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*J Immunol* 2002; 168:1190-1197; doi: 10.4049/jimmunol.168.3.1190

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Activation-Induced Nonresponsiveness: A Th-Dependent Regulatory Checkpoint in the CTL Response

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CD8 T cells undergo autocrine IL-2-dependent proliferation upon TCR engagement and costimulation, but within 3–4 days, they become activation-induced nonresponsive (AINR) and display a split anergy. They can lyse targets and secrete IFN-γ but they cannot produce IL-2 in response to TCR ligation and costimulation, due at least in part to an inability to up-regulate mitogen-activated protein kinases and IL-2 mRNA. Exogenous IL-2 can drive continued proliferation of AINR cells and nonresponsiveness is reversed within 1–2 days so that Ag-driven proliferation can resume. Mitogen-activated protein kinases and IL-2 mRNA can again be up-regulated, but “rewiring” has occurred so that these events no longer depend upon costimulation; TCR engagement is sufficient. Development of AINR appears to be a normal part of the differentiation program of CD8 T cells, providing a regulatory checkpoint to convert the initial helper-independent response to one that depends upon CD4 T cell help for continued expansion of the effector CTL. Once permission is given, in the form of IL-2, to pass this checkpoint, the CTL can make a prolonged response to persisting Ag in the absence of further CD4 T cell help. * The Journal of Immunology, 2002, 168: 1190–1197.

*†‡ 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.

This work is supported by National Institutes of Health Grants PO1 AI35296 (to M.F.M.) and RO1 AI34824 (to M.F.M.).

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4 Abbreviations used in this paper: AINR, activation-induced nonresponsiveness; ERK, extracellular-signal regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.
the CD8 response again becomes helper-independent once the checkpoint has been passed.

Materials and Methods

Mice and cell lines

OT-I TCR-transgenic mice (25), originally a gift from Dr. F. Carbone (Monash Medical School, Victoria, Australia), were bred to Thy-1.1/C57BL/6/PiL mice. Lymph node cells from these mice were used for in vitro experiments and as the source of transgenic T cells for adoptive transfer. C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained under specific pathogen-free conditions. All experiments were performed in compliance with the relevant laws and institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Minneapolis, MN). E.G7 tumor, EL-4 thymoma derived from the C57BL/6 mouse (H-2b) and transfected with the gene for OVA (26), was maintained in vitro in complete RPMI medium containing 400 μg/ml G418. Cell lines were periodically passaged in vivo.

Proliferation assays

Cell-size latex microspheres bearing H-2Kb-OVA264/265. H-2Kb-OVA257-264/B7-1, and H-2Kb-OVA257-264/B7-2 were prepared as described (27). Alternatively, microspheres were prepared by incubating 10² latex microspheres with 2 μg of DimerX H-2Kb fusion protein (BD Pharmingen, San Diego, CA) in 1 ml of PBS for 24 h at 4°C. Microspheres were then blocked with BSA, washed, and pulsed with OVA257-264 peptide at 2.0 μg/ml. After 2 h at 37°C, lymph node OT-I cells that were adherence depleted and the CD8 T cells were enriched by negative selection using CD8 Bellec columns (Cedarlane Laboratories, Hornby, Ontario, Canada). Purity of CD8 T cells was assessed by flow cytometry and was typically 90–95% with <1% CD4 T cells. A total of 5 × 10⁶ CD8 T cells were cultured with 1 × 10⁵ microspheres in 0.2 ml of complete RPMI medium. Cells were pulsed with 1 μCi of [³²P]thymidine/well for the last 1 h of the indicated time and incorporation of radioactivity was determined. Results are expressed as the mean ± SD of triplicate samples.

In vitro induction and reversal of AINR

Three to 4 days after initial stimulation of CD8 T cells, the AINR cells were harvested and dead cells were removed by Lympholyte M (Cedarlane Laboratories) gradient centrifugation. For all experiments, some of the cells were immediately restimulated to confirm that they were AINR, i.e., no longer responsive to stimulation with Kᵇ-OVA/B7-1 microspheres, but still responsive to IL-2. Reversal of AINR was conducted by stimulating continued proliferation of the AINR CD8 T cells using 2.5 U/ml human rIL-2 for 1–2 days. In each experiment, a proliferation assay was again done at the end of the IL-2 culture period to confirm that AINR had been reversed. The proliferation assay was done using 5 × 10⁴ CD8 T cells (naive, AINR, or reversed) with 1 × 10⁶ microspheres in 0.2 ml of complete RPMI medium.

IL-2 mRNA measurements

CD8 T cells (naive, AINR, or reversed) and Kᵇ-OVA/B7-1 microspheres were pelleted in 96-well V-bottom plates (1.5 × 10⁵ cells and 3 × 10⁶ microspheres/well) by centrifugation at 1250 × g for 10 min. The pellets were subsequently incubated at 37°C for 6–7 h. Trypan blue exclusion showed no significant change in cell viability at the end of the incubation period. Total RNA was isolated from the cells using the RNAqueous kit (Ambion, Austin, TX). IL-2 and actin mRNAs were determined by RT-PCR analysis using the same IL-2 and actin promoters and conditions as previously described, using a number of cycles confirmed to be within the linear amplification range for the assay (27). Experiments examining the effects of mitogen-activated protein kinase inhibitors on IL-2 mRNA expression were done by pretreating CD8 T cells with either DMSO (Fluka, Buchs, Switzerland), PD98059, or SB202190 (Calbiochem, San Diego, CA) at 37°C for 45 min before stimulation of the cells with microspheres.

In vitro kinase assays and immunoblotting

CD8 T cells (naive, AINR, or reversed) were pelleted with ligand-bearing microspheres by centrifugation at 1250 × g in a swinging bucket rotor for 10 min at 4°C. The pellets were then incubated in a 37°C water bath for 2.5 min (extracellular signal-regulated kinase [ERK] assay), 10 min (c-Jun N-terminal kinase [JNK] assay), or 7.5 min (phospho-p38 Western blot) and the cells were then lysed by the addition of ice-cold Triton-X buffer.

ERK and JNK activities were assayed as previously described (27) following precipitation with GST-Elk-1 (aa 307–428) and GST-c-jun (aa 1–69), respectively. For immunoblotting, whole cells lysates were resolved on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Blots were blocked with 5% skim milk powder and incubated with an anti-phospho-p38 Ab (Cell Signaling Technology, Beverly, MA) overnight at 4°C. Detection of a HRP-conjugated secondary Ab was done using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Adaptive transfer, tumor challenge, and IL-2 administration

Adaptive transfer of Kᵇ/OVA-specific TCR-transgenic CD8 T cells from OT-I mice and analysis of responding cells was done as previously described in detail (15, 24). Briefly, lymph node cells from OT-I mice on the Thy 1.1/C57BL/6/PiL background were adoptively transferred into C57BL/6 mice by i.v. injection. Mice were challenged 1 day later by i.p. injection of 3 × 10⁶ E.G7 cells in 0.5 ml of PBS (day 0). At varying times, mice were sacrificed and cells were isolated from the spleen, draining lymph nodes (peri-aortic, mesenteric, axillary, and brachial) and the peritoneal cavity. Adoptively transferred OT-I cells in the resulting cell populations were identified and quantitated by flow cytometry by staining with anti-Thy-1.1-PE and anti-CD8α-phycoerythrin mAbs and CD25 levels were determined using a third anti-CD25-FITC mAb. Flow cytometry was done using a FACSCaliber and CellQuest software (BD Biosciences, San Jose, CA). When used, murine IL-2 (R&D Systems, Minneapolis, MN) was administered by i.v. (tail vein) injection of 2000 IU/day on days 8 and 9 after inoculation with tumor.

Results

Reversal of AINR by exogenous IL-2

In vitro induction of AINR was examined using CD8 T cells from OT-I mice that have a transgenic TCR specific for OVA257-264 bound to H-2Kb. Stimulation of these cells with Kᵇ/OVA257-264 complexes immobilized on microspheres (Kᵇ-OVA) was not sufficient to cause proliferation unless IL-2 was also added to the cultures. However, the need for exogenous IL-2 was overcome if B7-1 ligand was immobilized along with Ag on the microspheres (Kᵇ-OVA/B7-1, Fig. 1A); the B7-1 provides costimulation to allow autocrine production and use of IL-2 by the cells. Proliferation measured by [³²P]thymidine incorporation peaks on day 3 and then declines and maximal numbers of OT-I cells are reached on day 4.

To examine responsiveness following the primary response, cells stimulated with Kᵇ-OVA/B7-1 were recovered from the cultures after 3.5 days, washed, and placed back into culture at the same starting cell density as in the primary stimulation (5 × 10⁶/well). Unlike naive cells, these previously stimulated cells responded well when just IL-2 was added to the cultures; Ag alone stimulated no response nor did it increase the response to exogenous IL-2. Also, in marked contrast to the naive cells, the previously stimulated cells could not respond to Ag even when B7-1 was present to provide costimulation. Thus, the cells become AINR following an initial response, even when effective costimulation is present during the primary response. Although AINR cells cannot be stimulated to make IL-2, some TCR-dependent signaling remains intact because these cells can lyse targets and make IFN-γ in response to Ag (20).

As discussed in more detail below, observations of in vivo responses of OT-I T cells raised the possibility that the AINR state might be reversible upon proliferation of the cells in response to exogenous IL-2. This was examined using OT-I cells harvested after 3.5 days of stimulation with Kᵇ-OVA/B7-1 microspheres. Less than 1% of the OT-I cells remain undivided at this time, as demonstrated in experiments examining dye dilution in cells labeled with CFSE before stimulation (data not shown). Following the initial stimulation, cells were washed and placed back into culture and IL-2 was added. After varying times from 0 to 4 days in culture with IL-2, cells were again harvested, washed, and placed into culture with various stimuli. As expected, the cells
were AINR at 3.5 days and responded only if IL-2 was added; they did not respond to Ag or Ag and costimulation alone (Fig. 2A). However, even after 1 day in culture with IL-2, the AINR cells had regained significant responsiveness that was further increased after 2 days and persisted through at least 4 days. The responsiveness of the “reversed” cells was distinctly different from that of naive cells in that Ag alone was sufficient to stimulate a strong proliferative response and coimmobilizing B7-1 to provide costimulation did not cause any further increase in the response. Essentially identical results have been obtained in experiments examining OT-I cells or CD8 T cells from C57BL/6 mice stimulated with microspheres having anti-TCR mAb and B7-1 immobilized on the surface (data not shown).

Proliferation following reversal is autocrine IL-2 dependent but costimulation independent

Stimulation of the reversed cells with Ag alone suggested that these cells had lost a requirement for costimulation to support proliferation. However, activated T cells express B7 ligands and it was important to determine whether transcostimulation was being provided by cell-cell interactions in the culture. Anti-B7-1 and anti-B7-2 mAb effectively block costimulation of naive cells when the respective ligands are coimmobilized on microspheres along with Ag (Fig. 2B). These same mAbs fail to block the response of reversed cells to Ag alone on the microspheres (Fig. 2B), thus demonstrating that costimulation provided by B7 ligands on the T cells is not contributing to this response.

Because production of IL-2 by naive cells requires costimulation, the independence from costimulation following reversal of AINR raised the possibility that this response did not depend on IL-2. However, proliferation of the reversed cells in response to Ag alone was completely dependent on IL-2, as demonstrated by the effective blocking of the response that occurred when anti-IL-2R mAb was added to the cultures (Fig. 3). Consistent with this, Ag alone is sufficient to up-regulate IL-2 mRNA expression in reversed cells (see below).

Altered requirements for activation of mitogen-activated protein kinases (MAPKs) following reversal of AINR

MAPKs are involved in regulating the transcription factors required for IL-2 gene expression in T cells and up-regulation of ERK, JNK, and p38 is defective in cells that have become AINR (27). Therefore, we examined cells that had recovered responsiveness following AINR to determine whether activation of the
MAPKs was also restored. ERK and JNK activities were measured in lysates of stimulated cells using GST-Elk-1 and GST-c-Jun fusion proteins as the substrates. Activation of p38 was assessed by Western blotting with a phospho-specific anti-p38 Ab to determine the amount of the active phosphorylated form of the enzyme. ERK is activated in naive cells in response to Ag and its activity does not increase further upon costimulation with B7-1 and/or ICAM-1 (Fig. 4A). Activation was greatly reduced in AINR cells, but restored in reversed cells to the same levels as in naive cells. In contrast to ERK, JNK activation in naive cells requires costimulation (Fig. 4B). Activation of JNK was also greatly reduced in AINR cells and restored in reversed cells. However, in the reversed cells, Ag alone was sufficient to fully activate JNK, and the presence of costimulatory ligands caused no further increase in activity. Active phospho-p38 increases in naive cells in response to just Ag (Fig. 4C). AINR cells were profoundly defective in phospho-p38 formation, but activation was restored in reversed cells. Thus, activation of all three MAPKs is defective in AINR cells and restored in reversed cells, and some rewiring occurs during the reversal such that activation of JNK no longer depends upon costimulation as it does in naive cells.

PD98059 is a specific inhibitor of mitogen-activated protein/extracellular signal-related kinase and, therefore, ERK, and SB202190 blocks p38 activation and, at somewhat higher concentrations, JNK activation. Both block proliferation of naive CD8 T cells (27). The restoration of activation of the MAPKs, and the parallel loss of a costimulation requirement to activate JNK and induce proliferation, suggested that the MAPK pathway was also involved in stimulation of cells following reversal from AINR. This was confirmed in experiments demonstrating that both PD98059 (Fig. 5, A and C) and SB202190 (Fig. 5, B and D) blocked IL-2 mRNA up-regulation and proliferation by reversed cells upon stimulation with either Ag alone or Ag and B7-1. These results support the conclusion that recovery from AINR involves, at least in part, recovery of the ability to activate the MAPKs to signal for IL-2 production.

In vivo reversal of AINR by IL-2 administration

Adoptive transfer of T cells from TCR-transgenic mice into normal recipients provides a means of directly visualizing and quantitating in vivo responses; the transgenic cells can be identified for flow cytometric analysis but are present in small numbers so that the immune response is not completely skewed as it is in the intact transgenic animal (28). We have used this approach to examine the response of transferred OT-I cells to i.p. E.G7 tumor that expresses OVA (15, 24). Within 3–4 days of i.p. injection of the tumor, OT-I cells migrate to the peritoneal cavity, undergo activation and clonal expansion, and control tumor growth. Clonal expansion peaks at about day 5 and the OT-I cells then migrate out of the peritoneal cavity and are found in the spleen and lymph nodes, even though the Ag-expressing tumor is still growing in the peritoneal cavity. The OT-I cells that have responded and migrated to the spleen have developed AINR; they exhibit direct ex vivo killing activity and can proliferate in response to exogenous IL-2 but not in response to Ag and costimulation.

The ability to reverse the AINR state in vitro by driving proliferation for 1–2 days with IL-2 predicted that it might also be possible to reverse the nonresponsive state in vivo with limited administration of IL-2 and achieve long-term responsiveness of the CD8 T cells once AINR was reversed. This was tested by challenging OT-I adoptive transfer recipients by i.p. injection of E.G7 tumor on day 0, allowing the initial response to occur unperturbed, and then administering low dose (2000 U/mouse) IL-2 systemically on days 8 and 9, the time at which the OT-I cells have become AINR (24). Mice were then sacrificed 10 days later (day 18 after tumor challenge) and the numbers of OT-I cells in the peritoneal cavity, spleen, and lymph nodes were determined, as well as the number of E.G7 tumor cells in the peritoneal cavity.

Adoptive transfer recipients that had not been challenged with tumor, OT-I cells were present in the spleen and lymph nodes in low numbers (Fig. 6A, OT-I) and had a naive phenotype (data not shown). In mice challenged with tumor and left untreated (Fig. 6A,
OT-I/E.G7), some OT-I cells were present in the peritoneal cavity but greater numbers were found in the spleen and lymph nodes (Fig. 6A) and tumor load was high (Fig. 6B). In contrast, mice treated with IL-2 on days 8 and 9 had a high number of OT-I remaining in the peritoneal cavity by day 18 (Fig. 6A, OT-I/E.G7/H11001/IL-2, days 8–9), as well as elevated numbers in the spleen and lymph nodes (Fig. 6A), and tumor load was substantially reduced in comparison to that in untreated mice (Fig. 6B). Essentially the same results have been obtained in similar experiments done using recipient mice depleted of CD4 T cells by in vivo mAb administration or CD4 knockout recipients, thus ruling out a role for host CD4 T cells in reversal of the AINR state by IL-2 administration (data not shown).

In addition to causing higher numbers of OT-I cells to be present at late times, brief IL-2 treatment resulted in prolonged responsiveness of the cells. Cells from both the lymph nodes and peritoneal cavities of mice treated on days 8 and 9 with IL-2 were continuing to respond at day 18, long after the exogenous IL-2 was gone, as evidenced by the fact that a large fraction of the cells had high forward scatter, indicating that they were blasting, and more than half expressed high CD25 (IL-2R/H9251) expression (Fig. 7). In contrast, few of the day 18 OT-I cells from mice that had not received IL-2 exhibited high forward scatter or high CD25 expression (Fig. 7). Thus, brief exposure to IL-2 is sufficient to reverse AINR and allow prolonged in vivo response to the tumor.

Discussion

Helper-independent initiation of CTL responses has been demonstrated in numerous models examining virus infections (9–13), tumors (14–16), and autoimmunity (17), but these responses often fail to persist in the absence of CD4 T cell help. Naive CD8 T cells respond to Ag and costimulation by producing IL-2 to drive their initial clonal expansion (6, 7, 19), but within a few days the cells become AINR (Fig. 1); they can still be signaled through the TCR to carry out effector functions but can no longer produce IL-2 to sustain expansion (20, 27). However, the CTL can continue to proliferate if provided with IL-2. This appears very likely to be the basis for the requirement for CD4 T helper cells to sustain effective CTL responses in many instances.

We would suggest that AINR is an integral part of the CD8 T cell developmental program, because it occurs both in vitro (Fig. 1; Ref. 20) and in vivo (20, 24), and occurs irrespective of the nature of the primary stimulus. In vitro stimulation results in the development of AINR within 3 days whether peptide/class I Ag or high-affinity anti-TCR mAb are used to provide signal one, and whether costimulation is provided by B7-1, ICAM-1, or by both ligands which synergize to provide potent costimulation (Fig. 1; Refs. 20.
and 27). AINR also develops when stimulation is with Ag or anti-TCR mAb in the absence of costimulatory ligands, with exogenous IL-2 added to drive proliferation (27). Thus, development of AINR does not depend upon signals delivered through the costimulatory receptors. Once AINR develops, costimulation by either B7-1, ICAM-1, or the combination is ineffective (Fig. 1; Refs. 20 and 27). AINR also develops in CD8 cells that have responded in vivo to allogeneic tumor, even when the tumor cells express B7-1 (20), or to syngeneic tumor (24). Thus, nonresponsiveness develops whether Ag is eliminated, as in the case of the allogeneic tumor, or persists, as in the case of the syngeneic tumor. Programmed development of AINR following the initial response of CD8 T cells would provide a consistent explanation for the lack of persistence of CTL responses in many situations, and is likely be the basis for the phenomenon of clonal “exhaustion” that has been shown to occur following a vigorous response to virus infection, thus allowing persistent infection (29).

CTL that have developed AINR become helper-dependent; they can no longer make IL-2, but can still respond if IL-2 is provided. However, this is not a permanent conversion to helper dependence. Rather, if proliferation is maintained by provision of exogenous IL-2 for a brief period (1–2 days), the cells regain their ability to up-regulate IL-2 mRNA (Fig. 5) and continue to proliferate in response to the IL-2 they produce (Figs. 2 and 3) without further exogenous growth factors being provided. Following reversal of AINR, the CTL continue to respond for a prolonged period of time, up to at least 14 days in vivo, the longest times examined (data not shown). This is in marked contrast to the initial response that ends in 3–4 days as the cells develop AINR (Fig. 1). Thus, it appears that development of AINR is not a mechanism for tolerizing CD8 T cells. Rather, it is a normal part of the CD8 developmental program that serves as a regulatory checkpoint for the potentially destructive CTL response, insuring that the cells can only continue to expand if regulatory helper CD4 T cells give permission in the form of IL-2. What determines whether a particular biological system appears to be “helper-independent” or “helper-dependent” may often depend upon whether or not the initial response is sufficient to clear Ag before development of AINR, e.g., due to sufficiently high precursor frequency, high avidity, or low Ag load.

Nonresponsiveness is not due to receptor down-regulation; AINR cells express essentially the same levels of TCR, CD28, and LFA-1 as naive cells (20). AINR cells also express CTLA-4, a receptor for B7 ligands that can deliver an inhibitory signal to cells (30–33). However, CTLA-4 does not mediate the nonresponsiveness that develops following initial stimulation. AINR cells cannot be costimulated via LFA-1/ICAM-I interactions when B7 ligands are not present (20) and where CTLA-4 would, therefore, not be engaged, and blocking anti-CTLA-4 mAb neither prevents nor reverses AINR in vitro (20) or in vivo (15). Rather, AINR appears to result from an intrinsic inability of the cells to signal for up-regulation of expression of IL-2 mRNA. Activation of MAPKs is required for IL-2 gene expression in T cells and anergic CD4 T cell clones have a markedly reduced ability to up-regulate ERK and JNK activities upon stimulation and cannot produce IL-2 (34–36). CD8 T cells also exhibit reduced up-regulation of ERK and JNK, as well as p38, upon development of AINR (Fig. 4). The enzymes remain present and can be activated by treatment of the AINR cells with phorbol ester and ionomycin (27), but they are not up-regulated in response to Ag and costimulation. The inability to up-regulate the MAPKs accounts, at least in part, for the inability of AINR cells to make IL-2 because use of specific inhibitors has demonstrated that activation of this pathway is essential for up-regulation of IL-2 mRNA by naive cells (27). Upon reversal of the AINR state, the ability to up-regulate the MAPKs is restored (Fig. 4) and IL-2 mRNA up-regulation and proliferation by the reversed cells depends upon these activities (Fig. 5). However, the signaling requirements are different in naive and reversed cells. Up-regulation of JNK in naive cells requires costimulation through either CD28/B7 or LFA-1/ICAM-I interactions, while JNK up-regulation in the reversed cells occurs when just the TCR is engaged (Fig. 4). Consistent with this, the reversed cells exhibit a decreased dependence on costimulation for increasing expression of IL-2 mRNA and proliferating (Figs. 3 and 5). Thus, once AINR is reversed, the CTL can more effectively respond to Ag that is not being expressed on professional APC. Memory cells also exhibit a decreased dependence on costimulation in comparison to naive cells; this may result from the rewiring that occurs upon reversal of the AINR state. The molecular basis for this change in signaling requirements remains to be determined.

**FIGURE 7.** OT-I CD8 T cells undergo prolonged activation following in vivo reversal of AINR. Cells recovered from the draining lymph nodes and peritoneal cavities of mice described in Fig. 6 were stained with anti-Thy 1.2 and anti-CD8 mAb to identify OT-I T cells (left panels). The OT-I populations identified by the boxes shown in the left panels were then analyzed for forward side scatter (middle panel) and CD25 expression (right panel). The numbers are the percentage of cells falling within the gates indicated by the horizontal bars. Similar results were obtained in more than four independent experiments.
There is an increasing appreciation of the importance of CD4 T cell help for generating effective anti-tumor responses by CD8 T cells and development of AINR in the responding CTL in the absence of help is likely to be an important factor in this. Evidence for this is accumulating in studies using models that make it possible to directly visualize the tumor-specific CTL during the course of a response. Using an adoptive transfer system employing OT-I T cells having a transgenic TCR specific for an OVA peptide bound to H-2K\(^d\), we showed that the OT-I cells could make an initial CD4-independent response upon i.p. challenge with an OVA-expressing E.G7 tumor. The OT-I cells clonally expanded in the peritoneal cavity developed effector function and controlled tumor growth for a few days. However, they then became AINR, migrated out of the peritoneal cavity, and stopped responding and tumor expansion resumed (24). In contrast, if OVA-specific CD4 helper cells were activated, the OT-I cells continued to make a prolonged response at the site of the tumor and control tumor growth and this was dependent on IL-2 (15). As shown in this study, brief administration of low dose IL-2 results in reversal of the nonresponsiveness (Figs. 6 and 7) and prolonged response of the OT-I cells at the site of the tumor (Fig. 6). A similar example is provided by studies of Marzo et al. (16) using the influenza hemagglutinin gene as a model tumor Ag. The tumor-specific CD8 T cells responded to tumor challenge and on day 14 their numbers and functions were comparable with or without Ag-specific CD4 T cells. However, more prolonged maintenance of the CD8 response required the presence of Ag-specific CD4 T cells and without them the number and function of the tumor-specific CD8 T cells was substantially reduced by day 28.

Thus, it appears that there are two distinct mechanisms by which CD4 T cells can provide help for a CD8 T cell response. CD4 cells can interact with APCs via CD40 to condition them to provide more effective activation of CD8 T cells and, thus, help in the initiation of a response (3–5). The mechanism of this conditioning has not been defined, but may involve stimulation of the production of inflammatory cytokines by the APC as a result of CD40 ligation. This type of help is clearly not always required, because there are numerous examples of generation of CTL responses in the absence of CD4 T cells. The requirement for CD4-dependent conditioning of APC may be bypassed if microbial or viral components are present to activate the APC and stimulate inflammatory cytokine production. There is also evidence that priming of CD8 cells is less dependent on help when the peptide epitope binds class I with high affinity (37). The studies described here have defined the second mechanism by which CD4 T cells can deliver help by providing IL-2 to reverse the AINR state that develops following the initial CTL response. Whether there are conditions that bypass AINR and allow a prolonged CTL response in the absence of help from CD4 T cells remains to be determined.

The ability to reverse CTL nonresponsiveness by brief administration of IL-2 (Figs. 6 and 7) has obvious therapeutic implications, but would appear to be at odds with the limited therapeutic efficacy that has been seen for IL-2 in animal models and in the clinic. However, in addition to its ability to promote T cell growth, IL-2 can also induce apoptotic death in activated T cells (38, 39). In another study (P. Shrikant and M. Mescher, manuscript in preparation), we have found that the reversal of AINR by low dose administration of IL-2 for a brief period (Fig. 6) can result in significant therapeutic benefit in the OT-I/E.G7 tumor model described above. However, when IL-2 administration is more prolonged or at high doses, as is often the case in clinical studies, then the number of CTL declines dramatically, with many of the cells dying by apoptosis, and the therapeutic benefit is lost. It will be important to determine how long CTL can persist in the AINR state and still be capable of having their nonresponsiveness reversed. We have found that IL-2 administration as long as 18 days after the peak of the primary response (the longest time examined in the other study) can still result in reversal (P. Shrikant and M. Mescher, manuscript in preparation). Some studies have shown that CTL that have made a primary response and then stopped expanding even though Ag remains present exhibit reduced effector function at longer times (13, 16, 40, 41), suggesting that CTL that have become AINR may undergo a gradual decline in function. Whether there is a concomitant loss of reversibility will be of critical importance in developing immunotherapeutic strategies for eliminating persistent viral infections and residual tumors.

Although CTL that have become AINR in the face of persisting Ag require help in the form of IL-2 to regain responsiveness (Figs. 6 and 7), the situation appears to be different if Ag is eliminated as a result of the primary response. When mice adoptively transferred with 2C CD8\(^+\) T cells specific for L\(^a\) alloantigen are challenged with allogeneic tumor, the 2C cells respond and the tumor is rapidly rejected (42). The 2C CTL that have responded have become AINR at this time, as demonstrated by the fact that they are unable to respond in vitro when restimulated with Ag and costimulation, but can respond if exogenous IL-2 is provided (20). In vivo, the number of these AINR 2C cells declines following elimination of Ag, but a long-term memory population develops that is capable of mounting a rapid secondary response upon rechallenge (43). These observations suggest that the fate of AINR CTL differs depending upon whether or not they continue to be exposed to Ag. Ehl et al. (44) have similarly observed that CTL unresponsiveness can be a consequence of persistent Ag. Clearly, further study will be needed to understand the mechanisms that determine the ultimate fate of CTL following a primary response to Ag and the programmed development of AINR.

The CD40-dependent help provided by CD4 T cells to initiate a CTL response (3–5) has been termed the “license” to kill (45). However, the primary stimulation of a CTL response, whether helper-dependent or -independent, appears to result in only a “temporary” license. The “permanent” license is only issued when the CTL successfully eliminate Ag or when CD4 T cells provide IL-2 to reverse AINR.

**Acknowledgments**

We thank D. Mueller, Y. Shimizu, and M. Jenkins for discussions and review of the manuscript and D. Lins and P. Champoux for technical assistance.

**References**


