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Transloading of tumor cells with foreign major histocompatibility complex class I peptide ligand: A novel general strategy for the generation of potent cancer vaccines

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ABSTRACT **The major hurdle to be cleared in active immunotherapy of cancer is the poor immunogenicity of cancer cells. In previous attempts to overcome this problem, whole tumor cells have been used as vaccines, either admixed with adjuvant(s) or genetically engineered to express nonself proteins or immunomodulatory factors before application. We have developed a novel approach to generate an immunogeneic, highly effective vaccine: major histocompatibility complex (MHC) class I-positive cancer cells are administered together with MHC class I-matched peptide ligands of foreign, nonself origin, generated by a procedure we term transloading. Murine tumor lines of the H2-K^d or the H2-D^b haplotype, melanoma M-3 and B16-F10, respectively, as well as colon carcinoma CT-26 (H2-K^d), were transloaded with MHC-matched influenza virus-derived peptides and applied as irradiated vaccines. Mice bearing a deposit of live M-3 melanoma cells were efficiently cured by this treatment. In the CT-26 colon carcinoma and the B16-F10 melanoma, high efficacies were obtained against tumor challenge, suggesting the universal applicability of this new type of vaccine. With foreign peptide ligands adapted to the requirements of a desired MHC class I haplotype, this concept may be used for the treatment of human cancers.**

Being of self origin, tumor cells are poorly or nonimmunogeneic and thus are often ignored by the immune system. However, in animal models, antitumor immunity has been shown to be very effective in the rejection of malignant cells in the context of minimal residual disease as soon as expression of a foreign gene is elicited in tumor vaccines—e.g., after viral infection (1) or after transfection of a viral gene (2). Tumor cells thus modified present foreign protein-derived epitopes in the form of processed peptides on their surface major histocompatibility complex (MHC) molecules and are thus efficiently recognized by the immune system (3–5), leading to rejection of unmodified, parental-type tumor cells. We sought to use this mechanism for the generation of cancer vaccines by using synthetic, nonself, but MHC-matched, virus-derived peptides to bring about immunogenicity in whole tumor cells. Tumor cells displaying foreign peptides should be efficiently recognized by the immune system and thus initiate an immune response against tumor-specific antigen(s) (6). To test this concept, we chose the N-terminal influenza hemagglutinin peptide derivative LFEAIEGFI (7), a putative ligand of the MHC class I haplotype H2-K^d. This allele is expressed on murine melanoma M-3 (8) and colon carcinoma CT-26 (9), two tumor cell lines used in animal studies. For efficient peptide delivery, we developed a protocol termed transloading in which peptides are applied as polylysine–DNA or polylysine/transferrin–DNA complexes. Here we show that foreign peptide transloaded vaccine (FPTV) elicits a long-lasting cellular immune response against preestablished M-3 "micrometastases" or CT-26 tumor challenge. To prove the general applicability of this new type of cancer vaccine, we chose a different MHC class I allele, H2-D^b on B16-F10 as target for the nonself peptide modification. An immunogeneic peptide of the influenza nucleoprotein, ASNENMETM (10), was transloaded onto the H2-D^b molecules of B16-F10 melanoma cells. Upon vaccination the same powerful anti-tumor activity was induced as in the H2-K^d tumor system. These results identify the FPTV strategy as a novel, general way for successful anti-tumor vaccination.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized on a peptide synthesizer (model 433 A with feedback monitoring; Applied Biosystems) using TentaGel S PHB (Rapp, Tübingen, Germany) as solid phase with the Fmoc-strategy (HBTU activation, 0.25-mmol scale, Fastmoc, Weiterstradt, Germany). Peptides were dissolved in 1 M triethylammonium acetate (pH 7.3) and purified by reverse-phase chromatography on Vydac C 18 column (model 218ATP54; Vydac, Hesperia, CA). Peptide identity was confirmed by time-of-flight mass spectrometry on MAT Lasermat (Finnigan-MAT, San Jose, CA). Influenza virus-derived peptide sequences used in this study were: LFEAIEGFI, a putative H2-K^d ligand motif; LPEAIEGFG, a negative control peptide in which the H2-K^d anchor amino acid residues of LFEAIEGFI have been altered to prevent binding to this MHC class I haplotype (alterations are indicated in boldface type); and ASNENMETM, a putative H2-D^b ligand (3, 5, 7).

Tumor Cells and Cell Culture. B16-F10 melanoma cells (H2-D^b) were kindly provided by A. Schneeberger (Vienna International Research Cooperation Center, Vienna). Cloudman S91 melanoma cells, clone M-3 (H2-K^d), were purchased from American Type Culture Collection, and colon CT-26 cells (H2-K^d) were kindly provided by M. Zöller (Deutsches Krebstorschungszentrum, Heidelberg). Highly tumorigenic variants of all three tumor cell lines were generated (T.S., unpublished results). The M-3 variant was termed M-3W for distinction from the parental M-3 line also used in this study. The other two tumor lines were not renamed.

Fluorescence-Activated Cell Sorter (FACS) Analysis of M-3 Cells Transloaded, Pulsed, or Admixed with Fluorescein Isothiocyanate (FITC)-Labeled Peptide. For transloading, 160 μg of FITC-labeled peptide LFEAIEGFI was complexed with 3 μg of transferrin/polylysine (11), 10 μg of polylysine (Sigma), and 6 μg of plasmid pSP65 (Boehringer Mannheim) in 500 μl of HBS (20 mM Hepes, pH 7.4/150 mM NaCl) and applied

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Abbreviations: MHC, major histocompatibility complex; FACS, fluorescence-activated cell sorter; FPTV, foreign peptide transloaded vaccine; FITC, fluorescein isothiocyanate; IL-2, interleukin 2.

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onto 10^6 M-3 cells in 20 ml of DMEM (GIBCO/BRL) supplemented with 10% fetal calf serum (FCS) at 37°C. After 3 h, cells were washed twice with PBS, detached with PBS/2 mM EDTA and resuspended in 1 ml of PBS/2% FCS for FACS analysis. Pulsing of 10^6 M-3 cells in 20 ml of DMEM was performed with 450 μg of FITC-labeled peptide for 3 h at 37°C. Cells were harvested as described above. For admixing, 10^6 detached tumor cells were incubated with 100 μg of FITC-labeled peptide in 1 ml of DMEM for 30 min at room temperature before first FACS analysis. Cells were washed once afterwards and analyzed again. FACS analysis was performed using a flow cytometer (FACS Vantage; Becton Dickinson) equipped with a 5-W argon laser tuned to 100 mW at 488 nm.

FPTV and Control Vaccine Preparation for Animal Studies. For the CT-26 vaccine, 160 μg of peptide LFEAIEGFI or LPEAIEGFG was admixed with 11.8 μg of polylysine (ref. 11; alternatively with 3 μg of transferrin/polylysine conjugate and 10 μg of polylysine) in 1 ml of HBS and complexed for 30 min at room temperature. The complex was diluted with 4 ml of medium DMEM/10% FCS to obtain a final volume of 5 ml and added to 1.5×10^6 cells, which had been plated 1 day before in a T75 cell culture flask. After 4 h, medium was removed and cells were washed once with PBS. Fresh medium (15 ml) was added and vaccine cells were incubated at 37°C in 5% CO_2 overnight. Four hours before injection, the vaccine was irradiated with 100 Gy and prepared for injection as described (13).

The protocol for the M-3 vaccine preparation with transloaded peptides was the same as for CT-26, except that 160 μg of peptide LFEAIEGFI or LPEAIEGFG was admixed with a complex consisting of 3 μg of transferrin/polylysine conjugate, 10 μg of polylysine, and 6 μg of psp65 (ref. 13; Boehringer Mannheim). Irradiation dose was 20 Gy. Preparation of FPTV in the B16-F10 model was the same as in the M-3 system, except that H2-D^b-matched peptide ASNENMETM was transloaded, and an irradiation dose of 100 Gy was applied. Interleukin 2 (IL-2)-secreting M-3 vaccines and IL-2- or granulocyte-macrophage colony-stimulating factor-secreting B16-F10 vaccines were generated by transfection as described (13).

For admixing of LFEAIEGFI in the M-3 system, cells were harvested after irradiation, and peptide was added at the amount indicated (see *Results and Discussion*), after which the cell density was adjusted to 10^5 cells per 100 μl. After 30 min at room temperature, vaccines were injected.

For pulsing experiments, 400 or 4000 μg of peptide LFEAIEGFI was dissolved in 10 ml of high-glucose DMEM and transferred into a T75 cells culture flask with 1.5×10^6 M-3 cells. After an incubation period of 24 h, cells were harvested and injected as described above.

Animal Experiments. C57BL/6 mice (H2-D^b) were bred inhouse. Balb/c and DBA/2 (H-2K^d) mice (6–12 weeks old) were obtained from Charles River Wiga (Sulzfeld, Germany). Groups of eight to 10 animals were immunized twice at a weekly interval under halothane anesthesia. All animals received a vaccine cell dose of 10^5 in 100 μl of Earle's balanced salt solution (GIBCO/BRL). C57BL/6 mice received 10^5 B16-F10 irradiated vaccine cells twice in a weekly interval and a contralateral challenge 1 week after the second vaccination with 10^4 viable B16-F10 cells (≥10 times the minimal tumorigenic cell dose). Balb/c mice received 10^5 irradiated CT-26 vaccine cells and a contralateral challenge with 5×10^4 viable tumor cells (≥10 times the minimal tumorigenic cell dose) was applied 1 week after the last immunization. $CD4^+$ cells were depleted by i.v. injection of 500 μg of monoclonal antibody GK1.5, and $CD8^+$ cells were depleted by i.v. injection of 500 μg of monoclonal antibody 2.43 (T.S., unpublished results) 24 h before FPTV application. In the M-3 system, DBA/2 mice were inoculated s.c. with 10^4 viable M-3 or M-3W cells (≥10 times the minimal tumorigenic cell dose) to model minimal residual disease. After 5 days, contralateral immunization with 10^5 irradiated vaccine cells was started and repeated once 7 days later. For challenge experiments, naive DBA/2 mice were

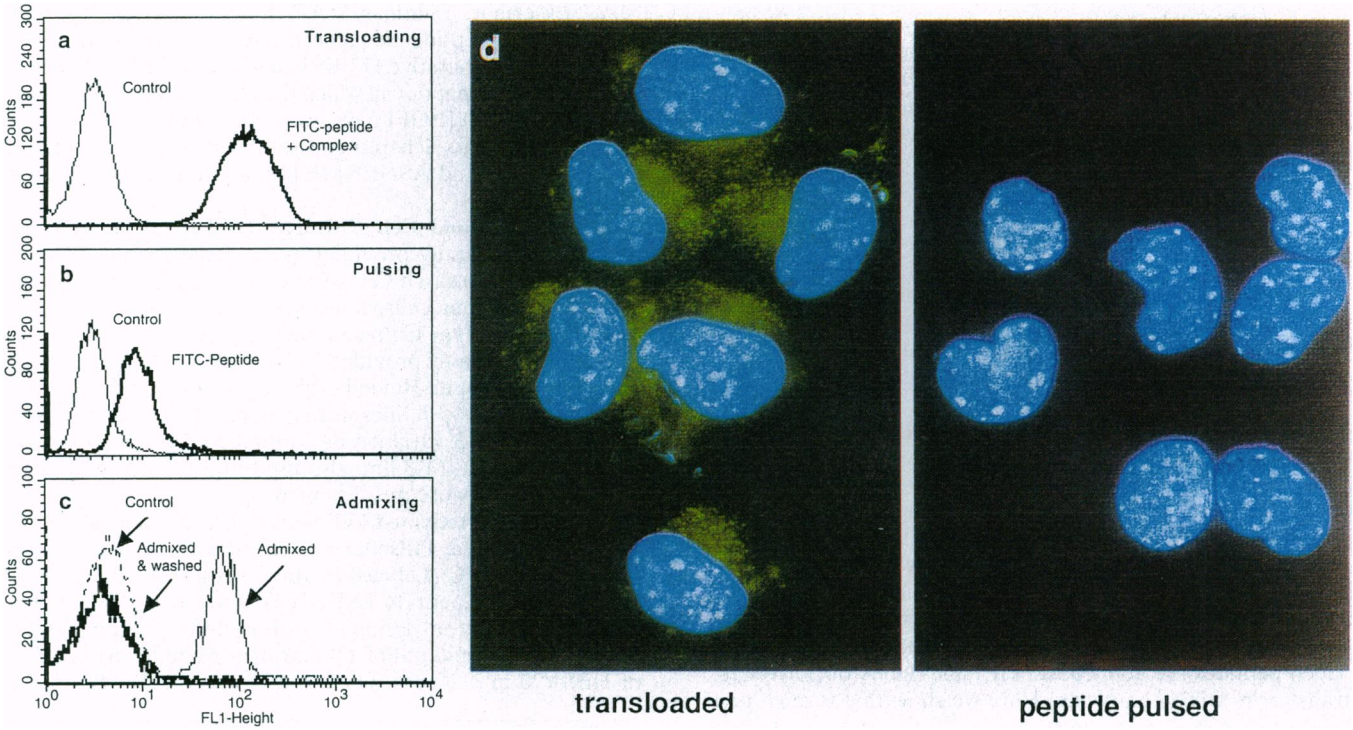

FIG. 1. FACS analysis of M-3 cells transloaded (*a*), pulsed (*b*), or admixed with FITC-labeled peptides (*c*). Also shown is fluorescence microphotography of M-3 cells (cytospin) transloaded (*d Left*) or pulsed (*d Right*) with peptide. 4,6′-Diamidino-2-phenylindole (DAPI) was used for counterstaining of the nucleus.

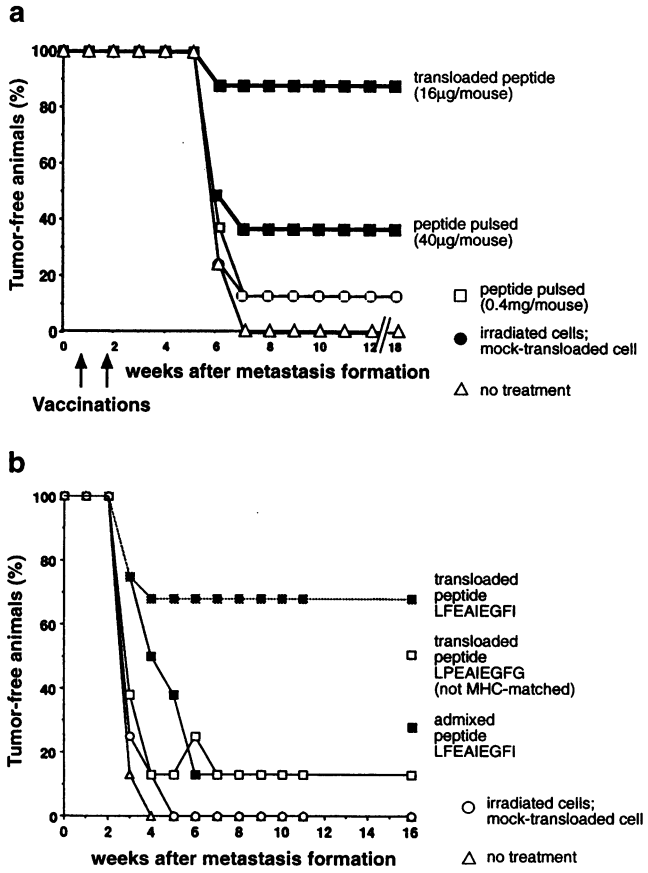

FIG. 2. Cure of micrometastasis-bearing mice. (*a*) Data for M-3 melanoma and peptide LFEAIEGFI are shown. (*b*) Data for clone M-3W and peptide LFEAIEGFI or peptide LPEAIEGFG.

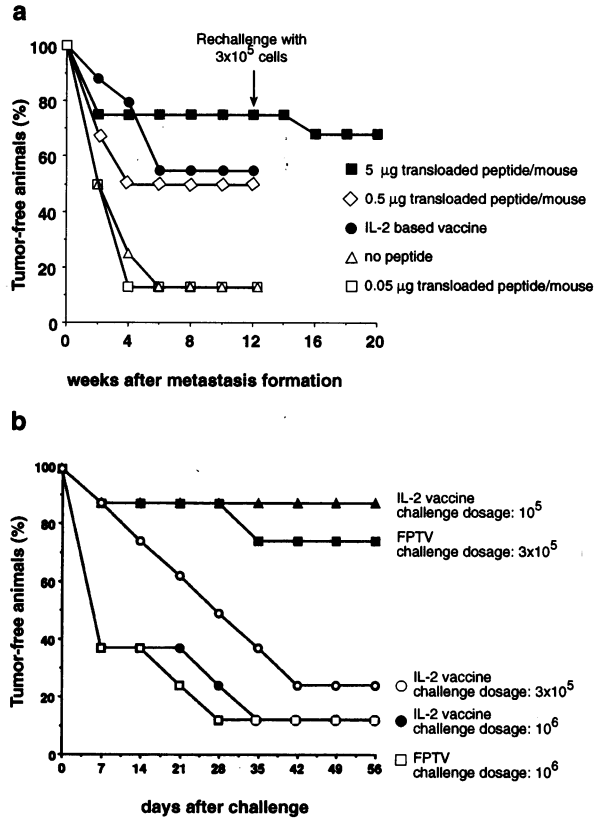

FIG. 3. Vaccination of metastasis-bearing and naive DBA/2 mice. (*a*) Titration of peptide applied in the FPTV (eight animals per group). Animals without detectable tumor burden 12 weeks after metastasis formation were considered disease-free. (*b*) Protection of naive DBA/2 mice against escalating challenge dose with viable M-3W tumor cells.

vaccinated twice with 10^5 cells at a weekly interval and challenged with the indicated cell number 1 week after the second immunization. All animals were inspected daily. Tumor development was scored in weekly intervals and followed up for the indicated time periods.

RESULTS AND DISCUSSION

Transloading Efficiently Delivers Small Peptides onto Cells in Culture. The modification of cancer vaccines with synthetic, nonself peptides calls for a protocol that efficiently delivers such peptides onto tumor cells to guarantee that they become highly immunogenic. To ensure efficient and tight binding of such peptides to tumor cells, we first developed a method termed transloading. In this novel approach the synthetic peptide is applied to tumor cells in conjunction with polylysine, with polylysine–DNA, or transferrin/polylysine–DNA complexes, resulting in a foreign peptide transloaded vaccine (FPTV). To demonstrate that transloading is superior to conventional pulsing of cells with peptides, we performed FACS analysis of M-3 cells, which were transloaded, pulsed (long-term exposure to peptide in culture), or admixed (short-term exposure to peptide in cell suspension) with FITC-labeled peptide LFEAIEGFI (Fig. 1). After transloading, M-3 cells showed a ≈100-fold shift in fluorescence compared with untreated or polylysine-administered control cells, indicating efficient transfer of the transloaded peptide (Fig. 1*a*). Pulsing cells with peptide resulted in only a small shift in fluorescence (Fig. 1*b*), reflecting low efficiency peptide delivery. At the cellular level, FITC-labeled peptide was clearly detectable after transloading, but not after pulsing (Fig. 1*d*). In contrast to the transloading or pulsing procedure, the shift in fluorescence from the admixed peptide disappeared after a washing step (Fig. 1*c* and unpublished results). These observations suggest that in this case, peptide binding was minimal at best. The two negatively charged glutamic acid residues in the sequence of peptide LFEAIEGFI might lead to the impression that the peptide is complexed to the positively charged polylysine via ionic interactions. Because this would limit the transloading approach to glutamic acid- or aspartic acid-containing peptides, we compared the loading of LFEAIEGFI to a peptide KYQAVTTTL, a tumor antigen peptide of the murine mastocytome P815 (12), omitting any negative charges but containing H2-K^d-compatible anchor amino acids (boldface type). Both peptides were delivered with the same efficiency, indicating that the transloading approach is not limited by specific peptide sequence requirements, because transloading of either peptide resulted in the same shift of fluorescence (data not shown).

Anti-Tumor Immunity Elicited by FPTV. To investigate the impact of FPTV on the induction of antitumor immunity, we transloaded M-3 vaccine cells with influenza hemagglutinin-derived peptide LFEAIEGFI. DBA/2 mice bearing a s.c. "micrometastasis," established by injecting a tumorigenic dose of 10^4 live M-3 melanoma cells, were vaccinated. As shown in Fig. 2*a*, FPTV efficiently cured animals (seven of eight animals) in this minimal residual disease model. The vaccines generated by pulsing an amount of peptide comparable to that used for transloading, or even 10 times that quantity, were less effective, indicating a correlation between the efficient peptide delivery (Fig. 1) and successful vaccination. Furthermore, control vaccines of irradiated cells or cells transloaded with polylysine–DNA in the absence of peptide (mock-transloaded

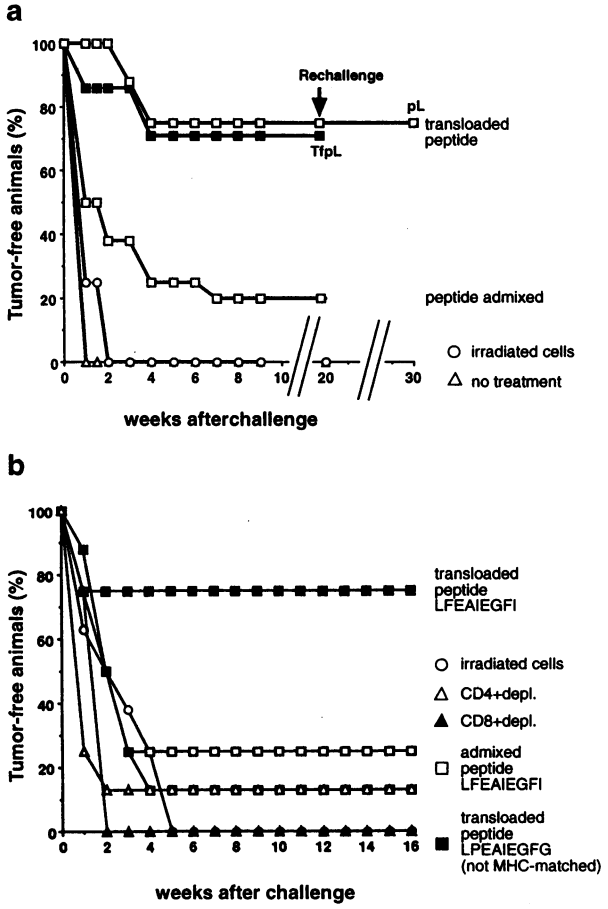

FIG. 4. FPTV-mediated protection against CT-26 challenge. (*a*) Data for immunization with FPTV, generated upon transloading of CT-26 vaccine cells with peptide LFEAIEGFI as polylysine (pL) or transferrin/polylysine (TfpL) complex. (*b*) Comparison of peptide LFEAIEGFI and peptide LPEAIEGFG complexed with polylysine; abrogation of antitumor protection after depletion of $CD4^+$ or $CD8^+$ cells before immunization with peptide LFEAIEGFI-based FPTV.

cells) evoked no protection. Fig. 2*b* demonstrates the importance of both the transloading protocol and the MHC class I ligand function of the peptide in an experiment with M-3W, a highly tumorigenic variant of the original M-3 melanoma. Again, significant numbers of animals were cured when treated with FPTV equipped with the H2-K^d ligand LFEAIEGFI but not when this peptide was simply admixed with the tumor cells.

We next examined whether binding of the peptide to MHC molecules is required. The two putative anchor amino acid residues (10, 12) of LFEAIEGFI were altered to prevent H2-K^d binding (F2 → P and I9 → G, resulting in peptide LPEAIEGFG). When animals were injected with FPTV prepared with LPEAIEGFG, no more protection was observed, indicating that the MHC class I ligand motif of peptide LFEAIEGFI was crucial for MHC class I binding and effective vaccination (Fig. 2*b*).

Furthermore, the curative effect of FPTV correlates with the amount of the effective peptide LFEAIEGFI applied during transloading, because antitumor effects were reduced or abolished when the vaccine was prepared with a lower amount of peptide (Fig. 3*a*). Fig. 3*a* also illustrates that treatment of minimal residual disease by FPTV generated with 5 μg of peptide affords similar if not better protection than a vaccine consisting of cytokine gene transfected cells secreting optimal levels of IL-2 (13). To further characterize the efficacy of the FPTV treatment, all mice resisting tumor in the 5 μg of peptide class were rechallenged with 3×10^5 live cells of the

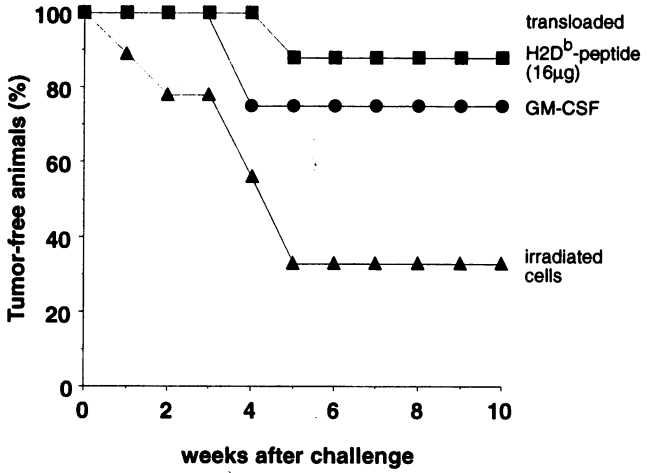

FIG. 5. FPTV-mediated protection against B16 challenge.

aggressively growing, M-3W cell line at week 12 after implantation of the first tumor inoculum. Five of the six mice rejected the tumor challenge (Fig. 3*a*), indicating that a long-lasting systemic anti-tumor immunity had been established by prior vaccination with the nonself peptide transloaded vaccine. In an escalating challenge dosage experiment, FPTV was also shown to be superior to the IL-2-secreting vaccine (Fig. 3*b*). Although naive mice, vaccinated with the IL-2-secreting vaccine, were efficiently protected against a challenge with 10^5 viable M-3W tumor cells, the capacity of the IL-2 secreting vaccine was exhausted when a challenge of 3×10^5 cells was applied, whereas this challenge dose was still rejected to a large degree in animals preimmunized with FPTV. A challenge dose of 10^6 cells was required to overcome FPTV-mediated protection.

General Validity of the FPTV Approach. To demonstrate the general utility of the FPTV concept, we transloaded CT-26 colon carcinoma vaccine cells (H2-K^d) and prevaccinated naive Balb/c mice against tumor challenge (≥10 times the minimal tumorigenic dose). As shown in Fig. 4 *a* and *b*, the FPTV generated with peptide LFEAIEGFI mediated protection in a substantial number of the animals (five of seven animals and six of eight animals, respectively). In this system, transloading required the use of polylysine or transferrin/polylysine rather than transferrin/polylysine–DNA complexes as in the M-3 system (see *Materials and Methods*). As expected from the results obtained with M-3 melanoma, peptide simply admixed afforded no protection. Again, application of FPTV conferred systemic immune memory. When the successfully FPTV-treated animals were rechallenged at week 20 after the first challenge, all six animals were still protected and eradicated the tumor inoculum (Fig. 4*a*). Fig. 4*b* demonstrates that with this H2-K^d positive vaccine nonmatched MHC peptide LPEAIEGFG was devoid of activity. Furthermore, Fig. 4*b* also demonstrates that T cells seem to be essential for the generation of anti-tumor immunity because depletion of either $CD4^+$ or $CD8^+$ cells with monoclonal antibodies before vaccination abrogated the tumor rejection in FPTV-treated animals. No differences were seen when polylsysine transferrin instead of polylysine was used in the complexes.

The above results clearly demonstrate that the FPTV approach is very effective in K^d-positive melanoma and colon carcinomas. We next wanted to extend this new approach to another MHC class I haplotype with diverse requirements for the peptide ligand to underline the universal applicability of our approach. We chose the H2-D^b allele expressed on murine B16 melanoma cell line (C57BL/6 mice) as a target for foreign peptide ligand modification. The transloaded, nonself peptide was ASNENMETM (anchor amino acids in boldface type) from influenza nucleoprotein (amino acids 366–374), a well-

characterized ligand for H2-D^b allele gene product (3, 5). This nonself peptide was used in vaccination experiments in the B16 system (Fig. 5). Transloading was performed to obtain ≈16 μg of peptide per 10^5 vaccine cells, the cell dose applied per animal (see *Materials and Methods*). Fig. 5 shows that this vaccine was very effective, protecting seven of eight animals against tumor challenge, compared with control vaccines consisting of irradiated B16-F10 cells alone (six of nine animals developed tumors) or compared with a granulocyte-macrophage colony-stimulating factor-secreting vaccine (six of eight animals protected), which was the most effective vaccine identified in this tumor system so far (14).

Our results show that tumor cells transloaded with MHC class I peptide ligand of foreign origin are highly effective vaccines in three different murine tumor model systems, the M-3 melanoma and the CT-26 colon carcinoma, as well as the B16 melanoma. M-3 melanoma and CT-26 colon carcinoma express the same MHC class I haplotype H2-K^d. Both tumors were successfully treated with FPTV based on the identical nonself peptide. These findings demonstrate that the FPTV strategy can be applied against different malignancies. The third tumor model investigated in this study, melanoma B16-F10, is derived from a completely different mouse strain C57BL/6, therefore expressing different MHC class I molecules requiring a different peptide ligand to be transloaded. Interestingly, application of the transloading strategy with a matched, nonself ligand in this system also conferred potent antitumor protection, suggesting that this new strategy is of universal applicability.

Although the exact mechanism of the peptide transfer induced by polylysine or transferrin/polylysine–DNA has yet to be determined, our results suggest that efficient transloading of the peptide is a prerequisite for the initiation of a strong antitumor immunity. Polylysine is known to permeabilize membranes (15). Although peptide LFEAIEGFI is contained in the amphipathic helix of the lytogenic peptide, which enhances DNA transport into cells (7), it is in itself not lytogenic or membrane-active (unpublished results). There may be importation of peptide through fluid transport via endosomes. Thus, peptides may actually be endocytosed and enter the MHC class I loading pathway in the endoplasmic reticulum. However, it cannot be excluded that influenza virus-derived peptides LFEAIEGFI and ASNENMETM peptide have the capacity to directly replace self peptide ligands from MHC class I molecules at the cell surface, although this seems less likely, because cells pulsed or admixed with FITC-labeled peptides are devoid of significant fluorescence (see above).

Conclusions. A highly efficient cancer vaccines consisting of whole tumor cells, transloaded with a synthetic, nonself peptide has been prepared. Recently, there has been a flurry of reports on the generation of tumor vaccines consisting of dendritic (16–18) or RMA-S (19) cells pulsed with tumor antigen or neoantigen peptides. Our study suggests that in these instances, the transfer of peptides might be considerably improved using our transloading procedure.

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1. Liebrich, W., Schlag, P., Manasterski, M., Lehner, B., Stohr, M., Möller, P. & Schirrmacher, V. (1991) *Eur. J. Cancer* **27,** 703–710.
2. Fearon, E. R., Itaya, T., Hunt, B. Vogelstein, B. & Frost, P. (1988) *Cancer Res.* **48,** 2975–2980.
3. Rötzschke, O., Falk, K., Wallny, H. J., Faath, S. & Rammensee, H. G.(1990) *Science* **249,** 283–287.
4. Van Bleek, G. M. & Nathenson, S. G. (1990) *Nature (London)* **348,** 213–216.
5. Townsend, A. R. M., McMichael, A. J., Carter, N. P., Huddleston, J. A. & Brownlee, G. G. (1984) *Cell* **39,** 13–25.
6. von Hoegen, P., Weber, E. & Schirrmacher, V.(1988) *Eur. J. Immunol.* **18,** 1159–1166.
7. Plank, C., Oberhauser, B., Mechtler, K., Koch, C. & Wagner, E. (1994) *J. Biol. Chem.* **269,** 12918–12924.
8. Zatloukal, K., Schneeberger, A., Berger, M., Schmidt, W., Koszik, F., Kutil, R., Cotten, M., Wagner, E., Buschle, M., Maass, G., Payer, E., Stingl, G. & Birnstiel, M. L.(1995) *J. Immunol.* **154,** 3406–3419.
9. Plautz, G. E., Yang, Z. Y., Wu, B. Y., Gao, X., Huang, L. & Nabel, G. J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4645–4649.
10. Rammensee H. G., Falk, K. & Rötzschke, O. (1993) *Annu. Rev. Immunol.* **11,** 213–244.
11. Wagner, E., Zenke, M., Cotten, M., Beug, H. & Birnstiel, M. L. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3410–3414 .
12. Rammensee, H. G., Friede, T. & Stepvanovic, S.(1995) *Immunogenetics* **41,** 178–228.
13. Schmidt, W., Schweighoffer, T., Herbst, E., Maass, G., Berger, M., Schilcher, F., Schaffner, G. & Birnstiel, M. L.(1995) *Proc. Natl. Acad. Sci. USA* **92,** 4711–4714.
14. Dranoff, G., Jaffe, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D. & Mulligan, R. C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3539–3543.
15. Bashford, C. L, Adler, G. M., Menestrina, G., Micklem, K. J., Murphy, J. J. & Pasternak, C. A. (1986) *J. Biol. Chem.* **261,** 9300–9308.
16. Mayordoma, J. I, Zorina, T., Storkus, W. J., Zitvogel, L., Celluzzi, C., Falo, L. D., Metlief, C. J., Ilstad, S. T., Martin Kast, W., Deleo, A. B. & Lotze, M. T. (l995) *Nat. Med.* **1,** 1297–1302.
17. Zitvogel, L., Mayordoma, J. I., Tjandrawan, T., Deleo, A. B., Carke, M. R. & Lotze, M. T. (l996) *J. Exp. Med.* **183,** 87–97.
18. Paglia, P., Chidoni, C., Rodolfo, M. & Colombo, M. P. (l996) *J. Exp. Med.* **183,** 317–322.
19. Mandelbaum, O., Vadai, E., Fridkind, M., Katz-Hillel, A., Feldman, M., Berke, G. & Eisenbach, L. (l996) *Nat. Med.* **1,** 1179–1183.