Proliferation and differentiation in isogenic populations of peripheral B cells activated by Epstein–Barr virus or T cell-derived mitogens

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Human B cells isolated from peripheral blood were activated and induced to proliferate by either Epstein–Barr virus (EBV) or the T cell-derived mitogens CD40 ligand (CD40L) plus interleukin (IL)-4. Although both populations initially proliferated as B-blasts, significant differences were revealed over a longer period. EBV infection resulted in continuously proliferating lymphoblastoid cell lines (LCLs), whereas most of the CD40L/IL-4-stimulated B cells had a finite proliferative lifespan of 3–4 weeks. Cell cycle analysis, trypan blue staining and Western blot analysis for cleavage of poly(ADP-ribose) polymerase (PARP) all demonstrated that the decrease in proliferation in CD40L/IL-4-stimulated B cells is not due to cell death. Instead, these cells arrest, accumulate in G0/G1 and undergo alterations in cell surface marker expression, cellular morphology and immunoglobulin production, all consistent with plasmacytoid differentiation. In contrast, B cells infected with EBV continued to proliferate and retained a blast-like phenotype. Differences in both cytokine production and the expression of cell cycle regulators were identified between the two B-cell populations, which might contribute to the differentiation of the CD40L/IL-4-stimulated B cells and suggest potential mechanisms by which EBV may overcome this. The study has also identified a window of opportunity during which a comparison of isogenic populations of EBV- and mitogen-driven B blasts can be made.

INTRODUCTION

The B-cell compartment in peripheral blood consists of quiescent naïve (70–80 %) and memory (20–30 %) B cells (Agematsu, 2000; Klein et al., 1997, 1998). In vitro, Epstein–Barr virus (EBV) has the ability to infect resting primary B cells, driving them into the cell cycle and sustaining their proliferation as continuously growing, B-blast-like, lymphoblastoid cell lines (LCLs) (Henle et al., 1967; Kieff & Rickinson, 2001; Pope, 1967). LCLs have been extensively studied to identify the mechanisms utilized by EBV in B-cell transformation; however, comparison with proliferating normal B cells is required to dissect EBV-specific effects on cell cycle entry and proliferation.

In vitro, CD4+ T cells, activated by specific antigen, express CD40 ligand (CD40L/CD154) (Armitage et al., 1992; Casamayor-Palleja et al., 1995; Lane et al., 1992; Noelle et al., 1992), which binds to CD40 on B cells. This interaction, in concert with B cell receptor (BCR) cross-linking and the action of T cell-derived cytokines interleukin (IL)-2, IL-5 and particularly IL-4, stimulates B-cell activation and proliferation (Croft & Swain, 1991; Gordon & Pound, 2000; Lohoff et al., 1992). CD40L is also a critical mediator of B-cell differentiation (Gordon & Pound, 2000; Schonbeck & Libby, 2001; van Kooten & Banchereau, 2000), which either involves formation of a germinal centre, producing either plasma cells (PCs) or memory cells, or direct differentiation of naïve B cells into PCs (Staudt & Brown, 2000). EBV is thought to mimic T cell-derived signals to stimulate B-cell proliferation and utilize B-cell differentiation to gain access to its in-vivo site of persistence, the memory B cell (Thorley-Lawson, 2001).

J. Banchereau and colleagues established a system of sustainable B-cell proliferation in vitro by mimicking T cell-dependent stimulation. They demonstrated that CD40 stimulation with monoclonal antibodies could induce primary B cells to proliferate in culture and these blast-like, EBV-free B-cell lines could be maintained for 10 weeks (Banchereau et al., 1991; Banchereau & Roussel, 1991; Roussel et al., 1995). Subsequent reports indicated IL-10, but not IL-4, could induce differentiation in CD40-stimulated B cells (Roussel et al., 1995) but that B cells stimulated with CD40L in conjunction with IL-4 do not maintain indefinite growth (Jung et al., 2001; Shvarts et al., 2002; Urashima et al., 1996). Current literature describing CD40 stimulation of B cells contains a number of contradictions, with studies reporting that CD40 ligation mediates differentiation into both memory and PCs (Arpin et al., 1995; Garrone et al., 1995; Jego et al., 2001; McCloskey et al., 1999; Urashima et al., 1996; Worm et al., 1998) or blocks differentiation in vitro (Callard et al., 1995;
Randall et al., 1998). Therefore, mitogenic stimulation of peripheral primary B cells in vitro required further characterization if they were to be compared with EBV-infected isogenic cells. Here, an analysis of cell surface phenotype, cellular morphology and Ig production, revealed that these mitogen-stimulated primary B cells are blast-like 7–14 days after initial stimulation. However, CD40L/IL-4-stimulated cells ceased to proliferate after a period of 3–4 weeks and showed features consistent with plasmacytoid differentiation, whereas EBV-infected cells proliferated well beyond this.

**METHODS**

**Cell isolation, culture and infection with EBV.** Primary B cells were isolated from mixed-donor platelet-depleted buffy coat residues (North London Blood Transfusion Service) by positive selection, using anti-CD19 monoclonal antibody (mAb)-coated magnetic beads (M450 pan-B Dynabeads and Detachabeads (Dynal)) as described previously (Cannell et al., 1996; Spender et al., 1999). Flow cytometric analysis was performed using fluorescein isothiocyanate (FITC)-conjugated anti-CD20 mAbs (Dako) to monitor cell purity. Purified B cells were incubated for 36 h at 37 °C before stimulation or infection, then passed in 10% (v/v) fetal calf serum (FCS)/RPMM 1640 medium (Gibco Invitrogen) at 37 °C.

For CD40 stimulation, 10^7 primary B cells were cultured with a layer of 1:5 × 10^6 gamma-irradiated (50 Gy) CD40L^+^-L cells (Garrone et al., 1995) and 10 U ml^-1 human recombinant IL-4 (R&D). L292 and LTK^-^ cells (lacking CD40L) were used as controls. Anti-IgM mAb (BU.1 ascites) (Greipp et al., 1985) was used at 1:2000 dilution. EBV purified by ultrafiltration from B95-8 cells was used to infect primary B cells as previously described (Spender et al., 1999; Wade & Allday, 2000).

**Cell proliferation and viability.** B cells (2 × 10^5) were pulse-labelled for 2 h with 1 μCi [^3]Hthymidine, collected onto a glass-fibre filter using a semiautomatic cell harvester (Skatron) and scintillations counted using a Beckman LS3801 scintillation counter. Cell viability was monitored by trypan blue staining.

**Flow cytometry.** Antibodies used for surface antigen analysis were: FITC-conjugated mAbs to CD19 (HD37), CD20, CD23 (MHM6), CD38 (AT 13/5) (Dako), mAbs to CD27 (LT-27) and CD138 (B-B4) (Cymbus Biotechnology Ltd) and mAb to CD40 (B-B20) (Serotec). CD38 (AT 13/5) (Dako), mAbs to CD27 (LT-27) and CD138 (B-B4) (Cymbus Biotechnology Ltd) and mAb to CD40 (B-B20) (Serotec). B cells (5 × 10^5) were incubated with primary antibody in 1% BSA/0.1% sodium azide/PBS on ice for 1 h. For unconjugated Abs, B cells were subsequently incubated with FITC-conjugated goat anti-mouse Ab (Dako) for 1 h.

To analyse cell cycle distribution, 2 × 10^6 B cells were fixed in 80% ethanol, incubated in PI solution [PBS containing 18 μg propidium iodide (PI) ml^-1 and 8 μg RNase A ml^-1 (Sigma Aldrich)] at 4 °C for 1 h before flow cytometric analysis as previously described (Parker et al., 1996; Wade & Allday, 2000). To quantify cells in S phase, cells were incubated with 10 μM 5-bromo-2′-deoxyuridine (Brdu) (Sigma Aldrich) for 1 h at 37 °C, fixed in 80% ethanol and co-stained with FITC-conjugated anti-Brdu mAb and PI (Recton Dickinson) (Wade & Allday, 2000).

**Immunofluorescence and analysis of cellular morphology.** Cytospins of 8 × 10^5 B cells were fixed in methanol/acetic acid (1:1) and rehydrated in PBS. For immunofluorescent detection of Ig or EBV nuclear antigen (EBNA) leader protein (EBNA-LP) (EBNA-LP), cytopsin were blocked with 1% BSA/PBS and incubated with FITC-conjugated anti-human IgG or IgG/A/M Ab (Dako) for 1 h or anti-EBNA-LP mAb (JF186) (Finke et al., 1987) followed by FITC-conjugated goat anti-mouse Ab. Stained slides were washed in PBS, mounted in Citifluor and analysed by fluorescence microscopy. To examine cellular morphology, cytopsin were stained with Giemsa stain (Accustain; Sigma Diagnostics).

**SDS-PAGE and Western blot analysis.** Protein extraction using radioimmunoprecipitation (RIPA) lysis buffer, protein quantification and Western blotting were performed as described previously (Wade & Allday, 2000). Membranes were immunoprobed with the following: mAbs against p21 (WAF1/CIP1) (SX118) and p53 (DO-1) were kindly gifts from Xin Lu; mAbs against Bcl-2 (Dako), cdk6 (C-21) (Santa Cruz), cyclin D2 (G132-4) (BD PharMingen), p18^INK4C (Ab-3) (Neomarkers) and polyclonal antibodies (pAbs) to p14^ARF (Ab-1) (Oncogene), p21^WAF1/CIP1 (N-20) (Santa Cruz) and poly(ADP-ribose)-polymerase (PARP) (Roche). Each Ab was used in accordance with the instructions of the supplier. Secondary Abs were rabbit anti-mouse Ig (Dako) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Dako). Bound proteins were visualized by enhanced chemiluminescence (Amersham Biosciences).

**Immunoglobulin and cytokine ELISAs.** Secreted human IgG, IgM, IgA and IgE were measured by sandwich ELISA. Briefly, Maxisorp Nunc-Immuno ELISA plates were coated with the appropriate capture pAbs (Dako) diluted in 0.16% (v/v) Na_2CO_3, 0.30% (v/v) NaHCO_3, pH 9.6 and incubated with serially diluted B-cell tissue culture supernatants for 2 h at 37 °C in a damp chamber. Plates were washed in PBS/0.05% Tween 20 and incubated with HRP-conjugated anti-IgG or IgG/A/M (Dako) antibody diluted in 10% FCS/PBS for 1 h. ELISAs were developed using O-phenylenediamine (OPD)/H_2O_2 substrate solution, terminated with 2 M H_2SO_4 and the absorbance read at 492 nm (Anthos Labtec Instruments). Serially diluted, purified human IgG, IgM, IgA (all from Sigma) and IgE (Serotec) were used to generate standard curves. To assay secreted human IL-6 and IL-10 in supernatants, human IL-6 or IL-10 OPTEIA ELISA kit II (BD PharMingen) was used, following manufacturer’s instructions.

**RESULTS**

**Primary B cells infected with EBV have a greater proliferative lifespan than those stimulated with the mitogens CD40L and IL-4**

The proliferation of primary B cells ( > 95% CD20^-^) was monitored during continuous stimulation with irradiated CD40L^+^-L cells and 10 U IL-4 (CD40L/IL-4) ml^-1 or after infection with EBV. Stimulation with CD40L/IL-4 or EBV both induced DNA synthesis as evidenced by an increase in the levels of incorporated [^3]Hthymidine (Fig. 1A). Dual-parameter flow cytometric analysis performed on BrdU-labelled B cells co-stained with PI 5 days after initial stimulation (Fig. 1B) confirmed that both stimuli induced cell cycle progression and that similar percentages of EBV-infected and CD40L/IL-4-treated cells were in the S phase (BrdU^-^) after 5 days (10-2 and 11-3 % respectively). Due to the stoichiometric nature of PI binding to DNA, apoptosis produces cells with a sub-G_1 DNA content (Nicoletti et al., 1991; Ormerod et al., 1993; Walker et al., 1991). Fig. 1(B) shows a proportion of EBV-infected B cells have a sub-G_1 DNA content at 5 days (not detected in CD40L/IL-4-stimulated cells), indicating apoptosis is occurring, consistent with other reports (Allday et al., 1995). The majority of CD40L/IL-4-stimulated cells expressed the B-cell marker CD20 (96%) and the activated B-cell marker CD23 (76%)
Fig. 1. Proliferation induced by EBV or mitogenic stimulation. (A) Primary B cells were infected with B95.8 EBV (◇), co-cultured with irradiated CD40L+L cells and 10 U ml⁻¹ IL-4 (□), or irradiated LTK cells. Equal numbers of cells were harvested at the indicated times after stimulation, pulsed with 1 μCi [³H]thymidine for 2 h and the levels of incorporated [³H]thymidine assayed as a measure of cellular proliferation. (B) After 5 days, equal numbers of cells from each experimental condition were pulsed with 10 μM 5-bromo-2'-deoxyuridine (BrdU) for 1 h and co-stained with an FITC-conjugated anti-BrdU Ab to measure BrdU incorporation and PI to show DNA content. All samples were analysed by flow cytometry. In each case the y-axis shows the fluorescence intensity and the x-axis shows DNA content. A schematic diagram depicts asynchronously growing cells labelled with BrdU and the distinct cell cycle phases detectable with this technique: a, unlabelled cells in G₀/G₁; b, labelled cells in early–mid S phase; c, labelled cells in late S phase/G₂/M; d, unlabelled cells in G₂/M; and e, cells with a < G₁ DNA content. (C) [³H]Thymidine incorporation was determined at the indicated intervals from 3 to 19 days post-stimulation. B cells infected with B95.8 EBV (○), co-cultured with irradiated CD40L+L cells and 10 U IL-4 ml⁻¹ (◇); untreated B cells (□) and those co-cultured with L cells that do not express CD40L (L929) (△) are included as controls. (D) Addition of anti-IgM mAb (BU.1) (◇) did not affect [³H]thymidine incorporation by CD40L/IL-4-stimulated (□) B cells.
after 5 days in culture, confirming that DNA synthesis measured was due to the outgrowth of B-blasts, not a minor contaminating population (data not shown). Isogenic B cells co-cultured with L929 or LTK cells, which lack CD40L, or left untreated did not proliferate (Fig. 1A, B, C).

Fig. 1(C) demonstrates that mitogen- or virus-driven primary B cells have distinctly different proliferative capacities. CD40L/IL-4-stimulated cells rapidly proliferated in the first 7 days, then slowed, before proliferation decreased significantly between 13–17 days until almost ceasing by 19 days (Fig. 1C). No recovery in proliferation after this period was detected (data not shown). In contrast, EBV-infected cells continued to proliferate throughout the time-course. Fig. 1(C) is representative of six independent experiments (summarized in Table 1), in which CD40L/IL-4-stimulated B cells underwent three distinct phases of growth; an ‘exponential phase’ lasting on average 7–10 days, a ‘plateau phase’ until 14–16 days and a ‘decline phase’ up to around 21 days. Some variation in \(^{3}H\)thymidine incorporation levels occurs between different experiments, probably due to batch variability between buffy coat residues.

EBV-encoded gene products, latent membrane protein (LMP)1 and LMP2, mimic the CD40 receptor and the BCR respectively (Caldwell et al., 1998, 2000; Gires et al., 1997; Thorley-Lawson, 2001) and cross-linking the BCR with anti-IgM has been reported to enhance CD40-mediated proliferation (Lam et al., 1999, 2000). However, we observed no long-term proliferative advantage by anti-IgM-mediated BCR cross-linking of CD40L/IL-4-stimulated B cells (Fig. 1D).

### Table 1. Proliferation of primary B cells following continuous stimulation with CD40L/IL-4

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Exponential/Plateau/Decline/</th>
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<th>2 weeks</th>
<th>3 weeks</th>
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<tr>
<td>1</td>
<td>68 619 (100 %)</td>
<td>41 474 (60-44 %)</td>
<td>2 861 (4-17 %)</td>
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<tr>
<td>2</td>
<td>41 910 (100 %)</td>
<td>36 064 (86-05 %)</td>
<td>7 602 (18-15 %)</td>
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</tr>
<tr>
<td>3</td>
<td>11 722 (100 %)</td>
<td>10 105 (86-21 %)</td>
<td>540 (4-60 %)</td>
<td></td>
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<tr>
<td>4</td>
<td>18 144 (100 %)</td>
<td>14 415 (79-45 %)</td>
<td>1 330 (7-33 %)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>37 073 (100 %)</td>
<td>26 064 (70-30 %)</td>
<td>1 866 (5-03 %)</td>
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<tr>
<td>6</td>
<td>43 754 (100 %)</td>
<td>36 429 (128 %)</td>
<td>8 375 (19-16 %)</td>
<td></td>
</tr>
</tbody>
</table>

In light of this, flow cytometric analysis was performed on PI-stained cells to investigate the cell cycle distribution of primary B cells following CD40L/IL-4 stimulation over 21 days (Fig. 2C). Isogenic EBV-infected cells, which proliferate continuously, were included for comparison. After 7 days, CD40L/IL-4- and EBV-driven B cells had cell distributions throughout the cycle, with the majority in G1, typical of asynchronous, proliferating B cell cultures. The mitogenically stimulated cells showed comparable PI profiles after 7 and 14 days (when \(^{3}H\)thymidine uptake is high but had plateaued); however, by 21 days (when \(^{3}H\)thymidine incorporation has decreased significantly), the G1 compartment increased from 62.8 % (7 days) to 77.9 % (21 days), concomitant with a decrease in cells in S (17.3 % to 8.8 %) and G2/M (16.4 % to 8.8 %), suggesting the cells were unable to re-enter S phase. No difference was observed in the EBV-driven B cells in 7–21 days. Furthermore, no increase in sub-G1 cells was detected after CD40L/IL-4 stimulation for 21 days, confirming no apoptosis had occurred. Instead, a correlation exists between G1 accumulation and the reduction in proliferation seen in CD40L/IL-4-stimulated B cells.

### Stimulation of primary B cells with CD40L/IL-4 does not result in cell death but ultimately in cell cycle exit

To determine whether the decrease in \(^{3}H\)thymidine incorporation consistently observed in CD40L/IL-4-stimulated B cells was due to cell death, the growth characteristics of these cells were investigated over 19 days (Fig. 2A). As before, B-cell proliferation reached a peak and plateaued before declining towards baseline levels. The total number of cells increased following CD40L/IL-4 stimulation and there was no significant decrease in cell viability. Western blotting for PARP cleavage, which is a marker of apoptosis, also showed no correlation between cell death and proliferative decline (Fig. 2B).

Dual parameter flow cytometric analysis was performed on cells pulsed with 10 \(^{3}H\)thymidine and stained for DNA content with PI prior to analysis of the cell cycle profile by flow cytometry. The \(y\)-axis shows relative cell number and the \(x\)-axis shows DNA content. B cells stimulated with CD40L and IL-4 accumulate in G1 over time, whereas EBV-driven B cells retain a profile consistent with an asynchronous population. (B) Equal amounts of protein extracted from CD40L/IL-4-stimulated and EBV-infected B cells over 3 weeks were analysed by SDS-PAGE and Western blotting for caspase-mediated proteolytic cleavage of PARP. The levels of cleaved PARP do not increase in either B cell population after 3 weeks. Bcl-2 is included as a control to demonstrate equal protein loading. (C) Cells were stained for DNA content with PI prior to analysis of the cell cycle profile by flow cytometry. The \(y\)-axis shows relative cell number and the \(x\)-axis shows DNA content. B cells stimulated with CD40L and IL-4 accumulate in G1 over time, whereas EBV-driven B cells retain a profile consistent with an asynchronous population. (D) Cells were harvested 7 and 21 days after the initial stimulation or infection. Dual parameter flow cytometric analysis was performed on cells pulsed with 10 \(\mu\)M BrdU for 1 h and then co-stained with FITC-conjugated anti-BrdU for BrdU incorporation into cellular DNA and PI for DNA content. After 21 days, BrdU incorporation has significantly declined in the CD40L/IL-4-stimulated cells but not in the EBV-infected population.

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Fig. 2. Primary B cells cease to proliferate and exit the cell cycle after prolonged stimulation with CD40L/IL-4. (A) B cells were harvested during co-culture with CD40L and IL-4. Cell proliferation was determined by \(^{3}H\)thymidine incorporation, the total number of cells in the population was counted and the proportion of dead cells was estimated by staining with trypan blue. (B) Equal amounts of protein extracted from CD40L/IL-4-stimulated and EBV-infected B cells over 3 weeks were analysed by SDS-PAGE and Western blotting for caspase-mediated proteolytic cleavage of PARP. The levels of cleaved PARP do not increase in either B cell population after 3 weeks. Bcl-2 is included as a control to demonstrate equal protein loading. (C) Cells were stained for DNA content with PI prior to analysis of the cell cycle profile by flow cytometry. The \(y\)-axis shows relative cell number and the \(x\)-axis shows DNA content. B cells stimulated with CD40L and IL-4 accumulate in G1 over time, whereas EBV-driven B cells retain a profile consistent with an asynchronous population. (D) Cells were harvested 7 and 21 days after the initial stimulation or infection. Dual parameter flow cytometric analysis was performed on cells pulsed with 10 \(\mu\)M BrdU for 1 h and then co-stained with FITC-conjugated anti-BrdU for BrdU incorporation into cellular DNA and PI for DNA content. After 21 days, BrdU incorporation has significantly declined in the CD40L/IL-4-stimulated cells but not in the EBV-infected population.
cells. Analysis of BrdU-labelled/PI-stained cells demonstrated that the number of cells in S phase was reduced in these cells; BrdU$^+$ cells fell from 24% (7 days) to 8% (21 days), in contrast to the EBV-infected cells, which continued to cycle (22% and 19% BrdU$^+$ after 7 and 21 days respectively) (Fig. 2D).

The effect of prolonged CD40L/IL-4 stimulation on B-cell surface antigen expression

In vitro, continued signalling through CD40 is required to maintain proliferation (Banchereau et al., 1991; data not shown). Therefore, we investigated whether loss of CD40 expression could account for the decrease in proliferation
observed. Expression of CD40 on B cells stimulated with CD40L/IL-4 for 26 days was analysed by flow cytometry at intervals corresponding to the different phases of exponential proliferation, plateau and decline (Fig. 3A). CD40 expression appears to be down-regulated, as the mean fluorescence decreased from 463 (10 days) to 210 (15 days) before falling to 98 (26 days). EBV-infected B cells were also monitored and CD40 expression was consistently higher at each time-point than in CD40L/IL-4-stimulated cells.

CD40 is a pan B-cell marker found on all B-cell subtypes except terminally differentiated PCs. During differentiation, B cells undergo sequential alteration of surface antigen expression and finally exit the cell cycle. As we had already demonstrated that CD40L/IL-4-stimulated cells arrest, we asked whether they were differentiating. The expression of surface markers was examined after 10 (high proliferation) and 26 (low proliferation) days co-culture with CD40L/IL-4 (Fig. 3B) and compared to isogenic EBV-infected B cells at 26 days (as a proliferating B-blast control) and resting B cells (day 0). Unstimulated B cells displayed a characteristic phenotype; CD20⁻, CD19⁻ and CD38⁺ (pan B-cell markers) but CD23⁺ (activation marker). Ten days after CD40L/IL-4 stimulation, expression of pan B-cell markers was decreased but retained and the cells were now CD23⁺ and activated. CD40L/IL-4 stimulation consistently induced greater expression of CD23 than EBV infection, probably due to synergistic up-regulation of the CD23 promoter by both CD40L and IL-4 (Richards & Katz, 1997). After 26 days, CD23 was down-regulated more than twofold on CD40L/IL-4-stimulated B cells, indicating they were losing activated status. Furthermore, CD20 and CD19 were also down-regulated, whereas, EBV-infected cells expressed these markers at a level similar to CD40L/IL-4-stimulated cells at 10 days, indicating both these populations are blast-like. CD38 expression remained unchanged on CD40L/IL-4-stimulated B cells. The memory B-cell marker, CD27, was lost and the PC-specific antigen, CD138, was never detected in either population. Therefore, CD40L/IL-4-stimulated B cells initially became B-blast-like, but then down-regulated all B-cell markers tested save CD38. This occurred concurrently with cell cycle arrest and was not seen in EBV-driven B cells.

### CD40L/IL-4 stimulation induces plasmacytoid-like morphology and immunoglobulin (Ig) production in primary B cells

Plasma cells are morphologically distinct, CD20⁻, CD38⁺, immunoglobulin-producing B cells (Harada et al., 1993; Jego et al., 2001). The effects of EBV infection and CD40L/IL-4 stimulation on the morphology and cytoplasmic Ig production of primary B cells was investigated (Fig. 4). At 7 days, the morphology of CD40L/IL-4-stimulated B cells is similar to the EBV-infected cells, with both displaying a blast-like appearance of high nucleus/cytoplasm ratio, covered in microvilli-like projections and existing in tight aggregations (Fig. 4A; upper and lower panels) (Janeway et al., 2001; Nilsson, 1979; Nilsson & Ponten, 1975). In contrast, at 24 days, CD40L/IL-4-stimulated B cells were larger, more ovoid, with a lower nucleus/cytoplasm ratio, fewer cell projections (Fig. 4A; middle panels) and forming looser clumps in culture. These features are characteristic of plasmacytes, which contain an extended Golgi network in order to produce antibodies.

The amount of cytoplasmic Ig increased in the mitogenically stimulated cells over time (Fig. 4B); cells examined at 7 and 15 days showed low levels of cytoplasmic Ig whereas after 26 days many cells had much higher levels concentrated in a perinuclear cuff, consistent with the polarized distribution of cytoplasm seen in Fig. 4(A). In contrast, 26 days after EBV infection, B cells had varying but generally low levels of cytoplasmic Ig, distributed throughout the cytoplasm, similar to the pattern seen in CD40L/IL-4-stimulated cells after 7 and 15 days and established LCLs, indicating this is typical of B-blasts. These experiments were repeated using a pan-specific anti-human Ig antibody, which yielded comparable results and in both cases an isotype-matched control showed no fluorescence (data not shown).

The level of Ig secreted by CD40L/IL-4- and EBV-driven B cells was assayed over a period of 28 days (Fig. 4C). All four Ig isotypes (M, G, A and E) were detected in supernatant from both CD40L/IL-4-stimulated and EBV-infected cultures from 7–12 days post stimulation, with IgM predominant. In mitogen-driven cultures, secreted Ig accumulated to high levels by 23 days and continued to accumulate up to 28 days post-stimulation; however, Ig concentration peaked

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**Fig. 3.** Expression of surface antigens on primary B cells stimulated with CD40L/IL-4. (A) Primary B cells from the same starting population were either infected with EBV or stimulated by CD40L/IL-4 and stained at the indicated times with an anti-CD40 mAb followed by an FITC-conjugated goat anti-mouse Ab. Stained cells were analysed by flow cytometry for surface expression of CD40 and representative results are shown. The mean fluorescence of stained cells (top right-hand corner of each profile) indicates the levels of positive staining in the populations, showing that surface expression of CD40 is down-regulated following co-culture with CD40L and IL-4. (B) Ten and 27 days after stimulation with CD40L/IL-4, primary B cells were stained for the expression of the pan B-cell markers CD20, CD19 and CD38, the B-cell activation marker CD23, the memory B-cell marker CD27 and the plasma cell (PC) marker CD138. The stained cells were analysed by flow cytometry and the mean fluorescence calculated. EBV-infected B cells harvested after 27 days are shown as representative of B-cell surface marker expression following infection with EBV. The levels of expression of all the markers analysed on the surface of untreated, resting B cells (day 0) is included for comparison.
at 23 days and began to decline in the supernatant of virally driven B cells, consistent with previously reported patterns of Ig secretion following EBV infection of primary B cells (Kataoka et al., 1997; Nilsson et al., 1971). It can be assumed that Ig secretion in EBV-infected B cells continues to decline after 28 days, as culture supernatants of two newly established LCLs contained much lower Ig levels (0.004–0.010 μg IgM/10^5 cells and 1.038–1.150 μg IgG/10^5 cells). Therefore, CD40L/IL-4-stimulated cells continue to secrete all isotypes beyond 23 days in culture, whereas Ig secretion peaks in EBV-infected cells at around 20 days.

**B cells induced to differentiate with CD40L/IL-4 can be infected but not immortalized with EBV**

To determine whether CD40L/IL-4-mediated proliferative decline could be rescued by EBV, B cells stimulated with CD40L/IL-4 for 19 days were infected with EBV and returned to culture with CD40L/IL-4. Cytospins prepared after 48 h were stained with an anti-EBNA-LP mAb (Fig. 5A). The presence of positive staining for EBNA-LP (≈25%) demonstrated that CD40L/IL-4-stimulated B cells could be successfully infected after 19 days. This was confirmed by Western blot analysis, showing that EBNA-LP is only expressed in infected cultures (Fig. 5B). As expected, proliferation continued at a low level in the uninfected CD40L/IL-4-stimulated cells but, surprisingly, in the infected population, proliferation decreased dramatically 2 days post-infection and completely ceased by 7 days (Fig. 5C). Therefore, EBV is unable to induce proliferation in these cells and override the differentiation-associated halt in proliferation in primary B cells stimulated with CD40L/IL-4.

**The effect of CD40L/IL-4 stimulation and EBV infection on cytokine production**

Cytokines continuously influence B-cell development. IL-6 and IL-10 are both autocrine and paracrine B-cell growth factors produced by activated (including EBV-infected) B cells (Burdin et al., 1995, 1997; Nakagomi et al., 1994; Tosato et al., 1990) and induce plasmacytoid differentiation and Ig secretion (Chen-Kiang, 1995; Rouset et al., 1995; Smeland et al., 1989; Urashima et al., 1996). ELISAs were performed to determine whether IL-6 or IL-10 are involved in the differential response of primary B cells following CD40L/IL-4 stimulation or EBV infection. Fig. 6 shows that IL-6 and IL-10 are produced in response to both stimuli; however, the levels and patterns of cytokine production differ in the two populations. The concentration of IL-10 is comparable at 7 days and peaks at 23 days in both cultures, but EBV infection induced approximately sevenfold more IL-10 secretion than did CD40L/IL-4 stimulation. Conversely, CD40L/IL-4 stimulation gave a greater production of IL-6 than EBV infection at all time-points assayed.
(Fig. 6B). These data suggest that IL-6, but probably not IL-10, plays an autocrine role in CD40L/IL-4-mediated differentiation.

The differences in cytokine production between CD40L/IL-4- or EBV-stimulated isogenic B cells raised the possibility that EBV infection produces a soluble factor capable of extending the replicative lifespan of mitogenically stimulated B cells. We therefore compared growth of primary B cells cultured with CD40L/IL-4 and 50% (v/v) LCL culture supernatant, or CD40L/IL-4 alone, and saw no enhancement of proliferation; in fact a negative effect was seen (Fig. 6C). This suggests there are no secreted factors responsible for the extended proliferation of EBV-infected cells that could be used to extend mitogen-induced proliferation.

**The effect of chronic CD40L/IL-4 stimulation on the expression of key cell cycle regulatory proteins**

The above data show that prolonged CD40L/IL-4 stimulation induces phenotypic changes in primary B cells consistent with differentiation, accompanied by cell cycle arrest in G1/G0. Protein extracts were prepared from CD40L/IL-4-stimulated B cells at intervals between 10 and 22 days and G1 regulatory proteins were analysed by Western blot (Fig. 7A). Extracts from isogenic, EBV-infected cells at 15 or 22 days were included for comparison as non-differentiating, EBV-positive B-blasts. The proliferation of these two populations is shown in Fig. 7(B). Normal B cells possess functional p53, which accumulates in response to stress (Allday *et al*., 1995; O’Nions & Allday, 2003; Vousden & Lu, 2002). Once the cells were activated, no change in the level of expression of p53 was detected in either EBV-infected or CD40L/IL-4-stimulated cells, consistent with differentiation, rather than stress, triggering the arrest observed after chronic CD40L/IL-4 stimulation. The expression of cyclin-dependent kinase inhibitors (CKIs), which regulate cell cycle progression and mediate G1 arrest, was also investigated. No variation in the level of p27KIP1 was detected in either population; however, p21WAF1/CIP1 accumulated in CD40L/IL-4-stimulated B cells after prolonged culture. A slower migrating band (indicated by * in Fig. 7A) was detected after 15 days using a pAb raised against the N-terminus of p21WAF1/CIP1, which could represent a modification to p21WAF1/CIP1.

p18INK4C is reported to be up-regulated during B-cell differentiation (Morse *et al*., 1997; Schrantz *et al*., 2000; Tourigny *et al*., 2002). An increase in p18INK4C was detected in CD40L/IL-4-stimulated B cells after 10 days post-stimulation. p16INK4A, another key INK4 CKI, was not detected in either population with the available antibodies (data not shown); however, the alternative protein expressed from the INK4A locus, p14ARF, showed a clear increase in CD40L/IL-4-stimulated cells by day 22. In addition, cyclin D2 increased by day 18 in CD40L/IL-4-stimulated B cells, but no increase occurred in its binding partner cdk6. No increase in any of the proteins investigated was detected in EBV-infected B cells (right-hand panel, Fig. 7A). Levels of Bcl-2 did not change in either population, indicating equal protein loading.
DISCUSSION

Banchereau and colleagues reported that EBV-free B-cell lines could be established by stimulation with anti-CD40 and IL-4 and maintained for at least 10 weeks (Banchereau et al., 1991; Banchereau & Rousset, 1991; Rousset et al., 1995); however, the mechanism behind the subsequent arrest in proliferation was not described. Our observation of a progressive reduction in proliferation after 3–4 weeks of continuous CD40L/IL-4 stimulation is consistent with more recently published studies reporting that primary B cells proliferate for less than 40 days following treatment with these mitogens (Jung et al., 2001; Shvarts et al., 2002).

Here we show that prolonged stimulation of primary B cells with CD40L/IL-4 can be characterized by three individual phases; a period of exponential proliferation, a 'plateau phase' and a final decline. No correlation was found between this decline and cell death; instead, flow cytometric analysis indicated that the decrease in proliferation corresponded to an arrest in G0/G1. Furthermore, during the decline phase, CD40L/IL-4-stimulated B cells showed increased synthesis of Ig and changes in morphology and cell surface phenotype consistent with differentiation from blastoid towards plasmacytoid, antibody-secreting cells. This is consistent with studies showing B-cell differentiation coupled to cell cycle arrest, including a recent study reporting that B cells chronically stimulated with CD40L/IL-4 show progressive decrease in proliferation concurrent with Ig accumulation (Altmeyer et al., 1997; Morse et al., 1997; Jung et al., 2001). Importantly, these changes were largely absent from the EBV-driven population, which maintained a blast-like phenotype and sustained proliferation.

Prolonged co-culture with CD40L/IL-4 resulted in down-regulation of all surface markers analysed, except CD38. During PC generation, B cells pass through pre-plasmablast (CD20\(^\pm\), CD38\(^\pm\), CD138\(^-\)), plasmablast (CD20\(^-\), CD38\(^++\), CD138\(^-\)) and early PCs (CD20\(^-\), CD38\(^+++\), CD138\(^++\)) (Jego et al., 2001). However, following prolonged culture with CD40L/IL-4, the expression of CD38 or CD138 was not increased, indicating that CD40L/IL-4 stimulation induces activation and differentiation of primary B cells to the plasmablast stage but that additional signals are probably required to produce CD138-expressing PCs. This is consistent with the findings of Jung and colleagues, who reported that CD40L/IL-4-stimulated B cells do not acquire a full PC-surface phenotype (Jung et al.,

![Fig. 7. Mitogenic stimulation of primary B cells results in up-regulated expression of cell cycle regulators. (A) Equal amounts of protein extracted from primary B cells stimulated with CD40L/IL-4 or infected with EBV at the indicated times after initial stimulation were subjected to SDS-PAGE and Western blotting. The levels of cellular proteins involved in cell cycle regulation were analysed by probing with the appropriate Ab. (B) Proliferation of the two B-cell populations, i.e stimulated with CD40L/IL-4 (□) or infected with EBV (○), was measured by \(^{3}H\)thymidine incorporation.](http://vir.sgmjournals.org)
2001). Differentiation of B-blasts to memory B cells also involves cell cycle exit, and CD40L induces differentiation of tonsillar germinal centre (GC) B cells to memory cells in vitro (Arpin et al., 1995). Memory B cells are CD27+, CD20+ (Agematsu, 2000); however, CD20 expression decreased and CD27 was not expressed at any stage following CD40L/IL-4 stimulation, indicating memory cells were not produced. Memory-cell production is thought to require GC formation, whereas PC production can occur independently of this. No expression of the GC cell marker, CD10, was detected on CD40L/IL-4-stimulated cells (not shown). Therefore, differentiation to antibody-producing PCs could represent a default pathway for peripheral primary B cells following prolonged stimulation and differentiation to memory cells may require additional signals.

Another marker of plasmacytoid differentiation is increased Ig synthesis. Cytoplasmic Ig increased in CD40L/IL-4-stimulated B cells. Neither high levels of cytoplasmic Ig nor morphological changes indicative of antibody-producing PCs were visible in comparable EBV-infected cells. However, the Ig level in culture supernatant did increase, consistent with previous studies (Crawford, 1986; Kataoka et al., 1997; Nilsson et al., 1971). This suggests that more cells are producing a lower amount of Ig in the EBV-infected cultures as B-blasts also secrete Ig, albeit at levels below that of PCs.

All Ig isotypes were present in both CD40L/IL-4- and EBV-driven cultures, consistent with previous reports (Kataoka et al., 1997; Urashima et al., 1996) and can be explained by isotype switching occurring in culture (perhaps due to cytokine action) or stimulation of memory cells, which have already undergone isotype switching. Both naive and memory cells were present in our quiescent B-cell population, demonstrated by the presence of CD27+ cells (Fig. 3B, day 0). The latter hypothesis is supported by recent work indicating that EBV can infect both memory and naive B cells in vitro with similar efficiency (Ehlin-Henriksson et al., 2003).

CD40L/IL-4-stimulated B cells secreted high levels of IL-6, correlating with proliferative decline, development of plasmacytoid morphology and increases in cytoplasmic and secreted Ig. It has been reported that CD40L/IL-4 induces Ig production, in part due to the autocrine action of IL-6 and induces differentiation of B-blasts (Chen-Kiang, 1995; Jung et al., 2001; Smeland et al., 1989; Urashima et al., 1996), consistent with our data. The EBV-infected cells, which did not differentiate, secreted threefold less IL-6 than CD40L/IL-4-stimulated B cells; however, the addition of IL-6 to CD40L/IL-4-stimulated 85 B blasts (Chen-Kiang, 1995) and in vitro studies have shown exogenous signals, such as IL-6 and CD40L, can induce differentiation of EBV-positive B-blasts (Chen-Kiang, 1995; Fukuda et al., 2000; Pokrovskaja et al., 2002). Infection of CD40L/IL-4-stimulated B cells with EBV could not reverse proliferative decline (Fig. 5), consistent with early reports that only small, resting (not large, activated) B cells can be immortalized by EBV (Aman et al., 1984). It should be remembered that B cells receive a plethora of additional signals in the GC milieu and it is possible that EBV cannot override these.

CD40L/IL-4-induced differentiation was associated with an increase in p18INK4C, p21WAF1/CIP1, cyclin D2 and p14ARF. This is consistent with previous reports that p18INK4C is induced in differentiating B cells (Franklin et al., 1998; Morse et al., 1997; Schrantz et al., 2000; Tourigny et al., 2002). In addition, IL-6-mediated up-regulation of p18INK4C has been proposed to mediate differentiation-associated arrest in the LCL, CESS (Morse et al., 1997). p21WAF1/CIP1 is up-regulated in differentiated cells of many lineages (Parker et al., 1995), including B cells (Morse et al., 1997), consistent with the increased levels of this protein following long-term CD40L/IL-4 stimulation. The concomitant increase in cyclin D2 levels in CD40L/IL-4-stimulated B cells is interesting in the light of a recent study demonstrating that p21WAF1/CIP1 is stabilized by interaction with cyclin D1 (Coleman et al., 2003; and our unpublished data). Cyclin D2 also binds and hence could stabilize p21WAF1/CIP1. Interestingly, p27KIP1 did not increase, a phenomenon generally associated with cell cycle exit; however, these data are consistent with another study showing no increase in p27KIP1 in differentiating B cells (Schrantz et al., 2000). p14ARF is not a CKI, but negatively regulates growth through p53 stabilization, leading to growth arrest (Kamijo et al., 1998; Quelle et al., 1995). p14ARF accumulation in CD40L/IL-4-treated B cells suggests either it is involved in B-cell differentiation or it accumulates as the cells arrest. In addition, the slower migrating band, detectable with the anti-p21WAF1/CIP1 antibody, may play a role in the differentiation phenotype.

Data presented here indicate that the replicative lifespan of normal B cells stimulated by CD40L/IL-4 in culture is determined by plasmacytoid differentiation. Since isogenic EBV-infected cells do not differentiate, latent EBV may suppress differentiation, at least in vitro. EBNA-2 has been reported to block c-myc-induced B-cell differentiation in vitro (Polack et al., 1996). It is unlikely that EBV abrogates B-cell differentiation per se, since studies on the latent virus life cycle in vivo suggest that EBV-infected cells differentiate to gain access to their site of persistence, the memory B-cell compartment (Thorley-Lawson, 2001). Furthermore, in-vitro studies have shown exogenous signals, such as IL-6 and CD40L, can induce differentiation of EBV-positive B-blasts (Chen-Kiang, 1995; Fukuda et al., 2000; Pokrovskaja et al., 2002). Infection of CD40L/IL-4-stimulated B cells with EBV could not reverse proliferative decline (Fig. 5), consistent with early reports that only small, resting (not large, activated) B cells can be immortalized by EBV (Aman et al., 1984). It should be remembered that B cells receive a plethora of additional signals in the GC milieu and it is possible that EBV cannot override these.
Cells undergoing senescence display a progressive reduction of proliferation and eventually arrest, characterized by an increase in the levels of markers including p53, p21WAF1/CIP1, p14ARF, and p16INK4A (Campisi, 2003; Mathon & Lloyd, 2001; Sherr & DePinho, 2000). It remains possible that senescent cells could provide the increases seen in p14ARF and p21WAF1/CIP1, thus altering the interpretation of these data; however, induction of p53 or p16INK4A was not seen in B cells chronically stimulated with CD40L/IL-4.

Collectively, the data presented here indicate that differentiation, rather than senescence or cell death, determines the replicative lifespan of CD40L/IL-4-stimulated B cells in culture. These data also suggest that EBV infection either provides additional signals to extend proliferation, which is lacking in CD40L/IL-4 stimulation, or overrides a pre-programmed, differentiation pathway – at least in vitro. In addition, this characterization of B-cell proliferation has highlighted CD40L/IL-4 stimulation as a useful system to enable comparisons to be made between isogenic populations of EBV-infected and -uninfected cycling B cells. The window of opportunity in which the two isogenic populations proliferate at a similar rate, and are at comparable stages of differentiation, is 7–14 days after stimulation.

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