

Shift from Systemic to Site-Specific Memory by Tumor-Targeted IL-2¹

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IL-2 has been approved for treatment of patients with cancer. Moreover, it has been used as a component of vaccines against cancer. In this regard, we have recently demonstrated that dendritic cell-based peptide vaccination in mice required IL-2 to mount an effective immune response against established melanoma metastases. In this study, we confirm this observation by use of tumor-targeted IL-2. However, the development of a protective systemic memory was substantially impaired by this measure, i.e., mice, which successfully rejected s.c. tumors of B16 melanoma after vaccination with dendritic cells pulsed with tyrosinase-related protein 2-derived peptides plus a boost with targeted IL-2, failed to reject a rechallenge with experimental pulmonary metastases. Detailed analysis revealed a change in the distribution of the tumor-reactive T cell population: although targeted IL-2 expanded the local effector population, tyrosinase-related protein 2-reactive T cells were almost completely depleted from lymphatic tissues. *The Journal of Immunology*, 2004, 172: 5843–5850.

Immunological memory is one of the most important properties of the immune system and is manifested physiologically by resistance to rechallenge with the same pathogen and experimentally by a secondary response that is much more rapid and intense than the primary response. The main property of the memory state that differentiates it from the naive state of the immune system is the presence of a much higher frequency of Ag-reactive cells that are equipped with an adequate homing phenotype (reviewed in Ref. 1). Moreover, memory T cells are not as dependent on costimulation as naive T cells and possess a lower activation threshold (2).

The major task of CTL is to kill infected or malignant cells that express viral or tumor Ags, respectively. However, this requires direct contact with the target cell. Because these cells may reside anywhere in the body, CTL must diversify their ability to migrate to different tissues. Consequently, it is well established that naive and memory cells have different capacities to traffic in lymphoid and nonlymphoid tissues (3). Recent reports further indicate that Ag-experienced T cells in humans comprise at least two functionally distinct subsets distinguishable by their preferential ability to home to lymphoid organs (central memory cells) or nonlymphoid tissues (effector memory/effector cells) (4). The first memory cell population is characterized by expression of CCR7 and CD62L,

whereas the latter possess a CCR7⁻CD62L^{low} phenotype. In analogy, two functionally distinct populations of CD8 memory cells have been demonstrated in mice: the designated lymphoid memory cells, residing in secondary lymphoid tissues; and the nonlymphoid memory cells, residing in tertiary tissues. Both populations are capable of producing IFN- γ , but only the latter exhibits immediate Ag-specific cytotoxicity *ex vivo* (5). The factors that govern CTL differentiation and their migratory properties are just starting to emerge. There is growing evidence that cytokines, which bind to receptors containing a common γ -chain, namely IL-2, IL-4, IL-7, and IL-15, are involved in T cell maintenance and homeostasis (6). The relative importance of these different γ -chain cytokines varies depending on their site of production as well as the expression and signaling capacity of the relevant cytokine receptors.

The therapeutic measure to induce a protective immunity to pathogens is vaccination. In this regard, vaccination played an important role in reducing the mortality and morbidity caused by infectious diseases (reviewed in Ref. 7). Additionally, therapeutic antitumor vaccination established itself in the therapeutic repertoire for cancer over the past decade. Active specific immunotherapy of cancer has focused on the induction of CD8⁺ CTL responses, primarily by using MHC class I-restricted tumor-derived peptides together with adjuvants or APCs (8).

Dendritic cells (DCs)³ are professional APC that effectively induce cellular immune responses through Ag presentation in the presence of appropriate costimulatory molecules (9). Recent evidence indicated that DCs pulsed with tumor Ags can stimulate the regression of established renal cell carcinoma and melanoma (10, 11). Despite such clinical efficacy, a significant percentage of patients with metastatic solid tumors remain unresponsive to immunomodulatory therapy (12), mandating a better understanding of DC function, T cell priming, and improved T cell activation as a means to enhance clinical efficacy.

A syngeneic B16 mouse melanoma model in combination with naturally processed peptides derived from melanoma-associated

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³ Abbreviations used in this paper: DC, dendritic cell; TRP-2, tyrosinase-related protein-2; LC, Langerhans cells.

AgS provides a valuable tool to further optimize DC-based therapeutic vaccinations to treat cancer. In this regard, we recently demonstrated that systemic IL-2 treatment following vaccination with tumor-associated Ag-pulsed DCs significantly improved the clinical efficacy of the vaccination, because it allowed the inhibition of melanoma growth in a therapeutic setting (13). In the present study, we scrutinized the effect of tumor-targeted IL-2 on DC-based peptide vaccination both with respect to therapeutic efficacy as well as to the development of immunological memory.

Materials and Methods

Animals

C57BL/6J mice were obtained from Charles River Laboratories (Sulzfeld, Germany) at the age of 6 wk. These animals were housed under specific pathogen-free conditions, and all experiments were performed according to National Institute of Health Guidelines for Care and Use of Laboratory Animals.

Cell line, Abs, peptides, and fusion proteins

The murine melanoma cell line B78-D14 has been described previously (14). B78-D14 was derived from B16 melanoma by transfection with genes coding for β -1,4-*N*-acetylglucosaminyltransferase and α -2,8-sialyltransferase, inducing a constitutive expression of the disialogangliosides GD2 and GD3. B78-D14 melanoma cells were maintained as monolayers in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 400 mg/ml G418, and 50 mg/ml Hygromycin B. Cells were passaged when subconfluent.

All Abs used (anti-CD4, clone RM4-5; anti-CD8a, clone 53-6.7; anti-CD45R/B220, clone RA3-6B2; anti-CD25, clone 7D4; and anti-CD62L, clone MEL-14) are commercially available (BD Biosciences, Heidelberg, Germany) and have been described in detail by the manufacturer.

The tyrosine-related protein-2 (TRP-2)₁₈₀₋₁₈₈ (SVYDFVWL) 9-mer peptide was synthesized with a free C terminus by Fmoc peptide chemistry on a Biosearch SAM2 peptide synthesizer (Biosearch Technologies, Novato, CA). The peptide, which was 90% pure, as indicated by HPLC, was dissolved in DMSO (Sigma-Aldrich, Taufkirchen, Germany) and stored at -20°C .

The mouse/human chimeric Ab directed against GD2 (ch14.18) was constructed by joining the cDNA for the variable region of the murine 14.18 Ab with the C regions of the human γ_1 H chain and the κ L chain (15). From this, the ch14.18-IL-2 fusion protein was constructed by fusion of a synthetic sequence coding for human IL-2—lacking the leader peptide—to the carboxyl end of the human *C γ 1* gene. The fused genes were inserted into the vector pHL2, which encodes for the dihydrofolate reductase gene. The resulting expression plasmids were introduced into Sp2/0-Ag14 cells and selected in DMEM supplemented with 10% FBS and 100 nM methotrexate. The fusion proteins were purified over a protein A-Sepharose affinity column (16).

Tumor model

Subcutaneous tumors were induced by s.c. injection of 2×10^6 B78-D14 melanoma cells in RPMI 1640, which resulted in tumors of $\sim 20\text{-}\mu\text{l}$ volume within 7 days.

Experimental lung metastases were induced by injection of single-cell suspensions of 2×10^6 B78-D14 cells into the lateral tail vein. To prevent pulmonary embolism caused by injection of tumor cells, mice were anesthetized by halothane inhalation; tumor cells were suspended in 500 μl of PBS containing 0.1% BSA and administered i.v. over a period of 60 s. At day 28, grossly visible metastases were present on the surface of the organ.

Dendritic cells

Epidermal Langerhans cells (LC) represent a DC type that can be cultured in the absence of FCS without losing any DC function or influencing their maturation. They were isolated from the mice ears as described with modifications (13). Briefly, split ears were trypsinized, and epidermal cells were released from the epidermal sheets by gentle knocking on a metal sieve. These cells were then cultured at 2×10^7 per Falcon 3003 dish (Schubert and Weiss, Munich, Germany) in 10 ml of serum-free IMDM supplemented with 5% of culture supernatant from the murine GM-CSF-secreting Ag8653 myeloma line, 200 μM glutamine, 100 $\mu\text{g}/\text{ml}$ penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin for 48 h before enrichment of LC by 13% Nycopenz (Nycomed Pharma, Oslo, Norway) gradient ($p = 1.068$). LC were stimulated overnight with anti-CD40 (mAb 3/23 at 5 $\mu\text{g}/\text{ml}$) and were pulsed for 2 h with 10–20 μM TRP-2₁₈₀₋₁₈₈ peptide before injection.

Treatment schedule

DC vaccinations were given intradermally into the lateral thigh of the mice on days 7 and 14 for s.c. tumors and on days 3 and 10 for pulmonary metastases. After the second vaccination, mice received for 5 days i.v. injections of PBS (control), 2.5×10^5 U of rIL-2, or 10 μg of ch14.18-IL-2 fusion protein, respectively.

Flow cytometry

For evaluation of the percentage and phenotype of TRP-2₁₈₀₋₁₈₈/H2-K^b-reactive T cells, analysis by flow cytometry was performed on cells isolated from spleens. Single-cell suspensions were prepared by transferring the tissues through a nylon filter. Erythrocytes were subsequently lysed by treatment with NH₄Cl lysing buffer, and the remaining cells were washed.

Samples of 5×10^5 cells were washed twice with a solution of PBS with 0.5% BSA and 0.02% NaN₃ (PBS/BSA) and incubated for 20 min with 20 μl of the appropriate dilutions of FITC-conjugated anti-CD8, PE-conjugated tetrameric TRP-2₁₈₀₋₁₈₈/H2-K^b complexes, and allophycocyanin-conjugated anti-CD25 or anti-CD62L Abs at 4°C . Cells were washed twice and resuspended in PBS/BSA. Data acquisition and analysis were performed on a BD Biosciences FACSCalibur using CellQuest software.

Confocal laser-scanning microscopy

Biotinylated K^b/ β_2 -microglobulin/peptide molecules were generated with peptides as previously described (17). Tetramers/multimers were generated by adding allophycocyanin-labeled ExtraAvidin (Sigma-Aldrich). Complexes were stored at -20°C in 0.5% BSA and 16% glycerol.

Frozen tissue sections (10- μm thick) were mounted onto silane-coated slides before staining. Tissue sections were incubated overnight at 4°C with FITC-labeled anti-CD8a (6 $\mu\text{g}/\text{ml}$) and allophycocyanin-labeled MHC tetramers (20 $\mu\text{g}/\text{ml}$) in a volume of 100 μl of PBS, 0.5% BSA, and 10% normal mouse serum on a rocking platform. This was followed by three washes with 1 ml of ice-cold PBS/0.5% BSA for 5 min. Sections were mounted in PBS/0.5% BSA/10% glycerol and covered with a cover slide before confocal laser-scanning microscopy.

FITC and allophycocyanin signals were collected on a Leica TCS NT confocal system (Leica Microsystems, Wetzlar, Germany) equipped with an argon-krypton laser. Images were taken using $\times 20$, $\times 40$, or $\times 63$, numerical aperture 1.4, objective. Possible cross talk among fluorochromes, which could lead to false-positive colocalization of signals, was routinely checked. Color photomicrographs were taken from electronic overlays.

ELISPOT assay

The ELISPOT assay has been described earlier (18) and was modified to detect TRP-2₁₈₀₋₁₈₈-specific CD8 T cells. First, 96-well filtration plates (Millipore, Schwalbach, Germany) were coated with rat anti-mouse IFN- γ Ab (clone R4-6A2; BD PharMingen, San Diego, CA). Peptide-pulsed target cells were generated by incubating RMA-S cells, a TAP-deficient T cell lymphoma line derived from C57BL/6J mice (B6, H-2^b), with the appropriate concentration of peptide for 45 min at room temperature. CD8⁺ T cells were isolated from PBL or tumor-infiltrating lymphocytes as described and kept in culture for 5 days in complete medium supplemented with 10 U/well of recombinant human IL-2 (Chiron, Marburg, Germany) before being added at indicated numbers to 5×10^4 target cells. After 24 h, the plates were washed, followed by incubation with biotinylated anti-mouse IFN- γ Ab (clone XMG 1.2; BD PharMingen). Spots were developed using freshly prepared substrate buffer (0.3 mg/ml amino-9-ethyl-carbazole and 0.015% H₂O₂ in 0.1 M sodium acetate (pH 5)).

Results

Therapeutic effect of DC-based TRP-2 peptide vaccination in conjunction with tumor-targeted IL-2

We recently demonstrated that DC vaccination with TRP-2 peptide-loaded DC cultured in the absence of FCS inhibits tumor growth efficiently in a therapeutic setting only if systemic IL-2 is used to boost T cell activity (13). Although day 3 pulmonary metastases could be cured in the majority of animals by this therapy, established s.c. tumors could only be delayed in their growth. Because prior studies indicated that tumor-targeted IL-2 is superior to systemic IL-2 and that tumor-targeted IL-2 is effective in boosting DNA-based tumor vaccination (16, 19), we tested the effect of an Ab-IL-2 fusion protein consisting of the GD2-specific Ab ch14.18 and IL-2 on therapeutic DC-based vaccinations. Subcutaneous tumors were induced by inoculation of 2×10^6 cells of the B16

melanoma subline B78-D14, which was genetically modified to express the ganglioside GD2 constitutively and thus serves as a docking site for the ch14.18-IL-2 fusion protein. Seven days thereafter, when the tumors reached a volume of $\sim 20 \mu\text{l}$, the C57BL/6J mice received the first vaccination with 2×10^4 TRP-2₁₈₀₋₁₈₈ loaded DCs, followed by a second vaccination 1 wk thereafter. After the second vaccination, mice received $10 \mu\text{g}$ of ch14.18-IL-2 i.v. for 5 consecutive days, which corresponds to only $\sim 1/100$ of the amount of systemic IL-2 used in our previous studies. Nevertheless, as depicted in Fig. 1A, the therapeutic effect obtained did result in complete tumor eradication in three of eight animals and a marked tumor growth inhibition in all of the remaining animals. In comparison, tumor growth in the group receiving only vaccination was nearly the same as in untreated naive mice (Fig. 1A). Similar results were observed for pulmonary metastases, i.e., almost no therapeutic effect for only vaccination, which was drastically improved by additional Ab-IL-2 fusion protein administration (Fig. 1B). However, it should be noted that this type of experimental metastases had already been successfully treated by the combination of DC-based vaccination with systemic IL-2.

Tumor-targeted IL-2 reduces systemic memory

These promising observations prompted us to determine whether the addition of tumor-targeted IL-2 to DC vaccinations would also improve the development of a protective immunity. To this end, established s.c. tumors were treated as described above either by DC vaccination alone or in combination with ch14.18-IL-2; the tumors not eradicated by therapy were surgically removed on day 28. On day 56, these animals were challenged i.v. with 2×10^6 B78-D14 cells. To our surprise, mice treated by DC vaccination alone were most effectively protected against this tumor challenge, whereas mice that received additional ch14.18-IL-2 displayed almost the same tumor burden as animals not treated at all (Fig. 1C). To investigate this observation in more detail, we tested for the presence of TRP-2₁₈₀₋₁₈₈-reactive T cells in the spleens of the challenged animals with an ELISPOT assay at the time of tumor rechallenge (Fig. 2). This analysis revealed that the number of T cells capable of producing IFN- γ in response to the peptide epitope used for vaccination was substantially larger in animals receiving peptide-pulsed DCs alone than those receiving additional ch14.18-IL-2. Indeed, the number of TRP-2₁₈₀₋₁₈₈-reactive cells present in the spleen was nearly as low as in control animals that did not receive any therapy (Fig. 2).

Activation of TRP-2-reactive T cells by tumor-targeted IL-2

To further analyze the effect of tumor-targeted IL-2 on vaccination-induced TRP-2-reactive T cells, TRP-2/K^b-tetramers were used to detect such cells in the circulation. The kinetic analysis revealed that, although TRP-2/K^b-tetramer-positive cells were slightly more frequent during and immediately after ch14.18-IL-2 therapy, their number in these animals decreased equally rapidly during the following 4 wk as in animals receiving DC vaccination alone (Fig. 3A). Phenotyping of TRP-2/K^b-tetramer-positive splenocytes clearly demonstrated that vaccination in combination with IL-2 fusion protein treatment led to an activation of TRP-2/K^b-tetramer-positive cells exemplified by expression of the α -chain of the IL-2R (CD25). The percentage of activated TRP-2/K^b-specific T cells was clearly increased compared with mice that received only vaccination or a combination of vaccination and systemic IL-2 (Fig. 3B). Notably, the TRP-2-reactive cells lost the expression of CD62L, i.e., the homing receptor for secondary lymphoid tissues, in response to ch14.18-IL-2 (Fig. 3C). In contrast, the expression level of CD62L remained essentially the same

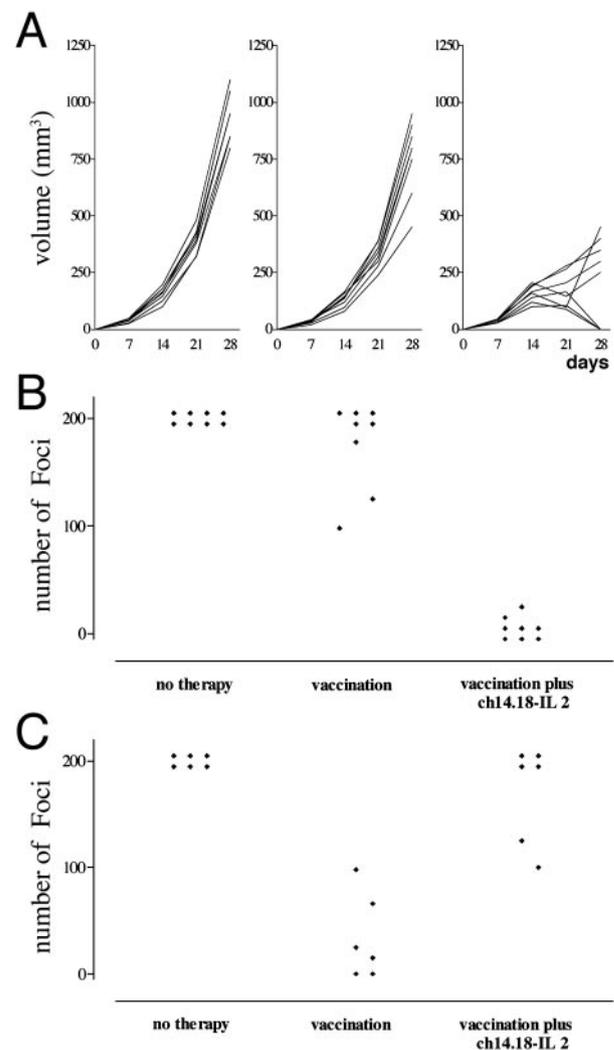


FIGURE 1. Effect of ch14.18-IL-2 on the growth of s.c. and pulmonary B78-D14 melanoma metastases. Animals received no therapy, or vaccination alone or together with tumor-targeted IL-2. *A*, A total of 2×10^6 B78-D14 melanoma cells was injected s.c. into C57BL/6J mice. Vaccinated animals received 2×10^4 TRP-2₁₈₀₋₁₈₈-pulsed DC on days 7 and 14. Therapy with i.v. ch14.18-IL-2 fusion protein ($10 \mu\text{g}/\text{day}$ for 5 days) was started after the second vaccination. Control animals received PBS over the same period. Individual tumor volumes of all animals are shown separately. *B*, C57BL/6J mice were injected with 2×10^6 B78-D14 melanoma cells into the lateral tail vein. Vaccinated animals received 2×10^4 TRP-2₁₈₀₋₁₈₈-pulsed DCs on days 3 and 10. Therapy with i.v. ch14.18-IL-2 fusion protein ($10 \mu\text{g}/\text{day}$ for 5 days) was started after the second vaccination. Control animals received PBS over the same period. Animals were killed 28 day after challenge and evaluated for metastatic disease. *C*, A total of 2×10^6 B78-D14 melanoma cells was injected s.c. into C57BL/6J mice. Vaccinated animals received 2×10^4 TRP-2₁₈₀₋₁₈₈-pulsed DCs on days 7 and 14. Therapy with i.v. ch14.18-IL-2 fusion protein ($10 \mu\text{g}/\text{day}$ for 5 days) was started after the second vaccination. Control animals received PBS over the same period. On day 28, tumors or, if completely regressed, the scar tissue was excised. Four weeks later, 2×10^6 B78-D14 melanoma cells were injected into the lateral tail vein. Animals were killed 28 days after tumor rechallenge and evaluated for metastatic disease.

on TRP-2/K^b-tetramer-reactive cells in animals not receiving ch14.18-IL-2.

The observed phenotypic changes triggered by tumor-targeted IL-2 suggested a differentiation of tumor-reactive T cells to effector or nonlymphoid memory cells. To test this hypothesis, we took

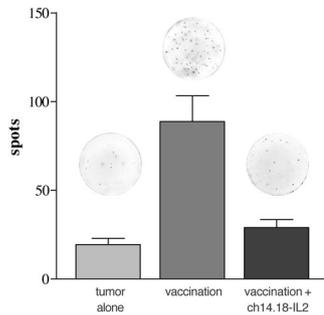


FIGURE 2. Quantification of circulating TRP-2-reactive IFN- γ -producing T cells after therapy. Subcutaneous tumors were induced by s.c. injection of 2×10^6 B78-D14 melanoma cells into C57BL/6J mice. Animals received either no therapy, or vaccination with 2×10^4 TRP-2-pulsed DCs on days 7 and 14 alone, or followed by i.v. injection of ch14.18-IL-2 fusion protein ($10 \mu\text{g}/\text{day}$ for 5 days) after the second vaccination. Control animals received PBS over the same period. Tumors or, if completely regressed, the scar tissue was excised on day 28. On day 56 after tumor inoculation, 10^5 CD8 T cells isolated from spleen were analyzed after 5 days of in vitro culture for their reactivity against the TRP-2_{180–188} epitope by ELISPOT assay. Each spot represents an IFN- γ -producing cell. Graphs depict the quantification of reactive cells; columns represent the average number of IFN- γ -producing cells in the presence of the TRP-2_{180–188} peptide subtracted by the number of respective cells in the absence of peptide. The analysis was performed in triplicate for two mice per group, and the error bars represent SD. The experiment was repeated once giving similar results.

advantage of a recently developed technique to detect T cells with a given TCR specificity in situ. Fig. 4, A–C, depicts the validation of this technique; to this end, cells of a TRP-2-reactive T cell line, splenocytes of a naive mouse or a mixture thereof were injected i.m., and the injection sites were excised 60 min thereafter and snap-frozen in liquid nitrogen. Cryosections of these samples were subjected to FITC-labeled anti-CD8 Abs and allophycocyanin-conjugated TRP-2/K^b-multimers. By confocal laser-scanning microscopy, TRP-2-reactive cells could be distinguished without difficulty (Fig. 4, A–C). By means of this technique, we first demonstrated the presence of TRP-2/K^b-reactive cells among tumor-infiltrating lymphocytes in tumors of vaccinated animals (Fig. 4, D and E) and in those receiving ch14.18-IL-2 in addition (F and G). Although the inflammatory infiltrate was substantially stronger in animals receiving ch14.18-IL-2, the frequency of TRP-2/K^b-reactive cells within the tumor infiltrating CD8 cells remained essentially the same, ranging from 3 to 6%. Nevertheless, this results in a higher absolute number of TRP-2-reactive cells in the ch14.18-IL-2-treated animals.

Depletion of TRP-2-reactive T cells from lymph nodes by tumor-targeted IL-2

If tumor-targeted IL-2 therapy would indeed trigger a shift to effector cells, TRP-2/K^b-reactive cells should be depleted from the secondary lymphoid tissue. To address this question, we analyzed inguinal lymph nodes, i.e., the draining lymph nodes of the inoculation sites of TRP-2_{180–188}-pulsed DCs, obtained from animals of both therapy groups. Serial sections of these lymph nodes were subjected to FITC-labeled anti-CD11c Abs and either allophycocyanin-labeled anti-CD8 Abs or allophycocyanin-conjugated TRP-2/K^b-multimers (Fig. 4, H–K). As depicted in Fig. 4I, TRP-2/K^b-reactive cells were reproducibly detectable in the T cell areas of lymph nodes of animals not receiving ch14.18-IL-2. Although the frequency of such cells was rather low with only two to three small clusters of TRP-2/K^b-tetramer-positive cells being detectable in

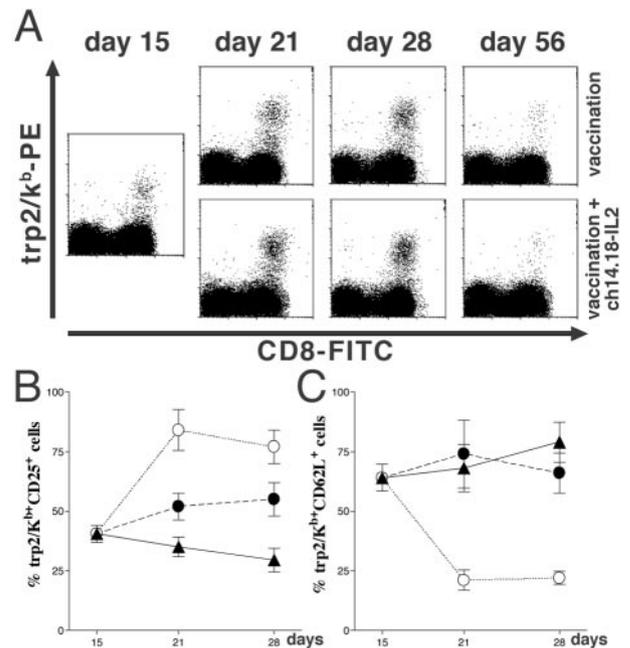


FIGURE 3. Characterization of circulating TRP-2-reactive lymphocytes in correlation to ch14.18-IL-2 therapy. Subcutaneous tumors were induced in C57BL/6J mice by s.c. injection of 2×10^6 B78-D14 melanoma cells. Animals received either vaccination with 2×10^4 TRP-2-pulsed DCs on days 7 and 14 alone or followed by i.v. injection of ch14.18-IL-2 fusion protein ($10 \mu\text{g}/\text{day}$ for 5 days) after the second vaccination. Control animals received PBS over the same period. Tumors or, if completely regressed, scar tissue was excised on day 28. A, Flow cytometry analysis using PE-conjugated TRP-2/k^b tetramer together with anti-CD8 FITC Ab on PBL obtained at the indicated time points. For each analysis, >100,000 events were collected, and 50,000 depicted. At each time point, one mouse was analyzed per group, and the experiment was performed twice with similar results. The y-axis represents tetrameric complex staining, and the x-axis represents staining with the anti-CD8 Ab. Both axes show fluorescence intensity on a log scale. Extent of CD25-positive (B) or CD62L-positive (C) cells among TRP-2/k^b tetramer-reactive cells in circulation measured by flow cytometry at the indicated time points of two mice per group per time point. ▲, Results obtained from animals treated by vaccination alone; ●, those from animals receiving additional systemic IL-2; and ○, those from animals receiving vaccination and ch14.18-IL-2. The error bars represent SD.

each analyzed section, it still seemed to be higher than in animals treated with tumor-targeted IL-2 (Fig. 4K), where only one cluster of TRP-2/K^b-positive cells in 10 examined sections could be observed. As double staining for CD8 and TRP-2/K^b-reactive T cell receptors strengthened this observation (Fig. 4, L and N), we performed flow cytometry to quantify the percentage of TRP-2/K^b-reactive T cells among the CD8⁺ cells within the lymph nodes. To this end, the frequency was already lower when vaccinated mice received soluble IL-2. This reduction was even more pronounced when the animals received tumor-targeted IL-2. In fact, on day 56, the frequency of TRP-2/K^b-reactive T cells in the lymph nodes of ch14.18-IL-2-treated mice was just above zero (Fig. 5).

Impact of tumor-targeted IL-2 on site-specific memory

Even though the development of a systemic protective memory, i.e., the protection against rechallenge with experimental pulmonary metastases, was hampered by IL-2 therapy targeted to s.c. tumors, the animals may nevertheless be able to mount a site-specific memory. Hence, animals that hosted the tumor for only 28 days that were additionally treated by vaccination alone or by vaccination in combination with ch14.18-IL-2 were challenged by s.c.

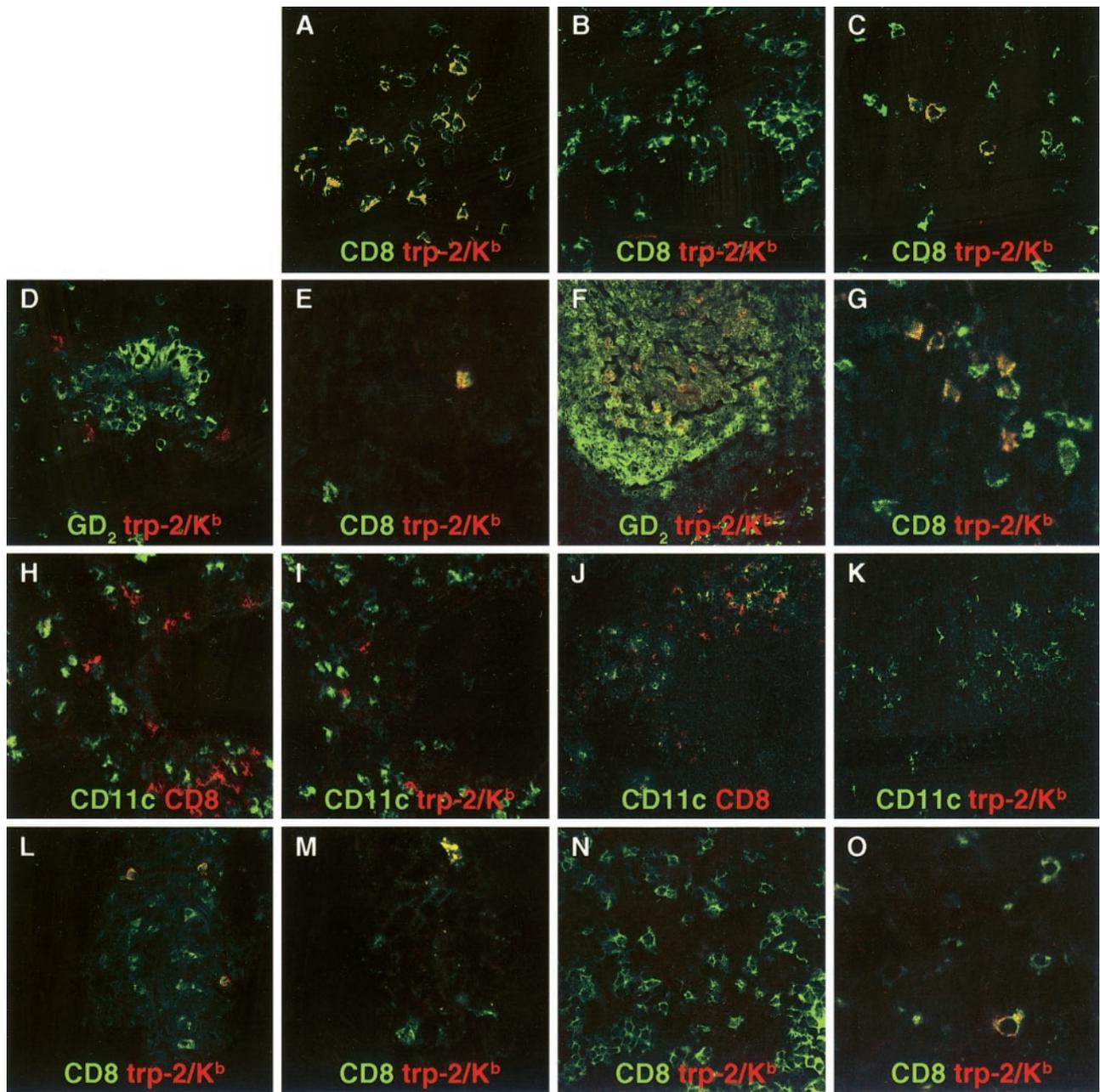


FIGURE 4. In situ detection of TRP-2_{180–188}-specific T cells in tumor tissue, lymph nodes, and skin. *A–C*, A total of 5×10^5 cells of either a TRP-2-reactive cell line (*A*), spleen cells from a naive mouse (*B*), or a mixture thereof (*C*) was injected i.m. in C57BL/6 mice. After 60 min, the injection sites were excised and snap frozen, and 10- μ m sections were subjected to anti-CD8 Ab (in green) and TRP-2/k^b-tetramers (in red); magnification, $\times 630$. *D–G*, Subcutaneous tumors obtained 21 days after induction were excised from animals receiving DC vaccination alone (*D* and *E*) or combined with ch14.18-IL-2 (*F* and *G*) as described above. Ten-micrometer cryosections were subjected to anti-GD2 (*D* and *F*) or anti-CD8 Ab (*E* and *G*; in green) and TRP-2/k^b-tetramers (in red); magnification, $\times 630$. *H–O*, Lymph nodes (*H–L* and *N*) or skin (*M* and *O*) were obtained at day 56 from animals that received vaccination alone (*H*, *I*, *L*, and *M*) or followed by ch14.18-IL-2 therapy (*J*, *K*, *N*, and *O*). Cryosections were subjected to anti-CD11c (*H–K*) or anti-CD8 Ab (*L–O*; in green) together with anti-CD8 Ab (*H* and *J*) or TRP-2/k^b-tetramers (*I* and *K–O*; in red). Magnification: $\times 200$ (*J* and *K*); $\times 400$ (*H* and *I*); and $\times 630$ (*L–O*). FITC and allophycocyanin signals were collected on a Leica TCS NT confocal system (Leica Microsystems). Color photomicrographs were taken from electronic overlays.

inoculation of B78-D14 cells on the contralateral site of the initial challenge. To this end, the tumor growth in both vaccinated groups was slower than in untreated mice (Fig. 6A). Thus, for s.c. tumor challenges, ch14.18-IL-2 did not impede the development of a protective local memory. As a matter of fact, although statistically not significant, there was a trend toward a stronger protection in ch14.18-IL-2-treated animals.

The development of a site-specific memory after tumor-targeted IL-2 was further confirmed by the detection of TRP-2/K^b-tetramer-

positive cells in the skin of vaccinated animals with or without ch14.18-IL-2 treatment (Fig. 4, *N* and *O*) and by the incidence of vitiligo-like depigmentation (Fig. 6B). Notably, loss of pigmented hair was observed only in animals treated by vaccination together with ch14.18-IL-2.

Discussion

T cells play an important role in immunological surveillance against cancer cells as well as in tumor destruction (20). Successful

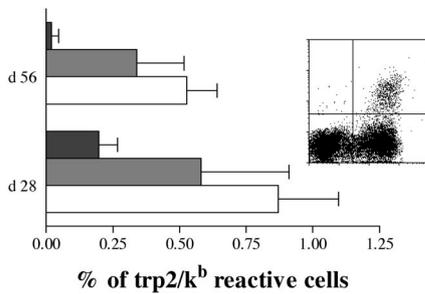


FIGURE 5. Quantification of TRP-2-specific T cells in the lymph nodes. Seven and 14 days after s.c. tumor induction, C57BL/6J received vaccinations with 2×10^4 TRP-2_{180–188}-pulsed DC alone (□), followed by rIL-2 (▨) or ch14.18-IL-2 treatment (■). Tumors or, if completely regressed, the scar tissue was excised on day 28. On day 28 or 56, respectively, lymph nodes were removed, and the percentage of TRP-2_{180–188}-reactive among the CD8-positive cells was determined by flow cytometry. The insert depicts an original flow cytometric analysis of a lymph node obtained at day 28 from a mice receiving only DC vaccination. The y-axis represents tetrameric complex staining, and the x-axis represents staining with the anti-CD8 Ab. Both axes show fluorescence intensity on a log scale. The flow cytometric analysis was performed for three mice per group per time point, and the error bars represent SD.

identification of a number of tumor Ags from both human and murine melanomas has allowed the development of Ag-specific cancer vaccines (21). However, the majority of these Ags are non-mutated self-Ags, which tend to elicit weak, self-reactive T cell responses (22). In this regard, the frequently used tumor-associated Ag of murine melanoma, TRP-2, is no exception. Thus, it is not surprising that vaccination with TRP-2_{180–188}-pulsed DCs failed to produce protective or therapeutic immunity against B16 tumor, even though CTL specific for the TRP-2 peptide were readily generated in vitro from the spleens of immunized mice. To this end, we recently demonstrated that IL-2 may be used as an adjuvant to meliorate the efficacy of vaccination-induced T cell responses (13). In an attempt to improve this effect, we used tumor-targeted IL-2, which was shown to enhance pre-existing T cell responses to tumors more efficiently than systemic IL-2 (16). This measure actually boosted the therapeutic effect of a TRP-2 vaccine, resulting in the partial or complete regression of established tumors. However, it also severely interfered with the development of a protective, systemic memory. Long-term immunological protection depends on both the quantity and the quality of memory T cells that are formed (23). The data presented here indicate that the localization where memory cells reside is also important. In fact, conditions of high local concentration of IL-2 at the dermal/s.c. tumor site seem to favor the development of nonlymphoid memory cells homing to the dermal/s.c. compartment, whereas lymphoid memory cells either did not develop at all or are depleted from lymph nodes by this measure. The impact of these changes was obvious from the effective protection against s.c. tumor challenges, whereas pulmonary challenges proved to be fatal. Notably, the observation of a site-specific protection following additional IL-2 fusion protein treatment seems to rule out the possibility that development of memory was generally impaired in the mice treated by DC vaccination and targeted IL-2, which could have been caused by incomplete immunization due to the smaller tumor volume of these mice at day 28, i.e., the time when the s.c. tumors were excised. It should be further noted that the regression of the tumor was immunologically mediated. Finally, the mere presence of a large s.c. tumor did not induce any immunization, because the control group that was treated with PBS did not display any protection against subsequent pulmonary nor s.c. tumor challenges.

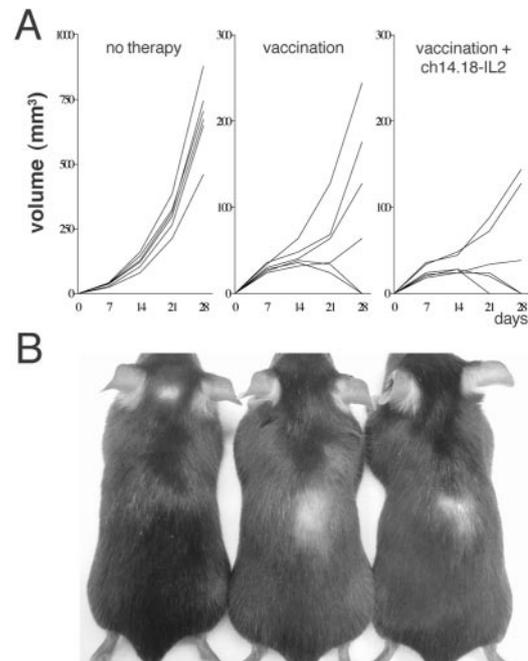


FIGURE 6. Effect of ch14.18-IL-2 on the development of site-specific memory. A total of 2×10^6 B78-D14 melanoma cells was s.c. injected into the left flank of C57BL/6J mice. Animals received no therapy, or vaccination with 2×10^4 TRP-2_{180–188}-pulsed DCs on day 7 and day 14 alone or followed by i.v. administered ch14.18-IL-2 fusion protein (10 μ g/day for 5 days) after the second vaccination. Control animals received PBS over the same period. Tumors or, if completely regressed, the scar tissue was excised on day 28. On day 56, 2×10^6 B78-D14 melanoma cells were injected s.c. into the right flank. *A*, Individual tumor volumes of all animals for the second tumor challenge are given separately. *B*, Vitiligo-like depigmentation in mice receiving TRP-2 vaccination followed by ch14.18-IL-2. Coat color changes varied in degree, site, and pattern.

Results from in vitro sustain the observed in vivo effects of tumor-targeted IL-2. In this regard, Manjunath et al. (24) demonstrated that TCR engagement together with high-dose IL-2 caused the generation of effector cells capable of immediate effector function. In contrast, IL-15 or low doses of IL-2 never allowed such cells to fully acquire the effector phenotype and function, but rather rendered them into memory-like cells. Thus, IL-2 at high concentrations as provided in the tumor microenvironment by targeted IL-2 therapy may have induced differentiation into potent effector cells, as opposed to IL-2 at low doses, e.g., produced by T cells or DC themselves, which would cause memory cell differentiation. Similarly, murine Ag-primed CD8⁺ T cells cultured in IL-15 resemble central memory cells in phenotype and function, whereas primed CD8⁺ T cells cultured in IL-2 became cytotoxic effector cells (25). These two distinct T cell populations were characterized by different migratory patterns: cells cultured in IL-15 preferentially homed to lymphoid organs and only moderately infiltrated sites of inflammation; in contrast, cells cultured in IL-2 accumulated in inflamed tissues, but were excluded from lymphoid organs. The observation that vaccine-induced TRP-2/K^b-tetramer-positive cells lost the expression of the homing receptor for high endothelial venules of lymph nodes in response to ch14.18-IL-2 is in agreement with these findings.

Recently, Masopust et al. (26) have demonstrated that, in response to viral or bacterial infection, Ag-specific CD8⁺ T cells migrated to nonlymphoid tissues and were detectable there over prolonged periods of time, suggesting that such cells either continuously recirculate through peripheral tissues or permanently reside in them. CD8⁺ memory T cells isolated from nonlymphoid

tissues exhibited effector levels of *ex vivo* lytic activity, in contrast to their splenic counterparts. Thus, a subset of memory cells with immediate effector ability is sequestered in peripheral tissues after the resolution of the initial Ag challenge, i.e., after an infection as reported by Masopust et al. (26) or after eradication of a tumor as reported in this study. Notably, the fast regression of s.c./dermal tumor challenges and the occurrence of vitiligo both at sites different from the initial s.c. tumor site imply the movement of these memory cells. However, this T cell subtype apparently possessed only a skin-homing phenotype, because the mice receiving additional IL-2 fusion protein treatment were not protected against pulmonary tumor challenges. The ability of memory cells in nonlymphoid tissue to exert immediate cytolytic activity provides a mechanism for improved survival of the organism via rapid containment of future challenges (27).

The strong polarizing effect of tumor-targeted IL-2 therapy on the differentiation of vaccine-elicited TRP-2-reactive T cells from systemic to site-specific memory could actually not be extrapolated from earlier studies using this form of immune therapy. Indeed, we have previously observed that tumor-targeted IL-2 therapy resulted in the induction of a long-lived and transferable immunity if applied in the absence of any tumor-specific vaccination (28). This immunity was based on boosting pre-existing T cell responses (29). These differences may be due to the following. First, it may be attributable to the amount and nature of Ag provided (i.e., a single epitope of an autoantigen induces a less robust immune response than a variety of tumor-associated Ags). Second, spontaneous pulmonary metastases, which were likely present during earlier studies, may account for the induction of a protective site-specific memory in the lungs; such pulmonary metastases were prevented by tumor-specific vaccination before initiation of targeted IL-2 therapy in the current study. Third, the observation that T cells seem to follow a naive→effector→nonlymphoid memory→lymphoid memory cell differentiation pathway if the specific Ag is cleared (30). Our previous studies with tumor-targeted IL-2 therapy demonstrating the induction of a long-lived and transferable immunity are more similar to the situation after clearance of viral infections as splenocytes were transferred from the treated into naive mice. Thus, tumor-specific T cells could differentiate into central memory cells and thereby exert systemic memory. In contrast, in the present study, the Ag used to pulse the DCs is also expressed by normal melanocytes, and the development of vitiligo in accordingly treated mice exemplifies the immunological relevance of this expression. Finally, the observation that TRP-2-reactive T cells were detectable before tumor rechallenge in the lymph nodes of mice receiving only DC vaccination, but not in mice receiving additional IL-2 fusion protein, implies that the cytokine milieu at the tumor site influences the development and/or differentiation of memory cells, and might reflect the situation that T cells differentiated at a slower rate when mice received high-dose infections compared with low-dose infections (30).

Our results further confirm earlier studies demonstrating that induction of autoreactivity to a nonmutated melanocyte differentiation Ag can lead to tumor destruction; however, this therapeutic effect was associated with an autoimmune disease, i.e., vitiligo. Approximately one-third of successfully treated animals exhibited a loss of pigment. Cutaneous lesions similar to those observed in the present study were described in a series of studies reporting on successful immune therapy of murine melanoma (31, 32). All of these patterns resembled vitiligo, which occurred in some melanoma patients who responded to IL-2 treatment (33). These observations suggest that the same T cell populations are responsible for the destruction for normal and neoplastic melanocytic cells. In this regard, we were recently able to demonstrate the presence of

identical T cell clonotypes in melanoma and melanoma-associated vitiligo (34). TRP-2/K^b-tetramer-positive cells present in the skin not directly affected by the tumor may be recruited for immune destruction of normal melanocytes. However, the observation that only a minority of animals developed vitiligo indicates that its initiation is dependent on the coincidence of at least two different events: the presence of specific lymphocyte populations as well as specific features of the skin disclosing a target for these cells. Accordingly, it has been shown that DCs consistently triggered autoimmune responses, which, however, led to clinical autoimmunity only in susceptible animals (35).

In conclusion, our data support and expand the concept that different Ag-experienced T cell populations exist, which possess distinct migratory properties. In the model used here, these properties are not acquired during the initial Ag encounter, but appear to be influenced by the cytokine milieu during a re-encounter of Ag. A high IL-2 concentration in the tumor microenvironment seems to favor a shift from lymphoid (or central) to nonlymphoid (or effector) memory cells. This shift is particularly obvious by a reduction of Ag-reactive T cells in the lymph nodes. Although nonlymphoid memory cells provide excellent protection to the site in which they reside, lymphoid memory cells provide a systemic memory to sites which did not have prior Ag encounter.

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