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Report

Tat mammaglobin fusion protein transduced dendritic cells stimulate mammaglobin-specific CD4 and CD8 T cells

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Key words: antigen-presenting cell, breast cancer vaccine, mammaglobin, protein transduction, T cells, Tat fusion protein

Summary

Proteins can be efficiently introduced into cells when fused to a protein transduction domain, such as Tat from the human immunodeficiency virus. We recently reported that dendritic cells transduced with a Tat fusion protein containing the extracellular domain of Her2/neu (Tat-Her2/neu) induced CD8 cytotoxic T lymphocytes (CTL) that specifically lysed Her2/neu-expressing breast and ovarian cancer cells. In the current study we further investigated the mechanism of protein transduction, utilizing the breast cancer-associated protein, mammaglobin-A, which is expressed in about 80% of breast cancers. Using a Tat-mammaglobin fusion protein, we tested the ability of Tat-mammaglobin transduced dendritic cells to stimulate antigen-specific CD4 and CD8 T cells. Low levels of serum considerably improved protein transduction as determined by Western blot, and also improved presentation of antigenic peptide as evidenced by functional studies using antigen-specific T cells. Confocal microscope analyses of antigen-presenting cells (APC) incubated with Tat-mammaglobin showed localized distribution in addition to diffuse distribution in the cytosol. In contrast, mammaglobin lacking Tat showed only a localized distribution. Simultaneous incubation with both proteins resulted in overlapping localized distributions, suggesting Tat fusion proteins are processed through both the MHC class I and class II pathways. Indeed, stimulation of T cells with Tat-mammaglobin transduced dendritic cells led to an expansion of mammaglobin-specific CD4 T helper-1 lymphocytes along with CD8 CTL.We conclude that Tat-mammaglobin transduced dendritic cells can induce *both* CD4 and CD8 mammaglobin-specific T cells. These findings could be further exploited for the development of a mammaglobin-based vaccine for breast cancer.

Abbreviations: Tat: trans-activating; APC: antigen-presenting cells; HIV: human immunodeficiency virus; MHC: major histocompatibility complex; MGB: mammaglobin; CTL: cytotoxic T lymphocyte; IL: interleukin; FITC: fluorescence isothiocyanate; PBMC: peripheral blood mononuclear cells; E/T: effector/target; IFNγ: interferon gamma

Introduction

Clinical application of vaccines requires that they are safe, well tolerated, easy to generate and administer, and applicable to virtually all patients. Attempts to generate effective vaccines have led investigators to use dendritic cells in the design of vaccines. The importance of dendritic cells in eliciting T cell responses is highlighted by studies in animal models in which protective immunity to viruses and tumors was induced. In addition, clinical trials involving dendritic cell-based immunization of cancer patients have been completed with promising results [1,2]. Successful immunization and clinical responses were achieved with minor or no toxicity with dendritic cells presenting CD8 and/or CD4 T cell epitopes. The data also suggest that immunization against non-mutated, self antigens can be successful, suggesting that T cell tolerance to self antigens such as the breast cancer protein, mammaglobin-A can be broken by using dendritic cells.

We recently developed a strategy for stimulating antigen-specific T cells using dendritic cells transduced with recombinant Tat fusion protein [3]. The Tat protein from the human immunodeficiency virus (HIV)-1 is used as a carrier for the specific delivery of antigenic protein into dendritic cells. The direct

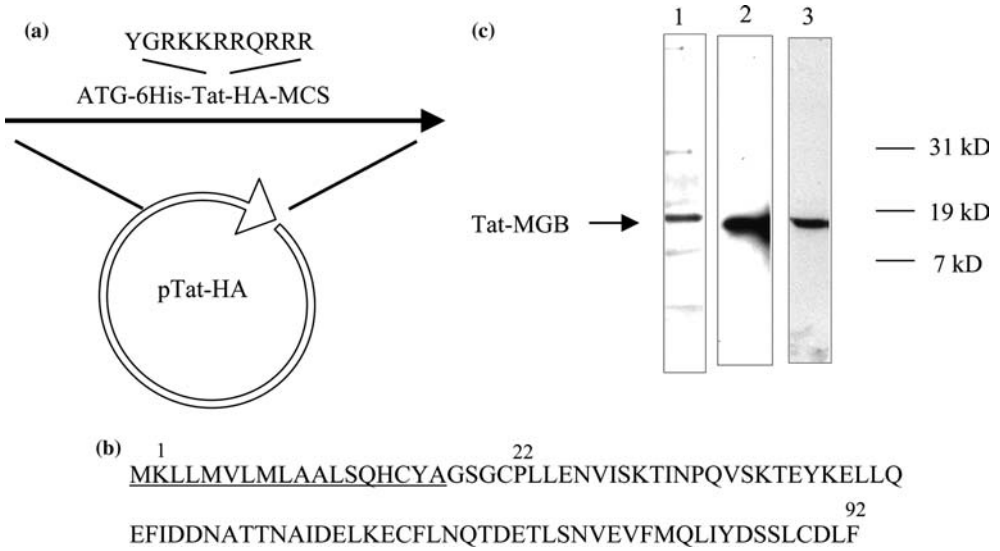

Figure 1. Schematic overview of the pTAT-HA vector (a) which after the initiation codon, ATG, has six histidines (6His), the Tat fragment, the hemagglutinin (HA) tag, and a multicloning site (MCS). The mammaglobin protein (b) contains 92 amino acids including an 18 amino acid signal sequence (underlined). A fragment encoding amino acids 22, proline, through 92, phenylalanine, was cloned into the pTAT-HA vector. Purified Tat-MGB has a molecular mass of ~17 kD (c) Clear bands at the predicted molecular weight were detected after gel electrophoresis using reducing conditions, and Coomassie staining (lane 1), and after immunoblotting with an anti-HA antibody (lane 2) or polyclonal anti-mammaglobin antibody (lane 3).

delivery of proteins to cells is now a well-documented and feasible methodology [4]. Proteins or peptides can be directly inserted into cells using viral-mediated protein delivery motifs such as Tat [4,5]. The latter process is called protein transduction [5,6]. Studies by Dowdy and colleagues have shown the Tat protein delivery motif can be easily tagged to the protein or peptide of interest [6,7]. The recombinant fusion protein is delivered to cells with nearly 100% efficiency when added exogenously to medium in concentrations as low as 1 nM [4,8]. Tat fusion proteins ranging in size of up to 120 kD have successfully been transduced into a large variety of mammalian cells and tissues in vivo [9]. The unique ability of the Tat protein to transport proteins into the cytosol of target cells such as dendritic cells permits MHC class I-mediated processing and presentation, i.e. cross-priming [10]. Consequently, antigen-specific CD8 T cells can be induced, as has been demonstrated by several groups [3,11–13]. However, recombinant Tat fusion protein could potentially also be taken up by dendritic cells through standard uptake mechanisms, e.g. endocytosis. This would lead to MHC class II-mediated processing and potentially to stimulation of CD4 T cells, as was shown for the model antigen, ovalbumin [14]. The simultaneous activation of antigen-specific CD4 and CD8 T cells may lead to an improved overall immune response. In the current study we further investigated the mechanism of Tat-mediated protein transduction. In addition, the possibility that protein transduced dendritic cells stimulate antigen-specific CD4 and CD8 T cells was studied using a Tat fusion protein containing the breast cancer-associated protein, mammaglobin [15].

Materials and methods

Recombinant proteins

Tat fusion proteins were generated using the Tat fusion vector, pTAT-hemagglutinin (pTAT-HA), a kind gift from Dr. S. Dowdy, UCSD, CA [4]. The plasmid contains an initiation codon, ATG, followed by six histidines (His-tag), the HIV Tat domain, an HA tag, and a multicloning site followed by a termination codon (Figure 1a). Both Tat-Her2/neu and Tat-M1 were described previously [3]. The influenza M1 peptide containing Tat fusion protein (Tat-M1) was initially named Tat-minigene as it also contains a peptide from the melanoma antigen gp100. However, because of the emphasis in the current study on M1, the fusion protein was renamed Tat-M1. Tat-mammaglobin (Tat-MGB) fusion protein was generated by cloning a nucleotide fragment encoding amino acids 22-92 of mammaglobin (Figure 1b) into pTAT-HA. For this, MDA-MB-415 DNA was isolated and two oligonucleotide primers were used to extend the desired fragment through PCR. The desired fragment was cloned as a *Kpn*I–*Eco*RI fragment, and its correct sequence was confirmed through sequence analysis. Full-length recombinant mammaglobin-A without Tat (MGB) was provided by Dr. T. Fleming.

Purification of Tat fusion proteins

Tat fusion proteins were purified as described [3,4] with a few modifications. Briefly, Tat fusion proteins were expressed in BL21-CodonPlus (DE3)-RP competent cells (Stratagene, La Jolla, CA). Bacteria were grown in

Terrific Broth (Sigma, St. Louis, MO) and sonicated in buffer Z (8 M urea/100 mM NaCl/20 mM HEPES, pH 8.0). Tat fusion proteins were purified from the bacterial supernatant on a Ni-NTA column (Qiagen, Valencia, CA) under denaturing conditions. Proteins were refolded and urea was removed by dialysis. Finally, purity and concentration were determined by gel electrophoresis (PAGE) and Coomassie staining, and the identity of the purified protein was confirmed by immunoblot, using an anti-HA antibody (Covance, Berkeley, CA). For Tat-MGB, a polyclonal anti-mammaglobin A-antibody (Dr. T. Fleming) was also used (Figure 1c).

Confocal microscope analysis

Tat-MGB, and Tat-M1 were fluorescently labeled with Fluorescein (FITC), and MGB (without Tat) was labeled with Alexa Fluor 546 using protein labeling kits (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Unbound fluorescent dye was removed by dialysis. Concentration of the labeled proteins was determined by comparison to protein standards after gel electrophoresis. Fluorescent-labeled proteins were incubated with APC for various lengths of time. The cells were subsequently washed twice in PBS, fixed in 4% paraformaldehyde buffer, and analyzed with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) at 1000× magnification. To obtain thin optical slices the confocal pinhole was kept small.

Peptide synthesis

Peptides MAM3 (p79–88) and MAM7 (p1–9) [16] were purchased from BioSynthesis (Lewisville, TX). The HLA-A2-restricted influenza matrix protein-derived peptide M1 (GILVFTFTL) was purchased from Genemed Synthesis (San Francisco, CA).

Cell lines

The HLA-A2-positive lymphoblastoid B cell line, JY; the HLA-A2-positive, TAP-deficient T cell-B cell hybrid, T2, and the human breast cancer cell line, MDA-MB-415, were purchased from ATCC (Manassas, VA). MDA-MB-415-A2.1 and MDA-MB-415-A24 cell lines are MDA-MB-415 transfected with the genes for HLA-A2.1 and HLA-A24, respectively [16]. All cell lines were cultured in RPMI (Mediatech, Herndon, VA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma), 1% glutamine, and 1% penicillin-streptomycin, and 1% HEPES (all Cellgro, Herndon, VA).

In vitro *generation of dendritic cells*

Dendritic cells were generated from adherent peripheral blood mononuclear cells (PBMC). PBMC were isolated after centrifugation of peripheral blood on Histopaque-1077 (Sigma) and cultured in 10 cm^2 culture dishes (1×10^8 cells/dish). Non-adherent cells were removed after 1 h at 37 °C and cryopreserved for later use. The adherent cells were predominantly monocytes and were cultured in X-VIVO 15 medium (BioWhittaker, Walkersville, MD) and 400 U/ml interleukin (IL)-4 plus 800 U/ml granulocyte-macrophage colony stimulating factor (both from Pierce, Rockford, IL). On day 3, new medium and cytokines were added. The monocytes differentiated into immature dendritic cells over a six-day period. Maturation was induced on day 6 using 100 ng/ml lipopolysaccharide (Sigma). After an additional 24 h, the cells were harvested and used as mature dendritic cells. The phenotype of the dendritic cells was confirmed using specific antibodies against CD83, CD11c, CD54, HLA-DR, and Lineage cocktail (Lin) 1, and analysis by flow cytometry, as described [3].

T cell stimulation

Heparinized peripheral blood from healthy donors was obtained from the American Red Cross (St. Louis, MO). CD4 T cells were isolated using RosetteSep enrichment mixtures (StemCell Technologies, Vancouver, BC). Purified T cells (containing <5% CD8 T cells, data not shown) were depleted of $CD25^+$ cells using magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). T cells were cultured in AIM-V medium (Gibco, Grand Island, NY) with 2.5% (v/v) pooled human AB serum (hABS; Valley Biomedical, Winchester, VA), 1% glutamine, and 1% penicillin/streptomycin. Autologous dendritic cells were co-cultured with protein for 4 h at 37 °C in AIM-V plus 2.5% hABS. The cells were subsequently irradiated with 5000 rad, washed, and added to T cells at a T cell to dendritic cell ratio of 10:1. Recombinant IL-2 (a kind gift from Amgen, Thousand Oaks, CA) was added every 3–4 days at a concentration of 50 IU/ml. The T cells were restimulated twice at weekly intervals at a ratio of 20:1. To generate M1-specific T cells, PBMC of $HLA\text{-}A2^+$ healthy donors were pulsed with M1 peptide (1 μg/ml), irradiated and co-cultured with autologous PBMC at a ratio of 1:1 for 12–14 days without restimulation. Before use, the presence of M1-specific CTL was confirmed by staining with an M1/HLA-A2 specific tetramer and anti-CD8 antibody (Beckman Coulter, Fullerton, CA), followed by FACS analysis (BD BioSciences, San Diego, CA).

Cytotoxicity assay

Standard 4-h ^{51}chromium (Cr) release assays were performed to test the antigen-specific recognition by stimulated T cells. Various effector to target (E/T) cell ratios were tested in triplicate. Target cells were the mammaglobin expressing MDA-MB-415 cell lines, T2, and JY. MDA-MB-415 and its transfectants were

exposed to 250 IU/ml IFNγ 24 h prior to culture with T cells to induce ICAM-1 expression, as described [16]. After exposure, tumor cells were extensively washed to remove all IFNγ. Peptide pulsing was carried out prior to labeling with ^{51}Cr by incubation for 2 h at 37 °C. Likewise, target cells were incubated with Tat fusion protein for 4 h at 37 °C before labeling with ^{51}Cr. In selected experiments, brefeldin A (Sigma) was added at a final concentration of 2.5 μg/ml during the pulsing period.

IFNγ ELISA

CD4 T cells (10^6/ml) were co-cultured with autologous dendritic cells for 24–48 h at a ratio of 5:1. Supernatant was collected, and cells and debris was removed. The supernatants were tested in duplicate for the presence of IFNγ by ELISA (BioSource). PBL cultures were co-cultured with irradiated MDA-MB-415 parental cells and the corresponding HLA-A2 and HLA-A24 transfected cell lines. Supernatants from T cell/tumor cell co-cultures were harvested and tested for the presence of IFNγ. Wells containing tumor cells only did not contain detectable levels of IFNγ (data not shown).

Results

Generation and purification of Tat fusion proteins

Both Tat-M1, containing the influenza virus-derived M1 peptide, and Tat-Her2/neu were purified using previously described protocols [3]. Tat-MGB (Figures 1a and b) was obtained through a slightly modified protocol, and its purity and concentration were determined by gel electrophoresis and Western blot, respectively (Figure 1c). An antibody to the HA tag that is unique to Tat fusion proteins as well as an anti-mammaglobin antibody detected a band at about 17 kD, identifying the purified protein as Tat-MGB.

Protein transduction is serum dependent

We and others showed earlier that protein transduction of antigen-presenting cells is concentration dependent [3,4]. Protein transduction appeared to be dependent also on serum, as transduction in the absence of serum was not nearly as efficient as in the presence of serum (Figure 2a). Addition of relatively small amounts of serum (2.5%) greatly increased the transduction efficiency. Enhanced transduction in the presence of low amounts of serum also improved cross-priming and recognition of the transduced cells (Figure 2b). Using Tat-M1, B cells transduced in the presence of 2.5% serum were lysed more efficiently by M1-specific CTL than B cells transduced in the absence of serum.

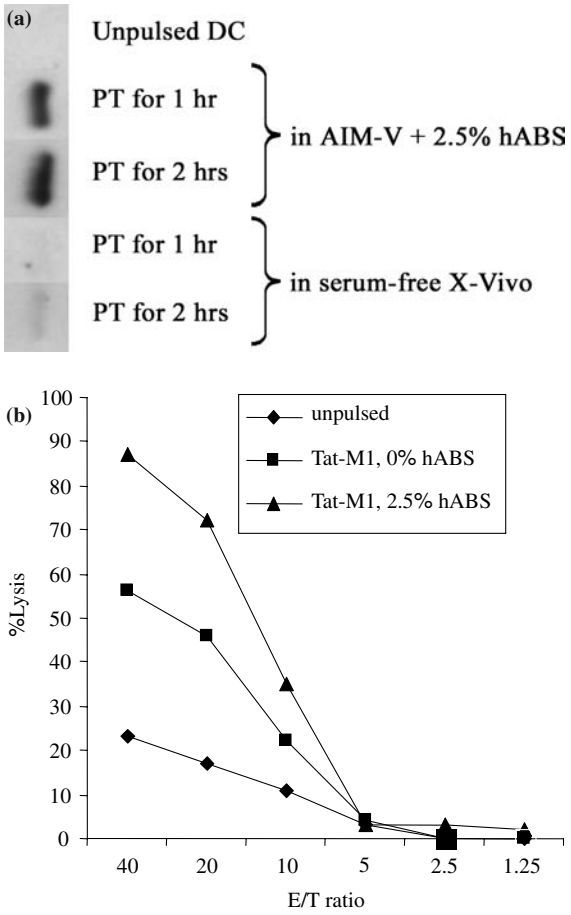

Figure 2. Tat fusion protein-mediated protein transduction (PT) and cross-priming is serum dependent. (a) Dendritic cells were transduced for 1 or 2 h with Tat-Her2/neu in the presence or absence of serum, and evaluated for the presence of fusion protein by Western blot using an anti-HA antibody. Protein concentrations were determined by Bradford assay, and identical amounts of protein were loaded in each lane. (b) T2 cells, transduced with Tat-M1 in the presence and absence of serum, were tested for lysis by M1-specific CTL in a standard 4-h chromium-release assay.

Confocal microscope analysis of protein transduced APC

To determine the cellular distribution of Tat fusion proteins, confocal analysis was performed on APC incubated with fluorescent-labeled fusion protein. After 30 min incubation, a diffuse cytoplasmic staining pattern was observed using FITC-labeled Tat-M1 (Figures 3a and b). Prolonged incubation for 3 h resulted in localized as well as diffuse staining patterns (Figures 3c and d). To compare the localization of antigenic proteins with and without Tat, proteins were labeled with different fluorescent dyes and equimolar amounts were added to APC. The transduction by FITC-labeled Tat-MGB was rapid, as fluorescence after 10 min was intense (Figure 4a) and similar to that observed after 3 h (Figure 4e). As with Tat-M1, Tat-MGB showed a clear diffuse cytoplasmic distribution as well as a localized distribution in APC after prolonged incubation (Figures 4a and e). In contrast, Alexa 546-labeled mammaglobin without Tat showed exclusively a localized distribution (Figure 4f), in agreement with known pro-

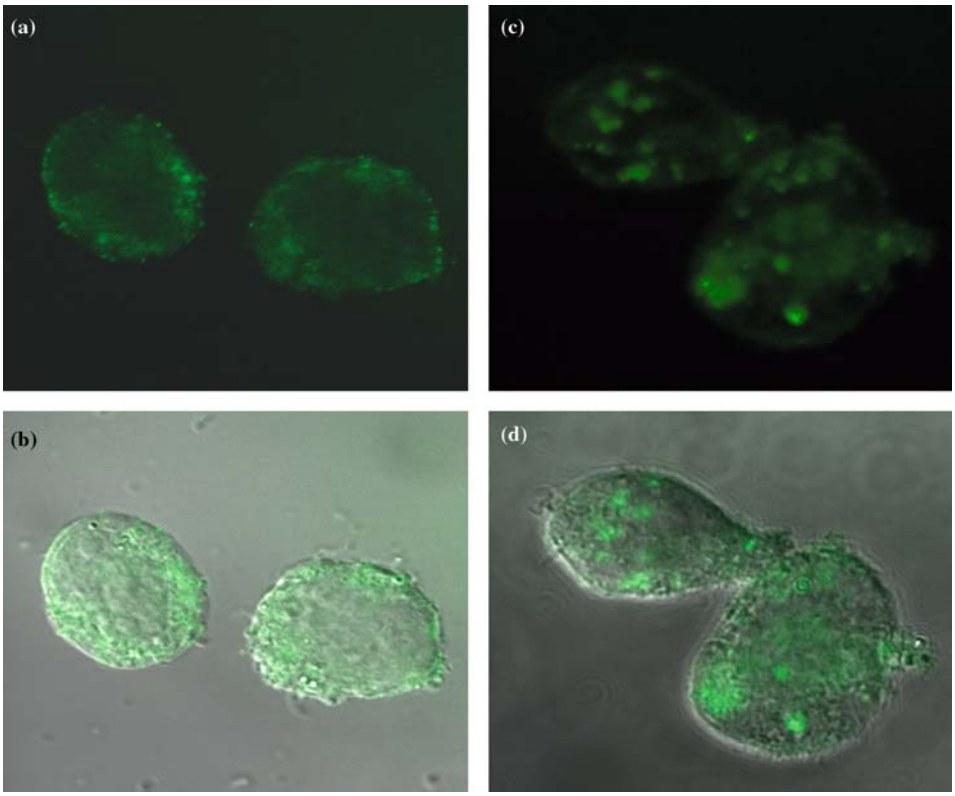

Figure 3. Tat fusion protein efficiently crosses membranes. Pulsing of APC with FITC-Tat-M1 resulted initially in a diffuse, cytoplasmic distribution. Prolonging the pulsing time additionally led to localized distribution. Confocal micrographs (1000×) of T2 transduced with 1 μM FITC-labeled Tat-M1 for 30 min (a/b) and 3 h (c/d). Top panels (a, c) show the epifluorescent picture, and the bottom panels (b, d) show an overlay on the corresponding bright field image.

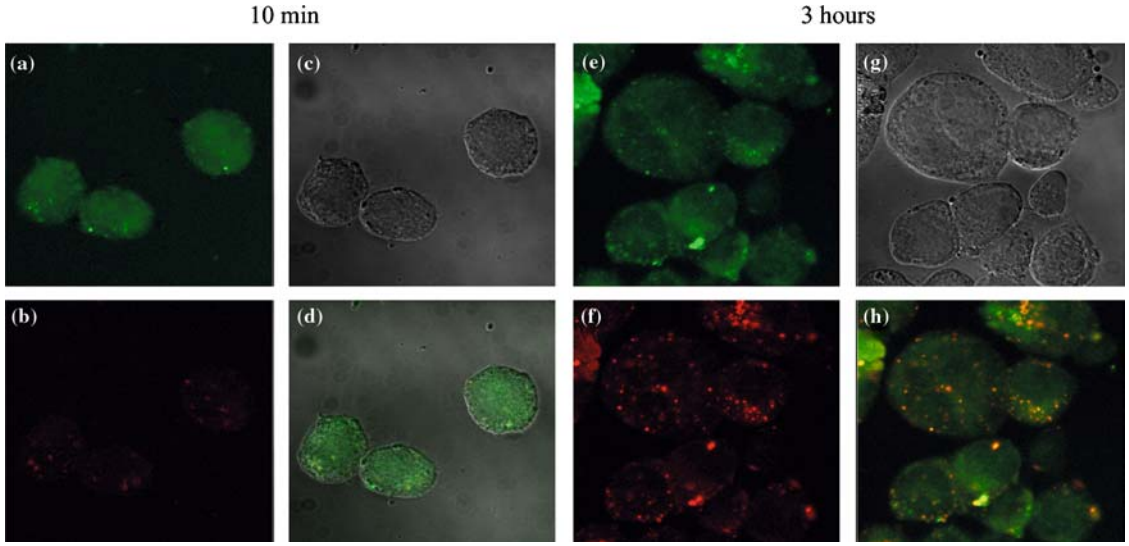

Figure 4. Intracellular localization of antigenic proteins with and without Tat. Confocal micrographs (1,000×) of T2 pulsed with equimolar amounts of FITC-Tat-MGB and Alexa 546-MGB without Tat for 10 min (a–d) or 3 hrs (e–h). Left panels are FITC (a and e) or Alexa 546 (b and f) channels alone, upper right panels (c and g) show bright field image, lower right panels (d and h) show overlay of all three aforementioned. Proteins with or without Tat co-localized in the same compartments (h, yellow).

tein uptake mechanisms that result in MHC class II-mediated antigen processing. Furthermore, uptake of mammaglobin without Tat occurred gradually as evidenced by a clear difference in fluorescence intensity between the 10 min evaluation (Figure 4b) and the 3 h time point (Figure 4f). Dual color analysis showed both mammaglobin and Tat-MGB localized to the same intracellular compartments (Figures 4d and h). These observations suggest that Tat-MGB is processed through both MHC class I and class II pathways. Similar studies using Alexa 546-labeled mammaglobin and FITC-Tat-M1 showed similar results (data not shown).

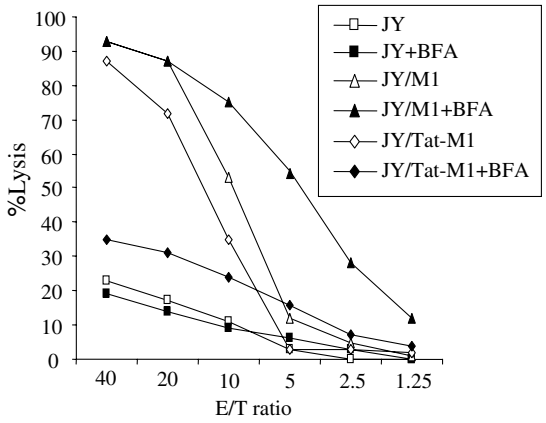

Figure 5. Brefeldin A (BFA) blocks presentation of M1-peptide after protein transduction. JY cells were transduced with Tat-M1 or pulsed with M1 peptide in the presence or absence of brefeldin. Recognition of M1 on JY was tested with M1-specific CTL in a 4-h chromium-release assay.

Tat-mammaglobin transduced dendritic cells stimulate both CD8 and CD4 T cells

Extending earlier observations that Tat promotes cross-priming, protein transduction was performed in the absence and presence of brefeldin A, a reagent known to block egress of MHC-peptide complexes from the endoplasmic reticulum. Protein transduced cells were subsequently tested for recognition by specific CTL. Using Tat-M1, lysis of Tat-M1 transduced B cells was strongly inhibited in the presence of brefeldin (Figure 5). By contrast, lysis of B cells pulsed with M1 peptide was not inhibited by brefeldin. In fact, brefeldin increased lysis of M1-pulsed B cells.

To evaluate if Tat fusion protein-transduced APC can stimulate both CD4 and CD8 T cells, T cells were stimulated with autologous, Tat-MGB transduced dendritic cells. After two restimulations at weekly intervals, T cell cultures were tested for mammaglobin-specificity. To demonstrate specific CD8 responses, HLA-A2.1$^+$ T cells were co-cultured with irradiated MDA-MB-415 breast cancer cells or with the HLA-A2.1 or HLA-A24 transfected MDA-MB-415. After 24 h, the supernatant was collected and tested for the presence of IFNγ by ELISA. Secretion of IFNγ was induced after stimulation with MDA-MB-415-A2.1, but not by MDA-MB-415 parental or HLA-A24-transfected MDA-MB-415 cells (Figure 6a). To test specificity for mammaglobin, the MDA-MB-415-A2.1 cells were pulsed with either MAM3 or MAM7 peptides. Both peptides are HLA-A2.1-binding peptides derived from mammaglobin [16]. Whereas MAM3 (p79–88) is included in Tat-MGB, MAM7 (p1–9) is not. The IFN secretion by CD8 T cells was increased after pulsing with MAM3, but not after pulsing with MAM7, suggesting the CD8 T cells recognized the mammaglobin-derived MAM3 peptide presented by HLA-A2.1 (Figure 6a). Likewise, MDA-MB-415-A2.1 was more susceptible to lysis by the CD8 T cells than MDA-MB-415 parental cells (Figure 6b).

To test if mammaglobin-specific CD4 T cells can be expanded, CD4$^+$ CD25$^-$ T cells were purified prior to stimulation with protein transduced dendritic cells. After three stimulations, CD4 T cells were tested for secretion of IFNγ. Autologous, irradiated dendritic cells transduced with Tat-MGB induced considerably higher levels of IFNγ than dendritic cells transduced with an irrelevant Tat fusion protein or unpulsed dendritic cells (Figure 6c). CD4 T cells from two consecutive healthy donors both showed specificity for mammaglobin after Tat-MGB stimulation.

Discussion

Dendritic cell-based tumor vaccines are being tested in clinical trials [17] because of their ability to induce antigen-specific T cell responses. One of the main challenges in designing dendritic cell vaccines is the introduction of tumor antigen for presentation. Many different strategies have been employed ranging from co-incubation of dendritic cells with tumor-derived peptide(s), protein, or tumor cells/lysates, to genetic modification using tumor antigen encoding DNA, RNA, or viral vectors [17]. Effective antitumor immune responses are thought to require the contribution of both CD4 and CD8 T cells [18,19]. Our goal to design cancer vaccines that induce antigen-specific CD4 and CD8 responses led us to explore the relatively novel strategy of protein transduction of APC. This strategy involves the use of a fusion protein consisting of a fragment of the HIV Tat protein, the protein transduction domain, and an antigenic protein of choice. Earlier studies demonstrated that Tat fusion proteins cross the membranes of virtually all mammalian cells at very high frequency [5]. Furthermore, using a model antigen, Kim et al. [10] showed that protein transduction through Tat fusion protein introduces protein into the MHC class I processing pathway. More recently, several groups have shown that tumor-specific CD8 T cells can be induced by protein transduced APC. Shibagaki and Udey [14] demonstrated proof-of-principle using the model antigen, ovalbumin. Others demonstrated that synthetic polypeptides encoding a protein transduction domain and an antigenic peptide transduced APC and induced peptide-specific CTL [13,20,21]. Our current data confirm and extend earlier observations that protein transduction using Tat fusion proteins leads to cross-priming and stimulation of MHC class I restricted CTL. Both Tat-M1 and Tat-MGB rapidly crossed the cell membranes of APC and localized, in part, in the cytosol of the APC (Figures 3 and 4). The Tat fusion proteins were processed through the MHC class I processing pathway, as evidenced by the ability of the transduced APC to stimulate MHC class I-restricted CTL (Figures 2b and 6ab). Cross priming occurred in T2 as well as in other cells, demonstrating it is independent of the presence of TAP. Furthermore, brefeldin A which is known to inhibit presentation of endogenous antigen by MHC class I, significantly inhibited the recognition of

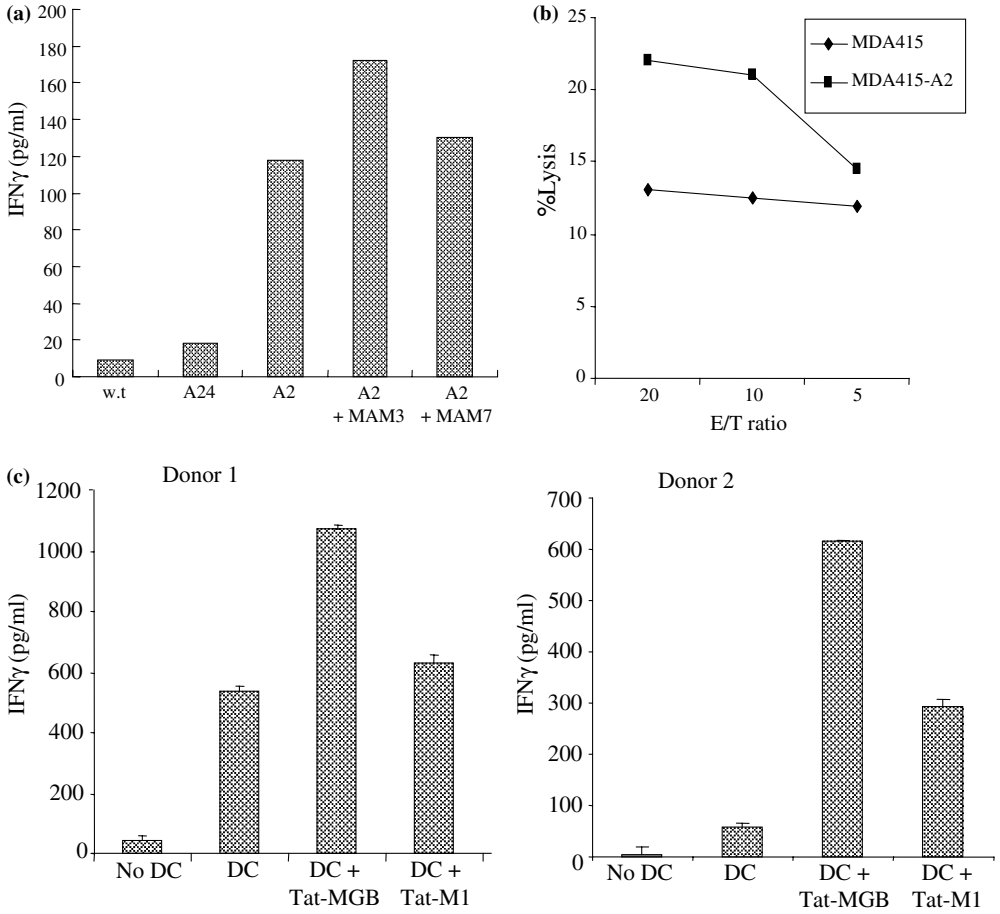

Figure 6. Dendritic cells transduced with Tat-MGB induce specific CD8 and CD4 T cells. (a) Three times stimulated T cells recognize the mammaglobin-expressing breast cancer cell line, MDA-MB-415-A2.1 significantly better than the MDA-MB-415 wild type (w.t.) cells or the MDA-MB-415-A24 cells. IFNγ secretion was enhanced in the presence of the HLA-A2.1-binding mammaglobin peptide, MAM3, but not in the presence of MAM7. (b) T cells stimulated with Tat-MGB lyse the mammaglobin-expressing MDA-415 cell line. Lysis is in part restricted by HLA-A2.1 as MDA-415-A2.1 is significantly better recognized than MDA-415 wild type. (c) Purified $CD4^+$ $CD25^-$ T cells from two healthy donors stimulated three times with Tat-MGB transduced dendritic cells were tested for secretion of IFNγ in response to various stimuli. IFNγ was detected by ELISA.

Tat fusion protein-transduced APC (Figure 5). Interestingly, brefeldin appeared to enhance the recognition of peptide-pulsed target cells (Figure 5), suggesting that by blocking the transportation of newly synthesized MHC-antigen complexes to the cell surface, the half-life of existing complexes at the cell surface is prolonged.

Our previous studies with Tat fusion proteins focused on induction of Her2/neu-specific CD8 T cells *in vitro* using a recombinant Tat-Her2/neu fusion protein containing a large fraction of the extracellular domain of Her2/neu [3]. Dendritic cells transduced with Tat-Her2/neu induced Her2/neu-specific CD8 T cells that recognized Her2/neu-expressing tumor cells. More recently, adoptive transfer experiments were performed with bone marrow-derived dendritic cells transduced with Tat-Her2/neu fusion protein. Immunized mice challenged with Her2/neu-expressing mouse breast tumor cells showed a delay in tumor development and grew significantly smaller tumors than non-immunized mice, or mice immunized with dendritic cells transduced with irrelevant Tat fusion protein (Viehl CT and Goedegebuure PS, submitted for publication). Similarly, Shibagaki et al. [11] and Lu et al. [12] demonstrated significant anti-tumor efficacy after immunization with dendritic cells transduced with a recombinant Tat fusion protein containing the melanoma antigen, TRP2, and a synthetic polypeptide containing TRP2 epitopes, respectively. These experiments demonstrate the potential of Tat fusion protein transduced dendritic cells to stimulate antigen-specific T cells. In theory, transduction of protein rather than peptide increases the chance that (i) multiple epitopes for both CD4 and CD8 T cells are included, resulting in a stronger overall immune response, and (ii) epitopes are included for the most common MHC class I as well as class II alleles which permits treatment of virtually all patients.

The current study provides evidence that in addition to CD8 T cells, antigen-specific CD4 T cells are induced by protein transduced dendritic cells. Analysis of the distribution of Tat fusion proteins by confocal microscope indicates endocytic uptake and processing through the MHC class II pathway (Figure 4). Although detection of Tat fusion protein in the cytosol precedes detection in specific compartments, prolonged exposure further increased endocytosis. More direct evidence for stimulation of antigen-specific CD4 cells

was obtained through functional analysis of Tat fusion protein stimulated CD4 T cells. Tat-MGB transduced dendritic cells induced specific CD4 T cells as evidenced by secretion of IFNγ (Figure 6c). Studies are ongoing to further characterize the CD4 response to mammaglobin.

Our studies currently focus on mammaglobin as a candidate molecule for a vaccine to breast cancer [15]. Several characteristics suggest that mammaglobin is an ideal candidate for immune targeting. First, approximately 80% of breast cancers express mammaglobin [22,23]. Its expression is almost exclusively confined to breast cells, and is reportedly higher on tumor cells than on normal cells. Second, mammaglobin is expressed in primary, occult, and metastatic disease, and even though it has not systematically been followed over time during tumor progression, available data suggest that expression is stable. Third, our earlier report [16] and reports from others [24–26] show that mammaglobin contains HLA-A2 and HLA-A3-binding peptides that stimulate CD8 T cells *in vitro* and *in vivo* in a CD8/HLA-A2 double transgenic mouse model. Thus, mammaglobin may be a suitable target for vaccine therapy of breast cancer.

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