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Immunization with non-replicating *E. coli* minicells delivering both protein antigen and DNA protects mice from lethal challenge with lymphocytic choriomeningitis virus

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Abstract

In the midst of new investigations into the mechanisms of both delivery and protection of new vaccines and vaccine carriers, it has become clear that immunization with delivery mechanisms that do not involve living, replicating organisms are vastly preferred. In this report, non-replicating bacterial minicells simultaneously co-delivering the nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) and the corresponding DNA vaccine were tested for the ability to generate protective cellular immune responses in mice. It was found that good protection (89%) was achieved after intramuscular administration, moderate protection (31%) was achieved after intranasal administration, and less protection (7%) was achieved following gastric immunization. These results provide a solid foundation on which to pursue the use of bacterial minicells as a non-replicating vaccine delivery platform.

Keywords

Minicells; Vaccine; Immune response; Mucosal delivery; LCMV

1. Introduction

A major goal in vaccine development is the production of safe and efficient delivery mechanisms capable of eliciting protective immune responses. In the past decade, the use of attenuated bacterial pathogens such as *Listeria monocytogenes*, *Shigella flexneri* and a variety of attenuated *Salmonella* serovars have proven useful in the generation of both antibody and cell-mediated protective immunity in animal infection or tumor models [1–3]. In most cases, constitutive expression of heterologous protein antigens by these bacterial carriers *in vivo* elicits protective immune responses [4–6]. In addition to this strategy, *Salmonella typhimurium* and *Shigella flexneri* have been used to deliver plasmid DNA vaccines to elicit protective immune responses in mice [2,7]. In light of these successes, it should be noted that major safety concerns such as pathogenic reversion, horizontal gene transfer and adverse inflammatory responses with respect to using these approaches in human beings still exist [8–10].

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The use of bacterial minicells presents a unique, alternative approach in furthering the development of safe and efficacious vaccine delivery. Minicells are small (100–400 nm), quasi-spherical, achromosomal bacterial particles that result from the disruption of the normal bacterial cell cycle [11,12]. Minicells contain many components of their parental cells including lipopolysaccharides (LPS), an intact peptidoglycan layer (cell wall), as well as any recombinantly expressed proteins and plasmid DNA molecules [11,13–16]. This is the same combination of components and properties that makes attenuated pathogenic strains effective as vaccine carriers. Minicells have already been used to express plasmid encoded antigens from a wide variety of pathogens [17–19] and have previously been used as vaccine carriers [20]. In those studies, heterologous protein antigens delivered by minicells resulted in antigen specific antibody responses. Studies using attenuated pathogenic bacteria to deliver DNA vaccines have been shown to elicit antigen specific cellular immune responses [2,21,22] suggesting that DNA vaccine delivery using minicells could potentially elicit similar responses.

There is mounting evidence to suggest that the administration of both DNA and the corresponding protein antigen vaccines, either sequentially or in combination, is more effective at generating robust immune responses [23–25]. Preliminary studies suggest that minicells co-delivering a eukaryotic expression plasmid and the corresponding protein antigen could generate much higher antigen-specific serum IgG antibody responses when administered intramuscularly (i.m.) [26]. More importantly, administration of minicells via the intranasal (i.n.) or oral (p.o.) routes of administration resulted in the production of both antigen-specific serum IgG and mucosal IgA.

In this report, bacterial minicells derived from a non-pathogenic *E. coli* K-12 strain capable of the simultaneous delivery of both recombinant protein antigen and the corresponding DNA vaccine are evaluated for their ability to elicit protective cell-mediated immunity against a lethal challenge with lymphocytic choriomeningitis virus (LCMV) in mice following i.m., i.n., or p.o. administrations. Eighty-nine percent of mice survived a lethal intracranial challenge when the vaccine was administered i.m., in comparison to 31 or 7% when administered i.n. or p.o., respectively. The investigation into the underlying cellular responses mediating immunity and survival is also described. Together, the results of this study demonstrate for the first time that non-replicating bacterial minicells can simultaneously deliver heterologous protein antigens and the corresponding plasmid DNA vaccine to a mucosal surface to elicit protective, systemic immunity.

2. Materials and methods

2.1. Bacterial strains

MPX1B9 [F^- , λ^- , *ilvG*, *rfb-50*, *rph-1*, *zac::aph*, *lacI^q*, P_{tac} *ftsZ20*, Δ *phoA*] [26,27] is a genetically stable, mini-cell producing *E. coli* strain that was obtained from Vaxiion Therapeutics, Inc. Growth of MPX1B9 in Luria-Bertani (LB) broth requires 15 μ M IPTG to support normal cell division and minicell induction requires additional IPTG inducer as described below.

2.2. Plasmid construction

The rhamnose inducible prokaryotic expression plasmid pRHA-67 was obtained from Vaxiion Therapeutics, Inc. This pUC18 derivative contains a multiple cloning site placed downstream of the *E. coli rhaB* promoter sequence as well as the two tandemly encoded *rhaB* regulatory genes *rhaR* and *rhaS* [28].

The coding sequence for the LCMV NP protein was PCR amplified using pCMV-NP as template DNA [29]. The forward primer was designed to introduce a *SalI* restriction

endonuclease site and the reverse primer designed to replace the natural stop codon with a FLAG[®] tag sequence positioned in front of a bacterial stop codon followed by an *Xba*I restriction endonuclease site. The appropriately sized 1712 bp product was subcloned into pRHA-67 that was previously digested with *Sal*I and *Xba*I to complete pMJG28. In a separate PCR reaction, the entire sequence for the eukaryotic expression cassette driving LCMV NP protein was amplified using pCMV-NP as a template. Both the forward and reverse primers of this reaction were designed to introduce flanking *Kpn*I sites. This 2774 bp product was subcloned into the unique *Kpn*I site of pMJG28 to complete pMJG30.

Prokaryotic expression of FLAG-tagged LCMV NP from pMJG30 was tested in *E. coli* Top 10 cells [F- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ**AM15* *AlacX74* *recA1* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG*] (Invitrogen, Carlsbad, CA) or in minicells derived from MPX1B9 harboring this plasmid as described below. Eukaryotic expression of LCMV NP from pMJG30 was confirmed in Cos-7 cells.

2.3. Minicell isolations

Plasmid DNA was introduced into MPX1B9 cells by electroporation using a Bio-Rad Gene Pulser[™] apparatus and transformants selected for on LB agar plates containing 100 μ g/mL ampicillin and 15 μ M IPTG. Cultures of MPX1B9 cells harboring pMJG30 were started from a single distinct colony and grown overnight in 3 mL LB broth containing 100 μ g/mL ampicillin and 15 μ M IPTG at 37 °C (LB-Amp/IPTG). After overnight incubation, cultures were diluted 1:125 into 400 mL of LB-Amp/IPTG in a 1 L baffled shake flask. Cultures were grown to an optical density of 600 nm (A_{600}) = 0.1 at which time minicell production was induced by the addition of IPTG for a final concentration of 45 μ M. MPX1B9 cells harboring pMJG30 were co-induced to produce minicells with C-terminus FLAG-tagged LCMV NP protein by the simultaneous addition of 1 mM rhamnose. Cultures were allowed to grow overnight for 15 h from time of induction.

Minicells were enriched by differential centrifugation and further purified by linear sucrose gradients as described previously [11] with LB broth containing 1 μ g/mL ciprofloxacin substituted as the buffer. Minicell quantification was performed measuring A_{600} and applying the equation: No. minicells/mL = $A_{600} \times 5.0 \times 10^{11}$ as previously described [27]. Viable parent cell counts were performed by plating serially diluted minicells on LB agar and growing at 37 °C overnight. Typically, there was ~1 contaminating parent cell per 2×10^8 minicells after purification in the presence of ciprofloxacin.

2.4. Western blotting

Detection of C-terminus FLAG tagged LCMV NP expressed in minicells from pMJG30 was performed by Western blot analysis. Minicells were collected by centrifugation and then boiled in 15 μ L of Laemmli buffer for 10 min prior to fractionation using SDS-PAGE [30]. In cases where eukaryotic expression of NP from pMJG30 was tested in Cos-7 cells, cells were transfected in 6-well cell culture plates using lipofectamine 48 h prior to western blot analysis (see below). Cos-7 cells were prepared by removal of the growth medium followed by lysis in 100 μ L of cell lysis buffer (50 mM HEPES, 0.1% CHAPS, 10 mM EDTA, pH 7.4). Following protein concentration determination by Bradford assay, cell lysates (10 μ g) were fractionated using SDS-PAGE.

Detection of the C-terminus FLAG tag was performed using mouse monoclonal anti-FLAG IgG (Sigma, St. Louis, MO) at a 1:5000 dilution in PBS with 0.1% Tween 20 (PBST) for 1 h. An HRP-conjugated rabbit anti-mouse monoclonal (Sigma) was used as the secondary antibody at 1:10,000 dilution in PBST. Where indicated, detection of NP with LCMV-infected guinea pig serum, was performed at a 1:500 dilution in PBST. An HRP-conjugated goat anti-

guinea pig monoclonal (Sigma) was used as the secondary antibody at 1:10,000 dilution in PBST. Western ECL reagent (Perkin-Elmer, Inc., Boston, MA) was used as the HRP substrate and densitometry performed using a StormTM densitometer (Molecular Dynamics; Amersham Biosciences, Piscataway, NJ)

2.5. Cell culture and media

Cos-7 cells were grown in DMEM containing 10% fetal bovine serum (FBS), penicillin G (50 U/L), streptomycin (50 µg/L), and 2 mM L-glutamine (Gibco, Rockville, MD) Transient transfection experiments using Lipofectamine[®] 2000 (Invitrogen) were performed in 6-well plates according to the manufacturers instructions. BHK cells were grown in MEM containing 10% FBS, penicillin G (50 U/L), streptomycin (50 µg/L), and 2 mM L-glutamine (Gibco). Vero cells were grown in RPMI-1640 with 10% FBS, penicillin G (50 U/L), streptomycin (50 µg/L), and 2 mM L-glutamine. MC-57 (*H-2^b*) cells were grown in RPMI-1640 with 10% FBS, penicillin G (50 U/L), streptomycin (50 µg/L), and 2 mM L-glutamine.

2.6. Viruses and viral infection

Stocks of the LCMV Armstrong 5 strain were grown on BHK cells. LCMV stock titers were determined by plaque assay on Vero cells in RPMI-1640 containing 3% FBS, penicillin G (25 U/L), streptomycin (25 µg/L), 1 mM L-glutamine, and 0.4% agarose as previously described [31]. In experiments requiring that mice be previously infected with LCMV as a positive control, mice were infected via intraperitoneal injection of 2×10^5 PFU of LCMV. All work involving the use of live virus was performed in Biosafety Level 2 (BSL-2) facilities.

2.7. Immunizations

For i.m. immunizations, groups of 6–9 female C57BL/6 mice, 6 weeks in age, were immunized in the right quadriceps with PBS containing naked plasmid DNA (pMJG30; concentrations indicated in the figures), minicells containing empty vector plasmid (pRHA-67), or minicells containing *both* soluble LCMV NP protein and the pMJG30 plasmid containing the eukaryotic LCMV NP cassette in a 50 µL volume of PBS, unless indicated otherwise. Intranasal immunizations were carried out by administration of minicells in a 20 µL volume via pipetman at 10 µL per nostril of mice anesthetized with isoflourane. For p.o. immunizations, mice were anesthetized with isoflourane and given minicells in a 200 µL volume of sodium bicarbonate buffer (pH 9.5) with a feeding cannula. A total of 10^{10} minicells were used in each individual immunization unless otherwise indicated.

Immunizations were given using a three-dose regimen in which immunizations took place on days 0, 14 and 28 and mice were challenged or sacrificed for analysis (described below) on day 35. All animal experiments were performed in strict accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

2.8. Intracellular cytokine staining (ICCS)

Splenocytes (4×10^6) were incubated for 12 h in 250 µL of RPMI-1640 containing 10% FBS and Golgi Stop (Pharmingen, San Diego, CA) in the presence of 2 µg/mL of the immunodominant *H-2^b* restricted CD8+ T cell epitope derived from NP (FQPQNGQFI; NP_{396–404}, Sigma). Negative controls were incubated without peptide. Following stimulation, cells were stained for CD8 and intracellular IFN-γ as specified by the manufacturer (Pharmingen). Following staining, cells were analyzed by flow cytometry using a FAC-Scan or FACSCalibur and the data analyzed for expression of CD8 and IFN-γ using CellQuestTM software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Percent of peptide-specific activation of CD8+ T cells was calculated by dividing the number of CD8+ T cells expressing IFN-γ by the total number of CD8+ T cells. As a positive control for the induction

of T cells to produce IFN- γ , an equivalent number of splenocytes from naïve control mice were incubated for 6 h in the presence of 20 ng/mL phorbol-12-myristate-13-acetate (PMA, Calbiochem, La Jolla, CA) and 3 μ M ionomycin (Calbiochem) prior to staining.

2.9. ⁵¹Chromium-release CTL assays

Primary *ex vivo* cytotoxic lymphocyte (CTL) assays were performed as previously described using ⁵¹Cr-labeled MC-57 cells incubated in the presence or absence of the immunodominant peptide (described above) as targets [32]. Results are determined by applying the following equation and multiplying by 100%:

$$\frac{\text{Experimentally lysis} - \text{spontaneous lysis}}{\text{Maximally lysis} - \text{spontaneous lysis}} \times 100\%$$

2.10. Intracranial challenges

Mice were challenged by an intracranial administration of 10³ plaque forming units (pfu) in 20 μ l RPMI, which is approximately 20 50% lethal dosage (20LD₅₀) of LCMV as described [5]. Mice previously infected intraperitoneally (i.p.) with LCMV (9 days prior to challenge) served as positive controls for protective immunity. Mice were observed daily for 14 days, a time point known to be adequate in the LCMV IC challenge model, and deaths occurred within 6–8 days after challenge. All LCMV infected animals were housed in BSL-2 facilities.

3. Results

3.1. Construction of recombinant minicell vaccine against LCMV nucleoprotein

Previous studies have shown that minicells delivering both heterologous protein and plasmid DNA encoding the protein induce immune responses significantly superior to those induced by minicells that carry protein or DNA alone [27]. However, these studies were done using green fluorescence protein as a general antigen and therefore protection from infection could not be measured. To determine if minicell vaccines can protect animals after challenge with a live virus, a minicell vaccine was designed to deliver both the LCMV-NP protein and a plasmid encoding LCMV-NP. Prior to the immunization of any animals it was necessary to test for the amount of both plasmid DNA and recombinant NP protein antigen encapsulated by the minicells. As shown in Fig. 1, prokaryotic expression of full length NP protein expressed from pMJG30 in *E. coli* Top 10 cells could be detected by either an anti-FLAG monoclonal antibody (Fig. 1a) or by polyclonal antisera raised in LCMV-infected guinea pigs (Fig. 1b, left panel). Eukaryotic expression of NP from pMJG30 in transiently transfected Cos-7 cells was also detectable when probed with LCMV-specific guinea pig sera (Fig. 1b, right panel). The amount of C-terminus FLAG-tagged NP recombinantly expressed from pMJG30 in minicells was quantitated by densitometry of Western blots using FLAG-tagged bacterial alkaline phosphatase (BAP; Sigma) to create a standard curve (Fig. 1c) as previously described [28]. Quantitation by this method suggests that $\sim 0.12 \pm 0.02$ μ g of recombinant NP protein was present in 10⁹ minicells such that $\sim 1.2 \pm 0.2$ μ g was present in the 10¹⁰ minicell immunization.

The plasmid pMJG30 also contains a eukaryotic expression cassette driving the expression of NP acting as a DNA vaccine. Plasmid DNA (pMJG30) was purified from 10¹⁰ minicells by alkaline lysis and quantified by spectrophotometry at a wavelength of 260 nm. As shown in Fig. 1d, $\sim 3.0 \pm 0.7$ μ g of plasmid DNA was recovered from 10¹⁰ minicells in three experiments. Fig. 1d summarizes all of the formulation testing results.

3.2. CD8⁺ T cell responses following minicell vaccination

The protective and immunodominant MHC class I restricted peptide epitopes have been identified for NP in *H-2^b* mice [33,34] and a subpopulation of antigen-specific CD8⁺ T cells

have been shown to quickly express and secrete cytokines after exposure to their cognate peptide bound to MHC class I in this system [35]. ICCS has been shown to detect the peptide-dependent production of IFN- γ by antigen experienced CD8⁺ T cells *ex vivo* in LCMV vaccine and infection models [36]. The ability to stimulate cellular immune responses from splenic CD8⁺ T cells following three i.m. inoculations of 10^{10} minicells containing a combination of both NP protein antigen and the corresponding DNA vaccine (pMJG30) was tested by ICCS as shown in Fig. 2. In these experiments, mice inoculated with minicells were compared to mice inoculated with either 3 or 50 μ g of pMJG30 plasmid DNA alone. The plasmid pMJG30 was derived from pCMV-NP (see Section 2) which has been shown to elicit long term cellular responses in mice when inoculated three times at a concentration of 50 μ g [29]. Thus, testing the newly cloned pMJG30 at a concentration of 50 μ g was an appropriate positive control. In contrast, 10^{10} minicells contain only ~ 3 μ g of pMJG30 (see Fig. 1d) and testing the ability of that amount of plasmid DNA to elicit immune responses was important for comparative purposes.

The average percentage of peptide-specific CD8⁺ effector T cells isolated from the spleens of mice immunized with minicells resulted in significant ($p < 0.05$) peptide-dependent CD8⁺ effector T cell responses as shown in Fig. 2. The plasmid pMJG30, at a concentration of 50 μ g, also resulted in significant ($p < 0.05$) peptide-dependent CD8⁺ effector T cell responses (Fig. 2). However, pMJG30 at a concentration of 3 μ g did not, supporting the theory that the co-delivery of recombinant protein antigen and the corresponding DNA vaccine by minicells is responsible for generating more optimal adaptive cellular immune responses.

3.3. Peptide-specific CD8⁺ T cell mediated lytic activity following minicell vaccination

Effective killing of virally infected cells by antigen-specific CD8⁺ cytotoxic lymphocytes is of paramount importance in controlling LCMV infection *in vivo*. Accordingly, mice were vaccinated with minicells containing both NP protein antigen and DNA and their ability to elicit cytolytic responses *ex vivo* was tested. Cytolytic activity of splenocytes from mice inoculated i.m. with minicells was analyzed using syngeneic target cells pulsed with the immunodominant H-2^b restricted peptide from the NP protein (NP₃₉₆₋₄₀₄). As shown in Fig. 3, splenocytes from mice immunized with minicells exhibit robust cytolytic activity against peptide pulsed target cells. Comparisons were made to groups of mice inoculated with naked plasmid DNA (pMJG30; 3 or 50 μ g) and vigorous responses were observed when 50 μ g was administered. In contrast, small but significant ($p < 0.05$) responses were detected in cases where 3 μ g was used. This result was unexpected based on the ICCS assay results (Fig. 2) and was only observed at an effector to target ratio (E:T) of 100 (see Fig. 3, bottom panel). LCMV infected and naïve (non-immunized) mice served as control groups for cytolytic activity.

3.4. Recombinant minicells protected mice from a lethal dose of LCMV

The LCMV challenge model was chosen for this cell-mediated immune response proof-of-concept study because antigen specific CD8⁺ T cells are essential in establishing protective immunity against LCMV in mice [36–38]. To determine if antigen specific cellular responses could confer protective immunity, mice were vaccinated three times i.m. with minicells containing both NP protein antigen and DNA vaccine (pMJG30) prior to receiving a normally lethal intracranial dose of live LCMV. As shown in Fig. 4, three i.m. immunizations with 10^{10} minicells (mc-NP) resulted in protective immunity in 89% (8 of 9 survived) of mice tested. Also shown is that protective immune responses were dose dependent as evidenced by a reduction in the survival rates of mice immunized three times i.m. with 10^9 minicells (40%, 2 of 5), 10^8 minicells (20%, 1 of 5), or 10^7 minicells (0%, 0 of 5).

Mucosal delivery is very important for vaccines that work against organisms that infect across mucosal surfaces and the development of delivery mechanisms that work mucosally is an

important goal. Theoretically, minicells should be good at mucosal delivery, due to the fact that bacteria have evolved to survive the hostile environment these surfaces represent. Therefore, minicells were tested for their ability to induce protective immune responses after mucosal vaccine delivery. Interestingly, p.o. delivery of the vaccine did not result in good protection of the animals, although 1 animal tested (7%, 1 of 13) did survive the challenge. The possible reasons for this are discussed further below. Importantly, mice immunized three times i.n. were partially protected (31%, 4 of 13). Control groups included mock-vaccinated PBS controls, empty vector minicell controls (no NP protein antigen and empty eukaryotic expression cassette), and mice previously infected i.p. with live LCMV. All mice previously infected i.p. with LCMV survived (100%, 9 of 9) in contrast to both empty minicell (0%, 0 of 9) and PBS-only controls (0%, 0 of 9). Taken together, these results are a good indication that minicells delivering heterologous protein antigen and the corresponding DNA vaccine are capable of eliciting protective systemic cellular immunity in mice after both i.m. and mucosal immunization, although it is clear that optimization will be a requirement for mucosal delivery.

4. Discussion

Infectious disease accounts for an estimated 19% of total deaths (an estimated 57 million) worldwide per annum (WHO website for estimates of death by cause, 2004). While vaccine development efforts have helped to curb those numbers, pathogens continue to take a significant toll on human populations, especially in undeveloped countries. Many of the molecular mechanisms behind pathogenesis and protective immunity have been identified with a large number of pathogen-specific protective antigens. The limiting factors in vaccine development as a whole have been in delivering antigens to the appropriate cell types and antigen processing pathways as well as generating T cell responses that will support the development of immunological memory in both B and T cells.

This work was designed to test the capacity of bacterial minicells to simultaneously package and deliver both heterologous protein antigens and the corresponding plasmid DNA vaccine to elicit protective cell-mediated immune responses to LCMV. Minicells given three times via the i.m. route were able to generate cell-mediated immune responses as determined by peptide-specific target cell lysis as well as the ability to secrete the cytokine IFN- γ upon restimulation with viral peptide *ex vivo*. Most importantly, at the highest dose of minicells given (10^{10} , three times), mice immunized i.m. were able to survive a normally lethal intracranial challenge with live LCMV (89% survival) demonstrating that the cell-mediated immune responses generated by minicells conferred protection.

While i.m. administration of minicells is an important experiment to perform from a proof-of-concept standpoint, the real potential in using bacterial minicells as a delivery vehicle is in delivering vaccines via the mucosal route of administration. Fortunately, testing the ability of minicells to elicit protective systemic immunity following mucosal administration can be tested directly using the intracranial LCMV challenge. Similar experiments have already demonstrated that the NP protein antigen can effectively induce protective systemic cellular immunity against LCMV after being delivered by an attenuated *Salmonella* strain that constitutively expresses the NP protein *in vivo* [5]. In the study presented here, it was found that minicells co-packaging NP protein antigens and the corresponding plasmid DNA vaccine could afford partial protection (31% of animals tested) from a lethal LCMV challenge after i.n. administration. On the other hand, minicells delivered via p.o. administration did not provide similar results. There may be several reasons for this disparity including the fact that *E. coli*, the bacterial strain from which the minicells were derived, resides naturally in the gut and as such may be tolerated there. *E. coli* is not a part of the normal lung microbiota in mice and may conversely be recognized as foreign by resident immune cells, leading to better uptake and processing. At the same time, one animal tested after p.o. administration did survive the

lethal challenge which may mean that further engineering of the minicell producing strain used in this study could improve delivery and/or efficacy.

Using bacterial minicells to deliver vaccines may have distinct advantages over other bacterial based delivery systems. First, minicells can package a wide array of different antigenic proteins and plasmid DNA vaccines and the levels of both can be easily modified and manipulated. In addition, minicells have a distinct safety advantage stemming from the fact that they are non-infectious and non-dividing. This may allow minicell vaccines to be developed that can accommodate certain patient populations such as children, the elderly and the immunocompromised. Although they contain all of the adjuvant properties of their parent cells (LPS, etc.), they do not contain a chromosome, eliminating the possibility of pathogenic reversion in this approach. The ability of minicells to contribute to horizontal gene transfer to other pathogenic strains of bacteria is minimal as it has been well documented that bacterial mating into minicells is possible while the opposite is not [11,39,40].

Ultimately, minicells may prove to be most useful in delivering vaccines to mucosal lymphoid tissues. Preliminary experiments using the minicells described in this report to immunize mice via mucosal administration have proven somewhat successful thus far. In addition to further engineering of the *E. coli* strain used here, it is well known that minicell producing strains can be isolated from almost any species of bacteria. This may help when a specific tropism may be more appropriate for a particular route of administration than another. For example, using minicells derived from *Salmonella enterica* may be more appropriate for delivering vaccines to the gut associated lymphoid tissues (GALT) because of their natural tropism [41,42]. The results as presented here indicate that bacterial minicells represent a unique approach to the safer and possibly more effective mucosal delivery of a wide variety of vaccines.

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Fig. 1.

Full length prokaryotic expression of C-terminus FLAG tagged LCMV-NP. (a) Prokaryotic expression of C-terminally FLAG-tagged LCMV-NP protein from pMJG30 was determined by Western blot. *E. coli* Top10 cells were grown in the presence or absence of 1 mM rhamnose as described in the Methods. A total of 5×10^7 cells were loaded per lane and purified FLAG-tagged BAP was used as a positive control. (b) LCMV-NP expression (appropriate size indicated by arrows) was detected by the antisera of LCMV infected guinea pigs. Prokaryotic expression of LCMV-NP was performed as described for (a) except that LCMV infected guinea pig antisera was used to detect expression. Eukaryotic expression was determined by the transient transfection of pMJG30 into Cos-7 cells as compared to empty vector control

transfections. (c) Quantitation of FLAG-tagged LCMV-NP in minicells. Quantitation was carried out by creating a standard curve of FLAG-tagged BAP protein in comparison to the indicated range of minicells tested (10^9 to 2.5×10^8). Intensities were determined by densitometry. (d) Summarization of minicell vaccine formulations showing the concentrations of both the protein antigen component and the DNA vaccine component in a single dose of 10^{10} minicells measured from three independent minicell preparations.

Fig. 2.

Intramuscular immunization of C57Bl/6 mice with minicells (mc-NP, $n = 6$) results in peptide-specific γ -IFN production following a 5 h *ex vivo* restimulation with the immunodominant NP peptide epitope (NP₃₉₆₋₄₀₄). Splenocytes were isolated, restimulated with peptides and stained for both CD8 and γ -IFN prior to analysis. The dot plots are a representative of the raw data collected. The histogram shown reflects the percentage of CD8+ splenocytes ($\pm$ S.E.M.) that produce γ -IFN following restimulation. The X and Y-axes indicate increasing fluorescence intensities. Splenocytes from mice infected 7 days prior to any assay served as positive controls ($n = 6$) while naïve mice (non-immunized) served as the negative control ($n = 6$). The ability of naked plasmid DNA (pMJG30) elicit immune responses after an intramuscular injection was tested at concentrations of 50 ($n = 6$, bottom panel only) or 3 μ g ($n = 3$). Due to space constraints, the representative raw flow cytometry data for the 50 μ g naked DNA group was not included in the top panel. Results were analyzed using a student's *t*-test, * $p < 0.05$.

Fig. 3.

Intramuscular immunization with minicells (mc-NP, $n = 6$) primes for NP specific CTL activity. The *ex vivo* cytotoxicity of splenocytes isolated 7 days after a third i.m. immunization of C57Bl/6 mice with minicells was tested using a standard ^{51}Cr release assay in the presence or absence of the immunodominant peptide (NP₃₉₆₋₄₀₄). Splenocytes from mice infected 7 days prior to any assay served as positive controls ($n = 6$) while naïve mice (non-immunized) served as the negative control ($n = 6$). The ability of naked plasmid DNA (pMJG30) to prime cytotoxic CTLs after an i.m. injection was tested at concentrations of 50 ($n = 6$) or 3 µg ($n = 3$). The latter concentration is equivalent to that delivered by each minicell vaccine. The 50

µg group was only tested at an E:T ratio of 100 as shown in the top panel. Results were analyzed using student's *t*-test, $*p < 0.05$. E:T = effector to target cell ratio.

Fig. 4.

Immunization of C57Bl/6 mice protects from a lethal challenge with live LCMV. Mice were immunized three times each via the indicated route of administration and dose of minicells at 2-week intervals. Naïve mice (non-immunized) and mice immunized with empty minicells (no protein antigen, no DNA vaccine) served as the negative controls. Mice previously infected with LCMV (9 days prior) served as positive controls. Mice were challenged on day 35 with an intracranial administration of 20 LD₅₀ of live LCMV and the percentage of mice which survived, as well as the number of animals used, are shown.