Current Research in Immunology 5 (2024) 100079

Contents lists available at ScienceDirect

Current Research in Immunology

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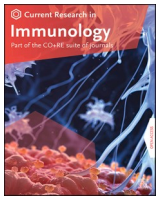

Lymphoblastoid cell lines do not recapitulate physiological circulating B cell subtypes

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ARTICLE INFO

Keywords:
Lymphoblastoid cells
LCL
EBV immortalised transformed B cells
B cell subtypes
Markers

ABSTRACT

Lymphoblastoid cell lines (LCLs) are immortalised peripheral B lymphocytes, transformed via infection with Epstein Barr virus (EBV). The use of LCLs to study B cell function remains controversial and core markers to define physiological B cell populations are not consistent between studies of physiological B cells and LCLs. A consensus on the nature of these commonly used cell lines has not been reached. Recently, a core set of markers to subtype peripheral B cells was proposed, addressing the lack of agreed markers for B cell characterisation. In this present study, the consensus panel was applied to describe the B cell subtypes in LCLs. We found that LCLs were generally not physiologically representative of B cells, with most cells harbouring marker combinations absent on peripheral B cells. Some B cell subtyping markers were fundamentally altered during EBV transformation to LCLs (e.g. CD19, CD21). Notably, most LCLs secreted IgG but the associated marker combinations were predominantly only present *in vitro* following EBV transformation. This study therefore informs interpretation of past investigations, and planning of future studies using LCLs, as these cells are unlikely to behave like their pre-transformed B cell subtype.

1. Introduction

Epstein Barr virus (EBV) can infect peripheral human B lymphocytes, resulting in the formation of immortalised lymphoblastoid cell lines (LCLs), the most notable feature of which is indefinite proliferation (Neitzel, 1986). These transformed B cells have been used extensively in laboratory-based research for decades and have been found to have many benefits experimentally, including: (i) easy preparation, (ii) minimal maintenance of cell culture, and (iii) minimal somatic mutation rate within the culture (Hussain and Mulherkar, 2012; Mohyuddin et al., 2004). Despite this, the ability of LCLs to recapitulate B cell subtypes and function remains controversial (Rodriguez et al., 2021; Mazzei et al., 2011; Zijno et al., 2010) and fundamental questions regarding the physiological B-cell features of these cell lines remain.

B cell development is a highly regulated and ordered selection process, originating from hematopoietic precursor cells in the bone marrow. CD19 and CD20 are markers traditionally used in the identification of global B cell populations due to their wide-ranging expression across almost all B cell subtypes in the development pathway. CD19 is expressed from pro-B cells to early plasma cells and CD20 is expressed from pre-B cells to early plasmablasts (Forsthuber et al., 2018); i.e. the longer lived matured humoral immune cells. Markers to identify specific B cell subtypes along this pathway have been identified but their expression levels are often less well defined between subtypes and have been inconsistently applied across studies (Rodgers et al., 2022).

Historically, the core markers used to define many of the specific physiological B cell subtypes have differed between studies (as summarised by Sanz et al. (2019)) and reviewed in the context of melanoma associated B cells (Rodgers et al., 2022)), which makes comparison of B cell subtypes between a large number of studies difficult. The question of whether some B cell phenotyping markers can be applied to the characterisation of LCLs has previously been explored (Supplementary Table 1). These studies have clearly indicated that the transformed B cell sub-populations were heterogeneous and remained so following *in vitro* expansion (Supplementary Table 1; (Megyola et al., 2011; Ozgyin et al., 2019; Yap et al., 2019). CD19 and CD20 are expressed on LCLs, however, expression of these fundamental markers was variable, with some cell lines downregulating CD19 and/or CD20 following EBV

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https://doi.org/10.1016/j.crimmu.2024.100079
Received 1 April 2024; Accepted 30 May 2024

Table 1
Summary of the FACS panel investigation of B cell subtype and results of the IgG ELISA using FMO gating strategy.

Cell Line	IgG Surface Marker Expression (−/+/++/+++)	Secretion (−/+/++/+++)						
		IgG	CD20	IgD	CD27	CD38	CD24	CD21
C001	++	−	+++	+	−	+++	+	+
C006	++	−	+++	−	+	+++	+	−
C013	+	−	+++	+	−	+++	+	+
C016	++	−	+++	−	+	+++	+	−
C017	++	−	+++	−	−	+++	+	+
C025	++	−	+++	−	−	+++	−	−
C027	++	−	+++	−	+	+	−	+
C039	++	−	+++	−	−	+++	+	+
C043	+	+	+++	−	−	+++	−	+
C045	++	−	+++	−	−	+++	+	−
C057	−	−	+++	+	−	+++	−	+
C058	++	+	+++	−	+	+++	+	+
C065	−	−	+++	+	−	+++	+	+
C071	+	−	+++	−	+	+++	+	+
C074	−	−	+++	−	−	+++	+	+
C089	+	−	+++	+	−	+++	+	+
C092	+	−	+++	+	−	+++	+	+
C094	++	−	+++	+	+	+++	+	+
C106	+++	−	+++	+	+	+++	+	+

IgG secretion results are displayed alongside the results for the FACS panel analysis using FMO gating strategy.
IgG secretion summarised by negative (−) positive; low (+), mid (++) and, high (+++). Ranges for IgG secretion categories: + = 1–60 ng/mL), ++ = 61–750 ng/mL and, +++ = >750 ng/mL. Marker expression classified as; negative (−) low (+), mid (++) and high (+++) dependant on the percentage of positive populations (i.e. - = <5%, + = 5–75%, ++ = 76%–90%, +++ = >90%.

transformation, without the typical upregulation of mature B cell markers (Wroblewski et al., 2002; Vockerodt et al., 2008; SoRelle et al., 2021); Supplementary Table 1. While inconsistencies between marker definitions for B cell subtypes hinders the comparison between previous studies, they have demonstrated variable expression in key B cell markers, including: CD21, CD23, CD30, CD38, CD54, CD69, CD71, CD77, CD95, IgA, IgD, IgE, IgG and IgM (Hur et al., 2005; Steinitz, 2014; Mrozek-Gorska et al., 2019; Karran et al., 1995; Ozgyin et al., 2019; Yap et al., 2019; SoRelle et al., 2021); Supplementary Table 1.

Class switching and the ability to secrete immunoglobulin are key features of B cell function and can also act as markers of B cell development. For example, secreted IgG could be indicative of B cells functioning as plasma cells, plasmablasts and, more controversially, B-1 cells (Quach et al., 2016). There is a consensus that LCLs are capable of secreting immunoglobulins, but the expression of the different isotypes has been variable between studies (Supplementary Table 1) (Steinitz, 2014; SoRelle et al., 2021; Heath et al., 2012; Lau et al., 1989; Burlingham et al., 1989; Ariga et al., 1985; Simmons et al., 1981; Guglielmi and Preud'homme, 1981). While some studies suggested the antibody repertoire of the original B cells were maintained in LCLs (Steinitz, 2014) and that class switching was not induced by EBV transformation (Heath et al., 2012), others showed a change in isotype occurred (Hur et al., 2005) and that LCLs are more clonal than those B cells present in the periphery (Rodriguez et al., 2021); Supplementary Table 1.

Recently, a consensus marker panel and gating strategy was proposed to conclusively phenotype peripheral B cells derived from peripheral blood mononuclear cells (PMBC) (Sanz et al., 2019). Key cell surface phenotypic markers CD19, CD21, CD24, CD27, CD38, IgM and IgD, along with optional additional markers, including CD20, CD95, IgA and IgG were proposed to be used in distinct combinations to define most agreed B cell subtypes (Sanz et al., 2019).

Given the inconsistencies in published data characterising features of cells present in LCLs, we aimed to investigate if B cell subtypes could be identified using a panel based on the recently published consensus B cell subtyping markers (Sanz et al., 2019). Our data provide conclusive evidence of B cell features retained by LCLs as well as the paucity of recognised defined B cell subtypes present in these transformed cells.

2. Methods

Nineteen lymphoblastoid cell lines were derived from stage III and IV cutaneous melanoma patients (O'Rourke et al., 2003; O'Rourke et al., 2007) using standard protocols (originally described by (Neitzel, 1986)). The LCLs have undergone low passage since the establishment of the transformed culture, stored long term at −152 °C and were cultured under standard conditions (37 °C, 5% CO2 and 21% O2).

Flow cytometry (Miltenyi Biotec, UK) was used to determine the B cell marker profile of LCLs (Supplementary Table 2), with 100μL/5 × 10^5 cells/test fixed in PBS, 1% BSA, 0.1% sodium azide. Due to the human IgG backbone on the REAffinity antibodies (Miltenyi Biotec, UK), the anti-IgG antibody was incubated first, then after washing, the remaining panel of antibodies were incubated. Cell pellets were resuspended in PBS, 1% BSA, 0.1% sodium azide and data were collected using a calibrated MACSQuant Analyser 10 (Miltenyi Biotec, UK) then analysed using FlowJo software (version 10.08.00, FlowJo, US). A fluorescence minus one (FMO) based gating strategy was used to characterise the presence or absence of markers and the undefined LCL populations. The gating strategy described by Sanz et al. (2019) was used to identify specific, established B cell subtypes. This gating strategy relies on classification of low (CD20), mid (CD38) or high (CD21, CD24 and CD38) fluorescence for some subtypes (Supplementary Figure 1).

Secreted IgG was detected in LCL supernatant using a commercial human IgG ELISA kit according to the manufacturer's instructions (catalogue #3850-1AD-6; MABTECH, UK) and optical density was read at 405 nM by a Thermo Varioskan LUX scanner (Thermo Scientific, UK).

3. Results/discussion

During panel optimisation, CD19 was not consistently expressed on all cells present in LCL culture (Supplementary Fig. 2), which is in agreement with some past studies (Wroblewski et al., 2002; Vockerodt et al., 2008; SoRelle et al., 2021) but not others (Hussain et al., 2012; Gellner et al., 2016; Ozgyin et al., 2019), although the sample numbers in these latter studies were low (Supplementary Table 1). CD19 is robustly expressed on nearly all primary B cell subtypes, except plasma cells (terminally differentiated, mature, somatic hypermutated, activated and antibody secreting) and is frequently co-expressed with CD20, except for early pro-B cells and plasmablast/plasma cells. In contrast

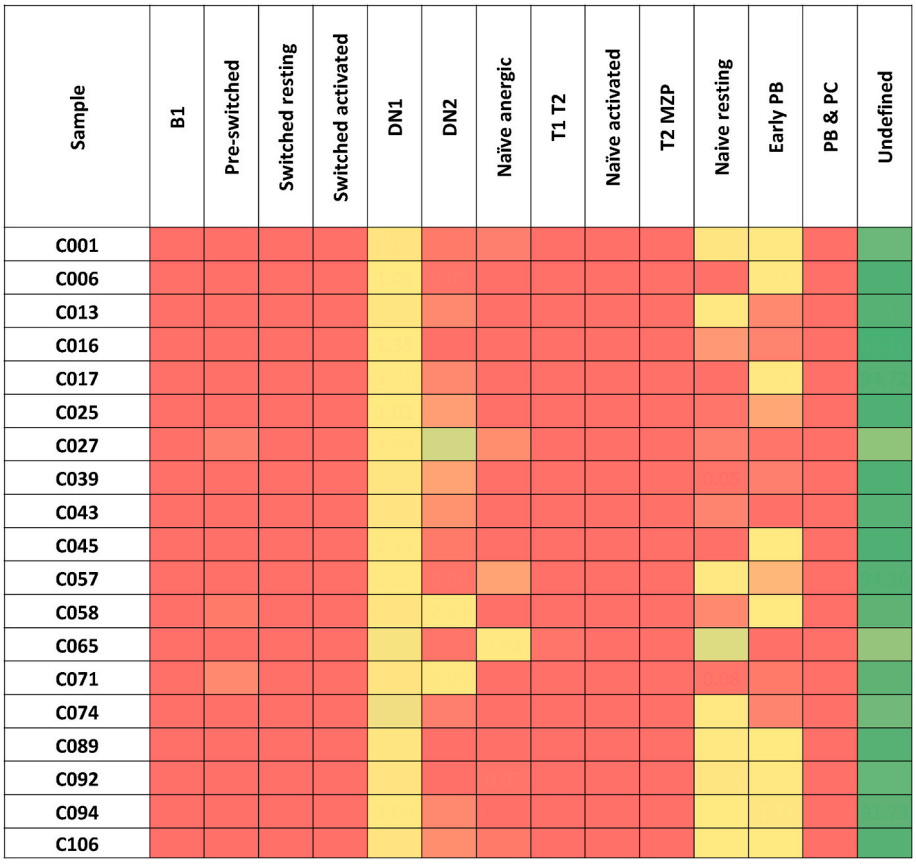

Fig. 1. Heat map of percentage of B cell subtypes.
B cell subtypes as determined using a gating strategy put forward by Sanz et al. (2019) for each cell line. The highest percentage is represented by a green colour and lower percentages/zero in red. 'Undefined' populations fell outside the defined gates.
DN1 = double negative 1, DN2 = double negative 2, T1/T2 = transitional 1/transitional 2, T2 MZP = transitional 2 marginal zone progenitor, PB = plasma blast, PC = plasma cell.

CD20 was highly expressed by all LCLs tested, including where CD19 was not (Table 1; Supplementary Fig. 2). Previous studies suggested that EBV transformation could down-regulate CD19 on B cells (Wroblewski et al., 2002; Vockerodt et al., 2008; SoRelle et al., 2021), which was consistent with these observations. As $CD20^{-}$ plasmablasts and plasma cells lack CD21, the receptor required for EBV internalisation (Jabs et al., 1999), it was unlikely that these B cell subtypes would be present within the LCL population. Therefore, CD20 is an appropriate pan-B cell marker in this study and was used in the final panel in place of CD19 (i.e. CD20, CD21, CD24, CD27, IgD and IgG).

A FMO quadrant-based gating strategy was used first to analyse the markers; Table 1. CD20 was universally highly expressed, in agreement with the hypothesis that plasmablasts and plasma cells would not be present in LCLs. Physiologically, in humans, immunoglobulins are either expressed on the B cell surface or are secreted, but not both. Two LCLs contained cells that expressed IgG on the cell surface and also secreted IgG (C043 and C058), suggesting a heterogeneous population of subtypes. There were three LCLs that contained cells neither expressing IgG on the surface nor secreted it (C057, C065 and C074). The remaining fourteen LCLs contained cells that secreted IgG but did not express it on their surface. CD38 was also highly expressed by all cell lines except for a single cell line (C027), where expression was low. The $CD20^{+}CD38^{+++}$ phenotype has been proposed as being indicative of a plasmablasts precursor, with some shared characteristics of plasmablasts, including spontaneous antibody secretion, Vh (heavy chain variable) utilisation and somatic mutation (Quach et al., 2016). This precursor phenotype was not included in the Sanz et al. (2019) B cell subtypes. $CD21^{-}$ cells were exclusively present in four cell lines and there were variable proportions of $CD21^{-}$ cells present in the remaining fifteen cell lines. As CD21 is well established as being required for EBV infection of B cells (Hussain and Mulherkar, 2012; Kuppers, 2003; Mrozek-Gorska et al., 2019), this suggests CD21 was downregulated following EBV infection, or in cell lines grown *in vitro*, rendering it uninformative as a B cell subtyping marker in this context. IgD, CD24 and CD27 expression was variable across cell lines, either showing low or no expression.

Using the gating strategy proposed by Sanz et al. (2019) (Supplementary Fig. 1), the B cell subtypes present within the LCLs were defined (Fig. 1). Most cells present in the LCL population were an 'undefined' subtype (61.52–98.10%) using this strategy; Fig. 1. There were, however, some definable B cell subtypes present within all the LCLs tested, notably double negative (DN)-1 (1.0–14.6%), DN2 (0.1–30.8%), naïve resting (0.1–23.5%) and early plasmablast (0.1–3.74%) subtypes (Fig. 1). C057, C065 and C074, which did not secrete IgG, were dissimilar to each other in the proportions of different subtypes present. C057 had only small populations of defined B cell subtypes, C065 had 23.5% naïve resting and 12.8% DN1 subtypes, and C074 had 2.93% naïve resting and 14.6% DN1 subtypes present. The majority of IgG secreting LCLs did not contain notable populations of antibody secreting physiological B cell subtypes as defined by Sanz et al. (2019). A previous study demonstrated some naïve B cells can undergo germinal centre-like activation and differentiation *in vitro* soon after EBV infection, resulting in transcriptomic and phenotypic features resembling plasmablasts and early plasma cells (Mrozek-Gorska et al., 2019). It is therefore possible these IgG secreting cells could have been either the $CD20^{+}CD38^{+++}$ precursor pre-plasmablasts described by Quach et al. (2016) or occurred as a result of EBV infection.

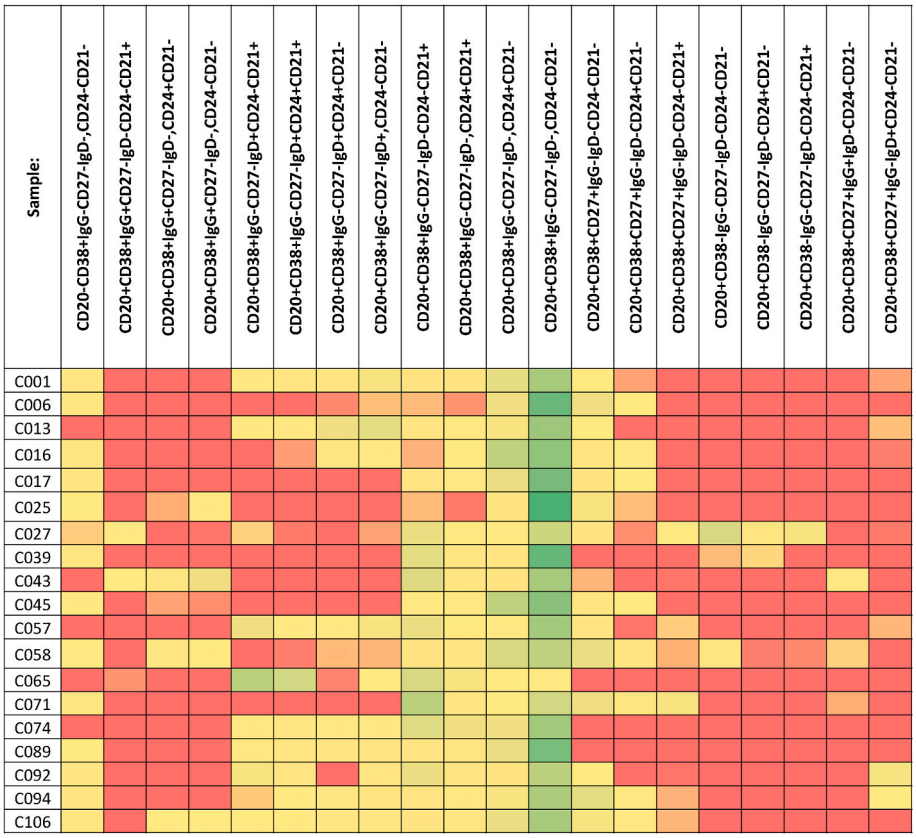

Fig. 2. Heat map of percentage of B cell population by FMO determined marker profiles.
The heatmap displays the percentage of each marker profile identified by FMO gating present in each LCL. The highest percentage is represented by green and lower percentages/zero in red.

Given the high proportion of undefined cells present in the LCL population, the markers defined by the FMO gating strategy were compiled into profiles to characterise the phenotypes present (Fig. 2). The most common marker profile identified was $CD20^{+}CD38^{+}IgG^{-}CD27^{-}IgD^{-}CD24^{-}CD21^{-}$, with other common marker profiles including $CD20^{+}CD38^{+}CD27^{+}IgG^{-}IgD^{-}CD24^{-}CD21^{-}$, $CD20^{+}CD38^{+}IgG^{-}CD27^{-}IgD^{-}CD24^{+}CD21^{-}$ and $CD20^{+}CD38^{+}IgG^{-}CD27^{-}IgD^{-}CD24^{+}CD21^{+}$ (Fig. 2). These marker combinations have not been previously described as physiological B cell subtypes; the proportions of different phenotypes present were largely different between the different LCLs, with only C017 and C045 being similar (Fig. 2).

4. Conclusions

This is the first study to systematically characterise the cells present in EBV transformed B cell lymphoblastoid cell lines using a consensus B cell marker panel (Sanz et al. (2019). Together, these data showed that LCLs were comprised of largely distinctively heterogeneous cell populations, which were frequently IgG secreting; however, only some cells were a defined B cell subtype, with the majority of cells displaying an unphysiological phenotype. Further investigation of the nature of the secreted antibodies is clearly warranted. These data unify published data (Supplementary Table 1), helping to explain some of the seemingly contradictory results, particularly given the observed heterogeneous nature of marker expression between cell lines, and the discrepancies with CD19 and CD21 expression and IgG secretion in LCLs. Consequently, it is unlikely that these cells will behave in a manner consistent with B cells *in vivo* or primary B cells *in vitro*. This study provides clarity on the nature of the B cell subtypes present in the LCLs, which helps to inform interpretation of past and planning for future studies.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antonia Pritchard reports financial support was provided by Highlands and Islands Enterprise. Connie MacKinnon reports financial support was provided by Highlands and Islands Enterprise. Ryan Maclean reports financial support was provided by Schizophrenia Association of Great Britain. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

CM and ALP were supported by Highland Island Enterprise (HMS 9353763). RTM was supported by the Schizophrenia Association of Great Britain. We are very grateful to Dr Chris Schmidt (formerly of QIMR Berghofer Medical Research Institute, Brisbane, Australia) who created and supplied the lymphoblastoid cells that were used in this project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crimmu.2024.100079.

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