

Constructions of hepatitis C Virus prophylactic vaccine candidate using *Berberis vulgaris* stimulated and nonstructural protein 3 loaded dendritic cells

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ABSTRACT

Introduction: Dendritic cells (DCs) have been recently employed as carriers for vaccines against several viral infections. The present study was designed to develop a prophylactic vaccine against hepatitis C virus (HCV) using DCs treated with *Berberis vulgaris* root extract (BRE), as a preclinical study. **Methods:** BRE was prepared and injected to female BALB/c mice for DCs expansion. The expanded splenocyte cells (EDC) were purified and efficiently loaded *ex vivo* with HCV-nonstructural protein 3, NS3, (EDC-NS3). Mice were subcutaneously inoculated with EDC-NS3 vaccine candidate thrice with 4-week-intervals and IL-12, IFN- γ , IL-4, IL-10, MHC class II, CD3, CD16, indoleamine 2, 3 dioxygenase (IDO) and total protein levels were measured, post-vaccination by PCR and flow cytometry. Moreover, cytotoxic T lymphocyte and humoral immune responses were examined. **Results:** Our data revealed that immunization with EDC-NS3 vaccine elevated IL-12, IFN- γ and IL-4 expressions as well as MHC II and CD16 at protein levels. It also elicited strong HCV-NS3-specific humoral and cellular immune responses. However, the expressions of CD3, IDO, and IL-10 were down-regulated, post-vaccination. **Conclusion:** EDC-NS3 immunization serves as an innovative modality for immunoprophylaxis against HCV infection.

KEYWORDS: *Berberis vulgaris*, Dendritic cells, HCV: NS3, Prophylactic vaccine.

INTRODUCTION

Hepatitis C virus (HCV) derives from *Hepacivirus* genus in *Flaviviridae* family. It is a severe health problem which infects 130-170 million people, globally. It is the main reason for liver damage and diseases. HCV open-reading-frame encodes 3 structural proteins and 5 nonstructural (NS) proteins; among them, the most conserved one is NS3. Nowadays, effective treatments for HCV are the direct acting drugs which give high cure rates with few side effects; however, there is no available vaccine to prevent the infection [1]. Several trials for HCV vaccine have been developed, including the use of dendritic cells (DCs) as an adjuvant.

DCs are expert antigen-presenting cells (APCs) which are critical for inducing a primary antiviral T cell response [2]. Optimizing the DC's function by facilitating their functional effects upon transfer into the host is essential. This can occur through expanding the DC numbers, before the administration of an antigen-based vaccine. DCs can be expanded by DC-poietins such as plasmid fms-like tyrosine kinase 3 ligand or granulocyte-macrophage-colony-stimulating factor [3]. In the

present study, *Berberis vulgaris* root extract (BRE) was used for DCs expansion *in vivo*. *B. vulgaris* is one of the well-known medicinal plants. Its root, bark, leaf and fruit have been used in the traditional medicine. The plant has been reported as one of the most common immunomodulating herbs. It grows in Europe and Asia and is exploited by many societies as a medicinal herbal remedy for homeopathic treatments [4].

The current study focused on the development of a prophylactic vaccine for genetically diverse populations of HCV, using CD11c+ DCs stimulated *in vivo* with BRE and loaded *ex vivo* with HCV-NS3 protein. In addition, this study evaluated the humoral and cellular immune responses, provoked by this vaccine in BALB/c mice.

MATERIALS and METHODS

Chemicals and kits

Cluster of differentiation (CD)11c MicroBeads kit, fluorescein IsoThioCyanate (FITC)-labeled anti-mouse CD11c antibody and FITC-labeled anti-mouse major histocompatibility class II (MHCII) antibody were from Miltenyi Biotec (Germany). Allophycocyanin (APC)-labeled anti-mouse Fc γ receptor (R) III/CD16 and phycoerythrin (PE)-labeled anti-mouse CD3

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antibody were from R&D systems (UK). Goat anti-mouse γ -chain specific peroxidase antibody was from KPL (USA). Anti-NS3 antibody, BioPROTER QuikEase protein delivery kit, Folin-Ciocalteu reagent, HCV-NS3 protein (recombinant, expressed in *Escherichia coli*), Gallic acid (GA), Ursolic acid (UA), Rutin (RU), Berberine (Be), Catechin, Naphthyl-ethylenediamine dihydrochloride, Coomassie brilliant blue G-250, Catalase, and p-Dimethylaminobenzaldehyde were purchased from Sigma-Aldrich (USA). Lactate dehydrogenase assay kit was purchased from Cayman (USA). Mouse interleukin (IL)-12 and interferon (IFN)- γ ELISA kits were supplied from Komabiotech (Korea). Maxime reverse transcriptase (RT) PreMix kit was obtained from Intron Biotechnology (Korea). Polymerase chain reaction (PCR) Master Mix (2X) kit was from Fermentas (Germany). Primers of IL-4, IL-10, IL-12, IFN- γ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Bioneer Co. (Korea). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein and albumin kits were bought from RAM (Egypt). Fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer) and Roswell Park Memorial Institute medium (RPMI)-1640 were from Lonza (Switzerland). Other chemicals were analytical grade.

Animals and cell line

Fifty-four inbred female BALB/c mice (18-20 g weight, 6-8 weeks old) were purchased from Theodor Bilharz Research Institute (Egypt). Mice were divided randomly into a group of 24 mice for DCs generation and a group of 30 mice for immunization with the prepared vaccine candidate. Animals were given tap water and a standard commercial diet. The mice were subjected to conventional conditions of temperature, humidity and 12 h light/dark cycles. All mice were adapted to the laboratory surroundings for 1 week before handling and were observed daily for abnormal signs.

EL4 murine lymphoblast cell line (Genethor GmbH, Germany), were cultured in RPMI-1640 basal medium supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine and 1% penicillin/streptomycin. The culture was incubated at 37°C in a humidified 5% CO₂ incubator.

Plant extraction

Dried *B. vulgaris* roots were brought from Iran. The roots were ground using a mixer to obtain *B. vulgaris* powder. Fifty grams of the dried powdered roots were defatted using petroleum ether, packed in a Soxhlet apparatus and extracted in 70% ethanol for 8 h. The ethanolic extract was lyophilized to obtain a powdered extract (BRE yielded 25% of root powder) then stored at -20°C until use [5].

Phytochemical analyses for BRE

Total phenolic, flavonoid, triterpenoid and alkaloid contents of BRE were quantified. Phenolics were determined as GA equivalents in mg/g BRE according to Taga method [6], using GA as standard and the absorbance of the obtained blue color solution was recorded at 750 nm. Flavonoid content was determined colorimetrically as RU equivalents in mg/g of BRE using 5% sodium nitrite and 10% aluminum chloride solutions to produce a colored product quantified at 510 nm [7]. Alkaloids were determined using bromocresol green dye (BCG) which formed a complex yellow colored product that was read at 470 nm [8]. The concentration of alkaloid content in BRE was expressed as BE equivalents in mg/g of the dry extract, using standard BE calibration curve. The content of triterpenoids in BRE as UA equivalents in mg/g BRE was

determined using the color reaction of triterpenoids with vanillin and the absorbance was measured at 520 nm [9].

HPLC analysis for phenolics

Twenty microliters of BRE were separated on Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μ m; Agilent Technologies, Germany) at pH 2.5, 320 nm and flow rate of 0.75 mL/min. The mobile phase was a mixture of 1% formic acid: 2-propanol: acetonitrile (70:8:22) [10,11].

Vaccine preparation

For the preparation of the vaccine, the splenic CD11c+ DCs were generated and expanded by BRE in BALB/c mice then were purified and loaded with HCV-NS3 protein ex vivo. The generation of DCs was carried out using BRE, as described previously [5]. Mice were divided into two groups (containing 12 mice each) of control untreated and BRE. Mice in BRE group were injected with 60 mg/kg b.w. intravenously every other day for 15 days (8 times). On day 16 from the 1st injection, all mice were sacrificed via cervical dislocation under aseptic condition and spleens were rapidly dissected and used for preparation of single-cell suspensions of the splenocytes.

Preparation of single-cell splenocyte suspension and CD11c+ DCs purification

The spleens of mice used for each dose were dissociated in 5 ml of RPMI-1640 medium containing 20% FBS (culture medium) using a 70- μ m metal mesh [12]. The cell suspensions were centrifuged at 2000 rpm for 5 min at 25°C and the RBC in the pellet were lysed then suspended in RPMI-1640 culture medium (1 ml). Cells were counted via trypan blue stain and then were directly used in CD11c+ DCs purification.

CD11c+ DCs were purified using MACS-anti-mouse CD11c-conjugated MicroBeads kit. According to the manufacturer's guidelines, the method was divided into two steps: Step 1: magnetic labeling of CD11c+ cells with CD11c MicroBeads. Step 2: magnetic separation of CD11c+ cells. The CD11c+ DCs purified from the BRE-untreated mice were named control DCs (CDC) and those expanded with BRE were named expanded DCs (EDC).

Identification and enumeration of CD11c on the DC surface

Evaluation of MACS separation was achieved by flow cytometric analysis for the identification and enumeration of CD11c molecules on DCs surface. For this analysis, 0.1x10⁶ cells (CDC or EDC) were centrifuged for 10 min at 1650 rpm. Pellets were then washed and fixed in 25 μ l paraformaldehyde. The amount of 10 μ l of FITC-labeled anti-mouse CD11c antibodies and 25 μ l of the paraformaldehyde fixed CD11c+ DCs were mixed and incubated at 4°C for 10 min. Control cells without antibodies treatments were analyzed similarly. Thereafter, cells were resuspended in MACS buffer (200 μ l) and examined using Becton Dickinson (B.D.) flow cytometer (USA) and results were analyzed using B.D. software version 5.56.2012.

Cell loading with HCV-NS3 protein

Two types of cells, EL4 (for cytotoxicity assay) and CD11c+ DCs (CDC and EDC) were loaded with HCV-NS3. EL4 cells were loaded with 10 μ g of HCV-NS3 protein by a BioPORTER QuikEase protein delivery kit following the manufacturer's protocol to produce NS3-loaded EL4 (EL4-NS3). However, CD11c+ DCs (EDC and CDC) were loaded with 0.1 μ g of the viral protein for 24 h, producing NS3-loaded CD11c+ DCs (EDC-NS3 {vaccine} and CDC-NS3).

Estimation of HCV-NS3 protein concentration

The concentration of HCV-NS3 protein after loading was determined in both culture supernatant and cells by an indirect ELISA technique [13] using anti-NS3 antibody and peroxidase-conjugated goat anti-mouse IgG. The control samples that contained no cells (total amount of HCV-NS3) were involved. The concentration of HCV-NS3 protein was expressed as a percentage of the control.

Immunization of mice

According to the World Health Organization (WHO) general aspects of vaccination, primary immunization programs should include at least two doses of the vaccine and continual at a time interval of 3-4 weeks. This program leads to the generation of consecutive influences of the immune response. Therefore, 30 female BALB/c mice were divided into 3 groups (Fig. 1B), 10 mice each. Mice were subcutaneously inoculated with 0.5 ml of only culture medium (RPMI-1640 containing 20% FBS, MC group) or culture medium containing EDC-NS3 (EDC-NS3 group) or CDC-NS3 (CDC-NS3 group). The inoculation was 3 times at 4 weeks intervals and the 1st inoculation was 100,000 cells/mouse while the 2nd and the 3rd inoculations were 50,000 cells/mouse. This immunization scheme was performed according to Ghareeb et al. 2016 [5] with modifications. At the 7th day following the last inoculation, the mice were sacrificed via cervical dislocation then blood and spleens were directly separated under aseptic environments. Blood samples were gained by cardiac puncture method then sera were separated and stored at -20°C until use. While the erythrocyte-depleted splenocyte suspensions were prepared from the spleens as described previously and utilized in the following assays.

Evaluation of the anti-HCV efficiency of the prepared vaccine

The CTL assay

The CTL assay was performed by lactate dehydrogenase (LDH) release cytotoxicity method [14]. In this assay, lysis of target cells (EL4-NS3) by effector cells (CTL from immunized and control mice splenocytes) was monitored. In brief, U-bottom 96-well microtiter plates received 0.1x10⁶ cells /well of total cells (the effector and the target) or only the target cells or only the effector cells or the target cells with 50 µl Triton X-100. The optimum target: effector co-culture ratio as demonstrated before was 1: 1.65 [5]. Cells were incubated in a CO2 incubator (New Brunswick Scientific Co., England) for 4 h then centrifuged for 10 min at 2000 rpm. The supernatants were used for determination of LDH leakage using LDH assay kit as directed by the manufacturer's manual. The percentage of cytotoxicity was calculated using the following equation:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100$$

Where, the experimental release is the LDH release from the co-culture cells (the effector and the target); Spontaneous release is the sum of the LDH release from the target cells alone and the effector cells alone. While the maximal release is the total LDH of the target cells (the target cell with Triton X-100).

Flow cytometric analysis for three immune cells markers

The protein levels of CD3, MHCII, and CD16 were examined by flow cytometry. For sample preparation, 0.1x10⁶ cells were centrifuged at 1700 rpm. The pellets were washed 3 times and resuspended in PBS (25 µl), containing BSA (0.5%; for CD3 and CD16) or in MACS buffer (for MHCII). Following the manufacturer's instructions, 10 µl of PE-labeled anti-mouse CD3, APC-labeled anti-mouse Fcγ RIII/CD16 or FITC-labeled

anti-mouse MHCII antibodies were incubated with 25 µl of the sample at 4°C. The excess antibody was removed by washing then cells were analyzed on a Becton Dickinson flow cytometer.

Assessment of the humoral immune response

The levels of the anti-HCV-NS3 antibody in the serum of each immunized mouse were quantified by indirect ELISA using recombinant HCV-NS3 (50 µl /well in 50 mM NaHCO₃/Na₂CO₃, pH 9.6) to coat a microtiter plate [15].

Quantification of cytokine production

The IL-12, IFN-γ, IL-4 and IL-10 gene expression

The cytokine expression was investigated by PCR. Total RNA was extracted from 1x10⁶ splenocytes using an acid guanidinium thiocyanate-phenol-chloroform mixture [16]. One microgram of each RNA sample was used to prepare the cDNA using Maxime RT PreMix kit following the manual protocol. Then the PCR was carried out by Master Mix (2x) kit using IL-12 forward (5'ATGTGTCAATCAGCTACCTC3'), reverse (5'TCAGGCGGAGCTCAGATAG3'); IFN-γ forward (5'GTCAACAACCCACAGGTCCAG3'), reverse (5'TTGGGACAATCTCTTCCCA3'); IL4 forward (5'TTGTCTCTCGTCACTGACGCA3'), reverse (5'TACGAAGCACCTTGAAGCC3'); IL10 forward (5'AGAGCTTGCTCTTGCACTACCAA3'), reverse (5'GTAAGAGCAGGCAGCATAGCAGT); GAPDH (internal control) forward (5'GGAGATTGTTGCCATCAACG3'), reverse (5'CACAATGCCAAGTTGTCATGG3') primers. After thermocycling, the PCR product of each sample was electrophoresed using 1.5% agarose gel. The gene expression was evaluated by myImageAnalysis software v.1 (Thermo Fisher Scientific Inc.) using the expression ratio of (target gene/GAPDH).

IFN-γ and IL12 protein levels

The protein levels of IFN-γ and IL-12 were determined in 1x10⁶ splenocytes by sandwich ELISA kits using corresponding specific capture and detection antibodies, following the manufacturer's protocol.

Indoleamine 2, 3-dioxygenase (IDO) activity assay

IDO activity was determined in splenocytes through determination of kynurenine level [17]. Briefly, 100 µl of splenocyte suspension was mixed with the same volume of the reaction buffer (100 mM sodium phosphate buffer, pH 6.5, 20 µM methylene blue, 40 mM ascorbate, 800 µM L-Trp, and 200 µg/ml catalase). The mixture was incubated at 37°C for 30 min to start the IDO reaction. Then, the reaction was inhibited using 40 µl of trichloroacetic acid, incubated for 30 min at 50°C and centrifuged for 10 min at 10,000 rpm. The supernatants were mixed with the same volume of the Ehrlich solution (0.4% p-dimethylaminobenzaldehyde in acetic acid) in a 96-well microtiter plate. Lastly, the absorbance of the resulted color was measured at 490 nm. One unit of the enzyme activity refers to the amount of enzyme needed to give 1 nmol of kynurenine product per 1 h. The specific activity of IDO was calculated as µmol/min/mg protein. Protein content in 20 µl of the splenocyte suspension was estimated using Bradford Coomassie brilliant Blue method [18] and the absorbance of the resulted color was read at 630 nm. The protein concentration was calculated from the standard curve of BSA.

Statistical analyses

The data are given as mean values ± standard error (SE). Comparisons between the studied groups were analyzed through their mean values by the least significant difference (LSD) test. The significant differences were considered at P <

0.05. The statistical analysis was conducted by SPSS statistical software, version 16 (SPSS Inc. USA).

RESULTS

Phytochemical composition

Quantitative determination of the phytochemical constituents of BRE showed the presence of different amounts of phenolics, flavonoids, alkaloids and triterpenoids (Table 1). HPLC analysis showed that the predominant phenolic constituents in the BRE were GA, chlorogenic acid, caffeic acid, catechin, RU and tannic acid (Fig. 1, Table 1).

Loading of CD11c+DCs and EL4 with HCV-NS3 protein

Results in Table 2 show that the CD11c+ DC loading efficiency was approximately 99%, while it was approximately 90% for EL4 cell line.

Immunological profiling of the splenocytes suspension in response to DCs immunization

CTL response

The results in Fig. 2A elucidated that the level of CTL activity against EL4-NS3 cell line was elevated significantly by 82.56% in mice splenocytes immunized with the prepared vaccine. This level was also elevated in the same group; however not significantly, by 5.42% against EL4 cell line as compared to the MC group. Similarly, mice in NS3-loaded control dendritic cell (CDC-NS3) group showed a significant increase in CTL activity against EL4 and EL4-NS3 cell lines by 15.01% and 25.15%, respectively.

IDO activity and total protein level

The results in Fig. 2B demonstrate non-significant changes in the splenic total protein levels after EDC-NS3 and CDC-NS3 immunizations. Whilst, IDO specific activity decreased significantly by 45.85% after EDC-NS3 immunization but CDC-NS3 immunization caused a non-significant decrease (3.14%) in its activity.

Immunophenotypic properties

The basic immunophenotyping panel for APC (MHCII), T cells (CD3) and natural killer cells (CD16) are summarized in Fig. 3. EDC-NS3 immunization induced significant up-regulation of MHCII (61.72%; Fig. 3A, B) and CD16 (208.16%; Fig. 3E, F) levels as compared to the MC group. In contrast, the CD3 level showed significant down-regulation with 63.92% (Fig. 3C, D) decrement. CDC-NS3 immunization induced significant down-regulation in MHCII level (42.24%) and nonsignificant changes in both CD3 (0.63%) and CD16 (0.00%) levels.

In vivo generation of CD11c+DCs by BRE

The flow cytometric analysis for CD11c+ expression (Fig. 3G, H) indicated that the percentage of expression for the control mice (CDC) was $1.6\% \pm 0.008$. However, this value increased to $17.3\% \pm 0.013$ for the BRE-treated mice.

Humoral immune response

The results in Fig. 4A showed that immunization with the prepared vaccine (EDC-NS3) induced an increase in the anti-NS3 antibody response by 77.14%, compared to MC group. Also, a strong anti-NS3 antibody response (25.71%) was found in mice immunized with CDC-NS3.

Cytokine secretion

The gene expressions of IL-12, IFN- γ , IL-4, and IL-10 by the splenocytes were estimated as shown in Fig. 4B, C. Our data showed that the immunization with EDC-NS3 and CDC-NS3 induced significant increases in the relative expression of IL-12 by 79.93% and 85.27%, respectively, as well as IFN- γ by 25.4% and 74.8%, respectively. In addition, both immunization models up-regulated IL-4 by 26.58% and 9.83%, respectively. In contrast, EDC-NS3 immunization decreased the relative expression of IL-10, significantly by 31.28% while a significant elevation of 65.64% of this cytokine was detected after CDC-NS3 immunization. In addition, our vaccine immunization showed significant depletion in IL-12 (0.14 pg/ml, 41.67%) and significant rise in IFN- γ (63.73 pg/ml, 49.71%) protein levels as compared to MC group. Meanwhile, CDC-NS3 immunization induced elevation in IL-12 (0.25pg/ml, 4.17%) and IFN- γ (45.32 pg/ml, 6.46%) protein levels, although not significantly ($P > 0.05$).

Table 1. Phytochemicals composition of *B. vulgaris* root extract (BRE).

Phytochemicals	Concentration
Total phenolics (mg GA eq/g extract)	5.641±0.003
Flavonoids (mg RU eq/ g extract)	3.132 ± 0.011
Alkaloids (mg BEeq/ g extract)	35.000 ± 0.980
Triterpenoids (mg UA eq/ g extract)	446.470 ± 0.551
HPLC analysis (Phenolics)	(mg/g extract)
Gallic acid	0.307± 0.000
Chlorogenic acid	0.201± 0.001
Caffeic acid	0.159± 0.000
Catechin	0.114± 4.881
Rutin	0.001± 0.000
Tannic acid	0.021 ± 0.000
Results are expressed as Mean ± SE (n=3).GA; gallic acid, RU; rutin, BE; berberine.	

Table 2. HCV-NS3 protein levels (%) inCD11c+DCs and EL4 after loading.

Protein location	HCV-NS3 protein		
	In medium	Inside cells	On membrane
CD11c+DC	1.101 ± 0.212 ^a	10.400 ± 1.200 ^b	88.502 ± 4.300 ^c
EL4	10.150 ± 1.255 ^a	37.352 ± 1.500 ^b	52.501 ± 2.300 ^c
Results are mean ±S.E. (n = 3). NS3, nonstructural protein 3; DCs, dendritic cells; EL4, murine lymphoblast cell line.Different letters refer to the significantly differentvalues ($P < 0.05$).			

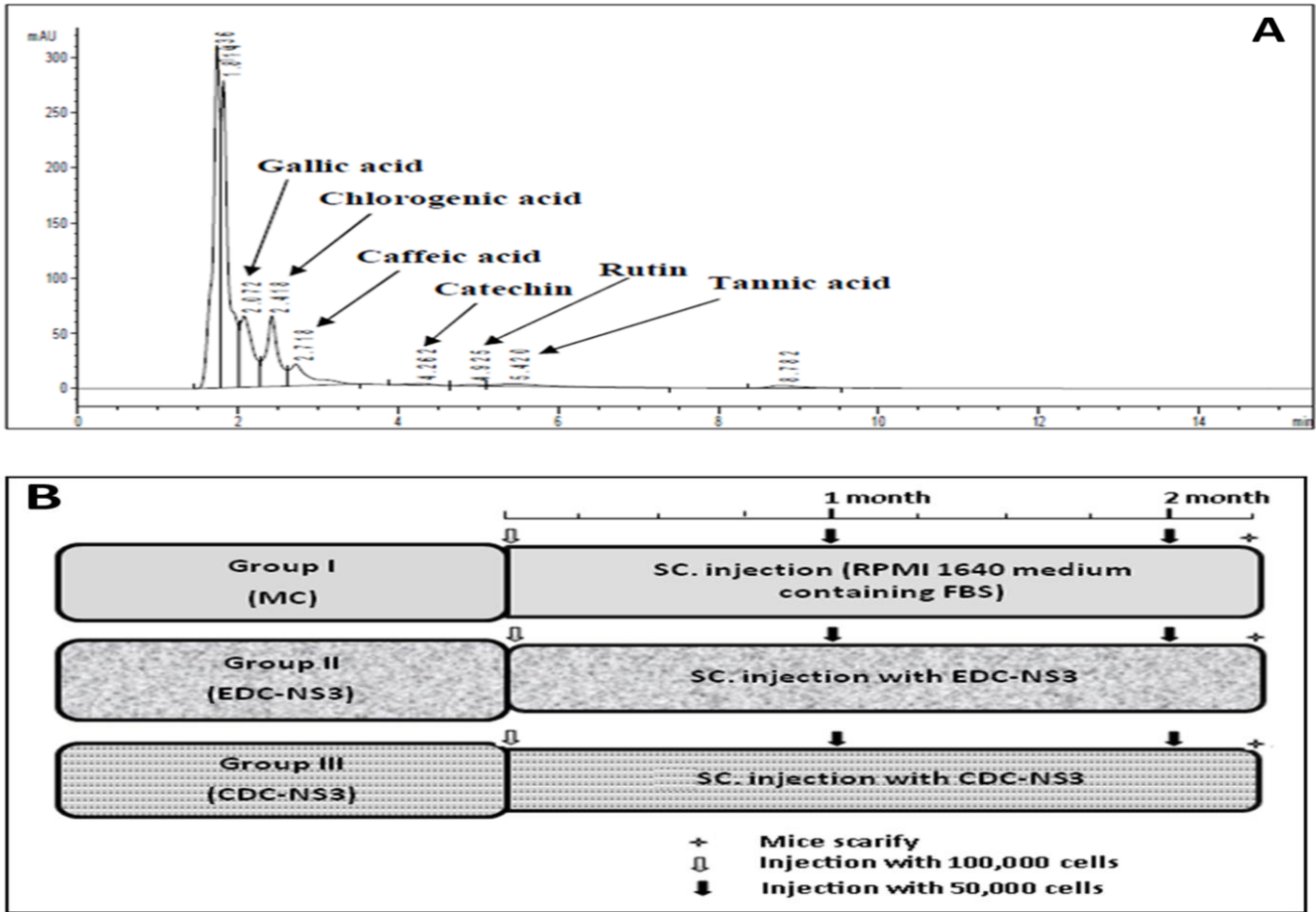


Fig. 1. HPLC chromatogram of *B. vulgaris* root extract (BRE) phenolic compounds (A) and an illustration of mice immunization protocol (B). FBS, fetal bovine serum; MC, medium control; EDC-NS3, expanded HCV-NS3-loaded dendritic cells; CDC-NS3, control HCV-NS3-loaded dendritic cells.

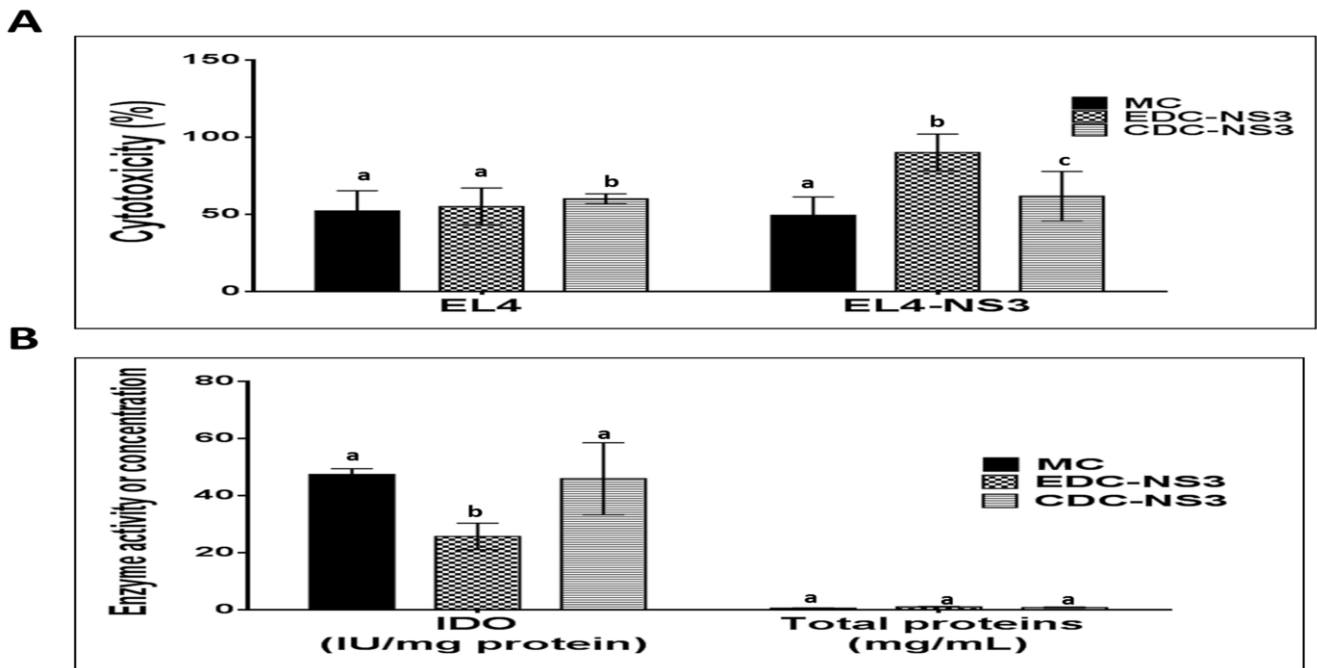


Fig. 2. Cellular immune response (Cytotoxicity against EL4 or EL4-NS3), Indoleamine 2, 3 dioxygenase (IDO) activities and total protein levels in the splenocyte suspension after mice immunization. MC, medium control: mice were immunized with medium alone (RPMI-1640 basal medium containing 20% FBS); EDC-NS3, mice were immunized with expanded HCV-NS3-loaded dendritic cells; CDC-NS3, mice were immunized with control HCV-NS3-loaded dendritic cells. Results are presented as mean \pm S.E. (n = 6). Different letters refer to the significant differences between groups (P < 0.05).

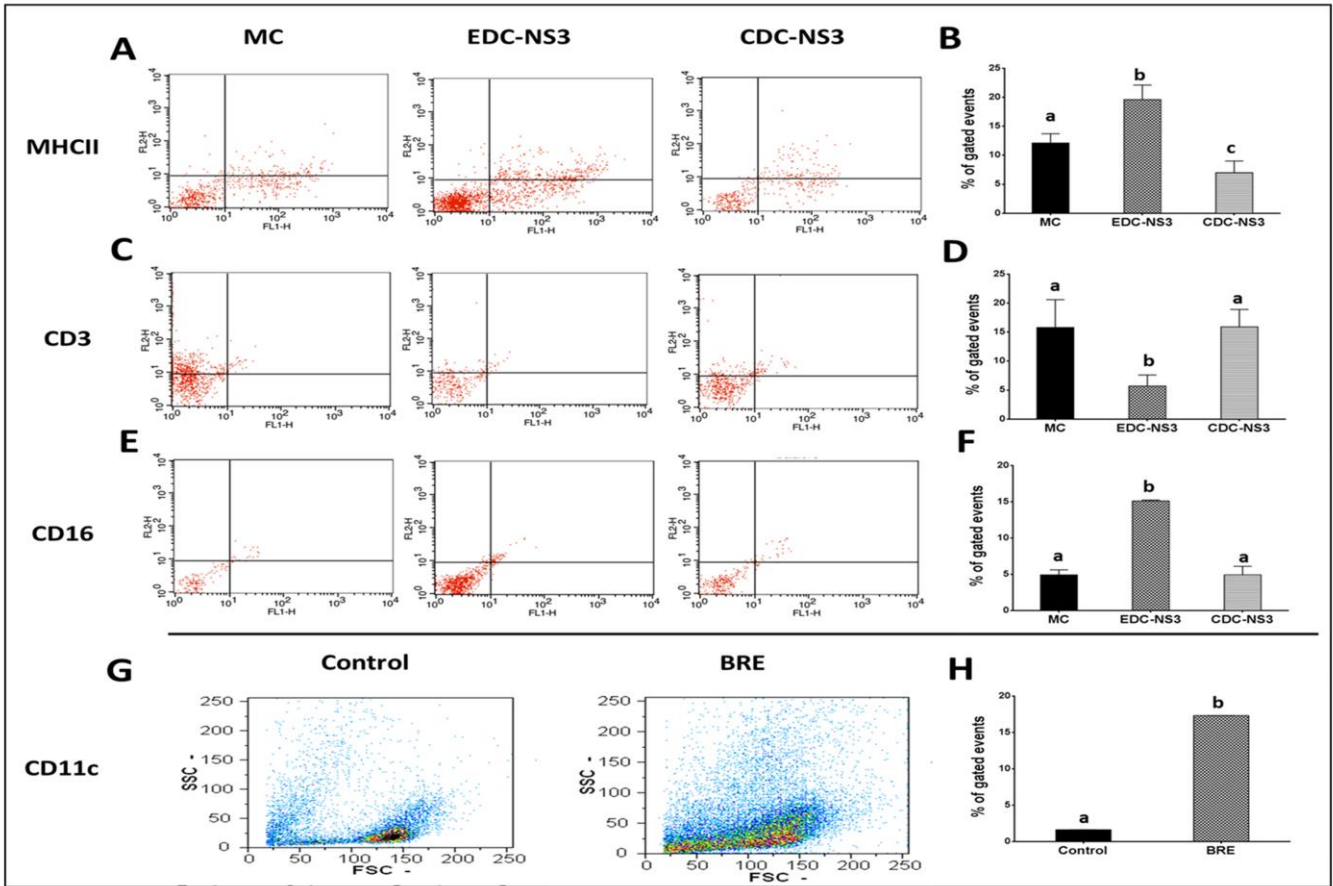


Fig. 3. Flow cytometric analyses for the immune cell markers in the splenocyte suspension after immunization. A, B) MHCII; C, D) CD3; E, F) CD16; G, H) CD11c protein levels on the surface of the purified DCs from untreated mice (control) and after iv injection of *B. vulgaris* root extract (BRE) at the dose of 45 mg/kg b.w. MHCII, major histocompatibility class II; CD, cluster designation; MC, medium control: mice were immunized with medium alone (RPMI-1640 basal medium containing 20% FBS); EDC-NS3, mice were immunized with expanded HCV-NS3-loaded dendritic cells; CDC-NS3, mice were immunized with control HCV-NS3-loaded dendritic cells. Results are presented as mean \pm S.E. (n = 6). Different letters refer to the significant differences between groups (P < 0.05).

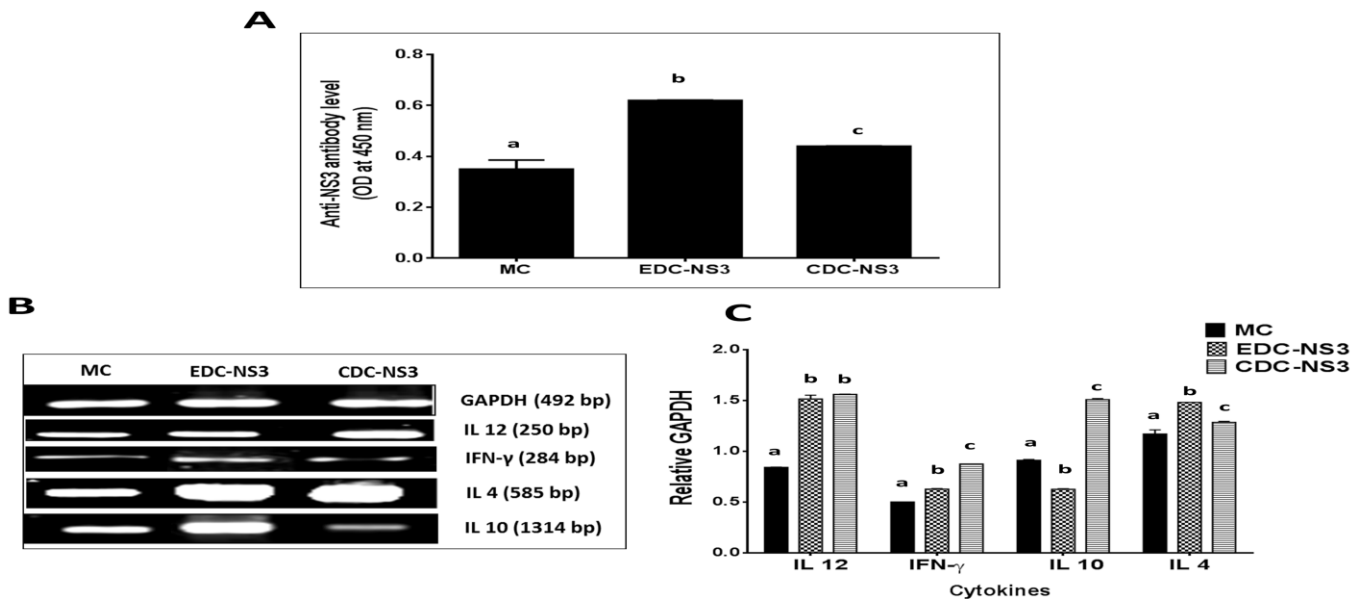


Fig. 4. PCR study for the expression of splenocyte cytokine genes and humoral immune responses after mice immunization. A) Gel electrophoresis images for the expression of GAPDH, IL-12, IFN- γ , IL-4 and IL-10 genes for the studied groups; B) Relative cytokines gene expression values; C) Anti-NS3 antibody levels in mice splenocytes of the studied groups. IL, interleukin; IFN, interferon; MC, medium control: mice were immunized with medium alone (RPMI-1640 basal medium containing 20% FBS); EDC-NS3, mice were immunized with expanded HCV-NS3-loaded dendritic cells; CDC-NS3, mice were immunized with control HCV-NS3-loaded dendritic cells. Results are presented as mean \pm S.E. (n=6). Different letters refer to the significant differences between the groups (P < 0.05).

DISCUSSION

The present study demonstrate the development of a HCV prophylactic vaccine candidate based on *in vivo* expanded and *ex vivo* HCV-NS3 loaded DCs. This vaccine shifts away from the traditional use of the whole inactivated or live attenuated viruses towards approaches based on protein antigens which are more easily characterized and defined. Moreover, it avoids the reduced and down-regulated DC function, as a consequence of HCV infection. This can be achieved by giving the essential maturation stimuli *in vivo* using BRE. Here we showed that the count of murine splenic CD11c+ DCs increased after BRE administration. This indicated that BRE had an ability to mature and proliferate murine splenic CD11c+ DCs *in vivo*. This may be related to the effect of its constituents such as berberine, chlorogenic acid, and triterpenoids [19,20]. Triterpenoid compounds are also able to promote maturation of DCs through enhancing MHCII and production of IL-12 [21].

In this study, CD11c+ DCs were incubated with HCV-NS3 protein *ex vivo* for vaccine preparation. These cells take up and process the protein antigens due to their abilities for internalization of macromolecules by macropinocytosis and receptor-mediated endocytosis. Exogenous antigens are usually ingested and processed for presentation to CD4+ T helper (Th) cells, in association with MHCII [22]. DCs can also cross-present endocytosed antigen on MHCI. The simultaneous appearance of antigen on MHC class I and II supports the generation of coordinated immune responses and the licensing of CTL by activated CD4+ T cells [23]. HCV-NS3 protein has been selected for CD11c+ DC loading, as it is one of the most conserved HCV proteases [1]. In addition, this viral antigen has been shown to be immunodominant and essential for the viral clearance [24]. Therefore, the development of a vaccine against HCV-NS3 protein has attracted considerable interests. The present study used this protein in combination with splenic DCs. Using this complex (protein-loaded DC), the immune responses were induced which exceeded the responses achieved by immunization with the protein only.

The results of the current study indicated that, immunization of mice with the prepared vaccine (EDC-NS3) and CDC-NS3 induced overexpression of IL-12 and IFN- γ which may be caused by the activation of these immunized cells [25]. Thus, the two immunization models activated Th1 response against HCV-NS3 protein [26]. Moreover, IL-12 protein level decreased significantly post-vaccination. Whilst, the protein levels of both IL-12 and IFN- γ did not increase significantly after CDC-NS3 immunization. This could be linked to the low half-life time of these cytokines [27]. The overexpression of IL-4 after immunization with the prepared vaccine and CDC-NS3 indicates the activation of Th2 response [28]. Therefore, both immunization models triggered Th1/Th2 differentiation and this could potentially play an important role in the outcome of HCV infection. Moreover,IDO specific activity decreased significantly after EDC-NS3 immunization that may be related to the overexpression of IL-4 [29]. The specific activity of this enzyme decreased after CDC-NS3 immunization; however, not significantly. This is possibly due to the overexpression of IL-10 in the animals which were administered with these cells [30]. The inhibition of IDO enhances T cell-activating ability of DCs [31] and increases the potency of this vaccine to overcome HCV infection.

We also showed that MHCII protein level was up-regulated significantly post-vaccination but decreased sharply after CDC-NS3 immunization. The upregulation of MHCII may be linked to the overexpression of IL-12, IFN- γ and IL-4, post-vaccination. The IFN- γ increases mRNA level of class II transactivator which is an essential factor for the expression of MHCII. Meanwhile, IL-4 can increase MHCII post-transcriptionally through decreasing its endocytosis and degradation; therefore, its stability and half-life are increased [32]. However, the overexpression of IL-10 after CDC-NS3 immunization may be the cause for the MHCII down-regulation [33]. The elevation in MHCII is important in CD4+ T cell responses through binding of T cell receptors (TCRs) to peptide/MHCII complexes expressed by APCs (DCs, macrophages and B cells). The nature and consequences of this response depend on the type of the APC. Hence, DCs prime naïve T cells and start immune responses and macrophages are recruited to the infection sites and activate the previously primed T cells. Whilst, presentation of B cell to T cells enhances the antibody production [34]. However, it appears that down-regulation of MHCII level makes the CDC-NS3 incapable of stimulating additional naïve CD4+ T cells. Furthermore, the CD16 level was significantly elevated as a result of the prepared vaccine immunization but did not change after CDC-NS3 immunization. This will lead to a strong cytolytic capacity of natural killer cells, post-vaccination through activation of the antibody-dependent-cell-mediated cytotoxicity pathway [35]. In contrast, no activation of this pathway after CDC-NS3 immunization was observed.

The down-regulation of IL-10 expression post-vaccination may be due to the overexpression of IFN- γ . A previous study has reported that IL-10 overexpression is detected during various persistent viral infections in humans. This is directly linked to the decreased T cell responsiveness and the disability to control the viral replication. Therefore, blockade of IL-10 during the persistent infection and after exhaustion of T cell leads to enhanced clearance of the virus due to the improved T cell functions [36]. Thus, the down-regulation of IL-10 expression post-vaccination boosts the host's immune response and helps to prevent the viral replication. On the other hand, there was a paradoxical overexpression of both IFN- γ and IL-10 after CDC-NS3 immunization. This could have occurred during the maximal activation of Th1 in the presence of IL-12 while Th1 overcame the increase of IL-12 by secreting IL-10 [37]. This difference between immunizations with EDC-NS3 and CDC-NS3 could be attributed to the role of BRE in the pre-activation of CD11c+ DC, *in vivo*.

The elevation in CTL activity, as well as the strong humoral immune response post-vaccination and after CDC-NS3 immunization are probably caused by a strong induction of CD4+ T cells and the activation of the immunized CD11c+ DCs. The activated CD11c+ DCs can stimulate B cells which need the second signal from the activated Th2 for antibody production [38]. Notably, the presence of strong CTL and humoral immune responses post-vaccination increase the potency of this vaccine for clearance of HCV infection. Moreover, the activation of CD4+ T cell and CTL responses post-vaccination were confirmed by the down-regulation of CD3. This may be due to the internalization and lysosomal degradation process that occurred to TCR/CD3 complex on the surface of these cells after their activation [39]. Conversely, the CD3 protein level was up-regulated after CDC-NS3 immunization although not significantly which may be related

to the overexpression of IL-10 that prevents this internalization process [40]. Interestingly, the prepared vaccine candidate may give effective immunoprophylaxis for different genotypes of HCV. This is owed to the inclusion of multiple epitopes from HCV-NS3 antigen in this vaccine which is conserved in all HCV genotypes. As a result, it is expected to be capable of eliciting broad-based cellular and humoral immune reactions which remains to be investigated.

In conclusion, BRE could effectively induce expansion of CD11c+ DCs *in vivo* and the immunization with these expanded cells after their loading with HCV-NS3, elicited strong cellular and humoral immune responses. We envisage such immune responses will achieve specific immune protection against HCV infection.

ACKNOWLEDGEMENT

This work was funded by The Science and Technology Development Fund (STDF; Egypt) with Grant number 1557. The authors acknowledge Prof. Salama El-darier, for providing the plant material for this study. Authors also thank Eiman El-wakeel and Marwa Abu-Serie for their kind assistances during this work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Guo X, Zhong JY, Li JW. Hepatitis C Virus Infection and Vaccine Development. *J Clin Exp Hepatol.* 2018;8(2):195-204. doi:10.1016/j.jceh.2018.02.003.
- Zhou Y, Zhang Y, Yao Z, Moorman JP, Jia Z. Dendritic cell-based immunity and vaccination against hepatitis C virus infection. *Immunology.* 2012;136(4):385-96. doi:10.1111/j.1365-2567.2012.03590.x.
- Colino J, Snapper CM. Dendritic cells, new tools for vaccination. *Microbes Infect.* 2003;5(4):311-9.
- Mokhber-Dezfuli N, Saeidnia S, Gohari AR, Kurepaz-Mahmoodabadi M. Phytochemistry and pharmacology of berberis species. *Pharmacogn Rev.* 2014;8(15):8-15. doi:10.4103/0973-7847.125517.
- Ghareeb DA, Elwakeel EH, Khalil R, Aziz MS, El Demellawy MA. Investigation of the Immunomodulatory effect of Berberis vulgaris on core-pulsed dendritic cell vaccine. *BMC Complement Altern Med.* 2016;16(1):325. doi:10.1186/s12906-016-1327-2.
- Taga MS, Miller EE, Pratt DE. Chia Seeds as a Source of Natural Lipid Antioxidants. *J Am Oil Chem Soc.* 1984;61(5):928-31. doi:10.1007/Bf02542169.
- Zhishen J MT, Jianming W. Research on antioxidant activity of flavonoids from natural materials. *Food Chem.* 1999;65:555-9.
- Shamsa F, Monsef H, Ghamooshi R, Verdian-rizi M. Spectrophotometric determination of total alkaloids in some Iranian medicinal plants. *Thai J Pharm Sci.* 2008;32:17-20.
- Bai XP, Qiu AY, Guan JJ. Optimization of microwave-assisted extraction of antihepatotoxic triterpenoid from Actinidia deliciosa root and its comparison with conventional extraction methods. *Food Technol Biotech.* 2007;45(2):174-80.
- Shaban NZ, El-Kersh MAL, El-Rashidy FH, Habashy NH. Protective role of Punica granatum (pomegranate) peel and seed oil extracts on diethylnitrosamine and phenobarbital-induced hepatic injury in male rats. *Food Chemistry.* 2013;141(3):1587-96. doi:10.1016/j.foodchem.2013.04.134.
- Habashy NH, Abu Serie MM, Attia WE, Abdelgaleil SAM. Chemical characterization, antioxidant and anti-inflammatory properties of Greek Thymus vulgaris extracts and their possible synergism with Egyptian Chlorella vulgaris. *J Funct Foods.* 2018;40:317-28. doi:10.1016/j.jff.2017.11.022.
- Zhu LX, Liu J, Ye Y, Xie YH, Kong YY, Li GD et al. A candidate DNA vaccine elicits HCV specific humoral and cellular immune responses. *World J Gastroenterol.* 2004;10(17):2488-92. doi:DOI 10.3748/wjg.v10.i17.2488.
- Coligan JE. Current protocols in immunology. New York: John Wiley and Sons. p. 101-4.
- Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods.* 1988;115(1):61-9.
- Encke J, zu Putlitz J, Geissler M, Wands JR. Genetic immunization generates cellular and humoral immune responses against the nonstructural proteins of the hepatitis C virus in a murine model. *J Immunol.* 1998;161(9):4917-23.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 1987;162(1):156-9. doi:10.1006/abio.1987.9999.
- Feng GS, Taylor MW. Interferon gamma-resistant mutants are defective in the induction of indoleamine 2,3-dioxygenase. *Proc Natl Acad Sci U S A.* 1989;86(18):7144-8.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-54.
- Kim TS, Kang BY, Cho D, Kim SH. Induction of interleukin-12 production in mouse macrophages by berberine, a benzodioxoloquinolizine alkaloid, deviates CD4+ T cells from a Th2 to a Th1 response. *Immunology.* 2003;109(3):407-14.
- Karasawa K, Uzuhashi Y, Hirota M, Otani H. A matured fruit extract of date palm tree (Phoenix dactylifera L.) stimulates the cellular immune system in mice. *J Agric Food Chem.* 2011;59(20):11287-93. doi:10.1021/jf2029225.
- Yu YL, Chen IH, Shen KY, Huang RY, Wang WR, Chou CJ et al. A triterpenoid methyl antcinat K isolated from Antrodia cinnamomea promotes dendritic cell activation and Th2 differentiation. *Eur J Immunol.* 2009;39(9):2482-91. doi:10.1002/eji.200839039.
- Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic Cells Use Macropinocytosis and the Mannose Receptor to Concentrate Macromolecules in the Major Histocompatibility Complex Class-II Compartment - down-Regulation by Cytokines and Bacterial Products. *J Exp Med.* 1995;182(2):389-400. doi:DOI 10.1084/jem.182.2.389.
- Nimmerjahn F, Milosevic S, Behrends U, Jaffee EM, Pardoll DM, Bornkamm GW et al. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. *Eur J Immunol.* 2003;33(5):1250-9. doi:10.1002/eji.200323730.
- Pape GR, Gerlach TJ, Diepolder HM, Gruner N, Jung M, Santantonio T. Role of the specific T-cell response for clearance and control of hepatitis C virus. *J Viral Hepat.* 1999;6 Suppl 1:36-40.
- Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev.* 2003;14(5):361-8.
- Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med.* 1996;184(2):747-52.
- Veeraseetukul P, Chutipongvivate S. Expression Patterns of Intracellular Cytokines in Splenic Dendritic Cells from BALB/c Mice Infected with Neospora caninum. *The Journal of Tropical Medicine and Parasitology.* 2007;30(2):43-50.
- Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. *Immunity.* 2003;19(5):739-48.
- Musso T, Gusella GL, Brooks A, Longo DL, Varesio L. Interleukin-4 inhibits indoleamine 2,3-dioxygenase expression in human monocytes. *Blood.* 1994;83(5):1408-11.
- Schandene L, Alonso-Vega C, Willems F, Gerard C, Delvaux A, Velu T et al. B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. *J Immunol.* 1994;152(9):4368-74.
- Grohmann U, Fallarino F, Bianchi R, Belladonna ML, Vacca C, Orabona C et al. IL-6 inhibits the tolerogenic function of CD8 alpha+ dendritic cells expressing indoleamine 2,3-dioxygenase. *J Immunol.* 2001;167(2):708-14.
- Pai RK, Askew D, Boom WH, Harding CV. Regulation of class II MHC expression in APCs: roles of types I, III, and IV class II transactivator. *J Immunol.* 2002;169(3):1326-33.
- Oh J, Wu N, Baravalle G, Cohn B, Ma J, Lo B et al. MARCH1-mediated MHCII ubiquitination promotes dendritic cell selection of natural regulatory T cells. *J Exp Med.* 2013;210(6):1069-77. doi:10.1084/jem.20122695.

34. Burrows PD, Cooper MD. Regulated expression of cell surface antigens during B cell development. *Semin Immunol.* 1990;2(3):189-95.
35. Galon J, Gauchat JF, Mazieres N, Spagnoli R, Storkus W, Lotze M et al. Soluble Fcγ receptor type III (FcγRIII, CD16) triggers cell activation through interaction with complement receptors. *J Immunol.* 1996;157(3):1184-92.
36. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol.* 2008;180(9):5771-7.
37. Gerosa F, Paganin C, Peritt D, Paiola F, Scupoli MT, AsteAmezaga M et al. Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-γ and interleukin-10. *J Exp Med.* 1996;183(6):2559-69. doi:DOI 10.1084/jem.183.6.2559.
38. Pavio N, Lai MMC. The hepatitis C virus persistence: how to evade the immune system? *J Biosciences.* 2003;28(3):287-304. doi:Doi 10.1007/Bf02970148.
39. Schrum AG, Turka LA, Palmer E. Surface T-cell antigen receptor expression and availability for long-term antigenic signaling. *Immunol Rev.* 2003;196:7-24.
40. Lowell CA. Src-family and Syk kinases in activating and inhibitory pathways in innate immune cells: signaling cross talk. *Cold Spring Harb Perspect Biol.* 2011;3(3). doi:10.1101/cshperspect.a002352.