Epstein-Barr virus persistence in the absence of conventional memory B cells: IgM+ IgD+ CD27+ B cells harbour the virus in X-linked lymphoproliferative disease patients

Sridhar Chaganti1*, Cindy S. Ma2*, Andrew I. Bell1, Debbie Croom-Carter1, Andrew D. Hislop1, Stuart G. Tangye2, and Alan B. Rickinson1

1 Cancer Research UK Institute for Cancer Studies, University of Birmingham, Vincent Drive, Edgbaston, Birmingham, B15 2TT, UK; 2 Garvan Institute for Medical Research, 384 Victoria St, Darlinghurst, Sydney, New South Wales 2010, Australia

*These authors contributed equally to the work
ABSTRACT

Epstein-Barr virus (EBV) persists in healthy virus carriers within the immunoglobulin (Ig)D\(^-\) CD27\(^+\) (class-switched) memory B cell compartment that normally arises through antigen stimulation and germinal centre transit. Patients with X-linked lymphoproliferative disease (XLP) lack such class-switched memory B cells but are highly susceptible to EBV infection, often developing fatal symptoms resembling those seen in EBV-associated haemophagocytosis (EBV-AHS), a disease caused by aberrant virus entry into the NK or T cell system. Here we show that XLP patients who survive primary EBV exposure carry relatively high virus loads in the B cell, but not the NK or T cell, compartment. Interestingly, in the absence of conventional class-switched memory B cells, the circulating EBV load was concentrated within a small population of IgM\(^+\) IgD\(^+\) CD27\(^+\) (non-switched) memory cells rather than within the numerically dominant naïve (IgM\(^+\) IgD\(^+\) CD27\(^-\)) or transitional (CD10\(^+\) CD27\(^-\)) subsets. In two prospectively studied patients, the circulating EBV load was stable and markers of virus polymorphism detected the same resident strain over time. These results provide the first definitive evidence that EBV can establish persistence in the B cell system in the absence of fully-functional germinal centre activity and of a class-switched memory B cell compartment.
INTRODUCTION

Epstein-Barr virus (EBV), a predominantly B-lymphotropic herpesvirus, is the causative agent of infectious mononucleosis (IM), a self-limiting lymphoproliferative disease seen in some cases of primary infection, and is linked to a range of malignancies that arise as rare accidents of long-term virus carriage. Despite its pathogenic potential, EBV is widespread in human populations, is usually acquired sub-clinically and is carried by the vast majority of individuals as a latent, asymptomatic infection of the re-circulating B cell pool. Fractionation of B cell subsets in the blood of such carriers has shown that the virus is harboured selectively within IgD+ CD27+ cells, i.e within the classical isotype-switched memory B cell population. Physiologically, this population is produced from naïve (IgD+ IgM+ CD27-) B cells by antigen stimulation and subsequent transit through a germinal centre (GC) reaction. Within the GC, diversification of IgV gene sequences by somatic hypermutation, coupled with Ig class switching, leads to the selection of high affinity antibody variants and the appearance of IgD- CD27+ memory cells expressing either IgG or IgA. Since EBV infects and transforms both naïve and memory B cells apparently equally well in vitro, how EBV achieves selective colonisation of the memory B cell pool in vivo remains unclear. One hypothesis envisages preferential entry of the virus into naïve B cells which, as a result of a transient growth-transforming infection mimicking antigen stimulation, are driven to form a GC; thereafter the physiologic processes of GC transit come into play and deliver latently-infected GC progeny cells into the long-lived memory compartment. Another hypothesis, based largely on the study of individual infected cells in IM lymphoid tissues, questions the involvement of the GC reaction in this context and envisages either the preferential infection of memory cells or the preferential expansion/survival of memory cells post-infection, compared to their naïve counterparts.
To inform this debate, one approach is to examine EBV infection in patients with immune deficiencies characterised by an inability to generate class-switched memory B cell responses. Typically, the circulating B cell pool of such patients is dominated by IgD$^+$ CD27$^-$ naïve B cells. The remaining cells are immature bone marrow-derived transitional B cells (distinguished by CD10 expression and normally present in very low numbers in healthy donor blood) and a small population of IgM$^+$ IgD$^+$ CD27$^+$ cells that express mutated IgV sequences$^{14-16}$; these latter cells have been referred to as IgM-memory or non-switched memory B cells$^{17,18}$. The origin of these atypical memory cells is unclear but, given their presence in such immune deficient patients, they may arise independently of a conventional GC pathway$^{18-20}$. Interestingly such IgM$^+$ IgD$^+$ CD27$^+$ memory B cells are also present in the blood and spleen of healthy individuals$^{21,22}$ but, like the naïve cell fraction, they are reportedly not a reservoir for EBV persistence$^3$. Here we focus on one such immune deficiency characterised by memory B cell impairment, X-linked lymphoproliferative disease (XLP). This is associated with mutations in $SH2D1A$ that encodes SAP$^{23-25}$, a protein involved in the regulation of B cell-T cell interactions mediated by members of the SLAM family of cell surface receptors$^{26-28}$. SAP is selectively expressed in T and NK cells, rather than B cells$^{15}$, and SAP deficiency abrogates the delivery of CD4$^+$ T cell help to B cells necessary for the generation of fully functional GCs and hence for the production of class-switched memory B cells$^{14,29,30}$. One of the clinical symptoms shown by XLP, therefore, is hypogammaglobulinaemia and an inability to mount IgG or IgA antibody responses; moreover, from the histologic analysis of splenic tissues, this is associated with an inability to form recognisable GC structures$^{15}$.

Virus carriage in boys with XLP has not previously been analysed in detail, not least because another consequence of SAP deficiency is an extreme susceptibility to primary EBV infection, such that many patients do not survive into the carrier state$^{31,32}$. Thus, exposure to EBV during
childhood or adolescence often (but not always) leads to severe IM-like symptoms, with high fever, lymphadenopathy and acute expansions of NK and CD8+ T cells in blood. Thereafter, in many cases the disease progresses to a fatal aplastic phase, first involving macrophage activation (thought to be initiated by NK- and T-cell derived cytokines), then haemophagocytosis and eventually bone marrow failure. At this point the clinical symptoms of XLP resemble those of another very rare complication of primary EBV infection, the EBV-associated haemophagocytic syndrome (EBV-AHS). Very interestingly, it now appears that EBV-AHS stems from the aberrant infection and virus-driven expansion of NK and/or T cell subsets in the blood, leading to a cytokine storm and ensuing macrophage activation and haemophagocytosis; survivors of the acute infection thereafter harbour EBV in circulating NK or T cells. The aim of the present work, therefore, was to examine XLP patients who had survived their primary EBV infection and to ask (i) is a stable virus carrier state established in these individuals?, (ii) if so, does this reflect virus persistence in the B cell pool in the absence of a class-switched memory compartment, or is the virus elsewhere?, and (iii) if there is persistence within B cells, is the virus colonising all available B cell subsets in the blood or just the “non-switched”, potentially GC-independent, memory pool?

MATERIALS AND METHODS

Donors

Peripheral blood samples were collected with informed consent from normal healthy donors, from XLP patients and from an EBV-AHS patient. All studies were approved by the Central Sydney Area Health Service Human Research Ethics Committee (Royal Prince Alfred Hospital, New South Wales, Australia) and South Birmingham Research Ethics Committee, United Kingdom.
Cell surface staining and cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Isopaque centrifugation. In initial experiments to isolate the B cell and non-B cell fractions from PBMCs, peripheral blood B cells were positively selected from PBMCs by immunomagnetic cell isolation using M-450 CD19 (Pan B) Dynabeads® (Dynal Biotech) followed by bead detachment. In subsequent experiments to identify transitional, naïve and memory B cell populations, PBMCs were labelled with FITC-conjugated anti-CD20 and PE-conjugated anti-CD27 monoclonal antibodies (mAbs, BD Pharmingen) and with one of the following antibodies, either APC-conjugated anti-CD10 or biotinylated anti-IgM, anti-IgD, anti-IgG, or anti-IgA mAbs followed by streptavidin (SA)-peridinin chlorophyll-a protein (BD Pharmingen) as previously described14. Data were collected on a FACScalibur flow cytometer (BD Biosciences) and analysed using FlowJo software (Tree Star Inc). PBMCs triple-stained for CD20, CD10 and CD27 as above were then sorted by FACS to isolate transitional (CD20+ CD10+ CD27−), naïve (CD20+ CD10− CD27−) and total memory (CD20+ CD10− CD27+) populations using a FACS Aria (BD Biosciences) cell sorter. In further experiments to identify naïve, non-switched memory and class-switched memory B cells, PBMCs were labelled with FITC-conjugated anti-CD20, PE-conjugated anti-IgD, PE-conjugated anti-IgM (BD Pharmingen) and APC-conjugated anti-CD27 (eBiosciences) mAbs and subsequently FACS-sorted to isolate naïve (CD20+ IgM+ IgD+ CD27−) and non-switched memory (CD20+ IgM+ IgD+ CD27+) populations.

Quantitative PCR assays for viral load

EBV genome load was assayed by quantitative real time PCR as described39,40. Briefly, total genomic DNA was extracted from PBMCs or isolated B cell subsets using a DNeasy Tissue kit (Qiagen) and triplicate aliquots of DNA (300ng) were then subjected to a multiplex PCR using
primer/probe combinations specific for the EBV BALF5 (POL) and the human β2 microglobulin (β2m) sequences. In parallel, serial DNA dilutions prepared from the Namalwa BL cell line (known to contain two integrated EBV copies per cell\textsuperscript{41}) were also amplified and used to generate standard curves for the absolute quantitation of POL and β2m copy numbers in the starting samples. In each case, the number of POL copies per sample was normalised using the corresponding β2m value, and the final result expressed as EBV genomes per 10\textsuperscript{6} cells.

**Quantitative reverse-transcriptase PCR assays for viral gene expression**

RNA extraction, cDNA synthesis and quantitative reverse transcriptase (RT)-PCR assays for the EBV lytic gene transcripts BZLF1, BVRF2 and BLLF1 were performed essentially as described\textsuperscript{42}, with the following modifications. To ensure that RNA preparations were free of residual genomic DNA (which might generate false positive signals for the unspliced BVRF2 and BLLF1 transcripts), total RNA was subjected to a second DNAse I treatment (DNA-free, Ambion). cDNA was prepared using a pool of primers specific for the viral transcripts BZLF1, BVRF2 and BLLF1, and the cellular GAPDH mRNA. In addition to the previously described primers and probe to amplify BZLF1 transcripts\textsuperscript{42}, we also designed new primer/probe combinations to amplify BVRF2 (forward primer 5′ CCACGGCAGTCTACGGTACA 3′, reverse primer 5′ GCGGCATTGGCGTCAT 3′, Taqman probe 5′ (FAM)-ACCTTGCGTGGGTCCTGAAGCATT-(TAMRA) 3′) and BLLF1 (forward primer 5′ AGAATCTGGGCTGGGACGTT 3′, reverse primer 5′ ACATGGAGCCCGGACAAGT 3′, Taqman probe 5′ (FAM)-AGCCCACCACAGATTACGCGGT-(TAMRA) 3′) lytic gene transcripts. PCR reactions were prepared in a final volume of 25µl containing 1x Taqman Universal PCR Mastermix (Applied Biosystems), 300nM primers, 200nM probe, 0.5µl human GAPDH endogenous control (Applied Biosystems P/N 4310884E) and 5µl cDNA. Relative
quantitation of gene expression was performed as described\textsuperscript{42}, using serial dilutions of cDNA derived from a suitable EBV-positive reference line Sal tr-LCL. Template-negative and RT-negative samples served as controls and were always undetectable. In cell reconstruction experiments using RNA preparations from serial dilutions of the semi-permissive B95.8 cell line (in which 10% cells were in lytic cycle) into an excess of EBV-negative BJAB cells, all three assays were capable of detecting the presence of a single lytically-infected cell (data not shown).

**Heteroduplex tracking assays**

EBV isolates were characterised on the basis of sequence polymorphisms within the EBNA2 and LMP1 latent genes using established heteroduplex tracking assays (HTA)\textsuperscript{43,44}. Aliquots of DNA from *ex vivo* PBMC samples were subjected to nested PCR amplification using primers specific for EBNA2 and LMP1. The subsequent product/probe binding reactions and heteroduplex analysis were performed as previously described\textsuperscript{44}.

**RESULTS**

**High EBV loads in XLP patients involve the B cell compartment**

A total of 8 XLP patients were studied during the course of this work. Table 1 gives their age and clinical phenotype at time of sampling, and indicates whether or not they had an earlier history of a fulminant IM episode. In an initial series of experiments, PBMCs from six of these patients (XLP1-6) were assayed for EBV DNA load by quantitative PCR alongside control samples from 12 healthy EBV carriers and, as a comparator, PBMCs from a rare patient who presented with acute EBV-AHS. As shown in Figure 1A, all six XLP patients’ samples were found to be EBV-positive with levels of infection spread across an unusually wide range, individual results being shown in Table 1. In particular three of the six XLP patients had loads either at the top of or (for XLP5)
greatly exceeding the normal donor range; the EBV-AHS patient also had a very high virus load. We then positively selected B cells from the PBMCs of four of these patients (XLP1-4) and of the EBV-AHS patient using CD19 antibody-coated Dynabeads, a procedure that routinely yields B cell-enriched preparations with >98% purity and leaves depleted preparations with <5% B cell contaminants, as judged by staining for a second B cell marker, CD20 (data not shown). These paired preparations were examined for EBV load, the data being shown in Figure 1B. Clearly the virus load in XLP patients was concentrated in the CD19+ B cell fraction, in contrast to the situation in EBV-AHS where the dominant virus load was in non-B cells. Positive selection of CD3+ T cells or of CD56+ NK cells confirmed infection of these subsets in the EBV-AHS patient, whereas the same procedure showed no evidence of T or NK cell infection in the XLP patients (data not shown).

While healthy individuals are known to carry EBV in the circulating B cell pool as an exclusively latent infection3, it remained possible that the situation in XLP patients may be different and that on-going virus replication might be contributing to the often-elevated viral loads seen in the above patients. To examine this, we developed sensitive quantitative RT-PCR assays for three EBV lytic cycle transcripts, the immediate early gene BZLF1, and two late genes BVRF2 (protease) and BLLF1 (gp350). These assays were each capable of detecting the presence of a single lytically-infected B95.8 cell in in vitro-reconstructed cell mixtures. However, these same assays never detected the presence of such transcripts in RNA isolated from the purified B cells of XLP patients, including B cells from XLP5, the patient with the highest viral load (data not shown). We infer from this that virus load assays are truly reflecting the latent genome load in the circulating B cells of these patients.
**EBV is concentrated in the non-switched memory B cells of XLP patients**

Four of the available XLP patients from whom larger blood samples could be obtained (XLP5-8) were chosen for more detailed analysis and compared to 6 healthy donor controls. Their PBMCs were co-stained with anti-CD20 and anti-CD27 mAbs to identify naïve (i.e. CD20⁺ CD27⁻) and memory (i.e. CD20⁺ CD27⁺) B cells, and with a third antibody against either surface IgD, IgM, IgG or IgA, or a marker that distinguishes the transitional B cell population, CD10⁺⁴⁵,⁴⁶. Representative FACS plots from one XLP patient and a healthy control are shown in Figure 2A and overall results are summarised in Figure 2B. In agreement with a previous study¹⁴, total B cell numbers were not significantly different between patients and healthy controls. However, the distribution of B-cell subsets was quite different. The percentage of circulating B cells with the CD10⁺ CD27⁻ transitional phenotype was significantly higher in XLP patients than controls (mean of 12% versus 4%)¹⁶, as was the percentage of CD10⁻ CD27⁻ naïve B cells (82% versus 61%). Conversely CD10⁻ CD27⁺ memory B cell numbers were greatly reduced in XLP patients compared to controls (5% versus 32%). Furthermore, confirming earlier observations on such patients¹⁴,¹⁵, the vast majority of these CD27⁺ B cells in XLP blood were non-switched memory cells that were IgD⁺ IgM⁺ and lacked IgG or IgA expression, in contrast to the situation in the control donor (Figure 2A).

The above PBMC samples from XLP patients that had been triple-stained for CD20, CD10 and CD27 were then sorted by FACS to isolate the transitional (CD20⁺ CD10⁺ CD27⁻), naïve (CD20⁺ CD10⁻ CD27⁻) and memory (CD20⁺ CD10⁻ CD27⁺) cell populations; these gave typical sort purities of 95-98% for each of the subsets, as published¹⁴⁻¹⁶. Figure 3 illustrates the data obtained when these different subsets were analysed for EBV DNA load and shows that, in each case, the virus was highly enriched in the memory subset. In particular, the virus load in memory cells was always...
>100-fold higher than in transitional cells and, depending on the patient, was between 10- and 300-fold higher than in naïve cells. As shown in earlier work\textsuperscript{15}, the CD27\textsuperscript{+} memory B-cell subset in the peripheral blood of XLP patients is dominated by cells co-expressing both IgM and IgD, with extremely small numbers of cells staining positively for IgG\textsuperscript{+} or IgA\textsuperscript{+}. It therefore seemed very unlikely that such a barely detectable class-switched population could account for the very high virus loads observed in these patients. However, to specifically address this point, we obtained further blood samples from two of these XLP patients (XLP5, XLP6) and in this case stained their PBMCs with anti-CD20, -IgM, -IgD and -CD27 mAbs. Figure 4A confirms that, in both patients, the circulating CD20\textsuperscript{+} B cell pool is dominated by naïve (IgM\textsuperscript{+} IgD\textsuperscript{+} CD27\textsuperscript{-}) cells, with smaller numbers of non-switched memory (IgM\textsuperscript{+} IgD\textsuperscript{+} CD27\textsuperscript{+}) cells and virtually no detectable class-switched (IgM\textsuperscript{-} IgD\textsuperscript{-} CD27\textsuperscript{+}) cells. Accordingly, in subsequent cell sorting experiments, we could not isolate a class-switched cell population from these samples, whereas we could readily obtain naïve and non-switched memory populations. With this sorting protocol, re-analysis showed that the naïve cell sorts were of high purity, while the non-switched memory cells gave purities of 91% for XLP5 and 77% for XLP6 (the lower XLP6 value reflecting the difficulty of sorting a population that is at low frequency in the initial sample); in both cases, however, the vast majority of the contaminants within the non-switched population are IgM\textsuperscript{+} IgD\textsuperscript{+} CD27\textsuperscript{-} naïve cells. Figure 4B shows the corresponding EBV DNA load data from these sorted populations compared to the load seen in unfractionated B cells from the same patient. In both cases the virus was predominantly found in the non-switched memory population.

**Stability of the virus load and of the resident EBV strain in XLP patients**

For these two patients (XLP5, XLP6) in whom the virus had been definitively localised to the non-switched memory B cell pool, successive bleeds had been taken and PBMCs stored over periods of
3 and 4 years respectively. These samples were screened in parallel by quantitative PCR and the EBV load data are shown in Figure 5A. While the two patients had quite different EBV load values, each retained their own characteristic load at a roughly stable level over the period of study. Such stability is consistent with the view that these patients are maintaining their EBV-positive status through persistence of a long-term infection rather than through a series of transient infections, each of which is cleared and then replaced by another exogenously acquired EBV strain. A more definitive resolution of the point became possible with the development of heteroduplex tracking assays to identify the resident EBV strain or strains within an individual. These assays involve amplifying viral DNA from *ex vivo* samples across non-linked polymorphisms in the EBNA2 and LMP1 regions in the virus genome and in each case identifying the allelic products through the mobility of heteroduplexes that form with a standard labelled probe. We applied these assays to the successive blood samples from the above two patients. Figure 5B shows the data obtained from XLP5 DNA samples taken over a 3 year period, the amplification products being run alongside those from reference EBV strains representing five different alleles of the EBNA2 locus commonly seen among type 1 EBV strains and five of the known alleles of the LMP1 locus. At each time point, the virus strain present in patient XLP5 had a 1.3B allele at the EBNA2 locus and a Med` allele at the LMP1 locus. The correct identification of these alleles was later confirmed by DNA sequence analysis (data not shown). The second prospectively studied patient gave similar results, with a single EBNA2 and a single LMP1 allelic sequence maintained throughout (data not shown).

**DISCUSSION**

The seminal observation that EBV persistence in healthy virus carriers is associated with selective colonisation of the IgD` CD27+ memory B cell pool has prompted much debate as to how that
selectivity is achieved. One hypothesis envisages that EBV infection of a IgD+ CD27 naïve B cell can recapitulate the physiologic process of antigen-induced maturation, such that transient activation of full virus latent gene expression drives the infected cell into a GC reaction, from which arise EBV-positive progeny cells that have acquired the IgV-mutated genotype and IgD− CD27+ phenotype typical of isotype-switched memory7. Another hypothesis denies any GC involvement in virus colonisation of the B cell system and envisages that the pre-existing memory B cell pool is either preferentially infected in vivo or is capable of preferential expansion/survival once infected, thereby outnumbering naïve B cell infections13. The present work sought to inform this debate by studying EBV infection in patients with XLP, an immune deficiency characterised by an inability to form conventional GC structures and to mount isotype-switched antibody responses. The main conclusions are (i) that XLP patients are capable of sustaining a persistent EBV infection in the absence of an isotype-switched memory population, and (ii) that this infection is associated with preferential colonisation of non-switched IgM+ IgD+ CD27+ memory B cells, an apparently GC-independent population that also exists in healthy individuals where, reportedly3, it does not harbour the virus.

The genetic defect in XLP involves the SH2D1A gene encoding SAP23-25, a protein normally expressed in T cells, NK cells and NK/T cells but, as far as is known, not in other lymphoid and myeloid lineages. SAP interacts with the cytoplasmic domains of members of the SLAM family of surface receptors26-28. This multiplicity of interactions with different cell signalling receptors produces a complex immunodeficiency characterised by three main features (i) a failure of NK-T cell development, (ii) a failure to generate conventional GCs and to mount isotype-switched antibody responses in response to antigenic challenge, and (iii) an unusual susceptibility to primary EBV infection, associated with aberrant polyclonal activation of CD8+T cell and NK cell responses
and leading to cytokine-mediated macrophage activation and an often fatal haemophagocytosis. Of these, the block in NK-T cell development$^{28,47}$ and the impairment in eliciting long-lived humoral immune responses$^{29,30}$ are reproduced in SAP-deficient mice; as to the susceptibility to primary infection, while there is no murine equivalent of EBV (a gamma-1 herpesvirus), challenging SAP-deficient animals with the murine gamma-2 herpesvirus MHV 68 or with a virus known to elicit strong CD8$^+$ T cell responses (lymphocytic choriomeningitis virus) likewise produces immunopathology from unrestrained responses of CD8$^+$ T cells$^{48,49}$. Just as the factors influencing susceptibility to IM in immunocompetent individuals are poorly understood$^{50,51}$, it is not clear why some SAP-deficient patients die from primary EBV infection, others develop a severe IM-like illness and survive, while others appear to acquire EBV sub-clinically$^{52}$. The rarity of the XLP trait and the focus on understanding the pathogenesis of the fatal IM has, until now, diverted attention away from any analysis of the longer-term fate of the EBV infection in surviving patients. Here we have addressed this issue in a cohort of patients with genetically confirmed SAP-deficiency, all of whom lacked a detectable isotype-switched memory B cell population in blood and displayed one or more clinical features of the XLP syndrome. Of these patients, some had a clinical history of a fulminant IM-like episode following primary exposure to EBV, others did not.

We first showed by Q-PCR for the viral genome that all patients did carry EBV in the circulating PBMC population. In some cases, the virus load was well within the normal range, while in other cases it was unusually high; although patient numbers were small, it is interesting to note that individuals with the higher loads were not necessarily those who had a prior history of fulminant IM (Table 1). As to which cells were infected, we were first prompted to compare B cells versus non-B cells because of the strong clinical parallels between the fulminant IM syndrome seen in XLP patients and primary EBV infection manifesting as EBV-AHS, a disease now shown to be
caused by rare entry of the virus into T and/or NK cells. We found that SAP-deficient patients did indeed carry the virus in B cells and not other cell types. Furthermore, as in healthy carriers, the virus appeared to be carried as a latent infection since we could find no evidence of lytic gene expression in circulating B cells even using very sensitive quantitative RT-PCR assays. Thereafter, sorting of B cell subsets clearly showed marked concentration of the virus in the small subset of circulating B cells with a CD10⁻ CD27⁺ memory phenotype. By comparison, loads were 10-300-fold lower in the numerically-dominant naïve B cell pool (CD10⁻ CD27⁻) and consistently more than 100-fold lower in transitional B cells (CD10⁺ CD27⁻), an immature population that is present as a minor subset in normal blood but is expanded in SAP-deficient patients. While the very low signals seen within sorted transitional cell populations almost certainly reflect low level contamination of these preparations by a few CD27⁺ cells, we cannot discount the possibility that where naïve B cell loads were only 10-fold lower than in the CD27⁺ subset, for example in XLP6, this could reflect some naïve B cell infection. The central point, however, is that the virus is being preferentially carried in a CD27⁺ B cell population which, as earlier work has shown and the present staining data confirm, is dominated by non-switched IgM⁺ IgD⁺ memory B cells and is essentially devoid of isotype-switched cells. Indeed in two cases we deliberately FACS-sorted this non-switched memory population away from any possible isotype-switched contaminants and showed that it did indeed harbour the virus.

There are some parallels between the above findings and those described in an earlier study of EBV infection in patients with hyper-IgM syndrome arising from CD40L deficiency. These patients also are unable to form conventional GCs and lack isotype-switched memory B cells. In that study, virus DNA could be detected on at least one occasion in the blood and/or throat washings of 6 of 9 patients analysed. However the levels were low, often close to the threshold of detection, and
only in one blood sample could viral infection be localised to the IgM+ IgD+ CD27+ population54. Furthermore prospective sampling showed that, in contrast to healthy carriers included as controls, most CD40L-deficient patients did not give consistent evidence of virus infection. Only one patient was positive on more than one occasion and, even in this case, the choice of an EBNA3C gene repeat sequence as the marker of strain identity meant that persistence of the same EBV strain could not be convincingly shown. The findings from CD40L-deficient patients were therefore interpreted to suggest that infections may be occurring transiently in these individuals and that persistence within the B cell pool requires entry into the isotype-switched memory compartment54. By contrast, here we have used a sensitive PCR assay to analyse B cell subsets sorted from four different XLP patients; the data clearly show that EBV can colonise the IgM+ IgD+ CD27+ population in patients lacking classical memory B cells and can do so at high levels. We also addressed the question of virus persistence using blood samples taken prospectively over 3-4 years from two different XLP patients, one with an unusually high load in IgM+ IgD+ CD27+ B cells, the other with a lower load. Those particular loads were maintained at roughly stable levels over the whole period of study. Moreover, using two independent and highly polymorphic markers of virus strain identity situated within the EBNA2 and LMP1 genes respectively, we found both patients retained one characteristic strain throughout. Such constancy of virus load and of virus identity is indicative of a persistent rather than a recurring infection.

The present findings therefore demonstrate that, in patients who lack conventional isotype-switched memory B cells and who from histologic analysis of splenic tissues15 cannot make conventional germinal centres, EBV can establish a stable infection and does so by selectively colonising an unusual B cell subset with an IgM+ IgD+ CD27+ phenotype. While such B cells are also present both in the blood and splenic marginal zone of healthy immunocompetent
individuals, their presence in XLP and CD40L-deficient patients has led some investigators to propose that they are not antigen-instructed memory cells in the true sense; rather, it is suggested, they are B cells that have undergone somatic hypermutation during generation of the pre-immune repertoire and later mount antibody responses to T cell-independent antigens only\textsuperscript{19,20}. As such, these cells would be GC-independent both in their generation and function. From this viewpoint, therefore, the detection of EBV within this population in XLP patients might be used to argue against any involvement of the GC reaction in virus colonisation of the B cell system. However we would caution against unequivocal interpretations until the issue of the ontogeny, specificity and function of IgM$^+$IgD$^+$CD27$^+$ B cells is fully resolved\textsuperscript{18,57,58}. In particular, while conventional GCs are absent from the spleens of SAP-deficient patients, it remains possible that atypical transient GC-like structures (like those seen in mice responding to T-independent antigens\textsuperscript{59}) can form in the absence of T cell help and support a degree of somatic hypermutation without subsequent isotype switching\textsuperscript{18}. That would be consistent with the lower number of cell divisions that IgM$^+$ IgD$^+$ CD27$^+$ cells have undergone \textit{in vivo}\textsuperscript{60}, and their lower levels of IgV mutation\textsuperscript{21,22}, compared to isotype-switched memory. It is therefore still possible that EBV infection of a naïve cell might commit that cell to initiate a GC-like reaction which, in a SAP-deficient patient, is transient and produces EBV-positive non-switched memory cells and, in an immunocompetent individual, goes through to completion and delivers the infected cell into the isotype-switched memory subset.

We would stress that the present findings were made in individuals with a complex immunodeficiency, XLP, where an impairment of specific cytokine production by TH2-like CD4$^+$ T cells abrogates isotype switching in antigen-driven B cell responses\textsuperscript{14}. The relevance of such findings to the situation in the normal healthy host remains to be seen. The evidence to date
suggests that EBV selectively colonises only class-switched (IgD⁻ CD27⁺) B cells in healthy carriers³. In light of our present findings, the EBV status of the non-switched subset also deserves close scrutiny. Interestingly, IgM⁺ IgD⁺ CD27⁺ B cell numbers are 3-5 times higher in the blood of healthy individuals than of XLP patients¹⁴. This has led to the suggestion that there are in fact two types of IgM⁺ IgD⁺ CD27⁺ B cells: the minority which arise independently of T cell help and are the only ones seen in XLP or CD40L-deficient patients; and the majority population which is T cell-dependent and is the numerically dominant population in normal individuals¹⁸. As yet, however, there is no obvious phenotypic or functional evidence of such heterogeneity¹⁸. If EBV were to selectively colonise one population but not the other, it may provide a means of their discrimination.
ACKNOWLEDGEMENTS

We would like to thank all patients for their participation in this study and the following clinicians for their cooperation: Drs S. Adelstein, F. Alvaro, P. Arkwright, J. Cohen, B. Gaspar, A. Klion, S. Riminton and P. Rohrlich. This work was supported by grants from Cancer Research UK, the National Health and Medical Research Council (NHMRC) of Australia and Cancer Council New South Wales. S.C. was funded by a Leukaemia Research Fund Clinical Research Fellowship. C.S.M and S.G.T. are recipients of Research Fellowships from the NHMRC.

AUTHORSHIP

S.C., C.S.M., A.I.B., D.C.-C. and A.D.H. performed the experiments, S.C., C.S.M. and A.I.B. analysed the data and prepared the figures, S.G.T and A.B.R. designed the research and wrote the paper. S.C. and C.S.M. contributed equally to this work. Correspondence: Alan Rickinson, Cancer Research UK Institute for Cancer Studies, University of Birmingham, Vincent Drive, Edgbaston, Birmingham, B15 2TT, United Kingdom; email: a.b.rickinson@bham.ac.uk.

Conflict-of-Interest statement: The authors declare no competing financial interests.
REFERENCES


FIGURE LEGENDS

Figure 1. Analysis of EBV genome load in healthy control donors and patients with EBV-associated disease. (A). Results of quantitative PCR analysis to determine the EBV load in PBMC samples isolated from control donors, from XLP patients or from an EBV-AHS (VAHS) patient. Data are reported as EBV genome copies per $10^6$ PBMCs, with the median value for each group denoted by the horizontal bar. (B). Results of quantitative PCR analysis to determine the EBV load in CD19$^+$ B cell (grey bars) and CD19$^-$ non-B cell (hatched bars) fractions isolated from XLP and VAHS PBMC samples. Data (mean and standard deviation of three replicates) are reported as EBV genome copies per $10^6$ cells.

Figure 2. Surface phenotype of B cells from healthy control donors and XLP patients. (A). PBMCs from a normal control donor and a representative XLP patient, XLP5, were stained with anti-CD20 mAb, and the frequency of B cells in the lymphocyte population determined (left-most panels, frequency shown as percentage). In the remaining panels, purified CD20$^+$ B cells were dual-stained with mAbs specific for CD27 and either CD10, IgD, IgM, IgG or IgA to obtain the FACS profiles shown. (B) Summary of B cell surface phenotype data obtained from 6 healthy control donors and 4 XLP patients (XLP5-8). Results are expressed (left to right) as the percentage of PBMCs that were CD20$^+$ B cells, and the percentage of CD20$^+$ B cells that had the transitional, naïve or memory cell phenotypes shown. The mean value for each group is denoted by the horizontal bar.

Figure 3. Analysis of EBV genome load in B cell subsets isolated from XLP blood. The histogram shows the results of quantitative PCR analysis to determine the EBV load in purified
CD20⁺ CD10⁺ CD27⁻ (transitional, open bars), CD20⁺ CD10⁻ CD27⁻ (naïve, hatched bars) and CD20⁺ CD10⁻ CD27⁺ (memory, black bars) B cells. Data (mean and standard deviation of three replicates) are reported as EBV genome copies per 10⁶ cells.

**Figure 4. Analysis of EBV genome load in IgM memory B cells isolated from XLP blood.**

(A). PBMCs from two XLP patients, XLP5 and XLP6, were stained with mAbs specific for CD20, IgM, IgD and CD27 to identify naïve (CD20⁺ IgM⁺ IgD⁺ CD27⁻), non-switched memory (CD20⁺ IgM⁺ IgD⁺ CD27⁺) and class-switched memory (CD20⁺ IgM⁻ IgD⁻ CD27⁺) B cell populations; numbers indicate the percentage distribution of CD20⁺ B cells between the three populations (left panel). Naïve and non-switched memory cells were subsequently isolated by FACS sorting and the isolated populations re-analysed, giving percentage purities as shown (centre and right panels). (B). The histogram shows the results of quantitative PCR analysis to determine the EBV load in unfractionated CD20⁺ B cells (grey bars), naïve B cells (hatched bars) and non-switched memory B cells (black bars) isolated from the two donors shown in (A) above. Data (mean and standard deviation of three replicates) are reported as EBV genome copies per 10⁶ cells.

**Figure 5. Analysis of EBV genome load and EBV sequences present in XLP patients.** (A).

The graph shows the results of quantitative PCR analysis to determine the EBV load in PBMC samples collected prospectively from patients XLP5 and XLP6 between 2003-2006. (B). Analysis of EBV sequences present in the same PBMC samples from patient XLP5 using EBNA2 and LMP1 HTAs. In the upper panel, PBMC DNA was PCR-amplified using type 1 specific EBNA2 primers and the resulting PCR products analysed by HTA using a 1.1 EBNA2 allele-specific probe. Also shown are the results from a panel of reference control isolates known to carry a 1.1,
1.2, 1.3A, 1.3B or 1.3E EBNA2 allele. In the lower panel, the same DNA samples were PCR-amplified using LMP1-specific primers and the resulting PCR products analysed by HTA using a Med+ LMP1 allele-specific probe. Also shown are the results from a panel of reference control isolates known to carry a B95-8, Ag876/Ch1, Ch2, Med+ or Med- LMP1 allele. The results show that each XLP5 PBMC sample contains detectable 1.3B EBNA2 and Med- LMP1 alleles.
**TABLE 1:** Features of XLP patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age yrs</th>
<th>Clinical phenotype</th>
<th>History of fulminant IM</th>
<th>EBV genomes per 10^6 PBMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>XLP1</td>
<td>12</td>
<td>Hypogammaglobulinaemia</td>
<td>Yes</td>
<td>5.2 \times 10^3</td>
</tr>
<tr>
<td>XLP2</td>
<td>10</td>
<td>Hypogammaglobulinaemia</td>
<td>No</td>
<td>1.9 \times 10^2</td>
</tr>
<tr>
<td>XLP3</td>
<td>31</td>
<td>T cell disorder, vasculitis</td>
<td>No</td>
<td>1.0 \times 10^2</td>
</tr>
<tr>
<td>XLP4</td>
<td>5</td>
<td>Hypogammaglobulinaemia</td>
<td>No</td>
<td>3.4 \times 10^3</td>
</tr>
<tr>
<td>XLP5</td>
<td>40</td>
<td>Hypogammaglobulinaemia</td>
<td>Yes</td>
<td>9.7 \times 10^4</td>
</tr>
<tr>
<td>XLP6</td>
<td>46</td>
<td>Hypogammaglobulinaemia</td>
<td>Yes</td>
<td>6.7 \times 10^2</td>
</tr>
<tr>
<td>XLP7</td>
<td>49</td>
<td>Hypogammaglobulinaemia, lymphoma, haemophagocytosis</td>
<td>Yes</td>
<td>nt</td>
</tr>
<tr>
<td>XLP8</td>
<td>28</td>
<td>Eosinophilia</td>
<td>No</td>
<td>nt</td>
</tr>
</tbody>
</table>

nt: not tested
Figure 1.
Figure 2.

A

Normal

XLP

B

CD20+ Total B

CD10+ CD27- Transitional B

CD10- CD27- Naive B

CD10- CD27+ Memory B
Figure 3.
Figure 4.
Figure 5.
Epstein-Barr virus persistence in the absence of conventional memory B cells: IgM + IgD+ CD27+ B cells harbour the virus in X-linked lymphoproliferative disease patients

Sridhar Chaganti, Cindy S Ma, Andrew I Bell, Debbie Croom-Carter, Andrew D Hislop, Stuart G Tangye and Alan B Rickinson