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 demonstrate 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 (huKS1/4-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 immune 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 huKS1/4-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-23 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. Immunological 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 (huKS1/4-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 immunocytoxine-induced tumor-specific T-cell response is followed by a long-lived tumor protective immunity. The therapeutic effect of the huKS1/4-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 syngenic 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-nitro-o-N-methyurethane 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
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 x 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. Naive syngeneic SCID mice were reconstituted with a total of 4.5 x 10^7 CD4+ and CD8+ T cells by i.v. injection of 1.5 x 10^7 T cells of each subset on days -3, -1, and +3. All mice were challenged by i.v. injection of 5 x 10^5 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 x 10^4 CT26-KSA colon carcinoma cells were treated with 7 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 x 10^6 CT26-KSA or 3 x 10^6 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 axillary, 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, 5X), chl7217-IL-2 fusion protein (15 /µg, 5X), 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 x 10^6) were each labeled with 0.5 mCi of ⁵¹Cr for 2 h at 37°C. The ⁵¹Cr-labeled tumor target cells (5 x 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 x 10⁴ 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-STIM 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. Purification 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 target cell lysis was calculated by using the formula: [(E - S)/E X 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 CT26KSA 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-
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 Naive 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 for the early T-cell activation observed in the draining lymph nodes of the fusion protein-treated mice, we determined whether this antitumor response involved the activation of T cells in the draining lymph nodes of these mice.

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for the persistent, protective immunity demonstrated here in BALB/c mice successfully treated with the huKSI/4-IL-2 fusion protein (Table 1), passive lymphocyte transfer studies were performed. Lymphocytes were obtained from spleens of BALB/c mice that previously rejected pulmonary CT26-KSA metastases after treatment with huKSI/4-IL-2. Subpopulations of CD4+ and CD8+ T cells were transferred to naive BALB/c scid/scid mice by i.v. injection before and after i.v. challenge with CT26-KSA tumor cells. As shown in Table 1, only those naive BALB/c scid/scid mice injected with CD8+ T cells from BALB/c mice previously cured of metastatic disease by treatment with huKSI/4-IL-2 showed a complete absence of macroscopic pulmonary metastases and exhibited normal lung weights. However, adoptive transfer of CD4+ T cells obtained from the same BALB/c mice that were cured of pulmonary metastases was far less effective in preventing pulmonary metastases in naive BALB/c scid/scid mice than CD8+ T cells and only partially suppressed tumor growth in two of four animals tested. In contrast, CD8+ T cells obtained from control animals treated with either PBS or a mixture of mAb huKSI/4 and rhIL-2 had no tumor-protective effect following passive transfer into naive syngeneic scid/scid mice (Table 1).}

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

<table>
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<th>Treatment</th>
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<th>Challenge</th>
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<td></td>
<td>16</td>
<td>CT26</td>
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</table>

*BALB/c mice with established pulmonary metastases 4 days after i.v. injection of 5 × 10^5 CT26-KSA colon carcinoma cells were treated daily with 15 μg of huKSI/4-IL-2 fusion protein for 7 consecutive days.*

### 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 (CD44^hi) 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 naive BALB/c mice showed the reverse phenotype (Fig. 4).
involves the reactivation of memory T cells in secondary lymphoid organs of mice successfully treated with huKSI/4-IL-2 fusion protein. Support for this type of mechanism comes from our finding of T-cell transcripts, indicative of T-cell activation, and expansion in draining lymph nodes of BALB/c mice with persistent tumor-protective immunity (Fig. 5). Specifically, these were mice with eradicated pulmonary metastases following huKSI/4-IL-2 fusion protein treatment that completely rejected a challenge with CT26 tumor cells after 6 months when boosted with noncurative doses of anti-TIR-IL-2 fusion protein (see 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. 5, B and C). This cytokine release occurred only in lymphocytes of mice boosted with noncurative doses of anti-TIR-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-TIR-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-TIR-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 106 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-
cells. KSA, recognized by the huKSI/4-IL-2 fusion protein, serves as a docking site to direct this fusion protein to the tumor microenvironment.

To follow up on this notion, we looked for a typical phenotype of antigen-activated T cells by induction of a persistent and long-lived tumor protective immunity. To do this, we first treated naive BALB/c mice with the huKSI/4-IL-2 fusion protein (chl72l7-IL-2) for 5 consecutive days. A control group of naive BALB/c mice was i.v. injected with 1×10⁶ CT26-KSA cells and, 4 days thereafter, received i.v. injections of PBS.

A boost for tumor-protection immunity was provided by daily i.v. injections of 15 µg of anti-TfR-IL-2 fusion protein (chi7217-IL-2) for 5 consecutive days. A group of BALB/c mice treated in parallel received five injections of PBS. These data suggest that tumor-bearing animals and that administration of the fusion protein significantly increased this immune response to the point where complete eradication of metastases occurred.

We now extended these findings by demonstrating that CD8+ T cells isolated from the spleens of mice treated with CT26-KSA colon carcinoma metastases in BALB/c mice treated with huKSI/4-IL-2 fusion protein (14). We now extended these findings by demonstrating that CD8+ T cells isolated from the spleens of 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 CT26-KSA tumor cells bearing mice and that 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 (14). 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 tumor cells lacked expression of KSA. These in vivo findings correlate with our 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 (14). 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.

Effect on the Tumor Microenvironment

Our data indicating long-lived tumor protective immunity and lung weight between the fusion protein-boosted group and all control groups were statistically significant (P < 0.01).

A boost for tumor-protection immunity was provided 4 days after tumor challenge by two i.v. injections of 5 µg of anti-TfR-IL-2 fusion protein (chi7217-IL-2) on days 4 and 6. A control group of animals treated with PBS. These data suggest that tumor-bearing animals and that administration of the fusion protein significantly increased this immune response to the point where complete eradication of metastases occurred.

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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 CD44high, CD45RBlow, and L-selectin (CD62L)low 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 demonstrated long-lived tumor-protective immunity, boosted by IL-2 fusion protein (C); mice receiving only PBS (B); and tumor-bearing control mice that received only noncurative doses of anti-TfR-IL-2 fusion protein ch17217-IL-2 (O).

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 ch17217-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 ch17217-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 ch17217-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 IL-12, IFN-γ, and TNF-α 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

**Fig. 6. Cytotoxicity induced by CD8+ T cells of BALB/c mice against CT26-KSA and CT26 tumor target cells.** Lytic activity of CD8+ effector cells was measured in a 4-h 32Cr-release assay against CT26-KSA (A) and CT26 (B) target cells at different E:T ratios. CD8+ T cells were isolated from the spleens of three experimental groups of BALB/c mice (n = 8) with demonstrated long-lived tumor-protective immunity, boosted by IL-2 fusion protein (C); mice receiving only PBS (B); and tumor-bearing control mice that received only noncurative doses of anti-TfR-IL-2 fusion protein ch17217-IL-2 (O).
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 huKS14/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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Induction of Persistent Tumor-protective Immunity in Mice Cured of Established Colon Carcinoma Metastases

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