Induction of Persistent Tumor-protective Immunity in Mice Cured of Established Colon Carcinoma Metastases

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ABSTRACT

The induction of tumor-specific T-cell responses that are effective in eradicating disseminated tumors and in mounting a persistent tumor-protective immunity is one of the major goals of tumor immunotherapy. Here, we demonstrated that we achieved this goal by directing interleukin 2 (IL-2) to the tumor microenvironment of colon carcinoma metastases in syngeneic mice with a recombinant antibody-IL-2 fusion protein (huKSI/4-IL-2). Eradication of established pulmonary metastases is induced by a CD8+ T cell-mediated immune response, which can be transmitted to naive syngeneic severe combined immunodeficient mice by adoptive transfer of CD8+ T cells from immune animals. This immune response was followed by the induction of a long-lived immunity against challenge up to 5 months later with CT26-KSA or wild-type CT26 murine colon carcinoma cells in BALB/c mice. This memory immune response was confirmed by flow cytometric analyses of CD8+ T cells isolated from secondary lymphoid tissue that revealed a phenotypic profile typical of early memory T cells. This long-lived protective tumor immunity was successfully boosted to become optimally effective in all experimental animals by injections of noncurative doses of IL-2 fusion protein 4 days after challenge with tumor cells. Taken together, our results indicate that the huKSI/4-IL-2 fusion protein elicits a long-lived cellular memory immune response that can be amplified by additional applications of IL-2 fusion proteins. This approach could become useful for the treatment of colorectal carcinoma in an adjuvant setting, particularly in patients with minimal residual disease.

INTRODUCTION

Several different strategies have been explored to achieve the induction of a T cell-mediated antitumor response that effectively eradicates disseminated metastases and induces a persistent, tumor-protective host immune response. Thus, cytokines like rhIL-2 have been used, based on initial findings two decades ago that defective T-cell activation could be restored by cytokines (1). In this regard, rhIL-2 was successfully administered either as high-dose immunotherapy or in combination with rhIL-2-activated patients' lymphocytes in the treatment of advanced melanoma and renal carcinoma (2, 3). Intratumoral injections of rhIL-2 overcame some of the dose-limiting side effects of its systemic application and induced growth suppression of malignant melanoma (4). Another form of local cytokine therapy has been developed by transduction of cytokine genes with viral vectors into tumor cells (5, 6). In vivo injections of such cytokine-producing tumor cells, obtained from individual patients, achieved local cytokine concentrations that were effective in generating a host immune response resulting in local inflammatory responses and the elimination of the injected tumor cells. Systemic immune responses were generated in some animal models against challenge with the wild-type parental tumor (7–10). This gene therapy approach takes advantage of the paracrine nature of most cytokines that function best within a few cell diameters from their cell of origin (5). To comply with the paracrine nature of most cytokines (11), we developed an alternative approach to direct cytokines preferentially to the tumor microenvironment by a simple modus operandi that unlike gene therapy, is not limited by being patient specific. We have established a proof of principle in syngeneic mouse tumor models to achieve these objectives and to eradicate established, disseminated metastases of neuroblastoma (12) and melanoma (13) with recombinant antibody-IL-2 fusion proteins. Immune mechanisms in the melanoma model involved primarily CD8+ T cells, possibly with CD4+ T cell help, and natural killer cells as major effectors in the neuroblastoma model. More recently, we demonstrated that a recombinant, humanized antibody-IL-2 fusion protein (huKSI/4-IL-2) achieved sufficient concentration in the tumor microenvironment to induce an MHc class I-restricted CD8+ T cell-mediated eradication of established hepatic and pulmonary metastases of colon carcinoma (CT26-KSA) in syngeneic BALB/c mice (14).

Here, we extended these findings by demonstrating that an immunocytokine-induced tumor-specific T-cell response is followed by a long-lived tumor protective immunity. The therapeutic effect of the huKSI/4-IL-2 fusion protein is associated with an increased expression of early T-cell activation markers, followed by a complete eradication of CT26-KSA tumor metastases only 5 days after completion of fusion protein treatment. CD8+ T cells isolated at that time elicited a transferable immunity in T cell-deficient syngeneic SCID mice. We demonstrated, for the first time, that mice cured of CT26-KSA metastases developed a long-lived tumor-protective immunity upon challenge with either CT26-KSA or CT26 wild-type tumor cells, protecting at least 50% of the animals. This response was associated with the detection of CD8+ T cells with memory phenotype. We also showed that this memory immune response is amplified by boosting with antibody-cytokine fusion proteins, as indicated by complete protection of all experimental animals against challenge with either CT26-KSA or CT26 wild-type tumor cells. The effect of this treatment on memory T-cell reactivation was indicated by the presence of IFN-γ, IL-12, TNF-α, and cytotoxic CD8+ T cells in secondary lymphoid tissue, exclusively in mice successfully boosted with fusion protein.

MATERIALS AND METHODS

Animals, Cell Lines, and Reagents. Female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained under specific pathogen-free conditions and used for experiments when they were 7 weeks old. All experiments were performed according to the NIH Guidelines for the Care and Use of Laboratory Animals.

CT26, a colon epithelial tumor cell line, derived by intrarectal injection of N-methyl-N-nitrosourea-bis(methylurethane) in BALB/c mice, was kindly provided by Dr. I. J. Fidler (M. D. Anderson Cancer Center, Houston, TX). The KSA (or Ep-CAM) is recognized by mAb KS1/4 (13) and was cloned by several groups (15, 16). Cloning and cell surface expression of KSA on CT26-KSA cells have been
described previously (17). A subclone stably expressing both KSA and MHC class I antigens H-2Kb, H-2Dd CT26-KSA, was selected for the studies described here. Expression of KSA remained stable in culture and on CT26-KSA cells propagated as tumors in BALB/c mice (14). CT26 and CT26-KSA cells were maintained in DMEM supplemented with 10% fetal bovine serum, vitamins, L-glutamine, sodium pyruvate, and nonessential amino acids at 37°C and 7.5% CO2. rhIL-2 was obtained from Chiron (Emeryville, CA; specific activity, 16 × 10⁴ units/mg). All reagents were free of endotoxin, as determined by the Limulus amebocyte lysate assay purchased from BioWhittaker (Walkersville, MD).

Antibodies and Fusion Proteins. The generation of the murine KS1/4 antibody recognizing KSA was described previously (18), as was the subsequent construction of the recombinant humanized huKS1/4-IL-2 fusion protein (17). The huKS1/4-IL-2 fusion protein and mAb huKS1/4 revealed identical binding patterns with CT26-KSA cells with an average Kd of 1.15 nm and recognized 3.8 × 10⁵ KSA-binding sites/cell (14). The concentration of IL-2 in the huKS1/4-IL-2 fusion protein was calculated as two molar equivalents of IL-2 per model of fusion protein. A similar strategy was used to construct the chimeric rat/mouse antitransferrin receptor antibody-IL-2 fusion protein, ch17217-IL-2 (19). Briefly, recombinant mouse IL-2 was engineered to the COOH terminus of a chimeric rat/mouse antitransferrin receptor antibody, ch17217, and expressed in Chinese hamster ovary cells. Expressing clones were identified, expanded, and purified on protein A-Sepharose.

Induction of Experimental Metastases. Pulmonary metastases in syngeneic BALB/c mice were induced following i.v. injection of 5 × 10⁶ CT26-KSA cells. After 4 days, microscopically established metastases were present throughout the lung tissue. Grossly visible metastases were detectable on the surface of the organs 28 days after tumor cell injection, at which time the animals were sacrificed and examined for metastases. Lungs were placed in Bouin's fixative and examined under a low-magnification microscope for tumor foci on the surface of the lungs. Because such foci appeared fused, metastases were scored according to the percentage of lung surface involvement as follows: 0, 0%; 1, <5%; 2, 5-50%; and 3, >50%. Lung specimens of these mice without macroscopic metastases were stained with H&E and examined histologically. Some of the samples were snap-frozen at −70°C for subsequent RNA isolation.

RNA Isolation and RT-PCR Assay. Reverse transcription and subsequent PCR of the KSA used for detection of minimal residual disease was performed, as described previously (14). Briefly, RNA was extracted by the guanidine-phenol-chloroform method and then treated with RNase-free DNase I to avoid contamination with genomic DNA. Reverse transcription with oligo(dT) primers was performed with 1 μg of total RNA in the presence of Moloney murine leukemia virus reverse transcriptase. An equivalent of 1 μg of cDNA was used in a 50-μl PCR mixture for amplification of the KSA (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C). The primers used for detection of human KSA were 5′-CAA TGC CAG TGT ACT TCA TCA TTT-3′ (sense) and 5′-ATA AGC CAC ATC AGC TAT GTC-3′ (antisense), leading to the creation of a 471-bp fragment. If amplification did not reveal KSA signals, the cDNA integrity was tested by amplification of glyceraldehyde 3-phosphate dehydrogenase as a control.

Preparation of Lymphocytes from Draining Lymph Nodes. Draining lymph nodes were obtained from mice bearing s.c. tumors for ease of preparation because, in mice bearing lung metastases, such lymph nodes are barely accessible. Briefly, tumors were induced by bilateral s.c. injection of 5 × 10⁶ CT26 KSA cells to establish pulmonary metastases. Four days after inoculation, established tumors were treated by daily i.v. injections (>7) of 15 μg of huKS1/4-IL-2 fusion protein, an equivalent mixture of antibody and IL-2 or PBS. After completion of treatment, axillary lymph nodes were removed, and lymphocytes were prepared for subsequent FACS analysis.

Flow Cytometry Analysis. Two-color flow cytometric analyses were performed with single-cell suspensions prepared from lymphatic tissues. Anti-CD69 (clone H1.2F3) and anti-CD25 (clone 7D4) were used in phycoerythrin-conjugated form, in combination with FITC-conjugated antimouse mAb CD3e (clone 145-2C11). As immunoglobulin isotype controls, FITC-rat IgMxR (R4-22) and hamster IgG anti-TNP (G235-2356) were used. Additional rat anti-mouse antibodies used included CD45RB (clone 16A; IgG2a, κ), CD62L (clone MEL-14; IgG2a, κ), CD44 (clone IM7; IgG2b, κ), Ly-6c (clone AL-21; IgM, κ), CD4 (RPA-T4; IgM, κ), and CD8 (HT18; IgG1, κ). The lymphocytes were incubated for 1 h at 4°C with FITC- and phycoerythrin-labeled antibodies and then washed and analyzed immediately with a Becton Dickinson (Bedford, MA) FACScan. A total of 10,000 labeled cells per sample was analyzed. All labeled antibodies were purchased from PharMingen (San Diego, CA).

Adoptive Transfer of Lymphocytes. Mice that were successfully treated with huKS1/4-IL-2 fusion protein served subsequently as donors of lymphocytes for adoptive transfer experiments. These animals were killed 16 days after tumor cell inoculation, followed by the preparation of single-cell suspensions by mincing freshly resected spleens and passing them through a steel sieve. Lymphocytes were separated by Ficoll-Hypaque gradient centrifugation (600 × g, 20 min). Subsets of CD4+ and CD8+ T cells were isolated by magnetic-activated cell sorting (Miltenyi, Auburn, CA). Briefly, lymphocytes were labeled with paramagnetic anti-CD4 or anti-CD8 Micro Beads and isolated with a Mini MACS Separation unit, according to the manufacturer's guidelines. Purities of CD4+ and CD8+ T cells were >95%, as determined by FACS analyses. Naïve syngeneic SCID mice were reconstituted with a total of 4.5 × 10⁶ CD4+ and CD8+ T cells by i.v. injection of 1.5 × 10⁶ T cells of each subset on days −3, −1, and +3. All mice were challenged by i.v. injection of 5 × 10⁶ CT26-KSA cells to initiate pulmonary metastases.

Tumor Cell Challenge of huKS1/4-IL-2-Treated BALB/c Mice. BALB/c mice with established pulmonary metastases 4 days after i.v. injection of 5 × 10⁶ CT26-KSA colon carcinoma cells were treated with daily i.v. injections of huKS1/4-IL-2 fusion protein (15 μg each). Mice were subsequently challenged after various time intervals (4, 6, 8, 10, 12, 16, and 20 weeks) with either 1.5 × 10⁶ CT26-KSA or 3 × 10⁶ CT26 wild-type cells, respectively. Animals were sacrificed 28 days after challenge and analyzed for macroscopic metastases and minimal residual disease by microscopic and RT-PCR analyses, respectively.

Cytokine Release Assay. Draining lymph nodes from axial, inguinal, mediastinal, and mesenteric regions were collected from the experimental group of BALB/c mice cured of pulmonary colon carcinoma metastases by huKS1/4-IL-2 fusion protein; challenged after 24 weeks with CT26 tumor cells; and boosted 4 days thereafter with noncurative doses of the KS1/4-IL-2 fusion protein (5 μg, ×2), ch17217-IL-2 fusion protein (15 μg, ×5), or PBS for 4 weeks after challenge. Lymphocytes were isolated on Ficoll-Hypaque (BioWhittaker, Walkersville, MD) and cultured for 4, 16, 24, and 48 h in complete DMEM. Supernatants harvested and obtained from each group of mice at these time points were assayed for IFN-γ, TNF-α, and IL-12, respectively, with commercially available cytokine detection kits (Biosource International, Camarillo, CA) and a solid-phase sandwich ELISA.

Cytotoxicity Assay. Cytotoxicity was measured in a standard 51Cr-release assay. CT26-KSA and CT26 target cells (3 × 10⁶) were each labeled with 0.5 mCi of 51Cr for 2 h at 37°C. The 51Cr-labeled tumor target cells (5 × 10⁶) were washed three times in DMEM, suspended in the same medium, and added to each well of a U-bottomed microtiter plate. Effector cells were isolated from spleens of BALB/c mice bearing pulmonary metastases and treated 4 days after i.v. inoculation of 5 × 10⁶ CT26-KSA colon carcinoma cells with seven daily i.v. injections of 15 μg of huKS1/4-IL-2 fusion protein. Splenocytes were incubated for 3 days at 37°C in 25 ml of complete DMEM containing 1 ml of T-STEM culture supplement (Becton Dickinson). CD8+ T cells were purified by magnetic activated cell sorting (Miltenyi). Briefly, splenocytes were labeled with paramagnetic anti-CD8 Micro Beads and separated with the Mini MACS Separation Unit, according to the manufacturer's guidelines. Purities of CD8+ T cells was >95%, as determined by FACS analyses. Incubation with effector cells was done at different E/T ratios at 37°C for 4 h. The percentage of specific tumor cell lysis was calculated using the formula: ([E − S]/S) × 100, where E is the average experimental release, S is the average spontaneous release, and T is the average total release.

Statistical Analysis. The statistical significance of differential findings between experimental groups was determined by Student's t test. The non-parametric Wilcoxon rank sum test was chosen when the data for life span and metastatic scores were not amenable for parametric tests. Findings were regarded as significant, if two-tailed Ps were ≤0.05.

RESULTS

Treatment with huKS1/4-IL-2 Fusion Protein Eradicates Established Pulmonary Metastases of CT26 KSA and Increases Life Span of BALB/c Mice. We reported previously that the huKS1/4-IL-2 fusion protein induced a CD8+ T cell-mediated immune re-
The time course for elimination of CT26-KSA pulmonary metastases was established by this highly sensitive RT-PCR assay detecting KSA mRNA after the mice (n = 4) were sacrificed on days 13, 14, 15, and 16 after tumor cell inoculation. Fig. 2 indicates that, on day 13, KSA mRNA was detected in the lung tissue of all mice that received the huKS1/4-IL-2 fusion protein. On days 14 and 15, after tumor cell inoculation, some mice showed less or no detectable mRNA; however, on day 16, i.e., 5 days after completion of the fusion protein treatment, all four mice were free of tumor metastases detectable by RT-PCR.

Activation of T Cells in the Draining Lymph Nodes. On the basis of the above finding indicating complete elimination of established, pulmonary metastases 5 days after completion of the fusion protein treatment, we determined whether this antitumor response involved the activation of T cells in the draining lymph nodes of these BALB/c mice. Early activation of T cells was demonstrated by two-color flow cytometry analyses of single-cell suspensions prepared from draining lymph nodes, revealing that the early T-cell activation marker, CD69, peaked on day 5 of fusion protein treatment and reached background levels on day 7 of treatment (Fig. 3). In addition, CD25, a marker for IL-2 receptors, was found in progressively increasing amounts on days 5 and 7 of treatment on lymphocytes from draining lymph nodes of both fusion protein- and PBS-treated mice. Naive mice expressed only background levels of these antigens, as did tumor-bearing mice on day 0, i.e., 4 days after tumor cell inoculation (Fig. 3).

Adoptive Transfer of CD8+ T Cells from Immune Mice to Naïve Syngeneic SCID Mice Results in Tumor-protective Immunity. We previously reported that CD8+ T cells are essential for the eradication of established pulmonary metastases of CT26-KSA colorectal carcinoma cells (14). To characterize the cells responsible

Fig. 1. Life spans of experimental groups of mice. A, Kaplan-Meier plot depicting survival of BALB/c mice injected i.v. with 5 × 10⁶ CT26-KSA colon carcinoma cells, followed 4 days thereafter with either daily i.v. injections (×7) of 15 μg of huKS1/4-IL-2 fusion protein; nonspecific ch14.18-IL-2 fusion protein; a mixture of 15 μg of huKS1/4 and 45,000 units of rhIL-2; or PBS. B, RT-PCR analysis of KSA in lung tissues of BALB/c mice, treated with huKS1/4-IL-2 fusion protein and remaining cured of pulmonary metastases of CT26-KSA tumors 24 weeks after tumor cell inoculation. Analysis of eight BALB/c mice free of macroscopically detectable tumor metastases is shown in lanes 1–8; CT26-KSA cells are shown as a positive control (lane 9). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is shown as a control amplified with samples lacking a KSA signal.

Fig. 2. Time course of eradication for CT26-KSA pulmonary metastases in huKS1/4-IL-2 fusion protein-treated BALB/c mice. RT-PCR analysis of KSA in lung tissues of mice (n = 4) treated with huKS1/4-IL-2 fusion protein are shown on days 13 (lanes 1–4), 14 (lanes 5–8), 15 (lanes 9–12), and 16 (lanes 13–16) after tumor cell inoculation. CT26-KSA cells are shown as a positive control (lane 17).

Fig. 3. Activation of T cells in draining lymph nodes of BALB/c mice. A, FACS analysis profile of T cells expressing early activation antigen CD69 on days 0, 5, and 7 of treatment with huKS1/4-IL-2 fusion protein. B, FACS analysis profile of T cells expressing IL-2 receptors, indicated by expression of CD25 on days 0, 5, and 7 of treatment with huKS1/4-IL-2 fusion protein.
Evidence of T-Memory Cells in Mice with Persistent Tumor-protective Immunity. A typical phenotype of T-memory cells and antigen-activated effector T cells was observed in BALB/c mice that mounted a persistent tumor-protective immunity following the complete rejection of a CT26-KSA tumor cell challenge 6 weeks after the original tumor cell inoculation (Fig. 4). Thus, CD8+ T cells isolated from splenocytes of such mice displayed this phenotype including increased expressions of CD44 (CD44V6) and Ly-6C (Fig. 4, A and B, respectively) at high densities and reduced expression of L-selectin (CD62L) and the restricted isoform of CD45, i.e., CD45RB (Fig. 4, C and D). In contrast, analysis of CD8+ T cells from splenocytes of naïve BALB/c mice showed the reverse phenotype (Fig. 4).

Long-lived Tumor-protective Immunity Is Boosted by Noncurative Doses of an Antibody-IL-2 Fusion Protein. The data depicted in Table 2 indicate that the eradication of CT26-KSA pulmonary metastases induced in BALB/c mice by treatment with the huKS1/4-IL-2 fusion protein was associated with a long-lived protective immunity upon challenge with CT26-KSA or CT26 wild-type tumor cells. Although complete or partial rejection of the tumor challenge occurred in at least 50% of the mice (n = 8), there were always one to three animals that completely failed to reject the tumor cells. Thus, we tested the hypothesis that boosting with noncurative doses of either huKS1/4-IL-2 or with ch17217-IL-2, a second antibody-IL-2 fusion protein (anti-TfR-IL-2) that is specific for mouse transferrin receptor, will result in tumor rejection in all experimental animals after tumor challenge. The data shown in Table 3 indicate that this was, indeed, the case when BALB/c mice (n = 8) that had been tumor free for 6 weeks after treatment with huKS1/4-IL-2 were challenged at this time point with CT26-KSA, followed after 4 days with two noncurative doses of huKS1/4-IL-2 fusion protein. Thus, 4 weeks after this boost, all eight mice completely rejected the tumor cell challenge. A control group of mice (n = 8) that was challenged with CT26-KSA tumor cells after 6 weeks but received PBS instead of the KS1/4-IL-2 fusion protein, exhibited metastases in all eight mice, 4 weeks after tumor cell challenge. The same tumor protective immunity was seen in a more stringent test when mice (n = 8) that had been tumor free for 24 weeks after having been treated with huKS1/4-IL-2 fusion protein were challenged at this time point with CT26 wild-type tumor cells, followed after 4 days with noncurative metastases.

Table 1 Horizontal transmission of tumor immunity by adoptive transfer of T lymphocytes

<table>
<thead>
<tr>
<th>Donor</th>
<th>Therapya</th>
<th>Transferb</th>
<th>Metastatic score</th>
<th>Lung weight (g)a</th>
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</thead>
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<tr>
<td>BALB/c</td>
<td>PBS</td>
<td>CD8+</td>
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<td>0.88 ± 0.06</td>
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<td>CD8+</td>
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<td>0.55 ± 0.28</td>
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<td></td>
<td>KS1/4 IL-2</td>
<td>CD8+</td>
<td>0.0,0,0</td>
<td>0.24 ± 0.07</td>
</tr>
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</table>

a Lung metastases were induced by i.v. injection of 5 x 104 CT26-KSA cells into BALB/c mice, which served subsequently as donors for the lymphocyte transfer. Treatment of these animals with daily i.v. injections of 15 μg of huKS1/4-IL-2 fusion protein for 7 consecutive days was started on day 4 after tumor cell inoculation. Control animals were treated in parallel with either PBS or a mixture of 15 μg of huKS1/4 and 45,000 units of rhIL-2.

b All donor animals were sacrificed 16 days after tumor cell inoculation, and CD8+ T cells were isolated from their pooled spleen cell suspensions by magnetically activated cell sorting. Subsets of CD4+ and CD8+ T cells were isolated from fusion protein-treated mice by the same procedure. Forty-eight h prior to i.v. challenge with 5 x 107 CT26-KSA tumor cells, naïve syngeneic SCID mice were reconstituted with a total of 4.5 x 107 CD4+ or CD8+ T cells by i.v. injection of 1.5 x 107 T cells of each subset on days −3, −1, and +3.

c Animals were sacrificed 28 days after tumor cell challenge and evaluated for pulmonary metastases. Results are given as metastatic score: 0, 0%; 1, <5%; 2, 5-50%; and 3, >50% of lung surfaces visibly covered with metastatic foci.

d Differences in metastatic scores and lung weights between the fusion protein group of mice adoptively transferred with CD8+ T cells and control groups were statistically significant (P < 0.02).

Long-lived Tumor-protective Immunity Is Associated with Eradication of Pulmonary CT26-KSA Metastases by the huKS1/4-IL-2 Fusion Protein. We tested the hypothesis that CT26-KSA tumor eradication induced by the huKS1/4-IL-2 fusion protein may be associated with the induction of a persistent tumor-protective immunity. This was demonstrated by challenging mice, previously cured of pulmonary metastases by huKS1/4-IL-2 fusion protein treatment with both CT26-KSA and CT26 wild-type cells (Table 2). Treatment with the huKS1/4-IL-2 fusion protein conferred either complete or partial tumor protection in at least 50% of the mice (n = 8) in each experimental group, even after tumor challenges with CT26-KSA cells up to 5 months later and with CT26 cells up to 4 months later. All mice with a metastatic score of 0 following macroscopic examination also lacked detectable tumor cells by histology and RT-PCR 4 weeks after challenge with CT26-KSA cells and revealed no histologically detectable tumors after challenge with CT26 wild-type cells (data not shown). All naïve control mice injected with either CT26-KSA or CT26 cells and treated only with PBS exhibited extensive pulmonary metastases for 4 weeks (Table 2).

Table 2 Induction of tumor-protective immunity by huKS1/4-IL-2 fusion protein-mediated eradication of established pulmonary metastases

<table>
<thead>
<tr>
<th>Initial tumor</th>
<th>Treatmenta</th>
<th>Interval (weeks)</th>
<th>Challengeb</th>
<th>Metastatic scorec</th>
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<tr>
<td>None</td>
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<td>None</td>
<td>CT26-KSA</td>
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<td>None</td>
<td>None</td>
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<td></td>
<td>16</td>
<td>CT26</td>
<td>0.0,1,1,3,3,3</td>
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</table>

a BALB/c mice with established pulmonary metastases 4 days after i.v. injection of 5 x 105 CT26-KSA colon carcinoma cells were treated daily with 15 μg of huKS1/4-IL-2 fusion protein for 7 consecutive days.

b Mice cured of established metastases were subsequently challenged after various time intervals with either 1.5 x 107 CT26-KSA or 3 x 107 CT26 wild-type tumor cells, respectively. BALB/c controls, injected i.v. with either 1.5 x 107 CT26-KSA or 3 x 107 CT26 wild-type tumor, received no treatment and were examined for macroscopic pulmonary metastases 28 days after tumor cell inoculation.

c Animals in each experimental group were sacrificed 28 days after tumor cell challenge and analyzed for macroscopic metastases. Results are given as metastatic score: 0, 0%; 1, <5%; 2, 5-50%; and 3, >50% of lung surfaces visibly covered with metastatic foci.
doses of anti-TfR-IL-2 fusion protein. In fact, 4 weeks after this boost, seven of eight mice completely rejected the tumor cell challenge, and only one animal had a single metastatic focus involving <5% of the lung surface. In contrast, a control group of mice (n = 8) that was challenged with CT26 cells at the 24-week time point but received PBS instead of the anti-TfR-IL-2 fusion protein exhibited metastases in seven of eight animals 4 weeks after CT26 tumor cell challenge (Table 3). Cytokine release assays of lymphocytes from secondary lymphoid tissues of these animals revealed maximal release of IFN-γ, TNF-α, and IL-12 following a 16-h incubation of these lymphocytes in DMEM at 37°C. Only IFN-γ was still released from lymph node lymphocytes after 48 h, albeit at decreasing levels (Fig. 5A), whereas release of TNF-α and IL-12 occurred mainly after 16 h of incubation (Figs. 5B and C). This cytokine release occurred only in lymphocytes of mice boosted with noncurative doses of anti-TfR-IL-2 fusion protein 4 days after CT26 tumor cell challenge. No cytokine release at all was observed in animals that received PBS instead of the fusion protein after tumor cell challenge (Fig. 5).

Another line of evidence indicates that tumor-specific cytotoxic CD8+ T cells were present in the spleens of BALB/c mice with persistent tumor-protective immunity, boosted by anti-TfR-IL-2 fusion protein. This is demonstrated by data shown in Fig. 6, where such CD8+ T cells specifically lysed CT26-KSA and CT26 wild-type tumor target cells, respectively. In contrast, no target cell lysis was obtained with CD8+ T cells from BALB/c mice (see Table 3) that did not receive a boost of tumor-protective immunity with IL-2 fusion protein or tumor-bearing control mice that only received noncurative doses of anti-TfR-IL-2 fusion protein (Fig. 6).

**DISCUSSION**

We demonstrate that IL-2 therapy, directed to the tumor microenvironment by a recombinant antibody-IL-2 fusion protein (huKSI/4-IL-2), effectively induces a long-lived tumor-protective immune response following the eradication of disseminated pulmonary colon carcinoma metastases in syngeneic BALB/c mice. We established two lines of evidence that completely rule out minimal residual disease in these animals. First, survival of all fusion protein-treated mice increased 5-fold over that of controls to 168 days, and second, highly sensitive RT-PCR analyses, detecting 1 tumor cell in 10^6 normal cells, indicated the absence of tumor cells in all animals sacrificed at this time point.

Time course studies of fusion protein therapy demonstrated that tumor metastases were completely eliminated 16 days after tumor inoculation, i.e., 5 days after completion of therapy, as indicated by RT-PCR analyses for KSA expression. In view of this rapid and effective eradication of metastases, we tested the hypothesis that this was accomplished by the activation of T cells, which is known to occur in the draining lymph nodes following tumor antigen processing by APC (20–23). In our experimental model, T cells are, indeed, activated in the draining lymph nodes of fusion protein-treated mice. Thus, FACS analyses of such cells revealed expression of the early T-cell activation marker CD69, which peaked briefly on day 5 of fusion protein treatment and then decreased to background levels on day 7, similar to those observed in naive mice. In this regard, it has been reported that IL-2 can rapidly induce expression of CD69 on lymphocytes following T-cell receptor/CD3 stimulation in vitro and that its expression is transient on recently activated lymphocytes located in lymphoid tissues (24). Also, expression of the CD69 membrane glycoprotein on T cells in the draining lymph nodes was reported as being indicative of IL-2 CD25-dependent T-cell prolifer-
Fig. 5. Cytokine release of lymphocytes from draining lymph nodes of BALB/c mice. Release of IFN-γ (A), TNF-α (B), and IL-12 (C) measured after 4-, 16-, 24-, and 48-h culture of lymphocytes isolated from BALB/c mice cured of established pulmonary CT26-KSA metastases by huKSI/4-IL-2 fusion protein. Twenty-four weeks thereafter, one group of these mice completely rejected a challenge with CT26 wild-type tumor cells when followed after 4 days by a boost of anti-TFR-IL-2 fusion protein (ch17217-IL-2). Another group of mice did not receive this boost and failed to reject the tumor cell challenge.

Table 3 Boost of tumor-protective immunity after tumor challenge of mice cured of established pulmonary colon cancer metastases

<table>
<thead>
<tr>
<th>Initial tumor</th>
<th>Treatment</th>
<th>Interval (weeks)</th>
<th>Tumor challenge</th>
<th>Boost</th>
<th>Metastatic score</th>
<th>Lung weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT26-KSA</td>
<td>huKSI/4-IL-2</td>
<td>6</td>
<td>CT26-KSA</td>
<td></td>
<td>0.00,0.0,1.2,2,3</td>
<td>0.28 ± 0.1</td>
</tr>
<tr>
<td>CT26-KSA</td>
<td>huKSI/4-IL-2</td>
<td>6</td>
<td>CT26-KSA</td>
<td>huKSI/4-IL-2</td>
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<td>0.19 ± 0.01</td>
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<tr>
<td>None</td>
<td>None</td>
<td>24</td>
<td>CT26</td>
<td>PBS</td>
<td>2.2,2,3,3,3,3,3</td>
<td>0.51 ± 0.11</td>
</tr>
<tr>
<td>CT26-KSA</td>
<td>huKSI/4-IL-2</td>
<td>24</td>
<td>CT26</td>
<td>ch17217-IL-2</td>
<td>0.00,0.0,0,0,0,1</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
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<td>None</td>
<td>24</td>
<td>CT26</td>
<td>PBS</td>
<td>0.1,1,1,1,2,2,3</td>
<td>0.37 ± 0.17</td>
</tr>
</tbody>
</table>

a Pulmonary metastases were initially induced by i.v. injection of 5 × 10^4 CT26-KSA cells into BALB/c mice. Treatment of established metastases was started 4 days thereafter by daily i.v. injections of 15 μg of huKSI/4-IL-2 fusion protein for 7 consecutive days.
b Mice free of tumor metastases after 6 and 24 weeks were challenged by i.v. injection of 1.5 × 10^6 CT26-KSA and 1 × 10^6 CT26 wild-type tumor cells, respectively.
c A boost for tumor-protective immunity was provided 4 days after tumor challenge by daily i.v. injections of 15 μg of chimeric rat/mouse antitransferrin receptor antibody-IL-2 fusion protein (ch17217-IL-2) for 5 consecutive days. A group of BALB/c mice treated in parallel received five injections of PBS.
d All mice were evaluated for macroscopic metastases 28 days after tumor cell challenge. Results are given as metastatic score. 0, 0%; 1, <5%; 2, 5-50%; and 3, >50% of lung surfaces visibly covered with metastatic foci.
e Differences in metastatic scores and lung weights between the fusion protein-boosted group and all control groups were statistically significant (P < 0.01).
f A boost of tumor-protective immunity was attempted 4 days after tumor challenge by two i.v. injections of 5 μg of KS1/4-IL-2 each on days 4 and 6. A control group of animals received five daily i.v. injections of PBS.
g A control group of naive BALB/c mice was i.v. injected with 1 × 10^6 CT26 wild-type tumor cells and, 4 days thereafter, received i.v. injections of 15 μg of anti-TFR-IL-2 fusion protein (ch17217-IL-2) for 5 consecutive days.

A summary of this article: In our study, we investigated the role of tumor-specific T cells in the eradication of established metastases in the context of adoptive immunotherapy. We found that T-cell-mediated tumor rejection was associated with an increase in IL-2 receptors, indicating a robust immune response. Furthermore, we demonstrated that T-cell priming in draining lymph nodes also occurred in these tumor-bearing animals, and administration of the fusion protein significantly increased this immune response to the point where complete eradication of metastases occurred.

We reported previously the involvement of CD8+ T cells in the eradication of disseminated CT26-KSA colon carcinoma metastases in BALB/c mice treated with huKSI/4-IL-2 fusion protein. We now extended these findings by demonstrating that CD8+ T cells isolated from the spleens of such mice are capable of exerting tumor-protective immunity in naive, syngeneic SCID mice. This was established by adoptive transfer studies indicating that only CD8+ T cells from huKSI/4-IL-2 fusion protein-treated mice are capable of completely rejecting a challenge with CT26-KSA tumor cells made in SCID mice 48 h after T cell transfer. In contrast, adoptive transfer of CD4+ T cells from these mice and CD8+ T cells from mice treated either with a mixture of huKSI/4 antibody and rhIL-2 or PBS were completely ineffective in this regard.

The most important consequence of the huKSI/4-IL-2 fusion protein-mediated eradication of established tumor metastases is the induction of a persistent and long-lived tumor protective immunity. This was achieved in at least 50% of experimental animals against challenges with CT26 and CT26-KSA tumor cells made 4 and 5 months, respectively, after eradication of tumor metastases. Challenges with these two tumor cell lines were rejected equally well, although the wild-type CT26 cells lacked expression of KSA. These in vivo findings correlate with prior in vitro data indicating that both tumor target cells were lysed equally well in vitro by CD8+ cytotoxic T lymphocytes isolated from the spleen of BALB/c mice cured of pulmonary metastases of CT26-KSA tumor cells following eradication by IL-2 fusion protein therapy. Taken together, these findings support the notion that the CT26-KSA and CT26 tumor cell lines share the same tumor antigen(s) that are recognized by CD8+ T cells. KSA, recognized by the huKSI/4-IL-2 fusion protein, serves merely as a docking site to direct this fusion protein to the tumor microenvironment.

Our data indicating long-lived tumor protective immunity against challenges with CT26 and CT26-KSA tumor cells suggest the involvement of T-memory cells. To follow up on this notion, we looked for a typical phenotype of antigen-activated T cells by we looked for a typical phenotype of antigen-activated T cells by we looked for a typical phenotype of antigen-activated T cells by we looked for a typical phenotype of antigen-activated T cells by
FACS analysis of CD8+ T cells isolated from splenocytes of BALB/c mice with demonstrated long-lived tumor-protective immunity. Although no definitive markers exist for memory T cells, numerous studies of T-cell phenotype during ontogeny and following immunization have led to a consensus view that mouse memory T cells are concentrated in a CD44hi, CD45RBlo, and L-selectin (CD62L)lo subset, whereas naive T cells express the reciprocal phenotype (26–28). We, indeed, observed this very same phenotype on CD8+ T cells isolated from the spleens of BALB/c mice with documented protective immunity against tumor cell challenges. However, caution must be used in equating CD44hi, CD45Rblo, and CD62Llo mouse T cells with memory T cells because this phenotype is also displayed by recently activated T cells (29). Because these memory cell markers were detected 6 weeks after initial tumor induction in our model, a contribution by recently activated T cells is somewhat unlikely. However, despite negative results in a highly sensitive RT-PCR detection system, we cannot rule out the possibility of persisting antigens, which could provide continuous stimulation of T cells, once persistently presented by APCs via MHC class II antigens (30, 31). Recent studies have demonstrated that the cure of CT26-KSA colon carcinoma metastases is followed by a functional memory response, which is primarily mediated by CD8+ T cells and efficient enough to protect half of the experimental animals against a challenge with CT26-KSA tumor cells. This observation is similar to our previously reported finding in a murine melanoma model in which an antigen-specific GDE2 antibody-IL-2 fusion protein also mediated eradication of metastases following by persistent tumor-protective immunity against challenge with GDE2+ murine melanoma cells in half of the experimental animals. Challenge with GD2+ wild-type tumor cells was not performed in these experiments (33).

Consequently, we attempted to achieve protection against tumor cell challenge in all experimental animals with eradicated pulmonary metastases after initial huKS1/4-IL-2 fusion protein therapy by boosting a partially protective memory immune response. Thus, we tested the hypothesis that aside from tumor antigens being required for activation of CD8+ T cell-mediated effector activities, IL-2 is necessary for amplification of memory in tumor-specific CD8+ cytotoxic lymphocytes. We found this to be the case when BALB/c mice, free of pulmonary colon carcinoma metastases 6 and 24 weeks after huKS1/4-IL-2 fusion protein therapy, received treatments with noncurative doses of KS1/4-IL-2 and the anti-TR-IL-2 fusion protein 4 days after challenge with CT26-KSA and CT26 wild-type tumor cells, respectively. In fact, eight of eight and seven of eight mice, respectively, showed complete rejection of these tumor cells at examination 4 weeks after challenge. One animal had a single metastasis, indicating that <5% of its lung surface was involved. These data clearly indicate that the provision of IL-2 in the tumor microenvironment by an antibody-cytokine fusion protein increases the efficiency of partially effective memory immune responses. The chl7217-IL-2 fusion protein (anti-TR-IL-2), directed against the murine transferrin receptor, was administered in these experiments because CT26 wild-type cells used for tumor cell challenge strongly express transferrin receptor but lack the KSA recognized by the KS1/4-IL-2 fusion protein. The presence of fulminating pulmonary metastases in naive mice challenged with CT26 tumor cells after treatment with chl7217-IL-2 fusion protein, shown as a control, clearly demonstrates that 15-μg doses (~5) of this fusion protein are noncurative. This is in contrast to mice that were cured previously of CT26-KSA colon carcinoma metastases by huKS1/4-IL-2 therapy in which CT26 challenges were completely rejected by a boost with chl7217-IL-2. We had previously shown that 5-μg doses (~2) of huKS1/4-IL-2 fusion protein are noncurative (14).

These findings indicate that, following the primary immune response, some CD8+ memory cells survived more than 24 weeks after pulmonary metastases were eliminated in fusion protein-treated mice. These memory CD8+ T cells were most likely reactivated by antigens provided by challenge with CT26 tumor cells and then were stimulated to proliferate by IL-2 provided by the huKS1/4-IL-2 and anti-TR-IL-2 fusion proteins, respectively. Support for this hypothesis comes from the finding of T-cell activation in secondary lymphoid organs, including spleen and axillary or inguinal lymph nodes. This was demonstrated by the detection of CD4+ and CD8+ T cells expressing the CD44hi, CD45Rblo, and L-selectin (CD62L)lo phenotype in lymphocytes from lymph nodes of BALB/c mice, the last two of which are well-known features of activated T cells. The up-regulation of these cytokines occurred only in lymph node cells from mice successfully boosted with anti-TR-IL-2 fusion protein to reject the challenge of CT26 wild-type tumor cells, in contrast to such cells...
isolated from mice treated with PBS. The notion that secondary lymphoid tissues of mice with successfully boosted tumor-protective immunity contained tumor-specific CD8+ T cells was supported by a second line of evidence. Thus, CD8+ T cells isolated from the spleen of such mice specifically lysed both CT26 and CT26-KSA tumor cells in vitro. It is noteworthy that this lysis was achieved without additional in vitro stimulation with IL-2, which previously had to be supplied to CD8+ T cells derived from the primary immune response following the initial huKSI1/4-IL-2 fusion protein-mediated eradication of CT26-KSA tumor metastases (14). CD8+ T cells isolated from spleens of the two control groups of BALB/c mice that were immune but did not receive a boost of IL-2 fusion protein or that were naive and received the boost failed to lyse these same tumor target cells to any significant extent.

In summary, we demonstrated the induction of a long-lived tumor-protective immune response against challenge with CT26-KSA and CT26 wild-type tumor cells in BALB/c mice with prior eradicated pulmonary metastases of colon carcinoma cells by antibody-IL-2 fusion protein therapy. This tumor immunity could be adoptively transferred to naive syngeneic SCID mice by CD8+ T cells. Long-lived protective tumor immunity against CT26-KSA and CT26 wild-type tumor cell challenges were each amplified by a boost with targeted tumor cell challenge, suggesting a more effective antigen-induced memory T-cell reactivation and differentiation to CD8+ cytotoxic T lymphocytes in the presence of IL-2. The data obtained in this model system of experimental colon cancer metastasis in syngeneic BALB/c mice suggest that repeated therapies with recombinant antibody-IL-2 fusion proteins may be effective for the treatment of human colon carcinoma in patients with minimal residual disease.

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REFERENCES


Induction of Persistent Tumor-protective Immunity in Mice Cured of Established Colon Carcinoma Metastases

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