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

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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_{hly}) 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.

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 protein 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.

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depending on both cytotoxic CD8 and CD4 Th1 T cells [20]. Our group published recently that a virulence-attenuated strain of *Lm* (*Lm* Δ*actA*) is safe and well tolerated in newborn mice and that immunization with *Lm* Δ*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* Δ(*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* Δ(*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* Δ(*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* Δ(*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* Δ(*trpS actA*) strains secreting OVA into the cytosol. But neonatal and adult mice immunized with *Lm* Δ(*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* Δ(*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
Listeria monocytogenes EGDe strains		
	Δ(*trpS actA*)/pTRPS	[30]
	Δ*trpS*/pTRPS	[30]
	Δ(*trpS actA*)/pSP0-PS_{actA}OVA	This work
	Δ(*trpS actA*)/pSP118-PS_{actA}OVA	This work
	Δ(*trpS actA*)/pSP0-PS_{hly}OVA	This work
	Δ(*trpS actA*)/pSP118-PS_{hly}OVA	This work
Listeria monocytogenes strain 10403s		
	Δ*actA*-OVA	[10]
	-OVA	Dr. H. Shen
Plasmids		
pSP0-PS_{actA}OVA	Em^R, *trpS*, $(PS)_{actA}$-*ova*-T_{inlA}	[23]
pSP118-PS_{actA}OVA	Em^R, *trpS*, P_{actA}-*ply*118, $(PS)_{actA}$-*ova*-T_{inlA}	[23]
pSP0-PS_{hly}OVA	Em^R, *trpS*, $(PS)_{hly}$-*ova*-T_{inlA}	[46]
pSP118-PS_{hly}OVA	Em^R, *trpS*, P_{actA}-*ply*118, $(PS)_{hly}$-*ova*-T_{inlA}	[46]