Research Article ISSN 2639-8478

Cancer Science & Research

Cytomegalovirus (CMV) Infection Suppresses The Expression Levels of Serum Cytokines in Follicular Lymphoma Patients

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Received: 10 August 2021; **Accepted:** 12 October 2021

Citation: Lugos MD, Perikala V, Banda JM, et al. Cytomegalovirus (CMV) Infection Suppresses The Expression Levels of Serum Cytokines in Follicular Lymphoma Patients. Cancer Sci Res. 2021; 4(3): 1-10.

ABSTRACT

Follicular lymphoma (FL) is an indolent B-cell neoplasm. Increasing evidence suggests that chronic cytomegalovirus infection impacts the immunity of FL patients. This study was designed to unveil the impact of CMV infection on FL biology. Pretreatment serum samples from 42 FL patients recruited into a phase 3 FL clinical trial to compare two alternative frontline chemoimmunotherapy regimens were used. Twenty-one (21) of these patients had tested positive for CMV IgG antibodies, whereas 21 patients were negative. A multiplex assay was employed to measure the expression levels of 27 cytokines in each of these samples. Results were then compared between CMV-positive and CMV- negative groups using the Mann-Whitney U test using IBM SPSS Statistics version 20. Sera from CMV-positive FL patients showed decreased expression levels of most cytokines than those from the CMV-negative population. Data shows a statistically significant down-regulation of IL-6, IL-8, IL-9, IL-17A, FGF-basic, MIP-1α, and MIP-1β expression in CMV-positive FL patients compared to the CMV-negative group. Overall, our results suggest that CMV infection down-regulates the expression levels of serum cytokines in FL. Complementary studies to investigate immune cells in these patients' blood and tissue biopsies could provide a comprehensive perspective on the impact of CMV infection on FL.

Keywords

Cytokines, Cytomegalovirus, Follicular lymphoma, Multiplex assay, and sELISA.

Introduction

Immune cells in both the tumour microenvironment and peripheral circulation are known to secrete cytokines that perform critical roles in controlling the immune system's fundamental pathways [1]. The cytokine types and concentrations may reveal some crucial biological tumour-host interplays [2]. Cytokines are incriminated in the pathogenesis of various lymphomas; for instance, high frequencies of several cytokines and angiogenic factors have been

documented as biomarkers for utility in the diagnosis and prognosis of Hodgkin and Non-Hodgkin lymphoma (NHL) [2-4]. In this perspective, high serum quantities of Vascular endothelial growth factor (VEGF) and Fibroblast Growth Factor-Basic (FGF-basic) at diagnosis have been associated with poor outcomes in NHL [5]. Also, low baseline serum IL-6 and VEGF levels have been related to a reasonable response rate and overall survival in NHL patients [2]. This has been further echoed by a significant reduction of the serum IL-6 and VEGF in NHL patients that achieved complete remission (CR) following CHOP [cyclophosphamide, doxorubicin hydrochloride (hydroxydaunorubicin), vincristine sulfate (Oncovin), and prednisone] in the same studies [2].

CMV infection has been shown to elevate end-stage T cell subtypes in patients with FL as (unpublished data), cytokines produced by those cells or other cell types may reflect functions and regulations of those T cells in FL. CMV infection, therefore, may alter the cytokine profiles in the serum of patients with FL. This study aimed to address this hypothesis by analyzing 27 serum cytokines secreted by T cells and other cells in patients with and without CMV infection before starting trial treatment.

Materials and Methods

Study Sample Size Estimation

Sample size calculation was carried out based on pilot data collected before the start of the main study. The previous investigation found that 8 out of the 27 cytokines studied had a significant difference between CMV-positive and CMV–negative patients with FL (unpublished). To replicate these results in a more extensive study, the sample size calculation was based on a Bonferroni adjusted alpha level of 0.00625 to control the overall type I error rate at 0.05. As cytokines are measured on different levels, sample size calculations assume the data are calculated on the standardized normal distribution. It was determined that difference would be significant if the difference in cytokine expression levels between positive and negative CMV patients were at least one standard deviation.

Given that each cytokine is measured on a different scale, each cytokine will have its standard deviation. Therefore, the parameters used for the sample size calculation for comparison between two groups with an equal number of patients include the statistical power at 80%, the alpha level at 0.00625, the standard deviation of 1, and the clinically relevant difference of 1. This gives an overall sample size of 42 patients.

Study population and Samples

The study benefited from 42 stored pretreatment serum samples of FL patients recruited into an FL phase 3 clinical trial (in Liverpool), which compared two alternative frontline chemoimmunotherapy regimens. Twenty-one (21) of these patients had tested positive for CMV IgG antibodies by the local site, whereas 21 patients were negative. The samples were stored at -80ºC prior to use. This study cohort's demographic and baseline characteristics are summarised in Table 1. There is no significant difference between CMV positive and CMV negative arms of the study patients due to the clinicopathological features.

Inclusion and Exclusion Criteria

The inclusion criteria for recruiting patients into the National Cancer Research Institute (NCRI) PACIFICO (Purine-Alkylator Combination In Follicular lymphoma Immuno-Chemotherapy for Older patients) phase III clinical trial includes established grades 1, 2, and 3a patients of mainly 60 years or above, at Ann Arbor stages II to IV without prior treatment, as well as minimal haematological and other health complications. The exclusion criteria included Grade 3b FL and above transformed FL and other health complications that may not withstand the adverse effects of chemotherapy.

Ethical Approval & Informed Consent

As part of the PACIFICO trial ethics application, this study was included in the context of translational research. This trial had approvals of the European Union Drug Regulating Authorities Clinical Trials (EudraCT) on a unique number 2008-004759-31 and the International Standard Randomised Controlled Trial (ISRCTN) number ISRCTN99217456. Written informed consent to participate in the NCRI PACIFICO trial was obtained before patients' recruitment to the study as required by the International Council for Harmonisation (ICH)-Good Clinical Practice (GCP) regulations.

Laboratory Methodologies

Multiplex (Luminex) Assay for Serum cytokines

The multiplex instrument, Bio-Rad Bio-Plex 200 system (Bio-Rad Laboratories, Hertfordshire, UK) powered by Luminex xMap Technology, was used for the Bio-Plex Pro™ Human Cytokine 27-plex assay. Consequently, all reagents comprising cytokine standards, coupled beads, 96-well plates, detection antibodies, streptavidin-PE, and diluents, as well as the software used for the analysis of data, were purchased from Bio-Rad Laboratories (Cat #: M500KCAF0Y: Bio-Rad Laboratories, Hertfordshire, UK).

As part of the validation protocol for Luminex assay and before data acquisition, each bead region number of the cytokine was entered against each cytokine in the Bio-plex manager software. In addition, the Human Cytokine Standard was reconstituted following the manufacturer's instructions and incubated on ice for 30 minutes before use.

The multiplex assay employs the Luminex platform to detect targets using antibody-coupled bead systems. The unique content of two addressing colours recognized each bead in the set. A third dye is used to ensure the coupling of the target analyte through a biotin-coupled antibody plus streptavidin-conjugated secondary step indicator. A dedicated flow cytometer of the Luminex Bio-Plex® Bio-Plex 200 system platform (Luminex MAP Technology, Texas, USA) was used to acquire the data.

Data were acquired in the Bioplex reader after calibrating the

Bioplex manager software. The values that fell way beyond the curves had an asterisk (*) and were removed before analysis. To evaluate assay reproducibility, the inter-assay percentage (%) coefficient of variation of most cytokines appears within the acceptable limit of 20% and below. However, cytokines with low levels of abundance that fall below the assay's detection limit expressed zero scores; for instance, IL-1b, IL-2, IL-5, IL-15 had unacceptably high %CV of above 100%. Also, for cytokines with elevated abundance levels, for example, RANTES, the %CV appeared very high. This is because the %CV can usually be higher due to cytokine levels that were 'out of range' (OOR). The intra-assay precision of triplicate wells for the 27-plex assay was excellent, with a %CV of ≤ 20% across most of the 27 analytes from the two Luminex plates, except IL-2, IL-15, and IL-17A that had %CV of 173.21% in plate-1, plate-2, and plate-1 respectively. Assay precision data were obtained using healthy serum control and test samples. The standard curves for the two plate assays appear comparable, indicating that raw MFI values for each point in the standard curve are highly reproducible from run to run.

Sandwich ELISA for Cytokines

The ELISA Development Kits contains essential components for the quantitative measurement of human cytokines in a sandwich ELISA format, including capture antibodies (CAs), detection antibodies (DAs), Wash Buffer, Block Buffer, and Diluent were supplied by PeproTech EC Ltd, London, UK. Additional materials and reagents required and not provided in the kit are ELISA microplates, ABTS Liquid Substrates solution (Sigma Cat. #: A3219), Bovine serum albumin (BSA; Sigma Cat. #: A-7030), Dulbecco's Phosphate buffered saline (PBS; [10 ×]) (Gibco BRL. Cat #: 14200-075), Wash Buffer, Block Buffer, Diluent and Tween-20 (Sigma Cat. #: P-7949). The sELISA assay used for validation was IL-9 (Cat. # 900-K20) (PeproTech EC Ltd, London, UK).

ELISA plate was aspirated and washed four times in 1 × Diluent. A multichannel pipette was used to add 100ul of ready-to-use ABTS substrate to each ELISA well. A SPECTRAmax PLUS 384 ELISA Plate Reader (Molecular Devices, California, USA) set at 405nm with correction wavelength at 650nm was used to monitor colour development at 5 minutes intervals at RT for up to 50 minutes. Colour development was recorded by reading optical density (OD) of the wells, and SOFTmax Pro software (Molecular Devices, California, USA) was used for endpoint reading. When readings fall between zero and the highest standard values, reliable standard curves are said to be obtained.

Statistical Analysis

Continuous variables normally distributed are summarised with their mean and 95% confidence interval, while continuous variables that did not reach normality are presented with their median and IQ range. P-values, obtained after performing the Chi-squared test (or Fisher's exact test, if any expected value was smaller than 5), t-test (or Mann-Whitney U test, if data was not normally distributed) using IBM SPSS Statistical software version 22.0 (SPSS, Inc., Chicago, IL, USA) are used to examine for any statistically significant differences in the outcomes between the groups.

All correlation assessments were determined using the Pearson correlation coefficient. A statistically significant difference was considered if the test P value was <0.05. The hierarchical clustering and heat maps were generated using Partek Genomic Suite Version 6.6 (Partek Inc., St. Louis, MO, USA), and all data were floored (i.e., 0 transformed to 0.01) and logged.

Results

The cytokine expression profiles in the CMV- negative and CMV- positive patients with FL

To understand whether the differences between the two cohorts presented in Table1 were statistically significant, Mann-Whitney U-test using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA) was used for comparison. Overall, the serum levels of 10 out of the 27 cytokines showed some difference between the CMV- negative and CMV- positive FL patients. IL-17A, IL-9, IL-8, IL-6, MIP-1α, MIP-1β, and FGF-basic significantly differed with a two-sided P value ranging from 0.01 to 0.04. The remaining IL-4, GM-CSF, and IL-1b also showed a trend of difference, although the P values (0.06 ~ 0.07) were slightly more significant than the pre-set α level. We presented whiskers graphs of the levels of ten cytokines measured by the Luminex method, showing significant differences between the two groups in Figure 1.

We showed the mean rank of the concentration of each of the 27 serum cytokines in the 21 CMV- positive patients are compared to that in the 21 CMV-negative cases (set as 0). The expression of cytokines marked by * was significantly different (P<0.05), and those by ** board-line different (P=0.066~0.070) between the two groups. In the heat map in Figure 3, cytokine data were log-transformed to 0.01, and cytokine expression was scaled from the minimum level (-3.90) in green and the maximum level (3.90) in red. Patients were coded red for CMV- positive and blue for CMV- negative. The hierarchical clustering of the ten cytokines of interest versus the 42 FL patients has grouped cytokines according to their functional combinations of chemokines (IL-8, MIP-1α, & MIP-1β) and IL-1b, T_H2 cytokines (IL-4, IL-6, and IL-9), T_H17 (IL-17A) and Vascular remodelling (FGF-basic & GM-CSF).

The correlation between IL-9 concentrations measured by Luminex and sELISA

As shown in Figure 4, Pearson's correlation analysis revealed a positive linear correlation between the two methods, although not perfect ($r = 0.374$, $p = 0.015$). Expectedly, the average concentration of the cytokine measured by sELISA was over seven folds of that as measured by the Luminex (280pg/ml versus 36pg/ml). This might partially explain repeated sample freezing and thawing effects, as noticed in our previous experiment [6]. Those results suggested that the relative cytokine levels in patients of this cohort were consistent with the results of the sELISA investigation, and therefore reliable.

Table 1: The comparison of baseline characteristics with CMV infection status of study patients.

Characteristics	Total (n=42) *	CMV Status (n=42)		
		Positive (n=21) *	Negative (n=21) *	P **
Age Median (IQR)	71.5 (68 – 75)	73.0 (70 – 76)	69 (66 – 73)	0.055
Gender N (%)				1.000
Male	17 (40.5)	9 (42.9)	8 (38.1)	
Female	25 (59.5)	12 (57.1)	13 (61.9)	
Haemoglobin (g/dL), Median (IQR)	13.2 (9.25 - 20.75)	13.2 (11.5-14.6)	13.2 (11.6 - 14.1)	0.812
Ann Arbor stage N (%)				1.000
2	6 (14.3)	3 (14.3)	3 (14.3)	
3	14 (33.3)	7 (33.3)	7 (33.3)	
4	22 (52.4)	11 (52.4)	11 (52.4)	
CIRS Score N (%)				1.000
≤5	34 (80.9)	17 (81.0)	17 (81.0)	
>5	8 (19.1)	4 (19.0)	4 (19.0)	
No. lymph nodes N (%)				0.666
1	1 (2.4)	0 (0.0	1 (4.8)	
2	1 (2.4)	1 (4.8)	0 (0.0)	
3	3 (7.1)	1 (4.8)	2 (9.5)	
4	4 (9.5)	2 (9.5)	2 (9.5)	
5	2 (4.8)	2 (9.5)	0 (0.0)	
6	31 (73.8)	15 (71.4)	16 (76.2)	
LDH Median (IQR)	385 (266-481)	388 (216-453)	382 (349-492)	0.584
FLIPI Score N (%)				1.000
0-1 (low risk)	3 (7.1)	1 (4.8)	2 (9.5)	
2 (intermediate risk)	10 (23.8)	5 (23.8)	5 (23.8)	
3-5 (high risk)	29 (69.1)	15 (71.4)	14 (66.7)	
Histology N (%)				0.541
1	12 (28.6)	5 (23.8)	7 (33.3)	
2	12 (28.6)	13 (61.9)	9 (42.9)	
3	8 (19.0)	3 (14.3)	5(23.8)	

*For continuous variables, this refers to the Mean (SD) or Median (IQR) if indicated.

**P-value by x2 test or Fisher's exact test for the difference between categorical variables or t-test for the difference between two means.

CIRS = Cumulative illness rating scale, FLIPI = Follicular lymphoma international prognostic index and LDH = Lactate dehydrogenase.

Table 2: Cytokine levels (pg/ml) in serum samples of FL patients in CMV- positive and negative patients with FL.

S#	Cytokines (pg/ml)	CMV Status			
		Total	Positive	Negative	P
		Median (IQR)	Median (IQR)	Median (IQR)	
1	PDGF-bb	5977.06 (4122.61-7437.84)	5630.0 (3676.3-6457.3)	6640.7 (4461.7-8224.6)	0.170
2	IL-1b	3.8 (3.1-5.6)	3.3 (2.8-4.8)	4.0 (3.5-5.8)	**0.068**
3	IL-1ra	194.0 (139.4-264.5)	182.6 (146.8-212.9)	229.3 (139.4-310.6)	0.119
4	IL-2	0.0 (0.0-9.8)	0.0 (0.0-6.9)	3.0 (0.0-13.6)	0.248
5	IL-4	4.3 (3.9-5.5)	4.2 (3.9-5.1)	5.1 (4.1-5.6)	**0.066**
6	IL-5	2.34 (1.67-2.82)	2.3 (1.7-2.7)	2.5 (1.7-3.1)	0.831
7	IL-6	16.2 (10.7-26.9)	11.5 (9.7-17.5)	19.6 (12.1-35.1)	**0.029**
8	IL-7	10.8 (8.2-15.4)	10.2 (8.4-13.7)	10.9 (7.5-16.2)	0.359
9	IL-8	268.2 (66.3-715.5)	168.9 (48.8-338.8)	534.2 (135.3-1077.1)	**0.037**
10	IL-9	28.8 (20.1-44.0)	22.2 (17.5-29.9)	42.6 (28.4-56.1)	**0.006**
11	IL-10	12.4 (6.1-19.4)	8.3 (5.7-19.8)	14.0 (9.0-18.5)	0.222
12	IL-12 p70	62.0 (49.8-97.1)	62.5 (35.7-75.9)	61.4 (52.5-123.6)	0.571
13	IL-13	8.4 (5.7-11.4)	8.2 (5.4-10.2)	9.1 (6.2-11.4)	0.443
14	IL-15	0.0	0.0	0.0	0.269
15	IL-17A	31.2 (9.5-66.8)	23.0 (6.3-35.7)	43.9 (19.7-69.1)	**0.020**
16	Eotaxin	160.5 (114.7-225.9)	158.9 (114.6-205.3)	191.0 (122.7-242.0)	0.333
17	FGF basic	61.7 (43.2-75.8)	55.9 (36.9-66.8)	72.3 (56.8-86.8)	**0.028**
18	G-CSF	60.3 (48.9-76.0)	55.6 (45.9-67.2)	64.2 (52.8-77.6)	0.213
19	GM-CSF	62.0 (18.4-86.5)	37.6 (7.9-73.0)	72.9 (50.1-92.9)	**0.070**
20	IFN-g	175.9 (149.0-225.1)	174.0 (146.3-202.3)	198.5 (155.2-231.4)	0.187
21	IP-10	1804.1 (1074.2-2894.2)	1827.5 (1319.1-3030.7)	1788.3 (1054.2-2253.2)	0.308
22	MCP-1(MCAF)	67.9 (44.3-124.3)	68.6 (36.8-93.9)	67.3 (57.2-178.4)	0.320
23	MIP-1α	8.7 (6.4-12.5)	7.4 (5.8-9.9)	10.1 (7.5-21.4)	**0.039**
24	MIP-1β	258.2 (209.2-330.3)	225.0 (198.6-296.8)	294.4 (228.2-419.6)	**0.029**
25	RANTES	61692.1 (32722.9-90443.7)	61249.3 (32722.2-75198.7)	62134.9 (37391.4-92008.7)	0.521
26	TNF-α	48.8 (35.5-59.8)	45.3 (35.8-57.0)	52.2 (35.5-62.9)	0.346
27	VEGF	197.1 (135.4-283.0)	170.0 (102.6-254.9)	243.5 (176.7-369.3)	0.110

P< 0.05 is considered statistically significant.

IQR = Interquartile Range, VEGF = Vascular endothelial growth factor, TNF-α = Tumor necrosis factor-alpha, RANTES = Regulated on Activation, Normal T Cell Expressed and Secreted, MIP-1α = Macrophage Inflammatory Protein-1 alpha, MIP-1β = Macrophage Inflammatory Protein-1 beta, MCP-1 = monocyte chemoattractant protein-1, MCAF = Monocyte chemotactic and activating factor, IP-10 = Interferon gamma-induced protein-10, GM-CSF = Granulocyte-macrophage colony-stimulating factor, G-CSF = Granulocyte colony-stimulating factor, FGF-β = Basic fibroblast growth factor and PDGF-bb = Platelet-derived growth factor bb.

The concentrations of IL-9 measured with the Luminex (Y-axis) and sELISA (X-axis) in all of the 42 patients studied are compared in the two-sided Pearson's correlation analysis. Note that the serum samples used for the sELISA test experienced two more cycles of thawing/freezing than those for the Luminex test.

Discussion & Conclusions

Being that T cells are known to be the major source of cytokines, a quantitative analysis of pretreatment serum cytokine levels of the PACIFICO trial patient samples was carried out as a strategy to evaluate the functional potential of the elevated terminally differentiated T cell subtypes observed in the CMV- positive FL patients (unpublished data).

Strikingly, Luminex data reveal decreased levels of all the 27 serum cytokines studied among the CMV- positive FL patients compared to the CMV- negative patients, except for IP-10, IL-12, and MCP-1(MCAF). As depicted in Table 2 and Figure 1, significantly diminished serum IL-9, IL-17A, FGF-basic, MIP-1α, MIP-1β, IL-6, and IL-8 were observed in the CMV- positive group compared to the CMV- negative counterparts ($P < 0.05$). Also, marginally significant inferior median quantities of IL-1b, IL-4, and GM-CSF are seen in the CMV-positive compared to the CMV- negative ($P = 0.066 \sim 0.070$). Moreover, there were no significant variations in clinicopathological features among patients with different statuses of cytokine production (inclusion criteria). Meanwhile, reduced CD4+ T cells can further compound this deficiency since CD4+ T cells are needed to support the activity of other immune cells,

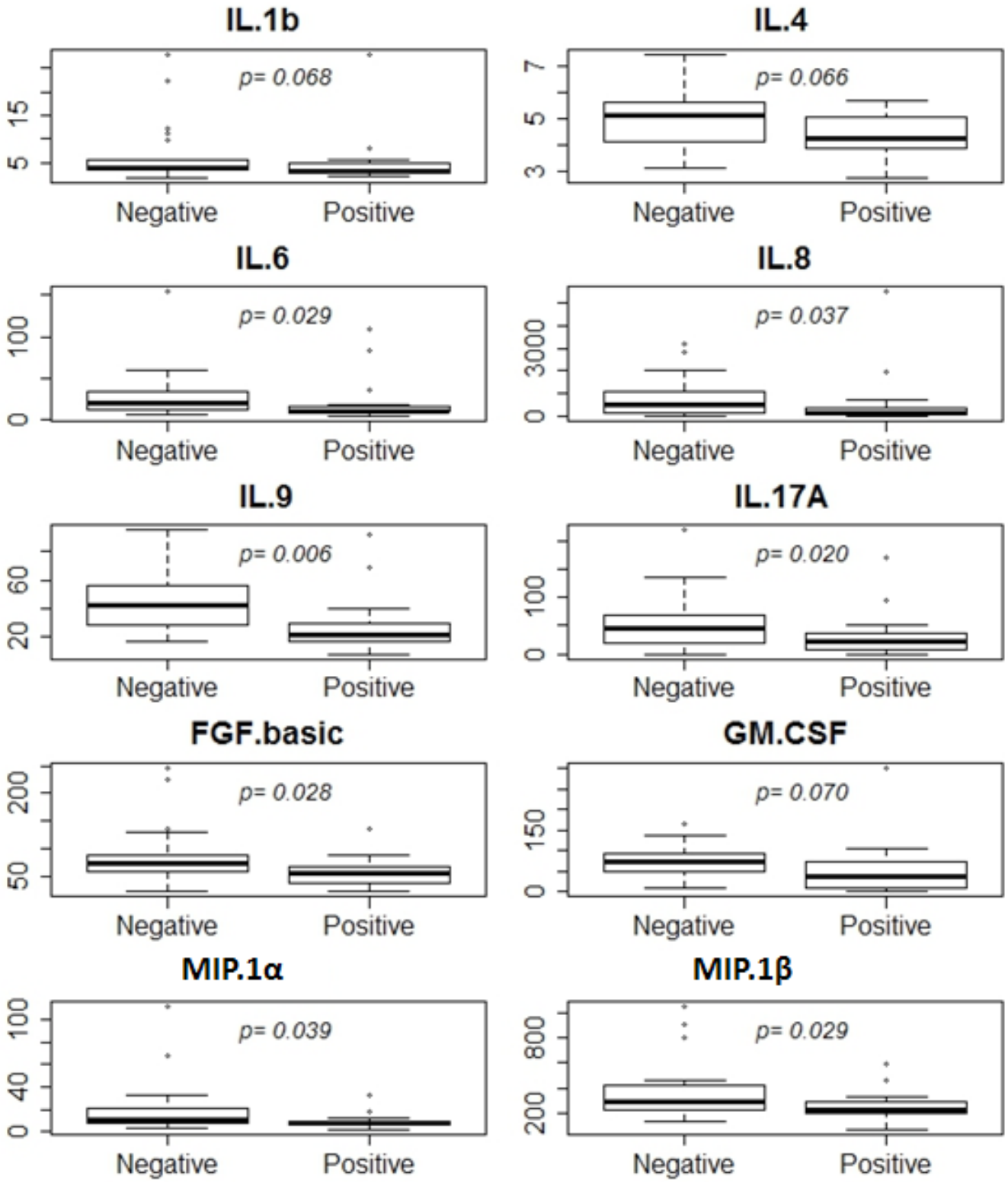

Figure 1: The box plot/whiskers graphs of concentrations of 10 cytokines measured using the Luminex method that show significant differences between CMV- positive (n=21) and CMV- negative (n=21) FL patients.

The P-values were from the two-sided Mann-Whitley U test.

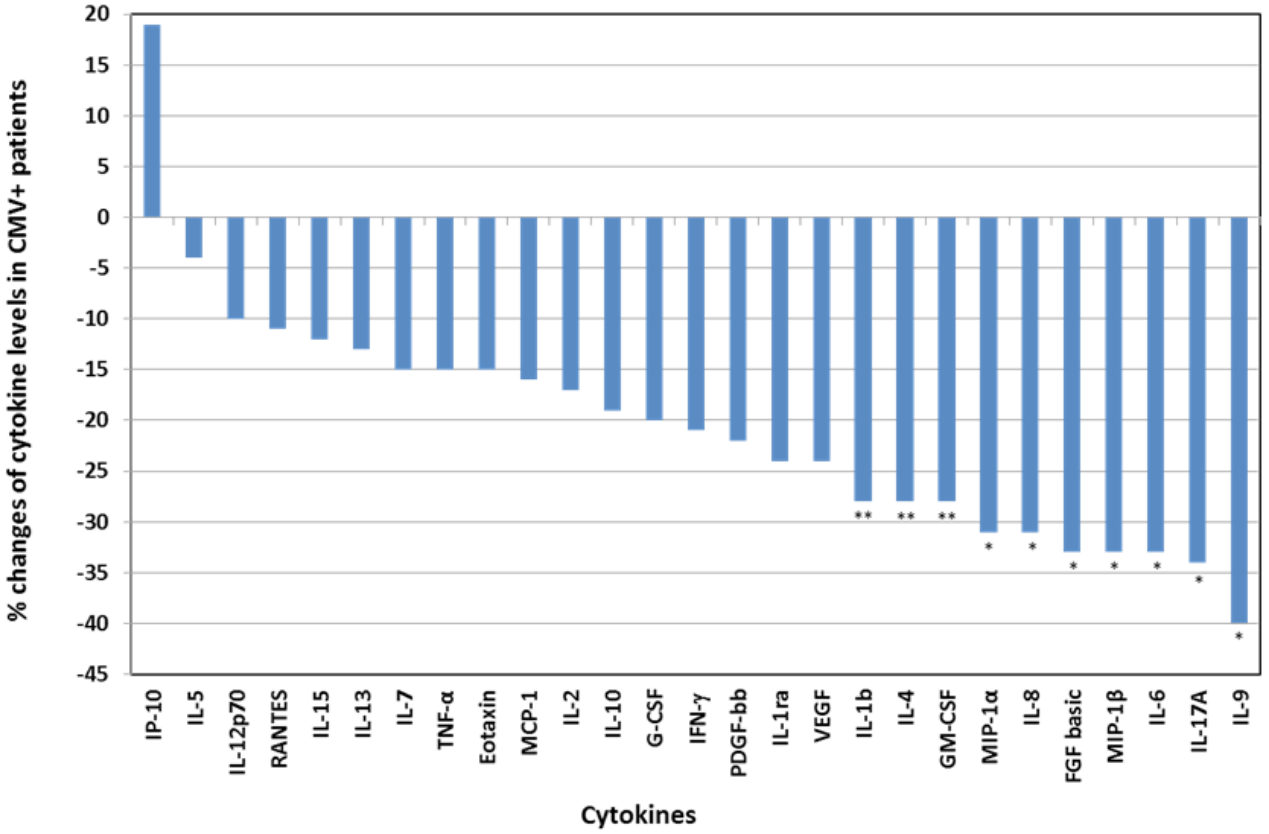

Figure 2: The relative changes in cytokine levels in patients with CMV infection as compared to those without the infection.

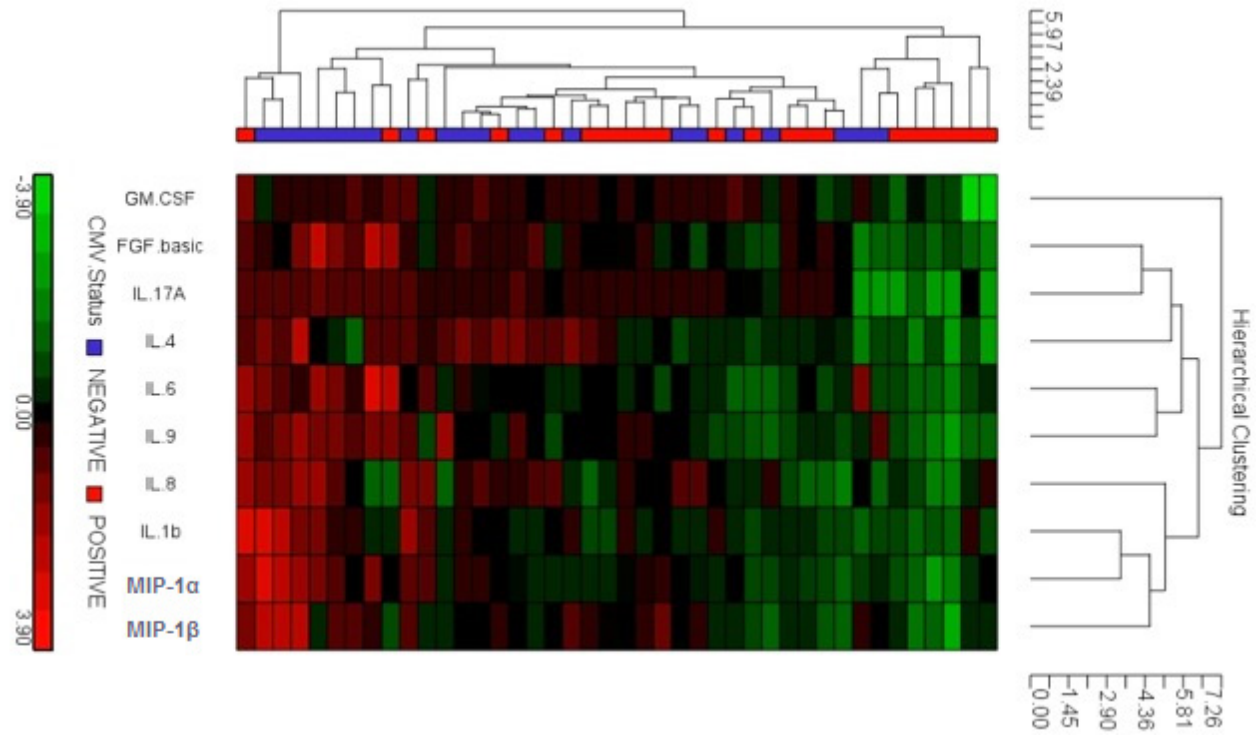

Figure 3: The unsupervised heat maps showing expression levels of the Ten cytokines with statistically differential expression between the two groups.

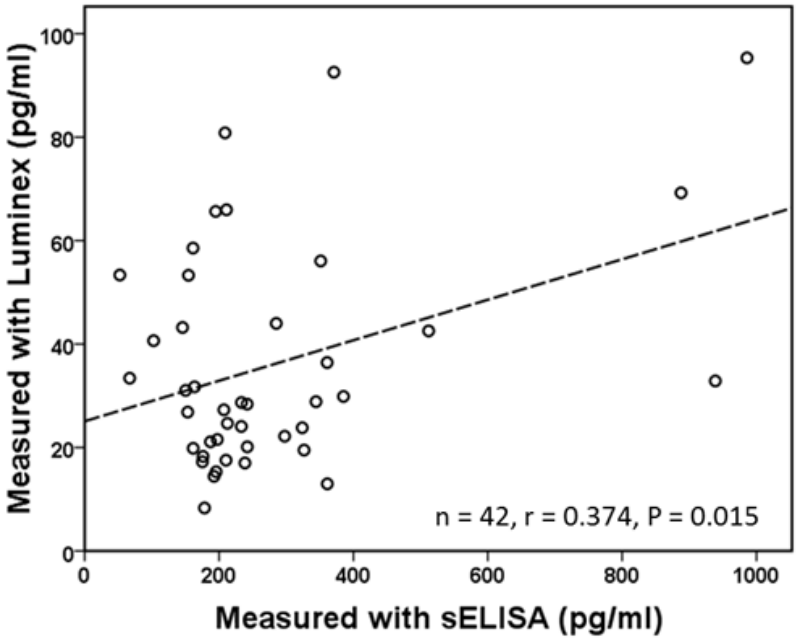

Figure 4: A positive correlation between the Luminex and sELISA in measuring IL-9 concentrations in patient serum samples.

including the production of cytokines to suppress or regulate immune responses. These observations could suggest that the presence of CMV infection in FL may portend some functional immune deficit for the patients, subjecting them to heightened vulnerability to other infections. The study suggests there could be some degree of homogeneity in the participating patients. It implies that trial patients' baseline clinical and pathological characteristics may not be responsible for the variations in the cytokine levels.

Although Multiplex assay (Luminex technology) has been shown to be comparable with other immunoassays [7,8] we have evaluated the impact of thawing and freezing of serum samples on the results of sELISA [6]. This was with the view to the validation of the Luminex cytokine data for use in the subsequent relevant studies.

The Luminex method was selected for the cytokine studies because Luminex is a multiplex platform that simultaneously measures 27 cytokines in a single experiment and avoids result alterations caused by the use of samples that experienced a repeated cycle of storage [9]. As a matter of fact, this method has been successfully applied in other cytokine studies. For instance, cytokine data from multiplex assays have been utilized in Wei-Lin-Weissfeld regression models to define the relationship between serum cytokine concentrations and oral HPV clearance [10]. In addition, a multiplex bead assay was employed to analyze stored serum samples collected at the time of delivery for cytokine levels, and results were used to suggest that elevated maternal levels of anti-inflammatory cytokines throughout the perinatal time may guard against psychotic episodes [11]. Data of 77 inflammation markers generated by Luminex bead-platform were subjected to weighted Cox models and conditional logistic regression to estimate cumulative risks and odds ratios (ORs), respectively, which revealed that certain levels of circulating inflammatory markers are associated with prospective lung cancer risk [12]. In another study, prediagnostic serum cytokine levels of B-cell NHL cases were measured by the Luminex platform. The relevant statistical analysis of data revealed an association between a moderately raised risk of all B-cells NHL in women and elevated cytokine levels [13].

The Luminex assay was well established in our GCLP laboratories for cytokine studies before this study. Finally, in this study, a positive linear correlation was found between IL-9 concentrations measured using this method and sELISA in all 42 patients studied ($r = 0.374$, $p = 0.015$). This result was in agreement with other studies. For instance, Wang et al. revealed perfect correlations between Luminex multiplex technology and ELISA platform in measuring levels of IL-1β, IL-4, IL-5, IL-6, IL-10, IFN γ, and TNF α [8]. Others also found the Luminex assay highly reproducible and reliable compared to ELISA in quantifying cytokines [14,15].

Therefore, the results in this research provided one more piece of evidence to support that both the Luminex and sELISA methods can be used to determine serum cytokine levels among patients. However, due to the limitation of using serum samples without repeated storage cycles, cytokine data from the Luminex assay was used for subsequent analysis in this work.

The hierarchically distinct functional clusters of cytokines in Figure 4 revealed ten cytokines down-regulated in CMV- positive patients. Among them, MIP-1α (CCL-3), MIP-1β (CCL_4), and IL-8 (CCL8) are members of a group of structurally related small molecules of about 8–14 kDa involved in the regulation of cell trafficking via interactions with a subgroup of transmembrane G-protein–coupled receptors [16,17]. They belong to the

inflammatory category of chemokines, which have been reported to be produced in high frequencies during infection or injury as they regulate the movement of inflammatory cells into the site of injury [18].

The second distinct cluster is shown in Figure 4, comprised of IL-6 and IL-9. It is not surprising that IL-6 and IL-9 clustered together since both cytokines perform similar functions and were initially grouped as T_H2 cytokines. The T_H2 cells secrete several cytokines such as IL-4, IL-6, and IL-9 to activate and sustain humoral immunity, mainly targeted at extracellular bacterial and parasitic challenges, plus allergens and toxins. Aside from being T_H2 cytokines, IL-6 and IL-9 are described as pleiotropic and further identified as pro-inflammatory cytokines [19]. The IL-6 and IL-9 as pleiotropic cytokines can influence the activities of multiple cell types leading to a broad spectrum of biological functions in oncogenesis, inflammation and autoimmunity, immune regulation, and hematopoiesis [19]. Therefore, the deficit observed in the frequencies of IL-6 and IL-9 among the CMV-positive FL patients could be thought to cause suppressed humoral immune responses, among others, thereby causing increased infection and disease vulnerability.

The third cytokine cluster identified on the heat map in Figure 4 includes IL-4, IL-17A, and FGF-basic. IL-4 is an autocrine cytokine that is marginally reduced among CMV- positive FL patients compared to the negative group. This is an essential cytokine for T_H2 priming and maturation, and high concentrations can prevent the production of T_H1 cells from the immature T cells. IL-4 is also vital in stimulating B cell proliferation and maturation of plasma cells and regulating antibodies' class switching. The IL-17A is a pro-inflammatory cytokine and a member of the IL-17 cytokine family and IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F [20]. It provides a strong defence against extracellular microbes and bacterial and fungal infections and adds to the pathogenesis of several autoimmune disorders [21,22]. It also regulates the formation and recruitment of neutrophils to the sites of inflammation due to their ability to cause the release of CXC chemokines [23] and coordinate G-CSF expression [24]. The FGF-basic and GM-CSF can be grouped under vascular remodelling cytokines. In a situation where there is no signal, FGF-basic is known to remain in basement membranes of the subendothelial extracellular matrix of blood vessels. It is assumed that during both the normal wound healing process and in tumour development, FGF-basic is activated by heparan sulfate degrading enzymes and triggers angiogenesis [25].

Studies have also demonstrated that FGF-basic protects the heart from injury connected to heart attack, reduced tissue death, and encouraged enhanced function following reperfusion [26]. Also, low levels of FGF-basic have been reported to play a vital role in the occurrence of extreme anxiety [27]. A monomeric glycoprotein, GM-CSF, operates as a cytokine by activating stem cells to produce granulocytes and monocytes [28]. The polymorphs are known to play pivotal roles, especially in the innate arm of immunity. At the same time, the monocytes egress the circulation and relocate into tissues where they develop into macrophages and dendritic cells and participate in the characteristic immunological processes of defending the body and combating infections [29]. GM-CSF can, therefore, be said to promote the development of the immune system.

Therefore, it is logical to speculate that the ability of the end-stage effector T cells reported among the CMV-positive patients (unpublished) to produce cytokine is poor. Therefore, by removing all other confounders, the reduced cytokine levels in the serum of the CMV-infected FL patients can be attributed to the disruptive influence of the virus on T cell repertoires, being the primary cellular source of cytokines.

By implication, the low expression levels of cytokines in this study can impact the immune system's innate and adaptive arms and pose a grave danger to patients exposed to CMV. They can be attributable to reduced secretions by relevant cells that produce them. The generally low cytokine levels observed in the CMV-positive patients may potentially make them more vulnerable to adverse events and recurrent infection in the course of therapy compared to the CMV- negative. It is speculated that a functional study on T-cell subtypes, especially the end-stage T cell types, may provide a further understanding of T cell plasticity and open the mystery of re-engineering and reverting end-stage T cells to functional subtypes. To further unravel the impact of CMV infection in FL lymphoma biology, probing into the FL microenvironment to evaluate quantitative intensities of accessory cells according to the CMV infection status is thought to be complementary.

Acknowledgements

This is an opportunity to thank Dr Melanie Oates (PhD), Dr Ke Lin (Bachelor of Medicine, MD, PhD), and Professor Andy R Pettitt (MA, MB BChir, PhD, MRCP, FRCPath), who supervised and provided reagents for this study. In addition, we are thankful to Dr Richard Jackson & Miss Silvia Cicconi of Biostatistics Department, Insitute of Translational Medicine, University of Liverpool, Mr Philip Jalo of the Intercountry Centre for Oral Health (ICOH) for Africa Jos, Nigeria, for helping out with statistical analysis. The content of this manuscript has been published [IN PART] as part of the thesis of Dr Moses Dabah Lugos [30].

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