

# T Cell Memory Against Colon Carcinoma Is Long-Lived in the Absence of Antigen<sup>1</sup>

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Eradication of established colon carcinoma metastases is a major goal for adjuvant immunotherapy of this disease. This was accomplished in a murine model by targeting IL-2 to the tumor microenvironment with a recombinant Ab-IL-2 fusion protein (huKS1/4-IL-2). The generation of a long-lived protective immunity was demonstrated by a 10- to 14-fold increase in CTL precursor (pCTL) frequency and induction of genes encoding Th1 cytokines, followed by the generation of tumor-specific CD8<sup>+</sup> T effector cells, some of which differentiated into long-lived T memory cells. The frequency of pCTL correlated with enhanced immune protection against tumor cell challenge, and long-lived T cell memory was maintained in syngeneic SCID mice in the absence of tumor Ag. Tumor cell challenge of these SCID mice, concomitant with a boost of two noncurative doses of huKS1/4-IL-2 fusion protein, resulted in the generation of primed CD8<sup>+</sup> T effector cells with concurrent release of Th1 cytokines. These events culminated in the complete rejection of the tumor cell challenge and prevention of pulmonary metastases. Taken together, the data suggest that T cell memory against colon carcinoma can be maintained in the absence of Ag. *The Journal of Immunology*, 1999, 163: 3676–3683.

Current efforts in tumor immunotherapy have been stimulated by the recognition that even some nonimmunogenic tumors encode tumor-rejection Ags capable of producing protective immunity (1). The cellular arm of the immune response, particularly CD8<sup>+</sup> CTL, together with cognate CD4<sup>+</sup> T cell help, appear to be best equipped to recognize tumor cells as foreign, leading to their elimination. In fact, CTL were shown capable of eradicating murine tumors and their metastases in several syngeneic tumor models (2–4). Several strategies focusing on CD8<sup>+</sup> T cells employed tumor cells transduced with cytokine genes to enhance CD8<sup>+</sup> T cell responses, while other approaches made use of tumor cells transfected with costimulatory molecules to deliver the Ag signal concomitantly with a second costimulatory signal to CD8<sup>+</sup> T cells (5).

We focused our efforts primarily on an alternative approach to direct cytokines, such as IL-2, to the tumor microenvironment with recombinant Ab-cytokine fusion proteins (immunocytokines), which take advantage of the paracrine nature of most cytokines that function best within a few cell diameters from their cell of origin (6). We previously reported that targeted IL-2 therapy with such immunocytokines induced tumor-specific CD8<sup>+</sup> T cell responses that were effective in eradicating established metastases of murine melanoma (7) and colon carcinoma (8) in syngeneic mice. This tumor eradication was followed by a long-lived tumor-protective immunity sufficiently effective to reject a secondary tumor cell challenge in 50% of mice that had been previously cured of experimental pulmonary metastases by Ab-IL-2 fusion proteins (9,

10). Importantly, in the colon carcinoma model, this long-lived tumor-protective immunity was successfully amplified to become optimally effective in 100% of experimental animals by injecting two noncurative doses of Ab-IL-2 fusion protein 4 days after the secondary tumor cell challenge (10).

The persistence of tumor-protective immunity up to 6 mo (10) indicated a long-lived T memory cell response. In this regard, it is well known that primary responses to T-dependent Ags are followed by long-lived immunological memory with the host giving heightened responses following secondary contacts with Ag (11–15). Long-term memory has been well documented at the T and B cell levels and reflects the combination of increased frequency of Ag-specific precursors and preferential survival of high-affinity cells (15).

Memory cells are thought to appear during the later stages of the primary immune response, and the majority of T lymphocytes participating in this response are short-lived and rapidly eliminated at the end of the response (15). In fact, memory cells were suggested to arise from precursor cells that avoid contact with high concentrations of Ag (16). The notion that low-level stimulation of T cells favors survival rather than death suggests that maintenance of long-lived T cell memory may require continuous exposure to residual Ag depots (15). This idea is supported by reports of poor memory cell survival on adoptive transfer unless accompanied by specific Ag (16). However, the notion that CD8<sup>+</sup> T cell memory cells require constant Ag stimulation was challenged by several reports indicating that such cells can survive for prolonged periods after adoptive transfer in the apparent absence of Ag (17, 18).

Here, we examine some of the cellular mechanism(s) involved in these events in our tumor model with emphasis on T cell memory reflected, in part, by specific fusion protein-induced increases in the frequency of precursor CTL (pCTL).<sup>3</sup> Treatment of tumor-bearing BALB/c mice with huKS1/4-IL-2 fusion protein increases frequency of pCTL concomitant with the induction of genes encoding primarily Th1 cytokines. Subsequent generation of tumor-specific CD8<sup>+</sup> T effector cells is followed by differentiation into

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<sup>3</sup> Abbreviations used in this paper: pCTL, precursor CTL; KSA, KS Ag; rhIL-2, recombinant human IL-2; RPA, RNase protection assay; LT, lymphotoxin.

long-lived T memory cells, which can be maintained in the absence of tumor Ags.

## Materials and Methods

### Animals, cell lines, and reagents

Female BALB/c and *scid/scid* mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). The mice were maintained under specific pathogen-free conditions and used for experiments when 7 wk old. All experiments were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

CT-26, a colon epithelial tumor cell line, derived by intrarectal injection of *N*-nitroso-*N*-methylurethane in BALB/c mice, was kindly provided by Dr. I. J. Fidler (MD Anderson Cancer Center, Houston, TX). The KS Ag (KSA) is an epithelial cell adhesion molecule recognized by mAb KS1/4 (19) that was cloned by several investigators (20, 21). KSA was cloned by PCR and expressed in CT26 colon carcinoma cells using a retroviral vector. A subclone of CT26-KSA stably expressing both KSA and MHC class I Ags H-2K<sup>d</sup>, H-2D<sup>d</sup> was selected for the studies described in this report. Expression of KSA remained stable in culture and on CT26-KSA cells propagated as tumors in BALB/c mice, as indicated by FACS analyses (8). CT26 and CT26-KSA cells were maintained in DMEM, supplemented with 5% FBS, vitamins, L-glutamine, sodium pyruvate, and nonessential amino acids at 37°C and 7.5% CO<sub>2</sub>. Recombinant human IL-2 (rhIL-2) was obtained from Chiron (Emeryville, CA) with a sp. act. of 16 × 10<sup>6</sup> IU/mg. All reagents were free of endotoxin, as determined by the *Limulus* amoebocyte lysate assay purchased from BioWhittaker (Walkersville, MD).

### Abs and fusion proteins

The generation of the murine KS1/4 Ab recognizing KSA was described previously (19), as was the subsequent construction of the recombinant humanized huKS1/4-IL-2 fusion protein (22). The huKS1/4-IL-2 fusion protein and mAb huKS1/4 revealed identical binding patterns with CT26-KSA cells with an average K<sub>d</sub> of 1.15 nM and recognized 3.8 × 10<sup>6</sup> KSA binding sites/cell (8). The concentration of IL-2 in the huKS1/4-IL-2 fusion protein was calculated as two molar equivalents of IL-2 per mole of fusion protein.

### Induction of experimental metastases

Pulmonary metastases in syngeneic BALB/c mice were induced following i.v. injection of 5 × 10<sup>4</sup> 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. Since such foci appeared fused, metastases were scored according to the percentage of lung surface involvement with 0, 0%; 1, <5%; 2, 5–50%; and 3, >50% (8). Lung specimens of these mice without macroscopic metastases were stained with hematoxylin/eosin and examined histologically. Some of the samples were snap frozen in –70°C for subsequent RNA isolation.

### Tumor cell challenge models

Tumor cell challenges were performed in two different experimental models. First, BALB/c mice, with established pulmonary metastases 4 days after i.v. injection of 5 × 10<sup>4</sup> CT26-KSA colon carcinoma cells, were administered daily i.v. injections (15 μg each) for 7 consecutive days. These mice, cured of pulmonary metastases, were challenged after 6 wk with 1.5 × 10<sup>4</sup> CT26-KSA cells, sacrificed 12 days thereafter, and analyzed for frequency of pCTL and for cytolytic activity of T effector cells. Second, the same treatment regimen was used except CD8<sup>+</sup> T cells were purified from splenocytes of BALB/c mice obtained 12 days after tumor cell inoculation and 1 day after completion of the 7-day treatment with huKS1/4-IL-2 fusion protein. However, in this case, the purified CD8<sup>+</sup> T cells were adoptively transferred to syngeneic SCID mice and parked there for 6 wk, a time after which these mice received a tumor cell challenge, followed 4 and 6 days thereafter by two boosts with noncurative doses (5 μg each) of either huKS1/4-IL-2 or a nonspecific fusion protein, ch14.18-IL-2. Additional control animals received no boost.

### Multiprobe RNase protection assay

The expression of total murine cytokine RNA by naive mice and mice treated with either PBS or KS1/4-IL-2 fusion protein was determined with an RNase protection assay (RPA; PharMingen, San Diego CA), which allows for the simultaneous quantification of several mRNA species in a

single sample of total RNA. The murine RPA probe sets used analyzed for mRNA of murine cytokines IL-2, IL-4, IL-10, IL-6, IFN-γ, GM-CSF, lymphotoxin (LT)-β, and TNF-α and a housekeeping gene transcript, L32. The latter encodes a ribosomal structural protein and facilitates assessments of total RNA levels for normalization of sampling or technical errors from each sample. Standard precautions were taken to avoid RNase contamination during all steps of the test. The probe synthesis uses [α-<sup>32</sup>P], GACU nucleotide pool and a set of templates supplied by the manufacturer (PharMingen). Sample RNA of high quality and purity was used to hybridize with the labeled probe, and 10 μg RNA was added to each tube, including one containing yeast total RNA as a background control. Following probe synthesis and overnight hybridization, the RNase treatment, purification of the protected probe, electrophoresis on denaturing polyacrylamide gel, autoradiography, and phosphorimaging were done according to the manufacturer's guidelines.

### Cytotoxicity of CD8<sup>+</sup> T effector cells

Splenocytes were isolated from either BALB/c mice or syngeneic SCID mice. To this end, splenocytes were labeled with paramagnetic anti-CD8<sup>+</sup> MicroBeads and separated with Mini MACS Separation Unit (Miltenyi Biotec, Auburn, CA) or T cell purification kits (R&D Systems, Minneapolis, MN) according to the manufacturer's guidelines. Purity of CD8<sup>+</sup> T cells was >95%, as determined by FACS analysis. CD8<sup>+</sup> T cells were incubated for 3 days at 37°C in 25 ml complete DMEM culture medium containing 1 ml of T-STIM culture supplement (Becton Dickinson, Bedford, MA).

Tumor-specific cytotoxicity was measured in a standard <sup>51</sup>Cr-release assay. Briefly, CT26-KSA tumor target cells (3 × 10<sup>6</sup>) were each labeled with 0.5 mCi of <sup>51</sup>Cr for 2 h at 37°C, washed three times in DMEM and aliquots of 5 × 10<sup>3</sup> labeled cells were added to each well of a U-bottom microfilter plate and incubated with effector cells 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)/(T – S)] × 100, where “E” is the average experimental release, “S” is the average spontaneous release, and “T” is the average total release of <sup>51</sup>Cr.

### Analysis of pCTL frequency

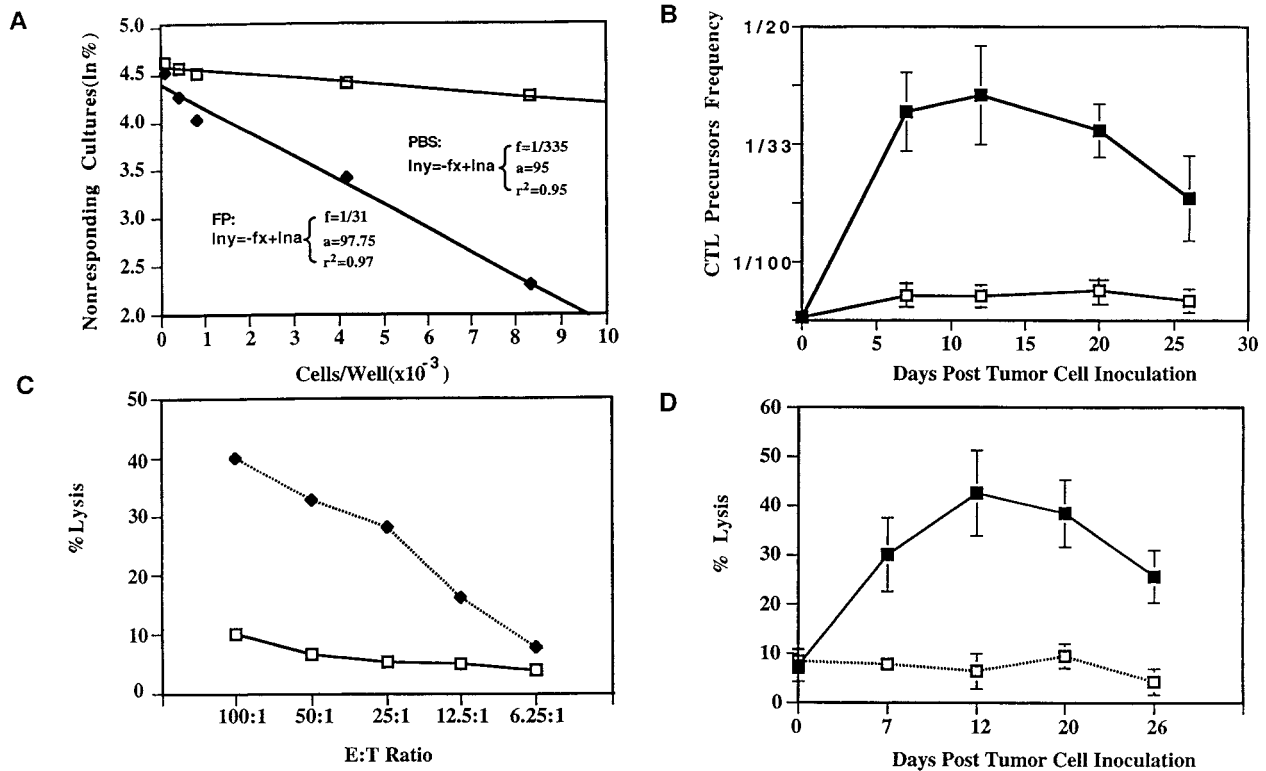
The frequency of pCTL was determined by a limiting dilution analysis. Briefly, spleen cells were harvested from tumor-bearing BALB/c mice on days 0, 7, 12, and 24 of treatment with huKS1/4-IL-2 fusion protein and from a control group treated only with PBS. These cells were diluted serially and cultivated in 96-well flat-bottom plates in the presence of T cell medium and incubated with irradiated (12,000 rads) feeder cells consisting of 1 × 10<sup>4</sup> cells each of splenocytes from naive BALB/c mice and CT26-KSA colon carcinoma cells. After 5 days, each well was assayed for cytotoxic activity against CT26-KSA target cells. Cultures were designated positive when specific lysis was three SDs above the lysis found in negative controls. The fraction of negative cell cultures was determined for each dilution with 48 cultures per dilution. The frequency of pCTL was assessed, as described previously (23). Briefly, the fraction of negative cultures was plotted on a semilogarithmic scale against the number of splenocytes per well. Frequencies of pCTL were defined by the slope of the linear regression between at least three separate data points. The formula used was  $\ln y = (-fx + \ln a)$ , where “f” is the frequency of responding precursors in a given cell population, “x” is the number of splenocytes added to each culture, “a” is the y-axis intercept (in our experiments, 100% ± 6%) and “y” is the percentage of negative cultures.

### Cytokine release assay

Splenocytes were collected from the experimental groups of BALB/c *scid/scid* mice cured of pulmonary colon carcinoma metastases by huKS1/4-IL-2 fusion protein, challenged after 6 wk with CT26-KSA tumor cells, and boosted 4 days thereafter with either noncurative doses (2 × 5 μg) huKS1/4-IL-2 fusion protein or with PBS. Lymphocytes were isolated on Hypaque-Ficoll (BioWhittaker) and cultured for 4, 12, 24, and 48 h in DMEM medium. Supernatants were harvested, obtained from each group of mice at these time points, and assayed for murine IFN-γ, GM-CSF, and IL-2, respectively, with commercially available cytokine detection kits (BioSource International, Camarillo, CA) using a solid-phase sandwich ELISA.

### Adoptive transfer of lymphocytes

Mice that were successfully treated with huKS1/4-IL-2 fusion protein, which eradicated pulmonary metastases of CT26-KSA tumor cells, served subsequently as donors of lymphocytes for adoptive transfer experiments.



**FIGURE 1.** CTL precursor frequency in CT-26KSA tumor-bearing mice after treatment with KS1/4-IL-2 fusion protein. *A*, Syngeneic BALB/c mice with established pulmonary metastases, 4 days after i.v. injection of  $5 \times 10^4$  CT-26KSA colon carcinoma cells were treated daily with  $15 \mu\text{g}$  of huKS1/4-IL-2 fusion protein for 7 consecutive days. pCTL frequencies were determined from splenocytes harvested from control mice ( $\square$ ) or huKS1/4-IL-2-treated mice ( $\blacksquare$ ) by limiting dilution assay, as described in *Materials and Methods*. Data shown are from one of three representative experiments on day 12 after tumor cell inoculation. *B*, pCTL frequency was determined over time. Each time point represents the mean  $\pm$  SD of at least three separate experiments; huKS1/4-IL-2-treated mice ( $\blacksquare$ ); PBS controls ( $\square$ ). *C*, Representative CT26-KSA target cell lysis by CTL (day 12) was determined with  $\text{CD8}^+$  T cells harvested from splenocytes of control mice ( $\square$ ) or huKS1/4-IL-2-treated mice ( $\blacklozenge$ ) as a function of E:T ratio in a 4-h  $^{51}\text{Cr}$ -release assay. *D*, Temporal assessment of CTL lytic activity of splenocytes from KS1/4-IL-2-treated mice ( $\blacksquare$ ) and PBS controls ( $\square$ ) (E:T, 1:100). Mean  $\pm$  SD from four individual mice each are plotted for each time point.

These animals were sacrificed 12 days after tumor cell inoculation, followed by the preparation of single cell suspensions obtained by mincing freshly resected spleens and passing them through a steel sieve. Lymphocytes were separated by Ficoll/Hypaque gradient centrifugation ( $600 \times g$ , 20 min). Subsets of  $\text{CD8}^+$  T cells were isolated at  $>95\%$  purity by magnetic-activated cell sorting (Miltenyi Biotec). Naive, syngeneic SCID mice were reconstituted with a total of  $4.5 \times 10^7$   $\text{CD8}^+$  T cells by i.v. injection of  $1.5 \times 10^7$   $\text{CD8}^+$  T cells on days  $-3$ ,  $-1$ , and  $+3$ , and their fate was followed by flow cytometry analyses at 1, 2, 3, 4, and 5 wk using anti- $\text{CD8}^+$  and anti- $\text{CD3}^+$  Abs, respectively. After 6 wk, individual groups of mice were challenged by i.v. injection of  $5 \times 10^4$  CT26-KSA cells to initiate pulmonary metastases, followed by a boost of two noncurative doses of huKS1/4 fusion protein ( $2 \times 5 \mu\text{g}$ ) on days 4 and 6 after CT-26KSA tumor cell challenge. Tumor-specific cytotoxicity, cytokines, and markers for T cell activation, i.e., CD25 and CD69, were determined 8 days after the boost with huKS1/4-IL-2 fusion protein. Animals were sacrificed 28 days after challenge and analyzed for macroscopic metastases and minimal residual disease by microscopic 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 PE-conjugated form in combination with FITC-conjugated anti-mouse mAb CD3e (clone 145-2c11). FITC-rat IgM (R4-22) and hamster IgG anti-trinitrophenol (G235-2356) were used as Ig isotype controls. Lymphocytes were incubated for 1 h at  $4^\circ\text{C}$  with FITC- and PE-labeled Abs and washed and analyzed immediately with a Becton Dickinson FACScan. A total of 10,000 labeled cells per sample were analyzed. All labeled Abs were purchased from PharMingen.

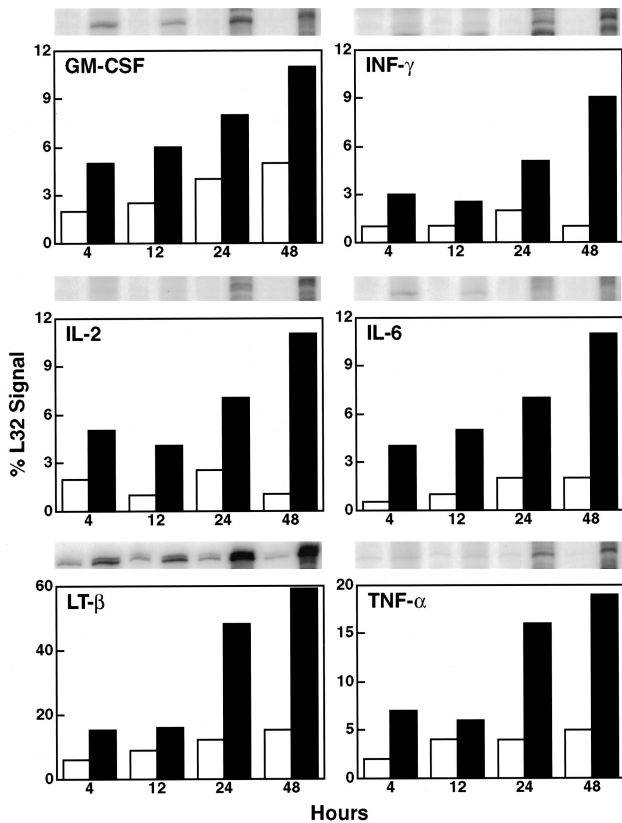
#### Statistical analyses

The statistical significance of differential findings between experimental groups was determined by the Student's *t* test. The nonparametric 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 *p* values were  $<0.05$ .

## Results

### Treatment of tumor-bearing mice with huKS1/4-IL-2 fusion protein increases frequency of precursor CTL and priming of $\text{CD8}^+$ T cells

We previously demonstrated that an huKS1/4-IL-2 fusion protein specifically induces a  $\text{CD8}^+$  T cell-mediated immune response in syngeneic BALB/c mice capable of completely eradicating established pulmonary metastases of CT26-KSA colon carcinoma (8, 10). We initially examined the cellular mechanisms involved in these events with emphasis on the frequency of pCTL and subsequent priming of  $\text{CD8}^+$  T effector cells. Determinations of the frequency of pCTL in splenocytes of BALB/c mice with established pulmonary metastases of CT26-KSA colon carcinoma using a limiting dilution assay at different time points during and after the huKS1/4-IL-2 fusion protein treatment indicated a 10-fold increase compared with mice treated with PBS (Fig. 1A). The increase in pCTL frequency reached a maximum on day 12 after tumor cell challenge, corresponding to 1 day after completion of

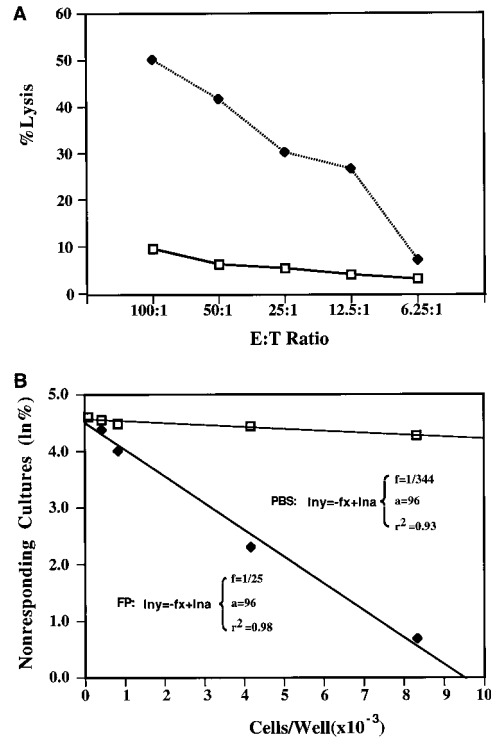


**FIGURE 2.** Multiprobe RPA for cytokine gene expression. Total RNA was extracted from CD8<sup>+</sup> T cells purified from splenocytes of tumor-bearing syngeneic BALB/c mice 12 days after treatment with either huKS1/4-IL-2 fusion protein (■) or PBS (□). Pooled RNA from four mice was used for a multiprobe RPA analysis of IL-2, IL-6, GM-CSF, IFN- $\gamma$ , LT- $\beta$ , and TNF- $\alpha$ , followed by quantitation of autoradiographic intensity compared with that of the constitutively expressed L32 control.

fusion protein treatment during 7 consecutive days, initiated 4 days after tumor cell inoculation (Fig. 1B). In contrast, pCTL frequency in splenocytes obtained from PBS-treated mice remained at background levels during the 4-wk period examined (Fig. 1B). Although the frequency of pCTL decreased somewhat from the maximum observed on day 12, it still remained substantially above the level observed in control animals even 26 days after tumor cell inoculation (Fig. 1B). The marked increase in the frequency of pCTL observed correlated with a distinct increase in cytolytic activity of the splenocytes against CT26-KSA tumor cell targets (Fig. 1C). Concordantly, the percent cytolysis achieved also reached a maximum on day 12 post-tumor cell inoculation (Fig. 1D). The splenocytes obtained from control animals treated with PBS showed only background levels (<10%) of cytolytic activity against CT26-KSA tumor target cells (Fig. 1, C and D).

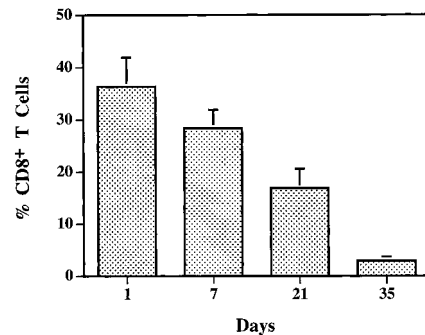
*Treatment with huKS1/4-IL-2 fusion protein induces the expression of genes encoding Th1 cytokines*

In an attempt to further characterize priming, proliferation, and maintenance of CD8<sup>+</sup> T cells induced by the IL-2 fusion protein, an RPA was found sensitive in detecting and quantitating cytokine genes at the RNA level. Analysis of total RNA extracted from splenocytes obtained from BALB/c mice 12 days after CT26-KSA tumor cell inoculation and quantitation with a multiprobe RPA of cytokine gene expression revealed markedly increased levels of genes encoding Th1 cytokines IL-2, IFN- $\gamma$ , TNF- $\alpha$ , LT- $\beta$ , and GM-CSF only in mice treated with the IL-2 fusion protein. This



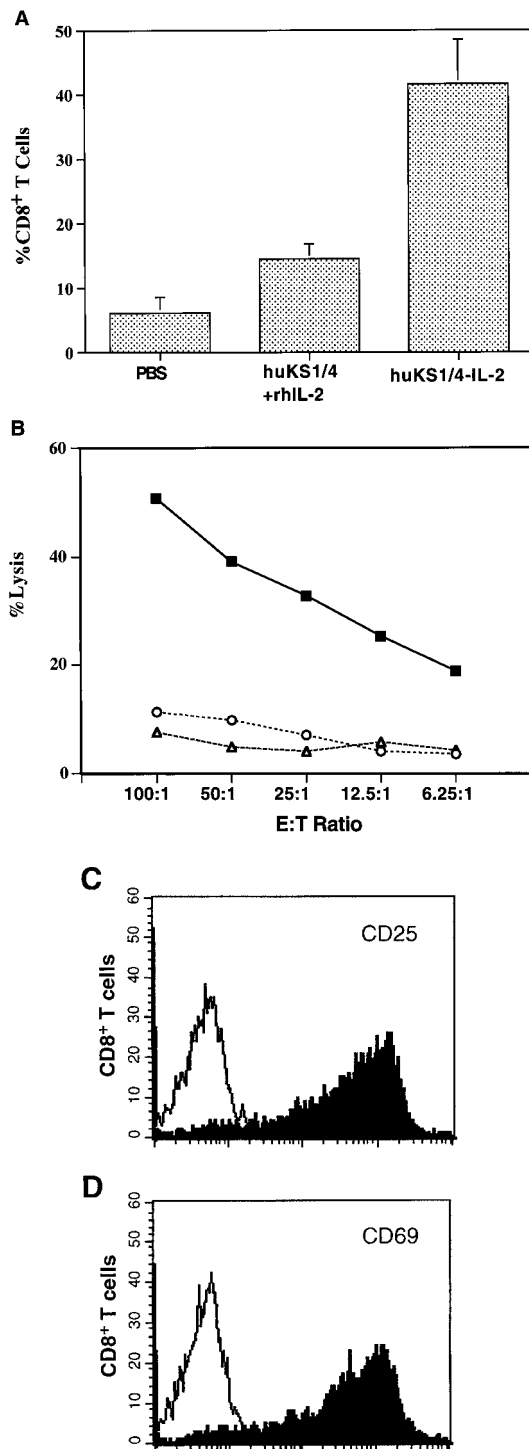
**FIGURE 3.** CTL precursor frequency in rechallenged mice. Syngeneic BALB/c mice that had been cured of CT-26KSA pulmonary metastases by treatment with huKS1/4-IL-2 were subsequently rechallenged with CT-26KSA tumor cells 6 wk after the original tumor cell inoculation. Twelve days after tumor cell challenge, pCTL activity was assessed among splenocytes harvested from either mice treated with PBS (□) or with huKS1/4-IL-2 (◆). *A*, Quantitation of lytic activity with a <sup>51</sup>Cr-release assay. *B*, Reassessment of pCTL frequency by limiting dilution assay.

was evident from quantitation of autoradiographic intensity compared with that of the constitutively expressed L32 control indicating a time-dependent increase over a period of 48 h (Fig. 2). Although expression of the gene encoding the Th2 cytokine IL-6 was also induced, genes encoding for the typical Th2 cytokines IL-4 and IL-10, also quantifiable by this assay, were not detected (data not shown). By comparison, the expression levels of genes encoding the cytokines shown in Fig. 2 were considerably less in RNA extracted from splenocytes of mice treated with PBS, suggesting that the priming of CD8<sup>+</sup> T cells induced by huKS1/4-IL-2



**FIGURE 4.** Fate of CD8<sup>+</sup> effector T cells after adoptive transfer into BALB/c scid/scid mice. FACS analysis of CD8<sup>+</sup> splenic T cells (4.5 × 10<sup>7</sup>) adoptively transferred to BALB/c mice cured of pulmonary metastases by huKS1/4-IL-2 fusion protein. CD8<sup>+</sup> T cells were monitored for 5 wk to determine the effect of apoptosis on these cells after parking them in the syngeneic SCID mice.





**FIGURE 5.** Amplification of CD8<sup>+</sup> effector T cells from CD8<sup>+</sup> memory T cells by tumor-targeted IL-2 in BALB/c scid/scid mice. CD8<sup>+</sup> T cells isolated from splenocytes of BALB/c mice cured of pulmonary CT26-KSA metastases by huKS1/4-IL-2 fusion protein were adoptively transferred to syngeneic SCID mice and parked there for 6 wk. These animals were then challenged with  $1.5 \times 10^4$  CT26-KSA tumor cells and treated 4 days thereafter with PBS, an equivalent mixture of huKS1/4 and rhIL-2, or two noncurative doses (5  $\mu$ g each) of huKS1/4-IL-2 fusion protein. **A**, FACS analysis of CD3<sup>+</sup>/CD8<sup>+</sup> T cells 12 days after tumor cell challenge. **B**, Lysis of CT26-KSA target cells by pooled CD8<sup>+</sup> T cells isolated on day 12 after tumor cell challenge from four BALB/c *scid/scid* mice of each experimental group: PBS ( $\Delta$ ); huKS1/4 + IL-2 mixture ( $\circ$ ); huKS1/4-IL-2 fusion protein ( $\blacksquare$ ). Lysis was determined at various E:T ratios in a 4-h <sup>51</sup>Cr-release assay. Mean  $\pm$  SD of triplicate determinations is shown. **C**, Expression of IL-2 receptor (CD25) on reactivated CD8<sup>+</sup> T effector cells

treatment specifically induces a marked increase in the expression of genes primarily encoding Th1 cytokines.

*Frequency of pCTL correlates with enhanced immune protection against tumor cell challenge*

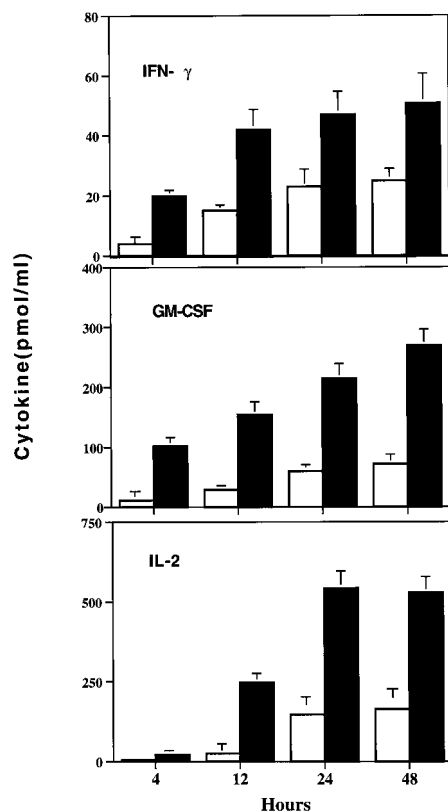
During examination of the mechanism(s) involved in the complete rejection of a secondary tumor cell challenge in mice cured of pulmonary metastases by huKS1/4-IL-2 fusion protein, we observed a 14-fold increase in the frequency of pCTL over that detected in splenocytes from naive mice by using the limiting dilution assay (Fig. 3B). However, in this case, the cured mice received a secondary tumor cell challenge 6 wk after the initial tumor cell inoculation. The animals then received a boost 4 days thereafter with two noncurative doses (5  $\mu$ g each) of huKS1/4-IL-2 fusion protein on days 4 and 6 after tumor cell challenge. Importantly, this boost induced not only the marked increase in pCTL frequency, but also correlated with a decisive increase in cytolytic activity of splenocytes of these treated mice against CT26-KSA tumor cell targets (Fig. 3A). In contrast, neither an increase in pCTL frequency nor in cytolytic activity was observed when splenocytes were obtained from mice boosted only with PBS (Fig. 3, A and B).

*Long-lived T memory cells are maintained in the absence of Ag and differentiate to CD8<sup>+</sup> T effector cells*

To determine the fate of CD8<sup>+</sup> T effector cells in the absence of tumor Ag and naive T cells, we adoptively transferred purified CD8<sup>+</sup> T cells from tumor-bearing BALB/c mice treated with huKS1/4-IL-2 fusion protein to syngeneic SCID mice ( $n = 4$ ). In this case, CD8<sup>+</sup> T cells were purified from splenocytes obtained from these mice 12 days after initial tumor cell inoculation and 1 day after completion of the 7-day treatment with huKS1/4-IL-2 fusion protein. The CD8<sup>+</sup> T cells were parked for 5 wk in the SCID mice, and then these animals' splenocytes were subjected to FACS analysis for the presence of CD8<sup>+</sup> T cells. As indicated in Fig. 4, there was a continuous decrease in the number of CD8<sup>+</sup> T cells, until after 5 wk <5% of CD8<sup>+</sup> T cells were detectable, suggesting that the majority of these T effector cells apoptosed.

The continuous presence of tumor Ags or naive T cells was not required to maintain long-lived CD8<sup>+</sup> T cell memory among CD8<sup>+</sup> T cells that were adoptively transferred into syngeneic SCID mice and then parked there for 6 wk. This is indicated by the data shown in Fig. 5A. In these experiments, the SCID mice were challenged with CT26-KSA tumor cells at the 6-wk time point and then received 4 days later boosts with either PBS, a mixture of huKS1/4 with rhIL-2, or the huKS1/4-IL-2 fusion protein. The putative T memory cells remaining in these mice after 6 wk recognized the tumor Ag presented, as shown by markedly increased generation of CD8<sup>+</sup> T cells in mice treated with the IL-2 fusion protein, whereas the controls revealed only a minor increase in CD8<sup>+</sup> T cells (Fig. 5A). Importantly, when the CD8<sup>+</sup> T cells from each of these experiments were examined for their ability to lyse CT26-KSA target cells, only the CD8<sup>+</sup> T cells obtained from mice boosted with the huKS1/4-IL-2 fusion protein were primed and effectively killed the tumor cells in vitro (Fig. 5B). Further evidence for strong priming of these same CD8<sup>+</sup> T cells is documented by their strong expression of CD25, a marker for IL-2

isolated 12 days after challenge with CT26-KSA tumor cells and boosted on days 4 and 6 thereafter with either PBS ( $\square$ ) or two noncurative doses (5  $\mu$ g each) of huKS1/4-IL-2 fusion protein ( $\blacksquare$ ). **D**, Expression of early T cell activation Ag (CD69) on these same reactivated CD8<sup>+</sup> T cells after boosts with PBS ( $\square$ ) or huKS1/4-IL-2 fusion protein ( $\blacksquare$ ).



**FIGURE 6.** Cytokine release from CD8<sup>+</sup> effector T cells adoptively transferred to BALB/c scid/scid mice following challenge with CT26-KSA tumor cells. CD8<sup>+</sup> T cells were isolated from splenocytes of mice 12 days after tumor cell challenge. One group of mice was boosted with two noncurative doses (5  $\mu$ g each) on days 4 and 6 after tumor cell challenge (■); the other group of mice were controls and received only PBS (□). CD8<sup>+</sup> T cells from each group of mice were subsequently cultured with feeder and target cells and monitored for production of cytokines IFN- $\gamma$ , GM-CSF, and IL-2 with cultured supernatants harvested at 4, 12, 24, or 48 h being assayed for cytokine levels by a sandwich ELISA. The mean and SD for triplicate determinations are shown.

receptors (Fig. 5C) and CD69, an early activation marker of T cells (Fig. 5D). The effective priming of these CD8<sup>+</sup> T effector cells was further substantiated by their release of Th1 cytokines IFN- $\gamma$ , IL-2, and GM-CSF measured by a sandwich ELISA 12 days after tumor cell challenge and boosts with the huKS1/4-IL-2 fusion protein.

tein. In contrast, release of these cytokines was markedly less from CD8<sup>+</sup> T cells of mice boosted only with PBS (Fig. 6).

#### Long-lived T memory cells in syngeneic SCID mice exhibit tumor-protective immunity in vivo

We determined whether CD8<sup>+</sup> T cells adoptively transferred from immune mice to syngeneic SCID mice and parked there for 6 wk could maintain effective and long-lived memory in the absence of tumor Ag and naive T cells. To this end, such SCID mice ( $n = 4$ ) were challenged with CT26-KSA tumor cells and boosted 4 days thereafter with two noncurative doses (5  $\mu$ g each) of either huKS1/4-IL-2 fusion protein or a nonspecific ch14.18-IL-2 fusion protein. The data depicted in Table I indicate that putative CD8<sup>+</sup> T memory cells that had been parked in SCID mice effectively recognized a secondary challenge with CT26-KSA cells only when boosted with noncurative doses of huKS1/4-IL-2. In fact, these cells were able to completely reject this challenge and prevent the establishment of pulmonary metastases (Table I). This effect was specific, since it did not occur when boosts were done with the nonspecific fusion protein, ch14.18-IL-2. Also, boosts with noncurative doses of huKS1/4-IL-2 proved ineffective unless the adoptively transferred CD8<sup>+</sup> T cells were obtained from BALB/c mice that had been successfully treated with huKS1/4-IL-2 fusion protein (Table I).

## Discussion

This study delineated some of the cellular mechanisms involved in the complete eradication of established murine colon carcinoma metastases and the generation of the long-lived tumor-protective immunity induced by targeting IL-2 to the tumor microenvironment with a recombinant Ab-IL-2 fusion protein (huKS1/4-IL-2) (8, 10).

We demonstrated that this treatment of tumor-bearing BALB/c mice resulted in a 10- to 14-fold increase in frequency of pCTL, followed by the induction of genes encoding Th1 cytokines (IFN- $\gamma$ , IL-2, TNF- $\alpha$ , LT- $\beta$ , GM-CSF) and the generation of tumor-specific CD8<sup>+</sup> T effector cells, some of which differentiated into long-lived T memory cells. The frequency of pCTL correlated with enhanced immune protection against tumor cell challenge, and long-lived T memory cells could be maintained in syngeneic SCID mice in the absence of tumor Ag. This was demonstrated when a tumor cell challenge and subsequent boost of these mice

Table I. Horizontal transmission of tumor immunity by adoptive transfer of CD8<sup>+</sup> T cells<sup>a</sup>

Therapy	Transfer	Boost	Metastatic Score <sup>b</sup>	Lung Weight (g)
None	None	None	3,3,3,3	0.99 $\pm$ 0.10
PBS	CD8 <sup>+</sup>	None	3,3,3,3	0.85 $\pm$ 0.10
PBS	CD8 <sup>+</sup>	huKS1/4-IL-2	3,3,3,3	0.91 $\pm$ 0.12
huKS1/4 + IL-2	CD8 <sup>+</sup>	huKS1/4-IL-2	1,2,2,2	0.41 $\pm$ 0.04
huKS1/4-IL-2	CD8 <sup>+</sup>	ch14.18-IL-2	0,1,1,2	0.37 $\pm$ 0.07
huKS1/4-IL-2	CD8 <sup>+</sup>	None	0,1,1,1	0.32 $\pm$ 0.03
huKS1/4-IL-2	CD8 <sup>+</sup>	huKS1/4-IL-2	0,0,0,0	0.21 $\pm$ 0.01

<sup>a</sup> BALB/c mice, cured of pulmonary CT26-KSA metastases after treatment with huKS1/4-IL-2 fusion protein 2 wk after tumor cell inoculation, served as donors for CD8<sup>+</sup> T cells adoptively transferred to syngeneic SCID mice. Three aliquots of  $1.5 \times 10^7$  CD8<sup>+</sup> T cells, purified from splenocytes by magnetic activated cell sorting, were injected i.v. into BALB/c scid/scid mice and parked there for 6 wk. Then, 48 h prior to i.v. challenge with  $1.5 \times 10^4$  CT26-KSA tumor cells, naive syngeneic SCID mice were reconstituted with a total of  $4.5 \times 10^7$  CD8<sup>+</sup> T cells by i.v. injection of  $1.5 \times 10^7$  T cells on days -3, -1, and +3. On days 4 and 6 after tumor cell challenge, seven groups of mice ( $n = 4$ ) received either no boost or two i.v. injections of a noncurative dose (5  $\mu$ g each) of huKS1/4-IL-2 fusion protein. One group of mice treated with the huKS1/4-IL-2 fusion protein received the same regimen, but with a nontumor-specific ch14.18-IL-2 fusion protein. All groups of mice were sacrificed 28 days after tumor cell challenge and evaluated for metastatic disease.

<sup>b</sup> Results are given as metastatic score. 0, no visible metastatic foci; 1, <5% of lung surfaces covered with metastatic foci; 2, between 5 and 50% of lung surfaces covered with metastatic foci; and 3, >50% of lung surfaces covered with metastatic foci.

with two noncurative doses of IL-2 fusion protein resulted in effective reactivation of CD8<sup>+</sup> T effector cells with concurrent release of Th1 cytokines IFN- $\gamma$ , IL-2, and GM-CSF, followed by complete rejection of the tumor cell challenge and prevention of pulmonary metastases.

The increase in pCTL frequency induced specifically by the huKS1/4-IL-2 fusion protein correlated with increased tumor-protective immunity in our tumor model. A critical assumption in the determination of pCTL frequency by the linear regression analyses of data obtained in the limiting dilution assay is a good fit of experimental data with the Poisson distribution. This is true for our data with a mean Y-intercept (a) equaling 96 and the mean coefficient of determination ( $r^2$ ) being 0.98. However, the efficiency of detection of these CTL precursors cannot be assessed with certainty, and the frequency of pCTL reported should be considered as minimal estimates (23). Importantly, the increase in pCTL frequency correlated well with an increase in cytolytic activity of mouse splenocytes against CT26-KSA tumor target cells, indicating effective priming of T effector cells. There was also direct evidence for the involvement of Th1 CTL by the concurrent induction of genes encoding such Th1 cytokines as IFN- $\gamma$ , IL-2, TNF- $\alpha$ , LT $\beta$ , and GM-CSF, as well as the Th2 cytokine IL-6. Since this up-regulation of Th1 cytokine genes occurred only in IL-2 fusion protein-treated mice, it appears that these cytokines were newly synthesized as a consequence of this therapy and were not released from pre-existing stores. In this regard, it was recently reported that proliferation and differentiation of CD8<sup>+</sup> T cells to generate cytotoxic lymphocyte responses is enhanced by inflammatory cytokines, such as IFN- $\gamma$ , produced by macrophages or dendritic cells, and that they can provide a third signal for activation of naive CD8<sup>+</sup> T cells (24). Although there is no direct evidence that this type of activation occurs in our tumor model, it cannot be ruled out.

It is of considerable interest that the generation of increased pCTL frequency could again be achieved by huKS1/4-IL-2 fusion protein 6 wk after the initial tumor cell inoculation, following a secondary challenge with CT26-KSA tumor cells. In this case, only two boosts with noncurative doses of the IL-2 fusion protein were required to achieve a 14-fold increase in frequency of pCTL compared with control mice that did not receive such a boost. Additionally, splenocytes from these BALB/c mice exhibited strong tumor-specific cytolytic activity against CT26-KSA tumor target cells in vitro that was not evident among control animals. These findings, together with our previous observation that under these circumstances secondary tumor cell challenges are completely rejected in 100% of experimental animals, lead to two conclusions. First, increased frequency of pCTL correlates with increased T cell memory. Second, there is an altered and more pronounced responsiveness of T memory cells following a second encounter with the same or related Ag. This "positive" memory occurring after secondary stimulation is known to occur faster, reaches higher peak levels of activity, maintains the response for longer periods, and requires less stringent conditions of costimulation than the primary immune response (25, 26).

The role of persisting Ag in T cell memory and the requirement for chronic exposure to residual deposits of Ag for maintenance of CD8<sup>+</sup> T cell memory have been the subject of much discussion and controversy (27–29). This is in contrast to CD8<sup>+</sup> effector T cells that absolutely require the presence of Ag consistent with the decrease in adoptively transferred CD8<sup>+</sup> T cells in the absence of Ag, a fact observed in our system (Fig. 4). Indeed, there are a number of reports indicating that memory cells survive poorly on adoptive transfer unless accompanied by specific Ag (12, 30, 31). However, the contention that memory cells require constant Ag

stimulation has been challenged by reports demonstrating that CD8<sup>+</sup> memory cells can survive for prolonged periods after adoptive transfer in the absence of Ag (17, 18, 32). These findings suggest that, at least for CD8<sup>+</sup> cells, some memory T cells do not require continuous stimulation with Ag for survival. To define the conditions for maintaining long-lived memory T cells in our tumor model, CD8<sup>+</sup> T cells from immune BALB/c mice were adoptively transferred to syngeneic SCID mice 12 days after tumor cell inoculation and parked there for 6 wk. In these animals, which lack mature T and B lymphocytes, the transferred CD8<sup>+</sup> T cells died by apoptosis after 5 wk, when <5% were detectable by FACS analysis. Apparently, CD8<sup>+</sup> T cell memory was maintained under these circumstances, since a secondary challenge with CT26-KSA tumor cells after 6 wk, followed by a boost with two small, noncurative doses of huKS1/4-IL-2 fusion protein, induced the differentiation of these resting CD8<sup>+</sup> T memory cells into CD8<sup>+</sup> effector T cells. This contention is supported by four lines of evidence. First, there was a pronounced increase of >40% in CD8<sup>+</sup> T cells that apoptosed to <5% of CD8<sup>+</sup> T cells that had been adoptively transferred to SCID mice and maintained there for 6 wk. Second, these were primed CD8<sup>+</sup> effector T cells that specifically lysed CT26-KSA target cells in vitro and strongly expressed markers for IL-2 receptors and early T cell activation indicated by CD25 and CD69, respectively. Third, these CD8<sup>+</sup> T cells were effectively primed, as they secreted Th1 cytokines IFN- $\gamma$ , IL-2, and GM-CSF. Fourth, and most importantly, the CD8<sup>+</sup> T effector cells exhibited tumor-protective immunity, as they completely rejected the secondary tumor cell challenge to SCID mice and prevented the formation of pulmonary metastases.

Taken together, these data strongly suggest that T cell memory was maintained in SCID mice in the absence of Ag and that tumor cell challenge and boost with huKS1/4-IL-2 fusion protein induced differentiation of resting CD8<sup>+</sup> T memory cells into tumor-specific CD8<sup>+</sup> T effector cells that rejected the tumor cell challenge. The data obtained in our mouse tumor model suggest that Ab-IL-2 fusion proteins could be of future benefit for treatment in an adjuvant setting of colon cancer patients with minimal residual disease.

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