

Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4

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CD40 ligand (CD40L) and IL-4 are sufficient to induce resting murine B cells to divide and switch isotypes from IgM and IgD to IgG1 and IgE. Tracking of cell division following (5- and 6) carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling revealed that B cells expressed IgG1 after three cell divisions, and IgE after five. The probability of isotype switching at each division was independent of both time after stimulation and of the dose of CD40L. IL-4 concentration regulated the number of divisions that preceded isotype switching. Loss of surface IgM and IgD was also related to cell division and appeared to be differentially regulated. B cell proliferation was typically asynchronous with the proportion of cells in consecutive divisions being markedly affected by the concentration of CD40L and IL-4. Simultaneous (5-bromo)-2'-deoxyuridine labeling and CFSE staining revealed that B cells in each division cycle were dividing at the same rate. Therefore, division cycle asynchrony resulted from dose-dependent variation in the time taken to enter the first division cycle. These results suggest that T-dependent B cell expansion is linked to predictable functional changes that may, in part, explain why IgE is produced in response to prolonged antigenic stimulation.

Key words: Cell division / Isotype switching / IgE

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1 Introduction

Antibody responses to thymus-dependent antigens require physical contact between T and B cells, as well as the delivery of soluble lymphokine-mediated signals (reviewed in [1–4]). The T cell contact-dependent signal is mediated by the CD40 ligand (CD40L) molecule on activated Th cells, engaging CD40 on the B cell surface [5–7]. A large body of data also indicates that IL-4 produced by Th cells plays an important role in B cell activation [8]. IL-4 is known to synergize with CD40 cross-linking in the stimulation of DNA synthesis by resting B cells [9] and induces B cell switching to the IgG1 and IgE isotypes [10–12], leading to its designation as a differentiation factor [13]. Studies of B cell proliferation and cytokine-induced class switching following activation by

LPS, T cells or T cell components have implied a complex picture of regulation where the B cell integrates numerous signals into a switching decision [14, 15]. Switching also has been shown to occur in activated B cells during S phase of the cell cycle [16, 17], suggesting that DNA replication might be necessary [18]. According to our recent studies, an additional mode of control may exist since switching to the IgG1 isotype in response to Th membrane and Th2 cell culture supernatant required at least three cell division cycles [19].

Here we extend these earlier observations by showing that recombinant CD40L and IL-4 are sufficient to induce switching to both the IgG1 and IgE isotype and that each of the switching events is related to division cycle number. The relationship between the frequency of isotype switching with time, dose of stimulation and division cycle number revealed that the B cell differentiation sequence was highly predictable.

[17806]

Abbreviations: CFSE: 5(6)-Carboxyfluorescein diacetate succinimidyl ester **BrdUrd:** (5-βromo)-2'-deoxyuridine **SA-TC:** Streptavidin-Tricolor **CD40L:** CD40 ligand

2 Results

2.1 CD40L and IL-4 induce division-dependent changes in IgM, IgD, IgG1 and IgE expression

We have previously demonstrated that switching to IgG1 induced by Th cell membranes together with IL-4 correlated with cell division number [19]. More recently agonistic CD40 binding reagents have allowed the effects of CD40 ligation to be examined in the absence of other cell surface interactions. The relationship between division and isotype expression was examined by stimulating B cells with membranes from insect cells infected with CD40L recombinant baculovirus [20] and recombinant IL-4 (Fig. 1). With this stimulus expression of IgM, IgD

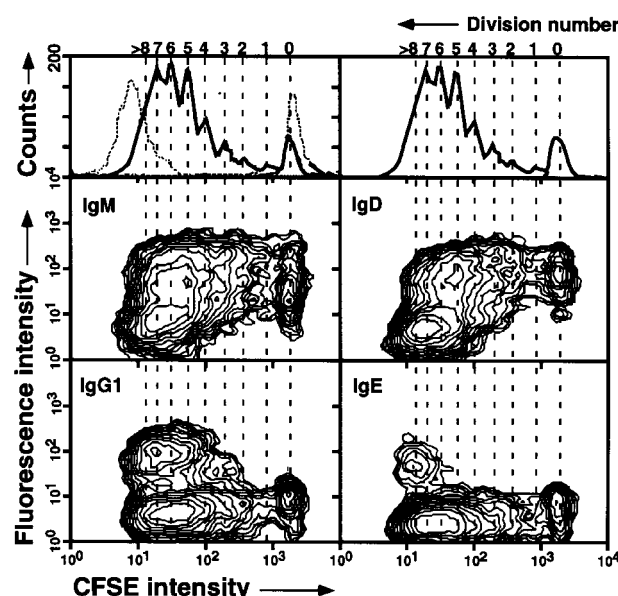


Figure 1. Surface Ig isotype expression induced by membrane CD40L and IL-4. CFSE-labeled resting B cells were activated with CD40L (1/1000) and IL-4 (1000 U/ml) for 5 days. The following mAb conjugates were used for the cell surface Ig staining: PE-conjugated 331.12 (anti-IgM), PE-conjugated AMS.15.1 (anti-IgD), biotinylated LOMG-1 (anti-IgG1) and biotinylated R1E4 (anti-IgE). SA-TC was used as a second step for the latter two mAb. Top panels show the histogram of CFSE-labeled cells for comparison to the contour plots. On the top left panel, the dotted histogram at 0 division shows the position of CFSE-labeled but unstimulated cells after 5 days incubation. The dotted histogram at the left shows the position of CFSE-unlabeled but CD40L- and IL-4-activated cells (autofluorescence). The lower four panels show contour plots of surface Ig-stained CFSE-labeled cells as indicated. Dashed lines show positions of cell division peaks for reference.

and IgG1 changed in a division-related manner that was similar to the previously described effect of Th membrane. Fig. 1 also shows that IgE expression is division related and only appears on cells that have divided at least six times.

2.2 The effect of CD40L and IL-4 dose on division cycle progression

Many variables that affect isotype switching, including time of culture, the mode of activation and the concentration of stimuli [15, 21–23], also influence B cell proliferation. This association raises the question of how the relationship between division and switching is affected by these variables. The baculovirus CD40L and recombinant IL-4 system together with 5(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling provided the opportunity to examine B cell differentiation induced by defined and controlled receptor-ligand interactions. The influence of the dose of CD40L and IL-4 on proliferation was initially tested by monitoring division cycle progression. Purified resting CFSE-labeled B cells were placed in culture for 4 days (Fig. 2) and then examined for CFSE intensity by flow cytometry. The CFSE profiles indicated that the dose of both CD40L and IL-4 profoundly affected the distribution of cells within the division cycles. In general a group of leading cells (the cells that had reached the greatest number of divisions, $D_{\max}$) were trailed by varying proportions of cells in each division cycle between undivided (D_0) and $D_{\max}$. At highly stimulatory combinations of CD40L and IL-4, the proportion in each division cycle diminished from $D_{\max}$ to D_0 (Fig. 2, columns e and f). At very low stimulatory doses the profile was reversed and the proportion of cells was maximal at D_0 and minimal at $D_{\max}$ (Fig. 2, columns a, b).

The $D_{\max}$ of B cells stimulated with different doses of CD40L alone for 4 days appeared constant at 6, although only a very small proportion reached this number at the lowest concentrations used (Fig. 2, column a). The addition of increasing concentrations of IL-4 resulted in a trend toward greater synchrony of cells in $D_{\max}$ (Fig. 2, columns b–f). Furthermore, IL-4 increased $D_{\max}$ by two divisions compared with CD40L alone. A comparison of these dose patterns revealed that both IL-4 and CD40L dose exert a marked influence on the distribution of cells in each division cycle.

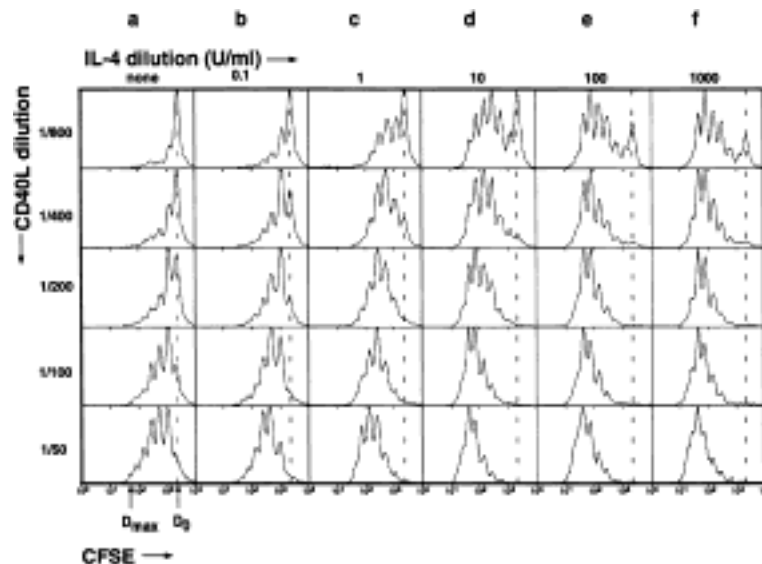


Figure 2. CD40L and IL-4 dose influence division cycle distribution. CFSE-labeled resting B cells were stimulated with various doses of CD40L and IL-4 as indicated for 4 days. The histograms represent data of 20 000 acquired events on FL1 channel with two smoothing cycles. Dotted lines show the positions of undivided cells (D_0). Maximum divided cells (D_{max}) are indicated by an arrow. Cells cultured with IL-4 alone did not divide and run as a single peak at the D_0 position.

2.3 The division number required for switching to IgG1 is independent of CD40L dose and is regulated by IL-4 dose

The influence of CD40L and IL-4 dose on B cell division implied that the effects of these variables on switching to IgG1 might be partly, or completely, explained by alterations in the rate of cell division. Thus, it was important to examine the effect of CD40L and IL-4 dose on isotype switching. B cells were stimulated with a range of doses of CD40L and IL-4 and surface IgG1 expression was determined simultaneously with CFSE division profiles after 4 days in culture. Results of division-based gating (see Sect. 4.5 and Fig. 7) for the CD40L and IL-4 titration are shown in Fig. 3. The lower panels illustrate the variation in the distribution of viable cells between the divisions. As described above, the dose of both CD40L and IL-4 markedly affected the distribution of cells among the division cycles. The upper panels show the percentage of IgG1-positive cells found within each division after stimulation with the various combinations of CD40L and IL-4 dose. At 1000 U/ml and 100 U/ml IL-4, the proportion of IgG1-positive cells was < 7 % in divisions 0, 1 and 2; however, for each consecutive division after the second there was a 7 % increase in IgG1⁺ cells, irrespective of the dose of CD40L. The maximum percentage of IgG1⁺ cells in this experiment reached 45 % at the latest cell divisions. Moreover, the proportion of IgG1⁺ cells was never greater than 50 % at any dose or time in

over 20 experiments (data not shown). A decrease in the IL-4 dose to 10 U/ml had the effect of delaying the division number at which the progressive increase in IgG1⁺ expression occurred. Interestingly, the slope of the increase (*i.e.* approximately 7 %/division) was similar for both the high and low doses of IL-4. Concentrations of IL-4 less than 10 U/ml enhanced B cell proliferation (Fig. 3), but did not initiate an increase in switching to IgG1 before the seventh division. In cultures incubated with high doses of CD40L and IL-4 the frequency of IgG1⁺ cells had apparently increased in divisions 0, 1 and 2 when compared to cells stimulated with CD40L only. This could indicate some division-independent switching in rapidly dividing cultures. However, it is also possible that some passive absorption of secreted IgG1 was responsible, as high concentrations of secreted IgG1 were found in these cultures (data not shown).

2.4 IgG1 and IgE expression is independent of culture time and related to division cycle number

The above results indicated that IgG1 switching was related to division number and was not affected by CD40L dose. IgE expression could not be examined on day 4 because it is expressed only after five divisions in a region of the CFSE plot that showed considerable overlap between divisions (Fig. 1). This overlap is due to

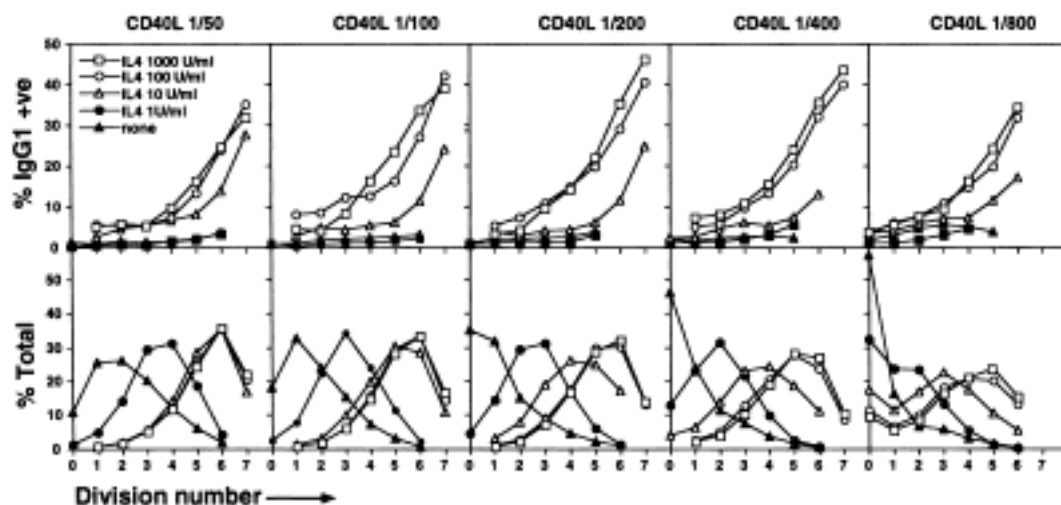


Figure 3. The switch to IgG1 is independent of CD40L dose, but low IL-4 dose delays switching. CFSE-labeled resting B cells were stimulated with various doses of CD40L and IL-4. Four days after initiation of culture, B cells were stained with anti-IgG1 mAb (biotinylated LOMG-1) followed by SA-TC. CFSE peaks were individually gated and peak distributions or IgG1 percents were calculated as described in Sect. 4.5 and Fig. 7. The top panels show IgG1 percent for each division. The lower panels show the percentage of the total live cells found in each division (% Total). In total 20 000 events were analyzed and individually gated peaks containing less than 100 events were excluded from analysis.

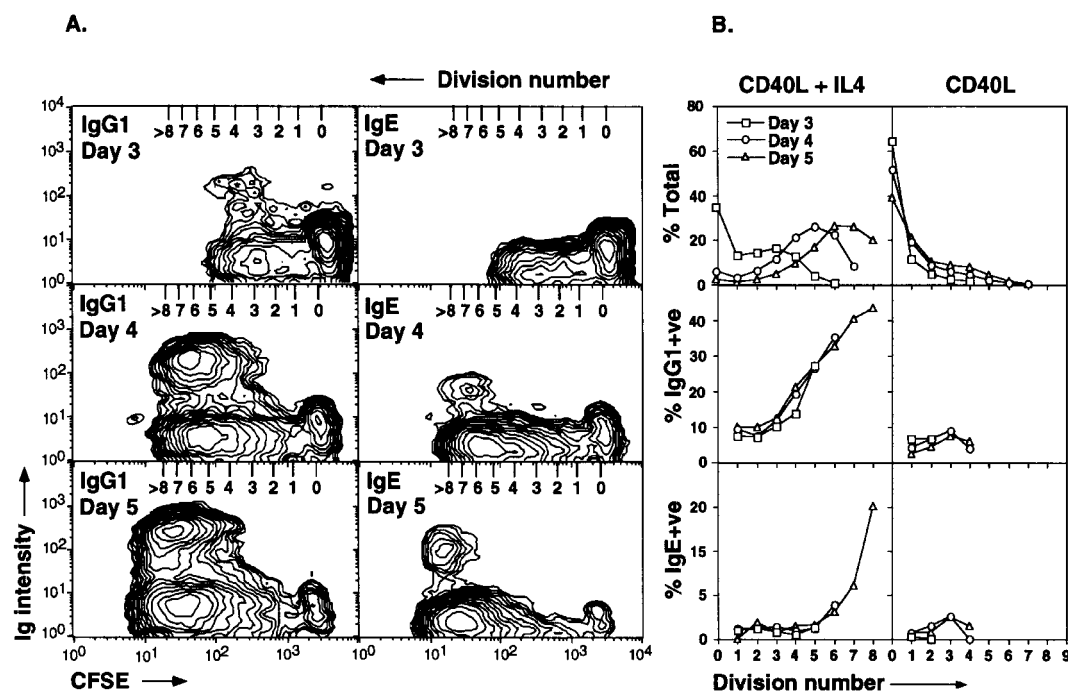


Figure 4. CD40L- and IL-4-induced IgG1 and IgE expression is independent of the culture time and related to division cycle number. CFSE-labeled small B cells were activated with CD40L (1/1000) in the presence or absence of IL-4 (1000 U/ml). (A) Contour plots of the CD40L + IL-4-induced IgG1 and IgE expression on days 3 to 5 are shown. Positions of the cell divisions are indicated above each contour plot. Because of the decrease in CFSE intensity with time, the cell division positions slightly shift left on consecutive days. (B) Peak analysis of data shown in (A). On day 3 (squares), 4 (circles), and 5 (triangles), CFSE histogram peaks corresponding to the cell divisions were individually gated and analyzed as described in Sect. 4.5 and Fig. 7. The top panels show the percentage of live B cells in each division. The middle and lower panels show cell division gated IgG1 and IgE isotype expression of B cells stimulated with CD40L in the presence or absence of IL-4.

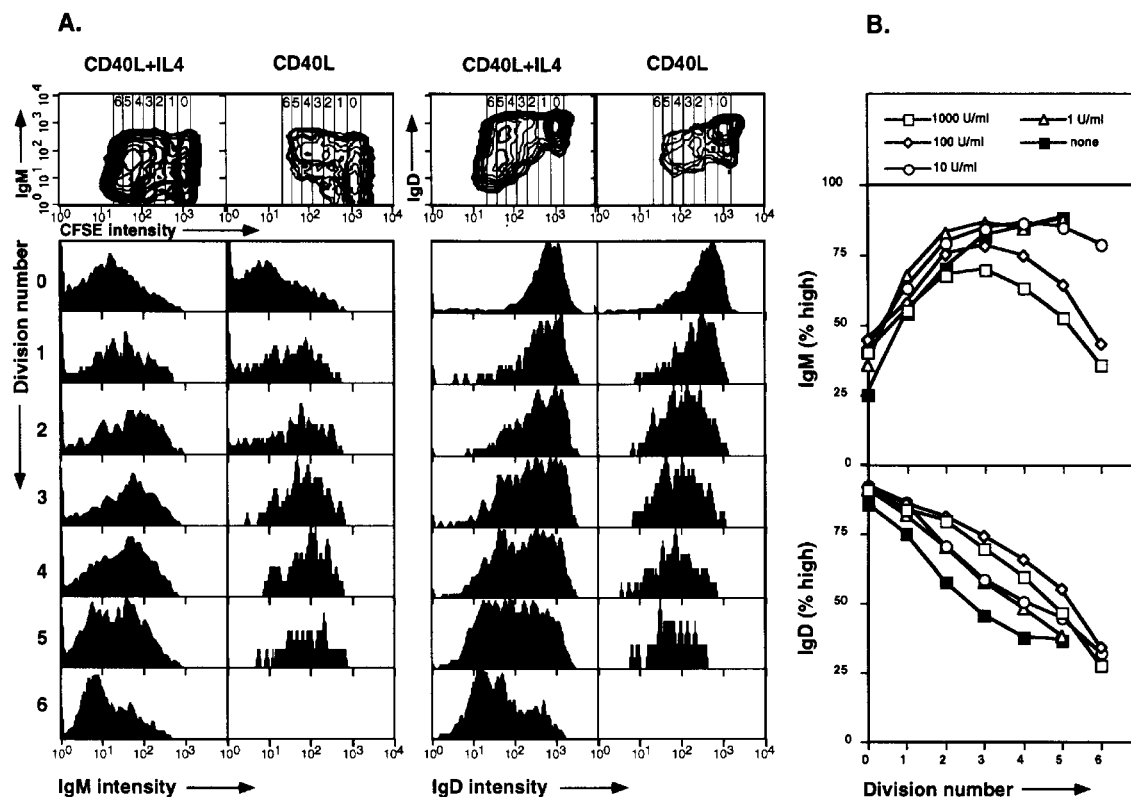


Figure 5. Differential down-regulation of surface IgM and IgD on CD40L-stimulated B cells. CFSE-labeled B cells were activated with CD40L (1/1000) and various doses of IL-4. On day 4 cells were harvested and stained with anti-IgM (PE-conjugated 331.12) or anti-IgD (PE-conjugated AMS15.1). FCM analyses of the gated CFSE peaks were performed as described in Sect. 4.5. (A) The top panel shows contour plots of IgM and IgD expression by the CFSE-labeled activated B cells. The numbered vertical lines show positions of the cell divisions. The lower panel sequence represents IgM and IgD histograms of the gated consecutive division peaks. (B) Quantitative plot of the division-dependent IgM and IgD loss in response to different doses of IL-4. Cell divisions were individually gated and analyzed as described in Sect. 4.5 and Fig. 7. As for IgG1, the time of culture did not affect the pattern of division-related changes to IgM and IgD expression (data not shown).

the small level of autofluorescence contributed by activated B cells. We therefore examined the kinetics of appearance of both IgG1 and IgE on dividing cells to determine whether switching to each isotype in this system was independent of time, as previously noted for Th membrane-induced IgG1 switching [19]. CFSE-labeled B cells were cultured with CD40L and IL-4, and surface IgG1 and IgE were assessed by flow cytometric staining on days 3–5. Fig. 4A shows the contour plots relating cell division to IgG1 and IgE expression. At day 3 the leading cells ($D_{\max}$) had reached only the fifth division with some IgG1- and no IgE-positive cells. At days 4 and 5, $D_{\max}$ was approximately 8 and a proportion of cells after division 5 had expressed IgE. Fig. 4B shows the same data gated and overlaid to reveal the influence of time on switching. IgG1-positive cells appeared after the third division and increased by a constant percentage until division 8 at each time point. Thus, the percentage

of CD40L- and IL-4-activated cells that switched to the IgG1 isotype in each division cycle remained constant from day 3 to day 5 (Fig. 4B, middle panel), although the proliferation profile of these cells had changed considerably during this time (Fig. 4B, upper panel). Expression of IgE also seemed to correlate with division number, and not time, increasing in frequency only after division gate 5 (Fig. 4B, lower panel).

2.5 Differential down-regulation of surface IgM and IgD

Freshly prepared small dense B cells express high levels of IgD and low to medium levels of IgM on the cell surface (data not shown). Concurrent with the division-related acquisition of IgG1 and IgE, CD40L- and IL-4-stimulated B cells also down-regulated and lost surface

IgM and IgD (Fig. 1). To quantitate the division cycle-dependent changes in surface IgM and IgD, and to allow comparisons with the IgG1 and IgE data already shown, CFSE division peaks were gated and analyzed as described in Sect. 4.5.

Fig. 5A shows the IgM and IgD expression histograms for each division for cells activated with CD40L with and without IL-4 during 4 days of culture. Undivided cells retained the broad, predominantly low, IgM distribution (Fig. 5A, division 0). In the absence of IL-4, cells in the first division exhibited a fivefold increase in IgM expression, which remained high for up to six divisions (Fig. 5A, IgM panel, CD40L column). In contrast, IgD expression was initially high on all undivided cells in the absence of IL-4 and consistently declined with each division, showing a fivefold reduction after five division cycles (Fig. 5A, IgD panel, CD40L column). The addition of IL-4 altered the pattern of IgM and IgD expression with division (Fig. 5A, CD40L + IL-4 columns). A clear transition from IgM- and IgD-positive to a negative population was observed and was related to division number. This transition is presented quantitatively for both IgM and IgD in Fig. 5B. In comparison with cultures activated with CD40L alone, IL-4 had little effect on IgM expression over the first three division cycles, after which the proportion of IgM-negative cells increased at the rate of approximately 7 % per division cycle. The IL-4-induced transition to the IgD-negative phenotype followed a different pattern than IgM (Fig. 5A and B). By the second division, cells with an intermediate level of IgD appeared, and then remained detectable during subsequent divisions. IgD-negative cells appeared at the third division with the proportion increasing in divisions four, five and six.

2.6 Stimulated B cells divide with a constant division rate

The above data showed that B cell differentiation correlated with the number of division cycles and depended upon the concentration of IL-4 and not CD40L. However, cells were typically distributed over a broad range of division cycle numbers and appeared to be dividing asynchronously. This could be explained in one of two ways: cells were either entering the first division at different times and dividing at a constant rate thereafter, or cells were dividing at different rates, such that the cells in $D_{\max}$ were dividing faster than the trailing cells. To distinguish between these possibilities, a method for simultaneously monitoring cell division number and rate of division was established. B cells were placed in culture for 3 days in the presence of CD40L and IL-4 and then pulsed with (5-bromo)-2'-deoxyuridine (BrdUrd) for the final 1, 3 or 9 h (Fig. 6A). After fixation and permeabilization, the cells

were stained intracellularly with anti-BrdUrd mAb and analyzed by FCM. The results showed that the percentage of cells incorporating BrdUrd was constant after division one (Fig. 6B), suggesting that the B cells proliferated at the same rate once they had entered the first division. Thus, switched cells appeared to divide at the same rate as unswitched cells. To examine this point more precisely, cells were counterstained with IgG1 and examined for dividing IgG1-positive and negative cells (Fig. 6C). The percentage and rate of BrdUrd incorporation was similar for both IgG1⁺ and IgG1⁻ cell populations, confirming that switching does not affect the rate of cell division.

3 Discussion

In a typical primary antibody response B cells undergo a progressive isotype switch from IgM to IgG, IgA or IgE. Antigen-specific IgG-positive cells usually appear *in situ* 3–6 days after immunization and become more numerous with time [24–26]. IgE-secreting cells are detectable by day 6 of immunization [27]. Many *in vitro* studies have established that isotype switching is controlled by T cell-derived cytokines, such as IL-4 and IFN- γ , delivered during cognate interaction with the antigen-presenting B cell [1]. However, we have previously reported that the number of cell divisions B cells undergo in the presence of the cytokines is an additional variable in the switching process [19]. To more precisely define how B cells integrate signals that affect both proliferation and switching, we used various combinations of rCD40L and IL-4. These two T-dependent B cell-activating signals induce naive B cells to proliferate and switch to both IgG1 and IgE. Application of the new CFSE labeling technique allowed the quantitative features of both growth and differentiation to be assessed simultaneously.

The proportion of B cells in each division cycle at a given time varied with the dose of CD40L. Although $D_{\max}$ remained relatively constant, the proportion of cells in $D_{\max}$ increased with increasing CD40L doses. The addition of IL-4 had three distinct effects on CD40L-induced B cell proliferation. First, the cytokine altered the distribution of cells found in each division round by increasing the proportion of cells in divisions close to $D_{\max}$. This effect of IL-4 was approximately equivalent to the change that would occur with a 30-fold increase in the CD40L concentration. At high CD40L and IL-4 concentrations almost all B cells entered division and proceeded to divide in relative synchrony. Hence, IL-4 was not obligatory for B cell growth but substantially enhanced the sensitivity to CD40L stimulation. Secondly, and as previously noted, the spontaneous rate of cell death of the unstimulated/undivided group of B cells

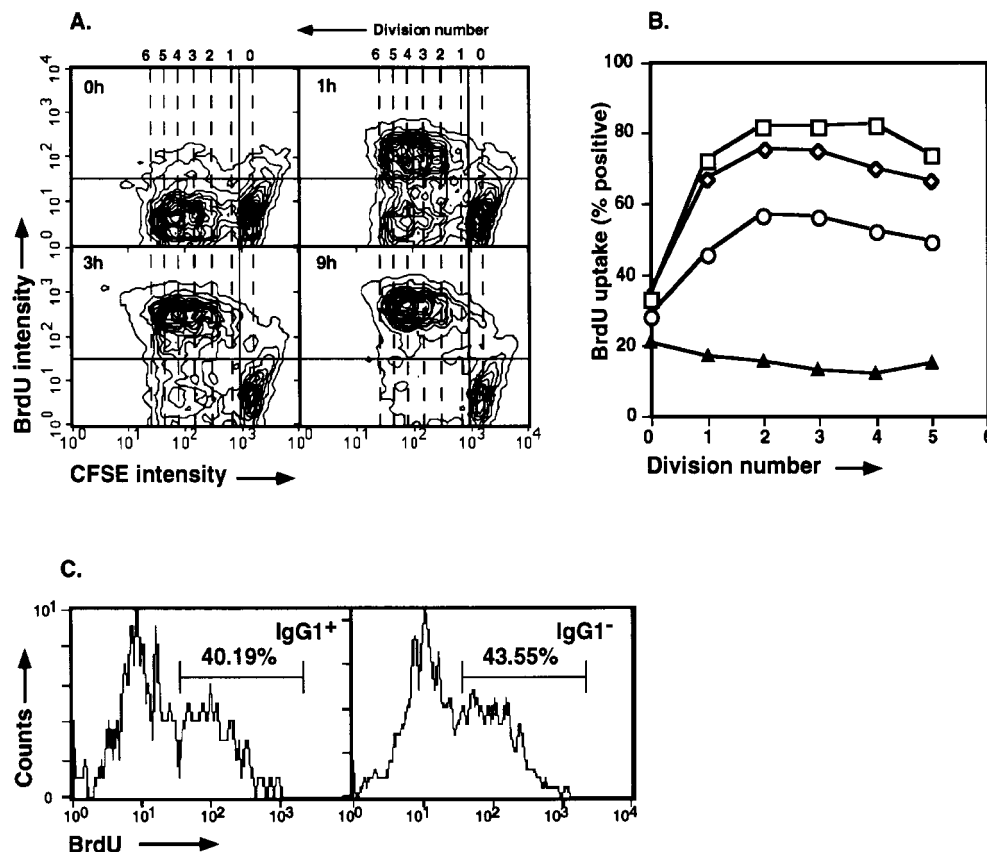


Figure 6. B cells enter the first division at different times and then proliferate with a constant division cycle rate. CFSE-labeled small B cells were activated with CD40L (1/1000) and IL-4 (1000 U/ml) for 3 days. For the last 1, 3 or 9 h of culture, B cells were pulsed with 50 μ g/ml BrdUrd. Intracellular staining by anti-BrdUrd mAb was performed as described in Sect. 4.6. (A) Contour plot of proliferating B cells pulsed with BrdUrd for different times, followed by anti-BrdUrd mAb staining. Dashed lines show cell division cycle numbers. (B) Peak analysis of the BrdUrd-positive cells in each cell division pulsed with BrdUrd for 9 h (open squares), 3 h (open diamonds), 1 h (open circles) and 0 h (background control, closed triangles). (C) Histograms of 3-h BrdUrd-pulsed cells gated on IgG1⁺ (left) and IgG1⁻ cells (right). Marker shows percent of BrdUrd⁺ cells.

was reduced by IL-4 [19]. This enhancement of the number of D₀ cells contributed to a higher proportion of undivided cells at each time point. Finally, IL-4 induced a small increase in D_{max} reached by the stimulated cells.

In contrast to the complex relationship found between CD40L and IL-4 dose on cell division, the effects on the differentiation program were remarkably simple. There was no CD40L dose dependence observed for differentiation to IgG1 in the presence of IL-4. In contrast, decreasing doses of IL-4 increased the number of divisions observed before switching to IgG1. Additionally it was found that switching to IgE was also related to cell division cycle, occurring with increasing frequency after five cell divisions. This appearance of IgE-positive cells in cell divisions after IgG1 is consistent with earlier reports

of kinetic differences in appearance of IgG1 and IgE in either LPS- or Th-stimulated cultures [23, 28], and with the evidence for sequential switching from IgG1 to IgE [29, 30]. Although at a first examination, time and CD40L dose appeared to directly influence isotype switching it is now clear that the increased number of switched cells which occurred at later times or at higher CD40L doses is simply an indirect measure of the number of cells progressing to the later division cycles. By contrast, the IL-4 dose response is a composite of two effects, *i.e.* one enhancing the number of cells that reach the later divisions, and a second increasing the proportion of those cells that switch to IgG1 and IgE.

The levels of expression of IgM and IgD were also related to cell division. Most notable was the gradual loss of high

levels of IgD expression with division on cells that otherwise retained high levels of IgM. This loss of IgD occurred even in the absence of IL-4 and therefore did not precede switching. In contrast, in the presence of IL-4 dividing B cells expressed high IgM and intermediate IgD levels and then upon further division lost both IgM and IgD. These results imply considerable independent control over the expression of the two forms of antigen receptor during the early cell divisions. A gradual reduction in IgD with division would progressively reduce the efficiency of antigen capture of the selected B cells, possibly ensuring that only the highest affinity B cells progress in their differentiation. This idea is consistent with the observation that an IgD homozygous null mouse has a slightly reduced level of affinity maturation [31]. Whether the expression of IgD and IgM is controlled by RNA synthesis, protein synthesis, RNA turnover or protein turnover is currently unknown. It has been reported that the differential expression of surface IgM and IgD is regulated both at the levels of transcription and post-translational modification [32].

Although proliferation of B cells after stimulation was previously shown to be asynchronous by the CFSE technique [19, 33], simultaneous monitoring of BrdUrd incorporation demonstrated that B cells in each division cycle between $D_{\max}$ and division 1 were dividing at the same rate. Therefore, asynchrony in division number arises because of variation in the time stimulated B cells take to enter into their first division cycle. The average time to first division decreased as the level of stimulation was increased. This is consistent with increased CD40 signaling leading to more rapid accumulation of a cellular product, such as the cyclin proteins, with the capacity to trigger cell division when they reach a critical threshold. It is possible that the log-normal distribution of CD40 observed on the resting B cell population is responsible for the widely varying times to first division in the same culture. This leads to the prediction that cells bearing low levels of CD40 receptor should take longer than cells with high levels of receptor to reach the triggering threshold for division. Currently we are testing this hypothesis by correlating receptor level with time to division. A relationship between receptor number, ligand dose and time to division has been reported previously for IL-2-driven T cell proliferation [34].

Together the results of the effects of varying CD40L and IL-4 signals on the B cell differentiation response can be summarized. Although many different outcomes were possible, the probability of losing cell surface IgD and IgM, and of expressing IgG1 followed by IgE was consistently related to division number and was independent of the time of stimulation or the dose of CD40L. Moreover, IL-4 concentration simply appeared to regulate the num-

ber of divisions preceding isotype switching. These conditions suggest that a mechanism for turning on the cellular switch machinery which is linked to an IL-4-sensitive division counting mechanism may exist. The data are also consistent with previous suggestions that chromosomal replication is necessary for switch recombination to occur [17, 18, 35]. Thus, integration of various combinations of signals and doses by B cells in this system follows simple rules that result in highly predictable sequential differentiation events.

The striking relationship between division number and switching presents an obvious mechanism for linking switching to affinity-based selection *in vivo*. Thus, early in an immune response, when antibody levels are low and antigen concentration high, both high- and low-affinity B cells can be activated by T cells to proliferate and make IgM. With time the antigen concentration will decline, due to dispersion or antibody-mediated clearance. At this time only the more competitive higher affinity cells will be selected for further division and undergo the transition from ten binding sites to the less avid two sites of IgG. Less clear is the logic of why IgE should follow IgG after further division. It is possible that the primary antibody response operates by a form of antigen-driven feedback. If the antigen is not removed by the early secreted IgM, or the later IgG, a switch in isotype to IgE and therefore a different clearance strategy may be warranted. Irrespective of the logic of producing IgE after IgG, the observed response suggests that IgE-secreting cells should display a higher affinity than IgG. This has been reported in at least one study of IgG and IgE binding to a common allergen in human serum [36]. Furthermore, the putative importance of antigen persistence for the switch to IgE could go some way to explaining why invasive parasites and environmental allergic antigens, such as house dust and pollens, induce this isotype.

4 Materials and methods

4.1 Animals

Male 8–12-week-old CBA/H inbred mice obtained from the Animal Resource Centre (Perth, Australia) were used for the experiments. Mice were maintained in a specific pathogen-free environment at the Centenary Institute animal facility.

4.2 Reagents and antibodies

CFSE (Molecular Probes Inc., Eugene, OR) was dissolved in DMSO at a concentration of 5 mM and kept at -20°C until further use. BrdUrd was purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Cell membranes expressing mouse CD40L were prepared as previously described [37]

from the Sf9 insect cell line infected by baculovirus vector containing a CD40L gene construction and kept frozen at -70°C . Recombinant murine IL-4 was kindly provided by R. Kastelein (DNAX, Palo Alto, CA).

The following mAb were used for cell surface labeling: RS 3.1 (anti-IgM^a); 331.12 (anti-IgM); AMS 15.1 (anti-IgD^a); and R1E4 (anti-IgE, [38]). These mAb were purified using a protein G (Pharmacia, Uppsala, Sweden) column from hybridoma cell culture supernatant and either biotinylated with NHS-Biotin (Pierce, Rockford, IL) or conjugated to R-PE using succinimidyl 4-(p-maleimidophenyl)butyrate (Pierce, Rockford, IL) according to the manufacturer's instructions. LOMG-1 biotin (anti-IgG1) was purchased from Serotec (Oxford, GB). Anti-BrdUrd-specific mAb (BR-3 biotin) was provided by Caltag (Caltag Laboratories, Burlingame, CA). Streptavidin-Tricolortm (SA-TC, Caltag Laboratories, Burlingame, CA) was used as the second step in staining biotinylated mAb.

4.3 Cell preparation and *in vitro* culture

Small resting B cells were prepared as described previously [39]. Briefly, single-cell suspensions were prepared by teasing mouse spleens through a stainless steel mesh. After lysing

RBC with hypotonic ammonium chloride solution, adherent cells were depleted by incubation on a plastic tissue culture dish. T cells were depleted by complement lysis using a mixture of CD4, CD8 and Thy-1 specific mAb. Small dense B cells were further purified by centrifugation on a Percoll density gradient. The B cells recovered from the 65/72 % Percoll gradient interface were considered to be resting B cells and were $\sim 95\%$ B220⁺ and $< 1\%$ CD4- and CD8-positive.

4.4 CFSE labeling

B cells to be labeled with CFSE were washed and resuspended at 1×10^7 cells/ml in PBS containing 0.1 % BSA (PBS/BSA). CFSE was then added to a final concentration of 10 nM and the cell suspension was incubated for 10 min at 37°C [40]. The cells were subsequently washed twice with PBS/BSA and resuspended into B cell culture medium (RPMI 1640 containing 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 mM Hepes, 5×10^{-5} M 2-ME, 100 $\mu\text{g/ml}$ streptomycin, 100 U/ml penicillin and 10 % FCS). Typically, 1×10^5 labeled B cells were activated and cultured in 1 ml cell culture medium at 37°C in a humidified atmosphere containing 5 % CO_2 .

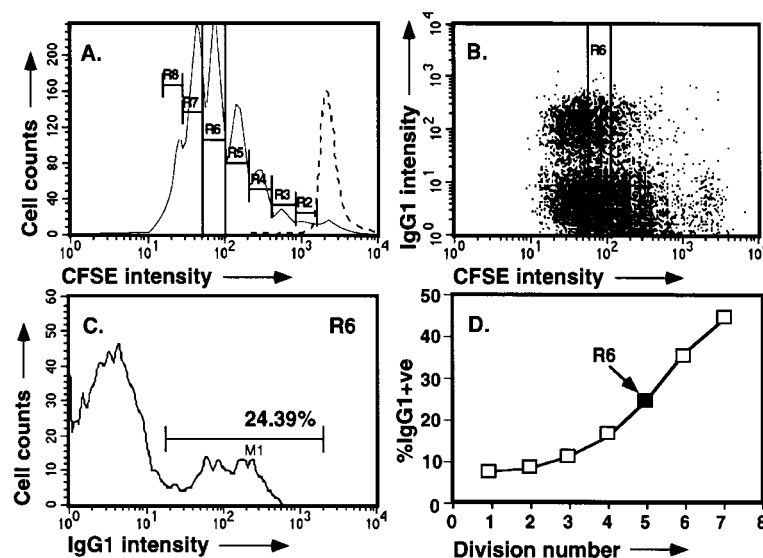


Figure 7. Quantitative analysis of division-related surface Ig expression. This figure illustrates the individual division gating method used to present the data in this report (the R6 gate is shown as an example). CFSE-labeled B cells were stimulated with CD40L (1/1000) and IL-4 (1000 U/ml), and after 4 days of culture cells were stained with anti-IgG1 mAb (biotinylated LOMG-1) followed by SA-TC. The viable B cell population is gated on forward and side scatter profiles. (A) Peaks on the CFSE profile are gated individually (note R6 gate). The dashed histogram on the right shows the position of unstimulated cells. (B) The R6 gate position on a dot plot of the CFSE-labeled B cells stained with anti-IgG1. The y-axis represents IgG1 fluorescence intensity. (C) Percentage of the IgG1⁺ cells gated with the R6 gate. IgG1 fluorescence intensity histogram of the R6 gated cell population was used to quantitate surface IgG1 expression. (D) This graph shows % IgG1⁺ cells in consecutive CFSE peaks (% IgG1⁺ of R6 gate is shown as a closed square).

4.5 Cell surface staining and FCM analysis

At various times after initiation of culture, activated B cells were harvested, washed and incubated with PE-labeled or biotinylated mAb for 45 min, followed by 30 min incubation with SA-TC conjugates. All incubations were performed on ice and the diluent or washing solution was PBS/BSA with 0.1 % sodium azide. Labeled B cells were then examined by FCM on FACScan (Becton Dickinson, Mountain View, CA) and analyzed using CellQuest 3.1 software (Becton Dickinson). Cell division profiles of CFSE-labeled cells were plotted as histograms of fluorescence intensity over four log decades. Two-dimensional data are presented as 75 % log contour plots with 1 % threshold and two smoothing cycles. To condense the data, each CFSE histogram was individually gated to calculate: 1) the proportion of total viable cells in culture within each division round (% total), and 2) the proportion of IgG1-positive cells within each division round. The method of gating on division peaks for IgG1 expression is illustrated in Fig. 7. The overlap between adjacent gaussian distributions of fluorescence for each division substantially increases as the CFSE level decreases and approaches the autofluorescence limit [19]. Hence the result of the gating analysis must be taken only as a guide, especially after the fifth division.

4.6 BrdUrd pulse and staining method

Purified small B cells (10^5 cells/ml) were cultured in 24-well plates (Falcon, Becton Dickinson) at a final volume of 1 ml. After 3 or 4 days of culture, cells were pulsed with 50 µg/ml BrdUrd and incubated further for the time indicated in the text. The pulsed cells were then harvested, washed twice in a PBS/BSA solution and fixed with 1 % paraformaldehyde (Merck, Darmstadt, Germany) for 10 min on ice followed by 10 min quenching with 0.1 M L-lysine at room temperature. The fixed cells were washed and subsequently permeabilized with PBS/0.1 % Tween-20 (Sigma, St. Louis, MO) overnight at room temperature. DNA from the fixed and permeabilized cells was digested with DNase I (Boehringer Mannheim) for 30 min at 37 °C. DNase I was used at 5 µg/ml final concentration diluted in a 20 mM Tris buffer (pH 8.2) with 10 mM NaCl, 1 mM MgCl₂. Finally the BrdUrd-pulsed cells were stained intracellularly with anti-BrdUrd mAb and analyzed on the FACScan flow cytometer.

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