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Quantitative Regulation of B Cell Division Destiny by Signal Strength

Marian L. Turner,*† Edwin D. Hawkins,*† and Philip D. Hodgkin1*

Differentiation to Ab secreting and isotype-switched effector cells is tightly linked to cell division and therefore the degree of proliferation strongly influences the nature of the immune response. The maximum number of divisions reached, termed the population division destiny, is stochastically distributed in the population and is an important parameter in the quantitative outcome of lymphocyte responses. In this study, we further assessed the variables that regulate B cell division destiny in vitro in response to T cell- and TLR-dependent stimuli. Both the concentration and duration of stimulation were able to regulate the average maximum number of divisions undergone for each stimulus. Notably, a maximum division destiny was reached during provision of repeated saturating stimulation, revealing that an intrinsic limit to proliferation exists even under these conditions. This limit was linked directly to division number rather than time of exposure to stimulation and operated independently of the survival regulation of the cells. These results demonstrate that a B cell population’s division destiny is regulable by the stimulatory conditions up to an inherent maximum value. Division destiny is a crucial parameter in regulating the extent of B cell responses and thereby also the nature of the immune response mounted.


A ctivation of B cells can occur in a T cell-dependent or -independent manner. In vitro, both stimulation conditions lead to a proliferative response comprising progression through multiple rounds of cell division and concurrent differentiation to Ab secreting and isotype-switched effector cells. The type of stimulatory signal and the features of the signal in terms of dose, affinity, physical structure, or duration can qualitatively and quantitatively regulate B cell responses in vivo and in vitro (1–7). Although it is intuitively sensible that a population of cells will respond better to stronger signals than to weaker ones, a thorough and quantitative description of how B cell proliferation is regulated in response to signals of varying strength and quality is lacking. Understanding the mode of regulation is important not simply because proliferation determines the overall size of the responding lymphocyte population but also because the differentiation of B cells to Ab secreting cells and the process of isotype switching are integrally linked to cell division (8–12). As a consequence of this, qualitative features of the response, such as type of Ab, will be influenced by the extent of division progression of the participating cells. Thus, progression through successive divisions and the control of the ultimate number of divisions reached is an important variable in the generation of an appropriate immune response.

Previous studies exploring the control of lymphocyte division progression have highlighted two broad classes of behavior. The first has been called the “autopilot” response (13), in which a brief encounter with Ag leads to a large number of successive divisions, as exemplified by CD8+ T cell behavior. In contrast, CD4+ T and B cell division progression appears to be more closely dependent on continual stimulation (14, 15). Rush and Hodgkin (14) showed that B cells are able to undergo only a few further divisions if the stimulus is removed, before they stop dividing and die. Clearly this behavior will influence the response mounted, as intrinsic division-linked differentiation events will only proceed as long as division is maintained. Rush and Hodgkin (14) used this information to suggest a feedback mechanism whereby the continued presence of Ag serves to progress the B cell Ab class through a series of changes to modify the type of response.

We recently presented a quantitative model of lymphocyte proliferation (16). In this model, we proposed a control unit in each cell made up of two independent operators or machines, one regulating the time for cell division and the other regulating the time to cell death. The mean values for the time to either outcome are regulated by internal and external influences and stochastic variation within the population gives the potential for many different outcomes. The interaction of these intracellular “timers” sensitively controls the proliferation dynamics of the lymphocyte population. A third control mechanism necessary to complete the model and to fully describe in vitro and in vivo data was that describing the population “division destiny,” or the maximum number of divisions each participating cell would undergo.

In this study, we examine in greater detail the regulation of division destiny during B cell proliferation. We demonstrate that in vitro B cell division progression is different for different stimuli and is further regulated by the concentration of the stimulatory molecule and the timing of exposure to stimulation. Our results reveal that B cells have an intrinsic division number restriction, even under the optimal stimulation conditions used in our experiments, which represents the maximum of the division destiny continuum. We also provide evidence supporting the hypothesis that the implementation of division destiny is likely to be achieved via division counting rather than a timing mechanism.
FIGURE 1. B cell proliferation in response to varying stimulus concentrations. CFSE-labeled naive resting B cells were stimulated in vitro with anti-CD40 and IL-4, LPS, or CpG at the concentrations shown. Cell numbers (A–C) and the proportion of cells in each division (D–F) were calculated at representative time points for each stimulation condition. Each data point represents the mean of cell numbers or division numbers calculated from three culture wells. Mean division number (G–I) was calculated at multiple time points as described in Materials and Methods.

Materials and Methods

Mice

B cells were obtained from mice that were bred and maintained at the Walter and Eliza Hall Institute (WEHI) animal facilities in specific pathogen-free conditions and in accordance with WEHI animal ethics committee regulations. Male C57BL/6 mice were used unless specified. Bcl-2 Vav transgenic mice were provided by Dr. Robert Kastelein (DNAX Research Institute, CA). LPS derived from Salmonella typhosa was obtained from Sigma-Aldrich. CpG DNA oligonucleotide CpG-1668, fully phosphothioated (sequence TCCATGACGTT CCTGATGCT) was synthesized by Geneworks (Adelaide, Australia). Stimuli were used at concentrations of 20 μg/ml 1C10, 500 U/ml IL-4, 15 μg/ml LPS, and 3 μM CpG unless otherwise stated. CFSE was purchased from Molecular Probes and PKH26 was purchased from Sigma-Aldrich. Hen egg lysozyme (HEL)2 was purchased from Sigma-Aldrich.

Reagents and Abs

Anti-CD40 mAb (1C10) was prepared from a hybridoma cell line provided by the DNAx Research Institute. Recombinant mouse IL-4 was a gift of Dr. Robert Kastelein (DNAX Research Institute, CA). LPS derived from Salmonella typhosa was obtained from Sigma-Aldrich. CpG DNA oligonucleotide CpG-1668, fully phosphothioated (sequence TCCATGACGTT CCTGATGCT) was synthesized by Geneworks (Adelaide, Australia). Stimuli were used at concentrations of 20 μg/ml 1C10, 500 U/ml IL-4, 15 μg/ml LPS, and 3 μM CpG unless otherwise stated. CFSE was purchased from Molecular Probes and PKH26 was purchased from Sigma-Aldrich. Hen egg lysozyme (HEL)2 was purchased from Sigma-Aldrich.

B cell isolation and cell culture

Single-cell suspensions were prepared from mouse spleens, subjected to red cell lysis, and separated on discontinuous Percoll density gradients as described previously (8). Small dense cells were harvested from the 65:95% IgM/H11022, IgD/H11022, and IgM/H9262 interface and B cells purified via negative selection using MACS beads (Miltenyi Biotec). B cells were typically 80% interface and B cells purified via negative selection using MACS beads (Miltenyi Biotec). B cells were typically 80% interface and B cells purified via negative selection using MACS beads (Miltenyi Biotec).

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Flow cytometry and data analysis

Flow cytometric data were collected on FACScan or LSR flow cytometers (BD Biosciences) and analyzed with FlowJo software (Tree Star). Propidium iodide was added to cell cultures before harvest to identify dead cells, which were excluded from analysis. To determine the absolute number of live cells per culture, a known number of CaliBRITE beads (BD Biosciences) was added directly to cell cultures before harvesting for flow cytometry. The proportion of cells in each division was calculated from CFSE profiles and bead counts as previously described (21). Mean division number was calculated by multiplying the number of cells in each division by the division number (taken to be 0.5, 1.5, 2.5, etc.). MoFlo or FACSDiva flow cytometers (BD Biosciences) were used for cell sorting.

Results

The stimulus dose determines B cell proliferation capacity by regulating division number

Naïve B cells labeled with CFSE and stimulated in vitro with polyclonal activators can be assessed at various time points during a stimulation experiment to provide information on the effects of culture conditions on multiple quantitative parameters of cell proliferation and survival (16). We first sought to assess the effect of signal strength on the proliferative response of B cells by varying the concentration of the stimuli. T cell-dependent stimulation was mimicked using an anti-CD40 mAb (1C10) and IL-4. The TLR agonists LPS and CpG were used as T cell-independent stimuli. B cell cultures were treated with varying concentrations of each of the three stimuli, and proliferation was assessed in terms of cell number and progression through subsequent divisions. The proliferation profiles followed the same pattern in each condition, showing an increase in cell number to a point at which population growth ceased and a gradual decline in cell number commenced. However, a clear effect of stimulus concentration was also observed, with the rate of population growth and the total number of live cells increasing with an increasing dose (Fig. 1, A–C). Analysis of CFSE profiles revealed that the proportion of cells entering division was affected by the stimulus dose, such that a larger number of cells remained undivided at low stimulus concentrations (Fig. 1, D–F). These populations had also progressed through

Abbreviations used in this paper: HEL, hen egg lysozyme; ASC, Ab-secreting cell.
fewer subsequent divisions. Further examination of division progression over the course of the experiment revealed that the mean population division number increased with increasing concentration of all stimuli (Fig. 1, G–I). Cells stimulated with the highest concentrations of anti-CD40 and LPS stimulation underwent divisions, such that CFSE autofluorescence was reached and peaks could no longer be discriminated (data not shown). These cells were considered as being in division 8 for the purpose of calculating mean division number. In contrast, CFSE profiles of CpG-stimulated cells showed maximum division progression within the range of CFSE resolution, as revealed by the plateau of mean population division number seen in Fig. 1I.

The duration of stimulation also regulates the division number reached by the responding population

Another variable of stimulation of a proliferative response that can be experimentally adjusted is the duration of the stimulus. Division bursts following transient T-dependent stimulation of B cells have been observed previously (14). Furthermore, the cessation of proliferation after limited-duration stimulation has been used to verify the independence of division progression and survival parameters (16). As in vivo Ag exposure and T cell help are also likely to be temporally regulated, we studied the effect of the duration of stimulation on B cell proliferation in more detail. B cells activated with each of the three stimuli tested were capable of entering division even if the stimulus was removed before the onset of mitosis; however, the maximum total cell numbers (Fig. 2, A–C) and the degree of division progression reached (Fig. 2, D–F) were less than when stimulus was continually present. The extent of proliferation correlated with the duration of stimulus exposure. The proportion of cells entering division was also lower in cultures receiving short stimulation than those in continual stimulation (Fig. 2, D–F). Notably, during the period of population decline, there was no further division progression, as indicated by the plateau of mean population division number (Fig. 2, G–I) and by the observation of no change in the proportion of cells in each division class (Fig. 2, J–L).

Stimulation dose and duration can coordinately regulate B cell division destiny

We next explored the interaction between stimulus concentration and duration in regulating proliferation. B cells were subjected to the same concentration range of stimuli as presented in Fig. 1, but the stimuli were removed before entry to division. The mean division number achieved was regulated by the dose of stimulus in all cases (Fig. 3, G–I) in a similar manner to that seen in continual stimulation. This indicates that the dose of the stimulus is also capable of regulating the division destiny of a B cell population that only encounters that stimulus before entry to division. However, the mean division number reached at all concentrations was considerably less when stimulation was removed early in the culture period (Fig. 3, G–I) than when compared with cell populations in which the equivalent dose was present throughout the culture period (Fig. 1, G–I).

A notable observation from this experiment was that while the mean division number of cells stimulated with anti-CD40 and IL-4 or LPS plateaued at maximum division progression and remained static during population decline, the mean division number of...
CpG-stimulated cultures appeared to decrease during this phase suggesting that, in this system, cells in later divisions may die more rapidly than those in earlier divisions.

The distribution of cells across divisions at the point of proliferation arrest following stimulus removal has been previously described as the population division destiny (16). The results presented in Figs. 1–3 demonstrate that the division destiny of responding B cells is able to be regulated in a progressive manner by both the concentration and the duration of all stimuli tested and is an important parameter in determining the overall proliferative outcome.

The extent of B cell proliferation depends on the mitogen but is intrinsically limited in all stimulation conditions

In the experiments presented above, it was observed that responding B cell populations reached a point at which proliferation ceased even when provision of the highest stimulus concentrations was maintained. These results suggested that an intrinsic limit to B cell proliferation capacity exists for a given stimulus, even in maximal stimulation conditions. This appeared to be true for all stimuli tested, although the onset of population decay occurred at different population division progression profiles (Fig. 4, A–C) and times (Fig. 4D) depending on the mitogen used. We hypothesized that this limit is an extension of the regulable division destiny parameter observed in conditions of restricted stimulus dose or duration.

We first sought to exclude the possibility that the limit to proliferation was imposed by cell overgrowth or insufficient access to mitogenic signals. Identical cell cultures in CpG stimulation were grown undisturbed or diluted by a factor of 10 before the point at which population decay commenced and then were supplemented with fresh medium alone. Cell numbers (A–C), the proportion of cells in each division (D–F) and the mean division number (G–I) were determined at various time points as described in Fig. 1. Each data point represents the mean of cell numbers or division numbers calculated from three culture wells.

**FIGURE 3.** The B cell proliferation response to defined stimulation concentration and duration. CFSE-labeled naive resting B cells were stimulated in vitro with anti-CD40 and IL-4, LPS, or CpG at the concentrations shown. At 40 (anti-CD40), 30 (LPS), or 27 h (CpG) cells were removed from culture, washed three times in medium, and returned to culture in medium alone. Cell numbers (A–C), the proportion of cells in each division (D–F) and the mean division number (G–I) were determined at various time points as described in Fig. 1. Each data point represents the mean of cell numbers or division numbers calculated from three culture wells.

**FIGURE 4.** B cell proliferation in response to repetitive in vitro stimulation. CFSE-labeled naive resting B cells were stimulated in vitro with 20 μg/ml anti-CD40 and 500 U/ml IL-4, 15 μg/ml LPS, or 3 μM CpG. Cells stimulated with CpG were resuspended after 70 h of stimulation and one tenth of the culture volume was transferred to new culture wells containing medium and CpG at the required concentration to return the cells to the original stimulatory conditions. Parallel cultures were maintained undisturbed. CFSE profiles of division progression (A–C) and calculated total live cell numbers (D, E) are shown at various time points during culture. Corrected cell numbers were determined by multiplying the actual cell number by the dilution factor (F). Each data point represents the mean ± SD from three replicate cultures.
Enforced survival does not extend B cell proliferation

Bcl-2 over-expressing B cells from transgenic mice have been used to demonstrate that survival and division progression are independently regulated (16). We used these transgenic cells to assess whether enforced survival could overcome the limit to division number observed during continual stimulation. Cultures of Bcl-2 transgenic cells proliferating in response to CpG stimulation were left undisturbed or split to reduce cell number and provided with additional stimuli. Cell number analysis revealed a plateau in population expansion in each case (Fig. 5A), in contrast with the cell death seen in cultures of identically stimulated nontransgenic cells. Our interpretation of this arrest is that the B cells were no longer cycling, however they remained alive due to the block in apoptosis caused by over-expression of Bcl-2. Indeed, CFSE profiles revealed that enforced survival did not lead to progression through >6 divisions (Fig. 5, B and C), the limit for CpG proliferation observed previously (Fig. 4). To ensure that division cessation in the Bcl-2 transgenic B cells was not complicated by dys-regulation of c-Myc (22), we repeated the experiment with Bim-deficient B cells, which are also long-lived (17). These B cells also stopped dividing at a similar time point and division profile to wild type B cells in CpG stimulation, yet remained alive for considerably longer (data not shown). These results indicate that the division destiny of the responding cells was not influenced by extending their survival.

B cells retain their division destiny even when proliferation is interrupted

The data presented in Figs. 4 and 5 revealed a limit to the in vitro proliferation of B cells under conditions of saturating stimulation. This feature of B cell responses operates independently of cell survival and can be well explained as representing the natural endpoint of a continuous scale of division destiny described in earlier sections. However, the existing data were insufficient to determine whether the proliferation limit was imposed by the time in culture or by the division number of responding cells. This is an important distinction as it may provide information on the mechanism by which B cell proliferation is regulated and insight into the control of contraction of B cell responses in vivo. A series of experiments was therefore designed to discriminate between these alternatives (Fig. 6).

Firstly, we used the observation that short stimulation duration leads to a truncated division destiny (Fig. 2) to conduct an experiment in which progression through divisions was interrupted for an interval of time. B cells were stimulated with LPS, removed from culture during the population expansion phase and washed thoroughly before being returned to culture in either medium alone or with LPS. As seen in Fig. 2, cells in cultures in which the stimulus was removed reached a lower total cell number than the restimulated cells and rapidly commenced dying (Fig. 6A). However, subsequent reprovision of LPS rescued these B cells from population decay and resulted in resumed proliferation (Fig. 6A). The second expansion phase also reached a maximal point, but at a later time point than in cultures stimulated continuously. CFSE profiles indicated that >8 divisions had been undergone in all cultures by the time at which maximum proliferation was reached (data not shown). This suggested that the reprovision of stimulus allowed cells in these cultures to reach a similar division destiny to those cells in undisturbed cultures, but that this occurred at a later time due to the proliferation arrest during the period of stimulus deprivation.

Low temperatures slow proliferation and delay the time of population contraction

The conundrum of whether cell fates are determined by counting division number or time has been addressed in other systems. During the examination of oligodendrocyte precursor cells it was found that changes in temperature could be used to explore the relative contribution of time and division based counting mechanisms (23). We adopted this strategy and investigated the effect of temperature on B cell proliferation. Replicate cultures of B cells identically stimulated with LPS were incubated at 32°C or 37°C. B cells cultured at the lower temperature took a significantly longer time to enter division (Fig. 6B). However, once proliferation commenced, the cells underwent multiple rounds of division in a normal fashion, as assessed by CFSE dilution (data not shown). Similar overall levels of proliferation were achieved by cell populations cultured at both temperatures, however the time at which the maximum cell number was reached occurred significantly later in populations of cells grown at the lower temperature (Fig. 6B). CFSE profiles revealed that cells grown at 32°C lagged behind in earlier divisions compared with cells cultured at 37°C at various time points throughout the experiment, but that these cells had also undergone 8 divisions by the time at which population decline began (data not shown). These results are consistent with the existence of a division-based regulator and argue against the conclusion that cells stop dividing after a given time.

B cells sorted from different divisions demonstrate a hierarchy of subsequent proliferation capacity

In a further experiment designed to distinguish between a time- or division-imposed limit to proliferation, proliferating B cells were
sorted according to the number of completed divisions and returned to separate cultures. Tracking the cells’ subsequent proliferation revealed that cells sorted from later divisions proliferated less extensively and entered population decay earlier than cells sorted from earlier divisions (Fig. 6C). All cell populations had reached CFSE autofluorescence by the time of growth cessation. This sorting experiment was repeated using B cells stimulated in T-dependent conditions using anti-CD40 and IL-4. As for LPS stimulation, cells sorted from early divisions had a greater proliferation capacity on return to culture than those sorted from later divisions (Fig. 6D).

The results of the experiments presented in Fig. 6 are all consistent with the hypothesis that a division-linked limit is the key regulator of the extent of B cell proliferation, not the time of response. This implies that populations of naive B cells have an intrinsic distribution of maximum division number and that this distribution is a continuation of the division destiny parameter described in earlier sections to be regulated by stimulation dose and duration.

**Division destiny is additionally influenced by B cell differentiation**

The differentiation of B cells to Ab-secreting cells (ASC) has been shown to be linked to division number (9, 11). Of the three stimulatory conditions used in this study, the number of ASC generated is most significant for LPS stimulation, reaching nearly 50% of the population after 4 days of culture (19). Thus, for the LPS stimulation system, it was possible that differentiation was contributing to the cessation of cell division. We used B cells from blimp-1-GFP reporter mice to assess whether ASC differentiation has an effect on B cell division destiny. LPS-stimulated cells were sorted into populations based on their relative division progression and GFP levels. Of the undifferentiated (GFP negative) cells, those sorted from earlier divisions exhibited the greater proliferative capacity on return to culture (Fig. 7A), an expected pattern in light of data presented in Fig. 6C. However, the behavior of the differentiated (GFP positive) cells was strikingly different, as these cells did not achieve any population expansion on return to culture (Fig. 7A). These results indicated that differentiated and undifferentiated
cells have very different division progression competencies and suggested that the process of differentiation is able to reprogram the division destiny of individual cells.

To assess whether the process of ASC differentiation has a direct influence on proliferation limitation, we exploited the ability of BCR stimulation to potently inhibit differentiation in LPS-stimulated cultures. Specific Ag HEL was added to cultures of HEL BCR-transgenic (SWHEL) B cells stimulated with LPS. Extensive proliferation occurs in these cultures but B cells do not progress to exhibit ASC phenotypic characteristics or secrete Ab (Ref. 24 and Fig. 7B). However, a limit to proliferation was still observed in these cultures, even when the cell density was reduced and additional stimulation provided (Fig. 7C). These results are in accordance with a model of B cell proliferation in which division destiny is an independent regulator of B cell division capacity but that differentiation may further alter the division destiny of a cell.

Discussion

Lymphocyte differentiation has been shown to be tightly linked to proliferation and cell division and is therefore a crucial determinant in the nature and effectiveness of the immune responses. The cyton model of Hawkins et al. (16) introduced a division destiny parameter to allow for description of lymphocyte proliferation cessation and population contraction. The inclusion of the division destiny parameter enabled the model to accurately fit data from both in vitro proliferation experiments and in vivo infection responses highly accurately and is therefore of significant relevance for a better understanding of lymphocyte behavior in vitro and physiologically. In this study, we sought to better understand the regulation of B cell division number and the rules governing the contraction of B cell responses.

We chose the strength of the activating signal as an appropriate starting point for investigating the regulation of the extent of immune responses. Naïve B cells responded to a variety of activating signals in vitro in a dose-dependent manner, manifested in the maximum cell number reached. The effect of stimulus dose on cell number did not appear to be due to significant differences in the relative survival rates of cells before first division, consistent with previous findings (16). However, it was clear that the number of subsequent divisions undergone was strongly affected by signal strength.

During these experiments, we noted that responding B cell populations followed a characteristic pattern of population growth, proliferation cessation, and subsequent cell death, even in conditions of continual stimulation at saturating doses. This pattern resembled a physiological immune response. The onset of population contraction occurred at different times under the various stimulation conditions and appeared to be an intrinsic feature of the responses, and not a consequence of restrictive culture conditions (Fig. 4). Furthermore, B cells forced to survive by transgenic over-expression of Bcl-2 also reached a maximum cell number and could not be driven to proliferate further (Fig. 5). These results suggested a limit to B cell proliferation that is imposed by the stimulation conditions and is independent of the survival capacity of the cells.

B cells have previously been shown to exhibit “division momentum”: a short period of continued in vitro proliferation following removal of T-dependent stimulation (14). However, these cells stop dividing earlier than continually stimulated cells. We extended these studies to show that naïve B cells were able to undergo multiple rounds of cell division following removal of T-dependent or -independent activating signals (Figs. 2 and 3). Furthermore, cells could enter and continue proliferation even when the stimulus was removed before the first mitotic event (Figs. 2 and 3). The striking conclusion for all three stimuli was that the duration of stimulus exposure was a strong regulator of the number of subsequent divisions achieved. Thus, both the concentration of stimulus and the time of exposure are able to regulate division number, and these variables can work in tandem to yield many possible quantitative outcomes.

These results can be compared with the reported behavior of T cells. Ag concentration has been shown to regulate the number of T cells recruited into the proliferative response and the subsequent number of divisions they reach (25–27). CD8+ T cells can continue to divide in vitro (28) or in vivo (29) even after the activating signal is removed. In fact, only a very brief pulse of stimulus is required to initiate multiple rounds of CD8+ T cell division and differentiation to cytotoxic effector and memory cells (30). CD4+ T cells that have experienced Ag encounter long enough to enter the cell cycle are also able to progress through further divisions in the absence of a continued TCR signal, although their subsequent proliferative program is not as striking as that of CD8+ T cells (15). Despite this “autopilot” behavior of proliferating T cells (13), there is ample evidence that the response can be regulated by stimulation conditions (15, 31, 32). We have shown in this study that the duration and dose of stimulation can operate alone or in tandem to determine the maximum B cell division number reached. These mechanisms for regulating division number are also seen in T cells (25, 33). B cell behavior appears to more closely mimic that of CD4+ than CD8+ T cells, in that proliferation can occur without prolonged or repeated Ag exposure, but that participating cells do not undergo as many divisions as would be achieved in the presence of continual stimulation.

A proliferation limit could be a cell-intrinsic characteristic or one imposed by extrinsic factors. Although extrinsic factors are highly likely to influence the behavior of B cells during physiological responses, the in vitro proliferation system used in these experiments is not cell density or contact dependent. In mixed cultures of B cells from late divisions with naive cells, there was no detrimental effect of the presence of the cycling cells on the proliferation capacity of the naive cells (data not shown). The data presented in Fig. 4 show that proliferating B cells reached the point of maximum population expansion at approximately the same time regardless of whether they were undisturbed or restimulated. This implied that either a time- or division-based limit is imposed on proliferating B cells. A series of experiments (Fig. 6) was designed to distinguish between the two possible mechanisms: division- or time-based control of proliferation cessation. Cells subjected to an interrupted stimulation regime (Fig. 6A) or cultured at low temperatures (Fig. 6B) proliferated to an equivalent degree to cells that received uninterrupted stimulation but achieved this over an extended time period. Furthermore, cells sorted from late divisions exhibited a lesser ability to continue proliferating than cells sorted from earlier divisions (Fig. 6C). These combined results strongly suggest that the basis for the contraction of B cell populations responding to continual stimulation is via a division counter. A plausible mechanistic explanation for this could be the dilution of an accumulated intracellular factor that occurs with each cell division (14). However, the data still do not formally exclude a time-based counter that operates from the time of entry to first division. In effect, these two mechanisms would produce very similar outcomes in B cell populations, given the asynchrony of time to enter first division (10, 16).

There are various examples of intrinsic intracellular timers that operate during developmental pathways and that may be triggered by time or division number (reviewed in Ref. 34). Normal somatic cells undergo only a finite number of cell divisions in vitro before reaching a state termed replicative senescence (35–37). The onset
of senescence is due to the effects of telomere shortening with each mitotic event and is therefore directly linked to cell division number and not time (38, 39). However, activated human and mouse lymphocytes up-regulate expression of telomerase, an enzyme that extends telomere length, which may function to support the capacity of lymphocytes to undergo extensive clonal expansion (reviewed in Ref. 40). Furthermore, Mus musculus telomeres are several times the length of those of humans, with mean lengths of 25–150 kb, while telomere shortening generally proceeds in lymphocytes at a rate of ~100 bp per population doubling (reviewed in Ref. 40, 41). In the system studied in this study, we are considering a relatively small number of cell divisions and telomere shortening is unlikely to play a significant role in initiating population contraction.

An intrinsic limit to T cell proliferation has been identified that is similar to that demonstrated in this study to exist in B cells (42). Badovinac and colleagues (42) showed that Ag-specific CD8+ T cells responding to infection in vivo were programmed to undergo contraction at a defined time and that the kinetics of contraction were independent of the magnitude of the response or the duration of the infection. This may appear contradictory to the results presented in this study in Fig. 2, in which the duration of stimulation dictated the division capacity of responding B cells. However, Badovinac and colleagues reported on an in vivo infection model and the time scale of their proliferation and contraction responses was understandably less fine than is possible for in vitro studies. Additionally, the shortest duration of infection they investigated was 24 h, which is likely to be long enough to induce very substantial CD8+ T cell proliferation. Furthermore, they demonstrated that contraction commenced at the same time point even in the presence of persistent viral infection. Badovinac and colleagues propose that a “hard-wired”, programmable mechanism exists to exert a limit to T cell proliferation, in agreement with our results for B cells.

We conclude that division number is a critical factor in determining the magnitude and duration of B cell responses. We refer to this intrinsic parameter as the population’s division destiny (16). We have shown in this study that division destiny may be regulated by the strength of stimuli (Figs. 1–3), the type of stimulus (Fig. 4) and the differentiation status of the cells (Fig. 7). We predict that other factors are likely to influence division destiny, including direct BCR stimulation, or the presence of cytokines or other costimulatory molecules, such as B cell activating factor of the TNF family. Exploring the effects of these molecules alone and in combination with the polyclonal stimuli used in this study on division destiny will complement these studies and provide further understanding of the role of division destiny in physiological B cell responses. The fact that Ag dose and duration of exposure primarily influence the recruitment of cells into a response and their subsequent division capacity is a characteristic that is shared by B and T cell proliferative responses. Division number strongly influences the differentiation and effector classes adopted by the responding B cells and it is therefore highly appropriate that this parameter be regulable by the stimulatory conditions. We have also shown that an intrinsic division-based limit operates to contain B cell proliferation; a feature that may have evolved as a safeguard to prevent against inappropriate overwhelming or recurrent responses.

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