Published in final edited form as:

Clin Immunol. 2009 October ; 133(1): 45–51. doi:10.1016/j.clim.2009.06.004.

Vaccination with tumor cells pulsed with foreign peptide induces immunity to the tumor itself

Tobias R. Schlingmann, Frauke H. Rininsland, Wolf C. Bartholomae, Haydar Kuekrek, Paul V. Lehmann, and Magdalena Tary-Lehmann

Department of Pathology, Case Western Reserve University, Cleveland, OH, USA

Abstract

EMT-6 mammary carcinoma and B16 melanoma (B16M) cells are lethal and barely immunogenic in syngeneic BALB/c and C57BL/6 mice, respectively. We show that mice vaccinated with tumor cells pulsed with a MHC class I-restricted peptide develop a T cell response, not only to the peptide, but also to the unpulsed tumor. These mice display protective immunity against the unpulsed tumor, and their T cells adoptively transfer tumor-specific protection to immunodeficient SCID mice. Our data have implications for cancer vaccine strategies. Grafting a single well-defined foreign peptide on tumor cells might suffice to trigger anti-tumor immunity.

Keywords

Tumor immunity; Vaccination; Antigens; Epitope spreading; T cells

Introduction

Current tumor vaccination regimens typically use tumor antigens with adjuvants or dendritic cells in order to elicit a T cell response to tumor cells. Since tumor antigens are highly variable and largely unknown, it would be ideal to find mechanisms that permit the induction of anti-tumor immunity without having to define the tumor antigens first. Adjuvant effects or epitope spreading mechanisms entail such intriguing possibilities.

Epitope spreading was first described in the context of experimentally induced allergic encephalomyelitis (EAE) where we showed that immunization with one autoantigen triggers second wave autoimmunity to unrelated epitopes presented in the same target organ [1]. In 1999, Disis *et al.* were the first to observe epitope spreading in tumor immunology [2], followed by several groups reporting confirmatory findings in both mice and humans [3–8]. Several other clinical trials have also implied that epitope spreading may in fact be crucial for a favorable clinical outcome [6,9–12].

All of these reports relied on using defined tumor antigens as vaccines. In a clinical setting, this would require the identification of a suitable tumor antigen for each patient. While major

© 2009 Elsevier Inc. All rights reserved.

Correspondence and Reprints: Magdalena Tary-Lehmann, M.D., Ph.D. Associate Professor of Pathology Department of Pathology, Wolstein Research Building, Room # 5128 10900 Euclid Ave. Cleveland, OH 44106 Tel: 216-368-1298 Fax: 216-368-1357 E-mail: mxt27@po.cwru.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

progress has been made in this field, it has also become clear that even within one type of tumor there is considerable inter- and even intra-individual variation of tumor antigen expression [13]. This variability of tumor antigens and the need to customize vaccines for different individuals continues to be a major challenge for the immune therapy of cancers.

Furthermore, in the approaches cited above, the tumor antigen itself is the major constituent of the vaccination, and epitope spreading is merely a welcome secondary amplificatory reaction. An intriguing alternative hypothesis for a therapeutic strategy, however, would be to specifically exploit the epitope spreading reaction as the primary mechanism for engaging an anti-tumor immune response.

According to the spreading hypothesis, it should be possible to graft any tumor cell with an immunogenic antigen. A first wave T cell response against this grafted “tumor antigen” would engage – via the epitope spreading reaction – a second wave T cell response against the variable antigens naturally expressed by that particular tumor. According to this hypothesis, one would not even need to know the relevant tumor antigens; any immunogenic antigen would suffice to trigger an immune response that will custom tailor a T cell response to the antigens expressed by the respective tumor. In this hypothesis, the antigen used for vaccination is not directly involved in rejecting the tumor, but merely serves as a trigger. In the epitope spreading model, cytokines secreted locally by the tumor specific T cells function as “adjuvants”, rendering the tumor immunogenic. A similar effect would be accomplished if an antigen grafted on the tumor has adjuvant effects on its own.

We loaded MHC class I molecules of tumor cells with foreign peptide and tested whether such cells would induce protective immunity against tumor cells that do not bear the foreign peptide. Murine B16 melanoma cells were pulsed with *Lymphocytic Choriomeningitis Virus* glycoprotein peptide (LCMVp) 33-41 and injected into C57BL/6 mice. Murine EMT-6 breast cancer cells were pulsed with *Plasmodium berghei* circumsporozoite peptide (CSp) 252-260 and injected into BALB/c mice. In both tumor models, the pulsed tumor cells not only induced an immune response to the foreign peptide but also to the unrelated tumor antigens, leading to the rejection of unpulsed tumor and providing specific tumor protection.

Materials and methods

Mice and tumors

C57BL/6 and BALB/c mice were purchased from Harlan (Indianapolis, IN) and The Jackson Laboratory (Bar Harbor, ME) and maintained at the animal facility of CWRU under specific pathogen-free conditions. Experiments were performed under Case Western Reserve University's Institutional Animal Care and Use Committee Office (IACUC/ARC) guidelines following approved protocols. Mice were used at 6-10 weeks of age. B16M is of C57BL/6 origin and was obtained from ATCC (American Type Culture Collection, Rockville, Maryland) [14]. For vaccination and challenge, 2×10^5 B16M cells per recipient were injected intravenously (*i.v.*) in a volume of 500 μ l PBS. EMT-6 is a mammary tumor of BALB/c origin and was also obtained from ATCC [15]. For vaccination, 5×10^4 peptide-pulsed EMT-6 cells per mouse were injected intraperitoneally (*i.p.*) in a volume of 500 μ l PBS. Balb/c SCID mice were challenged with 1,000 EMT-6 cells *i.p.* ELISPOT assays were performed two weeks after the injection of tumor cells.

Peptides

H-2K^d-restricted *Plasmodium berghei* circumsporozoite peptide (CSp) 252-260 (SYIPSAEKI) [16,17] and H-2K^b-restricted *Lymphocytic Choriomeningitis Virus* glycoprotein peptide (LCMVp) 33-41 (KAVYNFATC) [18] were purchased from Princeton

BioMolecules Corporation (Langhorne, PA). For immunizations with adjuvant, 1 mg of peptide was dissolved in 1 ml PBS with 300 µg CpG (Oligo Etc. Inc., Wilsonville, OR) and was emulsified with 1 ml IFA (Gibco BRL, Grand Island, NY). Each mouse was injected *i.p.* with 200 µl of this emulsion, thus receiving 100 µg of peptide. Peptide pulsing was performed as previously described [19]: 1×10^6 tumor cells per ml were incubated with 70 µg/ml peptide at 37°C for 30 minutes. After three washes in PBS, the cells were re-suspended in PBS and injected as specified above. H-2K^b-restricted TRP-2 peptide 180-188 (SVYDFVWL) [20] and H-2K^b-restricted mgp100 peptide 25-33 (EGSRNQDWL) [21] were purchased from GenScript Corporation (Piscataway, NJ) and used in ELISPOT assays at concentrations from 25 µg/ml to 50 µg/ml.

Cell separation

CD4⁺ cells and CD8⁺ cells were obtained by negative selection, passing spleen cells through murine CD4⁺ or CD8⁺ T Cell Enrichment Columns (R&D Systems, Minneapolis, MN). The efficacy of enrichment was controlled by FACS analysis, staining with labeled anti-CD4, anti-CD8, and anti-CD3 antibodies (PharMingen, San Diego, CA). More than 95% enrichment for the desired phenotypes was obtained.

ELISPOT assay

These assays were performed as previously described [22]. Briefly, ImmunoSpot® M200 plates (Cellular Technology Limited, Cleveland, OH) were coated overnight at 4° C with the cytokine-specific capture antibodies specified below. The plates were washed 3 × with PBS, then blocked with 1% BSA in PBS for 2h at room temperature. After washing 3 × with PBS, cells and antigens were plated as specified below and in the text. After 24h (for IFN-γ and IL-2) or 48h (for IL-4 and IL-5) of cell culture in an incubator, the cells were removed by washing 3 × with PBS and 3 × with PBS containing 0.05% Tween (PBST). Then the biotinylated detection antibodies were added and incubated at 4°C overnight. The plates were then washed 4 × with PBST, and Streptavidin-HRP conjugate (Dako Corp., Carpinteria, CA) was added at a 1:2000 dilution, incubated for 2h at room temperature, and removed by washing 3 × with PBST and 3 × with PBS. The spots were visualized by adding HRP substrate AEC (3-amino-9-ethylcarbazole) (Pierce, Rockford, IL). The plates were then washed with distilled water, air-dried and analyzed the next day with the ImmunoSpot® Image Analyzer (Cellular Technology Limited). We used the following combinations of monoclonal capture antibodies for the cytokines tested: IFN-γ (R46A2: 5 µg/ml), IL-2 (JES6-1A12: 3 µg/ml), IL-4 (11B11: 3 µg/ml), and IL-5 (TRFK5: 1.5 µg/ml). For their detection we used the following monoclonal antibodies: IFN-γ (XMG1.1-biotin: 0.25 µg/ml), IL-2 (JES6-5H4-biotin: 1 µg/ml), IL-4 (BVD6-24G2-biotin: 2 µg/ml), and IL-5 (TRFK4-biotin: 2 µg/ml).

For testing bulk spleen cells, freshly isolated splenocytes were plated at 1×10^6 cells/well in serum-free medium, HL-1 (BioWhittaker, Walkersville, MA), supplemented with L-glutamine, in the presence or absence of 2×10^4 tumor cells (irradiated with 10,000 rad). Where indicated, peptide was added at a final concentration of 20 µg/ml. As positive control, anti-CD3 (2C11) was used at 1 µg/ml. For testing purified T cell subsets, CD4 or CD8 cells were plated at 5×10^5 cells/well with 1×10^6 irradiated (3,000 rad) syngeneic spleen cells per well, obtained from naïve WT mice.

Results

EMT-6 and B16M do not induce a protective immune response in syngeneic mice

When EMT-6 breast cancer cells or B16M cells were injected into syngeneic BALB/c or C57BL/6 mice, the tumor cells underwent rapid growth and caused 100% lethality within 20 and 40 days, respectively (Fig. 1A). Testing at single-cell resolution with ELISPOT analysis,

we asked whether the injected tumors induce a T cell response in the recipient mice (Fig. 1B). When spleen cells of naïve BALB/c mice were challenged directly *ex vivo* with EMT-6 tumor cells, the numbers of IFN- γ spots elicited were < 10 per million cells; that is, the results were indistinguishable from those of unchallenged spleen cells. EMT-6-injected mice did not show an IFN- γ response over untreated controls (Fig. 1B); increased frequencies of tumor-specific IL-2, IL-4, and IL-5 producing T cells were also not detected (data not shown). Therefore, the tumor antigens expressed by EMT-6 were not constitutively immunogenic. In contrast, immunization with a well-defined foreign antigen - the H-2K^d-restricted *Plasmodium berghei circumsporozoite* peptide (CSp) 252-260 - induced an IFN- γ recall response in the frequency range of 20 per million (Fig. 1B). Cell separation experiments showed that the IFN- γ -producing cells resided in the CD8⁺ fraction (data not shown). The CSp-reactive T cells did not respond to the EMT-6 tumor (Fig. 1B), showing that this peptide does not cross-react with any of the tumor's antigens.

The above data were obtained in the Th2-biased BALB/c mice. We tested whether the findings also apply for Th1-biased C57BL/6 mice. In naïve C57BL/6 mice B16M did not elicit an IFN- γ response. In B16M-injected mice the tumor induced IFN- γ -producing cells at a frequency of $\sim 20/10^6$ (Fig. 1B). The cells that produced this IFN- γ were exclusively CD4⁺ (data not shown). B16M therefore was mildly immunogenic in C57BL/6 mice, but the magnitude/quality of the immune response was insufficient to control tumor growth. Immunization of C57BL/6 mice with a foreign antigen - the H-2K^b-restricted *Lymphocytic Choriomeningitis Virus* glycoprotein peptide (LCMVp) 33-41 - induced IFN- γ -producing cells in the $110/10^6$ frequency range (Fig. 1B). The LCMVp-specific cells were defined by cell separation as CD8⁺ (data not shown), and they did not cross-reactively recognize the B16M tumor (Fig. 1B).

Pulsing of tumor cells with a class I-restricted foreign peptide induces determinant spreading to tumor antigens

Because neither EMT-6 nor B16M induced a protective immune response on their own, we grafted a foreign antigenic determinant onto the tumor cells. Class I molecules on EMT-6 cells were loaded *in vitro* with CSp; those on B16M were loaded with LCMVp. Injection of the same numbers of tumor cells as above into naïve mice resulted in the doubling of survival time in the case of EMT-6:CSp and in 60% indefinite survival in the case of B16M:LCMVp (Fig. 2A). When fourteen days later spleen cells of such mice were tested directly *ex vivo* for recall responses to the respective peptides (Fig. 2B), the EMT-6:CSp-injected mice exhibited an IFN- γ response to CSp at a frequency of $\sim 39/10^6$ while the B16M:LCMVp-injected mice showed responses to LCMVp around $93/10^6$. This shows that the peptide-loaded tumor cells are clearly immunogenic and fully capable of immunizing the recipient mice to the peptide in both mouse/tumor models. Recall responses to peptide-pulsed tumor cells (Fig. 2B) were of even greater magnitude. EMT-6:CSp elicited IFN- γ responses at a frequency of $\sim 60/10^6$ splenocytes; responses to B16M:LCMVp had a frequency of $\sim 230/10^6$.

The spleen cells of those mice that were injected with peptide-pulsed tumor cells also mounted an IFN- γ response to the unpulsed tumor. EMT-6:CSp-injected mice responded to EMT-6 at a frequency of $\sim 40/10^6$; B16M:LCMVp-injected mice showed responses to B16M at $\sim 220/10^6$ (Fig. 2B). These responses were tumor-specific since neither the EMT-6:CSp-injected BALB/c mice responded over medium background to a third party tumor of BALB/c origin (L5178Y-R, a murine leukemia cell line), nor did the B16M:LCMVp-injected C57BL/6 mice respond to RMA tumor (a T cell lymphoma of C57BL/6 origin) with increased cytokine production (data not shown). Therefore, the grafted foreign peptide caused determinants of the tumor that had originally been cryptic to become immunogenic.

Interestingly, further ELISPOT analysis showed that two well-characterized and frequently cited melanoma antigens, TRP-2 and mgp100, do not seem to play a significant role in the

tumor rejection caused by epitope spreading. When spleen cells of mice treated with LCMV-pulsed B16M cells were challenged 14 days later with either TRP-2 peptide 180-188 (SVYDFVWL) or mgp100 peptide 25-33 (EGSRNQDWL), they showed no significant IFN- γ response, even at high peptide concentrations (data not shown). Therefore, less prominent tumor antigens, possibly synergistically, seem to be responsible for the observed anti-tumor immunity in this model.

These results are further supported by the fact that immunization of mice with TRP-2 peptide 180-188 in conjunction with CFA fails to provide protection from B16M tumor challenge despite a significant IFN- γ recall response to both peptide and tumor cells (data not shown).

T cells primed by peptide-pulsed tumor adoptively transfer protection to the unpulsed tumor

The protective effect seen after injecting the peptide-pulsed tumor might have resulted from the persistence of the foreign antigen on the tumor cells, with the response engaged to tumor antigens playing a minor protective role – or no role at all. To test for this possibility and to establish whether it is T cells that mediate the protection, we primed the immune-competent wild-type (WT) mice with the peptide-pulsed tumors as above. Two weeks later, T cells were isolated and injected into congenic immunodeficient mice, followed by a challenge with a lethal dose of the unpulsed tumor. Thirty-five million CD3⁺ cells purified from EMT-6:CSp-primed WT BALB/c mice completely protected BALB/c SCID recipients from the EMT-6 challenge (Fig. 3A). The CD4⁺ (20 million per recipient) and CD8⁺ (15 million per recipient) cell fractions from the EMT-6:CSp-primed WT BALB/c mice also provided 100% protection in the SCID recipients. In contrast, the adoptive transfer of 70 million spleen cells from naïve WT BALB/c mice failed to protect (Fig. 3A); the mice died at a similar rate as the WT mice (Fig. 3A vs. Figs. 1A & 2A). T cells from mice inoculated with non-pulsed tumor cells did not transfer protective immunity to naïve mice receiving the tumor cells (data not shown).

Therefore, peptide pulsing was required, and of the T cells engaged by the peptide-pulsed tumor both CD4⁺ cells (that can only mediate protection against this MHC class II negative tumor via cross-presentation) and CD8⁺ cells (that can directly recognize the MHC class I positive tumor) are capable of independently preventing tumor growth. In the B16M:LCMVp model (Fig. 3B), unseparated T cells (40 million per recipient), injected *i.p.*, protected 100% of the C57BL/6 SCID mice from the normally lethal *i.v.* challenge with 2×10^5 B16M cells. However, neither the CD4⁺ (20 million cells per recipient) nor the CD8⁺ (35 million cells per recipient) subfractions showed this effect. Therefore, in the B16M model, tumor protection required both CD4⁺ and CD8⁺ cells to be jointly engaged.. Unlike in the EMT-6 model, the CD4⁺ cell-mediated cross-presentation pathway alone was not sufficient to allow protection. Also, unlike the EMT-6 model, the rejection of B16M by CD8⁺ cells seemed to be dependent on CD4⁺ cell help. The observed protection was tumor-specific in both models. When the protected mice were challenged with a third party tumor, they died at the same rate as naïve mice (Fig. 4).

Discussion

Tumor antigens (being self-antigens) induce negative selection during T cell development, eliminating the high affinity end of the auto-reactive T cell repertoire [23]. Low affinity auto-reactive T cells, however, can escape negative selection and constitute the tumor antigen-reactive T cell repertoire that can be engaged for cancer vaccination [24,25]. Inducing immune responses to tumor antigens, therefore, requires the activation of these low avidity T cells. This is usually achieved by using high concentrations of the auto/tumor antigen for immunization so that during priming the T cell activation threshold is exceeded. In addition, such immunizations typically involve TLR activators that increase co-stimulatory molecules to further lower the T cell activation threshold [26,27].

Instead of using tumor/self antigens, however, we pulsed tumors with peptides derived from foreign antigens. These peptides are unrelated to self-proteins and show no cross-reactivity to tumor cells. Because the peptides did not induce negative selection, they engage a high affinity T cell repertoire at immunization. Naïve high affinity T cells have less stringent co-stimulatory requirements and tend to develop into IFN- γ secreting effector cells [28,29].

We believe that peptide pulsing rendered tumor cells immunogenic for two reasons: Firstly, the use of a foreign peptide engaged a high affinity T cell repertoire. Secondly, the direct extracellular loading of peptide created a much higher cell surface density of peptide:MHC complexes than would occur following natural antigen processing. Taken together, this might have provided a strong CD8 cell activation signal – strong enough for the tumor cells themselves to prime naïve CD8 cells without the need for cross-presentation by DC.

Alternatively, or in addition, the initial cytokine production by such CD8 cells might have activated local DC to cross-present, recruit CD4 cells, and thus sustain the peptide-specific CD8 cell response that otherwise would have been abortive. Whether or not cross-presentation played a role in the initial priming of the CD8 cells to the foreign peptide, it must have been involved in rendering the tumor immunogenic because we found that CD4 cells did in fact contribute to tumor protection. In our experiments, the tumor immunity that had been engaged could be adoptively transferred with CD8 cells in the EMT-6 model, and was also dependent on CD8 cells in the B16M model. In both models, the transfer of CD4 cells contributed to protection. Such CD4 cells must recognize cross-presented tumor antigens in order to contribute to tumor rejection via the indirect pathway [30,31].

An important test for any novel technique of tumor vaccination is the clearance of established tumors. While the rapid growth of these tumors and the fast lethality precluded advanced therapeutic vaccinations, the data provide indirect evidence that the peptide-pulsed tumor cells are effective in extending the survival of mice with established growing tumors. In our model, we inject viable tumor cells, pulsed with peptide, into mice. These tumor cells initially encounter a naïve host for both tumor antigens and peptide. This allows them to grow unrestrictedly for about seven days – in the lung (B16M) or in the peritoneal cavity (EMT-6) – before antigen specific T cells have been primed to become effector cells and begin to target the peptide-pulsed tumor. The tumor survival we document in our experiments, particularly in the case of B16M, therefore results from clearance of established tumor. These results are very encouraging and warrant further exploration.

Our data suggest that pulsing tumors with foreign peptides can be readily exploited to induce tumor specific and protective T cell immunity. Using our protocol, it would no longer be necessary to identify a tumor's (individually highly variable) antigen profile. Instead, it would suffice to know the patient's HLA type and to select a class I-binding foreign peptide. Tumor cells (obtained from biopsies or tumor resection) would be pulsed with that peptide, irradiated, and re-administered in a single step vaccination. The ease and presumed safety of this protocol and its universal clinical applicability could have a major impact on cancer therapy.

Acknowledgments

This work was supported by grants from the National Institutes of Health to M.T.L. (AI-47756) and to P.V.L. (DK-48799, AI-42635, AI/DK 44484), and to P.V.L. from the National Multiple Sclerosis Society (RG-2807). T.R.S., W.C.B., and H.K. were supported by a scholarship from the *Studienstiftung des deutschen Volkes*.

References

1. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 1992;358:155–157. [PubMed: 1377368]

2. Disis ML, Grabstein KH, Sleath PR, Cheever MA. Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin. Cancer Res* 1999;5:1289–1297. [PubMed: 10389911]
3. el-Shami K, Tirosh B, Bar-Haim E, Carmon L, Vadai E, Fridkin M, Feldman M, Eisenbach L. MHC class I-restricted epitope spreading in the context of tumor rejection following vaccination with a single immunodominant CTL epitope. *Eur. J. Immunol* 1999;29:3295–3301. [PubMed: 10540341]
4. Markiewicz MA, Fallarino F, Ashikari A, Gajewski TF. Epitope spreading upon P815 tumor rejection triggered by vaccination with the single class I MHC-restricted peptide P1A. *Int. Immunol* 2001;13:625–632. [PubMed: 11312250]
5. Ribas A, Timmerman JM, Butterfield LH, Economou JS. Determinant spreading and tumor responses after peptide-based cancer immunotherapy. *Trends Immunol* 2003;24:58–61. [PubMed: 12547500]
6. Butterfield LH, Ribas A, Disette VB, Amarnani SN, Vu HT, Oseguera D, Wang HJ, Elashoff RM, McBride WH, Mukherji B, Cochran AJ, Glaspy JA, Economou JS. Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. *Clin. Cancer Res* 2003;9:998–1008. [PubMed: 12631598]
7. Mittendorf EA, Gurney JM, Storrer CE, Shriver CD, Ponniah S, Peoples GE. Vaccination with a HER2/neu peptide induces intra- and inter-antigenic epitope spreading in patients with early stage breast cancer. *Surgery* 2006;139:407–418. [PubMed: 16546506]
8. Wierecky J, Muller MR, Wirths S, Halder-Oehler E, Dorfel D, Schmidt SM, Hantschel M, Brugger W, Schroder S, Horger MS, Kanz L, Brossart P. Immunologic and clinical responses after vaccinations with peptide-pulsed dendritic cells in metastatic renal cancer patients. *Cancer Res* 2006;66:5910–5918. [PubMed: 16740731]
9. Ranieri E, Kierstead LS, Zarour H, Kirkwood JM, Lotze MT, Whiteside T, Storkus WJ. Dendritic cell/peptide cancer vaccines: clinical responsiveness and epitope spreading. *Immunol. Invest* 2000;29:121–125. [PubMed: 10854179]
10. Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood* 2000;96:3102–3108. [PubMed: 11049990]
11. Jonuleit H, Giesecke-Tuettenberg A, Tuting T, Thurner-Schuler B, Stuge TB, Paragnik L, Kandemir A, Lee PP, Schuler G, Knop J, Enk AH. A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. *Int. J. Cancer* 2001;93:243–251. [PubMed: 11410873]
12. Lally KM, Mocellin S, Ohnmacht GA, Nielsen MB, Bettinotti M, Panelli MC, Monsurro V, Marincola FM. Unmasking cryptic epitopes after loss of immunodominant tumor antigen expression through epitope spreading. *Int. J. Cancer* 2001;93:841–847. [PubMed: 11519046]
13. Gilboa E. The promise of cancer vaccines. *Nat. Rev. Cancer* 2004;4:401–411. [PubMed: 15122211]
14. Silagi S. Control of pigment production in mouse melanoma cells in vitro. Evocation and maintenance. *J. Cell Biol* 1969;43:263–274. [PubMed: 4981070]
15. Rockwell SC, Kallman RF, Fajardo LF. Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative. *J. Natl. Cancer Inst* 1972;49:735–749. [PubMed: 4647494]
16. Romero P, Maryanski JL, Corradin G, Nussenzweig RS, Nussenzweig V, Zavala F. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature* 1989;341:323–326. [PubMed: 2477703]
17. Romero P, Corradin G, Luescher IF, Maryanski JL. H-2Kd-restricted antigenic peptides share a simple binding motif. *J. Exp. Med* 1991;174:603–612. [PubMed: 1714934]
18. Pircher H, Moskophidis D, Rohrer U, Burki K, Hengartner H, Zinkernagel RM. Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. *Nature* 1990;346:629–633. [PubMed: 1696684]
19. Adorini L, Muller S, Cardinaux F, Lehmann PV, Falcioni F, Nagy ZA. In vivo competition between self peptides and foreign antigens in T-cell activation. *Nature* 1988;334:623–625. [PubMed: 3261393]

20. Bloom MB, Perry-Lalley D, Robbins PF, Li Y, el-Gamil M, Rosenberg SA, Yang JC. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J. Exp. Med* 1997;185:453–459. [PubMed: 9053445]
21. Overwijk WW, Tsung A, Irvine KR, Parkhurst MR, Goletz TJ, Tsung K, Carroll MW, Liu C, Moss B, Rosenberg SA, Restifo NP. gp100/pmel 17 is a murine tumor rejection antigen: induction of “self”-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J. Exp. Med* 1998;188:277–286. [PubMed: 9670040]
22. Karulin AY, Hesse MD, Tary-Lehmann M, Lehmann PV. Single-cytokine-producing CD4 memory cells predominate in type 1 and type 2 immunity. *J. Immunol* 2000;164:1862–1872. [PubMed: 10657635]
23. Sebzda E, Mariathasan S, Ohteki T, Jones R, Bachmann MF, Ohashi PS. Selection of the T cell repertoire. *Annu. Rev. Immunol* 1999;17:829–874. [PubMed: 10358775]
24. Targoni OS, Lehmann PV. Endogenous myelin basic protein inactivates the high avidity T cell repertoire. *J. Exp. Med* 1998;187:2055–2063. [PubMed: 9625765]
25. Bouneaud C, Kourilsky P, Bousso P. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity* 2000;13:829–840. [PubMed: 11163198]
26. Lehmann PV, Targoni OS, Forsthuber TG. Shifting T-cell activation thresholds in autoimmunity and determinant spreading. *Immunol. Rev* 1998;164:53–61. [PubMed: 9795763]
27. Zinkernagel RM. Antiinfection immunity and autoimmunity. *Ann. N. Y. Acad. Sci* 2002;958:3–6. [PubMed: 12021079]
28. Ohashi PS, DeFranco AL. Making and breaking tolerance. *Curr. Opin. Immunol* 2002;14:744–759. [PubMed: 12413525]
29. Heath VL, Kurata H, Lee HJ, Arai N, O'Garra A. Checkpoints in the regulation of T helper 1 responses. *Curr. Top. Microbiol. Immunol* 2002;266:23–39. [PubMed: 12014201]
30. Rock KL, Shen L. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol. Rev* 2005;207:166–183. [PubMed: 16181335]
31. Jiang S, Herrera O, Lechler RI. New spectrum of allorecognition pathways: implications for graft rejection and transplantation tolerance. *Curr. Opin. Immunol* 2004;16:550–557. [PubMed: 15341998]

B

Mouse strain	In vivo treatment	In vitro recall		
		Medium	Peptide	Tumor
BALB/c	none	< 10	< 10	< 10
BALB/c	EMT-6	< 10	< 10	< 10
BALB/c	CSp*	< 10	22 ± 1	< 10
C57BL/6	none	< 10	< 10	< 10
C57BL/6	B16M	< 10	< 10	21 ± 1
C57BL/6	LCMVp*	< 10	110 ± 9	< 10

* immunization with CpG

Figure 1.

Lethality and immunogenicity of EMT-6 breast cancer cells and B16M cells in syngeneic mice. A, EMT-6 cells were injected *i.p.* into BALB/c mice (n=16, open squares). B16M cells were injected *i.v.* into C57BL/6 mice (n=16, open circles). The survival rate of the mice was monitored daily. The data were reproduced in 3 independent experiments. B, BALB/c or C57BL/6 mice were injected with EMT-6 or B16M tumor cells as above or remained uninjected, 4 mice in each group. A third group of mice was immunized with CSp or LCMVp in IFA with CpG oligonucleotide. Fourteen days later, single cell suspensions of spleen cells were tested in an IFN- γ ELISPOT assay as specified under “in vitro recall”. The data shown are the mean values and standard error of the frequencies of IFN- γ -producing cells from

duplicate cultures of representative mice. The data were reproduced in three independent experiments.

B

Mouse strain	In vivo treatment	In vitro recall		
		Medium	Peptide-Pulsed Tumor	Unpulsed Tumor
BALB/c	EMT-6:CSp	< 10	59 ± 16	39 ± 10
C57.BL/6	B16M:LCMVp	< 10	229 ± 2	218 ± 21

Figure 2.

Pulsing of EMT-6 or B16M cells with foreign antigenic peptide induces protective immunity and determinant spreading to tumor antigens. A, EMT-6 or B16M cells were cultured with CSp or LCMVp and injected into BALB/c (*i.p.*) or C57BL/6 (*i.v.*) mice (closed squares or circles), respectively. Unpulsed EMT-6 or B16M cells were injected at the same numbers (open squares or circles). Mice were monitored daily for survival. The survival rates shown are for 4 mice in each group. The data were reproduced in three independent experiments. B, Fourteen days after the injection of peptide-loaded tumor cells, spleen cells of respective mice were tested in IFN- γ ELISPOT assays for the response elicited by the peptide-pulsed and unpulsed

tumor as well as by peptide alone. Mean values from duplicate wells are shown for representative mice. The data were reproduced in two independent experiments.

Figure 3.

The T cell response engaged by epitope spreading to tumor antigens is protective. EMT-6 (A) or B16M cells (B) were pulsed with CS_p or LCMV_p and injected into BALB/c or C57BL/6 mice as described in legend to Fig. 2A. Fourteen days later, CD3⁺, CD4⁺ or CD8⁺ subpopulations were isolated at >95% purity by negative selection on affinity columns and were injected into congenic SCID mice as specified by the symbols (the numbers of T cells injected are stated in the text). 24 hours later, the unpulsed tumor was injected, and the survival rate of the recipient mice was monitored. Each group involved 6 mice, and the shown results were reproduced in two separate experiments.

	<u>Mouse strain</u>	<u>Adoptive transfer of primed T cells</u>	<u>Tumor challenge</u>	
			<u>Primary</u>	<u>Secondary</u>
—△—	BALB/c Scid	none	none	LR
—▲—	BALB/c Scid	EMT 6:CSp primed	EMT 6	LR
—◇—	C57BL/6 Scid	none	none	RMA
—◆—	C57BL/6 Scid	B16M:LCMVp-primed	B16M	RMA

Figure 4.

The protection induced by peptide-pulsed tumor cells is tumor-specific. The CD3⁺ cell-grafted BALB/c SCID mice that survived the challenge with EMT-6 tumor in Fig. 3A (closed triangles) and untreated BALB/c SCID mice (open triangles) were injected with a lethal dose of LR lymphoma cells (2×10^5 cells per recipient *i.p.*). The C57BL/6 SCID mice that survived the challenge with B16M in Fig. 3B were challenged 50 days later with RMA tumor cells (1×10^3 cells per recipient *i.p.*) (closed diamonds). Naïve C57BL/6 SCID mice (open diamonds) received the same challenge. Survival of the mice was monitored.