Dendritic Cells Transduced with Protein Antigens Induce Cytotoxic Lymphocytes and Elicit Antitumor Immunity[1]

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Dendritic cell (DC)-based vaccines are being developed for treatment of patients with cancer, in part because DC are potent inducers of $CD8^+$ CTL. DC MHC class I:antigenic peptide complexes that are required for CTL elicitation are most often generated by incubating DC with peptides or by transfecting (or transducing) DC with cDNAs (or viral vectors) that encode protein Ags. The former approach is feasible when MHC class I Ags and relevant peptides are known. The latter approach may be hampered by inefficient DC transfection (transduction) and/or difficulties associated with preparation and use of viral vectors. Herein we demonstrate that a bacterial recombinant model tumor-associated Ag (OVA) that contains the HIV TAT protein transduction domain (PTD) was readily engineered and purified, efficiently transduced murine lymphocytes and DC, and was processed by proteasomes for MHC class I-restricted presentation to CTL. In addition, PTD-containing rOVA was processed and presented by DC to CD4 T cells as efficiently as native OVA or rOVA lacking the PTD. PTD-OVA-transduced DC induced CTL in vivo in a Th cell-independent fashion and vaccinated against OVA-expressing tumors. In contrast, rOVA lacking the PTD did not enter the DC MHC class I presentation pathway and DC treated with this protein did not prime OVA-specific CTL in vivo. Treatment of mice harboring clinically apparent OVA-expressing tumors with PTD-OVA-transduced DC resulted in tumor regression in some animals. This straightforward vaccination strategy may translate into DC-based treatments for patients with cancer and other serious diseases. *The Journal of Immunology,* **2002, 168: 2393–2401.**

Dendritic cells (DC)[3] are accessory cells that bridge innate and adaptive immunity (1). Positioned at interfaces between organism and environment and in lymphoid tissues, DC are uniquely suited to recognize and acquire pathogens, process macromolecular Ags into peptides, and initiate immune responses in naive T cells, including $CD8^+$ cytotoxic lymphocytes (CTL). There is considerable interest in harnessing the potent Ag-presenting power of DC for patient benefit, and DC-based vaccine trials are ongoing. Although results from early experiences in cancer patients were encouraging (2, 3), DC vaccines are not routinely efficacious (4). Optimization of DC vaccine protocols in normal volunteers (5, 6) will likely enhance the chance of success of future clinical trials involving patients.

DC readily ingest and process exogenous Ags for presentation to $CD4^+$ Th cells in association with MHC class II Ags (1). In contrast, CTL epitopes preferentially derive from proteins that are synthesized endogenously, degraded by proteasomes, and presented as complexes with MHC class I. Several strategies have been used to load tumor Ag epitopes onto DC MHC class I molecules. If class I alleles and tumor Ag-derived class I binding peptides are known, DC class I loading can be achieved by coincubating DC and peptides (4). In this instance, CTL responses generated are restricted to the peptides used for immunization and Th cell priming is not an expected result. To generate CTL responses with as much antigenic complexity as possible, accessory cells expressing tumor Ags have been generated by fusing DC directly with tumor cells (7). Considerable immunogenic complexity can theoretically also result if DC are incubated with tumor-derived proteins or RNA (8, 9).

Expression of defined tumor Ags in DC can be achieved by transfection with cDNAs or infection with viruses encoding tumor Ags (10). However, a variety of practical and theoretical concerns may limit the utility of these vectors in patients.

We have explored protein Ag transduction of DC as an alternative to genetic modification to elicit CTL that can effect tumor rejection in vivo. We used bacterial recombinant proteins containing the 11-aa HIV TAT protein transduction domain (PTD; YGRKKRRQRRR), whose recent use has been pioneered by Dowdy et al. (11). TAT PTD-containing proteins indiscriminately translocate across cell membranes in vivo and in vitro (12, 13). Based on these and earlier (14) studies, we predicted that DC transduced with TAT PTD-containing recombinant Ags would express epitopes derived from these proteins, and that protein Ag-transduced DC would elicit CTL that could eradicate tumors. We chose to use chicken OVA as a model tumor-associated Ag for several reasons. First, the immunogenicity of OVA in $H\text{-}2^b$ mice is well characterized and an immunodominant MHC class I binding peptide that elicits $H\text{-}2K^b$-restricted CTL has been identified (SIINFEKL) (15). Second, a mAb that binds selectively to $H\text{-}2K^b$-SIINFEKL complexes has been described (25.D1.16), allowing quantitation of relevant CTL epitopes on the surfaces of accessory cells (16). Finally, the ability of OVA-specific CTL to reject tumor cells that express OVA (OVA cDNA-transfected EL-4 (E.G7) cells) can be readily assessed (17).

Materials and Methods

Generation and characterization of recombinant protein Ags

His_6-tagged TAT-hemagglutinin (HA)-OVA constructs were generated by inserting full-length OVA cDNA (18) into pTAT-HA plasmids (19). HA-OVA constructs were generated by *Bam*HI digestion and religation of

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[3] Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived DC; PTD, protein transduction domain; HA, epitope from influenza hemagglutinin; β-Gal, β-galactosidase.

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pTAT-HA-OVA plasmids. High expressing BL21 (DE3) (Novagen, Madison, WI) transformants were selected after blotting lysates of transformants with anti-HA mAb (Covance, Richmond, CA). pTAT-HA-β-galactosidase (β-Gal)-transformed bacteria were provided by S. Dowdy (14). Denatured TAT-HA-OVA, HA-OVA, and TAT-HA-β-Gal were purified by sequential Ni^{2+} NTA-agarose chromatography, fast protein liquid ion exchange chromatography, and gel filtration chromatography as described (19). Proteins were stored at -70°C in PBS/10% glycerol and thawed immediately before use. SDS-PAGE was performed with NuPAGE 4–12% Bis-Tris gels and MOPS running buffer (NOVEX, San Diego, CA). Endotoxin contamination of recombinant proteins was determined via *Limulus* lysate assay (BioWhittaker, Walkersville, MD). Recombinant proteins were treated with polymyxin B sulfate (50 μg/ml; Sigma-Aldrich, St. Louis, MO) before addition to DC.

Mice

Six- to 8-wk-old female C57BL/6 mice were purchased from the National Cancer Institute Animal Production Program and housed and used in accordance with institutional guidelines. C57BL/6 MHC class $II^{-/-}$ mice and controls were purchased from Taconic Farms (Germantown, NY).

Tumor cells and DCs

EL-4 cells were purchased from American Type Culture Collection (Manassas, VA) and OVA cDNA stable transfectants (EG.7 cells) (17) were provided by M. Bevan (University of Washington, Seattle, WA). Cell lines were cultivated in RPMI 1640 containing 10% FBS (Gemini Bio-Products, Woodland, CA), glutamine, and penicillin-streptomycin (Life Technologies, Gaithersburg, MD). EG.7 medium also contained G418 (400 μg/ml; Life Technologies). DC were obtained by culturing C57BL/6 bone marrow cells in RPMI 1640 containing 5% FBS, glutamine, penicillin/streptomycin, murine rGM-CSF, and rIL-4 (10 ng/ml each; PeproTech, Rocky Hill, NJ) for 6 days. After enrichment on 14.5% metrizamide gradients and an overnight incubation, nonadherent and loosely adherent cells were harvested and used in experiments (20).

Confocal laser microscopy

FITC-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and FITC-OVA was from Molecular Probes (Eugene, OR). After incubation with proteins (333 nM for 18 h), cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Nonidet P-40, blocked with PBS/1% BSA/0.1% skim milk, and stained with 5 μg/ml anti-HA mAb and secondary Ab. Stained cells were analyzed using a Personal Confocal Laser microscope (Nikon, Tokyo, Japan).

Flow cytometry

25.D1.16 (mouse IgG1) reactive with SIINFEKL:H-$2K^b$ complexes (16) was obtained from R. Germain (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Anti-CD16/CD32 (2.4G2), PE-conjugated goat anti-mouse Ig, FITC-conjugated anti-H-$2K^b$ (AF6-88.5), FITC-conjugated anti-I-A^b (AF6-120.1), PE-conjugated anti-I-A^b (M5/114.15.2), FITC-conjugated anti-CD11c (HL3), FITC-conjugated anti-CD86 (GL1), and FITC-conjugated anti-CD54 (ICAM-1) mAb were purchased from BD PharMingen (San Diego, CA). Cells were stained for surface Ag expression and analyzed using a FACScan flow cytometer equipped with CellQuest software (BD Biosciences, Mountain View, CA). DC FcR were blocked with anti-CD16/CD32 before staining.

Reagents

NH2-SIINFEKL-COOH peptide was purchased from Multiple Peptide Systems (San Diego, CA). Murine rIFN-γ was from Genzyme (Cambridge, MA) and anti-CD40 mAb (clone HM40-3) was purchased from BD PharMingen. MG132 was provided by A. Weissman (National Cancer Institute).

$CD4^+$ T cell proliferation assay

Primed $CD4^+$ T cells were elicited in naive mice by injecting right foot pads with 50 μg of native OVA (Sigma-Aldrich) in 30 μl of Titermax (Sigma-Aldrich) (21, 22) on day 0. On day 10, popliteal lymph node cells were harvested and $CD4^+$ T cells were isolated (>90% purity) using mouse CD4 cell enrichment columns (R&D Systems, Minneapolis, MN). $CD4^+$ T cells were subsequently cocultured in 96-well flat-bottom microtiter plates (3×10^5/well) with varying numbers of bone marrow-derived DC (BMDC) that had been pretreated with SIINFEKL peptide (10 μg/ml for 1 h), native OVA, or recombinant proteins (333 nM for 18 h each). [^{3}H]methylthymidine (Amersham Pharmacia Biotech, Piscataway, NJ) was added for the final 16 h of a 72-h incubation period and cell-associated radioactivity was measured by direct beta counting (Packard Instrument, Downers Grove, IL).

Immunization schedules

To generate SIINFEKL-specific CTL, mice were injected in the right foot pads on day 0 with 20 μg of SIINFEKL peptide in 30 μl of Titermax. On day 7, splenocytes and lymph node cells were harvested and restimulated with DC treated with SIINFEKL peptide (10 μg/ml for 1 h). In other experiments, mice were immunized (day 0) s.c. in the right flank with 5×10^5 DC that had been treated with peptide (10 μg/ml for 1 h) or recombinant proteins (333 nM for 18 h). On day 7, splenocytes and lymph node cells were harvested and restimulated with SIINFEKL-treated EL-4 or mitomycin C-pretreated (50 μg/ml for 45 min) EG.7 cells for 5 days. CTL activity was assessed on day 12.

Quantification of CTL

Calcein release assays were performed as previously described (23). Nonadherent spleen and lymph node cells were harvested from in vitro restimulation cultures and used as effector cells. EG.7 or EL-4 target cells were labeled with calcein (Molecular Probes), washed, and added to round-bottom microtiter plates with various numbers of effector cells. Plates were incubated for 3 h, supernatants were recovered, and calcein release was measured using a CytoFluor 2350 plate reader (Millipore, Bedford, MA.). Specific lysis = ((experimental − spontaneous)/(maximal − spontaneous)) × 100. Maximal lysis was achieved with 0.1% Triton X-100.

Tumor susceptibility and treatment studies

In tumor protection experiments, mice were vaccinated s.c. in the right flanks on day -7 (one time), or on days -14 and -7 (two times), with 5×10^5 DC that had been treated with peptide (10 μg/ml for 1 h) or recombinant proteins (333 nM for 18 h). On day 0, mice were challenged in shaved left flanks with 1×10^6 EG.7 cells. Tumor sizes were determined biweekly in a blinded fashion. Tumor index (in millimeters) = square root (length × width). To assess the ability of this vaccination strategy to elicit immune responses that could eradicate preexisting tumors, EG.7 cells (1×10^6) were injected into the subcutis of naive mice on day 0 followed by two weekly injections of DC into the opposite flanks on days 5, 6, or 7 (in different experiments). Tumor indices varied between 7 and 10 mm at the time of initial DC administration. In selected individual mice, EG.7-reactive CTL activity was quantified after in vitro restimulation of RBC-depleted spleen cells with mitomycin C-treated EG.7 cells (see above).

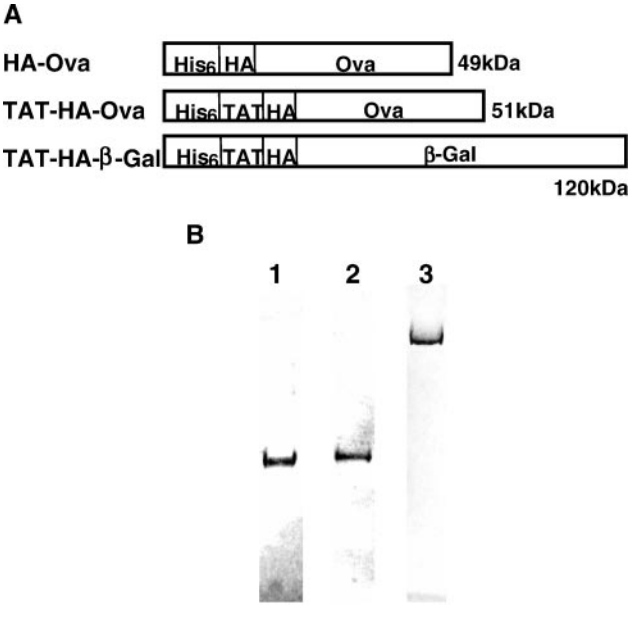

FIGURE 1. HIV TAT PTD-containing protein Ags. *A*, Schematic representation of bacterial recombinant proteins. *B*, Coomassie blue-stained 4–12% SDS-PAGE gels in which Ni-affinity- and ion exchange chromatography-purified HA-OVA (*lane 1*), TAT-HA-OVA (*lane 2*), and TAT-HA-β-Gal (*lane 3*) were resolved.

Results

Production and characterization of rHIV TAT peptide-containing protein Ags

Plasmids encoding TAT PTD-containing and control proteins (Fig. 1*A*) were engineered and recombinant proteins were purified from lysates of transformants by sequential Ni-NTA agarose chromatography, fast protein liquid ion exchange chromatography, and gel filtration chromatography (19). Characterization of purified proteins by SDS-PAGE revealed predominantly single species with appropriate sizes (Fig. 1*B*).

Generation of MHC class I:peptide complexes following protein Ag transduction of murine thymoma cells

Translocation of rOVA into cells was verified after incubation of EL-4 thymoma cells with HA-OVA or TAT-HA-OVA, fixation, permeabilization, and staining with anti-HA mAb and FITC-conjugated goat anti-mouse IgG. Anti-HA immunoreactivity was easily detected in cells exposed to TAT-HA-OVA while cells incubated with HA-OVA were not labeled (Fig. 2*A*).

Processing of TAT-HA-OVA and expression of OVA-derived epitopes on cell surfaces was assessed by staining EL-4 cells with a mAb (25.D1.16) that binds to H-2K^b:OVA SIINFEKL peptide complexes (16) and PE-conjugated goat anti-mouse mAb, and quantifying fluorescence intensities via flow cytometry (Fig. 2*B*). Although complexes were expressed at high levels on EL-4 cells incubated with SIINFEKL in the presence or absence of IFN-γ (panel 3), detection of complexes on TAT-HA-OVA-transduced EL-4 cells (panel 1) and OVA cDNA stable transfectants (EG.7 cells; panel 2) required IFN-γ pretreatment (16). Inhibition of expression of complexes on TAT-HA-OVA-transduced EL-4 cells by MG132 (CBZ-leu-leu-leu-CHO) suggested that EL-4 cells processed TAT-HA-OVA in a proteasome-dependent fashion (panel 4) (24, 25). H-2K^b:SIINFEKL complexes were not detected on HA-OVA-treated EL-4 cells (panel 5). Note that the levels of complexes on TAT-HA-OVA-transduced and EG.7 cells were similar and much lower than those on SIINFEKL-pulsed EL-4 cells.

Although levels of H-2K^b:SIINFEKL complexes on TAT-HA-OVA-transduced or EG.7 cells were very low if cells were not pretreated with IFN-γ, these cells were susceptible to lysis by CTL nonetheless (Fig. 2*C*). C57BL/6 mice were immunized with SIINFEKL, draining lymph node and spleen cells were restimulated in vitro with SIINFEKL-pulsed BMDC, and CTL activity was determined using a calcein release assay. SIINFEKL-primed CTL lysed peptide-pulsed targets, TAT-HA-OVA-transduced cells, and EG.7

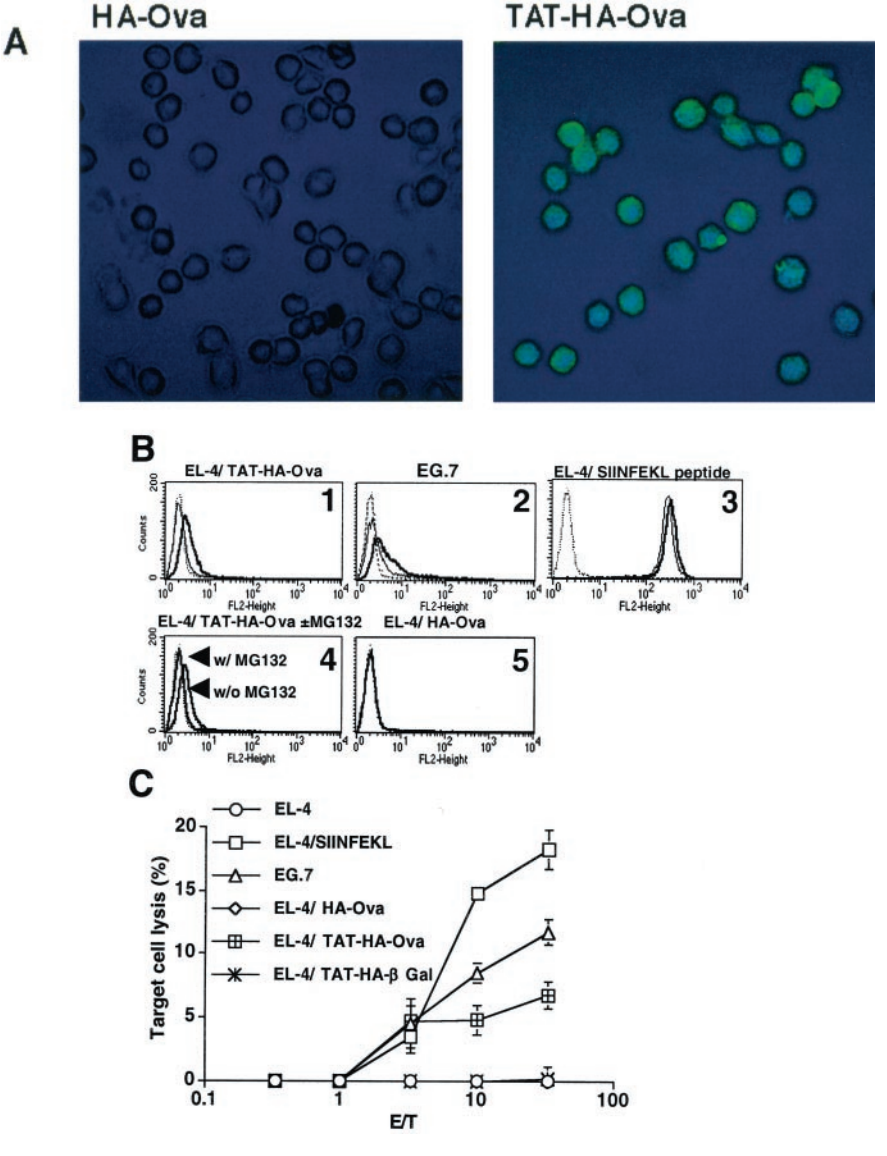

FIGURE 2. Protein Ag transduction and processing in murine thymoma cells. *A*, Transduction of intact cells with TAT-HA-OVA. EL-4 cells were incubated for 18 h with HA-OVA or TAT-HA-OVA (333 nM), washed, fixed, permeabilized, and stained with anti-HA mAb and FITC-conjugated goat anti-mouse IgG. *B*, Expression of H-2K^b:SIINFEKL complexes by peptide-treated, OVA-transduced, or OVA cDNA-transfected thymoma cells. Cells were pretreated with IFN-γ (1000 U/ml for 24 h) as indicated and exposed to rOVA (333 nM for 18 h) or SIINFEKL (10 μg/ml for 1 h), and H-2K^b:SIINFEKL complexes were detected using mAb 25.D1.16 plus PE-conjugated anti-mouse IgG. Proteasome inhibitor MG132 (10 μM) was also added 60 min before TAT-HA-OVA as indicated. Dotted lines, Isotype control mAb; thin lines, mAb 25.D1.16; thick lines; 25.D1.16 mAb reactivity with IFN-γ-treated cells. *C*, Susceptibility of OVA-transduced EL-4 cells to lysis by SIINFEKL-specific CTL. C57BL/6 mice were immunized with SIINFEKL peptide in Titermax, and lymph node and spleen cells were restimulated with peptide-pulsed DC and used as a source of CTL. Susceptibility of OVA protein-transduced and OVA cDNA-transfected thymoma cells to lysis was assessed using a fluorescent dye release assay.

cells but did not kill HA-OVA-exposed EL-4 cells, cells that had been transduced with TAT-HA-β-Gal, or unmodified EL-4 cells.

Formation of MHC class I:peptide epitopes following protein Ag transduction of BMDCs

Before initiating vaccination experiments, we characterized rTAT PTD protein handling by DC. Because recombinant bacterial proteins were contaminated with endotoxin ($\leq$300 ng/mg protein), we added polymyxin B to all proteins before addition to DC. The amount of polymyxin B added was sufficient to prevent phosphorylation of mitogen-activated protein kinase p38 and up-regulation of MHC class II or costimulatory molecules in response to recombinant proteins (and amounts of endotoxin determined to be present as contaminants) (data not shown).

Preparations of BMDC propagated in GM-CSF- and IL-4-supplemented media contained DC that expressed varying levels of MHC class II Ag, but DC were uniformly positive for CD11c, MHC class I, CD40, CD56, and CD86 after enrichment on density gradients (see Fig. 3*A*). To assess distribution of native and recombinant protein in DC, cells were incubated with FITC-conjugated native OVA and rHA-OVA or rTAT-HA-OVA, cells were fixed, permeabilized, stained with anti-HA mAb and Cy3-conjugated goat anti-mouse IgG and examined using confocal laser microscopy. FITC-OVA and HA-OVA colocalized (Fig. 3*B*) in discrete compartments (presumably endosomes or lysosomes). Although TAT-HA-OVA and FITC-OVA coconcentrated in similar structures, TAT-HA-OVA was also distributed more diffusely in DC, perhaps reflecting the ability of TAT PTD-containing proteins to translocate across plasma and/or vesicular membranes (12).

By quantifying cell surface levels of H-2K^b:SIINFEKL complexes with mAb 25.D1.16 after exposure of DC to TAT-HA-OVA at various concentrations and for various periods of time (at a single concentration), we determined that MHC class I loading was near maximal when DC were incubated with 333 nM TAT-HA-OVA for 18 h (Fig. 3*C*). Incubation of DC with HA-OVA did not result in formation of detectable H-2K^b:SIINFEKL complexes (Fig. 3*D*). Note that levels of H-2K^b:SIINFEKL complexes on optimally loaded TAT-HA-OVA-transduced DC were considerably lower than those achievable by incubating DC directly in SIINFEKL peptide (Fig. 3*D*).

Activation of CD4$^+$ T cells by PTD protein Ag-transduced DCs

The pattern of distribution of TAT-HA-OVA in transduced DCs suggested that this protein should be processed and presented to CD4 T cells like other exogenous Ags. To test this hypothesis, CD4$^+$ lymph node T cells were prepared from mice that had been immunized 10 days earlier with native OVA in adjuvant and then restimulated in vitro with DC that had been pretreated with native OVA, rOVA, or control Ags. DC that had been loaded with native OVA, HA-OVA, and TAT-HA-OVA were equipotent with regard to their ability to stimulate thymidine incorporation in OVA-primed T cells (see Fig. 4). DC treated with control Ags (TAT-HA-β-Gal or SIINFEKL peptide) did not activate T cells to a greater extent than untreated DC.

Priming of cytotoxic lymphocytes after administration of protein Ag-transduced DCs

After initial experiments demonstrated that TAT-HA-OVA-transduced DC could restimulate SIINFEKL peptide-primed CTL in vitro (data not shown), we tested the ability of TAT-HA-OVA-transduced DC to stimulate naive CTL precursors in vivo. DC were incubated with SIINFEKL peptide, TAT-HA-OVA, or control proteins and administered s.c. to naive mice. After 7 days,

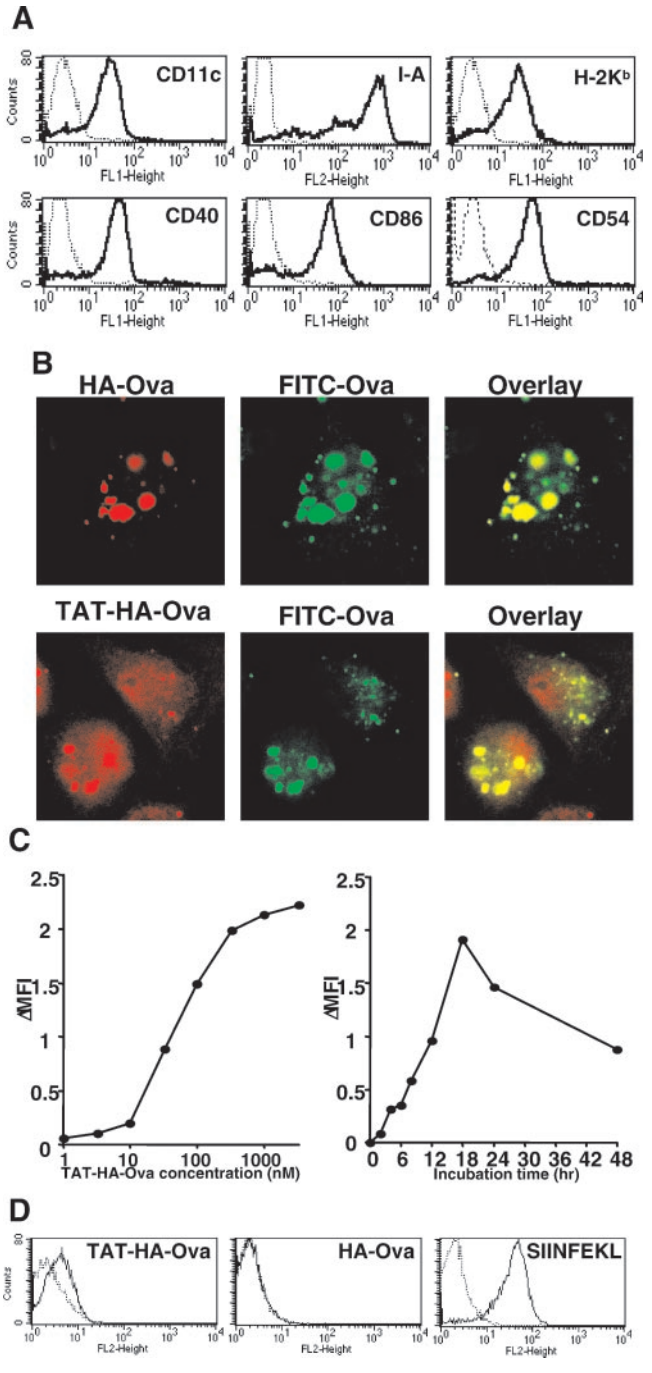

FIGURE 3. Protein Ag transduction and processing by BMDCs. *A*, Expression of surface molecules on BMDC. DC were expanded from bone marrow in GM-CSF and IL-4 and surface Ag expression was assessed via flow cytometry. *B*, Distribution of HA-OVA and TAT-HA-OVA in DC. DC were incubated with FITC-conjugated native OVA and rHA-OVA (or rTAT-HA-OVA) (333 nM each) for 18 h, fixed, permeabilized, stained with anti-HA mAb and Cy3-conjugated anti-mouse IgG, and analyzed via confocal laser microscopy. *C*, Expression of H-2K^b:SIINFEKL complexes by DC. DC were treated with various concentrations of TAT-HA-OVA for 18 h, or with 333 nM TAT-HA-OVA for the times indicated, and surface levels of H-2K^b:SIINFEKL complexes were detected using 25.D1.16 mAb plus PE-conjugated anti-mouse IgG and flow cytometry. ΔMFI = mean fluorescence intensity of 25.D1.16 $-$ mean fluorescence intensity of isotype control mAb. *D*, Relative levels of H-2K^b:SIINFEKL complexes expressed by DC. DC were incubated with TAT-HA-OVA or HA-Ova (333 nM for 18 h), or SIINFEKL (10 μg/ml for 1 h), and surface levels of H-2K^b:SIINFEKL complexes on CD11c$^+$ cells were assessed using flow cytometry. Dotted lines, Isotype control mAb; solid lines, mAb 25.D1.16.

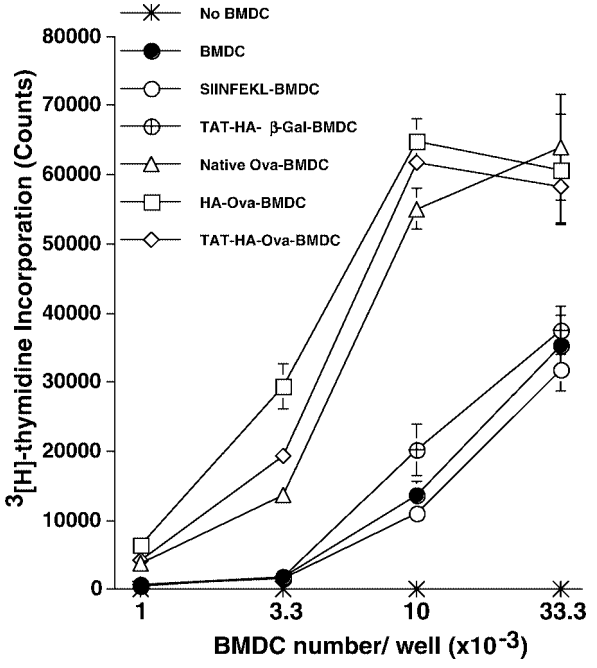

FIGURE 4. Ag-specific stimulation of CD4$^+$ T cells by protein Ag-transduced DCs. CD4$^+$ T cells from popliteal lymph nodes of mice primed with OVA in Titermax were cocultured with DC that had been treated with native OVA or recombinant proteins (333 nM for 18 h), or SIINFEKL peptide (10 μg/ml for 1 h), for 72 h. Cell proliferation was determined by measurement of [^{3}H]methylthymidine incorporation.

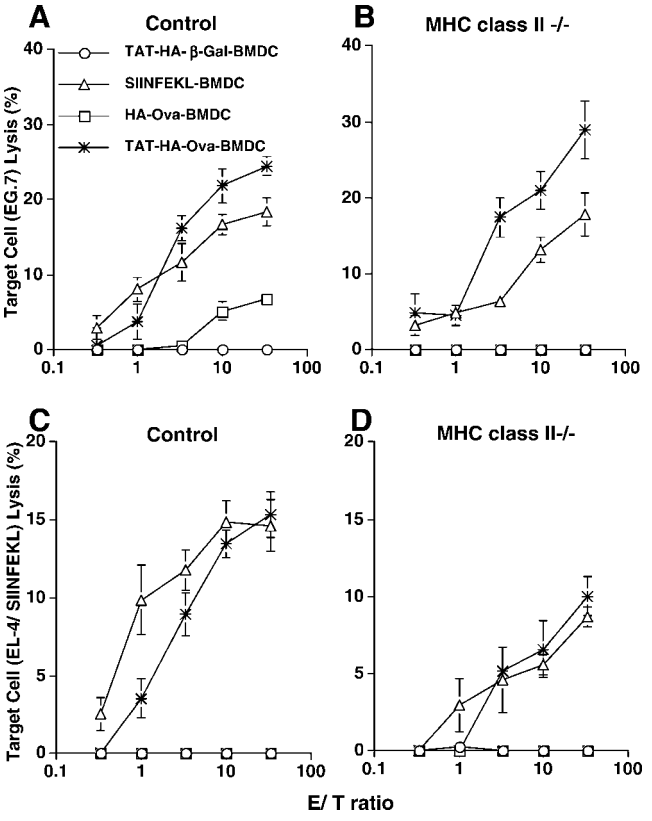

FIGURE 5. Induction of cytotoxic lymphocytes in normal and MHC class II-deficient mice by protein Ag-transduced DCs. Wild-type control (*A* and *C*) or MHC class II$^{-/-}$ (*B* and *D*) mice were immunized with C57BL/6 DC that had been treated with recombinant proteins (333 nM for 18 h) or SIINFEKL peptide (10 μg/ml for 1 h) (5×10^5 cells injected s.c.). Lymph node and spleen cells were restimulated in vitro with mitomycin C-treated EG.7 cells and CTL reactivity with EG.7 (*A* and *B*) or SIINFEKL-sensitized EL-4 (*C* and *D*) cells was determined. CTL reactivities with EL-4 cells were <2% (data not shown).

draining lymph node and spleen cells were restimulated in vitro with mitomycin C-treated EG.7 cells and CTL reactivity with EG.7 and SIINFEKL-treated EL-4 cells was measured. The requirement for CD4$^+$ cells for induction of CTL by TAT-HA-OVA-transduced DC was also assessed by attempting to immunize MHC class II knockout mice that contained <10% of the normal CD4$^+$ T cell number (data not shown).

Administration of SIINFEKL-treated and TAT-HA-OVA-treated DC to control and MHC class II knockout mice primed CTL that killed EG.7 and SIINFEKL-pulsed EL-4 targets (Fig. 5). Unmodified EL-4 cells were not lysed (data not shown). DC treated with TAT-HA-β-Gal did not elicit CTL responses, and the responses induced by HA-OVA-treated BMDC were only slightly above background when EG.7 cells were used as targets (Fig. 5*A*). HA-OVA-induced CTL were not observed in MHC class II knockout mice (Fig. 5*B*), and HA-OVA-induced CTL from normal animals did not kill SIINFEKL-pulsed EL-4 cells (Fig. 5, *C* and *D*). Subsequent studies indicated that EG.7 cells reacted to a limited extent with one of four anti-I-A^b mAb tested (AF6-120.1) and contained small amounts of I-A α- and invariant chain mRNA (data not shown). Thus, the CTL reactivity observed in HA-OVA-transduced, DC-immunized control mice may reflect interactions of primed CD4$^+$ cells with EG.7 cells.

Assessment of CTL priming in individual mice vaccinated with protein Ag-transduced DC revealed significant OVA-specific CTL responses in all animals treated with TAT-HA-OVA-transduced DC as well as those that received SIINFEKL-treated DC (see Fig. 6). The CTL responses in the TAT-HA-OVA-BMDC-immunized mice were perhaps somewhat more variable but were, on average, higher than those in the peptide-DC-immunized mice. As before, CTL priming in the HA-OVA-BMDC- and TAT-HA-β-Gal-BMDC-immunized animals was minimal and absent, respectively.

Induction of antitumor immunity in mice immunized with protein Ag-transduced DCs

To determine whether CTL elicited by protein-transduced DC could reject tumors, mice were immunized with DC, DC pulsed with SIINFEKL peptide, or DC treated with various recombinant protein Ags 14 and 7 days before s.c. challenge with EG.7 cells. Data from a representative experiment are depicted in Fig. 7 and aggregate data are presented in Table I. Tumors grew progressively in almost all mice that were not immunized (38 of 40), as well as mice treated with unmodified DC (23 of 24) and DC transduced with TAT-HA-β-Gal (19 of 20). In contrast, almost all mice (24 of 26) immunized with TAT-HA-OVA-transduced DC failed to develop tumors. Mice that failed to develop tumors after immunization also rejected tumors (8 of 8) when rechallenged with EG.7 cells 5 wk after the last administration of TAT-HA-OVA-transduced DC (data not shown). Mice that received SIINFEKL-pulsed DC were also protected to some extent (20 of 30 were tumor free), but the degree of protection did not equal that afforded by TAT-HA-OVA-treated DC. Although all mice that were inoculated with EG.7 cells after receiving injections of HA-OVA-treated DC developed tumors initially, 27% ultimately rejected them. The same conclusions follow if vaccine success is judged by assessing tumor burden rather than tumor-free survival.

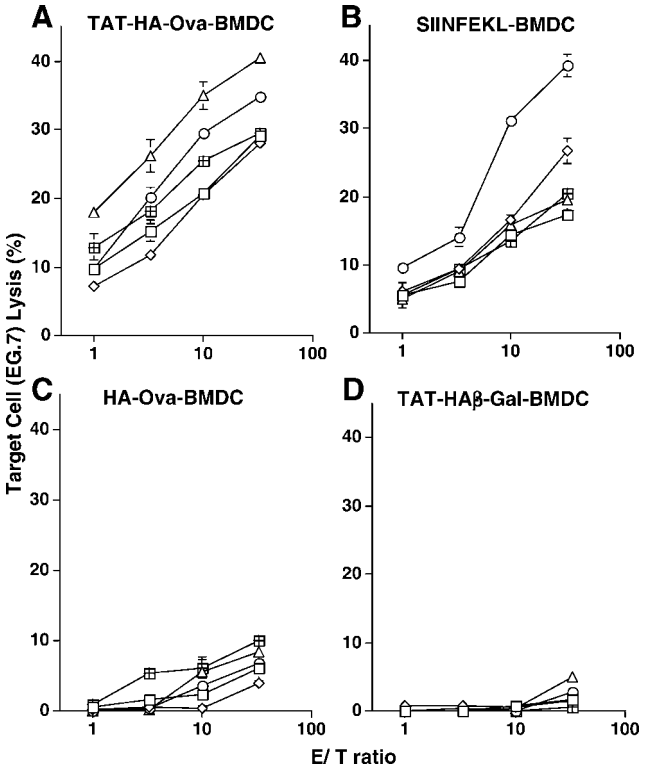

FIGURE 6. Induction of CTL in individual mice by protein Ag-transduced DCs. Mice were immunized with DC that had been treated with recombinant proteins (333 nM for 18 h) or SIINFKL peptide (10 μg/ml for 1 h) (5×10^5 cells injected s.c.). Lymph node and spleen cells from individual mice ($n = 5$ for each condition) were restimulated in vitro with mitomycin C-treated EG.7 cells and CTL reactivity with EG was determined using a calcein dye release assay.

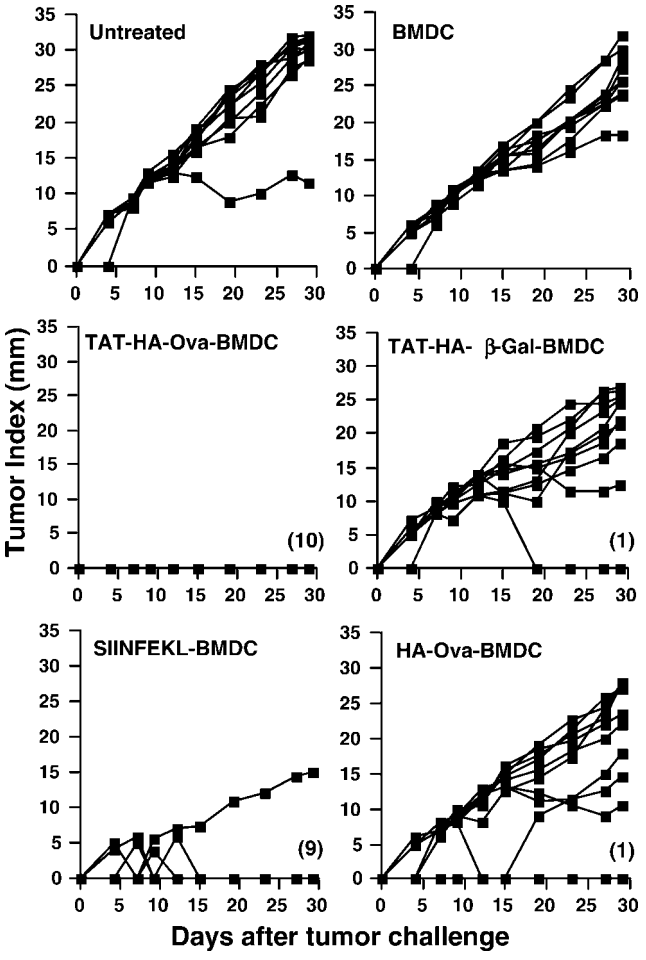

FIGURE 7. Vaccination with protein Ag-transduced DCs prevents tumor engraftment. C57BL/6 naive mice (6–10 animals per group) were immunized with DC that had been incubated with recombinant proteins or SIINFEKL peptide (5×10^5 cells injected on days -14 and -7) and challenged in the subcutis of the opposite flank (on day 0) with 1×10^6 EG.7 cells. Tumor sizes were assessed twice weekly in a blinded fashion. Tumor index = square root of the product of the vertical and horizontal dimensions of the tumor (in millimeters).

Single (rather than repeated) immunizations produced an identical pattern of responses, and efficacy was similar (Table I). Comparison of the numbers of tumor-free animals in the groups immunized once or twice with TAT-HA-OVA-transduced DC indicated a trend favoring multiple treatments (unadjusted $p = 0.02$). Such a trend might reflect the involvement of CD4 T cells in optimal CTL priming and/or survival of memory CTL in vivo (26, 27). TAT-HA-OVA-transduced DC effectively initiate OVA-specific CD4 T cell responses, whereas SIINFEKL-pulsed DC do not (see Fig. 4). It is also possible that TAT-HA-OVA-treated DC elicit higher avidity CTL than peptide-pulsed DC, and these cells expand more vigorously in vivo after a second exposure to Ag or reject tumors more efficiently (28).

Treatment of tumor-bearing animals with protein Ag-transduced DCs

The ability of protein Ag-transduced DCs to elicit immune responses that could reject established tumors was also tested. Naive mice were injected s.c. with EG.7 cells and subsequently immunized twice at weekly intervals with DC that had been incubated with recombinant proteins or SIINFEKL peptide as indicated. Initial injections of DCs were timed (days 5–7 in different experiments) such that tumor indices were 7–10 mm before immunization. Tumors grew progressively in mice immunized with DC or DC that had been treated with TAT-HA-β-Gal (Fig. 8). In contrast, 5 of 30 mice (in a total of three experiments) immunized with TAT-HA-OVA-transduced DC and 1 of 30 mice immunized with peptide-pulsed DC rejected their tumors. No mice injected with HA-OVA-treated DC experienced tumor regression. Hotchberg-adjusted p values were <0.05 (TAT-HA-OVA-DC vs DC alone) in each of the three experiments when changes in tumor indices after initiation of treatments were compared. A two-tailed Fisher's exact test using pooled data yielded a p value of 0.052 when numbers of tumor-free mice in the groups vaccinated with TAT-HA-OVA-DC (5 of 30) were compared with those in groups that received HA-OVA-DC or untreated DC (0 of 30).

To determine whether there was a relationship between the ability of the DC-based vaccines to elicit CTL responses and antitumor immunity, spleen cells were recovered from selected individual animals and restimulated with mitomycin C-treated EG.7 cells, and their ability to lyse EG.7 targets was assessed. For logistical reasons, it was not possible to quantify CTL in all mice from each experiment. A total of 54 mice from a possible 130 in three experiments were studied. The animals selected had variable tumor burdens at the time spleens were obtained. CTL data from the tumor therapy experiment depicted in Fig. 8 are presented in Table II. Results summarized in Table II indicate that mice that mounted EG.7-specific CTL responses had lower tumor burdens than those that did not. Pooled Spearman correlations between percentage of target cell lysis and day 22 tumor burdens (using data from all

Table I. *Tumor-free survival and tumor burden after vaccination with protein Ag-transduced DCs*[a]

Group	Immunogen	Tumor-Free Mice	Tumor Burden
A	None	2/40 (5%)	32.4 (31.1–33.7)
B-1	DC (1×)	0/28 (0%)	26.5 (21.5–29.7)
B-2	DC (2×)	1/24 (4.1%)	26.6 (24.1–30.6)
C-1	SIINFEKL-DC (1×)	21/30 (70%)	0 (0–14.0)
C-2	SIINFEKL-DC (2×)	20/30 (66.7%)	0 (0–14.9)
D-1	TAT-HA-OVA-DC (1×)	20/30[b] (66.7%)	0[c] (0–9.5)
D-2	TAT-HA-OVA-DC (2×)	24/26[d] (92.3%)	0[e] (0–0)
E-1	TAT-HA-β-Gal-DC (1×)	1/19 (5.2%)	28.4 (26.5–30.9)
E-2	TAT-HA-β-Gal-DC (2×)	1/20 (5%)	22.0 (19.3–25.2)
F-1	HA-OVA-DC (1×)	5/30 (16.7%)	24.9 (13.4–28.0)
F-2	HA-OVA-DC (2×)	8/30 (26.7%)	22.7 (0–27.8)

[a] Naive female C57BL/6 mice were vaccinated once (day −7), or twice (days −14 and −7) with 5×10^5 DC (6–10 animals per group) and challenged in the subcutis with 1×10^6 EG.7 cells. Presence or absence of tumors and tumor sizes were assessed in a blinded fashion in three individual experiments and data were pooled (day 30 data reported). Tumor burdens represent the median tumor indices (square root of product of horizontal and vertical dimensions) in millimeters. Values in parentheses represent the range of the middle 50% (25–75%) of the tumor indices measured.

[b] Different from A and B-1 ($p < 10^{-6}$), different from E-1 ($p < 2 \times 10^{-6}$), and different from F-1 ($p < 2 \times 10^{-4}$) using the Hochberg-adjusted exact Wilcoxon rank sum test.

[c] Different from A ($p < 10^{-6}$), B-1 ($p < 4 \times 10^{-6}$), E-1 ($p < 6 \times 10^{-6}$), and F-1 ($p < 8 \times 10^{-5}$) using the Hochberg-adjusted χ^2 test.

[d] Different from A, B-2, and E-2 ($p < 10^{-6}$), different from F-2 ($p < 5 \times 10^{-6}$), and different from C-2 ($p = 0.0197$) using the Hochberg-adjusted exact Wilcoxon rank sum test.

[e] Different from A, B-2, and E-2 ($p < 10^{-10}$), different from F-2 ($p < 5 \times 10^{-7}$), and different from C-2 ($p = 0.0085$) using the Hochberg-adjusted χ^2 test.

three experiments) revealed a moderately strong inverse correlation ($r = -0.59$; $p_2 = 0.005$).

Discussion

This study demonstrates that a recombinant model tumor-associated Ag (OVA) containing the TAT PTD entered murine thymoma cells and immature DC and was processed by proteasomes, and resultant peptides were displayed on cell surfaces bound to MHC class I. As expected, PTD-containing OVA was also processed and presented to $CD4^+$ T cells (in the context of MHC class II). TAT-HA-OVA-transduced DC initiated Th cell-independent CTL responses in vivo, and mice vaccinated with TAT-HA-OVA-transduced DC did not allow engraftment of OVA-expressing tumors. TAT-HA-OVA-transduced DC initiated CTL responses that were as vigorous as, or were more vigorous than, those initiated by SIINFEKL-pulsed DC. In addition, more mice treated with TAT-HA-OVA-treated DC resisted tumors than mice injected with peptide-pulsed DC. Although mice that received HA-OVA-treated DC did not mount a CTL response, some mice treated with this immunogen rejected tumors (but all tumors engrafted initially). Treatment of mice with established tumors was also somewhat

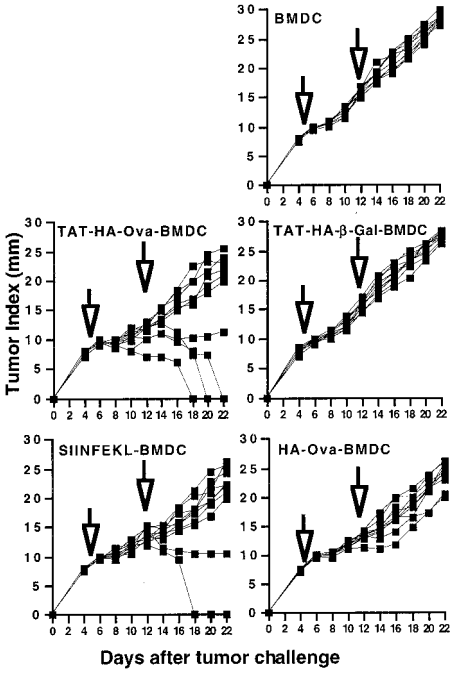

FIGURE 8. Treatment of tumor-bearing mice with protein Ag-transduced DCs. C57BL/6 naive mice (10 animals per group) were inoculated in the subcutis with 1×10^6 EG.7 cells on day 0 and subsequently immunized in the opposite flanks with DC that had been incubated with recombinant proteins or SIINFEKL peptide (5×10^5 cells injected on days 5 and 12 (arrows)). Tumor indices (in millimeters) were assessed twice weekly in a blinded fashion.

Table II. *Inverse relationship between tumor burden and ability of spleen cells in vaccinated mice to lyse tumor cells in vitro*[a]

Immunogen	Tumor Index	% EG.7 lysis
TAT-HA-β-Gal-DC	28.39	0.13 ± 0.22
	28.39	0.24 ± 0.42
SIINFEKL-DC	0	9.53 ± 0.69
	10.39	14.83 ± 1.18
	19.71	2.79 ± 0.44
	21.91	4.45 ± 0.61
	22.05	1.67 ± 0.48
	22.36	1.27 ± 0.73
TAT-HA-OVA-DC	0	31.64 ± 1.28
	0	15.03 ± 0.80
	0	14.83 ± 1.18
	11.22	19.85 ± 1.84
	20.78	5.23 ± 0.23
	21.78	3.19 ± 0.63
HA-OVA-DC	20.35	1.22 ± 0.17
	22.91	2.39 ± 0.34
	23.81	4.42 ± 0.07
	24.92	3.06 ± 0.76
	25.92	5.89 ± 0.56
	26.27	1.89 ± 0.54

[a] Nucleated spleen cells from selected tumor-challenged individual mice (see Fig. 8) were restimulated with mitomycin C-treated EG.7 cells and their ability to lyse EG.7 cells was assessed. Tumor indices (in millimeters) represent day 22 values, and target cell lysis is reported at an E:T ratio of 30.

successful. Tumors regressed completely in 5 of 30 mice treated with TAT-HA-OVA-transduced DC and 1 of 30 mice treated with SIINFEKL peptide-pulsed DC as compared with 0 of 30 mice treated with DC that had been loaded with control proteins or untreated DC.

Although the results obtained in this study were almost entirely consistent with our initial hypothesis, several observations warrant comment. TAT-HA-OVA-transduced DC expressed much lower levels of H-2K^b:SIINFEKL complexes than peptide-pulsed DC (Fig. 3*D*) and yet elicited comparable SIINFEKL-specific CTL activity (Figs. 5 and 6) and superior antitumor immunity in vivo (Fig. 7). It is noteworthy that APC that express very low levels of 25.D1.16 reactivity are able to stimulate SIINFEKL-reactive T cells very effectively in vitro (see Fig. 4 in Ref. 20). Indeed, in the present study TAT-HA-OVA-treated EL-4 cells that are devoid of 25.D1.16 reactivity remained sensitive to lysis by SIINFEKL-primed CTL (Fig. 2). Although we have not assessed the avidity of CTL in TAT-HA-OVA/DC-immunized mice, one could argue that TAT-HA-OVA-transduced DC expressing low levels of SIINFEKL:H-2K^b complexes might preferentially stimulate high-avidity CTL that exhibit potent antitumor activity (28). Alternatively, the increased antigenic complexity that results when DC are transduced with full-length OVA as opposed to being incubated with SIINFEKL peptide may be advantageous.

The incomplete success of our DC vaccine in the treatment of mice with clinically apparent tumors is also of interest (Fig. 8), and the inverse relationship between the ability to elicit CTL responses and therapeutic efficacy is striking (Table II). We cannot attribute this result to expected variation in the ability of TAT-HA-transduced DC (or peptide-pulsed DC) to elicit CTL in individual animals because naive mice are invariably immunized (see Fig. 6). It seems more likely that that the environment in some tumor-bearing mice is actively immunosuppressive. It is tempting to implicate IL-10 and/or CD4$^+$CD25$^+$ regulatory T cells in tumor-dependent immunosuppression (29), but we have no data to support this concept at present.

Vaccination with DC transduced with full-length recombinant tumor Ags may offer advantages over strategies that are in current use. As compared with administration of peptide-pulsed DC, MHC class I alleles and class I binding peptides need not be defined before vaccination with protein-transduced DC. In addition, the number of epitopes recognized by CTL in recipients of protein Ag-transduced DC should be greater than that achievable with peptide-pulsed DC. Protein Ag-transduced DC are also able to activate Th cells. Recent reports suggest that although Th cells may not mediate tumor rejection directly, they are required for persistence of memory CTL (27) and/or optimal antitumor immunity (30, 31).

Relative to standard viral infection and plasmid transfection methods, TAT PTD-mediated protein transduction of DC is more convenient and efficient. Bacterial recombinant proteins can easily be engineered and large amounts of Ags can be readily purified. Endotoxin contamination of the proteins used in this study is undesirable, and this will ultimately necessitate modification of the purification scheme or generation of proteins in eukaryotic cells. Consistent with results in other cells (13, 14), DC were uniformly transduced by TAT PTD-containing proteins and, if contaminating endotoxin had been neutralized, protein transduction did not alter DC phenotype. Thus, we expect that trafficking and function of protein-transduced DC in vivo will not be significantly different from their nontransduced counterparts.

It will be interesting to compare the utility of the methodology that we describe with that of two related approaches that have been reported recently. Treatment of accessory cells (including DC) with native OVA modified with positively charged peptides enabled them to stimulate MHC class I-restricted OVA-specific T cell hybridomas in vitro and to induce at least limited CTL activity in vivo (32). This approach does not appear to afford advantages over the one that we describe and is feasible only if purified Ags are available. Positively charged peptides have also been used to dramatically increase DC transfection efficiency (33). DC transfected with peptide-coated plasmids encoding Ags of interest induced Ab and CTL responses and vaccinated against tumors. Although these approaches, as well as the approach described in this study, hold promise, it will not be possible to judge their relative merits until each approach has been optimized and they can be compared directly in experimental animals and appropriate patient populations.

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