



Fine-tuning the safety and immunogenicity of *Listeria monocytogenes*-based neonatal vaccine platforms

Daniela I.M. Loeffler^{*,1}, Kinga Smolen¹, Laura Aplin, Bing Cai, Tobias R. Kollmann

UBC, Department of Paediatrics, Division of Infectious and Immunological Diseases, Child and Family Research Institute, Vancouver, BC, Canada

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ABSTRACT

We have developed virulence-attenuated strains of *Listeria monocytogenes* (*Lm*) that can be used as safe yet effective vaccine carriers for neonatal vaccination. Here we compare the vaccine efficacy of *Lm* based vaccine carrier candidates after only a single immunization in murine neonates and adults: *Lm* Δ (*trpS actA*) based strains that express and secrete multiple copies of the model antigen ovalbumin (OVA) either under the control of a phagosomal (P_{hty}) or cytosolic (P_{actA})-driven listerial promoter. While both strains induced high levels of antigen-specific primary and secondary CD8 and CD4 T cell responses, both neonatal and adult mice immunized with the phagosomal driven strain were significantly better protected against wildtype *Lm* challenge as compared to the naïve control group than mice immunized with the cytosolic driven strains. Interestingly, only neonatal mice immunized with the phagosomal driven strains generated high IgG antibody responses against OVA. Our phagosomal driven *Lm*-based vaccine platform presents the broadest (cellular & humoral response) and most efficient (highly protective) vaccine platform for neonatal vaccination yet described.

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1. Introduction

Neonates and infants have an increased susceptibility to infection and respond sub-optimally to most vaccines, resulting in over 2.2 million deaths due to vaccine preventable infections per year (reviewed in [1,2]). The urgent need for vaccines that induce protection early in life has been recognized for many years [2–4]. The challenge to develop effective neonatal vaccines arises from what some consider inherent limitations of the neonatal immune system [5]. It is believed that neonates exhibit functionally impaired antigen presentation, shorter lived and lower level antibody responses, and a Th2-type immune response bias and decreased cell-mediated immune responses overall when compared to adults [6]. However a number of studies have shown that neonates are able to generate an adult-like T cell response by using, for instance, a strong activator of the cell-mediated immune response or delivering pro-

tein antigens directly to the professional antigen presenting cells (APC) [1,7–10]. These observations suggest that under appropriate conditions, neonates can develop immune responses to vaccination that are similar in quality and quantity to their adult counterparts [5].

Vaccines based on recombinant live virulence-attenuated microorganisms have proven to induce long-lasting protective immunity, wherein both humoral and cell-mediated immune responses are often efficiently generated [11,12]. Particularly, *Listeria monocytogenes* (*Lm*)-mediated delivery of antigens has been established as a functional and versatile approach for vaccination against allergies, or malignancies in adult mice (reviewed in [13,14]). But particularly for infectious diseases, *Lm* has been successfully used as a vaccine carrier to deliver bacterial, viral, or parasitic antigens [15–18]. This model has been so successful that human clinical trials are already under way (Advaxis, Inc.; Cerus Corporation). The great appeals of *Lm* as a vaccine carrier are its intracellular life cycle and its strong associated immunomodulatory abilities. This Gram-positive bacterium escapes the phagolysosome through a process facilitated by the secreted pore-forming protein listeriolysin O (LLO). After its escape, *Lm* replicates efficiently within the cytosol of many host cells including macrophages and dendritic cells [19]. In addition, *Lm* spreads from cell to cell via an ActA-mediated process (ActA, actin nucleator protein of *Lm*), thereby evading the extracellular milieu where antibodies are found. Protective immunity against *Lm* is thus almost entirely cell-mediated,

Abbreviations: *Lm*, *Listeria monocytogenes*; LLO, listeriolysin O; ActA, actin nucleator protein; CTL, cytotoxic T lymphocyte; CFU, colony forming unit; Ig, immunoglobulin.

* Corresponding author at: Paediatric, Infectious & Immunological Diseases, BC Children's Hospital & UBC, CFRI A4-102, 938 West 28th Ave, Vancouver, BC, V5Z 4H4 Canada. Tel.: +1 604 875 2000x5986; fax: +1 604 875 2226.

E-mail address: dloeffler@cw.bc.ca (D.I.M. Loeffler).

¹ These authors contributed equally.

depending on both cytotoxic CD8 and CD4 Th1 T cells [20]. Our group published recently that a virulence-attenuated strain of *Lm* (*Lm* $\Delta actA$) is safe and well tolerated in newborn mice and that immunization with *Lm* $\Delta actA$ strain carrying protein antigens directly into the cytosol of neonatal host cells was successful in eliciting a life-long protective immune response in murine neonates after only a single immunization [10].

The use of a robust listerial promoter, which is activated within the host cell, is required to optimally express a heterologous vaccine antigen in an *Lm* vaccine carrier strain. Overall, there are two strategies used to introduce heterologous antigens into *Lm*. The first is by insertional integration of the expression cassette within the *Lm* chromosome; the second is by cloning an expression cassette into a multi-copy replicating vector that remains extra-chromosomal (reviewed in [13]). In the first strategy, the integration of the heterologous antigen expression cassette into the bacterial chromosome increases its stability but lowers the antigen expression levels [21]. Moreover, the integration of an expression cassette into the chromosome of the bacterial carrier is time-consuming and labour-intensive. On the other hand, extra-chromosomal multi-copy plasmids are often afflicted by instability, with resulting plasmid loss and marginal antigen expression diminishing the efficacy of these recombinant vaccines [22]. We have previously developed a balanced-lethal plasmid system *Lm* $\Delta(trpS)$ that represents a multi-copy, stable, high-expression extra-chromosomal vaccine platform that has proven to be a superb vaccine carrier in adult mice [23].

Both subsets of T cells, CD8 and CD4, are often required for efficient protection against pathogens [24]. Therefore, antigens must have access to both major histocompatibility complex (MHC) class I (for CD8) and class II (for CD4) presentation pathways. The presence of *Lm* as a vaccine carrier in both phagosomal (i.e. MHC-II) and cytosolic (i.e. MHC-I) host cell compartments, as well as its inherent immunostimulatory capacities, gives the antigen direct access to both MHC molecules for antigen presentation and stimulation of CD4 and CD8 T cells [20]. However, where the antigen should first be expressed (phagosome or cytosol) to have an optimal impact on primary and secondary T cell-mediated responses and on protective efficacy by bacterial vaccine carriers in neonatal mice has, to our knowledge never been investigated.

In this report, we describe a crucial improvement of our previously published system [10] by using the balanced-lethal plasmid system *Lm* $\Delta(trpS actA)$ which adds additional attenuation and safety check-points, and is easily manipulated to carry heterologous vaccine antigens into antigen-presenting cells. Using this system, we compared the relative vaccine efficacy in neonatal and adult mice which were immunized with several virulence-attenuated *Lm* $\Delta(trpS actA)$ strains that express and secrete multiple copies of the model vaccine, chicken egg albumin (ovalbumin, OVA). These vaccine strains express and secrete OVA protein under the control of a predominantly phagosomal (P_{hly}) or cytosolic (P_{actA}) listerial promoter [19]. We found that immunization with *Lm* $\Delta(trpS actA)$ secreting OVA into the phagosomal compartment elicited levels of antigen-specific primary and secondary CD8 and CD4 T cell responses comparable to *Lm* $\Delta(trpS actA)$ strains secreting OVA into the cytosol. But neonatal and adult mice immunized with *Lm* $\Delta(trpS actA)$ secreting OVA into the phagosome were better protected against wild-type *Lm* challenge after only a single immunization. Interestingly, only neonatal mice immunized with the phagosomal expression strain developed anti-OVA antibodies, while no antibodies were detected in adults immunized in the same manner. Our results with this *Lm*-based neonatal vaccine platform represent a major step forward in the overall goal of a single-dose neonatal vaccination able to induce protection from infectious diseases early in life.

2. Materials and methods

2.1. Animals

For all our animal experiments we used 5–7-day-old mouse pups (Neonates) and 6–12-week old (Adult) F1 mice (H-2^b × H-2^d) derived from matings between C57BL/6 (H-2^b) and C57B10.D2 (H-2^d), which were bred in our animal facility. H-2^b × H-2^d F1 mice were used because *Lm* class I immunodominant peptides have been described only in the mouse H-2^d haplotype and class II immunopeptides only in the H-2^b haplotype. All animals were housed under specific pathogen-free conditions at the Child and Family Research Institute of the University of British Columbia. All animal experiments were approved by the Institutional Animal Care and Use Committee.

2.2. Bacterial strains, plasmids, media, and growth conditions

The construction of the plasmids and *Lm* strains (kindly provided by W. Goebel (University of Wuerzburg, Germany)) has been described in detail previously [23]. The recombinant bacterial strains used in this work are listed in Table 1. Competent *Lm* cells were transformed by electroporation as described by Park and Stewart [25]. After transformation of *Lm* $\Delta(trpS actA)/pTRPS$ with the expression plasmids, the resulting recombinant *Lm* strains were cultured in an erythromycin-containing medium without tetracycline to remove the plasmid pTRPS. For immunization and infection experiments, *Lm* strains were grown to the late logarithmic phase (optical density at 600 nm (OD600), 1.0) at 37 °C in brain-heart infusion (BHI) medium, washed twice with endotoxin-free isotonic saline (0.9% NaCl), resuspended in 20% (vol/vol) glycerol in 0.9% NaCl, and stored at –80 °C prior to injection as described below.

2.3. Preparation of supernatant and cellular proteins of *L. monocytogenes* strains

For preparation of protein extracts of *L. monocytogenes*, all strains were grown to the logarithmic phase (OD600, 1.0) in BHI medium supplemented with 1% (w/v) Amberlite™ XAD-4. Addition of Amberlite™ XAD-4 into the BHI broth leads to the activation of the PrfA-dependent virulence gene expression [19,26]. Supernatants were precipitated on ice with 10% trichloroacetic acid, pelleted by centrifugation (5000 × g at 4 °C), washed in acetone, and resuspended in phosphate-buffered saline (PBS) to obtain a volume that was 0.2% of the original culture volume. For preparation of cel-

Table 1
Strains and plasmids used in this work.

Strains and plasmids	Relevant genotype	Reference or source
<i>Listeria monocytogenes</i> EGDe strains		
	$\Delta(trpS actA)/pTRPS$	[30]
	$\Delta trpS/pTRPS$	[30]
	$\Delta(trpS actA)/pSP0-PS_{actA}OVA$	This work
	$\Delta(trpS actA)/pSP118-PS_{actA}OVA$	This work
	$\Delta(trpS actA)/pSP0-PS_{hly}OVA$	This work
	$\Delta(trpS actA)/pSP118-PS_{hly}OVA$	This work
<i>Listeria monocytogenes</i> strain 10403s		
	$\Delta actA-OVA$	[10]
	-OVA	Dr. H. Shen
Plasmids		
pSP0-PS _{actA} OVA	Em ^R , <i>trpS</i> , (PS) _{actA} - <i>ova</i> -T _{inlA}	[23]
pSP118-PS _{actA} OVA	Em ^R , <i>trpS</i> , P _{actA} - <i>ply118</i> , (PS) _{actA} - <i>ova</i> -T _{inlA}	[23]
pSP0-PS _{hly} OVA	Em ^R , <i>trpS</i> , (PS) _{hly} - <i>ova</i> -T _{inlA}	[46]
pSP118-PS _{hly} OVA	Em ^R , <i>trpS</i> , P _{actA} - <i>ply118</i> , (PS) _{hly} - <i>ova</i> -T _{inlA}	[46]

lular proteins, the cell pellet was washed twice in PBS, resuspended in cold lysis buffer (PBS supplemented with protease inhibitors (Sigma)), and transferred into a 2-ml BLUE TUBE (Q-Biogene) filled with silica sand. The tube was shaken 3 times for 45 s each in a mini-beadbeater (Biospec Products). This was followed by ultrasonication for 45 s. The cell debris was removed by centrifugation at 14,000 rpm for 30 min at 4 °C. Total protein concentrations were determined by defining the amount of proteins at the wavelength-ratio of 260/280 in a spectrophotometer (Bio-Rad). A total amount of 150 µg/0.025 ml protein suspension in SDS-PAGE loading buffer was heated to 110 °C for 7 min before they were loaded on sodium dodecyl sulphate (SDS)-PAGE gels.

2.4. SDS-PAGE and immunoblotting

SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to standard protocols [27]. After SDS-PAGE, cellular proteins and proteins from the culture supernatant were subjected to Western blotting onto nitrocellulose membranes. OVA proteins were detected using rabbit polyclonal anti-OVA antibody (Sigma), peroxidase-conjugated secondary goat anti-rabbit antibody (Sigma) and ECL chemiluminescent kit (GE Healthcare).

2.5. Immunization and Infection of animals

Groups of 3–5 mice were immunized intraperitoneally (*i.p.*) with 1×10^7 bacteria (unless otherwise noted), resuspended in 0.1 ml endotoxin-free 0.9% NaCl. Ten days post immunization, spleens were collected for flow cytometric analysis. In protection experiments, groups of 3–5 mice were infected intravenously with 5×10^6 wild-type *Lm*-OVA in 0.1 ml endotoxin-free 0.9% NaCl 6 weeks after immunization. Spleens and livers of infected mice were harvested on day 3 and 5 after challenge to determine the number of *Lm* in each organ (day 3 post-challenge (*p.c.*)) and for the enumeration of Ag-specific T cells in spleens of immunized mice (day 5 *p.c.*). Viable bacterial counts of intracellular bacteria (colony-forming units (CFU)) were determined by plating on BHI agar serial dilutions of mechanically lysed cell suspensions as described above.

2.6. Enumeration of Ag-specific T cells

Ag-specific T cells were detected as described [10]. Briefly, splenocyte suspensions were prepared by homogenizing spleens between two sterile glass slides, subjected to red blood cell lysis, and filtered through a 70 µm cell strainer. A total of 2×10^6 splenocytes was cultured for 5 h in 200 µl of complete medium (RPMI 1640 supplemented with 10% FCS, streptomycin, penicillin), along with Brefeldin A (10 µg/ml) in either the presence or absence of 0.5 µM LLO₉₁₋₉₉, P60₂₁₇₋₂₂₅, OVA₂₅₇₋₂₆₄ (SIINFEKL) or LLO₁₉₀₋₂₀₁ peptide. Cells were then incubated with Cytofix/Cytoperm (BD PharMingen) to permeabilize the plasma membrane. Staining for intracellular cytokines was performed using allophycocyanin-labeled anti-IFN-γ for 30 min at room temperature together with surface staining for CD3, CD8 and CD4 using FITC-labeled anti-CD3 and PerCP-labeled anti-CD8 or anti-CD4 (BD PharMingen). Stained cells were acquired on a FACSAria flow cytometer and analyzed using FlowJo software (Tree Star).

2.7. Enzyme-linked immunosorbent assay (ELISA) for mouse IgG antibodies

ELISA assays were performed two weeks after challenge with WT *Lm*-OVA. Briefly, maxisorb 96-well ELISA plate (Nunc Inc.)

were coated overnight at 4 °C with 2 mg/ml OVA (Worthington, Biochemical Corporation) in 100 µl of freshly prepared carbonate buffer (pH 9.6 at 4 °C). The wells were washed 5 times with 0.05% (vol/vol) Tween-20 in PBS (PBST) and blocked for 2 h at room temperature with 200 µl of 10% FCS in PBS. After an additional 5 washes, 100 µl of two-fold serial dilutions (in PBS with 10% FCS) of the serum aliquots were transferred to the coated and blocked wells and incubated for 2 h at room temperature. Measurements were obtained in duplicate. Naïve mouse and anti-OVA serum were included as negative and positive controls, respectively. After this, the plates were washed 5 times with PBST, incubated with 1:5000 of peroxidase-conjugated AffiniPure Goat anti-mouse IgG antibody (JacksonImmunoResearch) for 1 h at RT. After five washes in PBST, bound antibody was exposed to 100 µl/well of TMB substrate solution (eBioscience), the reaction was stopped with 50 µl 2 M H₂SO₄, and then measured by subtracting the optical readings at 570 nm from those at 450 nm.

2.8. Statistics

Data are expressed as mean ± standard deviation. One-way ANOVA followed by Dunnett's post-test was used for statistical analysis. *p*-Values < 0.05 were considered statistically significant.

3. Results

3.1. Multi-copy plasmid-containing *Lm* Δ(*trpS actA*) strains produce more OVA proteins in comparison to integrated, single-copy *Lm* Δ*actA*-OVA strains

Based on our previous data [10], we hypothesized that *Lm* Δ(*trpS actA*), a derivative of *Lm* Δ*actA* that is more easily manipulated to carry heterologous vaccine antigens on an extra-chromosomal multi-copy expression plasmid, might serve as a vaccine vehicle for neonatal immunization that is at least as effective as the *Lm* Δ*actA* with a chromosomally-integrated, single-copy, antigen-expressing cassette. As a first step towards testing this hypothesis, we compared the new strain *Lm* Δ(*trpS actA*)/pSP0-PS_{actA}OVA with *Lm* Δ*actA*-OVA through *in vitro* expression profiling. Expression of OVA protein was detected by Western blot analysis in supernatants (*i.e.* secreted form) and cell pellets (*i.e.* non-secreted form) only in strains expressing OVA, not in the control strains *Lm* Δ(*trpS actA*)/pSP0 and *Lm* Δ*actA* (Fig. 1). The new strain, *Lm* Δ(*trpS actA*)/pSP0-PS_{actA}OVA, displayed a higher level of OVA production as compared to *Lm* Δ*actA*-OVA, both in the cell-bound as well as in the secreted fraction.

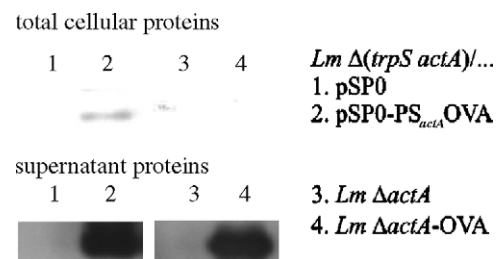


Fig. 1. Multi-copy plasmid-containing *Lm* Δ(*trpS actA*)/pSP0-PS_{actA}OVA strains produce more OVA protein (truncated OVA ~25 kDa) in comparison to chromosomally-integrated single-copy *Lm* Δ*actA*-OVA strains (full-length ~50 kDa). OVA protein expression and secretion of indicated *Lm* strains was determined by Western blot analysis using a purified rabbit anti-ovalbumin antiserum. Shown here are total cellular proteins and secreted proteins in supernatants of *Lm* cultures grown in BHI broth supplemented with 1% (w/v) Amberlite™ XAD-4. *Lm*, *Listeria monocytogenes*.

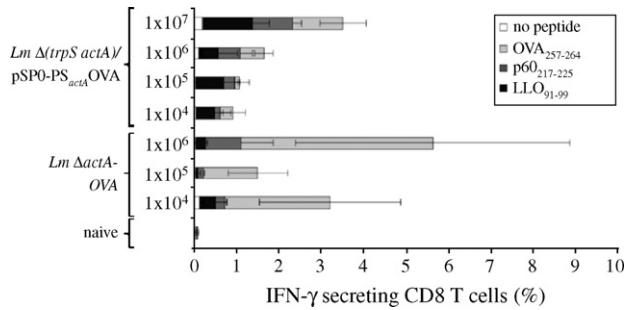


Fig. 2. A dose of 10^7 CFU *Lm* Δ (*trpS actA*)/pSP0-PS_{actA}OVA induces similar primary CD8 T cell responses in mouse neonates as the *Lm* Δ actA-OVA strain. Six-day-old mice were immunized *i.p.* with respective strains at the indicated dose or with saline (Naive). On day 10 after immunization, spleens were harvested to obtain splenocytes. Splenocytes were stimulated for 5 h with *Lm*- and OVA-peptides, fixed and stained for IFN- γ -secreting CD8 T cells. These data represent means \pm standard deviation for groups of 3–5 mice from one representative out of two experiments.

3.2. A high dose of *Lm* Δ (*trpS actA*)/pSP0-PS_{actA}OVA induces primary total CD8 T cell responses in mouse neonates similar to a low dose of the *Lm* Δ actA-OVA strain

We next compared the capacity of the new strain *Lm* Δ (*trpS actA*)/pSP0-PS_{actA}OVA to induce a vaccine-specific primary T_C1 response in mouse neonates to that of the older *Lm* Δ actA-OVA strain in a direct, dose-dependent analysis. For this we injected 10^4 , 10^5 , 10^6 or 10^7 CFU of either strain intraperitoneally (*i.p.*) into neonatal mice and analyzed their splenocytes for OVA- and *Lm* peptide-specific CD8 T cells 10 days post infection. At immunization doses of 10^4 – 10^5 CFU, neonates immunized with *Lm* Δ actA-OVA contained a higher number of antigen-specific IFN- γ secreting CD8 T cells as mice immunized with the same dose of *Lm* Δ (*trpS*

actA)/pSP0-PS_{actA}OVA (Fig. 2). Since we discovered that 10^7 CFU of *Lm* Δ actA-OVA per mouse was the 50% lethal dose (LD50) for neonates, we were not able to determine the amount of CD8 T cells in neonatal mice immunized with a dose higher than 10^6 CFU of *Lm* Δ actA-OVA. While always displaying lower OVA reactive fraction, neonatal mice immunized with *Lm* Δ (*trpS actA*)/pSP0-PS_{actA}OVA obtained similar overall percentages of *Lm*- and OVA-reactive CD8 T cells as mice immunized with *Lm* Δ actA-OVA when they were immunized with 10^6 or 10^7 CFU of *Lm* Δ actA-OVA. Thus, our new strain *Lm* Δ (*trpS*)/pSP0-PS_{actA}OVA is comparable to the *Lm* Δ actA-OVA strain as a neonatal vaccine carrier when used at a dose of 10^7 CFU.

3.3. Compared to adult mice, neonatally immunized mice develop a stronger *Lm*- and OVA-specific primary CD8 and CD4 T cell response

Given the above observation, we next assessed in detail the capacity of the *Lm* Δ (*trpS*)/pSP0-PS_{actA}OVA strain to generate an efficient *Lm*- and OVA-specific primary CD8 and CD4 T cell response in neonatal and adult mice by carrying out a dose-dependent analysis. To evaluate the optimal immunization dose of multi-copy plasmid strain *Lm* Δ (*trpS*)/pSP0-PS_{actA}OVA, mice were immunized with 10^4 – 10^8 CFU. Ten days after immunization, we enumerated *Lm*- and OVA-specific CD8 and CD4 T cells by intracellular IFN- γ staining of splenocytes after restimulation *in vitro* with *Lm*-specific MHC-class-I LLO₉₁₋₉₉, p60₂₁₇₋₂₂₅ and OVA₂₅₇₋₂₆₄ or MHC-class-II LLO₁₈₉₋₂₀₁ restricted peptides. We observed a dose-dependent increase in *Lm*- and OVA-specific primary CD8 and CD4 T cells in adult as well as in neonatally immunized mice (Fig. 3). Remarkably, neonatally immunized mice developed a higher percentage of *Lm*- and OVA-specific primary CD8 (Fig. 3B) and CD4 T cells (Fig. 3C) than mice immunized as adults. We also determined that 10^7 CFU as an

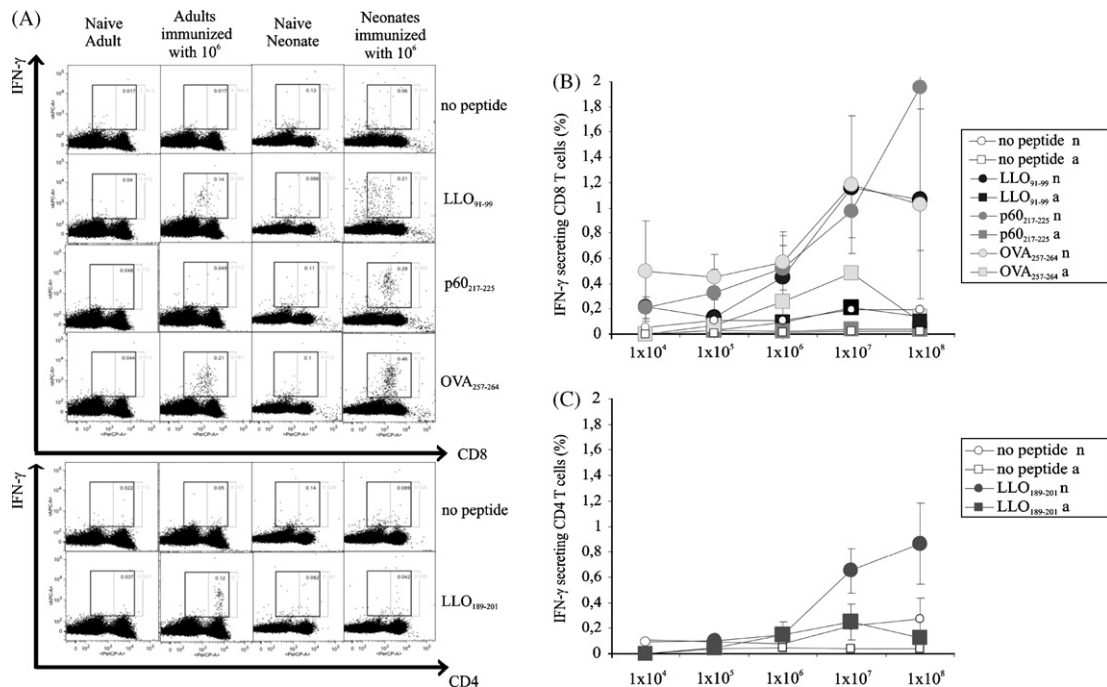


Fig. 3. Neonatally immunized mice develop a stronger *Lm*- and OVA-specific CD8 (A and B) and CD4 (A and C) T cell primary response than mice immunized as adults. Neonatal and adult mice were immunized *i.p.* with increasing doses (10^4 to 10^8 CFU) of *Lm* Δ (*trpS actA*)/pSP0-PS_{actA}OVA. Ten days after immunization, spleens were obtained from these mice, and splenocytes were stimulated with the indicated MHC class I- and MHC class II-restricted *Lm*- and OVA-specific peptides before analysis of intracellular IFN- γ cytokine secretion by flow cytometry. Shown in A for CD8 and CD4 are examples of the flow cytometric analysis. The mean percentage of IFN- γ -producing splenocytes are shown in (B) for CD8 and in (C) for CD4. Unstimulated controls are included in each experiment. These data present means \pm standard deviation for 3–5 mice per age-group from one representative out of two experiments. n, neonates; a, adults.

immunizing dose led to the highest percentage of reactive T cells during the primary T cell response for both adult as well as neonatally immunized mice. We detected no pathologically appreciable side effects at any of the administered doses.

3.4. Independent of *in vivo* subcellular location of antigen expression, neonatally immunized mice develop a broader primary T cell response than adult immunized mice

To further define the use of *Lm* $\Delta(trpS actA)$ as a neonatal vaccine platform, we determined the impact of *in vivo* subcellular location of vaccine antigen expression on primary T cell-mediated responses in neonatal and adult mice. To our knowledge, there are no published reports on the impact of subcellular location of vaccine antigen expression on primary T cell-mediated responses by *Lm* vaccine carriers in neonates. We constructed variants of the *Lm* $\Delta(trpS actA)$ (Table 1), each with a different *Lm* promoter that is predominantly active in the phagosomal (P_{hly}) or cytosolic (P_{actA}) compartment of the host cell [28,29]. We also set out to test the impact of an added virulence-attenuation (*Lm*-specific phagelysin Ply118 under the control of P_{actA}) on the primary response [23], with the goal of adding additional safeguards for future potential clinical applications. We thus compared a total of four different bacterial expression plasmids. Both neonatal and adult mice were immunized *i.p.* with 10^7 CFU of *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}OVA$, *Lm* $\Delta(trpS actA)/pSP118-PS_{actA}OVA$, *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}OVA$ or *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}OVA$. Ten days after immunization splenocytes were stimulated with *Lm*- or OVA-specific peptides before analysis of intracellular IFN- γ secretion by flow cytometry. Strikingly, neonatally immunized mice generated significantly more antigen-specific CD8 and CD4 T cells in comparison to adult immunized mice in response to all of the four different *Lm* strains

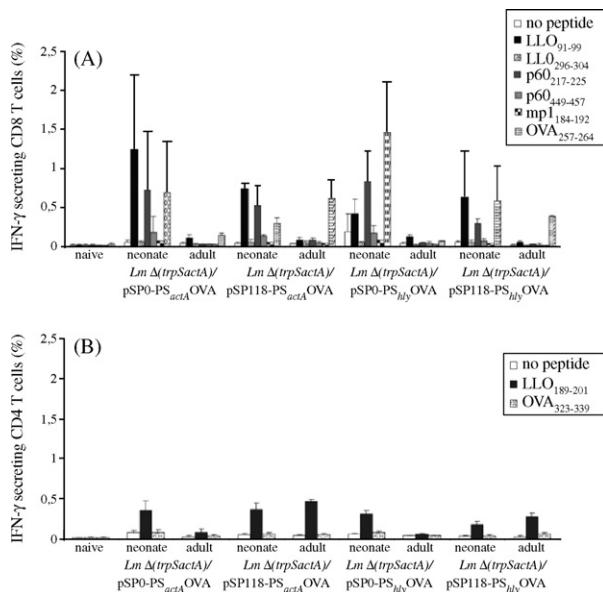


Fig. 4. Neonatally immunized mice develop a broader *Lm*- and OVA-specific primary T cell response than adult immunized mice, which is not dependent on subcellular location of antigen expression. Neonatal and adult mice were immunized *i.p.* with 10^7 CFU of *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}OVA$, *Lm* $\Delta(trpS actA)/pSP118-PS_{actA}OVA$, *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}OVA$ or *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}OVA$. Ten days after immunization, spleens were removed and the splenocytes stimulated with the indicated MHC class I- and MHC class II-restricted *Lm*- and OVA-specific peptides before analysis of intracellular IFN- γ cytokine secretion by flow cytometry. Unstimulated controls are included. CD8 T cell responses are shown in (A) and CD4 T cell responses in (B). These data are expressed as means \pm standard deviation and represent 3–5 mice per age-group from one representative out of two experiments.

(Fig. 4A and B). In addition, neonatal immunized mice developed a broader spectrum of antigen specific CD8 T cells than adult mice immunized in the same manner: neonates recognized multiple epitopes of the heterologous antigen (LLO₉₁₋₉₉, p60₂₁₇₋₂₂₅ or OVA₂₅₇₋₂₆₄) in a co-dominant fashion, while mice immunized as adults generated predominantly OVA₂₅₇₋₂₆₄ reactive CD8 T cells (Fig. 4A). Detection of the subdominant epitopes LLO₂₉₆₋₃₀₄, p60₄₄₉₋₄₅₇ and mpl₁₈₄₋₁₉₂ was similarly low in both adult and neonatally immunized mice (Fig. 4A). The MHC-class-II restricted peptide OVA₃₂₃₋₃₃₉ was not recognized by adult and neonatal CD4 T cells, whereas the dominant CD4 epitope LLO₁₈₉₋₂₀₁ was strongly and equally recognized by both (Fig. 4B). Furthermore, mice immunized with self-destructing strains *Lm* $\Delta(trpS actA)/pSP118-PS_{actA}OVA$ and *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}OVA$ harboured similar amounts of IFN- γ secreting CD8 and CD4 T cells compared to mice immunized with the non-self-destructing strains *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}OVA$ and *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}OVA$ (Fig. 4A and B). There was no significant difference detectable between the levels antigen-specific CD8 and CD4 T cells induced in mice immunized with *Lm* secreting OVA into the phagosomal compartment vs. those that express OVA predominantly in the cytosol of the host cell. Taken together, neonatal immunized mice developed a stronger and broader *Lm*- and OVA-specific primary T cell response than adult immunized mice. The self-destructing Ply118-mediated lysis did not negatively impact the induction of a primary immune response. Furthermore, the secretion of the antigens by the respective carrier strains into phagosomal vs. cytosolic compartment had no appreciable impact on the generation of primary CD8 and CD4 T cell responses in either neonates or adults.

3.5. Neonatally immunized mice develop protective *Lm*- and OVA-specific secondary response that is dependent on the subcellular location of antigen expression

We next wished to characterize which of the four strains—*Lm* $\Delta(trpS actA)/pSP0-PS_{actA}OVA$, *Lm* $\Delta(trpS actA)/pSP118-PS_{actA}OVA$, *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}OVA$ and *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}OVA$ —would provide the most efficient protection against challenge with wild-type *Lm*. To this end, we immunized neonates and adults with 10^7 CFU and challenged them, along with naive control mice, six weeks later with 5×10^6 CFU of *Lm*-OVA. Three days after challenge, we determined CFUs in spleen and liver. Five days after challenge we also enumerated *Lm*- and OVA-specific T cells in splenocytes by intracellular IFN- γ staining. As shown in Fig. 5A and B, both immunized adults and neonates displayed a robust CD8 and CD4 T cell secondary response. There was no appreciable difference in immunodominance for the secondary response between mice immunized as neonates or as adults. Neonatal and adult *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}OVA$ - and *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}OVA$ -immunized mice displayed a CD8 T cell secondary response completely dominated by the OVA₂₅₇₋₂₆₄ response, whereas neonatal and adult immunized mice immunized with *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}OVA$, *Lm* $\Delta(trpS actA)/pSP118-PS_{actA}OVA$ elicited a broader CD8 T cell memory response to LLO₉₁₋₉₉, p60₂₁₇₋₂₂₅ and OVA₂₅₇₋₂₆₄ (Fig. 5A1 and A2). In summary, subcellular location of expression strongly affects immunodominance. Strikingly, mice immunized as neonates with *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}OVA$ and *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}OVA$ displayed significantly better protection than mice immunized as neonates with *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}OVA$, *Lm* $\Delta(trpS actA)/pSP118-PS_{actA}OVA$ (Fig. 5C1 and C2) in direct comparison to the naive control group. Furthermore, only the adult mice immunized with *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}OVA$ showed a significant reduction in bacterial counts in liver and spleen in comparison to the naive control group (Fig. 5C2). In conclusion, expression

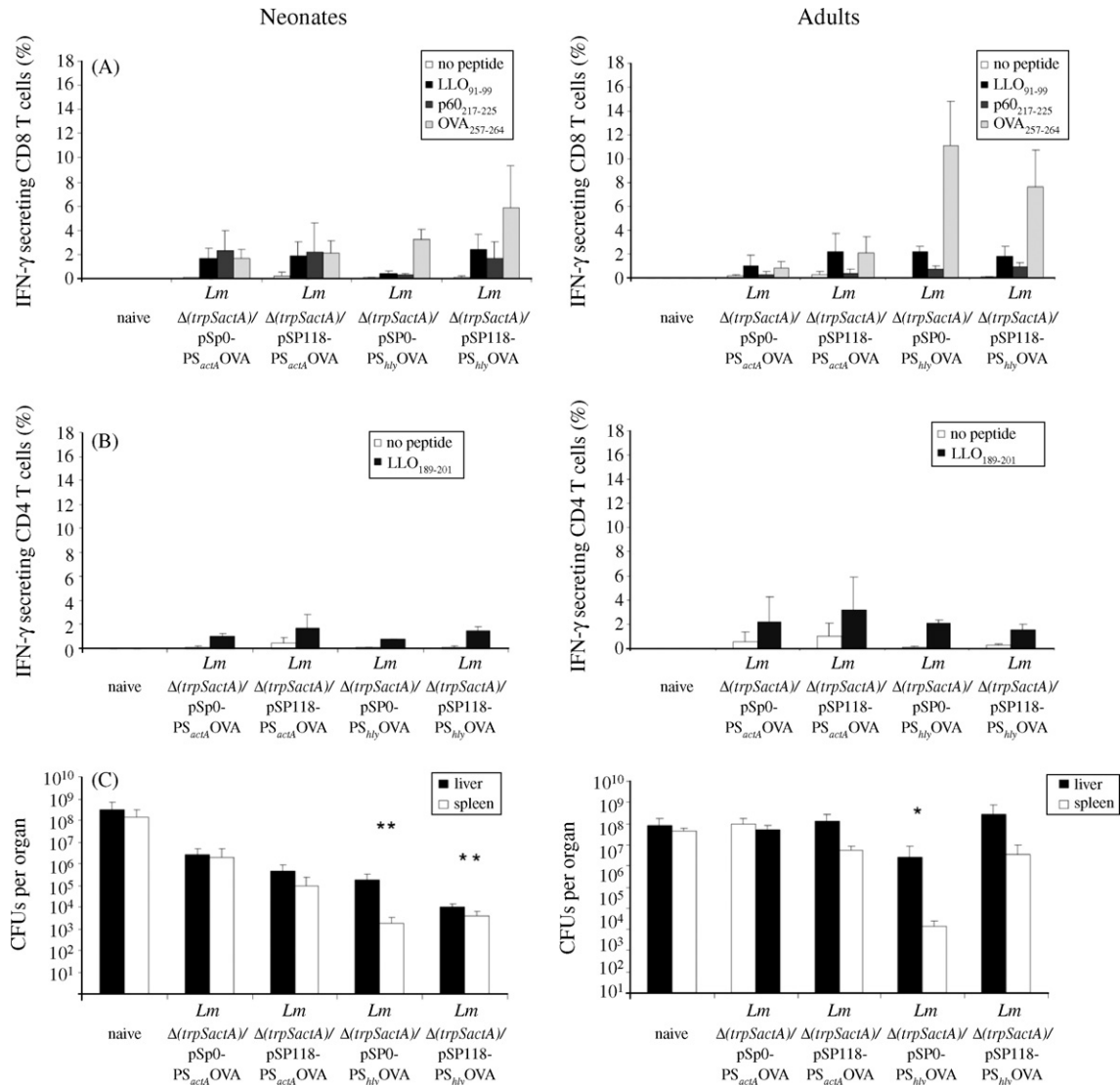


Fig. 5. Neonatally immunized mice develop an *Lm*- and OVA-specific secondary response that provides protection, which is dependent on subcellular location of antigen expression. Mice immunized *i.p.* with 10^7 of *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}$ OVA, *Lm* $\Delta(trpS actA)/pSP118-PS_{actA}$ OVA, *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}$ OVA or *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}$ OVA on day 6 of life (Neonate) or at 6 weeks of age (Adult) were infected *i.v.* with 5×10^6 CFU of wild-type *Lm*-OVA 6 weeks after immunization. Splenocytes were obtained from the indicated mice 5 days after infection along with age-matched non-immune mice (Naive) and stimulated with the indicated MHC class I- and MHC class II-restricted *Lm*- and OVA-specific peptides before analysis of intracellular IFN- γ cytokine secretion by flow cytometry. Unstimulated controls are included. CD8 T cell responses are shown in (A), CD4 T cell responses in (B) and bacterial counts in liver and spleen in (C). These data represent 3–5 mice per age-group from one representative out of two experiments. The results are expressed as means \pm standard deviation. An asterisk indicates a statistically significant difference ($p < 0.05$ and $**p < 0.01$ as determined by one-way ANOVA) between experimental and control group.

of bacterial antigens into the phagosomal compartment promoted protective immune responses more effectively than expression in the cytosol.

3.6. Neonates, not adults, generate a strong IgG antibody response against ovalbumin after immunization with *Lm*

Finally, we investigated if our two most promising strains, *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}$ OVA and *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}$ OVA, i.e. those that protected neonatal mice most efficiently *in vivo*, could also generate a humoral immune response against the model vaccine antigen ovalbumin. Serum from mice immunized as neonates or adult with 10^7 CFU of *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}$ OVA or *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}$ OVA was examined by ELISA for the presence of IgG antibodies against OVA 14 days after challenge with 5×10^6 CFU of wild-type *Lm*-OVA 6 weeks after primary

immunization. Surprisingly, only neonates immunized with *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}$ OVA and *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}$ OVA developed significant titers of IgG antibodies against ovalbumin (Fig. 6).

4. Discussion

Effective yet safe vaccines to be administered in the first days to weeks of life are urgently needed. This report confirms that virulence-attenuated strains of *Lm* can be used as safe and effective vaccine carriers for newborns. Our present study also showed the following: First, *Lm* $\Delta(trpS actA)$ -based strains, which express and secrete multiple copies of OVA either under the control of a phagosomal (P_{hly})- or cytosolic (P_{actA})-driven listerial promoter, elicited similarly high levels of antigen-specific primary CD8 and CD4 T cell responses with just a single immunization. Second,

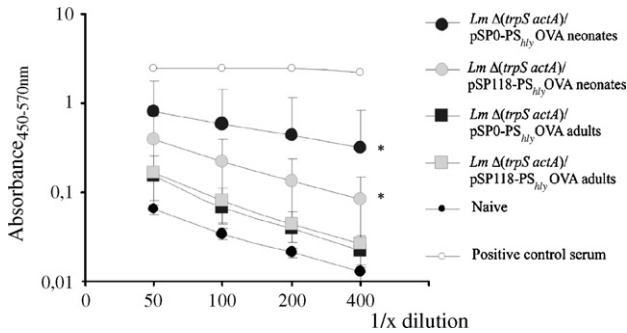


Fig. 6. Only neonatally immunized mice, not mice immunized as adults, generate a strong IgG antibody response against ovalbumin. Mice were immunized *i.p.* with 10^7 CFU of *Lm* $\Delta(trpS actA)/pSP0-PS_{hly}$ OVA and *Lm* $\Delta(trpS actA)/pSP118-PS_{hly}$ OVA, six weeks later challenged with 5×10^6 CFU of wild-type *Lm*-OVA given *i.v.* and sera were collected and analyzed for anti-OVA IgG antibodies 14 days after challenge. Absorbance at 450 nm minus at 570 nm of different serial dilutions of serum is shown. Serum from mice boosted three times with WT *Lm*-OVA served as the positive control, and serum from a non-infected mouse served as negative control. Each symbol indicates the means for the experimental groups ($n = 4$ each). An asterisk indicates a statistically significant difference ($p < 0.05$ as determined by one-way ANOVA) between experimental and control groups. The results of one representative out of two experiments are shown.

neonatally immunized mice developed a stronger and broader dose-dependent antigen-specific primary T cell response than mice immunized as adults. Third, both neonatal and adult mice immunized with the phagosomal-driven strains were significantly better protected against a lethal wild-type *Lm* challenge as compared to mice immunized with the cytosolic-driven strains. Lastly, only neonatal (not adult) mice immunized with the phagosomal-driven strains generated high IgG antibody responses against the model vaccine antigen OVA.

We have recently shown that *Lm* is suitable as a neonatal vaccine vehicle, requiring only a single immunization at birth to induce life-long protection [10]. We now wished to develop highly attenuated *Lm* strains to increase safety for clinical applications of *Lm* as a vaccine vehicle in neonates. Furthermore, the integration of vaccine antigen expression cassettes into the chromosome as required for *Lm* $\Delta actA$ is time-consuming and labour-intensive. To increase ease of manipulation, we set out to develop a plasmid-encoded *Lm*-based vaccine antigen expressing system. For these purposes (safety and ease of vaccine antigen expression) we focused on the virulence-attenuated *Lm* $\Delta(trpS actA)$ carrier strains that harbour the antigen expression cassette as well as the essential listerial *trpS* gene (*TrpS*, tryptophanyl-tRNA-synthetase) on a balanced-lethal plasmid. A plasmid-based, balanced-lethal system absolutely requires the stability of the plasmid since the essential protein required by *Listeria* to survive is expressed on the plasmid. The loss of the plasmid results in cell death [22]. The *Lm* $\Delta(trpS actA)$ balanced-lethal plasmid system has already proven its stability and ability as a vaccine carrier in adult mice [23,30]. We found that carrier strains of *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}$ OVA were indeed able to elicit primary antigen-specific CD8 T cell responses in neonatal and adult mice comparable to strain *Lm* $\Delta actA$ -OVA. A comparable primary immune response required a higher immunizing dose for *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}$ OVA (Fig. 2). This dose-dependent difference was expected, indeed desired, since the *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}$ OVA parental strain is anticipated to be more attenuated than the *Lm* $\Delta actA$ -OVA's parental strain *Lm*-OVA. The higher level of antigen expression driven by the multi-copy plasmids creates a metabolic burden reducing the overall fitness of *Lm* $\Delta(trpS actA)$ as compared to *Lm* $\Delta actA$ (reviewed in [31]). We confirmed these assumptions by Western blot analysis of cell pellet and supernatant fractions of both *Lm* $\Delta(trpS actA)/pSP0-$

PS_{actA} OVA and *Lm* $\Delta actA$ -OVA strains (Fig. 1), and by detecting lower numbers of bacteria three days after infection with the respective parental strains, *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}$ OVA in comparison to *Lm*-OVA (data not shown). We thus had found what we were looking for: an easy to manipulate, high-level vaccine antigen expressing strain of *Lm* that is greatly attenuated yet effective as a neonatal vaccine carrier.

During these initial investigations, we also determined that an immunizing dose of 10^7 CFU of *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}$ OVA leads to the most efficient primary T cell response for both adult and neonatally immunized mice (Fig. 3). These results are in accordance with literature in that the CD8⁺ T cell expansion after *Lm* infection is primarily dependent on the initial infection dose or amount of antigen displayed [32,33]. Similarly to what we had described for *Lm* $\Delta actA$ -OVA already, mice immunized with *Lm* $\Delta(trpS actA)/pSP0-PS_{actA}$ OVA as neonates developed stronger *Lm*- and OVA-specific primary CD8 and CD4 T cell responses than mice immunized as adults (Fig. 3).

The use of *Lm* strains that secrete the foreign protein under the transcriptional control of a phagosomal (P_{hly})- or cytosolic (P_{actA})-driven listerial promoter results in bacterial proteins having access to both MHC class II and class I molecules for antigen presentation to CD4 and CD8 T cells, respectively [13,20,28,29]. For many pathogens, both subsets of T cells are needed to provide optimal protective immunity [24,34]. Therefore, we investigated the impact the subcellular location of antigen expression would have on the subsequent immune response. Surprisingly, the subcellular location of antigen expression had no impact at all on the generation of the primary immune response in all four strains tested (Fig. 4). In sharp contrast, immunodominance of the secondary response and, more importantly, protection were significantly affected by phagosomal vs. cytosolic antigen expression (Fig. 5A and C). In comparison to the naive control group, neonates, which had been immunized with *Lm* $\Delta(trpS actA)$ strains that secrete OVA into the phagosome, were much better protected against wild-type *Lm* challenge than neonatal mice immunized with strains that secrete OVA into the cytosol (Fig. 5C1). We are currently in the process of elucidating which of the antigen-specific immune responses (*Lm* or OVA) are primarily responsible for this surprising observation. We hypothesize that the various *Lm* carrier strains may have triggered different signalling pathways in the infected antigen-presenting cell, which in turn promoted a differential CD8 T cell response. For example, bacterial ligands generated in the phagosomal compartment are also targets of the cytosolic innate immune system receptors, such as NOD2 [35], and distinct differences in signalling patterns, intensities, enhancement of dendritic cell maturation and T cell differentiation and function after infection with phagosomal vs. cytosolic localized *Lm* have been demonstrated before [20,36–39].

The subcellular location of antigen expression did affect the epitope hierarchy of the CD8 T cell response to *Lm*. CD8 T cells often focus on a few epitopes out of thousands available. In this study, we confirmed our previous findings that neonates have broader epitope recognition. LLO₉₁₋₉₉, p60₂₁₇₋₂₂₅, and OVA₂₅₇₋₂₆₄ were recognized similarly by neonatal CD8 T cells in the primary response. On the other hand, the adult response was severely restricted to OVA₂₅₇₋₂₆₄, with LLO₉₁₋₉₉, p60₂₁₇₋₂₂₅, p60₄₄₉₋₄₅₇, and mpl₈₄₋₉₂ barely recognized at all. This type of adult response is in accordance with the published record [40]. Adult IFN- γ KO mice also develop a broader CD8 T cell response [41,42] akin to the broad neonatal response we have observed. Neonates are known to produce reduced levels of IFN- γ as compared to their adult counterparts [43]. We are currently in the process of identifying the responsible mechanisms for these observations, but hypothesize that IFN- γ is centrally involved and may influence, e.g. T cell repertoire selec-

tion, T cell sensitivity, or specific T cell effector functions involved in the neonate's broader and wider epitope recognition.

Surprisingly, we found that immunized neonates are able to generate IgG antibodies against OVA compared to immunized adults (Fig. 6). Why adult mice immunized in the same manner did not mount a detectable humoral response is not clear. It is known that exposure to antigen during the neonatal period leads to 'imprinting' of Th2 dominance that is maintained into adulthood. Th2 cytokines promote the preferential production of Th2-associated IgG1 [44,45]. Thus, the net effector function associated with different Ig isotypes is expected to be different in mice immunized as adults vs. those immunized as neonates [44]. The possibility that our *Lm* Δ (*trpS actA*) strains secreting vaccine antigens into the phagosome induces both protective CD4 and CD8 T cell memory response, as well as a robust antibody response is of great importance in our long term goal of developing a single-dose, broadly protective neonatal vaccine platform. Further studies on the subclass, affinity, affinity maturation, and neutralizing capacity of these neonatal antibody responses are currently under way.

In this study we also characterized the use of self-destructing carrier strains in order to provide additional safeguards for employing *Lm*-based vaccine vehicles for neonatal immunization. The use of bacterial carriers that destroy themselves after several rounds of replication via Ply118 listerial phage-mediated lyses [23] did not affect the primary or secondary CD8 and CD4 T cell response, or the resulting protection (Figs. 4 and 5). We believe the *Lm*-specific phagelysin Ply118 under the control of P_{actA} encoded on the balanced-lethal plasmid designed to lyse bacteria upon entry of the cytosolic compartment of the infected cell adds an important safety component to this platform.

In summary, we identified *Lm* Δ (*trpS actA*) as an ideal vaccine vehicle for neonatal immunization. It is safe at high doses, and induces a strong primary and secondary immune response. Most importantly, it induces protection from challenge with wild-type *Lm* after only one immunization given around birth. In this report, we confirm and extend our previous observation of broader epitope recognition by CD8 T cells in neonates as compared to adults, and add to the advantages of neonatal *Lm*-based vaccination a stronger IgG response to the vaccine antigen in neonates as compared to adults. *Lm* Δ (*trpS actA*) is highly attenuated, and given the multi-copy balanced-lethal plasmid platform available in this strain, allows easy manipulation to rapidly fine-tune the desired vaccine response. In this study, we have shown this advantage and demonstrated that (a) a predominantly phagosomal vaccine antigen plasmid expression cassette provides the most optimal protection, and; (b) additional attenuation through a plasmid-encoded phage-mediated suicide vector does not negatively affect the immune response or protection. We believe that *Lm* Δ (*trpS actA*) will rapidly allow dissection of many parameters important for successful neonatal immunization, and will promote the development of rational vaccine design for early life immunization against infectious diseases such as malaria or whooping cough.

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