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IL-2 During In Vitro Priming Promotes Subsequent Engraftment and Successful Adoptive Tumor Immunotherapy by Persistent Memory Phenotypic CD8+ T Cells

Oliver F. Bathe, Nava Dalyot-Hereman, and Thomas R. Malek

Adoptive T cell tumor immunotherapy potentially consists of two protective components by the transferred effector cells, the immediate immune response and the subsequent development of memory T cells. The extent by which adoptively transferred CD8+ CTL are destined to become memory T cells is ambiguous as most studies focus on the acute effects on tumor shortly following adoptive transfer. In this study we show that a substantial fraction of the input CTL develop into memory cells that reject a s.c. tumor challenge. The use of exogenous IL-2 or a combination of IL-2 and IL-4, but not solely IL-4, during the ex vivo culture for the CTL inoculation was necessary for efficient development of CD8+ memory T cells. Thus, an important component of adoptive immunotherapy using CTL is the production of CD8+ Ag-specific memory cells which is primarily favored by IL-2 receptor signaling during ex vivo generation of the effectors CTL.

Materials and Methods

Animals

OT-I TCR transgenic mice (13) were maintained by breeding heterozygous OT-I TCR transgenic mice to wild-type C57BL/6J mice. The progeny were screened by PCR for the expression of the TCR transgene. All recipient mice were C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) and were used between 6 and 9 wk of age.

Biological reagents

EL-4 is a thymoma derived from the C57BL/6 mouse (H-2b). E.G7 cells consist of EL-4 cells transfected with OVA cDNA (14), and these were a gift from Dr. M. Bevan (University of Washington, Seattle, WA). These cell lines were maintained in complete medium (CM)3 consisting of RPMI 1640 containing 5% FCS, glutamine (30 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 2-ME (5 × 10^-3 M). OVA257-264 (SII-NFEKL) (13) was synthesized by Research Genetics (Huntsville, AL). CyChrome-anti CD8α, PE-anti-Vo2-TCR, FITC-Vαβ2,15.2-TCR, FITC-anti-CD8α (53.6.7), biotin-anti-CD44 (Pgp-1), biotin-anti-CD62L (MEL-14), and biotin-anti-CD69 were purchased from BD PharMingen (San Diego, CA). Biotin-anti-CD25 (7D4) was prepared in our laboratory. The PE-labeled MHC-peptide tetramer (H-2Kb/SHI-INFEKL) was provided by the National Institute of Allergy and Infectious Diseases MHC Tetramer Facility (Atlanta, GA).

Cell culture

Unless indicated otherwise, OT-I CTL used for adoptive transfers were generated by the culture of OT-I splenocytes (1 × 10^6/well) in 24-well plates containing 1 ml of CM containing OVA257-264 (1 nM), IL-4 (175 U/ml), and IL-2 (50 U/ml). After 3 days in culture, the cells were washed and recultured at 0.5 × 10^6 cells/well in 24-well plates containing 1 ml of CM without OVA257-264, but with the same cytokines that were present during initial culture. After an additional 2 days in culture, the cells were harvested and used for in vitro analyses or for adoptive transfer. CTL assays were performed as previously described (15) using 51Cr-labeled E.G7 and EL-4 cells as targets. For T cell proliferation, spleen cells (2 × 10^6 cells/well) were cultured with the indicated concentration of OVA257-264 in flat-bottom 96-well culture plates for 1–3 days. Each well was pulsed with 1 µCi of [H]thymidine, and 5 h later, the cells were harvested with an automated harvester. The mean of triplicate values that varied by less than 10% was calculated. Data are expressed as Δcpm (i.e., cpm from experimental condition − cpm from cultures containing culture medium alone).

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2 Abbreviation used in this paper: CM, complete medium.
Cells were stained with the various Ab conjugates as previously described (15). Between 50,000 and 100,000 events were collected and analyzed for each sample using a FACSscan flow cytometer (BD Biosciences, San Jose, CA) and CellQuest software (BD Biosciences).

Adoptive immunotherapy

OT-I CTL were injected via the tail vein in 0.5 ml of HBSS into normal C57BL/6J mice. Control mice for the adoptive transfers received only HBSS. The proportion of input cells that consisted of OT-I CTL was determined by staining for CD8, Vα2, -TCR, and Vββ1.5.2,-TCR, and this information was used to calculate the number of OT-I CTL adoptively transferred. E.G7 or EL-4 cells (1 x 10⁶) were injected s.c. in 0.2 ml of HBSS at the midline of the lower abdomen 21–28 days after adoptive transfer of OT-I CTL. The tumor cells were freshly thawed within 6 days of inoculation. Tumor cell growth was recorded over time. Detectable tumor was considered to be >0.5 cm². Tumor-free survival curves were compared by log-rank test. A p value of <0.05 was considered significant.

Results

Persistence, phenotype, and function of adoptively transferred CTL

We recently reported that naive T cells from the OT-I TCR transgenic mice, specific for an OVA peptide, OVA257-264, in the context H-2Kb, failed to recognize and respond to the OVA-affected E.G7 thymoma, i.e., were immunologically ignorant, after adoptive transfer into mice bearing E.G7 as a solid tumor (15). However, the adoptive transfer of OT-I effector cells, generated in short-term cultures, inhibited the growth of E.G7 in a dose-dependent manner (15). In the current study we have continued to use this model system to study the ability of in vitro-derived OT-I CTL effector cells to develop into memory cells in vivo and to then function in antitumor immunity.

Our past work indicated that the generation of primary tumor-specific CTL in vitro was favored by culture with exogenous IL-2 and IL-4 (16). Therefore, splenocytes from naive OT-I transgenic mice were stimulated with OVA257-264 in the presence of exogenous IL-2 and IL-4 for 3 days and were expanded in these exogenous cytokines for an additional 2 days. On the fifth day, greater than 90% of the cells consisted of CD8⁺ cells that coexpressed Vα2 and Vβ5, characteristic of the OT-I TCR (not shown). FACS analysis revealed that these activated cells typified effector T lymphocytes, i.e., increased size, up-regulated expression of CD69, CD25, and CD44, and down-regulated expression of CD62L and Ly-6C (Fig. 1).

These CTL effector cells were adoptively transferred to syngeneic C57BL/6 recipient mice. The number of OT-I CTL effector cells that persisted 21–28 days after adoptive transfer was assessed by the fraction of cells that were “triple-stain positive” for CD8, Vα2, and Vβ5 (Fig. 2A). This analysis indicated that the number of triple-stain-positive cells in the spleen and lymph nodes was proportional to the number of cells administered (Fig. 3, A and B). In each animal, the spleen, two axillary lymph nodes, and two inguinal lymph nodes were analyzed. In sum, in animals that received 10 x 10⁶ CTL, the mean number of triple-stain-positive lymphocytes recovered from a pool of these organs was 4.4 ± (1.6) x 10⁶ (Fig. 3B). Given that the mean number of triple-stain-positive cells in control mice is 0.4 ± (0.2) x 10⁶, the estimated recovery of OT-I cells in these organs is 40% of the input cells. If one were to extrapolate this number to the entire peripheral lymph tissue, ~68% of the OT-I cell persisted at this time, assuming there are 25 lymph nodes in a mouse, all containing an equal number of OT-I cells in each node.

FACS analysis revealed that these triple-stain-positive cells were smaller than the adoptively transferred CTL as assessed by forward light scatter (Fig. 1). The phenotype of the OT-I cells was further characterized for other surface markers by gating on CD8⁺ and Vα2⁺ cells (Fig. 1). Unlike the in vitro-generated OT-I CTL effector cells, the in vivo-retained cells no longer expressed elevated levels of CD69 or CD25, but Ly6C was substantially up-regulated. In addition, CD44 remained up-regulated, whereas CD62L reverted to a higher degree of expression. This cell surface phenotype is consistent with that attributed to memory T cells (17–19). In three mice tested at 70–75 days after adoptive transfer, triple-stain-positive (Fig. 2B) or MHC-tetramer-positive (Fig. 2C) OT-I T cells were readily detected with a cell surface phenotype identical (not shown) to that observed at 21–28 days after adoptive transfer. All of these features except the elevated Ly6C expression were detected as early as 7 days after adoptive transfer (not shown). At 21–28 days after adoptive transfer, five of five mice tested expressed a high level of Ly6C. However, in two mice tested at earlier time points, the high Ly6C expression was not seen, suggesting that this aspect of the phenotype appears relatively late.

Memory CD8 T cells often show more rapid proliferation to a lower concentration of Ag when compared with naive T cells (18–21), and they retain CTL activity (22–24). When compared with naive T cells, the recovered adoptively transferred “memory” OT-I T cells proliferated to an ~10-fold lower concentration (Fig. 4A) of OVA257-264 and more rapidly (Fig. 4B). Furthermore, the freshly prepared effector cells exhibited potent and specific CTL
activity to OVA-expressing targets just before adoptive transfer (Fig. 4C). With respect to CTL activity by the "memory" OT-I T cells, the level of cytotoxicity was proportional to the number of OT-I CD8^+ cells that persisted in the spleen (Fig. 4D). Thus, collectively these data demonstrate that 21 days after adoptive transfer, the OT-I effector cells also exhibited functional properties characteristic of memory CD8^+ T cells.

To assess the antitumor activity of the persistent OT-I T cells, mice received E.G7 s.c. 21–28 days after adoptive transfer of the OT-I CTL. Unlike control-treated animals, mice that received OT-I CTL were generally resistant to E.G7 tumor inoculation (Fig. 5). This protection was specific to OVA-expressing tumors, as these mice did not mount an antitumor response when inoculated with the parental EL-4 thymoma. Furthermore, mice (n = 2) adoptively transferred with OT-I CTL that rejected a primary challenge with E.G7 remained tumor-free after a second challenge with E.G7 when reinoculated 56 and 66 days after the first inoculation (not shown). Thus, long-lasting protective tumor-specific immunity was not prevented by the initial response by the persistent OT-I T cells.

Development of OT-I memory T cells and antitumor response is dependent upon the cytokines used to generate the effector cells

Recent data suggest that generation of CTL in the presence of IL-4 enhances their survival following adoptive transfer (25). Therefore, we examined the effect of IL-2 and IL-4 during the in vitro culture on the long-term fate and function of OT-I CTL. In these experiments the OT-I T cells were stimulated with a lower dose (0.1 nM) of OVA257–264, as this was found to be necessary for optimal cytokine expansion of these CTL (data not shown). The proportion of cells staining for CD8, Vα2-TCR, and Vβ5.1.5.2-TCR did not differ significantly between conditions and was typically 87–95% of the cells (not shown). When compared with naive OT-I T cells (see Fig. 1), all effector cells up-regulated CD69, CD25, and CD44, whereas the levels of CD62L and Ly-6C did not differ significantly between conditions and was typically 87–95% of the cells (not shown). When compared with naive OT-I T cells, each group consisted of three to five recipient mice that were individually analyzed.
irrespective of the exogenous cytokine added to the cultures. Furthermore, although all effector groups displayed strong CTL activity to E.G7, effector cells generated in the presence of IL-4 or both IL-2 and IL-4 showed stronger activity than cells expanded with only IL-2 (Fig. 6B).

These effector OT-I CTL were adoptively transferred into C57BL/6 mice, and their persistence in the spleen and lymph nodes was assessed 21–28 days later. The largest number of OT-I cells was detected in recipients of CTL generated with either IL-2 or a combination of both IL-2 and IL-4 (Fig. 7). In contrast, a markedly lower number of triple-stain-positive cells was detected in recipients of CTL generated in IL-4. The use of a higher dose of IL-4 did not improve the degree of persistence of the OT-I. These

![FIGURE 4.](image)

**FIGURE 4.** Functional properties of naive, effector, and adoptively transferred OT-I T cells. A and B, Proliferative responses by naive and persistent adoptively transferred OT-I T cells. Dose response to OVA257–264 48 h after culture initiation. B, Time course of proliferative response using 100 pM OVA257–264. Persistent OT-I T cells were analyzed 21–28 days after adoptive transfer. Splenocytes from a naive OT-I mouse were mixed with normal C57BL/6 splenocytes to contain a fraction of OT-I cell equivalent to that in the spleen from the adoptively transferred recipient (typically ~4%). C and D, CTL activity by ex vivo effector and persistent adoptively transferred OT-I T cells. C, OT-I effector cells were prepared as described in Materials and Methods and were directly tested for CTL activity against E.G7 or EL4 targets. D, CTL activity by splenocytes from adoptively transferred mice that received the indicated number of OT-I CTL 21–35 days previously. Data shown represent specific CTL activity against E.G7 target cells at an E:T ratio of 100:1. Data are representative of three experiments (A–C) or three mice per group (D).

![FIGURE 5.](image)

**FIGURE 5.** Antitumor activity of memory OT-I T cells. Twenty-one to 28 days after adoptive transfer of 10 × 10⁶ OT-I CTL (prepared as described in Materials and Methods), recipient mice were inoculated with 1 × 10⁶ E.G7 or EL4 cells, s.c., as indicated. As a control, some mice received HBSS rather than OT-I CTL. Data are represented as tumor-free survival for ≥6 mice/group.

![FIGURE 6.](image)

**FIGURE 6.** Phenotype and function of OT-I effector cells generated with IL-2 and/or IL-4. OT-I splenocytes were stimulated with OVA257–264 (0.1 nM) and IL-2 (50 U/ml) and/or IL-4 (50 U/ml) or with IL-4high (175 U/ml) as described in Materials and Methods for 5 days. A, FACS analysis before adoptive transfer. Staining for the indicated markers (dark line) or negative controls (light line) is shown. B, CTL activity just before adoptive transfer. Data are representative of two experiments.
findings were confirmed by staining for the donor cells with MHC-peptide tetramer (Fig. 7). These data indicate that stimulation with IL-2 during the generation of CTL optimally promotes the survival of these cells upon adoptive transfer in vivo.

FACS analysis revealed that the phenotype of the persistent OT-I CTL generated in either IL-2 or both IL-2 and IL-4 was typical of a memory phenotype, i.e., CD25low, CD44high, and Ly6Chigh (Fig. 8). By contrast, only a small proportion of the persistent OT-I cells generated in IL-4 expressed high levels of CD44 and Ly6C (Fig. 8). This finding, in conjunction with the lower yield of persistent OT-I cells (Fig. 7), indicates that there are 4- to 6-fold fewer cells with a memory phenotype when the naive OT-I were solely primed ex vivo with Ag and exogenous IL-4.

To examine Ag-specific responsiveness, spleen cells from adoptively transferred mice were stimulated with OVA257–264 in vitro. In general, the magnitude of the resulting proliferative response was proportional to the number of donor cells identified in the spleen. However, this relationship was not linear, as the magnitude of this response by the splenocytes in recipients of OT-I cells generated in IL-4 was disproportionately low (Fig. 9A).

At 21–28 days after adoptive transfer, recipients of OT-I CTL generated in each cytokine condition were inoculated s.c. with E.G7 cells. When compared with untreated cells, all treatment groups exhibited a significant increase in tumor-free survival (IL-2, p < 0.001; IL-4, p < 0.001, IL-2/IL-4, p < 0.001; and IL-4high, p = 0.006; Fig. 9B). It is noteworthy that the IL-4-generated OT-I effector cells developed a therapeutic effect even though these cells inefficiently persisted and developed a memory phenotype. Thus, these residual cells were still competent to affect E.G7 growth. Between the treatment groups, the difference in tumor-free survival between mice that received OT-I effector cells generated with IL-2 and recipients of CTL generated in IL-2/IL-4 and IL-4 (50 U/ml) was not significant. However, the greater tumor-free survival in mice that received OT-I effector cells generated in IL-2 when compared with mice that received CTL primed in a high dose of IL-4 (175 U/ml) was statistically significant (p = 0.04). The greater therapeutic efficacy of OT-I generated in IL-2 may be related to its ability to optimally promote differentiation of the effector CTL into persistent memory phenotypic cells, as these effector cells exhibited the weakest CTL activity upon adoptive transfer to tumor-bearing mice (Fig. 6).

Discussion
Many previous studies have demonstrated that protective memory responses against tumor cells occur in vivo. In these cases, the memory response is typically demonstrated after either naive or effector tumor-specific T cells have first elicited a protective primary antitumor response. For adoptive immunotherapy, it has been difficult to ascertain whether the memory response is dependent
upon encountering tumor Ags in vivo or whether it reflects an intrinsic potential of the transferred effector cells. Based on both phenotypic and functional characteristics, our data support the notion that a substantial portion of adoptively transferred CTL differentiates into memory T cells that are then competent to reject a tumor challenge. At 21–28 days after adoptive transfer, the persistent OT-I T cells expressed a cell surface phenotype of memory cells, i.e., CD44$^{high}$, CD62L$^{high}$, Ly-6C$^{high}$, CD25$^{neg}$, and CD69$^{neg}$ (17–19). Furthermore, unlike naive OT-I T cells, these persistent OT-I cells expressed intrinsic CTL activity and exhibited rapid proliferation to a relatively low dose of OVA257$^{-}$ upon in vitro challenge. These functional properties have been reported to be features that distinguish memory T cells (6, 18–21, 24).

When studying cell populations thought to represent memory T cells, it is imperative that the cells under study be distinguished from chronically stimulated effector T cells that persist secondary to Ag. Even cells present several months after adoptive transfer may display characteristics more consistent with effector cells (27). Identification of CD8$^+$ memory cells is also problematic in some models in which T cells are adoptively transferred to lymphopenic mice. In this situation, naive CD8$^+$ T cells acquire the phenotypic and functional characteristics of memory T cells in the absence of Ag by the process of homeostatic proliferation (28–30). In our model, we have avoided potential complications due to either Ag or homeostatic proliferation by removing extraneous Ag by washing the effector cells several days before adoptive transfer to mice with a normal pool of lymphocytes.

A number of studies have documented that exogenous cytokines influence the magnitude and quality of the resulting CD8$^+$ effector CTL generated during in vitro culture (16, 31–34). More recently, we demonstrated an essential role for cytokines for the generation of effector CTL. In the absence of IL-2R and IL-4R signaling in vitro, TCR-activated CD8$^+$ T cells failed to differentiate into CTL, in part due to lack of expression of granzyme B, and they exhibited limited proliferative capacity (3–4 cell divisions) before apoptosis by the vast majority of the cells (35). Comparatively little is known concerning the long-term consequences of these culture conditions on CTL effector cells in vivo. Our findings demonstrate a critical role for IL-2 during in vitro generation of the effector CTL for the optimal in vivo persistence of CTL with characteristics of memory T cells. Analysis of allospecific T cells in autoimmune-prone IL-2-deficient mice and an Ag-specific CD8$^+$ cytotoxic T cell line have suggested that IL-2 favors the development of T memory cells (36, 37). Our data directly implicate signaling through the IL-2R for development of CD8 T memory cells from naive normal Ag-specific precursor T cells. In this regard, it is important to note that the sole use of exogenous IL-4 resulted in the production of optimal numbers of CTL. However, upon adoptive transfer, these effector cells did not readily persist or express a memory phenotype. These IL-4-driven effector CTL were correspondingly less effective in antitumor immunity.

Several other studies have reported findings consistent with our results. In a model of viral infection, Aung et al. (38) showed that the presence of IL-4 at the time of activation diminishes the expansion of viral Ag-specific CD8$^+$ T cells and inhibits the development of CD8$^+$ T cell memory. Similarly, Villarces and Bergmann (39) demonstrated that the absence of IL-4 at the time of activation of CTL increases the frequency of CD8$^+$ memory cells 8 wk later and enhances the effector functions of these memory cells. In our experiments, coculture of T cells with exogenous IL-2 alone or both IL-2 and IL-4 resulted in enhanced long-term survival and differentiation to the memory phenotype, suggesting that signal transduction through the IL-2R is sufficient to promote memory CTL upon adoptive transfer in vivo. This result also indicates that IL-4 does not actively prevent engraftment of CTL that persist with a memory phenotype. However, our experiments do not rule out that signals through other cytokine receptors or T cell surface proteins might also favor the engraftment and expression of the memory phenotype by CD8$^+$ effector cells. Furthermore, our data do not address whether IL-2 also functions in vivo to promote memory CTL. IL-15, whose receptor also uses IL-2Rβ and the common γ-chain for signaling, has been shown to play an important role in the homeostasis of CD8$^+$ memory cells (5, 9–11) and is also a candidate to promote memory CTL in vivo.

Although IL-2 and IL-4 redundantly function as T cell growth factors and promote differentiation into CTL in vitro (35, 40, 41), our data suggest that IL-2 is mandatory for promoting memory CTL. This finding was somewhat surprising because Huang et al. (25) reported that CD8$^+$ T cells activated in the presence of IL-4 in vitro persisted to a greater degree after adoptive transfer than CTL activated in IL-2. These persistent cells expressed high levels of CD44 and produced large amounts of IFN-γ following re-exposure to peptide, suggesting that they consisted of memory cells. However, it should be emphasized that although these investigators initially stimulated the CD8$^+$ T cells in either IL-2 or IL-4 for 3 days, all cells were expanded in IL-2 for an additional 2 days before adoptive transfer. Thus, analogous to our experimental design, these T cells also received signaling through the IL-2R before adoptive immunotherapy, which we believe likely contributed to promoting memory CTL in vivo.

Our study reveals an unappreciated potential value of adoptive antitumor immunotherapy. Besides the initial antitumor response by effector CTL, in vitro expansion using IL-2 promotes the production of memory CD8$^+$ T cells, which may favor long-term protection against tumor recurrence. Furthermore, adoptive immunotherapy with effector CTL has been shown to overcome immunological ignorance (15), a phenomenon by which naive tumor-specific T cells simply ignore tumor-associated Ag. Nevertheless, despite these beneficial features, adoptive immunotherapy with ex vivo IL-2-expanded tumor-infiltrating lymphocytes that contain CTL is often unsuccessful in cancer patients. In addition to tumor-induced immunosuppression and outgrowth of tumor Ag escape variants, growth factor deprivation is a problem after adoptive transfer, particularly when CTL have been generated and expanded over longer periods of time (1). In the model described in this study, unlike in most therapeutic protocols, large numbers of effector cells were generated over a much shorter time frame. Therefore, successful adoptive T cell therapy may be linked to rapid production of effector T cells, using the appropriate cytokines during the ex vivo culture.

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