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Memory T Cells Originate from Adoptively Transferred Effectors and Reconstituting Host Cells after Sequential Lymphodepletion and Adoptive Immunotherapy

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Adoptive transfer of tumor-specific effector T cells induces regression of advanced tumors and induces a long term memory response; however, the origin of this response has not been clearly defined. In this study Thy1.2+ mice bearing advanced MCA-205 tumors were treated with sublethal total body irradiation, followed by adoptive transfer of congenic Thy1.1+ T cells that had been sensitized to tumor in vivo and then activated ex vivo with anti-CD3, IL-2, and IL-7. Splenocytes were recovered >140 days after the initial therapy, and the L-selectinlow memory cell subset was separated into host Thy1.2+ and transferred Thy1.1+ cells and restimulated ex vivo. Both adoptively transferred Thy1.1+ cells as well as reconstituted host Thy1.2+ cells could specifically eliminate MCA-205 pulmonary metastases. Interestingly, hosts with partial responses followed by tumor recurrence nevertheless harbored memory cells that could be isolated and numerically amplified ex vivo to regenerate potent effector function. Memory cells were recovered after adoptive transfer into lymphodepleted nontumor-bearing hosts, indicating that they were not dependent on continued Ag exposure. These experiments establish that rapid ex vivo expansion of tumor Ag-primed T cells does not abrogate their capacity to become long-lived memory cells. Moreover, immune-mediated tumor regression coincident with lymphoid reconstitution produces another wave of host memory cells. These data suggest an approach to rescuing antitumor immune function even in hosts with long-standing progressive tumor through restorative ex vivo activation. The Journal of Immunology, 2004, 172: 3462–3468.

T cell memory is a crucial feature of the immune response to exogenous pathogens. The increased precursor frequency, heightened sensitivity to antigenic stimulation, accelerated kinetics of proliferation, and enhanced trafficking to tissues augment host protection upon exposure to previously experienced pathogens (1, 2). The selective pressure imposed by infectious pathogens has molded the features of the memory response, and its potency underpins the success of vaccination against viral diseases. Unfortunately, it has been difficult to effectively use the immune memory response for cancer therapy. Prophylactic vaccination for cancer is impractical because the shared tumor Ags defined to date are constituents of normal tissue (3, 4). Antigenic targets in commonly subverted signaling pathways, such as receptor tyrosine kinases and their downstream targets, are also shared with normal proliferating cells. Thus, it would be hard to maintain effective antitumor prophylaxis without the potential risk of adverse effects. An added difficulty arises with strategies for vaccination of patients with established tumors, because presentation of tumor Ags typically induces tolerance among T cells with the potential to recognize tumor Ags (5, 6). In addition, tumors lack the pathogen-associated molecular patterns that contribute to a robust immune response through activation of Toll-like receptors (7). As a consequence, the extant T cells detected with tumor Ag/MHC tetramer probes have functional defects (8, 9). Strategies to reverse tolerance through active immunotherapy have been partially successful, in that they are able to boost the frequency of tumor-reactive T cells. However, this has not translated into effective therapy of established metastatic disease, which remains the unsolved, clinically relevant problem.

We have used adoptive transfer of T cells obtained from mice with progressively growing tumors to define the requirements for optimal ex vivo activation of Ag-primed pre-effector T cells and to elucidate the mechanism of tumor regression. The Ag-primed T cells reside within the L-selectinlow subset in tumor-draining lymph nodes (LN)3 and are at a peak level between 9 and 12 days after tumor inoculation (10). The preferential trafficking of naïve T cells through LNs and the short time period between tumor inoculation and T cell isolation are consistent with L-selectin down-regulation as a marker of recent Ag exposure (11, 12). Tumor sensitization alone, however, is insufficient to retard tumor growth in the primary host, and adoptive transfer of primed LN T cells does not mediate regression of even minimal tumor burdens in secondary recipients (13). This state of functional tolerance is reversible through ex vivo stimulation with anti-CD3 mAb and IL-2, which induces rapid proliferation, acquisition of Ag-specific IFN-γ production, and in vivo therapeutic efficacy against established tumor (14). Mice cured of established tumors by adoptively transferred effector T cells exhibit long term memory against tumor rechallenge, but the relative contributions of transferred cells, host cells, or Ab responses have not yet been defined.

Our previous studies demonstrated that although the tumors express only MHC class I molecules, adoptive transfer of either CD4+ or CD8+ T cells alone is sufficient to mediate tumor regression (15, 16). Indeed, cross-presentation of specific tumor Ags

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3 Abbreviations used in this paper: LN, lymph node; CM, complete medium; TBI, total body irradiation; ACK, ammonium chloride.

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to effector T cells by bone marrow-derived APC within the tumor plays a key role in regression (17, 18). This raised the question of whether the process of immune-mediated tumor regression leads to the generation of a second wave of memory cells from host T cells. In this study we demonstrate that transferred effector T cells as well as host T cells regenerating in the context of immune tumor regression each contribute to the memory response.

Materials and Methods

Mice and tumors

Female C57BL6N (B6) mice were purchased from the Biologic Testing Branch, National Cancer Institute (Frederick MD), and female B6.PL Thy1.1 mice were purchased from The Jackson Laboratory (Bar Harbor ME). They were maintained in a specific pathogen-free environment according to National Institutes of Health guidelines using an Institutional Animal Care and Use Committee approved protocol. The MCA-205 and MCA-207 fibrosarcomas, syngeneic to B6 mice, were serially passaged in vivo s.c. as described previously (19). Single-cell suspensions were prepared from minced s.c. tumor by enzymatic digestion for 3 h at room temperature with 0.1% collagenase type IV, 0.01% DNase I, and 2.4 U/ml hyaluronidase type V (Sigma-Aldrich, St. Louis, MO) in HBSS.

Preparation and culture activation of tumor-sensitized CD62Llow cells

Tumors were established in Thy1.1 mice by s.c. flank inoculation of 1.5 × 106 MCA-205 cells. Twelve days after inoculation, the tumor-draining inguinal LNs and spleens were removed and mechanically disrupted to obtain a single-cell suspension. Splenocytes were subjected to ammonium chloride (ACK) lysis of erythrocytes. The lymphoid cells were incubated with 100 μl of anti-CD62L microbeads/107 cells and applied to MACS columns (Miltenyi Biotech, Auburn, CA), and the flow-through fraction was collected as previously described (20). CD62Llow cells, containing <50% TCR-positive and 50% B220-positive subsets, were suspended in complete medium (CM) and incubated for 2 days at 4 × 107/well in 24-well culture plates coated with anti-CD3 (145-2C11). Activated cells were washed, counted, and suspended at 0.5 × 107/ml in CM with recombinant human IL-2 (2 U/ml) and recombinant mouse IL-7 (10 ng/ml; R&D Systems, Minneapolis, MN) and subsequently adjusted to 107/ml on day 5 of activation.

Isolation and activation culture of memory T cells

Splenocytes were prepared from recipients of activated Thy1.1 cells at 94 days after transfer for mice with persistent tumor. 144 days for cured mice, or 208 days for nontumor-bearing mice. ACK lysis and separation of CD62Llow cells are described above. The CD62Llow cells were then incubated with Thy1.2 microbeads and applied to MACS columns (Miltenyi Biotech) to separate the Thy1.2low cells. The flow-through fraction was incubated with PE-conjugated anti-CD90.1 (Thy1.1; BD Biosciences, Minneapolis, MN) and subsequently adjusted to 107/ml on day 5 of activation.

IFN-γ and FACS analysis

T cells were stimulated with a single-cell suspension of either MCA-205 or MCA-207 tumors at a 1:1 ratio or with immobilized anti-CD3. Brefeldin A was added at 5 h, and the cells were harvested at 14 h and stained for intracellular IFN-γ according to the manufacturer’s instructions (BD Biosciences). Abs for phenotype analysis were obtained from BD Biosciences and were used according to the manufacturer’s instructions. Cells were analyzed on a FACS using CellQuest software (BD Biosciences).

Adoptive immunotherapy

Mice were inoculated with MCA-205 or MCA-207 tumor cells (3 × 106) i.v. to establish pulmonary metastases. Subcutaneous tumors were established by inoculation of 1.5 × 106 cells. Mice bearing 10-day s.c. tumors were treated with 5 Gy of total body irradiation (TBI) delivered from a 137Cs irradiator (J. L. Shepard, San Fernando CA) before i.v. transfer of T cells, whereas mice with pulmonary tumors were not irradiated. For pulmonary tumors, mice were sacrificed on day 20, the lungs were inflated with India ink, and the number of surface tumor nodules was enumerated using a dissecting microscope. Subcutaneous tumors were measured in two perpendicular dimensions three times per week, and mice with progressive tumors were sacrificed when the product of dimensions exceeded 200 mm2.

Statistical analysis

Treatment groups consisted of five individuals. Analysis of bidimensional s.c. tumor size was performed by the Mann-Whitney rank sum test. For pulmonary tumors, a t test was performed on paired samples, and p < 0.05 was considered significant.

Results

Adoptive transfer of memory T cells mediates tumor regression

A constant feature of successful T cell adoptive immunotherapy is the development of long term memory, manifested as protection against tumor challenge. However, protection does not clearly define the requirements for cellular or humoral components, nor does it indicate whether the donor or host cells are critical. We harvested spleens from 10 naive mice and 10 cured mice, 70 days after successful T cell adoptive immunotherapy of 10-day s.c. MCA-205 tumors. The cured mice had no visible evidence of tumor at the original inoculation site or in other organs. The splenocytes were treated with ACK to eliminate erythrocytes and washed, and 7 × 106 cells were immediately transferred to hosts bearing 3-day MCA-205 pulmonary metastases. Whereas control mice and mice treated with naive splenocytes had >250 pulmonary metastases, the recipients of splenocytes from cured donors had no metastases (p < 0.01). This indicates that a cellular component of spleens from cured mice could function without the requirement for ex vivo culture activation. This characteristic is similar to that of memory splenocytes derived from hyperimmune mice (21).
three of five mice in each treatment group achieving complete tumor regression, but was statistically significant. In addition, the onset of tumor regression required several days, but was completed by day 33 in cured mice, similar to previous observations using hyperexpanded effector T cells (20).

Memory cells arise from reconstituting host T cells as well as transferred effector T cells

Mice achieving complete tumor regression were observed for 144 days, and at the time of sacrifice no detectable tumor was present at the inoculation site or in other organs. L-selectinlow memory cells (26% of total splenocytes) were isolated from spleen and were further segregated into subsets of Thy1.2+/H11001 (10% of initial cell yield) and Thy1.1+ (2.8% of initial cell yield). A substantial number of Thy1.1 and Thy1.2 double-negative cells was initially present in the Thy1.1 culture, but did not proliferate, whereas Thy1.1+ cells rapidly expanded to predominate the culture. After activation with anti-CD3 and culture in IL-2/IL-7-containing medium for 15 days, there was 82-fold amplification in the Thy1.1+ cultures and 95-fold expansion in the Thy1.2+ cultures. The stimulated T cells were subjected to MACS selection to remove low level cross-contamination resulting in >99% purity (Fig. 3A). There was a marked difference in the composition of the cultures, with the adoptively transferred Thy1.1 cells consisting of >90% CD4 and 6% CD8 cells that were L-selectinlow and TCR+.

Antitumor reactivity of memory cells

To determine the frequency of responding tumor-reactive T cells among the transferred or host memory cells they were incubated with MCA-205 tumor and assayed for intracellular IFN-γ production. Although there was minimal production of IFN-γ in the absence of stimulation, the majority of the Thy1.1+ subset produced IFN-γ specifically in response to MCA-205 tumor stimulation, but was nonreactive against the MCA-207 tumor (Fig. 4A). Similarly, the Thy1.2+ cells had minimal spontaneous IFN-γ production, but CD4 and CD8 cells reacted specifically against MCA-205 (Fig. 4B).

Days Post Tumor Inoculation

FIGURE 2. Therapy of advanced s.c. MCA-205 tumors with activated Thy 1.1 effector cells. Mice (n = 5/group) bearing 10-day tumors were treated with 5 Gy of TBI, followed by no cell transfer (A), 1.5 × 10⁷ activated LN effector cells (B), 3 × 10⁷ activated LN effector cells (C), or 5 × 10⁷ activated spleen effector cells (D). Tumor size was measured in two perpendicular dimensions and is plotted for individual mice. Two mice in group D experienced slowly progressive tumor growth and were sacrificed on day 94, with the final tumor size indicated. Significant differences in tumor size were observed (A vs B, p = 0.015; A vs C, p = < 0.01; A vs D, p < 0.01).
Thy1.2 subsets were isolated, culture-activated, and analyzed by FACS. Thy1.1 subsets were isolated, culture-activated, and analyzed by FACS for expression of CD4, CD8, TCR. At the completion of 15-day ex vivo expansion, Thy1.1 or Thy1.2 subsets were gated into Thy1.1 or Thy1.2 subsets before culture activation. At the time of sacrifice, the s.c. tumors were 188 and 149 mm², but there was no detectable metastatic disease similar to numerous other observations with host bearing advanced s.c. MCA-205 tumors (Fig. 2D). Despite the large s.c. tumor burden, the spleens were hyperplastic with 164 × 10⁶ cells per spleen. The L-selectinlow memory subset comprised 25% of the total splenocytes and was additionally segregated into Thy1.2+ (6.25% of initial cell yield) and Thy1.1+ (1.2% of initial cell yield). Isolated T cells were stimulated with anti-CD3 mAb and cultured in medium containing IL-2 and IL-7. T cells responded with vigorous proliferation over 16 days. As demonstrated in Fig. 3B, CD4 T cells again remained as the majority of the Thy1.1 cultures, but the ratio of CD4:CD8 cells was 2:1, unlike the highly skewed CD4 predominance in the cured mice. The ratio of CD4:CD8 in the host Thy1.2 cells was similar to the Thy1.1 cells with a 2:1 predominance. Again, the cultures were overwhelmingly TCR+ and CD62Llow. Thy1.1 or Thy1.2 T cells were purified to >99% homogeneity before adoptive transfer to hosts bearing 3-day lung metastases. As shown in Fig. 5C, a dose of 10⁷ Thy1.1 cells was curative, and 2 × 10⁶ cells nearly completely eliminated metastases. This indicates that the transferred tumor-reactive T cells were not exhausted by prolonged exposure to Ag or tumor progression. Although the T cells were not effective in the tumor-bearing host, potent ex vivo activation signals could restore full effector function.

Long term persistence of effector cells in nontumor-bearing recipients
Thy1.1 effector T cells (1.5 × 10⁷) were transferred into 5 Gy of TBI-conditioned recipients that were nontumor bearing. The spleens were harvested 208 days later, and the L-selectinlow cells were fractionated as described above into the Thy1.1+ and Thy1.2+ subsets and culture-activated as previously described. The long term survival and ex vivo proliferation of CD4 and CD8 cells was equivalent in Thy1.1 effector cells that had been parked for 2 weeks. Thy1.1 cells demonstrated higher therapeutic activity on a per cell basis than Thy1.2 cells at a lower cell dose (2 × 10⁶). This may reflect the presence of a higher percentage of memory cells with irrelevant Ag specificity among the host T cells.

Hosts with progressive tumor contain T cells that can be restored to effector function
Two recipients of adoptively transferred Thy1.1 effector cells developed incomplete tumor regression, followed by tumor growth that was much slower than in naive mice, but nonetheless progressive. Spleens were removed 94 days after adoptive transfer of Thy1.1 effector T cells to determine whether tumor progression resulted from depletion vs anergy of transferred immune cells. At the time of sacrifice, the s.c. tumors were 188 and 149 mm², but there was no detectable metastatic disease similar to numerous other observations with host bearing advanced s.c. MCA-205 tumors (Fig. 2D). Despite the large s.c. tumor burden, the spleens were hyperplastic with 164 × 10⁶ cells per spleen. The L-selectinlow memory subset comprised 25% of the total splenocytes and was additionally segregated into Thy1.2+ (6.25% of initial cell yield) and Thy1.1+ (1.2% of initial cell yield). Isolated T cells were stimulated with anti-CD3 mAb and cultured in medium containing IL-2 and IL-7. T cells responded with vigorous proliferation over 16 days. As demonstrated in Fig. 3B, CD4 T cells again comprised the majority of the Thy1.1 cultures, but the ratio of CD4:CD8 cells was 2:1, unlike the highly skewed CD4 predominance in the cured mice. The ratio of CD4:CD8 in the host Thy1.2 cells was similar to the Thy1.1 cells with a 2:1 predominance. Again, the cultures were overwhelmingly TCR+ and CD62Llow. Thy1.1 or Thy1.2 T cells were purified to >99% homogeneity before adoptive transfer to hosts bearing 3-day lung metastases. As shown in Fig. 5C, a dose of 10⁷ Thy1.1 cells was curative, and 2 × 10⁶ cells nearly completely eliminated metastases. This indicates that the transferred tumor-reactive T cells were not exhausted by prolonged exposure to Ag or tumor progression. Although the T cells were not effective in the tumor-bearing host, potent ex vivo activation signals could restore full effector function.

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of the Thy1.1 cells produced IFN-γ in response to MCA-205 tumor, but had minimal reactivity against MCA-207 tumor (Fig. 4C).

Adoptive transfer of $4 \times 10^6$ Thy 1.1 eliminated MCA-205 pulmonary metastases, and $2 \times 10^6$ cells had a substantial therapeutic effect (Fig. 5D). As anticipated, because there was no source of tumor Ags during host lymphoid reconstitution, there was no detectable therapeutic activity among the Thy 1.2 cells even at a dose of $10^7$ cells (Fig. 5D).

**Discussion**

These experiments examine the development of memory T cells from adoptively transferred donor effector cells and from host lymphocytes that were regenerating coincident with an immune anti-tumor response. We used a broad polyclonal population of Agprimed pre-effector T cells to study their in vivo development into memory cells in the settings of regressing tumor, progressing tumor, or absence of tumor Ag. The tumor model used in this study contributes to the body of in vivo work on memory cell development in that it examines weakly immunogenic tumor Ags rather than antiviral responses or systems with strong Ags and high frequencies of T precursors (22, 23).

There is a linear differentiation of T cells from naive, to Agprimed, to effector, and then memory cells with different phenotypic and functional characteristics at each stage (24, 25). We chose to study the transition from Ag-primed pre-effector cells to long term memory cells because of the clear implications for clinical applications of cancer immunotherapy. To isolate Ag-primed pre-effectors, L-selectinlow cells were harvested 12 days after tumor inoculation. In mice, L-selectin is expressed at high levels on naive T cells and facilitates migration from the bloodstream into secondary lymphoid organs, but is rapidly down-regulated upon Ag activation and remains at low levels on most long term memory cells (11, 12, 26).

FIGURE 5. Therapeutic efficacy and antigenic specificity of culture activated memory cells. Thy 1.1 or Thy 1.2 memory cells with CD62Llow phenotype were isolated from spleens of cured mice 144 days after adoptive transfer of Thy 1.1 effector cells and activated in vitro for 15 days. A, Culture-activated memory cells from either the Thy 1.1 or Thy 1.2 purified subsets were adoptively transferred to recipients bearing 3-day MCA-205 pulmonary metastases at the indicated doses, and tumors were enumerated on day 20, with metastases >250 being assigned a value of 250. Statistically significant differences ($p < 0.01$) were observed for groups 2, 3, and 5 vs group 1 or 4. B, Culture-activated memory cells had no efficacy against 3-day MCA-207 pulmonary metastases. C, Regeneration of therapeutic efficacy by ex vivo activation of CD62Llow cells recovered from mice with recurrent MCA-205 tumors. Thy 1.1 or Thy 1.2 activated cells were transferred to mice with 3-day MCA-205 tumors at the indicated dose ($p < 0.01$ for groups 2, 3, and 5 vs group 1 or 4). D, Long term memory cells were recovered from nontumor-bearing hosts 208 days after adoptive transfer and culture-activated. Thy 1.1 cells (groups 2 and 3) were efficacious against 3-day pulmonary metastases, whereas Thy 1.2 cells had no therapeutic activity (groups 4 and 5).
tumor regression upon immediate adoptive transfer (G. E. Plautz, unpublished observations) (13) indicates that the L-selectin<sup>low</sup> T cells were still at a pre-effector stage. It is likely that the enriched L-selectin<sup>low</sup> T cells from the initial Thy1.1 host also contained some passenger memory cells of various irrelevant specificities. However, the specificity of tumor immunotherapy exhibited by L-selectin<sup>low</sup> T cells indicates that any pre-existing memory cells transferred as passenger cells do not have any detectable anti-tumor function and thus should not interfere with the functional analysis presented in this study (10). Even though the polyclonal population of effector cells precludes tracking with MHC/peptide tetramers, the highly focused immune response and functional competence allow monitoring of the persistence of antitumor memory cells.

The observation that adoptively transferred effector T cells can undergo further differentiation and persist in the recipient as memory cells was anticipated based on TCR transgenic models demonstrating linear differentiation of CD4<sup>+</sup> or CD8<sup>+</sup> effector cells to memory cells (24–28). Even though the transferred T cells were highly activated by anti-CD3 stimulation and rapidly proliferating, they were able to persist after Ag and IL-2 withdrawal in nontumor-bearing hosts. This is consistent with recent findings demonstrating the persistence of memory cells in MHC-deficient hosts, where Ag restimulation is not possible (29, 30).

The predominant isolation of CD4<sup>+</sup> cells from among the surviving adoptively transferred Thy1.1 cells was somewhat surprising considering that most of the transferred effector cells were CD8<sup>+</sup>. This suggests either that CD4<sup>+</sup> effector cells were better suited to withstand activation-induced cell death upon encountering tumor in vivo, or that long term surviving CD8<sup>+</sup> cells are preferentially located in other anatomic compartments. Studies of the anatomic distribution of murine CD8<sup>+</sup> memory cells responding to viral or bacterial Ags have, in fact, demonstrated their preferential localization in nonlymphoid tissues (31, 32). In addition, the rapid ex vivo proliferation of CD4<sup>+</sup> compared with CD8<sup>+</sup> memory cells after anti-CD3/IL-2/IL-7 activation was unanticipated because the Ag-primed L-selectin<sup>low</sup> CD8<sup>+</sup> cells from LNs proliferate much faster than CD4<sup>+</sup> cells. It is possible that the optimal cytokine requirements for expansion of CD8<sup>+</sup> memory cells were not used in these experiments. Several recent studies of CD8<sup>+</sup> memory cells have documented the importance of either IL-7 or IL-15 for acute homeostatic proliferation and IL-15 for maintenance through basal proliferation with inhibitory effects of IL-2 (33, 34). It is important to note that basal or homeostatic proliferation is independent of strong TCR/CD3 activation induced by Ag challenge or in our system by anti-CD3 mAb. Under the activation conditions used in this study, IL-2 does promote the proliferation of CD8<sup>+</sup> T cells that produce IFN-γ and mediate effector function. In future studies we will compare the effects of IL-15 with IL-2 on the proliferation of memory CD8<sup>+</sup> cells after anti-CD3 stimulation.

The finding that a new cohort of host memory T cells arose from reconstituting T cells has relevance to clinical applications of T cell immunotherapy for cancer. It is evident that the process of tumor regression allowed tumor Ags to be acquired by DC under activating, not tolerizing, conditions. Sensitized host T cells were competent to complete their differentiation to long term memory cells that were harvested >90 days after the disappearance of tumor Ag. Vaccination with tumor Ag during lymphoid reconstitution has been demonstrated to augment T cell sensitization in other systems (35, 36). It remains to be tested whether regression of established tumors provides a quantitatively or qualitatively different immune response in regenerating T cells (37). Host lymphodepletion with nonmyeloablative chemotherapy is an important component of several clinical protocols of T cell adoptive immu-


