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Induction of Antigen-Specific CTL by Recombinant HIV *Trans*-Activating Fusion Protein-Pulsed Human Monocyte-Derived Dendritic Cells[1]

Yoshiyuki Tanaka,* Steven F. Dowdy,† David C. Linehan,* Timothy J. Eberlein,* and Peter S. Goedegebuure[2]*

Several systems have been tested for introduction of Ags into human dendritic cells (DC). Most of them to date, however, are complex and possess limited efficiency. Recent advances in HIV *trans*-activating (TAT) fusion protein technology permit extremely high transduction efficiencies for a majority of mammalian cell types. Here we report our attempts to develop a simple, but highly efficient, protocol for loading of antigenic protein into DC using TAT fusion technology. A TAT-minigene fusion protein was generated, encoding both the HLA-A2-restricted influenza matrix protein-derived epitope (GILVFTFTL, Flu-M1) and a melanoma Ag gp100-derived modified epitope (YLEPGPVTV, $G9_{280}$-9V). In addition, both a TAT-Her2/neu extracellular domain (ECD) fusion protein and a TAT-green fluorescence protein fusion protein were generated. Over 95% of DC stained positively for TAT-green fluorescence protein within 20 min of coculture. DC treated with TAT-minigene were efficiently recognized by both Flu-M1 and $G9_{280}$-9V-specific T cells in cytotoxicity assays and IFN-γ ELISPOT assays. In contrast, DC pulsed with minigene fusion protein lacking TAT were either poorly recognized or not recognized by the T cells. DC pulsed with TAT-minigene also efficiently induced Flu-M1-specific T cells from naive lymphocytes. Similarly, DC treated with TAT-Her2/neu ECD stimulated patient-derived lymphocytes that specifically recognized Her2/neu$^+$ ovarian and breast cancer cell lines. The CTL induced by TAT-Her2/neu ECD-pulsed DC specifically recognized the Her2/neu ECD-derived immunogenic peptide E75 (KIFGSLAFL). Our data suggest that TAT fusion proteins efficiently transduce DC and induce Ag-specific T cells. This could prove to be a useful method for treatment of infectious diseases and cancer. ***The Journal of Immunology*****, 2003, 170: 1291–1298.**

Recent advances in tumor immunology have facilitated the induction of T cell immune responses against cancer. In particular, the discovery of tumor-associated Ags (TAA)[3] and the development of protocols for the ex vivo generation of dendritic cells (DC) permit the induction of strong tumor-specific T cell responses (1). Vaccination with HLA class I-restricted synthetic peptides pulsed onto DC has become a common method to induce Ag-specific CTL. However, this method has several limitations, including the dependence on particular haplotypes and the relatively small number of known HLA class I-restricted TAA in certain tumors. Although tumor cell preparations such as tumor cell lysates (2, 3), apoptotic tumor cells (4, 5), or DC-tumor cell fusions (6, 7) could overcome these limitations, only weak immune responses against each TAA might be induced because of the small amount of each antigenic protein. An alternative approach to express full-length TAA in DC involves transfer of DNA or RNA, or viral vectors containing TAA genes (8–10). However, DNA and RNA transfection is relatively inefficient. In vitro-transcribed RNA or naked DNA combined with liposomes can be introduced into only ~5% of DC (9). In contrast, transduction of DC by viral vectors has been successfully performed with a very high transduction efficacy of >90%. However, this method contains several potential problems, such as the complex generation of the recombinant viral particle and the immunogenicity of the virus itself (10).

To overcome these disadvantages, we developed a relatively simple and unique method to induce antigenic protein into DC by using recombinant HIV *trans*-activating (TAT) fusion proteins (FP). HIV type 1-derived TAT comprises 86 aa and is an early RNA-binding protein that regulates transcription (11). Green et al. (12) and Frankel et al. (13) independently demonstrated that HIV TAT protein is able to cross cell membranes. Fawell et al. (14) showed that chemical cross-linking of only a 36-aa domain of TAT to various kinds of proteins permitted the transfer into cells. Subsequently, Kim et al. (15) showed that the conjugation of this TAT peptide to OVA protein resulted in efficient stimulation of MHC class I-restricted mouse T cell responses in vitro. Recently, Dowdy et al. (16) developed a system for transduction of full-length proteins using urea-denatured genetic in-frame TAT FP. This method, called protein transduction, has been applied to a broad spectrum of proteins and cells and demonstrates a remarkably high transduction efficiency (16). We used this system, therefore, for the induction of antigenic proteins into DC, followed by T cell stimulation. We show here that Ag-specific T cells can be efficiently induced with both viral and tumor Ag-containing TAT FP.

Materials and Methods

Peptide synthesis

Peptides were purchased from Genemed Synthesis (San Francisco, CA). Syntheses were conducted by a standard solid phase method based on

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[3] Abbreviations used in this paper: TAA, tumor-associated Ag; CD40L, CD40 ligand; DC, dendritic cells; ECD, extracellular domain; FP, fusion protein; GFP, green fluorescence protein; HA, hemagglutinin; TAT, *trans*-activating protein; CEA, carcino embryonic Ag.

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fluorenylmethoxycarbonyl chemistry. Recovered lyophilized peptides were purified by HPLC on C_{18} columns. Peptide identity and purity (>95%) were demonstrated by mass spectrometry.

Cell lines

The Her2/neu overexpressing breast cancer cell line, EF192A, was purchased from Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). The HLA-$A2^+$ peptide TAP-deficient T-B cell hybrid, T2 cell line; the Her2/neu-overexpressing ovarian cancer cell line, SKOV3; the breast cancer cell lines MCF-7 and MDA-415; and the leukemia cell line, K562, were all purchased from American Type Culture Collection (Manassas, VA). HLA-A2 and HLA-A24 genes were transfected into SKOV3 to yield SKOV3-A2 and SKOV3-A24, respectively, using lipofectin (Life Technologies, Gaithersburg, MD). HLA-A2 and A24 expression vectors were gifts from Drs. Kawakami and Shichijo, respectively. All cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 1% penicillin-streptomycin (all from Mediatech, Herndon, VA).

In vitro generation of DC

DC were generated from HLA-$A2^+$ PBMCs from normal donors or ovarian cancer-associated mononuclear cells by centrifugation on Histopaque (Sigma-Aldrich, St. Louis, MO). PBMCs were plated in 10-cm^2 culture dishes (1×10^8 cells/dish), and monocyte-enriched adherent cells were observed after a 1-h incubation at 37°C. The nonadherent cells were removed and cryopreserved, and the adherent cells were cultured in the presence of 800 U/ml rGM-CSF and 400 U/ml rIL-4 (both from Pierce, Rockford, IL) in AIM-V medium (Gibco Invitrogen, Grand Island, NY). On day 3, the medium was replenished, and additional GM-CSF and rIL-4 were added. On day 6, 100 ng/ml LPS (Sigma-Aldrich) was added to the cells, and after a 12-h incubation the cytokine-treated cells were harvested and used as mature DC. In some experiments 10 μg/ml CD40 ligand (CD40L trimer; Alexis Biochemicals, San Diego, CA) or 10 μg/ml polyinosinic-polycytidylic acid (Sigma-Aldrich) was added for 12 h to induce DC maturation.

Phenotype analysis of DC

DC were labeled using FITC- or PE-conjugated mAb specific to a Lineage cocktail (Lin 1), CD83, CD11c, ICAM, and HLA-DR (all from BD Biosciences, San Jose, CA). The fluorescence intensity was measured with a FACSCalibur (BD Biosciences)

In vitro T cell stimulation using TAT FP-transduced DC

DC were pulsed with each FP for 1 h at room temperature. The FP-loaded DC were irradiated with 5000 rad and mixed with PBMCs at a ratio of 1:20 in the presence of 10 ng/ml rIL-7 and 50 U/ml rIL-2 (a gift from Amgen, Thousand Oaks, CA) in AIM-V medium supplemented with 2.5% human AB serum. In selected cases, the responder cells were stimulated at a ratio of 10:1 with peptide-pulsed DC in medium with 10 ng/ml rIL-7 and 50 U/ml rIL-2 on day 7, and weekly thereafter.

Vector construction

The bacterial expression TAT fusion vector, pTAT-hemagglutinin (pTAT-HA), contains an initiation codon, atg, followed by six histidines (His-tag), the HIV TAT domain (YGRKKRRQRRR), an HA tag, and a multicloning site (16). A minigene was constructed encoding the Flu-M1 (17) and $G9_{280}$-9V (18) peptides using PCR extension of two overlapping oligonucleotides (5′-ctc gag aag ctt gcc gcc acc atg gga atg cag gtg cag atc cag agc ctg ttt ctg ctc ctc ctg tgg gtg ccc ggg tcc aga gga gcc aa-3′ and 5′-ctc gag cca tgg cga cag tga ctg ggc cag gct cca ggt agg cga gcg tga aca caa acc cta aaa tcc cct tgg ctc ctc tgg ac-3′) under the following conditions: 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min, for a total of 30 cycles. After the PCR reaction, the products were subcloned into the pGEM-T vector (Promega, Madison, WI). Part of the cloned gene was amplified by PCR with the following primers (5′-ggc cat ggg agc caa ggg gat ttt a-3′ and 5′-ggg aat tca tgg cga cag tga ct-3′) under the following conditions: 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min, for a total of 20 cycles, and subcloned into the *Nco*I-*Eco*RI site of pTAT-HA. The Her2/neu extracellular domain (ECD) was excised by *Nco*I-*Eco*RI digestion of the Her2/neu expression vector, pSV2-Her2/neu. The fragment was subcloned into the *Nco*I-*Eco*RI site of pTAT-HA (pTAT-Her2/neu ECD). The pTAT-green fluorescence protein (GFP) fusion vector was generated previously (16)

TAT FP purification

Recombinant TAT FP were generated in high expressing *Escherichia coli* BL21 codon plus cells. The cell pellet obtained from a 6-h, 500-ml culture inoculated with a 50-ml overnight culture was sonicated in buffer A (8 M urea/20 mM HEPES (pH 8.0)/100 mM NaCl). Cellular lysates were obtained by centrifugation and loaded onto a 5-ml nickel-nitrolotriacetic acid column in buffer A plus 10 mM imidazole. The column was washed, and proteins were eluted with imidazole in buffer A at increasing concentrations (100, 250, and 500 mM). After analyzing the elutriate by immunoblotting with an anti-HA Ab, rapid dialysis was performed to remove urea and salt. FITC-labeled TAT FP was generated by fluorescein labeling of TAT FP, followed by rapid dialysis. The FITC-labeled TAT FP was added directly to cells in culture medium. The purity of the FP generally reached >80% as determined by gel electrophoresis and Coomassie staining. Purified FP were always checked by immunoblot using an anti-HA Ab.

Confocal microscope analysis of TAT FP-transduced DC

Confocal epifluorescence microscopy was performed with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) and a ×100/1.3 oil objective. The confocal pinhole was kept small to obtain thin optical slices. DC were incubated with FITC-labeled TAT-minigene at 26°C for 30 min. Excess FP was removed by a single PBS wash, and cells were then mounted on a microscope slide.

Cytotoxic assay

Standard ^{51}Cr release assays and cytokine (IFN-γ) release assays were performed to evaluate the Ag specificity of T cells. Peptide-pulsed T2 as well as breast and ovarian tumor cell lines were used as target/stimulator cells. ^{51}Cr-labeled T2 cells were preincubated with 100 μg of peptide for 1 h at 37°C. These cells were then used as targets in cytotoxicity assays (2000 targets/well). Target cells and effector cells were coincubated for 4 h at 37°C, and the radioactivity in supernatants was determined with a gamma counter. The percent specific lysis of target cells by CTL was calculated. Spontaneous release never exceeded 25% of the maximum. Unlabeled K562 cells (1×10^5 cells/well) were added to neutralize nonspecific lysis.

ELISPOT assay

Ninety-six-well plates with nitrocellulose membrane inserts (Millipore, Bedford, MA) were coated overnight at 4°C with 5 μg/ml of the primary anti-IFN-γ mAb (Mabtech, Stockholm, Sweden). After five washings with PBS, the plates were blocked with PBS containing 5% human AB serum for 1 h at 37°C. Various numbers of effector and target cells were mixed and placed in the wells and incubated overnight (~18–20 h) at 37°C. Wells were washed five times in PBS with 0.1% Tween 20 (Sigma-Aldrich), followed by 2-h incubation with 1 μg/ml of the secondary Ab (biotin-conjugated anti-IFN-γ mAb; Mabtech). Plates were washed five times in PBS with 0.1% Tween 20. Streptavidin-alkaline phosphatase (Mabtech) was added to the wells for 1 h at room temperature. The plates were washed five times in PBS with 0.1% Tween 20, followed by an ~5- to 15-min incubation in stable 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich) to develop the reaction. Tap water was added to stop the reaction. The spots were counted with a microscope using a ×40 magnification. Only spots with a fuzzy border and a clear blue color were counted, and results were expressed as the number of spot-forming cells per 5×10^4 effector cells.

Flu-M1-specific tetramer

The Flu-M1 streptavidin-PE-labeled tetramer used in this study was obtained from the Tetramer Core Facility at National Institute of Allergy and Infectious Disease (Atlanta, GA). Two-color staining and flow cytometric analyses were performed with anti-CD8-FITC and tetramer-PE. Cells were considered positive for tetramer staining when they formed a clear population with an intensity at least 1 log above the mean fluorescence of the negative population. Events up to 100,000 were collected progressively after live gating on lymphocytes by forward and side scatter.

Results

Purification and detection of recombinant TAT FP

Five different FP were generated for this study: minigene with/without TAT domain, Her2/neu ECD with/without TAT domain, and TAT-GFP (Fig. 1*A*). The FP were purified using the urea-denaturing protein purification protocol described by Nagahara et al. (19) The purified proteins were shown to have a molecular mass of ~15 kDa for the minigene FP, ~60 kDa for the Her2/neu FP, and ~36 kDa for the TAT-GFP FP. Clear bands were detected by

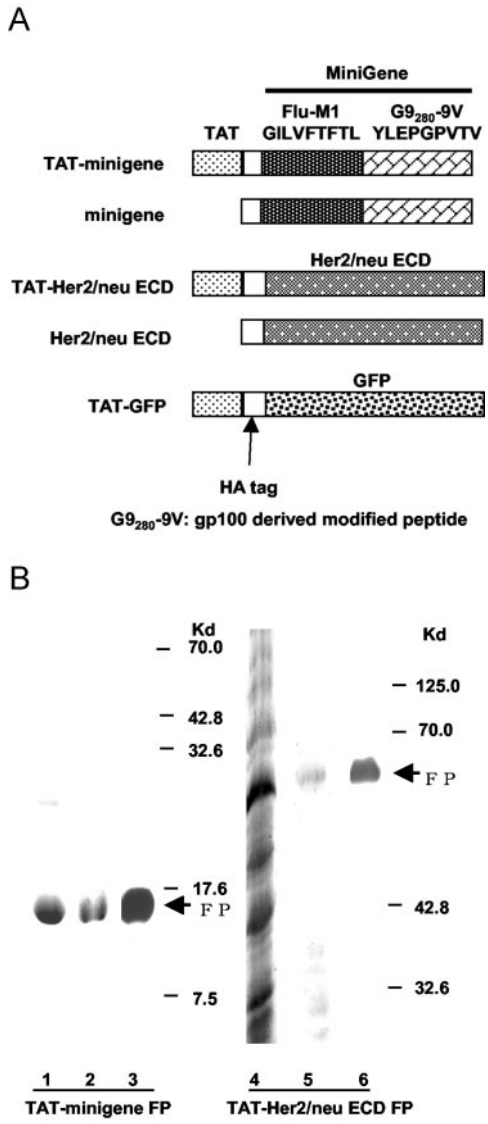

FIGURE 1. *A*, Schematic overview of the TAT FP used. *B*, Purification of TAT-minigene FP and TAT-Her2/neu FP over nickel-nitrolotriacetic acid resin. SDS-polyacrylamide gels were loaded with a crude extract from the *E. coli* strain BL21 codon plus bearing pTAT-minigene (*lane 1*), pTAT-Her2/neu ECD (*lane 4*), and purified TAT-minigene FP (*lane 2*) and TAT-Her2/neu FP (*lane 5*). Western blotting was performed using an anti-HA-specific Ab against HA tag for each FP (*lanes 3* and *6*).

both Coomassie staining and immunoblotting with an anti-HA Ab (Fig. 1*B*).

Successful transfer of denatured TAT FP into DC

To analyze the ability of TAT FP to transduce DC, TAT-GFP was added to the culture medium of DC, and DC were analyzed by FACS at various time points for the presence of the protein. TAT-GFP was detected in DC within 20 min of addition to the culture medium, and the transfer efficiency of the protein was shown to increase with higher concentrations of protein (Fig. 2*A*). Confocal microscopic analysis of DC pulsed with FITC-labeled TAT-minigene revealed protein localization in both the cytoplasm and the nucleus of DC (Fig. 2*B*). Immunostaining and FACS analysis performed on DC both before and after coculture with TAT FP further demonstrated that protein transduction using Tat FP did not significantly change the cell surface expression levels of CD83, CD11c, HLA-DR, and CD54, nor did it change the percentage of Lin1-positive cells (Fig. 2*C*).

Flu-M1- and $G9_{280}$-9V-specific T cell lines efficiently recognize TAT-minigene-transduced DC

To assess the ability of T cells to recognize the FP-transduced DC, the response of a Flu M1-specific T cell line (Fig. 3*A*) to FP-transduced DC stimulation was measured. As evidenced by specific release of IFN-γ, the Flu M1-specific T cell line was able to recognize TAT-minigene transduced DC (Fig. 3*B*). This recognition appeared to be TAT dependent, since DC pulsed with the same FP lacking the TAT domain failed to induce a T cell response, as measured by IFN-γ release. T cell recognition was also dose dependent (Fig. 3*B*). In addition, $G9_{280}$-9V-specific T cells recognized DC transduced with TAT-minigene (Fig. 4). The inclusion of TAT in the minigene FP similarly enhanced the T cell response as with the Flu M1-specific T cell line (Fig. 4*B*).

TAT-minigene-transduced DC efficiently induce Flu-M1-specific T cells

Primary T cells were then assessed for their ability to respond to the FP-transduced DC. PBMCs were stimulated by Flu-M1 peptide, TAT-minigene FP, minigene FP, or TAT-GFP FP-pulsed DC. After 10 days the presence of Flu-M1-specific T cells was evaluated by ^{51}Cr release and IFN-γ ELISPOT assays. DC pulsed with TAT-minigene or Flu-M1 peptide elicited Flu-M1-specific recognition. DC pulsed with Flu-M1 peptide were more potent in inducing Flu-M1-specific recognition than DC transduced with TAT-minigene (Fig. 5, *A–C*). In contrast, DC transduced with minigene FP lacking the TAT domain and TAT-GFP FP did not induce Flu-M1-specific T cells. Flu-M1-specific tetramer analyses of each T cell culture showed a clear tetramer-positive cell population within the $CD8^+$ lymphocytes stimulated with DC and TAT-minigene or DC and Flu-M1 peptide (Fig. 5*C*). Although $G9_{280}$-9V-specific recognition was evaluated simultaneously, we could not detect any $G9_{280}$-9V-specific T cells even after four rounds of weekly stimulation with TAT-minigene-transduced DC (data not shown).

To evaluate the effects of different DC maturation-inducing factors on the induction of Flu-M1-specific T cells, DC were treated with LPS, CD40L, or polyinosinic-polycytidylic acid. The results showed that all methods of DC maturation followed by TAT-minigene pulsing induced Flu-M1-specific, tetramer-positive T cells (Fig. 5*D*).

TAT-Her2/neu ECD FP induces HLA-A2-restricted, Her2/neu-specific T cells

We further tested whether TAT FP can be used to stimulate T cells against tumor Ags. We focused on Her2/neu and generated a TAT-Her2/neu ECD FP and a Her2/neu ECD FP without TAT (Fig. 1*A*). T cells induced by TAT-Her2/neu-transduced DC lysed the Her2/neu-overexpressing ovarian cancer cell line, SKOV3-A2 (Fig. 6*A*), and the Her2/neu$^+$, HLA-A2$^+$ breast cancer cell line, MCF-7 (Fig. 6*B*). CTL activity was partially blocked by anti-HLA-A2 and anti-HLA class I mAb, but not by anti-HLA-A24 mAb (data not shown). In contrast, SKOV3, SKOV3-A24, and the Her2/neu$^+$, HLA-A2$^-$ breast cancer cell line, MDA415, were poorly recognized by the CTL line. T cells stimulated with DC pulsed with Her2/neu FP without TAT domain or TAT-GFP poorly recognized SKOV3, SKOV3-A2, SKOV3-A24, MCF7, and MDA415. Since the HLA-A2-restricted p369–377 (E75) from Her2/neu has been described as an immunodominant peptide (20), we tested the Her2/neu-specific CTL for recognition of T2 pulsed with E75 peptide. The HLA-A2-restricted, Her2/neu-derived p654–662 (GP2) (21),

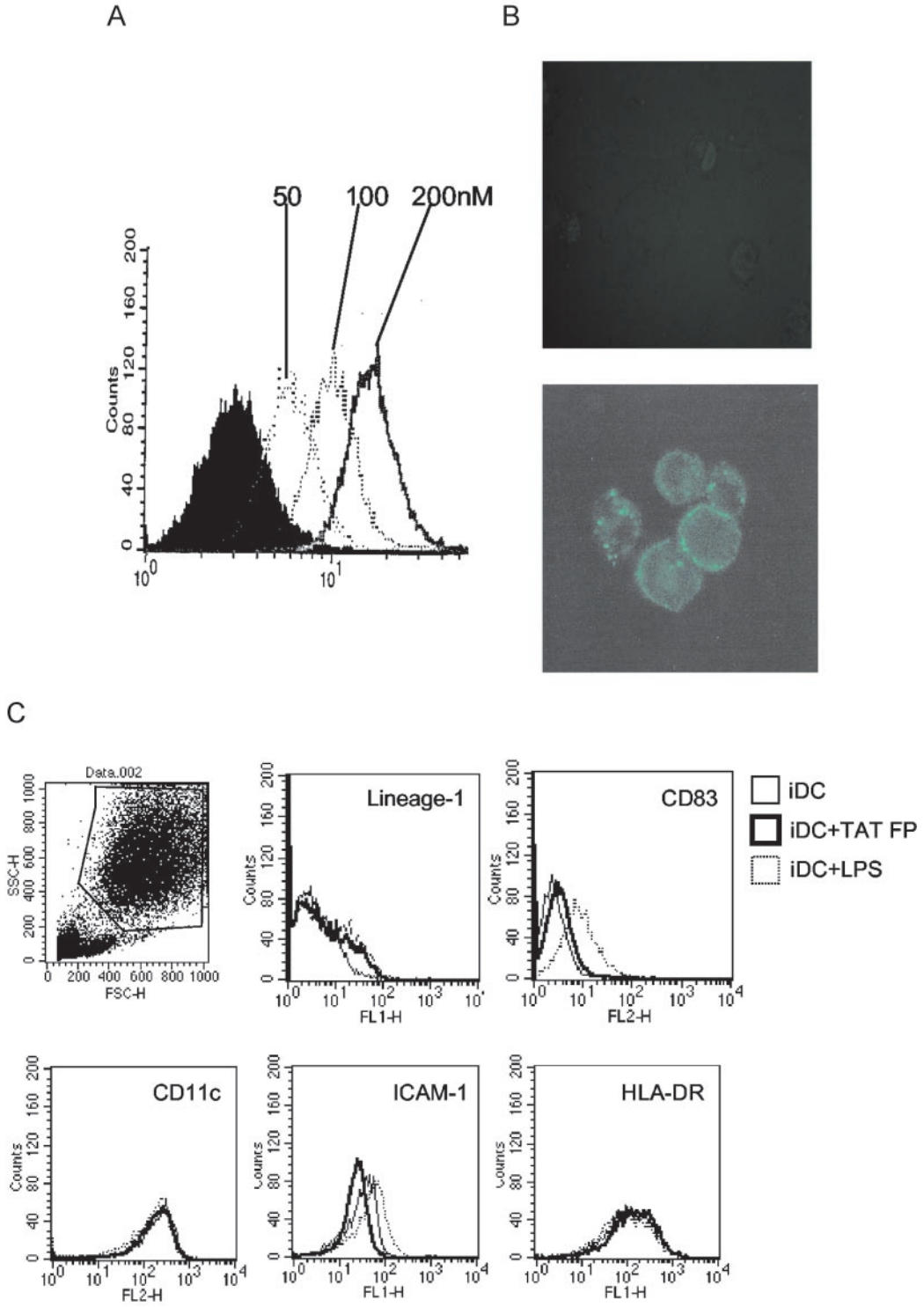

FIGURE 2. Pulsing of DC with TAT FP. *A*, Dose-dependent analysis of TAT-GFP added to DC 30 min after addition. *B*, Confocal microscope photographs (original magnification, ×1000) of cells pulsed with control (PBS) and 200 nM FITC-labeled TAT-minigene. *C*, DC phenotype analysis before and after TAT-minigene pulsing. Immature (i) DC, iDC pulsed with TAT-minigene, and iDC stimulated by LPS were stained with FITC- or PE-labeled mAb specific to Lin-1, CD83, CD11c, ICAM, and HLA-DR. The fluorescence intensity was measured by flow cytometry.

which was not included in the TAT-Her2/neu FP, was tested as control. The CTL line specifically recognized E75 peptide, but not GP2, as evidenced by IFN-γ ELISPOT assay (Fig. 7).

Discussion

It is well known that DC can take up exogenous Ags through phagocytosis and other mechanisms. These Ags are processed intracellularly and are presented on the cell surface by MHC class II molecules. However, some exogenous Ags find their way to the cytosolic area and are loaded onto MHC class I molecules after processing. This process, called cross-presentation (22), may lead to the induction of Ag-specific $CD8^+$ CTL. To enhance cross-presentation, several strategies have been applied, including the use of specific carrier molecules such as gp96 (23), cationic and

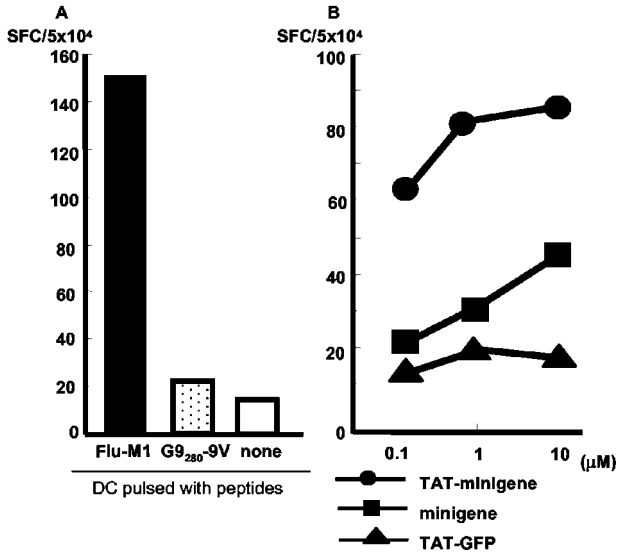

FIGURE 3. Recognition of TAT-minigene-transduced DC by Flu-M1-specific T cells. Flu-M1-specific T cells were generated by stimulating CD8-enriched PBL with 1 μM Flu-M1 peptide-pulsed DC. After 10 days of culture with IL-2, T cells were harvested. *A*, Peptide recognition by Flu M1 peptide-stimulated T cells. DC pulsed with Flu-M1 (■), $G9_{280}$-9V peptide (▨), or no peptide (□) were used as target cells, and Flu-M1 peptide-stimulated T cells were used as effector cells. *B*, Dose-dependent recognition of DC pulsed with TAT-minigene (●), minigene FP without TAT (■), or TAT-GFP (▲) by Flu-M1-specific T cells. DC pulsed with FP were used as target cells, and Flu-M1-specific T cells were used as effector cells. The recognition of target cells by Flu-M1-specific T cells was analyzed by IFN-γ ELISPOT assay and expressed as the number of spot forming cells (SFC) per 5×10^4. One representative experiment of two performed is shown.

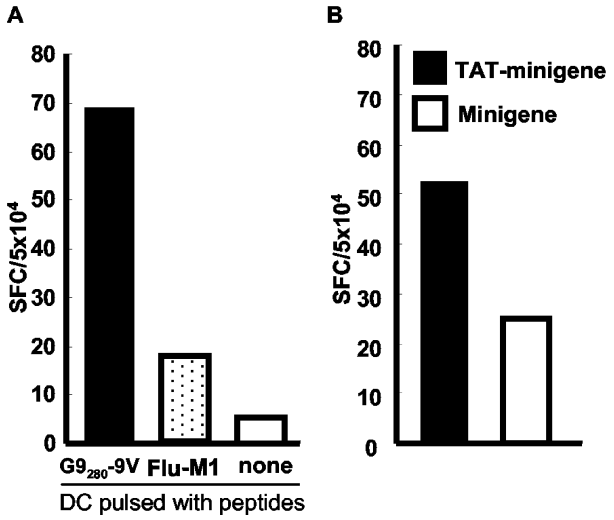

FIGURE 4. Recognition of TAT-minigene transduced DC by $G9_{280}$-9V-specific T cells. $G9_{280}$-9V-specific T cells were generated by stimulating CD8-enriched PBL with DC pulsed with 1 μM $G9_{280}$-9V peptide. After three weekly rounds of stimulation, T cells were tested for peptide-specific recognition. *A*, Recognition of DC pulsed with 1 μM $G9_{280}$-9V (■), Flu-M1 peptide (▨), or no peptide (□) by $G9_{280}$-9V-specific T cells. DC pulsed with Flu-M1 or $G9_{280}$-9V peptide were used as target cells, and $G9_{280}$-9V-stimulated T cells were used as effector cells. *B*, Recognition of 1 μM FP-pulsed DC by $G9_{280}$-9V-specific T cells. DC transduced with TAT-minigene (■) or minigene FP without TAT (□) were used as target cells, and $G9_{280}$-9V-specific T cells were used as effector cells. One representative experiment of two performed is shown.

fusogenic peptides (24), and outer membrane protein A from *Klebsiella pneumoniae* (25). Various methods have been employed to make FP, such as chemical cross-linking, *E. coli or baculovirus* expression systems, and artificial peptide synthesis. The system developed by Dowdy et al. (16) relies on the TAT protein of HIV-1. TAT has the ability to cross the membrane of almost every mammalian cell with an efficiency close to 100% (19). The TAT expression vector pTAT-HA contains a His tag, TAT domain, and HA tag in a small segment. The His tag facilitates purification of the final FP, and the HA tag permits detection of the final FP by Western blot. Furthermore, the short carrier protein keeps the size of the final FP small and, therefore, easily expressible in *E. coli*. The *E. coli* protein expression system is one of the most simple and cost effective and, therefore, is suitable for large scale production. The main advantage of TAT FP is the remarkably high transduction efficiency. Although the mechanism underlying this system's tremendous transduction efficiency is still unclear, urea denaturing during purification of the TAT FP is known to play an important role (16). Our data suggest that TAT FP successfully reach the cytoplasm within 20 min and are effectively processed through the HLA class I presentation pathway. Recent efforts demonstrated that T2 cells pulsed with TAT linked to the HLA-A2-restricted carcino embryonic Ag (CEA) epitope p691–699 were recognized by a CEA peptide-specific CTL clone to the same extent as CEA_{691} peptide-pulsed T2 cells (26). This means that TAT FP can deliver peptides to HLA class I molecules for presentation in a TAP-independent fashion. However, TAT FP is also taken up and processed by DC through the MHC class II pathway, as was recently shown for a TAT-OVA FP, which, after uptake by DC, induced OVA-specific CD4 T cells (27)

One of our unique approaches in this study was to fuse TAT with a minigene. The minigene strategy is one potential approach to induce multiple responses against conserved T cell epitopes. Since the minigene is a completely artificial gene, the sequence was customized to our purposes. For example, to increase the HLA binding affinity, a modified gp100-derived peptide sequence, $G9_{280}$-9V, was used to facilitate a peptide-specific CTL response instead of the wild-type sequence. Furthermore, by using a minigene, large amounts of a relatively small, multi-T cell epitope-containing protein are easily generated by the *E. coli* expression system.

In this study TAT-minigene FP-transduced DC were recognized by both Flu-M1- and $G9_{280}$-9V-specific CTL lines. The TAT-minigene FP induced a CTL response against Flu-M1, but, surprisingly, not against the $G9_{280}$-9V epitope. Similarly, a previous study showed that a retrovirus containing a minigene that encoded MART-1 and Flu-M1 transduced into bone marrow-derived DC generated only Flu-M1-specific CTL (28). Since the $G9_{280}$-9V peptide is an already proven immunogenic peptide (18), we anticipated that DC transduced with TAT-minigene would induce $G9_{280}$-9V-specific T cells. We speculate that the Flu-M1 epitope suppressed the expansion of $G9_{280}$-9V-specific T cells from normal donors because of its rapid induction of Flu-M1-specific T cells. Another possible explanation is that the proteasome in DC might not efficiently excise $G9_{280}$-9V peptide from TAT-minigene FP, so that DC present insufficient numbers of HLA-peptide complexes on the surface to induce specific T cells. Considering that the TAT-minigene FP is a totally artificial protein, it might be possible to customize the amino acid sequence for the proteasome in DC to readily produce $G9_{280}$-9V peptide from the minigene sequence. However, there is no reliable way to predict the processing patterns of protein by the proteasome.

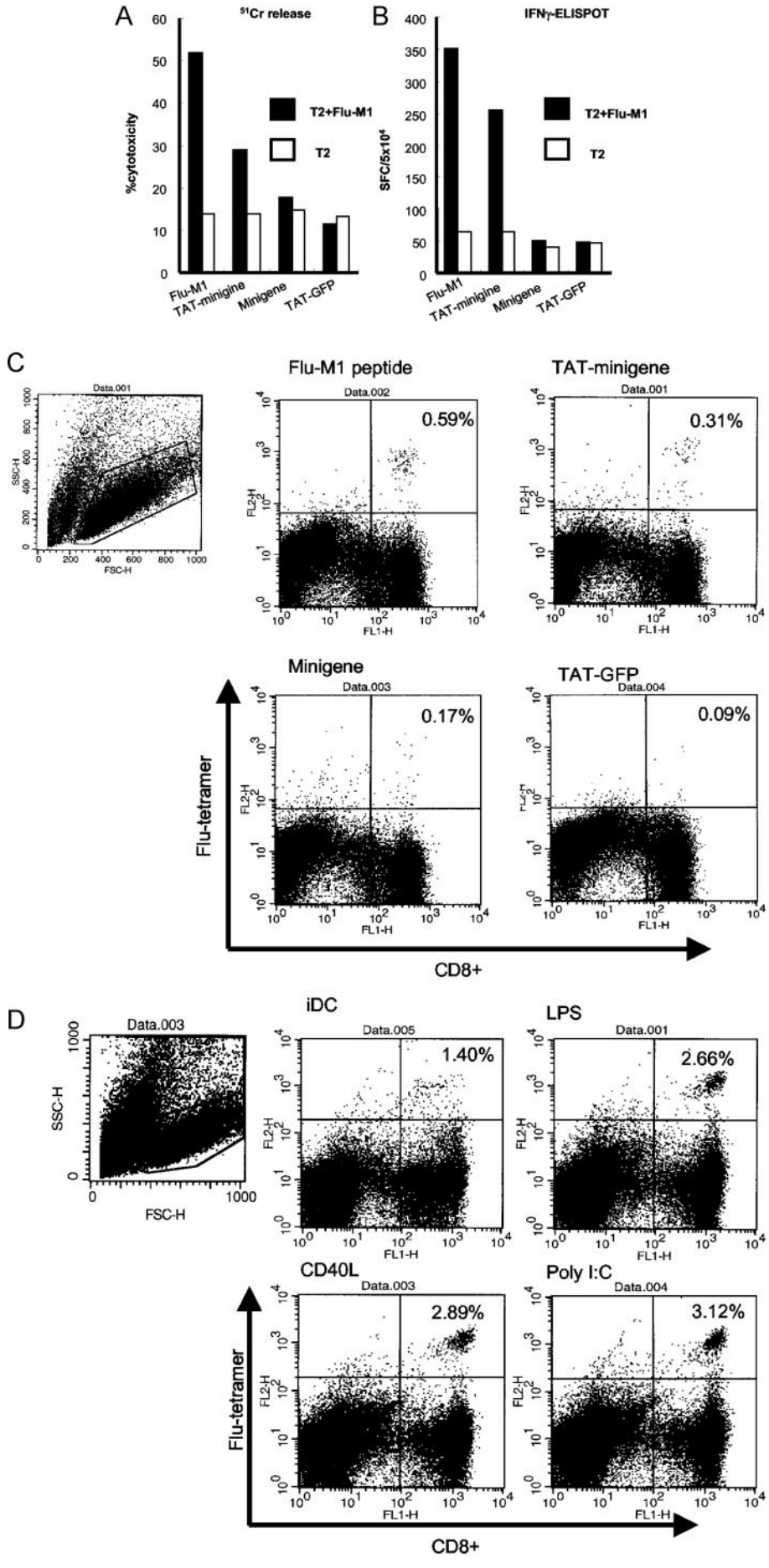

FIGURE 5. Induction of Flu-M1-specific T cells by DC pulsed with Flu-M1 peptide, TAT-minigene, minigene without TAT, and TAT-GFP. After pulsing DC with 1 μM Flu-M1 peptide or FP for 1 h, CD8-enriched PBL were stimulated with DC. After 10 days of culture with IL-2, bulk T cells were tested for Flu-M1-specific activity: *A*, ^{51}Cr release assay; *B*, IFN-γ ELISPOT assay. T2 cells pulsed with (■) or without (□) 1 μM Flu-M1 peptide were used as target cells. *C*, Flu-M1-specific tetramer analysis. T cells were stained with Flu-M1-specific tetramer and analyzed by FACS. The percentage in each FACS profile represents tetramer-positive and CD8$^+$ T cells. *D*, Induction of tetramer-positive, CD8$^+$ T cells by iDC or DC stimulated with LPS, CD40L, or polyinosinic-polycytidylic acid pulsed with TAT-minigene. Stimulated T cells were evaluated for tetramer-positive cells by flow cytometry. One representative experiment of three performed is shown.

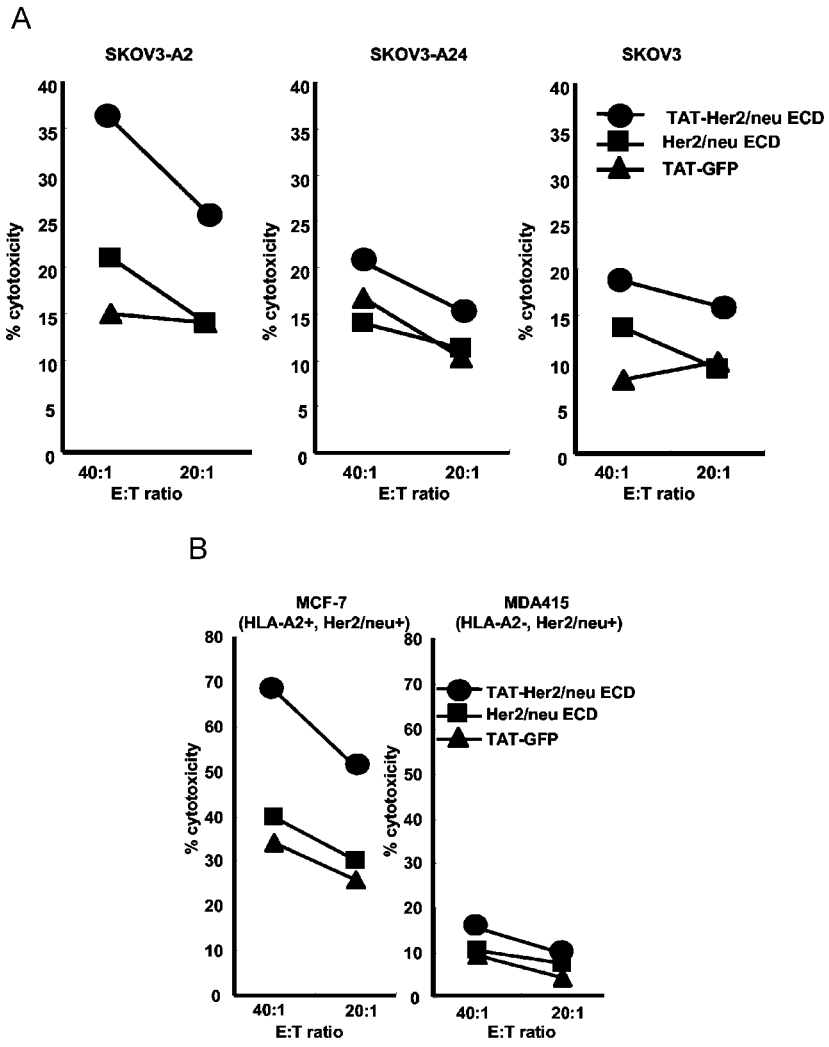

FIGURE 6. Induction of Her2/neu ECD-specific T cells by DC transduced with TAT-Her2/neu ECD. T cells stimulated by DC pulsed with Her2/neu FP with (●) or without TAT (■) or TAT-GPF FP (▲) were analyzed for CTL activity by ^{51}Cr release assay after four weekly stimulations. *A*, The HLA-A2 transfected ovarian cancer cell line, SKOV3-A2, and the control cell lines, SKOV3 and SKOV3-A24, were used as target cell lines. *B*, Breast cancer cell lines, MCF-7 (HLA-A2$^+$, Her2/neu$^+$), and MDA415 (HLA-A2$^-$, Her2/neu$^+$) were used as target cell lines. Each cell line was sensitized by 500 U of IFN-γ for 48 h before assay.

In addition, our results show that peptide pulsing was superior to TAT-minigene pulsing in terms of induction of Flu-M1-specific T cells. TAT FP require processing by the proteasome to generate peptides. This process may reduce the efficiency of the peptide presentation by HLA molecules compared with peptide pulsing. Nonetheless, the naturally processed TAT-minigene FP induced significant numbers of Flu-M1-specific T cells. It should be taken into account that the strength of TAT FP lies in the ability to generate multiple T cell epitopes that could result in a stronger overall T cell response than when an individual peptide is used. This was recently demonstrated by Shibagaki et al. (27) using the model tumor Ag OVA. Moreover, it is possible that the difference between peptide-pulsing and transduction with TAT FP is smaller or even absent when a peptide with a lower binding affinity and/or immunogenicity than Flu-M1 is used, as was recently shown for a CEA-derived peptide and an OVA-derived peptide by Lu et al. (26).

To investigate whether TAT FP can be applied to epithelial cancer-related tumor Ags, we selected the Her2/neu proto-oncogene and fused the TAT domain with Her2/neu ECD. The Her2/neu gene is overexpressed in a proportion of breast, ovarian, lung, pancreas, and renal cell carcinomas, and Her2/neu-derived peptides are recognized by CTL (21, 29–31) and Th cells (32). TAT-Her2/neu-transduced DC efficiently induced a Her2/neu-specific immune response in an HLA-A2-restricted fashion. We are currently investigating whether TAT-Her2/neu-transduced DC induce T cell responses restricted by other HLA class I allotypes as well as HLA class II alleles.

In conclusion, since TAT FP can transduce almost all types of mammalian cells within minutes, TAT FP containing tumor Ag(s) can potentially be applied to a wide variety of purposes, such as Ag

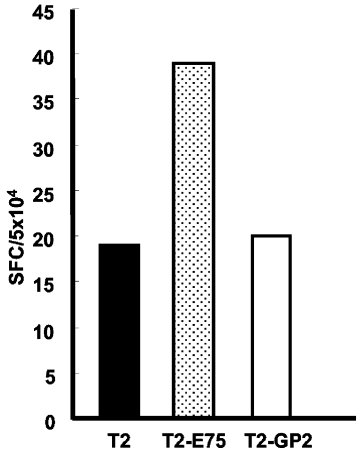

FIGURE 7. Peptide recognition by Her2/neu ECD-specific T cells stimulated by DC transduced with TAT-Her2/neu ECD. The peptide recognition of Her2/neu-specific T cells was evaluated by IFN-γ ELISPOT assay. T2 (■) and T2 pulsed with the Her2/neu ECD-derived immunodominant peptide E75 (▨) or the transmembrane portion-derived peptide GP2 (□) were used as target cells.

or antigenic peptide discovery, DC-based vaccine strategies, and basic research on MHC class I Ag presentation.

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