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Original Article

Comparison of the inhibitory and stimulatory effects of Core and NS3 candidate HCV vaccines on the cellular immune response

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Abstract: Currently, hepatitis C virus (HCV) infects nearly 3% of the global population, the majority of whom are chronically infected; however, hepatitis C vaccines are still in the developmental stage. Numerous studies suggest that the spontaneous resolution of HCV infection and the design of its vaccine are reliant on vital contributions from CTL cell responses and T regulatory cells. Multiple researchers have identified both Core and nonstructural protein 3 (NS3) proteins as crucial immune genes and potential candidates for HCV DNA vaccine design. In this study, Core and NS3 were subcloned and inserted into pcDNA3.1 to construct HCV DNA vaccines administered in mouse models. Furthermore, the effects of Core and NS3 on the induction of CTL and NK were compared in spleen mouse models using the LDH method. Additionally, flow cytometry was employed to investigate the percentage of T regulatory cells (Treg cells) and cells expressing PD-1 in the spleens of the mouse models. Our data indicated that pcDNA3.1+NS3 and pcDNA3.1+Core could enhance CTL and NK activity in mouse models. Importantly, the Treg and PD-1 analysis in mouse models revealed a substantial reduction in the proportions of CD4+/CD25+/Foxp3+ T cells and PD-1+ cells in experimental subjects treated with HCV NS3 along with 5 mg/kg of lenalidomide, utilized as a novel adjuvant, compared to those administered an equivalent dosage of lenalidomide in conjunction with HCV Core. In conclusion, our observations indicated that the NS3-HCV gene had a limited impact on the activation of inhibitory factors. Therefore, NS3 is considered a more suitable candidate for DNA vaccine design compared to Core HCV.

Keywords: HCV, Core, NS3, vaccine, CTL cell, T regulatory cells

Introduction

HCV is among the main factors in acute and chronic liver diseases such as hepatocellular carcinoma and cirrhosis [1-3]. According to studies conducted by the WHO, 170 million people currently suffer from HCV, and 3 to 4 million, mostly in developing countries, are newly affected by this disease each year globally. Despite significant efforts in producing an HCV vaccine, the development of such a vaccine has encountered several obstacles, namely a lack of animal models, genetic heterogeneity, and several effective immune escape strategies. Although neutralizing antibodies against HCV can be identified within 7-8 weeks post-infection, they cannot successfully protect the infected person against reinfection. However, cellular immunity is capable of clearing HCV infection, implying its importance in spontaneous resolution of acute HCV and long-term protection from persistent infection [4-6]. Furthermore, regulatory T cells have a pathologic role in viral infection, especially in chronic infections and vaccine design in humans. It has been shown that the number of circulating CD4+/CD25+/Foxp3 cells in HCV carriers is larger than that of healthy persons. Treg cells also inhibit CTL cells and IFN-γ secretion, which results in delayed virus clearance and persistent infection. In most cases, CD8 cytotoxic T-lymphoid (CTL) responses were associated with the control of infectious agents, especially against viral infection or tumor Ag-derived pep-

tide epitopes [7, 8]. Indeed, exhaustion of T cells and increased PD-1+ cells significantly contributed to the spread of chronic disease [9].

It has been reported that T-cell exhaustion leads to dysfunction and depletion of T cells in viral infections in mouse models [10]. PD-1 is also an inhibitory receptor that plays a vital role in limiting the effective performance of T cells for viral infection and cancer. Several studies have illustrated that reduction of PD-1 cells is a strategic therapy for controlling tumor immunity in humans [9, 11]. On the other hand, natural killer (NK) cells are crucial in the host's innate immunity in viral infection, as NK cells normally constitute 20 to 30 percent of the liver lymphocytes [12]. As a result, the design of the HCV vaccine that is capable of activating NK and CTL cells, as well as reducing Treg and PD1 cells, is of utmost importance. The NS3 and the Core of HCV are considered highly conserved among HCV isolates, which have been applied to the design of various vaccines against the virus. Additionally, these proteins can be attributed to stimulating HCV-specific CTL populations [13, 14]. Previous studies have shown that HCV Core and NS3 can stimulate the CD4+ and CD8+ T cells and secrete IFN-γ in patients with self-limited infection [15].

NS3 acts as a multifactorial protein in the HCV life cycle and exhibits serine protease activity within the N-terminus and RNA helicase activity within the C-terminus. Indeed, NS3 cleaves HCV polyproteins and contains the highest immune dominant epitopes for stimulation of T helper CD4+ and cytotoxic CD8+ T cells [16, 17]. The correlation of NS3-specific T-cell responses with the resolution of acute infection has been approved in previous studies. In addition, NS3 consists of low genetic variables and relatively large-size epitopes, which makes it a suitable target for T-cell stimulating vaccines [18]. HCV Core protein is widely used in the design of HCV DNA vaccines due to containing conserved regions in various HCV genotypes. However, it has been reported that the HCV Core protein induces the expansion of natural CD4+CD25+ Tregs and can promote pro-inflammatory and anti-inflammatory cytokines. Nonetheless, the opposite role in the secretion of anti-viral cytokines such as IFNs has been observed for Core protein [19, 20].

It is well recognized that DNA vaccines are a highly applicable technology as they are safe and affordable stimulators of cellular immunity. Nevertheless, the low efficiency of DNA vaccines, especially in immune-deficient groups, is a major hurdle that prevents their sustainable development to infections [21]. Recently, adjuvants have been utilized to boost DNA vaccine efficiency; hence, the selection of suitable adjuvants is a leap forward for designing the HCV DNA vaccine. This paper compares the effects of Core and NS3 on the induction of CTL, NK, PD-1, and Treg cells through HCV DNA-based vaccine in mice models. Additionally, the impacts of the Core and NS3 HCV DNA vaccines on immune response were investigated to determine candidate recombinant DNA vaccines for HCV. Furthermore, we used lenalidomide, a novel FDA-approved immunomodulatory agent that recently proved to impact Treg reduction and act as an adjuvant for immune enhancement, along with NS3HCV DNA and Core HCV DNA.

Materials and methods

Ethics statement

All animal studies used in this study were approved by the Institutional Ethics Committee and Research Advisory Committee of Tarbiat Modares University based on the National Specific Ethical Guidelines for Biomedical Research issued by the Ministry of Health and Medicinal Education (MOHME) of Iran.

Plasmid construction

The whole Core-1a protein coding region (kindly provided by Dr. Hosseini, Shiraz University of Medical Sciences, Shiraz, Iran) was subcloned and inserted into pcDNA3.1. Briefly, the plasmid covering the complete Core-1a sequences was used as the template for amplification of Core-1a sequences using design-specific primers covering the restriction enzyme site for *BamHI* and *EcoRI* (Forward: 5-AAAAGGATCCATGAGCACGAATCCTAAACC-3, Reverse: 5-TTTTGAATTCTCATCAATTGCGCACTTGGTAGGCTGA-3). After agarose gel electrophoresis, the PCR product was purified by the gel extraction method (QIAquick Gel Extraction Kit, Qiagen). The

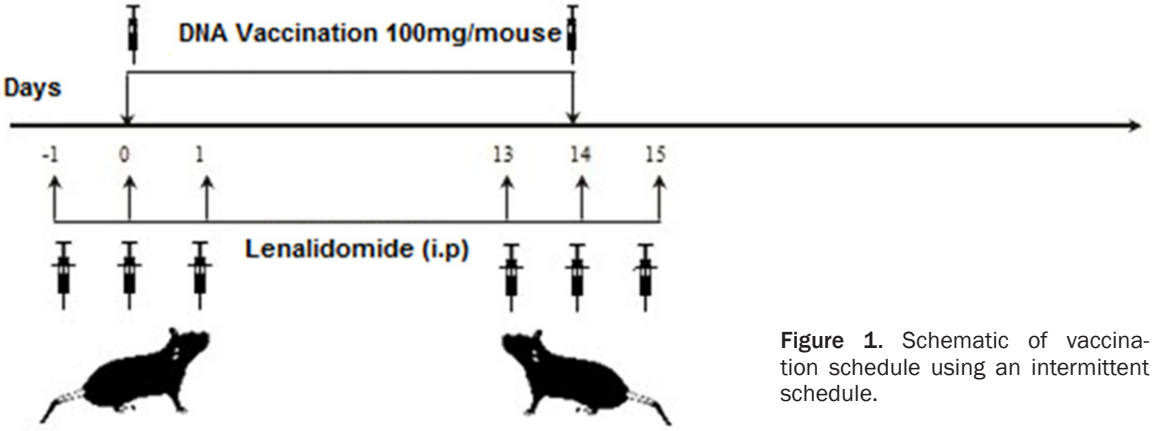

Figure 1. Schematic of vaccination schedule using an intermittent schedule.

purified PCR product and pcDNA3.1 expression plasmid were digested sequentially with *BamHI* and *EcoRI* to produce sticky ends. The digested PCR product was ligated into pcDNA3.1, followed by verification and purification with an Endo Free Plasmid Giga Kit (Qiagen).

NS3, amino acids 1095 to 1384, which encode immunogenic and conserved epitopes, were subcloned to pcDNA3.1, as described in a previous study [22]. The constructed plasmids were purified with an Endo Free Plasmid Giga Kit (Qiagen).

Animals and drug

Inbred Female 6-8-week-old Balb/c mice were purchased from the Pasteur Institute of Iran (Karaj, Iran). Mice were used for individual experiments; all the mice were maintained for one week before the experiment in a pathogen-free mouse facility according to Tarbiat Modares University guidelines. There were no unexpected deaths; they were given free access to food and water and maintained in a light/dark cycle with lights on from 6:00 to 18:00. For in-vivo studies, lenalidomide (NATCO, India) was dissolved at a concentration of 5 mg/ml in 0.5% DMSO in PBS and stored at 4°C for the duration of the experiment.

Cell cultures

The NK cell cytotoxicity-sensitive cell line K562 (human erythroleukemia) was obtained from the Pastor Institute (Tehran, Iran). The K562 cells were cultured in RPMI 1640 without phenol red, 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin (all from Gibco, USA) at 37°C in an incubator containing 5% CO_2 and humidified air.

The inbred mouse myeloma cell line SP2/0, a CTL-sensitive cell line, was propagated in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (all from Gibco, USA) and 100 U/mL penicillin and maintained in a 5% CO_2 atmosphere at 37°C. The cells were cultured in 96-well round-bottom plates (Nunc, Denmark) and used as a target cell for the cytotoxicity assay.

Vaccination

The DNA plasmid, pcDNA3.1+Core, and pcDNA3.1+NS3 were given intramuscularly (IM) at 100 mg/mouse on days 0 and 14, as shown in **Figure 1**. The control mice received 100 mg/kg pcDNA3.1+ twice on days 0 and 14, respectively. It is worth noting that other control mice received PBS alone. In this schedule, a low dose of lenalidomide (five mg/kg), which was shown to be the optimal dose in our earlier studies, was applied [22]. Two weeks after the second immunization, the mice were anesthetized and sacrificed. Subsequently, the spleen cells were collected to study immune assays. The experimental schedule is shown in **Table 1**.

CTL cell assay

The immunized mice were sacrificed, and their spleens were isolated one week after the second immunization. In the experimental protocol, single-cell suspensions were prepared from individual mouse splenocytes and subsequently employed as the effector cells. These

Table 1. Groups of immunized mice

Group	Vaccine formulation
Rec NS3	pcDNA3.1+NS3 (100 mg/mouse)
Rec Core	pcDNA3.1+Core (100 mg/mouse)
Vector	pcDNA3.1 (100 mg/mouse)
PBS	PBS
Rec NS3+Lena	pcDNA3.1+NS3 (100 mg/mouse) and low dose of lenalidomide (5 mg/kg)
Rec Core+Lena	pcDNA3.1+Core (100 mg/mouse) and low dose of lenalidomide (5 mg/kg)
Lena	lenalidomide (5 mg/kg)

effector cells were cultivated in RPMI 1640 medium. As target cells, SP2/0-derived cells were utilized, which had been genetically modified to express HCV NS3 stably [23]. Cells were pulsed with 10 μg/ml purified HCV Core protein 72 hrs before coculture with the spleen cells. The effector-to-target cell (E/T) ratios were maintained at 50:1 during the experiments. This setup was designed to facilitate the investigation of cytotoxic immune responses associated with the interaction between the effector and target cells in the context of HCV infection. Lysis of the target cells was evaluated by measurement of lactate dehydrogenase (LDH) release using an LDH cytotoxicity assay kit according to the procedures stated by the manufacturer (LDH Cytotoxicity Detection Kit, Roche, Germany). All the results expressed in this study were repeated at least three times. A plate reader measured the product at 490 nm after 8 hours of incubation. The following formula was used to determine the percentage of specific cytolysis: Cytotoxicity = [(experimental value-effector spontaneous) - Low control High Control-Low control] ×100.

NK assay

One week after the last immunization, mouse models were sacrificed, and their spleens were isolated. To evaluate NK cell lytic activity, the cytotoxicity was measured by the LDH activity of cellular culture supernatants against the K562 cell line as a highly sensitive target for NK cells. For this purpose, the mice spleen, as effector cells, and the K562 cells, as target cells, were combined with the 50:1 dilution ratio. The assay was performed in 96 round bottom microwell plates (Sigma), which were incubated at 37°C in a humidified atmosphere containing 5% CO_2. All experimental procedures and assays were performed three or more times, with similar results. The percentage of cytotoxic activity was determined using the same formula as described.

Flow cytometry for Treg assay

The mouse models were killed seven days after the last immunization. Based on the manufacturer's recommended procedure, single cell suspension of splenocytes prepared from each inbred mouse after isolation of the spleens, 1×10^6 mononuclear cells were harvested and stained with eBioscience Mouse Regulatory T-cell staining kit #2, containing anti-mouse CD4-FITC, anti-mouse CD25-PE, and anti-mouse Foxp3-APC (FJK-16s; eBioscience, USA).

Flow cytometry for PD-1 assay

One week after the final vaccination, the mice were killed to collect the spleen. For PD-1, after isolating the spleens for all study groups, we harvested 5×10^5 mononuclear cells and stained them with a mouse PD-1 staining kit (Biolegend, USA) consisting of anti-mouse PD-1-PE and isotype control, according to the manufacturer's instructions.

Statistical analysis

The analyses were performed using GraphPad Prism software Version 6 (GraphPad, La Jolla, CA, USA), and one-way analysis of variance (ANOVA) was employed for statistical comparison. All experimental data are shown as the mean ± SE in the text, as well as in the figures. Data are presented as the means ± standard deviations (SD). Differences were considered statistically significant when $P < 0.05$.

Results

CTL cytotoxic response in vaccinated mice

The results of the LDH test conducted on different groups in **Figure 2** show that both HCV Core

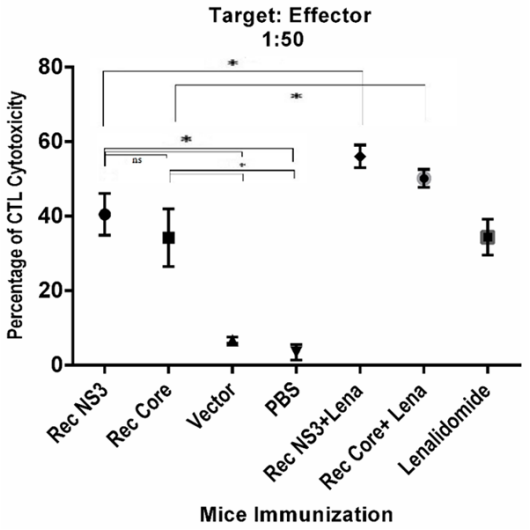

Figure 2. Effect of HCV DNA vaccines on the mouse CTL response by LDH assay. The data are presented as the means ± SDs. *P < 0.0001.

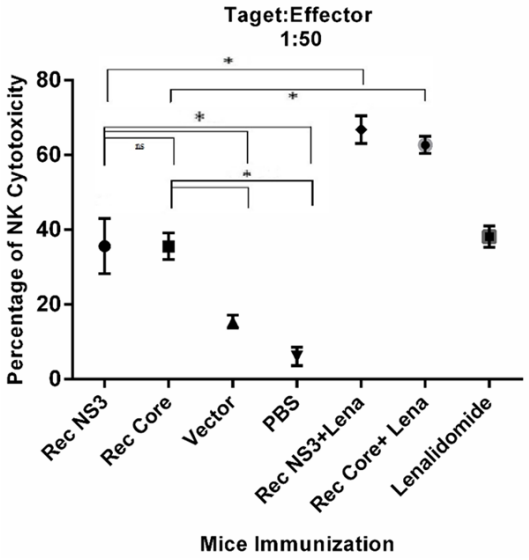

Figure 3. Effect of HCV DNA vaccines on mouse NK cells by LDH assay. The data are presented as the means ± SDs. *P < 0.0001.

and NS3 can induce a CTL response. A nonsignificant difference in the parameters mentioned above was noticed between DNA vaccine-treated samples in the Rec NS3 and Rec Core groups; however, the response was higher in the NS3-stimulated CTLs, along with lenalidomide treatment as an adjuvant candidate. The highest CTL cytotoxic activity equaled 56.05%, obtained in the case of the Rec NS3+Lena group, which received pcDNA3.1+NS3 and intermittent administration of 5 mg/kg of lenalidomide. In comparison, the PBS group, as a control group, showed a CTL cytotoxic activity as low as 3.46% ± 0.80%. It was also found that the CTL cytotoxic activity of the Rec NS3+Lena group was 7.22% higher than those of the group treated with pcDNA3.1+Core and 5 mg/kg lenalidomide in the Rec Core+Lena group. Moreover, for the Lena group (drug-treated samples) and the Rec NS3 Rec Core (vaccine-treated samples), the values of CTL cytotoxic activities were greatly lower than that of the Rec NS3 and Rec Core groups (DNA vaccine drug-treated samples). Successful results were obtained when compared to the control group, which consisted of SP2 cells that were not stimulated with Core or NS3 expression. This confirmed the expression of both proteins on the surface of SP2 cells. Furthermore, the absence of a cytotoxic response from splenocytes toward unstimulated SP2 cells confirmed that the target cells were derived from the same strain of mice.

NK cytotoxic response in vaccinated mice

In the mouse model, as depicted in **Figure 3**, the NK cell cytotoxic activity in the case of group Rec NS3 was reasonably close to that of the group Rec Core. Both HCV Core and NS3 can induce a greater NK cell response in comparison to the control groups (PBS and Vector groups). **Figure 4** also shows that the NK cell cytotoxic activity of group B, which was treated with lenalidomide alone, was 2.54% higher than the corresponding values of the Rec NS3 and Rec Core groups, which were treated with pcDNA3.1+NS3 alone or pcDNA3.1+Core. It was also observed that higher NK cell cytotoxic activities were observed in the Rec NS3+Lena group and Rec Core+Lena, respectively.

Analysis of Treg response in vaccinated mice

As shown in **Figure 4A** and **4B**, the percentage of CD4+/CD25+/Foxp3+ cells was analyzed using flow cytometry in the spleen. This cell population was higher in the Rec NS3 and Rec Core groups than those in the control groups (PBS and Vector). A noticeably lower percentage of CD4+/CD25+/Foxp3+ cells was observed in the Rec NS3+Lena group, which was treated with DNA vaccine and intermittent administration of 5 mg/kg lenalidomide as an adjuvant candidate. Moreover, the percentage of CD4+/CD25+/Foxp3+ in the Rec Core+Lena

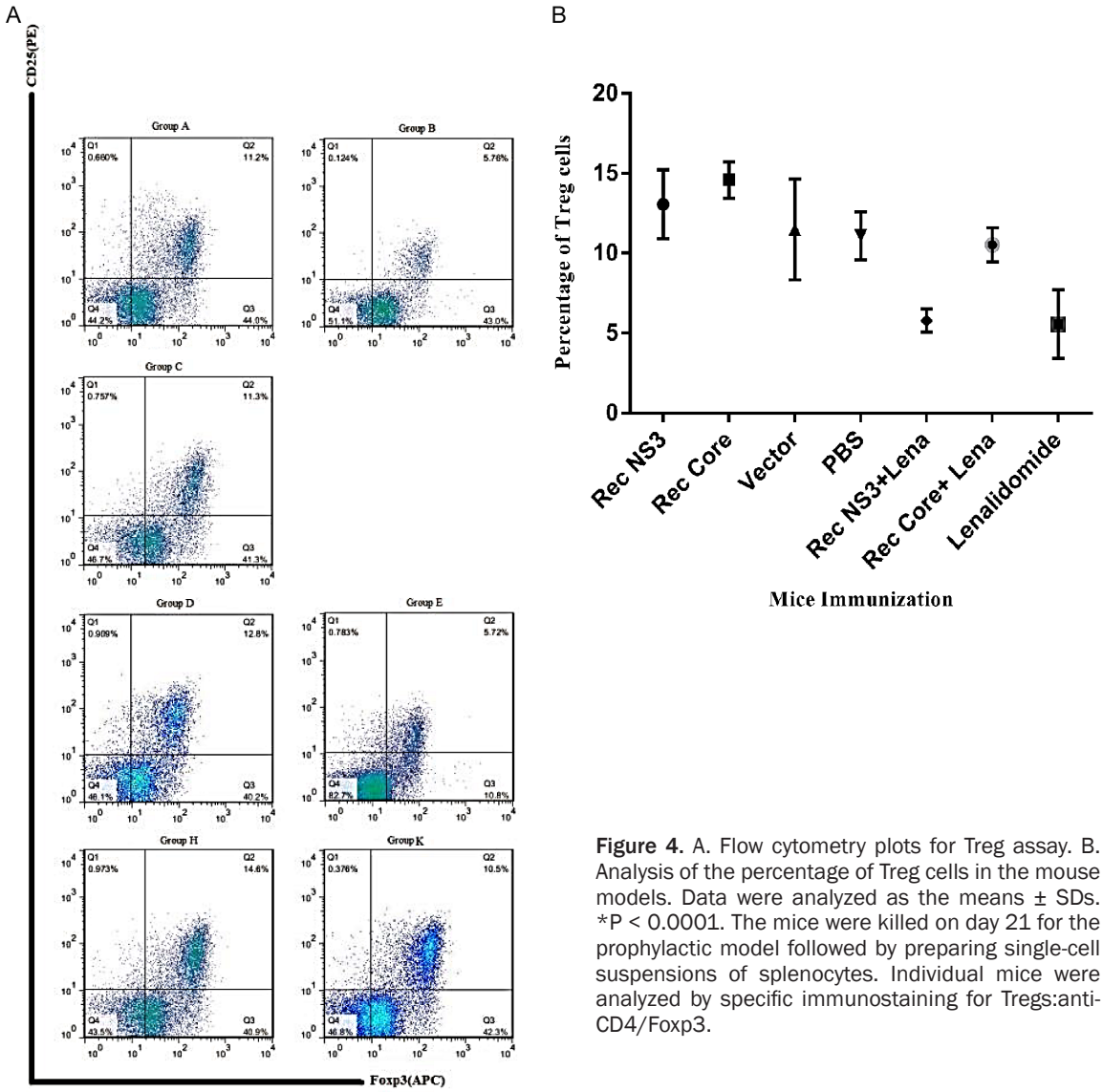

Figure 4. A. Flow cytometry plots for Treg assay. B. Analysis of the percentage of Treg cells in the mouse models. Data were analyzed as the means ± SDs. *P < 0.0001. The mice were killed on day 21 for the prophylactic model followed by preparing single-cell suspensions of splenocytes. Individual mice were analyzed by specific immunostaining for Tregs:anti-CD4/Foxp3.

group, which received 5 mg/kg lenalidomide, along with pcDNA3.1+Core, decreased in comparison to the Lena group. In mouse models, the percentage of CD25+/Foxp3+/CD4+ cells in the Lena group, treated with 5 mg/kg lenalidomide alone, was determined to be 6.15 ± 0.55. However, the corresponding value in the Rec Ns3 group treated with pcDNA3.1+NS3 alone or in the Rec Core group treated with pcDNA3.1+Core was found to be 12.08 ± 1.58 and 15.06 ± 1.39.

Analysis of PD-1 expression in vaccinated mice

As shown in **Figure 5A** and **5B**, the number of cells with PD-1+ expression in the Rec NS3 group, which received pcDNA3.1+NS3 injection alone, was 50.01 ± 2.25%, while it was 56.89 ± 1.25% in the Rec Core group, which received pcDNA3.1+Core alone. Furthermore, the corresponding values were 40.27% ± 3.80% in the PBS and 40.90% ± 3.11% in the Vector group as the control group. A minimum number of PD-1-positive cells were detected in the DNA vaccine, and intermittent administration of low-dose lenalidomide (group Rec NS3+Lena), which was 26.93% ± 1.12%. In addition, we observed 21.90% and 27.78% augmentation of the cells with PD-1+ expression in the Rec NS3 and Rec Core (single vaccine-treated samples) compared with the Lena group (single-drug-treated samples). A significant difference in the parameters mentioned above was noticed between the Rec NS3+Lena group and the Rec Core+Lena group.

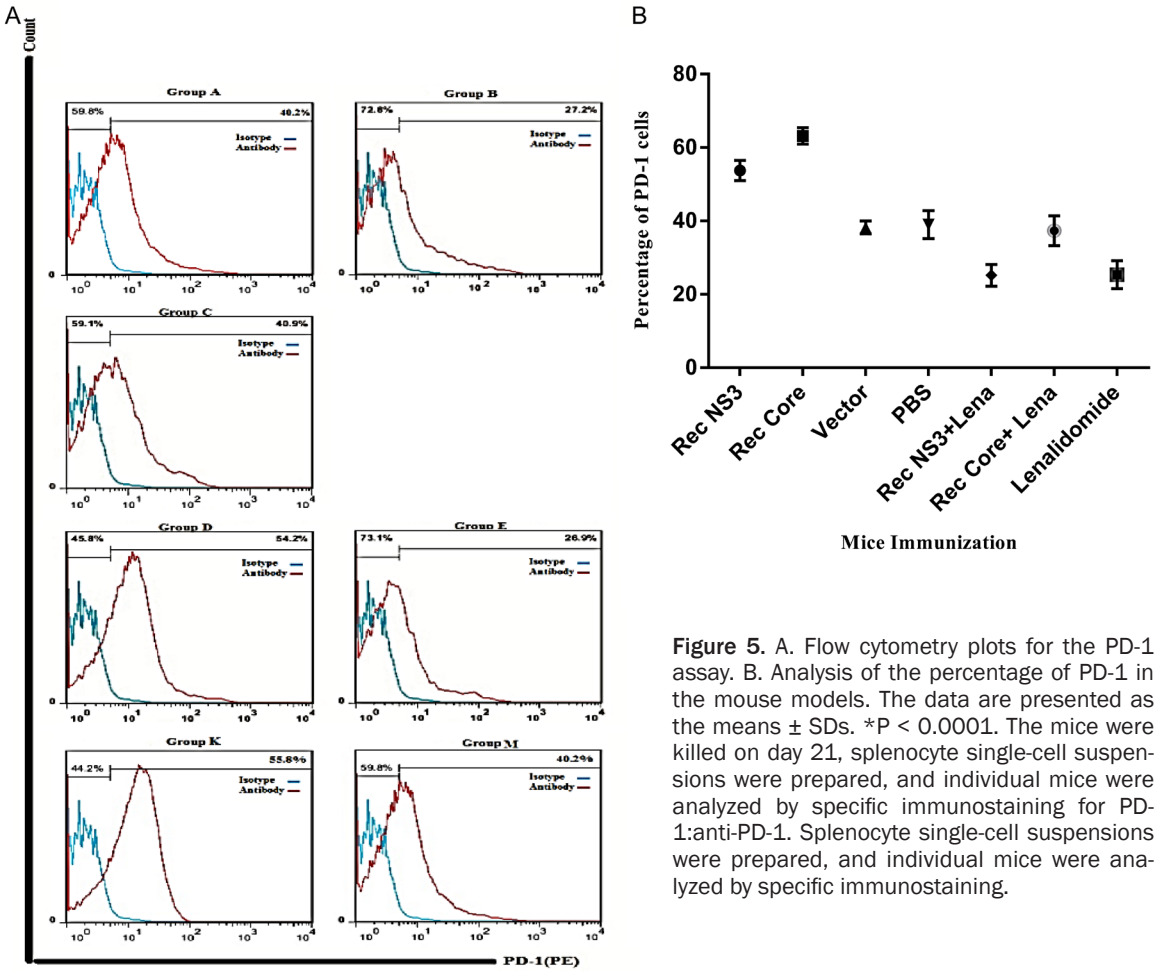

Figure 5. A. Flow cytometry plots for the PD-1 assay. B. Analysis of the percentage of PD-1 in the mouse models. The data are presented as the means ± SDs. *P < 0.0001. The mice were killed on day 21, splenocyte single-cell suspensions were prepared, and individual mice were analyzed by specific immunostaining for PD-1:anti-PD-1. Splenocyte single-cell suspensions were prepared, and individual mice were analyzed by specific immunostaining.

Discussion

Infectious diseases are still a major human health problem in all societies that pose great catastrophes in human history; hence, the need for vaccines is an essential request to control them [24-26]. It has been reported that plasmid DNA encoding antigenic HCV protein(s) can trigger long-lasting cell-mediated immune responses against pathogenic agents such as HBV, HIV, tuberculosis, and malaria [27].

According to the literature, cytotoxic T lymphocyte (CTL) response against NS3 HCV protein has been associated with viral clearance after acute infection. In contrast, the absence of this T-cell response leads to viral persistence. Recent studies have confirmed that in-vitro induction of HCV-NS3 and HCV-Core Ags stimulates various cytokines and lead to the reactivation of human T cell responses in healthy individuals [28]. In this research, we showed that recombinant DNA encoding HCV NS3 and HCV Core protein are highly effective in specific CTL induction in the spleen of Balb/c mice models; hence, they can be considered candidate DNA vaccines for improving cellular immunity. To investigate which of the Core and NS3 proteins are more effective in CTL and Treg induction and can inhibit PD-1 expression better, we vaccinated the mice with NS3 and Core vaccine constructs. Arribillagal et al. [29] showed that vaccination of mice with Rad NS3 could protect 66% of the mice challenged with Vhcv-3011. In this research, we showed that pcDNA3.1+NS3 provided lower stimulation of CD4+/CD25+/Foxp3+ Tregs compared to pcDNA3.1+Core alone. Shuo et al. [30] reported that Treg epitopes could decrease the chance of successful vaccines development. Another study showed that injection of plasmid HPV DNA vaccine and subsequent depletion of Tregs could promote the antigen-specific CD8+ T-cell immune response [31]. Furthermore, Casares et al. [32] used a recombinant replicative vaccinia vHCV1-3011 to downregu-

late the Treg activity in vivo and improve immunogenicity of the vaccine.

Based on the literature above, NK cells provide an early defense against pathogens, especially in viral infection. Moreover, these cells can rapidly attack the sites of virus entry and play a critical role in the restriction of acute viral infections [33]. In this research, the results obtained from animal models showed that NS3 and Core HCV vaccine candidates were able to boost the NK cell cytotoxicity. Leblanc et al. showed that lenalidomide was able to stimulate the NK cell activity by increasing the Thelper1-type cytokine response, such as IFN-γ and IL-2 [34, 35]. Another study indicated that lenalidomide, as an immunomodulatory drug, promoted the NK cell activity in vitro [36]. Our experiments illustrated that NK cell activity could notably increase after immunization with Core HCV DNA or NS3 HCV DNA vaccine plus 5 mg/kg lenalidomide in mouse models.

Furthermore, it has been reported that the upregulated expression of PD-1 was part of the major factor leading to T-cell exhaustion, especially in LCMV mouse models and chronic infections such as HBV, HIV, and HCV [37]. Researchers have shown that additional PD-L1 blockade, along with HBV vaccination, yielded a higher efficiency of the vaccine through increasing T-cell functional capacities [38, 39]. The results of this study showed that treatment with HCV Core could remarkably increase the expression of PD-1 in comparison to HCV NS3 treatment in the spleen of mouse models. However, we demonstrated that 5 mg/kg of lenalidomide, along with the HCV NS3 vaccine, could greatly decrease PD-1+ expression in comparison to the HCV Core vaccine in mouse models. In addition, researchers have shifted their focus to the application of adjuvants to improve the efficiency of the HCV vaccine and enhance the cellular response. In this research, we used 5 mg/kg lenalidomide along with pcDNA3.1(+)/NS3 and pcDNA3.1(+)/Core and observed that lenalidomide dramatically increased the CTL activity response in mouse models. Knobloch et al. [40] showed that lenalidomide stimulated different parts of the immune system in tumor mouse models by improving anti-inflammatory cytokines such as IL-6, IL-1, and IL-10 in human PBMC samples. In another study, it was reported that CTL responses increased in mice models that received HCV-NS3 along with lenalidomide, suggesting that lenalidomide can promote the CTL activity in response to the vaccine candidate [22].

Previous studies suggest that lenalidomide can decrease the Treg cells and enhance T helper 1 by producing cytokines in myeloma mouse models [22]. Several publications have shown that the percentage of CD4+/CD25+/Foxp3+ T cells decreased in T cells obtained from healthy donors by stimulation of anti-CD3/CD28 in the presence of lenalidomide [41]. We found that treatment with 5 mg/kg lenalidomide along with HCV NS3 or HCV Core vaccine candidates induced an inhibitory effect on the CD4+/CD25+/Foxp3+ T cells more efficiently than HCV vaccines alone. Moreover, the analysis of flow cytometry data on the Treg cells in the spleen of the mice revealed that the percentages of CD4+/CD25+/Foxp3+ T cells were significantly different from those of HCV Core and HCV NS3 plus injection of 5 mg/kg lenalidomide. Interestingly, examination of the CD4+/CD25+/Foxp3+ T cells in mouse models showed significantly lower percentages in cases that received HCV NS3 plus 5 mg/kg lenalidomide compared with the cases that received HCV Core plus 5 mg/kg lenalidomide.

Conclusion

Our data suggest that the NS3 sequence of the HCV genome is an attractive candidate for DNA vaccine design. At the same time, it has a limited impact on the activation of inhibitory factors in comparison to Core HCV. This study concluded that adjuvants were paramount components in vaccines in terms of enhancing and directing immunity to vaccine antigens. Overall, lenalidomide, along with the NS3 HCV DNA vaccine, might serve as an effective adjuvant candidate to improve the efficiency of vaccine, in comparison with Core antigen. Finally, the adjuvant lenalidomide decreased the activated Tregs more effectively in the NS3-vaccinated mice.

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Disclosure of conflict of interest

None.

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