RESEARCH ARTICLE

Ex vivo generation and maturation of dendritic cells from peripheral blood mononuclear cells of patients with chronic HCV or hepatocellular carcinoma using Toll-like receptor 3 ligand poly(I:C)

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ABSTRACT

Chronic hepatitis C virus (HCV) infection and hepatocellular carcinoma (HCC) resulted in dysfunction of the immune response, in particular, dendritic cells (DCs). Stimulation and reactivation of DCs could restore the immune responses in this disease. Our previous study revealed that a synthetic double-stranded RNA (polyI:C) recognized by toll-like receptor 3 (TLR3) induced a potent innate immune response and had an impact on DCs both ex vivo and in vivo. The current study was aimed to generate and maturate DCs from peripheral blood mononuclear cells (PBMCs) of healthy controls and patients with HCV and HCC. Fresh peripheral blood (PB) was collected from healthy controls, patients with chronic HCV or patients with hepatocellular carcinoma (HCC). PBMCs were isolated and cultured for 6 days in RPMI-1640 supplemented with GM-CSF and rhIL-4 (10 ng/mL each). Poly(I:C) was added to the culture on day 6 and the cells were harvested on day 7. Phenotypic analysis of DCs and their maturation markers were assessed using flow cytometry. DCs were generated from adherent PBMCs of healthy donors or patients with HCV or HCC. Interestingly, the addition of poly(I:C) induced expansion and maturation of DCs as evidenced by the expression of HLA-DR and CD11bCD11c surface molecules on the DCs generated from all groups. In conclusion, DCs can be ex vivo generated from control or patients with HCV or HCC in response to external stimulation. These results may be a promising tool for a therapeutic vaccine against HCV infection.

Keywords: Poly(I:C), Dendritic cells, HCV, HCC.

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INTRODUCTION

Induction and maintenance of specific immune responses against chronic hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) require the active participation of dendritic cells (DCs), which play a crucial role in linking innate and adaptive immunity (Yoneyama *et al.,* 2001; Iyonaga *et al.,* 2002). The function of DCs is impaired in both chronic HCV-infected and HCV-infected patients (Beckebaum *et al.,*

2003; Dolganiuc *et al.,* 2003; Ormandy *et al.,* 2006). The interaction between chronic HCV and DCs is not clearly understood; however recent studies have revealed reduced numbers of DCs, reduced cytokine production, as well as disruption of antigen presentation in patients who cannot clear the virus (van der Molen 2004; Duan *et al.,* 2005). This could explain the low virus-specific T- and B-cell responses in chronic HCV patients.

One reason of the dysfunctional activity of DCs in HCV and HCC patients could be due to the lower level expression of the Toll-like receptors (TLRs), which are important for pathogen recognition (Kanto and Hayashi, 2007). Several studies have reported that the treatment with different TLR agonists can induce activation of DCs, while other studies have shown that poly(I:C) is an excellent inducer for DCs activation and maturation (Salem *et al.,* 2009; Salem *et al.,* 2010). Thus, it is possible that stimulation and reactivation of DCs could restore specific immune responses against HCV disease. The use of DCs as cell-based therapy in cancer and other diseases are intensively being studied (Qiu *et al.,* 2005; Akbar *et al.,* 2006; Ma *et al.,* 2007; Cohen *et al.,* 2009). E*x vivo*-generated and matured DCs might be a potent candidate for cell-based HCV vaccine (Garcia *et al.,* 2005; Hong *et al.,* 2006). Therefore, this study aimed to *ex vivo* generate and maturate DCs from peripheral blood mononuclear cells (PBMCs).

MATERIALS AND METHODS

Study Population

Peripheral blood samples were collected either from control or from patients with HCV or HCC (n=20 each group). The patients were recruited from the Department of Tropical Medicine (Tanta University Hospital, Tanta University, Tanta, Egypt) upon the approval of the ethical committee of the Faculty of Medicine, Tanta University, Tanta, Egypt. Written consent was obtained from all patients and healthy control volunteers before blood sampling.

Inclusion and exclusion criteria

The inclusion criteria of HCV patients included the evidence for HCV infection using PCR (viral titer) and liver function tests and the inclusion criteria for HCC patients included cirrhosis. The exclusion criteria included any concomitant infectious diseases such as HBV, HIV, schistosomiasis, auto-immune diseases, or chemotherapy, as well as the previous intervention of HCC patients such as radiotherapy, chemotherapy, immunosuppressive therapy and other medical or surgical intervention.

Reagents and antibodies

Ficoll-Histopaque was purchased from Corning cellgro® (Mediatech, Inc., Manassas, VA, USA) and fluorescence-activated cell sorting (FACS) lysis buffer was purchased from BD Bioscience (San Diego, USA). RPMI-1640 medium (Biowest, South America) and Ca^{2+} Mg^{2+} free-phosphate buffered saline (PBS) were purchased from Biowhittaker® (Belgium). Recombinant human interleukin-4 (rhIL-4) and granulocyte macrophage-colony stimulating factor (GM-CSF) were purchased from BD Bioscience (San Diego, USA) and reconstituted in phosphate-buffered saline (PBS). Penicillin-streptomycin solution (Pen/Strep) and fetal bovine serum (FBS) were obtained from Euro-lone® (Italy). The toll-like receptor 3 ligands (TLR3L) poly(I:C) was purchased from Sigma Aldrich (St. Louis, MO, USA) and reconstituted in PBS. The following human monoclonal antibodies (hAbs) were purchased from eBioscience (San Diego, CA): anti-HLA-DR, anti-CD11b/MAC-1, and anti-CD11c.

Hematological analysis:

Leucocytes (WBC) ($10^{3}/cm^{3}$), hemoglobin percentage (HB%) and platelet count (PLT) ($10^{6}/cm^{3}$) was analyzed (Nihon Kohden automated hematology analyzer, Japan). Serum α-fetoprotein (AFP) concentration (µg/L) was measured by enzyme-linked immunoassay (EIA, Smith-Kline Biosciences, Philadelphia, USA).

Generation of PBMCs-derived DCs

Blood samples (10 mL) from healthy controls, HCV patients or HCC patients were collected and used to generate DCs as previously described (Kim *et al.* 2010). Briefly, PBMCs were isolated from blood samples by Ficoll-Paque density gradient centrifugation. Isolated

PBMCs were plated at 2×10^6 cells/mL in the complete RPMI-1640 medium. Cells were allowed to adhere for 2 hours at 37°C, and then the non-adherent cells were removed by pipetting medium gently over the adherent cells. Fresh medium containing 10 ng/mL of rhIL-4 and 10 ng/mL of GM-CSF were added, and the cells were cultured for 6 days to generate immature DCs (iDCs). During the day 6 of culture, 10 µg/mL of polyI:C was added to rhIL-4 and GM-CSF as maturation stimuli. On day 7, cell differentiation was monitored by inverted microscopy.

Flow cytometry analysis

Flow cytometry analysis for measuring the expression of different DCs surface marker molecules: CD11c, CD11b, and HLA-DR was performed with FACS Calibur (BD Biosciences, USA). Data analysis was done by FlowJo software. For staining, 1×10^6 cells were incubated in staining-buffer, with or without 1 µl of specific antibodies, for 30 min on ice in the dark. Stained cells were pelleted for 3 minutes at 2000 rpm and were washed twice with staining-buffer. A negative control with unstained cells was run first to determine the baseline fluorescence.

Statistical analysis

The data were expressed as a mean ± standard error (SE) of the mean. Differences between the groups were assessed using the one-way analysis of variance (ANOVA) and chi-square. Significant differences were indicated when $p < 0.05$.

RESULTS

Patient demographics

The present study was conducted on twenty patients with chronic HCV and twenty patients with HCC compared to twenty matched healthy controls. The age of patients ranged from 41 to 64 yrs with a mean of 53.3 ± 6.9 yrs in HCV patients, ranged from 46 to 66 yrs with a mean of 54.1 ± 7.1 years in HCC patients and ranged from 40 to 65 years with a mean of 51.3 ± 6.1 yrs in healthy controls (Table 1).

Complete Blood Count analysis and AFP concentration

The complete blood count (CBC) data and Serum α-fetoprotein (AFP) concentrations (µg/L) are summarized in Table 2 and Figure 1, respectively. The data showed a non-significant decrease in the total numbers of WBCs, however HB concentration, PLT count showed a significant decrease in patients with HCV or with HCC (Table 1), however, AFP levels showed significant increases in HCC as compared to control and HCV patients (Fig. 1).

Table 1: Demographic data of healthy control, HCV, and HCC patients

variables		Control	HCV	HCC
Age		51.3±6.1	53.3±6.9	54.1±7.1
Sex	**Male**	15 (75%)	11(55%)	13(65%)
	Female	5(25%)	9 (45%)	7(35%)

Data are represented as mean ± SE.

Table 2: CBC of control, HCV patients, and HCC patients.

Index	Control	HCV	HCC
WBC ($\times10^3/cm^3$)	7.20 ± 1.55	5.00 ± 2.15	4.92 ± 1.53
PLT ($\times 10^6/cm^3$)	0.22 ± 0.04	0.09 ± 0.04*	0.10 ± 0.04*
HB %	13.11 ± 0.87	9.64 ± 1.58*	10.12 ± 1.30*

Data are represented as mean ± SE. * $p < 0.05$.

***Ex vivo* generation of DCs from PBMCs**

By microscopic examination of PBMCs from a healthy control, HCV and HCC patients, cells underwent morphological changes on day 3 and differentiated to immature DCs on day 6. Addition of the TLR3 agonist poly(I:C) on day 6 for 24 hrs enhanced maturation DCs that showed clear branched projections at higher magnification when harvested on day 7 as compared to those without poly(I:C) (Fig.2).

Phenotypic characterization of DCs

To identify the differentiation of PBMCs towards DCs after 7 days of culturing with GM-CSF and IL-4, flow cytometric analysis of generated DCs was performed and their phenotypic analysis was identified by measuring the expression of CD11b CD11c.

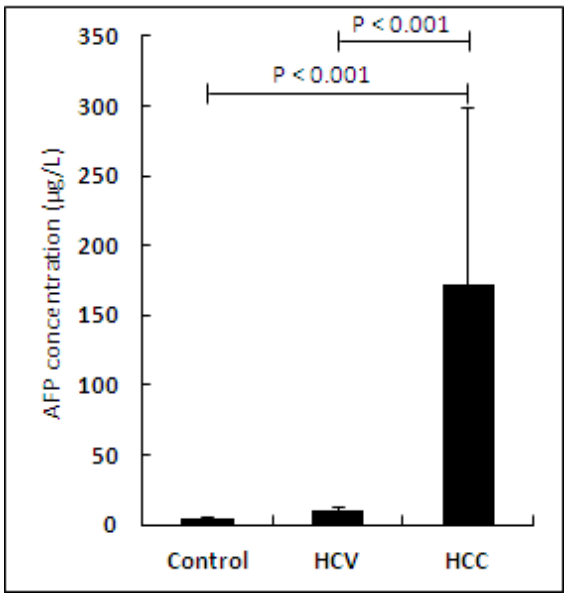

Figure 1: Serum α-fetoprotein (AFP) concentrations (µg/L) of control, HCV patients, and HCC patients. All data are represented as mean ± SE.

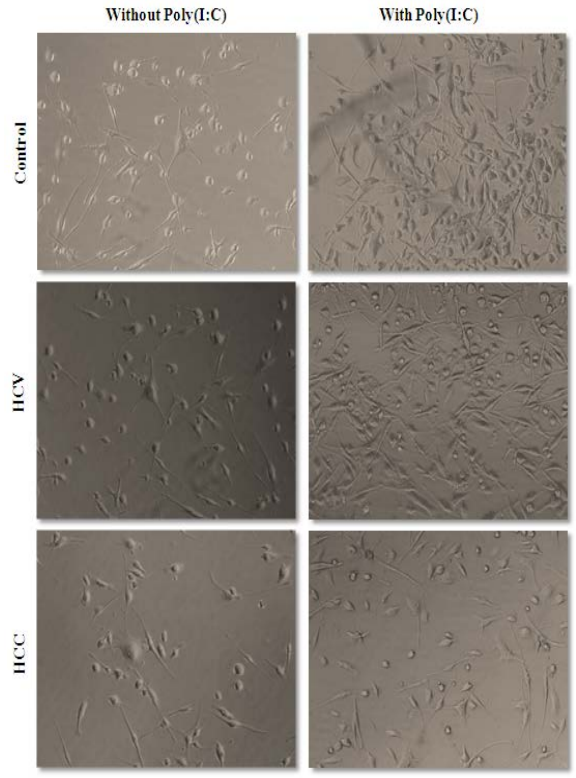

Figure 2: Morphology of DCs *ex vivo* generated from PBMCs of control or patients with HCV or with HCC loaded with or without Poly(I:C) on day 6 showing mature DCs with branched projections (10x).

The phenotypic analysis of DCs generated from controls, patients with HCV or with HCC showed high expression of $CD11b^+$, $CD11c^+$ with 53.06%, 43.92%, and 36.22% respectively (Fig. 3 A). Interestingly, the addition of poly(I:C) to culture medium on day 6 highly induced expression of $CD11b^+CD11c^+$ markers with 75.58% in healthy controls, 66.68 % in HCV patients and 57.92% in HCC patients (Fig. 3 B).

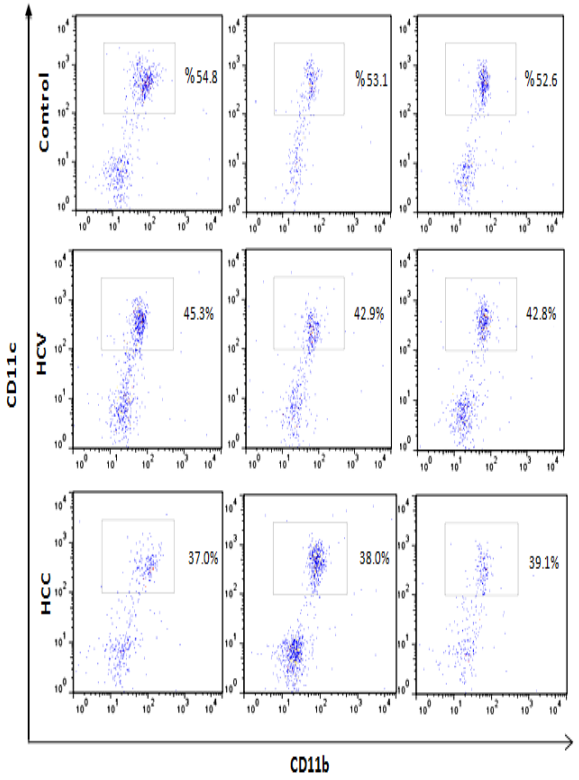

Figure 3A: Phenotypic analysis of DCs *ex vivo* generated from $PBMC_S$ of controls or from patients with HCV or with HCC unloaded with poly(I:C), showing expression of $CD11b^+CD11c^+$.

Maturation of DCs in the presence or absence of poly(I:C) was analyzed on day 7 as shown in Figures 4 and 5. DCs from healthy control, HCV and HCC patients expressed low level of HLA-DR typical marker for DC maturation (12.0%, 11.4% and 10.6% respectively), whereas addition of poly(I:C) highly induced the expression levels of $HLA\text{-}DR^+$ on DC with 83.5% in healthy control, 79% in HCV patients and 66.6% in HCC patients (Figs. 4 and 5).

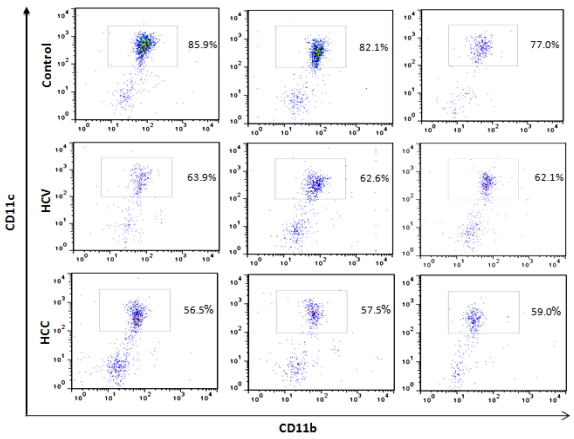

Figure 3B: Flow cytometric analysis of DCs *ex vivo* generated from PBMCs of controls or patients with HCV or with HCC loaded with poly(I:C) on day 6, showing expression of $CD11c^+$ $CD11b^+$.

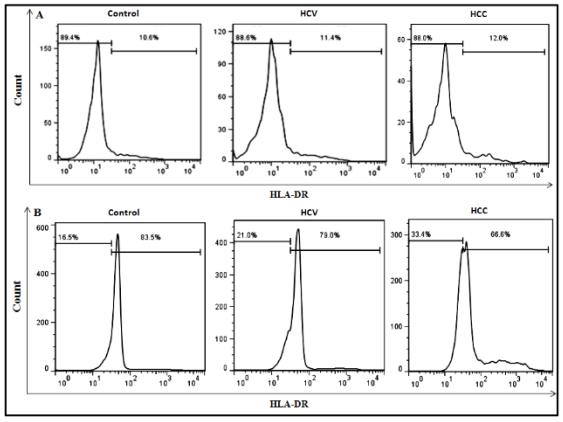

Figure 4: Flow cytometric analysis of HLA-DR expression of DCs *ex vivo* generated from PBMCs of control, patients with HCV or with HCC loaded with Poly(I:C) (A) or unloaded with Poly(I:C) (B).

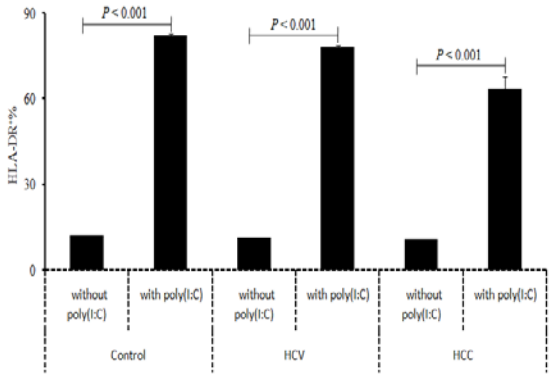

Figure 5: Flow cytometric analysis of $HLA\text{-}DR^+$ expression percentage of DCs *ex vivo* generated from PBMCs of control, patients with HCV or with HCC loaded or unloaded with Poly(I:C).

DISCUSSION

Induction of an effective antiviral immune response requires the active participation of host antigen presenting cells (APCs) such as DCs and macrophages (Steinman, 1991). As such defective antiviral immune response might be an important reason for HCV persistence (Larrubia *et al.,* 2014). In this regard, weak cellular immunity to HCV seen in individuals with chronic HCV infection could be due to alteration of DC function by the virus leading to weak, absent, or inappropriate T cell responses to HCV (Dolganiuc *et al.,* 2003; MacDonald et al., 2007). Strategies using DCs have been effective in overcoming self-tolerance when they are used as antitumor vaccines that may help to manipulate DCs differentiation and function exploiting the full potential use of DCs in clinical immunotherapeutic strategies (Kugler *et al.,* 2000; Haniffa *et al.,* 2013; Osorio *et al.,* 2015). Indeed, few studies analyzed the role of DCs in chronic HCV infection (Chehimi *et al.*, 1994; Chehimi *et al.*, 2002) and HCC patients using variety of maturation cocktails, antigen sources, treatment schedules and routes of administration providing a promising strategy for DC-based immunotherapy (Prieto *et al.,* 2015; Nakamoto, 2017).

In this study, we evaluated the role of poly(I:C) as a stimulatory agent to DCs *ex vivo*-generated from PBMCs of patients with chronic HCV or HCC as compared to healthy controls. This study can be considered as a foundation for the application of DC-based vaccine for HCV and HCC in future clinical trials.

DCs subjected to several changes during their maturation, including morphological changes, upregulation of costimulatory molecules, loss of endocytic and phagocytic receptors (Martín-Fontecha *et al.,* 2004; Trombetta and Mellman 2005; El-Ashmawy *et al.,* 2015) and these changes prepared DCs to interact successfully with T cells. Further, the direct activation of antigen-specific T cell by DCs resulted in colonies expansion and differentiation of T-cells leading to successful adaptive immunity (Piqueras *et al.,* 2006).

The current results showed that DCs generated from PBMCs of HCV or HCC patients expressed low levels of $CD11b^+$, $CD11c^+$ as compared to

their expression in subjects control, however, the addition of poly(I:C) enhanced the expression of $CD11b^+$, $CD11c^+$ in all groups. This finding is consistent with Ormandy *et al.* (2006); Rovati *et al.* (2008) who reported that *ex vivo*-generated DCs were positive for CD11c and CD11b as typical surface markers for myeloid DCs (Mellman, 2013).

The current results indicated that there was nonsignificant change in numbers of WBCs among HCV, HCC, or control. This agrees with Aino *et al.* (2014) and El-Zefzafy *et al.* (2015) who reported that WBC count was not significantly changed in both HCC and HCV patients in comparison to the control individuals. The present results further indicated that the number of DCs generated from PBMCs of HCC patients was lower than that generated from control individuals or HCV patients. This finding is in line with Chehimi *et al*., (2002) who indicated that numbers of DCs reduced in chronic HCV patients and this defect is subtle and can be modulated by the addition of maturational stimulus such as poly(I:C).

The results here displayed that DCs generated from PBMCs of HCV or HCC patients for 6 days and stimulated with poly(I:C) resulted in up-regulation of the expression of the maturation marker $HLA\text{-}DR^+$ on DCs.

In agreement with our findings, Barnes *et al.* (2008) postulated that poly(I:C) stimulated maturation of DCs. Moreover, several preclinical and clinical settings demonstrated that the use of TLR agonist-evoked rapid induction of DCs and in particular the TLR3 agonist poly(I:C) can induce activation of DCs (Wang *et al.,* 2008; Salem *et al.,* 2009; Salem *et al.,* 2010). Specifically, treatment of DCs *ex vivo*-generated from peripheral blood with the TLR3 agonist poly(I:C) enhanced DC activation (Renn *et al.,* 2006; Boullart *et al.,* 2008; Navabi *et al.,* 2009).

In conclusion, DCs can efficiently be *ex vivo* activated using TLR3 agonist poly (I:C) as it was seen by upregulation expression of their surface markers (i.e. $CD11c^+$, $CD11b^+$ and $HLA\text{-}DR^+$) in chronic HCV infection or HCC patients compared to control. In sum, these findings raise the possibility of using this potent strategy in DC-based therapy and may be a promising tool for a therapeutic or prophylactic vaccine against the hepatitis C virus or HCC. Further studies are needed to optimize the timing and concentration of poly(I:C) addition.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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