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Reduced Type 1 and Type 2 Cytokines in Antiviral Memory T Helper Function Among Women Coinfected with HIV and HCV

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Abstract

Bias in cytokine responses has been proposed as a contributing mechanism to pathogenesis in persistent HIV or hepatitis C virus (HCV) infections. We investigated whether coinfection with HCV modifies the profile of antigen-specific cytokine secretion in women persistently infected with HIV compared to women with single HIV or HCV infection. The T helper response to HIV, HCV and cytomegalovirus (CMV) as a positive viral control was dominated by type 1 cytokines (interleukin- [IL] 2, interferon- [IFN] γ and tumor necrosis factor- [TNF] α), with IFN- γ as the most abundantly secreted. IL-4, IL-5 and IL-10 were low in healthy controls and patients. Robust CMV-specific responses contrasted with curtailed HCV-specific responses in HCV-infected women. The overall anti-viral profile was dominated by Th1 cytokines even in coinfecting women but both type 1 and type 2 responses were reduced in HIV-infected women and more extensively in women with HCV/HIV coinfection.

Keywords

T helper cells; cytokines; infectious diseases; hepatitis C virus; HIV

INTRODUCTION

Factors produced by T helper cells are important for the immune control of persistent viral infections such as HIV and HCV. Since the initial description of a dichotomy in the factors, e.g. cytokines produced by T helper cells (1), the role of polarized type 1 or type 2 responses in antiviral immunity, particularly during the chronic phase of infection has been debated. Potent HIV-specific T helper responses are detected during the acute phase of HIV (2–4) infection, or in subjects with long-term nonprogression (4, 5). For HCV, resolution of infection is associated with robust CD4+ responses to HCV (6–8). In contrast, memory T helper responses can rarely be detected during chronic infection with either HIV (3, 4) or HCV (7–9) infection.

A feature of HIV infection is the progressive loss of CD4+ T cells (10, 11). Consequently, polyclonal as well as antigen-specific proliferative responses are compromised, a functional alteration likely preceding any detectable decline in CD4+ T cell numbers (12–14). The

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dysfunction in the T cell compartment is also evident in substantially reduced cytokine expression, prominently IL-2 and to a lesser extent IL-4 and IL-10 (15–17).

The incidence of HCV coinfection is relatively high in HIV-infected patients (18, 19), and progression to HCV-mediated liver disease is faster in coinfecting individuals (20–22). Recent studies indicate that T helper cell proliferation to HCV is more extensively compromised compared to HIV in coinfecting patients (23). However, whether the cytokine response is involved in accelerated disease progression during coinfection or whether the cytokine profile is altered by HCV infection in HIV-infected patients has not been extensively investigated. We aimed to further the understanding of cell-mediated reactivity in HCV/HIV coinfection by investigating the Th1/Th2 cytokine profile in blood lymphocytes. This study compared the capacity for secretion of IL-2, IL-4, IL-5, IL-10, TNF- α and IFN- γ ; in response to HIV, HCV and CMV as positive control among women with single HCV or HIV infection and women dually infected with both viruses and in healthy subjects seropositive for CMV. Responses to HCV and HIV were detected in about one third of women infected with HCV or HIV respectively. Responses to CMV were more frequent and more potent than responses to HCV or HIV, with a clear type 1 profile and IFN- γ as the cytokine most abundantly secreted. The frequency of IFN- γ , TNF- α and IL-10 responders was significantly reduced among coinfecting (HCV+ HIV+) women. Levels of TNF- α and IL-10 were also significantly reduced in coinfecting women compared to women with single HIV infection. Potent cytokine responses to CMV contrasted sharply with curtailed responses to HCV in women singly infected with HCV. Overall, type 1 cytokines dominate the profile of antigen-specific T helper responses both in patients persistently infected with the highly pathogenic viruses HCV and HIV, and in healthy CMV-seropositive controls.

MATERIAL AND METHODS

Subjects

This is a cross-sectional study nested within the Women's Interagency HIV Study (WIHS, 24). HIV+ and HIV- women with confirmed HCV infection status enrolled in the Southern California WIHS were evaluated as part of one of their scheduled visits. Inclusion criteria were confirmed HIV or HCV infection and female gender, and the patients were sequentially added to the study during Nov 2000 and Oct 2003. Immune responses to viral antigens were investigated in a group of 70 infected women, of which 33 were coinfecting (HIV+/HCV+), 27 were HIV+ only and 10 were HCV+ only. Healthy donors ($n = 43$) recruited for a vaccine study and without any medical condition were used as a control group. The median age for the control group was 48.5 years, 38/43 were women (88.4%) and 38/43 were Caucasians (88.4%). Written consent was obtained from all subjects, and the institutional review boards of the University of Southern California and the City of Hope Medical Center approved the study. For analysis of responses to CMV, serostatus of all participants was verified by ELISA (Sigma Diagnostics).

HIV and HCV Viral Loads

Plasma HIV RNA levels were determined using NASBA (Nucleic Acid Sequence Based Amplification) assay (NucliSens, bioMerieux, Inc., lower limit of detection 25 copies/mL). HCV RNA was detected by PCR assay using Amplicor HCV Monitor 2.0 Roche Diagnostics®, with a dynamic range of 600–850,000 IU/mL.

PBMC Stimulation

Fresh PBMC were cultured in serum-free medium (X-vivo 20, BioWhittaker) at a density of 1×10^5 cells/well in 96-well round-bottomed plates. Antigens used in cell proliferation were

recombinant HIV-1 p25/p24 gag (5 µg/mL, Viral Therapeutics Inc), recombinant HCV NS3 (1 µg/mL, Fitzgerald Industries), CMV whole virus lysate (10 µg/mL, Microbix Biosystems Inc) or medium alone for 3–5 days. Both recombinant p24/p25 and NS3 were produced in yeast. Phytohaemagglutinin (1 µg/mL, PHA, Murex Biotech Limited) was used as polyclonal control.

Cytometric Bead Array (CBA) Assay

Cell culture supernatants for cytokine profile were recovered after 3 and 5 days of stimulation and kept frozen until batch analysis. The CBA assay was performed in undiluted tissue culture supernatants, except for samples after PHA stimulation that were tested at 1:2 dilutions, as described by the supplier (BD Biosciences). For this assay, soluble cytokines are captured on microparticles and then measured using a fluorescence-based detection system and flow cytometry analysis as described previously (25, 26). A series of 10 dilutions from cytokine standards were run in each assay for generation of standard curves. Samples were analyzed in a FACSCalibur flow cytometer using the BD CBA Analysis Software. For statistical analysis, results are presented as cytokine indexes by calculating the ratio between stimulated and nonstimulated cultures. For the differentiation between responders and low/nonresponders, responders were classified as such if a cytokine result was $\geq \text{mean} \pm 3\text{SD}$ of background release in PBMC from healthy controls ($n = 43$). For each study subject, the highest value for each cytokine (either day 3 or day 5) was used for statistical analysis.

Depletion of CD4+ T Cells

PBMC were depleted of CD4+ T cells by immunomagnetic separation. Briefly, cell suspensions were incubated for 30 min at 4°C with anti CD4-coated magnetic beads (Dynabeads, Dynal) at a 10:1 ratio. Bound cells were removed by application of a magnetic field. The resulting preparation after one-round of separation contained 1.5–2% CD4+ T cells. PBMC depleted of CD4+ T cells were recounted and stimulated with antigens or PHA control, and supernatants were tested for secreted cytokines as described above.

Study Design and Statistical Analyses

Student's *t*-test was used for the comparison of continuous variables between two groups. Analysis of variance (ANOVA) was used for the comparison of continuous variables between three or more groups. Further comparisons between any two groups within these multiple groups were conducted using Scheffe's method. Proportions between categorical groups were compared using Chi-squared test or Fisher's exact test. Pearson's correlation coefficient was used to study the relationship between Th1 and Th2 cytokines. General linear regression model was used to explore the association between Th1 responses and other parameters. In these analyses, when $\log_{10}$ transformation was used, the value of 0 was assigned for those with undetectable measures. Two-tailed *p* value < 0.05 was considered statistically significant.

RESULTS

Viral Load and CD4+ Counts in Women with Single HIV or HCV Infection and Coinfected Women

A group of 70 women singly or dually infected with HIV and/or HCV were evaluated for virus-specific T helper cell function. Of these, 33 were coinfecting (HIV+/HCV+), 27 were infected only with HIV (HIV+) and 10 were infected only with HCV (HCV+). Demographic and clinical characteristics of the study groups are described in Table I. Age and ethnicity were similar among patient groups. IV drug use was higher among HCV+ women ($p <$

0.0001), either coinfecting with HIV or not. PBMC from 43 healthy individuals were used as control group, of which 22 were seropositive for CMV. Among study patients, 97% of HCV+/HIV+, 93% of HIV+ and 90% of HCV+ were seropositive for CMV.

CD4+ T cell counts were similar in HIV+/HCV+ and HIV+ women ($p = 0.56$), but lower than in HCV+ women ($p < 0.0001$). Also, mean CD4 nadirs were similar between HIV+/HCV+ and HIV+ women but lower than in HCV+ women ($p < 0.0001$). Mean ALT value among HIV+/HCV+ women was significantly higher than in HIV+ women ($p < 0.05$), but similar to HCV+ women ($p = 0.81$).

Mean log₁₀ HIV-1 viral load was similar in HIV+/HCV+ and HIV+ women (2.70 and 2.52 respectively, $p = 0.59$). Also, mean log₁₀ HCV RNA levels were similar in HIV+/HCV+ women and HCV+ women (5.33 and 5.19 respectively, $p = 0.80$).

CD4 Responses Specific for HCV and HIV

The cytokine profile of T helper cells in HIV and/or HCV infected women in response to HIV p24 and HCV NS3 proteins was investigated. For each cytokine, responders were classified as such if a cytokine result was $\geq$ mean $\pm$ 3SD of background release in PBMC from healthy controls ($n = 43$). Thirty nine % (13 of 33) of HIV+/HCV+ women and 30% (3 of 10) in the HCV+ group responded to NS3 stimulation with secretion of 1 or more cytokines (Table II). Among responders to NS3, TNF- α was the most commonly secreted cytokine in HIV+/HCV+ women (33%) and IFN- γ along with IL-2 in HCV+ women (20% for both cytokines). Despite detectable responses in some patients, the group means were not statistically different from healthy controls.

For HIV-specific memory, stimulation with p24 elicited responses in 24% (8 of 33) in the HIV+/HCV+ group and 37% (10 of 27) in the HIV+ group. Among responders to p24, the most frequently secreted cytokine in the HIV+/HCV+ and HIV+ groups was IFN- γ (Table II). However, group means were not statistically different from healthy controls. Taken together, these results indicate a relatively low frequency as well as low levels of responses to HCV and HIV in women with single HCV or HIV infection, or coinfection. PBMC from healthy controls did not respond to stimulation with NS3 or p24 (data not shown).

CD4 Responses to CMV

Responses to CMV were comparatively more frequent than responses to p24 or NS3 in all infected groups, with 75%, 92% and 100% of overall responses among CMV-seropositive HIV+/HCV+ ($n = 32$), HIV+ ($n = 25$) and HCV+ ($n = 9$) women respectively (Table II). The frequency of responders for IFN- γ , TNF- α and IL-10 was significantly reduced in HCV+/HIV+ women compared to all other groups, whereas the frequency of IL-2 responders was low for both HCV+/HIV+ and HIV+ women. IFN- γ was the most abundant cytokine among HIV+/HCV+, HCV+ and healthy CMV-seropositive controls, whereas TNF- α was higher in the HIV+ group (Fig. 1A and Table III). Levels of IFN- γ were similar among HCV+ women and healthy controls but significantly higher compared to the HIV+/HCV+ and HIV+ groups. Levels of TNF- α were higher in HIV+ women compared to HIV+/HCV+ women, but similar to HCV+ women and healthy controls. Levels of IL-10 were significantly lower among HIV+/HCV+ women compared to all other groups (Fig. 1A). Levels of IL-2 were similar in HIV+ and HCV+/HIV+ women but significantly reduced compared to HCV+ women and controls. Overall, secretion of the type 2 cytokines IL-4 and IL-5 was low and no significant differences were observed among groups (data not shown). The cells responding to antigens were confirmed to be of the T helper type by depletion experiments: removal of CD4+ T cells resulted in >99% reduction in cytokine responses (Fig. 1B).

Type 1 Responses Dominate the Cytokine Profile of CMV-Specific T Helper Cells

The substantial level of responses to CMV allowed for a more detailed analysis of the cytokine profile specific for this antigen. For this, the indexes for type 1 (IL-2, IFN- γ and TNF- α) and type 2 cytokines (IL-4, IL-5 and IL-10) were calculated for each patient (Fig. 2). Interestingly, we found both in infected women and healthy controls that only those women with relatively high secretion of type 1 cytokines had also substantial secretion of type 2 cytokines ($p < 0.0001$ for the overall Th1/Th2 correlation). Among HIV-infected and HIV+/HCV+ coinfecting women, levels of secreted cytokines were low for both type 1 and type 2 but still type 1 was detected to higher extent.

The role of covariates on the levels of Th1 cytokines among HIV-infected women was investigated in multivariate analysis. For CMV, Th1 responses in HIV+/HCV+ women were significantly associated with HIV viral load ($p = 0.038$) even after adjusting for HCV viral load (data not shown). However, no significant association was observed for CD4 counts. Similarly, no significant association was observed for responses to NS3 and p24 in multivariate analysis. Together, these results implicate that HIV viremia in coinfecting women has a larger impact than HCV viremia on the levels of cytokine responses to CMV.

DISCUSSION

This is, to our knowledge, the first study investigating extensively the Th1/Th2 cytokine profile in patients with multiple viral infections and demonstrates a significant dominance of type 1 responses specific for three persistent viral infections. Our data also highlight an important quantitative difference in levels of cytokine responses in single HIV or HCV infections compared to coinfection, and appear to reflect the combined impact of the dual pathogenic infection on the immune system. For HIV, HCV and CMV, either in single or multiple infections, IFN- γ was the most commonly and abundantly secreted cytokine, although TNF- α was also readily detectable among women with single HIV infection. The comparatively high levels of IFN- γ and TNF- α secreted in response to viral proteins indicate that, even in patients with reduced CD4 counts because of HIV infection, T helper cells contribute significantly to viral control by direct secretion of anti-viral cytokines.

The quantitative difference in cytokine levels was most evident in responses to CMV, which were more readily detected than responses to HIV or HCV. CMV is a ubiquitous member of the herpes virus family, eliciting potent memory responses in asymptomatic but chronically infected individuals (27–29). Although CMV-specific T helper responses are reduced in HIV infection compared to healthy subjects, the responses are relatively stronger than HIV-specific responses (30–32). Thus, responses to CMV are useful to monitor anti-viral responses in patients with T cell dysfunction.

As observed before, responses specific for NS3 in chronic HCV infection were low compared to responses observed during acute HCV infection (7–9, 33). Contrasting the scarcity of HCV-specific responses in HCV+ women, cytokine secretion in response to CMV was robust. This dichotomy may be due to loss of HCV-specific CD4+ responses, which has been described during persistent HCV viremia (7). Because CD4 counts were normal in HCV+ women, it is likely that populations of CMV-specific CD4 memory cells were intact. Activity of regulatory T cells specific for HCV may also limit immune responses, although this remains to be investigated. Other factors may also have an impact on immune function in single HCV infection. It is conceivable that activation of monocytes/macrophages by HCV may favor CMV replication in those cells, leading to higher CMV antigenic load in these patients. In addition, several studies indicate that HCV proteins have the capacity to modulate lymphocyte survival by pro- and anti-apoptotic mechanisms (34, 35), which may also have an impact on the level of CMV-specific memory cytokine

response. Although HCV can infect monocytes and dendritic as well as T and B cells besides hepatocytes (36–38), the effect of HCV infection on the function of those lymphocytes remains to be investigated.

Immune responses, particularly cytotoxicity by CD8⁺ T cells, have been implicated in the pathogenesis of HCV infection. Studies in subjects with chronic HCV infection indicate that secretion of IFN- γ might be linked to the degree of liver damage (39, 40). In our study, HCV-specific responses were detectable in 30–40% of coinfecting or HCV-infected women and IFN- γ responses only in 9% of coinfecting women. Overall our results were in agreement with low proliferation to NS3 but better preserved responses to other antigens reported earlier (41). However, our multivariate analysis did not detect any significant association between HCV viral load and responses to NS3. Noteworthy, serum levels of ALT were similar among coinfecting and HCV-infected women, which suggest less extensive liver damage in coinfecting women and contrasted with reported higher ALT values in a predominantly male population (41). Further investigations are needed to clarify whether the low frequency of cytokine responses, including IFN- γ , in coinfecting women is indeed reflecting less extensive liver damage in our patients.

Scarcity of memory T cell responses has also been observed in chronic HIV infection (3, 4), which in the presence of HIV replication may be due to exhaustion of antigen-specific T cells, as shown in other infection models (42, 43). Other mechanisms affecting T cell function, such as altered tryptophan catabolism by the enzyme indoleamine 2,3-deoxygenase (44) have not been ruled out. However, Th1 cells appear to be more susceptible to tryptophan starvation than Th2 cells (45) which does not correlate with our results of decline in both Th1 and Th2 functions. Despite similar CD4 counts in HIV+/HCV+ and HIV+ women, cytokine secretion specific for CMV was more extensively compromised in coinfecting women, suggesting that monitoring of T cell function should complement the routine control of CD4 counts in these patients. Further long-term studies are required to clarify whether the reduced cytokine responses in coinfecting women may be associated with increased risk for disease progression.

In summary, this study demonstrates a profile dominated by Th1 cytokines in anti-viral responses to HIV, HCV and CMV during persistent infection, a pattern consistently observed even in multiple-infected subjects. It also indicates that compared to singly infected women, the T helper response of women coinfecting with HIV and HCV is markedly reduced. Finally, this study highlights the importance of monitoring simultaneous responses to unrelated viruses such as CMV in subjects infected with HCV or HIV, where responses specific for HIV and HCV are clearly curtailed. Additional studies are needed to evaluate mechanism(s) behind reduced HCV-specific T helper responses, as well as the clinical implications of reduced cytokine responses in coinfecting women.

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Fig 1.

Analysis of memory cytokine responses to CMV by infection group. (A) Cell culture supernatants from PBMC stimulated with CMV were analyzed for secreted IFN- γ , TNF- α , IL-2 and IL-10 and as described in the section on "Materials and Methods." Levels of secreted IL-4 and IL-5 were low or undetectable, and not significantly different between the groups (data not shown). Bars represent group means of individual cytokine indexes. Note the different scales used to allow for visualization of responses in all groups. Levels of secreted IFN- γ , TNF- α , IL-2 and IL-10 were not statistically different between HCV+ women (black bars) and healthy controls (striped bars). Above the indicated bars: *1, $p < 0.05$ compared to HIV+/HCV+ women (gray bars); *2, $p < 0.05$ compared to HIV+ women (white bars). (B) Depletion of CD4+ T cells abolishes the cytokine responses to CMV (IL-4 and IL-5 were below the detection limit of the assay both before and after depletion). Data (from a HCV+ patient) representative of 8 control experiments with similar results performed in singly infected women and healthy controls.

Fig 2.

Substantial secretion of type 2 cytokines is only observed in subjects with high type 1 secretion. Cytokine indexes in response to CMV stimulation were grouped in type 1 (IL-2, IFN- γ and TNF- α) and type 2 (IL-4, IL-5 and IL-10), and plotted for each subject. The correlation between the sum of type 1 and type 2 cytokines was statistically significant ($p \leq 0.01$) for healthy controls but not for HCV+ women ($p = 0.137$, panel A) as well as for HIV+ ($p \leq 0.0001$) and HIV+/HCV+ women ($p \leq 0.01$, panel B).

Table 1

Demographic and Clinical Characteristics of the Study Population

Characteristics	Group	HCV+/HIV+	HIV+	HCV+	p
<i>N</i>		33	27	10	
IV drug use	Ever	26 (79)	1 (4)	9 (90)	<0.0001
CMV sero-reactivity		32 (97)	25 (93)	9 (90)	
HAART-last 6 months	Yes	21 (66)	21 (78)		ns
Length of HAART ^a (years)		2.81	3.61		ns
CD4 (cells/ μ l) ^a		459	499	1043 ^b	<0.0001
Nadir CD4 ^a		268	250	863 ^b	<0.0001
ALT (U/L) ^a		69 ^c	39	76	<0.05
HIV (Log 10 RNA copies/mL) ^a		2.7	2.52		ns
HCV (Log 10 IU/mL) ^a		5.33		5.19	ns

Note. Data are no. (%) of women, unless otherwise indicated. Ns: not significant.^aGroup mean.^bSignificantly higher than the other 2 groups^cSignificantly higher compared to the HIV+ group.

Table II
Frequency of Cytokine Responses Among HIV and/or HCV Infected Women and Healthy Controls

Antigen	Group	Number of cases	Cytokine responders (%)					
			IL-2	IL-4	IL-5	IL-10	IFN- γ	TNF- α
NS3 (HCV)	HCV/HIV	33	0	0	0	4 (12.1)	3 (9.1)	11 (33.3)
	HCV	10	2 (20.0)	0	0	1 (10.0)	2 (20.0)	1 (10.0)
p24 (HIV)	HCV/HIV	33	1 (3.0)	1 (3.0)	1 (3.0)	1 (3.0)	4 (12.1)	2 (6.1)
	HIV	27	1 (3.7)	0	2 (7.4)	1 (3.7)	8 (29.6)	3 (11.1)
CMV ^a	HCV/HIV	32	6 (18.8)	1 (3.1)	7 (21.9)	9 (28.1)	17 (53.1)	13 (40.6)
	HIV	25	4 (16.0)	0	9 (36.0)	17 (68.0) ^b	20 (80.0) ^b	22 (88.0) ^b
	HCV	9	9 (100) ^b	0	5 (55.6)	6 (66.7)	9 (100) ^b	8 (88.9) ^b
	Control	22	10 (45.5) ^b	1 (4.6)	10 (45.5)	18 (81.8) ^b	19 (86.4) ^b	18 (81.8) ^b

Note. For each cytokine, responders were classified as such if the result was $\geq$ mean $\pm$ 3SD of the spontaneous cytokine release by unstimulated PBMC from healthy controls.

^a Data from subjects seropositive for CMV.

^b Significantly higher frequency ($p < 0.05$) for each group compared to HCV+/HIV+.

Table III

CMV-Specific T Helper Cytokines Indexes

Test group	Geometric mean	95% confidence interval	p value		
			HCV	HCV	Control
IFN- γ : HCV/HIV	7.49	3.16–17.76	ns	<0.05	<0.05
HIV	15.96	6.47–39.35		<0.05	ns
HCV	315.07	94.17–1054.16			ns
Control	83.97	38.46–183.31			
TNF- α : HCV/HIV	4.44	2.71–7.26	<0.05	ns	ns
HIV	21.86	11.24–42.50		ns	ns
HCV	15.92	7.58–33.47			ns
Control	11.30	6.91–18.48			
IL-10: HCV/HIV	2.01	1.43–2.84	<0.05	ns	<0.05
HIV	4.95	3.34–7.33		ns	ns
HCV	3.94	2.59–6.01			ns
Control	5.81	4.34–7.77			

Note. Cytokine indexes were calculated for each patient as the ratio of CMV-specific secretion and spontaneous release of the same cytokine. Values expressed as geometric mean of each patient group. Ns: not significant.