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Research Article Immunology

Individuals with X-linked lymphoproliferative disease (XLP) display defects in B cell differentiation in vivo. Specifically, XLP patients do not generate a normal number of CD27$^+$ memory B cells, and those few that are present are IgM$^-$. Recent studies have suggested that IgM$^+$CD27$^+$ B cells are not true memory cells, but rather B cells that guard against T cell–independent pathogens. Here we show that human XLP IgM$^+$CD27$^+$ B cells resemble normal memory B cells both morphologically and phenotypically. Additionally, IgM$^+$CD27$^+$ B cells exhibited functional characteristics of normal memory B cells, including the ability to secrete more Ig than naive B cells in response to both T cell–dependent and –independent stimuli. Analysis of spleens from XLP patients revealed a paucity of germinal centers (GCs), and the rare GCs detected were poorly formed. Despite this, Ig variable region genes expressed by XLP IgM$^+$CD27$^+$ B cells had undergone somatic hypermutation to an extent comparable to that of normal memory B cells. These findings reveal a differential requirement for the generation of IgM$^+$ and Ig isotype–switched memory B cells, with the latter only being generated by fully formed GCs. Production of affinity-matured IgM by IgM$^+$CD27$^+$ B cells may protect against pathogens to which a normal immune response is elicited in XLP patients.



# Selective generation of functional somatically mutated IgM$^+$CD27$^+$, but not Ig isotype–switched, memory B cells in X-linked lymphoproliferative disease

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**Individuals with X-linked lymphoproliferative disease (XLP) display defects in B cell differentiation in vivo. Specifically, XLP patients do not generate a normal number of CD27$^+$ memory B cells, and those few that are present are IgM$^+$. Recent studies have suggested that IgM$^+$CD27$^+$ B cells are not true memory cells, but rather B cells that guard against T cell–independent pathogens. Here we show that human XLP IgM$^+$CD27$^+$ B cells resemble normal memory B cells both morphologically and phenotypically. Additionally, IgM$^+$CD27$^+$ B cells exhibited functional characteristics of normal memory B cells, including the ability to secrete more Ig than naive B cells in response to both T cell–dependent and –independent stimuli. Analysis of spleens from XLP patients revealed a paucity of germinal centers (GCs), and the rare GCs detected were poorly formed. Despite this, Ig variable region genes expressed by XLP IgM$^+$CD27$^+$ B cells had undergone somatic hypermutation to an extent comparable to that of normal memory B cells. These findings reveal a differential requirement for the generation of IgM$^+$ and Ig isotype–switched memory B cells, with the latter only being generated by fully formed GCs. Production of affinity-matured IgM by IgM$^+$CD27$^+$ B cells may protect against pathogens to which a normal immune response is elicited in XLP patients.**

## Introduction

The humoral immune response plays a crucial role in the elimination of both intracellular and extracellular pathogens. This is mediated by the differentiation of mature B cells into plasma cells (PCs), which secrete large amounts of Ig (1, 2). Following activation with T cell–dependent antigen (Ag), mature B cells can yield PCs by 2 separate differentiation pathways. Activated B cells can enter the extrafollicular proliferative foci, where they rapidly differentiate into short-lived PCs that secrete predominantly unmutated IgM (3–6). Activated B cells can also seed a germinal center (GC), where molecular events such as somatic hypermutation (SHM), Ig isotype switching, and selection of high affinity variants occurs (3, 6–8). Ag-selected GC B cells give rise to 2 types of progeny — high-affinity memory B cells and long-lived PCs — that are responsible for long-term humoral immunity (1, 9).

Analyses of humans and mice with mutations in specific genes have led to the identification of a number of molecules that are required for the formation of GCs. These include the receptor/ligand pairs CD40 and CD40 ligand (CD40L), ICOS and its ligand ICOSL, and CD28 and CD80/CD86 as well as the transcription factor Bcl-6, the cytoplasmic adaptor protein signalling lymphocytic activation molecule–associated (SLAM-associated) protein (SAP), and cytokines such as TNF or lymphotoxin (LT) (10–14). In humans and/or mice harboring defects in the genes encoding these proteins, 1 or more of the processes that generate high-affinity Ag-specific B cells and GC formation (i.e., SHM and Ig isotype switching) are impaired.

In humans, memory B cells can be identified by the expression of CD27 (15, 16). Studies of the X-linked and autosomal-recessive forms of the hyper IgM syndrome (HIGM), resulting from mutations in *CD40L* and *CD40*, respectively (10), have shown impaired memory B cell development, with only a small population of IgM$^+$CD27$^+$ B cells detected in these patients, revealing an absence of Ig isotype–switched memory B cells (17–19). However, despite the lack of apparent GCs, these residual IgM$^+$ memory B cells had mutated Ig variable (V) region genes, leading to the suggestion of the existence of a GC-independent mechanism for SHM, but not for Ig isotype switching (18–21). Based on these findings, it was hypothesized that there are 2 pathways for diversification of the Ab repertoire: a T cell–dependent pathway, which occurs in GCs, and a T cell–independent pathway occurring outside of GCs (18, 19). Although the precise function of this second pathway, and of the IgM$^+$CD27$^+$ B cells generated, had not previously been determined, these cells have been proposed to play a role in the humoral response against T cell–independent pathogens such as encapsulated bacteria (19).

X-linked lymphoproliferative disease (XLP) is a human immunodeficiency resulting from mutations in the *SH2D1A* gene,

**Nonstandard abbreviations used:** Ag, antigen; CD40L, CD40 ligand; CDR, complementarity determining region; GC, germinal center; GIT, gastrointestinal tract; HIGM, hyper IgM syndrome; LT, lymphotoxin; PB, peripheral blood; PC, plasma cell; SAP, signalling lymphocytic activation molecule–associated protein; SHM, somatic hypermutation; V, variable; XLP, X-linked lymphoproliferative disease.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Table 1**
Features of XLP patients

| Patient | Clinical phenotype | EBV infection | i.v. Ig | Mutation | Effect on SAP expression |
|---|---|---|---|---|---|
| XLP#6 | Healthy | No | No | Missense | I84T, reduced SAP |
| XLP#12 | Neonatal cord blood | No | No | Missense | R55X, truncated SAP |
| XLP#13 | FIM, lymphadenopathy | Yes | Yes | Missense | S28R, reduced SAP |
| XLP#18 | FIM, hemophagocytosis | Yes | No | Insertion, frameshift | fs82X103, truncated SAP, reduced expression |

FIM, fulminant infectious mononucleosis. The features of the other XLP patients included in this study have previously been described (27).

which encodes SAP (22–24). XLP patients are highly susceptible to infection with the human herpesvirus EBV (25, 26). Like individuals with HIGM, patients with XLP have a marked reduction in circulating $CD27^+$ B cells with the majority of residual memory cells expressing IgM (27). However, it was not previously known whether these cells undergo SHM and possess other characteristics typical of memory B cells or, alternatively, have been aberrantly induced to express CD27 in vivo. We now demonstrate that $CD27^+$ B cells in XLP patients resemble classical memory B cells with respect to morphology and phenotype, as well as their ability to proliferate and differentiate in response to T cell–dependent and –independent stimuli in vitro. Furthermore, analysis of Ig V region genes expressed by $CD27^+$ B cells from XLP patients and normal donors showed similar SHM frequencies and patterns, consistent with Ag-driven selection. On the other hand, there was a paucity of GCs in the spleens of XLP patients. Together, these results suggest that $IgM^+CD27^+$ B cells in XLP patients are bona fide memory B cells that can be generated under conditions that do not favor the generation of classic Ig isotype–switched memory B cells, which require intact GCs. The production of affinity-matured IgM by these cells may provide a defense against some pathogens to which a normal immune response is elicited in XLP patients (25, 26).

## Results

### *XLP patients*

We examined peripheral blood (PB) B cells from 17 XLP patients from 11 different families (age range, 6 months–49 years). We also examined tissue sections from several XLP patients (28). With the exception of patients XLP#6, XLP#12, XLP#13, and XLP#18 (Table 1), the clinical features of these patients, their *SH2D1A* mutations, the effect of these mutations on SAP expression, and the patients' EBV status have been previously described (27, 28).

### *SAP is expressed in primary human T cells but not in primary B cells*

SAP has been reported to be expressed in T cells, NK cells, NKT cells, and some transformed human B cell lines (22–24, 29, 30). It may also be expressed in a small subset of human and mouse GC B cells (30–32) and human memory B cells (33). Such expression of SAP by B cells could explain the reported defect in B cell differentiation in humans and mice deficient in SAP (13, 25, 27, 32, 34, 35). However, the previous conclusions regarding SAP expression were based on immunofluorescence, colocalization studies, and mRNA analysis, without confirmation at the protein level (30–33). To investigate this possibility further, we examined SAP expression in human GC B cells by Western blotting. T cells ($CD3^+CD20^-CD38^+$; population 1), mature B cells ($CD20^+CD38^+$; population 2), and GC B cells ($CD20^{hi}CD38^{hi}$; population 3) (7, 36) were isolated by

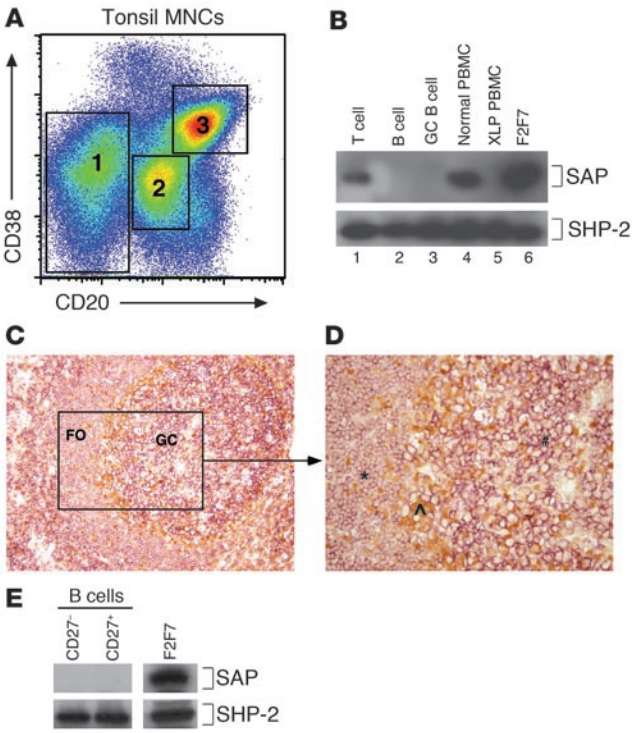


**Figure 1**
SAP is expressed in human T cells but not B cells. (**A**) Human tonsil mononuclear cells were stained with CD20 and CD38 mAbs. T cell ($CD20^-CD38^+$; population 1), B cell ($CD20^+CD38^+$; population 2), and GC B cell ($CD20^{hi}CD38^{hi}$; population 3) populations were isolated by cell sorting. Following sorting, the purity of populations 1, 2, and 3 was 98%, 98% and 95%, respectively. (**B**) Sort-purified tonsillar T cells, B cells, and GC B cells as well as activated PBMCs from a normal donor or an XLP patient (XLP#10; ref. 27) were solubilized in 1% NP-40 lysis buffer in 10 mM Tris-HCl and 150 mM NaCl (pH 7.8) supplemented with protease and phosphatase inhibitors. Lysates containing ~5–10 × $10^5$ cell equivalents were analyzed for SAP expression by SDS-PAGE and Western blotting using rabbit anti-human SAP polyclonal antiserum (top). Lysates from the human T cell line F2F7 were examined concomitantly, and detection of SHP-2 expression was used to demonstrate equivalent loading of cellular proteins (bottom). Results are representative of data obtained by analyzing sort-purified B cell populations isolated from 3 different donor tonsils. (**C** and **D**) Immunohistology was performed on reactive tonsil sections using anti-CD20 mAb (pink/purple) and anti-SAP polyclonal Ab (orange/brown). B cells in the follicle (FO) ($CD20^+$; asterisk) and GC ($CD20^{hi}$; pound symbol), as well as SAP-expressing non–B cells (caret), are indicated. Boxed region in **C** (magnification, ×20) is shown in **D** (magnification, ×40). (**E**) Sort-purified PB naive and memory B cells were prepared and analyzed for SAP expression as described in **A**.

sorting from normal tonsils (Figure 1A). Expression of SAP by these populations was compared with that in activated PBMCs from a normal donor, as well as those from XLP#10, who has a nonsense mutation in the *SH2D1A* gene (R55X; ref. 27) that abrogates SAP expression. Western blot analysis revealed SAP expression in primary tonsillar T cells as well as activated normal PBMCs and the F2F7 T cell line (Figure 1B, lanes 1, 4 and 6). In contrast, SAP could not be detected in mature B cells or GC B cells (Figure 1B, lanes 2 and 3). In fact, the reactivity of the anti-SAP antiserum with lysates from tonsillar B cells was no different from that with lysates of activated PBMCs from a SAP-deficient XLP patient (Figure 1B, compare lanes 2 and 3 with lane 5), thus demonstrating a virtual absence of SAP from resting B cells as well as GC B cells.

A recent study reported detectable expression of SAP in GC B cells present in murine spleens (32). Based on this, we next examined in situ expression of SAP in human lymphoid tissue by staining sections of reactive tonsils with anti-SAP and anti-CD20 Abs. Even though SAP was clearly detectable in human T cells, we failed to observe any expression in B cells present in the follicle or in the GC (Figure 1, C and D). Lastly, expression of SAP was assessed in sort-purified populations of PB naive ($CD20^+CD27^-$) and memory ($CD20^+CD27^+$) B cells. SAP could not be detected in either B cell subset (Figure 1E). Therefore, SAP expression appears to be confined to human T cells, NK cells, and NKT cells, but not B cells, regardless of their stage of differentiation.

### *XLP patients lack Ig isotype–switched B cells*

In normal individuals, the majority of human memory B cells express CD27 (15, 16, 37). In XLP patients there was a severe reduction in both the number (Figure 2A) and frequency (27) of circulating $CD27^+$ memory B cells. By analyzing the few detectable $CD27^+$ B cells present in XLP patients, we also found an absence of $IgG^+$ and $IgA^+$ cells, revealing an inability of naive B cells to differentiate into Ig isotype–switched classical memory B cells in vivo (Figure 2, B and C, right; previously described in ref. 27 but shown here for comparative purposes). Further analysis of $CD27^-$ B cells from normal individuals revealed the presence of a small but consistently detectable population of cells that were $IgG^+$ or $IgA^+$ (3.7% ± 0.44% and 1.6% ± 0.23% of $CD27^-$ B cells, respectively; mean ± SEM; $n = 29$; Figure 2, B and C, left, and Figure 2D). This revealed a subpopulation of putative memory B cells in the $CD27^-$ compartment, a finding consistent with our previous studies in which approximately 3% and 1.5% of $CD27^-$ B cells in human spleen expressed IgG and IgA, respectively (37), as well as a recent study that identified a subset of $CD27^-$ memory B cells predominantly expressing IgG or IgA in human tonsils, defined by expression of the inhibitory receptor FcRH4 (38). Hence, to examine whether some Ig isotype–switched B cells persist in the $CD27^-$ B cell population of XLP patients, we determined the frequency of $CD27^-$ B cells from XLP patients that were either $IgG^+$ or $IgA^+$. In contrast to normal individuals (Figure 2D, left), less than 0.4% of XLP $CD27^-$ B cells were $IgG^+$ or $IgA^+$ (mean; $n = 15$; Figure 2D, right). Thus, XLP patients have an absolute deficiency in Ig isotype–switched memory B cells, as revealed by analysis of both $CD27^-$ and $CD27^+$ B cell compartments.

### *$CD27^+$ B cells from XLP patients have morphological and phenotypic characteristics typical of normal memory B cells*

Memory B cells differ from naive B cells not only in the expression of switched Ig isotypes, but also with respect to morphological and other phenotypic characteristics. Specifically, memory B cells are larger and more granular than naive B cells and express different levels of a suite of surface molecules (15, 16, 36, 38–41). Based on this, PB $CD27^+$ B cells from XLP patients were next examined for these morphological and phenotypic characteristics.

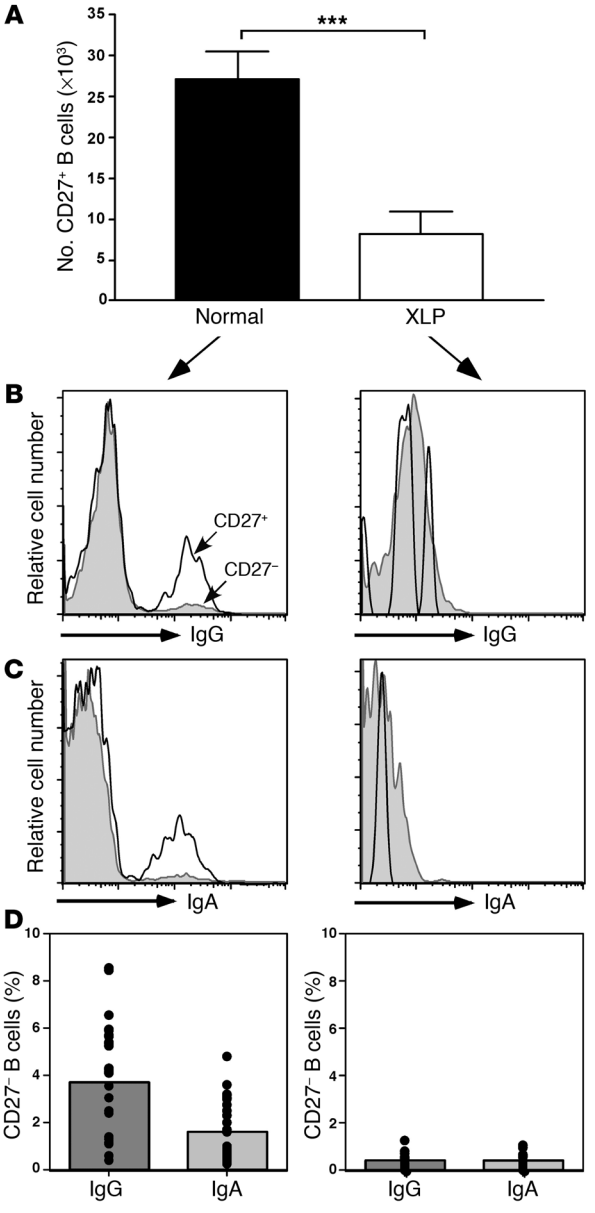


**Figure 2**

Complete absence of Ig isotype–switched B cells in XLP. PBMCs were isolated from 29 normal individuals and 15 XLP patients and labeled with mAbs against CD20, CD27 and either IgG, IgA or an isotype control Ab. (**A**) The number of $CD20^+CD27^+$ cells per milliliter of blood was quantitated. Expression of IgG (**B**) and IgA (**C**) on $CD20^+CD27^-$ (filled) and $CD20^+CD27^+$ (open) cells isolated from a normal donor (left) and an XLP patient (right) was determined. (**D**) The frequency of $IgG^+$ and $IgA^+$ cells within the $CD20^+CD27^-$ compartment of normal donors (left) and XLP patients (right) was determined by setting gates on $CD20^+CD27^-$ cells. $^{***}P < 0.001$.

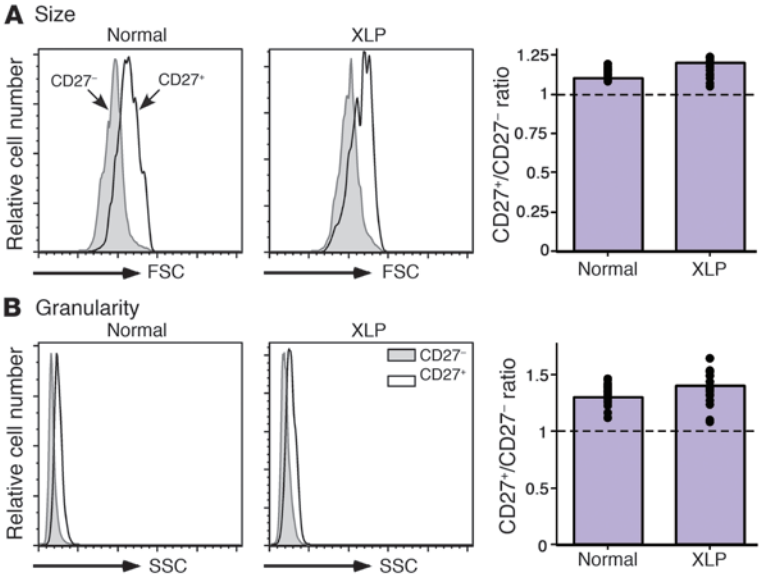


**Figure 3**
Size and granularity of CD27⁺ B cells in XLP patients resembles that of normal memory B cells. PBMCs from 30 normal individuals and 17 XLP patients were labeled with anti-CD20 and anti-CD27 mAbs. Size (**A**) and granularity (**B**) of $CD20^+CD27^-$ (filled) and $CD20^+CD27^+$ cells (open) were determined by measuring the forward scatter (FSC) and side scatter (SSC), respectively, by flow cytometry. The ratio of the size and granularity of CD27⁺ to CD27⁻ B cells was determined (right).

*Size and granularity*. The size and granularity of CD27⁻ and CD27⁺ B cells were determined by measuring the forward scatter and side scatter, respectively, by flow cytometry. In normal individuals CD27⁺ memory B cells were found to be larger and more granular than CD27⁻ B cells (Figure 3, A and B, left). When the ratio of size and granularity of memory and naive B cells from normal individuals was calculated, PB memory B cells proved to be 10–20% larger and approximately 35% more granular than naive B cells (Figure 3, A and B, right; $n = 30$). On repeating this analysis on CD27⁻ and CD27⁺ B cells from XLP patients, it was revealed that CD27⁺ B cells were similarly larger (~20%) and more granular (~40%) than CD27⁻ B cells from the same patient (Figure 3, A and B). Thus, the residual CD27⁺ B cells from XLP patients resemble classical memory B cells morphologically in that they are both larger and more granular than CD27⁻ (i.e., naive) B cells.

*Cell surface molecules*. CD27⁻ and CD27⁺ B cells from XLP patients were next analyzed for expression of surface molecules that are differentially expressed by naive and memory B cells (9, 15, 36). In normal individuals, PB CD27⁻ B cells uniformly expressed IgM, while its expression on CD27⁺ B cells was bimodal, such that one population was IgM⁻ (i.e., Ig isotype–switched) and a second population had upregulated IgM compared with CD27⁻ B cells to become $IgM^{hi}$ memory B cells (Figure 4A). Normal naive B cells uniformly expressed IgD and CD23, while these molecules were downregulated on the majority of memory B cells (Figure 4A). In contrast, memory B cells expressed low but detectable levels of the activation molecules CD80 and CD95, yet these molecules were largely absent from the surface of naive B cells (Figure 4A).

Expression of CD1d, CD10, CD21, CD24, and CD38 on human PB B cell subsets was also determined because these molecules are expressed differentially throughout B cell development and differentiation (15, 36, 42–44). CD10 was absent from circulating B cell subsets in normal healthy donors (Figure 4A). In contrast, CD1d and CD21 were expressed by normal PB B cells, yet there was no difference in the level of expression between CD27⁻ and CD27⁺ B cells (Figure 4A). On the other hand, CD24 was expressed at a higher level on CD27⁺ B cells than on CD27⁻ cells, while CD38 was weakly expressed on CD27⁺ B cells and upregulated on CD27⁻ B cells (Figure 4A).

CD27⁻ B cells from XLP patients shared a phenotype with normal CD27⁻ B cells in that they were $IgM^{lo}IgD^{hi}CD1d^+CD21^+CD23^+CD24^+CD38^+$ and lacked CD80 and CD95 (Figure 4B). One notable difference was the presence of a population of B cells within the CD27⁻ subset from XLP patients that was $CD10^+$ and also expressed a higher level of CD38 and CD24 (Figure 4B; circles). These cells were found to comprise approximately 15% of circulating B cells in XLP patients and less than 3% of B cells from normal donors (45) and are likely to be human transitional B cells (43, 45, 46). Strikingly, XLP CD27⁺ B cells were essentially identical to normal CD27⁺ memory B cells, as this population contained cells that were $IgM^{hi}IgD^{lo}CD23^{-/lo}$ and had acquired detectable expression of CD80 and CD95 (Figure 4B). CD27⁺ B cells from XLP patients also resembled those from normal donors with respect to expression of CD1d and CD21, which was similar to that observed for CD27⁻ B cells, and CD38 and CD24, which were found to be expressed at a lower and higher level, respectively, compared with CD27⁻ B cells (Figure 4B).

The human memory B cell compartment is composed of cells that are either $IgM^{hi}IgD^{lo}$, IgM only, IgD only, or have undergone Ig class switching to express IgG or IgA (15, 16, 19, 36, 37, 40, 47). It was therefore of interest to determine the pattern of expression of IgM and IgD on the few memory B cells present in XLP patients. B cells from normal donors and XLP patients were labeled with mAbs specific for CD20, CD27, IgM, and IgD, and the frequency of $CD20^+CD27^+$ (i.e., memory) B cells that were $IgM^{hi}IgD^{lo}$, IgM only, IgD only, or $IgM^-IgD^-$ was determined. In normal individuals, $IgM^{hi}IgD^{lo}$ and $IgM^-IgD^-$ cells accounted for 38.2% ± 3.3% and 58.7% ± 4.2%, respectively, of the total population of PB memory B cells (Figure 4A, dot plot; mean ± SEM; $n = 7$). In contrast, only 1.8% ± 0.5 % and 1.3% ± 0.4% of normal memory B cells were IgM only or IgD only (Figure 4A, dot plot; $n = 7$). When this analysis was applied to CD27⁺ B cells from XLP patients, 89.4% ± 2.2% of them were found to be $IgM^{hi}IgD^{lo}$, while only 6.0% ± 1.4% had undergone isotype switching (Figure 4B, dot plot; $n = 4$). In contrast to previous findings (16), neither in the normal donors nor in the XLP patients were IgM-only B cells found to comprise a significant proportion of the total memory B cell population. Collectively, with the exception of an isotype-switched phenotype, the CD27⁺ B cells detected in XLP patients resembled those cells present in the PB of normal donors, suggesting that these cells are indeed memory B cells.

***XLP CD27⁺ B cells exhibit normal memory-type responses in vitro***

The data presented thus far indicated that the CD27⁺ B cells present in XLP patients, although present at a low frequency, were phenotypically and morphologically similar to those in normal indi-

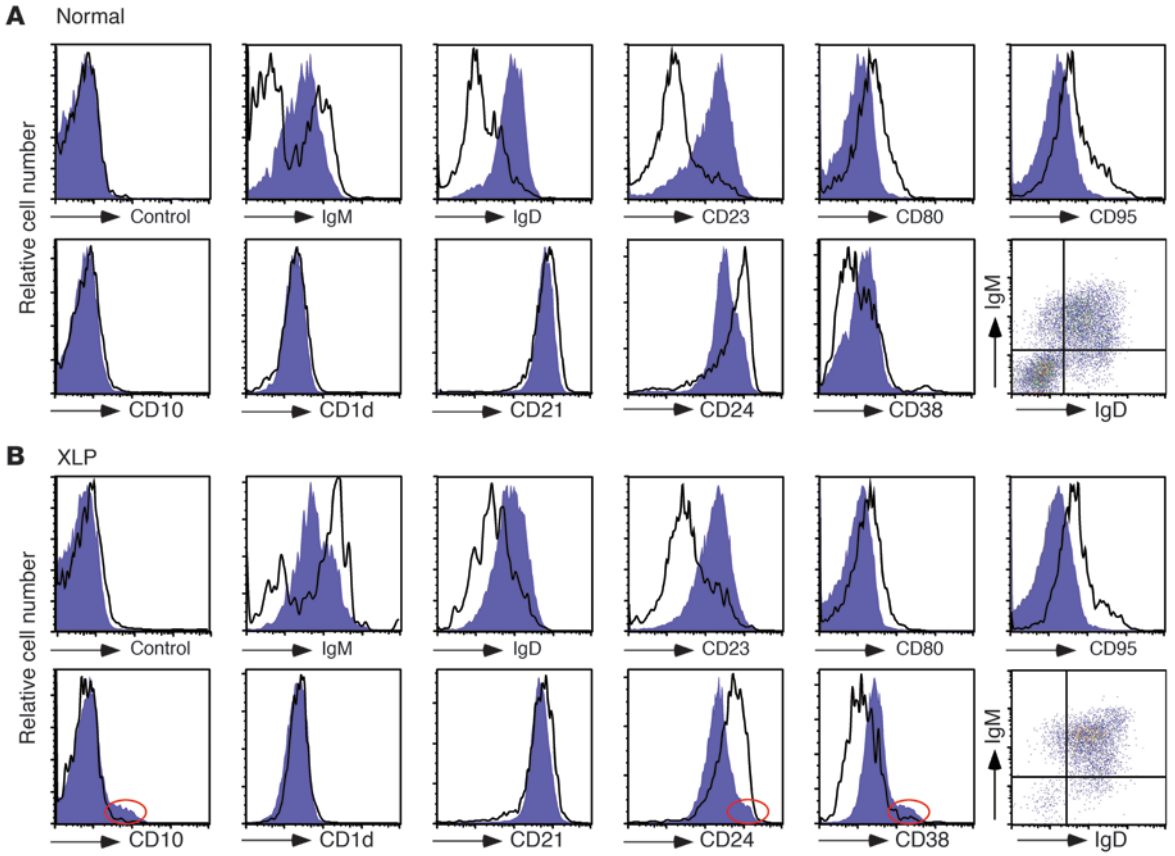


**Figure 4**

Surface phenotype of CD27$^+$ B cells from XLP patients corresponds to normal memory B cells. PBMCs from (**A**) a normal donor and (**B**) an XLP patient were stained with mAbs specific for CD20, CD27 and IgM, IgD, CD1d, CD10, CD21, CD23, CD24, CD38, CD80, CD95, or an appropriate isotype control Ab. The surface expression of these molecules on CD27$^-$ (filled) and CD27$^+$ (open) B cells was determined. The coexpression of IgM and IgD on gated CD27$^+$ B cells was determined and is presented as a dot plot. Data represent experiments performed using B cells from 4–7 normal donors and 4 XLP patients. The population of B cells present in the CD27$^-$ subset from XLP patients that corresponds to transitional B cells (i.e., CD10$^+$CD24$^{hi}$CD28$^{hi}$) is circled in **B**.

viduals. However, human memory B cells can also be distinguished from naive B cells according to their functional characteristics. In particular, memory B cells undergo more rounds of cell division, preferentially differentiate into plasmablasts, and secrete higher levels of Ig compared with naive B cells (15, 41, 48–51). To investigate cell function, we next examined the ability of XLP CD27$^+$ B cells to proliferate, differentiate, and secrete Ig in vitro compared with CD27$^-$ B cells.

*XLP CD27$^+$ B cells have a proliferative advantage over CD27$^-$ B cells.* B cell proliferation was investigated by isolating CD27$^-$ and CD27$^+$ B cells, labeling them with the division tracking dye CFSE, and culturing them under 2 different culture conditions. First, the B cells were stimulated with the T cell–dependent stimulus of CD40L, IL-2, and IL-10 (48, 49). Under these conditions, CD27$^+$ B cells from normal individuals underwent greater proliferation than CD27$^-$ B cells, as revealed by dilution of CFSE (Figure 5A). By setting gates on CFSE peaks, it was found that the majority of normal naive CD27$^-$ B cells had divided 3 times, whereas the majority of memory CD27$^+$ B cells had undergone at least 4 rounds of cell division (Figure 5A, left). When this analysis was performed on CFSE-labeled XLP CD27$^-$ and CD27$^+$ B cells cultured identically, the CD27$^+$ B cells also underwent at least 1 more round of cell division than did CD27$^-$ B cells (Figure 5A, right).

Memory B cells also respond more robustly than naive B cells following stimulation through TLR-9 with CpG DNA (52). Furthermore, the level of B cell responsiveness can be augmented by ligation of the B cell receptor (52). Thus, the combination of CpG DNA and anti-Ig was employed as a model of T cell–independent stimuli and also to examine whether signals through the B cell receptor preferentially favor the proliferation of memory B cells. In response to stimulation with CpG and anti-Ig, approximately 30% of CD27$^-$ B cells from normal individuals underwent 3 or more divisions, compared with more than 70% of normal CD27$^+$ B cells (Figure 5B, left). When B cells from XLP patients were cultured under these conditions, similar differences were observed for the proliferative responses of CD27$^-$ and CD27$^+$ B cells: approximately 20% of CD27$^-$ B cells underwent 3–6 divisions, compared with approximately 65% of CD27$^+$ B cells (Figure 5B, right). Overall, these results demonstrate that CD27$^+$ XLP B cells, like those from normal donors, have a proliferative advantage over CD27$^-$ B cells regardless of whether the stimulus involves triggering the B cells through CD40, the B cell receptor, or TLRs.

*XLP CD27$^+$ B cells differentiate into CD38$^+$ plasmablasts in vitro.* Lymphocyte differentiation is linked to cell division: when stimulated in vitro, human memory B cells differentiate into plasmablasts

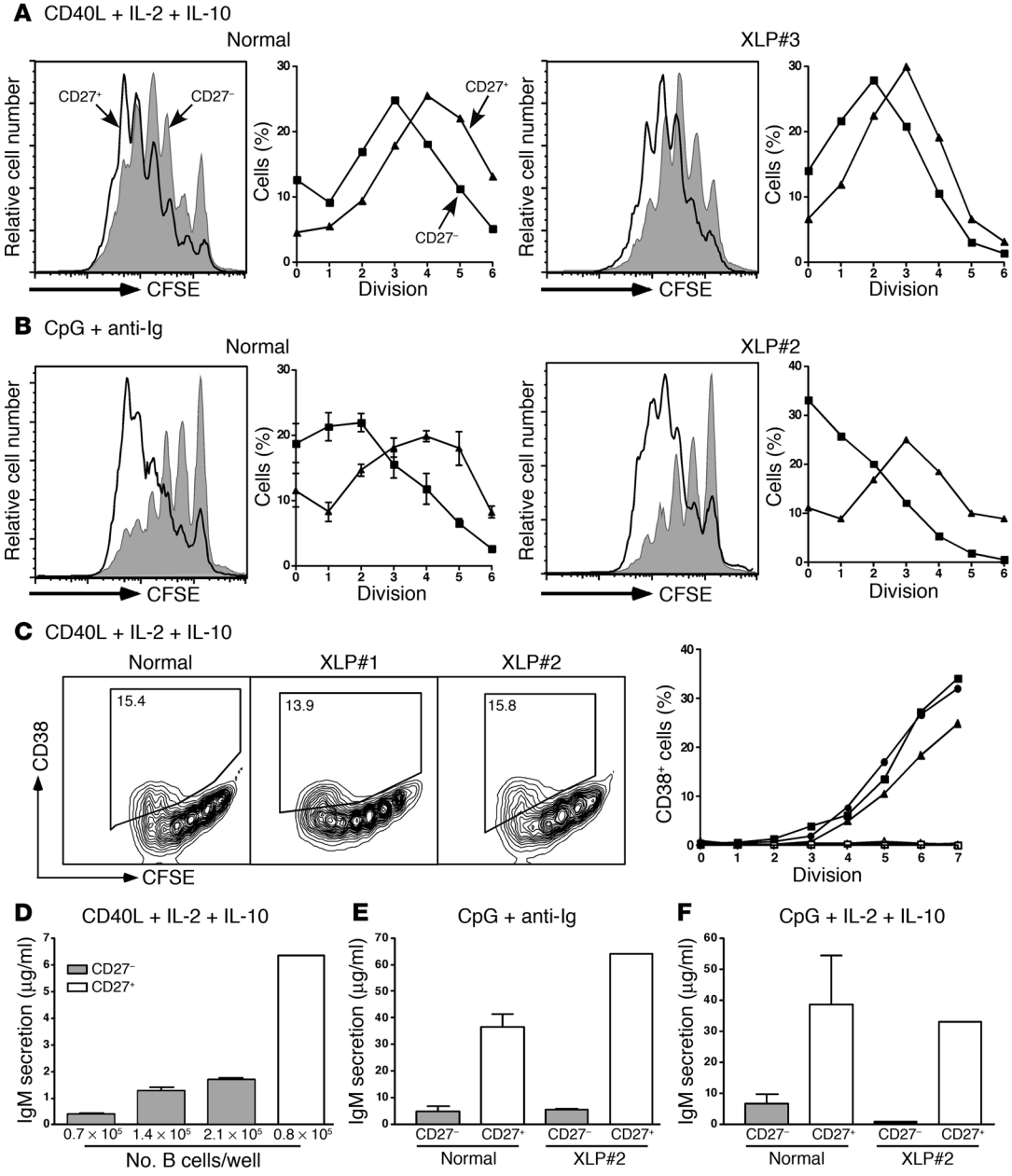


**Figure 5**

CD27+ XLP B cells have a greater capacity to proliferate, differentiate, and secrete Ig in vitro than do CD27− B cells. (**A** and **B**) CD27− (filled histogram and squares) and CD27+ (open histogram and triangles) B cells were isolated from normal donors and XLP patients, labeled with CFSE, and cultured with (**A**) CD40L, IL-2 and IL-10, or (**B**) CpG and anti-Ig. After 5 days, the cells were harvested, and CFSE profiles were analyzed for cell division. By setting gates on CFSE peaks, the frequency of cells within each division was determined. (**C**) CD27+ B cells from 1 normal donor (squares) and 2 XLP patients (XLP#1, triangles; XLP#2, circles) were cultured with CD40L, IL-2, and IL-10 for 5 days and then harvested and stained with anti-CD38 mAb to detect plasmablasts. Numbers indicate the percentage of activated CD27+ B cells that differentiated to become CD38+ plasmablasts during in vitro culture. (**D**) CD27− and CD27+ B cells isolated from XLP#3 were cultured with CD40L, IL-2, and IL-10 at different cell densities. (**E** and **F**) Normal and XLP CD27− and CD27+ B cells were cultured with CpG and anti-Ig (**E**) or CpG, IL-2, and IL-10 (**F**). After 5 days, culture supernatants were harvested, and the amount of IgM secreted was determined by ELISA. Data for normal donors in **B**, **E**, and **F** represent the mean ± SEM of 3 independent experiments. The data for XLP B cell cultures is from 1 experiment; however, similar results were obtained in a second experiment using B cells from patient XLP#7.

after approximately 3 rounds of cell division (48). To examine the ability of CD27+ B cells from XLP patients to differentiate into effector cells, CD27+ B cells were isolated from normal donors and 2 XLP patients, labeled with CFSE, and cultured with CD40L, IL-2, and IL-10, a culture known to induce the generation of plasmablasts, as revealed by the acquisition of CD38 expression (48, 49). After 5 days of culture the frequency of CD38+ cells generated from CD27+ B cells isolated from XLP patients was comparable

to that of normal individuals (~15%; Figure 5C), and CD38$^+$ cells appeared in a similar division-linked manner in both normal and XLP CD27$^+$ B cells (Figure 5C, right).

*Compared with XLP CD27$^-$ B cells, XLP CD27$^+$ B cells have an enhanced ability to secrete Ig in vitro*. Another characteristic of normal memory B cells is their ability to secrete greater amounts of Ig than naive B cells (9, 15, 41, 48–51). To investigate this, XLP CD27$^-$ and CD27$^+$ B cells were isolated and cultured with CD40L, IL-2, and IL-10 for 5 days. When the B cells were cultured at approximately the same density (~8 × $10^4$ B cells/well), approximately 16 times more IgM was detected in supernatants collected from CD27$^+$ B cells than from CD27$^-$ B cells (Figure 5D). Furthermore, small amounts of IgG (48 ng/ml) and IgA (62 ng/ml) were also detected in cultures of XLP CD27$^+$ B cells, but not CD27$^-$ B cells (<5 ng/ml), demonstrating that XLP CD27$^+$ B cells can be induced to secrete switched Ig isotypes in the presence of a sufficient T cell–dependent stimulus (data not shown). When the starting density of cultured CD27$^-$ B cells was increased 2- and 3-fold above that of CD27$^+$ B cells, the amount of IgM secreted increased, but remained approximately 5-fold less than that observed from the smaller number of CD27$^+$ B cells (Figure 5D). In additional experiments, CD27$^+$ B cells from several XLP patients produced amounts of IgM that were comparable to normal CD27$^+$ B cells (normal CD27$^+$, 45,079 ± 13,565 ng/ml; XLP CD27$^+$, 33,769 ± 18,055 ng/ml; mean ± SEM; $n$ = 4). In a second series of experiments, CD27$^-$ and CD27$^+$ B cells from normal donors and XLP patients were stimulated with CpG DNA in combination with either anti-Ig or IL-2 plus IL-10. Both populations of B cells secreted detectable levels of IgM in response to these stimulatory conditions (Figure 5, E and F). However, regardless of whether the B cells were from normal donors or XLP patients, the

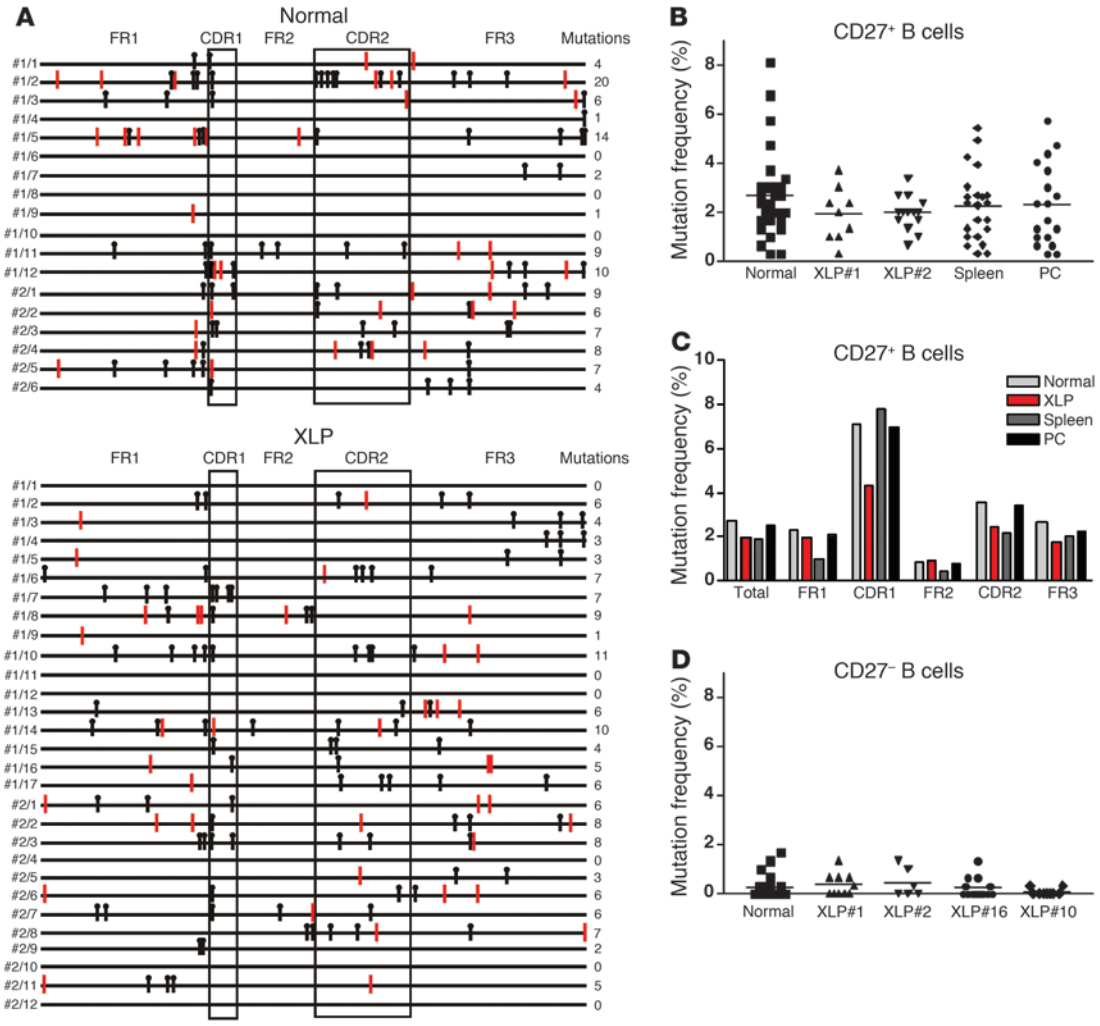


**Figure 6**

CD27$^+$ B cells in XLP patients undergo somatic hypermutation to a similar extent as normal memory B cells. CD27$^+$ B cells were isolated from 4 normal donors and 2 XLP patients (XLP#1 and XLP#2), mRNA was extracted, and cDNA was synthesized. Ig $V_H5$ genes were amplified from cDNA by PCR, cloned, and then sequenced. (**A**) Each line represents a single Ig $V_H5$ gene; vertical bars represent silent mutations; vertical bars with closed circles represent replacement mutations. The total number of mutations detected in the different cloned genes is shown to the right of each sequence. Data representative of 2 of 4 normal donors and 2 XLP patients. FR, framework region. (**B**) Mutation frequency of Ig $V_H5$ genes expressed by CD27$^+$ B cells from 4 normal donors and 2 XLP patients. (**C**) Mutation frequency of the entire Ig $V_H5$ gene, as well as the individual FRs and CDRs. Shown for comparative purposes are data of SHM in normal splenic CD27$^+$ B cells ($n$ = 2) (15) and PCs ($n$ = 2) (39). (**D**) Mutation frequency of Ig $V_H5$ gene sequences isolated from CD27$^-$ B cells from 4 normal donors and 4 XLP patients.

$CD27^+$ B cells secreted 5- to 10-fold more IgM than the corresponding $CD27^-$ B cells (Figure 5, E and F). Taken together, these results show that similar to normal memory B cells, $CD27^+$ XLP B cells secrete substantially higher levels of Ig than $CD27^-$ B cells in response to stimulation with T cell–dependent or –independent signals.

***$IgM^+CD27^+$ B cells from XLP patients undergo normal SHM***

In normal secondary lymphoid tissue, SHM and isotype switching occur in GCs, resulting in the generation of high-affinity memory B cells and PCs (7, 9, 53). The deficiency in isotype-switched memory B cells in XLP patients raised the question of whether the residual $CD27^+$ B cells had undergone SHM. This was important to answer given the reported lack of GCs and a deficiency in Ag-specific memory B cells and long-lived PCs in *Sap*$^{-/-}$ mice (13, 32).

This issue was investigated by examining the frequency of SHM within the Ig $V_H5$ region genes of $CD27^+$ B cells isolated from 2 XLP patients (XLP#1 and XLP#2) and 4 normal individuals (Figure 6A; data from 2 normal donors presented diagrammatically). By comparing each of the cloned sequences to those of germline $V_H5$ genes, the total number of mutations, the nature of the mutation (i.e. silent or replacement), and their position within the framework regions and complementarity determining regions (CDRs) was determined. Twenty-nine of 35 (83%) and 23 of 29 (79%) unique sequences from normal and XLP $CD27^+$ B cells, respectively, contained mutations (range: normal, 1–24 mutations; XLP, 1–11 mutations; Figure 6A). On average, the mutation frequency (normal, 2.7% ± 0.3%; XLP#1, 1.9% ± 0.4%; XLP#2, 2.0% ± 0.2%; mean ± SEM) and number of mutations per sequence (normal, 8 ± 1; XLP#1, 5.9 ± 0.8; XLP#2, 5.7 ± 0.7) in Ig V region genes from $CD27^+$ B cells from the 2 sources was similar ($P > 0.05$; Figure 6, A and B). In previous studies, we and others have investigated the frequency of SHM in IgM-expressing memory B cells and PCs isolated from human spleen (15, 39, 54). Comparison of the number of mutations in these different B cell populations revealed that the level of SHM in XLP $CD27^+$ B cells was comparable not only to that of normal PB $CD27^+$ B cells but also to that of splenic memory B cells (5.6 ± 1.1 mutations per sequence; refs. 15, 54) and splenic PCs (7.4 ± 1.1 mutations per sequence; ref. 39; Figure 6B). Thus, SHM appears to occur at a normal rate in XLP $CD27^+$ B cells.

Seventy percent of mutations in $CD27^+$ B cells from XLP patients were replacement mutations, which represented a similar frequency to that reported for normal memory B cells in PB (65%; Figure 6A) and spleen (>60%) (15, 37), as well as in splenic PCs (69%) (39). Furthermore, there was an increase in the incidence of replacement mutations in CDR1 for both normal (77.5%) and XLP (95%) $CD27^+$ B cells (Figure 6A). Another feature of Ag selection is an increase in the frequency of mutations in CDR1 compared with the total gene and other regions of the Ig V region gene (16, 38, 53). Interestingly, when compared with the total gene, the mutation frequency within CDR1 of normal PB and splenic $CD27^+$ B cells, as well as splenic PCs, was increased approximately 3-fold, from 2.3% to 7.3% (Figure 6C). However, the increase in frequency of mutations in CDR1 of Ig V region genes isolated from XLP $CD27^+$ B cells increased only 2-fold (2.0% to 4.3%; Figure 6C), suggesting a less efficient affinity maturation process in XLP $CD27^+$ B cells compared with normal $CD27^+$ B cells.

Within the Ig V genes there are "hot spot" sequences that are preferentially targeted by the SHM machinery. One of these is the RGYW/WRCY sequence (R = A/G, Y = C/T, W = A/T; G:C is the targeted nucleotide), which is present in both DNA strands. Within this motif, the G:C nucleotide is mutated at a frequency that is 4-fold greater than would be expected if SHM were a random process (16). Thus, mutations in this motif are believed to result from Ag-induced selection (16). Using this as an indicator of Ag selection, as well as of the nature of SHM occurring in $CD27^+$ B cells obtained from normal donors and XLP patients, we calculated the frequency of mutations that occurred in this known hot spot. Of the 231 point mutations identified in the individual Ig $V_H5$ gene sequences isolated from 4 healthy donors, 76 of them (33%) occurred at the G or C nucleotide in the RGYW/WRCY sequence. Strikingly, a similar frequency (34 of 133; 26%) of mutations detected in the Ig $V_H5$ gene sequences from the 2 XLP patients resulted in a replacement of the G or C in RGYW/WRCY to another nucleotide. These data confirm that XLP $IgM^+CD27^+$ B cells undergo SHM at a rate comparable to that observed in normal $IgM^+CD27^+$ B cells and suggest that SHM results from Ag-induced selection of these B cells in XLP.

Ig V region genes isolated from $CD27^-$ B cells were also examined for SHM. Unlike $CD27^+$ B cells, $CD27^-$ B cells isolated from both normal individuals ($n = 4$) and XLP patients ($n = 4$) exhibited minimal SHM (mutation frequency: normal, 0.24%; XLP, 0.28%; Figure 6D). This represents a 7- to 10-fold reduction in the incidence of SHM compared with $CD27^+$ B cells. The very low level of SHM in $CD27^-$ B cells from XLP patients is further evidence that memory B cells, defined as cells expressing mutated Ig V region genes, do not aberrantly accumulate in the $CD27^-$ B cell compartment in this disorder.

***Perturbed formation of GC in spleens of XLP patients***

Analysis of spleens from *Sap*$^{-/-}$ mice revealed defective formation of GCs in response to T cell–dependent Ag (13, 32). To extend this observation to SAP-deficient humans, immunohistology was performed on spleen sections from XLP patients who had not been exposed to EBV. These patients (XLP#10 and XLP#11; ref. 27) had normal frequencies of peripheral B cells (XLP#10, ~7%; XLP#11, 27%); however, less than 1.5% (XLP#10) and less than 5% (XLP#11) of B cells exhibited a memory phenotype (i.e., were $CD27^+$). Serum levels of IgM and IgA in XLP#10 were normal, while IgG was slightly reduced and IgE was undetectable; XLP#11 had normal levels of serum Ig. The assessment of EBV-negative patients allowed us to eliminate the possibility that: (a) GCs develop in XLP patients but are then destroyed as a result of tissue damage following EBV infection (25) or (b) GCs can no longer form following viral infection. Staining spleen sections from normal donors with anti-IgD mAb alone or in combination with anti-CD27 mAb identified naive B cells within the primary follicle ($IgD^{hi}CD27^-$), memory B cells in the marginal zone ($IgD^{lo/-}CD27^+$), and T cells in the T cell zone ($IgD^-CD27^+$; Figure 7, A and B) (15, 19, 54). Secondary follicles containing GCs could also be identified by the presence of B cells that were $IgD^-$ (Figure 7, A and B) and expressed the transcription factor Bcl-6 (Figure 7C; refs. 1, 36, 55).

Primary follicles in spleen sections from XLP patients were detected with anti-IgD (Figure 7, D and E) and anti-CD20 mAbs (data not shown). Although $IgD^+$ follicular (i.e., naive) B cells were evident, there was a severe reduction in the frequency of surrounding $CD27^+$ B cells (Figure 7D). This confirms the absence of memory B cells not only from the PB of XLP patients (27), but also from the spleen. Despite the formation of normal primary follicles in the spleens of these patients, there was a deficiency of $IgD^-Bcl\text{-}6^+$ GC B cells (Figure 7, D–F). The few cells detected that weakly expressed Bcl-6 (Figure 7F) appeared disorganized. Collectively, these data

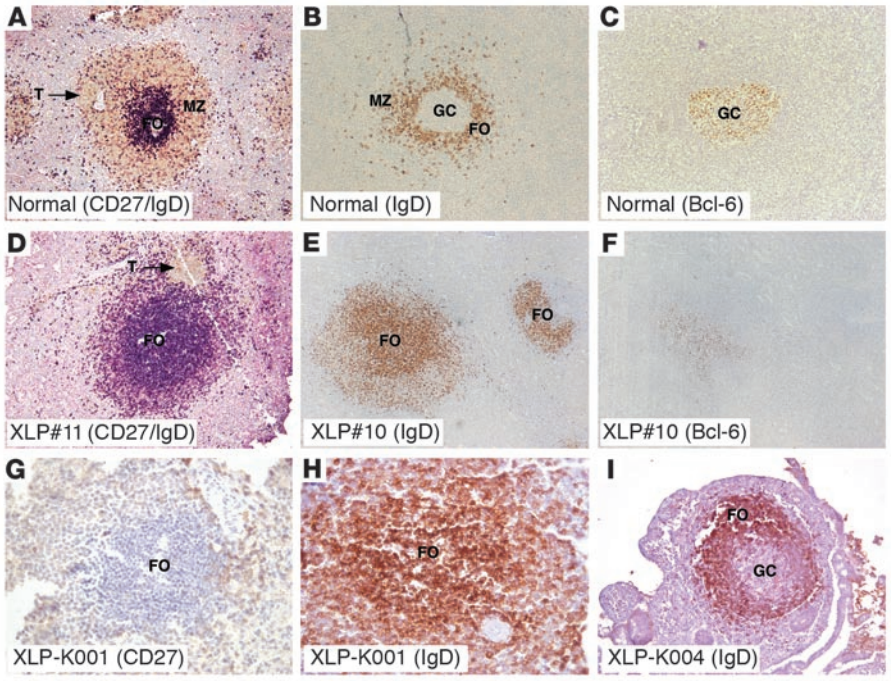


**Figure 7**

Deficiency of GCs in spleens of XLP patients. Immunohistology was performed on spleen sections obtained from normal donors (**A**–**C**), EBV$^-$ XLP patients (**D**, XLP#11; **E** and **F**, XLP#10), and EBV$^+$ XLP patients obtained from the XLP Registry (**G** and **H**, XLP-K001; see ref. 28). Gastrointestinal tissue obtained from another XLP patient (**I**, XLP-K004; see ref. 28) was also examined. Anti-IgD (**A**, **B**, **D**, **E**, **H**, and **I**), anti-CD27 (**A**, **D**, and **G**), and anti–Bcl-6 (**C** and **F**) were used to identify the B cell follicle (IgD$^+$CD27$^-$Bcl-6$^-$), marginal zone (MZ; IgD$^{lo}$CD27$^+$Bcl-6$^-$), GC (IgD$^-$Bcl-6$^+$), and T cell zone (T; IgD$^-$CD27$^+$) in sections of human spleen and GIT. Rare Bcl-6$^+$ secondary follicles were noted in XLP#10; in addition, the absence of IgD$^-$ B cells within a B cell follicle (XLP#11, XLP-K001) revealed the paucity of appropriately formed GCs in the spleens of these XLP patients.

suggest a paucity of well-formed GCs and secondary follicles in the spleens of XLP patients. To extend these findings, EBV-positive XLP patients were examined (XLP-K001 and XLP-K004; see ref. 28). Similar to EBV-negative XLP patients, those infected with EBV had normal architecture of primary splenic follicles; however, there was an absence of CD27$^+$ memory B cells (XLP-K001; Figure 7G). This supports our earlier finding that the deficiency in circulating memory B cells is evident in EBV-negative as well as -positive XLP patients (27). Furthermore, consistent with the original description of XLP (25), IgD$^-$ GC B cells were not detected or only infrequently detected in the spleens of EBV-infected patients (XLP-K001; Figure 7H). Interestingly, when tissue from the small intestine was examined, GCs were detected in the lamina propria (XLP-K004; Figure 7I). Thus, although GCs may form inefficiently in the spleens of XLP patients, the ability to form GCs in mucosal-associated lymphoid tissue may not be compromised in the absence of SAP.

## Discussion

Several characteristics have been identified that distinguish human naive and memory B cells. Traditionally, B cells expressing the switched Ig isotypes IgG, IgA, and IgE were considered to be memory B cells, while those with an IgM$^{lo}$IgD$^{hi}$ phenotype were naive B cells (9). Subsequent studies demonstrated CD27 expression to be a more reliable marker of memory B cells (15, 16, 37). Moreover, other phenotypic differences between naive and memory B cells have emerged, including elevated expression of CD23 by naive B cells and expression of activation markers on memory B cells (15, 36, 37, 39, 40). Naive and memory B cells also differ functionally, as revealed by the increased capacity of memory B cells to proliferate and produce Ig (15, 41, 48–51). However, the hallmark of memory B cells is the accumulation of mutations within Ig V regions (15, 16, 38, 53, 54, 56).

In several human immunodeficiencies, patients present with perturbed development of memory B cells. For example, in HIGM resulting from mutations in *CD40L* (HIGM-1) or *CD40* (HIGM-3) (17–19), and in common variable immunodeficiency due to ICOS deficiency (57), there is a reduction in memory B cells, in particular those that express switched Ig isotypes. This has been attributed to the absence of GCs in the secondary lymphoid tissues of these patients. However, B cells expressing mutated Ig V region genes have been detected in patients with HIGM-1 and HIGM-3 (18–21), and the mutation frequency of Ig V region genes in IgM$^+$CD27$^+$ B cells from these patients is similar to that of normal B cells (18, 19). The finding that some B cells from HIGM patients expressed mutated Ig V region genes led to 2 proposals. The first proposal was that some mature B cells undergo SHM via a GC-independent pathway (18, 19). The alternative proposal was that IgM$^+$CD27$^+$ B cells represent a diversified arm of the preimmune repertoire that arises independently of GCs and is involved in responses to T cell–independent Ag, rather than representing memory B cells per se (19). However, the study by Weller et al. did not formally exclude the possibility that IgM$^+$CD27$^+$ B cells are in fact bona fide memory B cells generated via Ag-driven selection outside of the GC (19), which is possible given the distribution and pattern of mutation throughout the Ig V region genes expressed by these B cells (20, 21). Furthermore, rodent models have provided convincing evidence for the existence of IgM$^+$ memory B cells and thus support the proposal that IgM$^+$CD27$^+$ B cells are memory B cells. First, memory B cells resulting from immunization of rats with T cell–dependent Ag exhibit a phenotype (IgM$^{hi}$IgD$^{lo}$) and anatomical localization (splenic marginal zone) (58) analogous to human IgM$^+$CD27$^+$ B cells (15, 54). Second, studies in mice have identified Ag-specific IgM$^+$ B cells, with characteristics of conventional memory cells, that appear to make a substantial contribution to the humoral recall response (59, 60). Third, Bcl-6–deficient mice, which fail to develop GCs, can generate IgM and IgG1 memory B cells, albeit with low affinity for the immunizing Ag (61). Together, these studies support the proposal that memory B cells may exist within the population of human IgM$^+$CD27$^+$ B cells. An extension of this, based on the detection of IgM$^+$CD27$^+$ B cells in HIGM patients (18, 19), would be that these cells may arise independently of GCs.

We have recently reported a deficiency in CD27$^+$ memory B cells in patients with XLP (27) similar to that observed in HIGM patients (17–19). The current study was aimed at determining whether the small residual population of CD27$^+$ B cells detectable in XLP patients were conventional memory B cells or whether memory B cells existed in a population with a different phenotype. Overall, the CD27$^+$ B cells present in XLP patients resembled those present in normal donors with respect to cell morphology, phenotype (CD1d$^+$CD10$^-$CD21$^+$CD23$^{lo/-}$CD24$^{hi}$CD38$^-$CD80$^+$CD95$^+$), and function. The finding of similar functional behavior in vitro of CD27$^+$ B cells from XLP patients and normal donors is further evidence that B cells in XLP patients are intrinsically normal, which is consistent with (a) the lack of expression of SAP within the B cell lineage and (b) previous observations that EBV-transformed B cell lines derived from XLP patients secrete Ig in vitro in amounts comparable to EBV-transformed B cell lines obtained from healthy donors (62).

The major difference between normal and XLP CD27$^+$ B cells was the lack of cells expressing IgG or IgA in XLP patients. Indeed, the majority (~90%) of CD27$^+$ B cells in XLP patients were IgM$^{hi}$IgD$^{lo}$, while IgM-only, IgD-only and isotype-switched B cells comprised less than 10% of this population. In normal donors, we found that IgM-only and IgD-only cells represented only approximately 3% of the CD27$^+$ B cell subset; the majority of normal CD27$^+$ B cells were either IgM$^{hi}$IgD$^{lo}$ (~40%) or IgM$^-$IgD$^-$ (~60%), indicative of isotype switching. Although it has been previously reported that IgM-only B cells represent as much as 10% of the memory population (16, 40), our findings and those of others (19, 47) suggest that these cells are only a minor component of the memory B cell pool. Thus, CD27$^+$ B cells in XLP patients correspond to the non-switched (i.e., IgM$^{hi}$IgD$^{lo}$) population of CD27$^+$ B cells present in normal donors, rather than a minor subset of cells, such as IgM-only or IgD-only memory cells.

By investigating SHM, we found that the frequency of mutations, as well as the targeting of mutations to known hot spots, in Ig V region genes expressed by CD27$^+$ B cells from XLP patients was similar to that of normal memory CD27$^+$ B cells, despite the paucity of GCs in splenic tissue. Furthermore, the majority of these mutations were replacement mutations, with an enrichment of mutations in CDR1. On the other hand, the overall incidence of mutations in CDR1 of Ig V region genes expressed by XLP CD27$^+$ B cells was lower than that in normal memory B cells. This may be a consequence of the reduction in the number of GCs in the spleens of XLP patients, a site in which high-affinity B cells can be selected by Ag-presenting follicular dendritic cells (FDCs) (1). This is consistent with observations in mice deficient in LT or TNF receptor or mice treated with anti-ICOS mAb, where B cells remain capable of undergoing SHM but low-affinity, rather than high-affinity, memory B cells are generated in the absence of GCs (63–65). Thus, in XLP as well as other immunodeficiencies, Ag-selected memory B cells may still be generated in the spleen; however, those with greatest affinity most likely require a well-formed GC microenvironment, including an FDC network, for their selection.

Our results, which suggest a paucity of GCs in spleens of XLP patients, raises the question of the origin of IgM$^+$IgD$^+$CD27$^+$ B cells. One possibility is that they are generated independently of GCs, analogous to the detection of these cells in HIGM patients, which are presumably deficient in GCs (18, 19). Another possibility is that the few GC-like structures detected in XLP patients (based on weak Bcl-6 staining) represent T cell–independent GCs similar to those that have been detected in mice (66), and these give rise to IgM$^+$, but not Ig isotype-switched, memory B cells. Although this is feasible, it is perhaps unlikely because T cell–independent GCs do not yield affinity-matured memory B cells (66). Alternatively, GCs may form in XLP patients in secondary lymphoid tissues other than the spleen, and such structures may generate IgM$^+$CD27$^+$ B cells. Indeed, analysis of lymphoid tissue in the gastrointestinal tract (GIT) of 4 XLP patients revealed the presence of B cell follicles containing GCs. However, the significance of the detection of GC in the GIT of XLP patients remains unclear. Interestingly, studies in several gene-targeted mice have revealed a similar scenario to what we have observed in XLP and suggest that the formation and function of GC in distinct anatomical sites may be differentially regulated. For instance, mice globally deficient in CXCR5 (67) or CD19 (68) or mice specifically lacking LT-β in B cells (69) or those unable to signal through the CD28/B7 pathway (70) all lack splenic GC yet have normal numbers of well-formed GCs and IgA$^+$ B cells, in Peyer's patches and lamina propria. Despite this, these mice exhibit poor responses to immunization to Ag targeted to mucosal lymphoid tissues. Thus, although GCs can develop in mucosal tissues under conditions that impede their formation in spleens, the function of such GCs may be compromised (67–70). This finding also resembles XLP because, although GCs form poorly in the spleen yet normally in GIT, a significant proportion of XLP patients have hypogammaglobulinemia (26). It is curious to consider the finding of normal GCs in the GIT, but markedly fewer in the spleen, in the context of the development of lymphoma in XLP patients. Because many B cell lymphomas resemble GC B cells (71), it is tempting to speculate that the reason why B cell lymphomas in XLP are predominantly extranodal and restricted to the gut is because the microenvironment permissive to their formation is intact and well established in this tissue compared with the spleen. Based on our present findings, it will be interesting to determine whether GCs form in mucosal-associated lymphoid tissue of *Sap*$^{-/-}$ mice.

In conclusion, our data provide compelling evidence that the IgM$^+$CD27$^+$ B cells in XLP patients are genuine and functional memory B cells, rather than B cells involved strictly in T cell–independent immune responses. Our results also reveal distinct requirements for the generation of IgM$^+$ versus isotype-switched memory B cells, with the latter being more dependent on the presence of well-formed GCs in spleens and the former arising independently of GCs or from GCs present in the GIT. It is possible that the production of affinity-matured IgM by these cells may provide a defense against some pathogens which XLP patients do not display the same degree of vulnerability (25, 26). The selective targeting of these cells in vivo may improve the hypogammaglobulinemic state, and overall humoral immunity, of XLP patients.

## Methods

*Reagents.* PE-conjugated anti-CD23, anti-CD24, and anti-CD38 mAbs were purchased from Caltag. Biotinylated anti-CD27 mAb was purchased from eBioscience. Allophycocyanin-conjugated anti-CD10, allophycocyanin-conjugated anti-CD38, biotinylated anti-IgD, anti-IgM, anti-IgG, anti-IgA, FITC-conjugated anti-CD20, PE-conjugated anti-CD1d, anti-IgD, anti-CD80 and anti-CD95 mAbs, and SA-Peridinin Chlorophyll were from BD Biosciences — Pharmingen. Purified and biotinylated goat anti-human IgM, IgG and IgA polyclonal antisera were purchased from SouthernBiotech. Recombinant human IL-2 (rIL-2) was purchased from Endogen, and human IL-10 was provided by R. de Waal Malefyt (DNAX Research Institute, Palo Alto, California, USA). Anti–SHP-2 mAb was purchased from Santa Cruz Biotechnology Inc., and rabbit anti-human SAP

polyclonal antiserum has been previously described (72). CFSE was purchased from Invitrogen Corp.

*Isolation and characterization of PBMCs*. PB samples were collected from normal healthy donors and XLP patients (XLP#1—XLP#16 and XLP#18) after obtaining informed consent, and PBMCs were isolated by Ficoll-Paque centrifugation. All studies described were approved by the Central Sydney Area Health Service Human Research Ethics Committee (Royal Prince Alfred Hospital, New South Wales, Australia) and the Children's Hospital of Philadelphia Institutional Review Board. PBMCs were surface stained with mAb to characterize B cell subsets as previously described (27, 37). Data was collected on a FACScalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 6.1.1; Tree Star Inc.).

*Isolation of B cells*. $CD19^+$ B cells were isolated from PBMCs using the CD19-DYNA/detach-a-bead system or the CD19 negative isolation kit (Dynal Biotech). $CD27^-$ and $CD27^+$ B cells were then isolated using CD27 MACS Microbeads (Miltenyi Biotec), or by sorting $CD20^+CD27^-$ and $CD20^+CD27^+$ populations, respectively (39, 48). Purified human B cells were then labeled with CFSE as previously described (48).

*Expression of SAP*. F2F7 cells, activated PBMCs (72), and sort-purified human tonsil T and B cells were solubilized in cold lysis buffer (1% NP-40 prepared in 10 mM Tris-HCl, 150 mM NaCl, pH 7.8) containing protease and phosphatase inhibitors (37, 72). Whole cell lysates were electrophoresed through a 15% SDS polyacrylamide gel, transferred to a PVDF membrane (Gelman Sciences Inc.), and analyzed for SAP expression with a rabbit anti-human SAP polyclonal anti-serum (72). Detection of SHP-2 expression was used to demonstrate equivalent loading of cellular proteins. The membranes were developed using enhanced chemiluminescence (Pierce Biotechnology Inc.) and autoradiography (72).

*B cell cultures*. CFSE-labeled $CD27^-$ and $CD27^+$ B cells were cultured for 5 days in 48-well plates (BD) with CD40L (48), rIL-2 (50 U/ml), and rIL-10 (100 U/ml) or with CpG2006 (1 μg/ml; Progen) (52) alone, in the presence of $F(ab)'_2$ fragments of goat anti-human Ig (2.5 μg/ml; Jackson ImmunoResearch Laboratories Inc.), or with IL-2 and IL-10. In vitro–activated $CD27^+$ B cells were harvested and surface stained for expression of CD38 (48). The levels of secreted Ig in culture supernatants were determined using Ig heavy chain–specific ELISA (39).

*SHM*. RNA was extracted from sort-purified naive and memory B cells using the QIAGEN RNeasy Kit (QIAGEN), transcribed into cDNA and then used as a template to amplify Ig $V_H5$ genes by nested PCR using Pfu DNA polymerase (PerkinElmer). Primers (Sigma-Aldrich) for the initial PCR corresponded to the 5′ region of the $V_H5$ leader sequence (ATGGGGTCAACCGCCATCCT) and the 3′ Cμ constant region (GTCCTGTGCGAGGCAGCCAA). Primers for the second PCR were: 5′-CTCCTGGCTGTTCTCCAAGG and 3′-AGGAGACGGTGACCAGGGTT (39). PCR was performed using a DNA Engine Thermocycler (GeneWorks) for 35 cycles, with each cycle consisting of 1 minute denaturation at 94°C, 1 minute annealing at 55°C, and 1 minute extension at 72°C. Amplified PCR products were cloned into PCRblunt (Invitrogen Corp.) and transformed into TOP10F′ bacteria (Invitrogen Corp.). Individual clones were selected, and plasmid DNA was recovered and sequenced (SUPAMAC). Nucleotide sequences were analyzed and compared with germline Ig $V_H5$ region genes using Sequencher (Gene Codes Corporation).

*Immunohistology*. Tissue sections from different XLP patients were obtained either by the authors (K.E. Nichols and A.D. Klion) or through the XLP Registry. These were formalin fixed and paraffin embedded according to conventional procedures. Spleen sections were then stained with hematoxylin and eosin using standard procedures. Immunohistochemical studies were performed on sections using immunoperoxidase staining procedures after Ag retrieval (citrate buffer 10 mMol, pH 6.0, in a microwaveable pressure cooker) and automated immunostainers (Ventana Medical System Inc. and DakoCytomation), according to the manufacturers' instruction and as previously described (73). The Ab panel included CD20, CD3, IgD, and Bcl-6 (DakoCytomation) and CD27 (Novocastra). The double immunostains were performed sequentially using different peroxidase substrates diaminobenzidine (DAB) and Vector-VIP (Vector Laboratories) and the Envision Plus detection system (DakoCytomation).

*Statistics*. All statistics were performed using Prism software (version 3.0; GraphPad Software). ANOVA was performed to determine statistical significance between data sets. $P$ values of less than 0.05 were considered significant.

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1. Calame, K. 2001. Plasma cells: finding new light at the end of B cell development. *Nat. Immunol.* **2**:1103–1108.
2. Slifka, M.K., Antia, R., Whitmire, J.K., and Ahmed, R. 1998. Humoral immunity due to long-lived plasma cells. *Immunity.* **8**:363–372.
3. Jacob, J., Kassir, R., and Kelsoe, G. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* **173**:1165–1175.
4. Liu, Y.J., Zhang, J., Lane, P.J., Chan, E.Y., and MacLennan, I.C. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur. J. Immunol.* **21**:2951–2962.
5. Toellner, K.M., et al. 1996. Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B cell activation. *J. Exp. Med.* **183**:2303–2312.
6. McHeyzer-Williams, M.G., McLean, M.J., Lalor, P.A., and Nossal, G.J. 1993. Antigen-driven B cell differentiation in vivo. *J. Exp. Med.* **178**:295–307.
7. Liu, Y.J., et al. 1996. Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation. *Immunity.* **4**:241–250.
8. MacLennan, I.C. 1994. Germinal centers. *Annu. Rev. Immunol.* **12**:117–139.
9. Banchereau, J., and Rousset, F. 1992. Human B lymphocytes: phenotype, proliferation, and differentiation. *Adv. Immunol.* **52**:125–262.
10. Gulino, A.V., and Notarangelo, L.D. 2003. Hyper IgM syndromes. *Curr. Opin. Rheumatol.* **15**:422–429.
11. Grimbacher, B., Warnatz, K., and Peter, H.H. 2003. The immunological synapse for B-cell memory: the role of the ICOS and its ligand for the longevity of humoral immunity. *Curr. Opin. Allergy Clin. Immunol.* **3**:409–419.
12. Fu, Y.X., and Chaplin, D.D. 1999. Development and maturation of secondary lymphoid tissues. *Annu. Rev. Immunol.* **17**:399–433.

13. Crotty, S., Kersh, E.N., Cannons, J., Schwartzberg, P.L., and Ahmed, R. 2003. SAP is required for generating long-term humoral immunity. *Nature.* **421**:282–287.
14. Vinuesa, C.G., Tangye, S.G., Moser, B., and Mackay, C.R. 2005. Follicular B helper T cells in antibody responses and autoimmunity. *Nat. Rev. Immunol.* **5**:853–865.
15. Tangye, S.G., Liu, Y.J., Aversa, G., Phillips, J.H., and de Vries, J.E. 1998. Identification of functional human splenic memory B cells by expression of CD148 and CD27. *J. Exp. Med.* **188**:1691–1703.
16. Klein, U., et al. 1998. Somatic hypermutation in normal and transformed human B cells. *Immunol. Rev.* **162**:261–280.
17. Agematsu, K., et al. 1998. Absence of IgD-CD27(+) memory B cell population in X-linked hyper-IgM syndrome. *J. Clin. Invest.* **102**:853–860.
18. Weller, S., et al. 2001. CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc. Natl. Acad. Sci. U. S. A.* **98**:1166–1170.
19. Weller, S., et al. 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood.* **104**:3647–3654.
20. Razanajaona, D., et al. 1996. Somatic mutations in human Ig variable genes correlate with a partially functional CD40-ligand in the X-linked hyper-IgM syndrome. *J. Immunol.* **157**:1492–1498.
21. Chu, Y.W., et al. 1995. Somatic mutation of human immunoglobulin V genes in the X-linked HyperIgM syndrome. *J. Clin. Invest.* **95**:1389–1393.
22. Nichols, K.E., et al. 1998. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc. Natl. Acad. Sci. U. S. A.* **95**:13765–13770.
23. Coffey, A.J., et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat. Genet.* **20**:129–135.
24. Sayos, J., et al. 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature.* **395**:462–469.
25. Purtilo, D.T., Cassel, C.K., Yang, J.P., and Harper, R. 1975. X-linked recessive progressive combined variable immunodeficiency (Duncan's disease). *Lancet.* **1**:935–940.
26. Nichols, K.E., et al. 2005. Molecular and cellular pathogenesis of X-linked lymphoproliferative disease. *Immunol. Rev.* **203**:180–199.
27. Ma, C.S., et al. 2005. Impaired humoral immunity in X-linked lymphoproliferative disease is associated with defective IL-10 production by $CD4^+$ T cells. *J. Clin. Invest.* **115**:1049–1059. doi:10.1172/JCI200523139.
28. Sumegi, J., et al. 2000. Correlation of mutations of the SH2D1A gene and epstein-barr virus infection with clinical phenotype and outcome in X-linked lymphoproliferative disease. *Blood.* **96**:3118–3125.
29. Nichols, K.E., et al. 2005. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat. Med.* **11**:340–345.
30. Nagy, N., et al. 2000. SH2D1A and SLAM protein expression in human lymphocytes and derived cell lines. *Int. J. Cancer.* **88**:439–447.
31. Shlapatska, L.M., et al. 2001. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. *J. Immunol.* **166**:5480–5487.
32. Morra, M., et al. 2005. Defective B cell responses in the absence of SH2D1A. *Proc. Natl. Acad. Sci. U. S. A.* **102**:4819–4823.
33. Feldhahn, N., et al. 2002. Silencing of B cell receptor signals in human naive B cells. *J. Exp. Med.* **196**:1291–1305.
34. Wu, C., et al. 2001. SAP controls T cell responses to virus and terminal differentiation of TH2 cells. *Nat. Immunol.* **2**:410–414.
35. Czar, M.J., et al. 2001. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc. Natl. Acad. Sci. U. S. A.* **98**:7449–7454.
36. Liu, Y.J., et al. 1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity.* **2**:239–248.
37. Tangye, S.G., van de Weerdt, B.C., Avery, D.T., and Hodgkin, P.D. 2002. CD84 is up-regulated on a major population of human memory B cells and recruits the SH2 domain containing proteins SAP and EAT-2. *Eur. J. Immunol.* **32**:1640–1649.
38. Ehrhardt, G.R., et al. 2005. Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. *J. Exp. Med.* **202**:783–791.
39. Ellyard, J.I., et al. 2004. Ag-selected Ig-secreting cells persist in human spleen as well as bone marrow. *Blood.* **103**:3805–3812.
40. Klein, U., Kuppers, R., and Rajewsky, K. 1997. Evidence for a large compartment of IgM-expressing memory B cells in humans. *Blood.* **89**:1288–1298.
41. Agematsu, K., et al. 1997. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. *Eur. J. Immunol.* **27**:2073–2079.
42. Gagro, A., et al. 2003. Naive and memory B cells respond differentially to T-dependent signaling but display an equal potential for differentiation toward the centroblast-restricted CD77/globotriaosylceramide phenotype. *Eur. J. Immunol.* **33**:1889–1898.
43. Carsetti, R., Rosado, M.M., and Wardmann, H. 2004. Peripheral development of B cells in mouse and man. *Immunol. Rev.* **197**:179–191.
44. Amano, M., et al. 1998. CD1 expression defines subsets of follicular and marginal zone B cells in the spleen: beta 2-microglobulin-dependent and independent forms. *J. Immunol.* **161**:1710–1717.
45. Cuss, A.K., et al. 2006. Expansion of functionally immature transitional B cells is associated with human immunodeficient states characterised by impaired humoral immunity. *J. Immunol.* In press.
46. Sims, G.P., et al. 2005. Identification and characterization of circulating human transitional B cells. *Blood.* **105**:4390–4398.
47. Kruetzmann, S., et al. 2003. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J. Exp. Med.* **197**:939–945.
48. Tangye, S.G., Avery, D.T., and Hodgkin, P.D. 2003. A division-linked mechanism for the rapid generation of Ig-secreting cells from human memory B cells. *J. Immunol.* **170**:261–269.
49. Arpin, C., Banchereau, J., and Liu, Y.J. 1997. Memory B cells are biased towards terminal differentiation: a strategy that may prevent repertoire freezing. *J. Exp. Med.* **186**:931–940.
50. Kindler, V., and Zubler, R.H. 1997. Memory, but not naive, peripheral blood B lymphocytes differentiate into Ig-secreting cells after CD40 ligation and costimulation with IL-4 and the differentiation factors IL-2, IL-10, and IL-3. *J. Immunol.* **159**:2085–2090.
51. Jelinek, D.F., Splawski, J.B., and Lipsky, P.E. 1986. Human peripheral blood B lymphocyte subpopulations: functional and phenotypic analysis of surface IgD positive and negative subsets. *J. Immunol.* **136**:83–92.
52. Bernasconi, N.L., Traggiai, E., and Lanzavecchia, A. 2002. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science.* **298**:2199–2202.
53. McHeyzer-Williams, M.G., Nossal, G.J., and Lalor, P.A. 1991. Molecular characterization of single memory B cells. *Nature.* **350**:502–505.
54. Dunn-Walters, D.K., Isaacson, P.G., and Spencer, J. 1995. Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells. *J. Exp. Med.* **182**:559–566.
55. Liu, Y.J., Grouard, G., de Bouteiller, O., and Banchereau, J. 1996. Follicular dendritic cells and germinal centers. *Int. Rev. Cytol.* **166**:139–179.
56. Siekevitz, M., Kocks, C., Rajewsky, K., and Dildrop, R. 1987. Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. *Cell.* **48**:757–770.
57. Grimbacher, B., et al. 2003. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat. Immunol.* **4**:261–268.
58. Liu, Y.J., Oldfield, S., and MacLennan, I.C. 1988. Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones. *Eur. J. Immunol.* **18**:355–362.
59. White, H., and Gray, D. 2000. Analysis of immunoglobulin (Ig) isotype diversity and IgM/D memory in the response to phenyl-oxazolone. *J. Exp. Med.* **191**:2209–2220.
60. Soenawan, E., et al. 2004. Maintenance of long-term immunological memory by low avidity IgM-secreting cells in bone marrow after mucosal immunizations with cholera toxin adjuvant. *Vaccine.* **22**:1553–1563.
61. Toyama, H., et al. 2002. Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. *Immunity.* **17**:329–339.
62. Lindsten, T., et al. 1982. Immune deficiency in the X-linked lymphoproliferative syndrome. II. Immunoregulatory T cell defects. *J. Immunol.* **129**:2536–2540.
63. Matsumoto, M., et al. 1996. Affinity maturation without germinal centres in lymphotoxin-alpha-deficient mice. *Nature.* **382**:462–466.
64. Wang, Y., et al. 2000. Antigen persistence is required for somatic mutation and affinity maturation of immunoglobulin. *Eur. J. Immunol.* **30**:2226–2234.
65. Inamine, A., et al. 2005. Two waves of memory B-cell generation in the primary immune response. *Int. Immunol.* **17**:581–589.
66. de Vinuesa, C.G., et al. 2000. Germinal centers without T cells. *J. Exp. Med.* **191**:485–494.
67. Forster, R., et al. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell.* **87**:1037–1047.
68. Gardby, E., and Lycke, N.Y. 2000. CD19-deficient mice exhibit poor responsiveness to oral immunization despite evidence of unaltered total IgA levels, germinal centers and IgA-isotype switching in Peyer's patches. *Eur. J. Immunol.* **30**:1861–1871.
69. Tumanov, A., et al. 2002. Distinct role of surface lymphotoxin expressed by B cells in the organization of secondary lymphoid tissues. *Immunity.* **17**:239–250.
70. Gardby, E., Lane, P.J., and Lycke, N.Y. 1998. Requirements for B7-CD28 costimulation in mucosal IgA responses: paradoxes observed in CTLA4-H gamma 1 transgenic mice. *J. Immunol.* **161**:49–59.
71. Shaffer, A.L., Rosenwald, A., and Staudt, L.M. 2002. Lymphoid malignancies: the dark side of B-cell differentiation. *Nat. Rev. Immunol.* **2**:920–932.
72. Tangye, S.G., Nichols, K.E., Hare, N.J., and van de Weerdt, B.C. 2003. Functional requirements for interactions between CD84 and Src homology 2 domain-containing proteins and their contribution to human T cell activation. *J. Immunol.* **171**:2485–2495.
73. Quintanilla-Martinez, L., et al. 1998. Mantle cell lymphomas lack expression of p27Kip1, a cyclin-dependent kinase inhibitor. *Am. J. Pathol.* **153**:175–182.