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Unique Cytokine/Chemokine Signatures for HIV-1 and HCV Mono-infection versus Co-infection as Determined by the Luminex® Analyses

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Abstract

Liver disease caused by HIV-1/HCV co-infection is characterized by the inflammation and cell-death. The co-existence of these two chronic viral infections also alters the cytokine production in vivo. The ability to visualize changes in cytokine networks with the onset and progression of disease or treatment is critical to advance our understanding of the immune response to pathogens. The recent Luminex® technology has revolutionized the simultaneous detection and quantitation of several cytokines and chemokines in clinical samples that are generally available in small quantities. We have applied this technology to analyze the plasma samples from patients who have either HIV-1 or HCV mono-infection or HIV-1/HCV co-infection and monitored the presence of 23 cytokines and chemokines. Of these, 8 (IFN-α2, IL-2, IL-3, IL-6, IL-8, IL-12p70, IL-15 and RANTES) cytokines were expressed at higher levels in the co-infected individuals. Interestingly, in case of HIV-1 mono-infected individuals, the levels of the proinflammatory cytokines IFN-γ and TNF-α were increased. Standard correlation clustering of the normalized data demonstrated unique plasma cytokine signatures for HIV-1/HCV co-infected individuals. These signatures were characterized not only by an up regulation of the aforementioned antiviral mediators but also by a marked down regulation in the chemokines Eotaxin and MIP-1α when compared to mono-infected individuals. Luminex®- based analyses have proven to be a powerful tool for therapeutic immunomonitoring, but may have an even greater impact in the discovery of the underlying immune response at all phases of infection. The study presented herein has potential to offer insight into the underlying mechanisms of immunopathogenesis of HIV-1/HCV co-infection.

Keywords: Luminex® assay; HIV-1/HCV co-infection; Multiplex cytokine analyses

Abbreviations: APC: Antigen-Presenting Cell; ELISA: Enzyme-Linked Immuno Sorbent Assay; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; HAART: Highly Active Antiretroviral Therapy; HCV: Hepatitis C Virus; HIV-1: Human Immunodeficiency Virus; IDU: Intravenous Drug User; IFN: Interferon; IL: Interleukin; MCP-1: Monocyte Chemoattractant Protein; MIP-1α: Macrophage Inflammatory Protein-1α; PBMC: Peripheral Blood Mononuclear Cell; RANTES: Regulated upon Activation Normal T cell Expressed and Secreted; RPE: R-Phyco Erythrin; TNF-α: Tumor Necrosis Factor-α

Introduction

Currently the global burden of both human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV) is quite significant. HCV currently infects 3% of the world's population (greater than 170 million people), with approximately 38,000 new infections occurring annually in the United States alone [1]. Currently 33 million people globally are estimated to be living with HIV-1 [2]. Due to similar routes of transmission of these viruses, co-infection with the two is quite common. Intravenous drug use, in particular, has resulted in an increase in rates of HIV-1/HCV co-infection, with incidence reaching or exceeding 90% prevalence [3]. Furthermore, progression to chronic HCV infection following acute infection is increased from 70 - 85% as seen in HIV-1 seronegative individuals to 90% in those individuals co-infected with HIV-1 [3]. Progression of HCV-related disease is further enhanced in HIV-1 positive individuals with advanced immunosuppression [4,5]. Co-infected individuals are known to have higher HCV RNA levels, which may result in greater risks of transmission [3]. Prior to the antiretroviral therapy (ART) era, HCV-related chronic liver disease mortality was masked by extra-hepatic mortality in HIV-1 co-infected individuals [6]. Now that ART has decreased HIV-related morbidity; liver disease constitutes a high proportion of mortalities among HIV-1 patients [7].

The increased levels of immune activation have been demonstrated in HIV-1/HCV co-infected individuals in contrast to HIV-1 mono-infected individuals [8,9], which may account for the high rate of disease progression in these individuals. Immune defects caused by either HIV-1 or HCV can alter the course of secondary infection and deregulated innate immune responses can contribute to more rapid disease progression. In addition, whether HIV-1 or HCV is the primary infection can also lead to different rates of HIV-1 disease progression as observed in various HIV-1/HCV coinfection studies [10,11]. Studies in the past have hinted that pre-existing HIV-1 infection thwarts the ability of the host to clear secondary HCV infection or worsens the individual's condition as compared to HCV mono-infected individuals [3,12-16]. This may possibly be due to the pre-established immunosuppression that occurs subsequent to primary HIV-1 infection. However, it is still remains unclear how chronic HCV infection affects secondary HIV-1 infection. Recently, it was found that IL-15 levels were enhanced in HIV-1/HCV co-infected individuals, suggesting a pro-fibrotic role for this cytokine [17]. With respect to inflammation and fibrosis, Blackard et al. [18] demonstrated that suppression of intrahepatic cytokines

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Code	Gender	Age	Infection status	Therapy Status	CD4 counts	HIV load	HCV load
HI1	M	35	HIV	Naïve	670	42600	NUL
HI2	F	42	HIV	Naïve	323	15017	NUL
HI3	M	51	HIV	Naïve	527	10700	NUL
HIC1	M	40	HIV/HCV	Failed	601	<75	116000
HIC2	F	49	HIV/HCV	Failed	223	10729	117000
HIC3	M	53	HIV/HCV	Failed	632	<75	>850000
HC1	F	44	HCV	Failed	516	NUL	500
HC2	M	40	HCV	Failed	397	NUL	3510000
HC3	M	48	HCV	Naïve	NA	NUL	1900000

NA = not available

Table 1: Patients utilized in the study.

ID	Eotaxin	GM-CSF	IFN-α2	IFN-γ	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12p40	IL-12p70	IL-13	IL-15	IP-10	MCP-1	MIP-1 α	RANTES	TNF-α
HIV monoinfection																							
HI1	13	ND	ND	3.2	17	0.5	ND	0.4	ND	0.4	ND	ND	3.0	1.6	26	ND	ND	ND	1050	143	23	1669	11
HI2	156	ND	ND	0.3	ND	0.7	ND	ND	ND	0.4	ND	ND	0.8	2.2	ND	ND	ND	ND	951	434	ND	1877	6.7
HI3	92	ND	ND	17	64	0.3	ND	ND	ND	0.4	ND	ND	3.6	1.9	ND	ND	ND	ND	5013	278	163	925	11
HIV/HCV coinfection																							
HIC1	13	ND	11	ND	99	0.5	0.1	0.4	ND	0.4	ND	ND	3.6	1.1	ND	ND	ND	ND	2074	124	ND	4261	3.4
HIC2	44	ND	ND	0.5	29	0.8	0.1	ND	ND	0.4	ND	ND	15	3.8	ND	ND	ND	1.5	1176	169	140	2202	7.2
HIC3	12	ND	34	1.1	21	1.8	11	9.7	ND	0.6	3.3	ND	27	1.4	ND	11.8	ND	6.8	1955	347	44	1873	6.9
HCV monoinfection																							
HC1	66	ND	ND	4.0	80	0.8	0.8	ND	ND	0.4	ND	ND	7.1	3.0	ND	0.2	ND	3.4	3289	243	11	1729	4.4
HC2	36	ND	ND	2.8	72	0.3	ND	ND	ND	0.4	ND	ND	ND	1.0	ND	ND	ND	2.3	1815	234	129	971	7.0
HC3	96	ND	ND	0.8	58	1.2	ND	ND	ND	0.7	ND	ND	11	2.9	ND	ND	ND	1.3	2304	457	16	3330	9.0
Normal																							
NC	23	10.3	11.5	28	10	2.9	5.6	ND	ND	0.4	5,5	ND	16	1.0	34	6.5	15.8	2.8	363	224	72	2491	5.6

Table 2: Absolute plasma concentrations of various cytokines and chemokines from mono HIV-1 and HCV mono- and co-infected individuals.

during HIV-1/HCV co-infection results in an imbalance between pro-fibrogenic and anti-fibrogenic cytokines, favoring HCV replication and fibrosis within the liver. Another study demonstrated impaired IL-2 production by HCV-specific $CD4^+$ T cells and impaired IL-2 and IFN-γ production from HCV-specific $CD8^+$ T cells in co-infected patients [19]. Barrett et al. [20] examined cytokine profiles within PBMCs from uninfected, HIV-infected, HCV-infected and HIV-1/HCV-co infected individuals in response to HCV proteins. Exposure to HCV antigens was found to increase IL-10 production by PBMCs in uninfected and HIV-monoinfected individuals, but this response was attenuated in chronic HCV infection and HIV-1/HCV-co infection. This selective induction was suggested to play a role in establishing chronic HCV infection, thus contributing to liver pathology [20]. However, a more detailed analysis of steady-state cytokine profile to compare mono-infected individuals to co-infected ones is required. In this regard, the ability to visualize changes in cytokine networks with the onset and progression of disease or treatment is critical to advance our understanding of the immune response to pathogens. The recent Luminex® technology has revolutionized the simultaneous detection and quantitation of several cytokines and chemokines in clinical samples that are generally available in small quantities, making it difficult to perform individual ELISAs for each cytokine of interest. Here we have utilized Luminex® technology to compare cytokine and chemokine profiles of mono-infected HIV-1 or HCV individuals and co-infected HIV-1/HCV individuals.

Materials and Methods

Patients and plasma samples

Plasma samples from a well-characterized cohort of patients were utilized as per the institutional guidelines. The relevant clinical information related to these patient samples is given in Table 1.

Multiplex-25 bead Luminex® assay

The human cytokine multiplex-25 bead array assay kit for Luminex was purchased from Invitrogen (Carlsbad, CA) to measure the following cytokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IP-10, MCP-1, MIP-1α, RANTES, TNF-α, IFN-α2, IFN-γ, GM-CSF and Eotaxin. The protocol was performed as per the manufacturer's instructions and as previously described [21,22]. Appropriate dilutions of the human plasma samples in assay diluents were made. The assay was performed in a 96-well filter plate, using all the assay components provided. All incubation steps were performed at room temperature and in the dark to protect the beads from light. All washes were performed using a vacuum manifold. For the detection of cytokines and chemokines, the samples were finally incubated for 30 minutes with streptavidin conjugated to the fluorescent protein, R-phycoerythrin (Streptavidin-RPE, diluted 1:10). After washing to remove the unbound Streptavidin-RPE, the beads (minimum of 50 beads per cytokine) were analyzed in the Luminex 100 instrument, which monitored the spectral properties of the beads while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin. Raw data was analyzed using Bio-Plex Manager software, v4.1 (Bio-Rad) [23]. Each undiluted plasma sample was assayed in duplicate, and cytokine standards supplied by the manufacturer were used to calculate the concentrations of the samples.

Results and Discussion

Plasma samples from patients who have either HIV-1 or HCV mono-infection or HIV-1/HCV co-infection were analyzed for the presence of 23 cytokines and chemokines by Luminex® (Table 2). Cytokine concentrations for all analytes across all samples were derived using a 5PL curve fit algorithm. Out of the total 23 cytokines measured, 8 (IFNα2, IL-2, IL-3, IL-6, IL-8, IL12-p70, IL-15 and RANTES) were highest in patients with HIV-1/HCV co-infection (Figures 1 and Figure 2) by comparing the trend of the median value. These included important APC derived antiviral effectors such as IFN-α2 and IL-12p70, mediators of T-cell expansion and memory differentiation IL-2 and IL-15 (which was previously found to be elevated in HIV-1/HCV co-infection [17] as well as the key APC chemokine RANTES

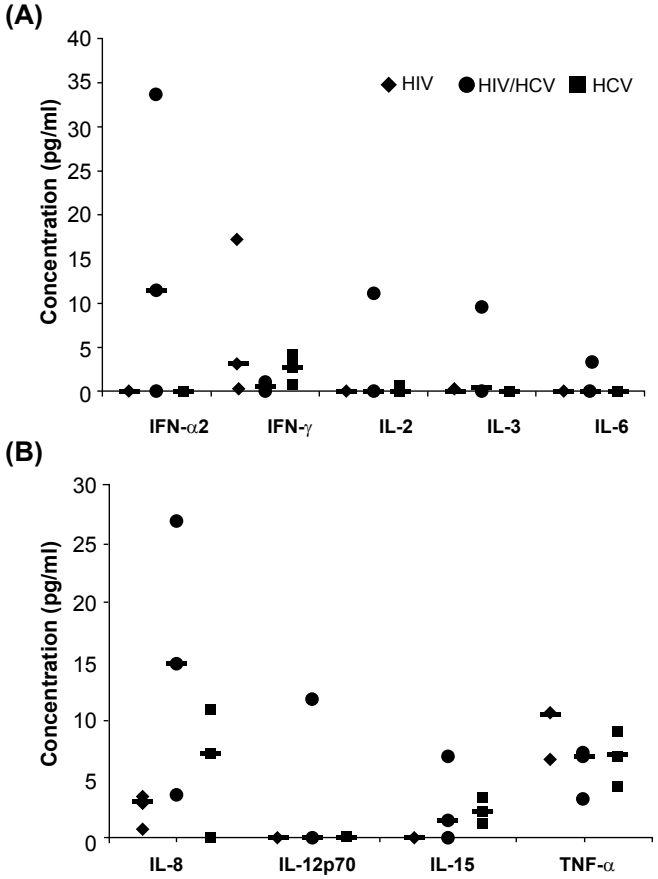

Figure 1: Plasma concentrations of various cytokines. The individual and median concentrations of various cytokines from HIV-1 mono-infected, HIV-1/HCV co-infected, and HCV mono-infected individuals (n=3 for each condition and are represented by diamond, circle and square symbols, respectively). The median value is represented by a bar.

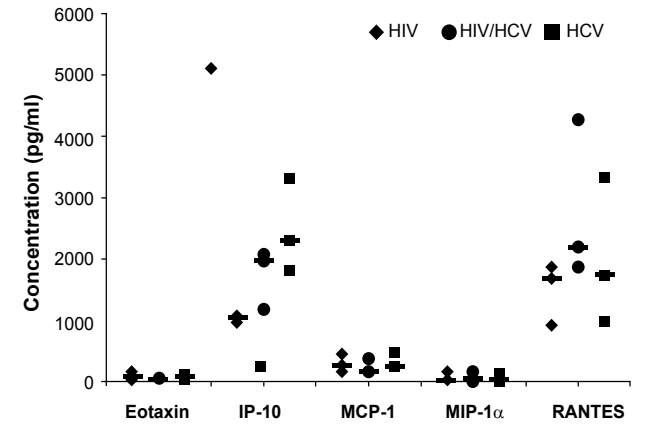

Figure 2: Plasma concentrations of various chemokines. The individual and median concentrations of various chemokines from HIV mono, HIV-1/HCV co-infected, and HCV mono-infected individuals (n=3 for each condition and are represented by diamond, circle and square symbols, respectively). The median value is represented by a bar.

that is known to be involved in the recruitment of leukocytes to sites of inflammation. Expression of RANTES from one mono-infected individual, HC3, was higher than the two co-infected individuals HIC2 and HIC3 and must be looked at in further detail. The levels of the proinflammatory cytokines IFN-γ and TNF-α were higher in the HIV-1 mono-infected individuals as compared to the other groups (Figure 1), which correlates with previous findings from Ciuffreda et al. [19] demonstrating that $CD8^+$ T cells from HIV-1/HCV co-infected individuals were found to have impaired IFN-γ secretion [19]. However, when comparing each HIV-1 mono-infected individual's IFN-γ and TNF-α response with the respective proviral loads, no direct relationship was observed. Interestingly, with respect to IP-10, the HIV-1 mono-infected individual with the lowest HIV-1 viral load, HI3, demonstrated the highest IP-10 expression. Nevertheless, this trend did not follow as individual HI1 had the next highest expression of IP-10, but the overall highest HIV-1 viral load of the 3 HIV-1 mono-infected individuals. Additionally, with respect to the HCV mono-infected individuals' cytokine expression, no correlation with proviral load was observed. Cluster analysis was performed on the derived concentrations of the 19 cytokines (without normalization) to determine a pattern or unique signature (Figure 3A). The cluster analysis was better evaluated by normalizing the derived concentrations for all cytokines to healthy donor plasma and then uploaded to an in-house software suite for complex pattern analysis and visualization of multiplex cytokine and chemokine data (Figure 3B). Standard correlation clustering of the normalized data demonstrated a unique plasma proteomic signature for HIV-1/HCV co infected individuals (Figure 3B, upper dendrogram),

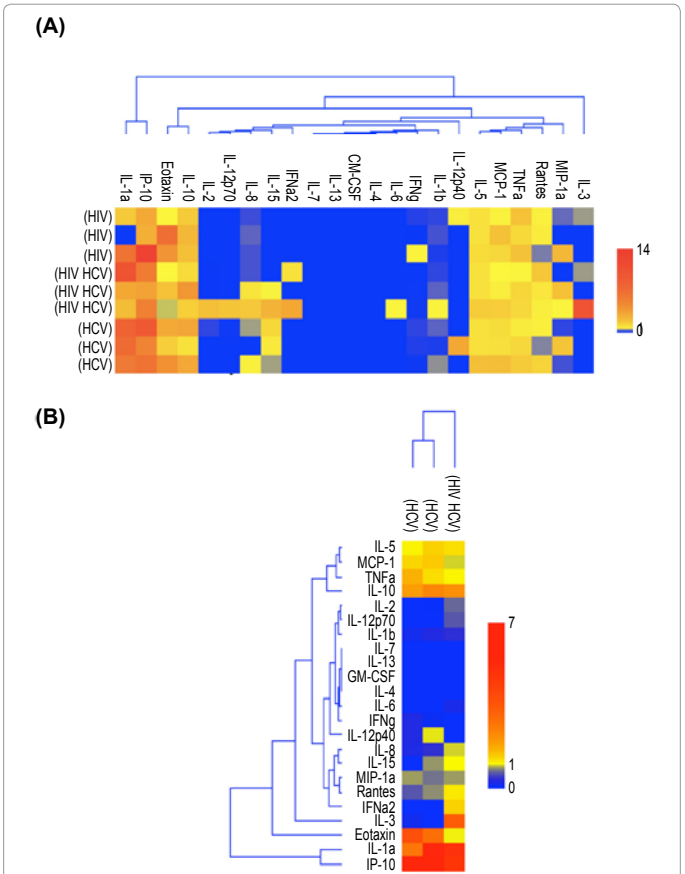

Figure 3: Cluster and pattern analysis of plasma cytokine and chemokine expression in infected individuals. (A) Cluster representation of the absolute concentrations of various cytokines and chemokines. (B) The derived concentrations were normalized to healthy donor plasma and a pattern analysis was performed using a core developed software suite specific for Luminex® analysis. Results are presented as the mean plasma cytokine value for the sample (S) over the concentration from normal donor plasma (D) for that cytokine ({S/n)}/(D)) and arranged as an unsupervised distance clustered intensity plot.

demonstrating that in addition to the key cytokines that were highly expressed in these patients, the levels of Eotaxin and MIP-1α were contrastingly decreased in the same individuals when compared to mono-infected individuals (Figure 2). Interestingly, the co-infected individual with the highest HCV viral load (but not the highest HIV-1 viral load), HIC3, demonstrated the greatest expression of 7 of the 8 aforementioned elevated cytokines. With respect to RANTES and IL-1α, the opposite was observed in which this same individual expressed the lowest amount compared to the other two co infected individuals, suggesting that HCV may have role in with stimulating or suppressing release of these particular cytokines/chemokines. Furthermore, the co-infected individual who had both high HIV-1 and HCV viral loads demonstrated the greatest expression of IL-10, similar to previous findings of HCV-antigen stimulation [20] and MIP-1α (Tables 1 and Table 2). Overall, cytokine expression levels may allow distinguishing co-infected individuals and assisting evaluation of the immune impact of therapeutic strategies in reversing immune activation changes associated with chronic mono or dual infection in such individuals.

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