Protective T Cell and Antibody Immune Responses against Hepatitis C Virus Achieved Using a Biopolyester-Bead-Based Vaccine Delivery System

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Hepatitis C virus (HCV) infection is a major worldwide problem. Chronic hepatitis C is recognized as one of the major causes of cirrhosis, hepatocellular carcinoma, and liver failure. Although new, directly acting antiviral therapies are suggested to overcome the low efficacy and adverse effects observed for the current standard of treatment, an effective vaccine would be the only way to certainly eradicate HCV infection. Recently, polyhydroxybutyrate beads produced by engineered Escherichia coli showed efficacy as a vaccine delivery system. Here, an endotoxin-free E. coli strain (ClearColi) was engineered to produce polyhydroxybutyrate beads displaying the core antigen on their surface (Beads-Core) and their immunogenicity was evaluated in BALB/c mice. Immunization with Beads-Core induced gamma interferon (IFN-γ) secretion and a functional T cell immune response against the HCV Core protein. With the aim to target broad T and B cell determinants described for HCV, Beads-Core mixed with HCV E1, E2, and NS3 recombinant proteins was also evaluated in BALB/c mice. Remarkably, only three immunization with Beads-Core+CoE1E2NS3/Alum (a mixture of 0.1 µg Co.120, 16.7 µg E1.340, 16.7 µg E2.680, and 10 µg NS3 adjuvanted in aluminum hydroxide [Alum]) induced a potent antibody response against E1 and E2 and a broad IFN-γ secretion and T cell response against Core and all coadministered antigens. This immunological response mediated protective immunity to viremia as assessed in a viral surrogate challenge model. Overall, it was shown that engineered biopolyester beads displaying foreign antigens are immunogenic and might present a particulate delivery system suitable for vaccination against HCV.

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been tested in a wide range of veterinary and wildlife species (17–19). Bioengineered nano/microstructures manufactured by microorganisms are becoming increasingly attractive as delivery systems for use in vaccines because of their inherent properties of biocompatibility with living tissue, versatility, small size, low cost, ease of production, and mode of functionalization of the surface antigens (20–22).

Recently, polyhydroxyalkanoate granules were conceived as stable subcellular structures enabling the display of foreign protein functions and showing potential as specific and tailor-made devices for medical and biotechnological applications (23). Particular vaccine carriers appear to have adjuvanting effects, with uptake by dendritic cells (DCs) and consequential activation of the NALP-3 inflamasome (24).

In this study, we evaluated the functionality of specific T cell immune responses induced by polyhydroxybutyrate beads displaying the Core antigen on their surface (Beads-Core) formulated in aluminum hydroxide (Alum) using a challenge viral model based on recombinant vaccinia virus for HCV expressing either Core (vvCo) or HCV structural proteins (vvRE). In addition, with the aim to target broad T and B cell determinants described for HCV, Beads-Core mixed with recombinant HCV E1, E2, and NS3 proteins was evaluated. Our study demonstrated that polyester Beads-Core particles induce a functional T cell immune response against the Core antigen and could serve as an adjuvant to elicit humoral and T cell immune responses against coadministered HCV antigens.

**MATERIALS AND METHODS**

**Proteins and polyester beads.** HCV protein variants Co120 (Co), E1.340, and NS3 are HCV Core, E1, and NS3 protein variants encompassing amino acids 1 to 120, 192 to 340, and 1192 to 1457 of the HCV polyprotein from a genotype 1b Cuban isolate, respectively. These proteins were obtained from recombinant *Escherichia coli* cells and purified by a combination of washed pellet procedures and gel-filtration chromatography to 95% purity as described previously (25–27). E2.680 is an HCV E2 protein variant encompassing amino acids 384 to 680 of the viral polyprotein, obtained from recombinant *Pichia pastoris* cells. This recombinant protein was purified by heparin-Sepharose affinity chromatography and gel-filtration chromatography at 95% purity as described previously (28). The HCV recombinant antigens were prepared as a single large batch for the immunization schedules and had an endotoxin level under 0.1 endotoxin units per ml.

Concanavalin A (ConA; Sigma-Aldrich, USA) (5 µg/ml) was used as a positive control in cellular immune response evaluation.

The polyester Beads-Core beads were produced either in endotoxin-free ClearColi BL21(DE3) (Lucigen, Middleton, WI, USA), an endotoxin-free *E. coli* mutant, or in recombinant *Lactococcus lactis* (29). The bacterial production strains were transformed with plasmids mediating the production polyhydroxybutyrate beads displaying the Core antigens. The beads were purified and characterized as previously described (29). The Core antigen displayed on polyester beads was identified by SDS-PAGE and matrix-assisted laser desorption ionization–time of flight tandem mass spectrometry (MALDI-TOF MS-MS) and was also quantified by densitometry.

**Viruses and cell lines.** The Western Reserve (WR) vaccinia virus strain was used to generate recombinant vaccinia viruses for HCV Core (vvCo) and HCV structural proteins (vvRE) (31).

African green monkey kidney BSC40 cells (32) were grown in monolayers and used for *in vitro* studies and *ex vivo* determination of vaccinia virus titer in mouse ovaries. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 U/ml penicillin. During vaccinia virus infections, FBS was used at 2% (vol/vol). Cells were maintained at 37°C with 5% CO₂.

**Mouse immunization schedules.** Groups of BALB/c mice 6 to 8 weeks of age weighing 18 to 20 g were purchased from CENPALAB (Centro Nacional para la Producción de Animales de Laboratorio, La Habana, Cuba). The housing, maintenance, and care of the animals were in compliance with all relevant CIGB, Havana, Cuba, guidelines and requirements.

In the first immunization schedule, groups of 10 mice received intramuscular (i.m.) injections into the quadriceps muscle of Beads-Core/Alum (polymer beads displaying 30 µg of Core protein on the surface) (29) or 30 µg of Co120 protein (25) in alum. Animals injected with alum or beads without antigen in alum (Beads/Alum) served as negative controls. The four groups were injected at weeks 0, 2, and 4, as summarized in Table 1.

In the second immunization schedule, groups of 10 mice received i.m. injections into the quadriceps muscle of CoE1E2NS3/Alum (a mixture of 0.1 µg Co120, 16.7 µg E1.340, 16.7 µg E2.680, and 10 µg NS3 adjuvanted in alum) as described before (15) or Beads-Core+E1E2NS3/Alum (Beads-Core mixed with 16.7 µg E1.340, 16.7 µg E2.680, 10 µg NS3, and alum). Animals injected with alum or beads without antigen in alum (Beads/Alum) were used as negative controls. The groups of animals inoculated with CoE1E2NS3/Alum or alum were injected five times, at weeks 0, 2, 4, 6, and 8, while groups inoculated with Beads-Core+E1E2NS3/Alum or Beads/Alum were injected three times, at weeks 4, 6, and 8, as summarized in Table 2.

All immunogens were formulated in aluminum hydroxide (Alum) (0.5 mg Al³⁺ for 20 µg of antigen). Blood samples were collected 2 weeks after the last immunization from retro-orbital sinus, and sera were analyzed for antibodies. Five animals per group were euthanized after the final blood collection, and spleens were taken for proliferative response analysis. The remaining five mice were challenged with vvCo in the first immunization schedule or with vvRE in the second immunization schedule.

**Evaluation of IgG antibody response.** To detect specific IgG antibodies against E1 and E2 proteins, an in-house enzyme-linked immunosorbent assay (ELISA) was performed (33). E2.680 and E1.340 (10 µg/ml) were used to coat microtiter plates (Costar, USA). Titration was carried out by interpolation of a curve constructed by serial dilution of a positive-control sample of known reactivity. Measurement of absorbance at 492 nm (A₄₉₂) was performed in a plate reader (Sensidet Scan; Merck, Darmstadt, Germany). The cutoff value to identify a positive antibody response was established as twice the mean OD₄₉₂ of the negative-control sera.

**Measurement of cells secreting IFN-γ.** The enzyme-linked immunospot (ELISPOT) assay was performed essentially as previously described.

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**TABLE 1 First immunization schedule**

| Group | Immunogen | Frequency (wks of injection) |
|-------|-----------|-----------------------------|
| 1     | Beads-Core/Alum | 0, 2, 4                     |
| 2     | Beads/Alum    | 0, 2, 4                     |
| 3     | Core/Alum     | 0, 2, 4                     |
| 4     | Alum          | 0, 2, 4                     |

*Intramuscular injections were employed for all groups. Each group consisted of 10 mice.*

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**TABLE 2 Second immunization schedule**

| Group | Immunogen | Frequency (wks of injection) |
|-------|-----------|-----------------------------|
| 1     | Beads-Core + E1E2NS3/Alum | 4, 6, 8                     |
| 2     | Beads/Alum    | 4, 6, 8                     |
| 3     | CoE1E2NS3/Alum | 0, 2, 4, 6, 8               |
| 4     | Alum          | 0, 2, 4, 6, 8               |

*Intramuscular injections were employed for all groups. Each group consisted of 10 mice.*
FIG 1 IFN-γ secretion response against HCV core antigen. Data are shown as numbers of HCV-specific IFN-γ spots per 10^6 cell and represent the mean values for spots from five individual mice, determined in duplicate tests after subtracting the background value (incubation without antigen). The percentage values above the bars indicate the numbers of animals with positive results per group. The positive value always exceeded twice the number of spots detected in the nonstimulated cells for each group and represented at least 40 spots per million splenocytes. The error bars represent standard deviations of the means. Numbers of spots from negative-control groups were always below 5 (P < 0.01 [Kruskal-Wallis test and Dunn’s multiple-comparison test]).

RESULTS

Beads-Core vaccination induces a specific cell-mediated immune response able to control the viremia after vvCo challenge. Following immunization, no overt toxicity was observed in any of the animals. Weights of mice did not differ significantly between groups during the time course of the experiment, and mice in all groups gained weight (data not shown). Mice immunized with polyester beads developed small lumps (2.5 mm in diameter) at the immunization sites that disappeared after a short period of about 5 days, but no abscess or suppuration was observed, and all mice were healthy throughout the trial, with normal behavior and good-quality fur (data not shown).

Quantification of specific IFN-γ cytokine-secreting cells was performed by ELISPOT assay. Although we did not separate CD8+ cells from the spleen cells, the cells responding to proteins in the ELISPOT assay were most likely CD4+ cells (36). As Fig. 1 shows, three dose injections with polyester Beads-Core/Alum and Core/Alum elicited positive IFN-γ secretion responses (100% and 80%, respectively). The mean number of specific spots per 10^6 splenocytes, elicited by polyester Beads-Core/Alum, was significantly higher than that seen with an equivalent dose of the Core protein in the alum (Core/Alum) and negative-control groups (P < 0.01 [Kruskal-Wallis and Dunn’s multiple-comparison test]). No positive response against Core was detected in animals injected with wild-type Beads/Alum or Alum.

To analyze the in vivo functionality of the cell-mediated immune response induced by Beads-Core vaccination, a challenge with vvCo was carried out 15 days after the last immunization. Viremia suppression was observed in mice vaccinated with Core/Alum and Beads-Core/Alum compared to the respective Alum and Beads/Alum control groups (P < 0.001 [ANOVA and Newman-Keuls multiple-comparison test]) (Fig. 2). Beads-Core/Alum-immunized mice showed a remarkable reduction in viral titer compared to those vaccinated with Core/Alum and wild-type beads (Beads/Alum). Three of five (60%) Beads-Core/Alum-immunized animals had no detectable viremia level after challenge with vvCo. The two other animals in this group showed significantly (3 log-lower) reduced viremia titers in ovaries compared to the viral titers detected in animals vaccinated with Core/Alum and the control Beads/Alum, respectively (P < 0.001 [ANOVA and Newman-Keuls multiple-comparison test]). The results of these experiments point out that polyester Beads-Core induces a functional cellular immune response able to control viremia after challenge with recombinant vvCo.
A broad immune response was induced in mice vaccinated with Beads-Core mixed with recombinant hepatitis C virus E1, E2, and NS3 proteins. In order to assess a formulation able to induce an immune response targeting a variety of HCV epitopes, an immunization schedule with Beads-Core mixed with recombinant HCV E1, E2, and NS3 proteins (Beads-Core/H11001 E1E2NS3/Alum) was carried out in mice. In this experiment, we compared the specific immune responses induced by three doses of a vaccination regimen of Beads-Core/H11001 E1E2NS3/Alum to those induced by five doses of a mix of HCV recombinant proteins Core, E1, E2, and NS3 formulated in Alum (CoE1E2NS3/Alum) (15). Mice injected with Alum and control Beads/Alum were used as negative controls. The immune response was determined in immunized animals to assess both the potential of polyester beads to act as vaccine delivery agents and the contribution to the immunogenicity of coadministered HCV antigens.

Beads-Core enhances specific antibody responses against HCV E1 envelope protein. All groups assayed with the protein formulations developed a positive IgG antibody response against E1 and E2 envelope proteins, with statistically significant differences from the control group results \( (P < 0.05 \text{ [ANOVA and the Newman-Keuls test as a multiple-comparison test]}) \) (Fig. 3). Regarding the magnitude of the response, significantly higher IgG antibody titers against E1 were observed in the group vaccinated with Beads-Core+E1E2NS3/Alum than in the group vaccinated with CoE1E2NS3/Alum \( (P < 0.05 \text{ [ANOVA and the Newman-Keuls test as a multiple-comparison test]}) \). High IgG antibody titers against E2 were induced in both studied groups, and no statistically significant differences between them were detected.

Beads-Core stimulated a broad and specific cell-mediated immune response in vaccinated mice. Quantification of specific IFN-\( \gamma \) cytokine-secreting cells was performed by ELISPOT assay. All mice vaccinated with Beads-Core+E1E2NS3 showed a 100% positive IFN-\( \gamma \) secretion response to the assayed HCV antigens, with statistically significant differences compared to the control group results \( (P < 0.01 \text{ [Kruskal-Wallis test and Dunn’s multiple-comparison test]}) \) (Fig. 4). In contrast, no positive response was detected in animals in the control groups. The mean number of spots per million cells, against Core and E1 proteins, reached in animals vaccinated with Beads-Core/E1E2NS3/Alum, was significantly higher than that reached in the CoE1E2NS3/Alum group \( (P < 0.05 \text{ [Kruskal-Wallis test and Dunn’s multiple-comparison test]}) \). No statistically significant differences in the levels of IFN-\( \gamma \) response were detected between the two vaccination regimens regarding E2 and NS3 proteins.

To analyze the in vivo functionality of the T cellular immune response induced by vaccination, a challenge with vvRE was carried out. As shown in Fig. 5, mice vaccinated with CoE1E2NS3/
Alum and Beads-Core+E1E2NS3/Alum showed significantly reduced virus titers in ovaries compared with animals inoculated with Alum and Beads/Alum (P < 0.01 [ANOVA and Newman-Keuls multiple-comparison test]).

The group of mice vaccinated with Beads-Core+E1E2NS3/Alum showed greater control of viremia with only three vaccination doses than with five doses of CoE1E2NS3/Alum. The differences between these groups with regard to the protective response were statistically significant, according to results of ANOVA and the Newman-Keuls multiple-comparison test (P < 0.01). This study demonstrated that polyester beads displaying Core stimulated a functional T cell immune response against the displayed Core antigen and the coadministered antigens, enabling control of viremia in mice after challenge with vvRE.

**DISCUSSION**

The demand for an affordable prophylactic HCV vaccine to prevent viral transmission to reduce the future disease burden worldwide remains urgent. One of the challenges regarding the development of an HCV vaccine is that HCV exhibits extensive genetic diversity (37, 38). Hence, the conserved core antigen represents an attractive vaccine target. The core antigen is the most conserved HCV protein across the various genotypes, and Core-specific cytotoxic T lymphocytes (CTLs) can recognize and lyse target cells expressing Core derived from most HCV genotypes (39). Therefore, the inclusion of Core in an HCV vaccine might broaden its effectiveness, as the immune responses elicited should be relevant for most, if not all, HCV genotypes and HCV quasispecies.

In the present work, we successfully engineered an endotoxin-free mutant of *E. coli* (ClearColi) to produce polyhydroxybutyrate beads displaying the Core antigen. Core antigens displaying polyhydroxybutyrate beads isolated from recombinant ClearColi as well as from *Lactococcus lactis* were immunologically characterized. Here we demonstrated that a three-dose vaccination protocol of bioengineered polyester beads displaying hepatitis C Core protein induced significantly stronger IFN-γ responses than vaccination with an equivalent dose of the soluble Core protein in alum (Fig. 1). Immunization with subunit protein vaccines adjuvanted in alum or oil/water emulsions can usually elicit CD4+ T cells and antibodies but is generally inefficient at priming major histocompatibility complex (MHC) class I-restricted CTLs, as proteins in the extracellular fluid are generally processed through the exogenous processing pathways and are degraded into peptides that bind MHC class II molecules (30). Cell-mediated immune responses induced by Beads-Core vaccination suppressed viremia after challenge with vvCo (60% protection) (Fig. 2). Since Core amino acid sequences present in vvCo and Beads-Core are highly conserved among HCV genotypes, the T cell immune response induced by Beads-Core might be broadly protective. It has been demonstrated that protection against challenge with recombinant vvRE...
binant vaccinia virus is associated with the induction of a specific response mediated by T cells, fundamentally involving CD4+ T cells as inducers and CD8+ T cells as effectors for viral clearance (40). Our results are in agreement with a previously described immune response analysis of polyester beads displaying the hep-atitis C core antigens, which induced an antigen-specific Th1 re-
response as confirmed by production of IFN-γ and interleukin-17A (IL-17A) as well as the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) and IL-6 (29). Th1 immunity has long been associated with IFN-γ production and was found to play a critical role in vaccine-induced protective immunity to infectious dis-
eases, in particular, those caused by intracellular pathogens such as, e.g., viruses. Additionally, an antigen-specific IFN-γ response and a reduction in mean virus titers of vvCo were also observed in Core/Alum-immunized mice. The Co.120 protein variant in-
cluded in Core/Alum is obtained as VLPs (41) and was found to
induce a strong and specific humoral response with a Th1 pattern (42). It is known that macrophages and DCs are able to cross-

present VLPs and prime an effective CD8+ T cell response (43). In
the context of HCV infection, patients with infection controlling
immunity as well as responders to antiviral therapy have been
shown to develop specific CD4+ T cells and CD8+ T cells corre-
sponding to IFN-γ responses to the HCV Core protein (9, 44, 45).
In fact, the CD4+ T cell response to Core protein has been associ-
ated with a benign course of infection (46).

However, it has been shown that HCV Core protein can modu-late multiple cellular processes, such as apoptosis, lipid metabolism, and transcription, and can induce cellular transfor-
mation (47). Therefore, there are potential concerns about elicit-
ing Core-specific immune responses in patients using a genetic vaccine (naked DNA; viral, retroviral, or bacterial vec-
tors; replicons). Nevertheless, although most licensed subunit
vaccines are inefficient at inducing CTLs, there has been con-
siderable advancement in the field of adjuvant research. For
example, classical and nonclassical ISCOMs have been shown to
prime CD4+ and CD8+ mediated immune responses and
memory cellular immune responses (48, 49). Recently, we de-
monstrated that Core-VLP immunization coadministered with
E1, E2, and NS3 proteins induced broad T cell proliferation
and IFN-γ responses in mice and monkeys (15).

Accordingly, the nature of antigens and the association be-
tween antigen and adjuvant are thought to be crucial for induction
of functional T cell immune responses (50). The use of novel ad-
juvants in vaccine development could help simplify vaccination by
decreasing the amount of administered purified antigen (antigen
sparring) and/or the number of doses required. One of the main
advantages of the expression and carrier system based on polyester
particles is that the antigens are covalently attached to the particle
surface and are presented in homogenous orientation. The use of
particles smaller than 2 μm in size as vaccines has the advantage
that the particles are readily phagocytosed by macrophages and
dendritic cells (51). The particles appear to have adjuvanting ef-

fects due to efficient uptake by dendritic cells and the subsequent
activation of the NALP-3 inflammasome (24). Delivery of drugs
or vaccines using biocompatible particulate vehicles is an area
of study currently gaining significant momentum. Cell-free vaccine
delivery systems are often particulate (using, e.g., emulsions, mi-
cro/nanoparticles, and liposomes), and the particle dimensions
are similar to those of pathogens, which the immune system has
evolved to inactivate (18). However, such particulate systems have
to be chemically synthesized and processed to enable adsorption
or chemical cross-linking of the separately produced and purified
protein antigen (52). However, polyester beads obtained using
engineered bacteria as production hosts for polymeric carriers of
protein subunit vaccines seem to represent an affordable and ef-

ficient alternative. The disadvantage of using standard E. coli
strains as production hosts for human and animal vaccines is the
potential contamination of products with lipopolysaccharides
(LPS) (23). Therefore, LPS-free production hosts are desirable for
production of antigen-displaying polyester beads. The absence of
LPS in the production strain does significantly reduce the need for
extensive downstream processing and hence reduces production
costs (29). Here we used two LPS-free production strains, food-
grade Gram-positive Lactococcus lactis and an E. coli mutant,
ClearColi. This novel vaccine delivery system based on biopolys-
tester beads produced in E. coli has previously been shown to stimu-
late an immune response to HCV antigens (53).

There is evidence that priming of CD4+ and CD8+ T cells
specific for various HCV gene products might increase the ef-
ficacy of a vaccine conferring immunity to HCV (54–56). Since
Core and NS3 antigens comprise the most relevant epitopes
related to the cell-mediated immune response against HCV (9,
57) and the viral E1 and E2 glycoproteins contain epitopes
inducing neutralizing antibody responses found to be protect-
ive (58) and associated with the resolution of hepatitis C in-
fec tion (59), Beads-Core was coadministered with recombin-
ant E1, E2, E2, and NS3 proteins. It had been demonstrated that CoE1E2NS3 (MixprotHC) vaccination in-
duces broad HCV-specific humoral and cellular immune re-
sponses after immunization with five doses in mice and mon-
keys (15, 37). A MixprotHC formulation previously showed
induction of cross-neutralizing antibodies in mice and African
green monkeys, and the specific T cell-induced immunity pro-
vided protection in a surrogate vaccinia virus-HCV challenge
model (15). In the present work, Beads-Core + E1E2NS3/Alum
showed significantly increased IgG antibody titers in response
to E1 protein compared to CoE1E2NS3/Alum (Fig. 3). The
cross-neutralizing nature of antibodies against E1, E340, E1, E680,
and NS3 proteins has been previously demonstrated in the con-
text of CoE1E2NS3 immunization (15). Nevertheless, further
investigations using HCV pseudoparticles (HCVPp) and the
cell culture-derived HCV (HCVCc) neutralization system are
needed to study the activity, range, and magnitude of cross-
neutralization induced by Beads-Core + E1E2NS3/Alum im-
munization.

As expected, no statistically significant differences in the anti-
body and T cell responses against E2,680 were observed between
CoE1E2NS3/Alum and Beads-Core + E1E2NS3/Alum vaccina-
tion (Fig. 3 and 4). Since E2,680 is produced as an aggregated
antigen and has been proven to effectively activate the immune
response (27), the presence of E2,680 in both formulations might
contribute to these results in terms of capture, processing, and
presentation by antigen-presenting cells (60, 61).

Immunization with Beads-Core + E1E2NS3/Alum induced a
specific IFN-γ response against Core, E1, E2, and NS3 (Fig.
4). This was similar to results previously observed, where a
positive and broad IFN-γ response was also induced after
vaccination with CoE1E2NS3/Alum. However, the IFN-γ re-

sponse against Core and E1 was significantly higher in the
group vaccinated with only 3 doses of Beads-Core + E1E2NS3/
Alum than in the group vaccinated with 5 doses of CoE1E2NS3/Alum. Additionally, mice immunized with 3 doses of Beads-Core+E1E2NS3/Alum showed the greatest reduction of vRE titer in ovaries of vaccinated mice (Fig. 5). Parlane et al. also demonstrated the induction of a protective response in mice vaccinated with similar polyester particles expressing antigens Ag85A-ESAT6 which was superior to that induced by soluble antigens (62). The versatility and potential of the bead antigen delivery system in eliciting different complementary facets of the immune response could be applied to the development of multivalent vaccines (52). Other approaches using particles for vaccine delivery that express HCV proteins, HCV-like particles (HCV-LP), and a DNA–prime/modified vaccinia Ankara (MVA)-boost strategy have been able to induce broad and robust HCV-specific T cell immune responses (17, 63). Nevertheless, Beads-Core induced a specific T cell immune response and served as an adjuvant to potentiate antibody and T cell immune responses against coadministered antigens.

Overall, this study showed that polyester beads are safe and efficient delivery systems for immunization purposes. Further studies are needed to characterize CD4+ and CD8+ T cell responses and long-lasting immunity induced by Beads-Core+E1E2NS3/Alum or beads displaying a broad range of HCV antigens.

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