Cytokine 46 (2009) 43–50

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Effect of immunological adjuvants: GM-CSF (granulocyte-monocyte colony stimulating factor) and IL-23 (interleukin-23) on immune responses generated against hepatitis C virus core DNA vaccine

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ARTICLE INFO

Article history:
Received 19 July 2008
Received in revised form 3 December 2008
Accepted 13 December 2008

Keywords:
Cytokine adjuvants
GM-CSF
IL-23
HCV
DNA vaccine

ABSTRACT

The use of cytokines as adjuvants has been shown to be a promising approach for enhancing DNA vaccine induced-immune responses. In this report, we investigate the administration of cytokines to modulate both humoral and cell-mediated immune responses elicited by an HCV-core plasmid DNA vaccine in Balb/c mice. Our studies indicate that the HCV-core DNA vaccine has been able to induce both antibody and cellular immunity in a DNA prime-protein boost regimen. GM-CSF (granulocyte-monocyte colony stimulating factor) which is considered to be a cytokine displaying both Th1 and Th2 characteristics, and plays an important role in augmenting antibody and cell-mediated immunity was also administered. The induction of cellular immunity was not as striking as humoral immunity in this case. To obtain a stronger cellular response, IL-23, a Th1 cytokine belonging to the IL-12 family, was also included in the regimen. Spleen cell proliferation, IFN-γ production from spleen cells and specific serum IgG2a, all demonstrate the enhancement of cell-mediated immunity without any observable suppressive effect on antibody and humoral immune responses. We also examined the timing of plasmid IL-23 administration on the phenotype of the resultant T cell responses in a 3 day interval, before and after plasmid GM-CSF administration. The results did not indicate any change in theTh1/Th2 balance as compared with simultaneous IL-23 administration.

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1. Introduction

DNA vaccines have the potential to be a safer and more effective alternative to current vaccine modalities. However, several problems must be addressed before there is a licensed DNA vaccine [1]. Increasing the immunogenicity of DNA vaccines is the most important challenge facing the field. While DNA vaccines have proved effective at stimulating immune responses in small animals, as the size of the animal increases the efficacy of the vaccine decreases [2]. Cytokines are powerful mediators of innate and adaptive immunity and attractive candidate vaccine adjuvants. Beneficial effects of cytokines as adjuvants include stimulation of T cell-mediated immunity at the level of Ag presentation and T-cell proliferation. Therefore, cytokine adjuvants have been co-delivered with DNA vaccines as a means of increasing their immunogenicity [3].

The HCV-core protein is highly conserved among the various HCV genotypes [4]. Several B cell and T cell epitopes have been characterized within this antigen [5–9]. Because of its conserved nature across viral genotypes, the core protein is a promising candidate antigen for vaccine development, as it can induce a broad immune response. Plasmid encoding core protein is known to induce strong cell-mediated immunity but a weaker humoral response [10], thus the immunogenicity of this antigen should be enhanced using adjuvants.

Granulocyte-monocyte colony stimulating factor (GM-CSF) is secreted by a variety of cells, it is able to recruit, activate and enhance the function of professional antigen presenting cells, which makes it useful as an adjuvant to vaccines. A number of studies,

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doi:10.1016/j.cyto.2008.12.007

in different animal models, have clearly shown that plasmid expressing GM-CSF can augment the immune responses generated against DNA vaccines [11–16]. Importantly, it has been shown that GM-CSF is neither a Th1 nor a Th2 cytokine, and can exert its adjuvant effect without skewing the Th1/Th2 balance, facilitating the generation of both antibody and cell-mediated immune responses [12,13]. Nevertheless, Chiang et al. demonstrated that in co-delivery of GM-CSF gene with HCV DNA vaccine, enhancement of cell-mediated immunity was not as striking as humoral immunity [12]. Such results were obtained by Okuda et al. where co-injection of HIV-1 specific DNA vaccine and GM-CSF gene promoted low levels of cell-mediated immunity despite higher induced levels of specific antibody [13].

Interleukin-23 (IL-23) is a heterodimeric cytokine composed of a p40 subunit, shared with IL-12, and a unique p19 subunit. It is secreted by activated dendritic cells (DC) and enhances in vitro proliferation and IFN-γ production. Although there are similarities between IL-12 and IL-23, this novel cytokine shows unique characteristics different from IL-12 [17]. A number of studies showed that IL-12, along with enhancement of cell-mediated immunity, suppresses antibody production and downplays the humoral immune response [18,19]. While IL-23 as a vaccine adjuvant, has displayed a Th1-like immune response without any suppression in the Th2 paradigm [20,21].

To compensate the shortcomings of GM-CSF in enhancing the cellular immune response, we contemplated employing IL-23 with GM-CSF in a HCV-core DNA prime-recombinant protein boost vaccination regimen to augment both humoral and cell-mediated immunity simultaneously.

Prime boost immunization protocols consisting of DNA priming and protein boosting in several infectious disease models have shown robust induction of immune responses, favoring the humoral responses [22–25].

Shih et al. indicated that administration of DNA encoding HCV core did not raise specific IgG anti-core antibody even after plasmid booster and only IgM anti-core levels rose after DNA vaccine prime and boost. However, after boosting with HCV-core protein, the IgG antibody levels increased immediately in HCV-core DNA vaccine primed mice [25]. Considering this criteria, in this study, HCV core DNA-prime-recombinant core protein-boost approach was employed to achieve rapid rise in specific IgG titer in immunized mice.

2. Materials and methods

2.1. Constructs for DNA immunization

2.1.1. Preparation of HCV-core DNA vaccine

2.1.1.1. RNA extraction and cDNA synthesis. Serum sample was prepared from a chronic hepatitis C patient. RNA was extracted from positive anti-HCV serum by RNA extraction solution (RNX-Plus, Cinagene Company, Iran). Serum sample (50 μl) was added to 450 μl cold RNX-Plus solution and mixed to dissolve the clumps followed by 10 min incubation on ice. Chloroform/isoamylalcohol (100 μl) (24:1) was then added, the samples were vigorously mixed and centrifuged at 12,000*g* for 5 min. Equal volume of isopropanol was added to aqueous phase, incubated at −20 °C for 20 min and centrifuged at 12,000g for 5 min. The pellet was washed with 75% ethanol and dried for 20 min at room temperature, and then it was dissolved in 30 μl of DEPC-treated water.

cDNA was synthesized from core region of the HCV genome by using gene specific primers of 1b subtype. Forward primer (CTC**GAATTC**GGGAGGTCTCGTAGA) with EcoRI restriction and reverse primer (TCTC**GGATCC**TTAGCTAACAGC) with BamHI site were designed and synthesized. Complete cDNA was synthesized using 5′ RACE System kit (Gibco-BRL, Bethesda, MD), according to manufacturers' protocol. cDNA was amplified by polymerase chain reaction with specific primers. PCR product to be cloned was first excised from the gel with a sterile scalpel and then purified following the QIAquick Gel Extraction kit protocol (Qiagen, Germany). It was cloned into the EcoRI, BamHI sites of pUC18 using conventional procedures. The recombinant clones were selected and confirmed by PCR and restriction enzyme analysis. Sequencing reactions were carried out using the ABI Prism DNA Sequencing kit (Applied Biosystem, UK). Sequencing was performed using an ABI 377 from both directions of gene (Applied Biosystem, UK).

2.1.1.2. Construction of HCV-core DNA vaccine. The core gene of HCV was subcloned into the EcoRI/XbaI sites of **pcDNA3** eukaryotic expression vector (Invitrogen). The correctness of pcDNA3-core (summarized here as **pCore**) construct was verified by sequencing and enzyme digestion analysis and expression of core protein was detected by Western blot. HEK 293T cells were transected by **pCore** or mock vector as negative control by using calcium-phosphate precipitation method. Cell lysate was used to detect core protein utilizing anti-HCV core monoclonal antibody (Biogenesis, Germany) followed by addition of a HRP-conjugated anti-mouse antibody (DAKO, Denmark).

2.1.2. Cytokine constructs

Plasmid expressing murine GM-CSF (summarized here as **pGM-CSF**) and plasmid expressing murine single chain IL-23 (summarized here as **pIL-23**) were kindly provided by Dr. Young-Chul Sung and Dr. Masanori Matsui, respectively. All vectors were used to transform *Escherichia coli* DH5α for amplification, and then they were extracted and purified using Giga Scale NucleoBond PC10000 Endotoxin free plasmid DNA purification kit (BIOKE', The Netherlands).

2.2. Recombinant protein

HCV-core protein (aa 1–191 of HCV polyprotein, CIGB, Cuba) used as antigen in protein booster injection, in vitro lymphocyte proliferation assay & in vitro spleen cell cytokine secretion assay.

2.3. Mice immunization

Six to eight-week old, Balb/c (H^2d) female mice were purchased from Razi institute (Karaj, Iran). The mice were divided into 7 groups each one containing 7 mice, to receive different regimens of DNA immunization. One hundred micrograms of each plasmid in final 100 μl volume of 0.9% sterile saline was injected directly intramuscularly into the quadriceps muscles of both legs.

Mice were injected according to the regimen described below: All plasmids were injected in final concentration of 100 μg/ml.

Group 1: sterile saline (control) day 0;
Group 2: mock vector (control) day 0;
Group 3: **pCore** day 0;
Group 4: (**pCore** + **pGM-CSF**) day 0;
Group 5: (**pCore** + **pGM-CSF** + **pIL-23**) day 0;
Group 6: **p IL-23** 3 days before day 0 (day −3) + (**pCore** + **pGM-CSF**) day 0;
Group 7: (**pCore** + **pGM-CSF**) day 0 + **pIL-23** 3 days after day 0 (day +3).

To enhance and accelerate the production of anti-core IgG antibody, all mice receiving pCore plasmid, were injected by 5 μg of core antigen sub-cutaneously (SC) as protein booster injection 3 weeks after day 0 of immunization.

Two weeks after antigen booster, mice were anesthetized, bled and sacrificed. Spleen cells collected for immunological assays and serum collected for antibody titration.

2.4. Serological assays

Collected serums samples were analyzed for the presence of specific antibody by indirect ELISA on 96-well plates (MaxiSorb, Nunc, Denmark). In brief, the sera were diluted in 1:100. Microtiter plates were coated with 0.5 μg core antigen in 100 μl carbonate coating buffer (0.1 M $NaHCO_3$, pH 9.6) per well and incubated at 4 °C overnight. The plates were washed 3 times with washing buffer (0.05% Tween 20 in 1× PBS), blocked with a blocking solution (5% BSA–0.05% Tween 20 in 1× PBS) for 2 h at 37 °C, and then washed 3 times with washing buffer. Diluted serum samples (100 μl) was added to each well and incubated at room temperature for 2 h and washed 6 times after reaction. Then 100 μl HRP-conjugated anti-mouse IgG antibody (DAKO, Denmark) diluted in 1:4000 was added and incubated at 37 °C for 1 h and then washed 6 times. Finally 100 μl of TMB (tetramethylbenzidine) substrate was added to develop the stain. The OD data was measured at 450 nm following the addition of 100 μl stop solution ($H2SO_4$ 2N). All tests were performed in triplicate for each mouse.

Specific IgG antibody isotypes were measured as described above using mouse IgG1 and IgG2a specific HRP-labeled conjugates (Sigma).

2.5. Lymphocyte proliferation assay (LPA)

Single cell suspension of spleen cells obtained from immunized mice was used for lymphocyte proliferation assay. Briefly, the suspension of isolated spleen cells was treated with ACK lyses buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, and pH 7.2) in order to clear the red blood cells. 2×10^5 cells/well were cultured in 96-well flat-bottom culture plates (Nunc, Denmark). The preparations were cultured with complete RPMI-1640 medium RPMI-1640 supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 0.1% penicillin/streptomycin and incubated in the presence of 0.01 μg/ml core antigen per well at 37 °C in 5% CO_2. T cell mitogen PHA (phytohemaglotinin, Sigma chemicals) at a concentration of 5 μg/ml, was used in as positive control. After 3 days, MTT (3-(4,5-dimethyl tetrazolyl-2)2,5 diphenyl) tetrazolyumbromide (Sigma chemicals) in concentration 5 μg/ml added per well and incubated for 5 h at 37 °C in 5% CO_2. DMSO (dimethyl sulfoxide) (100 μl) added to dissolve produced formazan crystals. Plates were read with a microtiter reader (Eppendorf, Germany) at 540 nm, and the results were expressed as stimulation index (SI). The SI was determined as follows: OD values of stimulated cells (C_s) minus relative cell numbers of unstimulated cells (C_u) by relative OD values of unstimulated cells.

$$SI = (C_s - C_u)/C_u$$

All tests were performed in triplicate for each mouse.

2.6. Cytokine release assay

Splenocytes from immunized mice at a concentration of 2 × 106 cells/well in 24-well plates (Nunc, Denmark) were incubated for 2 days in a total volume of 1.5 ml of RPMI-1640 supplemented with 10% Fetal Bovine Serum, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 0.1% penicillin/streptomycin and pulsed with 0.01 μg/ml core protein at 37 °C in 5% CO_2. The cell supernatants were collected and assayed for the presence of IFN-γ and IL-4 using commercially available sandwich-based ELISA kits (R&D, USA) following manufacturer's instruction. All tests were performed in triplicate for each mouse.

2.7. Statistical analysis

To compare results between the different groups, a one way ANOVA test was used. The statistical software SPSS 11.0 was utilized for statistical analyses. Differences were considered statistically significant when P value <0.05.

3. Results

3.1. Construction of HCV-core DNA vaccine

The accuracy of PCR product cloned into **pUC18** and also the **(pCore)** was confirmed by sequencing and restriction enzyme digestion (Data not shown). The expression of ~21.5 kDa core protein by **pCore** construct in HEK293T cells was detected by western blotting as shown in Fig. 1.

3.2. Serological assays

3.2.1. Total specific IgG Ab responses

To assess the specific total IgG antibody response to HCV core, mouse sera were collected and tested for total IgG level by ELISA. As seen in Fig. 2, for anti-HCV core IgG, the group 3 (vaccinated with **pCore**) showed rise in anti-core IgG, which indicates that HCV core DNA prime and protein boost can enhance specific IgG against this antigen, whereas the control mice in groups 1 and 2 showed no increase in specific IgG titer. Co-administration of **pGM-CSF** and **pCore** in group 4 resulted in significant higher core specific IgG antibody. Addition of **pIL-23** to **pCore** and **pGM-CSF** in group 5 simultaneously, did not make any change in the total IgG titer when compared with group 4. To investigate the temporal effect of IL-23 administration on biasing of immune responses, **pIL-23** also was injected either 3 days before (group 6) or 3 days after (group 7) administration of **pCore** and **pGM-CSF**. As seen in figure, there was no significant difference in specific anti-core IgG titer observed among groups 5–7 which received IL-23 in 3 different time intervals relative to **pCore** and **pGM-CSF** administration.

3.2.2. Antibody isotype responses

As IgG1 is a Th2-dependent subclass and IgG2a is Th1 dependent subclass of IgG antibody, the isotype of the specific anti-core IgG induced by DNA vaccination in different groups were

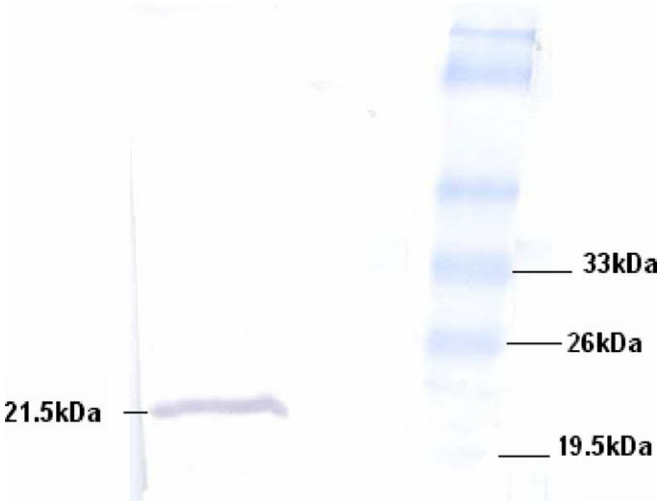

Fig. 1. Intracellular expression of HCV-core protein in transiently transfected 293T cells by core expressing DNA vaccine. The successful transfection and expression of 21.5 kDa core protein was detected by western blotting with anti-core monoclonal antibody using transfected cell lysate.

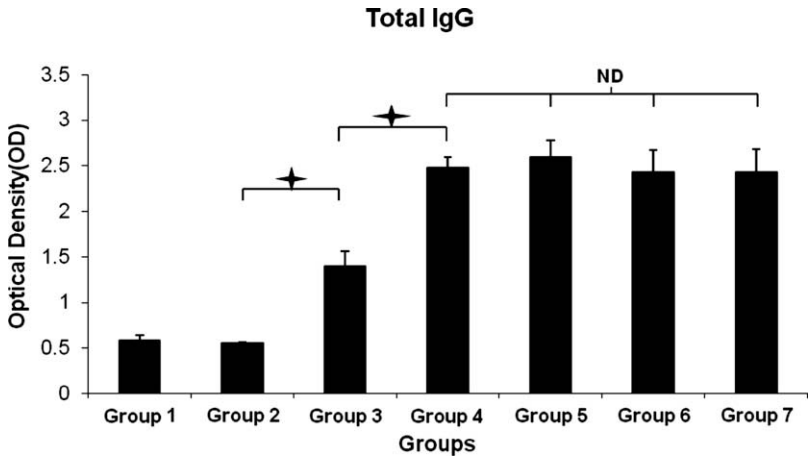

Fig. 2. Anti-core total IgG antibody titers throughout DNA immunization. Mice sera were collected from tail vein. Anti-core total IgG titer was assayed by indirect ELISA and OD measured in 450 nm. The OD represented in each group is a median of mice sera, each one performed in triplicate. The asterisks indicate the groups which were significantly different ($P < 0.05$) with each other and the ND indicates not detectable differences between groups ($P < 0.05$). Error bar represents SD.

measured to investigate modulation of immune responses qualitatively. Specific IgG1 and IgG2a antibody subtypes were measured using specific secondary antibodies. Fig. 3 shows the IgG1 and IgG2a titers. With HCV-core DNA vaccine immunization (group 3); IgG2a rose slightly more than IgG1 when compared with control groups, implying that DNA vaccine with a protein booster favored a Th1 prone immune response. Whereas administration of GM-CSF in group 4 resulted in a balanced increase of both IgG1 and IgG2a titers. Simultaneous addition of **pIL-23** to **pCore** and **pGM-CSF** in group 5, resulted in increase of IgG2a, whereas titer of IgG1 did not changed significantly when compared with group 4. There were no significant differences in IgG2a/IgG1 patterns, between the groups receiving **pIL-23** in 3 days intervals, (groups 5–7) which denotes that these intervals did not induce any change in IgG isotype switching.

3.3. Lymphocyte proliferation

To determine whether core-specific lymphoproliferative responses were induced in immunized animals, spleen cells from immunized mice and control groups were isolated and in vitro stimulation with 0.01 μg/ml HCV-core protein was performed. As

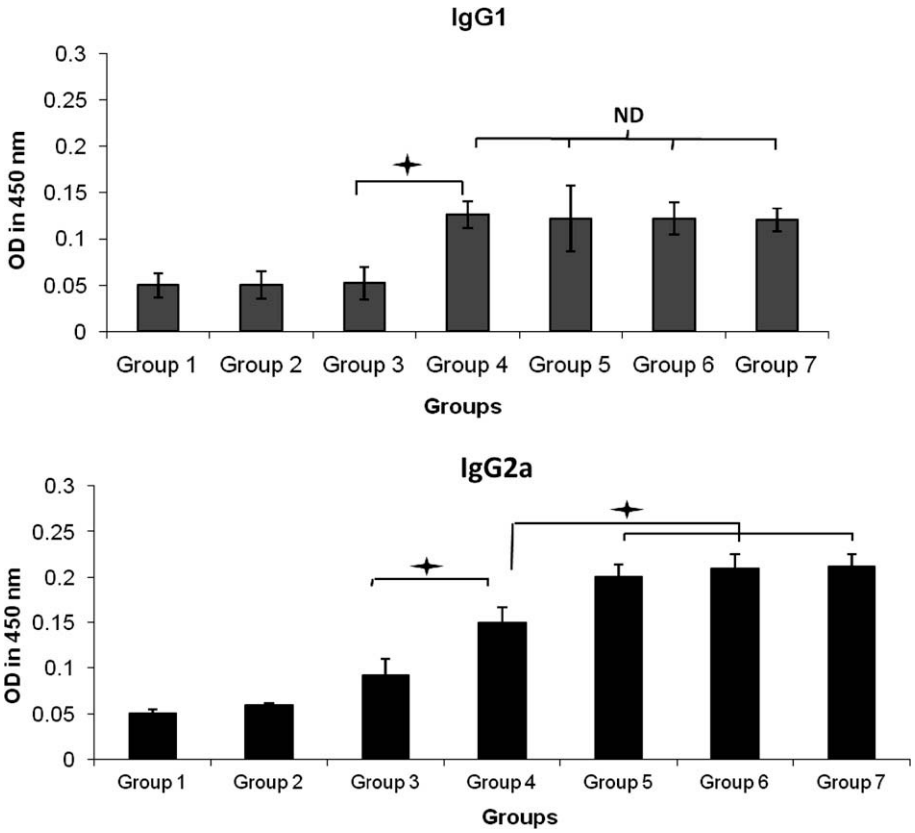

Fig. 3. Analysis of isotype specific IgG antibody levels in mice sera. IgG1 and IgG2a titers have been represented in two distinct graphs. Each mouse sera analyzed in duplicate by indirect ELISA using IgG isotype specific HRP labeled detection antibodies. Values for individual isotypes are expressed in OD 450 nm (mean ± SD) of mice in each group. Asterisks indicate the groups which were significantly different and ND indicates not detectable differences ($P < 0.05$).

shown in Fig. 4, mice co-immunized with HCV-core plasmid DNA and adjuvant GM-CSF gene (group 4), induced a much better core specific proliferation response, in comparison with group 3 which received HCV-core DNA vaccine alone. This shows the ability of GM-CSF to induce spleen cell proliferation as a marker of cellular immune responses. All groups receiving **pIL-23** either 3 days before, at the same time or 3 days after **pCore** and **pGM-CSF** co-administration (groups 5–7, respectively), had specific proliferative responses significantly higher than group 4 which received only **pCore** and **pGM-CSF**. These results did not differ significantly among groups receiving pIL-23 in various time intervals.

3.4. Cytokine release assays

To measure cytokine secretion, splenocytes were cultured as described above and re-stimulated in vitro with HCV-core protein. Collected supernatants were screened for the presence of IFN-γ and IL-4 to determine the phenotype (Th1 versus Th2) of the immune responses. As shown in Fig. 5, the splenocytes taken from immunized mice that received HCV-core DNA vaccine, produced higher levels of IFN-γ in comparison to the control groups; without any change in IL-4 levels, indicating that HCV-core DNA prime-protein boost regimen displays a predominantly Th1 cytokine profile. **pCore** and **pGM-CSF** administration (group 4) showed increases in both IFN-γ and IL-4, denoting the effect of GM-CSF in enhancing both Th1 and Th2 immune responses. Nevertheless, this group displayed a predominant Th1 cytokine profile, because such cells secreted high levels of IFN-γ and low amounts of IL-4.

Co-delivery of **pIL-23** with **pCore** and **pGM-CSF** in group 5 resulted in a significant increase in the IFN-γ secretion by spleen cells, whereas no change was observed in the IL-4 titer, in comparison with group 4. A similar pattern in Th1 development without any change in Th2 was resulted in groups 6 and 7 which received pIL-**23** 3 days before or after administration of **pGM-CSF** and **pCore**, respectively. These results suggested that administration of plasmid encoding IL-23 can boost the Th1 cytokine profile without any effect on Th2 cytokine secretion. On the other hand, the timing of **pIL-23** administration does not impact the Th1 versus Th2 cytokine balance.

4. Discussion

This study was performed with the objective of understanding the effect of cytokine adjuvants in boosting immune responses

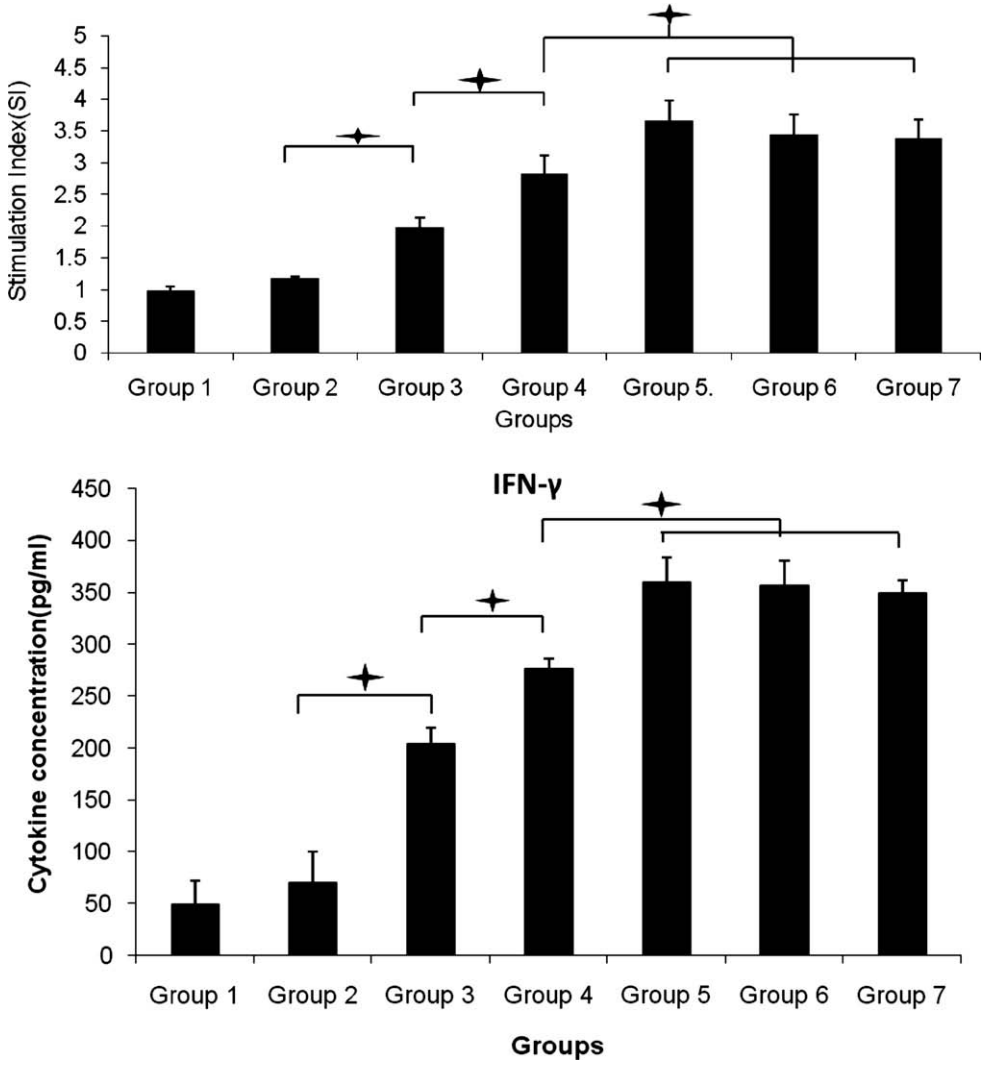

Fig. 4. Splenocyte proliferation levels after in vitro stimulation with core protein. Mice of each group were sacrificed 5 weeks after DNA priming and 2 weeks after core protein boosting and spleen cells were cultured in triplicate for each mouse stimulated with 0.01 μg/ml of core protein for 3 days in vitro. Formazan crystal formation after 3 h incubation of MTT was determined by solving the crystals in DMSO and OD was read at 540 nm. The Asterisks indicate the groups which were significantly different ($P < 0.05$).

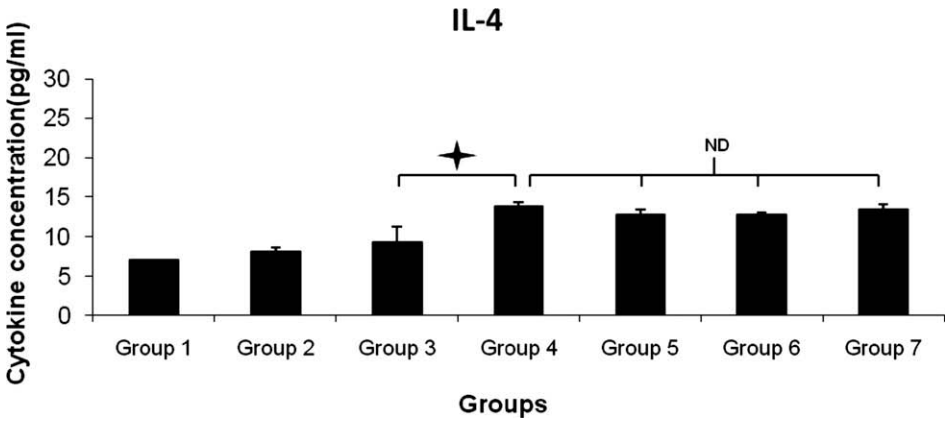

Fig. 5. Levels of cytokine production from mice splenocytes in vitro. Spleens of mice were isolated and the splenocytes were cultured in triplicate for each mouse in the presence of 0.01 μg/ml core protein. After 48 h supernatants were collected and analyzed by sandwich ELISA method to measure the IFN-γ and IL-4 titers separately. Data are the mean ± SD of mice in each group. The Asterisks indicate the groups which were significantly different ($P < 0.05$) while ND indicates not detectable differences between groups ($P < 0.05$).

generated against candidate DNA vaccine encoding HCV core antigen in an intramuscular immunization regimen. The outcome of this study indicated that addition of IL-23 to GM-CSF enhanced cell-mediated immunity. Previous studies of the GM-CSF plasmid co-inoculation with DNA vaccines against HIV [13,26], influenza [27] and HCV [12] have confirmed the boosting effect of this cytokine on both humoral and cellular immune responses to plasmid encoded antigens. Our results resemble to that of the above-mentioned studies. Administration of GM-CSF in group 4 activated both Th1 and Th2 immune responses as manifested by the increased antigen-specific serum total IgG, IgG1 and IgG2a levels, improved proliferation and significantly, increased IFN-γ and IL-4 production by splenic cells when compared with the DNA vaccine alone (group 3). The difference of our approach with Chen et al., who co-administered GM-CSF and HCV-core DNA vaccine [12], was that we utilized a recombinant protein boost to increase anti-core specific IgG. The rationale of employing a protein booster was that as previously mentioned, administration of HCV-core DNA vaccine, even by boosting with the same construct, did not raise the anti-core specific IgG while levels of the IgM isotype of antibody increased. However, after protein boost, specific IgG rose rapidly [25]. It is likely that induction of anti-core IgG in the Chen et al. study without protein booster might be due to the secreted nature of the core protein by the expression vector [28].

In spite of such evidence, there are controversial reports which reveal that GM-CSF stimulates only either Th1 or Th2 responses [29,30] or has a weak immune-stimulatory effect in a malaria DNA vaccine model in Rhesus monkeys [31]. From the other point of view, co-delivery of plasmids encoding GM-CSF and Human CEA (carcinoembryonic antigen) expressing plasmids via epidermal particle bombardment, decreased antibody and T-cell proliferative responses compared to CEA plasmid alone, whereas administering CEA plasmid enhanced antibody and T cell responses [32]. Further study of this cytokine is needed to determine the reasons for this apparent inconsistency. Also, in many studies, it has been clearly observed that route of administration, amount of administered plasmid, nature of antigen, number of immunizations and animal species all influence the polarization of immune responses. With more consideration, it is noticeable that for group 4, in which GM-CSF is co-administered with core expressing DNA vaccine, rise of antibody titers, is more obvious than increase in IFN-γ levels and the proliferative response when compared with group 3 which received the DNA vaccine alone. Our results are comparable with Chen et al. and Kusakabe et al. findings which showed that co-administration of GM-CSF with HCV core and HIV(1) DNA vaccine, respectively, enhanced both humoral and cellular immune responses, but the induction of cellular immunity was not as striking as humoral one [12,13]. Even though, in our study administration of a protein booster also could be a reason for such a result. Based on the findings of the Chen et al. study, we expected similar results and sought to compensate for lower cell-mediated immunity so that humoral and cellular immunity would be enhanced simultaneously. Therefore, IL-23 was co-administered as a Th1 cytokine to GM-CSF in group 5. Since the total IgG titer did not change remarkably, it can be inferred that addition of IL-23, had no effect on antibody production. This finding clearly confirms the results of Britton et al. that showed co-immunization of a *Mycobacterium Tuberculosis* DNA vaccine with IL-23 was not associated with changes in the antibody titer, while co-administration of IL-12, the other member of this cytokine family, had suppressed specific antibody production. On the other hand, addition of IL-12 to GM-CSF and HIV-specific DNA vaccine decreased the total IgG titer while significantly enhancing Th1 type immunity when compared with the group receiving GM-CSF and HIV-specific DNA vaccine. These outcomes implicate the advantages of IL-23 as a vaccine adjuvant which, along with enhancement of cellular immunity, does not decrease the humoral immune response. On the other hand, there is another study of IL-23 effect on antibody response, showing that genetically fused influenza virus hemagglutinin (HA) antigen to IL-23 was able to mount IgG1 isotype responses but not higher than HA DNA vaccine alone [33]. Since these authors fused the antigen and cytokine gene to produce a chimeric protein and delivered DNA vaccines via a gene gun, this inconsistency is somewhat explicable. More detailed studies on the effect of IL-23 on antibody production is required before any precise judgment is possible.

Enhanced antigen-specific proliferative response, increased IFN-γ release and increase in IgG2a/IgG1 ratio, all suggest that addition of IL-23 could enhance Th1 and cell-mediated immunity. Similar investigations on DNA vaccines for infectious disease and cancer have proved the validity of these results [20,21,34,35]. In our work negligible differences in IL-4 levels demonstrate that IL-23 has no effect on Th2 immune response as also described above in antibody production. Others have stated that mice co-immunized with IL-23 and HCV E2 DNA vaccine showed higher IFN-γ and IL-4 producing CD4+ T cells than DNA vaccine alone 27 weeks after DNA immunization which shows IL-23 is involved in generation of Th2 cells as well as Th1 cells [14]. However, higher IFN-γ to IL-4 CD4+ T cells ratio shows Th1 prone immunity rather than Th2. It seems that long term studies will be needed to clarify the role of IL-23 in Th2 responses since we evaluated the immune responses in only 5 weeks.

It has been well demonstrated that antigen and cytokine timing also is a critical parameter in determining the overall biologic effect of the cytokine. Administration of GM-CSF 3 days before HIV-1 specific DNA vaccine markedly enhanced Th2 immunity; simultaneous administration stimulated both Th1 and Th2 responses. Administration of GM-CSF 3 days after DNA vaccination preferentially enhanced Th1 immunity indicating that the Th1/Th2 balance of an antigen-specific immune response can be dramatically affected by the timing of cytokine plasmid administration [13]. Such results have been obtained by another group who expressed it is likely that immunostimulstory cytokines in general operate in this fashion [36]. In our work, timing effect of IL-23 administration to the GM-CSF/HCV-core DNA vaccine constructs was studied in a 3 days interval. No significant qualitative and quantitative difference in both humoral and cell-mediated immunity was observed among the groups which received IL-23 3 days before (group 6), simultaneously (group 5) and 3 days after (group 7) GM-CSF and DNA vaccine administration. Our results are in contrast with the above mentioned statement and imply that IL-23 does not follow the same pattern. Concerning memory CD4+ T cell responses, it has been reported that the effect of IL-23 co-delivery could be observed in the late phase rather than early phase of immune response induction [21]. Therefore, it is possible to explain that a 3 days interval is too short a window of time for IL-23 to display its immunomodulatory effects on the microenvironment and likely, studies of longer intervals will be required to closely investigate the timing effect of IL-23 administration.

In addition, the negative side effects of such cytokines should not be overlooked. IL-23 induces the production of IL-17 which is a proinflammatory cytokine and enhances Th17 cells which have a critical role in autoimmunity [35]. Recently, it has been shown that limited toxicity of IL-23 was due to weight loss, apparently due to a loss of appetite that was reversed upon IL-23 discontinuation. The therapeutic and side effects of IL-23 were linked across the entire dose response range. Interestingly, local delivery of IL-23 prevents weight loss while preserving antitumor activity [37]. Reports on the side effects of GM-CSF also indicate that it is relatively well tolerated in the clinic [38]. Other complementary studies are needed to elucidate the role of locally expressed cytokines as vaccine adjuvants.

As both humoral and cellular immune responses are important in inducing protective immunity for HCV infection, our results suggest that such formulation can induce a balanced immune response against HCV core antigen. Of the various regions of HCV, antibodies against hepatitis C viral core protein are among the first antibodies appearing during HCV natural infection [39,40], even though the core antigen is not placed on the surface of the virus and antibodies against core are not neutralizing. In our further studies we will attempt to evaluate the ability of such antibodies in ADCC (antibody dependent cell-mediated cytotoxicity) and to explore the value of such antibodies in preventive and/or therapeutic vaccines. On the other hand, cellular immune responses against HCV core often tend to be reduced in individuals with chronic infection [41] and many clinical studies have been suggested that a vigorous cellular immune response is readily detectable in acute self-limited hepatitis C, but that this response is weak or undetectable in patients with chronic hepatitis C [42,43]. Since our cytokine combination regimen with DNA vaccine can induce both humoral and cellular immunity, it may have gained the merit to be further evaluated in non-human primate models for viral challenge experiments. As IL-23 induces long lasting memory T cell immunity such as specific CTL and Th1 immune responses [21], another issue which deserves to be addressed in future is to investigate whether these effects, which are an important determinant of a candidate vaccine, persist for the longer periods.

Taken together, we demonstrated that co-administration of IL-23 with GM-CSF can result in induction of both humoral and cellular immune responses generated against HCV-core DNA prime-protein boost vaccination regimen. We have revealed that IL-23 holds potent adjuvant effects in combination with GM-CSF, these results appear promising for both microbial and cancer DNA vaccines.

Acknowledgments

This study was supported by a grant from Tarbiat Modares University, Faculty of Medical Sciences. We also thank Mr. Abbas Jamali and Mr. Saeed Bayanolhagh for their technical support.

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