Mechanisms of cell division as regulators of acute immune response

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Abstract

The acute adaptive immune response is complex, proceeding through phases of activation of quiescent lymphocytes, rapid expansion by cell division and cell differentiation, cessation of division and eventual death of greater than 95% of the newly generated population. Control of the response is not central but appears to operate as a distributed process where global patterns reliably emerge as a result of collective behaviour of a large number of autonomous cells. In this review, we highlight evidence that competing intracellular timed processes underlie the distribution of individual fates and control cell proliferation, cessation and loss. These principles can be captured in a mathematical model to illustrate consistency with previously published experimentally observed data.

Introduction

The immune system of vertebrates is markedly complex. Broadly, the system is organised into innate and adaptive components with the former characterized by rapid responses triggered by common features of infectious organisms(1), while the latter is responsible for slower but highly efficient pathogen-specific responses and the generation of long-lived protection and memory. The strength and speed of the adaptive immune response is in large part determined by the sheer number of different pathogen-specific B and T lymphocytes that must be made and maintained. To pre-empt the possibility of infection by millions of possible pathogens it is impossible to code for a target-specific clone for each possible pathogen in the germ-line. Instead, DNA coding for receptors is shuffled uniquely in each cell to create a broad range of specificities combinatorially as predicted theoretically by Burnet in 1957 (2). Then, during the acute immune response, the small number of specific pathogen-specific lymphocyte clones are selected, activated and expanded many fold by cell division resulting in cell numbers sufficiently large to clear the pathogen (Figure 1)(3). As such, cell division is one of the key mechanisms behind a successful immune response via an on-demand expansion of a pathogen-specific clone.

From a control perspective these features appear logical. The clonal expansion of lymphocytes must be rapid to address the threat on time and limited to a level sufficient for an effective outcome without overwhelming the host or commanding more resources than necessary. Furthermore, the expanded cellular pool must be reduced post infection or frequent responses would result in a massive accumulation of unnecessary cells. Recent studies show that these elements of control are implemented via parameters of individual cell division, survival or differentiation (4-8). For example, the speed of clonal expansion can be modulated by the average time it takes individual cells to divide or by interplay between cell division and death.

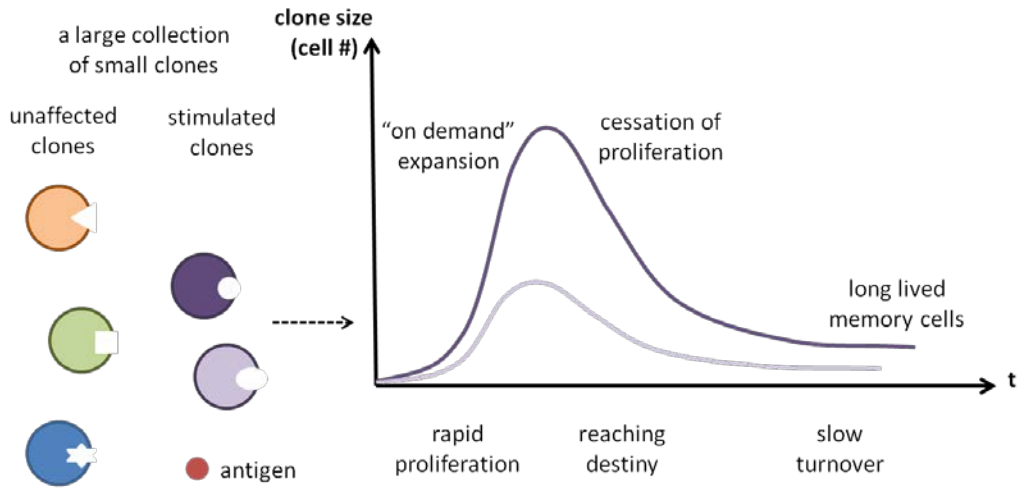

Figure 1: A triggered clonal expansion as a result of antigen stimulation. Resting cells have different receptor affinities. Clones with sufficiently high affinity get stimulated and proliferate. The level of affinity modulates the extent of proliferation.

A further prominent feature of cell responses is that significant variation underlies division and death times by homogeneous cell types. Times to first division after activation vary broadly, and after division of a B cell, the two daughter cells have different times until the next division, and in some cases, one daughter cell dies while another divides further. Therefore, this variation must be accounted for in models of the acute immune response. In this review, we first describe the hypothesis that competing division and death could underlie these complex features of immune regulation and how it could be extended to include differentiation as well. We then review recently published experiments (7-11) that directly test this hypothesis and serve to inform development of more sophisticated models of the adaptive immune response consistent with responses at both single cell and population levels. Our goal is to illustrate how this coupled theoretic-experimental approach can guide future research directions.

Competing division and death: The Cyton model

The Cyton model introduced by Hawkins et al (10) and adapted further by other authors (3, 12, 13) posited a hypothesis for how the complex proliferation, cessation and death curves, illustrated in Fig 1, could be explained by competing fates with stochastic times. In this model all cells are viewed as containing two independent molecular machines each with observable stochastic behaviour (10). The key concepts of the model are *competition*, *censorship* and *division limit* (Figure 2). In the original model, each cell can have either of two fates: division and death. The likelihood of each fate is the result of the corresponding molecular machinery. Furthermore, time it takes from cell birth (i.e., the previous division) to division and time it takes to death are described with two independent random variables. This assumption of independence of the two fate timers within the same cell is based on experimental results from both T cells and B cells (9) . When the model was formulated the pattern of inheritance of such times, and how they would proceed through multiple generations was unknown. The model proposed that both times are reset at birth, with no inheritance of times taken by parents, and whichever time runs out first determines the observed fate of the cell (i.e., ongoing competition within each generation). The other fate is

left unobserved (censored). For example, a particular molecular configuration at cell birth can predetermine durations of division and death processes to be 8 and 10 hours respectively. These processes then run independently, and the cell divides after 8 hours. Clearly, it is then not possible to observe death.

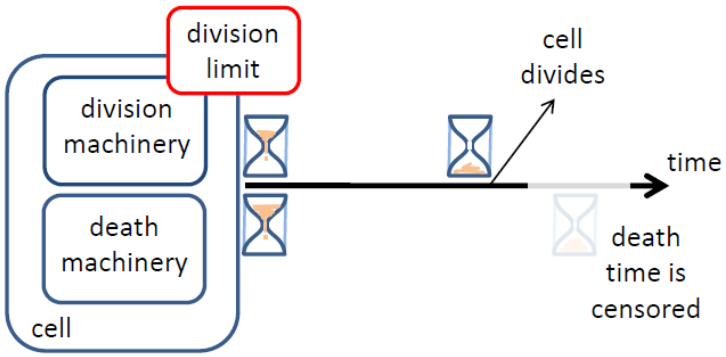

Figure 2: The operation of the Cyton model can be illustrated with two independent timers (hourglasses). Each timer is reset at cell birth with a different time. Whichever timer runs out first determines the fate of the cell, while the other fate remains unobserved. The division machinery is subject to division limit for a given clone. If the limit is reached (e.g., 3 divisions) then the division timer is not set, and death is the only fate.

Numerical solutions of this mechanism illustrated that variable rates of growth can be achieved by interleaving times, and that the system is highly sensitive to small differences (5).

The final feature of the original Cyton model is that the division machinery is subject to further internal regulation and a *division limit* for the given cell clone can be imposed by the stimulation conditions. This limit applies to the total number of times a progeny cell and its descendants can divide. Once the limit is reached, cells remain in G1 phase of the cell cycle, and death becomes the only possible fate for the cell. This model feature was supported by experimental evidence for B lymphocytes stopping dividing after a few rounds even if cell medium is sufficiently rich to support growth of the culture (8, 9). Evidence for a limit on division was also observed *in vivo* by Sze et al. (14). Incorporating this limit into the model offered a simple way to explain the growth and loss curves illustrated in Fig. 1 and typical of adaptive immune responses. The model could be used to fit directly to cell tracking data and time courses to extract features such as times to first and subsequent division, division times and the numbers of times to divide before stopping (5).

On the molecular level, such a model can emerge if there are two independent division and death machineries that start acting from cell birth. Here each molecular pathway implements a sequence of events leading to the corresponding cell fate. Due to a stochastic nature of intercellular reactions, or due to the stochastic nature of protein expression in each cellular machine, the time required to transit either pathways varies, but can be described with an appropriate probability distribution for a population of similar cells. As yet there is no theoretical reason to expect a particular probability density for the population, however empirically, for both division and death, long-tailed distributions such as lognormal or gamma are found to give excellent fits to time series data (7, 8).

While the initial Cyton model was consistent with lymphocyte proliferation kinetics and behaviour at single cell and population levels, the model was a mixture of experimentally

validated rules and educated guesses. Such guesses included the randomisation and resetting of times after each generation, the independence of all cells, including siblings, for times chosen and the setting of a division limit. The model also was ambiguous on some important mechanistic questions that did not alter predictions at population level. For example, the carriage of a division limit could have been a family property imposed on descendants of a single cell or operate as a stochastic choice regulated independently in all cells. To directly test the guesses and clarify the ambiguities, single cell imaging over time was developed and used(15). Furthermore, direct imaging allowed additional fates to be monitored offering a method to explore the interleaving and allocation of complex fate maps within families and populations more generally. As this series of experiments use B lymphocytes we briefly summarise the essential elements of their biology. Note that both T and B lymphocytes mediate the acute type response illustrated in Fig. 1 and the models developed below can be applied to both cell types.

B cell regulation

In vivo, resting B cells can become activated through different pathways(16). In general, the B cell receptor (BCR) captures foreign material and initiates B cell proliferation if additional activating signals are available (4, 17). Broadly, there are two types of additional signalling. In the T cell independent (TI) pathway, signals are provided via ligands of the Toll-like receptor (TLR) family(18, 19). For T cell dependent (TD) stimulation, B cells internalize the antigen bound to BCR and present protein fragments in context of class II Major histocompatibility complex (MHC) on the cell surface(20). Such a combination can now be recognised by T helper cells. Stimulated T cells in turn activate B cells via CD40 ligation and provision of secreted cytokines.

In vitro, TI and TD stimulation can be reproduced by treating naïve B cells with the activating ligands directly without BCR involvement. TI stimuli include CpG DNA which is an agonist of TLR9(17) and TLR4 stimulation by lipopolysacharide (LPS). B cells can be activated in TD manner using CD40 agonists along with cytokines such as interleukins 4 and 5 (IL-4, IL-5)(4). The dynamics of proliferating lymphocytes can be followed by flow cytometry using division tracking dyes (21, 22) and antibody markers to detect differentiated cells. B cells provided with cytokines such as IL-4, can switch from expressing immunoglobulin M (IgM) to IgG, and differentiate into antibody secreting cells (plasma cells)(23). These additional fates could be naturally incorporated in the Cyton framework by postulating the existence of additional internal timing mechanisms for isotype switch and differentiation. If correct the combinatorial features would ensure considerable heterogeneity (24). Thus, direct filming was used to test this hypothesis for additional fates, noting the important additional censorship rule that once a plasma cell switching no longer occurs (25). The state diagram for B cell censorship is illustrated in Fig. 3.

The goal of tracking single cells is complicated as dividing B cells in culture express strong adhesion molecules and form large aggregates, even before division, preventing unambiguous tracking. Two approaches were developed to avoid this constraint. In the first approach, Hawkins et al. (8) reported that CpG stimulated B cells do not differentiate and do not self adhere allowing individual cells to be followed for multiple generations. In the second, B cells are stimulated as usual in bulk culture then disaggregated and undifferentiated cells from different generations sorted by flow cytometry and allowed to settle into microwells. A single generation is then followed, cells are manually observed to divide and then the two siblings can be followed to their next fates. By limiting to one round, fates can be observed,

but family history is not possible. Here, differentiation to a plasma cell can be detected using the blimp1-GFP reporter (26), and isotype switch can be detected using IgG-APC stain (7).

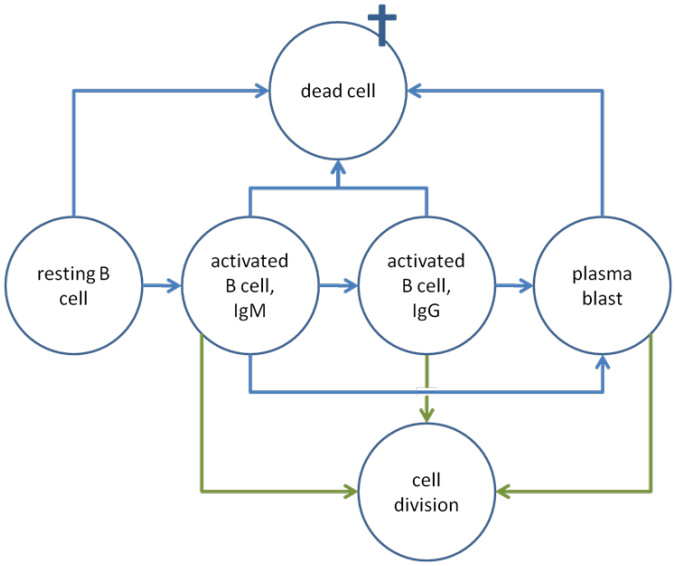

Figure 3: State diagram for B cells under certain conditions (see text). Arrows indicate potential state transitions rather than necessary transitions. The diagram illustrates that death can occur at any point, and division can occur for activated cells. Both death and division censor all other events, whereas differentiation into a plasmablast censors isotype switching.

In the next sections, we review the experimental testing of the Cyton model by direct imaging of *in vitro* stimulated B lymphocytes under different conditions.

Tracking CpG stimulated B cells

The filming of CpG B cells successfully reported new features and confirmed some of the basic premises of the Cyton model. The idea that division times would be randomised each generation and not inherited from parents held correct to a reasonable level. A surprising finding was that sibling cells had very similar division times, suggesting the molecular identity of the two daughters governed, and ensured, very similar fates and that randomisation of times was determined by the mother. In response further studies noted that this correlation does not affect model predictions of mean population responses, although variance would be underestimated (12, 27). A further important feature of this system was that cells divide 3-4 times and then lose motivation to divide again. Cells progressively get smaller before entering quiescence and eventually dying. The times to die in this final generation were considerably longer than the average times to divide and so it was not possible to use this system to confirm the presence of competition for the two fates. Rather cells died when motivation for division was lost and death was the only possible fate, which was consistent with competition, but could have other explanations. More confidently the experiments could remove the ambiguity regarding how the division limit was set. For B cells the number of divisions reached was found to be a strong family property and could be traced to the original founder cell (8).

Hallmarks of competition and censorship

Recall that among basic premises of the Cyton model are competition and censorship. Given these premises, the following effects were expected as a logical consequence (24). First, consider two cell fates A and B, such that fate B censors fate A (for example, B is division and A is isotype switching), and two associated probability distributions. The times to these fates are independently drawn from the corresponding distributions. Note that if time to fate A happens to be shorter than time to fate B, then both fates are observed for the cell (t_A, t_B, e.g., differentiation followed by division), otherwise, only one fate is observed due to censorship (t_B, e.g., division only). It follows that the stronger the competition (i.e., the larger is the overlap between the distributions), the more censoring happens and fewer pairs of fates are expected to be observed. Second, even though the original times to fates are independent, in the case when both fates are observed, there is an induced correlation between the observed times(24). Intuitively, if time to fate A is long, in order to be able to observe both fates, time to fate B must also be long.

Furthermore, consider mutually exclusive fates, division and death, in sister cells. In some cases, sister cells exhibit non-concordant fates for such cells: one cell divides and another one dies. If times to divide of sister cells are correlated and so are times to death then the times to non-concordant fates are expected to be correlated as well. That is, times to divide of one sister are expected to correlate with times to die of another, even though division is assumed to be independent to death. This happens because if division wins over death in one sibling, and loses in another, then times to divide and die for this pair of siblings must be close to each other.

It is such hallmarks of competition that were investigated by Duffy et al. (7). Activated B cells were cultured for 72 hours then sorted for non-switched non-differentiated cells into generations 0, 2, 4 and 6. These sorted populations were then filmed such that individual cells in divisions 1, 3, 5 and 7 could be tracked for one division. In total, several thousand cells were tracked and the times from their birth to isotype switch (becoming APC^+), differentiation (becoming GFP^+), division and death were recorded.

First, there are more cells that isotype switch and divide than cells that differentiate and divide as expected because division times distribution overlaps more with differentiation than with isotype switching, and hence censors differentiation more strongly. Second, there is a positive correlation in times to differentiate and times to divide for cells that experienced both these fates. Finally, there is a positive correlation between times to die of one sibling and times to divide of another sibling, which was expected by the model as explained above. Together these results demonstrate that experimental results are consistent with the Cyton model predictions and provide strong evidence that division and death, switching and differentiation are operating autonomously and in competition within each single cell(7, 24).

Different activation patterns in B cells

Having established the feasibility of the competing fates as a mechanism underlying extraordinary cellular heterogeneity at the single cell level as well as a family program for setting division limits, we now summarise how this model can be used to draw strong conclusions from population time series studies without the need to laboriously track single cells (4). In this study, B cells were activated *in vitro* via different pathways: TLR9 ligation by CpG, TLR4 ligation by LPS and CD40 ligation in presence of IL-4 and IL-5. Importantly, while total cell numbers varied with concentration of all these stimuli, division progression was remarkably different. The division progression was monitored using Cell Trace Violet

(CTV) which is a fluorescent division tracking dye (22). During CpG and LPS stimulation (through TLRs) proliferation patterns as uncovered by CTV profiles did not change with concentration. Furthermore, the distribution of times from activation to first division did not depend on concentration. Instead, the proportion of cells entering first division was dose dependent. In addition, LPS stimulation induced cell differentiation, and the proportion of differentiated cells again did not depend on concentration.

In contrast, during αCD40 stimulation the distribution of times to first division was dose-dependent, and cells appear to progress through each subsequent division faster with increased concentration. In particular, the proportion of differentiated cells decreased with increasing concentration, consistent with the idea of faster division times leading to stronger censorship of differentiation. Together, these results show that the evolutionary more ancient TLR ligation pathways lead to a simpler proliferation pattern where stimulation strength mainly affects only division progression, while a TD stimulation alters times to divide (Figure 4).

These conclusions were further validated using mathematical modelling. Predictions of the Cyton model for a set of single cell parameters were computed by a MATLAB software package. This software also included a procedure for deducing the most likely parameters for a given observed dataset. It was shown that the model that has all, but division limit related parameters fixed across concentrations offers a reasonable fit to CpG and LPS data, whereas a model with varying times to division was required in order to explain αCD40 data(4).

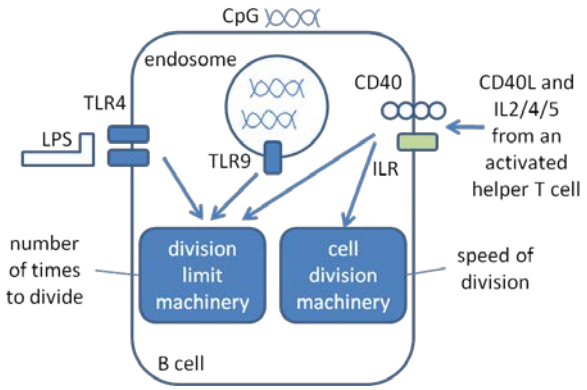

Figure 4: Different activation patterns of B cells. Cell division machinery is a hypothetical system associated with the speed of division, whereas division limit applies to the number of times a cell (clone) can divide. There is experimental evidence that the two machineries operate independently: CpG and LPS activation affects only division limit, whereas αCD40 activation affects both.

Relating single cell behaviour to population dynamics

Recall that the Cyton model describes times to different fates with random variables drawn from some empirically chosen distributions, such as the lognormal. Each distribution is then characterised by parameters, and consequently a particular population of cells can be concisely described numerically. This allows us to quantitatively relate individual cell behaviour with the dynamics of proliferation of stimulated cells. For example, consider a cell with two fates, division and death, and suppose that the distributions of times to divide and die are modulated by the strength of stimulus. Then small changes in relative mean positions of these distributions can lead to dramatic consequences for the expansion of a group of such

cells (10). In other words, changes in single cell machinery result in changes in population response patterns.

Interestingly, despite postulated independence of different fate machineries, cell division can affect, for example, differentiation rate. Indeed, consider a system in which cells have times to differentiation drawn from a fixed distribution, while the times to divide depend on the strength of the stimulus. Here, where division time gets shorter, division will censor more and more differentiation, and hence a smaller proportion of cells will differentiate. This possibility is currently being tested, but illustrates how interlinking multiple fates can evolve in complex ways and the consequences might be hard to predict.

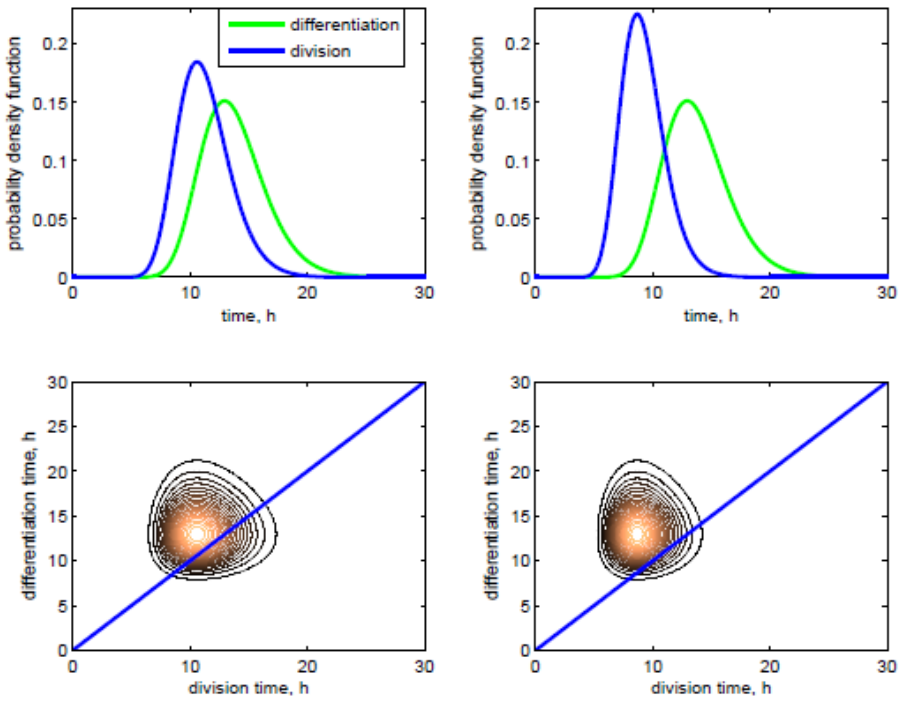

Figure 5: Differentiation rate can be controlled through division mechanism. In this artificial example, a distribution for times to differentiate is fixed (lognormal, mean 13.7h, st.dev. 2.8h), but distributions for times to divide vary (both lognormal; left mean 11.2h, st.dev. 2.3h; right mean 9.2h, st.dev. 1.9h). The bottom panels show corresponding bivariate distributions (contour plots, darker lines correspond to lower probabilities). Assuming no death or any other fates, cells with time to differentiate smaller than time to divide (below the blue line on the bivariate plots) will differentiate. In the first case (left), differentiated rate is about 24%, whereas in the second case (right), the rate is only 8%.

Discussion

In this report, we focus on the Cyton model and competing intracellular fates in general, as an explanation for how global features of the immune response can arise as a consequence of single cell parameters with emphasis on division. This model is based on three principles: competition, censorship and division limit. Recent findings show that predictions of these principles agree with experimental evidence, and hence the model offers a plausible explanation of observed cell population dynamics. As such, further explorations of molecular mechanisms that implement these principles offer an exciting future research path.

Specific research questions of interest now include identification of the molecular machinery for setting up the division limit and the mechanism for randomising the length of division and other fate regulating timed processes. Regardless of the final resolutions of the above research questions, one thing is already certain - competition for intracellular fates offers a powerful framework to link molecular determinism, cell based stochastic behaviour and reproducible population outcomes.

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