Tumor-Primed, In Vitro-Activated CD4\(^+\) Effector T Cells Establish Long-Term Memory without Exogenous Cytokine Support or Ongoing Antigen Exposure

Li-Xin Wang and Gregory E. Plautz

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Tumor-Primed, In Vitro-Activated CD4⁺ Effector T Cells Establish Long-Term Memory without Exogenous Cytokine Support or Ongoing Antigen Exposure

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T cell adoptive immunotherapy of cancer has shown promise but has not achieved its full potential (1, 2), in part, because of difficulty in achieving long-term persistence of cells that have previously been activated and numerically expanded in vitro, thereby acquiring effector properties. Clinical studies have shown an association between persistence of transferred T cells and therapeutic response, so this is a critical parameter to optimize (3–6). Many factors influence the survival of adoptively transferred T cells, including the stage of differentiation, conditions used during in vitro activation, host lymphodepletion, and exogenous cytokine support. The typical conditions of in vitro activation are designed to induce rapid proliferation and are accompanied by acquisition of phenotypic characteristics of effector cells. To maintain a highly activated functional state and to protect against apoptosis from cytokine withdrawal, several protocols involve host lymphodepletion and high-dose IL-2 administration. However, strategies to induce rapid in vitro growth or cytolytic function might not permit optimal differentiation to long-term memory cells. In a search for controllable parameters that could improve the survival of adoptively transferred T cells, several recent reports compared adoptive transfer of in vitro-activated CD8⁺ CTLs at various stages of differentiation and concluded that central memory T cells (T<sub>CM</sub>) provide superior persistence (7) and antitumor function vis-à-vis effector memory T cells (T<sub>EM</sub>) (8). In one experimental model, the source of CMV-specific CD8⁺ T cells was from primate hosts with chronic latent CMV infections (7). The other model was dependent on in vivo restimulation of transferred T cells with fowlpox vector, which occurred in lymphoid tissues, thus favoring T<sub>EM</sub> with preferential lymphoid trafficking (8). Because cancer patients have chronic exposure to tumor Ags, and dysfunctional CD8⁺ cells may require augmentation with tumor vaccines, such studies identify T<sub>CM</sub> as an important subset of T cells to activate. Particular details of various experimental models, such as the activation history of T cells or requirements for homing to lymph organs, are critical because the interactions that maintain homeostasis of immune cells are complex and differ between CD4⁺ and CD8⁺ T cells, as well as between naive and memory cells (9, 10).

We and other investigators have been interested in using experimental models to analyze the roles that CD4⁺ T cell plays in the immune response against tumors (11–13). CD4⁺ T cells provide help for CD8⁺ CTLs during Ag priming. Moreover, the establishment and maintenance of CD8⁺ memory cells are facilitated by CD4⁺ T cells (14, 15). Our recent studies indicated that tumor-reactive CD4⁺ T cells provide synergistic effector responses with CD8⁺ CTLs. Intriguingly, CD4⁺ effector T cells, without CD8⁺ cells, can even mediate regression of tumors that do not express MHC class II molecules, implicating indirect Ag presentation by tumor-associated APCs (11, 16). For our experiments, we used s.c. inoculation of a weakly immunogenic tumor into naive hosts to sensitize T cells in tumor-draining lymph nodes (TDLNs). This generates a primary immune response and among the changes associated with Ag sensitization, it is well established that responding T cells downregulate CD62L expression (17). Lymph node (LN)-resident naive T lymphocytes and T<sub>CM</sub> that are unresponsive to specific tumor Ags retain high expression of CD62L. Therefore, it is possible to segregate the tumor-sensitized T cells from irrelevant cells by enrichment of the CD62L<sup>low</sup> subset. Experiments confirmed that the CD62L<sup>low</sup> subset of TDLNs contains cells with antitumor efficacy, whereas the reciprocal CD62L<sup>high</sup> subset is devoid of activity (18, 19). Consistent with features of the primary immune response and similarly to other
TUMOR-PRIMED EFFECCTOR T CELLS ESTABLISH LONG-TERM MEMORY

investigators, we were able to activate tumor-primed T cells in vitro under conditions that induce rapid proliferation; even after 10^3-fold numerical expansion, such cells retain potent effector function (11, 20). To extend our previous studies and to determine the long-term potential of such cells to mediate antitumor responses, we transferred effector T cells to naive hosts and then challenged with tumor after an extended period. Our results indicated that adoptively transferred effector T cells could persist and acquire the phenotype and functions of TCM and TEM without ongoing exposure to Ag or exogenous cytokine support.

Materials and Methods

Animals and cell lines

Female C57BL/6N (B6, Thy1.2) mice were purchased from the biologic Testing Branch, Frederick Cancer Research and Development Center, National Cancer Institute (Frederick MD), and female B6.PL.Thy1.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a specific pathogen-free environment according to National Institutes of Health guidelines, using an Institutional Animal Care and Use Committee-approved protocol. The 3-methylcholanthrene (MCA)-induced fibrosarcomas MCA 205 and MCA 207, originally derived in B6 mice, were maintained in vivo by serial s.c. transplantation in syngeneic mice, as described previously (21). Single-cell tumor suspensions were prepared from solid tumors by enzymatic digestion (11).

Isolation and in vitro activation of tumor-sensitized C62Llow LN T cells

Mice were immunized by s.c. inoculation of 1.5×10^5 MCA 205 or MCA 207 tumor cells in the flank region bilaterally. A single-cell suspension was prepared from draining LNs 12 d later. Anti-mouse CD62L, CD4, or CD8 or anti-PE microbeads, purchased from Miltenyi Biotec (Auburn, CA), were used for negative selection of CD62Llow cells. CD62Llow/CD4+ T cells were subsequently purified by depletion of CD8+ cells, followed by positive selection with CD4 MACS beads. Alternatively, CD62Llow/CD8+ cells were purified by depletion of CD4+ cells, followed by positive selection of CD8+ cells, as previously described (11). CD62Llow/CD4+ or CD62Llow/CD8+ T cells were suspended in complete medium at 2×10^6/ml, activated with plate-bound anti-CD3 mAb (145–2C11, American Type Culture Collection, Manassas, VA) for 48 h, and cultured in complete medium supplemented with a mixture of 4 U/ml IL-2 plus 10 ng/ml recombinant mIL-7 (rIL-7) or 10 ng/ml rIL-7 plus rIL-23 (2 ng/ml) (all from R&D Systems, Minneapolis, MN). For long-term expansion, cultures were restimulated with anti-CD3 Ab for 14 h on day 21, and CD4+ cells were repurified.

FACS analysis and intracellular IFN-γ detection

FITC, CyChrome, or PE-conjugated anti-CD8, anti-TCR, anti–IFN-γ, anti-CD4, anti-CD62L, anti-CD69, anti-CD25, anti-CD44, Thy1.1, anti-Thy1.2, and isotype mAbs were purchased from BD Biosciences (San Jose, CA). PE-conjugated anti-CD127 (IL-7Rα) was purchased from eBioscience (San Diego, CA). Cell surface phenotypes were measured by direct immuno-fluorescence staining with conjugated mAbs, and stained cells were analyzed using CellQuest Software (BD Biosciences). In vitro-activated T cells were incubated with a single-cell tumor digest at a 1:1 cell ratio, stained for intracellular IFN-γ, and analyzed by FACS, as previously described (11).

Tumor challenge and in vivo expansion assay

Culture-activated CD62Llow/CD4+ or CD62Llow/CD8+ T cells derived from MCA 205 or MCA 207 TDLNs were adoptively transferred into irradiated (5 Gy) or nonirradiated B6 hosts. Mice surviving for >75 d received 3×10^5 MCA 205 or 3×10^6 MCA 207 tumor cells by tail vein injection. Mice were sacrificed on days 19–21 after tumor challenge, the lungs were insufflated with india ink, and the number of tumor nodules on the surface was enumerated.

In some experiments, Thy1.1+CD62Llow/CD4+ T cells were injected i.v into irradiated B6 (Thy1.2) hosts. At the indicated number of days after adoptive transfer, the hosts received MCA 205 or MCA 207 tumor challenge. At the designed time points following i.v. tumor injection, mice were anesthetized prior to removal of 0.5 ml blood and then euthanized for removal of spleen, lung, and LNs. A single-cell suspension was prepared by mechanical separation of spleen or LNs or enzymatic digestion for lungs. Small lymphoid cells in the lungs were isolated with Percoll (Pharmacia, Peapack, NJ) density gradient centrifugation, as described (22). Cells were stained with the indicated FITC, CyChrome, or PE-conjugated Abs, and phenotypes were analyzed by FACS. The percentage of donor Thy1.1+ T cells in the lungs, spleen, LNs, and blood was determined by FACS assay; the number of memory CD4+ T cells was also calculated by multiplying the total lymphoid cell count by their percentage.

Isolation and culture activation of CD4+ TCM or CD4+ TEM T cells

Spleens were removed from recipients of Thy1.1+CD62Llow/CD4+ T cells at the indicated time points. A single-cell suspension was incubated with Thy1.2 microbeads and applied to MACS columns (Miltenyi Biotec) to delete host (Thy1.2) cells. The flow-through fraction was incubated with anti-CD62L microbeads to positively isolate CD62Llow cells (TCM), and the flow-through fraction contained the CD62Llow T cell subset (TEM). After anti-CD3 activation for 48 h, Thy1.1+ cells were positively selected on a MACS column. Thy1.1+ CD62Llow or CD62Lhigh subsets were cultured in IL-2 plus IL-7, as described above. Thy1.1 purification in each subset was repeated on days 7 and 14, and CD8+ T cells were depleted on day 14. After two cycles of stimulation with anti-CD3/IL-2/IL-7, expanded Thy1.1+ memory T cell subsets were used for adoptive immunotherapy.

Adoptive immunotherapy

Pulmonary metastases were established by i.v. inoculation of 3×10^5 MCA 205 tumor cells suspended in 1.0 ml HBSS. Three days later, mice were treated with the indicated number of purified CD4+ TCM or CD4+ TEM cells by i.v. injection. Mice were sacrificed on days 19–21 after tumor inoculation, the lungs were harvested, and tumor nodules on the surface were enumerated, as described above.

Statistical analysis

The significance of differences between groups was analyzed by the two-tailed Student t test or by the Wilcoxon rank-sum test. A two-tailed p value <0.05 was considered significant.

Results

Adoptive transfer of tumor-reactive effector T cells into a tumor-free host establishes memory

We previously demonstrated that LNs draining s.c. tumors contain tumor-sensitized T cells and that this response peaks 9–12 d after tumor inoculation. The kinetics of this response in previously tumor-naive animals, coupled with the phenotype of the sensitized T cells (CD62Llow), are consistent with recently Ag-stimulated T cells. We harvested MCA 205 TDLNs 12 d after inoculation and depleted CD62Lhigh cells, obtaining 14% of the original cell number. The CD4+ or CD8+ subsets were additionally purified by depletion of the reciprocal subset, followed by positive selection. Although the multiple selection steps decreased the final yield of CD8+ cells to only 4% of the initial CD62Llow subset and the CD4+ subset to 14% of initial cells, the purity was high. The CD4+ and CD8+ T cell subsets were separately activated with anti-CD3 mAb for 48 h and again overnight on day 21. T cells were cultured in medium supplemented with IL-2 plus IL-7 or IL-7 plus IL-23 for a total of 29 d. The CD4+ cultures required an additional depletion of residual CD8+ T cells, because the CD8+ cells are capable of more rapid proliferation (23). However, at the time of adoptive transfer, the CD4+ or CD8+ cultures were >95% pure, with <1% of the reciprocal population, and they had proliferated >1000-fold. Table I shows the overall proliferation of each T cell culture and its phenotype. In addition, the T cells were tested for IFN-γ production when stimulated with a single-cell digest of tumors, which contains numerous infiltrating MHC-II+ APCs that can activate CD4+ cells. As previously observed, CD4+ or CD8+ cells cultured with IL-7 plus IL-23 displayed greater production of IFN-γ than did the T cells cultured in IL-2 plus IL-7 (20). Similar to our previous experiments, the cultured T cells did not produce IFN-γ spontaneously or in response to a distinct tumor cell line MCA 207, but they were highly polarized to produce IFN-γ in response to anti-CD3 stimulation.

The T cells were adoptively transferred to naive hosts, conditioned with 5 Gy of total body irradiation to achieve lymphodepletion, as well as nonirradiated hosts. Notably, recipient mice were

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not treated with exogenous cytokines. The mice were challenged 75 d later with i.v. MCA 205 tumor cells, which embolize in the pulmonary vasculature and induce lung metastases. Recipients were sacrificed 20 d later, and the number of lung metastases was determined. As demonstrated in Fig. 1, a dose of 10^7 CD4+ cells, cultured with IL-2 plus IL-7 cytokine support in vitro, protected hosts from tumor challenge. Host lymphodepletion was not required, because protection was effective in nonirradiated as well as irradiated hosts. This is interesting, because many T cell adoptive immunotherapy strategies are postulated to require profound lymphodepletion, as well as exogenous IL-2, to support lymphocyte viability (24–26). Fig. 1 also demonstrates that CD8+ cells cultured with IL-2 plus IL-7 were less effective per cell than similarly prepared CD4+ cells. IL-23 supplementation during in vitro culture with IL-2 plus IL-7 were less effective per cell than similarly pre-

<table>
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<th>IFN-γ (%) Among CD4+ or CD8+ Cells</th>
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CD62Llow T cells were isolated from TDLNs, and CD4+ or CD8+ subsets were additionally purified and activated with anti-

Effector T cells progressively decrease in frequency and acquire the phenotype of memory T cells

We prepared MCA 205 TDLNs in Thy 1.1+ hosts and, after 29 d of in vitro stimulation, 4 × 10^7 activated CD4+ T cells were transferred to congenic Thy 1.2+ hosts to track their frequency and phenotype in lymph tissues. We observed that after 82 d, spleen contained 2% Thy 1.1+ cells that were CD44+, CD44high and that had heterogeneous expression of CD62L, with ~50% of cells expressing high levels (Fig. 2A). In LNs, the frequency of transferred T cells was lower (0.3%); however, 80% of these cells were CD44high, CD62Lhigh. By day 143 (Fig. 2B), the frequency of Thy 1.1+ cells had declined to 0.2% in spleen, 0.12% in LNs, and 0.3% in blood, but the relative frequency of CD62Lhigh cells was similar, with 50% in spleen, 80% in LNs, and 66% in blood. Interestingly, lung tissue contained far fewer T lymphocytes, but 1% of them were the transferred Thy 1.1+ cells, among which 90% were CD62Llow, in contrast to lymph tissues. These findings are consistent with a preferential distribution of cells with TEM in peripheral tissues distinct from TCM in LNs, with spleen containing a mixed population, reflecting contributions from white pulp and red pulp compartments.

**FIGURE 1.** Adoptively transferred CD4+ or CD8+ effector T cells establish memory. Purified CD62Llow CD4+ or CD8+ T cells derived from MCA 205 TDLNs were culture activated with anti-CD3/IL-2/IL-7 or anti-

**FIGURE 2.** CD62Llow/CD4+ effector T cells differentiate into TEM and TCM in vivo. Purified Thy1.1+/CD62Llow+CD4+ T cells were activated in vitro, and 4 × 10^7 cells were adoptively transferred to irradiated tumor-free Thy 1.2+ congenic mice. A, The percentage of Thy 1.1+ cells in the spleen and LNs was determined on day 82 posttransfer. Among donor T cells, 100% were CD44high, and >95% were CD4+, with high expression of CD62L on 50.2% in spleen or 81.3% in LN. B, On day 143 posttransfer, Thy 1.1+ T cells were analyzed for CD62L expression in spleen, LNs, blood, and lung. The numbers represented the percentage of donor cells in the dot-plot quadrants. Each experiment included two mice.
Memory T cells convert to an activated phenotype and proliferate upon tumor challenge

One of the hallmarks of memory T cells is their ability to undergo rapid proliferation upon re-encounter with Ag. To monitor the fate of the transferred effector T cells, we harvested TDLNs from Thy 1.1+ hosts inoculated with MCA 205 or MCA 207 tumors 12 d earlier. The CD62L\text{low}/CD4+ subset was activated in vitro for 29 d, and 4 × 10^7 CD4+ effector T cells were adoptively transferred to congenic Thy 1.2+ hosts. Approximately 9 mo later (273 d), the mice were challenged with i.v. tumor cells to establish lung metastases, and the number and phenotype of Thy 1.1+ cells were determined at several time points. As demonstrated in Fig. 3A, recipients had detectable, but very low, numbers of Thy 1.1+ T cells in lung, spleen, LN, and blood prior to challenge. There was an increase in spleen Thy 1.1+ cells after tumor challenge; however, there was a dramatic increase in the number of Thy 1.1+ cells isolated from lung on day 7, which subsequently declined but remained substantially above background levels 32 d after tumor challenge. A similar experiment using a distinct tumor (MCA 207) reproduced the pattern of rapid T cell expansion in lung tissue 7 d after tumor challenge (Fig. 3B). In addition to their vigorous proliferation, the memory T cells prevented the appearance of tumor at each time point, indicating their effector function.

On day 273 following adoptive transfer and immediately prior to tumor challenge, the phenotype of the Thy 1.1+ T cells in the lung was exclusively CD44\text{high}, CD62L\text{low}, CD127+, without expression of CD25 or CD69, consistent with a tissue-resident TEM phenotype (Fig. 4, day 0). Three days after seeding of lung metastases, there was a dramatic increase in CD69 expression on Thy 1.1+ T cells. CD127 showed transient downregulation, with different kinetics and maximal change on day 7 posttumor challenge. Expression of CD62L remained low on pulmonary T cells throughout the period of tumor rejection. In contrast, Thy 1.1+ T cells in the spleen prior to tumor challenge showed heterogeneous expression of CD62L, high levels of expression of CD44 and CD127, and the absence of activation markers, such as CD25 or CD69. Interestingly, 50% of the T cells re-expressed high levels of CD62L, consistent with a TCM phenotype. There was a different response among splenic T cells to tumor challenge, with a decrease in the proportion of CD62L\text{high} TCM between days 3–18, followed by a return to the basal level on day 32. Unlike pulmonary T cells, the splenic T cells displayed minimal expression of CD69, indicating that Ag stimulation was principally occurring in lung and bronchial lymph tissue.

**T_{CM} and T_{EM} populations can be reactivated in vitro and convert to effector function**

Despite activation and proliferation of Thy 1.1+ CD4+ T cells within lung tissue after tumor challenge, it is possible that the principal function of adoptively transferred cells is to provide helper functions during sensitization of host Thy 1.2+ CD8+ T cells. In addition, the in vivo tumor-challenge experiments did not identify whether the effector T cells for tumor rejection arose from T_{EM} resident in the lung and circulation or recruitment of T_{CM} from specialized lymph tissue. To explore the capability of T_{CM} to differentiate again into secondary-response effector T cells, we separated spleen T cells, based on CD62L expression, into two populations on day 276 after adoptive transfer. As demonstrated in Fig. 5A, this process effectively segregated the Thy 1.1+ cells into CD62L\text{high} and CD62L\text{low} fractions. For these experiments, it was necessary to deplete host Thy 1.2 T cells two additional times to yield a pure population of Thy 1.1+ cells. This is because the in vitro activation uses anti-CD3 mAb, which is an Ag-independent signal that can also stimulate host Thy 1.2+ T cells (Fig. 5B). Notably, although the T_{CM} subset was initially selected as CD62L\text{high}, these cells converted to a CD62L\text{low} phenotype during in vitro activation. Two additional distinguishing features became apparent during culture activation. First, there was a 1000-fold proliferation of the T_{EM} subset in contrast to a 190-fold proliferation of the T_{CM}, suggesting a more rapid entry into cell cycle following in vitro activation. Second, although the initially transferred Thy 1.1+ T cells were <1% CD8+, there emerged a subset (5%) of CD8+ Thy 1.1+ T cells, but only in the T_{CM} cultures.

The T_{EM} and T_{CM} activated T cells were able to mediate regression of established pulmonary metastases upon adoptive transfer (Fig. 6A). In addition, both populations displayed similar frequencies of T cells that could produce IFN-γ, specifically in response to MCA205 tumor digest (Fig. 6B). An independent experiment of similar design performed 285 d after the initial adoptive transfer of T cells also showed augmented proliferation of T_{EM} compared with T_{CM}, as well as a small proportion of CD8+ cells arising in the T_{CM} in vitro-activated cells and a similar proportion of IFN-γ–producing cells in the T_{EM} and T_{CM} cultures. The therapeutic efficacy of the T_{CM} cultures was moderately better than the T_{EM} cultures in this second experiment, but T_{EM} and T_{CM} were fully competent to undergo reactivation and acquire secondary effector function.

**Discussion**

Despite the theoretical appeal of harnessing the adaptive immune response for cancer therapy, there are significant barriers to its effective and routine clinical use. First, the extant immune response in patients with progressive metastatic disease is, by definition, inadequate. Recent elucidation of the process of tumor immunoeediting, as well as the complex regulation of the immune response to self-Ags, suggests that high-avidity TCR responses may be subverted during tumor development (27–29). Lower-avidity T cells may receive inadequate stimulation during priming to generate a robust and sustained response. Moreover, chronic persistence of tumor Ags may render tumor-reactive T cells in a state of exhaustion, much as in chronic viral infections (30). These features of
CD4+ T cells were in a resting state, CD62L was expressed on cells. Open graphs represent isotype control. On days 0 and 32, when CD8+ T cells were depleted on day 14. At the completion of the in vitro posttumor challenge. Graphs are gated on Thy1.1+/CD4+ double-positive tumor cells. Lungs and spleen were harvested at the indicated time points from tumor-free hosts. On day 273, mice were challenged by i.v. injection of MCA 205 tumor cells and their subsequent capacity to replicate and provide effector function are similar. Thy1.1+/CD4+ memory

FIGURE 5. Isolation and in vitro activation of Thy1.1+/CD4+ memory cells. A. Spleens were harvested 276 d after adoptive transfer of 4x10^7 Thy1.1+/CD62L^low/CD4+ effector T cells to tumor-free Thy1.2 mice. Thy1.2+ cells were depleted, and the negative fraction was separated into TCM (CD62L^high) and TEM (CD62L^low) subsets with MACS columns before culture activation. The percentage of Thy1.1+ TEM and TCM is indicated to the right of the dot-plot quadrants. B. On days 2, 7, and 14 of culture, Thy1.1+ cells in each subset were further purified using MACS columns; CD8+ T cells were depleted on day 14. At the completion of the in vitro activation with anti-CD3/IL-2/IL-7, cultures originally derived from TCM or TEM were analyzed for purity and phenotype.

FIGURE 6. Reactivated TEM and TCM CD4+ cells have similar therapeutic efficacy. Thy1.1+ TCM or TEM cells isolated 276 d after adoptive transfer to tumor-free hosts were activated in vitro with anti-CD3/IL-2/IL-7. A. The indicated number of cells initially derived from TCM or TEM was adoptively transferred to hosts bearing 3-d MCA 205 pulmonary metastases. On day 20, lungs were harvested, and tumors were enumerated. Transfer of 5x10^6 TCM or TEM cells effectively eradicated pulmonary metastases (p < 0.01 compared with control group). There was no significant difference between the TCM and TEM groups at the two doses. B. Culture-activated TCM or TEM cells were incubated alone (no stimulation), with a single-cell MCA 205 or MCA 207 tumor digest, or with anti-CD3 and stained for intracellular IFN-γ. A similar percentage of TCM or TEM produced IFN-γ in response to MCA 205 stimulation (13% versus 14%).

The dampened immune response in cancer patients might account for the difficulty in obtaining objective clinical responses with cancer vaccines, despite stimulating measurable immunologic responses and increases in precursor frequency.

Suboptimal conditions for active immunotherapy have rekindled interest in adoptive T cell transfer, which has advantages of in vitro generation of T cells with defined Ag reactivity and strict control over conditions of activation (31). Cell extrinsic regulatory mechanisms can, thereby, be bypassed. Moreover, the strength and duration of TCR/CD3 stimulation, as well as the composition and concentration of exogenous cytokines, can be optimized for growth and long-term survival of effector T cells. Additionally, conditions for competitive survival of transferred T cells can be improved through host lymphodepletion, or even myeloablation and hematopoietic stem cell rescue, combined with exogenous high-dose cytokine support (24, 25). However, intensive concomitant treatments have their own inherent toxicity, and limits on host tolerability may eventually outweigh gains to T cell survival. It would be advantageous to have a source of T cells that were activated under conditions that support effector function and preserve long-term persistence by virtue of intrinsic epigenetic and metabolic properties, rather than extrinsic manipulations.

Our data indicated that tumor-reactive T cells could be activated in vitro under conditions that promote their numerical expansion and acquisition of effector phenotype and function but that also preserve their capacity to subsequently differentiate into TEM and TCM. This linear differentiation of CD4+ effector cells to memory cells and their subsequent capacity to replicate and provide effector function are consistent with data from CD8+ effector cells (32). Interestingly, this differentiation did not require host lymphodepletion or extrinsic cytokine support after adoptive transfer. Consistent with well-established models of CD4+ T cell differentiation, it also did not require Ag restimulation in vivo (33).

There are several aspects of our experimental model that likely contributed to the emergence of long-term memory cells from effector T cells. First, T cells were obtained from healthy naive hosts at the peak of the priming response. At first pass, this does not seem applicable to clinical situations. However, it suggests that MHC-compatible normal donors, as used in this experimental model, might prove to be a superior source for tumor-reactive T cells. This is partly because the repertoire of tumor-reactive CD4+ and CD8+ T cell is intact, but more importantly, the T cells can be selected and manipulated during the primary immune response, before they have been functionally impaired by a suboptimal priming event. Using transgenic CD4+ T cells specific for a lymphocytic choriomeningitis virus epitope, it was recently demonstrated that diminished strength of the primary sensitization did not influence the extent of expansion or effector function, but it had a profound effect, preventing differentiation to memory cells (34). Interestingly, transgenic CD4+...
cells that were effectively primed by lymphocytic choriomeningitis virus infection and became memory cells did not have a defective response during secondary stimulation. In our experiments, the strength of TCR/CD3 signaling could be controlled in vitro with the anti-CD3 mAb to provide full activation, which does not require pre-existing knowledge of the Ag(s). The duration of TCR signaling is also important for programming the developmental pathways of CD4+ and CD8+ T cells, and this is another parameter that can be controlled in vitro (35, 36).

The provision of cytokines to T cell cultures during in vitro activation provided for numerical expansion and may have contributed to the development of long-term persistence after adoptive transfer. The initial proliferative signal was delivered with immobilized anti-CD3 mAb for 48 h at a high-density of T cells. Then T cells were removed from the stimulus, washed, and diluted to a low concentration with cytokine supplementation. T cells underwent rapid expansion over the subsequent 7 d in the presence of low concentrations of IL-2 (4 U/ml), IL-7 (10 ng/ml), or IL-23 (2 ng/ml). In CD4+ cells, we observed equivalent proliferation over the first 9 d in the presence of IL-7, with or without IL-2, suggesting a cell-intrinsic source. IL-23 alone does not augment the intrinsic proliferative signal. Subsequently, CD4+ T cells in the absence of a proliferative signal. Subsequently, CD4+ T cells in the absence of IL-7 decline in number. In contrast, when the cultures are maintained with IL-7, they convert to a low-proliferative state, and their viability is sustained until day 23, when they are again stimulated with anti-CD3 mAb and undergo another proliferative burst. It is possible that periodic strong TCR/CD3 stimulation, with a subsequent period of quiescence after the proliferative phase is completed, permits the acquisition of properties that enhance memory cell formation. Intriguing recent data using Bim-/- OT-1 transgenic mice indicated that cell-intrinsic factors, rather than competition for limited cytokine pools, regulate the contraction phase of effector CD8+ T cells and that all effector T cells have the capacity to become fully functional memory cells (37). It seems that a shift in the metabolic state of T cells regulates conversion from effector to memory status. In particular, modulation of the mTORC1 complex and a change from an anabolic glycolytic metabolism to a catabolic metabolism underlie the differentiation from effector to memory status (38). Although the proximal signals that regulate this conversion have not been defined, it is anticipated that such signals could be optimized during in vitro culture activation. Provision of high-dose exogenous cytokines may have a paradoxically negative effect on effector T cell survival following adoptive transfer if it hinders metabolic transition to a memory state.

Our experiments focused on CD4+ T cells because we have an interest in gaining a greater understanding of the mechanism by which they eliminate tumors that are MHC class II+. Tumor Ags are indirectly presented to CD4+ T cells on MHC class II+ APCS that infiltrate tumors, and this provides sufficient restimulation to initiate a process of tumor rejection. Presumably, tumor cell death occurs through cytokine release and activation of tumoricidal function in tumor-associated macrophages. Our focus on CD4+ T cells does not ignore the importance of CD8+ T cells, and the data in Fig. 1 indicate that they too were able to persist and provide memory function. Interestingly, the conditions of in vitro activation provide for more rapid proliferation of CD8+ T cells and, even in the CD4+ cultures, a small number of CD8+ T cells (<1%) that were adoptively transferred established themselves within the long-term TCM pool (Fig. 2). This is consistent with the stable persistence of TCM CD8+ T cells and the declining persistence of CD4+ TCM T cells specific to viral Ags (39). When CD8+ TCM were activated in vitro, they again proliferated more rapidly than CD4+ T cells to become a 5% presence within cultured TCM cells. Although we were not specifically examining the long-term response of the CD8+ TCM, it is evident that they survived adequately in the presence of CD4+ TCM. Because tumor-specific CD4+ T cells can provide synergistic antitumor effects to CD8+ effector cells in the period shortly after adoptive transfer, adapting in vitro activation conditions to optimize CD4+ T cell growth is important. The current experiments indicated that effector CD4+ T cells transition to become memory cells and their capacity to provide persistent help to CD8+ memory cells should augment the overall efficacy of adoptive immunotherapy for cancer.

Disclosures
The authors have no financial conflicts of interest.

References


