**Superior immunogenicity of HCV envelope glycoproteins when adjuvanted with
cyclic-di-AMP, a STING activator or archaeosomes**

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Abstract

Three decades after the discovery, hepatitis C virus (HCV) is still the leading cause of liver transplantation and poses a major threat to global health. In spite of recent advances in the development of direct acting antivirals, there is still a need for a prophylactic vaccine to limit the virus spread and protect at-risk populations, especially in developing countries, where the cost of the new treatments may severely limit access. The use of recombinant HCV glycoproteins E1E2 (rE1E2) in combination with the MF59, an oil-in-water emulsion-based adjuvant, has previously been shown to reduce the rate of chronicity in chimpanzees and to induce production of cross-neutralizing antibodies and cellular immune responses in human volunteers. To further improve neutralizing antibody responses in recipients along with robust T cell responses, we have explored the immunogenicity of different adjuvants when formulated with the HCV rE1E2 vaccine in mice.

Our data show that cyclic di-adenosine monophosphate (c-di-AMP) and archaeosomes elicit strong neutralizing antibodies similar to those elicited using aluminum hydroxide/monophosphoryl lipid A (Alum/monophos. /MPLA) and MF59. However, both c-di-AMP and archaeosomes induced a more robust cellular immune response, which was confirmed by the detection of vaccine-specific poly-functional CD4⁺ T cells. We conclude that these adjuvants may substantially boost the immunogenicity of our E1E2 vaccine. In addition, our data also indicates that use of a partial or exclusive intranasal immunization regimen may also be feasible using c-di-AMP as adjuvant.

Introduction

Chronic hepatitis C is the common consequence of infection with the hepatitis C virus (HCV). It is the leading cause for liver transplant and still a major threat to global health [1]. Despite recent progress in direct antiviral drugs (DAA) that significantly improved the cure rate to higher than 90% [2], the high cost of DAA, especially in developing countries is a major obstacle to control the disease or to stop the spread of the infection among humans [3]. In addition, the potential of occurrence of resistant strains of HCV after use of the DAAs [4], reinfection of individuals that cleared the virus after

1 therapy, and the existence of a major population of undiagnosed silent carriers [5, 6] all
2 make the eradication of the disease by the use of DAA alone very unlikely. Indeed, no
3 infectious disease has yet been eradicated or efficiently controlled through the use of
4 therapeutic drugs alone without the introduction of an effective vaccine. Thus,
5 vaccination may be the only solution for controlling the spread of HCV as has been
6 previously shown for other human-specific pathogens [7].

7 Recently, the use of recombinant antigens-, subunit- or even peptide-based
8 vaccines are becoming more favorable than killed or attenuated viruses as they could be
9 tolerated better and designed more specifically. However, as these antigens are less
10 immunogenic, the presence of adjuvants in the formulation turns out to be more crucial
11 for optimal and adequate immune responses, while also helping to reduce the amount of
12 antigen required. Adjuvants can serve either as a delivery system or as an immune
13 stimulator or both and can skew the immune response toward a Th-2 type humoral
14 response such as aluminum hydroxide (Alum) or a Th-0 type response as in MF59 or a
15 Th-1 type cellular response such as with monophosphoryl lipid A (MPLA).

16 Our previous efforts on the development of a vaccine for HCV have shown that
17 the use of recombinant HCV glycoproteins E1E2 (rE1E2) in combination with MF59-
18 type adjuvants, oil-in-water based emulsions, reduces the rate of chronicity in
19 chimpanzees and induces production of neutralizing antibodies and cellular immune
20 responses in humans [8-10]. In addition, we have also shown that the neutralizing
21 antibodies elicited by rE1E2/MF59 in humans are capable of cross-neutralizing many
22 different clades of HCV *in vitro*, thus validating the use of rE1E2 as an optimum
23 immunogenic antigen [11]. To enhance these antibodies in recipients along with a robust
24 T cell response, we have explored the efficacy of other adjuvants in combination with
25 HCV rE1E2. Various adjuvants operating via different pathways such as Alum/MPLA,
26 MF59, cyclic di-adenosine monophosphate (c-di-AMP), and archaeosomes were
27 formulated to immunize mice and the immunogenicity was measured and compared
28 among groups using neutralizing antibody assays and intracellular cytokine production in
29 T cell assays.

30 Alum adjuvants are the oldest and most commonly used adjuvants in humans and
31 have been used with great success with very well established safety and efficacy profiles.

1 Alum is primarily effective in promoting humoral immunity as a result of its depot
2 effects. Moreover, Alum increases the antigen uptake and stability and induces pro-
3 inflammatory reactions [12-15]. However, Alum is a poor inducer of Th-1 type immunity
4 and for that reason may be supplemented with other adjuvants such as MPLA.

5 MPLA is a derivative of lipid A from bacterial lipopolysaccharide (LPS) and a
6 well-known agonist for toll-like receptor 4 (TLR4). MPLA could skew the immunity
7 toward a Th1-type in combination with other adjuvants [16] and facilitate optimal
8 humoral and cellular responses when it is used in combination with Alum [17]. Another
9 advantage of using MPLA is that it is now approved for use in combination with Alum in
10 a vaccine for human papilloma virus [18].

11 MF59 is a licensed adjuvant. It is an oil-in-water emulsion-based adjuvant that is
12 safe and potent in generating antibodies. MF59-adjuvanted vaccine has recently been
13 used to control a 2009 pandemic of H1N1 influenza. It has been shown that MF59
14 promotes a CD4⁺ T cell response that leads to production of protective antibodies against
15 the H5N1 Influenza strain [19]. In addition, MF59 induces the recruitment of Th-2 type
16 antigen-presenting cells (APCs), increases the antigen uptake by dendritic cells, and
17 activates multiple inflammatory pathways resulting in production of inflammatory
18 cytokines [20, 21].

19 Stimulator of interferon genes (STING) is a signalling molecule that is essential
20 for the production of pro-inflammatory cytokines including type 1 interferon (IFNs). It
21 has been demonstrated that STING can be strongly activated by cyclic dinucleotide
22 molecules and acts as a direct sensor for them when produced by bacteria [22, 23]. Cyclic
23 dinucleotide adenosine monophosphate (c-di-AMP) is a member of the cyclic
24 dinucleotides and acts as a pathogen-associated molecular pattern (PAMP) to induce
25 immunity when it is used in vaccine formulations [24]. In other studies, c-di-AMP was
26 used as a mucosal adjuvant, where it primarily induced dendritic cells and shown to be
27 capable of promoting both humoral and cellular immune responses [25, 26]. This
28 encompasses the stimulation of Th1, Th2 and Th17 cells, as well as the induction of
29 cytotoxic T lymphocytes.

30 Archaea are the third domain of life, distinct from eubacteria, and possess unique
31 highly stable membrane lipids. Different archaeal species optimally grow in harsh

conditions such as high temperature, extreme pH, high-salt water, and anaerobic habitats; they are also stable in the presence of lipases and bile salts [27-29]. Liposomes are closed vesicles that have been used as delivery vehicles for drug and/or antigen cargo. The term archaeosomes was ascribed to liposome vesicles comprised of ether lipids derived from *Archaea*. Archaeosomes constitute stable antigen delivery vesicles for vaccines. A key feature of archaeosomes is their dual ability to target antigen for processing on the MHC class I pathway, while concomitantly activating APCs costimulation and cytokine production [30]. Entrapment of antigen in archaeosomes induces both robust antibody and cell-mediated immunity through induction of CD4⁺ and CD8⁺ T cells [27, 31].

Here we compare the strength and type of immunity that is elicited by a HCV candidate vaccine when associated with different adjuvants in mice.

Materials and Methods

Antigen and vaccine preparation: HCV H77 rE1E2 heterodimer was prepared and purified to > 90% purity as described before [32]. Then, various adjuvants including Alum/MPLA, MF59, c-di-AMP, and archaeosomes were formulated with H77 rE1E2 to immunize mice. In general, 20 µl of adjuvant was added to 20 µl of antigen solution (containing 1 µg of rE1E2 in PBS) and mixed well before injecting the animal. The amount of each adjuvant was 100 µg of Alum + 10 µg of MPLA, 10 µg c-di-AMP or 100 µg archaeosomes. Archaeosomes containing E1E2 protein were prepared by the method of detergent removal using the hydrophobic polystyrene Bio-beads SM adsorbent [33, 34]. Where needed, the total volumes of adjuvants were adjusted to 20 µl per dose by adding PBS. In case of MF59, 20 µl of in-house prepared adjuvant was used.

Immunization: Female CB6-F1 mice (8 mice per group, 6-8 week-old, Charles River laboratories, MA, USA) were immunized with 1-2 µg of antigen mixed in a 1:1 ratio with adjuvants three times on day 0, day 14, and day 42. All groups received the vaccine intramuscularly (IM) with the exception of the c-di-AMP group that received either the first vaccine IM and the second and third intranasally (IM-IN-IN) or all vaccinations IN (IN-IN-IN). Previous work with other vaccines has shown these regimens to be superior to IM-IM-IM protocols (Guzman; unpublished). Experiments were done in accordance with the Canadian Council on Animal Care guidelines.

Experimental methods were reviewed and approved by the University of Alberta Health Sciences Animal Welfare Committee. Pre-vaccination serum was collected at day 0 before the first immunization and the final test bleed was collected two weeks after the last immunization (Fig. 1). Serum was collected after centrifugation of the blood samples at 5,000 xg for 15 minutes. Serum was heat-inactivated by incubation at 56°C for 30 minutes and stored in aliquots at -80°C until use.

rE2 ELISA: ELISA assays were performed as we described previously . Briefly, 96-well plates were coated with soluble rE2 (from amino acid #384 to 656 of the H77 sequence) overnight at 4°C. Plates were washed and blocked for 1 h in 4% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). Then, diluted antisera from vaccinated mice were added to the plates for 1 h. Finally, E2-specific antibodies from mouse antisera were detected by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Cedarlane Laboratories, ON, Canada) and peroxidase substrate (KPL, Gaithersburg, MD, USA). Absorbance values from three independent experiments are expressed as means $\pm$ standard errors of the means (SEM). Recombinant E2 H77 was kindly provided by Joseph Marcotrigiano.

Neutralization assay: The ability of antibodies to neutralize HCV was evaluated using HCV pseudo-particles (HCVpp) expressing genotype 1a (H77) E1E2 and carrying a luciferase reporter gene. HCVpp was generated as described previously [35]. For neutralization assay, human hepatoma cell line (Huh7.5) was seeded on collagen-coated 96-well plates 1 day prior to infection. The HCVpp stock was diluted 1:10, premixed with either heat-inactivated (56°C, 30 min) mouse sera or mouse anti-human CD81 monoclonal antibody (BD Biosciences, CA, USA) for 1h at 37°C, and then added to Huh7.5 cell culture. The 96-well plates were spinoculated with a bench-top swing-bucket centrifuge (Beckman Coulter, CA, USA) at 1,200 RPM at 37°C for 1 h, and then kept in a 37°C cell culture incubator (Thermo Fisher Scientific, MA, USA) for 6 h. After inoculation, media was replaced and the cells were incubated for additional 48 h. Finally, the cells were lysed and assessed for expression of luciferase using the Bright-Glo™ Luciferase Assay System (Promega, WI, USA) according to manufacturer's instructions. Luminescence was measured with an EnSpire Multimode Plate Reader (PerkinElmer, MA, USA). Neutralization activity was calculated as described previously [32].

T cell assays: After collecting the test bleeds, the spleens were immediately extracted and collected in culture media. Splenocytes were immediately isolated and red blood cells were lysed using ACK RBC Lysis Buffer (Affymetrix eBiosciences, CA, USA). The splenocytes from each group was pooled together (4 spleens per pool, 2 pools per group) and dispensed in three replicates in the wells of a round bottom 96-well plate (Corning, NY, USA) for negative control, positive control, and test groups. Each test group was stimulated using a pool of 55 peptides spanning the whole homologous H77 HCV E1E2 region. Each peptide was 20 amino acid (aa) long overlapping 10 aa with up- and down-stream peptides. The positive control groups received phorbol myristate acetate (PMA) (250 ng/ml; Sigma, MO, USA) and Ionomycin (1 µg/ml; Sigma) in culture media and the negative control received media alone. DMSO was added at the same volume as the test group (0.4% v/v) to the both negative and positive control groups. Brefeldin A (Biolegend, CA, USA) and Monensin (Biolegend) were added to the culture after 1 hour of incubation at 37°C and 5% CO₂. After additional 5 h of incubation the cells were centrifuged and washed with PBS. The cells were then stained for dead/live marker (Biolegend), surface markers (CD3, CD4, and CD8), intracellular cytokines (IFN-γ and TNF-α) and finally were re-suspended in flow cytometry buffer and read using Fortessa-SORP flow cytometer analyzer (BD Biosciences, CA, USA) flow cytometer. The percentage of the T cells expressing both IFN-γ and TNF-α were compared between groups as an indicator of activated T cell responses to HCV rE1E2 antigen. The number of collected CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were supportive of a statistic *p* value of 0.05 or less for each plot as described on Fig. 4 and Fig. 5.

Statistical Analysis: Data were analyzed using statistical software (GraphPad Prism Version 5.00, Prism Software Corporation, Irvine, CA, USA). As outcome variables from each group were not distributed normally, the medians between each pairs of groups were compared using non-parametric Mann-Whitney test, where the ranks are compared. Differences were considered significant if the two-tailed *P* value was lower than 0.05 with confidence intervals of 95%.

Results

Highly significant anti-E2 antibodies titers were detected by ELISA in all vaccinated groups with exception of c-di-AMP (IN-IN-IN): As compared to the control group (rE1E2 antigen alone), the sera collected from immunized mice with rE1E2 mixed with different adjuvants showed significantly increased titers of anti-E1E2 (p value < 0.001) with the exception of the group rE1E2 + c-di-AMP (IN-IN-IN), where anti-E1E2 was not detected and the level of antibody was comparable to the group that only received rE1E2 antigen with no adjuvant (Fig. 2). In the case of rE1E2 + c-di-AMP (IM-IN-IN), the level of antibody was significantly lower than rE1E2 + MF59.

Antisera collected from immunized mice is able to neutralize and prevent the entry of HCV pseudo-particles into human hepatoma cell-lines in vitro: As compared to the controls, the sera collected from immunized mice with adjuvanted H77 rE1E2 showed significant increases in preventing the entry of HCVpp in the *in vitro* neutralization assay (Fig. 3)

When the antigen was formulated with Alum/MPLA or MF59 or c-di-AMP, this effect was highly comparable to neutralization activity using a control anti-CD81 antibody that effectively blocks entry of HCVpp via the CD81 receptor. However, although comparable to MF59 and Alum/MPLA, somewhat lower neutralizing antibodies were measured in the case of c-di-AMP (IM-IN-IN) and archaeosomal vaccine formulations when compared to anti-CD81 antibody. Comparison of c-di-AMP (IM-IN-IN) and c-di-AMP (IN-IN-IN) groups showed that the route of immunization plays a significant role, since mice vaccinated with three intranasal administrations (IN-IN-IN) of rE1E2 + c-di-AMP did not neutralize HCVpp, in contrast to the regimen comprising an intramuscular immunization followed by two intranasal boosts (IM-IN-IN). The relative neutralizing activities of the different vaccine groups showed a similar trend to that revealed in the ELISA assay.

Robust CD4⁺ T cell immune responses were observed in c-di-AMP- and archaeosomes-adjuvanted rE1E2 groups: *In vitro* stimulation of mice splenocytes with a pool of 55 peptides that span the whole length of homologous HCV H77 E1E2 induced strong activated CD4⁺ T cell responses in the groups where the antigen was formulated with c-di-AMP or archaeosomes (p value < 0.005). A moderate response was also detected in the Alum/MPLA group (p value < 0.05). Our data showed that c-di-AMP

(IM/IN/IN) and archaeosomes elicit strong neutralizing antibodies comparable to MF59 and Alum/MPLA, and additionally both induced a more robust cellular immune response, which was confirmed by the detection of vaccine specific poly-functional CD4⁺ T cells expressing both IFN- γ and TNF- α following *in vitro* stimulation (Fig. 4 and 5). Interestingly, c-di-AMP elicited strong CD4⁺ T cell responses, even when given IN/IN/IN unlike neutralizing antibody. None of the test groups induced a CD8⁺ T cellular immune response (Fig.4 and 5).

Discussion

The data from this study indicates that modified H77 rE1E2 that is formulated with Alum/MPLA, MF59, c-di-AMP, or archaeosomes is immunogenic in mice and can induce the production of E1E2-specific antibodies with significant neutralization activity. The presence of neutralizing activity is more indicative of protection against the pathogen in comparison to ELISA antibody assays. Interestingly however, the data in both assays correlated well. Strong neutralizing antibody activity is accompanied with very robust T cell immune responses when the antigen was formulated with c-di-AMP administered IM/IN/IN or archaeosomes given IM while a moderate T cell response was observed when formulated with Alum/MPLA. This vaccine-specific cellular response was biased toward CD4⁺ T cells and was not detected in the mice that were vaccinated with MF59-adjuvanted H77 rE1E2. This is highly compatible with our previous report, where the sera collected from mice vaccinated with rE1E2-MF59 showed strong neutralizing activity, but vaccination did not elicit any CD4⁺ or CD8⁺ T cellular immune response, which was only detected when the CpG was added to the vaccine cocktail or where the immunization protocol included at least one boost with replicon particles [36].

The absence of anti-E1E2 antibodies titers in ELISA and neutralizing assays (as compared with antigen alone controls) in mice antisera in the rE1E2+c-di-AMP (IN-IN-IN) group was surprising in view of previous data obtained with other antigens [37] but this was a consistent finding in our hands.

With the exception of MF59, all other adjuvanted vaccines elicited multifunctional CD4⁺ T cells producing both IFN- γ and TNF- α production indicating that the cellular response to rE1E2 is accompanied with the generation of Th-1 type CD4⁺

1 T cells. In the sense of immune protection against HCV, this is important as it is strongly
2 suggested that the presence of HCV-specific Th-1 type CD4⁺ T cells correlates with the
3 spontaneous resolution of HCV infection, by facilitating the generation of CD8⁺ T cells
4 along with neutralizing antibodies [38-41]. Moreover, studies with Chimpanzee have
5 shown that the resolution of HCV during reinfection is highly dependent on CD4⁺ T cells
6 even though the CD8⁺ T cells have been developed during the primary infection [42].
7 The detected CD4⁺-specific cellular response in immunized mice may agree with our
8 previous finding in rE1E2-vaccinated humans [8], where we detected strong E1E2-
9 specific CD4⁺ T cell proliferation in peripheral blood mononuclear cells. The absence of
10 a CD8⁺-specific cellular response could be due to the absence of CD8⁺ T cell epitopes in
11 E1E2 in the inbred strain of mice used (CB6-F1) in comparison to human, where the
12 genetic diversity of MHC molecules in human may allow for additional CD8⁺ T cell
13 responses as suggested in a recent study [43].

14 Potent adjuvant activities of the di-nucleotide family (c-di-AMP and c-di-GMP)
15 have been shown before for the model antigen ovalbumin and recombinant antigens
16 derived from pathogens such as *Streptococcus pneumoniae*, *Trypanosoma cruzii*, or
17 influenza virus [25, 26, 44-47]. The use of the adjuvant c-di-AMP in a partial intranasal
18 immunization regimen has some advantages in avoiding repeated IM needle injections
19 for the recipient. However, no adjuvanted mucosal vaccines have been approved so far
20 partly due to prior safety concerns with other mucosal adjuvants [48-50]. The use of c-di-
21 AMP leads to the STING-dependent stimulation of both TNF-alpha and type I IFNs. In
22 general, the first cytokine seems to be critical for the stimulation of antibody responses
23 and the activation of Th2 cells [51], whereas type I IFNs are required for stimulation of
24 CTL responses and modulation of Th1 cells activity. Interestingly, c-di-AMP promotes
25 self-limited type I IFN activation as a result of an activation of STING degradation
26 (unpublished data). This feature, together with the capacity of c-di-AMP to promote a
27 type I IFN induction restricted at the local site of administration [52] reduces the
28 likelihood of systemic side effects related to immune activation thereby making c-di-
29 AMP a promising candidate adjuvant.

30 Archaeosome adjuvants comprised of total polar lipids of archaea target the
31 antigen effectively to APCs, and additionally provide co-stimulation and activation

1 signals. The robust antigen-specific immune response including CD4+ T cells is
2 consistent with previous studies utilizing immunization of mice with model antigens
3 entrapped in various archaeosomes comprised of total polar lipids and/or a mixture of
4 semi-synthetic archaeal lipids. In this study, archaeosomes were comprised of a single
5 semi-synthetic negatively charged lipid (sulfated lactosyl archaeol), demonstrating the
6 advantage of a simple formulation. Archaeosomes traditionally also evoke robust CD8 T
7 cell response; however, the absence of the response may be related to lack of antigenic
8 epitopes in the mix. Moreover, archaeosomes in previous studies have been
9 demonstrated to evoke long-lasting functional responses to antigen, resulting in
10 prolonged efficacy against infectious or tumor challenge. Thus, comparing memory
11 immune responses to the various adjuvants may help select the appropriate formulation
12 for HCV vaccine.

13 In this study, we have presented a side-by-side comparison of the immunogenicity
14 of HCV rE1E2 formulated with c-di-AMP and archaeosomes versus two broadly used
15 and licensed adjuvants, MF59 and Alum/MPLA. We have demonstrated that in mice, c-
16 di-AMP and archaeosomes offer the advantage of inducing a superior cellular response.
17 This may indicate the use of c-di-AMP and archaeosomes as potential adjuvants to elicit
18 an optimal dual humoral and cellular immune response. In addition, our data also
19 indicates that use of a partial (but not exclusive) intranasal immunization regimen (IM-
20 IN-IN) may also be feasible using c-di-AMP as adjuvant.

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Figure Legends

Figure 1. The experimental plan

Figure 2. Detection of anti-HCV E2 in mice immunized with rE1E2. Highly significant anti-E2 antibodies titers were detected by ELISA in all vaccinated groups with exception of c-di-AMP (IN-IN-IN). Horizontal lines are the medians in each group. *** = p value < 0.001; ** = p value < 0.01; and * = p value < 0.05.

Figure 3. Neutralization activity of rE1E2 in combination with adjuvant in mice. The sera from mice immunized with rE1E2 are able to neutralize the entry of HCV pseudo particle *in vitro*. Percentage of neutralization was calculated based on the neutralizing activity for post-vaccination bleed divided by neutralization activity of pre-vaccination bleed. Horizontal lines are the medians in each group. ** = p value < 0.01; and * = p value < 0.05.

Figure 4. Differential detection of T cells immune response in mice. The splenocytes from vaccinated mice with rE1E2 in combination with different adjuvants were stimulated *in vitro* and intracellular production of cytokine was detected by multi-color flow cytometry. IM = Intramuscular; IN = Intranasal; Control = Negative control splenocytes; Peptide pool = Splenocytes that are stimulated with a pool of 55 peptides spanning E1E2.

Figure 5. The percentage of CD4⁺ and CD8⁺ T cells that are expressing INF- γ , TNF- α or both. Graphs **a**, **b**, and **c** showing CD4⁺ T cells and graphs **d**, **e**, and **f** showing CD8⁺ T cells. The splenocytes from vaccinated mice with rE1E2 in combination with different adjuvants were stimulated *in vitro* and intracellular production of cytokine was detected by multi-color flow cytometry. IM = Intramuscular; IN = Intranasal; Control = Negative control splenocytes; Peptide pool = Splenocytes that are stimulated with a pool of 55 peptides spanning E1E2.

Figure 1. Landi *et al.*

Figure 2. Landi *et al.*

Figure 3. Landi *et al.*

Figure 5. Landi *et al.*