

The influence of North American Aboriginal ethnicity on pro-inflammatory and anti-inflammatory cytokine responses to IFN- α

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SUMMARY. North American Aboriginals have an enhanced propensity to clear HCV infection. Interferon (IFN)- α is a critical agent in the clearance of hepatitis C virus (HCV) and other viruses; therefore the influence of Aboriginal ethnicity on IFN- α responses was investigated in healthy Caucasian population control and Aboriginal cohorts. Cohort peripheral blood mononuclear cells produced similar levels of IFN- α upon culture with reovirus, an innocuous virus capable of triggering IFN- α synthesis. In addition, similar IFN- γ synthesis was observed in the presence IFN- α or reovirus. In contrast, Caucasian supernatants exhibited greater IL-10 levels ($P < 0.005$), contributing to the overall cytokine balance as assessed by IFN- γ /IL-10 ratios being consistently elevated in the Aboriginal cohort. The potential of HCV proteins to alter IFN- α cytokine induction was also investigated. Although there was some indication that HCV proteins might increase IFN- α induced IL-10 synthesis in Caucasians and conversely,

IFN- γ synthesis in Aboriginals, the addition of HCV proteins did not influence IFN- γ /IL-10 ratios. Finally, signal transducer and activator of transcription (STAT) 3 nuclear translocation was examined by western blot because it is a required intermediate in IFN- α induced IL-10 synthesis. Supporting the differential IL-10 production, IFN- α and core synergistically enhanced STAT3 nuclear translocation in Caucasian ($P < 0.05$); whereas, nuclear translocation of STAT3 remained unchanged in Aboriginal cells. Taken together, these findings suggest that ethnicity may influence certain responses to IFN- α , possibly even in the presence of viral agents. These differences could impact early immune events allowing for enhanced viral clearance in Aboriginal populations.

Keywords: HCV, IFN- α induced cytokine responses, North American Aboriginal ethnicity.

INTRODUCTION

The hepatitis C virus (HCV) infects approximately 170 000 000 people worldwide. In North America, 65–85% of acutely infected individuals develop chronic infection [1]. Progression from acute to chronic infection has been linked to the ability of HCV to alter interferon (IFN)- α activity and other immune responses [2]. In addition to inhibiting viral replication, IFN- α participates in the earliest defence against viral infections by promoting pro-inflammatory immunity through the production of IFN- γ and other cytokines [3,4].

Abbreviations: HCV, hepatitis C virus; NS, nonstructural; PBMC, peripheral blood mononuclear cells; STAT, signal transducer and activator of transcription; β -gal, β -galactosidase.

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Clearance of HCV infection has been associated with the induction of pro-inflammatory IFN- γ synthesis [5–7]. Conversely, in progression to chronic HCV infection, the cytokine balance is often shifted in favour of production of the anti-inflammatory cytokine IL-10 by peripheral blood mononuclear cells (PBMC) [8–10]. The potential for IL-10 to influence the outcome of HCV infection is further supported by a greater frequency of IL-10 polymorphisms in the promoter region of the IL-10 gene associated with high IL-10 production in individuals with chronic infection [11–14]. IFN- α induction of IL-10 acts as a natural feedback mechanism limiting the detrimental aspects of IFN- α activity [15]. However, HCV augmentation of IFN- α induced IL-10 may limit IFN- α based treatment [13] and may also impair the ability to develop effective immunity early in infection.

Thus, it is of interest that North American Aboriginal groups who are reported to have an enhanced ability to clear HCV infection, also display a reduced genetic tendency to produce IL-10 [16,17]. Genetics and environment likely cooperate in the higher prevalence of acute HCV infection

within North American Aboriginal populations [18–21]. Nonetheless, observations by our group and others suggest that Aboriginal populations have higher rates of spontaneous HCV clearance, as indicated by a lower than predicted prevalence of detectable HCV RNA in HCV antibody positive individuals [18,22–24]. Although the mechanism(s) responsible for this finding remains/remains to be elucidated, in recent studies, we demonstrated that Aboriginal people have a lower genetic predisposition to produce IL-10 according to defined single nucleotide polymorphisms [16,17].

This study sought to investigate the impact of ethnicity on cytokine synthesis in response to exogenous IFN- α and reovirus, a strong inducer of IFN- α . The ability of HCV proteins to modulate IFN- α induced cytokine production was also investigated. PBMC isolated from viral-naïve Caucasian (control population) and Aboriginal subjects were evaluated, allowing the study to be focused on early innate immunity prior to immune deregulation that occurs within weeks of HCV infection.

MATERIAL AND METHODS

Subjects

This study was approved by and conducted according to the policies of the University of Manitoba Ethics Board. Healthy, viral-naïve Caucasian ($n = 32$) and Aboriginal ($n = 23$) volunteers were recruited. Aboriginal volunteers were recruited from First Nation groups in Manitoba. Inclusion criteria included subjects 18 years of age or older, negative for antibody to HCV (by enzyme immunoassay and recombinant immunoblot assay) and hepatitis B core antigen, the absence of immunosuppressive or anti-inflammatory treatment and no evidence of other conditions known to alter IL-10 synthesis (pregnancy, infections, diabetes or cancer).

Cellular assays

Peripheral blood mononuclear cells were isolated as previously described and cultured at 2.5×10^5 cells/well in 96-well plates with 10% foetal calf serum/RPMI [16]. To determine the influence of ethnicity on IFN- α production, PBMC were cultured with live reovirus (kindly provided by Dr Kevin Koombas, UM). Like HCV, reovirus is a positively stranded RNA virus. To examine the influence of ethnicity on IFN- α induced cytokine synthesis and signal transducers and activator of transcription (STAT)3 expression, PBMC were cultured as indicated with IFN- α (1000 IU/mL). The impact of HCV proteins on IFN- α responses was evaluated by the addition of purified proteins (1 μ g/mL, Virogen) β -galactosidase (β -gal) or β -gal-linked recombinant HCV proteins, core or nonstructural (NS)3.

ELISA

Tissue culture supernatants were collected at 24 h for the analysis of IFN- α and IL-10 concentrations by ELISA (Biolegend, San Diego, CA, USA). IFN- γ levels were examined in supernatants collected at day 6. ELISAs were performed using internal standards calibrated against recombinant human cytokine (Preprotech Inc., Rock Hill, NC, USA). Sample concentrations of cytokine were calculated from a minimum of three points falling on the linear portion of titration curves calibrated against recombinant cytokine standards serially diluted on each plate. Data were evaluated by Mann–Whitney.

Western blot

Peripheral blood mononuclear cells (1×10^6 cells/well) were cultured with β -gal, IFN- $\alpha \pm \beta$ -gal, core, IFN- $\alpha \pm$ core. As previously described [16], cultures were washed, lysed and total protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Mississauga, ON, USA). Nuclear fractions were extracted. The amount of STAT3 translocated to the nucleus was detected by Western blot with antibodies purchased from Santa Cruz Biotech (Santa Cruz, CA, USA) [25]. Western blots were scanned with ImageJ version 1.37, NIH software. Computed optical densities were evaluated by Mann–Whitney [26].

RESULTS

Ethnicity does not affect PBMC production of IFN- α

North American Aboriginals appear to clear HCV infection more readily than Caucasians [18,22–24]. Here, we evaluated whether ethnic differences in the ability of PMBC to produce the endogenous antiviral agent IFN- α might participate in the disparity reported in HCV outcomes. In the laboratory, PBMC can produce IFN- α when stimulated with live virus [27]. To evaluate the capacity of Caucasian and Aboriginal cells to produce IFN- α , PBMC were cultured with live reovirus, a positive stranded RNA virus known to activate IFN- α synthesis [27]. Reovirus enhanced IFN- α production in Caucasian and Aboriginal cells 20- and 12-fold, respectively (Fig. 1). However, no significant difference in IFN- α synthesis was observed between the two cohorts.

Ethnicity selectively influences IL-10 synthesis in the presence of IFN- α

The influence of ethnicity on IFN- α responses was examined following the culture of PBMC with IFN- α (Fig. 2a). Reovirus was included in this assessment to evaluate potential responses to endogenous IFN- α synthesis. Culture supernatants were harvested and analysed for IFN- γ and IL-10 levels. Although reovirus was capable of inducing 2–3 times

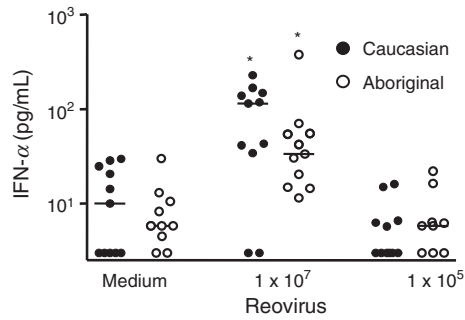


Fig. 1 Ethnicity does not influence the production of IFN- α . PBMC from healthy Caucasian and Aboriginal individuals were cultured with medium alone or reovirus. IFN- α levels were assessed in supernatants harvested at 24 h by ELISA. Medians indicated by line. Significant differences in IFN- α synthesis between culture medium and reovirus within a cohort indicated by * $P < 0.05$.

the amount of IFN- γ than IFN- α , IFN- γ secretion was similar between the cohorts independent of stimulation. In contrast, IFN- α and reovirus had a comparable affect on IL-10 resulting in elevated IL-10 levels in Caucasian cultures relative to Aboriginal cultures (IFN- γ , $P \leq 0.005$; reovirus, $P \leq 0.05$).

In HCV infection, IFN- α activity has been shown to be counter regulated by viral proteins. The impact of HCV proteins on cytokine activity in response to IFN- α was determined by co-culture with IFN- α (Fig. 2b). IFN- α induced IFN- γ synthesis was slightly enhanced by the addition of HCV proteins in Caucasian cells (2.4-fold). However, in the Aboriginal cohort, IFN- α induced IFN- γ synthesis was significantly enhanced ($P < 0.05$) by core resulting in a 4.5-fold increase in IFN- γ levels. The co-culture of IFN- α with core or NS3 resulted in greater increases in IL-10 synthesis by Caucasian cells; whereas, in Aboriginal samples, IL-10 was only enhanced by co-culture with core.

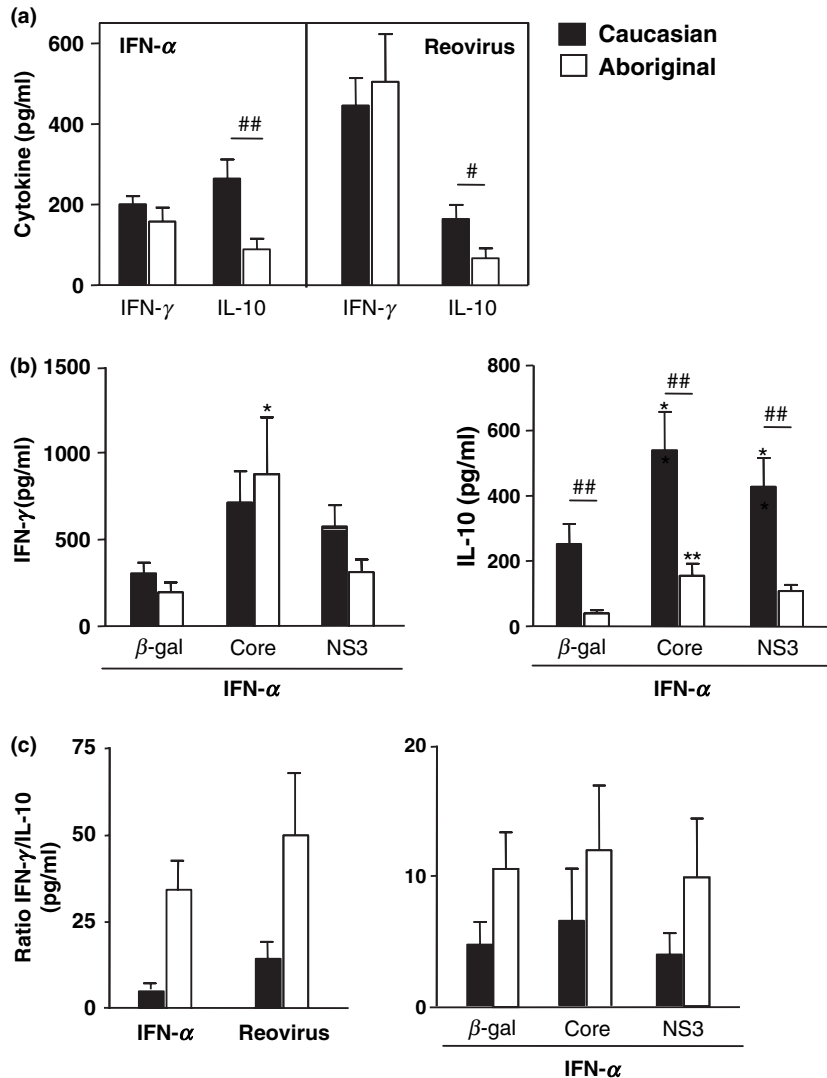


Fig. 2 The influence of HCV proteins on cytokine production in response to IFN- α . (a) PBMC isolated from healthy Caucasians (■) and Aboriginals (□) were cultured with IFN- α (1000 U/mL) and reovirus (10^7). Supernatants were analysed for IFN- γ and IL-10 concentrations by ELISA. (b) Caucasian and Aboriginal PBMC were cultured with IFN- α (1000 U/mL) and HCV proteins (1 μ g/mL). Supernatant cytokine concentrations were determined for IFN- γ (day 6) and IL-10 (day 1). (c) For each individual, the IFN- γ /IL-10 ratio was calculated. For all graphs, mean \pm SE are shown. Significant differences between β -gal and HCV proteins within a cohort indicated by * $P < 0.05$, ** $P < 0.005$. Significant differences between cohorts indicated by # $P < 0.05$, ## $P < 0.005$.

To estimate how ethnicity might affect the overall cytokine milieu in response to IFN- α and reovirus, IFN- γ /IL-10 ratios were calculated. Although the values did not reach statistical significance, Aboriginal's cell cytokine profiles appeared skewed in favour of IFN- γ /IL-10 relative to Caucasian cells (Fig. 2c). HCV proteins did not appear to affect these values.

IFN- α and core synergistically enhance STAT3 nuclear translocation in PBMC from Caucasian subjects

IFN- α induced IL-10 production is dependent on STAT3 and interferon regulatory factor (IRF)1 activation [15]. Hence, the participation of these upstream signalling molecules in the observed ethnic differences in IL-10 synthesis was evaluated. The capacity of IFN- α and core either individually or synergistically to alter phosphorylated STAT3 nuclear translocation was assessed by the addition of IFN- α , core and IFN- α \pm core to PBMC isolated from healthy Caucasian and Aboriginal individuals. Protein nuclear fractions were isolated and evaluated for the presence of STAT3 by Western

blot analysis. As shown in Fig. 3, IFN- α and core synergistically enhanced STAT3 nuclear translocation in Caucasian ($P < 0.05$), but not Aboriginal, PBMC. No differences in IRF1 activation were observed (data not shown).

DISCUSSION

Emerging data strongly suggest that North American Aboriginal ethnicity influences the outcome of HCV infection, potentially reflecting differences in host immunity against the virus [18,22–24]. Host defence against many viral infections is reliant on IFN- α [3]. Modulation of IFN- α responses by HCV and other viruses appears to contribute to adverse outcomes. Here, we observed that Caucasian and Aboriginal ethnicity influenced IFN- α induced cytokine synthesis and the capacity of HCV proteins to differentially regulate those responses. The participation of virally naïve, healthy Caucasian and Aboriginal individuals in this study enabled us to focus on ethnic differences in innate immunity that would be relevant upon initial exposure to the virus.

Interferon-alpha activity is initiated by the production of endogenous IFN- α upon viral replication. Therefore, we evaluated whether ethnic differences in the capacity to produce IFN- α existed, by infecting PBMC with live reovirus, a positive stranded RNA virus like HCV. Reovirus is also a strong inducer of IFN- α synthesis. Reovirus infection resulted in similar levels of IFN- α secretion by Caucasian and Aboriginal cells, suggesting that differences in viral outcomes between the ethnic cohorts do not appear to involve the ability to produce IFN- α (Fig. 1).

As IFN- α production between the ethnic cohorts did not differ; IFN- γ and IL-10 synthesis in response to the addition of exogenous IFN- α and reovirus were evaluated (Fig. 2a). No significant cohort differences were observed for IFN- γ synthesis. In contrast, IL-10 production was substantially enhanced from Caucasian compared with Aboriginal samples. To evaluate the potential of ethnicity to influence HCV modulation of cytokine synthesis in response to IFN- α , IFN- α and HCV proteins were co-cultured because of the limited HCV culture models available (Fig. 2b). IFN- α induced IFN- γ synthesis was somewhat enhanced by HCV proteins. However, a more substantial impact on IL-10 production was observed in that there was significantly higher IL-10 synthesis by Caucasian compared with Aboriginal cells. The overall influence of ethnicity on the pro-inflammatory/anti-inflammatory profile was estimated by calculating the IFN- γ /IL-10 ratios (Fig. 2c). The trend suggested that Aboriginal cytokine profiles favour IFN- γ over IL-10 synthesis relative to Caucasian cells. A greater tendency towards a pro-inflammatory cytokine milieu, as in the case of IFN- γ synthesis, is considered a key factor in the ability to clear HCV infection [5,28].

IFN- α induced IL-10 synthesis requires STAT3 and IRF1 activation [15]. HCV core protein is capable of activating STAT3 in mouse and human hepatocyte models [29–31].

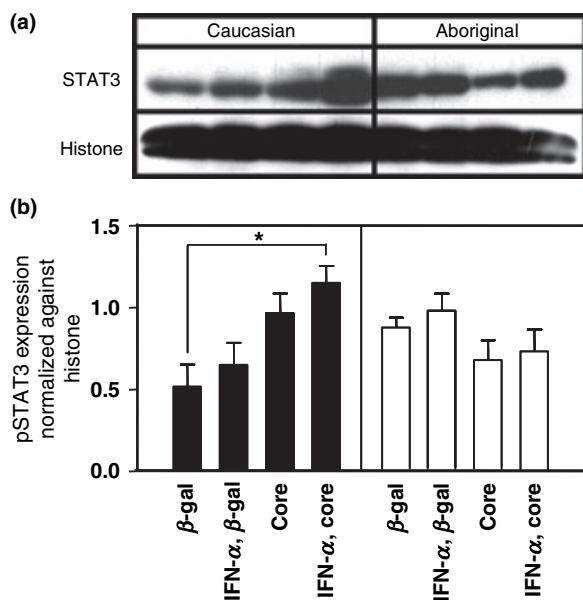


Fig. 3 Core and IFN- α synergistically enhance STAT3 translocation into the nucleus of Caucasian, but not Aboriginal, cells. PBMC were cultured with β -gal, IFN- α plus β -gal, core or IFN- α plus core. Cells were harvested at 24 h. The presence of STAT3 and histone in nuclear proteins fractions was detected by western blot. (a) One experiment representative of three for Caucasians and four Aboriginals is shown. (b) Nuclear pSTAT3 expression normalized against histone loading control. Mean \pm SE of normalized values are shown. Significant difference between IFN- α and core induced pSTAT3 translocation relative to β -gal indicated by * $P < 0.05$.

Therefore, the potential influence of ethnicity on STAT3 nuclear translocation in the presence of IFN- α and core was evaluated here (Fig. 3). The enhanced IL-10 synthesis by PBMC in the presence of IFN- α and core in the Caucasian cohort was supported by the observation that IFN- α and core together synergistically enhanced STAT3 nuclear translocation in Caucasian PBMC. In marked contrast, STAT3 nuclear translocation was not altered in Aboriginal cells following culture with IFN- α and/or core. No cohort differences in IRF1 activation were observed (data not shown). It should be noted that STAT3 also influences IL-6 production and acute-phase responses [32]. These aspects of STAT3 regulation will be investigated in future studies.

Deregulation of IFN- α activity early in infection is thought to negatively influence the development of effective immunity against HCV thereby promoting chronic infection [5–7]. The capacity of HCV to redirect IFN- α activity in support of increased IL-10 synthesis could limit the pro-inflammatory influence of IFN- α , contributing to HCV persistence. Recent clinical observations by our group and others suggest that despite increased rates of HCV exposure in Aboriginal populations [18–21], rates of progression to chronic infection are lower in these individuals [18,22–24]. The higher rates of clearance in Aboriginals may reflect a decreased susceptibility to HCV redirection of early IFN- α responses during the early phase of infection. It is tempting to speculate that in chronic infection, redirection of IFN- α activity towards IL-10 synthesis would reduce the activation of IFN-induced genes that directly inhibit viral replication [33] or that promote effective T-cell responses.

In conclusion, these findings suggest that ethnicity can influence cytokine responses to IFN- α responses, and in some cases, subsequent modulation by HCV proteins. These findings highlight the capacity of ethnicity to affect immune events and potentially influence the natural history of HCV infection. Ongoing studies in our laboratory are investigating whether these findings in a model of IFN- α responses early in infection translate into clinical outcomes in HCV chronically infected Aboriginal and Caucasian patients treated with IFN-based therapy.

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