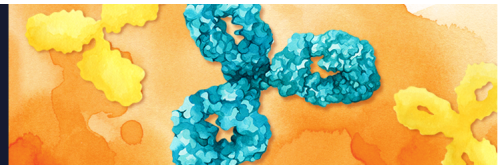


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Regression of Extensive Pulmonary Metastases in Mice by Adoptive Transfer of Antigen-Specific CD8⁺ CTL Reactive Against Tumor Cells Expressing a Naturally Occurring Rejection Epitope

Mary Hilburger Ryan, J. Andrew Bristol, Elwood McDuffie, and Scott I. Abrams¹

In this study, we developed a mouse model of adoptive immunotherapy reflecting immune recognition of syngeneic tumor cells naturally expressing an endogenous rejection Ag. Specifically, in a pulmonary metastases model, we examined the potency and maintenance of an antitumor CD8⁺ CTL response *in vivo*, as well as its effectiveness against an “extensive” tumor burden. The approach taken was to first generate tumor-specific CTL from mice challenged with the CMS4 sarcoma coadministered with anti-CTLA4 mAb, which has been shown to facilitate the induction of Ag-specific T cell responses *in vivo*. An H-2L^d-restricted nonamer peptide, derived from an endogenous murine leukemia provirus was identified as a CMS4-reactive CTL epitope based upon the following: CTL cross-recognition of another syngeneic tumor cell line (CT26 colon carcinoma) previously characterized to express that gene product; sensitization of Ag-negative lymphoblasts or P815 targets with the peptide; and by cold target inhibition assays. *In vivo*, the adoptive transfer of CMS4-reactive CTL ($\geq 1 \times 10^6$) resulted in nearly the complete regression of 3-day established lung metastases. Furthermore, mice that rejected CMS4 following a single adoptive transfer of CTL displayed antitumor activity to a rechallenge 45 days later, not only in the lung, but also at a s.c. distal site. Lastly, the adoptive transfer of CTL to mice harboring extensive pulmonary metastases (>150 nodules) led to a substantial reduction in tumor burden. Overall, these data suggest that the adoptive transfer of tumor-specific CTL may have therapeutic potential for malignancies that proliferate in or metastasize to the lung. *The Journal of Immunology*, 2001, 167: 4286–4292.

Despite extensive experimental and clinical efforts, the treatment of patients with advanced metastatic disease of solid tumors is frequently ineffective. Chemotherapy remains the only clinically relevant modality for therapy of metastatic cancer. Unfortunately, chemotherapy is highly immunosuppressive and has numerous side effects, which can be detrimental to normal tissues. These limitations reinforce the need for the development of safer and more effective strategies that better target neoplastic cells while sparing normal host cells.

The requirement for more selective cancer therapies, along with the molecular identification of tumor-specific Ags (TSA)² and tumor-associated Ags, has led to the development of immune-based strategies for the treatment of metastatic disease, which are being tested in both animal models and clinical trials (1–5). Adoptive cellular immunotherapy of cancer involving the reinfusion of patient-derived effector cells that have been generated, propagated, and expanded *in vitro* is an example of one such promising experimental therapy. However, adoptive immunotherapy in clinical studies has achieved success only in limited patient populations, the exact reasons for which remain unclear (1, 2, 6).

The principles for developing adoptive immunotherapy for human disease can be guided by insights derived from experimental animal models. In fact, a number of animal studies have been performed that show the effectiveness of adoptive cellular immunotherapy against several different tumor types (7–10). However, the vast majority of those models have not generally reflected what is seen in human disease. For example, tumor cell lines used in animal studies are often transfected with surrogate rejection Ag (7–9), which would not necessarily reflect expression or tissue distribution of an endogenously derived, relevant tumor Ag. The level of expression of these transfected Ags may also be higher than what is seen with an endogenously derived TSA. Because a surrogate Ag, such as OVA or β -galactosidase, would represent a foreign Ag in a TCR nontransgenic mouse, the T cells specific for those model Ags may also have an affinity different from that of T cells specific for naturally occurring tumor rejection Ags. Additionally, the adoptive transfer of resting CD8⁺ T cells derived from TCR transgenic animals is highly effective (7, 10), yet large numbers of T cells with the same phenotype are not typically found in a host or individual with a normal, heterogeneous T cell repertoire. Moreover, in an effort to collect adequate numbers of Ag-specific T cells in clinical situations, it would be necessary to propagate immune effector cells *in vitro* before the initiation of adoptive immunotherapy. Taken collectively, these models do not accurately parallel what would be faced in a clinical paradigm of adoptive immunotherapy.

We sought to develop a mouse model for adoptive immunotherapy that would more closely mimic the nature of the biologic components (e.g., cancer cells and T cells) confronted in a clinical setting. For example, we used a tumor cell line expressing a naturally occurring tumor rejection Ag and established an Ag-specific CD8⁺ T cell line against that tumor from TCR nontransgenic

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² Abbreviation used in this paper: TSA, tumor-specific Ag.

mice. Using this T cell-tumor cell model, we examined several fundamental principles of adoptive immunotherapy in a pulmonary metastases setting that have remained largely uncharacterized. Specifically, we explored 1) the potency of Ag-specific CTL, both in vitro and in vivo; 2) the maintenance of an antitumor response following adoptive transfer, not only at the site of primary tumor rejection, but also at a s.c. distal site based on rechallenge experiments; and 3) the antitumor activity of Ag-specific CTL in mice with extensive disease.

Materials and Methods

Mice

Female BALB/c mice were obtained from the Frederick Cancer Research Animal Facility (Frederick, MD) and were used at >6 wk of age.

Tumor cells

The CMS4 sarcoma (11) was provided by Dr. A. DeLeo (University of Pittsburgh, Pittsburgh, PA). The CT26 carcinoma was provided by Dr. N. Restifo (National Institutes of Health, Bethesda, MD). The P815 mastocytoma and BALB/3T3 fibroblast lines were obtained from the American Type Culture Collection (Manassas, VA).

Production of CMS4-specific CD8⁺ CTL

A CD8⁺ CTL line reactive with the CMS4 (H-2^d) sarcoma was established from BALB/c mice using an immunization strategy consisting of a viable tumor challenge (5×10^5 cells given s.c. in one flank) coadministered with anti-CTLA4 mAb (affinity-purified hamster anti-mouse clone UC10-4F10-11 (12); hybridoma line provided by Dr. J. Bluestone, University of California, San Francisco, CA) at 100 μ g/inoculation/mouse given i.p. on days 0, 3, and 6 posttumor transplant in a manner similar to that described in Ref. (13). Mice exposed to this regimen that failed to display evidence of primary tumor growth were rendered "tumor-immune" based on resistance to a subsequent live rechallenge on the contralateral flank (given in the absence of anti-CTLA4 mAb). Rejection of the primary and/or secondary CMS4 tumor challenge correlated with the induction of a tumor-specific CD8⁺ CTL response demonstrable from immune splenocytes. A spleen-derived CD8⁺ CTL line ($1-2 \times 10^5$ /well) was propagated in vitro in 24-well plates (Costar, Cambridge, MA) by weekly stimulation with irradiated (20 Gy) syngeneic BALB/c splenocytes (5×10^6 /well) as APC, irradiated (200 Gy) CMS4 tumor cells (1×10^5 /well) as a source of cognate Ag, and IL-2 (60 IU/ml, Tecin; Hoffman-LaRoche, Nutley, NJ).

Peptides

The AH1 peptide (SPSYVYHQF) (14) and other experimental peptides were synthesized in our laboratory using an Applied Biosystems 432A peptide synthesizer (Foster City, CA) by F-moc chemistry. Peptides were purified and analyzed by reversed-phase HPLC using a C₁₈ column (>90% purity). The peptides were dissolved in distilled water at 2 mg/ml, filter-sterilized, and stored in aliquots at -80°C .

s.c. tumor growth

CMS4 tumor cells were washed three times with HBSS before injection. Mice were given an injection of 5×10^5 tumor cells s.c. in the flank. Tumor growth was measured weekly in two dimensions, using a digital caliper. Tumor volume was calculated as previously described (15).

Cytotoxicity assays

CTL activity was assessed using a standard 4-h ⁵¹Cr-release assay. Target cells were labeled with 250 μ Ci of Na₂⁵¹Cr]O₄ (Amersham, Arlington Heights, IL). CTL were recovered from culture by centrifugation over a Ficoll-Hypaque gradient (LSM; ICN Biomedicals, Aurora, OH). CTL and radiolabeled target cells were then coincubated in 96-well, U-bottom plates (Costar), either at a constant E:T ratio with/without differing concentrations of peptide or with/without a constant peptide concentration at graded E:T ratios. For recovery of CTL following adoptive transfer, lungs from mice receiving CTL (adoptively transferred, AT, lungs) or control mice (control lungs) were enzymatically digested for 4–6 h at room temperature with a sterile enzyme mixture consisting of hyaluronidase (0.1 mg/ml), collagenase (1 mg/ml), and DNase (30 U/ml), all obtained from Sigma (St. Louis, MO). After incubation, the single cell suspension was collected, washed, and centrifuged over a Ficoll-Hypaque gradient to recover viable cells before in vitro stimulation, as described above. For cold target inhibition

assays, Con A-induced lymphoblasts, precoated with 1 μ g/ml peptide for 90 min, were used as cold targets. Peptide-coated cold targets were added in increasing numbers and incubated with CTL 40 min before the addition of ⁵¹Cr-labeled targets. Following a total incubation of 4 h, supernatants were collected using a Supernatant Collection System (Skatron, Sterling, VA). Radioactivity was quantitated using a gamma counter. Percent specific ⁵¹Cr release was calculated according to the following formula: percent specific lysis = [(experimental cpm – spontaneous cpm)/(total cpm – spontaneous cpm)] \times 100. Total ⁵¹Cr release was obtained by adding 0.2% Triton X-100 (final concentration) to the wells.

Cytokine production

Ficoll-Hypaque-purified CTL were incubated with tumor cells at a 1:3 or 1:10 ratio for 24 h in a 24-well plate (1 ml final volume). Supernatants were collected by centrifugation and maintained in aliquots at -80°C until use. Cytokine analysis was performed by ELISA using matched pairs of anti-cytokine-specific mAb (BD PharMingen, San Diego, CA) for IL-4, IL-5, IL-10, GM-CSF, TNF- α , and IFN- γ . Recombinant murine cytokine standards for IL-4, IL-5, IL-10, and TNF- α were also purchased from BD PharMingen. Recombinant murine IFN- γ was purchased from R&D Systems (Minneapolis, MN). Recombinant GM-CSF was obtained from PeproTech (Rocky Hill, NJ). Limit of detection in the assay for IL-4 was 50 pg/ml; IL-5 was 100 pg/ml; IL-10 was 500 pg/ml; TNF- α was 750 pg/ml; GM-CSF was 200 pg/ml; and IFN- γ was 200 pg/ml.

Adoptive transfer experiments

CMS4 tumor cells, previously selected by one in vivo passage in the lungs of normal, nonimmunized mice, were washed three times, resuspended in HBSS, and injected i.v. into the lateral tail vein (26-gauge needle, $1-1.5 \times 10^5$ cells in 100- μ l total volume). Three or 10 days later, CTL (4–5 days following in vitro stimulation) were prepared by centrifugation over a Ficoll-Hypaque gradient, washed three times, and resuspended in HBSS. Cells or HBSS alone were injected i.v. into the tail vein (26-gauge needle, varying numbers of cells in a 100- μ l total volume). Mice were sacrificed at the indicated times following T cell transfer. For enumeration of lung metastases, lungs were inflated with a 15% solution of India ink, resected, and fixed in Fekete's solution as described (16).

Statistical analysis

The exact Wilcoxon rank sum test was used to compare tumor growth in the naive control vs rechallenged mice. In the case of the lung metastasis setting, a two-sided *p* value was shown. In the case of the s.c. setting, two-sided *p* values were adjusted at each time point using the Hochberg method (17).

Results

Use of anti-CTLA4 mAb for induction of tumor immunity against the CMS4 sarcoma

The s.c. growth of CMS4 tumor cells is shown in Fig. 1A. Ten BALB/c mice received an inoculation of 5×10^5 CMS4 tumor cells and all of these 10 control mice exhibited s.c. tumor growth. In contrast, of 15 mice injected with 5×10^5 CMS4 tumor cells in combination with a course of anti-CTLA4 mAb (13), only one grew a tumor (Fig. 1B). Five immune and another set of five control (naive) mice were given a subsequent injection of 5×10^5 CMS4 tumor cells and monitored for tumor growth. All control mice developed tumors, whereas 0/5 mice that had previously rejected a CMS4 tumor challenge on the contralateral side developed tumors (Fig. 1C). To further confirm the establishment of systemic immunity following treatment with anti-CTLA4 mAb, CMS4-immune mice that had also rejected a second s.c. challenge with CMS4 tumor cells were inoculated i.v. with 1.5×10^5 CMS4 tumor cells; their lungs were examined for tumor nodules 2 wk later. As shown in Fig. 1D, control mice (3/3) developed tumors, whereas there was limited tumor growth in the lungs of CMS4-immune mice (average <2 nodules/lung).

Production of a murine sarcoma (CMS4)-reactive CD8⁺ CTL line

Spleen cells from mice that had rejected both a primary challenge with CMS4 tumor cells (following anti-CTLA4 mAb treatment)

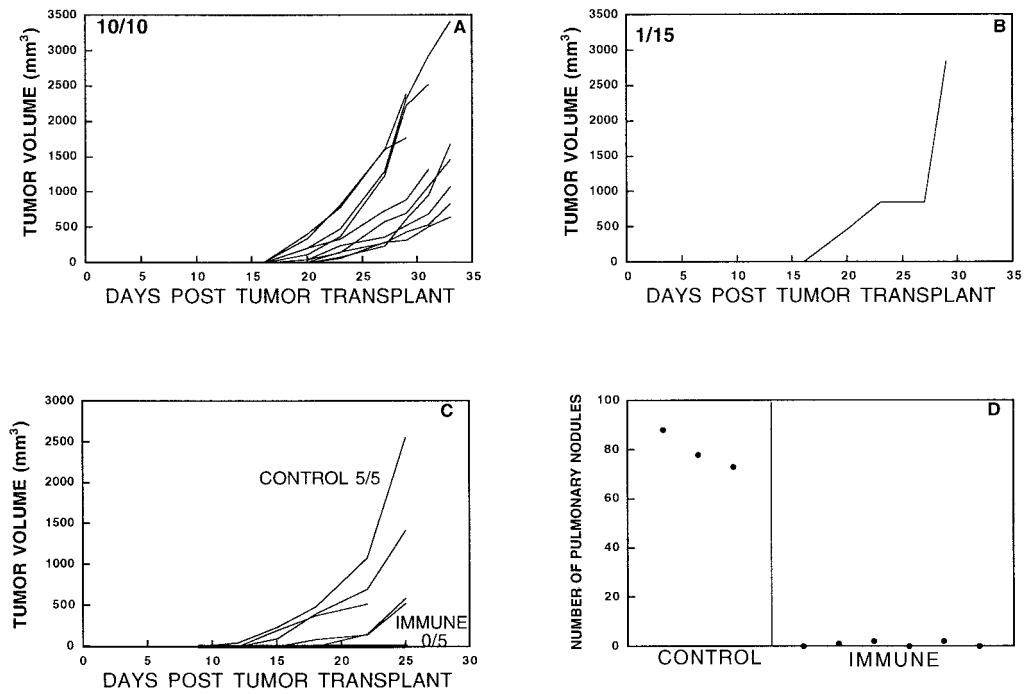


FIGURE 1. Growth of CMS4 tumor cells in vivo. *A*, Ten BALB/c mice were injected with 5×10^5 CMS4 tumor cells s.c., and tumor measurements were recorded for ~5 wk. *B*, Effect of anti-CTLA4 mAb on growth of CMS4 cells in vivo. Mice received 100 μ g i.p. of anti-CTLA4 mAb on days 0, 3, and 6 following tumor inoculation. Growth was monitored for 5 wk. *C*, Rechallenge of CMS4-immune mice. Five control BALB/c (naive) and five immune BALB/c mice that had rejected CMS4 tumor cells following anti-CTLA4 mAb treatment from *B* were inoculated with 5×10^5 CMS4 tumor cells s.c. Tumor growth was monitored for 4 wk. *D*, To further confirm the induction of systemic immunity in these mice, control mice or CMS4-immune mice that had rejected a second s.c. challenge with CMS4 tumor cells were inoculated i.v. with 1.5×10^5 CMS4 tumor cells, and the lungs were processed for enumeration of lung metastases 2 wk later.

and a secondary s.c. challenge with the same tumor cells were cultured in vitro with irradiated CMS4 tumor cells as a source of cognate Ag, and IL-2. When tested for cytolytic activity in a 4-h ⁵¹Cr-release assay, these splenocytes demonstrated specific lysis against CMS4 tumor cells (Fig. 2A). They did not lyse P815 or BALB/3T3 fibroblast cell lines. Interestingly, these cells were cross-reactive with the CT26 colon carcinoma cell line (Fig. 2A). Continued restimulation of the CTL cultures resulted in the establishment of a stable CD8⁺ CTL line, termed CMS4-reactive CTL.

CT26 tumor cells were previously shown to express a TSA, reflecting an endogenous murine retrovirus (14). An H-2L^d-restricted peptide nonamer (termed AH1, SPSYVYHQF) derived from that TSA was identified as one rejection epitope for these tumor cells. Due to the cross-reactivity of our CTL line for recognition of CT26, we tested the CMS4-reactive CTL line for recognition of the AH1 peptide epitope. As shown in Fig. 2B, the CMS4-reactive CTL line recognized and lysed P815 target cells or Con A-stimulated lymphoblasts incubated in the presence of AH1 peptide. Lysis of these targets in the absence of exogenous peptide was <10%. The peptide specificity of the CTL line for AH1 was demonstrated by cytotoxicity assays using a panel of peptides (Fig. 2C). Lysis of P815 target cells was observed only when these targets were incubated with the AH1 peptide and not a panel of peptides reflecting other tumor Ags or viral epitopes. Dose-response experiments revealed that half maximal lysis occurred at ~1 ng/ml exogenous AH1 peptide (data not shown).

Cold target inhibition assays using peptide-coated lymphoblasts as cold cells to compete for lysis of ⁵¹Cr-labeled CMS4 tumor cells confirmed that lysis of CMS4 targets by the CMS4-reactive CTL line involved immune recognition of the AH1 epitope on these tumor cells. Increasing numbers of AH1 peptide-coated lymphoblasts, but not uncoated cells or cells coated with an irrelevant

H-2L^d peptide epitope (P1A), were capable of inhibiting lysis of ⁵¹Cr-labeled CMS4 tumor cells (Fig. 3). Eighty-four percent inhibition of lysis was observed using 8×10^4 cold AH1-coated lymphoblasts, compared with 11% inhibition using the same number of P1A-coated lymphoblasts.

In addition to cytotoxicity, this CTL line was capable of producing type 1 cytokines in response to either tumor cells or peptide as a source of Ag. High levels of IFN- γ were detected in 24-h culture supernatants when the CMS4-reactive CTL line was stimulated with CMS4 tumor cells, APC/AH1 peptide, or Con A, as a positive control (Table I). No detectable IFN- γ was produced if the cell line was stimulated with P815 or APC presenting a control H-2L^d peptide, P1A. GM-CSF was also detected in the same culture supernatants (Table I), and although TNF- α was not detected in an ELISA, functional "TNF-like" activity (most likely TNF- β) was shown by a TNF bioassay using L929 cells (data not shown). No detectable IL-4, IL-5, or IL-10 was found in any of the supernatants tested.

Adoptive immunotherapy of CMS4 lung metastases by CMS4-reactive CTL line

To test the ability of the CMS4-reactive CTL line to mediate antitumor activity in vivo, mice bearing 3-day CMS4 lung metastases were treated with varying numbers of CTL. Two weeks following the adoptive transfer, lungs were removed and processed for the enumeration of lung metastases. As shown in Fig. 4, lungs of control mice had an average of 214 nodules. The adoptive transfer of as few as 3×10^4 CMS4-reactive CTL was able to reduce tumor burden in the lung by approximately half (112 ± 17.2), and optimal reduction of tumor burden was seen between 1×10^6 and 3×10^6 adoptively transferred CMS4-reactive CTL (2–6 nodules/lung). As an additional negative control, no reduction of tumor

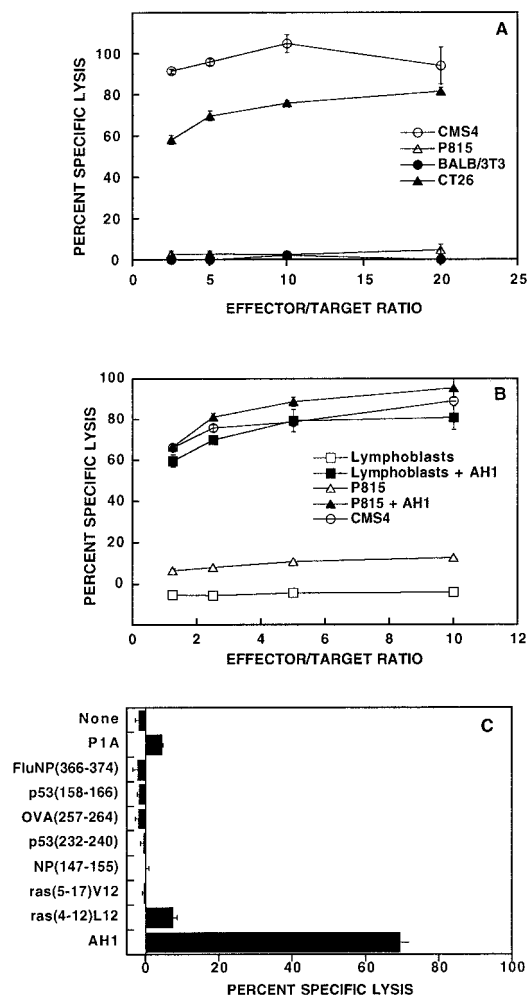


FIGURE 2. Production of a CMS4-reactive CD8⁺ CTL line. *A*, Cytotoxic activity of splenocytes cultured from CMS4-immune mice was tested against the indicated target cells. Data are the mean \pm SEM of triplicate wells. *B*, Lysis of tumor targets in the absence (open symbols) or presence (closed symbols) of AH1 peptide. Data represent the mean \pm SEM of triplicate wells. *C*, The CMS4-reactive CTL line was examined for Ag specificity by cytotoxicity against ⁵¹Cr-labeled P815 targets in the presence of a panel of peptides (10 μ g/ml) that have been reported to bind to H-2L^d Ags (AH1, P1A), H-2K^d Ags (NP₁₄₇₋₁₅₅, ras_{4-12L12}), H-2D^b Ags (p53₂₃₂₋₂₄₀, FluNP₃₆₆₋₃₇₄), H-2K^b (p53₁₅₈₋₁₆₆, OVA₂₅₇₋₂₆₄), and E α A β ^d Ags (ras_{5-17V12}). Data are the mean \pm SEM of triplicate wells for both cytotoxicity and proliferation. E:T ratio was 2:1. Data shown are representative of two independent experiments.

burden was observed when 1×10^6 Flu peptide-specific CTL were transferred to mice bearing 3-day CMS4 lung metastases. No evidence of overt toxicity was observed by adoptive transfer of Ag-specific CTL (up to 3×10^6 cells per mouse) under these conditions. Furthermore, in an ongoing long-term survival experiment, up to 4 mo post adoptive transfer of 3×10^6 CTL per mouse, no apparent evidence of toxicity caused by the immunotherapy protocol is noted ($n = 6$ mice); on the contrary, these mice still appear to be active and thriving as well as naive control mice (data not shown).

Enzymatic digestion of lungs from mice without detectable tumor burden by adoptive transfer were used to determine whether viable CTL could be recovered from the lungs following rejection of tumors. These digests were incubated with APC/AH1 peptide and IL-2 for 6 days and tested for cytotoxicity against ⁵¹Cr-labeled CMS4. Lungs from age-matched control animals were processed

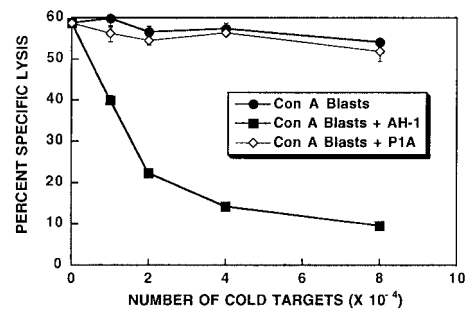


FIGURE 3. Identification of AH1 as a dominant CD8⁺ T cell peptide epitope presented endogenously by the CMS4 sarcoma line. To test the hypothesis that tumor cell killing involved AH1 expression by CMS4 tumor cells, 2×10^4 CMS4-reactive CTL were preincubated with graded numbers of cold, peptide-coated targets before the addition of 1×10^4 ⁵¹Cr-labeled CMS4 tumor cells. Data are representative of 10 separate experiments and are expressed as percent specific lysis (mean \pm SEM of triplicate cultures).

in a similar fashion and used as a negative control. As shown in Fig. 5, no detectable lysis of CMS4 tumor cells was observed with cells recovered from control lungs. However, cells digested from the lungs of mice that rejected CMS4 cells lysed $\sim 40\%$ CMS4 tumor cells at an E:T ratio of 18:1. The CMS4-reactive CTL line taken directly from culture was used as a positive control for the assay.

To determine whether the adoptive transfer of the CMS4-reactive CTL line would result in the establishment of a longer term systemic immune response, mice that had been treated for CMS4 lung metastases by the adoptive transfer of 3×10^6 CMS4-reactive CTL were divided into two groups; one group was rechallenged with CMS4 tumor cells i.v. (Fig. 6A), whereas the other group was rechallenged with CMS4 tumor cells s.c. (Fig. 6C), 45 days following the original adoptive transfer. In the first i.v. experiment, control mice challenged with CMS4 tumor cells developed an average of 161 ± 38 pulmonary nodules compared with 3 ± 2 nodules in the rechallenged mice (Fig. 6A). When the experiment was repeated, control mice developed tumors that were too numerous to count. However, the rechallenged mice developed an average of 17 ± 7 pulmonary nodules. Overall, the difference between tumor growth in naive control mice vs the rechallenged mice was highly significant ($p = 8.2 \times 10^{-5}$). In the s.c. setting, in two independent experiments, control (Fig. 6B) mice challenged with CMS4 tumor cells s.c. developed measurable tumors 11–14 days later. All mice (10/10) developed tumors, the vast majority of which continued to grow progressively. In the rechallenge group (Fig. 6C),

Table I. Cytokine production by CMS4-reactive CTL

Stimulus	Cytokine Production ^a (ng/ml/ 10^6 cells/24 h)	
	IFN- γ	GM-CSF
CMS4 ^b (1:10)	186.6 \pm 5.9	7.98 \pm 0.85
P815 ^b (1:10)	<0.2 ^c	<0.2 ^c
AH-1 ^d	294.4 \pm 47.5	28.2 \pm 4.6
P1A ^d	<0.2 ^c	<0.2 ^c
Con A ^d	475.6 \pm 21.8	19.6 \pm 2.0
None	<0.2 ^c	<0.2 ^c

^a Cytokine production by the CMS4-reactive CTL was measured in culture supernatants by ELISA as described in *Materials and Methods* and is expressed as the average \pm SEM of triplicate wells.

^b T cells were stimulated with tumor cells at an E:T ratio of 1:10.

^c Level of cytokine production was below the limit of detection of the assay.

^d T cells were stimulated with peptide (1 μ g/ml) or Con A (2.5 μ g/ml) in the presence of irradiated syngeneic spleen cells as APC.

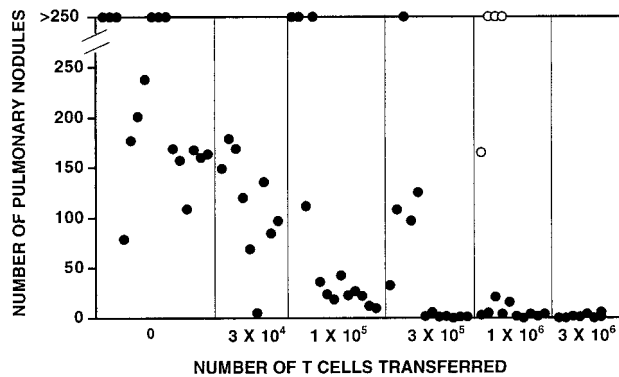


FIGURE 4. Adoptive transfer of the CMS4-reactive CD8⁺ CTL line mediates regression of 3-day established pulmonary metastases. To test the ability of the CMS4-reactive CTL line to mediate antitumor activity in vivo, BALB/c mice bearing 3-day established metastases were treated with HBSS, various numbers of CMS4-reactive CTL (filled circles), or, in one experiment, 1×10^6 FluNP-specific CTL as an irrelevant CTL control (open circles). Fourteen days following adoptive transfer of CTL, lungs were removed and processed for enumeration of metastases. Data are a summary of three independent experiments.

there was a significant difference in tumor size compared with control mice at the earlier time points ($p = 0.02$ at day 11 or 12). Although the average tumor size in the rechallenged group remained smaller than that of the control group at later time points, this was not statistically significant ($p = 0.08$ at day 17 and $p = 0.16$ at day 23). Average tumor sizes are given in Fig. 6. However, it is important to note that in the group of eight mice that were rechallenged s.c., two remained tumor-free for the duration of the experiment (Fig. 6C).

The efficacy of adoptive transfer of the CMS4-reactive CTL line to mice bearing a larger tumor burden was tested in mice with 10-day established CMS4 metastases. Three million CMS4-reactive CTL were adoptively transferred to mice that had been inoculated with 1×10^5 CMS4 tumor cells i.v. 10 days earlier (Fig. 7). Lungs were removed and the number of metastases enumerated 7, 14, or 21 days following the adoptive transfer. In the first experiment, the average number of pulmonary nodules in control mice at the time of adoptive transfer on day 10 was 189 ± 26 ($n = 5$ mice). By day 17, the number of metastases in control (HBSS) mice at day 17 was >250 , whereas mice receiving the CMS4-reactive CTL line had an average of 47 ± 8 ($n = 5$). This represented an 80% reduction of the tumor burden in these animals (Fig. 7). To examine the efficacy of this adoptive transfer paradigm in more detail, a second experiment was performed and the number of pulmonary nodules enumerated at several time points following the adoptive transfer. As shown by the open circles in Fig. 7, the pulmonary nodules in the control mice at the time of adoptive transfer on day 10 were too numerous to count and increased in size by day 17. However, the mice that received the CMS4-reactive CTL line had 117 ± 12 ($n = 5$) nodules per lung on day 17. By day 24 the number of pulmonary nodules in mice receiving the CMS4-reactive CTL line was further reduced to 35 ± 8 ($n = 4$). This number remained steady at day 31 ($n = 4$); however, the size of the nodules on day 31 was larger than on day 24 (data not shown).

Discussion

Adoptive cellular immunotherapy for metastatic disease has shown efficacy in diverse animal models, yet limited success in clinical settings has been demonstrated (1–5). Initial studies by Cheever and Greenberg (reviewed in Refs. 4 and 5) examined various prin-

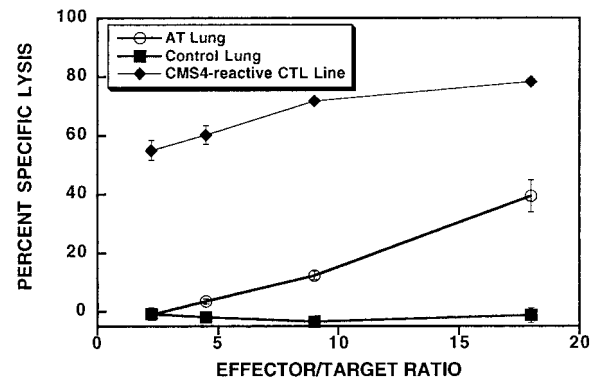


FIGURE 5. Recovery of CTL activity from the lungs of mice treated for CMS4 lung metastases by adoptive transfer. Two weeks following the adoptive transfer of the CMS4-reactive CTL line to mice bearing 3-day established CMS4 metastases, lungs were removed and processed as described in *Materials and Methods*. Control lungs from unimmunized, age-matched animals were processed in a similar manner. Cells cultured for 6 days in the presence of syngeneic splenocytes as APC, IL-2, and AH1 peptide were then tested for the ability to lyse CMS4 tumor cells in a 4-h ⁵¹Cr-release assay. The CMS4-reactive CTL line taken directly from in vitro culture was included as a control for the assay. Data represent the mean \pm SEM of triplicate wells.

ciples of adoptive immunotherapy using Ag-specific T cells in combination with a potent anti-neoplastic agent, cyclophosphamide, in a murine leukemia model. However, the principles for therapy of solid tumors may be inherently different. Subsequent studies by Shu and coworkers (18) began to address the principles for therapy of both early and later visceral metastases. In those studies, the Ag specificity of the transferred cells was undefined, and later experiments made use of polyclonally activated cells derived from tumor-draining lymph nodes (19). More recent studies of adoptive immunotherapy have used animal models that do not necessarily accurately reflect the human disease condition (i.e., tumors transduced with surrogate rejection Ag or T cells derived from TCR transgenic mice) (7–10, 20). Therefore, we sought to develop and characterize a model for adoptive immunotherapy that would more closely mimic the biology of the T cell/tumor interaction seen in the clinical setting.

An Ag-specific CD8⁺ CTL line was produced from normal BALB/c mice challenged with a syngeneic tumor (CMS4 sarcoma) coinjected with anti-CTLA4 mAb, which has previously been reported to facilitate the induction of Ag-specific T cell responses in vivo (13). Anti-CTLA4 mAb presumably functions by disallowing or disengaging the transduction of inhibitory signals triggered during the process of T cell costimulation (12, 21). Importantly, these T cells were derived from a donor host with a normal, unmanipulated TCR repertoire. An H-2L^d-restricted peptide derived from an endogenous ectopic murine leukemia provirus, previously described as a dominant rejection epitope for the CT26 colon carcinoma cell line (14), was shown to be recognized by the CMS4-reactive CTL line (Fig. 2). This is the first description of this peptide (AH1) as an epitope expressed by the CMS4 sarcoma cell line. Cold target inhibition assays confirmed that AH1 was a dominant CD8⁺ T cell epitope presented endogenously by the CMS4 sarcoma cell line (Fig. 3). Furthermore, these CTL could be stably maintained, both phenotypically and functionally, in vitro by culture with autologous tumor cells or AH1 peptide (data not shown) as a source of cognate Ag and expanded proficiently to large numbers. These are both important prerequisites for the production of

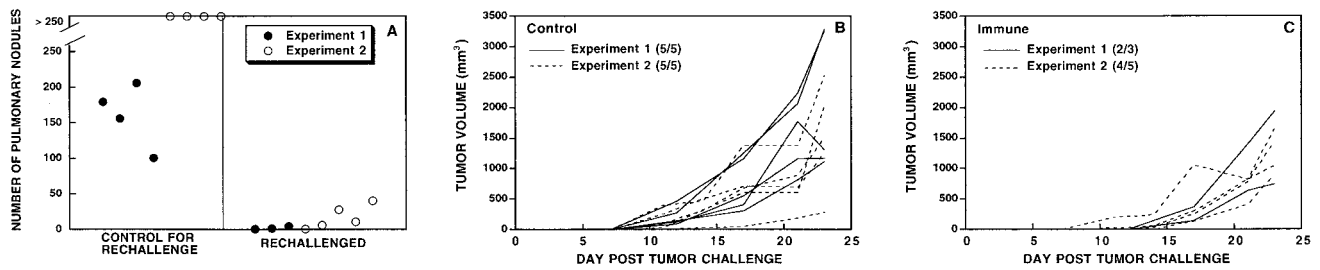


FIGURE 6. Rechallenge of mice treated for lung metastases by adoptive transfer. Mice bearing 3-day established CMS4 tumors in the lung were treated with 3×10^6 CMS4-reactive CTL and rechallenged 45 days following the initial tumor challenge. *A*, Enumeration of lung metastases 14 days following rechallenge. s.c. growth of CMS4 tumors in control (*B*) and rechallenged (*C*) mice. Average tumor sizes (in mm³) were 190.9 ± 49.1 for controls and 28.5 ± 24.7 for the rechallenged group at day 11 or 12; 716.7 ± 136.7 for the controls and 284.9 ± 120.2 for the rechallenge group at day 17; and 1783.6 ± 311 for controls and 971.8 ± 253.6 for the rechallenged group at day 23. Two of eight mice in *C* remained tumor-free, and the data for those two mice are superimposed along the x-axis.

immune effector cells for adoptive immunotherapy in clinical settings. The Ag specificity of the CMS4-reactive CD8⁺ CTL reflected CTL-mediated lysis of, or cytokine production in response to, syngeneic CMS4 tumor cells endogenously expressing the TSA. The notion that AH1 was involved in the rejection response in this T cell tumor model was further supported by the observation that immunization of BALB/c mice with AH1 peptide in adjuvant led to the production of a peptide-specific CD8⁺ CTL line, which also efficiently lysed CMS4 sarcoma cells in vitro and expressed antitumor activity in vivo by adoptive transfer (data not shown). Thus, the biological components of this model resemble more closely what would be available in a clinical setting. Using this model system, we then examined 1) the potency and efficacy of CMS4-reactive CD8⁺ CTL in adoptive therapy of pulmonary metastases; 2) the maintenance of this antitumor response following adoptive transfer, based on tumor rechallenge at the original or distal sites of neoplastic growth; and 3) the effectiveness of antitumor activity of Ag-specific CTL in mice with extensive disease.

The potency of the antitumor response mediated by the CD8⁺ CMS4-reactive CTL line in vivo was shown in the titration experiments (Fig. 4). As few as $0.3\text{--}1 \times 10^5$ CMS4-reactive CTL resulted in a reduction of tumor burden in mice with 3-day established pulmonary metastases. One million CMS4-reactive CTL resulted in the nearly complete elimination of detectable metastases, whereas the adoptive transfer of the same number of a control, influenza peptide-reactive CTL had no effect on tumor burden. CTL activity was recovered from the lungs of mice, which rejected tumor growth by adoptive transfer, suggesting that the antitumor response in vivo correlated with the presence of functional effectors in the lung (Fig. 5). Previous work from our laboratory has shown that Ag-specific CD4⁺ or CD8⁺ T cells can be recovered from spleen and lung up to 10 wk following adoptive transfer in nontumor-bearing mice, provided that these cells are exposed to Ag in vivo subsequent to the adoptive transfer (22). In the present model, Ag stimulation of the adoptively transferred cells was likely provided through recognition of tumor cells in vivo.

The efficacy of adoptive transfer for therapy of mice with more extensive pulmonary metastases was also examined. The CMS4-reactive CTL was efficient at causing tumor regression in mice with this large tumor burden (Fig. 7). Although regression was not complete and residual metastatic lesions eventually began to grow in size (data not shown), the mice survived at least 3 wk longer than control animals, which were moribund by day 17.

Hansen et al. (10) examined the effectiveness of adoptive transfer of uncultured, resting CD8⁺ T cells for therapy of s.c. tumors expressing an endogenous tumor rejection Ag. They were able to show that adoptive transfer was effective in mice with 3-day (ear-

ly), but not 5-day (late), s.c. established tumors. In our model, therapeutic efficacy by adoptive transfer against 3-day established s.c. tumors could not be demonstrated (data not shown). However, it is important to point out that there was at least partial immunity against a s.c. tumor cell challenge in mice that had previously rejected tumor growth in the lung by adoptive transfer (Fig. 6C). In addition, the CMS4-reactive CTL line was highly effective in the treatment of both early (day 3) and late (day 10) visceral tumors (Figs. 4 and 7). Although both models use Ag-specific T cells against tumors expressing endogenous tumor Ag, Hansen et al. (10) used resting CD8⁺ splenocytes from a TCR transgenic mouse. However, in a clinical setting, patient-derived T cells would need to be expanded in vitro to generate adequate numbers of Ag-specific T cells for adoptive transfer. Thus, it is important to study the in vivo properties of T cells cultured under those conditions.

It is also important to note that the adoptive transfer experiments described here were conducted without exogenous cytokines (e.g., IL-2), Ag or peptide boosting, or immunosuppressive drugs (e.g., cyclophosphamide), and with only one cycle of adoptive transfer. The addition of these reagents or multiple cycles of adoptive transfer may potentially improve therapeutic responses in cases of more

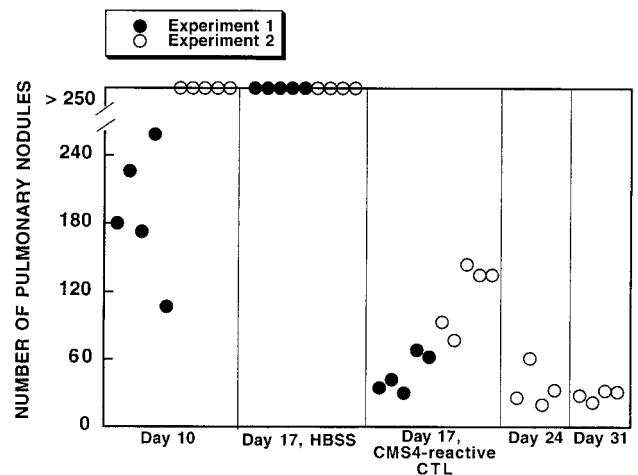


FIGURE 7. In vivo antitumor effect of CMS4-reactive CTL on a more extensive tumor burden. To determine the efficacy of adoptive transfer of the CMS4-reactive CTL line to mice bearing a larger tumor burden, mice with 10-day established CMS4 metastases were given 3×10^6 CTL or HBSS as a control. Lungs were removed, and the number of metastases were enumerated 7, 14, or 21 days later.

aggressive tumor-bearing conditions, such as an extensive or systemic tumor burden (Figs. 6 and 7). Although the exact mechanisms of tumor rejection in the lung, as well as at the s.c. distal site, require further elucidation, the adoptive transfer of Ag-specific CD8⁺ CTL can play an important regulatory and/or effector role in the eradication of pulmonary metastases. In our model, the initial rejection response also renders these mice tumor-immune, based on the rejection of new tumor transplants given several weeks later (Fig. 6). This was evident not only in the lungs of these animals (Fig. 6A), but also at distal (s.c.) sites (Fig. 6C), albeit to a lesser extent. This finding may have implications for the control of tumor growth in the event of disease recurrence at systemic sites. The fact that these CTL produce copious amounts of immunoregulatory cytokines in response to Ag stimulation, such as IFN- γ and GM-CSF, suggests that these, as well as other lymphokines, may be involved in the tumor rejection response.

Overall, we have described a model of adoptive immunotherapy for advanced pulmonary metastases using Ag-specific CTL and a tumor expressing an endogenous tumor rejection Ag. Highly potent, Ag-specific CTL were generated from tumor-immune mice and were stably propagated *in vitro*. Using this T cell-tumor model, we have shown that 1) tumor-reactive CD8⁺ CTL are highly effective in the adoptive therapy of pulmonary metastases; 2) adoptively transferred CTL are important for promoting or eliciting antitumor responses, not only at the initial site of tumor burden, but also at a s.c. distal site; and 3) tumor-reactive CD8⁺ CTL can mediate antitumor activity in mice with extensive pulmonary disease (day 10 metastases). Overall, these data suggest that the adoptive transfer of tumor-specific CTL may have therapeutic potential for immunotherapy of certain types of malignancies, such as those that might metastasize to the lung.

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