated by granzymes, which are serine proteases found in the cytoplasmic granules of activated CD8+ T cells that gain access to the cytoplasm of the target cell and induce PCD by activation of the caspase cascade. Although the mechanism(s) by which perforin and granzymes are involved in cytolysis remain controversial (Froelich *et al*., 1996; Shi *et al*., 1997), the dependence of some cytolytic activity on perforin is clear (Kagi *et al*., 1994; Kojima *et al*., 1994; Lowin *et al*., 1994; Walsh *et al*., 1994). The other pathway by which CD8+ T cells are capable of efficient *in vitro* cytolysis is dependent upon interactions between CD95 ligand (CD95L, Fas ligand) on the activated CD8+ T cell and CD95 on the target cell. Ligation of CD95, in some cell types, leads to intracellular signaling events that also activate the caspase cascade and induce PCD (Nagata, 1997).

1.4.2.1.2. H2-K^d-restricted recognition by T cells

Four major *Listeria*-derived epitopes of CD8+ T cells presented by H2-K^d have been identified. Attempts to quantitate the presentation of these peptides by infected cells have been made. Two of the peptides derive from p60, a constitutively secreted protein of *Listeria*: p60 (residues 449-457) is presented at a high density by infected cells but is subdominant based on the size of the T cell population specific for this peptide; in contrast a stronger T cell

response is specific for p60 (residues 217-225), even though this epitope is presented at a lower level. A third epitope derives from LLO (residues 91–99); although presented in smaller amounts than the p60 epitopes, it dominates the T cell response. The fourth epitope is from a secreted metalloprotease, mpl (residues 84–92). A major contribution in understanding the response to *Listeria* has been made by following the development of the CD8+ T cell response to these four peptides, an accomplishment made by Eric Pamer's laboratory (Busch *et al*., 1998; 1999) using the MHC tetramer approach developed by Davis and Altman (Altman *et al*., 1996).

Pamer's group developed four tetramers that were shown to recognize CD8+ T cells specific for the *Listeria*-derived epitopes described above. Following primary infection, the T cell populations specific to each epitope increased albeit in different amounts. The largest number of cells was specific to $LLO_{91–99}$, reaching about 1.4% of all CD8+ cells in the spleen at day 7 post-infection, while the response to the subdominant epitopes ($p60_{449–457}$ and $mpl_{84–92}$) were about 0.05%. The T cell response to the p60 217–225 epitope was intermediate, at 0.25%. These responses all declined noticeably by day 35. A secondary challenge resulted in the expansion of all of these T cell populations but cells specific for the two major epitopes proliferated most dramatically: 17% of all CD8+ spleen cells recognized $LLO_{91–99}$, while about 4% were specific to $p60_{217–225}$. The T cell populations again contracted after the secondary infection was resolved; however they persisted at a level higher than that seen after the initial primary infection. Therefore, the burst and subsequent decline of the response to each epitope occur in parallel, despite the wide range in the size of the T cell populations specific for these four epitopes. A point to be noted in the overall studies from *Listeria* is the lack of correlation between the quantity of MHC–peptide display by the antigen-presenting cell and the magnitude of the T cell response. This could indicate that a number of regulatory mechanisms are present or that the magnitude of the response is dependent on the existing TCR repertoire prior to stimulation (Busch *et al*., 1998; 1999).

1.4.2.1.3. H2-M3-restricted recognition by T cells

Other antigenic components in the anti-*Listeria* response are the bacterial peptides presented by H2-M3. Presentation of peptides by this molecule requires the presence of N-formyl-methionine at their amino termini. Consequently, it was expected that bacteria-derived peptides could be selected by this special class I molecule and presented to T cells. Several *Listeria*-derived peptides presented by H2-M3 have been identified (Gulden *et al*., 1996; Lenz

et al., 1996; Princiotta *et al*., 1998). A recent paper (Kerksiek *et al*., 1999) followed the T cell response to one of these peptides by using MHC tetramer technology. An important finding was that these T cells expanded rapidly during primary infection, peaking at days 5 –7 post-infection. In comparison, the response to the $LLO_{91–99}$ peptide (presented by Kd) peaked on days 7–9. Surprisingly, however, the H2-M3-restricted T cell response to a secondary infection was rather small compared with the vigorous recall response to the LLO peptide described previously.

1.4.2.2. Role of humoral immunity in *Listeria* infection

In the past few years provocative studies showing the role of antibody in protection against some intracellular pathogens have been reported, most notably to *Cryptococcus neoformans* (Yuan *et al*., 1995). Protective mAbs were shown to recognize the capsular polysaccharide of the organism, with protection depending on epitope specificity and isotype of the mAb (Yuan *et al*., 1995; Nussbaum *et al*., 1997).

The human humoral immune response directed against proteins of *Listeria monocytogenes* have been examined in both healthy individuals and listeriosis patients. Two major targets for an antibody response were found in individuals that did not suffer from listeriosis: listeriolysin O (LLO) and internalin-related protein (IrpA). In contrast, the humoral response in listeriosis patients appears to be more heterogeneous and included LLO, IrpA, InlB, and ActA as major targets (Grenningloh *et al*., 1997) indicating that these proteins can be used to probe for and identify listerial infections in patients and, in particular, to assess the incidence of innocuous infection by pathogenic listeria in the general population.

There are at least four potential mechanisms for antibody mediated protection against intracellular pathogens: firstly opsonization of the pathogen when it is present in the extracellular space, leading to complement activation and/or increased phagocytosis; secondly binding to key surface molecules on the pathogen, preventing entry into host cells; thirdly neutralization of toxins elaborated by the pathogen in the extracellular environment; fourthly antibody action at an intracellular location inside of a pathogen-containing phagocyte (Cossart and Lecuit, 1998). It should be noted that on some occasions polyclonal antisera can fail to have a protective effect whereas a mAb of a defined specificity and isotype can be shown to provide protection. That is to say, natural infection may not result in the production of protective antibodies. This means that studies employing serum transfers (as was the case for

Listeria) should not be taken as evidence that no role exists for antibody in the context of a particular organism.

Two recent reports (Edelson *et al*., 1999; Ochsenbein *et al*., 1999) have demonstrated a surprising role for natural antibodies and for anti-LLO antibody in the context of *Listeria* infection. Ochsenbein *et al*. showed that naive C57BL/6 mice possess low but detectable levels of *Listeria*-specific natural IgM in their serum (reactive to *Listeria* surface components at a titer of 1:16) although there was no detectable *Listeria*-specific IgG (Ochsenbein *et al*., 1999). Mice lacking antibody production (μMT mice) as expected did not have this natural anti-*Listeria* IgM and the early dissemination of *Listeria* was characterized in these antibody-deficient mice compared with wild-type counterparts after a high dose intravenous infection.

Recent work of Edelson *et al*; has involved antibody neutralization of the *Listeria* pore-forming toxin LLO. Mice were passively treated with mouse anti-LLO mAb and then infected with *Listeria*. Antibody treatment resulted in significantly lower bacterial titers in the spleen and liver at both six hours (titers were 1 log lower) and two days (2–3 logs) post-infection; increased survival after a lethal challenge also resulted (Edelson *et al*., 1999). Increased survival was also seen in SCID mice, indicating that the antibody could work in the absence of B and T cells although antibody-treated SCID mice still developed a chronic *Listeria* infection, demonstrating the need for T cells for full *Listeria* clearance. Protection by mAb depended on toxin neutralization, as a non-neutralizing mAb did not affect *Listeria* resistance. Authors suggested that LLO was neutralized either extracellularly or within the phagosomes of infected cells. However, older studies in which anti-*Listeria* serum was taken following infection or immunization with killed bacteria and then transferred to naive animals did not demonstrate protection against *Listeria* infection (Osebold and Sawyer, 1957; Mackaness, 1962; Cheers and Ho, 1983). The ability of mAb to provide resistance over serum antibody has been explained to be due to a low abundance of serum Ab to protective Ags, or as a result of serum Ab being of an inappropriate isotype to mediate resistance (Edelson *et al*., 1999).

1.5. Cholesterol-binding cytolytic protein toxins

Cholesterol-binding cytolysins (CBCs) are a large family of 50- to 60-kDa single-chain proteins produced by 23 taxonomically different species of gram-positive bacteria from the genera *Streptococcus, Bacillus, Clostridium, Listeria* and *Arcanobacterium* (Billington *et al*., 2000). Apart pneumolysin, which is an intracytoplasmic toxin, all the other toxins are secreted in the extracellular medium. Among the species producing CBCs, only *L. monocytogenes* and

L. ivanovii are intracellular pathogens which grow and release their toxins in the phagocytic cells of the host. CBCs are lethal to animals and highly lytic toward eukaryotic cells, including erythrocytes. Their lytic and lethal properties are suppressed by sulfhydryl-group blocking agents and reversibly restored by thiols or other reducing agents. These properties are irreversibly abrogated by very low concentrations of cholesterol and other 3-hydroxysterols (Alouf, 2000). Membrane cholesterol is thought to be the toxin-binding site at the surface of eukaryotic cells. Toxins molecules bind as water-soluble monomers to the cholesterol-containing lipid bilayer of eukaryotic cells. Upon binding they oligomerize into arc- and ring-shaped structures comprising about 50–80 toxin molecules followed by the generation of large oligomeric transmembrane pores (15–30 nm diameter) leading to cell lysis (Bhakdi *et al*., 1985; Bayley, 1997; Rossjohn *et al*., 1998; 1999; Shepard *et al*., 1998; Alouf and Palmer, 1999; Gilbert *et al*., 1999; Mitchell, 1999). The exception to this is found in the *Listeria monocytogenes* CBC, listerolysin O, which lyses phagosomal membranes but not the cell membrane due to its low pH activity optimum at around pH 5.5 (Geoffroy *et al*., 1987). As for other bacterial membrane-damaging proteins, a key function of CBCs is likely to enable access to free iron in host cells for bacterial growth purposes. In many cases cytolysin production is upregulated by low environmental iron (Alouf, 2000).

Thirteen structural genes of the toxins have been cloned and sequenced to date. The deduced primary structure of the proteins shows obvious sequence homology particularly in the C-terminal part and a characteristic common consensus sequence containing a unique Cys residue (ECTGLAWEWWR) near the C-terminus of the molecules (except pyolysin and intermedilysin) known as the Trp-rich loop and shown to be involved in cholesterol and membrane binding (Nakamura *et al*., 1995; Rossjohn *et al*., 1998; Jacobs *et al*., 1999). However, another Cys residue outside this undecapeptide and closer to the C-terminus occurs in ivanolysin. Genetic replacement of the Cys residue in the consensus undecapeptide by certain amino acids demonstrated that this residue was not essential for toxin function. Other residues in the undecapeptide have been mutagenized, particularly the Trp residues. One of these Trp appeared critical for lytic activity (Alouf, 2000).

1.5.1. Pneumolysin

Pneumolysin (PLY) is an important virulence factor from the pathogenic bacterium *S. pneumoniae*. The pneumococcus is responsible for many serious diseases such as bacterial

meningitis, pneumonia and otitismedia which cause significant mortality and morbidity of human populations worldwide. Various pneumococcal proteins allow efficient propagation of bacterium within the host, including pneumolysin (PLY), hyaluronatelyase (SpnHL) (Jedrzejas *et al*., 1998a; 1998b; Li *et al*.,2000) and pneumococcal surface protein A (PspA) (Jedrzejas *et al*., 2000).

Pneumolysin is a 53-kDa protein produced by all clinical isolates of the pathogen (Paton *et al*., 1986; 1993). Unlike other pneumococcal antigen, this molecule is not surface exposed. It is a cytoplasmic enzyme that is released due to the action of surface pneumococcal autolysin. The virulence properties of pneumolysin are therefore directly dependent on the action of autolysin. Pneumolysin has several distinct functions, especially in the early pathogenesis of pneumococcal infection. The enzyme is cytotoxic to ciliated bronchial epithelial cells, slows ciliary beating in organ culture, and disrupts tight junctions and the integrity of the bronchial epithelial monolayer (Steinfort *et al*., 1989; Rayner *et al*., 1995). Due to pneumolysin function, the ability of ciliated bronchial cells to clear mucus from the lower respiratory tract is reduced, which facilitates the spread of pneumococcal infection. In addition, pneumolysin interactions with alveolar epithelial cells and pulmonary endothelial cells probably cause alveolar edema and hemorrhage during pneumococcal pneumonia. Pneumolysin action during pneumococcal infection disrupts the alveolar-capillary boundary, which produces an alveolar flooding providing nutrients for bacterial growth and facilitates penetration through the epithelium into the pulmonary interstitium and ultimately into the blood stream (Rubins and Janoff, 1998). The virulence and multiple function of pneumolysin, especially in early stages of infection by pneumococci, are crucial to the pneumococcal colonization of a host (Jedrzejas, 2001). For pneumolysin, its membrane pore assembly consists of 30 to 50 monomeric pneumolysin molecules with the assembly diameter of 35 to 45 nm (Rubins and Janoff, 1998).

Although the structure of PLY is not known, it is expected to be similar to the structures of CBC molecules from other bacterial organisms that have been structurally elucidated by X-ray crystallography. Of these CBC molecules, the structure of perfringolysin (PFO) from *Clostridium perfringens* probably is the most similar to that of PLY, based on the high sequence similarity of 60% (Feil *et al*., 1996; Rossjohn *et al*., 1997). The PFO molecule was divided into four domains. Three domains are arranged in a row, giving an elongated shape. Domain 3 is covalently connected to the N terminal domain 1 and packed laterally against domain 2. Membrane interaction of the monomer appears to be mediated by domain 4, while, oligomerization involves several sites scattered throughout the sequence. The Trp-rich region

around the conserved Cys residue within domain 4 is assumed to conformationally adapt to cholesterol, and domain 3 is envisaged to move across the "hinge" by which it is connected to domain 1 (Jedrzejas, 2001).

1.5.1.1. Immunomodulatory effects of pneumolysin

Cholesterol binding cytolysins were shown to possess, at its sublytic doses, a regulatory activity in target cell networks. As mentioned before, Listeriolysin O elicits IL-1, IL-6, IL-10, IL-12, TNF-α (Nishibori *et al*., 1996), chemokines (IL 8, monocyte chemotactic protein-1) expression, adhesion molecules (ICAM-1, E-selectin), NF-κB nuclear translocation (Kayal *et al*., 1999) and induction of apoptosis of mouse dendritic cells (Guzman *et al*., 1996). On the other hand, the immunomodulatory effects of pneumolysin are reflected by the stimulation of human IL-1 and TNF-α release *in vitro* (Hackett and Stevens, 1992; Houldsworth *et al*., 1994) and *in vivo* (Shanley *et al*., 1996); and complement activation (Bhakdi and Tranum-Jensen, 1985). Pneumolysin binds to immunoglobulin Fc fragment, leading to classical complement pathway activation in absence of pneumolysin-directed antibodies. Activation could divert complement away from intact bacteria and thereby leading to inflammation. Pneumolysin also shares limited sequence homology to C-reactive protein involved in acute-phase response to infection or injury (Mitchell *et al*., 1991; Paton *et al*., 1984).

Moreover, the cytotoxic effects of pneumolysin can directly inhibit phagocyte and immune cell function, which leads to suppression of the host inflammatory and immune responses. Low concentrations of pneumolysin are able to inhibit human neutrophil and monocyte respiratory bursts, chemotaxis, bactericidal activity, and production of lymphokines and immunoglobulins (Rubins and Janoff, 1998). The striking effects of pneumolysin on the host cell upon exposure to *S. pneumoniae* were recently investigated with the aid of cDNA microarrays to identify genes which are responsive to *S. pneumoniae* in a pneumolysin-dependent and -independent fashion in THP-1 human monocytic cell line. Of 4133 genes evaluated, 142 were found to be responsive in a pneumolysin-dependent fashion, whereas 40 were found to be responsive independent of pneumolysin. Genes that were up-regulated in cells exposed to the virulent type 2 *S. pneumoniae* strain relative to those exposed to the isogenic strain, which does not express pneumolysin included genes encoding proteins such as mannose binding lectin-1, lysozyme, α-1 catenin, cadherin-17, caspases 4 and 6, macrophage inflammatory protein-1β (MIP-1 β), interleukin 8 (IL-8), monocyte chemotactic protein 3 (MCP-3), IL-2 receptor-β (IL-2R-β), IL-15 receptor-α (IL-15R-α), interferon receptor-2, and

prostaglandin E synthase. Down-regulated genes included those encoding complement component receptor 2/CD21, platelet-activating factor acetylhydrolase, and oxidized low-density lipoprotein receptor 1 (OLR1). Pneumolysin-independent responses included down-regulation of the genes encoding CD68, CD53, CD24, transforming growth factor β2, and signal transducers and activators of transcription 1 (Rogers *et al*., 2003). Recent report showed that pneumolysin is recognized by Toll-like receptor 4 which mediates innate immune response to pneumococcal infection (Malley *et al*., 2003).

1.6. Regulation of iron uptake and storage in *L. monocytogenes*

The essential nature of iron for virtually all organisms is a double-edged sword that requires a careful balancing act to ensure survival. On the one hand, iron is a required cofactor of many enzymes involved in the maintenance of basic metabolic functions such as electron transport. On the other hand, interaction of free oxygen with iron leads to the Fenton reaction, which results in the production of oxygen radicals that can cause severe damage to the majority of cellular biomolecules (Touati, 2000). Due to these two opposing phenomena, it is perhaps no surprise that organisms have developed tightly regulated systems for both uptake and sequestration of iron. Within the context of mammalian systems this is accomplished in part through the action of iron binding molecules such as transferrin, heme and lactoferrin (Otto *et al*., 1992; Nuijens *et al*., 1996). In addition to protecting cells from the harmful effects of oxygen radicals, the ability to sequester iron also limits bacterial growth within the mammalian host. This is evidenced by extensive epidemiological data suggesting that there is a strong link between the iron status of the host and susceptibility to a number of bacterial pathogens (Wanachiwanawin, 2000; Collins, 2003). In light of the requirement for iron and the stiff competition for this nutrient, it is no surprise that successful pathogenic microorganisms have developed intricate systems that allow them to acquire and store iron within the bacterial cell. In fact, genes that encode factors involved in these processes are often considered to be virulence factors and have been the subject of intense study by numerous groups (Braun, 2001). Intrinsic to the ability to acquire iron is the fact that microbes must also be able to sense and respond to changing iron concentrations within the environment. This is crucial to help maintain the homeostatic relationship between having enough iron to grow and avoiding iron toxicity. In a number of organisms, this regulation is accomplished by the ferric uptake regulator (Fur) protein (Crosa, 1997). Found in both gram-negative and gram-positive bacteria, Fur functions by binding to promoter regulatory

elements, called Fur boxes, in an iron-dependent manner. Typically, Fur-regulated promoters are repressed under iron-replete conditions and derepressed under iron-depleted conditions. Moreover, it is critically important for infecting bacteria to have the ability to store iron intracellularly while in an iron-rich environment and utilize the stored iron under iron depleted conditions. Micro-organisms have developed two types of iron storing proteins: ferritins and bacterioferritins (Andrews, 1998). The former contain iron, whereas the latter contain heme. Amongst prokaryotes, ferritins have been isolated from *Bacteroides fragilis* (Rocha *et al*., 1992), *Escherichia coli* (Hudson *et al*., 1993), *Helicobacter pylori* (Frazier *et al*., 1993) and *Campylobacter jejuni* (Wai *et al*., 1995, 1996). In addition, ferritin-encoding genes have been found in the genomes of *Haemophilus influenzae*, *Clostridium acetobutylicum*, *Thermotoga maritima*, *Archaeoglobus fulgidus*, *Mycobacterium tuberculosis* and *Vibrio cholerae* (Andrews, 1998). However, the complete genome sequences of *Bacillus subtilis*, *Methanococcus jannaschii* and *Mycoplasma pneumoniae* do not contain a ferritin-encoding gene. This indicates that the iron storage system varies amongst bacterial species. Evidence to demonstrate the contribution of ferritin to protection against oxidative stress has also been provided by analysis of a ferritin-deficient mutant of *Cam. jejuni* (Wai *et al*., 1996). In *E. coli*, however, a mutation in the ferritin-encoding gene (*ftnA*) confers no sensitivity to oxidative stress on the cell (Abdul-Tehrani *et al*., 1999).

In eukaryotes, Ferritins are polymers of 24 identical or similar subunits forming a spherical protein shell where up to 4500 iron atoms can be sequestered (Harrison and Arosio, 1996; Polidoro *et al*., 2002). They mainly composed of two subunit types, termed H and L. The ferritin H subunit has a potent ferroxidase activity that catalyses the oxidation of ferrous iron, whereas ferritin L plays a role in iron nucleation and protein stability (Orino *et al*., 2001). An unusual dodecameric ferritin has been isolated from the gram-positive bacterium *Listeria innocua* (Bozzi *et al*., 1997). *L. innocua* ferritin is an oligomeric, spherical protein complex containing up to 50–100 iron atoms per oligomer and the functional properties of an authentic ferritin (Ilari *et al*., 2000). It binds and incorporates iron as an authentic ferritin, although its sequence and structure are related to the DNA-binding proteins designated DNA-binding proteins from starved cells (Dps) that are expressed by bacteria under conditions of oxidative or nutritional stress (Almiron *et al*., 1992; Pena and Bullerjahn, 1995; Yamamoto *et al*., 2000). The apoferritin shell of *L. innocua* does not contain 24 subunits like all ferritins, but is characterized by the dodecameric assemblage described for the Dps protein from *E. coli* (Bozzi *et al*., 1997; Grant *et al*., 1998). Each subunit contains one high affinity iron-binding site that resembles known ferroxidase sites (Ilari *et al*., 2000). The way of iron incorporation

by the dodecameric ferrirtin in *Listeria innocua* was recently investigated (Stefanini *et al*., 1999). The polypeptide chain that assembles into the unusual dodecameric shell of *Listeria innocua* apoferritin lacks the ferroxidase centre characteristic of H-type mammalian chains, but is able to catalyse both Fe (II) oxidation and nucleation of the iron core. A cluster of five carboxylate residues, which correspond in part to the site of iron core nucleation typical of L-type mammalian ferritins, has been proposed to be involved in both functions. Interestingly, the iron ligands in *Listeria* ferritin are conserved in the Dps proteins and belong to the group of amino acids forming the so-called DNA-binding signature, an observation in line with the recently reported iron binding capacity of a neutrophil-activing dodecameric protein from *H. pylori* (Tonello *et al*., 1999). At variance with the Dps proteins, *Listeria* ferritin does not appear to bind DNA (Bozzi *et al*., 1997). Hebraud M. and Guzzo J. have recently reported that the main cold shock protein of *Listeria monocytogenes* belongs to the family of ferritin-like proteins. Its N-terminal sequence shared a complete sequence identity with a *Listeria innocua* non-heme iron-binding ferritin. The purification of this protein revealed a native molecular mass of about 100-110 kDa which indicates a polypeptide composed of six 18 kDa-subunits (Hebraud and Guzzo, 2000). As a part of the *Listeria spp.* genome project, the ferritin gene (fri) has been identified at 970,638 bp in the 3,010,209 bp *L. innocua* chromosome and at 979,044 in the 2,944,528 bp *L. monocytogenes* chromosome (Glaser *et al*., 2001).

1.7. Aim of this work

In the first part of this study, due to the need for highly purified cytolysins in performing several biological and immunological assays, listeriolysin O (LLO) and pneumolysin (PLY) were expressed in the non-pathogenic species *Listeria innocua* followed by their purification by ion exchange chromatography.

In the second part, a novel *Listeria monocytogenes* ferritin (Frm) was identified as a target of the humoral response following infection of mice with pathogenic *Listeria monocytogenes* but not with the non-pathogenic *Listeria innocua*. A *Listeria monocytogenes* Δ*frm* mutant lacking the entire *frm* gene was generated in order to simplify identification of the role of this novel protein during *Listeria* infection as well as in mediating protection against reactive oxygen intermediates (ROI).

In the third part, the suitability of some *Listeria* mutants to be used as live vaccines against the corresponding virulent pathogen or as a carrier for introducing heterologous antigen into

animals and humans was investigated. Two categories of mutants were tested, the first includes the isogenic *Listeria monocytogenes* mutant strains that harbour either a specific deletion within the actin nucleator gene (*act*A) and/or multiple deletions within the *act*A and phosphlipase B (*plc*B) genes. The second category includes a recombinant of the non-pathogenic *Listeria innocua* harbouring the complete virulence gene cluster (vgc) from the wild type *Listeria monocytogenes*. The attenuation of these mutants, their ability to survive in mouse listeriosis model and to induce a protective as well as inflammatory T- cell mediated immunity was tested.

In the fourth part, the question if replacement of listeriolysin O (LLO) with another related cholesterol-binding cytolysin can maintain the listerial capacity to escape the host cell vacuole and gain access into the cytosole as well as to induce a protective antilisterial immunity was addressed. In this respect, prokaryotic shuttle vector harbouring the coding region for pneumolysin downstream from the *hly* promoter and regulated by *prf*A was constructed and transformed into the isogenic *Listeria monocytogenes* deletion mutant EGD-eΔ*hly*. The ability of the mutant to express and secret pneumloysin into the extracellular environment, its intracellular survival both *in vitro* and *in vivo* as well as induction of humoral and cellular responses to this mutant was examined.

Finally, the role of the putative PEST-like sequence in listeriolysin O in mediating induction of protective antilisterial immunity was analysed by studying the intracellular survival of *L. monocytogenes* mutant, lacking the PEST like sequence, both *in vitro* and *in vivo* as well as its ability to induce production of an effector and protective cellular antilisterial response.

2. Materials and Methods

2.1. Bacterial strains and plasmid vectors

Bacterial strains as well as plasmid vectors used during this work are shown in tables 2.1. and 2.2. respectively with their specific features as well as their resources and/or references.

Table 2.1. Bacterial stains.

Strain	Genotype/phenotype	Resource/Reference
Escherichia coli:		
INVαF'	F' *end*A1, *rec*A1, *hsd*R17(r_k^-,m_k^+), *sup*E44, *thi-1*, *gyr*A96, *rel*A1, F80*lac*ZΔ M15, Δ (*lac*ZYA-*arg*F)U169.	Life technologies co., Karlsruhe
DH5α^{TM}	F^-Φ*80*Δ*lac*ZΔM15,Δ*(lac*ZYA-*arg*F*)*U169,*deo*R *rec*A1,*end*A1,*hsd*R17(r_k-,r_k+),*pho*A,*sup*E44,λ^-*thi*-1, *gyr*A96, *rel*A1.	Life technologies co., Karlsruhe
XL2-Blue	*Rec*A1, *endA1, hsdR17, SupE44, thi*-1, *gyrA96*, *rel*A1, *lac* [F^1 *lac*I^qZΔM15 *Tn*10 (Tet^R) Amy Cam^R].	Stratagene co., Amsterdam; Holland
Listeria monocytogenes:		
EGD-e	Serotype 1/2a	Mackaness, 1962
EGD-eΔ*hly*2	Deletion of amino acids 61 to 420 coding nucleotides in *hly* gene.	Guzman *et al*., 1995
EGD-eΔ*act*A2	Deletion of amino acids 31 to 425 coding nucleotides in *act*A gene.	Chakraborty *et al*., 1995
EGD-eΔ*act*A Δ*plc*B	Deletion of amino acids coding nucleotides starting from aa 31 in *act*A to aa 264 in *plc*B.	Darji *et al*., 2003

Strain	Genotype/phenotype	Resource/Reference
Listeria innocua		
L. innocua	Serotype 6a NCTC 11288	Francis and Stewart, 1997
L. innocua + pUvBBAC::vgc	Serotype 6a NCTC11288 *harboring a* 6, 6 kb fragment encoding the *prf*A virulence gene cluster (*prf*A-*plc*A-*hly-mpl-act*A-*plc*B) of *L. monocytogenes* on a BAC-based shuttel vector (pUvBBAC).	Hain *et al*., unpublished

Table 2.2. Plasmid vectors.
AP^R = Ampicillin resistant, Km^R = Kanamycin resistant, Em^R = Erythromycin resistant, Cm^R = Chloramphenicol resistant, MCS = multiple cloning site, origin (ori) = origin of replication, ts = temperature sensitive.

Plasmid	Features	Antibiotic resistance	Reference
pCR®2.1-TOPO®	*lacZ*α,M13-andT7-Primer binding position,MCS,colE1-Origin	Ap^R, Km^R	Invitrogen co., Groningen; Holland
pERL3	*lacZ*α,MCS,*erm*C,ColE1-origin,pAMβ1-origin	Em^R	Leimeister-Wächter *et al*.,1990
pERL3-503	*prf*A and *hly* genes from *L. monocytogenes* in pERL3	Em^R	Darji *et al*., 1995
pAUL-A	*Lac*Za`,MCS,*erm*C,pMPa-origin,ori^{ts} from pE194	Em^R	Chakraborty *et al*., 1992
pSOG304	Shuttel vector *E. coli-L. monocytogenes, ErmC from pE194,*ColE1 from LITMUS™, pIP501-Replicon without *cop*R, *prf*A gene from *L. monocytogenes*	Em^R	Otten S., unpublished
pSOG306	promoter and signal peptide of *hly* gene *from L. monocytogenes* in pSOG304	Em^R	Otten S., unpublished
pPL2	Shuttel vector *E. coli-L. monocytogenes,* MCS*,Com*C *,col* p15A *ori,*RP4 *ori*T,U153 integrase, *L. monocytogenes* p60 promoter	Cm^R	Lauer, *et al*. 2002

Plasmid	properties	Antibiotic resistance	Reference
pUvBBAC	Shuttel BAC-based Gm+ve/Gm-ve vector , MCS, *Lac*Zα, single-copy mini-F replicon in Gm-ve host, low-copy pIP501 replicone in Gm+ve host, lox-sites for chromosomal integration of BAC-recombinant	Em^R, Cm^R	Hain *et al*., unpublished

In this work, the *Escherichia coli* host for pCR®2.1-TOPO®, pERL3-503, pSOG304, as well as pSOG306 plasmid vectors was INVαF' while DH5α™ and XL2-Blue was the host for pAUL-A and pPL2 plasmid vectors respectively.

2.2. Chemicals and biochemicals

Standard chemicals purchased from Amersham Pharmacia Biotech (Freiburg), Boehringer Mannheim (Mannheim), Life Technologies (Karlsruhe), Merck (Darmstadt), New England Biolabs (Schwalbach), Roth (Karlsruhe), Serva (Heidelberg) und Sigma-Aldrich (Deisenhofen) were used during this work. Synthetic oligonucleotides were purchased from Sigma-ARK scientific (Steinheim). Enzymes for restriction digestion from New England Biolabs and MBI Fermentas (St. Leon-Rot) as well as *Taq*-Polymerase from Life Technologies were used.

2.3. Culture media, supplements, buffers and solutions

2.3.1. Culture media

E. coli strains were grown in Luria-Bertani (LB) medium (Sambrook *et al*., 1989). LB medium consists of:

1.0 % [w/v] tryptone	10 g	
0.5 % [w/v] yeast extract	5 g	
0.5 % [w/v] NaCl	5 g	(H_2O ad 1000 ml)

L. monocytogenes EGD-e, *L. innocua* wild type strains as well as its mutant derivatives were grown in Brain Heart Infusion (BHI) medium containing 37.0 g brain heart infusion (Difco co., Augsburg) in 1.0 l H_2O. For preparing LB and BHI agar medium, 1.5% [w/v] bacteriological agar (Life Technologies, Karlsruhe) was added. For detecting the hemolytic activity of *L. monocytogenes* and *E. coli* strains, enterohemolytic washed blood agar plates from Oxoid co. (Wesel) were used.

L. monocytogenes were grown in minimal medium (Premaratne *et al*., 1991) containing glucose as a defined carbon source. Minimal medium is composed of:

Salt component A (see below)	100 ml
Salt component B (see below)	10 ml
13 mM ferric citrat	20 ml
20% [w/v] glucose	50 ml
1 mg/ml Amino acids solution	10 ml
5 mg/ml L-cysteine	20 ml
30 mg/ml L-glutamine	20 ml
10 mg/ml Riboflavin (Vitamin B2)	50 µl (in 1 N formic acid)
10 mg/ml Thiamine (Vitamin B1)	100 µl
10 mg/ml (+)-Biotin	50 µl (in 0.1 N NaOH)
1 mg/ml DL-6.8-Thioctic Acid:	5 µl (in 70% [v/v] Ethanol)
H_2O	ad 1000 ml

Salt component A:

KH_2PO_4	6.56 g
Na_2HPO_4.7 H2O	30.96 g
H_2O	ad 100 ml

Salt component B:

$MgSO_4$.7 H_2O	4.09 g
H_2O	ad 100 ml

Amino acids solution:

L-Arginine	0.1 g
L-Isoleucine	0.1 g
L-Leucine	0.1 g

L-Methionine	0.1 g
L-Valine	0.1 g
H_2O	ad 100 ml

All components were autoclaved separately and/or sterile filtered. Amino acids solution were stored at 4°C, vitamins at -20°C while all other components were kept at RT. Cysteine and glutamine solutions must be freshly prepared due to its oxidative instability.

For production of competent *E. coli* cells, *E. coli* were grown in SOB-medium (Inoue *et al*., 1990):

2.0% [w/v] tryptone	5.0 g
0.5% [w/v] yeast extract	1.25 g
10 mM NaCl	0.145 g
2.5 mM KCl	0.045 g
10 mM $MgCl_2.6\ H_2O$	0.5 g
10 mM $MgSO_4.7\ H_2O$	0.615 g
H_2O	ad 250 ml

The SOC-Medium in which *E. coli* cells are suspended after managing the transformation contains 20 mM of D- glucose in addition to the components of the SOB medium.

2.3.2. Media supplements

For inoculation of culture media by antibiotic resistant strains, the following antibiotics were added: Ampicillin (stock solution 100 mg/ml in 50% (v/v) ethanol, end concentration 100 µg/ml), erythromycin for selecting plasmid-containing *E. coli* strains (stock solution 50 mg/ml in 96% (v/v) ethanol, end concentration 300 µg/ml), erythromycin for selecting plasmid-containing *L. monocytogenes* strains (stock solution 5 mg/ml in 96% (v/v) ethanol, end concentration 5 µg/ml) and chloramphenicol (stock solution 5 mg/ml in 70% (v/v) ethanol, end concentration 8 µg/ml for selecting plasmid-containing *L. monocytogenes* strains or 20 µg/ml for selecting plasmid-containing *E. coli* strains). For production of electrocompetent *L. monocytogenes* cells, penicillin was used (Stock solution 10 mg/ml in 50% [v/v] ethanol, end concentration 10 µg/ml). For killing the extracellular bacteria after monolayer eukaryotic cell line infection in case of invasions or plaque-assays, gentamicin

was used (Stock solution 50 mg/ml in H_2O, end concentration 20 µg/ml or 10 µg/ml in soft agar into the over layer of eukaryotic cells respectively).

In *E. coli* strains containing plasmids harbouring the multiple cloning site (mcs) in the *lacZ*α gene, the medium must contain 5-Bromo-4-chlorindoxyl-β-D-galactoside (XGal; Stock solution 20 mg/ml in Dimethylformamid, end concentration 15 µg/ml) for detecting the successful insertion of the desired gene in the mcs region of the plasmid.

For production of electrocompetent *Listeria*, bacteria were grown in BHI medium containing 0.5M D-sucrose. For electroporation purpose, BHI medium with 0.5M D-sucrose was used.

2.3.3. Buffers and solutions

TE Buffer:

10 mM Tris.HCl (pH 8.0)
1.0 mM EDTA

GES-Reagent:

60 g	guanidiniumthiocyanate	
0.5 M	EDTA	20 ml
10% [w/v]	sodium-N-lauroylsarcosinate	5 ml
H_2O		ad 100 ml

Sterile filter with 0.45 µm-Filter and
keep at room temperature away from light .

50X TAE-Puffer:

242.3 g	Tris
57.1 ml	96% [v/v] acetic acid
100 ml	0.5 M EDTA (pH 8,0)
H_2O	ad 1000 ml

5X loading buffer (for agarose gel electrophoresis):

25.0% [w/v]	ficoll Type 400
0.25% [w/v]	bromphenol blue
in TE buffer	

DNA standard marker:

The 1 Kb Plus DNA Ladder ™ (delivered from Life Technologies, Karlsruhe as aliquots of 1µg/µl) was diluted to be used at an end concentration of 50 ng/µl as follows:

1 Kb Plus DNA Ladder ™	20 µl
5X loading buffer	100 µl
TE buffer	ad 400 µl

From this dilution, 10-12 µl were loaded onto the agarose gel.
The ladder contains a total of twenty bands; ten bands ranging in size from 3000 bp to 12000 bp in 1000-bp increments while the other ten bands ranges in size from 100 to 2000 bp.

CCMB80 solution:

$CaCl_2.2H_2O$	11.8 g
$MnCl_2.4H_2O$	4.0 g
$MgCl_2.6H_2O$	2.0 g
Potassium acetate	0.98 g
Glycerin (sterile)	100 ml
H_2O	ad 1000 ml

10X PCR buffer:

		End conc.:
1 M Tris.HCl (pH 8.3)	200 µl	200 mM
1 M $MgCl_2$	20 µl	20 mM
1 M KCl	250 µl	250 mM
10% [v/v] tween 20	50 µl	0.5% [v/v]

10 mg/ml gelatin	100 µl	1 mg/ml
H_2O	ad 1000 µl	

10X PBS:

NaCl	80 g
KCl	2.0 g
Na_2HPO4	6.1 g
KH_2PO4	2.0 g
H_2O	ad 1000 ml (pH 7.4 with 1M NaOH)

2X sample buffer (SDS-PAGE):

100% [v/v] glycerin	2.0 ml
10% [w/v] SDS	1.0 ml
100% [v/v] β-Mercaptoethanol	0.2 ml
0.5 M Tris.HCl (pH 6.8)	0.29 ml
0.2% [w/v] bromphenolblue	0.15 ml
(in 0.1 M Tris.HCl [pH 7,5])	
H_2O	6.9 ml

Polyacrylamide resolving gel (10 or 12%):

1.5 M Tris.HCl (pH 8.8)	5 ml
Rotiphorese® Gel 30	6.45 or 8.4 ml
10% [w/v] SDS	0.2 ml
TEMED	10 µl
25% [w/v] APS	60 µl
H_2O	8.15 or 6.2 ml

Polyacrylamide stacking gel (5.7 %):

0.5 M Tris.HCl (pH 6.8)	2.5 ml
Rotiphorese® Gel 30	1.9 ml

10% [w/v] SDS	0.1 ml
TEMED	3.5 µl
25% [w/v] APS	40 µl
H_2O	5.4 ml

10X SDS-running buffer:

Tris	30.3 g
Glycine	144 g
SDS	10 g
H_2O ad	1000 ml

Coomassie blue (for protein staining):

0.2%[w/v] coomassie R250
10% [v/v] acetic acid (96% [v/v] glacial acetic acid)
10% [v/v] ethanol
H_2O ad 1000 ml

Destaining solution for SDS-PAGE:

10% [v/v] acetic acid (96% [v/v] glacial acetic acid)
40% [v/v] methanol
H_2O ad 1000 ml

Overnight destaining solution for SDS-PAGE:

5% [v/v] acetic acid (96% [v/v] glacial acetic acid)
7.5% [v/v] methanol
H_2O ad 1000 ml

10X Blot buffer:

Tris	58 g

Glycine	29 g
SDS	3.7 g
H_2O	ad 1000 ml

10X TBS:

Tris	24.2 g
NaCl	80 g
H_2O	ad 1000 ml (pH 7.6 with HCl)

1X TBS-T:

100 ml	10X TBS
0.1% [v/v]	tween 20
H_2O ad	1000 ml

Blocking solution (for immunoblotting assay):

Skimmed milk	5 g
1X TBS	100 ml

BCIP-solution:

0.1 M glycine (pH 10)

1 mM $ZnCl_2$

1 mM $MgCl_2$

1 mg/ml 5-Brom-4-chlor-3-indolylphosphat-*p*-toluidine (BCIP)

Sodium phosphate loading buffer for protein purification (cation exchange chromatography):

0.05M NaH_2PO4 pH 6.2 (with 0.05 Na_2HPO4)

Sodium Phosphate elution buffer for protein purification
(cation exchange chromatography):

0.05M NaH_2PO4	pH 5.6 (with 0.05 K_2HPO4)
1M NaCl	

Tris-HCl loading buffer for protein purification
(anion exchange chromatography):

0.05M Tris	pH 7.5 (with 37% HCl)

Tris-HCl elution buffer for protein purification
(anion exchange chromatography):

0.05M Tris	pH 7.5 (with 37% HCl)
0.5M NaCl	

Mowiol solution:

Mowiol	2.4 g
Glycerin 100 %(v/v)	4.8 ml
H_2O	6.0 ml $\rightarrow$ keep the mixture at room temperature
0.2M Tris.HCl (pH 8.5)	12.0 ml$\rightarrow$10 min at 50°C; 15 min at 5000g, take the supernatant, store at 4°C

Diethanolamine buffer for ELISA:

$MgCl_2$	10mg	
Diethanolamine	10ml	
H_2O to	100ml	pH 9.8 (with37% HCl)

Erythrocytes lysis buffer:

Tris	2.06 gm	
NH_4Cl	7.49 gm	
H_2O to	1000.0 ml	pH 7.2 (with37% HCl)

FACS buffer:

FCS (100%)	2.0 ml
PBS (1X)	98.0 ml

2.4. Bacterial storage

To keep the bacteria for the routine use over a short period of time, bacteria were inoculated onto fresh agar plates every 4 to 8 weeks, incubated at 37°C, then stored at 4°C. *E. coli* species were grown on LB plates while *Listeria* strains were grown on BHI plates. In case of antibiotic resistance as a result of plasmid transformation, *E. coli* and *L. monocytogenes* were grown on LB and BHI agar plates supplemented with the corresponding antibiotics.
For longer-term storage, 1.0 ml bacterial culture was mixed with 0.5 ml sterile 100 % (v/v) glycerin in a 2 ml cryotubes (Nalge Nunc, Hamburg), freezed in fluid nitrogen and subsequently stored in -80°C.

2.5. Bacterial growth conditions

For the most purposes as for example plasmid DNA or chromosomal DNA isolation, infection assays, *E. coli* and *L. monocytogenes* were grown in 10 ml medium in 100 ml conical flasks at 37 °C with shaking at 180 rpm either in a water bath (Infors, Bottmingen, Swiss) or on a shaking apparatus (GFL co., Burgwedel) placed in a 37 °C incubation room. In case of all *in vitro* and *in vivo* infection experiments, subcultures were performed by 1:50 dilution of overnight culture (200 µl) in a fresh medium (10 ml) followed by further incubation at 37 °C for 2-3 hours corresponding to the bacterial exponential growth phase. 2 ml of bacterial culture were transferred to a sterile 2.0 ml reaction tube, spinned down at 8000 rpm for 2 minutes. The bacterial pellet was saved, resuspended in 2.0 ml sterile PBS and spinned down again. This washing step was repeated twice. The pellet was resuspended in PBS and the bacterial concentration was calibrated by measuring its optical density at wavelength of 600. Further dilutions were prepared in PBS to obtain required numbers of bacteria for infection (for details, see section 2.9.6.1).

2.6. Bacterial growth measurement

The optical density of bacterial culture was measured with a spectrophotometer (Ultrospec 3000; Amersham Pharmacia Biotech, Freiburg).1.0 ml of bacterial culture or suspension was added in a disposable microcuvet and the optical density was measured at a wave length of 600 nm. In case of optical density over 0.6, bacterial culture must be 1:10 diluted with the respective buffer or culture medium before measurement.

2.7. Molecular biological and molecular genetics methods

Chromosomal as well as plasmid DNA isolation, restriction enzyme analyses and amplification by PCR were performed according to standard protocols (Sambrook *et al*., 1989; Ausubel *et al*., 1990; McClure *et al*., 2000).

2.7.1. DNA isolation

27.1.1. Plasmid DNA isolation from *E. coli*

It took place by means of the GFX^{TM} micro plasmid prep kit (Amersham pharmacia biotech. co., Freiburg) following instructions of the manufacturer. 3.0ml of bacterial culture were divided into two halves, centrifuged after each other for 1 minute at 13000 rpm in a 1.8ml reaction tube and treated according to the manufacturer instructions submitted for a 2-3 ml bacterial culture. Plasmid DNA was eluted with 50 µl 10 mM Tris.HCl (pH 8.5). If the plasmid DNA was intended to be used in sequencing reaction, double distilled H_2O (pH 7.5) was used in elution. Plasmid DNA can be stored at -20 °C.

2.7.1.2. Chromosomal DNA isolation from gram-positive bacteria

Chromosomal DNA was isolated from *Listeria monocytogenes* and *Streptococcus pneumonia* following the protocol of Pitcher *et al*. (1989) as follows:
1.5 ml bacterial culture was spinned down in 1.8 ml reaction tube for 4 minutes at 1500 g. The supernatant was flicked out and the pellet was washed with 0.85% (w/v) NaCl solution, centrifuged at 15.000 g for 5 minutes, then suspended in 100 µl TE buffer containing 50 mg/ml lysozyme and finally incubated in a thermomixer (Eppendorf, Hamburg) at 37°C and 1200 rpm for 30 minutes. 1.0µl Dnase-free Rnase (Boehringer Mannheim co., Mannheim) was added to the pellet, then incubated for 5 minutes at room temperature (RT) before mixing with 500µl GES-reagent by inverting the reaction tube 5 times. After 5 minutes incubation at RT followed by 2 minutes on ice, 250µl ice-cooled 7.5M ammonium acetate was added for protein precipitation. After 10 minutes incubation on ice, the reaction tube was inverted 5 times for mixing. The DNA was extracted by adding 500µl phenol/chloroform/isoamyl alcohol (25:24:1) to the reaction tube. The reaction tube was inverted gently for 15 minutes, then centrifuged for another 15 minutes at 18.000g and 4°C. The upper aqueous layer was removed with a pipette and placed in a new microfuge tube. DNA was precipitated by adding

0.5 volume of 2-propanol followed by careful horizontal turning of the tube, careful and slow inverting and finally strong shaking so that the chromosomal DNA could be rolled up to a well visible coil. The received DNA coil would be a pellet through 5 minutes centrifugation at 18.000 g and 4°C. The DNA pellet was washed five times, each with 1.0 ml 70% (v/v) ethanol and then centrifuged. Thereafter, the DNA pellet was vacuum dried for 5-10 min and resuspended in 100µl of filtered TE buffer. The DNA was dissolved by incubating the samples overnight at 37°C. The chromosomal DNA was stored at 4°C. According to the described method, 10µg of chromosomal DNA could be obtained from 1.5 ml of stationary grown bacteria.

2.7.2. Enzymatic treatment of DNA

2.7.2.1. DNA digestion with restriction enzymes

In order to digest plasmid DNA for fragment isolation or further cloning, DNA quantities between 100 ng and several micrograms in a volume of 15 to 100 µl would be incubated for 1 to 4 h with the respective restriction enzymes (about 5 to 25 Units per µg). The restriction digestion took place in the manufacturer enzyme specific reaction buffer. In case of restriction digestion with two restriction endonucleases working in two different buffer systems, the digested DNA would be purified after the first digestion by means of the QIAquick PCR Purification Kits (Qiagen co., Hilden) then incubated with the second enzyme.

2.7.2.2. Ligation of a DNA fragment with a DNA vector

In general, a 1:3 (vector: insert) ratio is recommended for the ligation reaction. The ligation is carried out in presence of three ingredients in addition to water: Two or more fragments of DNA that have either blunt or compatible cohesive ends (the vector and the insert), a 1X buffer containing ATP which is provided as a 10X concentrate, and the T4 DNA ligase (Life Technologies co., Karlsruhe). The volume of the ligation mixture depends on the DNA concentration ranging from 15-40µl using 1-2 units from the T_4-ligase for 50ng-2µg of DNA. The optimal incubation conditions for T4 DNA ligase is overnight at 16°C.

2.7.3. Agarose gel electrophoresis

Agarose gel electrophoresis is employed to check the progression of a restriction enzyme digestion, to quickly determine the yield and purity of a DNA isolation or PCR reaction, and

to size fractionate DNA molecules, which then could be eluted from the gel. Prior to gel casting, dried agarose is dissolved in 1X TAE buffer by heating. 0.5µg/ml ethidium bromide was included in the warm gel solution to enable fluorescent visualization of the DNA fragments under UV light. The warm gel is poured into a mold (made by wrapping clear tape around and extending above the edges of an 18 cm x 18 cm plastic plate), which is fitted with a well-forming comb. The percentage of agarose in the gel varied between 0.8-1.2 percent [w/v] depending on the expected size(s) of the fragment(s). Agarose gels are submerged in 1X TAE electrophoresis buffer in a horizontal electrophoresis apparatus. The DNA samples are mixed with 5X gel tracking dye (1:1 v/v) and loaded into the sample wells. Electrophoresis is usually carried out at 150 - 200 mA for 30-60 minutes at RT, depending on the desired separation. When low-melting agarose is used for preparative agarose gels, electrophoresis is carried out at 100-120 mA for 30-60 minutes, again depending on the desired separation. 1 kb plus DNA LADDER^{TM} (Life Technologies co., Karlsruhe) size markers were co-electrophoresed with DNA samples for fragment size determination. After electrophoresis, the gel is placed on a UV light (254nm) box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern is taken with the aid of a gel documentation system of Cybertech CSI (Cybertech co., Berlin) and a video copy processor (P68E; Mitsubishi Electric, Ratingen). If the DNA would be eluted from the gel for size fractionation purposes, the amount of time at which the DNA is exposed to the UV transilluminator has to be minimised because the UV mutagenises the DNA at a measurable rate. A clean scalpel blade is used to cut around the band of interest. The transilluminator is switched off and the white light is switched on. The band is carefully removed from the gel and placed on the glass. It is recommended to trim off as much empty agarose as possible. The excised band is transferred into a 1.8 ml microfuge tube.

2.7.4. Extraction of DNA fragments from the agarose gel

QIAquick gel extraction kit (Qiagen co., Hilden) following producer's instructions was used. DNA is eluted with 20-30µl 10mM Tris.HCl (pH8.5) and can be stored at –20 °C for further use.

2.7.5. Quantification of DNA concentration

For cloning or sequencing purposes, the amount of DNA must be firstly measured using the Nanodrop ND-1000 spectrophotometer (G. Kisker GbR¸ Steinfurt). 1µl of the DNA sample is

pipetted directly onto the measurement surface. Using surface tension, a column is drawn between the ends of two optical fibers to establish the measurement path. DNA measurement took place at a wave length 260 nm and the amount of DNA is given as ng/µl.

2.7.6. Transformation

2.7.6.1. Transformation in *E. coli*

2.7.6.1.1. CCMB80 method

Chemically competent *E. coli* is prepared following the protocol of Hanahan *et al*. (1991). 20 ml LB medium was inoculated with *E. coli* INVαF' strain and incubated overnight at 37°C. A 1 ml overnight culture was inoculated in 50 ml SOB-medium and incubated at 25-30°C until the optical density at 550nm was approximately 0.3. The culture was chilled for at least 10 min on ice. In the following steps, the cell suspension should be kept on ice as much as possible. The cell suspension was centrifuged for 10 min at 3.000 rpm (Sorvall GSA rotor, GMI co., Minnesota; USA) at 4°C. The pellet was gently resuspend in 17 ml ice-cold CCMB80 buffer and incubated for 20 minutes on ice then centrifuged for 15 minutes at 3000 rpm (Sorvall GSA rotor) at 4°C. Again, the pellet is gently resuspended in 4.2 ml ice-cold CCMB80 buffer. The cell suspension is aliquoted at 200 µl per cryotube (Nalge Nunc co., Hamburg). The cell suspension is shock-freezed in liquid nitrogen and tubes are stored at 80°C where they will be competent for at least 6 months. For transformation, competent *E. coli* are thawed out on ice. 100-200µl of competent *E. coli* are gently mixed with the DNA preparation (usually 10pg to 10ng DNA) in a 1.8ml microfuge tube, incubated on ice for 30 minutes and heat shocked at 42 °C for 90 seconds. The transformation mixture is added to 800 µl SOC medium and incubated at 37 °C with shaking at 180-200 rpm for 1-3 hours depending on the antibiotic selection marker in the transforming DNA. If the transforming DNA has a phenotypic expression of ampicillin or chloramphenicol resistance, one hour will be enough for incubating the transformed *E. coli* but in case of erythromycin resistance, the incubation time will be extended to 3 hours. Finally, 50-200 µl of the transformed bacteria will be plated out on suitable selection media and incubated for 1-2 days at 37°C. In case of pPL2 and pAUL-A transformation, it is recommended that plates have to be incubated overnight at 30 °C due to its temperature sensitivity.

2.7.6.2. Transformation in *L.monocytogenes* by electroporation

Electrocompetent *L. monocytogenes* was prepared following the protocol of Park and Stewart

(1990). A single colony of *L. monocytogenes* or its isogenic mutants was inoculated in 10 ml BHI. The bacterial culture was grown overnight with shaking at 180 rpm and 37 °C. 2.0 ml is inoculated into 200 ml 0.5 M sucrose containing BHI in 1 L flask and incubated with shaking at 180 rpm and 37 °C for 2-3 hours till reaching OD_{600} of about 0.2. 200 µl of 10mg/ml penicillin G stock solution is added and bacteria were further incubated for 2 hours. Bacteria were spinned down at 5000 rpm for 10 minutes at 4°C (Sorvall GSA-rotor) and the supernatant was decanted. At this time point, bacteria must be kept on ice during work. The bacterial pellet was resuspended in 20 ml ice cold HEPES buffer (1 mM HEPES [pH 7.0], 0.5M sucrose) and centrifuged at 6000 rpm and 4°C for 10 minutes. The pellet was again resuspended in 10ml HEPES buffer and centrifuged at 6000 rpm and 4°C for 15 minutes. This step was repeated three times. In the last time, the supernatant was decanted and the bacterial pellet was mixed thoroughly with 500 µl glycerin containing HEPES buffer (1mM HEPES [pH 7.0], 0.5M Sucrose, 10% [v/v] glycerin). The bacterial suspesion is aliquoted as 50 µl cell suspension in 1.8 ml cryotubes (Nalge Nunc co., Hamburg), shock-freezed in a liquid nitrogen and kept at –80°C. Electroporation took place by mixing 50µl of thawed electrocompetent bacteria with 1-10µl (containing 1-2µg) of plasmid preparation. The mixture was pipetted in a pre-cooled electroporation cuvette (0.1 cm slit; Invitrogen, Groningen; Holland). The electroporator is set at 1 KV, 400 ohms, 25 µFD. Cuvette was placed and pulsed. The time constant for electroporation is usually about 5 minutes. Immediately 1 ml pre warmed 0.5M sucrose containing BHI was added and bacteria was incubated for 1-3 hours, depending on the antibiotic selection, at 37°C and 180 rpm. 100-250 µl of bacterial suspension are plated out on selective media for 1-2 days at 37°C.

2.7.7. Polymerase chain reaction (PCR)

2.7.7.1. Amplification of DNA fragments for further cloning

The DNA fragment, to be cloned in a vector, has to be amplified to an amount sufficient to be cloned with a minimum nucleotide mismatch. This can be done with the aid of the expand™ high fidelity PCR system (Boehringer Mannheim co., Mannheim) which increase both the yield and accuracy of the PCR product (Mullis *et al*., 1986). It contains the *Pwo*-DNA-polymerase proofreading enzyme which increases the fidelity of PCR threefold over Taq DNA Polymerase. It prevents truncated products from mis-incorporated nucleotides as well as limiting primer degradation by the 3' to 5' exonuclease activity of the proofreader. According to the producer's instructions, the volume of of PCR preparation for DNA fragment smaller

than 1kb is smaller than that of DNA fragment bigger than 1kb.The dNTP-mixture used in the hifi PCR preparation was prepared from stock solution of 100mM dNTP set (Life technologies co., Karlsruhe). The concentration of each nucleotide in the dNTP-mixture is 5mM while its end concentration in the PCR preparation is 200µM. The end concentration of each primer in the PCR preparation is 30nM. 1-2µl of isolated chromosomal DNA(100ng-1µg) is used as a template. It is common to use 0.2ml "thin walled" tubes (Biozym Diagnostic co, Hess. Oldendorf) for polymerase chain reactions. These tubes transmit the temperature changes faster. PCR took place in thermocyclers Gene Amp PCR system2400 (Perkin Elmer co., Langen).

The amplified DNA has to be purified from buffer, primers and other proteins prior to cloning or restriction digestion. The purification is done by means of QIAquick PCR purification kits (Qiagen co., Hilden) according to the manufacturer's instructions. The DNA is eluted with 20-30µl of 10mM Tris.HCl (pH 8.5). After purification, 3-5µl DNA can be tested with agarose gel electrophoresis for the right size of the PCR amplified DNA.

Usually , the amplified DNA fragment is subcloned in PCR®2.1-TOPO® vector with the aid of TOPO™ TA Cloning® kit (Invitrogen co., Groningen, Holland) and transformed in *E. coli* INVαF'. Now, the DNA insert in the new constructed PCR®2.1-TOPO® derivative is available for further restriction digestion and cloning in other vectors. Sometimes, the amplified DNA fragment can be used directly in further restriction digestion and ligation in the desired vector.

2.7.7.2. Amplification of DNA fragments for testing the recombinant clone

After cloning a DNA fragment in the desired vector by ligation or in the PCR®2.1-TOPO® vector by topoisomerisation, PCR analysis is performed to confirm the identity of the right clone using forward and reverse primers possessing sequences complementary either to those of the vector lying up-and down stream from the DNA insert or to the sequences at the 5` and 3`ends of the DNA insert. One colony of the transformed bacteria is picked by means of a tooth pic and placed in a 0.2ml reaction tube (Biozym Diagnostic co., Oldendorf). 50µl of PCR preparation is added in the tube as follows:

		End conc.:
10X PCR buffer	5.0 µl	1X
5 mM dNTPs	2.0 µl	200 µM
20 µM forward primer	0.5 µl	200 nM

20 µM reverse primer	0.5 µl	200 nM
Taq-Polymerase (5 U/µl)	0.25 µl	1.25 U/50 µl
H_2O	ad 50.0 µl	

The thermostable *Taq* polymerase was obtained from Life Technologies (Karlsruhe).
The temperature program of PCR reaction was adjusted as follows:

1)	2 min	94°C
2)	20 sec	94°C
3)	30 sec	55°C
4)	90 sec	72°C
5)	3 min	72°C
6)	∞	4°C

Steps 2) to 4) are repeated 25 times. The PCR product is tested for the right size through agarose gel electrophoresis.

2.7.8. Construction of a site-directed insertion mutation

The isogenic deletion mutants were produced by means of chromosomal integration of a pAUL-A vector containing a portion of the desired gene coding sequence (Chakraborty *et al*., 1992; Schäferkordt and Chakraborty, 1995). The pAUL-A vector is a plasmid containing a temperature sensitive origin of replication from plasmid pE194, lacZa` multiple cloning site surrounded by transcriptional terminators and erythromycin resistance marker expressed in both *E. coli* and *Listeria* sp. Two fragments from the chromosomal DNA, one is (often about 1000bp) upstream and the other (often about 500 bp) downstream from the gene of interest were amplified with high fidelity PCR followed by PCR product purification and restriction at the 3` end of the upstream fragment as well as the 5`end of the downstream fragment with the same endonuclease. Thereafter, the two restricted fragments were ligated together and the ligation product lacking the gene of interest was amplified with high fidelity PCR and restricted at its 5` and 3` ends with specific endonucleases. The pAUL-A plasmid was isolated from *E. coli* DH5α and restricted with the same endonucleases followed by ligation with the PCR amplified and restricted chromosomal DNA fragment. The ligation mixture was transformed into *E. coli* INVαF' followed by overnight incubation at 30°C. Recombinants were identified as Lac⁻ (white coloured) colonies and the plasmid DNA was analysed by PCR

analysis using the forward primer for the upstream fragment and the reverse primer for the downstream fragment to identify the insert with the right size followed by DNA sequencing to identify plasmids carrying the correct insert. These plasmids were isolated from *E. coli* INVαF' and electroporated into *L. monocytogenes* EGD-e followed by overnight incubation at 30°C. Again, PCR analysis was performed with grown EGD-e colonies to confirm the presence of plasmids carrying the right size of insert. Integration of the plasmid was achieved by streaking out the PCR positive colonies onto BHI plates containing 5µg/ml erythromycin and overnight incubation at 42°C. At this temperature, pAUL-A can not multiply but will integrate into the chromosomal DNA by homologous recombination disrupting the whole gene in the chromosomal DNA with its insert possessing the deleted gene sequence. Several transformants were purified by re-streaking colonies onto BHI plates containing 5µg/ml erythromycin and further incubation at 42°C. Colonies were tested by PCR analysis using universal primers of pAUL-A (M100/M101), which bind few nucleotides up and downstream of the multiple cloning site of pAUL-A, to identify integration of pAUL-A into the chromosomal DNA. The absence of the band corresponding to the insert indicates a successful integration. A weak band refers to incomplete integration. So, re-streaking must be continued for further few days till complete integration is achieved. The homologous recombination between the chromosomal and pAUL-A DNA sequences is accomplished by two-step gene replacement procedure. The plasmid is first integrated in the chromosome corresponding to the site on the plasmid that was cleaved by a restriction endonuclease. Homologous recombination results in two copies of the gene, separated by the plasmid sequences. The second step involves homologous crossing over in the repeated DNA segment to loop-out the plasmid, along with the intact gene. In other words, the wild-type chromosomal allele can be replaced by the mutant allele from pAUL-A. To exice pAUL-A that carry either the wild type or the mutant gene out of the bacterial cell, PCR positive colonies were overnight incubated in BHI containing 5µg/ml erythromycin at 30°C. At the next day, the bacterial culture was 1:1000 diluted and further incubated at the same conditions till reaching OD_{600} of 0.3-0.5 followed by further 1:20 dilution in BHI containing 10µg/ml erythromycin and incubation for 2.5 hours at 30°C. Addition of 200µg/ml ampicillin and overnight incubation at 30°C gives selection for bacteria that already lost pAUL-A to live over bacteria that still containing pAUL-A. Several dilutions from the bacterial culture (10^{-1} –10^{-5}) were plated out on BHI plates without erythromycin and overnight incubated at 30°C. The colonies that grow only on BHI plates but not on those containing 5µg/ml erythromycin were selected for PCR analysis using the forward primer for the upstream fragment and the

reverse primer for the down stream fragment to find out if the homologous recombination managed to exchange the wild type with the mutant gene. Colonies that carry the mutant gene underwent sequencing analysis to confirm the correct insert sequence.

2.7.9. DNA sequencing

Identification of nucleotides sequence of a DNA fragment was carried out using a strength of grouping cycle sequencing with the aid of thermosequenase fluorescent labelled primer cycle sequencing kit and ALFexpress (both purchased from Amersham Pharmacia Biotech co., Freiburg) following the manufacturer's instructions. For each sequencing reaction 1µl of Cy5-labelled primer(1-2pmol) was combined with 6µl of the tested DNA fragment (0.5µg) and added to each of four labelled microfuge tubes containing 2µl of G,C,T or A dNTPs and introduce the four sequencing reaction mixtures to the thermocycler pre-programmed as follows:

1) 5 min.	95°C
2) 30 secs.	98°C
3) 40 secs.	60°C
5) 5 min	60°C
6) ∞	4°C

Steps 2) and 3) were repeated 25 times. Addition of 5µl stop buffer to each reaction tube could stop the reaction.

2.7.10. Computer programs

Nucleotides sequences were processed with the aid of " Heidelberg Unix Sequence Analysis Resources"(HUSAR) program and the lasergene 99-sequence analysis system (DNA STAR, Inc; purchased from GATC Biotech, Konstanz). Figures of plasmid and new clones were created with the aid of "Clone Manager 5" while graphics and pictures were processed with the aid of Corel Draw (Scientific and Educational Software co., Durham; USA). Different statistical data were converted to figures using Sigma plot 2000 (SPSS Inc., Chicago; USA).

2.7.11. Primers

All primers used in this work either for purpose of amplification or sequencing was combined in the following list:

Table 2.3. A list of primers used either in DNA amplification or in sequencing. Sites for restriction digestion are underlined

Primer	Nucleotide sequence (5`→3`)	Restriction site
hly-gene for	5'- AATTCCCTCGAGCCTCCTTTGATTAGTATATTC –3'	*Xho*I
hly-gene rev	5'- AGCCACCTACAACTAGTCTGACAGAGAG -3'	*Spe*I
hly-P for	5'- CTTGACTAGAGGGTACCTCC-3'	*Kpn*I
PEST rev	5'-CTGAAGCAAAGCATATGTCTGC-3'	*Nde*I
PEST for	5'-AAGACGCATATGGAAAAGAAACACG-3'	*Nde*I
*hly*ΔPEST rev	5'-TATGGATCTCGAGTCAGATATTC -3'	*Xho*I
ply-gene for	AGGTAGCATATGGCAAATAAAGCAG	*Nde*I
ply-gene rev	CCCTGTATCTCGAGGAATTGG	*Xho*I
Lmo-fri for1	TTGTTCTCTCGGATCCGTTGACAAGGTTTCTTTATC	*BamH*I
Lmo-fri rev 1	AAATTCCTTTGCGGCCGCTGAGTTGATTGTTTTC	*Not*I
Lmo-fri for 2	CAAACATATCGCGGCCGCCAAAGCATTCTTAGGAAAA GC	*Not*I
Lmo-fri rev 2	GTTTTTGGTTCAAGCTTGATTTTCCAGTCGTGGTC	*Hind*III
fri for	CCTGAAAGCGGTGAGCTCAATTTTCCATA	*Sac*I
fri rev	CATTTATTTGCTTGGTCGACTACCTCGATATTC	*Sa*lI
M100	GTAAAACGACGGCCAGT	/
M101	CAGGAAACAGCTATGAC	/

2.8. Protein biochemical methods

2.8.1. Protein isolation from *Listeria* species

2.8.1.1. Proteins in bacterial supernatant

A fresh colony of *Listeria* sp. was inoculated in 250-300 ml conical flask containing 50 ml BHI and incubated at 37°C and 180 rpm till reaching OD_{600} of 1.0. The bacterial culture was transferred into 50 ml reaction tube. Bacteria were removed by centrifugation for 20 minutes

at 2.800g and 4°C. The culture supernatants were carefully aspirated and transferred into a fresh tube. Supernatant proteins were precipitated overnight by the addition of 10% (v/v) trichloroacetic acid (TCA) at 4°C on ice. The supernatant was centrifuged for 20 minutes at 6.200g and 4°C. The precipitated proteins were suspended in Tris.HCl (pH 8.8) and stored at –20°C.

2.8.1.2. Somatic soluble antigens

Bacteria were cultured overnight in 20ml BHI broth and transferred to 50 ml reaction tube. The supernatant was removed by centrifugation for 5 minutes at 8000 rpm and RT. Bacterial pellet was washed twice with PBS, and subsequently subjected to ultrasonication. 1g (wet weight) of bacterial cells was suspended in 10 ml of PBS and sonicated (87.5% output, degree 7 on a sonifier [Model S-125; Branson Sonic Power Co., Danbury, Conn; USA]) 6 times for 1 minute followed by 1 minute incubation on ice per time to avoid elevation of temperature. The sonicated suspension was centrifuged at 39000g for 50 minutes, and the supernatant was filter sterilized (pore size 0.45μm [Millipore co. Eschborn]), aliquoted and stored at –20°C.

2.8.2. Protein analysis

2.8.2.1. SDS-Polyacrylamide Gel Electrophoresis

Two glass plates (one notched and the size of both is 10 x 10cm.) were attached together using fixed spacers and a one-piece profiled silicone rubber seal and fixed with clamps. The assembly was kept to stand upright using clamps as supports. Resolving gel was prepared in 0.25 M Tris.HCl pH 8.8 with a final concentration of 10 or 12% depending on the molecular weight of the separated proteins. The gradients of the resolving gel were mixed gently ensuring no air bubbles form and subsequently poured carefully into glass plate assembly. The gel was overlayed with water to ensure a flat surface and to exclude air and left one hour to dry. Water was decanted before adding the stacking gel. Stacking gel was prepared in a final concentration of 5.7% in 0.125 M Tris.HCl pH 6.8, mixed and poured carefully onto the top of set resolving gel, comb was inserted and allowed to set for 30 minutes to dry. The assembled glass plates were fitted in a gel casting stand (Biometra co., Göttingen). The comb was removed and the gel casting stand was filled with 1X SDS running buffer so as to completely cover the gels. Protein samples were mixed with 2X sample buffer (3:1 v/v), heated at 95°C for 5 minutes and loaded into the wells in the stacking gel by layering them

under the running buffer using a micropipet. The gel casting stand was covered with the protection cover and connected to the power supply with a voltage of 150v and a current of 200mA for 60-80 minutes. As a molecular weight marker, 5.0µl of a pre-stained SDS-PAGE standard marker (Bio-Rad co., Philadelphia; USA) possessing a wide molecular weight range covered by broad range pre-stained standards ranging from 6.0 to 200.0 kDa was also loaded. The gel was covered with Coomassie blue staining solution, sealed in plastic box and left on a shaker for 2 to 3 hours at RT with agitation. The stained gel was destained with destaining solution with agitation.

2.8.2.2. Immunoblotting (Western blot)

2.8.2.2.1. BCIP immunodetection procedure

After separation of proteins by the SDS-PAGE electrophoresis, the gel was transferred on a 6 x 9 cm piece of PVDF transfer membrane (Millipore co., Eschborn) previously soaked in 100 % (v/v) methanol. Both gel and PVDF membrane were sandwiched between two Whatmann 3MM papers (Biometra co., Göttingen) previously soaked in 1X blot buffer. The assembled gel with membranes were carefully transferred into Fastblot^{TM} B44 semidry blotting system (Biometra co., Göttingen) in a manner that the electric current passes from the cathod through the gel and then through the PVDF membrane to the anode. The blot casting was connected to the standard power pack supply P25 (Biometra co., Göttingen) and blot running parameters were adjusted to voltage of 200v and a current of 250mA per gel for 45 minutes. The blotted nitrocellulose membrane was blocked in freshly prepared 5% skimed milk in 1X TBS buffer for 2 hours at RT with constant agitation. The primary antibody was diluted to the recommended concentration in a fresh TBS containing 0.2% tween 20 (TBS/T). The nitrocellulose membrane was soaked in the primary antibody solution and incubated for 1 to 2 hours at RT or overnight at 4ºC with agitation. The primary antibody used in this work may be monoclonal or polyclonal antibodies (Table 2.4.) as well as natural antisera isolated from mice previously infected with *Listeria monocytogenes* or its derived mutants. The nitrocellulose membrane was washed five times for 3 to 5 minutes each with TBS/T. The nitrocellulose membrane was incubated in TBS/T solution containing a 1:1000 diluted secondary antibody for 30-60 minutes at RT. For a mouse monoclonal antibody, alkaline phosphatase conjugated goat-anti-mouse IgG was used while for a rabbit polyclonal antibody, alkaline phosphatase conjugated goat-anti-rabbit IgG (Dianova co., Hamburg) was used. The nitrocellulose membrane was washed five times for 3 to 5 minutes each with TBS/T.

Detection of proteins was performed using BCIP solution as an enzyme substrate. As soon as the protein bands were detected, membrane was washed 4-5 times with water and left to air dry.

2.8.2.2.2. Enhanced chemiluminescence (ECL) immunodetection procedure

This method is more sensitive than that using BCIP as a substrate. So, it is often used for detection of minor amounts of proteins. The sequence of procedure was the same as that mentioned above with some differences; the secondary antibody used in this technique was either horseradish peroxidase-conjugated goat-anti-mouse or anti-rabbit IgG (1:1000 diluted). After the last wash, the membrane was transferred to a clean glass plate and covered with a membrane of 10 ml of developing solution (ECL from Amersham International, Piscataway; USA) for 2 minutes. The excess of developing solution was drained and membrane was wrapped in Saran Wrap (Dow chemical co. South Carolina ;USA), fixed in an x-ray film cassette with the proteins facing up and exposed to an x-ray film (Kodak co. ,Stuttgart, Germany) in a dark room for few seconds or up to several minutes.

Table 2.4. A list of first antibodies used in Immunoblotting during this work

Detected protein(s)	Type of antibody	Dilution in TBS-T
LLO (Listeriolysin O)	monoclonal (mouse ab)	1:15000
PLY (Pneumolysin)	monoclonal(mouse ab)	1:15000
Frm (Ferritin-like protein	monoclonal(mouse ab)	1:15000
from *L. monocytogenes*)	polyclonal (rabbit ab)	1:10000

2.8.3. Protein Purification

Bacterial cytolysins were hyper-expressed in *L. innocua* through co-expression of the positive regulatory factor *prf*A in the plasmid vector in conjunction with the structural cytolysin gene (Darji *et al*., 1995). One bacterial colony was inoculated in 20 ml BHI overnight with shaking at 180 rpm and 37ºC. 10 ml of the overnight bacterial culture was used to inoculate one liter of minimal medium in a 3 liter volume conical flask. The bacterial culture was incubated without shaking at 30ºC for 48 hours. The bacterial pellet was removed by centrifugation at 6000 rpm for 30 minutes at 4ºC in a floor F6-6X 250ml Sorvall centrifuge (GMI, Minnesota; USA). The supernatant was concentrated by transferring at 4ºC to Amicon stirred cell series

8000 (Millipore co., Eschborn) with a cut-off point of 30 kDa and allowed to be concentrated to about 25 ml. In case of cation exchange chromatography used for purification of listeriolysin O, the concentrated supernatant was patch absorbed with Q-sepharose (pharmacia co., Freiburg), pre-equilibrated with loading buffer (20mM sodium phosphate, pH6.2) for 60 minutes. The non-absorbed fraction was centrifuged, filtered by 0.45μm Millipore filter and transferred through a super loop, which allows introduction of larger sample volumes into a pressurized fluid system, to a HiPrep™ 26/10 desalting column intended for desalting and buffer exchange using Äkta explorer which is a high-performance chromatography system (all tools and components used in connection with ÄKTA explorer were purchased from Pharmacia biotech AB, Uppsala; Sweden) and UNICORN™ control system version 3.0 provided with a program for protein desalting. Proteins were eluted from the desalting column with the loading buffer that used in the ion exchange chromatography in order to carry the same charge as the loading buffer. Purification of the desired protein from supernatant fluids was achieved by ion exchange chromatography. Separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. In cation exchange chromatography, pH of the loading buffer (sodium phosphate loading buffer) must be lower than the isoelectric point of the desired protein by at least 0.5 units while must be higher with the same value when applying to anionic exchanger (Tris.HCl loading buffer). Resource S column was used as a cation exchange column while Resourse Q was used as an anion exchange column depending on the isoelectric point of the separated protein. With the aid of Äkta explorer and UNICORN™ control system version 3.0 provided with a program for ion exchange chromatography, the column was firstly equilibrated with the loading buffer at a flow rate of 4ml/min followed by introduction of the sample. The protein of interest was bound with the oppositely charged chromatographic medium leaving the other proteins carrying the same charge to elute out of the column. When the elution buffer start to drop in the column, conditions were then altered by increasing salt concentration so that the bound substances were eluted differentially and collected in a fraction collector with the solution eluting from the column being monitored by an absorbance device for measuring the protein concentration in the effluent. The quantity of the isolated protein in mg/ml could be determined by measuring the effluent at OD_{280} and dividing the optical density value by 1.25. Also, the haemolytic activity of the purified cytolysin could be identified qualitatively and quantitatively by the aid of hemolysin titre test. The cytolysin was stored in aliquots at –80ºC. Under these conditions, they were stable for more than 6 months.

2.8.4. Screening of hemolytic activity

With the aid of hemolysin titre test, the haemolytic activity of a protein can be identified by measuring the magnitude of lysis of (1% v/v) sheep erythrocytes suspension (Young *et al*., 1986). 1 ml of defibrinated erythrocytes (Oxoid co., Wesel) was diluted with gentle mixing in 9 ml of 1X PBS (pH 5.6 or pH 7.0) in 15 ml reaction tube followed by centrifugation for 5 minutes at 2900 rpm and 4ºC. The supernatants was gently decanted and the washing step was repeated once. 100 µl from the washed erythrocytes was added to 9.9 ml of 1X PBS (pH 5.6 or pH 7.0) containing 10 mM DTT. In case of measuring the haemolytic activity in bacterial culture supernatant, the supernatant was separated from bacteria by centrifugation at 14000 rpm for 2 minutes. The test was carried out in 96-well microtiter plates with tapered (v-shaped) well base. 50µl of 1X PBS (pH 5.6 or pH 7.0) was added to each of the 12 well of the row except the first well where 100 µl of the protein suspension was added. 1:2 serial dilution was carried out in the next 11 wells by gentle mixing of 50 µl from the protein suspension in the first well with the 50µl of 1X PBS (pH 5.6 or pH 7.0) in the second well followed by mixing 50 µl from the second well with that in the third and so on till reaching the end of the row where 50 µl from the last well was flicked out. A negative control sample (100 µl of 1X PBS [pH 5,6 or pH 7.0]) and a positive control sample (100 µl of distilled water) were included in all tests. 50 µl of washed defibrinated (1% v/v) sheep erythrocytes suspension was added to each well followed by incubation for 1-3 hours at 37ºC and 5%CO_2. The non-lysed erythrocytes would aggregate and the intact erythrocytes could be seen as a pellet on the base of plate wells. To measure the haemolytic activity of a purified protein, 100µl of the purified protein was added in the first well followed by 1:2 serial dilutions in 1X PBS as previously discussed. To quantify the haemolytic activity, the number of wells containing lysed erythrocytes was counted. The hemolytic units were expressed as the reciprocal dilution of toxin that is required for 50% lysis of erythrocytes compared to the lysis obtained by distilled water.

2.9. Cell culture

2.9.1. Eukaryotic cell lines

Infection assays were carried out in a variety of eukaryotic cell lines of different types and derived from several sources (Table 2.5.). Furthermore, the P3-X63-Ag8-653 myeloma cell line was included in production of monoclonal antibodies (Table 2.5.).

Table 2.5. Eukaryotic cell lines used in this study

Cell line	Cell Type	Organ	Source	Catalog. Nr.
HeLa	Epithelial	Uterus	Human	DSMZ ACC 57
J774A.1	Monocytes	/	Mouse	DSMZ ACC 170
L-929	Fibroblasts	subcutaneous tissue	Mouse	DSMZ ACC 2
P3-X63.Ag8–653	Myeloma cells	/	Mouse	DSMZ ACC 43
P3-X63.Ag8–653 *	Myeloma cells	/	Mouse	DSMZ ACC 43

* X63.Ag8–653 myeloma cell line transfected with BCMG-Neo vector harbouring mouse IFN-γ cDNA.

2.9.2. Cell culture media and supplements

DMEM: Dulbecco's Modified Eagle Medium; with sodium pyruvate, 1 g/l glucose and pyridoxine (Life Technologies co.)

MEM: Minimum Essential Medium; with Earle's salts; L-glutamine (Life Technologies, Karlsruhe co.)

RPMI 1640: RPMI 1640; with L-glutamine, and 2.0 g/l NaHCO3 (PAN Biotech, Aidenbach co.)

FCS: 100% Foetal Calf Serum (Sigma-Aldrich, Deisenhofen co.)

L-Glutamine: 100X; 29.3 mg/ml in normal saline, 200 mM (PAA Laboratories, Linz; Österreich)

NEA: Non-essential amino acids (100X) (Biochrom co., Berlin)

Hanks' salt solution: Hanks' salt solution (1X); w/o Ca^{2+}, Mg^{2+}, w/o Phenol Red (Biochrom co., Berlin)

Trypsin/EDTA: Trypsin/EDTA (1X) (PAA Laboratories, Linz; Österreich)

2X MEM: Modified Eagle Medium (2X); with L-glutamine, w/o phenol red (Life Technologies co.)

2% [w/v] Agar: Agar, cell culture tested (Sigma-Aldrich co.), 2% [w/v] in H_2O

Neutral red: Neutral red solution (Sigma-Aldrich co.)

HeLa cells were grown in MEM , J774A.1 were grown in DMEM , and L929 cells as well as X63.Ag8–653 myeloma cell line were grown in RPMI 1640. To prepare a complete medium, all media were supplemented with 10 % (v/v) foetal calf serum, and 2mM L-glutamine and

$5x10^{-5}$ M 2-mercaptoethanol. MEM was additionally supplemented with 1% (v/v) non-essential amino acids (NEA). HAT selection medium is prepared by supplementing a 500 ml complete RPMI 1640 medium with 2 % (v/v) of 50X HAT (Gibco # 21060-017). X63.Ag8–653 cells producing IFN-γ were grown in complete RPMI medium supplemented with 10μg/ml G418 as a selection antibiotic (Life technologies co., Karlsruhe).
In Plaque assay, the infected monolayer of L-929 cells was incubated in MEM medium containing the following components:

2X MEM	12.9ml
100% [v/v] FCS	1.5ml
100X NEA	0.3ml
100X L-glutamine	0.3ml
2% [w/v] Agar	15.0ml

2.0 ml from this mixture were applied to each well.

2.9.3. Counting the eukaryotic cells using a microscope counting chamber

For cell culture applications that require the use of cell suspensions it is necessary to determine cell concentration. A device used for cell counting is called a counting chamber (Becton Dickinson co., Frankfurt/Main). To prepare the counting chamber, the mirror-like polished surface is carefully cleaned with lens paper. The coverslip is also cleaned. Coverslips for counting chambers are specially made and are thicker than those for conventional microscopy since they must be heavy enough to overcome the surface tension of a drop of liquid. The coverslip is placed over the counting surface prior to putting the cell suspension. The cell suspension was 1:10 diluted in a sterile trypan blue solution (Sigma-Aldrich co., Munich),which can stain only the living cells, and 10μl of diluted cell suspension was introduced into one of the V-shaped wells by means of a 10μl pipet (Eppendorf co., Hamburg). The suspension was allowed to be drawn into the chamber by capillary action. Care should be taken not to overfill or underfill the chamber. The opposite chamber was filled in the same manner. The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low power. The counting chamber consists of sixteen 1 mm squares divided into smaller squares. Each square represents a volume of 10^{-4} . The whole cells in the sixteen squares were counted. To determine the number of cells per ml,

the total number of cells counted in all squares was multiplied by 10^4 and by the dilution factor (10).

2.9.4. Culture of eukaryotic cells

The eukaryotic cells were cultured in 15ml of its suitable medium in rounded cell culture dishes (Nalge Nunc co., Hamburg) and incubated at 37ºC in presence of 5% CO2 (Steri-Cult incubator, Forma scientific/Labotect, Göttingen) for 3 to 4 days when the cells are about 80% confluent. Then, the cells are detached from the surface by trypsinization. The cells were washed twice with 5ml Hanks' salt solution (Biochrom AG co., Berlin) followed by addition of 1.0ml Trypsin/EDTA and incubated for 5 minutes at 37ºC. 500µl of detached cell suspension was reseeded in a new cell culture plate containing 15ml of fresh corresponding medium. If cells were intended to be used in an infection assay or a plaque assay, the Trypsin/EDTA cell suspension was taken, one day before performing the assay, in 15 ml of the corresponding medium, counted using a microscope counting chamber and adjusted to 10^5cells/ml. Cell suspension was seeded in 24-well plate (1ml/well) for invasion assay or 6-well plate (2ml/well) for plaque assay.

2.9.5. Storage of eukaryotic cells

In order to keep the eukaryotic cells viable for a long time, cells were washed with Hanks' salt solution followed by addition of 1 ml Trypsin/EDTA and incubated for 5 minutes at 37ºC. Cells were detached from the plate surface by trypsinization. The cells were counted and the number was adjusted to 10^6/ml. 1ml aliquots of cell suspension was added separately in 15ml conical tubes (BD bioscience co., Heidelberg) and washed with 10ml of its corresponding medium by centrifugation for 10 minutes at 1100rpm and 37ºC. The medium was removed while the cell pellet was resuspended in 500µl of FCS containing 8% dimethyl sulfoxide (DMSO) in 2ml-cryotubes (Nalge Nunc co., Hamburg) and kept in a freeze box (Nalge Nunc co., Hamburg) with 250ml 2-propanol overnight at -80ºC. At the next day the cryotubes were transferred into a tank of liquid nitrogen for long period storage. When needed, the cryotube was taken out from the N_2 tank, thawed at 37ºC and added to 15ml of its corresponding medium in a cell culture plate and incubated at 37ºC and 5% CO2. When 50% confluent growth was obtained, the medium must be changed with fresh one in order to eliminate any traces of DMSO because of its toxic effect on the cells.

2.9.6. Infection of eukaryotic cell lines with *Listeria* strains

2.9.6.1. Invasion assay

The ability of the wild type *Listeria monocytogenes* or its mutant derivatives to invade and/or proliferate in eukaryotic cells can be identified and quantitatively estimated by performing the invasion assay. Eukaryotic cells were seeded and grown in its corresponding tissue culture media in cell culture plates. The number of 24-wells cell culture plates used in the assay was equal to the number of time points at which the bacteria will be isolated and plated out after eukaryotic cell infection. In all assays performed during this work four plates, each corresponding to one time point, (ranging from 1 to 8 hours) were used. Each bacterial species were used to infect three wells containing eukaryotic cells in each plate, with an additional fourth well containing a slide cover for detecting the bacterial invasion through fluorescent microscopy. Carrying out the assay independently in triplicates is necessary to confirm the number of isolated bacteria from each well separately. One day before the screening assay, the cells were collected and counted by the aid of the counting cell chamber. 10^5 cells were seeded per well. At the same day, single colony from each bacterial species were picked with a sterile toothpick and used to inoculate 20 ml of BHI, with or without antibiotics, in a 250ml conical flask followed by overnight incubation. At the day of assay, the overnight bacterial culture was 1:50 diluted in BHI medium and incubated for further 3 hours at 180 rpm and 37ºC till reaching OD_{600} of 0.8-1.0. 2 ml of the exponentially growing bacteria were centrifuged for 2 minutes at 8000 rpm and RT. The supernatants were flicked out and the bacterial pellets were washed twice by 2 ml of PBS by centrifugation at the same conditions to remove any traces of BHI. The number of bacteria in PBS will be quantitatively determined through measuring the optical density at OD_{600}. The bacterial suspension will be diluted to be equivalent to OD_{600} of 0.1. By the aid of *L. monocytogenes* growth curve, it was estimated that OD_{600} of 0.1 for *Listeria monocytogenes* is equivalent to $1.6x10^8$cfu/ml. Then bacterial suspensions were further 1:10 diluted in the eukaryotic cell culture medium and 300µl from this suspension (Approximately $5X10^6$ cfu) were pipetted into its corresponding four wells containing the eukaryotic cell monolayer in each of the four assay plates to be equivalent to MOI of 10. The bacteria were spinned down for 1 minute at 2800 rpm to come in contact with the eukaryotic cells. The plates were incubated at 37ºC and 5% CO2 for a period depending on the invaded cell line; In case of J774 macrophages, 30 minutes will be enough for bacteria to be phagocytosed but in case of HeLa cells, the time will be elongated to one hour for bacteria to be internalised into the eukaryotic cells. The inoculating bacterial suspensions

were diluted to $1.6x10^3$ cfu/ml by 1:10 serial dilutions in 1X PBS and 20 µl would be used for plating out BHI agar plates to confirm equal inoculating numbers of bacteria for all inoculating bacterial species. After incubation, plates were taken out and washed three times, each with 1ml of eukaryotic cell culture medium per well followed by addition of 1 ml of cell culture medium containing gentamicin (20µg/ml) in order to kill the extracellular bacteria. The plates were incubated for a period ranging from one hour to eight hours under the same conditions. At each determined time point after incubation, a single plate was taken out from the incubator, washed 3 times with 1ml 1X PBS per well. The well containing the coverslip was fixed by treating with 0.5ml of cold 3.7% formaldehyde in 1X PBS for 10 minutes at RT followed by washing twice with 1X PBS and incubation in 1 ml 1X PBS at 4ºC for further processing for immunofluorescent microscopy preparation (see section 2.9.6.3).

Permeablisation of eukaryotic cell membrane in the other 3 wells took place by addition of 1.0ml of a cold sterile distilled water containing 0.2% (v/v) triton X-100 followed by incubation for 10 minutes at RT. The internalised bacteria diffused out of lysed eukaryotic cell line through strong pipetting in each well. The bacterial suspensions were 1:10, 1:100, and 1:1000 diluted in PBS and each dilution was plated out in dublicate on BHI agar plates followed by overnight incubation at 37ºC. Bacterial colonies were counted and the numbers of colony forming units per well were calculated by multiplying the colonies number by its corresponding dilution factors. The mean value of cfu/well and the standard error was estimated from the values of cfu in each of the 3 wells. Growth kinetics for each bacterial strain is represented through drawing a growth curve for each bacterial species by the aid of Sigma plot software.

2.9.6.2. Plaque assay

Performance of plaque assay (Sun *et al*., 1990) is similar to invasion assay in terms of bacterial or viral invasion and proliferation in eukaryotic cell lines but plaque assay can qualitatively determine these parameters. So, plaque assay gives indication for bacterial multiplication in eukaryotic cells as well as cell to cell spread by counting the number of clear plaques in a continuous sheet of cultured cell monolayer infected with bacteria. One day before performing the assay, L-929 cell line culture was diluted, and adjusted to 5 $x10^5$cells/ml. 2 ml was seeded in each well of 6-well cell culture plates. Inoculating bacterial suspensions were prepared as described in invasion assay and 10µl from $1.6x10^8$ cfu/ml bacterial cell suspension was used to inoculate each well of the 6-well plate. The plates were

incubated for 2 hours at 37ºC and 5% CO2 followed by washing twice with Hanks' salt solution and addition of 2 ml of complete RPMI 1640 medium containing 20µg/ml gentamicin. The MEM-soft agar mixture (see section 2.9.2) must be prepared just before use. A sterile 2% agar was melted completely in a microwave. Agar was allowed to cool slightly, to near 70°C and 20 ml was aliquoted to a 50 ml conical tube. 20 ml of pre-warmed 2X MEM containing 5% FCS, non-essential amino acids, glutamine, 10µg/ml gentamicin, as well as the recommended concentration of the selection antibiotic (when assay was performed for plasmid containing bacteria) was added to the 20 ml aliquot of 2% agar, and incubated in a 37°C water bath. When ready, the plates were tipped up and let drain to one side. The entire medium was aspirated, the plates were turned to level and quickly the overlay agar was removed from the water bath and approximately 2 ml of molten overlay mixture was added to each well by allowing it to slide down the far wall of the well and onto the plate. The plates were incubated for 3 days at 37ºC and 5%CO_2. This time period gave plaques the chance to be big enough to be detected. The plaques were stained by adding 1ml sterile neutral red (1:10 diluted in 1X PBS, pH 7.4) to each well followed by further incubation for 3-4 hours at 37ºC. Destaining was performed with 1 ml of 1X PBS/well and subsequent 30 minutes incubation. Now plaques could be easily counted. The size and number of plaques can give information about the ability of bacteria to escape phagosomes, multiply and invade the neighbour cells.

2.9.6.3. Immunofluorescence microscopy

The coverslips with attached infected eukaryotic cells (see section 2.9.6.1.) were prepared for immunofluorescence microscopy by flicking out the PBS followed by permeablization of eukaryotic cell membrane by only one minute incubation with 600µl 1X PBS containing 0.2%(v/v) triton X-100 per well at RT with subsequent washing twice with 1X PBS. The coverslips were taken out of the 24-well plates and placed on a wet Whatman®3MM paper (Biometra co. ,Göttingen). The intracellular infecting bacteria were stained by incubation with 20µl of undiluted primary monoclonal antibody (a mixture of both specific α-ActA-antibody N4 and N81) for 30 minutes at RT. The coverslips were washed 3 times by immersing them in a beaker containing 1X PBS followed by removing the buffer by a careful attaching the coverslip to a Whatman®3MM paper. 20 µl of 1:100 diluted Oregon Green 488 conjugated phalloidin (green) and anti-mouse antibody conjugated with Cy3 (red) mixture was added to each coverslip followed by incubation for 30 minutes at RT and 3 times washing with 1X PBS. Thereafter, 4 µl of 1:1 mowiol/antifade mixture (The antifade solution was purchased

from Biorad Lab., München) was pipetted as a drop on a preparation slide and the coverslip was carefully placed on the drop with the attached cells side coming in contact with the drop. The slides were kept away from light for 15 minutes allowing mowiol to dry. The coverslips were fixed to the slide by surrounding the coverslip with a layer of a nail polish, thus avoiding the movement of coverslips during microscopic examination and preventing air trapping between the coverslip and the slide. The coverslips were examined under a fluorescence microscope and images were captured and processed using KS 300 software (Carl Zeiss co., Berlin). Under the microscopic field, the green fluorescent eukaryotic cell actin skeleton and actin polymerisation processed by *Listeria monocytogenes* together with the red fluorescent bacterial cells could be detected.

2.10. Immunological methods

2.10.1. Experimental mice infection

Mice used in all experiments were six to eight week-old female BALB/c mice, purchased from Harlan Winkelmann co., Borchen and were kept at breeding facilities in specific-pathogen-free conditions.

Primary infection with *Listeria* strains was performed by an intravenous or intraperitoneal injection of viable bacteria in a volume of 0.2 ml of PBS (for preparation of bacterial suspension, see section 2.5). In all experiments, the dose of primary infection with the wild type EGD-e was approximately 1000-2000 cfu/mouse while 50.000 cfu/mouse (equal to 10 times the LD_{50} of the wild type *L. monocytogenes*) was the challenge dose. The primary infection dose of all *L. monocytogenes* mutants was approximately (10^6-10^7) cfu/mouse except for EGD-eΔ*frm* (2000 cfu/mouse).

2.10.2. Determination of bacterial load in infected organs

At certain time points after infection, spleens and livers were aseptically removed from mice in a biohazard facility. Shortly, mouse was euthanised by CO_2 inhalation and submerged in 70% ethanol. The mouse was left to air dry on its back on a paper towel. Spleens and livers were removed using sterile instruments and carefully suspended in 3ml and 5ml 0.2% NP40 in 1X PBS respectively. The organs were aseptically homogenized by means of polytron homogenizer (Kinematica AG, Littau-Lucerne; Switzerland.) at a speed of 7000 rpm. Bacterial growth in spleens and livers was determined by plating 10-fold serial dilutions in 1X

PBS (10^{-1}-10^{-3}) of the organ homogenates on BHI agar. The detection limit of this procedure was 10^2 colony forming units (cfu) per organ. Colonies were counted after 24 hours of incubation at 37°C.

2.10.3. Production of protein-specific antibodies

2.10.3.1. Polyclonal antibodies

Specific polyclonal antisera against various listerial proteins were produced in rabbits. 6 week old rabbits (New Zealand White) were used for immunization. The purified protein is diluted to a final concentration of 200 µg in 1.0 ml PBS and combined with 1.0 ml of the appropriate adjuvant. The protein and adjuvant are mixed thoroughly to form a stable emulsion which is injected subcutaneously and provides enhanced immune response from the sustained presence of the immunogen. Blood is collected from the central ear artery with a 19-gauge needle and allowed to clot and retract at 37°C overnight. The clotted blood is then refrigerated for 24 hours before the serum is decanted and clarified by centrifugation at 2500 rpm for 20 minutes. The schedule of immunization was carried out according to Richard *et al*. (2004) as follows:
At the day of immunization (step A), 5.0 ml of blood is drawn from the rabbit's ear to prepare about 2.0 ml of pre-immune serum utilizing as a negative control. For each rabbit, 200µg of antigen in PBS are emulsified with complete Freund's adjuvant (CFA) and injected subcutaneously. At day 21 (step B), 200 µg of antigen in PBS are emulsified with CFA and injected subcutaneously into each rabbit. At day 28 (step C), 200 µg of antigen in PBS are emulsified with incomplete Freund's adjuvant (IFA) and injected subcutaneously into each rabbit. At day 35 (step D), 1.0 ml of blood is drawn from the rabbit's ear to prepare about 0.5 ml of serum. This sample is tested by ELISA or immunoblotting assay, against the protein used for immunization, for its positivity versus the pre-immune serum. A positive signal allows proceed to Step E, a negative signal results in the process returning to Step B. At day 37 (step E), 100 µg of antigen in PBS are emulsified with CFA and injected subcutaneously into each rabbit. At day 44 (step F), 100 ml of blood are drawn from each rabbit. At least 40 ml of serum is then obtained which can then be utilized at a dilution from 1:1000 to 1:10000.

2.10.3.2. Monoclonal antibodies (mAb)

Monoclonal antibodies utilized in this study were generated by hybridoma fusion (Berry *et al*., 2003). Mice were immunized i.p. with 5µg of purified protein in PBS emulsified with

complete Freund's adjuvant. In general, mice were immunized every 2-3 weeks. When a sufficient antibody titer is reached in serum, the immunized mouse was euthanized by CO_2 inhallation, spleen was removed in a biohazard facility and kept in 10 ml of complete RPMI1640 medium for transport back to the lab. Additionally, peritoneal cells were removed from naive mouse by peritoneal lavage for use as feeder cells. Spleen was placed into sterile petri dish and cells were perfused out of the spleen by poking the spleen 8-10 times with an 18 ga needle followed by injecting the complete RPMI medium slowly in the spleen by means of a 3ml-syringe with 21 gauge needle. The injection process was repeated many times until nearly all the spleen cells were washed out. The spleen was discarded into the biohazards bag. Spleen cells were carefully collected, transferred to a 50 ml conical tube and spinned down at 900 rpm for 12 minutes at 4°C (Eppendorf centrifuge 5810 R, Eppendorf; Hamburg). The pellet was resuspended in 30 ml serum free complete RPMI 1640 medium and spinned down again as above. At the day of fusion, approximately 10^7 cells of P3-X63-Ag8–653 mouse myeloma cell line were spinned down at 850 rpm for 12 minutes at 4°C and washed with 30 ml serum free complete RPMI 1640 medium. The cells were spinned down again, resuspended in the same medium and left at 37°C until spleens are retrieved. Both immune spleen cells and myeloma cells were counted using a microscope counting chamber and combined at a ratio of 5:1 (spleen cells: myeloma cells). In addition, peritoneal cells were washed twice as above in serum-free complete RPMI and counted. The cell combination was washed 2 times more with 30 ml serum-free complete RPMI 1640 medium and spinned down as above. The supernatant was aspirated and the pellet was broken by gentle tapping on the flow hood surface. The tube was placed in container of warm water (37°C). Gradually, over a period of 30 seconds, 1 ml of 37°C PEG 1500 (Roche diagnostics, Mannheim) was added while tapping the side of the tube to achieve thorough mixing. Mixing was continued over the next 90 seconds. After approximately 1 minute 40 seconds, mixing was stopped and a 5 ml pipet was filled with warm serum-free complete RPMI. When exactly 2 minutes had elasped, the PEG/cell mixture was slowly diluted by adding dropwise 1 ml of serum-free complete RPMI over a 1 minute time span. During the next 1 minute, 2 ml of the same medium was added dropwise. The remaining 2 ml in the pipet was added during the next 40 seconds. Next a 10 ml pipette was used to add 14 ml of 37°C pre-warmed serum-free complete RPMI during the last 1 minute period. The total volume was brought to 50 ml using a complete RPMI medium supplemented with 5% fetal calf serum, centrifuged at 4°C and resuspended in a selective HAT medium at the appropriate volume to bring the cells to a concentrations of 1.5 x 10^6 cells per ml. HAT medium is a selection medium for hybrid cell lines; contains

hypoxanthine; aminopterin; thymidine. Only hybridoma cell lines (B-lymphocytes+myeloma cells), but not unfused meyloma cells or B-lymphocytes, can express both hypoxanthine phosphoribosyl transferase (HPRT+) and thymidine kinase (TK+) and subsequently survive in this medium. Peritoneal cells were added to the fused cells at 2.5 x 10^4 cells per ml and the cells are dispensed into 96 well plates in a volume of 150µl. 24-48 hours later, all wells are treated with 50µl of 4X HAT. Fusion plates were examined at 24-48 hours for any abnormalities (i.e. bacterial contamination). On day 7, wells were inspected under microscope and then fed. One half of the volume in each well was aspirated using a sterile Pasteur pipet. A new pipet was used for each plate. Wells were fed with 125 µl of complete RPMI1640 supplemented with 1X HAT on days 7, 11 and thereafter as needed. Cultures were examined visually at each feeding. Once a majority of wells appear 50% confluent for growth, supernatants were harvested for screening by ELISA or by immunoblotting assay against the antigen used in immunization. Plates were fed at this time. Supernatants from Positive clones were isotyped using "hybridoma subisotyping kit" (Merck Biosciences co., Schwalbach), purified using standard methods on protein A-Sepharose (Sigma, St. Louis, MO). Antibody concentration was determined by the measurement of absorbance at OD 280. Supernatants were stored at –20°C. Positive hybridomas were expanded in 6-well plates till 50 % confluent growth, harvested and stored in liquid nitrogen.

2.10.4. Detection of *Listeria*-specific antibodies

Type specific IgG anti-*listeria* antibodies were detected in mice antisera 7-9 days after *Listeria* infection. *Listeria* specific antibodies were either qualitatively detected using immunoblotting assay (see section 2.8.2.2) or quantitatively measured using ELISA assay.
ELISA measures type specific IgG anti-*Listeria* specific antibodies present in mice serum. When dilutions of mice sera are added to type-specific *Listeria* antigen coated microtiter plates, antibodies specific for that antigen bind to the microtiter plates. The antibodies bound to the plates are detected using a goat anti-mouse IgG alkaline phosphatase-labeled antibody followed by a *p*-nitrophenyl phosphate substrate. The optical density of the coloured end product is proportional to the amount of anti-*Listeria* antigen antibody present in the serum.
Blood was collected from mice by cardiac puncture. Cardiac puncture must be carried out on an anesthetized animal only. This is a terminal procedure used to acquire the maximum volume of blood from a mouse (approximately 1.5 ml). Mice were anesthetized with an inhalant diethyl ether and checking that the pedal response be absent before starting. The

mouse was laid flat on its back. An area on the left side of the mouse at the base of the elbow was landmarked where the heartbeat is the strongest. A 23ga needle was inserted, attached to a syringe, between 2 ribs into the heart. The needle was slowly advanced down and forwards. When the heart is pierced, blood would be visible in the hub of the needle which must be steady in this case with thumb and index fingers and the syringe was gently aspirated. Blood samples were transferred to a sterile 2.0 ml reaction tube and centrifuged at 14.000 rpm for 10 minutes. Serum was carefully aspirated and stored at −20°C before analysis.

For ELISA, 96-well NUNC Maxisorb plates (Nalge Nunc, Hamburg) were incubated at 4°C overnight with 100 µl of somatic soluble antigen from *L. monocytogenes* diluted 1:2 in 0.5 M sodium carbonate buffer, pH 9.6. Plates were sealed to prevent evaporation. After two washes with TBS-0.2% Tween (TBS/T), non-specific binding was blocked by adding 150 µl of 2% bovine serum albumin. Plates were sealed and incubated at RT for 1-2 h. Individual sera were added to the samples starting at a dilution of 1:10 and serially diluted 1:2 in 0.5% BSA in TBS/T for determining the end point titers. Plates were sealed. After 1.5 h at 37°C, plates were washed three times with TBS/T, and 100 µl of alkaline phosphatase-coupled goat anti-mouse IgG (BD bioscience co., Heidelberg) diluted 1:1000 in TBS/T were added. Plates were sealed and incubated at RT for 30 minutes followed by 5 times washing with TBS/T. One tablet of alkaline phosphatase substrate (Sigma-Aldrrich co., Deiesenhofen) was dissolved in 10 ml diethanolamine buffer by vortexing. Immediately 50 µl of substrate solution was dispensed into each well. Plates were incubated at RT for 5-10 minutes for colour development. Optical densities were measured with a SpectraMax 250 ELISA reader (MWG Biotech, Ebersberg) at 405 nm. Each assay was performed in duplicates, and data represent means ±SD.

2.10.5. DTH response to somatic listerial antigen

For determination of DTH (Delayed Type Hypersensitivity) responsiveness, somatic soluble *L. monocytogenes* EGD-e antigen diluted in sterile PBS (final protein concentration: 60 ng/ml) was injected into the left hind footpads of mice while the right one was injected with only PBS and acts as a control. Twenty-four hours later, thickness of the left and right footpads of individual mice were measured with dial-gauge calipers (Kröplin co., Schlüchtern). DTH-induced footpad swelling was calculated by subtracting the mean differences between left and right footpad thickness of injected naive control mice from those of immune mice (Mielke *et al*., 1998). Non-specific footpad swelling never exceeded 0.2 mm.

2.10.6. Stimulation of spleen cells *in vitro* for cytokine production

Infection of mice with *Listeria* strains gives rise to T cell stimulation for proliferation and production of cytokines. For cytokines to be detected in cytokines immunoassayes, T cells have to be restimulated *in vitro* by synthetic *Listeria* derived antigens.

Spleens of naïve (as a control) or previously infected mice were removed and used to prepare *in vitro* cell cultures. Single-cell suspensions were generated by cell perfusion out of spleen through injecting the spleens with PBS and dissociating the tissue mechanically (for details, see section 2.10.3.2). After spontaneous sedimentation of cell clusters, the single-cell suspension was filtered through a 100 W metal sieve and washed three times in culture medium RPMI 1640. Erythrocytes were lysed by osmotic shock in 3 ml erythrocyte lysis buffer. The cell population was stained with trypan blue solution and counted under microscope. At least 95% of the cells must be viable (for details, see section 2.9.3). Cells were incubated on the basis of spleen equivalents ($1x10^8$ cells/5 ml) in 25 cm^2 cell culture flasks in complete RPMI 1640 medium. Lymphokine secretion was stimulated by either a synthetic peptide derived from *L. monocytogenes* EGD-e for CD8+ T lymphocytes stimulation or the released soluble antigens of *L. monocytogenes* EGD-e for CD4+ T lymphocytes stimulation (10 -100 ng). Peptides used in this work were derived from either listeriolysin O (LLO_{91-99}) or P60 ($P60_{217-225}$). At the end of 5 days incubation, cells were harvested and centrifuged in order to produce cell-free culture supernatant. The supernatant was passed through a 0.45 W filter, fractionated, and stored at -70°C until it was thawed only once before use in cytokine assays (Peters *et al*., 2003). Cells were resuspended in RPMI 1640 complete medium, counted and transferred directly to be analysed through ELISPOT assay.

2.10.7. Cytokines ELISA

Cytokine sandwich ELISA is a sensitive enzyme immunoassay that can specifically detect and quantitate the concentration of soluble cytokine and chemokine proteins. The basic cytokine sandwich ELISA method makes use of highly-purified anti-cytokine antibodies (capture antibodies) which are noncovalently adsorbed (coated) primarily onto plastic microwell plates as a result of hydrophobic interactions. After plate washings, the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples which were applied to the plate. After washing away unbound material, the captured cytokine proteins are detected by biotin-conjugated anti-cytokine antibodies (detection

antibodies) followed by an enzyme-labeled avidin or streptavidin stage. Following the addition of a chromogenic substrate-containing solution, the level of coloured product generated by the bound, enzyme-linked detection reagents can be conveniently measured spectrophotometrically using an ELISA plate reader at an appropriate optical density.
In details, the purified anti-cytokine capture antibody (BD bioscience co., Heidelberg) was diluted to 10 µg/ml in binding solution (0.1M NaHCO3, pH 9.6). 50 µl/well diluted antibody was added to the wells of an enhanced protein Nunc Maxisorb binding ELISA plate (Nalge Nunc, Hamburg). Plates were sealed to prevent evaporation and incubated overnight at 4°C. Plates were brought to room temperature; the capture antibody solution was removed. The non-specific binding was blocked by adding 150 µl of 2% BSA or 10% of FCS. Plates were sealed and incubated at RT for 1-2 h. Plates were washed 3 times with TBS containing 0.2% Tween20 (TBS/T). 50µl/well of standards and samples were added. Plates were sealed and incubated for 2-4 h at RT or overnight at 4°C. Plates were washed 3 times with TBS/T. The biotinylated anti-cytokine detection antibody (BD bioscience co., Heidelberg) was diluted to 10µg/ml in TBS/T and 50 µl of diluted antibody was added to each well. Plates were sealed and incubated for 1 h at RT followed by 5 times washing with TBS/T. 50µl/well of 1:1000 diluted Streptavidin-Alkaline phosphatase conjugate (Sigma-Aldrrich co., Deiesenhofen) in TBS/T was added. Plates were sealed and incubated at RT for 30 minutes followed by 5 times washing with TBS/T. One tablet of Alkaline phosphatase substrate (Sigma-Aldrrich co., Deiesenhofen) was dissolved in 10 ml diethanolamine buffer by vortexing. The wells were emptied and immediately 50 µl of substrate solution was dispensed into each well, incubate at RT for 5-10 minutes for colour development. The optical density for each well was read with a microplate ELISA-reader set to 405 nm.

2.10.8. Cytokines ELISPOT

The ELISPOT assay is designed to enumerate cytokine producing cells in a single cell suspension. This method has the advantage of requiring a minimum of *in vitro* manipulations allowing cytokine production analysis as close as possible to *in vivo* conditions. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation, and the follow-up of such frequency during a treatment and/or a pathological state. After cell stimulation, locally produced cytokines are captured by a specific monoclonal antibody. After cell lysis, trapped cytokine molecules are revealed by a secondary biotinylated detection antibody, which is in turn recognised by streptavidin conjugated to alkaline

phosphatase. PVDF-bottomed-well plates are then incubated with BCIP/NBT substrate. Coloured "purple" spots indicate cytokine production by individual cells.

In details, PVDF-bottomed-96-well plates (Millipore co. Eschborn) were incubated with 100µl of 70% ethanol for 10 minutes at RT. Wells were emptied and washed three times with 100µl of PBS. 100µl of 10µg/ml of the capture antibody in 1X PBS was dispensed in each well. The plate was covered and incubated overnight at 4°C. The wells were emptied and washed 3 times with 100 µl of PBS. The wells were blocked by dispensing 100 µl of 2% BSA in PBS into wells. The plate was covered and incubated for 2 hours at RT. The wells were emptied by flicking the plate over a sink and tapping it on absorbent paper. The plate was washed once with PBS. 100 µl of complete RPMI 1640 medium containing *in vitro* stimulated spleen cells (10^4-10^6 cells) (see section 2.10.6.) and appropriate concentration of the stimulator peptide (10 ng) was dispensed into triplicate wells. As a positive control, when analysing CD8+ T cells producing IFN-γ, 100 µl of complete RPMI medium containing standard IFN-γ-X63.Ag8–653 producing cells (50-100 cells) was included. The plate was covered and incubated at 37°C in a 5% CO2 for 48 hours. During this period the plate could not be agitated or moved. The wells were emptied by flicking the plate over a sink and gently tapping it on absorbent paper. The wells were washed three times with PBS followed by addition of 100µl distilled water and 5 minutes incubation at RT to allow cell lysis. The wells were further washed twice with PBS. The biotinylated anti-cytokine detection antibody (BD bioscience co., Heidelberg) was diluted to 10.0 µg/ml in PBS and 50 µl of diluted antibody was added to each well. The plate was covered and incubated 90 minutes at 37°C. The wells were emptied and washed three times with PBS. 50µl/well of 1:1000 diluted Streptavidin-Alkaline phosphatase conjugate (Sigma-Aldrich co., Deisenhofen) in PBS was added. The plate was covered and incubated at RT for 30 minutes followed by 5 times washing with PBS. 100µl of ready-to-use Alkaline phosphatase conjugate substrate kit (Biorad lab., München) were distributed in wells and the reaction was let to go for about 5-20 minutes at RT. Spots formation was monitored by eyes. The wells were rinsed three times with distilled water and left to dry for 2 hours at RT or overnight at 4ºC away from direct light. The spots were counted with the aid of a magnifying lens or under microscope.

2.10.9. Flow Cytometry Analysis

Flow cytometry employs instrumentation that scans single cells flowing past excitation sources in a liquid medium. This technology can provide rapid, quantitative, multiparameter

analysis on living or dead cells based on the measurement of visible and fluorescent light emission. Flow cytometry is a widely used method for characterizing and separating individual cells. This basic protocol focuses on a measurement of fluorescence intensity produced by fluorescent-labelled antibodies and ligands that bind specific cell-associated molecules. Spleens of naïve (as a control) or previously infected mice were removed and used to prepare *in vitro* cell cultures. Single-cell suspensions were generated by injecting the spleens with PBS and dissociating the tissue mechanically. After spontaneous sedimentation of cell clusters, the single-cell suspension was filtered through a 100 W metal sieve and washed three times in culture medium RPMI 1640. Erythrocytes were lysed by osmotic shock by suspending them in 3 ml of erythrocytes lysis buffer followed by 5 minutes incubation at 4 ºC and 5 minutes centrifugation at 1000 rpm and 20ºC. The cell population was stained with trypan blue solution and counted under microscope. At least 95% of the cells must be viable. Spleen cells were suspended on the basis of spleen equivalents ($1x10^8$ cells/5 ml) in a 5 ml FACS buffer. 50 µl from the single cell suspension was added to a 96 well V-bottom plate (Nalge Nunc, Hamburg).

100 µl of appropriately diluted labeled antibody was added to the cells, mixed gently and incubated at 4^oC in dark for 30 minutes. The cells were washed three times by adding 100µl of FACS buffer to each well followed by centrifugation at 900 rpm and 4ºC for 5 minutes. The stained cell pellets were resuspended in 300 µl FACS buffer for flow cytometry. As a control, unstained cells must be included. Flow cytometry was performed using a FACS Calibur flow cytometer and further analysed with CELL Quest software (BD bioscience co., Heidelberg).

3. Results

3.1. Purification and characterization of thiol-activated cytolysins hyper-expressed in the non-pathogenic species *Listeria innocua*

The thiol-activated (oxygen-labile) cytolysin listeriolysin O (LLO) produced by pathogenic *Listeria monocytogenes* as well as pneumolysin (PLY) produced by *Streptococcus pneumonia* belong to a family of related toxins that are expressed by diverse species of gram-positive bacteria including streptolysin, alveolysin, cereolysin, perfringolysin, produced by *Streptococcus pyogenes*, *Bacillus alvei*, *Bacillus cereus*, and *Clostridium perfringens* respectively (Smyth and Duncan, 1978). Molecular cloning and sequencing of these various toxins has shown that apart from the commonly shared properties such as being inactivated by free cholesterol and their presumed receptors on cell surface, they all contain a conserved undecapeptide sequence that includes a single conserved cystein located at the C-terminal end of the respective molecules (Nato *et al*., 1991).

3.1.1. Listeriolysin O

Several isolation procedures have been previously reported for purification of listeriolysin. The majority of these protocols suffer from the disadvantage that the protein is isolated from the bulk quantities of culture supernatants expressing various other virulence factors indigenous to pathogenic *L. monocytogenes* strains (Darji *et al*., 1995). Although expression and secretion of listeriolysin in the non-pathogenic species of *B. subtilis* has been reported (Bielecki *et al*,. 1990), the secretion of proteases by this species restricts its use as a source for efficient purification of this protein. Expression of this protein in *E. coli* has been hampered by lack of haemolytic activity levels, poor secretion of the polypeptide to the external medium and the probable contamination of the purified listeriolysin with the lipopolysaccharide (LPS) from *E. coli* (Mengaud *et al*,. 1988). Moreover, in the previous procedures, listeriolysin was purified from bacteria that were grown in enriched media containing high levels of proteins thus decreasing the efficiency of purification process. Therefore, listeriolysin was purified in this study from the closely related non-pathogenic species *Listeria innocua* engineered to hyperexpress listeriolysin and grown in a minimal medium that contains the lowest amounts of amino acids, and essential elements required for growth of *Listeria* giving the advantage of avoiding contamination of listeriolysin with other external proteins. In addition, expression of *L. monocytogenes hly*, *plc*B, *act*A, and *plc*A and production of their respective proteins is

upregulated by incubation under stress conditions, such as a shift from rich to minimal medium, heat shock, or growth within mammalian cells (Sokolovic *et al*., 1993; Bohne *et al*., 1994; Klarsfeld *et al*., 1994) giving the advantages of higher yield production of listeriolysin O. Moreover, it was previously established that co-expression of the positive regulatory factor *prf*A in the plasmid vector in conjunction with the structural gene *hly* increased the expression over 500-fold (Darji *et al*., 1995). In addition, strains of the species *L. innocua* are devoid of extracellular proteases and permitted the development of a simple and rapid procedure for the purification of large quantities of listeriolysin.

3.1.1.1. Bacterial strain and growth conditions

Listeriolysin O was purified from *Listeria innocua* serotype 6a strain NCTC 11288 electroporatically transformed with pERL3-503 gram-positive/gram-negative schuttle vector containing the intact gene of listeriolysin O under the regulatory control of *prf*A gene (Darji *et al*., 1995). This strain was grown on brain heart infusion agar, broth, or in minimal medium supplemented with 5 μg/ml erythromycin. 10 ml of BHI overnight culture of the recombinant *L. innocua* strain was used to inoculate one liter of minimal medium.

Table 3.1. Expression of listeriolysin O and pneumolysin in BHI and minimal medium culture supernatants of the recombinant *L. innocua* strain

Medium	Hemolytic activity [a]	
	Listeriolysin O	Pneumolysin
BHI	1024	64
Minimal medium	2048	128

[a] Hemolytic activity are estimated from supernatants of overnight cultures starting with an equal inocula and calculated as the reciprocal of the highest dilution required to lyse sheep erythrocytes.

To determine the effect of growth in minimal medium on activity of listeriolysin O, 200μl of BHI overnight culture of *L. innocua* expressing LLO was used to inoculate 10 ml of either BHI or minimal medium. After further overnight incubation at 37°C, hemolytic activity of both culture supernatants, isolated from equal number of bacteria, was measured. In accordance with previous reports (Bohne *et al*., 1994; Klarsfeld *et al*., 1994), the shift of this strain from enriched BHI medium to minimal medium resulted in a 2 fold increase of

haemolytic activity in culture supernatant of minimal medium over that of BHI medium (Table 3.1.).

3.1.1.2. Purification and characterization of listeriolysin O

Listeriolysin was purified to homogeneity from the supernatant of the *L. innocua* strain harbouring plasmid pERL3-503 by two consecutive steps of ion exchange chromatography separated by a desalting step. As can be seen in Table. 3.2., already the concentration step resulted in a 15.9-fold enrichment of specific activity probably due to the loss of low molecular weight materials during the procedure.

Listeriolysin O has an isoelectric point of 6.7 (Nomura *et al*., 2002) and shows an optimum activity at acidic pH. So, it was purified using cation exchange chromatography. Efficient elution of the toxin from the column was achieved with a buffer combining high salt (1M NaCl) with acidic pH (5.6). Listeriolysin eluted as a sharp peak between 0.18-0.23 M NaCl.

Table 3.2. Purification of listeriolysin O from recombinant *Listeria innocua* cultures

Purification steps	Volume (ml)	Total protein (mg)	Total activity (HU[a] x 10^7)	Specific activity (HU/mg)	Enrichment (fold)	Recovery (%)
Crude sup.	1000	2412	2.21	$9.2x10^3$	_	100
Conc. Sup.	25	145	2.11	$1.46x10^5$	15.9	95.5
Q-sepharose Na-Fr[b]	25	95	2.05	$2.16x10^5$	23.5	92.8
Resource-S purified Fr.	4	5.4	1.66	$3.07x10^6$	333.7	75.1

[a] One haemolytic unit is defined as the reciprocal dilution of the toxin that is required for 50% lysis of erythrocytes compared to the lysis obtained by distilled water .
[b] Na-Fr = Non-absorbed fraction

The eluate containing the peak of haemolytic activity exhibited a specific activity of $3.07x10^6$ HU per mg protein (Table 3.2). The homogeneity and purity of the protein was confirmed by SDS-PAGE electrophoresis. A protein of 58 kDa was visualised as a single band after coomassie brilliant blue (Fig.3.1.A). The monoclonal antibody (M275) that neutralised the haemolytic activity (Darji *et al*,. 1995) also recognized this polypeptide in an immunoblot (Fig.3.1.B) confirming the identity of the protein as listeriolysin O.

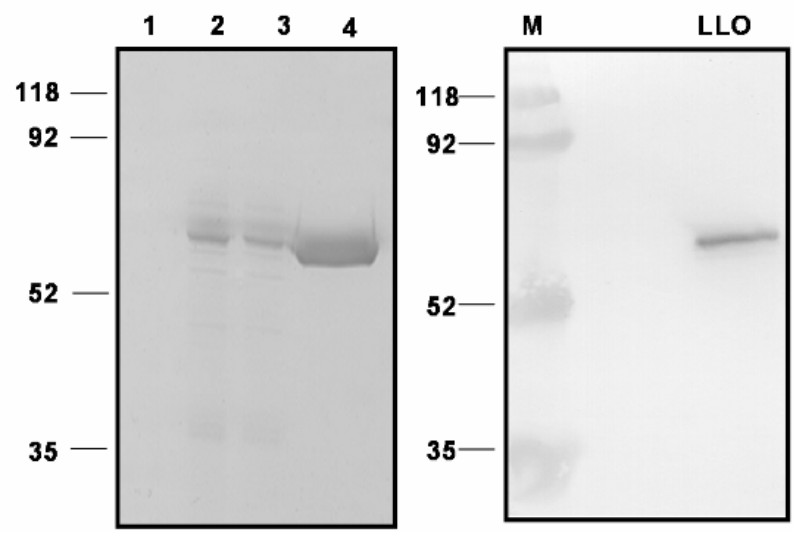

Fig. **3.1. A)** Analysis of the purification steps of listeriolysin by SDS-PAGE. Crude unconcentrated supernatant of *L. innocua* transformed with PERL3-503 (1), concentrated supernatant (2), fraction unabsorbed on Q-sepharose (3), fraction eluted from the Resource-S column (4). **B)** Immunoblot developed with a monoclonal mouse anti-listeriolysin antibody (M275) using 1 μg of the fraction eluted from Resource-S column. Lane (M) corresponds to the running marker protein while the numbers to the left of the both figures correspond to apparent molecular weights (in kDa).

3.1.2. Pneumolysin

All cholesterol-binding cytolysins are produced in the extracellular medium except pneumolysin which is an intracytoplasmic toxin that lacks a leader peptide and thus released due to the action of surface pneumococcal autolysin (Jedrzejas, 2001). All previous procedures that reported pneumolysin purification have used the wild type *Streptococcus pneumonia* as a source of pneumolysin which is released in a low amount as a consequence of cell lysis along with other virulence factors including both proteins and proteases. Furthermore, Not all strains of *S. pneumonia* were found to produce pneumolysin (Kanclerski and Mollby, 1987). To further investigate the properties of the pneumolysin and to use it as an antigen in a serological assay of pneumococcal disease, it was necessary to improve the methods of production and purification of this protein. Here, the structural gene of pneumolysin was over-expressed in the non-pathogenic *L. innocua* species under the control of listeriolysin O promoter and *prf*A regulator and was efficiently exported to the extracellular medium by the aid of Listeriolysin O signal peptide and purified using a single step of anion exchange chromatography.

3.1.2.1. Bacterial strain and growth conditions

Bacterial strain used in this study is the wild type *Listeria innocua* 6a strain NCTC 11288 electroporatically transformed with pSOG306 gram-positive/gram-negative schuttle vector which contains the intact promoter of listeriolysin O followed by its signal peptide coding sequence under the regulatory control of *prf*A gene (Otten *et al*., unpublished) and harbours the structural gene of pneumolysin which was cloned downstream from listeriolysin O signal peptide (Fig. 3.25.A). Bacteria were grown in brain-heart infusion (BHI) broth as well as in minimal medium in presence of 5 μg/ml erythromycin. As described above for listeriolysin O, shift of *L. innocua* expressing pneumolysin from BHI to minimal medium also resulted in a 2 fold increase in haemolytic activity of pneumolysin in culture supernatants (Table 3.1.).

3.1.2.2. Purification and characterization of pneumolysin

A simple one step anion exchange chromatography procedure was performed for purifying pneumolysin. The concentrated crude supernatant underwent desalting and buffer exchange in HiPrep™ 26/10 Desalting column. The desalted product was subjected to anion exchange

Table 3.3. Purification of pneumolysin from recombinant *Listeria innocua* culture

Purification steps	Volume (ml)	Total protein (mg)	Total activity (HU^a x 10^6)	Specific activity (HU/mg)	Enrichment (fold)	Recovery (%)
Crude sup.	1000	1920.0	4.67	$2.43x10^3$		100
Conc. Sup.	25	162.25	3.96	$2.44x10^4$	10	84.8
Resource-Q purified Fr.	4	2.0	3.27	$1.6x10^6$	673.9	70.0

[a] One haemolytic unit is defined as the reciprocal dilution of the toxin that is required for 50% lysis of erythrocytes compared to the lysis obtained by distilled water .

chromatography using resourse-Q column. Chromatography of the concentrated supernatant fluid on the resource Q-anion-exchange column produced two major peaks of absorbance. Pneumolysin eluted reproducibly from the column at 0.21 to 0.28 M NaCl, pH 7.5, as the second major peak and was collected in fraction 16. This fraction retained a hemolytic activity of approximately $1.6x10^6$ hemolytic units per mg and contained approximately 500 μg of

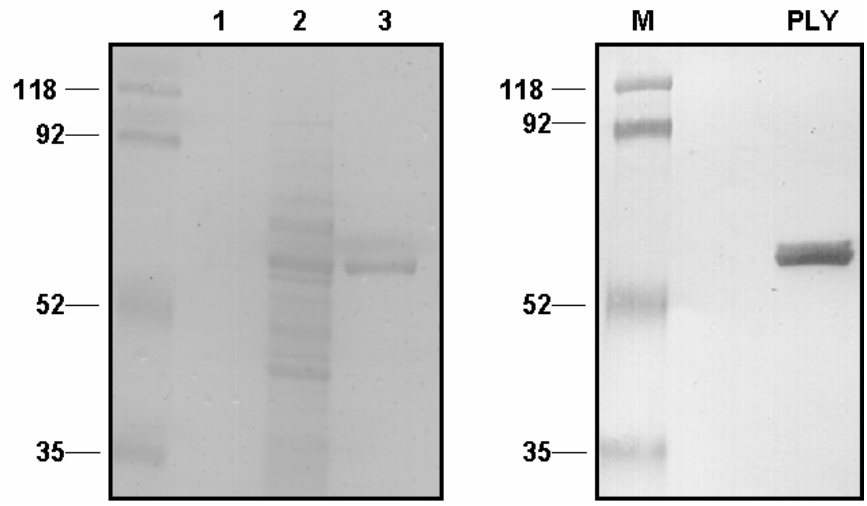

Fig. 3.2.A) Analysis of the purification steps of pneumolysin by SDS-PAGE. Crude unconcentrated supernatant of *L. innocua* transformed with pSOG306 harbouring pneumolysin gene (1), concentrated supernatant (2), fraction eluted from the Resource-Q column (3). B) Immunoblot developed with a monoclonal mouse anti-pneumolysin antibody using 5 μg of the fraction eluted from Resource-Q column. Lane (M) corresponds to the running marker protein while the numbers to the left of both figures correspond to apparent molecular weights (in kDa).

protein per ml, representing approximately 70.0 % recovery of lytic activity and a 674.0-fold purification (Table 3.3.). In SDS-PAGE, fraction 16 was shown by coomassie blue staining to contain a single homogeneous protein with a molecular mass of 53 kDa (Fig. 3.2.A). The protein was recognized in immunoblots by a specific monoclonal antibody (Fig. 3.2.B).

3.2. The ferritin protein Frm, a novel listerial antigen, mediates hydrogen peroxide resistance and is required for efficient intracellular growth of *L. monocytogenes*

Iron is an essential element for mammalian cell growth. It is a required constituent of numerous enzymes, including iron sulphur and haem proteins of the respiratory chain, as well as ribonucleotide reductase, which catalyses the rate-limiting step in DNA synthesis (Cammack *et al*., 1990). However, free iron has the capacity to participate in oxygen free radical formation via Fenton chemistry (Linn, 1998). Balancing the deleterious and beneficial effects of iron thus emerges as an essential aspect of cell survival. Therefore, bacteria have evolved complex iron acquisition systems that allow them to maintain this delicate intracellular iron balance (Theil, 1990; Harrison and Arosio, 1996; Chasteen, 1998). Bacteria possess three different kinds of iron storage proteins that belong to distinct, albeit distantly related families. Ferritins and heme-containing bacterioferritins which share the molecular architecture of mammalian ferritins (Harrison and Arosio, 1996; Andrews, 1998) in addition to the recently discovered prototype of ferritin from the gram-positive bacterium *L. innocua* (Bozzi *et al*., 1997). These ferritins have the ability to sequester iron atoms which is not required for immediate metabolic needs. Iron is taken up by ferritins in the ferrous form, oxidised in its ferroxidase center and stored in the central cavity of the molecule in the ferric form (Harrison and Arosio, 1996).

In an attempt to identify the role of humoral immunity in response to *Listeria* infection, the respective target antigens recognized by antisera of mice previously infected with the wild type *L. monocytogenes* were examined. A cross-reactivity with a 110 kDa polypeptide species present in the soluble antigen fraction of *L. monocytogenes* were detected in the antisera of mice infected with pathogenic *L. monocytogenes*. N-terminal amino acid sequencing of this species revealed identity to the previously described ferritin Fri from *L. innocua* as well as the ferritin-like protein, Flp of *L. monocytogenes*. Here the role of the listerial ferritin in response to oxidative stress as well as during systemic *Listeria* infection was assessed.

3.2.1. Bacterial strains and culture

Wild type *Listeria monocytogenes* strain EGD-e (Glaser *et al*., 2001) and its isogenic derivatives EGD-eΔ*frm,* EGD-eΔ*frm*: :pPL2-*frm* and EGD-eΔ*hly* were used in this study. Bacteria were grown either in brain-heart infusion (BHI) broth or in minimal medium at indicated temperatures in presence or absence of antibiotics.

3.2.1.1. Generation of the Δ*frm* mutant and its complementation

The isogenic deletion mutant *L. monocytogenes* EGD-eΔ*frm* was generated following a published procedure (Schäferkordt and Chakraborty, 1995). For the generation of the isogenic deletion mutant, a 1000 bp long PCR fragment comprising *frm* upstream sequences was

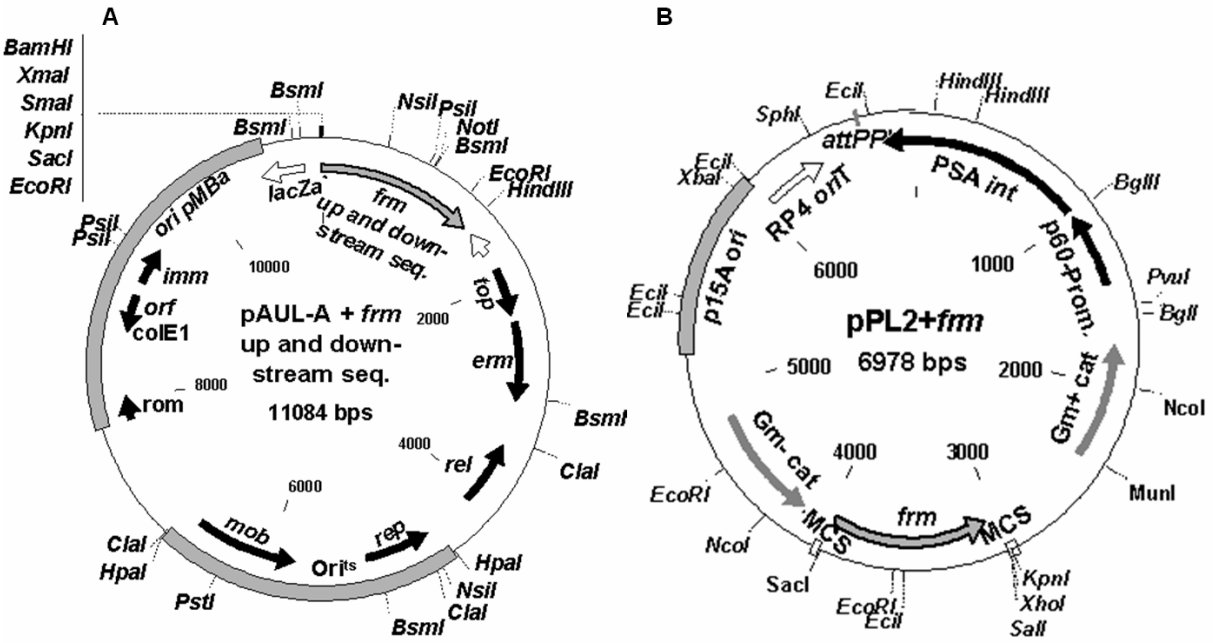

Fig. 3.3. Generation of the Δ*frm* mutant and its complementation. **A)** Construction of the pAUL-A integration vector carrying approximately 1000 and 500 pbs upstream and downstream fragments from the *frm* gene, respectively. Both fragments are ligated at the *Not*I restriction site and cloned into the mcs of the lacZa` gene (empty arrow) in pAUL-A using *BamH*I and *Hind*III restriction sites. B) Plasmid map of the pPL2 integration vector harbouring the structural gene of ferritin-like protein (Frm) of *L. monocytogenes*. The structural gene encoding ferritin-like protein (Frm) of *L. monocytogenes* with *Sac*I and *Sal*I restricted ends was cloned in the multiple cloning site of pPL2.

amplified with oligonucleotides (*Lmo-fri* for. 1, *Bam*HI) (-105) and (*Lmo-fri* rev. 1, *Not*I) (-1036). A second PCR fragment harbouring *frm* downstream regions was amplified with oligonucleotides (*Lmo-fri* for 2, *Not*I) (1419) and (*Lmo-fri* rev 2, *Hind*III) (1965) (see section 2.7.11). Both fragments were restricted with endonuclease *Not*I and used in a ligation reaction. The entire PCR fragment of 1423 bp, lacking *frm* gene, was amplified with –105 and 1965 oligonucleotides, restricted with *BamH*I and *Hind*III and was cloned into the *Bam*HI and *Hind*III restriction sites of the pAUL-A plasmid (Fig. 3.3.A). Transformation of this pAUL-A::Δ*frm* plasmid into *E. coli* and subsequently into *L. monocytogenes* EGD-e followed by generation of chromosomal in-frame deletion of the *frm* gene was performed as described

in section 2.7.8. The desired deletion of the *frm* gene was confirmed by nucleotide sequencing and immunoblotting.

For the construction of EGD-eΔ*frm* strains complemented with *frm* gene (EGD-eΔ*frm*: :*frm*), pPL2 plasmid was used (Lauer *et al*., 2002). A 935 bp long fragment containing entire *frm* gene was cloned on to the multiple cloning site of pPL2 plasmid (Fig. 3.3.B) using (*fri* for. *Sac*I) and (*fri* rev. *Sal*I) oligonucleotides primers (see section 2.7.11). Transformation of EGD-eΔ*frm* strains with pPL2 plasmid containing *frm* gene was performed by electroporation (Schäferkordt and Chakraborty, 1995). The desired recombinants were selected using chloramphenicol (8µg/ml) on BHI agar plates and subsequently verified by sequencing.

3.2.2. Detection of Frm during infection

Antisera obtained from mice 9 days after infection with the wild type *L. monocytogenes* EGD-e (2000 cfu) were detected using immunoblotting assay against a soluble antigen fraction of *L. monocytogenes*. Antisera reacted with a 110 kDa polypeptide species derived from the soluble antigen fraction. This crossreactivity was not observed with antisera from mice infected with a non-pathogenic *L. innocua* strain (Fig. 3.4.A). To identify the novel 110 kDa polypeptide species that was specifically recognized by the antisera, the soluble antigen fraction was subjected to separation by SDS-PAGE and transferred by blotting to a PVDF filter. The filter was stained with Coomassie Blue and the band corresponding to the 110 kDa species excised, and subjected to N-terminal sequencing.

The sequence obtained MKTINSVDTKEFLN was identical to the N-termini of two previously described 18 kDa polypeptides described as *L. innocua* ferritin (Fri) and the ferritin-like protein (Flp) of *L. monocytogenes* respectively (Bozzi et al, 1997; Hebraud and Guzzo, 2000). In keeping with the nomenclature describing the ferritin gene from *L. innocua* as *fri*, the designation *frm* for ferritin from *L. monocytogenes* was proposed. Rabbit polyclonal antibodies were produced against the purifed Fri protein from *L. innocua* and immunoblotting experiments revealed the presence of a 18 kDa polypeptide in both *L. monocytogenes* EGD-e and *L. innocua* soluble antigen fractions (Fig.3.4.B). Additional, distinct multimeric forms of the protein were also recognised by the antibody in soluble antigen preparations of either strains.

The Fri-specific antibodies were also used to monitor expression of the Frm-protein under various growth conditions. Expression of Frm at different growth temperatures was complex; Frm was observed in bacteria grown at 37 °C but was not detectable in cultures grown at

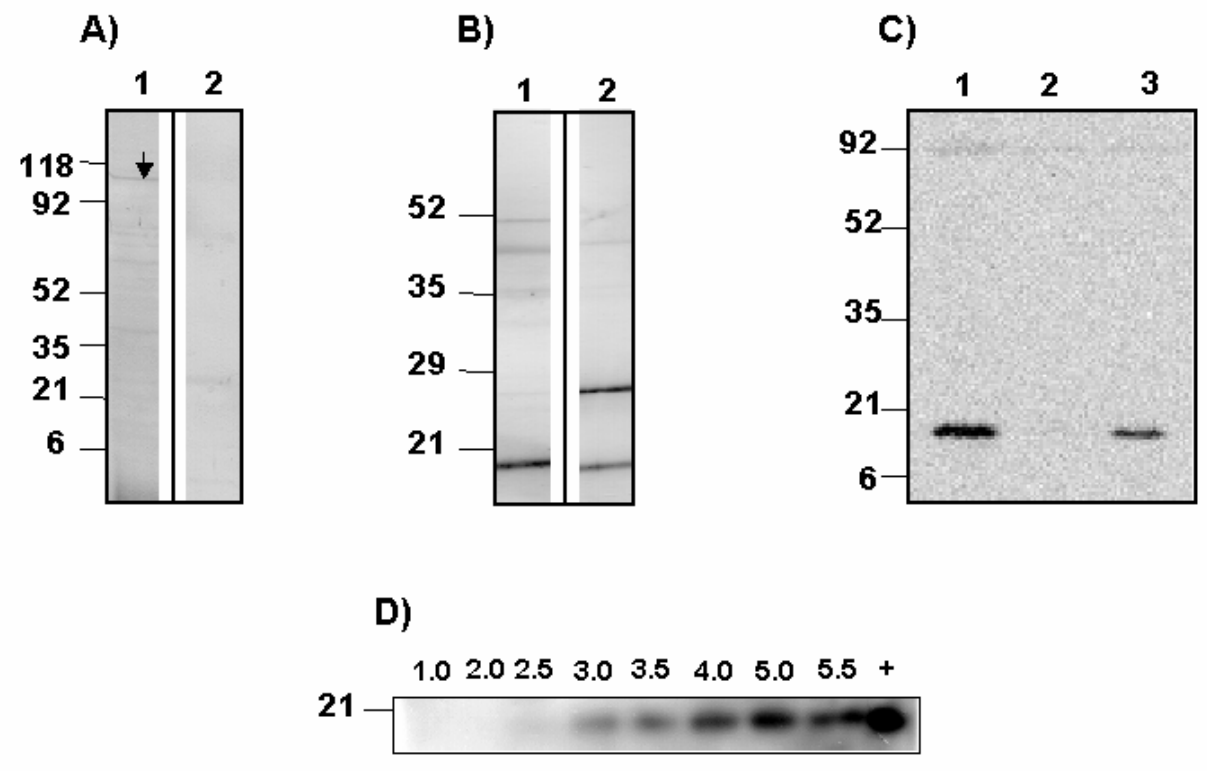

Fig. 3.4. Immunoblot analysis and physical characterization of Frm. Soluble antigen fractions (15 µg) either from *L. monocytogenes* (Lane 1) or *L.innocua* (Lane 2) were transferred onto a PVDF filter and incubated with antisera obtained from mice infected with the wild type *L. monocytogenes* EGD-e (Lane 1), *L.innocua* (Lane 2) **(A)** or with a polyclonal rabbit antibody raised against purified ferritin **(B)** of *L. innocua* followed by AP-conjugated secondary antibody. Blots were visualised using BCIP as a substrate. The arrow refers to the position of the 110 kDa polypeptide. Equal amounts of soluble fractions obtained from *L. monocytogenes* EGD-e cultures grown at 37°C at different time intervals over a period of 5.5 hours and purified ferritin as a positive control (+) were transferred onto a PVDF filter **(D)**. Equal amounts of soluble fractions obtained from *L. monocytogenes* EGD-e grown at 37°C (Lane 1) , 20 °C (Lane 2) and 5 °C (Lane 3) were blotted onto PVDF filter **(C)**. Blots (C and D) were developed with the Fri polyclonal rabbit antibody using the ECL chemiluminescence kit. The numbers to the left of the figures correspond to apparent molecular weights (in kDa).

20°C (Fig. 3.4.C). Nevertheless, expression of Frm was regained in cultures grown at 5°C. As can be seen in data presented in Fig. 3.4.D, expression of Frm also increased with the age of the cultures indicating growth-phase dependent regulation.

3.2.3. Properties of the Δ*frm L. monocytogenes* mutant

Further evidence that the protein recognized by antisera of *L. monocytogenes* EGD-e is indeed Frm was sought by generating a Δ*frm* mutant lacking the entire gene. Fri-specific antibodies

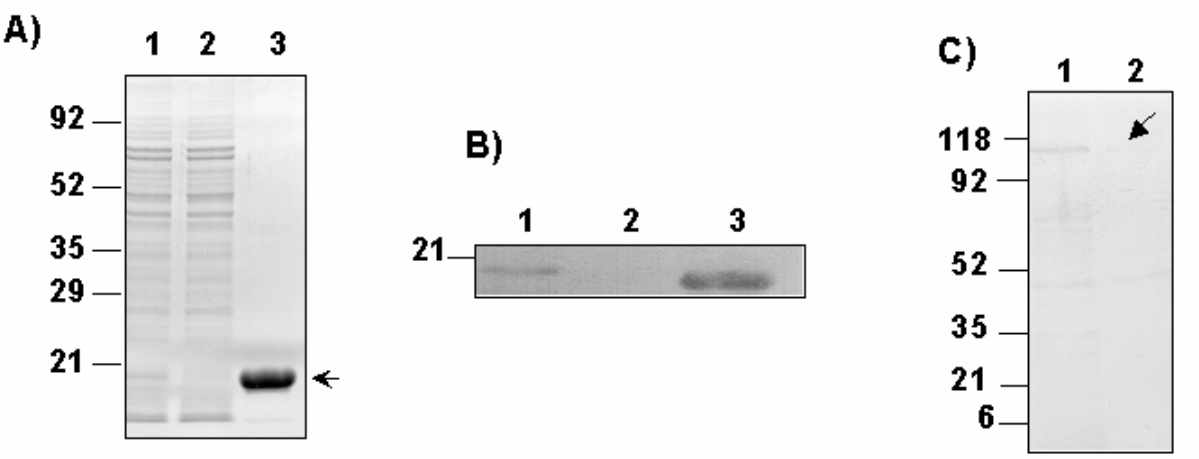

Fig. 3.5. Immunological characterization of the Δ*frm* mutant. Equal amounts of soluble antigen fractions from *L. monocytogenes* EGD-e (Lanes 1), the isogenic deletion mutant *L. monocytogenes* EGD-eΔ*frm* (Lane 2), and 1µg purified bacterial ferritin from *L. innocua* (Lane 3) were loaded onto 12 % SDS-polyacrylamide gel. Gels were stained with Coomassie Blue **(A)** or electrophoretically blotted and developed with rabbit anti-ferritin polyclonal antibody followed by AP-conjugated anti-rabbit IgG **(B)**. The arrow indicates the position of the monomeric Fri polypeptide (18kDa). Proteins in Fig. A were transfered electrophoretically onto a PVDF filter and incubated with antisera obtained from mice infected with the wild type *Listeria monocytogenes* followed by AP-conjugated anti-mouse IgG. Blots were visualized using BCIP as a substrate **(C)**. The arrow refers to the absence of the 110.0 kDa polypeptide in EGD-eΔ*frm*.

were used to confirm its absence in the resulting mutant strain. As can be seen from the results depicted in Fig. 3.5.A and B, the 18 kDa protein observed in the wild type strain was lacking in the mutant. In addition, antisera obtained from mice following infection with *L. monocytogenes* EGD-e also do not react with the soluble fraction preparations from the mutant strain indicating that the 110 kDa species observed is indeed a multimeric form of the Frm monomer (Fig. 3.5.C).

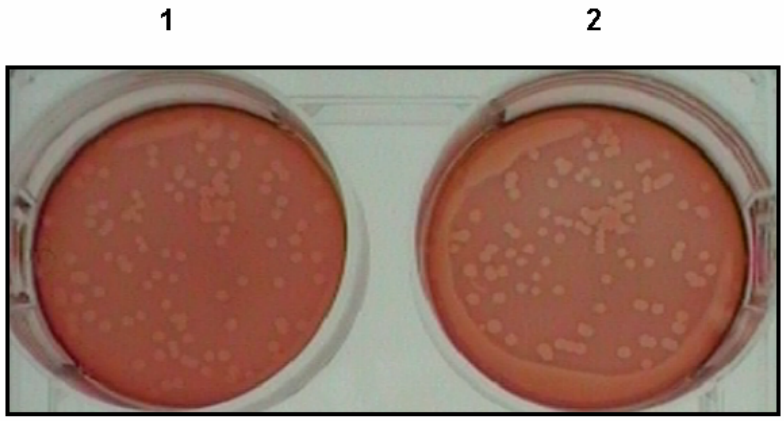

Fig. 3.6. Plaque formation of EGD-eΔ*frm* mutant strain in mouse fibroblasts. L-929 mouse fibroblast monolayer was infected with the wild type *L. monocytogenes* EGD-e (1) and mutant *L. monocytogenes* EGD-eΔ*frm* (2) for 2 hours followed by further 30 minutes incubation with 20µg/ml gentamicin. The overlay agar was added to the infected cells followed by 3 days incubation at 37ºC and 5%CO_2.

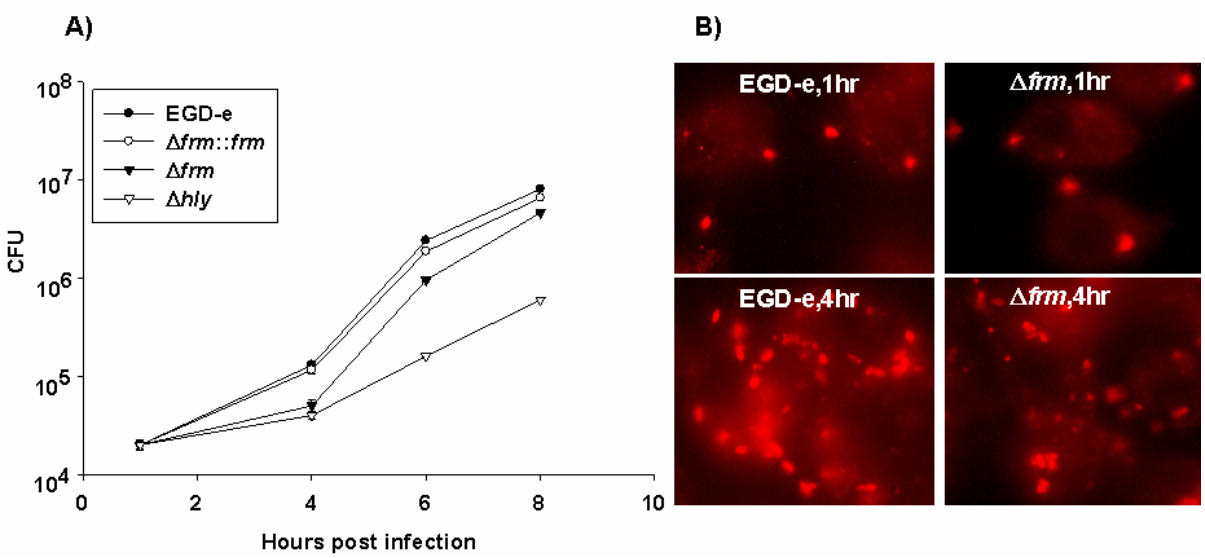

Fig. 3.7. Impaired intracellular growth of EGD-eΔ*frm* mutant strain in eucaryotic cells. **A)** Human HeLa cell line was infected with the wild type *L. monocytogenes* EGD-e, mutant *L. monocytogenes* EGD-eΔ*frm, L. monocytogenes* EGD-eΔ*frm* complemented with the ferritin gene (Δ*frm: :frm*) and mutant *L. monocytogenes* EGD-e Δ*hly* strains. Cells were lysed at certain time intervals and plated on BHI plates. The viable bacteria were enumerated. **B)** The mouse macrophage like cell line J774 was infected with the wild type *L. monocytogenes* EGD-e and the mutant *L. monocytogenes* EGD-eΔ*frm* strains. Bacteria were labelled with Cy3 conjugated anti-*Listeria* antibody (red). The kinetics of infection were monitored by immunofluoresence microscopy at 1 and 4 hours post infection.

When grown in laboratory media, growth of the Δ*frm* mutant was indistinguishable from that obtained with the wild type strain (Fig. 3.8.A). Further properties of the mutant were examined using tissue culture infection assays. Both the wild type *L. monocytogenes* and its mutant strain Δ*frm* showed the same efficiency to form plaques upon infection of mouse fibroblasts L-929 (Fig. 3.6) reflecting the same ability to spread from cell to cell. Also, the invasion rate of the Δ*frm* mutant was not significantly different from that obtained with its parental wild type strain for HeLa cells. When examined for its ability to replicate intracellularly it was observed that although the overall generation time of the mutant was similar to the wild type strain, a significant lag phase was observed before intracellular growth was initiated (Fig. 3.7.A) indicating that Frm may be involved in initiating efficacious intracellular growth. To obtain information on the nature of the defect we compared the intracellular growth properties of the Δ*frm* mutant to that of the Δ*hly* strain. It has been previously shown that Δ*hly* is unable to escape from the phagolysosome of J774 macrophages and therefore does not replicate in this cell line (Lety *et al.,* 2001). For the HeLa cell line, intracellular growth of Δ*hly* occurs, but is delayed primarily because the bacterium escapes only slowly into the cytoplasmic milieu (O´Riordan *et al*., 2002). In HeLa cells growth of the Δ*hly* strain is initiated following an extended lag phase and is more

pronounced than that observed with the Δ*frm* strain. Extrapolating from this information, it appears that the Δ*frm* mutant may have a defect in exiting from the host vacuole to the cytoplasm. Indeed, immunofluoresence staining of bacterial growth in J774 macrophages using both strains showed that growth of the mutant is retarded at early times of infection (Fig. 3.7.B).

Also a single copy chromosomally located *frm*-complementing mutant was constructed by inserting the *frm* gene into the PSA bacteriophage attachment site at $tRNA^{Arg}$- *attBB´* using the integration vector pPL2. From the growth properties of these mutants in HeLa cells, it is

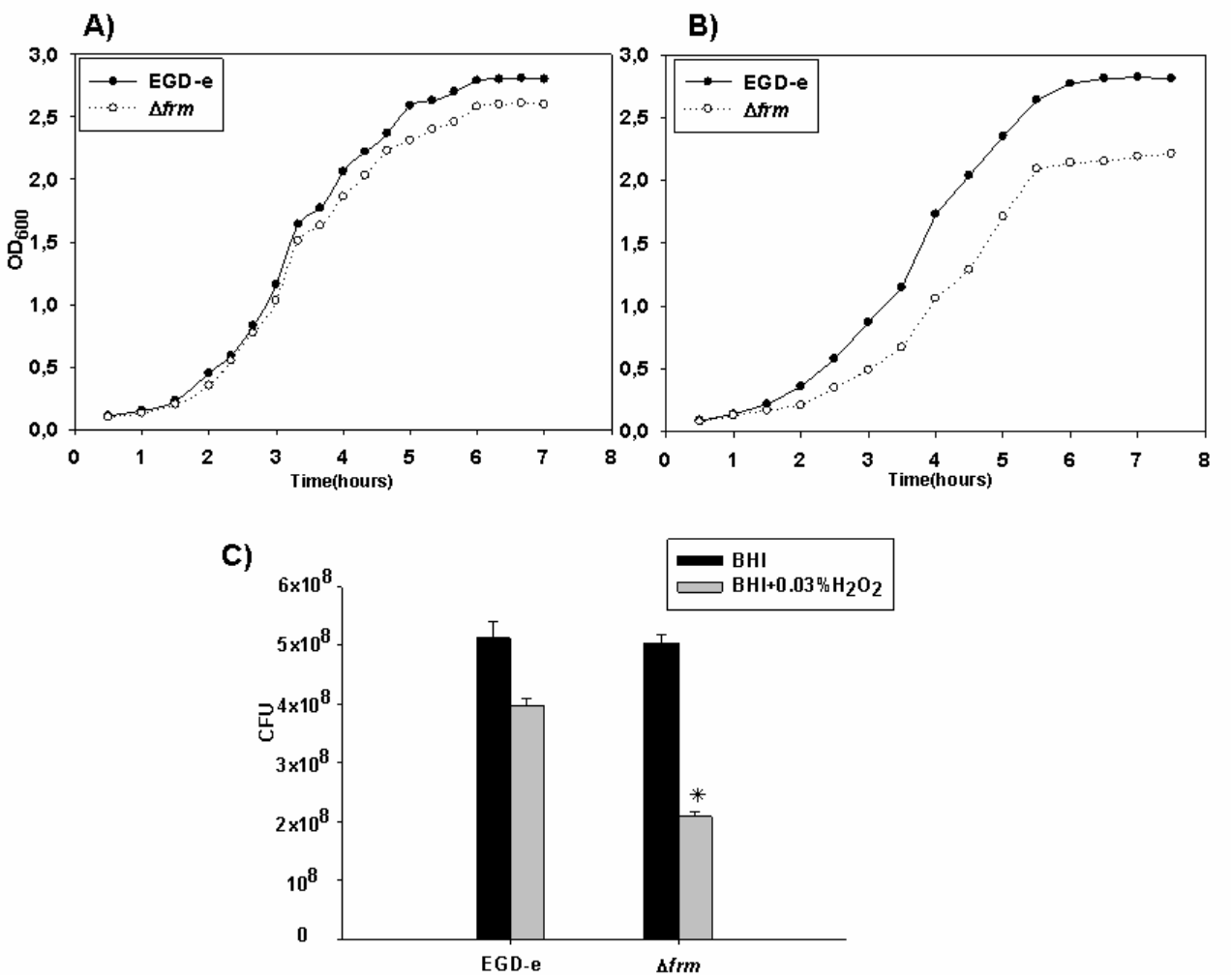

Fig. 3.8. The EGD-eΔ*frm* mutant is sensitive to hydrogen peroxide–mediated killing. Overnight cultures in BHI of the wild type *L. monocytogenes* EGD-e and the mutant *L. monocytogenes* EGD-eΔ*frm* strains were diluted 1:50 in normal BHI medium **(A)** and in BHI medium containing 0.03% hydrogen peroxide **(B)** and the bacterial growth was followed over a period of time by measuring the optical density of bacterial culture at 600nm.Sensitivity to hydrogen peroxide was also monitored by plating both the wild type *L. monocytogenes* EGD-e and the mutant *L. monocytogenes* EGD-eΔ*frm* strains after 3 hrs of 0.03% hydrogen peroxide treatment **(C)**. *P<0.05 (EGD-e vs EGD-eΔ*frm* both treated with 0.03% H_2O_2)

clear that single-copy complementation restored the growth capabilities of these strains to essentially wild type levels (Fig. 3.7.A).

3.2.4. Frm mediates resistance to the effects of hydrogen peroxide

The growth properties of the wild type and mutant strains were examined under a variety of abiotic stress conditions. Strains were grown at high and low temperatures, in acidic media, in the presence of sub-lethal concentrations of SDS, under high osmolarity conditions involving salt and in the presence of hydrogen peroxide. In all of the conditions tested, no differences were observed excepting for cultures grown in the presence of low concentrations (0.03%) of hydroxide peroxide in the growth medium (Fig. 3.8.B). Indeed, using plating assays it was found that the percentage of viable bacteria of the Δ*frm* mutant to be about 50% of the parental EGD-e strain when exposed to 0.03% hydrogen peroxide (Fig.3.8.C).

3.2.5. Frm promotes bioaccessibility of mineralized iron

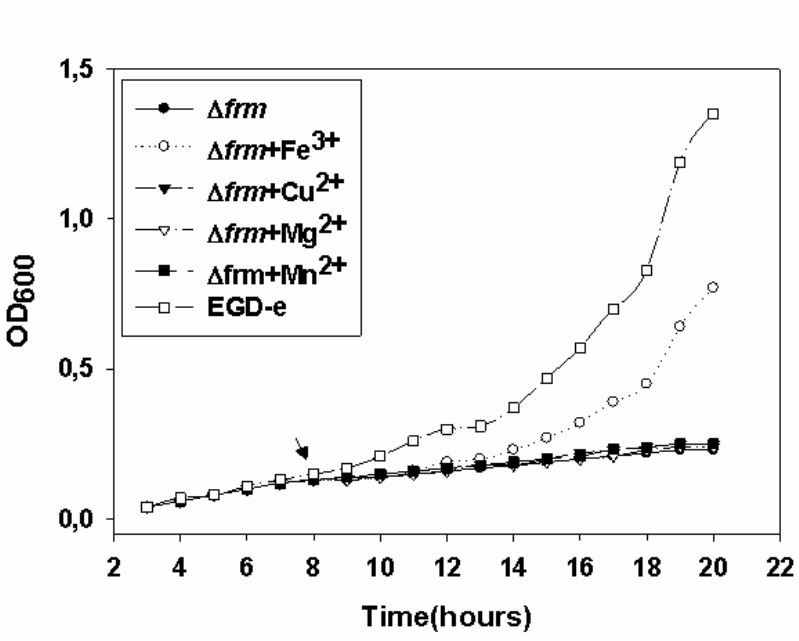

Fig. 3.9. Ferric citrate restores growth of EGD-eΔ*frm* mutant strain in chemically defined minimal medium. Parallel cultures (controlled by equal optical densities) of both the wild type *L. monocytogenes* EGD-e and the mutant *L. monocytogenes* EGD-eΔ*frm* strains were grown in a minimal medium at 37°C. At 8h of growth (indicated by an arrow), equal volumes of cultures of both the *L. monocytogenes* EGD-e and the mutant *L. monocytogenes* EGD-eΔ*frm* strains were supplemented with different divalent cations (citrate salts 260 μM) or left untreated. The bacterial growth was followed over a period of 22 hours by measuring the optical density of the cultures at 600nm. Addition of different salts has no significant effect on the growth of the wild type *L. monocytogenes* EGD-e strains. For clarity only the supplemented cultures of the mutant *L. monocytogenes* EGD-eΔ*frm* strains are plotted.

When the strains were grown in a minimal medium containing iron citrate virtually no growth was observed for the Δ*frm* mutant (Fig. 3.9.) while the parental strain grew on to reach stationary phase. The addition of further iron citrate to the culture of non-growing Δ*frm* bacteria after 8 hours promoted bacterial growth. To further determine the Δ*frm* strain's selectivity for iron, a number of divalent cations such as Cu^{2+}, Mn^{2+}, Mg^{2+} were added to the non-growing Δ*frm* cultures. As indicated in Fig. 3.9. none of them was able to additionally support the growth of non-growing Δ*frm* suggesting that the bio-availability of iron is limiting to the Δ*frm* strain. Increase in the amount of other components of the minimal medium i.e. essential amino acids did not affect growth of the Δ*frm* mutant.

3.2.6. The Δ*frm* mutant exhibits defects at early stages of infection

Studies were performed comparing the virulence properties of the mutant and the Δ*frm*::pPL2-*frm* complemented strain to that of the wild type strain EGD-e in the mouse model of infection. At all time points studied the number of bacteria isolated from an EGD-e infected mouse was set arbitrarily at 100%. Although a similar inoculum was used for all

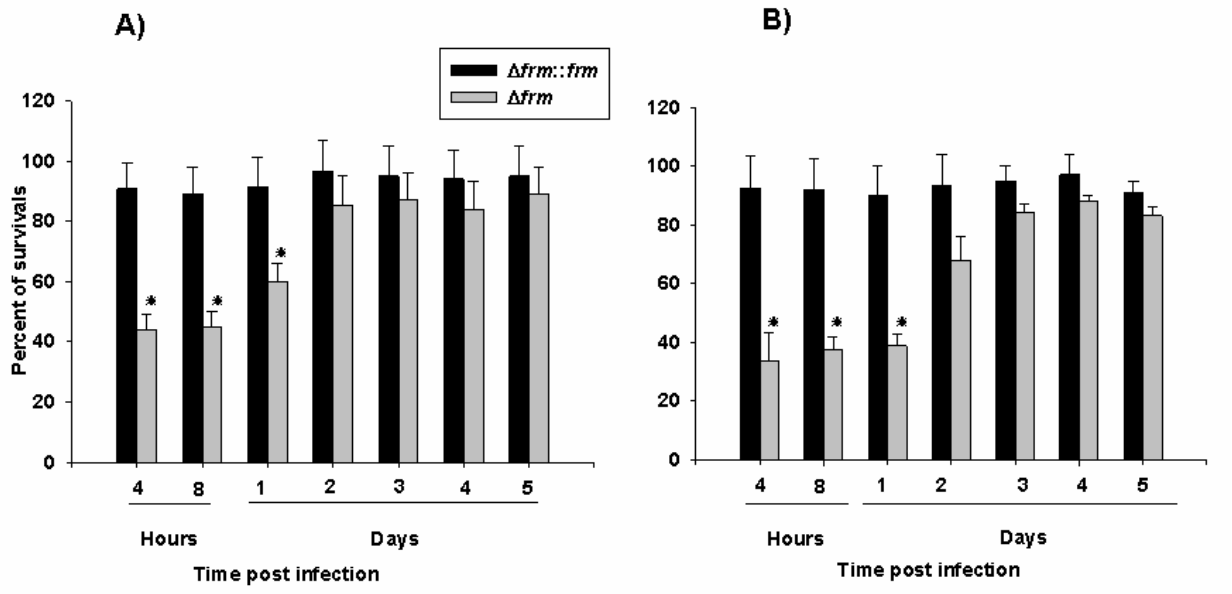

Fig. 3.10. Frm is required for efficient growth at early stages of listerial infections. The kinetics of bacterial growth was followed in organs of mice infected with the wild type *L. monocytogenes* EGD-e, *L. monocytogenes* EGD-eΔ*frm* and *L. monocytogenes* EGD-eΔ*frm* complemented with the ferritin gene (EGD-eΔ*frm*::*frm*). Mice were injected (i.v.) with 2000 viable bacteria for all strains. Bacterial survival was followed in both spleens **(A)** and livers **(B)** of infected mice over 5 days. The number of surviving EGD-eΔ*frm* and EGD-eΔ*frm*::*frm* are calculated as a percent of survival number of the wild type *L. monocytogenes* at each time point examined. *P<0.05 (EGD-eΔ*frm* vs EGD-eΔ*frm*::*frm*).

three bacteria we found that the Δ*frm* mutant had consistently lower numbers of bacteria present in both spleens and livers of the infected mice as compared to the complemented and wild type strain at all of the experimental time points examined (Fig. 3.10.). This effect was most visible at 4 hours and 8 hours as well as one day post infection, where although roughly equivalent initial doses of either bacteria were used, significantly lower numbers of bacteria were recovered from the spleens (Fig. 3.10.A) and livers (Fig. 3.10.B) of mice infected with the Δ*frm* mutant as compared to the complemented mutant or the wild type strain.

3.3. Induction of immune responses by attenuated isogenic mutant strains of *Listeria monocytogenes*

Listeria monocytogenes has been used for decades for the induction and analysis of T cell-mediated immunity (Mackaness, 1962; Hahn and Kaufmann, 1981; Kaufmann, 1993; Mielke *et al*., 1997; 1998;). These studies have led to extensive and detailed information on the host response to infection, and have triggered strong interest in bacterial virulence factors that allow entry into the host, support intracellular survival and facilitate dissemination of these bacteria during infection (Portnoy *et al*., 1992; Chakraborty and Wehland, 1997). Studies of cell biology revealed that invading bacteria use active mechanisms to escape from the encapsulating phagosome, and that they can replicate in the cytoplasm of both professional phagocytes and parenchymal cells, such as hepatocytes. These studies have also explained the cellular basis of *Listeria* potent intrinsic capacity to induce major histocompatibility complex (MHC) class I-restricted protective CD8+ T cells (Mielke *et al*., 1989; Kaufmann, 1993; Pamer *et al*., 1997). This in turn resulted in the current interest in developing *L. monocytogenes* as a viable bacterial T cell vaccine vector as an alternative to *Salmonella*, which mainly induces CD4+ T cell responses (Hess *et al*., 1996). Indeed, recombinant *L. monocytogenes* expressing foreign antigens has already been shown to be a highly effective vector for the induction of specific T cells active in the prevention of tumor growth and for protection against viral infections (Schafer *et al*., 1992; Ikonomidis *et al*., 1994; Frankel *et al*., 1995; Goossens *et al*., 1995; Pan *et al*., 1995; 1996; Shen *et al*., 1995; Jensen *et al*., 1997). However, one drawback to the use of virulent *L. monocytogenes* as a vector is that *Listeria* infection induces a strong T cell responses mediating DTH and granulomatous inflammation that may result in extensive tissue destruction which make their use as vaccine vectors undesirable (Darji *et al*., 2003). For increased safety, however, a more appropriate vaccine vector would be a well-characterised mutant that is reliably attenuated in virulence but that still retains the favourable immunological CD8+ T cell-inducing properties of the parental strain. In addition, the accompanying CD4+ T cell response mediating delayed type hypersensitivity (DTH) and granulomatous inflammation (Mielke *et al*., 1989; 1992) should be downmodulated in order to prevent inflammatory tissue destruction. Here, the immunological properties of isogenic *Listeria monocytogenes* mutants carrying a specific deletion within the actin nucleator (*act*A) or multiple deletions within *act*A and phospholipase B (*plc*B) were systemically examined for their suitability for use as delivery vehicles.

3.3.1. Survival and persistence of wild type *L. monocytogenes* and isogenic *L. monocytogenes* mutant strains *in vivo*

The presence of viable microorganisms during the early phase of infection is critical in the induction and the establishment of cell-mediated immunity. *L. monocytogenes* EGD-e mutant strains (*L. monocytogenes* Δ*act*A2 and *L. monocytogenes* Δ*act*AΔ*plc*B) were examined whether they display similar virulence attenuation *in vivo* compared to their parental wild type *L. monocytogenes* EGD-e strain. A group of BALB/c mice (20 mice) were infected i.v. with sub-lethal doses of either wild type *L. monocytogenes* EGD-e (10^3), or the isogenic *L. monocytogenes* mutant strains (10^7). *In vivo* survival and growth kinetics of bacteria were followed by determining the number of bacteria in the spleen and liver of infected mice at various time points for several days. As indicated in Fig. 3.11., bacterial count of the mutant *Listeria* strains dropped rapidly from day 2 after the infection onwards and levelled off at a low number, persisting up to day 8 post-infection. On the other hand, the bacterial number of the wild type *L monocytogenes*, both in spleen and liver, increased between day 2 and day 4 of the infection and declined rapidly from day 5 post-infection onwards. Since higher dose of the wild type *Listeria* strain is lethal, growth kinetics was performed with a lower infection dose (10^3). In all cases, both in the wild type EGD-e and in the isogenic EGD-e mutant strains, bacteria were cleared from the spleen and liver by days 8 -10 after the infection.

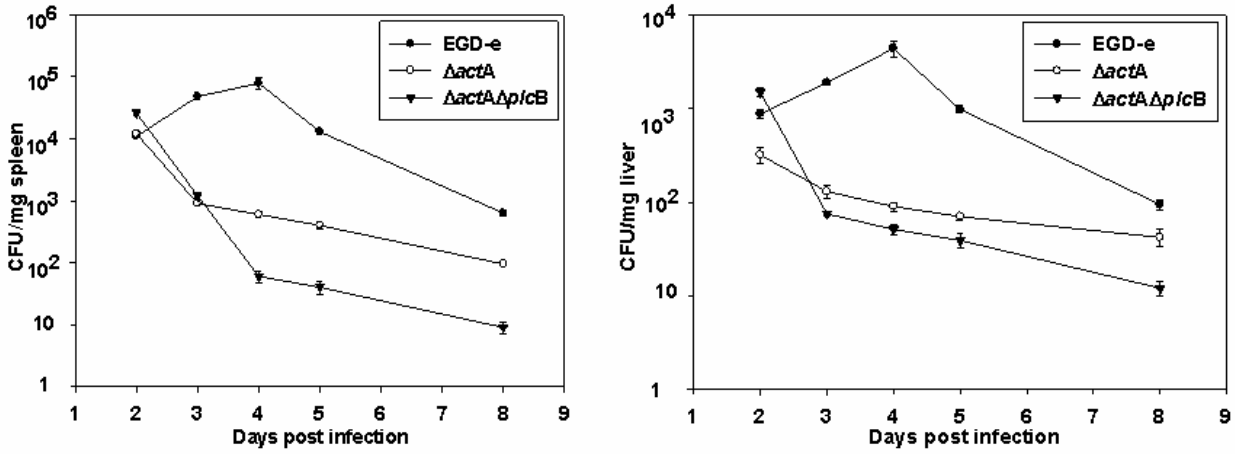

Fig. 3.11. Kinetics of primary infection in mice with the wild type *L. monocytogenes* and isogenic *L. monocytogenes* mutant strains. Mice were infected i.v. with either 10^3 wild type *L. monocytogenes* or 10^7 isogenic *L. monocytogenes* mutant strains Δ*act*A2 and Δ*act*AΔ*plc*B. Different time intervals after the infection, 3 mice per group were sacrified and the number of viable bacteria in the organs enumerated. Data presented are representative of three independent expirements.

3.3.2. Spleen morphology on day 4 post-infection

L. monocytogenes infection often elicits host inflammatory reaction accompanied by a cascade of tissue alterations, such as splenomegaly and granulomatous inflammation that finally result in the eradication of bacteria. These responses are cell-mediated and predominantly involve activation of non-specifically invading CD4+ T cells at the site of infection. Since the bacterial number of isogenic EGD-e mutant strains *in vivo* declined faster (Fig. 3.11.) than the parental wild type EGD-e strain, the morphological alterations in the spleens, following i.v. infection of mice with either the wild type EGD-e strain or isogenic EGD-e mutant strains were examined. As shown in Fig. 3.12., marked differences were observed in the morphological structure of spleens obtained from mice infected with these strains. Spleens obtained from mice infected with the wild type EGD-e strain displayed a pronounced strong granuloma formation from day four post-infection onwards. These granulomas probably resulted from intense and vigorous monocytes infiltration. In contrast, granuloma formation was hardly visible in spleens of mice infected with both *L. monocytogenes* Δ*act*A and Δ*act*A Δ*plc*B mutant strains.

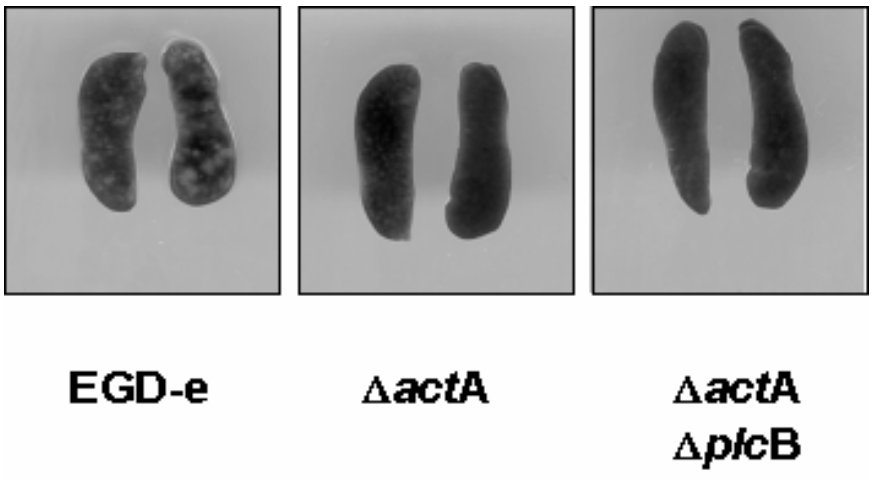

Fig. 3.12. Morphological alterations in the spleens of mice infected i.v. with the wild type *L. monocytogenes* and isogenic *L. monocytogenes* mutant strains. Spleens of mice (infected i.v. as mentioned in Fig. 3.11.) were removed on day 4 after infection. Shown is the scanned picture of spleens from the wild type *L. monocytogenes* and its isogenic mutant strains. Infiltration of monocytic cells and granulomatous lesions are only detectable in the spleens isolated from mice infected with the wild type *L. monocytogenes*.

3.3.3. *Listeria*-induced IFN-γ production of spleen cells *in vitro* and DTH-response *in vivo*

Antigen-induced CD4+ T cell-derived IFN-γ production of spleen cells was measured as an *in vitro* correlate of DTH to determine whether immunization with the various mutants may lead to quantitative differences in the proinflammatory T cell response. Day 9 after infection was chosen for the analysis of the primary immune effector phase. Spleen cells were isolated and stimulated *in vitro* with the released soluble antigen of *L. monocytogenes* EGD-e (100 ng). Spleen cells from mice immunized with *L. monocytogenes* Δ*act*A2 and *L. monocytogenes* Δ*act*AΔ*plc*B mutant produced significantly lower levels of IFN-γ in comparison to splenocytes of mice pre-immunized with the wild type strain (Fig. 3.13.A). Furthermore, the observations obtained *in vitro* were confirmed *in vivo* by antigen-elicited skin responses showing corresponding results (Fig. 3.13.B). Six mice per group were injected into the left hind footpads with 50 µl of somatic soluble *L. monocytogenes* EGD-e antigen (60 ng/ml) at day 9 post-infection. Twenty-four hours later, thickness of the left and right footpads of individual mice were measured. Footpads of mice pre-immunized with both *L. monocytogenes* Δ*act*A2 and *L. monocytogenes* Δ*act*AΔ*plc*B mutant strains showed a markedly lower thickness than those of mice pre-immunized with the wild type EGD-e strain.

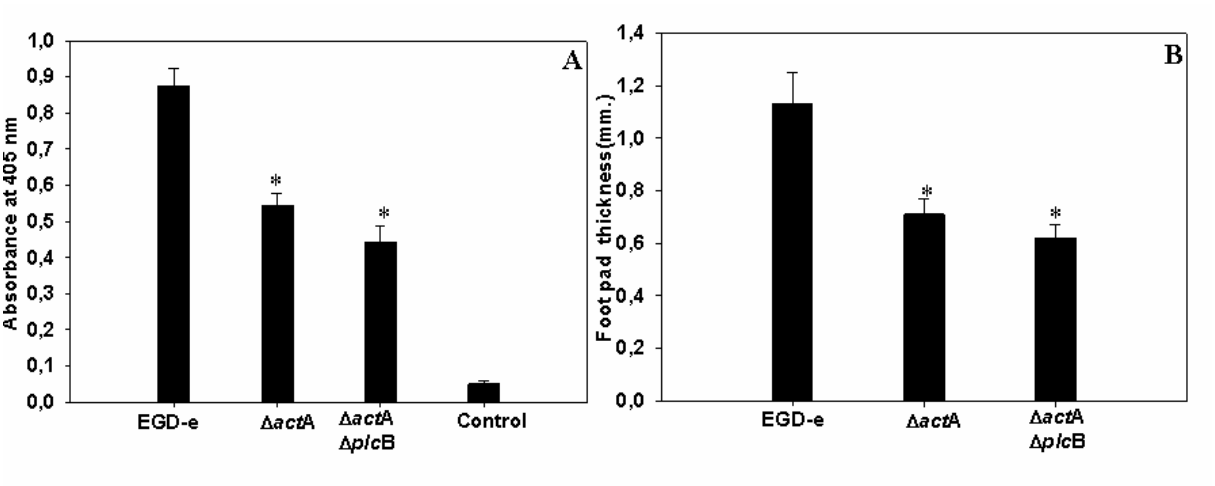

Fig. 3.13. A) *Listeria*-induced IFN-γ production by spleen cells 9 days after infection (i.v.). Mice were infected with 10^3 CFU of *L. monocytogenes* EGD-e or with 10^7 CFU of its isogenic mutants. On day 9 after infection, mice were killed and spleens were removed. Single cell suspensions were stimulated *in vitro* with secreted soluble *Listeria* antigen (100 ng) to produce IFN-γ. After 48 h, culture supernatants were tested for presence of IFN-γ by ELISA.*P<0.05 (EGD-e vs Δ*act*A2 and Δ*act*AΔ*plc*B). **B)** DTH response to listerial antigen 9 days after primary infection. Mice were infected as in Fig. 3.13.A. 9 days after infection, DTH was triggered through injection of soluble somatic listerial antigen. Twenty-four hours later, specific skin response was determined. Experiments were repeated twice. The mean value ± S.D. of five animals of a representative experiment is shown.*P<0.05 (EGD-e vs Δ*act*A2 and Δ*actA*Δ*plc*B).

3.3.4. Acquired protection conferred by isogenic attenuated mutants

Sub-lethal infecting doses of wild type *L. monocytogenes* strains are generally controlled by mice in the initial infection and result in the induction of protective immunity to further

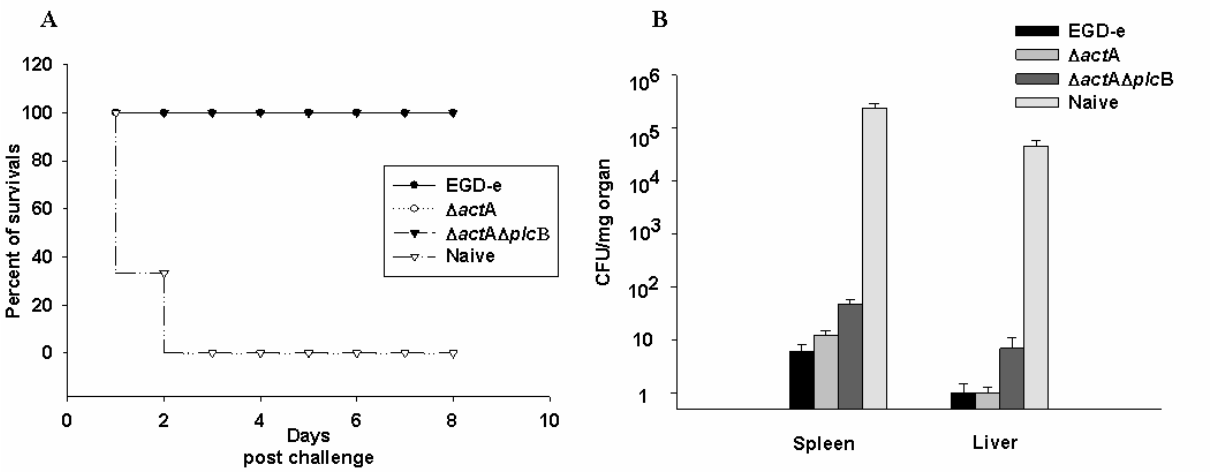

Fig. 3.14. A) Induction of protective immunity conferred after infection with the wild type *L. monocytogenes* and its isogenic mutant strains. Groups of mice were infected i.v. as described in Fig.3.11. Two months later all mice were challenged with a lethal dose (10X LD_{50}) of the wild type *L. monocytogenes*. As a control, a group of uninfected normal mice was included. Survival of mice after the challenge was monitored up to 8 days. **B)** Bacterial titers in spleens and livers of 3 mice per group of mice challenged in Fig. A were monitored at day 2 after challenge.

infections. Such an acquired protective immunity is exclusively T cell-mediated and requires specific cytotoxic CD8+ T cells. So, most noteworthy is to investigate whether infection of mice with the highly attenuated isogenic EGD-e mutant strains *L. monocytogenes* Δ*act*A2 and Δ*act*AΔ*plc*B confers a protective immunity to re-infection. Groups of BALB/c mice were infected with the wild type *L. monocytogenes* EGD-e strain (10^3) or with the isogenic *L. monocytogenes* mutant strains (10^7). Two months later, all mice were challenged with a lethal i.v. dose ($5x10^4$), corresponding to 10X LD_{50}, of the wild type *L. monocytogenes* EGD-e strain, and the survival of mice in all groups was monitored. As a control, a group of untreated normal BALB/c mice that have received a similar lethal dose ($5x10^4$) of the wild type *L. monocytogenes* EGD-e strain were included. As shown in Fig. 3.14.A, immunization with isogenic EGD-e mutant strains protects mice against a lethal infection with the wild type EGD-e strain. The protective response was significant as all mice, pre-immunized with the isogenic EGD-e mutant strains, survived. Not surprisingly, all mice that were pre-immunized with sub-lethal doses of the wild type EGD-e strain were also protected against a lethal

listerial infection and survived. All control mice that were not pre-immunized succumbed to the infection and died within 3 days after re-infection. In addition, the bacterial titres were determined in spleens and livers at day 2 post-challenge in each mice group. Naïve unprimed mice were not able to control challenge infection as indicated by the high bacterial load in spleen and liver. In contrast, mice primed with the wild type as well as the isogenic mutant strains *L. monocytogenes* Δ*act*A2 and *L. monocytogenes* Δ*act*AΔ*plc*B managed to restrict listerial growth in the first 2 days after infection (Fig. 3.14.B).

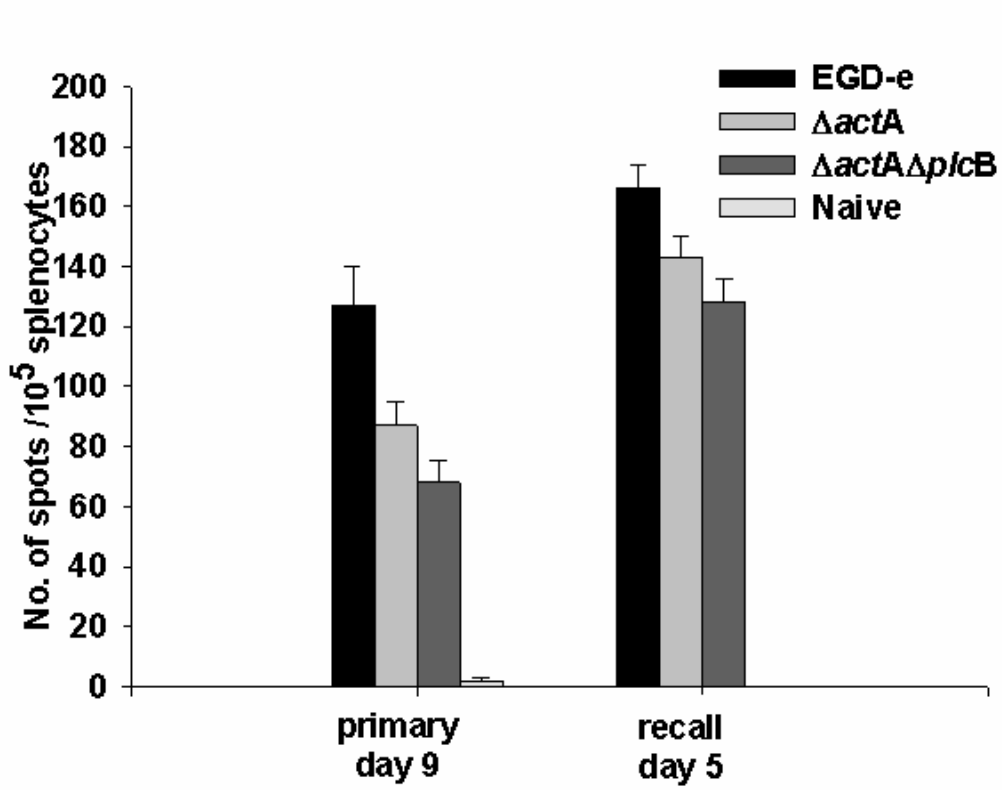

Fig. 3.15. Number of antigen-specific IFN-γ producing CD8+ T cells in the spleens of mice infected i.v. with the wild type *L. monocytogenes* and isogenic *L. monocytogenes* mutant Δ*act*A and Δ*act*AΔplcB strains, determined by ELISPOT. Spleen cells from infected mice were isolated either 9 days after the primary infection or 5 days after the recall challenge infection and stimulated with immunodominant MHC class I peptide $LLO_{91\text{-}99}$ in triplicates in nitrocellulose based 96-well culture plates. Number of specific IFN-γ producing cells against the dominant H-$2K^d$ restricted $LLO_{91\text{-}99}$ epitope were determined by counting the number of spots under microscope.

The ability of *L. monocytogenes* to gain access to the cytoplasm of infected cells allows processing and presentation of bacterial antigens through the MHC class I pathway. Infection of mice with *L. monocytogenes* leads therefore to the generation of MHC class I restricted CD8+ T cells, specific for listerial antigens, which transfer protective immunity and are also a major component of the adaptive immune response of the host to re-infection by this

intracellular pathogen. Mice, pre-immunized with isogenic EGD-e mutant strains were protected against a lethal challenge of the wild type EGD-e strain. The impact of previous immunization with attenuated isogenic EGD-e mutant strains on the generation of antigen-specific MHC class I restricted CD8+ T cells were quantitatively determined. The numbers of IFN-γ producing CD8+ cells, after immunization with either the wild type or mutant EGD-e strains, against the dominant H-2K^d restricted LLO_{91-99} epitope (Wipke *et al*., 1993) in *in vitro* ELISPOT assay were evaluated. The number of LLO_{91-99}-specific CD8+ T cells in spleens of mice infected with the two isogenic mutant strains (*L. monocytogenes* Δ*act*A2, *L. monocytogenes* Δ*act*AΔplcB) was very similar. It was however approximately 1.5-fold lower than that observed in spleens of mice primarily infected with the wild type EGD-e strain (Fig. 3.15.). The number of LLO_{91-99}-specific CD8+ T cells however, was significantly raised during the recall challenge infection in spleens of mice primarily infected with the two isogenic mutant strains (*L. monocytogenes* Δ*act*A2, *L. monocytogenes* Δ*act*AΔ*plc*B). It was almost as high as that observed in spleens of mice infected with the wild type *L. monocytogenes* EGD-e strain (Fig. 3.15.).

Sub-lethal infections of *L. monocytogenes* in mice are generally cleared by innate and specific immune responses, providing the animals with long-term immunity to re-infection. Such infections usually resulted in a rapid activation and an expansion of cytotoxic T lymphocytes (CD8+), specific for multiple peptides derived from *L. monocytogenes* (Mercado *et al*., 2000). The number of cells in the spleen, particularly CD8+ T cells, increases during the recall response to *L. monocytogenes*. Antigen specific T cells were monitored in mice 9 days after immunization (primary infection) with the wild type EGD-e and the isogenic mutant strains (*L. monocytogenes* Δ*act*A2, *L. monocytogenes* Δ*act*AΔ*plc*B). They were found to be predominantly CD8+ effector T cells, as determined by the rapid induction of IFN-γ (ELISPOT) following antigen exposure (Fig. 3.15.). It was previously shown that pathogen specific T cells are programmed during the first days of infection and subsequently undergo proliferation and differentiation into effector T cells (Mercado *et al*., 2000). All mice, pre-immunized either with the wild type EGD-e, or with the mutant *L. monocytogenes* Δ*act*A2 and *L. monocytogenes* Δ*act*AΔ*plc*B strains survived a lethal challenge with the wild type EGD-e strain (Fig. 3.14.A).The contribution of CD8+ T cells to the conferred protection was therefore further investigated through quantifying the expression of CD62L (as a memory T cell marker) on CD8+ T cells before and after the recall infection with *L. monocytogenes* (Fig. 3.16.). CD62L (L-selectin) is expressed at high levels on the surface of naïve T lymphocytes, but rapidly down-regulated upon T cell activation. As indicated in Fig. 3.16., a striking

increase in the number of $CD8^{+}CD62L^{lo}$ T cells was observed in the spleens of mice, pre-immunized with the wild type EGD-e and the isogenic mutant (*L. monocytogenes* Δ*act*A2, *L. monocytogenes* Δ*act*AΔ*plc*B) strains after the recall infection, as determined by flow

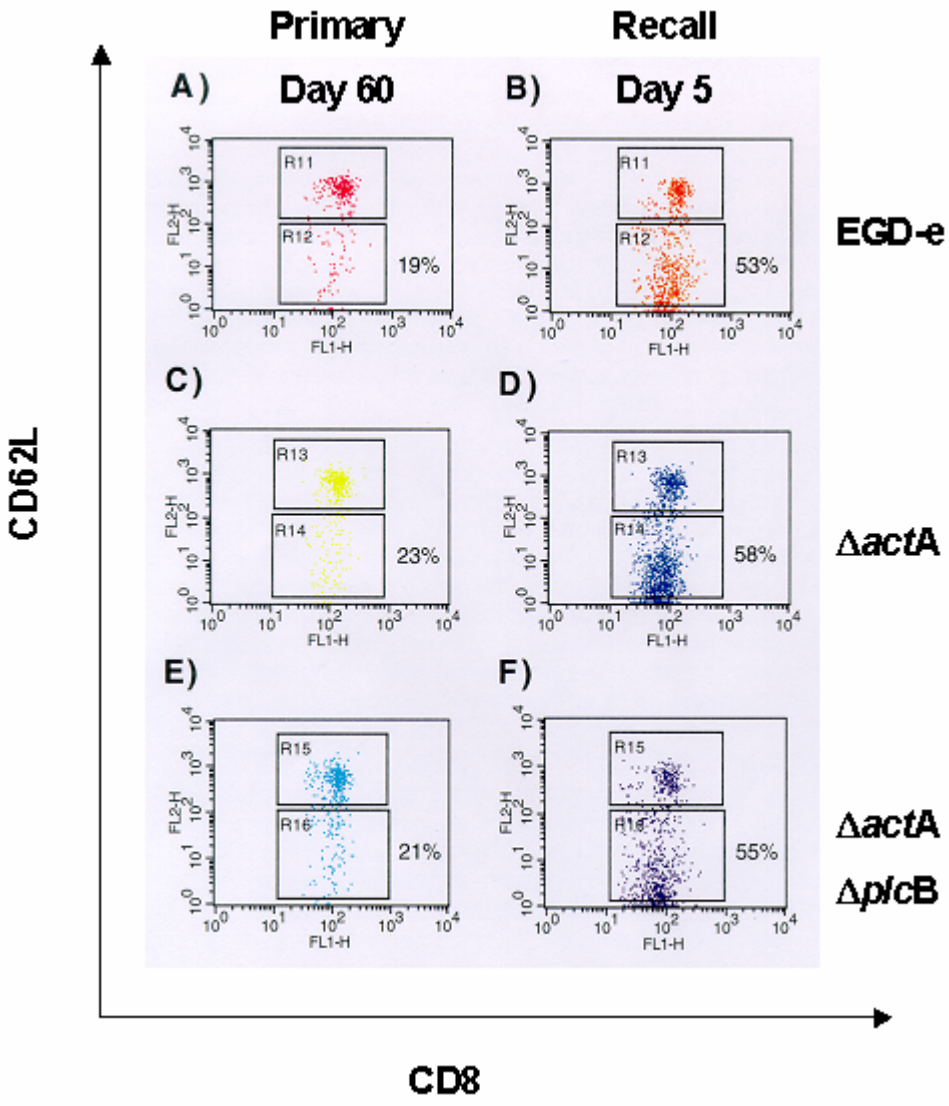

Fig. 3.16. Expression levels of CD62L on CD8+ splenocytes following primary and recall infection with *L. monocytogenes* and its isogenic mutant strains. Flow cytometry was performed with spleen cells, isolated from mice at day 9 after the primary infection or day 5 after the challenge. Cells were stained with FITC-labeled anti-Lyt-2 and biotinylated anti-CD62L, and the binding of anti-CD62L on the cell surface was detected with PE-conjugated streptavidin. Numbers shown are gated $CD8^{+}CD62^{lo}$ T cells (lower panel) and analysed with CELLQuest software.

cytometric analysis. Two months after the primary infection, all groups of mice showed approximately 19-23% of $CD8^{+}CD62L^{lo}$ expressing T cell population which is relatively similar to that expressed in naïve uninfected mice (Mercado *et al*., 2000). During the recall infection however the population of $CD8^{+}CD62L^{lo}$ expressing T cells increased from about

20% to over 50%. The increase in the percentage of $CD8^{+}CD62L^{lo}$ T cell population, attributed to the isogenic mutant (*L. monocytogenes* Δ*act*A2, *L. monocytogenes* Δ*act*AΔ*plc*B) strains, was similar or higher than that observed in animals primarily infected with the wild type *L. monocytogenes* EGD-e strain and positively correlated with the increase in the number of LLO_{91-99}-specific CD8+ T cells (Fig. 3.15.).

3.3.5. Induction of listeriolysin O-specific antibodies against isogenic *L. monocytogenes* deletion mutant strains

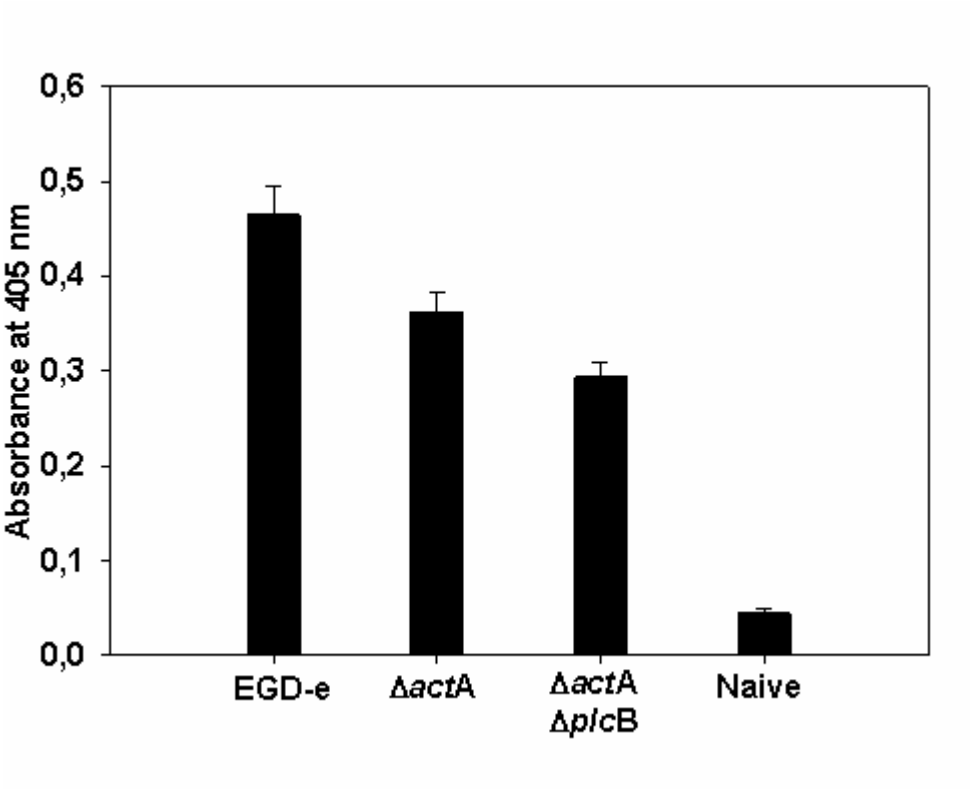

Fig. 3.17. Induction of specific antibody response against isogenic *Listeria* mutants. Listeriolysin O-specific antibodies in sera of mice 9 days post infection with the wild type *L. monocytogenes* or its isogenic deletion mutants (Δ*act*A2 and Δ*act*AΔ*plc*B) were measured with ELISA at 405 nm. Results from 3 mice per group are given.

It was previously established that listeriolysin O is a main protein target of the human humoral response against *L. monocytogenes* (Grenningloh *et al*., 1997). Moreover, the monoclonal antibody against listeriolysin O was found to be protective from a lethal dose of the wild type *L. monocytogenes* (Edelson *et al*., 1999; Edelson and Unanue, 2001). The ability of mice infected with the *L. monocytogenes* isogenic deletion mutants (Δ*act*A2 and Δ*act*A Δ*plc*B) to produce listeriolysin O-specific antibody was examined. Groups of 3 mice were injected i.v. with the wild type *L. monocytogenes* (10^3) cfu, its isogenic mutants (Δ*act*A2

and Δ*act*A Δ*plc*B) (10^7) cfu, or left uninfected. 9 days later, blood was collected from all groups by heart puncture, sera were isolated and incubated in ELISA plates with 100 ng of purified listeriolysin O. The amount of antibody in mice sera was measured by means of ELISA reader at 405 nm. As shown in Fig. 3.17., the isogenic *Listeria monocytogenes* mutant strains managed to induce listeriolysin O-specific antibodies in an amount comparable to the wild type strain while no significant antibody production was demonstrated in naïve unprimed mice.

3.4. Immunological characterization of a *L. innocua* recombinant strain carrying the virulence gene cluster (vgc) of *L. monocytogenes*

As previously discussed, the virulence of pathogenic *L. monocytogenes* wild type strain is attributed to the presence of the virulence gene cluster (vgc) comprises genes (*hly*, *plc*A, *plc*B, *mpl*, *act*A , *inl*A , *inl*B) required for survival in host vacuoles following uptake as well as replication and intracellular motility in the host cell cytoplasm (Chakraborty *et al*., 2000). The virulence gene cluster is, however, totally absent from the non-pathogenic *L. innocua* strain (Gouin *et al*., 1994; Kreft *et al*., 1999; Chakraborty *et al*., 2000) explaining its non-invasivness. However, It was interesting to examine the immunological properties of a recombinant strain of *L. innocua* transformed with a gram-positive/gram-negative schuttle BAC-based vector (pUvBBAC) harbouring a 96.9 kb fragment encoding the *prf*A virulence gene cluster (*prf*A-*plc*A-*hly*-*mpl*-*act*A-*plc*B) of *L. monocytogenes* (Hain *et al.*, unpublished). The suitability of this recombinant strain to be an appropriate vaccine vector was systemically examined through testing its ability to induce sufficient CD8+ T cell response as well as lower levels of the unfavourable accompanying CD4+ T cell response mediating delayed type hypersensitivity (DTH) and granulomatous inflammation.

3.4.1. Growth kinetics of the recombinant *L. innocua* strain *in vivo*

It is well-known that the ability of *listeria* to survive *in vivo* at the early stage of infection is crucial for induction of cell-mediated immunity. The *in vivo* survival of the recombinant *L. innocua* strain harbouring the complete virulence gene cluster of *L. monocytogenes* was compared to that of the wild type *L. monocytogenes* EGD-e in both spleen and liver. A group of BALB/c mice (16 mice) were infected i.v. with sub-lethal doses of wild type *L. monocytogenes* EGD-e (10^3), the recombinant *L. innocua* mutant strain, (designated *L. innocua*: :vgc) (10^7), or the wild type *L. innocua* strain (10^7). *In vivo* survival and growth kinetics of bacteria were followed by daily determining the number of bacteria in the spleen and liver of infected mice beginning at day 1 till day 4 after infection. As expected, regardless of the dose of infection, the wild type *L. innocua* strain was rapidly cleared from both organs from the outset (Fig. 3.18.) whereas *L. innocua*: :vgc strain successfully survived in both spleen and liver during the first two days after infection as indicated by the bacterial number that increased in both spleen and liver till day 2 and falled gradually over days 3 and 4 post-

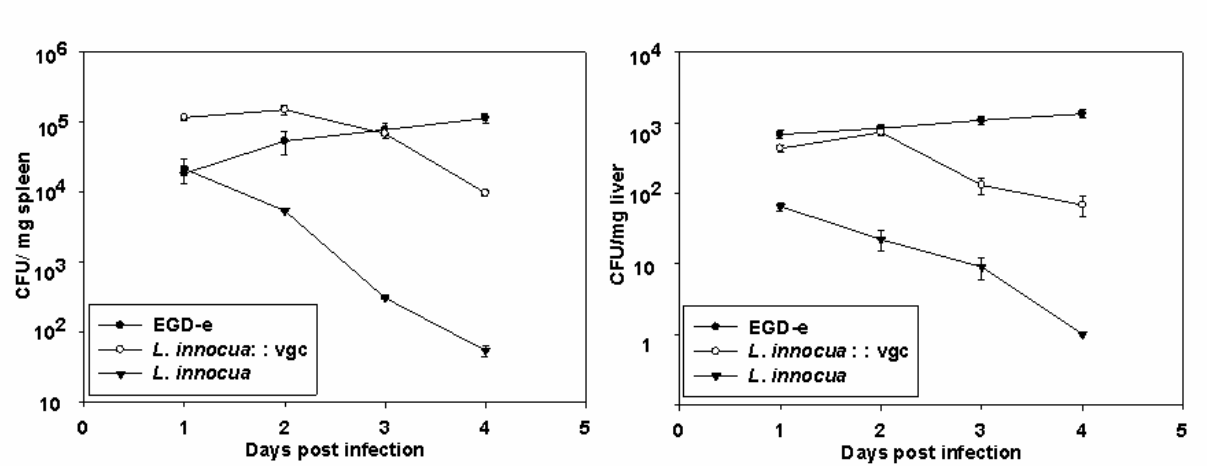

Fig. 3.18. Course of primary infection in mice with the wild type *L. monocytogenes* and the recombinant *L. innocua* strain. Mice were infected i.v. with 10^3 wild type *L. monocytogenes*, 10^7 wild type *L. innocua*, or 10^7 recombinant *L.innocua* strain (*L.innocua*: :vgc). Different time intervals after the infection, 3 mice per group were sacrified and the number of viable bacteria in the organs was enumerated. Data presented are representative of three independent experiments.

infection onwards. On the other hand, the bacterial number of the wild type *L. monocytogenes*, both in spleen and liver, increased from day 1 till day 4 of the infection onwards. Since higher dose of the wild type *listeria* strain is lethal, growth kinetics was performed with a lower infection dose (10^3).

3.4.2. Downmodulation of CD4+-mediated inflammatory responses by the recombinant *L. innocua* derivative

To determine the degree of CD4+-mediated inflammatory response induced by the recombinant *L. innocua* strain (*L. innocua*: :vgc), morphological changes were examined in the spleens on day 3 after i.v. infection with the wild type EGD-e(10^3), the wild type *L. innocua* strain and its recombinant derivative (*L. innocua*: :vgc) (10^7). Although the number of bacteria survived from spleens at day 3 post-infection for both the wild type EGD-e and *L. innocua*: :vgc were approximately the same, marked difference in the morphological appearance between spleens isolated from mice primarily infected with the wild type EGD-e and those isolated from mice primarily infected with *L. innocua*: :vgc (Fig. 3.19.). Splenomegaly associated with extensive granuloma formation was characteristic to spleens of EGD-e infected mice, as a result of intensive leukocyte infiltration, whereas only splenomegaly (without granuloma) was the case in spleens of *L. innocua*: :vgc infected mice. Infection with the wild type *L. innocua* strain did not result in any morphological changes in spleens on day 3 post infection.

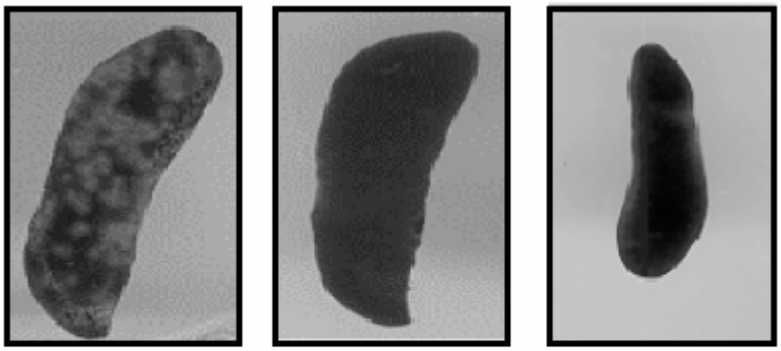

Fig. 3.19. Morphological appearance of the spleens of mice infected i.v. with the wild type *L. monocytogenes* and the recombinant *L.innocua* strain. Spleens of mice (infected i.v. as mentioned in Fig.3.18.) were removed on day 3 after infection. Shown is the scanned picture of spleens from the wild type *L. monocytogenes*, the wild type *L.innocua* and its recombinant mutant strain *L.innocua*: :vgc. Infiltration of monocytic cells and granulomatous lesions are only detectable in the spleens isolated from mice infected with the wild type *L. monocytogenes*.

Moreover, antigen-induced CD4+ T cell-derived IFN-γ production of spleen cells was measured as an indication for DTH and proinflammatory T cell response induced against EGD-e, *L. innocua*, or its recombinant derivative. Spleen cells were isolated at day 9 post-infection and stimulated *in vitro* with the released soluble antigen of *L. monocytogenes* EGD-e (100 ng). Spleen cells from mice immunized with the *L. innocua*: :vgc mutant produced significantly lower levels of IFN-γ when compared with that produced after immunization with the wild type EGD-e strain. The wild type *L. innocua* strain failed to prime T cells for the production of IFN-γ (Fig. 3.20.A.). Most notably, the observations obtained *in vitro* were confirmed *in vivo* by antigen-elicited skin responses showing corresponding results (Fig. 3.20.B). 6 mice per group were injected into the left hind footpads with 50 µl of somatic soluble *L. monocytogenes* EGD-e antigen (60 ng/ml) at day 9 post-infection. Twenty-four hours later, thickness of the left and right footpads of individual mice were measured. Footpads of mice pre-immunized with *L. innocua*: :vgc mutant strain showed a markedly lower thickness than those of mice pre-immunized with the wild type EGD-e strain. The wild type *L. innocua* strain could not able to induce DTH response in footpads.

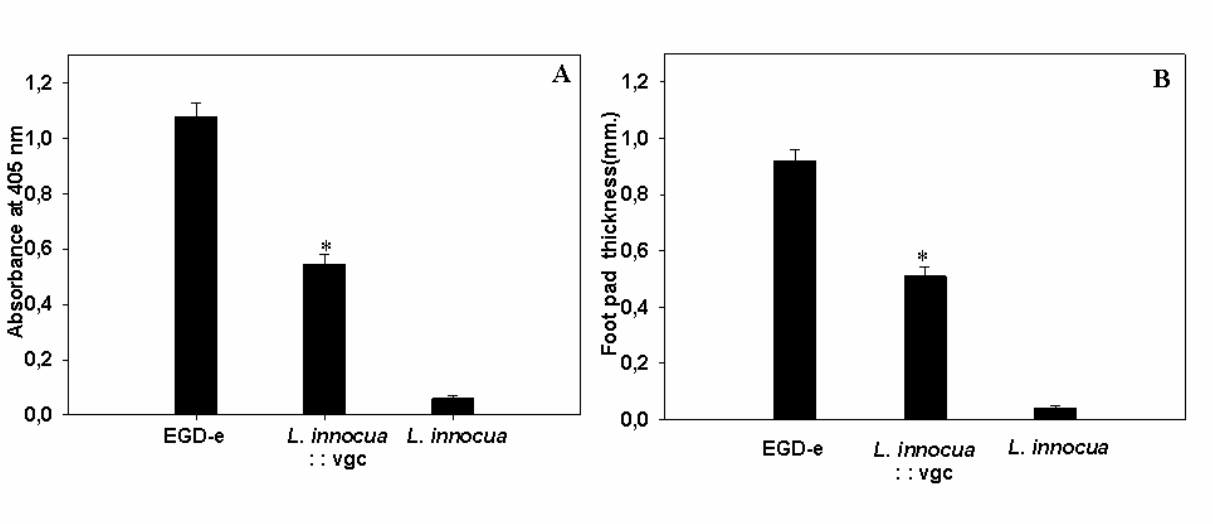

Fig. 3.20. A) *Listeria*-induced IFN-γ production by spleen cells 9 days after infection (i.v.). Mice were infected with 10^3 CFU of *L. monocytogenes* EGD-e, 10^7 CFU wild type *L. innocua*, or with 10^7 CFU its recombinant mutant strain *L.innocua*: :vgc. On day 9 after infection, mice were killed and spleens were removed. Single cell suspensions were stimulated *in vitro* with secreted soluble *Listeria* antigen to produce IFN-γ. After 48 hours, culture supernatants were tested for presence of IFN-γ by ELISA.*P<0.05 (EGD-e vs *L.innocua*: :vgc). **B)** DTH response to listerial antigen 9 days after primary infection. Mice were infected with 10^3 CFU of *L. monocytogenes* EGD-e , 10^7 CFU wild type *L.innocua*, or 10^7 CFU its recombinant mutant strain *L.innocua*: :vgc. 9 days after infection, DTH was triggered through injection of soluble somatic listerial antigen. Twenty-four hours later, specific skin response was determined. Experiments were repeated twice. The mean value ± S.D. of five animals of a representative experiment is shown.*P<0.05 (EGD-e vs *L.innocua*: :vgc).

3.4.3. Expression of T cell-mediated immune response to the recombinant *L. innocua* strain

A number of cell types are involved in host defenses to *Listeria*. Antigen-specific T lymphocytes mediate recovery from primary listerial infections and protective immunity to subsequent infections (Lane and Unanue, 1972; North, 1973). Both CD4+ (helper, MHC class II restricted) and CD8+ (cytotoxic, MHC class I restricted) T cell subpopulations have been implicated (Kaufmann *et al*., 1985; Ladel *et al*., 1994). Recent experimental evidence indicates, however, that CD8+ T cells play the predominant role (Mielke *et al*., 1988; Berche *et al*., 1989 ; Baldridge *et al*., 1990; Goossens *et al*., 1992; Roberts *et al*., 1993; Ladel *et al*., 1994). The ability of the recombinant *L. innocua* strain to induce T cell mediated immunity was examined. Groups of BALB/c mice were infected with the wild type EGD-e (10^3), the wild type *L. innocua* strain (10^7) and its recombinant derivative (*L. innocua*: :vgc) (10^7). Two months later, all mice were challenged with a lethal i.v. dose ($5x10^4$), corresponding to 10X LD_{50}, of the wild type *L. monocytogenes* EGD-e strain and the survival of mice in all groups was monitored. As a control, a group of untreated normal BALB/c mice that have received a

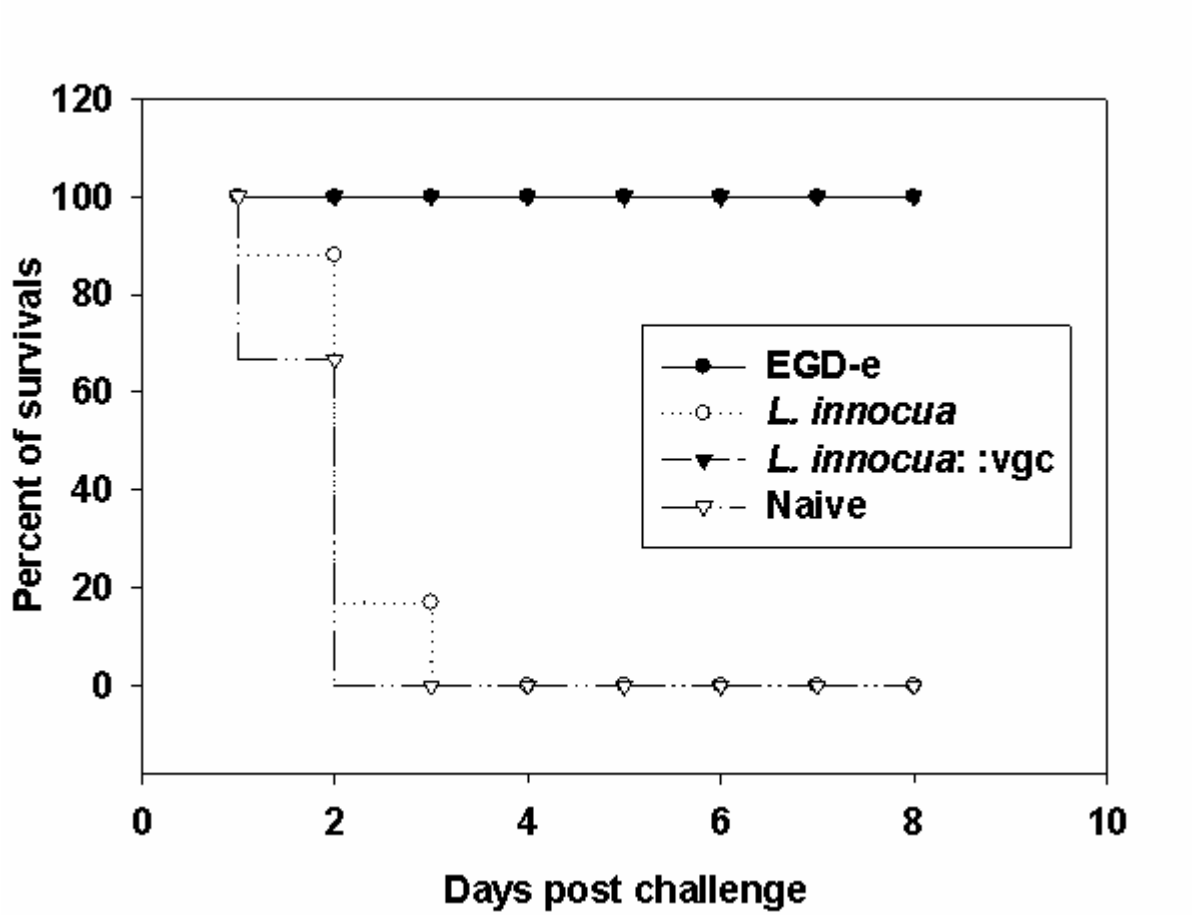

Fig. 3.21. Induction of protective immunity conferred after infection with the *L.innocua* recombinant mutant strain *L.innocua*: :vgc. Groups of mice were infected i.v. as described in Fig. 3.20. Two months later all mice were challenged with a lethal dose (10X LD_{50}) of the wild type *L. monocytogenes*. As a control, a group of uninfected normal mice was included. Survival of mice after the challenge was monitored up to 8 days.

similar lethal dose ($5x10^4$) of the wild type *L. monocytogenes* EGD-e strain were included. Primary infection with *L. innocua*: :vgc strain protected mice against a lethal infection with the wild type EGD-e strain. The protective response was significant as all mice, pre-immunized with the recombinant *L. innocua* strain, survived. As expected, all mice that were pre-immunized with sub-lethal doses of the wild type EGD-e strain were also protected against a lethal listerial infection and survived whereas all the non-immunized mice as well as those pre-immunized with wild type *L. innocua* strain died within 4 days after challenge (Fig. 3.21.).

Entry of *Listeria* into the cytosol is a critical event for CD8+ T-cell recognition and the induction of immunity (Brunt *et al*., 1990). In order to establish the correlation between the protection of mice previously infected with the wild type EGD-e strain or the *L. innocua* recombinant strain upon lethal challenge with the wild type *L. monocytogenes* EGD-e

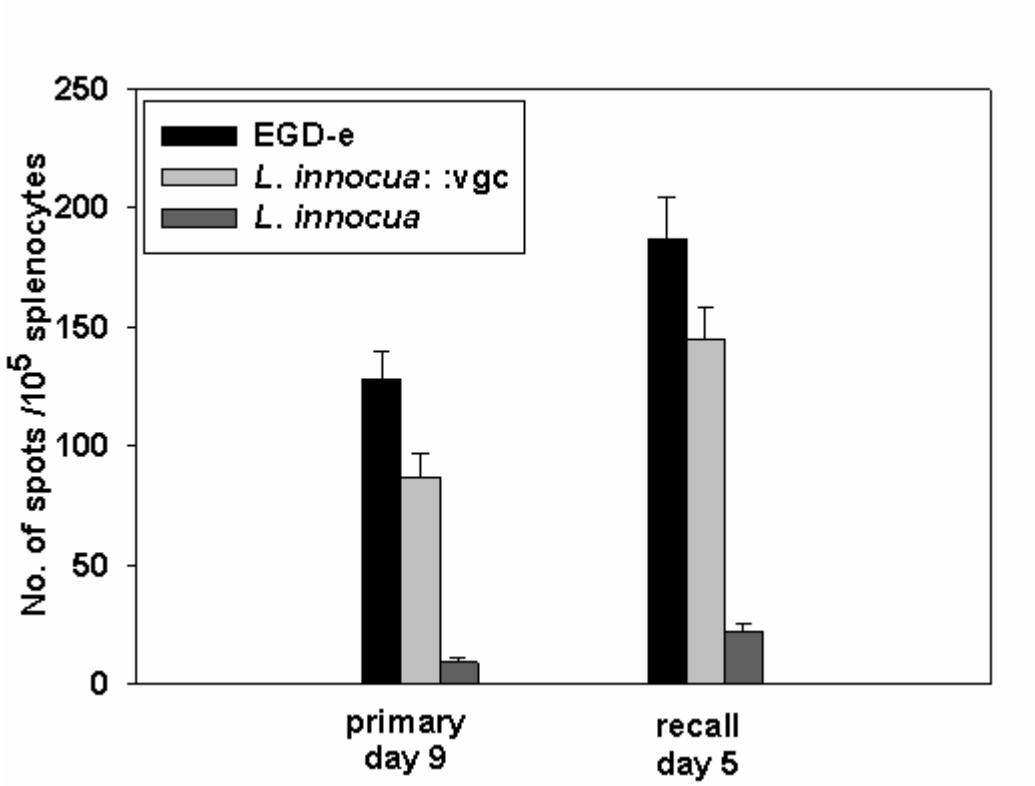

Fig. 3.22. Number of antigen-specific IFN-γ producing CD8+ T cells in spleens of mice infected i.v. with the wild type *L. monocytogenes, L.innocua* and *L.innocua* recombinant mutant strain (*L.innocua*: :vgc), determined by ELISPOT. Spleen cells from infected mice were isolated either after the primary infection or after recall challenge infection and stimulated with the immunodominant MHC class I peptide LLO_{91-99} in triplicates in nitrocellulose based 96-well culture plates. Number of specific IFN-γ producing cells against the dominant H-$2K^d$ restricted LLO_{91-99} epitope was determined by counting the number of spots under microscope.

strain and the induction of CD8+ cells in response to infection, the generation of antigen-specific MHC class I restricted CD8+ T cells were quantitatively examined. The amount of antigen specific MHC class I restricted effector CD8+ T cells induced in spleens of mice 9 days after the primary infection and 5 days after the challenge with the wild type *L. monocytogenes* ($2x10^3$) was determined through evaluation of the number of IFN-γ producing CD8+ T cells induced against the dominant H-$2K^d$ restricted LLO_{91-99} epitope (Wipke *et al*., 1993) in *in vitro* ELISPOT assay. As shown in Fig. 3.22., infection with wild type *L. monocytogenes* as well as the recombinant *L. innocua* derivative (*L. innocua*: :vgc) managed to induce a highly detectable population of LLO_{91-99} specific CD8+ T-cells. This number raised significantly during the recall infection. On the other hand, Infection with the wild type *L. innocua* strain failed to induce a significant level of CD8+ T-cells population either primarily or after challenge.

It was recently established that activated CD8+ T cells seem to be programmed to develop into memory T cells, because CD8+ T cells that were stimulated briefly (~24 hours), proliferated and differentiated into CTLs without further antigenic stimulation and continued to develop into long-lived, protective memory CD8+ T cells (Mercado *et al*., 2000; Kaech and Ahmed, 2001). Memory T cells can be subdivided into two categories on the basis of activation markers, homing receptor expression, and effector function (Lanzavecchia and Sallusto , 2000). Central memory T cells, which express high levels of the chemokine receptor CCR7 and the adhesion molecule CD62L and do not express effector functions, may differentiate into effector memory T cells, which express low levels of CCR7 and CD62L (also known as L-selectin or Mel-14) but produce cytokines (Sallusto *et al*., 1999). Whether these memory T cell subsets differ in their capacity to mediate protective immunity is unknown. Furthermore, the stimuli that generate central versus effector memory T cells remain undefined (Lauvau *et al*., 2001). In order to address the contribution of effector memory CD8+ T cells in mediating long last immunity after re-infection with the wild type *Listeria monocytogenes*, the expression level of CD62L, an effector memory CD8+ T cell surface marker, was quantified. Two months after the primary infection, the number of $CD8^{+}CD62L^{lo}$ lymphocytes was approximately identical in all groups of primarily infected mice. This number increased dramatically upon re-infection with the wild type *L. monocytogenes* ($2x10^{3}$) in mice pre-immunized with EGD-e as well as *L. innocua*: :vgc mutant strain while pre-immunization with the wild type *L. innocua* strain was not able to induce a significant CD62L down-regulation (Fig. 3.23.).

3.4.4. Induction of listeriolysin O specific antibody in response to the recombinant *L. innocua*: :vgc strain

As previously discussed (section 3.3.5.), listeriolysin O (LLO) was efficiently recognized with antisera of mice primarily infected with the wild type *L. monocytogenes* as well as its isogenic deletion mutants, Δ*act*A2 and Δ*act*AΔ*plc*B, while antisera from unprimed mice failed to recognize the purified listeriolysin O. In an attempt to examine if primary infection with *L. innocua*: :vgc recombinant strain can induce a humoral immune response against listeriolysin O, groups of 3 mice were injected i.v. with the wild type *L. monocytogenes* (10^{3}), *L. innocua*: :vgc (10^{7}), the wild type *L. innocua* (10^{7}), or left uninfected. 9 days later, blood were collected from all groups, sera were isolated and used to detect purified listeriolysin O (100 ng) in an immunoblotting assay. As expected antisera from mice primarily infected

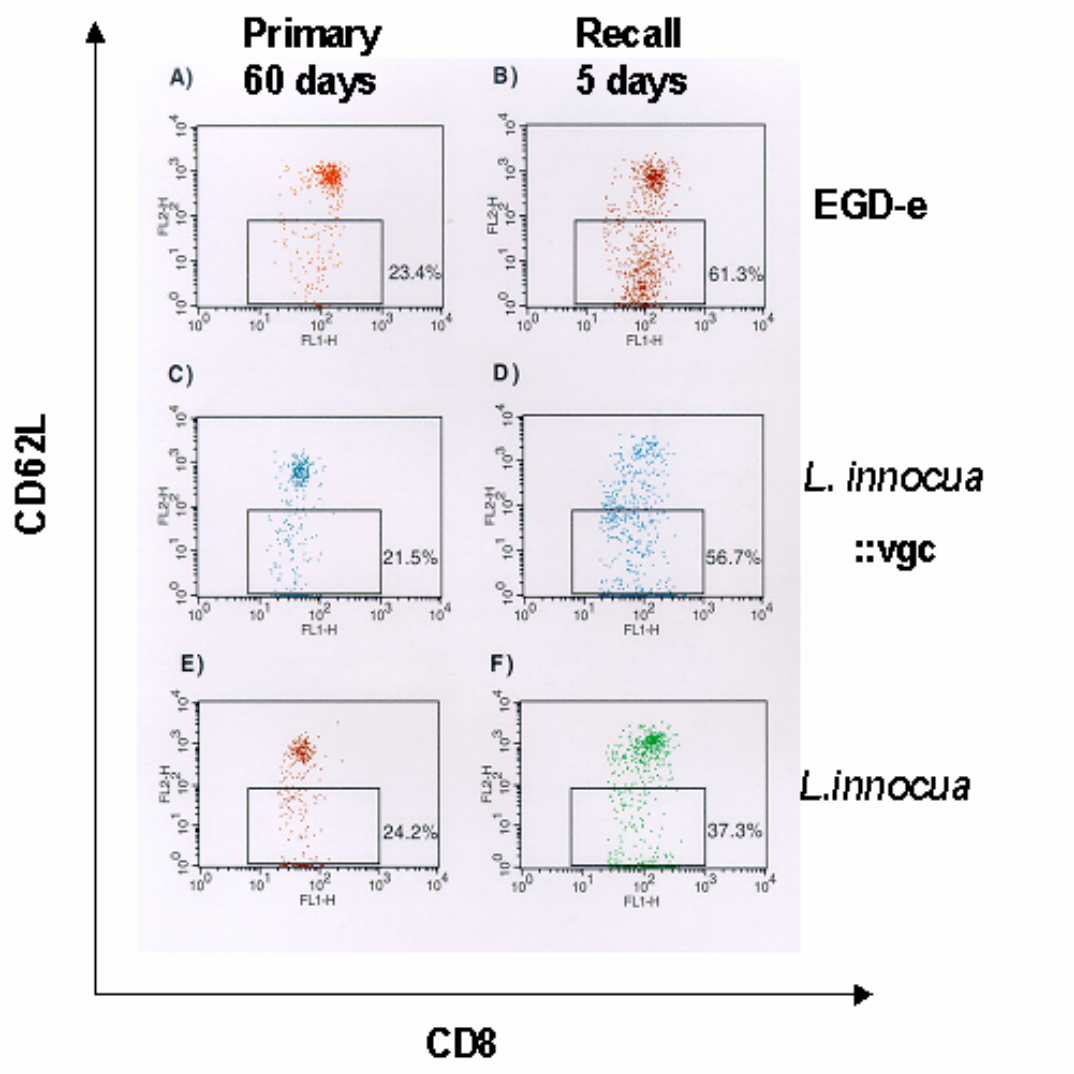

Fig. 3.23. Expression levels of CD62L on CD8+ splenocytes following primary and recall infection with *L. monocytogenes, L.innocua* and *L.innocua*: :vgc. Flow cytometry was performed with spleen cells, isolated from mice at day 9 after the primary infection or day 5 after the challenge. Cells were stained with FITC-labeled anti-Lyt-2 and biotinylated anti-CD62L. The binding of anti-CD62L on the cell surface was detected with PE-conjugated streptavidin. Numbers shown are gated $CD8^{+}CD62^{lo}$ T cells and analysed with CELLQuest software.

with the wild type *L. monocytogenes* can recognize listeriolysin O. Antisera from mice primarily infected with *L.inn*: :vgc recombinant strain can also efficiently recognize LLO while antisera from unprimed mice as well as mice primarily infected with the wild type *L. innocua* failed to recognize LLO (Fig. 3.24.).

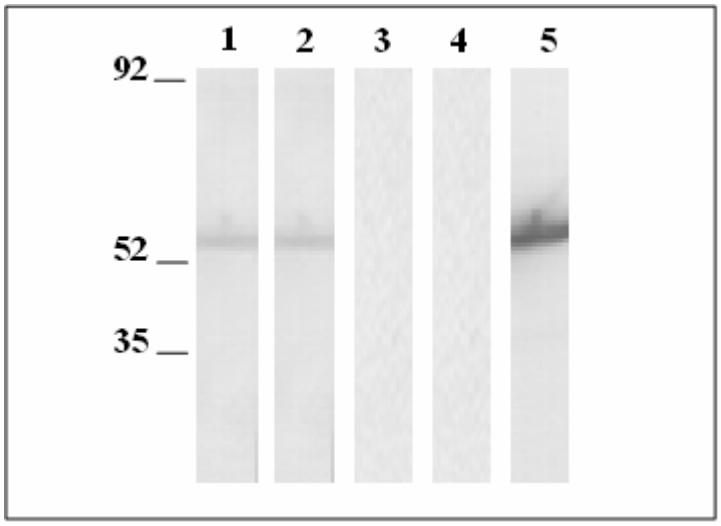

Fig. 3.24. Induction of listeriolysin O-specific antibodies against the recombinant *L. innocua*: :vgc strain. 100 ng of purified LLO were loaded onto 10 % SDS-polyacrylamide gel, transferred electrophoretically onto a PVDF filter and incubated with antisera obtained from mice 9 days after infection with the wild type *Listeria monocytogenes* (1), *L. innocua*: :vgc (2), wild type *L.innocua* (3), unprimed mice (4), and M275 antibody (as a control) (5), followed by AP-conjugated anti-mouse IgG. Blots were visualized using BCIP as a substrate. The numbers to the left of the figure correspond to apparent molecular weights (in kDa).

Based on its ability to induce effector CD8+ T lymphocytes as well as humoral immunity in addition to the downmodulation of unfavourable CD4+ inflammatory response, *L. innocua* complemented with the virulence gene cluster of *L. monocytogenes* is shown to be a promising vaccine or delivery vector.

3.5. Molecular and immunological characterization of a *Listeria monocytogenes* strain harbouring a gene of pneumolysin in instead of listeriolysin O

Lysis of the host cell vacuole is mediated in large part by the thiol-activated cytolysin listeriolysin O (LLO) (Gaillard *et al*., 1987; Tilney and Portnoy, 1989; Bielecki *et al*., 1990). Transposon insertions in *hly*, the structural gene encoding LLO, result in a non-hemolytic phenotype, complete avirulence and inability to lyse the host vacuole (Gaillard *et al*., 1986; Kathariou *et al*., 1987; Kuhn *et al*., 1988; Portnoy *et al*., 1988). Introduction of *hly* on a plasmid restores both haemolytic capacity and virulence (Cossart *et al*., 1989). Thiol-activated hemolysins, so named for the the unique cysteine highly conserved undecapeptide, or in other wards, cholesterol-binding cytolysins (CBCs), due to their ability to bind cholesterol, have been identified in 23 taxonomically different species of gram-positive bacteria (Alouf, 2000) including for example streptolysin O (SLO) produced by *Streptococcus pyogenes*, pneumolysin (PLY) produced by *Streptococcus pneumonia*, suilysin (SLY) produced by *streptococcus suis* and perfringolysin O produced by *Clostridium perfringens* (Smyth and Duncan, 1978; Alouf and Geoffroy, 1991). LLO, however, is the only one that is produced by intracellular bacterium (Jones and Portnoy,1994). Apart from pneumolysin, which is an intracytoplasmic toxin, all other toxins are produced in the extracellular medium (Alouf, 2000). It was previously shown that *L. monocytogenes* expressing perfringolysin O in place of listeriolysin O was able to lyse the host cell vacuole, grow in the cytoplasm, and spread from cell to cell to a limited extent *in vitro* followed by damage of the cells, preventing the intracellular proliferation of bacteria (Jones and Portnoy,1994).

Subclinical infection of BALB/c mice with virulent *Listeria monocytogenes* leads to the induction of *Listeria*-immune T-cell populations and protective immunity. LLO production appears to be required for induction of protective immunity, since preparations of killed *L. monocytogenes* or mutants of *L. monocytogenes* which lack LLO expression remain within the phagocytic vacuole of the host cell, do not replicate intracellularly, and do not trigger a protective antilisterial immune response (Barry *et al*., 1992; Cossart , 1988; Mengaud *et al*., 1989). Here, a related cytolysin, pneumolysin (PLY) was chosen to be tested if it is able to complement LLO for intracellular survival, virulence, and induction of protective immunity.

3.5.1. Bacterial strains and growth conditions

Bacterial strains used in this study are the wild type *Listeria monocytogenes* EGD-e serotype 1/2a (Glaser *et al*., 2000), its isogenic deletion mutant EGD-eΔ*hly* (Leimeister-Wächter *et al*.,

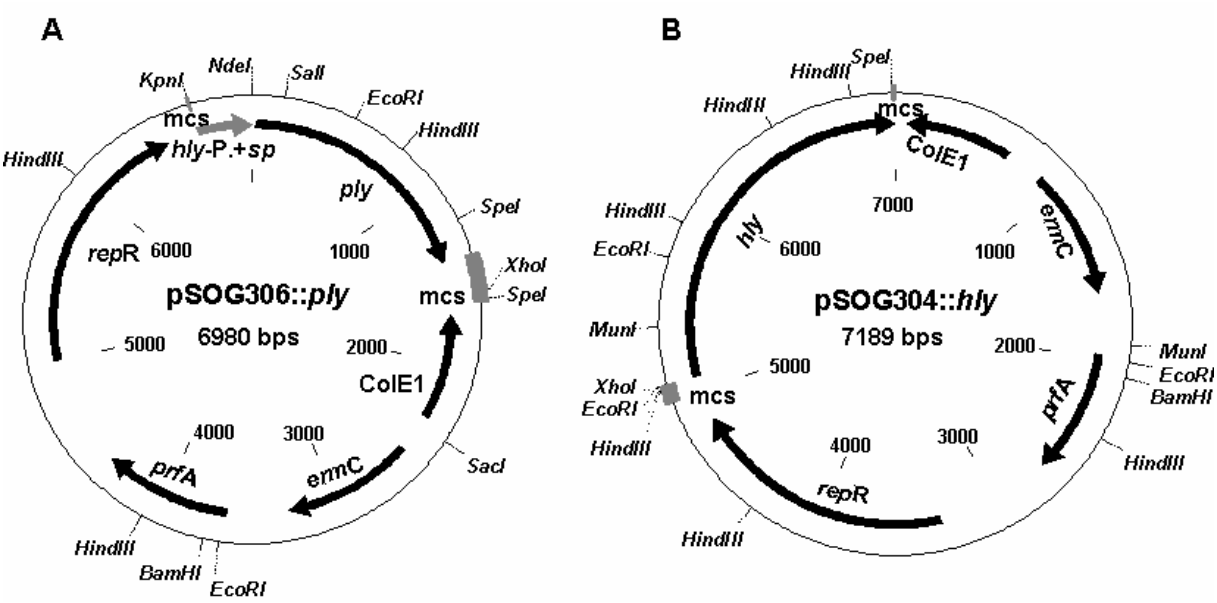

Fig. 3.25. A) The structural gene encoding pneumolysin with *Nde*I and *Xho*I restricted ends was cloned in the multiple cloning site (mcs) of pSOG306, containing the same restriction ends, downstream from the promoter and signal peptide sequence of listeriolysin O (*hly* P+SP). **B)** The intact gene encoding listeriolysin O with *Xho*I and *Spe*I restricted ends was cloned in the multiple cloning site of pSOG304 restricted with the same enzymes.

1990; Guzman *et al*., 1995), both were electroporatically transformed with pSOG306 gram-positive/gram-negative schuttle vector, containing the intact promoter of listeriolysin O followed by its signal peptide coding sequence under the regulatory control of *prf*A gene (Otten *et al*., unpublished) and its isogenic derivatives EGD-eΔ*hly*::pSOG304-*hly* (designated EGD-eΔ*hly*: :LLO), and EGD-eΔ*hly*::pSOG306-ply (designated EGD-eΔ*hly*: :PLY). Bacteria were grown in brain-heart infusion (BHI) broth in presence of 5μg/ml erythromycin.

3.5.2. Construction of plasmid-based strains

For the construction of EGD-eΔ*hly* strains complemented with either intact *ply* or *hly* gene, pSOG306 or pSOG304 plasmid, respectively, was used.

3.5.2.1. EGD-eΔ*hly*::pSOG306-*ply*

The structural gene for PLY were amplified from the *S. pneumonia* (Genbank accession no. X52474) chromosomal DNA by means of (*ply*-gene for., *Nde*I) and (*ply*-gene rev., *Xho*I) oligonucleotides primers (see section 2.7.11) which contained restriction sites for the enzymes *Nde*I and *Xho*I, respectively. The 1397 bp fragment was cloned into pSOG306 that was

restricted with the same enzymes, downstream from the signal peptide sequence of *hly* (Fig. 3.25.A).

3.5.2.2. EGD-eΔ*hly*::pSOG304-*hly*

By means of (*hly*-gene for., *Xho*I) and (*hly*-gene rev., *Spe*I) oligonucleotides primers (see section 2.7.11), a 2086 bp long fragment containing entire *hly* gene including its promoter was amplified from the *Listeria monocytogenes* chromosomal DNA using high fidelity PCR procedure followed by cloning on to pSOG304 plasmid, harbouring the *prf*A regulator (Otten, *et al*., unpublished) using *Xho*I and *Spe*I restriction sites (Fig. 3.25.B). Both constructions were checked by DNA sequence analysis. Transformation of EGD-eΔ*hly* strains with pSOG306 or pSOG304 plasmid containing *ply* or *hly* genes respectively was performed by electroporation (Schäferkordt and Chakraborty, 1995). The desired recombinants were selected using erythromycin (5µg/ml) on BHI agar plates and subsequently verified by sequencing and western blot analysis.

3.5.3. Expression and hemolytic activity of pneumolysin

Pneumolysin was expressed and efficiently secreted into the culture supernatent of EGD-eΔ*hly*, as shown by Western blot using monoclonal anti-PLY antibody (Fig. 3.26.).

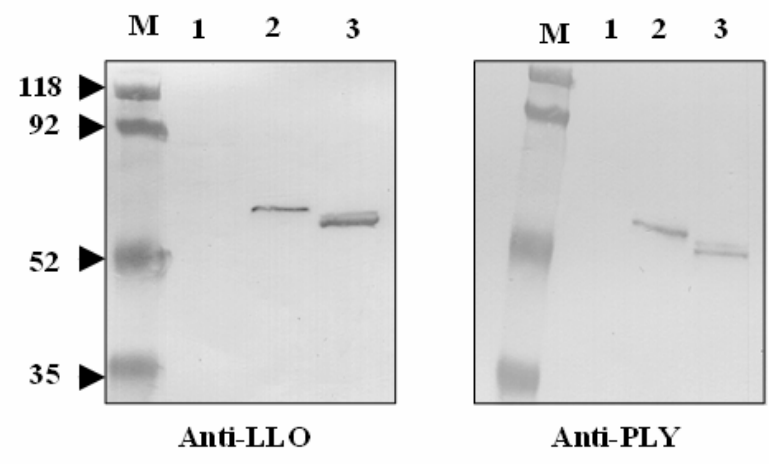

Fig. 3.26. Immunoblot analysis. Equal amounts of proteins precipitated by 10% trichloroacetic acid from BHI culture supernatants of *L. monocytogenes* EGD-eΔ*hly* (Lanes 1) and the isogenic deletion mutant *L.monocytogenes* EGD-eΔ*hly* expressing either LLO (Lanes 2) or PLY (Lanes 3) were loaded onto 10 % SDS-polyacrylamide gel. Proteins were electrophoretically blotted and developed with either mouse anti-LLO monoclonal antibody (M275) or mouse anti-PLY monoclonal antibody followed by AP-conjugated anti-mouse IgG. Lane (M) corresponds to the running marker protein while the numbers to the left of the figure correspond to apparent molecular weights (in kDa).

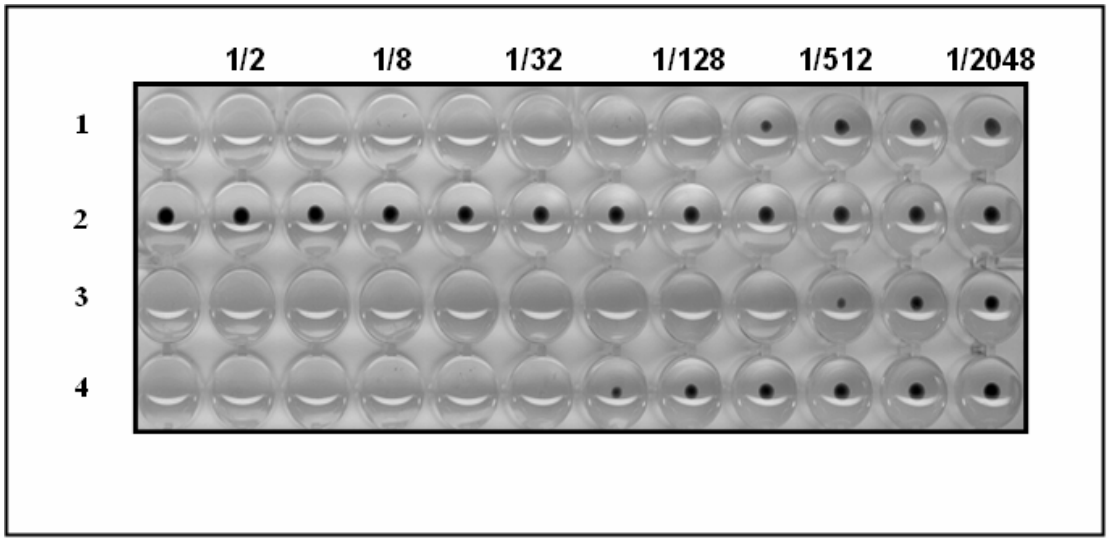

Fig. 3.27. Hemolytic activity of recombinant pneumolysin. Serial twofold dilutions of each culture supernatant (isolated from bacterial cultures at OD_{600} of 1.0) from *L. monocytogenes* EGD-e (Lane 1), the isogenic deletion mutant *L. monocytogenes* EGD-eΔ*hly* (Lane 2), and EGD-eΔ*hly* expressing either LLO (Lane 3) or PLY (Lane 4) (starting from 50 µl of non-diluted sample) were incubated with defibrinated sheep erythrocytes at pH 5.6 and 37°C for 2 hours. Cytolysis of erythrocytes is visualized by a uniform grey colour of the well (corresponding to haemoglobin release). A dark spot at the bottom of the well corresponds to sedimented intact erythrocytes.

Interestingly, pneumolysin was recognized with anti-LLO monoclonal antibody (M275) while LLO was also recognized with anti-PLY monoclonal antibody. The cross reactions of LLO and PLY against both antibodies are more likely due to the close structure homology between the two proteins. The regulated expression of the cloned gene products was examined by assaying the hemolytic activity of bacterial culture supernatants on sheep erythrocytes at pH 5.6. As expected, EGD-eΔ*hly* had no detectable hemolytic activity. Pneumolysin showed a comparable hemolytic activity to that of LLO (Fig. 3.27.).

3.5.4. *Listeria* expressing pneumolysin shows a limited intracellular growth in J774 macrophages

The ability of this strain to grow inside the mouse macrophage like cell line J774 was examined. During the first 8 hours of intracellular growth, wild type EGD-e as well as the mutant strain EGD-eΔ*hly* complemented with LLO doubled approximately 5 times, whereas the pneumolysin expressing EGD-eΔ*hly* mutant strain doubled only once during the first 4 hours followed by a decrease in the growth rate suggesting the induction of host cell cytotoxicity by pneumolysin, resulting in entry of extracellular gentamicin and bacterial death (Fig. 3.28.).

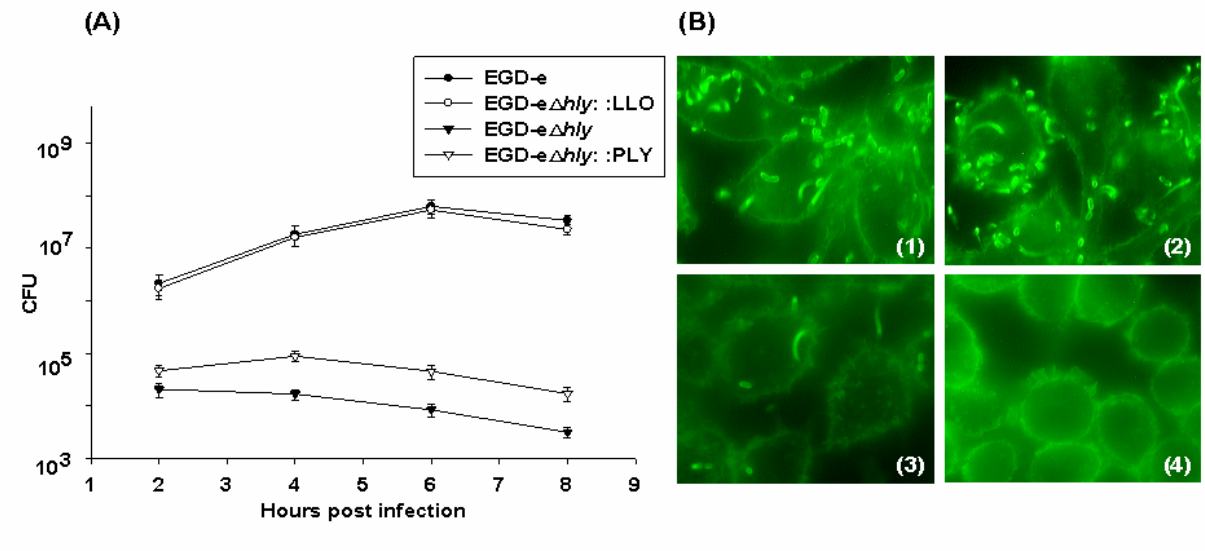

Fig. 3.28. A) Intracellular growth kinetics in macrophages. A) macrophage-like J774 cell line was infected with the wild type *L monocytogenes* EGD-e, mutant *Lmonocytogenes* EGD-eΔ*hly* expressing either LLO or PLY, and mutant *L. monocytogenes* EGD-eΔ*hly* strains at MOI of 10. Viable bacteria were enumerated at indicated time intervals following incubation at 37°C. **B)** macrophage-like J774 cell line was infected with the wild type *L.monocytogenes* EGD-e (1), mutant *L.monocytogenes* EGD-eΔ*hly* expressing either LLO (2) or PLY (3), and mutant *L. monocytogenes* EGD-eΔ*hly* strains (4). 4 hours after gentamicin treatment, cells were fixed with 4% formaldehyde and visualised by staining F-actin using Oregon Green 488 conjugated phalloidin under a fluorescence microscope.

In order to confirm this suggestion, the infected macrophages were examined 4 hours post-infection by flourescent microscopy after staining with beta-phalloidin to visualize the F-actin. The wild type *L. monocytogenes* as well as mutant bacteria expressing wild type LLO had multiplied and were found associated with tails of polymerized actin, propelling bacteria to adjacent cells, whereas bacterial escape as well as actin polymerisation for the EGD-eΔ*hly* mutant strain expressing pneumolysin was significantly lower (Fig. 3.28.). As expected, the EGD-eΔ*hly* strain was unable to multiply or to polymerize actin.

3.5.5. *L. monocytogenes* expressing pneumolysin shows a reduced *in vivo* survival

In order to correlate the *in vitro* intracellular growth of the *L. monocytogenes* expressing pneumolysin strain to its *in vivo* survival, the virulence of this strain in comparison to the wild-type was assessed by using the i.v. mouse model. The number of bacteria in both spleens and livers of infected mice was determined over 9 days after infection. A group of BALB/c mice were intravenously (i.v) injected with sublethal doses of either wild type EGD-e strain (10^3), or the isogenic *L. monocytogenes* derivatives EGD-eΔ*hly*, EGD-eΔ*hly*: :LLO, and EGD-eΔ*hly*: :PLY ($8x10^6$). As shown previously, regardless of the dose of infection, the

mutant EGD-eΔ*hly* is rapidly cleared from both organs from the outset (Lety *et al*., 2001; Peters *et al*., 2003). On day 3 post-infection, bacterial load had already fallen to a level of approximately 40 cfu/mg of spleen and 10 cfu/mg of the liver even after infection with $8x10^6$ viable bacteria per mouse (Fig. 3.29.). Although the survived numbers of EGD-eΔ*hly*: :PLY mutant strain increased from day one to day three post infection in both spleen and liver, it remained 10–60 as well as 10 folds lower than the number of wild type survived from spleen or liver respectively over the first 3 days after the infection. Then, the number of survived mutant strain EGD-eΔ*hly*: :PLY decreased rapidly from day 4 post-infection onwards.
Survival was fully restored in EGD-eΔ*hly* complemented with plasmid-encoded LLO to an extent seems to be identical to that of the wild type *L. monocytogenes* where the number increased between day 1 and day 3. On day 3, the number of wild type and EGD-eΔ*hly* +LLO peaked at the same level and declined rapidly from day 4 post-infection onwards. In all cases, both in the wild type EGD-e and in its mutant derivatives, bacteria were cleared from the spleen and liver by day 8 -10 post-infection.

3.5.6. Induction of a protective antilisterial immune response by *Listeria monocytogenes* expressing pneumolysin in place of listeriolysin O

Analysis of the specificity of antilisterial cytotoxic cells from *Listeria* immune BALB/c mice donors has shown a dominant response to an epitope corresponding to amino acids 91 to 99 of LLO (Pamer *et al*., 1991; Harty and Bevan, 1992**)**. Pamer and co-workers have identified four major *Listeria*-derived epitopes of CD8+ T cells presented by H2-k^d (Busch *et al*., 1998; 1999). Two of the peptides derive from p60, a constitutively secreted protein of *Listeria*: $p60_{449–457}$ is presented at a high density by infected cells but is subdominant based on the size of the T cell population specific for this peptide; in contrast a stronger T cell response is specific for $p60_{217–225}$, even though this epitope is presented at a lower level. A third epitope derives from LLO (residues 91–99); although presented in smaller amounts than the p60 epitopes, it dominates the T cell response. The fourth epitope is from a secreted metalloprotease, $mpl_{84–92}$. A major contribution in understanding the response to *Listeria* has been made by following the development of the CD8+ T cell response to these four peptides (Busch *et al*., 1998; 1999) using the MHC tetramer approach developed by Davis and Altman (Altman *et al*., 1996). Demonstration of antilisterial T cells with specificity to non-LLO-derived epitopes had been difficult to achieve because of the requirement of LLO in

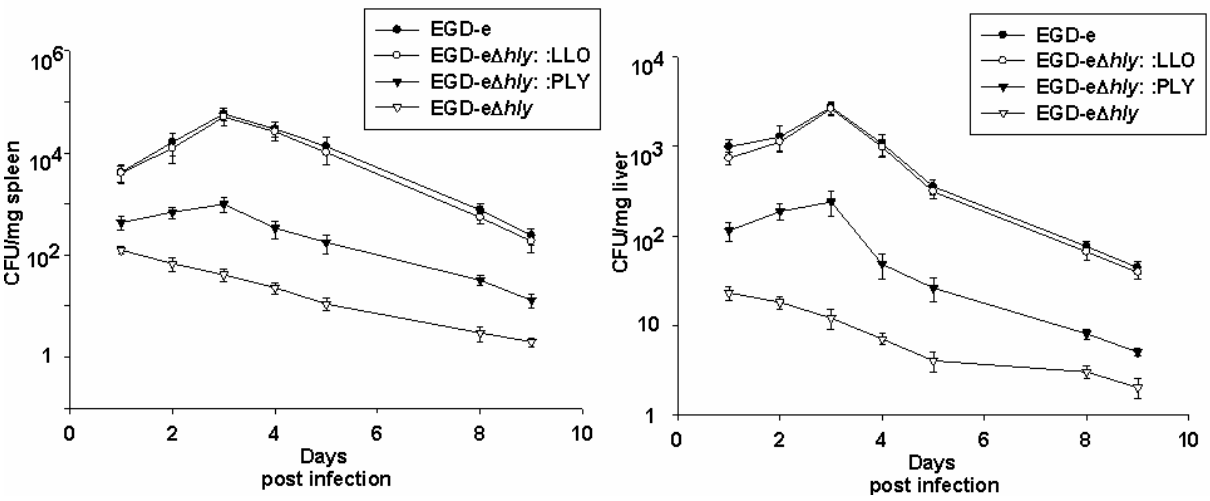

Fig. 3.29. *In vivo* kinetics of primary infection. The kinetics of bacterial growth was followed in organs of mice infected i.v. with either 10^3 wild type *L. monocytogenes* EGD-e or $8x10^6$ isogenic *L. monocytogenes* mutant strains EGD-eΔ*hly* expressing either LLO or PLY, and mutant *L. monocytogenes* EGD-eΔ*hly* strains. On days 1, 2, 3, 4, 5, 8, and 9 after infection, the numbers of viable bacteria in the spleens and the livers of three animals per group were determined. Data presented are representative of three independent experiments.

facilitating escape of the bacteria to the cytoplasm of the host cell and the apparent dominance of an anti-LLO response in antilisterial immunity. In this study, it was examined if the ability of pneumloysin expressing EGD-eΔ*hly* mutant strain to escape phagolysosome and gain access to the cytoplasm can induce a protective immunity against a lethal infection with the wild type *L. monocytogenes* by facilitating endogenous antigen processing and presentation of non LLO-determinant epitopes for association with MHC class I molecules for surface display to CD8+ T cells. Groups of BALB/C mice were infected i.v. with the wild type strain (10^3) or its isogenic derivatives ($8x10^6$). 2 months later, all mice were challenged with a lethal i.v. dose ($5x10^4$), corresponding to 10X LD_{50} of the wild type *L. monocytogenes*, and survival was monitored. As a control, a group of non-immunized normal BALB/C mice that have received a similar lethal dose of the wild type *L. monocytogenes* were included. As expected, all the non-immunized mice died in 3 days as well as those pre-immunized with the isogenic mutant strain EGD-eΔ*hly* were dead in 4 days after the challenge infection with *L. monocytogenes* whereas those pre-immunized with the wild type as well as EGD-eΔ*hly* complemented with LLO were protected against a lethal *Listeria* infection and survived (Fig. 3.30.A). Surprisingly, all the mice pre-immunized with EGD-eΔ*hly* mutant strain expressing PLY can also survive the lethal infection of the wild type EGD-e strain.

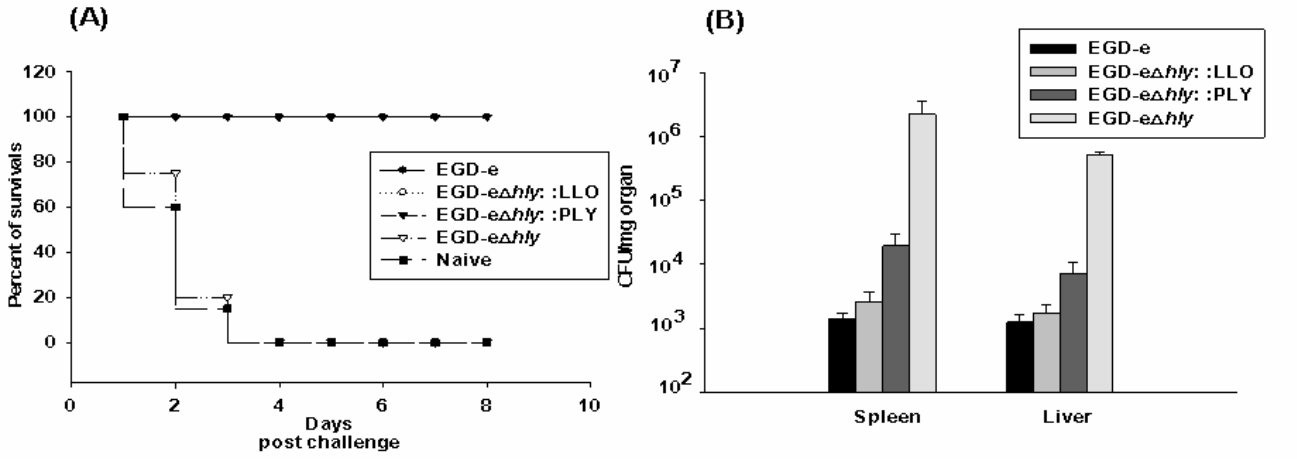

Fig. 3.30. Induction of protective immunity by *Listeria monocytogenes* expressing pneumolysin. **A)** Mice were infected i.v. with either 10^3 wild type *L. monocytogenes* EGD-e or $8x10^6$ isogenic *L. monocytogenes* mutant strains EGD-eΔ*hly* expressing either LLO or PLY, and mutant *L. monocytogenes* EGD-eΔ*hly* strains. Two months later all mice were challenged with a lethal dose (10X LD_{50}) of the wild type *L. monocytogenes*. As a control, a group of uninfected normal mice was included. Survival of mice after the challenge was monitored up to 8 days. Data were pooled from two independent experiments with a total of 15 mice per group. **B)** Bacterial titres in spleens and livers of 3 mice per group of mice challenged in Fig. A were monitored at day 2 after challenge.

In addition, the bacterial titres were determined in spleens and livers at day 2 post-challenge in each mice group. Mice primed with the isogenic mutant strain EGD-eΔ*hly* as well as naïve unprimed mice were not able to control challenge infection as indicated by the high bacterial load in spleen and liver. In contrast, mice primed with the wild type EGD-e or the mutant strain EGD-eΔ*hly* complemented with either LLO or PLY managed to restrict listerial growth in the first 2 days after infection (Fig. 3.30.B). Hence, to this point, pneumolysin seems to manage to induce a protective immunity in absence of listeriolysin O.

In order to determine whether T cells with specificity for non-LLO-derived epitopes of *L. monocytogenes* are a component of a protective antilisterial immune response, the amount of antigen specific MHC class I restricted effector CD8+ T cells induced against a P60 derived peptide, $P60_{217-225}$, in mice spleens 9 days after the primary infection as well as 5 days after the challenge with a sub-lethal dose ($2x10^3$) of *L. monocytogenes* was quantitatively determined through evaluation of the number of IFN-γ producing CD8+ T cells induced against the dominant H-$2K^d$ restricted $P60_{217-225}$ subdominant epitope (Vijh and Pamer, 1997) in *in vitro* ELISPOT assay. As shown in Fig. 3.31., infection with the PLY expressing mutant strain managed to induce a highly detectable population of $P60_{217-225}$ specific CD8+ T-cells comparable to those induced in response to infection with the wild type *L. monocytogenes* as

well as the mutant complemented with LLO. This number raised significantly during the recall infection. On the other hand, Infection with the isogenic mutant strain EGD-eΔ*hly* failed to induce a significant level of CD8+ T-cells population either primarily or after

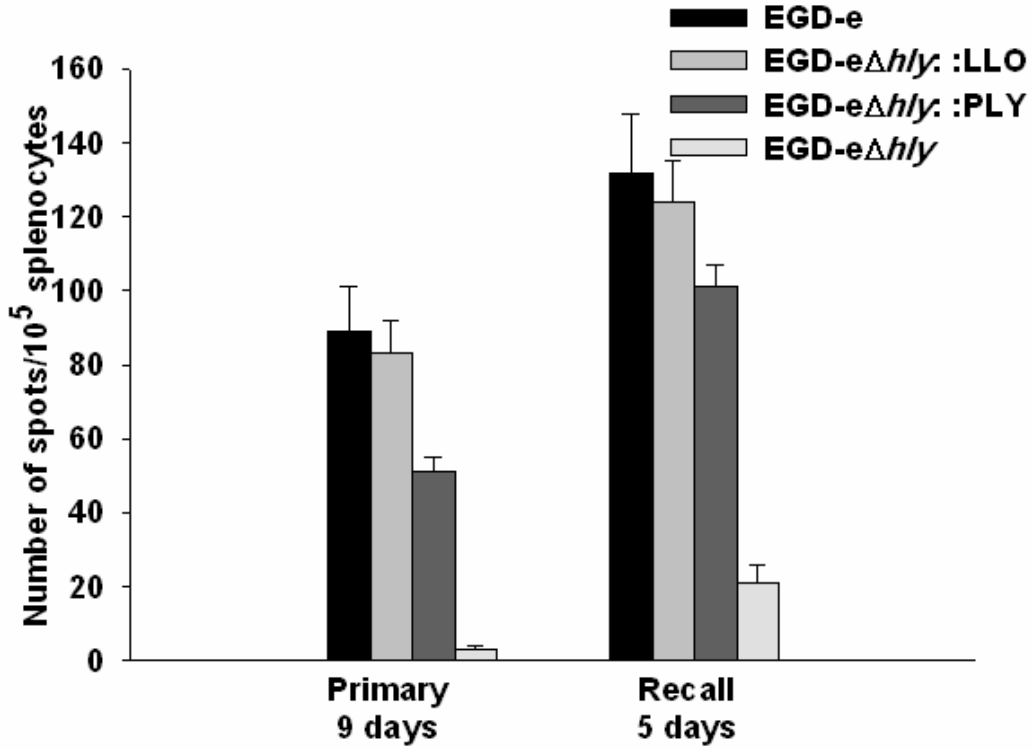

Fig. 3.31. Number of antigen-specific IFN-γ producing CD8+ T cells in the spleen of mice infected i.v. with *L.monocytogenes* or its mutants, determined by ELISPOT assay. Spleen cells from infected mice were isolated either at day 9 after the primary infection or day 5 after recall challenge infection and stimulated with the immunosubdominant MHC class I peptide $P60_{217-225}$ in triplicates in nitrocellulose based 96-well culture plates. Number of specific IFN-γ producing cells was determined by counting the number of spots under microscope.

challenge. These data reveal that pneumolysin can replace listeriolysin for induction of protective anti-listerial immunity developed against non-listeriolysin derived epitopes.

The induction of antilisterial protective immunity with PLY expressing *Listeria* was addressed by identifying the production of memory T cells in response to challenge infection. The magnitude of down regulation of CD62L T cell surface marker was quantitatively determined 2 months after the primary infection and 5 days after the recall infection. The expression of CD62L on the T cells was shown to be downregulated in response to primary *Listeria* infection and is upregulated after bacterial clearance (Busch and Pamer, 1999). However, during the recall infection with *L. monocytogenes*, CD62L was significantly down regulated. Day 5 post challenge was chosen because maximum spleen cellularity as well as

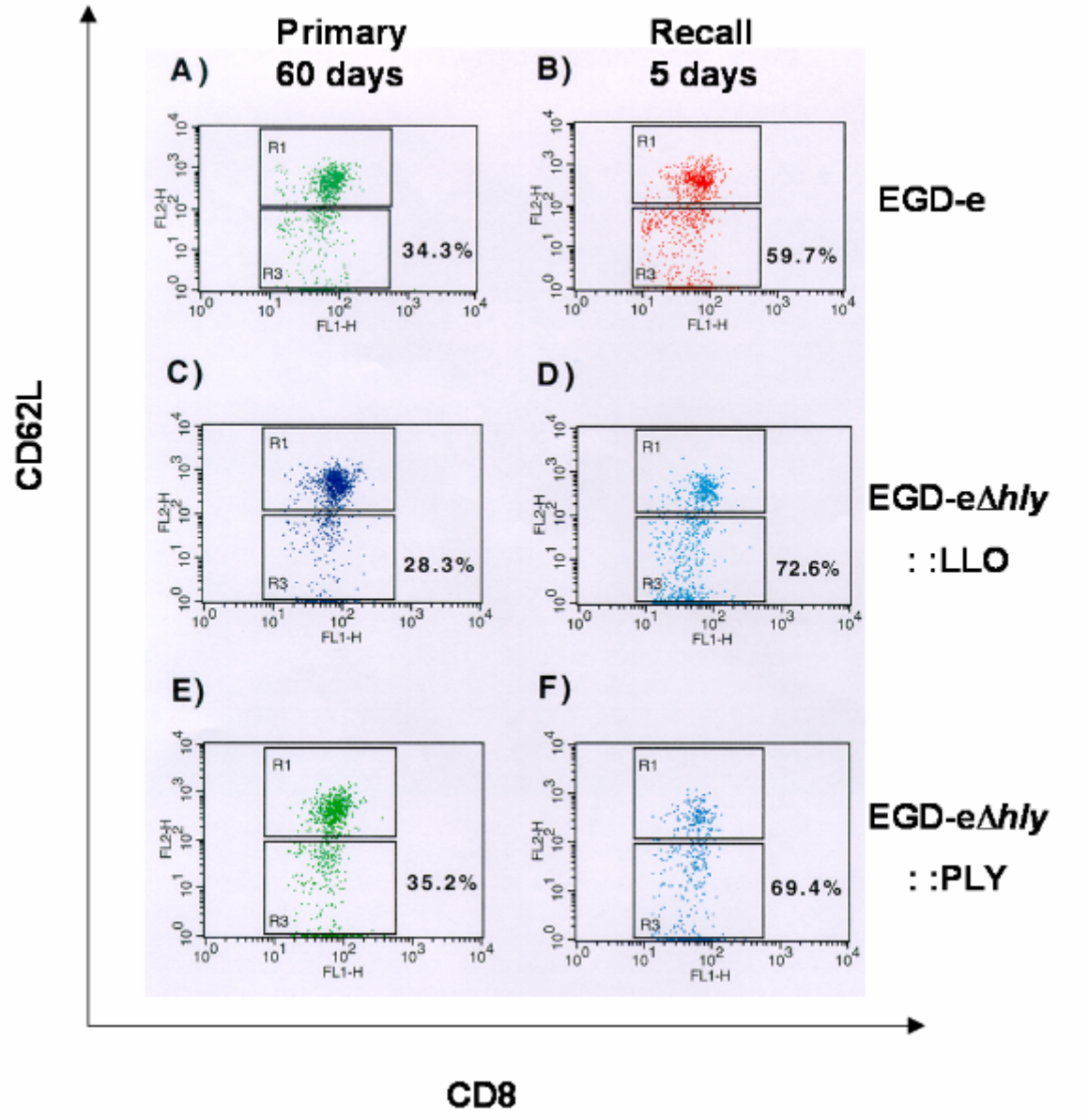

Fig. 3.32. Expression levels of CD62L on CD8+ splenocytes following primary and recall infection with *L. monocytogenes* strains. Flow cytometry was performed with spleen cells, isolated from mice 2 months after the primary infection or 5 days after the challenge. Cells were stained with FITC-labeled anti-Lyt-2 and biotinylated anti-CD62L, and the binding of anti-CD62L on the cell surface was detected with PE-conjugated streptavidin. Numbers shown are gated $CD8^{+}CD62L^{lo}$ T cells (lower panel) and analysed with CELL Quest software.

maximum number of CD8+ T cells was established on this day (Busch and Pamer, 1999). As shown in Fig. 3.32., challenge with the wild type *L. monocytogenes* managed to induce a significant down regulation of CD62L on CD8+ splenocytes in mice pre-immunized with PLY expressing EGD-eΔ*hly* strain similar to that induced in mice pre-immunized with the wild type *L. monocytogenes* as well as with EGD-eΔ*hly*: :LLO suggesting that CD8+ T

lymphocytes induced against non-listeriolysin determinants are able to confer anti-*Listeria* protective immunity.

3.5.7. Humoral response is induced against both listeriolysin O and pneumolysin

It was previously shown that Listeriolysin O and irpA are major protein targets of the human humoral response against *L. monocytogenes* (Grenningloh *et al*., 1997). So, it was important to examine if mice pre-immunized with either the wild type *L. monocytogenes* or its isogenic derivatives can produce antibodies against either listeriolysin O or pneumolysin. Blood was obtained from mice by heart puncture at day 8 post-infection, corresponding to the plateau phase of antibody response to antigen. Sera were separated from blood cells by ultracentrifugation at 15.000 rpm for 15 minutes. Purified listeriolysin O or pneumolysin

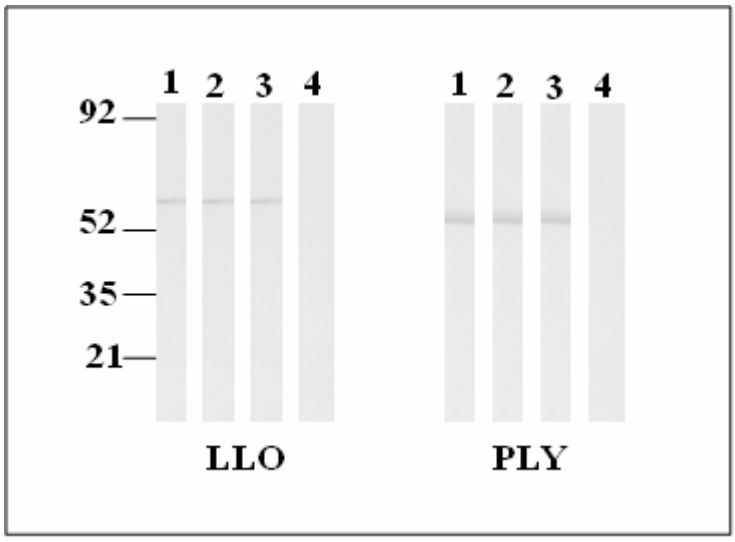

Fig. 3.33. Induction of humoral immunity against thiol-activated cytolysins. Equal amounts (1µg) of purified listeriolysin O (LLO) or pneumolysin (PLY) were loaded onto 10 % SDS-polyacrylamide gel. Gels were electrophoretically blotted and incubated with antisera obtained from mice 9 days after infection with 10^3 wild type *L. monocytogenes* EGD-e (Lane 1), $8x10^6$ isogenic *L. monocytogenes* mutant strains EGD-eΔ*hly* expressing either LLO (Lane 2) or PLY (Lane 3), and $8x10^6$ mutant *L. monocytogenes* EGD-eΔ*hly* strains (Lane 4) followed by AP-conjugated anti-mouse IgG. Blots were visualized using BCIP as a substrate. The numbers to the left of the figure correspond to apparent molecular weights (in kDa).

(1µg/serum tested) were used as antigens in immunoblots and incubated with mice sera. Surprisingly, Sera from mice pre-immunized with the wild type EGD-e as well as the isogenic mutant strain complemented with either listeriolysin O or pneumolysin have strongly recognized the two cytolysins while those isolated from mice pre-immunized with the

isogenic EGD-eΔ*hly* mutant strain failed to detect the two toxins as shown in Fig. 3.33. The cross reactivity of monoclonal antibodies as well as natural antibodies against both listeriolysin O and pneumolysin was suggested to be due to the structural similarities between the two toxins. So, pneumolysin expressing EGD-eΔ*hly* mutant strain acts as a target for both antilisterial cellular and humoral immune responses.

3.6. The PEST-like region in Listeriolysin O is critical for induction of effective long-term immunity

Listeriolysin O (LLO), a major virulence factor secreted by the bacterial pathogen *Listeria monocytogenes,* contains near to its N-terminus a putative PEST-like sequence (P, Pro; E, Glu; S, Ser; T, Thr) which was recently identified to be essential for the virulence and intracellular compartmentalization. The first study concerning the role of PEST like motif in the pathogenicity of *Listeria monocytogenes* was conducted with Decatur *et al*, and concluded that the PEST like motif may target eukaryotic proteins for phosphorylation and/or degradation by the proteasome, and may generally represent sites of protein-protein interactions (Decatur and Portnoy, 2000) although the actual mechanism by which the PEST-like sequence acts to negate LLO toxicity was not clear. The critical role of this sequense in mediating escape from the host cell phagolysosome was recently established (Lety *et al*., 2001; 2002).

Listeria monocytogenes has been used for decades for the induction and analysis of T cell-mediated immunity (Mackaness, 1962; Mielke *et al*., 1998). Among the listerial virulence factors, Listeriolysin O is critical for generating a protective anti-listerial T lymphocyte response (Dunn and North, 1991). In an attempt to analyze if the PEST like sequence lacking listeriolysin O can induce a T cell-mediated immunity, 28 amino acid residues at the N-terminus of listeriolysin O harbouring the PEST like sequence was deleted and the growth kinetics of the isogenic *Listeria* mutant lacking the PEST-like sequence as well as its immunological properties were systemically examined.

3.6.1. Generation of the LLO mutant protein

3.6.1.1. Bacteria

Bacterial strains used in this study are wild type *Listeria monocytogenes* EGD-e serotype 1/2a (Glaser *et al*., 2001), its isogenic deletion mutant EGD-eΔ*hly* (Leimeister-Wächter *et al*., 1990; Guzman *et al*., 1995), both were electroporatically transformed with pSOG304 gram-positive/gram-negative schuttle vector (Otten *et al*., unpublished), and its isogenic derivatives EGD-eΔ*hly*: :pSOG304-*hly* (designated EGD-eΔ*hly*: :LLOwt), and EGD-eΔ*hly*: :pSOG304-*hly*ΔPEST(designated EGD-eΔ*hly*: :LLOΔPEST). Bacteria were grown in brain-heart infusion (BHI) broth in presence of 5μg/ml erythromycin. In all *in vitro* and *in vivo* experiments, fresh cultures of bacteria, prepared from an overnight culture, were used.

3.6.1.2. Construction of the mutants

For the construction of EGD-eΔ*hly* strains complemented with either intact *hly* gene or *hly* gene lacking the PEST-like sequence, pSOG304 plasmid was used.

3.6.1.2.1. EGDeΔ*hly*::pSOG304-*hly*ΔPEST

A 28 amino acids region (a`a` 26 - a`a` 53) harbouring the PEST-like sequence at the N-terminus of Listeriolysin O was removed using a method of site-directed mutagenesis called gene splicing by overlap extension (Ho *et al*., 1989). A 309 bp long PCR fragment comprising PEST-like region upstream sequences was amplified with (*hly*-P for., *KpnI)* and (PEST rev., *Nde*I) oligonucleotides primers. A second 1595 bp PCR fragment harboring PEST-like region downstream sequences was amplified with (PEST for., *Nde*I) and (*hly*ΔPEST rev., *Xho*I) (see section 2.7.11). Both fragments were restricted with endonuclease *Nde*I and used in a ligation reaction. So, the deleted sequence was substituted with a *Nde*I restriction site encoding a (h) and (m) residues (Fig. 3.34.B). The entire PCR fragment, lacking the PEST-like region , was amplified with oligonucleotides containing *Kpn*I and *Xho*I restriction sites, restricted with *KpnI* and *XhoI* restriction enzymes and was cloned into the *Kpn*I and *Xho*I restriction sites of the pSOG304 plasmid vector (Fig. 3.34.A). Both constructions were checked by DNA sequence analysis.

3.6.1.2.2. EGD-eΔ*hly*: :pSOG304-*hly*

EGD-eΔ*hly* expressing the intact wild type listeriolysin O was used in all experiments as a positive control. By means of (*hly*-gene for., *Xho*I) and (*hly*-gene rev., *Spe*I) oligonucleotides primers (see section 2.7.11), a 2086 bp long fragment containing entire *hly* gene and its promoter was amplified from *Listeria monocytogenes* chromosomal DNA using high fidelity PCR procedure followed by cloning on to pSOG304 plasmid using *Xho*I and *Spe*I restriction sites (Fig. 3.25.B).

Transformation of EGD-eΔ*hly* strains with pSOG304 plasmid containing intact *hly* gene or *hly* lacking PEST-like sequence was performed by electroporation (Schäferkordt and Chakraborty, 1995). The desired recombinants were selected using erythromycin (5µg/ml) on BHI agar plates and subsequently verified by sequencing and western blot analysis.

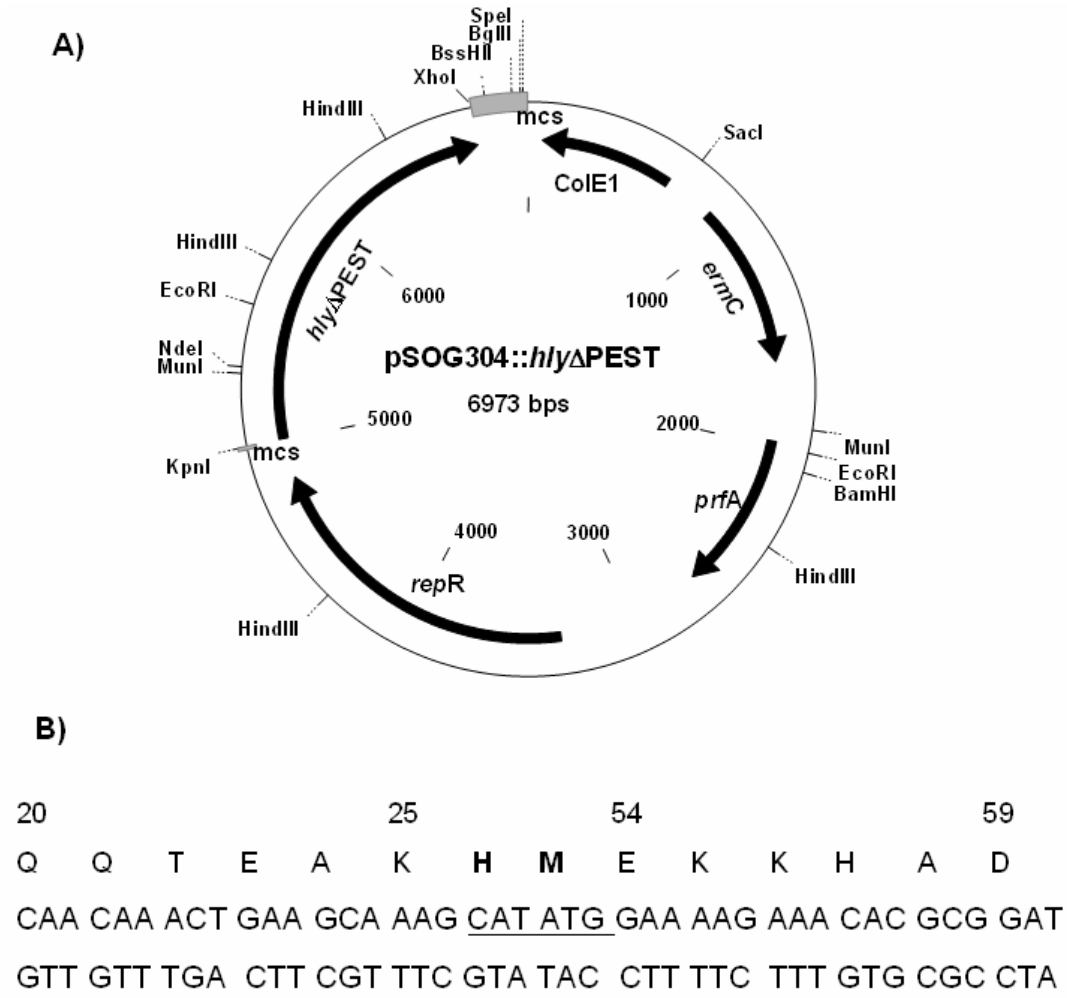

Fig. 3.34. A) Cloning of the PEST-knock out listeriolysin O in pSOG304. The structural gene encoding listeriolysin O lacking the PEST-like sequence with *Kpn*I and *Xho*I restricted ends was cloned in the multiple cloning site (mcs) of pSOG304 restricted with the same enzymes. **B)** Sequence of the mutated protein. DNA and amino acid sequences of the LLOΔPEST mutant. The created *Nde*I site is underlined, and the corresponding residues are in bold. The numbers above the sequences refer to the amino acid position.

3.6.2. Expression and hemolytic activity of the LLO mutant protein

The LLO mutant protein was expressed and efficiently secreted into the culture supernatant of EGD-eΔ*hly*, as shown by western blot using monoclonal anti-LLO antibody (M275) (Fig. 3.35.A). The PEST-like sequence lacking LLO was normally recognized by the monoclonal antibody which appeared to react more strongly with LLOwt than with the mutant protein. Moreover, several additional bands of lower molecular weight were clearly detected with LLOwt but poorly recognized with the mutant protein suggesting more instability of LLOwt than of the mutant protein.

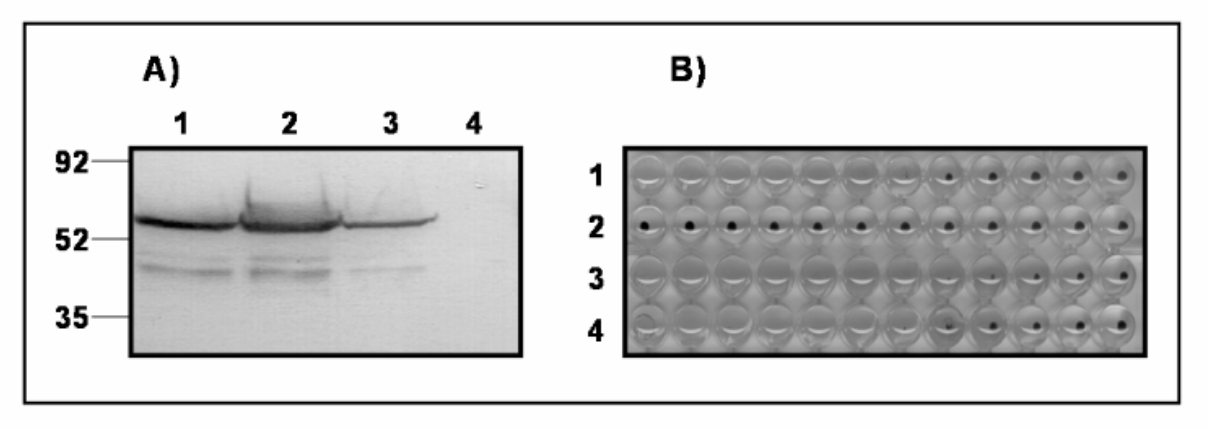

Fig. 3.35. A) Immunoblot analysis. Equal amounts of precipitated proteins from culture supernatants of *L. monocytogenes* EGD-e (Lane 1), the isogenic deletion mutant *L. monocytogenes* EGD-eΔ*hly* expressing either LLOwt (Lane 2) or LLOΔPEST (Lane 3), and EGD-eΔ*hly* (Lane 4) were loaded onto 10 % SDS-polyacrylamide gel. Proteins were electrophoretically blotted and developed with mouse anti-LLO monoclonal antibody (M275) followed by AP-conjugated anti-mouse IgG. The numbers to the left of the figure correspond to apparent molecular weights (in kDa). **B)** Hemolytic activity of the truncated protein. Serial two-fold dilutions of each culture supernatant from *L. monocytogenes* EGD-e (Lane 1), the isogenic deletion mutant *L.monocytogenes* EGD-eΔ*hly* (Lane 2), and EGD-eΔ*hly* expressing either LLOwt (Lane 3) or LLOΔPEST (Lane 4) (starting from 50 µl of non-diluted sample) were incubated with defibrinated sheep erythrocytes at pH 5.6 and 37°C for 2 hours. Cytolysis of erythrocytes is visualized by a uniform grey colour of the well (corresponding to haemoglobin release). A dark spot at the bottom of the well corresponds to sedimented intact erythrocytes.

The regulated expression of the cloned gene products was examined by assaying the hemolytic activity of bacterial culture supernatants on sheep erythrocytes at pH 5.6. As expected, EGD-eΔ*hly* had no detectable hemolytic activity. The mutant protein show a hemolytic activity appears to be identical to that of LLOwt (Fig. 3.35.B).

3.6.3. Deletion of the PEST-like sequence of LLO inhibits bacterial phagosomal escape

To understand the role of PEST-like sequence in mediating survival of *L. monocytogenes in vivo*, the interaction of mutant *L. monocytogenes* lacking the PEST-like sequence with J774 macrophages was examined *in vitro*. It has been previously shown that EGD-eΔ*hly* is unable to escape from the phagolysosome of J774 macrophages and therefore does not replicate in this cell line (Decatur and Portnoy, 2000, Lety *et al*., 2001; 2002). During the first 6 hours of intracellular growth, the wild type EGD-e as well as the mutant strain EGD-eΔ*hly* complemented with LLOwt doubled approximately 5 times (Fig. 3.36.A), whereas the number

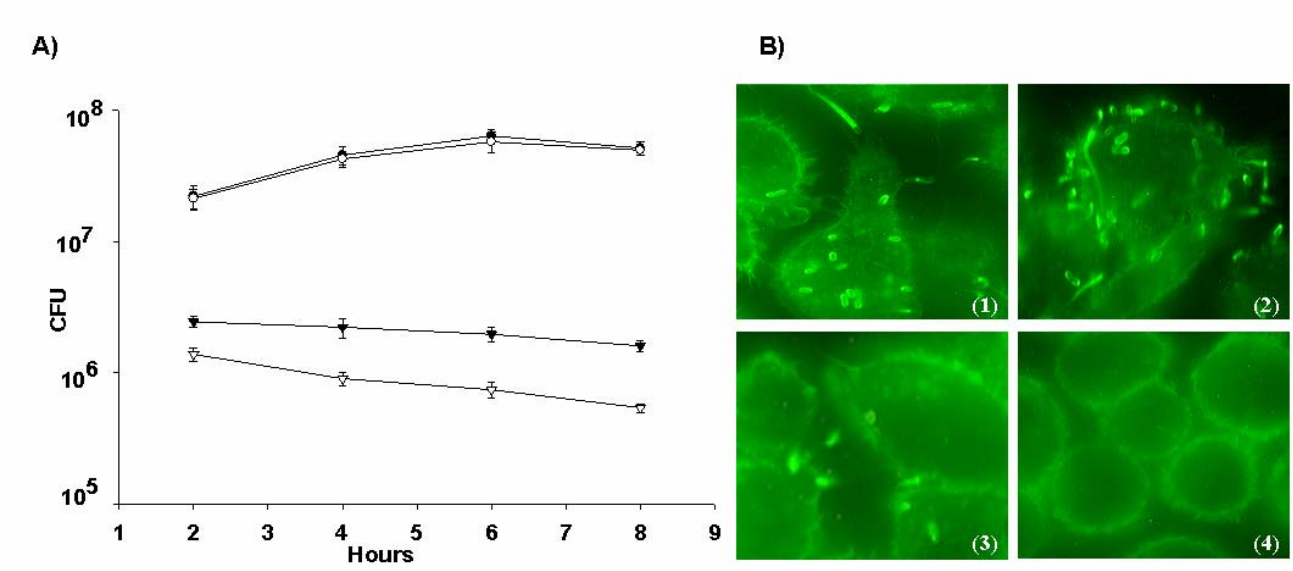

Fig. 3.36. Intracellular growth kinetics in eukaryotic cells. **A)** macrophage-like J774 cell line was infected with the wild type *L. monocytogenes* EGD-e (filled circles), mutant *L. monocytogenes* EGD-eΔ*hly* expressing either LLOwt (open circles) or LLOΔPEST (closed triangles), and mutant *L. monocytogenes* EGD-eΔ*hly* strains (open triangles) at MOI of 10. Viable bacteria were enumerated at indicated time intervals following incubation at 37°C. **B)** macrophage-like J774 cell line was infected with the wild type *L. monocytogenes* EGD-e (1), mutant *L. monocytogenes* EGD-eΔ*hly* expressing either LLOwt (2) or LLOΔPEST (3), and mutant *L. monocytogenes* EGD-eΔ*hly* strains (4). 4 hours after gentamicin treatment, cells were fixed with 4% formaldehyde and visualised by staining F-actin using Oregon Green 488 conjugated phalloidin under a fluorescence microscope.

of both mutant strain lacking the PEST-like sequence and EGD-eΔ*hly* neither increased nor severely decreased during the 8 hours infection path although the number of colony forming units of the former remained slightly higher than that of the latter suggesting that both strains remained trapped, but viable, in phagosomes of the J774 macrophages. The infected macrophages were examined 4 hours post-infection by flourescent microscopy after staining with beta-phalloidin to visualize the F-actin. The wild type *L. monocytogenes* as well as mutant bacteria expressing wild type LLO had multiplied and were found associated with tails of polymerized actin, propelling bacteria to adjacent cells (Fig. 3.36.B), whereas bacteria expressing LLOΔPEST showed a severely reduced replication with very low capacity to polymerize actin in most of the infected cells. As expected, the EGD-eΔ*hly* strain was unable to multiply or to polymerize actin. These data reflects the importance of PEST-like sequence in mediating disruption of the phagosomal membrane and bacterial intracellular multiplication.

3.6.4. PEST-like sequence mediates survival of *L. monocytogenes in vivo*

The first and crucial step in the *in vivo* characterization of mutants was the determination of their growth kinetics in spleen and liver. The presence of viable microorganisms during the early phase of infection is critical in the induction and establishment of cell-mediated immunity (Darji *et al*., 2003). Therefore, the virulence of EGD-eΔ*hly* transformants

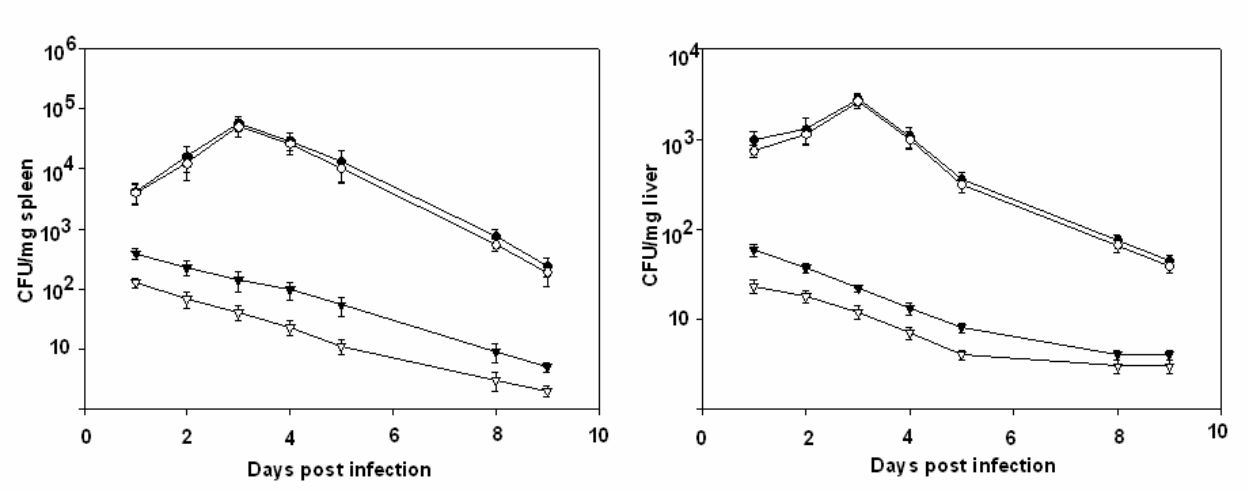

Fig. 3.37. *In vivo* kinetics of primary infection in mice with the wild type *L. monocytogenes* and its isogenic mutants. Mice were infected i.v. with either 10^3 wild type *L. monocytogenes* EGD-e (filled circles) or 10^6 isogenic *L. monocytogenes* mutant strains EGD-eΔ*hly* expressing either LLOwt (open circles) or LLOΔPEST (closed triangles), and mutant *L. monocytogenes* EGD-eΔ*hly* strains (open triangles). On days 1,2,3,4,5,8,and 9 after infection, the numbers of viable bacteria in spleens and livers of three animals per group were determined. Data presented are representative of three independent experiments.

expressing LLOwt and LLOΔPEST was compared with that of wild type *L. monocytogenes* and EGD-eΔ*hly*. A group of BALB/c mice were intravenously (i.v) injected with sublethal doses of either wild type EGD-e strain(10^3), or the isogenic *L. monocytogenes* derivatives EGD-eΔ*hly* , EGD-eΔ*hly*: :LLOwt, and EGD-eΔ*hly*: :LLOΔPEST (10^6). *In vivo* survival and growth kinetics of bacteria were followed by determining the number of bacteria in spleens and livers of infected mice at various time points for several days. As shown previously, regardless of the dose of infection, the mutant EGD-eΔ*hly* is rapidly cleared from both organs from the outset (Lety *et al*., 2001; Peters *et al*., 2003). On day 3 post-infection, bacterial load had already fallen to a level of approximately 40 cfu/mg of spleen and 10 cfu/mg of the liver even after infection with 10^6 viable bacteria per mouse (Fig. 3.37). Although the PEST-like sequence lacking strain showed a slightly more survival than EGD-eΔ*hly* in spleen and

liver of infected mice, bacterial counts remains very low up the first day after infection and dropped more after day 2 onwards. Survival was fully restored in EGD-eΔ*hly* complemented with plasmid-encoded LLOwt to an extent identical to that of the wild type *L. monocytogenes* where the number increased between day 1 and day3. On day 3, the number of the wild type EGD-e and EGD-eΔ*hly*+LLOwt peaked at the same level and declined rapidly from day 5 post-infection onwards. In all cases, both in the wild type EGD-e and in its isogenic mutant strains, bacteria were cleared from spleens and livers by day 8-10 post-infection.

3.6.5. Spleen morphology on day 3 after infection

Because the spleen is the lymphatic organ where the immune response is initiated following an i.v. inoculum, morphological alteration in the spleens on day 3 after i.v. infection with the wild type EGD-e and its isogenic derivatives was monitored. As shown in Fig. 3.38., marked differences were observed in the morphological structure of spleens obtained from mice infected with these strains. Spleens obtained from mice infected with the wild type EGD-e strain showed a pronounced strong granuloma formation on day 3 post-infection resulted from intense and vigorous leukocyte infiltration of the white pulp, whereas

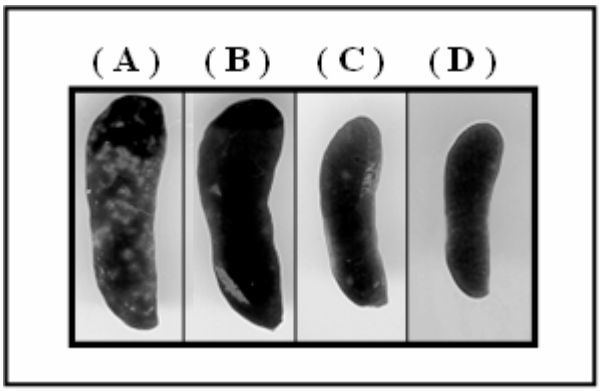

Fig. 3.38. Morphological changes in spleens of mice infected with *Listeria* strains. Spleens of mice (infected i.v. as mentioned in Fig. 3.37) were removed on day 3 after primary infection. Shown is the scanned picture of spleens from the wild type *L. monocytogenes* EGD-e (A), mutant *L. monocytogenes* EGD-eΔ*hly* expressing either LLOwt (B) or LLOΔPEST (C), and mutant *L. monocytogenes* EGD-eΔ*hly* strains (D).Vigourous monocytic infiltration and intense granulomatous lesions are detectable only in the spleens isolated from mice infected with the wild type *L.monocytogenes*(A) while splenomegaly was detected in the spleens isolated from mice infected with mutant *L. monocytogenes* EGD-eΔ*hly* expressing LLOwt (B). No morphological alterations were detected in spleens of mice infected with mutant *L. monocytogenes* EGD-eΔ*hly* expressing LLOΔPEST (C) or those infected with the isogenic mutant *L. monocytogenes* EGD-eΔ*hly* (D).

EGD-eΔ*hly*: :LLOwt displayed a splenomegaly without granuloma. Infection with both EGD-eΔ*hly* and EGD-eΔ*hly*: :LLOΔPEST did not result in morphological changes on day 3 post infection.

3.6.6. PEST-like sequence truncated Listeriolysin O fails to induce IFN-γ either in serum or by splenocytes during primary infection

It was previously reported that IFN-γ produced at the first stage of infection with *L. monocytogenes* is critical for the generation of host resistance (Xiong *et al*., 1994). The induction of IFN-γ was, therefore, measured after infection with wild type and its isogenic derivatives. Mice were i.v. injected with (10^3) cfu of *L. monocytogenes* or (10^6) cfu of its

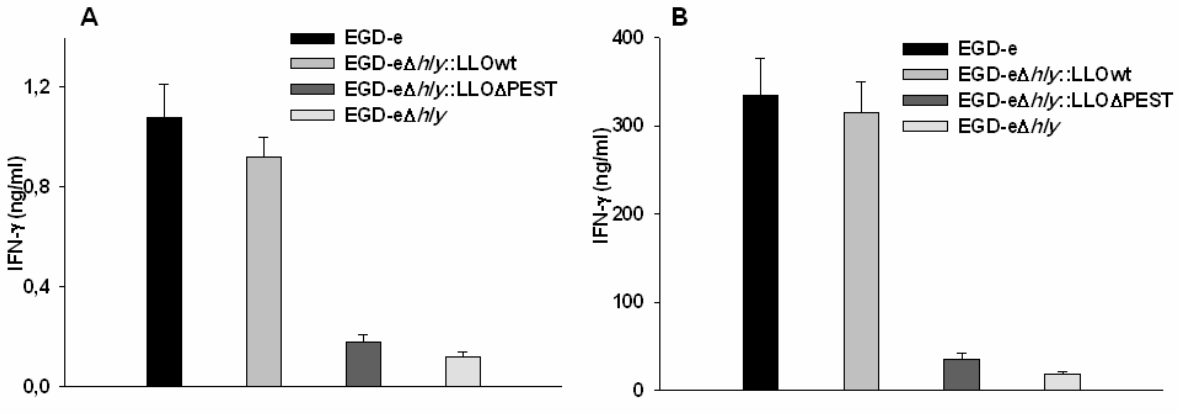

Fig. 3.39. A) IFN-γ concentration in the serum of mice infected with *Listeria* strains. Mice were infected with 10^3 CFU of *L. monocytogenes* EGD-e or 10^6 CFU of the various *Listeria* mutants. On day 2, animals were killed and blood was obtained by heart puncture. Serum concentration of IFN-γ was determined by ELISA. **B)** *Listeria*-induced IFN-γ production by spleen cells 9 days after infection. Mice were infected with 10^3 CFU of *L. monocytogenes* EGD-e or with 10^6 CFU of various *Listeria* mutants. On day 9 after infection, mice were killed and spleens removed. Single cell suspensions were stimulated *in vitro* with secreted soluble *Listeria* antigens to produce IFN-γ. After 48 hours, culture supernatants were tested for presence of IFN-γ by ELISA.

isogenic derivatives, and IFN-γ concentration was measured in serum samples on day 2 post-infection. As shown in Fig. 3.39.A, infection with the wild type and EGD-eΔ*hly*: :LLOwt resulted in high serum levels of IFN-γ while a significantly low amount of IFN-γ was detected in sera of mice infected with either EGD-eΔ*hly* or the ΔPEST strain. In addition, the IFN-γ level *in vitro* in culture supernatants of spleen cells isolated from the same groups mice on day 9 post-infection was measured. Only the wild type strain and the mutant EGD-eΔ*hly*

complemented with LLOwt induced detectable levels of IFN-γ whereas EGD-eΔ*hly* and ΔPEST strains failed to induce production of IFN-γ (Fig. 3.39.B).

3.6.7. Expression of acquired immunity by the wild type *L. monocytogenes* and its isogenic derivatives

It was established that sub-lethal primary infection with the wild type *L. monocytogenes* was controlled by mice and resulted in the induction of protective immunity to further infections (Darji *et al*., 2003). Such an acquired protective immunity is absolutely mediated by cytotoxic CD8+ T lymphocytes. It was therefore noteworthy to investigate whether the induction of protective immunity is affected with absence of the PEST-like sequence from

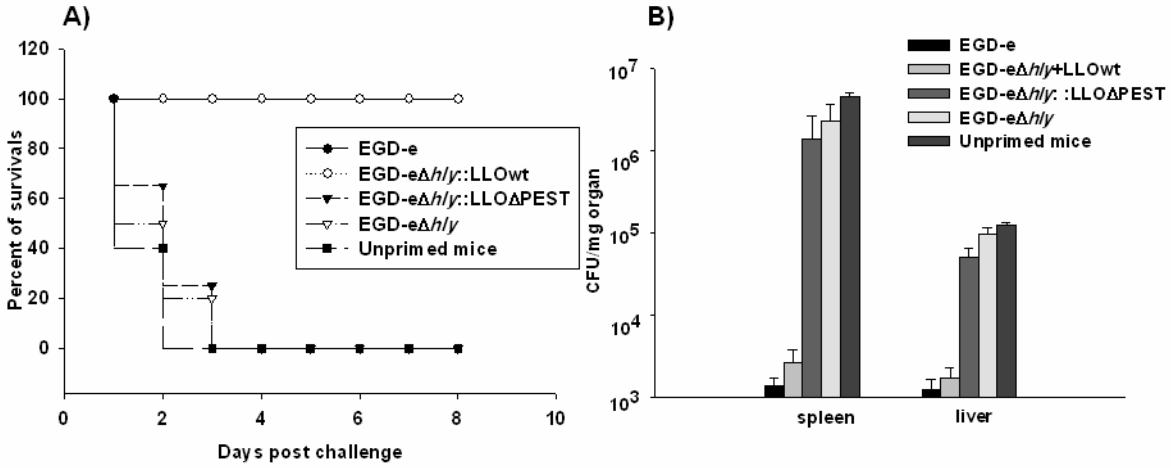

Fig. 3.40. Primary infection with ΔPEST mutant *Listeria* strain fails to induce a protective immunity against a lethal wild type *L. monocytogenes* infection. **(A)** Mice were infected i.v. with either 10^3 wild type *L. monocytogenes* EGD-e or 10^6 isogenic *L. monocytogenes* mutant strains EGD-eΔ*hly* expressing either LLOwt or LLOΔPEST, and mutant *L. monocytogenes* EGD-eΔ*hly* strain. Two months later all mice were challenged with a lethal dose (10X LD_{50}) of the wild type *L. monocytogenes*. As a control, a group of uninfected normal mice was included. Survival of mice after the challenge was monitored up to 8 days. Data were pooled from two independent experiments with a total of 15 mice per group. **(B)** Bacterial titer in spleens and livers of 3 mice per group of mice challenged in Fig. A were monitored at day 2 after challenge.

Listeriolysin O. Groups of BALB/C mice were infected i.v. with the wild type strain(10^3) or its isogenic derivatives (10^6). 2 months later, all mice were challenged with a lethal i.v. dose ($5x10^4$), corresponding to 10X LD_{50} of the wild type *L. monocytogenes*, and survival was monitored. As a control, a group of non-immunized normal BALB/C mice that have received a similar lethal dose of the wild type *L. monocytogenes* were included. As shown in Fig. 3.40.A, all the non-immunized mice died in 3 days as well as those pre-immunized with

mutant strains EGD-eΔ*hly* and EGD-eΔ*hly*: :LLOΔPEST were dead in 4 days after the challenge infection with *L. monocytogenes* whereas those pre-immunized with either the wild type or EGD-eΔ*hly* complemented with LLOwt were protected against a lethal *Listeria* infection and survived. In addition, the bacterial titer was determined in spleens and livers at day 2 post-challenge in each mice group. Mice primed with mutant strains EGD-eΔ*hly* and EGD-eΔ*hly*: :LLOΔPEST as well as naïve unprimed mice were not able to control challenge infection as indicated by the high bacterial load in spleens and livers. In contrast, mice primed with either the wild type or the mutant strain EGD-eΔ*hly* complemented with LLOwt managed to restrict listerial growth in the first 2 days after challenge infection (Fig. 3.40.B). Hence, absence PEST-like sequence abrogates the induction of a protective *Listeria*-specific T cell response.

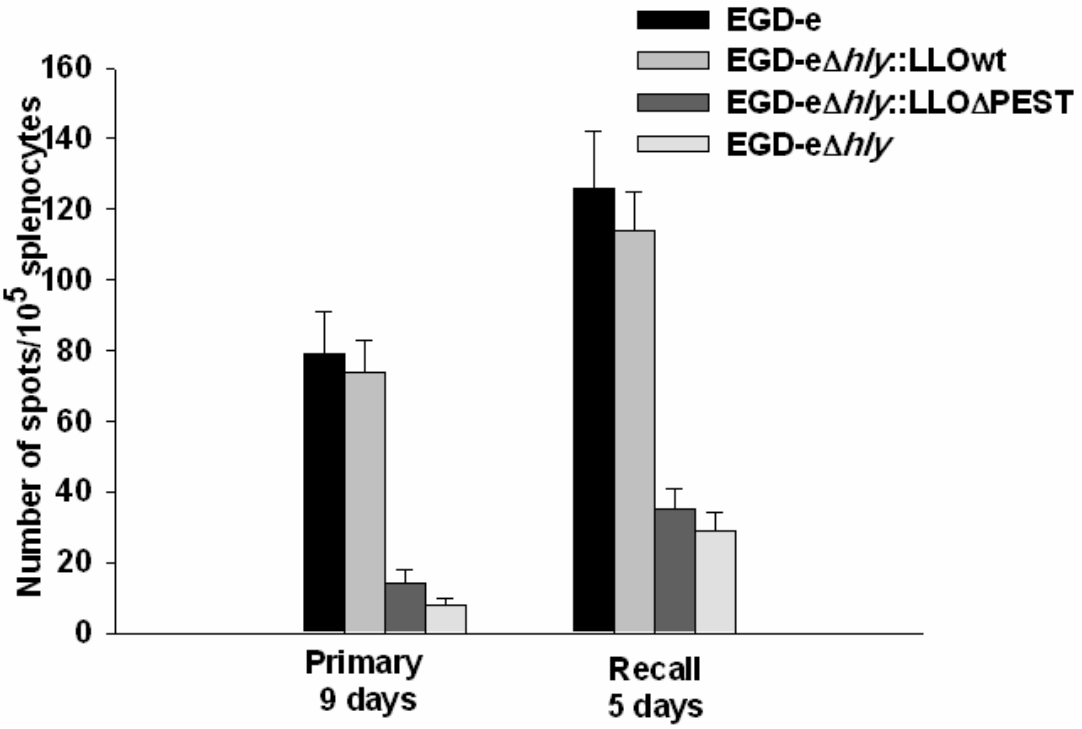

Fig. 3.41. Number of antigen-specific IFN-γ producing $CD8^+$ T cells in the spleen of mice infected i.v. with the wild type *L.monocytogenes* and its mutant strains, determined by ELISPOT assay. Spleen cells from infected mice were isolated either at day 9 after the primary infection or day 5 after recall challenge infection and stimulated with immunodominant MHC class I peptide LLO_{91-99} in triplicates in nitrocellulose based 96-well culture plates. Number of specific IFN-γ producing cells were determined by counting the number of spots under microscope.

To obtain information on the nature of the inability of mutant strain lacking PEST-like sequence to confer a protective immunity upon re-infection, the amount of antigen specific

MHC class I restricted effector CD8+ T cells induced in mice spleens 9 days after the primary infection and 5 days after the challenge with a sublethal dose ($2x10^3$) of *L. monocytogenes* was quantitatively determined through evaluating the number of IFN-γ producing CD8+ T cells induced against the dominant H-$2K^d$ restricted LLO_{91-99} epitope (Wipke *et al*., 1993) in an *in vitro* ELISPOT assay. As shown in Fig. 3.41., infection with the wild type *Listeria monocytogenes* as well as the mutant complemented with LLOwt managed to induce a highly detectable population of LLO_{91-99} specific CD8+ T-cells. This number raised significantly during the recall infection. On the other hand, Infection with both mutant strains EGD-eΔ*hly* and EGD-eΔ*hly*: :LLOΔPEST failed to induce a significant level of CD8+ T-cells population either primarily or after challenge. So, the lack of PEST-like sequence seems to influence the antigen presentation efficiency through the MHC class I pathway due to the inability of PEST-like sequence lacking strain to gain access to the cytosole of the infected cell and to undergo further processing and antigen presentation.

It was recently established that the pathogen specific T lymphocytes are programmed during the first day of infection and subsequently undergo proliferation and differentiation into effector T cells without further calibration by the progressing inflammatory response but the magnitude of the LLO_{91-99}-specific response correlates with the duration of *in vivo* bacterial growth during the first 24 hours after infection (Mercado *et al*., 2000).

To correlate the *in vivo* growth kinetics of bacteria in spleens of infected mice with the magnitude of memory T cell response, the expression of CD62L (L-selectin) on CD8+ T cells, as an effector memory T cell marker, 2 months after the primary infection and 5 days after the recall infection has been quantified. CD62L is expressed at high levels on the surface of naïve T lymphocytes, but its expression is rapidly downregulated upon T cell activation (Bush and Pamer, 1999). 2 months after the primary infection, all groups of mice showed approximately 19-22 % of $CD8^+CD62L^{lo}$ expressing T cell population which is relatively similar to that expressed in naïve uninfected mice (Mercado *et al*., 2000). During the recall infection however the $CD8^+CD62^{lo}$ T cell population showed more than three folds increment in mice pre-immunized either with the wild type or with EGD-eΔ*hly*: :LLOwt whereas pre-immunization with EGD-eΔ*hly* or EGD-eΔ*hly*: :LLOΔPEST fails to induce CD62L downregulation on the surface of T-lymphocytes (Fig. 3.42.) as indicated by the slight increase in the percent of $CD8^+CD62^{lo}$ T cells during recall infection suggesting that the restricted *in vivo* growth and intracellular replication of EGD-eΔ*hly*: :LLOΔPEST as well as EGD-eΔhly inhibits the capability of naïve T cells to come into contact with APCs in order to elicit a full T cell response.

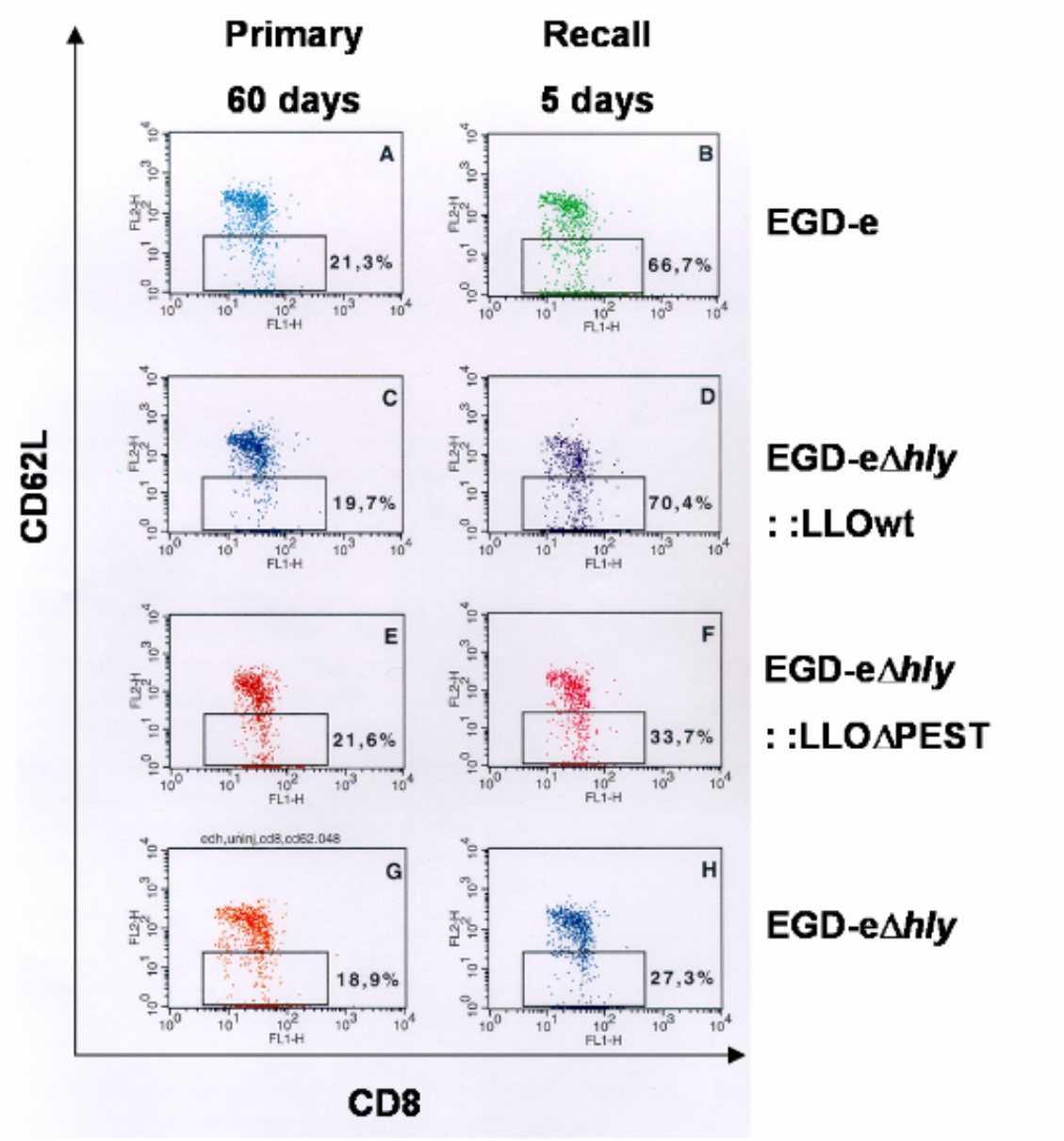

Fig. 3.42. Quantification of CD62L expression on CD8+ splenocytes following primary and recall infection with *L. monocytogenes* and its mutant strains. Flow cytometry was performed with spleen cells, isolated from mice two months after the primary infection or day 5 after the challenge. Cells were stained with FITC-labeled anti-Lyt-2 and biotinylated anti-CD62L, and the binding of anti-CD62L on the cell surface was detected with PE-conjugated streptavidin. Numbers shown are gated $CD8^{+}$ $CD62^{lo}$ T cells analysed with CELLQuest software.

4. Discussion

4.1. Antibody responses are important in defense during infection with intracellular bacteria

The view that antibody-mediated immunity against many prokaryotic and eukaryotic intracellular pathogens is not important was popular until recently. The concept of a division whereby antibody-mediated immunity protected against extracellular pathogens and cell-mediated immunity protected against intracellular pathogens may have had its intellectual origins in the great debate between the advocates of humoral and cellular immunity at the turn of the 20th century. The humoralists viewed immunity as being conferred by soluble substances in the blood and the generation of an effective antibody response, with phagocytic cells functioning primarily to clean up microbial debris while the cellularists viewed immunity as being conferred by macrophages and other phagocytic cells, with the role of humoral factors being to provide opsonins (Silverstein, 1979; Casadevall, 1998). This debate was fuelled by the success and difficulties associated with demonstrating antibody-mediated protection against certain pathogens in passive immunization studies. Administration of immune serum protected against toxin-mediated diseases such as tetanus and diphtheria and a certain subset of extracellular bacterial pathogens like *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* (Casadevall and Scharff, 1994). A recent study showed that monoclonal antibody raised against PLY protected mice against pneumococcal pneumonia (Garcia-Suarez *et al*., 2004). However, passive immunization provided little or no protection against intracellular microbes such as *Mycobacterium* tuberculosis (Glatman-Freedman and Casadevall, 1998) and *Listeria monocytogenes* (Osebold and Sawyer, 1957; Mackaness, 1962; Miki and Mackaness, 1964).

The fact that a microbe inside a cell is separated from serum antibody has contributed to the belief that serum antibody cannot be effective against an intracellular pathogen. However, a recent surprising finding concluded that a neutralizing mAb to LLO provided resistance to *Listeria* infection (Edelson *et al*., 1999). Antibody-mediated protection was evident very early (within 6 hr) after infection and occurred in SCID mice, indicating that the effects of antibody were independent of the adaptive immune response. The authors reasoned that anti-LLO antibody-mediated protection was occurring through intracellular neutralization of LLO within phagocytic cells which would block *Listeria* escape to the host cell cytosol and thereby block the rapid bacterial replication which takes place at this site (Edelson and Unanue, 2001).

Indeed, both LLO (Low *et al*., 1992) and PLY (Cima-Cabal *et al*., 1999) were found as major targets of serum antibodies in listeriosis or pneumococcal pneumonia patients. In addition, due to their potential role in bacterial virulence as well as in immunoregulation against their corresponding pathogens, it was noteworthy to purify these toxins to facilitate understanding their role in bacterial pathogenicity and immune responses elicited against them.

In this study an improved production and a conventional column method for the bulk purification of pure cytolysins was reported. Listeriolysin O as well as pneumolysin were currently isolated from the supernatant of pathogenic *L. monocytogenes* and *S. pneumonia* strains respectively. There are 2 basic problems associated with this; large quantities of the pathogen have to be cultivated, restricting large scale purification of the toxin to designated facilities (Walton *et al*., 1999). Previous attempts to purify pneumolysin depended on its passive release in culture supernatants as a result of autolysis phenomena, since pneumolysin is an intracytoplasmic protein lacking a leader peptide to be exported to the extracellular medium, giving rise to a poor yield (Kanclerski and Mollby, 1987). Furthermore, the pathogenic *L. monocytogenes* and *S. pneumonia* strains secret a large number of additional proteins into its supernatant fluids including proteases (Courtney, 1991, Domann *et al*., 1993). To circumvents these problems, either listeriolysin O or pneumolysin gene was expressed in a non-pathogenic strain, *Listeria innocua*, a species found to lack all known virulence factors present in pathogenic *L. monocytogenes* (Leimeister-Wächter and Chakraborty, 1989). By including the positive regulator of listeriolysin production, the *prf*A gene, on the same plasmid it was possible to express large quantities of listeriolysin O in the culture supernatants of recombinant *Listeria innocua* strains. This 27 kDa cytoplasmic protein is a positively acting transcriptional activator required for all known listerial virulence genes (Leimeister-Wächter *et al*., 1990). In addition, by introducing both promoter and signal peptide of listeriolysin O gene under the regulatory control of *prf*A, expression of pneumolysin as well as its active export to the extracellular medium was facilitated.

In all previous reports concerning of LLO purification, *Listeria* was grown in an enriched Brain Heart Infusion (BHI) medium for production of LLO. In addition to containing a high amounts of proteins that may interfere with efficiency of purification process, expression of virulence genes of *L. monocytogenes*, including LLO, are not induced in BHI but highly up regulated upon shift of *L. monocytogenes* from enriched medium to minimal medium (Sokolovic *et al*., 1993; Bohne *et al*., 1994; Klarsfeld *et al*., 1994) which has also the advantage of containing the minimum amount of amino acids required for bacterial growth,

thus enhancing both quantity and quality of purified LLO. Indeed, hemolytic activity of both toxins showed a 2-fold increment upon shift from BHI to minimal medium (Table 3.1).
Listeriolysin O has been previously purified by thiol-disulphide exchange chromatography on thiopropyl/sepharose-6B, by immunoaffinity chromatography or by conventional methods using several column chromatographic steps (Geoffroy *et al*., 1987, Low *et al*., 1992, Matar *et al*., 1992, Schoel *et al*., 1994). The method described here used batch absorption of concentrated bacterial supernatants to Q-sepharose, followed by column chromatography of the non-absorbed fraction on a Resource-S column. The use of a buffer with an acidic pH was instrumental in improving the yields of pure listeriolysin. Using this procedure, approximately 5.4 mg of purified protein per liter of original culture supernatant was obtained (Table 3.2). This is a substantial improvement to the methods with the highest yields reported previously (Nato *et al*., 1991 , Schoel *et al*., 1994, Darji *et al*., 1995).

On the other hand, earlier purification methods for pneumolysin have used preparative electrophoresis as a last step (Shumwory and Klebanoff, 1971; Johnson, 1972 ; Paton *et al*., 1983) resulted in a poor yield. Other methods such as gel filtration and sucrose density gradient centrifugation have resulted in findings of different molecular weights (Kreger and Bernheimer, 1969). The method developed by Kanclerski and Mollby resulted in a highly purified pneumolysin but several column chromatographic steps were required (Kanclerski and Mollby, 1987). In the present study, pneumolysin underwent a simple one purification step resembles that used for LLO but due to the difference in pH activity and isoelectric points (LLO=6.7; PLY=5.2), pneumolysin was subjected to anion exchange chromatography on Resource-Q column. This procedure gave a pure protein yield of 2.0 mg per liter of original culture supernatant with a specific activity of $1.6x10^6$ Hu/mg (Table 3.3).
The relative ease with which listeriolysin O and pneumolysin can be purified from recombinant *L. innocua* strains will simplify the isolation and characterization of novel mutants of both toxins that are defective for hemolysis or for binding to its putative receptor on the eukaryotic cells. In addition, it will facilitate studies in pathogenicity of their parent strains as well as serological diagnosis of both listeriosis and pneumococcal infections.

To date, studies on specific antilisterial antibodies following infection with *L. monocytogenes* have revealed cross-reactivity to only a few proteins, including listeriolysin, IrpA, InlB, actA, and P60 as targets of the humoral immune response (Gentschev *et al*., 1992; Low *et al*., 1992; Gholizadeh *et al*., 1996; Grenningloh *et al.*, 1997). A pivotal study by Edelson and Unanue showed that prior treatment of mice with neutralizing monoclonal antibody to listeriolysin

prevents subsequent infection with this bacterium suggesting that listerial antigens targeted by the humoral response are required for successful host infection and thus warrant investigation (Edelson *et al*., 1999). Here it was shown that specific antibodies are directed against Frm, a newly discovered ferritin-like protein in *L. monocytogenes*, during infection by this bacterium. Physiological studies suggest that ferritin is required for making environmental iron available to these bacteria and in mediating protection against hydrogen peroxide and the reactive oxygen intermediates derived thereof. The properties exhibited by an isogenic Δ*frm* strain provide evidence that the listerial ferritin is involved in efficacious bacterial growth at early times in the infectious process.

The identity of a 110 kDa species seen by the immune antisera from *L. monocytogenes* infected mice as the *frm* gene product was revealed in several ways. First, antisera from *L. monocytogenes*-immunized mice recognised the 110 kDa polypeptide species derived from the *L. monocytogenes* (Fig. 3.4.A). This cross-reactivity was not observed with antisera from mice infected with a non-pathogenic *L. innocua* strain indicating that antibodies are directed to this protein during infection. Second, antisera from *L. monocytogenes*-infected mice showed cross-reactivity to the 110 kDa polypeptide species in soluble antigen extracts derived from the wild type, but not from the Δ*frm* mutant strain (Fig.3.5.C). Third, specific rabbit antisera raised against *L. innocua* ferritin also cross-reacted with the *L. monocytogenes* counterpart but was absent in a mutant lacking *frm* (Fig.3.5.B).

Immunoblotting experiments revealed distinct differences in the multimeric molecular species detected in *L. innocua* and *L. monocytogenes* (Fig.3.4.B). One explanation for these differences could possibly be the two amino acid substitutions in *L. monocytogenes* Frm relative to the *L. innocua* Fri protein, namely Lys114→Gln and Asp126→Asn both of which are located at the entrance of the three-fold channels pores and are hydrogen-bonded to each other. Similarly, changes in the subunit arrangement and spatial structures could account for differences in multimeric species of Frm observed in the *L. innocua* and *L. monocytogenes*. In a study on proteins induced by cold shock in *L. monocytogenes*, a ferritin-like protein (Flp) has been described and purified to homogeneity (Hebraud and Guzzo, 2000). Flp is identical in its N-terminal sequence and molecular weight to Frm described in this study. Based on its elution properties, these authors suggested a native molecular mass for Flp of about 110 kDa, composed of six identical 18 kDa subunits (Hebraud and Guzzo, 2000).

Inoculation of mice with a sub-lethal dose of the virulent *L. monocytogenes* EGD-e strain invariably led to the production of Frm-specific antisera in the infected mice. Although the non-pathogenic *L. innocua* strain also produces a related ferritin no antibody is directed to this

protein during infection with this strain (Fig.3.4.A), suggesting that some level of extracellular bacterial growth early in systemic infection is required for adequate amounts of Frm to be recognized by cells regulating the humoral response. Indeed the rapid elimination of non-pathogenic bacteria from the host could explain the absence of Frm-specific antisera in the infected host.

Listerial ferritin levels were strongly dependent on growth phase (Fig.3.4.D). A complex pattern of Frm expression was observed in cultures growing at 5, 20 and 37°C. Thus, in bacteria grown at 20°C Frm could not be detected, but was readily detectable in cultures grown at either 5° or 37°C (Fig.3.4.C) suggesting that Frm is subjected to multiple forms of regulation. This phenomenon was also described in the study of Hebraud and Guzzo who found that transcription of the ferritin gene was barely detectable at 30°C but was induced in cells that were either heat- or cold- shocked at 49° and 5°C respectively (Hebraud and Guzzo, 2000). Similarly, in a study on adaptive changes to high and low temperatures in *Listeria*, Phan-Thanh and Gormon described a 17.6kDa polypeptide that was induced under conditions of both cold and heat shock (Phan-Thanh and Gormon, 1995).

In previous studies, transcription of the *L. innocua fri* gene was shown to be dependent on iron in growth medium (Polidoro *et al*., 2002). In brain heart infusion cultures depleted for iron by chelation, induction of RNA corresponding to the *fri* gene was detected. In contrast, *fri* was poorly transcribed in cultures growing in brain heart infusion showing that the availability of iron regulates Fri expression. Promoter mapping studies indicate the presence of two sites at which transcription is initiated, one of which is probably a *sig*B-dependent transcript (Polidoro *et al*., 2002). In that study, similar results were detected using the *L. innocua fri* gene as a probe to monitor expression of the corresponding gene in *L. monocytogenes*. Since *sig*B-dependent genes are expressed as bacteria enter a nutrient limiting stage it is indeed likely that both Fri and Frm expression is also dependent on this alternative sigma factor (Ferreira *et al*., 2003; Fraser *et al*., 2003; Moorhead and Dykes, 2003).

Unlike the parental wild type strain, a Δ*frm* strain is capable of very limited growth in minimal media containing 260μM ferric citrate. Reconstruction experiments revealed that the arrested growth of Δ*frm* strain in minimal medium can only be restored upon addition of further ferric citrate (260μM) to the cultures (Fig.3.9). Addition of other divalent citrate salts like, Cu^{2+},Mg^{2+},or Mn^{2+} (260μM) to growth-arrested cultures of the Δ*frm* strain in minimal medium failed to promote further growth. These data strongly emphasize reduced bioavailability of iron in the Δ*frm* mutant strain grown in minimal medium and exclude the

possibility of induction of a citrate-inducible iron acquisition system as previously described (Adams *et al.,* 1990).

Evidence that Frm is required for early survival in the infected host was suggested by the observation that although the Δ*frm* mutant strain was as proficient as the wild type strain for invasion and cell to cell spread as indicated by the plaque assay (Fig.3.6), it exhibited an early defect in intracellular growth in both the HeLa and J774 macrophage cell lines (Fig.3.7). Complementation of the Δ*frm* strain restored the intracellular proliferative ability of these bacteria to wild type strain levels (Fig.3.7.A). This deficiency in growth at early times was also manifested in the mouse model of infection (Fig.3.10) indicating that expression of the listerial ferritin is required for efficient systemic growth and spread.

It has been suggested that the reaction of ferrous ions with metabolically generated reactive oxygen intermediates leads to killing of the bacterial cell (Harrison and Arosio, 1996). Based on the most recent *in vitro* (Zhao et al., 2002) and *in vivo* evidence (Ishikawa *et al*., 2003; Ueshima *et al*., 2003) that the ferritin-like activity of Dps proteins leads to protection against the toxic combination of Fe(II) and hydrogen peroxide, it was reasoned that the early defect observed following cellular infection was related to the inability of the Δ*frm* mutant to remove excess ferrous ions thereby inducing cell damage, in particular during transit through the host cell vacuole. Indeed, hydrogen peroxide is a constituent of the host vacuole and, among the various bacterial stresses examined; it was the only deleterious one for growth of the Δ*frm* mutant bacteria during bacterial growth (Fig.3.8). Reintroduction of a single copy of the frm gene to mutant Δ*frm* bacteria restored the growth ability to wild type levels in the presence of this agent.

In conclusion, the present data indicate that listerial ferritin appears to be a general survival factor required for overcoming changes in cellular redox state in the diverse habitats that *Listeria* spp. occupy. Thus, regulated expression of the *frm* gene is probably of great consequence for the lifestyle of this bacterium. A large number of iron and other metal transporters are present in the genome of *L. monocytogenes* to ensure uptake of iron from both the extracellular and intracytoplasmic environment of the infected host (Glaser *et al*., 2001). Clearly other mechanisms for iron homeostasis must exist in the absence of the listerial ferritin because mutant bacteria lacking *frm* are still capable of intracytoplasmic growth. Further analysis of the role of iron in listerial growth and physiology would provide valuable information on the ability of *L. monocytogenes* to adapt to different ecological niches.

4.2. Tailored bacterial vehicles as vaccine strains

In this study, it was shown that selective manipulation of virulence genes in *L. monocytogenes* can generate mutant bacteria that retain the marked protective immunogenic potential of the wild type strain but are attenuated in virulence as well as their capacity to induce CD4+-mediated inflammation. Two approaches were conducted to generate these mutants; the first was the deletion of certain virulence genes from the wild type *L. monocytogenes*. The second was the complementation of the non-pathogenic *L. innocua* strain with the virulence gene cluster isolated from the wild type *L. monocytogenes*.

Here, the induction of a protective cell-mediated immunity elicited by attenuated isogenic *Listeria* mutant strains was reported. Two highly attenuated isogenic *L. monocytogenes* mutant strains, *L.monocytogenes* EGD-eΔ*act*A and Δ*act*AΔ*plc*B that have retained the desired immunological characters of a wild type *L. monocytogenes* strain were characterized. The mutant EGD-eΔ*act*A2 strain is identical to the wild type *L. monocytogenes* strain, except for lacking the entire *act*A gene, crucial for actin polymerisation, and thus it devoids of the *Listeria*'s intracellular actin-based motility (Chakraborty *et al*., 1995). The EGD-eΔ*act*AΔ*plc*B lacks both the *act*A gene and the gene encoding the phosphatidyl choline-specific phospholipase (*plc*B), located in the lecithinase operon. The plcB protein modulates host signalling pathway and its absence impairs the mechanisms by which *Listeria* escapes from being translocated in the host vacuoles (Bannam and Goldfine, 1999; Wadsworth and Goldfine, 1999). Although both mutant strains lack genes responsible for intracellular motility and cell to cell spread, they persist long enough in the host to induce a potent CD8+ T cell-mediated immunity, accompanied by a reduced capacity to induce helper T cell-mediated inflammatory response.

As revealed by the *in vivo* growth kinetics, both mutants were slowly and continuously eliminated from the host, although could still be detected at a lower number in the spleen and liver up to day 8 after infection. This is in contrast to the non-pathogenic *Listeria innocua* strain (Fig.3.18) or a *L. monocytogenes* strain lacking the *hly* gene, encoding the toxin listeriolysin (Fig.3.29), where bacteria are completely eliminated 3 days after infection. The latter strains were unable to provide a protective immunity to re-infection (Fig.3.21 and 3.30.A), in accordance with the findings that the persistence and the number of viable microorganisms are important factors for efficient T cell-mediated immunity (North *et al*., 1981). Here, the data also show that even a few hundred bacteria, as in the case of

Δ*act*AΔ*plc*B mutant, persisting in spleens or livers of infected mice are sufficient for the generation of sterile immunity (Fig. 3.11).

Even at higher doses (10^7) of infection with the mutant strains, no obvious morphological alterations in spleens and livers were detected. Thus, pathologically these mutants behave similarly to the non-pathogenic *L. innocua* strain that is efficiently cleared by the host (Fig.3.19), without inducing any detectable morphological changes. On the other hand, strong monocytic infiltrations were observed in spleens of mice infected with the wild type EGD-e. The morphological difference observed between spleens of mice infected with the wild type EGD-e and its isogenic mutant strains were not due to the absence of bacteria, as comparable numbers of mutant EGD-e strains were present on day 3. The intensity of detectable changes in the spleen morphology (Fig.3.12) was associated with the level of the induced DTH response, a reaction mainly associated with helper CD4+ T cell activation, as indicated with the skin reactions (Fig.3.13.B) and the production of IFN-γ after *in vitro* stimulation of spleen cells (Fig.3.13.A). Significant reduction in DTH response and in production of IFN-γ was observed in mice infected with the mutant EGD-e strains in comparison to the wild type EGD-e strain.

Dynamics and early programming of T cells, responding to antigen, during *L. monocytogenes* infection has recently been reported (Busch and Pamer, 1999). It has been shown that during the primary phase of infection, the cytotoxic T cells specific for listerial antigens express low levels of CD62L. This expression of CD62L on the cytotoxic T cells (CD8+) is up-regulated during the memory phase. The expansion of antigen-specific T cells and generation of the T cell memory were reported to be independent of the duration and severity of *in vivo* bacterial infection (Mercado *et al*., 2000). The *in vivo* growth kinetics of isogenic mutants (EGD-eΔ*act*A2 and Δ*act*AΔ*plc*B) and the induction of memory cytotoxic cells ($CD8^+CD62^{lo}$) presented here are in agreement with this report (Fig. 3.16). Both isogenic mutant strains have functional listeriolysin genes that enable the bacteria to escape from the phagosome to the cytosole. The data of this study therefore further suggest that transient infection with isogenic mutant strains is sufficient for the induction and expansion of protective CD8+ T cells (Fig. 3.15).

Significant levels of antiserum to the haemolytic toxin, listeriolysin, were observed in mice infected with the wild type EGD-e and both mutant strains (Fig.3.17). There were no significant differences between these strains even though there were differences in the numbers of viable bacteria at the later time points during infection. Thus generation of anti-listeriolysin antibodies may be an early event during infection where larger number of

bacteria is present during hematogenous spread. Neutralizing antibodies to listeriolysin can also confer immunity (Edelson *et al*., 1999).

In an attempt to increase immunogenicity, a number of approaches, taking advantage of the properties of a particular cytokine (for example, IL-12, IFN-γ) have recently been employed. Many of these approaches were unable to produce satisfactory results simply because they led to a strongly dominant CD4+ T cell response (Miller *et al*., 1995), or developed into a Th2 recall as well as Th1 response (Wolf *et al*., 1995). Others considered approaches to *in vivo* modulation of Th1 response by using neutralising antibodies against cytokines (IFN-γ) resulting, beside exacerbated infection, in a dramatic increase of IL-4 and a switch from Th1 to Th2 cells (Nakane *et al*., 1996). Genetically defined mutants, on the other hand, display differential capacities to induce IFN-γ levels and activation of CD4+ T cells and, in this study, led to a reduction in both granuloma formation and DTH. At the same time, these bacteria were able to produce sufficient immunomodulators that retain their ability to induce a potent, protective cell-mediated T cell response. Indeed, these two strains have advantages over strains lacking metabolic genes like *L. monocytogenes* auxotrophic mutant bearing deletions in alanin racemase (*daI*) and D-amino acid aminotransferase (*dat*), two genes required for the biosynthesis of bacterial cell walls (Thompson *et al*., 1998). This strain showed, in absence of D-alanine, a defective growth in mice and a failure to induce a protective immunity against challenge infection. Another example is *L. monocytogenes* Δ*phe*A, a gene encoding the prephenate dehydratase (*phe*A), an enzyme acting late in the pathways for biosynthesis of phenylalanine. This strain was not efficiently attenuated and had elicited the same level of CD4+ inflammatory responses as the wild type (Alexander *et al*., 1993). In addition, *Salmonella typhimurium* Δ*aro*A, although attenuated, induced mainly CD4+ T cell responses (Hess *et al*., 1996).

Isogenic mutants of wild type *L. monocytogenes* that are highly attenuated for virulence were identified. They showed a strong reduction in the undesirable localized inflammatory capacities, but are nevertheless capable of providing effective protective responses. The highly reduced virulence of the Δ*act*AΔ*plc*B mutant, in particular, indicates that a very low number of persisting bacteria is required for immunity. Based on these studies, it will be interesting to determine, through the introduction of further mutants, whether there is a minimum number of persisting live replicating bacteria needed for mediating immunity. As shown above, the Δ*act*AΔ*plc*B mutant is able to induce sufficient IFN-γ to prevent a Th1 to Th2 switch and retains its ability to induce a strong CD8+-mediated T cell response. Recently initial clinical studies in healthy human volunteers with similar mutants have been carried out

indicating no serious long-term health consequence (Angelakopoulos *et al*., 2002). These virulence-attenuated isogenic mutant strains can not only be used as live vaccines against the corresponding virulent pathogen, but also as carriers for introducing heterologous protective antigens of other pathogenic microorganisms into animals and humans.

The gene products of the PrfA-dependent gene cluster, which are crucial for the release from the host cell phagosome, intracellular replication, intracellular movement, and the cell-to-cell spread of pathogenic *Listeria monocytogenes* strain (Portnoy *et al*., 1992), are lacking in the non-pathogenic *L. innocua* strain. The present study investigated that expression of the prfA-dependent virulence gene cluster of the wild type *Listeria monocytogenes* strain in the non-pathogenic *L. innocua* strain can generate a recombinant attenuated strain that can efficiently induce a protective immunogenic responses with a capacity to downmodulate CD4+ T cell-mediated inflammation. The recombinant *L.innocua* strain transformed with a gram-positive/gram-negative schuttle BAC-based vector (pUvBBAC) harbouring the chromosomal region of *Listeria monocytogenes* that encodes the gene cluster *prf*A-*plc*A-*hly*-*mpl*-*act*A-*plc*B was assessed.

Investigation parameters were selected to correspond to critical steps of the host response during the pre-immune phase, the primary immune effector phase, and the memory immune effector phase of the infection (Mielke *et al*., 1997). Day 3 of a *Listeria* infection marks the end of the pre-immune phase. It is the last day before the expansion of specific T cells in this model (Kaufmann, 1986; Ehlers *et al*., 1992). The presence of viable bacteria on this day has been shown to be critical for the successful induction of T cell-mediated immunity (North *et al*., 1981). On day 3, therefore, bacterial load as well as spleen morphology was investigated. Day 9 corresponds to the primary immune effector phase, that is, acquired resistance. On this day, DTH to soluble antigen was measured *in vivo* as well as T cell-derived IFN-γ production by spleen cells, known to be an *in vitro* correlate of DTH reaction and a measure of CD4+ T cell activity (Mielke *et al*., 1998). Moreover, the induction of antigen specific IFN-γ producing CD8+ cytotoxic T cells was estimated on this day. Day 60 as well as day 5 post-challenge were chosen to refer to the memory immune effector phase (Busch and Pamer, 1999) when the number of memory effector T- cells were quantitated.

Bacterial growth kinetics *in vivo* indicate not only the virulence of a bacterium but are also associated with the induction of T cell-mediated host responses (Kaufmann, 1984). Experiments that temporally abrogated *Listeria* infection with antibiotics (North *et al*., 1981) have revealed that the persistence and number of viable microorganisms are important

parameters for efficient induction of T cell-mediated immunity. The recombinant *L. innocua*: :vgc strain showed a significant survival pattern in the first 3 days after infection in both spleens and livers (Fig.3.18.). It was present in a substantial number for at least 3 days which was comparable to that of the wild type *L. monocytogenes*. The wild type *L. innocua*, lacking the vgc, was rapidly eliminated from day 1 onwards even when high inocula were used for infection. Most noteworthy, although the recombinant *L. innocua*::vgc strain was injected in mice in a dose of 10^7 cfu, mice can efficiently control the infection. Morphological appearance of infected spleens have shown that the wild type *L. monocytogenes* induces granuloma as a result of monocytic infiltrations of the white pulb which is most intensive on day 3 post infection while only splenomegaly without any detectable granulomas was the case after *L. innocua*: :vgc infection (Fig.3.19). The wild type *Listeria innocua* strain did not induce any detectable changes in spleen morphology. The intensity of the morphological alterations in spleens paralleled the level of *Listeria*-induced DTH responses. In accordance with data presented by Yang *et al*., IFN-γ turned out to be a most helpful *in vivo* and *in vitro* parameter predicting the strength of the proinflammatory CD4+ T cell-mediated immune response to the bacteria (Yang *et al*., 1997). A previous study showed that only vaccination with strains with the capacity to induce T cell-mediated immunity, i.e. the wild type *L. monocytogenes* strain resulted in appreciable levels of type I cytokines transcripts like IL-12 and IFN-γ (Peters *et al*., 2003). In the present study, the wild-type *L. monocytogenes* strain could induce a significant level of IFN-γ in the supernatants of spleen cell cultures from immunized mice which was approximately 2 folds more than IFN-γ induced in response to *L. inn*: :vgc infection (Fig. 3.20.A). This finding was supported with the measurement of the *in vivo* induction of DTH after recombinant *L. inn*: :vgc strain infection which was also significantly lower than DTH induction after *L. monocytogenes* infection (Fig. 3.20.B). In both cases, the wild type *L. innocua* failed to induce any proinflammatoty CD4+ T cell-mediated immune response. So, infection with the recombinant *L. inn*: :vgc strain seems to abrogate the induction of undesirable CD4+-mediated inflammatory responses.

Recent advances in techniques to investigate the specific T cell response, in particular of the CD8+ subpopulation with peptide loaded tetrameric MHC, intracellular cytokine staining and the use of transgenic animals (Busch *et al*., 1999; Mercado *et al*., 2000; Huleatt *et al*., 2001; Lauvau *et al*., 2001; Badovinac *et al*., 2002; Yajima *et al*., 2002) have provided tremendous insight as to how the specific immune response is mounted. It is the elegant work by Pamer's group that also revealed that priming of CD8+ T cells by heat-killed bacteria must be differentiated from differentiation into effector cells (Lauvau *et al*., 2001). Therefore, the

most reliable approach to assess the complex ability of T cells to protect animals against a lethal dose of microorganisms remains the demonstration of the *in vivo* function of T cells by the effect of T cell subset depletion (CD4+ and CD4+ plus CD8+) in previously immunized and subsequently challenged animals (Mielke *et al*., 1998). In contrast to animals immunized with the wild type *L. innocua* as well as unimmunized mice, all animals immunized with *L. monocytogenes* EGD-e or *L. inn*: :vgc were protected against 10X LD_{50} of virulent *Listeria* (Fig. 3.21), despite significant differences in their DTH responses. T cell subset depletion revealed that this protection was CD4+ T cell independent, but that it was severely impaired when both CD4+ and CD8+ T cells were depleted (Mielke *et al*., 1998). In accordance to this report, *L. inn*: :vgc strain was efficiently able to induce LLO_{91-99}-specific CD8+ T cells producing IFN-γ after both primary and recall infection (Fig. 3.22).

During an immune response, antigen-specific T cells proliferate enormously and develop into effector T cells capable of immediate effector functions, such as cytotoxicity and IFN-γ production (Butz and Bevan, 1998; Murali-Krishna *et al*., 1998). Following a successful immune response, activated effector T cells undergo large-scale apoptosis, presumably to maintain homeostasis in T cell numbers (Van Parijs and Abbas, 1998). However, the process leaves behind an enhanced pool of relatively quiescent antigen-experienced memory T cells that persist over long periods of time and mount a rapid and augmented response upon re-challenge with antigen (Van Parijs and Abbas, 1998). The entry into memory stage, however, is accompanied by changes in gene expression profile of antigen-specific CD8+ T cells. This includes the ability to rapidly expand their population during recall responses and to downregulate expression of some cell surface markers such as CD62L (L-selectin) and CCR7 which, therefore, have been widely used to define memory and naive phenotypes of T cells in humans and mice (Dutton *et al*., 1998; Kaech *et al*., 2002b; Gett *et al*., 2003; Wherry *et al*., 2003). The expression of CD62L, an adhesion cell surface molecule, is used for identifying naive T cells, and the loss of CD62L expression correlates with T-cell priming. The presence of live bacteria over the first 48 hours after immunization was found to be critical for induction of effector CD8+ T cell mechanisms (Mercado *et al*., 2000). It was previously reported that primary infection with the wild type *L. monocytogenes* induces down regulation of CD62L on surface of effector CD8+ T cells which reaches its downregulation peak at day 8 post infection (Kaech *et al*., 2002a; Wherry *et al*., 2003). However, over the next several weeks, expression of CD62L is up regulated. During recall infection, CD62L was massively and rapidly downregulated on the surface of memory CD8+ T cells (Mercado *et al*., 2000; Darji *et al*., 2003). Here, infection with the recombinant *L. inn*: :vgc strain managed to induce

a significant population of cytotoxic CD8+ T lymphocytes (Fig. 3.22) which, upon challenge with the wild type *L. monocytogenes*, showed a CD62L expression pattern similar to that shown upon infection with the wild type *L. monocytogenes* (Fig. 3.23) while primary infection with the wild type *L. innocua* failed to induce a significant down regulation of CD62L during recall infection. These data together with the successful *in vivo* survival pattern of the recombinant *L. inn*: :vgc strain support the previous findings. Moreover, a significant induction of humoral immunity against listeriolysin O was observed in mice primarily infected with *L. inn*: :vgc as well as the wild type *L. monocytogenes* but not with the wild type *L. innocua* (Fig.3.24) as indicated by the recognition of listeriolysin O by the immune mice antisera in immunoblotting assay proposing that the persistence of viable bacteria over the first three days after infection is critical for induction of humoral immunity.

Most noteworthy, the inability of *L. monocytogenes* strain lacking only listeriolysin O (Tanabe *et al*., 1999) as well as *L. innocua* strain expressing only LLO (Peters *et al*., 2003) to induce a protective T cell response reflects the critical coordination role of virulence gene cluster elements in conferring a long lasting immunity. The delineation of its immunological properties shows that the recombinant *L. inn*: :vgc strain retains the favourable immunological properties of the wild type *L. monocytogenes* and fulfils appropriate criteria for a suitable live bacterial T cell vaccine vector.

4.3. Listeriolysin O is not absolutely essential for induction of long term cellular immunity against *Listeria monocytogenes*

The role of the major virulence factor in *L. monocytogenes*, listeriolysin O, in mediating escape from host cell vacuole as well as in pathogenicity of *L. monocytogenes* was elucidated (Kathariou *et al*., 1987; Gaillard *et al*., 1986; Cossart *et al*., 1987; 1989; Portnoy *et al*., 1988; Tilney and Portnoy, 1989). Moreover, listeriolysin O production by infecting bacteria is essential for generating a protective T lymphocyte response against *L. monocytogenes* (Mengaud *et al*., 1988; Mielke *et al*., 1997). The results of the present study indicate that although the related hemolysin, pneumolysin, can not restore a full virulence to *L. monocytogenes*, it can functionally mediate lysis of the host cell vacuole and induce a protective immunity against a lethal dose of the wild type *L. monocytogenes*. Unlike other cytolysins, pneumolysin molecule is not surface exposed. It is a cytoplasmic enzyme that lacks a signal peptide and is, therefore, released due to the action of surface pneumococcal autolysin (Paton *et al*., 1983; 1986; 1993). So, the functional gene structure of pneumolysin was cloned downstream to the putative LLO signal peptide sequence in a plasmid-borne genes

under the control of LLO promoter and *prf*A regulator and expressed in EGD-eΔ*hly*. In comparison to LLO, pneumloysin was efficiently secreted in culture medium (Fig.3.26) and showed a clear haemolytic phenotype comparable to that of the LLO expressing strain (Fig.3.27).

In primary amino acid structure, thiol activated cytolysins are defined by an almost invariant undecapeptide sequence ECTGLAWEWWR, which is important for cytolytic activity (Billington *et al*., 2000) giving rise to a possibility that both LLO and PLY share some cross-reacting epitopes. This structure homology can explain the cross-reactivity of both LLO and PLY proteins with either monoclonal antibodies produced against them (Fig.3.26). This cross reactivity was extended to include the immune sera from mice infected with either LLO or PLY-expressing strain which could recognize both purified LLO and PLY proteins (Fig.3.33). Definition of listeriolysin O as well as pneumolysin by sera of patients with listeriosis or pneumococcal diseases might be of great diagnostic value.

L. monocytogenes has emerged as a model system for the molecular study of intracellular parasitism. It can enter into a wide variety of cells by phagocytosis. Subsequent to entry into a host cell, *L. monocytogenes* lyses its vacuole and escapes into the cytosol, where it can multiply and spread from cell to cell (Cossart and Lecuit, 1998). There is an overwhelming evidence that the primary *L. monocytogenes* determinant responsible for escape from a vacuole and thus entrance in the cytosol, two key events for virulence, is LLO, encoded by the *hly* gene. First, mutants lacking LLO fail to escape from a vacuole and are absolutely avirulent. Complementation with *hly* restores virulence (Cossart *et al*., 1989). Second, expression of LLO by *Bacillus subtilis* confers to these extracellular non-pathogenic bacteria the capacity to escape from a vacuole and grow in the cytosol (Bielecki *et al*., 1990). Third, purified LLO encapsulated into pH-sensitive liposomes can mediate dissolution of a vacuole (Lee *et al*., 1996).

Several members of the pore-forming CBCs family such as perfringolysin O (PFO) of *Clostridium perfringens* have been characterized in detail (Rossjohn *et al*., 1997; Shatursky *et al*., 1999). The major known difference between LLO and other members of the family is that LLO has an optimum activity at acidic pH (Geoffroy *et al*., 1987). This raises the question of whether this unique trait has any physiological relevance in the infectious process. To start to address this question, PFO had been cloned and expressed in *L. monocytogenes* in place of LLO under the control of the endogenous *hly* promoter (Jones and Portnoy, 1994). PFO was able to mediate vacuolar escape at approximately 50% of the efficiency of LLO. However, after a small number of bacterial divisions in the cytosol, the host cell became permeabilized

and died. In accordance with these findings, results of this study showed that the PLY-expressing strain is able to enter the host cell cytoplasm, grow, and spread from cell to cell to a limited extent over the first 4 hours post *in vitro* infection followed by inhibition of growth (Fig.3.28). These results indicate that LLO has evolved some specific properties to prevent cytotoxicity that are not shared by PLY, a protein from an extracellular pathogen that normally acts on cells from outside (Alouf and Geoffroy, 1991). It is unclear at this point what particular properties allow LLO but not PLY to mediate *L. monocytogenes* survival. Two striking different parameters between LLO and PLY were supposed to be responsible for *L. monocytogenes* survival pattern. The first parameter is their pH optima (Geoffroy *et al*., 1987; Portnoy *et al*., 1988). LLO has a pH optimum in the acidic range, while PLY is equally active at both acidic and neutral pHs. Accordingly, one interpretation of these results is that released LLO is relatively inactive in the cytoplasm, while PLY is fully active. The low pH optimum of LLO may represent a protective mechanism to prevent host cell damage in the cytoplasm. Conceivably, *L. monocytogenes* has adopted a strategy used by eukaryotic cells to compartmentalize the potentially toxic activity of lysosomal acid hydrolases (Jones and Portnoy, 1994). The second parameter includes the protein instability in the cytosole. A recent study demonstrated that a PEST-like sequence is present at the NH_2 terminus of LLO while absent in both PFO and PLY and is responsible for the rapid degradation of LLO in the host cell cytosole, thus decreasing the intracytoplasmic half life time of LLO (Decatur and Portnoy, 2000). PEST-like sequences are thought to target eukaryotic proteins for phosphorylation and degradation, and deletion or specific amino acid substitution of this sequence in LLO led to increased cytotoxicity and lower virulence in a mouse model. When the sequence was introduced in PFO and the chimeric toxin expressed in *L. monocytogenes,* bacteria were less toxic than those expressing wild-type PFO and were able to multiply intracellularly in J774 macrophages. Thus, introduction of a PEST motif in LLO is a strategy used by *L. monocytogenes* to restrict the activity of this powerful toxin to the host cell vacuole, thereby preserving the intracellular niche for bacterial multiplication. Based on these postulations, the limited survival of pneumolysin expressing strain in J774 macrophage-like cell line is supposed to be due to cytotoxicity of pneumolysin that induced permeability of cell membrane resulting in entry of extracellular gentamicin inside the macrophages and killing the bacteria.

Determination of virulence in mice showed that, in contrast to the wild type, the isogenic mutant EGD-eΔ*hly* strains expressing either LLO or PLY was avirulent. Mice can control a dose of $8x10^6$ cfu suggesting that some levels of regulation were missing in plasmid based

strains. However, the strain expressing LLO followed the same *in vivo* survival pattern as the wild type strain in both spleens and livers (Fig.3.29) while in accordance to the *in vitro* survival, the pneumolysin expressing strain showed a slight increase in level of replication and survival *in vivo* over the first 3 days after infection followed by slow and continuous elimination from the host, although could still be detected at a lower number in the spleen and liver up to day 8 after infection. One speculation for slow *in vivo* replication of pneumolysin expressing strain is that cytotoxicity of pneumolysin causes the host cells to become permeable to the extracellular bactericidal factors in serum that enter the host cells and kill PLY-expressing strain. In contrast, the isogenic mutant EGD-eΔ*hly* strain was rapidly cleared from both organs over the first 3 days after infection.

Survival of mice primarily infected with pneumolysin-expressing strain upon recall infection with a lethal dose of the wild type *L. monocytogenes* supports previous reports showing that the persistence and the number of viable microorganisms over the first 48-72 hours after infection are important factors for efficient T cell-mediated immunity (North *et al*., 1981; Mercado *et al*., 2000) and that the presence of few hundreds of bacteria viable in spleens and livers of infected mice was sufficient for induction of protective immunity (Darji *et al*., 2003). Moreover, immunization of mice with a PFO-expressing strain led to a protective immunity (Bouwer *et al*., 1994) indicating that T cells with specificity for non-LLO-derived epitopes of *L. monocytogenes* are a component of a protective antilisterial immune response. Here, in agreement with these findings, the ability of pneumolysin-expressing strain to confer a protective immunity and to induce a significant number of CD8+ cytotoxic T cells specific to the subdominant *Listeria* epitope, $P60_{217\text{-}225}$, either primarily or after challenge (Fig.3.30 and 3.31) confirm that they do indeed enter the cytoplasm facilitating procession and presentation of *Listeria*-derived peptides by H-$2K^d$ MHC class I molecules for cytotoxic T cells that are expected to domain the protective immune response (Berche *et al*., 1987; Portnoy, 1992; Pamer , 1993). Analysis of the specificity of antilisterial cytotoxic cells from *Listeria* immune BALB/c donors has shown a dominant response to an epitope corresponding to amino acids 91 to 99 of LLO (Busch *et al*., 1998). Previously, demonstration of antilisterial T cells specific to non-LLO-derived epitopes has been difficult to achieve, because of the crucial requirement of LLO in facilitating escape of the bacteria to the cytoplasm of the host cell, which may be responsible for the apparent dominance of an anti-LLO response in antilisterial immunity (Beattie *et al*., 1990; Pamer *et al*., 1991; Safley *et al*., 1991; Harty and Bevan, 1992). In addition, LLO but not PFO-expressing Bacillus subtilis were able to confer immunity against the wild type *L. monocytogenes* (Bouwer *et al*., 1992) indicating that anti-

listerial immunity has specificity to both LLO and non-LLO derived epitopes. Induction of $P60_{217-225}$ specific effector T cells by pneumolysin-expressing strain (Fig.3.31) was also supported by the ability of this strain to initiate a successful programming of T cells leaving, following a successful eradication of the strain, a significant pool of CD8+ T cells that acquire memory cell properties. One of memory cell surface markers is the adhesion molecule, L-selectin (CD62L), whose expression is upregulated on naïve CD8+ T cells but rapidly down regulated upon cell stimulation (Busch and Pamer , 1999). The *in vivo* growth pattern of PLY-expressing strain together with its ability to induce a significant number of $CD8^{+}CD62L^{lo}$ T cells (Fig.3.32) was in agreement with the finding that induction of an efficient T cell stimulation and programming is dispensable for duration and severity of *in vivo* bacterial infection (Mercado *et al*., 2000).

L. monocytogenes has been described as a potential vaccine carrier for induction of protective cell-mediated immune responses to virus-specific or pathogenic bacterium-specific epitopes (Schafer *et al*., 1992). The premise for this vaccine design is that following uptake by the host cell, *L. monocytogenes* escapes to the cytoplasm of the host cells, thereby introducing peptides into the intracellular environment for processing through the endogenous pathway for presentation, in association with MHC class I at the cell surface, to reactive cytotoxic CD8+ T cell populations. Indeed, the results of this study are consistent with a model in which a protective immunity can be specifically induced against non-LLO derived antigens in absence of the pore-forming listeriolysin O.

4.4. Cytosolic localisation of *L. monocytogenes* is critical for induction of protective immunity

Several virulence factors associated with *Listeria* infection have been identified. One of these is listeriolysin O (LLO). LLO is not only required as a virulence factor that enables *L. monocytogenes* to escape from the vacuole formed upon initial internalization but it is also required in the immunization process during infection through generation of a protective T lymphocyte response to *L. monocytogenes*.

The critical role of the PEST-like region at the N-terminus of Listeriolysin O in pathogenesis of *Listeria monocytogenes* was previously studied (Decatur and Portnoy, 2000; Lety *et al*., 2001; 2002). On the other hand, nothing is known about the role of this motif in the induction of a T cell response against *Listeria monocytogenes*. In this study the critical role of PEST-like sequence of Listeriolysin O in mediating induction of Th1-type immune response and conferring a protective T cell antilisterial immunity was established. The 28 amino acids

region at the N-terminus of Listeriolysin O that harbours the PEST-like sequence was deleted and the mutant protein was expressed in EGD-eΔ*hly*. As shown by Western blotting with a monoclonal anti-LLO antibody (Fig.3.35.A), the mutant protein, designated LLOΔPEST, was secreted in comparable amounts in the culture supernatants. The additional recognized bands of lower molecular weight in case of the wild type LLO may reflect its instability over the mutant protein supporting the earlier prediction of Decatur and Portnoy that the PEST-like sequence may target LLO for degradation (Decatur and Portnoy, 2000).

In vitro, the culture supernatants containing the mutant protein (LLOΔPEST) exhibited a haemolytic activity comparable to that of the wild type (Fig.3.35.B). This finding was in agreement with previous observations (Lety *et al*., 2002) indicating that modifications in the proximal portion of LLO do not affect protein secretion and hemolytic activity. Evidence that PEST-like sequence is crucial for mediating the disruption of phagolysosomal membrane and the escape of *Listeria* into the cytosole of phagocytic cells was confirmed by following the *in vitro* growth kinetics and the immunofluorescence microscopy analysis of the LLOΔPEST mutant strain in J774 macrophages (Fig.3.36). These data revealed that the escape of the LLOΔPEST mutant from the vacuole was severely impaired. This deficiency in growth was also manifested in the mouse model of infection where the LLOΔPEST mutant strain was rapidly removed from spleens and livers of infected mice up to day 3 post-infection (Fig.3.37).

It is well-known that a Th1-type immune response is induced in mice upon infection with *L. monocytogenes* and that antigen specific T cells play a central role in protective immunity against challenge infection (Darji *et al*., 2003; Kimoto *et al*., 2003). Day 3 of *Listeria* infection represents the end of the pre-immune phase of host response against *L. monocytogenes*. It is the last day before the expansion of specific T cells in this model (Kaufmann, 1986; Ehlers *et al*., 1992). The presence of viable bacteria on this day has been shown to be critical for successful induction of T cell-mediated immunity (North *et al*., 1981). It was recently established that the pathogen specific T lymphocytes are programmed during the first day of infection and subsequently undergo proliferation and differentiation into effector T cells without further calibration by the progressing inflammatory response but the magnitude of the LLO_{91-99} specific response correlates with the duration of *in vivo* bacterial growth during the first 24 h after infection (Mercado, *et al*., 2000).

These findings prompted the analysis of the relation between the *in vivo* survival of the PEST-like sequence mutant strain and its ability to induce a protective immunity. The bacterial load in spleens and livers of infected mice was measured at certain time points for several days.

The ΔPEST mutant strain and the ΔLLO isogenic mutant strain were rapidly cleared from spleens and livers although the former showed a slightly more survival than the latter (Fig.3.37). The slight increase in survival pattern of ΔPEST mutant strain over the LLO lacking strain both *in vitro* and *in vivo* suggests that the removal of the PEST-like motif extremely inhibit, but not completely abolish, the disruption of phagolysosomal membrane and intracellular bacterial survival. Furthermore, either ΔPEST mutant strain or the ΔLLO isogenic mutant strain failed to induce any detectable changes in spleen morphology 3 days after infection (Fig.3.38) while infection with the wild type and LLO producing mutant showed an intensive granuloma and splenomegaly respectively due to induced monocytic infiltration which paralleled the level of DTH responses, a reaction mainly associated with helper CD4+ T cell activation.

LLO was shown to possess the ability to induce various cytokines, including IL-1α and IFN-γ. The early production of IFN-γ following infection was shown to be important for the generation of protective T cells indicating that listeriolysin O is critical for generating a protective anti-listerial T lymphocyte response (Nishibori *et al*., 1995; 1996; Xiong *et al*., 1994; 1998). Mitsuyama and co-workers reported that the inability of a LLO-lacking strain or killed bacteria to induce the generation of protective T cells is due not to the lack of a central T cell epitope(s) but to the lack of ability to induce the production of endogenous cytokines during the early stage of immunization (Tanabe *et al*., 1999). It has been reported that a significant level of IFN-γ production in the murine spleen occurred within 24 to 48 h of immunization with a virulent strain (Nakane *et al*., 1989; 1990). Moreover, when endogenous IFN-γ was neutralized by administration of anti- IFN-γ monoclonal antibody for the initial 2 days in mice immunized with viable *L. monocytogenes*, the generation of protective T cells on day 6 was completely blocked (Yang *et al*., 1997). As expected, early production of IFN-γ was successfully induced by wild type and LLO producing mutant (Fig.3.39.A). On the other hand, both ΔPEST mutant strain and the LLO lacking strain were unable to induce IFN-γ in mice serum at day 2 post infection indicating that the *in vivo* growth deficiency of this mutant may contribute to its deficiency to induce IFN-γ production.

In an attempt to confirm this indication, the amount of IFN-γ produced in culture supernatants of splenocytes at day 9 post-infection (Fig.3.39.B) as well as the number of specific MHC class I restricted effector CD8+ T cells in spleens at either day 9 post-infection or day 5 post-challenge (Fig.3.41) were estimated. Day 9 post-infection corresponds to the primary immune effector phase, that is, acquired resistance. It was found that the ΔPEST as well as the ΔLLO mutant strain fails to induce the number of CD8+ T cells, after either the primary infection or

the challenge, or to prime splenocytes to produce a detectable level of IFN-γ. So, the removal of PEST-like sequence seems to influence the antigen presentation efficiency through the MHC class I pathway due to the poor ability of PEST-like sequence lacking strain to gain access to the cytosole of the infected cell and to undergo further processing and antigen presentation.

Earlier studies suggested that differentiation of CD8+ T cells into effector cells during primary immune response has important consequences for the development of long-lived memory cells (Lauvau *et al*., 2001) and that the immunization with avirulent *Listeria* with an *in vivo* persistence of less than 48 hours (Baldridge *et al*., 1988) or with a heat-killed *Listeria* (Lauvau *et al*., 2001) induced only low levels of protection. There was a consensus that the virulence factors of *L. monocytogenes* may contribute directly to the induction of protective immunity (Xiong *et al*., 1998). It was previously confirmed that the deletion of both actin nucleator (*act*A) and phospholipase B (*plc*B) genes from *L. monocytogenes* has extremely lowered its virulence but had no influence on its ability either to induce and maintain effector memory T cells or to confer a long-lasting protective immunity against the wild type *L. monocytogenes* (Darji *et al*., 2003) whereas the isogenic mutant strain EGD-eΔ*hly* was shown to lack the ability to induce a protective immunity (Xiong *et al*., 1994; Tanabe *et al*., 1999; Peters *et al*., 2003) indicating that listeriolysin is essential for induction of protection mediating T cells. All other virulence factors even if over-expressed cannot compensate for the absence of listeriolysin (Marquis *et al*., 1995). Most noteworthy, the ability to produce listeriolysin O was not sufficient to convert *L. innocua* into a protection inducing bacterial vector (Peters *et al*., 2003).

In this study, these findings were extended by testing the effect of PEST-like sequence deletion from Listeriolysin O on T cell mediated protection. It was found that mice immunization with ΔPEST mutant strain was not protective against a secondary lethal wild type listerial infection (Fig.3.40.A). These mice could not control the challenge infection as indicated by either the high bacterial load in their spleens and livers (Fig.3.40.B) or the poor induction of $CD8^{+}CD62L^{lo}$ effector memory splenocytes (Fig.3.42). In contrast, mice that were previously primed with mutant strain expressing the intact listeriolysin O as well as the wild type *L.monocytogenes* showed a full survival and an extreme effector memory T cell protective response. Hence, these findings confirm the role of PEST-like sequence in mediating escape of *L. monocytogenes* from phagosomal vacuole into the cytosole as well as targeting the listeriolysin O for degradation and presentation on MHC I molecules.

Collectively, the ability of *L. monocytogenes* to gain access into the cytosole of antigen presenting cells is crucial for protection and for its use as a potential live vaccine vector.

4.5. Outlook

4.5.1. Antibody-mediated immunity against intracellular pathogens

For intracellular bacteria, the role of antibody-mediated immunity is still poorly understood. This is due to the fact that the bacteria inside the cell are protected from the serum antibody. Indeed, the recognition of some listerial proteins by the immune sera of listeriosis patients (Grenningloh *et al.*, 1997) in addition to the recent finding that monoclonal antibody produced against LLO can limit the intracellular listerial growth in macrophages (Edelson and Unanue, 2001) have paved the way for thinking about the contribution of humoral response to *Listeria* infection.

However, the two-dimensional separation and categorization of microbes as either intracellular and extracellular pathogens was never absolute, since tissue examination often revealed that pathogens classified as intracellular could be found in the extracellular space and vice versa. Furthermore, at some points in the infectious cycle, most intracellular pathogens reside in the extracellular space, where they are vulnerable to antibody action, and Fc receptor cross-linking can have profound effects in the intracellular milieu through signal transduction.

Based on these information, it is suggested that some level of extracellular listerial growth early in systemic infection is required for adequate amounts of antigens to be recognised by cells regulating the humoral response. Indeed, generation of monoclonal antibodies against secreted or surface-exposed listerial antigens like LLO, P60, actA, and irpA will be helpful as a therapeutic tool where antibiotic resistance are common or for infectious listerial diseases that could arise in the setting of severe immunosuppression such as during bone marrow and organ transplantation, and AIDS.

4.5.2. Requirements for the creation of novel vaccine vectors

There is a need to develop vaccines with the potential for global use against bacterial infectious diseases. These should be inexpensive to produce, stable in the absence of refrigeration, safe and efficacious, and able to be given orally or intranasally rather than by injection.

A vaccine should be sufficiently attenuated through deletion of certain virulence as well as metabolic genes. Attenuation should be sufficient to decrease if not eliminate induction of undesirable disease symptoms. In this regard, the nutritional status and health of the population to be vaccinated should be considered. The attenuation should be an inherent property of the bacterial vaccine and not be dependent on fully functional host defences and immune response capabilities. The attenuation should not be reversible by diet or by host modification of diet constituents, including by host-resident microbial flora. The attenuation should not lead to the development of a persistent carrier state for the vaccine. The attenuated vaccine should be sufficiently invasive and persistent to stimulate both strong primary and lasting memory immune responses.

A vaccine should also be designed to minimize tissue damage that is not needed to induce an effective immune response. For example, vaccines for enteric pathogens must access the gut-associated lymphoid tissue (GALT) via invasion and transcytosis through M cells. However, attachment to and invasion into enterocytes can lead to undesirable diarrhoeal episodes that do not contribute to the desired immune response. As even attenuated vaccine strains may cause disease in a few unlucky individuals, safety considerations dictate that any live bacterial vaccine should be susceptible to all clinically useful antibiotics. Lastly, the attenuated vaccine should possess some containment features to reduce its shedding and/or survival in nature to preclude vaccination of individuals who did not elect to be vaccinated. However, persistence of a live vaccine with potential for individual-to-individual spread could have a positive public health benefit, as is probably the case for vaccination against polio.

When such an attenuated bacterial mutant is used as a delivery carrier for heterologous antigens, the plasmid vector component has to be optimised. Indeed, stability of protective antigen expression *in vivo* is essential for recombinant vaccine efficacy. Insertion of genes into the chromosome can increase stability, but the level of antigen expression is generally too low to stimulate an adequate immune response. Since the level of protein synthesis in bacteria is very much dependent upon gene copy number, antigen production can be vastly increased by use of multicopy plasmid vectors. In this case, the use of a balanced-lethal host-vector system wherein the plasmid possesses a gene complementing a chromosomal deletion mutation of a vital gene, such as for cell wall synthesis or DNA stability or replication, ensures that the plasmid is maintained (Nakayama *et al.*, 1988). An alternate approach to ensuring retention of the plasmid vector is the "Hok-Sok" strategy, which also results in bacterial cell death if the plasmid is lost (Galen *et al.*, 1999). When a plasmid vector with a high copy number is used, the level of expression of the gene encoding the vector-selective

marker can be far in excess of that necessary for maintenance of the vector. In these instances, overexpression of such a gene product further attenuates the vaccine, presumably due to the added energy drain on the recombinant vaccine. To address this problem, the selective marker gene can be designed to include a ribosome-binding recognition sequence but no promoter (Kang et al., 2002). As a safety consideration, it is desirable that plasmid vectors possess some containment features to minimize the possibility of transfer to and maintenance in other bacterial species. The vector should therefore be non-conjugative, should preferably be non-mobilizable, should possess a narrow replicon host range, and should not specify resistance to any antibiotic.

4.5.3. Role of Listeriolysin O in *Listeria monocytogenes* infection

In spite of the great number of studies dealing with the role of LLO in pathogenesis of *Listeria monocytogenes*, more researches have to be conducted to understand how LLO interacts with phagolysosmal membranes and allows bacterial phagosomal escape. The patch clamp technique may be helpful to elucidate the precise molecular mechanisms by which the PEST-like sequence participates in the opening of the vacuole. Indeed, the role of the PEST-like sequence in mediating escape of *Listeria* monocytogenes into the cytosol can be demonstrated through its fusion to the coding sequences of particular proteins like Green Fluorescent Protein (GFP). The intracellular localisation of the fusion proteins can be followed in phagocytic cells using fluorescent microscopy.

A potential screening system for the ability of the PEST-like sequence to permit cytosolic transfer of LLO could exploit the Cre–*lox* recombination system. The Cre–*lox* recombination system has the potential to become a powerful tool for the conditional and cell-specific deletion of genes. The introduction of this system into transgenic mice should facilitate studies on the loss of function of genes in a particular cell type. The Cre recombinase from bacteriophage P1 (Sternberg and Hamilton, 1981) excises intervening DNA sequences located between two unidirectional *lox* sites positioned on the same linear DNA segment, leaving one *lox* site behind. Fusion of the PEST-like sequence from listeriolysin O to the sequence encoding the Cre recombinase may facilitate its escape from the phagolysosme and gaining access to the cytosole of eukaryotic cells for further translocation into the nucleus where Cre-mediated gene deletion takes place. In cells that have previously engineered to express the Green Fluorescent Protein (GFP) flanked by *lox*-sites, facilitation of entry of the Cre recombinase by the PEST-like sequence would therefore result in GFP-negative cells.

5. Summary

In this study, the cholesterol-binding cytolysins, listeriolysin O (LLO) as well as pneumolysin (PLY), were expressed in the non-pathogenic species *Listeria innocua*. Growth of *L. innocua* recombinant strains in the chemically defined minimal medium resulted in 2-fold increment in the expression of both cytolysins. Purification from supernatant fluids was achieved by ion exchange chromatography. The procedure resulted in about 75 % (LLO) or 70 % (PLY) yield of a hemolytically active, highly purified homogenous 58 (LLO) or 52 (PLY) kDa protein which can be used to produce monospecific antibodies as well as in many diagnostic and serological procedures including procedures which were examined or developed in this study.

The identity and role of listerial antigens recognized by antibodies following listerial infection is largely unknown. In this study, a new and previously uncharacterized target of the humoral immune response, the listerial ferritin protein (Frm), was discovered and characterized. Specific antibodies to Frm are detected in antisera of mice infected with the *L. monocytogenes* wild type strain but not in antisera of mice infected with a non-pathogenic *L. innocua* strain. Antibodies raised to purified listerial ferritin allowed demonstration that expression of Frm is both growth phase- and temperature- dependent. Using an isogenic Δ*frm* mutant, ferritin was found to be essential for bacterial growth in chemically-defined minimal media but not in complex media such as BHI. Mutant bacteria also exhibited a defect in intracellular survival, probably in exiting from the phagosomal vacuole to the cytoplasm. The Δ*frm* strain is hypersensitive to hydrogen peroxide indicating that Frm mediates protection to reactive oxygen intermediates under various growth conditions. Mouse infection studies revealed that the listerial ferritin is required for efficacious bacterial growth early in the infectious process.

Although attenuated strains of microbial pathogens have triggered vaccine development from its origin, the role of virulence factors in determining host immunity has remained largely unexplored. In this study, using the murine listeriosis model, it was investigated whether the induction and expansion of protective and inflammatory T cell responses may be modified by selective manipulation of virulence genes. This was accomplished in two ways; the first was through generation of isogenic *Listeria monocytogenes* mutant strains that harboured either a specific deletion within the actin nucleator (*act*A) and/or multiple deletions within the *act*A and phospholipase B (*plc*B) genes while the second way was through the complementation of the non-pathogenic *Listeria innocua* strain with the PrfA-dependent virulence gene cluster (vgc) of the wild type *L. monocytogenes*. In comparison to the wild type *L. monocytogenes* EGD-e strain, the mutant strains were extremely low in virulence and were rapidly eliminated

by the host during the first days of infection. Nevertheless, a single immunization with mutant strains (EGDeΔ*act*A, EGD-eΔ*act*AΔ*plc*B and *L. innocua*: :vgc) has efficiently induced and maintained effector memory CD8+ T cells and provided animals with a state of long-lasting protective immunity against wild type *L. monocytogenes*. Moreover, these mutants were shown to exhibit a significantly reduced ability to induce CD4+ T cell-mediated inflammation. Therefore, they can be used as live vaccines against the corresponding virulent pathogen and as carriers for introducing heterologous protective antigens into animals and humans. Among the three mutants tested, the double-deletion mutant (EGD-eΔ*act*AΔ*plc*B) showed, beside its profound ability to induce protective CD8+ T cells, the most significant level of *in vivo* attenuation as well as the lowest ability to induce unfavourable CD4+ T cell-mediated inflammatory responses. Thus, this mutant, *L. monocytogenes* Δ*act*AΔ*plc*B, appears to be the most promising mutant as a bacterial vaccine vector.

In an attempt to address the role of the pore forming listeriolysin O (LLO) in intracellular survival of *L. monocytogenes* as well as in mediating a protective T- cell response against the wild type *L. monocytogenes* strain, two approaches were conducted in this study. Firstly, the structural gene for the related cytolysin pneumolysin (PLY) was cloned on a plasmid vector downstream from the promoter and signal peptide sequences of *hly*, the gene encoding LLO, and expressed in the isogenic EGD-eΔ*hly* mutant strain. The resultant recombinant strain secreted active PLY in culture supernatants and was able to escape phagosomes of phagocytic cells *in vitro* and spread from cell to cell to a limited time after which growth was aborted because of a cytotoxic effect on the host cell. This strain also showed a restricted *in vivo* survival in a mouse model of listeriosis but was able to protect mice against a lethal dose of the wild type *L. monocytogenes* and induced a specific T-cell responses against *Listeria* derived epitopes other than LLO such as the subdominant $P60_{217-225}$ epitope indicating that during the immune response to *L. monocytogenes*, immune splenocytes with specificity for LLO and other non-LLO-derived epitopes develop. These non-LLO epitopes serve as targets for antilisterial cytotoxic cells that confer antilisterial immunity.

The second approach was to study the role of the putative PEST-like sequence (P, Pro; E, Glu; S, Ser; T, Thr), found near the N-terminus of LLO, in virulence and intracellular compartmentalization of the wild type *L. monocytogenes* strain as well as in induction of a strong cell-mediated protective immunity to the wild type *L. monocytogenes*. Therefore, the 28 amino acids harbouring the PEST-like sequence at the N-terminus of LLO were deleted and the mutant LLO protein was expressed in a *hly*-negative isogenic mutant of *L. monocytogenes*. The mutant protein was secreted in normal amounts in the culture supernatant

and was fully haemolytic. *L. monocytogenes* expressing this truncated LLO showed a reduced capacity to escape the phagosomes of J774 cell line macrophages and showed a 1000-fold decrease in virulence in the mouse model. Moreover, the mutant strain showed a low ability to induce both an early serum level of gamma interferon and gamma interferon-secreting T cells following infection of BALB/c mice and exhibited reduced levels of protection against virulent *L. monocytogenes*. These results suggest that the PEST-like sequence is crucial for conferring a long lasting immunity against the wild type *Listeria monocytogenes* through facilitating the bacterial escape into the cytosole of host cells for further processing and antigen presentation.

6. Zusammenfassung

In der vorliegenden Arbeit wurden die Cholesterin-bindenden Cytolysine, Listeriolysin O (LLO) oder Pneumolysin (PLY) in dem apathogenen Stamm *L. innocua* exprimiert. Die Kultivierung von rekombinanter *L. innocua*-Stämme in einem chemisch definierten Minimalmedium führte zu einer zweifachen Steigerung der Expression von beiden Cytolysinen. Mit Hilfe der Methode der Ionenaustausch-chromatographie wurden die Proteine aus dem Überstand der Kultur aufgereinigt. Damit erhielt man in etwa 75% (LLO) bis 70% (PLY) Ausbeute von hämolytisch aktiven, mit hochreinen, homogenen Proteinen LLO (58kDa) und PLY (52kDa), die Verwendung bei der Produktion von spezifischen Antikörpern und in der Diagnostik und Serologie haben können.

Die Identität und Rolle von listeriellen Antigenen, die nach einer Infektion mit *L. monocyogenes* von Antikörpern erkannt werden , ist bisher noch weitgehend unbekannt.

In dieser Arbeit wurde das listerielle Ferritin (Frm) als neuer Angriffspunkt für die humorale Immunantwort nach einer Infektion von Mäusen mit pathogenen *L. monocytogenes* identifiziert. Spezifische Antikörper gegen Frm werden im Antiserum von mit dem Wildtyp *L. monocytogenes* infizierten Mäusen nachgewiesen, nicht aber im Antiserum von Mäusen, die mit dem apathogenen Stamm *L. inoccua* infiziert wurden. Antikörper, die aus aufgereinigtem Ferritin aus *Listeria* gewonnen wurden, zeigten, dass die Expression von Frm sowohl von Wachstumsphase als auch von Temperaturbedingungen abhängt. Bei der Verwendung einer isogenen Mutante Δfrm stellte sich heraus, dass Ferritin für das Bakterienwachstum in einem chemisch–definierten Minimalmedium essentiell ist, nicht aber in Komplexmedium wie z. B. BHI. Die Deletionsmutante wies außerdem einen Defekt im Überleben innerhalb der Wirtszelle auf, wahrscheinlich beim Schritt des Austritt aus dem Phagosom in das Cytoplasma. Die Mutante Δ*frm* ist hypersensitiv gegenüber Wasserstoffperoxid, was darauf schließen lässt, dass Frm für den Schutz gegen reaktive Sauerstoff-Intermediate unter unterschiedlichen Wachstumsbedingungen sorgt. Versuche am Mausmodell zeigten, dass das listerielle Ferritin für ein effizientes Bakterienwachstum in der frühen Infektionsphase notwendig ist.

Obwohl attenuierte Stämme pathogener Mikroorganismen die Entwicklung von Impfstoffen schon zu ihrem Beginn anführte, ist die Rolle von Virulenzfaktoren in Hinblick auf die Immunität des Wirtes größtenteils noch immer nicht erforscht. In dieser Arbeit wurde, mit Hilfe eines Listeriose-Mausmodells, untersucht, ob die Auslösung einer schützenden oder inflammatorischen T-Zell-Antwort durch die selektive Manipulierung von Virulenzgenen

modifiziert werden kann. Dies wurde auf zwei Wegen erreicht: Zum Einen erfolgte durch die Herstellung von isogenen *Listeria monocytogenes* Mutanten, die entweder eine spezifische Deletion im Gen für das Aktin-akkumulierende Protein (*actA*) und/oder multiple Deletionen im *actA* Gen und Phospholipase B (*plcB*) Gen trugen. Zum Anderen erfolgte eine Komplementierung eines nicht-pathogenen Stammes *Listeria inoccua* mit dem PrfA-abhängigen Virulenzgencluster (vgc) eines Wildtyps von *L. monocytogenes*.

Im Vergleich zum wildtypischen *L. monocytogenes* EGD-e waren die Virulenz der Mutanten extrem herabgesetzt, und sie wurden während der ersten Tage der Infektion rasch von Wirt eliminiert. Dagegen wurde durch die einmalige Immunisierung mit den Mutanten (EGDeΔ*act*A, EGD-eΔ*act*A Δ*plc*B and *L. innocua*: :vgc) sehr effizient eine Gedächtnis T-Zell-Antwort (CD8+) ausgelöst, die die Tiere mit einem lang-anhaltenden Immunschutz gegen den Wildtyp *L. monocytogenes* ausstattete. Darüber hinaus war die Fähigkeit der Mutanten signifikant reduziert, eine CD4+- T-Zell- vermittelte Inflammation zu stimulieren. Daher können sie als Lebendimpfstoff gegen die entsprechenden Erreger verwendet werden, und als Träger für die Bildung heterologer schützender Antikörper in Tier und Mensch dienen.

Unter den drei Mutanten, die getestet wurden, zeigte die Doppelmutante (EGD-eΔ*act*AΔ*plc*B), neben seiner ausgeprägten Fähigkeit, schützende CD8+-T-Zellen zu induzieren, den signifikantesten Grad an Attenuierung *in vivo* als auch die geringste Fähigkeit, eine ungewollte CD4+- T-Zell-vermittelte inflammatorische Antwort auszulösen.

Daher wird diese Mutante *L. monocytogenes* Δ*act*A Δ*plc*B zur Zeit als die vielversprochenste Mutante für einen bakteriellen Impf-Vektor angesehen.

In einem Versuch, die Rolle des Poren-bildenden Listeriolysin O (LLO) beim Überleben von *L. monocytogenes* in der Zelle als auch bei der Vermittlung einer schützende T-Zell-Antwort gegen den Wildtyp *L. monocytogenes* zu bestimmen, wurden in dieser Arbeit zwei Ansätze verfolgt. Erstens, wurde das Strukturgen für das ähnliche Cytolysin Pneumolysin (PLY) in ein Plasmid kloniert, downstream vom Promoter und der Sequenz des Signalpeptids von *hly,* dem für das LLO codierenden Gen, und das Konstrukt wurde in der isogenen EGD-eΔ*hly* Mutante exprimiert. Der resultierende rekombinante Bakterienstamm sezernierte aktiv PLY in den Kulturüberstand und war im *in-vitro* Experiment befähigt, den Phagosomen phagozytierender Zellen zu entkommen. Er konnte sich außerdem von Zelle zu Zelle ausbreiten. Der Stamm zeigte zudem im *in vivo* Versuch mit einem Listeriose-Mausmodell eine verringerte Überlebensrate, er war jedoch zugleich dazu in der Lage, Mäuse gegen eine letale Dosis des Wildtyps von *L. monocytogenes* zu schützen. In der Maus induzierte der

rekombinante Bakterienstamm eine spezifische T-Zellantwort gegen von Listeria-stammende Epitope, und zwar nicht gegen Epitope von LLO sondern solche wie das subdominante $p60_{217-225}$ Epitop. Dies lässt darauf schließen, dass sich während der Immunantwort auf *L. monocytogenes*, immungewordene Milzzellen mit Spezifität für LLO und andere nicht-LLO Epitope bilden. Diese nicht-LLO Epitope dienen als Angriffspunkte für anti-listerielle cytotoxische Zellen die zur Immunität gegen Listerien beitragen.

Der zweite Ansatz war die Untersuchung der Rolle der putativen PEST-ähnlichen Sequenz, die sich nahe des N-Terminus des LLO-Proteins befindet, in Bezug auf die Virulenz und auf die intrazelluläre kompartimentierung des Wildtyps *L. monocytogenes*, sowohl in Bezug auf die Induktion einer starken zell-vermittelten Immunität gegen den Erreger. Die 28 Aminosäuren am N-Terminus von LLO, die die PEST-ähnliche Sequenz beinhalteten wurden somit deletiert und die LLO Variante wurde in einer *hly*-negativen isogenen Mutante exprimiert. Das mutierte Protein wurde in normalen Mengen in den Kulturüberstand sezerniert und war in vollem Maße hämolytisch. Der Listerienstamm, der dieses veränderte LLO exprimierte, zeigte eine reduzierte Kapazität den Phagsosomen von J774 Makrophagen zu entkommen, und wies eine tausendfache Senkung der Virulenz im Mausmodell auf.

Darüber hinaus zeigte die Mutante eine herabgesetzte Fähigkeit, sowohl ein frühes Serumlevel an γ-Interferon als auch an γ-Interferon-sezernierenden T-Zellen nach der Infektion von BALB/c Mäusen zu induzieren, und dadurch kam es zu einem reduzierten Schutz gegen den virulenten Stamm *L. monocytogenes*. Diese Ergebnisse weisen darauf hin, dass die PEST-ähnliche Sequenz des reifen LLO entscheidend für die Erhaltung einer lang anhaltenden Immunität gegen den Wildtyp *L. monocytogenes* ist.

7. References

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Hereby, I declare on oath, that the thesis "*Listeria monocytogenes* as a vaccine vehicle: generation of attenuated mutants and their immunological characterization" is the product of my original research, and I did not use other sources or methods than those I have cited.
In addition, I declare that this thesis is not submitted to any another evaluation, neither in this form nor in another.
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