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TCR Affinity Promotes CD8\(^+\) T Cell Expansion by Regulating Survival\(^1\)

Mirja Hommel\(^2\) and Philip D. Hodgkin

Ligation with high affinity ligands are known to induce T lymphocytes to become fully activated effector cells while ligation with low affinity ligands (or partial agonists) may result in a delayed or incomplete response. We have examined the quantitative features of CD8\(^+\) T cell proliferation induced by peptides of different TCR affinities at a range of concentrations in the mouse OT-I model. Both the frequency of cells responding and the average time taken for cells to reach their first division are affected by peptide concentration and affinity. Consecutive division times, however, remained largely unaffected by these variables. Importantly, we identified affinity to be the sole regulator of cell death in subsequent division. These results suggest a mechanism whereby TCR affinity detection can modulate the subsequent rate of T cell growth and ensure the dominance of higher affinity clones over time. The Journal of Immunology, 2007, 179: 2250–2260.

One of the most important regulators of T cell responses is the nature of the Ag. Variations as subtle as a single amino acid exchange in a given antigenic peptide/MHC ligand can drastically alter its ability to bind to the TCR (affinity) and hence induce T cell activation (1). Based on their effects on T cell activation and differentiation, these altered peptides can be classified as partial (weak) agonists and antagonists (2, 3). Furthermore, with some exceptions their binding affinity for the TCR correlates with the spectrum of biological activity they elicit; the higher its affinity or, more accurately, the longer the half-life of the complex the better a ligand is at inducing a T cell response (4–7). Although the natural/wild-type Ag is able to elicit a T lymphocyte’s full program of activation events including proliferation and acquisition of effector functions, variant ligands may only be able to induce early activation events such as \(\zeta\)-chain phosphorylation or partial calcium influx (2, 8). Various models have been proposed to explain how affinity is detected, with the concept of kinetic proofreading being widely accepted (2, 8, 9). This theory is based on “timing” the duration of binding by a sequence of downstream events and was first proposed for the TCR by McKeithan (9). Given that a cascade of such events is required for most functional T cell responses, signal transmission would depend on the kinetic stability of intermediate products, e.g., phosphorylation. In recent years the original model has been extended by the concept of T cell responses being triggered by a cumulative signal that is reached at different time points for altered ligands (10) as well competing positive and negative feedback loops (11, 12). On a more functional basis, TCR stimulation with variant ligands may result in altered cytokine profiles, induction of clonal anergy, or target cell lysis without the production of cytokines (reviewed in Ref. 3). However, while most of these studies have focused on mechanistic early and late activation events within the cellular machinery, only limited data is available on the effects of affinity on individual kinetic features of T cell proliferation.

When T cells interact with Ag-derived peptides on an APC there will be, inevitably, a range of concentration and affinities of TCR interaction that lead to varying levels of stimulation of reactive T cells. As a consequence of these differences, over time some TCR clonotypes come to dominate the response. How this selection occurs is unclear. It is known that early in a response there is a more diverse usage of TCR that narrows down with time, suggesting kinetic differences in the proliferation response of individual clones (13, 14). Repeated immunizations were also shown to reduce the oligoclonality of cells, with surviving cells generally being of higher affinity (15–17). This narrowing of the repertoire over time is difficult to reconcile with the data supporting the “autopilot model” where limited stimulation of CD8\(^+\) T cells results in large numbers of rapid proliferation cycles (18–22). Thus, by this autopilot model the stimulation of low and high affinity T cells would result in multiple rounds of division that would see no change in the relative number of each.

In an attempt to gain deeper insight into the interplay of affinity and concentration, we used the well-defined OT-I system to assess how a T cell incorporates variations in these parameters into its survival and proliferation programs. Our results show that both concentration and affinity determine the frequency of responding cells, their average entry time into first division, and, to some extent, the predivision rate of cell death. Most importantly, we identified affinity as the primary variable regulating the survival of T cells as they progress through consecutive division rounds in this system.

Materials and Methods

Mice

Female OT-I (CD8\(^+\)) TCR transgenic mice specific for chicken OVA in the context of H-2\(^b\) (23, 24) were obtained from Walter and Eliza Hall Institute (Parkville, Australia) animal facility (Kew, Victoria, Australia). Mice were maintained under specific pathogen-free conditions and used between 6 and 15 wk of age.

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Immunology Division, Walter and Eliza Hall Institute, Parkville, Victoria, Australia

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Peptides

The chicken OVA-derived peptide SIINFEKL (85% purity) and variants D7 (77%), E1 (92%), G4 (95%), R4 (93%), S4 (93%), and V4 (98%) were obtained from Auspep.

Cell preparation and culture

Cell suspensions were prepared from total lymph nodes and CD8+ T cells were enriched by depleting other lymphoid subsets using MACS according to the manufacturer’s protocol (CD8+ T cell isolation kit, catalog no. 130-090-862, Miltenyi Biotec). Enrichment of naive cells was verified by flow cytometry. Typically, ≥93% were naive CD8+CD62Lhigh population. However, despite the higher purity similar frequencies of responding cells were recorded as well as similar proliferation and survival rates.

Labeling of T cells with CFSE (Molecular Probes) was conducted as described previously (25). Cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) comprising 2 mM l-glutamine (ICN Pharmaceuticals), 10% heat-inactivated FCS (CSL), 5 × 10^-5 M 2-ME (Sigma-Aldrich), 100 μg/ml streptomycin, and 100 U/ml penicillin (both from Invitrogen Life Technologies).

Flow cytometry and cell number determination

Flow cytometry was performed on a FACScan, LSR, or FACSCalibur cytometer (BD Biosciences) and data were analyzed using FlowJo (Tree Star) or WEASEL software (Walter and Eliza Hall Institute) software. The absolute number of cells in culture at each time point was determined by reference to a known number of CaliBRITE beads (BD Biosciences) added to each well before harvest. The ratio of live cells to beads was then used to calculate the total number of cells in each culture (26). Dead cells were identified by staining positive to propidium iodide, which was added just above conditions in the presence of 25 μg/ml colcemid (Roche). At various times cells were briefly pulsed with 1 Ci/well [3H]TdR (Amersham Biosciences). 3H-TdR was measured in the supernatant using a scintillation counter (Topcount NXT, Packard). Log-Gaussian distributions were fitted to the data using nonlinear regression analysis in GraphPad Prism.

Cytokine expression

For direct measurement of entry into first division, cells were cultured under the above conditions in the presence of 25 ng/ml colcemid (Roche). At various times cells were briefly pulsed with 1 μCi/well [3H]TdR (Amersham Biosciences) before harvesting. Incorporation of radioactivity was measured using a scintillation counter (Topcount NXT; Packard). Log-Gaussian distributions were fitted to the data using nonlinear regression analysis in GraphPad Prism.

Results

The magnitude of proliferation is dependent on peptide concentration and affinity

The OT-I model provides an excellent tool to assess the effect of affinity on survival and proliferation. In this system a wild-type ligand, the chicken OVA-derived peptide SIINFEKL (N), as well as a number variants have been defined and classified as agonists (N), weak agonists/antagonists (D7, E1, G4, S4, and V4), and antagonists (R4) based on their ability to induce or inhibit specific lysis of target cells (29). In addition, the affinities for several of the peptides is known: Keq values of 6.5 μM for N, 22.6 μM for E1,
57.1 μM for R4, and 10.0 μM for G4 (7, 10). Initially we prepared class I MHC complexes that we anchored to streptavidin-coated wells via a biotin-linker. However, as was also observed by others (30, 31) we found that the predominant stimulation was due to peptide eluted from the complex and self-presented by T cells to each other. Thus, we took advantage of this simpler system of self-presentation and sought to determine how CD8+ T lymphocyte proliferation and survival were regulated by both concentration and affinity. To constrain the system further and to eliminate the complication of different levels of IL-2 being produced over time, we added saturating levels of this growth factor to each culture (27).

When stimulating T cells of a given specificity with titrated amounts of peptide, the absolute number of live cells at a given time correlates well with the stimulus; cultures stimulated with higher peptide concentrations give rise to higher cell numbers over time (Fig. 1A). This was found to be true for the primary OVA-derived agonist N as well as for a panel of derivatives with weaker agonistic properties. Furthermore, the response to N was much stronger than to the lower affinity variant G4 and the other altered ligands assessed. The differences observed can be the result of numerous possible combinations of effect on the proliferation rate or survival.

Division profiles of CFSE-labeled cells allow us to draw some initial conclusions (Fig. 1B). As observed previously, cell division is asynchronous and cells can be found across a range of divisions (27, 32). This heterogeneity is in large part due to differences in the time that cells need to enter the first division. At higher concentrations of a given peptide the window of time of division entry is narrower and the cells behave more synchronously, as indicated by their spread over a smaller number of peaks. As the concentration is lowered the cells are spread over more divisions, hence indicating...
FIGURE 2. Quantitative analysis can be used to reveal the effects of affinity on proliferation. A, CFSE-labeled OT-I T cells were stimulated with 0.01 μg/ml N or G4 and cultures were harvested and analyzed at 42, 50, 57, 65, and 73 h. From their CFSE profiles the number of cells in each division was determined and plotted against division number for each harvest time (left). The proportion of parent cells (“precursor cohorts”) having given rise to cells in the next division was then calculated by dividing by 2^i, with i being the division number (right). B, Gaussian distributions are shown excluding cells in division 0. The means of Gaussian distributions were plotted against the division number for both peptides and a linear regression analysis was performed (for details see Materials and Methods). Extrapolation to the intercept with division 1 gives the mean time taken for lymphocytes to enter their first division (B, left). Areas (middle) and SD values (STDEV; right) were plotted against mean division number as well.

Entry into division/division time

With the aim of determining the underlying kinetic changes in proliferation and survival under different stimulatory conditions, we undertook a quantitative analysis of the above data using a series of graphical methods adapted and improved from the previous method of Gett and Hodgkin (27). The method is illustrated in Fig. 2. When plotting the number of cells in each division against harvest time following stimulation with N, an increase in the area under the curves can be observed (Fig. 2A, upper left panel), as expected for an expanding population. The same holds true for a culture stimulated with the same concentration of the lower affinity agonist (G4; Fig. 2A, lower left panel). The effect of cell proliferation can be removed by dividing cell numbers by 2^i where i is the division number. We refer to these numbers as precursor cohorts after Gett and Hodgkin (27). Each experimental series was performed in triplicate and averaged. Plots of precursor cohorts are shown to the right of Fig. 2A. For N the areas are visually similar, indicating that the cells are not dying to an appreciable level as they divide. For G4 the areas are much smaller to begin with, indicating that fewer cells enter the first division and then clearly decline as they progress through division (Fig. 2A, upper and lower right panels). This progressive change in area provides an indication of the amount of cell death through division.

The precursor cohort plots were explored further to estimate the average time to first division and the subsequent division times of T lymphocytes cultured as described above. Gaussian distributions were fitted to precursor cohort numbers (as described in Materials and Methods) and the mean division numbers, SD values, and areas under the curves were recorded. (We are aware that log-normal distributions are more accurate; nevertheless, the mathematical complications introduced by using log-normal distributions offer, for this evaluation, little additional benefit (32).) By plotting the mean division numbers against harvest time as done in Fig. 2B (left), the intercept of the resulting line for each peptide with division 1 gives an indication of the mean time to first division while the reciprocal of its slope gives an average subsequent division time. When lowering the affinity of a given peptide by a single amino acid exchange (N→G), the average time cells need to enter division is prolonged (~30 h for N (black) and 43.5 h for G4 (blue), while the average division time appears to be shorter (N, 9 h; G4, 7 h)). When the fitted areas are plotted against the mean division number a striking difference between N and G4 becomes apparent. Clearly the area and therefore the representation of the cohort of cells that started dividing are being progressively lost through division when cultures are stimulated with G4. The pattern of loss is potentially informative and will be discussed further below. Extrapolating to division 1 we can also see that more of our starting cells have entered first division when stimulated by N compared with G4. This could be due to different death rates leading up to division or to changes in the frequency of cells stimulated by the peptides. These possibilities will also be explored further below. Plotting the SD values of the Gaussian fits against the mean division numbers reveals that it is higher for G4 than for N, consistent with their reduced synchronicity when entering the first cell division. Finally, increases in SD values with division number are
FIGURE 3. Data from cultures as described in Fig. 1 were analyzed as described previously for precursor cohorts in Fig. 2. a, Mean division number vs time. b, Area of Gaussian vs mean division number. c, SD of Gaussian vs mean division number. Because the standard variations did not change much through divisions, we used the average value to fix the SD at the early time points for better fitting. d, Adjusted and normalized (≥2 divisions) areas are shown to illustrate the rate of cell loss per division.
an indication of additional variation in times through subsequent division, adding to the initial variation in entry to the first division. (For the Gaussian approximation of variation in division times used in this analysis we expect a linear relation between variance and mean division number for this plot. The slope of the line yields the variance of the time to subsequent division distribution. For more information see Deenick et al. (32)). Thus, there were numerous quantitative differences in proliferation and survival due to the affinity difference between the N and G4 peptides.

**The effects of changing concentration and affinity on kinetic parameters**

The different quantitative outcomes for two peptides with varying affinities led to the question of whether lowering the concentration of a peptide had the same effect as lowering affinity. To test this, the above analysis was performed with varying concentrations of N and G4. In addition we included three other weaker agonists in the analysis (D7, S4, and V4) for which the affinities are unknown but are presumably weaker than that of N. Cultures simulated with a very weak agonist/antagonist (E1) did not yield enough cells to be included in a thorough analysis, whereas cells stimulated with an antagonist (R4) hardly proliferated at all. The data were plotted in numerous ways (Fig. 3) and the resulting parameter values are given in Table I.

Linear regression analysis of mean division number vs time of harvest plots reveals that when lowering the concentration of a given peptide the average time cells need to enter the first division is prolonged. This is also true for each of the weaker agonists tested (Fig. 3a). In contrast, the average division time through subsequent divisions remains largely unaffected for most peptides and concentrations. The fact that the slopes of the linear regression analysis are occasionally dropping at low peptide concentrations can be the result of either of two scenarios; first, it might be an indication of the division time becoming longer or, second, cells are dying as they proceed through divisions because a significant amount of cell loss per division can change the slope (data not shown).

The cohort areas vs mean division number plots (Fig. 3B) provide further information. The intersection of these plots with division 1 can be taken as a measure of the number of cells that enter the first division. The data clearly indicate that concentration affects the numbers of cells entering division, as does the affinity of the peptide (N vs G4). These plots also give an indication of cell survival as division progresses. This feature shall be discussed in more detail below.

In Fig. 3c the SD values are plotted against mean division number. The fitted SD values were higher for the weaker agonist G4 and for lower concentrations. However, they did not change markedly with progress through divisions, indicating that variation in the times through subsequent divisions is relatively small and independent of either peptide concentration or affinity. We used this finding to obtain a more accurate estimate of the areas by averaging the deviation and fixing it in the fitting procedure. The results were similar to those depicted in Fig. 3b (not shown). The effects observed for the weak agonists of unknown affinity (D7, S4, and V4) were similar to those of the low affinity ligand G4.

**FIGURE 4.** Time to first division entry is directly linked to concentration and affinity. Purified OT-I T cells ($10^6$) were placed in culture in the presence of colcemid and stimulated with an array of peptides and concentrations. At the indicated time points cells were briefly pulsed with $[^3]$H]TdR and thymidine incorporation was measured (note: pulse was 1 h for N and G4 and 1.5 h for D7, S4 and V4; cultures stimulated with D7 also comprised $2 \times 10^4$ starting cells to obtain sufficient counts at low concentrations). A series of log-Gaussian distributions was fitted to the data with GraphPad Prism. Except for D7, peptide concentrations were the same as those described for Fig. 1.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>MTTFD</th>
<th>95% CI</th>
<th>Division Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>N 0.1</td>
<td>29.22</td>
<td>0.41</td>
<td>10.73</td>
</tr>
<tr>
<td>0.01</td>
<td>29.26</td>
<td>0.34</td>
<td>10.49</td>
</tr>
<tr>
<td>0.001</td>
<td>30.02</td>
<td>0.67</td>
<td>10.67</td>
</tr>
<tr>
<td>0.0001</td>
<td>36.23</td>
<td>0.92</td>
<td>9.29</td>
</tr>
<tr>
<td>0.00001</td>
<td>44.06</td>
<td>0.92</td>
<td>8.14</td>
</tr>
<tr>
<td>D7 6</td>
<td>40.44</td>
<td>0.71</td>
<td>8.12</td>
</tr>
<tr>
<td>2</td>
<td>42.85</td>
<td>0.33</td>
<td>8.45</td>
</tr>
<tr>
<td>0.6</td>
<td>48.81</td>
<td>0.85</td>
<td>8.50</td>
</tr>
<tr>
<td>0.2</td>
<td>49.06</td>
<td>1.33</td>
<td>9.49</td>
</tr>
<tr>
<td>G4 1</td>
<td>39.41</td>
<td>0.42</td>
<td>8.06</td>
</tr>
<tr>
<td>0.1</td>
<td>40.30</td>
<td>0.40</td>
<td>7.80</td>
</tr>
<tr>
<td>0.01</td>
<td>43.50</td>
<td>0.38</td>
<td>6.95</td>
</tr>
<tr>
<td>0.001</td>
<td>55.13</td>
<td>1.06</td>
<td>11.16</td>
</tr>
<tr>
<td>S4 2</td>
<td>34.20</td>
<td>1.12</td>
<td>6.88</td>
</tr>
<tr>
<td>0.6</td>
<td>34.17</td>
<td>0.47</td>
<td>7.47</td>
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<td>36.97</td>
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<td>7.03</td>
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<td>0.06</td>
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<td>7.66</td>
</tr>
<tr>
<td>0.02</td>
<td>37.54</td>
<td>1.23</td>
<td>17.21</td>
</tr>
<tr>
<td>V4 0.2</td>
<td>30.05</td>
<td>0.73</td>
<td>6.87</td>
</tr>
<tr>
<td>0.06</td>
<td>30.54</td>
<td>0.81</td>
<td>7.04</td>
</tr>
<tr>
<td>0.02</td>
<td>30.52</td>
<td>0.84</td>
<td>7.66</td>
</tr>
<tr>
<td>0.006</td>
<td>31.79</td>
<td>0.84</td>
<td>7.40</td>
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<tr>
<td>0.002</td>
<td>38.20</td>
<td>1.31</td>
<td>12.10</td>
</tr>
</tbody>
</table>

* CI, Confidence interval.
sured (note: pulse was 1 h for N and G4 and 1.5 h for D7, S4 and V4; cultures of OT-I cells stimulated to divide with various concentrations of N is a measure of the number of cells in S phase (33). The distributions points during culture allows detection of replicated DNA and thus

trations of N (black, 0.1

of the cell cycle so that they are able to replicate their DNA but do
division can be given as N

although this rate of death may be exaggerated in in vitro culture,

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the results clearly indicate that survival before division is affected

The hierarchy of the potential to promote preprolifer-

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moted during this early stage when compared with cultures with-

out peptide (high (N) or low (G4) affinity) or the peptide does not

The fitted curves are log-normal, which give a more accurate fit to

to divide and the area until the concentration is lowered below a

with peptide affinity and concentration.

Death

We have previously divided the deaths of cells placed in in vitro culture into two components: 1) an initial death rate, approximately exponential, that operates upon both stimulated and unstimulated cells before their first division (27, 32), presumably initiated by withdrawal from survival cytokines in vivo such as IL-7 (34); and 2) a second death component that reflects the loss of cells as they are progressing through divisions. This subsequent death rate might be constant or it could change in magnitude with successive divisions. Both death rates are potentially affected by peptide concentration and affinity.

Initial death rate. When assessing and comparing total live cell numbers in cultures in the presence/absence of peptide over the first 24 h of culture, two patterns emerge: either survival is pro-

moted during this early stage when compared with cultures with-

out peptide (high (N) or low (G4) affinity) or the peptide does not

support survival above the level observed in control cultures even

at relatively high concentrations (D7; Fig. 5). Thus, despite being a weak agonist capable of inducing proliferation, D7 does not im-

prove survival in these early times. Both S4 and V4 are able to

promote cell survival in a concentration-dependent manner, above

the level found in unstimulated cultures but below that for N (data

not shown). The hierarchy of the potential to promote preprolifer-

ation survival can be given as N

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at relatively high concentrations (D7; Fig. 5). Thus, despite being a weak agonist capable of inducing proliferation, D7 does not im-

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promote cell survival in a concentration-dependent manner, above

the level found in unstimulated cultures but below that for N (data

not shown). The hierarchy of the potential to promote preprolifer-

ation survival can be given as N > V4 > S4 > G4 > D7. Although this rate of death may be exaggerated in in vitro culture, the results clearly indicate that survival before division is affected to different degrees by the strength of stimulation.

Table II. Summary of areas and mean time to first division (MTTFD)

from colcemid analysis.*

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Amplitude</th>
<th>SEM</th>
<th>95% CI</th>
<th>MTTFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.1</td>
<td>3.511</td>
<td>646</td>
<td>2,155–4,867</td>
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<td>1.266</td>
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<td>878–1,653</td>
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<tr>
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<td>41</td>
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<td>43.28</td>
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<td>535</td>
<td>62</td>
<td>406–665</td>
<td>44.26</td>
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<td>5</td>
<td>6,143</td>
<td>381</td>
<td>5,419–6,867</td>
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<td>DNC</td>
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<td>40</td>
<td>294–463</td>
<td>45.06</td>
</tr>
<tr>
<td>V4</td>
<td>0.2</td>
<td>5,263</td>
<td>309</td>
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</tr>
<tr>
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<tr>
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<tr>
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<td>620</td>
<td>114</td>
<td>380–850</td>
<td>24.99</td>
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* Purified OT-I T cells (10^4) were placed in culture in the presence of colcemid and stimulated with an array of peptides and concentrations. At the indicated time points, cells were briefly pulsed with [3H]TdR and thymidine incorporation was measured (note: pulse was 1 h for N and G4 and 1.5 h for D7, S4 and V4; cultures stimulated with D7 also comprised 2 x 10^4 starting cells to obtain sufficient counts at low concentrations). A series of log-Gaussian distributions was fitted to the data with GraphPad Prism. Except for D7, peptide concentrations were the same as those described for Fig. 1. CI, Confidence interval; DNC, did not converge.

Direct measure of entry into the first division

The above cohort analysis indicated that the average times T cells take to enter the first division, the number of cells involved, and the variation over time were all affected by peptide affinity and concentration. We tested these conclusions directly by use of the cell cycle inhibitor colcemid, which arrests cells in the G2/M phase of the cell cycle so that they are able to replicate their DNA but do not undergo division. Briefly pulsing with [3H]TdR at various time points during culture allows detection of replicated DNA and thus a measure of the number of cells in S phase (33). The distributions of OT-I cells stimulated to divide with various concentrations of N or its derivatives in the presence of colcemid is shown in Fig. 4. The fitted curves are log-normal, which give a more accurate fit to these data than the Gaussian distributions (27, 32). Again, we find that the mean time to divide varies with both peptide concentration and affinity and so does its variation, thus confirming our previous findings. The same is true for the series of weak agonists. The area under these curves can also be taken as a measure of the proportion of cells dividing, reflecting the overall precursor frequency. Clearly, lowering concentration or affinity (G4) results in reduced areas. This is also observed for weak agonists. These changes can be due to either increased cell death before division or reduced frequency of cells responding to the stimulus by progressing to the next division. (A summary of the fitting results is given in Table II.) Interestingly the response to V4 differs from that to all other peptides in that there appears to be little effect on both the mean time to divide and the area until the concentration is lowered below a threshold value.

FIGURE 5. Concentration and affinity affect the early death rate. Purified and CFSE-labeled OT-I T cells (10^4) were stimulated with various concentrations of N (black, 0.1 μg/ml; blue, 0.001 μg/ml; green, 0.00001 μg/ml), G4 (black, 1 μg/ml; green, 0.1 μg/ml; blue, 0.001 μg/ml), D7 (black, 6 μg/ml; blue, 2 μg/ml; green, 0.6 μg/ml), or no peptide (red) and harvested at various time points. Viability was measured by propidium iodide uptake, total live number of cells in cultures in the presence/absence of peptide over the first 24 h of culture, two patterns emerge: either survival is pro-

moted during this early stage when compared with cultures with-

out peptide (high (N) or low (G4) affinity) or the peptide does not

support survival above the level observed in control cultures even

at relatively high concentrations (D7; Fig. 5). Thus, despite being a weak agonist capable of inducing proliferation, D7 does not im-

prove survival in these early times. Both S4 and V4 are able to

promote cell survival in a concentration-dependent manner, above

the level found in unstimulated cultures but below that for N (data

not shown). The hierarchy of the potential to promote preprolifer-

ation survival can be given as N > V4 > S4 > G4 > D7. Although this rate of death may be exaggerated in in vitro culture, the results clearly indicate that survival before division is affected to different degrees by the strength of stimulation.

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Subsequent death rate. As mentioned earlier, an indication of the rate of cell death as the cells progress through divisions can be obtained from Fig. 3b, where the area of the precursor cohorts is plotted against mean division number. The plots clearly show that the overall areas are smaller as concentration or affinity is lowered or when weak agonists are assessed. Extrapolating the area plots to intersect with division 1 reflects the number of cells entering the first division and correlates well with the previous effect of affinity/potency and concentration observed in the colcemid experiment described above. Due to the different starting areas for each concentration it is difficult to compare the rate or the proportion of cells lost through each division under each culture condition. To enable this comparison the areas were normalized to the area found closest to mean division number 2 (Fig. 3d). This division number was chosen because mean values <2 were considered inaccurate. For N there is no or negligible death as cells progress through divisions. This was true for all concentrations tested. For all other peptides the loss of cells as they divide is substantial, even at the highest concentrations assessed. Of particular note is that the rate of subsequent cell death is clearly not, or only weakly, concentration-linked, thus appearing to be a direct function of the peptide only. It is also apparent that the proportional loss of cells is increasing with progression through division, suggesting that the rate of loss is not constant.

CD8$^+$ T lymphocytes have the potential to differentiate into cytotoxic effector cells, and it was possible that the cell death observed during divisions induced by G4 (low affinity) or the other weak agonists occurred by fratricide as a result of the self-presentation of the peptide. Although T cells are generally not considered to be potent APCs, strong activation after the addition of a peptide had been shown previously (30). To address this issue, proliferating OT-I cells that had been stimulated with N, D7, or G4 for 68 h ($\geq$5 divisions) were incubated with either $^{51}$Cr-labeled lymphoblasts from the same culture or freshly prepared, CFSE-labeled naive lymphocytes. These cells were incapable of lysing either target during a culture period of 4 h, hence excluding fratricide as the cause of the death observed (data not shown). As we were interested in whether the minimal T-T stimulation resulted in acquisition of some effector function at all, cells from 68-h cultures that had been stimulated as described above were also analyzed for production of the effector cytokines IFN-γ and TNF-α. As reported previously, cytokine production was found to be division linked (35, 36). Interestingly, though, all cultures produced TNF-α at equivalent levels and while all produced IFN-γ also, the mean fluorescence of cells staining positive for this cytokine was reduced for a low concentration of G4 as well as for both concentrations of D7 assessed (data not shown).

Effect of IL-2

Having determined the kinetic parameters affected by peptide affinity and concentration under saturating IL-2 concentrations, we asked how proliferation in this system was regulated when this growth factor was limiting. To examine the quantitative effects of IL-2 on CD8$^+$ T cell proliferation, we stimulated OT-I cells with a strong (N) agonist in the presence of an anti-mouse IL-2 mAb (S4B6) and various concentrations of IL-2. It was shown previously that although hIL-2 is active on mouse lymphocytes it is not neutralized by the S4B6 mAb (37, 38). Analyzing the data as described above revealed that reducing the IL-2 concentration had little effect on the mean time to first division and division time (Fig. 6a) and, like affinity, was an important regulator of survival (Fig. 6b). However, although IL-2 concentration had no effect on the predivision survival over the first 24 h of culture (Fig. 6b), it exerted a potent influence on the survival of dividing cells as revealed by the changes in cohort areas with successive division in Fig. 6, b and d. Clearly, reducing the amount of IL-2 in the culture translates into greatly enhanced loss of cells across divisions (Fig. 6d). The SD values of the fitted cohort plots for varying IL-2 concentrations are distinct from the effect of changing peptide concentrations. Here the value increases with successive divisions at a greater rate for low concentrations compared with high ones (Fig. 6c). This is consistent with greater variation in times to divide at the lower levels of the growth factor.

Discussion

In this study, we examined in a minimal system the kinetic variables of proliferation that are affected by peptide affinity and concentration. We generated a large set of CFSE time series data for OT-I CD8$^+$ T cells stimulated at different affinities and concentrations that we then analyzed using graphical methods that were an extension of those described by Gett and Hodgkin (27). The principles behind this method revolve around the cohort plot where the mean division number, the SD, and the area are determined for cell numbers taken at different harvest times. We favor this analysis approach as it is intuitive and clearly illustrates the important points of difference that result from changes in concentration and affinity. The alternative would be to fit a series of parametric models such as those of Deenick et al. (32), DeBoer et al. (39), Leon et al. (40), and Hawkins et al. (41). However, this introduces a discussion of the strengths and weaknesses of individual models that we believe is unnecessary for our major conclusions here, but our data will be made available to
others who wish to investigate it using their own models (see supplementary data). 4  
In general, stimulated CD8+ T cells took between 29 and 55 h to enter into their first division. The average time and variance for cells to enter the first division were both affected to a large extent by the strength of the agonist and the concentration. The first division was followed by a prolonged series of successive divisions, each taking 7–10 h. The times through successive divisions were relatively constant for most peptides and concentrations examined, indicating that once cell division is initiated the programmed cycle time is relatively insensitive to peptide affinity (G4) or that the peptide is a strong vs a weak agonist. This finding was in striking contrast to the discovery that the survival of cells progressing through successive division rounds was markedly different for each peptide. OT-I T cells stimulated by the high affinity agonist N showed little evidence of death for up to six division rounds. In contrast, all of the weaker agonists lost cells through division to varying degrees. The pattern of cell loss revealed by the changing area of the cohort plot as cells divide was notably insensitive to the peptide concentration. Although reducing the concentration of peptide usually resulted in fewer cells entering division overall, the proportion of the cells lost in successive divisions was usually the same. The peptide variants V4 and G4 gave the most rapid loss of cells and D7 the least. For N and G4 the difference in survival clearly correlated with affinity and with the time TCR and MHC spent in complex once engaged. The half-life of TCR association with N-Kb is reported to be 533.2 s and for G4-Kb it is 69.3 s (10). Recently, Yachi et al. (42) showed that stimulation with G4 vs N resulted in a lag of conjugate formation, TCR recruitment into the synapse, interaction between CD3ε and CD8β, and thus a delayed polarization of pERK to the synapse. The latter has been shown to play a role in defining an antagonist vs an agonist signal (11). We conclude that the CD8+ T cell is able to integrate differences in affinity into a division-associated survival pattern that favors the growth of the higher affinity T cells. The enhanced survival pattern cannot be reproduced by high concentrations of the low affinity peptide. Furthermore, survival rather than time between divisions is the primary affinity-driven regulator of clonal growth in this system. There is limited information regarding the affinity of the other peptides for the TCR, making it difficult to map the effect on survival precisely to an affinity level. However, with the ability of a peptide to elicit a T cell response generally being linked to the half-life of the peptide/TCR complex, it is likely that the half-lives of S4 and V4 will be in between those of N and G4. Furthermore, variant peptides may bind less avidly to the MHC, potentially further reducing the ability of a TCR to remain attached to a MHC-peptide complex. In a study by Chen et al. (43) it was shown that D7 bound less efficiently to the MHC than the wild-type peptide N. Therefore, the effects seen for this peptide cannot be attributed to the TCR alone.

Our data provide support for the concept that TCR stimulation duration drives T cells through hierarchical thresholds affecting cell responses (10, 18–22, 44, 45). However, triggering cell division and “programming” survival of the T cell appear to operate differently. For cell division our data reconcile nicely with a TCR signaling model that envisages the accumulation of a signal-derived counter such as c-Jun (10). The authors were able to show that differences in T cell activation by either N or its weak agonist variant G4 were due to the duration of TCR/MHC-peptide interaction. Both were able to induce maximal CD69 up-regulation in vitro, but 1,000- to 10,000-fold higher doses of G4 were required to reach equivalent responses. The expression of CD69 was similar at 24 h, but up-regulation in response to G4 was found to lag at least 5 h behind that of N. CFSE dilution assays also revealed a similar lag in the proliferative response. These in vitro results are in concordance with our observations for triggering cell division. The concentration of a peptide and affinity both influence the number of complexes available to the T cells such that the lower the concentration or affinity the longer time on average a cell will take to trigger division. As the number of complexes is reduced further, some cells never accumulate a threshold level of stimulus and, hence, are not triggered into division at all. Thus, both peptide concentration and affinity affect the average time to first division as well as the frequency of cells that participate in proliferation. Those cells that are triggered into division even at low concentrations undergo numerous consecutive rapid divisions. In contrast, the survival attributes of the T lymphocyte appear more sensitive to the duration of TCR/peptide/MHC engagement, and longer periods may be required to fully promote cell survival. This is consistent with the kinetic proofreading concept (2, 8–11), which provides a mechanism for understanding why increasing affinity (or more accurately, association times) will not behave in the same way as increasing concentration. This interplay between sensitive triggering of a full proliferation response coupled with an affinity-regulated survival rate carried through subsequent division rounds can explain how different clonotypes can be selected over time, even when activated cells run through many division rounds on “autopilot” (18–22). We illustrate this point in Fig. 7. In this example the same division time but altered survival through division leads to different rates of cell expansion. It is interesting to note that a 20% change in cell loss per division results in a 9-fold (2.7-fold) difference in cell number after ~10 divisions (~6 divisions). Thus, this mechanism alone can play a major role in clonal selection and outgrowth of dominant clones. It is also possible that this difference is amplified by division-linked increases in the rate of cell death or in affinity-regulated differences in the programmed number of divisions.

4 The online version of this article contains supplementary material.
To explain how survival rates change for proliferating T cells, we suggest two possible mechanisms. First, the programming of cell survival following interaction through the TCR may be an intrinsic feature of the now activated T cell. Proliferation survival could be due to the more rapid expression and accumulation of high concentrations of antiapoptotic proteins such as Bcl-2 or Bcl-xL. Bcl-2 is expressed in resting T cells (46) and levels are progressively up-regulated during stimulation, reaching a maximum concentration immediately before division. Bcl-xL levels also increase upon proliferation (21, 47). Thus, stronger affinity interactions would result in greater inherent survival that is then less influenced (or diluted) by progression through cell division. A related possibility is that the weaker affinity interactions result in lower levels of IL-2 receptor expression and, hence, relative insensitivity to the growth factor present in the culture. This explanation is consistent with our data showing that, as a consequence of lowering the IL-2 concentration, there is more death in subsequent divisions (Fig. 6d). This later dependence on IL-2 was also reported by others, (18, 48, 49), and recently the presence of IL-2 during the primary response was shown to be important for the survival and expansion of subsequently arising memory cells (50). Furthermore van Stipdonk et al. (20) were able to show that a prolonged stimulus resulted in sustained CD25 expression while a short stimulus led to transient CD25 expression. Determining the relative contribution of affinity-related “programming” of cell survival vs that of the regulated expression of survival enhancing receptors and how these attributes are altered through successive cell divisions is the subject of further quantitative experiments and kinetic model development in our laboratory.

At very low concentrations the apparent division times of the T cells is shown to be increasing as the IL-2 concentration is lowered. This results in more division times when stimulated under different conditions. Although we are dealing here with a transgenic system in which all T cells are specific for the provided ligands, the natural immune response is much more complex. It is not only shaped by the participation of T lymphocytes at a very low frequency and with a diverse TCR/peptide affinity but also by the presence of a variety of costimulatory molecules and growth factors. This complexity as well as the difficulty of accounting for all T cells taking part in an in vivo response make it very difficult to precisely assess the effect of individual variables on the parameters of T cell proliferation and survival in an in vivo setting. However, we believe that careful quantitative studies like the one presented here are helpful for interpreting kinetic growth effects in vivo and that our findings are an important step forward in developing fully predictive models of immunomodulation.

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Disclosures
The authors have no financial conflict of interest.

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