Peptide Vaccine Induces Enhanced Tumor Growth Associated with Apoptosis Induction in CD8+ T Cells


J Immunol 2010; 185:3768-3776; Prepublished online 23 August 2010;
doi: 10.4049/jimmunol.0903649
http://www.jimmunol.org/content/185/6/3768

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/08/20/jimmunol.0903649.DC1

References
This article cites 35 articles, 14 of which you can access for free at:
http://www.jimmunol.org/content/185/6/3768.full#ref-list-1

Why The JI? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2010 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Peptide Vaccine Induces Enhanced Tumor Growth Associated with Apoptosis Induction in CD8+ T Cells

Daisuke Muraoka,* Takuma Kato,† Linan Wang,* Yuka Maeda,* Takuro Noguchi,* Naozumi Harada,* Kazuyoshi Takeda,§ Hideo Yagita,§ Philippe Guillaume,¶ Immanuel Luescher,¶ Lloyd J. Old,‖ Hiroshi Shiku,*# and Hiroyoshi Nishikawa* 1

CD8+ CTLs play a critical role in antitumor immunity. However, vaccination with synthetic peptide containing CTL epitopes has not been generally effective in inducing protective antitumor immunity. In this study, we addressed the detailed mechanism(s) involved in this failure using a new tumor model of BALB/c transplanted tumors expressing NY-ESO-1, an extensively studied human cancer/testis Ag. Whereas peptide immunization with an H2-Db–restricted CTL epitope derived from NY-ESO-1 (NY-ESO-1 p81–88) induced NY-ESO-1 157–165–specific CD8+ T cells in draining lymph nodes and spleens, tumor growth was significantly enhanced. Single-cell analysis of specific CD8+ T cells revealed that peptide immunization caused apoptosis of >80% of NY-ESO-1 157–165–specific CD8+ T cells at tumor sites and repetitive immunization further diminished the number of specific CD8+ T cells. This phenomenon was associated with elevated surface expression of Fas and programmed death-1. When peptide vaccination was combined with an adjuvant, a TLR9 ligand CpG, the elevated Fas and programmed death-1 expression and apoptosis induction were not observed, and vaccine with peptide and CpG was associated with strong tumor growth inhibition. Selection of appropriate adjuvants is essential for development of effective cancer vaccines, with protection of effector T cells from peptide vaccine-induced apoptosis being a prime objective. The Journal of Immunology, 2010, 185: 3768–3776.
and used at 7–10 wk of age. DUC18 mice, transgenic for αβ-TCR reactive with the K†-restricted mERK2136–144 were established as described previously (13). Mice were maintained at the Animal Center of Mie University Graduate School of Medicine (Mie, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine.

**Tumors**

CT26 is a colon epithelial tumor cell line derived by intrarectal injections of N-nitroso-N-methylurethane in BALB/c mice (14). CMS5 is a 3-methylcholanthrene-induced sarcoma cell line of BALB/c origin (15). CMS5a is a subcloned cell line obtained from CMS5, a tumor expressing mERK2 (15). CT26 and CMS5a expressing NY-ESO-1, a human CT Ag were established as described previously (16). Groups of five mice were inoculated s.c. in the right hind flank with 1 × 10⁶ CT26 or 3 × 10⁶ CMS5a and monitored three times a week. Tumor volume was calculated by the following formula: tumor volume = 0.5 × length (mm) × width (mm)².

**Abs and reagents**

Anti-CD8 (19/178, mouse IgG2a) and anti-Fas ligand (FasL) (MFLA, an anti-human hamster IgG3) (17) Abs were produced from hybridomas and were purified by protein G columns. Anti-PD-L1 (MIH5, rat IgG2a) mAb (18) was kindly provided by Dr. M. Azuma (Tokyo Medical and Dental University, Tokyo, Japan). mAb was i.p. injected as described (15, 16). PerCP-Cy5.5-conjugated anti-CD8 (53-6.7), FITC-conjugated anti-CD45RB (16A), FITC-conjugated anti-CD80 (16-10A1), FITC-conjugated anti-CD86 (GL1), FITC-conjugated anti-Fas (Jo2), purified anti-programmed death-1 (PD-1, J34), FITC-conjugated anti-Armenian and Syrian Hamster IgG mixture (G70-204, G94-56), allophycocyanin-conjugated anti–IFN-γ (XM1G1.2), and PE-Cy7–conjugated anti–TNF-α (MP6-XT22) mAbs were purchased from BD Bioscience (Franklin Lakes, NJ). Allophycocyanin-conjugated anti-CD62L (MEL-14) and PE-conjugated anti-TRA1 (N2B2) mAbs were purchased from eBioscience (San Diego, CA). Allophycocyanin-PC conjugated annexin V and the vital dye 7-aminoactinomycin D were purchased from BD Bioscience. Synthetic mERK2136–144–9m peptide QYIHSANVL (15, 19), AH-1 peptide SPSYYVHYQF (20), and NY-ESO-181–88 peptide RGPESRLL (D. Murakoa, H. Nishikawa, and H. Shiku, manuscript in preparation) were obtained from Sigma-Aldrich (St. Louis, MO). Cpg ODN 1668 (5’-TCCCCAGGTTCTTCGTAGCT-3’) was synthesized by Hokkaido System Science (Sapporo, Japan).

**Immunization**

A total of 100 µg (unless otherwise noted) NY-ESO-181–88 or AH-1 peptides emulsified in Montanide (incomplete Freund’s adjuvant [IFA]) were injected s.c. in the right flank of mice at weekly intervals. A total of 50 µg purified by protein G columns. Anti–PD-L1 (MIH5, rat IgG2a) mAb (18) was kindly provided by Dr. M. Azuma (Tokyo Medical and Dental University, Tokyo, Japan). mAb was i.p. injected as described (15, 16). PerCP-Cy5.5-conjugated anti-CD8 (53-6.7), FITC-conjugated anti-CD45RB (16A), FITC-conjugated anti-CD80 (16-10A1), FITC-conjugated anti-CD86 (GL1), FITC-conjugated anti-Fas (Jo2), purified anti-programmed death-1 (PD-1, J34), FITC-conjugated anti-Armenian and Syrian Hamster IgG mixture (G70-204, G94-56), allophycocyanin-conjugated anti–IFN-γ (XM1G1.2), and PE-Cy7–conjugated anti–TNF-α (MP6-XT22) mAbs were purchased from BD Bioscience (Franklin Lakes, NJ). Allophycocyanin-conjugated anti-CD62L (MEL-14) and PE-conjugated anti-TRA1 (N2B2) mAbs were purchased from eBioscience (San Diego, CA). Allophycocyanin-PC conjugated annexin V and the vital dye 7-aminoactinomycin D were purchased from BD Bioscience. Synthetic mERK2136–144–9m peptide QYIHSANVL (15, 19), AH-1 peptide SPSYYVHYQF (20), and NY-ESO-181–88 peptide RGPESRLL (D. Murakoa, H. Nishikawa, and H. Shiku, manuscript in preparation) were obtained from Sigma-Aldrich (St. Louis, MO). Cpg ODN 1668 (5’-TCCCCAGGTTCTTCGTAGCT-3’) was synthesized by Hokkaido System Science (Sapporo, Japan).

**In vivo CTL assay**

Cells were prepared from spleens and pulsed with NY-ESO-181–88 or control peptide. These cells were labeled with a high concentration (2.5 M) of CFSE (CFSEhigh cells) or with a low concentration (0.25 M) of CFSE (CFSELow cells). Recipient mice were injected i.v. with 5 × 10⁶ of target cell populations. Twenty hours later, splenocytes were analyzed by flow cytometry.

Percent-specific lysis was normalized to the results observed in naive BALB/c mice: % specific lysis = (1 – [(CFSElow/CFSEhigh)/(CFSElow/BALB/c)]) × 100.

**Cell isolation**

To isolate splenic CD11c⁺ dendritic cells (DCs), spleen cell suspensions were mixed with CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated into CD11c⁺ DCs by positive selection on a MACS column. CD11c⁺ DCs were preincubated with/without Cpg ODN 1668 (5 µg/ml) for 2 d and then cultured with CD8⁺ cells.

To collect tumor infiltrating T cells, tumors were minced and treated with 1 mg/ml collagenase IA (Sigma-Aldrich) in HBSS media for 90 min at room temperature.

**Statistical analysis**

Statistical significance was evaluated by Student t test. The p values <0.05 are considered significant.

**Results**

**NY-ESO-1 peptide vaccination enhances tumor growth**

We examined the impact of vaccination with peptide emulsified in IFA, a standard method used in human clinical trials, in our animal system (3, 4, 10). To this end, we used two tumor transplantation models; CT26 (a murine colon carcinoma) and CMS5a (a murine fibrosarcoma) with stable expression of NY-ESO-1, a human CT Ag that has been the focus of considerable studies in humans (10). BALB/c mice were inoculated with CT26-NY-ESO-1 or CMS5a-NY-ESO-1, and injected with NY-ESO-181–88 peptide (RGPESRLL), an H2-D^d–restricted CTL epitope of NY-ESO-1. Both in prophylactic (vaccinated on day −21, −14, and −7) and in therapeutic (vaccinated on day +3, +10, and +17) models, peptide vaccination significantly augmented tumor growth compared with control mice (Fig. 1A). In contrast, vaccination with plasmids encoding the whole sequence of NY-ESO-1 by gene gun prevented growth of both CT26-NY-ESO-1 and CMS5a-NY-ESO-1 (Fig. 1A). To address whether this enhanced tumor growth induced by peptide vaccination was unique to the NY-ESO-1 system, mice with parental CT26 tumors were vaccinated with AH-1 peptide derived from the envelope protein (gp70) of an endogenous ectotropic murine leukemia provirus expressed by CT26 and shown to be the target of CD8⁺ T cells (20). CT26 tumor growth was enhanced after vaccination with AH-1 peptide, indicating that the enhanced tumor growth induced by peptide vaccination may be a more general phenomenon of peptide vaccine strategies (Fig. 1B). We next examined whether this enhancement of tumor growth was seen in mice vaccinated with lower amounts of peptide. The enhancement of tumor growth was observed in a dose-dependent manner and was detected as little as 1 µg of NY-ESO-181–88 peptide (Fig. 1C).

We next inquired whether this effect of short peptide vaccination was due to malfunction of CD8⁺ T cells. As a first step, nude mice were inoculated with CT26-NY-ESO-1 and vaccinated with NY-ESO-181–88 peptide. Tumor growth was comparative between control and vaccinated nude mice (Fig. 1D). To further confirm the involvement of CD8⁺ T cells, BALB/c mice were inoculated with CT26-NY-ESO-1 and vaccinated with NY-ESO-181–88 peptide. Groups of mice were additionally administered with anti-CD8 mAb to examine the involvement of CD8⁺ T cells. Mice administered with anti-CD8 mAb did not exhibit further enhancement of tumor growth by vaccination with NY-ESO-181–88 peptide, indicating a clear correlation between the enhanced tumor growth and CD8⁺ T cells (Fig. 1E).

**Peptide vaccine-induced Ag-specific CD8⁺ T cells are not energetically, but exhibit an apoptotic phenotype**

To gain insight into the mechanism(s) of this tumor growth enhancement induced by peptide vaccinations, we analyzed the effector function of peptide vaccine-induced CD8⁺ T cells. BALB/c tumor-free mice were injected with NY-ESO-181–88 peptide three times at weekly intervals.
In addition, a group of mice was inoculated with CT26-NY-ESO-1 and injected with NY-ESO-1<sub>181-88</sub> peptide (100 μg) emulsified in IFA in prophylactic (vaccinated on day −21, −14, and −7) or therapeutic (vaccinated on day +3, +10, and +17) schedule. Mice immunized with plasmids encoding the entire NY-ESO-1 by gene gun in prophylactic (vaccinated on day −21 and −7) schedule served as a positive control. B, BALB/c mice were inoculated with 1 × 10<sup>6</sup> CT26 and injected with AH-1 peptide (100 μg), which is derived from the envelope protein (gp70) of an endogenous ecotropic murine leukemia provirus, emulsified in IFA in prophylactic or therapeutic schedule. C, BALB/c mice were inoculated with 1 × 10<sup>6</sup> CT26-NY-ESO-1 and injected with graded amounts of NY-ESO-1<sub>181-88</sub> peptide in prophylactic schedule. D, BALB/c<sub>nmu</sub> mice were inoculated with 1 × 10<sup>6</sup> CT26-NY-ESO-1 and injected with NY-ESO-1<sub>181-88</sub> peptide (100 μg) in prophylactic schedule. E, BALB/c mice were inoculated with 1 × 10<sup>6</sup> CT26-NY-ESO-1 and vaccinated with NY-ESO-1<sub>181-88</sub> peptide (100 μg) in therapeutic schedule. In addition, groups of mice received the administration with anti-CD8 mAb (19/178, 100 μg) 2 d before tumor inoculation (resulting in >99% of CD8<sup>+</sup> T cell depletion). Tumor size was monitored three times a week. Each group consisted of five mice. These experiments were repeated two to four times with similar results. Data are mean ± SD. *p < 0.05 as compared with control. CMS5a-NY-ESO-1, CMS5a with stable expression of NY-ESO-1; CT26-NY-ESO-1, CT26 with stable expression of NY-ESO-1.
Peptide vaccine induced Ag-specific CD8+ T cells are not anergic, but exhibit an apoptotic phenotype. BALB/c mice were injected with NY-ESO-181–88 peptide three times at 1 wk intervals. In addition, a group of mice were inoculated with $1 \times 10^6$ CT26-NY-ESO-1 and injected with NY-ESO-181–88 peptide (100 $\mu$g) in therapeutic schedule. BALB/c mice immunized with plasmids encoding the entire NY-ESO-1 by gene gun were served as a positive control. After 7 d of the last vaccination, mice were sacrificed. A, dLN cells were prepared and incubated with NY-ESO-181–88 or control peptide, and cytokine secretion was analyzed with flow cytometry. Production of TNF-α and IFN-γ was negligible, when dLN cells were stimulated control peptide. B, CFSE-labeled target cells were prepared as described in Materials and Methods and transferred to mice vaccinated as indicated. Twenty hours later, target cells were harvested from spleens and analyzed by flow cytometry. Percent specific lysis was calculated as described in Materials and Methods. C, Surface and apoptosis markers (D) of NY-ESO-181–88–specific CD8+ T cells, identified as CD8+NY-ESO-1/D1 tetramer+ T cells in dLNs, were analyzed with flow cytometry. E, After 3 d of the last vaccination, TIL were prepared and apoptosis markers of NY-ESO-181–88–specific CD8+ T cells were analyzed with flow cytometry. These experiments were repeated twice with similar results. TIL, tumor infiltrating lymphocytes.
vaccine and DNA vaccine-induced NY-ESO-181–88–specific CD8+ T cells identified as CD8+NY-ESO-1/Dd tetramer+ T cells, the effector/memory status defined by CD62L and CD45RB was analyzed (21). Although DNA vaccination elicited both NY-ESO-181–88–specific CD8+ T cells, peptide vaccination dominantly induced NY-ESO-181–88–specific CD8+ CD62LlowCD45RBlow T cells at tumor local sites (Fig. 2E). In sharp contrast, 80% of NY-ESO-181–88–specific CD8+ T cells by DNA vaccination showed a vigorous nonapoptotic phenotype at tumor sites (Fig. 2E).

Elevated expression of PD-1 and Fas is associated with the apoptotic phenotype observed in tumor Ag-specific CD8+ T cells

Given that peptide vaccinations induce apoptosis in NY-ESO-181–88–peptide-specific CD8+ T cells, we examined the kinetics of induction/augmentation of NY-ESO-181–88–specific CD8+ T cells in dLN and spleens. BALB/c mice were injected with NY-ESO-181–88 peptide (100 μg) twice at weekly intervals. Some groups of mice received the administration with anti–PD-L1 mAb (MIH5, 200 μg) and/or anti-FasL mAb (MFL4, 200 μg) on day 1, 2, 6, 9, 13, 16. After 3 d of the last vaccination, dLN cells were isolated and apoptosis markers of NY-ESO-181–88–specific CD8+ T cells were analyzed with flow cytometry. These experiments were repeated two to four times with similar results. Data are mean ± SD. *p < 0.05 as compared with control. MFI, mean fluorescent intensity.

FIGURE 3. Elevated expression of PD-1 and Fas is associated with the apoptotic phenotype observed in tumor Ag-specific CD8+ T cells. A, BALB/c mice were injected with NY-ESO-181–88 peptide (100 μg) as the indicated times and CD8+ T cells were purified from dLN and spleen 7 d after the last vaccination. The number of NY-ESO-181–88–specific CD8+ T cells was assessed as CD8+NY-ESO-1/Dd tetramer+ T cells by flow cytometry. B, BALB/c mice were inoculated with 1 × 106 CT26-NY-ESO-1 and vaccinated with NY-ESO-181–88 peptide (100 μg) in prophylactic schedule as the indicated times. Tumor size was monitored three times a week. Each group consisted of five mice. C, BALB/c mice were injected with NY-ESO-181–88 peptide (100 μg) three times at weekly intervals. In addition, a group of mice was inoculated with 1 × 106 CT26-NY-ESO-1 and injected with NY-ESO-181–88 peptide in therapeutic schedule. After 7 d of the last vaccination, dLN cells were isolated, and Fas, PD-1 and TRAIL expression of NY-ESO-181–88–specific CD8+ T cells was analyzed. D, BALB/c mice were injected with NY-ESO-181–88 peptide (100 μg) twice at weekly intervals. Some groups of mice received the administration with anti–PD-L1 mAb (MIH5, 200 μg) and/or anti-FasL mAb (MFL4, 200 μg) on day 1, 2, 6, 9, 13, 16. After 3 d of the last vaccination, dLN cells were isolated and apoptosis markers of NY-ESO-181–88–specific CD8+ T cells were analyzed with flow cytometry. These experiments were repeated two to four times with similar results. Data are mean ± SD. *p < 0.05 as compared with control. MFI, mean fluorescent intensity.
FIGURE 4. The combination of peptide vaccination with CpG abrogates the elevated expression of PD-1 and Fas and exhibits strong antitumor immunity. BALB/c mice were inoculated with $1 \times 10^6$ CT26-NY-ESO-1 and injected with NY-ESO-181–88 peptide (100 µg) emulsified in IFA with/without 50 µg CpG in prophylactic or therapeutic schedules. A. Tumor size was monitored three times a week. Each group consisted of five mice. B. After 7 d of the last vaccination, dLN cells were isolated, and Fas, PD-1, and TRAIL expression of NY-ESO-181–88-specific CD8+ T cells was analyzed. C, BALB/c mice were injected with NY-ESO-181–88 peptide (100 µg) with/without 50 µg CpG twice at weekly intervals. After 3 d of the last vaccination, dLN cells were isolated and apoptosis markers of NY-ESO-181–88-specific CD8+ T cells were analyzed with flow cytometry. D and E, After 7 d of the last vaccination, spleen cells were prepared and incubated with NY-ESO-181–88 or control peptide, and cytokine secretion was analyzed with flow cytometry (D). The number of NY-ESO-181–88-specific CD8+ T cells was assessed as CD8+NY-ESO-1-Dd tetramer+ T cells (E). BALB/c mice immunized with plasmids encoding the entire NY-ESO-1 by gene gun were served as a positive control. These experiments were repeated two to four times with similar results. Data are mean ± SD. *p < 0.05 as compared with control. MFI, mean fluorescent intensity.
vaccination, the percentage of NY-ESO-181–88–specific CD8+ T cells decreased upon repetitive vaccinations (Fig. 3A). This decay of NY-ESO-181–88–specific CD8+ T cells by repetitive vaccinations correlated with enhancement of tumor growth (Fig. 3B).

Next, we explored the molecular event(s) involving this apoptosis induction by peptide vaccination. BALB/c mice were injected with NY-ESO-181–88 peptide three times at 1 wk intervals. An additional group of mice were also inoculated with CT26-NY-ESO-1 and injected with NY-ESO-181–88 peptide in a therapeutic vaccination schedule (vaccinated on day +3, +10, and +17). Seven days after the last vaccination, dLN cells were isolated and subjected to analyses of three apoptosis-associated molecules: Fas, PD-1, and TRAIL. Expression of Fas and PD-1, but not TRAIL, was elevated on NY-ESO-181–88–specific CD8+ T cells induced by NY-ESO-181–88 peptide vaccination, but not in those induced by DNA vaccination (Fig. 3C). Consistent with the enhancement of Fas and PD-1 expression in NY-ESO-181–88–specific CD8+ T cells, blocking of either Fas or PD-1 signal by neutralizing mAb against FasL or PD-1 ligand 1 (PD-L1) Ab partially abrogated the induction of apoptosis on NY-ESO-181–88–specific CD8+ T cells (Fig. 3D). Blocking of both Fas and PD-1 signals additively inhibited the apoptosis induction in NY-ESO-181–88–specific CD8+ T cells (Fig. 3D), suggesting that Fas and PD-1 signals control apoptosis induction by peptide vaccination in different ways.

We next analyzed whether blocking of Fas and PD-1 signals provided antitumor effects. Mice were immunized with peptide and injected with neutralizing mAb against FasL or PD-L1. Although the frequency of apoptotic cells was decreased by blocking of these signals as shown in Fig. 3D, the enhancement of tumor growth by peptide vaccination was not reversed by mAb injection (Supplemental Fig. 1).

Combination of peptide vaccination with an adjuvant, a TLR9 ligand CpG, abrogates the elevated expression of PD-1 and Fas and induces strong antitumor immunity

We next examined whether the failure of peptide vaccination could be overcome by combination with appropriate adjuvants. Among several adjuvants, we chose CpG, a TLR9 ligand, because of its strong antitumor activity (22). BALB/c mice were inoculated with CT26-NY-ESO-1 and injected with NY-ESO-181–88 peptide with or without CpG in prophylactic (vaccinated on day −21, −14, and −7) or therapeutic (vaccinated on day +3, +10, and +17) vaccination schedule and tumor growth was monitored. CT26-NY-ESO-1 growth was significantly inhibited by peptide vaccination combined with CpG in both prophylactic and therapeutic models (Fig. 4A). CD8+ T cells purified from dLNs of these mice revealed the addition of CpG abrogated the elevation of Fas and PD-1 expression on NY-ESO-181–88–specific CD8+ T cells (Fig. 4B). Furthermore, the number of NY-ESO-181–88–specific CD8+ T cells with the apoptotic phenotype was comparable with those of DNA vaccination (Fig. 4C), indicating that peptide vaccination combined with appropriate adjuvants, such as CpG, rescues its inherent antitumor activity. In accordance with this, induction of NY-ESO-181–88–specific CD8+ T cells was augmented by co-administration of CpG (Fig. 4D, 4E).

CpG provides suitable signals to Ag-specific CD8+ T cells by promoting DC maturation

To explore the mechanism(s) by which CpG affects Ag-specific CD8+ T cells, we analyzed the Fas and PD-1 expression on Ag-specific T cells after in vitro stimulation with immature or CpG-matured splenic DC11c+ DCs. To this end, we examined maturation status of DC11c+ DCs after treatment with CpG. CpG-treated DCs exhibited higher expression of CD80 and CD86 (Fig. 5A), indicating that CpG treatment induced the maturation of DCs. Then naive Ag-specific CD8+ T cells were prepared from spleens of DUC18 mice, transgenic for αβ-TCR reactive with the Kd-restricted mutated MAP kinase, mERK2(136–144) (13), and stimulated with immature or CpG-matured DCs pulsed with cognate peptide. mERK2(136–144)–specific CD8+ T cells stimulated with immature DCs exhibited significantly elevated expression of Fas on day 6 and PD-1 on day 14 compared with those stimulated with CpG-matured DCs (Fig. 5B).

Discussion

Melief et al. (7, 8) reported that vaccination with peptides derived from the adenovirus type 5 early region 1 (Ad5E1) oncoregines tolerizes T cells and causes enhanced tumor outgrowth. They showed this enhancement is associated with peptide-induced loss of Ag-specific CTL activity (7, 8). It has also recently been reported that peptide vaccine induces the death of adoptively transferred Ag-specific
CD8+ T cells (23). Although these studies demonstrated the decay of Ag-specific CD8+ T cells, they did not elucidate the molecular mechanism(s). Using our new animal model, we addressed this issue and found that Ag-specific CD8+ T cells were induced by peptide vaccination in dLNs and spleens, although to a lesser extent as compared with DNA vaccine, and that these Ag-specific CD8+ T cells secreted cytokines and exhibited killing activity. However, these Ag-specific CD8+ T cells present at tumor sites exhibited an apoptotic phenotype, which was associated with elevated expression of death signal molecules, such as Fas and PD-1. The tumor enhancement effect induced by peptide vaccination was observed in a dose-dependent manner. We attempted to find an optimal dose that induced antitumor immunity, but not apoptosis of Ag-specific effector CD8+ T cells. However, although tumor enhancement was reduced with lower peptide doses, no tumor growth inhibition was observed, indicating that peptide vaccination without proper adjuvants is not able to induce protective antitumor immunity.

The percentage of Ag-specific CD8+ T cells with the apoptotic phenotype increased in tumors compared with dLNs. As peptides express PD-L1 (data not shown), it is possible that apoptosis induction of Ag-specific CD8+ T cells is augmented in tumor sites by PD-1-related signals. Alternatively, Ag-specific CD8+ T cells that have received apoptosis signals at dLNs may exhibit this phenotype at variable time courses. These data indicate that Ag-specific loss of CD8+ T cell function was not due to anergy induction, rather Ag-specific death of CD8+ T cells as occurred in tolerance induction against self-Ag (24). Consistent with this notion, repetitive vaccinations actually decreased the number of Ag-specific CD8+ T cells in dLNs and spleens. Furthermore, when mice immunized with NY-ESO-1ls-ss peptide received secondary immunization with NY-ESO-1ls-ss peptide or with DNA vaccine, NY-ESO-1–specific CD8+ T cells were augmented by DNA vaccine, but not NY-ESO-1ls-ss peptide (Supplemental Fig. 2). This indicates that a proportion of NY-ESO-1–specific CD8+ T cells induced by peptide vaccination indeed responded to the proper secondary stimulation and was not in an anergic state.

In clinical studies, it has been shown that rapid and strong Ag-specific CD8+ T cells are elicited by peptide, IFA and CpG vaccination compared with previous studies using peptide and IFA (25). In our study, peptide vaccination combined with an adjuvant CpG showed strong antitumor immunity and caused >50% complete tumor regression. Peptide vaccination with CpG also inhibited the elevated expression of Fas and PD-1 that was associated with the apoptotic phenotype. These augmented antitumor responses were correlated with CpG-induced DC maturation, because CpG-matured DCs, but not immature DCs, namely DCs that can provide suitable costimulatory signals to CD8+ T cells, could rescue Ag-specific CD8+ T cells. It has been reported that T cell priming versus tolerance induced by peptide vaccination depends on the route of peptide injection (6). Although difference of peptide injection route may activate different DCs, our data clearly demonstrate that appropriate costimulatory signals were essential for determining the outcome of peptide vaccination.

PD-1 has been reported as a negative regulator of activated T cells associated with functional impairment when upregulated on the surface of CD8+ T cells (26–29). It has further been shown that PD-1 ligation inhibits the induction of Bcl-xL, an important cell survival factor (29). Blocking PD-1 signals with anti–PD-L1 restores the activated phenotype of CD8+ T cells (26–29). It has been recently reported that spontaneously induced tumor Ag, particularly NY-ESO-1–specific CD8+ T cells exhibit high level of PD-1 expression, and that blockade of PD-1 signals augments the number of cytokine-producing and proliferating NY-ESO-1–specific CD8+ T cells (30). In the case of Fas, this molecule plays a major role in activation-induced cell death (31). It has been shown that Fas-induced activation-induced cell death is triggered by limited TCR signaling compared with killing of target cells (32), indicating that nonprofessional APCs, such as tumor cells, may also contribute Ag presentation in our peptide vaccine and Fas-induced activation-induced cell death. In our study, peptide vaccination-induced CD8+ T cells with higher expression of PD-1 and Fas were partially rescued by blocking of either of these signals, and blocking of both PD-1 and Fas signals additively inhibited apoptosis induction. Furthermore, when naive CD8+ T cells were exposed to immature DCs, Fas expression preceded PD-1 expression. Although PD-1 has not been linked to activation-induced cell death, such as Fas signaling (29, 31), our data suggest that Fas and PD-1 signals provide distinct signals and synergistically contribute to cell death.

Although the frequency of apoptotic Ag-specific CD8+ T cells was decreased after blocking Fas and PD-1 signals, enhancement of tumor growth by peptide vaccination was not reversed. One plausible explanation is that Fas–FasL signal may be an effector molecule of CD8+ T cells (33) and blocking this signal may hinder CD8+ T cell function. Although we used anti–PD-L1 for blocking the PD-1 signal, other PD-1 associated signals may contribute to enhance tumor growth (34). Alternatively, we have recently found that CD8+ T cells protected from apoptosis by blocking Fas and PD-1 signals still exhibited an exhausted phenotype, such as higher expression of LAG3 and CD160, and lower expression of NKG2D (35) (data not shown). This suggests that although Fas and PD-1 signals are critical for peptide vaccine-induced apoptosis, other signals through LAG3, CD160, and NKG2D may also be important to generate sufficient effector CD8+ T cell function for tumor lysis, namely, blocking the repertoire of immunosuppression signals is necessary to acquire antitumor activity.

We found that vaccination with peptides emulsified in IFA enhanced tumor growth compared with growth in control animals. From this and other reports (7, 8), there is now a clear evidence for enhancement of tumor growth in mice immunized with peptides. Peptide vaccination-mediated tumor enhancement with its associated effect on CD8+ T cells mimics the effect of CD8+ T cell depletion on tumor growth, indicating that both maneuvers abrogate the CD8+ T cell restraint, albeit incomplete, that CD8+ T cells normally exert on tumor growth. Although vaccine-mediated tumor enhancement has not been reported in humans (3, 10), the variable course of cancer growth and progression in humans makes establishing such an association very difficult. Assessing the activation state, cytokine profile and apoptotic characteristics of CD8+ T cells generated by peptide vaccines in humans may provide surrogate markers for a state of tumor enhancement and this needs to be carefully monitored in clinical cancer vaccine trials. Also, induction of regulatory T cells and other immunosuppressive mechanisms in peptide-vaccine patients, requires attention. The safest approach, however, is to add appropriate adjuvants (costimulatory signals) to peptide vaccines that, from our study, abrogate the induction of T cell apoptosis and tumor enhancement.

**Acknowledgments**

We thank Dr. M. Azuma for providing anti–PD-L1 mAb, Dr. T. Takahashi for helpful discussion, and K. Mori and K. Sasada for technical assistance.

**Disclosures**

D.M. and N.H. are employees of ImmunoFrontier, Inc. The other authors have no financial conflicts of interest.

**References**

Enhanced Tumor Growth by Peptide Vaccine


Supplemental Figure 1. Blocking PD-1 and Fas signals does not abrogate enhanced tumor growth.
BALB/c mice were inoculated with $1 \times 10^6$ CT26-NY-ESO-1 and vaccinated with NY-ESO-1$_{81-88}$ peptide (100 μg) in prophylactic schedule. Some groups of mice received the administration with anti-PD-L1 mAb (MIH5, 200 μg) and/or anti-FasL mAb (MFL4, 200 μg) on day -1, 2, 6, 9, 13, 16. Tumor size was monitored three times a week. Each group consisted of 5 mice. These experiments were repeated two times with similar results. Data are mean ± SD.

Supplemental Figure 2. NY-ESO-1 peptide vaccination does not induce an anergic state in NY-ESO-1$_{81-88}$-specific CD8$^+$ T cells.
BALB/c mice were immunized with NY-ESO-1$_{81-88}$ peptide. 7 days later, some groups of mice received secondary immunization with NY-ESO-1$_{81-88}$ peptide or DNA vaccine. Cells were purified from dLN and spleens 7 days after the last vaccination and the number of NY-ESO-1$_{81-88}$-specific CD8$^+$ T cells was assessed as CD8$^+$NY-ESO-1/D$^d$ tetramer$^+$ T cells by flow cytometry. These experiments were repeated two times with similar results. Data are mean ± SD.
Supplementary Figure 1.
Supplementary Figure 2.