Dynamics and Requirements of T Cell Clonal Expansion In Vivo at the Single-Cell Level: Effector Function Is Linked to Proliferative Capacity

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Dynamics and Requirements of T Cell Clonal Expansion In Vivo at the Single-Cell Level: Effector Function Is Linked to Proliferative Capacity

Hrefná Gudmundsdóttir, Andrew D. Wells, and Laurence A. Turka

The adoptive transfer of TCR-transgenic T cells into syngeneic recipients allows characterization of individual T cells during in vivo immune responses. However, the proliferative behavior of individual T cells and its relationship to effector and memory function has been difficult to define. Here, we used a fluorescent dye to dissect and quantify T cell proliferative dynamics in vivo. We find that the average Ag-specific CD4+ T cell that undergoes division in vivo generates >20 daughter cells. TCR and CD28 signals cooperatively determine the degree of primary clonal expansion by increasing both the proportion of Ag-specific T cells that divide and the number of rounds of division the responding T cells undergo. Nonetheless, despite optimal signaling, up to one-third of Ag-specific cells fail to divide even though they show phenotypic evidence of Ag encounter. Surprisingly, however, transgenic T cells maturing on a RAG-2-/- background exhibit a responder frequency of 95–98% in vivo, suggesting that maximal proliferative potential requires either a naive phenotype or allelic exclusion at the TCR locus.

An early limitation of the examination of in vivo responses was the inability to identify, independently of some functional response parameter, those T cells that have the potential to participate in an Ag-specific immune response. This limitation has now been overcome with the use of bacterial superantigens (2), or more recently, TCR-transgenic T cells (3). The response of T cells in TCR-transgenic mice, however, is often altered by the unnaturally high frequency (30–80%) of Ag-reactive cells. The adoptive transfer of small numbers of TCR-transgenic T cells to congeneric, nontransgenic mice offers the opportunity to create a system in which the frequency of Ag-reactive cells is more physiologic (0.1%), and yet the cells can still be identified using a mAb specific for the transgenic TCR Id (4). Studies using such a system have helped to define the localization of Ag-reactive cells following antigenic challenge, the role of the route of Ag administration in the ability of T cells to initiate and sustain a response, and the role of adjuvants and costimulatory molecules in the response (4–8).

In vivo T cell responses are the summation of the function (proliferation and cytokine production) and fates (survival versus apoptosis) of individual cells that comprise the Ag-specific population. In vivo, cytokine production can be assessed on an individual cell basis using flow cytometry; however, determination of proliferation is generally indirect, measured either as the accumulation of clonotype T cells and/or by the incorporation of the nucleotide analogue 5-bromo-2'-deoxyuridine (BrdU) (9). A major limitation of these approaches is the inability to determine the percentage of Ag-specific T cells that actually participate in the immune response (i.e., actual responders) as a function of their frequency in the lymphocyte pool (i.e., potential responders), and to quantitatively assess the “strength” of the proliferative response (i.e., how often each responding T cell divides). Clearly, it is important to relate these factors to other functional parameters of the immune response, such as cytokine production, and to determine how these parameters are affected by such variables as costimulation or previous Ag exposure.

Recently, we have used the fluorescent dye 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE), which labels live cells and segregates equally between daughter cells during mitosis (10), to quantitatively assess the dynamics of a T cell proliferative response in vitro (11). These studies showed that signals from TCR and CD28 differentially contribute to proliferation in response to polyclonal T cell activation using agonistic Abs. TCR engagement...
quantitatively influenced proliferation at the induction phase by controlling the proportion of T cells that divided following stimulation. CD28 costimulation functioned both at the induction phase by recruiting additional T cells into the proliferating pool under conditions of suboptimal TCR occupancy, and at the expansion phase by allowing the responding T cells to progress through multiple cycles of cell division. We also found that the frequency of T cells that responded to coligation of TCR and CD28 was limited to multiple cycles of cell division. We also found that the frequency of T cells that responded to coligation of TCR and CD28 was limited to multiple cycles of cell division. We also found that the frequency of T cells that responded to coligation of TCR and CD28 was limited to multiple cycles of cell division. We also found that the frequency of T cells that responded to coligation of TCR and CD28 was limited to multiple cycles of cell division. We also found that the frequency of T cells that responded to coligation of TCR and CD28 was limited to multiple cycles of cell division. We also found that the frequency of T cells that responded to coligation of TCR and CD28 was limited to multiple cycles of cell division. We also found that the frequency of T cells that responded to coligation of TCR and CD28 was limited to multiple cycles of cell division.

Here, we have applied this system to the more complex dynamics of an Ag-specific response in vivo and show that the proliferative fate of individual T cells can be modeled using this approach. We find that each proliferating, Ag-reactive T cell generates an average of >20 daughter cells within a 48-h period, traversing the cell cycle in ~10.6 h. Costimulatory signals through CD28 regulate both the frequency of Ag-specific T cells that divide and the number of rounds of division the responding cells undergo. Surprisingly, only ~60% of the transgenic T cells that encounter Ag in vivo actually participate in clonal expansion. However, RAG-2-deficient transgenic T cells exhibit a responder frequency of 95–98% in vivo, suggesting that proliferative potential may depend upon complete allelic exclusion at the TCRα locus. Finally, we demonstrate a division cycle dependence in the expression of memory markers such as CD44, CD45RB, and CD62L and show that effector functions including the production of IL-2 and IFN-γ correlate with division cycle rather than with the receipt of co-stimulatory signals. These results provide a quantitative assessment of in vivo T cell proliferative dynamics and define a relationship between cell division and other parameters of the immune response including cytokine production, the availability of co-stimulation, and the capacity for memory.

Materials and Methods

Mice

DO11.10 (3) and RAG-2−/− DO11.10 (William Lee, State University of New York, Albany) mice have been described previously. Both sets of mice were maintained as breeding colonies in our animal facility. BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were used at 6–12 wk of age.

Peptide, Abs, and CFSE labeling

CTLA4 Ig (12) was provided by P. Linsley (Bristol-Myers Squibb Pharmaceuticals, Princeton, NJ). The anti-CD28 mAb (37.51) was provided by J. Allison (University of California, Berkeley). Control hamster IgG was obtained from The Jackson Laboratory, and OVA peptide 323–339 was synthesized by the Protein Chemistry Lab, University of Pennsylvania (Philadelphia). Fluorochrome-labeled Abs against CD4, CD44, CD69, L-selectin, IL-2, IFN-γ, CD16/CD32 (Fc block), and isotype controls were purchased from Becton Dickinson Immunocytometry Systems, San Jose, CA, and were analyzed using CellQuest acquisition/analysis software. Between 20 × 10³ and 10⁶ events were collected.

Intracellular cytokine staining

Briefly, lymphocytes obtained from BALB/c mice that had received DO11.10/RAG-2−/− cells were restimulated in vitro with 5 μg/ml OVA peptide 323–339 in the presence of 2 μg/ml anti-CD28 and 2 μM melenin (Boehringer Mannheim, Indianapolis, IN) (13). After staining for cell surface receptors, the cells were fixed in final concentration of 1% formaldehyde at 4°C, washed in PBS, and then washed in PBS containing 2% FCS, 0.02% azide, and 0.1% saponin (Sigma). PE- or APC-conjugated anti-IL-2 and PE conjugated anti-IFN-γ mAb (PharMingen) mixed in 30 μl PBS/saponin buffer was used to stain each sample for 60 min (30 min at 4°C followed by 30 min at room temperature).

Quantitation of T cell proliferative dynamics from CFSE profiles

The quantitative analysis of proliferation using CFSE has been described previously (11). In these studies, we apply the same principles but take a slightly different approach that more accurately models the dynamics of clonal expansion in vivo. To measure the “size” of a proliferative burst, we first relied on the fact that the size of the Ag-specific daughter T cell subset (measured at 72 h postimmunization) and the size of the precursor subset are related by a function of 2ⁿ, where n is the number of division cycles achieved during clonal expansion. Therefore, for the events under each CFSE fluorescence peak (E), which represent a sample of the total population of Ag-specific T cells present in the mouse which have undergone n division cycles, the number of precursor T cells (P) which must have divided n times to generate them can be extrapolated by dividing E by 2ⁿ.

The sum of the extrapolated precursors for each division cycle gives the total number of precursor T cell subset present in the mouse. Mice were treated on day 0, 1, 2, and 3 days (relative to immunization) with 200 μg or 500 μg of either CTLA4 Ig or human IgG i.p.

Flow cytometry

Cells were washed in PBS containing 2% FCS and 0.02% azide at 4°C. Unlabeled anti-CD16 (anti-FcγRIII) and anti-CD32 (anti-FcRII) were used to block Fc receptor binding. Between 0.2 and 3.0 × 10⁶ cells were stained with either 1) PE-conjugated anti-CD4 and Cy-chrome conjugated anti-CD44 or 2) PE-conjugated CD45RB or L-selectin, and Cy-chrome conjugated CD4. In both cases, cells were also stained with biotinylated KJ1-26 followed by APC-conjugated streptavidin to identify the transgenic cells. Four-color flow cytometry was performed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA), and cells were analyzed using CellQuest acquisition/analysis software. Between 20 × 10³ and 10⁶ events were collected.

Adoptive transfer and immunization

Labeled splenocytes were plated at 10⁵ cells per well in a round-bottom 96-well plate in RPMI 1640 medium containing 10% FCS (HyClone, Logan, UT), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5 μM 2-ME, and stimulated with varying concentrations of OVA peptide 323–339 in the presence of anti-CD28 mAb or control hamster mAb.

Adaptive transfer and immunization

A total of 2.5–5.0 × 10⁶ CFSE-labeled transgenic cells (obtained from the lymph nodes and spleen of DO11.10 or DO11.10/RAG-2−/− mice) were injected i.v. into BALB/c mice in a total volume of 0.2 ml. One to 4 days later the mice were immunized with 50 μg of OVA peptide 323–339 into two sites in the lower back and at the base of the tail (total of 150 μg/mouse). Mice were treated on day −1, 0, and 2 days (relative to immunization) with 200 μg or 500 μg of either CTLA4 Ig or human IgG i.p.
The time required for the average responding T cell to achieve a single cell division, or the doubling time \( T_d \), can be determined from the proliferative capacity and the period of time between the onset of proliferation and the peak of clonal expansions \( T \) (Equation 7). In all experiments, the value of \( T \) was 48 h.

\[
T_d = \frac{(T)(\ln 2)}{\ln C_p}
\]  

Because both the frequency and proliferative capacity of Ag-specific T cells that participate in clonal expansion can be inferred directly from the pattern of cell division exhibited by a sample of daughter cells taken at a given time during or after the response, the quantitation of these components of clonal expansion is therefore independent of the net yield of Ag-specific T cells measured at the peak of the response. In this way, the “expected” yield of daughter cells (projected from the precursor number as described above) can be compared with the observed yield measured at the peak of clonal expansion using the anti-clonotypic Ab.

**Results**

**Quantitative assessment of Ag-specific T cell clonal expansion in vitro**

CFSE segregates equally between daughter cells upon cell division, resulting in sequential halving of fluorescence intensity with each generation (10). By monitoring how individual T cells divide in response to a given stimulus, key parameters of a proliferative response such as the proportion of T cells that participate in clonal expansion (precursor or responder frequency), the number of daughter cells generated by each precursor T cell (proliferative capacity), and the absolute number of cell divisions accumulated within a population over time can be assessed quantitatively (11). To assess the determining factors for clonal expansion in response to a physiologic stimulus, splenocytes from DO11.10 mice, which carry a transgenic TCR specific for an OVA-derived peptide presented in the context of I-A\(^d\) (3), were labeled with CFSE and cultured in vitro with various doses of OVA peptide. In this culture system, splenic APCs provide the ligand for TCR, as well as endogenous costimulatory interactions. Alternatively, DO11.10 splenocytes were cultured with OVA peptide in the presence of agonistic anti-CD28 Ab, to provide maximal CD28-mediated costimulation. OVA-specific CD4\(^+\) T cells were identified using an anti-clonotypic Ab (3), and cell division (CFSE fluorescence) was monitored by flow cytometry (Fig. 1). Cell division began \( \sim 48 \) h following stimulation, and the phase of clonal expansion in vitro was complete within 48–72 h (data not shown). The dynamics of Ag-specific T cell proliferation were dependent on the dose of OVA peptide, as the frequency of OVA-specific T cells that participated in clonal expansion (responder frequency) increased 4-fold, and proliferative capacity of the responders increased 2-fold when the dose of OVA peptide was raised from 5 ng/ml to 50 \( \mu \)g/ml (Fig. 1 and Table I). The rate of clonal expansion also increased with higher peptide concentration, as cells stimulated at low peptide dose exhibited a doubling time of 20.7 h, whereas cells stimulated at high peptide dose exhibited a doubling time of 14.7 h (Table I). CD28 costimulation augmented each of these response parameters at low peptide concentrations; however, provision of maximal CD28 costimulation signals did not have any effect when OVA-specific T cells were stimulated at a high peptide dose (Fig. 1 and Table I). Therefore, consistent with our previous studies (11), either a combination of low TCR occupancy and high CD28 costimulation (Fig. 1D) or high TCR occupancy and low/endogenous CD28 costimulation (Fig. 1A) could result in optimal in vitro clonal expansion. The combination of high TCR occupancy and high CD28 costimulation (Fig. 1C), although leading to an efficient proliferative response, was not optimal as it resulted in reduced survival of the expanded population compared with optimal TCR/CD28 coligation (Table I). Notably, and consistent with our findings using anti-CD3 mAb as a stimulus (11), the responder frequency never exceeded 80%, even with maximal TCR and CD28 signaling. This was not due to failure of Ag-reactive T cells to receive these signals, as we observed a peptide dose-dependent decrease in clonotype TCR expression (data not shown), indicating that the entire specific T cell population had been exposed to Ag (14, 15).

**Quantitative assessment of Ag-specific T cell activation and clonal expansion in vivo**

We were next interested in how signals from TCR and CD28 might quantitatively influence the dynamics of clonal expansion in a physiologic, in vivo microenvironment. CFSE-labeled spleen and lymph node cells from DO11.10 mice were adoptively transferred into syngeneic BALB/c mice, and the recipients were immunized s.c. with OVA peptide in adjuvant (IFA or CFA).
Twenty hours after peptide immunization, >98% of the OVA-specific CD4⁺ T cells in the draining lymph nodes showed evidence of activation, as indicated by up-regulation of CD69 and down-regulation of CD62L (16) (Fig. 2, A and B). By this route of immunization, essentially every OVA-specific T cell in the mouse had apparently been exposed to Ag, as this degree of activation was evident not only in the regional lymph nodes but also in the distant lymph nodes and in the spleen (data not shown). This extent of early T cell activation is consistent with previous observations in this adoptive transfer model (4). As mentioned above, in vitro stimulation of OVA-specific T cells resulted in extensive TCR down-modulation. In contrast, despite evidence of extensive TCR signaling at 20 h postimmunization, we detected no down-modulation of the transgenic TCR on OVA-specific CD4⁺ T cells in vivo (Fig. 2C). Blockade of B7-CD28 interactions did not appear to interfere quantitatively with the early stages of activation in vivo (Fig. 2, A and B); however, individual OVA-specific T cells immunized in the presence of CTLA4Ig expressed a lower surface density of CD69 as compared with OVA-specific T cells immunized in the presence of control Ig (Fig. 2A).

Examination of OVA-specific CD4⁺ T cell CFSE profiles at serial time points revealed the onset of cell division approximately 30 h after immunization (data not shown). Over the 42- to 48-h period from the beginning of cell division to the peak of expansion, each responding OVA-specific precursor generated greater than 20 daughter cells (Fig. 3A and Table II). Therefore, during this period, responding T cells underwent between four and five cell divisions and traversed the cell cycle in 10.6 h (Table II). To illustrate, in a representative experiment each peripheral lymph node contained ~20,000 OVA-specific CD4⁺ T cells (0.3–0.5% of total lymph node cells) before the onset of proliferation. Seventy-two hours after immunization, the division pattern of individual Ag-specific T cells indicated that ~280,000 mitotic events had occurred in each draining lymph node, resulting in the generation of 300,000 daughter cells. The proliferation observed in these experiments occurred in response to peptide, as no cell division was observed in the OVA-specific CD4⁺ T cell population from unimmunized mice or from mice immunized with PBS in adjuvant (Fig. 3C).

**Table I. Quantitative dynamics of OVA-specific CD4⁺ T cell proliferation and survival in vitro**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Survival⁺</th>
<th>Responder frequency⁺</th>
<th>Proliferative capacity⁺</th>
<th>Doubling time⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.005</td>
<td>50</td>
<td>0.005</td>
<td>50</td>
</tr>
<tr>
<td>+Control Ig</td>
<td>73 26</td>
<td>0.77 0.22</td>
<td>9.6 4.6</td>
<td>14.7 20.7</td>
</tr>
<tr>
<td>+Anti-CD28</td>
<td>50 90</td>
<td>0.81 0.62</td>
<td>9.7 10.1</td>
<td>14.6 14.4</td>
</tr>
</tbody>
</table>

*Expressed as the proportion of CD4⁺ KJ1-26⁺ T cells excluding the vital dye TOPRO-3.
⁺Expressed as the percentage of CD4⁺ T cells responding to stimulation by dividing (see Materials and Methods).
⁺Expressed as the mean number of daughter cells generated per CD4⁺ KJ1-26⁺ precursor T cell (see Materials and Methods).
⁺Defined as the number of hours required for the average CD4⁺ KJ1-26⁺ T cell to undergo one round of division (see Materials and Methods). The data shown are representative of at least four separate experiments.

**FIGURE 2.** Early activation of OVA-specific CD4⁺ T cells following stimulation in vivo with nominal Ag under varying costimulatory environments. DO11.10 BALB/c lymphocytes were labeled with CFSE and adoptively transferred into normal BALB/c mice (5 × 10⁶ transgenic T cells per recipient). After 24 h, the recipients were injected s.c. with either PBS in IFA (dotted lines) or OVA peptide in IFA, followed by i.p. injection of either CTLA4Ig (thin solid lines) or control human IgG (thick solid lines) as described in Materials and Methods. Twenty hours later, regional (draining) lymph node cells were harvested and the expression of CD69 (A), CD62L (B) and TCR (C) on the CD4⁺ KJ1-26⁺ T cell subset was assessed by flow cytometry. The data shown are representative of at least four separate experiments.

**Individual Ag-specific T cell proliferative potential: influence of allelic exclusion at the TCRα locus**

Interestingly, despite the fact that essentially every OVA-specific CD4⁺ T cell was activated as a result of Ag encounter in vivo (Fig. 2), only 65% of these cells actually participated in the phase of clonal expansion described above (Table II and Fig. 3A, note undivided population). This apparent limitation in the potential of OVA-specific CD4⁺ T cells to undergo cell division following activation in vivo is similar to that observed in OVA-specific CD4⁺ T cells stimulated in vitro (Fig. 1) and to our previous results using wild-type T cells subjected to polyclonal stimulation in vitro (11). Although this constraint could be due to many factors, one possibility is that heterogeneity in TCRα-chain usage might influence T cell proliferative potential. Allelic exclusion at the TCRα locus is relatively inefficient compared with allelic exclusion at the TCRβ locus (17); therefore a significant proportion...
of the mature T cells in the DO11.10 mouse express an endogeneously rearranged α-chain in addition to the transgenic α- and β-chains (18). The expression of an additional, endogenous α-chain has two important consequences: 1) the endogenous α-chain may pair with the transgenic β-chain, displacing the transgenic α-chain and effectively reducing the surface density of the transgenic TCRαβ, and 2) these “hybrid” TCR extend the specificity of those transgenic T cells which express them, such that a subset can react to environmental Ags and differentiate into memory cells. Indeed, the proportion of transgenic T cells from a DO11.10 mouse that exhibit a phenotype consistent with previous Ag exposure can approach 30% (19, 20). To determine whether the expression of an alternate TCRα-chain affects the dynamics of OVA-specific T cell clonal expansion in response to OVA peptide in vivo, we performed adoptive transfer experiments using DO11.10 mice which lack a functional RAG-2 gene (RAG-2−/−DO11.10) as a source of donor T cells. As the rag-2 gene is required for recombination at Ag receptor loci, T cells from RAG-2−/−DO11.10 mice lack endogenous α-chains, express the transgenic TCRαβ exclusively, and should maintain a fully naive phenotype in the absence of expression of OVA peptide. After stimulation in vivo, RAG-2−/− OVA-specific T cells showed a proliferative capacity similar to that of normal (i.e., recombination-competent) OVA-specific T cells, with each responder generating ~25 daughter cells (Table III). However, the absence of endogenous TCRα-chains was associated with an increased responder frequency, as 95% of the RAG-2−/− OVA-specific T cells from immunized mice participated in clonal expansion (Table III), compared with only 65% for normal OVA-specific T cells (Table II). The difference in responder frequency between normal and RAG-2−/− OVA-specific T cells is not due to differential density of the transgenic OVA-specific TCRαβ, as the level of this TCR on DO11.10 versus RAG-2−/− DO11.10 T cells was equivalent as determined by the binding of the anti-clonotypic Ab KJ1-26 (data not shown). These results suggest that incomplete allelic exclusion at the TCRα locus can interfere with the full proliferative potential of an Ag-specific T cell population, possibly by decreasing the frequency of cells with a naive phenotype.

Contribution of CD28 signals to the dynamics of Ag-specific T cell proliferation in vivo

OVA-specific CD4+ T cells from recipient mice immunized with OVA peptide and treated with CTLA4Ig exhibited a drastic reduction in proliferation (Fig. 3B). Analysis of cell division at the single-cell level revealed that this impairment was associated with a 2-fold reduction in the responder frequency, a 4-fold reduction in

Table II. Quantitative dynamics of OVA-specific CD4+ T cell accumulation and proliferation in regional and distant lymph nodes following immunization with OVA peptide in vivo

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Lymph Node</th>
<th>OVA-Specific T Cell Accumulation* (×10^5)</th>
<th>Responder Frequencyb</th>
<th>Proliferative Capacityb</th>
<th>Doubling Timec</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA/IFA</td>
<td>Regional</td>
<td>228 ± 113c</td>
<td>0.67 ± 0.11c</td>
<td>20 ± 4c</td>
<td>10.6</td>
</tr>
<tr>
<td>+Control Ig</td>
<td>Regional</td>
<td>49 ± 13</td>
<td>0.65 ± 0.08</td>
<td>5 ± 2</td>
<td>20.7</td>
</tr>
<tr>
<td>+CTLA4Ig</td>
<td>Regional</td>
<td>36 ± 18</td>
<td>0.35 ± 0.10</td>
<td>5 ± 1</td>
<td>20.7</td>
</tr>
<tr>
<td>PBS/none</td>
<td>Pooled</td>
<td>14 ± 5</td>
<td>0.14 ± 0.05</td>
<td>3 ± 1</td>
<td>30.3</td>
</tr>
</tbody>
</table>

* Expressed as the absolute number of CD4+ KJ1-26+ T cells yielded per regional lymph node 72 h postimmunization. A total of four to five regional lymph nodes and six to seven distant lymph nodes was collected from each recipient. Within each experiment, each group of recipient mice (OVA/IFA + control Ig, OVA/IFA + CTLA4Ig, PBS/IFA + control Ig) received equal numbers of donor transgenic T cells, varying from 2 × 10^6 to 5 × 10^6 between experiments.

b See legend to Table I.

c Values represent the mean ± SE of the data obtained from three separate experiments.

d Not applicable.
the proliferative capacity, and a 2-fold decrease in the rate of expansion (Table II). CTLA4Ig treatment had a similar effect on the proliferative dynamics of RAG-2−/− OVA-specific T cells (Table III). This abortive proliferative response occurred despite the fact that Ag encounter and early activation was not impaired under conditions of CD28 blockade (Fig. 2). These results also demonstrate that the small proliferative response observed in the presence of CTLA4Ig is due not to the phenotypically normal proliferative response of a small subset of cells that “escaped” costimulatory blockade, but rather to a global reduction in the proliferative potential and proliferative capacity of all OVA-specific T cells.

Dynamics of Ag-specific T cell proliferation in nondraining lymph nodes
As noted above, immunization of recipient mice with OVA peptide in adjuvant resulted in the activation of all OVA-specific T cells, even those in distant (nondraining) lymph nodes. Consistent with this observed activation, we measured a slight accumulation of OVA-specific CD4+ T cells in the distant lymph nodes 72 h after immunization, and this accumulation was associated with a quantitatively small proliferative response in which only five daughters were generated per responder (Table II). However, the frequency of OVA-specific CD4+ T cells that entered the proliferating pool was as high (65%) as that observed in regional lymph nodes. This distant response was CD28-dependent, as systemic administration of CTLA4Ig blocked the majority of proliferation (Table II). Similar results were obtained using RAG-2−/− OVA-specific T cells (data not shown).

Measuring the efficiency of Ag-specific T cell clonal expansion in vivo
Enumeration of OVA-specific (KJ1-26+) CD4+ T cells in the regional lymph nodes of mice immunized with OVA peptide, indicated an approximate 10-fold increase in the relative number of Ag-specific CD4+ T cells by 72 h postimmunization (Tables II and III). However, the accumulation of cell divisions within the pool of Ag-specific T cells at the peak of clonal expansion (day 3) suggested that a 15-fold increase in the number of OVA-specific T cells (Table II), and a 25-fold increase in the number of RAG-2−/− OVA-specific T cells (Table III) must have occurred (the fold increase in OVA-specific T cells is the product of the responder frequency and the proliferative capacity ($R \times C_p$); see Materials and Methods). The apparent contradiction between the degree of clonal expansion measured by the direct detection of clonotypic T cells present in the regional lymph nodes vs the degree of clonal expansion extrapolated from the proliferative history of OVA-specific daughter cells can be reconciled by the fact that the majority of effector cells generated during an immune response ultimately undergo programmed cell death (21, 22). That a high rate of cell death may be operative in this model is supported by our in vitro experiments in which up to 50% of OVA-specific CD4+ T cells had died during the response to high levels TCR and CD28 engagement (Table I), and that the majority of the dead T cells had undergone several rounds of division (data not shown). Therefore, although the OVA-specific daughter cells that remained after the peak of the response bore the imprint of extensive clonal expansion, our results suggest that roughly 50% of the daughter cells generated during the expansion phase ultimately died, resulting in a net 10-fold increase in the number of OVA-specific CD4+ T cells at the peak of the response. In this context, the “efficiency” of this proliferative burst (in the production of live effector cells) was only 50%, in that the generation of ~20 daughter cells from a single precursor T cell was required to yield 10 Ag-specific effector cells.

In the absence of CD28 costimulation, we observed little or no expansion of the OVA-specific CD4+ T cell pool (Tables II and III). Although the failure to expand under these conditions was due largely to reduced proliferation, the residual number of mitotic events that did occur should have resulted in an ~2-fold increase in the OVA-specific T cell pool. Therefore, the rate of OVA-specific T cell clonal expansion in the presence of CD28 costimulatory blockade was balanced by the rate of cell death.

Division cycle-associated T cell differentiation in vivo
Naïve T cells express a low surface density of the homing molecule CD44, a high density of the homing molecule CD62L, and a high density of the receptor tyrosine phosphatase CD45 isoform RB (16). Following encounter with Ag, the expression pattern of these surface molecules becomes reversed, and consequently, a CD44highCD45RBlowCD62Llow surface phenotype is generally associated with the differentiation of T cells toward an effector, or eventually, a memory phenotype (16). To determine how T cell differentiation, as defined by the expression of these surface markers, might be influenced by the dynamics of clonal expansion in response to nominal Ag in vivo, we measured the expression of CD44, CD45RB, and CD62L on individual, adoptively transferred OVA-specific CD4+ T cells following immunization with peptide. At the population level, most OVA-specific T cells from mice given PBS in adjuvant expressed surface levels of CD44, CD45RB, and CD62L consistent with a naïve phenotype (Fig. 4A), whereas the surface phenotype of OVA-specific T cells from mice immunized with OVA peptide indicated that the majority had differentiated into CD44highCD45RBlowCD62Llow effector cells (Fig. 4B). At the single-cell level, the differentiation of OVA-specific CD4+ T cells was quantitatively associated with the number of cell divisions achieved during the preceding phase of clonal expansion (Fig. 4D). CD44 was up-regulated after a single mitosis, and remained elevated throughout at least seven rounds of cell division.

Table III. Quantitative dynamics of RAG-2−/− OVA-specific CD4+ T cell accumulation and proliferation in regional lymph nodes following immunization with OVA peptide in vivo

<table>
<thead>
<tr>
<th>Immunization</th>
<th>OVA-Specific T Cell Accumulation</th>
<th>Responder Frequency</th>
<th>Proliferative Capacity</th>
<th>Doubling Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA/IFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Control Ig</td>
<td>123 ± 15</td>
<td>0.93 ± 0.04</td>
<td>26.2 ± 2.9</td>
<td>10.2</td>
</tr>
<tr>
<td>+CTLA4Ig</td>
<td>6.8 ± 0.9</td>
<td>0.63 ± 0.09</td>
<td>4.4 ± 1.7</td>
<td>22.4</td>
</tr>
<tr>
<td>PBS/NONE</td>
<td></td>
<td></td>
<td></td>
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* See legend to Table II.
* See legend to Table I.
* Values represent the mean ± SE of the data obtained from three separate experiments.
* Not applicable.
 Seventy-two hours later, regional lymph node cells were harvested and cell division as well as the expression of CD44 (left panel), CD45RB (middle panels) and CD62L (right panels) by individual CD4 T cells were assessed by flow cytometry. D. The relative levels of CD44, CD45RB, and CD62L on OVA-specific CD4 T cells (shown in A–C) are plotted as a function of primary division cycle. The data shown are representative of three separate experiments (PBS in IFA, △; OVA peptide plus control Ig, ○; OVA peptide in IFA plus CTLA4Ig, ◊).

**FIGURE 4.** Cell division cycle-associated expression of differentiation markers by OVA-specific CD4 T cells following stimulation with nominal Ag in vivo under varying costimulatory environments. DO11.10 BALB/c lymphocytes were labeled with CFSE and adoptively transferred into normal BALB/c mice. After 24 h, the recipients were injected s.c. with either PBS in IFA (A) or OVA peptide in IFA, followed by i.p. injection of either control human IgG (B) or CTLA4Ig (C). Seventy-two hours later, regional lymph node cells were harvested and cell division as well as the expression of CD44 (left panel), CD45RB (middle panels) and CD62L (right panels) by individual CD4 T cells were assessed by flow cytometry. D. The relative levels of CD44, CD45RB, and CD62L on OVA-specific CD4 T cells (shown in A–C) are plotted as a function of primary division cycle. The data shown are representative of three separate experiments (PBS in IFA, △; OVA peptide plus control Ig, ○; OVA peptide in IFA plus CTLA4Ig, ◊).

(Fig. 4D, left panel). This pattern of CD44 expression was observed regardless of the CD28 costimulatory environment during immunization (Fig. 4D, left panel). The surface density of CD45RB decreased 2-fold with each cell division, exhibiting a second-order decay consistent with the equal segregation of the molecule during each mitotic event (Fig. 4D, middle panel). This pattern of CD45RB expression was also independent of CD28 co-stimulation (Fig. 4D, middle panel). OVA-specific T cell populations from mice immunized under physiologic costimulatory conditions contained relatively more CD62Llow cells than populations from mice immunized in the presence of CTLA4Ig (Fig. 4, B vs C, right panels). The surface density of CD62L was also associated with division cycle; however, its expression at the single-cell level followed more complex dynamics than CD44 and CD45RB. The mean surface density of the undivided population at 72 h postimmunization remained low relative to the density on unimmunized T cells (Fig. 4D, right panel), reflecting the initial down-modulation of CD62L observed on all OVA-specific CD4 T cells 20 h after immunization (Fig. 2B). With each subsequent division cycle, there was a greater increase in the mean density of CD62L on the surface of OVA-specific CD4 T cells (Fig. 4D, right panel). This pattern of CD62L down-modulation immediately following TCR engagement, then up-regulation during the effector phase in vivo, has been described (6, 23).

**Division cycle-associated cytokine production in vivo**

The data above show that the reduction in the relative number of OVA-specific T cells in mice treated with CTLA4Ig is associated with impaired clonal expansion. The apparent linkage between the relative proliferative history of an individual CD4 T cell and its quantitative expression of these markers suggests that CD4 T cell differentiation may be driven in part by the process of clonal expansion. To determine whether a similar association exists between cell division and actual effector function, we adoptively transferred OVA-specific CD4 T cells into normal BALB/c mice. To ensure that all the donor cells were naive, we used OVA-specific T cells on a RAG-2/− background as donor cells, thus eliminating dual receptor T cells that are not naive because of previous encounter with an environmental Ag. OVA-specific CD4 T cells from the regional or distant lymph nodes of immunized recipients were restimulated in vitro for 5 h with OVA peptide and the frequency of cytokine-secreting cells was assessed by flow cytometry. The cytokine production measured in these experiments specifically reflects T cells primed by prior encounter with Ag, as naive OVA-specific CD4 T cells isolated from unimmunized recipients exhibited essentially no IL-2 or IFN-γ production during the assay period (data not shown). At the bulk level, >60% of the total OVA-specific CD4 T cells immunized in vivo under physiologic costimulatory conditions were capable of producing IL-2, and >15% were capable of producing IFN-γ (Fig. 5A and Table IV). In the distant lymph nodes of these mice, only 25% of the OVA-specific CD4 T cells produced IL-2, whereas <3% were capable of producing IFN-γ (Fig. 5B and Table IV). This decrease in the proportion of OVA-specific T cells capable of producing cytokines in the distant compared with the regional lymph nodes...
FIGURE 5. Cytokine production by individual OVA-specific CD4+ T cells following stimulation with nominal Ag in vivo under varying costimulatory environments. DO11.10/RAG-2−/− lymphocytes were labeled with CFSE and adoptively transferred into normal BALB/c mice. After 24 h, the recipients were injected s.c. with OVA peptide in IFA, followed by i.p. injection of either control human IgG (A and B) or CTLA4Ig (C and D). Seventy-two hours later, regional (A and C) and distant (B and D) lymph node cells were harvested and cultured with 5 μg/ml OVA peptide, anti-CD8, and monensin for 5 h and IL-2 (left panels) or IFN-γ (right panels) production by individual CD4+KJ1-26+ T cells was assessed by flow cytometry (see Materials and Methods). Dotted lines represent the fluorescence of the CD4+KJ1-26+ subset stained with fluorochrome-matched isotype control Ab, and solid lines represent the fluorescence of this subset stained with specific anti-cytokine Ab. The histograms shown are from a single representative experiment, and the values in each panel represent the mean percent cytokine-producers compared with OVA-specific T cells from the same recipient mice (Fig. 6A, inset). To verify that the increase in IL-2 production with cell division is a true phenomenon and not an artifact due to CFSE brightness, we performed parallel experiments using PE-(FL-2) and APC-conjugated (FL-4) anti-IL-2 Abs, as CFSE does not bleed into flow cytometry channel FL-4 (data not shown). These experiments showed identical results, supporting the finding that increasing IL-2 production with cell division is

translates to a 10-fold and 40-fold reduction in the absolute number of IL-2 producers and IFN-γ producers, respectively (Table IV). CD28 blockade during immunization resulted in at least a 2-fold reduction in the proportion of OVA-specific T cells capable of producing IL-2, and a >10-fold reduction in the proportion of IFN-γ-producers compared with OVA-specific T cells from the regional lymph nodes of mice immunized under physiologic costimulatory conditions (Fig. 5, C and D, Table IV). These differences in the proportion of cytokine producers translate to greater than a 30-fold reduction in the absolute number of OVA-specific T cells capable of producing IL-2, and more than a 100-fold reduction in the absolute number of OVA-specific T cells capable of producing IFN-γ (Table IV). Interestingly, the degree of clonal expansion influenced not only the absolute quantity of cytokine-producing T cells, but also appeared to affect cytokine potential at the single-cell level. Individual OVA-specific CD4+ T cells from mice immunized under physiologic costimulatory conditions were more likely to produce IL-2 with each round of division achieved during primary clonal expansion (Fig. 6A), such that <10% of undivided cells produced IL-2, whereas 40% of the cells that had divided twice, and >80% of the cells that had divided five or more times, were capable of producing IL-2 (Fig. 6G, filled symbols). This direct proportionality between IL-2 production and cell division can be visualized more clearly in the population of OVA-specific T cells obtained from the distant lymph nodes of the same recipient mice (Fig. 6A, inset). To verify that the increase in IL-2 production with cell division is a true phenomenon and not an artifact due to CFSE brightness, we performed parallel experiments using PE-(FL-2) and APC-conjugated (FL-4) anti-IL-2 Abs, as CFSE does not bleed into flow cytometry channel FL-4 (data not shown). These experiments showed identical results, supporting the finding that increasing IL-2 production with cell division is

<table>
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<th>Immunization</th>
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<th>OVA-Specific T Cells at 72 h (×10^3)</th>
<th>Cytokine-Producing OVA-Specific T Cells at 72 h (×10^3)</th>
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<tr>
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Table IV. Absolute numbers of cytokine-producing OVA-specific (RAG-2−/−) T cells per lymph node at the peak of the expansion phase in vivo

a Absolute number of CD4+KJ1-26+ T cells detected per lymph node at 72 h postimmunization.
b Absolute number of CD4+KJ1-26+ T cells capable of producing cytokine on a per lymph node basis 72 h postimmunization, obtained by multiplying the absolute number of CD4+KJ1-26+ T cells per lymph node by the frequency of CD4+KJ1-26+ T cells that produced cytokine during a 5-h antigenic boost in vitro (see Fig. 5).

c Values represent the mean ± SD of the data obtained from three separate experiments.
d Values represent the mean ± SE of the data obtained from two separate experiments.
a true phenomenon and not an artifact. The production of IFN-γ by these cells was also markedly linked to cell division (Fig. 6B), such that only those OVA-specific T cells that had divided five or more times were capable of appreciable IFN-γ production (Fig. 6H, filled symbols). Again, the relationship between IFN-γ production and cell division is more apparent in OVA-specific T cells obtained from the distant lymph nodes of the same recipient mice (Fig. 6B, inset). The fluorescence of OVA-specific CD4+T cells in this assay specifically reflects cytokine content, as the same populations stained with fluorochrome-conjugated isotype control Abs exhibited no positive events (Fig. 6C and F). The proportion of CD4+KJ1-26+ T cells positive for IL-2 (G) or IFN-γ (H) is shown plotted as a function of primary division cycle.

Discussion

Over the past several years, the system of adoptive transfer of TCR-transgenic T cells first introduced by Kearney et al. (4) has been widely utilized to examine Ag-specific T cell responses in vivo (reviewed in Ref. 24). While a significant advance over previous approaches, this approach by itself cannot define quantitative parameters of T cell proliferation such as the responder frequency of the population or the proliferative capacity of the responding cells, nor can it assess directly the relationship of these parameters to effector function. To address these questions in this study, we have coupled the adoptive transfer of TCR-transgenic T cell cells to the use of the fluorescent dye CFSE, permitting us to follow the proliferative history of individual cells as well as to examine how they differentiate and acquire functional capacity as they pass through multiple rounds of cell division.

While it is possible that the lower than predicted cell yields in regional lymph nodes are the result (at least partially) of sequestration of clonotypic T cells in other anatomic sites, examination of likely sites such as the spleen and distant lymph nodes demonstrated only a minor accumulation of OVA-specific T cells. However, we cannot exclude some emigration to nonlymphoid sites.
We considered also that TCR down-regulation as a result of Ag exposure could prevent detection of Ag-specific cells, resulting in an underestimation of their number. TCR down-regulation clearly occurs in vitro in this system (Fig. 1); however, it is not complete. Furthermore, and consistent with other reports, we find no evidence for significant in vivo TCR down-regulation at the time points examined, despite the fact that the T cells bear markers of Ag activation, such as CD69 and CD44 expression (4, 5, 25). In vitro, we have noted that when peptide-pulsed APCs are used to activate OVA-specific T cells, TCR down-regulation is transient, and reexpression occurs by 72 h of culture (data not shown). It is likely that Ag concentration in draining lymph nodes is below the level required to maintain TCR down-regulation.

The accumulation of Ag-specific T cells during an in vivo immune response reflects input into the compartment as a result of cell division, as well as loss due to cell death. Studies using either anti-clonotypic Abs (in a TCR-transgenic system) or MHC-peptide tetramers can determine the size of the Ag-reactive T cell compartment, but cannot evaluate the relative contribution of cell proliferation and apoptosis (24, 26), a determination which is possible with CFSE labeling. The number of transgenic T cells measured in the draining lymph nodes at the peak of expansion in our model was consistently about half of that estimated from the division profile of this same population. Implicit in the calculation of cell loss is the assumption that emigration from the lymph nodes and TCR down-regulation do not play a significant role, but also that the cells that died during the course of the immune response exhibit a roughly equivalent proliferative profile (responder frequency and proliferative capacity) as the cells that survived and were available for analysis. In support of this, we have observed that live and dead CD4\(^+\) T cells exhibit extremely similar patterns of cell division after an in vitro response (where the division of the dead T cells can be specifically tracked and quantified (Ref. 11; data not shown)). Furthermore, cells that undergo apoptosis during immune response in vivo are known to consist of both proliferating and quiescent cells, susceptible to passive cell death or AICD (activation-induced cell death), respectively (21, 22). However, if the relationship between cell death and cell division during an in vivo response is very different from that observed in vitro, then our estimate of the size of the dead T cell pool would be affected proportionately. For instance, if the “missing” T cells had divided to a lesser extent before they died as compared with the remaining live population, then our original calculation would represent an overestimate. In any event, our results suggest that cell death during the initial phase of the immune response is a significant limiting feature of the efficiency of the response, at least as defined by the accumulation of Ag-reactive cells.

One of the most powerful features of CFSE labeling is the ability to calculate the T cell responder frequency, i.e., the proportion of the Ag-reactive population that undergo proliferation following Ag-stimulation. Previously, we have shown that polyclonal stimulation of lymph node T cells from normal mice, leads to a maximal responder frequency of only 50–65%, even under conditions of optimal TCR and CD28 stimulation, despite demonstrable activation of >95% of T cells as determined by CD25 and CD69 expression (11). Here, we find a similar responder frequency to nominal Ag, both in vitro and in vivo. Perhaps surprisingly, the responder frequency approached 95% (i.e., all activated cells proliferate) when TCR-transgenic cells from mice with RAG-2\(^{-/-}\) background were used. As the levels of transgenic TCR expression on the two sets of cells were comparable, differences in the availability of TCR signaling are not likely to account for this finding. Rather, we favor the interpretation that the lower responder frequency seen in the recombination competent mice reflects the presence of previously stimulated T cells within the transgenic pool. If so, these cells may represent a “terminally differentiated” effector population with a low proliferative potential, a possibility currently under investigation.

With CFSE labeling, we also were able to determine the proliferative capacity of individual T cells which had responded to Ag by dividing. Here, we find that each dividing Ag-specific T cell generates on average 20 daughter cells during an in vivo immune response (Table II), but only 10 daughter cells when stimulated in vitro by soluble Ag in the presence of APCs and agonistic anti-CD28 mAbs (Table I). Interestingly, the responder frequency is roughly comparable in each instance, 60–80% (depending on the peptide concentration used). This finding suggests that signals which regulate the initiation of the mitotic program following cell activation are similar in vivo and in vitro, but that the in vivo microenvironment is superior in sustaining the response.

The use of fluorescent labeling to track T cell division also has helped define the role of CD28 costimulatory signals during in vivo responses. As was suggested by in vitro studies, CD28 signaling both increased the number of cells that participated in the proliferative response as well as enhanced their proliferative capacity. In vitro, CD28 costimulation was particularly important at low peptide concentration. Previously, using the system of adoptive transfer of TCR-transgenic T cells, it was shown that following Ag immunization, Ag-specific T cells accumulate in both regional and distant nodes, although only transiently so in the latter (4). Here, we directly demonstrate that the OVA-specific T cells found in the distant lymph nodes have proliferated, although less dramatically than cells in the regional lymph nodes. This is unlikely to be explained solely by recirculation of OVA-specific T cells stimulated by OVA peptide in the regional lymph nodes, as all the reactive T cells, regardless of location, were activated as early as 20 h after immunization. It is more likely that peptide is absorbed into the systemic circulation and finds its way to the distant lymph nodes where it can stimulate potentially reactive T cells to proliferate. The observed decreased proliferation of OVA-specific T cells in the distant compared with the regional lymph nodes therefore most likely reflects dose-dependent effects of OVA peptide on the proliferative response.

Consistent with published data we find the appearance of a more ‘mature’ phenotype as the cells progress through division (27–29). Previously, it has been shown that cell cycle blockade does not prevent up-regulation of CD44 (30). Here, we show that CD44 expression is maximally up-regulated after only one round of division, and that subsequent mitoses do not affect CD44 surface levels. As CD44 is required for recruitment of effector cells into inflammatory sites, this suggests that divided cells are preferentially suited to carry out local immune functions (31). In contrast, we observe sequential halving of CD45RB with each cell division, suggesting dilution of a stable protein product with progressive cell division. This finding suggests that multiple rounds of cell division are required to generate a population with a surface phenotype consistent with memory function (32, 33).

Our results help clarify the role of proliferation in cytokine production and the mechanism by which deprivation of costimulatory signals inhibits the development of cytokine secreting effector cells. Recent studies have shown that the production of IL-2 by in vitro-stimulated T cells was cell cycle-independent, while the frequency of IFN-γ producing cells increased with successive cell mitoses and was controlled through epigenetic modification of genes involving demethylation and acetylation (30). Khoruts et al. (34) also have suggested that IL-2 production can precede proliferation based on kinetic analyses of the two events. Both studies examined cytokine production during the early phases of primary
clonal expansion of naive T cells. Here we are studying a time point late in clonal expansion when activated effector cells expressing cell cycle-dependent genes, such as IFN-γ (30), are emerging. Thus we are able to establish a linkage between the initial replicative history of a naive cell and its likelihood of IL-2 and IFN-γ production when faced again with Ag. In our own studies, cells were “boosted” by culture with OVA peptide for 4–6 h. This type of stimulation does not elicit a cytokine response in naive T cells within the time period of the assay (Ref. 13 and our unpublished observations), meaning that only cells which were already activated in vivo are responding. As the assay is performed 3 days after in vivo Ag immunization, we consider it reflective of the terminal phases of the primary response, although we cannot formally exclude that a small number of cells may have returned to their resting state and are now being restimulated.

These studies also provide new insights into the role of costimulatory signals during in vivo immune responses. Recently, Khoruts et al. (34) showed that clonal expansion and the normal induction of IL-2-producing cells during the course of an immune response in this model required CD28 costimulation. However, overall IL-2 production (as measured by ELISA in previously published papers) depends not only on the proportion of cells that make IL-2, but also on the number of IL-2-producing cells and how much each of them make (Ref. 35; reviewed in Ref. 36). Here we show that the ability of CTLA4Ig to block IL-2 production is tied to its antiproliferative effect. In any given division cycle, the proportion of IL-2-producing cells, and the amount of intracellular IL-2 protein per cell, was not impaired by CTLA4Ig. However, maximally proliferating cells were most likely to produce IL-2, and the absolute number of dividing cells was greatly reduced by CTLA4Ig. In the case of IFN-γ, CTLA4Ig also reduced the likelihood that a dividing cell would make the cytokine. This observation suggests that the ability of costimulatory blockade to inhibit cytokine production of naive T cells is a direct consequence of its ability to block cell expansion. In contrast, cytokine production by memory cells may be relatively CD28-independent (37, 38).

Dividing cells making IL-2, and IFN-γ, during a primary response despite B7/CD28 blockade may still exhibit an anergic phenotype when subsequently restimulated. Previous studies from our laboratory have suggested that cells expanding in vivo in the absence of costimulation fail to produce IL-2 or IFN-γ upon restimulation (35). Recent and elegant studies by Malvey et al. (39) support this finding with regard to IL-2, but suggest that in vivo “anergized” cells can be primed for IFN-γ production, and that costimulatory blockade inhibits responses by suppressing the expansion of primed cells, rather than by inhibiting priming per se. A longer recovery period after initial Ag challenge in their study may contribute to the differences observed. In either instance, however, despite relatively high proportions of IL-2-producing cells in the proliferating compartment (Fig. 6), the absolute numbers of cytokine producers is reduced 30- to 100-fold by costimulatory blockade with CTLA4Ig (Table IV), facilitating immunologic hyporesponsiveness.

From this and other studies, a model emerges in which TCR and CD28 signals cooperate to support both clonal expansion and cytokine production (Fig. 7). In this model, T cells with the most extensive proliferative history exhibit the greatest potential for cytokine production upon reencounter with Ag (Fig. 6; Ref. 30). CD28 costimulation promotes the generation of cytokine producing effector cells mainly by allowing naive cells to progress through multiple rounds of division. Overall, our studies provide a quantitative assessment of T cell proliferation in vivo and define the interrelationship between cell division and other immune response parameters, such as cytokine production and the availability of costimulation. The results provide unexpected insights into the regulation of proliferative responsiveness, the link between proliferation and cytokine production, and the role of costimulatory signals in regulating these responses.

**Acknowledgments**

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**References**