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Flow Cytometric Analysis Revealed a Significant Accumulation of Terminally Differentiated T cell Subtypes in the Circulating Lymphocytes of Cytomegalovirus (CMV) Positive Follicular Lymphoma Patients

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ABSTRACT

Follicular lymphoma (FL), a non-Hodgkin lymphoma, is an indolent cancer of the B cell lineage that runs a chronic deterioration course that can result in multiple treatment episodes leading to resistance and possible transformation to diffuse large B cell lymphoma. Cytomegalovirus (CMV) reactivation during chemotherapy or after an organ or hematopoietic stem cell transplantation is a major cause of morbidity and mortality. This study tests the hypothesis that some of the heterogeneity of FL might result from chronic infection with Cytomegalovirus (CMV). This research was intended to appraise the impact of CMV infection on the subtypes of T cells in follicular lymphoma patients. We accessed stored peripheral blood mononuclear cells (PMBCs) from patients of known CMV serostatus recruited into an FL clinical trial. We undertook a multicolour flow cytometric analysis of the PBMCs and compared the number of lymphocyte subtypes of CMV-positive and CMV-negative FL patients. Data showed a significant increase in the quantity of terminally differentiated (TEMRA) T cell subsets, including EM3-CD8 (P=0.005), EM3-CD4 (P=0.018), E-CD4 (P=0.029), E-CD8 (P=0.033) and pE2-CD4 (P=0.046) phenotypes, as well as increased NKT cells (P=0.031) among CMV-positive patients compared to the negative group. Our findings support the hypothesis that recurrent infections characterise CMV infection in FL due to accelerated immune senescence and the accumulation of exhausted T cells. Based on the data, a case could be argued for the routine application of CMV screening in FL before treatment with chemo-immunotherapy to implement enhanced infection surveillance in CMV-positive patients. These discoveries can eventually help improve the treatment approaches in the management of FL toward a combinatorial viewpoint for direct cytotoxic and indirect immunomodulatory outlook.

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Introduction

A variety of cell types and subtypes such as granulocytes, macrophages, dendritic cells, T cells, and B cells at different populations constitute an essential part of the immune system [1]. In CMV infection, a large fraction of T cell subtypes have been reported to be committed to keeping the virus under checks, thereby causing alterations in the dynamics of T-cell populations, which strongly influence T cell immunity [2]. Therefore, quantitation, defining and ascribing specific roles played by the diverse T cell populations, for instance, in the immunity of different pathological conditions, will help provide diagnostic and prognostic evidence for patient management in FL Multicolour flow cytometry can be used to quantify different subtypes of T cells and other cell types. Further research evidence has explained the events leading to cellular differentiation, acquisition of effector potential and eventual death of T cells. Activation of immature CD4+ T cells has been reported to present a linear differentiation model, in which cells continuously acquire functional abilities by every additional differentiation step [3]. However, persistent antigenic provocation over time can result in a gradual decline of memory and cytokine secretion capacities, and consequently, accumulation of short-lived CD4+ T cells (TEMRA cells) with poor cytokine production ability. Furthermore, other antigenic assault on the CD4+ TEMRA cells can activate the CD4+ T cell at this stage of differentiation, causing apoptosis. On the other hand, two alternative differentiation patterns have been reported to produce CD8+ TCM cells and CD8+ TEM cells: linear differentiation and fixed lineage [4, 5]. A continued antigenic challenge will lead to accumulation of CD8+ TEMRA cells and death similar to the

CD4+ T cells pattern; however, CD8+ TEM cells can regain IL-2 expression and become CD8+ TCM cells, unlike CD4+ TEM cells [4]. NK cells are a subcategory of lymphocytes of the innate immune system that form about 10% of the circulating blood mononuclear cells in humans they are usually described as CD3−CD56+ cells and can be subgrouped by the intensity of CD56 on the cell surface [6,7]. The distinct subtypes of NK cells can be classified based on CD56 and CD16 surface markers [8]. The majority of circulating NK cells (about 90%) exhibit low expression for CD56 (CD56dim) with high levels of CD16 and perforin and are described as terminally differentiated NK cells [9]. NK cell development is characterised by the reduction in the expression of CD56 and the addition of CD16 expression. However, activated CD56dimCD16+ has been shown to represent the highest level of NK cell activation, which implies that CD56dimCD16+ cells represent the most mature NK cells subtype [10-12]. Therefore, this study was set out to evaluate the impact of CMV infection on circulating peripheral T and NK cell populations in FL patients.

Materials and Methods

The flow cytometer - BD LSRFortessa™

Cells were acquired and analysed using the BD LSRFortessa™ Special Order Research Product (BD Biosciences, Oxford, UK). It is an air-cooled multi-laser benchtop flow cytometer, equipped with Red, Yellow/Green, Blue, Violet and UV lasers, capable of analysing 18 parameters. The equipment is housed in the Liverpool Good Clinical Laboratory Practice (GCLP) Facility, Liverpool.

The Multicolour FACS Design

We used a 3-tube multicolour FACS analysis design to quantify several T and NK cell subtypes in the peripheral mononuclear blood cells (PBMCs) of FL patients, as illustrated in Figure 1. Tube 1 contains the ten fluorochrome-conjugated monoclonal antibodies (mAbs) listed in Figure 1, designed to analyse for senescent T cells (CD57+CD28-), NKT and NK cells (CD56 & CD16), B cells (CD19+) and monocytes (CD14+) of the study cohort. Tube 2 contained 12 mAbs and was dedicated to examining the frequencies of the different differentiation stages to T cells, including the naïve (CD45RA+CD45RO-CCR7+CD62L+), central memory (CD45RA-CD45RO+CCR7+CD62L+), effector memory (CD45RA-CD45RO+CCR7-CD62L-) and effector (CD45RA+CD45RO-CCR7-CD62L-) T cells. Also, we evaluated terminally differentiated T cells (TEMRA) subtypes using CD27 and CD28 as well as those of regulatory T cells (Tregs) (CD4+CD25+). Tube 3, an 11 mAbs panel, committed to analysing T cell exhaustion markers, including PD-1, LAG-3, and TIM-3.

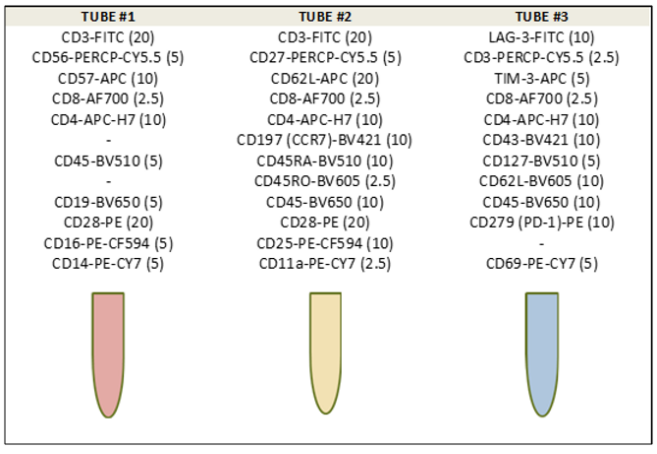

Figure 1: Illustrating the 3-tube FACS design showing the respective panels of Fluorochrome-Conjugated Monoclonal Antibodies (mabs).

The values in brackets represent the respective optimal working volumes of the mAbs (in microliters) as validated in our lab.

The Fluorochrome-conjugated Monoclonal Antibodies (mAbs)

A total of 26 fluorochrome-labelled monoclonal antibodies were used for this study, including CD3-FITC (BD Biosciences, Oxford, UK), CD3-PerCP-Cy5.5 (BD Biosciences, Oxford, UK), CD4-APC-Cy7 (BD Biosciences, Oxford, UK), CD8-Alexa Fluor 700 (BD Biosciences, Oxford, UK), CD11a-PE-Cy7 (BD Biosciences, Oxford, UK), CD14-PE-Cy7 (BD Biosciences, Oxford, UK), CD16-PE-CF594 (BD Biosciences, Oxford, UK), CD19-BV650 (BD Biosciences, Oxford, UK), CD25-PE-CF594 (BD Biosciences, Oxford, UK), CD27-PerCP- Cy5-5 (BD Biosciences, Oxford, UK), CD28-PE (BD Biosciences, Oxford, UK), CD43-BV421 (BD Biosciences, Oxford, UK), CD45-BV510 (BD Biosciences, Oxford, UK), CD45-BV650 (BD Biosciences, Oxford, UK), CD45RA-BV510 (BD Biosciences, Oxford, UK), CD45RO-BV605 (BD Biosciences, Oxford, UK), CD56-PerCp.-Cy5.5 (BD Biosciences, Oxford, UK), CD57-APC (BD Biosciences, Oxford, UK), CD62L-APC (BD Biosciences, Oxford, UK), CD62L-BV605 (BD Biosciences, Oxford, UK), CD69 –PE-Cy7 (BD Biosciences, Oxford, UK), CD127-BV510 (BD Biosciences, Oxford, UK), CD197 (CCR7)-BV421 (BD Biosciences, Oxford, UK), CD279 (PD-1)-PE (BD Biosciences, Oxford, UK), LAG3-FITC (Enzo Life Sciences, UK), and TIM3-APC (R & D Systems, UK). We also had CD45- BV650 & BV510 isotype (BD Biosciences, Oxford, UK) controls. Fluorochrome-conjugated mAbs were kept in the fridge until usage.

Sample Size Estimation

Sample size calculations were 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 to be studied were shown to show a significant difference between CMV-positive and CMV–negative patients with FL. Based on replicating these results, sample size calculations are 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 measured on the standardised normal distribution. It was determined that difference would be interesting if the difference in cytokine expression levels between positive and negative CMV 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 variables used for the sample size calculation for comparison between two groups with an equal number of patients include the statistical power at 80%, an alpha level at 0.00625, a standard deviation of 1 and a clinically relevant difference of 1. This gives an overall sample size of 42 patients.

Study Population and Samples

This study benefited from 42 stored pretreatment PBMC samples from FL patients recruited into an FL phase 3 clinical trials, which compared two alternative frontline chemoimmunotherapy regimens comprising 21-CMV- positive and 21-CMV negative. The study samples were stored at -80oC before use. Aside from the strict inclusion and exclusion criteria applied in the recruitment of FL trial participants, a careful selection of study patients based on balanced clinical and pathological baseline characteristics as in Table 1 between the CMV groups was adopted. This approach aimed to minimise the possible influence of confounders in the analysis and enhance the chances of accurate evaluation of the biological effect of CMV infection status on FL.

The demographic and pretreatment features of the study population are described in Table 1. CMV- positive patients are marginally older compared to CMV- negative with a median age of 73 years versus 69 years (p=0.055). Patients' risk factors and the prognosis are stratified according to the Follicular Lymphoma International Prognostic Index (FLIPI) score into three risk categories: good (0 or 1), Intermediate (2) and poor (3-4). The FLIPI score is determined using five adverse prognostic factors, including Ann Arbor stage (III-IV vs I-II), age (> 60 years vs ≤ 60 years), haemoglobin level (< 120 g/L vs ≥ 120 g/L), number of nodal areas (> 4 vs ≤ 4), and serum LDH level (above normal vs normal or below) [13]. A slightly higher proportion of CMV- positive patients is on the high-risk grade compared to the CMV- negative (71% vs 67%). The Cumulative Illness Rating Scale (CIRS) is used for the physical assessment of impairment of patients under 13 near independent areas. It is a 5-point rating of the degree of severity scale that ranges from none (1) to extremely severe (5) [14]. The proportion of patients in Table 1 did not show a significant difference due to CIRS. Also, as shown in Table 1, there is no significant difference in the clinicopathological variables of patients included in the two arms of study cohorts, the CMV- positive and CMV- negative.

Table 1: The comparison of pretreatment features by CMV infection status of patients whose PBMC samples were analysed

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 x^2 test or Fisher's exact test for the difference between categorical variables or t-test for the difference between two means.

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 can be summarised to include Grade 3b FL and over, cases of transformed FL to DLBCL 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 recruitment to the study as per the International Council for Harmonisation (ICH)-Good Clinical Practice (GCP) regulations.

The laboratory techniques

The Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

In a biological class II Safety Cabinet, Lymphoprep (Product #: 1114740, Axis-Shield, Alere Ltd., Stockport-UK) was pipetted into prelabeled tubes in a 2:1 ratio of blood to Lymphoprep. Blood was gently layered on top of the Lymphoprep using a serological pipette. The tubes were centrifuged at 800g for 30 minutes at room temperature (RT) with the brake setting at low. Following centrifugation, mononuclear cells in the buffy coat layer were carefully removed into a tube, which was filled up with Roswell Park Memorial Institute (RPMI)-1640 medium (LM-R1641/500, Labtech International Ltd, East Sussex, UK) and centrifuged at 550g for 10 minutes to wash and pellet the cells with the brake on. The supernatants were discarded, and cells were resuspended in the appropriate volume of freezing solution A (10% foetal calf serum (FCS) in RPMI 1640 media. Cells were counted to determine live and dead cells using ChemoMetec Nuclear Counter in the GCP Lab facility, and an equal volume of medium B (10% FCS, 20% Dimethyl sulfoxide (DMSO in RPMI) was added slowly. Cells were aliquoted in vials and placed in Nalgene cryofreezing containers in the -80oC freezer for a minimum of 12 hours before moving to -150oC freezer in the GCP Lab Facility freezer room.

The Cytometer Setup and Tracking (CS&T)

The CS&T is a fully automated BD FACSDiva software and reagent research system unique to BD digital cytometers designed to provide Characterisation, Setup, and Tracking for baseline settings. This system optimises and standardises cytometer setup and tracks cytometer performance, ensuring consistency and reproducibility of FACS data by offsetting the routine instrument variability. The BD FACSDiva CS&T Research Bead set (Cat #: 655051, BD Biosciences, Oxford UK) consists of uniform beads of different intensities (bright, mid, and dim beads) designed to characterise the flow cytometer fully. For daily performance checks, a CS&T passed result was considered essential for running the FACS experiment for the day.

The Compensation Set up

After vortexing the BD™ CompBeads(Cat #: 552843, BD Biosciences, Oxford UK) had one drop each of the negative control and anti-Mouse Ig, κ was added to each of 12 prelabelled compensation tubes representing each fluorochrome. The optimal working volume of each primary conjugated antibody was added to the respective tube. The tubes were gently mixed and incubated for 30 minutes in the dark at RT. Subsequently, 2ml of CellWash (Cat# 349524; BD Biosciences, Oxford UK) was added to each tube and centrifuged for 5 minutes at 300 × g with a low brake setting to wash beads. Supernatants were discarded, and the bead pellets were resuspended in 500µL BD FACSFlowTM Sheath Fluid and vortexed thoroughly before analysis. Five thousand events were acquired from each of the 12 compensation tubes. By using the software, the bead population was carefully gated, and the positive and negative populations were identified before the compensation values were calculated using BD FACSDiva software version 7.

The PBMC Samples: Thawing and Recovery of Cells

When ready to use, the frozen specimens were thawed by being held in a closed fist before transferring the cells to pre-label Universals on ice. Each 1mL of thawed cell suspension was slowly diluted with 10mL of RPMI- 1640 medium supplemented with 10% foetal calf serum (FCS). The RPMI 1640 solution was added to cells gradually in a drop-wise manner with constant and careful agitation throughout the process. The Universals were centrifuged at 500 × g for 5 minutes, and the supernatants were discarded.

Then the cell pellets were resuspended, counted and assessed for viability using ChemoMetec NucleoCounter (ChemoMetec A/S, Denmark) by detecting total and dead cell counts. Cells were resuspended in BD FACSFlow Sheath Fluid (Cat #: 342003, BD Biosciences, Oxford UK) for lymphocyte immunophenotyping.

The Staining and Acquisition of Cells for Facs

Following the addition of optimal working volumes of the respective fluorochrome-conjugated monoclonal antibodies (data not published), 100µL containing about 2×10^5 to 1×10^6 well-mixed washed PBMCs were placed in the prelabelled FACS tubes. Tubes were incubated for 30 minutes in the dark, at room temperature, after which 2ml of CellWASH (Cat #:349524, BD Bioscience Oxford, UK) was added, and the tubes centrifuged at 300g for 5 minutes with low brake. The supernatants were discarded before repeating the washing process. Finally, the cells were resuspended in 500µL of BD FACSFlowTM Sheath Fluid before being gently vortexed.

Due to variations in the availability of material and the number of gated T cells (CD3+), the number of events acquired per tube was between 100,000 and 2,000,000, with most samples yielding 500,000 events and above. Isotype controls and single stained fluorescence histograms were used to gate for cut-offs for distinct lymphocyte populations on the Diva software platform using respective surface markers.

The Hierarchical Illustrations of the Approach used for the Analysis of Cell Types

A PACIFICO trial stored PBMC sample, pac0108, was used to illustrate the contour plot gating approach and the hierarchy adopted to analyse the subsets of lymphocytes. We used FACSDiva software, which provides ready information on the number of events (#Events), per cent parent cell (%Parent), and per cent total (%Total) as displayed on illustrations of hierarchical analysis of different cell types in tubes 1-3 depicted in figures 2-4 respectively. In all three tubes, gating began by identifying the CD45+ population. For instance, in tube 1, four main populations were gated from the common leukocyte antigen (CD45-Amcyan), including CD3-Alexa Fluor-488 (T cells), CD14-PE-Cy7 (Monocytes), CD19-Qdot-655 (B cells) and CD56-PerCP (NK cells).

Tube: pac0108-1

Population	#Events	%Parent	%Total
All Events	500,000	####	100.0
CD45	303,282	60.7	60.7
CD3	62,326	20.6	12.5
CD4	32,316	51.8	6.5
CD3CD4CD57+/CD28- [Senescent CD4]	3,498	10.8	0.7
CD8	37,043	59.4	7.4
CD3CD8CD57+/CD28- [Senescent CD8]	17,182	46.4	3.4
CD3CD56+/CD16+ [NKT cells]	150	0.2	0.0
CD56	6,105	2.0	1.2
CD56 minus CD3	5,655	92.6	1.1
CD56+/CD16+ [NK cells]	3,600	63.7	0.7
CD56+/CD16- [NK cells]	2,095	37.0	0.4
CD14+ [Monocytes]	98,760	32.6	19.8
CD19+ [B cells]	21,308	7.0	4.3

Figure 2: The hierarchical approach adopted to analyse cell types tested in tube 1 shows the #Events, %Parent and %Total

pac108 -1 represents a PACIFICO trial stored PBMC sample used to illustrate the hierarchy adopted to analyse cell types in tube 1. The cell types of interest are represented in brackets.

Tube: pac0108-2

Population	#Events	%Parent	%Total
All Events	500,000	####	100.0
CD45	291,941	58.4	58.4
CD3	63,649	21.8	12.7
CD4	28,439	44.7	5.7
CD45RA	16,909	59.5	3.4
CCR7	14,484	85.7	2.9
CD11a	13,936	96.2	2.8
CD27+CD28+ [N CD4]	13,235	95.0	2.6
CCR7-	2,425	14.3	0.5
CD27+CD28+ [pE1 CD4]	427	17.6	0.1
CD27+CD28- [pE2 CD4]	410	16.9	0.1
CD27-CD28- [E CD4]	1,067	44.0	0.2
CD45RO	20,246	71.2	4.0
CCR7+	13,183	65.1	2.6
CD27+CD28+ [CM CD4]	11,287	85.6	2.3
CCR7--	7,063	34.9	1.4
CD27-CD28+ [EM4 CD4]	2,722	38.5	0.5
CD27+CD28+ [EM1 CD4]	2,209	31.3	0.4
CD27-CD28- [EM3 CD4]	1,617	22.9	0.3
CD27+CD28- [EM2 CD4]	515	7.3	0.1
CD4+CD25+ [Tregs]	2,870	10.1	0.6
CD8	32,195	50.6	6.4
CD45RA+	24,274	75.4	4.9
CCR7++	1,400	5.8	0.3
CD11a+	1,358	97.0	0.3
CD27+CD28+ [N CD8]	788	58.0	0.2
CCR7---	22,874	94.2	4.6
CD27+CD28+ [pE1 CD8]	1,386	6.1	0.3
CD27+CD28- [pE2 CD8]	1,732	7.6	0.3
CD27-CD28- [E CD8]	16,936	74.0	3.4
CD45RO+	21,212	65.9	4.2
CCR7+++	1,204	5.7	0.2
CD27+CD28+ [CM CD8]	664	55.1	0.1
CCR7----	20,008	94.3	4.0
CD27-CD28+ [EM4 CD8]	4,518	22.6	0.9
CD27+CD28+ [EM1 CD8]	2,135	10.7	0.4
CD27-CD28- [EM3 CD8]	11,897	59.5	2.4
CD27+CD28- [EM2 CD8]	1,458	7.3	0.3

Figure 3: The hierarchical approach adopted for analysis of cell types tested in tube 2, showing the #Events, %Parent and %Total

pac108 -2 represents a PACIFICO trial stored PBMC sample used to illustrate the hierarchy adopted for the analysis of cell types in tube 2. The cell types of interest are represented in brackets.

Tube: pac0108-3

Population	#Events	%Parent	%Total
All Events	500,000	####	100.0
CD45	193,128	38.6	38.6
CD3	61,893	32.0	12.4
CD4	28,244	45.6	5.6
CD43+/CD62L+	2,552	9.0	0.5
CD127+/CD69+	4	0.2	0.0
PD-1	661	25.9	0.1
LAG3	4	0.2	0.0
TIM3	8	0.3	0.0
CD8	31,539	51.0	6.3
CD43/CD62L	925	2.9	0.2
CD127/CD69	4	0.4	0.0
PD1	125	13.5	0.0
LAG-3	4	0.4	0.0
TIM-3	5	0.5	0.0

Figure 4: The hierarchical approach used to analyse cell types tested in tube 3 shows the #Events, %Parent and %Total

pac108 -3 represents a PACIFICO trial stored PBMC sample used to illustrate the hierarchy adopted for the analysis of cell types in tube 3.

The number of events, per cent parent and per cent total events of the various cell types and subtypes were defined automatically and displayed using FACSDiva software. Conventionally, this provides the needed values for measuring the per cent of lymphocyte subpopulations within the lymphocyte gate. A comparative analysis of the median values of per cent parent (relative) frequencies of the different lymphocyte subtypes according to the CMV status was carried out using a nonparametric Mann-Whitney U-test analysis to determine the significant difference. A p-value of < 0.05 is considered significant.

Results

The analysis of cell surface markers has been used to define the various lymphocyte populations in stored PBMC samples using multiparametric flow cytometry. Essential FACS optimisation steps aimed at minimising errors and generating reliable results and subsequent interpretations were strictly adopted. Of note, the right approach to FACS data analyses and presentation of the cell population of interest is as crucial as cells acquisition design. Before the FACS experiments commence, optimal working volumes of fluorochrome-conjugated antibodies were determined, contour and dot plots were evaluated against polygon and rectangle gating strategies, respectively, gating strategies for the three FACS tubes were evaluated, and fresh versus frozen FACS assessed.

The Lymphocyte Subtypes

Using the established flow cytometry method and analysis strategies, we successfully quantified relative percentages of T and NK cells from parent cells (as in Figures 2,3 and 4) in samples taken from a cohort of patients with FL (see Table 2). Of interest, statistically significant elevation of effector (E) and effector memory (EM) T cell subtypes, representing end-stage CD4 and CD8 T cell populations among CMV-positive patients compared to the CMV-negative counterparts was observed. Statistically significant higher frequencies of pre-effector-2 CD4 (pE2-CD4; CD45RA+CD27+CCR7-CD28-), effector-CD4 (E-CD4; CD45RA+CD27-CCR7-CD28-), effector memory-3 CD4 (EM3-CD4; CD45RO+CD27-CCR7-CD28-), effector-CD8 (E-CD8; CD45RA+CD27-CCR7-CD28-) and effector memory-3-CD8 (EM3-CD8; CD45RO+CD27-CCR7-CD28-) phenotypes were observed in CMV- positive compared to CMV-negative

patients as shown in Table 2. These T cell subcategories' most striking common features are the loss of CD28 and CCR7 (CD28-CCR7-) surface markers. The subtypes of effector cells express CD45RA+ isoform; those of the effector memory cells express CD45RO+.

Also, looking at the results in Table 2, a statistically significant increase in the frequency of NKT cells (p=0.031) was detected among CMV-positive patients compared to CMV- negative cohort. However, NKT cells are known to perform both protective and destructive consequences due to their abilities to produce cytokines that support either inflammatory responses or immune tolerance.

The other cell types with marginal differences between CMV groups include CD4+ T cells population and CD4-LAG3 (p=0.051 and p=0.074; Table 2). Also, a marginal difference is observed in the ratio of CD4+ and CD8+ T cell populations between CMV- Positive and CMV- negative groups (1.2 vs 1.5, p=0.054; Table 2).

Table 2: The comparison of cell subtypes between patients with and without CMV infection

S#	Cell types	CMV Status			
		Total	Positive	Negative	P
		Median (IQR)	Median (IQR)	Median (IQR)	
1	Viability	78.4 (70.8-83.2)	77.9 (69.6-83.2)	80.6 (73.0-82.8)	0.458
2	CD4	72.4(56.1-88.4)	65.8 (46.3-75.0)	85.6 (68.6-93.0)	**0.051**
3	CD8	48.6 (28.8-66.1)	49.9 (38.3-64.5)	42.3 (22.5-70.0)	0.466
4	CD4:CD8	1.4 (0.9-2.6)	1.2 (0.7-1.7)	1.5 (1.0-3.3)	**0.054**
5	Snt-CD4	3.4 (0.7-10.8)	5.0 (1.0-13.2)	2.7 (0.6-7.1)	0.134
6	Snt-CD8	24.7 (15.5-46.3)	35.1 (15.5-55.1)	24.3 (16.5-37.2)	0.218
7	NKT Cells	1.15 (0.3-2.8)	1.6 (0.6-3.8)	0.5 (0.1-1.6)	**0.032**
8	Monocytes	9.9 (4.7-24.4)	15.9 (4.7-24.4)	8.6 (6.0-17.8)	0.571
9	B cells	9.2 (6.2-168)	9.3 (7.0-25.7)	9.1 (6.2-12.0)	0.589
10	N-CD4	91.5 (82.0-95.0)	90.7 (84.6-94.5)	91.5 (82.0-97.3)	0.850
11	pE1-CD4	33.9 (17.5-51.0)	33.3 (17.6-50.2)	34.5 (14.3-51.0)	0.920
12	pE2-CD4	10.6 (5.5-17.1)	10.8 (8.2-25)	7.3 (0.3-12.2)	**0.048**
13	E-CD4	18.8 (10.5-42.1)	20.2 (13.8-47.8)	11.9 (5.4-26.9)	**0.030**
14	CM-CD4	84.2 (69.6-91.7)	84.6 (71.2-91.3)	83.9 (69.6-91.7)	0.860
15	EM4-CD4	22.6 (4.0-39.0)	22.1 (9.2-35.7)	23.1 (0.4-57.6)	0.792
16	EM1-CD4	46.4 (15.8-65.7)	46.7 (37.7-57.9)	39.9 (13.2-70.0)	0.473
17	EM3-CD4	11.9 (2.3-22.1)	13.9 (9.4-33.6)	7.3 (0.7-13.7	**0.019**
18	EM2-CD4	6.2 (1.6-11.2)	6.8 (2.3-11.2)	5.2 (0.6-10.8)	0.521
19	Tregs	6.2 (2.4-15.7)	7.9 (3.8-10.9)	5.6 (1.1-17.5)	0.940
20	N-CD8	53.5 (12.0-76.5)	34.0 (12.0-63.4)	63.7 (26.8-81.6)	0.163
21	pE1-CD8	14.4 (6.1-34.6)	11.5 (6.3-31.1)	16.0 (2.1-36.1)	0.782
22	pE2-CD8	13.9 (5.4-28.9)	15.3 (6.2-25.2)	10.1 (0.8-31.1)	0.208
23	E-CD8	41.4 (14.6-64.1)	54.9 (38.1-72.1)	20.8 (7.4-49.1)	**0.035**
24	CM-CD8	62.2 (25.8-75.7)	59.4 (17.8-74.4)	63.6 (42.8-84.5)	0.521
25	EM4-CD8	10.9 (1.3-22.6)	7.1 (1.9-16.8)	17.6 (0.7-35.0)	0.279
26	EM1-CD8	25.6 (10.9-47.9)	20.9 (12.1-41.0)	26.6 (6.9-47.9)	0.850
27	EM3-CD8	31.6 (12.1-53.7)	46.5 (27.5-63.1)	15.5 (7.1-34.2)	**0.005**
28	EM2-CD8	8.7 (5.3-20.0)	9.4 (6.8-16.9)	7.6 (1.5-20.4)	0.563
29	PD1-CD4	49.7 (16.5-91.3)	62.5 (25.5-91.8)	43.3 (5.3-84.6)	0.151
30	LAG3-CD4	31.2 (2.6-83.3)	42.0 (11.1-95.7)	15.5 (2.0-66.7)	**0.074**
31	TIM3-CD4	0.3 (0.0-16.7)	2.0 (0.0 -26.0)	0.1 (0.0- 2.6)	0.172
32	PD1-CD8	25.0 (4.5-70.5)	29.5 (9.1-65.2)	12.5 (4.4- 70.5)	0.358
33	LAG3-CD8	6.4 (0.8-51.6)	12.8 (1.9- 82.5)	4.1 (0.0- 31.9)	0.278
34	TIM3-CD8	0.5 (0.0-25.0)	1.6 (0.0- 25.7)	0.4 (0.0 -5.6)	0.435
35	CD56+/CD16+	87.2 (68.8-95.4)	88.6 (69.5-95.3)	83.7 (68.8-95.9)	0.935
36	CD56+/CD16-	12.9 (4.6-31.9)	11.5 (4.7-30.5)	16.4 (4.1-31.9)	0.935

The table shows median relative percentages of cells types as determined from the parent populations in the hierarchical procedure in Figures 2, 3 and 4. The cell markers analysed included CD4, CD8, the ratio of CD4 to CD8 (CD4: CD8) calculated, senescent CD4 (Snt-CD4), CD8 (Snt-CD8), natural killer T cells (NKT cells), monocytes, B cells, Naïve CD4 (N-CD4), pre-effector - 1 CD4 (pE1-CD4), pre-effector - 2 CD4 (pE2-CD4), effector CD4 (E-CD4), central memory CD4 (CM-CD4), effector memory -1 - CD4 (EM1-CD4), effector memory -2 - CD4 (EM2-CD4), effector memory -3 - CD4 (EM3-CD4), effector memory -4 - CD4 (EM4-CD4), regulatory T cells (Tregs), naïve CD8 (N-CD8), pre-effector - 1 CD8 (pE1-CD8), pre-effector - 2 CD8 (pE2-CD8), effector CD8 (E-CD8), central memory CD8 (CM-CD8), effector memory -1 - CD8 (EM1-CD8), effector memory -2 - CD8 (EM2-CD8), effector memory -3 - CD8 (EM3-CD8), effector memory -4 - CD8 (EM4-CD8), Programmed cell death protein- 1 CD4 (PD1-CD4), PD1-CD8,

Lymphocyte-activation gene -3 CD4 (LAG3-CD4), Lymphocyte-activation gene -3 CD8 (LAG3-CD8), T-cell immunoglobulin and mucin-domain containing-3 CD4 (TIM3-CD4), T-cell immunoglobulin and mucin-domain containing-3 CD8 (TIM3-CD8), natural killer cells (CD56+/CD16+ and CD56+/CD16-).

The CD4+ T cells have shown a reduced frequency in the CMV- positive FL patients than CMV- negative group. Notably, CD4+ T cells alongside CD8+T cells forms the bulk of T-lymphocytes. The CD4+T cells are critical in attaining a coordinated, efficient immune response to pathogenic agents. Following activation, CD4+T cells differentiate into distinct effector subtypes that actively mediate immune response by secreting different cytokines and performing many functions. The functional activities of CD4+ T cells cover the activation of the cells of the natural immune system, B-lymphocytes, cytotoxic T cells, and nonimmune cells, and also play a crucial part in the elimination of immune response. Although no striking observation is made on the values of exhaustion surface markers analysed, higher expression values of CD4+ and CD8+ PD-1, LAG-3 and TIM-3 (Table 3-3) phenotypes are seen among CMV- positive cohort compared to CMV- negative arm. The CMV-negative group presents a marginal increase in the median value of CD4: CD8 ratio of 1.5 against 1.1 of CMV- positive FL patients.

Also, no significant difference in quantities is observed of senescent CD4+ and CD8+ T cells in the study population. However, the CMV-positive patients show an increased expression trend for senescent CD4+ and senescent CD8+ T cell populations compared to the negative FL patients.

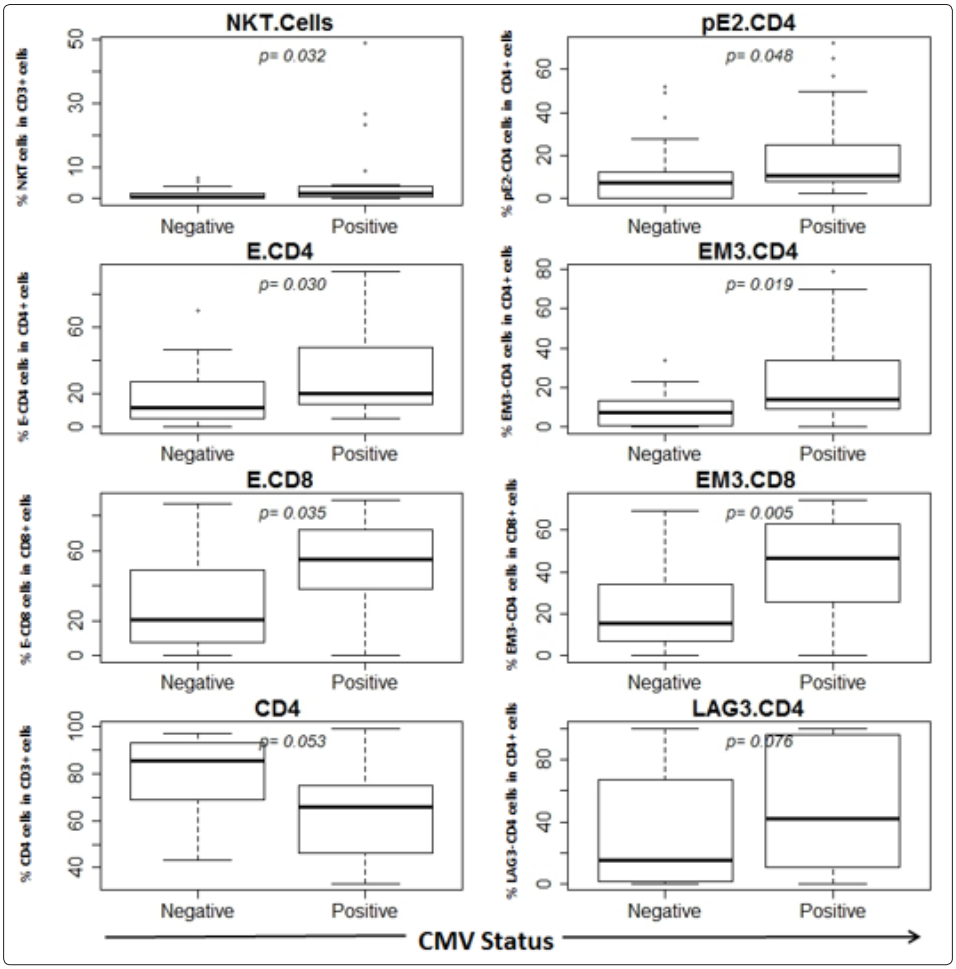

Figure 5: Boxplots of end-stage T cell subtypes and NKT cells showing significant difference between CMV-positive and -negative patients

Discussion

The FACS analysis of the stored PBMC samples of FL patients has revealed high quantities of subtypes of end-stage terminally differentiated (TEMRA) T cells from both the CD4+ and CD8+ T cell compartments as well as NKT cells in the CMV-positive patients compared to the CMV- negative group. In details, these data show that pre-effector-2 (pE2) (CD45RA+CD27+CCR7-CD28-), effector (CD45RA+CD27-CCR7-CD28-) and effector memory-3 (EM3) (CD45RO+CD27-CCR7-CD28-) subtypes of the CD4+ T cell compartment, effector (CD45RA+CD27-CCR7-CD28-) and effector memory-3 (EM3) (CD45RO+CD27-CCR7-CD28-) phenotypes of the CD8+ T cell compartment accumulated more in the CMV-positive patients. Phenotypically, the terminally nonproliferating effector T cell subsets usually lack the costimulatory receptors CD27 and CD28 as well as lymph node homing receptors CCR7 and CD62L on the cells surface, and are variously referred to as TEMRA, CD45RA+ memory, terminally differentiated (TTD) or end-stage or persisting effector T cells [15]. As a result, these cells are characterised by diminished long-term memory potential, limited effector function and poor cytokine production, resulting in impaired immune functions, leading to increased vulnerability to infections.

CD4+ and CD8+T cells constitute most T-lymphocytes in the peripheral blood, both of which possess specific T cell receptors for efficient adaptive immunological responses. The high accumulation of nonproliferating versions of these cells portends immune deficits for the patients. Moreover, effector T cells that display TEMRA phenotypes have been reported to demonstrate senescence and end-stage differentiation and are found eminently increased in both CD4+ and CD8+ T cell arms, especially in association with ageing and CMV infection [16, 17]. A close look at the differentiation phases of T cells identifies terminal effector T cells (TEMRA) as the most differentiated subtype of memory T cells, which are known to be extremely susceptible to apoptosis and display high levels of perforin and Fas ligand (cytotoxic molecules) [18].

Although a high proportion of NKT cells is observed in the CMV-positive FL patients, a functional analysis of these cells would be required to help in assessing the functional potential of the high-frequency cells in FL However, NKT cells are known to share properties of both NK cells and T cells. They act rapidly to stimulus, a characteristic of the innate arm of the immunity, and can produce cytokines such as IL-2, IFN-gamma, TNF-alpha, and IL-4 typical of adaptive immunity to promote or suppress different immune responses [19]. Of interest, the NKT cells have been shown to play a role in tumour immunity. Notably, it has been demonstrated that poor IFN-gamma production due to defective NKT cells was observed in patients with progressive malignant multiple myeloma but not in non-progressive myeloma or premalignant gammopathy [20, 21]. Furthermore, an analysis of peripheral blood has reported a statistically significant reduction in the quantities of NKT cells in patients with some solid tumours in relation to healthy persons [20, 22]. Also, decreased production of IFN-gamma or proliferation by the type-I NKT cells associated with cancer patients and low circulating levels of type I NKT cells was an independent predictor of poor overall survival (O/S) and disease-free survival (DFS) in patients with head and neck squamous cell carcinoma [23].

Conversely, the type II NKT cells mediate an opposite regulatory role in tumour immunity; for instance, activation of type II NKT cells by sulfatide enhanced tumour burden. However, because we did not stratify the NKT cells into their subtypes, it is impossible to conclude which subtype of NKT cells are elevated in the CMV-positive FL patients and the impact of such an increase in these clinical outcomes patients. However, drawing from the functional profile of NKT cells, it is also sensible to infer that the CMV-positive FL will either suffer more recurrent infections and adverse events (AEs) or poor response to therapies. It is also suggested that the increase in NKT cells in CMV- positive cases might be a compensatory response to the reduction in adaptive immunity resulting from reduced CD4+ T cells and accumulation of exhausted T cells.

Contrarily, although marginally significant, elevated CD4+T cells (p=0.051, Table 3-3) were associated with CMV-negative FL patients compared to the CMV-positive group. Of note, CD4+ T helper (Th) and cytotoxic CD8+ T lymphocytes (CTL) constitute the bulk of T-lymphocytes. Importantly, CD4+ T cells that comprise different lineages elicit immune responses via the production of distinct cytokines and perform multiple functions, including activating a host of immune and nonimmune cells such as B-lymphocytes and cytotoxic CD8+ T cells. In this regard, the CMV- negative FL patients possibly have some immunologic advantage over the CMV-positive patients. Furthermore, although there is no significant difference in the quantities of the Naïve-CD4+ and -CD8+ T cell phenotypes due to CMV status, the CMV- negative patient have shown a trend of higher median values for both naïve CD4+ and CD8+ T cells compared to the CMV-positive group (Table 2). Finally, it is essential to mention that having sufficient quantities of naïve T cells provide a more virile response and protection against infections by novel pathogens [24].

The multicolour flow cytometric analysis employed by the current study seems to demonstrate for the first time an analysis of a large number of subpopulations of T cells in FL, especially in relation to CMV infection. However, to secure a clear-cut interpretation of flow cytometric analysis of circulating lymphocyte subtypes, a functional analysis of the various T cell subsets is suggested. In the meantime, an exploratory analysis relating the quantities of the cell types to clinical data, involving treatment response status and toxicities records of the trial patients will help provide a further understanding of the clinical implications of findings. Also, it is a well-known fact that in a deliberate effort to keep the CMV under checks, the immune profile of a latent CMV-infected individual is adversely affected, particularly leading to an expanded population of CMV-specific memory CD8+ T cells, in a memory inflation phenomenon [25]. Unfortunately, it was not within the scope of this study to investigate this important hallmark of CMV infection by evaluating for CMV-specific CD8+ T cells and functional studies that would have further support the findings.

Although not much has been reported on CD4 TEMRA cells, the phenomenon of expanded end-stage T cells in the circulating peripheral blood has been reported by researchers in relation to other clinical conditions. For instance, a study by Reinke et al. has reported a significant correlation between elevated levels of end-stage CD8 (+) effector memory T (TEMRA) cells in peripheral blood with delayed fracture healing [26]. This study has provided a clear illustration of the role of T cells in modulating endogenous bone fracture repair even in the absence of infection. Another study that employed the Cox regression model reported a 2-fold higher risk of late graft dysfunction among kidney transplant recipients who had increased levels of differentiated Temra CD8+ T cells before transplantation [27]. Also, an increased accumulation of terminally differentiated CD8 T cells (TEMRA) has been reported in chronic graft-versus-host disease (cGVHD) as a consequence of continuous differentiation from naïve/central memory T cells to TEMRA cells following prolonged alloantigen exposure [28]. However, a study that also determined the frequency of subtypes of peripheral T-cells, including CD8 TEMRA cells before kidney transplantation, showed that advanced end-stage renal disease (ESRD) related with T-cell dysregulation is associated with a relative expansion of CD8 TEMRA cells guards against the acute rejection of kidney allografts [29]. Elevated percentages and absolute numbers of TEMRA cells in the subsets of CD4 and CD8 T that express CD26 were observed in type 1 diabetes, and the number of TEMRA cells associated positively with indices of intermediate and long-term glycaemic control [30]. The significant accumulation of TEMRA T cells in type 1 diabetes patients is thought to be caused by a long-term and sustained antigenic stimulation consequent to a prolonged presentation of viral or other antigenic particles or could be a homeostatic deficit in the regulation/contraction of immunological responses.

Judging from the significant accumulation of end-stage T cells and high senescence and exhausted T cells in the CMV-positive FL patients, one may speculate that the presence of CMV infection in FL patients can alter the immune dynamics of patients. Thus, these findings support the hypothesis that CMV infection results in perturbation of the immune system in FL in a way that might

influence disease course. These revelations can be factored in the management approach for Follicular lymphoma patients towards an improved outcome.

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