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Acquisition of MHC:Peptide Complexes by Dendritic Cells Contributes to the Generation of Antiviral CD8\(^+\) T Cell Immunity In Vivo

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There is an increasing body of evidence suggesting that the transfer of preformed MHC class I:peptide complexes between a virus-infected cell and an uninfected APC, termed cross-dressing, represents an important mechanism of Ag presentation to CD8\(^+\) T cells in host defense. However, although it has been shown that memory CD8\(^+\) T cells can be activated by uninfected dendritic cells (DCs) cross-dressed by Ag from virus-infected parenchymal cells, it is unknown whether conditions exist during virus infection in which naive CD8\(^+\) T cells are primed and differentiate to cytolytic effectors through cross-dressing, and indeed which DC subset would be responsible. In this study, we determine whether the transfer of MHC class I:peptide complexes between infected and uninfected murine DC plays a role in CD8\(^+\) T cell priming to viral Ags in vivo. We show that MHC class I:peptide complexes from peptide-pulsed or virus-infected DCs are indeed acquired by splenic CD8\(^+\) T cells. Furthermore, the acquired MHC class I:peptide complexes are functional in that they induced Ag-specific CD8\(^+\) T cell effectors with cytolytic function. As CD8\(^+\) DCs are poor cross-presenters, this may represent the main mechanism by which CD8\(^+\) DCs present exogenously encountered Ag to CD8\(^+\) T cells. The sharing of Ag as preformed MHC class I:peptide complexes between infected and uninfected DCs without the restraints of Ag processing may have evolved to accurately amplify the response and also engage multiple DC subsets critical in the generation of strong antiviral immunity. The Journal of Immunology, 2012, 189: 000–000.

During the immune response to a virus infection, it is generally accepted that APCs display Ag to CD8\(^+\) T cells by two distinct mechanisms. Direct presentation in the context of a MHC class I:peptide complex derived from endogenous gene products synthesized by a virus-infected APC (1). Additionally, there is indirect presentation in which Ag is transferred from live or apoptotic virus-infected cells to uninfected APC and cytosolic peptide cross-presented by the MHC class I molecules expressed by the recipient APC (2–8). Recently, a third mechanism known as cross-dressing has been proposed to contribute to antiviral immunity, whereby an uninfected APC acquires pre-existing MHC class I:peptide molecules from a virus-infected cell (9–11). In support of such a role, we, and others, have shown that MHC class I:peptide complexes can be acquired by immature and mature dendritic cells (DCs) both in vitro and in vivo (10–15). Furthermore, the transferred MHC:OVA peptide complexes were functional in that they stimulated OVA-specific CD8\(^+\) T cell proliferation and IL-2 production (9). Likewise, we have observed that DCs can acquire MHC:peptide complexes from epithelial cells in vitro (10). This suggested that DCs trafficking through a virus-infected tissue could de facto acquire Ag in the form of preformed MHC class I:peptide complexes from virally infected parenchymal cells and subsequently present the acquired MHC:peptide complexes to T cells in lymph nodes (LNs) (10). Consistent with this hypothesis, Wakim and Bevan (11) recently provided compelling evidence that virus-infected parenchymal cells cross-dress DC with peptide-loaded MHC class I and drive T cell proliferation in vivo. However, this mechanism was not universally efficient in driving T cell activation, because naive T cells were selectively excluded from expansion, suggesting the number of transferred MHC:peptide complexes and/or accessory costimulatory molecules derived from parenchymal cells disfavors priming of naive T cells. Given the possibility that viruses could evolve the capacity to evade or shut down the cross-presentation pathway, we hypothesized that during infection subpopulations of APCs may have evolved the capacity to faithfully share MHC:peptide complexes of an infected APC with the aim of spreading Ag to uninfected APC and increase the efficiency of priming rare naive T cells. Such a scenario for multiple Ag-processing pathways activating naive CD8\(^+\) T cells in vivo is not inconceivable (16). Evidence that this may occur comes from our previous observations that ex vivo splenic CD8\(^+\) and CD8\(^-\) DC subsets can acquire MHC:peptide complexes from bone marrow-derived DCs (BM-DCs) in vitro and stimulate Ag-specific T cells (10). Likewise, Qu et al. (14) reported that migratory MHC class I-expressing monocyte-derived DCs transfer intact MHC class I:

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Abbreviations used in this article: BM-DC, bone marrow-derived DC; DC, dendritic cell; DT, diphtheria toxin; DTR, DT receptor; LN, lymph node; vp, virus particle.
peptide complexes to MHC class I-deficient splenic DCs. Clarification of the capacity of DC subsets to promote cross-dressed Ag presentation and also act as recipients of cross-dressed Ag remains a key question in unraveling antiviral immunity and could aid in the design of improved viral and tumor vaccines.

In this study, we demonstrate that naive Ag-specific CD8+ T cells are primed by splenic DCs that acquired viral Ag via cross-dressing in vivo. Ag recognized in this way, in the absence of direct or cross-presentation, was sufficient to induce cytolytic function by CD8+ effector T cells. Furthermore, the CD8α− subset of splenic DC populations supported activation of naive CD8+ T cells via cross-dressed Ag presentation in vivo. This observation suggests that CD8α− DCs expressing acquired MHC-peptide complexes may play a key role in activating viral specific naive CD8+ T cells in vivo.

Materials and Methods

Mice and Abs

C57BL/6 (H-2b, B6) and BALB/c (H-2d) mice, 6–10 wk of age, were purchased from Harlan Olac (Bicester, U.K.). B6-CD11c-GFP-DTR mice (which express a GFP-diphtheria toxin [DT] receptor [DTR] fusion protein under the control of the CD11c promoter [DTR mice]) were a gift of D. Kiovossis (National Institute for Medical Research). OT-1 mice were purchased from The Jackson Laboratory. OT-1 Rag-/-/ H-2Km1 (Kbm1), and B6.SJL-PtprcaPep3bBoyJ (H-2b, CD45.1) mice were a gift of S. Diebold (Harvard Medical School, Boston). OT-1 Rag-/- mice were s.c. injected with 5 × 10^5 melanoma-expressing FLT-3L cells. After 7–10 d, spleens were disaggregated using collagenase (Sigma-Aldrich) in the presence of DNase (Roche) and CD11c splenic DCs were isolated using a FACSCalibur. DCs were incubated with an anti-CD16/CD32 (anti-FcRγII/FcRγIII, clone 2.4G2) Ab before each experiment, a negative control (no addition of peptide) was included to experiment, a negative control (no addition of peptide) was included to experiment, a negative control (no addition of peptide) was included to calculate the percentage of Ag-specific killed target cells with the following formula: killing (%) = 1 − ([no. of targets/no. of control cells in immunized animal]/[no. of targets/no. of control cells in control animal]) × 100.

Antigens

DCs were incubated with OVA257–264 (SIINFEKL) peptide, at either 1 or 5 μg/ml, for 2 h in the presence of 1 μg/ml LPS, in RPMI 1640 containing 10% FCS, 50 μM 2-ME, 100 IU/ml penicillin, 100 mg streptomycin, 2 mM l-glutamine, and 1% HEPES. Cells were washed several times before being used.

Preparation of responder T cells and CFSE labeling

Responder T cells were purified from RBC-depleted spleenocytes isolated from either OT-1, OT-1 Rag-/-, or CD45.1+ OT-1 mice and then depleted with rat mAbs to B220 (RA3-6B2 hybridoma), MHC class II (MS1/14.52 hybridoma), anti-CD16/CD32 (2.4G2 hybridoma), and anti-CD4 (YTS191 hybridoma), followed by an incubation period with sheep anti-rat IgG-coated Dynabeads (Dynal Biotech). For in vitro studies, T cells from OT-1 Rag-/- mice were isolated using a CD8+ T cell isolation kit (Miltenyi Biotec). However, for in vivo studies, RBC-depleted splenocytes used were purified. The purity of responder cells was consistently between 90% and 95%. T cells were labeled with 1 μM CFSE-SA (Invitrogen), according to the manufacturer’s instructions, before being injected.

MHC class I transfer experiments

In certain experiments, recipient B6 mice were injected i.v. with 200 μg dsRNA (polyinosinic-polycytidylic acid; Invivogen) to block cross-presentation (20). In other experiments, recipient H-2Km1 mice received 250 μg purified anti-Thyl.1.2 Ab (eBioscience) to remove endogenous T cells to inhibit an alloresponse to H-2β10 (12). After 24 h, recipient mice received i.v. injection 2 × 10^6 head and flow cytometry-purified, DTR+ GFP(high) DCs either pulsed with or without OVA257–264 peptide (SIINFEKL) or infected with Ad-OVA or Ad-GFP. Eighteen to 20 h later, some mice received 4 ng/g body weight of DT (Sigma-Aldrich) via i.v. and i.p. injection to remove the DTR+ GFP(high) DCs, as described by Prlic et al. (22). One day later, 2 × 10^7 CFSE-labeled T cells isolated from OT-1 or OT-1 Rag-/- mice were adoptively transferred i.v. T cell proliferation was measured on day 3 or 4 after adoptive transfer of the labeled T cells via flow cytometry following staining with an anti-Vα2-PE Ab specific to the transgenic T cells.

In vitro cross-presentation assay

Recipient B6 mice were either untreated or injected i.v. with 200 μg dsRNA to block cross-presentation 24 h prior to adoptive transfer of 2 × 10^7 CFSE-labeled CD8+ T cells isolated from OT-1 Rag-/- mice and either 2 × 10^6 BALB/c (H-2d) irradiated spleen cells previously o mmunized with soluble OVA peptide (10 μg/ml) or 2 × 10^5 irradiated CD11c+ BALB/c splenic DCs infected with Ad-OVA at 3000 vp/cell. After 3 d, spleens were harvested and cell suspensions depleted of RBCs were stained with anti-CD8 and anti-Vα2 Abs, washed, and then analyzed by flow cytometry.

CFSE killing assay

To generate peptide-pulsed target cells, splenocytes from naive B6 mice were pulsed with 10 μg/ml OVA257–264 peptide for 90 min at 37°C, washed, and labeled with 0.25 μM CFSE (CFSElow). Control target cells were splenocytes cultured in medium alone and labeled with 2.5 μM CFSE (CFSEhigh). Both the control and peptide-pulsed target cells were mixed at a 1:1 ratio prior to injection into recipient mice at a dose of 1 × 10^7 cells/animal. Recipient mice received DTR+ DC pulsed with OVA257–264 peptide or DTR+ DC infected with Ad-OVA and responder OT-1 T cells 7 previously. Control animals received uninfected DTR+ DCs and responder OT-1 T cells alone. Eighteen hours later, spleens were harvested and single-cell suspensions were analyzed for CFSEhigh and CFSElow populations by flow cytometry. CFSE+ cells were gated using the FL2 versus FL1 dot blot to exclude autofluorescent cells. Numbers of target (CFSEhigh) versus control (CFSElow) cells recovered were used to calculate the percentage of Ag-specific killed target cells with the following formula: (no. of targets/no. of control cells in immunized animal)/[no. of targets/no. of control cells in control animal]) × 100.

CD107 mobilization assay

Spleen-cell suspensions from recipient mice were isolated and cultured 6 h with naive B6 spleen cells pulsed with 10 μg/ml OVA257–264 peptide, at an E:T ratio of 1:2 in the presence of FITC-conjugated anti-CD107a and anti-CD107b mAbs (or a FITC-conjugated isotype control mAb) at 2.5 μg/ml plus anti-CD28 and anti-CD49d (each at 2 μg/ml) and GolgiPlug (BD Biosciences) during the stimulation period. In each experiment, a negative control (no addition of peptide) was included to control for spontaneous CD107a/b expression. Cells were then stained with anti-CD11c/CD32 Ab, followed by fluorescent-labeled Abs to CD8 and CD45.1. Flow cytometric analysis for CD107a/b expression on CD8+ CD45.1+ cells was performed on a BD Biosciences FACSCalibur.

Statistical analysis

For comparison of means between two groups, the data were analyzed using the unpaired Student t test with Welch’s correction. The p values < 0.05 were considered significant. All statistical analysis was performed using GraphPad Prism 5.0d.
Results

MHC class I:peptide complexes are transferred between DCs in vivo

To address whether MHC class I:peptide complexes are transferred between DCs in vivo, we used a system in which MHC donor DCs derived from C57BL/6 (B6)-CD11c-GFP-DTR mice (which express a GFP-DTR fusion protein under the control of the CD11c promoter) (23) were transferred into nontransgenic mice. Because wild-type cells of the recipient do not express the high-affinity DTR, the transgenic DCs are the only cells susceptible to the toxin following DT injection (22). Using this approach, we observed that following adoptive transfer into nontransgenic B6 recipient mice, both FACS-purified CD11cGFPDCs (DTRhighDCs) and also CFSE-labeled DTRhighDCs (as a further stringent test) were depleted both in spleen and LNs 24 h after DT injection, but not in the absence of DT treatment (Fig. 1). To exclude the possibility that small numbers of functional DCs could remain undetected (by flow cytometry) after DT treatment (and hence a source of direct Ag presentation), we established by titration that the minimum number of functional peptide-pulsed DTRhighDCs required to trigger naive T cell proliferation in this system was within the limits of flow cytometric detection (data not shown). Collectively, the data confirm that DT treatment eliminates functional donor DTRhighDCs below the threshold required to stimulate proliferation in this setting. We were therefore able to test in vivo whether endogenous DCs acquired MHC: peptide complexes from adoptively transferred donor DCs. DTRhighDCs loaded with OVA257–264 peptide were transferred into B6 recipient mice, followed by DT injection after cell transfer. To determine whether the number of transferred MHC–peptide complexes was sufficient to stimulate an Ag-specific CD8+ T cell response, CFSE-labeled H-2Kbm1-OVA257–264 peptide-specific CD8+ T cells derived from OT-1 Rag−/− mice were injected into the same B6 recipient mice. Efficient Ag-specific T cell proliferation occurred in mice receiving DT 24 h after OVA257–264 peptide DTRhighDC transfer (Fig. 2). Because all donor DTRhighDCs were depleted by the toxin, the only MHC: OVA257–264 peptide available to stimulate OT-1 CD8+ T cell proliferation would have been donor-derived MHC:OVA257–264 peptide complexes acquired by endogenous DCs. As expected, OT-1 CD8+ T cells proliferated in control B6 mice injected with OVA257–264 peptide DTRhighDCs in the absence of DT treatment (Fig. 2), indicative of direct priming by the donor DCs. OT-1 CD8+ T cells failed to proliferate under conditions where control unpulsed DTRhighDCs were transferred (Fig. 2).

Priming of CD8+ T cells by virally infected DCs can occur in the absence of cross-presentation and direct priming

Although the previous data suggested that recipient DCs cross-dressed by donor DCs primed naive OT-1 T cells in vivo, the experimental system had the caveat of using DTRhighDCs loaded ex vivo with peptide. In this setting, the donor DCs may have expressed supraphysiologic numbers of MHC class I:peptide complexes per cell, possibly unrepresentative of Ag processing and presentation in the context of virus infection. Therefore, we next investigated the capacity of virus-infected MHC donor DC to drive proliferation of naive CD8+ T cells via MHC class I:peptide transfer in vivo and thereby contribute to the antiviral CD8+ T cell response. To this end, we established an experimental system in which DTRhighDCs infected with a recombinant adenovirus vector expressing a nonsecreted OVA (Ad-OVA) were adoptively transferred to either B6 or H-2Kbm1 recipients. To exclude the

![FIGURE 1](http://www.jimmunol.org/DownloadedFrom/301x187to539x326)

**FIGURE 1.** Adaptorically transferred GFPhighDTRhigh DCs are depleted in vivo following DT administration. (A) DCs were isolated from mice treated with a FLT3 ligand-expressing melanoma cell line using positive bead selection and were sorted by flow cytometry on the basis of GFPhigh and CD11c expression. A total of $2 \times 10^6$ GFPhigh (thus DTRhigh, expressing)-sorted DCs was adoptively transferred into individual B6 mice that were left untreated or were treated with 100 ng DT at 24 h. Control mice received no DCs or GFPhigh DCs only. The spleens and LNs were harvested 24 h after the DT injection. Histograms show GFP expression for CD11c-positive cells. The data shown are representative of six independent experiments. (B) A total of $2 \times 10^6$ GFPhigh-sorted DCs [derived as in (A)] labeled with CFSE was adoptively transferred into individual B6 mice that were left untreated or were treated with 100 ng DT at 24 h. Control mice received no DCs or CFSE-labeled GFPhigh DCs only. Histograms show CFSE expression for CD11c-positive cells isolated from spleen and LNs 24 h after DT injection. The data are representative of five independent experiments.

![FIGURE 2](http://www.jimmunol.org/DownloadedFrom/342x135to567x359)

**FIGURE 2.** Cross-dressed DCs stimulate Ag-specific naive CD8+ T cells in vivo. DCs were isolated from mice treated with a FLT3 ligand-expressing melanoma cell line using positive bead selection and were sorted by flow cytometry on the basis of GFPhigh and CD11c expression. A total of $2 \times 10^6$ GFPhighDCs pulsed for 2 h with 5 μg/ml OVA257–264 peptide and 1 μg LPS or LPS only (control DCs) was adoptively transferred into B6 mice. After 24 h, mice were left untreated or received 100 ng DT. A total of $2 \times 10^6$ CFSE-labeled Ag-specific OT-1 Rag−/−CD8+ T cells was adoptively transferred 24 h after DT injection. Spleen cells were harvested 4 d after OT-1 T cell transfer and stained with anti-CD8 and anti-Va2 Abs. CFSE dilution was measured by flow cytometry and used as readout of T cell proliferation. Panels represent the CFSE division of Va2-positive cells for each individual mouse gated on CD8+ cells. The data shown are representative of eight independently performed experiments.
contribution of cross-presentation in this setting, we made use of reports that pretreatment of mice with dsRNA drives apoptosis of splenic CD8α− DC (24) and inhibits CD8α− DC-mediated cross-presentation of cell-associated Ag (20). In accordance with these reports, flow cytometry of spleen suspensions derived from dsRNA-treated mice showed a significant decrease in the CD8α+ DCs as compared with spleen suspensions isolated from untreated mice in our system, p < 0.001 (Fig. 3A). Moreover, we did not observe cross-presentation of OVA by CD8α+ DCs from dsRNA-treated B6 mice. In a three-cell in vitro assay, BALB/c BM-DC (H-2k) loaded with soluble OVA protein via electroporation and cultured with CD8α− DCs isolated from dsRNA-treated B6 (H-2b) mice did not trigger OT-1 proliferation (Fig. 3B). Of note, CD8α− DCs derived from the spleen of untreated mice do not cross-present cell-associated OVA in this assay (10). In parallel, we did not observe proliferation of OT-1 T cells adoptively transferred to B6 mice pretreated with dsRNA and injected with Ad-OVA–infected BALB/c spleen-derived CD11c+ DCs (Fig. 3C). Thus, we were able to test under conditions where cross-presentation is abrogated, if priming of CD8+ T cells can occur by MHC class I peptide transfer. In the absence of cross-presentation, OT-1 T cells proliferated in mice that were recipients of Ad-OVA–infected DTRhigh DCs that were eliminated following the administration of DT (Fig. 4A). In sharp contrast, OT-1 T cells failed to proliferate when DTRhigh DCs were omitted in recipient mice (Fig. 4A) and also when DTRhigh DCs infected with an adenovirus expressing a noncognate Ag (GFP) were transferred under the same conditions (data not shown). This observation suggested that OVA Ag, in the form of MHC class I peptide complexes, was transferred between Ad-OVA–infected DCs and endogenous recipient DCs. To rigorously confirm these observations and exclude endocytosis of donor DCs as opposed to the transfer of MHC:peptide complexes from DTRhigh DCs, we used H-2Kbm1 mice as recipients. These mice are congenic with B6 apart from mutations in the Kbm allele (Kbm1), which prevents OT-1 T cells from recognizing the OVA257–264 peptide presented by Kbm1, as shown in Supplemental Fig. 1. Thus, we were able to test whether proliferation of the adoptively transferred OT-1 T cells was driven only by the MHC:peptide transferred from DTRhigh DCs, because OT-1 T cells in this setting should still proliferate in Kbm1 mice. To circumvent an allogeneic response against wild-type Kbm expressed on the transferred cells, recipient mice were treated with anti-Thy1.2 Ab to deplete endogenous CD4+ and CD8+ T cells prior to transfer of the DTRhigh DCs. Nonetheless, priming of OT-1 T cells after adoptive transfer of Ad-OVA–infected DTRhigh DCs and DT administration was observed, suggesting that intact MHC class I peptide complexes were transferred between Ad-OVA–infected MHC donor cells and endogenous APCs in vivo (Fig. 4B). Collectively, these data demonstrate that transfer of preformed MHC:peptide complexes from virally infected DCs to uninfected DCs occurs in vivo and these transferred molecules are functional in as much as they induce Ag-specific CD8+ T cell proliferation.

**Endogenous DCs, including the CD8α− subset, are cross-dressed by virus-infected DC and present Ag to CD8+ T cells in vivo**

We previously reported that CD8α+ and CD8α− DCs are both capable of acquiring MHC:peptide complexes in vitro; however, the CD8α− DC population was identified as superior at presenting acquired intact MHC:peptide complexes (10). This led to the hypothesis that cross-dressing provides the main mechanism by which CD8α− DCs present exogenous Ag (10, 11). Therefore, to determine the capacity of CD8α− DCs cross-dressed with Ag from virus-infected DCs in vivo to drive naive T cell proliferation, we purified CD8α− DCs from dsRNA-treated B6 mice previously injected with Ad-OVA–infected DTRhigh DCs, followed by DT ablation. These DCs were then used to stimulate OT-1 T cells as a readout in vitro. Under these conditions, CD8α− DCs purified from the spleen of dsRNA-treated mice induced a robust expansion of OT-1 T cells from recognizing the OVA257–264 peptide presented by Kbm1, as shown in Supplemental Fig. 1. Thus, we were able to test whether proliferation of the adoptively transferred OT-1 T cells was driven only by the MHC:peptide transferred from DTRhigh DCs, because OT-1 T cells in this setting should still proliferate in Kbm1 mice. To circumvent an allogeneic response against wild-type Kbm expressed on the transferred cells, recipient mice were treated with anti-Thy1.2 Ab to deplete endogenous CD4+ and CD8+ T cells prior to transfer of the DTRhigh DCs. Nonetheless, priming of OT-1 T cells after adoptive transfer of Ad-OVA–infected DTRhigh DCs and DT administration was observed, suggesting that intact MHC class I peptide complexes were transferred between Ad-OVA–infected MHC donor cells and endogenous APCs in vivo (Fig. 4B). Collectively, these data demonstrate that transfer of preformed MHC:peptide complexes from virally infected DCs to uninfected DCs occurs in vivo and these transferred molecules are functional in as much as they induce Ag-specific CD8+ T cell proliferation.

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**FIGURE 3.** CD8α+ DCs isolated from dsRNA-treated mice do not cross-present OVA Ag. (A) B6 mice were treated with either 200 μg dsRNA or PBS. After 24 h, the spleens were harvested and the absolute number of CD8α+ and CD8α− DCs per spleen was determined by flow cytometry. Data are shown as the absolute cell number per spleen from seven individual mice treated with dsRNA and from two untreated controls and are representative of two independent experiments. (B) CD8α+ DCs purified from the spleen of B6 mice treated 24 h earlier with or without 200 μg dsRNA were cocultured with irradiated BALB/c BM-DCs (electrically pulsed with 4 mg/ml soluble OVA) and CD8α− T cells derived from OT-1 mice at a 1:1:1 ratio. T cell proliferation was measured on day 3 with 3H-thymidine added into the culture media for the final 24 h. Data represent the mean ± 1 SD for triplicate samples and are representative of two independent experiments. (C) B6 mice, treated or untreated 24 h earlier with 200 μg dsRNA, were injected with 2 × 106 irradiated BALB/c-derived CD11c+ splenic DCs infected with Ad-OVA (3000 vp/cell) and 2 × 106 CFSE-labeled OT-1 Rag−/− T cells. A control group received CFSE-labeled OT-1 Rag−/− T cells only. After 3 d, the spleens were harvested and stained with Abs to CD8 and Vα2, and the CFSE profile of labeled cells was analyzed. Panels represent the CFSE profile of CD8+ Vα2+ cells only (gated on live cells) for individual animals and are representative of two individual experiments.
in vitro, which was significantly greater than that induced by CD8α− DCs derived from naive B6 mice (p = 0.0009) or CD8α− DCs derived from B6 recipients of uninfected DTRhigh DCs (p = 0.0105) (Fig. 5A). These data strongly suggest that the proliferation of OT-1 T cells observed in dsRNA-treated B6 mice injected with Ad-OVA–infected DTRhigh DCs followed by DT (Fig. 5A) is due to the acquisition of MHC class I:OV A257–264 peptide complexes by CD8α− DCs in vivo. To test this conclusion further, we investigated the capacity of virus-infected DTRhigh DCs ablated by DT to drive OT-1 T cell proliferation in the setting of Batf3−/− mice. These mice have a selective loss of CD8α+ DEC205+ DCs and are defective in cross-presentation (17), but express normal populations of CD8α− DCs (17). Nonetheless, in the Batf3 null setting, recipients of Ad-OVA–infected DTRhigh DCs ablated by DT (to eliminate direct presentation) demonstrated sizable proliferation of the adoptively transferred OT-1 T cells under conditions in which cross-presentation was blocked by dsRNA and direct presentation eliminated by DT treatment. After 7 d, B6 spleen cells loaded with OVA257–264 peptide (CFSElow) and control unpulsed spleen cells (CFSEhigh) were injected in equal numbers as target cells. At this time point, no DTR high GFPhigh DCs were detected in recipient mice (data not shown). Eighteen hours later, the presence of CFSElow and CFSEhigh cells was analyzed by flow cytometry. In mice that received peptide-loaded DTRhigh DCs as MHC donor cells under conditions in which cross-presentation was blocked by dsRNA and direct presentation eliminated by DT treatment, 15.3% of the OVA257–264 peptide-pulsed targets were specifically killed relative to that of control naive mice (representative experiment shown in Fig. 6A), mean 18.77 ± 1.94% (data pooled from three independent experiments, Fig. 6B). When this experiment was repeated using Ad-OVA–infected DTRhigh DCs as MHC donor cells, 30% of the OVA257–264 peptide-pulsed targets were specifically killed relative to that of control naive mice (representative experiment shown in Fig. 6A), mean 32.5 ± 1.64% (data pooled from three independent experiments, Fig. 6B). This result suggests that Ag presentation via cross-dressing efficiently induces CTL effector cells in vivo and contributes to the overall effector CTL response elicited by a viral Ag. To further confirm the effector function of

**FIGURE 4.** Cross-dressed DCs stimulate Ag-specific CD8+ T cell responses to viral Ags in vivo. DTRhigh DCs infected with Ad-OVA (3000 vp/cell) for 3 h were adoptively transferred into (A) B6 mice previously left untreated or treated with 200 µg dsRNA, or (B) H-2Kbm1 mice previously treated with anti-Thy1.2 Abs. Control mice received uninfected DTRhigh DCs. After 24 h, mice were either untreated or treated with 100 ng DT, followed after an additional 24 h by adoptive transfer of 2 × 106 CFSE-labeled OT-1 Rag−/− CD8+ T cells. Spleens were harvested either 3 or 4 d after OT-1 transfer (H-2Kbm1 and B6 recipients, respectively), and proliferation was measured by flow cytometry after staining with anti-CD8 and anti-Vα2 Abs. Histograms show the CFSE profile of spleen cells gated on CD8+ and Vα2+ cells from individual mice. The percentage of CD8+ Vα2+ OT-1 cells in the indicated gates for each individual mouse is shown. The data shown are representative of eight independently performed experiments.

Presentation of transferred MHC class I:peptide complexes in vivo induces an effector response

Although we have shown that functional MHC class I:peptide complexes are transferred between DCs in vivo and induce CD8+ T cell proliferation, the question remained whether the amount of MHC class I:peptide transferred was sufficient to prime a functional CTL response. To address this, we examined CTL killing in vivo by mice primed by MHC class I:peptide transfer. OVA257–264 peptide-loaded or Ad-OVA–infected DTRhigh DCs were transferred into B6 recipient mice either untreated or treated with dsRNA. In some mice, the transferred DTRhigh DCs were removed by DT administration prior to OT-1 T cell transfer. After 7 d, B6 spleen cells loaded with OVA257–264 peptide (CFSElow) and control unpulsed spleen cells (CFSEhigh) were injected in equal numbers as target cells. At this time point, no DTRhigh GFPhigh DCs were detected in recipient mice (data not shown). Eighteen hours later, the presence of CFSElow and CFSEhigh cells was analyzed by flow cytometry. In mice that received peptide-loaded DTRhigh DCs as MHC donor cells under conditions in which cross-presentation was blocked by dsRNA and direct presentation eliminated by DT treatment, 15.3% of the OVA257–264 peptide-pulsed targets were specifically killed relative to that of control naive mice (representative experiment shown in Fig. 6A), mean 18.77 ± 1.94% (data pooled from three independent experiments, Fig. 6B). When this experiment was repeated using Ad-OVA–infected DTRhigh DCs as MHC donor cells, 30% of the OVA257–264 peptide-pulsed targets were specifically killed relative to that of control naive mice (representative experiment shown in Fig. 6A), mean 32.5 ± 1.64% (data pooled from three independent experiments, Fig. 6B). This result suggests that Ag presentation via cross-dressing efficiently induces CTL effector cells in vivo and contributes to the overall effector CTL response elicited by a viral Ag. To further confirm the effector function of
CD8+ T cells responding to cross-dressed Ag, an additional experiment was undertaken. This time, surface mobilization of CD107 (an integral membrane protein in cytolytic granules) was quantitated from spleen cells isolated from B6 recipients of Ad-OVA-infected DTRhigh DCs and OT-1 T cells and then stimulated with OVA257-264 peptide in vitro. To clearly distinguish the effector function of the transferred OT-1 T cells from endogenous T cells, we made use of mice where the congenic CD45.1 marker is expressed on OT-1 T cells and CD45.2 is expressed on recipient B6 mice. In a representative experiment (Fig. 6C), we observed that 13.9% of the CD45.1+CD8+ T cell population isolated from dsRNA-treated B6 mice injected with Ad-OVA–infected DTRhigh DCs expressed CD107 when stimulated in the presence of OVA257-264 peptide-pulsed APC in vitro. At the same time, 5.65% of CD45.1+CD8+ T cells isolated from dsRNA-treated B6 mice infected with Ad-OVA–infected DTRhigh DCs expressed CD107 when primed by cross-dressed Ag presentation represents ~40% of that primed under conditions where the direct and cross-dressed Ag presentation pathways are intact (Fig. 6C) and as summarized in Fig. 6D from data pooled from three independent experiments. Surface mobilization of CD107 was specific to priming recipients with Ad-OVA–infected DTRhigh DCs, because no expression was detected from CD45.1+CD8+ T cells isolated from control mice injected unpulsed DTRhigh DCs (Fig. 6D). No background staining was observed following incubation with a FITC-labeled isotype Ab (data not shown).

Collectively, the data demonstrate that uninfected DCs cross-dressed with MHC class I:peptide complexes acquired from virus-infected DCs prime naive CD8+ T cells to effector CTL in vivo. Thus, we conclude that the cross-dressing pathway of Ag recognition contributes toward CTL priming in vivo.

Discussion
This study advances several important and novel insights into Ag transfer between DC subsets in vivo and extends our previous in vitro observations (10). To our knowledge, we show in this work for the first time that intact MHC class I:peptide complexes from virally infected DCs are acquired in vivo by uninfected spleen resident DCs, including the CD8α+ DC subset. Furthermore, this cross-dressing of DC in vivo with MHC class I:viral peptide induced priming of naive CD8+ T cells that developed effector functions in vivo.

During viral infection, it is currently thought that subpopulations of tissue resident DCs, within skin or lung tissue, are either directly infected (e.g., the CD11b+ DCs) or capture viral Ag from infected parenchymal cells by cross-presentation or cross-dressing (e.g., via the CD103+ DCs) (11, 25, 26). Migration of these DCs to the LN may induce the activation of CD8+ T cells by direct Ag presentation as well as through cross-presentation by LN resident CD8α+ DCs (27, 28). Nonetheless, at the same time our data may suggest that Ag transfer from migratory CD103+ DCs or CD11b+ DCs may occur through the donation of intact MHC class I:viral peptide complexes to resident uninfected DCs, even if en route the DC undergo virus-induced apoptosis or cytolysis (13). Similarly,
within the spleen, it could be envisaged that blood-borne viral Ag captured by splenic marginal zone macrophages or viral Ag acquired by CD8α+ DCs located in the outer marginal zone (29, 30) may be donated as MHC class I:viral peptide complexes to spleen resident CD8α+2 DCs unable to cross-present viral Ag directly (31). Consistent with this concept, Backer et al. (29) reported that CD8+ T cells are primed by Ag transfer between splenic marginal metallophilic macrophages and DCs in vivo, although the precise mechanism, whether this involved MHC class I:viral peptide or Ag alone, was not addressed (29). Additionally, Yewdall et al. (32) reported induction of CD8+ T cell responses by endogenous DCs following adoptive transfer of peptide-pulsed MHC:donor DCs. However, they suggested that this occurred due to the transfer of peptide alone, rather than the transfer of intact MHC class I:peptide complexes from the donor DCs (32). In sharp contrast, our experimental evidence indicates that activation of OVA 257–264-specific TCR transgenic CD8+ T cells occurred in B6 and H-2Kbm1 recipient mice following adoptive transfer of either H-2b DTR expressing DCs pulsed with OVA257–264 peptide or infected with Ad-OVA (3000 vp/cell) was adoptively transferred into B6 recipient mice either left untreated or treated 24 h previously with 200 μg dsRNA. Control mice received uninfected DTRhigh DCs. At 24 h, some mice received 100 ng DT. All mice received 2 × 106 OT-1 or CD45.1 × OT-1 CD8+ T cells after an additional 24 h. (A) Mice were challenged with CFSE-labeled B6 splenocytes pulsed with OVA257–264 peptide (CFSElow) or no Ag (CFSEhigh) 7 d after OT-1 T cell administration. Eighteen hours later, spleens were harvested and the number of CFSElow and CFSEhigh cells was measured by flow cytometry. Killing of CFSEhigh targets in animals receiving unpulsed DCs was assessed against targets injected into mice receiving only OT-1 T cells. Upper panels, Indicate recipients of DTRhigh DCs pulsed with OVA257–264 peptide (or control mice); lower panels, indicate recipients of Ad-OVA-infected DTRhigh DCs (or control mice). Data show killing of OVA257–264 peptide-pulsed targets (left peaks) and of control targets (right peaks) and are representative of three independent experiments. (B) Percentage of specific in vivo killing of OVA257–264 peptide targets by each of the experimental groups shown in (A); error bars represent the mean ± 1 SD pooled from three independent experiments; p values indicated for comparisons between each experimental group. (C) Spleen cells isolated from B6 recipients (treated as in A) and injected with Ad-OVA-infected DTRhigh DCs (or control DTRhigh DCs), followed by DT (or not) and CD45.1 × OT-1 CD8+ T cells, were cultured either with RBC-depleted B6 APCs pulsed with OVA257–264 peptide or untreated APCs in the presence of anti-CD107a and anti-CD107b Abs for 6 h. Cells were stained with anti-CD8 and anti-CD45.1 and analyzed by flow cytometry. The percentage of CD8+ CD45.1+OT-1 cells in the indicated gates for each individual mouse is shown. Data are representative of three independent experiments. (D) Percentage of CD107+ OT1 T cells (within the CD8+CD45.1+ gate) by each of the experimental groups shown in (C) after in vitro stimulation with peptide-pulsed DC; error bars represent the mean ± 1 SD pooled from three independent experiments and p values indicated for comparisons between each experimental group and the control group.
strong proliferation of OT-1 T cells (Fig. 4A). Because few DCs may efficiently drive CD8\(^+\) T cell proliferation, we considered the formal possibility that the residual CD8\(^+\) DCs remaining in the spleens of dsRNA-treated mice may have contributed to the CD8\(^+\) T cell priming observed. This would seem unlikely, given that CD8\(^+\) DCs activated in vivo by dsRNA under the same conditions display a selective impairment in cross-presentation (20). Thus, the most probable explanation for the observed CD8\(^+\) T cell proliferation (Fig. 4A) was Ag presented by cross-dressed CD8\(^+\) DCs. Findings from two separate models studied in this work also support this hypothesis. First, CD8\(^+\) DCs purified from the spleen of dsRNA-treated mice injected with virus-infected donor DC eliminated by toxin administration were alone sufficient in driving significant expansion of OT-1 T cells (Fig. 5A). Secondly, in Batf3 null mice that lack the dominant cross-presenting CD8\(^+\) DC subset (17), MHC class I peptide transfer from exogenous to endogenous DCs under conditions in which direct Ag presentation was ablated was associated with CD8\(^+\) T cell proliferation (Fig. 5B). Although we cannot entirely exclude a residual cross-presentation capacity of other DC (34) or macrophage subsets (35) in the Batf3 null mice, this contribution to the residual cross-presentation capacity of other DC (34) or macrophage subsets (35) in the Batf3 null mice, this contribution to the total cytolytic killing observed by our adoptive DC transfer system in the K\(^{b}\) bm mouse (Fig. 6B) in which cross-presentation is eliminated, this suggests that endogenous DCs, including the CD8\(^+\) subset of DCs, are efficient at acquiring cross-dressed Ag.

Is cross-dressing effective in eliciting fully functional effector CD8\(^+\) T cells in vivo? Data analyzing the in vivo killing of OVA\(_{257-264}\) peptide-loaded target cells in mice primed by viral Ag presented in this way showed that cross-dressed Ag presentation represented a significant contribution to the total cytolytic killing (Fig. 6A, 6B) in agreement with CD107a/b expression (Fig. 6C, 6D). Together these data indicate that transfer of MHC-peptide complexes in the absence of cross-presentation is an efficient method of priming naive CD8\(^+\) T cells to cytolytic effectors against virus-encoded Ag.

Recently, Wakim and Bevan (11) showed that cross-dressing of MHC class I peptide from parenchymal cells activates resting memory T cells rather than naive CD8\(^+\) T cells (11). At first glance, the data reported in this work may seem at odds with this observation, as we are able to induce proliferation and cytolytic effector activity of naive OT-1 T cells in vivo through cross-dressed Ag presented by recipient DCs. Nonetheless, the differences in the two studies may reflect the relative levels of MHC class I peptide complexes displayed on the surface of TLR ligand peptide-pulsed and virus-infected donor DCs injected in vivo as compared with that displayed by parenchyma cells infected with virus in vivo. Alternatively, it may reflect the amount of plasma membrane exchange between DC and parenchyma cells as compared between donor and recipient DC, or the likely transfer of molecules required for costimulation provided by donor DCs in our system, but not by parenchymal cells. Lastly, the inflammatory cytokine environment induced by the different viral pathogens used in the two studies, adenovirus (in this study) and lymphocytic choriomeningitis virus and vesicular stomatitis virus by the Wakim and Bevan study (11), may likely be very different and impart different signals to DCs.

Finally, by what mechanism are DCs cross-dressed with MHC class I peptide complexes? Conceivably, this may be mediated through the uptake of MHC class I peptide-containing exosomes released from donor cells (9, 13, 14, 37–39). However, Wakim and Bevan (11) find no evidence in support of this. In fact, these authors suggest that MHC class I peptide complexes present on the surface of exosomes are degraded within the recipient DC such that exosome-derived Ag is presented by the recipient’s own MHC molecules. Alternatively, MHC class I peptide complexes may be transferred through acquisition of plasma membrane from living or dying cells (40, 41). Regardless of the mechanism involved, what is evident from our earlier in vitro data, and that of others, and also the data presented in this work, is that transferred MHC class I peptide complexes are capable of activating Ag-specific CD8\(^+\) T cells (10, 11, 15, 42).

In summary, we propose a model whereby Ag-specific naive CD8\(^+\) T cells are primed following interaction with uninfected CD8\(^+\) DCs that present cross-dressed viral Ags on their surface. During virus infection, the sharing of MHC class I viral peptide complexes between infected and uninfected DCs or to a DC unable to cross-present the Ag will help initiate and amplify the CD8\(^+\) T cell immune response well before acquired Ags are cross-presented. The sharing of Ag as preformed MHC class I peptide complexes between infected and uninfected DCs without the restraints of Ag processing may have evolved not only to accurately amplify the response, but also to engage multiple DC subsets critical in the generation of strong antiviral immunity (43, 44). Finally, what makes the CD8\(^+\) DCs efficient at acquiring Ag via this route is a key question that remains to be answered.

Our increased understanding of how Ags are processed by DC subsets will be important for the rational development of CD8\(^+\) T cell vaccines for HIV, malaria, and tuberculosis.

Disclosures

The authors have no financial conflicts of interest.

References