Tailor the adaptive immune response with
Vaccine Adjuvants
InvivoGen

The Journal of Immunology

This information is current as of January 23, 2018.

Antagonistic Roles for CTLA-4 and the Mammalian Target of Rapamycin in the Regulation of Clonal Anergy: Enhanced Cell Cycle Progression Promotes Recall Antigen Responsiveness

Tracy L. Vanasek, Alexander Khoruts, Traci Zell and Daniel L. Mueller

J Immunol 2001; 167:5636-5644; ;
doi: 10.4049/jimmunol.167.10.5636
http://www.jimmunol.org/content/167/10/5636

Why *The JI*?

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

**average*

References This article **cites 45 articles**, 29 of which you can access for free at:
http://www.jimmunol.org/content/167/10/5636.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2001 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

Antagonistic Roles for CTLA-4 and the Mammalian Target of Rapamycin in the Regulation of Clonal Anergy: Enhanced Cell Cycle Progression Promotes Recall Antigen Responsiveness[1]

Tracy L. Vanasek,* Alexander Khoruts,* Traci Zell,† and Daniel L. Mueller[2]*

CD4$^+$ T cells that undergo multiple rounds of cell division during primary Ag challenge in vivo produce IL-2 on secondary Ag rechallenge, whereas cells that fail to progress through the cell cycle are anergic to restimulation. Anti-CTLA-4 mAb treatment during primary Ag exposure increases cell cycle progression and enhances recall Ag responsiveness; however, simultaneous treatment with rapamycin, an inhibitor of the mammalian target of rapamycin and potent antiproliferative agent, prevents both effects. The data suggest that cell cycle progression plays a primary role in the regulation of recall Ag responsiveness in CD4$^+$ T cells in vivo. CTLA-4 molecules promote clonal anergy development only indirectly by limiting cell cycle progression during the primary response. ***The Journal of Immunology,*** **2001, 167: 5636–5644.**

Lymphocyte cell cycle progression is carefully regulated in the peripheral immune system. Because effector cell precursor frequencies for any individual Ag are so low in the naive repertoire, an intense clonal expansion of Ag-specific lymphocytes must precede any attempt to clear a pathogen. Similarly, immune tolerance to peripheral self-Ag relies to a great degree on the maintenance of autoreactive lymphocytes at low frequencies through various control mechanisms that prevent T cell proliferation. These mechanisms include the induction of clonal anergy, a state in which T cells lose the capacity to produce IL-2 and proliferate in response to Ag rechallenge (1–3). APC are primarily responsible for determining the response of T cells to Ag, both through their display of peptide Ag-MHC complexes, as well as through their surface expression of other costimulatory ligands that can bind receptors on the T cells. Only upon the recognition of a sufficient number of Ag-MHC complexes on an APC that has been previously conditioned to also express high levels of costimulatory ligands (in the setting of infection or tissue injury) will a T cell proliferative response be maximally induced. Following such a clonal expansion, memory phenotype CD4$^+$ Th cells are left highly responsive to secondary Ag challenge. In contrast to this immunogenic form of T cell activation, recognition of Ag-MHC complexes in the presence of only limiting amounts of accessory cell-derived costimulatory molecules leads to an abortive proliferative response that is quickly followed by the development of a persistent state of Ag unresponsiveness (4).

The molecular basis for these Ag/APC-induced alterations in T cell clonal frequency and function in vivo is only incompletely understood. In addition to TCR occupancy, the costimulation of CD4$^+$ T cells by APC-derived B7 ligands (CD80 and CD86) through CD28 receptors appears essential for Ag-stimulated clonal expansion (5). The production of IL-2 and signaling by IL-2Rs during the Ag encounter may be one consequence of this costimulation that can act to promote cell cycle progression (6, 7). However, other T cell growth factors and receptors are probably also important, since IL-2$^{-/-}$ and IL-2R$\alpha^{-/-}$ T cells can proliferate in vivo (8, 9). In contrast to CD28, another B7-binding protein called CTLA-4 (CD152) is induced on T cells during the course of the primary Ag challenge, and when it is ligated, this molecule acts to limit the extent of the clonal expansion (5, 10, 11). Furthermore, CTLA-4 signals appear necessary for the induction of clonal anergy (12–14).

The mechanism by which CTLA-4 signaling promotes anergy induction is unknown. CTLA-4 ligation can limit the production of IL-2, the expression of IL-2Rs, and entry of T cells into cell cycle (14–16). Since the avoidance and/or reversal of T cell clonal anergy have been shown in several in vitro systems to be associated with IL-2R signaling and proliferation (17–19), the capacity of CTLA-4 to place a brake on cell cycle progression may explain its importance to anergy development. Nevertheless, a more straightforward model may be that CTLA-4 signals synergize with the TCR to directly induce a putative anergy gene product.

Given the correlation observed in vivo between effective T cell clonal expansion in the primary Ag response and the apparent avoidance of clonal anergy, we questioned whether cell cycle progression per se plays a direct role in the regulation of Ag responsiveness. Specifically, we examined the hypothesis that a biochemical signaling event that occurs when naive T cells are induced to progress through cell cycle leads to a reversal of the clonal anergy state. Such a model predicts that the anergy state is induced whenever the TCR is productively engaged, and that CTLA-4 molecules only indirectly promote the maintenance of tolerance through an inhibition of cell cycle progression. To control the rate of cell cycle progression without influencing TCR, CD28, or CTLA-4 ligation,

Departments of *Medicine and †Microbiology and Center for Immunology, University of Minnesota Medical School, Minneapolis, MN 55455

Received for publication August 1, 2001. Accepted for publication September 14, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants P01 AI35296 and R01 GM54706 (to D.L.M.). T.L.V. is also a recipient of a National Institutes of Health HeaHea Immunology Predoctoral Training Grant (T32 AI07313).

[2] Address correspondence and reprint requests to Dr. Daniel L. Mueller, Center for Immunology, University of Minnesota Medical School, 312 Church Street SE, Room 6-120, Basic Science and Biomedical Engineering Building, Mayo Mail Code 334, Minneapolis, MN 55345. E-mail address: dmuell@lenti.med.umn.edu

[3] Abbreviations used in this paper: mTOR, mammalian target of rapamycin; CMC, carboxymethylcellulose; GFP, green fluorescence protein; OVAp, chicken OVA peptide 323–339; SAPK, stress-activated protein kinase.

Copyright © 2001 by The American Association of Immunologists 0022-1767/01/$02.00

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

we have made use of the T cell immunosuppressant rapamycin (a specific inhibitor of the cell cycle regulator called mammalian target of rapamycin (mTOR)[3]; Ref. 20), and monitored its effects with the cell division-sensitive fluorescence-tracking dye CFSE. Our results suggest that in $CD4^+$ T cells undergoing a primary Ag challenge, an mTOR-dependent signaling pathway leading to cell cycle progression is primarily responsible for establishing the level of recall Ag responsiveness within daughter cells. Furthermore, our experiments indicate that CTLA-4 signals are not necessary for clonal anergy induction in vivo if cell cycle progression is controlled with rapamycin. Thus, CTLA-4 acts indirectly to promote the development of clonal anergy through its inhibition of cell cycle progression.

Materials and Methods

Mice

The DO11.10 TCR-transgenic mice (21) were bred to homozygosity and maintained in our animal facility, as previously described (22). $CD4^+$ T cells in these mice are uniformly reactive to chicken OVA peptide 323–339 (OVAp)/I-A^d complexes and express a clonotypic TCR detectable with the KJ1-26 mAb (23). BALB/c mice, 6–8 wk old, were purchased from Charles River Breeding Laboratories (Wilmington, MA) through a contract with the National Cancer Institute at the National Institutes of Health (Frederick, MD). All mice were housed under specific pathogen-free conditions and used in accordance with National Institutes of Health guidelines and the University of Minnesota Institutional Animal Care and Use Committee. Mice were age and sex matched for all experiments. For some experiments, DO11.10 mice were backcrossed to BALB/c RAG-$2^{-/-}$ (Taconic Farms, Germantown, NY) to generate TCR-transgenic mice that lacked endogenous TCR gene rearrangements.

Abs and staining reagents

The following Abs and fluorochrome conjugates were purchased from BD PharMingen (San Diego, CA): CyChrome-labeled anti-CD4, allophycocyanin-labeled anti-CD4, PE-labeled anti-IL-2, PE-labeled irrelevant IgG2, PE-labeled anti-CD69, PE-labeled anti-CD25 mAb, biotin-labeled anti-mouse IgG1, FITC-labeled streptavidin, and CyChrome-labeled streptavidin. FITC-conjugated KJ1-26 mAb, biotin-labeled anti-rabbit IgG, and PE-labeled streptavidin were obtained from Caltag Laboratories (South San Francisco, CA). Anti-phospho c-Jun Ab (KM1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho p38 polyclonal Ab was purchased from New England Biolabs (Beverly, MA). Anti-FcR mAb 2.4G2 (American Type Culture Collection, Manassas, VA) was used as a 10% spent hybridoma cell culture supernatant. FCS, rat serum, formaldehyde, and saponin were obtained from Sigma-Aldrich (St. Louis, MO).

Adoptive transfer and in vivo treatments of mice

Lymph node and spleen cells containing $2.5–5 \times 10^6$ KJ1-26^+ $CD4^+$ cells from DO11.10 or DO11.10 RAG-$2^{-/-}$ TCR-transgenic mice were prepared for adoptive transfer (4), as described previously (22), and injected into the tail vein of recipient mice. In most experiments, cells were labeled with CFSE (Molecular Probes, Eugene, OR) before transfer, using a modification of a technique previously described (24). Briefly, lymph node and spleen cells at a concentration of 1×10^7 cells/ml were incubated in 2.5 μM CFSE for 5 min at 37°C. The labeling reaction was stopped with the addition of a 1:1 mixture of Eagle's Hanks' amino acids medium (Biofluids, Rockville, MD) and RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 5×10^{-5} M 2-ME. The CFSE-labeled T cells were washed twice in PBS before adoptive transfer. $OVAp_{323–339}$ was produced in our microchemical facility and dissolved in PBS (either 100 or 250 μg), and then delivered systemically to mice by i.v. injection. The hamster anti-mouse CTLA-4 mAb 4F10 (10) was purified from hybridoma cell culture supernatants using standard protein A-Sepharose chromatography techniques. Anti-CTLA-4 mAb or control hamster IgG were injected i.v. into mice at the time of Ag administration (250 μg/injection). Rapamycin was obtained from S. N. Sehgal (Wyeth-Ayerst Research, Princeton, NJ). A stock solution of 1 mg/ml rapamycin in 100% ethanol was prepared. Rapamycin was then suspended in 0.2% carboxymethylcellulose (CMC), as previously described (25), and delivered i.p. at a dose of 0.5–0.75 mg/kg per day for 4–8 days beginning on the day of T cell adoptive transfer.

Detection of surface marker expression and intracellular cytokines in DO11.10 T cells

Adoptive transfer recipients containing naive DO11.10 T cells were challenged with an i.v. injection of OVAp (100 μg) and sacrificed at various later times. To detect cell surface activation markers, lymph node and spleen cells were washed with staining buffer (PBS containing 2% FCS and 0.2% azide), and then incubated with anti-Fc mAb 2.4G2 and 10% rat serum to block FcR. Cells were then stained with the combination of CyChrome-labeled anti-CD4 mAb, biotinylated KJ1-26 mAb, FITC-labeled streptavidin, and PE-labeled anti-CD25 mAb. Alternatively, cells were stained with a mixture of allophycocyanin-labeled anti-CD4 mAb, biotinylated KJ1-26 mAb, CyChrome-labeled streptavidin, and PE-labeled anti-CD69 mAb. Intracellular IL-2 was detected essentially as previously described (8, 22). Briefly, washed and Fc-blocked lymph node or spleen cells were first incubated with allophycocyanin-labeled anti-CD4 mAb, biotinylated KJ1-26 mAb, and CyChrome-labeled streptavidin. Cells were then washed one time in PBS, fixed in 2% formaldehyde for 20 min at room temperature, permeabilized in 0.5% saponin, and incubated at room temperature for 30 min with PE-labeled anti-IL-2 mAb or PE-labeled irrelevant IgG2a. Finally, cells were washed twice in 0.5% saponin and once in staining buffer. For all experiments, more than 1000 KJ1-26^+CD4^+ events were collected using the FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star, San Carlos, CA) software.

Detection of intracellular phosphoproteins in DO11.10 T cells

Phosphorylated c-Jun and p38 were measured by flow cytometry, as previously described (26). In some experiments, spleens were removed and fixed immediately by preparing a single-cell suspension in PBS containing 2% formaldehyde. Following a 20-min incubation at room temperature in the fixative, cells were washed twice in PBS and incubated for 15 min in 1% mouse serum, 1% rat serum, and 10% anti-FcR mAb 2.4G2 culture supernatant. Cells were then stained with anti-CD4 CyChrome and FITC-labeled KJ1-26 mAb in staining buffer for 15 min at room temperature. Following a wash in staining buffer, cells were permeabilized with two washes in staining buffer containing 0.5% saponin and incubated for 30 min with PE-labeled anti-IL-2 mAb, PE-labeled anti-CD69, anti-phospho c-Jun mAb KM1, or anti-phospho p38 polyclonal Ab. Anti-IL-2- and anti-CD69-stained cells were then washed sequentially with saponin buffer, PBS, and staining buffer. Anti-phospho c-*jun* or anti-phospho p38-stained cells were washed in saponin buffer and then incubated for 30 min with biotin-labeled anti-mouse IgG1 or biotin-labeled anti-rabbit IgG Abs, respectively, washed, and then incubated for 30 min with PE-labeled strepavidin. Cells were then washed, as described above, before analysis. The fluorescence intensities of more than 1000 KJ1-26^+ $CD4^+$ events were collected using the FACSCalibur flow cytometer and analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star) software.

Measurement of cell cycle progression in vivo

Cell cycle progression was monitored using the CFSE dye as a marker of cell division (24). FL1 fluorescence was measured in the KJ1-26^+CD4^+ T cells at various times after stimulation, and cells were found to demonstrate a 2-fold decrease in FL1 fluorescence with each successive round of cell division. Based on these peaks of CFSE fluorescence intensity within the population, each T cell was assigned to a particular cell division group d (with $d = 0$ to n cell divisions), and the number of T cell events (E) observed within each cell division group (E_d) was determined. To calculate the average number of cell divisions within a population, the following equation was used:

$$\text{Average divisions} = \frac{\sum_{d=1}^{n}(d)(2^{-d})E_d}{\sum_{d=0}^{n}(2^{-d})E_d}$$

Results

Despite rapid and synchronous activation of $CD4^+$ T cells in vivo, IL-2 production and cell cycle progression are heterogenous

A single i.v. bolus of $OVAp_{323–339}$ was previously shown to induce a significant clonal expansion of adoptively transferred

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

OVAp-specific KJ1-26$^+$CD4$^+$ T cells within 3 days (4). Thereafter, the number of OVAp-reactive T cells fell to near baseline (presumably as a consequence of Ag clearance and apoptosis of the lymphocytes), and the remaining T cells demonstrated a persistent hyporesponsiveness to Ag rechallenge consistent with the induction of clonal anergy (27). To further investigate this activation event and the tolerance elicited by it, we studied cell cycle progression in relation to other activation events following the i.v. administration of OVAp. Fig. 1*A* shows that within 3 min of Ag infusion, the stress-activated protein kinase (SAPK) p38 becomes phosphorylated in virtually all of the KJ1-26$^+$CD4$^+$ T cells, as previously reported (26). In contrast, host T cells were generally unaffected by the OVAp infusion and preparation of the cells for flow cytometry. Over the course of the next 5 h of stimulation, the OVA-specific T cells uniformly expressed increasing levels of phosphorylated c-Jun, consistent with the actions of another SAPK called c-Jun N-terminal kinase (Fig. 1*B*, and data not shown). With i.v. peptide stimulation, KJ1-26$^+$CD4$^+$ T cells also demonstrated a unimodal increase in both CD25 and CD69 surface marker expression. Consistent with our previous observations (8, 22), however, IL-2 accumulated in the cytoplasm of Ag-stimulated T cells in a heterogenous fashion. At no time after Ag exposure were more than about half of the splenic T cells positive for intracellular IL-2 (Fig. 1*C*, and data not shown). Thus, infusion of aqueous peptide Ag into the bloodstream resulted in a remarkably rapid and synchronous activation of virtually all CD4$^+$ T cells bearing an Ag-reactive TCR. Nevertheless, some of these activated T cells failed to synthesize significant amounts of IL-2.

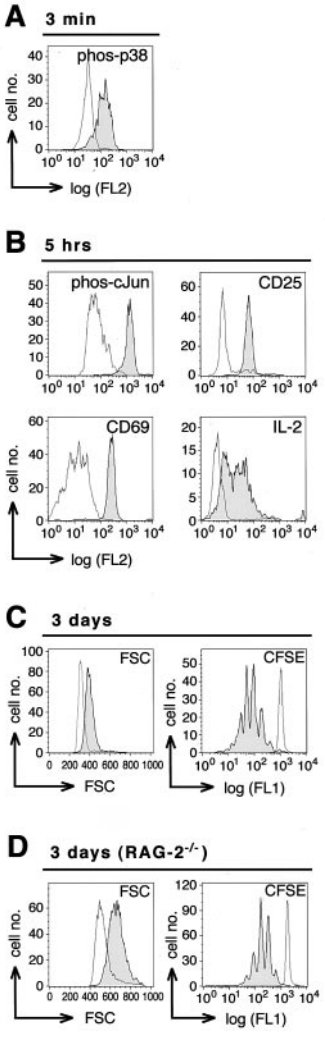

FIGURE 1. Heterogeneous IL-2 production and proliferation in splenic T cells in vivo despite synchronous TCR ligation. *A*, Phosphorylation of intracellular p38 in KJ1-26$^-$ (faint tracing) and KJ1-26$^+$ (dark tracing) CD4$^+$ T cells in an adoptive transfer recipient mouse 3 min after i.v. infusion of 100 μg of OVAp. *B*, Phosphorylation of c-Jun, expression of CD25 and CD69, and accumulation of intracellular IL-2 in KJ1-26$^-$ (faint tracings) and KJ1-26$^+$ (dark tracings) CD4$^+$ T cells in an adoptive transfer recipient mouse 5 h after i.v. infusion of 100 μg of OVAp. *C* and *D*, Size enlargement (forward scatter profile; *left panels*) and CFSE dye dilution (log(FL1)) of KJ1-26$^+$CD4$^+$ wild-type (*C*) or RAG-2$^{-/-}$ (*D*) T cells 3 days after adoptive transfer and i.v. infusion of either PBS alone (faint tracings) or 100 μg of OVAp in PBS (dark tracings).

Cell size enlargement and CFSE dye dilution indicative of cell cycle progression were also examined during a 3-day period following the i.v. infusion of either OVAp, or PBS as a control. Both host CD4$^+$ cells in OVAp-treated animals and donor TCR-transgenic T cells in PBS-treated animals remained small in size during the course of the experiment based on their forward scatter profile (Fig. 1*C*, and data not shown). Likewise, OVA-specific T cells in PBS control animals demonstrated no evidence of CFSE dye dilution. However, KJ1-26$^+$CD4$^+$ T cells from OVAp-treated animals became uniformly enlarged, and CFSE fluorescence was reduced in a pattern, indicating that cell divisions had occurred. Like the synthesis of the autocrine growth factor IL-2 many hours earlier, proliferation in response to the OVAp challenge appeared to be heterogenous, with some cells dividing only once and others dividing as many as four times during the 3-day period. Transgenic TCR expression did not correlate with either the level of IL-2 accumulation or degree of CFSE dye dilution within individual cells (data not shown). Furthermore, similar results were obtained using donor T cells from DO11.10 RAG-2$^{-/-}$ (Fig. 1*D*, and data not shown). Therefore, the failure of some of the activated T cells to produce IL-2 and proliferate was not the result of a heterogeneity in the level of TCR transgene expression because of endogenously rearranged TCR α-chains, or because of previous environmental Ag recognition.

CD4$^+$ T cells that proliferate only poorly to primary Ag stimulation are unresponsive to Ag rechallenge

As described above, a number of in vitro studies have suggested that TCR stimulation that is unaccompanied by proliferation leads to the development of clonal anergy. To determine whether a similar relationship exists in vivo between proliferation in the primary stimulation and recall Ag responsiveness, we rechallenged the KJ1-26$^+$ T cells with Ag and measured their capacity for IL-2 production as a function of cell cycle history using the CFSE fluorescence as a marker. CFSE-labeled DO11.10 T cells were adoptively transferred into normal BALB/c mice, and then given a single infusion of OVAp. Three and 7 days after primary challenge (Figs. 2 and 3, respectively, as well as Table I), the animals received a second injection of OVAp i.v., and 3 h later the cells were recovered and examined for intracellular IL-2 accumulation. Recall IL-2 production in the bulk KJ1-26$^+$ T cell population was always higher at the 7-day time point after primary Ag challenge, suggesting that the *IL-2* gene in day 3-activated T cells is relatively refractory to stimulation (Figs. 2 and 3, and data not shown). However, the single i.v. infusion of OVAp in PBS was not found to effectively induce clonal anergy in the bulk population of KJ1-26$^+$ T cells, as measured by the accumulation of intracellular IL-2 during a 3-h period following secondary Ag infusion. This is in fact consistent with our previous observation that multiple OVAp infusions are necessary to achieve a significant level of tolerance to Ag restimulation in vivo (22). It should also be noted that the level

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

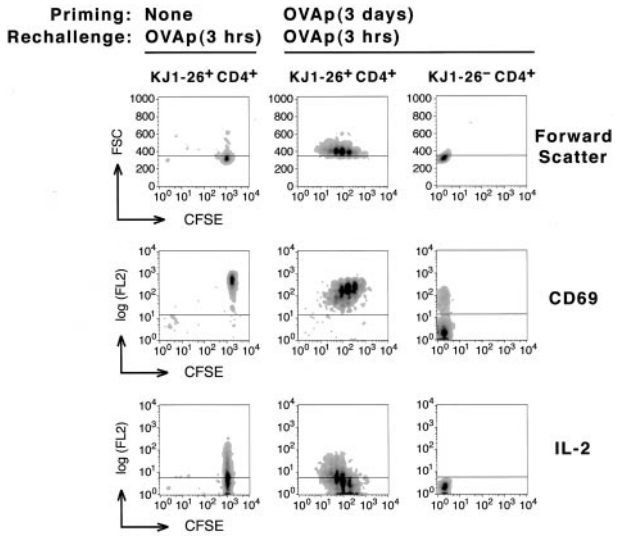

FIGURE 2. Direct relationship between day 3 cell division history and rechallenge IL-2 production after i.v. OVAp treatment. Forward scatter, CD69 expression, and intracellular IL-2 accumulation (as a function of CFSE dye content) were examined in splenic KJ1-26$^-$ and KJ1-26$^+$CD4$^+$ T cells on day 3 after adoptive transfer and primary treatment with either i.v. PBS alone or 100 μg of OVAp in PBS (as indicated). In this experiment, duplicate animals received a rechallenge infusion of 250 μg of OVAp 3 h before harvesting of the spleen T cells and staining. Horizontal lines in the forward scatter profiles were arbitrarily placed. Lines within the CD69 and IL-2 panels indicate the log(FL2) fluorescence of cells stained with an isotype control Ab-PE conjugate. Results shown are representative of 18 individual animals.

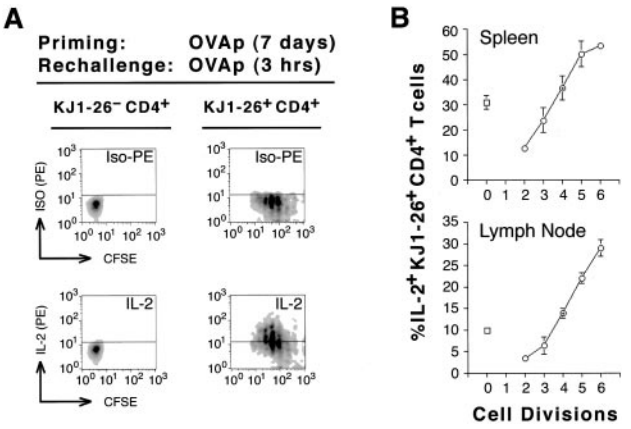

FIGURE 3. Direct relationship between day 7 cell division history and rechallenge IL-2 production after i.v. OVAp treatment. *A,* Adoptive transfer recipient animal treated with 100 μg OVAp for a 7-day period, and then rechallenged i.v. with 250 μg of OVAp. Three hours later, splenic KJ1-26$^-$ and KJ1-26$^+$CD4$^+$ T cells were recovered and stained with either PE-conjugated isotype control Ab or PE-conjugated anti-IL-2 mAb, as indicated. Horizontal lines in the plot indicate log(FL2) fluorescence of the isotype control-stained cells. *B,* Plot of relationship between cell division history and mean recall Ag-induced IL-2 production in KJ1-26$^+$ spleen and lymph node CD4$^+$ T cells 3 h after i.v. rechallenge with 250 μg of OVAp. Animals were pretreated i.v. 7 days earlier with either PBS alone (□) or 100 μg of OVAp (○) in PBS. The $*$ each plot represents the mode number of cell divisions observed in the KJ1-26$^+$CD4$^+$ T cell population as a result of the OVAp pretreatment. Error bars represent the range in responses seen in duplicate animals in an individual experiment. These duplicate animals were representative of 14 individual mice.

of tolerance in the bulk population may be underestimated in this study because IL-2 synthesis was measured in these Ag-experienced T cells 3 h after reexposure to Ag, whereas naive T cell IL-2 accumulation peaks only after 5 h of stimulation (26). When compared with KJ1-26$^+$ T cells primed with OVAp plus LPS, these T cells exposed to OVAp alone consistently demonstrated a deficit in IL-2 production (data not shown).

When recall IL-2 synthesis was compared with the level of CFSE that remained in the T cell, at both the 3- and 7-day time points IL-2 production on rechallenge was found to be greatest in those T cells that had undergone the highest number of cell divisions during the primary Ag stimulation (Figs. 2 and 3). This observation of low recall IL-2 inducibility in lymphocytes that had experienced only weak cell cycle progression during the primary Ag stimulation indicated the possibility of a causal relationship between the primary and secondary responses. KJ1-26$^+$ T cells may be an intrinsically heterogenous population with regard to their capacity to produce IL-2 and proliferate, and poor responsiveness in the primary immunization may simply predict a poor capacity to respond in the rechallenge. Alternatively, T cells stimulated to vigorously progress through the cell cycle in the primary response may maintain or increase their capacity to later respond on Ag rechallenge with the production of IL-2. It should be noted that the defect in *IL-2* gene expression observed in T cells that had undergone little previous cell cycle progression was not a consequence of some intrinsic inability of the cells to recognize OVAp-MHC complexes. Poorly dividing T cells underwent a size enlargement by day 3 that was indistinguishable from the majority of the Ag responders (Fig. 2). Furthermore, CD69 expression could still be markedly up-regulated in such T cells during Ag rechallenge (Fig. 2, and unstimulated control data not shown). Thus, our data again suggested that TCR occupancy after i.v. peptide Ag infusion is relatively uniform for all Ag-reactive T cells. Nevertheless, IL-2 production and cell cycle progression are heterogenous, with responsiveness in the primary and secondary Ag stimulations highly correlated.

Rapamycin inhibits cell cycle progression in vivo and promotes the development of clonal anergy

This observation of a direct correlation between cell cycle progression in the primary Ag stimulation and the level of *IL-2* gene responsiveness in the secondary Ag challenge was reminiscent of data obtained using cloned Th1 cells in vitro. Th1 cells stimulated with Ag and normal APC lose their capacity to make IL-2, if their cell cycle progression during the incubation is prevented by the addition of neutralizing Ab to IL-2 and CD25 (17). Furthermore, anergic Th1 cells regain their capacity to produce IL-2 when driven to proliferate with exogenous growth factor (18). Therefore, we speculated that the poorly dividing OVA-reactive T cells were induced into clonal anergy in vivo as a consequence of TCR occupancy that was unaccompanied by vigorous growth factor-dependent cell cycle progression. However, it remained possible that a significant fraction of KJ1-26$^+$ T cells is intrinsically deficient in their ability to secrete IL-2 and, as a result, they failed to proliferate well during the primary challenge. To experimentally address these two possibilities, we made use of the T cell antiproliferative agent rapamycin (20). If cell cycle progression does not in fact positively regulate recall Ag responsiveness, then a reduction of T cell proliferation by inhibiting mTOR during the primary response should not alter IL-2 production in the rechallenge.

Treatment of mice with rapamycin during the time of the primary i.v. OVAp administration significantly inhibited cell cycle progression by the OVA-specific T cells (Fig. 4 and Table I). By day 3 of the OVAp-induced clonal expansion, KJ1-26$^+$CD4$^+$ T

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

Table I. *Day 7 proliferation and recall IL-2 production profile of OVA-specific TCR-transgenic T cells*[a]

Lymphoid Organ	Primary Treatment (i.v.)	Additions (i.p.)	% KJ1-26$^+$CD4$^+$ T Cells (±SEM)	Average Divisions (±SEM)	% IL-2$^+$KJ1-26$^+$CD4$^+$ T Cells (±SEM)
Spleen	None (PBS alone)	Vehicle alone	0.19 ± 0.03	0.01 ± 0.00	30.39 ± 3.00
		Rapamycin	0.11 ± 0.01	0.01 ± 0.00	36.55 ± 1.41
	OVAp	Vehicle alone	0.32 ± 0.05	2.48 ± 0.09	34.74 ± 2.93
		Rapamycin	0.14 ± 0.00	0.74 ± 0.02	15.04 ± 0.52
	OVAp plus anti-CTLA-4 mAb	Vehicle alone	1.59 ± 0.10	3.98 ± 0.10	54.69 ± 0.21
		Rapamycin	1.04 ± 0.10	1.45 ± 0.06	23.12 ± 0.50
Lymph node	None (PBS alone)	Vehicle alone	0.45 ± 0.01	0.01 ± 0.00	9.91 ± 0.57
		Rapamycin	0.47 ± 0.04	0.01 ± 0.00	11.29 ± 0.28
	OVAp	Vehicle alone	0.51 ± 0.12	2.43 ± 0.06	11.56 ± 1.84
		Rapamycin	0.46 ± 0.06	0.67 ± 0.01	3.90 ± 0.35
	OVAp plus anti-CTLA-4 mAb	Vehicle alone	2.33 ± 0.28	3.59 ± 0.06	19.06 ± 0.38
		Rapamycin	1.19 ± 0.19	1.23 ± 0.14	9.90 ± 1.69

[a] Adoptive transfer recipient mice were treated for 7 days as indicated. Three hours before the end of the experiment, animals received a single i.v. infusion of 250 μg of OVAp, after which lymphocytes were recovered and analyzed by flow cytometry.

cells were reduced in frequency in the lymph nodes and spleen 61 ± 15% as a result of the rapamycin treatment, and this result was statistically significant ($p < 0.01$; $n = 9$). Consistent with this, the mean number of KJ1-26$^+$ cell divisions observed after the OVAp infusion (based on CFSE dye dilution) was decreased after rapamycin treatment. In contrast, rapamycin only modestly inhibited T cell size enlargement, suggesting that TCR signaling was still effective even in the presence of the drug (Fig. 4*B*). In addition to its effects on cell cycle progression during the primary OVAp response, rapamycin treatment clearly also led to an overall decrease in recall IL-2 production in the Ag-primed KJ1-26$^+$ T cells (Fig. 4*A* and Table I). Importantly, even in the presence of rapamycin, intracellular IL-2 accumulation following Ag rechallenge remained directly proportional to the number of cell divisions achieved during the primary response. Thus, Ag stimulation in the presence of rapamycin (and unaccompanied by a vigorous cell cycle progression) induced even the most highly responsive T cells to become more anergic.

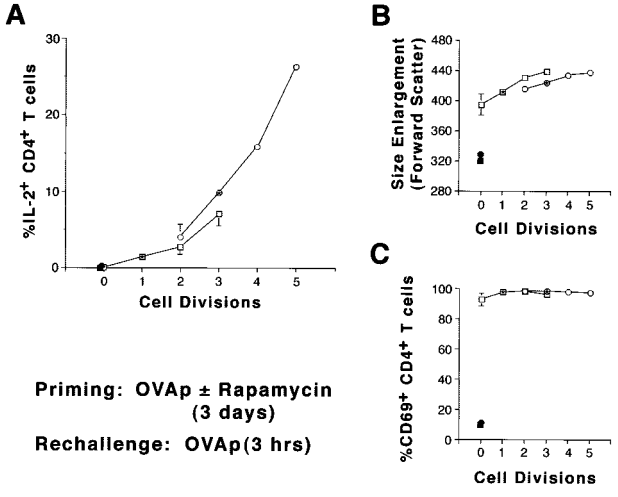

FIGURE 4. Rapamycin blocks cell cycle progression and promotes anergy induction in response to aqueous peptide Ag treatment. Splenic KJ1-26$^-$ (■ and ●) and KJ1-26$^+$ (□ and ○) CD4$^+$ T cells were recovered from animals pretreated 3 days earlier with i.v. OVAp (100 μg) either with (■ and □) or without (● and ○) the addition of daily i.p. injections of rapamycin. All animals received an i.v. infusion of OVAp (250 μg) 3 h before sacrifice and flow cytometry for intracellular IL-2 accumulation (*A*), forward scatter profile (*B*), and CD69 expression (*C*). The * and error bars are as in Fig. 3. Flow cytometry density plots from one of the OVAp-pretreated animals included here are shown in Fig. 2. Results shown are representative of six animals tested.

This rapamycin effect supported the hypothesis that mTOR-dependent cell cycle progression is a primary regulator of *IL-2* gene inducibility. Had there existed an intrinsic variability in the capacity of naive KJ1-26$^+$ T cells to produce IL-2 that accounted for the direct correlation between cell division and IL-2 production, one should have expected the rapamycin to inhibit cell division without affecting the recall IL-2 inducibility of the population. Instead, we observed that the majority of Ag-experienced KJ1-26$^+$ T cells in rapamycin-treated animals now remained CFSE bright and had become profoundly unresponsive to further Ag stimulation (Fig. 4*A*). This loss of Ag responsiveness was not the result of rapamycin directly inhibiting intracellular IL-2 accumulation, as rapamycin had no effect on signaling to the *IL-2* gene in naive animals responding to OVAp (Table I). Rather, the hyporesponsiveness observed in animals treated with rapamycin depended on an infusion of OVAp 3 to 7 days earlier. Finally, there was no evidence that rapamycin was specifically toxic to Ag-experienced cells. OVAp/rapamycin-pretreated KJ1-26$^+$ T cells remained fully competent to express the activation marker CD69 during the Ag rechallenge (Fig. 4*C*, and control unstimulated cell data not shown). Thus, this experiment indicates that a vigorous cell cycle progression is required during the primary Ag response to sustain a high level of responsiveness for a later Ag challenge.

CTLA-4 signaling promotes clonal anergy induction through its inhibitory effects on cell cycle progression

The CTLA-4 inhibitory receptor acts to limit T cell responsiveness in vivo (5). This may result in part from its capacity to compete effectively with CD28 for costimulatory B7 molecules; CTLA-4 signaling may also directly inhibit cell cycle progression (16, 28). Interestingly, neutralizing mAb against CTLA-4 has also been shown to prevent the induction of clonal anergy in vivo (12). Furthermore, CTLA-4$^{-/-}$ T cells are resistant to tolerance induction (14). This could suggest that signals from the CTLA-4 molecule are directly responsible for inducing the anergy state. An alternative hypothesis is that CTLA-4 signals affect the level of recall Ag responsiveness by controlling the intensity of cell cycle progression during the primary immune response. Donor DO11.10 OVA-specific T cells were found to undergo more rounds of cell division when challenged with OVAp in the presence of the anti-CTLA-4 mAb 4F10 (Fig. 5 and Table I). T cell clonal expansion in the Ag-stimulated 4F10-treated animals was similarly increased. Therefore, the CTLA-4 molecule was indeed acting as a brake on

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

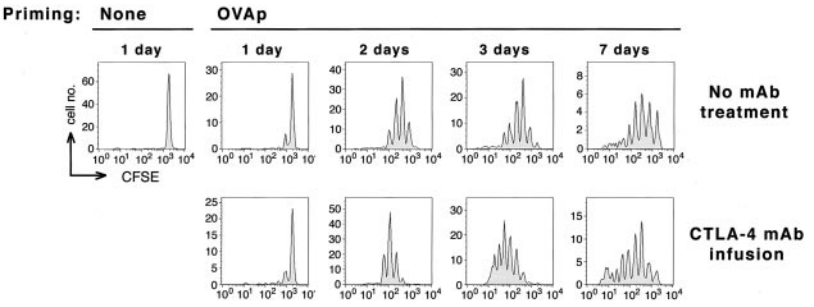

FIGURE 5. CTLA-4 signaling acts to reduce cell cycle progression in response to peptide Ag infusion. CFSE-labeled KJ1-26$^+$CD4$^+$ T cells were examined for FL1 channel fluorescence at various times after adoptive transfer and i.v. priming with 100 μg of OVAp, either in the absence or presence of the 250 μg of anti-CTLA-4 mAb 4F10, as indicated. Duplicate animals were examined in this experiment, and the time course was repeated once with similar results.

cell cycle progression in our model system, and the 4F10 mAb was an effective antagonist of its function. Consistent with Perez et al. (12), treatment of animals with the anti-CTLA-4 mAb during Ag stimulation also led to an enhanced recall Ag responsiveness at the level of the *IL-2* gene in the OVAp-reactive KJ1-26$^+$CD4$^+$ T cell population (Table I).

A direct relationship between cell division history and IL-2 inducibility was again observed even in the presence of the anti-CTLA-4 mAb (Fig. 6). The typical T cell (mode of six cell divisions) found in anti-CTLA4 mAb-treated mice on day 7 demonstrated the same level of recall Ag responsiveness as a T cell from a control animal that had divided to a similar extent during the primary Ag challenge. Likewise, the more rare T cell that had undergone only four rounds of cell division in 4F10-treated mice produced the same amount of IL-2 on rechallenge as similarly divided T cells from animals treated with OVAp alone. This result suggested that CTLA-4 signals promote clonal anergy induction by limiting cell cycle progression. In the presence of the 4F10 mAb, recall Ag responsiveness is improved, but only in those T cells that have undergone additional rounds of cell division.

Rapamycin promotes clonal anergy induction in vivo, even in the presence of CTLA-4 blockade

Is the relationship that exists between enhanced Ag-stimulated cell cycle progression and the avoidance of clonal anergy in anti-CTLA-4 mAb-treated animals coincidental or causal? Once again, we examined the effect of rapamycin on the response. Rapamycin was found to effectively block cell cycle progression induced by OVAp treatment even in the presence of 4F10 mAb (Table I and Fig. 6). Clonal expansion measured at day 7, however, was not as dramatically affected by the addition of rapamycin as would be expected based on cell division history alone (Table I). This perhaps suggests that the inhibition of the mTOR signaling molecule with rapamycin promotes T cell survival during the clonal expansion. Despite this enhanced survival of T cells responding to the OVAp in the presence of anti-CTLA-4 mAb and rapamycin, the Ag responsiveness of the survivors was significantly reduced. In fact, T cells from OVAp plus 4F10/rapamycin-treated animals accumulated less intracellular IL-2 on Ag rechallenge than did T cells from animals treated with OVAp alone. When this recall IL-2 production was examined as a function of cell division history, Ag-experienced KJ1-26$^+$ T cells from anti-CTLA-4 mAb and rapamycin-treated animals maintained the strict relationship between cell division history and IL-2 inducibility that has been observed throughout these experiments. Thus, signaling by the CTLA-4 molecule during primary Ag challenge does not directly affect recall IL-2 responsiveness. Rather, CTLA-4 signals promote anergy induction by limiting Ag-stimulated and mTOR-dependent cell cycle progression. Furthermore, rapamycin can promote the development of clonal anergy even in the absence of CTLA-4 signals because of its capacity to directly block cell cycle progression.

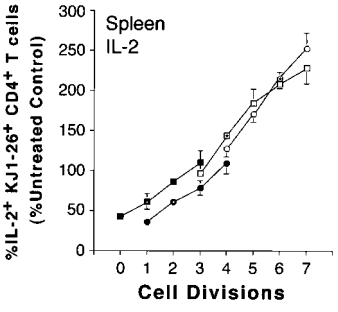

FIGURE 6. Rapamycin promotes anergy induction in aqueous peptide Ag-treated T cells even in the absence of CTLA-4 signaling. KJ1-26$^+$ CD4$^+$ splenic T cells were examined for CFSE dye dilution and recall Ag-induced IL-2 production 7 days after pretreatment with either 100 μg of OVAp in combination with 250 μg of control hamster IgG (■ and □) or OVAp in the presence of the 250 μg of anti-CTLA-4 mAb 4F10 (● and ○). Some animals were treated with simultaneous i.p. infusions of 0.5 mg/kg rapamycin in CMC (■ and ●), whereas other mice received only the CMC vehicle as a control (□ and ○). IL-2 production is calculated as the percentage of the T cell response observed in control animals receiving no pretreatment. The $*$ and error bars are as in Fig. 3. The data shown are representative of results obtained in three independent experiments examining a total of six mice per group.

Discussion

In these experiments, we have used an i.v. bolus infusion of peptide Ag to examine activation events within CD4$^+$ T cells in vivo. Specifically, we have investigated the relationship between cell cycle progression during the primary Ag response, and later recall Ag responsiveness to stimulation. Although this experimental model of peptide stimulation may not faithfully mimic all aspects of T cell responses to the typical Ag, it does provide the opportunity to synchronously assess the inherent reactivity of T cells within their natural lymphoid environments under conditions in which APC-derived costimulatory signals can be expected to be limiting. Activation of the SAPK p38 and c-Jun N-terminal kinse was shown to occur rapidly and uniformly in the responder cell population soon after peptide infusion, perhaps indicating that virtually every T cell in the lymph node and spleen is in close proximity to an APC capable of presenting a newly formed OVAp-I-A^d complex. We have not explicitly examined the identity of the relevant APC in these experiments; however, the extremely rapid response may indicate that the myeloid and lymphoid dendritic cells that are resident to the T cell-rich areas of the lymph node and spleen (the parafollicular region of the cortex and the periarteriolar lymphoid sheath, respectively) are most relevant to this Ag presentation event.

The rapid activation of the SAPK was found to be closely paralleled by a profound change in T cell phenotype. Specifically,

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

surface expression of the CD69 and CD25 molecules was uniformly and highly induced upon exposure to the soluble Ag, and all of the T cells enlarged in size. Although the functional importance of these activation markers has not been formally tested in this study, one can speculate that a rapid increase in CD25 surface molecule expression on all of the Ag-specific T cells would contribute to the development of competence to proliferate in the presence of IL-2. Therefore, cell cycle progression in response to this TCR occupancy may be limited more by growth factor production (or availability) than by growth factor receptor expression. We have in fact observed that only a fraction of the Ag-stimulated KJ1-26$^+$ T cell population is capable of producing high levels of IL-2 during the primary Ag challenge. Furthermore, this heterogeneity in the production of IL-2 is accompanied by a highly variable pattern of cell cycle progression, with some cells dividing as often as seven times during the course of the response, and others in the same population dividing only once.

Our experiments cannot determine whether those T cells accumulating the highest levels of intracellular IL-2 early in the primary response are also the ones that undergo the highest number of cell divisions. However, when taken together with several previous in vitro studies, this would seem to be a logical extension. Weaver and colleagues (29) examined *IL-2* gene responsiveness using DO11.10 double-transgenic T cells that also carry a green fluorescence protein (GFP)-based reporter gene containing DNA sequences from the 5′ *IL-2* gene promoter/enhancer. Coordinate expression of both GFP protein and IL-2 was induced in a heterogenous pattern by in vitro challenge with syngeneic APC and OVAp. Interestingly, GFP$^+$ cells sorted at the end of the experiment were found to be competent to produce IL-2 on rechallenge, whereas GFP$^-$ T cells were eventually found to be anergic to further stimulation. Wells et al. (30) have shown that, like the in vivo activation model presented in this study, recall IL-2 production following in vitro anti-CD3 mAb-induced proliferation is heterogenous and enriched in those T cells that have previously undergone the highest number of cell divisions. Therefore, the results of these experiments predict that in vivo those T cells induced to secrete the highest amounts of IL-2 during the primary challenge undergo the most vigorous cell cycle progression and retain the highest level of *IL-2* gene inducibility upon rechallenge.

The contrast between the unimodal increase in forward scatter, CD69, and CD25, and the variable extent to which the naive cells could be induced to produce IL-2 and proliferate during the primary challenge supports the notion that growth factor production and cell cycle progression are more stringently regulated in naive CD4$^+$ T cells than other phenotypic changes. The nature of this regulation, however, remains uncertain. It is possible that within a population of naive Ag-specific T cells, some cells will simply lack the capacity to secrete IL-2 and proliferate in response to effective TCR ligation. Alternatively, the activation of naive Ag-specific T cells may not be as uniform as the SAPK activation and surface marker expression data would lead one to believe. Such activation markers may have a relatively low threshold for TCR serial engagements, while IL-2 production and cell cycle progression may require more intense or prolonged TCR signaling events to reach threshold in naive T cells (31, 32). It should be noted that simply increasing the dose of OVAp in the primary Ag infusion does not eliminate the heterogeneity in IL-2 production observed (data not shown). The hypothesis we favor is that costimulatory ligands such as CD80 and/or CD86 are available in excess to only a fraction of the T cells during the primary stimulation. Although these costimulatory ligands can be detected in the secondary lymphoid tissues of these mice at the time of the Ag challenge, the levels remain low relative to CD80 and CD86 expression that is induced with adjuvants such as LPS (33). Consistent with this hypothesis, the CD28 antagonist CTLA-4Ig, while having no effect on SAPK activation in naive CD4$^+$ T cells (26), is a potent inhibitor of IL-2 production and clonal expansion in vivo (5, 8). Likewise, T cells from CD28$^{-/-}$ mice demonstrate defective IL-2 production and proliferation, and yet are capable of normal increases in forward scatter following Ag stimulation (8). Regardless of the underlying nature of this heterogeneity in IL-2 production and cell cycle progression, our data strongly indicate that after a single bolus i.v. infusion of aqueous OVAp, each of the OVA-reactive T cells experiences a stimulation of its TCR.

In the peripheral immune system, exposure of CD4$^+$ T cells to aqueous peptide Ag in the absence of infection or adjuvant leads to an abortive clonal expansion of the Ag responders (4). The end result is death for the majority of the expanded T cells and reduced Ag responsiveness within the bulk of the survivors. As a consequence, T cells with that particular Ag specificity are left at only low frequency and in a defective state in which Ag-dependent autocrine growth is ineffective (27). Such a mechanism may normally be important to limit further T cell aggression directed against peripheral tissues that persistently express peptide Ag (34). In vitro examinations of T cell activation events have lent insight into the nature of this Ag-induced tolerance. Intense, continuous stimulation of Th1-like cloned CD4$^+$ T cells in the absence of APC using immobilized anti-TCR or anti-CD3 mAbs leads to a suboptimal proliferative response and increased rate of apoptosis (35). In addition, surviving T cells demonstrate a long-lived unresponsiveness to further Ag stimulation characterized by defective IL-2 production and proliferation. This unresponsiveness can last indefinitely; however, exposure to rIL-2 induces cell cycle progression in the anergic cells and causes a reversal of the tolerant state (18). The notion that IL-2Rs and downstream biochemical signaling cascades play an important role in regulating the level of Ag responsiveness is also supported by the observation that stimulation of cloned CD4$^+$ Th1 cells with Ag plus normal APC in the presence of neutralizing IL-2 and IL-2R mAbs leads to clonal anergy (17). Furthermore, ligation of the IL-2R common γ-chain has been shown to prevent the development of clonal anergy (19). Taken together, the results suggest that TCR stimulation unaccompanied by IL-2 production or IL-2R engagement promotes clonal anergy induction, whereas TCR occupancy that leads to IL-2R signaling promotes continued Ag responsiveness.

This regulation of clonal anergy by growth factors and their receptors indicates that cell cycle progression may itself be the important cellular event that controls Ag responsiveness. The role of cell cycle progression in the regulation of Ag responsiveness has previously been examined in vitro. The antiproliferative cytokine TGF-β1 has been shown to cause the development of clonal anergy in naive CD4$^+$ T cells undergoing a primary response to Ag and APC (36). Likewise, Gilbert and Weigle (37) have demonstrated that the G_{1a} blocker *n*-butyrate, a histone deacetylase inhibitor, can cause the development of clonal anergy in Th1 cells when present during stimulation with Ag and normal APC. In contrast, drugs that blocked Th1 cell cycle progression in G_0, G_{1b}, or S-G_2 phases did not reduce their Ag responsiveness. Powell et al. (38) later showed that Th1 cells responding to Ag and APC would develop an anergic phenotype when treated with the G_1 phase inhibitor rapamycin, but not the S phase inhibitor hydroxyurea.

Rapamycin forms a molecular complex with the FK506-binding protein in T cells (39), and inhibits the activity of the serine/threonine protein kinase called mTOR (20, 40). One function of mTOR activity during G_1 phase is thought to be the down-regulation of the cyclin-dependent kinase-negative regulator p27^{Kip1}. High constitutive levels of p27^{Kip1} prevent G_1 to S

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

phase progression by binding to and inhibiting the activity of cyclin E-cyclin-dependent kinase 2 complexes. Rapamycin prevents the degradation of $p27^{Kip1}$ that normally occurs in response to growth factor receptor signals (41). This maintenance of high $p27^{Kip1}$ levels in rapamycin-treated T cells may be responsible for both the block in cell cycle progression observed, as well as the development of Ag unresponsiveness. Boussiotis et al. (42) have in fact shown in vitro that $p27^{Kip1}$ levels rise in T cells during the induction of clonal anergy, and the accumulation of this repressor protein can lead to a defect in IL-2 production as a result of its capacity to bind the *IL-2* gene transcriptional coactivator JAB1 (43). Whether increased $p27^{Kip1}$ levels can account for all of the defects in signaling to the *IL-2* gene that have previously been reported in anergic T cells remains to be seen. Regardless, the relationship between cell cycle progression and the elimination of one *IL-2* gene repressor factor is a paradigm worthy of further investigation.

We now speculate that Ag recognition in vivo is always accompanied by a TCR-mediated signal to induce clonal anergy. This model requires that, like the expression of CD69 and CD25, the induction of clonal anergy is a relatively low-threshold event. Nevertheless, only some of the T cells will develop the capacity to produce autocrine growth factor when they encounter Ag in a B7-rich microenvironment, and it is these cells that efficiently progress through cell cycle and retain the capacity to respond vigorously to a secondary Ag challenge. Biochemical events occurring within the G_1 phase of the primary response as a consequence of this growth factor signaling (e.g., activation of mTOR and the degradation of $p27^{Kip1}$) not only promote further cell cycle progression, but also restore the Ag responsiveness of the T cells by eliminating a molecular repressor of *IL-2* gene transcription. Although the CTLA-4 molecule is an important modulator of this process as a result of its capacity to limit cell cycle progression (12, 14) (data shown in this study), we do not imagine that it mediates those signals from the TCR that directly induce the putative clonal anergy repressor molecule (44). The ability of CTLA-$4^{-/-}$ T cells to develop clonal anergy in vitro is against a causal role for CTLA-4 in the induction of Ag unresponsiveness (45). Furthermore, the capacity of rapamycin to promote the induction of Ag unresponsiveness even in the absence of CTLA-4 signals provides strong evidence for a direct role of cell cycle progression in the avoidance or reversal of clonal anergy.

Results of our study seemingly differ from those of Adler et al. (34), who varied the concentration of a transgenic self Ag and observed that T cells would become tolerant independent of the rate of cell division induced by the Ag. In fact, these two studies are complementary and point out the importance of two signals in the regulation of recall Ag responsiveness. Ag unresponsiveness appears to develop as a function of the ratio of anergy-inducing TCR signals (signal 1) to cell cycle progression (signal 2). Accordingly, chronic intense TCR ligation in high self-Ag-expressing mice can eventually dominate over signals driving cell cycle progression, and clonal anergy develops. For the case of low self-Ag-expressing mice, the anergy-inducing TCR signals will be weaker; nevertheless, tolerance is again induced because these signals are accompanied by only a low rate of cell cycle progression. In the experiments described in this work, OVAp-induced TCR signals are demonstrated to be uniform across the population of responding T cells. Therefore, variations in cell cycle progression had the opportunity to independently affect the level of recall Ag responsiveness within individual T cells.

Acknowledgments

We thank Dr. Stephen Jameson and Dr. Matthew Mescher for reviewing this manuscript. We also thank Dr. Marc Jenkins for helpful discussions during the planning and execution of the experiments.

References

1. Jenkins, M. K., and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med. 165:302.*
2. Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol. 7:445.*
3. Malvey, E.-N., D. G. Telander, T. L. Vanasek, and D. L. Mueller. 1998. The role of clonal anergy in the avoidance of autoimmunity: inactivation of autocrine growth without loss of effector function. *Immunol. Rev. 165:301.*
4. Kearney, E. R., K. A. Pape, D. Y. Loh, and M. K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity 1:327.*
5. Kearney, E. R., T. L. Walunas, R. W. Karr, P. A. Morton, D. Y. Loh, J. A. Bluestone, and M. K. Jenkins. 1995. Antigen-dependent clonal expansion of a trace population of antigen-specific $CD4^+$ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J. Immunol. 155:1032.*
6. June, C. H., J. A. Ledbetter, M. M. Gillespie, T. Lindsten, and C. B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol. 7:4472.*
7. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature 356:607.*
8. Khoruts, A., A. Mondino, K. A. Pape, S. L. Reiner, and M. K. Jenkins. 1998. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J. Exp. Med. 187:225.*
9. Van Parijs, L., A. Biuckians, A. Ibragimov, F. W. Alt, D. M. Willerford, and A. K. Abbas. 1997. Functional responses and apoptosis of CD25 (IL-2Rα)-deficient T cells expressing a transgenic antigen receptor. *J. Immunol. 158:3738.*
10. Walunas, T., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity 1:405.*
11. Krummel, M. F., and J. P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J. Exp. Med. 182:459.*
12. Perez, V. L., L. V. Parijs, A. Biuckians, X. X. Zheng, T. B. Strom, and A. K. Abbas. 1997. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity 6:411.*
13. Chai, J. G., S. Vendetti, E. Amofah, J. Dyson, and R. Lechler. 2000. CD152 ligation by CD80 on T cells is required for the induction of unresponsiveness by costimulation-deficient antigen presentation. *J. Immunol. 165:3037.*
14. Greenwald, R. J., V. A. Boussiotis, R. B. Lorsbach, A. K. Abbas, and A. H. Sharpe. 2001. CTLA-4 regulates induction of anergy in vivo. *Immunity 14:145.*
15. Krummel, M. F., and J. P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J. Exp. Med. 183:2533.*
16. Brunner, M. C., C. A. Chambers, F. K. Chan, J. Hanke, A. Winoto, and J. P. Allison. 1999. CTLA-4-mediated inhibition of early events of T cell proliferation. *J. Immunol. 162:5813.*
17. DeSilva, D. R., K. B. Urdahl, and M. K. Jenkins. 1991. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J. Immunol. 147:3261.*
18. Beverly, B., S. M. Kang, M. J. Lenardo, and R. H. Schwartz. 1992. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. *Int. Immunol. 4:661.*
19. Boussiotis, V. A., D. L. Barber, T. Nakarai, G. J. Freeman, J. G. Gribben, G. M. Bernstein, A. D. D'Andrea, J. Ritz, and L. M. Nadler. 1994. Prevention of T cell anergy by signaling through the γc chain of the IL-2 receptor. *Science 266:1039.*
20. Abraham, R. T., and G. J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu. Rev. Immunol. 14:483.*
21. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{low}$ thymocytes in vivo. *Science 250:1720.*
22. Malvey, E.-N., M. K. Jenkins, and D. L. Mueller. 1998. Peripheral immune tolerance blocks clonal expansion, but fails to prevent the differentiation of Th1 cells. *J. Immunol. 161:2168.*
23. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med. 157:1149.*
24. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods 171:131.*
25. Blazar, B. R., P. A. Taylor, A. Panoskaltsis-Mortari, and D. A. Vallera. 1998. Rapamycin inhibits the generation of graft-versus-host disease- and graft-versus-leukemia-causing T cells by interfering with the production of Th1 or Th1 cytotoxic cytokines. *J. Immunol. 160:5355.*

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018

26. Zell, T., A. Khoruts, E. Ingulli, J. L. Bonnevier, D. L. Mueller, and M. K. Jenkins. 2001. Analysis of *jun* N-terminal kinase and p38 mitogen-activated protein kinase phosphorylation in CD4 T cells stimulated by antigen in vivo. *Proc. Natl. Acad. Sci. USA 98:10805.*
27. Pape, K. A., R. Merica, A. Mondino, A. Khoruts, and M. K. Jenkins. 1998. Direct evidence that functionally impaired $CD4^+$ T cells persist in vivo following induction of peripheral tolerance. *J. Immunol. 160:4719.*
28. Carreno, B. M., F. Bennett, T. A. Chau, V. Ling, D. Luxenberg, J. Jussif, M. L. Baroja, and J. Madrenas. 2000. CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. *J. Immunol. 165:1352.*
29. Saparov, A., F. H. Wagner, R. Zheng, J. R. Oliver, H. Maeda, R. D. Hockett, and C. T. Weaver. 1999. Interleukin-2 expression by a subpopulation of primary T cells is linked to enhanced memory/effector function. *Immunity 11:271.*
30. Wells, A. D., M. C. Walsh, D. Sankaran, and L. A. Turka. 2000. T cell effector function and anergy avoidance are quantitatively linked to cell division. *J. Immunol. 165:2432.*
31. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science 273:104.*
32. Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity 8:89.*
33. De Smedt, T., B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. De Baetselier, J. Urbain, O. Leo, and M. Moser. 1996. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J. Exp. Med. 184:1413.*
34. Adler, A. J., C. T. Huang, G. S. Yochum, D. W. Marsh, and D. M. Pardoll. 2000. In vivo $CD4^+$ T cell tolerance induction versus priming is independent of the rate and number of cell divisions. *J. Immunol. 164:649.*
35. Jenkins, M. K., C. A. Chen, G. Jung, D. L. Mueller, and R. H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol. 144:16.*
36. Gilbert, K. M., M. Thoman, K. Bauche, T. Pham, and W. O. Weigle. 1997. Transforming growth factor-β1 induces antigen-specific unresponsiveness in naive T cells. *Immunol. Invest. 26:459.*
37. Gilbert, K. M., and W. O. Weigle. 1993. Th1 cell anergy and blockade in G_{1a} phase of the cell cycle. *J. Immunol. 151:1245.*
38. Powell, J. D., C. G. Lerner, and R. H. Schwartz. 1999. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J. Immunol. 162:2775.*
39. Choi, J., J. Chen, S. L. Schreiber, and J. Clardy. 1996. Structure of the FKBP12-rapamycin complex interacting with binding domain of human FRAP. *Science 273:239.*
40. Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber. 1994. A mammalian protein targeted by G_1-arresting rapamycin-receptor complex. *Nature 369:756.*
41. Nourse, J., E. Firpo, W. M. Flanagan, S. Coats, K. Polyak, M. H. Lee, J. Massague, G. R. Crabtree, and J. M. Roberts. 1994. Interleukin-2-mediated elimination of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature 372:570.*
42. Boussiotis, V. A., G. J. Freeman, A. Berezovskaya, D. L. Barber, and L. M. Nadler. 1997. Maintenance of human T cell anergy: blocking of *IL-2* gene transcription by activated Rap1. *Science 278:124.*
43. Claret, F., M. Hibi, S. Dhut, T. Toda, and M. Karin. 1996. A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. *Nature 383:453.*
44. Telander, D. G., E.-N. Malvey, and D. L. Mueller. 1999. Evidence for repression of interleukin 2 gene activation in anergic T cells. *J. Immunol. 162:1460.*
45. Frauwirth, K. A., M. L. Alegre, and C. B. Thompson. 2000. Induction of T cell anergy in the absence of CTLA-4/B7 interaction. *J. Immunol. 164:2987.*

Downloaded from http://www.jimmunol.org/ by guest on January 23, 2018