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# Tumor-Specific CD4<sup>+</sup> T Cells Are Activated by “Cross-Dressed” Dendritic Cells Presenting Peptide-MHC Class II Complexes Acquired from Cell-Based Cancer Vaccines<sup>1</sup>

Brian P. Dolan, Kenneth D. Gibbs, Jr., and Suzanne Ostrand-Rosenberg<sup>2</sup>

Tumor cells that constitutively express MHC class I molecules and are genetically modified to express MHC class II (MHC II) and costimulatory molecules are immunogenic and have therapeutic efficacy against established primary and metastatic cancers in syngeneic mice and activate tumor-specific human CD4<sup>+</sup> T lymphocytes. Previous studies have indicated that these MHC II vaccines enhance immunity by directly activating tumor-specific CD4<sup>+</sup> T cells during the immunization process. Because dendritic cells (DCs) are considered to be the most efficient APCs, we have now examined the role of DCs in CD4<sup>+</sup> T cell activation by the MHC II vaccines. Surprisingly, we find that DCs are essential for MHC II vaccine immunogenicity; however, they mediate their effect through “cross-dressing.” Cross-dressing, or peptide-MHC (pMHC) transfer, involves the generation of pMHC complexes within the vaccine cells, and their subsequent transfer to DCs, which then present the intact, unprocessed complexes to CD4<sup>+</sup> T lymphocytes. The net result is that DCs are the functional APCs; however, the immunogenic pMHC complexes are generated by the tumor cells. Because MHC II vaccine cells do not express the MHC II accessory molecules invariant chain and DM, they are likely to load additional tumor Ag epitopes onto MHC II molecules and therefore activate a different repertoire of T cells than DCs. These data further the concept that transfer of cellular material to DCs is important in Ag presentation, and they have direct implications for the design of cancer vaccines. *The Journal of Immunology*, 2006, 176: 1447–1455.

The potential efficacy of immunotherapy for the treatment of cancer has led to the development of novel experimental strategies. Many of these innovative approaches focus on the activation of tumor-specific T lymphocytes because the cell-mediated arm of the immune system can be effective in destroying tumor cells and in providing long-term protection (memory) against the recurrence and/or outgrowth of primary and/or metastatic tumor cells (1–3). Because optimal activation of tumor-specific, cytotoxic CD8<sup>+</sup> T cells requires coactivation of CD4<sup>+</sup> T lymphocytes, we are developing cell-based cancer vaccines that activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Many tumor cells constitutively express MHC class I (MHC I)<sup>3</sup> molecules and are therefore capable of interactions with CD8<sup>+</sup> T cells. Several studies have shown that coexpression of T cell costimulatory molecules in the tumor cell is sufficient for activation of tumor-specific CD8<sup>+</sup> T cells (4–8). However, these cells do not coexpress MHC class II (MHC II) molecules and therefore cannot activate CD4<sup>+</sup> T cells to provide the requisite help. We have reasoned that genetic manip-

ulation of MHC I<sup>+</sup> tumor cells to coexpress MHC II and T cell-costimulatory molecules would create a cell-based vaccine (MHC II vaccine) that is capable of activating tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes (9–11).

In vivo studies with three independent mouse tumors (Sa1 sarcoma, B16 melanoma, and 4T1 mammary carcinoma) have shown that vaccination/treatment with MHC II vaccines provides prophylactic protection (11), mediates rejection of established primary tumor (9), and reduces established spontaneous metastatic disease while extending survival (12). Although the MHC II vaccines have not as yet been tested clinically, in vitro studies demonstrated that HLA-DR- and CD80-modified human ocular melanoma and breast cancer cells are potent activators of tumor-specific HLA-DR- and HLA-A-matched CD4<sup>+</sup> and CD8<sup>+</sup> human T cells, suggesting that the vaccines also may be effective in patients (13, 14).

The MHC II vaccines were designed to facilitate T cell activation by direct presentation of endogenously synthesized tumor Ags and bypass the need for host APCs to capture and present tumor Ags. Several lines of evidence support the premise that the vaccine cells are the relevant APCs in vivo: 1) bone marrow (BM) chimeric mice and nude mice inoculated with genetically disparate vaccine cells develop tumor-specific CD4<sup>+</sup> T cells restricted to the genotype of the tumor (15, 16); 2) vaccine cells activate naive T cells in vitro (17); and 3) coexpression of the MHC II chaperone protein invariant (Ii), which blocks loading of endogenous Ags onto MHC II molecules, blocks both in vitro presentation of endogenous Ags and abolishes vaccine efficacy (16, 18, 19). Although there also is evidence from other systems that tumor cells directly activate T cells (8, 20), most studies indicate that dendritic cells (DCs) are the principal APCs that activate tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes (21, 22). DCs activate T cells by capturing exogenous Ags released from tumor cells, processing them into peptides, and presenting the peptides to T cells via the process of cross-presentation. Because of their superior Ag

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<sup>3</sup> Abbreviations used in this paper: MHC I, MHC class I; MHC II, MHC class II; BM, bone marrow; Ii, invariant; DC, dendritic cell; BMDC, BM-derived DC; pMHC, peptide-MHC; DTx, diphtheria toxin; DTR, DTx receptor; HEL, hen egg lysozyme; PI, propidium iodide; DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine.

presentation capabilities, DCs are considered the most important APCs *in vivo*.

Although our previous experiments indicated that MHC II vaccine cells are the principal APCs during vaccination (15, 16), these studies do not exclude a role for DCs in MHC II vaccine efficacy. We now report that DCs are essential for MHC II vaccine efficacy; however, they mediate their effect through a novel mechanism that is distinct from cross-presentation. The process involves the generation of peptide-MHC (pMHC) complexes within the vaccine cells, and their subsequent transfer to DCs, which then present the intact, unprocessed complexes to CD4<sup>+</sup> T lymphocytes. This process of "cross-dressing" produces DCs that are the functional APCs; however, the pMHC complexes that are presented are generated by the MHC II vaccine cells. This result is consistent with our earlier findings that MHC II vaccine genotype governs CD4<sup>+</sup> T cell restriction and provides a mechanistic explanation for the potent therapeutic efficacy of the MHC II vaccines.

## Materials and Methods

### Mice

Original breeding stocks of A/J, BALB/c, and Itgax-diphtheria toxin (DTx) receptor/enhanced GFP (CD11c-DTR) (23) transgenic mice were from The Jackson Laboratory. Original breeding stock of female FVB mice (3–6 mo of age) were from Charles River Laboratories. 3A9 transgenic mice (24) were maintained on a C3H/HeJ background or crossed once with A/J mice to yield (C3H-3A9 × A/J)<sub>F1</sub> mice. Offspring were screened as described (17). F<sub>1</sub> generations of CD11c-DTR mice were obtained by mating female transgenic mice on a BALB/c background with male A/J mice. Offspring were screened for transgene expression as described (23). Mice were housed according to National Institutes of Health guidelines for the humane treatment of laboratory animals and bred in the University of Maryland Baltimore County animal facility. All animal procedures were approved by the University of Maryland Baltimore County Institutional Animal Care and Use Committee.

### Cells and Abs

SaI (11), SaI/hen egg lysozyme (HEL) (19), SaI/A<sup>k</sup> (11), SaI/A<sup>k</sup>/HEL (19), MELF10/A<sup>b</sup> (25), 4T1 (12), and B16.BL6/A<sup>b</sup> (26) tumor cells were grown as described. The I-A<sup>k</sup>-restricted, HEL-specific T cell hybridomas A2.A2 (27) and 3B11.1 (28) and the B cell lymphoma M12.C3.F6 (29) were grown as described (16, 30). The B cell lymphoma A20 was grown in RPMI 1640 medium (Biofluids) supplemented with 10% FCS (HyClone), 1% penicillin, 1% streptomycin, 1% gentamicin (Biofluids), and 1% GlutaMAX (Life Technologies). 3LL tumor cells were grown in DMEM (Biofluids) supplemented with 10% fetal clone I serum (HyClone), 1% penicillin, 1% streptomycin, 1% gentamicin, and 1% GlutaMAX. The following Abs directly coupled to FITC or PE were purchased from BD Pharmingen: CD11c (clone HL3), I-A<sup>k</sup> (11-5.2), I-A<sup>d</sup> (39-10-8), I-A<sup>b</sup> (AF6-120.1), I-A/I-E (2G9), K<sup>k</sup> (36-7-5), K<sup>b</sup> (AF6-88.5), D<sup>d</sup> (34-2-12S), CD80 (1G10), and CD86 (GL1). CD4-PE was purchased from Caltag Laboratories. All purchased Abs were diluted 1/50 in 0.5% BSA in PBS for staining. B220 and HO-13-4-9 were used as undiluted culture supernatants of hybridoma cells. 10-2.16 and 3JP Abs were purified from hybridoma culture supernatants and used as described (19). For some experiments, 10-2.16 Ab was directly coupled to Alexa Fluor 488 (Molecular Probes) following the manufacturer's protocols (3 mol of dye per mole of Ab).

### Tumor challenges

(CD11c-DTR × A/J)<sub>F1</sub> transgenic and nontransgenic littermates were injected i.p. with DTx at 5 ng/g body weight (Sigma-Aldrich) or left untreated and 8 h later inoculated i.p. with 8 × 10<sup>5</sup> SaI or SaI/A<sup>k</sup> tumor cells. In some experiments, ~5 × 10<sup>6</sup> CD8<sup>+</sup> T cells from nontransgenic littermates were injected i.v. into the tail vein of transgenic mice 24 h before DTx administration and tumor challenge. The SaI variant used in these studies is an ascites tumor that grows progressively in A/J mice and is lethal before day 20 after tumor inoculation. Tumor-inoculated mice were monitored every other day by visual inspection for development of ascites tumor and were sacrificed when they became moribund. Tumor incidence is the number of mice that developed lethal ascites tumors and were moribund by day 20, divided by the total number of mice inoculated. In some instances, male CD11c-DTR mice developed a solid tumor that caused paralysis of one hindleg. Such tumors caused mice to become moribund an

average of 37 days after tumor inoculation. Because these tumors resulted in animals becoming moribund long after the ascites tumors were lethal and tumor growth was drastically altered from the parental tumor growth pattern, these mice were not considered susceptible to ascites tumors.

### DC isolation

DCs were purified from the spleens of 3- to 6-mo-old mice by magnetic bead sorting. Spleens were harvested, minced, and incubated in collagenase D (Sigma-Aldrich) for 30 min at 37°C. Resulting cells were washed in serum-free RPMI 1640 medium, and RBC were depleted by treatment with ACK lysing buffer (Biofluids). Splenocytes were resuspended in 0.5% BSA in PBS (1–2 × 10<sup>8</sup> cells/ml), incubated for 30 min on ice with 100 μl of CD11c microbeads (Miltenyi Biotec) per spleen, and applied to a magnetic column to isolate CD11c<sup>+</sup> cells. Purified CD11c<sup>+</sup> cells were cultured for 1 h at 37°C in DC medium (IMDM supplemented with 5% FCS, 1% penicillin, 1% streptomycin, 1% gentamicin, and 1% GlutaMAX), followed by a gentle washing with warm DC medium to remove residual dead cells and contaminating lymphocytes. Following purification, cells were routinely 80–90% CD11c<sup>+</sup>. BM-derived DCs (BMDCs) were obtained by isolating marrow from the femurs of 6-mo-old mice. RBC were removed by treatment with ACK lysing buffer. B cells, T cells, and MHC II<sup>+</sup> cells were depleted by incubation with ~1 μg each of B220, HO-13-4-9, and 3JP Abs, followed by addition of rabbit complement (Cedarlane Laboratories) as described (31). Remaining cells were resuspended at 10<sup>7</sup> cells per ml in BMDC medium (RPMI 1640 medium supplemented with 10% FCS, 1% penicillin, 1% streptomycin, 1% gentamicin, 1% GlutaMAX, 5 × 10<sup>-4</sup> M 2-ME (Sigma-Aldrich), 20 ng/ml rGM-CSF, and 10 ng/ml rIL-4 (both from RDI)) and cultured at 37°C in 5% CO<sub>2</sub>. Medium was replaced every 2 days, and loosely attached cells were removed. After 6–8 days of culture, cells were routinely >70% CD11c<sup>+</sup> as measured by flow cytometry.

### MHC transfer experiments

Cells were freeze-thaw lysed by freezing 10<sup>7</sup> cells/ml of serum-free RPMI 1640 medium at –80°C for 20 min, followed by rapid thawing at 37°C. The cycle was repeated up to three times until lysis was complete as determined by trypan blue uptake. Osmotic lysates of cells were obtained by resuspending 5 × 10<sup>6</sup> cells/ml in 0.9 vol of distilled water for 30 min on ice, followed by the addition of 0.1 vol of 10× PBS. Cells were heat killed by resuspending to 5 × 10<sup>6</sup> cells/ml in PBS and incubation at 65°C for 30 min. Freeze-thawed, osmotically lysed, or heated-killed tumor cells were added to DCs that had been plated 1 h earlier in six-well dishes at 1–2 × 10<sup>6</sup> cells/well/2 ml of DC medium at a ratio of two to four tumor cells per DC. Following a 3-h incubation at 37°C in 5% CO<sub>2</sub>, tumor cell material was removed from the cultures by extensive washing of the attached DCs with PBS. For confocal experiments, tumor cells were labeled before freeze-thaw lysis with the lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD, excitation, 648 nm; emission, 670 nm; Molecular Probes) according to the manufacturer's recommendations. For experiments with BMDCs, tumor cell material was removed after the 3-h incubation by centrifugation at 1100 × g through Ficoll-Paque (Pharmacia Biotech) for 15 min at room temperature. DCs were recovered at the Ficoll-Paque medium interface. For Transwell experiments, 1–2 × 10<sup>6</sup> DC were plated in 2 ml of DC medium in the bottom chamber of an 8-μm Transwell (Corning Glass) and two to three cell equivalents of freeze-thawed tumor cells in 1 ml of serum-free RPMI 1640 medium were added to the top chamber. In some experiments, 1.0-μm FITC beads (Polysciences) also were added to the top chamber.

### CFSE labeling and pronase treatments

BMDCs were treated with 3 μM CFSE (Molecular Probes) at 1 × 10<sup>7</sup> DC/ml in PBS for 10 min at room temperature. The reaction was quenched by the addition of calf serum and washing with excess PBS. DCs were treated with pronase (type XIV; Sigma-Aldrich) by incubating 10<sup>6</sup>–5 × 10<sup>6</sup> DC with 2 mg/ml pronase in PBS for 20 min at 37°C. Treated cells were washed extensively with DC medium.

### Flow cytometry and confocal microscopy

Cells were labeled for immunofluorescence and analyzed by flow cytometry using an XL instrument (Beckman Coulter) as described (32) and analyzed using Expo32 ADC software (Beckman Coulter). For confocal microscopy, DCs were fluorescently labeled with CD11c-PE and I-A<sup>k</sup>-Alexa Fluor 488 Abs, washed with PBS to remove unbound Ab, and then adhered to slides coated with poly-L-lysine (Sigma-Aldrich) for 30 min at 4°C. Attached cells were fixed by a 5-min incubation with 2% paraformaldehyde in PBS. Fixative was quenched by the addition of 1 M glycine (Sigma-Aldrich), and slides were washed once with excess PBS and once



with excess water. Coverslips were mounted with 20  $\mu$ l of a 50% solution of Fluoromount-G (Southern Biotechnology Associates) in PBS. Slides were visualized using a confocal microscope (LSM 510 META; Zeiss) located at the Johns Hopkins University Integrated Imaging Center (Baltimore, MD) and analyzed using LSM Image Browser software (Zeiss). Fluorescent images were collected sequentially, with each scan repeated four times and then averaged. Approximately 1- $\mu$ m optical slices through the z-plane were collected. A minimum of 25 labeled cells was analyzed for each cell type for each data set.

#### T cell isolation and Ag presentation

CD4<sup>+</sup> 3A9 TCR transgenic T cells were isolated by negative selection using a CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec) according to the manufacturer's directions. Purified populations were >90% CD4<sup>+</sup> as measured by flow cytometry. Ag presentation assays using DCs as APCs were performed as described previously for tumor cells (33) with the following modifications. Splenic DCs ( $1 \times 10^5$  cells per well) were incubated with 2.5–3 times as many freeze-thawed tumor cells. Following a 3-h incubation (37°C, 5% CO<sub>2</sub>), wells were washed extensively with PBS to remove tumor cell material, and 200  $\mu$ l of RPMI 1640 medium supplemented with 10% FCS was added to each well. Ag presentation experiments with hybridomas ( $5 \times 10^4$  per well) (33) and with transgenic T cells ( $10^5$  per well) (17) were performed as described. IL-2 release was quantified by ELISA (Endogen) as described (33).

#### In vivo T cell priming and in vitro measurement of T cell activation

(CD11c-DTR  $\times$  A/J)F<sub>1</sub> mice were untreated or injected i.p. with DTx (5 ng/g body weight) and 6 h later immunized i.p. with either  $8 \times 10^5$  SaI/HEL or SaI/A<sup>k</sup>/HEL cells. Six days later, splenic CD4<sup>+</sup> T cells were isolated by negative selection using magnetic beads (Miltenyi Biotec) according to the manufacturer's directions. Purified cells were >90% CD4<sup>+</sup> as measured by flow cytometry. In vivo priming of purified CD4<sup>+</sup> T cells was assessed by measuring their in vitro response to HEL presented by professional APCs as described (16), with the following modifications. One million purified CD4<sup>+</sup> T cells were cultured overnight with  $10^5$  M12.C3.F6 cells that had been pulsed previously for 15 h with 2 mg/ml HEL protein (Sigma-Aldrich) in RPMI 1640 medium containing an additional 1 mg/ml HEL. IL-2 release was determined by ELISA (Endogen). Background levels of IL-2 were determined by culturing T cells without APCs and were subtracted from experimental values.

#### Statistical analyses

SDs and Student's *t* test were calculated using Excel 2002 software (Microsoft).

## Results

### DCs are required for efficacy of MHC II vaccines

To determine whether host DCs are required for MHC II vaccine-mediated T cell activation and tumor rejection, mice were depleted for DCs before tumor inoculation and observed for tumor progression. CD11c-DTR transgenic mice express the simian DTx receptor under control of the DC-specific CD11c promoter. When CD11c-DTR mice are given DTx, their DCs are deleted for 2 days and return to normal levels by day 5 (23). The mouse SaI sarcoma and its corresponding MHC II vaccine, SaI/A<sup>k</sup>, are derived from A/J mice. Therefore, CD11c-DTR mice (BALB/c background) were mated to A/J mice to produce offspring semisyngeneic to SaI. When challenged i.p. with live wild-type SaI cells, CD11c-DTR and nontransgenic littermates developed ascites tumors and were moribund by day 20 (Table I). In contrast, neither transgenic nor nontransgenic littermates developed ascites tumors after inoculation of live SaI/A<sup>k</sup> vaccine cells (Table I), in agreement with previous studies showing that expression of syngeneic MHC II enhances tumor cell immunogenicity. However, if DCs are deleted from the CD11c-DTR mice by a single dose of DTx 6 h before tumor challenge, then SaI/A<sup>k</sup> cells cause a lethal ascites tumor, demonstrating that DCs are necessary for the rejection of MHC II vaccine cells.

Because CD11c expression has been detected on activated CD8<sup>+</sup> T cells (23), it is possible that DTx-treated CD11c-DTR

Table I. Vaccine efficacy provided by MHC class II vaccines requires host DC<sup>a</sup>

CD11c-DTR	DTx	Tumor Challenge	Tumor Incidence
–	–	SaI	5/5
+	–	SaI	4/5
–	+	SaI/A <sup>k</sup>	1/7
+	–	SaI/A <sup>k</sup>	1/9
+	+	SaI/A <sup>k</sup>	7/8

<sup>a</sup> CD11c-DTR or nontransgenic littermates were given a single dose of DTx or left untreated 6 h prior to tumor challenge. Mice were monitored daily for ascites tumor growth and sacrificed when they became moribund. Tumor incidence is the number of mice who became moribund by day 20 divided by the total number of mice challenged.

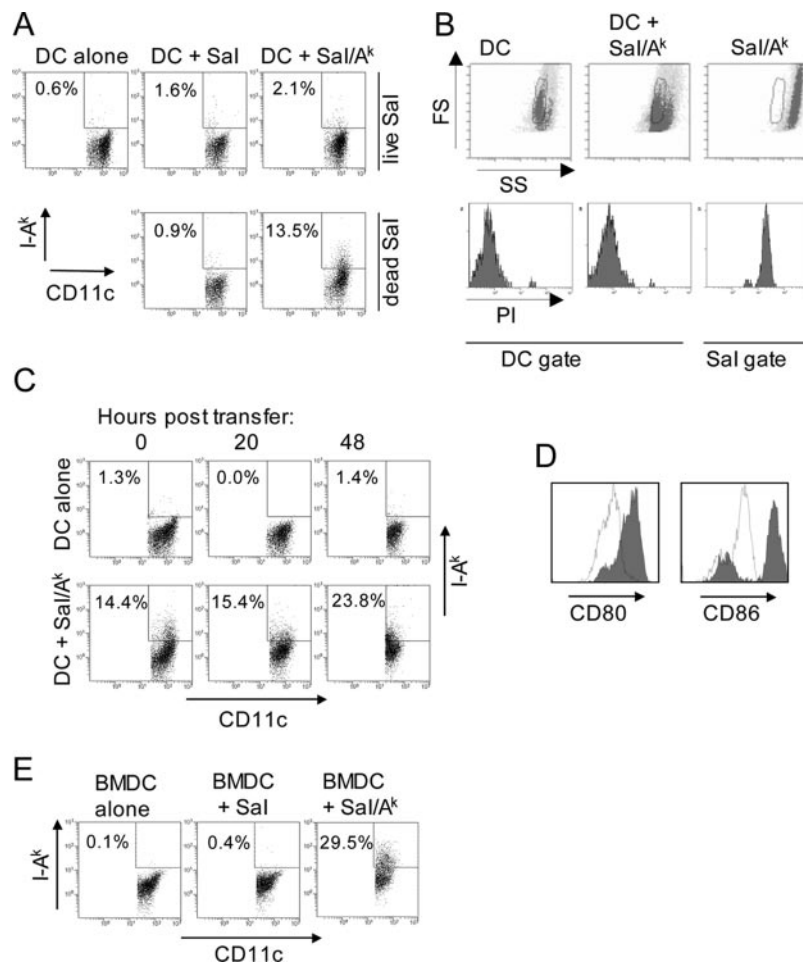
mice develop tumors because they are CD8 depleted. To control for this possibility, CD8<sup>+</sup> T cells from nontransgenic littermates were adoptively transferred to CD11c-DTR mice before DTx administration and tumor challenge. These reconstituted mice also were susceptible to SaI/A<sup>k</sup> tumor growth (data not shown), indicating that tumor susceptibility was due to DC depletion.

### DCs acquire MHC II molecules from dead tumor cells

The dual requirement for MHC II expression by tumor cells and the presence of host DCs suggested that interactions between these two cell populations might be essential for maximum T cell activation. Because recent reports have shown the physical transfer of plasma membrane molecules between multiple cell types, including DCs (34–36), we examined whether MHC II proteins could transfer from tumor cells to DCs. Splenic DCs were obtained from FVB mice (*H-2<sup>g</sup>*) by magnetic bead selection, incubated with SaI or SaI/A<sup>k</sup> cells for 3 h, and then analyzed for I-A<sup>k</sup> expression using an I-A<sup>k</sup>-specific mAb (11-5.2) and flow cytometry. When FVB DCs were incubated with live SaI or SaI/A<sup>k</sup> tumor cells, no I-A<sup>k</sup> expression is seen on the DCs (Fig. 1A). However, if FVB DCs are mixed with the lysates of freeze-thawed SaI/A<sup>k</sup> tumor cells, then the DCs acquire I-A<sup>k</sup> at their cell surface. Incubation with freeze-thawed SaI cells does not result in DC expression of I-A<sup>k</sup>. Transfer of I-A<sup>k</sup> molecules was confirmed using a second I-A<sup>k</sup>-specific mAb (10-2.16) and also was seen when recipient BALB/c splenic DCs were used (data not shown). Transfer of I-A<sup>k</sup> molecules from SaI/A<sup>k</sup> vaccine cells to FVB DCs also was seen when tumors were killed by osmotic lysis but not when cells were heat killed (data not shown), suggesting that cells must be disrupted for transfer to occur.

To eliminate artifacts due to contamination of the DC population with freeze-thawed or residual live SaI/A<sup>k</sup> cells, DCs were adhered to plastic and washed extensively to remove tumor cell debris before labeling for flow cytometry. In addition, DCs and SaI/A<sup>k</sup> cells have different forward- and side-scatter profiles, allowing for precise bitmapping of the DCs and elimination of SaI/A<sup>k</sup> cells and/or material (Fig. 1B). DCs also were distinguishable from SaI/A<sup>k</sup> material because they are propidium iodide (PI) negative, whereas freeze-thawed SaI/A<sup>k</sup> cells are PI positive (Fig. 1B).

Transfer of MHC II to DCs may represent a transient display of MHC II molecules that occurs during endocytosis. To determine whether the observed transfer events were transient, FVB DCs were exposed to freeze-thawed SaI/A<sup>k</sup> cells for 3 h and then washed extensively to remove tumor debris as in Fig. 1, A and B. DCs were then incubated in medium containing GM-CSF and IL-4 and analyzed for I-A<sup>k</sup> expression 20 and 48 h later. As seen in Fig. 1C, DCs expressed tumor-encoded MHC II molecules during the 48-h incubation, indicating that MHC II transfer is probably not



**FIGURE 1.** MHC II complexes transfer from dead tumor cells to DCs. *A*, Splenic DCs from FVB mice (*H-2<sup>d</sup>*) were cocultured with live or freeze-thawed Sal or Sal/A<sup>k</sup> cells for 3 h, and adherent DCs were washed extensively to remove tumor material. DCs were then stained for CD11c and I-A<sup>k</sup> and analyzed by flow cytometry. *B*, Splenic DCs can be resolved from tumor cells by forward scatter and side scatter and are PI negative. All of the DC flow cytometry data presented used the DC bitmap shown in the upper panels. *C*, Transferred MHC II is stable on recipient DCs. Splenic DCs from FVB mice were cocultured with freeze-thawed Sal/A<sup>k</sup> cells, and adherent cells were washed to remove tumor material and incubated in medium containing GM-CSF and IL-4 for 0, 24, or 48 h. DCs were then analyzed for I-A<sup>k</sup> expression. *D*, DCs mature during culture. Cells from *C* were stained for CD80 and CD86 at 0 (open histograms) or 20 h (shaded histograms). *E*, MHC II transfers from dead tumor cells to BMDCs. BMDCs were generated from the BM of FVB mice, cocultured with freeze-thawed Sal or Sal/A<sup>k</sup> cells as in *A*, and analyzed for I-A<sup>k</sup> expression. These data are from one of three or more independent experiments.

due to transient endocytosis. DCs also underwent maturation during the 48-h incubation as determined by the up-regulation of the costimulatory molecules CD80 and CD86 (Fig. 1*D*), demonstrating that transferred MHC II was retained and stably expressed during the maturation process.

To ascertain whether MHC II transfer is restricted to splenic DCs, experiments were performed with BMDCs. FVB BMDCs were obtained by incubation of BM cells in the presence of GM-CSF and IL-4 for 6–8 days until cells were >70% CD11c<sup>+</sup> as assessed by flow cytometry. The resulting cells were then incubated with lysates of freeze-thawed Sal/A<sup>k</sup> cells as in previous experiments with splenic DCs, and transfer of I-A<sup>k</sup> molecules to BMDCs was assessed by flow cytometry. As seen in Fig. 1*E*, tumor-derived I-A<sup>k</sup> molecules also are transferred to BMDCs.

#### MHC II transfer occurs from a variety of tumor cells

To determine whether MHC II transfer from tumor cells to DCs is a general phenomenon, we examined whether other MHC II vaccine cells and class II-positive tumor cells could be donors. The B16 melanoma-derived cell lines B16.BL6 and MELF10 were previously transfected with I-A<sup>b</sup> genes and shown to have decreased tumorigenicity and to activate immune responses (25, 26). When either cell type is lysed by freeze-thawing and mixed with FVB splenic DCs, I-A<sup>b</sup> molecules are detected on the surface of recipient DCs as determined by flow cytometry (Fig. 2*A*, upper panels). In agreement with the data presented for Sal/A<sup>k</sup>, no I-A<sup>b</sup> is detected when live tumor cells are used (data not shown). Similarly, I-A<sup>d</sup> and I-A<sup>k</sup> molecules are transferred from the B cell lymphoma lines A20 and M12.C3.F6, respectively, following incubations of

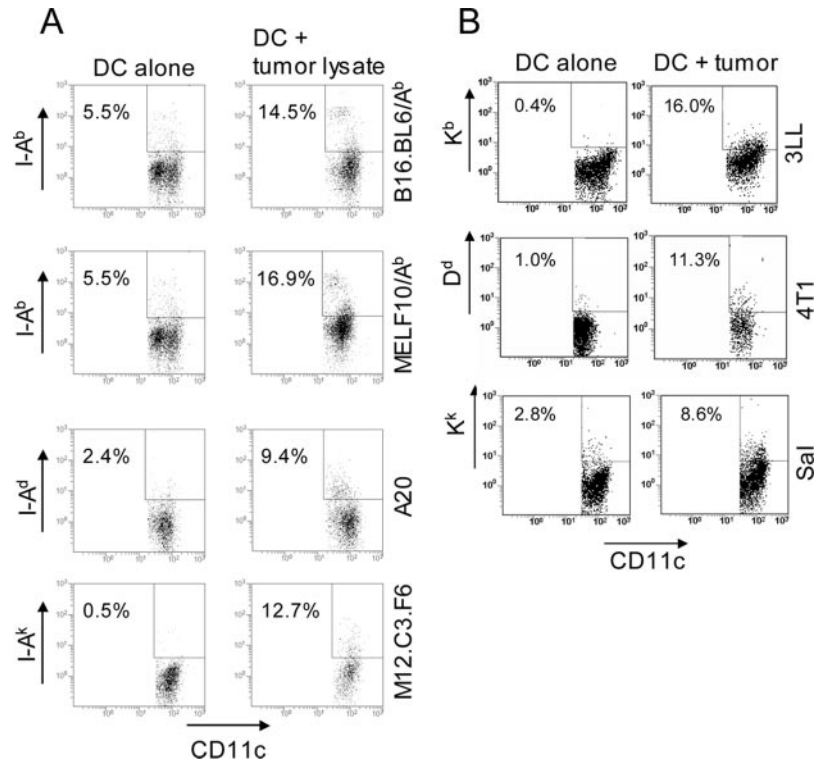
these freeze-thawed cells with FVB DCs (Fig. 2*A*, lower panels). Therefore, MHC II transfer from dead tumor cells to DCs appears to be characteristic of multiple tumors and is not restricted by tissue type or MHC II genotype.

#### MHC I transfers from necrotic tumor cells to DCs

To determine whether MHC I molecules are similarly transferred and retained, three independent tumor cell lines with different MHC I genotypes were tested. FVB splenic DCs were incubated with lysates of freeze-thawed 4T1 mammary carcinoma (*D<sup>d</sup>*), 3LL Lewis lung carcinoma (*K<sup>b</sup>*), or Sal sarcoma (*K<sup>k</sup>*) and analyzed for transferred MHC I molecules using the same procedure as used for the MHC II experiments. As shown in Fig. 2*B*, tumor MHC I expression is detected on recipient DCs. Transferred MHC I was stably expressed for up to 2 days (data not shown). These data indicate that, like MHC II, MHC I molecules can be transferred from necrotic tumor cells to DCs.

#### Transfer of MHC II molecules is cell-contact dependent and requires DC surface receptors

To determine whether direct contact between donor tumor cells and recipient DCs was necessary for MHC II transfer or whether MHC II transfer was mediated through soluble factors or exosomes, FVB DCs were placed in the bottom half of a Transwell chamber containing an 8- $\mu$ m semipermeable membrane, and freeze-thawed lysates of Sal/A<sup>k</sup> tumor cells were placed in the top half. Following a 3-h incubation, DCs were analyzed for I-A<sup>k</sup> expression by flow cytometry. To ensure that soluble material could traverse the semipermeable membrane, 1- $\mu$ m FITC-coated latex



**FIGURE 2.** MHC I and II molecules transfer from a variety of tumor cells to DCs. *A*, Splenic DCs from FVB mice were cocultured with freeze-thawed lysates of B16.BL6/A<sup>b</sup>, B16 MELF10/A<sup>b</sup>, A20, or M12.C3.F6 cells as in Fig. 1*A* and analyzed by flow cytometry for I-A<sup>b</sup> (B16 lines), I-A<sup>d</sup> (A20), or I-A<sup>k</sup> (M12.C3.F6) expression. *B*, Splenic DCs from FVB mice were cocultured with freeze-thaw lysates of the indicated tumor cells and subsequently analyzed for K<sup>b</sup> (from 3LL), D<sup>d</sup> (from 4T1), and K<sup>k</sup> (from SaI) expression. These data are from two or more independent experiments for each cell line.

beads were added to the tumor lysate in the upper chamber. In the presence of the semipermeable membrane, MHC II transfer to DCs is blocked (Fig. 3*A*), whereas DC uptake of FITC-coated latex beads is only 16% less than uptake in the absence of the membrane. Although exosome-mediated transfer of MHC II cannot be ruled out, these data suggest that transfer of MHC II molecules from tumor cells to DCs requires cell-to-cell contact or exposure to >8  $\mu$ m of tumor-derived particulate matter.

Acquisition of molecules by DCs can occur by several mechanisms, including receptor-mediated endocytosis or macropinocytosis. If surface receptors are necessary for MHC II transfer to DCs, then treatment of DCs with pronase, a protease that destroys surface receptors without affecting macropinocytosis (37), should eliminate MHC II transfer. CFSE-labeled BMDCs were pronase treated before incubation with freeze-thawed lysates of SaI/A<sup>k</sup> cells and subsequent analysis for I-A<sup>k</sup> transfer by flow cytometry. BMDCs were used in these experiments because they were more resistant to CFSE labeling and pronase treatment than splenic DCs. As shown in Fig. 3*B*, pronase treatment inhibited transfer of MHC II from tumor cells to DCs. As a control to demonstrate that pronase treatment does not significantly affect nonreceptor-mediated uptake, pronase-treated BMDCs were incubated with FITC-dextran, which is internalized predominantly by nonreceptor-mediated processes. Pronase treatment diminished dextran uptake by 17% as measured by flow cytometry (data not shown), indicating that nonreceptor-mediated uptake was largely unaffected. These results indicate that MHC II transfer to DCs requires expression of DC cell surface proteins and is not mediated by bulk fluid uptake by the DCs.

#### *DCs acquire discrete regions of tumor plasma membrane containing MHC II*

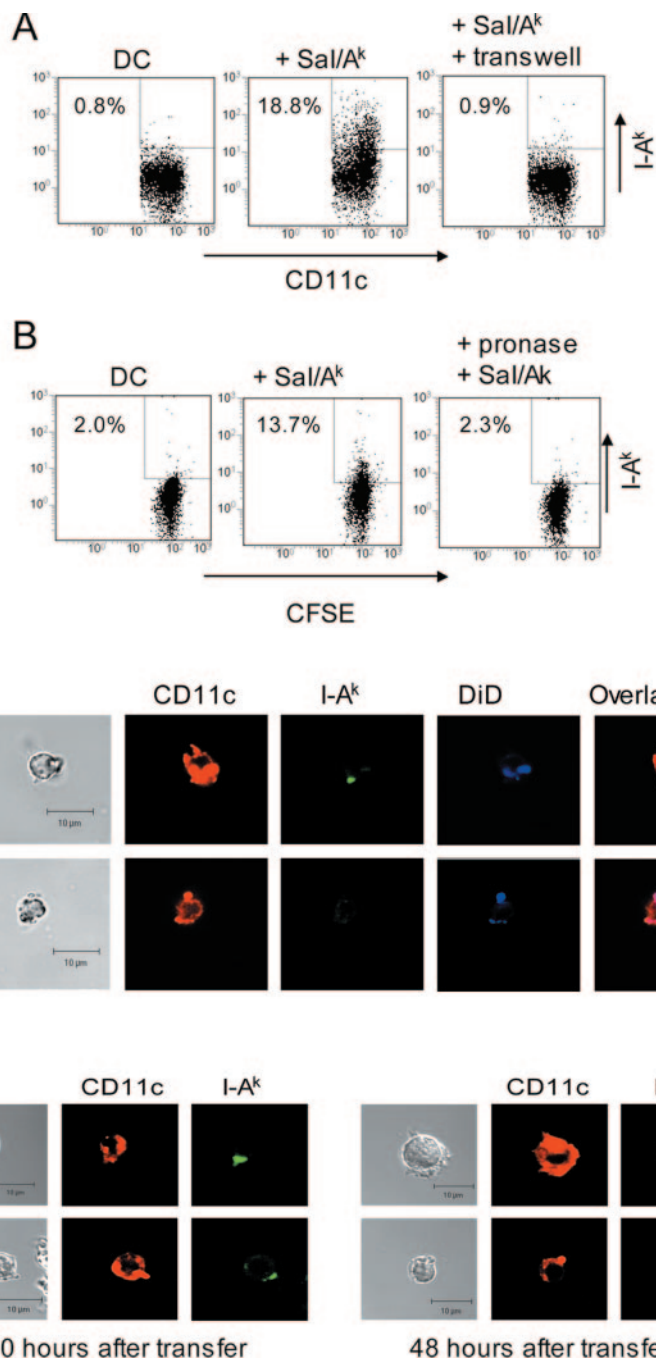
DCs can acquire plasma membrane lipids from other cells (34). To determine whether plasma membrane lipids from SaI/A<sup>k</sup> vaccine cells transferred to DCs, confocal microscopy was used. Splenic DCs from FVB mice were exposed to freeze-thawed lysates of SaI

or SaI/A<sup>k</sup> cells whose plasma membranes were labeled previously with the fluorescent lipophilic dye DiD. After transfer, the DCs were stained with mAbs to I-A<sup>k</sup> and CD11c. DCs incubated with labeled SaI/A<sup>k</sup> lysates contain discrete patches of colocalized tumor-derived lipid and MHC II molecules or patches of lipid without MHC II, whereas DCs incubated with SaI lysates exclusively contain transferred lipid devoid of I-A<sup>k</sup> (Fig. 3*C*). To determine the stability of transferred MHC II over time, FVB DCs exposed to SaI/A<sup>k</sup> lysates for 3 h were extensively washed to remove tumor material and either stained for CD11c and I-A<sup>k</sup> or incubated in GM-CSF and IL-4 for 48 h and then stained and visualized. As seen in Fig. 3*D*, patches of transferred I-A<sup>k</sup> are detected on the recipient DCs immediately after transfer, and by 48 h, I-A<sup>k</sup> is dispersed over the DC cell surface.

#### *Preformed pMHC II complexes transfer to DCs and activate CD4<sup>+</sup> T cells*

If MHC II transfer from genetically modified tumor cells to DCs is necessary for immunogenicity, then recipient DCs should activate T cells to tumor-encoded Ags restricted to the MHC genotype of the tumor cells. To test this hypothesis, we asked whether DCs containing transferred tumor pMHC II complexes activate tumor specific CD4<sup>+</sup> T cells restricted to the tumor cell genotype and specific for tumor-encoded Ags. SaI/HEL or SaI/A<sup>k</sup>/HEL cells were killed by freeze-thaw lysis and cultured with FVB splenic DCs as in previous experiments. DCs were then washed extensively to remove residual tumor material and cultured with I-A<sup>k</sup>-restricted, HEL-specific T hybridoma cells (3B11.1 or A2.A2) or with transgenic T cells expressing I-A<sup>k</sup>-restricted, HEL-specific TCR (3A9). T cell activation was measured by IL-2 release. The T cell hybridomas (Fig. 4*A*) or transgenic T cells (Fig. 4*B*) were activated only if the donor tumor cells were I-A<sup>k</sup> positive and DCs were present, or if intact, live I-A<sup>k</sup>-transfected tumor cells were used as controls (data not shown). Therefore, tumor vaccine-derived Ags and MHC II acquired by DCs activate tumor-specific CD4<sup>+</sup> T cells restricted to the genotype of the tumor.





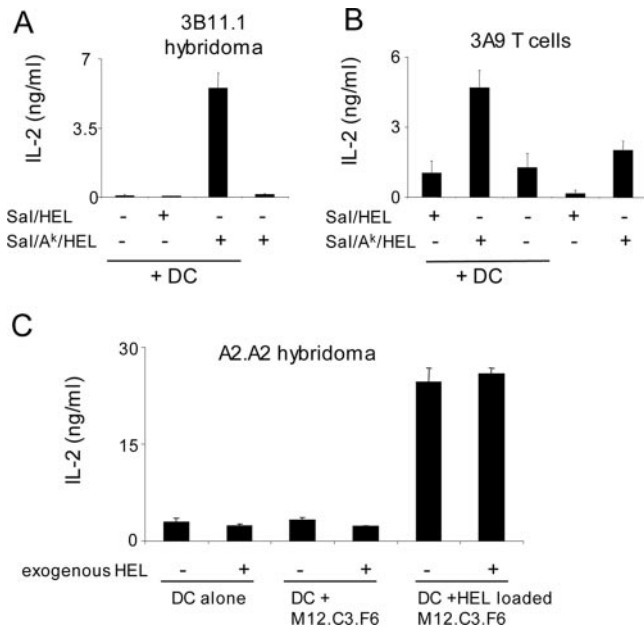
**FIGURE 3.** MHC II and lipid transfer from tumor cells to DCs requires cell-cell contact and DC-expressed plasma membrane receptors. *A*, Splenic DCs from FVB mice and freeze-thawed Sal/A<sup>k</sup> lysate were cultured in the lower and upper chambers, respectively, of a Transwell separated by an 8- $\mu$ m semipermeable membrane. After 3 h of culture, DCs were stained for CD11c and I-A<sup>k</sup> expression and analyzed by flow cytometry. *B*, MHC II transfer requires DC-expressed plasma membrane receptors. CFSE-labeled splenic DCs from FVB mice were treated with pronase before their incubation with freeze-thawed Sal/A<sup>k</sup> lysate. These data are from one of three independent experiments. *C*, Plasma membrane lipids are transferred from tumor cells to DCs. Splenic DCs from FVB mice were incubated with freeze-thawed Sal or Sal/A<sup>k</sup> cells that were labeled with the lipophilic dye DiD, processed as in Fig. 2A, and examined by scanning laser confocal microscopy. *D*, Transferred MHC II is retained on recipient DCs but becomes dispersed with time. Splenic DCs from FVB mice were incubated with freeze-thawed Sal/A<sup>k</sup> cell lysates for 3 h, separated from tumor material, and cultured for an additional 48 h in the absence of tumor lysates. DCs were then stained for CD11c and I-A<sup>k</sup> and analyzed by confocal microscopy. A minimum of 25 cells was examined for each cell type.

MHC II and tumor Ag could be transferred as preformed complexes, or transferred MHC II may assemble in the DCs with transferred tumor Ags or with other Ags acquired by the DCs. If pMHC II complexes are preformed in the tumor cells, then DCs exposed to HEL after MHC II transfer will not form pMHC II complexes and will not activate CD4<sup>+</sup> T cells. Conversely, if the pMHC II complexes form in the recipient DCs after MHC II transfer, then DCs containing transferred MHC II and pulsed with Ags will activate CD4<sup>+</sup> T cells. To test these alternatives, M12.C3.F6 cells were pulsed overnight with intact HEL protein to allow for the formation of I-A<sup>k</sup>-HEL complexes. Control M12.C3.F6 cells were not incubated with HEL. M12.C3.F6 cells were then freeze-thawed and incubated with FVB DCs. Following extensive washing to remove residual tumor material, recipient DCs were cultured with the T cell hybridoma A2.A2 in the presence or absence of exogenous HEL protein. As shown in Fig. 4C, CD4<sup>+</sup> T cells are

activated only if I-A<sup>k</sup> and HEL are present in the tumor cells before transfer. DCs that were fed exogenous HEL after the transfer of I-A<sup>k</sup> did not activate T cells. These data demonstrate that pMHC II complexes form in the tumor cells and transfer to DCs to stimulate CD4<sup>+</sup> T cells.

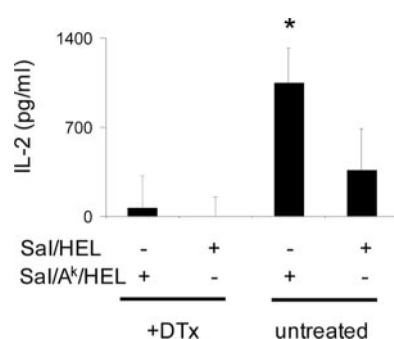
#### *Tumor MHC II expression and DCs are required for maximum in vivo CD4<sup>+</sup> T cell activation*

Previous in vivo studies have demonstrated enhanced activation of tumor-specific CD4<sup>+</sup> T cells in response to MHC II vaccines. If pMHC transfer from tumor cells to DCs is required for in vivo induction of tumor immunity, then MHC II vaccine-enhanced CD4<sup>+</sup> T cell activation would be dependent on the presence of DCs. Conversely, if pMHC transfer did not occur or was irrelevant, then T cell activation would require neither tumor cell MHC II expression nor the presence of DCs. To address this question, we



**FIGURE 4.** Complexes of MHC II and tumor-encoded peptides transfer from tumor cells to DCs and activate CD4<sup>+</sup> T cells. FVB splenic DCs were incubated with freeze-thawed Sal/HEL or Sal/A<sup>k</sup>/HEL cells as in Fig. 1A and used as APCs with the I-A<sup>k</sup>-restricted, HEL-specific T hybridoma 3B11.1 (A) or 3A9 transgenic T cells (B). C, Transferred MHC II complexes contain tumor-generated peptides. I-A<sup>k</sup>-restricted, HEL-specific hybridoma cells (A2.A2) were cultured in the presence or absence of exogenous HEL with freeze-thawed M12.C3.F6 cells that were loaded with exogenous HEL before freeze-thaw. IL-2 release was quantified by ELISA. Error bars are the SD of the mean. These data are from one of three independent experiments (A and B) or two independent experiments (C).

mock-treated or DC-depleted mice before tumor challenge with Sal/HEL or Sal/A<sup>k</sup>/HEL cells. One week later, CD4<sup>+</sup> T cells were isolated from animals and tested for their response to HEL presented by an I-A<sup>k</sup> B cell lymphoma, M12.C3.F6. Maximum activation of HEL-specific CD4<sup>+</sup> T cells occurred when the immunizing tumor cells expressed MHC II and DCs were present, although there was some CD4<sup>+</sup> T cell activation when mice were immunized with MHC II-negative tumor cells (Fig. 5). Therefore cross-priming results in some T cell activation; however, maxi-



**FIGURE 5.** In vivo CD4<sup>+</sup> T cell activation requires both DCs and MHC II expression by tumor cells. DTx treated or untreated CD11c-DTR mice were immunized with  $8 \times 10^5$  Sal/HEL or Sal/A<sup>k</sup>/HEL cells on day 0, and their splenic CD4<sup>+</sup> T cells isolated on day 6 and cocultured with I-A<sup>k</sup>-positive APCs (M12.C3.F6 B lymphoma cells) and exogenous HEL. Supernatants were harvested on day 7 and assayed for IL-2 release by ELISA. Error bars are the SD of the mean. These data are from one of two independent experiments. The asterisk indicates statistical significance ( $p \leq 0.05$ ) from other treatments.

mum CD4<sup>+</sup> T cell activation requires DCs and MHC II expression by vaccine cells, supporting the hypothesis that pMHC II complexes generated by tumor cells are acquired by DCs for T cell activation.

## Discussion

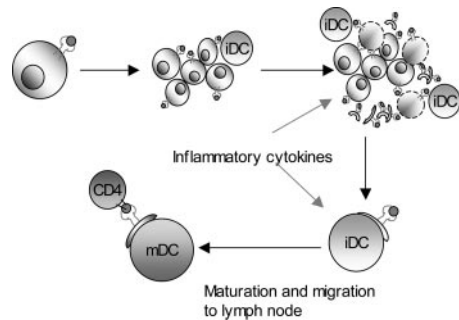
We have concluded from previous experiments that MHC II vaccine cells are the principal APCs in vivo and that they activate tumor-specific CD4<sup>+</sup> T cells by directly presenting endogenously synthesized tumor Ags. This conclusion was based on experiments in which activated tumor-specific CD4<sup>+</sup> T cells were MHC restricted to the class II genotype of the vaccine cells, and not to the genotype of host DCs (15, 16). The findings reported here confirm the genotype restriction of activated CD4<sup>+</sup> T cells but suggest that the process of activation is more complex than we originally proposed. The requirement for DCs, coupled with the transfer of pMHC complexes from the vaccine cells to DCs, makes it likely that DCs are the primary APCs and that they activate CD4<sup>+</sup> T cells by presenting pMHC complexes acquired from MHC II vaccine cells. Yewdell and Haeryfar (38) have speculated that this mechanism of Ag transfer occurs during the activation of CD8<sup>+</sup> T cells and have coined the term “cross-dressing” to describe it. The results presented here validate the concept of cross-dressing and demonstrate that it occurs during the activation of CD4<sup>+</sup> T cells by MHC II cancer vaccines.

DCs are considered the most efficient APCs (22). Their primary mechanism for acquiring soluble protein Ag is by endocytosis, although they also acquire Ag through exosome uptake (39–41). In both processes, DCs degrade acquired Ag to peptides and load the resulting peptides onto DC-encoded MHC I and II molecules for presentation at their cell surface. Because the antigenic epitopes presented by DCs are not synthesized by the DCs, these mechanisms of Ag uptake and presentation are called cross-presentation. Cross-presentation was first described by Bevan (42) and is well accepted as a mechanism for initiating T cell responses (21, 43). The studies presented here identify the acquisition of pMHC complexes by DCs as a potential additional process for activating T cells. Although DCs are the APCs in both cross-presentation and cross-dressing, the two processes have fundamental differences. In cross-dressing, donor cells synthesize and generate both the antigenic peptides and MHC molecules that are subsequently presented by DCs. In contrast, during cross-presentation, Ag is synthesized by donor cells and acquired by DCs, which then process and load the peptides onto DC-synthesized MHC proteins. Therefore, although DCs actively generate pMHC complexes in cross-presentation, their role in cross-dressing is more passive and is limited to presenting pMHC complexes acquired from donor cells.

The transfer of MHC molecules from donor to recipient DCs has been observed previously. For example, T cell responses against transferred allogeneic MHC molecules have been reported (35, 44, 45), and the transfer of exosomes containing donor-cell MHC molecules is well established (40, 46, 47). This study establishes that antigenic peptides are an integral component of the transferred MHC molecules and that T cells are activated to the intact transferred complexes. These data contribute to the growing evidence that the transfer of macromolecules from donor cells to DCs is an important mechanism for priming T cells.

Fig. 6 is a schematic model of how MHC II vaccines may activate CD4<sup>+</sup> T cells by cross-dressing. Initial inoculation of vaccine cells induces local inflammation, which causes vaccine cell necrosis and persistent low-grade inflammation at the inoculation site. The necrotic vaccine cells release pMHC complexes, which are picked up by DCs and without further processing are presented





**FIGURE 6.** Schematic model for the activation of tumor-specific T cells by MHC II vaccines. MHC II vaccine cells contain MHC complexes loaded with tumor-generated peptides. At the site of injection, the vaccine cells trigger an inflammatory response that causes the release of plasma membrane fragments containing pMHC complexes. Immature DCs acquire the pMHC complexes and concurrently receive maturation signals from the inflammatory milieu. DCs mature and migrate to the draining lymph nodes where they activate tumor-specific T cells.

on the DC plasma membranes. As the cross-dressed DCs mature in response to the local proinflammatory signals, they become activated, express increasing levels of costimulatory molecules, and migrate to the draining lymph nodes. In the lymph nodes, the activated DCs present the pMHC complexes plus costimulatory signals, and T cells with the appropriate TCR are primed and activated. Cross-presentation of tumor Ags by DCs also is likely to occur; however, by itself, it is not sufficient for the induction of optimal tumor immunity. This model is consistent with earlier observations that activated T cells are tumor specific and restricted to the MHC genotype of the vaccine cells and explains why vaccine cell expression of MHC II molecules is essential (15, 33). This model also is consistent with the activation of T cells in the lymph nodes and explains why MHC II vaccine cells are not found in lymph nodes after vaccination.

Cross-dressing of DCs may enhance MHC II vaccine efficacy because peptides in the transferred complexes are novel tumor Ag epitopes to which the host is not tolerant. Individuals with progressively growing tumors are frequently tolerant to the Ags of their tumors (48–50). However, tumor-bearing individuals may not be tolerant to tumor Ag epitopes generated by MHC II vaccines, because the vaccine cells may process and present novel peptides that are not generated by DCs. Professional APCs, such as DCs, coexpress MHC II, Ii, and DM. Expression of Ii favors the presentation of exogenously acquired Ags (51). However, MHC II vaccine cells do not express Ii, so their peptide repertoire is skewed toward peptides derived from endogenous sources. DM influences the peptide repertoire by editing peptides as they bind to MHC II molecules in endosomal compartments, and genetically manipulated APCs that do not express Ii and DM produce a different repertoire of peptides than that presented by DM-positive cells (52). Therefore, DCs carrying pMHC complexes acquired from MHC II vaccine cells may present novel tumor Ag epitopes and prime an expanded repertoire of tumor-specific CD4<sup>+</sup> T cells.

Although it is unclear whether cross-dressing occurs in other types of immunizations, the transfer of novel pMHC complexes from MHC II<sup>+</sup>Ii<sup>-</sup>DM<sup>-</sup> MHC II vaccine cells to DCs may be an important step in activating CD4<sup>+</sup> T cells that facilitate tumor immunity. Further studies are necessary to determine to what extent cross-dressed DCs activate T cells, which subset of DCs is most important for cross-dressing, and what role, if any, cross-dressing has in tolerance induction.

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## Disclosures

The authors have no financial conflict of interest.

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