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Increased lymphocyte viability after non-stimulated peripheral blood mononuclear cell (PBMC) culture in patients with X-linked lymphoproliferative disease (XLP)

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Abstract Survival of lymphocytes after prolonged culture was studied in two asymptomatic XLP patients. Viability of XLP PBMC after 30 days of non-stimulated culture was higher than that of normal controls (N), mainly due to the persistence of CD8 memory lymphocytes. IFNγ high CD8 T lymphocytes remained higher in XLP than in N after 30 days. The number of perforin+ CD8 lymphocytes was markedly reduced after 30 days in XLP and in N. Increased viability was not related to CD127, PD-1, CD27, or CD62L expression. Concerning B lymphocytes, memory CD27+ CD19+ cells prevailed over CD27− cells after 30 days in both XLP and N, with far more surviving cells in XLP. In N, few CD19+ B lymphocytes were viable after prolonged culture. In XLP, these cells were also IgD+, IgM+ and EBNA2+. These results demonstrate that IFNγ-positive memory CD8 T cells persist in XLP after prolonged culture in association with a subset of viable memory CD27+ B cells expressing latent EBV antigens. The survival advantage of XLP cells might be related to increased frequency of extranodal lymphoma in XLP patients.
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Introduction

Occurrence of malignant T or B cell proliferation in individuals with X-linked lymphoproliferative (XLP) disease who survive acute Epstein Barr virus (EBV) infection is one of the complications associated to deficiency of SLAM associated protein (SAP) controlled by the SH2D1A gene [1]. It has been established that the development of lymphoma or other lymphoproliferative diseases is a major determinant in the

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[1] The contribution of LB and CP to this study was equal.

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reduced life expectancy of this group of patients. The mechanisms that lead to the development of neoplasia are not fully understood. Since the oncogenic potential of EBV has been established [2], it is thought that defects in EBV control may cause immortalization and transformation of infected clones in XLP patients. In this regard, a persistently high EBV viral load has been detected in some XLP patients with hypogammaglobulinemia without evidence of B or T cell lymphoma [3,4]. However, it is not known if SH2D1A deficiency directly affects cell survival, contributing to cell immortalization.

Along the studies designed to analyze the development of memory T and B lymphocytes in adult XLP patients [5], we observed a survival advantage for XLP lymphocytes after culture. Because this fact could be related to lymphocyte immortalization and transformation leading to malignancy, we have studied cell viability and the phenotypic characteristics of XLP lymphocytes that survive in vitro after non-stimulated culture [6]. Our results indicate that survival of memory CD8 T lymphocytes was higher in XLP patients compared to normal controls (N). After 30 days the surviving memory CD8 T cells in XLP cultures corresponded to those with high IFNγ and low perforin [5]. Regarding B lymphocytes, while at the beginning of the culture B cells with a naïve phenotype predominated in XLP, mainly B cells with a memory CD27+, IgD+, IgM+ phenotype [7] prevailed in XLP after 30 days of culture. Interestingly, these cells were also EBNA2 positive at the end of the culture. It is not known if expansion of CD8 T lymphocytes and enrichment of memory CD27+, IgD+, IgM+ B cells are related events, but it will be important to explore this possibility in relation to the mechanisms that lead to the occurrence of malignancy in XLP.

Material and methods

XLP patients #9 and #4

Two surviving hypogammaglobulinemic siblings of an established XLP family were studied. Four siblings had died previously of different causes related to the XLP condition. The inactivating mutation identified in this family resulted from the substitution of a G for a C nucleotide at position 383 within SH2D1A exon 1 [3]. Patient #4, now 43 years old, developed a tonsilar lymphoma at 4 years of age while patient #9 (30 years old) developed severe acute infectious mononucleosis (AIM) at 26 years of age and was successfully treated with humanized anti-CD20 monoclonal antibody (Rituximab) [8] in combination with acyclovir. Since both patients became hypogammaglobulinemic, they currently receive monthly intravenous IgG infusions. EBV infection had been confirmed before by determination of EBV viral load above 500 viral copies/10^6 leukocytes [3,4]. The blood samples used in these investigations were obtained on a monthly basis before the IgG infusion over a period of 2–4 years. Informed consent was obtained. For these studies, normal control individuals (N, $n=25$) consisted of asymptomatic adults (25–40 years of age) with positive EBV serology (IgG anti-VCA+1:16–1:32), indicative of past EBV infection, whose blood samples were drawn and processed at the same time than XLP samples.

Non-stimulated PBMC culture

PBMC were obtained by Ficoll–Hypaque (FH) centrifugation of heparinized blood and resuspended to 1×10^6/ml in RPMI tissue culture medium containing 10% fetal calf serum (GIBCO, Grand Island, USA), streptomycin and penicillin (RPMI-FCS). PBMC cultures were carried out in round bottom 5 ml polystyrene tubes (Falcon) containing 2×10^6 PBMC that were suspended in 2 ml RPMI-FCS [6]. For each patient 4–30 different tubes (depending on the lymphocyte yield) were set up and left undisturbed in the CO_2 incubator in order to observe cellular morphology and viability. For each experiment, PBMC cultures from different N donors were set up in parallel (1–2 different N donors per experiment). Beginning on days 5–6, partial changes of RPMI-FCS (1 ml every 3–4 days) were carried out without centrifugation, by gentle aspiration above the cell pellet. PBMC cultures were maintained for 30–60 days (XLP patients) or until complete loss of viable cells (N, days 20–40).

Cell surface and intracellular staining and viability of cultured PBMC from XLP patients and N controls

PBMC were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and perdidin chlorophyll protein (PerCP)-labelled monoclonal antibodies according to the manufacturers' instructions, and flow cytometry was performed using a FacScan (Becton Dickinson, San Jose, CA). FACS data were analyzed with CELLQuest software. Anti-CD4, CD8, CD19, CD45RA, CD45RO, CCR7, CD27, CD127, Programmed-death 1 (PD-1) and CD62L were purchased from BD PharMingen (BD Biosciences, San Jose, CA). Anti-IgM, IgD and IgG were purchased from Dako (Dako A/S, Denmark). The phenotype of viable PBMC from XLP and N donors was analyzed after different periods of non-stimulated culture (0, 7, 14, 30 and >40 days). Lymphocyte viability was calculated taking into account the proportion of live lymphocytes in the SSC/FSC dot plots (R1). Alternatively, cell viability was determined by dye exclusion in cells stained with acrydine orange-ethidium bromide.

For intracellular staining, cells were fixed and permeabilized with Fix and Perm cell permeabilization reagents (Caltag Laboratories, Burlingame, CA) according to the instructions of the provider. After permeabilization, cells were stained with anti FoxP3 (BioScience), anti-perforin (BD Pharmingen) or anti-EBNA2 (Dako). The proportion of CD8 T cells with intracellular perforin was determined gating in the CD8 region. To establish the expression of intracellular EBNA2 in B lymphocytes, CD19+ cells were gated. Tregs staining for surface CD25 (Becton Dickinson, San Jose, CA) and intracellular FoxP3 were determined gating in the CD4 region. Results are shown as a percentage of viable lymphocytes for each culture date, or in absolute values calculated on the basis of the % and the number of each class of cells (cells/mm^3) for 0 or 30 days of culture.

Intracellular IFNγ staining

PBMC were stimulated with phorbol-12-myrisytate-13-acetate (PMA) (50 ng/ml) and ionomycin (0.5 μg/ml) during 4 h in the presence of Brefeldin A (BD Golgi Plug) (10 μg/ml). After

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washing, the cells were stained with CD4 and CD8 monoclonal antibodies for 20 min at room temperature, and then fixed and permeabilized with the Fix and Perm cell permeabilization reagents (Caltag Laboratories, Burlingame, CA). Cells were finally stained with anti-IFNγ (BD Pharmingen) and the appropriate isotype control for 45 min at 4 °C, washed again and the CD4+ IFNγ+ or CD8+ IFNγ+ cell count was then calculated by flow cytometry on the basis of % values.

Statistical analysis

All statistics were performed using Prism software (version 3.0; GraphPad Software). The unpaired *t* test or the Mann–Whitney test was used for statistical comparisons between data sets from XLP patients and N. *p* values of less than 0.05 were considered significant.

Results

Viability of lymphocytes from XLP patients

In order to study survival of lymphocytes from XLP patients, long term non-stimulated cultures of PBMC [6] were set up and the viability and phenotype of the persisting cells were compared to that of normal controls. Each experiment corresponds to culture of PBMC drawn from XLP at different monthly extractions over a 2–3 year period, excluding the acute phase and the subsequent 24 months following primary EBV infection in patient #9 and more than 30 years after the initial XLP marker disease in patient #4. Lymphocyte viability fell to 30–35% after 14 days of culture in both XLP patients and in the normal controls. At 30 days, while viability was below 10% in the normal PBMC cultures, around 25–30% of the lymphocytes remained viable in both #4 and #9 PBMC cultures (Fig. 1A) and continued to be viable after more than 40 days (40–60 days) of non-stimulated culture compared to less than 2% viable lymphocytes in the control N PBMC cultures (data not shown) (2/3 of the original N PBMC cultures could not be evaluated at that time because viable cells were insufficient for cytometry studies).

T lymphocytes persisting after non-stimulated PBMC culture

In order to determine if the survival advantage associated to lymphocytes from patients with SH2D1A deficiency, was present in all T lymphocytes, we analyzed the composition of the persisting viable cells at different times of culture and compared it to that of non SAP-deficient PBMC (N). The CD4+ T cell count fell to around 10% of the original day 0 value on day 14 in XLP patients and was slightly higher in N (Fig. 1B). At 30 days the CD4+ cell count decreased further in #4 and N than in #9. In contrast, after an initial loss up to day 14, CD8+ T lymphocyte count remained stable in XLP patients, even after 30 days of culture, while it was further reduced in N. At 30 days of culture viable CD8+ T lymphocytes were 72–91% of the viable cells in XLP cultures and around 37% in N cultures (Fig. 1C). As expansion of CD8 T cells could be the result of their response to viral antigens (EBV) during the culture, we studied co-expression of CD4 and CD8 on T cells, as this has been observed in response to viral stimuli [9]. The % of CD4+, CD8+ T cells, that was initially similar in XLP and in N (#4, 1.8±0.25, n=5%; #9, 2.32±0.39%, n=5; N, 2.43±0.50%, n=5) increased after 30 days of culture (#4, 8.64±1.57%, n=4; #9, 7.25±2.49%, n=4; N, 18.15±5.64, n=4).

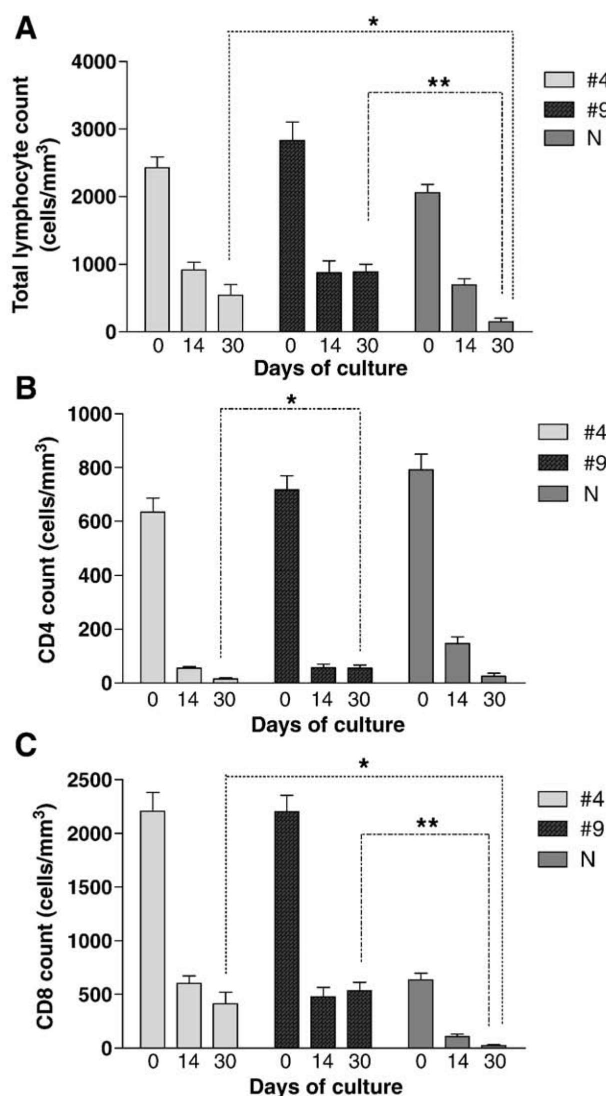

Fig. 1 Lymphocyte viability in XLP and N after non-stimulated culture. (A) Total viable lymphocyte count (cells/mm^3) at days 0, 14 and 30 of culture is shown for XLP patient #4 (light grey bars), XLP patient #9 (black bars) and N controls (dark grey bars). Cell viability was calculated on the basis of FSC and SSC parameters in flow cytometry dot plots. Each experimental result corresponds to analysis of samples obtained on a monthly basis at different dates during a 2–3 year period. N controls correspond to individual N PBMC samples processed and cultured in parallel. (B) CD4+ T lymphocyte count (cells/mm^3) in the viable lymphocyte region at days 0, 14 and 30 of culture is shown for XLP patient #4 (light grey bars), XLP patient #9 (black bars) and N controls (dark grey bar). C) CD8+ T lymphocyte count (cells/mm^3) in the viable lymphocyte region at days 0, 14 and 30 of culture is shown for XLP patient #4 (light grey bars), XLP patient #9 (black bars) and N controls (dark grey bars). Statistical differences were calculated (*t* test) between XLP patients and N. *p<0.05; **p<0.0001. Mean±SEM, n=7 is given.

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Because a subgroup of CD4 T cells expressing CD25 and the transcription factor FoxP3 (regulatory T cells, Treg) is important in the regulation of the immune response [10], we analyzed if the proportion of Tregs was different in XLP and in N. The percentage of Treg in XLP and N was similar at the beginning (#4, 5.70 ± 0.16%; #9, 5.63 ± 0.74%; N, 5.22 ± 0.82%) and did not vary significantly after 30 days of culture (#4, 7.60 ± 1.65%; #9, 6.18 ± 1.35%; N, 9.29 ± 2.34%) suggesting that differences in the proportion of CD8 T cells at the end of the culture were not related to Treg action.

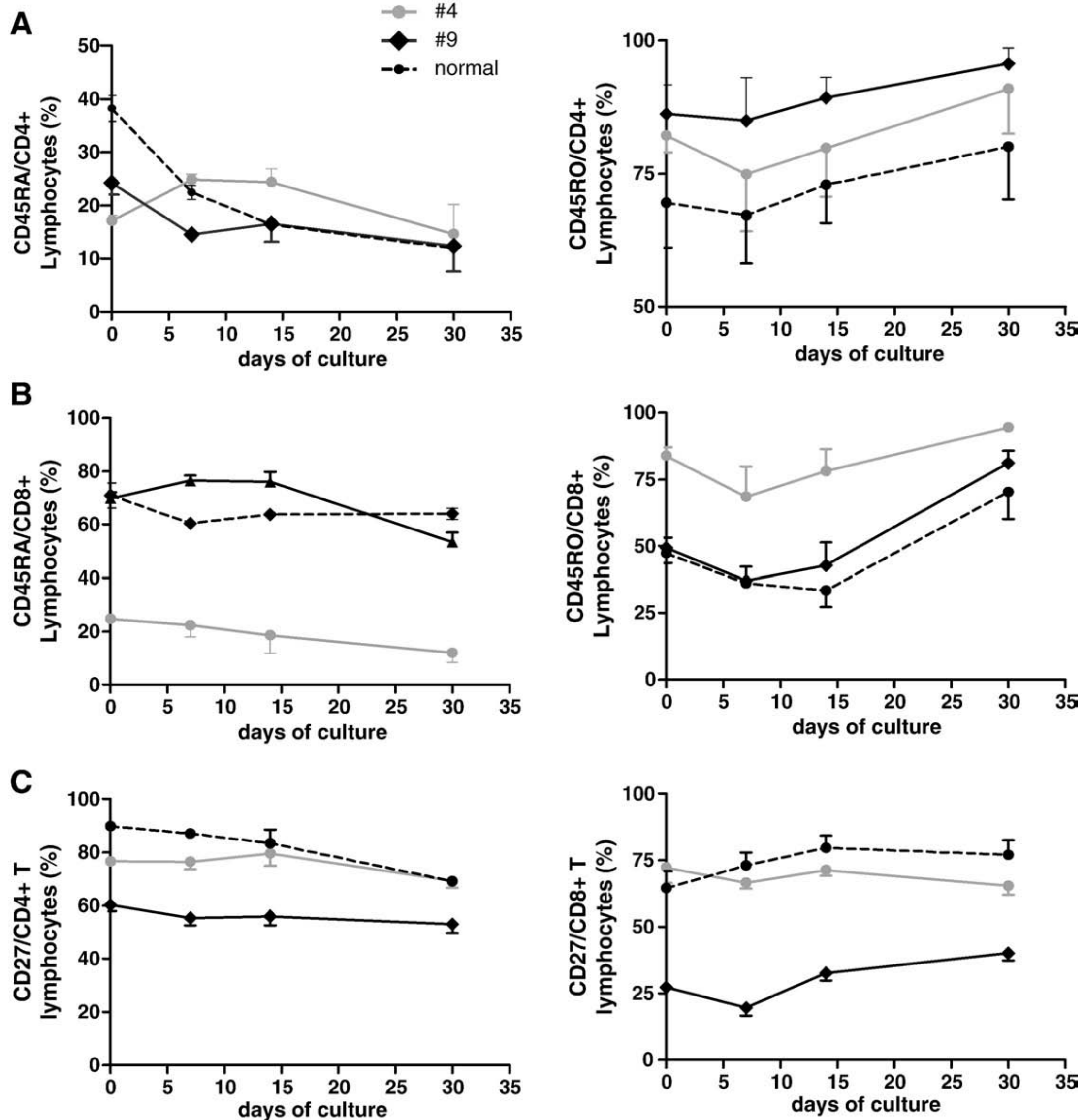

Fig. 2 Markers of cell memory (CD45RA, CD45RO) and differentiation (CD27) in XLP and N after non-stimulated culture. Percentage of CD4+ or CD8+ T cells expressing CD45RA, CD45RO or CD27 is shown. Mean ± SEM of different assays performed at 7, 14 and 30 days of culture in XLP patients #4 (grey dots), #9 (black diamonds) and N (black dots, broken line) are shown. (A) Percentage of CD4 T cells expressing CD45RA or CD45RO. CD45RA expression was significantly lower in #4 and #9 compared to N at day 0. At day 30, CD45RA expression was not different in XLP compared to N. However, at day 30 CD45RA expression in CD4 cells from #9 and N were significantly reduced when compared to their own initial values ($p = 0.03$ #9 day 0 vs #9 day 30; $p = 0.0002$ N day 0 vs N day 30). No significant differences were observed between #4, #9 and N in CD45RO expression at any time tested. (B) Percentage of CD8 T cells expressing CD45RA or CD45RO. CD45RA expression was significantly lower in #4 compared to #9 or N at day 0, while no differences were observed between #4, #9 and N at day 30. CD45RO expression was significantly higher in #4 compared to #9 and N at day 0 ($p < 0.0001$) and were not different at day 30. Expression of CD45RO was significantly increased in #9 and in N when compared to their own initial values ($p = 0.0026$ #9 day 0 vs #9 day 30; $p = 0.02$ N day 0 vs N day 30). (C) Percentage of CD27+ CD4 and CD8 T cells. CD27 expression was significantly lower in CD4 T lymphocytes from #9 than in #4 and N at day 0 ($p < 0.01$). At day 30 it was still significantly lower in #9 vs #4 and N ($p < 0.05$). Expression of CD27 was also lower ($p < 0.001$) in CD8 T cells from #9 compared to N and #4 at day 0 and this difference was maintained at day 30 ($p < 0.0001$). At day 30, only in N CD4 T lymphocytes expression of CD27 decreased significantly compared to day 0 ($p = 0.0008$).

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Markers of cell memory (CD45RA, CD45RO), differentiation (CD27) and homing (CD62L) in T lymphocytes after non-stimulated PBMC culture

The stage of differentiation may be a factor determining survival of lymphocytes in culture. Therefore, we examined the expression of memory and differentiation markers in XLP and N after culture. CD45RA was lower while CD45RO was higher in both XLP CD4 T lymphocytes than in N at the beginning of the culture. As the PBMC culture progressed, CD45RA expression in CD4 T lymphocytes tended to decrease as CD45RO expression increased in the three groups (Fig. 2A). In CD8 T lymphocytes, CD45RA did not change in N, but it decreased in XLP patients. CD45RO expression in CD8 T lymphocytes followed a similar pattern in both XLP and N, increasing as the culture progressed. Therefore, at the end of the culture (>30 days) most CD4 and CD8 T lymphocytes were CD45RO+ (Fig. 2B), indicating that memory cells were enriched after culture. On the other hand, expression of the differentiation marker CD27 was fairly stable in XLP CD4 T cells along the culture, and decreased in N after 30 days. CD27 expression in CD8 T cells increased in N and #9 after 30 days, but it did not vary in patient #4 CD8 T lymphocytes (Fig. 2C).

In order to study the homing characteristics of T lymphocytes that survived prolonged non-stimulated culture, expression of L selectin (CD62L) was surveyed in XLP and N PBMC. Results shown in Table 1 indicate that CD62L expression (initially lower in XLP than in N) tended to increase in XLP both in CD4 and CD8 T lymphocytes, while it remained stable in N T lymphocytes.

Expression of cell death or survival receptors in viable T cells after non-stimulated PBMC culture

Because survival of T lymphocytes in culture may be associated to expression of receptors involved in initiation of the cell death program (PD-1) [11] or of the IL-7 receptor (CD127) [12], we analyzed expression of PD-1 and CD127 in CD4 and CD8 lymphocytes along the culture. As shown in Table 2, expression of PD-1 in CD4 T cells was unchanged in XLP patients while it increased in N CD4 T lymphocytes. In CD8 T cells no significant variations in PD-1 expression were observed up to day 30 of culture, either in N or XLP patients. On the other hand, expression of CD127 decreased in CD4 T lymphocytes of both XLP patients and N, but it did not vary significantly in CD8 T lymphocytes, either from N or from XLP patients.

Table 1 Expression of CD62L in CD4 and CD8 T lymphocytes from XLP and N controls after culture.

	Days of culture	CD62L/CD4 (%)	CD62L/CD8 (%)
#4	0	56.2 ± 3.6	30.4 ± 4.7
	30	70.3 ± 5.9	45.9 ± 3.6
#9	0	35.5 ± 2.8	16.9 ± 1
	30	48.7 ± 4	28 ± 4.2
N	0	71.9 ± 6.8	39 ± 4.2
	30	78.2 ± 4.1	38.2 ± 4.6

CD62L expression was assayed by flow cytometry as described in Materials and methods. The results represent the percentage of CD62L+ CD4 or CD8 lymphocytes at 0 or 30 days of culture (CD62L/CD4 or CD62L/CD8 %). Significant differences were observed between #9 when compared to #4 and N at day 0, both in CD4 and in CD8 T lymphocytes ($p<0.01$). At day 30 CD62L expression significantly increased in CD4+ lymphocytes from #9 and in CD8 T lymphocytes from #4 and #9 when compared to their own values at day 0 ($p<0.04$).
Mean ± SEM of 4–7 experiments are shown.

Table 2 Expression of PD-1 and CD127 in CD4 and CD8 T lymphocytes from XLP patients and N controls after culture.

	Days of culture	PD1/CD4 (%)	PD1/CD8 (%)
#4	0	23 ± 7.7	17 ± 6
	30	20 ± 8	7.5 ± 2
#9	0	19 ± 6	16.5 ± 3.8
	30	24 ± 9	12.5 ± 4.6
N	0	8 ± 3	12 ± 3.2
	30	24 ± 7	14.2 ± 5
	Days of culture	**CD127/CD4 (%)**	**CD127/CD8 (%)**
#4	0	94.6 ± 0.4	85 ± 2
	30	77.6 ± 8	66.5 ± 7
#9	0	89.5 ± 0.9	25.7 ± 3.5
	30	66 ± 10	33 ± 4.3
N	0	92.8 ± 0.7	68 ± 10
	30	63.8 ± 8.2	72 ± 15

PD-1 and CD127 were assayed by flow cytometry in CD4 and CD8 T lymphocytes at days 0 and 30 of culture. The results represent the percentage of PD-1+ or CD127+ CD4 or CD8 lymphocytes at 0 or 30 days of culture PD-1/CD4 or PD-1/CD8; CD127/CD4 or CD127/CD8. No significant differences were observed for PD-1 expression in CD4 or CD8 T lymphocytes from XLP patients or N at 0 or 30 days of culture. CD127 expression was lower in #9 CD4 T lymphocytes than in #4 and N at day 0 ($p<0.02$) and no significant differences were observed at day 30. In CD8 T lymphocytes, CD127 was significantly lower in #9 compared to #4 or N, both at day 0 and at day 30 ($p<0.01$). Mean ± SEM of 5–8 experiments are shown.

Intracellular IFNγ and perforin expression in XLP and N CD4 and CD8 T cells

In order to get an insight into the function of cells that survive after prolonged culture, the proportion of CD4+ or CD8+ T cells with intracellular IFNγ or perforin was investigated. At day 0, the percentage of cytokine secreting cells was higher in XLP patients than in controls (around 30% IFNγ CD4 T cells for #4 and #9 vs 10% for N and 45% IFNγ+ CD8 T cells for #4 and #9 vs 20% for N) yielding higher absolute counts of IFNγ+ CD4 and CD8 T cell counts at the beginning of the culture, as shown in Table 3. After 1 month of culture, around 30% of the original IFNγ+ CD8 T cells persisted in XLP cultures, while they were lost in N cultures. Since we had shown that the basal percentage of expression of CD45RA and perforin in CD8+ memory cells from patients #4 and #9 differed, probably in relation to the initial EBV impact and treatment of these two patients [5], we evaluated the persistence of perforin+ CD8 T cells after culture. As previously shown, at day 0 the percentage of perforin+ CD8

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Table 3 IFNγ+ CD4 and CD8 lymphocytes and perforin+ CD8 lymphocytes at 0 and 30 days of culture.

	IFNγ, CD4 cells/mm^3		IFNγ, CD8 cells/mm^3		Perforin, CD8 cells/mm^3	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
#4	203.8±24.0** (n=8)	2.4±0.8 (n=7)	817±173# (n=7)	295±19.9# (n=3)	328±32.7 (n=6)	6.3±2.1* (n=3)
#9	254.1±44.0* (n=7)	36.3±26.8 (n=3)	1035±74.5# (n=5)	240.1±40.8# (n=3)	317±26.9 (n=6)	28.9±8.5** (n=5)
N	78.3±14.4 (n=6)	3.1±0.3 (n=3)	108.2±24.7 (n=5)	2.2±0.4 (n=3)	260.4±67.8 (n=6)	1.2±0.5 (n=6)

Intracellular IFNγ was assayed in permeabilized CD4+ or CD8+ cells from XLP patients #4 and #9 and N controls, after PMA stimulation in the presence of Brefeldin, at initiation of the culture and after 30 days. Intracellular perforin was determined in CD8 lymphocytes at days 0 and 30 of culture. Reactions were carried out as described in Materials and methods and the results are expressed in terms of IFNγ+ CD4 or CD8 cells/mm^3, calculated on the basis of the number of CD4 and CD8 lymphocytes (cells/mm^3) at the beginning and at the end of the culture (these values are shown in Fig. 1). Mean±SEM of the results of experiments performed in samples obtained at different times over a two year period are given; *n* is shown between parentheses. Statistical differences (*t* test) between XLP and N, $^*p<0.05$; $^{**}p<0.01$; $^{\#}p<0.001$. Differences between 0 day and 30 days of culture were significant for each XLP patient and for N ($p<0.001$).

T cells was lower in XLP patient #4 than in #9 and N (around 15% for #4 compared to 40% in #9 and 35% in N), but since the total CD8 count was higher in XLP than in N, the absolute numbers of perforin+ CD8 T cells were not statistically different in XLP and N. However, at the end of the culture (30 days), although the absolute count of perforin+ CD8 T cells was reduced in both XLP PBMC cultures when compared to the initial values (Table 3), they were still higher than in N. Persistence or expansion of cytokine secreting cells in culture could be related to the initial activation status or differences in growth factor secretion between XLP and N PBMC. This does not seem to be the case, as expression of activation markers CD38 (Mean±SEM %, *n*=6, CD38, CD4/CD4, #4: 45.15±2.53; #9: 24.69±7.8; N: 42.9±8.2; CD38, CD8/CD8, #4: 35.2±5.4; #9: 37.4±14.5; N: 43.9±9.1) and CD69 (Mean±SEM %, *n*=5, CD69, CD4/CD4, #4: 3.6±2.0; #9: 8.4±1.8; N: 6.9±1.3; CD69, CD8/CD8, #4: 16.8±5.0; #9: 12.1±2.7; N: 16.0±3.6) was similar in N and in XLP. Likewise, release of IL-2 to the culture supernatants was similar in both XLP and N during the culture (data not shown).

B lymphocyte markers after prolonged culture

CD19+ B lymphocytes decreased rapidly after 1–2 weeks of culture both in patients #4 and #9 and in N. After 30 days, only 1–7% of the original B lymphocytes remained viable in #9 and #4 PBMC cultures. In the N group, B lymphocytes were reduced to 0.3% of the original value after 30 days and in only 1/3 of the initial N PBMC cultures, there were enough viable cells to perform cytometry studies (Fig. 3). In order to determine if the surviving B lymphocytes had a naïve or memory phenotype, we analyzed the expression of the B cell memory marker CD27. Although at the beginning of the culture, the number of B lymphocytes expressing CD27 was lower in XLP than in N, after 30 days the CD27, CD19 lymphocyte count was significantly higher in XLP (especially in #4) than in N (Fig. 3).

As described before [7] the % of IgM+ and IgD+ cells was initially higher in XLP (especially in #9) than in N (Table 4). After culture, the proportion of these cells increased both in XLP and in N PBMC, and after 30 days around 78–90% of the viable XLP B lymphocytes were CD27+, IgD+ and IgM+. Interestingly most of these cells (>70%) could be stained with antibodies to Epstein Barr virus latency antigen EBNA2.

Discussion

Survival of lymphocytes after culture depends on a number of factors: stage of differentiation, response to growth factors and cytokines, response to apoptosis signals, etc. In addition to other factors, if the weight of cell survival factors is higher than that of cell death signals, a greater chance of immortalization and transformation leading to malignancy could be possible. Association of XLP with increased susceptibility to develop hematologic cancers has been reported in XLP individuals who overcome the acute phase of EBV infection [1]. It is not known if this depends of impaired immune response to this oncogenic virus, or if SH2D1A deficiency directly affects cell survival or the response to death signals.

We addressed this question by analyzing the survival of lymphocytes from XLP patients in comparison to that of N controls in a non-stimulated culture system [6]. Our results demonstrate that while in the first 2 weeks of culture viable lymphocyte loss was similar in XLP and in N, the viable lymphocyte count was higher in both XLP patients than in N after 30 days of culture. Sustained lymphocyte viability (around 20–30%) even after more than 40 days of culture contrasts with the results of the normal PBMC cultures where viable N PBMC represented 7% at 30 days and less than 2% at 40 days, suggesting that absence of SH2D1A provided a survival advantage to these lymphocytes in culture. We focused on the phenotypic markers of cells that remain viable in XLP when compared to N after prolonged non-stimulated culture.

CD8 T lymphocytes continued to prevail over CD4 lymphocytes in XLP cultures, while CD4 T lymphocytes decreased after 30 days of culture. The number of both CD4 and CD8 T lymphocytes was greatly reduced in N after 30 days. In fact, in XLP PBMC cultures, persistence of viable CD8 T lymphocytes accounts for increased viability of total lymphocytes after 30 days. Greater viability could not be related to differences in initial activation or IL-2 secretion.

We had recently shown that CD8 cells differed in the expression of cell markers of memory and differentiation in XLP patients with the same SAP mutation [5]. In one of these patients (#9), peripheral blood CD8 T lymphocytes appeared to be end-stage differentiated effector cells expressing perforin, CD45RA and low CD27, while 70% of the CD8 T cells in patient #4 corresponded to an early/intermediate effector

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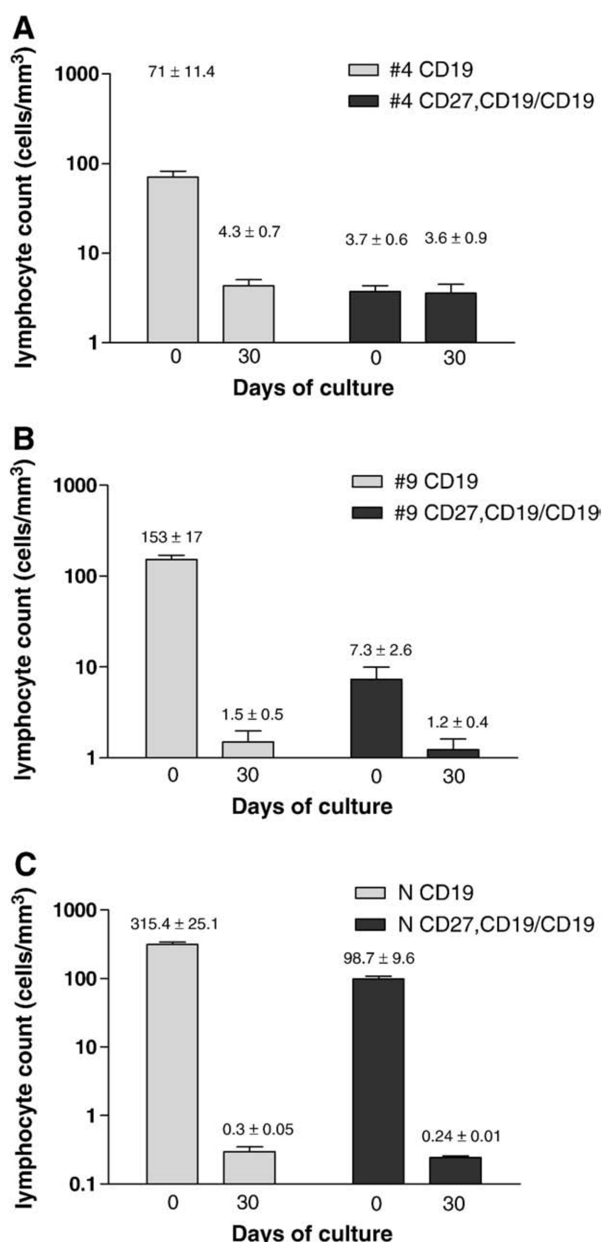

Fig. 3 Viability of B lymphocytes and memory B cells after non-stimulated culture of XLP and N PBMC. (A) #4 CD19+ lymphocyte count (cells/mm^3), grey bars; #4 CD27, CD19/CD19 lymphocyte count (cells/mm^3), black bars. Mean ± SEM of 6 experiments are shown in parentheses above the bars. (B) #9 CD19+ lymphocyte count (cells/mm^3), grey bars; #9 CD27, CD19/CD19 lymphocyte count (cells/mm^3), black bars. Mean ± SEM of 6 experiments are shown in parentheses above the bars. (C) N CD19+ lymphocyte count (cells/mm^3), grey bars; N CD27, CD19/CD19 lymphocyte count (cells/mm^3), black bars. Mean ± SEM of 6 experiments are shown in parentheses above the bars.

memory phenotype as did CD8 T cell of N. It had been suggested that primed CD8+ T cells re-expressing CD45RA were approaching terminal differentiation [13]. However, it has been recently shown that these cells were not terminally differentiated end-stage effector cells and could be part of a stable apoptosis-resistant memory pool retaining functional and replicative capacity [14]. Since CD8+ memory cells from patients #4 and #9 differed in the expression of CD45RA [5], we tried to determine if their viability was different after prolonged non-stimulated culture. There were no differences in the viability of CD8+ T lymphocytes of patients #4 and #9. Concerning the expression of CD45RO, considered as a marker of memory cells, most cells were CD45RO+ after prolonged culture in both N and XLP, while CD45RA expression was reduced, indicating that survival of memory cells was favoured after prolonged culture. As for the expression of CD27, it remained stable in patient #4 and in N CD8+ T lymphocytes but it increased in patient #9 CD8+ T lymphocytes after 30 days of culture. Thus, CD45RA+, CD27− CD8 T cells could have been replaced in part by CD45RO+, CD27+ CD8 T cells in patient #9 long term cultures, suggesting either that they were more susceptible to cell death, or that death resistant cells had divided recovering CD45RO and CD27 expression.

Co-expression of CD4 and CD8 in mature T lymphocytes occurs after viral stimulation [9]. Because control of expanding EBV+ B cell clones during culture could provide such a stimulus, we looked for T lymphocytes co-expressing both molecules and observed that the percentage of CD4+, CD8+ T lymphocytes increased after culture both in XLP and in N, suggesting that culture conditions rather than differences in SAP expression influenced changes in the co-expression of CD4 and CD8. Likewise, the proportion of CD4 regulatory T cells (CD25+Foxp3+) [10] was the same for XLP and N before and after the culture, suggesting that neither the absence of SAP nor factors depending on the culture conditions affected the expression of markers associated to Tregs. Since IL7 receptor (CD127) in T cells has been associated to their ability to survive [11], we analyzed CD127 expression in CD4 and CD8 T lymphocytes from XLP and N. When compared to initiation of the culture, CD127 decreased in CD4 T lymphocytes of both XLP patients and N at 30 days while its expression did not vary significantly in CD8 T cells. These results suggest that CD127 alone could not account for the observed survival advantage of XLP T cells. Concerning expression of PD-1, known to be involved in cell death [12], there was no relationship between PD-1 expression and the ability to survive both in CD4 and in CD8 T lymphocytes of XLP when compared to N. Increased viability of T lymphocytes in XLP patients after culture seems to be more related to SH21A deficiency and expansion of CD8 memory T lymphocytes than to over expression of IL-7 receptors or impaired death cell programming.

As previously suggested [5,15], IFNγ producing cells were higher in XLP than in N at the beginning of the culture. Furthermore, at 30 days 25–35% of the original IFNγ producing CD8 T cells remained viable in XLP, while only 2% persisted in N. The perforin+ CD8 T cell count, that was initially similar in both XLP patients and in N, was reduced after 30 days to 1–2% of the original value in #4 and N, but remained higher (30%) in #9. Thus, XLP CD8 lymphocytes that survived after prolonged culture were relatively enriched in IFNγ^{high}, perforin^{low} CD8 T cells (Table 3). It is tempting to speculate that in XLP, early effector cells or central memory cells [16,17] (IFNγ+, perforin^{low} CD8 T cells) had a survival advantage in culture over those of N.

B lymphocytes were greatly reduced in XLP and most of them were lost in N PBMC after 1 month in culture. The

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Table 4 Phenotype of B lymphocytes from XLP and N after culture.

	CD27 (%)		IgD (%)		IgM (%)		EBNA2 (%)	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
# 4	11.3±2.1 (*n*=7)	98.6±1.4 (*n*=3)	97.7±0.8* (*n*=7)	95.0±3.5 (*n*=4)	14.5±5.4 (*n*=7)	78.1±14.7 (*n*=3)	1.3±1.1 (*n*=3)	94.2±3.8 (*n*=3)
# 9	11.7±0.9 (*n*=7)	72.0±14.4 (*n*=4)	98.2±0.5* (*n*=7)	96.6±0.9 (*n*=3)	69.0±8.3** (*n*=6)	90.6±6.6* (*n*=3)	0.7±0.4 (*n*=3)	74.8±16.3 (*n*=4)
N	27.0±4.9 (*n*=7)	61.2±12.7 (*n*=5)	74.7±13.2 (*n*=4)	62.8±10.9 (*n*=7)	3.7±1.2 (*n*=4)	35.3±14 (*n*=7)	1.2±0.4 (*n*=3)	63.5±3.3 (*n*=3)

Expression (%) of CD27, IgD, or IgM was determined by flow cytometry on viable B lymphocytes (CD19+ cells) of XLP (#4 and #9) and controls (N), at 0 and 30 days of culture, as described in Materials and methods. EBNA2 determination was performed on permeabilized cells at 0 and 30 days of culture. Mean±SEM of the results of experiments performed on samples obtained on different occasions are shown; the number of experiments is shown between parentheses. Statistical differences (*t* test) between XLP and N, *$p<0.05$; **$p<0.01$. Differences of IgM expression at day 0, #4 vs #9: $p=0.0002$. Differences of EBNA2 expression between 0 day and 30 days of culture were significant for all groups ($p<0.001$).

remaining viable B lymphocytes corresponded to memory cells (CD27+), both in XLP and in N. Most of these cells also expressed IgD, IgM and intracellular EBNA2. Chaganti et al. [18] have recently shown that EBV can establish persistence in non switched IgM+, IgD+ CD27+ B lymphocytes in XLP. These patients lack functional germinal centers in the spleen. In our series, IgM+, IgD+ B lymphocytes were present in patient #9 in a higher proportion than in #4 and N at day 0. This is interesting, since the circulating EBV viral load was also persistently higher in this patient than in #4 and in N [3]. At 30 days, IgM+, IgD+ B lymphocytes had expanded (or resisted) in XLP (as well as in N although in a smaller proportion). At the end of the culture, 75–94% in XLP and 64% of the remaining viable B lymphocytes in N could also be stained intracellularly with anti EBNA2 antibodies. It is not known if enrichment of EBNA2+, IgM+, IgD+ memory B lymphocytes could also be favoured in gut associated germinal centers (GC) in XLP patients (18). This poses an important question, since it has been suggested that the presence of normal GC in gastrointestinal follicles in spite of their absence in the spleen of these patients [7] could be related to the occurrence of extranodal, gut restricted lymphoma in these patients.

It is also tempting to link the expansion or persistence of SAP-deficient CD8 memory effector T cells expressing IFNγ with enrichment of the particular subset of memory B cells expressing EBV latency antigens after prolonged culture both in N and in XLP. Because expression of SAP in peripheral B lymphocytes has not been demonstrated, it is difficult to attribute increased survival of memory B cells to a direct effect of SAP deficiency. Predominance of IFNγ producing CD8 T lymphocytes in cultured XLP PBMC, could be the combined consequence of the immune response to a higher EBV load or to continuous antigenic stimuli during culture and/or increased viability of XLP lymphocytes. Taken together, these results suggest that the overall cell survival advantage associated to SH2D1A deficiency should be taken into account as one of the factors that may determine the higher incidence of hematologic neoplasia in XLP patients.

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References

[1] M. Morra, D. Howie, M.S. Grandi, J. Sayos, N. Wang, C. Wu, P. Engel, C. Terhorst, X-linked lymphoproliferative disease: a progressive immunodeficiency, Annu. Rev. Immunol. 19 (2001) 657–682.

[2] D.A. Thorley-Lawson, A. Gross, Persistence of Epstein–Barr virus and origins of associated lymphomas, N. Engl. J. Med. 350 (2004) 1328–1337.

[3] A. Malbran, L. Belmonte, B. Ruibal-Ares, P. Bare, I. Massud, C. Parodi, R. Hodinka, K. Haines, K.E. Nichols, M.M.E. de Bracco, Loss of circulating CD27+ memory B cells and CCR4+ T cells occurring in association to elevated EBV viral loads in XLP patients surviving primary EBV infection, Blood 103 (2004) 1625–1631.

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[4] C.S. Ma, N.J. Hare, K.E. Nichols, L. Dupré, G. Andolfi, M.G. Roncarolo, S. Adelstein, P.D. Hodgkin, S.G. Tangye, Impaired humoral immunity in X-linked lymphoproliferative disease is associated with defective IL-10 production by CD4+ T cells, J. Clin. Invest. 115 (2005) 1049–1059.

[5] L. Belmonte, C. Parodi, A. Mabran, B. Ruibal-Ares, M.M.E. de Bracco, Factors involved in the generation of memory CD8+ T cells in patients with X-linked lymphoproliferative disease (XLP), Clin. Exp. Immunol. 147 (2007) 456–464.

[6] B. Ruibal-Ares, L. Belmonte, P. Baré, C. Bayo-Hanza, G. Mendez, R. Pérez Bianco, M. Tezanos Pinto, M.M.E. de Bracco, Monocyte differentiation and HIV replication after prolonged culture of peripheral blood mononuclear cells from HIV-infected individuals, Cell. Immunol. 210 (2001) 11–20.

[7] C.S. Ma, S. Pittaluga, DT. Avery, N.J. Hare, I. Maric, A.D. Klion, K.E. Nichols, S.G. Tangye, Selective generation of functional somatically mutated IgM+CD27+, but not Ig isotype-switched, memory B cells in X-linked lymphoproliferative disease, J. Clin. Invest. 116 (2006) 322–333.

[8] M.C. Milone, D.E. Tsai, R.L. Hodinka, L.B. Silverman, A. Malbran, M.A. Wosik, K.E. Nichols, Treatment of primary EBV infection in patients with X-linked lymphoproliferative disease using B-cell directed therapy, Blood 105 (2005) 994–996.

[9] M. Nascimbeni, E.C. Shim, L. Chiriboga, D.E. Kleiner, B. Rehermann, Peripheral CD4 (+) CD8 (+) T cells are differentiated effector memory cells with antiviral functions, Blood 104 (2003) 2156–2164.

[10] Q. Tang, J.A. Bluestone, The Foxp3+ regulatory cell: a jack of all trades, Nat. Immunol. 9 (2008) 239–244.

[11] K.S. Lang, M. Recher, A.A. Navarini, N.L. Harris, M. Lohning, T. Junt, H.C. Probst, H. Hentgartner, R.M. Zinkernagel, Inverse correlation betweenIL7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation, Eur. J. Immunol. 35 (2005) 738–745.

[12] A.H. Sharpe, E.J. Wherry, R. Ahmed, G.J. Freeman, The function of programmed cell death-1 and its ligands in regulating autoimmunity and infection, Nat. Immunol. 8 (2007) 239–245.

[13] J.M. Faint, N.E. Annels, S.J. Curnow, P. Shields, D. Pilling, A.D. Hislop, L. Wu, A.N. Akhbar, C.D. Buckley, P.A. Moss, D.H. Adams, A.B. Rickinson, M. Salmon, Memory T cells constitute a subset of the human CD8+CD45RA+ pool with distinct phenotype and migratory characteristics, J. Immunol. 167 (2001) 212–220.

[14] P.J. Dunne, J.M. Faint, N.H. Gudgeon, J.M. Fletcher, F.J. Plunkett, M.V.D. Soares, A.D. Hislop, N.Z. Annels, A.B. Rickinson, M. Salmon, A.N. Akhbar, Epstein Barr Virus specific CD8 T cell that re-express CD45RA are apoptosis resistant memory cells that retain replicative potential, Blood 100 (2002) 933–940.

[15] M. Okano, G.M. Thiele, D.T. Purtilo, Variable presence of circulating cytokines in patients with sporadic X-linked lymphoproliferative disease with fatal infectious mononucleosis, Pediatr. Hematol. Oncol. 10 (1993) 97–99.

[16] V. Appay, P.R. Dunbar, M. Callan, P. Klenerman, G.M. Gillespie, L. Papagno, G.S. Ogg, A. King, F. Lechner, C.A. Spina, S. Little, D.V. Havlir, D.D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salñio, V. Cerundolo, A.J. MacMichael, S. Rowland-Jones, Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections, Nat. Med. 8 (2002) 379–385.

[17] D. Masopust, S.M. Kaech, E.J. Wherry, R. Ahmed, The role of programming in memory T cell development, Curr. Opin. Immunol. 16 (2004) 217–225.

[18] S. Chaganti, C.S. Ma, A.I. Bell, D. Croom-Carter, A.D. Hislop, S.G. Tangye, A.B. Rickinson, Epstein–Barr virus persistence in the absence of conventional memory B cells: IgM+IgD+CD27+ B cells harbor the virus in X-linked lymphoproliferative disease patients, Blood 112 (2008) 672–679.

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