Gene delivery by attenuated *Salmonella typhimurium*: Comparing the efficacy of helper *versus* cytotoxic T cell priming in tumor vaccination

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Using the murine B16F1 melanoma, we compared a CTL- *versus* helper T cell (TH)-directed vaccination approach. Mice were either orally vaccinated with attenuated *Salmonella typhimurium* (SL) or subcutaneously with dendritic cells (DCs) loaded with gp100 peptides predicted to bind to H2-Kb/H2-Db molecules. SL were transformed with the murine gp100 cDNA (SL-gp100) or with a fusion construct of gp100 and a fragment of invariant chain cDNA (SL-gp100/Ii). Transcription of these genes *in vivo* has been readily observed in monocytes and DC. Retardation of B16F1 growth was more efficiently achieved by vaccination with SL-gp100 than with DC. Vaccination with SL-gp100/Ii aiming at preferential presentation by MHC II molecules provided some further improvement due to a stronger expansion of TH and CTL. The importance of help was further sustained by a prolongation of the survival time when mice concomitantly received IL2. Notably, prophylactic, compared to therapeutic, vaccination had no additional impact on survival time/rate. This was due to a striking decrease in frequencies of gp100-specific TH, CTL, and cytokine-expressing cells during tumor growth. Thus, the efficacy of vaccination was limited by tumor-induced immunosuppression. Our data demonstrate the oral route of vaccination *via Salmonella* as a most convenient transfer regimen and confirm the superiority of protocols aiming at preferential activation of TH. **Cancer Gene Therapy (2001) 8, 599–611**

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The isolation of melanoma-associated antigens, as started by Van denEynde and Boon,¹ has provided a rather comprehensive map of antigens setting the ground for therapeutic vaccination. Such an extrinsic stimulus, however, is mandatory because patients with malignant melanoma obviously do not mount an antitumor response spontaneously.²

One of the melanoma-associated antigens, gp100, is a melanocyte differentiation antigen.^{3,4} Despite its nature as a self-antigen, cytotoxic T cell precursors can be readily detected in the peripheral blood of healthy donors and melanoma patients.^{5,6} Because expression of gp100 is largely restricted to melanocytes, induction of a gp100-specific response does not lead to life-threatening autoaggression, i.e., no side effects besides vitiligo have been described.^{6,7} First clinical trials aiming at an induction of a CTL response showed limited success.^{8–11} Meanwhile, several studies demonstrated that vaccination with mixtures of peptides¹² and particularly with gp100 cDNA-transfected dendritic cells (DCs)^{13–16} provides a superior vaccination regimen. It is supposed that this is due to the activation of multiple CTL

clones, which recognize so far nonidentified peptides of gp100 binding to multiple HLA alleles. Murine gp100 has been demonstrated to be less immunogenic than human gp100.^{17,18} With the exception of one study, which used attenuated *Salmonella* transformed with a peptide-ubiquitin construct,¹⁹ a protective response against murine gp100 has only been observed after vaccination with human gp100.¹⁷

Currently, much effort is spent in elaborating optimal protocols of active immunization as a module in cancer therapy.^{20,21} *Ex vivo* loaded DCs have been shown to most efficiently activate T cells.^{22–26} This could be due to the preferential activation of TH cells, which are known to account for a number of advantages in initiating an immune response.^{27–33} Thus, an optimal activation of CTL depends on the right cytokines being provided by TH cells,^{34,35} and recruitment as well as activation of nonadaptive defense mechanisms can only be achieved by activation of TH cells.^{36,37} Recently, vaccination with RNA has come into focus because of the easy access to the “vaccine.” Liposome-encapsulated RNA has been applied directly or has been used for the transfection of DC.^{38–40} Similarly, naked cDNA^{40–45} can be delivered intramuscularly or intradermally or by transfection of, e.g., DC. Packaging of DNA in attenuated *Salmonella* or *Listeria* has opened another technically easy to handle and noninvasive mode of vaccination *via* the oral route.^{46–48} These bacteria can cross the epithelial barrier of the gut and will be taken up by

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macrophages and other mucosa-associated phagocytes, where they survive for a limited number of cell cycles. It has been described that the DNA introduced into the bacteria will be transcribed in the host cells, which either present the antigen directly or eventually may die such that DC will take up the cell debris and present resulting peptides preferentially (though not exclusively) on MHC class II molecules. Monocytes that have ingested the bacteria will present peptides mainly in the context of MHC class I molecules. These features explain why after DNA vaccination *via* a bacterial transporter, TH as well as CTL responses and antibody production can be observed.^{49,50}

Animal studies comparing the efficacy of different vaccination protocols will be rather helpful to define which strategy is best suited for clinical application. Here we compared the efficacy of vaccination with murine gp100 by transformed *Salmonella* versus peptide-loaded DC. The efficacy of vaccination was judged by tumor takes in mice, which had been prophylactically vaccinated, and in mice where vaccination was started at the day of tumor cell inoculation. The latter protocol was thought to mimic clinical settings, where immunotherapy may be approached in patients with a minimal residual tumor load. Therefore, we termed this protocol as “therapeutic” vaccination, even though we did not intend to cure established tumors. We addressed concomitantly the question of potential advantages by recruiting gp100 peptides toward the MHC class II loading compartment. The latter has been achieved by transformation of *Salmonella* with an invariant chain–antigen fusion cDNA. Invariant chain–antigen fusion proteins have been described repeatedly to function as a vehicle for directed and enhanced MHC class II antigen processing and presentation.^{51–54} The far cheaper and technically easier to handle oral vaccination with transformed attenuated SL actually proved to be superior to vaccination with loaded DCs, particularly when the former were transformed with a gp100/Ii fusion construct. This most likely is due to the strong and prolonged transcription of the gene in antigen-presenting cells.

MATERIALS AND METHODS

Mice and tumors

C57BL6 mice were obtained from WIGA (Sulzfeld, Germany). Animals were kept under specific pathogen-free conditions. They were fed sterilized food and water *ad libitum*. Animals were used for experiments at the age of 8–10 weeks. The C57BL6-derived melanoma line, B16F1,⁵⁵ was used for cloning of the murine gp100 cDNA and for all *in vivo* experiments. Where indicated, YAC cells were used as NK target. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS).

Monoclonal antibodies

The following mAb were used: YTA 3.1.2 (anti-CD4), YTS169.4.2.1 (anti-CD8), YBM 6.1.10 (anti-Mac-1), 33.1 (anti-DC), H2-D^b (K7-65)- and H2-A^b (K25-137)-specific mAb^{56,57} were kindly provided by G. Hämmerling (German Cancer Research Center, Heidelberg, Germany).

Anti-CD28, anti-CD40, anti-CD40L, anti-CD80, anti-CD86, anti-CD152, anti-IL2, anti-IL4, anti-IL6, anti-IL10, anti-IL12, anti-IFN γ , anti-TNF α , and secondary, dye-labeled (FITC, Rhodamin, or PE) antibodies were obtained commercially (Pharmingen, Hamburg, Germany).

For flow cytometry, $3\text{--}5 \times 10^5$ cells were stained according to standard protocols. When assaying cytokine-producing cells, lymphocytes were fixed with formaldehyde and permeabilized with PBS/1% Tween-20. Fluorescence was determined using a FACStar (Becton Dickinson, Heidelberg, Germany).

Cloning of the gp100 cDNA

Total mRNA was isolated from the B16F1 murine melanoma line and was reversely transcribed into cDNA. The cDNA was amplified by reverse transcription polymerase chain reaction using different primer pairs for either full-length gp100 cDNA or for a gp100 cDNA missing the starting 72 nucleotides at the 5' end (gp100-72) according to the sequence of mgp100 (EMBL database, accession no. U414133) (5' end primer for gp100: ATAAGCT-TATGGGTGTCCAGAGAAGG, 5' end primer for gp100-72: ATAAGCTTGGATCCAGGAATCAGGAC, 3' end primer (reverse): ATGAATTCTCAGACCTGCTGT CCACT; the recognition sequences for *Hind*III and *Eco*RI are underlined). The amplification products of 1881 bp (gp100) and 1808 bp (gp100-72) were ligated into the PCR II TOPO vector. After amplification in the *Escherichia coli* K12/DH5 α strain and partial digestion with *Hind*III and *Eco*RI, mgp100 was ligated in the pVAX1 vector, which had been digested with *Hind*III and *Eco*RI (pVAXmgp100).

To direct mgp100 into the MHC compartment, cDNA coding for the 78 N-terminal AA of the murine invariant chain (mIi) was fused to the cDNA of mgp100-72. The pVAX1 vector was digested with *Hind*III and the ends were filled to destroy the *Hind*III site. Thereafter, the plasmid was religated (pVAX-H⁺). pVAX-H⁺ was hydrolyzed with *Eco*RI, the 5' phosphate was removed with CIP, and ligated with the complete sequence of the murine Ii chain p41, which was isolated from the pFM332.1p41 vector by digestion with *Eco*RI (pVAXp41). pVAXp41 was digested with *Hind*III (complete) and *Eco*RI (partial) to elute a 3260-bp fragment. The pCRII TOPO-gp100-72 vector was digested with *Hind*III (partial) and *Eco*RI (complete) to elute the 1808-bp fragment. The fragment was fused in frame to the N-terminal sequence of the Ii chain (pVAXmIi-gp100-72). Constructs were sequenced to confirm fidelity of their translational reading frames.

To control for the functionality of the invariant chain fragment containing fusion construct, a second fusion protein was constructed where the N-terminal sequence of the Ii chain was fused to the EYFP (enhanced yellow fluorescent protein). After digestion of the pEYFP-N1 plasmid, the EYFP DNA was fused in frame to the Ii fragment as described above (pVAXmIi-EYFP).

Transformation of Salmonella typhimurium aroA (SL7207)

The attenuated *S. typhimurium* aroA strain SL7207⁵⁸ was grown in LB medium. Competent bacteria were transformed

with the plasmid pVAXmgp100 (SL-gp100), pVAXmli-gp100-72 (SL-gp100-li), and pVAXmli-EYFP (SL-EYFP/li) by electroporation (2500 V, 25 μ F). Transformation was controlled by selection with Kanamycin. After 1 hour at 37°C in LB medium, cells were cultured overnight on agar plates containing 50 μ g/mL Kanamycin. For vaccination *via* the oral route, transformed SL were cultured overnight in LB medium containing 2% NaCl and 50 μ g/mL Kanamycin at 37°C. After determination of OD₆₀₀ (OD₆₀₀ 1 roughly corresponds to 1×10^8 SL/mL), cells were centrifuged at 6000 rpm and washed two times with PBS. Cells were resuspended in PBS (containing 10% bicarbonate, pH 9.6), pH 8.3–8.5, to neutralize the acid pH of the stomach. Mice received $1\text{--}2 \times 10^8$ bacteria in 100 μ L *via* probang.

Generation of DCs

A modification of the protocol of Inaba et al⁵⁹ has been used. In brief, freshly harvested bone marrow cells (BMC) ($1.5\text{--}2 \times 10^6$ /mL) were suspended in IMEM containing 5% FCS and were seeded in 50 mL culture flasks. After an incubation period of 2 days, nonadherent cells were removed by washing and the plastic-adherent cells were cultured in IMEM containing 5% FCS and 8 ng/mL mGM-CSF, 2.5 ng/mL IL4, and 50 U/mL IFN γ (Stratmann, Hanover, Germany). The medium was exchanged every third day. After 7 days of culture, DCs were loaded either with a mixture of gp100 peptides selected after epitope prediction⁶⁰ (H2-K^b motif: GSQSYVPL, TWGKYWQV, VLYRYGSF, QRRSFLPV; H2-D^b motif: EGSRNQDWL, GLGENSPLL, LRVINDGPTL, HFLRNHPLI) (10 μ g/mL) or with lysates of B16F1 cells (lysate of 1×10^6 cells/mL). Loading was performed for 2 days. The protein-containing medium was sucked off and DCs were carefully washed with IMEM/5% FCS. DCs were allowed to mature for an additional 2 days in the medium described above. The maturity of DC was verified by FACS analysis (high expression of MHC class II and a DC marker) and the microscopic appearance of veiled cells.

In vitro assays

Proliferative activity of lymph node cells (LNCs) was evaluated by culturing titrated numbers of cells ($2 \times 10^5\text{--}2.5 \times 10^4$ /well) in the presence or absence of 10 μ g/mL gp100 peptide mix or B16F1 lysate (as described above) in 96-well round-bottom plates. Cells were cultured for 3 days by adding ³H-thymidine (10 μ Ci/mL) during the last 16 hours of culture. Plates were harvested using an automatic harvester, and ³H-thymidine incorporation was evaluated in a β counter.

Antibody production was determined by enzyme-linked immunosorbent assay. Culture supernatants were seeded in 96-well enzyme-linked immunosorbent assay plates, which had been coated with antimouse Ig. After overnight incubation at 4°C, plates were washed and alkaline phosphatase-conjugated antimouse IgG was added for 4 hours. Substrate was added after washing and the optical density of the enzyme reaction was measured after 30–60 minutes at 405 nm.

Cytotoxicity of lymphokine activated killer cells (LAKs) and cytotoxic T cells was performed according to the protocol described by Matzinger.⁶¹ In brief, target cells were labeled overnight with ³H-thymidine (10 μ Ci/mL) and were seeded (10^4 cells/well) after washing on titrated numbers ($5 \times 10^5\text{--}6 \times 10^4$) of effector cells in round-bottom microtiter plates. After an incubation for 6 hours at 37°C, plates were harvested and the remaining radioactivity was determined in a β counter. LAK activity was determined using YAC as target cells. CTL activity was defined by the lysis of B16F1 in the presence of a 10-fold excess of unlabeled YAC to compete out LAK activity. Activity of LAK was determined using SC, which had been cultured for 48 hours in the presence of 10 U/mL IL2. CTL activity was determined after culturing LNC for 10 days in the presence of 10 U/mL IL2 plus either the gp100 peptide mix or the B16F1 lysate (at the concentrations described above). Cytotoxicity is presented as % cytotoxicity = $100 \times (\text{counts in control wells} - \text{counts in test wells}) / (\text{counts in control wells})$. The spontaneous release of B16F1 and YAC cells was in the range of 6–12%; SD values of triplicate cultures were in the range of 3–5%.

The frequencies of proliferating and cytotoxic cells were evaluated under limiting dilution (LD) conditions. Cells were titrated using 24 replicates of 200–25600 cells/well. Irradiated C57BL6 thymocytes were added as feeder cells (1×10^5 /well) in medium containing the gp100 peptide mix. Proliferative activity was determined after 5 days of culture and cytotoxic activity after 14 days of culture. In the latter case, $3\text{--}5 \times 10^3$ ³H-thymidine labeled target cells were added during the last 6 hours of culture. The frequencies of proliferating and cytotoxic T cells were calculated according to the formula: F_0 (fraction of non-responding cultures) = e^{-u} , where $u = c/w$ (number of c cells distributed in w wells).⁶²

In vivo studies

C57BL6 mice received a subcutaneous or intravenous injection of 5×10^4 B16F1 cells. Tumor growth was controlled by measuring the mean diameter twice per week. Mice were killed when the mean tumor diameter reached 2.5 cm or when animals became anemic, cachectic, or short-breathing. These time points were defined as survival time.

Where indicated, mice were vaccinated in 3-week intervals. For vaccination, mice received either 1×10^8 SL, p.o. or 5×10^5 DC, i.v. Tumors were inoculated either concomitantly with the first vaccination or after the third vaccination. The vaccination schedule was maintained during tumor growth. In some experiments, mice received, in addition, IL2 (10,000 U) twice per week. IL2 was given subcutaneously in the vicinity of the tumor cell inoculum or intratumorally whenever tumor growth was observed.

Statistical analysis

Significance of differences was calculated according to the Student's t test (*in vitro* assays) or the Wilcoxon rank sum test (*in vivo* assays).

RESULTS

*Controlling in vivo transcription after oral application of transformed *S. typhimurium**

After oral application of SL-EYFP/Ii, PEC, PPL, mesenteric and peripheral LNC, SC and PBMC were collected at 24-hour intervals for a period of 14 days. Already 3 days after application, fluorescent cells were detected in the peritoneal cavity. The expression of EYFP in PEC increased strongly until day 5. It was still visible at day 10. EYFP was also strongly expressed and with similar time kinetics in PP and mLNC. In PBMC, a sharp peak of EYFP⁺ cells was seen 6 days after SL application. A small percentage of SC and peripheral LNC became EYFP⁺ at 8–10 days after vaccination (Fig 1A). When gating the cells according to size and granulation and counterstaining with leukocyte markers (Fig 1B), it became apparent that 5 days after SL application in the peritoneal exudate, the peripheral blood, the mesenteric lymph nodes, and the Peyer's patches, the vast majority of large and granulated cells which expressed the monocyte marker CD11b, were EYFP⁺. Within a medium-sized, less-granulated population, which stained with anti-CD4, anti-CD8, anti-sIgM, and a DC-specific antibody, it was a minority of CD4⁺ cells, but all DCs that consistently were EYFP⁺. Finally, a small and strongly granulated population of cells (data not shown), which was FcR⁺, also expressed EYFP. Taking into account that this expression profile was seen already 48 hours after detection of the first translation products, it is tempting to speculate that the cDNA was not only transcribed in monocytes, but likely also in DC and possibly in neutrophils.

We also checked by immunofluorescence microscopy different organs, like the gut, PP, mesenteric and peripheral lymph nodes, lung, kidney, liver, muscle, and spleen for

EYFP expression. We detected EYFP⁺ cells in the lamina propria of the small intestine, the PP, and in the mesenteric lymph nodes. Starting at day 8–10 after SL application, few EYFP⁺ cells were also seen in the spleen and the peripheral lymph nodes. EYFP⁺ cells were mostly found in clusters and exhibited a macrophage-like morphology, particularly in the spleen. In all these organs, EYFP⁺ cells were still detectable 14 days after vaccination. In other organs, including the colon, EYFP expression could not be detected (data not shown).

Evidences for peptide presentation by MHC class II molecules after vaccination with SL-gp100/Ii

It has been described that fusion constructs containing an invariant chain fragment direct the nascent protein toward the MHC class II loading compartment.^{51–54} To control this for vaccination with SL-gp100/Ii or SL-EYFP/Ii, PEC and PPL were harvested 4 or 5 days after oral application of SL-gp100/SL-EYFP or SL-gp100/Ii/SL-EYFP/Ii. PEC/PPL from nonvaccinated mice and from mice receiving SL transformed with the empty vector served as controls. First to mention, only with PPL (and PEC, data not shown) from animals receiving SL-EYFP/Ii, but not with any of the controls, we could detect by immunohistology intracellular compartments enriched in MHC class II molecules as well as EYFP. In controls, such double-stained vesicle-like structures could not be detected, i.e., MHC class II staining appeared equally distributed and EYFP was enriched in a ring-like structure (Fig 2). With respect to costimulatory molecules and their ligands (Table 1), it was particularly expression of CD40 that was strongly increased by application of SL-gp100/Ii. Furthermore, IL2 and IFN γ expressions were much stronger after vaccination with SL-

Figure 1. Expression of EYFP after oral application of transformed *S. typhimurium*. C57BL/6 mice received 1×10^8 SL-EYFP/Ii via probang. **A:** PEC, PPL, peripheral and mesenteric LNC, SC and PBMC were collected at days 2–7 and 10 after application of SL. Expression of the EYFP protein was evaluated by flow cytometry. A three-dimensional single parameter overlay (front: day 2, back: day 10) of the ungated total populations of leukocytes is shown. **B and C:** PEC, PPL, peripheral and mesenteric LNC, SC and PBMC were gated according to size and granulation as demonstrated for PEC (**B**). **C:** In the population of large plus granulated cells, expression of EYFP and Mac-1 is shown; in the population of middle-sized, less-granulated cells, staining with anti-CD4, anti-CD8, anti-sIgM, and a DC-specific antibody (33D1) (all counterstained with antirat IgG-PE) is presented.

Figure 2. Double staining of PPL for EYFP and MHC class II molecules. C57BL6 mice received 1×10^8 SL-EYFP or SL-EYFP/Ii via probang. PPL were collected after 4 days. Cells were fixed and permeabilized. Cells were stained with anti-H2-A^b and counterstained with antimouse IgG-Rhodamin. After washing, they were stained with anti-EGFP and counterstained with antirabbit IgG-FITC. Staining was analyzed by confocal microscopy. Overlays of PPL from mice vaccinated with SL-EYFP (A) and of mice vaccinated with SL-EYFP/Ii (B) are shown.

gp100/Ii than with SL-gp100. Finally, vaccination with SL-gp100/Ii induced a superior proliferative response and an increased antibody production compared to controls. Although these are indirect evidences, they all point toward a preferential activation of TH when transforming SL with a gp100/Ii fusion construct.

T-cell activation by gp100 peptide and B16F1 lysate-loaded DC

As described in Materials and Methods, a set of gp100 peptides potentially binding to the K^b and D^b molecules has been selected by epitope prediction using the SYFPEITHI database.⁶⁰ After *in vitro* maturation of bone marrow-derived DC and pulsing with either the peptides or the lysate, freshly harvested LNCs were added to control for proliferative activity and cytokine expression (Table 2). All eight peptides induced a proliferative response, which could be further improved when DCs were pulsed with the mixture of all eight peptides. Surprisingly, the proliferative response to the peptides was stronger than the proliferative response to lysate-pulsed DC, which may have been due to the lower amount of gp100-derived peptides in the whole cell lysate. In fact, cytokine expression was quite strongly induced by lysate-loaded DC. This accounted for IL12, IFN γ , but also for TNF and IL10. DCs loaded with the individual peptides or the peptide mix stimulated expression of IL2 and IFN γ , and to a lower degree IL12, but did not induce IL10 or TNF expression. Furthermore, DC induced cytokine expression more efficiently when loaded with the peptide mix than with the individual peptides. Because the

proliferative response toward protein (lysate)-pulsed DC was weak, this setting was not included in the *in vivo* studies.

Impact of gp100 vaccination on B16F1 growth retardation and rejection

In the first “therapeutic” trial, vaccination of C57BL6 mice was started concomitantly with subcutaneous application of tumor cells. Vaccinations were repeated in 3-week intervals (Fig 3A). Mice were vaccinated with either peptide-loaded DC or with SL-gp100 or SL-gp100/Ii. All three vaccination protocols proved to be quite efficient in retarding tumor growth. Some of the features should be noted in particular. First, although only borderline statistically significant, the efficacy of vaccination with SL-gp100 slightly exceeded the efficacy of peptide-loaded DC (SL-gp100 *versus* peptide-loaded DC: *P* value of .079). Second, when vaccinating with SL-gp100/Ii compared to SL-gp100, a further prolongation of the survival time and an increased survival rate were monitored (SL-gp100/Ii *versus* SL-gp100: *P* value of .006). Third, some of the tumor nodules became partly or totally amelanotic. The phenomenon was mostly seen after vaccination with DC and was more frequently observed after intravenous, than after subcutaneous, application of tumor cells.

Table 1. Activation Profile of PEC after Vaccination with SL-gp100 Versus SL-gp100/Ii

Stimulus	Percent stained PEC*				
	CD25	CD40	CD40L	CD86	CD28
None	8.3	7.6	15.5	15.7	12.7
SL	15.0	25.8	25.5	16.4	12.3
SL-gp100	26.4	32.8	32.3	28.9	21.7
SL-gp100/Ii	37.9	52.8	39.5	35.2	32.7

	Percent stained PEC*				
	IL2	IL10	IL4	IL12	IFN γ
None	16.1	5.0	35.5	24.5	4.3
SL	25.8	9.2	35.3	25.3	8.3
SL-gp100	36.1	12.7	47.1	35.6	9.3
SL-gp100/Ii	40.7	14.5	42.6	41.5	24.5

	Proliferative activity† (cpm/10 ⁵ PEC)	Antibody production† (OD ₄₀₅)
None	5,164±287	0.589±0.03
SL	8,893±531	0.737±0.04
SL-gp100	15,328±677	1.022±0.04
SL-gp100/Ii	27,337±945	1.305±0.05

*PECs were collected 5 days after oral application of transformed SL. Expression of costimulatory molecules and their ligands was evaluated on freshly harvested PEC; cytokine expression was determined after 2 days of culture in the presence of 20 U/mL IL2.

†PECs were cultured in the presence of B16F1 lysate. Proliferative activity was determined after 3 days of culture by adding ³H-thymidine during the last 16 hours. For the evaluation of antibody production, supernatants were harvested after 5 days of culture and were transferred to antimouse Ig-coated plates.

Table 2. Induction of Proliferative Activity and of Cytokine Expression by Murine gp100-Derived Peptides

Stimulus*	Proliferation (cpm/ 10^5 LNC)	Cytokine expression: percent stained cells				
		IL2	IL12	IFN γ	TNF α	IL10
None	5,131 $\pm$ 236	10.7	8.6	10.4	5.8	4.9
EGSRNQDWL	33,642 $\pm$ 1,122	17.4	18.1	19.1	4.1	5.0
GLGENSPLL	25,324 $\pm$ 1,087	19.0	16.7	14.3	3.9	4.8
HFLRNHPLI	40,001 $\pm$ 1,201	19.6	25.5	21.1	4.6	7.1
LRVINDGPTL	41,245 $\pm$ 1,536	12.2	11.1	24.1	3.8	5.0
GSQSYVPL	47,943 $\pm$ 1,422	14.6	13.7	12.9	2.7	5.2
VLYRYGSF	52,884 $\pm$ 1,765	26.9	33.6	27.2	7.2	9.8
TWGKYWQV	39,048 $\pm$ 1,226	16.8	13.6	16.1	4.1	8.4
QRRSFLPV	45,200 $\pm$ 1,833	17.8	11.4	23.6	4.5	5.1
Peptide mix	76,463 $\pm$ 2,045	29.7	30.3	29.3	5.5	10.7
B16F1 lysate	15,057 $\pm$ 386	25.4	43.9	51.4	17.6	25.3

*DCs were generated as described in Materials and Methods and were loaded with 10 μ g/mL peptides (individually or pooled) or a lysate of B16F1 cells (corresponding to 10^6 cells/mL). Two days after loading, DCs were washed and freshly harvested LNCs were added. Proliferative activity was determined after 3 days of culture by adding 3 H-thymidine during the last 16 hours. Cytokine expression was determined after 2 days of culture in the presence of 10 U/mL IL2.

Encouraged by the rather strong effect of a “therapeutic” vaccination protocol, we wanted to improve the efficacy of

vaccination by inducing an immune response before tumor cell application. Mice received transformed SL or loaded DC

Figure 3. Influence of gp100 vaccination on survival time and survival rate. C57BL6 mice were vaccinated either with unloaded DC, DC loaded with MHC class I binding peptides, SL transformed with the empty vector, or the vector containing the murine gp100 cDNA or the vector containing the gp100 cDNA plus the li chain cDNA. **A and B:** Mice received the first vaccination concomitantly with the subcutaneous tumor challenge. In **(B)**, mice received, in addition, twice per week a peritumoral/intratumoral injection of IL2. **C and D:** Mice were vaccinated for three times and received a subcutaneous **(C)** or intravenous **(D)** tumor challenge 5 days after the last vaccination. Vaccination was continued every third week after tumor grafting. Survival time and rate are shown.

Figure 4. Activation of proliferating and cytotoxic T cells by gp100 vaccination. Mice were vaccinated as described above. Lymph nodes and the spleen were excised 5 days after the second vaccination (tumor-free) or 21 days after tumor cell application (tumor bearer). **A:** LNCs were cultured for 3 days by adding ^3H thymidine during the last 16 hours of culture. Proliferation in the absence of an antigenic stimulus and in the presence of the gp100 peptide mix is shown. **B:** SCs were cultured in the presence of IL2 (10 U/mL) for 48 hours. LAK activity was evaluated using ^3H -thymidine labeled YAC cells. The percentage of lysed cells at an E:T ratio of 50:1 is shown. **C:** LNCs were cultured for 10 days in medium containing 10 U/mL IL2 and the gp100 peptide mix. Blasts were separated by Ficoll gradient centrifugation. Cytotoxic activity was evaluated using ^3H -thymidine labeled B16F1 cells and a 10-fold excess of YAC (for cold target inhibition of LAK activity). The percentage of lysed cells at an E:T ratio of 50:1 is shown. Significance of differences as compared to mice vaccinated with unloaded DC or vector only transformed SL is indicated by asterisk.

as described above, i.e., three cycles at 21-day intervals. The tumor was inoculated subcutaneously or intravenously 5 days after the third vaccination (Fig 3C and D). The intravenous route of application was chosen to see whether targeting of effector cells toward intrapulmonary processes may be more effective than toward subcutaneous nodules. Targeting of effector cells apparently plays a role because the overall survival rate of vaccinated mice was 3/54 in animals receiving the tumor subcutaneously, but 9/54 in mice receiving the tumor intravenously. Irrespective of the route of tumor cell application, the response pattern of the different vaccination schedules was similar to what was observed with “therapeutic” vaccines, i.e., vaccination with SL-gp100/Ii was the most efficient and vaccination with SL-gp100 was still slightly more efficient than vaccination with peptide-loaded DC. Yet, contrary to our expectation, the therapeutic efficacy was not improved by a prophylactic vaccination.

Whereas tumor growth was not retarded by a prophylactic, compared to a “therapeutic,” vaccination, a local supply of IL2 clearly was beneficial for animals receiving either transformed SL or peptide-loaded DC (Fig 3B). IL-2 was given locally, i.e., in the surroundings of the tumor cell

inoculum or intratumorally, as far as tumor growth was observed. In this setting, the mean survival time of mice receiving peptide-loaded DC or SL-gp100 or SL-gp100/Ii could be significantly prolonged by the concomitant application of IL2. The cytokine by itself had no effect on tumor takes and tumor growth rate. Thus, it is likely an IL2-mediated support of adoptive defense mechanisms which improved the vaccination efficacy.

Murine gp100 has been described to be rather weakly immunogenic.^{17,18} Thus, we were surprised to see retardation of tumor growth and increased survival rates with all three vaccination schedules. Yet, the above outlined features were peculiar and required an exploration of the underlying mechanism.

Analysis of immune responses initiated by SL and DC vaccination

To evaluate the response patterns initiated by the different vaccination protocols, proliferative responses and cytotoxic activity were evaluated in tumor-free and tumor-bearing animals.

Table 3. Frequencies of gp100-Specific Helper and Cytotoxic T Cell in Vaccinated Tumor-Free and Tumor-Bearing Mice

Vaccination	Frequency/10 ⁶ LNC* (<i>P</i> value)			
	TH		CTL	
	Tumor-free	Tumor bearer	Tumor-free	Tumor bearer
None	16.4±6.5	16.5±5.1	12.3±5.7	13.8±5.4
DC	26.0±7.1 (ns)	30.3±5.2 (ns)	13.1±4.3 (ns)	20.1±5.9 (ns)
DC peptide	220.3±58.9 (.006)	90.3±14.2 (.015)	38.8±9.4 (.038)	27.4±7.1 (ns)
SL	23.1±5.7 (ns)	26.8±5.3 (ns)	14.7±7.1 (ns)	15.0±4.5 (ns)
SL-gp100	99.3±14.9 (.014)	74.6±11.8 (.020)	42.0±9.6 (.032)	28.3±7.0 (ns)
SL-gp100/li	504.0±81.6 (.006)	102.3±14.3 (.014)	123.5±14.5 (.010)	40.2±5.7 (.059)

*Mean values of four experiments each consisting of LNC pooled from two mice are shown.

Proliferative activity was determined in LNC and draining LNC, respectively, of tumor-free and tumor-bearing mice. In tumor-free animals, good proliferative responses were induced by all three vaccines, SL-gp100/li being superior to DC peptide and SL-gp100. This also accounted for LNC cultured in the absence of an antigenic stimulus, which may have been due to persistence of the peptides on APC harvested 5 days after vaccination. A similar profile was seen in draining LNC of tumor-bearing mice, but the proliferative response was reduced compared to LNC of tumor-free mice. When mice received, in addition, peritumoral/intratumoral injections of IL2, no or only a minor reduction in proliferative activity was observed. Notably, too, IL2 exerted the strongest effect on the proliferative activity of LNC from nonvaccinated mice, whereas it was of minor efficacy when mice had been vaccinated (Fig 4A).

LAK activity was most strongly induced by SL-gp100/li and was unchanged in the tumor bearer compared to the tumor-free host. With the exception of mice vaccinated with SL-gp100/li, LAK activity was slightly improved by the concomitant application of IL2 (Fig 4B). Unexpectedly, the profile of CTL activity was again similar to the one described for proliferative responses, in as much as SL-gp100/li were somewhat more efficient than SL-gp100 and both were more efficient than DC peptide. Notably, CTL responses were strongly reduced in the tumor bearer compared to the tumor-free host and application of IL2 did not suffice for the maintenance of cytotoxic activity (Fig 4C).

The reduction in CTL activity was even more strikingly seen at the single cell level (Table 3). Whereas in the SL-gp100/li-vaccinated, tumor-free animals an expansion of CTL by a factor of 10 (compared to the nonvaccinated mouse) was observed, there was only a less than 3-fold expansion in the tumor-bearing mouse. A similar reduction accounted also for TH cells, i.e., in the tumor-free mouse an over 30-fold expansion and in the tumor-bearing mouse a 6-fold expansion was observed. In the nonvaccinated mouse, frequencies of TH and CTL were not influenced by tumor cell application. This implies that gp100 expressed by the tumor cells most likely did neither induce an immune response nor did it provoke suppression.

We interpret these *in vitro* analyses in the sense that all three vaccines initiated activation of TH and CTL, i.e., none of the regimen led to a selective activation of either TH or CTL. Yet, there was a preferential activation of TH by

vaccination with SL-gp100/li. The accompanying activation of LAK and the stronger CTL responses may have been supported by this efficient TH activation. Notably, CTL as well as TH responses were weaker or became partially suppressed in the tumor-bearing host.

Evidence for tumor-induced immunosuppression

To elucidate the putative mechanism of suppression, we explored expression of cytokines as well as of costimulatory molecules and their ligands in draining LNC and TIL of the vaccinated, tumor-bearing host (Table 4).

Expression of the CD40/CD40L pair, being one of the first triggers in costimulation, was strongly induced by vaccination with DC peptide and SL-gp100/li. Up-regulation of expression appeared to be stronger in draining LNC than in TIL. Expression of CD86 and its ligand CD28 was up-regulated in draining LNC and, weakly in TIL of tumor-bearing mice vaccinated with either of the three

Table 4. Expression of Costimulatory Molecules and Their Ligands in Vaccinated Tumor-Bearing Mice

Vaccination	Percent stained cells*					
	CD86	CD28	CD80	CTLA4	CD40	CD40L
LNC						
None	31.9	45.9	22.6	6.3	10.4	9.8
DC	30.6	44.8	21.0	6.8	10.3	11.8
DC peptide	43.4	58.0	35.1	15.5	34.8	27.6
SL	35.9	48.6	25.6	6.6	10.1	9.1
SL-gp100	56.8	63.0	42.6	22.6	23.3	18.2
SL-gp100/li	54.6	61.7	31.3	9.2	30.1	21.3
TIL						
None	8.6	13.2	17.0	6.1	8.3	4.1
DC	8.2	13.2	16.1	6.1	8.0	6.6
DC peptide	11.5	17.6	27.1	13.7	15.1	7.4
SL	9.7	14.2	15.9	8.2	10.2	6.7
SL-gp100	19.2	17.1	22.2	23.3	10.4	10.3
SL-gp100/li	17.3	29.3	27.8	29.1	16.3	15.2

*Expression of costimulatory molecules and their ligands was evaluated by flow cytometry of draining LNC and TIL, which have been enriched by Ficoll gradient centrifugation. Contaminating tumor cells have been excluded by gating. Mean values of three independently performed experiments are shown.

Table 5. Expression of Cytokines in Vaccinated Tumor-Bearing Mice

	Percent stained cells*										
	IL6	TNF α	IL12	IFN γ	IL2	IL6	TNF α	IL12	IFN γ	IL2	IL2 $\dagger$
	LNC (tumor-free)					Draining LNC (early stage of tumor growth)					
None	8.6	10.8	24.9	13.2	16.6	11.7	6.2	12.1	9.0	15.4	19.3
DC	11.2	12.2	24.1	17.4	17.2	10.0	6.9	16.5	13.5	12.7	
DC peptide	20.5	24.2	38.2	31.5	32.9	15.3	25.8	28.1	35.8	27.5	32.8
SL	10.9	12.7	24.6	20.5	17.4	11.1	9.8	17.5	14.6	16.6	
SL-gp100	21.7	25.8	33.7	46.3	28.8	20.9	15.4	28.8	29.8	33.3	38.1
SL-gp100/li	22.3	30.4	39.9	53.2	39.0	23.8	17.3	39.0	38.1	44.1	45.6
	TIL (early stage of tumor growth)					TIL (late stage of tumor growth)					
None	17.8	8.2	10.6	7.6	10.3	6.9	5.2	8.7	7.5	10.7	6.6
DC	17.9	8.0	12.3	10.7	12.2	8.4	7.9	10.8	10.1	11.9	
DC peptide	23.1	16.7	12.9	22.8	20.0	1.3	1.6	2.5	3.6	14.1	8.3
SL	16.5	10.8	15.8	13.3	14.0	7.4	3.7	8.2	9.2	12.9	
SL-gp100	26.3	21.8	28.1	22.1	22.4	0.5	5.9	9.4	3.7	7.8	6.3
SL-gp100/li	31.7	22.3	36.7	27.1	28.3	0.2	3.5	5.9	4.1	8.8	5.8

*Expression of cytokines was evaluated by flow cytometry of LNC and TIL, which have been fixed and permeabilized in advance. TILs were enriched by Ficoll gradient centrifugation and remaining contaminating tumor cells were excluded by gating. Mean values of three independently performed experiments are shown;

$\dagger$ IL2 expression in draining LNC (early stage of tumor growth) and TIL (late stage of tumor growth) in mice which had received peritumoral/intratumoral applications of IL2.

drugs. A different pattern was seen with CTLA4 and CD80, the supposedly preferential receptor for CTLA4. In draining LNC, CD80 and CTLA4 were more strongly up-regulated after vaccination with DC peptide and SL-gp100 than after vaccination with SL-gp100/li. On TIL, however, expression of CTLA4 was strongly up-regulated in mice vaccinated with either of the three drugs, although most strongly after vaccination with SL-gp100/li. The low expression of CD40 and CD86/CD28 as well as the high expression of CTLA4 in TIL pointed toward a mechanism of active immunosuppression within the tumor environment.

Up-regulation of CD95/CD95L could be another possible reason for the decrease in gp100-specific CTL and TH in the tumor-bearing host. We did not observe significant changes in the expression of CD95 in draining LNC and TIL of vaccinated compared to nonvaccinated tumor-bearing mice. Expression of CD95L was slightly, but consistently up-regulated, particularly by vaccination with peptide-loaded DC. Yet expression levels remained rather low and hardly could explain the decrease in effector cells. Also, propidium iodine and annexin FITC staining of freshly harvested draining LNC of tumor-bearing mice did not provide evidence for an increased rate of dead cells. Furthermore, B16F1 cells derived from the tumor-bearing host did not or only at a low percentage express CD95L, thus excluding direct lysis of effector cells by the tumor (data not shown).

Instead, the analysis of cytokine expression provided convincing evidence of tumor-induced immunosuppression. In the vaccinated, tumor-free animal, the monocyte-derived inflammatory cytokines TNF α and IL6 were strongly up-regulated. There was also a clear up-regulation of IL2, IL12 (stronger by DC than by SL), and IFN γ (stronger by SL than by DC). At early stages of tumor growth, the cytokine expression profile was unaltered in draining LNCs. In TIL,

expression of IL6, TNF α , and IFN γ was up-regulated. Yet, at late stages of tumor growth, expression of all cytokines was strikingly down-regulated, i.e., irrespective of the vaccination regimen, hardly any cytokine expression could be observed in draining LNC (data not shown) and in TIL of vaccinated mice (Table 5). Because no decrease in the frequency of cytokine-expressing cells was seen in the nonvaccinated, tumor-bearing mouse, it appears that the tumor-mediated suppression attacks selectively activated lymphocytes. In line with this interpretation is the observation that cytokine expression, as shown for IL2, was further decreased when mice received intratumoral applications of IL2.

Thus, the B16F1 tumor apparently drives activated lymphocytes in a state of anergy, which could well explain the failure to improve therapeutic efficacy by prophylactic vaccination. Despite these tumor-related limitations, we observed an overall quite efficient and encouraging response against B16F1 by vaccination with the "self-antigen" gp100.

DISCUSSION

The melanoma-associated antigen gp100 has been shown to efficiently induce a cytotoxic T cell response *in vitro*^{5,6} and its therapeutic efficacy is currently explored in several clinical studies.⁹⁻¹³ Yet, with respect to gp100 as well as tumor-associated antigens in general, it is obvious that vaccination protocols require further optimization.^{20,21} Thus, although protein- or peptide-loaded DCs have been shown to induce T cell activation with high efficacy,^{22,23} the retention of the peptide in the MHC groove remains to be a problem when transferring *ex vivo* loaded DC. The problem currently is being attacked by the transfer of virally

transduced DC.^{13–16,63,64} Oral application of attenuated *Salmonella* or *Listeria* transduced with the cDNA of tumor-associated antigens provides another means for a prolonged antigen presentation,^{46–48} which would be cheaper and by far easier to handle than *in vitro* matured and transduced DCs. Therefore, we considered it as important to evaluate the duration of antigen expression after vaccination with SL and to define which leukocyte subsets express the relevant protein and in which lymphatic compartments. Furthermore, because presentation of tumor-associated antigens/peptides by MHC class II molecules appears to be advantageous,^{34–37} we addressed a second question: Can a nascent intracellular protein be directed toward the MHC class II loading compartments *via* a fusion construct containing part of the invariant chain cDNA?^{52–54,65} The failure to strengthen the power of immune defense by a prophylactic, compared to a therapeutic, vaccination confronted us with a third question on the maintenance of defense mechanisms in the tumor-bearing host.

For the analysis of gene transcription after oral application of attenuated SL, we used the cDNA of EYFP. As described before,^{49,50,66} EYFP was readily detected after 2–3 days in peritoneal M ϕ and DC. Expression increased until days 5–6 and was still measurable at 14 days after SL application. During the first 4 days after application of SL, the gene product was detected exclusively in the gut-associated and gut-adjacent lymphatic tissues, while later on fluorescent cells were also detected in distant lymph nodes and the spleen. Thus, the cDNA is efficiently transcribed in host cells and the “antigen” is presented for a sufficient length of time to guarantee activation of lymphocytes. Notably, expression of EYFP was not only detected in M ϕ , but also in a population of small and strongly granulated cells, likely granulocytes, and in DC. It has been described that vaccination *via* application of transformed SL leads in the second instance, i.e., after decay of phagocytes as the first host, to uptake of cell debris and consecutively to antigen presentation *via* DC.⁴⁹ We actually observed expression of EYFP simultaneously in M ϕ and DC. Whether this finding implies that SL may have been taken up and gp100 may have been directly transcribed by DC remains to be explored.

Presentation of peptides derived from tumor-associated antigens by MHC class II molecules and, accordingly, activation of TH have been demonstrated in various models to increase therapeutic efficacy.^{15,29,30,32–35,67} One possibility to redirect intracellular proteins toward the MIIC compartment consists in the generation of invariant chain–antigen fusion proteins.^{51–54} In order to induce an efficient gp100-specific TH response, we have transformed SL with such a fusion construct. Although we have not proven explicitly that gp100-derived peptides actually are presented in the groove of MHC class II molecules, there are several evidences that point toward a successful transport of the nascent protein into the lysosomal compartment: (a) staining of permeabilized PPL provided evidence for colocalization of MHC class II molecules with EYFP in vesicle-like structures only when mice had received SL transformed with the EYFP/Ii fusion protein, but not when transformed with EYFP by itself; (b) up-regulation of costimulatory mole-

cules and their ligands on PEC was stronger after application of SL-gp100/Ii than of SL-gp100. The same accounted for cytokine expression; (c) activation and expansion of TH in LNC and TIL were much stronger after vaccination with SL-gp100/Ii than with SL-gp100; (d) activation and expansion of CTL were most efficiently achieved and activation of LAK was only observed by vaccination with SL-gp100/Ii. Thus, TH were more efficiently activated by vaccination with SL-gp100/Ii than by vaccination with SL-gp100, a finding which supports the assumption that the transcribed fusion construct was directed toward the MIIC compartment. A superior therapeutic effect has also been described for vaccination with DCs, which have been loaded with protein or MHC class II compared to MHC class I binding peptides.^{67–71} We also observed that vaccination with DC loaded with protein has been more efficient than vaccination with transformed SL if the gene product was not guided into the MIIC compartment.⁷² Thus, directly or indirectly guiding the peptide into the MHC class II groove of DC has proven superior in many instances, irrespective of the RNA *versus* DNA *versus* protein nature of the antigenic stimulus.

In this context, we also would like to mention our trial to improve the efficacy of vaccination by local application of IL2. Application of cytokines to circumvent the requirement for TH activation or to support activation of TH has been demonstrated in many instances to be efficacious in cancer immunotherapy (reviewed in Refs.^{8–11,73}). Although peritumoral/intratumoral application of IL2 led to a significant increase in the proliferative and LAK activity of draining LNC and TIL (data not shown) of mice, which had not been vaccinated, the overall effect of IL2 on tumor growth and survival was negligible in these animals. This implies that, at least in the B16 tumor, unspecific stimulation by IL2 does not suffice to initiate a tumor (gp100)-specific response *in vivo*. The strongest *in vivo* effect of IL2 was seen in mice vaccinated with SL-gp100, which is in line with our observations that activation and expansion of TH, though initiated, are suboptimally supported by this vaccination scheme. Yet, even in mice vaccinated with peptide-pulsed DC or SL-gp100/Ii, the survival time could be significantly prolonged by the application of IL2. However, the overall survival rate remained unaltered. The reasons for the latter apparently do not rely on the vaccination protocol, but — as discussed below — rather on the B16 tumor. Taken together, we interpret our data in the sense that a low dose of cytokines may be especially advantageous for supporting the clonal expansion of antigen-specific TH.

Besides the aspect of TH activation, another important feature of vaccination as a mode of cancer therapy should be discussed. Vaccination with SL-gp100 was more efficient than vaccination with peptide-loaded DC. Although the differences were minor, this ranking was consistently observed in *in vitro* as well as *in vivo* studies. Because both vaccination protocols are supposed to support preferentially the activation of CTL, we consider it as most likely that the superior efficacy of vaccination with SL relies on the prolonged period of peptide presentation. The importance of the time period of peptide presentation to achieve an optimal response has also been demonstrated by using different protocols of DC vaccination. Superior results have been

consistently observed when DCs were either fused with tumor cells or when DCs were transformed by DNA or RNA compared to externally loaded DC (Refs.,^{13–16,74–76} reviewed in Refs.^{38,40,77}). Thus, besides the aspect of TH activation, the lasting availability of peptide for T cell activation appears to be of major importance.

Finally, we want to mention the superior therapeutic efficacy when giving the tumor intravenously compared to subcutaneously. There is ample evidence that targeting of activated effector cells toward a solid tumor mass provides an additional and serious problem in cancer immunotherapy.⁷⁸ Our finding supports these observations and points toward the necessity of targeting drugs, e.g., cytokine fusion proteins, to improve therapeutic effects.^{79–81}

If SL-gp100/Li provides such an optimal vaccination module, why did we not observe superior therapeutic effects after prophylactic vaccination and why was only a minority of mice cured? As already mentioned, murine gp100 is weakly immunogenic such that in several studies, a therapeutic effect was only seen after crosspriming with human gp100.¹⁷ Second, the B16 melanoma appears to lose expression of gp100 under the selection pressure of the immune system as apparent by the development of partly amelanotic tumor nodules. This feature, i.e., loss of gp100, has also been observed in human therapeutic trials.^{82,83} Furthermore, as also described for human melanoma,⁸⁴ B16F1 has a tendency to shut down MHC class I expression during an immune attack. Because neither antigen loss nor MHC class I loss was complete, B16F1-induced immunosuppression may be the more serious problem. With progression of tumor growth, we noted a decrease in the frequency of gp100-specific TH and CTL. Furthermore, at late stages of tumor growth, we hardly could detect cytokine expression by TIL at all. Because the decrease in TH, CTL, and cytokine expressing cells was only observed in vaccinated mice and was most pronounced in TIL, it appears that the tumor attacks exclusively activated lymphocytes and drives them into a state of anergy, rather than into AICD, i.e., we did not observe expression of CD95L by B16F1 and neither the number of TIL nor of draining LNC decreased significantly at late stages of tumor growth. Whether the strong up-regulation of CTLA4 at late stages of tumor growth has to be considered as a system-inherent regulatory mechanism or is provoked also by tumor-related factors remains to be explored. Irrespective of this open question, it can be expected that signaling *via* CTLA4 contributes to counteract the therapeutic efficacy.⁸⁵

In conclusion, we could induce a strong and therapeutically efficient immune response toward murine gp100 by vaccination with transformed *S. typhimurium*. Likely due to the persistence of antigen loading, vaccination with SL-gp100 was more efficient than vaccination with peptide-loaded DC. The strength of response could be further increased by directing gp100 to the MHC compartment, i.e., supporting in particular a TH cell activation. Limitations in the therapeutic effect are, at least in the gp100-B16F1 model, not due to the weak immunogenicity of murine gp100. Instead, B16F1 cells develop escape mechanisms and, most importantly, counteract gp100-specific effector cells.

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