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Stimulation of CD8$^+$ T Cells following Diphtheria Toxin-Mediated Antigen Delivery into Dendritic Cells

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Recognition and clearance of many intracellular pathogens requires the activation and subsequent effector functions of CD8$^+$ T lymphocytes. To stimulate CD8$^+$ T cells by immunization, the target antigens must be delivered into the cytosol of host cells. There they can be processed into peptides and presented in the context of major histocompatibility complex class I molecules to antigen-specific CD8$^+$ T cells. One method of delivering antigens into the cytosol is to fuse them to modified bacterial toxins that are able to enter mammalian cells. The expression pattern of the toxin receptors in the host will determine the cell population that the toxin fusion protein targets and will thus restrict antigen-specific T-cell recognition to the same population. In this study we describe the development and characterization of a diphtheria toxin (DT)-based antigen delivery system. Using CD11c-DTR transgenic mice that express the DT receptor in dendritic cells (DC), this system allows for targeted delivery of CD8$^+$ T-cell antigen to DC. We show that antigen-specific CD8$^+$ T cells proliferate in CD11c-DTR mice following immunization with catalytically inactive DT-antigen fusion proteins. We also show that a toxin-based system that restricts antigen delivery to DC results in more robust antigen-specific CD8$^+$ T-cell proliferation than a toxin-based system that does not restrict delivery to a particular cell type. These results have implications for vaccine design, and they suggest that use of a toxin-based vector to target antigen to DC may be an effective way to induce a CD8$^+$ T-cell response.

CD8$^+$ T lymphocytes are important for the effective clearance of many intracellular pathogens (27, 28). These lymphocytes are activated in response to foreign protein present in the cytoplasm of host cells. The source of this foreign protein could be microbial antigens produced in an infected cell or otherwise delivered into the host cell cytoplasm. Antigen-specific CD8$^+$ T cells recognize peptides that are derived from cytoplasmic proteins and are loaded onto major histocompatibility complex class I (MHC-I) molecules (35). Recognition of these peptide: MHC-I complexes results in T-cell activation, proliferation, and differentiation into effector and memory cells. Effector cells secrete cytokines and lyse infected cells, helping to clear the primary infection (26). Memory cells remain in the host for extended periods of time and provide enhanced control of subsequent challenges with the same pathogen.

Because CD8$^+$ T cells are critical for controlling and clearing infections with intracellular microbes, there has been much interest in developing safe and effective vaccines that specifically stimulate memory CD8$^+$ T cells. Vaccines designed to stimulate CD8$^+$ T cells must deliver the target antigens into the cytosol of host cells. One successful method of achieving cytosolic delivery is to use bacterial toxins as delivery vectors (43). Because many toxins are able to translocate across a host cell membrane, cytosolic delivery of heterologous antigens can be accomplished by fusing antigens to a translocation-competent toxin. Once the toxin fusion protein enters the cytosol, it is degraded by cellular proteases, and antigenic peptides are loaded onto MHC-I molecules for presentation to antigen-specific CD8$^+$ T cells. Catalytically inactive forms of the toxin can deliver antigens into cells without any resulting cytotoxicity. These modified toxins are both nontoxic and nonreplicative and are therefore free of many safety issues inherent with other vaccine vectors.

We have published extensively on a system that uses a nontoxic form of anthrax toxin (AT) to deliver antigens into murine host cells in order to stimulate antigen-specific CD8$^+$ T cells (2–4, 14, 29). The two cellular receptors for AT, ANTXR1 (7) and ANTXR2 (40), are widely expressed in murine tissue (6, 7, 40). AT, therefore, has the potential to direct the delivery of antigen into many cell types. While such widespread antigen delivery is advantageous in some ways, one potential disadvantage is the effective dilution away from professional antigen-presenting cells, most notably dendritic cells (DC). DC are highly specialized antigen-presenting cells that are very efficient at antigen uptake, processing, and MHC-restricted presentation to T cells (20). We considered whether using a toxin to target delivery of CD8$^+$ T-cell antigens to DC would improve T-cell stimulation following toxin fusion protein immunization.

In this study we describe the development and characterization of a toxin-based antigen delivery system that specifically delivers CD8$^+$ T-cell antigen to DC in mice. This delivery system is based on diphtheria toxin (DT) and uses recently developed transgenic mice that express the simian DT receptor (DTR) under the control of the murine *CD11c* promoter (CD11c-DTR mice) (23). Because mice do not naturally express DTR (10, 32), and CD11c is primarily expressed by DC (31), DTR expression in these mice is largely restricted to DC (23). Using DT-antigen fusion proteins, we were able to target delivery of antigen to DC in vivo. We demonstrated that a detoxified version of DT could deliver a heterologous antigen

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to the MHC-I processing and presentation pathway of DTR-expressing cells. As a result, antigen-specific $CD8^+$ T cells were stimulated both in vitro and in CD11c-DTR transgenic mice. Furthermore, in vivo antigen targeting to $CD11c^+$ DC by detoxified DT appeared to stimulate a more robust $CD8^+$ T-cell proliferative response than ubiquitous antigen delivery by detoxified AT.

MATERIALS AND METHODS

Mice. C57BL/6J (H-2^b) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). B6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J and C57BL/6-Tg(TcraTcrb)1100Mjb/J mice (referred to below as CD11c-DTR and OT-I mice, respectively) were originally obtained from the Jackson Laboratory. CD11c-DTR and OT-I mice (both on a C57BL/6 background; H-2^b) were maintained by backcrossing to C57BL/6 mice in a specific-pathogen-free barrier facility at Harvard Medical School. Offspring were screened by PCR amplification of tail DNA using transgene-specific primers. CD11c-DTR mice express a simian DTR-green fluorescent protein (GFP) fusion protein under the control of the murine *CD11c* promoter (23), and those used in this study have been backcrossed to C57BL/6 mice for at least 7 generations. DTR has been identified previously as the precursor of the heparin-binding epidermal growth factor (EGF)-like growth factor (proHB-EGF) (33). Simian proHB-EGF (encoded by the DTR transgene used in this study) binds DT, but endogenous murine proHB-EGF does not, because it lacks residues critical for toxin binding (10).

Cell lines and cell culture. B3Z cells, a $CD8^+$ T-cell hybridoma engineered to secrete β-galactosidase when its T-cell receptor engages an $Ova_{257-264}$:K^b complex (24, 25), were a gift of N. Shastri (University of California, Berkeley) and were maintained in a medium (RP-10) consisting of RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum, L-glutamine, HEPES, 50 μM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. DC were harvested from the bone marrow of C57BL/6 or CD11c-DTR mice and plated in a medium (DC+) consisting of Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum, 1 ng/ml interleukin-4 (Roche Diagnostics, Indianapolis, IN), 10 ng/ml granulocyte-macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ), 25 mM HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin. Nonadherent and semiadherent cells were harvested on the indicated days, and the percentage of $CD11c^+$ cells was determined by flow cytometry. Where indicated, $CD11c^+$ DC were incubated with anti-CD11c-conjugated magnetic cell sorting (MACS) microbeads and purified using magnetic separation columns as indicated by the manufacturer (Miltenyi Biotec, Auburn, CA). All cells were incubated at 37°C with 7% CO_2.

Recombinant diphtheria and anthrax toxins. $Ova_{257-264}$-DTm (Ova-DTm) was generated as follows. pET22b-DT-51E148K, a plasmid containing detoxified DTm inserted between the BamHI and XhoI sites of the multiple cloning region of a pET22b vector (Novagen, Madison, WI), was a gift of R. J. Collier (Harvard Medical School, Boston, MA). The antigen tag was generated by annealing single-stranded DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA) to form double-stranded DNA including (from 5′ to 3′) an NdeI site, the sequence encoding the K^b-restricted $Ova_{257-264}$ (SIINFEKL) peptide antigen, an EcoRI site, and a BamHI site. This DNA fragment was ligated to pET22b-DT-51E148K that had previously been digested with NdeI and BamHI. The resulting plasmids were used to transform *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA), and clones containing the insert were identified by EcoRI restriction digestion and DNA sequence analysis. One positive clone (pCS60) was transformed into *E. coli* BL21(DE3) (Novagen) for expression of the fusion protein. The vector encoding Ova-DTmE349K (pCS61) was generated with the QuikChange site-directed mutagenesis kit (Stratagene), using primers specific for the region of the gene to be mutated and using pCS60 as a template.

The expressed proteins, Ova-DTm and Ova-DTmE349K, contain a pET22b vector-encoded His_6 tag at the carboxyl terminus and therefore could be purified using a Ni^{2+} affinity chromatography column. Specifically, cultures of BL21 containing pCS60 or pCS61 were grown in Luria-Bertani broth (Difco Laboratories, Detroit, MI) containing 50 μg/ml ampicillin to an optical density at 600 nm of 0.6 to 0.8. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Cell pellets were sonicated, and the sonicate was passed over a Ni^{2+} column according to the manufacturer's instructions (Novagen). The bound protein was eluted with 0.5 M imidazole and exchanged into 20 mM Tris (pH 8) using a PD-10 gel filtration column (Amersham Biosciences, Piscataway, NJ). Recombinant protein was stored in 20 mM Tris (pH 8) and 150 mM NaCl. The amino acid sequence of the N-terminal fusion to DTm is M**SIINFEKL**ANSDP.

LFn (a nontoxic fragment of lethal factor)-$Ova_{257-264}$ was made and purified as described elsewhere (4). The fusion protein contains an amino-terminal His_6 tag and a carboxyl-terminal fusion encoding $Ova_{257-264}$. Protective antigen (PA) protein was a gift of R. J. Collier.

Flow cytometry. Antibodies used for surface staining of cells include phycoerythrin-conjugated anti-CD11c (HL3), phycoerythrin-conjugated anti-Vα2 (B20.1), and allophycocyanin-conjugated anti-CD8α (53-6.7). All antibodies were obtained from PharMingen (San Diego, CA). Cells were analyzed on a BD Biosciences FACSCalibur flow cytometer (San Jose, CA) and analyzed using CellQuest software.

Cytotoxicity assay. CD11c-DTR or C57BL/6 DC (5×10^4 in a total of 100 μl DC+/well) were incubated in a round-bottom 96-well plate with the indicated concentrations of DT (Sigma-Aldrich, St. Louis, MO) or Ova-DTm for 25 h at 37°C with 7% CO_2. Leakage of lactate dehydrogenase (LDH) from the DC cytoplasm into the medium (cytotoxicity) was quantified colorimetrically using the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI). Briefly, the absorbance at 490 nm (A_{490}) was measured using a microtiter plate reader (SpectraMax; Molecular Devices, Sunnyvale, CA) after the conversion of a tetrazolium salt into a red formazan product. The percentage of cytotoxicity was calculated as (100%) [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Spontaneous release is the amount of LDH released from the cytoplasm of untreated cells, whereas the maximum release is the amount released after treatment with lysis solution. In this assay, the percentage of cytotoxicity is limited by the percentage of $CD11c^+$ cells in the culture that express functional DTR.

Antigen presentation assay. Presentation of the $Ova_{257-264}$:K^b complex by toxin fusion protein-treated DC to B3Z cells was determined as follows. CD11c-DTR or C57BL/6 DC were treated with the indicated concentrations of toxin fusion protein for 45 min or 3 h in 1 ml DC+. When DC were treated with the AT-based antigen delivery system, both LFn-$Ova_{257-264}$ and PA proteins were added at the indicated molar concentrations. Where specified, DC were preincubated with 0.6 μM bafilomycin A1 (bafA1; Calbiochem, La Jolla, CA) for 25 min before toxin fusion protein was added. After three washes with RPMI, cells were resuspended in RP-10, and approximately 1.5×10^5 cells of each group were plated per triplicate well of a 96-well round-bottom plate. A total of 1×10^5 B3Z hybridomas were added to the DC in a final volume of 200 μl RP-10/well. After 24 h at 37°C with 7% CO_2, the medium was replaced with 150 μl lysis buffer (phosphate-buffered saline [PBS], 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP-40, and 0.15 mM chlorophenol red-β-D-galactopyranoside [Calbiochem]) per well. Following color change, the absorbance at 570 nm (A_{570}) was read using a microtiterplate reader.

Adoptive transfer and toxin immunization. Splenocytes and peripheral lymph nodes were harvested from OT-I mice as a source of $CD8^+$ T cells expressing the Vα2/Vβ5 T-cell receptor (TCR) specific for $Ova_{257-264}$ in the context of H-$2K^b$ (21). Erythrocytes were lysed by hypotonic shock using ammonium chloride. Twenty to twenty-five percent of the harvested cells were $V\alpha2^+$ $CD8^+$ as determined by flow cytometry. Total splenocytes were labeled with the intracellular fluorescent dye carboxyfluorescein diacetate succimidyl ester (CFSE) by incubation with 5 μM CFSE in PBS with 0.1% bovine serum albumin for 10 min at 37°C. Loading was stopped by addition of 5 volumes of cold RP-10. Cells were washed three times with RP-10, and 1.5×10^6 $V\alpha2^+$ $CD8^+$ double-positive cells (6×10^6 to 7.5×10^6 total splenocytes) were injected intravenously into tail veins of CD11c-DTR transgenic or C57BL/6 mice. Mice were immunized intraperitoneally (i.p.) 1 day after OT-I adoptive transfer with 30 pmol of either Ova-DTm or a mixture of 30 pmol LFn-$Ova_{257-264}$ and 30 pmol PA, all in PBS and in a total volume of 200 μl.

Statistical analysis. Data are expressed as means ± standard deviations (SD). Statistical significance of differences was analyzed using a two-tailed Student *t* test for independent samples (followed by Bonferroni's correction to adjust for multiple comparisons in Fig. 2B). A P value of <0.05 was considered statistically significant.

RESULTS

DTR-expressing DC treated with a nontoxic form of DT fused to a $CD8^+$ T-cell antigen stimulate antigen-specific T cells in vitro. To specifically target DT to DC, we used transgenic mice in which DTR expression is controlled by the murine *CD11c* promoter (CD11c-DTR mice) (23). In CD11c-

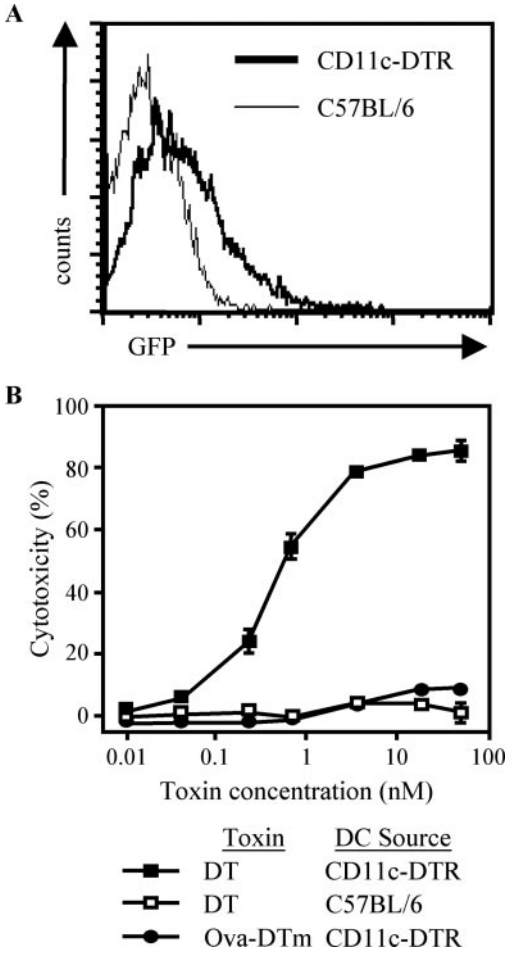

FIG. 1. CD11c+ DC cultured from the bone marrow of CD11c-DTR transgenic mice express functional DTR. (A) DC were cultured from the bone marrow of CD11c-DTR transgenic (thick line) and C57BL/6 control (thin line) mice. After 6 days, cells were incubated with anti-CD11c-conjugated MACS microbeads and purified using magnetic separation columns (>95% CD11c+). Flow cytometry was used to analyze DTR protein expression by the two cell populations. GFP fluorescence is used as a proxy for DTR expression, since the transgene encodes a DTR-GFP fusion protein. Histograms are gated on CD11c+ cells, and each histogram displays the same number of events. The mean fluorescence values for CD11c-DTR and C57BL/6 DC are 11.06 and 3.27, respectively. (B) CD11c-DTR (filled symbols) or C57BL/6 (open symbols) DC were cultured and purified as for panel A (>95% CD11c+) and were incubated with the indicated concentrations of DT (squares) or Ova-DTm (circles) for 25 h. Cytotoxicity was determined by LDH activity in the medium. Data are presented as means ± SD of triplicate wells and are representative of three independent experiments.

DTR mice, DTR expression by splenic CD11c+ cells has been demonstrated previously (23). To characterize antigen delivery by DT in vitro, we chose instead to use CD11c+ DC cultured from the bone marrow of the transgenic mice, and we showed that these DC also express the DTR protein (Fig. 1A). To determine whether native DT can target bone marrow-derived DC expressing DTR, we measured the ability of enzymatically active DT to kill these cells. In order for DT to kill a cell in this system, the heterodimeric protein must bind surface-expressed DTR and be internalized into an endosome via endocytosis. In the acidified vacuole, the toxin undergoes a conformational change and the amino-terminal portion containing the catalytic domain translocates across the vacuolar membrane into the cytosol (12). There it ADP-ribosylates elongation factor-2, inhibiting protein synthesis and ultimately killing the cell (12). We found that DT killed CD11c-DTR transgenic DC in a dose-dependent manner but did not kill control C57BL/6 DC, even at the highest DT concentration tested (Fig. 1B). Therefore, it is likely that DTR expression by DC cultured from the bone marrow of CD11c-DTR mice is able to function as a DT receptor, allowing toxin entry into the cytosol.

To create a DT-antigen fusion protein, we genetically fused DNA encoding the well-characterized H-2K^b-restricted CD8+ T-cell epitope from chicken ovalbumin ($Ova_{257-264}$) to the 5′ end of the full-length DT gene. We chose this orientation because previously published evidence indicated that peptides fused to the amino terminus of DT do not block translocation of the recombinant toxin into cells (44). In order to safely deliver antigen into DTR-expressing cells, the fusion was made incorporating mutations encoding two amino acid substitutions (K51E and E148K) that inactivate the ADP ribosylation activity of DT (DTm) (17). The resulting fusion protein, $Ova_{257-264}$-DTm (Ova-DTm), was expressed in *E. coli* and purified from cell extracts. As expected, this antigen-tagged catalytic mutant exhibited no cytotoxic effect when incubated with CD11c-DTR DC (Fig. 1B).

To determine whether DTR-expressing cells treated with antigen-tagged DTm could stimulate antigen-specific CD8+ T cells in vitro, we incubated CD11c-DTR transgenic DC with Ova-DTm and monitored the stimulation of Ova-specific B3Z T cells. B3Z is a CD8+ T-cell hybridoma engineered to produce β-galactosidase when its T-cell receptor engages the $Ova_{257-264}$:K^b complex (24, 25). We observed a robust B3Z response following Ova-DTm treatment of CD11c-DTR DC but not following identical treatment of C57BL/6 DC (Fig. 2A). These results suggest that Ova-DTm gains access to the host MHC-I pathway of antigen presentation to CD8+ T cells in a DTR-dependent manner. Furthermore, B3Z cells did not respond to CD11c-DTR DC incubated with 30 nM DTm fused to a CD8+ T-cell epitope from lymphocytic choriomeningitis virus (LCMV). This demonstrates that sequences within the DTm backbone were not responsible for B3Z stimulation (data not shown). We also observed Ova-specific CD8+ T-cell stimulation following Ova-DTm treatment of Cos/K^b cells (monkey-derived cells that naturally express DTR and are transfected with the murine K^b molecule) (data not shown), indicating that the toxin fusion protein can deliver antigen to the MHC-I processing and presentation pathway of cells that express a native form of DTR from a *DTR* promoter.

Ova-specific CD8+ T-cell stimulation is dependent on Ova-DTm translocation into the cytosol. Because an acidified endosome is needed for DT translocation, toxin entry into the cytosol can be blocked by incubating cells with a vacuolar ATPase proton pump inhibitor, bafA1 (45, 46). We found that when CD11c-DTR DC were pretreated with bafA1 and then incubated with Ova-DTm, B3Z stimulation was dramatically reduced compared to the level observed in the absence of the drug (Fig. 2B). Therefore, in vitro delivery of Ova-DTm to the MHC-I processing and presentation pathway is dependent on translocation of the toxin fusion protein into the cytosol.

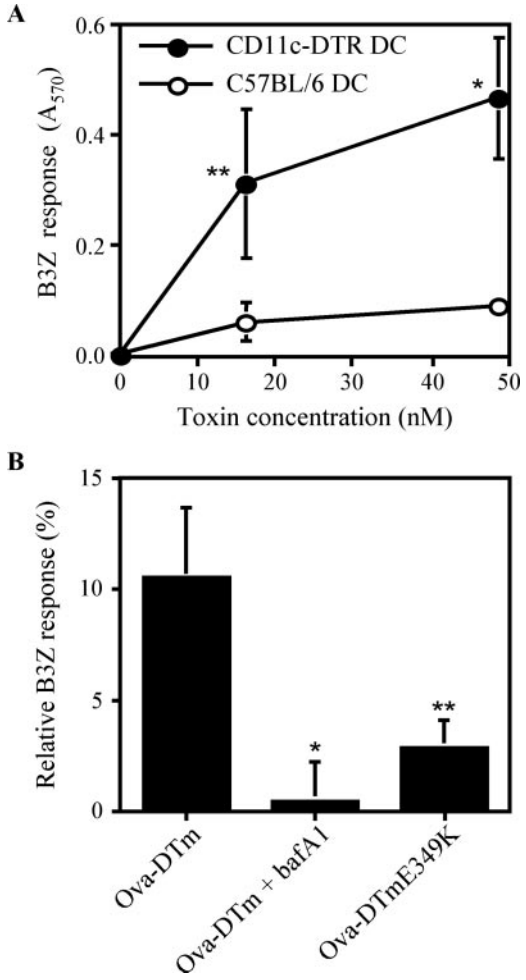

FIG. 2. Ova-DTm-treated CD11c-DTR DC stimulate Ova-specific $CD8^+$ T cells in vitro in a DTR- and translocation-dependent manner. (A) CD11c-DTR DC (filled circles) or C57BL/6 DC (open circles) were incubated for 45 min with the indicated concentrations of Ova-DTm. Presentation of the $Ova_{257–264}$ peptide by MHC-I was measured by coincubating the toxin-treated DC with B3Z cells and measuring β-galactosidase production 24 h later as indicated by an increase in A_{570}. As positive controls, A_{570} measurements were made for CD11c-DTR DC and C57BL/6 DC treated with 100 nM synthetic $Ova_{257–264}$ peptide and were 1.631 ± 0.125 and 1.588 ± 0.055, respectively. (B) CD11c-DTR DC were preincubated with or without 0.6 μM bafA1 for 25 min, followed by incubation with 16.3 nM Ova-DTm or Ova-DTmE349K (translocation mutant) for 45 min. B3Z activity was measured as for panel A. To account for any effects of bafA1 on DC-mediated peptide presentation to T cells, B3Z activity following bafA1/Ova-DTm treatment is reported relative to activity following pretreatment of DC with bafA1 and subsequent incubation with 100 nM $Ova_{257–264}$ peptide. B3Z activity following Ova-DTm or Ova-DTmE349K treatment is reported relative to activity following treatment of DC with 100 nM $Ova_{257–264}$ peptide (without bafA1). Results are presented as means ± SD of triplicate wells and are representative of two experiments. *, $P < 0.01$; **, $P < 0.05$ (compared with C57BL/6 DC treated with the same toxin concentration [A], or compared with Ova-DTm [B]).

DT mutants that fail to translocate into the cytosol but still bind host cells have been described (34, 42, 45). We incorporated one such translocation-blocking mutation, E349K (34), into Ova-DTm to generate a second toxin fusion protein, Ova-DTmE349K. This fusion protein remains nontoxic and is additionally unable to enter the cytosol. We used the E349K mutant to further confirm that delivery of Ova-DTm to the MHC-I pathway requires translocation of the toxin into the cytosol. We observed a 3.5-fold drop in B3Z activity when CD11c-DTR DC were incubated with Ova-DTmE349K, compared to the activity seen following treatment with the translocation-competent Ova-DTm (Fig. 2B). In summary, both bafA1 pretreatment and the E349K substitution inhibited access of Ova-DTm to the MHC-I pathway of CD11c-DTR transgenic DC, as measured by a reduction in B3Z stimulation. Therefore, we conclude that translocation of Ova-DTm to the host cytosol is required for maximal antigen processing, presentation, and stimulation of $CD8^+$ T cells specific for the antigenic peptide fused to these modified DT molecules.

Ova-specific $CD8^+$ T cells are stimulated in Ova-DTm-immunized CD11c-DTR mice. We next wanted to immunize CD11c-DTR mice with Ova-DTm and analyze the resulting Ova-specific $CD8^+$ T-cell response. However, we found that naïve CD11c-DTR mice had a previously unreported defect in the number of T cells resident in the spleens compared to C57BL/6 mice (25% fewer $CD8^+$ TCR^+ cells [$P < 0.05$] and 40% fewer $CD4^+$ TCR^+ cells [$P < 0.001$]). We were concerned that this defect might preclude effective immunization of these mice or complicate the interpretation of results we might obtain following immunization. Therefore, we analyzed the behavior of T cells adoptively transferred into DTR transgenic mice from donor mice that do not express DTR. By labeling the donor cells with the fluorescent dye CFSE, we were able to analyze the proliferation of the T cells transferred in the graft, since the dye is equally diluted to daughter cells during cell division. Specifically, we adoptively transferred CFSE-labeled, $Ova_{257-264}$-specific $CD8^+$ T cells from OT-I TCR transgenic mice (21) into the tail veins of CD11c-DTR transgenic mice. The following day, groups of recipient mice were immunized with 30 pmol (1.84 μg) of either Ova-DTm or Ova-DTmE349K. Two days later we used flow cytometry to analyze OT-I T-cell proliferation. Adoptively transferred OT-I T cells proliferated extensively in Ova-DTm-immunized CD11c-DTR mice but not in a similarly immunized group of C57BL/6 control mice and not in unimmunized CD11c-DTR or C57BL/6 mice (Fig. 3). The observed proliferation in CD11c-DTR mice was reduced following immunization with the translocation mutant Ova-DTmE349K compared to immunization with the translocation-competent Ova-DTm (Fig. 3). Furthermore, there was no detectable proliferation of OT-I T cells in CD11c-DTR mice injected with either commercially synthesized $Ova_{257-264}$ peptide (at the same molar concentration contained in the Ova-DTm immunization) or $Ova_{257-264}$ peptide plus DTm fused to a $CD8^+$ T-cell epitope from LCMV (data not shown). Cumulatively, these results demonstrate that Ova-specific $CD8^+$ T cells are stimulated in Ova-DTm-immunized CD11c-DTR mice and that this T-cell stimulation is dependent on DTR expression by host cells, translocation competence of the toxin fusion protein, and covalent linkage between DTm and the antigenic peptide.

CD11c-DTR DC treated with similar molar concentrations of DT- or AT-based fusion proteins stimulate $CD8^+$ T cells in vitro. After characterizing the DTm-based antigen delivery system, we wanted to directly compare DT-mediated delivery of antigen to AT-mediated delivery of antigen. Like DT, AT

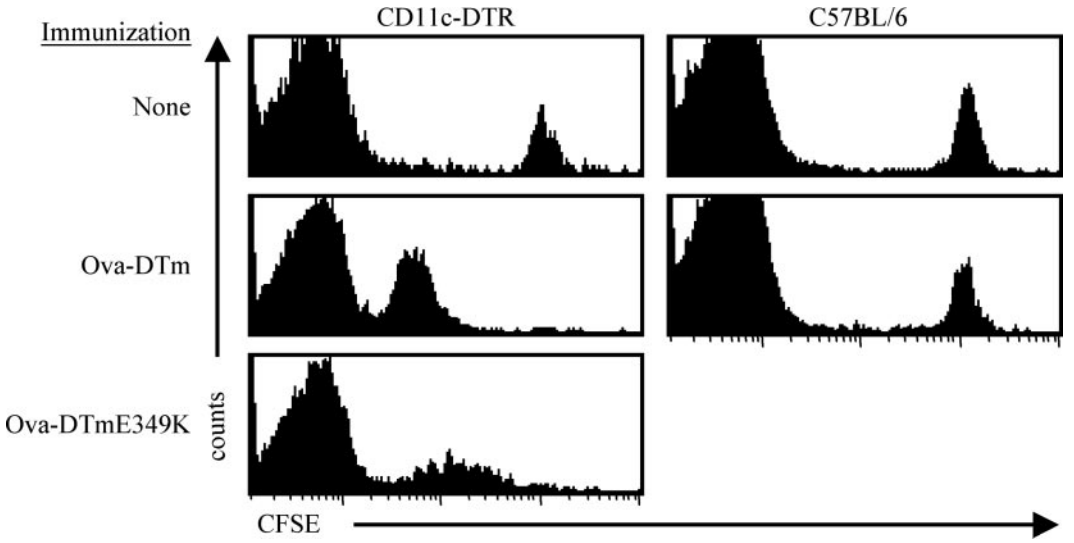

FIG. 3. Adoptively transferred Ova-specific $CD8^+$ T cells proliferate in Ova-DTm-immunized CD11c-DTR transgenic mice. CD11c-DTR transgenic and C57BL/6 mice were injected with 1.5×10^6 CFSE-labeled OT-I T cells ($V\alpha2^+$ $CD8\alpha^+$). The following day, groups of mice were i.p. immunized with 30 pmol Ova-DTm or Ova-DTmE349K. Two days later, spleens were harvested from the mice, and flow cytometry was used to analyze the proliferation of the transferred T cells. Histograms are gated on $V\alpha2^+$ $CD8\alpha^+$ cells, and the CFSE-negative peaks represent endogenous $V\alpha2^+$ $CD8\alpha^+$ T cells. Data are representative of 2 to 4 mice per group.

binds specific receptors on the surfaces of host cells, is internalized by receptor-mediated endocytosis, forms a transmembrane pore in an acidified endosomal compartment, and translocates a domain into the cytosol (13). However the DT- and AT-based antigen delivery systems differ in several ways. While a single DTm fusion protein is required to deliver antigen using the DT system, two proteins are required for the AT system. The first is protective antigen (PA), which contains the receptor-binding and pore-forming domains (13). The second is a nontoxic fragment of lethal factor (LFn [1]) fused to a heterologous antigen. The LFn portion of the fusion protein mediates binding to PA heptamers on the host cell surface. PA then facilitates translocation of the fusion protein into the cytosol (13). Differential expression of their specific receptors in mice also results in targeting of different cell populations by the two toxin systems. While DTR is not expressed by wild-type mice (10, 32) and is expressed only by $CD11c^+$ cells in CD11c-DTR transgenic mice (23), ANTXR1 and -2 are ubiquitously expressed in both wild-type (6, 7, 40) and CD11c-DTR mice. Therefore, in CD11c-DTR hosts, the DT-based system should restrict antigen delivery to $CD11c^+$ cells while the AT-based system should not restrict delivery to a particular cell type.

We first compared the abilities of the two toxin-based systems to deliver antigen to the intracellular MHC-I processing and presentation pathway of DC in vitro. We cultured $CD11c^+$ DC from the bone marrow of CD11c-DTR mice and treated the DC with either Ova-DTm or a 1:1 molar ratio of PA and LFn fused to the $Ova_{257-264}$ epitope (referred to below as AT-Ova). Because these DC express both toxin receptors (DTR because the cells are derived from a CD11c-DTR transgenic mouse, and ANTXR1 and -2 because mice naturally express these receptors), this experiment allowed us to compare B3Z stimulation following entry of the two toxins into the same population of cells. As expected, B3Z cells were stimulated following treatment of CD11c-DTR DC with either toxin fusion protein. Remarkably, while the magnitude of the B3Z response may have been slightly higher following AT-Ova treatment, a similar molar concentration range of either toxin was required to stimulate B3Z cells (Fig. 4A).

DTm-mediated targeting of antigen to $CD11c^+$ cells in vivo stimulates a more robust $CD8^+$ T-cell response than AT-mediated delivery. In the in vitro experiments described above, AT and DT fusion proteins were not limiting and had the potential to deliver antigen into every cell in the culture. However, the scenario following in vivo immunization is quite different: the toxin fusion proteins become limiting and target significantly different cell populations. Using CD11c-DTR transgenic mice, we were able to compare antigen-specific $CD8^+$ T-cell stimulation following targeted delivery of antigen to $CD11c^+$ cells by the DT-based system with that following more widespread delivery of the same antigen by the AT-based system. CFSE-labeled OT-I T cells were adoptively transferred into groups of CD11c-DTR transgenic mice that were immunized the following day with 30 pmol Ova-DTm or 30 pmol AT-Ova (30 pmol LFn-$Ova_{257-264}$ plus 30 pmol PA). Two days later, flow cytometry was used to measure the proliferation of transferred OT-I T cells present in the spleens of the recipient mice. As expected, OT-I T cells proliferated in mice immunized with either toxin fusion protein but not in unimmunized mice (Fig. 4B). However, OT-I T cells proliferated more rapidly and extensively following Ova-DTm immunization than following AT-Ova immunization (Fig. 4B). The kinetic and amplitude differences in T-cell proliferation seen 2 days postimmunization were also evident 3 days postimmunization, when OT-I T cells in Ova-DT-immunized mice had undergone several more rounds of cell division, while those in AT-Ova-immunized mice had expanded only slightly (data not shown). These in vivo results suggest that compared to ubiquitous delivery by the AT-based system, restricted delivery of antigen to $CD11c^+$ cells by the DT-based system results in more rapid

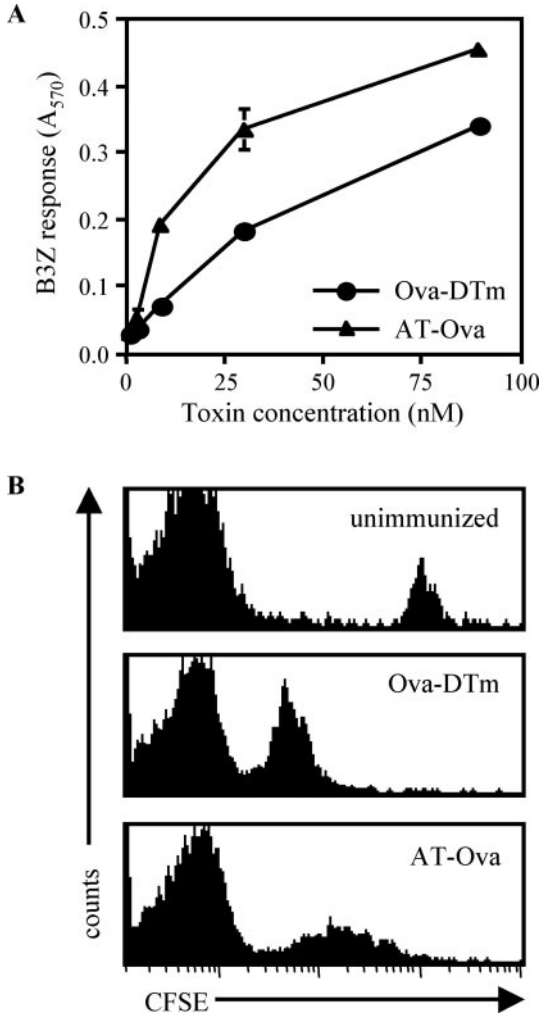

FIG. 4. Comparison of antigen delivery by the AT- and DT-based systems in vitro and in vivo. (A) DC derived from the bone marrow of CD11c-DTR mice were incubated with anti-CD11c-conjugated MACS microbeads (after 8 days in culture) and purified using magnetic separation columns (>90% $CD11c^+$). Cells were then incubated for 3 h with the indicated concentrations of Ova-DTm (circles) or AT-Ova (triangles). Presentation of the $Ova_{257–264}$ epitope was measured by coincubating the treated DC with B3Z cells and measuring β-galactosidase production 24 h later. A measurable B3Z response was detected following treatment of CD11c-DTR DC with concentrations of Ova-DTm or AT-Ova greater than 3 nM. Results are presented as means ± SD of triplicate wells and are representative of two independent experiments. (B) CD11c-DTR transgenic mice were injected with 1.5×10^6 CFSE-labeled OT-I T cells ($V\alpha2^+$ $CD8\alpha^+$). The following day, groups of mice were i.p. immunized with 30 pmol Ova-DTm or AT-Ova. Two days later, spleens were harvested from the mice, and flow cytometry was used to analyze the proliferation of the transferred T cells. Histograms are gated as in Fig. 3 and represent 2 to 4 mice per group.

and extensive proliferation of antigen-specific T cells. Therefore, we conclude that using a toxin to target the delivery of antigen to DC may be advantageous in eliciting a robust antigen-specific $CD8^+$ T-cell response.

DISCUSSION

We have generated and characterized an antigen delivery system consisting of nontoxic DT protein (DTm) fused to a heterologous $CD8^+$ T-cell antigen, in this case an antigenic peptide derived from ovalbumin. We have isolated $CD11c^+$ DC expressing the DT receptor from CD11c-DTR transgenic mice, treated them with Ova-DTm, and shown that this treatment results in Ova-specific $CD8^+$ T-cell stimulation in vitro. It appears that the observed T-cell stimulation required translocation of the toxin fusion protein into the host cell cytosol.

We have also characterized the antigen delivery system in CD11c-DTR transgenic mice and have shown that in these mice, Ova-specific $CD8^+$ T cells proliferate following a single i.p. injection with as little as 30 pmol Ova-DTm. T-cell proliferation was observed only in mice that expressed DTR, was dependent on physical fusion between DTm and the heterologous antigen, and was inhibited following immunization with the toxin translocation mutant Ova-DTmE349K. The T-cell proliferation observed following Ova-DTm immunization of CD11c-DTR transgenic mice was rapid and robust. However, some residual proliferation was seen following immunization of the transgenic mice with Ova-DTmE349K. Similarly, residual B3Z activity was seen following Ova-DTmE349K treatment of DC cultured from the transgenic mice. One explanation for these results is that the Ova antigen is cross-presented after the toxin fusion protein is associated with DTR-expressing cells. An alternative explanation is that the E349K mutation does not completely block translocation (45), and a small number of Ova-DTmE349K molecules gain access to the cytosolic MHC-I pathway of antigen processing. Cumulatively, the in vivo results parallel those obtained in vitro and suggest that DTm delivers antigen into the cytosol of cells expressing DTR, where the fusion protein then gains access to the host MHC-I pathway of antigen processing and presentation to $CD8^+$ T cells.

Because DTR expression in CD11c-DTR transgenic mice is restricted to $CD11c^+$ cells (23) and immunization of these mice with Ova-DTm resulted in proliferation of Ova-specific T cells, we conclude that DTm-mediated delivery of antigen to $CD11c^+$ cells is sufficient to result in antigen-specific $CD8^+$ T-cell stimulation in vivo. DC are the predominant CD11c-expressing cell type in mice and, on the basis of expression of additional cell surface markers, can be further classified into subsets such as $CD11c^+$ $CD8\alpha^{hi}$ DC, $CD11c^+$ $CD8\alpha^-$ DC, and $CD11c^{lo}$ $B220^+$ plasmacytoid DC. We postulate that in CD11c-DTR mice, cells of each of these DC subsets express DTR and are targeted for antigen delivery by DTm. This is supported by data from Scumpia et al., who demonstrated that all three of the DC subsets mentioned above are depleted in CD11c-DTR transgenic mice following DT injection (41). Although CD11c is commonly used as a marker for DC (30, 31, 47), its expression on other cells has also been reported, including activated intraepithelial lymphocytes and lymph node T cells (22), as well as a fraction of natural killer cells (36). Therefore, cell types other than DC may express DTR in CD11c-DTR transgenic mice. Indeed, a proportion of $CD8^+$ T cells, as well as marginal zone and metallophilic macrophages in the transgenic mice, have been shown to be sensitive to DT-mediated cell death (23, 37). It seems possible that, in vivo, DTm can deliver antigen into cell types other than DC and that this may influence the Ova-specific $CD8^+$ T-cell response we observed.

We have also compared the stimulation of antigen-specific

$CD8^+$ T cells in mice following immunization with AT- and DT-based antigen delivery systems. Using CD11c-DTR transgenic mice, we observed more robust Ova-specific $CD8^+$ T-cell proliferation following Ova-DTm immunization than following AT-Ova immunization. We hypothesize that these results are due to the selective delivery of antigen to DTR-expressing $CD11c^+$ DC by DTm. While most nucleated cells are capable of processing and presenting peptides on MHC-I molecules to effector $CD8^+$ T cells, optimal T-cell stimulation is seen when antigen is presented and costimulation is provided by $CD11c^+$ DC (20). Accordingly, the T-cell response following AT-Ova immunization may be weaker because entry of the fusion protein into other cell types not only reduces the effective concentration of fusion protein delivered to DC but may also result in partial T-cell anergy or nonresponsiveness if naïve $CD8^+$ T cells recognize peptide:MHC-I complexes in the absence of costimulatory signals (20). Besides differential receptor expression, other properties of the AT- and DT-based antigen delivery systems may contribute to the in vivo results, including inherent adjuvant properties of the toxin backbones or their stability in blood or tissue. Furthermore, intoxication of host cells by AT fusion protein requires seven PA molecules and at least one LFn-$Ova_{257-264}$ molecule to assemble on the same cell surface, whereas intoxication by DT fusion protein requires only one Ova-DTm molecule per cell. This difference may be more relevant in mice, where limiting toxin molecules become diluted throughout the body cavity, than in cell culture, where the toxin molecule-to-cell ratio is much greater. In fact, following in vitro treatment of CD11c-DTR DC with one or the other of the toxin fusion proteins, we found that the AT-based system was as efficient as the DT-based system at delivering Ova antigen to the MHC-I pathway for presentation to Ova-specific $CD8^+$ T cells.

Besides the DT- and AT-based antigen delivery systems developed in our laboratory, targeted delivery of antigen has been accomplished using other toxin-based systems (43). For example, detoxified adenylate cyclase toxin (CyaA) fused to heterologous antigens and injected into mice results in antigen-specific $CD8^+$ T-cell stimulation (16, 18). The cellular receptor for CyaA, the CD11b/CD18 integrin (19), is expressed by a variety of cell types, including a subset of $CD11c^+$ DC (38, 47). These studies, together with our findings, support an emerging picture in which antigen presentation by DC allows for optimal $CD8^+$ T-cell stimulation. This has important implications for the design of vaccines that aim to stimulate this T-cell population.

While our study has focused on using DTm to deliver antigen specifically to DTR-expressing DC, the DTm-based delivery system that we have developed could be used to deliver antigen or protein into other DTR-expressing cells. Recently, several lines of DTR transgenic mice with restricted DTR expression by different types of cells, such as macrophages (9, 15), Langerhans cells (5), or hepatocytes (39), have been developed. Mice with Cre-inducible DTR expression have also been generated (8, 11) and are quite versatile, because they can be crossed to existing transgenic mice expressing Cre under the control of a cell type-specific promoter, generating offspring in which DTR expression is limited to the Cre-expressing cell type. To date, DTR transgenic mice have been used only as tools for study of the effects of cell lineage ablation following treatment with native DT. However, investigators might alternatively treat available DTR transgenic mice with nontoxic DTm fusion proteins, permitting delivery of heterologous antigen into the cytosol of DTR-expressing cells. The effect of antigen presentation by a particular cell type on the overall stimulation of antigen-specific T cells in mice could then be determined. This approach could be useful both in infection models and in autoimmune models. DTm might also be used to safely deliver therapeutic or regulatory proteins into DTR-expressing cells in transgenic mice in a targeted and temporally controlled manner.

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