Production of a Particulate Hepatitis C Vaccine Candidate by an Engineered \textit{Lactococcus lactis} Strain\textsuperscript{v}

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Vaccine delivery systems based on display of antigens on bioengineered bacterial polyester inclusions can stimulate cellular immune responses. The food-grade Gram-positive bacterium \textit{Lactococcus lactis} was engineered to produce spherical polyhydroxybutyrate (PHB) inclusions which abundantly displayed the hepatitis C virus core (HCc) antigen. In mice, the immune response induced by this antigen delivery system was compared to that induced by vaccination with HCc antigen displayed on PHB beads produced in \textit{Escherichia coli}, to PHB beads without antigen produced in \textit{L. lactis} or \textit{E. coli}, or directly to the recombinant HCc protein. Vaccination site lesions were minimal in all mice vaccinated with HCc PHB beads or recombinant protein, all mixed in the oil-in-water adjuvant Emulsigen, while vaccination with the recombinant protein in complete Freund’s adjuvant produced a marked inflammatory reaction at the vaccination site. Vaccination with the PHB beads produced in \textit{L. lactis} and displaying HCc antigen produced antigen-specific cellular immune responses with significant release of gamma interferon (IFN-\gamma) and interleukin-17A (IL-17A) from splenocyte cultures and no significant antigen-specific serum antibody, while the PHB beads displaying HCc but produced in \textit{E. coli} released IFN-\gamma and IL-17A as well as the proinflammatory cytokines tumor necrosis factor alpha (TNF-\alpha) and IL-6 and low levels of IgG2c antibody. In contrast, recombinant HCc antigen in Emulsigen produced a diverse cytokine response and a strong IgG1 antibody response. Overall it was shown that \textit{L. lactis} can be used to produce immunogenic PHB beads displaying viral antigens, making the beads suitable for vaccination against viral infections.

The food-grade Gram-positive bacterium, \textit{Lactococcus lactis} has been increasingly considered as a production host for recombinant therapeutic proteins (6, 9, 49). The recent advances toward the development of efficient gene expression systems in \textit{L. lactis} and the established safety profile of \textit{L. lactis} based on long-term use in dairy food processing has led to new potential applications in protein production, therapeutic drug delivery, and vaccine delivery (5, 27, 30, 38).

Recently, it was shown that \textit{L. lactis} can be engineered to produce spherical polyhydroxybutyrate (PHB) inclusions which display the \textit{Staphylococcus aureus} protein A-derived IgG binding region, the Z domain, and that these can be isolated for \textit{in vitro} use in purification of IgG (26). This was achieved by establishing the PHB biosynthesis pathway in \textit{L. lactis} and by overproducing a Z domain-PHB synthase fusion protein which remained attached to the PHB inclusion surface. The PHB synthase represents the only essential enzyme required for PHB inclusion formation (39, 40). This strategy utilized protein engineering of the PHB synthase from \textit{Ralstonia eutropha} for the display of various protein-based functions, such as technical enzymes, binding domains, or a fluorescent protein, at the surfaces of PHB beads as had been previously established in recombinant \textit{Escherichia coli} (13, 15, 34, 35, 37). The successful display of various technically relevant protein functions as well as the \textit{in vitro} performance of the respective isolated PHB beads suggested a wide applicability of this bead display technology (12, 19, 41). Only recently have these PHB beads formed by recombinant \textit{E. coli} been considered for the display of antigens for \textit{in vivo} use as a particulate vaccine (32). PHB beads simultaneously displaying the \textit{Mycobacterium tuberculosis} antigens Ag85A and ESAT-6 were produced in recombinant \textit{E. coli}, isolated, and injected into mice to assess the immune response. The Ag85A–ESAT-6 beads induced significantly stronger humoral and cell-mediated immune responses than only the fusion protein Ag85A–ESAT-6. This antigen delivery system based on PHB beads has been considered relevant in the quest for an effective tuberculosis vaccine (31). A significant cell-mediated immune response is considered to be important for protection not only against intracellular pathogenic bacteria but also against viruses (44, 45). Therefore, it would be important to determine whether PHB beads displaying viral antigens also demonstrate immunogenic properties making the beads suitable for vaccination against viral infections. The downside of using \textit{E. coli} for recombinant protein production, vaccines, or other \textit{in vivo} uses is the copurification of lipopolysaccharide (LPS) endotoxins. LPS removal is costly, and the processes can destroy surface proteins and hence functionality of the beads (50). Therefore, the LPS-free \textit{L. lactis} might be the preferred production host for antigen-displaying PHB beads. The practicality of using \textit{L. lactis} as a production system for vaccine antigens is also based on extensive use in the fermentation industry, an abundance of genetic tools, and high expression levels of genes encoding recombinant proteins (5). Hepatitis C is a disease with worldwide...
distribution transmitted by blood-blood contact, often through inadequately sterilized drug injection equipment, and coinfection with HIV is common (24). It often leads to permanent liver damage, cirrhosis, and cancer. Not only is treatment limited and of variable efficacy (3), but there is no vaccine available. Research efforts have been limited because there is no cell culture system or effective small-animal model, with chimpanzees being the only model in which challenge studies can be performed (46). A number of new vaccine approaches are currently being explored for control of hepatitis C virus, including recombinant protein-, peptide-, DNA-, and virus vector-based vaccines, and some have reached phase I/II human clinical trials (14). Recombinant protein hepatitis C virus vaccines have the advantages of being well tolerated with low toxicity and inducing cross-neutralizing antibodies, and proof of concept has been established with hepatitis B virus vaccine; however, they suffer from the disadvantage of generally eliciting only weak T cell responses. The hepatitis C virus genome encodes three structural (core, E1, and E2) and six nonstructural proteins, and vaccines which target one or several of these proteins are being developed (47).

In this study, L. lactis and E. coli were genetically engineered to produce PHB beads which displayed the hepatitis C virus core antigen (HCc). The resulting beads were analyzed and subjected to vaccination trials to determine whether a significant immune response could be generated and to what extent the production host affects the immunogenic properties of the PHB beads displaying HCc antigen.

**MATERIALS AND METHODS**

**Plasmids, bacterial strains, and growth conditions.** All bacterial strains and plasmids are listed in Table 1. General cloning procedures were performed as described elsewhere (44). E. coli strains were grown in Luria broth (LB) (Difco, Detroit, MI) supplemented with 1% (wt/vol) glucose, ampicillin (75 μg/mL), and chloramphenicol (30 μg/mL). L. lactis strains were grown in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% glucose, 0.3% l-arginine, and chloramphenicol (10 μg/mL).

**Construction of plasmids for production of hepatitis C core antigen.** To display HCc on the surfaces of PHB beads produced by E. coli, the gene encoding the HCc with amino acid sequence MSTNPKPQRKTKRSTNRRPQDVKFPGGGQIVGVGVVLPRLPPRPLVRAKTRKESORPGRKOPRIPKAROGREAWAAAQPGWPWPLNGEWMGAVLLSPGRSPWGGPTPRRRLSRNLGKVIDTLCQFDMLMGYIIPLVAPLGGAAARALAHGVRLVEDGVNYATGNLPSCSIFLLALLCTIPASA was synthesized by DNA2.0 (CA), adapting it to the codon usage bias of E. coli and avoiding rarely used codons. An SpeI restriction site was inserted at the 5' end of the HCc gene, and a BsiWI restriction site and a sequence encoding five glycine residues were added at the 3' end. In order to accelerate cloning, part of phaC was included in the synthesis, enabling direct subcloning of the synthesized piece of DNA into pHAS-scfV13R4 (13) with SpeI and NotI, replacing the scFv gene with the HCc gene. The resulting plasmid, pET-HCc-phaC, encodes the HCc-PhaC fusion protein under the control of the T7 promoter, with HCc and PhaC connected by the pentaglycine linker. In addition to the polyester synthase gene (phaC), PHA biosynthesis requires the enzymes PhaA and PhaB for precursor synthesis, and these enzymes were encoded by plasmid pMCS69, which contains the phaA and phaB genes. pET-HCc-phaC and pMCS69 were transformed into E. coli BL21(DE3). Control PHB beads were produced using E. coli BL21(DE3) containing pET-phaC and pMCS69.

For display of HCc on the surfaces of PHB beads produced by L. lactis, the gene encoding HCc with the amino acid sequence as used above for E. coli was synthesized with the codon usage adapted to L. lactis by Genescript Corporation. The HCc gene was designed with a small proportion of the DNA encoding the N terminus of PhaC linked to the DNA corresponding to the antigen's C terminus, with flanking restriction sites (NcoI and NdeI), in order to allow easy subcloning into a preexisting vector, pNZ-CAB (26). Plasmid pNZ-CAB harbors the codon-optimized PHB biosynthesis operon, containing the phaA, phaB, and phaC genes, from Ralstonia eutropha under pM15 control. The HCc gene was ligated into pNZ-CAB downstream of the mck promoter, generating an HCc-phaC hybrid gene, and this was transformed directly into L. lactis NZ9000 by electroporation.

**Culture and isolation of PHB beads.** PHB beads, which displayed HCc or control PHB beads alone were produced in E. coli and L. lactis as previously described (26, 32). Briefly, E. coli was grown at 30°C in LB, induced with 1 mM isopropyl-β-D-thiogalactopyranoside to produce protein, and cultured for a further 48 h at 30°C to allow accumulation of particles. L. lactis cultures were produced in M17 broth, induced with 10 mM nisin to produce protein, and cultured for a further 24 h at 30°C. The presence of PHA/polyester was determined by staining the cultures with Nile Red lipophilic dye and observed under fluorescence microscopy. Transmission electron microscopy (TEM) was used to assess the size and shape of PHB beads formed. Bacteria were then mechanically disrupted, and E. coli lysate was centrifuged at 4,000 × g or L. lactis lysate was centrifuged at 8,000 × g for 15 min at 4°C to sediment the polymer particles. All beads were then purified via glycerol gradient ultracentrifugation as described elsewhere (15). To confirm functionality of the PhaC enzyme, the PHB content of the cells was quantitatively determined by gas chromatography-mass spectrometry (GC-MS) (7).

**Analysis of proteins attached to the PHB beads.** The concentration of proteins attached to the beads was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by SDS-PAGE using NuPAGE Bio-Tris 4 to 12% gels (Invitrogen) and stained with SimplyBlue Safe stain (Invitrogen). The amount of HCc-PhaC fusion protein relative to the amount of total proteins attached to the particles was detected using a Gel Doc XR and analyzed using Quantity One software (version 4.6.2) (Bio-Rad Laboratories, Hercules, CA). Proteins of interest were excised from the gels and subjected to tryptic peptide fingerprinting using matrix-assisted laser desorption

**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacI F' ompT hsdS29 (rB mB ) gal dcm (DE3)</td>
<td>Statragen Novagen</td>
</tr>
<tr>
<td>L. lactis NZ9000</td>
<td>MG1363 derivative, pepN::nisRK</td>
<td>18</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMCS69</td>
<td>pPBR1MCs derivative containing phaA and phaB genes from Cupriavidus necator</td>
<td>2</td>
</tr>
<tr>
<td>pET-phaC</td>
<td>pET-14b derivative containing phaC gene from C. necator</td>
<td>51</td>
</tr>
<tr>
<td>pHAS-scfV13R4</td>
<td>pET-14b derivative containing the gene scFv13R4+phaC</td>
<td>13</td>
</tr>
<tr>
<td>pET-HCc-phaC</td>
<td>pET-14b derivative containing HCc-phaC gene</td>
<td>This study</td>
</tr>
<tr>
<td>pNZ8148</td>
<td>Cm’, pSH71 origin, PhaA</td>
<td>25</td>
</tr>
<tr>
<td>pNZ-AB</td>
<td>pNZ8148 derivative, PphaAphaB</td>
<td>26</td>
</tr>
<tr>
<td>pNZ-CAB</td>
<td>pNZ8148 derivative, PphaAphaB</td>
<td>26</td>
</tr>
<tr>
<td>pNZ-HCc-CAB</td>
<td>pNZ-8148 derivative containing HCc-phaC, phaA and phaB</td>
<td>This study</td>
</tr>
</tbody>
</table>
ionization-time of flight mass spectrometry (MALDI-TOF MS). Specific activity of the HCc protein on the PHB beads was determined by enzyme-linked immunosorbent assay (ELISA). Micronol high-binding plates (Greiner) were coated overnight at 4°C with purified PHB beads diluted from 1 μg/ml to 60 μg/ml protein using 0.2 M phosphate coating buffer, pH 6.5. The plates were washed with phosphate-buffered saline (PBS) containing 0.05% (vol/vol) Tween 20 (PBST) and blocked with 1% (wt/vol) bovine serum albumin (BSA) in PBS for 1 h at 25°C. The plates were then washed in PBST and incubated for 1 h with mouse antibody to hepatitis C core protein (Devatal, NJ) diluted in 1% (wt/vol) BSA in PBS. Following washing with PBST, plates were incubated for 1 h at room temperature with biotinylated anti-mouse IgG (Sigma-Aldrich) diluted in 1% (wt/vol) BSA in PBS, incubated for 1 h, and washed with PBST, and streptavidin-horseradish peroxidase was added. After another hour of incubation, plates were washed and o-phenylenediamine (OPD) substrate (Sigma-Aldrich) was added and incubated for 30 min at room temperature. The reaction was stopped with 0.5 M H2SO4 and the absorbance was recorded at 490 nm on a VERSAmax microplate reader.

**Vaccination of mice.** Vaccines comprising control wild-type PHB beads produced in *E. coli*, control wild-type PHB beads produced in *L. lactis*, and PHB beads displaying HCc produced in *E. coli* and *L. lactis* were adjusted to contain 30 μg of the HCc-PhaC protein as calculated from the densitometry profile. Emulsigen (MVP Laboratories, Omaha, NE) adjuvant (20%, vol/vol) was mixed 30% at weekly intervals (200 μg purchased from the animal breeding facility of the Malaghan Institute of Medical Research (Wellington, New Zealand) and then vaccinated 3 times subcutaneously at weekly intervals (200 μl/injection; n = 6 per group). A positive-control group (n = 6) receiving 30 μg recombinant hepatitis C virus core protein (rHCc) (Devatal, NJ), or PBS. Female C57BL/6 mice aged 6 to 8 weeks were purchased from the animal breeding facility of the Malaghan Institute of Medical Research (Wellington, New Zealand) and then vaccinated 3 times subcutaneously at weekly intervals (200 μl/injection; n = 6 per group). A positive-control group (n = 6) receiving 30 μg recombinant hepatitis C virus core protein (rHCc) (Devatal, NJ) or PBS. Female C57BL/6 mice aged 6 to 8 weeks were purchased from the animal breeding facility of the Malaghan Institute of Medical Research (Wellington, New Zealand) and then vaccinated 3 times subcutaneously at weekly intervals (200 μl/injection; n = 6 per group). A positive-control group (n = 6) receiving 30 μg recombinant hepatitis C virus core protein (rHCc) (Devatal, NJ) was used as a positive control.

**Measurement of cytokines.** Levels of gamma interferon (IFN-γ) in culture supernatants were measured by ELISA according to the manufacturer’s recommendations (BD Biosciences [BD], CA). The assay used o-phenylenediamine substrate and was read at 495 nm on a VERSAmax microplate reader. A standard curve was constructed using SOFTmax PRO software, and averages of duplicate sample cytokine values were determined from the curve. Levels of other cytokines in culture supernatants were determined with a cytofaretic bead array (mouse Th1-Th2 cytokine kit; BD) according to the manufacturer’s instructions. Fluorescence was measured using a FACScalibur flow cytometer (BD) and analyzed using FCEO array software (BD). All results were calculated as the cytokine value of the PBS-stimulated sample subtracted from that of the rHCc-stimulated sample.

**Measurement of serum antibody.** Antibody in sera was measured by ELISA using Micronol high-binding plates (Greiner) coated overnight with 3 μg/ml rHCc and then blocked using 1% (wt/vol) BSA in PBS. After washing in PBST, dilutions of serum were added and incubated for 1 h. Following washing with PBST, anti-mouse IgG1-horseradish peroxidase (HRP) or IgG2c-HRP (ICL, Newberg, OR) was added and plates incubated. Plates were washed and tetramethylbenzidine was used as a substrate prior to reading at 450 nm on a VERSAmax microplate reader. Monoclonal HCc antibody (Devatal) was used as a positive control. Results were expressed as optical density (OD) at 450 nm for sera diluted 1/250.

**Statistical analysis.** Analyses of the cytokine and antibody responses were performed by Kruskal-Wallis one-way analysis of variance.

**RESULTS**

**Microbial production and characterization of PHB beads displaying hepatitis C core antigen.** Plasmids encoding PHB synthase with or without HCc were successfully introduced into both production strains, which enabled production of PHB beads alone or PHB beads displaying HCc. GC-MS analysis showed that PHB was produced by both recombinant *E. coli* and *L. lactis* strains, which in turn indicated in vivo functionality of the PHB synthase domain in the fusion protein (data not shown). The presence of intracellular polyester inclusions was further confirmed by fluorescence microscopy using Nile Red staining (data not shown) and TEM (Fig. 1). *E. coli* cells accumulated large numbers of intracellular beads with a diameter of about 150 to 250 nm (Fig. 1A), whereas *L. lactis* cells produced smaller intracellular particles (50 to 150 nm) (Fig. 1B).

**Statistical analysis.** Analyses of the cytokine and antibody responses were performed by Kruskal-Wallis one-way analysis of variance.
of 50% and an ion score of >100 for *L. lactis*-produced HCC-PhaC and a sequence cover of 46% and an ion score of >100 for *E. coli*-produced HCC-PhaC (data not shown). Densitometry analysis of the gels indicated that the HCC-PhaC protein accounted for 25.6% of total bead protein associated with *L. lactis* HCC beads, whereas this protein accounted for only 6.7% of that associated with the *E. coli* HCC beads. The presence of HCC at the surfaces of *E. coli* and *L. lactis* beads was assessed by ELISA. The results indicated that HCC beads from both bacterial hosts bound to the anti-HCC antibody in a dose-dependent manner (Fig. 3).

**Vaccination responses.** Mouse weights did not differ significantly between groups during the time course of the experiment, and mice in all groups gained weight; an average of 2.6 g was gained over 5 weeks (data not shown). Mice vaccinated with PHB beads developed small lumps of up to 2.5 mm in diameter at the vaccination sites, with no signs of an abscess or suppuration. All mice were healthy throughout the trial and displayed normal behavior. In contrast, 3 out of 6 mice vaccinated with recHCC in CFA showed skin sloughing at the injection site.

IFN-γ is an important marker of the development of Th1 cell-mediated immunity and was assessed by measuring the release of IFN-γ in splenocytes restimulated in vitro with proteins used for immunization (Fig. 4A). This study showed that vaccination of mice with HCC PHB beads produced by both *L. lactis* and *E. coli* hosts stimulated the generation of a significant antigen-specific cellular immune response compared to that in the PBS-vaccinated group (*P* < 0.05). Vaccination with recHCC in either Emulsigen or CFA also induced a significant increase in IFN-γ levels (*P* < 0.05). The vaccine groups receiving *E. coli* PHB beads, recHCC in Emulsigen, and recHCC in CFA produced significantly more interleukin-10 (IL-10) than the groups receiving PBS and *L. lactis* PHB beads (*P* < 0.05) (Fig. 4B).

Tumor necrosis factor alpha (TNF-α) was significantly increased in the *E. coli* HCC PHB bead-vaccinated group and the CFA control group compared to PBS-vaccinated mice (*P* < 0.05) (Fig. 4C). For the *E. coli*-produced wild-type control bead-vaccinated group, TNF-α values were not significantly increased, although there was a positive trend. IL-6 levels were significantly increased in both the group vaccinated with *E. coli* PHB beads and that vaccinated with recHCC in CFA (*P* < 0.05) (Fig. 4D). IL-17-A release was significantly increased in groups vaccinated with PHB beads produced in *E. coli*, HCC PHB beads from *L. lactis*, and recHCC in CFA (*P* < 0.05) (Fig. 4E). IL-2 increased only in the control group vaccinated with recHCC in CFA (data not shown). IL-4, a Th2 cytokine, was not detected in any of the groups (data not shown).

Antigen-specific serum antibody levels were assessed by measuring IgG1 and IgG2 (Table 2). IgG1 results are indicative of Th2 immune responses, and the results indicate that antigen-specific serum IgG1 to HCC was significantly increased only in the recHCC-in-Emulsigen vaccine group (*P* < 0.05) and was not increased in any vaccine groups receiving PHB beads. A small but significant increase in IgG2 antibody levels to HCC antigen was detected in the groups receiving *E. coli*-produced
Bioengineered PHB beads have previously been used to display proteins with a variety of potential end uses. Here further evidence was provided for the versatility of bioengineered PHB beads to be used for medical applications as viral antigen-displaying beads by allowing custom antigen display and the subsequent use as particulate antigen carrier systems. In this study, it was shown that the generally regarded as safe (GRAS) bacterium L. lactis as well as E. coli could be engineered as production hosts for PHB bead-based particulate vaccines which displayed HCc antigens. This antigen was used because it is a prime candidate antigen for inclusion in both therapeutic and prophylactic hepatitis C vaccines (42). However, the disadvantage of using E. coli as the production host for human biological products, including vaccines, is potential contamination of products with LPS. This precludes the use of such products for human vaccination without costly depyrogenation, a process which may also destroy protein function (50). L. lactis is a Gram-positive bacterium which does not contain LPS and has been extensively used in manufacture of dairy products. More recently it has been investigated as a production host for recombinant proteins (28) and as a mucosal vaccine for hepatitis B (52). The study described in this paper combined the production of recombinant protein, i.e., the viral antigen HCc, and the polymeric carrier in a one-step process. This new vaccine delivery system has the advantage that vaccine antigens are produced on beads rather than as soluble proteins. Particulate vaccines have been shown to be more immunogenic (20), and the size of particles is likely to play a role in the type of immune response, with nanoparticles stimulating cell-mediated immunity and larger particles stimulating antibody responses (16). The TEM images show differences in the sizes of beads produced in E. coli and L. lactis (Fig. 1), which may account for different antibody responses being obtained.

It was demonstrated that L. lactis was able to produce PHB beads displaying a substantial amount HCc antigen as shown in Fig. 4. Cytokine responses in mice (n = 6) vaccinated 3 times with control wild-type PHB beads produced in E. coli (EcWT), PHB beads displaying HCc produced in E. coli (EcHCc), control wild-type PHB beads produced in L. lactis (LcWT), PHB beads displaying HCc produced in L. lactis (LcHCc), recombinant hepatitis C virus core protein (recHCc), or PBS, all in Emulsigen. A single vaccination was used for mice vaccinated with recHCc emulsified in complete Freund's adjuvant (CFA). Three weeks after the final vaccination, splenocytes were cultured for 3 days with 5 μg recHCc. Release of IFN-γ (A) was measured by ELISA, and those of IL-10 (B), TNF-α (C), IL-6 (D), and IL-17A (E) were measured with cytometric bead arrays. Results were calculated as the value for the PBS-stimulated sample subtracted from that for the PBS-stimulated sample. Each data point represents the mean for 6 mice ± SEM. * significantly greater than the value for the PBS-vaccinated control group (P < 0.05).

**TABLE 2. Serum IgG1 and IgG2c antibody responses to HCc**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean (SEM) antibody response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
</tr>
<tr>
<td>PBS</td>
<td>0.001 (0.001)</td>
</tr>
<tr>
<td>EcWT</td>
<td>0.045 (0.011)</td>
</tr>
<tr>
<td>EcHCc</td>
<td>0.110 (0.065)</td>
</tr>
<tr>
<td>LcWT</td>
<td>0.001 (0.004)</td>
</tr>
<tr>
<td>LcHCc</td>
<td>0.017 (0.003)</td>
</tr>
<tr>
<td>recHCc in Emulsigen</td>
<td>1.126 (0.079)*</td>
</tr>
<tr>
<td>recHCc in CFA</td>
<td>0.089 (0.044)</td>
</tr>
</tbody>
</table>

* EcWT, control wild-type PHB beads produced in E. coli; EcHCc, PHB beads displaying HCc produced in E. coli; LcHCc, PHB beads displaying HCc produced in L. lactis; recHCc, recombinant hepatitis C core protein.
* Sera were collected 5 weeks after the initial vaccination. IgG1 and IgG2c antibodies to HCc were measured by ELISA. Results are expressed as mean (SEM) optical density at 450 nm for sera diluted 1/250. * significantly greater than response of the PBS-vaccinated control (P < 0.05).
by the SDS-PAGE gel (Fig. 2). In comparison, significantly less fusion protein was seen on the surfaces of the PHB beads produced in E. coli, which indicated that utilization of the nisin-controlled gene expression system by L. lactis enabled efficient overproduction of functional heterologous proteins (25). The strong overproduction of HCC-PhAc fusion proteins on the beads correlated with relatively fewer contaminating host proteins in the L. lactis-produced beads. An advantage of a purer product would be the reduction in the need for extensive downstream processing for the removal of host cell proteins and hence reduced production costs.

Mice vaccinated with PHB beads produced by L. lactis which displayed HCC antigens were found to initiate an antigen-specific Th1 immunity pattern shown by production of IFN-γ as well as a IL-17A (Fig. 4). Th1 immunity has long been associated with IFN-γ production (29), and IL-17A plays a critical role in vaccine-induced immunity against infectious diseases (21). Th17 cells are the major source of IL-17A, and it is reported that following vaccination, Th17 cells release IL-17A, which promotes the induction of chemokines to recruit effector Th1 cells and neutrophils to control pathogens (17, 48). While it has been established that a Th1 immunity pattern is important for protective immunity against hepatitis C virus (1), the significance of IgG1 antibodies in viral neutralizing activity remains controversial, since a high titer of anti-HCc antibodies can coexist with viremia (42). Therefore, the nonsignificant antibody responses (Table 2) measured in animals vaccinated with L. lactis HCC might be less relevant. The immune responses following vaccination using HCC PHB beads from the E. coli production host also demonstrated a Th1 immune pattern as evidenced by increased IFN-γ and serum IgG2c titers. However, animals vaccinated with either wild-type control or HCC beads produced in E. coli also showed increased levels of the proinflammatory cytokines TNF-α and IL-6, which can lead to tissue damage (10). It has been shown that IL-6 combined with transforming growth factor β (TGF-β) is a strong inducer of Th17 T cells in mice, leading to the production of IL-17, and that the combination of IL-6 and TGF-β induces CD4+ T cells to produce both IL-17 and IL-10 (23). In addition, Lombardi et al. have shown sequential production of IL-10 and IFN-γ, and eventually IL-17A, by CD4+ T lymphocytes after stimulation with dendritic cells stimulated via Toll-like receptor 4 (TLR4) and TLR7/8 (22). The coproduction of IL-10 is likely important in restraining the potentially destructive Th17 cell-mediated response. The results from the current study provided evidence for coexpression of IL-6, IL-10, and IL-17A in vaccine groups receiving E. coli-produced PHB wild-type and HCC beads. The responses of mice vaccinated in E. coli-produced bead vaccine groups may be due to LPS or contaminating E. coli proteins causing a nonspecific adjuvant effect following vaccination. In comparison, vaccination with L. lactis HCC PHB beads generated a specific Th1 immune response which is needed for many diseases for which there is no effective vaccine (4, 11, 33).

The use and assessment of a suitable adjuvant are important components of vaccine development. Adjuvants need to be assessed for each different antigen and are used to skew the immune response in the desired cell-mediated or humoral direction (8). Additionally, the presence of host cell proteins also can skew the immune response to enhance a Th1 or Th2 response (36). CFA is generally known to enhance Th1 immunity but cannot be used in humans due to severe side reactions. Vaccination with recHCC in Emulsigen induced a very strong IgG1 response (Table 2) associated with a Th2 immune response and also caused a significant induction of IFN-γ and IL-10 (Fig. 4). The present study using HCC PHB beads in Emulsigen showed Th1 but no Th2 responses, which is different from the results of a previous tuberculosis vaccination study which showed both Th1 and Th2 immune responses after vaccination using mycobacterial antigen PHB beads in Emulsigen (32). Therefore, it is worthwhile to investigate the use of different adjuvants and immunomodulators with PHB bead vaccines to determine the effect of adjuvant or host cell proteins on the immune response.

The vaccine production system described herein eliminates the need for the costly two-step process of manufacturing a purified recombinant antigen which is subsequently chemically conjugated to a particulate carrier. Combining this advantage with the advantage of using a GRAS bacterium as the production host and flexibility for antigen display, this vaccine system holds promise for future development and use.

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REFERENCES
