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Stochastic Model of T Cell Proliferation: A Calculus Revealing IL-2 Regulation of Precursor Frequencies, Cell Cycle Time, and Survival[1]

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The outcome of Ag exposure is dictated by complex regulation of T cell proliferation. The rates of proliferation and survival are altered by numerous signals that the cell receives and integrates to achieve a net response. We have illustrated previously how small changes in kinetic parameters can lead to large differences, even under conditions of saturating IL-2. In this study, we examine the effect of varying IL-2 concentration on T cell response and develop a model incorporating additional parameters of proliferation and survival. Strikingly, the proportion of cells that enter the first division, but not the time at which they enter, is dramatically altered by IL-2. Furthermore, the survival and average division time of cells in later divisions are also altered by IL-2 concentration. Together, the small simultaneous effects on these parameters result in large differences in total cell number. These results reveal how in vitro systems may exaggerate the contribution of IL-2, and thus how costimuli or additional helper cells that alter IL-2 concentration, even by relatively small amounts, will generate large in vitro differences in cell number and therefore appear obligatory. Furthermore, they illustrate how a quantitative model of T cell activation can clarify how complex signal integration is handled by T cells in situ, and therefore more appropriately aid development of a theory of behavior. ***The Journal of Immunology*****, 2003, 170: 4963–4972.**

The analysis of T cells in vitro has driven much of our development of theories of activation, including the concept of helper cells and APC-supplied costimulation (1–3). However, this approach has yielded consistent conceptual difficulties when explored in detail. For over 20 years, a central concept in our understanding of T cell activation has been that an Ag signal alone is insufficient, while an Ag signal plus another costimulatory signal leads to activation (4–8). The search for the molecular identity of the costimulatory signal has uncovered many candidates, both soluble and APC surface bound (3, 9–14). However, in most cases, the deletion of the costimulatory gene does not eliminate T cell responses in vivo (14–22). As a result, the meaning of the term costimulator has altered and is no longer used to imply an essential second signal. Typically, the term is now taken to mean any molecule that can increase activation of T cells under suboptimal stimulation conditions in vitro. This change in understanding of costimulation has created a gulf between our experiential understanding of T cell activation and one of our cherished theoretical paradigms, the two-signal theory (4–8). Adding to this confusing picture is the role of the T cell growth factor IL-2. In vitro experiments have long identified this autocrine cytokine as a critical, possibly essential, element in T cell proliferation (23–26). Furthermore, potent costimuli, such as agonists of CD28, act to a large extent by promoting production of this factor (9, 27–29). Nevertheless, mice deficient in IL-2 are able to mount diminished, but effective, immune responses (30–32). At best, it seems, qualitative models based on in vitro analyses have been misleading for the development of a theoretical understanding of the correct role for costimuli and growth factors in T cell activation.

To resolve these difficulties, we have explored the possibility of a quantitative understanding of T cell behavior that would enable us to assess the contribution of TCR engagement, costimulation, and IL-2 to the individual kinetic features of T cell proliferation. This more quantitative framework, we believe, will allow us to reconcile, and explore experimentally, questions of signal integration, and therefore achieve better insight into the regulation of T cells in vivo. Previously, we used a four-parameter model for describing T cell proliferation (33). Our approach assumed independent operation of the survival and proliferation components to behavior, and that variation in the population could be described by appropriate probability distributions. Thus, one parameter described the initial exponential rate of cell death exhibited by the population when cells were placed in culture, two parameters gave the Gaussian probability distribution for the time that the cells took to enter the first division, and the final (deterministic) parameter described the subsequent division rate (33). This model was developed to describe T cell proliferation in a system in which IL-2 was saturating; however, a complete model of T cell proliferation must also be able to describe the T cell behavior under conditions of changing IL-2 concentration, as occurs over time and with alterations to the range of costimulatory signals. When the four-parameter model was applied to data from T cells stimulated at different IL-2 concentrations, there were inconsistencies that could only be solved by incorporation of new parameters. In this study, we present a refined model that better accommodates the observed features of T cell proliferation when IL-2 levels are suboptimal.

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This amended model incorporates two new parameters: a precursor frequency for entry into the first division and a death rate for cells in division 1 or greater ($D1^+$ cells).[4]

Materials and Methods

Mice

Female B10.BR mice were obtained from the Animal Resource Center (Canning Vale, Australia). Mice were maintained under specific pathogen-free conditions and were used between 8 and 18 wk of age.

Reagents and Abs

CFSE was obtained from Molecular Probes (Eugene, OR). Human rIL-2 (rhIL-2) was purchased from Endogen (Woburn, MA). Murine IL-2 (mIL-2) was a gift of G. Zurawski (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). Anti-mIL-2 was purified from the hybridoma S4B6 (34, 35). Demecolcine was purchased from Sigma-Aldrich (St. Louis, MO). PE-conjugated anti-5-bromo-2′-deoxyuridine (BrdU)[5] and isotype control were purchased from BD PharMingen (San Diego, CA).

Cell preparation and culture

Cell suspensions were prepared from lymph nodes (axillary, brachial, cervical, inguinal, and para-aortic). Cells were enriched for $CD4^+$ T cells by complement lysis using a mixture of B220-specific (RA3.3A1), CD8-specific (31 M), and heat stable Ag-specific (J11d) mAbs. In some experiments, a CD25-specific (7D4) mAb was also included. The remaining cells were labeled with a mixture of Abs against B220 (RA3.6B2), I-A (b, d, q haplotypes) and $I\text{-}E^{dk}$ (M5/114.15.2), CD25 (PC61), and CD8 (YTS-169). Labeled cells were then depleted using anti-rat IgG DYNAbeads (Dynal Biotech, Oslo, Norway). The resulting purified $CD4^+$ T cells were further fractionated on the basis of CD62L expression by labeling with CD62L-biotin (MEL-14), followed by streptavidin MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were then passed through a MACS MS column held in a magnetic field by a MiniMACS magnet (Miltenyi Biotec). The resulting population was typically >93% $CD4^+CD62L^+$.

Cells were labeled with CFSE, as described previously (36). Culture medium was RPMI 1640 medium with L-glutamine (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Life Technologies), 5×10^{-5} M 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin, all from Sigma-Aldrich. T cells were stimulated at 3×10^4/200 μl with plate-bound anti-CD3 (145-2C11) in flat-bottom 96-well plates (BD Labware, Franklin Lakes, NJ).

For costimulation experiments, an anti-CD28 mAb (37.51) was added in soluble form.

Determining cell number

The absolute number of cells in culture at each time was determined by adding a known number of CaliBRITE beads (BD Biosciences, San Jose, CA) to each well before harvest. CaliBRITE beads and cells can be distinguished by flow cytometry on the basis of their forward and side scatter properties. The ratio of live cells to beads was then used to calculate the total number of cells in each culture (37). Flow cytometry was performed on a FACScan or FACSCalibur (BD Biosciences), and analysis was done using CellQuest (BD Biosciences) or FlowJo (Tree Star, San Carlos, CA).

The number of dead blasts found in each culture was an important additional data set to help constrain the fitting of the model to time course data. Previous studies have shown that CFSE-labeled dead cells retain the dye and can be identified by flow cytometry (38, 39). To determine the number of dead cells at each time of harvest, gates were set based on their distinctive forward and side scatter characteristics. Cells falling in the dead cell gate could clearly be categorized as having died as either an undivided cell, or while proliferating as a blast. Using these gates, the absolute number of dead undivided cells and blasts in each culture was determined with reference to CaliBRITE beads as for live cells (37). Using these methods, the fate of most of the input cells (>85%) could be accounted for over 4 days (data not shown).

Determining cells in each division

CFSE Modeler (ScienceSpeak, Canberra, Australia) was used to determine the number of cells in each division from CFSE profiles (33). Precursor cohort analysis was performed, as described previously (33). Briefly, precursor numbers were calculated by dividing the number of cells in division i by 2^i. A Gaussian distribution was fitted to the plots of precursor number against division number using nonlinear regression analysis in GraphPad Prism (GraphPad Software, San Diego, CA). The means of the fitted Gaussian distributions were plotted against time, and straight lines were fitted using linear regression analysis in GraphPad Prism.

Modeling

The number of cells expected in each division was calculated in a modified manner to that described previously (33). The new model incorporated the additional parameters p, the proportion of the cell population that will participate in division (the precursors), and d, the proportion of a cohort of cells that die while traversing a complete cell division (for division numbers ≥ 1). As previously described (33), the variation in the time of entry of cells into first division within the population is assumed to follow a Gaussian (or, in some instances, a log-Gaussian) distribution given as ϕ with mean and SD μ and σ, respectively. Once the cells have entered their first division, it is assumed they divide with equal subsequent division time (b), although this is a simplification. As for the previous model, it is assumed that the rate of death of undivided cells proceeds according to an exponential decay rate constant (k) independently of activation up until the first division (33).

When a number of starting cells (N_0) are placed in culture, the number of live and dead cells that are to be found in each division i (in which i is an integer) when the culture is harvested at time T_f (given in hours) can be calculated if all parameters are given.

Live undivided cells ($i = 0$) will comprise nonprecursor cells and precursor cells for whom their designated time to division is greater than T_f. It is assumed both are subject to the same rate of exponential death. Therefore, at T_f, the number of live nonprecursors (NP_0) is given by:

$$\mathrm{NP}_0(T_f) = N_0(1-p)e^{-kT_f}$$

And the number of undivided precursor cells (P_0) is essentially:

$$\mathrm{P}_0(T_f) = N_0 p e^{-kT_f} \int_{T_f}^{\infty} \phi(t)dt$$

To calculate the above term and subsequent values of cells in each division, the appropriate integrals were solved numerically using the trapezoidal rule. To do this, the distribution was split into 15-min intervals (up to 240 h), with time of entry into first division given by t (in which t is in hours). Therefore, we calculated the total number of precursors in division 0 (P_0), as follows:

$$\mathrm{P}_0(T_f) = N_0 p e^{-kT_f} \sum_{t=T_f}^{240} \left(\frac{\phi_t + \phi_{(t+0.25)}}{2} \times 0.25 \right)$$

And the total number of live cells in division 0 (L_0) is:

$$\mathrm{L}_0 = \mathrm{NP}_0 + \mathrm{P}_0$$

All precursor cell cohorts with a time to divide less than T_f will have divided at least once. For these cells, we need to calculate how many have entered division, which division they are now in, how many remain alive, and how many dead cells have been left behind in each division.

Thus, the total number of precursors in division i (for $i > 0$) is given by:

$$\mathrm{P}_i(T_f) = N_0 p \int_{T_f-ib}^{T_f-(i-1)b} e^{-kt}\phi(t)dt$$

The e^{-kt} term is used instead of e^{-kT_f}, as these cells have entered division and are therefore no longer subject to the exponential decay of undivided cells.

For $i > 0$, the number of live cells in division i (L_i) is based on this precursor number, but also takes into account the doubling of cell number

[4] Abbreviations used in this paper: BrdU, 5-bromo-2′-deoxyuridine; hIL, human IL; mIL, murine IL.

with division and the proportion of cells that die each division. Cells are assumed to die evenly across the division.

$$L_i(T_f) = 2N_0 p\,(2(1-d))^{i-1} \int_{T_f-ib}^{T_f-(i-1)b} e^{-kt}\,\phi(t)\left(1-d\left(\frac{(T_f-t)-(i-1)b}{b}\right)\right)dt$$

Where the term $(2(1-d))^{i-1}$ accounts for the cell death and doubling in previous divisions, the term

$$\frac{(T_f-t)-(i-1)b}{b}$$

gives the fraction of how far the cells are through the current division, and thus

$$d\left(\frac{(T_f-t)-(i-1)b}{b}\right)$$

gives the proportion of cells that have died in the current division.

The number of dead blasts that are found in division i is given by:

$$D_i(T_f) = 2N_0 pd\,(2(1-d))^{i-1}\left(\int_0^{T_f-ib} e^{-kt}\,\phi(t)dt + \int_{T_f-ib}^{T_f-(i-1)b} e^{-kt}\,\frac{(T_f-t)-(i-1)b}{b}\,\phi(t)dt\right)$$

Thus, the total number of dead blasts is given by:

$$\mathrm{D_b}(T_f) = \sum_{i=1}^{\left\lceil \frac{T_f}{b} \right\rceil} \mathrm{D_i}(T_f)$$

Where

$$\left\lceil \frac{T_f}{b} \right\rceil$$

gives the least integer greater than or equal to

$$\frac{T_f}{b}.$$

A Microsoft Excel (Microsoft, Redmond, WA) spreadsheet (xcelmodel) was used to give numerical solutions to the above equations. The model predicted values for total number of live cells per division and total number of dead blasts, and was then compared with experimental data, and the parameters were fitted to obtain the least sum of squares between predicted and experimental data. The values for N_0 and k were predetermined by the number of cells known to be added to culture and the exponential decay seen in nonstimulated cultures

BrdU analysis

Cultured T cells were pulsed at day 4 with 100 μg/ml BrdU (Sigma-Aldrich) for 4 h before harvesting. Cells were fixed in 2% formaldehyde for 10 min. Tween 20 (ICN, Irvine, CA) was then added to give a final concentration of 0.5% formaldehyde and 0.1% Tween 20 and incubated overnight. DNase I (Boehringer Mannheim, Mannheim, Germany) was added at 100 μg/ml (in 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 100 μg/ml BSA) for 30 min at 37°C before BrdU incorporation was detected using an anti-BrdU Ab.

Demecolcine analysis

For direct analysis of time of entry into first division, cells were stimulated in the presence of 5 ng/ml demecolcine (40). Flow cytometric analysis of CFSE-labeled cells confirmed that there was no division in the presence of demecolcine (data not shown). After various times, cultures were pulsed with [^{3}H]TdR (ICN; 1 μCi/well) for several hours before harvesting. Incorporation of radioactivity was measured using a Betaplate counter (Pharmacia-LKB, Uppsala, Sweden). Log-Gaussian distributions were fitted to the data using nonlinear regression analysis in GraphPad Prism.

Results

Quantitative effects of IL-2 on T cell proliferation kinetics

To examine the quantitative effects of IL-2 concentration on T cell proliferation, CFSE-labeled cells were stimulated with anti-CD3 in the presence of an anti-mouse IL-2 mAb (S4B6) and various concentrations of hIL-2. hIL-2 is active on mouse T cells while being resistant to the inhibitory effects of the S4B6 Ab. As noted previously (42), cells divided asynchronously so that at any time point cells can be found spread across a range of divisions (Fig. 1*A*). At higher concentrations of IL-2, the cells progressed through a greater number of divisions so that after 99 h, most cells in cultures activated in the presence of 50 U/ml of IL-2 were found in division 4 or 5. In contrast, in cultures exposed to only 1.25 U/ml, very few cells had reached division 5 by this time, with most cells being found in divisions 2–4. This increased progression through division may result from a decrease in the time to first division, from a decrease in subsequent division time, or from a combination of the two.

As a first attempt to determine the average time to first division (μ_{tdl}) and the subsequent division time (b) of T cells in the above cultures, precursor cohort plots were constructed (Fig. 1*B*) and Gaussian curves were fitted, as described previously (33). The mean division numbers were then plotted against time, and linear regression analysis was performed (Fig. 1*C*). The reciprocal of the slope of the resulting line gives an indication of the average subsequent division times (b) and the intercept with division 1 the mean time to first division (μ_{tdl}) (33). By this method, the intercept with division 1 was relatively constant at ~50 h for 50, 5, and 2.5 U/ml of hIL-2. In contrast, the slope of the line decreased as IL-2 concentration was lowered consistent with progressively longer division times. When plotted in this way, data for 1.25 U/ml of IL-2 were difficult to fit with Gaussian curves, as the cells had not moved far enough into division and the fitting was therefore subject to unacceptably large errors. Nevertheless, this analysis suggests that IL-2 concentration was not altering the average time to first division, while exerting a profound effect on the subsequent division rate.

A six-parameter model: a variable precursor frequency and variable rate of death in dividing cells

It was our intention to develop a useful quantitative model that could predict the number of stimulated T cells found in culture at various IL-2 concentrations. Previous analysis of cell numbers after stimulation using the four-parameter model of proliferation was done in the presence of saturating IL-2, and did not take account of death of cells in divisions after 0 ($D1^+$ cells). In the experiments described in this work, cultures containing low IL-2 concentrations displayed a proportionally large number of dead cells (data not shown). Thus, the earlier described four-parameter model required modification to resolve the proliferation behavior of cells in the presence of suboptimal IL-2. For this reason, a more complete model of proliferation was developed that incorporated a parameter describing the proportion of $D1^+$ cells that die in each division (d). This was assumed to be constant for all divisions after the first. The death rate per division of $D1^+$ cells was subtracted from each division and assumed to operate in a linear manner such that cells harvested halfway through a division will have lost one-half of the designated proportion of cells.

In addition to the above modification, a precursor frequency parameter (p) for cells entering the first division was introduced, as preliminary investigations revealed that, under suboptimal stimulation conditions, not all cells will be stimulated to divide (data not shown). The precursor frequency was introduced as a proportional multiplier of the total input cell number and essentially altered the area under the time to first division probability distribution. The method for calculating the predicted number of cells found in each division at a nominated culture harvest time based

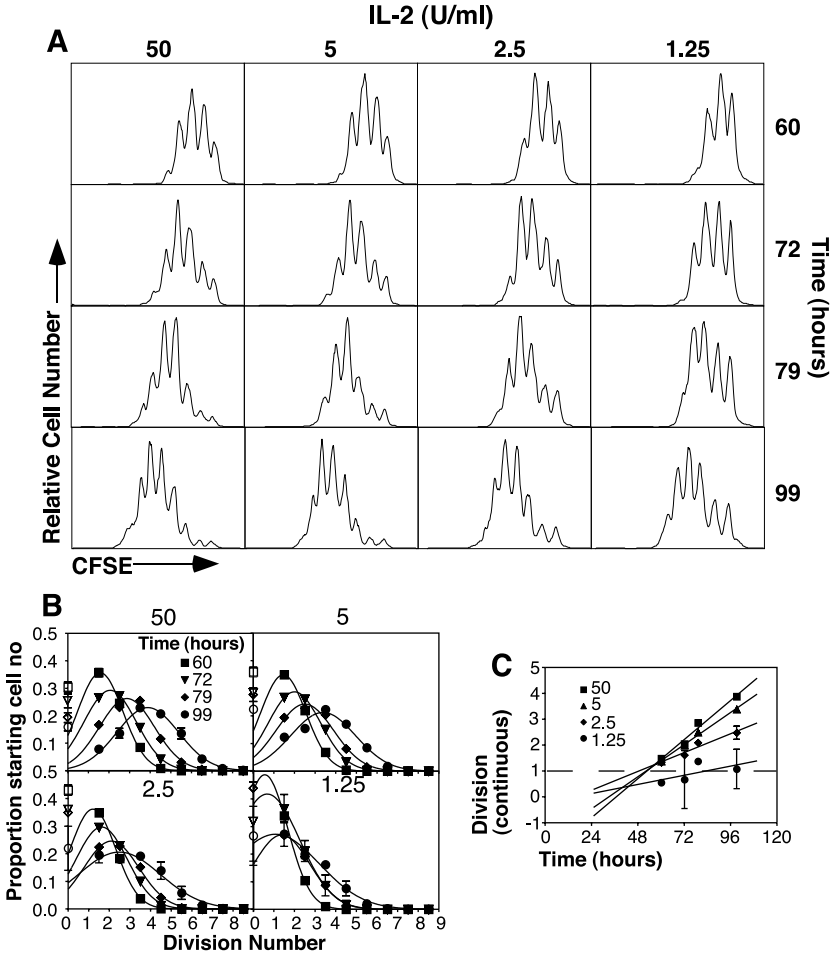

FIGURE 1. Time to first division analysis of the effect of IL-2 concentration on $CD4^+$ T cell proliferation. CFSE-labeled naive $CD4^+$ T cells were stimulated with anti-CD3 (40 μg/ml) and various concentrations of hIL-2, as indicated in the presence of anti-mIL-2 (S4B6, 50 μg/ml). Cells were harvested at 60, 72, 79, or 99 h, as shown. *A*, CFSE profiles of stimulated cells. *B*, Precursor cohort plots. The proportion of cells in each division was determined from CFSE plots for each time point. The proportion of starting cells in each division was calculated by dividing this number by 2^i (in which i is the division number) and normalizing to 1. These values were then plotted against continuous division number for each harvest time, as indicated, and Gaussian distributions were fitted using GraphPad Prism. The proportion of cells in division 0 was not used for this fitting and is shown by the open symbols. *C*, The means of the fitted Gaussian distributions were plotted against time for each IL-2 concentration. Lines were fitted to the data using GraphPad Prism for linear regression analysis. Results are representative of three independent experiments.

on the use of six parameters is described in detail in *Materials and Methods*.

*IL-2 concentration does not alter the rate of initial cell death (*k)

The new six-parameter model described above was to be used to analyze experimental data to extract values for each parameter, if possible. When fitting a complex model in this way, it is useful to constrain as many parameters as possible. One possible parameter that we reasoned might be fixed was the rate of cell death when cells were first placed in culture. Previously, we found that cell survival followed an exponential curve and was not affected for the first 30 h in culture by the presence or absence of anti-CD3 (33). To examine the effect of different concentrations of hIL-2 on the rate of cell death, CFSE-labeled naive $CD4^+$ T cells were placed in culture in the presence of varying concentrations of hIL-2. The cultures were harvested at various time points, and the absolute number of live cells was determined. As shown in Fig. 2*A*, a significant proportion of cells dies within the first 24–48 h of culture; however, this rate of death did not appear to be altered by the concentration of IL-2 present. Exponential decay curves were fitted to the data using GraphPad Prism (Fig. 2*A*), and the decay constants obtained were plotted against IL-2 concentration (Fig. 2*B*). The results of this fitting confirm that IL-2 does not significantly alter the initial rate of death when cells are placed in culture. Thus, the value of the exponential decay constant could be determined for unstimulated cultures and assumed to be identical for cultures containing various concentrations of IL-2.

Fitting of data to six-parameter model

The new six-parameter model was then used to more closely examine the effect of IL-2 concentration on all aspects of T cell proliferation. CFSE-labeled naive $CD4^+$ T cells were stimulated with anti-CD3 in the presence of the mIL-2-neutralizing Ab S4B6 and various concentrations of hIL-2. The cells were harvested at various times, and the total number of live and dead cells (both blast and undivided) was determined, as described in *Materials and Methods*. As observed previously, there was a steep decrease in cell number at the beginning of culture (Fig. 3*A*). Consistent with the above data and previous results, this cell death was similar for all cultures until ~40 h. At high concentrations of IL-2, however, the cell number began to increase after 48 h as the cells began to divide. This increase in live cell number was accompanied by the appearance of dead blasts. In contrast, at low concentrations of IL-2, the cell number did not increase again, but remained in decline. As observed earlier, IL-2 concentration also affected the progression of cells through division (Fig. 3*B*).

The six-parameter model was fitted to the data for total cells found in each division by reducing the sum of squares for live cells per division and total dead blasts. Values for each parameter could be found such that fitted lines closely followed the experimental data both in terms of total cell numbers (Fig. 3*A*) and cells per division (Fig. 3*B*). The optimum values for each parameter determined for each IL-2 concentration are plotted in Fig. 3, *C–G*. The fitting again suggested that IL-2 did not alter the mean time taken to enter the first division (Fig. 3*C*). In contrast, IL-2 concentration

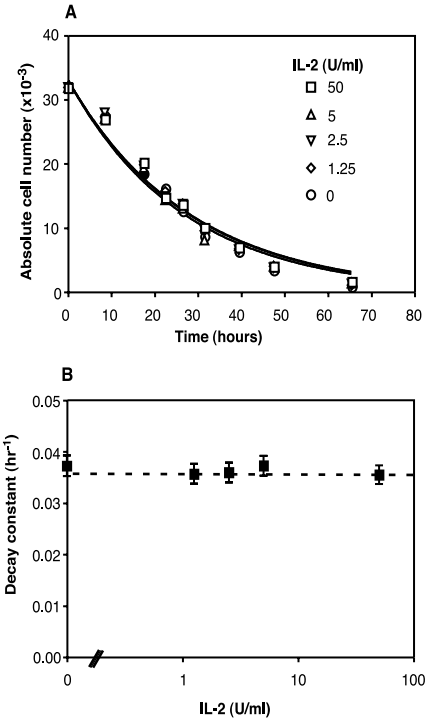

FIGURE 2. IL-2 concentration does not affect the initial rate of death when cells are placed in culture. CFSE-labeled naive $CD4^+$ T cells were placed in culture without anti-CD3 stimulation. hIL-2 was added at concentrations shown. *A*, The points (mean ± SEM of triplicate cultures) represent the number of live cells at various harvest times. The solid lines represent fitted exponential decay curves ($y = e^{-kt}$) determined by finding the least sum of squares using GraphPad Prism. *B*, Shows a plot of the *k* values obtained by fitting for various concentrations of IL-2. The absolute value of *k* varied between experiments; however, IL-2 was not found to affect this variable in four independent experiments.

dramatically affected the precursor frequency, division time, proportion dying per division, and SD of time to first division (Fig. 3, *D–G*).

Independent testing of parameters confirms predictions of model fitting

Although the six-parameter model fitted well to the experimental data, independent tests of the predictions and assumptions of the model were conducted to verify the accuracy of the model and the conclusions reached.

IL-2 affects the division rate of T cells

Initially, the prediction of the model that increasing IL-2 concentration would decrease the average division time consistently across all divisions was tested by exposing cells to a brief pulse (4 h) with BrdU. As the cells are unsynchronized with respect to position in cell cycle, only those that enter S phase within the pulse time are labeled. The faster the cell population is dividing, the more cells that will enter S phase in the pulse period, and thus the higher the percentage of $BrdU^+$ cells will be. Cells were labeled with CFSE so that BrdU incorporation and division number could be assessed simultaneously (Fig. 4*A*). The proportion of $BrdU^+$ cells was constant across divisions greater than 1 (Fig. 4*B*), as previously reported for saturating concentrations of IL-2 (42), reflecting the relatively constant rate of proliferation once the cells have begun proliferating. At lower concentrations of IL-2, the percentage of $BrdU^+$ cells was decreased, revealing a reduced proportion of cells in S phase consistent with a decreased division rate in these cultures. This change in BrdU incorporation with IL-2 concentration (Fig. 4*C*) mirrors the effect seen on division time in our model fitting (Fig. 3*F*).

IL-2 regulates the proportion of cells entering first division

Next, the entry into the first division was investigated directly. To measure the time the cells took to enter the first division, a previously reported system that uses the cell cycle inhibitor demecolcine (40) was used. Demecolcine inhibits cells in metaphase of the cell cycle; thus, the cells are able to replicate their DNA, but do not undergo cell division. As a consequence, DNA replication is only possible from cells entering their first division. Cultures containing demecolcine were pulsed briefly with [^{3}H]TdR at varying times to detect DNA replication. Fig. 5*A* shows that cells cultured in the presence of higher concentrations of IL-2 incorporated greater levels of TdR; however, the time at which the cells began to incorporate TdR was not noticeably altered. Furthermore, the increased accuracy of the demecolcine method of measuring time to first division allowed a finer resolution of the distribution of cells entering division. Fitting analysis with GraphPad Prism revealed that this distribution more accurately approximated a log-Gaussian rather than the Gaussian curve we had used previously. When log-Gaussian curves were fitted to the data (Fig. 5*A*), the mean, width, and area under the curve for each IL-2 concentration could be extracted. The mean and width of the time to first division distribution decreased slightly with decreasing IL-2 concentration (Fig. 5, *B* and *D*). In contrast, the area under the curve was dramatically reduced at lower concentrations of IL-2 (Fig. 5*C*). The area under the curve reflects the precursor frequency, so this result confirms the prediction from fitting the six-parameter model that IL-2 would act to increase the number of cells that are able to enter the first division.

Anti-CD3 concentration affects time to first division and precursor frequency

The result that IL-2 did not greatly affect time to first division was surprising, so the ability of the demecolcine system to detect changes in time to first division was tested. Cells were stimulated with suboptimal concentrations of anti-CD3, and time to first division was measured using the demecolcine system. Reducing the concentration of anti-CD3 decreased the level of TdR incorporated (Fig. 5*E*) and seemed to delay the onset of proliferation. Fitting of log-Gaussian curves to the data revealed that, in contrast to the effect of IL-2, decreasing the anti-CD3 concentration increased the mean time the cells took to enter the first division (Fig. 5*F*). However, in common with IL-2, the strength of stimulation through CD3 also altered the proportion of cells able to enter the first division (Fig. 5*G*).

A restricted window for entry into division: delayed addition of IL-2 cannot rescue cells

Given that the IL-2 concentration determined the proportion of cells that could enter division at a time determined by the anti-CD3 concentration, we examined the outcome on entry of cells into division when IL-2 addition was delayed by up to 48 h. We reasoned that if sufficient IL-2 was not available at the time cells were due to enter division, later addition of IL-2 would rescue these cells, recruiting them into division. As a result, we would expect to see the same area under the TdR incorporation curve, regardless of when the IL-2 was added. Cells were stimulated with anti-CD3, and IL-2 was added at the start of culture or after 24, 34, or 48 h, and the entry of cells into division was measured using the demecolcine system. Cultures that received delayed addition of IL-2 displayed reduced incorporation of TdR for at least 20 h after the

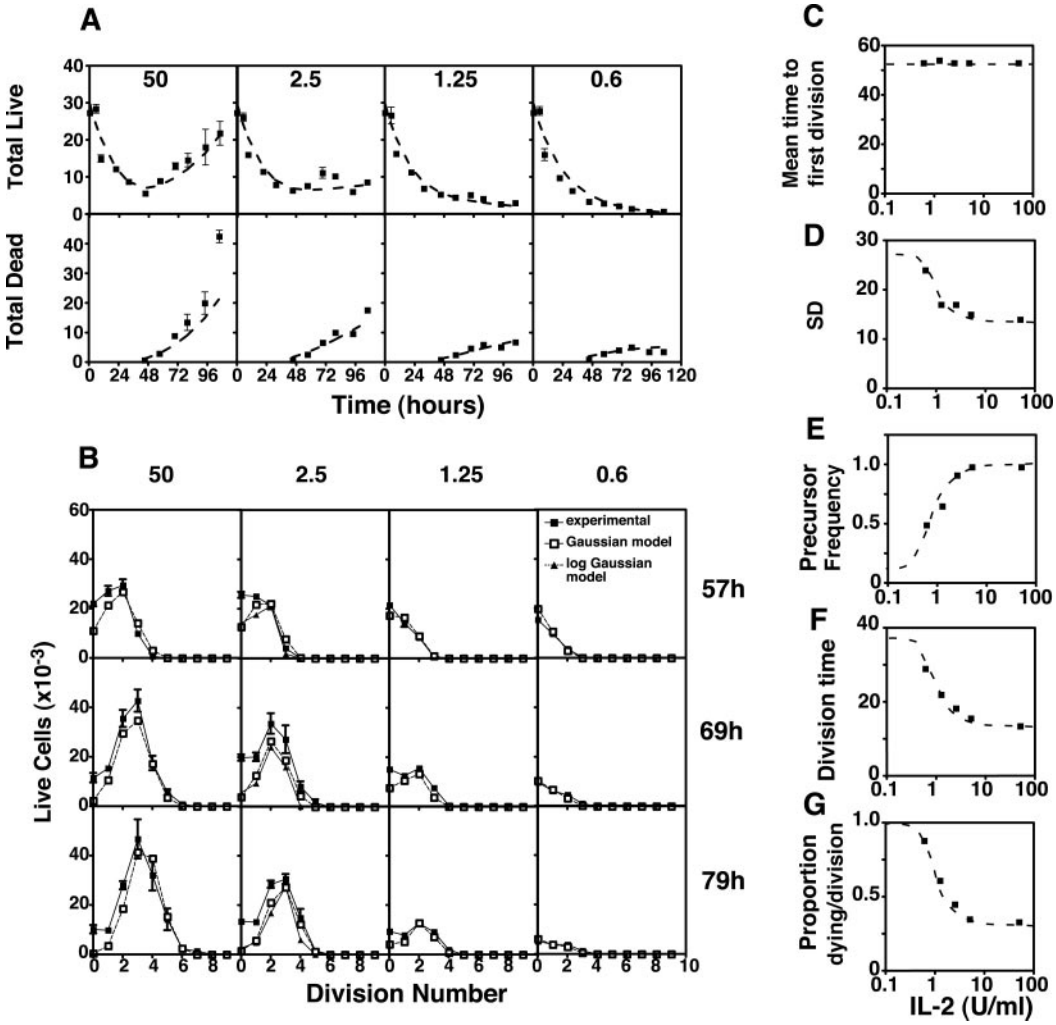

FIGURE 3. Fitting of data to six-parameter model. CFSE-labeled naive $CD4^+$ T cells were stimulated with anti-CD3 in the presence of anti-mIL-2 (S4B6, 50 μg/ml) and various concentrations of hIL-2, as shown. Cells were harvested at various time points, and the total number of live cells, dead blast, and dead undivided cells was determined for each harvest time. CFSE plots were used to calculate the number of live cells in each division. The best fit of the six-parameter proliferation model was found by reducing the sum of squares with reference to the total number of dead blasts and the number of live cells in each division. The same value, 0.043 h^{-1}, was used for the decay constant for all IL-2 concentrations based on the death curves, while the other five parameters were varied. *A,* Plots show the absolute number of live cells (*top panels*) and dead blasts (*lower panels*) at various times. Points represent the mean ± SEM of triplicate cultures. Dashed lines represent the numbers generated from the fitted model. *B,* Plots show the number of live cells in each division at various time points, as indicated. The experimental data (■) and fitted Gaussian model (□) are shown for each IL-2 concentration. Fitted values for the log-Gaussian model (△) are shown only for the 2–5 U/ml IL-2 culture. Points for experimental data represent the mean ± SD of triplicate cultures. *C–G*, Plots represent effect of IL-2 concentration on mean (*C*) and SD (*D*) of time to first division, precursor frequency (*E*), division time (*F*), and proportion of cells dying by division (*G*), as determined by fitting of the model.

IL-2 was added before reaching similar levels as the control cultures (Fig. 5*H*). This result indicates that 20 h was required for IL-2 to achieve its full effect on promoting the entry of cells into division. Furthermore, when IL-2 addition was delayed, the area under the curve was much lower than that of cultures that were continuously exposed to IL-2, implying that cells were unable to be rescued. That is, if a cell has not been exposed to sufficient IL-2 when its time to divide occurs (as determined by factors such as the level of anti-CD3 stimulation), the opportunity to divide is lost.

Discussion

A combination of modeling methods and additional confirmatory experiments was used in this study to dissect the manner in which IL-2 promotes T lymphocyte growth. A summary of the conclusions is depicted in Fig. 6. T cells when placed in culture begin to die in a manner that can be approximated to an exponential decay curve. The presence of IL-2 does not alter this rate of death over the first 24 h. Strikingly, the average time that cells took to enter the first division was also not greatly altered by the concentration of IL-2. In contrast, the apparent precursor frequency was dramatically altered, such that far fewer cells within the population entered division in the presence of low levels of IL-2. Furthermore, cells cultured at low concentrations of IL-2 were found to take longer to complete subsequent divisions, and a larger fraction of them died before the next division was undertaken. The quantitative outcome of these changes could be accommodated within a six-parameter model that accurately described total cell numbers and number of live cells in each division at various times after initiation of culture (Fig. 3).

When the precise value of each parameter is determined for each IL-2 concentration, as shown in Fig. 3, the manner in which subtle simultaneous effects on entry into division, division rate, and survival converge to induce large changes in cell number is revealed. For example, 30,000 T cells placed in culture with anti-CD3 and

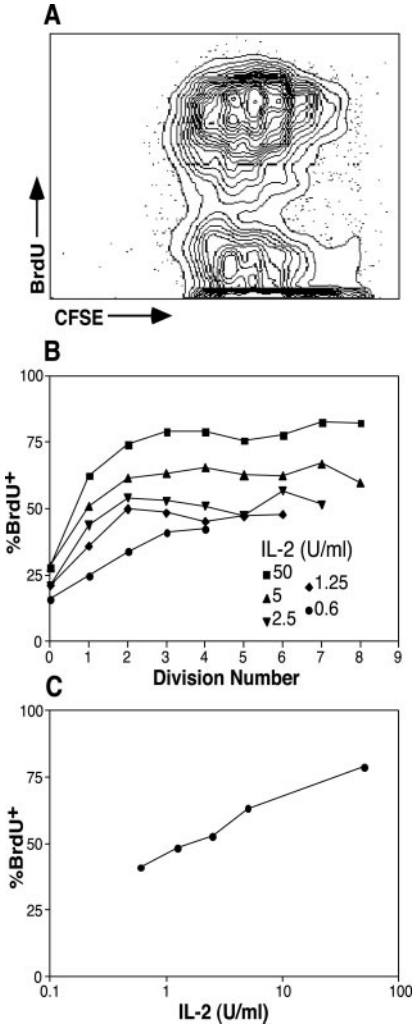

FIGURE 4. BrdU incorporation is decreased at low IL-2 concentrations. Naive CD4$^+$ T cells were stimulated with anti-CD3 (40 μg/ml) in the presence of anti-mIL-2 (50 μg/ml) and various concentrations of hIL-2. After 4 days, cells were pulsed with BrdU for 4 h. Cells were then harvested, fixed, permeabilized, and stained with anti-BrdU. *A*, Contour plot shows BrdU staining against CFSE for cells stimulated with 5 U/ml IL-2. *B*, The percentage of BrdU$^+$ cells is plotted against division number for each IL-2 concentration, as indicated. *C*, The percentage of BrdU$^+$ cells in division 3 plotted against IL-2 concentration. Data are representative of three independent experiments.

an IL-2 concentration in culture of 1 U/ml will yield only 1,700 live cells after 5 days. However, raising the IL-2 concentration to 3 U/ml will increase the number of live cells after 5 days to over 18,000. This 10-fold increase in cell number results from only a 30, 40, and 40% change in the parameters death per division, precursor frequency, and division time, respectively. This example helps illustrate how in vitro systems are highly sensitive to manipulations that alter, even a small amount, the total IL-2 concentration. The amount of IL-2 produced by TCR-stimulated T cells alone is weak. Nevertheless, it is not zero, and high levels of stimulation and/or high cell culture densities are able to generate sufficient IL-2 to initiate proliferation (Fig. 5*H*). Costimulation assays favor the use of low density and suboptimal stimulation, such that the endogenous IL-2 concentration will not sustain significant proliferation. Under these conditions, even small increases in IL-2 production induced by a costimulator, such as CD28 ligation, can yield significant increases in cell number. As a result, data that

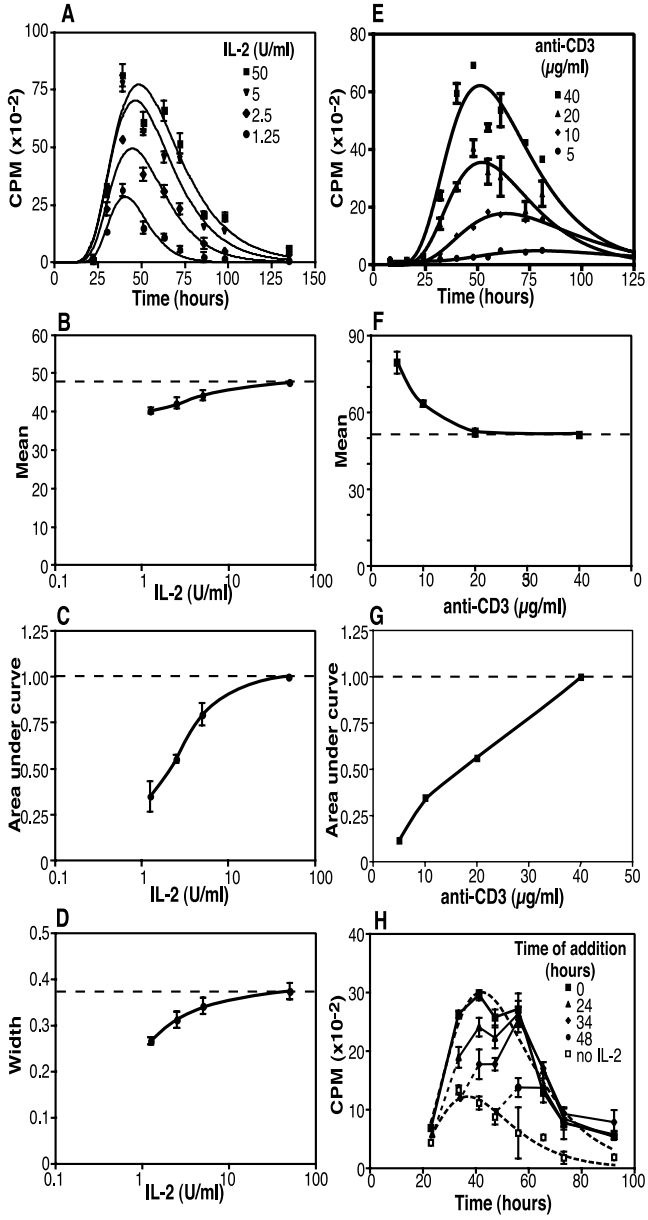

FIGURE 5. Direct measurement of entry into the first division. *A–D*, Naive CD4$^+$ T cells were stimulated with anti-CD3 (40 μg/ml) in the presence of various concentrations of hIL-2, as indicated. Demecolcine (5 ng/ml) was also added to cultures to inhibit cell proliferation, and anti-mIL-2 (50 μg/ml) was added to block endogenously produced IL-2. At various time points, cultures were pulsed with [^{3}H]TdR for 2 h and then harvested. *A*, Points represent cpm at each IL-2 concentration, as indicated. Each point represents the mean ± SEM of triplicate cultures. A series of log-Gaussian curves (solid lines) was fitted to the data using GraphPad Prism. The mean (*B*), area under curve (*C*), and width (*D*) of these fitted log-Gaussian curves are plotted against IL-2 concentration. Each point represents the mean ± SEM of three independent experiments. *E–G*, CFSE-labeled naive CD4$^+$ T cells were stimulated with various concentrations of anti-CD3, as shown in the presence of 100 U/ml mIL-2 and demecolcine (5 ng/ml). Cultures were pulsed with [^{3}H]TdR for 4 h at various times. Counts are plotted against time (*E*). Each point represents mean ± SEM of triplicate cultures. Lines are fitted log-Gaussian curves. The mean (*F*) and area under the fitted curves (*G*) are plotted against anti-CD3 concentration. *H*, Cells were stimulated with anti-CD3 in the presence of demecolcine, and mIL-2 (5 U/ml) was added to the culture at 0, 24, 34, or 48 h. Cultures were pulsed with [^{3}H]TdR for 2 h at various times. Each point represents the mean ± SEM of triplicate cultures. Log-Gaussian curves were fitted to the no IL-2 and IL-2 added at 0 h data using GraphPad Prism.

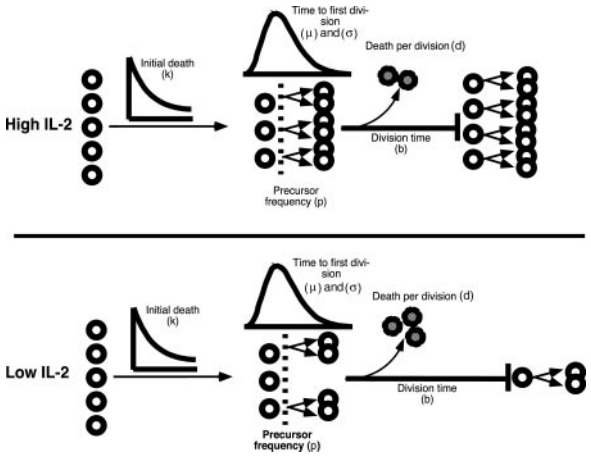

FIGURE 6. The six-parameter model of the effect of IL-2 on proliferation. A model was developed that incorporated the six parameters shown in brackets. If $CD4^+$ T cells are cultured, they will undergo initial cell death, which can be described by an exponential decay constant. This rate of death will be the same whether the cells are cultured in high or low concentrations of IL-2. The cells will enter the first division with a variability that can be described by a log-Gaussian distribution (given by a mean and width). The time of entry into division will not be altered by the concentration of IL-2. In contrast, the proportion of cells that are able to enter division (the precursor frequency) is affected by IL-2 concentration such that at low concentrations of IL-2, fewer cells will be able to enter division. IL-2 concentration also affects proliferation in subsequent divisions, and proportion of cells dying per division. At low concentrations of IL-2, the time taken to undergo subsequent divisions will be increased, and the proportion of cells that die before the next division will also be increased. The combined effect of these changes to the proliferation parameters results in the number of live cells in cultures with high IL-2 rapidly increasing above that seen in cultures with low IL-2.

show marked differences in cell number or TdR incorporation in cultures with and without the costimulus are possible, leading to the somewhat misleading qualitative interpretation that an added stimulus is essential for T cell activation and proliferation.

The quantitative view of multiple signal integration can be extended to accommodate accessory cell licensing by CD4 cells recently reported to serve as a replacement, or adjunct to Th cell action for promoting CTL activation (43–45). Thus, we propose increased expression of costimulatory molecules, such as CD40, on the APC induced by CD4 helpers can enhance T cell proliferation in ways additional to cytokines (such as altering the time to division and/or the survival through divisions). The quantitative sum of all of these effects (costimulators and soluble molecules) dictates the subsequent rate of T cell proliferation. The relative contribution of costimulatory molecules and soluble growth factors will depend on the stimulation conditions, and neither should be considered as obligatory pathways, despite the practical reality that an experimental system can be manipulated to yield convincing data consistent with such a view.

These studies may help explain the paradox concerning the difference in requirement for IL-2 in vitro compared with in vivo (23–26, 30–32, 46–48). Potentially multiple stimuli in vivo, including other common γ-chain ligands, may be summed by the T cell to alter the parameters of proliferation and thereby lessen the single contribution of IL-2-induced changes to these parameters. Furthermore, there is accumulating evidence that TCR engagement with costimulation is sufficient to induce some rounds of T cell proliferation in the absence of IL-2, or other γ-chain ligands (49, 50). Thus, the in vitro/in vivo paradox can be resolved by noting three features of IL-2 that lead to its markedly exaggerated importance in vitro. First, IL-2 is the major potential growth-promoting factor produced in vitro and may not be so in vivo; second, rapid accumulation of the cytokine within the confines of the in vitro culture allows high, stable concentrations to develop that probably never occur in vivo; and third, as we have seen, relatively small changes in IL-2 can have large effects after a few days in culture. Thus, while in vitro analyses are useful and even essential to demonstrate the quantitative operation of IL-2, studies of mice deficient in IL-2, or components of the IL-2R must complement this work to more appropriately apportion the contribution of this cytokine to in vivo responses.

Our experiments determined that IL-2 did not alter the time at which entry into the first division occurred, although it did alter the time to traverse subsequent divisions. The former observation was surprising, and appeared to be inconsistent with the important early studies of Cantrell and Smith (26, 51). These authors have previously shown that IL-2 concentration, IL-2R levels, and the duration of the IL-2/IL-2R interaction determine the time that activated T cells take to enter S phase (26). These earlier experiments, however, were conducted on T cells that had already been activated with mitogens and then rested without IL-2. Their reported regulation of entry into divisions, therefore, probably more closely reflects what is occurring in divisions after 1 than it does the entry of naive cells into the first division. Thus, these reports are consistent with our finding that IL-2 does not alter the time taken to enter the first division, but does alter the time taken to enter subsequent divisions.

Wells, Gudmundsdottir, and Turka (39) observed previously that the proportion of cells entering division following anti-CD3 stimulation is regulated by both the strength of TCR engagement and the provision of costimulatory signals. They also noted that only ~60% of T cells were able to enter division under maximum stimulatory conditions (39). Our analysis extends this work to reveal that IL-2 concentration is a further variable in determining the frequency of cells entering cycle even when all cells show signs of activation. Additionally, our experiments reveal that if IL-2 is not present at the time that cells are designated to divide by the strength of CD3 stimulation, they cannot be rescued by later addition of IL-2 (Fig. 5*H*). This finding raises the question of the fate of these cells. Perhaps they become anergic or undergo apoptosis after a further culture period. Our attempts to test the latter possibility by detecting increased death in the undivided population in the absence of IL-2 were hampered by the high intrinsic rate of death (data not shown). Thus, no conclusion could be reached, and further investigation will be required to determine the fate of these below-precursor threshold cells.

The addition of two new parameters was not the only modification we propose for the original Gett/Hodgkin model. We suggest that the original Gaussian distribution describing entry into the first division should also be replaced with a log-Gaussian function. This adjustment is a result of the greater accuracy in measuring time to first division with the demecolcine method. Such a pattern of entry into division is still consistent with a stochastic model of T cell proliferation and is reminiscent of the log-Gaussian distribution of many receptors, including CD3 and IL-2R, on the surface of T cell populations (26, 52). Thus, a simple explanation is that there may be a linear relation between the log-Gaussian growth factor receptor number and log-normal time to division consistent with the original experiments of Cantrell and Smith (26, 51). Whether the variation in receptor number and/or time to division results from differences in additional factors, such as the age of the cell, its activation history, or level of self reactivity, or is inherent to the construction of the cell, is currently unknown.

Nevertheless, it is noteworthy that this variability within the population ensures a full range of quantitative outcomes following stimulation, such that the summation of a large series of all or none decisions for division or death for each cell in the population results in a smooth transition from low cell to high cell numbers generated as IL-2 is increased. Thus, by this example, variability should not be viewed as an imperfection in the population, but potentially as an essential element in the smooth quantitative operation of the T cell response.

The six-parameter model presented in this work has three probabilistic parameters that are used to describe the variability within the cell population: two describing time to first division and one for the rate of initial cell death. Clearly, the pattern of death (d) and the time to divide in subsequent divisions (b) would also be better described by probability functions once the shape of these distributions can be experimentally determined. An example in which this may be relevant is demonstrated by the results of our fitting of the six-parameter model, which implied that decreasing IL-2 should increase the variation in the time to first division distribution. Our demecolcine data, however, revealed that, if anything, there was a slight decrease in the spread of entry into first division as IL-2 concentration was reduced. The solution to this inconsistency is presumably that a degree of variability also exists in the time that cells take to reach subsequent divisions. As a result, over time the spread of cells through division is the net result of compounding the variation in time to first and subsequent divisions. The above discrepancy in the effect of IL-2 on time to first division variability can thus be explained by the reasonable assumption that decreasing IL-2 concentration increases the variability in time taken to enter subsequent divisions. The variable b, therefore, gives the average time to next division, and thus its value may be skewed by the accumulation of very slowly dividing (or division-arrested) cells. Methods for investigating variation in time to enter subsequent divisions and the presence of nondividing cells are currently being developed.

A further potential variable not addressed as yet in our model is the possibility that the value of the death rate d may change with consecutive divisions. Renno et al. (53) found that superantigen-stimulated T cells in vivo exhibit an increase in death rate at later divisions. Furthermore, although our model incorporated a linear rate of cell death across division, it is possible that this death may be better described by other functions, such as the exponential decay or a log-normal distribution. We have not found it necessary to include this additional level of complexity to fit T cell proliferation data in vitro; however, given the results of Renno et al., attempts to model other T cell activation systems may require this additional amendment to our model.

In conclusion, these studies help to resolve some of the discrepancies observed between in vitro and in vivo results. Quantitative modeling of in vitro systems allows exploration of the complex signal integration while keeping a more appropriate perspective on the importance of each stimuli. Thus, in vitro analysis continues to be of use in exploring the response to multiple stimuli.

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