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Macrophages and Dendritic Cells Use the Cytosolic Pathway to Rapidly Cross-Present Antigen from Live, Vaccinia-Infected Cells[1]

Maria Carmen Ramirez and Luis J. Sigal[2]

Professional APCs (pAPC) can process and present on their own MHC class I molecules Ags acquired from Ag donor cells (ADC). This phenomenon of cross-presentation is essential in the induction of CD8$^+$ T cell responses to viruses that do not infect pAPC and possibly contributes to the induction of CD8$^+$ responses to many other viruses. However, little is known about the mechanisms underlying this process. In this study, we show that dendritic cells and macrophages cross-present a model Ag supplied by vaccinia virus-infected ADC via the cytosolic route. Strikingly, we also found that cross-presentation of Ags provided by vaccinia-infected cells occurs within a couple of hours of pAPC/ADC interaction, that the duration of cross-presentation lasts for only 16 h, and that cross-presentation can occur at early times of infection when the ADC are still alive. ***The Journal of Immunology*****, 2002, 169: 6733–6742.**

Activated CD8$^+$ T cells can have their target in any cell expressing MHC class I and their cognate peptide. However, the initiation of CD8$^+$ T cell responses requires Ag presentation by bone marrow-derived professional APCs (pAPC)[3] (1–4) most likely because they express costimulatory molecules that provide a crucial second signal to CD8$^+$ T cells (5–11), and because they can carry Ags from the sites of infection into secondary lymphoid organs where T cell responses are initiated (12, 13). Although the definitive identification of the pAPC that initiates CD8$^+$ T cell responses in vivo is still unknown, in the past few years there has been a large body of literature assigning this function to dendritic cells (DC) (14, 15). Nonetheless, macrophages (Mϕ) are also good candidates since they share many of the characteristics of DC including common precursors, expression of costimulatory molecules, and the ability to move to and from sites of inflammation. In addition, they can reconstitute CTL responses in vivo (16).

Because pAPC play such an important role in the orchestration of antiviral CD8$^+$ T cell immunity, it becomes important to understand the mechanisms whereby these cells present viral Ags. As with all other cells, infected pAPC can present endogenously synthesized viral Ags in vitro and in vivo (17). In this way, they can initiate responses by a process known as direct presentation. However, pAPC also have the unique ability to acquire Ag from exogenous sources and present them on their own MHC class I molecules. This ability of pAPC to present exogenous Ags can be readily observed in vitro by coculturing DC or Mϕ with many different antigenic formulations, and also in vivo upon inoculation of mice with those same Ags (18).

In this paper, we use the term "cross-presentation" to refer exclusively to a subtype of exogenous Ag presentation that involves the transfer of Ag from an Ag donor cell (ADC) to pAPC. The important physiological function of cross-presentation in viral infections became clear when we demonstrated that cross-presentation is necessary and sufficient to initiate CTL responses to viruses that do not infect pAPC (3). Moreover, it is also becoming apparent that cross-presentation is required to induce CTL responses to viruses that suppress MHC class I Ag presentation on infected cells (19, 20). In addition, it is very likely that, concurrent with direct presentation (17), cross-presentation contributes somewhat to the induction of CTL toward most other viruses. Significantly, it has been shown that cultured DC phagocytose apoptotic or necrotic cells that had been infected with influenza virus or irradiated cells that had been infected with recombinant vaccinia virus and cross-present viral Ags supplied by these cells (21–23). However, in those same reports, Mϕ phagocytosed the apoptotic/necrotic cells, but were unable to cross-present the associated Ags. This led the authors to propose that cross-presentation of cell-associated Ags is a function of DC and not of Mϕ. Furthermore, they proposed that by competing for Ag uptake, Mϕ actually inhibit DC cross-presentation. It should be noted that this model contrasted with the ability of Mϕ to present on MHC class I and also on MHC class II molecules other types of exogenous Ags (not cell-associated) such as soluble proteins, proteins bound to beads, or recombinant proteins expressed by bacteria (24–27) and to reconstitute CTL responses in vivo (16). However, the view that Mϕ cannot cross-present Ags supplied by virus-infected cells is very widely held.

To gain a better understanding of the mechanisms of cross-presentation during the course of a viral infection, we developed an in vitro assay of MHC class I Ag cross-presentation where Ag production is restricted to vaccinia virus-infected ADC, and Ag presentation is restricted to pAPC (see Fig. 1 for a diagram of the assay). Using this assay, we show that Mϕ as well as DC are capable of cross-presenting viral Ags supplied by infected cells. This cross-presentation is the direct result of viral infection as it occurs in the absence of further treatment of the ADC to induce

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[3] Abbreviations used in this paper: pAPC, professional APC; ADC, Ag donor cell; CRPMI, complete RPMI media; DC, dendritic cell; Mϕ, macrophage; β-gal, β-galatosidase; ER, endoplasmic reticulum.

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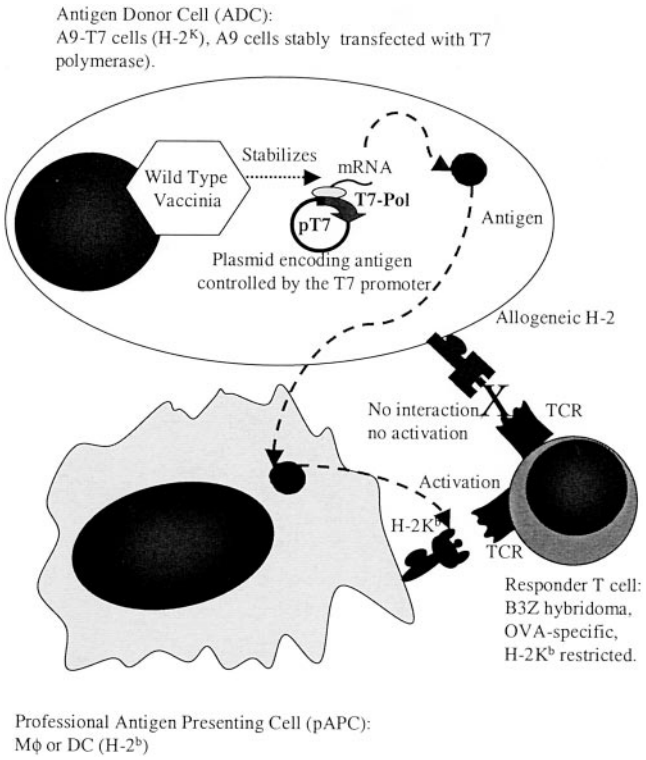

FIGURE 1. Diagram of the cross-presentation assay. ADC cells are prepared by transfecting A9-T7 cells (H-2^k) with a plasmid controlled by the T7 promoter (PBS-$OVA_{197–386}$) and infecting with wild-type vaccinia. We prepared Mϕ and DC by culturing mouse bone marrow with M-CSF or GM-CSF, respectively, and responder cells were the B3Z hybridoma that is reactive with the OVA peptide SIINFEKL in the context of H-$2K^b$ and produces β-gal when activated.

apoptosis, necrosis, or to inactivate the virus. In addition, we show that our model Ag follows the cytosolic route within the pAPC, since it requires proteasome processing, TAP transport, and is inhibited by brefeldin A. Moreover, we also found that Mϕ and DC cross-present Ag with fast kinetics since cross-presentation is detectable within 1 h of ADC-pAPC encounter, peaks between 2 and 5 h, and decreases rapidly to almost nil within the next 10–15 h. Strikingly, cross-presentation does not require apoptosis or necrosis of the ADC, since live-infected cells are capable of supplying Ags to pAPC within few hours of becoming infected.

Materials and Methods

Cells lines

All cells were maintained in complete RPMI media (CRPMI) that consisted of RPMI supplemented with 10% FCS (Atlanta Biologicals, Norcross, VA), 2 mM L-glutamine, penicillin-streptomycin, 0.01 M HEPES buffer and nonessential amino acids (all from Invitrogen, Carlsbad, CA), and 5×10^{-5} 2-ME (Sigma-Aldrich, St. Louis, MO). A9 cells (H-2^K) are an APRT and HPRT negative derivative of strain L cells (no. CCL-1.4; American Type Culture Collection (ATCC), Manassas, VA). A9-T7 cells (28, 29) are A9 cells stably transfected with T7 polymerase (a kind gift from Dr. B. Moss, National Institutes of Health, Bethesda, MD). B3Z (30) is a CTL hybridoma that produces β-galatosidase (β-gal) upon recognition of the OVA epitope SIINFEKL in the context of the H-$2K^b$ molecule (a kind gift from Dr. N. Shastri, University of California, Berkeley, CA). All cells were grown and assays incubated at 37°C in an atmosphere of 5% CO_2 unless otherwise indicated.

Mice

All experiments using animals were performed under protocols approved by the Institutional Animal Use and Care Committee. All the mice used in the experiments were bred at Fox Chase Cancer Center's Laboratory Animal Facility (Philadelphia, PA). $TAP^{0/0}$ (B6-$Tap^{1tp1Arp}$) breeders were originally purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 (B6) mice were from the Fox Chase Cancer Center colony.

Viruses

The WR strain of vaccinia virus and WR expressing the phage T7 polymerase was a gift from Dr. B. Moss. Vaccinia virus expressing the minigene (M)SIINFEKL was a kind gift of Dr. J. Yewdell (National Institutes of Health). All vaccinia stocks were grown in Hela S3 cells (no. CCL-2.2; ATCC) in T150 tissue culture flasks and virus titers determined using BS-C-1 cells (no. CCL-26; ATCC) growing in 6-well plates following published procedures (29). Virus titers were adjusted to 10^9 PFU in CRPMI.

Plasmids

We generated a PCR fragment of $OVA_{197–386}$ preceded by a Kozac sequence and ending in a stop codon using as template a plasmid containing the full cDNA of OVA (a generous gift from Dr. L. Shen, University of Massachusetts Medical Center, Worcester, MA). As forward and reverse primers we used oligos with the sequences CACCATGCCTTTCAGAGTG ACTGAGCA and TTAAGGGGAAACACATCTGCC, respectively. The PCR fragment was directionally cloned into pCDNA 3.1-TOPO (Invitrogen) following manufacturer's instructions to obtain pCDNA-$OVA_{197–386}$. The cloned fragment was excised from pCDNA-$OVA_{197–386}$ with *Bam*HI and *Not*I and inserted into the *Bam*HI-*Not*I sites of plasmid pBlueScript II SK (pBS; Stratagene, La Jolla, CA) to generate pBS-$OVA_{197–386}$. Transcription and translation from $OVA_{197–386}$ generates a fragment of chicken OVA (residues 197–386) lacking the endoplasmic reticulum (ER) transfer signal sequence but containing the K^b-restricted OVA epitope SIINFEKL (amino acid single letter code) corresponding to positions 258–265.

ELISA

The presence of $OVA_{197–386}$ was determined by a sandwich ELISA in lysates of ADC that had been prepared as described in the next section and lysed at 10^6 cells/ml in PBS 0.1% Triton X-100 buffer. For the ELISA, 96-well flat-bottom RIA/ELISA plates (Costar; all other plasticware was from BD Biosciences, Mountain View, CA) were incubated overnight with 50 μl of a 1/2,500 solution of goat anti-OVA Ab (ICN Pharmaceuticals, Costa Mesa, CA), washed, and blocked with 200 μl ELISA buffer (PBS 0.5% nonfat milk, 0.1% Triton X-100, 0.025% Tween 20) for 2 h at 37°C. A total of 50 μl of cell lysates in ELISA buffer were added to triplicate wells as sets of seven 10-fold serial dilutions and incubated at room temperature for 3 h. The plates were thoroughly washed and 50 μl of a 1/16,000 dilution of rabbit anti-OVA (ICN Pharmaceuticals) diluted in ELISA buffer was added and incubated at room temperature for 2 h. The plates were washed again, incubated with 1/8,000 dilution of goat anti-rabbit IgG conjugated with HRP (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in ELISA buffer, washed, incubated with tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories) for 20 min and read at 450 nm in a μ Quant 96-well spectrophotometer (Bio-tek Instruments, Watford Herts, U.K.). As a positive control, we used purified chicken OVA (ICN Pharmaceuticals). However, the exact quantification of the amount of $OVA_{197–386}$ present in the cell lysates was not possible because the polyclonal Abs that we used were raised against full-length OVA. Therefore, some epitopes present in the full-length OVA might be absent in $OVA_{197–386}$, which may skew the signal. As a guide, the absorbance for 10 pg/well of purified OVA was 0.259 at 450 nm of Abs. All ELISA experiments were repeated at least three times. Each data point represents the mean of triplicate wells for one of the dilutions. The value of the signal decreased linearly with the decrease in the concentration of the cell lysate or of the standard.

Ag cross-presentation assay

Preparation of ADC

A9-T7 or A9 cells were seeded in 60-mm tissue culture dishes (1.2×10^6 cells/dish) or 6-well plates (4×10^5 cells/well). Following overnight incubation at 37°C, the cells were transfected with the indicated DNA using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Four or 6 h later, the monolayer was washed twice with PBS and the cells infected for 2 h with 10 PFU/cell (the number of cells was an estimation calculated as twice the initial number of cells) of the indicated vaccinia virus in Optimem media (Invitrogen). Next, the monolayer was washed twice with complete media and incubated in CRPMI for the indicated times. When indicated, the cells were irradiated for 5 min with a short wave (254 nm) Spectroline germicidal UV lamp EF180 (Spectronics, Westbury, NY) placed at 15 cm over the cells. The cells were harvested with a rubber

policeman, centrifuged, and the pellet resuspended to an estimated concentration of 10^6 or 10^7 cells/ml according to the requirements of the particular experiment.

In some experiments (see Fig. 4), the ADC cells were split in aliquots and frozen at −80°C. Aliquots were thawed at different times, vortexed, and used in the cross-presentation assay. In other experiments (see Fig. 5), the cells were transfected with the indicated PBS-$OVA_{197–386}$ and the infections were performed at different times, the first infection being 4 h before transfection and the last infection just immediately before harvesting. In this way, all the ADC cultures containing cells that had been transfected at the same time but infected at different times were harvested together.

Generation of bone marrow-derived pAPC

To obtain DC, bone marrow cells obtained from the C57BL/6 or $TAP^{0/0}$ mice were incubated overnight in CRPMI. Nonadherent cells were collected, seeded at 10^6 cells/ml, and cultured for 5–6 days in CRPMI supplemented with 7 ng/ml GM-CSF (BD PharMingen, San Diego, CA) which was replaced every 2–3 days (BD PharMingen). To obtain Mϕ, the procedure was similar, but instead of GM-CSF, the media contained 20% of L cell supernatant as a source of M-CSF (16).

Cross-presentation assays

In some experiments, pAPC were grown in 10-mm bacteriological grade petri dishes (BD Biosciences), collected with a rubber policeman, washed, counted, and 10^5 placed at the indicated ADC:pAPC ratio in duplicate wells of white, flat-bottom 96-well plates (BD Biosciences). In other experiments, pAPC were grown in 24-well plates and cross-presentation was initiated by adding 5×10^5 ADC (10^7 cells/ml). In some cases, 20 min before adding the ADC, the media of the pAPC was replaced with 200 μl of CRPMI that contained 16 μM lactacystin, 1 μg/ml brefeldin A (both from Sigma-Aldrich), or diluent. In this case ADC were added directly, without media replacement. To measure direct presentation by Mϕ and DC infected with vaccinia (M)SIINFEKL, 5 PFU/cell of virus stock was added instead of the ADC. To allow for cross-presentation or direct presentation, the ADC-pAPC cocultures or infected pAPC were incubated for 4 h or the indicated times, harvested using the plunger of a 1 ml syringe as a rubber policeman. When indicated, the harvested cells were spun and fixed for 15 min in 200 μl of 0.5% paraformaldehyde, washed twice with PBS, neutralized for 20 min in 0.5 mM L-lysine in CRPMI, washed twice in CRPMI, and serially diluted in white 96-well plates. In some cases, the procedure was similar but the cells were not fixed. The concentration of pAPC in these conditions was estimated by counting the number of pAPC in wells that did not receive ADC. In other experiments, the process was reversed and the pAPC were added to the ADC cultures. For this purpose, the ADC were prepared by transfecting and infecting A9-T7 cells in 6-well plates. After 9 h, any detached ADC cells were removed by gently washing the monolayer with CRPMI after which 10^6 pAPC that had been grown in 100-mm petri dishes were added to the ADC that remained attached to the wells. Following a 4-h incubation, the pAPC-ADC cocultures were harvested using a rubber policeman, fixed, and seeded in 96-well plates as before.

To determine cross-presentation, 10^5 B3Z responder cells were added to each well of the 96-well plates above and incubated for an additional 16–24 h to allow for hybridoma activation and β-gal production. To detect β-gal activity, we used the Galactostar chemiluminescent kit (Applied Biosystems, Foster City, CA) following the instructions of the manufacturer except that we used 15 μl/well of lysis buffer and 40 μl/well of substrate diluted 1/150 in reaction buffer. The assays were read using a Topcount instrument (Packard Instrument, Meriden, CT).

All experiments of cross-presentation were repeated at least three times and each figure corresponds to a representative experiment. Each data point in the figures represents the average of duplicate wells. Positive controls included pAPC incubated with 2-fold serial dilutions of SIINFEKL at an initial concentration of 200 or 100 pM and a final concentration of 3.2 or 1.56 pM. This range of concentrations induced a nonsaturating, linear response by the hybridoma. The maximal stimulation through cross-presentation fluctuated around the values obtained between 3.12 and 6.25 pM and never reached the β-gal activity induced with 50 pM SIINFEKL.

Ab staining for flow cytometry

For cell surface staining 0.5×10^6 cells were incubated in a well of a U-bottom 96-well microtiter plate with 0.1 μg of the corresponding primary labeled Ab in 50 μl FSB (PBS 2% FCS, 0.04% sodium azide). The following primary labeled Abs used were PE-labeled anti-MHC class II ($I\text{-}A^b$-specific clone AF6-120.1), FITC-labeled anti-CD11c (clone HL3), FITC-labeled anti-Vb8.1,8.2 (clone MR5-2), PE-labeled anti-CD8 (clone 53-6.7) from BD PharMingen, and FITC-labeled anti-CD11b (a kind gift of Dr. R. Hardy, Fox Chase Cancer Center). For immediate analysis, the cells were washed with FSB three times and resuspended in 0.4 ml SB. When the analysis was postponed, the cells were washed three times with PBS and resuspended in 0.2 ml PBS containing 0.5% paraformaldehyde.

Detection of cell death

Ethidium bromide and acridine orange staining for microscopy

ADC well prepared in 6-well plates as above. After a 13-h infection, 10 μl of ethidium bromide (Sigma-Aldrich) and acridine orange (Fisher Scientific, Pittsburgh, PA) (both stocks at 100 μg/ml in PBS) were added, and the cells were immediately observed under FITC (green) and Rhodamine (red) filters using a Nikon Diaphoto microscope (Nikon, Melville, NY). Pictures were taken with a Pixera Pro ISOES camera (Nikon) coupled to the Nikon microscope at ×100 magnification. The green and red images were merged using Picture Publisher 10.0 software (Micrograph, Dallas, TX).

TUNEL assay for apoptotic death

Apoptotic death was assessed using the TUNEL kit (Roche Diagnostic Systems, Somerville, NJ). Briefly, ADC were prepared in 6-well plates, and following a 13-h infection ADC were washed in PBS, fixed in 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate for 2 min on ice. Fixed and permeabilized ADC were washed with PBS and incubated for 1 h at 37°C in a solution containing 25 mM Tris (pH 6.6), 200 mM cacodylate, 1 mM $CoCl_2$, 0.6 nM fluorescein-12dUTP, and 25 U of TdT (Roche Diagnostic Systems). ADC were analyzed by fluorescent microscopy in a drop of PBS using a FITC band path filter.

Results

Mϕ and DC can cross-present Ags shed by vaccinia-infected cells

To study cross-presentation in viral infections, it is absolutely necessary to restrict Ag synthesis to the ADC and Ag presentation to the pAPC. Vaccinia can infect pAPC and cannot be used to study cross-presentation as a consequence of viral infection if the Ag is encoded within the viral genome because direct presentation would mask cross-presentation. Therefore, to study cross-presentation as a consequence of vaccinia virus infection, we restricted Ag synthesis to the ADC by adapting a method originally used to produce recombinant proteins in mouse cells. In this system, A9-T7 cells, a derivative of the mouse fibroblast cell line A9 (an HPRT and APRT negative mouse subclone of the L strain, $H\text{-}2^K$) stably expresses the bacteriophage T7 polymerase. These cells produce a protein encoded within a transiently transfected plasmid and controlled by the T7 promoter only under conditions of vaccinia virus infection (28). The ability of vaccinia virus to induce expression of a T7 controlled protein in the presence of T7 polymerase has been attributed to the capacity of the virus to stabilize the plasmid encoded RNA in the cytoplasm. For our purposes, we transfected A9-T7 cells with pBS-$OVA_{197–386}$ and infected with wild-type vaccinia virus. The cells were then incubated for 24 h, harvested, lysed, and the presence of $OVA_{197–386}$ was determined by ELISA. Fig. 2*A* shows that A9-T7 cells that had been transfected with pBS-$OVA_{197–386}$ and infected with wild-type vaccinia produced large amounts of $OVA_{197–386}$ while transfection of A9-T7 cells with pBS-$OVA_{197–386}$ in the absence of vaccinia infection or parent A9 cells (T7 negative) that had been transfected with PBS-$OVA_{197–386}$ and infected with wild-type vaccinia did not produce $OVA_{197–386}$. Further controls showed that A9 cells produced as much OVA as A9-T7 cells when they were transfected with pBS-$OVA_{197–386}$ and infected with recombinant vaccinia virus carrying the T7 polymerase, indicating that parent A9 cells were an appropriate negative control since they could be transfected and infected with similar efficiency as A9-T7 cells. In addition, $OVA_{197–386}$ was not detected in lysates of A9-T7 cells that had been infected with vaccinia wild type, but transfected with

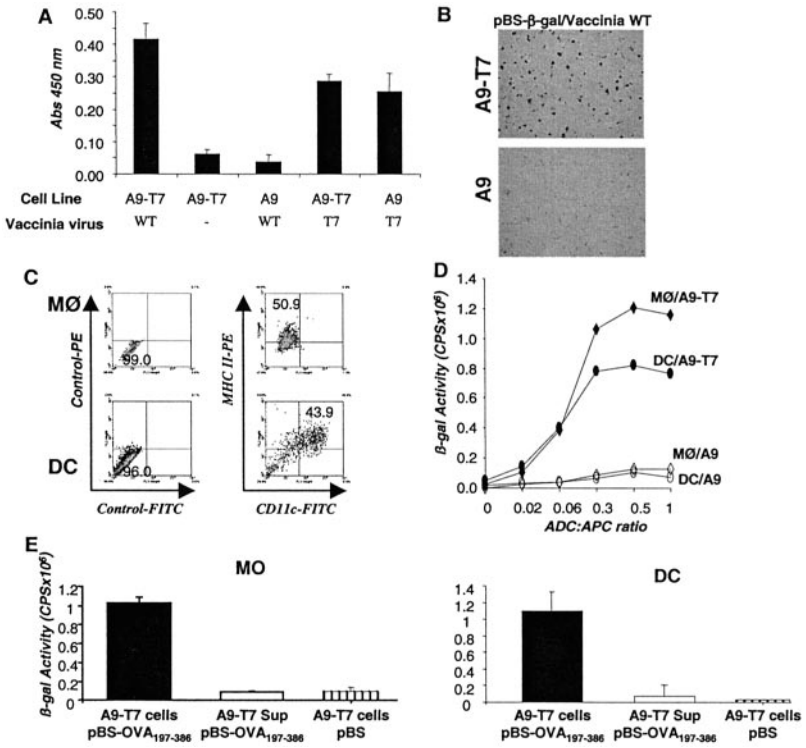

FIGURE 2. Mϕ and DC cross-present Ag from vaccina-infected cells. *A*, A9-T7 or A9 cells (as indicated) were transfected with pBS-$OVA_{197–386}$ and infected as indicated. The expression of $OVA_{197–386}$ in 50 μl of cell lysates (10^6 cells/ml) was determined by ELISA. All samples in the figure were processed at the same time. The A_{450} for 10 pg of purified OVA in 50 μl was 0.259. *B*, X-gal staining of A9-T7 and A9 cells transfected with pBS-β-gal and infected with wild-type vaccinia virus. *C*, Flow cytometry of Mϕ and DC cultures using anti-CD11c-FITC and anti-MHC class II-PE mAbs. Results with control-irrelevant Abs are also shown. *D*, A total of 10^5 Mϕ or 10^5 DC were incubated with different concentrations of A9-T7 cells or control A9 cells that had been transfected with pBS-$OVA_{197–386}$ and infected with wild-type vaccinia virus, and with 10^5 B3Z hybridoma cells in duplicate wells of 96-well plates. The three-cell culture was incubated for 18 h after which β-gal production by the B3Z cells was measured by chemiluminescence. *E*, A total of 10^5 Mϕ or DC were incubated with 10^5 A9-T7 cells or their supernatants that had been transfected with pBS-$OVA_{197–386}$ or pBS vector as indicated, and infected with wild-type vaccinia virus. B3Z hybridoma and detection of β-gal activity as in *D*.

vector alone (data not shown). In related experiments, we transfected A9-T7 and A9 cells with pBS-β-gal instead of pBS-$OVA_{197–386}$ to find that most (80–100%, varying with each experiment) of the A9-T7 cells that had been infected with vaccinia wild type stained blue very rapidly (within a few minutes) and very strongly with X-gal while no staining was found in A9 cells infected with wild-type vaccinia (Fig. 2*B*). In addition, strong staining was observed in control A9-T7 and A9 cells that had been infected with vaccinia-T7 but not in uninfected A9-T7 cells (data not shown). Therefore, A9-T7 cells transfected with pBS-$OVA_{197–386}$ and infected with wild-type vaccinia are an excellent source of ADC to study cross-presentation. In this system, $OVA_{197–386}$ constitutes a model of a viral Ag as it is only produced upon vaccinia infection. In addition, ADC irradiation is not required because the transfer of live virus to the pAPC or even unwilling transfection of the pAPC by carryover plasmid (an unlikely but theoretically possible problem that may occur when using vaccinia-T7 instead of A9-T7 cells) should not result in production and direct presentation of $OVA_{197–386}$ by the pAPC.

With a reliable method in hand to generate ADC, our first objective was to determine whether different pAPC could cross-present viral Ags supplied by vaccinia-infected cells. As pAPC we used Mϕ and DC prepared from bone marrow cells obtained from C57BL/6 (B6) mice. The identity of the pAPC was confirmed by microscopic morphology (data not shown) and by flow cytometry, which indicated that the bone marrow cells cultured in the presence of M-CSF expressed MHC class II but not CD11c as is expected for Mϕ, while the cells cultured in the presence of GM-CSF expressed MHC class II and CD11c as expected for DC (Fig. 2*C*). It should be noted that we consistently found that the Mϕ cultures were highly homogeneous, while the DC were more heterogeneous with day to day variations (40–70%) in the proportion of cells expressing CD11c. In addition, both cell types expressed CD11b, CD16, H-$2K^b$, and the Mϕ scavenger receptor A. DC expressed moderate levels of B7-2 and low B7-1 while Mϕ expressed low B7-2 and no B7-1. Both cell types increased MHC and B7 expression upon activation with LPS or UV- irradiated vaccinia-infected A9-T7 cells (data not shown). To determine cross-presentation, A9-T7 or A9 control ADC (H-2^K) were transfected and infected as indicated in *Materials and Methods*, harvested, washed, and plated at different concentrations in 96-well plates. To these cells, B6-derived (H-2^b) pAPC (Mϕ or DC) and B3Z responder T cells (OVA-specific, H-$2K^b$-restricted) were added. The cells were incubated overnight and the activation of B3Z was determined by measuring β-gal activity. Our results show that both Mϕ and DC cross-presented $OVA_{197–386}$ when cocultured with A9-T7 cells that had been transfected with pBS-$OVA_{197–386}$ and infected with wild-type virus (Fig. 2*D*). Importantly, no signal was detected when we used similarly treated A9 cells that did not produce $OVA_{197–386}$ as ADC controls. It should be noted that in this experiment as well as others, the cross-presentation signal obtained with Mϕ was higher than with DC, typical of most but not all our experiments. Although an interpretation could be that Mϕ cross-present better, it is more likely that the day to day variation in the purity of the DC cultures results in this difference. In additional controls, A9 cells and A9-T7 cells transfected with pBS-$OVA_{197–386}$ and infected with recombinant vaccinia-T7 cross-presented similarly (data not shown). Also, no signal was detected when A9-T7 cells were infected with wild-type vaccinia and transfected with empty pBS vector (see Fig. 2*E*). Furthermore, when we used ADC supernatants no cross-presentation was observed indicating that the cross-presented Ag is cell associated (see Fig. 2*E*). Altogether, these experiments demonstrate that Mϕ can cross-present viral Ags from vaccinia-infected cells similar to DC, and that cross-presented Ags are cell associated. It also demonstrates that vaccinia-infected cells provide Ag for cross-presentation without a requirement to artificially induce apoptosis or necrosis of the infected cells. This cross-presentation is highly efficient as only 0.06 ADC are required for each pAPC to detect cross-presentation, and 0.25 ADC per pAPC provides saturating amounts of Ag (Fig. 2*D*).

Cross-presentation of $OVA_{197–386}$ follows the cytosolic route

Previous work with proteins bound to beads and bacteria expressing recombinant proteins led respectively to the discovery of the cytosolic and vacuolar routes of exogenous Ag presentation (26, 31). The cytosolic route is characterized by Ags gaining access to the cytosol of the pAPC and, similar to the presentation of endogenous proteins (32–36), the Ags are degraded by the proteasome and the resulting peptides transported by the TAP to the ER. In the ER, the peptides bind to newly synthesized MHC class I molecules and are transported to the cell surface via the secretory pathway. In the vacuolar route, the exogenous Ags do not gain access to the cytosol and are most likely degraded by proteases within endosomal or lysosomal compartments and the resulting peptides bind to MHC class I molecules that recycle from the cell surface (26, 37). We have previously shown that bone marrow chimeric mice with TAP-deficient pAPC were inefficient in generating CTL responses to several viruses, including vaccinia, suggesting that the dominant route of cross-presentation in viral infections is cytosolic (2, 3). However, the use of TAP deficiency as the single method to demonstrate that an Ag follows the cytosolic route has been criticized because TAP deficiency reduces the number of MHC class I molecules at the cell surface that could be available for recycling and could indirectly affect the vacuolar route (38). Therefore, these results may require confirmation in a more controlled environment where each of the steps of the cytosolic route can be individually evaluated. We first tested the role of TAP in the cross-presentation of Ags supplied by vaccinia-infected cells. For this purpose, A9-T7 ADC were prepared as before and pAPC were obtained from either TAP-deficient or wild-type B6 mice as controls. As seen in Fig. 3*A*, Mϕ and DC from wild-type B6 mice were able to cross-present $OVA_{197–386}$ delivered by vaccinia-infected cells while those from TAP-deficient mice did not cross-present, strongly suggesting a dominant role for the cytosolic route. To confirm this, we blocked other steps of the cytosolic route by treating B6 Mϕ and DC with lactacystin to inhibit the proteasome or with brefeldin A to block the secretory pathway. To minimize any toxic or nonspecific effects of the drugs on the pAPC or the T cell hybridoma, the pAPC were only exposed to the inhibitors for 20 min before the incubation with the ADC and during the 4 h of the processing period. After this, the pAPC were fixed with paraformaldehyde and thoroughly washed before mixing with B3Z cells. These experiments showed that both lactacystin and brefeldin A completely inhibited cross-presentation (Fig. 3*B*). As a control, we infected pAPC with recombinant vaccinia virus expressing the minigene (M)SIINFEKL which does not require proteasome processing but requires de novo MHC class I synthesis. As expected, we found that brefeldin A but not lactacystin inhibited direct presentation by Mϕ or DC (Fig. 3*B*, *small insets*). In additional controls we found that Mϕ and DC still cross-presented when they were not fixed and brefeldin A was removed by thorough washing before adding the B3Z responder cells (Fig. 3*B*). Altogether, these data demonstrate that Mϕ and DC use the cytosolic route to cross-present $OVA_{197–386}$ delivered by vaccinia-infected cells.

Cross-presentation by Mϕ and DC is rapid and transient

To address how soon and for how long pAPC cross-present Ag following pAPC-ADC contact, we prepared ADC as before although they were frozen in aliquots which, at different times points within a 16-h period, were thawed, and saturating numbers (1 ADC:1 pAPC) were immediately added to cultures of Mϕ or DC. All ADC-pAPC cocultures were harvested together at the end of the assay, fixed with paraformaldehyde to stop further Ag processing, thoroughly washed, and serially diluted in duplicate wells of 96-well plates to which B3Z responder cells were added. Cross-presentation was determined following overnight incubation. The results indicated that Mϕ and DC cross-presented Ag acquired from vaccinia-infected cells with fast and similar kinetics (Fig. 4*A*). Maximal cross-presentation by both pAPC was reached between 2 and 5 h of ADC-pAPC contact and decreased thereafter with very little Ag remaining at the surface of pAPC after 16 h of ADC-pAPC coculture. It was reasonable to think that cross-presentation extinguished relatively quickly because vaccinia virus carried over by the ADC may have infected the pAPC and may have affected their ability to present Ags at later time points adversely. To test for this possibility, we performed a set of experiments where carry-over virus was inactivated by irradiating the ADC with UV light before dividing in aliquots and freezing while all other conditions remained the same. This irradiation rendered ADC that did not transfer virus since their lysates did not infect BSC-1 cells nor did they induce expression of β-gal in A9-T7 that

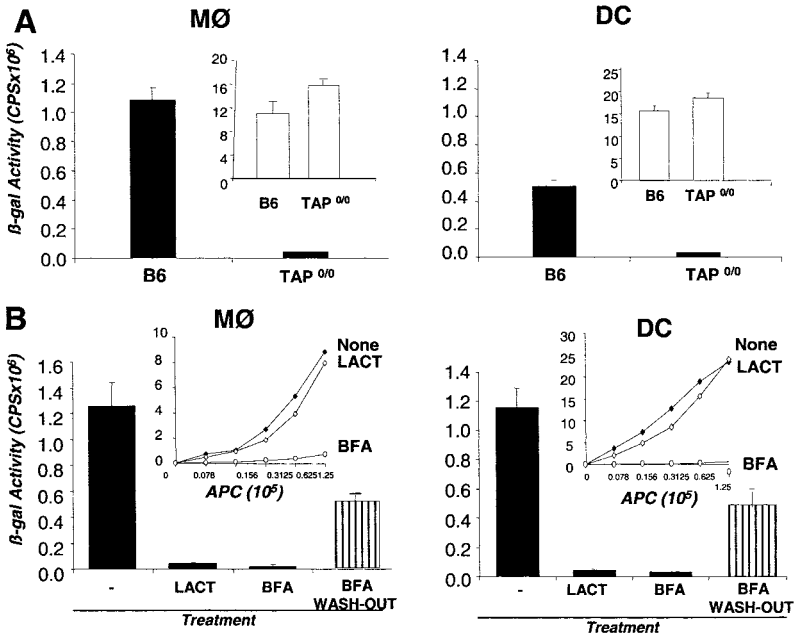

FIGURE 3. Cross-presentation of $OVA_{197–386}$ follows the cytosolic route. ADC were prepared by transfection of A9-T7 cells with PBS-$OVA_{197–386}$ and infection with vaccinia wild type. *A*, Titrated numbers of ADC were coincubated with 10^5 B3Z cells and 10^5 Mϕ or DC obtained from TAP or B6 mice. Cross-presentation was measured after a 24-h incubation. Only the data representing the 1:1 ADC:pAPC ratio are shown. The *small insets* correspond to control pAPC pulsed with X mM SIINFEKL. *B*, A total of 10^6 ADC (main figure) or 5 PFU/cell recombinant vaccinia virus expressing (M)SIINFEKL as a minigene (*small insets*) were added to 10^6 B6 Mϕ or DC that had been preincubated for 20 min with and in the presence of lactacystin (16 μM) or brefeldin A (1 μg/ml) as indicated. Following a 4-h incubation, the ADC-pAPC cocultures were harvested, fixed with paraformaldehyde, thoroughly washed, plated at different concentrations in duplicate wells of 96-well plates, and used in cross-presentation assays with B3Z cells as in Fig. 2*C*. Although multiple dilutions of pAPC were analyzed, the main figure only depicts the data corresponding to wells containing 10^5 pAPC.

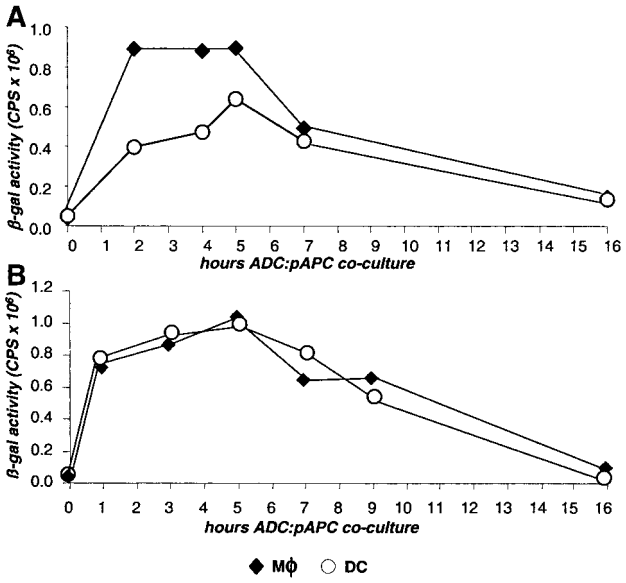

FIGURE 4. Cross-presentation by Mϕ and DC is rapid and transient. *A*, Cultures of ADC were prepared by transfection of A9-T7 cells with PBS-$OVA_{197–386}$ and infection with vaccinia wild type. Twenty-four hours after infection the ADC were frozen in aliquots of 10^6 cells. At different times, aliquots were thawed and half of each added to cultures of 10^6 Mϕ or DC that had been growing in 24-well plates. Sixteen hours after the first aliquot was added, all ADC-pAPC cocultures were harvested, fixed, thoroughly washed, plated at different concentrations in duplicate wells of 96-well plates and used in cross-presentation assays with B3Z cells as in Fig. 2*C*. *B*, As in *A*, but the ADC were irradiated with UV light to inactivate the virus before making aliquots and freezing.

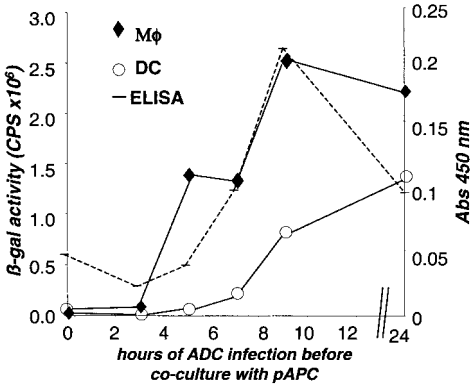

FIGURE 5. ADCs provide Ag for cross-presentation soon after infection. To prepare ADC infected for different times, several cultures of A9-T7 cells in 6-well plates were transfected with pBS-PBS-$OVA_{197–386}$ and then infected with wild-type vaccinia at various times. Twenty-four hours after the infection of the first culture, all the ADC cultures were harvested and added to 10^6 Mϕ or DC that had been growing in 24-well plates. Four hours later all ADC-pAPC cocultures were harvested, fixed, thoroughly washed, plated at different concentrations in duplicate wells of 96-well plates, and used in cross-presentation assays with B3Z cells as in Fig. 2*C*. The relative strength of cross-presentation (as measured by β-gal production by the B3Z hybridoma) is indicated on the left ordinate. In addition, for each time point a sample of ADC was removed, lysed, and the relative concentration of $OVA_{197–386}$ in 50 μl of lysate (10^6 cells/ml) was determined by ELISA and is indicated in the right ordinate.

had been transfected with pBS-β-gal while lysates of infected, nonirradiated controls did (data not shown). In addition, Mϕ and DC that had been incubated with irradiated ADC for 24 h remained viable and became activated as revealed by increased expression of MHC and B7 molecules by FACS similar to the activation induced by incubation with LPS (data not shown). Despite virus inactivation, the kinetics of cross-presentation remained the same (Fig. 4*B*). From these results we conclude that Mϕ and DC cross-present viral Ag acquired from vaccinia-infected cells rapidly and transiently in the presence or in the absence of active virus. However, it should be noted that in these last experiments the ADC were obviously dead as a consequence of the freeze-thaw and, in Fig. 4*B*, the irradiation. Nonetheless, this was the only way to keep our Ag source identical from one data point to the other without having to process each set of cells at different times and storing the fixed pAPC for prolonged times.

ADC provide Ag for cross-presentation at early times of infection

Another important aspect in the kinetics of cross-presentation was to determine how soon after infection vaccinia-infected ADC transfer Ag to pAPC. For this purpose, we transfected A9-T7 cells with pBS-$OVA_{197–386}$ as in previous experiments, but the cells were infected with wild-type vaccinia at different times. All ADC that had been infected for different times were collected at the same time and added to cultures of Mϕ or DC. After a 4-h incubation at 37°C, the ADC-pAPC cocultures were collected, fixed with paraformaldehyde to stop any additional cross-presentation, thoroughly washed, and serially diluted into duplicate wells of 96-well plates to which B3Z responder cells were added. Our results show that vaccinia-infected ADC could transfer Ag to Mϕ after only 5 h of infection, whereas DC were somewhat slower (Fig. 5, unbroken lines and *left ordinate*). Maximal cross-presentation was reached when the ADC had been infected for 9 h. Interestingly, the kinetics of cross-presentation followed the kinetics of Ag expression by the ADC as determined by ELISA (Fig. 5, dotted line and *right ordinate*). Altogether, these data indicate that ADC provide Ag for cross-presentation at early times of infection. That vaccinia-infected ADC can deliver Ag to pAPC at this very early time of the infectious process is surprising because at this stage ADC death should be minimal.

Cross-presentation does not require death of the ADC

Death of the ADC by either apoptosis or necrosis is one of the hallmarks of the current model of cross-presentation and has been argued to play a principal role in the recognition of ADC by pAPC. That pAPC can cross-present Ags from dead cells is confirmed by our experiments in Figs. 2–4 where many or all of the ADC were dead because they were used at late stages of infection and in Fig. 4, they were freezed and thawed and even irradiated. However, while vaccinia is a highly cytopathic virus that exerts very early morphological changes in infected cells (39), it has also developed strong antiapoptotic strategies (40–42) and death of vaccinia-infected cells should not occur for many hours after infection. The experiments in Fig. 5 showing cross-presentation at early times after infection suggested that cell death might not be required for the transfer of Ag from vaccinia-infected ADC to pAPC. However, the conditions for this experiment included harvesting the ADC, which could have induced some degree of ADC death. To carefully test whether live-infected cells could serve as a source of Ag for cross-presentation, we modified our assay to avoid harvesting the ADC before cross-presentation. For this purpose, we prepared ADC by transfecting and infecting A9-T7 in 6-well plates. Nine hours after infection, the ADC monolayer was gently washed with CRPMI to remove all detached ADC. Next, pAPC (to determine cross-presentation) or CRPMI (to determine cell viability) were

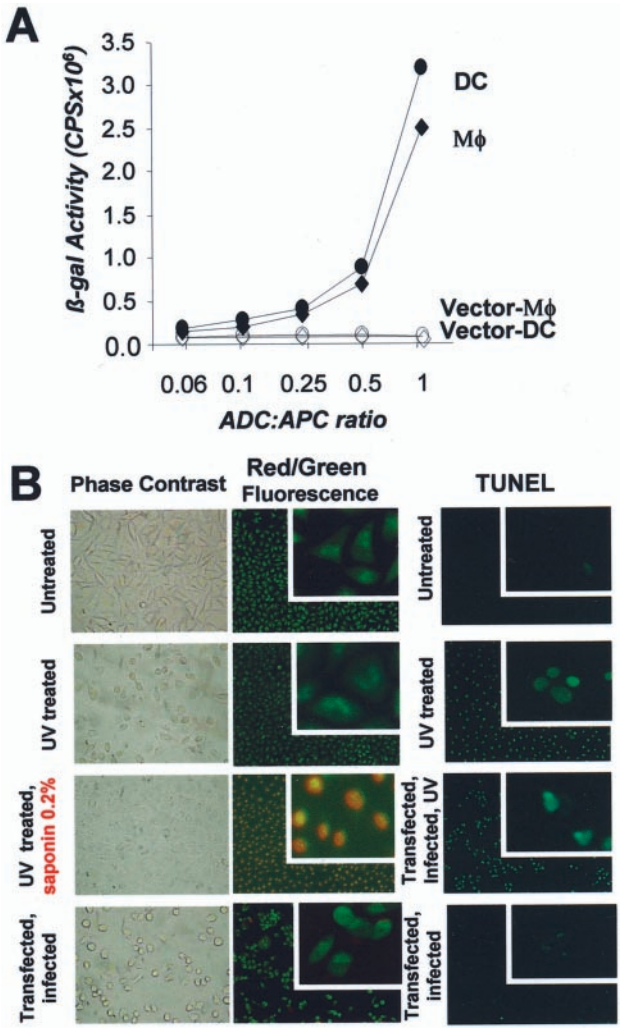

FIGURE 6. Cross-presentation does not require death of the ADC: ADC were prepared with A9-T7 cells in 6-well plates that were transfected with pBS-OVA$_{197–386}$ or empty pBS II vector as control, and infected with wild-type vaccinia virus. Nine hours after the infection, the ADC were gently washed to remove detached cells. *A*, the ADC cultures received 10^6 Mϕ or DC and were incubated for an additional 4 h after which the ADC-pAPC cocultures were harvested, fixed, thoroughly washed, plated at different concentrations in duplicate wells of 96-well plates, and used in cross-presentation assays with B3Z cells as in Fig. 2*C*. *B*, The ADC cultures received CRPMI and 4 h later 10 μl stocks of acridine orange, a supravital dye that stains all cells green, and ethidium bromide that stains cells with permeable plasma membrane orange, or were stained for apoptosis using a TUNEL assay. Positive controls for apoptosis were irradiated for 10 min with UV light before the 4-h incubation period and positive controls for membrane permeability were treated with 0.2% saponin. The cells were observed by phase contrast (*left column*), for acridine orange and ethidium bromide fluorescence staining (*center column*) using green and red band path filters that were merged for the figure, and for TUNEL fluorescence (*right column*) using the green band path filter. Experimental and controls as indicated. Note that the dim fluorescence that is observed in the experimental "transfected, infected" cells by TUNEL is localized to the cytoplasm and most likely labels viral DNA. This cytoplasmic fluorescence is also observed in the "transfected, infected, UV-treated" but not in the "UV-treated" controls both of which show strong nuclear staining.

added to the ADC that remained attached to the wells. After a 4-h incubation, the ADC-pAPC cocultures were harvested, immediately fixed, and cross-presentation determined as usual. Fig. 6*A* shows that both Mϕ and DC cells effectively cross-presented Ag under these conditions. At the same time that the ADC-pAPC cocultures were harvested, the cells that received only CRPMI were stained with acridine orange and ethidium bromide or using the TUNEL assay. Acridine orange is a supravital dye that intercalates in the DNA and stains the nuclei of all cells green. With this staining, apoptosis can be determined by comparing the morphology of the stained nuclei. Ethidium bromide stains the nuclei of nonviable cells bright orange. Fig. 6*B* demonstrates that A9-T7 cells that had been transfected and infected 13 h earlier (this time includes the 9 h of infection before adding the ADC to the cross-presentation wells and the 4 h of pAPC/ADC coculture) exhibited the typical rounding and membrane roughness of vaccinia-infected cells. However, they were >97% viable as revealed by exclusion of ethidium bromide, and they were not apoptotic as revealed by the morphology of the nuclei and the absence of nuclear fluorescence in the TUNEL assay. For comparison, the staining of normal, apoptotic, and saponin-permeabilized apoptotic cells are included in the figure. The low level of death (<3%) cannot explain the cross-presentation observed, because these ADC induced cross-presentation signals similar to those where 100% of the cells were dead. Therefore, live-infected ADC can transfer Ag to pAPC for cross-presentation.

Because Fig. 4 indicated that dead cells could transfer Ag to pAPC following only 1 h of coculture, we tested whether the same occurred when using live-infected cells as ADC. Therefore, A9-T7 cells were prepared as in Fig. 6 and cocultured with pAPC for 1 or 2 h. The results in Fig. 7 show that, similar to what occurs with dead-infected ADC, pAPC can cross-present Ag from live-infected ADC within 1 h of coculture.

Discussion

Cross-presentation is a kind of exogenous Ag presentation whereby pAPC acquire Ags from other cells and present them on their own MHC class I molecules. This process might have its foremost physiological importance in the generation of antiviral immunity. Much of what is currently known about exogenous presentation was obtained from the study of other types of exogenous Ags such as soluble and particulate proteins, immune complexes, and bacteria (24–27, 43). Although the studies with these antigenic formulations greatly advanced our knowledge about exogenous presentation, cross-presentation of Ags delivered by virus-infected cells involves greater complexity as it involves a multitude of parameters that may affect the ADC, the pAPC, and the interactions of both cell types with the virus. For example, cross-presentation might be affected by the quantity and timing of Ag production, virus-induced ADC death, the production of cytokines or other alarm signals by ADC, the kinetics with which pAPC acquire the Ag from infected cells, the need for Ag to compete with a multitude of other Ags produced by the ADC, the ability of the virus to

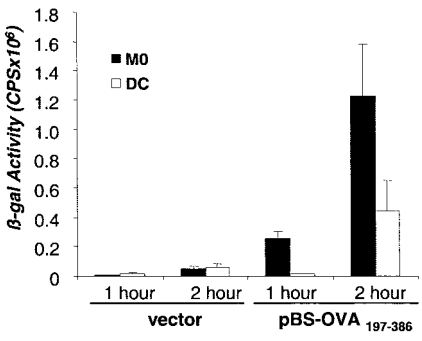

FIGURE 7. Cross-presentation by Mϕ and DC is also rapid when ADC are still alive. ADC were prepared as in Fig. 6 but they were cocultured with pAPC for the indicated times instead of 4 h.

infect the pAPC, the route followed by the Ag within the pAPC, etc. A major problem in studying cross-presentation in viral infections has been the difficulty in establishing reliable models where one can be certain that the measured phenomenon is cross-presentation and not direct presentation. We have previously achieved this in vivo by using a poliovirus system that restricted virus infection to non-pAPC and showed that cross-presentation is necessary and sufficient to induce CTL responses (3). However, the careful dissection of many important aspects of cross-presentation such as those that we describe in this study requires a reliable in vitro system that could limit Ag production to ADC and Ag presentation to pAPC. The use of allogeneic ADC is a simple approach to restrict Ag presentation to pAPC. However, restricting the synthesis of Ag to ADC is more challenging. Other authors have used human monocytes infected with recombinant vaccinia virus as ADC. However, this required the UV irradiation of the ADC to avoid transfer of live virus to the pAPC (23). Therefore, under these conditions cross-presentation was a result of irradiation and vaccinia was a vector for Ag production. In this study, we designed a powerful cross-presentation assay using vaccinia virus that undoubtedly restricts Ag production to ADC and presentation to pAPC without the need to artificially kill the ADC. Therefore, it allows measuring cross-presentation while maintaining intact the complex interaction between the virus, the ADC, and the pAPC. Using this assay, we have confirmed that DC can cross-present Ag shed by vaccinia-infected cells, and also demonstrated for the first time that Mϕ can also cross-present Ag supplied by vaccinia-infected ADC with high efficiency. Moreover, our results show that for the mechanisms and kinetics of cross-presentation that we have analyzed, Mϕ and DC are almost identical. In the past few years, there has been much emphasis placed on the exclusive role of DC as the primary cell type involved in Ag presentation while the idea of Mϕ as major player in this process has been almost abandoned. One possible reason for our results being different from those reported by Albert and coworkers (21, 22) is that human Mϕ and mouse Mϕ may differ in their ability to cross-present. It is also possible that Mϕ of differing tissue origin (peripheral blood monocyte-derived vs bone marrow-derived) differ in their ability to cross-present. Another possibility for this difference is that different pAPC could handle diverse Ags or cells infected by various viruses differently since we used OVA and vaccinia virus while Albert et al. (21, 22) used influenza virus. This notion is reinforced by the demonstration that Mϕ generated under similar conditions as ours failed to present exogenous OVA in the form of immune complexes (44). Nonetheless, our finding that Mϕ cross-present Ags contradicts a model generalizing the view that cross-presentation of Ags provided by infected cells is an exclusive ability of DC and that Mϕ are scavengers with an enzymatic machinery that destroys these Ags rather than uses them to generate antigenic peptides for MHC class I cross-presentation (22). Therefore, the role of Mϕ as pAPC should be revisited. However, it should be noted that our assay measures Ag presentation and not priming of naive $CD8^+$ T cells. Therefore, it remains possible that the activation of naive cells in the lymph nodes is an exclusive function of DC. This would agree with the observation that DC but not Mϕ can be found in the T cell areas of lymphoid organs (45). However, cross-presenting Mϕ could be involved in the amplification of the response in the periphery, or in the rapid activation of memory cells in tissues during recall responses. Alternatively, there is the provocative possibility that cross-presenting Mϕ that localize to the subcapsular region of lymph nodes (17, 45) could rapidly differentiate to DC and migrate to the lymph nodes to activate naive T cells.

In this manuscript, we also show that $OVA_{197–386}$ as a model of a vaccinia Ag is cross-presented by pAPC using the cytosolic route. This result agrees and confirms our previous work in vivo using poliovirus also expressing a truncated, cytosolic form of OVA, and with the results from other laboratories using a different virus and Ag (22). Nevertheless, it is also interesting to note that for Ags delivered in the form of bacteria or protein bound to microbeads, exogenous presentation of SIINFEKL followed the cytosolic route when expressed in the context of the native OVA sequence (as in our experiments). However, it could use the vacuolar pathway when expressed in a recombinant form, fused very close to the C terminus of the *Escherichia coli* Crl protein. Whether these findings are mirrored when the exogenous Ag is provided by vaccinia infected cells is currently under investigation (26).

The current model of cross-presentation during viral infections is that DC acquire Ag from apoptotic- or necrotic-infected cells in the periphery and migrate to lymphoid organs (13, 46, 47). This process is accompanied by the maturation of the DC, which involves a decreased ability to phagocytose and increased expression of MHC, B7, and other molecules allowing for a more effective presentation of Ag to T cells (48, 49). Our experiments show that pAPC cross-present Ags released by vaccinia-infected cells very soon after contacting ADC, that cross-presentation lasts for a short period of time, and that cross-presentation starts at early times of infection when the ADC are still alive.

That pAPC cross-present Ag from virus-infected cells very soon after ADC-pAPC contact is in agreement with the finding that cross-presenting cells migrate to the draining lymph nodes within 6 h after scarification of the epidermis with HSV (50). However, our finding that cross-presentation of Ags from vaccinia-infected cells is also short-lived even in the presence of saturating amounts of Ag and in the absence of pAPC infection is surprising. Indeed, it would be expected that direct presentation by pAPC infected with cytopathic viruses would be short-lived but not during cross-presentation when the pAPC infection is spared as when we irradiated the ADC. Still, it is very likely that our results reflect the duration of cross-presentation for individual cells in vivo, and is consistent with the measured half-life of MHC class I peptide complexes of high affinity at the surface of cells, which is ~6–12 h (51, 52). If this is correct, it implies that the activation of pAPC and their ability to stimulate naive T cells should also occur very fast, and that the activation of a sufficient number of precursor CTL in vivo may require the continuous arrival of pAPC from the site of infection. An alternative explanation for the short time of cross-presentation during vaccinia infection may be that vaccinia may produce cross-presentation inhibitory factors. Poxviruses are known to encode multiple immune evasion molecules such as cytokine and chemokine-like proteins (53, 54). If this were the case, the immune evasion molecules acting in our assay should be transferred with the infected cells because we discarded the supernatants. Moreover, these factors should work in *trans* (i.e., produced in the ADC but functions in the pAPC) because virus inactivation did not alter the kinetics of cross-presentation. To our knowledge, inhibition of MHC class I Ag presentation in *trans* has not yet been described. If this were the reason for the short time that cross-presentation lasts, it might be of interest to identify the responsible molecules. Another alternative is that some mechanisms that prolong Ag cross-presentation in vivo may be absent in our assay. For example, cytokines or inflammatory molecules could increase the half-life of the MHC class I peptide complexes at the cell surface (48) or retard the intracellular degradation of Ag within the pAPC.

It is widely held that cross-presentation in viral infections required either necrotic or apoptotic cells as the source of Ag. Therefore, our present findings that cross-presentation occur at early

times of vaccinia infection and without the need for cell death is striking. It will now be important to identify the signals used by the infected ADC to alert pAPC and to determine the mechanisms whereby pAPC incorporate the Ags supplied by live-infected ADC. One possible mechanism is that pAPC phagocytose and process whole live-infected ADC. It is also possible that Mϕ and DC cells recognize signals imparted by the infected cells and kill them just before phagocytosis. Other possibilities are that live-infected ADC release Ag-loaded exosomes (55) or antigenic peptides bound to heat shock proteins (56). These could be taken up by phagocytosis or through receptor-mediated endocytosis and cross-presented by pAPC. We also speculate that the ability of pAPC to acquire Ag from live ADC at early times of infection could be advantageous because at this time the replication cycle of the virus might not have been completed and pAPC could avoid becoming infected themselves.

Together, our findings that cross-presentation during vaccinia infection occurs very rapidly following ADC-pAPC contact and that ADC provide Ag before dying and at early phases of infection should contribute to rapid Ag presentation and a headstart in the initiation of the $CD8^+$ T cell response. This timing could be critical to tip the balance in favor of the host in its race against the virus. Together, our findings and those of others (17, 50) point to a model where direct presentation and cross-presentation are both fast and brief. This would also require pAPC-T cell contact to be brief and occur at a very early phase of the immune response. This model would also be in agreement with findings that a short exposure of $CD8^+$ T cells to Ag bearing pAPC is sufficient to start a program of maximal proliferation and differentiation into effector and memory cells (57–60).

Acknowledgments

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