

Host Lymphodepletion Augments T Cell Adoptive Immunotherapy through Enhanced Intratumoral Proliferation of Effector Cells

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Abstract

T-cell adoptive immunotherapy for stringent murine tumor models, such as intracranial, s.c., or advanced pulmonary metastases, routinely uses lymphodepletive conditioning regimens before T-cell transfer, like recent clinical protocols. In this study, we examined whether host lymphodepletion is an obligatory component of curative T-cell therapy; we also examined the mechanism by which it augments therapy. Mice bearing intracranial, s.c., or 10-day pulmonary metastases of MCA 205 received total body irradiation conditioning or were nonirradiated before i.v. transfer of tumor-reactive T cells. Total body irradiation was not required for immunologically specific curative therapy and induction of memory provided that a 3- to 12-fold higher T-cell dose was administered. The mechanism involved enhanced intratumoral proliferation of T-effector cells in total body irradiation-conditioned recipients. In this tumor model, intratumoral T_{reg} cells were not detected; consequently, intratumoral T-effector cells produced identical amounts of IFN- γ upon *ex vivo* antigen stimulation irrespective of total body irradiation conditioning. Thus, host lymphodepletion augments T-cell immunotherapy through enhanced antigen-driven proliferation of T-effector cells, but curative therapy can be achieved in nonconditioned hosts by escalation of T-cell dose. These data provide a rationale for dose escalation of T-effector cells in situations where single or repeated lymphodepletion regimens are contraindicated. (Cancer Res 2005; 65(20): 9547-54)

Introduction

The adoptive transfer of tumor-reactive T lymphocytes is an effective approach to eradicate advanced tumors in preclinical animal models and in some patients with metastatic diseases (1-4). Tumor-draining lymph nodes have been established as a highly enriched source of T cells that have been sensitized to tumor antigens (5). There is a minor subset (10-15%) of CD62L^{low} T cells in the tumor-draining lymph nodes that contains all of the antitumor activity, whereas the reciprocal subset of CD62L^{high} cells are completely ineffective (6). The kinetics of the appearance of CD62L^{low} cells in draining lymph node following tumor inoculation, coupled with its physiologic down-regulation following antigen stimulation of naïve T cells (7), indicate that this subset is highly enriched for T cells recently sensitized by tumor antigens. *In vitro* activation of tumor-draining lymph node CD62L^{low} T cells with anti-CD3/interleukin 2 (IL-2) induces the tumor-sensitized

T cells to proliferate and differentiate into T-effector cells that can mediate regression of established tumors (8).

In vitro activation conditions can be modified to optimize T-cell proliferation; however, following adoptive transfer, T cells must traffic to every site of metastatic disease and perform effector function within a potentially hostile tumor environment. One strategy to maintain functional support of *in vitro*-cultured immune cells, particularly CD8⁺ T cells, is to administer IL-2 to the host following adoptive transfer (9, 10). Clinical studies also show that IL-2 enhances the persistence of adoptively transferred tumor-infiltrating lymphocytes and *in vitro* expanded CD8⁺ clones (11-13). However, concomitant IL-2 support is not without some disadvantages. High-dose IL-2 has toxicity that limits duration of use to several days and it causes toxicity in patients with brain metastases (14). IL-2 also has complex effects on T cells with specificities irrelevant to tumor regression, as well as CD4⁺CD25⁺ regulatory T cells (15-18). Moreover, our studies using *in vitro* activated tumor-draining lymph node cells show that treatment of hosts with systemic IL-2 inhibits the trafficking of adoptively transferred T cells to certain anatomic sites, particularly brain tumors (19, 20). For these reasons, we have developed the approach of combined tumor-reactive CD4⁺ and CD8⁺ T-cell transfer, which is effective in all anatomic sites and does not require concomitant IL-2 (21).

Another strategy that has been used for nearly three decades to support the survival and activation of transferred effector T cells is to perform lymphodepletion of the host before adoptive transfer to eliminate "suppressor activity", currently described as T_{reg} cells (22-25). Recently, there has been a resurgence in interest in lymphodepletion before active immunotherapy or adoptive cell transfer (26, 27). Lymphodepletion induces homeostatic proliferation of residual T cells through improved competition for cytokines such as IL-7 and IL-15 (28-30). It also transiently eliminates T_{reg} cells, which are likely to account for some of the previously described suppressor activity (31). In the current study, we show that host lymphodepletion is not absolutely required to cure advanced pulmonary metastases or challenging sites of disease, provided that a higher number of effector T cells is administered. The operative mechanism in this tumor model is greater intratumoral T-cell proliferation in irradiated versus nonirradiated hosts.

Materials and Methods

Mice and tumors. Female C57BL/6N mice were purchased from the Biological Testing Branch, Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD), and green fluorescent protein (GFP) transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in a specific pathogen-free environment and used at 8 to 12 weeks of age. The 3-methylcholanthrene-induced fibrosarcomas MCA 205 and MCA 207 originally derived in B6 mice (32) have been maintained *in vivo* by serial s.c. transplantation and were between passages 4 to 8. Single-cell suspensions were prepared from solid tumors by

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digestion with a mixture of 0.1% collagenase type IV, 0.01% DNase I, and 2.5 units/mL hyaluronidase type V (Sigma-Aldrich, St. Louis, MO) in HBSS for 2 to 3 hours at room temperature. Then, tumor cells were filtered through a 100 μ m nylon mesh, washed, and suspended in HBSS for i.v., s.c., or intracranial inoculation.

Isolation and activation of CD62L^{low} tumor-draining lymph node T cells. B6 mice were inoculated s.c. with 1.5×10^6 MCA 205 tumor cells in the lower flank region bilaterally and 12 days later tumor-draining lymph nodes were removed, mechanically teased apart with 20 G needles, and a single-cell suspension was prepared. CD62L^{low} cells were isolated by depletion of CD62L^{high} cells using MACS beads (Miltenyi Biotec, Auburn, CA) as previously described (33). CD62L^{low} T cells were suspended in complete medium at 2×10^6 /mL and activated with plate-bound anti-CD3 monoclonal antibody (mAb; 145-2C11; American Type Culture Collection, Rockville, MD) for 48 hours in 24-well plates at 37°C in 5% CO₂. Complete medium consists of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mmol/L nonessential amino acids, 1 μ mol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 μ g/mL streptomycin, 100 units/mL penicillin, 0.5 μ g/mL amphotericin B (all obtained from Life Technologies, Grand Island, NY), and 5×10^{-5} mol/L 2-ME (Sigma-Aldrich). Recombinant human IL-2 was provided by Chiron (Emeryville, CA) and recombinant mIL-7 was purchased from R&D Systems (Minneapolis, MN). After 2 days of anti-CD3 stimulation, T cells were cultured in complete medium supplemented with IL-2 (4 units/mL) or a mixture of IL-2 plus rmIL-7 (10ng/mL) at a concentration of 0.5 to 1×10^5 cells/mL in 24-well plates for 3 days. On day 5, the cells were diluted to 2×10^5 /mL and incubated for 4 additional days. On day 9, the activated and expanded CD62L^{low} T cells were harvested, washed, and used for adoptive immunotherapy.

Fluorescence-activated cell sorting analysis and intracellular IFN- γ staining. Phycoerythrin-conjugated anti-CD8, anti-TCR, or anti-IFN- γ , and FITC-conjugated anti-CD4, or anti-CD62L, and isotype-matched mAbs were purchased (BD Biosciences PharMingen, San Diego, CA). Cell surface phenotypes were measured by direct immunofluorescence staining with conjugated mAbs, and stained cells were analyzed using the CellQuest Software (BD Biosciences Immunocytometry, San Jose, CA). Intracellular IFN- γ staining was done as previously described (33). Briefly, activated T cells were stimulated with a single-cell suspension of either MCA 205 or MCA 207 tumor digest at a 1:1 ratio, or with anti-CD3. Brefeldin A (10 μ g/mL) was added at 5 hours and cells were harvested at 20 hours. The cells were then washed and pretreated with FcR block, followed by staining for 30 minutes with a mixture of FITC-conjugated anti-CD8 and cychrome-conjugated anti-CD4. Washed cells were fixed with 2% paraformaldehyde for 20 minutes, permeabilized with 0.3% saponin, and incubated for 40 minutes with phycoerythrin-conjugated IFN- γ at 4°C. Unbound mAbs were removed by two washes with 0.3% saponin in PBS.

Adoptive immunotherapy. Therapeutic efficacy of activated T cells expanded with IL-2 or IL-2 plus IL-7 for 9 days was assessed in three tumor models. Pulmonary metastases were established by i.v. inoculation of 3×10^5 MCA 205 or MCA 207 tumor cells suspended in 1.0 mL of HBSS. Ten days later, mice received i.v. transfer of the indicated number of *in vitro* activated T cells. Mice were sacrificed on days 18 or 21 after tumor inoculation, and the lungs were insufflated with India ink and the number of tumor nodules on the surface was enumerated. S.c. tumors were established by injecting 1.5×10^6 MCA 205 cells in 50 μ L of HBSS into the right hind flank. Mice received T cells i.v. on day 3 of tumor growth and tumor size was measured in two perpendicular dimensions twice to thrice per week with Vernier calipers, and was recorded as tumor area (mm²). Brain tumors were established by transcranial inoculation of 1×10^5 MCA 205 tumor cells in 10 μ L of HBSS as previously described (34). Mice received i.v. transfer of T cells on day 3. Mice were followed for survival or were sacrificed when neurologic symptoms were apparent. In each adoptive transfer experiment, tumor-bearing mice were pooled and randomly divided into treatment groups that were not irradiated or which received sublethal total body irradiation (5 Gy) from a ¹³⁷Cs irradiator (J.C. Shephard & Associates, Glendale, CA) several hours before adoptive immunotherapy.

In vivo trafficking. *In vitro* activated CD62L^{low} tumor-draining lymph node cells derived from GFP transgenic mice were injected i.v. into B6 hosts

with established 10-day pulmonary metastases. Alternatively carboxyfluorescein succinimidyl ester (CFSE)-labeled T-effector cells were injected. Mice were pretreated with total body irradiation or remained nonirradiated before adoptive immunotherapy. At the designated time points after adoptive transfer, mice were sacrificed and peripheral blood, lung, and spleen were harvested. Single cell lymphocyte suspensions were prepared from lungs by enzymatic digestion followed by Percoll density gradient centrifugation as described (35, 36). Cells were counted and stained with anti-CD8 or anti-CD4 and analyzed by fluorescence-activated cell sorting (FACS). The number of GFP- or CFSE-labeled T cells in the lungs was calculated by multiplying the total lymphoid cell count by their percentage. In the same experiments, lungs were harvested, snap-frozen, and cryostat sections (8 μ mol/L) were examined by fluorescent microscopy.

Statistical analysis. The significance of differences between groups was analyzed by the Wilcoxon rank-sum test, or by Student's *t* test. A two-tailed *P* value of <0.05 was considered significant.

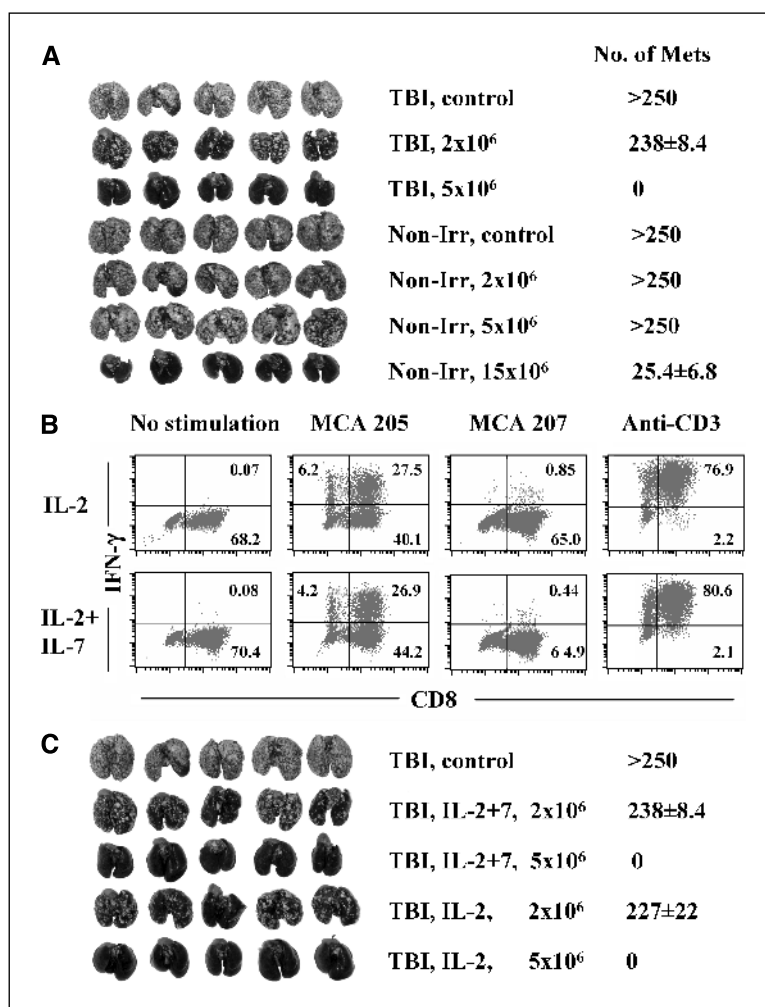
Results

Total body irradiation is not required but augments therapy of advanced pulmonary metastases. The capacity of *in vitro* activated CD62L^{low} tumor-draining lymph node cells to mediate rejection of a large tumor burden in the absence of total body irradiation was tested in hosts with 10-day pulmonary metastases. To generate large numbers of T-effector cells required for these experiments from the typical yield of 10^6 CD62L^{low} cells in a single tumor-draining lymph node, we modified the previously described *in vitro* anti-CD3/IL-2 activation protocol to include IL-7 and extended the duration in culture to 9 days. In 12 independent experiments, T cells cultured for 9 days in IL-2/IL-7 expanded 166 ± 18.3 -fold, whereas those cultured in IL-2 alone had 54 ± 4.1 -fold proliferation (*P* < 0.01). Although total proliferation differed, the phenotype was similar for T-effector cells cultured in IL-2 alone versus IL-2/IL-7 and consisted of CD8⁺ (60-80%) and CD4⁺ (10-20%) T cells, lacking expression of natural killer markers.

As shown in Fig. 1A, large numbers of pulmonary tumors (>250 per animal) are visible in the lungs of total body irradiation-conditioned or nonirradiated controls, which did not receive T cells. Adoptive transfer of 2×10^6 T cells in total body irradiation-conditioned hosts was subtherapeutic, whereas 5×10^6 T cells completely eliminated metastases. By contrast, in nonirradiated hosts 5×10^6 T cells were subtherapeutic, whereas 15×10^6 cells provided effective therapy. This experiment shows that total body irradiation conditioning provides a therapeutic augmentation that is approximately compensated by 3-fold increase in T-cell dose. Thus, host lymphodepletion through total body irradiation conditioning was not absolutely required in this stringent tumor model. Interestingly, equivalent potency for T cells cultured in IL-2 compared with IL-2/IL-7 was readily apparent at partially effective T-cell doses of 2×10^6 cells and each set of T-cell cultures mediated complete tumor regression at higher doses (Fig. 1C). Thus, exposure to IL-7 during *in vitro* activation augmented the number of T-effector cells without altering their subsequent *in vivo* trafficking capacity or intrinsic therapeutic efficacy.

Specificity of anti-CD3/IL-2/IL-7-activated T cells in nonirradiated hosts. One of the hallmarks of T-cell immunotherapy for methylcholanthrene-induced tumors is the exquisite specificity for unique tumor antigens that remain predominantly uncharacterized. The adoptive transfer of 3.5×10^7 anti-CD3/IL-2/IL-7 activated CD62L^{low} T cells derived from MCA 205 tumor-draining lymph node led to complete regression (*n* = 0 tumors, five mice per group) of MCA 205 pulmonary metastases, but no therapeutic effect was observed in hosts bearing MCA

Figure 1. Total body irradiation augments immunologically specific T-cell therapy of 10-day pulmonary tumors. **A**, mice were inoculated with 3.0×10^5 MCA 205 tumor cells, and on day 18 or 21 lungs were harvested and insufflated with India ink to highlight the solid tumor nodules. Control total body irradiation-conditioned mice (*first row*) or nonirradiated controls (*fourth row*) without T-cell transfer had extensive tumor burden (>250 metastases as indicated by average number of metastases \pm SE) by day 18. In total body irradiation-conditioned mice, partial response was observed with 2×10^6 T cells and complete response with 5×10^6 cells. In nonirradiated hosts, a partial response was observed with 5×10^6 cells, and nearly complete response with 15×10^6 cells. **B**, specific antitumor response is highly polarized toward IFN- γ production. CD62L^{low} T cells (10^6), activated for 9 days with anti-CD3/IL-2 (*top row*) or anti-CD3/IL-2/IL-7 (*bottom row*), were incubated for 20 hours without stimulation with a single cell digest of MCA 205 (10^6 cells), MCA 207 tumor digest (10^6 cells), or anti-CD3 mAb. T cells were stained for intracellular IFN- γ and anti-CD8 and the percentage of positive versus negative CD8⁺ cells is indicated. **C**, adoptive transfer of 2×10^6 CD62L^{low} tumor-draining lymph node cells activated with anti-CD3/IL-2 alone or anti-CD3/IL-2/IL-7 mediated partial regression and 5×10^6 cells mediated complete regression of established 10-day pulmonary metastases in total body irradiation-conditioned hosts.



207 ($n > 250$ tumors) similar to control mice bearing MCA 205 or MCA 207 that did not receive T cells ($n > 250$ tumors). Specificity was also apparent in an *in vitro* assay of IFN- γ production in response to single cell tumor digest of MCA 205 but not MCA 207 (Fig. 1B). It is evident that there is minimal spontaneous cytokine production; however, cultured T cells uniformly respond to anti-CD3, indicating a phenotype that is highly polarized to IFN- γ production. In addition, a similar percentage of cells cultured in IL-2 (27.5%) or IL-2/IL-7 (26.9%) produced IFN- γ after stimulation with MCA 205 digest. This assay indicates a high level of enrichment of tumor-reactive T cells among the CD62L^{low} subset of the tumor-draining lymph node cells. Because the percentage of tumor-reactive T cells and *in vivo* function were not significantly different between the two culture conditions, and because anti-CD3/IL-2 and anti-CD3/IL-2/IL-7 cultures showed equivalent potency *in vivo* in the above experiments and in s.c. tumor models (21), anti-CD3/IL-2/IL-7 was used to generate T cells for the subsequent experiments.

Total body irradiation enhances immunotherapy of established subcutaneous or intracranial tumors. Regression of established weakly immunogenic s.c. tumors is difficult to achieve with adoptive immunotherapy. Previous immunotherapy experiments for s.c. MCA 205 tumors used total tumor-draining lymph node and required pretreatment of the host with total

body irradiation and 5×10^7 T cells (37). This precluded the ability to perform dose escalation to assess the requirement of total body irradiation. CD62L^{low} T cells derived from MCA 205 tumor-draining lymph nodes were activated with the anti-CD3/IL-2/IL-7 method, in irradiated and nonirradiated recipients bearing s.c. tumors. Although a dose as low as 5×10^6 T cells was curative in hosts conditioned with total body irradiation, transfer of 6×10^7 T cells was required to completely inhibit tumors in nonirradiated hosts (Fig. 2A). Therefore, the requirement for total body irradiation to achieve complete regression of established s.c. tumors could be replaced by using a 12-fold higher dose of effector T cells.

Intracranial tumors are also relatively refractory to adoptive immunotherapy, and local cranial irradiation or sublethal total body irradiation has been routinely used in prior experiments (38). Mice bearing 3-day intracranial MCA 205 tumors were treated with total body irradiation or remained nonirradiated then received systemic adoptive transfer of activated T cells. Total body irradiation plus 10^7 T cells was curative, whereas total body irradiation alone or 10^7 T cells without total body irradiation had no therapeutic effect (Fig. 2B). However, even in nonirradiated hosts, adoptive transfer of 4×10^7 T cells was curative in four of five mice ($P < 0.01$). Mice cured of intracranial tumors developed a protective memory response and all nine cured mice rejected a second intracranial tumor challenge of 10^5 tumor cells 80 days

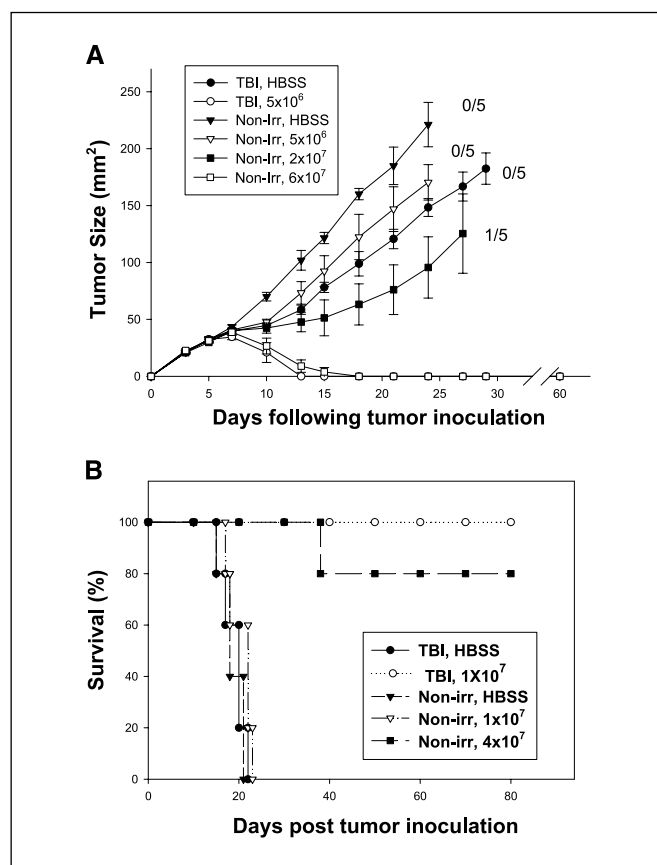


Figure 2. Escalated dose of T cells overcomes requirement for total body irradiation for immunotherapy of s.c. or intracranial tumors. **A**, mice were inoculated with 1.5×10^6 MCA 205 cells s.c. then on day 3 were conditioned with total body irradiation or remained nonirradiated. Mice received i.v. HBSS, 5×10^6 , 2×10^7 , or 6×10^7 CD62L^{low} tumor-draining lymph node cells activated with anti-CD3/IL-2/IL-7 as indicated. Number of cured mice with no tumor on day 60 out of total is indicated ($P < 0.01$ for total body irradiation 5×10^6 , and nonirradiated 6×10^7 versus all other groups). **B**, effective therapy of intracranial tumors does not require host total body irradiation. Mice were inoculated intracranial with 1×10^5 MCA 205 tumor cells and on day 3 received total body irradiation or remained nonirradiated then received HBSS, 10^7 , or 4×10^7 CD62L^{low} T cells as indicated ($P < 0.01$ for total body irradiation 10^7 cells or nonirradiated 4×10^7 cells versus all other groups).

later, whether or not they had previously received total body irradiation. By contrast, naïve mice succumbed to the MCA 205 tumor by day 20.

Total body irradiation augments proliferation of transferred T-effector cells. To determine the mechanism by which total body irradiation augments adoptive immunotherapy of tumors, we evaluated direct effects of total body irradiation on the tumor and effects on trafficking and proliferation of transferred T cells. Irradiation of some tumors can up-regulate expression of Fas, making tumor cells more susceptible to CTL-mediated apoptosis (39–41). Likewise, chemotherapy-induced up-regulation of Fas and TRAIL provides for a synergistic antitumor effect when used with immunotherapy (42, 43). We did not observe an increase in the low basal level of expression of Fas on MCA 205 tumor cells following irradiation with 2 to 15 Gy *in vitro* over 4 days. Likewise, Fas was not expressed at increased levels following 5 Gy irradiation of tumors *in vivo* (data not shown).

Our previous studies showed that T-cell infiltration of tumor metastases and antigen-specific proliferation were required for

triggering an antitumor effector mechanism (35, 44). To examine the intratumoral accumulation of T-effector cells, tumor-draining lymph nodes were prepared from GFP transgenic mice. The phenotype, GFP⁺TCR⁺ (84%), CD8⁺ (63%), CD4⁺ (14%), was similar to B6 effector T cells. Total body irradiation conditioned or nonirradiated hosts that were either tumor-free or bearing 10-day lung metastases received a therapeutic dose of 3.5×10^7 GFP⁺ effector T cells and the number of transferred TCR⁺GFP⁺ cells and host TCR⁺GFP^{neg} within tumors was measured. As shown in Fig. 3A, at 24 hours posttransfer the total number of GFP⁺ T cells was similar in total body irradiation tumor-bearing hosts (1.8×10^6) and nonirradiated tumor-bearing hosts (1.5×10^6) as was the number of CD4⁺ (Fig. 3B) and CD8⁺ (Fig. 3C) T cells. As shown in Fig. 4, GFP⁺ T cells were already localized to tumor metastases in total body irradiation as well as nonirradiated hosts by 24 hours and were in contact with tumor antigens. At later time points, the number of donor GFP⁺ T cells dramatically increased to a maximum on day 7 posttransfer, in both total body irradiation (13×10^6) and nonirradiated tumor-bearing animals (6.4×10^6). By contrast, the number of GFP⁺ T cells did not increase in tumor-free hosts whether they received total body irradiation or not. The number of intratumoral host GFP^{neg} T cells actually exceeded the number of transferred GFP⁺ cells in the nonirradiated host but displayed different kinetics and minimal change in number between days 3 to 7 postadoptive transfer. By contrast, total body irradiation-conditioned or non-tumor-bearing hosts had minimal numbers of host T cells (Fig. 3D–F). An independent experiment of similar design shows a similar pattern of augmented intratumoral accumulation of transferred T cells in total body irradiation conditioned hosts 5 days posttransfer (Fig. 3G–I). The preferential accumulation of T-effector cells was confined to the tumor and there was no difference in the number of GFP⁺ T cells in the spleen of tumor-bearing versus non-tumor-bearing hosts (data not shown).

The intratumoral location of the GFP⁺ T cells and greater number of T cells in irradiated hosts 5 days after transfer was confirmed by histologic examination of tissue sections (Fig. 4). By contrast, there were a low number of GFP⁺ T cells in tumor-free lungs at each time point in irradiated mice and nonirradiated hosts.

To confirm that the higher number of intratumoral T cells was due to enhanced proliferation of T-effector cells, Thy 1.1 congenic tumor-draining lymph node cells were labeled with CFSE before adoptive transfer into Thy 1.2 tumor-bearing hosts. As shown in Fig. 5, on day 1 posttransfer <3% of the CD8 or CD4 Thy 1.1 cells isolated from lung tumors had proliferated in either irradiated or nonirradiated hosts irrespective of whether they were tumor-bearing or non-tumor bearing. By contrast, on day 7 postadoptive transfer 93% of CD8⁺ T cells in irradiated hosts had undergone multiple cell divisions versus 56% in nonirradiated hosts. CD4⁺ T cells showed a similar pattern of proliferation. Homeostatic proliferation was evident in irradiated non-tumor-bearing hosts; however, only 32% of CD8⁺ T cells underwent multiple cell divisions, compared with 8% of T cell proliferation in nonirradiated non-tumor-bearing hosts, similar to CD4 proliferation. In an independent experiment, BrdUrd was injected i.p. into mice at 1 hour or 120 hours after adoptive transfer and mononuclear cells were harvested from lungs 24 hours later. This experiment similarly showed incorporation of BrdUrd by <1% of T cells in any condition, on day 1 whereas 60% of Thy 1.1 cells in irradiated hosts and 50% of Thy 1.1 cells in nonirradiated hosts were BrdUrd positive at the later time point.

Total body irradiation does not augment the *ex vivo* reactivity of donor T cells isolated from tumors. Another proposed function of lymphodepletion by either total body irradiation or chemotherapy is to eliminate regulatory T cells and there is considerable evidence that regulatory T cells are present within certain types of tumors (45–47). Hosts conditioned with total body irradiation were compared with nonirradiated hosts to determine whether there was evidence for differential suppression of the function of transferred tumor-reactive T cells. Tumor infiltrating lymphocytes were isolated from lung metastases 5 days after adoptive transfer and the function of these cells was assessed directly *ex vivo*. As shown (Fig. 6A–D), GFP⁺ cells account for 73% of the cells isolated by density gradient separation from total body irradiation-conditioned hosts. By contrast, in nonirradiated hosts (Fig. 6E–H), there are 29.3% GFP⁺ T cells. The percentage of donor GFP⁺ T cells that produce IFN- γ in response to *ex vivo* MCA 205 stimulation is similar between hosts conditioned with total body

irradiation (15.1%) and nonirradiated hosts (18.1%; Fig. 6B and F). Thus, there is no demonstrable functional inhibition of transferred effector T cells, at least within the initial 5 days. Tumor regression in nonirradiated hosts is also a functional indication of the preservation of immune competence of transferred T-effector cells. The transferred T cells retain their strong IFN- γ response to anti-CD3 stimulation as do the majority of the GFP^{neg} host cells recovered from total body irradiation hosts (Fig. 6D). By contrast, the majority of host cells in nonirradiated mice do not produce IFN- γ upon *ex vivo* anti-CD3 stimulation, indicating that they have not been effectively primed in hosts with progressive tumors (Fig. 6H).

Discussion

The results presented here show that adoptive transfer of *in vitro* activated tumor-reactive T lymphocytes, alone, to lymphoreplete

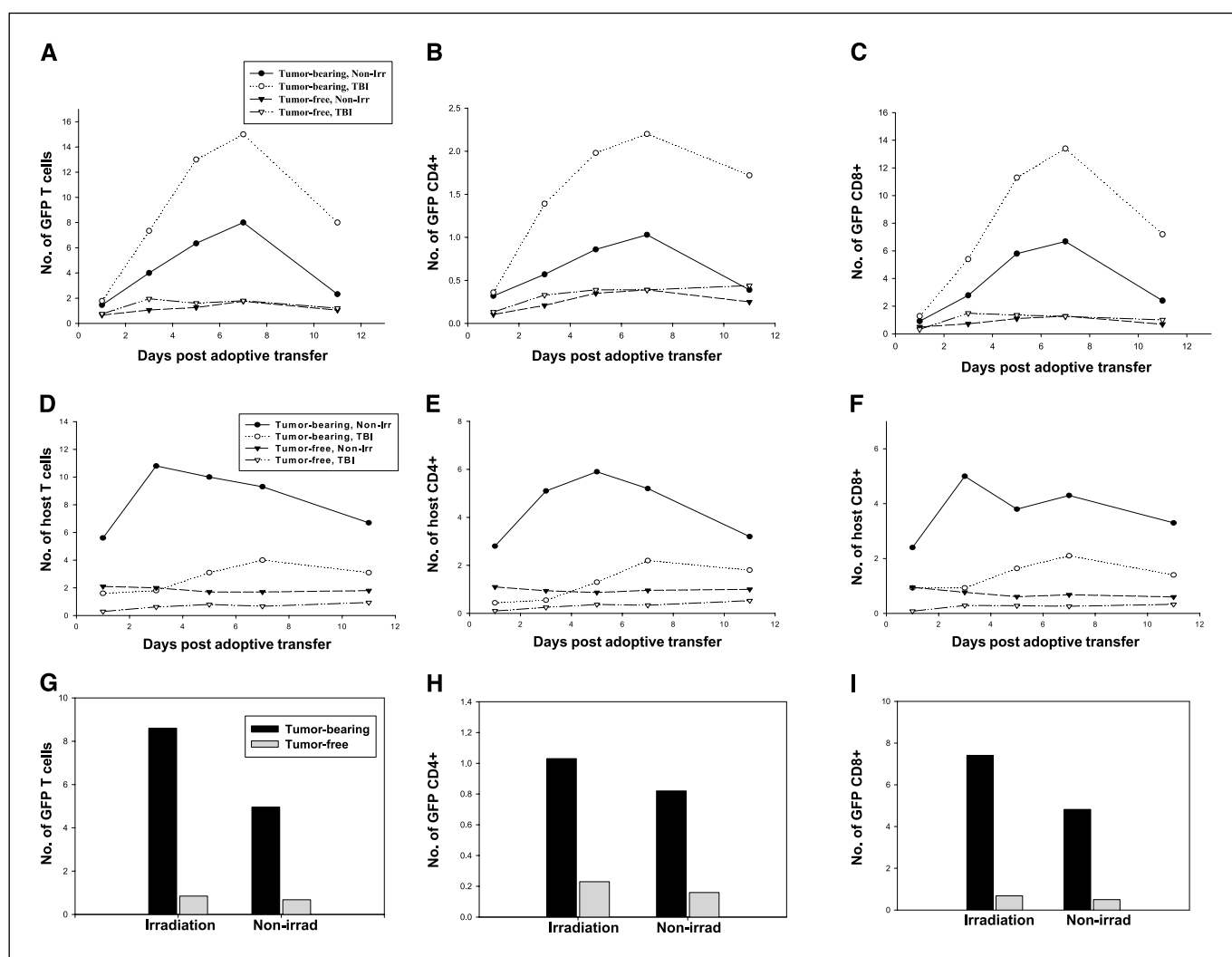


Figure 3. Kinetics of donor GFP T-cell accumulation in the lung following adoptive transfer. A, 35×10^6 activated CD62L^{low} tumor-draining lymph node cells derived from GFP mice were transferred i.v. to total body irradiation-conditioned or nonirradiated mice bearing 10-day established pulmonary tumors or tumor-free controls. At indicated time points, lungs were harvested and the number of TCR⁺GFP⁺ donor T cells was enumerated. The number of donor T cells was significantly higher in tumor-bearing compared with tumor-free hosts at every time point (irradiated hosts $P = 0.012$, nonirradiated hosts $P = 0.016$). B, higher number of donor CD4⁺ T cells in tumor-bearing versus tumor-free hosts ($P < 0.01$ for irradiated, $P = 0.033$ for nonirradiated). C, higher number of donor CD8⁺ T cells in tumor-bearing mice ($P = 0.015$ irradiated, $P = 0.031$ nonirradiated). D, E, and F, the number of TCR⁺GFP^{neg} host T cells and CD4⁺ or CD8⁺ subsets isolated from pulmonary metastases of total body irradiation-conditioned or nonirradiated tumor-bearing or tumor-free hosts is indicated. G, H, and I, an independent experiment of similar design demonstrating increased number of TCR⁺GFP⁺ donor T cells 5 days after transfer in irradiated versus nonirradiated and tumor-bearing versus tumor-free.

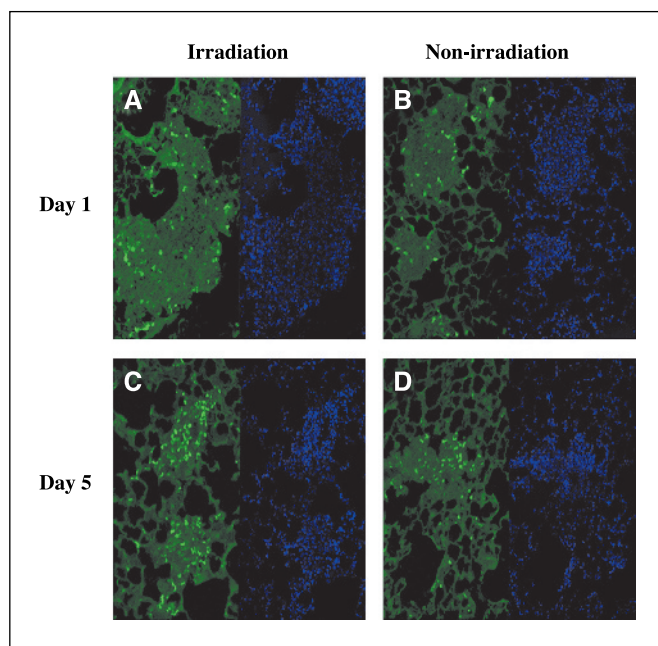


Figure 4. Adoptively transferred GFP⁺ T lymphocytes infiltrate pulmonary metastases. Mice bearing 10-day MCA 205 pulmonary metastases received total body irradiation or were nonirradiated, and then were injected with 35×10^6 CD62L^{low} T cells derived from GFP tumor-draining lymph nodes. *Left*, green fluorescent view of lung under a fluorescent microscope ($\times 200$). *Right*, 4',6-diamidino-2-phenylindole nuclear stain showing total cellular content ($\times 200$). *A* and *B*, donor T cells infiltrate pulmonary metastases in irradiated or nonirradiated hosts 24 hours after adoptive transfer. *C* and *D*, more donor T cells are present within pulmonary metastases in irradiated versus nonirradiated hosts 5 days after transfer.

hosts with advanced tumors is curative. In our previous studies, total body irradiation conditioning of hosts was routinely used for the three stringent tumor models used here. Total body irradiation was needed to achieve curative responses at the conventional doses of T cells that were prepared using our previous protocol of anti-

CD3/IL-2 activation for 5 days. Now, with the enhanced T-cell proliferation provided by anti-CD3/IL-2/IL-7 and 9-day activation period (150-fold versus 10-fold previously), it was feasible to escalate treatment doses up to 6×10^7 cells to determine the necessity of host lymphodepletion. These results clearly indicate that, although not absolutely necessary, host lymphodepletion augments the efficacy of adoptively transferred T cells. In the case of pulmonary metastases, a 3-fold higher number of T cells was required to cure nonirradiated compared with total body irradiation conditioned hosts. Similarly, for intracranial tumors, a 4-fold higher number of T cells and for s.c. tumors 12-fold higher number of T cells were required in the absence of lymphodepletion. Thus, in situations where the number of T-effector cells is limited, host lymphodepletion might convert subtherapeutic effects into curative therapy.

The augmentation of T-cell adoptive transfer by lymphodepletion is consistent with all of our previous experiments as well as findings from a number of investigators, dating back to the pioneering investigations of North et al. (24, 48). Thus, in preclinical or clinical situations where lymphodepletion is feasible, it would be anticipated to improve the overall therapeutic efficacy of T-cell adoptive immunotherapy. Indeed, there may be a role for repetitive cycles of lymphodepletion followed by adoptive transfer of aliquots of *in vitro* activated T cells. However, it is important to recognize that there are potential limitations of host lymphodepletion. Whereas lymphodepletion provides T cells with a competitive advantage for limiting growth factors such as IL-7 and IL-15, it also depletes the host immune repertoire. In older cancer patients with diminished thymic capacity for immune reconstitution, this may be a relevant consideration (49, 50).

Another putative function of host lymphodepletion is to transiently eliminate T_{reg} cells. Thus, it is possible that agents that selectively target T_{reg} cells could alleviate the detrimental effects on the immune repertoire induced by global lymphodepletion. However, selective depletion of T_{reg} cells would not provide the transferred T cells with a competitive advantage for growth factors (51). Persistence of agents that target the CD25 molecule or other

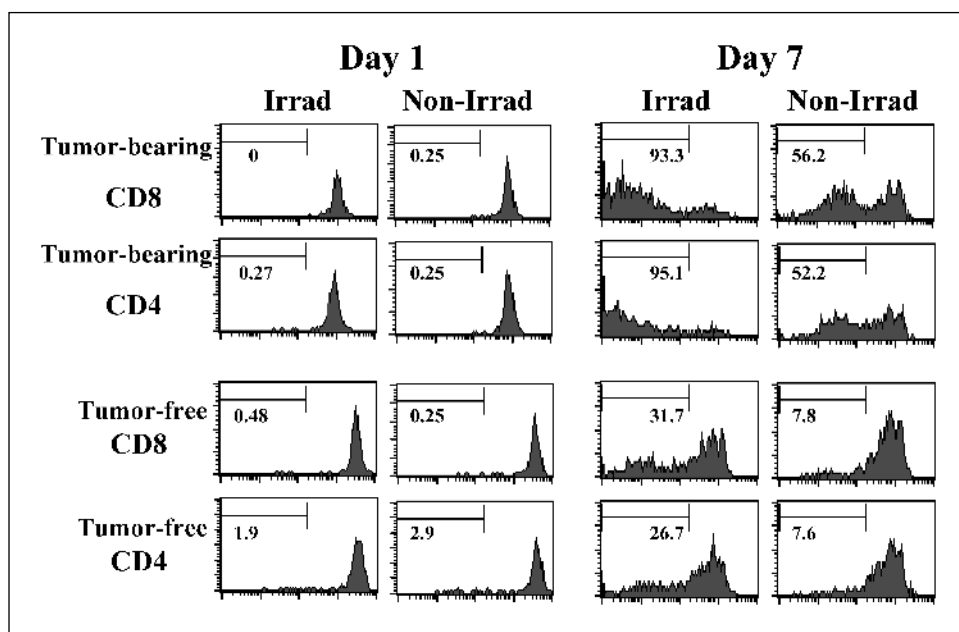


Figure 5. Proliferation of intratumoral CD4 and CD8 T-effector cells is enhanced in total body irradiation hosts. Activated T-effector cells were labeled with CFSE before adoptive transfer to mice bearing 10-day pulmonary metastases. Lungs were harvested 1 or 7 days after T-cell transfer, enzymatically digested, stained for CD4 or CD8 expression, and analyzed by FACS. The percentages of CFSE-labeled T cells that did not undergo greater than two cycles of cell division are indicated in each graph.

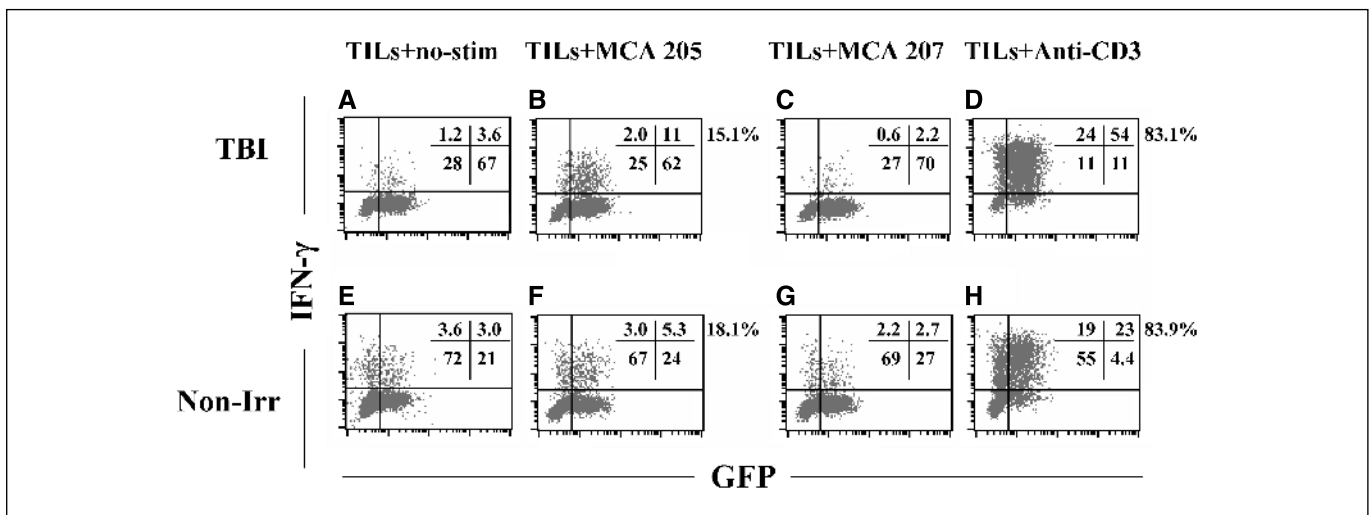


Figure 6. IFN- γ production by tumor-infiltrating donor T cells. Lymphoid cells were isolated from the lung of irradiated or nonirradiated hosts bearing 10-day established MCA 205 pulmonary tumors 5 days after adoptive transfer of GFP⁺ T cells. Freshly harvested donor T cells from total body irradiation-conditioned hosts (A-D) or nonirradiated hosts (E-H) were not additionally stimulated (A and E), stimulated with MCA 205 tumor digest (B and F), stimulated with MCA 207 tumor digest (C and G), or stimulated with anti-CD3 mAb (D and H), and analyzed for IFN- γ production. The percentage of GFP⁺ cells that secrete IFN- γ in response to tumor is similar between total body irradiation-conditioned and nonirradiated tumor-bearing hosts (15.1% versus 18.1%). The percentage of GFP⁺ T cells that responds to anti-CD3 stimulation is similar between total body irradiation-conditioned and nonirradiated hosts (83.1% versus 83.9%).

membrane proteins that are shared by T_{regs} and activated T_{eff} cells might prove counterproductive. In addition, the long-term consequences of selective depletion of T_{reg} cells have not been extensively explored, particularly in the setting of adoptive transfer of T cells reactive with normal tissue-restricted antigens. Although tissue-restricted autoimmune phenomena are considered an acceptable toxicity in the setting of an effective therapy of a life-threatening disease (13), animal models indicate that prolonged interference with regulatory networks could increase the development of serious autoimmunity.

A third theoretical advantage of host conditioning before adoptive immunotherapy is that it allows for direct cytotoxic therapy of the tumor while sparing the sequestered T cells from the toxic effects of conditioning regimen. It remains to be determined whether the doses of radiation or chemotherapy used for conventional host conditioning are sufficient and persist long enough to augment the susceptibility of tumor cells to cytolytic effector T cells. In addition, there is a paucity of data on whether recurrent tumors, which frequently acquire resistance to chemotherapy, would have the capacity to modulate Fas or TRAIL expression.

The potential difficulties in uniform application of lymphodepletion regimens in clinical situations due to patient age or prior

therapy provided one impetus for this study. These experiments were specifically designed to test, in challenging tumor models, whether effector T-cell dose escalation alone could circumvent the need for host lymphodepletion with total body irradiation. The results in each tumor model clearly show that simply providing a sufficient number of tumor-reactive T cells, even in lymphoreplete hosts with extensive tumor burden, leads to complete tumor regression. In a recent study, we have developed an approach to amplify the number of either CD4⁺ or CD8⁺ CD62L^{low} tumor-draining lymph node cells to >10⁸-fold whereas retaining polyclonality and potent effector function against tumors in intracranial or s.c. locations (21). Such an approach relieves the constraint on generating an adequate number of T cells to treat advanced disseminated tumors and the data presented here relieves the constraint of obligatory host lymphodepletion for successful adoptive immunotherapy.

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