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Immunology

Tumor-targeted IL-2 amplifies T cell-mediated immune response induced by gene therapy with single-chain IL-12

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ABSTRACT **Induction, maintenance, and amplification of tumor-protective immunity after cytokine gene therapy is essential for the clinical success of immunotherapeutic approaches. We investigated whether this could be achieved by single-chain IL-12 (scIL-12) gene therapy followed by tumor-targeted IL-2 using a fusion protein containing a tumor-specific recombinant anti-ganglioside GD_2 antibody and IL-2 (ch14.18-IL-2) in a poorly immunogenic murine neuroblastoma model. Herein, we demonstrate the absence of liver and bone marrow metastases after a lethal challenge with NXS2 wild-type cells only in mice (five of six animals) vaccinated with scIL-12-producing NXS2 cells and given a booster injection of low-dose ch14.18-IL-2 fusion protein. This tumor-protective immunity was effective 3 months after initial vaccination, in contrast to control animals treated with a nonspecific fusion protein or an equivalent mixture of antibody and IL-2. Only vaccinated mice receiving the tumor-specific ch14.18-IL-2 fusion protein revealed a reactivation of $CD8^+$ T cells and subsequent MHC class I-restricted tumor target cell lysis *in vitro*. The sequential increase in the usage of TCR chains Vβ11 and -13 in mouse $CD8^+$ T cells after vaccination and amplification with ch14.18-IL-2 suggests that the initial polyclonal $CD8^+$ T cell response is effectively boosted by targeted IL-2. In conclusion, we demonstrate that a successful boost of a partially protective memory T cell immune response that is induced by scIL-12 gene therapy could be generated by tumor-specific targeting of IL-2 with a ch14.18-IL-2 fusion protein. This approach could increase success rates of clinical cancer vaccine trials.**

The induction and amplification of an effective T cell-mediated immune response in malignancies characterized by poor immunogenicity is the most challenging aspect of tumor vaccine development. Many tumor vaccine strategies, including cytokine-transduced tumor cells, commonly referred to as gene therapy (1), tumor-peptide-pulsed dendritic cells (2), and DNA vaccines (3, 4) are currently under preclinical and clinical investigation but have yielded only marginal immunological and clinical responses thus far. It appears that the measurable induction of a T cell immune response in cancer patients after such vaccination strategies does not necessarily translate into suppression of primary tumor growth and metastases, to say nothing of their eradication. The missing link between the T cell immune response frequently induced in patients by such immunotherapeutic approaches and clinical effects are potent adjuvants that can switch a weak T cell activation into an effective T cell-mediated immune response.

Recombinant human IL-2 is a good candidate in this regard, because it is the T cell growth factor necessary for priming and expansion of this effector cell population. In fact, it was shown recently that melanoma patients receiving a gp100 melanoma-associated antigen peptide vaccine, designed to increase binding to HLA-A2 molecules, resulted in clinical cancer regression in 42% of the patients when high-dose recombinant human IL-2 was administered as a bolus (5). This was in contrast to patients who received only the vaccine and showed no, or minor, cancer regressions (5). These findings clearly demonstrate a role for IL-2 as a potent vaccine adjuvant. However, the systemic application of IL-2 in clinically effective doses is associated with life-threatening side effects that limit its use in cancer immunotherapy. Our previous work demonstrated that tumor-specific targeting of IL-2 by means of a mAb-cytokine fusion protein is a superior alternative to systemic IL-2 application. Specifically, we showed that an anti-ganglioside GD_2 antibody-cytokine fusion protein effectively suppresses and eradicates established disseminated metastases in GD_2-positive neuroectodermal tumor models (6, 7). In contrast, systemic IL-2 given as an equivalent mixture of antibody and cytokine was entirely ineffective (6, 7). Based on these findings, we hypothesized that tumor-specific targeting of IL-2 into the tumor microenvironment is more effective in amplifying a cancer vaccine-induced T cell-mediated immune response than systemic IL-2.

This hypothesis was tested with a vaccine consisting of NXS2 neuroblastoma cells genetically engineered to produce single-chain IL-12 (scIL-12); we named the engineered cells scIL-12 NXS2 (8). We demonstrated that s.c. injection of scIL-12 NXS2 cells induces a $CD8^+$ T cell-mediated protective immunity that decreases over time against a lethal intravenous challenge with wild-type NXS2 neuroblastoma cells.

Herein, we demonstrate effective amplification of the initial T cell response by tumor-specific targeting of IL-2 with an anti-GD_2 antibody-IL-2 fusion protein (ch14.18-IL-2) after vaccination with scIL-12 NXS2 cells. This was verified by the complete absence of liver and bone marrow metastases in the majority of scIL-12 NXS2 vaccinated animals challenged with wild-type NXS2 tumor cells 90 days after the vaccination and given booster injections beginning 5 days thereafter of repeated noncurative injections of tumor-specific ch14.18-IL-2 fusion protein. In contrast, administration of nonspecific ch225-IL-2 fusion protein or an equivalent mixture of ch14.18 antibody and IL-2 were completely ineffective. A mechanism of ch14.18-IL-2-mediated reactivation for $CD8^+$ memory T cells initially induced by the vaccine was provided by increases in $CD25^+/CD8^+$ T cells, MHC class I-restricted target cell killing, and expansion of Vβ11 and -13 expressing T cells. Consequently, the results of this study clearly demonstrate a superior role for tumor targeted IL-2 as an adjuvant for cancer vaccines.

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Abbreviations: scIL, single-chain IL; mIL, mouse IL; RT-PCR, reverse transcriptase–PCR; TCR, T cell receptor.

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MATERIALS AND METHODS

Construction of ch14.18-IL-2 Fusion Protein and Mouse scIL-12. The ch14.18-IL-2 fusion protein was constructed by fusion of a synthetic sequence coding for human IL-2 to the carboxyl-terminal end of the human Cγ1 gene of the ch14.18 antibody, as described (9). The fused gene was introduced into the vector pdHL2 and expressed in Sp2/0-Ag14 cells in the presence of increasing concentrations of methotrexate (100 nM to 5 μM). The ch14.18-Il-2 fusion protein (1 μg) contains a molar equivalent of 3,000 units of recombinant human IL-2 (10, 11). Mouse scIL-12 was constructed by generating the p35 and p40 chains of mouse IL-12 (mIL-12) by reverse transcriptase-PCR (RT-PCR) and linkage of the p35 and p40 cDNAs with a synthetic 48-bp linker encoding 15 amino acids $(Gly_4\text{-}Ser)_3$ (8). The pBK-CMV vector (Stratagene) was used for expression of scIL-12 in NXS2 cells in the presence of G418 (Sigma; 500 μg/ml) (8). Specific mIL12 bioactivity of the scIL-12 construct was one-sixth that of the mIL-12 standard (Hoffmann–La Roche), as determined by measuring the ability to induce mouse IFN-γ after incubation with mouse splenocytes, as described (12). The mIL-12 protein content was measured by an ELISA for mIL-12-p70 (Genzyme). One NXS2 clone secreting 4.0 ng of mIL-12 per 10^6 cells per 24 h, as determined by ELISA, was chosen for vaccination experiments and compared with an empty vector control.

Experimental Bone Marrow and Liver Metastases Model of Neuroblastoma. Syngeneic female A/J mice were obtained at 8 weeks of age from The Jackson Laboratory. Animal experiments were performed according to the National Institutes of Health Guide for *The Care and Use of Laboratory Animals*. NXS2 cells (13) were harvested and used for induction of metastases only if their viability exceeded 95%, as determined by trypan blue staining. Experimental bone marrow and liver metastases were induced by tail vein injection of 5×10^4 NXS2 cells and mice were sacrificed for evaluation after 28 days. The percentage of liver surface covered by fused metastases was determined and liver weights were measured with fresh tissue specimen. For evaluation of bone marrow metastases, the bone cavities of both femurs and tibiae of each animal were flushed with 3 ml of PBS (pH 7.4). The cell pellet was used for total RNA isolation (Rneasy, Qiagen, Chatsworth, CA) and subsequent RT-PCR for the detection of tyrosine hydroxylase, as described (7). According to results of both high- and low-sensitivity PCR assays, bone marrow metastases were designated as stage 0 with no PCR signal, stage 1 with an exclusive signal for high-sensitivity PCR, and stage 2 in the presence of both high- and low-sensitivity PCR signals.

Cytotoxicity Assays. Effector cells were prepared from mouse spleen cells by hypotonic lysis of RBCs with ACK lysis buffer (GIBCO). These cells were cultured in the presence of irradiated NXS2 cells in complete DMEM with 10% FCS and 5% T-STIM culture supplement (Becton Dickinson) for 3 days and either used for cytotoxicity assays or for flow cytometry analyses. Target cells were incubated in the presence of 0.5 mCi of $Na_2{}^{51}CrO_4$ (Amersham Pharmacia) for 2 h at 37°C, washed three times, and seeded in a U-shaped 96-well plate at a density of 5,000 cells per well. Effector cells were added at 50:1 effector-to-target cell ratio in a final volume of 200 μl per well and incubated for 18 h. Anti-$H2K^k$ MHC class I antibodies (clone 36–7-5; PharMingen) were used at a concentration of 25 μg/ml. Total ^{51}Cr release was induced with 5 μl of 10% SDS and radioactivity in the supernatant was measured. The percentage of specific lysis was determined, as described (8).

Flow Cytometry Analysis. Two-color flow cytometric analyses were performed with splenocytes after lysis of RBCs with ACK lysis buffer. Lymphocytes were incubated for 1 h at 4°C with FITC-conjugated anti-CD25 (7D4) and phycoerythrin-labeled anti-CD8 (HIT8a) mAb (PharMingen), washed three times by centrifugation ($400 \times g$, 5 min), and analyzed immediately with a Becton Dickinson FACScan. A total of 10,000 labeled cells per sample were analyzed.

T Cell Receptor (TCR) Vβ Chain Analysis. The usage of the TCR Vβ chain of T cell subpopulations in mice was analyzed and quantified with a multiprobe RNase protection assay, as described (14, 15). Briefly, total RNA was isolated from $CD4^+$ and $CD8^+$ T cells purified by magnetic-activated cell sorting (Mini MACS Miltenyi, Auburn, CA) (8). RNA was hybridized overnight with labeled probes specific for murine TCR Vβ sequences, as described (15). After digestion of unhybridized RNA and ethanol precipitation, samples were electrophoresed on polyacrylamide sequencing gels for autoradiography, and radioactivity was measured by direct β counting (Radioanalytical Imaging Apparatus, AMBIS). Results for each of the 18 gene probes were expressed as percentages of total measured TCR Vβ. The clonality of a preferential TCR Vβ usage was determined by RT-PCR, as described (16).

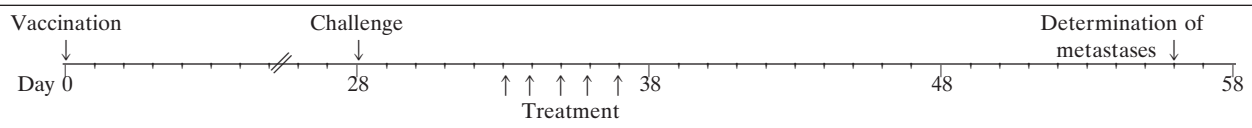
Statistical Methods. The statistical significance of differential findings of metastatic scores between experimental groups of animals was determined by the nonparametric Mann–Whitney rank sum test. The parametric Student's *t* test was used to evaluate the statistical significance of FACScans, cytotoxicity assays, and TCR Vβ chain analyses. Findings were regarded as significant if two-tailed *P* values were ≤ 0.01.

RESULTS

Effect of Targeted IL-2 on Protective Immunity After scIL-12 Gene Therapy. The induction of a $CD8^+$ T cell-mediated protective immunity after scIL-12 gene therapy has been demonstrated by the complete absence of experimental liver and bone marrow metastases after a lethal challenge with NXS2 wild-type cells, i.e., 7 days after initial vaccination (8). This vaccination effect was most likely due to the paracrine production of scIL-12, because no systemic scIL-12 could be detected by ELISA up to 14 days after initial vaccination at a sensitivity of 10 pg/ml (data not shown). Partial tumor protection was still evident in vaccinated mice challenged on day 28 (Table 1). However, all mice eventually presented with detectable macroscopic liver disease and bone marrow metastases, as verified by tyrosine hydroxylase RT-PCR. This was in contrast to scIL-12 NXS2-vaccinated animals receiving subcurative amounts of disialoganglioside GD_2-specific ch14.18-IL-2 (10 μg, five times) 5 days after challenge with GD_2-positive NXS2 neuroblastoma cells. Notably, mice receiving both the scIL-12-NXS2 vaccine and the ch14.18-IL-2 booster injections presented with a complete absence of liver metastases in all animals, and five of six mice were also free of bone marrow metastases. Only one mouse revealed a signal with the high-sensitivity tyrosine hydroxylase RT-PCR detecting one NXS2 cell in 100,000 naïve bone marrow cells (7). It is important to note that vaccinated mice injected with a nonspecific ch225-IL-2 fusion protein recognizing the human epidermal growth factor receptor (17) showed no boost in the immune response, indicating that specific targeting of IL-2 into the tumor microenvironment was required for effective amplification. This was also demonstrated in control experiments with animals receiving an equivalent mixture of ch14.18 antibody and IL-2 cytokine (Table 1); the absence of an increased immune response in this group convincingly demonstrates the superiority of targeted versus systemic IL-2 for amplification of anti-tumor immune responses by the scIL-12 NXS2 cell vaccine.

Mice injected with NXS2 neuroblastoma cells carrying the empty vector produced no protective immunity upon lethal challenge with NXS2 wild-type cells in the presence or absence of additional ch14.18-IL-2 injections, a control for the protective immunity only observed with scIL-12-secreting NXS2 cells. The lack of tumor eradication in naïve mice injected with only 5×10 μg of ch14.18-IL-2 (Table 1) represented an

Table 1. Effect of a tumor-specific antibody-IL-2 fusion protein on the amplification of tumor-protective immunity induced by scIL-12 gene therapy



Vaccine	Depletion	Treatment	Bone marrow metastasis	Liver metastasis	Liver weight, mg
None	None	PBS	2,2,2,1,1,1	4,4,4,4,4,4	3,593 ± 313
None	None	ch14.18-IL-2	1,1,1,1	3,2,2,1	1,310 ± 67
pBK-CMV NXS2	None	PBS	2,2,2,2,2,2	4,3,3,2,2,2	1,705 ± 431
pBK-CMV NXS2	None	ch14.18-IL-2	2,2,1,1,1,0	4,3,2,2,2,1	1,896 ± 765
scIL-12 NXS2	None	PBS	2,2,2,1,1,0	4,4,3,2,1,1	2,011 ± 986
scIL-12 NXS2	None	ch14.18 + IL-2	2,2,1,1	3,2,2,1	1,530 ± 320
scIL-12 NXS2	None	ch225-IL-2	2,2,2,1,1,0	4,3,2,2,1,1	1,610 ± 357
scIL-12 NXS2	None	ch14.18-IL-2*	0,0,0,0,0,1	0,0,0,0,0,0	912 ± 126
scIL-12 NXS2	$CD8^+$ T cells	ch14.18-IL-2	2,2,1,1,1,0	3,3,2,1,1,1	1,792 ± 632
scIL-12 NXS2	$CD4^+$ T cells	ch14.18-IL-2	1,1,1,0,0,0	2,2,1,1,0,0	1,366 ± 475

Mice were vaccinated by s.c. injection of 5×10^6 NXS2 cells genetically engineered to produce scIL-12 and challenged by a lethal injection of 5×10^4 NXS2 wild-type cells 28 days after the initial vaccination. T cells were depleted by i.p. injection of 500 μg of anti-CD4 and anti-CD8 antibodies at days 26, 31, 39, and 46. Treatment was initiated at day 5 after tumor cell challenge by five daily i.v. injections of PBS; 10 μg of ch14.18 antibody + 30,000 units of rhIL-2, 10 μg of the nonspecific ch225-IL-2 fusion protein, or 10 μg of the tumor-specific ch14.18-IL-2 fusion protein. For each mouse, bone marrow metastases were staged according to results obtained by high- and low-sensitivity tyrosine hydroxylase RT-PCR. For each mouse, liver metastases were staged according to the percentage of metastatic liver surface as follows: 0, 0%; 1, <0–25%; 2, 25–50%; 3, 50–75%; 4, 75%.

*Differences in bone marrow staging, liver metastasis, or liver weights between fusion-protein-treated mice and all control groups were statistically significant ($P < 0.05$). Data for liver weight are the mean ± SD.

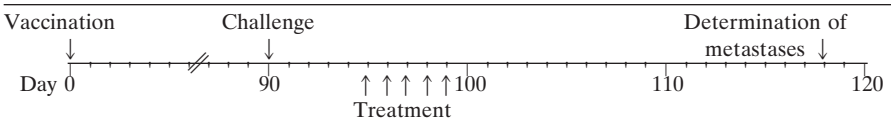
important experimental control, because synergy was only observed in animals that also received the scIL-12 NXS2 vaccine.

Our previous finding of decreased immune protection after scIL-12 vaccination over time (8) led us to determine whether the amplification was effective after long intervals between vaccination and ch14.18-IL-2 boost. We observed that the amplification of the scIL-12 NXS2 cell vaccine-induced immune response was effective up to 3 months after initial vaccination (Table 2). This is in contrast to mice injected with the nonspecific ch225-IL-2 fusion protein or an equivalent mixture of ch14.18-antibody and IL-2. This result confirmed the requirement of specific IL-2 targeting for an effective boost.

Reactivation of $CD8^+$ T Cells After Targeted IL-2-Mediated Amplification of the Immune Response. The mechanism involved in the amplification of the scIL-12 NXS2 cell vaccine-induced immune response by ch14.18-IL-2 was addressed in mice depleted of $CD4^+$ or $CD8^+$ T cells. Depletion of $CD8^+$ T cells almost entirely abrogated the amplification effect of the ch14.18-IL-2 boost, whereas depletion of $CD4^+$ T cells resulted in only a partial abrogation, indicating that the boosted immune response is primarily mediated by $CD8^+$ T cells and aided by the helper function of $CD4^+$ T cells.

Several lines of evidence support the contention that $CD8^+$ T cells are the effector cells mediating the amplification of the scIL-12 NXS2-induced vaccine after targeting of IL-2 by the ch14.18-IL-2 fusion protein (Fig. 1). (*i*) Only mice receiving scIL-12 NXS2 cells developed a memory T cell phenotype (18) 28 days after vaccination, as indicated by the increased frequency of $CD44^{hi}$, $Ly6C^{hi}$, and $CD62L^{lo}$ $CD8^+$ T cells (Fig. 1*A*). This is in contrast to naïve mice or mice injected with NXS2 cells carrying the empty vector, which essentially featured the reverse or naïve phenotype. (*ii*) A doubling in

Table 2. Long-lived amplification of tumor-protective immunity by a tumor-specific antibody IL-2 fusion protein



Vaccine	Treatment	Bone marrow metastasis	Liver metastasis	Liver weight, mg
scIL-12 NXS2	PBS	2,2,2,2,2,1	4,4,4,2,2,1	2,451 ± 723
scIL-12 NXS2	ch14.18 + IL-2	2,2,2,1,1,1	3,3,2,2,2,1	2,192 ± 823
scIL-12 NXS2	ch225-IL-2	2,2,2,1,1,0	4,3,3,1,1,1	1,810 ± 650
scIL-12 NXS2	ch14.18-IL-2*	0,0,0,0,0,1	0,0,0,0,0,0	981 ± 88

Mice were vaccinated by s.c. injection of 5×10^6 NXS2 cells genetically engineered to produce scIL-12 and challenged by a lethal i.v. injection of 5×10^4 NXS2 wild-type cells 90 days after initial vaccination. Treatment was initiated at day 5 after tumor cell challenge by five daily i.v. injections of PBS; 10 μg of ch14.18 antibody + 30,000 units of rhIL-2, 10 μg of the nonspecific ch225-IL-2 fusion protein, or 10 μg of the tumor-specific ch14.18-IL-2 fusion protein. Bone marrow metastases were staged according to results obtained by high- and low-sensitivity tyrosine hydroxylase RT-PCR. Liver metastases were staged according to the percentage of metastatic liver surface as follows: 0, 0%; 1, <0–25%; 2, 25–50%; 3, 50–75%; 4, >75%. Data for liver weight are the mean ± SD.

*Differences in bone marrow staging, liver metastasis, and liver weights between fusion-protein-treated mice and all control groups were statistically significant ($P < 0.01$).

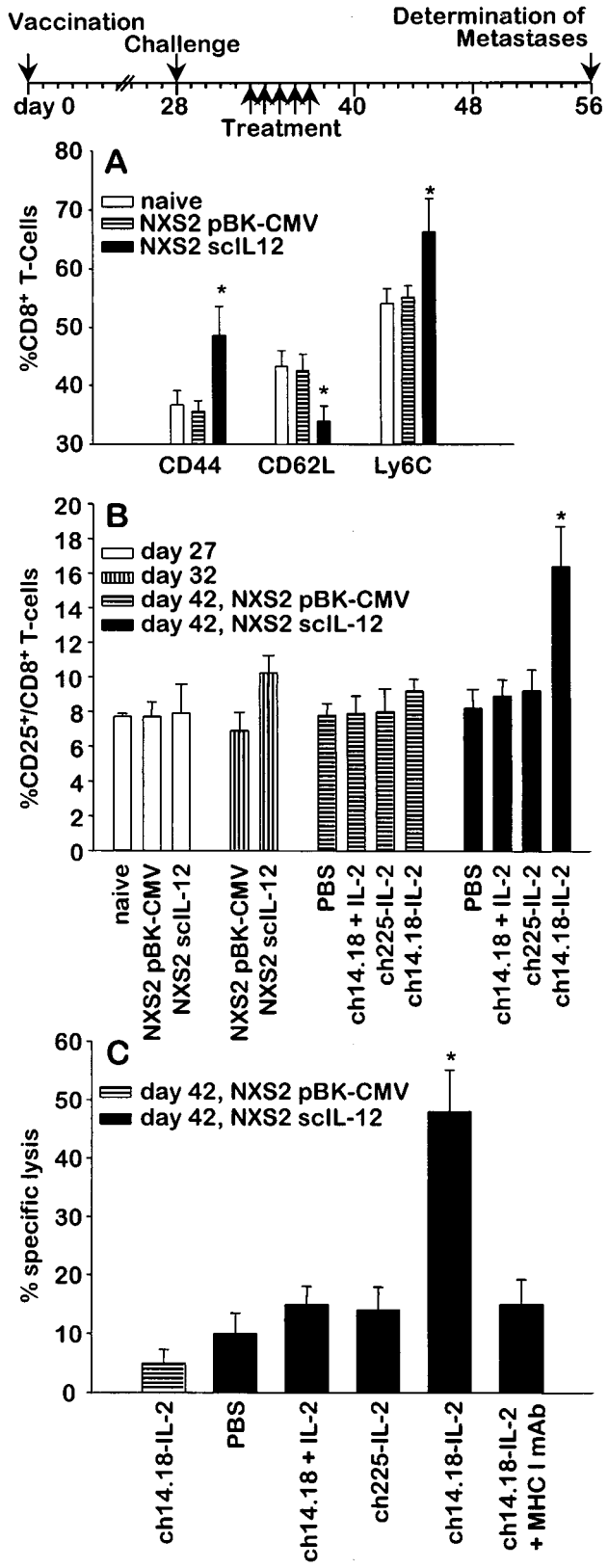


FIG. 1. Effect of ch14.18-IL-2 treatment on reactivation of $CD8^+$ T cells after initial vaccination with scIL-12 NXS2 cells. (*A*) Phenotype of $CD8^+$ T cells after vaccination of A/J mice with NXS2 cells genetically engineered to secrete scIL-12. Splenocytes of mice ($n = 4$) injected with scIL-12 NXS2, NXS2 cells carrying the empty vector, or naive mice were analyzed 28 days after vaccination by two-color flow cytometry. Differences between scIL-12 NXS2 vaccinated and naive mice or mice receiving the irrelevant vaccine were statistically significant. *, $P < 0.02$. (*B*) Detection of activated $CD8^+$ T cells after vaccination, challenge, and booster injection with ch14.18-IL-2 fusion protein. Splenocytes of naive mice ($n = 4$), scIL-12 NXS2-vaccinated mice, and mice injected with NXS2 cells carrying the empty vector were analyzed for $CD25^+/CD8^+$ T cells 5 days after five daily i.v. injections of 10 μg of ch14.18-IL-2, an equivalent mixture of 10 μg of ch14.18 antibody and 30,000 units of IL-2, or 10 μg of nonspecific ch225-IL-2 fusion protein. Differences among scIL-12 NXS2-vaccinated mice and mice receiving a ch14.18-IL-2 booster injection, and all control groups was statistically significant. *, $P < 0.01$. (*C*) Determination of $CD8^+$ T cell-mediated cytotoxicity after vaccination and booster injections with ch14.18-IL-2 fusion protein. $CD8^+$ T cells of scIL-12 NXS2-vaccinated mice ($n = 4$) and mice injected with NXS2 cells carrying the empty vector were isolated 5 days after the booster injections as described in *B* and used in a standard ^{51}Cr release assay with NXS2 target cells. Differences among scIL-12 NXS2-vaccinated mice and mice receiving ch14.18-IL-2 booster injections, and all control groups was statistically significant. *, $P < 0.001$.

reactivated $CD8^+$ T cells 5 days after the booster injection was indicated by the $CD8^+/CD25^+$ phenotype observed in animals receiving both the scIL-12 NXS2 vaccine and injections of ch14.18-IL-2 (Fig. 1*B*). This increase of $CD8^+/CD25^+$ T cells was observed in mice vaccinated with scIL-12 NXS2 cells receiving the nonspecific fusion protein ch225-IL-2 or an equivalent mixture of ch14.18 antibody and IL-2 cytokine. (*iii*) $CD8^+$ T cells from splenocytes of mice vaccinated with scIL-12 NXS2 cells and receiving the tumor-specific booster injections with ch14.18-IL-2 revealed a unique 3-fold increase in MHC class I-restricted tumor target cell lysis. This is in contrast to controls treated with nonspecific ch225-IL-2 or an equivalent mixture of ch14.18 antibody and IL-2 (Fig. 1*C*). Importantly, only background lysis was observed in control mice injected with NXS2 cells carrying the empty vector and receiving a booster injection with ch14.18-IL-2, a finding that demonstrates the absence of an amplified T cell immune response with an ineffective vaccine.

Expansion of $CD8^+$ T Cells with a Distinct TCR Vβ Chain Repertoire. The question of whether the reactivated T cells, after a tumor-specific booster injection with ch14.18-IL-2 fusion protein, originate from T cells initially activated by the scIL-12 NXS2 cell vaccine was addressed by analysis of TCR Vβ chain usage. A distinct increase in $CD8^+$ T cells using TCR Vβ11 and Vβ13 was observed 14 days after vaccination with scIL-12 NXS2 cells, which was doubled 5 days after completion of the booster injections with tumor-specific ch14.18-IL-2 fusion protein. This increase revealed a polyclonal pattern after TCR amplification with Vβ11- and Vβ13-specific primers (data not shown). The analysis of the TCR Vβ repertoire of $CD4^+$ T cells revealed no change between all experimental groups (data not shown). In contrast, $CD8^+$ T cells of controls receiving the nonspecific fusion protein or the equivalent mixture of ch14.18 antibody and IL-2 cytokine (Fig. 2) showed no such increase in Vβ11 and Vβ13, as did mice injected with the irrelevant vaccine of NXS2 cells carrying the empty vector but receiving booster injections with tumor-specific ch14.18-IL-2. Thus, these data suggest that the initial induction of $CD8^+$ T cells with TCR Vβ11 and Vβ13 by the scIL-12 NXS2 cell vaccine is a necessary requirement for further amplification by tumor-specific ch14.18-IL-2 fusion protein.

DISCUSSION

Effective amplification of immune responses induced by a cancer vaccine is an important strategy to achieve clinical responses in cancer patients receiving immunotherapeutic treatment. The important role of effective immune response amplifiers, called adjuvants, has captured the imagination of immunologists for decades and has led to important developments, such as the use of killed *Bordetella pertussis* for diphtheria and tetanus toxoids (19), muramyl dipeptide extracted

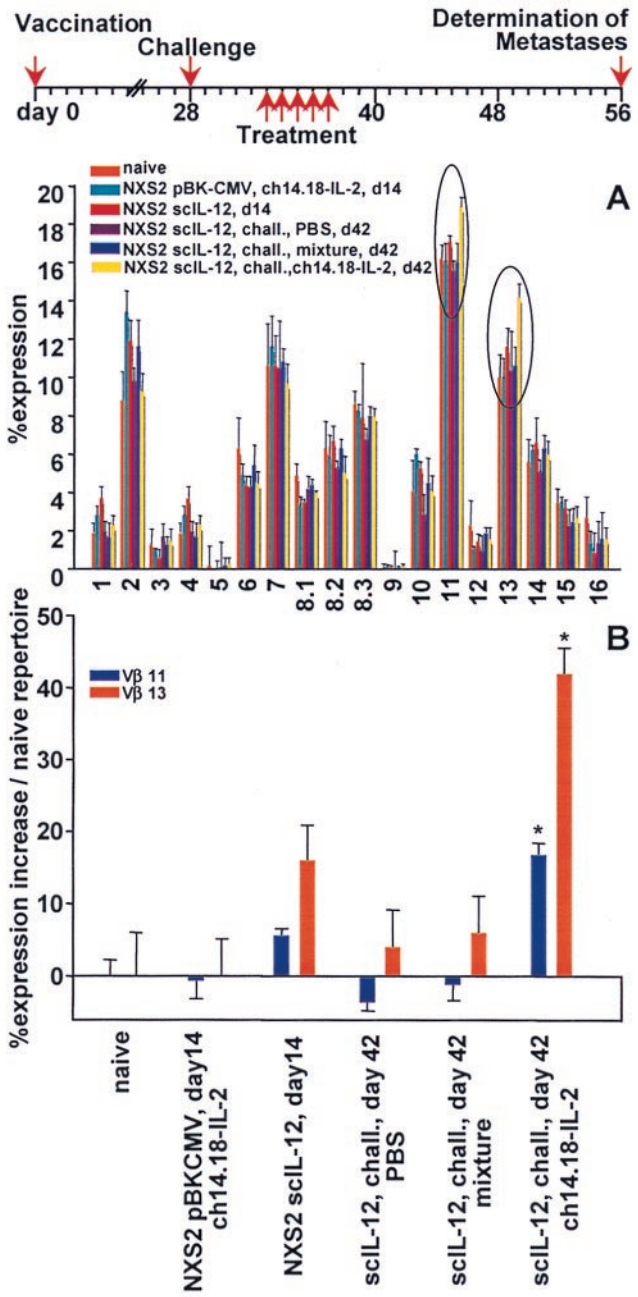


FIG. 2. Effect of ch14.18-IL-2 treatment on the TCR Vβ chain usage after vaccination with scIL-12 NXS2 cells. (*A*) Determination of the relative expression of TCR Vβ chain usage in CD8$^+$ T cells of scIL-12-vaccinated mice after booster injections with ch14.18-IL-2 fusion protein. CD8$^+$ T cells of scIL-12 NXS2-vaccinated mice, mice injected with NXS2 cells carrying the empty vector, and naive mice ($n = 4$) were analyzed for TCR Vβ chain usage 5 days after five daily i.v. injections of 10 μg of ch14.18-IL-2, an equivalent mixture of 10 μg of ch14.18 antibody and 30,000 units IL-2, or PBS. (*B*) Relative expression increase of TCR Vβ11 and Vβ13 over naive repertoire of mice treated as described in *A*. Differences among scIL-12 NXS2-vaccinated mice, mice receiving ch14.18-IL-2 booster injections ($n = 4$), and all control groups was statistically significant. *, $P < 0.02$.

from the mycobacterial cell wall (20), and immune stimulatory complexes called ISCOMs that simultaneously serve as antigen carriers and adjuvants (21). Additional adjuvants are cytokines, such as granulocyte–macrophage colony-stimulating factor or IL-2 (5), the latter also used successfully as an adjuvant in a clinical trial of melanoma patients with a respectable response of 42% in patients receiving a gp-100 peptide vaccine. However, all of these agents operate by nonspecific activation of the immune system via mechanisms that are poorly understood.

Herein, we demonstrate the efficacy of an approach to specifically amplify a T cell-mediated immune response initially induced with a cellular tumor vaccine genetically engineered to secrete scIL-12. Thus, tumor-specific targeting of the T cell growth factor IL-2 into the tumor microenvironment was shown to effectively boost a CD8$^+$ T cell-mediated immune response, as indicated by increased CD8$^+$ T cell activation, MHC class I-restricted tumor cell killing, and increased usage of Vβ11 and Vβ13 TCRs by CD8$^+$ T cells in mice receiving both the vaccine and tumor-specific IL-2 boost. The absence of such a response with systemically administered IL-2 or a nonspecific fusion protein clearly proves the concept of tumor-specific booster injection with ch14.18-IL-2 fusion protein specific for ganglioside GD_2, which is highly expressed in the neuroblastoma tumor microenvironment (22). Interestingly, the Vβ11 and Vβ13 TCR-positive CD8$^+$ T cells appear polyclonally expanded, reflecting heterogeneity of immunogenic T cell antigens present on the scIL-12 NXS2 cell vaccine. The increase in effective concentrations of IL-2 in the tumor microenvironment appears to be the crucial step in effective CD8$^+$ T cell reactivation, which can be achieved with systemic injections of tumor-specific antibody-IL-2 fusion proteins (6, 18, 23–25). Thus, the presence of both tumor-associated T cell antigens and adequate costimulation in the tumor microenvironment, provided by targeting of the tumor cell surface with IL-2, fulfill the necessary requirement for amplification of the CD8$^+$ T cell-mediated immune response.

This principle of amplifying an immune response initially induced by a cancer vaccine with a tumor-specific antibody-cytokine fusion protein could find broad application in immunotherapeutic cancer treatments. Indeed, additional treatments with tumor-specific antibody-IL-2 fusion protein could benefit many clinical trials with cancer vaccines based on cytokine gene therapy approaches, dendritic cells pulsed with immunogenic tumor-associated peptides, or DNA vaccines encoding for tumor-associated peptide antigens. Such effective adjuvants are of major interest based on the clinical response rates observed thus far in vaccine trials (1). The use of tumor-specific antibody-IL-2 fusion proteins in combination with other immunotherapeutic strategies is also justified, because these fusion proteins have proven to be effective tools for cancer immunotherapy when used alone. This observation has been demonstrated with ch14.18-IL-2, KS1/4-IL-2 and chS5A8-IL-2 fusion proteins in four syngeneic tumor models for neuroblastoma (26), melanoma (24), colon carcinoma (18), and B cell lymphoma (27). In three solid tumor models, eradication of established tumor micrometastases after intravenous injection with antibody-IL-2 fusion proteins also led to a long-lived protective immunity in preclinical models of murine melanoma and colon carcinoma (18, 24). Based on the data in this report, antibody-IL-2 fusion proteins are also effective adjuvants for immunotherapeutic gene therapy aiming at the induction of protective and therapeutic immune responses mediated by CD8$^+$ T cells.

In summary, we demonstrate that targeting of IL-2 into the tumor microenvironment with a ch14.18-IL-2 fusion protein effectively amplifies a CD8$^+$ T cell immune response induced by scIL-12 gene therapy at least 3 months after initial vaccination. This effect was specific, because equivalent mixtures of ch14.18 antibody and IL-2 or nonspecific ch225-IL-2 were ineffective in this regard. A mechanism for reactivated CD8$^+$ memory T cells was provided by increased CD8$^+$ T cell activation, MHC class I-restricted tumor cell killing, and appearance of Vβ11- and Vβ13-positive CD8$^+$ T cells only in mice that received both the vaccine and the tumor-specific boost. Thus, these data suggest that tumor-targeted IL-2 may overcome weak immune responses induced by cancer vaccines

and, therefore, lead to further improvement in the adjuvant treatment of patients with minimal residual disease.

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