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Recombinant adenoviral vector expressing HCV NS4 induces protective immune responses in a mouse model of Vaccinia-HCV virus infection: A dose and route conundrum



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ABSTRACT

Hepatitis C virus (HCV) leads to chronic infection in the majority of infected patients presumably due to failure or inefficiency of the immune responses generated. Both antibody and cellular immune responses have been suggested to be important in viral clearance. Non-replicative adenoviral vectors expressing antigens of interest are considered as attractive vaccine vectors for a number of pathogens. In this study, we sought to evaluate cellular and humoral immune responses against HCV NS4 protein using recombinant adenovirus as a vaccine vector expressing NS4 antigen. We have also measured the effect of antigen doses and routes of immunization on the quality and extent of the immune responses, especially their role in viral load reduction, in a recombinant Vaccinia-HCV (Vac-HCV) infection mouse model. Our results show that an optimum dose of adenovirus vector (2×10^7 pfu/mouse) administered intramuscularly (i.m.) induces high T cell proliferation, granzyme B-expressing CD8⁺ T cells, pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-2 and IL-6, and antibody responses that can significantly reduce the Vac-HCV viral load in the ovaries of female C57BL/6 mice. Our results demonstrate that recombinant adenovirus vector can induce both humoral and cellular protective immunity against HCV-NS4 antigen, and that immunity is intricately controlled by route and dose of immunizing vector.

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1. Introduction

Hepatitis C virus (HCV) infects human liver and about 150 million people worldwide are infected chronically [1,2]. The people who clear HCV infection exhibit strong and multi-specific T cell responses, while those who develop persistent infection lack this immunity [3–6]. Exhaustion of the antigen-reactive T cells and modulation of T cells by various HCV proteins have been suggested to play a major role in T cell failure in chronic HCV [7–11]. Further, due to highly evolving antigenic variability in structural proteins, this virus evades recognition by generated antibodies [12]. Currently available treatment includes a combination of Interferon- α and antiviral drugs ribavirin and protease inhibitors. However, this treatment also has several limitations and the search to find new therapies is ongoing [13–17].

From multiple studies, it is clear that a vaccine that would induce broad, robust and effective T cell and antibody responses would be able to protect against HCV infection [18–20]. The vehicle for the delivery of antigens plays a critical role in the induction of strong cellular, as well as humoral responses. Replication-defective recombinant adenoviral vectors have been shown to generate very strong transgene-specific cellular and humoral immune responses [21–26]. However, there have been inconsistencies in results obtained in various studies, where adenoviral vectors have been found to correlate with seemingly contrasting exhausted and chronically over-activated, or sustained and polyfunctional CD8⁺ T cell responses [27,28].

It is well known that both the quality and quantity of immune responses depend upon antigen doses and routes of immunization [29]. The route of immunization can also influence the trafficking of antigen-specific T cells [30]. Surprisingly, these important aspects of adenoviral vector based immunizations are still not well understood. Intramuscular (i.m.) and intraperitoneal (i.p.) routes are among the most utilized routes of immunization in several studies using recombinant adenoviral vectors [31–38]. These

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strategies become even more important for pathogens whose clearance depends upon the generation of robust immune responses. Previously, pre-existing immunity against adenoviruses has discouraged the use of adenoviral vectors as a tool for antigen delivery in humans [39]. However, it has been shown that the encounter of the adenoviral vector with pre-existing neutralizing antibodies can be circumvented by choosing the right route of immunization [39,40].

Various vaccine strategies for HCV have focused on generating strong multi-specific cellular immunity against various structural and nonstructural proteins of HCV because they have been correlated with viral clearance [3,5,15,17–21,41–44]. Meta-analysis of all chimpanzee studies has suggested that antibodies against structural proteins play a significant role in protection from HCV infection [45]. Therefore, the role of antibody responses cannot be ruled out in protection from HCV infection.

In this study, we used replication-defective recombinant adenovirus vectors containing HCV non-structural protein 4 (rAd-NS4) to induce immune responses in C57BL/6 mice. We compared the effects of different doses of adenoviral vector (2×10^5 pfu to 2×10^8 pfu/mouse) delivered i.m. or i.p. on the induction and longevity of both humoral and cellular immune responses, and their subsequent role in reduction of viral load in a Vac-HCV infection model [46]. Our studies demonstrate significant reduction in viral loads that is dependent on both route and dose of rAd-NS4 influencing the induced immune responses.

2. Materials and methods

2.1. Recombinant adenoviral vector

HCV (genotype 1a) NS4 (amino acids 1658–1972) gene was cloned into replication-defective AdenoVator Vector (QBiogene Inc.) as per the standard method provided in the QBiogene AdenoVator manual. Recombinant and control adenoviruses were prepared, propagated, purified and stored as reported by our laboratory [47].

2.2. Vaccinia virus

The Western Reserve (WR) strain of VV containing the HCV NS3-NS4-NS5 gene of the HCV BK strain (genotype 1b) (Vac-HCV) was kindly provided by Dr. A. Prince, U.S.A. The virus was grown and assayed in BHK-cells [48].

2.3. Mice immunizations

Six- to seven-week old inbred female C57BL/6 mice were purchased from Charles River laboratories (Sherbrooke, ON, Canada) and housed in the Health Sciences Lab Animal Facility (HSLAS) at the University of Alberta. Groups of six mice were immunized with varying doses of rAd-NS4 (2×10^5 – 2×10^8 pfu/mouse) by either i.m. or i.p. routes on days 0 and 14. Each group was divided in two subgroups (three mice each). One set of mice was euthanized after 8 days of single immunization and the remaining set was euthanized 8 days after second immunizations. The time points of immunizations were chosen from several pilot experiments performed in the lab. Animal immunization experiments were repeated more than three times. All procedures were approved by the University of Alberta Animal Care and Use Committee in accordance with Canadian Council of Animal Care guidelines.

2.4. T cell proliferation assay

Antigen-specific T cell proliferation assays were performed using nylon wool purified splenocytes and inguinal lymph node

cells as reported previously by us [47]. Briefly, 4×10^5 T cells and equal number of antigen presenting cells (γ -irradiated splenocytes from un-immunized mice) were added in each well in 3–4 replicates, and stimulated with 1 and 5 μ g/ml of recall recombinant HCV NS4 protein antigen (kindly provided by Chiron Corp.). Plates were incubated at 37 °C with 5% CO₂, and on the fourth day, 75 μ l culture supernatants were collected from each well and replicates were pooled together and stored at –20 °C until used for cytokine analysis. Thymidine labeled with radioactive tritium (Perkin Elmer, Canada) was added to each well. After overnight incubation, cells were transferred on filters using a harvester (Tomtec Inc. Connecticut, USA) and counted in Microbeta β -scintillation counter (Perkin-Elmer Inc., Massachusetts, USA). Average counts per minute (CPM) of replicates with SD were plotted in bar graphs (representative of three individual experiments).

2.5. Cytokine ELISA

Culture supernatants from T cell proliferation assay plates were collected and used to quantify cytokine production using mouse cytokine ELISA kits obtained from eBiosciences (eBiosciences Inc. San Diego, USA) according to the manufacturer's instructions. The plates were read and analyzed in a FluoStar ELISA reader (BMG Labtech GmbH, Ortenberg, Germany). Graphs represent averages of duplicate values.

2.6. T cell phenotype analyses by flow cytometry

Mouse splenocytes were cultured for 4 days in the presence of 1 μ g/ml HCV NS4 antigen. After 4 days of culture, cells were collected, counted and stained for CD3, CD4, CD8, Granzyme B (Biolegend Inc. San Diego, USA), FoxP3, PD1 and CTLA4 markers with fluorophore-labeled antibodies (eBiosciences Inc. San Diego, USA) using previously reported procedures [49]. Stained cells were run in a FACS Canto and analyzed in FACS Diva software (BD Biosciences, CA, USA).

2.7. T cell killing assay

Enriched spleen T cells were cultured in the presence of γ -irradiated splenocytes and 1 μ g/ml NS4 protein. After 5 days of culture, the effector cells were collected and washed with RPMI media. EL4 target cells (ATCC TIB 39, VA, USA) were incubated overnight with an NS4 peptide pool (20 amino acid long peptides overlapping in five amino acids at 5 μ g/ml of each peptide) obtained from Genscript Inc. After incubation, EL4 targets stained with 3 μ M/ml CFSE (CFDA) (Life Technologies Corp., NY, USA) and incubated with effector cells (E:T ratios of 50:1, 25:1 or 12.5:1) for 6 h. Next, 20 μ l (20,000 beads) of Count Bright Absolute Counting Beads (Invitrogen, Oregon, USA) were added to each tube. Each sample was run for 10,000 events of count beads in a BD FACS Canto and data were analyzed using FlowJo v.7 software (Tree Star Inc., USA). For the calculation of percentage killing, following formula was used: % Killing = [(Average live cells in PBS control – live cells in immunized group)/Average live cells in PBS control] $\times$ 100. The killing assay was done in triplicates and the % killing was calculated for individual replicates and data are shown as avg. $\pm$ SD.

2.8. Antigen specific antibody ELISA

Blood from mice of the same group was pooled and serum was prepared and stored at –20 °C until use. For the detection of IgG, IgG1, IgG2a and IgM antibodies against NS4 or adenoviral vector, 96-well plates were coated with HCV NS4 protein (1 μ g/ml in 1 $\times$ PBS) or Adeno-vector infected HEK-293A cell lysate (100 μ l/well), and incubated overnight at 4 °C. Plates were washed and blocked

with 1% BSA followed by addition of diluted pooled serum in duplicate. The plates were incubated for 2 h, washed with $1 \times$ PBST, and added with secondary antibody (anti-mouse IgG, IgG1, IgG2a or IgM, AP labeled) (Southern Biotech, Alabama, USA) and color was developed with PNPP substrate. Absorbance was read using a FluorStar ELISA Reader. The background (OD obtained from the wells coated with uninfected HEK-293 A cell lysate) was subtracted from OD values obtained from experimental wells coated with adenovector infected HEK-293 A cell lysate.

2.9. Neutralizing antibodies against recombinant adenovirus vectors

96-well plates were seeded with HEK-293A cells at 1×10^4 cells/well and incubated overnight. Serial 10-fold dilutions of Ad-vector (alone) were prepared and incubated with a 1:100 dilution of serum from various groups of immunized mice for 2 h. 100 μ l of neutralized Ad-vector was added in triplicate to the plates. After 10 days, the cell monolayer was fixed by adding 50 μ l of 10% formalin to each well and stained with 0.3% crystal violet solution. Reduction in the end-point titer due to neutralization was determined by observing the cytopathic effects of Ad-vector on monolayers

(disruption or removal of monolayer). Lower end-point titer of rAd-vector indicates more neutralizing antibodies.

2.10. Chimeric Vac-HCV challenge

Vac-HCV (NS3-NS5) chimeric virus was used to challenge the immunized C57BL/6 mice according to procedure reported previously [48,49]. Briefly, 8 days after immunization, mice were injected with 1×10^5 pfu of chimeric Vac-HCV i.p. Five days after Vac-HCV challenge, mice were euthanized, ovaries were removed and processed individually to quantitate the Vac-HCV virus load. Vac-HCV virus samples were titrated by standard plaque assay on TK-1 cell line (ATCC no. CRL8303).

2.11. Statistical analysis

Data were analyzed by Graph-pad Prism software (Graph-pad Software Inc., CA, USA). Student's *t*-test and one-way ANOVA were used to determine the significant difference between two groups. *p*-value less than 0.05 (<0.05) was considered to be statistically significant.

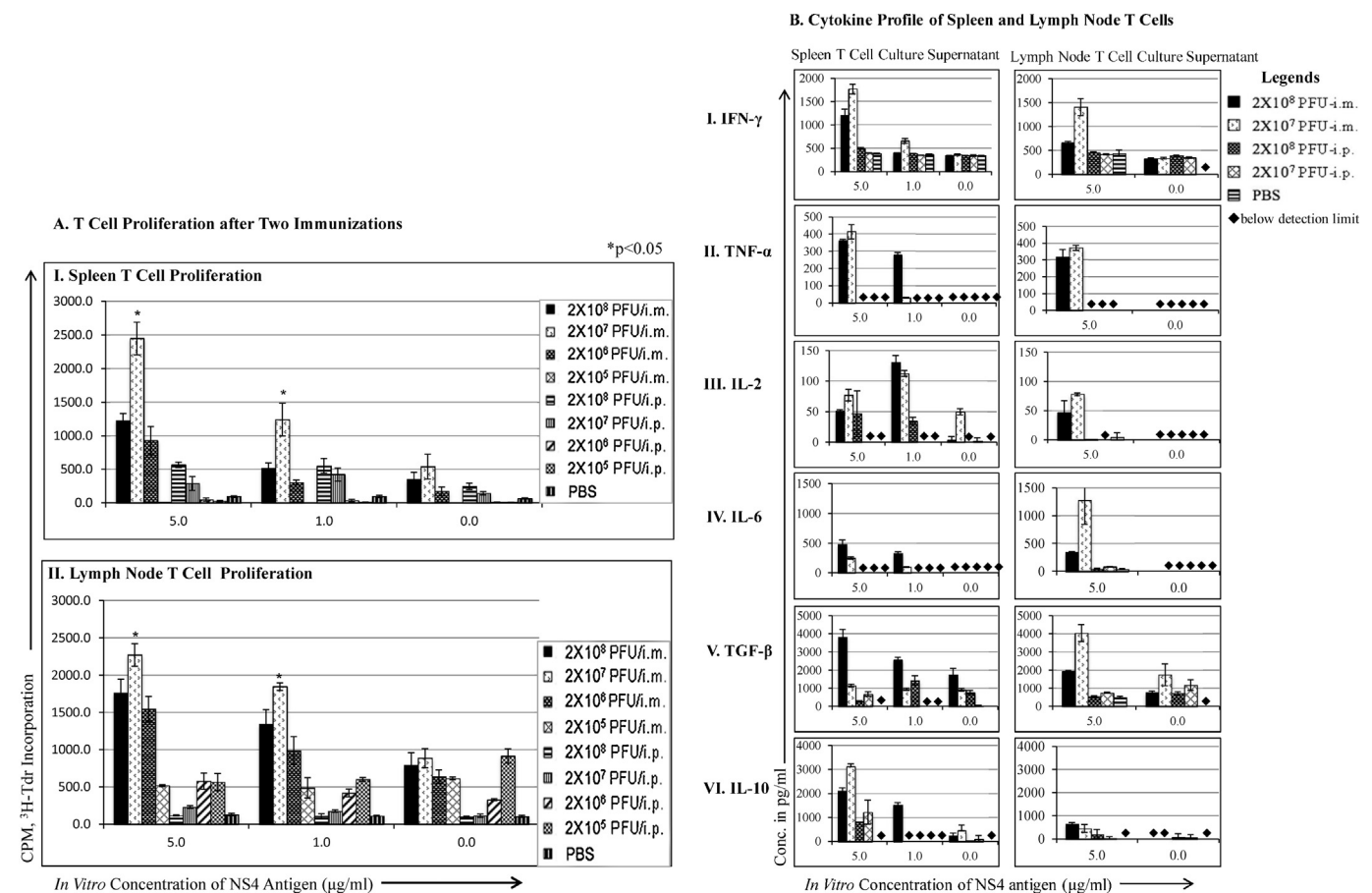


Fig. 1. Immune responses after two immunizations. (A) T cell proliferation. Antigen-specific T cell proliferation after immunization with various doses (2×10^5 – 2×10^8 pfu/mouse) of recombinant adenovirus vector (rAd) containing HCV-NS4 antigen, by intramuscular (i.m.) and intraperitoneal (i.p.) routes in C57BL/6 mice. After 8 days of two immunizations, mouse splenocytes and lymph nodes were harvested and T cell proliferation was evaluated in each group of immunized mice by a ^3H -thymidine-uptake assay. Data with spleen (I) and draining lymph node (II) T cells are shown. Mean $\pm$ standard deviation of CPM (counts per minutes) from quadruplicate (I) triplicate (II) wells are shown. The data are representative of more than three different repeated experiments. (B) Cytokine profile of spleen and lymph node T cells. Spleen and draining lymph nodes were collected 8 days after two immunizations with higher (2×10^8 pfu/mouse) or lower (2×10^7 pfu/mouse) doses of rAd-NS4, followed by culturing the T cells with recombinant NS4 antigens and irradiated syngeneic APCs. Culture supernatants were collected at day 4 and examined for IFN- γ (I) TNF- α (II), IL-2 (III), IL-6 (IV), TGF- β (V) and IL-10 (VI). Mean $\pm$ standard deviation of cytokine concentration from triplicate wells are shown. The data are representative of three different repeated experiments.

3. Results

3.1. Intramuscular immunization with an optimum dose of rAd-NS4 induces high antigen specific T cell proliferation and cytokine responses

Mice were immunized with four different doses of rAd-NS4 (2×10^8 , 2×10^7 , 2×10^6 or 2×10^5 pfu/mouse) either i.m. or i.p. at day 0 and 14. Using both i.p. and i.m. routes of immunizations, the two lower doses of rAd-NS4 (2×10^6 and 2×10^5 /mouse) exhibited low NS4-specific responses (Fig. 1A I and II). We found that the higher doses of immunizations (i.e., 2×10^7 and 2×10^8 /mouse) via the i.m. route induced higher T cell proliferation in comparison to the i.p. route (Fig. 1A). Further, after both one (data not shown) and two i.m. immunizations, highest antigen dose (2×10^8 pfu/mouse) induced less T cell proliferation, whereas 2×10^7 pfu/mouse induced significantly higher ($p < 0.05$) T cell proliferation in both spleen and lymph node T cells (Fig. 1A). Therefore, in the next experiments, we compared the effects of only two doses of rAd-NS4 (high 2×10^8 pfu/mouse and low 2×10^7 pfu/mouse) administered through i.m. or i.p. routes.

Upon examining the production of various cytokines in culture supernatants, after two immunizations, both the spleen and lymph node T cells produced higher levels of IFN- γ , TNF- α , IL-2 and IL-6 when mice were immunized intramuscularly (Fig. 1B). From splenocytes, the TGF- β levels were higher in high-dose and i.m. route as compared to the i.p. route. In lymph node T cells; TGF- β was higher in the lower dose group given via i.m. route (Fig. 1B). In antigen unstimulated groups TGF- β and IL-6 were also induced to levels similar or little lower than the antigen stimulated group; it could be reflective of the in vivo status of cells independent of ex vivo stimulation with antigen. With a lower antigen dose, more IL-10 was detectable in comparison to the higher dose (Fig. 1B).

3.2. Phenotypic characteristics and target killing activity differ in T cells obtained from mice immunized with high vs. low doses of rAd-NS4 given by intramuscular or intraperitoneal routes

T cells obtained from mice after immunization with low and high doses of rAd-NS4 via i.m. and i.p. routes, were examined for expression of granzyme B, FoxP3, PD-1 and CTLA-4 to determine if effector and/or regulatory T cells are induced, and also if T cells are negatively regulated due to the expression of inhibitory receptors. Low i.m. antigen dose (2×10^7 pfu/mouse) induced the highest levels of Granzyme B expression in CD8 $^+$ T cells compared to the higher i.m. antigen dose, or both i.p. doses (Fig. 2A, panel I). Interestingly, the higher i.m. dose generated a higher frequency of CD4 $^+$ FoxP3 $^+$ T regulatory cells compared to the lower i.m. dose or to both i.p. doses (Fig. 2A, panel II). Both i.m. and i.p. routes induced similar patterns of PD1-expressing CD4 $^+$ T cells; the lower dose of antigen induced less PD1-expressing CD4 $^+$ T cells compared to the higher dose (Fig. 2A, panel III). Numbers of CTLA4-expressing CD4 $^+$ T cells were higher in the mice immunized with the higher i.p. dose when compared to either i.m. dose or the lower i.p. dose (Fig. 2A, panel IV). PD1 and CTLA4 expression on CD8 $^+$ T cells showed similar patterns with both doses and routes (Data not shown).

In accordance with Granzyme B expression, T cells induced by i.m. immunization showed higher percent killing of NS4 peptide loaded EL4 target cells in comparison to i.p. immunization (Fig. 2B).

3.3. Induction of antigen-specific humoral immune responses in mice immunized with higher vs. lower doses of rAd-NS4 given by intramuscular or intraperitoneal routes

After two immunizations, the high dose of rAd-NS4 by both i.m. and i.p. routes induced significantly higher levels of anti-NS4 IgG

antibodies in comparison to the lower immunizing dose (p values < 0.05). Low i.m. dose induced slightly higher IgG (p value = 0.1252) and significantly higher IgG1 (p value = 0.021) responses than did low i.p. dose (Fig. 3A). We also determined the induction of adeno-vector derived antigens-specific IgG antibodies in each route of immunization. The dose of immunization showed an interesting pattern of anti-adenovector antibodies. After one immunization, low i.m. dose (2×10^7 pfu/mouse) provided the highest adeno-vector specific IgG responses, whereas after two immunizations, high i.m. dose (2×10^8 pfu/mouse) provided the highest IgG titers (p values < 0.05 , in comparison to all other groups) (Fig. 3B). IgG1 shows a similar pattern with both one and two immunizations. IgG2a was undetectable after single immunization, however it was detectable after two immunizations, but was not significantly different in various immunization groups ($p > 0.05$). After single immunization, IgM was not significantly different among various groups ($p > 0.05$). However, after two immunizations, adeno-vector specific IgM was comparable in all rAd-NS4 immunized groups but higher in comparison to PBS control ($p < 0.05$).

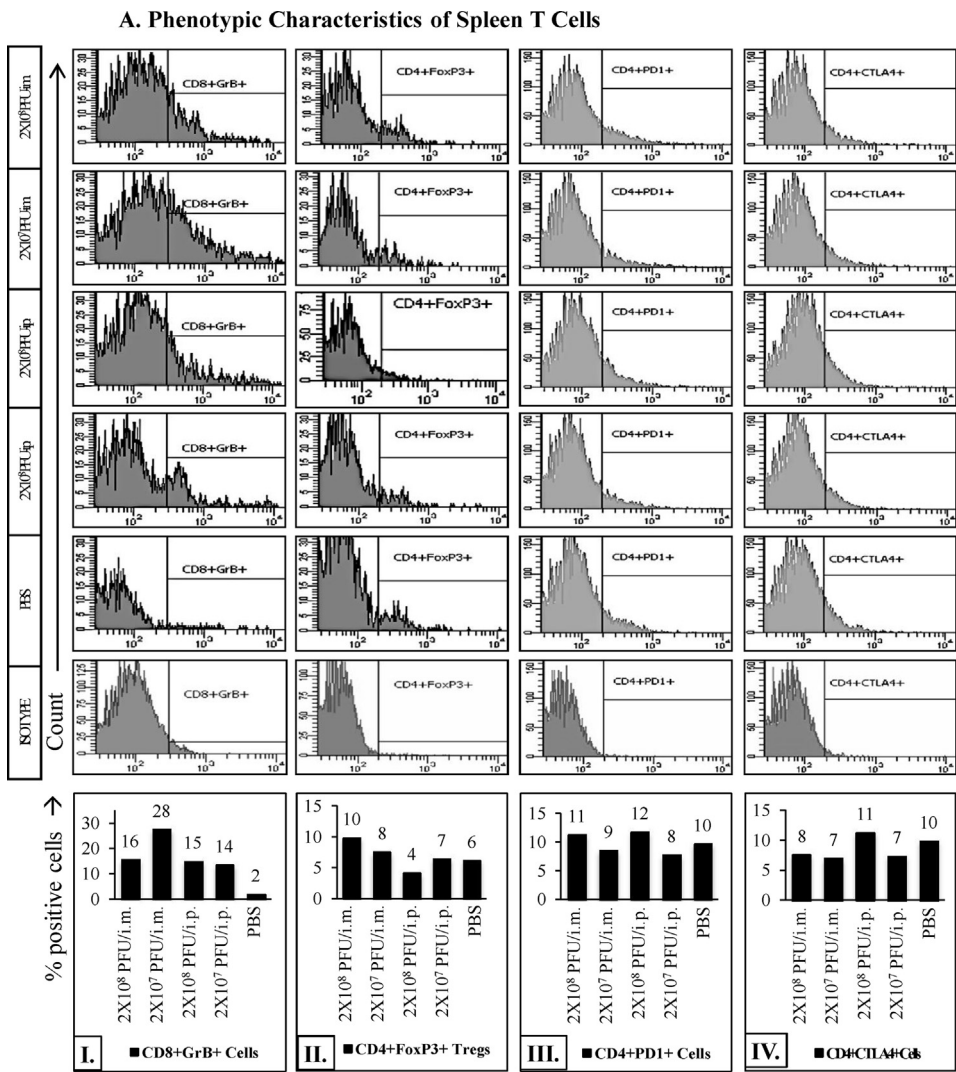
We also analyzed neutralizing antibodies against adeno-vector in mice immunized i.m. or i.p. with high and low rAd-NS4, where lower viral titers represent the presence of higher levels of neutralizing antibody titers (Fig. 3C). Interestingly, sera from mice immunized with rAd-NS4 with both high and low doses and by both routes, demonstrated some neutralization of adenovirus compared to the PBS control group (Fig. 3C). However, we observed the least neutralization by the sera from the mice immunized with low i.m. dose and the highest levels of neutralizing antibodies by high i.p. dose ($p < 0.05$).

3.4. Intramuscular immunization with rAd-NS4 induces higher long-term cellular and humoral memory responses than intraperitoneal immunization

We examined antigen-specific T cell and antibody responses two months after two i.m. or i.p. immunizations with low doses of rAd-NS4 (2×10^7 pfu/mouse) to determine the longevity of the immune responses induced. The i.m. route of immunization showed higher antigen-specific T cell proliferation in comparison to the i.p. route, however, the target cell killing activity was comparable with T cells obtained from both routes of immunization (Fig. 4A and B). Antigen-specific antibodies were also present two months after immunization. Anti-NS4 IgG and IgG1 were higher in mice immunized i.m., whereas IgM levels were higher in mice immunized i.p. (p values < 0.05) (Fig. 4C).

3.5. Immunization with low intramuscular dose rAd-NS4 provides the maximum reduction in Vac-HCV viral load

We challenged mice with recombinant Vac-HCV 8 days after first and second immunizations to examine prevention of infection or reduction in viral load after immunization with low and high doses of rAd-NS4, via i.p. and i.m. routes. Mice immunized once only failed to control Vac-HCV load in ovaries compared to controls (data not shown). However, after two immunizations, all of the groups were able to significantly reduce Vac-HCV load compared to PBS group (Fig. 5). Interestingly, maximum reduction in viral load was observed in mice immunized i.m. with low antigen dose. Moreover, out of three mice, one mouse successfully cleared the virus. Overall, this group was significantly different than groups with immunization by both i.p. doses and high i.m. dose ($p < 0.05$) (Fig. 5). Table 1 summarizes various factors that together impact the reduction in viral load and depicts the overall picture of immune status of mice immunized with higher or lower antigen dose administered by i.m. or i.p. routes.



B. T Cell Killing Activity

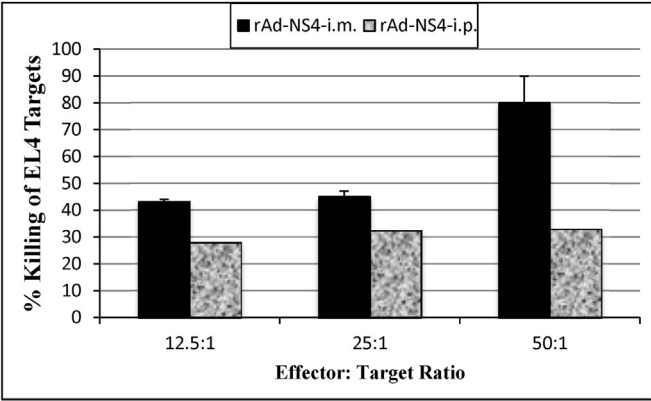


Fig. 2. (A) Panels I–IV Phenotype of T cells. Spleens were collected 8 days after two immunization with higher (2×10^8 pfu/mouse) or lower (2×10^7 pfu/mouse) doses of NS4-rAd and cultured in the presence of $1 \mu\text{g/ml}$ NS4 antigen for 5 days, followed by staining for various markers such as Granzyme B (GrB), FoxP3, PD1 and CTLA4. (I) Frequency of Granzyme B-expressing CD8⁺ T cells (II) FoxP3⁺ CD4⁺ T regulatory (Tregs) cells (III) PD1⁺CD4⁺ T cells. (IV) CTLA4⁺CD4⁺ T cells. The data are representative of three different repeated experiments. (B) T cell killing activity. Spleen T cells obtained from i.m. and i.p. immunized mice were cultured in vitro for 5 days with recombinant NS4 protein and irradiated syngeneic APCs, followed by a CFSE killing assay using EL4 target cells loaded with an NS4 peptide pool. For the calculation of percentage killing, following formula was used: % Killing = [(Average live cells in PBS control – live cells in immunized group)/Average live cells in PBS control] $\times$ 100. The killing assay was done in triplicates and the % killing was calculated for individual replicates and data are shown as avg. $\pm$ SD. The data are representative of three different repeated experiments.

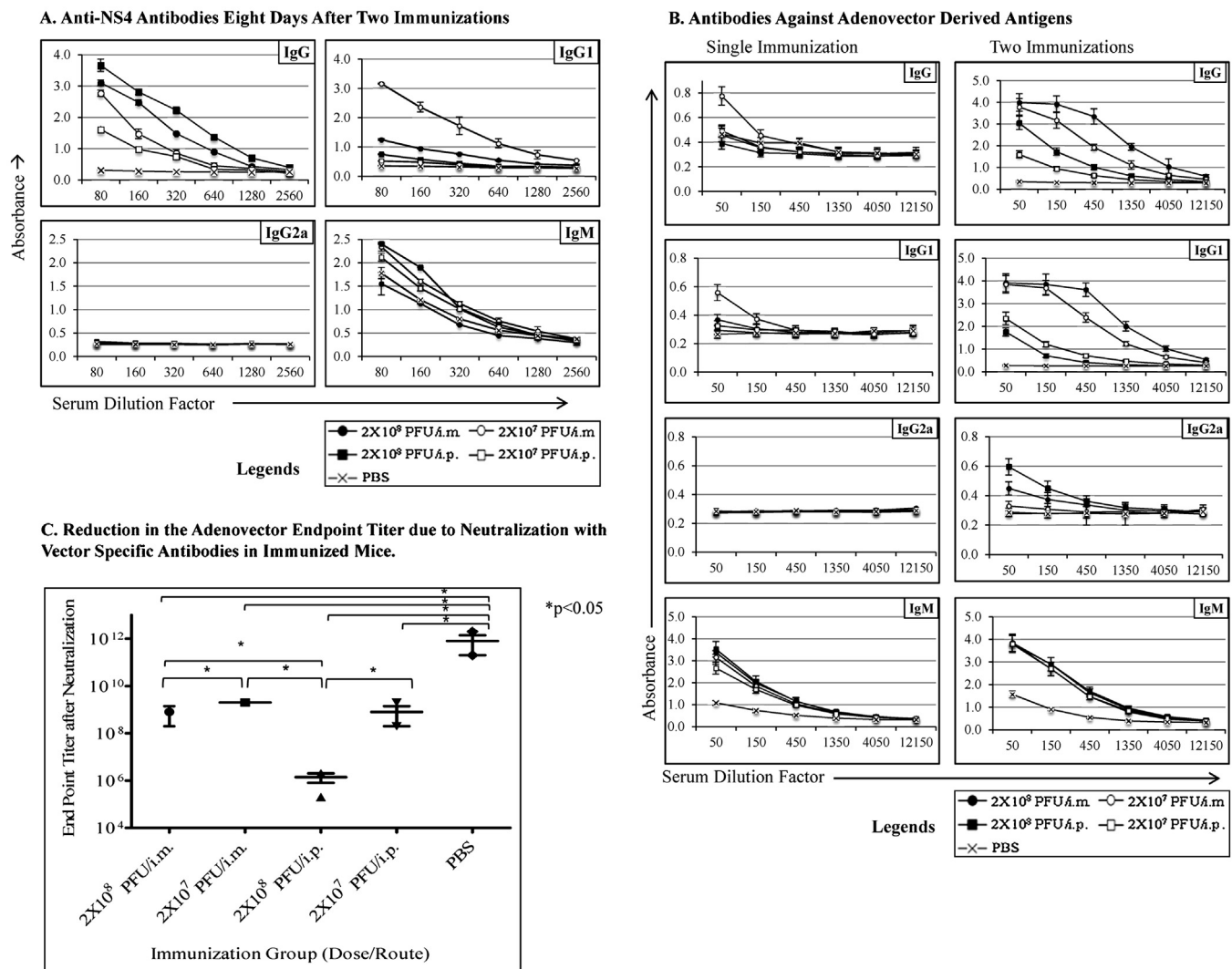


Fig. 3. Assays for antibodies in immunized mice. (A) Anti-NS4 antibodies. Recombinant NS4 antigen was used as the coating antigen in an ELISA; (B) Adeno-vector specific antibodies: lysate from adenovirus-infected HEK-293A cells was used as the coating antigen in an ELISA; Mean $\pm$ SD of duplicate OD is plotted against serum dilution factor. (C) Adenovirus vector neutralizing antibodies: Ad virus stock was preincubated with sera from various immunized mice, followed by infecting the HEK-293A cells and examining the viral plaques/cytopathic effects for end-point titer determination (lower end-point titer of Ad-vector indicates more neutralizing antibodies). Mean $\pm$ SD of viral titers from four replicates is shown. Data are representative of three different repeated experiments.

4. Discussion

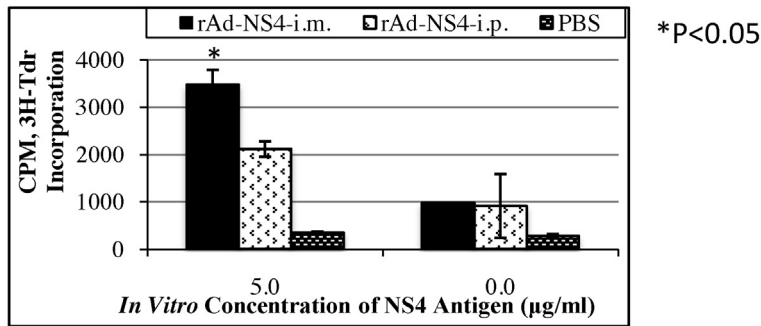
Adaptive immunity against HCV includes humoral (antibodies), and cellular (CD4+ and CD8+ T cells) responses, and all three components have been shown to be important for viral clearance [3–5,45]. Therefore, vaccine/immunotherapeutic strategies for HCV should include both types of adaptive immune responses.

Several studies have reported the importance of dose and route for effective immunization by the adeno-vector expressing the transgene antigens [29–38]. Both intramuscular and intraperitoneal routes of immunization have been examined using recombinant adenovirus vectors, but not compared directly. In this study, we investigated the induction of humoral as well as cellular responses after immunization with rAd-NS4 by i.m. and i.p. routes and correlated them with reduction of Vac-HCV viral load. The results and various immunization parameters obtained are summarized in Table 1. Our results demonstrated intriguing effects of dose and route of immunizations with replication-defective recombinant adenovirus vector expressing HCV-NS4 antigen. While intramuscular immunization fared better in inducing immune responses and reducing viral load compared to intraperitoneal

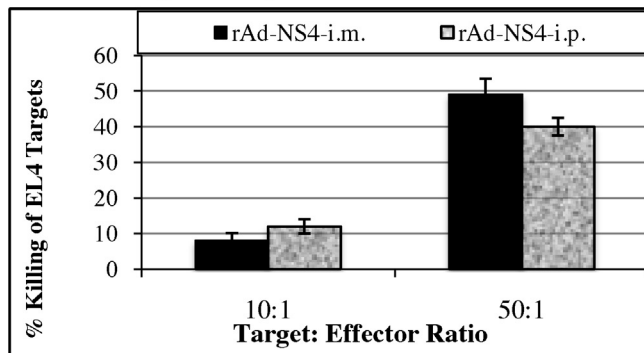
route, a lower dose of rAd-NS4 vector (2×10^7 pfu/mouse) provided better immunity and protection via i.m. route compared to a higher dose (2×10^8 pfu/mouse) of immunization.

Initially, we optimized the antigen dose by immunizing the mice with 2×10^5 to 2×10^8 pfu/mouse (at one log differences) given by i.m. or i.p. routes (Fig. 1A). After two immunizations, the lower i.m. dose (2×10^7 pfu/mouse) induced higher proliferation in both spleen and inguinal lymph node T cells in comparison to the higher i.m. dose (2×10^8 pfu/mouse) or both i.p. doses (Fig. 1A). In our experiments, only inguinal lymph nodes were harvested; the results could have been different if Peyer's patches or mesenteric lymph nodes were harvested for i.p. immunized mice. However, since the systemic spleen T cells also provided very low T cell responses after i.p. immunizations, it is possible that 8 days after immunizations, the local lymph nodes may have given response similar to spleen. With the i.m. route of immunization, various pro-inflammatory cytokines such as IFN- γ , TNF- α , and IL-6, were expressed at higher levels in both the spleen and lymph node T cells (Fig. 1B). In contrast, IL-10 was produced at comparable levels in both antigen doses via both routes. Higher IL-10 production may be due to an intrinsic property of HCV NS4, as shown in a previous

A. Proliferation of spleen T cells two months after 2nd immunization



B. T cell killing activity two months after 2nd immunization



C. Anti-HCV NS4 antibodies two months after 2nd immunization

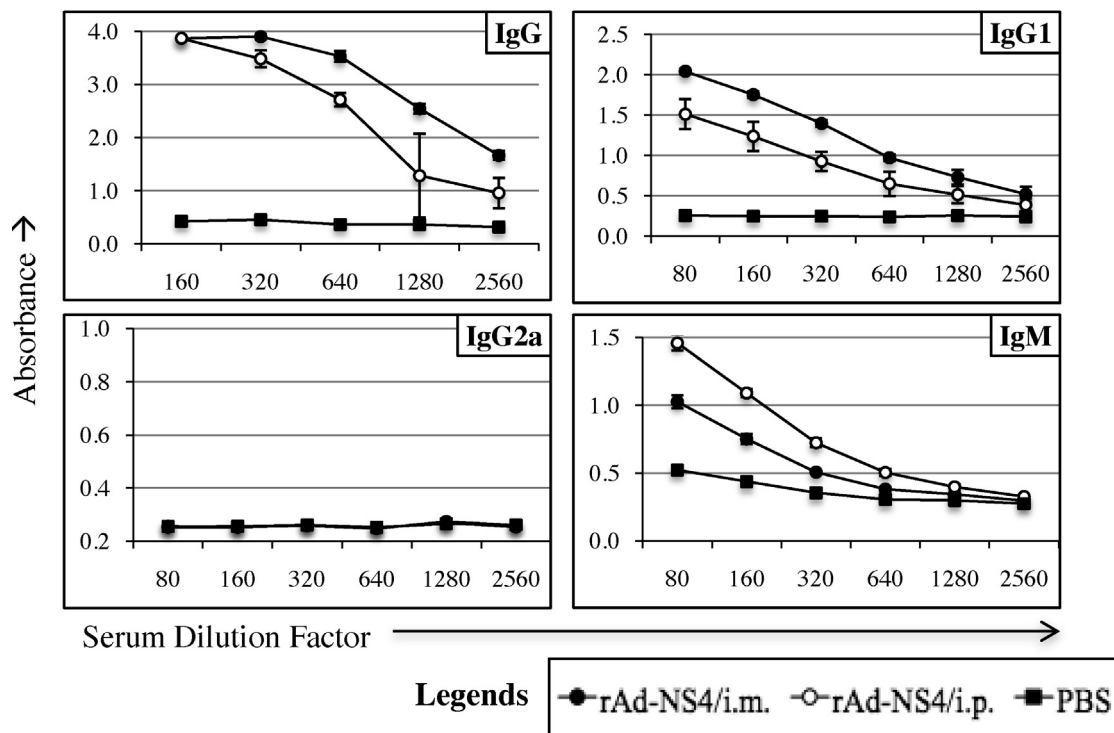


Fig. 4. Memory responses two months after two immunizations. (A) T cell proliferative responses. The proliferation assay was done in triplicates and data are shown as avg. $\pm$ SD. (B) Antigen-specific EL4 target killing. The killing assay was done in triplicates and the % killing was calculated for individual replicates, and data are shown as avg. $\pm$ SD. (C) NS4 antigen-specific antibody responses. The antibody ELISA was done in duplicates and data are shown as avg. $\pm$ SD. The data are representative of three different repeated experiments.

Table 1

Comparison of various immune parameters in mice immunized with rAd-NS4 with different doses and routes of Immunization.

Parameters	2×10^8 pfu/mouse i.m.	2×10^7 pfu/mouse i.m.	2×10^8 pfu/mouse i.p.	2×10^7 pfu/mouse i.p.	PBS
T cell proliferation	+++	++++ ($p < 0.05$)	++	+	+
IFN- γ	+++	++++	++	++	–
TNF- α	+++	++++	–	–	–
IL-2	+++	++++	++	+	–
IL-6	+++	++++	+	+	–
IL-10	+++	++++	+++	++++	–
TGF- β	+++	++	–	–	–
CD8 $^+$ GrB $^+$ cells	+++	++++	+++	+++	+
CD4 $^+$ PD1 $^+$ cells	+++	++	+++	++	++
CD4 $^+$ CTLA4 $^+$ cells	++	++	+++	++	++
CD4 $^+$ FoxP3 $^+$ Tregs	+++	++	+	++	++
NS4 specific antibodies (IgG)	+++	++	++++	+	–
NS4 specific antibodies (IgG1)	++++	++	–	–	–
NS4 specific antibodies (IgG2a)	–	–	–	–	–
NS4 specific antibodies (IgM)	+	+++	+++	+++	–
Ad vector specific antibodies (IgG)	++++	+++	++	+	–
Ad vector specific antibodies (IgG1)	++++	+++	+	++	–
Ad vector specific antibodies (IgG2a)	++	–	+++	–	–
Ad vector specific antibodies (IgM)	+++	+++	+++	+++	–
Neutralization of Ad vector by serum	++	+	++++	+++	–
Vac-HCV load	+++	+	+++	+++	++++

Key: symbol '+' denotes; High (++++), medium (+++), low (++) magnitude of the parameter compared to lowest (+) value among the groups; (–) indicated undetectable or absent.

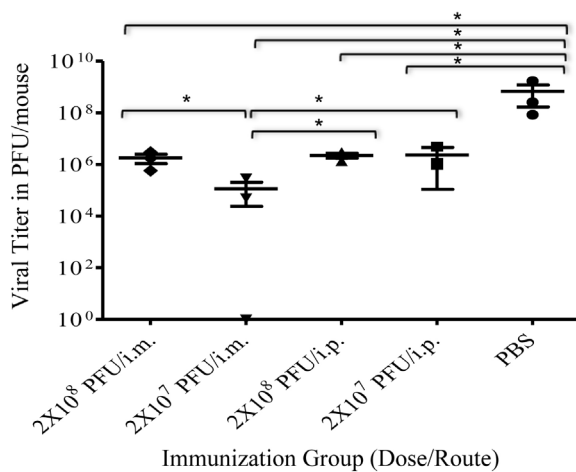


Fig. 5. Vac-HCV challenge of immunized mice. After two immunizations (Day 0 and 14) with high (2×10^8 pfu/mouse) or low (2×10^7 pfu/mouse) antigen doses via i.m. or i.p. route, mice were challenged with 1×10^5 pfu/mouse i.p. Five days after challenge, mice were euthanized and viral load was determined in the ovaries of individual mouse by plaque assay using TK-1 cell line. Mean $\pm$ Standard deviation of viral titers from four replicates is shown. The data are representative of three different repeated experiments.

study [50]. Expression of granzyme B by CD8 $^+$ T cells is correlated to effector function [51]. Interestingly, GrB expression was the highest in CD8 $^+$ T cells in mice immunized with low i.m. dose (Fig. 2A, panel I), which again correlated to peptide-loaded target killing activity (Fig. 2B) and antigen-specific proliferation (Fig. 1A).

Various inhibitory markers such as CTLA-4, PD-1 and CD4 $^+$ FoxP3 $^+$ T regulatory cells have been found to be up-regulated during persistent infection with HCV (7–11). In our study, we observed that high antigen dose of immunization via i.m. route led to low T cell response, therefore, we examined the expression of these inhibitory/regulatory markers among various antigen doses and routes of immunization. It was interesting to note that numbers of CD4 $^+$ FoxP3 $^+$ T regulatory cells were higher in i.m. immunized mice, perhaps representing a feedback regulatory mechanism (Fig. 2A, panel II). The expression of negative regulators of T cells, PD1 and CTLA4, was higher in the high dose group by both routes and by high i.p. dose, respectively. It is possible that

due to early timing of examining the inhibitory marker expression, we did not observe a significant difference, and small differences were observed. In addition, multiple immunizations over a longer time than we did here, may be necessary to induce stable inhibitory molecule expression on T cells, as is the case in chronic HCV.

High titers of IgG specific to NS4- and adenovirus antigens were obtained after two immunizations with the higher dose of rAd-NS4 by either i.m. or i.p. routes. Interestingly, with lower doses of rAd-NS4, the i.m. route provided significantly higher IgG titers against NS4 and adenovector derived antigens compared to the i.p. route (Fig. 3A and B). These results provided a conundrum as it is generally believed that presence of antibodies against adenoviral vector antigens negatively influences the overall immune responses due to rapid clearance of the vector [39]. We hypothesized that the induction of neutralizing vs. viral protein-specific antibodies may be differentially induced by different routes and doses of rAd-NS4. To test this theory, we also examined neutralizing antibody against adenovector by using a virus neutralization assay (Fig. 3C). In this assay, interestingly, the serum from mice immunized with the high i.p. dose of NS4-rAd had the highest virus neutralization ability, in contrast to the antibody responses against NS4 or intracellular adenovirus derived antigens (Fig. 3A and B). These data suggest that in the case of intraperitoneal immunization, induction of higher levels of neutralizing antibodies negatively influences the induction of cellular immune responses. However, when the adenovector is immunized in the muscle, leading to rapid intramuscular expression of viral proteins and transgene product, neutralizing antibodies are induced at a lower level and the pre-existing antibodies are not able to access the adenovirus particles immediately. The efficient antigen expression by the vector is followed by stimulation of T cell responses. Interestingly, after high dose of immunization by both i.m. and i.p. routes, significantly higher levels of IgG were obtained, compared to low dose immunizations, but T cell responses were reduced. Another curious result was the detection of high levels of IgG1 against NS4 in mice immunized with low i.m. dose of rAd-NS4 (Fig. 3A). IgG2a levels were relatively very low and comparable in all four groups (Fig. 3A). High IgG1 titers are generally considered indicative of a TH2 type immune response [52]. However, in our experiments, high levels of IFN- γ and TNF- α were obtained from splenocyte cultures of these immunized mice, suggesting a TH1 type cellular immunity.

It has been reported previously that in chronic HCV patients, IgG1 antibodies are predominant against all of the structural and non-structural proteins [53]. In addition, in chronic HCV patients treated with IFN- α and ribavirin, HCV-specific IgG1 predominates, irrespective of induction of TH1 cellular responses, suggesting their independence from TH1 and/or TH2 [54]. It has further been suggested that IgG1 production may be IL-2 dependent, and IFN- γ or IL-4 independent [55]. Murine IgG1 has been shown to facilitate phagocytosis of IgG1-bound immune complexes into macrophages and dendritic cells through Fc γ RIII [56], which would allow efficient processing and presentation of antigens to T cells.

We also investigated the longevity of the immune responses in mice after two i.m. or i.p. immunizations with low dose of rAd-NS4 and these showed a pattern of T cell and antibody responses similar to those after 8 days of immunization, with i.m. route showing higher overall immune response compared to i.p. (Figs. 3 and 4).

We challenged mice with recombinant Vac-HCV 8 days after first and second immunizations, so we could observe viral clearance or viral load reduction after immunization with low and high doses, i.p. and i.m., of rAd-NS4. Mice immunized once only failed to control Vac-HCV load in ovaries compared to controls (data not shown). However, after two immunizations, all of the groups were able to significantly reduce Vac-HCV load (Fig. 5). Interestingly, maximum reduction in viral load was observed in mice immunized i.m. with low antigen dose. This was significantly different than viral loads seen by both i.p. doses and by high i.m. dose ($p < 0.05$) (Fig. 5). Our results strongly indicated that optimum reduction in the viral load in these mice is associated with strong cellular and humoral immune responses and high levels of pro-inflammatory cytokines induced upon immunization with the low i.m. dose of rAd-NS4 (Table 1).

The differences we observed in the induced immune responses by i.m. vs. i.p. routes were significant and have not been reported previously. Upon intramuscular immunization, the transgene is expressed in muscle cells, and start declining after 4 days [57]. There is no clear report on the site of transgene expression upon intraperitoneal immunization with rAd vector. It could be expressed by peritoneal macrophages, followed by processing and presentation of antigens. Alternatively, upon i.p. injection, the adenovirus can gain entry into the blood circulation through mesenteric veins, followed by expression within splenic cells, and/or other distant tissues. However, this may result in a loss of efficiency of antigen expression due to non-replicative nature of the virus vector. It is possible that the efficiency of antigen expression in muscle immediately at the injection site allows for better antigen presentation by APCs and stimulation of T cells.

In our study, immunization with rAd-NS4 is shown to induce strong cellular and humoral immune responses, and pro-inflammatory cytokines, which are critically dictated by both route and dose of immunizations. Although, in our studies, pre-immunization with rAd-NS4 did not completely prevent the infection, it was able to significantly reduce viral load. Therefore, rAd-NS4 can be effective as immune-therapeutic vaccine to reduce viremia and viral load. Further, therapeutic rAd-NS4 vaccine can be used in combination with anti-viral drugs to reduce the viremia/viral load to undetectable ranges.

5. Conclusions

We found that an optimum dose of adenoviral vector expressing HCV NS4 antigen (rAd-NS4) administered through i.m. route, induces very strong cellular and humoral immune responses which are able to significantly reduce the viral load in Vac-HCV challenged mice. In contrast, high dose intraperitoneal immunization with rAd-NS4 induces high vector-neutralizing antibodies (Table 1). Therefore, route and dose of immunization should be selected and

optimized carefully in all adenoviral vector-based immunization strategies.

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References

- [1] Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. *Seminars in Liver Disease* 2000;20(01):1–16.
- [2] Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* 1990;87:6547–9.
- [3] Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, Houghton M, Parham P, Walker CM. Analysis of a successful immune response against hepatitis C virus. *Immunity* 1999;10:439–49.
- [4] Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, Govindarajan S, Purcell RH, Chisari FV. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proceedings of the National Academy of Sciences of the United States of America* 2002;99:15661–8.
- [5] Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nature Reviews Immunology* 2005;5(3):215–29.
- [6] Dustin LB, Rice CM. Flying under the radar: the immunobiology of hepatitis C. *Annual Review of Immunology* 2007;25:71–99.
- [7] Large M, Kittleson D, Hahn Y. Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. *Journal of Immunology* 1999;162:931–8.
- [8] Sarobe P, Lasarte J, Zabaleta A, Arribillaga L, Arina A, Melero I, et al. Hepatitis C virus structural proteins impair dendritic cell maturation and inhibit in vivo induction of cellular immune responses. *Journal of Virology* 2003;77:10862–71.
- [9] Pavo N, Lai M. The hepatitis C virus persistence: how to evade the immune system. *Journal of Biosciences* 2003;28:287–304.
- [10] Krishnadas D, Ahn J, Han J, Kumar R, Agrawal B. Immunomodulation by hepatitis C virus-derived proteins: targeting human dendritic cells by multiple mechanisms. *International Immunology* 2010;22:491–502.
- [11] Bengsch B, Seigel B, Ruhl M, Timm J, Kuntz M, Blum H, et al. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8⁺ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathogens* 2009;6:e1000947.
- [12] Simmonds P. Genetic diversity and evolution of hepatitis C virus—15 years on. *The Journal of General Virology* 2004;85:3173–88.
- [13] Aman W, Mousa S, Shih G, Mousa S. Current status and future directions in the management of chronic hepatitis C. *Virology Journal* 2011;9:57–68.
- [14] Liang T, Ghany M. Current and future therapies for hepatitis C virus infection. *The New England Journal of Medicine* 2013;368:1907–17.
- [15] Feinstone S, Hu D, Major M. Prospects for prophylactic and therapeutic vaccines against hepatitis C virus. *Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of America* 2012;55(Suppl 1):S25–32.
- [16] Fauvel C, Lepiller Q, Felmlee D, Fofana I, Habersetzer F, Stoll-Keller F, et al. Hepatitis C virus vaccines—progress and perspectives. *Microbial Pathogenesis* 2013;58:66–72.
- [17] Law J, Chen C, Wong J, Hockman D, Santer D, Frey S, et al. A hepatitis C virus (HCV) vaccine comprising envelope glycoproteins gpE1/gpE2 derived from a single isolate elicits broad cross-genotype neutralizing antibodies in humans. *PLoS One* 2012;8:e59776.
- [18] Lauer G, Barnes E, Lucas M, Timm J, Ouchi K, Kim A, et al. High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 2004;127:924–36.
- [19] Thimme R, Oldach D, Chang K, Steiger C, Ray S, Chisari F. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *The Journal of Experimental Medicine* 2001;194:1395–6.
- [20] Neumann-Haefelin C, Timm J, Spangenberg H, Wischniowski N, Nazarova N, Kersting N, et al. Virological and immunological determinants of intrahepatic virus-specific CD8⁺ T-cell failure in chronic hepatitis C virus infection. *Hepatology* 2008;47:1824–36.
- [21] Arribillaga L, de A, Sarobe P, Casares N, et al. Vaccination with an adenoviral vector encoding hepatitis C virus (HCV) NS3 protein protects against infection with HCV-recombinant vaccinia virus. *Vaccine* 2002;21:202–10.
- [22] Bassett J, Swift S, Bramson J. Optimizing vaccine-induced CD8⁺ T-cell immunity: focus on recombinant adenovirus vectors. *Expert Review of Vaccines* 2011;10:1307–19.

- [23] Liu M. Immunologic basis of vaccine vectors. *Immunity* 2010;33:504–15.
- [24] Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. *Annual Review of Immunology* 1996;15:617–48.
- [25] Tatsis N, Fitzgerald J, Reyes-Sandoval A, Harris-McCoy K, Hensley S, Zhou D, et al. Adenoviral vectors persist in vivo and maintain activated CD8⁺ T cells: implications for their use as vaccines. *Blood* 2007;110:1916–23.
- [26] Holst Peter Johannes, Thomsen Allan Randrup. Harnessing the potential of adenovirus vectored vaccines. In: Xu Ke, editor. *Viral gene therapy*. InTech; 2011. Available from (<http://www.intechopen.com/books/viral-gene-therapy/harnessing-the-potential-of-adenovirus-vectored-vaccines>), 978-953-307-539-6.
- [27] Krebs P, Scandella E, Odermat B, Ludewig B. Rapid functional exhaustion and deletion of CTL following immunization with recombinant adenovirus. *Journal of Immunology* 2005;174:4559–66.
- [28] Yang T, Millar J, Groves T, Grinshtein N, Parsons R, Takenaka S, Wan Y, Bramson J. The CD8⁺ T cell population elicited by recombinant adenovirus displays a novel partially exhausted phenotype associated with prolonged antigen presentation that nonetheless provides long-term immunity. *Journal of Immunology* 2006;176:200–10.
- [29] Holst P, Ørskov C, Thomsen A, Christensen J. Quality of the transgene-specific CD8⁺ T cell response induced by adenoviral vector immunization is critically influenced by virus dose and route of vaccination. *Journal of Immunology* 2010;184:4431–9.
- [30] Kaufman D, Bivas-Benita M, Simmons N, Miller D, Barouch D. Route of adenovirus-based HIV-1 vaccine delivery impacts the phenotype and trafficking of vaccine-elicited CD8⁺ T lymphocytes. *Journal of Virology* 2010;84:5986–96.
- [31] Zhi Y, Gao G. Muscle as a target for genetic vaccine. *Muscle gene therapy*, 14; 2010. p. 231–47, http://dx.doi.org/10.1007/978-1-4419-1207-7_14.
- [32] Lin S, Cun A, Harris-McCoy K, Ertl H. Intramuscular rather than oral administration of replication-defective adenoviral vaccine vector induces specific CD8⁺ T cell responses in the gut. *Vaccine* 2007;25:2187–93.
- [33] Guan J, Wen B, Deng Y, Zhang K, Chen H, Wu X, et al. Effect of route of delivery on heterologous protection against HCV induced by an adenovirus vector carrying HCV structural genes. *Virology Journal* 2011;8:506–14.
- [34] Ogura T, Mizukami H, Mimuro J, Madoiwa S, Okada T, Matsushita T, et al. Utility of intraperitoneal administration as a route of AAV serotype 5 vector-mediated neonatal gene transfer. *The Journal of Gene Medicine* 2006;8:990–7.
- [35] Berraondo P, Ochoa L, Crettaz J, Rotellar F, Vales A, Martínez-Ansó E, et al. IFN- α gene therapy for woodchuck hepatitis with adeno-associated virus: differences in duration of gene expression and antiviral activity using intraperitoneal or intramuscular routes. *Molecular Therapy: The journal of the American Society of Gene Therapy* 2005;12:68–76.
- [36] Estcourt M, Létourneau S, McMichael A, Hanke T. Vaccine route, dose and type of delivery vector determine patterns of primary CD8⁺ T cell responses. *European Journal of Immunology* 2005;35:2532–40.
- [37] Lehner T, Wang Y, Ping L, Bergmeier L, Mitchell E, Cranage M, et al. The effect of route of immunization on mucosal immunity and protection. *The Journal of Infectious Diseases* 1999;179(Suppl 3):S489–92.
- [38] Valdenegro-Vega V, Crosbie P, Vincent B, Cain K, Nowak B. Effect of immunization route on mucosal and systemic immune response in Atlantic salmon (*Salmo salar*). *Veterinary Immunology and Immunopathology* 2013;151:113–23.
- [39] Ahi Y, Bangari D, Mittal S. Adenoviral vector immunity: its implications and circumvention strategies. *Current Gene Therapy* 2011;11:307–20.
- [40] Richardson J, Pillet S, Bello A, Kobinger G. Airway delivery of an adenovirus-based Ebola virus vaccine bypasses existing immunity to homologous adenovirus in nonhuman primates. *Journal of Virology* 2013;87:3668–77.
- [41] Bruña-Romero O, Lasarte J, Wilkinson G, Grace K, Clarke B, Borrás-Cuesta F, et al. Induction of cytotoxic T-cell response against hepatitis C virus structural antigens using a defective recombinant adenovirus. *Hepatology* 1997;25:470–7.
- [42] Li W, Krishnadas D, Li J, Tyrrell D, Agrawal B. Induction of primary human T cell responses against hepatitis C virus-derived antigens NS3 or core by autologous dendritic cells expressing hepatitis C virus antigens: potential for vaccine and immunotherapy. *Journal of Immunology* 2006;176:6065–75.
- [43] Lang Kuhs K, Toporovski R, Ginsberg A, Shedlock D, Weiner D. Induction of intrahepatic HCV NS4B, NS5A and NS5B-specific cellular immune responses following peripheral immunization. *PLoS One* 2011;7:e52165.
- [44] Krishnadas D, Ahn J, Han J, Kumar R, Agrawal B. Immunomodulation by hepatitis C virus-derived proteins: targeting human dendritic cells by multiple mechanisms. *International Immunology* 2010;22:491–502.
- [45] Dahari H, Feinstone SM, Major ME. Meta-analysis of hepatitis C virus vaccine efficacy in chimpanzees indicates an importance for structural proteins. *Gastroenterology* 2010;139(3):965–74.
- [46] Murata K, Lechmann M, Qiao M, Gunji T, Alter H, Liang T. Immunization with hepatitis C virus-like particles protects mice from recombinant hepatitis C virus-vaccinia infection. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100:6753–8.
- [47] Li W, Krishnadas D, Kumar R, Tyrrell D, Agrawal B. Priming and stimulation of hepatitis C virus-specific CD4⁺ and CD8⁺ T cells against HCV antigens NS4, NS5a or NS5b from HCV-naïve individuals: implications for prophylactic vaccine. *International Immunology* 2007;20:89–104.
- [48] Pancholi P, Perkus M, Tricoche N, Liu Q, Prince AM. DNA immunization with hepatitis C virus (HCV) polycistronic genes or immunization by HCV DNA priming-recombinant canarypox virus boosting induces immune responses and protection from recombinant HCV-vaccinia virus infection in HLA-A2.1 transgenic mice. *Journal of Virology* 2003;77(1):382–90.
- [49] Krishnadas D, Li W, Kumar R, Tyrrell D, Agrawal B. HCV-core and NS3 antigens play disparate role in inducing regulatory or effector T cells in vivo: Implications for viral persistence or clearance. *Vaccine* 2010;28:2104–14.
- [50] Brady M, MacDonald A, Rowan A, Mills K, Hepatitis. C virus non-structural protein 4 suppresses Th1 responses by stimulating IL-10 production from monocytes. *European Journal of Immunology* 2003;33:3448–57.
- [51] Mahajan SD, Aalinkel R, Schwartz SA, Chawda RP, Nair MP. Effector cell mediated cytotoxicity measured by intracellular Granzyme B release in HIV infected subjects. *Biological Procedures Online* 2003;5:182–8.
- [52] Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology* 1989;7:145–73.
- [53] Chen M, Sällberg M, Sönnnerborg A, Weiland O, Mattsson L, Jin L, Birkett A, Peterson D, Milich DR. Limited humoral immunity in hepatitis C virus infection. *Gastroenterology* 1999;116(1):135–43.
- [54] Hempel G, Galle PR, Löhr HF. Quantitative analysis of specific Th1/Th2 helper cell responses and IgG subtype antibodies in interferon- α -treated patients with chronic hepatitis C. *Journal of Medical Virology* 2001;64(3):340–9.
- [55] Kawano Y, Noma T, Yata J. Regulation of human IgG subclass production by cytokines IFN- γ and IL-6 act antagonistically in the induction of human IgG1 but additively in the induction of IgG2. *Journal of Immunology* 1994;153(11):4948–58.
- [56] Hazenbos WL, Heijnen IA, Meyer D, Hofhuis FM, Renardel de Lavalette CR, Schmidt RE, Capel PJ, van de Winkel JG, Gessner JE, van den Berg TK, Verbeek JS. Murine IgG1 complexes trigger immune effector functions predominantly via Fc gamma RIII (CD16). *Journal of Immunology* 1998;161(6):3026–32.
- [57] Tatsis N, Fitzgerald JC, Reyes-Sandoval A, Harris-McCoy KC, Hensley SE, Zhou D, Lin SW, Bian A, Xiang ZQ, Iparraguirre A, Lopez-Camacho C, Wherry EJ, Ertl HC. Adenoviral vectors persist in vivo and maintain activated CD8⁺ T cells: implications for their use as vaccines. *Blood* 2007;110(6):1916–23.