Baculovirus-Infected Insect Cells Expressing Peptide-MHC Complexes Elicit Protective Antitumor Immunity

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Evaluation of T cell responses to tumor- and pathogen-derived peptides in preclinical models is necessary to define the characteristics of efficacious peptide vaccines. We show in this study that vaccination with insect cells infected with baculoviruses expressing MHC class I linked to tumor peptide mimotopes results in expansion of functional peptide-specific CD8+ T cells that protect mice from tumor challenge. Specific peptide mimotopes selected from peptide-MHC libraries encoded by baculoviruses can be tested using this vaccine approach. Unlike other vaccine strategies, this vaccine has the following advantages: peptides that are difficult to solubilize can be easily characterized, bona fide peptides without synthesis artifacts are presented, and additional adjuvants are not required to generate peptide-specific responses. Priming of antitumor responses occurs within 3 days of vaccination and is optimal 1 wk after a second injection. After vaccination, the Ag-specific T cell response is similar in animals primed with either soluble or membrane-bound Ag, and CD11c+ dendritic cells increase expression of maturation markers and stimulate proliferation of specific T cells ex vivo. Thus, the mechanism of Ag presentation induced by this vaccine is consistent with cross-priming by dendritic cells. This straightforward approach will facilitate future analyses of T cells elicited by peptide mimotopes. The Journal of Immunology, 2008, 180: 188–197.

Identification of MHC class I- and MHC class II-binding epitopes have expedited the use of peptides in immunotherapy. Peptide vaccine strategies target T cells with fine specificity and, in combination with the appropriate adjuvants, generate immune responses to pathogens and cancers. Peptides in combination therapies may be key in the next generation of vaccines. Many studies have used insect cells infected with baculoviruses (BV) for production of proteins used in vaccines (1–3). Due to the large viral genome and strong promoters, BV vectors accommodate large gene inserts (> 1 kb) and produce high yields of mammalian proteins (4). Furthermore, posttranslational modifications, such as glycosylation and phosphorylation, in insect cells are similar to mammalian processes, allowing expression of proteins that biochemically resemble those of mammalian origins (5). For example, Spodoptera frugiperda (Sf9) and High Five insect cells infected with rBV produce soluble immunogenic viral proteins and viral-like particles from HIV and foot and mouth disease virus for use in vaccines (6, 7). Both serological (8) and cellular responses (2) are elicited by purified HIV proteins produced by BV-infected insect cells, which protect animals against subsequent viral challenge. Although vaccination with protein produced by BV-infected insect cells induces Ag-specific immune responses, this vaccination strategy requires protein purification and appropriate adjuvants.

Because the BV polyhedron promoter is not active (9–11) and BV cannot replicate in mammalian cells (12), injection of rBV may provide effective and safe means for delivery of vaccines. rBV expressing immunogenic proteins linked to the transmembrane domain of gp64 for viral surface expression elicit Ag-specific responses (13–15). However, rBV are inactivated by complement proteins in vivo (16) and may be damaged during purification processes, particularly by ultracentrifugation (17).

Injection of insect cells infected with rBV is an attractive method for vaccine delivery because it combines benefits from both the protein and viral vaccines. It has been shown that these vaccines elicit humoral immune responses to surface-expressed viral Ags (18). For example, vaccination with infected insect cells expressing foot and mouth disease virus Ag elicits seroneutralizing Abs, resulting in protection from viral challenge (7). The recombinant proteins are produced in culture, where complement proteins do not interfere with Ag production. In addition, the Ag can be quantified before injection, and preparation of infected insect cells for vaccination requires only low-speed centrifugation. Thus, we hypothesized that vaccination with insect cells infected with BV-encoding tumor-specific Ags would be a promising technique for priming specific CD8+ T cell responses.

Peptide-MHC complexes and peptide-MHC libraries used for the discovery of novel peptide Ags are successfully produced by insect cells infected with rBV (19–23). These peptide libraries are screened for binding to soluble TCR and activation of T cells in vitro before testing the peptides in vivo. Peptides produced in the BV peptide-MHC library are soluble and not easily oxidized, which permits screening of all amino acid residues, including cysteine and tryptophan. In theory, peptide epitopes

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3 Abbreviations used in this paper: BV, baculovirus; β-gal, β-galactosidase; βM, β2-microglobulin; CT, colorectal tumor; DC, dendritic cell; MFI, mean fluorescence intensity; Sf9, Spodoptera frugiperda; Tg, transgenic.

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or peptide mimotopes identified using this library system may regulate the T cell response and thus the disease progression in autoimmune, cancer, and infectious diseases (reviewed in Ref. 24).

We are using BV peptide-MHC libraries to identify novel cancer mimotopes, or mimics of tumor peptides, which stabilize the peptide-MHC/TCR complex and elicit T cells that cross-react with the tumor Ag (23). Like the peptide mimotopes we have identified (27), most mimotopes used in clinical trials of cancer vaccines have alterations in the MHC-anchor residues (reviewed in Ref. 25). We are characterizing mimotopes that improve antitumor immunity to the CT26 mouse colon carcinoma, specifically to the immunodominant tumor Ag AH1 (gp7023–431) (26), restricted by the MHC class I molecule H-2Ld. We previously showed that antitumor activity is improved by vaccinating with mimotope-liposome complexes (27) or mimotope-loaded dendritic cells (DCs) (28). Because some mimotopes are insoluble in water, sensitive to oxidation, and cannot be characterized using these methods, we developed a vaccine using infected insect cells expressing peptide-MHC molecules.

We demonstrate in this study that vaccination with insect cells infected with rBV-encoding peptide-MHC complexes generates peptide-specific cytotoxic T cell responses, and, when the appropriate peptide is used, protects mice from subsequent tumor challenge. Our results indicate that the infected insect cells activate APCs in vivo, which effectively present the expressed peptides to T cells. This vaccination strategy is advantageous for the following reasons: it ensures the bone fide peptide is presented, it does not require adjuvants in addition to those produced by BV and insect cells, it greatly reduces the cost of in vivo studies by eliminating the need to synthetically generate peptides, and it expedites the direct evaluation of peptides identified in BV peptide-MHC libraries.

Materials and Methods

Cells

S9 and High Five insect cells (22) (Invitrogen Life Technologies) and CT26 tumor cells (29) were cultured, as described. Splenocytes from vaccinated mice were expanded in vitro with AH1 peptide and IL-2, as described (27). DCs were prepared from collagenase-digested spleens or mesenteric lymph nodes for flow cytometric analyses or in vitro proliferation assays, as described (200 μg/ml collagenase D (Roche) and 40 μg/ml DNase-I (Sigma-Aldrich)) (30). Mesenteric lymph node cells were harvested 20 h later, and the number of CFSE-labeled cells in each peak was determined by flow cytometry. Mice were depleted with i.p. injections of Ab specific for CD8 (53.6.72; American Type Culture Collection) 3 days (500 μg) and 1 day (250 μg) before vaccination. Depletion was maintained with weekly injections of 250 μg of Ab and was confirmed by flow cytometry (<99.8% depletion before vaccination and >79% depletion before tumor challenge; data not shown).

Infection of S9 insect cells

A total of 3 × 10⁶ S9 insect cells was cultured in T175 flasks in complete Grace’s Insect medium (Invitrogen Life Technologies) containing 10% FCS (Atlanta Biologicals), 1% F-68 detergent (Invitrogen Life Technologies), and 1% antibiotic-antimycotic (Invitrogen Life Technologies).

Vaccination

Infected S9 insect cells were resuspended in HBSS, and 5 × 10⁶ cells were injected i.p. on days 0 and 7. The number of responding Ag-specific T cells is similar following i.v. and s.c. injection. Splenocytes or PBMCs were harvested for flow cytometric analyses on days 10, 14, and 17. Statistical analyses were performed with Prism version 4.0 (GraphPad), using unpaired two-tailed Student’s t test. A p value of <0.05 was considered statistically significant.

Immunoprecipitation of H-2Ld from whole cell lysates

Whole cell lysates were prepared by incubating infected S9 insect cells in lysis buffer and protease inhibitors, as previously described (34), at a concentration of 1 × 10⁶ cells/ml for 4 h at 4°C. H-2Ld was immunoprecipitated from whole cell lysates with the 28.14.8s Ab and protein A-Sepharose beads (35). Precipitated proteins were separated by SDS-PAGE (5–20% Tris–HCl; Bio-Rad) under reducing conditions using a standard protocol and stained with Coomassie blue.

In vivo killing assays

Mice were vaccinated with 39-LdTM-infected or uninfected insect cells on days 0 and 7. Thirty days after the last vaccination, target cells (BALB/c splenocytes) were incubated with either β-gal or AH1 peptide (10 μg/ml) for 2 h at room temperature. Cells were washed and labeled with 0.2 μM or 2 μM CFSE, respectively, and injected i.v. Splenocytes were harvested 20 h later, and the number of CFSE-labeled cells in each peak was determined (percentage of specific killing = 1 – percentage of survival; percentage of survival = (number of AH1 targets remaining)/(number of β-gal targets remaining)). Groups were compared using Prism version 4.0 (GraphPad), using unpaired two-tailed Student’s t test. A p value of <0.05 was considered statistically significant.
Insect cells infected with rBV express functional peptide-H-2Ld complexes that reflect their relative avidity. a. Insect cells infected with the indicated BV were stained with a mAb specific for H-2Ld (28.14.8s) and fluorescent CT-TCR and analyzed by flow cytometry. To control for the amount of BV infection, an MHC-Ab, but not with the CT-TCR, demonstrating specificity of the CT-TCR reagent (Fig. 1a). Importantly, as shown with other BV-encoded peptide-MHC complexes (22), the CT-TCR fluorescence intensity directly correlated with its affinity for the peptide-MHC molecules (Fig. 1b). Specifically, 15-LdTM stained most intensely with CT-TCR, followed by 39-LdTM and AH1-LdTM.

These results show that peptide-Ld complexes produced by insect cells are processed and folded to resemble those produced in mammalian cells. Furthermore, the binding properties of the covalently linked peptides directly correlate with the binding properties of soluble peptides, suggesting that insect cell-produced peptide-Ld complexes bind Ag-specific T cells. Finally, these results confirm the results of Crawford et al. (22), as follows: binding affinity of the peptide-MHC/TCR interaction can be readily analyzed using these BV constructions.

**BV-infected insect cells stimulate peptide-specific immune responses in vivo**

We hypothesized that the infected insect cells provide both an Ag-specific signal and adjuvant from the combination of BV and foreign insect cells. To determine whether infected insect cells induce antitumor responses in vivo, we analyzed AH1-specific CD8 T cells following injection of these cells. To produce this vaccine, we infected S9 insect cells for 3 days, harvested and washed the cells, then injected them i.p. Although the insect cells were washed, remaining free virus and dead insect cells were also included.

We previously showed that vaccination with the intermediate affinity mimotope 39 in liposomes elicits tumor-specific T cells and protects ~50% of mice from tumor growth (27). Although the high-affinity mimotope 15 elicits tumor-specific T cells, it does not protect mice from tumor growth. Thus, for simplicity we used insect cells infected with 39-LdTM throughout the following experiments. As shown in Fig. 2a, AH1-specific (tumor-specific), but not β-gal-specific, T cells were elicited after injection of insect
cells infected with BV-encoding mimotope 39, as determined by tetramer staining. AH1-specific T cells were not elicited with the β-gal peptide vaccine, the negative control.

We next determined the optimal number of insect cells needed to prime the T cell response and calculated the corresponding amount of peptide-Ld\textsuperscript{a} produced in infected insect cells by ELISA. Mice were injected with increasing numbers of 39-Ld\textsuperscript{a}TM-infected Sf9 insect cells on days 0 and 7. Splenocytes were harvested on day 14 and cultured for 1 wk with AH1 peptide and IL-2. Quadrants were set using β-gal-Ld\textsuperscript{a} tet-stained cells (left); the percentages of CD8\textsuperscript{+} AH1-Ld\textsuperscript{a} tet\textsuperscript{+} cells are indicated. b, Mice were vaccinated with 5 × 10\textsuperscript{6} 39-Ld\textsuperscript{a}TM-infected insect cells, and the frequency of CD8\textsuperscript{+} AH1-Ld\textsuperscript{a} tet\textsuperscript{+} cells in the blood was determined by flow cytometry on the indicated days (x-axis). The bars indicate the average percentage of CD8\textsuperscript{+} AH1-Ld\textsuperscript{a} tet\textsuperscript{+} T cells minus background β-gal-Ld\textsuperscript{a} tet staining (average percentages ± SD were 1.7 ± 0.9, 4.2 ± 0.7, 1.4 ± 0.4 for days 10, 14, and 17, respectively; *, p = 0.0178, using unpaired two-tailed Student’s t test). c, Splenocytes were labeled with CFSE and incubated with either the β-gal peptide (left peak) or AH1 peptide (right peak), and equal numbers of cells were transferred into mice vaccinated with the indicated insect cells. Splenocytes were harvested 20 h later, and the number of CFSE\textsuperscript{+} cells in each peak as indicated was determined by flow cytometry. d, The percentage of specific killing was determined as in c for multiple mice. The bars indicate the average percentage of specific killing (39-Ld\textsuperscript{a}TM, 45.9 ± 7.9; n = 8; uninfected, 0.4 ± 1.6, n = 6; **, p = 0.0004 using unpaired two-tailed Student’s t test).

We next determined whether vaccine-elicited T cells were functional in an in vivo killing assay (Fig. 2, c and d). Splenocytes from BALB/c mice were incubated with AH1 or β-gal peptides and labeled with a high or low concentration of CFSE, respectively. These labeled splenocytes were transferred into vaccinated BALB/c mice 30 days following the second injection of 39-Ld\textsuperscript{a}TM-infected insect cells. AH1 peptide-loaded target cells were specifically eliminated in mice vaccinated with 39-Ld\textsuperscript{a}TM-infected insect cells, but not with uninfected insect cells (Fig. 2d). The number of β-gal-loaded targets remained similar in both samples. These results indicate that 39-Ld\textsuperscript{a}TM-infected insect cells elicit effector T cells that specifically kill Ag-loaded target cells in vivo. Thus, the same viral constructions can be used for in vivo and in vitro analyses.

### Persistence of peptide-Ld\textsuperscript{a} TM Ag in vivo

To analyze T cell responses to this vaccine and ultimately to design mimotopes to tumor-associated Ags, we derived Tg mice that express the TCR from the CT-T cell clone (28), a clone that recognizes the AH1 peptide restricted by H-2L\textsuperscript{d}. Like many other T cells that recognize tumors, this T cell clone recognizes a self Ag, and therefore is subject to negative selection in the thymus and peripheral tolerance after leaving the thymus. We developed this new Tg mouse model, rather than using an established Tg strain, such as the OT-1 mice, to better mimic T cell tolerance encountered by tumor vaccines. We backcrossed the transgenes onto BALB/c mice for 12 generations and then crossed the transgenes onto RAG2-deficient mice. Approximately 90% of the T cells in the thymus of these Tg mice are coreceptor negative (data not shown), indicating the following: 1) strong negative selection of the T cells during development; 2) a developmental block in the T cells at the double-negative stage, because the CT-TCR is specific for a self peptide derived from an endogenous retroviral gene product, gp70; and/or 3) the Ig enhancer used to drive gene expression is suboptimal (31).
As in other models of self tolerance, some T cells escape negative selection and are found in the periphery (37). Some of the peripheral Vβ8.3+/H9253 T cells from the Tg mice express CD8 molecules, and these T cells are functional, as determined by tetramer binding and other assays (Fig. 3). The coreceptor-negative cells bind a Vβ8.3 Ab (Fig. 3b), but they do not all bind to AH1-Ld tet (Fig. 3a), suggesting that T cells lacking coreceptor may require a higher affinity peptide to form a complex. Consistent with this possibility, more coreceptor-negative T cells bind 39-Ld TM-infected (black lines) or uninfected (dashed lines) insect cells 7, 3, or 1 days before transfer of 1 × 10^7 CFSE-labeled CT-TCR Tg RAG splenocytes. Proliferation of Tg T cells was compared on the indicated days using unpaired two-tailed Student’s t test (day −1, p = 0.0014; day −3, p = 0.0106; day −7, p = 0.0884). The percentages shown are the average proliferation in two mice vaccinated with 39-Ld TM-infected insect cells minus the background proliferation observed in mice vaccinated with uninfected insect cells.

FIGURE 3. Peptide 39 is presented to transferred TCR Tg T cells for at least 3 days after vaccination. a, T cells from the CT-TCR Tg mice on a BALB/c RAG knockout genetic background were characterized. Splenocytes from CT-TCR Tg RAG mice were gated on MHC class II− cells and stained with a CD8 Ab and AH1-Ld tet or β-gal-Ld tet. b, Splenocytes from a CT-TCR Tg mouse were gated on CD3+ MHC class II− cells, and the staining with Abs to CD8, CD4, and Vβ8.3 is shown. c, T cells from the CT-TCR Tg RAG mice produce IFN-γ when stimulated with the AH1 peptide. Tg mice were injected twice (days 0 and 7) with irradiated CT26 tumor cells. Splenocytes were stimulated ex vivo (day 14) with AH1 or β-gal peptides and stained with Abs specific for Vβ8.3, CD8, and IFN-γ. Data shown are gated on Vβ8.3+ splenocytes; percentages indicate the percentages of Vβ8.3+/CD8+ IFN-γ+ cells. d, T cells from the CT-TCR Tg RAG mice proliferate in response to the AH1 peptide and peptide 39. CFSE-labeled splenocytes from CT-TCR Tg mice were incubated with increasing concentrations of synthesized peptide in vitro. Percentage of proliferation was determined by CFSE dilution of CD8+ T cells. e, BALB/c mice were injected with 5 × 10^6 39-Ld TM-infected (black lines) or uninfected (dashed lines) insect cells 7, 3, or 1 days before transfer of 1 × 10^7 CFSE-labeled CT-TCR Tg RAG splenocytes. Proliferation of Tg T cells was determined by flow cytometric analysis (Fig. 3e). Vaccination with 39-Ld TM-infected insect cells 1 day before transfer induced proliferation of a significant number of Tg T cells (37%). Proliferation in mice vaccinated 3 days before transfer was reduced (16%), although it was significantly higher than in mice vaccinated with uninfected insect cells. Vaccination 7 days before transfer did not induce proliferation of Tg T cells, indicating that most of the Ag is cleared between 3 and 7 days after vaccination. The persistence of infected insect cells is similar to vaccination with Ag and IFA, which are cleared 6–8 days following injection (39).

Infected insect cells do not directly stimulate T cells in vivo

Although rBV that express both peptides and MHC molecules is convenient and effective for both in vitro and in vivo analyses, we wanted to determine whether Ag is presented directly by insect cells, cross-presented by APCs, or presented using a novel mechanism. To determine whether insect cells directly
molecules may be detectable in whole cell lysates (Fig. 4).

As expected, both 39-Ld and 39-Ld2m are not detectable by immunoprecipitation or ELISA because infected insect cells expressing membrane-bound peptide Ld (39-LdTM) with nonmembrane-bound peptide 39 (39-LdBirA and 39-Ld2m) were not detectable by immunoprecipitation with the indicated BV encoding 39-LdTM, 39-LdBirA, or 39-β2m (no H-2Ld), respectively. Insect cells were infected with the indicated BV and stained with the 28.14.8s Ab for H-2Ld surface expression. The long dashed line represents H-2Ld staining of uninfected insect cells. Splenocytes from mice vaccinated with insect cells infected with the indicated BV on days 0 and 7 were harvested on day 14 and analyzed by flow cytometry for CD8+ AH1-Ld tet+ T cells. Quadrants were set using β-gal-Ld tet-stained cells (left), and the percentages shown are the average CD8+ AH1-Ld tet+ cells from two mice minus the background β-gal-Ld tet staining (right three panels).

**FIGURE 4.** Infected insect cells do not directly present peptide-H-2Ld to T cells in vivo. a, H-2Ld was immunoprecipitated from whole cell lysates of insect cells expressing soluble or membrane-bound 39-H-2Ld1, separated by SDS-PAGE under reducing conditions, and stained with Coomassie blue. Lanes 1 and 2, Controls of purified monomeric LdBirA and 28.14.8s Ab, respectively. Lanes 3, 6, and 8, Whole cell lysates; lanes 4, 5, and 7, immunoprecipitated lysates from insect cells infected with the indicated BV encoding 39-LdTM, 39-LdBirA, or 39-β2m (no H-2Ld), respectively. b, Insect cells were infected with the indicated BV and stained with the 28.14.8s Ab for H-2Ld surface expression. The long dashed line represents H-2Ld staining of uninfected insect cells. c, Splenocytes from mice vaccinated with insect cells infected with the indicated BV on days 0 and 7 were harvested on day 14 and analyzed by flow cytometry for CD8+ AH1-Ld tet+ T cells. Quadrants were set using β-gal-Ld tet-stained cells (left), and the percentages shown are the average CD8+ AH1-Ld tet+ cells from two mice minus the background β-gal-Ld tet staining (right three panels).

present peptide-Ld to T cells in vivo, we compared vaccination with insect cells expressing membrane-bound peptide-Ld (39-LdTM) with nonmembrane-bound peptide 39 (39-LdBirA and peptide-β2m). The BV-encoding 39-LdBirA is identical with 39-LdTM, but encodes a BirA peptide tag rather than the transmembrane domain of gp64. The sequence encoding H-2Ld was removed from these BV to produce 39-β2m. Insect cells infected with membrane- and nonmembrane-bound peptide 39 produce a similar amount of protein, as detected by immunoprecipitation (Fig. 4a) and ELISA (data not shown) of whole insect cell lysates 3 days after infection. Although 39-β2m molecules may be detectable in whole cell lysates (Fig. 4a), they are not detectable by immunoprecipitation or ELISA because these assays are specific for H-2Ld. As expected, both 39-LdBirA and 39-β2m are not detectable on the surface of infected insect cells with the H-2Ld Ab (Fig. 4b), confirming that these infected insect cells do not present MHC-restricted Ags.

If insect cells present Ags directly to T cells, we would expect Ag-specific responses to the insect cells expressing the membrane-bound Ag and not to the insect cells expressing nonmembrane-bound Ag. If Ags are cross-presented by APCs, then we would expect little difference between the specific T cell response to insect cells expressing membrane- and nonmembrane-bound Ag. We vaccinated mice with insect cells infected with BV-expressing 39-LdTM, 39-LdBirA, or 39-β2m, and analyzed the frequency of CD8+ AH1-Ld tet+ T cells in the spleen. Insect cells infected with membrane- or nonmembrane-bound peptide induced a similar number of AH1-specific T cells (Fig. 4c). In addition, surface expression on insect cells infected with 39-LdTM that also express costimulatory molecules ICAM-1 and B7.1, facilitators of direct Ag presentation and T cell priming, did not increase the number of responding AH1-specific T cells (data not shown). These results suggest that infected insect cells do not present Ag directly to T cells, but are processed by APCs, which present the peptides in the context of H-2Ld to CD8+ T cells.

**Vaccination with infected insect cells induces maturation of DC subsets**

To determine whether the insect cell vaccine activates APCs as expected, we examined surface markers on DCs after vaccination. Mice were vaccinated with 39-LdTM-infected insect cells, and DCs from the draining mesenteric lymph nodes were characterized over time. DCs were stained with Abs against the DC subset markers CD11c, CD8, and CD11b, and the maturation markers MHC class II, CD80, CD86, and CD70. We observed an increase in the expression of MHC class II and the costimulatory molecules CD80 and CD86 in both DC populations (Fig. 5a). Expression of CD70, a molecule expressed by activated DCs that was recently shown to be necessary for optimal T cell stimulation (40), also increased (Fig. 5a). Similar results were obtained for DC populations in the spleen (data not shown). Although the vaccine stimulated both CD8+ CD11c+ and CD11b+ CD11c+ cells, up-regulation of the costimulatory molecules CD80 and CD86 was more pronounced in the CD8+ CD11c+ subset, suggesting that these cells respond more vigorously to the vaccine. The infected insect cell vaccine induces maturation of DCs, particularly the CD11c+CD8+ DCs, consistent with a function in cross-presentation (41).

**DCs cross-present insect cell-derived peptides**

To confirm that Ags from infected insect cell vaccines can be cross-presented by DCs, we determined whether DCs isolated from mice vaccinated with 39-LdTM-infected insect cells induce proliferation of CT-TCR Tg T cells ex vivo. Mice were vaccinated with 39-LdTM- or β-gal-LdTM-infected insect cells, and spleens were harvested 24 h later. Purified CD11c+ splenocytes from vaccinated mice (average 88% CD11c+ MHC class II+) were incubated with CFSE-labeled Tg T cells for 3 days ex vivo. Like Tg T cells incubated with DCs exogenously loaded with peptide 39, Tg T cells proliferated when incubated with CD11c+ cells from mice vaccinated with 39-LdTM-infected insect cells (Fig. 5b, black lines).
Fected insect cells (Fig. 5b). We previously showed that vaccination with peptide 39 protects BV-infected insect cells induce protective antitumor immunity

We next determined the therapeutic efficacy of vaccination with infected insect cells. We injected mice with 5 × 10^6 peptide-LdTM-infected insect cells 14 and 7 days before s.c. injection of 5 × 10^6 CT26 tumor cells. Mice were measured every 2 days. Mice were sacrificed when the tumor reached 100 mm². Tumors were measured every 2 days, and tumor sizes were compared from each group on individual days with unpaired two-tailed Student’s t test (*, p ≤ 0.0001 for unvaccinated vs 39-LdTM and p ≤ 0.0044 for β-gal-LdTM vs 39-LdTM).

In vivo assessment of a water-insoluble mimotope with high affinity in the peptide-MHC/TCR interaction identified in a BV peptide library

Finally, we determined whether peptides identified in the BV peptide library could be analyzed in vivo using this method. The synthetic peptide designated WMF (SPTYAYWMF) (23), a mimotope of the AH1 Ag, was identified in a BV peptide library with substitutions in the MHC-contact residues. This peptide is insoluble in water and is difficult to synthesize, indicated by the heterogeneity of HPLC and mass spectrometry profiles (data not shown). However, the WMF peptide produced in infected insect cells binds to CT-TCR with high affinity relative to peptide 39 (Fig. 7a) and stimulates a corresponding amount of proliferation of the CT-T cell clone (Fig. 7b). These experiments demonstrate that the WMF peptide-H-2Ld complex is produced in infected cells and specifically binds to both soluble CT-TCR molecules and T cells expressing CT-TCR.
FIGURE 7. The BV-infected insect cell vaccine can be used to evaluate peptides identified from the BV peptide library. a, Insect cells infected with the indicated BV were analyzed as in Fig. 1a. The MFI of the CT-TCR at a specific MHC expression level were 150, 98.8, 22.7, and 2.2 for WMF-L<sup>L</sup>dTM, 39-L<sup>L</sup>dTM, AH1-L<sup>L</sup>dTM, and β-gal-L<sup>L</sup>dTM, respectively. These data are representative of three independent experiments. b, T cells expressing the CT-TCR were incubated with infected insect cells expressing ICAM and B7 costimulatory molecules and AH1-L<sup>L</sup>dTM, 39-L<sup>L</sup>dTM, β-gal-L<sup>L</sup>dTM, or WMF-L<sup>L</sup>dTM in vitro at a 5:1 ratio. Proliferation was determined by incorporation of [3H]thymidine and is shown as mean cpm ± SD, n = 3. c, Mice were injected with the indicated vaccines, as in Fig. 2b, and the frequency of CD8<sup>+</sup> AH1-L<sup>L</sup>d tet<sup>+</sup> splenocytes was determined by flow cytometry 14 days after the initial injection. The data shown are representative plots, and the numbers indicate the average percentage of CD8<sup>+</sup> AH1-L<sup>L</sup>d tet<sup>+</sup> T cells minus background β-gal-L<sup>L</sup>dTM tetramer staining (39-L<sup>L</sup>dTM, n = 2; WMF-L<sup>L</sup>dTM, n = 4; unvaccinated, n = 2). d, Tumor protection assays were performed as in Fig. 6a. Groups were compared using the log rank test (*, p < 0.0044 comparing 39-L<sup>L</sup>dTM with β-gal-L<sup>L</sup>dTM).

We next vaccinated mice with insect cells infected with BV-encoding WMF-L<sup>L</sup>dTM to analyze the tumor-specific T cell responses and to determine whether antitumor activity is afforded by this high-affinity mimotope. Vaccination with 39-L<sup>L</sup>dTM- and WMF-L<sup>L</sup>dTM-infected insect cells elicited tumor Ag-specific T cells, as determined by tetramer staining (Fig. 7c). Although the affinity of the WMF peptide in the peptide-MHC/TCR interaction is higher than the intermediate-affinity 39 peptide, fewer AH1-specific T cells were detected in the blood after vaccination with WMF-L<sup>L</sup>dTM-infected insect cells. In addition, vaccination with 39-L<sup>L</sup>dTM-infected insect cells protected the majority of mice from subsequent CT26 tumor challenge, whereas vaccination with β-gal-L<sup>L</sup>dTM-, AH1-L<sup>L</sup>dTM-, or WMF-L<sup>L</sup>dTM-infected insect cells failed to protect (Fig. 7d). These results are consistent with those in Fig. 6 and our previous results showing that peptides that form an intermediate-affinity peptide-MHC/TCR complex with substitutions in the MHC-contact residues, such as the 39 peptide, protect mice from tumor challenge (27), whereas those that form a low (AH1)- or high (peptide 15 or WMF)-affinity peptide-MHC/TCR complex do not.

Discussion

We have shown that vaccination with BV-infected insect cells expressing peptide-MHC complexes generates functional Ag-specific CD8<sup>+</sup> T cell responses. This vaccine, which protects against tumor challenge and delays growth of tumors, is convenient in that it does not require additional adjuvants to those provided by the BV and insect cells; it eliminates the need, expense, and potential artifacts of synthesizing peptides identified in the BV peptide library before evaluation; and it does not require purification of proteins or BV before injection. Vaccination with infected insect cells expressing other recombinant proteins also results in specific immune cell responses. For example, we have used this vaccination strategy to develop TCR-specific Abs (data not shown). Like with the peptide-MHC vaccine, vaccination with infected insect cells expressing recombinant proteins eliminates the need for protein purification.

This vaccine strategy is unique and cannot be practically achieved using other strategies because it provides a method to analyze peptides identified in BV peptide-MHC libraries that are otherwise technically difficult to evaluate, such as the WMF peptide (Fig. 7). Amino acids such as cysteine, methionine, and tryptophan are often avoided in peptide libraries due to disulfide bonding, insolubility, and sensitivity to oxidation (27, 42). Small molecular changes in amino acid residues of the peptide, such as oxidation or alkyl-chain modifications, can alter epitopes and thus elicit different T cell responses (43, 44). Furthermore, vaccination with synthetic peptides does not always stimulate T cells that recognize endogenously processed peptides, possibly due to oxidation or cysteinylation of amino acid residues during peptide synthesis or handling (45–47). Although oxidation does not affect the T cell response to all Ags, in the BV-infected insect cell vaccine strategy, the peptide is produced, processed, and cross-presented in a reduced intracellular environment similar to natural tumor Ags.

In vitro characterization of peptides derived from libraries requires coexpression of MHC molecules and peptides. Infect cells infected with RV-encoding peptide, H-2L<sup>d</sup>, and β<sub>2m</sub> molecules bind H-2L<sup>d</sup>-specific Abs and the AH1-specific CT-TCR, suggesting that the protein structure is similar to that of mammalian cells. Furthermore, the avidity of the peptide-MHC/TCR complexes correlates with the affinity of the soluble mimotope-MHC/TCR complexes (Fig. 1) (27). In addition to binding assays for characterization of peptides, other in vitro studies using infected insect cells examine T cell function, such as cytokine production (21, 22). In vivo, insect cells expressing peptide 39 restricted by H-2L<sup>d</sup> elicit a population of T cells that binds Ag-loaded tetramer (Fig. 2, a and b), kills Ag-loaded target cells (Fig. 2, c and d), and protects 67% of mice from subsequent tumor challenge (Fig. 6a). These proof-of-concept experiments indicate that the T cells elicited by infected insect cells recognize native peptide on tumor cells.
To analyze the mechanism of priming by this vaccine, we developed a new Tg mouse that expresses the α- and β-chains of a TCR specific for the AH1/H-2Ld Ag. This TCR was derived from a BALB/c mouse vaccinated with irradiated CT26 tumor cells expressing GM-CSF (28), i.e., a T cell clone that had escaped negative selection in the thymus. Because T cells from this mouse recognize a tumor/self Ag, we are including them in our analyses to determine the requirements of peptide vaccines that break tolerance. Not all tumor-specific T cells generated in this mouse express CD8 molecules, suggesting that down-regulation of the CD8 molecule is a consequence of tolerance, as reported by others (48, 49). Alternatively, aberrant expression of the TCR during T cell development may disrupt the expression of the CD8 molecule. The Ag-specific response of the CD3+CD8− T cells is less robust relative to the CD3+CD8+ cells, suggesting that the coreceptor contributes to the binding avidity of the TCR complex. Consistent with this possibility, more coreceptor-negative T cells bind 39-4L tet than AH1-L1 tet (data not shown).

Although the BV constructions require the MHC molecules for peptide studies in vitro, it is not required to elicit specific T cells in vivo. Like other effective antitumor CD8+ T cell responses (41, 50), the results we show in this study are consistent with tumor-specific T cells elicited by cross priming. 1) Direct recognition of Ag on the surface of infected insect cells is not required to elicit T cells, suggesting that the coreceptor contributes to the binding avidity of the TCR complex. Consistent with this possibility, more coreceptor-negative T cells bind 39-4L tet than AH1-L1 tet (data not shown).
