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Control of Syngeneic Tumor Growth by Activation of CD8\(^+\) T Cells: Efficacy Is Limited by Migration Away from the Site and Induction of Nonresponsiveness\(^1\)

Protul Shrikant and Matthew F. Mescher\(^2\)

Activation of Ag-specific CD8\(^+\) T cells in response to syngeneic tumor has been visualized by adoptive transfer of CD8\(^+\) T cells from OT-I mice, with a transgenic TCR specific for H-2K\(^b\) and an OVA peptide, into Thy-1 congenic recipients. Intraperitoneal challenge with E.G7, the EL-4 thymoma transfected with OVA, results in activation and clonal expansion of the OT-I cells in the peritoneal cavity and transient control of tumor growth. However, within 2 days after becoming activated, the OT-I cells migrate out of the peritoneal cavity into the spleen and lymph nodes, and tumor growth resumes in the peritoneal cavity. The OT-I cells in lymph nodes and spleen have lytic effector activity, but exhibit split anergy in that they cannot proliferate in response to Ag unless exogenous IL-2 is provided. The failure to remain at the tumor site and continue to control tumor growth is not due to selection of Ag loss variants or development of suppression. These results suggest that effective CD8-targeted immunotherapy may depend less on enhancing the initial activation and more on sustaining the response at the appropriate location and/or reactivating cells that have left the site of tumor growth and become nonresponsive. *The Journal of Immunology*, 1999, 162: 2858–2866.

Numerous peptides that serve as tumor-specific CTL epitopes have been identified (1–3), encouraged by a great deal of evidence suggesting that a CTL response can be effective in eliminating tumor. Prior immunization in murine tumor models can establish sufficient immunity so that the host is protected against a subsequent challenge with live tumor (4), and adoptive transfer of CTL lines or clones can eliminate growing tumors (4–7). Tumor-specific CD8\(^+\) T cells can clearly be present in the animal, as evidenced by in vitro expansion or protective immunization, but nevertheless fail to protect when the host is challenged with live tumor. This is probably also the case in at least some human cancers where tumor-specific CTL can be isolated from the patient (8–10), yet the tumor is progressing. There are numerous reasons why a CTL response may fail to occur or may occur but fail to control tumor growth. Various mechanisms of tolerance or suppression may be operative by which the or may occur but fail to control tumor growth. Various mechanisms of tolerance or suppression may be operative by which the host is challenged against a subsequent challenge with live tumor (4), and adoptive transfer of CTL lines or clones can eliminate growing tumors (4–7). Tumor-specific CD8\(^+\) T cells can clearly be present in the animal, as evidenced by in vitro expansion or protective immunization, but nevertheless fail to protect when the host is challenged with live tumor. This is probably also the case in at least some human cancers where tumor-specific CTL can be isolated from the patient (8–10), yet the tumor is progressing. There are numerous reasons why a CTL response may fail to occur or may occur but fail to control tumor growth. Various mechanisms of tolerance or suppression may be operative by which the naive CD8\(^+\) T cells may remain ignorant of the presence of tumor in a peripheral site, or a response may occur but be too weak and/or too short-lived to be effective. Therapeutic approaches using cytokines, costimulatory molecules, or peptides to increase response levels can give dramatic results in murine models and are beginning to be evaluated in the clinic.

A major difficulty in attempting to understand why an effective CD8\(^+\) CTL response to syngeneic tumor does not normally occur and in developing novel therapeutic approaches that target the CTL response is that the Ag-specific CD8\(^+\) T cells are present in very low numbers in the animal and cannot be specifically identified. Thus, their trafficking and response to Ag in vivo cannot be directly tracked. Instead, the location and status of the relevant tumor-specific CD8\(^+\) cells can only be assessed by indirect methods that rely on in vitro restimulation and assay of cytolytic activity. These are cumbersome at best and rely upon the ability of the cells to respond in vitro to the stimulus used.

Adoptive transfer of T cells from TCR transgenic mice into normal recipients provides a means of monitoring Ag-specific T cells during a response (11). The transferred cells can be identified by unique surface markers and serve as an indicator of the endogenous host response. Provided that the cells are transferred in small numbers, to constitute 0.2–0.5% of the spleen and lymph node (LN)\(^3\) cells in the recipient, the presence of the transferred cells does not aberrantly skew the normal response, as is the case when attempting to study responses in intact TCR transgenic mice. Adoptive transfer of CD8\(^+\) TCR transgenic T cells has been used to study both virus-specific (12, 13) and allogeneic CTL responses (14). Recent studies of virus-specific responses by adoptive transfer (13) or direct visualization of endogenous Ag-specific cells using tetrameric Ag (15) have shown that the number of specific cells is dramatically underestimated by approaches that rely on in vitro restimulation of the cells, emphasizing the limited information that can be gained by such indirect approaches.

To begin to develop a more detailed understanding of CD8\(^+\) T cell responses to syngeneic tumor, we have applied the TCR transgenic adoptive transfer approach to study the response to E.G7, the EL-4 thymoma transfected with the gene for OVA (16). E.G7 cells grow rapidly in C57BL/6 mice and the host dies within 25–35 days. This cell line, expressing OVA as a pseudo-tumor Ag, has been extensively used in studies of immunotherapy procedures (17–22). CD8\(^+\) T cells from OT-I mice with the C57BL/6 background express a transgenic TCR that recognizes H-2K\(^b\) and OVA\(^{257–264}\) peptide (23). By adoptively transferring these cells into Thy-1 congenic C57BL/PL mice and challenging them with...
E.G7, it is possible to identify the Ag-specific cells and determine whether they can respond to the tumor, and if so where and when the response occurs and if it is effective in reducing tumor growth. As described in this report, the OT-I cells do respond at the site of the tumor by clonally expanding and developing cytolytic function, and as a result they transiently control tumor growth. However, at a point where tumor is still present, the OT-I cells migrate from the tumor site into the spleen and LN where they still retain lytic effector function but appear to be anergic to restimulation; the tumor progresses and the mice die. These results clearly define the in vivo induction and progression of a CD8⁺ T cell immune response to a syngeneic tumor. Furthermore, they identify novel mechanisms involved in the circumvention of the immune response by the tumor and have important implications for devising strategies for effective immunotherapy of cancer.

**Materials and Methods**

**Animals**

OT-I TCR transgenic mice (23), a gift from Dr. Francis Carbone (Monash Medical School, Victoria, Australia), were bred to wild-type C57BL/6 mice to generate mice heterozygous for the OT-I TCR transgene, and these mice were used as the source of transgenic T cells in all experiments. C57BL/PL mice congenic for the Thy-1 marker were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used as recipients in all the experiments described. Animals were housed under specific pathogen-free conditions at all times.

**Cell lines**

EL-4, a thymoma derived from the C57BL/6 mouse (H-2b), was maintained in vitro in complete RPMI medium; RPMI 1640 (Cellgro, Herndon, VA), 10% FCS (Tissue Culture Biologicals, Tulare, CA), 0.2% l-glutamine, 0.1% penicillin/streptomycin, 0.1% HEPES (BioWhittaker, Walkersville, MD), 0.1% nonessential amino acids, 0.01% sodium pyruvate (Cellgro), and 0.05% 2-ME. E.G7 (OVA-transfected EL-4) (16) was maintained in complete RPMI medium containing 400 μg/ml of G418 (Cellgro). Both cell lines were periodically passaged in B3, a K⁺/OVA257-264-specific CTL cell line was a gift from Drs. S. Jameson and K. Hogquist (University of Minnesota, Minneapolis, MN).

**Abs and reagents**

Directly conjugated mAbs including anti-CD8ε-chychochrome, anti-Thy-1.2-PE, anti-CD62L-FITC, anti-CD44-FITC, anti-CD49d-FITC, anti-CD25-FITC, and anti-CD69-FITC were purchased from PharMingen. Purified anti-CD16/CD32 (Fc Block) was also purchased from PharMingen.

**Adoptive transfer of transgenic cells and tumor challenge**

LN cells (axillary, brachial, mesenteric, inguinal, cervical, periaticor, and mediastinal) from heterozygous OT-I transgenic mice were removed, homogenized, and washed three or four times in PBS. CD8⁺/Thy-1.2⁺ cells (3–4 × 10⁷) were transferred into sex-matched naive C57BL/PL mice by tail vein injection. Recipient mice were rested for a day and were then challenged by i.p. injection of 4 × 10⁶ EL-4 or E.G7 cells in 0.5 ml of PBS or PBS alone as a control.

**Analysis by flow cytometry**

Cells were sacrificed at varying times, and the spleen and LN were collected (periaticor, mesenteric, axillary, and brachial), homogenized, and treated with ammonium chloride to remove RBCs. The peritoneal cavity was washed twice with 25 ml of PBS each time, and the resulting peritoneal exudate lymphocytes were adherence-depleted for 90 min in complete medium at 37°C. The total number of cells obtained from each site was determined by counting using a hemocytometer.

**Abs and reagents**

Mature exudate lymphocytes were adherence-depleted for 90 min on ice and were then stained with anti-CD8ε-chychochrome, anti-Thy-1.2-PE and a third Ab-FITC specific for phenotypic marker (see below). After a 1 h incubation on ice, the cells were washed twice, resuspended in 0.2 ml of 1% formaldehyde, and analyzed by three-color flow cytometry using the CellQuest software package (Becton Dickinson, San Jose, CA). Transferred OT-I cells were identified as CD8⁺/Thy-1.2⁺ cells. Cells from C57BL/PL mice stained in the same way showed no events in the CD8⁺ Thy-1.2⁺ gate, and cells from mice that had received OT-I cells by adoptive transfer had <0.05% of events in this gate when the Thy-1.2-PE mAb was replaced with an isotype-matched control Ab labeled with PE. These controls were included in every experiment.

**Results**

**CD8⁺ OT-I T cells respond in vitro to Ag on E.G7 tumor**

E.G7 cells are EL-4 thymoma transfected with the OVA gene (16) and express about 10⁴ K⁺/OVA257-264 complexes/cell (24). Thus, the OVA peptide is present at a low density, as is likely to be the case for physiologically relevant tumor Ags. To insure that T cells from OT-I mice with a TCR-specific for K⁺/OVA257-264 (23) could respond to Ag on E.G7, proliferation in response to irradiated E.G7 cells was examined (Fig. 1A). OT-I cells made a strong proliferative response to OVA257-264-pulsed spleen cells, and a weaker but still substantial, response to E.G7, while no response occurred to the parental EL-4 cells. E.G7 cells could also be killed by OT-I effector CTL, while lysis of EL-4 was minimal (Fig. 1B).

The ability of E.G7 to stimulate Ag-specific responses by OT-I CD8⁺ T cells confirmed that this system should be suitable for examining in vivo response to syngeneic tumor by adoptive transfer. Almost all Thy-1.2⁺ cells from the LN of OT-I mice are also CD8⁺, and these cells have a naive phenotype: CD25 and CD69 negative, low expression of CD44 and VLA-4, high expression of L-selectin, and forward scatter (FSC) consistent with small resting cells (Fig. 2A). Adoptive transfer was performed by i.v. (tail vein) injection of cells from OT-I mice into Thy-1 congenic C57BL/PL recipients to allow detection of the transferred cells by staining with anti-Thy-1.2-mAb. In C57BL/PL mice that had not received OT-I, virtually no Thy-1.2⁺ CD8⁺ or CD8⁻ CD8⁺ OT-I cells could
be readily detected in spleen and LN (Fig. 2C). The naive phenotype of the OT-I cells (Fig. 2A) was maintained in the recipients following adoptive transfer (Fig. 2C).

**CD8\(^+\) OT-I T cells respond to E.G7 tumor in vivo**

EL-4 tumor expresses Ags that can be recognized by C57BL/6 CD8\(^+\) T cells (25, 26), and the E.G7 line expresses in addition the OVA\(_{257-264}\) CD8\(^+\) T cell determinant. Nevertheless, i.p. injection of live E.G7 results in progressive tumor growth and death of the host in about 25–35 days. This could be due to failure to generate a CD8\(^+\) CTL response or failure of the response to control tumor growth. To examine this, OT-I cells were adoptively transferred by i.v. (tail vein) injection in C57BL/PL recipients and allowed to equilibrate for 24 h so that their distribution in lymphoid organs reflected that of the endogenous CD8\(^+\) T cells. One day later the recipients were challenged by i.p. injection of live E.G7, and the number of OT-I cells (CD8\(^+\)/Thy-1.2\(^+\) cells) in the LN, spleen, and peritoneal cavity was determined at subsequent times. By day 4 after challenge, the number of OT-I cells had declined modestly in the spleen and LN (Fig. 3, A and B) and increased substantially in the peritoneal cavity (Fig. 3C), suggesting that they were migrating to the latter site and undergoing clonal expansion. By day 6, OT-I cell numbers had begun to decline in the peritoneal cavity and were increasing in the spleen. Few detectable OT-I cells remained in the peritoneal cavity by day 10, while increasing numbers were present in the LN and spleen and remained present at these sites through days 18 (Fig. 3, A and B) and 22 (data not shown). In mice that were not challenged with E.G7 (Fig. 3, OT-I), the number of OT-I cells declined for the first 4 days and then remained constant, with no expansion at any sites. Similarly, no expansion of OT-I cells occurred in adoptively transferred mice that were challenged with EL-4 tumor (data not shown). Thus, OT-I cells exhibit an Ag-specific increases in number at the site of tumor growth in mice challenged with tumor.

The increase in OT-I cells in the peritoneal cavity by day 4 suggested that the cells were clonally expanding there, as opposed to simply migrating into the site, and this conclusion was supported by the phenotype of the cells. In contrast to cells before transfer (Fig. 4A), the majority of OT-I in the peritoneal cavities of E.G7-challenged mice had an activated phenotype (Fig. 4C), with increased CD44, CD25, and VLA-4 and decreased L-selectin surface levels, and about half were blasts as judged by FSC. However, the OT-I cells in the spleen and LN on day 4 maintained a naive phenotype, with the exception of increased VLA-4 expression on OT-I cells in the LN (data not shown). When challenge was with EL-4, very few OT-I cells appeared in the peritoneal cavity on day 4 or later, and they did not increase as a percentage of the total CD8\(^+\) T cells. The small numbers made precise estimates of phenotypic distribution difficult, but it is clear that the majority of OT-I cells did not become activated in response to the EL-4 tumor (Fig. 4B). Thus, OT-I cells undergo Ag-dependent proliferation in response to the E.G7 tumor, and clonal expansion occurs preferentially at the site of tumor growth rather than in draining LN.
Activated CTL transiently control tumor growth and then leave the tumor site

While they were present in expanded numbers in the peritoneal cavity (days 4–6), OT-I cells limited the rate of growth of the tumor compared with tumor growth in normal C57BL/PL mice (Fig. 5A). After day 6, when OT-I cells were declining in the peritoneal cavity, tumor growth rate increased (Fig. 5A) and paralleled the growth rate in normal, untransferred C57BL/PL mice. Control of tumor growth by the OT-I cells is Ag specific; transferred OT-I cells have no effect on the growth of EL-4 tumor (Fig. 5B). In five of six experiments the time course of the response, the numbers and kinetics of clonal expansion followed by decline in OT-I cells and the numbers and kinetics of tumor cells in the peritoneal cavity were essentially the same as shown in Figs. 3 and 5. Thus, the Thy-1 difference between the OT-I cells and the C57BL/PL recipients cavity during the response. The numbers and kinetics of clonal expansion followed by decline in OT-I cells and the numbers and kinetics of tumor cells in the peritoneal cavity were essentially the same as shown in Figs. 3 and 5. Thus, the Thy-1 difference between the OT-I cells and the C57BL/PL recipients

The above experiments used OT-I cells from C57BL/6 mice for transfer into C57BL/PL Thy-1 congenic recipients. There was therefore the possibility that a response by the recipient to the Thy-1 difference might influence the results. Experiments were thus performed in which OT-I cells were transferred into normal C57BL/6 recipients and detected by staining for CD8 and Vα2, one of the transgenic TCR chains. This allows detection of OT-I cells with reasonable accuracy over the low background of CD8⁺Vα2⁺ host cells, particularly in the peritoneal

FIGURE 3. Adoptively transferred OT-I CD8⁺ T cells initially expand in the peritoneal cavity upon Ag challenge and subsequently increase in number in the LN and spleen. OT-I LN cells (3–4 × 10⁶) are transferred i.v. into C57BL/PL recipients. Twenty-four hours later the mice received an i.p. injection of 0.5 ml of PBS (OT-I) or 4 × 10⁶ E.G7 cells in 0.5 ml of PBS (OT-I/E.G7). Two animals from each group were sacrificed on the indicated days after tumor challenge, and cells harvested from draining and peripheral LN (A), spleen (B), and peritoneal cavity (C) were analyzed by flow cytometry to determine the total number of OT-I cells (CD8⁺/Thy-1.2⁺) at each site. The results shown are the mean ± SD of three separate experiments examining two mice per group at each time.

FIGURE 4. Phenotypic characterization of OT-I cells from the peritoneal cavities of adoptively transferred recipients 4 days after challenge with E.G7, but not EL-4, display an activated phenotype. Cells were stained with CD8α-cychrome and Thy-1.2 PE mAbs, and the double-positive population of cells (OT-I cells) was gated as shown in the top panels and analyzed for expression of CD44, CD25, VLA-4, and L-selectin as described in Materials and Methods. Naive OT-I cells before adoptive transfer are shown in the left panels for comparison (A, pretransfer). The middle and right panels show analysis of peritoneal lavage cells from adoptive transfer recipients challenged by i.p. injection of 4 × 10⁶ EL-4 cells in 0.5 ml of PBS (B) or 4 × 10⁶ E.G7 cells in 0.5 ml of PBS (C).

FIGURE 5. Adoptive transfer of OT-I cells results in transient control of the growth of E.G7, but not EL-4, during the time that CD8⁺ OT-I cells are present in the peritoneal cavity. A, E.G7 tumor cells (4 × 10⁶) were injected i.p. on day 0 into either normal C57BL/PL mice (E.G7) or C57BL/PL mice that had received OT-I cells by adoptive transfer (OT-I/E.G7). B, EL-4 tumor cells (4 × 10⁶) were injected i.p. on day 0 into either normal C57BL/PL mice (EL-4) or C57BL/PL mice that had received OT-I cells by adoptive transfer (OT-I/EL-4). For both A and B, the number of tumor cells and the number of OT-I cells (A only) in the peritoneal cavities of the mice were determined on the indicated days by flow cytometry as described in Materials and Methods.
OT-I effector CTL migrate out of the peritoneal cavity

The increasing numbers of OT-I cells in the LN and spleen after day 4 concomitant with the decline in the peritoneal cavity suggested that the cells might be migrating out of the peritoneal cavity. Alternatively, a second wave of clonal expansion might occur in the LN and spleen. Examination of the phenotype of the OT-I cells in spleen and LN on day 10 showed the majority to have a phenotype consistent with having been previously activated. The cells had high CD44 and low L-selectin levels, but few were blasts as judged by FSC (Fig. 6), and they did not have increased CD25 levels (data not shown), indicating that they were not actively proliferating. Similar results were obtained when OT-I cells from the LN and spleen were examined on day 8 (data not shown).

The phenotype of the OT-I cells in the LN and spleen suggested that they had proliferated in the peritoneal cavity and then migrated out. To directly examine this, cells were recovered on day 4 from the peritoneal cavities of mice that had received OT-I cells and been challenged with E.G7. The CD8+ cells (that include OT-I T cells) were purified. The purified cells were then transferred into the peritoneal cavities of C57BL/PL mice that had received either PBS or E.G7 tumor cells by i.p. injection 4 days earlier. Three days later, the OT-I cells placed in mice bearing E.G7 had left the peritoneal cavity and appeared in the LN and spleen (Fig. 7, E.G7). In contrast, OT-I cells placed in the peritoneal cavities of mice that received PBS remained in the peritoneal cavities (Fig. 7, PBS). These results are consistent with the suggestion that OT-I cells respond to E.G7 in the peritoneal cavity, control tumor growth transiently, but then migrate out of the site of tumor growth and into the spleen and LN. Furthermore, the retransferred CD8+ T cells did not proliferate in response to tumor, as evidenced by their forward scatter (data not shown).

Growing E.G7 tumor remains antigenic following the transient OT-I response

Exit of activated OT-I cells from the site of tumor and resumed tumor growth could occur if cytolyis by the OT-I CTL resulted in selection of an Ag loss variant, E.G7 cells that no longer expressed OVA. However, a K+bOVA257-264-specific cloned CTL line could kill E.G7 cells isolated on day 18 from the peritoneal cavities of OT-I-transferred, E.G7-challenged mice (Fig. 8A), demonstrating that the majority of E.G7 cells still express sufficient OVA peptide to act as targets.

Antigenicity of the E.G7 tumor following the transient OT-I response was more directly demonstrated by transferring fresh, naive OT-I cells into mice after an initial OT-I response to E.G7. In the initial response, OT-I cells reached high numbers in the peritoneal cavity on day 4 and declined to a low level by day 8 as expected (Fig. 8B). When fresh OT-I cells were then injected i.v., a second wave of OT-I expansion occurred in the peritoneal cavity that peaked 3–4 days after the cells were injected and then declined. Thus, the cells freshly injected on day 8 responded identically to cells present on day 0 at the time of the initial tumor challenge. Tumor growth was transiently controlled during both the first and the second response by OT-I cells (Fig. 8C).

A response also occurred when tumor was allowed to grow uncontrolled in normal C57BL/PL mice (no adoptive transfer) for 8 days before i.v. injection of the OT-I cells (Fig. 8B). Again, clonal expansion of OT-I cells occurred in the peritoneal cavity 4 days after injection, and tumor growth was controlled while OT-I cells were present at the site of the tumor (Fig. 8C). Thus, even when tumor load has reached a high level before introduction of the OT-I cells, they are still able to respond by clonal expansion and to transiently control further tumor growth.

OT-I cells from day 10 spleen have lytic effector function but are anergic

When cells were recovered from the spleens and LN of mice on day 10 following the response to tumor, and the CD8+ cells (which included OT-I cells) were purified, they demonstrated potent cytolytic activity for E.G7 tumor (Fig. 9A, OT-I/E.G7). In contrast, killing of E.G7 was low by cells from mice that had received OT-I by adoptive transfer but had not been challenged with tumor (Fig. 9A, OT-I) or from mice challenged with EL-4...
FIGURE 8. Persisting E.G7 tumor is antigenic in vitro and in vivo. A. Persisting tumor is lysed by anti-K\(^{b}\)/OVA\(_{257-264}\) CTL. C57BL/PL mice received OT-I LN cells by adoptive transfer and were challenged by i.p. injection of 4 \times 10^6 EL-4 or E.G7 tumor cells. Tumor cells were harvested from the peritoneal cavities of the mice 18 days later, labeled with \(^{51}\)Cr, and used as targets in a standard 4-h \(^{51}\)Cr release assay with B3 effectors, a cloned CTL line specific for H-2\(^{b}\)/OVA\(_{257-264}\). B. Naive OT-I cells can respond in vivo following an initial response. C57BL/PL mice received OT-I cells and were challenged on day 0 by i.p. injection of PBS (OT-I), EL-4 (OT-I/EL-4), or E.G7 (OT-I/E.G7). CD8\(^{+}\) T cells were purified by adherence cell depletion and negative selection columns, and used immediately as effector cells in a standard 4-h \(^{51}\)Cr release assay using E.G7 (A) or EL-4 cells (B) as targets. E:T cell ratios are expressed as the number of OT-I cells per number of target cells.

FIGURE 9. OT-I cells from the LN and spleen on day 10 have Ag-specific cytolytic activity. Cells from LN and spleens were harvested on day 10 from OT-I-transferred mice that had been challenged on day 0 by i.p. injection with PBS (OT-I), EL-4 (OT-I/EL-4), or E.G7 (OT-I/E.G7). CD8\(^{+}\) T cells were purified by adherence cell depletion and negative selection columns, and used immediately as effector cells in a standard 4-h \(^{51}\)Cr release assay using E.G7 (A) or EL-4 cells (B) as targets. E:T cell ratios are expressed as the number of OT-I cells per number of target cells.

tumor (Fig. 9A, OT-I/EL-4). For all populations there was essentially no lysis of EL-4 tumor cells (Fig. 9B). Thus, the OT-I cells that have migrated out of the peritoneal cavity retain Ag-specific cytolytic activity and could presumably continue to control tumor growth, but they are no longer in the right location to perform this function.

When the OT-I cells stop expanding and exit the peritoneal cavity, the tumor is clearly still antigenic (Fig. 8). This suggested that although the OT-I cells on day 10 can still lyse target cells (Fig. 9), they may no longer be able to proliferate in response to Ag. We therefore examined whether cells recovered from LN and spleen on day 10 could respond in an in vitro proliferation assay. Cells were taken from mice that had received OT-I cells by adoptive transfer but not been challenged with E.G7 (OT-I), mice that received OT-I cells and were challenged with EL-4 (OT-I/EL-4), or mice that received OT-I cells and were challenged with E.G7 (OT-I/E.G7).

Cells from OT-I or OT-I/EL-4 mice made a vigorous proliferative response to C57BL/6 spleen cells pulsed with OVA\(_{257-264}\) peptide, as measured by [\(^{3}\)H]TdR incorporation at 48 h (Fig. 10A). In contrast, the OT-I cells from E.G7-challenged mice did not respond (Fig. 10A, OT-I/EL-4). Addition of exogenous IL-2 to the cultures restored the response of the cells from the OT-I/E.G7 mice (Fig. 10A). IL-2 in the absence of peptide Ag stimulated some response by the cells from the OT-I/E.G7 mice, but the response was much stronger when Ag was also present. To confirm that the [\(^{3}\)H]TdR incorporation measured in these experiments reflected the proliferative capacity of the OT-I cells, we also determined the changes in absolute numbers of OT-I cells in the cultures after 3 days (Fig. 10B). This analysis yielded the same conclusion; cells from OT-I/E.G7 can respond in the presence of exogenous IL-2, but not in its absence. Thus, the cells from the OT-I/E.G7 mice display an apparent split anergy, able to lyse targets but unable to undergo proliferation unless exogenous IL-2 is provided. Induction of this state depends upon Ag recognition in vivo, since OT-I cells from mice challenged with EL-4 tumor respond without IL-2 addition.

Discussion

It has become apparent that most tumors express peptides bound to class I proteins that can be recognized by CD8\(^{+}\) T cells, and this provides considerable encouragement for the possibility of inducing therapeutic immune responses to these epitopes. It has long been recognized, however, that immunogenic tumors grow progressively in the syngeneic host; even in cases where some CD8\(^{+}\) T cell response occurs, the immune system ultimately fails to control the tumor. Numerous suggestions have been made to attempt to explain the inability to mount an effective CTL response, including tolerance due to ignorance or other mechanisms, failure of the antigenic peptide to be presented in a stimulatory context, i.e.,
with costimulatory ligands and/or cytokines present, selection of Ag loss variants, production by the tumor of Ab-dependent blocking factors that block cytotoxicity, and generation of suppressor cells. The results described here examining the EG.7 tumor with OVA as a pseudo-tumor Ag suggest the additional possibility that activation and development of effector function can be effective, but that the CTL fail to control tumor growth because they do not remain at the site of the tumor.

When adoptively transferred mice are challenged by i.p. injection of EG.7, OT-I CD8\(^+\) T cells migrate to the peritoneal cavity within 3 days, undergo substantial clonal expansion, develop lytic activity, and control tumor growth over the next 2 days (Figs. 3 and 5). Thus, the OT-I cells do not remain ignorant and are not tolerant to the Ag; in fact, they make a vigorous response. By day 6, however, the CTL are leaving the peritoneal cavity and migrating to the LN and spleen. They continue to have lytic activity, and control tumor growth over the next 2 days (Figs. 3 and 5). Thus, the environment present in the tumor-bearing host by day 8 and beyond is not inhibitory to the generation of an Ag-specific CD8\(^+\) T cell response and does not prevent lytic effector cells from being active in the peritoneal cavity, nor is the tumor resistant to control by the CTL that are generated.

The first, somewhat surprising, observation in this system was that full activation of the OT-I cells, as assessed by CD25 up-regulation and blast transformation, was first detected in the peritoneal cavity at the site of tumor rather than in draining LN (Fig. 4 and data not shown). In fact, most of the clonal expansion that was observed occurred in the peritoneal cavity, with the cells subsequently migrating to the LN and spleen (Fig. 7) where they expressed a high level of CD44, indicating that they had already responded to Ag, but where few were blasts. Failure to detect full activation and expansion in the draining LN was unexpected. However, although blast transformation was not detected in LN at early times, the level of VLA-4 expression on the OT-I cells did increase in an Ag-dependent manner (data not show). This occurred at the same time that OT-I cells were decreasing in number in the spleen and LN and appearing in the peritoneal cavity. Similar changes were seen when examining 2C TCR transgenic CD8\(^+\) T cells responding to allogeneic tumor challenge in the peritoneal cavity (14). These observations suggest the hypothesis that recognition of Ag by naive CD8\(^+\) T cells results initially in altered expression of receptors involved in trafficking, including VLA-4, and that this promotes selective migration of the Ag-specific cells to the peripheral site of tumor growth where they become fully activated. This could potentially explain the somewhat paradoxical observation that expression of B7-1 on a tumor cell enhances the CD8\(^+\) T cell response to the tumor, but that the responding T cells are restricted only by class I expressed by host APC; T cells specific for class I expressed just by the tumor make only a small contribution (28). Ag-specific CD8\(^+\) T cells may need to recognize Ag presented by host APC in the draining LN, i.e., by cross-priming, so that they then migrate to the tumor site and undergo potent activation and expansion in response to the tumor expressing the B7-1 costimulatory ligand. Most T cells specific for class I on the tumor that is not expressed by the host APC may never gain access to the tumor.

Although the EG.7 tumor used in these experiments does not express B7 ligands, vigorous clonal expansion of OT-I cells occurs. This may involve cross-priming (28, 29). Alternatively, ICAM expressed by the tumor may provide sufficient LFA-1-dependent costimulation to support proliferation (30, 31). Host CD4\(^+\) T cells do not appear to be involved, since the OT-I response through at least day 10 is essentially identical in recipients that have been depleted of CD4\(^+\) T cells by in vivo anti-CD4 mAb administration (data not shown).

Following the peak of clonal expansion on day 4, the number of OT-I cells declines in the peritoneal cavity (Fig. 3). This is probably due in part to cell death; by using mAb staining for annexin V (32), about 30% of OT-I cells in the peritoneal cavity were found to be positive on days 4–6 (data not shown). It is also clear, however, that much of the decline in number of effector cells at the tumor site is due to migration out of the peritoneal cavity into LN and spleen (Fig. 7). Additional experiments have shown that migration out of the peritoneal cavity is not Ag specific; activated OT-I cells placed in the peritoneal cavities of mice that have been injected i.p. with either EL-4 tumor or LPS also leave and appear in the LN and spleen (P. Shrikant and M. F. Mescher, manuscript in preparation). Thus, it appears that exit from the peritoneal cavity requires inflammation, but not recognition of specific Ag by the OT-I cells. OT-I cells placed in the peritoneal cavities of EG7-bearing mice can be found in LN and spleen as early as 2 days after injection (data not shown).
Why do the active lytic effector OT-I cells leave the peritoneal cavity while tumor cells are still present in large numbers? This is not because the tumor no longer expresses Ag (Fig. 8). It also does not appear to be due to the tumor creating an environment that either prevents T cells from entering the peritoneal cavity or causes them to leave rapidly, since naive OT-I transferred late can migrate there, respond, and again transiently control tumor growth (Fig. 8, B and C). The response of 2C CD8\(^+\) T cells to allogeneic tumor in the peritoneal cavity shows almost identical kinetics of clonal expansion followed by exit from the peritoneal cavity (14) as does the response of OT-I cells to EG.7, the difference being that allogeneic tumor has been eliminated by the time the CTL leave, while the syngeneic tumor is still present in large numbers.

Taken together, these observations raise the possibility that the CTL developmental program may dictate this behavior and that it is largely Ag independent once the initial activation has occurred. What regulates the location of the CTL is unclear. One possibility is largely Ag independent once the initial activation has occurred. The OT-I cells that have responded to tumor and then exited the peritoneal cavity, while in the absence increased numbers of CTL are still generated but are ineffective because they leave the site. The initial activation results in the cells being held at the site of the tumor, but that within a short time of gaining effector function the cells lose this ability. Thus, once they exit the peritoneal cavity they would be unable to efficiently reenter the site. Another possibility is that Ag recognition during the initial activation results in the cells being held at the site of the tumor, perhaps through constitutive up-regulation of adhesion receptors. Subsequent down-regulation would then allow the cells to release and exit the site; the effector cells might continue to migrate through the peritoneal cavity, but fail to accumulate there after the initial activation period. Consistent with this suggestion, we have found that CD8\(^+\) T cells display constitutively active binding to ICAM-1 and fibronectin within about 24 h of in vitro stimulation with Ag, and this disappears by about 72 h when the cells develop effector function (J. M. Curtisinger, D. Lins, and M. F. Mescher, manuscript in preparation).

Whatever the mechanism responsible, it is clear that failure of the cytolytic effector cells to remain at the site of the tumor is the major limitation in the ability of the CD8\(^+\) T cell response to control tumor growth. A similar phenomenon may occur for CD8\(^+\) T cell responses to human tumors. In recently reported trials (33), administration of melanoma-specific peptides resulted in increased Ag-specific reactivity of cells from the peripheral blood of the majority of patients, but few tumor responses were seen. In contrast, administering peptide along with IL-2 had little effect on the reactivity of peripheral blood cells, but induced tumor responses in a significant fraction of the patients. The investigators suggested that IL-2 administration might cause the Ag-specific effector CTL to traffic to the tumor site, while in its absence increased numbers of CTL are still generated but are ineffective because they leave the tumor site. We have, in fact, found that IL-2 administration at the appropriate time can cause OT-I cells to remain in the peritoneal cavity in large numbers and control tumor growth for longer times than in the absence of IL-2 (P. Shrikant and M. F. Mescher, unpublished observations).

The OT-I cells that have responded to tumor and then exited the peritoneal cavity are anergic, in that they cannot respond to Ag by proliferating unless exogenous IL-2 is provided (Fig. 10). This somewhat resembles the split anergy described for cloned CD8\(^+\) T cell lines (34), where a signal 1 stimulus is sufficient to mediate cytolyis but renders the cells unresponsive to subsequent costimulation. It also resembles the anergy described for CD4\(^+\) T cells (35–37). In both of those cases, however, the cell are rendered anergic when they recognize Ag in the absence of costimulation and fail to make an initial response. In contrast, the results described here demonstrate that OT-I cells become nonresponsive following a vigorous initial response to the EG.7 tumor. This induction of anergy may not be unique to tumor Ag, since CD8\(^+\) T cell nonresponsiveness has also been observed following in vivo stimulation with peptides and superantigen (38–42). Whether activation-induced nonresponsiveness limits the ability of CD8\(^+\) T cells to control tumor growth is unclear, given that the cells are not at the site of Ag in any case.

Being able to visualize the response of Ag-specific CD8\(^+\) T cells to a tumor has provided some novel insights into the factors that lead to the immune system failing to control tumor growth. In the system studied here, this is clearly not a result of a failure of the CD8\(^+\) T cells to become activated. Rather, it is a failure of the activated cells to remain at the site of tumor growth, where they could continue to control or eliminate it. Thus, successful immunotherapy in this case would not require manipulations to initiate a response as this occurs anyway, but, rather, manipulations to sustain the response and, critically, to sustain it at the appropriate location.

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References

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