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RESEARCH ARTICLE

Bactofection of mammalian cells by Listeria monocytogenes: improvement and mechanism of DNA delivery

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Bacteria-mediated transfer of plasmid DNA into mammalian cells (bactofection) is a potent approach to express plasmid-encoded heterologous proteins (protein antigens, toxins or enzymes) in a large set of different cell types including phagocytic and nonphagocytic mammalian cells. Previously, we have described a Listeria monocytogenes-mediated DNA delivery system, which releases plasmid DNA directly into the cytosol of mammalian cells by partial self-destruction of the carrier bacteria. Here we report on a second generation of this phage lysin supported bactofection system, which is greatly improved with respect to plasmid stability, transfer efficacy and biosafety. In this case, DNA release is initiated by spontaneous bacterial lysis in the infected cells cytosol which is subsequently enhanced by the simultaneously released phage lysin produced by the intracellular carrier bacteria. Bacteria that are capable of cell-to-cell spread are found to be much more efficient in bactofection than their nonspreading counterparts.
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Introduction

Naked plasmid DNA encoding antigens under the control of strong eucaryotic promoters can induce antigen-specific humoral and cellular immune responses when injected intramuscularly[1] or intradermally. [2] Using this vaccination approach, protection of mammalian hosts against a variety of viral, bacterial and parasitic agents and even tumors has been demonstrated (for recent reviews see Donnelly *et al*[3] and Gūrūnathan *et al* [4]). However, this method requires highly purified DNA, and when applied without adjuvants leads to rather weak or even adverse immune responses probably due to limited costimulatory activity.[5]

Recent studies have indicated that bacteria, like *Shigella flexneri*,[6–9] *Salmonella* spp,[9,10] *E. coli*,[7,11] *Yersinia enterocolitica*[12] and *Listeria monocytogenes*,[9,13,14] can be used as carriers for transporting plasmids similar to those used as DNA vaccines into a variety of mammalian cells including antigen-presenting cells (APC). The term 'bactofection' has been recently coined for this bacteria-mediated DNA delivery into mammalian cells.[15] In addition to the easy manufacture and application, the carrier bacteria can also provide the necessary costimulatory effects when used as live vaccines.

Furthermore, intracellular bacteria can be applied to deliver eucaryotic expression plasmids to introduce relevant transgenes into somatic tissues to treat, cure or prevent diseases that result from genetic disorders.[16] One already reported example is the transfer of the human cystic fibrosis transmembrane conductance regulator (*cftr*) into epithelial cells, which recently was shown by using *L. monocytogenes* as carrier.[17] Likewise, *Salmonella typhimurium* were used to deliver genes encoding therapeutic proteins like IL-12 or GM-CSF to achieve antitumor effects in mice.[18]

The use of bacterial systems for gene therapy has the added advantage that bacteria often have an innate tropism for specific target tissues.

Shigella spp and *L. monocytogenes* reach the cytosol of infected host cells and deliver the DNA directly into this compartment upon lysis. *Salmonella, Yersinia* and *E. coli* remain in phagosomal compartments of infected host cells and it is basically unknown how the released DNA reaches the host cells nuclei where transcription of the antigen-encoding DNA occurs. In most reported cases, disruption of the bacterial carriers with subsequent release of the plasmid DNA occurs either spontaneously[9] within the infected cell or is facilitated by treatment with antibiotics[14] or use of specific auxotrophic mutants[6–8,10,11] that have a higher tendency of disintegration in the host cell intracellular milieu. The suicide *L. monocytogenes* carrier system that we have recently developed for DNA

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delivery into mammalian cells takes advantage of a phage lysin[19] that is specifically expressed once the bacteria enter the host cell cytosol.[13]

The highly improved system reported here is at least as efficient as all other previously described bacteria-mediated DNA delivery systems[6–14] with respect to the efficacy of bactofection and by far superior with respect to stability and biosafety.

Results

Construction of a novel balanced-lethal system for bactofection of mammalian cells

When applying the previously constructed suicide *L. monocytogenes* DNA delivery system[13] to BALB/c mice, we noticed high loss of the expression plasmid leading to a large population of 'empty' carrier bacteria. To stabilize the plasmid in the *L. monocytogenes* carrier strain, we first tested a number of different replication origins for stable replication and found that the one from pAMβ1[20] was most stable. This origin was taken for the construction of pUNK1 – the new parental plasmid for all further derivatives. Next, the *trpS* gene encoding tryptophanyl-tRNA synthetase was introduced into pUNK1, resulting in plasmid pSP0. We then inserted into pSP0 the previously described listerial autolysis cassette consisting of the lysis gene of phage A118 (*ply*118)[19] under the control of the *actA* promoter (P_{actA}) which is activated in the cytosol of infected mammalian host cells;[13] this plasmid is called pSP118. The two plasmids (pSP0, pSP118) are present in 95–100 copies per listerial cell as determined by real-time PCR (data not shown).

Additionally, we deleted the chromosomal *trpS* gene copy of the carrier strain *L. monocytogenes* EGD-e[21] which was only possible in the presence of the *trpS*-carrying plasmid pTRPS (for details, see Experimental protocol) indicating the expected essential function of this gene (Figure 1a and b). After growth of this strain (WL-140) for 50 generations in different culture media, we observed no plasmid loss as shown by replica-plating of colonies obtained from a brain–heart-infusion (BHI)-grown culture on tetracycline-containing agar plates (*tet* gene is carried by the plasmid). After insertion of various eucaryotic expression cassettes, all under the control of the immediate-early promoter of hCMV (P_{CMV}) into pSP0 and pSP118, the generated expression plasmids were transformed into WL-140 with subsequent removal of pTRPS (Figure 1c) by cultivation of WL-140 in medium without tetracycline.

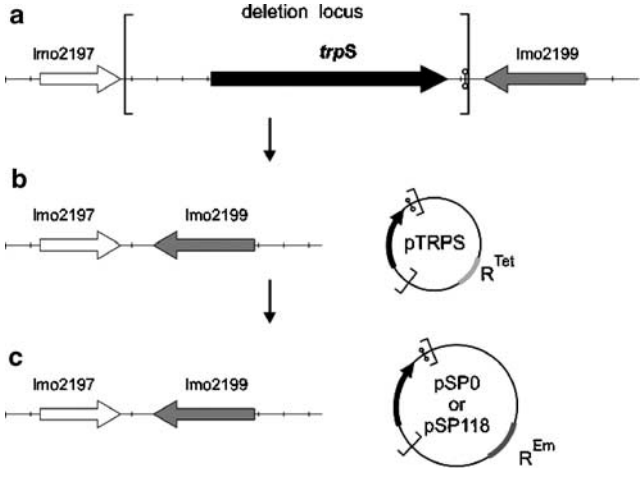

Figure 1 *Deletion of the trpS-containing region on the chromosome of the (a) L. monocytogenes strain EGD-e, (b) complementation of this region in the balanced-lethal ΔtrpS mutant strain WL-140 with plasmid pTRPS. The plasmid pTRPS contains a chromosomal fragment that comprises the promotor region of trpS, the trpS gene and its transcription terminator. (c) This plasmid can be replaced by the eucaryotic expression plasmids pSP0 and pSP118 due to different resistance markers: pTRPS – R^{Tet}, pSP0 and pSP118 – R^{Em}.*

Table 1 Bacterial strains and plasmids

Strains and plasmids	*Relevant genotype*	*Reference or source*
Listeria monocytogenes		
Sv1/2a EGD-e		Glaser *et al*[21]
WL-140	Δ*trpS*/pTRPS	This work
WL-141	Δ(*trpS aroA*)/pTRPS	J Stritzker
WL-142	Δ(*trpS actA*)/pTRPS	This work
Plasmids		
pTRPS	Tet^R, *trpS*	This work
pUNK1	Em^R, *ori*E1, *ori* pAMβ1	This work
pSP0	Em^R, *trpS*	This work
pSP118	Em^R, *trpS*, P_{actA}-*ply*118	This work
pSP0-EGFP	Em^R, *trpS*, P_{CMV}-*egfp*	This work
pSP118-EGFP	Em^R, *trpS*, P_{actA}-*ply*118, P_{CMV}-*egfp*	This work
pSP0-KMP	Em^R, *trpS*, P_{CMV}-KMP-11	This work
pSP118-DsRed	Em^R, *trpS*, P_{actA}-*ply*118, P_{CMV}-*rfp*	This work
P_{actA}-*gfp*	Tet^R, P_{actA}-*gfp*	Dietrich *et al*[13]

This *trpS*-based balanced-lethal plasmid carrier system was also found to be stable after a 4-day passage through BALB/c mice in contrast to the isogenic wild-type strain, both carrying the plasmid pSP0. The Δ*trpS* bacteria were found in equal amounts in liver and spleen as the wild-type bacteria indicating that the displacement of the *trpS* gene from the chromosome to the plasmid does not considerably affect the intracellular growth rate of this strain (see also Figure 6). While 37% of wild-type carrier bacteria lost this plasmid *in vivo*, all isolated Δ*trpS* bacteria still harbored plasmid pSP0.

For additional attenuation of the *L. monocytogenes* Δ*trpS* carrier strain, we deleted the *aroA* gene, which encodes the first enzyme in the biosynthesis of aromatic amino acids, rendering this strain dependent on all aromatic amino acids, resulting in strain WL-141. All strains and plasmids used in this study are summarized in Table 1.

Efficacy of bactofection of nonphagocytic cells by phage lysin-positive and -negative L. monocytogenes carrier strains

L. monocytogenes Δ*trpS* or Δ(*trpS aroA*) strains harboring either pSP0-EGFP or pSP118-EGFP (each carrying the cDNA for enhanced green fluorescent protein (EGFP) adapted to eucaryotic cells as a model antigen under the control of P_{CMV}) were used for bactofection of several nonphagocytic cell lines including Caco-2, HeLa, HepG2 (all epithelial cells) and COS-1 (fibroblasts). Efficacy of bactofection was determined by the number of EGFP-expressing cells, and the fate of the carrier bacteria inside

infected HeLa cells was followed by staining the bacteria with FITC-labelled anti-ActA antibodies. The highest number of EGFP-expressing cells (defined as bactofection rate) was observed with all cell lines 3 days after infection (Figure 2). All bactofections were carried out with a multiplicity of infection (MOI) of 5 since optimal bactofection rates were achieved using this ratio.

The Δ(*trpS aroA*) carrier strain harboring pSP118-EGFP yielded the highest bactofection rate in Caco-2 and COS-1 cells, while the *aroA*$^+$ carrier strain was more efficient in HeLa and HepG2 cells possibly due to the rather low growth rate of the Δ(*trpS aroA*) strain in these host cells (Figure 2a). Figure 2b shows also the rate of viable target cells (measured by the colorimetric reaction with MTT) after infection compared to uninfected cells which was highest for all four cell lines with the Δ(*trpS aroA*) carrier strain harboring pSP118-EGFP. Thus this carrier strain not only yields high bactofection rates but is also least harmful to the different mammalian cell lines tested.

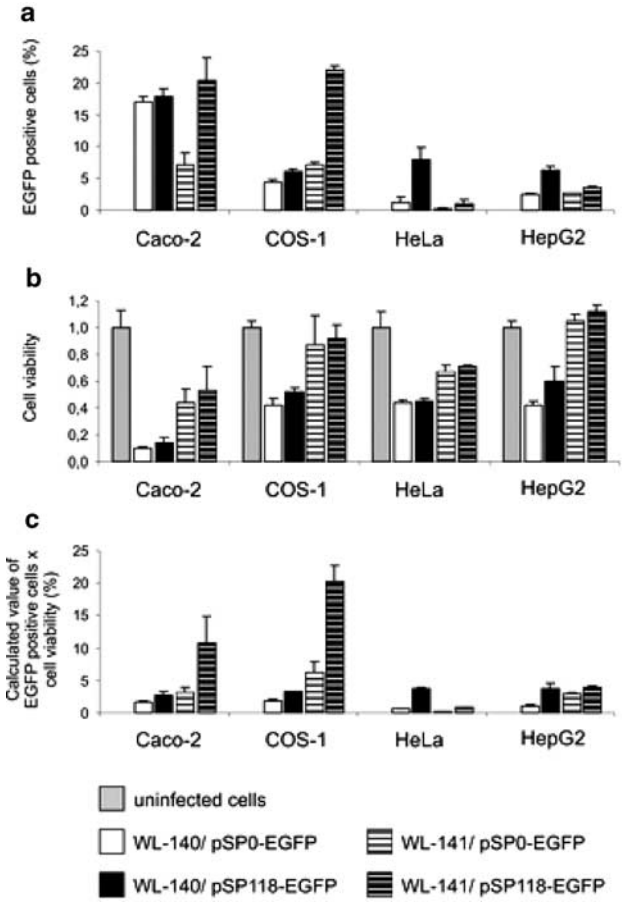

Figure 2 *Bactofection efficiency and cytotoxic effect of the applied L. monocytogenes strains carrying plasmids with or without phage lysin (pSP0-EGFP and pSP118-EGFP, respectively) in various cell lines (both parameters were determined 3 days after infection). (a) Propidium iodide (PI)-negative, EGFP-expressing cells (calculated as percentage of PI-negative cells), measured by flow cytometry. (b) Rate of viable cells after infection in comparison with uninfected cells (set to 1), measured with MTT. (c) Owing to the unequal cell toxicity of the different bacterial strains, the bactofection efficiencies were compared accurately by multiplying the percentage of PI-negative (viable), EGFP-expressing cells with the ratio of total viable cells.*

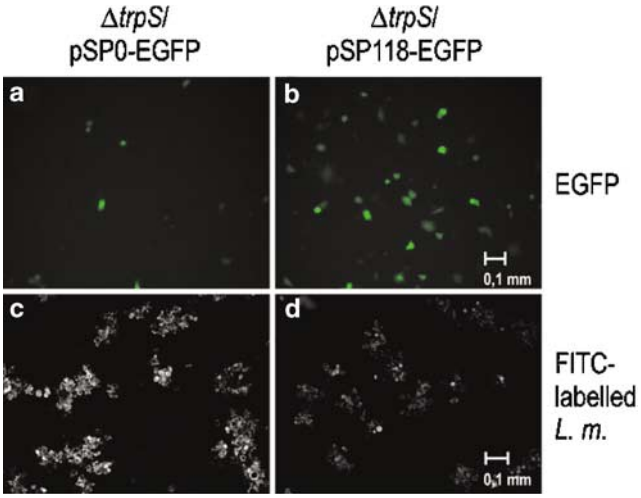

Figure 3 *Bactofection of HeLa cells with the L. monocytogenes ΔtrpS strain carrying the EGFP expression plasmids pSP0-EGFP (a + c) and pSP118-EGFP (b + d); plasmid pSP0-EGFP is without the phage lysin gene (ply118) and pSP118-EGFP harbors the lysin gene cassette P_{actA}-ply118. (a + b) EGFP expression in HeLa cells 24 h after bactofection; cells were infected with 5 bacteria per cell. (c + d) Staining of the intracellular carrier bacteria with FITC-labelled anti-ActA antibodies 24 h after bactofection; infection was performed with 0.1 bacteria per cell.*

EGFP expression and the fate of the intracellular carrier bacteria harboring either pSP0-EGFP or pSP118-EGFP were followed in more detail in HeLa cells (Figure 3). Uptake of both bacterial strains was similar (about 20% of the cell population were infected) when infection was performed with 5 bacteria/HeLa cell; the internalized *L. monocytogenes* pSP0-EGFP bacteria multiplied extensively within the next 24 h as visualized by the FITC-mediated fluorescence. Interestingly, spreading of these carrier bacteria was rather restricted in the HeLa cells (indicated by the rather small spreading halos shown in Figure 3c) compared to the other three nonphagocytic mammalian cell lines used (not shown). After 24 h, EGFP expression was observed in about 1% of the infected cells (Figure 3a) apparently by spontaneous lysis of the carrier bacteria in the infected HeLa cell. An approximately three-fold higher number of EGFP-expressing cells was obtained with *L. monocytogenes* carrying pSP118-EGFP (Figure 3b); in other experiments, we could also observe higher numbers of EGFP-expressing cells (Figure 2). Only weakly fluorescent bacteria were observed in the infected cells (Figure 3d), but the number of colony-forming bacteria was comparable to that of *L. monocytogenes* carrying pSP0-EGFP (data not shown) indicating surprisingly efficient replication of these phage lysin-expressing bacteria inside HeLa cells. We assume that the weak staining of the pSP118-EGFP-carrying bacteria is caused by reduced production of ActA due to titration of PrfA by the high copies of the *actA* promoter in front of *ply*118 carried on pSP118-EGFP rather than being a sign of bacterial lysis.

Bactofection of phagocytic cells and presentation of EGFP epitopes by P338.D1 macrophages

In vitro bactofection of macrophages was unsuccessful or was obtained with very low efficacy using the previously described listerial carrier systems[9,14] including our first *L. monocytogenes* suicide system.[13]

In contrast, bactofection of different phagocytic cells could be successfully achieved with the new *L. monocytogenes* balanced-lethal system. The efficiencies of EGFP expression were always considerably higher (10-fold and more) with the *L. monocytogenes* strains carrying pSP118-EGFP than with the same carrier strain carrying pSP0-EGFP (Figure 4a and b), while the numbers of intracellular bacteria producing phage lysin were strongly reduced (Figure 4c). With this improved system, EGFP expression could be obtained in up to 2.5% of infected P388.D1 macrophages which is more than 10-fold higher than with the previously described system.[13] As shown in Figure 4b, the number of EGFP-expressing macrophages, infected with the carrier strain harboring pSP118-EGFP, increases within 3 days after infection. The highest bactofection rate (about 1–2%) was obtained with the macrophage line P388.D1, while isolated human blood-derived dendritic cells (DC) yielded bactofection rates of about 0.3–1% (Figure 5a and c). In the latter case, the obtained bactofection rates varied considerably and depended strongly on the individual donator of the DCs. The bactofection rate always seems to coincide with the efficiency of infection of the DC by the carrier bacteria

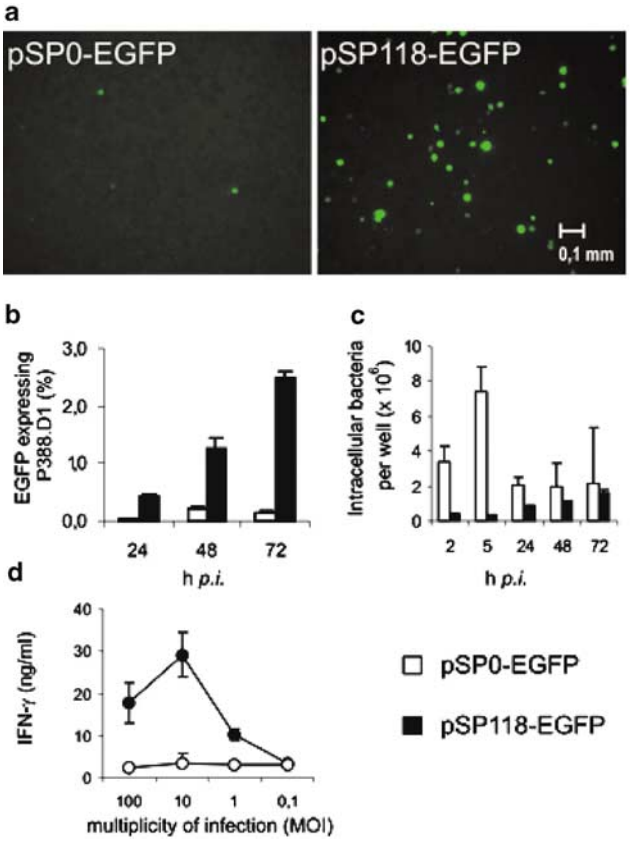

Figure 4 *Bactofection of phagocytic cells. (a) EGFP expression after bactofection of P388.D1 macrophages with L. monocytogenes ΔtrpS carrying plasmids pSP0-EGFP or pSP118-EGFP, 72 h postinfection (p.i.). (b) PI-negative, EGFP-expressing P388.D1, 24–72 h p.i. (calculated as percentage of PI-negative cells measured by flow cytometry). (c) Number of intracellular bacteria per well, 2–72 h p.i. (d) MHC class I restricted presentation of the EGFP-derived CD8 T cell epitope HYLSTQSAL by P388.D1 macrophages after bactofection with L. monocytogenes ΔtrpS carrying plasmids pSP0-EGFP or pSP118-EGFP. Activation of the T cells by the bactofected macrophages was determined by the amount of released IFN-γ.*

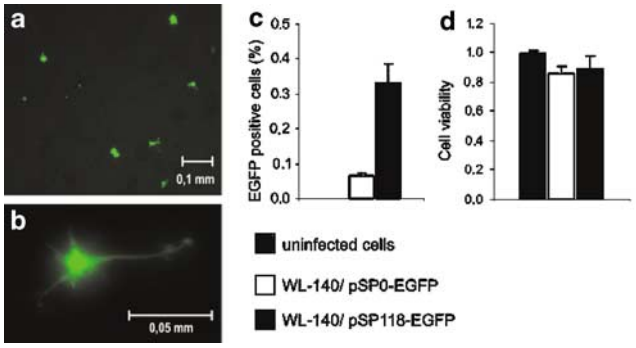

Figure 5 *Bactofection of human dendritic cells (DC). (a,b) EGFP expression after bactofection of human DC with L. monocytogenes ΔtrpS carrying plasmids pSP0-EGFP or pSP118-EGFP, 24 h p.i. (c) EGFP-expressing DC, 24 h p.i. (calculated as percentage of all CD83-positive cells measured by flow cytometry). (d) Rate of viable cells after infection in comparison with uninfected cells (set to 1), measured with MTT.*

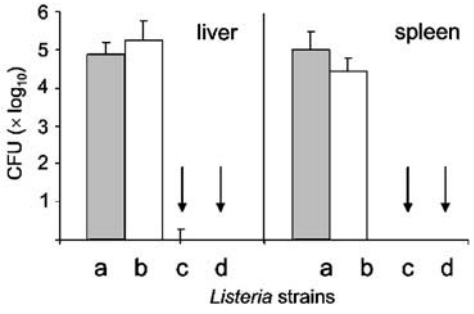

Figure 6 *Bacterial load in liver and spleen of orogastrically infected BALB/c mice (4 days p.i.) with 10⁹ bacteria of (a) L. monocytogenes wild-type, (b) ΔtrpS strain (WL-140) with plasmid pSP0, (c) ΔtrpS strain carrying plasmid pSP118 and (d) Δ(trpS aroA) strain (WL-141) carrying plasmid pSP0.*

and their rate of survival within the DC (data not shown).

Remarkably, DC infected with bacteria carrying pSP0-EGFP showed virtually no EGFP expression. EGFP expression in DC was notably stronger compared to other cell types (Figure 5b) and was already observed 20 h after infection with WL-140 carrying pSP118-EGFP. The viability of DC after infection was comparable to that of uninfected DC and was independent of the listerial strain used (Figure 5d). Primary murine bone marrow macrophages (BMM) also yielded bactofection rates of about 1% with this carrier strain (data not shown).

Using an EGFP-specific CD8 T cell line recognizing the epitope HYLSTQSAL,[22] we could show that P388.D1 macrophages bactofected with *L. monocytogenes* containing pSP118-EGFP are recognized by T cells (measured by the production of gamma interferon (IFN-γ); Figure 4d) indicating that EGFP-expressing macrophages can present EGFP epitopes after bactofection as expected in the context of MHC class I molecules. Optimal activation of the EGFP-specific CD8 T cell line was obtained when the macrophages were infected at a ratio of 10 carrier bacteria per macrophage (Figure 4d). Lower IFN-γ values were obtained when higher (MOI of 100) or lower infection ratios (MOI of 100) were used which is probably due to increased cell toxicity in the former case

and inefficient bactofection rates of the macrophages in the latter case.

Virulence of the L. monocytogenes plasmid-delivery strains in the mouse model

Previous bactofection studies with *L. monocytogenes* as carrier of eucaryotic expression plasmids have used wild-type strains in order to obtain significant transfection rates.[9] Such bacterial carriers can hardly be applied for gene therapy or vaccination due to their high virulence.

To test the virulence of our new *L. monocytogenes* plasmid delivery strains, we performed orogastric infections in BALB/c mice using 2×10^9 bacteria of *L. monocytogenes* EGD-e wild-type and the Δ*trpS* mutant strain each carrying pSP0 or pSP118 and the Δ(*trpS aroA*) strain with plasmid pSP0. At 4 days after infection, the bacterial load was determined in spleen and liver. As shown in Figure 6, roughly equal viable bacterial counts were observed in both organs after infection with the wild-type strain and the Δ*trpS* strain carrying pSP0. No viable bacteria were detected in both organs after infection with the Δ*trpS* strain carrying pSP118, indicating strong virulence attenuation of the Δ*trpS* carrier strain with pSP118. Similar high attenuation was obtained with the Δ(*trpS aroA*) mutant carrying pSP0; this attenuation is expected to be further enhanced in the pSP118-carrying strain. Thus the Δ*aroA* mutation of the *L. monocytogenes* carrier WL-141 (Δ(*trpS aroA*) harboring pSP118 as expression plasmid may represent not only a very efficient vector system for bactofection, at least *in vitro*, but may warrant also the highest biosafety for *in vivo* studies.

Mechanism of bacterial lysis and plasmid release in the cytosol of mammalian cells

The above-described data indicate that release of plasmid DNA can occur in the absence of phage lysin. The extent of this apparently spontaneous bacterial lysis with subsequent plasmid release seems to differ in the used mammalian cells (Figure 2); similar observations were also made with other bacterial carriers.[9] One may therefore argue that disruption of the listerial carrier does not solely occur in the cytosol of the bactofected cell but may be initiated already by partial damage of the bacteria in the phagosomal compartment which they have to pass before entering the cytosol.

To avoid passage of the bacterial carrier through phagosomal compartments, we microinjected a spreading-deficient Δ*actA* mutant of the *L. monocytogenes* Δ*trpS* strain (WL-142) harboring either pSP0-EGFP or pSP118-EGFP into Caco-2 cells as recently described.[23] By coinjection of Texas red-labelled dextran together with the bacterial carrier strains (Figure 7, right panel) we observed that EGFP expression occurred in the microinjected cell 24 h after microinjection (indicated by the yellow fluorescence caused by the overlap of the green EGFP and the red fluorescence of Texas red). As summarized in Figure 7, about 5% of the cells microinjected with the *L. monocytogenes* Δ(*trpS actA*) strain harboring pSP0 and more than 13% of those harboring pSP118 expressed EGFP. There was no further increase but even a decrease in the number of EGFP-expressing cells after extended incubation (48 and 72 h) of the

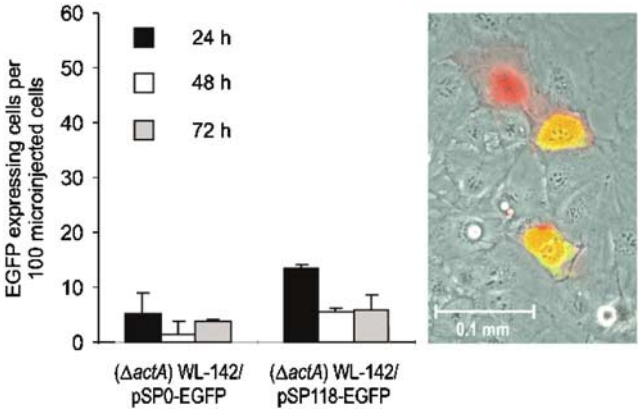

Figure 7 *EGFP expression of Caco-2 cells after microinjection with Δ(trpS actA) mutant Listeriae (WL-142) carrying pSP0-EGFP or pSP118-EGFP. EGFP-expressing Caco-2 cells were counted 24, 48 and 72 h after bactofection. The micrograph shows Caco-2 cells 24 h after microinjection with the Δ(trpS actA) strain carrying pSP118-EGFP. Primarily microinjected Caco-2 cells appear red due to coinjection of Texas red-labelled dextran together with the bacteria, and yellow cells indicate EGFP expression in the primarily microinjected cells due to the overlay of red (Texas red) and green (EGFP) fluorescence.*

microinjected cells, which seems to be due to enhanced cell death with time. These data support the assumption that DNA release is initiated in the cytosol by spontaneous lysis of at least some carrier bacteria.

To further analyze the mechanism of lysis of *L. monocytogenes* in bactofected cells, we replaced in pSP118 the cDNA of EGFP by cDNA of the red fluorescent protein (DsRed) or by the gene encoding the *Leishmania* antigen KMP-11 (the expression of which was monitored by a red-fluorescent LRSC-labelled monoclonal antibody). The intracellular bacteria were tagged either with a plasmid containing the previously reported P_{actA}-*gfp* cassette which results in GFP expression when *L. monocytogenes* replicates in the mammalian cell cytosol[13] or with an FITC-labelled anti-ActA antiserum. After infection into HeLa cells, we could now simultaneously determine the fate of the carrier bacteria and the expression of the two delivered genes. As shown in Figure 8, most infected HeLa cells that did not express DsRed or KMP-11 carried many bacteria (green fluorescent) after 24 h, whereas those cells that expressed either of the two (red fluorescent) proteins contained only few or no detectable bacteria. Qualitatively similar results were obtained regardless of whether the bacteria harbored pSP0 or pSP118 suggesting that spontaneous lysis of a single carrier bacterium may release not only plasmid DNA but also the phage lysin (in the case of pSP118) or other bacteriolytic enzymes (in the case of pSP0 and pSP118), which subsequently lyse all other carrier bacteria in the cytosol of the mammalian cell. The more efficient lysis of the pSP118-harboring bacteria then leads to higher bactofection rates.

Bactofection rate is strongly enhanced by spreading-competent bacteria

The rather low bactofection of Caco-2 cells observed by microinjection of the spreading-incompetent *L. monocytogenes* Δ(*trpS actA*) mutant strain (Figure 7) prompted us to further analyze the effect of spreading on bactofection. For this, we microinjected the *actA*$^+$ listerial carrier

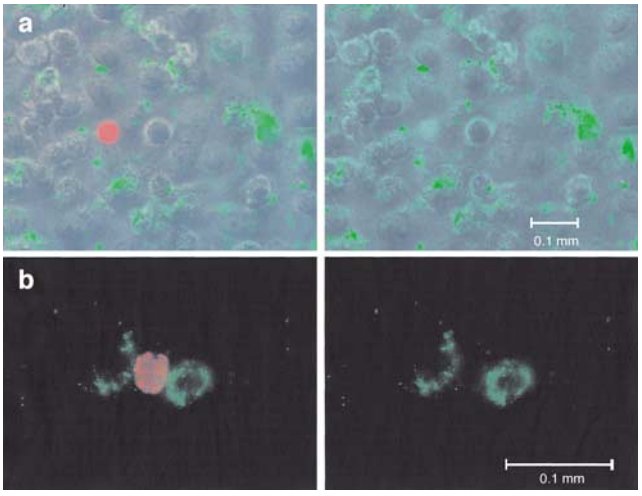

Figure 8 *Colocalization of HeLa cells expressing either red fluorescent protein (DsRed) or the leishmanial antigen KMP-11 and the intracellular Listeriae (green) 24 h after bactofection. (a) The listerial ΔtrpS carrier was equipped with plasmid pSP0-DsRed for eucaryotic DsRed expression and plasmid P_{actA}-gfp for bacterial expression of GFP. The phase contrast microscopic picture shows cell infected with many green fluorescent bacteria and one cell expressing DsRed with no or few green fluorescent bacteria. (b) The ΔtrpS carrier strain harbored plasmid pSP118-KMP for eucaryotic expression of the leishmanial antigen KMP-11, which was determined by staining with an LRSC-labelled anti-KMP-11 mAb (red) and the bacteria were visualized with FITC-labelled anti-ActA antiserum (green). The picture shows one cell expressing KMP-11 visualized with red fluorescent antibodies but no bacteria and neighboring cells containing many bacteria visualized by green fluorescent antibodies, but not expressing KMP-11.*

strain (WL-140) harboring pSP0-EGFP or pSP118-EGFP into Caco-2 cells as described in Figure 7 for the Δ(*trpS actA*) mutant. As shown in Figure 9, low numbers of EGFP-expressing cells were obtained 24 h after microinjection, but in contrast to the Δ(*trpS actA*) mutant strain these numbers increased very significantly at later time points (48 and 72 h). Interestingly, at these time points, EGFP expression was rarely found in the primary microinjected Caco-2 cells (red fluorescent due to coinjected Texas red) but rather in cells in the vicinity of the microinjected ones (green fluorescent Caco-2 cells due to EGFP expression). These cells can be accessed only by spreading carrier bacteria. Such events were never observed with the isogenic Δ(*trpS actA*) mutant strain, ruling out the possibility that reinfection of Caco-2 cells by bacteria released from lysed primary microinjected cells is responsible for these events. In addition, reinfection is highly unlikely due to the presence of gentamicin in the culture media during the entire experiment.

To better quantify this 'spreading effect', we compared the efficiency of bactofection of Caco-2 cells by the *L. monocytogenes* Δ(*trpS actA*) mutant strain harboring pSP0-EGFP or pSP118-EGFP with that of the isogenic *actA*$^+$ strain. Both carrier strains (applied at an MOI of 5) were initially taken up by about 20% of the Caco-2 cells at similar frequencies (Figure 10a). While the Δ(*trpS actA*) mutant strain remained in the primarily infected cells (Figure 10c), the *actA*$^+$ carrier strain infected virtually all cells in the cell layer by spreading during the course of infection (Figure 10d). The number of EGFP-expressing cells obtained after 48 h postinfection with the Δ(*trpS*

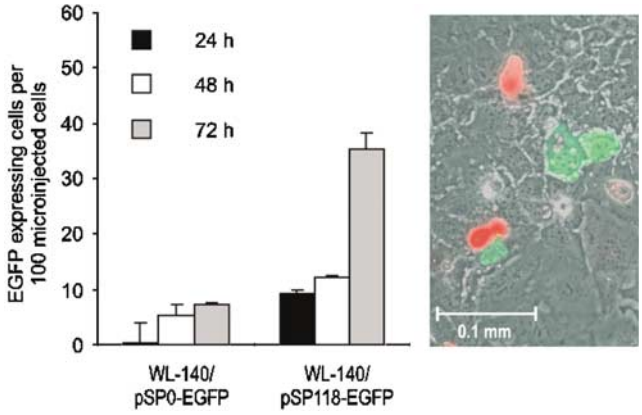

Figure 9 *EGFP expression of Caco-2 cells after microinjection with the spreading-competent ΔtrpS strain (WL-140) carrying pSP0-EGFP or pSP118-EGFP. The experiment was performed as described in the legend to Figure 2. Primarily microinjected Caco-2 cells appear red due to coinjection of Texas red-labelled dextran together with the bacteria, and green fluorescent cells indicate EGFP-expressing Caco-2 cells in the vicinity of the primarily microinjected cell.*

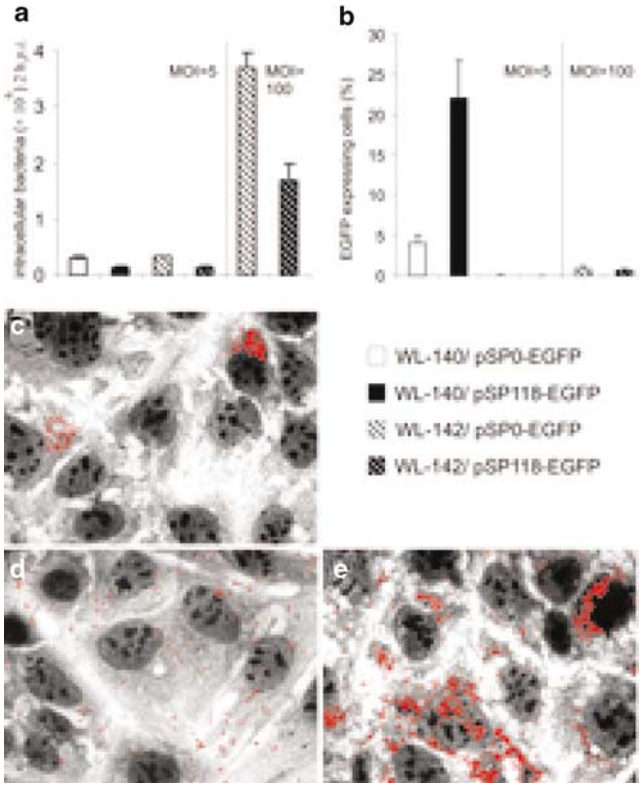

Figure 10 *Efficiencies of bactofection of Caco-2 cells by the L. monocytogenes ΔtrpS (WL-140) and Δ(trpS actA) (WL-142) mutant strains both carrying plasmids pSP0-EGFP or pSP118-EGFP after infection of Caco-2 cells with 5 or 100 bacteria per cell. (a) Average number of intracellular bacteria per well containing 5×10^5 Caco-2 cells 2 h after bactofection (performed in triplicate). (b) EGFP-expressing cells (% of all cells measured by flow cytometric analysis) 48 h after bactofection. (c) L. monocytogenes Δ(trpS actA) (WL-142) mutant bacteria, growing in microcolonies in initially infected Caco-2 cells (applied at an MOI of 5). (d) ΔtrpS (WL-140) bacteria, also applied at an MOI of 5, infected nearly all Caco-2 cells by cell-to-cell spread. (e) Δ(trpS actA) (WL-142) mutant bacteria infect nearly all cells when applied at an MOI of 100. All pictures were taken 6 h p.i. after Giemsa staining. Bacteria were highlighted in red.*

actA) mutant strain was more than 20-fold lower (Figure 10b) than with the *actA*$^+$ strain.

However, even infection with 100 Δ(*trpS actA*) mutant bacteria per Caco-2 cell, which resulted in at least as many infected Caco-2 cells (Figure 10e) and even more intracellular bacteria than obtained with the *actA*$^+$ strain by spreading (Figure 10a), yielded still at least 10-fold less EGFP-expressing Caco-2 cells than the *actA*$^+$ strain (Figure 10b). This result further supports the notion that not only the number of intracellular carrier bacteria or the number of initially infected cells but also their spreading capacity are important factors determining the efficiency of bacterial lysis and release of plasmid DNA.

Discussion

This report describes a greatly improved *L. monocytogenes*-mediated plasmid DNA delivery system for mammalian cells (bactofection[15]). Our new bactofection system consists of the *L. monocytogenes* EGD-e strain[21] with deletion in *trpS* and for additional attenuation in *aroA*, carrying a plasmid pSP118 with four characteristic features: (1) a reasonably small size (9.2 kb); (2) an origin of replication highly stable in *L. monocytogenes*; (3) the *trpS* gene with its own promoter allowing *in vitro* and *in vivo* growth of the thus stabilized Δ*trpS* carrier strain at a similar rate as the wild-type strain; (4) the previously reported lysis cassette[13] which expresses the phage lysin 118[19] once the carrier bacteria enter the cytosol of the infected mammalian cells. Although this phage lysin remains in the cytoplasm of the producing carrier bacteria and does not lyse all intracellular bacteria, it proved to be sufficient for lysis of the intracellular bacteria, particularly in combination with the Δ*aroA* mutation.

Bactofection of several nonphagocytic and phagocytic mammalian cells with the optimized *L. monocytogenes* system (determined mainly by the expression of the plasmid-encoded EGFP cDNA under the control of P_{CMV}) was more than 10-fold enhanced compared to our previously described system[13] and at least comparable to other reported bactofection systems.[9,14] Bactofection efficacy was highly dependent on the mammalian cell lines used as also observed in other studies[9,14] and was between 5 and 20% for the nonphagocytic cells tested.

Bactofection of phagocytic cell lines, primary murine macrophages and human DC with this new system was also much higher than with our previously reported system,[13] but still less efficient than that of the nonphagocytic cell lines. The fact that lipofection or direct microinjection of the same plasmid into these cells also yielded lower transfection rates than into nonphagocytic cells (data not shown) suggests that enhanced degradation or inefficient expression of the introduced DNA may be responsible for the reduced bactofection of the phagocytic cells. Nevertheless, efficient antigen (EGFP) presentation together with MHC class I molecules was observed in bactofected macrophages as determined by the activation of an EGFP-specific CD8 T cell line.

Bactofection requires the disruption of the carrier bacteria. Our data obtained by microinjection and infection of Caco-2 cells with the new vector system indicate that bactofected cells expressing DsRed or a leishmanial antigen contained only few or no detectable intracellular bacteria, whereas the nonexpressing cells carried large numbers of bacteria. This suggests that bacterial cell disruption and subsequent plasmid release is an autocatalyzing process probably initiated by the spontaneous lysis of a single bacterial cell which releases phage lysin (in the case of pSP118) and other peptidoglycan-hydrolyzing enzymes subsequently disrupting most other carrier bacteria present in the target cell thus resulting in the release of sufficient plasmid DNA to warrant expression of the plasmid-encoded gene by the target cell. The observation that microinjection of *S. typhimurium* carrying the same plasmid (pSP118) yields considerably fewer EGFP-expressing cells than even the *L. monocytogenes* Δ*trpS* strain with pSP0 supports this assumption; *S. typhimurium* is virtually unable to replicate in Caco-2 cell cytosol after microinjection[23] and hence the amount of plasmid DNA released by spontaneous lysis of these carrier bacteria will be rather low and hence EGFP expression less efficient.

A highly interesting observation is the strongly increased bactofection rate (defined by the number of EGFP-expressing mammalian cells) by spreading-competent *L. monocytogenes* carrier bacteria compared to isogenic spreading-deficient ones. This is not solely due to the larger number of infected target cells obtained by spreading carrier bacteria since the same number of cells infected with spreading-deficient bacteria (obtained by a higher MOI of the latter carrier bacteria) yield still a considerably lower number of bactofected cells. In addition, microinjection of Caco-2 cells with spreading-competent *L. monocytogenes* carrying pSP118 showed that most EGFP-positive cells were not the microinjected cells but cells located in the vicinity of primary microinjected cells. Such EGFP-positive satellite cells were not observed with spreading-incompetent *actA* mutant carrier bacteria ruling out the possibility that reinfection of Caco-2 cells by bacteria released from lysed primary microinjected cells is responsible for the generation of these EGFP-expressing satellite cells. These results suggest that spreading *L. monocytogenes* bacteria (especially when harboring pSP118) are (for yet unknown reasons) more lysis sensitive than bacteria remaining in the infected target cell.

It seems difficult to imagine a mechanism by which a listerial strain yields the optimal DNA release when it has (a) to be lysed in the cytosol, (b) to grow intracellularly and (c) to spread from cell to cell. A possible explanation may be that more the bacteria are in the cytosol, the higher the probability of destructing a bacterial cell of this intracellular bacterial population which will release the produced phage lysin. This lysin will destruct more bacteria thus amplifying the release of additional phage lysin and of plasmid DNA. Spreading bacteria that have to pass again phagosomal compartments may become more sensitive to lysis than bacteria that remain only in the host cells cytosol. Thus we propose that intracellular listeriae are gradually lysed during the course of infection of a cell layer. During an *in vivo* infection, all these processes are occurring and the production of the phage lysin causes a very efficient destruction of the carrier bacteria.

Taken together, our results indicate that bactofection with the optimized *L. monocytogenes* vector system is

highly efficient in transfering eucaryotic expression plasmids into mammalian cells. In addition to the high DNA delivery capacity, the *L. monocytogenes* Δ*trpS* carrier strain harboring pSP118 and the Δ(*trpS aroA*) carrier strain harboring pSP0 are well attenuated in the mouse model. If the attenuation of this carrier should prove to be too rigorous under *in vivo* conditions, we can lower its attenuation by modulating the expression of the phage lysin within the host cell cytosol by using different variants of the *actA* promoter. This live vector system, which seems to be not only suitable for the release of DNA but also of RNA (C Schoen *et al*, unpublished results) and of biologically active proteins (J Stritzker *et al*, unpublished results), may thus be applicable without risk in genetic therapy approaches[17,18] and vaccine development against infectious agents and tumors.[31]

Experimental protocol

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* DH10b was used as host for all DNA manipulations.

For bactofection, *Listeria* strains were grown to a late logarithmic growth phase (180 Klett units resp. 150 Klett units using the Δ(*trpS aroA*) strain) at 37°C in BHI, washed with phosphate-buffered saline pH 7.4 (PBS), resuspended in 20% (v/v) glycerol/PBS and stored at −80°C.

Plasmid pUNK1 was constructed by amplifying fragment OriE1 (681 bp) with primers ori1 (5′-AAAAAAGAATTCGCCAGCAAAAGGCCAGGA-3′) and ori2 (5′-AAAAAAGAATTC-ACTGAGCGTCA GACCCCG-3′) and pUC18 as template. The fragment OriE1 and plasmid pIL253 (Gene Bank accession no. AF041239) were cut with *Eco*RI and ligated, resulting in pUNK1 (5586 bp). To construct the complementation plasmid pTRPS, the *trpS*$^+$ expression cassette was amplified using primers trpS1 (5′-TGTTATGTC GACTAGTATTTTATG-3′) and trpS2 (5′-GGTAACGTC GACGTGGAAATT-AAA-3′) and genomic DNA of *L. monocytogenes* EGD-e as template. After digestion with *Sal*I, this cassette was cloned into shuttle plasmid pFLO1 (with a tetracycline resistance marker), resulting in pTRPS.

Construction of Listeria mutant strains

The Δ*trpS* and the Δ(*trpS actA*) deletion mutant (called WL-140 and WL-142) were constructed using *L. monocytogenes* EGD-e as parental strain. Deletion mutagenesis were performed by a homologous recombination technique using constructs derived from the mutagenesis vector pLSV1.[24] Primers trpS-A1 (5′-AA GAAA-TGTGGATCCGAATTACTATTT-3′) and trpS-A2 (5′-AGTTTACAACCCGGGTTGTCA-ATCACA-3′) were used to amplify a 342 bp fragment which was localized upstream of the deletion locus and a second downstream fragment (382 bp) was amplified with the primers trpS-B1 (5′-CGATGTTACCCCGGGTTGCTTTAGAAT-3′) and trpS-B2 (5′-AATTAGGAG-GAATTCAAAATGAA AAAA-3′). Both fragments were digested with *Psp*AI, ligated and further amplified with primers trpS-A1 and trpS-B2 mentioned above. The double fragment was then cloned into pLSV1 using *Eco*RI and *Bam*HI restriction and the resulting knockout plasmid was transformed into *L. monocytogenes* EGD-e. After cultivation at 42°C and with 5 μg/ml erythromycin, clones with a chromosomally integrated knockout plasmid could be selected which were additionally transformed with a *trpS*$^+$ expression plasmid (pTRPS) for *trans*-complementation of the gene. These resulting clones were further cultivated at 30°C without erythromycin to obtain the Δ*trpS* deletion mutant, which has a chromosomal deletion of the *trpS* gene but harbors a plasmid-coded *trpS*$^+$ expression cassette.

WL-140 (Δ*trpS*) was used as parental strain for the construction of the double-mutant strain WL-142 which harbors an additional Δ*actA* mutation. First, a specific knockout plasmid was made with a similar strategy illustrated above: primers actA-A1 (5′-AAAAAAGGATCC-AATCGCTTCCACTCACAGAGG-3′) and actA-A2 (5′-AAAAAACCCGGGCACTTATACTC CCTCCTCGTG-3′) were used to amplify an upstream fragment (467 bp), and a second 346 bp fragment which is localized downstream of the *actA* gene locus was amplified with primers actA-B1 (5′-AAAAAACCCGG GAATAATTAAAAACAC-AGAACG-3′) and actA-B2 (5′-AAAAAAGAATTCCCTTGAGCTATTTGTTTATCG-3′). Both fragments were cut with *Psp*AI, ligated and a large fragment was then obtained by PCR amplification with primers actA-A1 and actA-B2 using the ligation mixture as template. The large fragment was introduced into pLSV1 with the same restriction enzymes mentioned above, resulting in a specific Δ*actA* knockout plasmid. After electroporation of this plasmid into WL-140, plasmid integration and excision were executed as described above, resulting in the Δ(*trpS actA*) double-mutant strain WL-142.

Cell culture and infection experiments

Caco-2 (human colon adenocarcinoma), COS-1 (monkey African green kidney), HeLa (human cervix epitheloid carcinoma) and P388.D1 (murine lymphoid macrophage) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine (Gibco) and 10% fetal calf serum (FCS, Biochrom, Berlin, Germany); HepG2 (human hepatocyte carcinoma) were grown in Eagle's minimal essential medium with Earl's salts supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 0.15% sodium bicarbonate (all from Gibco) and 10% FCS. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere. For bactofection, cells were seeded into 24-well plates 1 day prior to infection. After a wash step with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS(Ca^{2+}/Mg^{2+})), 2×10^5 cells were infected with an MOI of 5 (if not otherwise indicated) bacteria per cell for 1 h. The cells were washed three times with PBS(Ca^{2+}/Mg^{2+}) and cultivated with gentamicin-containing medium (100 μg/ml) which was replaced with medium containing 10 μg/ml gentamicin after 1 h. Viable bacterial counts of intracellular bacteria were determined by plating serial dilutions of mechanically lysed cell suspensions on BHI-agar.

Isolation of human DCs from peripheral blood and infection assay

Human MoDC were prepared from peripheral blood mononuclear cells (PBMC) as described in detail in

Kolb-Mäurer *et al*.[25] On day 7, cells were transfered to 24-well plates in RPMI supplemented with 3% human autologous plasma at a density of 5×10^5 cells/ml and bacteria were added. After incubation for 1 h, medium was replaced by RPMI 1640 medium containing 3% human autologous plasma and 100 μg/ml gentamicin. After 1 h, medium was replaced by RPMI 1640 medium containing 3% human autologous plasma and 15 μg/ml gentamicin.

Cell viability assay

The amount of viable cells after infection with bacteria was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), following the manufacturer's instructions. At 1, 2 or 3 days after infection of cells, medium was replaced by 0.2 ml MTT solution at a concentration of 2.5 mg/ml MTT dissolved in RPMI 1640 without phenol red and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. After removal of the MTT solution, the color reaction was stopped by adding 1 N HCl diluted in isopropanol. The probes were measured at a wavelength of 570 nm. Uninfected cells were used as a reference and were considered as 100% viable.

Flow cytometry analysis

At 1, 2 or 3 days after infection, cells were washed with PBS, trypsinized and resuspended in PBS. Cell viability was determined by staining cells with propidium iodide (PI) (1 μg/ml). Since nonviable cells tend to fluoresce at a similar wave length as EGFP, PI-positive cells were gated out from the measurement. A minimum of 5×10^4 cells (or 10^5 phagocytic cells) were then measured using an Epics XL flow cytometer (Beckman Coulter). DC were additionally stained with PE anti-human CD83 (BD Biosciences Pharmingen).

Immunofluorescence analysis

According to a previously described procedure,[26] cells were grown on glass cover slides and infected as described above. After 12 h, the cell layer was washed and fixed as described in Kuhn *et al*.[26] Cells were stained with an antiserum against ActA[27] or monoclonal antibodies (mAb) directed against kinetoplastid membrane protein-11 (KMP-11) (Cedarlane Laboratories) for 1 h, followed by a wash step with PBS and afterwards stained with an FITC- or Lissamine Rhodamine (LRSC)-labelled goat anti-rabbit IgG or (Dianova) for another hour. Cells were then pictured with a fluorescence-equipped microscope (Leica DMR HC) and an electronic camera (Diagnostic Instruments Inc.). Digital images were processed using META-MORPH software (Universal Imaging, Media, PA, USA).

Microinjection protocol

Microinjection procedure was exactly performed as recently described.[23] Additionally, bacteria were microinjected after resuspension with PBS buffer containing 0.5 μg/ml Texas red-labelled dextran (70 000 MW, Molecular Probes).

T cell lines and antigen presentation assay

The CD8 T cell line specific for the *gfp*-derived H-2K^d-restricted epitope HYLSTQSAL[22] was established from spleens of DBA/2 mice 3 weeks after immunization with 5×10^6 P815 cells transfected with the pEGFP-N1 vector (according to the instructions supplied by the manufacturer; Clontech Laboratories, Palo Alto, CA, USA). Spleen cells (15×10^6 cells/well) were cultured in 24-well plates in 2 ml alpha-modified Eagle's medium (Gibco) supplemented with 1 mM Hepes, 1 mM glutamine, 2×10^{-5} M 2-mercaptoethanol, penicillin, streptomycin, 10% FCS and 10^{-9} M synthetic HYLSTQSAL peptide (Jerini Biotools, Berlin, Germany). After 5 days of culture, 1 ml of medium was exchanged with IL-2 medium supplemented with 10 ng/ml murine recombinant IL-2 (R&D, Wiesbaden, Germany). CD8 T cell lines were further restimulated every 3–4 weeks.[28] In 2 ml IL-2 medium, 0.8×10^6 T cells were cultured with 0.4×10^6 mitomycin D-inactivated P815/B7 cells (P815 cells transfected with the human B7.1 gene[29]) in the presence of 10^{-9} M HYLSTQSAL peptide. T cell recognition of *Listeria*-infected P388.D1 as APC was measured by the detection of IFN-γ in culture supernatants as described.[28] Briefly, APC were infected in 96-well flat bottom microwell plates by 10 min × 200 *g* centrifugation. After 2 h at 37°C, infected APC were washed twice and culture medium supplemented with 10 μg/ml gentamicin was added. After 18 h at 37°C, cells were fixed for 10 min with 1% paraformaldehyde in PBS and after thorough washing, 5×10^4 T cells were added to each well. Supernatants were harvested 12–18 h after addition of T cells and the IFN-γ concentration was measured by means of an IFN-γ-specific ELISA that binds and detects IFN-γ with a pair of specific mAb. Results were corrected for dilution of the sample to yield the sample concentration in ng/ml. The sensitivity and specificity of T cell lines was monitored with APC loaded with graded amounts of the target peptide. The detection limit of T cells was between 10^{-10} and 10^{-11} M HYLSTQSAL (data not shown).

Infection of animals

All animal experiments were approved by the government of Unterfranken and conducted according to the German animal protection guidelines. Female BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories, Germany. Mice in groups of five animals were fed with 10^9 bacteria via a flexible orogastric feeding tube. The number of viable bacteria in the inoculum and in liver and spleen homogenates was determined by plating serial dilutions on BHI agar plates. Plates were incubated at 37°C and numbers of colony forming units (CFU) were counted after 24 h.

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